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

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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

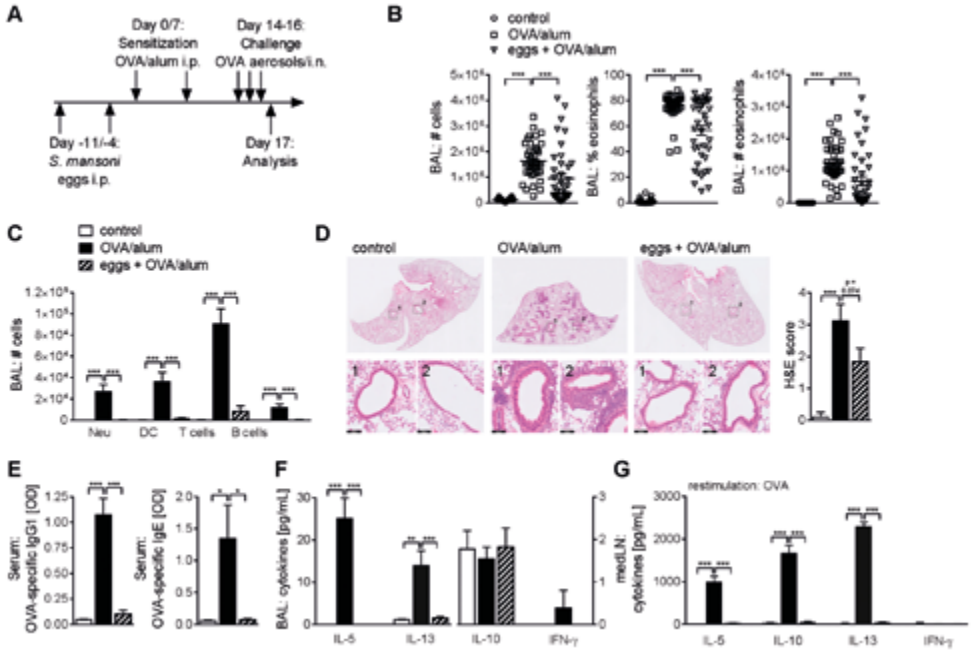

Figure 1. ***S. mansoni*** **eggs protect against OVA/alum-induced AAI.** (**A**) Schematic representation of the experimental model. (**B**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. Summary of multiple experiments. (**C**) Total 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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

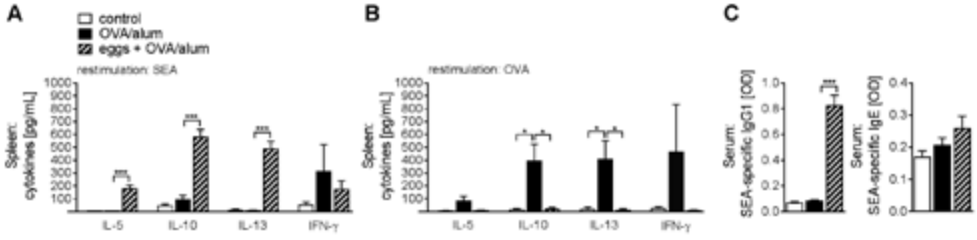

Figure 2. Protection occurs despite the induction of an egg-specific type 2 induction. (**A, B**) Cytokine concentration in spleen cell supernatants after 4d re-stimulation with SEA (10μg/mL; **A**) or OVA (10μg/mL; **B**). Representative of 2 experiments, n=3-4. (**C**) SEA-specific IgG1 and SEA-specific 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

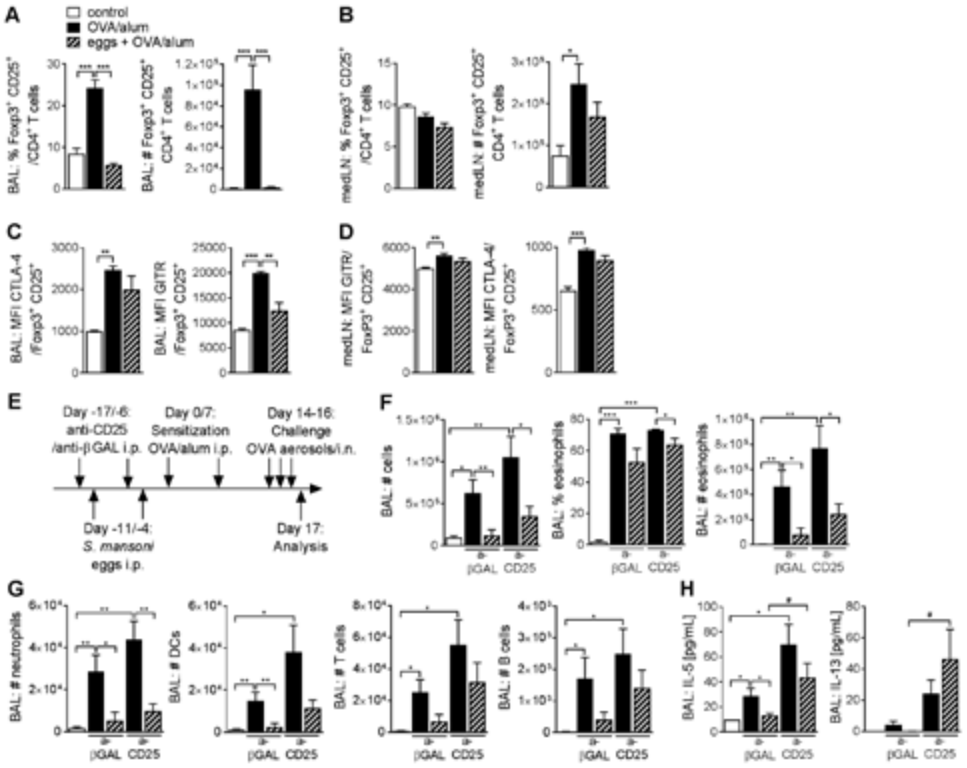

Figure 3. Regulatory T cells are not involved in egg-induced protection against AAI. (**A, B**) Percentage and total number of Foxp3$^+$ CD25$^+$ Treg cells in BAL fluid (**A**) and medLNs (**B**) assessed by FACS. Representative of multiple experiments, n=4-5. (**C, D**) Geometric mean expression of 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

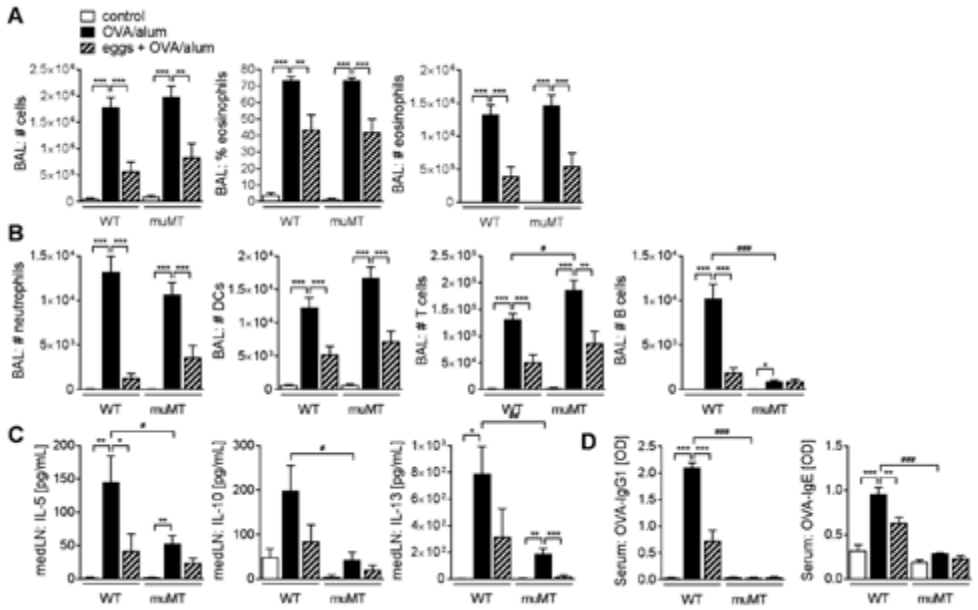

Figure 4. Mature B cells are not involved in egg-induced protection against AAI. (**A**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed by FACS. (**B**) Total number of neutrophils, dendritic cells (DCs), T cells and B cells in BAL fluid as assessed 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.

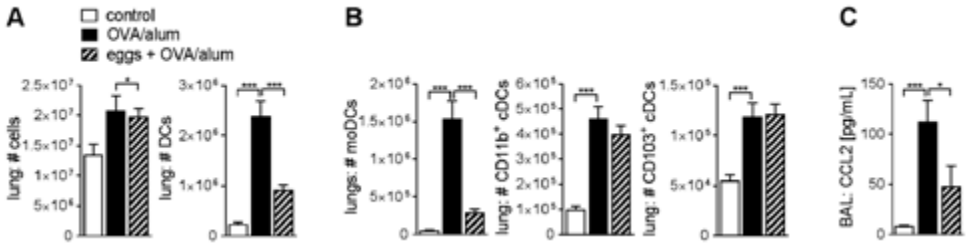

Figure 5. Egg treatment impairs the lung moDC, but not cDC, compartment. (**A**) Total number of lung cells, and total number of lung DCs ($CD11c^+$ $MHCII^+$), assessed by FACS. Representative of 2 experiments, n=4-6. (**B**) Total number of moDCs ($CD11c^+$ $MHCII^+$ $CD11b^+$ $CD103^-$ $CD64^+$), $CD103^+$ 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.

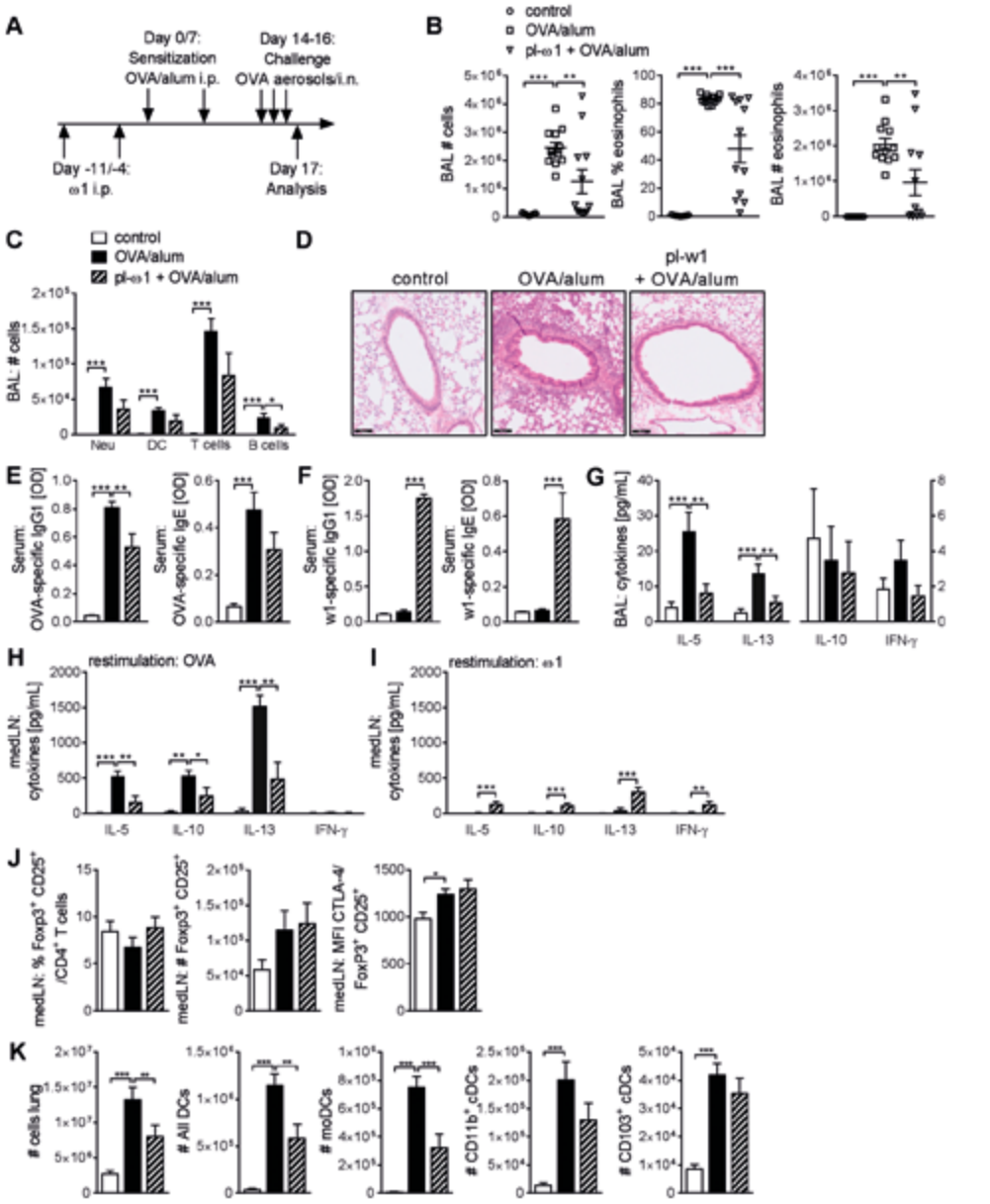

Figure 6. The suppressive effect of *S. mansoni* egg on AAI can be reproduced by one of the major egg antigens, the glycoprotein ω-1. (**A**) Schematic representation of the experimental model. (**B**) Total number of cells, percentage of eosinophils and total number of eosinophils in BAL fluid as assessed 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

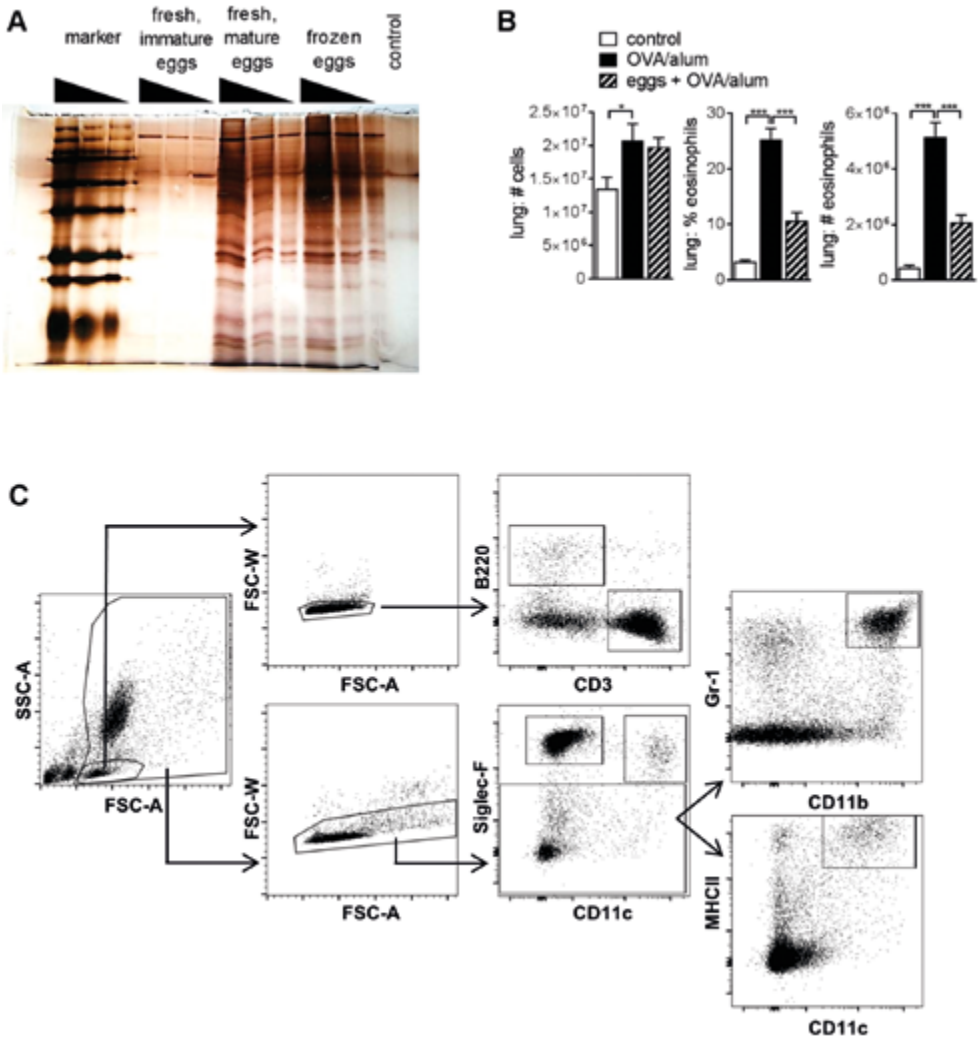

Figure S1. (**A**) Eggs were isolated from the liver of infected hamsters, washed extensively and either separated into immature and mature eggs following by culture, or frozen at -80°C prior to culture. Freshly isolated and freeze-thawed eggs were cultured at a density of 200.000 eggs/mL in medium for 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 ($CD3^-$ $B220^+$).

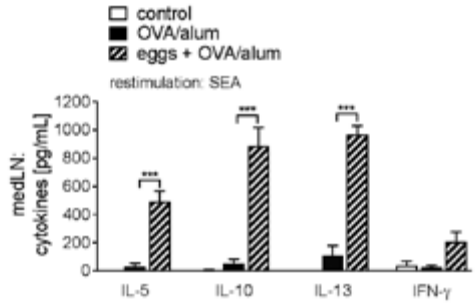

Figure S2. Cytokine concentration in medLN cell supernatants after 4d re-stimulation with SEA (10µg/mL), n=5. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with *** $p < 0.001$.

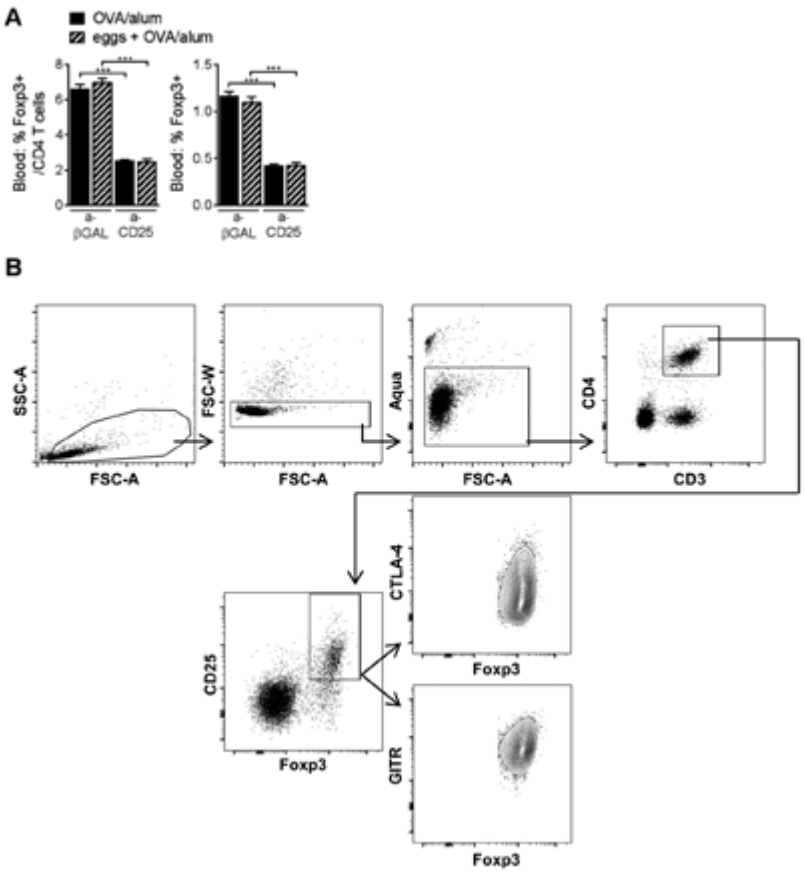

Figure S3. (**A**) Mice were treated with anti-CD25 depleting antibody (clone PC61) as described in Figure 4. Five days after the first antibody injection, tail blood was collected and the percentage of $Foxp3^+$ Treg cells of all CD4 T cells and of all cells assessed by FACS. Representative of two experiments, 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.

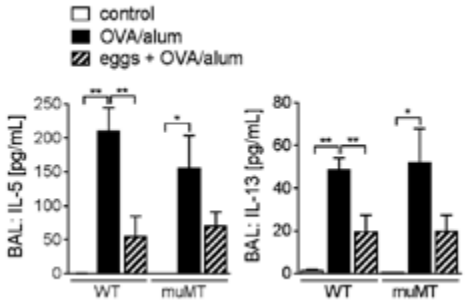

Figure S4. Cytokine concentration in BAL fluid measured by ELISA, n=3-6. Significant differences were determined by one-way ANOVA following Dunnett's multiple comparisons test and are indicated with * $p < 0.05$, ** $p < 0.01$.

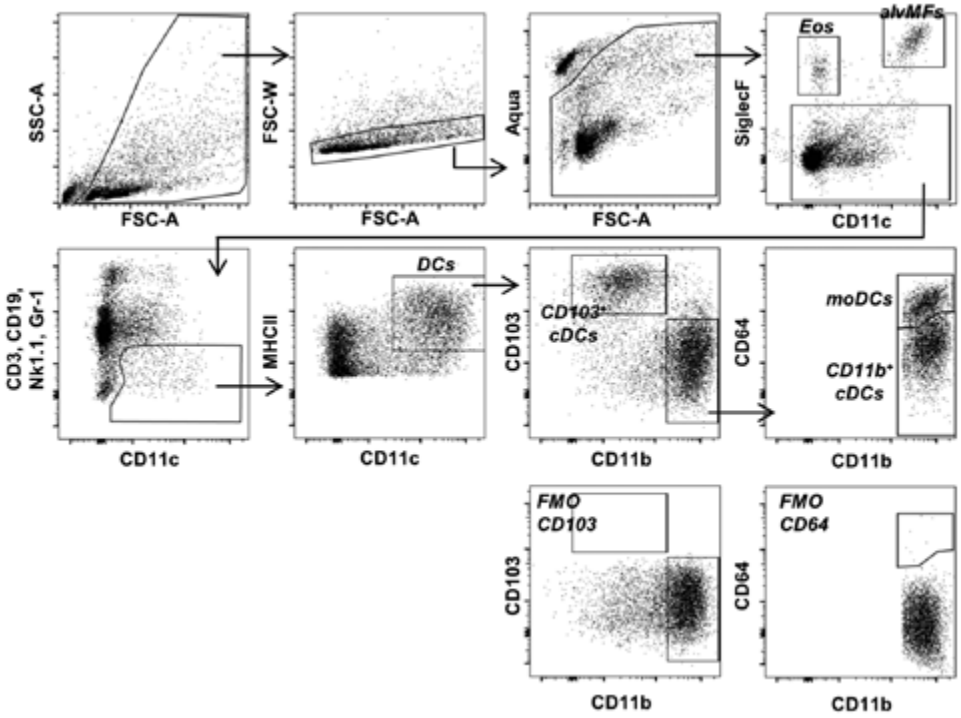

Figure S5. FACS gating strategy for the analysis of DC subsets in lung tissue. Single, live cells were identified, and all SiglecF, CD3, CD19, NK1.1 and Gr-1 expressing cells excluded. DCs were identified as CD11c$^+$ MHCII$^+$, and subdivided into CD103$^+$ cDCs (CD103$^+$ CD11b$^-$ CD64$^-$), CD11b$^+$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