

Research paper

Immune responses to fungal aeroallergen in *Heligmosomoides polygyrus*-infected mice vary by age

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ABSTRACT

Parasite infections in the developing world have been considered to promote resistance to immune-mediated diseases such as asthma. Mouse studies have shown that helminths and their products reduce the development of allergic asthma. Since epidemiologic studies that show similar protection are in relation to geohelminth infections that occur in early life, we hypothesized that the parasite-mediated protection against asthma may differ by age. Mice infected with *Heligmosomoides polygyrus* at 3-weeks of age had similar asthma phenotype compared to mice infected at 28-weeks of age wherein airway eosinophilia was unaltered but tissue inflammation and GC metaplasia were reduced. In contrast, mice infected at 18-weeks of age had elevated macrophagic airway inflammation with accompanying tissue pathology. The presence of $\gamma\delta$ T cells and T_{reg} cells in the airways was also regulated by age at worm infection. Our findings demonstrate the importance of age in immune responses that may regulate gut and lung diseases.

1. Introduction

The incidence of allergen-associated diseases including allergic rhinitis, food and skin allergies, allergic asthma, and insect allergies has increased dramatically in the past few decades and is cause for increased health care costs worldwide [1,2]. Allergic immune responses (eosinophils, T_H2 cells and cytokines, IgE) are commonly thought to have evolved in defense against macroparasites, and proposed as a defense mechanism against noxious environmental antigens [3,4]. Considering the embryonic lineage association between the gut and lung [5], along with evidence suggesting possible associations between the gut and lung microbiomes [6], the “Old Friends” hypothesis [7,8] may hold true for allergic diseases of the respiratory system such as asthma.

Allergic asthma is a disease of the airways in which there are changes to lung function from increased (eosinophil predominant) inflammation and structural changes. Epidemiologic studies have shown both positive and negative associations between parasite infections and the development of allergy [9–11]. Experimental induction of allergic asthma in mice pre-infected with helminths have supported a role for worms as protectors against pulmonary allergy through immunoregulatory mechanisms [12,13]. The use of helminths in the

clinical setting as therapeutics for inflammatory bowel disease has demonstrated a benefit [14–16]. The immunologic responses during helminth infections are often T_H2 biased with abundant IgE. While these immune responses are directed at worm expulsion and clearance, most parasitic worms have evolved to co-exist in the host possibly through mediation of immune tolerance. As helminths are envisioned as therapeutics, the importance of identifying relevant protective antigens is considered along with the ideal helminth and their gender [17]. Another important factor should be the age of the host as immune development, maturation, and senescence are affected by age [18–20]. Herein, we investigated the impact of age on gastrointestinal helminth-mediated suppression of characteristics of fungal asthma.

2. Materials and methods

2.1. Mice and experimental groups

Female C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were maintained in a biosafety level 2 facility. Mice were housed in microisolator cages with alpha-dri bedding and exposed to standardized 12-h light/dark cycles and had free access to food and water. All experiments were approved by the Animal Care and Use

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Committee of St. Jude Children's Research Hospital. Animals in the 'Naïve' groups were administered PBS in place of larvae and allergen. Those in the 'Worm' groups were administered larvae and PBS in place of allergen. Mice in the 'Asthma' groups were administered PBS in place of larvae, and those in the 'W + A' (worm and asthma) groups received larvae and allergen as detailed below.

2.2. *Heligmosomoides polygyrus* infection

Mice were infected with 300 L3 stage larvae of *H. polygyrus* (kindly gifted by Dr. DeBroski Herbert) via oral gavage as previously described [21] at 3-, 18-, or 28- weeks of age. Mock- or worm-infected mice were subjected to the fungal asthma model four weeks later. Since evidence suggests that *H. polygyrus* worm burden may be indirectly reflected by the egg burden in feces [22], we measured the fecal egg count starting at 8 days after inoculation then once weekly afterward until the second allergen challenge.

2.3. Fungal asthma model

Mice that were mock- or parasite-infected were subjected to the fungal asthma model at 4-weeks after infection per previously published protocol [23,24], at which time they were 8-, 22-, or 32-weeks old. Briefly, *Aspergillus fumigatus* antigen (Greer Labs, Lenoir, NC) was adsorbed in Imject Alum (Thermo Scientific, Rockford, IL) and administered subcutaneously and intraperitoneally to deliver 20 µg/mouse, and mice were allowed to rest for two weeks. Following this, *A. fumigatus* antigen was administered intranasally to deliver 4 µg/mouse once weekly for three weeks to complete sensitization to the allergen. Sensitized mice were anesthetized and laid supine for nose-only delivery of dry conidia from a mature *A. fumigatus* culture for 10 min by natural inhalation route [25]. An additional inhalation exposure to fungal conidia occurred in these animals following a two week rest period. We have previously demonstrated hallmarks of allergic airways diseases including eosinophilic inflammation, GC metaplasia, airway hyperresponsiveness, and airway wall remodeling events occur in mice following this protocol [23,24]. The entire experimental protocol is provided in Fig. 1.

2.4. Cell isolation from tissue

Mice were euthanized at one week after the second allergen challenge at which time they were 15-, 30-, or 40-weeks old. Bronchoalveolar lavage (BAL) was performed by inserting an 18-gauge catheter through the incised trachea and lavaging with 1 mL of PBS for 3 times and contents were centrifuged to separate BAL cells from fluid. BAL fluid was stored at -80 °C until use. Contaminating erythrocytes in the BAL cell pellet were lysed and leukocytes resuspended in complete tumor media (CTM). Cell number was determined by Trypan Blue dye

exclusion method and an aliquot of cells were cytospun (Thermo, Grand Island, NY) and differentially stained.

Mediastinal lymph node (MLN), thymus, and mesenteric lymph node (MesLN) from each mouse were isolated and ground tissue was filtered through a 100 µm cell strainer to obtain single cell suspensions. After centrifugation, the cells were resuspended 1 mL of CTM and enumerated as above.

2.5. Tissue harvest and goblet cell enumeration

Blood was collected from the thoracic cavity and centrifuged to obtain serum. Collected sera were placed at -80 °C until use. Left lobes of the lungs were harvested and fixed with 1 mL of 10% neutral buffered formalin *ex vivo* and constant pressure and 4 µm sections were fixed onto slides and stained with hematoxylin and eosin and periodic acid Schiff's stain. Goblet cells (GCs) in 10 high power fields (800×) were counted for every 30 cells lining the large airways to calculate the percentage of GCs in each airway. Slides were analyzed by an investigator blinded to the study conditions. The mean and standard error were calculated for each experimental condition in each age group category.

2.6. Small intestinal lavage

The small intestines from each mouse were removed and gently cleaned to remove luminal material. Intestines were lavaged with 1 mL of PBS to collect luminal fluids. Contents were clarified by centrifugation and intestinal lavage fluid (ILF) was frozen at -80 °C until use.

2.7. Cell surface staining, FACS analysis

The numbers of neutrophils, macrophages, eosinophils, and lymphocytes (CD4⁺, CD8⁺, CD25⁺, FoxP3⁺, and γδ T cells) present in the BAL, MLN, and MesLN were examined by FACS analysis. Single cell suspensions from each niche were incubated in Fc block (BD biosciences, San Jose, CA) to limit non-specific binding of antibodies. Extracellular markers were then stained with antibodies at the dilution of 1:200 in the dark on ice. Fixed and permeabilized cells were then stained for intracellular marker FoxP3. Data on stained cells were acquired using a FACS Canto II (BD) and analyzed using FlowJo software (v8, FlowJo LLC, Ashland, OR). Please see [Supplementary Table 1](#) for details on antibodies and [Supplementary Table 2](#) for gating strategies.

2.8. Total and *A. fumigatus*-specific antibody quantification

Total IgE (BD), IgG₁, and IgA (Bethyl Labs, Montgomery, TX) were quantified in the serum, BALF, and ILF using commercially available enzyme-linked immunosorbent assays (ELISA) per manufacturer's

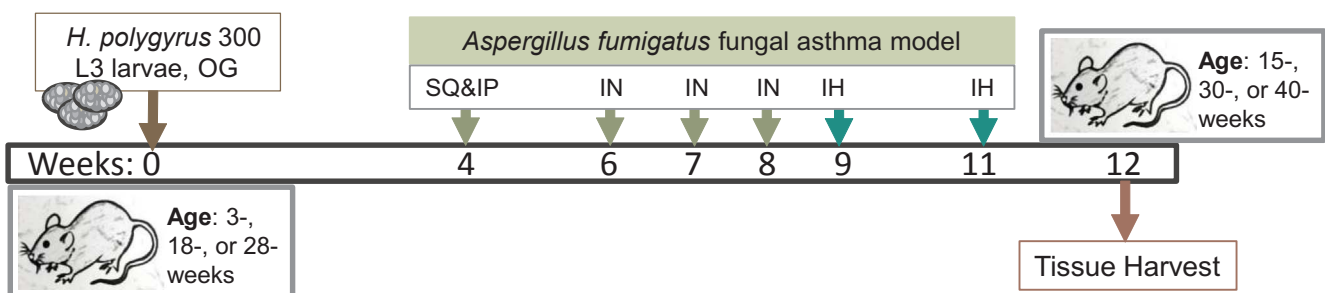


Fig. 1. Schematic representation of experimental model. C57BL/6 female mice were infected with 300 *Heligmosomoides polygyrus* larvae via oral gavage (OG) at 3-, 18-, or 28-weeks of age. Mice were separated into four groups as PBS-ctr, Worm-ctr, Asthma-ctr, and Worm + Asthma indicative of the treatments administered. After four weeks, mice in the Asthma-ctr and Worm + Asthma groups were administered antigen from *Aspergillus fumigatus* subcutaneously (SQ) and intraperitoneally (IP). Two weeks later, these mice received weekly intranasal (IN) administrations of *A. fumigatus* antigen followed by two inhalation (IH) exposures of *A. fumigatus* conidia two weeks apart. Samples were harvested one week after the second fungal inhalation exposure at which time mice were 15-, 30-, or 40-weeks of age.

guidelines. *A. fumigatus* specific antibody titers were measured by ELISA as previously described [59]. Briefly, plates were coated overnight with 20 µg/mL *A. fumigatus* antigen (Greer Labs) and serially diluted samples (serum (10^{-1} to 10^{-8}), BAL fluid and ILF (2^{-1} to 2^{-6})) were added. Horseradish peroxidase-conjugated antibodies against IgE (ThermoFisher, Waltham, MA), IgG₁ (Abcam, Cambridge, MA), and IgA (Abcam) were diluted at 1:500 and 1:10,000 respectively and added. Colorimetric changes were measured at 650 nm in the presence of tetramethylbenzidine substrate (BD Biosciences).

2.9. Statistical analysis

All data are represented as the mean and standard deviation of one representative study of two. Data from each of the experimental conditions within the age group and between age groups were compared by two-way ANOVA followed by Tukey's multiple comparisons posttest. Significant differences ($P < 0.05$) are denoted by different letters when comparing experimental conditions within an age group or between age groups. For example, 'a' vs 'b' is a significant difference whereas 'a' vs 'a' is not. Statistical analyses performed on GraphPad Prism v6.01 software (LaJolla, CA).

3. Results

Immune responses to foreign antigens may vary by age. Gut dwelling worms are known to regulate immune responses and the age at which worms begin their life cycle in the host may have an impact on immune programming to subsequently affect sensitization and tolerance to allergens. This study was designed to investigate changes in allergic responses in hosts infected with a gut parasite at three different ages.

3.1. Allergen sensitization and challenge did not have an impact on the helminth life cycle

H. polygyrus egg load in feces is a marker of helminth presence and health, as adult worms produce eggs continuously throughout their lifespan [26]. We measured the egg burden in the feces weekly beginning at eight days after oral gavage of L3 larvae and ending at the conclusion of the fungal asthma protocol (Fig 2A). There were no significant differences between the 'Worm' and 'Worm + Asthma' experimental conditions at each of the age groups (Fig 2A, a–b). We then compared the egg load in mice infected at each age group individually in the 'Worm' and 'Worm + Asthma' groups (Fig 2A, d & e) and noted significant differences in fecal egg load. Mice infected with *H. polygyrus* at 3-weeks of age had significantly greater egg burden by the 4th week of infection irrespective of allergen exposure (Fig 2A, d & e). These mice also maintained a higher egg load compared to the other age groups at last measurement (Fig 2A, d & e). While the trends in fecal egg load were similar between mice infected at 18- and 28-weeks irrespective of allergen exposure, the egg load was higher in mice infected at 28-weeks in the absence of allergen exposure, although this difference was only statistically significant at week 4 (Fig 2Ad).

3.2. Inflammatory responses against helminth and allergen exposures were altered by age

Since we expected an immune response against helminth infection and allergen exposure, we enumerated cells in the airways (BAL), lung-draining MLN, and gut-draining MesLN one week after the second allergen challenge (Fig 3A). The number of cells in the naïve condition was a marker of basal levels. Allergen exposure caused significant inflammation in the lungs in all age groups with peak inflammation occurring in the 30-week old mice. While this age group also had the highest number of cells in the 'Worm + Asthma' group, the number of

cells was significantly lower than in the 'Asthma-only' condition. The number of cells in the BAL of 'Worm + Asthma' was increased in similar levels to 'Asthma' condition at the other age groups tested. Interestingly, cells in the MLNs of the experimental conditions did not show an increase over basal levels in the 30-week age group, but there was a significant increase in the MLN cell content in the 40-week old 'Asthma' and 'Worm + Asthma.' *H. polygyrus*-infection resulted in a significant increase in cell number within the MesLN in mice that were 40-weeks old, but none of the experimental conditions at any of the other age groups had an impact of MesLN cell numbers.

Goblet cell metaplasia is a marker of lung epithelial response to allergen exposure. Periodic acid Schiff's stain makes these cells visible by magenta staining of mucins within them (Fig 3B). GCs were not present in naïve and helminth-infected mice at any of the age groups. Allergen exposure resulted in the increase of GCs within the airway epithelia in all age groups with no notable differences among the age groups in the 'Asthma' controls. However, mice that were infected with *H. polygyrus* prior to allergen sensitization and challenge had an age-associated change in GC metaplasia. Mice that were 15- and 40-weeks old had a reduction in the GC numbers, with a more prominent change in the younger age group. Mice that were 30-weeks old had equivalent levels compared to 'asthma' alone (Fig 3B).

3.3. Pre-existing helminth infection affected allergic airway inflammation

Since there were age- and condition-associated differences in airway inflammation (Fig 3), we investigated the types of cells that were largely responsible for the inflammatory response. Eosinophils were recruited into the airways in mice that were allergen exposed and there was a significant reduction in eosinophils in the 'Worm + Asthma' condition compared to 'Asthma' alone in the 30-week old mice (Fig 4A). The number of macrophages in the airways only increased in the 'Asthma' and 'Worm + Asthma' groups in the mice that were 30-weeks in age, with significantly more macrophages being present in the 'Asthma' group (Fig 4B). Neutrophil recruitment into the airways was minimal, and was only significantly increased in the 'Asthma' group of mice that were oldest (Fig 4C).

3.4. Expansion of T_{reg} and $\gamma\delta$ T cells was differentially regulated based on experimental condition and age

Since we did not notice a change in the total number of lymphocytes based on experimental condition and age group, we investigated regulatory T (T_{reg}) cell populations that are known to be important in immunomodulation during helminth infections, noting that $CD4^{+} T_{reg}$ cells were more abundant than $CD8^{+} T_{reg}$ cells (Fig 5). Regulatory T cell numbers and trends differed between the BAL and the LNs. While parasite infection did not have a significant impact on $CD4^{+} T_{reg}$ cells in the airways, *H. polygyrus* infection affected the number of $CD8^{+} T_{reg}$ cells in this niche (Fig 5A & B). The number of T_{reg} cells in the MLN was similar between the experimental conditions except in the 40-week age group in which the 'Asthma' group had a significantly higher number of T_{reg} cells (Fig 5C & D). Untreated mice in the 15-week age group had more $CD4^{+} T_{reg}$ cells in the MesLN than at other age groups (Fig 5E), but this was not observed for the $CD8^{+} T_{reg}$ population (Fig 5F). A significant increase in $CD4^{+}$ and $CD8^{+} T_{reg}$ cells occurred in the 'Asthma' group at 30-weeks and 'Worm' group at 40 weeks in the MesLN (Fig 5E & F).

Treatment and age had an impact on the $\gamma\delta$ T cells in the BAL and LNs. There was no expansion of airway $\gamma\delta$ T cells in response to helminth infection alone. Allergen exposure led to an increase in BAL $\gamma\delta$ T cells in an age-dependent manner. Helminth infection prior to allergen exposure resulted in a significant expansion of $\gamma\delta$ T cells in the 15- and 30-week old mice, but not the 40-week old group (Fig 6A). The $\gamma\delta$ T cells in the MLNs were significantly elevated in the 40-week old mice that were allergen sensitized and challenged while these cells

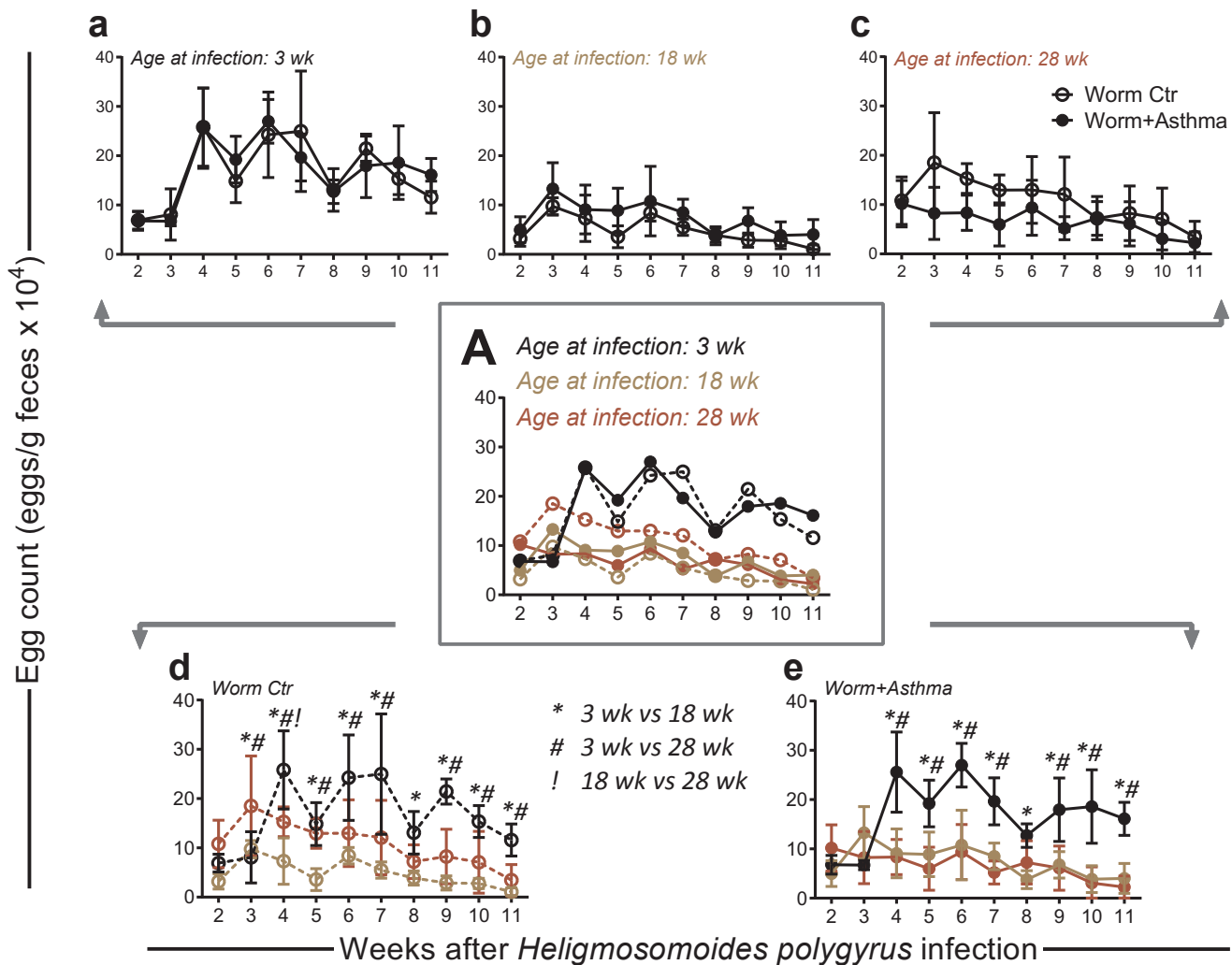


Fig. 2. *Heligmosomoides polygyrus* eggs were found in feces of infected mice throughout the experimental model. Eggs in the feces were measured starting at 2-weeks after the helminth infection until the second allergen challenge. While there was no apparent difference between helminth-infected mice that received allergen exposure or not, there were differences between the age groups (A). Mice infected at 3-weeks of age had multiple peaks in egg load (a), whilst those infected at 18- and 28- weeks of age generally had the highest egg burden early during the infection but tapered towards the end (b and c). When helminth-infected mice at the three age groups were compared, mice infected at 3-weeks clearly had a significantly higher egg load beginning on the 4th week of infection which was maintained in high levels throughout the time course (d). While the *H. polygyrus*-infected mice also subjected to the fungal asthma model had similar levels of eggs in the feces of mice infected at 18- and 28- weeks, those infected at 3-weeks of age had a significantly greater egg burden throughout the model (e). Data represented as the mean and standard deviation of one of two experiments and compared by two-way ANOVA with Tukey's multiple comparisons test. *p < 0.05 between 3- and 18-weeks; #p < 0.05 between 3- and 28-weeks; and !p < 0.05 between 18- and 28-weeks.

were significantly reduced in the MLN of 30-week old 'Asthma' group (Fig 6B). The 15-week old mice did not have an expansion of MesLN $\gamma\delta$ T cells in response to any of the treatments. Mice that were 30-weeks old and were in the 'Asthma' and 'Worm + Asthma' groups had increased $\gamma\delta$ T cells in the MesLN. The most striking increase in MesLN $\gamma\delta$ T cells occurred in response to *H. polygyrus* infection in the 40-week old mice (Fig 6C).

3.5. Humoral immune responses against helminth and allergen exposures were altered by age

Since both helminth infections and allergens can activate humoral immune responses, we measured levels of total immunoglobulins E, G₁, and A in the serum and mucosal fluids in the airways and small intestine. IgG₁ was the most abundant isotype in all niches tested, followed by IgA (Fig 7). Serum IgE was increased in 'Worm' and 'Asthma' alone and levels were significantly elevated in the 'Worm + Asthma' group (Fig 7A). While 'Asthma' only mice at 40-weeks did not have elevated serum IgE, this antibody response was similar to that of the younger age groups for 'Worm' and 'Worm + Asthma' (Fig 7A).

IgE concentration in the BALF was elevated in animals that were allergen exposed with an age-associated reduction in the 'Asthma' treatment condition (Fig 7B). An increase in IgE in the ILF was only evident in the 'Worm' and 'Worm + Asthma' at 30-weeks (Fig 7C).

All treated mice at 15-weeks had significantly elevated IgG₁ in the serum compared to naïve controls (Fig 7D). At 30-weeks, none of the groups had elevated IgG₁ in the serum whereas by 40-weeks, a significant increase in serum IgG₁ occurred in the 'Worm' and 'Worm + Asthma' groups (Fig 7D). There was an increase in IgG₁ in the BALF of 'Worm + Asthma' groups at 15- and 40-weeks (Fig 7E), while this increase was only evident at 30-weeks in this group in the ILF (Fig 7F). The downregulation of IgG₁ in the serum and BALF in the 30-week group correlated with their lack of inflammation in the LNs.

Serum IgA levels only increased over basal expression in 'Worm' and 'Worm + Asthma' groups at 30-weeks (Fig 7G). 'Worm + Asthma' groups had elevated IgA levels in the BALF at all age groups tested, whereas this was only evident in the 'Asthma' group at 15- and 40-weeks (Fig 7H). IgA concentration in the ILF followed a similar trend to as levels in the serum (Fig 7I).

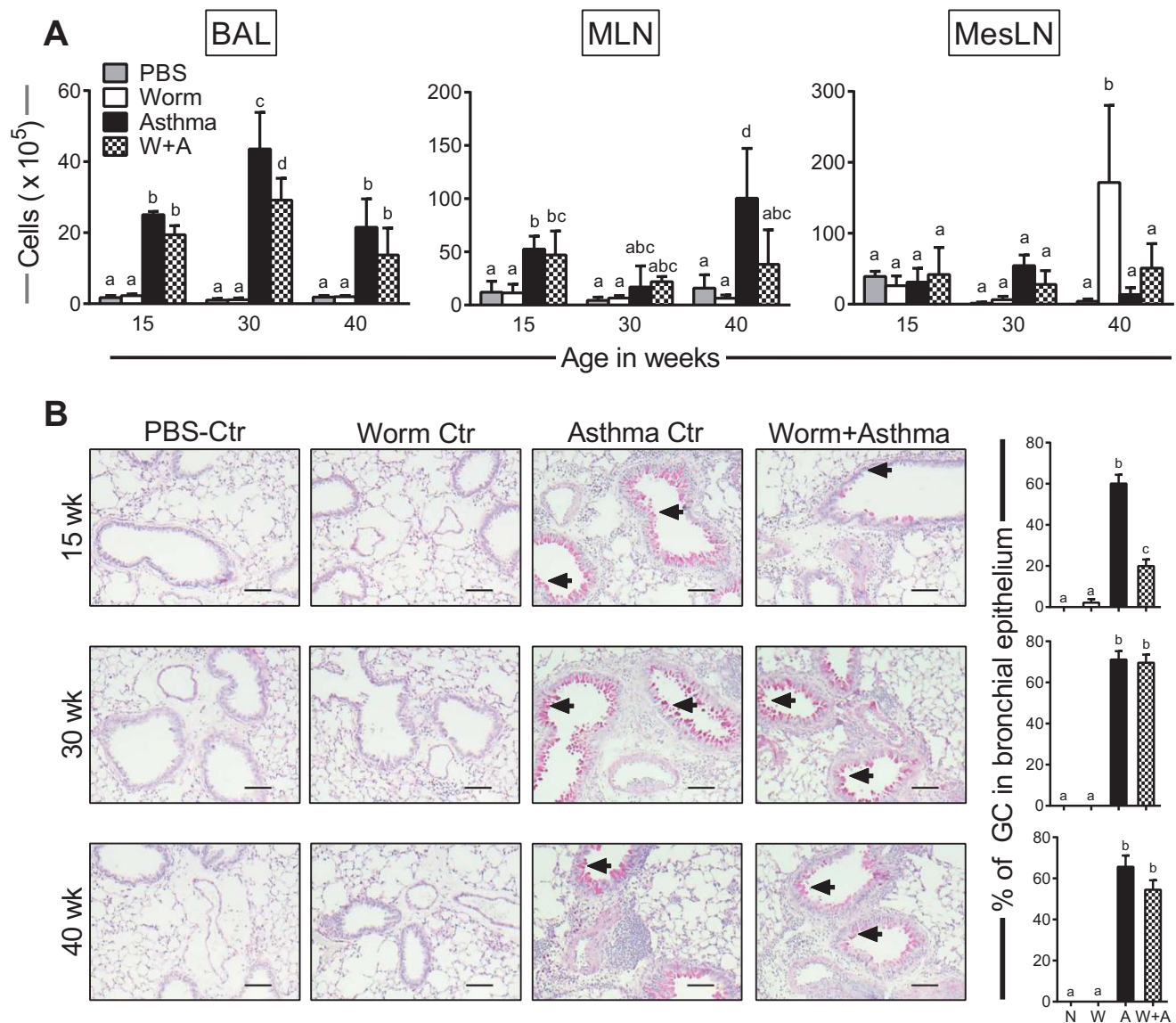


Fig. 3. Pre-existing helminth infection affected inflammation and goblet cells in lungs of allergic mice. Leukocytes in the bronchoalveolar lavage (BAL) content, mediastinal lymph nodes (MLN), and mesenteric lymph node (MesLN) were enumerated. Increased cell influx was most prominent in the BAL after fungal exposure and pre-existing helminth infection appeared to reduce this inflammation especially in the 30-week group. Interestingly, the 30-week group had the least change in MLN cell number. Cells in the MesLN increased after helminth infection, but this only occurred in the 40-week group. GCs in the airways (arrows) were prominent in all age groups after allergen exposure, but more inflammatory foci were visible in the 15- and 40-week groups compared to the 30-week group. A reduction in inflammation and GCs in the airways occurred in the 15- and 40-week age animals pre-infected with *H. polygyrus* prior to the *A. fumigatus* sensitization and challenge. Data represented as the mean and standard deviation and are representative of one of duplicate studies. Data from experimental groups were compared to one another within each age group and experimental groups were compared between age groups with two-way ANOVA with Tukey's multiple comparisons test. Goblet cell percentage data from experimental groups in each age group were compared using one-way ANOVA with Dunn's multiple comparisons test. $P < 0.05$ was considered significant when letters above bars are different. Scale bars = 100 μ m.

3.6. Allergen-specific antibody titers varied by age and helminth infection

We measured antibodies generated against the sensitizing antigens to determine age-associated changes that may occur in the humoral response because of the changes noted in the total antibody content (Fig 7). As expected, the 'Naïve' and 'Worm' control mice did not have *A. fumigatus*-specific antibodies (Fig 8). All isotypes tested were in abundance in the serum of allergen exposed groups with IgG₁ taking precedence in the 15- and 30-week old mice (Fig 8). Allergen-specific IgE was not significantly increased in the BALF of 40-week old mice (Fig 8) in spite of the total IgE present (Fig 7B). As in the serum, *A. fumigatus*-specific IgG₁ was abundant in the 15- and 30-week old mouse BALF while allergen-specific IgA was available in high titers in all age groups (Fig 8). *A. fumigatus*-specific antibodies were limited in the ILF with low availability of IgA in select groups (Fig 8).

4. Discussion

Epidemiologic evidence suggesting an inverse correlation between helminthic infections and immune-mediated diseases have led to the exploration of gut parasites and/or their components (secreted/excreted/surface) as therapeutics against diseases like allergy and inflammatory bowel disease [17]. Previous studies have established that gut parasites are able to alleviate disease severity in mouse models of colitis, asthma, and arthritis [13,27,28] through activation of regulatory cells and cytokines [12,13,29] or by skewing immune responses towards a T_H2 phenotype [27,30,31]. Reproducible benefits in mouse models led to the successful clinical use of parasites to treat immune-mediated gut diseases [14,15,32]. The hygiene hypothesis proposes that pathogen (in this case, parasite) exposure early in development is necessary for appropriate immune programming to prevent the immune

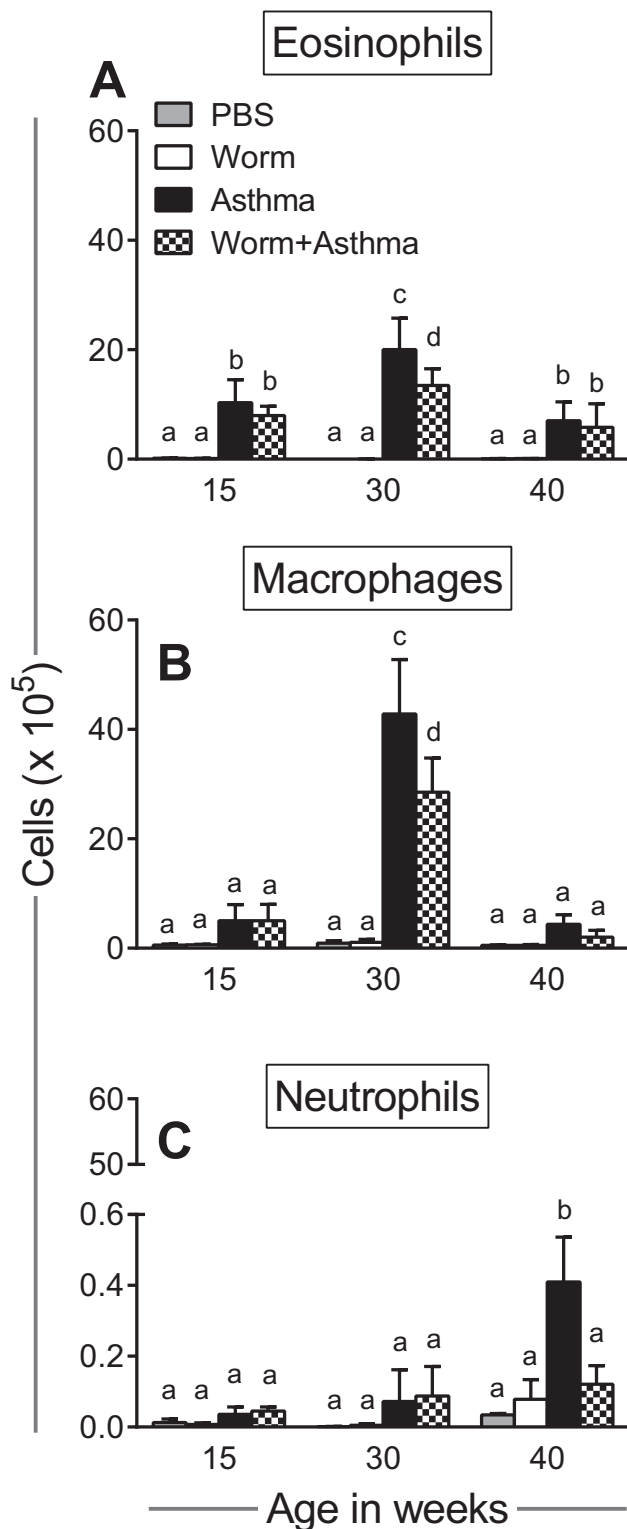


Fig. 4. Inflammatory cells were increased in the airways as a result of allergen exposure. Cells in the bronchoalveolar lavage were analyzed by flow cytometry. Eosinophils were found in the allergen exposed groups with highest numbers in the 30-week age group. Macrophages were also highest in this age group. While neutrophils were very limited in the airways compared to eosinophils and macrophages, they were highest in the 40-week age group. Eosinophils and macrophages were significantly reduced in mice that were infected with *H. polygyrus* prior to allergen sensitization and challenge in the 30-week group while the difference in the neutrophil population was significant between these experimental conditions in the 40-week group. Data from experimental groups were compared to one another within each age group and experimental groups were compared between age groups with two-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered significant when letters above bars are different.

activation against innocuous agents later in life [33]. However, animal studies investigating the impact of gut parasite infection on asthma are typically performed using mice between six and eight weeks of age which generally corresponds to teenage years in humans [34]. Therefore, we investigated the possible impact that age may have on the hygiene hypothesis in the context of asthma. Our model system failed to show a complete reduction in hallmarks of allergic airways disease contrary to previous studies but in support of others [35,36]. However, we did note that age-related differences occurred in the overall immune responses mediated by allergen in worm-infected mice. Our experimental model had several differences from previous models in addition to the age groups, including high *H. polygyrus* larvae content for infection and the *Aspergillus fumigatus* model of allergic asthma which utilizes live fungal conidia exposure by inhalation challenge resulting in robust immune responses and structural changes in the lungs [23,24].

Both the lungs and the gut arise from the embryonic foregut and differentiate into organ systems that serve separate physiologic functions. Gnotobiotic mice have aberrant immune development [37,38] highlighting the importance of the gut microbiome in immune cell development/programming, which may be especially important at younger ages. Since *H. polygyrus* remains solely in the gut, this parasite may function in a similar manner to gut dwelling bacteria wherein a high antigen load *in situ* promotes the function of residing and draining immune cells. Recently, Zaiss et al., showed that *H. polygyrus* infection alters the gut microbiome which in turn prevents the development of allergic asthma [39]. While information on *H. polygyrus* egg burden was not measured in other mouse models of lung allergy [12,13,40], we found that worm-infected mice continued to shed eggs throughout the course of the study despite immune modulations that occurred in response to fungal allergy. While immune responses were generally similar between the youngest and oldest age groups, the highest egg burden occurred in the youngest age group with an increase occurring at first allergen sensitization. Therefore, parasite infection at weaning age may promote parasite tolerance/ignorance, whereas the gut milieu after immune maturation at later ages may be more adept at controlling parasite burden. The similarity in egg burden between allergic and non-allergic mice suggests that immune responses associated with pulmonary allergy do not influence gut immune responses to parasites.

Allergic asthma is considered to be a T_H2 -skewed disease with eosinophilic inflammation, mucus production, and IgE-dominant humoral immunity. Immune responses to parasite infections are also T_H2 skewed as parasite expulsion depends on IgE-mediated activation of granulocytes [41]. Therefore, the concept that parasite infections can benefit allergic asthma is enigmatic. Yet, this hypothesis has been clearly supported in mouse studies [12,13,40,42]. We only noted a parasite-induced reduction in airway inflammation and eosinophilia in mice infected with *H. polygyrus* larvae at 18 weeks of age, but not in other age groups. However, reduced pulmonary tissue inflammation and GC metaplasia were noted in mice that were infected at younger and older ages. Regulatory $CD4^+$ T cells increased in response to allergen mostly in the airways and were unaffected by worm infection, while worm infection promoted IgE levels in the serum. Our study differed from previous mouse models in the selected age groups, the dose of parasite, the choice and model of allergen exposure, and the time point that was studied. Specifically, we infected mice at three different age groups with more larvae than other studies, we subjected mice to a clinically relevant fungal allergen that induces robust allergic inflammation and airway wall remodeling events [23,24], and we harvested tissue for analysis one week after the allergen challenge. Therefore, it is possible that while gut parasites may be able to negate/control the development of immune responses against allergens, this ability may be dependent on immune maturity and the robustness of the allergen.

The complex molecular interactions that occur in a parasite-infected host together with the age at which exposure occurs, may contribute to the immune-programming and pathophysiology of disease. Allergic

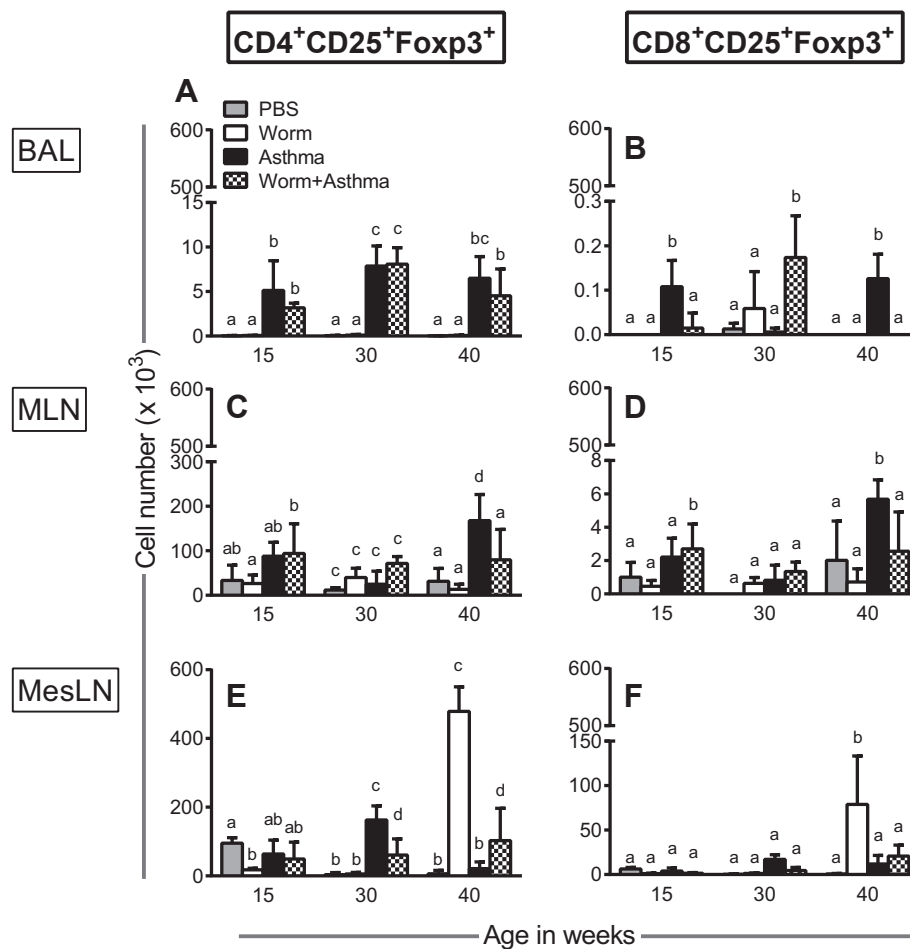


Fig. 5. Regulatory T (T_{reg}) cells were increased as a result of treatment conditions. Treatment conditions resulted in the presence of more CD4⁺ T_{reg} cells in all niches tested compared to CD8⁺ T_{reg} cells. Allergen exposure resulted in an increase in CD4⁺ T_{reg} cells in the bronchoalveolar lavage (BAL) in all age groups (A), while allergen-induced changes in the CD8⁺ T_{reg} cells differed by age group (B). The trends in both T_{reg} populations in the mediastinal lymph nodes (MLN) were similar for all age groups (C & D). A marked increase in CD4⁺ and CD8⁺ T_{reg} cells in the mesenteric lymph nodes (MesLN) occurred in response to worm infection in the oldest age group (E & F). Data from experimental groups were compared to one another within each age group and experimental groups were compared between age groups with two-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered significant when letters above bars are different.

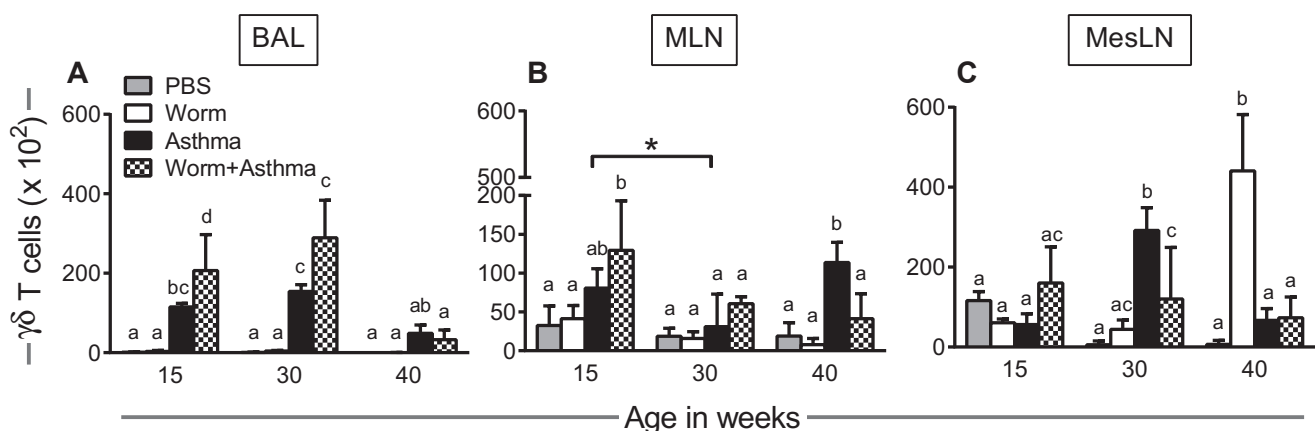


Fig. 6. $\gamma\delta$ T cells were increased in the airways and lymphoid organs after treatment. Bronchoalveolar lavage (BAL) contained $\gamma\delta$ T cells after allergen exposure, although pre-existing helminth infection resulted in a further increase in these cells in the 15- and 30-week age groups. There was no change in $\gamma\delta$ T cells in the BAL of 40-week mice as a result of helminth or allergen exposure. The number of $\gamma\delta$ T cells in the mediastinal lymph nodes (MLN) increased significantly in the allergen exposed mice at 40-weeks but were unaltered in the other age groups. Allergen exposed mice had significantly elevated $\gamma\delta$ T cells in the mesenteric lymph node (MesLN) at 30-weeks but not at other time points. The number of $\gamma\delta$ T cells in the helminth-infected mouse MesLN at 40-weeks was significantly higher than any other group or niche. Data from experimental groups were compared to one another within each age group and experimental groups were compared between age groups with two-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered significant when letters above bars are different and as marked by asterisk.

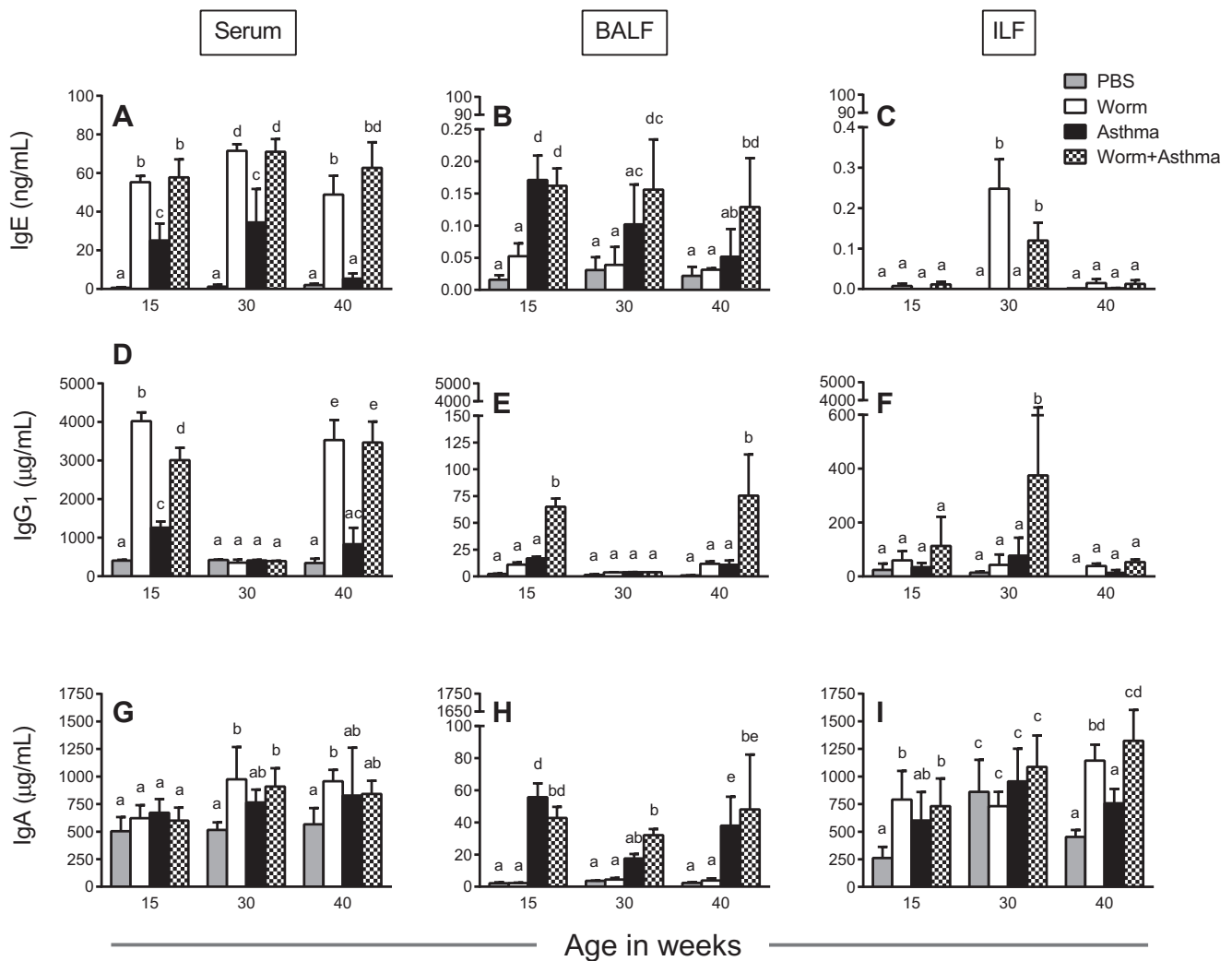


Fig. 7. Total antibodies were differentially regulated by experimental condition and age. Total levels of immunoglobulins E, G₁, and A were measured in the serum, bronchoalveolar lavage fluid (BALF), and intestinal lavage fluid (ILF). Antibodies were most abundant in the serum and least abundant in the BALF. IgE was increased as a result of helminth or allergen exposure at varying levels in all three fluids tested. IgG₁ were prominent in the serum while IgA was abundant in the serum and ILF. There were age-associated differences in all three antibodies and helminth infection appeared to regulate the availability of these antibodies. Data from experimental groups were compared to one another within each age group and experimental groups were compared between age groups with two-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered significant when letters above bars are different.

rhinitis is a disease of the upper respiratory tract that affects more people globally than asthma. Its pathophysiology includes inflammation and mucus production, and can progress into nasal polyposis [43]. Like asthma, allergic rhinitis prevalence increases with age until adulthood prior to decreasing in the older population [44]. Similar to asthma, the use of parasites as therapeutics for rhinitis has been considered [45], although not successfully [45]. Since allergen sensitization is a prerequisite for these allergic diseases, the impact of age on allergen sensitization is an important consideration. While allergen sensitization has been shown to be inversely proportional to age [44], parasites can reduce allergen sensitivity in asthmatics [46]. Some parasites that appear to worsen asthma may have antigens that mimic allergens thereby adding to the sensitization process [47]. We noted transient increases in the worm egg burden at initial allergen sensitization and challenge in the youngest age group of mice that had reduced overall inflammation and GC numbers compared to the middle age group. Therefore, host age, the time that lapses between parasite infection and initial allergen encounter, and the sensitizing allergens may all contribute to the outcome of the resulting allergic disease.

Airway inflammation in response to allergen also appears to vary by age. We propose that worm-mediated reduction in asthma severity may

not be universal for all antigens and all ages. In young mice, worm infection clearly reduced GC metaplasia. Since we noted the highest egg burden in this age group, this reduction may be an effect of secreted molecules by worms that may be resistant to asthma pathogenesis [48,49]. The fact that improved lung tissue pathology in this age group was not associated with reduction in eosinophil number needs further exploration although tissue pathology may not be solely dependent on eosinophils. Helminth products could interfere TLR-mediated inflammation in asthma [49], a mechanism that may be crucial in disease pathogenesis with our fungal asthma model. An alternative mechanism which may explain improved lung pathology in young mice is that worm-infected asthmatic mice had a higher number of $\gamma\delta$ T cells in the BAL and MLNs, than allergic mice alone, and $\gamma\delta$ T cells can be protective against airway hyperresponsiveness [50], and $\gamma\delta$ T cells in lung tissue can produce IL-17, which suppresses airway inflammation [51]. However, 15-week old mice in the 'Worm + Asthma' group had increased airway hyperresponsiveness (data not shown) in spite of there being significantly greater numbers of $\gamma\delta$ T cells in the airways. Others have shown that although allergen sensitivity can be reduced in helminth infected asthmatics, pulmonary function remains equivalent to un-infected asthmatics [46]. As such, mechanisms that lead to airway

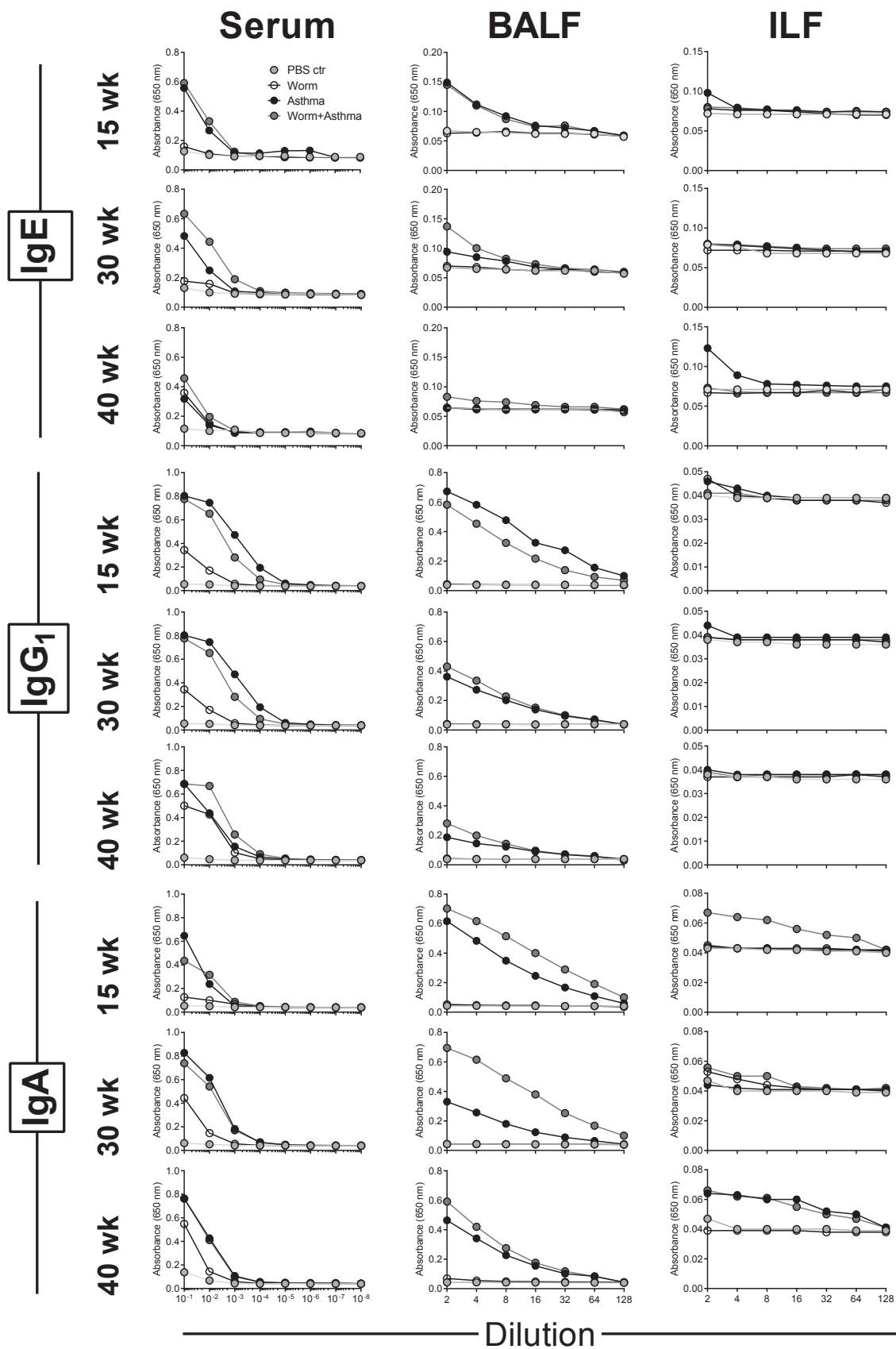


Fig. 8. *A. fumigatus*-specific antibodies are regulated in parasite infection. Immunoglobulins E, G₁, and A that had specificity to the allergen were measured in the serum, bronchoalveolar lavage fluid (BALF), and intestinal lavage fluid (ILF). The highest amount of *A. fumigatus*-specific antibody titers was found in the serum while the lowest was in the ILF. There was an age-associated impact in allergen-specific IgE content while IgG₁ was equivalent between the age groups tested. The highest *A. fumigatus*-specific IgA content occurred in the BALF with higher titers in the 'Worm + Asthma' group compared to 'Asthma' alone at 30-weeks. Data are represented as the mean for each group at each dilution.

physiologic responses are unlikely to result from a single mediator. Worm infection also reduced airway GC metaplasia in older mice to some extent, though this was not as robust as in the young group. Since the egg burden was similar in this group compared to the 30-week age group, worm burden could not explain this discrepancy. We did note neutrophil influx in the airways of the allergic 40-week old mice which was unique to this age group. Severe asthma can be associated with neutrophils [52], and it is possible that severe asthma in older mice has a mixed granulocyte content as noted here. Since these cells have been proven to induce airway GC metaplasia [53], blunting neutrophil infiltration may help improve asthma severity in this age group.

In spite of changes induced by the presence of gut parasites, in our model, worms were unable to mitigate asthma development. Although we did note a significant reduction in airway eosinophilia in 30-week old mice infected with *H. polygyrus*, there was no noted improvement in lung inflammation or GC metaplasia in these animals that also had increased $\gamma\delta$ T cells and serum IgE. Interestingly, this age group also had the greatest influx in airway macrophages which remained higher compared to the other age groups even with the reduction induced by worm infection. Therefore, the mechanism by which macrophages contribute to fungal asthma pathology based on age and how these responses may be modulated by gut parasites is worthy of investigation.

Immune responses to helminths are dictated by the type, timing, and intensity of parasite infection and host genetic factors [54]. Epidemiologic studies showing geohelminth infections have no effect or increase allergy [11,36,55] in contrast to those that show clear benefits [56–58] underscore the intricacies between the parasite, host, and environment that may contribute to disease outcome. Our findings lend themselves to the understanding of dynamics of immune regulation between the gut and the lung with antigen sources that are restricted to each organ. It may be important to consider the age of the host and the type of sensitizing allergen when developing helminth-based therapeutics for asthma.

Conflict of Interest statement

The authors have no conflicts of interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2017.04.009>.

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Supplementary Table 1. Fluorescence-tagged antibodies utilized for cell identification by FACS.

Antibodies	Clone	Manufacturer
APC-CD193	83103	BD Biosciences
V450-Ly6G	1A8	BD Biosciences
PE-Mac3	ebioABL-93	BD Biosciences
FITC-CD25	7D4	Pharmingen
eFluor450-CD8	eBioH35-17.2 (H35-17.2)	eBioscience
APC- $\gamma\delta$ TCR	eBioGL3 (GL-3, GL3)	eBioscience
PE-FoxP3	NRRF-30	eBioscience
PECy7-CD4	GK1.5	eBioscience
eFluor780-CD3	17A2	eBioscience

Supplementary Table 2. Gating strategies used to identify cell populations by FACS.

Cells	Gating
Neutrophil	SSC ^{hi} CD193 ⁻ Mac3 ⁻ Ly6G ⁺
Eosinophil	SSC ^{hi} CD193 ⁺
Macrophage	FSC ^{hi} CD193 ⁻ Ly6G ⁻ Mac3 ⁺
CD4 T cell	CD3 ⁺ CD4 ⁺
CD8 T cell	CD3 ⁺ CD8 ⁺
$\gamma\delta$ T cell	CD3 ⁺ $\gamma\delta$ TCR ⁺
Regulatory T cell	CD4 ⁺ CD25 ⁺ FoxP3 ⁺