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

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IMMUNE MODULATION BY SCHISTOSOMES:

mechanisms of regulatory B cell induction and inhibition of allergic asthma

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Chapter 1

GENERAL INTRODUCTION

The human immune system has evolved to fulfil a myriad of functions. It has to confer protection from potentially harmful infectious agents including bacteria, viruses and parasites and fight malignant cells, but tolerate self-tissue and foreign, but harmless substances introduced via e.g. the lungs or the digestive system. This is reflected in the complexity of the immune system, with various innate and adaptive, often highly specialized effector functions at interplay. Helminth (greek: 'intestinal worm') infections are likely as old as humanity itself. The co-evolution of helminths and humans has not only resulted in specified immune responses against helminths, but also in the development of immune evasion mechanisms by these parasites and even a selective pressure to alter the host's genetic composition and thereby shape the immune system [1-3].

1. AN INTRODUCTION TO *SCHISTOSOMA*

Schistosoma (S.) spp. are parasitic flatworms of the genus trematode. In humans, they cause an acute and chronic disease known as schistosomiasis/bilharzia. The species causing most human schistosomiasis cases are *S. mansoni*, *S. japonicum* and *S. haematobium* [3]. In 2016, an estimated 206.4 million people, mainly in poor and rural communities of tropical and subtropical areas, required preventative treatment [4]. *Schistosoma spp.* parasites can cause severe disease, but can also survive in the human host up to 20 years, meaning both mortality and morbidity are major concerns.

1.1. Immunity to *Schistosoma spp.*

Many features of schistosomiasis and the underlying immunological processes can be replicated well in animal models, most commonly infected with *S. mansoni* or *S. japonicum*. The immunological response to *Schistosoma* can be divided into an acute and a chronic phase. The acute phase, in response to premature life cycle stages that penetrate the host skin and subsequently migrate through several tissues including the lung, is initially characterized by a type 1 immune response. A much stronger type 2 immune response is however induced in response to eggs released by mating adult worm pairs starting 5-6 weeks post infection. *S. mansoni* and *S. japonicum* adult worm pairs reside in the mesenteric vein, and part of the released eggs penetrate through the tissue into the intestinal lumen where they are released into the environment with the faeces. Importantly, many eggs are transported with the blood flow into the liver and other organs. Tissue-entrapped eggs and the resulting inflammatory granulomatous response are the main drivers of pathology [5]. The egg-specific type 2 response predominates the earlier type 1 response [6]. In the chronic phase of infection, regulatory responses are induced which contribute to limiting immunopathology despite the continuous release of eggs, and allow long-term parasite survival in the host.

Type 2 immune responses have a major role in both the host defence against helminths or venoms and in allergic disorders. The central features of a type 2 response shared by immune responses to helminth and allergens include the induction of $CD4^+$ T helper (Th) 2 cells and their innate counterpart, type-2 innate lymphoid cells (ILC2s), which both produce the type 2 cytokines interleukin-4 (IL-4), IL-5, IL-9 and IL-13, the induction of B cell IgE production and the recruitment and activation of granulocytes and alternatively activated macrophages (AAMs) [7]. The type 2 immune response to helminth is diverse and adapted to the type of helminth, with the classical 'weep and sweep' response including increased smooth-muscle-cell contractility, intestinal permeability and goblet-cell mucus production that has developed to expel intestinal helminths [8]. The type 2 response to *Schistosoma spp.*, resident in the vasculature, is induced in response to antigens released by the eggs [6, 9]. Characteristic for this type 2 response is the formation of granulomas around tissue-entrapped eggs, which mainly consist of $CD4^+$ Th2 cells, ILC2s as well as eosinophils and macrophages. The central, and dichotomous, role of type 2 immunity in schistosomiasis becomes apparent in transgenic mouse models. Mice deficient in IL-4 display intestinal and hepatic pathology and increased mortality around the time of egg production

onset [10, 11], highlighting the modulatory and host-protective role of Th2 responses especially during the acute stage of disease. Mice deficient in IL-13 or IL4Rα, the shared receptor for both IL-4 and IL-13, do not develop severe hepatic fibrosis as wild-type (WT) animals do, resulting in prolonged survival [10, 12]. This in turn demonstrates that prolonged type 2 responses during chronic infection contribute to morbidity.

1.2. *Schistosoma spp.* eggs & egg antigens

In the context of experimental intraperitoneal (i.p.) administration, eggs can be considered as 'antigen reservoirs'. After intravenous (i.v.) injections, eggs transported with the blood stream get trapped in the lung [13], which serves as a model to study lung inflammation in response to *Schistosoma spp.* In contrast, eggs likely remain in the peritoneal cavity after i.p. injection. *S. mansoni* soluble egg antigens (SEA), which is the soluble fraction of homogenized eggs, is a complex mixture of hundreds of antigens, many of which are glycoproteins [14, 15]. The *S. mansoni* egg excretory-secretory product (ES), which represents the entirety of molecules released by cultured eggs, has been described to still contain 188 proteins [16]. Some of the most abundant proteins, including IPSE/alpha-1 [17-21] and omega-1 [22-27], have been well-characterized and described to fulfil distinct functions. Others, such as kappa-5 [28, 29], lyso-PS [30] or SmCKBP [31] are less well characterized, and for many the function is completely unknown. Some immunomodulatory molecules of *Schistosoma spp.* life cycle stages other than eggs have been described, including Smteg, Sm22.6, PIII and Sm29, all of which have been described to prevent allergic airway inflammation (AAI) [32, 33], and cyclophilin A [34]. The only description of a *Schistosoma spp.* egg-derived molecule inhibiting AAI is the *S. japonicum* molecule SjP40 [35]. *Schistosoma spp.* single molecules and their described functions are summarized in **Table 1.**

1.3. The regulatory network

The immune regulatory network, induced during the chronic phase of infection, suppresses the host immune system to allow parasite survival while preventing excessive tissue damage. Immunomodulation is thus advantageous for both the parasite and the host. The induction of regulatory T (Treg) cells, the most prominent component of the regulatory network, by *Schistosoma spp.* has been described in both human and murine studies [36-38]. Treg cells suppress effector T cell responses in murine schistosomiasis [38, 39], and depletion of Treg cells during the acute or chronic phase of infection results in increased granulomatous immunopathology [40, 41]. An adoptive transfer of *S. mansoni*-primed Treg cells or a retroviral transfer of the *Foxp3* gene suppresses granuloma formation and ameliorates immunopathology [40-42]. More recently, additional members of the regulatory network, such as regulatory B (Breg) cells (see section 2) and AAMs [43], have been described. Importantly, cells of the regulatory network are interrelated and synergize, e.g. Breg cells induce Treg cell generation. The aforementioned regulatory immune cells possess various effector functions. One of the most prominent ones is immune modulation through the regulatory cytokine IL-10 [44, 45]. As a result of the induced regulatory network, both antigen-specific and non-specific, more generalized immune modulation occurs. Importantly, the regulatory network also results in 'spill-over suppression' and diminished responses to allergens (see section 3), vaccinations and co-infections [46]. Another member of the regulatory network are AAMs, which play a central role in tissue remodelling [47]. This rapid wound healing response is a central component of type 2 immunity, as it prevents excessive host tissue damage and pathology caused by large, multicellular parasites such as helminths [48].

2. REGULATORY B CELLS

The first description of B cells with suppressive capacities stems from 1974, when it was observed that B cells suppress delayed-type hypersensitivity (DTH; [49, 50]). More than 20 years later, the first descriptions

TABLE 1. Literature on *Schistosoma spp.* single molecules and described functions

Helminth species	Name	Molecule	Source	Suggested immunological function	Reference
S. mansoni	Cyclophilin A	Peptidyl-prolyl cis-trans isomerase	adult worms, secretory	immunomodulatory; regulation of APC activity	(34)
	IPSE/alpha-1	glycoprotein	eggs (sub-shell area), secretory	binding of IgE and DNA; induction of basophil IL-4 production; alters transcription profile of DCs	(17-21)
	Kappa-5	glycoprotein	eggs, secretory	interaction with CLRs	(28, 29)
	Lyso-PS	lyso-phosphatidylserine	tegument	induction of Treg cells via TLR2; Th2 polarization	(30, 125)
	Omega-1	T2 RNase, glycoprotein	eggs, secretory	Th2 polarization of DCs; induction of Treg cells; inflammasome activation and IL-1β induction in MFs; IL-33/ILC2-mediated improvement in metabolic homeostasis	(22-27)
	PIII	fraction of soluble adult worm antigen	adult worm antigen	immunomodulatory; inhibition of AAI	(32)
	Sm22.6 (=SmTAL-1)	tegument-allergen-like (TAL)	tegument of all life cycle stages except eggs, soluble	immunomodulatory; inhibition of AAI	(32)
	Sm29	glycoprotein	tegument of adult worms during lung stage, membrane-bound	immunomodulatory; inhibition of AAI	(32)
	SmCKBP	chemokine-binding protein	eggs, secretory	inhibition of CXCL8 and suppression of neutrophil recruitment	(31)
	Smteg	soluble fraction of schistosomula tegument	tegument	induction of IL-10 in lung monocytes & inhibition of AAI	(33)
S. japonicum	SjP40		eggs	induction of Th1 cytokines & inhibition of AAI	(35)

of suppressive B cells in murine models of autoimmune diseases were published [51-53] and the term 'regulatory B cell' introduced.

2.1. Phenotype & origin

Breg cells is an umbrella term for a heterogeneous group of B cells that comprise immunomodulatory capacity. Various Breg cell subsets have been described both in humans and mice, depending on their origin, the tissue they reside in, their phenotype and mode of action. To date, it is unknown whether Breg cells develop from a committed precursor or whether any B cell can acquire suppressive capacity in response to certain environmental stimuli, and different models are discussed in the literature [54, 55]. Extensive overviews over different Breg cell subsets and the range of described suppressor mechanisms has also been provided in the literature [44, 55, 56]. In brief, both murine B cell lineages, innate-like B1 B cells abundant in peritoneal and pleural cavity [57] and B2 B cells that populate secondary lymphoid organs, can give rise to Breg cells. Of B2 B cells, especially marginal zone (MZ) B cells [58, 59] and their precursors (transitional type MZ precursors, T2-MZP) [60, 61] have been described to acquire regulatory functions. More recently, plasma blasts and plasma cells have also been acknowledged to produce regulatory cytokines and act suppressive [62, 63]. In humans, Breg cells are mainly characterized within the $CD24^{hi}CD38^{hi}$ immature B cell and the $CD24^{hi}CD27^{+}$ B cell compartment [64, 65]. Suppressive functions of Breg cells other than IL-10 production, include e.g. the release of other regulatory cytokines including IL-35 and transforming growth factor beta (TGF-β), the induction of Treg cells and suppression of effector T cells through expression of ligands such as FasL, ICAM-1/LFA-1, GITRL or PD-L1 [66].

2.2. Signals for the induction and expansion of Breg cells

Breg cells are present in naïve individuals and mice, but often expand and display increased suppressive activity in the context of autoimmunity and infection [44, 67, 68]. Various signals have been described to play a role in Breg cell development and activation, of which stimulation through the B cell receptor (BCR) [53, 69, 70], CD40 [53, 60, 71, 72], and Toll-like receptors (TLRs; especially TLR4 [73-75], TLR7 [76] and TLR9 [58, 73]) are considered most central. Moreover, different cytokines including IL-15 [77], IL-21 [78], IL-35 [62, 79], BAFF [80], APRIL [81] and type I interferons [63] have been described to support Breg cell development and activation.

Recently, it has also been acknowledged that microbiota-derived signals contribute to Breg cell activation. The microbiome induces Breg cells in the spleen and the mesenteric lymph node (LN) in an IL-1β- and IL-6-dependent manner. Mice with a disrupted microbiome have an impaired Breg cell compartment and develop exacerbated autoimmunity [82]. Other studies confirmed that alterations of the gut microbiome, either by increased estrogen levels or housing animals under specific-pathogen-fee conditions, induce IL-10-producing Breg cells in the spleen and the mesenteric LN [83, 84].

It is likely that more than one stimuli and converging signalling pathways are needed to achieve maximal Breg cell development and activation. A two-step model has been proposed for the acquisition of regulatory properties by B cells, with the exposure to innate stimuli (e.g. TLR ligands) as first step initiating IL-10 production, and BCR or CD40 ligation as a second step promoting B cell survival and activation and thus amplifying suppression [61, 73]. Other studies suggest that TLR and CD40 ligation may enable B cell IL-10 expression by promoting differentiation into IL-10-competent plasmablasts or plasma cells [62, 63].

It is worth noting that the signals required for the development and activation of Breg cells likely differ *in vivo* and *in vitro*. During an inflammatory response *in vivo*, multiple processes occur in parallel at any given time, while *in vitro* systems allow to dissect the role of individual signals and pathways, but often fail to mimic the complexity *in vivo*.

2.3. Breg cells in helminth infection

The first indication that B cells with regulatory properties are induced during helminth infection has been made 20 years ago in μMT mice lacking mature B cells, which develop augmented pathology following *S. mansoni* infection [85]. Subsequently, different helminth species have been shown to induce Breg cells [86-89]. These helminth-elicited Breg cells have immunomodulatory capacities in a variety of autoimmune and inflammatory conditions, including anaphylaxis [90], experimental autoimmune encephalomyelitis (EAE) [87, 91] and AAI [86, 87, 92]. In humans, Breg cells have also been identified in helminth-infected individuals [88, 93].

Options of how helminth or their products induce Breg cells are a) the direct ligation of above mentioned receptors, e.g. the BCR or TLRs on B cells by helminth molecules, and b) signals derived from accessory cells (e.g. via the ligation of CD40) or the general inflammatory environment (e.g. cytokines) induced by helminth infection. That Breg cells can be directly induced by *Schistosoma spp.* products in both mice and humans has been shown by *in vitro* co-culture of murine, purified splenic B cells with living *S. mansoni* worms [86], and by *in vitro* stimulation of PBMC-derived B cells with SEA [94], respectively. *S. mansoni* infection has been described to induce the expression of *Tlr7* in Breg cells. TLR7 ligation was moreover found to facilitate IL-10 production, it remains unclear however whether this is the consequence of direct TLR7 ligation by a *S. mansoni* molecule [76]. Despite these studies suggesting a direct Breg cell induction by helminths and their products is possible, it is plausible that *in vivo*, Breg cells also receive signals from accessory cells and their environment.

The description of helminth-specific signals that induce Breg cells is limited. In the context of *Schistosoma spp.*, *in vitro* exposure of murine splenocytes to live worms [86, 90] and of human peripheral blood B cells from helminth-infected individuals to SEA [94] has been reported to induce IL-10-producing Breg cells. In all these cases the molecular identity of the stimulus has however not been identified. The milk oligosaccharide lacto-*N*-fucopentaose III (LNFP-III), which carries a glycosylation pattern that also occurs on *Schistosoma spp.* products and is therefore cross-reactive, has been described to induce B cell IL-10 production [95]. Except for the stimulation of isolated human B cells from helminth-infected individuals with SEA [94], which may be a recall reaction of memory B cells, it remains unclear whether the observed Breg cell induction is the result of a direct interaction of with receptors on B cells. For helminth other than *Schistosoma spp.*, the glycoprotein ES-62 from *Acanthocheilonema viteae* and an extract from *Brugia malayi*, both filarial nematodes, also induce IL-10 production by B cells [96, 97].

2.4. Breg cell transcriptomics

Transcriptomics allows to study the complete set of genes transcribed at a certain time point and potentially allows insight into the whole breadth of cellular processes at interplay. To date, only few studies have utilized transcriptomics approaches to gain insight into the signals required for the development and activation of Breg cells. Khan et al. reported that *Tlr7* is significantly upregulated on *S. mansoni*-induced $CD19^{+}CD1d^{hi}$ B cells compared to naïve control cells and $CD19^{+}CD1d^{lo}$ B cells from infected animals, and that TLR7 ligation increases their IL-10 production [76]. Another study by Sun et al. identifies CD9 as a marker of murine IL-10-competent, $CD19^{+}CD1d^{hi}CD5^{+}$ Breg cells induced by *in vitro* polyclonal stimulation. Although the exact role of CD9 remains unclear, the study also suggests that CD9 might play a role in the immunosuppressive function of Breg cells, as blocking CD9 *in vitro* impaired their ability to suppress T cell proliferation [98]. $CD9^{+}$ B cells have subsequently been described to suppress house dust mite-induced AAI [99].

3. ALLERGY AND ASTHMA

Asthma is an atopic, chronic inflammatory disorder that is estimated to affect 315 million people worldwide [100]. Asthma is characterized by chronic lung inflammation, bronchial hyper-responsiveness

and airway obstruction, resulting in episodes of wheezing and breathlessness. First episodes of atopic disease often occur during childhood following sensitization to inhaled allergen, and infants who suffer from atopic disease often develop allergic asthma later in life. Recent studies suggest that the incidence of asthma has now reached a plateau in high prevalence countries [101]. The global burden of asthma however continues to rise as incidence rates in Africa, South America, and parts of Asia still rapidly increase [102]. Asthma is a multivariable disease in which genetic predispositions are certainly important risk factors. Genome-wide association studies (GWAS) have identified different susceptibility loci, most prominently the chromosome 17q21 region and various genes of the type 2/IgE cluster, including e.g. *Il33* and *Tslp* (thymic stromal lymphopoeitin), which are genes encoding type 2 innate cytokines produced by bronchial epithelial cells [103, 104]. However, the rapid increase in incidence rates over the last few decades points toward a strong contribution of environmental factors to disease development.

3.1. Hygiene & Old Friends hypothesis, epidemiological evidence

The 'hygiene hypothesis' already postulated in 1989 [105] suggests that a decrease in childhood exposure to infectious agents as a result of increasing sanitation standards, improved health care, and life style changes contributes to the increase in incidence of allergies and asthma. This conceptual framework has been developed further into the 'old friends hypothesis', suggesting an evolutionary adaptation of our immune system to the continuous encounter with microbes and infectious agents, and a tendency towards an over-reactive immune system in their absence. Both concepts suggest that a reduction in infectious pressure over time leads to an immune system imbalance promoting allergic and other immune-mediated disorders such as inflammatory bowel disease (IBD) or multiple sclerosis (MS) [106].

The increase in asthma and other immune-mediated diseases correlates with urbanization and economic development, but a causal link has not been demonstrated so far. As one possible factor, changes in the exposure to pathogenic microbes, including helminths, have been suggested. The incidence of helminth infections is inversely correlated with increasing asthma rates in westernized countries. The ability of helminths to establish chronic infections and induce regulatory immunity has generated strong interest in the possibility that helminths or their products suppress hyper-inflammation and could be used as new anti-inflammatory treatment strategy [107].

The association between helminth infections and the reduced incidence of allergic disorders has been addressed in numerous epidemiological studies, yielding heterogeneous results. Meta-analyses show that there is no overall effect of helminth infections on asthma, but that hookworm infections do protect against allergic sensitization [108-110]. Individual studies have also suggested that infections with *S. mansoni* reduce the severity of asthma [111] and allergic skin reactions [112, 113]. While early clinical trials using eggs of the pig whipworm *Trichuris suis* (TSO) or experimental hookworm infections, both of which are intestinal helminths, resulted in promising safety and efficacy data in patients with intestinal inflammatory diseases, efficacy could not be demonstrated in larger studies or studies with allergic or asthmatic subjects [107]. Potential reasons for the heterogeneity in results from epidemiological studies and clinical trials, apart from the helminth species, include a range of other factors such as time, location, intensity and chronicity of infection and host genetics [114].

3.2. Immunity in asthma

The asthmatic immune response is complex, with a multitude of innate and adaptive, cellular and humoral processes at interplay. During allergic sensitization, epithelial cells produce cytokines including TSLP, IL-25 and IL-33, which activate DCs and ILC2s to induce Th2 cell polarization. Upon renewed allergen exposure, Th2 cells and ILC2s quickly produce large amounts of type 2 cytokines including IL-4, IL-5 and IL-13, which in turn cause class-switching of B cells to IgE and the recruitment and activation of eosinophils, mast cells and basophils [115, 116]. Mast cells and basophils release inflammatory mediators

such as histamines following cross-linking of allergen-specific IgE bound to surface IgE Fc-receptors, amplifying the allergic response and resulting in an acute reaction including mucus production and bronchoconstriction [115, 117].

3.3. Helminth-mediated protection against AAI

Helminths have developed various strategies to modulate the host immune system. They amplify the natural immune regulatory network of the host and modify pro-inflammatory immunity, thereby preventing exaggerated immune responses to ubiquitous antigens such as allergens.

Early insight into the protective effects of helminth infections stems from research using a variety rodent and human nematodes and *Schistosoma spp*. The latter provides a unique model of helminth infection as it, in contrast to the other models of mainly gastrointestinal nematodes used, allows to study both the role of egg deposition and chronicity of infection on protection against AAI. The literature published thus far on the protective effect of *Schistosoma spp*. infections is summarized in **Table 2**. Building up on this work, several groups have successfully employed excretory-secretory products (ES) or, in the case of *Schistosoma spp*., SEA and eggs instead of natural infections. Ultimately, a range of single molecules that harbour protective effects against AAI have already been identified and their mode of action characterized, including *Ancylostoma caninum* AIP-2, *Anisakis simplex* MIF-like protein, *Ascaris suum* PAS-1, *Acanthocheilonema vitae* cystatin and ES-62 as well as *Heligmosomoides polygyrus* HpARI. For schistosomes, *S. mansoni* Smteg, Sm22.6, Sm29 and PIII as well as *S. japonicum* SjP40 have been described in the literature as single molecules with protective effects against AAI. The literature published thus far on the protective effect of helminth-derived molecules is summarized in **Table 3**.

3.4. Mechanisms of protection

A range of different mechanisms by which helminths and their molecules protect against AAI have been described, including the induction of Treg cells, regulatory macrophages and regulatory cytokines (IL-10, TGF-β), the induction of Th1 responses and IFN-γ which result in a shift in the Th1/ Th2 balance, as well as the inhibition of pro-inflammatory processes such as IL-33 release and mast cell degranulation (also see **Table 2** and **Table 3**).

Different mechanisms of protection have been suggested to be involved in conferring *Schistosoma spp*.-mediated protection from AAI. *Schistosoma spp*. provides an especially interesting model for experimental studies as its life cycle in the host includes the formation of adult worm pairs and continuous deposition of eggs. Infections with either mixed sex worms or only male worms allows to dissect the contribution of eggs to protection. Various studies identified Treg cells and Breg cells, respectively, as mediators of protection [32, 86, 88, 92, 118-120]. Only some of these studies however provide functional experiments to formally prove their role in protection rather than solely showing an association between reduced AAI and elevated numbers or activity of these cells. One recent study links protective effects against AAI to an increased IFN-γ production [35].

The variety of proposed mechanisms most likely reflects the variety of different species, molecules and models studied. It is plausible that helminths have evolved distinct ways of altering the host immune response and promote their own survival.

3.5. Development of novel therapies & outlook

Despite the drastic worldwide increase in asthma prevalence, not many novel therapies have been developed. The current treatment of asthmatic patients still consists of inhaled steroids and bronchodilators, which only alleviates symptoms and is impeded by the development of steroid resistance. New treatment approaches that alter the immune response and promote long-lasting

tolerance are needed. Exploiting the modulatory capacities of evolutionary conserved 'old friends' like helminths might serve as a promising strategy towards the development of novel treatment options. Although early clinical trials investigating the use of natural helminth infections initially showed promising results in intestinal inflammatory diseases [121, 122], follow-up studies have shown little effect in allergy [123, 124] and efforts to use live infections as treatment strategies have since diminished. Treatment with live helminth infections also bears risks, and efforts have therefore intensified to identify single, helminth-derived molecules that mediate protection.

4. SCOPE OF THIS THESIS

The induction of Breg cells by helminth parasites and the implication of helminth-induced immune modulation for providing protection from hyper-inflammatory disorders such as allergies has been extensively studied over the last decades. However, due to the complex immunity to helminth infections, it proved difficult to a) dissect the molecular signals for induction of Breg cells, and b) fully characterize cellular mechanisms of protection in allergic airway inflammation. Experimental *S. mansoni* infection and isolated *S. mansoni* eggs have provided us with tools to further address these research areas.

The first part of this thesis focusses on the role of Breg cells in protection from AAI by chronic *S. mansoni* infections and aims at identifying molecular signals required for schistosome-induced Breg cell development.

In **chapter 2** we build up on previous work and show that not only splenic B cells, but also pulmonary B cells induced during chronic *S. mansoni* infection can provide protection against airway inflammation. In **chapter 3**, we sought to identify *S. mansoni*-derived antigens that induce Breg cells and describe that the egg glycoprotein IPSE/alpha-1 directly interacts with splenic MZ B cells, induces IL-10 production and promotes Treg cell expansion. In **chapter 4**, we aimed to identify molecular signals contributing to Breg cell induction *in vivo* by performing transcriptomics on splenic B cell subsets from chronically infected mice. In **chapter 5**, we describe that type I interferons enhance Breg cell IL-10 production in response to *S. mansoni* antigens *in vitro*, but are dispensable *in vivo*.

In the second part of this thesis, we build up on earlier work by us and others showing a protective effect of *S. mansoni* on experimental AAI, and aimed to identify protective, single *S. mansoni*-derived molecules.

Chapter 6 summarizes and discusses the evidence for helminth-induced protection from AAI, mechanisms of protection, efforts towards the identification of protective, single helminth-derived molecules and the implications for the development of novel treatment strategies (as of 2014). In **chapter 7**, we describe that isolated *S. mansoni* eggs and the single egg-derived glycoprotein omega-1, in the absence of adult worms, also protect from AAI.

The main findings of this thesis are summarized and discussed in **chapter 8,** including directions for future research towards understanding of the link between *Schistosoma*, Breg cells and allergic asthma.

TABLE 2. Literature on the protection from AAI by *Schistosoma spp.* infection

Helminth species	Sex of cercariae	Time of AAI induction	Mouse strain	Cerc. strain	No. of cerc./ mouse	Suggested mechanism	Reference
S. mansoni	mixed	wk 1 (pre-patent); wk 5 (patent)	Balb/c	Brazilian	90	dependent on egg deposition; Treg cells	(118)
	mixed	wk 14 (chronic)	C57BL/6	Puerto Rican	40	Breg cells	(88)
	male only	wk 8 (acute)	Balb/c	Puerto Rican	?	Breg cells	(86)
	mixed	wk 8 (acute)	Balb/c	LE	30		(119)
	mixed	wk 8 (acute); wk 12 (intermed.); wk 16 (chronic)	C57BL/6	Puerto Rican	15/30/45	dependent on infection intensity and chronicity	(126)
	mixed & male only	wk 7-11 (acute); wk12-16 (chronic)	Balb/c	Puerto Rican	30	protection only in 'male only' group; B cell-/IL-10 dependent	(92)
S. japonicum	mixed & male only	wk 4	Balb/c	?	25	protection both in mixed and 'male only' group	(127)

TABLE 3. Literature on the protection from AAI by helminths-derived molecules

Helminth species	Molecule	Time of immunization	Suggested mechanism	Reference
***Schistosoma spp.* products**				
S. mansoni	Smteg	during sensitization	IL-10	(33)
	Sm22.6,Sm29, PIII	during sensitization + pre-challenge	Treg cells	(32)
	eggs	during sensitization	Treg cells; IL-10 independent	(119)
S. japonicum	SjP40	during sensitization	induction of IFN-γ	(35)
	SEA/eggs		Treg cells	(120)
Products from other helminths				
A. caninum	AIP-2	during challenge	Treg cells	(128)
A. simplex	MIF-like protein	during challenge	Treg cell-/IL-10-/TGF-β-dependent	(129)
A. suum	PAS-1	during sensitization and challenge	IFN- γ- and IL-10-dependent	(130)
		during sensitization and challenge	Treg cells, CD8 γδ T cells	(131)
A. vitae	Cystatin	during sensitization	Shift to IFN-γ/Th1 response	(132)
		during sensitization or pre-challenge	IL-10-producing macrophages	(133)
	ES-62	during sensitization and challenge	Shift to IFN-γ/Th1 response	(134)
		during sensitization and challenge	Inhibition of mast cell degranulation	(135)
H. polygyrus	ES	during sensitization	Inhibition of IL-33 release and ILC2s	(136)
	HpARI	during sensitization	Inhibition of IL-33 release	(137)
N. brasiliensis	ES	during sensitization and challenge	TLR2-, TLR4- and IL-10-independent	(138)

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Chapter 2

SCHISTOSOME-INDUCED PULMONARY B CELLS INHIBIT ALLERGIC AIRWAY INFLAMMATION AND DISPLAY A REDUCED TH2-DRIVING FUNCTION

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ABSTRACT

Chronic schistosome infections protect against allergic airway inflammation (AAI) via the induction of IL-10-producing splenic regulatory B (Breg) cells. Previous experiments have demonstrated that schistosome-induced pulmonary B cells can also reduce AAI, but act independently of IL-10. We have now further characterized the phenotype and inhibitory activity of these protective pulmonary B cells. We excluded a role for regulatory T (Treg) cell induction as putative AAI-protective mechanisms. Schistosome-induced B cells showed increased CD86 expression and reduced cytokine expression in response to Toll-like receptor (TLR) ligands compared with control B cells. To investigate the consequences for T cell activation we cultured ovalbumin (OVA)-pulsed, schistosome-induced B cells with OVA-specific transgenic T cells and observed less Th2 cytokine expression and T cell proliferation compared with control conditions. This suppressive effect was preserved even under optimal T cell stimulation by anti-CD3/28. Blocking of the inhibitory cytokines IL-10 or TGF-β only marginally restored Th2 cytokine induction. These data suggest that schistosome-induced pulmonary B cells are impaired in their capacity to produce cytokines to TLR ligands and to induce Th2 cytokine responses independent of their antigen-presenting function. These findings underline the presence of distinct B cell subsets with different stimulatory or inhibitory properties even if induced by the same type of helminth.

INTRODUCTION

Chronic infections with the helminth *Schistosoma mansoni* are associated with immune hyporesponsiveness and an enhanced regulatory network [1]. Regulatory B (Breg) cells, which are predominantly characterized by an enhanced production of IL-10 [2], are part of this network. Their functionality was first demonstrated in mouse models of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), lupus and chronic colitis [3]. Interestingly, helminth-induced Breg cells also can inhibit inflammation and were shown to protect against EAE, systemic fatal anaphylaxis and ovalbumin (OVA)- or house dust mite allergen (Der p1)-induced allergic airway inflammation (AAI) [4-6].

Murine Breg cells were mostly detected within splenic B cell subsets [2]. Breg cells have also been found within the mesenteric lymph node (LN), highly expressing the low-affinity IgE Fc receptor CD23 during *Heligmosomoides polygyrus* infection [6], and within the B-1a B cell compartment of the peritoneal cavity [7]. Various Breg cell-associated markers and suppressive mechanisms have been reported, however most of these studies are based on splenic populations. Apart from their capacity to produce IL-10, Breg cells have been described to induce other members of the regulatory network, e.g. regulatory T (Treg) cells [4, 5, 8], thereby amplifying the protective effect. B cells expressing the membrane-bound T cell immunoglobulin and mucin domain-1 (Tim-1) were able to control auto-immune [9] and allergic diseases [10]. Expression of CD25 by human Breg cells correlated with their IL-10 production [11], and in mice $CD25^+$ Breg cells attenuated inflammatory bowel disease (IBD) [12]. Furthermore, B cells expressing suppressive cytokines other than IL-10 have been described. TGF-β-producing Breg cells controlled inflammation in inhalation tolerance [13] and diabetes models [14], and IL-35-producing B cells regulated immunity during EAE and *Salmonella* infection [15, 16]. B cells also have the capacity to suppress T cell proliferation and cytokine production via cell-cell interactions that involve inhibitory receptors, and result in T cell hypo-responsiveness or the induction of apoptosis. Examples of such inhibitory receptors are T cell-expressed PD-1, Fas as well as CTLA-4, and their respective ligands PD ligand 1 (PD-L1), PD-L2, Fas ligand (FasL) and CD80/CD86 on antigen-presenting cells (APCs) including B cells.

Apart from their role as regulators, B cells play an important role in the induction and maintenance of Th2 immunity. B cells not only produce IgE and IgG1 antibodies, they can also directly interact with Th2 T cells and act as APCs to drive their expansion and cytokine production. Indeed, presentation of allergen by pulmonary B cells has been shown to be required for full Th2 cytokine production in AAI models using μMT and $JH^{-/-}$ mouse models [17, 18], and immunization experiments with the cysteine protease allergen papain suggest that B cells induce T cell/T follicular helper (Tfh) cell IL-4 production in the draining LN [19].

We have previously shown that *S. mansoni*-infected mice are protected against OVA-induced AAI, and that both splenic and pulmonary B cells from infected mice were able to transfer protection to OVA-sensitized mice [5, 20]. Intriguingly, splenic B cells inhibited AAI via IL-10 and the induction of Treg cells, while pulmonary B cells essentially acted in an IL-10-independent manner *in vivo* [5]. We hypothesized that schistosome infections support the development of distinct Breg cells in the lungs. Therefore, we further explored the effector mechanism by which pulmonary B cells can protect against AAI. Here, we demonstrate that pulmonary B cells from OVA-allergic mice which were infected with schistosomes are phenotypically and functionally distinct from splenic Breg cells. They have a reduced cytokine response to Toll-like receptor (TLR) ligands and a reduced Th2 cell priming capacity, which seems to be independent of their antigen-presentation function.

RESULTS

Schistosome-induced pulmonary B cells do not share phenotypical characteristics of classical Breg cells

We first set out to investigate whether a specific pulmonary B cell subset or the expression of specific surface markers linked to Breg cell activity was selectively expanded during schistosome infection compared with uninfected mice. Pulmonary B cells did not contain typical Breg populations that have been described in the spleen, such as CD1d^{hi}CD5^{+}, CD21^{hi}CD23^{lo} MZ or CD1d^{hi}CD21^{hi}CD23^{hi}IgM^{hi} transition type 2 MZ B cells (less than 0.5% of all pulmonary CD19^{+} B cells during infection, data not shown). Therefore, we analyzed several other cell-surface markers as putative markers of Breg cell phenotype and activity, e.g. the membrane-bound marker LAP, as part of a latent TGF-β complex, CD25, Tim-1, CD5 and CD23 [6, 9, 10, 13, 14, 21]. Pulmonary B cells from chronically *S. mansoni*-infected, OVA-allergic (infected/OVA) mice which are known to be protective against AAI [5] had a similar increase in LAP-1^{+} and CD25^{+} cell frequencies over B cells from uninfected, non-allergic mice (uninfected/PBS) as B cells from uninfected, OVA-allergic (uninfected/OVA) mice (**Figure 1A**, **suppl. Figure S1A**), suggesting that TGF-β- or CD25-expressing B cells are not involved in protection against AAI. Although the fold increase in Tim-1^{+} B cell frequencies was significantly higher (**Figure 1A**, **suppl. Figure S1A**) in infected/OVA compared with uninfected/OVA mice, the total percentages of Tim-1^{+} cells remained rather low (less than 7% of all pulmonary CD19^{+} B cells; **suppl. Figure S1A**). CD5 is one of the markers that defines the B-1a subclass of B cells which have been described to have regulatory properties [7]. The fold increase of CD5^{+} B cell frequencies was significantly reduced in infected/OVA compared with uninfected/OVA mice (**Figure 1A**), indicating that this subclass of B cells is probably not important in mediating protection against AAI. The only marker assessed that was clearly enhanced on most pulmonary B cells in infected/OVA mice was CD23 (**Figure 1B**, **suppl. Figure S1B**). As the majority of pulmonary B cells in naïve mice already express CD23, the fold increase in % CD23^{+} cells is only moderate. However, the per cell expression level of CD23 strongly increases on infected/OVA B cells compared with their uninfected/OVA counterparts. The increase in CD23 expression is not surprising as enhanced frequencies of CD23-expressing B cells were already demonstrated for schistosome infections [22] and most probably reflects the general and modified type 2 inflammation present during chronic helminth infection [23]. Furthermore, adoptive transfers of sorted CD23$^{low/intermediate}$ or CD23^{hi} B cells from infected/OVA mice into OVA-sensitized mice remained inconclusive (data not shown), suggesting that the expression level of CD23 on pulmonary B cells does not correlate with suppressive function in our system. Collectively, we found that pulmonary B cells of chronically *S. mansoni*-infected animals do not show enhanced expression of phenotypic markers characteristic to classical Breg cell subsets.

Pulmonary B cell-induced protection against AAI during schistosomiasis is independent of FoxP3^{+} Treg cells

We previously described that pulmonary B cells transfer protection to OVA-sensitized mice in an IL-10-independent manner [24]. We therefore set out to study the involvement of alternative inhibitory mechanisms. One of the major effector functions of murine Breg cells centers around the induction and/or recruitment of FoxP3^{+} Treg cells. In our previous studies, we observed that adoptive transfer of pulmonary B cells did not induce increased numbers of FoxP3^{+} Treg cells *in vitro* nor *in vivo* [5]. However, this does not exclude the possibility that, despite equal numbers, the activity of Treg cells on a per cell basis had increased. We therefore transferred pulmonary B cells to FoxP3-DTR transgenic DEREG mice, in which Treg cells can be temporarily depleted by DT injections (**Figure 2A**), to investigate the contribution of Treg cell activity during pulmonary B cell-induced protection against AAI. BAL eosinophil numbers remained equally reduced in both PBS- and DT-treated DEREG mice when receiving pulmonary B cells from infected/OVA compared with uninfected/OVA mice (**Figure 2B**). We also did

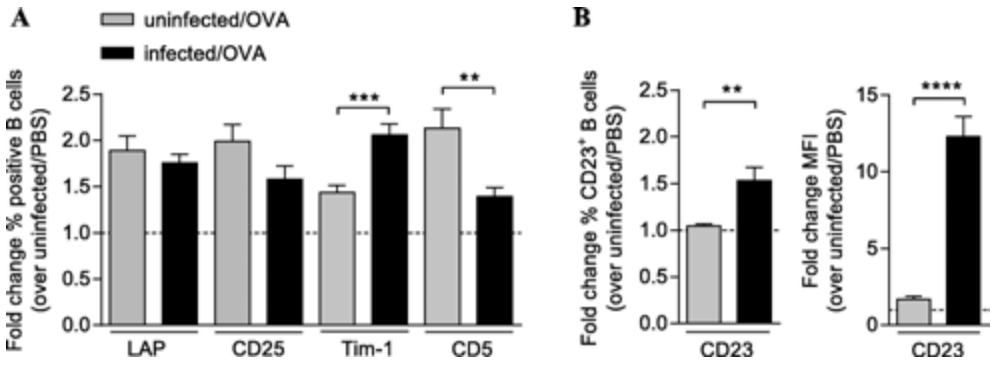


Figure 1. Schistosome-induced pulmonary B cells do not share phenotypical characteristics of classical regulatory B (Breg) cells. Mice were chronically (15 weeks) infected with 36-40 *Schistosoma mansoni* cercariae. Allergic sensitization was induced by two i.p. injections of ovalbumin (OVA)/alum 1 week apart and control mice received PBS. Seven days after the last injection, mice were challenged by OVA aerosol exposure on three consecutive days. Mice were sacrificed 24 h after the last challenge. The perfused lungs were minced, digested and the single cell suspension from 2-3 mice pooled. Next, B cells were purified using anti-CD19 MicroBeads and stained for different Breg cell-associated markers. (**A**) The fold change of percentage surface latency-associated peptide (LAP-), CD25-, T cell immunoglobulin and mucin domain-1 (Tim-1)- and CD5-expressing B cells from uninfected- and infected/OVA mice over control (uninfected/PBS) are shown, representing a summary of 2-4 independent experiments (n=11-17/group). (**B**) Fold change of percentage and geometric mean fluorescence intensity (MFI) of CD23 expression over control (uninfected/PBS). Summary of four independent experiments (n=14-17/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: $^{**}P$ <0.01, $^{***}P$ <0.001, $^{****}P$ <0.0001, as tested by two-tailed unpaired Student's t-test.

not observe an enhanced induction of Treg cells by infected/OVA pulmonary B cells in *in vitro* co-cultures with naïve T cells compared with co-cultures with B cells from allergic control mice (data not shown). These data indicate that AAI is not restored when Treg cell activity is abolished, and suggests that schistosome-induced pulmonary B cells do not drive protection against AAI via enhanced Treg cell activity.

Schistosome-induced pulmonary B cells express elevated levels of CD86 *ex vivo*, and secrete less IL-10 after *in vitro* TLR ligation

T cell-derived Th2 cytokines play a dominant role in the induction and maintenance of AAI [25]. We therefore aimed to investigate the role of pulmonary B cells as APCs and as modulators of effector T cell activation. Important signals that can influence T cell activation/induction of apoptosis, proliferation and cytokine production are, for example, provided by the co-stimulatory molecule CD86, antigen-presentation molecule MHCII, inhibitory receptors such as PD-L1, PD-L2, FasL or various cytokines such as IL-10 and IL-6. To investigate a putative role for those (co-)stimulatory molecules and/or inhibitory receptors on schistosome-induced pulmonary B cells, we first analyzed the expression of the above-mentioned molecules. Pulmonary B cells from infected/OVA mice showed a significantly increased CD86 expression compared with B cells from uninfected/OVA mice. Expression levels of MHCII, PD-L1 and PD-L2 were equal between the groups (**Figure 3A**, **suppl. Figure S2**). FasL expression was not induced on pulmonary B cells in response to AAI or *S. mansoni* infection (data not shown). Furthermore, we analyzed the capacity of pulmonary B cells to produce cytokines, which may support or suppress T cell activation, following stimulation by either SEA or TLR-4 ligand LPS and TLR-9 ligand CpG-ODN 1826 as strong B-cell activators. While B cells from infected/OVA mice produced more

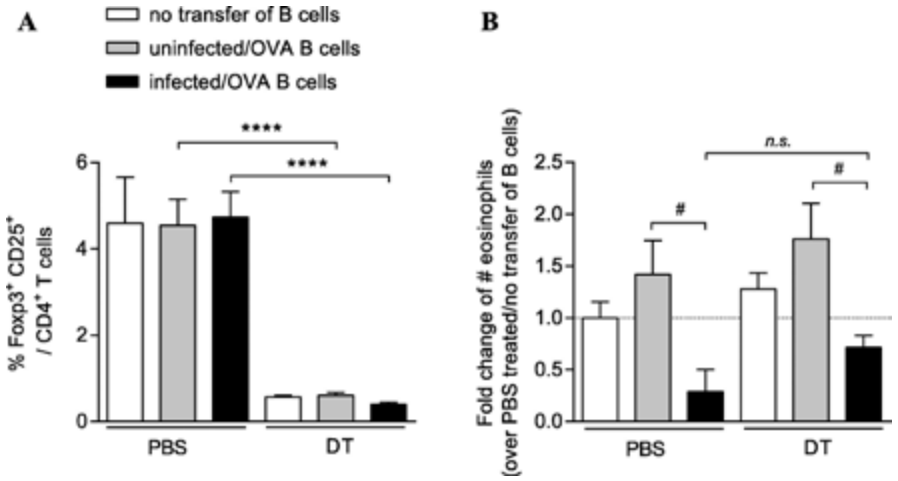


Figure 2. Pulmonary B cell-induced protection against allergic airway inflammation (AAI) during schistosomiasis is independent of Foxp3⁺ regulatory T (Treg) cells. Ovalbumin (OVA)-sensitized DEREG (DEpletion of REGulatory T cells) mice, which carry a diphtheria toxin (DT) receptor-eGFP transgene under the control of an additional Foxp3 promoter, were treated with two PBS or DT (1 µg/mouse) i.p. injections in order to deplete the Foxp3⁺ Treg cells, 1 day before and 2 days after the adoptive transfer of 5 x 10^6 CD19⁺ lung B cells. After 2 days, mice were challenged for three consecutive days and sacrificed 24 h after the last challenge. (**A**) The percentage of Foxp3⁺ CD25⁺ Treg cells in the lungs are shown, representing a summary of 1-2 independent experiments (*n*=2-8/group). (**B**) Fold change of bronchoalveolar lavage (BAL) eosinophil numbers over PBS-injected DEREG mice that did not receive B cell adoptive transfer. A summary of two independent experiments (*n*=3-7/group) is shown. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: ****P <0.0001, as tested by two-tailed unpaired Student's *t*-test (A) or #P <0.05 as tested by One-way ANOVA following Tukey's multiple comparisons test (B). n.s., not significant.

IL-10 in response to SEA as previously described [5], they interestingly secreted significantly less IL-10 and IL-6 in response to LPS or CpG-ODN 1826 compared with B cells from uninfected/OVA mice (**Figure 3B**). Recently, B cells have been reported to secrete the regulatory cytokine IL-35 in response to TLR4 and CD40 ligation [15]. Pulmonary B cells in this study were MACS-sorted for CD19, but they nevertheless contained CD138-expressing plasma cells (**suppl. Figures S3A, B**), which downregulate CD19 expression and have been described as the main IL-35 expressing B cells. We measured a clear gene expression of the IL-35 subunits Ebi3 and IL-12p35 by qPCR after stimulation of pulmonary B cells with LPS/anti-CD40 or CpG-ODN 1826 (Ct-values: 22-25 for *Ebi3* and *IL-12p35* after LPS/aCD40 and *IL-12p35* after CpG-ODN 1826 stimulation; 27-29 for *Ebi3* after CpG-ODN 1826 stimulation), stimuli that were reported to optimally enhance the expression of the separate IL-35 subunits. However, we did not observe an increased expression of either IL-35 subunit by infected/OVA compared with uninfected/OVA pulmonary B cells (**suppl. Figure S3C**), suggesting that it is not likely that IL-35 mediates protection in our model. Taken together, these data show that during chronic schistosomiasis pulmonary B cells have an increased CD86 expression, equal expression levels of MHCII, lower IL-10 and IL-6 production upon TLR ligation and no increase in gene expression of the subunits for IL-35, suggesting that pulmonary B cells do not suppress Th2 cytokine production by an increased expression of those inhibitory molecules or regulatory cytokines.

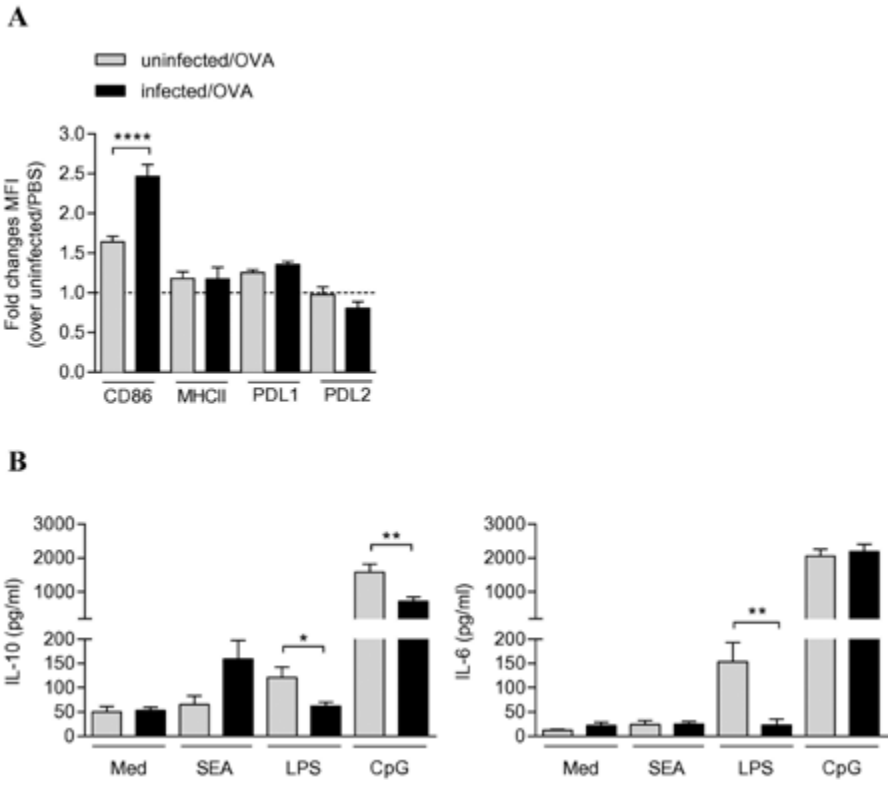


Figure 3. Schistosome-induced pulmonary B cells express elevated levels of CD86 *ex vivo,* and secrete less IL-10 after *in vitro* Toll-like receptor (TLR) ligation. Pulmonary B cells were isolated as described in **Figure 1**. For both phenotypical characterization and co-culture, pulmonary B cells from 2-3 mice were pooled to obtain sufficient cell numbers. (**A**) Fold changes of geometric mean fluorescence intensity (MFI) expression of indicated antigens on B cells from uninfected/ovalbumin (OVA) and infected/OVA mice over control (uninfected/PBS). A summary of 2-4 independent experiments (*n*=6-17/group) is shown. (**B**) Uninfected/OVA and infected/OVA B cells were stimulated with supplemented RPMI medium (Med), schistosomal egg antigen (SEA, 20 µg/ml), lipopolysaccharide (LPS, 100 ng/ml) or CpG-ODN 1826 (5 µg/ml) for 5 days to determine the presence of IL-10 and IL-6 in the supernatant. Data represents a summary of 2-4 independent experiments (*n*=4-12/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: **P* <0.05, ***P* <0.01, *****P* <0.0001, as tested by two-tailed unpaired Student's *t*-test.

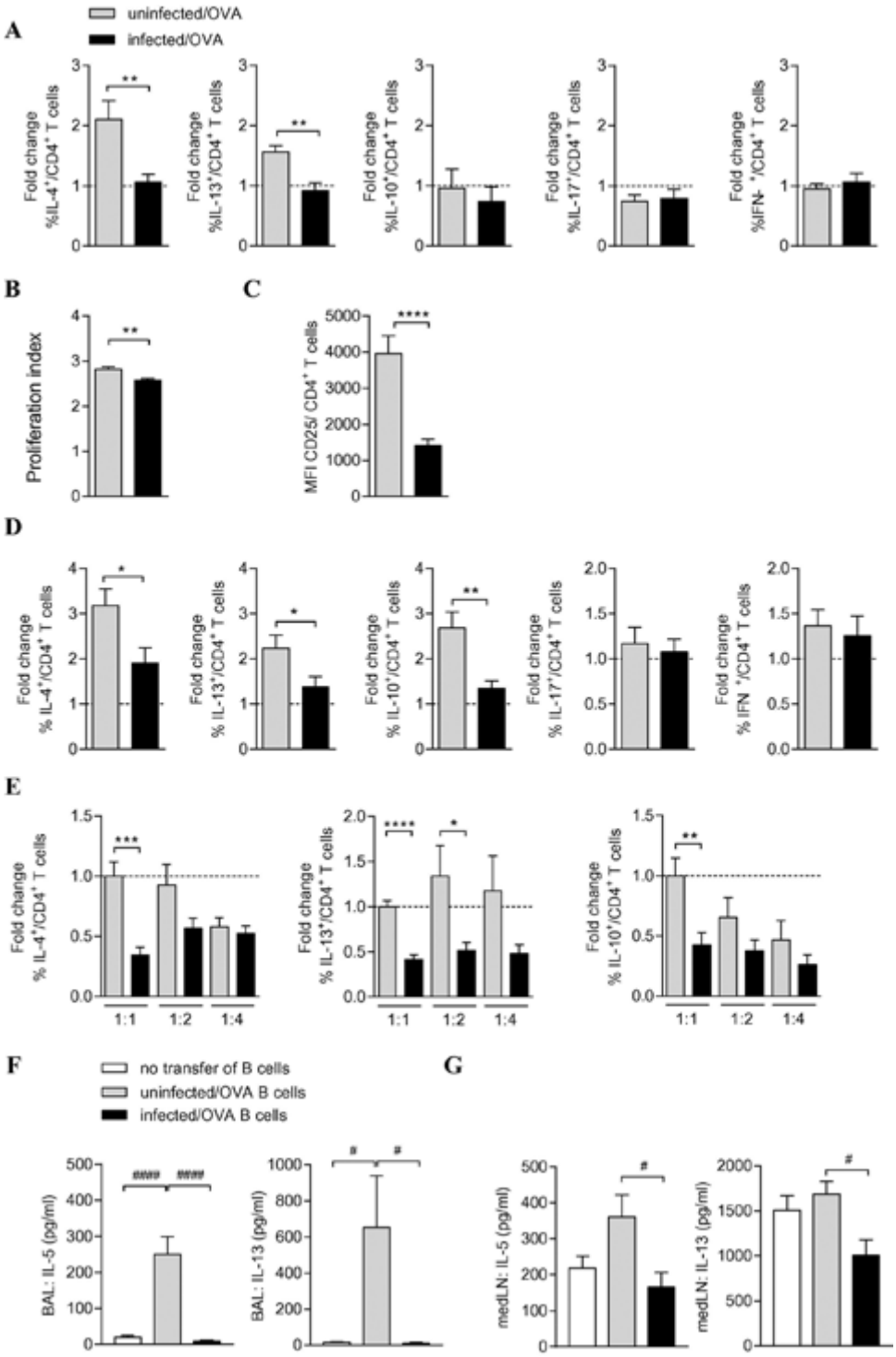
Schistosome-induced pulmonary B cells induce less Th2 cytokine secretion *in vitro*

Because schistosome-induced pulmonary B cells did not show the typical regulatory features of Breg cells such as Treg cell induction or increased production of regulatory cytokines, we next investigated their potential to reduce AAI through a reduced APC function that would lead to less T cell proliferation and/or less T cell cytokines. To this end, T-cell activation was examined under a condition where antigen-presentation by B cells was essential to drive T-cell activation. OVA-specific T-cell activation of $CD4^+$ T cells from OT-II mice was achieved by culture with OVA peptide-pulsed pulmonary B cells. After 3 days, OVA-presentation by B cells from uninfected- or infected/OVA mice did not affect intracellular expression of T-cell IL-4, IL-13 or IL-17 production, whereas IFN-γ seemed to be slightly up- and IL-10 downregulated (**Figure 4A**, **suppl. Figure S4A**). We also cultured OVA-pulsed B cells and OT-II T cells in the presence of anti-CD28 to bypass differences in CD80 and CD86, and ensure optimal

Figure 4. Schistosome-induced pulmonary B cells have an impaired capacity to induce Th2 cytokine secretion *in vitro*. Pulmonary B cells were isolated as described in **Figure 1**. Pulmonary B cells, pooled from 2-3 mice per group were co-cultured at a 1:1 ratio with either naïve, ovalbumin (OVA)-peptide loaded OT-II CD4+ T cells (**A**) or naïve C57BL/6 CD4+ T cells (**B-E**). (**A**) B cells were loaded with OVA_{17} peptide, washed and co-cultured at a 1:1 ratio with OVA-specific $CD4^+$ OTII T cells in the presence of anti-CD28 (1 µg/ml) for 3 days. Intracellular cytokine staining for the Th2 cytokines IL-13, IL-4 and IL-10, Th1 cytokine IFN-γ and Th17 cytokine IL-17 cells was performed following phorbol 12-myristate 13-acetate (PMA)/Ionomycin and Brefeldin A stimulation for the last 4 h. Data are expressed as fold change over co-culture with control B cells (uninfected/PBS). A summary of 2-3 independent experiments (*n*=6-12/group) is shown. Results are expressed as mean ± S.E.M. (**B-E**) B cells were co-cultured at a 1:1 ratio with naïve, CFSE-stained (0.5 uM CFSE, 15 min) C57BL/6 CD4+ T cells in the presence of anti-CD3/anti-CD28 (both (1 µg/ml) for 3 days. (**B**) The proliferation index was calculated within FlowJo. A summary of three independent experiments (*n*=15-20/group) is shown. (**C**) The mean fluorescence intensity (MFI) of the activation marker CD25 on T cells assessed by flow cytometry. (**D**) Fold changes of percentage IL-4, IL-13, IL-10, IL-17 and IFN-γ-producing T cells over control (B cells from uninfected/PBS mice). A summary of four independent experiments (*n*=17/group) is shown. (**E**) Naïve C57BL/6 CD4+ T cells ($1x10^5$) were cultured at ratios of 1:1, 1:2 and 1:4 (B cell:T cell) with B cells from uninfected/OVA and infected/OVA mice. Bar graphs represent fold changes of percentage Th2 cytokines production (with uninfected/OVA 1:1 ratio set to 1). A summary of two independent experiments (*n*=6-7/group) is shown. (**F**, **G**) IL-5 and IL-13 measured by ELISA in supernatants of bronchoalveolar lavage (BAL) fluid (**F**) and OVA-restimulated mediastinal lymph node (medLN) cells (**G**) from recipient mice after adoptive transfer of either uninfected/OVA or infected/OVA B cells. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: $*P$ <0.05, $**P$ <0.01, $***P$ <0.001, $****P$ <0.0001, as tested by two-tailed unpaired Student's *t*-test. $^{\#}P$ <0.05, $^{\#\#\#\#}P$ <0.0001, as tested by One-way ANOVA and Tukey's multiple comparisons test.

co-stimulation. In the presence of anti-CD28, the fold change expression of the Th2 cytokines IL-4 and IL-13 was significantly lower in co-cultures with infected/OVA B cells compared with co-cultures with B cells from uninfected/OVA mice (**Figure 4A**, **suppl. Figure S4A**). The reduced IL-10 production found under stimulation conditions of infected/OVA B cell co-cultures was overcome in the presence of sufficient co-stimulation and may point to an impaired expression during sub-optimal stimulation.

Additionally, we performed co-culture experiments of pulmonary B cells from uninfected/OVA and infected/OVA mice with $CD4^+$ T cells from naive C57BL/6 mice in the presence of anti-CD3/28 to bypass the need for B cell antigen presentation and co-stimulation for T cell activation, and cultured them for 3 days. We still observed higher Th2 cytokine production in co-cultures with B cells from uninfected/OVA mice (**Figure 4D**, **suppl. Figure S4B**), together with slightly more T-cell proliferation (**Figure 4B**) and an enhanced T-cell CD25 expression (**Figure 4C**) compared with co-cultures with infected/OVA B cells. The ability of B cells from uninfected/OVA mice to induce Th2 cytokines was found to be dose-dependent and most effective at a 1:1 ratio (**Figure 4E**), while the percentage of Th2 cytokine-producing T cells induced by infected/OVA B cells was similarly low at all indicated ratios. To evaluate whether B cells could also inhibit Th2 cytokines *in vivo*, we turned to our previous studies, where adoptive transfer of pulmonary B cells from *S. mansoni*-infected, but not uninfected, mice protected recipient mice from AAI[24]. We now analyzed the abundance of Th2 cytokines in both the BAL fluid and mediastinal LN cell cultures of recipient mice after OVA restimulation. While the transfer of uninfected/OVA B cells strongly increased the concentration of Th2 cytokines in the BAL fluid compared with control mice (**Figure 4F**), we found both IL-5 and IL-13 to be significantly reduced in both tissues after transfer of infected/OVA B cells compared with cells transferred from uninfected mice (**Figure 4F, G**). Collectively, we found a lesser induction of Th2 cell proliferation and cytokine production by infected/OVA B cells, which is also evident in a setting independent of B cell antigen-presentation and with optimal co-stimulation. These findings suggest that schistosome-induced pulmonary B cells may not dampen CD4 T cell responses via a reduced APC function, but that this may be the result of both reduced Th2-driving signals and alternative inhibitory factors.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The impaired capacity of schistosome-induced pulmonary B cells to induce Th2 cytokine secretion is largely independent of IL-10 and TGF-β

We next aimed to investigate what other suppressive factors were involved in the reduced Th2 cell driving capacity of schistosome-induced pulmonary B cells. In that context, the roles of IL-10 and TGF-β were investigated by adding anti-IL-10R or anti-TGFβ blocking antibodies to the co-cultures of pulmonary B cells and $CD4^+$ T cells. We focused on the production of IL-4 and IL-13 due to the activity of these Th2 cytokines in boosting allergic responses in the airways. Blocking IL-10 signaling or neutralizing TGF-β only slightly, but significantly, increased the IL-4 production in T cells cultured with pulmonary B cells from infected/OVA mice (**Figure 5**). IL-13 production was only increased upon blocking IL-10 signaling (**Figure 5**). This may suggest that of the two inhibitory cytokines, the influence of IL-10 seemed to be slightly more pronounced. Nevertheless, infected/OVA B cells still induce significantly less IL-4 and IL-13 in co-cultures where IL-10 signaling is ablated or TGF-β neutralized, suggesting that either one of these cytokines, if at all, only play a minor role.

DISCUSSION

Helminths drive strong immunoregulatory processes that limit immunopathology during chronic infection, and Breg cells seem to be important players. Notably, *S. mansoni*-induced splenic and pulmonary B cells also attenuate allergic diseases such as AAI upon adoptive transfer. Splenic Breg cells mediate their suppressive effect through IL-10- and Treg cell-dependent mechanisms [4, 5, 8]. Here, we demonstrate that helminth-induced pulmonary B cells are phenotypically and functionally distinct from splenic Breg cells that we previously studied [5], by showing a reduced capacity to initiate Th2 cytokine responses. The question remains whether this is truly the result of an increased inhibitory, or simply a reduced Th2 stimulatory, activity.

By definition, Breg cells suppress inflammatory processes and induce tolerance by various mechanisms of which production of immunosuppressive IL-10 is the most widely studied. While IL-10

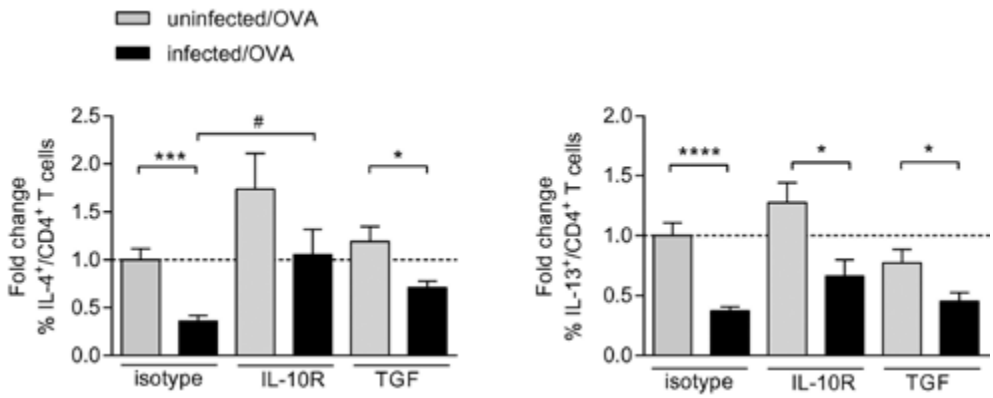

Figure 5. The impaired capacity of schistosome-induced pulmonary B cells to induce Th2 cytokines is independent of IL-10 and TGF-β. *In vitro* co-cultures were performed as described in **Figure 4** in the presence of blocking anti-IL-10R, anti-TGF-β or isotype control antibodies. Bar graphs represent fold changes of IL-4 and IL-13 (with uninfected/ovalbumin (OVA) isotype set to 1). A summary of two independent experiments (n=6-8/group) is shown. Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: *P <0.05, ***P <0.001, ****P <0.0001, as tested by two-tailed unpaired Student's t-test, and #P <0.05 as tested by One-way ANOVA following Dunnett's multiple comparisons test.

has a pleiotropic suppressive effect on most hematopoietic cells such as T cells and APCs, it also indirectly suppresses immune responses via supporting the generation and maintenance of Treg cell subsets [4, 5, 8]. We adoptively transferred pulmonary B cells from uninfected/OVA and infected/OVA mice into DEREG recipient mice and did not observe a significant change in the protective effect of B cells from infected mice in the absence of Treg cells (**Figure 2**). We have not formally addressed where these adoptively transferred B cells migrate to in recipient mice. However, we have previously examined that i.v. injected splenic B cells migrate to the spleen and lung of allergic mice, and assume that the pulmonary B cells studied here also migrate, at least in part, to the lung (unpublished findings). Nevertheless, we cannot exclude that systemic signals have also contributed to the observed protective effect.

Recently, it has become evident that Breg cells utilize a number of IL-10-independent suppressive mechanisms in order to control inflammation [26]. For example, B cells can contribute to the maintenance of tolerance via the production of TGF-β [13, 14], IL-35 [15, 16] or by induction of T cell hypo-responsiveness [14]. The schistosome-induced pulmonary B cells studied here did not utilize any of the Breg effector mechanisms described in the above-mentioned studies. In our study, the majority of the B cells in the lungs of infected/OVA mice had elevated levels of CD23 (**Figure 1B**) compared with uninfected/OVA mice. Interestingly, mesenteric LN $CD23^{hi}$ B cells from *H. polygyrus*-infected mice were shown to suppress Der p1-induced airway inflammation by an unknown mechanism, but independently of IL-10 [6]. Although it was unclear whether this suppression was accomplished through CD23, it may point to similarities with the pulmonary B cells in the infected/OVA mice described here. As several adoptive transfer experiments with sorted pulmonary CD23+ B cells, compared with CD23low/int B cells from OVA/infected mice, did not show clear differences in their protection against AAI, the elevated expression of CD23 may merely serve as a proxy for a certain inhibitory phenotype without being actively involved. Alternatively, CD23 expression may not define a specific Breg population but may be more the consequence of the local cytokine milieu [27], as IL-4 and IgE drive CD23 expression [28, 29] on B cells during helminth infections [30]. Indeed, the CD23 expression seemed to be elevated on almost all pulmonary B cells of infected/OVA mice. Interestingly, we found elevated concentrations of total IgG1 and IgG2a in infected/OVA compared with uninfected/OVA mice (data not shown). Earlier studies have suggested an inhibitory role for IgG1 ligating the inhibitory FcγRIIB in OVA-induced AAI [31]. However, adoptive transfers of pulmonary B cells from uninfected- and infected/OVA mice into OVA-sensitized $FcγRIIB^{-/-}$ mice suggested that pulmonary B cell-induced protection against AAI was independent of FcγRIIB ligation (data not shown).

Schistosome-induced pulmonary B cells expressed enhanced levels of CD86, whereas MHC Class II expression levels remained unchanged and in some experiments were even enhanced. Each of these markers has been suggested to affect T cell activation. For example, down-regulation of B cell CD80 and CD86 expression during *Brugia pahangi* larval infection restricted T cell proliferation [32]. Using B cell $B7^{-/-}$ mice, expression of CD86 was shown to be essential for B cell-mediated recovery of EAE by induction of Treg cells and IL-10 [33]. In our study, co-cultures of schistosome-induced pulmonary B cells presenting OVA to OVA-specific T cells resulted in reduced Th2 cytokine production compared with control conditions. However, since similar results were observed in co-cultures of B cells from C57BL/6 mice supplemented with anti-CD3/28 to bypass the role of B cells as APCs, this may suggest the involvement of other molecules or mechanisms.

We additionally examined different inhibitory molecules for their contribution to the protective effect observed. Studies in mice with *S. mansoni* or *Litomosoides sigmodontis* infections showed that PD-1 and interaction with its ligands (PDL-1/2) was important for T(h2)-cell hypo-responsiveness [34, 35], and PD-1-PD-L1/2 interactions have also been reported to be involved in regulating autoimmune and allergic Th2 responses [36, 37]. Other studies however suggested that Th2 hypo-responsiveness during

S. mansoni infection was not related to PD-1-PD-L1/2 interaction [38]. The latter is in agreement with our own data, as we observed that blocking of PD-L1 or PD-L2 expression on infected/OVA B cells did not restore Th2 cytokine production or proliferation in co-cultures with T cells (data not shown). We also investigated a potential role for the enhanced expression of CD86 on the pulmonary B cells from infected/OVA mice. Human $CD25^{hi}$ Breg cells have been reported to increase the expression of the inhibitory receptor CTLA-4 on $FoxP3^{+}$ Treg cells *in vitro* [11], suggesting that the interaction of B cell co-stimulatory molecules CD80/CD86 and CTLA-4 could be important in controlling inflammation. However, blocking of CTLA-4 in *in vitro* co-cultures did not restore Th2 cytokine production in our study (data not shown). Collectively, the pulmonary B cells studied are phenotypically and functionally different from classical Breg cells, and rather show a reduced Th2-driving capacity. It remains to be established whether this is the result of a reduced capacity to drive Th2 stimulation, or rather a suppressive signal by which schistosome-induced pulmonary B cells actively reduce Th2 polarization and inhibit AAI.

Alternatively, local Treg cells, induced during helminth infection, may influence B cell function leading to reduced B-cell activation, antibody production and the APC function of B cells via e.g. TGF-β or IL-35 [39-41]. Secreted helminth products from *Schistosoma* may also directly attenuate the T cell stimulatory capacity of B cells as described for DCs [42]. As pulmonary B cells from infected/OVA mice produced less cytokines after stimulation with TLR ligands (**suppl. Figure S3**), the B cells might become hypo-responsive themselves in the context of chronic *S. mansoni* infection, resulting in a reduced capacity to stimulate T cells. In addition to their role as APCs and cytokine producers, B cells have also been reported to remodel the LN architecture to facilitate DC-dependent Th2 induction and be required for the formation of a Tfh cell response in the context of nematode infections [43, 44].

Very little is known with regard to B cell migration to the respiratory system, and the permanence of their residence in the tissue, in contrast to other mucosal sites such as the gut or the peritoneal cavity. The lung B cell population in chronically *S. mansoni*-infected mice that are subjected to AAI is likely to be heterogeneous, consisting both of resident and infiltrating cells. Further studies are needed to examine the origin and migration pattern of the protective pulmonary B cells in *S. mansoni* infection.

Recently, various Breg cell populations have been identified in peripheral blood of humans infected with schistosomiasis or other helminths [5, 45]. Furthermore, in several inflammatory diseases, Breg cells were impaired in terms of their number and/or their regulatory function, i.e. in patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) or allergic asthma [46-48]. Since most human studies are restricted to peripheral blood B cells, these results might not fully reflect the processes that occur in inflamed organs. Therefore, further studies on B cell biology and its activity in the inflamed organs are needed to better understand the importance and relative contribution of the various Breg cell subsets in peripheral blood and local tissues. Collectively, we identified that murine infection with schistosomes induces potent IL-10-producing suppressive Breg cells in the spleen [5] and impaired Th2-driving pulmonary B cells in the inflamed tissue (this study) which both contribute to the suppression of AAI. Identifying the mechanisms that influence pulmonary B cell function and impair their capacity to induce Th2 cytokines may be an interesting novel strategy to prevent or control allergic inflammatory responses.

MATERIAL AND METHODS

Animals

Six week-old female C57BL/6 OlaHsd mice were purchased from Harlan Sprague Dawley Inc. (USA). DEREG (depletion of regulatory T cells) mice [49] were kindly provided by Dr. T. Sparwasser (Twincore/ Centre for Experimental and Clinical Infection Research, Germany) and bred in the animal facilities of the Leiden University Medical Center (LUMC) Leiden, The Netherlands. Mice were housed under

specific-pathogen-free (SPF) conditions in the animal facilities of the LUMC. All animal studies were performed in accordance with the guidelines and protocols (DEC-11166, 12182) approved by the Ethics Committee for Animal Experimentation of the University of Leiden, The Netherlands.

Parasitic infection and AAI induction

Mice were infected percutaneously with 36-40 *S. mansoni* cercariae and kept until the chronic phase of infection (15 weeks). For AAI induction, mice were sensitized twice by i.p. injections of OVA (10 µg/mL, Worthington Biochemical Corp, USA) in Imject Alum (2 mg/ml; Pierce, USA) at weeks 13 and 14. Seven days after the last injection, mice received OVA aerosol challenges (10 mg/ml in PBS) for three consecutive days. Mice were sacrificed 24 h after the last challenge. Bronchoalveolar lavage (BAL) fluids were collected and phenotyped by flow cytometry [5, 20].

Cell purification

Perfused lungs were minced to ~1 mm pieces and digested by collagenase III (Worthington) and DNase (Sigma-Aldrich, USA) for 1 h. The digested lungs were sequentially dispersed through 70 µm sieves. Erythrocytes were removed from the lung single cell suspensions by lysis. Adhesive cells were removed from cell suspensions by passage over LS columns (Miltenyi Biotec, Germany). Next, B cells were purified using anti-CD19 MicroBeads (Miltenyi Biotec). Untouched splenic $CD4^+$ T cells were enriched using negative selection with MicroBeads (Miltenyi Biotec) and were ~95% pure.

Adoptive transfer of isolated pulmonary B cells

Recipient mice were sensitized with two injections of OVA/Alum at day 0 and day 7, as described in Section 2.2. Ten days after the last injection, the OVA-sensitized animals received an i.v. injection of 5 x 10^6 $CD19^+$ lung B cells from uninfected- or infected/OVA mice, or PBS as a control. DEREG mice were treated with two diphtheria toxin (DT, 1 µg/ml) i.p. injections or PBS as a control, 1 day before and 2 days after the adoptive transfer of B cells in order to deplete the $FoxP3^+$ Treg cells. After 2 days, mice were challenged for three consecutive days and sacrificed 24 h after the last challenge.

Phenotypic characterization

Ex vivo pulmonary B cells were characterized using CD25-FITC (clone: 3C7; BD Biosciences), Tim-1-PE (RMT1-4), B220-V510 (RA3-6B2), PD-L1-Pe-Cy7 (10F.9G2; all Biolegend, USA), B220-APC-eF780 (RA3-6B2), B220 eF450 (RA3-6B2), LAP-1-PerCP-eFluor710 (TW7-16B4), CD5-APC (53-7.3), CD23-PeCy7 (B3B4), CD86-PE-Cy5 (GL1), FasL-APC (MFL3), major histocompatibility complex Class II (MHCII)-APC-eF780 (M5/114.15.2), PD-L2 Biotin (TY25; all eBioscience, USA) combined with streptavidin-Qdot525 (Life Technologies, USA) and LIVE/DEAD fixable Violet or Aqua stain (eBioscience). For all flow cytometric measurements, FcγR-binding inhibitor (2.4G2) was added and fluorescence minus one (FMO) controls were used for gate setting for all surface markers and cytokines. All antibody stainings were performed as surface stainings for 30 min at 4ºC.

Cytometric bead array

Cytokines were measured in BAL fluid and in cell culture supernatant of mesenteric lymph node (LN) cells (0.3x10^6 cells) restimulated for 3 days with 10 µg/mL of OVA using BD cytometric bead array Flex-set kits (BD Biosciences, USA).

***In vitro* B cell stimulation**

Pulmonary $CD19^+$ B cells and B cell subsets ($1x10^5$ cells) were cultured in medium (RPMI 1640 glutamax; Invitrogen Life Technologies, USA), containing 5% heat-inactivated FBS (Greiner Bio-One, Austria), 5×10^{-5} M 2-Mercaptoethanol (Sigma-Aldrich) and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin; Invitrogen). Cells were cultured either in the presence of schistosomal egg antigen (SEA; 20 µg/ml), lipopolysaccharide (LPS; 100 ng/ml) or CpG-oligodeoxynucleotides (CpG-ODN) 1826 (5 µg/ml) for 5 days for the detection of IL-10 and IL-6 in culture supernatants by ELISA (BD Biosciences), or in the presence of LPS (1 µg/ml) and anti-CD40 (clone 1C10; 10 µg/ml) or CpG-ODN 1826 (1 µg/mL) for the detection of IL-35 mRNA expression.

Immunoglobulin measurements

Total and OVA-specific IgG1, IgG2a and total IgA were measured from the first 1 ml of collected BAL fluid by ELISA (BD Biosciences).

***In vitro* B cell stimulation and co-culture with $CD4^+$ T cells**

Pulmonary $CD19^+$ B ($1x10^6$/ml) cells were loaded with 10 µg/ml of OVA_{17} peptide ($OVA_{323-339}$: ISQAVHAAHAEINEAGR, kindly provided by M.G.M. Camps (Leiden University Medical Center, The Netherlands) for 1 h at 37 ° C, washed, and subsequently co-cultured with OT-II $CD4^+$ T cells ($1x10^5$ cells/well) at a 1:1 ratio in the presence or absence of anti-CD28 (1 µg/ml). Additionally, $CD19^+$ B cells were co-cultured with $CD4^+$ T cells at 1:1, 1:2, 1:4 ratios, in the presence of medium or anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml). To assess proliferation, T cells (10×10^6/ml) were incubated with carboxy- fluorescein diacetate, succinimidyl ester (CFSE; 0.5 µM) for 15 min. Under some conditions, the following blocking antibodies were added to the cultures: 10 µg/ml of isotype control anti-βGal and anti-TGF-β (kindly provided by L. Boon, Bioceros, The Netherlands). T cells were incubated for 30 min at 37 °C with 10 µg/ml of anti-IL-10 receptor (kindly provided by L. Boon). After 3 days, CFSE-labelled T cell co-cultures were stained with anti-CD3-eFluor450 (17A2), CD25-PE (PC61.5), B220-eFluor780 (RA3-6B2), 7-AAD, CD4-biotin (GK1.5) (all eBioscience) with streptavidin-Qdot525 to measure proliferation of activated T cells. The proliferation index, being the total number of cell divisions divided by the number of cells that went into division, was calculated within the FlowJo proliferation platform. For cytokine analysis, the cells were restimulated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 µg/ml of ionomycin for 6 h in the presence of 10 µg/ml of Brefeldin A (all Sigma-Aldrich) for the last 4 h, followed by fixation using 1.9% paraformaldehyde (PFA; Sigma-Aldrich). Next, the cells were stained for viability (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, ThermoFisher Scientific, USA) IL-4-PE (BVD4-1D11; BD Biosciences), CD3-eFluor710 (17A2), IFN-γ-FITC (XMG1.2), IL-17-PeCy7 (eBio17B7), IL-10-APC (JES5-16E3), IL-13-eFluor450 (eBio13A), and B220-eFluor780 (RA3-6B2; all eBioscience).

Quantitative PCR (qPCR)

Isolated pulmonary B cells were snap-frozen in liquid nitrogen, and RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. cDNA was synthetized and quantitative real-time PCR performed on a C1000 Thermal Cycler (BioRad, USA) using GoTaq qPCR MasterMix (Promega, USA). Transcripts were quantified using the following forward (FP) and reverse (RP) primers (Eurogentec, Belgium): β-2-microglobulin FP: 5'-CACTGAATTCACCCCCACTGA -3', β-2-microglobulin RP: 5'-TGTCTCGATCCCAGTAGACGG-3'; Ebi3 FP: 5'-CATTGCCACTTACAGGCTCG-3', Ebi3 RP: 5'-TGATGATTCGCTCAGCCACA-3'; IL-12p35 FP: 5'-GGTGAAGACGGCCAGAGAAA-3', IL-12p35 RP: 5'-GTAGCCAGGCAACTCTCGTT-3'. mRNA expression was normalized to the reference gene β-2-microglobulin, and expressed as the fold change to B cells from uninfected, non-allergic mice by using the ΔΔ comparative threshold (ΔΔCt) method.

Statistical analysis

All results were analyzed using GraphPad Prism (version 5.00/6.05 for Windows, GraphPad Software, La Jolla USA) and are expressed as mean ± S.E.M. Statistical analysis was performed using the two-tailed unpaired or paired Student's *t*-test for comparison of two experimental groups, and One-Way ANOVA followed by Tukey's multiple comparisons test for comparisons between more than two groups. Differences between groups were considered significant at $P < 0.05$.

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SUPPLEMENTARY MATERIAL

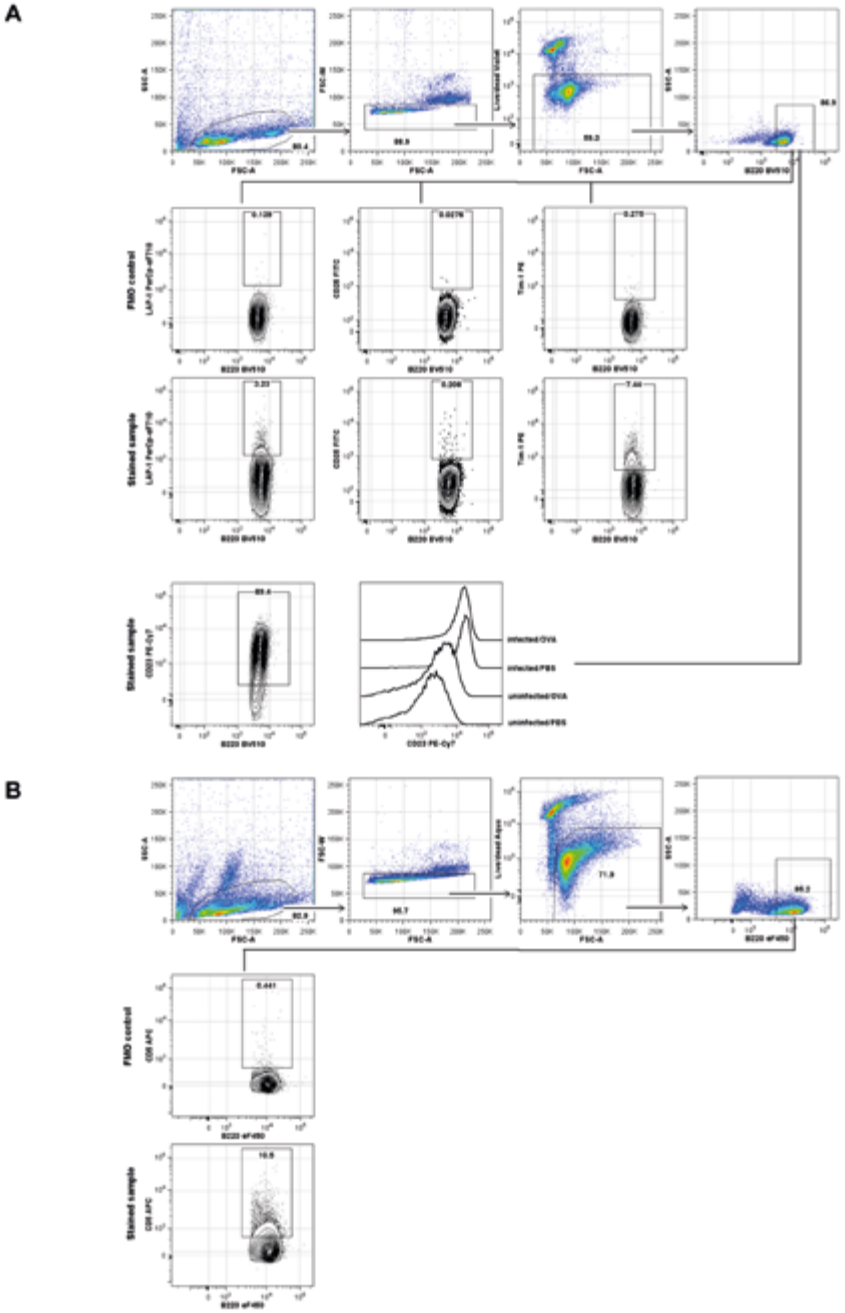

Figure S1. Gating strategy and representative flow cytometry plots (relating to **Figure 1**). (**A**) Percentage of latency-associated peptide (LAP$^+$), CD25$^+$, T cell immunoglobulin and mucin domain-1 (Tim-1$^+$) and CD23$^+$ pulmonary B cells, as well as mean fluorescence intensity (MFI) of CD23. (**B**) Percentage of CD5$^+$ pulmonary B cells. OVA, ovalbumin; FMO, fluorescence minus one control.

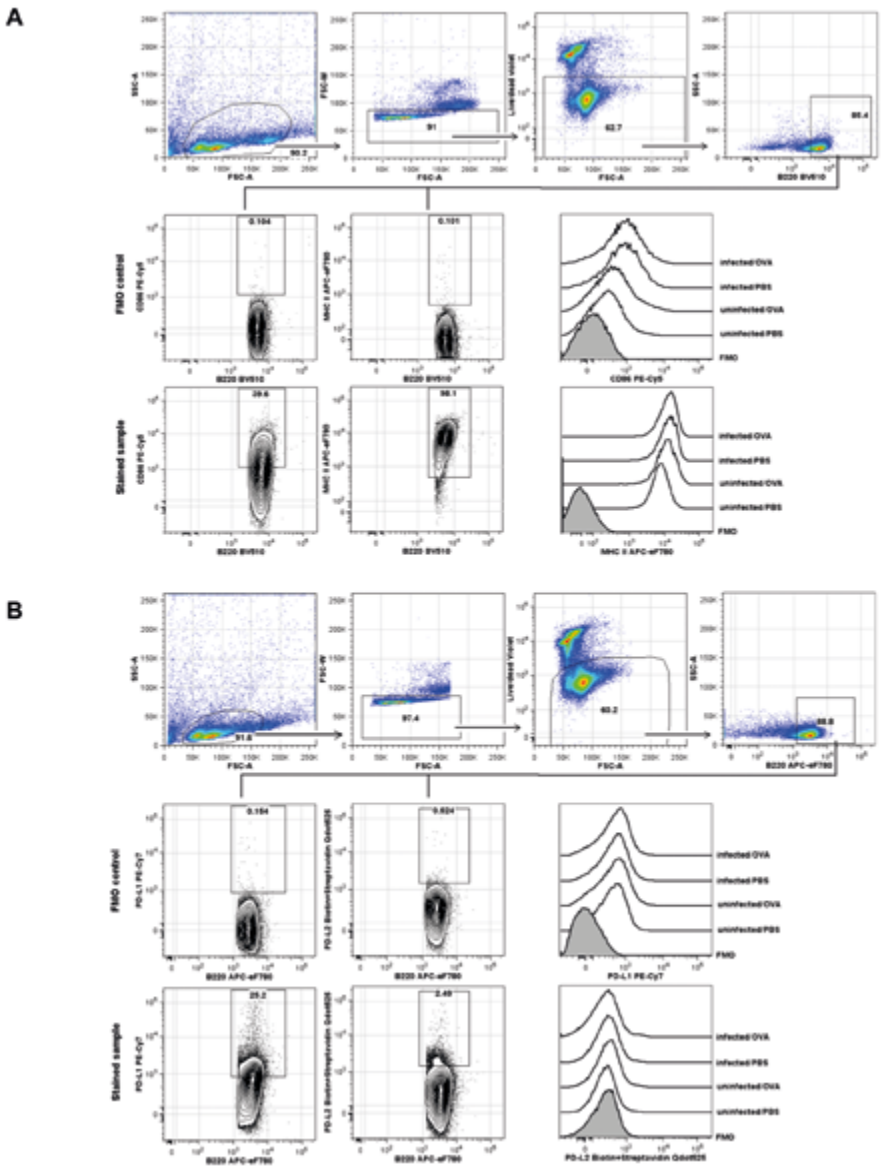

Figure S2. Gating strategy and representative flow cytometry plots (relating to **Figure 3A**). (**A**) Percentage of CD86^{+}, MHCII^{+}, and (**B**) PD-L1^{+} and PD-L2^{+} pulmonary B cells in addition to the mean fluorescence intensities (MFIs) presented in **Figure 3A**. OVA, ovalbumin; FMO, fluorescence minus one control.

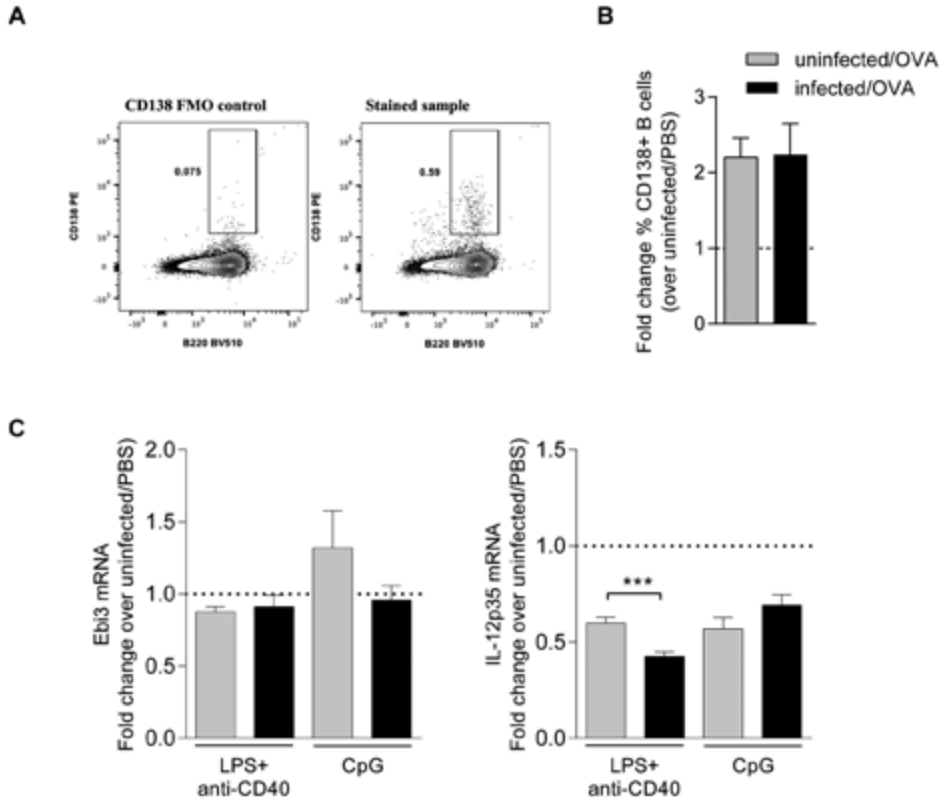

Figure S3. Pulmonary B cells were isolated as described in **Figure 1**. Cells from 2-3 mice were pooled to obtain sufficient cell numbers. (**A**) Representative flow cytometry plots showing the expression of the plasma cell marker CD138 on pulmonary B cells. (**B**) Fold change of percentage CD138-expressing B cells from uninfected/ovalbumin (OVA) and infected/OVA mice over control (uninfected/PBS). A summary of three independent experiments (n=11-14/group) is shown. (**C**) Fold change of mRNA expression of the IL-35 subunits Ebi3 and IL-12p35 as analyzed by quantitative PCR using β-2-microglobulin as a reference gene and the ΔΔ comparative threshold method. Data represent one experiment (n=3-6/group). Results are expressed as mean ± S.E.M. Significant differences are indicated as follows: ***P <0.001, as tested by a two-tailed unpaired Student's t-test. FMO, fluorescence minus one control; LPS, lipopolysaccharide.

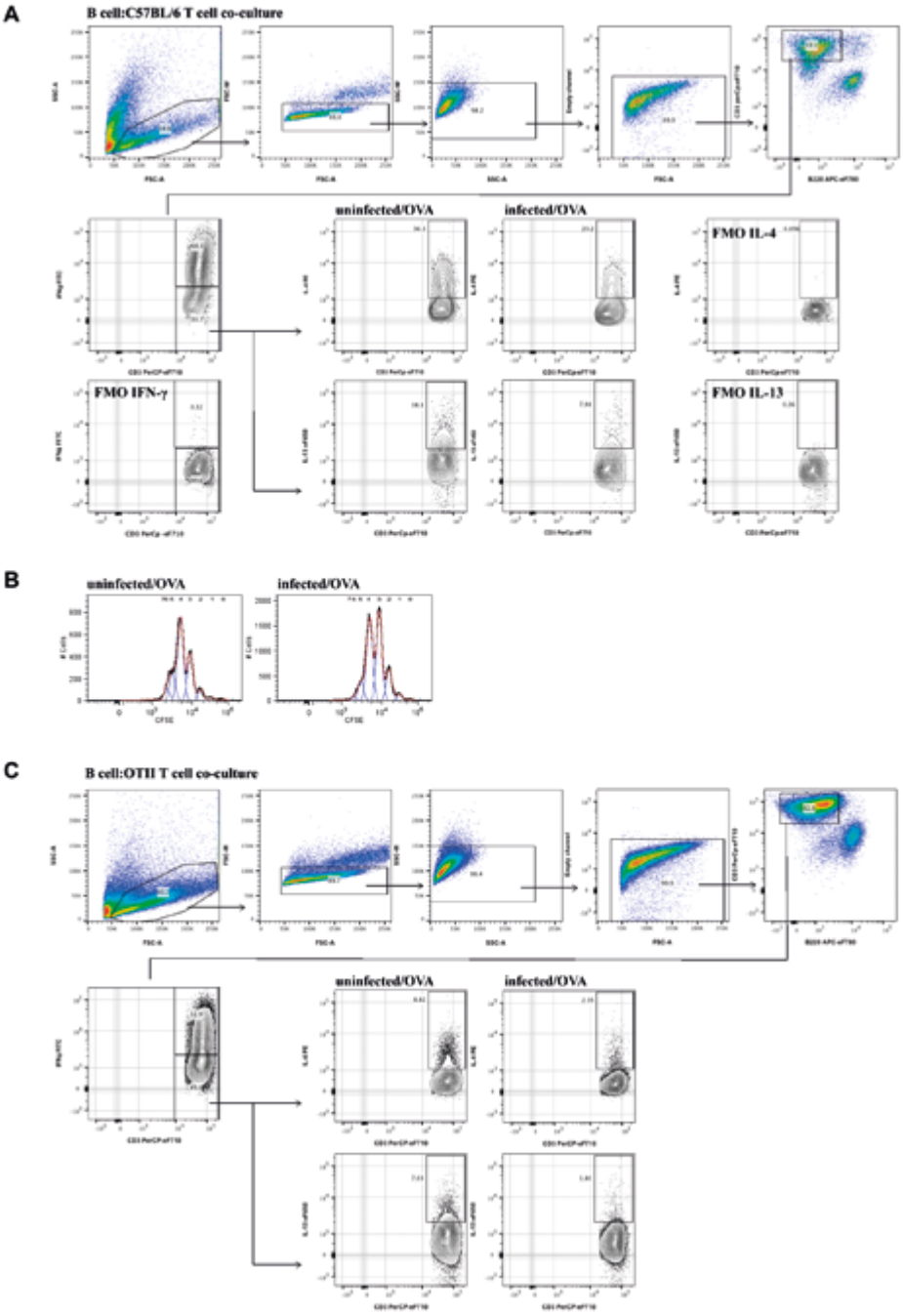

Figure S4. Gating strategy and representative flow cytometry plots (relating to **Figure 4**), showing (**A**) the expression of IL-4 and IL-13 by C57BL/6 T cells after co-culture with pulmonary B cells in the presence of anti-CD3 and anti-CD28, (**B**) the proliferation as assessed by CFSE dilution, and (**C**) the expression of IL-4 and IL-13 by OTII T cells after co-culture with ovalbumin $(OVA)_{17}$ peptide-loaded pulmonary B cells in the presence of anti-CD28. FMO, fluorescence minus one control.

Chapter 3

SCHISTOSOME EGG ANTIGENS, INCLUDING THE GLYCOPROTEIN IPSE/ALPHA-1, TRIGGER THE DEVELOPMENT OF REGULATORY B CELLS

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ABSTRACT

Infection with the helminth *Schistosoma (S.) mansoni* drives the development of interleukin (IL)-10-producing regulatory B (Breg) cells in mice and man, which have the capacity to reduce experimental allergic airway inflammation and are thus of high therapeutic interest. However, both the involved antigen and cellular mechanisms that drive Breg cell development remain to be elucidated. Therefore, we investigated whether *S. mansoni* soluble egg antigens (SEA) directly interact with B cells to enhance their regulatory potential, or act indirectly on B cells via SEA-modulated macrophage subsets. Intraperitoneal injections of *S. mansoni* eggs or SEA significantly upregulated IL-10 and CD86 expression by marginal zone B cells. Both B cells as well as macrophages of the splenic marginal zone efficiently bound SEA *in vivo*, but macrophages were dispensable for Breg cell induction as shown by macrophage depletion with clodronate liposomes. SEA was internalized into acidic cell compartments of B cells and induced a 3-fold increase of IL-10, which was dependent on endosomal acidification and further enhanced by CD40 ligation. IPSE/alpha-1, one of the major antigens in SEA, was also capable of inducing IL-10 in naïve B cells, which was reproduced by tobacco plant-derived recombinant IPSE. Other major schistosomal antigens, omega-1 and kappa-5, had no effect. SEA depleted of IPSE/alpha-1 was still able to induce Breg cells indicating that SEA contains more Breg cell-inducing components. Importantly, SEA- and IPSE-induced Breg cells triggered regulatory T cell development *in vitro*. SEA and recombinant IPSE/alpha-1 also induced IL-10 production in human $CD1d^+$ B cells. In conclusion, the mechanism of *S. mansoni*-induced Breg cell development involves a direct targeting of B cells by SEA components such as the secretory glycoprotein IPSE/alpha-1.

AUTHOR SUMMARY

Infection with helminth parasites is known to be inversely associated with hyper-inflammatory disorders. While *Schistosoma (S.) mansoni* has been described to exert its down-modulatory effects on inflammation by inducing a network of regulatory immune cells such as regulatory B (Breg), the mechanisms of Breg cell induction remain unclear. Here, we use *in vivo* and *in vitro* approaches to show that antigens from *S. mansoni* eggs, among which the major glycoprotein IPSE/alpha-1, directly interact with splenic marginal zone B cells of mice which triggers them to produce the anti-inflammatory cytokine IL-10 and their capacity to induce regulatory T (Treg) cells. We also found that IPSE/alpha-1 induces IL-10 in human $CD1d^+$ B cells, and that both natural and recombinant IPSE/alpha-1 are equally effective in driving murine and human Breg cells. Our study thus provides insight into the mechanisms of Breg cell induction by schistosomes, and an important step towards the development of helminth-based treatment strategies against hyper-inflammatory diseases.

INTRODUCTION

Helminths can persist for up to decades in the human host. This is hypothesized to be, at least in part, because of their evolutionarily adapted relationship with the host [1]. Helminths are well-known for their strong capacity to promote the regulatory arm of the hosts immune system, thereby prolonging their survival within the host [2]. As a bystander effect, helminths can also suppress immune responses to other antigens, such as allergens and auto-antigens, and other pathogens. This bystander effect seems to be so pronounced that it may prevent the development of inflammatory diseases. Indeed, both epidemiological studies and mouse models show a clear protective role of helminths against various forms of auto-immunity, allergic airway inflammation, colitis etc. [3,4,5,6,7]. The formation of a network of regulatory immune cells plays a crucial role for the protective effect. Helminth infection, and in particular infection with schistosomes such as *Schistosoma (S.) mansoni* are well-known to induce regulatory B (Breg) cells [8-15], a relatively new member in the network of regulatory immune cells. Breg cells have gained considerable attention due to their ability to down-modulate inflammation in a variety of conditions ranging from autoimmune disorders such as experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), lupus and chronic colitis to anaphylactic and allergic airway inflammation [8,10-12,16-23]. Regulatory B cells suppress pro-inflammatory immune responses via several mechanisms, of which the ones best described are the expression of the regulatory cytokine interleukin-10 (IL-10) and induction of regulatory T (Treg) cells [24].

We previously reported the induction of Breg cells by schistosome infection in both mouse and human, and found the most potent IL-10-producing Breg cells within the human $CD1d^+$ B cell subset. This corresponds to the $CD1d^+CD23^{low}CD21^+$ marginal zone (MZ) B cell subset in mice, which efficiently reduced experimental allergic airway inflammation in our model [12]. The cellular mechanisms to achieve Breg cell induction as well as the nature of the B cell-activating *S. mansoni* antigens however remain largely unknown. The identification of relevant stimulatory molecules and optimal Breg cell-inducing conditions is a critical step in enhancing the activity of Breg cells for use as a new therapeutic tool against inflammatory diseases.

Both an indirect induction of a regulatory phenotype in B cells by activation of accessory cell types, as well as a direct binding and interaction between *S. mansoni* antigens and B cells via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed on B cells [25] are plausible options. In the splenic MZ, located at the border of white and red pulp, MZ B cells are nested between SIGN-$R1^+$ MZ macrophages and Siglec-1^+ metallophilic macrophages [26]. MZ macrophages not only fulfill a main function in sensing blood-borne pathogens, but also perform functional interactions with MZ B cells. These interactions have important implications for the maintenance of the MZ itself and the function of MZ B cells and macrophages [27-29]. Hence, it is therefore tempting to speculate that MZ macrophages are a prime candidate as Breg cell induction partner. On the other hand, the direct ligation of various TLRs on B cells, including TLR2, TLR4, TLR7 and TLR9, has been described to induce IL-10 production [30,31]. In addition, BCR and CD40 engagement were described to be involved in IL-10-dependent regulatory B cell function in models of EAE, CIA, and contact hypersensitivity [17-19,32].

In the current study, we tested the hypothesis that eggs and/or egg-derived excretory-secretory molecules from *S. mansoni*, without the context of natural infection, are sufficient to drive Breg cell development by activating splenic MZ B cells. In addition, we investigated whether Breg cells are induced indirectly by activation of accessory cell types in the MZ, or by direct binding and interaction via PRRs on B cells. We found that egg antigens drive Breg cell development *in vivo* and *in vitro* by direct interaction with splenic B cells, which after binding and internalization of egg antigens secrete elevated levels of IL-10 and are capable of driving Treg cell development. The egg antigen-induced Breg cell development was independent of macrophages of the marginal zone but was enhanced by CD40 ligation. Most importantly, we identified the egg glycoprotein IPSE/alpha-1 as a single molecule

from *S. mansoni* that is capable to induce Breg cells both in mice and man. This knowledge will assist to further define helminth-specific conditions for the generation of Breg cells to be used in therapeutic approaches.

RESULTS

Schistosome egg antigens drive the development of Breg cells *in vivo*

To elucidate the mechanism by which *S. mansoni* can drive the development of Breg cells, we first investigated whether schistosome eggs or their soluble antigens were sufficient to drive Breg cell development *in vivo*, without the context of natural infection. Intraperitoneal treatment of C57BL/6 mice with two doses of 5000 *S. mansoni* eggs or 100 µg of soluble egg antigens (SEA) one week apart efficiently induced IL-10 protein expression in splenic $CD19^+$ B cells one week after the last injection. IL-10 protein secreted during 2 days *ex vivo* restimulation with SEA was 3 to 4-fold increased compared to the amount secreted by restimulated B cells from control-treated animals (**Figure 1A**), while IL-6 was unchanged. This indicates a typical cytokine expression pattern characteristic for Breg cells. The frequencies of B cells expressing intracellular IL-10 protein were likewise significantly 2-fold increased (**Figure 1B and 1C**). Also the surface activation marker CD86, often upregulated on activated B cells and Breg cells [33-35], was increased on splenic B cells by egg or SEA treatment, while CD40 expression was not significantly changed (**Figure 1D**). To verify that the observed effects are specific and exclude a general influence of protein solutions on B cell IL-10 production and activation, we treated mice with human serum albumin (HSA) as infection-unrelated control protein. We did not observe an increased IL-10 secretion (**suppl. Figure S1A**) or CD86 expression (**suppl. Figure S1B**) by B cells compared to the PBS group, and concluded that PBS is a suitable control for subsequent experiments. Furthermore, injection of eggs or egg antigens was as efficient in increasing the frequency of IL-10-expressing B cells as was chronic infection with *S. mansoni* (**suppl. Figure S1C**), and egg-injected mice continued to have an elevated B cell IL-10 production until at least 4 weeks after the last egg injection (**suppl. Figure S1D**), indicating that this phenotype is persisting over longer periods. SEA was purified from liver eggs and can contain LPS to variable extent. Since the TLR4 ligand LPS is known for its capacity to drive B cell IL-10 expression and Breg cell development [33,36,37], it is crucial to exclude that the Breg driving capacity by SEA *in vivo* was due to LPS contamination of the schistosome antigen preparations. Therefore, the same experiment was repeated in TLR4-deficient animals and compared to wild-type. Upon SEA treatment, IL-10 secretion of B cells as well as intracellular IL-10 and surface CD86 expression was overall comparable in both groups (**suppl. Figure S2**), indicating that the Breg-inducing capacity by SEA was largely not attributable to a putative LPS contamination. To confirm the regulatory function, splenic B cells from the various groups were tested for their capacity to drive Treg cell development, an acquired phenotype previously described for splenic B cells during natural schistosome infections [12]. Indeed, splenic B cells from egg- or SEA-injected, but not control-treated, mice induced the development of $CD25^+Foxp3^+$ Treg cells during 4 day co-culture with CD25-depleted CD4 T cells (**Figure 1E and 1F**), which confirms the regulatory capacity of egg antigen-activated B cells *ex vivo*. As expected, IL-10 protein concentration in co-culture supernatants was only increased in presence of egg antigen-activated but not control B cells (**Figure 1G**). Collectively, these data show that schistosome eggs or their soluble antigens on their own are sufficient to induce IL-10-producing Breg cells *in vivo*, without the context of natural infection, and that these B cells are bona fide Breg cells that can drive Treg cell development.

Schistosome antigens activate marginal zone B cells in the spleen

Different B cell subsets have been described to give rise to Breg cells, especially in spleen where subsets differ e.g. in tissue localization and pathogen recognition receptor expression [25,38]. We and

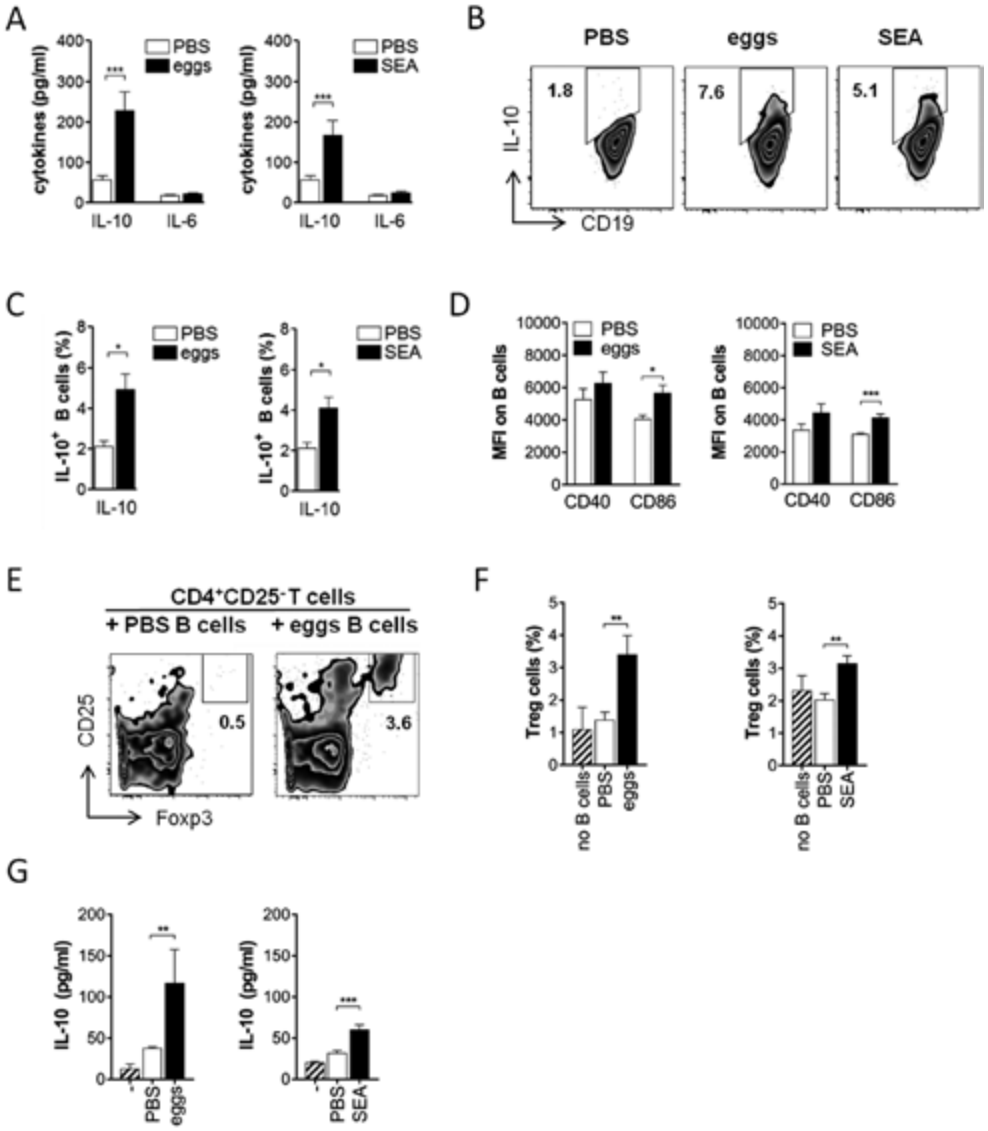

Figure 1. Schistosome eggs and SEA (soluble egg antigen) drive development of Breg cells *in vivo*. C57BL/6 mice were i.p. injected with two doses of 5000 *S. mansoni* eggs or 100 µg SEA in PBS, or PBS as control. At day 14, $CD19^+$ MACS-isolated splenic B cells were restimulated with SEA (20 µg/ml) for 2 days. **(A)** Cytokine concentration in culture supernatants as determined by ELISA. **(B, C)** Representative FACS plots (B) and summary (C) for intracellular IL-10 expression of B cells after addition of Brefeldin A to the last 4 hours of the culture. **(D)** Mean fluorescence intensity of CD40 and CD86 expression. **(E-G)** SEA-restimulated B cells were co-cultured for 4 days with CD25-depleted CD4 T cells. Frequency of $CD25^+Foxp3^+$ Treg cells after co-culture in representative FACS plots **(E)** and summary **(F)** is shown. **(G)** IL-10 concentration in culture supernatants after co-culture. Summary of 4 experiments with N = 12-16 (A-D) or 2 experiments with N = 8-9 (E-G). Significant differences by Mann-Whitney test are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

others had previously identified $CD23^{low}CD21^{+}$ marginal zone B cells as the major IL-10-producing splenic B cell subset during chronic *Schistosoma* infection and mediating protection in a mouse model of airway inflammation [8,12]. To test whether soluble egg antigens act on the same splenic subset, we sorted splenic $CD23^{low}CD21^{+}$ marginal zone B cells from egg-, SEA-treated, or control mice for subsequent *ex vivo* restimulation and cytokine analysis, and compared this with the major splenic B cell subset, $CD23^{hi}CD21^{-}$ follicular B cells (**Figure 2A**). Only marginal zone B cells but not follicular B cells showed significantly increased IL-10 secretion as was measured in culture supernatants after 2 day restimulation with SEA (**Figure 2B**). As for total B cells (**Figure 1A**), also for the individual subsets IL-6 expression was not increased (**Figure 2C**). Intracellular IL-10 expression and CD86 expression was likewise significantly upregulated in marginal zone B cells of egg- or SEA-treated mice compared to control-treated mice. SEA seemed to be more potent than egg injection in activating follicular B cells, as SEA-injection also significantly increased intracellular IL-10 and CD86 expression in this subset, although expression levels remained significantly lower compared to marginal zone B cells (**Figure 2D and 2E**). For analysis of B cell activation we generally restimulated cells *ex vivo* with SEA. This increased the baseline expression of CD86 in all groups compared to medium (average MFI of follicular B cells: 1013-1632±62.2; for marginal zone B cells: 2640-4118±176.9, for all groups and without significant differences between groups), but was required for detection of B cell cytokines as a result of the *in vivo* antigen exposure. Without SEA restimulation, we found a trend of increased IL-10 production by B cells which only reached significance upon additional restimulation (**suppl. Figure S1E**), indicating that renewed exposure to antigen is required to achieve detectable B cell activity and cytokine production. This is also supported by experiments in IL-10 GFP reporter mice, in which IL-10 (GFP) accumulates in B cells during the entire *in vivo* treatment period. Here, increased IL-10 (GFP) expression, without *ex vivo* restimulation, was only detectable in B cells of 14 weeks chronically infected mice, but not in B cells of mice that received two injections of eggs within a relatively short period of 2 weeks (**suppl. Figure S1F and S1G**). Altogether, these data indicate that the result of *in vivo* development of IL-10 producing B cells is in principle detectable without restimulation (**suppl. Figure S1E and S1G**), but the data from egg-injected IL-10 reporter mice also suggest that an *ex vivo* SEA restimulation is required to visualize its full IL-10 potential, something which will happen *in vivo* during a natural infection due to the constant production of eggs and the high levels of circulating antigens. Taken together, these data support the notion that B cells, and in particular marginal zone B cells, are responsive to *in vivo* schistosome antigen stimulation thus supporting the findings in natural schistosome infections.

Macrophage subsets of the marginal zone bind SEA but are dispensable for Breg cell induction

Molecules secreted by schistosome eggs are highly glycosylated and known to bind to C-type lectin receptors [39,40]. Since B cells show a very restricted expression of those receptors [41], we hypothesized that other C-type lectin receptor-expressing cell types in the splenic marginal zone, such as macrophages or dendritic cells, bind SEA and provide additional signals to the marginal zone B cells to support Breg cell development. Among the accessory cell types, macrophages of the splenic marginal zone were of particular interest because of their known interactions with marginal zone B cells [28,42] and schistosome antigens [43,44]. However, it was unknown whether marginal zone macrophages can capture SEA *in vivo* and are important for B cell IL-10 expression. To evaluate this, fluorescently labeled SEA was injected i.v. and 30 minutes to 24 hours later various splenic cell types were analyzed for bound SEA using fluorescence microscopy and flow cytometry. Already after 30 minutes of injection, SEA clustered along the marginal zone area of the spleen as detected by fluorescence microscopy of splenic tissue sections (**Figure 3A**). SEA localized predominantly within two specialized macrophage subsets of the marginal zone: SIGN-R1-expressing MZ macrophages and Siglec-1-expressing marginal metallophilic macrophages (**Figure 3B**). In contrast, after injection of labeled ovalbumin

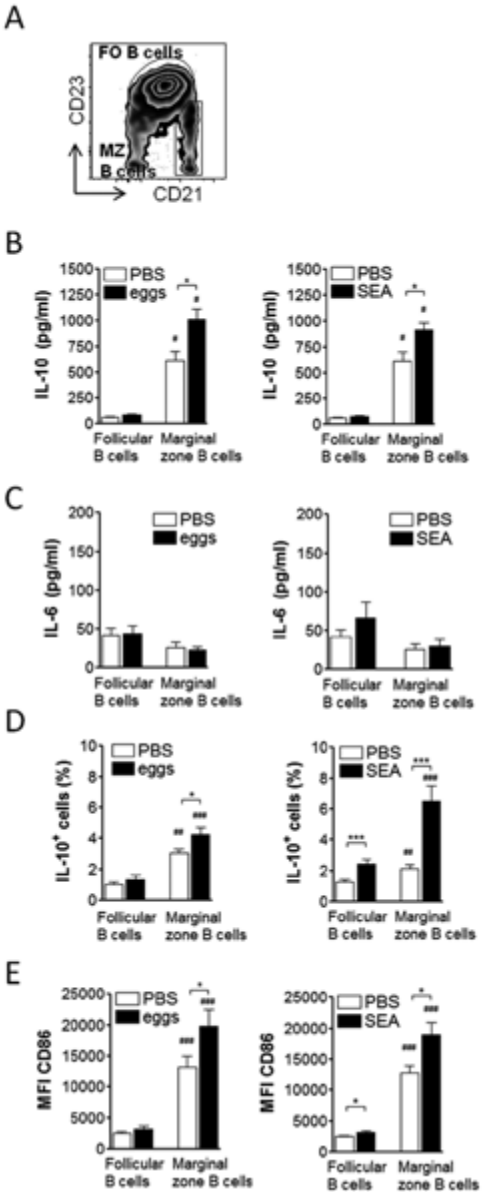

Figure 2. Schistosome antigens mainly activate the marginal zone B cell subset of the spleen. C57BL/6 were treated as in Figure 1, splenic follicular (FO) B cells ($CD19^+CD23^+CD21^{low}$) and marginal zone (MZ) B cells ($CD19^+CD23^-CD21^{hi}$) FACS sorted, and restimulated for 2 days with SEA (20 µg/ml). **(A)** Gating scheme for both B cell subsets within the $CD19^+$ gated B cell population. **(B, C)** Cytokine concentration in culture supernatants as determined by ELISA. **(D)** Intracellular IL-10 expression of B cell subsets after addition of Brefeldin A to the last 4 hours of the culture. **(E)** Mean fluorescence intensity of CD86 expression of B cell subsets. Summary of 2 experiments with N = 6 (B, C) or 3-5 experiments with N = 13-21 (D, E). Significant differences by Mann-Whitney test are indicated with * $p < 0.05$, *** $p < 0.001$. Significant differences between B cell subsets by Wilcoxon paired test are indicated with $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

(OVA) as non-schistosomal control protein no fluorescence signal was detected in the spleen (**suppl. Figure S3**). Flow cytometry confirmed that 83% of metallophilic macrophages were positive for SEA only 30 minutes after injection, and still 53% of cells after 24 hours (**Figure 3C**). In addition, F4/80-expressing red pulp macrophages and Ly6C^{hi} monocytes efficiently bound SEA (81.7 and 82.6%, respectively), while only a small fraction of dendritic cells and neutrophils did bind SEA (**Figure 3C, suppl. Figure S4A-S4C** for gating scheme of cell types). Metallophilic macrophages not only bound SEA abundantly, they also significantly upregulated typical surface activation markers such as CD11c and CD86, but not MHCII, at 30 minutes and 2 hours after SEA injection, respectively (**Figure 3D**). Because macrophages of the marginal zone were most potent in binding SEA, we next addressed their role for SEA-mediated marginal zone Breg cell induction. To this end, macrophages were depleted *in vivo* by i.p. injection of clodronate-containing liposomes [45] prior to injection of schistosome eggs. Eggs were injected 3 and 4 weeks after clodronate treatment, at time-points when only macrophages, including metallophilic and MZ subsets, were significantly reduced in spleens, but no other cell types (**suppl. Figure S4D** and [46]). Successful and specific depletion of splenic macrophages was also confirmed by fluorescence microscopy of tissue sections (**Figure 3E**) and flow cytometry (**suppl. Figure S4E**) at 7 days after the last egg injection, when B cell activity was analyzed. Unexpectedly, IL-10 secretion of splenic B cells from macrophage-depleted mice was equal to that from control liposome-treated mice (**Figure 3F**). Also the upregulation of intracellular IL-10 (**Figure 3G**) and CD86 expression in B cells, as well as the induction of Foxp3$^+$CD25$^+$ and IL-10$^+$CD25$^+$ CD4 T cells (**suppl. Figure S4F and S4G**) following SEA injection was not affected by the absence of macrophages. In conclusion, splenic macrophages are not essential for schistosome antigen-induced Breg cell development, despite the high binding of SEA by different macrophage subsets.

MZ B cells bind and take up schistosome antigens

To test whether B cells directly bind and interact with schistosome antigens without the help of surrounding accessory cell types, fluorescently labeled SEA was injected i.v. and its co-localization with B cells analyzed by fluorescence microscopy of splenic tissue sections. Indeed, egg antigens were found to co-localize with some splenic B220$^+$ B cells (**Figure 4A**). Flow cytometry, a more sensitive method compared to fluorescence microscopy, showed that only MZ B cells but not follicular B cells did bind SEA *in vivo*, with a maximum of 56.4% of cells being positive at 2 hours after SEA injection. SEA was still detectable on 11.5% of marginal zone B cells at 24 hours (**Figure 4B**). Marginal zone B cells also showed an increased CD86 surface expression following SEA injection, which was significant at 6 hours after SEA injection (**Figure 4C**). Interestingly, marginal zone B cells that bound SEA showed a higher CD86 expression compared to cells that were found to be negative for SEA, i.e. approximately 3-fold (SEA positive) versus only 1.5-fold (SEA negative) compared to B cells from untreated animals (**Figure 4C**). This suggests that marginal zone B cells not only efficiently bind egg antigens *in vivo*, but also become (more) activated as a consequence of this interaction. Binding of SEA to B cells was confirmed *in vitro* by culturing splenic B cells with fluorescently labeled SEA for 60 minutes, after which up to 16% were positive for SEA as measured by flow cytometry (**Figure 4D**), which is a similar percentage as found for total B cells after *in vivo* application of labeled SEA (**Figure 4B**). By using SEA labeled with the pH-sensitive dye pHrodo, it was shown that egg antigens were not only bound to the surface but were also internalized by B cells into acidic cellular compartments (**Figure 4D**). As *in vivo*, also *in vitro* the marginal zone B cell subset bound SEA more efficiently than the follicular B cell subset, with in average 15.9% versus 6.9% of cells being positive for SEA (**Figure 4E**). Collectively, these data show that B cells, and in particular MZ B cells, are capable of directly interacting with schistosome antigens by binding and internalization of those antigens, both *in vivo* and *in vitro*.

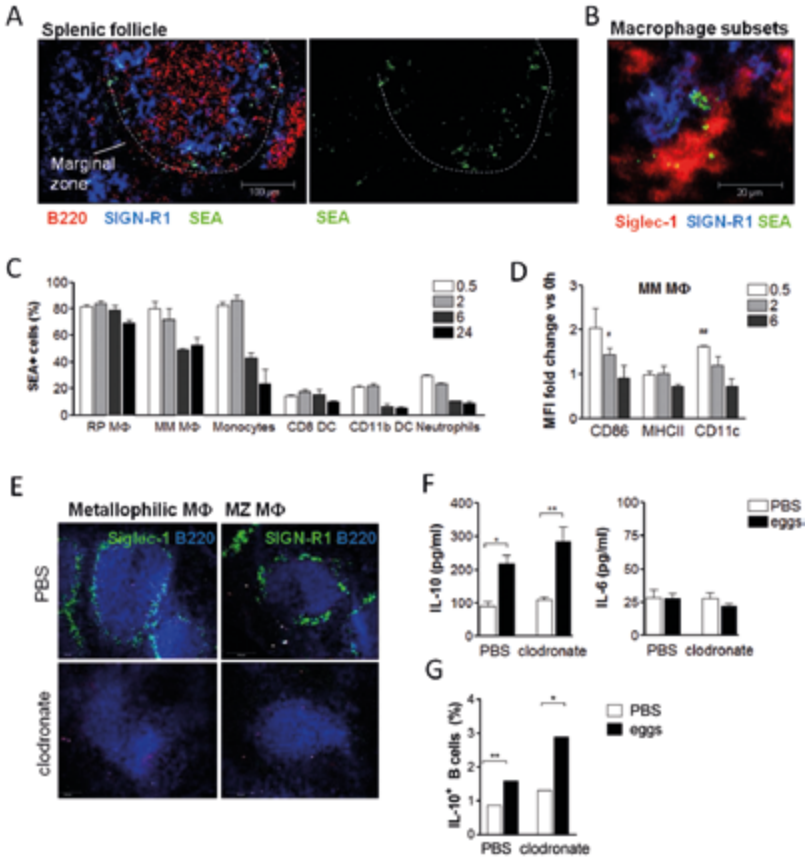

Figure 3. Macrophage subsets of the MZ bind SEA but are dispensable for schistosome antigen-mediated Breg cell induction. (A, B) Spleens were snap-frozen 30 minutes after i.v. injection of 200 µg fluorescently labeled SEA, and binding of SEA analyzed by fluorescence microscopy. Images are representative for 2 experiments with N = 5 mice and 3 follicles per section imaged. **(A)** SEA clustered around the marginal zone of the spleen (indicated by white dashed line). **(B)** SEA localized in the marginal zone to macrophages expressing Siglec-1 (marginal metallophilic macrophages) and SIGN-R1 (MZ). **(C, D)** Mice were i.v. injected with fluorescently labeled SEA and splenocytes harvested 30 minutes-24 hours later. **(C)** Frequency of SEA-positive cells in various splenocyte subsets as determined by flow cytometry (gating schemes see **suppl. Figure S4)**. MF, macrophages; RP, red pulp; MM, marginal metallophilic; DC, dendritic cells. **(D)** Mean fluorescence intensity of surface markers on MM macrophages calculated as fold increase versus the expression at time-point 0 hours. Summary of 2 experiments with N = 2. **(E-G)** Mice were i.p. injected with 200 µl clodronate-containing liposomes or PBS control liposomes. Three weeks later, mice were i.p. injected with two doses of 5000 eggs. Seven days after the last egg injection, splenic B cells were restimulated for 2 days with SEA. **(E)** Absence of splenic Siglec-1 and SIGN-R1-expressing macrophage subsets at the time-point of spleen collection was confirmed by fluorescence microscopy. B cells were stained with B220. **(F)** Cytokine concentration in culture supernatants as determined by ELISA. **(G)** Intracellular IL-10 expression of B cells after addition of Brefeldin A to the last 4 hours of the culture. One representative (N = 5) out of 2 similar experiments is shown. Significant difference by Mann-Whitney test is indicated with * $p < 0.05$, ** $p < 0.01$. Significance as determined by one-sample t-test of log-transformed data is indicated with $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

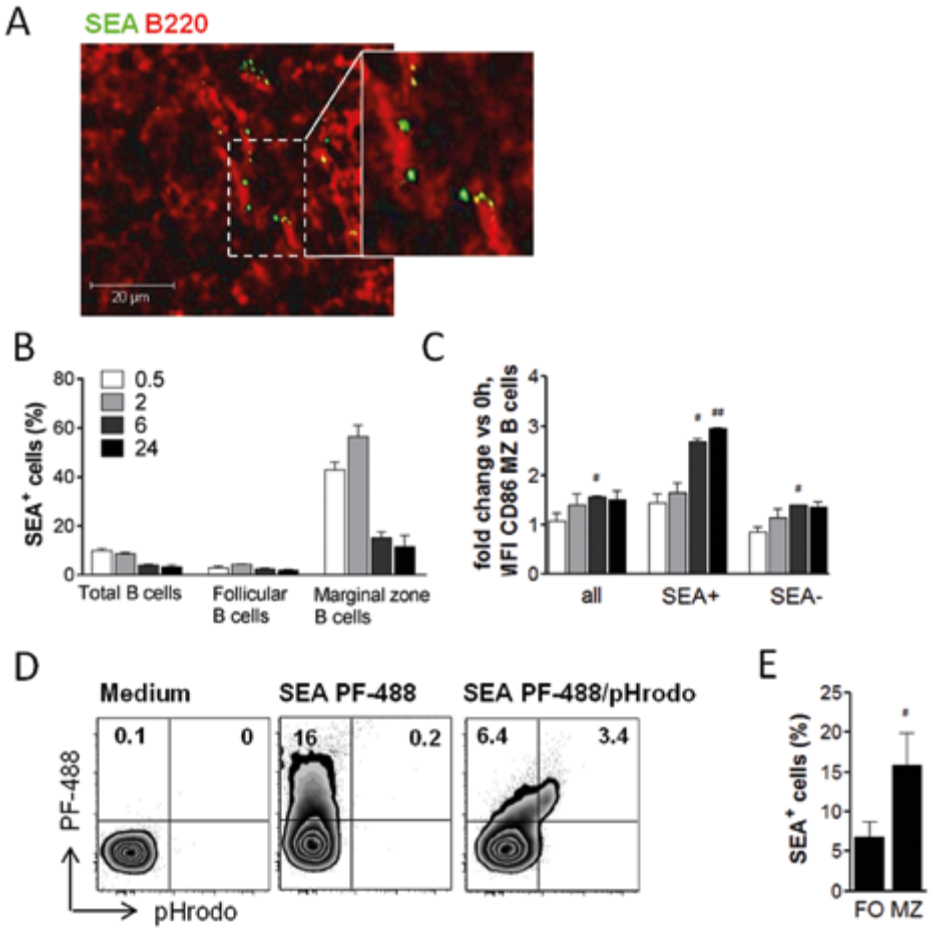

Figure 4. Schistosome antigens bind to B cells and are internalized into acidic compartments. (A) C57BL/6 mice were i.v. injected with 200 µg of fluorescently labeled SEA and spleens snap-frozen 30 minutes later. Fluorescence microscopic image shows localization of SEA to B220⁺ B cells and is representative for 2 experiments with N = 5 mice and 3 viewing fields per section imaged. **(B, C)** Splenic B cells were analyzed by flow cytometry at 30 minutes to 24 hours after i.v. injection of labeled SEA for **(B)** frequency of SEA⁺ cells within total B cells or B cell subsets as gated in **Figure 2A**, and for **(C)** fold increase of CD86 expression on total MZ B cells compared to MZ B cells gated for SEA⁺ and SEA⁻ populations. Summary of 2 experiments with N = 2. **(D, E)** Splenic B cells from naïve mice were cultured *in vitro* with SEA (20 µg/ml) labeled with a green dye (PF-488) alone, or co-labeled with a pH-sensitive dye (pHrodo Red). After 60 minutes, the frequency of SEA⁺ B cells was determined by flow cytometry. **(D)** Gated for total CD19⁺ B cells (1 representative out of 2 experiments shown). **(E)** Gated for follicular (FO) and marginal zone (MZ) B cell subsets (summary of 3 experiments). Significant differences indicated with $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ are determined by one-sample t-test of log-transformed data (C) or between B cell subsets by Wilcoxon paired test.

Breg cells can be generated *in vitro* by direct interaction with schistosome egg antigens

Next, we investigated whether the observed direct interaction of B cells with SEA can drive IL-10 expression and induction of regulatory B cell function *in vitro*. To this end, CD19⁺ splenic B cells from naïve mice isolated using MicroBeads were cultured for 3 days with SEA. SEA-stimulated B cells secreted significantly more IL-10, but not IL-6, compared to non-stimulated B cells (**Figure 5A**), showing a typical cytokine pattern characteristic for schistosome-induced Breg cells. Separate cultures

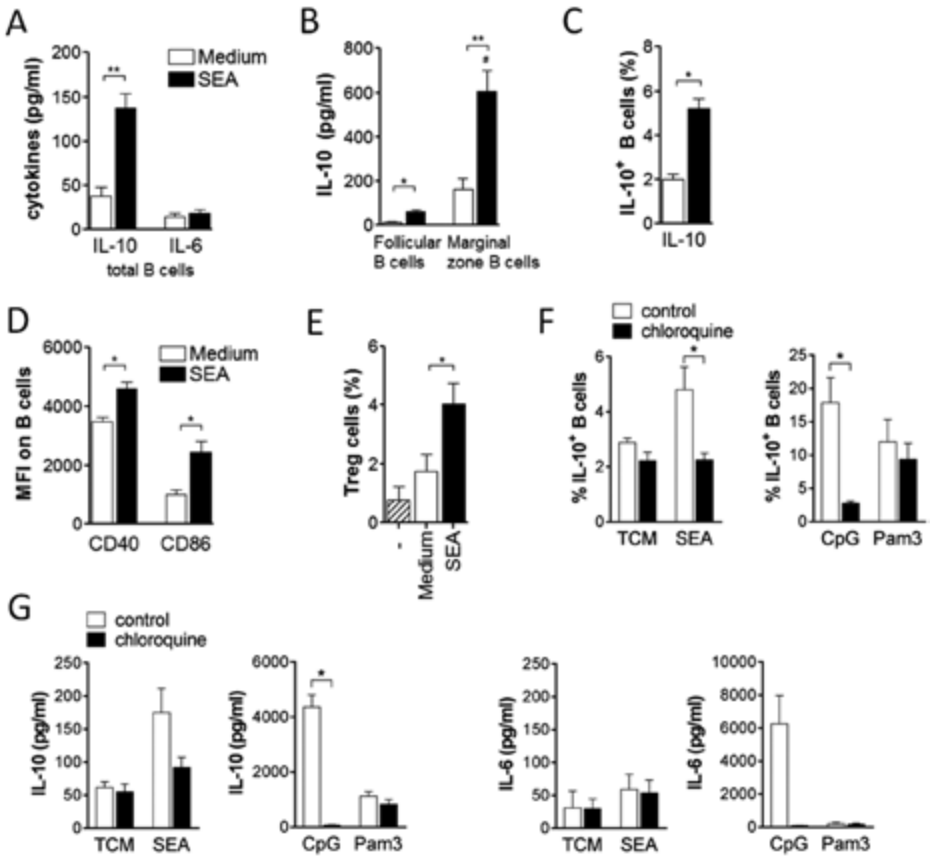

Figure 5. Breg cells can be generated *in vitro* by stimulation with schistosome egg antigens which involves lysosomal processing. (A-E) Splenic B cells from naïve mice were cultured for 3 days with 20 µg/ml SEA or medium as control. Cytokine concentration in culture supernatants of **(A)** total B cells (6 experiments) or **(B)** sorted B cell subsets (2 experiments with N = 6). **(C)** Intracellular IL-10 expression of total B cells after addition of PMA, ionomycin and Brefeldin A to the last 4 hours of the culture (4 experiments). **(D)** Mean fluorescence intensity of the activation markers CD40 and CD86 (4 experiments). **(E)** Splenic B cells were stimulated *in vitro* as above, and subsequently co-cultured with $CD4^+CD25^-$ sorted splenic T cells. After 4 days, the frequency of $CD25^+Foxp3^+$ Treg cells within the CD4 T cell population was determined by flow cytometry. Summary of 4 experiments. **(F, G)** Splenic B cells from naïve mice were cultured *in vitro* with SEA (20 µg/ml), CpG (5 µg/ml) or Pam3Cys (10 µg/ml) for 2 days. Every day, chloroquine (5 µM) was added to the culture. Intracellular IL-10 expression after addition of PMA, ionomycin and Brefeldin A during the last 4 hours of the culture **(F)**, and cytokine concentrations in culture supernatants **(G)**. Summary of 2 experiments with N=3-4. Significant differences are indicated with * $p < 0.05$, ** $p < 0.01$ and tested by Mann-Whitney. # $p < 0.05$ indicates significant difference between B cell subsets by Wilcoxon paired test.

of sorted MZ and follicular B cells showed once more that the MZ B cell subset reacted more potently to SEA, e.g. with a significantly higher IL-10 secretion compared to the follicular subset (**Figure 5B**). In addition, frequencies of B cells expressing intracellular IL-10 were likewise increased (**Figure 5C**). To ensure that the IL-10 phenotype is not reliant on artificially high levels of stimulation with PMA/ ionomycin which is added to facilitate detection of intracellular IL-10, we repeated the assay using cells from IL-10-GFP reporter (TIGER) mice with similar results **(S5 Figure**). CD40 and CD86 expression were upregulated compared to cultures in medium alone (**Figure 5D**). Importantly, *in vitro* SEA-activated B

cells were also capable of driving Treg cell development during a 4 day co-culture with CD25-depleted CD4 T cells (**Figure 5E**), thus providing further evidence for a bona fide regulatory function of the *in vitro* induced Breg cells. Because we had seen internalization of egg antigens into acidic compartments (**Figure 4D**), we wondered whether lysosomal processing is necessary for induction of IL-10 expression. Addition of chloroquine, an inhibitor of endosomal acidification [47], significantly reduced the IL-10 secretion and frequency of IL-10$^+$ B cells induced by SEA and CpG (ligand for endosomal TLR9), but not by Pam3Cys (ligand for surface TLR2) (**Figure 5F and 5G**). This suggests that internalization and endosomal processing of SEA is required for B cell IL-10 induction. The type of receptor involved in direct activation of Breg cells by SEA remains unknown. Egg antigens are abundantly glycosylated and known to bind to C-type lectin receptors [39,40]. Le^x-motifs, one of the most abundant glycan structures present in SEA, bind to the C-type lectin receptor SIGN-R1. However, when treating SIGN-R1-deficient mice with SEA, IL-10 expression was equally well induced compared to wild-type mice (**suppl. Figure S6A**), suggesting no involvement of the Le^x-motifs. Furthermore, stimulation of various TLRs on B cells, including TLR2, TLR4, TLR7 and TLR9, has been described to induce IL-10 production [30,31]. Because SEA has been reported to contain TLR2 activity [48,49], we compared SEA-induced Breg cell responses in wild-type and TLR2-deficient B cells. We did however not observe any difference in IL-10 secretion between the two strains (**suppl. Figure S6B**), excluding a role of TLR2-triggering SEA components in SEA-induced B cell IL-10 production. This is further supported by the fact that TLR2-mediated B cell activation was independent of endosomal processing while SEA-mediated activation was dependent on it (**Figure 5F and 5G**). Collectively, these data demonstrate that Breg cells can be generated *in vitro* by culture with schistosome antigens, that endosomal processing is involved in this process, and that these Breg cells are functional in the sense that they support Treg cell development.

CD40 ligation enhances the schistosome antigen-induced Breg cell development

Previous studies highlighted a role for CD40 ligation during *in vitro* Breg cell induction [50,51]. We therefore tested whether CD40 ligation could increase the SEA-mediated effect on Breg cell development. Addition of anti-CD40 stimulatory antibody (Ab) to the 3 day SEA culture increased IL-10 secretion of splenic B cells by 1.7-fold compared to SEA alone. A similar enhancing effect was observed for IL-6 secretion (**Figure 6A**) and CD86 expression (**Figure 6B**). In contrast to anti-CD40 Ab, addition of anti-IgM Ab did not significantly enhance the SEA-mediated B cell activation (**suppl. Figure S7**). To exclude stimulatory effects from LPS *in vitro*, B cells from TLR4-deficient mice were stimulated with SEA with or without addition of anti-CD40 Ab. The fold increase of IL-10 secretion compared to B cells cultured in medium was similar for wild-type and TLR4-deficient cells, thus excluding a major effect of the TLR4 ligand LPS (**suppl. Figure S6C**). Finally, co-culture of CD25-depleted CD4 T cells with anti-CD40-stimulated B cells increased the frequency of CD25$^+$Foxp3$^+$ Treg cells, which was further increased if B cells had been stimulated with SEA plus anti-CD40 Ab (**Figure 6C**). Thus, SEA stimulation plus CD40 ligation of B cells further enhanced the capacity to drive the development of IL-10-producing Breg cells *in vitro*.

Previous reports only addressed the role of CD40 ligation *in vitro*, but the relevance for *in vivo* Breg cell induction was not investigated. We therefore blocked CD40 ligand *in vivo* by i.p. injection of a hamster anti-mouse CD40 ligand blocking mAb (200 µg; every 4 days starting at day -1 prior to 1st SEA injection) during SEA treatment of mice and analyzed the effect on Breg cell activation. In hamster IgG-injected control mice, SEA treatment increased the amount of B cell-derived IL-10 secretion by 3.3-fold compared to PBS treatment. Upon anti-CD40 ligand administration this increase was only 1.6-fold and thereby significantly lower (**Figure 6D**). Importantly, the SEA-mediated upregulation of intracellular IL-10 and CD86 expression by B cells was even fully abolished when CD40 ligand was blocked (**Figure 6E and 6F**). Taken together, CD40 ligation enhances the Breg cell-inducing effect of SEA both *in vitro* and *in vivo*.

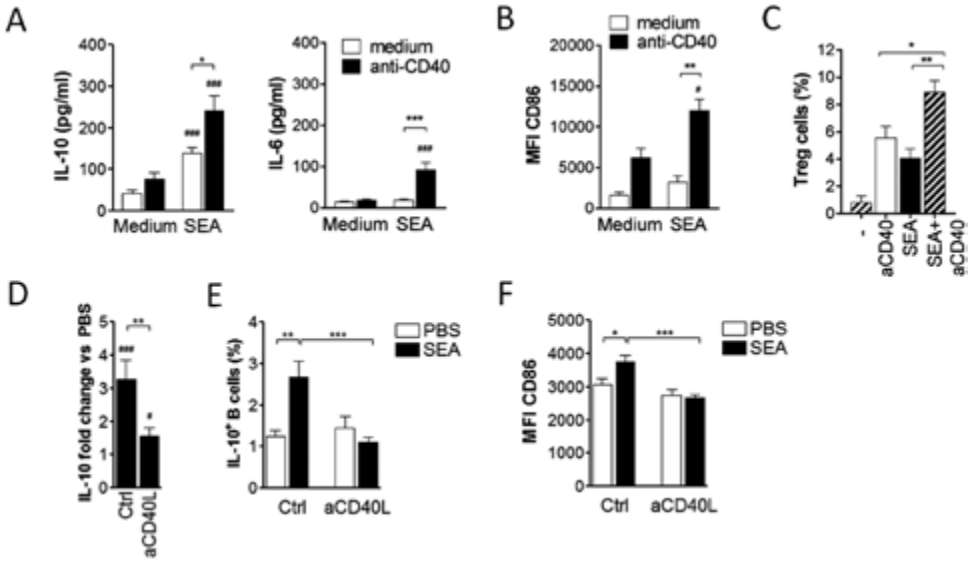

Figure 6. CD40 ligation enhances SEA-induced Breg cell development. (A, B) Splenic B cells from naïve mice were cultured with 20 µg/ml SEA or medium as control, with or without addition of anti-CD40 co-stimulatory antibody. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 by ELISA **(A)** and B cell CD86 expression by flow cytometry **(B)**. Summary of 6 experiments. **(C)** Splenic B cells were stimulated for 3 days with anti-CD40 mAb (2 µg/ml) and SEA (20 µg/ml) or anti-CD40 alone, and subsequently co-cultured with $CD4^+CD25^-$ sorted splenic T cells. After 4 days, the frequency of $CD25^+Foxp3^+$ Treg cells within the CD4 T cell population was determined by flow cytometry. Summary of 4 experiments. **(D-F)** Mice were i.p. injected with two doses of 100 µg SEA or PBS as control. CD40 ligand was blocked *in vivo* by i.p. injection of 200 µg hamster anti-mouse CD40 ligand or 200 µg hamster IgG as control (Ctrl) for 4 times, every 4 days starting at day -1 before SEA/PBS treatment. At day 14 after the first SEA/PBS injection, splenic B cells were *in vitro* restimulated with SEA for 2 days. Summary of 2 experiments (N = 8). **(D)** Fold increase of IL-10 in supernatants from B cells of SEA-injected versus PBS-injected mice. **(E)** Intracellular IL-10 expression of B cells after addition of Brefeldin A to the last 4 hours of the culture. **(F)** Mean fluorescence intensity of CD86 on B cells. Significant differences are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as tested by Mann-Whitney test. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ indicates significant difference by one-sample t-test of log-transformed data (D) or to respective medium control by Mann-Whitney test.

The secretory schistosome egg antigen IPSE/alpha-1 induces Breg cell development in mice and humans

SEA is a complex mixture of several different antigens. In the next step, we therefore aimed to identify specific antigens in SEA that are relevant for Breg cell induction. We focused on three major antigens that provoke an antibody response in nearly all infected patients: omega-1, kappa-5 and IPSE/alpha-1 [52,53]. B cells were able to bind fluorescently labelled natural IPSE/alpha-1 (nIPSE), a secreted egg antigen we purified from egg extracts, in a dose-dependent manner during 60 minutes *in vitro* culture (**Figure 7A**). During 3 days culture however, nIPSE induced significantly elevated IL-10 but not IL-6 secretion by B cells in a concentration dependent manner (**Figure 7B and S8A Figure**). Importantly, recombinant IPSE/alpha-1 expressed in tobacco plants (pIPSE), which behaves as nIPSE in terms of protein dimerization and human basophil activation (**suppl. Figure S9**), had similar effects to the natural molecule on B cell IL-10 and IL-6 secretion (**Figure 7C**). Both nIPSE and pIPSE-stimulated B cells were capable of driving Treg cell development during B cell-T cell co-culture (**Figure 7D**). Interestingly, SEA depleted of IPSE/alpha-1 (SEADIPSE) was as efficient as total SEA in inducing IL-10 secretion and CD86 expression by B cells, which suggests that also SEA antigens other than IPSE/alpha-1 can activate B cells (**Figure 7B**).

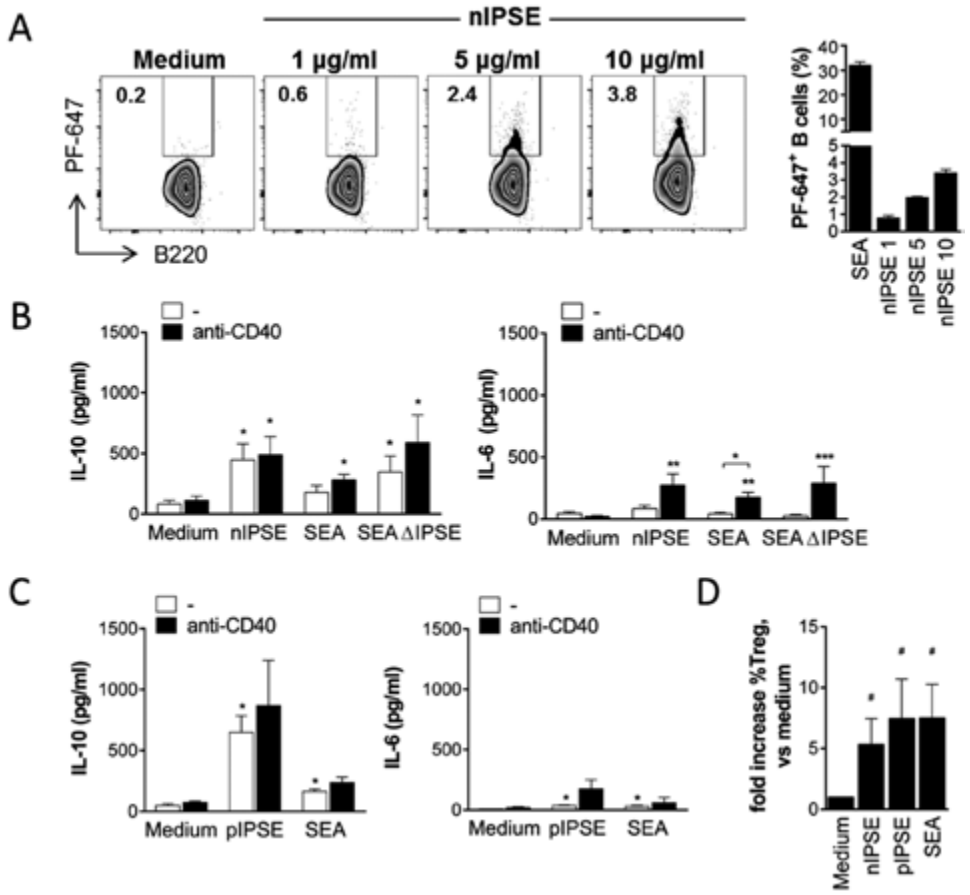

Figure 7. The secretory SEA component IPSE/alpha-1 induces Breg cell development. (A) Splenic B cells were incubated with PF-647-labeled SEA (20 µg/ml), natural IPSE/alpha-1 (nIPSE, 1 – 5 - 10 µg/ml) or left untreated and the frequency of SEA- and IPSE-positive cells measured after 60 minutes by flow cytometry. Representative FACS plots and summary of 3 experiments is shown (N = 3). **(B)** Splenic B cells from naïve mice were cultured for 3 days with 10 µg/ml nIPSE, 20 µg/ml SEA, 20 µg/ml SEAΔIPSE or medium as control, with or without addition of anti-CD40 mAb (2 µg/ml). Cytokine concentration in supernatants after 3 days as determined by ELISA. Summary of 4 experiments. **(C)** Splenic B cells from naïve mice were cultured for 3 days with 10 µg/ml recombinant, plant-derived IPSE (pIPSE), 20 µg/ml SEA or medium as control. Cytokine concentration in supernatants after 3 days as determined by ELISA. Summary of 4 experiments. **(D)** Splenic B cells were cultured for 3 days with nIPSE (10 µg/ml), SEA (20 µg/ml) or medium alone as control, and subsequently co-cultured with $CD4^+CD25^-$ sorted splenic T cells from C57BL/6 or DEREG mice. After 4 days, the frequency of $CD25^+Foxp3^+$ Treg cells within the CD4 T cell population was determined by flow cytometry. Summary of 3 experiments. Significant differences to medium control are indicated with * $p < 0.05$, as tested by Mann-Whitney test. # $p < 0.05$ indicates significant difference by one-sample t-test of log-transformed data.

However, as opposed to IPSE/alpha-1, other major components in SEA, such as omega-1 and kappa-5, did not increase IL-10 in any of the tested concentrations (1-20 µg/ml) (**suppl. Figure S8B**), thus excluding a role for these antigens in SEA-mediated Breg cell induction. Notably, omega-1 is toxic to B cells at concentrations of 5 µg/ml and above, and was therefore only tested at 1 µg/ml. For better comparability, we determined the following average relative amounts of IPSE/alpha-1, omega-1 and kappa-5 within SEA, based on the yields of several purifications of these molecules from SEA: 1.2%

IPSE/alpha-1, 0.6% omega-1 and 1.8% kappa-5. Furthermore, to proof that the Breg cell-inducing effect is specific for molecules in SEA, we stimulated B cells *in vitro* with adult worm antigen (AWA) as control of an *S. mansoni*-derived antigen mixture not containing IPSE. AWA was unable to induce IL-10 secretion when tested in the same concentration as used for SEA (**suppl. Figure S8C**). For a possible future therapeutic application of antigen-activated Breg cells against e.g. allergic diseases, it is crucial to confirm the IL-10-inducing effect in human B cells. After 3 days *in vitro* stimulation with SEA and anti-CD40, we found a significant increase in the percentage of total human IL-10$^+$ CD19$^+$ B cells compared to cells cultured with anti-CD40 alone. Comparing different B cell subsets, which have previously been attributed with regulatory properties [54,55], we found the increase in IL-10$^+$ B cells after SEA stimulation to be most pronounced among CD1d$^+$ B cells rather than CD24$^+$CD27$^+$ and CD24$^+$CD38$^+$ B cells. Both nIPSE and pIPSE significantly increased the fraction of IL-10-expressing cells among CD1d$^+$ B cells, whereas neither had an effect on the other two subsets investigated. As CD1d$^+$ B cells only comprise a very small fraction of all B cells (CD24$^+$CD27$^+$ B cells >> CD24$^+$CD38$^+$ B cells > CD1d$^+$ B cells), the effect of nIPSE and pIPSE does not translate into an increase in the percentage of IL-10$^+$ cell in the total B cell pool in contrast to SEA (**Figure 8**). This also suggests that additional molecules in SEA may have an IL-10-inducing effect. Collectively, we demonstrated that SEA was bound to and internalized by B cells, and that this direct interaction drives the development of Breg cells. Furthermore, we identified IPSE/alpha-1 as a single molecule of SEA that induces Breg cells in mice and humans, both as a natural and a recombinant molecule.

DISCUSSION

In this study, we sought to identify the molecules and mechanisms involved in the induction of IL-10-producing B cells by the helminth *S. mansoni*. We found that soluble antigens derived from schistosome eggs, amongst which the secretory antigen IPSE/alpha-1, directly interacted with B cells. This led to the development of Breg cells characterized by IL-10 secretion and Treg cell-inducing capacity. Next to the potentially therapeutic relevance of how to generate regulatory, anti-inflammatory cells, this study also provides mechanistic insight into how schistosomes interact with the host immune system, expanding the regulatory arm of immunity and thereby prolonging its survival in the host.

While we and others have shown IL-10 expression in splenic Breg cells during natural infection with *S. mansoni* [8,11,12], the contribution of *S. mansoni*-derived egg antigens was not yet studied. SEA is highly immunostimulatory and well-known to promote Th2 as well as Treg cell responses in the host [56]. Here, we found that *S. mansoni* egg antigens are also able to induce IL-10-producing B cells *in vivo*, without the context of natural infection. Because SEA is a complex mixture of several different antigens, it is not unexpected that different types of immune cells and qualities of immune responses are induced. The use of Breg cells as a therapeutic tool against inflammatory diseases is especially attractive because Breg cells in turn can induce Treg cell development [57], which would thus amplify the beneficial regulatory effect. It was therefore important to investigate whether SEA-induced IL-10-producing B cells have the capacity to trigger Treg cell development. This was indeed the case as we could show by *in vitro* co-cultures of T cells with egg antigen- or IPSE-activated B cells. Similarly, Breg cells isolated from naturally schistosome-infected mice and humans were previously shown to drive Treg cell development *in vitro* [8,12,58], thus indicating a common feature of schistosome-induced Breg cells.

Our finding that egg antigens could induce splenic Breg cell development *in vivo* raised the question whether those antigens can directly interact with B cells in the spleen. In *in vivo* binding studies using fluorescently labeled SEA and fluorescence microscopy, egg antigens were indeed found to directly bind to splenic B cells. Although various egg antigens are abundantly glycosylated, the restricted and low expression of C-type lectin receptors by B cells [41] argues rather for the involvement of non-C-

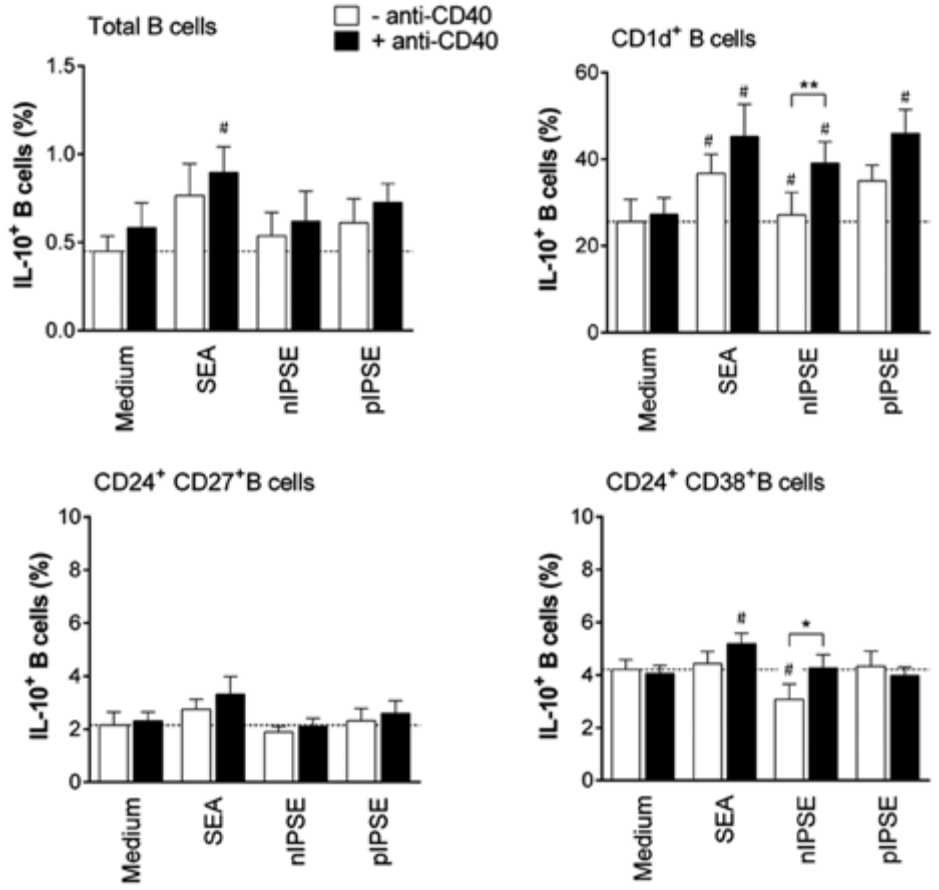

Figure 8. Human CD1d$^+$ Breg cells increase IL-10 expression after SEA and IPSE/alpha-1 stimulation. B cells were isolated from PMBCs of healthy volunteers and stimulated for 3 days with SEA (20 µg/ml), natural IPSE (nIPSE, 10 µg/ml), plant-derived IPSE (pIPSE, 10 µg/ml), or left untreated. Different Breg cell subsets and intracellular IL-10 expression were assessed by FACS. Summary of 3 donors. Significant differences to the respective medium control are indicated with $^{\#}$ $p < 0.05$, or between conditions with and without anti-CD40, with * $p < 0.05$, ** $p < 0.01$ as tested by paired t-test.

type lectin PRRs expressed by B cells in the direct binding of SEA components. Indeed, preliminary experiments with B cells of SIGN-R1-deficient mice, showed an equal IL-10 expression in response to SEA compared to wild-type littermates, suggesting no involvement of the Le^x-motifs, one of the most abundant glycan structures present in SEA and known to bind SIGN-R1. Instead, we found that within a mixture of schistosome antigens, at least the egg glycoprotein IPSE/alpha-1 was capable of driving Breg cell development *in vitro* by directly interacting with B cells, equipping them with Treg cell-inducing capacity. Two independent experimental approaches suggested that egg antigens are taken up and processed in acidic lysosomes. Previous reports on *in vitro* induction of Breg cells by helminth antigens did not use highly sort-purified B cells as in our study, but total splenocyte preparations [8,10] or merely B cell-enriched cultures [13]. Hence, it was impossible to exclude indirect, accessory cell type-mediated B cell stimulation or IL-10 production by other cell types [59,60]. Other reports addressed B cell activation by IL-10 production, but did not study the regulatory activity of schistosome antigen-

exposed B cells compared to unstimulated B cells [14]. It must be emphasized that the sole demonstration of upregulated IL-10 expression is not sufficient to characterize B cells as Breg cells, as IL-10 can fulfill other roles in B cell biology independent from a regulatory function. We thus present the first report on direct induction of functional Breg cells with *in vitro* regulatory activity by helminth antigens.

With respect to the development of therapeutic applications, it would be interesting to see whether SEA-induced Breg cells are more qualified than Breg cells induced by other compounds, like TLR7 or TLR9 ligands. Opposite to SEA, stimulants like R848 and CpG also induce substantial amounts of B cell proliferation and pro-inflammatory cytokines like IL-6 in addition to inducing high levels of IL-10. It is currently unknown which side-effects would result in a therapeutic application, but it is tempting to speculate that compounds that selectively induce IL-10 are more preferable. We tried to compare IL-10-producing B cells induced *in vitro* by different agents, including SEA, for their capacity to inhibit allergic airway inflammation *in vivo*. We were however not able to confirm a suppressive capacity for any of the conditions despite the usage of a published model [31]. In the past, we have successfully applied adoptive transfers of *in vivo*, schistosome-induced Breg cells (generated during a natural infection) in allergic airway inflammation models [12]. Therefore, we assume that underlying differences between *in vivo* and *in vitro* stimulation of Breg cells may be crucial for the activity in a disease model. This may be related to issues like a differential homing or to the strength and kinetics of activation and cytokine production which determine the suppressive capacity of Breg cells on bystander immune activation in the host. Knowing that schistosome antigens can directly induce Breg cell development, we next addressed signals that regulate or enhance antigen-induced B cell IL-10 expression. In previous studies, CD40 engagement was described to induce B cell IL-10 expression [18,19,50,51,59]. This is in line with our results showing that SEA-induced IL-10 expression was significantly increased by addition of agonistic CD40 Ab. This points to the potential of a combined therapy for inflammatory diseases using helminth antigens together with anti-CD40 Ab treatment. Indeed, a report by the group of Mauri *et al.* provided evidence that experimental therapy with an agonistic Ab against CD40 can ameliorate autoimmune disease [61], as did cellular therapy with Breg cells [50], although a combined treatment was not yet tested. The groups of Fillatreau and Mauri proposed a two-step model for the acquisition of regulatory properties by B cells, with exposure to innate stimuli – such as TLR ligands – as one step and CD40 or BCR engagement as second step to establish Breg cell function [36,50]. We found a similar dependency for *in vivo* Breg induction by helminth antigen, for which CD40 ligation was crucial. Our data also show that, although Breg cell induction *in vitro* can be achieved alone without additional stimuli, engagement of CD40 further enhances this effect. Several cell types including T cells, B cells, DCs, basophils, NK cells, mast cells and macrophages express CD40 ligand (CD40L, CD154) [62] and could in principle serve as interaction partner ligating CD40 on B cells. It is however tempting to speculate that neutrophils play a role as they have been reported to express CD40L and activate MZ B cells for immunoglobulin production in a contact-dependent manner [63].

As we found MZ B cells to be the main IL-10-producing B cell subset, it was tempting to speculate that accessory cell types of the splenic marginal zone interact with schistosome antigens, and subsequently drive the development of MZ Breg cells. Macrophages of the splenic MZ were of particular interest because of their known interactions with MZ B cells during steady state [28,42] and their expression of SIGN-R1. This C-type lectin receptor was found to bind schistosome antigens *in vitro* by using SIGN-R1-overexpressing fibroblasts [43,44]. However, it was unknown whether MZ macrophages can capture SEA *in vivo* and are important for B cell IL-10 expression. As hypothesized, we found macrophages of the MZ to efficiently bind SEA upon *in vivo* administration. However, Breg cell induction was not affected upon *in vivo* depletion of macrophages, thus excluding a major role of macrophages in this process. Indirectly, also Mangan *et al.* [10] showed that macrophages were dispensable for Breg cell induction during schistosomiasis, as macrophage depletion did not affect the B cell-mediated control

of anaphylaxis. The immunological role of SEA-binding MZ macrophage subsets and the identity of the binding receptor remain to be determined.

A limited number of reports is available that used specific helminth antigens to induce B cell IL-10 expression, namely the filarial antigen ES-62 [64] and the oligosaccharide lacto-N-fucopentaose III (LNFP III) that contains the Le^X trisaccharide antigen present on various schistosome glycoproteins [13]. Both antigens were either applied *in vivo* or used *in vitro* for stimulation of B cell-enriched cultures that still contained other cells, which means it remains unclear whether those antigens can directly bind to and interact with B cells, without indirect support from other cell types. Our study therefore identified IPSE/alpha-1 as the first helminth molecule with direct Breg cell-inducing capacity in mice and humans. Importantly, this capacity was resembled by recombinant IPSE, which is an important prerequisite for a possible therapeutic use. The use of helminth molecules for therapeutic purposes has gained renewed interest as controlled human infections with helminths showed disappointing effects in recent phase II and phase III trials (reviewed in [65]). More studies are required to define the optimal antigen, dose, time point and length of treatment as well as the suitability to treat specific inflammatory diseases [66,67]. Therefore, the identification of helminth-specific Breg-inducing antigens is warranted, even more so as the availability of active recombinant forms will ultimately allow its production under GMP conditions.

IPSE/alpha-1 was originally described as basophil IL-4-inducing principle of *Schistosoma* eggs [53] and was in addition shown to induce a mixed Th1/Th2 type of immune response in spleen upon *in vivo* administration [68]. In our assays, IPSE/alpha-1 directly interacted with murine B cells via a still unknown receptor, which led to activation, induction of B cell IL-10 secretion and Treg cell induction *in vitro.* Although IPSE/alpha-1 is a highly glycosylated protein, we consider a role of IPSE-related glycans as unlikely because both, pIPSE and nIPSE were capable to induce B cell IL-10 expression despite differences in glycosylation (native IPSE contains Le^x motifs [69], while plants per definition cannot make Le^x motifs [70]. In addition, natural omega-1 and IPSE share a similar glycosylation (Le^x related) but have opposing activities both in B cells (here) and on DCs [71]. IPSE/alpha-1 has been shown to not only bind to IgE but also to IgG, both to Fc and Fab fragments [53]. We therefore hypothesize that IPSE could bind to B cells via the B cell receptor or surface-exposed IgG. Particularly important for a possible therapeutic use is our finding that both natural and plant-derived IPSE/alpha-1 induced IL-10 expression also in human $CD1d^+$ Breg cells. This is even more intriguing as the $CD1d^+$ B cell subset has been previously described to be increased in number and activity both in experimental infections in mice and in people living in endemic areas [12,58]. We propose a mechanism of schistosome-induced Breg cell induction in which B cells directly interact with schistosome egg antigens by binding and internalizing antigen, lysosomal processing and subsequent up-regulation of CD86 and IL-10 expression. The MZ B cell subset appeared to be particularly responsive, and CD40 engagement further enhanced Breg cell activity. Furthermore, we have successfully identified the secreted egg antigen IPSE/alpha-1 as one of the Breg-inducing antigens. These egg antigen-induced Breg cells were potent in driving Treg cell development, allowing for induction of two potent regulatory responses by the same antigen. To our knowledge, our study provides the first description of a helminth-specific molecule that interacts with and induces Breg cells, and a mechanistic insight into how schistosomes interact with their host, influence its regulatory immunity and thereby promoting their prolonged survival in the host.

MATERIAL AND METHODS

Animals

Female C57BL/6OlaHsd mice from Harlan, TLR4-deficient mice (on C57BL/6 genetic background, kindly provided by Dr. S. Akira, Osaka, Japan), TLR2-deficient mice (on C57BL/6 genetic background, kindly provided by the group of Dr. K. Willems van Dijk), SIGN-R1-deficient mice (on C57BL/6 genetic background, kindly provided by the group of Dr. W. Unger), DEREG (DEpletion of REGulatory T

cells) mice (on C57BL/6 genetic background, kindly provided by Dr. T. Sparwasser) and IL-10-GFP reporter (TIGER) mice (on C57BL/6 genetic background, kindly provided by Dr. R.A. Flavell) were housed under SPF conditions in the animal facilities of the Leiden University Medical Center in Leiden, The Netherlands, and used for experiments at 8-14 weeks of age. Percutaneous infection of mice with *S. mansoni* was performed as described elsewhere [12], and mice sacrificed in the chronic phase (14-15 weeks) post infection.

Preparation and purification of schistosome eggs, egg and worm antigens

Freshly isolated *S. mansoni* eggs from trypsinized livers of hamsters infected for 50 days were washed in RPMI medium with 300 U/ml penicillin, 300 µg/ml streptomycin (both Sigma-Aldrich, Zwijndrecht, The Netherlands) and 500 µg/ml amphotericin B (Thermo Fisher Scientific, Breda, The Netherlands) and then kept at -80°C. SEA, AWA, omega-1, kappa-5 and IPSE/alpha-1 were prepared and isolated as described previously [53,71,72,73]. The purity of the antigen preparations was checked by SDS-PAGE and silver staining, and protein concentrations determined using the BCA procedure. The antigen preparations had an endotoxin content of less than 150 ng/mg protein (SEA) or 3 ng/mg protein (purified molecules) as tested by Limulus Amoebocyte Lysate (LAL) test and TLR4-transfected HEK-reporter cell lines (kindly provided by Prof. Golenbock, University of Massachusetts Medical School, Boston, USA).

Production and purification of recombinant IPSE from *N. benthamiana* plants

Recombinant IPSE was produced by transient expression in *Nicotiana benthamiana* and purified according to the methods described in [70]. In short, the complete sequence encoding the 134 AA mature *Schistosoma mansoni* IPSE (Smp_112110) was codon optimized and preceded by a signal peptide from the *Arabidopsis thaliana* chitinase gene (cSP) and a N-terminal 6x histidine-FLAG tag (H6F) was included. The full sequence was synthetically constructed at GeneArt and cloned into a pHYG expression vector. In all experiments the silencing suppressor p19 from tomato bushy stunt virus in pBIN61 was co-infiltrated to enhance expression. For gene expression the two youngest fully expanded leaves of 5-6 weeks old *N. benthamiana* plants were infiltrated by injecting *Agrobacterium tumefaciens* containing the IPSE expression plasmid. *N. benthamiana* plants were maintained in a controlled greenhouse compartment (UNIFARM, Wageningen) and infiltrated leaves were harvested at 5-6 days post infiltration. Plant-produced recombinant IPSE was obtained by applying leaf apoplast fluid containing IPSE to Ni-NTA Sepharose (IBA Life Sciences) in 50 mM phosphate buffered saline (pH 8) containing 100 mM NaCl. Bound IPSE was eluted with phosphate buffered saline (pH 8) containing 0.5M imidazole. Total soluble apoplast proteins and purified IPSE were separated under reducing/non-reducing conditions by SDS-PAGE on a 12% Bis-Tris gel (Invitrogen) and subsequently stained with Coomassie brilliant blue staining.

Isolation of splenocytes, B cells and T cells

Single cell suspensions of murine spleens were prepared by dispersion through a 70 µm cell strainer (BD Biosciences, Breda, The Netherlands), and erythrocytes depleted by lysis. For analysis of splenic myeloid cell populations, spleens were digested for 1 hour at 37°C by incubation with collagenase D (2 mg/ml; Roche, Woerden, The Netherlands) and DNase I (2000 U/ml; Sigma-Aldrich) before dispersion. B cells were purified from spleens by using anti-CD19 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands) following the manufacturer's protocol. Purity was routinely ~95-98%. After a typical CD19 MACS sort, circa 83% of all contaminating cells were $CD3^+$ T cells, 7% $CD11b^+CD11c^+$ cells, 2% $CD11b^+$ $CD11c^-$ cells, and 8% other cells. To determine cytokine secretion of splenic B cell subsets,

CD19$^+$ B cells were subsequently sorted by flow cytometry for follicular B cells (CD23$^+$CD21^{low}) and marginal zone B cells (CD23$^-$CD21^{hi}) which resulted in purities of > 98%. CD4$^+$ T cells were purified from spleens by negative selection and depleted of CD25-expressing cells using anti-CD25 MicroBeads (Miltenyi Biotec).

Isolation of human B cells from PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy volunteers by Ficoll gradient centrifugation, and B cells were purified from PBMCs by using anti-CD19 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands) following the manufacturer's protocol.

***In vitro* murine and human B cell stimulation**

Mouse splenic CD19$^+$ B cells (1.5x10^6/ml) were cultured in medium (RPMI 1640 glutamax; Thermo Fisher Scientific), containing 5% heat-inactivated Fetal Bovine Serum (FBS; Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 5 × 10^{-5} M 2-Mercaptoethanol (Sigma-Aldrich) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Sigma-Aldrich). Human B cells (1.5 x 10^6/ml) were cultured in medium (RPMI 1640; Thermo Fisher Scientific), containing 10% heat-inactivated Fetal Bovine Serum, pyruvate (1 mM), glutamate (2 mM) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; all Sigma-Aldrich). The following stimuli were added as indicated in the figure legends: SEA (20 µg/ml), SEA depleted of IPSE (SEADIPSE, 20 µg/ml), natural (1, 5, 10, 20 µg/ml) or plant-derived IPSE (10 µg/ml), omega-1 (1 µg/ml), kappa-5 (1, 5, 10, 20 µg/ml), AWA (5, 10, 20 µg/ml). For some conditions, co-stimulatory rat anti-mouse CD40 antibody (2 µg/ml; clone 1C10; BioLegend, Uithoorn, The Netherlands) or goat anti-mouse IgM (0.5, 1, 2 µg/ml; Jackson ImmunoResearch, Suffolk, UK) was added to the culture. After 3 days culture at 37°C, supernatants were collected for cytokine analysis by ELISA. Cells were restimulated 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) for flow cytometric analysis of intracellular IL-10. In experiments addressing involvement of lysosomal acidification, the inhibitor chloroquine (5 µM; Sigma) was added at the start of a two days culture and refreshed after 24 hours, and the TLR ligands CpG ODN 1826 (5 µg/ml; Invivogen) or Pam3Cys (10 µg/ml; Invivogen) used as control stimuli next to SEA.

***In vivo* experiments**

For *in vivo* stimulation of B cells, mice were i.p. injected with two doses of 5000 eggs or 100 µg SEA in PBS, determined as optimal doses where B cell IL-10 production plateaued in prior dose-titration experiments, and PBS or 100 µg human serum albumin (HSA) in PBS as control 7 days apart. At day 14 after the first injection, splenic B cells were harvested and cultured in medium at 1.5 x 10^6 cells/ml or restimulated for 2 days with SEA (20 µg/ml) to allow detection of cytokines, as established for *in vivo* schistosome-exposed B cells before [12]. Supernatants were collected to determine cytokine concentration by ELISA. Cells were cultured for additional 4 hours with Brefeldin A (10 µg/ml; Sigma-Aldrich) to detect intracellular IL-10 by flow cytometry. In some experiments, mice were treated i.p. with 200 µg of hamster anti-mouse CD40 ligand blocking antibody (clone: MR1) or 200 µg hamster IgG as control (Jackson ImmunoResearch) 4 times, every 4 days starting one day before SEA or PBS treatment. For *in vivo* depletion of macrophages, mice were i.p. injected with 200 µl clodronate-containing liposomes and control mice with 200 µl of PBS liposomes (ClodronateLiposomes.com, Amsterdam, The Netherlands) [45] three weeks prior to egg antigen treatment. Successful and specific depletion of splenic macrophage subsets was confirmed by fluorescence microscopy and flow cytometry.

Co-culture with $CD4^+CD25^-$ T cells

In vitro or *in vivo* SEA-stimulated $CD19^+$ B cells were co-cultured with MACS-sorted $CD4^+CD25^-$ T cells at 1:1 ratio (each 1 x 10^6/ml) to test for *in vitro* Treg cell induction. After 4 days, Treg cell frequencies were determined by flow cytometry by gating for $Foxp3^+CD25^+$ cells in the $CD3^+CD4^+$ T cell population, and culture supernatants collected for subsequent ELISA.

Antigen binding assays

SEA, IPSE/alpha-1 and ovalbumin (OVA) were fluorescently labeled with PF-488 or PF-647 using the PromoFluor labeling kits (PromoCell, Heidelberg, Germany) according to the manufacturer's protocol. For some experiments, SEA was co-labeled with the pH-sensitive pHrodo Red dye (Thermo Fisher Scientific). After protein labeling, non-reacted dye was removed using Zeba desalt spin columns (Thermo Fisher Scientific). For analysis of binding *in vitro*, $CD19^+$ splenic B cells were cultured for 60 minutes at 37°C with 20 µg/ml fluorescently labeled SEA or 1-10 µg/ml of IPSE antigen, then washed in ice-cold PBS before analysis by flow cytometry. For analysis of *in vivo* binding, mice were i.v. injected with 200 µg of fluorescently labeled SEA or OVA as non-schistosomal control protein and spleens snap-frozen 30 minutes to 24 hours later. Binding of SEA to B cells was analyzed by confocal fluorescence microscopy of tissue sections and by flow cytometry.

Induction of IL-4 release from human basophils

Basophils were purified from 250 ml of peripheral blood of healthy human donors to a mean purity of 99% by a three-step protocol consisting of a density gradient centrifugation via Ficoll/Percoll (100/6, density 1.080 g/l), followed by enrichment of the basophils via counter flow elutriation and final purification by magnetic cell sorting using the basophil isolation kit II for negative selection of basophils (Miltenyi-Biotech). Purified basophils were cultured in Iscove's Modified Dulbecco's Media (IMDM; PAA) containing 2 mM glutamine (PAA), 5 µg/ml insulin (Gibco), 50 µg/ml apo-transferrin (Sigma-Aldrich), 100 µg/ml Pen/Strep (PAA), 10% heat-inactivated Fetal Calf Serum (FCS-Gold; PAA) and 2.5 ng/ml IL-3 (kind gift of Kirin Brewery, Japan). Basophils were pre-incubated for regeneration for 30 min at 37°C, 6% CO_2, and then stimulated at a concentration of 0.025 x 10^6 basophils /ml in 96well flat-bottom culture plates in 100 µl at 37°C, 6% CO_2. Concentration of stimuli was as indicated. Culture supernatants were collected after 18h and stored at -20°C.

Flow cytometry

Flow cytometric analysis of murine B cells was performed by staining with fluorochrome-labeled antibodies against CD19, CD21 (both BD Biosciences), CD23, CD40, CD86 or IL-10 (all eBioscience) after fixation with 1.9% paraformaldehyde and permeabilization with 0.5% saponin (Sigma-Aldrich). Human B cells were stained for CD19, CD38 (both BD Biosciences), CD24, CD27 (both eBioscience), CD1d, IL-10, TNF (all Biolegend), and CD39 (Sony Biotechnology, San Jose, USA). Splenic myeloid cell subsets were discriminated using fluorochrome-labeled antibodies against CD11b, CD11c (both eBioscience), CD8, Ly6C (both Biolegend), F4/80 (AbD Serotec, Puchheim, Germany), Gr-1 (BD Biosciences), and Siglec-1 (Dr. J. den Haan, VUMC, Amsterdam, The Netherlands). Treg cells were fixed and permeabilized with the eBioscience Foxp3 fixation/permeabilization kit and stained using fluorochrome-labeled antibodies against CD3, CD4, Foxp3 (all eBioscience) and CD25 (BD Biosciences). All cells were stained with Aqua dye (Thermo Fisher Scientific) prior to fixation to discriminate dead cells. For all flow cytometric stainings, FcgR-binding inhibitor (2.4G2) was added and FMOs were used for gate setting. Flow cytometry was performed using a FACSCanto or Fortessa (BD Biosciences).

ELISA

The concentration of murine IL-6 and IL-10 as well as human IL-4 present in culture supernatants was quantified by commercial ELISA kits according to the manufacturer's instructions (BD Biosciences or Eli-Pair, Diaclone).

Confocal microscopy

Spleens were snap-frozen in O.C.T. medium (Tissue-Tek; Sakura, Alphen aan den Rijn, The Netherlands). Cryosections (10 µm) were fixed in ice cold acetone for 10 minutes, air-dried, and blocked in 1% BSA plus 20% FBS in PBS before staining with Abs at room temperature. Cryosections were incubated with rat anti-mouse Siglec-1 (clone SER-4; provided by Dr. J. den Haan, VUMC, Amsterdam, The Netherlands) followed by Alexa555-conjugated goat anti-rat IgG (Invitrogen), anti-SIGN-R1 Alexa647 (clone 22D1; Dr. J. den Haan) and anti-B220 eFluor450 (eBioscience). Images were acquired using a Zeiss LSM 710 confocal laser scanning microscope with Zen software (Carl Zeiss Microimaging, Jena, Germany).

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) using nonparametric Mann-Whitney U test to compare different groups and Wilcoxon paired test to compare B cell subsets. One-sample t-test of log-transformed data was applied to calculate significant changes for data which are expressed as fold increase. All p-values < 0.05 were considered significant.

Ethics statement

All animal studies were performed in accordance with the Animal Experiments Ethical Committee of the Leiden University Medical Center (DEC-12204). The Dutch Experiments on Animals Act is established under European Guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). For the isolation of B cells from PBMCs, human subjects were recruited within the framework of the study P09.170, which was approved by the Medical Ethical Committee of the Leiden University Medical Center. For the isolation of basophils, donors were recruited under approval by the Ethics Committee of the University of Luebeck (AZ-12-202A). Studies were performed according to the declaration of Helsinki and all participants were adults and have given written informed consent.

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SUPPLEMENTARY MATERIAL

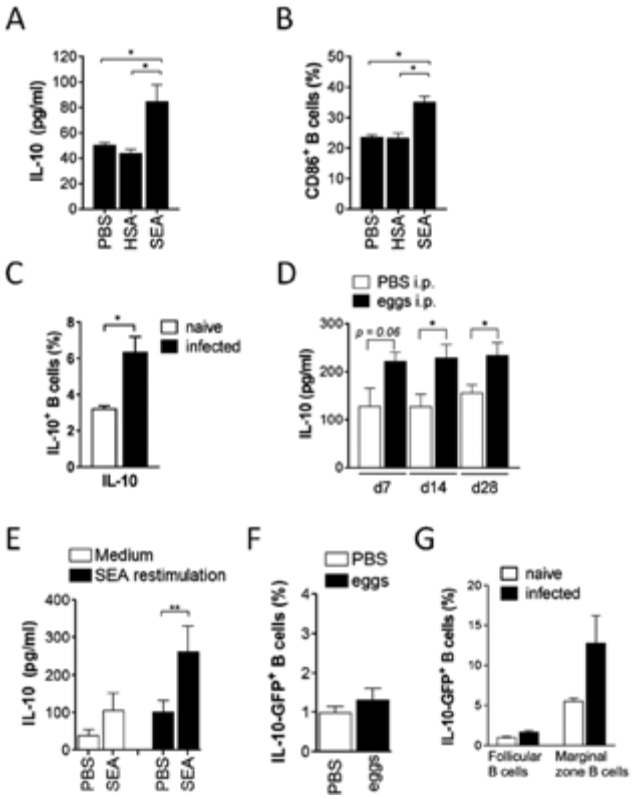


Figure S1. Additional data on the specificity and persistence of egg antigen-induced B cell activity. (A-B) A non-schistosomal control protein does not activate B cells. Wild-type mice were i.p. injected with two doses of 100 µg SEA in PBS, 100 µg human serum albumin (HSA) in PBS or PBS alone. At day 14, $CD19^+$ sorted splenic B cells were restimulated with SEA (20 µg/ml) for 2 days. **(A)** IL-10 concentration in culture supernatant as determined by ELISA. **(B)** Mean fluorescence intensity of CD86 expression as determined by FACS. One out of 2 similar experiments is shown. **(C)** Intracellular IL-10 expression of splenic B cells isolated from C57BL/6 mice infected chronically (14 weeks) with *S. mansoni*, compared to naïve control mice. **(D)** IL-10-producing B cells can be detected at least 28d after egg injection. Wild-type mice were injected twice with 5000 *S. mansoni* liver eggs (d-7, d0) by i.p. injection. Splenic B cells were isolated on d7, d14 and d28 after the last egg injection and re-stimulated *in vitro* with SEA (20 µg/ml) for 2 days. Supernatants were harvested and the concentration of IL-10 determined by ELISA. One experiment with n=5 mice per group. * $p < 0.05$ as determined by students t-test. **(E)** Wild-type mice were treated twice with SEA (100 µg) or PBS by i.p. injection. On day 14, splenic B cells were isolated and re-stimulated for 2 days with SEA or left untreated (medium). IL-10 concentration in culture supernatants as assessed by ELISA. Summary of 5 experiments. **(F, G)** IL-10 reporter (TIGER) mice were treated twice with 5000 *S. mansoni* eggs **(F)** or infected with *S. mansoni* until the chronic phase of infection (14 weeks) **(G)**. The percentage of IL-10-GFP+ total B cells or follicular (FO) and marginal zone (MZ) B cells within the spleen as assessed by FACS. Summary of three experiments with N=15 mice per group (F) or one experiment with N=2-4 mice per group (G). Significant differences are indicated with * $p < 0.05$ and ** $p < 0.01$ as tested by Mann-Whitney test.

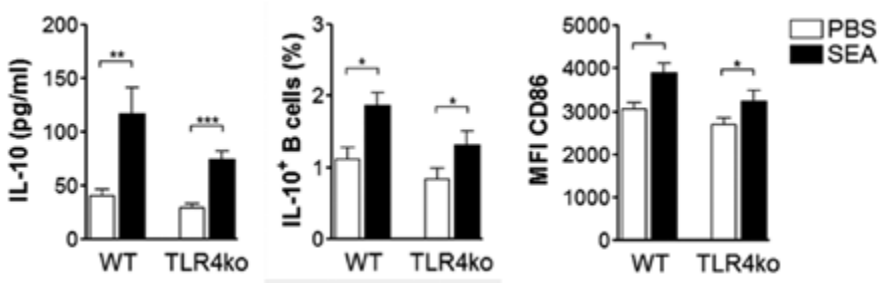


Figure S2. B cell IL-10 induction by egg antigens is independent of TLR4. C57BL/6 wild-type and TLR4-deficient mice were i.p. injected with two doses of 100 µg SEA in PBS, or PBS as control. At day 14, $CD19^+$ sorted splenic B cells were restimulated with SEA (20 µg/ml) for 2 days. Secreted IL-10, intracellular IL-10 and CD86 expression of B cells are shown. Significant differences are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as tested by Mann-Whitney test.

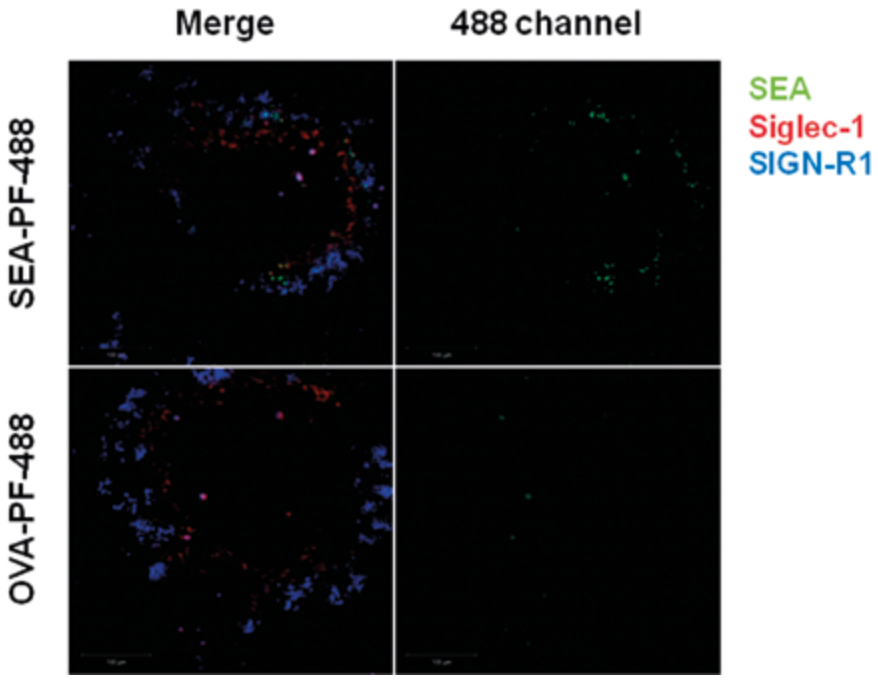


Figure S3. Macrophage subsets of the MZ do not bind the control protein ovalbumin. Spleens were snap-frozen 30 minutes after i.v. injection of 200 µg fluorescently labeled SEA or ovalbumin (OVA), and binding analyzed by fluorescence microscopy. SEA but not OVA localized in the marginal zone to macrophages expressing Siglec-1 (marginal metallophilic macrophages) and SIGN-R1 (MZ macrophages). Images are representative of N = 5 mice and 3 follicles per section imaged.

Figure S4. Depletion control for splenic cell subsets and Treg cell activity after clodronate treatment. (A-C) Gating scheme and representative FACS plots of splenocyte subsets which were subsequently analyzed for *in vivo*-captured fluorescently labeled egg antigens (shown in **Figure 3**). Splenocytes were pre-gated for living singlets. **(A)** Red pulp macrophages were gated as F4/80^{+}CD11b^{int}, marginal metallophilic (MM) macrophages are Siglec-1-positive and highly autofluorescent. **(B)** Dendritic cells were gated CD11c^{hi}CD11b$^{int/+}$ and further divided into CD8^{+}CD11b^{int} and CD8^{-}CD11b^{+} subsets. **(C)** Monocytes were gated as CD11b^{+}Ly6C^{hi}Gr-1^{int}, neutrophils as CD11b^{+}Ly6C^{int}Gr-1^{hi}. **(D)** Frequency of splenic cell types before (0 days) and at 1-28 days after i.p. injection of clodronate-containing liposomes. Only macrophage frequencies were significantly reduced at day 21 and 28. **(E)** Frequency of splenic cell types at the time-point of B cell analysis in egg-treated mice (day 7 after the second egg injection, i.e. day 35 after clodronate treatment). **(F,G)** Macrophage depletion prior to SEA treatment does not change the frequency of Treg cells. Mice were treated with chlodronate and injected with SEA i.p. The percentage of Foxp3^{+} CD25^{+} Treg cells in splenocytes **(F)** and IL-10^{+} CD25^{+} T cells **(G)** as assessed by FACS. Summary of 2 experiments with N=4-6 mice per group (D), N=6-12 (E), or data from one experiment with N=5 mice per group (F, G). Significant differences to the respective control (D, day 0; E-G, PBS or eggs) are indicated with $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ as obtained by Mann-Whitney test.

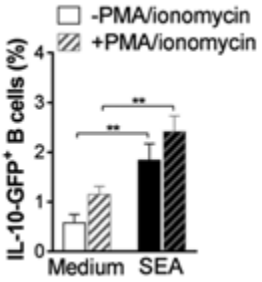


Figure S5. The IL-10 phenotype is not reliant on artificially high stimulation with PMA/ionomycin. Splenic B cells from IL-10-GFP (TIGER) mice were cultured for 2 days with 20 µg/ml SEA or medium as control. Intracellular IL-10 expression of total B cells as assessed by GFP signal in the presence or absence of PMA and ionomycin during the last 4 hours of the culture. Data are from 1 experiment with N=5 per group.

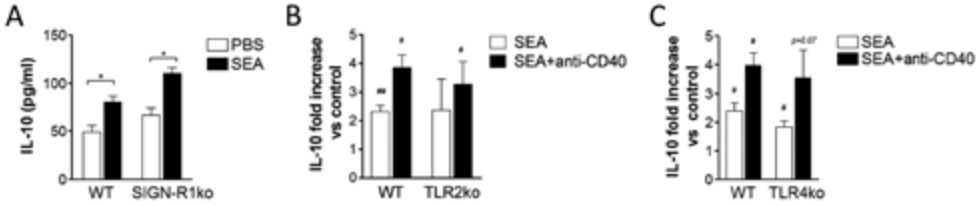
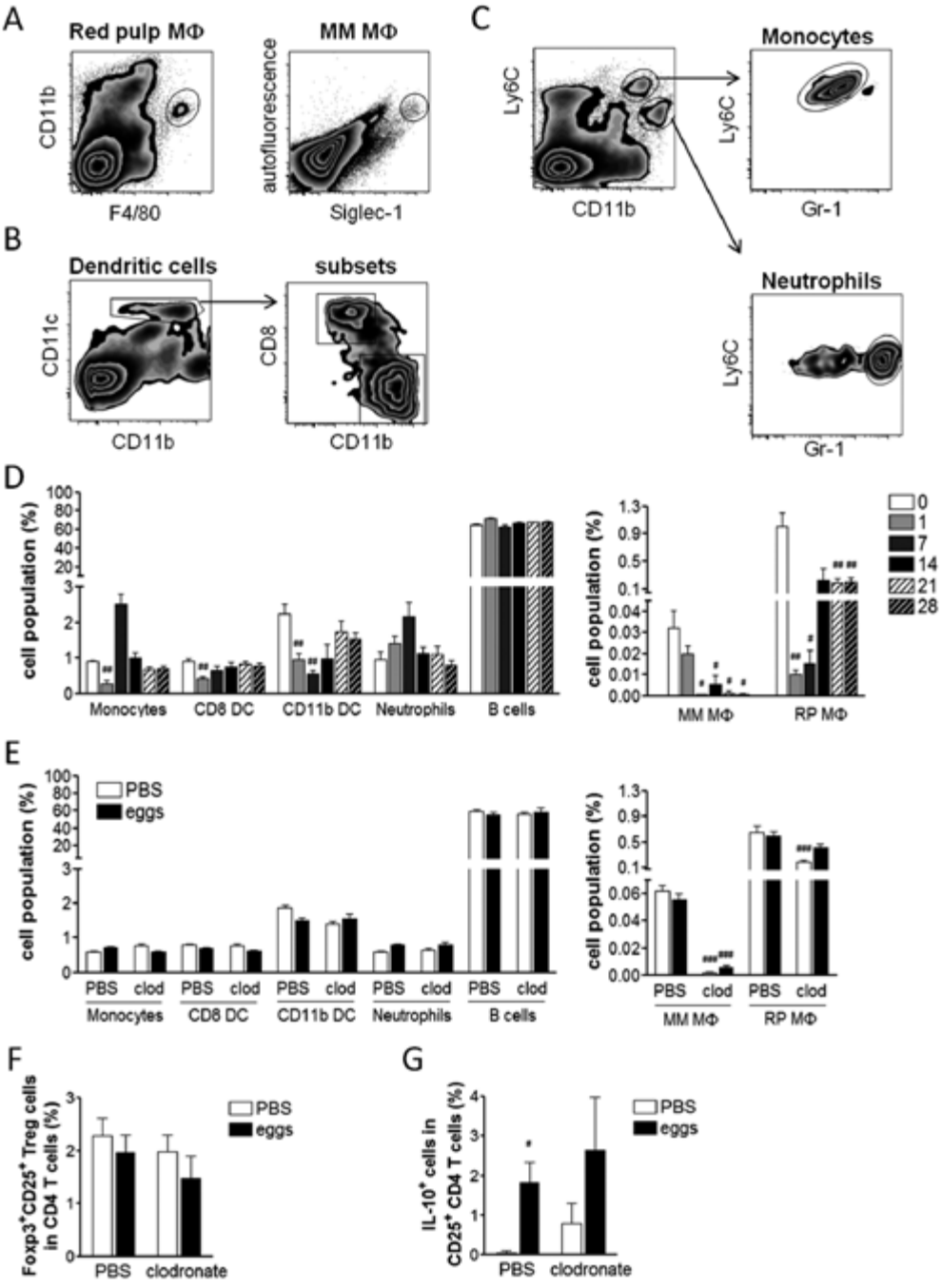


Figure S6. **B cell IL-10 induction by SEA is independent of SIGN-R1, TLR2 and TLR4. (A)** C57BL/6 wild-type or SIGN-R1-deficient mice were treated with 2 doses of SEA (each 100 µg) for 2 weeks or PBS as control. Secreted IL-10 was detected by ELISA after 2 days restimulation of splenic CD19^{+} B cells with SEA (20 µg/ml). Summary of N=2-6 mice per group. **(B, C)** Splenic B cells from naïve wild-type, TLR2-deficient **(B)** and TLR2-deficient **(C)** mice were cultured for 3 days with 20 µg/ml SEA or medium as control, with or without addition of anti-CD40 (0.5 µg/ml). IL-10 concentration in culture supernatants is expressed as fold increase versus the medium or anti-CD40 control in a summary of 3 experiments. Significance was tested by one-sample t-test of log-transformed data and is indicated by $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$.


A
Red pulp MΦ
CD11b
F4/80
MM MΦ
autofluorescence
Siglec-1
B
Dendritic cells
CD11c
CD11b
subsets
CD8
CD11b
C
Ly6C
CD11b
Monocytes
Ly6C
Gr-1
Neutrophils
Ly6C
Gr-1
D
cell population (%)
Monocytes
CD8 DC
CD11b DC
Neutrophils
B cells
MM MΦ
RP MΦ
0
1
7
14
21
28
E
PBS
eggs
cell population (%)
PBS clod
Monocytes
CD8 DC
CD11b DC
Neutrophils
B cells
MM MΦ
RP MΦ
F
Foxp3+CD25+ Treg cells in CD4 T cells (%)
PBS
clodronate
G
IL-10+ cells in CD25+ CD4 T cells (%)
PBS
clodronate

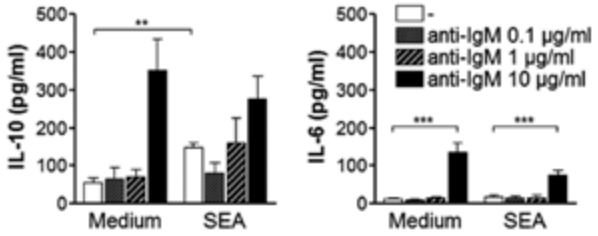


Figure S7. Anti-IgM Ab does not further increase SEA-induced IL-10 expression of B cells. Splenic B cells from naïve mice were cultured with 20 µg/ml SEA or medium as control, with or without addition of anti-IgM Ab in different concentrations. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 by ELISA. Summary of 3 experiments. Significant differences are indicated with ** $p < 0.01$ and *** $p < 0.001$ as tested by Mann-Whitney test.

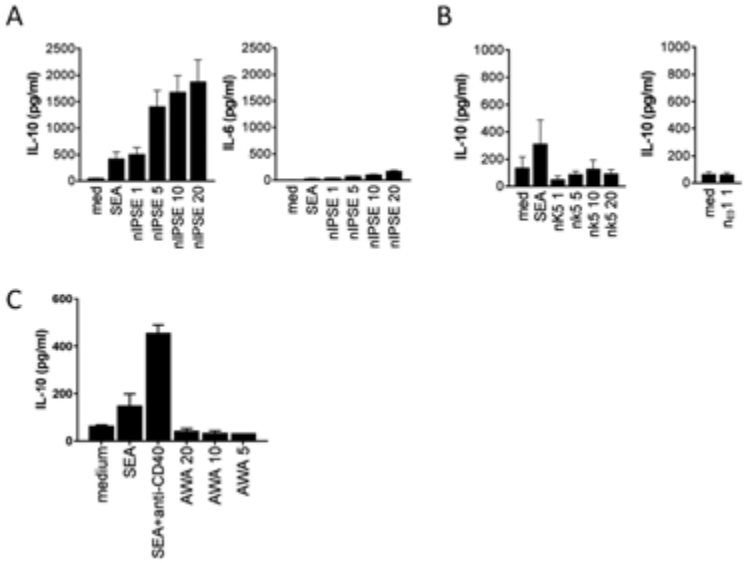


Figure S8. IPSE/alpha-1 activates Breg cells dose-dependently while the major egg antigens omega-1 and kappa-5 as well as worm antigen are ineffective. Splenic B cells from naïve mice were cultured for 3 days with different concentrations (indicated by numbers in the x-axis label) of natural (n) IPSE/alpha-1, omega-1 or kappa-5, or medium as negative and 20 µg/ml SEA as positive control. **(A)** IL-10 and IL-6 concentration in culture supernatants after nIPSE stimulation as measured by ELISA. Average of duplicates from one experiment shown. **(B)** IL-10 concentration in supernatants of kappa-5 or omega-1 stimulated B cells. Average of duplicates from one experiment shown. **(C)** Splenic B cells were stimulated *in vitro* with SEA (20 µg/ml), anti-CD40 (2 µg/ml) or adult worm antigen AWA (5 – 10 – 20 µg/ml) for 3 days. IL-10 in culture supernatant as determined by ELISA. Average of duplicate values from one experiment shown.

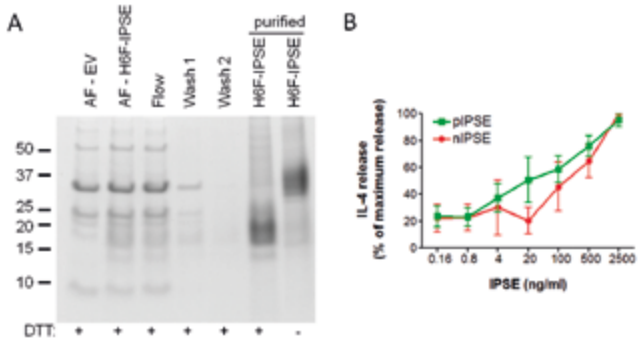


Figure S9. Plant-based production of IPSE. (A) SDS-PAGE and Coomassie blue staining of apoplast fluids (AF) from empty vector (EV), N-terminally tagged H6F-IPSE infiltrated *N. benthamiana* plants and subsequent small-scale purification of IPSE using Ni-NTA resin and the Äkta Prime purification system. Purified IPSE was analysed under reducing and non-reducing conditions (± DTT). **(B)** IL-4 release from isolated human basophils during 18h stimulation with pIPSE and nIPSE (0.16-2500 ng/ml), as determined by ELISA. Data are presented as percentage of maximum IL-4 release. Summary of N=4-6 donors.

Chapter 4

COMPARISON OF MURINE SPLENIC MARGINAL ZONE AND FOLLICULAR B CELL TRANSCRIPTOMES IN *S. MANSONI* INFECTION – SIMILARITIES AND DIFFERENCES

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Hailiang Mei, and Hermelijn H. Smits

Manuscript in preparation

ABSTRACT

Chronic *Schistosoma mansoni* infections induce a network of regulatory immune cells, including interleukin (IL)-10-producing regulatory B (Breg) cells. The signals that drive Breg cell development and activation are however not well characterized, and multiple signals likely synergize to induce optimal Breg cell induction *in vivo*. We here compared the transcriptome of splenic marginal zone (MZ) B cells, which preferentially acquire a Breg cell phenotype, and follicular (FO) B cells of chronically *S. mansoni*-infected mice using RNA sequencing technology, in order to gain a global picture of signals and pathways involved in *S. mansoni*-induced Breg cell development *in vivo*. We report that MZ and FO B cells display distinct transcriptional profiles, and that both B cell subsets moreover undergo transcriptional changes in response to infection which are partially cell-type specific. Comparing the MZ and FO B cell transcriptome suggests the cytokines IL-1β, IL-6 and type I interferons (IFN-I) as well as pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as preferentially important for MZ B cells during chronic schistosome infections. This analysis thus suggests that inflammatory signals, including pro-inflammatory cytokines and the ligation of PRRs preferentially activate MZ B cells, and provides several leads for further studies.

INTRODUCTION

B cells with regulatory properties, called regulatory B (Breg) cells, are a subset of B cells with immunomodulatory capacity. Breg cells suppress immunity in a range of different contexts, including autoimmunity [1], allergy [2] and cancer [3]. Breg cells are most often characterized by their production of the regulatory cytokine interleukin (IL-)10, although various other mechanisms of suppression have been described [4-6]. Breg cells are moreover a heterogeneous group of cells, and apart from their suppressive function as defining characteristic, various different subsets have been described based on origin and phenotype [6, 7]. Amongst others, marginal zone (MZ) B cells of the spleen and their precursors are well known to acquire regulatory functions [8-10]. Breg cells have been described in a range of infectious diseases [11], including experimental [12-15] and human [12, 16] helminth infections.

Breg cells expand and acquire increased suppressive activity in the context of infection and autoimmunity [1, 6, 17]. The signals for Breg cell development and activation that have been most widely studied are ligation of the B cell receptor (BCR) [18-20], CD40 [10, 18, 21, 22] and toll-like receptors (TLRs), especially TLR4 [23-25], TLR7 [26] and TLR9 [8, 23]. Additionally, a range of cytokines, including IL-1β and IL-6 [27], IL-15 [28], IL-21 [29], IL-35 [30, 31], the tumour-necrosis factor (TNF) family members B-cell-activating factor (BAFF) [32] and a proliferation-inducing ligand (APRIL) [33], as well as type I interferons (IFN-I) [34, 35] have been described to support Breg cell activation. The variety of signals described in Breg cell induction suggests different sources of activating signals. An inflammatory environment seems central and is a shared feature of infection and autoimmunity. The role of BCR- and TLR-derived signals shows that in infection, pathogen-derived molecules are candidates for Breg cell-activating signals. Interestingly, it has recently been shown that pathogen-derived signals that induce Breg cells can also stem from the microbiota, as microbiota-derived signals induced splenic and mesenteric lymph node Breg cells in an IL-1β- and IL-6-dependent manner in arthritis [27], and multiple studies have shown that alterations of the gut microbiota affect the Breg cell compartment [27, 36, 37].

We and others have previously demonstrated that chronic infection with the helminth *Schistosoma* (*S.*) *mansoni* induces IL-10-producing Breg cells in both humans and mice. Most of these cells are found within the human $CD1d^{+}$ B cell subset and the murine $CD1d^{+}CD21^{+}CD23^{lo}$ MZ B cell subset [12, 14]. The number of identified, *S. mansoni*-specific signals that induce Breg cells is however very limited. Breg cells can be induced by schistosomal egg antigens [38, 39], but this is notably less potent than the induction of Breg cells during chronic infection. The *S. mansoni* egg glycoprotein IPSE/alpha-1 is the only single helminth-derived molecule that has been shown to directly interact with splenic B cells and induce IL-10 production, but this has so far only been demonstrated *in vitro* [39]. Chronic *S. mansoni* infection induces a multitude of different immune responses in the host, and it is thus likely that multiple signals synergize *in vivo* to induce optimal Breg cell induction. Herein, we therefore studied the transcriptome of splenic MZ B cells isolated from chronically *S. mansoni*-infected mice.

The transcriptome of certain murine Breg cell subsets has been studied previously. Through such studies, CD9 has been identified as a marker of murine IL-10-competent, $CD19^{+}$ $CD1d^{hi}$ $CD5^{+}$ Breg cells induced by *in vitro* polyclonal stimulation [40]. In addition, by filtering a microarray library for secreted factors produced by splenic B cells activated with LPS and anti-CD40 agonistic antibodies *in vitro*, IL-35 was identified as suppressive cytokine produced by these Breg cells [30]. Interestingly, IL-35 production was confined to $CD138^{hi}$ $CD22^{-}$ regulatory plasma cells described in the context of *Salmonella* infection [30]. A further transcriptomics analysis of a subset of these natural regulatory plasma cells characterized as $LAG\text{-}3^{+}$ $CD138^{+}$ has revealed a distinct expression profile for several transcriptional regulators, including *Klf4*, *Fos*, *Junb*, *Irf8* and *Foxm1* [41]. For Breg cells in the context of acute helminth infections, microarray analysis identified TLR7 as overexpressed in *S. mansoni*-induced $CD19^{+}$ $CD1d^{hi}$ Breg cells compared to $CD19^{+}$ $CD1d^{lo}$ B cells. *In vitro* TLR7 stimulation of splenic B cells from naïve mice promoted Breg cell activation and IL-10 production [26], but it remains unclear from this study whether TLR7 ligands are present and whether TLR7 ligation is central to Breg cell expansion during *S. mansoni* infection.

In this study, we aimed to identify the similarities and differences between MZ and classical follicular (FO) B cells in their response to chronic *S. mansoni* infection, in order to better understand signals, receptors and pathways that lead to the preferential development of MZ B cells into Breg cells. We have compared the transcriptome of MZ B cells and FO B cells from chronically *S. mansoni*-infected and -uninfected control mice.

RESULTS

Chronic *S. mansoni* infection induces Breg cells within the splenic MZ B cell pool

In order to study the transcriptome of splenic MZ and FO B cells, we first isolated total splenic $CD19^+$ B cells and confirmed the capacity of these cells to produce IL-10 by *in vitro* restimulation with *S. mansoni* soluble egg antigen (SEA). As expected, splenic B cells isolated from chronically *S. mansoni*-infected mice secreted significantly more IL-10 protein, but only low levels of IL-6 protein, compared to uninfected controls (**Figure 1A**). The frequency of B cells with intracellular IL-10 expression was also significantly increased (**Figure 1B**). Total non-restimulated splenic $CD19^+$ B cells were sorted into $CD21^+$ $CD23^{lo}$ MZ B cells and the major splenic B cell subset, $CD21^{lo}$ $CD23^{hi}$ FO B cells, for subsequent RNA isolation and RNAseq analysis (**Figure 1C**).

To investigate transcriptional changes that are associated with the response of splenic B cells to *S. mansoni*, we compared the gene expression profile in MZ B cells and FO B cells from chronically *S. mansoni*-infected and uninfected control mice in a principal component analysis (PCA; **Figure 1D**). Biological replicates of all four experimental groups clustered well. MZ and FO B cells were segregated across the first principal component (PC1), representing the cell type difference and accounting for 66.8% of the total data variability. The second principal component (PC2) represented the treatment effect, accounting for 12% of the total data variability. Notably, while PC1 separates MZ and FO B cells to a similar degree irrespective of treatment, PC2 separates FO B cells from *S. mansoni*-infected (FO. Inf) mice and from control (FO.PBS) mice stronger than MZ B cells from *S. mansoni*-infected (MZ.Inf) mice and from control (MZ.PBS) mice. PC3, which does not clearly separate by cell type or treatment condition, only accounts for 6.2% of total variability.

Both MZ and FO B cells from control and *S. mansoni*-infected mice constitutively expressed the B cell markers *Cd19*, *Cd22*, *Cd79a*/*Cd79b* and *Ptprc* (B220) along with other B cell-associated markers including *Tnfrsf13b* (TACI) and *Tnfrsf13c* (BAFF-R). MZ B cells expressed lower levels of *Sdc1* (coding for CD138) than FO B cells (**Figure 1E**), and *Lag3* was not detectable in the dataset, indicating that the B cells assessed here are phenotypically different from the natural regulatory plasma cells recently described [41]. T cell genes including *Tcra*, *Tcrb*, *Cd3e*, *Cd4*, *Cd8a* and *Cd28* were also not detected in the dataset, showing that the isolated B cell populations were not contaminated with T cells. MZ B cells strongly overexpressed *Cd1d1* and *CD1d2* (together CD1d), *Cd9*, and to a lesser extent also *Cd5, Cd24a*, *Cd27* and *Cd38*, all markers characteristic of a Breg cell phenotype (**Figure 1E**). The strong overexpression of *Cd9* in MZ B cells is in line with previous reports on this marker as identifier of murine IL-10 competent, $CD19^+$ $CD1d^{hi}$ $CD5^+$ Breg cells [40]. With respect to cytokines, MZ B cells strongly overexpressed *Il10*, confirming that this splenic B cell subset is IL-10-competent. MZ B cells also mildly overexpressed *Ebi3*, in addition to expressing *Il12a*, which together form the regulatory cytokine IL-35 described to be produced by certain Breg cell subsets [30] (**Figure 1E**). In chronically *S.mansoni*-infected animals, both B cell subsets moreover strongly overexpressed *Ighe* and *Ighg1* compared to steady-state (**Figure 1E**). B cells from all experimental groups constitutively expressed the B cell lineage transcription factor (TF) *Pax5*, while FO B cells overexpressed *Bcl6* exclusively expressed in germinal centers and MZ B cells overexpressed *Prdm1* (Blimp-1) which drives B cell differentiation into plasma cells. The TFs IRF4, NFAT and STAT1/STAT3 have previously been suggested to be important for B cell IL-10 expression [20, 34, 35, 42], but expression of these genes did not differ between MZ and FO B cells or undergo changes with

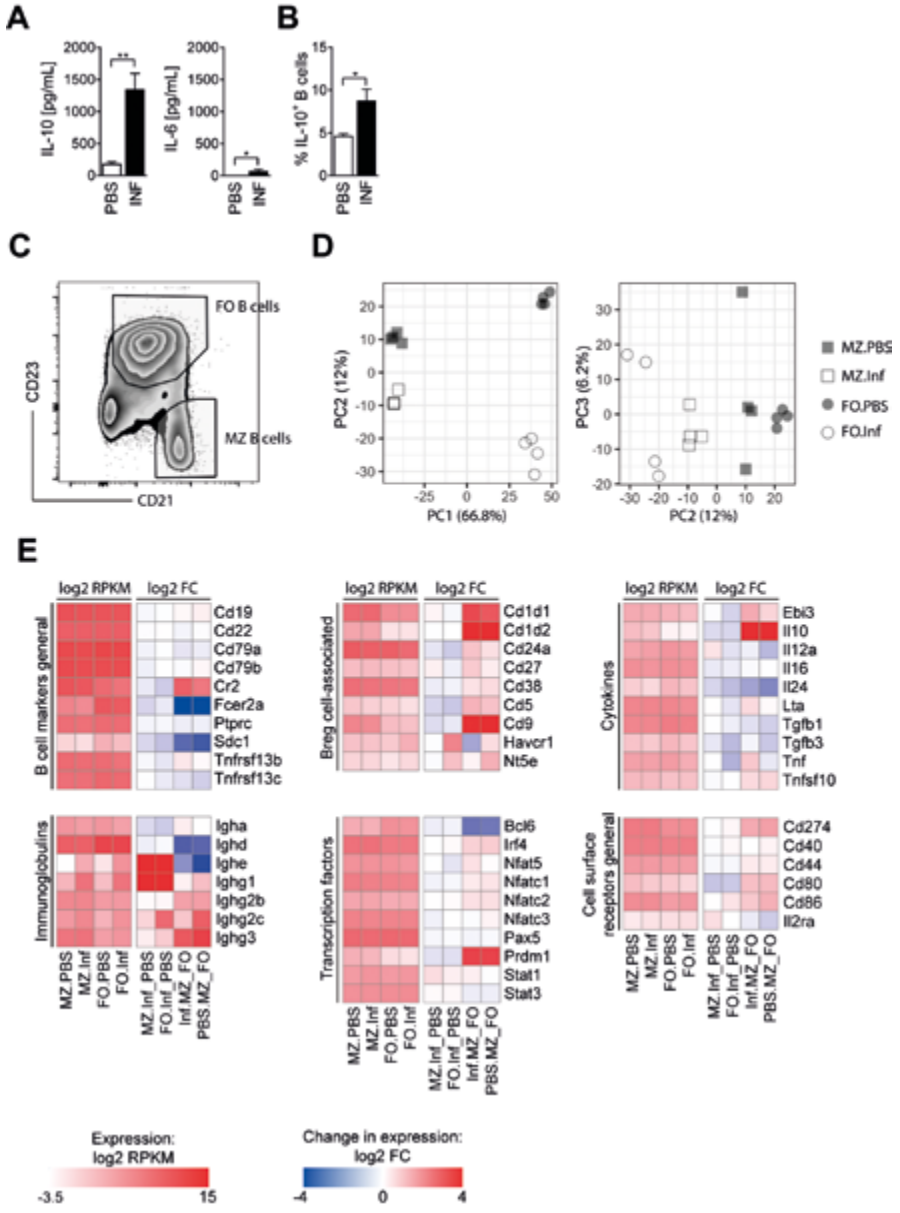

Figure 1. Chronic *S. mansoni* infection induces Breg cells within the splenic MZ B cell pool. Splenic B cells from chronically (14 weeks) *S. mansoni*-infected mice and uninfected control mice were isolated by CD19 MACS isolation. (**A-C**) Cells were restimulated with SEA (20µg/ml) for 2 days, n=5-6/group. Statistical significant differences were determined by unpaired t-test and are indicated by * $p < 0.05$, ** $p < 0.01$. (**A**) Cytokine concentrations in culture supernatant determined by ELISA. (**B**) Intracellular IL-10 expression after addition of Brefeldin A during the last 4 hours of culture, determined by FACS. (**C**) Representative FACS plot depicting the gating strategy to identify MZ B cells ($CD21^{+}CD23^{lo}$) and FO B cells ($CD21^{lo}CD23^{hi}$) applied during sorting. (**D-E**) Sorted MZ and FO B cells were subjected to RNA isolation and RNAseq analysis. (**D**) Principal component analysis (PCA) including all 16 samples. (**E**) Heatmaps depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pair-wise comparisons.

infection (**Figure 1E**). B cells from all experimental groups moreover constitutively expressed *Cd40*, whereas *Cd274* (PD-L1), *Cd80* and *Cd86* were slightly overexpressed in MZ B cells (**Figure 1E**).

MZ and FO B cells display distinct transcriptional profiles and undergo transcriptional changes that are partially cell-type-specific in response to chronic *S. mansoni* infection

To investigate the genes and signalling pathways associated with B cell responses to *S. mansoni*, we performed a differential expression analysis either comparing *S. mansoni* infection and control condition for both B cell subsets (denoted as MZ.Inf_PBS and FO.Inf_PBS, respectively), or comparing MZ and FO B cells in uninfected and *S. mansoni*-infected mice (denoted PBS.MZ_FO and Inf.MZ_FO, respectively). Chronic infection with *S. mansoni* resulted in transcriptional changes in both MZ and FO B cells, albeit to a different degree. MZ B cells displayed less differentially expressed genes (DEGs) than FO B cells in response to infection (**Figure 2A**, upper panel). The transcriptional profile strongly differed between MZ and FO B cells, both after chronic *S. mansoni* infection and in control mice (**Figure 2A**, lower panel). A list of all DEGs per condition is provided as **suppl. Table S1**.

A total of 438 DEGs were shared between MZ and FO B cells in response to infection, of which 19 DEGs were differentially expressed in opposing direction. A total of 252 DEGs (37%) in MZ B cells and 1982 DEGs (82%) in FO B cells were uniquely regulated for the respective B cell subset in response to infection (**Figure 2B**, upper panel). The transcriptional profile of MZ and FO B cells clearly differs, both after chronic *S. mansoni* infection and in control mice, with the majority of DEGs (4492 DEGs; 79% and 75%, respectively) being shared in both conditions (**Figure 2B**, lower panel).

Canonical Pathways differentially activated in MZ and FO B cells of chronically *S. mansoni*-infected animals

To evaluate the response of MZ and FO B cells to *S. mansoni* infection more broadly, we used the Canonical Pathway function of Ingenuity Pathway Analysis (IPA) [43] to search for common and discordant signalling pathways in MZ and FO B cells. To identify pathways that are more related to MZ or FO cells, respectively, we made use of the activation z-score calculated by IPA, which is a statistical measure to infer the likely activation state of the pathway, with z-scores of > 2 or < -2 suggesting significant activation or inhibition, respectively. Both MZ and FO B cells proliferate in infected mice, as apparent from cell cycle-related pathways being significantly activated in both cell types in response to infection (**Figure 3**). Interestingly, immune-related functions are found in the list of pathways significantly downregulated after infection for both cell types, which may suggest that these pathways are negatively regulated following previous activation. The list of pathways found to be significantly inhibited in the comparison MZ.Inf_PBS but not in FO.Inf_PBS (**Figure 3**, left panel) contained 'Toll-like Receptor Signaling', 'STAT3 Pathway', 'B Cell Activating Factor Signaling' (B-cell activating factor = BAFF) and 'April Mediated Signaling', which is in line with previous reports suggesting TLR-, STAT3-, BAFF- and APRIL-signalling to be associated with Breg cells [23, 32, 35, 44]. The pathways 'p38 MAPK Signaling', which is downstream of receptors for inflammatory cytokines, FasL, TGF-β, G-protein coupled receptors as well as DNA damage signals [45], and 'Acute Phase Response Signaling', which is a rapid inflammatory response pathway triggered by pro-inflammatory cytokines [46], likely reflect the response of MZ B cells to the inflammatory environment in chronic *S. mansoni* infection. Pathways found to be significantly inhibited in the comparison FO.Inf_PBS but not in MZ.Inf_PBS (**Figure 3**, right panel) contained 'IL-6 Signaling', 'IL-8 Signlling' and 'HMGB1 Signaling'. In human infection and cancer, B cells have been suggested to express the IL-6 receptor [47, 48], whereas little is known of B cell responses to IL-8. HMGB1 is secreted by immune cells, and mediates inflammatory responses via interaction with TLR4 [49, 50].

We did not only identify Canonical Pathways for the pairwise comparisons of cellular responses to infection, but also for cell type differences both at steady-state and after chronic *S. mansoni*

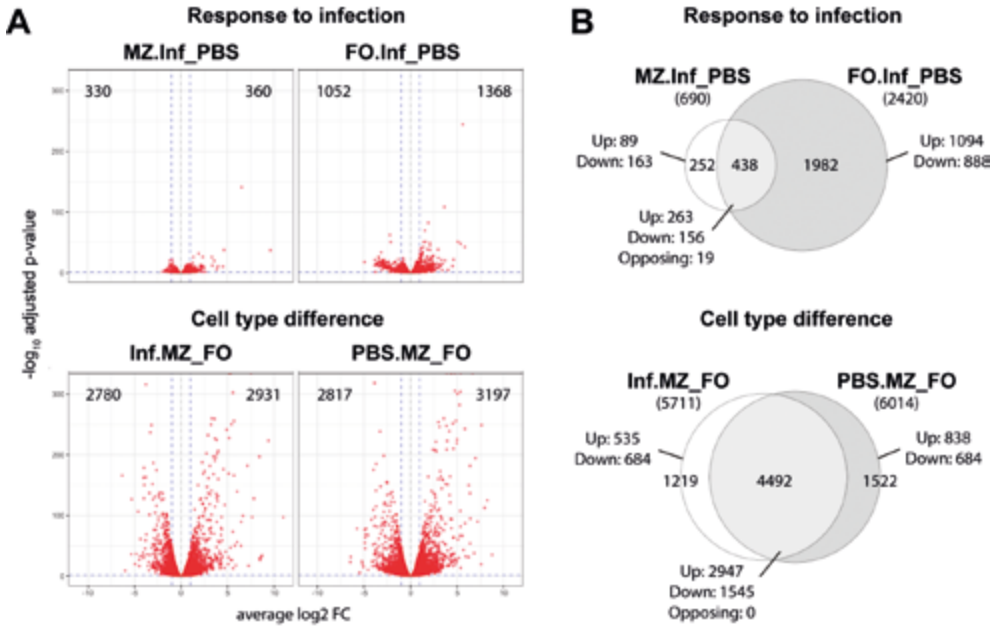

Figure 2. MZ and FO B cells display distinct transcriptional profiles and undergo transcriptional changes that are partially cell-type-specific in response to chronic *S. mansoni* infection. (**A**) Volcano plots comparing differential gene expression for the response of MZ and FO B cells to infection, and we as for cell type difference in control and infected condition. The number of differential up- and downregulated genes for each comparison is depicted in the plot. avgLog2FC = average log2 fold change; minusLog10AdjPVal = -log10 adjusted p-value. (**B**) Venn diagrams depicting the overlap of differentially expressed genes (DEGs) for the response of MZ versus FO B cells to infection, and for the cell type difference after infection versus at steady-state. The number of DEGs that are unique or shared, and whether they were upregulated ('up'), downregulated ('down'), or opposing for shared DEGs ('opposing') is indicated.

infection. A list of significantly activated or inhibited pathways for these comparisons is provided as **suppl. Figure S1**.

Predicted Upstream Regulators with differential activation status in MZ and FO B cells of chronically *S. mansoni*-infected animals

The Upstream Regulator analysis of IPA [43] examines the presence of known targets and their direction of change in the dataset compared to what is expected based on the literature in order to predict likely relevant upstream signals that explain the observed gene expression changes in the dataset. These Upstream Regulators do not have to be part of the dataset itself. As many Upstream Regulators passed the regular z-score threshold, we applied a more stringent cut-off of z-scores > 4 or < -4 to identify highly activated or inhibited Upstream Regulators (**suppl. Figure S2**). These lists still contained many hits of a range of different molecule types, which is why we focussed on cytokines and cell surface receptors as most informative molecule types with respect to identifying extracellular signals important for Breg cell activation in chronic *S. mansoni* infection. The list of significant (z-score > 2 or < -2) cytokines predicted as Upstream Regulators that are unique to the response of MZ B cells to infection includes IL-1β as IL-6 as strongly activated and highly significant hits (**Figure 4A**, left panel). In contrast, the list of Upstream Regulators that are unique to the response of FO B cells to infection

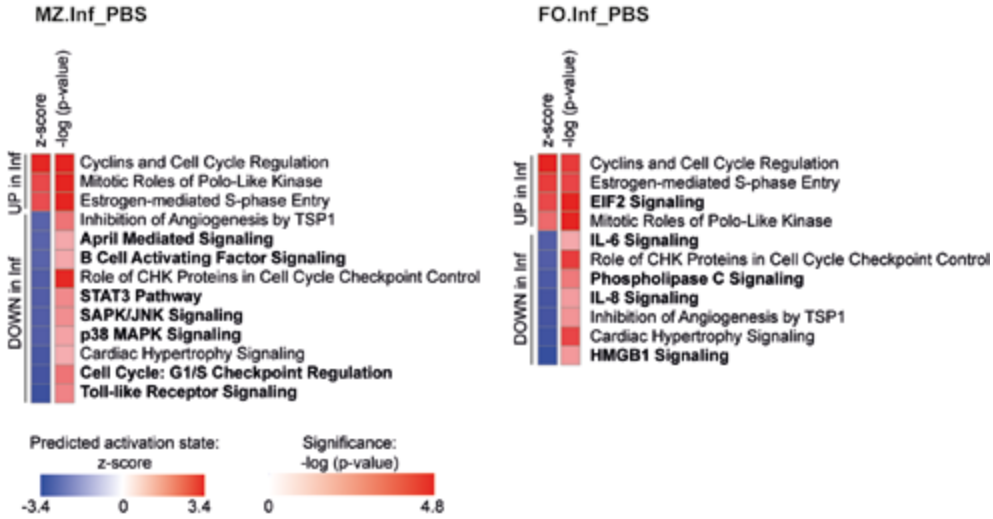

Figure 3. Canonical Pathways differentially activated in MZ and FO B cells of chronically *S. mansoni* infected animals. IPA software was used to identify Canonical Pathways significantly activated (z-score > 2; -log(p-value) > 1.3) or significantly inhibited (z-score < -2; log(p-value) > 1.3) for the response of MZ B cells to infection and of FO B cells to infection. Canonical Pathways that are not shared between the two pair-wise comparisons are indicated in bold font. Canonical Pathways significant for the comparison of MZ and FO B cells at steady-state and after infection is given in **suppl. Figure S1**.

includes amongst others the Th2 cytokines IL-4 and TSLP as activated Upstream Regulators (**Figure 4A**, right panel). In comparison of MZ and FO B cells at steady-state and after infection, it becomes apparent that the list of Upstream Regulators predicted to be more activated in MZ B cells contains several interferons, including members of the IFN-I family and IFNγ as type II interferon (**Figure 4B**). Notably, Upstream Regulators are signals solely predicted by IPA to cause the observed transcriptional pattern, irrespective of whether e.g. corresponding receptors are expressed. We therefore evaluated the expression of receptors for predicted Upstream Regulators in our dataset. The IL-6R subunit *Il6ra* and the IL-1R accessory protein *Il1rap* were both expressed in both B cell subsets, with *Il1rap* being slightly overexpressed in MZ B cells and *Il6ra* being slightly overexpressed in FO B cells. Moreover, the IFNAR subunits *Ifnar1* and *Ifnar2* are both clearly expressed in MZ and FO B cells irrespective of infection status (**Figure 4C**). Collectively, these data might indicate that, whereas FO B cells respond to the Th2 environment to undergo a germinal center reaction and differentiate into long-lived plasma and memory B cells, MZ B cells might be more responsive to pro-inflammatory, non-Th2-related cytokines such as IL-1β, IL-6 and IFN-I.

With respect to surface receptors, only few Upstream Regulators were predicted as significantly activated or inhibited for the response of MZ and FO B cells to infection, respectively (**Figure 4D**). The comparison of both cells types at steady-state and after infection yielded TNFRSF17 and TLR4 as significantly more activated in MZ than FO B cells at steady-state, and TLR9 as well as TLR7 as significantly more activated in MZ compared to FO B cells after infection (**Figure 4E**). These data suggest that TNFRSF17 (BCMA), which binds APRIL with high affinity and BAFF with low affinity, as well as the toll-like receptors TLR4, TLR7 and TLR9 are more important for the activation of MZ than FO B cells. This goes hand in hand with the fact that these receptors are known to be important for the induction of Breg cells [8, 23, 24, 26, 32]. In our dataset, *Tlr4* and *Tlr9* were constitutively expressed across all conditions with little changes between cell types or in response to infection, whereas *Tlr7*

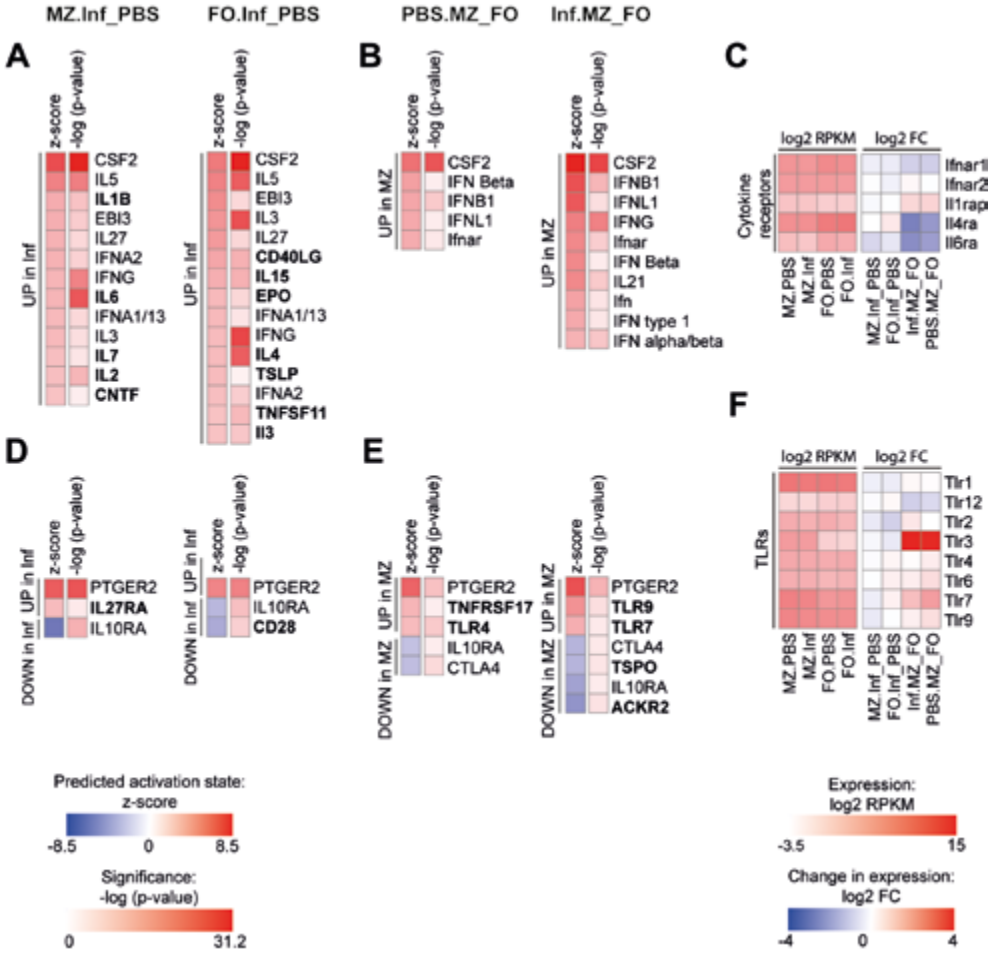

Figure 4. Predicted Upstream Regulators with differential activation status in MZ and FO B cells of chronically *S. mansoni*-infected animals. IPA software was used to identify predicted Upstream Regulators significantly activated (z-score > 2; p-value < 0.05) or significantly inhibited (z-score < -2; p-value < 0.05). An overview over all identified Upstream Regulators is given in **suppl. Figure S2**. Upstream regulators were filtered either for 'cytokines' manually including cytokines listed under molecule type 'group' (**A**, **B**), or for 'transmembrane receptor' and 'G-protein coupled receptor' to select for cell surface receptors (**D**, **E**). (**A**, **D**) Upstream Regulators for the response of MZ and FO B cells to infection. (**B**, **E**) Upstream Regulators for the cell type difference at steady-state and after infection. Upstream Regulators that are not shared between pair-wise comparisons for cellular response to infection and cell type difference are indicated in bold font. (**C**, **F**) Heatmaps depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pair-wise comparisons.

was overexpressed in MZ B cells. Interestingly, *Tlr3* was found to be strongly overexpressed on MZ B cells (**Figure 4F**), and TLR3 was also predicted by IPA as Upstream Regulator for MZ B cells, although not passing the p-value significance threshold (data not shown). In summary, TLR ligation might thus, in addition to signals provided by cytokines, be important for the response of MZ B cells to *S.mansoni* infection.

A cluster identifies genes with opposing transcriptional changes in MZ and FO B cells

We also compared the DEG lists for the response of MZ and FO B cells to infection, and the cell type difference in control condition and after infection, in plots comparing DEG fold changes between conditions. Interestingly, both plots for cell type difference and for response to infection displayed a distinct cluster of 64 and 71 genes, respectively (lists provided as **suppl. Table S4**). Almost all genes (n=63) were contained in both clusters (**suppl. Figure S3**). These genes are upregulated in MZ B cells, but downregulated in FO B cells, in response to infection (**Figure 5A**), or upregulated in MZ B cells from infected animals, but downregulated in steady-state MZ B cells, compared to the respective FO B cell condition (**Figure 5B**). The cluster contained the genes *C1qa*, *C1qb*, *C1qc* and *C6* coding for the complement factors C1 and C6. The cluster also contained *Clec4a3*, *Clec4n* and *Mrc1*, the latter ones coding for the C-type lectin receptors (CLRs) dectin-2 and mannose receptor (MR; CD206), respectively (**Figure 5C**). Thus, the cluster identified additional signals that might preferentially activate MZ B cells in the context of *S. mansoni* infection.

DISCUSSION

Breg cells are a relatively new member in the network of immune regulatory cells that have only been extensively studied in the recent decades, and many open questions around their origin, induction as well as phenotypical and functional characteristics remain. In this study, we focused on the identification of signals that may drive the preferential development of splenic MZ B cells over FO B cells into IL-10-producers in chronic *S.mansoni* infection in order to identify leads for further investigation.

We and others have previously reported that chronic *S. mansoni* infection induces IL-10$^+$ B cells within the murine and human CD1d$^+$ B cell subset [12, 14], which corresponds to the CD21$^+$ CD23^{lo} MZ B cell subset. In our dataset, MZ B cells as expected overexpressed several markers characteristic for splenic MZ Breg cell subsets, but are phenotypically distinct from regulatory plasma cells. Breg cells are most widely recognized for their production of IL-10 [5, 6]. We found *Il10* to be strongly overexpressed in MZ B cells compared to FO B cells, both at steady-state and after infection, identifying MZ B cells as IL-10-competent. The expression of *Il10* did not further increase after infection in either of the B cell subsets, indicating that optimal IL-10 expression requires an additional *ex vivo* restimulation with a mitogen, as described previously [51].

From the analysis of DEGs it became apparent that MZ and FO B cells display clearly distinct transcriptional profiles, not only after infection but also at steady-state. This difference likely reflects the distinct roles of MZ B cells as innate-like cells that mediate responses to antigens in blood-borne pathogens, and of FO B cells undergoing germinal center reactions to differentiate into long-lived plasma cells producing class-switched antibodies [52]. Both B cell subsets underwent transcriptional changes in response to chronic *S. mansoni* infection, whereby FO B cells displayed about 3.5-times more DEGs than MZ B cells. Again, these differences might be related to the unique function of both cell types rather than a differential activation status of MZ and FO B cells after infection, which is supported by the fact that Canonical Pathways identified by IPA to be highly activated in both cell types in response to infection include proliferation-related pathways. The analysis within IPA also suggested several pathways which have previously been associated with Breg cell induction [23, 32, 44] as more relevant for MZ B cells in response to infection, including TLR-, STAT3- BAFF- and APRIL-signalling. This indicates that these pathways are also induced in the context of *S. mansoni* infection.

The Upstream Regulator analysis performed in IPA identified several regulators as predicted to be more activated in MZ B cells of chronically *S.mansoni* infected mice than in FO B cells. With respect to cytokines, IPA identified IL-1β and IL-6 as potential Upstream Regulators, and their receptors are part of the transcriptome dataset and expressed in MZ and FO B cells. Rosser *et al.* showed that both of these cytokines directly promote suppressive Breg cells, as mice with B cell-specific deficiency in

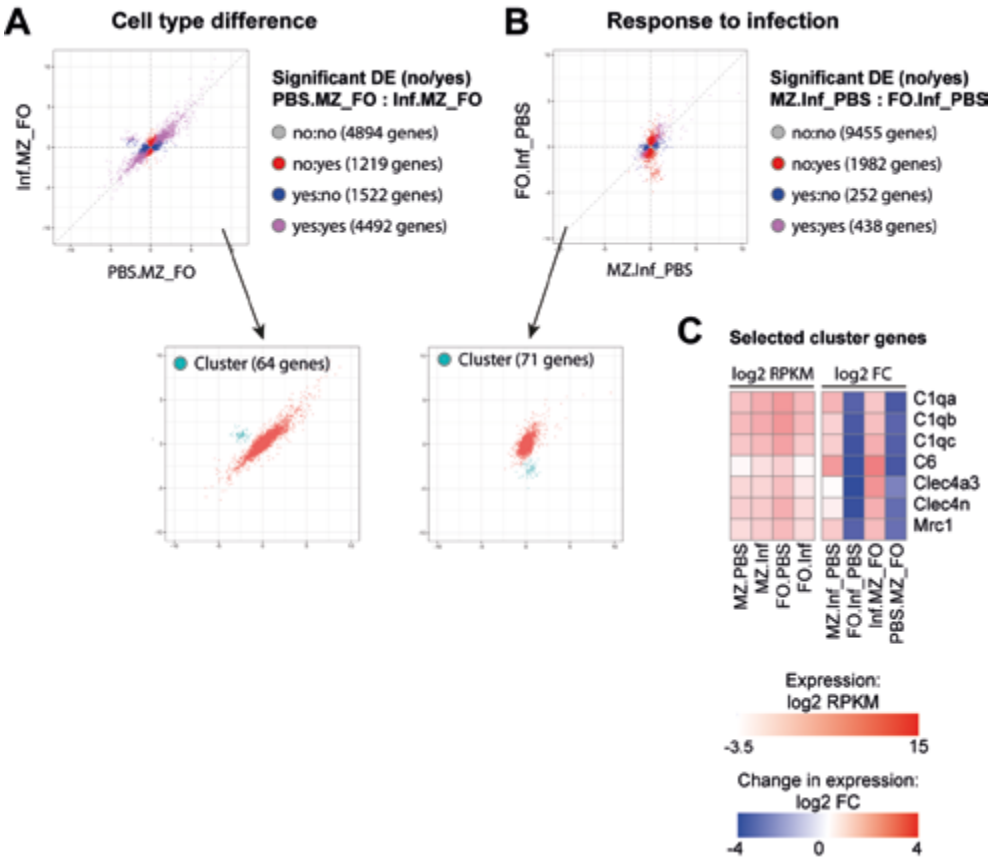

Figure 5. A cluster identifies genes with opposing transcriptional changes in MZ and FO B cells. (**A**, **B**) Plots depict the fold change (FC) of pair-wise comparisons for the cellular response to infection (**A**) and for cell type differences (**B**). (**C**) A heatmap depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pairwise comparisons for selected genes included in the identified clusters.

the IL-1- or the IL-6-receptor (IL-1R, IL-6R) develop more severe experimental arthritis. The production of IL-1β and IL-6 by macrophages and DCs was moreover found to be dependent on an intact gut microbiome [27]. With respect to *S. mansoni*, it is tempting to speculate that an infection and especially eggs penetrating the intestinal wall may cause translocation of bacteria across the intestinal wall and systemic exposure to microbial products, potential providing signals for Breg cell induction. In humans, it has already been shown that schistosomiasis can cause high endotoxemia [53].

Apart from IL-1β and IL-6, IPA also identified several members of the IFN-I cytokine family as well as IFNγ as Upstream Regulators predicted to be more activated in MZ B cells compared to FO B cells. Menon *et al.* showed that IFNα as a IFN-I family member induces Breg cells in humans [35]. B cells are widely recognized to express the IFNα/β receptor (IFNAR) and to respond to IFN-I [54-56], and both IFNAR subunits *Ifnar1* and *Ifnar2* are expressed in MZ and FO B cells irrespective of infection status. Moreover, recent reports have highlighted that helminths including *S. mansoni* or their products can induce IFN-I production in mouse models. Gastrointestinal helminths of rodents have been shown to induce IFN-I

in gut and lung in an microbiota-dependent manner [57], and in skin DCs [58], respectively. *S. mansoni* eggs and egg antigens induce an IFN-I signature both in splenic DCs *in vivo* [59] and in bone marrow DCs *in vitro* [60]. Collectively, this analysis suggests the cytokines IL-1β, IL-6 and members of the IFN-I family as Upstream Regulators more important for MZ than FO B cells in *S. mansoni* infection, and the literature provides interesting initial observations that they might be involved in Breg cell induction, making them promising targets for further investigation.

With respect to cell surface receptors, IPA identified TLR7 and TLR9 as Upstream Regulators predicted to be significantly more activated in MZ B cells than FO B cells after infection. Both TLR7 and TLR9 are endosomal TLRs that recognize nucleic acids. TLR7 has been described to be overexpressed in $CD19^+$ $CD1d^{hi}$ B cells compared to $CD19^+$ $CD1d^{lo}$ B cells, and to increase the capacity of $CD19^+$ $CD1d^{hi}$ B cells to produce IL-10 [26]. TLR9 expressed on B cells has been reported to recognize DNA-containing complexes on apoptotic cells, resulting in IL-10 production and protection from experimental autoimmune encephalitis (EAE) [8], whereas another study suggests B cell TLR9 is not required for the recovery from EAE [23]. The notion that TLR9 induces Breg cells is supported by numerous studies using synthetic TLR9 ligands to induce murine and human Breg cells *in vitro* [61, 62], but the role of TLR9 for Breg cell induction has so far not been studied in the context of *S. mansoni*. As chronic *S. mansoni* infection and especially egg migration causes tissue damage, granuloma formation and potentially also systemic exposure to microbial pathogen-associated molecular patterns (PAMPs), it is however likely that endogenous ligands released following apoptosis and/or microbial ligands for nucleic acid-binding TLRs including TLR9 get exposed. In addition to *Tlr7* and *Tlr9*, the third endosomal TLR recognizing nucleic acids, *Tlr3,* is strongly overexpressed in MZ B cells. The Upstream Regulator analysis does however not suggest a differential signalling through TLR3 in MZ and FO B cells in chronic *S. mansoni* infection to be responsible for the differential expression patterns found in these cell types. Collectively, these data suggest that *Tlr3* is predominantly expressed on MZ B cells compared to FO B cells, but that TLR7 and TLR9 rather than TLR3 might play a role in the differential response of both cell types to chronic *S. mansoni* infection.

A cluster of genes with opposing expression pattern in MZ and FO B cells identified complement factors and CLRs as upregulated in MZ B cells, but downregulated in FO B cells, after *S. mansoni* infection. B cells express various complement receptors including complement receptor 2 (CR2 = CD21), but little is known about the expression of complement factors by B cells [63, 64]. CD21 on B cells recognizes complement factor C3d-opsonized microbial products, resulting in enhanced BCR signalling [65]. To our knowledge, the expression of complement factors by B cells has not been described before, and our findings should be supplemented by gene and protein expression data before potentially investigating the role of complement expression by B cells further.

Apart from TLRs, CLRs are a second class of pattern recognition receptors (PRRs). Others have previously reported that B cells express CLRs including dectin-1 and MR [66], and we have found CLRs including dectin-2 and MR to be preferentially expressed by MZ B cells after infection. Notably, *S. mansoni* molecules have been described to ligate CLRs. Omega-1 induces DC Th2 polarization via MR [67], but failed to induce Breg cell development *in vitro* in naïve splenic B cells [39], likely arguing against a potent role for MR in Breg development. SEA induces prostaglandin E_2 synthesis by DCs through dectin-1 and dectin-2, also promoting DC Th2 polarization [68]. Apart from one study suggesting that signalling via dectin-1 induced IgG1 class switching by LPS-activated B cells [69], the role of these dectins in B cell and specifically Breg cell activation has not been addressed.

Collectively, comparing the transcriptome of MZ and FO B cells identified cytokines including IL-1β, IL-6 and IFN-I, as well as PRRs including the TLR7 and TLR9, Dectin-1 and MR as predicted upstream regulators that might cause a differential activation of these B cell subsets in *S. mansoni* infection. Apart from cytokine receptors and TLRs, the BCR and CD40 have been most well-described as cell

surface receptors involved in the induction of Breg cells, at least in the context of autoimmunity [10, 18-22]. The analysis performed herein however suggests that these receptors are not differentially activated in MZ and FO B cells of chronically *S. mansoni* infected animals.

Transcriptomics allow to study the entirety of genes transcribed at a certain time point and potentially allows insight into the breadth of cellular process at interplay. Such a dataset allows to address very different points of interest, including but not limited to predicted upstream signals and pathways that may lead to the observed transcriptional pattern. Others, such as e.g. the identification of cell-type-specific TFs or metabolism-related regulators and pathways were not the focus of this study, albeit the fact that they may be of interest in the context of Breg cells. Transcriptomics as performed in this study also bear limitations. Gene expression does not necessarily reflect the translation into functional protein, and key findings therefore will need to be supplemented e.g. by data on protein expression. It also has to be taken into consideration that this analysis is a snapshot of the splenic MZ and FO B cell transcriptome after 14 weeks of *S. mansoni* infection. At this time point, the peak of Th2 response to egg deposition has passed, and regulatory responses are on the rise. Moreover, the pool of MZ B cells and FO B cells isolated for analysis and carefully sorted into subsets on the basis of expression of selected markers might still comprise a heterogeneous population of cells at different stages of maturation and differentiation. In order to fully understand changes of MZ and FO B cells as the infection progresses from the acute, Th2-dominated to a more chronic state, the transcriptome of cells isolated form different time points of infection should be compared. Making use of single cell RNAseq technologies would also allow to better assess cellular differences.

In this study, we assessed the transcriptome of B cells during chronic *S. mansoni* infection and gained insight into the global pattern of differential signals and pathways important for MZ and FO B cells, respectively. We identified several inflammatory signals that seem to preferentially drive the activation of MZ B cells over FO B cells, but we cannot dissect from these data whether these signals are *S. mansoni*-specific or the result of the general inflammatory environment caused by chronic infection. A strategy to further address this topic could be to compare this transcriptome of MZ and FO B cells from chronically infected mice to the one of mice injected with *S. mansoni* eggs in the absence of full infection, a model we have previously shown to also induce IL-10^{+} splenic MZ B cells [39]. This model induces less inflammation as a result of tissue damage and pathology, and could therefore potentially allow to distinguish schistosome-specific and general inflammatory signals.

Collectively, this study identified several interesting leads with respect to signals that might differentially activate MZ and FO B cells in *S. mansoni* infection, but follow-up studies will be needed to verify the findings and test functional consequences of individual pathways and regulators for Breg cell induction.

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). All animals were used for experiments at 6-12 weeks of age. All animal studies were performed in accordance with the Animal Experiments Ethical Committee of the Leiden University Medical Center.

S. mansoni infection & isolation of splenic B cell subsets

Mice were infected percutaneously with 36 cercariae and readouts were performed during the chronic phase of infection (14 weeks p.i.). Spleens were homogenized by passage through a 70μM cell strainer (BD Biosciences) and erythrocytes depleted from the single cell suspension by osmotic lysis. B cells were

purified from splenocytes by anti-CD19 MicroBeads (Miltenyi Biotech) following the manufacturer's instructions. Subsequently, B cells were labelled with fluorescent antibodies against CD21 (clone 7G6) and CD23 (clone B3B4) and sorted into MZ B cells ($CD21^{+}$ $CD23^{lo}$) and FO B cells ($CD21^{lo}$ $CD23^{hi}$). Sorted cells were washed twice with PBS and snap-frozen and transferred to -80°C until further use.

RNA isolation

RNA was isolated from snap-frozen cell pellets by use of NucleoSpin RNA isolation kit (Machery-Nagel). A small aliquot of RNA was used to quantify RNA content using a Qubit Fluorometer (Invitrogen) and to confirm sample quality by Bioanalyzer total RNA pico kit (RIN 7.4-9.2). Library preparation and sequencing were contracted out to ServiceXS BV (now GenomeScan BV; Leiden, The Netherlands). The NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. mRNA was isolated from total RNA using oligo-dT magnetic beads and used for library preparation.

RNA sequencing, read mapping and differential expression analysis

For each of the 4 experimental groups (MZ_PBS, FO_PBS, MZ_Inf, FO_Inf), 4 biological replicates were generated, resulting in a total of 16 samples. Paired-end stranded RNA sequencing was performed on a Illumina HiSeq 2500, and generated approximately 17 million (range: 15.7-40.8 million) paired-end fragments per sample. Consistent across all samples, approximately 75% of the fragments could be aligned to annotated transcripts (UCSC annotation, dated 2014-11-25) over *Mus musculus* mm10 reference genome using GSNAP aligner (version dated 2014-12-23). The Trimmed Means of M values (TMM) method was used to produce normalization factors correcting raw counts for different library sizes. The initial count table (ca. 25.000 genes) was filtered to only include genes which had at least 2 aligned fragments per million of aligned fragments (CPM) in at least 4 out of the 16 samples, resulting in a final count table of ca. 12.000 genes included in further analysis. For each gene, its reads per kilobase of transcript per million mapped reads (RPKM) values have been obtained by normalizing CPM values to the transcript lengths approximated by sums of lengths of all exons of the gene. Analysis of differential gene expression was performed with the edgeR package in the R environment. A design matrix for a model with no baseline and 4 groups has been constructed. Next, a genewise negative binomial generalized linear model, with the design matrix and gene-specific dispersions, was fitted followed by a likelihood ratio tests for 4 contrasts (MZ.Inf_PBS contrasting MZ.Inf vs. MZ.PBS, and similarly: FO.Inf_PBS, Inf.MZ_FO and PBS.MZ_FO). Finally, in order to correct for multiple testing, we used the False Discovery Rate control method of Benjamini–Hochberg at a $p < 0.05$ threshold.

IPA Canonical Pathway and Upstream Regulator analysis

Canonical Pathway and Upstream Regulator analysis were performed using Ingenuity Pathway Analysis (IPA; Qiagen Inc.; [43]). The input were all up- and down-regulated genes using a cut-off for the adjusted p-value of < 0.05. A core analysis was performed for each of the pair-wise comparisons followed by a comparison analysis. For the analysis of Canonical Pathways, the list of available pathways was filtered prior to analysis to contain only the category 'signalling pathways' and exclude 'metabolic pathways'. Canonical Pathways were considered significant if log(p-value) > 0.5 and z-score > 2 or <-2. Upstream Regulators were considered significant if p-value < 0.01 and z-score > 2 or < -2 or highly significant if z-score > 4 or <-4.

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SUPPLEMENTARY MATERIAL

Online supplemental material

Suppl. Table S1 shows a list of all DEGs for each of the four analysed contrasts.

Suppl. Table S2 contains results of the Canonical Pathway analysis performed in IPA.

Suppl. Table S3 contains results of the Upstream Regulator analysis performed in IPA.

Suppl. Table S4 shows a list of DEGs within the identified cluster.

Suppl. Tables S1-S4 are available as Excel files under https://www.dropbox.com/sh/zj1c4b3iaubb3ki/AABiGi6nVT-Qe4tz6syTYsOSa?dl=0.

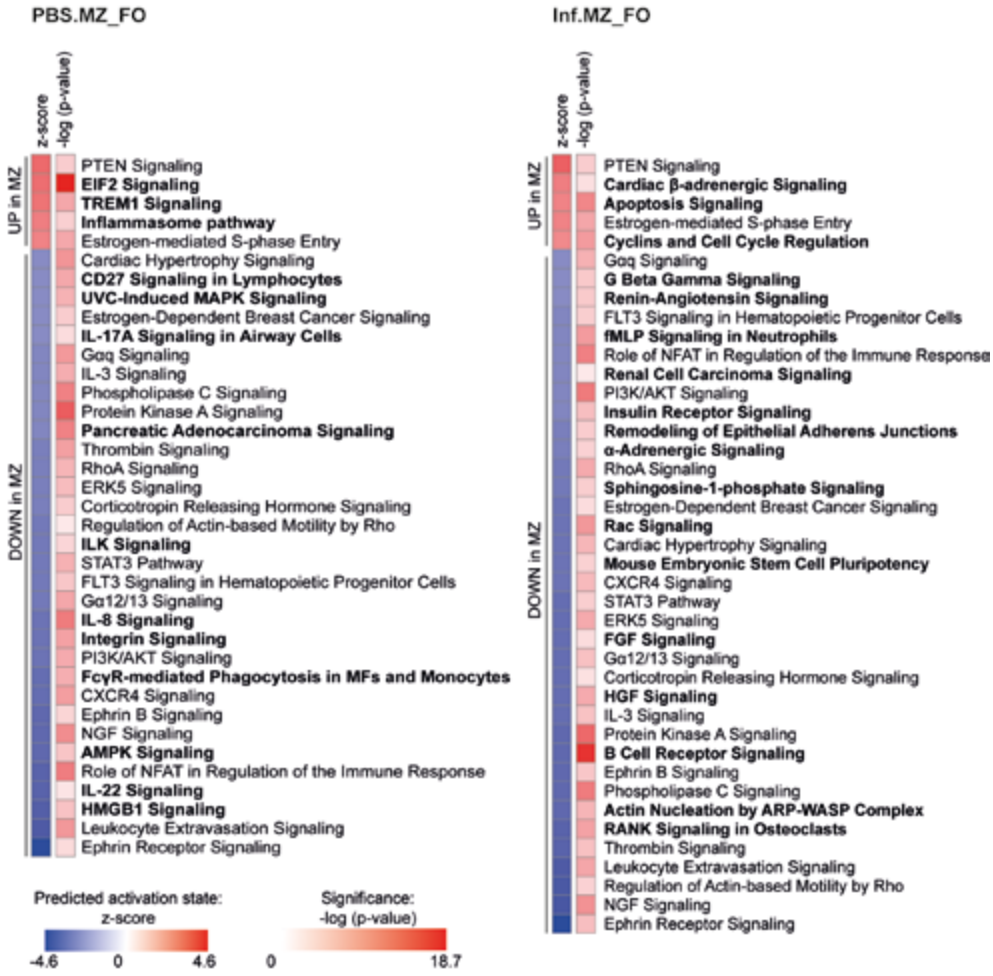

Figure S1. Canonical Pathways significantly activated or inhibited for the difference of MZ and FO B cells at steady-state and after infection. Also see Figure 3.

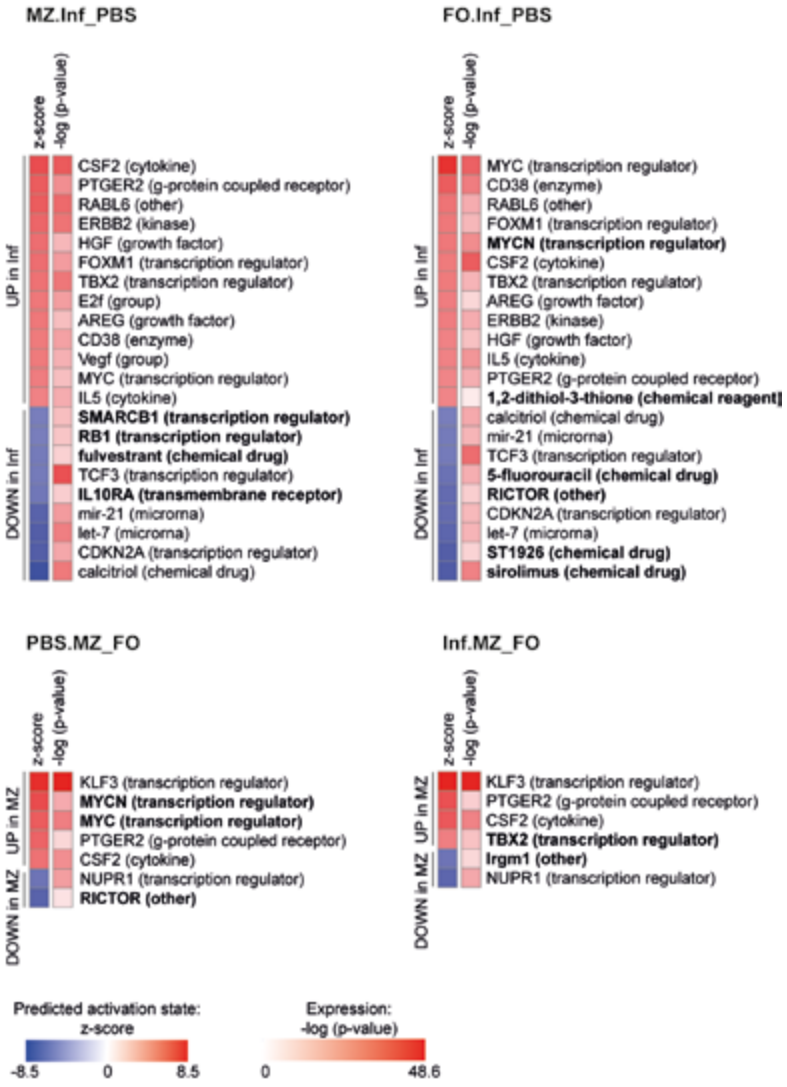

Figure S2. Upstream Regulators significantly activated or inhibited for all pair-wise comparisons and containing all molecule types. Also see Figure 4.

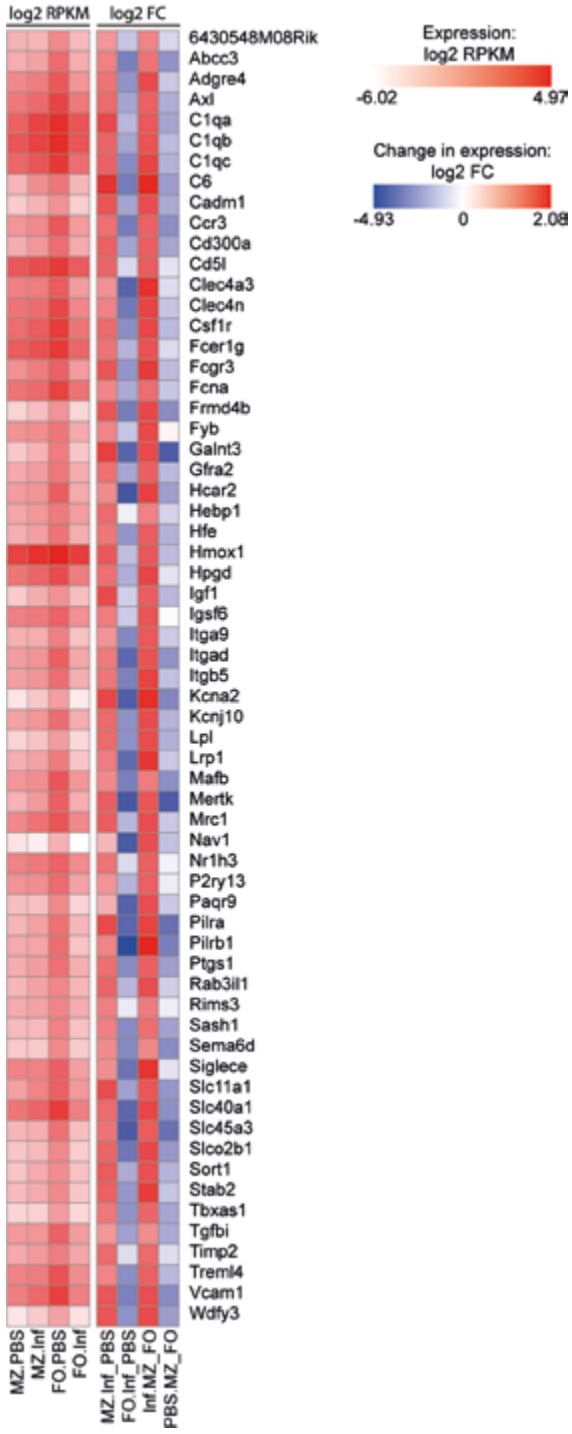

Figure S3. Clusters of genes with opposing fold change for both cell type difference and response to infection comparisons. Also see Figure 5.

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.

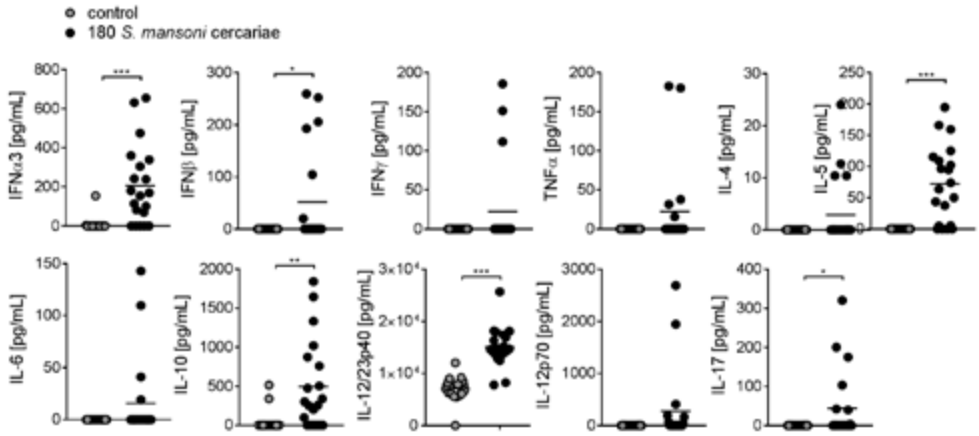

Figure 1. *S. mansoni* induces a systemic type I IFN signature. Mice were infected with 180 *S. mansoni* cercariae and serum samples taken at d49 of infection for assessment of cytokine levels by ELISA/CBA. Pooled data from 2 experiments, n=20/group. Significant differences were determined by unpaired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

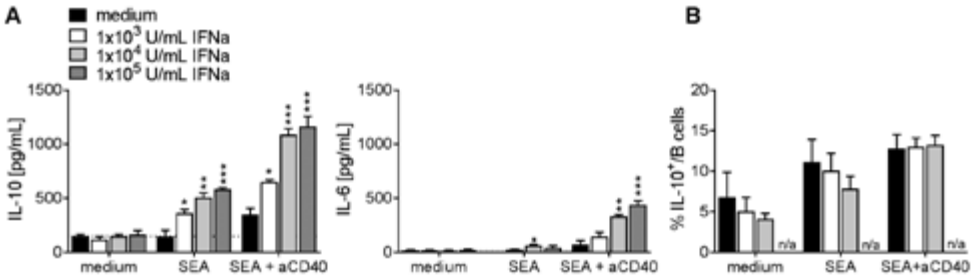

Figure 2. Recombinant IFNα enhances SEA/aCD40-induced B cell IL-10 production. B cells were isolated from the spleen of naïve mice and stimulated *in vitro* with SEA (20µg/mL), aCD40 (0.5 µg/mL) and IFNα (10^3-10^5U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

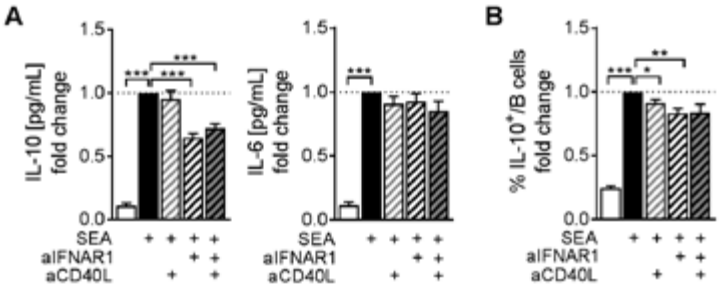

Figure 3. *Ex vivo* block of IFNAR1 reduces B cell IL-10 production. Splenocytes from SEA-injected mice (100µg SEA i.p. on d0 & d7; section d14) were re-stimulated *ex vivo* with SEA (20ug/mL) for 2 days in the presence or absence of anti-CD40L (aCD40L; 10µg/mL) and anti-IFNAR1 (aIFNAR1; 10µg/mL) blocking 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,

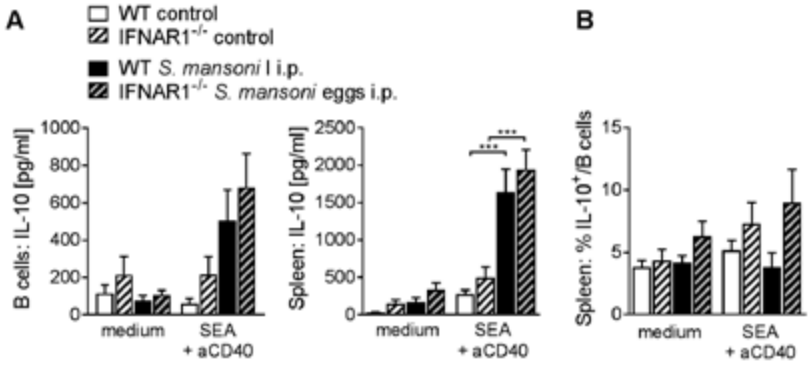

Figure 4. IFNAR1 signalling is dispensable for Breg cell induction *in* vivo. Splenocytes and MACS-isolated CD19$^+$ B cells from *S. mansoni* egg-injected mice (5000 *S. mansoni* eggs i.p. on d0 & d7; section d14) were re-stimulated *ex vivo* with SEA (20µg/mL) and aCD40 (2µg/mL) for 2 days. After 2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

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

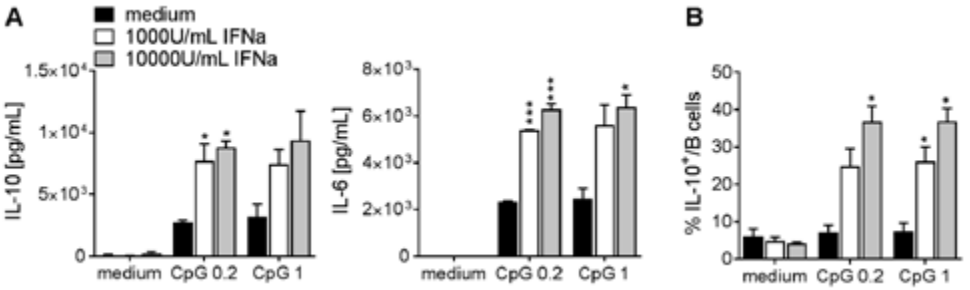

Figure S1. Recombinant IFNα enhances CpG-induced B cell IL-10 and IL-6 production. B cells were isolated from the spleen of naïve mice and stimulated *in vitro* with CpG ODN1826 (class B; 0.2-1μM) and IFNα (10^3-10^4 U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and 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. * $p < 0.05$, *** $p < 0.001$.

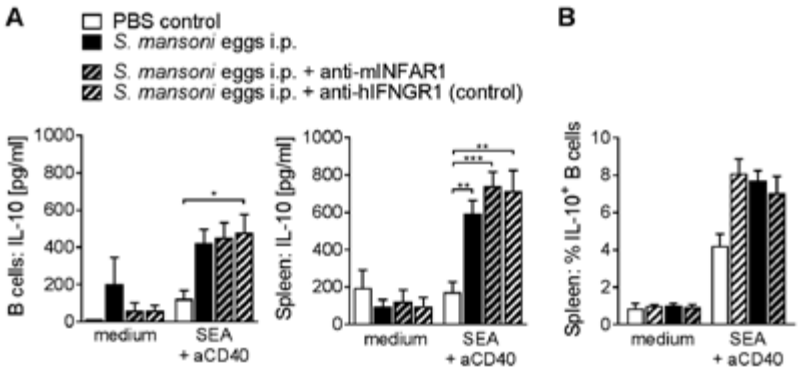

Figure S2. IFNAR1 signaling is dispensable for Breg cell induction *in vivo*. Mice were treated as depicted in **A.** On day 14, spleens were harvested and total splenocyte cell suspensions and isolated $CD19^+$ B cells restimulated with SEA (20ug/ml) and aCD40 (2ug/ml) for 2 days. Supernatants were 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter 6

MICROORGANISM-INDUCED SUPPRESSION OF ALLERGIC AIRWAY DISEASE: NOVEL THERAPIES ON THE HORIZON?

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ABSTRACT

Allergic airway diseases are a major global health burden, and novel treatment options are urgently needed. Numerous epidemiological and experimental studies suggest that certain helminths and bacteria protect against respiratory allergies. These microorganisms are strong regulators of the immune system, and a variety of potential regulatory mechanisms by which they protect against allergic airway inflammation have been proposed. Whereas early studies addressed the beneficial effect of natural infections, the focus now shifts toward identifying the dominant protective molecules and exploring their efficacy in models of allergic airway diseases. In this article we will review the evidence for microbe-mediated protection of allergic airway diseases, the potential modes of action involved, and discuss advances as well as limitations in the translation of this knowledge into novel treatment strategies against allergic airway disease.

INTRODUCTION

Asthma is an atopic, chronic inflammatory disorder that is estimated to affect 315 million people worldwide [1]. First episodes of atopic disease usually occur during childhood following sensitization to inhaled allergens. Infants that suffer from atopic dermatitis (AD) and/or allergic rhinitis (AR) often develop allergic asthma (AA) later in life. Different risk factors for the development of asthma have been identified in children including infection with respiratory viruses (respiratory-syncytial virus, rhinovirus) and impaired lung development in children born prematurely [2].

AA, the most common form of asthma, is characterized by bronchial hyperresponsiveness and airway obstruction, resulting in episodes of wheezing and breathlessness. These symptoms are fueled by chronic inflammatory responses against allergens, in which T helper (Th) 2 cells, eosinophils and elevated immunoglobulin (Ig) E levels play a central role [3]. Dendritic cells (DCs) are important in the initiation phase when they take up allergens, migrate to the draining lymph nodes and induce the differentiation of allergen-specific Th2 cells (**Figure 1**). Renewed exposure to the allergens induces a cascade of Th2-cytokine production (interleukin (IL)-4, IL-5, IL-13), which promotes class-switching of B cells to IgE production, the development of eosinophilic airway inflammation, goblet cell hyperplasia and airway hyperresponsiveness. Notably, the recently discovered innate lymphoid cells type 2 (ILC2s), activated by innate cytokines from damaged epithelial cells, have been described as a significant early source of Th2-cytokines after local allergen exposure [4].

The prevalence of childhood asthma has increased dramatically in westernized countries in the second half of the 20th century. Recent studies suggest that the incidence of asthma has now reached a plateau in high prevalence countries [5], the global burden of asthma however continues to rise as incidence rates in Africa, South America, and parts of Asia still rapidly increase [6]. Asthma is a multivariable disease in which genetic predispositions are certainly important risk factors. However, the rapid increase in incidence rates over the last few decades points toward a strong contribution of environmental factors to disease development. One explanation is provided by the 'hygiene hypothesis' suggesting that a decrease in childhood exposure to infectious agents as a result of increasing sanitation standards, improved health care, and life style changes attributes to the increase in incidence of allergies and asthma [7].

Microbial organisms such as commensal bacteria and helminths are part of human evolutionary history. These microorganisms have developed various strategies to modulate the host immune system, often by amplifying the natural immune regulatory network of the host and simultaneously reducing immunopathology. Many of these organisms establish chronic infections, which may also reflect the assumption that the host immune system tolerates these non-lethal organisms rather than inducing potent inflammatory responses at the cost of severe tissue damage and immunopathology [8]. As such, the immune system categorizes the degree of danger associated with invading pathogens and only reacts strongly to the most threatening organisms such as certain viruses, bacteria and protozoan parasites. In contrast, mainly repair and tissue integrity responses are activated following exposure to less dangerous microorganisms. More recently, the concept has been advocated that certain helminths and bacteria, which are tolerated by the host, may actually imply benefits. Bacteria of the intestinal microbiome for example ferment food, produce essential vitamins and prevent colonization by other, more harmful bacteria [9]. Furthermore, these helminths and microbiota prevent exaggerated immune responses to ubiquitous antigens, as they are strong inducers of the regulatory arm of the immune system. In line with that, the 'hygiene hypothesis' implies that a reduced infectious pressure leads to an immune imbalance, which promotes allergic disorders in genetically susceptible people [10]. In addition, potential benefits of certain microorganisms for the human body are being lost as the diversity of the microbiome decreases [9].

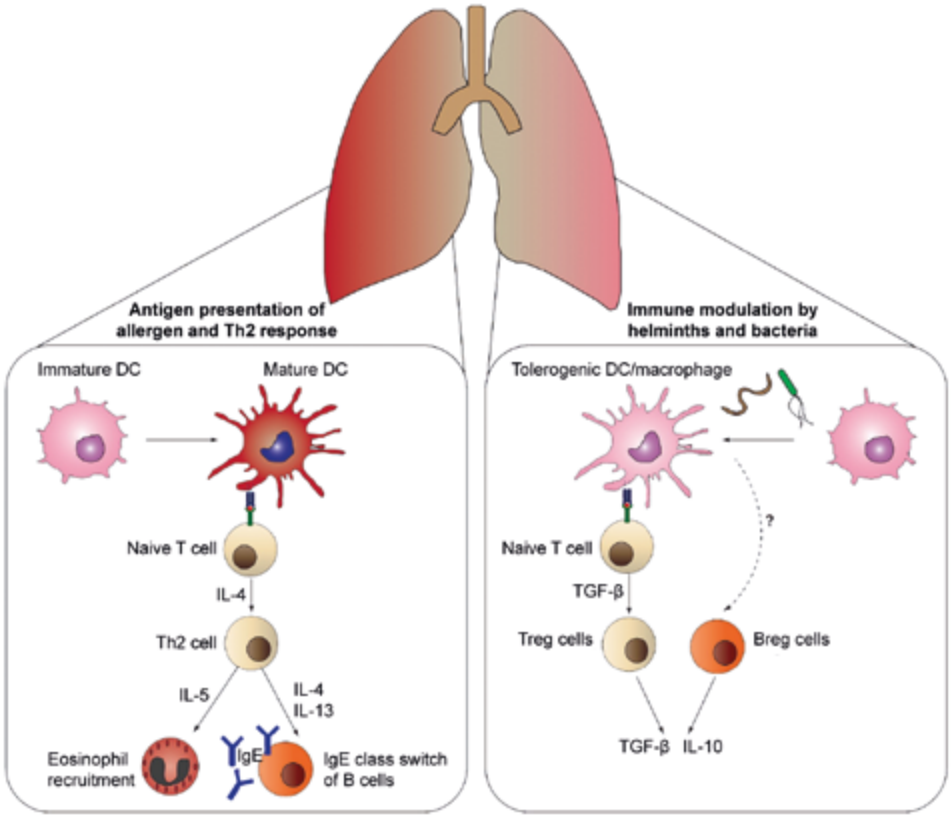

Figure 1. Bacteria and helminths induce T regulatory and B regulatory cell responses that dampen allergic airway inflammation. Left panel: Allergic airway inflammation is most often characterized by a predominant Th2 response. This is initiated by mature dendritic cells that after allergen recognition and presentation, skew T cells towards a Th2 phenotype. Th2 cells initiate effector responses by in particular recruitment and activation of eosinophils and by class switching B-cells to produce IgE antibodies.
Right panel: Bacteria and helminths can intervene in this response at various levels. An example is given in which they induce T regulatory and B regulatory cells. These cells can dampen ongoing inflammation or prevent induction of Th2 mediated inflammation.

Despite the increase in asthma prevalence worldwide in the last decades not many novel therapeutic options have been developed. Current therapies for asthma patients still consist of inhaled steroids and bronchodilators. These medications only alleviate symptoms, which will reoccur after medication has stopped. The treatment of severe asthma proves to be difficult and available therapeutic options are limited to systemic steroids and anti-IgE treatment. Thus, new therapeutic approaches which alter the immune response and promote long-lasting tolerance against allergens are needed. Exploiting the modulatory capacities of evolutionarily-conserved 'old friends' [11], like bacteria and helminths, is a promising strategy to design novel treatments for airway allergies such as asthma.

In this review, we will highlight the current evidence for the protective effect of helminths and certain bacteria in allergic disorders from both epidemiological studies and animal models. We will address both life infections and antigens derived from these organisms, and summarize the proposed mechanisms of protection. Finally, we will discuss the recent advances in the field and give recommendations for future microbial-based treatments against allergic diseases.

Evidence for inhibition of allergic airway inflammation (AAI) by microorganisms

The association between microbial infections and the reduced incidence of allergic disorders has been addressed in numerous epidemiological studies. Meta-analyses consistently show that several helminth species, e.g., *Acaris, Trichuris*, hookworm and Schistsoma can protect against allergic sensitization [12-14]. Hookworm infection is most clearly associated with a reduced risk of developing asthma [14], while other species give either no significant protective effect or have not been extensively studied. Nevertheless, individual studies have suggested that infection with *Schistosoma mansoni* reduces the severity of asthma [15] and allergic skin reactions [16;17]. In addition, a range of other factors have been suggested to determine the protective effect of helminth infection on atopic disease, such as time, intensity and chronicity of infection and host genetics [18]. Of note, worm infections have also been linked to protection against other inflammatory non-communicable diseases, such as inflammatory bowel disease (IBD), multiple sclerosis (MS) and type-1 diabetes [19].

Infection with or exposure to certain bacteria can also interfere with the development of allergies and airway disease. For example, there is mounting evidence that environmental factors such as living on a farm protect from developing asthma [20-22] and this protective effect is attributed to the environmental exposure to different bacterial and fungal microorganisms [23]. In contrast to previous belief, the lung is not sterile but colonized by certain bacterial species, and it was suggested that the composition of the lung microbiome was different in patients with asthma compared to healthy individuals [24;25]. Changes in the bacterial composition may have consequences for disease severity as patients with steroid resistance displayed a different composition of the lung microbiome compared to patients responsive to steroids [26]. Whether these changes are an effect of pathophysiological processes in the airways of patients with asthma or a predisposing factor for the development of asthma is not clear. Nevertheless, it is clear that colonization of the airways with certain bacteria increased the risk of developing allergic airway disease in children [27;28]. In contrast, exposure and infection with a range of other microorganisms may protect from the development of asthma. In epidemiological studies the prevalence of asthma was negatively associated with Herpes simplex virus I, Hepatitis A, and *Toxoplasma gondii* infection [29]. Not only exposure to external bacteria but also the composition of the microbiome can affect the development of asthma. The total diversity of the gut microbiota during the first month of life has been associated with the development of AA in infancy and childhood [30] and a higher prevalence of asthma has been found in children treated with antibiotics [31;32]. These associations must however be reviewed carefully as the causal relationship can be overestimated as reverse causation and confounding factors may have impacted the analysis [33]. Further examples are provided by infections with the gut bacterium *Helicobacter pylori* (*H. pylori*), which is reversely linked with asthma prevalence, particularly in children [34-37]. Interestingly, infection with *H. pylori* is also negatively associated with the development of other non-communicable disease such as IBD and MS [38]. The association between the decreasing prevalence of infection with *H. pylori* [39] and the increase in non-communicable diseases such as asthma has been linked to the so called `disappearing microbiota hypothesis` [9]. Experimental studies in models of allergic airway disease have confirmed this relation and furthermore unraveled the underlying mechanisms of this protective effect [40-42].

Protective, microbial-induced mechanisms against respiratory allergies

A number of different mechanisms by which helminths, certain bacteria and their molecules protect against AAI have been described. The variety of proposed mechanisms most likely reflects the variety of different molecules and models studied. Although part of these mechanisms may have overlapping features, it is plausible that helminths and bacteria have evolved distinct ways of altering the immune responses of the host to promote their survival and use different molecules to do so.

In general, helminths are recognized as potent regulators of the host immune system. They strongly induce Th2 cells, IgE class-switching and eosinophil-rich tissue infiltrations, but at the same

time promote regulatory cells, like regulatory T (Treg) and B (Breg) cells, tolerogenic/immature DCs and alternatively activated macrophages (AAMs). The regulatory cytokines IL-10 and transforming growth factor (TGF)-β play a key role in their capacity to regulate the host immune system. Bacteria of the microbiome are associated with various types of pro-inflammatory immune responses. However, as certain bacteria try to maintain their niche, they produce factors which are able to inhibit inflammation. *H. pylori* infection in the stomach for example can induce a strong Th1 response, but also secretes molecules that induce tolerogenic DCs and inhibit T cell proliferation as reviewed by Müller [43]. These factors may simultaneously affect immune responses to bystander antigens such as allergens. Collectively, both helminths and bacteria as well as their products have various regulatory effects on the host immune system. A large number of protective mechanisms have been described by which microbes and/or their products can inhibit experimental airway allergies (see **Table 1** and **Figure 2** for an overview of the discussed literature) The following main mechanisms appear to be important in microbial-induced protection against allergies and airway disease:

1) T cell polarization

AA is a predominantly Th2-mediated disease. Initial observations of protective effects from microbial compounds were thought to be mediated by influencing the Th1/Th2 balance. Indeed, many microorganisms and some microbial molecules that protect against AA induce Th1 cells and/or the Th1 cytokine IFN-γ, and/or reduce Th2 cytokines [44-48]. Interestingly, a similar shift from allergen-specific Th2 to Th1 cytokines was demonstrated *in vitro* in peripheral blood mononuclear cells from patients allergic to timothy grass pollen in response to cystatin from the filarial helminth *Acanthocheilonema viteae* [49]. Other helminth-derived material dampens allergen-specific Th2 immunity while simultaneously inducing a strong helminth antigen-specific Th2 response [50]. CpG oligodeoxynucleotides (CpG ODNs) [51] and *H. pylori* neutrophil-activating protein (HP-NAP) [52] were shown to inhibit AAI and to skewe human allergen specific Th2 cells to Th1 cells *in vitro* [53;54].

2) Induction of Treg/Breg cells

Anti-microbial pro-inflammatory responses are often accompanied by compensatory regulatory responses to prevent excessive damage as a result of inflammatory responses. Different regulatory cells are involved in protection against AAI induced by helminth parasites and bacteria. Treg cells are currently the most well-studied regulatory cell type and include the thymus derived, peripheral derived, and *in vitro* induced regulatory T cells (tTreg, pTreg, and iTreg respectively) [55;56]. They suppress other effector T cells via the regulatory cytokines IL-10 and TGF-β, as well as through cytokine-independent or cell-cell contact-mediated mechanisms. A role for Treg cells induced by natural helminth [57-64] or microbial infection [40;41;46;65;66] in the suppression of AAI has been demonstrated by different groups (**Figure 1**). In addition to live infection, a number of helminth- [67-69] and bacteria-derived molecules also mediate protection in association with the induction of Treg cells [42;70-74]. Infection with many helminths and certain microbiota is associated with the induction of IL-10 and TGF-β [66;75], and the protective effects of these microorganisms or their molecules on AA are often, but not always, dependent on these cytokines. One study with schistosome eggs for example described a Treg cell-mediated protection against AAI which appeared to be independent of these cytokines [76].

Another suppressive cell type, which has been increasingly studied is the Breg cell. Various model systems suggest that Breg cells can mediate protection against AA. Both IL-10-dependent and -independent effects have been described depending on the tissue source of these Breg cells: e.g., spleen IL-10-dependent; [77] versus gut/lungs IL-10-independent; [78;79]. Moreover, Breg cells enforce their protective effects not only by inhibiting effector T cell responses, but also by inducing Treg cells and inhibiting the effector function of antigen-presenting cells (APCs) [80]. Importantly, both helminths and

a number of bacteria have shown to be strong inducers of Breg cells [78;79;81-84]. For some of these species, it has been demonstrated in experimental models that their protective effect against allergic airway disease critically depends on Breg cells (**Figure 1**) [78;79;81]

Parasites or bacteria and their products may not only work via the direct induction of regulatory cells but may also influence the availability of other molecules that exert this effect. For example, certain strains of the human gut bacterium *Clostridia* can protect mice from developing colitis and allergic diarrhoea by the production of short chain fatty acids (SCFAs) [85]. These SCFAs stimulate epithelial cells to produce TGF-β, which is likely to contribute to the induction of IL-10-producing Treg cells. Especially the SCFA butyrate was able to induce Treg cells [86]. Interestingly, the gut microbiome and the production of SCFAs is dependent on diet. A high-fiber diet induces more SCFAs compared to one low-in-fiber via alterations of the microbiota with a direct inhibitory effect on the development of AAI [87].

In summary, many studies report a microbial-mediated induction of Treg/Breg cells, and show that either blocking their effector functions inhibits the protective effect or that the adoptive transfer of

Table 1. An overview of mechanisms by which bacteria and helminths and their products may modulate allergic inflammatory responses. Various cell types seem to be modulated by microorganisms or their compounds during their inhibition of an allergic response. Both results obtained in animal models and from *in vitro* experiments on human cells are summarized in this table.

Affected cell	Mechanism	Microorganism	Composition or molecule	Reference
T cell	Skewing to Th1 rather than Th2 cells	*Ascaris suum*	PAS-1	[44]
		Acanthocheilonema viteae	Cystatin	[49]
			ES-62	[47]
		Caenorhabditis elegans	Crude extract	[45]
		Nippostrongylus brasiliensis	ES	[50]
		Helicobacter pylori	HP-NAP	[52;53]
		Lactobacillus paracasei with *Lactobacillus rhamnosus*	Live infection	[46]
		Salmonella typhimurium SL7207	Attenuated	[48]
		N/A	CpG ODNs	[51;54]
	Induction Treg cells	*Anisakis simplex*	MIF-like protein	[69]
		Ascaris suum	PAS-1	[68]
		Heligmosomoides polygyrus	Live infection	[59;60;63]
		Litomosoides sigmodontis	Live infection	[58]
		Nippostrongylus brasiliensis	Live infection	[64]
		Schistosoma mansoni	Live infection	[61]
			Sm22.6/Sm29/PIII	[67]
			eggs	[76]
		Trichinella spiralis	Live infection	[57;62]
		Alcaligenes faecalis	β-glucan curdlan	[70]
		Clostridium leptum	Live infection	[66]
		Helicobacter pylori	Live infection	[40;41]
			VacA and GGT	[42]
		Lactobacillus rhamnosus GG or *Bifidobacterium lactis*	Live infection	[65]
		Lactobacillus paracasei with *Lactobacillus rhamnosus*	Live infection	[46]

Table 1. (continued)

Affected cell	Mechanism	Microorganism	Composition or molecule	Reference
		Mycobacterium bovis BCG	Freeze-dried preparation	[71;72]
		Multiple bacteria	BV, a lysate	[73]
		Streptococcus pneumonia	Type-3 polysacharide and pneumolysoid	[74]
	Induction γδTCR^{+} T cells	*Ascaris suum*	PAS-1	[68]
		Escherichia coli	Live infection	[106]
B cell	Induction Breg cells	*Heligmosomoides polygyrus*	Live infection	[79]
		Schistosoma mansoni	Live infection	[78;81]
	Induction IgE cross-reactivity	*Schistosoma haematobium*	Live infection	[105]
DC or macrophage	Alteration activation status	*Acanthocheilonema viteae*	Cystatin	[95]
		Clonorchis sinensis	Total protein	[90]
		commensals in general	Live infection	[100]
		Bacillus cereus	Synthetic lipopeptide	[96]
	Induction DC IL-18 secretion	*Helicobacter pylori*	Live infection	[41]
Mast cell	Blockage mast cell degranulation	*Acanthocheilonema viteae*	ES-62	[104]
Epithelial cell	Activation epithelial cells	*Pseudomonas aeruginosa* transfected with type 1 fimbiae	Inactivated	[107]
Various	Blockage IL-33 release and reduction ILC2 cells	*Heligmosomoides polygyrus*	ES	[103]
	Induction regulatory cytokines (IL-10, TGF-β)	*Clostridium leptum*	Live infection	[66]
		Lactobacillus paracasei NCC	Live infection	[75]
	Reduction chemotaxis eosinophils	*Heligmosomoides polygyrus*	Live infection	[63]
		Nippostrongylus brasiliensis	Live infection	[64]
		Strongyloides stercoralis,	Live infection	[97]
		Escherichia coli	LPS	[99]
	Reduction chemotaxis iNKT	commensals in general	Live infection	[101]
	Reduction development basophil precursors	commensals in general	Live infection	[102]
	Alteration microbiota by diet induced SFAs	Effect on multiple bacteria	Live infection	[87]

these regulatory cells is sufficient to exert protection. Despite the clear notion that Treg or Breg cells are important mediators of suppressive effects in AAI, the precise mechanisms by which these cells function is not yet well-described. Treg and Breg cells inhibit other effector T cells and APCs but may also have a range of other immunomodulatory functions in AAI [3;88].

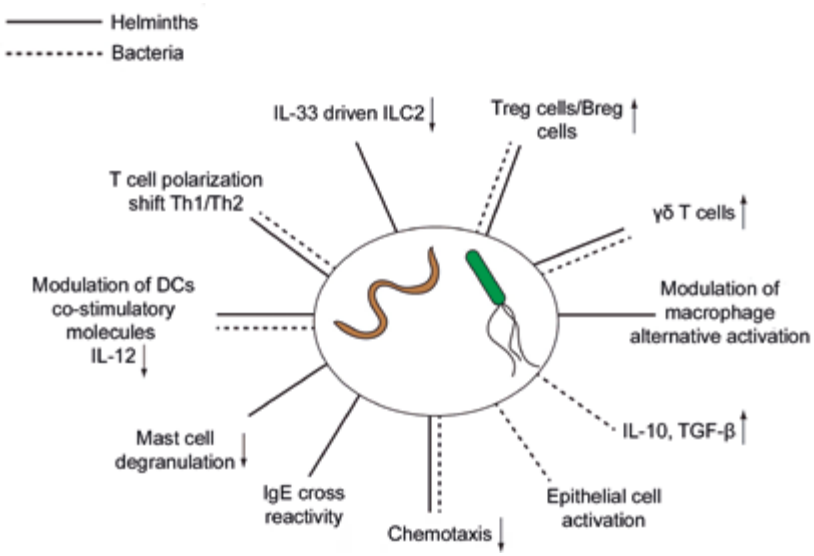

Figure 2. Mechanisms by which bacteria and helminths may modulate allergic inflammatory responses. The proposed mechanisms in which bacteria and helminths can reduce allergic airway inflammation are various in their origin and involve multiple immune cell types. In some studies that are discussed in this review, a combination of multiple mechanisms is proposed.

3) Modulation of DC/macrophage function

While both DCs and macrophages have been primarily implicated in the priming of pro-inflammatory Th1 responses, they are also important contributors to the initiation of regulatory (and Th2) immune responses. Antigen processing in the absence of danger signals retains DCs in an immature state and preferentially induces Treg cells. Several microorganisms have evolved this feature to their benefit. A molecule from Schistosoma directly acts on DCs via the toll-like receptor 2 to promote the polarization of Treg cells [89], while other helminth molecules downregulate co-stimulatory molecules thereby possibly influencing subsequent T cell polarization [90]. AAMs, induced by IL-4 and IL-13, are elicited during polarized type 2 immune responses. They are important innate effectors in anti-parasite immunity and wound healing, especially during helminth infections [91]. Interestingly, AAMs have also been described to possess immunosuppressive activities in models of schistosomiasis and filariasis [92;93]. Lung-stage helminth infections strongly induce alternative activation of alveolar macrophages [94]. A protein from filarial helminths has been reported to protect against experimental AAI in a macrophage- and IL-10-dependent manner [95], further pointing toward the importance of innate immune cells with regulatory properties in protection against allergic inflammation (**Figure 1**). *H. pylori* infection strongly reduces allergic airway disease in experimental models [40], and this protective effect was associated with the production of the cytokine IL-18 by tolerogenic DCs which skew the T cell phenotype toward Treg cells rather than Th17 cells [41]. This effect could be further pinned down to two specific virulence factors of *H. pylori*, the vacuolating cytotoxin (VacA) and the γ-glytamyl transpeptidase (GGT) [42]. A bacterial synthetic lipopeptide was also able to inhibit the disease by keeping the DCs in a tolerant state [96].

4) Other mechanisms

Inhibition of cell migration to the lungs is another immunosuppressive effect of microbial exposure. AAI is characterized by the influx of eosinophils, which is mediated by specific chemokines such as

eotaxin (**Figure 1**). Different studies have described that helminth infection reduces the levels of eotaxin in the bronchoalveolar lavage fluid [63;64;97] and the expression of its receptor CCR3 on eosinophils [98]. Prenatal microbial exposure can modulate AAI in the offspring by inhibition of eosinophil infiltration [99]. Interestingly, mice receiving antibiotics or totally lack gut commensal bacteria show a stronger AAI compared to mice with normal gut flora. This phenomenon has been associated with e.g. a dysregulation in DC recruitment and maturation [100] and an accumulation of invariant natural killer T (iNKT) cells in the lung [101]. Furthermore these mice have an increased frequency of circulating basophils, which was explained by the finding that the basophil precursors of these mice have a higher expression of the IL-3 receptor which regulates their development [102]. Interestingly, the blockage of IL-33 release and subsequent suppression of ILC2 induction by secreted helminth molecules [103] has recently been described in mediating protection against AAI, adding ILCs to the list of effector cells modulated by helminth molecules. Other mechanisms of protection include the blockage of FcεRI-induced degranulation of human mast cells by the helminth-derived glycoprotein ES-62 [104] as well as the induction of carbohydrate cross-reactive, allergen-specific IgE during helminth infections with a reduced capacity to induce basophil degranulation [105]. Furthermore, the induction of γδTCR+ T cells by both helminth and bacteria has been reported [68;106]. A bacterial compound able to inhibit AAI has also been shown to e.g. induce proliferation and wound repair of bronchial epithelial cells [107].

Clinical trials utilizing helminths and microbiota for treatment of respiratory allergies

Modulation of the host immune response against helminths or certain bacteria allows the establishment of chronic infection and a mutual beneficial co-existence in the host. As a bystander effect, immunoregulation often results in suppression of immunity to other antigens such as allergens. Although it is conceivable that part of the activity from life microbes on the immune system can be condensed to the activity of single or multiple molecules, it is elaborate to identify them and it will be a long process before they can be applied in the clinic. Therefore, several researchers have taken the approach of applying clinically controlled full infections in clinical trials, bypassing the process of identifying the responsible molecules.

These controlled infections have been applied now in a number of inflammatory diseases with mixed results. At present, the eggs of the pig whipworm *Trichuris suis* are approved for therapy of IBD, the first helminth-based therapy being used [108-110]. Randomized, controlled trials have shown promising results in terms of safety and efficacy in MS patients [111]. However, no beneficial effect of *T. suis* eggs was observed in patients with AR after 3 weekly doses of 2500 *T. suis* eggs over a 6 month period [112;113]. It remains to be established whether a longer treatment, more or higher dosages, different worms and/or treatment of a younger study population would be more effective. Probiotics and various inactivated bacterial strains have also been widely examined for their health-promoting activities and treatment of various inflammatory disorders. However, probiotics are also not routinely used as a treatment in allergic patients. A meta-analysis of application of probiotics to pregnant women or babies concluded that probiotics do not prevent the development of asthma [114], although it is suggested that probiotics might be effective in preventing AD [115]. For example, AD could be reduced by oral application of a lysate of heath-killed *Escherichia coli* and *Enterococcus faecalis* in a subgroup of children with at least one parent with AD [116]. Broncho-Vaxom (BV), an extract which contains a mixture of multiple bacteria is already used to treat patients from recurrent respiratory tract infections, but it is not yet applied against asthma [73]. Other bacterial preparations showed some effectiveness in clinical trials. For example, newly diagnosed adult patients with moderate persistent asthma experienced some improvement of lung function and asthma symptoms when treated with inactivated *Mycobacterium phlei* [117]. In conclusion, several trials have applied helminths, bacteria or complete extracts/lysates in clinical studies, of which some showed promising results for the treatment of certain inflammatory

diseases. There is little evidence so far for a therapeutic effect of controlled microbial infections on respiratory allergies. Although this is a relative young field and conceptually very exciting, it needs to be further explored and developed in the near future with help from small-medium enterprises (SMEs) and pharmaceutical companies.

Helminth- and microbial-derived molecules for the treatment of respiratory allergies

Although some of the clinical trials showed promising results, treatment options based on natural infections bear risks and the first trials for respiratory allergies showed that further testing and optimization is needed before implementation in the clinic is possible. Therefore several groups have instead focused their efforts on studying the effects of (single) helminth- or bacteria-derived molecules as opposed to natural infections. However, as this is a more elaborate path to follow, this field is still in its infancy and the current data available are mainly from animal models.

Molecules derived from helminths that show protective effects in models of AA include whole antigen generated from adult worms [45;90;118] or other live cycle stages such as eggs [119], excretory-secretory products (ES) [50;120;121] and isolated [44;47;68;104;122] as well as recombinantly expressed [49;67;69;95] single antigens. Different strategies are also examined with regard to the protective effects of bacterial derived products. Common components of bacteria such as the cell wall LPS [99;123;124], curdlan [70] and CpG sequences in the DNA [51] have been reported to be protective. Importantly, in many of the applied experimental models exposure to LPS actually induces rather than inhibits AAI. These findings demonstrate that exposure to bacteria is not universally protective but may rather depend on certain bacterial traits. Some groups use live-attenuated or inactivated bacteria [48;107;117;125], a lysate [73] or frieze-dried bacteria [71;72]. In some cases more specific single molecules were tested [42;52], however this field is not yet strongly evolved. The type of preparation of the compound may be of great importance, as it was shown that inhibition of AAI was only effective when using frieze-dried but not heat-killed or alive *Mycobacterium bovis* BCG [71].

From the bench to the clinic

Nearly all of the experimental studies have been conducted in models of AAI based on the model allergen ovalbumin that is isolated from chicken egg white. While this model is well established and allows the use of genetically modified mice to study antigen-specificity, there is a great need to apply models based on natural allergens (house dust mite, grass, tree pollen) as it allows to more easily relate findings from the model to the field and the clinic. Furthermore, these models use a natural route of sensitization. First studies show protective effects of both helminth- [49;126] and bacteria-derived products [73;75] in models based on natural allergens. It is of interest that some cases of asthma in humans are induced by fungal antigens, and some studies have also suggested a protective role of helminth and bacterial compounds in fungus-induced AAI in mice [103;127].

Another aspect of interest when studying the protective effect of microorganisms and their molecules is the timing and duration of administration. Allergen-sensitization often occurs early in life, followed by life-long suffering from asthmatic disease. Any preventative treatment approach would therefore be most beneficially applied during infancy. Of interest, it has been suggested in various studies that treatment of neonatal mice with bacteria or their compounds is more effective in inducing tolerance compared to adult mice [40;41;65]. The treatment of newborns might however be difficult to achieve in practice. Interestingly, a number of papers we have discussed have applied treatment to the pregnant and later lactating mothers, showing protection against features of AAI in the offspring [75;99;128]. The rationale for the success of these early treatment approaches might come from epigenetic changes in immune-associated genes. In regard to this, it was shown that epigenetic changes had occurred in mice following contact with environmental bacteria, leading to a reduced

susceptibility to allergies [128]. Altogether, it is clear that microbial molecules should be best applied as early as possible to prevent the development of allergic diseases, it nevertheless is of great importance to investigate microbial molecules for their potential to dampen already established disease.

Although there are some promising results that indicate treatment of ongoing asthmatic disease is possible [45;69], the majority of the data suggests that this is much more difficult to achieve than the prevention of disease onset. But as patients are repeatedly exposed to allergens, testing potential therapeutic molecules in a setting of repeated challenge is thus important. While some molecules fail to protect upon re-challenge [73] others maintain their protective effect [71;74], making them more suitable as a putative treatment for established disease. Working toward the implementation of a novel treatment for asthmatic patients, not only the timing but also the route of administration needs to be considered. In allergen immunotherapy, alternative routes of local (e.g. sublingual, intranasal) and systemic (e.g. oral) allergen administration to mucosal surfaces show promising results in terms of immunogenicity and safety in clinical trials [129] compared to the standard method of subcutaneous injections. In mouse models the same, but also different and less obvious routes of molecule application are applied (intraperitoneal, often in combination with an adjuvant; intravenous; intranasal/aerosol; intratracheal), when testing the therapeutic potential of microbial-derived molecules. In view of human application, we believe that administration routes that are most likely to induce tolerance should be favored also in the animal models to allow extrapolation of the results to humans.

From a clinical point of view, there are several issues that need to be addressed when working toward the implementation of helminth- and microbial-derived molecules in the clinics. As summarized elsewhere [130;131], potential side effects need to be considered. These include anaphylaxis and atopic reactions to helminth parasite-derived molecules, as well as the risk of general immune suppression in the patient. Trials of the recombinant Na-ASP-2 hookworm vaccine for example have shown important differences in the immune response between individuals not exposed to hookworm and individuals from endemic areas that have previously been infected [132;133]. In order to avoid general immune suppression, it is favorable to be able to induce antigen-specific tolerance, or local tolerance restricted to the site of allergen-induced inflammation (e.g. the lungs). Furthermore, it should be ensured that tolerance induced by microbial-derived molecules does not inhibit immune responses to natural infections with potential pathogenic microorganism. Interestingly, in mice the application of freeze-dried BCG to inhibit AAI did not interfere with the diagnosis of live infection with the bacteria [71]. Finally, the risk of cross-reactivity between antigens of microorganisms and allergens needs to be taken into account.

It is becoming clear that asthma is a term that describes a collection of symptoms. Personalized medicine tries to tailor treatment regimens specifically to a patient condition or characteristic by investigating individual-based treatments, as some patients might benefit from therapies that only show efficacy in a subgroup of the asthmatics. In greatest need for novel treatment options are probably patients that suffer from severe asthma, which are often resistant to treatment with steroids and cannot be subjected to specific immunotherapy. For these patients, it would be of special importance to develop a treatment that cures ongoing disease, which is harder to achieve than preventative approaches as discussed above. This may prove to be difficult as advanced local tissue degeneration has occurred which will probably not be restored even when inflammation has been dampened. However, it is important to realize that the larger group of asthma patients with milder forms of disease, which are now well controlled by steroids, form a realistic target group to apply a 'treatment to cure' therapy with microbial molecules. Altogether, we believe that, rather than applying natural infections, the isolation of modulatory, protective components from bacteria and helminths may enable the development of more sustained and controlled preventive or therapeutic strategies.

EXPERT COMMENTARY AND FIVE YEAR VIEW

Despite the increasing worldwide prevalence of asthma little has changed in the last years in the treatment or prevention of this disease. The main therapy is still based on inhaled steroids and bronchodilators. Despite the development of monoclonal antibodies targeting IgE or Th2 cytokines (IL-5, IL-13, IL-4 receptor alpha chain) no ground-breaking advancement has been made in prevention or treatment of asthma. Epidemiological and experimental research has increased our understanding of mechanisms how microorganisms can evade the immune system and suppress the development of allergy and airway disease. Several mechanistic pathways how these microorganisms actively suppress immune reactions have indeed been discovered. In our opinion, these novel insights are promising and open up a novel avenue for the development of innovative therapeutics. As live infection with bacteria and parasites is not always perceived as a desirable strategy, focus has been shifted to isolating molecules from microorganisms. Several molecules have been identified and are currently being evaluated in experimental models. We expect that in the following years the number of identified suppressive molecules will further increase and that these molecules will be evaluated in preclinical models. The predictive values of these models in regard to the effectiveness in human disease may be limited. Nevertheless, these model data will provide interesting and valuable insights about the underlying mechanisms involved in the suppressive capacity of these molecules, timing of treatment and route of administration. One major issue is if these molecules can be used solely for prevention or also for treatment of people with already developed allergic disease. This will certainly also critically determine the therapeutic windows in which treatment is feasible. Next, crucial steps need to be made toward the clinic in which active involvement of SMEs and pharmaceuticals will help to initiate clinical trials in patients with allergic disease such as AA. These trials should then reveal if a molecular approach can advance our therapeutic options in allergic disease and asthma and if we can effectively modulate immune responses to allergens long-term.

KEY ISSUES

- Atopic diseases (e.g. allergic rhinitis, allergic asthma) are a major global health burden today. Medication options are limited and only alleviate the symptoms, therefore novel treatment options need to be developed urgently.
- Helminths/microbiota modulate the host immune system, suppressing the immune response to themselves and bystander antigens such as allergens. The 'hygiene hypothesis' suggests that the drastic increase in incidence rates for atopic diseases can be ascribed to a reduction in infectious pressure and the resulting imbalance of the immune system.
- A body of epidemiological evidence underpins that helminths (e.g. hookworms, schistosomes) and certain bacteria (e.g. *H. pylori*) protect against allergic asthma in humans.
- *In vivo* studies suggested mechanisms by which microorganisms or their products protect against allergic airway disease, including shifting of the Th1/Th2 immune balance, induction of Treg/Breg cells, modulation of DC/macrophage function and impairment of chemotaxis and effector cell (e.g. mast cell, basophil) function.
- Helminth- and microbial-derived molecules have been identified which efficiently protect against experimental allergic airway inflammation, and which include whole antigen preparations, excretory-secretory products and single molecules (single/recombinant) of helminths as well as live-attenuated and freeze-dried bacteria, bacterial lysates and single bacterial molecules.
- At the moment microbial-derived treatments are not routinely applied in the clinics to treat allergic airway disease. Working toward a novel treatment option for atopic patients based on microbial-

derived molecules, a range of factors such as safety, mode of action (general vs. antigen-specific tolerance), timing and route of administration need to be taken into account.

- We expect that more immune suppressive molecules will be described and that these molecules will further progress into clinical trials.

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Chapter 7

ISOLATED *SCHISTOSOMA MANSONI* EGGS AND EGG-DERIVED GLYCOPROTEIN OMEGA-1 PREVENT ALLERGIC AIRWAY INFLAMMATION

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ABSTRACT

Chronic helminth infection with *Schistosoma mansoni* protects against allergic airway inflammation (AAI) in mice and is associated with reduced Th2 responses to inhaled allergens in humans, despite the presence of schistosome-specific Th2 immunity. Schistosome eggs strongly induce type 2 immunity and allow to study the dynamics of Th2 versus regulatory responses in the absence of worms. Omega-1 (ω-1) is a glycoprotein secreted by *S. mansoni* eggs, and represents one of the major type-2 immunity-inducing components within soluble egg antigen (SEA). Treatment with isolated *S. mansoni* eggs by i.p. injection prior to induction of AAI to ovalbumin (OVA)/alum led to significantly reduced AAI as assessed by less BAL and lung eosinophilia, less cellular influx into lung tissue, less OVA-specific Th2 cytokines in lungs and lung-draining mediastinal lymph nodes, and less circulating allergen-specific IgG1 and IgE antibodies. While OVA-specific Th2 responses were inhibited, treatment induced a strong systemic Th2 response to the eggs. The protective effect of *S. mansoni* eggs was unaltered in μMT mice lacking mature (B2) B cells, and unaffected by Treg cell depletion using anti-CD25 blocking antibodies during egg treatment and allergic sensitization. Notably, prophylactic egg treatment resulted in a reduced influx of pro-inflammatory, monocyte-derived dendritic cells into lung tissue of allergic mice following challenge. Treatment with recombinant, *Nicotinia benthamiana*-expressed ω-1 replicated the protective effect observed by eggs. Altogether, *S. mansoni* eggs can protect against the development of AAI, despite strong egg-specific Th2 responses, and protection can be achieved by the egg-derived glycoprotein ω-1.

INTRODUCTION

The prevalence of allergies and asthma has dramatically increased in developed countries over the last decades, and the incidence rates continue to increase especially in low and middle-income countries [1]. It has been suggested that environmental factors, such as an increased exposure to air pollutants and tobacco smoke [2], but also an overly sanitary life-style with decreased exposure to parasites may play an important role in the increased prevalence of asthma.

The protective effect of parasitic infections against allergic asthma has been introduced as one of many elements in the so-called 'old friends hypothesis' [3-6]. The relationship between helminths and asthma is complex, with factors such as worm species, timing, intensity and chronicity of infection as well as host genetics at interplay [7], and a causal link in humans has yet to be demonstrated. Acute or light helminth infections seem to promote allergic sensitization and allergic symptoms, while chronic helminth infections are more often associated with protection [7, 8]. This may also explain why deworming at population level has been shown to result in enhanced skin-prick test positivity or rates of eczema in some cases, while having no effect in others [8]. A large body of epidemiological and experimental studies has shown that, despite heterogeneity in the results, especially hookworm infections have been consistently found to reduce allergic sensitization [9, 10]. *Schistosoma* spp. has also been reported to be protective against allergic sensitization in humans [9, 11].

Schistosoma spp. infections consist of an acute phase dominated by a strong Th2 response to the eggs, and a chronic phase with a diminished Th2 response and increased activity of regulatory immune cells [12]. To distinguish between egg-induced and worm-induced protection from allergic airway inflammation (AAI), experimental infections with mixed sex or male *Schistosoma* worms were performed [13-19]. However, these reports revealed conflicting results as some indicated a reduction in AAI in the presence of egg-producing infections [14, 16, 18, 19], whereas others showed a reduction in the absence of eggs [13, 17]. In addition, some studies show protection from AAI during the acute (5-11 weeks) [17, 19], and others during the chronic (12-16 weeks) [14, 18] phase of infection, which elicit characteristically different immune responses.

Omega-1 (ω-1) is a glycoprotein secreted by *S. mansoni* eggs, and represents one of the major type-2 immunity-inducing components within soluble egg antigen (SEA). The glycoprotein is a T2 RNase and has been well-described to condition DCs for Th2 priming in a protein- and glycosylation-dependent manner [20, 21]. Ω-1 gets bound and internalized via its glycans by a member of the C-type lectin receptor (CLR) family, called mannose receptor (MR), and subsequently interferes with protein synthesis by RNA degradation [20]. Moreover, ω-1 has been described to be a hepatotoxin [22, 23], to induce inflammasome activation and interleukin (IL)-1β secretion in macrophages [24], and to induce Foxp3$^+$ regulatory T (Treg) cells in non-obese diabetic (NOD) mice via retinoic acid and transforming growth factor (TGF)-β production by DCs [25]. Interestingly, treatment of obese mice with ω-1 improves their metabolic homeostasis by promoting IL-33 release in white adipose tissue and subsequent accumulation of ILC2s [26].

From an immunological perspective, the conundrum that Th2-inducing helminth infections can dampen symptoms linked to allergic Th2 responses, as observed in humans and mouse models [7, 8], is still subject to discussion. Often, the immunomodulatory activity of helminths is associated with the induction of a regulatory network. In mouse models, the rodent nematodes *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* revealed important insights into the role of Treg [27] and regulatory B (Breg) [28] cells as well as the regulatory cytokine IL-10 [29, 30] in protection against AAI. Treg and Breg cells as well as IL-10 have also been described to mediate protection induced by *S. mansoni* infections [14, 16-19]. However, data showing that the acute phase *S. mansoni* infections and/or the presence of eggs are important for protection suggests that the induction of a regulatory network is not the sole determinant of immunomodulation.

To further explore the dynamics and interplay between Th2 responses and regulatory responses in the protective effect of *S. mansoni* infections against AAI, we used isolated eggs instead of a full natural infection. We show that eggs are equally protective as a natural *S. mansoni* infection in a prophylactic setting, despite the induction of a strong egg-specific Th2 response. Egg treatment did not lead to Treg cell expansion or enhanced activity markers on Treg cells following allergen challenge. The observed protection was subsequently found to be independent of both Treg cells and B cells. Instead, *S. mansoni* egg-induced protection was associated with a reduced pulmonary influx of pro-inflammatory monocyte-derived dendritic cells (moDCs). Importantly, we could demonstrate that the protective effect of eggs can be replicated by recombinant, *N. benthamiana*-expressed ω-1. This study shows that, although inducing egg-specific Th2 responses, *S. mansoni* eggs can protect from AAI, closely resembling the human situation. This study moreover provides the first description of protective effects of ω-1 in the context of AAI.

RESULTS

S. mansoni eggs protect against OVA/alum-induced AAI

We and others have previously reported that chronic, but not acute, mixed infection with *S. mansoni*, which most closely resembles the natural situation in humans, protects mice from AAI [14, 16, 18, 19]. A plausible explanation for the differential effect of acute and chronic infection might be the changing balance between Th2 and regulatory responses. To further explore the dynamics and interplay between Th2 and regulatory responses in helminth infections, we first tested whether *S. mansoni* liver-derived eggs can confer protection from AAI in the absence of worms. These eggs were isolated from livers of infected hamsters and frozen prior to use. We have confirmed that the excretory-secretory product (eggES) of these freeze-thawed eggs is similar to that of freshly isolated, mature liver eggs (**suppl. Figure 1 A**). Mice were treated twice with $5x10^3$ eggs by i.p. injection prior to allergic sensitization with OVA emulsified in alum adjuvant (**Figure 1 A**). This treatment resulted in a profound suppression of overall cellularity and eosinophilia both in the bronchoalveolar lavage (BAL) fluid (**Figure 1 B**) and in lung tissue (**suppl. Figure 1 B**), accompanied by a reduction in various other leukocyte populations (**Figure 1 C**). The reduction in AAI in treated mice was also reflected by the reduction of cellular infiltration around the airways (**Figure 1 D**) as assessed by histology. Assessment of serum immunoglobulins revealed that egg treatment ablates the OVA-specific IgG1 and IgE response (**Figure 1 E**). We also assessed local cytokine production in BAL fluid, as well as local recall responses to the allergen by restimulation of mediastinal lymph nodes (medLNs) with OVA. In both BAL fluid and OVA-restimulated medLN cell cultures, the production of allergic Th2 cytokines IL-5 and IL-13 was greatly increased in allergic mice, but blocked upon egg treatment (**Figure 1 F, G**). IL-10, whilst not detectable in BAL fluid, followed the same pattern as IL-5 and IL-13 in OVA-restimulated medLN cell cultures (**Figure 1 G**). Additionally, IFN-γ could hardly be detected in both BAL fluid and OVA-restimulated medLN cell cultures (**Figure 1 F, G**). Collectively, these data show that *S. mansoni* egg administration prior to allergic sensitization inhibits the development of OVA-induced AAI.

Protection occurs despite the induction of an egg-specific Th2 response

Human and animal hosts are known to mount a strong type 2 immune responses to egg deposition in live infections. To determine whether egg treatment induced a systemic, antigen-specific cytokine response in our model, we restimulated spleen cell cultures with soluble egg antigens (SEA). SEA restimulation profoundly increased the production of IL-5, IL-10 and IL-13, but not IFN-γ, in mice that had received isolated eggs compared to naïve or allergic, untreated mice (**Figure 2 A**). In the medLNs, similar cytokine profiles were observed following SEA restimulation (**suppl. Figure 2**). Furthermore, OVA-restimulated spleen cell cultures induced a strong Th2 cytokine production in the allergic group

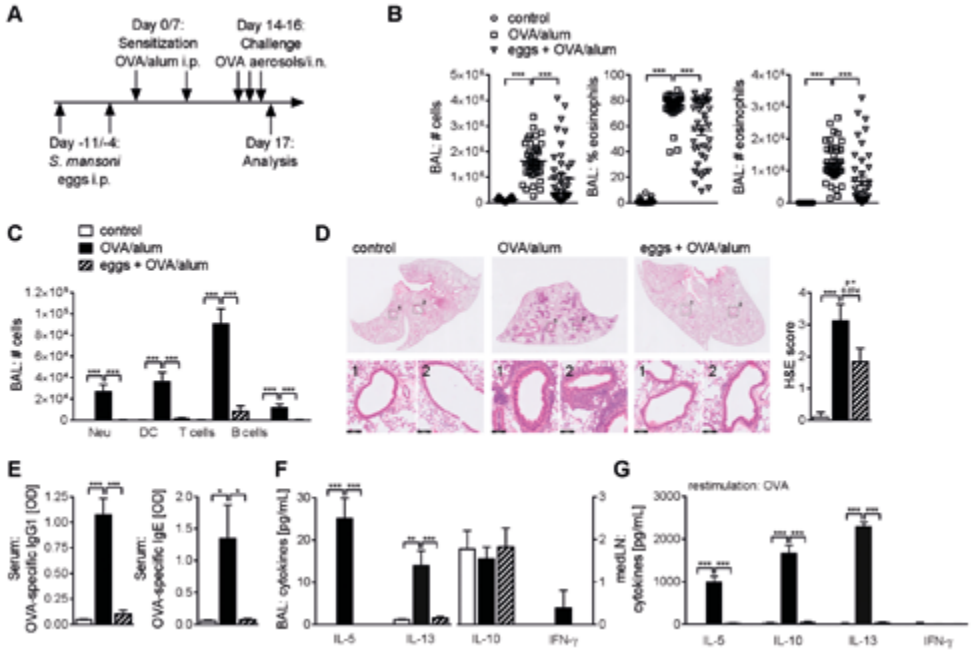

Figure 1. ***S. mansoni*** **eggs protect against OVA/alum-induced AAI.** (**A**) Schematic representation of the experimental model. (**B**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. Summary of multiple experiments. (**C**) Total number of neutrophils (Neu), dendritic cells (DC), T cells and B cells in BAL fluid as assessed by FACS. Representative of multiple experiments, n=4-5. (**D**) Representative images of haematoxylin and eosin (H&E) histology staining from PFA-fixed sections (scale bar = 100μm). Scores for severity of cellular infiltration around the airways on a scale of 0-4 were assessed by two blinded observers. The average of both scores is displayed. Significant difference was determined by unpaired t-test, * $p < 0.05$. (**E**) OVA-specific IgG1 and OVA-specific IgE antibodies in serum measured by ELISA. Representative of multiple experiments, n=4-5. (**F**) Cytokine concentration in BAL fluid measured by CBA. Representative of multiple experiments, n=4-5. (**G**) Cytokine concentration in medLN cell supernatants after 4d re-stimulation with OVA (10μg/mL) measured by CBA. Representative of multiple experiments, n=4-5. Significant differences were determined by two-way ANOVA following Tukey's multiple comparison test (B) or one-way ANOVA following Dunnett's multiple comparisons test (C-F) and are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

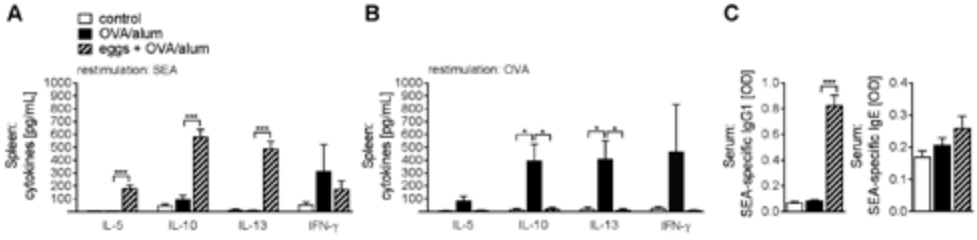

Figure 2. Protection occurs despite the induction of an egg-specific type 2 induction. (**A, B**) Cytokine concentration in spleen cell supernatants after 4d re-stimulation with SEA (10μg/mL; **A**) or OVA (10μg/mL; **B**). Representative of 2 experiments, n=3-4. (**C**) SEA-specific IgG1 and SEA-specific IgE antibodies in serum measured by ELISA. Representative of 3 experiments, n=4-5. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with * $p < 0.05$, *** $p < 0.001$.

(**Figure 2 B**). Strikingly, these data show a systemic inhibition of OVA-specific type 2 immunity in addition to the local inhibition observed in **Figure 1**. Additionally, high levels of IgG1 were observed, the SEA-specific IgE response was however found to be weak (**Figure 2 C**). These data show that egg treatment induces a fully developed Th2 response to egg antigens in the absence of an allergic Th2 response to OVA.

Egg-induced protection against AAI is independent of Treg cells

S. mansoni infection [16, 19] and antigens [31, 32] have been described to induce Treg cells in mice and humans. Therefore, we addressed whether egg treatment enhanced the number or activation state of Treg cells following allergen challenge in our model. The frequency and number of Treg cells was significantly increased in the BAL fluid of allergic mice, but remained at baseline in mice treated with eggs (**Figure 3 A**). While the frequency of Treg cells remained unchanged in the medLNs, total numbers increased in allergic mice irrespective of egg treatment (**Figure 3 B**). Additionally, extracellular regulatory markers CTLA-4 and GITR showed enhanced expression on Treg cells in the BAL (**Figure 3 C**) and medLNs of allergic animals (**Figure 3 D**), but were not further increased by egg treatment. To further dissect the role of Treg cells in egg-mediated suppression, we depleted Treg cells by means of monoclonal, anti-CD25 depleting antibodies (clone PC61) during egg treatment and allergic sensitization (**Figure 3 E**). Successful depletion of Treg cells was confirmed by flow cytometry (**suppl. Figure 3 A**). Mice depleted of CD25-expressing Treg cells still displayed significantly reduced BAL cellularity and number of BAL eosinophils, comparable to control mice treated with antibodies against anti-β-galactosidase (anti-βGAL) (**Figure 3 F**), as well as reduced numbers of neutrophils (**Figure 3 G**). In contrast, in egg-treated mice Treg cell depletion did seem to affect the number of DCs, T cells and B cells in the BAL fluid at least to some extent, as their numbers were increased and not significantly different anymore between allergic controls and egg-treated mice (**Figure 3 G**). We observed a similar trend in the secretion of type 2 cytokines IL-5 and IL-13 in BAL fluid following Treg cell depletion, which were restored compared to those in allergic control mice (**Figure 3H**). These data may suggest a selective effect on the lung T cell compartment following anti-CD25 treatment, resulting in enhanced T cell activation. Although there is a general trend towards increased airway inflammation following anti-CD25 treatment in both allergic control and egg-treated mice, this effect seems to be more pronounced in the egg-treated group with respect to the lung T cell compartment. Anti-CD25 treatment did however not restore eosinophilic inflammation in egg-treated mice. Collectively, these data suggest that depletion of Treg cells does not have a major influence on inhibition of AAI by egg treatment and thus cannot explain egg-induced protection against AAI.

Mature B cells are not crucial for egg-induced protection against AAI

In addition to Treg cells, we also sought to investigate the role of B cells in egg-induced protection against AAI. B cells possess various functions ranging from antibody production, formation of memory and antigen presentation to the production of pro- and anti-inflammatory cytokines. The production of regulatory cytokines such as IL-10 and the production of inhibitory immunoglobulins are widely recognized regulatory functions exerted by B cells [33]. Here, we used μMT mice, which lack mature (B2) B cells [34], to test whether B cells are required for the protective effect on OVA/alum-induced AAI observed after egg treatment. Both WT and μMT mice responded equally well to induction of AAI as shown by total BAL cellularity and the presence of eosinophils in BAL fluid (**Figure 4 A**). Egg treatment significantly inhibited eosinophilia both in WT and μMT mice (**Figure 4 A**), and a similar pattern could be observed for BAL neutrophils, DCs and T cells (**Figure 4 B**). Additionally, while the abundance of B cells sharply increased in WT mice and decreased with egg treatment, the number of B cells was expectedly low in μMT mice (**Figure 4 B**). In BAL fluid, μMT mice showed a tendency towards reduced

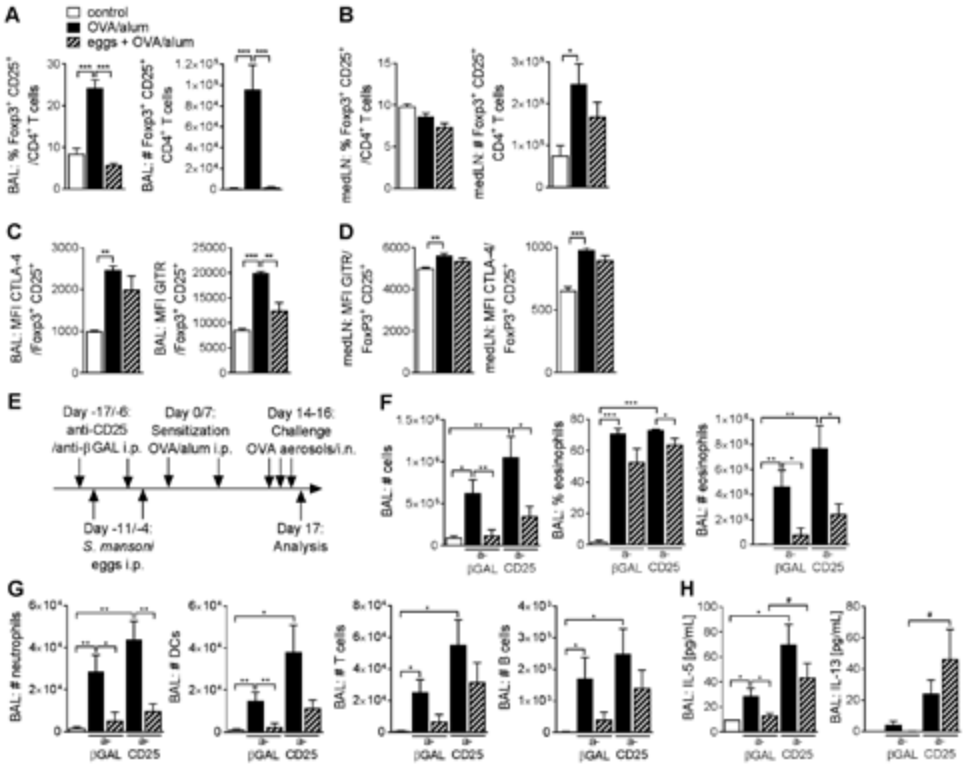

Figure 3. Regulatory T cells are not involved in egg-induced protection against AAI. (**A, B**) Percentage and total number of Foxp3$^+$ CD25$^+$ Treg cells in BAL fluid (**A**) and medLNs (**B**) assessed by FACS. Representative of multiple experiments, n=4-5. (**C, D**) Geometric mean expression of CTLA-4 and GITR on Foxp3$^+$ CD25$^+$ Treg cells in BAL fluid (**C**) and medLNs (**D**) assessed by FACS. Representative of multiple experiments, n=4-5. (**E**) Schematic representation of experimental model. (**F**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. Representative of 2 experiments, n=4-6. (**G**) Total number of neutrophils, dendritic cells (DCs), T cells and B cells in BAL fluid as assessed by FACS. Representative of 2 experiments, n=4-6. (**H**) Cytokine concentration in BAL fluid measured by CBA. Representative of 2 experiments, n=4-6. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test as indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or by unpaired t-test as indicated by $^{\#}$ $p < 0.05$.

IL-5 and IL-13 concentrations upon egg treatment similarly to WT animals, albeit not significant (**suppl. Figure 4**). The expression of Th2 cytokines in supernatants of *in vitro* OVA-restimulated medLN cell cultures from WT mice was highly elevated in AAI mice and significantly reduced after egg treatment (**Figure 5 C**). Allergic μMT mice produced significantly less IL-5, IL-10 and IL-13 compared to their WT counterparts (**Figure 4 C**), like recently also shown in a house dust mite model of asthma [35]. As expected, OVA-specific IgG1 and IgE antibodies in μMT mice remained at baseline values observed in naïve WT animals (**Figure 4 D**), excluding a major role of inhibitory antibodies in protection. These data show that B2 B cells, while contributing to Th2 cytokine production and the production of antigen-specific antibody responses, are not required for egg-mediated protection from AAI.

Egg treatment is associated with decreased recruitment of moDCs into the lung compartment

Different studies have shown that helminths not only affect T and B cells, but can also mediate important effects by acting on DCs [36, 37]. Pulmonary DCs play a central role in the immune response

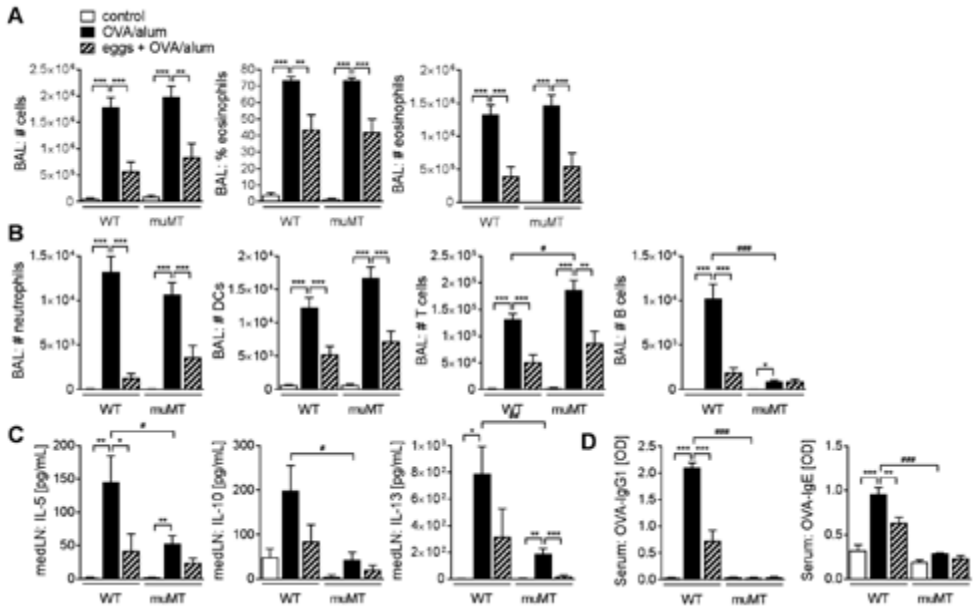

Figure 4. Mature B cells are not involved in egg-induced protection against AAI. (**A**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. (**B**) Total number of neutrophils, dendritic cells (DCs), T cells and B cells in BAL fluid as assessed by FACS. (**C**) Cytokine concentration in medLN cell culture supernatants after 4d re-stimulation with OVA (10µg/mL) measured by ELISA. (**D**) OVA-specific IgG1 and OVA-specific IgE antibodies in serum measured by ELISA. All data are a summary of 2 experiments, n=5-12. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test as indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or by unpaired t-test as indicated by $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

to allergens [38]. Under steady state conditions, CD103$^+$ and CD11b$^+$ conventional DC (cDC1 and cDC2, respectively) populations can be distinguished, whereas allergic inflammation triggers a strong influx of inflammatory moDCs [39]. Both CD11b$^+$ cDC2 and moDCs can drive allergic Th2 responses in a model of HDM allergy, whereby moDCs were only sufficient in a high-dose HDM model of AAI [40]. moDCs produce various chemokines and present allergen locally in the lung especially in a model of high-dose allergen exposure [40]. Next, we investigated whether prophylactic egg treatment alters the presence and function of different DC subsets in the lung of OVA/alum-allergic mice. The number of CD11c$^+$ MHCII$^+$ DCs strongly increased in allergic compared to control mice (**Figure 5 A**). Interestingly, egg treatment significantly impaired the number of lung DCs following challenge, whereas the total number of cells in the lung remained unaffected (**Figure 5 A**). The reduction in the number of DCs seems to be solely attributable to an abrogated expansion of the moDC compartment, as both the numbers of CD11b$^+$ cDC2 and CD103$^+$ cDC1s proved to be unaffected by egg treatment (**Figure 5 B**). Monocytes, which can differentiate into moDCs under inflammatory conditions, migrate in a CCR2/CCL2-dependant manner, whereas CD11b$^+$ cDC2 and CD103$^+$ cDC1s do not [40]. We found the concentration of CCL2 in BAL fluid to be strikingly increased in allergic mice, and significantly reduced upon egg treatment (**Figure 5 C**), providing an indication that the reduced number of moDCs in lung tissue is the result of a reduced CCL2-mediated influx.

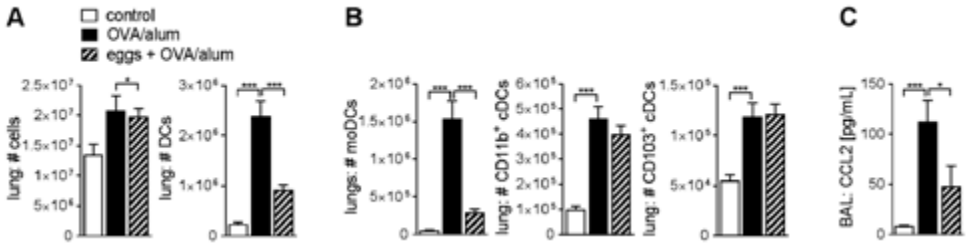

Figure 5. Egg treatment impairs the lung moDC, but not cDC, compartment. (**A**) Total number of lung cells, and total number of lung DCs ($CD11c^+$ $MHCII^+$), assessed by FACS. Representative of 2 experiments, n=4-6. (**B**) Total number of moDCs ($CD11c^+$ $MHCII^+$ $CD11b^+$ $CD103^-$ $CD64^+$), $CD103^+$ cDC1 ($CD11c^+$ $MHCII^+$ $CD11b^-$ $CD103^+$ $CD64^-$) and $CD11b^+$ cDC2s ($CD11c^+$ $MHCII^+$ $CD11b^+$ $CD103^-$ $CD64^-$) in the lung, assessed by FACS. Representative of 2 experiments, n=4-6. (**C**) Concentration of CCL2 in BAL fluid measured by CBA. Summary of 2 experiments, n=9-10. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and as indicated with * $p < 0.05$, *** $p < 0.001$.

The suppressive effect of *S. mansoni* eggs on AAI can be reproduced by one of the major egg antigens, the glycoprotein ω-1

S. mansoni eggs contain hundreds of proteins and glycoproteins, of which a subset constitutes eggES that can potentially modulate host immunity [41]. We therefore aimed to identify single molecules within eggES that could mediate AAI suppression. The glycoprotein ω-1 is abundant in eggES and most well-known as major Th2-inducing component of *S. mansoni* eggs [20, 21], but has also been reported to have a range of other effects including the induction of $Foxp3^+$ Treg cells in NOD mice [25]. Moreover, ω-1 is known to act on myeloid cells, binding and being internalized by MR, which makes ω-1 an interesting candidate molecule in the light of changes within the lung myeloid compartment observed following egg treatment. As the natural molecule is difficult to isolate in large quantities, we treated mice with recombinant ω-1 (2x 50μg/mouse) expressed in plants, which has a similar Th2-inducing activity as the native protein [42]. Treatment was performed prior to allergic sensitization, following the same protocol as for the egg treatment model (**Figure 6A**). We found ω-1 treatment to significantly impair overall cellularity and eosinophilia in OVA/alum-induced AAI (**Figure 6B**). Albeit not significant except for B cells, the influx of various other cell population also seemed affected (**Figure 6C**). ω-1-treated mice showed less cellular infiltration around the airways (**Figure 6D**). Treatment with ω-1 furthermore significantly reduced the production of OVA-specific IgG1, and showed a tendency towards also reducing OVA-specific IgE (**Figure 6E**), while clearly inducing IgG1 and IgE production in response to ω-1 (**Figure 6F**). Similar to eggs, ω-1 treatment also significantly reduced the concentration of IL-5 and IL-13 in BAL fluid (**Figure 6G**) and the OVA-specific production of IL-5, IL-10 and IL-13 in *in vitro* re-stimulated medLN cultures compared to allergic, untreated mice (**Figure 6H**). In contrast, re-stimulation of medLN cells with ω-1 induced the production of IL-5, IL-10, IL-13 and IFNγ, albeit at a much lower concentration that OVA re-stimulation (**Figure 6I**). As we have observed for treatment with eggs, ω-1 also did not alter the abundance of $Foxp3^+CD25^+$ Treg cells or their CTLA-4 expression in lung-draining medLNs (**Figure 6J**). While we have not performed Treg depletion experiments in ω-1-treated mice, like we did for the egg-treated mice, these data suggest that Treg cells might also not play a major role in ω-1-mediated suppression of AAI. Like observed for egg treatment, ω-1 treatment reduced the number of moDCs, but not $CD11b^+$ cDC2s and $CD103^+$ cDC1s (**Figure 6K**). Collectively, these data show that ω-1 can suppress AAI with a similar pattern of immune characteristics as observed for egg-mediated protection from AAI.

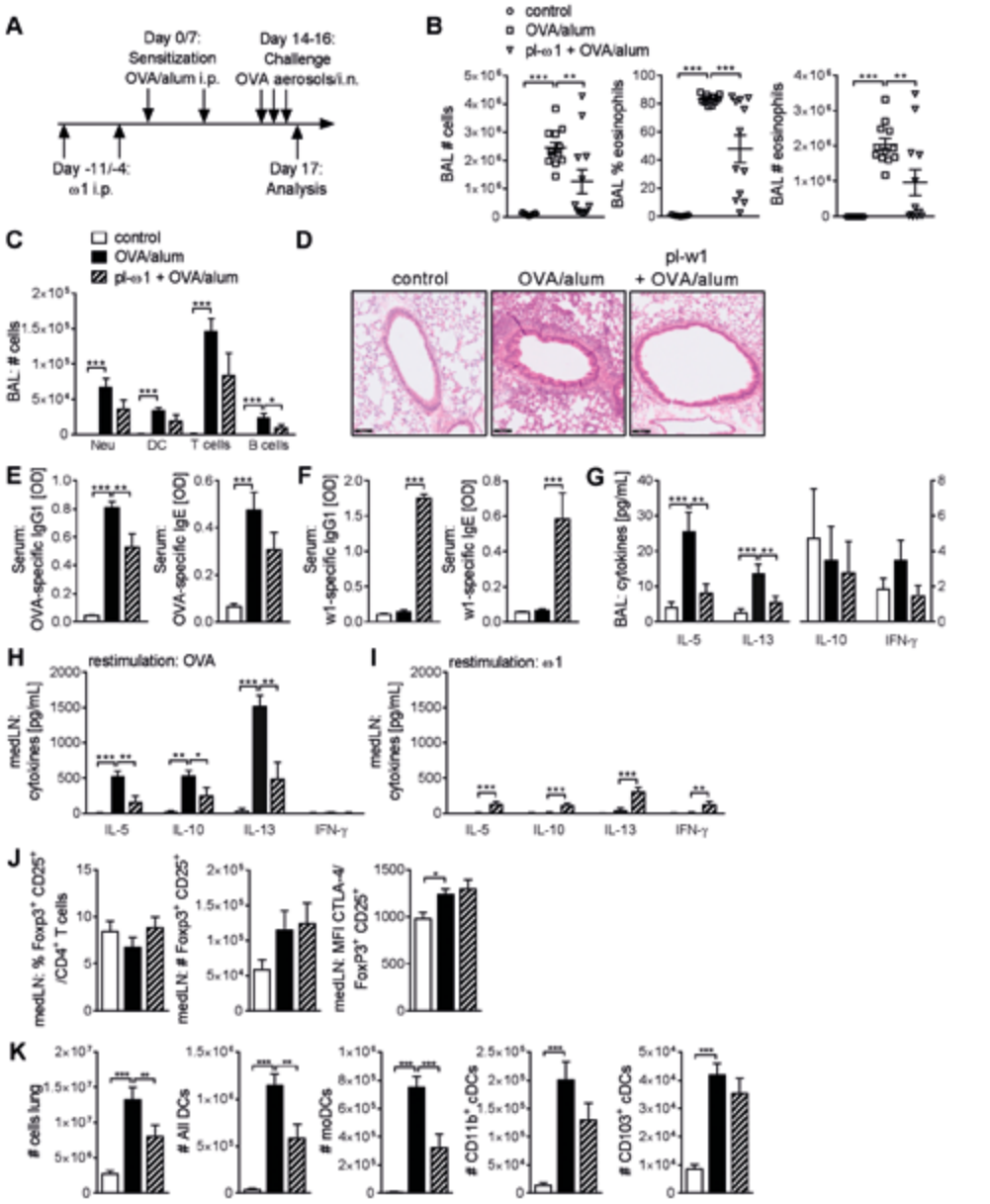

Figure 6. The suppressive effect of *S. mansoni* egg on AAI can be reproduced by one of the major egg antigens, the glycoprotein ω-1. (**A**) Schematic representation of the experimental model. (**B**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. (**C**) Total number of neutrophils (Neu), dendritic cells (DC), T cells and B cells in BAL fluid as assessed by FACS. (**D**) Representative images of haematoxylin and eosin (H&E) staining from PFA-fixed lung sections, and scores for severity of cellular infiltration around the airways on a scale of 0-4. Scoring was performed blinded by two different individuals, the average of both scores is displayed. Bars = 200μm (**E**) OVA-specific IgG1 and OVA-specific IgE antibodies in serum measured by ELISA. (**F**) ω-1-specific IgG1 and ω-1-specific IgE antibodies in serum as measured by ELISA. (**G**) Cytokine concentration in BAL fluid measured by CBA. (**H**) Cytokine concentration in medLN cell supernatants after 4d re-stimulation with OVA (10μg/mL) measured by CBA. (**I**) Cytokine concentration in medLN cell supernatants after 4d restimulation with ω-1 (10μg/mL) measured by CBA. (**J**) Percentage and total number of Foxp3$^+$ CD25$^+$ Treg cells in medLNs as well as

geometric mean expression of CTLA-4 on Foxp3$^+$ CD25$^+$ Treg cells as assessed by FACS. (**K**) Total number of lung cells, total number of lung DCs (CD11c$^+$ MHCII$^+$) as well as total number of moDCs (CD11c$^+$ MHCII$^+$ CD11b$^+$ CD103$^-$ CD64$^+$), CD103$^+$ cDC1s (CD11c$^+$ MHCII$^+$ CD11b$^-$ CD103$^+$ CD64$^-$) and CD11b$^+$ cDC2s (CD11c$^+$ MHCII$^+$ CD11b$^+$ CD103$^-$ CD64$^-$) in the lung, assessed by FACS. Summary of 2 experiments, n=9-12. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

DISCUSSION

In this study, we sought to further characterize the potential of *S. mansoni* to protect from allergic asthma, despite profound egg-specific Th2 responses, by using both isolated *S. mansoni* eggs in a setting without adult worms and the egg glycoprotein ω-1. We show that *S. mansoni* eggs are capable of protecting against experimental AAI, which is in line with previous reports [16, 43] and in contrast to earlier work postulating that protection can only be achieved in the absence of female, egg-laying worms. We found that protection from AAI can also be achieved by eggs isolated from infected mice instead of hamsters (data not shown), excluding confounding factors from a contamination of egg preparations with traces of hamster tissue. Importantly, the protective effect of eggs can be replicated by ω-1 as single, egg-derived antigen. Data on the administration of isolated eggs, and the cellular mechanisms eggs can induce in the context of an allergic inflammation, are still very limited. Moreover, a protective effect of ω-1 on AAI has not been reported previously. This study aims to advance the current knowledge by providing new insight into the putative mechanism of protection in the presence of egg-specific Th2 responses.

We show that egg treatment induces a fully developed Th2 response to egg antigens, while the OVA-specific Th2 response, normally induced by alum, is completely absent. This is in line with earlier observations in chronic *S. mansoni* infections [14] and after treatment with excretory-secretory products of *T. suis* [44]. Mangan et al. describe a 'helminth-modified pulmonary Th2 response' in *S. mansoni* infection, characterized by elevated pulmonary IL-10 and IL-13, but reduced IL-5 [13, 45]. We found the production of OVA-specific Th2 cytokines to be reduced upon egg treatment, which argues against a putative 'modified Th2 response' in our egg-treatment model and is similar to what has been described in humans. People in schistosome-endemic areas, for which a negative association between chronic infection and allergic sensitization has been shown, often have elevated Th2 responses to the eggs alongside reduced allergic symptoms [7, 8]. However, a recent study on a fishing community in Uganda, with a low prevalence of allergy-related diseases, found a positive correlation between *S. mansoni*-specific Th2 cytokines and atopy, and *S. mansoni*-specific IgE and atopy, respectively. A significant inverse associations was observed in relation to wheeze, keeping with the original hypothesis [46].

Previous reports describe Treg cells, Breg cells and IL-10 to be important for protection by natural *S. mansoni* infections [14, 16-19]. We observed that the number of pulmonary Treg cells was increased in the BAL fluid of allergic mice during the challenge phase, which has similarly been reported by others [44, 47], but returns to baseline rather than continues to rise in treated animals. Following allergen challenge, egg-treated mice did not induce Treg cell numbers or enhance the expression of regulatory activity markers in the lung compartment compared to untreated, allergen-challenged mice. In addition, IL-10 in BAL fluid of egg-treated animals was unchanged following egg treatment. The fact that Treg cells did not exceed the baseline levels of naïve control mice, combined with the lack of any activity markers, suggests that there is no active suppression by Treg cells during the allergen challenge phase. Mice depleted of CD25-expressing Treg cells during egg treatment and allergic sensitization display a similar degree of AAI suppression, despite the inflammation being generally increased upon Treg depletion, which is probably as a result of a dysregulated Treg to effector T cell balance. This suggests that egg-induced Treg cells do not play a decisive role in egg-mediated protection from AAI in our

hands. These findings seem, at least in part, contrary to a previous report on the putative role of Treg cells in a similar model of egg administration [16, 19]. Discrepancies in the results may be related to factors like the length of exposure to parasitic products (infection versus isolated injections) or the use of different mouse strains.

To study the role of B cells in protection, we treated both WT and μMT mice with *S. mansoni* eggs. μMT mice lack mature, conventional B2 B cells [34]. Most studies report that μMT mice mount an allergic response similar to their WT counterparts [48-50]. In line with these data, we also found the allergic response to be unaffected in μMT mice, apart from a significant reduction in the allergen-specific Th2 cytokine production by medLN cells. This could be, at least in part, due to the amount of allergen used, as we have previously reported an important role for B cells in low dose HDM-induced AAI [35]. Despite the difference in OVA-specific Th2 responses, egg treatment equally protects from AAI both in WT and μMT mice, indicating that mature B cells are not crucial for protection. While this does not formally exclude a role for all Breg cell subsets, which can be present in both the B1 and B2 B cell compartment [51-53], we believe they are unlikely to play a major role. We have previously studied the induction of Breg cells by *S. mansoni* infection, SEA and the single egg molecule IPSE/alpha-1, and predominantly identified Breg cells within the splenic marginal zone (MZ) B cell compartment as well as the pulmonary B cell compartment [54]. Both MZ B cells, which belong to the B2 B cell lineage, and pulmonary B cells are absent in μMT mice and thus unlikely crucial for protection. Additionally, μMT mice are also unable to mount an allergen-specific antibody response [53], excluding a role of inhibitory antibodies in protection.

DCs play a central role in the induction of adaptive immune responses in the context of AAI. Both $CD11b^+$ cDCs and moDCs can drive allergic Th2 responses in a model of HDM allergy, with moDCs being sufficient in models of high-dose allergen exposure [40]. Moreover, *Schistosoma* infection has been shown to functionally impair myeloid DCs in humans [36]. Here, we show that, during the challenge phase of OVA/alum-induced AAI, control mice depict a sharp influx of moDCs as well as increased numbers of $CD103^+$ cDC1 and $CD11b^+$ cDC2s. Interestingly, egg treatment selectively affects the lung moDC compartment, whereas both cDC populations remained unchanged. The reduced concentration of CCL2 in BAL fluid of egg-treated mice suggests that the recruitment of moDCs into the lung is impaired. During allergic sensitization with alum-supplemented allergen by i.p. injection, inflammatory monocytes are recruited to the peritoneal cavity within hours and ingest allergens in a uric acid-dependent manner. They migrate to the lung-draining medLNs and there develop into moDCs that contribute to the development of a Th2 response [55]. It is unclear whether this inflammatory monocyte response to allergic sensitization in the peritoneal cavity was targeted by egg treatment, or whether the reduced pulmonary moDC levels are the consequence of reduced inflammation and reduced CCL2 levels. Preliminary data suggest that moDCs isolated from allergic and egg-treated mice behave similar and both have a poor T cell stimulatory capacity (data not shown). This may suggest that the observed reduced number of moDCs is the consequence rather than the cause of reduced AAI in egg-treated mice.

While protection from AAI by *S. mansoni* eggs has been described before, this is the first description of a suppressive effect of ω-1 in a model of AAI. The only other *Schistosoma* spp. molecules described to inhibit AAI are the egg-derived *S. japonicum* molecule SjP40, which seems to induce splenic IFNγ production [56], and *S. mansoni* Sm22.6, Sm29 and PIII, all from life cycle stages other than eggs [32]. Treatment with ω-1 resulted in a very similar pattern of AAI suppression as previously observed for egg treatment. Treatment with ω-1 did not induce medLN Treg cells, similarly to what we have observed for eggs. Treg depletion experiments in the context of ω-1 treatment will however be necessary to formally exclude a role of Treg cells in ω-1-mediated protection. Treatment with ω-1 affected the lung

DC compartment as observed for egg treatment, but also in this case it is currently unclear whether this is a cause or consequence of reduced pulmonary inflammation.

Using plant-derived ω-1 offers unique tools to further study the mechanisms of protection. While the recombinant ω-1 used here is the active protein carrying wild-type plant glycans, this expression system can be engineered to express the protein H58F mutant that lacks T2 RNase activity and/or Lewis X (LeX) glycan motifs found on native ω-1 [42]. Ω-1 is internalized via its glycans by the MR expressed on macrophages, DCs and endothelial cells and conditions for Th2 polarization in an RNase-dependent manner [20]. Using ω-1 lacking the ribonuclease enzymatic activity will allow to assess whether the absence of ω-1-mediated Th2 polarization influences protection. The comparison of ω-1 carrying wild-type plant glycans versus glycans with engineered LeX glycan motifs will allow to discern whether the glycan composition is important for the observed protective effect. Furthermore, tracking experiments with labelled antigen should be carried out to assess which tissues ω-1 drains to after i.p. injection, and which cell types take up the molecule. The MR seems to be central in this process, but ω-1 has also been shown to bind to the CLR DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) and to induce DC IL-10 mRNA expression [42]. In addition to ω-1, several other helminth antigens have been reported to bind to MR, which is often associated with the induction of an anti-inflammatory or Th2 response [57, 58]. Studying the effect of ω-1 treatment on AAI in mouse lines deficient in MR or the murine DC-SIGN-equivalent SIGN-R1 will therefore be of interest to gain further insight into the mechanism of protection.

In summary, the here presented data show that the suppressive effect of *S. mansoni* infection on allergic asthma can be replicated by isolated eggs. This effect occurs despite a strong Th2 response to the eggs itself and is likely independent of Treg and B cells. Egg treatment strongly and selectively affects the lung moDC compartment. The protective effect of eggs can moreover be replicated by the egg-derived glycoprotein ω-1. Understanding the complex interactions early during allergic sensitization, and how helminths interfere there, is critical for the development of preventative strategies for allergies and allergy-related diseases.

MATERIAL AND METHODS

Mice

Female C57BL/6 mice (Harlan) were housed under SPF conditions in the animal facility of the Leiden University Medical Center (Leiden, The Netherlands) and used for experiments at 6-12 weeks of age. All animal studies were performed in accordance with the Animal Experiments Ethical Committee of the Leiden University Medical Center. The Dutch Experiments on Animals Act is established under European Guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). B6.129S2-Ighmtm1Cgn/J (μMT) mice (C57BL/6 background) were kindly provided by B. Lambrecht, Ghent University (Belgium) and originally purchased from Jackson Laboratory (Bar Harbor, USA).

Preparation of *S. mansoni* eggs and recombinant ω-1

Eggs were isolated from trypsinized livers of hamsters infected for 50 days with a Puerto Rican-strain of *S. mansoni*, washed with RPMI medium containing 300U/mL penicillin, 300μg/mL streptomycin (both Sigma-Aldrich) and 500μg/mL amphotericin B (ThermoFisher Scientific) and frozen at -80ºC until use. To investigate whether freeze-thawed eggs still release a comparable protein content to that of freshly cultured eggs, excretory-secretory product (eggES) of freeze-thawed and fresh eggs was compared by silver staining after 48h of egg culture. Recombinant ω-1 was produced as previously described in plants [42]. Briefly, ω-1 was expressed in *Nicotiana benthamiana* and purified from apoplast fluid by

cation exchange chromatography. Purity was routinely assessed by SDS-PAGE and Coomassie brilliant blue staining, and protein concentration assessed by BCA assay.

AAI model, egg and ω-1 treatment, and Treg cell depletion

Mice were sensitized by i.p. injection of OVA (10μg/mL; Invivogen) emulsified in alum adjuvant (2mg/mL; ThermoFisher Scientific) on day 0 and 7. Seven to 10 days after the last injection, mice were challenged for 3 consecutive days by either exposure to OVA aerosols (10mg/mL in PBS, 30mins) or by intranasal (i.n.) administration of 50μg OVA/50μL PBS. Mice were sacrificed 24 hours after the last challenge. Animals in the treatment group received two i.p. injections of 5000 *S. mansoni* eggs or 50μg ω-1 diluted in sterile PBS on day 11 and day 4 prior to allergic sensitization. To deplete Treg cells from egg-treated mice, mice were treated i.p. with anti-CD25 depleting (clone PC61) or control (anti-β-galactosidase, clone GL113) antibody (500μg/mouse) 6 days prior to the first egg injection, and again 6 days prior to the first allergic sensitization (2 days before second egg injection) [59].

Tissue preparation

BAL fluid was collected by flushing the lungs with 1mL PBS/2mM EDTA (Invitrogen), followed by additional two lavages to collect remaining cells. The 1st BAL flush was kept separate for cytokine analysis in cell-free supernatant, the cells from all flushes were pooled for flow cytometry. Perfused lungs were cut into small pieces and digested using collagenase III (100U/mL; Worthington) and DNAse (2000U/mL; Sigma-Aldrich) for 1 hour at 37ºC. Digested lungs were homogenized through 70μm cell strainers (BD Biosciences) and remaining red blood cells lysed. In some cases, one side of the lung was tied off with surgical suture and removed, and the other side inflated with and collected into 3.9% PFA/PBS. Mediastinal lymph nodes and spleens were homogenized through 70μm filters, and spleens were subjected to red blood cell lysis. Blood for assessment of Treg cells was collected from the tail vein 6 days after the first injection of anti-CD25 or control antibody, and red blood cells were lysed. For serum collection, blood was collected by heart puncture, spun down and the serum stored at -20ºC until further analysis.

Flow cytometry

The cellular composition of BAL fluid was determined by staining with fluorescently labelled antibodies against B220 (RA3-6B2), CD3 (17A2), CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), MHCII (M5/114.15.2) and Siglec-F (E50-2440) directly *ex vivo*. Treg cells in BAL fluid, medLNs and blood were identified by staining with live/dead fixable aqua dead cell stain kit (ThermoScientific) and fluorescently labelled antibodies against CD3 (17A2), CD4 (GK1.5), CD25 (PC61.5), CTLA-4 (UC10-4B9), Foxp3 (FJK-16s) and GITR (YGITR 765). DCs in lung tissue were identified by staining with live/dead fixable aqua dead cell stain kit (ThermoScientific) and fluorescently labelled antibodies against CD3 (17A2), CD11b (M1/70), CD11c (HL3), CD19 (MB19-1), CD64 (X54-5/7.1), CD103 (2E7), Gr-1 (RB6-8C5), MHCII (A5/114.15.2), Nk1.1 (PK136) and Siglec-F (E50-2440). For all stainings, FcγR-binding inhibitor (2.4G2, kind gift of L. Boon, Bioceros) was added. Flow cytometry was performed using a FACS Canto II and FACSDiva software (BD Biosciences) followed by data analysis using FlowJo.

Histology

Lungs were collected into 3.9% PFA/PBS and the tissue transferred into 70% ethanol after 1-2 days. Lungs were then embedded in paraffin, sliced and stained for inflammatory cell infiltration using haematoxylin and eosin (H&E; both Klinipath). Stained slices were analysed under a Olympus BX41 light microscope (Olympus). Peribronchial inflammation as assessed by H&E staining was scored on a scale 0-4 by two blinded, independent investigators.

ELISA and CBA

OVA- and SEA-specific IgG1 and IgE antibodies were measured in serum. 96-well Nunc Maxisorp plates (ThermoFisher Scientific) were coated with 25µg/mL of the respective antigen diluted in buffer (1M sodium carbonate) at 4°C overnight and subsequently incubated with serial dilutions of sera, biotinylated detection antibodies against IgG1 and IgE (BD Biosciences) and horseradish peroxidase-conjugated streptavidin (BD Biosciences). Optical densities were measured after addition of TMB peroxidase substrate (KPL). The concentration of the cytokines IL-5, IL-10, IL-13 and IFN-γ were detected in cell-free supernatants of BAL fluid and cell cultures using either ELISA kits or BD cytometric bead array (CBA) Flex-set kits (BD Biosciences) followed by flow cytometry measurement on a FACS Canto II (BD Biosciences). The chemokine CCL2 was also measured using a CBA Flex-set kit.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 7.02) using unpaired t-test for comparison of 2 groups, one-way ANOVA for comparison of more than two groups, and two-way ANOVA for comparison of more than two groups while correcting for a batch effect between different experiments. 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

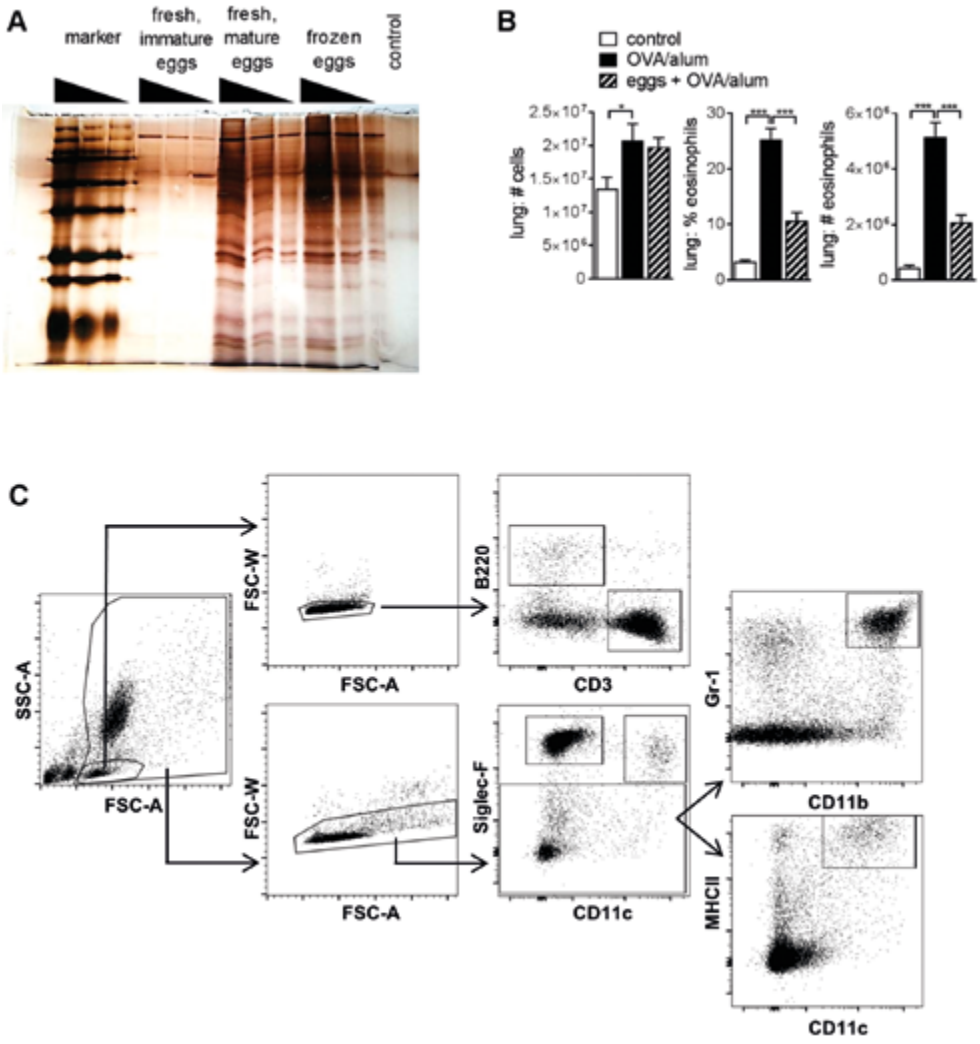

Figure S1. (**A**) Eggs were isolated from the liver of infected hamsters, washed extensively and either separated into immature and mature eggs following by culture, or frozen at -80°C prior to culture. Freshly isolated and freeze-thawed eggs were cultured at a density of 200.000 eggs/mL in medium for 48h before egg-free culture supernatant was collected and subjected to silver staining. (**B**) Total number of cells, percentage of eosinophils and total number of eosinophils in lung tissue as assessed by FACS. Representative of 2 experiments, n=4-6. (**C**) FACS gating strategy for the analysis of cells in BAL fluid. All single cells were gated for the identification of eosinophils ($SiglecF^+$ $CD11c^-$), alveolar macrophages (alvMFs; $SiglecF^+$ $CD11c^+$), neutrophils ($SiglecF^-$ $CD11c^-$ $CD11b^+$ $Gr\text{-}1^+$) and DCs ($SiglecF^-$ $CD11c^+$ $MHCII^+$). A lymphocyte gate was used to identify T cells ($CD3^+$ $B220^-$) and B cells ($CD3^-$ $B220^+$).

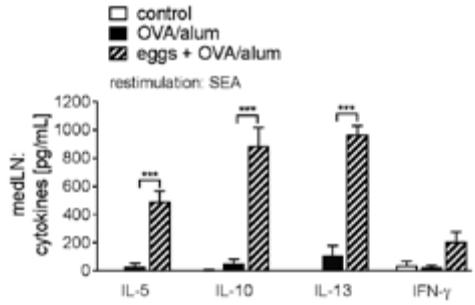

Figure S2. Cytokine concentration in medLN cell supernatants after 4d re-stimulation with SEA (10µg/mL), n=5. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with *** $p < 0.001$.

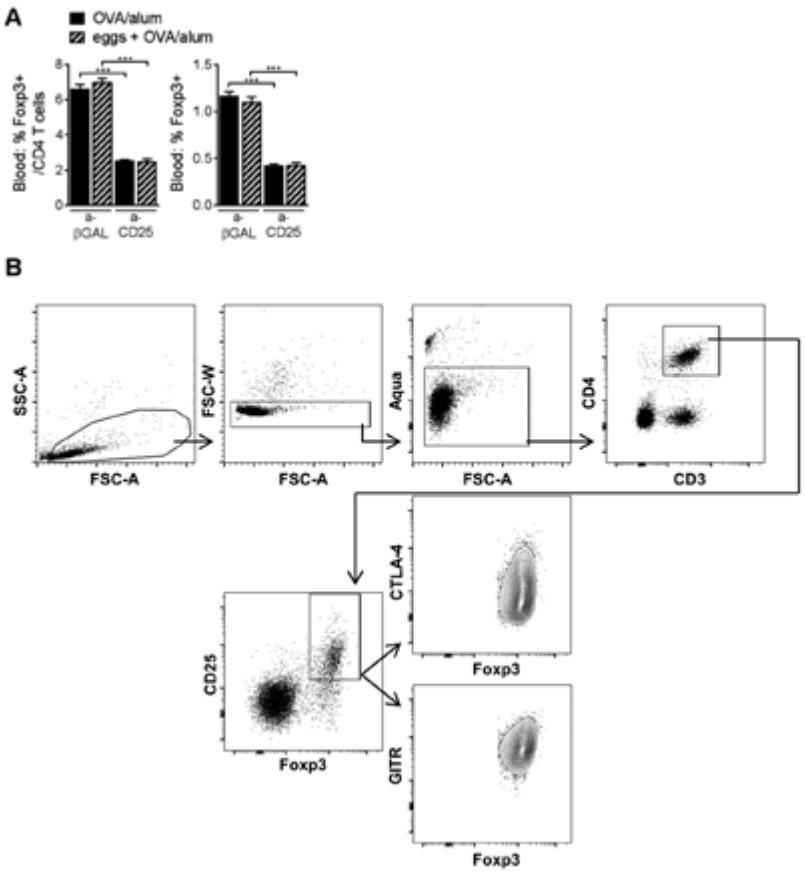

Figure S3. (**A**) Mice were treated with anti-CD25 depleting antibody (clone PC61) as described in Figure 4. Five days after the first antibody injection, tail blood was collected and the percentage of $Foxp3^+$ Treg cells of all CD4 T cells and of all cells assessed by FACS. Representative of two experiments, n=6. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with *** $p < 0.001$. (**B**) FACS gating strategy for the analysis of Treg cells. Treg cells were identified as single, live $CD3^+$ $CD4^+$ $Foxp3^+$ $CD25^+$ cells. The geometric mean of fluorescence intensity was determined on all Treg cells.

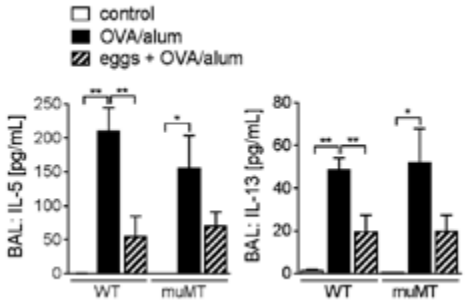

Figure S4. Cytokine concentration in BAL fluid measured by ELISA, n=3-6. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with * $p < 0.05$, ** $p < 0.01$.

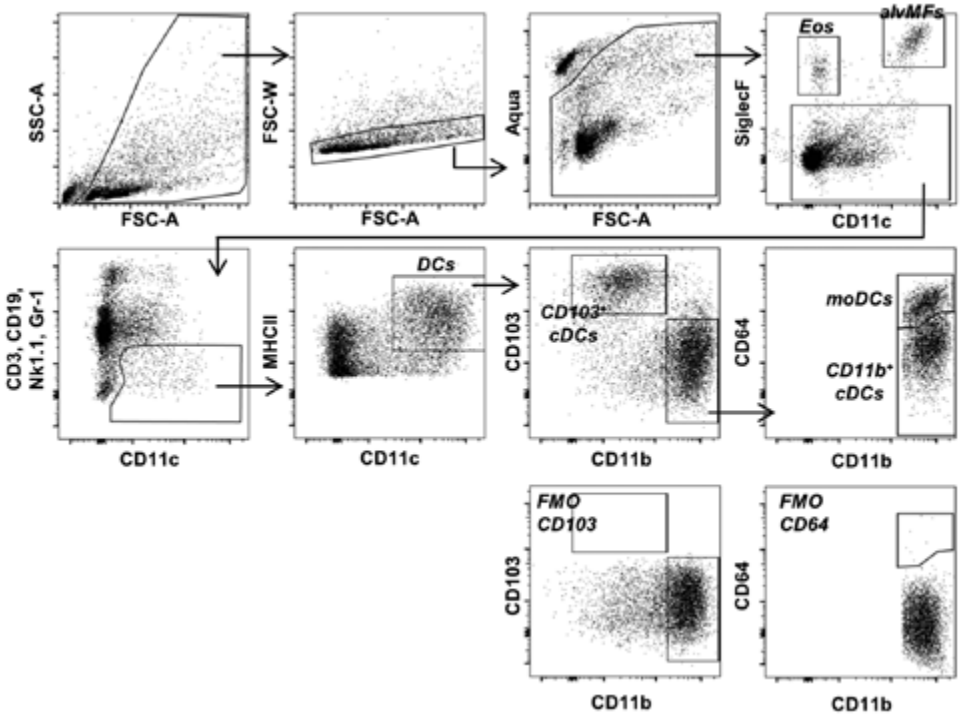

Figure S5. FACS gating strategy for the analysis of DC subsets in lung tissue. Single, live cells were identified, and all SiglecF, CD3, CD19, NK1.1 and Gr-1 expressing cells excluded. DCs were identified as CD11c$^+$ MHCII$^+$, and subdivided into CD103$^+$ cDCs (CD103$^+$ CD11b$^-$ CD64$^-$), CD11b$^+$ cDCs (CD103$^-$ CD11b$^+$ CD64$^-$) and moDCs (CD103$^-$ CD11b$^+$ CD64$^+$).

Chapter 8

SUMMARIZING DISCUSSION

WHAT WAS ALREADY KNOWN

Helminths, being well-known inducers of type 2 immune responses, also promote immune regulatory networks. The resulting immune modulation conveys 'spill-over' suppression to hyper-inflammatory conditions such as allergy [1].

Schistosomes, which establish chronic infection in both humans and mice, have been widely used to dissect the mechanisms underlying the development and activation of regulatory B (Breg) cells, a prominent member of the immune regulatory network. It is widely recognized that Breg cells are induced by schistosomes in both humans and mice [2, 3]. Breg cells comprise a heterogeneous group of cells of different cellular origin as well as phenotypical and functional characteristics. Multiple stimuli are known to contribute to Breg cell activation, of which most-well described are signalling via the B cell receptor (BCR), CD40 and Toll-like receptors (TLRs). Schistosome antigens have been described to induce Breg cells [4-6], but the molecular identity of the stimulus and the signalling pathways engaged remain to be characterized.

Hyper-inflammatory conditions such as allergy are less common in helminth-endemic areas. This relationship has both been studied in humans and in animal models. Human studies have yielded heterogeneous results over the years [7], suggesting that many factors including helminth species, time, location, intensity and chronicity of infection as well as host genetics influence helminth-mediated immunomodulation. Advances in understanding helminth-mediated protection from allergy have been made in animal models, where antigen mixtures and single molecules have been shown to protect from experimental allergic airway inflammation (AAI) in the absence of infection. A range of mechanisms mediating protection have been described, including the induction of various components of the immune regulatory network and suppression of pro-inflammatory responses [8]. The description of single, schistosome-derived molecules that mediate protection against AAI are however limited.

HOW DID OUR STUDIES ADVANCE THE FIELD

Molecular signals for schistosome-induced Breg cell development

Although Breg cells have been most well-characterized in the murine spleen, B cells with regulatory properties can also be found at other sites. We show that B cells with regulatory properties, protecting mice from AAI, can be found in the lung of chronically *S. mansoni*-infected mice (**chapter 2**). While we and others have previously shown that *S. mansoni*-induced splenic Breg cells exert their suppressive function through interleukin (IL)-10 production and induction of regulatory T (Treg) cells [2, 4], we found these schistosome-induced pulmonary B cells to be phenotypically different from splenic Breg cells and to suppress in an IL-10- and Treg cell-independent manner, rather displaying a reduced T helper 2 (Th2)-driving function. This study suggests that B cells with inhibitory properties exist at the site of inflammation, which are phenotypically and functionally different from 'classical' Breg cell subsets described in the spleen. Our study contributes to the body of evidence that Breg cells reside not only in lymphoid organs, but can also be found at other sites including human adipose tissue [9] and nasal polyps [10].

While it is well-established that helminths induce Breg cells, no single helminth-derived molecule had been described that mediates this effect. We show that the egg glycoprotein IPSE/alpha-1 induces splenic Breg cells *in vitro*, as assessed by IL-10 production and Treg cell induction, following direct interaction (**chapter 3**). Importantly, SEA depleted of IPSE/alpha-1 is also capable of Breg cell induction, highlighting that there are other, yet unidentified factors in SEA that induce Breg cells. We found IPSE/alpha-1 to also induce IL-10 production in human $CD1d^+$ B cells, thus identifying IPSE/alpha-1 as the first helminth molecule with direct Breg cell-inducing capacity in both mice and humans. That the Breg cell-inducing effect can be replicated by a recombinant, plant-expressed version of

the molecule, as previously described for the *S. mansoni* egg glycoprotein omega-1 (ω-1) [11], is an important point of departure for further investigations into the therapeutic potential of this molecule.

We could identify IPSE/alpha-1, previously mainly recognized for its capacity to activate basophils [12, 13], as one Breg cell-inducing factor within SEA *in vitro*, but *in vivo* multiple signals likely synergize to induce Breg cells. To study the molecular signals required for Breg cell induction in schistosomiasis more globally, we analysed the transcriptome of splenic MZ and FO B cells from chronically *S. mansoni* infected mice (**chapter 4**). We found marginal zone (MZ) and follicular (FO) B cells to display clearly distinct transcriptional profiles, both at steady-state and after infection. Both cell types moreover clearly responded to infection. We analysed genes, pathways and upstream regulators that are more unique to one B cell subset or the other, and identified interesting leads for further investigation. Amongst those, the cytokines IL-1β and IL-6, as well as members of the type I interferon (IFN-I) family, seem to preferentially activate MZ B cells, which is interesting as initial observations in the literature suggest their involvement in Breg cell induction [14, 15]. Furthermore, our data suggest TLR7 and TLR9 as upstream regulators that are more active in MZ than FO B cells, which also aligns with the literature [16, 17] and overall suggests the involvement of several innate signals in MZ B cell activation.

As IFN-I have been suggested as one upstream regulator of the transcriptional changes that MZ B cells undergo in *S. mansoni* infection, we further investigated whether IFN-I also provide signals for Breg cell induction in the context of *S. mansoni* (**chapter 5**). Schistosomes [18, 19] and other helminths [20, 21] induce IFN-I, and a recent report shows that IFN-I provide signals for the induction of human Breg cells [15]. We found Breg cell IL-10 production in response to *S. mansoni* antigens to be enhanced by IFN-I *in vitro*, but not *in vivo*. Stimulation with *S. mansoni*-derived antigens might provide a pre-activation signal *in vitro*, but the identification of optimal conditions for Breg cell expansion *in vivo* warrants further investigation.

In conclusion, our studies contribute to the understanding of how Breg cells develop and are activated in response to *S. mansoni*. *S. mansoni* actively secretes molecules to target host immunity, including molecules that directly bind and induce Breg cells such as IPSE/alpha-1. *In vivo*, multiple signals synergize leading to optimal Breg cell induction and activation, which seem to be a combination of both helminth-derived and host-derived factors. The further identification of such factors, their synergistic effects and the role of tissue-specific niches warrants further investigation.

Identification of single, schistosome-derived molecules for the inhibition of AAI

As discussed in the 2014 review (**chapter 6**), various helminths and helminth-derived molecules have been described to modulate allergic disease, and a range of different mechanisms of suppression have been suggested. However, the role of schistosome eggs in AAI protection was not well defined and hardly any protective, single molecules had been described. We subsequently showed that isolated *S. mansoni* eggs, in the absence of infection, protect from AAI when administered during the allergic sensitization phase (**chapter 7**). In contrast to several other studies that previously reported helminth- and, more specifically, *Schistosoma* spp.-mediated protection to be dependent on Treg and/or Breg cells, we still observed protection in the absence of Treg and B cells, respectively. Interestingly, protection from the allergic type 2 response occurred despite a strong antigen-specific type 2 response to the eggs. We could reproduce the protective effect of eggs on AAI by treatment with a plant-derived, recombinant version of the single, egg-derived glycoprotein ω-1, for which no such role had been described before. As for *S. mansoni* eggs, protection by ω-1 also seemed independent of regulatory lymphocytes and occured despite a strong antigen-specific Th2 response. Protein mutants lacking T2 RNase activity and protein variants with modified glycosylation pattern will help to further assess e.g. the importance of the ribonuclease activity of ω-1 and consequently the (partial) breakdown of mRNA [22] in mediating protection. Moreover, tracking experiments as well as mouse lines with mutation in ω-1-

binding receptors mannose receptor (MR) and SIGN-R1 will help to understand which tissues and cells take up ω-1, potentially allowing further insight into the mechanism of protection.

Collectively, our work on the protection from AAI by schistosome-derived eggs or single molecules has contributed to revealing the diversity in mechanisms involved in mediating bystander suppression in hyper-inflammatory settings such as allergic asthma. This adds to the expanding realization that helminths have developed various mechanisms of modulating host immunity which go beyond the development of regulatory lymphocytes.

DIRECTION FOR FUTURE RESEARCH

Molecular signals for schistosome-induced Breg cell development

It is currently unclear whether Breg cells develop from a committed precursor, or whether any B cell can acquire suppressive capacity in response to environmental stimuli. It becomes more and more clear that B cells at different stages of maturation and differentiation (from transitional B cells [23, 24] to plasma cells [25-27]), and B cells at different sites (from secondary lymphoid organs [28, 29]) to the peritoneal cavity [30] and the lung [31] in mice, and in human adipose tissue [9] and nasal polyps [10]) possess suppressive capacities, which argues against a committed precursor. Most recently, natural regulatory plasma cells have been identified that develop at steady-state and respond by IL-10 production within hours of stimulation [27].

Breg cells comprise a heterogeneous group of cells that lack a specific marker. The markers currently used to identify Breg cells are likely not specific enough to selectively target them in cellular immunotherapy. Novel technologies such as mass cytometry will allow a better characterization of Breg cell heterogeneity in both animal models and humans, similarly to studies already performed for Treg cells [32].

Breg cell activation i*n vivo* is more complex than the *in vitro* Breg cell-activating stimuli which have been extensively studied. A variety of receptors including BCR, CD40 and TLRs have been described to be involved in Breg cell activation in different models both *in vitro* and *in vivo*. While an inflammatory environment seems a shared feature between all models and central to Breg cell induction, it has been suggested that different Breg cell subsets require different additional stimuli for their development and activation. Innate-like Breg cells may require the ligation of TLRs, while other Bregs especially in autoimmunity seem to require BCR and CD40 ligation [33]. Work from us and others suggests that Breg cells induced during infection - and in particular during schistosome infections - might be of the innate-like type that develop in response to inflammatory cytokines and TLR ligation.

With respect to stimuli that might contribute to Breg cell activation *in vivo*, the gut microbiome is of particular interest. A recent study shows changes in the gut microbiome following *S. mansoni* infection [34]. Some studies moreover suggest that gut microbiota can support Breg cell induction [14, 35, 36], whereas others describe the development of regulatory plasma cells to be microbiota-independent [27]. Although changes in the composition of the lung microbiome have both been shown to promote airway disease [37] and to induce Treg cells that can suppress AAI [38], the influence of the lung microbiota on local B cells has so far not been addressed. It will be of special interest to further define whether and how changes in microbiome composition, and the subsequent interaction with the host immune system, contribute to Breg cell induction.

The importance of Breg cell antigen-specificity for maximal and focussed suppressive function, or for minimizing side effects due to non-specific immune suppression, needs to be addressed in more detail. It is clear from animal models that BCR signalling is one of the critical stimuli for Breg cell development at least in models of autoimmunity, as mice with a BCR fixed for a non-relevant antigen or with impaired BCR signalling harbour less Breg cells and cope less well with EAE [39, 40]. However, stimulation with mitogenic anti-IgM antibody alone does not induce Breg cells [41], suggesting that low

affinity BCR ligation induces regulatory properties rather than the strong signal provided by anti-IgM. Whereas the importance of B cell specificity in autoimmune models has been acknowledged, the nature of the antigen(s) remains to be defined. Interestingly, the frequency of IL-10- and IgG4-producing Breg cells specific for the bee venom allergen phospholipase A2 (PLA) was increased in patients allergic to bee venom after successful allergen immunotherapy, and reached levels comparable to tolerant beekeepers, whereas B cells not specific for PLA produced only little IL-10 and IgG4 and did not respond to allergen immunotherapy [42]. In this study, B cells specific for a defined antigen are the ones to acquire regulatory properties. In helminth infection, the importance of antigen-specificity for Breg cell development is less defined.

Helminths release a multitude of antigens, which can potentially activate B cells through the BCR and innate receptors like TLRs simultaneously. While signalling through TLRs has been repeatedly reported to induce Breg cells in helminth infection, the relative contribution of BCR signalling should be further defined by using e.g. mouse models with fixed BCRs as done in the field of autoimmunity. In addition, studies on Breg cell antigen-specificity are possibly hampered by technical challenges, such as low abundance of these cells and the co-staining of other, non-specific B cells with labelled antigens. This could potentially be overcome in the future through advancements in the field of single cell analysis.

It is furthermore not well understood whether Breg cells acquire suppressive capacity in a tissue-dependent manner, and what their migration pattern is. Many studies assess splenic Breg cells after intraperitoneal injection of a stimulus, which does not resemble the physiological situation well. Studying Breg cells at the site of inflammation and/or tracking B cell residency and migration would help to understand the role of the tissue micro-environment in Breg cell development and activation. Interestingly, it has been shown that tumour-infiltrating B cells acquire suppressive capacity upon exposure to metabolites of the 5-lipoxygenase pathway and growth factors in the tumour micro-environment [43, 44], suggesting an important role of local signals for Breg cells. Lipoxygenase derivatives were found in the lipidome of several *S. mansoni* life-cycle stages [45], and it would be interesting to further investigate those in the context of Breg cell induction during schistosome infections.

In view of potential therapeutic applications of Breg cells in conditions such as allergy and autoimmunity, it is important to identify conditions for optimal Breg cell activation and to study if suppression by Breg cells is a lasting phenotype, or whether these cells require frequent (re)stimulation to exert their function. To this end, the study of Breg cells should always include the characterization of their suppressive capacity e.g. by adoptive transfer into animal models of allergy or autoimmunity. This might pose challenges as active suppression via IL-10 is likely a transient feature, with recent cell activation necessary for active cytokine secretion [46]. In addition, proof of suppressive capacity might be more challenging to confirm in humans as it largely relies on *in vitro* assays [47, 48] that only yield limited information about *in vivo* suppressive capacity.

B cells are important therapeutic targets in many diseases with an immune component, including autoimmunity and cancer. Most prominently, B-cell depletion by anti-CD20 monoclonal antibody (rituximab) has been approved for the treatment of certain forms of leukaemia and rheumatoid arthritis (RA), and is under investigation for e.g. treatment of multiple sclerosis (MS) [49]. Treatment with anti-CD20 affects the balance of CD20-expressing and non-expressing B cells. It preferentially leaves immature, transitional B cells - a population that contains progenitor Breg cells [50, 51] - intact, thus targeting the Breg cell compartment indirectly. Another monoclonal antibody that, in addition to inhibiting pro-inflammatory B cells, might affect the Breg cell compartment is an IL-6R antagonist (tocilizumab), which has been shown to increase the TGF-β expression of $CD25^+$ Breg cells when used in RA patients [52]. Finally, IFNβ therapy commonly applied in patients with certain types of MS increases IL-10 production not only by monocytes and T cells [53, 54], but also by B cells and plasmablasts [55, 56].

None of these treatments have been developed to directly target Breg cells and the results are merely off-target effects. Proof-of-concept studies in mice have shown that suppressive Breg cells can be expanded, either *in vivo* by agonistic anti-CD40 antibody [23], or *ex vivo* by stimulation with CD40L, IL-4 and IL-21 [57]. More research is however necessary to understand the conditions that lead to optimal Breg cell activation and targeted suppressive function especially *in vivo* before novel therapies based on Breg cells can be developed.

With respect to applying treatment that targets the Breg cell compartment in allergy or asthma, any such treatment would have to be advantageous, meaning be of higher efficacy or lower side effects, compared to the currently applied options including anti-IgE therapy and allergen-specific immunotherapy. Anti-IgE treatment targets IgE produced by pro-allergic B cells as one of the central players in allergy and asthma, but is only short-lived [58]. Allergen-specific immunotherapy (AIT) aims to restore tolerance to the allergen by shifting the Th2 and IgE-dominated response to a more balanced one including IgG4 isotype switch and regulatory cells like Treg and Breg cells, achieving more long-lasting effects [59, 60]. Drawbacks of AIT are that it takes 3-5 year before AIT treatment reaches its full beneficial effect, and that this curative effect gradually declines over time [61]. Furthermore, the question remains how much of the symptom suppression is dependent on the induction of a long-lasting pool of (memory) Breg cells. Nevertheless, an interesting strategy could be to develop a new generation of allergen immunotherapeutics including an (helminth-derived) adjuvant component that specifically boosts the Breg cell compartment to perhaps shorten treatment time and increase the timespan of the curative effect.

Identification of single, schistosome-derived molecules for the inhibition of AAI

Although some of the clinical trials using *Trichuris suis* eggs (TSO) to treat patients with allergic and autoimmune conditions initially showed promising results, the progress in the field has slowed down in recent years. Larger trials have failed to confirm the early results, and efforts to use live infections as treatment strategies have diminished. Single, well-defined helminth-derived molecules circumvent the risks that full infections bear and are therefore advantageous. The identification of such immunomodulatory candidate molecules benefits from systemic approaches using computational tools and screening in high-throughput assays, such as already applied for the excretory-secretory products of *H. polygyrus* and *S. mansoni* adult worms [62-64]. The search for candidate molecules can by guided by the immunological activity of parasite-derived antigen mixtures such as excretory-secretory products, followed by molecule identification using e.g. fractionation, screening assays or proteomics approaches. Additionally, molecules of interest can also be identified in the absence of data from antigen mixtures, by e.g. identifying cytokine and chemokine homologues or family member of proteins already known to be of interest, such as proteases and protease inhibitors, members of the venom-allergen-like (VAL) family and lectins [65-68]. Notably, potential immunomodulatory molecules do not only include (glyco)proteins, but also lipids [69, 70], short chain fatty acids [71], and exosomes containing proteins, lipids and nucleic acids [72-74].

A growing body of literature shows that helminths including *S. mansoni* modulate allergic asthma via various mechanisms that are not limited to the induction of regulatory lymphocytes [75]. Bioinformatics-guided approaches such as dual transcriptomics and genomics databases such as WormBase ParaSite will not only aid the identification of immunomodulatory candidate molecules, but can also support the prediction of functional characteristics, interactions with host immunity and ultimately the putative mechanisms of action based on the presence of known homologues, predicted folding structures and functional domains [76-80].

Helminth parasites do not only directly interact with host immune cells to modulate immune responses, but also do so indirectly by their effect on the intestinal microbiome. Rodent intestinal nematodes have been shown to change the microbiome composition and thereby reduce AAI severity, or confer protection to respiratory syncytial virus (RSV) infection [20, 81, 82]. Whether helminths, and in particular *Schistosoma spp.* residing in the vasculature, alter the intestinal microbiome in humans, e.g. by the release of eggs in the intestinal lumen, is currently less well understood as studies come to conflicting conclusions [34, 83-86]. Future studies should therefore address the role of the microbiome in Breg cell induction in more detail.

Studies investigating the role of single, helminth-derived molecules are often limited by the amount and purity of the natural, isolated protein, and heterologous expression systems often lead to unintended alterations in the glycan composition. The development of a plant-derived expression system for schistosome molecules allows to produce large amounts of recombinant, helminth-glycosylated proteins [11], and the generation of protein mutants and variants with altered glycosylation will help to understand the role of protein activity and glycosylation pattern in protection.

There are multiple factors that might explain the mixed results obtained in clinical trials so far, and that thus are crucial points of consideration for the development of helminth-based therapies in the future, such as the timing, intensity/dose and localization of helminth infection or exposure to helminth-derived products. The body of evidence from studies in both animal models and humans suggest that, while exposure to helminths is able to prevent disease onset, treatment of established allergic disease is much harder to achieve. The importance of early-life exposure to microbial agents is supported by studies showing that growing up in a traditional farming environment with exposure to certain bacteria and fungi [87, 88] as well as unprocessed farm milk [89] protects against allergies and asthma. These studies suggest that stimuli shaping the immature immune system are critical for the protection against allergy and asthma later in life, and that in consequence also helminth-based therapies might have the most impact at a stage where the immune system can still be shaped. Supporting evidence also comes from studies on the impact of maternal helminth exposure on the offspring's susceptibility to allergy and asthma [90, 91]. As a prophylactic application of helminth-based therapies to the general public is likely not feasible and bears risks of side effects, such approach would require the identification of people at risk of developing allergy or asthma later in life, e.g. because of their genetic pre-disposition or aberrant immune reactions to allergens.

Most clinical trials so far have assessed helminth infections or their products that are restricted to the intestine, such as *T. suis* OVA. While intestinal exposure might be sufficient to alleviate local inflammation such as observed in inflammatory bowel disease, it might prove insufficient to induce systemic immunomodulation and suppress inflammation at distal sites such as the lung. With respect to treatment based on helminth-derived molecules, the choice of administration route could potentially overcome questions of local versus systemic exposure. Regarding the dose of treatment, animal studies naturally use higher infection or molecule doses than found in humans, where strict ethical regulations require dose escalation studies to determine that maximum tolerated dose and to minimize side effects [92]. The intraperitoneal administration of large doses of compound as employed in the majority of animal studies, in which the principles guiding human clinical trials are rarely applied, therefore provide only very limited guidance for translation into the human situation. Furthermore, once immunomodulatory candidate molecules are identified, it has to be assessed whether they can directly be applied in humans or whether alternative strategies are needed. For example, reproduction by small molecules or modified molecules that contain only the active motifs needed for interaction with host cells [93, 94] might be warranted to minimize side effects due to high immunogenicity of foreign, parasite-derived proteins in the human host. It however remains to be established whether

immunogenicity is a concern, as there are examples of pathogen-derived molecules where this is not the case. The hookworm molecule AIP-2 is an example of a helminth product that is minimally immunogenic in mice [95], and the bacteria-derived product streptokinase has been successfully used in humans despite its immunogenicity [96].

Additional efforts to fully understand the multiple variables at interplay that determine the effect of helminth infections and helminth-derived products on hyper-inflammatory conditions such as allergy may provide valuable leads for the development of novel pharmaceutical agents for the treatment of allergic disorders.

CONCLUDING REMARKS

The human immune system successfully distinguishes between benign and harmful agents, and mounts the appropriate response, in the majority of cases. It is however not without error, as apparent from e.g. allergic and autoimmune disorders. The rapid pace at which our living environments change, faster than any evolutionary adaptation can take place, poses challenges for the immune system. A deeper understanding of fundamental immunological processes, including immunomodulation such as induced by parasitic agents, might open up opportunities to develop novel treatment options based on targeted intervention in immune processes.

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Addendum &

ENGLISH SUMMARY

NEDERLANDSE SAMENVATTING

CURRICULUM VITAE

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ENGLISH SUMMARY

The immune system is our body's defence, protecting us from potentially harmful pathogens such as bacteria, viruses and parasites. It thereby has to differentiate between different microorganisms that can cause disease and should be expelled, and harmless substances that also enter the body but are not dangerous, such as food we eat or substances in the air we breathe. The immune system has developed a range of different responses, and most of the time mounts the appropriate reaction. Sometimes however, it fails to clear pathogens before they can do harm to the body, or it overreacts to harmless substances like pollen in the air or even or own body, causing e.g. asthma or autoimmune diseases. Asthma is a chronic inflammatory disease of the lung, which in characterized by symptoms such as breathlessness, chest tightness and wheezing. Allergic asthma, a common form of asthma and subject of this thesis, is caused by a reaction of the immune system to certain inhaled substances (allergens) such as dust mite or pollen.

The parasitic worm *Schistosoma* (commonly known as bilharzia), is a flatworm which can be found in freshwater snails and penetrates the host through the skin. Adult worms live and mate in the blood vessels of the intestine or bladder. There they lay eggs that are excreted with the faeces or urine. Worm parasites including *Schistosoma* are a unique class of parasites, as they usually remain in the human body for extended periods of time. These chronic infections do not only have adverse effects for the human host, but can in certain circumstances also be beneficial. Worms down-modulate the host immune response to promote their own survival, thereby providing protective effects against a spectrum of diseases with an overactive immune system, such as aforementioned asthma and autoimmune diseases. Worm parasites modify various aspects of the immune system, including the promotion of immune cells that act tolerogenic, such as regulatory B cells. While 'normal' B cells produce antibodies and protect us from invading microorganisms, regulatory B cells are immune cells that help to down-modulate the immune system when it is too active. A better understanding of how these cells get activated and how they work might enable us to make use of these cells for the treatment of e.g. asthma and autoimmune diseases.

Research conducted in this thesis

The first part of this thesis focusses on the role of regulatory B cells in protection from allergic asthma by chronic *S. mansoni* infections, as well as on the identification of molecular signals required for schistosome-induced development of regulatory B cells.

Chronic *S. mansoni* infection induces regulatory B cells both in the spleen and the lung. While splenic B cells are known to protect from allergic asthma by producing the immunomodulatory molecule interleukin (IL)-10, lung B cells act independent of IL-10. We further characterized these lung B cells with regulatory properties and show that they are phenotypically and functionally different from classical regulatory B cells in the spleen (**chapter 2**). This work therefore suggests the presence of distinct regulatory B cell subsets, even if induced by the same type of worm parasite.

Chronic *S. mansoni* infections are known to induce regulatory B cells. Single molecules that are produced and secreted by the worm to influence surrounding immune cells and mediate this protective effect had however not been identified yet. Moreover, the cellular mechanisms driving regulatory B cell development remained to be defined. We showed that a subset of B cells in the spleen, which preferentially develop into regulatory B cells, directly bound molecules released by the worm. No neighboring cells were necessary for these B cells to respond to the worm molecules. We could also identify a single worm protein, called IPSE/alpha-1, that can induce IL-10 production, both in B cells of mice and humans. We moreover found evidence that there are additional molecules which can also have this effect (**chapter 3**).

The induction of regulatory B cells by a chronic *S. mansoni* infection can thus also be achieved by a single worm molecule such as IPSE/alpha-1. This induction of regulatory B cells by isolated molecules is however less strong compared to the full infection, which suggests that there are several components working together in the case of an infection. To identify more signals and processes that contribute to the induction of regulatory B cells in an infected host, we compared which genes are expressed in the different B cell subsets from chronically *S. mansoni*-infected mice. We could identify several interesting signals and processes that seem more important for one of the B cell subsets than the other and might therefore play a role in the development of regulatory B cells. Amongst those were members of the type I interferon cytokine family, but also several elements of the innate immune system (**chapter 4**).

Type I interferons were thus suggested to be preferentially important for the activation of regulatory B cells in chronic *S. mansoni* infection. It had also been reported that different worms can trigger the production of these type I interferons, and that they are important in the activation of regulatory B cells in humans. We therefore further investigated whether type I interferons also provide signals for the induction of regulatory B cells in the context of *S. mansoni*. We found that type I interferons enhance regulatory B cell IL-10 production in response to *S. mansoni* molecules in a culture plate, but that they are not important in the body (**chapter 5**). Further research is therefore needed to identify the conditions that do lead to an optimal development and activation of regulatory B cells, and to better understand which role type I interferons play in parasitic worm infection.

The second part of this thesis focuses on the identification of *S. mansoni*-derived molecules that are protective in experimental models of allergic asthma. **Chapter 6** summarizes and discusses the scientific literature with respect to earlier findings for worm-induced protection from allergic asthma and the mechanisms of protection. In addition, studies are described that have identified protective worm molecules as well as their implications for the development of novel treatment strategies for allergic asthma.

Chronic *S. mansoni* infection had been shown to protect from allergic asthma in animal models, and infected humans are less likely to have allergies or asthma. We could show that isolated *S. mansoni* eggs, in the absence of infection, protect from experimental allergic asthma when administered to mice before they are exposed to allergenic substances for the first time. We could also reproduce the protective effect of eggs on allergic asthma in mice by a similar treatment with the worm-derived protein omega-1, for which no such role had been described before (**chapter 7**).

In conclusion, the work presented in this thesis shows that both the induction of regulatory B cells, as well as the protection from allergic asthma by a chronic worm infection can also be achieved by single molecules isolated from the worm. This work however also shows that immunological processes in response to a chronic worm infection are complex and often act in synergy. This thesis contributes to the understanding of how regulatory B cells are induced, and how molecules secreted by the parasite provide protection against allergic asthma. A deeper understanding of these fundamental immunological processes is necessary and will open up opportunities to develop new treatment options based on targeted interventions in immune processes.

NEDERLANDSE SAMENVATTING

Continu krijgen we allerlei stoffen en microben binnen via bijvoorbeeld het voedsel dat we eten of de lucht die we inademen. Ons afweersysteem beschermt ons lichaam tegen mogelijk schadelijke stoffen of ziekteverwekkers zoals bacteriën, virussen en parasieten. Om dit te kunnen bewerkstelligen heeft het afweersysteem verschillende strategieën ontwikkeld om onderscheid te maken tussen schadelijke deeltjes, die ziekten veroorzaken, en onschadelijke deeltjes en goede bacteriën. Meestal kiest het afweersysteem de juiste respons. Soms slaagt het er echter niet in om ziekteverwekkers te elimineren voordat ze schade aan het lichaam kunnen toebrengen, of reageert het juist te heftig op onschadelijke stoffen of zelfs op het eigen lichaam. Hierdoor kunnen bijvoorbeeld astma of auto-immuunziekten ontstaan. Astma is een chronische ontsteking van de longen, waarbij klachten zoals benauwdheid en een piepende ademhaling voorkomen. Allergisch astma, een veel voorkomende vorm van astma en onderwerp van dit proefschrift, wordt veroorzaakt door een reactie van het afweersysteem tegen bepaalde ingeademde stoffen (allergenen) zoals uitwerpselen van de huisstofmijt of stuifmeelkorrels.

Schistosoma (in de volksmond bekend als bilharzia), is een parasitaire platworm die in zoetwaterslakken te vinden is en waarvan de larven de gastheer via de huid binnendringen. De volwassen wormen leven en paren in het bloedvatenbed van de darm of blaas. Hier leggen zij vervolgens tientallen eieren per dag die uitgescheiden worden via de feces of urine. Wormen zoals *Schistosoma* zijn unieke parasieten omdat ze een zeer lange tijd in het menselijk lichaam kunnen verblijven. Deze langdurige infecties hebben echter niet alleen nadelige effecten voor de menselijke gastheer, maar kunnen soms ook juist een gunstige werking hebben. Wormen onderdrukken namelijk de afweerreacties van de gastheer om hun eigen overleving te veilig te stellen. En passant worden ook andere afweerreacties onderdrukt waardoor bescherming wordt geboden tegen een spectrum van ziekten veroorzaakt door een overactief afweersysteem. Hieronder vallen de hierboven genoemde astma en auto-immuunziekten. Parasitaire wormen beïnvloeden het afweersysteem op verschillende manieren, waaronder de aanmaak van afweercellen die tolerantie opwekken, zoals regulatoire B cellen. Terwijl 'normale' B-cellen antilichamen produceren en ons tegen binnendringende micro-organismen beschermen, onderdrukken regulatoire B cellen het afweersysteem. Een beter begrip van de activatie en functie van deze cellen kan helpen om de behandeling van bijvoorbeeld astma en auto-immuunziektes te optimaliseren.

Onderzoek in dit proefschrift

Het eerste deel van dit proefschrift richt zich op de rol van regulatoire B cellen in de bescherming tegen allergisch astma tijdens chronische *Schistosoma mansoni* infecties. Tevens wordt geprobeerd de signalen en moleculen afkomstig van de schistosoma worm parasieten te ontrafelen die de ontwikkeling van regulatoire B cellen verder aansturen.

Een chronische infecties met *S. mansoni* bevordert de aanmaak van regulatoire B cellen zowel in de milt als in de longen. B cellen uit de milt onderdrukken de ontwikkeling van allergisch astma door het uitscheiden van het tolerantie-opwekkend molecuul interleukine (IL)-10, terwijl B cellen uit de long dit onafhankelijk van IL-10 lijken te doen. Wij hebben de eigenschappen van deze long B cellen verder gekarakteriseerd en vonden dat zij zowel qua uiterlijke kenmerken als in hun afweer onderdrukkende werking verschillend zijn van de klassieke regulatoire B cellen uit de milt (**hoofdstuk 2**). Dit werk toont het bestaan aan van verschillende typen regulatoire B cellen die opgewekt kunnen worden door een en dezelfde parasiet.

Hoewel bekend is dat chronische *S. mansoni* infecties regulatoire B cellen opwekken, zijn de worm-specifieke moleculen die dit beschermende effect kunnen bewerkstellingen nog niet geïdentificeerd. Bovendien is het niet bekend in hoeverre andere cellen betrokken zijn bij de ontwikkeling van regulatoire B cellen. Wij tonen aan dat de ontwikkeling van regulatoire B cellen in de milt tot stand

komt door *direct* contact met moleculen die door wormeieren worden uitgescheiden. Wij hebben geen betrokkenheid van andere afweercellen kunnen vinden, die nodig zijn om op de wormmoleculen te kunnen reageren. Tevens hebben we één worm-specifiek eiwit, IPSE/alpha-1, geïdentificeerd dat kan aanzetten tot de productie van het kenmerkende IL-10 in B cellen afkomstig van zowel muizen als mensen. We hebben ook aanwijzingen dat er nog meer moleculen zijn die dit kunnen (**hoofdstuk 3**).

De aanmaak van regulatoire B cellen wordt dus niet alleen tijdens een chronische worminfectie aangedreven, maar kan ook tot stand komen door één enkel molecuul zoals bijvoorbeeld IPSE/alpha-1. De ontwikkeling van regulatoire B cellen als reactie op geïsoleerde wormmoleculen is echter minder sterk in vergelijking met die als reactie op een volledige infectie. Om de bijdrage van verschillende signalen en routes in de aanzet van regulatoire B cellen te onderzoeken, hebben wij de genen in kaart gebracht die tot expressie komen in de verschillende typen B cellen van muizen met een chronisch *S. mansoni* infectie. Wij laten zien dat de ontwikkeling van regulatoire B cellen wordt gedreven door verschillende categorieën genen. Wij vonden aanwijzingen voor de betrokkenheid van de type I interferon-cytokinefamilie evenals bepaalde elementen van het aangeboren afweersysteem (**hoofdstuk 4**).

In navolging van de bevindingen in hoofdstuk 4 onderzochten wij de relatieve bijdrage van type I interferonen in de activering en aanmaak van regulatoire B cellen tijdens een chronische *S. mansoni* infectie. Eerder onderzoek heeft aangetoond dat verschillende typen wormen de productie van deze type I-interferonen in gang kunnen zetten en dat ze belangrijk zijn voor de aanzet van menselijke regulatoire B cellen. Wij laten zien dat de aanwezigheid van type I interferonen de IL-10 productie door regulatoire B cellen als reactie op moleculen van *S. mansoni* wormen verbetert, wanneer dit wordt getest met behulp van celkweken. Echter, in muizenstudies lijkt het vooralsnog geen belangrijke bijdrage te leveren (**hoofdstuk 5**). Verder onderzoek is daarom nodig om de exacte omstandigheden te identificeren die leiden tot een optimale ontwikkeling en activering van regulatoire B cellen in het lichaam.

Het is bekend dat chronische *S. mansoni* infecties kunnen beschermen tegen allergisch astma in proefdiermodellen en dat ook geïnfecteerde mensen minder kans op het ontwikkelen van allergieën of astma lijken te hebben. Het tweede deel van dit proefschrift richt zich op de rol van moleculen afkomstig van *S. mansoni* wormen die bescherming bieden tegen de ontwikkeling van allergisch astma in proefdiermodellen. **Hoofdstuk 6** is een samenvatting en discussie van de wetenschappelijke literatuur met betrekking tot worm-gerelateerde bescherming tegen allergisch astma, met in het bijzonder de hierbij betrokken beschermingsmechanismen. Daarnaast worden studies beschreven die beschermende wormmoleculen hebben geïdentificeerd, evenals de rol van deze moleculen in de ontwikkeling van nieuwe behandelstrategieën voor allergisch astma.

In **hoofdstuk 7** tonen wij aan dat geïsoleerde eieren van *S. mansoni*, bij afwezigheid van een natuurlijke infectie met wormen, beschermen tegen de ontwikkeling van allergisch astma wanneer zij worden toegediend voorafgaand aan de blootstelling aan allergische stoffen. Daarnaast laten wij zien dat een vergelijkbaar beschermend effect bereikt kan worden door de behandeling met alleen het eiwit omega-1. Dit eiwit wordt uitgescheiden door eieren van *S. mansoni*. Een dergelijke rol voor omega-1 is nog niet eerder beschreven.

Concluderend laat het werk in dit proefschrift zien dat zowel de aanmaak van regulatoire B cellen als de bescherming tegen allergische astma door een chronische worminfectie ook bereikt kan worden met individuele moleculen die door de eieren van *S. mansoni* wormen worden uitgescheiden. Tevens laat dit werk zien dat immunologische processen in reactie op een chronische worminfectie complex zijn en vaak in synergie optreden. Dit proefschrift draagt bij aan het begrip hoe parasiet-uitgescheiden moleculen enerzijds regulatoire B cellen kunnen opwekken en anderzijds bescherming kunnen bieden tegen allergisch astma. Een beter begrip van deze fundamentele immunologische

processen is noodzakelijk voor de ontwikkeling van nieuwe, op wormmoleculen gebaseerde, astma behandelmethoden.

CURRICULUM VITAE

Katja Obieglo was born on 7th April 1988 in Berlin (Germany). She completed her primary and secondary education in Germany, and studied Molecular Medicine at the Georg-August Universität Göttingen (Germany), where she obtained her Bachelor of Science (B.Sc.) degree in 2010. Katja subsequently moved to Sweden and completed her Master of Science (M.Sc.) degree in Biomedicine at Karolinska Institutet in Stockholm (Sweden). During her Master's, Katja participated in an Erasmus exchange program, which allowed her to join the Honours class of immunology at the University of Edinburgh (UK) for 4 months. Throughout her studies, Katja was supported by a scholarship from a foundation that is part of the german scholarship system for outstanding students. After completion of the M.Sc. degree program, Katja stayed in the lab where she has conducted her Master thesis for another year and continued to work on this research project, funded by a Karolinska Institutet scholarship. In 2013, Katja went to the Netherlands to start as a PhD candidate at the Leiden University Medical Center under supervision of Dr. Hermelijn H. Smits and Prof. Maria Yazdanbakhsh. In 2017, Katja joined the cystic fibrosis team of ProQR Therapeutics NV in Leiden as Scientist Biomarker Project Coordinator, while simultaneously finishing her PhD thesis. The results of the research performed during Katja's PhD trajectory are described in this thesis.

LIST OF PUBLICATIONS

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