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Intestinal Helminths Protect in a Murine Model of Asthma[1]

Kunihiko Kitagaki, Thomas R. Businga, Doina Racila, David E. Elliott, Joel V. Weinstock,[2] and Joel N. Kline[3]

**Underdeveloped nations are relatively protected from the worldwide asthma epidemic; the hygiene hypothesis suggests this is due to suppression of Th2-mediated inflammation by increased exposure to pathogens and their products. Although microbial exposures can promote Th2-suppressing Th1 responses, even Th2-skewing infections, such as helminths, appear to suppress atopy, suggesting an alternate explanation for these observations. To investigate whether induction of regulatory responses by helminths may counter allergic inflammation, we examined the effects of helminth infection in a murine model of atopic asthma. We chose *Heligosomoides polygyrus*, a gastrointestinal nematode, as the experimental helminth; this worm does not enter the lung in its life cycle. We found that *H. polygyrus* infection suppressed allergen-induced airway eosinophilia, bronchial hyperreactivity, and in vitro allergen-recall Th2 responses in an IL-10-dependent manner; total and OVA-specific IgE, however, were increased by worm infection. Finally, helminth-infected mice were protected against eosinophilic inflammation induced by adoptive transfer of OVA-stimulated CD4$^+$ cells, and transfer of cells from helminth-infected/OVA-exposed mice suppressed OVA-induced eosinophilic inflammation, suggesting a role for regulatory cells. Increased CD4$^+$CD25$^+$Foxp3$^+$ cells were found in thoracic lymph nodes of helminth-infected/OVA-exposed mice. Helminthic colonization appears to protect against asthma and atopic disorders; the regulatory cytokine, IL-10, may be a critical player. *The Journal of Immunology, 2006, 177: 1628–1635.*

C haracteristic features of asthma include eosinophilic airway inflammation and airway hyperresponsiveness, both orchestrated by Th2 cytokines (1). Numerous studies show a substantial increase in the prevalence and morbidity of asthma and atopic disorders over the past few decades, especially in Westernized countries (2). Interestingly, as developing countries become more urbanized, and as populations migrate to Western nations, their asthma prevalence increases. In addition, within countries, asthma rates are often lowest among children raised on farms (3, 4). These data suggest that exposure to an extremely hygienic environment may increase the risk of children to develop atopic disorders such as asthma later in their life, which has been codified as the hygiene hypothesis (5). This hypothesis is supported by reduced atopic disorders among children with increased numbers of older siblings (6), those attending day care from a very early age (7), and those subjected to fecal-oral infections, such as salmonellosis, during infancy (8).

Mechanistic explanations for the hygiene hypothesis have focused on the role of microbes and microbial products on suppression of proatopic immune responses. In general, bacterial and viral infections promote protective Th1 cytokines; as Th2 and Th1 responses are counterregulatory (9), reduced Th1 could promote development of Th2 responses to Ags in the environment. Deviation toward Th1 responses by exposure to microbial products does not,

however, adequately explain all aspects of the hygiene hypothesis. Importantly, the prevalence of type I diabetes (10), inflammatory bowel disease (11), and multiple sclerosis (12), all Th1-mediated diseases, have increased in concert with allergic disorders. Finally, a consistent negative relation between helminth infections and the prevalence of atopic responses has been seen (13–17), despite the fact that helminth infections strongly induce Th2-type responses, including high levels of IgE, and circulating eosinophils and mast cells (18). The induction of regulatory responses, characterized by IL-10-releasing lymphocytes, has been speculatively linked to immune modulation by helminths in a number of disease models (19–21).

Intestinal helminths that infect humans include roundworms (nematodes) and flatworms (cestodes): these are a diverse group of multicellular organisms that often use intermediate hosts during larval stages and a mammalian host for adult worms. Unlike other nematodes, such as hookworms and *Ascaris*, whipworms (*Trichuris*) do not require a pulmonary phase in their life cycle, nor is an intermediate host required: eggs are passed into the intestinal lumen, and fecal contamination of food allows ingestion of the infectious eggs and a new cycle of infection. *Heligosomoides polygyrus*, a naturally occurring murine nematode, is similarly restricted to the intestines of its host. Its infective stage is the third-stage larva (L3)[4] that enters the muscular layer of the proximal small intestine within 24–48 h of ingestion (22); within 7–10 days, adult worms exit into the gut lumen, residing in the proximal small intestine and living in reasonable harmony with its host until being expelled (23).

In this study, we examined the effect of infection with intestinal helminths on manifestations of a murine model of asthma, to determine whether these unhygienic influences can suppress the development of atopic responses. We hypothesized that such protection would be characterized by induction of regulatory responses. We chose the organism *H. polygyrus*, a parasite that does not enter

[4] Abbreviations used in this paper: L3, third-stage larva; BAL, bronchoalveolar lavage.

the lung during its life cycle and yet induces systemic inflammatory responses. We found that helminthic colonization prevents the subsequent development of allergen-induced eosinophilic airway inflammation and allergen-specific Th2 cytokine production, despite promoting overall increases in the general Th2 inflammatory milieu. These effects are dependent on the regulatory cytokine, IL-10.

Materials and Methods

Animals

Six-week-old female BALB/c mice were obtained from The Jackson Laboratory. Mice on a BALB/c genetic background with a disrupted gene for IL-10 were a gift from D. Berg, University of Iowa (Iowa City, IA) (24). All protocols were reviewed and approved by the University of Iowa Animal Care and Use Committee; all animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed.

Parasites

Infective, ensheathed L3 of *H. polygyrus* (U.S. National Helminthological Collection no. 81930) were propagated and obtained by the modified Baermann method and stored at 4°C until used.

Murine models of asthma and H. polygyrus infection

Helminth infection and atopic asthma model. Mice were infected with 200 L3 of *H. polygyrus* in 200 μl of saline by gastric gavage on day -14 (*H. polygyrus*-colonized mice) or equal volumes of saline alone (noncolonized mice). All mice were sensitized to OVA (Sigma-Aldrich; 10 μg with 1 mg of alum, days 0 and 7). Mice were challenged with aerosolized OVA (1% solution, 30 min) or saline (sham challenge) on days 14 and 16, and sacrificed on day 18. Mice were grouped according to treatment: control, sham-challenged mice; worm, *H. polygyrus*-colonized/sham-challenged mice; OVA, OVA-challenged mice; worm/OVA, *H. polygyrus*-colonized/OVA-challenged mice (Fig. 1*A*).

Modulation of eosinophilic airway inflammation induced by transfer of OVA-activated CD4$^+$ cells

Donor BALB/c mice were sensitized by OVA/alum and challenged with aerosolized OVA, as above. At the time of sacrifice (day 18), splenocytes were isolated and stimulated in vitro with OVA (100 μg/ml) for 72 h, after which CD4$^+$ cells were separated using anti-mouse CD4 mAb (clone GK1.5; Miltenyi Biotec) in a magnetic cell sorting system (Miltenyi Biotec). Recipient BALB/c mice were inoculated with 200 L3 of *H. polygyrus* or saline on day -14, as above. The CD4$^+$ cells (5 $\times$ 10^6/mouse) isolated from donor mice were transferred to the recipients by tail vein injection on day 0. All recipient mice were challenged with inhaled OVA (1% solution, 30 min/day) on days 0–2, then sacrificed on day 4 (Fig. 1*B*).

Adoptive transfer of splenocytes before allergen inhalation challenge

Donor mice (control, worm, OVA, worm/OVA) were prepared, as described above; splenocytes were isolated on day 18 of the protocol, and 1 $\times$ 10^7 splenocytes/mouse were transferred to OVA-sensitized recipient mice on day 13 of the protocol; all mice were then challenged with inhaled OVA and sacrificed, as above (Fig. 1*C*).

General murine treatment

At the time of euthanasia, the trachea was cannulated, and saline washings, consisting of a total of 3.0 ml of 0.9% NaCl delivered by gravity feed in three aliquots, were collected; these bronchoalveolar lavages (BAL) were processed for BAL fluid (frozen for subsequent analysis) and total and differential cell counts (using Diff-Quik staining). Differential cell counts were performed twice by independent investigators who were blind to the grouping of the mice. Lungs of representative mice from each group were Formalin fixed, and sections were stained with H&E stain for examination by light microscopy. Phlebotomy was performed, and serum was isolated.

Airway hyperresponsiveness

Some mice were evaluated for nonspecific airway hyperreactivity to inhaled methacholine. Airway physiology was assessed using a computer-controlled small-animal ventilator (Flexivent; Scireq) (25). For these studies, 48 h after the final OVA inhalation, mice were anesthetized with

xylazine (12.5 mg/kg) and ketamine (87.5 mg/kg), and their trachea cannulated using a 20-gauge angiocath connected to the ventilator. After ventilation (at a rate of 150 breaths/min and a tidal volume of 10 ml/kg, with passive exhalation through a water trap maintained at a positive end-expiratory pressure of 2–3 cm H_2O) was initiated, mice were paralyzed with pancuronium (1 mg/kg). The Flexivent system measures respiratory system impedance through application of a sinusoidal perturbation in inspiratory airflow. Measurements of ventilator piston position and airway pressure are used in a constant phase model to produce an index of resistance in the major conducting airways (Newtonian resistance). This forced oscillation technique correlates well with direct measures of airway resistance (26, 27). Mice were exposed to increasing concentrations of aerosolized methacholine (0, 12.5, 25, 50 mg/ml), delivered in-line, and Newtonian resistance was measured at each dose level, allowing for determination of the methacholine concentration that doubles the airway resistance (methacholine PC_{200}).

In vitro assays

Cell culture. Single-cell suspensions of splenocytes (5 $\times$ 10^6 cells/ml/well) were plated in 24-well tissue culture plates in RPMI 1640 supplemented with 2 mM glutamine, 25 mM HEPES, 10% heat-inactivated FCS, 50 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cultured alone or with OVA (100 μg/ml) or Con A (5 μg/ml; Sigma-Aldrich) at 37°C in an atmosphere of 5% CO_2 in air. The supernatants were harvested after 72 h of culture and stored at -70°C until the measurement of cytokines.

ELISA. Murine IL-5, IFN-γ, IL-10, total IgE, and total IgG2a were measured by ELISA (OptEIA; BD Biosciences), according to the manufacturer's instructions. Serum levels of OVA-specific IgE and IgG2a were measured by ELISA in which plates were coated with OVA, and biotinylated rat anti-mouse IgE (clone R35-118) or anti-mouse IgG2a (clone R19-15, which recognizes an epitope in the C_H3 domain of mouse IgG, with strong reactivity to the *Igh-1a* allotype) were the detection Abs (BD Biosciences). Units were OD readings at 405 nm.

Flow cytometry. Single-cell suspensions were prepared from lung, spleen, and thoracic lymph nodes, and the cells were washed and suspended in a buffer containing 3% FBS and 0.1% sodium azide in PBS and FcR Ab (FcR block; BD Pharmingen); all staining was conducted in the dark at 4°C. Cells were phenotyped by expression of surface markers (CD4, CD25) and intracellular staining (Foxp3). Cells were incubated with fluorescent probe-conjugated mAb (PerCP-CD4, RM4-5; PE-CD25, PC61; BD Pharmingen). For Foxp3 detection, cells were permeabilized and stained with FITC-Foxp3, FJK-16s (eBioscience). Stained cells were washed three times, and fluorochrome intensity was measured by multiparameter flow cytometry (FACScan; BD Biosciences). Surface marker analysis was conducted, after gating for forward and side scatter, using CellQuest software.

Statistics

Statistical significance was evaluated using the program SPSS 11.0 for Windows (SPSS). All data are expressed as the mean ± SE. Statistical differences between the groups were determined by a single factor ANOVA, followed by a post hoc Tukey test. Correlation was assessed using a bivariate analysis with calculation of Spearman's coefficient. A $p <$ 0.05 was considered significant.

Results

H. polygyrus infection suppresses atopic asthma

As helminths are known to induce strong systemic eosinophilic inflammatory responses, it is counterintuitive to link infection with suppression of Th2-mediated atopic inflammation. We first examined whether enteral infection (Fig. 1*A*) with the gastrointestinal nematode, *H. polygyrus*, would modulate airway eosinophilia in a murine model of OVA-induced atopic asthma. In OVA-sensitized mice, OVA inhalation induces marked airway eosinophilia; infection with *H. polygyrus* alone did not induce airway inflammation, but in OVA-challenged mice significantly blunted airway eosinophilia (Fig. 2*A*). BAL eosinophil levels paralleled histopathologic demonstration of peribronchial airway inflammation (representative sections shown in Fig. 2, *B–E*). Airway neutrophilia (modestly increased by OVA inhalation) was also suppressed in the helminth-infected mice (data not shown).
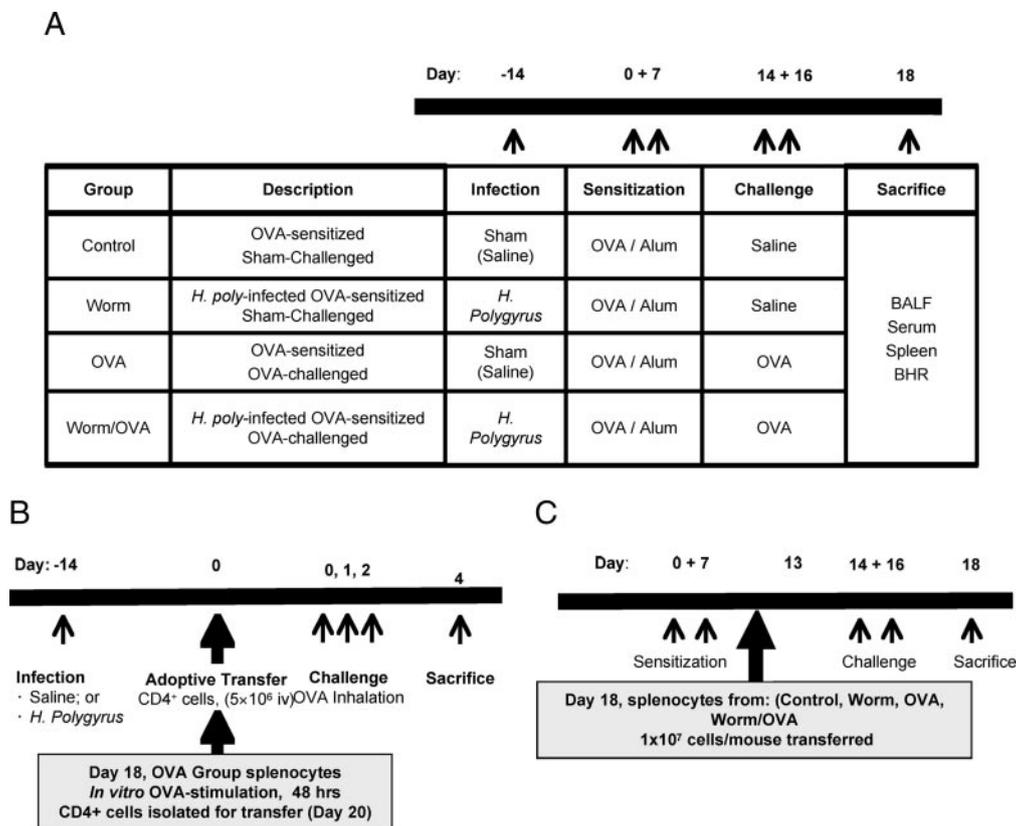
FIGURE 1. *H. polygyrus* colonization and murine models of atopic asthma. *A*, Mice were grouped and studied based on their infection with *H. polygyrus* (sham or active, by gastric gavage) and inhalation challenge with aerosolized OVA: control, worm, OVA, and worm/OVA. *B*, *H polygyrus*- and sham-infected mice underwent adoptive transfer to induce passive sensitization to OVA. CD4$^+$ lymphocytes were isolated from splenocytes of OVA-sensitized/challenged mice that were stimulated in vitro with OVA: recipient mice were then challenged with aerosolized OVA before sacrifice. *C*, Splenocytes were isolated from each of the four groups of mice, then transferred to OVA-sensitized recipient mice 1 day before inhalation challenge with OVA.

To correlate airway inflammation with the local cytokine milieu, we measured BAL fluid cytokine levels, and found that OVA-stimulated BAL IL-5 was significantly suppressed in mice infected with *H. polygyrus* (control, 20.1 ± 7.4 pg/ml; worm, 35.3 ± 7.4 pg/ml; OVA, 61.6 ± 7.0 pg/ml; worm/OVA, 35.5 ± 7.8 pg/ml; $p = 0.004$ for OVA vs control and $p = 0.038$ for OVA vs worm/OVA; $n = 8$/group in two separate experiments). BAL IL-10 and IFN-γ levels were not significantly changed.

Although eosinophilia is a critical marker of atopic inflammation, the diagnosis of asthma also requires bronchial hyperreactivity. OVA-challenged mice demonstrate significantly increased methacholine-induced airway resistance, compared with control mice (Fig. 2*F*). This increase is suppressed by infection with *H. polygyrus*, resulting in a methacholine doubling dose (PC$_{200}$) no different from control mice (methacholine PC$_{200}$ control, 56.7 ± 7.3 mg/ml; OVA, 27.5 ± 4.8 mg/ml; worm/OVA, 63.9 ± 13.9 mg/ml; $p = 0.03$, OVA vs worm/OVA group). Comparing parameters of airway inflammation and bronchial hyperreactivity, we found a nearly significant inverse correlation between BAL eosinophilia and methacholine PC$_{200}$ with a Spearman's ρ of -0.32, at a significance level of 0.10. In aggregate, these data show that enteral *H. polygyrus* infection confers protection in a murine model of atopic asthma.

*Infection with* H. polygyrus *modulates Igs*

Atopy is characterized by elevations in specific IgE, and modulation of IgG subsets is associated with successful immunotherapy; thus, we examined the effect of *H. polygyrus* infection on Ig levels, specifically Th2-associated IgE and Th1-associated IgG2a. In con-

trast to the suppression of OVA-induced eosinophilia and, as expected, we found that mice infected with the helminths demonstrated significant elevations of IgE, substantially overshadowing the increase associated with OVA inhalation in these OVA-sensitized mice (Fig. 3*A*). IgG2a, in contrast, was significantly suppressed by helminth infection (Fig. 3*B*).

Some have suggested that polyclonal induction of IgE may prevent the effect of Ag-specific IgE; in that case, we may not see differences in Ag-specific Igs between helminth-infected and uninfected atopic subjects, but these specific Igs would have reduced functional effect. Thus, we next examined the concentrations of OVA-specific IgE and IgG2a. Unexpectedly, we found that OVA-specific Igs were modulated similarly to total Ig levels, with significant induction of OVA-specific IgE (Fig. 3*A*) and suppression of OVA-specific IgG2a (Fig. 3*B*) among the mice infected with *H. polygyrus*. OVA-specific IgG1, another Th2-associated Ig, was increased both by helminth infection and by OVA inhalation, but there was no significant difference between the OVA and worm/OVA groups in this case (data not shown). Clearly, the protective effects of helminth infection in atopic asthma cannot be accounted for by modulation of the Ig response, and suppression of airway inflammation by helminth infection occurs despite the setting of increased levels of Ag-specific IgE.

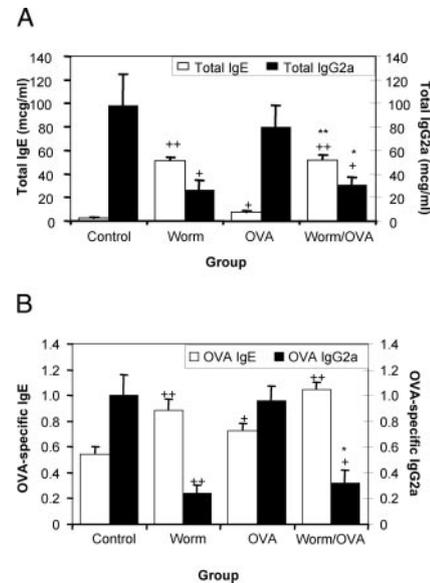H. polygyrus *infection modulates splenocyte cytokine responses*

Suppression of eosinophilia by helminth infection might reflect modulation of the systemic inflammatory milieu, such as suppression of IL-5 release. We examined both nonspecific (following stimulation with the mitogen Con A) and Ag-specific (after OVA
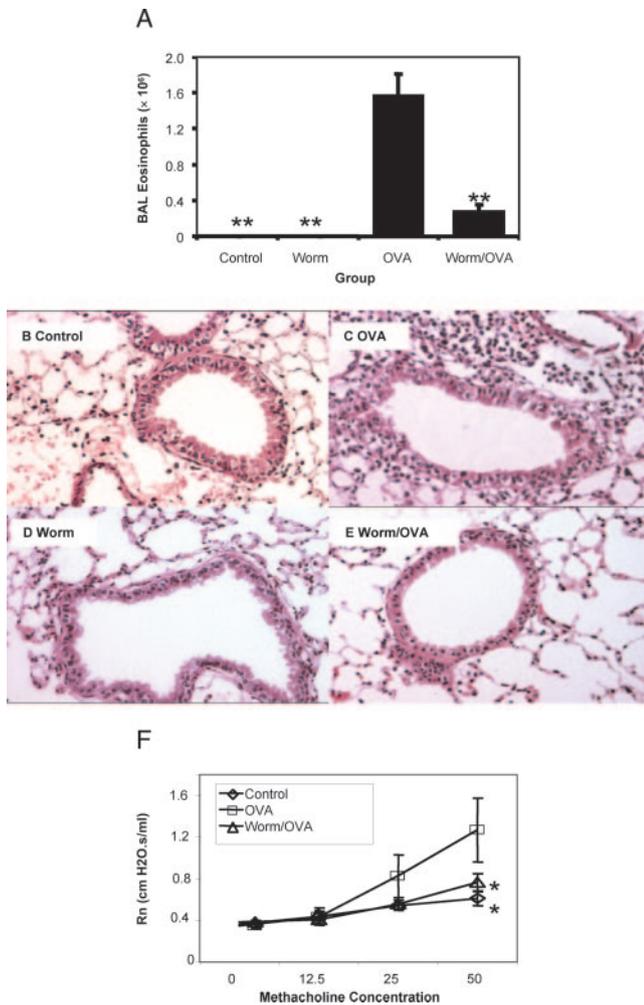
FIGURE 2. *H. polygyrus* colonization prevents the induction of OVA-induced eosinophilic airway inflammation and bronchial hyperreactivity. *A*, No BAL eosinophilia was seen in mice in the control or worm groups; marked eosinophilia induced by OVA inhalation was significantly suppressed in *H. polygyrus*-infected mice. **, $p < 0.01$ vs OVA-group mice. Each column indicates the mean and SEM of 6–10 mice in two separate experiments. *B–E*, In comparison with control mice (*B*), OVA-challenged mice (*C*) developed marked peribronchial and perivascular airway inflammation that was substantially reduced in worm/OVA mice infected with *H. polygyrus* before sensitization (*E*). Worm mice (infected with *H. polygyrus* and not subjected to OVA inhalational challenge) likewise did not demonstrate marked airway inflammation (*D*). Representative sections were stained with H&E, photographed at ×20 magnification. *F*, Bronchial hyperresponsiveness to inhaled methacholine was significantly greater in OVA-challenged mice, compared with sham-challenged control mice; bronchial hyperresponsiveness was suppressed by *H. polygyrus* infection in the worm/OVA group. *, $p < 0.05$ vs OVA-challenged mice. Each data point indicates the mean and SEM of eight mice in two separate experiments.

FIGURE 3. Modulation of Igs by *H. polygyrus* infection. *A*, Total serum IgE is modestly, but significantly increased in OVA group, in comparison with control mice (sensitized to, but not challenged with, OVA), and markedly further increased in both groups infected with *H. polygyrus* (worm and worm/OVA). Total serum IgG2a significantly suppressed in mice previously infected with *H. polygyrus*. *B*, OVA-specific IgE is significantly increased to a similar degree by OVA inhalation and by prior infection with *H. polygyrus*. OVA-specific IgG2a is significantly suppressed by prior infection with *H. polygyrus*. +, $p < 0.05$; ++, $p < 0.01$ vs control mice; *, $p < 0.05$; **, $p < 0.01$ vs OVA mice; $n = 6$–10 mice/group, in two separate experiments.

rechallenge) cytokine release by splenocytes; in the absence of stimulation, splenocytes from none of the groups consistently released detectable levels of cytokines.

Con A-induced splenocyte IL-5 release was significantly and similarly increased in helminth-infected, OVA-challenged, and helminth-infected/OVA-challenged mice, suggesting a nonspecific systemic Th2 bias induced by both the parasites and the atopic inflammation. In contrast, Ag-induced IL-5 release was not increased in the helminth-infected group, was markedly elevated in OVA-challenged mice, and was relatively suppressed (although

elevated in comparison with control mice) in the worm/OVA group, demonstrating a clear reduction in Ag-specific Th2 responses following helminth infection (Fig. 4*A*). Like IL-5, OVA-stimulated IL-4 release was not significantly increased in the helminth-infected group, markedly enhanced ($p < 0.0001$) in OVA-challenged mice, and this release was significantly suppressed in the helminth-infected/OVA-challenged mice (data not shown).

IFN-γ levels were greater following Con A stimulation than after OVA rechallenge, but the response patterns were similar: IFN-γ release by splenocytes from helminth-infected mice was no different from control mice, and release was suppressed in OVA-challenged mice, regardless of their prior exposure to helminths (Fig. 4*B*). Therefore, the increased Con A-induced IL-5 in helminth-infected mice is not due to reduced Th1 bias, nor is the reduced OVA-stimulated IL-5 release in helminth-infected/OVA-challenged mice due to a Th1 response.

Finally, we measured IL-10, a cytokine that can be associated with both Th2 and regulatory responses. We found that nonspecific stimulation with Con A increased release of IL-10 in helminth-infected and OVA-challenged mice, and IL-10 release was greatest in helminth-infected/OVA-challenged mice (Fig. 4*C*). OVA rechallenge, in contrast, induced IL-10 in OVA-challenged, but not in worm-infected mice; IL-10 release was relatively suppressed in the group of helminth-infected/OVA-challenged mice. These data, which parallel the changes in IL-5 release, are most consistent with the explanation that helminth-infected mice demonstrate reduced allergen-specific and enhanced nonspecific Th2 responses. They do not clearly point to the existence of helminth-induced regulatory mechanisms, but any such response could be obscured by the potent allergen-specific and nonspecific Th2 influence of OVA challenge and parasitic infection, respectively.
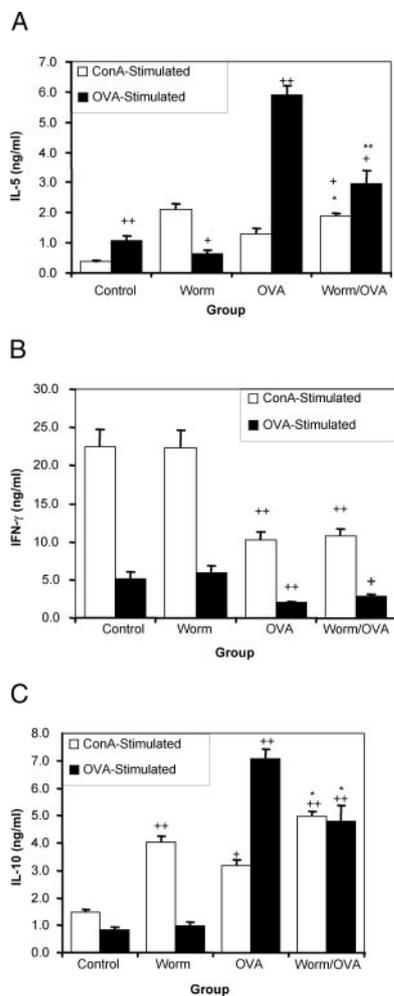
FIGURE 4. Modulation of mitogen- and allergen-stimulated cytokine responses by *H. polygyrus* infection. *A*, OVA-stimulated IL-5 is significantly enhanced in OVA group splenocytes compared with control cells; this is significantly suppressed by *H. polygyrus* infection in worm/OVA mice. Mitogen-stimulated IL-5 release is increased in parasite-infected (worm) mice as well as those challenged with OVA; IL-5 induction is not suppressed in worm/OVA group. *B*, Both OVA-stimulated and mitogen-stimulated IFN-γ release is significantly suppressed by OVA challenge; no effect of helminth infection was seen. *C*, OVA-stimulated IL-10 release is significantly increased in OVA and (to a lesser degree) OVA/worm groups. Mitogen stimulation enhances IL-10 release from splenocytes of mice from worm, OVA, and worm/OVA groups. +, $p < 0.05$; ++, $p < 0.01$ vs control mice; ∗, $p < 0.05$; ∗∗, $p < 0.01$ vs OVA-challenged mice; $n = 8$–10 mice/group, in two separate experiments.

IL-10 is critical to the protective effects of H. polygyrus

Despite the inconclusive in vitro cytokine data, we chose to conclusively evaluate the importance of IL-10 in the protective effect of the helminth infection by subjecting *H. polygyrus*-infected and noninfected IL-10$^{-/-}$ mice to the atopic asthma protocol. Unlike wild-type mice, mice lacking the ability to produce IL-10 demonstrated no suppression of airway eosinophilia in response to helminth infection (Fig. 5*A*), nor any reduction in airway hyperreactivity (data not shown). In vitro OVA rechallenge of IL-10$^{-/-}$ splenocytes induced IL-5 release that was not reduced in the helminth-infected group (Fig. 5*B*); IFN-γ responses resembled those of wild-type BALB mice, with the exception that OVA alone did not suppress levels of this Th1 cytokine (Fig. 5*C*). Ig responses were similar in wild-type and IL-10$^{-/-}$ mice, with enhancement of IgE in worm-infected mice (data not shown).
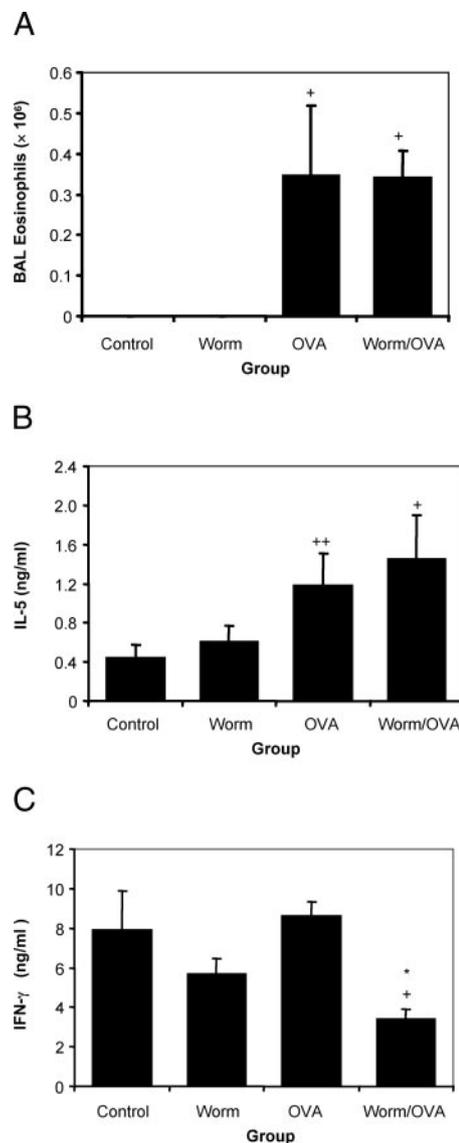
FIGURE 5. Suppression of atopic responses by *H. polygyrus* requires IL-10. *A*, OVA-induced BAL eosinophilia in IL-10$^{-/-}$ mice challenged is not suppressed in worm/OVA mice previously infected with *H. polygyrus*. *B*, OVA-rechallenged splenocytes from OVA group IL-10$^{-/-}$ mice release significantly more IL-5 than do splenocytes from control mice. IL-5 release is not suppressed by infection with *H. polygyrus*. *C*, OVA-rechallenged splenocytes from OVA group IL-10$^{-/-}$ mice do not demonstrate suppression of IFN-γ release, but those from the worm/OVA group do. +, $p < 0.05$; ++, $p < 0.01$ vs control mice; $n = 8$ mice/group, in two separate experiments.

H. polygyrus *infection induces regulatory mechanisms*

The requirement for IL-10 in the protection against atopic asthma provided by enteric parasitic infection increases the likelihood that the helminths act through induction of a population of regulatory cells. First asking whether increased regulatory T cells could be identified in worm-infected mice, we evaluated cells isolated from spleen, lung parenchyma, and thoracic lymph nodes for CD4$^+$CD25$^+$ Foxp3$^+$ cells. We found that expression of Foxp3 by CD4$^+$ cells was increased (Fig. 6*A*), as was the percentage of CD4$^+$CD25$^+$ cells that expressed Foxp3 (Fig. 6*B*), among cells isolated from thoracic lymph nodes: this pattern was not seen in cells isolated from the lung or from the spleen.
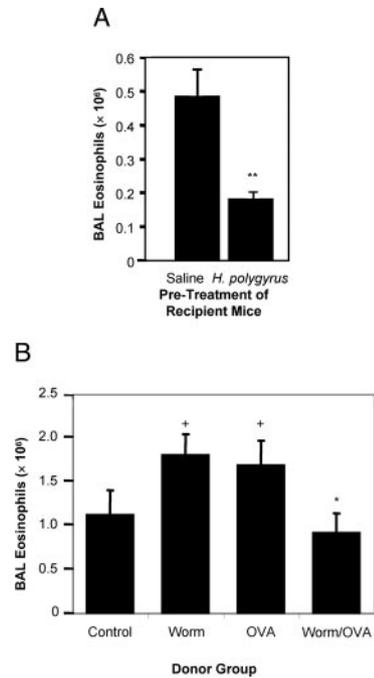
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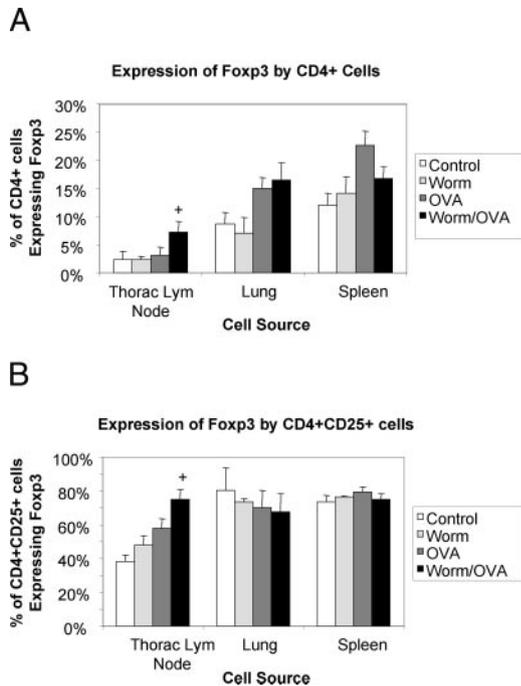
FIGURE 6. Expression of the transcription factor Foxp3 is increased in cells isolated from thoracic lymph nodes of worm/OVA group mice. *A*, Expression of Foxp3 by $CD45^+$ cells isolated from thoracic lymph node, minced lung, or spleen of control, worm, OVA, and worm/OVA group mice. A summary of percentage of positive cells taken from groups of four mice is shown. *B*, Expression of Foxp3 as a percentage of $CD4^+CD25^+$ cells. The data represent the mean and SE from four mice in each group. +, $p < 0.05$ vs control group.

A

B

FIGURE 7. Adoptive transfer studies suggest *H. polygyrus* induces regulatory cells. *A*, Transfer of OVA-activated $CD4^+$ cells to naive mice, followed by OVA inhalation, induces airway eosinophilia; this response is significantly suppressed by prior infection with *H. polygyrus*. Each column indicates the mean and SEM of seven mice in two separate experiments. **, $p < 0.01$ vs sham-infected group. *B*, Transfer of splenocytes from worm and OVA mice, but not worm/OVA mice, enhances OVA-induced airway eosinophilia. +, $p < 0.05$ vs control donor group; *, $p < 0.05$ vs OVA donor group. $n = 6$ mice/group, in two separate experiments.

Protection against passive atopic transfer (as opposed to a response that only inhibited sensitization) would functionally support a role for regulatory cells; thus, we examined the effect of prior infection with *H. polygyrus* on airway eosinophilia resulting from adoptive transfer of OVA-sensitized $CD4^+$ lymphocytes, followed by OVA inhalation (Fig. 1*B*). We found that mice infected with *H. polygyrus* develop significantly less airway eosinophilia than noninfected mice (Fig. 7*A*), suggesting induction of active or regulatory response by the parasites. In parallel with the number of eosinophils, OVA-induced IL-5 in BAL fluid was also significantly suppressed in the infected mice (uninfected mice, 89.5 ± 9.6 pg/ml; helminth-infected mice, 36.6 ± 6.5 pg/ml, $p < 0.01$).

We next examined whether the protective effect engendered by parasite infection could be transferred. For these studies, splenocytes were isolated from each of four groups of mice (control, worm, OVA, and worm/OVA) and transferred to OVA-sensitized recipient mice 1 day before the first of two OVA inhalation challenges (Fig. 1*C*). We found that transfer of cells from either helminth-infected (worm) mice or OVA-challenged (OVA) mice significantly increased OVA-induced eosinophilia in the recipient mice, but that transfer of splenocytes from helminth-infected/OVA-exposed (worm/OVA) mice did not induce this response (Fig. 7*B*), suggesting induction of a population of cells that requires exposure to Ag as well as the stimulation of helminth infection for optimal benefit.

Discussion

Recent epidemiological studies support the hygiene hypothesis, that children raised in extremely clean environments lack protective anti-atopic mechanisms, resulting in increased susceptibility to atopic disorders (5). Based on the immunological framework of

Th1 and Th2 balance (9), an increased prevalence of atopic disease could be due to unopposed Th2 responses from reduced exposure to (microbial-induced) Th1 responses. However, this explanation does not account for the observations that even Th2-skewing organisms such as helminths appear to protect against asthma and atopy (13). Although experimental models of Th1-skewing infections, such as mycobacteria, have demonstrated suppression of Ag-induced eosinophilic airway inflammation in murine models of asthma (28, 29), few studies have examined the protective effect of helminths in allergic inflammation (20, 30). Wohlleben et al. (20) used the hookworm *Nippostrongylus brasiliensis*, which traverses the lung during its life cycle, as an immune stimulus; in contrast, the effects of *H. polygyrus* do not require its presence in the lung. Quite recently, Wilson et al. (30) reported that *H. polygyrus* is effective in suppressing atopic inflammation in a murine model of asthma; in this study, we confirm and extend their findings.

In this study, we demonstrate that intestinal helminths protect against atopic responses in a murine model of asthma. We first showed that prior helminth infection suppressed OVA-induced airway eosinophilic inflammation, bronchial hyperreactivity, and in vitro OVA-induced IL-5 release from splenocytes. This was not associated with a Th1-type immune response: indeed, both total and Ag-specific IgE levels were enhanced, and IgG2a levels suppressed, in the worm-infected mice. Stimulation of splenocytes with either mitogen or Ag showed that cells from OVA-challenged mice have a Th2-skewed response; OVA-stimulated (but not Con A-stimulated) IL-5 release is suppressed in the helminth-infected OVA-challenged mice. Induction of IL-10 by Con A is enhanced, and by OVA is suppressed, in the helminth-exposed mice. We did not find a convincing elevation of IL-10 (nor of TGF-β) in the

BAL fluid of infected C57BL6 or BALB/c mice. Nevertheless, the protective effects of *H. polygyrus* infection are not seen in the absence of IL-10; this is in contrast to the findings of Wilson et al. (30), who reported no significant reduction in the anti-inflammatory response to *H. polygyrus* following administration of anti-IL-10R Abs. This discrepancy could be due to an incomplete ablation of IL-10 responses using an Ab, in comparison with the complete abrogation of the cytokine in the knockout mouse; however, the ability of cells transferred from *H. polygyrus*-infected IL-10$^{-/-}$ mice in that study suggests that other pathways, such as TGF-β responses, may also be induced.

IL-10 plays a complex role in the modulation of inflammation and airway responses in asthma. In regard to eosinophilia, by reducing expression of IL-5, IL-10 generally functions to suppress inflammation. Nevertheless, reports in the literature do not consistently find increases in Ag-induced airway eosinophilia in the absence of IL-10; some find reduced levels of eosinophils (31, 32), whereas others report modest to significant increases in this parameter of atopic inflammation (33–35). In part, this may be due to modulation of the Th1 arm, as IL-10 also suppresses expression of IFN-γ (36, 37); indeed, we have shown marked enhancement of the Th1 response to the TLR9 agonist, CpG DNA, in the absence of IL-10 (38). But mice genetically deficient in IL-10 do not necessarily have an overactive Th1 response in the absence of Th1 inducers. In this study, IFN-γ release by splenocytes of mice genetically deficient in IL-10 is similar to that from splenocytes of wild-type BALB/c mice.

The effect of IL-10 on airway hyperreactivity seems independent of effects on inflammation. Although some investigators have reported that airway hyperreactivity is not seen in the absence of IL-10 (31, 39), others have found similar (34) or only moderately reduced (40) levels of hyperreactivity in IL-10$^{-/-}$, as well as elevated baseline airway resistance (33) compared with wild-type mice. We have found (using IL-10$^{-/-}$ mice on the BALB/c background) that these mice develop airway hyperreactivity in conjunction with airway inflammation; levels of both responses are modestly reduced compared with wild-type mice.

Regulatory mechanisms are suggested by the ability to transfer protection and the effect of helminth infection on passively transferred sensitization. CD4$^+$CD25$^+$Foxp3$^+$ regulatory cells were increased within the population of CD4$^+$ (and CD4$^+$CD25$^+$) cells within the thoracic lymph nodes of *H. polygyrus*-infected, OVA-challenged mice. These may be the cellular mediators for the protective effect of helminth infection, although other cell populations (including CD4$^-$ cells) were found by Wilson and Maizels (41) to offer some protection.

The protective responses engendered by *H. polygyrus* in BALB/c mice were not strain specific; we found (data not shown) that C57BL/6 mice were equally protected against atopic airway inflammation. We further examined whether CpG-rich DNA potentially released from worms was involved in the immunomodulation, and saw that TLR9$^{-/-}$ mice (on a C57BL/6 background) were equally protected against eosinophilic airway inflammation as their parent strain (data not shown).

As previously reported, we found that colonization with *H. polygyrus* enhances total IgE (42). It has been suggested (the IgE blocking hypothesis) that elevated polyclonal IgE saturates the FcεR (IgE receptor), which may prevent the binding of Ag-specific IgE to mast cells and other cells central to atopic inflammation, resulting in reduced atopic symptoms. In our current study, we found that IL-10$^{-/-}$ mice developed equally high levels of IgE as was seen in wild-type mice, suggesting that induction of polyclonal IgE is not likely to be an important mechanism of helminth-induced protection in this model; moreover, recent clinical studies

have cast doubt on the IgE blocking hypothesis (15, 43–45). In addition to increasing IgE, helminth colonization suppressed Th1-associated IgG2a; similar patterns were seen in OVA-specific and in total Ig levels. These effects support a systemic Th2-type (and relative suppression of Th1-type) skewing of the immune system by worm infection. Thus, regulation of Ig responses did not parallel regulation of airway eosinophilia. Supporting our conclusion that induction of Th1 responses is not involved in helminth-provided anti-atopic responses, mice deficient in IFN-γ and IL-12 (IFN-$\gamma^{-/-}$IL-12$^{-/-}$ on a C57BL/6 background (46)) demonstrated equal protection and similar immune responses (data not shown).

It has been shown that heat-killed *Mycobacterium vaccae* suppress Ag-induced eosinophilic airway inflammation by induction of Ag-specific CD4$^+$CD45RB^{low} regulatory T cells (47) that depend on IL-10 and TGF-β. Although, in our study, the protective effects of colonization with *H. polygyrus* required the ability to generate IL-10, we did not find Ag-specific regulatory responses; IL-10 release by OVA-restimulated splenocytes was actually somewhat suppressed in helminth-infected mice. However, in these studies, all of the mice were first sensitized to OVA; the suppression of IL-10 release in helminth-infected mice may represent reductions in Ag-responsive Th2 cells. Indeed, the induction of IL-10 by mitogen stimulation could either reflect a larger non-Ag-specific Th2 population, or alternatively, release of cytokine from active regulatory cells. A number of reports have demonstrated the induction of T cells with non-Ag-specific regulatory properties. These include so-called type 1 T regulatory cells that may release both IL-10 and IL-5, but whose effects are IL-10 dependent, as we have seen in these studies (48–50).

Our findings that infection with a helminth that does not traverse the lung during its life cycle can prevent atopic airway inflammation are consistent with the hygiene hypothesis. This study bolsters, using an animal model, the epidemiologic observations that Th2-skewing helminth infections are associated with a reduced prevalence of clinically significant atopic disorders. In this model, Th1-type responses do not play a role, supporting the growing consensus that such responses are not central to the protection against atopic inflammation associated with microbes and their products; induction of a regulatory cell population is more likely the responsible mechanism. We have previously reported that the anti-asthmatic protective effects of CpG oligodeoxynucleotides, which mimic exposure to microbial DNA, induce, but are not dependent on Th1 responses (46). In that model system as well, IL-10 plays an important role in suppressing Th2-skewed responses (38). Increasing evidence supports the speculation that antiallergic effects of both Th1- and Th2-skewing microbial stimuli may be due to a common cellular mechanism, although the proximal innate immune receptors may differ. Clarification of these common mechanisms remains an important line of research.

Acknowledgments

Disclosures

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