

Heligmosomoides polygyrus infection down-regulates eotaxin concentration and CCR3 expression on lung eosinophils in murine allergic pulmonary inflammation

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SUMMARY

There is growing evidence that helminth infections might suppress allergic responses by mechanisms potentially involving regulatory T lymphocytes, cytokines, helminth molecules and polyclonal IgE. Heligmosomoides polygyrus infection in mice is associated with reduced local and systemic immune responses, thus providing an excellent model to study the mechanisms of immune regulation. In this research, we examined the way that nematode infection modulates the influx of eosinophils into the airways of asthmatic mice. We observed a reduction in the total number and percentage of lung eosinophils that coincided with decreased levels of eotaxin in bronchoalveolar lavage fluid (BALF), lower expression of the CCR3 receptor on eosinophils and impaired chemotaxis of these cells toward eotaxin. We conclude that allergen-induced immune response was down-regulated as production of Th1 (IFN- γ)-, Th2 (IL-4, IL-5)- and Treg (IL-10)-related cytokines as well as IL-6 and TNF- α was diminished upon nematode infection. We postulate that attenuation of allergic inflammation during H. polygyrus infection is a consequence of the dichotomy of the immune response in the face of concurrent antigenic challenge.

Keywords CCR3 chemokine receptor, cytokines, eotaxine, *Heligmosomoides polygyrus*, ova

INTRODUCTION

Every fifth human suffers from the clinical symptoms of allergy and there has been a worldwide increase in the prevalence of allergic disease (1). Helminth infections modulate the expression of allergic disease (2) or might lead directly to the development of allergic symptoms (3). Whether infection with a particular species of helminth would protect or exacerbate allergic reaction is a complex issue and strongly depends on the immune status of the host upon worm's presence (4,5). Several studies have reported increased protection from allergic disorders after helminth infections. Children harbouring *Schistosoma haematobium* exhibited decreased atopy (6) and *Strongyloides stercoralis* infection suppressed pulmonary allergic responses to ovalbumin (ova) (7). Many factors might be implicated in helminth-mediated suppression of allergy such as regulatory T lymphocytes as well as cytokines, helminthic molecules and polyclonal IgE.

Heligmosomoides polygyrus infection in BALB/c mice is useful to study the mechanisms of immune regulation (8). The course of this nematode infection might be divided into two phases: acute and chronic that exhibit different intensity and quality of host immune response. In the acute, prepatent phase, local inflammatory reaction is observed due to innate immune mechanisms and enhanced early parasite-specific activation of Th2 type of immunity in mesenteric lymph nodes (9). Worms, however, are able to resist this acute action of the immune system and reside for several months in their host giving rise to chronic infection. During this phase, the immune response is relatively quiescent. The adult nematodes locally reduce the number of eosinophils and mast cells infiltrating the lamina propria (10,11) as well as the level of proinflammatory cytokines in the periphery (12).

In vitro, eosinophils and immune serum are sufficient to activate eosinophils to kill helminth parasites by release of their toxic granule contents (13,14). Eosinophils act not only as immune effectors but could also contribute to the induction of immune response, because of their ability to act

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as APC (15) and their rapid production of IL-4 and IL-13 (16) that might accelerate the Th2 type immune response. Therefore, to survive, parasites might have evolved a mechanism of preventing the eosinophilic response or down-regulation of the number and activity of these cells.

IL-5 and eotaxin are pivotal in eosinophil production in the bone marrow and the attraction of these cells to the site of allergic inflammation, respectively. Eotaxin is up-regulated in endothelial and respiratory cells upon TNF- α (17), IL-4 and IL-13 stimulation (18), and is produced predominantly by T cells and eosinophils (19). This chemokine specifically binds to the CCR3 receptor (20). CCR3 is found both on human and murine eosinophils, and mediates their chemotaxis and adhesion to endothelial cells *in vitro* and *in vivo*. CCR3^{-/-} mice sensitized and challenged with aerosol ova had fewer eosinophils in the lung when compared with similarly treated wild-type controls (21). Hence, minimization of the number of eosinophils through the blockade of eotaxin-CCR3 axis might have potential therapeutic effects in asthma (22). Very late antigen-4 (VLA-4) binds to vascular cell adhesion molecule-1 (VCAM-1) on activated endothelial cells, and mediates selectively tethering, rolling and accumulation of eosinophils. After intranasal treatment with a small VLA-4 antagonist, eosinophils in the lungs and their activation products were substantially decreased (23).

In the present study, we investigated the way that *H. polygyrus* decreases the attraction of eosinophils to the site of inflammation. BALB/c mice infected chronically with *H. polygyrus* or uninfected control mice were treated to develop an ova-induced airway inflammation allergen typically associated with robust influx of eosinophils into the airways. The changes provoked by the nematode in the expression of CCR3 and VLA-4 on lung eosinophils and the migration ability of these cells were assessed. Concentration of eotaxin in the airways as well as ova and *H. polygyrus* antigen-induced IL-4, IL-5, IL-12, IFN- γ , IL-6, TNF- α , MCP-1, IL-10 production from splenocytes were studied.

MATERIALS AND METHODS

Animals

Male BALB/c mice were maintained under standard laboratory conditions and at 8 weeks of age were treated in accordance with the experimental protocol. All experiments were approved by and conducted in accordance with the guidelines of the local and national authorities.

Parasites and experimental infection

A strain of *H. polygyrus* was maintained and passed in BALB/c mice. Mice were infected by the oral route with 120

L3 larvae of *H. polygyrus*, 28 days prior to the first intraperitoneal (IP) immunization with ova (100 μ g; Sigma-Aldrich, St. Louise, MO, USA) in 100 μ L alum adjuvant (Imject Alum, Pierce Biotechnology, Rockford, IL, USA). Seven days later, mice were boosted IP with 100 μ g ova/alum. Five and six days after the second IP immunization mice were anaesthetized by an IP injection of a mixture of ketamine (2 mg, Ketavet, Pharmacia, Erlangen, Germany) and xylazine (0.4 mg, Rompun 2%, Bayer, Leverkusen, Germany) and challenged with 100 μ g of ova in 20 μ L PBS intra nasally (IN). Mice were sampled 3 days after the second IN application of ova.

Evaluation of eosinophils response in bone marrow, blood, peritoneal cavity, bronchoalveolar lavage fluid (BALF) and small intestine

From the tail vein of individual mice, 5 μ L of blood was taken and smears were performed. Animals were killed via CO₂ asphyxiation. Subsequently, blood samples were collected after disruption of vein. To obtain the BALF, a glass Pasteur pipette was inserted into trachea of mice. Lung lavage was performed three times with 1 mL of PBS pH 7.2. Cells from the peritoneal cavity were harvested by injection of 5 mL of PBS. The abdomen was gently massaged and a blood-free suspension was carefully withdrawn with glass Pasteur pipette. The left and right femurs were flushed with 2 mL of RPMI 1640 + 10% FCS each in order to collect bone marrow cells. The isolated tissue were pressed through needles of different diameters 0.5–1.1 mm. Peritoneal fluids and cells isolated from bone marrow as well as BALF were centrifuged at 600 g, for 7 min, at 4°C, the supernatants were stored at -70°C prior to cytokine determination and cell pellets were resuspended in 1 mL of cold PBS. Smears were made with 40 μ L of cell suspension. Differential cell counts were performed by staining smears with May-Grunwald-Giemsa or by staining 50 μ L of cell suspension with 150 μ L 0.04% solution of eosin. Two hundreds white cells in smears from blood and 1000 white cells in smears from peritoneal wash, BALF and bone marrow were counted per mouse, and the number of eosinophils per 200 or 1000 white cells was determined. A 1-cm length section of the small intestine was taken 5 cm proximal to the pylorus, frozen in liquid nitrogen and stored at -70°C. The number of cells was estimated from 6 μ m cryostat tissue sections. Eosinophils were assessed using DAB (diaminobenzidine tetrahydrochloride, Sigma-Aldrich) as a substrate for eosinophil peroxidase. The slides were examined with a fluorescent microscope with a 470-nm filter. The tissue cross-sections were counterstained with Evans Blue in PBS.

Expression of CCR3 and VLA-4 on eosinophils

The mixed cell population of BALF was analysed by flow cytometry. One million cells were resuspended in 50 μ L of PBS + 0.5% BSA (Sigma-Aldrich) and incubated with saturating concentrations of R-PE conjugated rat anti-CD49d mAb and Alexa Fluor 647 conjugated rat anti-mouse CCR3 mAb for 30 min on ice and washed. Nonbinding control mAbs of IgG2a and IgG2b subclasses were used to determine nonspecific binding (all from Pharmingen, San Diego, CA, USA). Samples were held on ice until FACS analysis.

Eosinophil chemotaxis assay

Chemotaxis assay was carried out in a Transwell® 24-well polystyrene plate, tissue culture treated, with a 3.0- μ m pore size polycarbonate membrane (Costar). The lower chamber contained 500 μ L of 2% FCS in HBSS with 4 ng/mL eotaxin (R&D Systems, Abington, UK). Leukocytes from BALF (5×10^5) in a 200 μ L volume of 2% FCS in HBSS were placed in the upper chamber and the plate incubated at 37°C with 5% CO₂ for 4 h. The contents of the lower and upper chambers were then collected, centrifuged and eosinophils were counted. The percentage of migrating eosinophils has been calculated according to the formula: $100 \times (\text{number of eosinophils found in the lower chamber}) / (\text{the number of eosinophils initially placed on the upper chamber})$.

Lymphocyte culture and cytokine responses

Spleen or mediastinal lymph nodes were removed from mice, and single-cell suspensions were prepared. Cells were resuspended in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U of penicillin per millilitre and 100 μ g of streptomycin per millilitre (all from Gibco, Inchinnan, UK), and red blood cell lysis were performed with Tris-NH₄Cl pH 7.2. After washing, cells at a concentration of 5×10^6 per millilitre were plated onto 96 wells flat bottom plates (Costar) and left untreated or stimulated at 37°C and 5% CO₂ with ova (100 μ g/mL, Sigma) or 10 μ g/mL *H. polygyrus* somatic extract. After 72 h, cell-free supernatants were harvested, aliquoted and stored at -70°C.

Cytokine assays

Cytokine analysis was carried out by sandwich enzyme-linked immunosorbent assay (ELISA) using the OptEIA Mouse sets for quantification of IL-5 and IL-4 (Pharmingen) and EotaxinKit (R&D Systems) according to the manufacturer's instructions. Mouse Inflammation Kit (Pharmingen) was

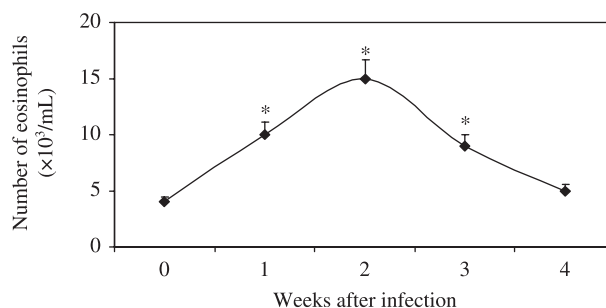


Figure 1 *Heligmosomoides polygyrus* infection in BALB/c mice evoked only short-lived eosinophilia. Number of eosinophils has been estimated in blood of four mice infected with 120 L3 *H. polygyrus*. * $P < 0.05$ compared to noninfected animals.

used to determine the concentration of IL-10, TNF- α , IFN- γ , IL-12, IL-6 and MCP-1 by Cytometric Bead Assay (CBA).

Statistical analysis

Data are presented as a mean $\pm$ standard deviation (SD). Analysis was carried out between ova vs. *Hp* + ova and *Hp* vs. *Hp* + ova groups with the use of Mann-Whitney test <faculty.vassar.edu/lowry/utest.html>. Five mice per group were used and differences were considered statistically significant when the P -value was less than 0.05.

RESULTS

Eosinophilic response during *H. polygyrus* infection in BALB/c mice

Heligmosomoides polygyrus evoked eosinophils response in the blood that peaked 2 weeks after infection ($P < 0.01$). However, in the chronic phase of infection, eosinophil number dropped to the control level (Figure 1). In the sixth week of infection, *H. polygyrus* adults induced only weak eosinophil responses in the bone marrow and the cell number was enhanced only in the peritoneum. Interestingly, the number of eosinophils in the intestine of infected mice was reduced to a lower level even than in the uninfected animals (Table 1).

Effect of *H. polygyrus* infection on cellular composition of BALF

In the airways of *H. polygyrus* infected and control animals, only a few cells were found; less than $1.0 \times 10^4 \pm 1.0 \times 10^3$. Lung lavage of asthmatic mice was typically composed of macrophages, lymphocytes and especially eosinophils (7).

Table 1 Eosinophilic response 6 weeks after infection of BALB/c mice with *H. polygyrus*. The number of eosinophils in five mice per group. **P* < 0.05 compared to non infected mice

No. of eosinophils	Bone marrow ($\times 10^5$)	Blood ($\times 10^2/\text{mL}$)	Peritoneum ($\times 10^5$)	BALF	Intestine (per VCU)
Noninfected	11 $\pm$ 1.0	37 $\pm$ 2.0	5 $\pm$ 1.0	undetectable	11 $\pm$ 2.0
<i>Heligmosomoides polygyrus</i>	20 $\pm$ 1.0	38 $\pm$ 7.0	22 $\pm$ 2.0	undetectable	6 $\pm$ 0.9

Table 2 Three days after last intranasal application of ova airway challenge BALFs were prepared and the number of leukocytes and eosinophils determined. Shown are the mean number of leukocytes, eosinophils and % of eosinophils $\pm$ SD determined in five individual mice per group (mice analysed were from one experiment; the same pattern of the cells response were obtained at least twice in independent experiments). **P* < 0.05, compared to values obtained in ova-only treated mice

BALF	'ova'	'Hp + ova'
Number of leukocytes	2.4 $\times 10^6 \pm 5.0 \times 10^4$	1.0 $\times 10^6 \pm 6.0 \times 10^4$
Number of eosinophils	1.1 $\times 10^6 \pm 2 \times 10^5$	3.4 $\times 10^6 \pm 1.8 \times 10^5$
% of eosinophils	46.2 $\pm$ 6.1	33.4 $\pm$ 3.9

Less than 1% neutrophils could be detected in both groups of mice. No basophils were found in BALF.

Infection with *H. polygyrus* significantly reduced the total leucocyte number in BALF as well as the eosinophil number in mice sensitized to ova (Table 2). The influx of eosinophils was selectively inhibited as in infected animals the percentage of eosinophils was lowered in comparison with noninfected littermates.

Down-regulation of CCR3 but not VLA-4 expression on eosinophils

BALF of asthmatic mice was composed of granulocytes, macrophages and lymphocytes as visualized by plotting the forward scatter (FSC) vs. side scatter (SSC). Granulocytes were represented predominantly by eosinophils as all cells expressed VLA-4, and almost no neutrophils and no basophils were detected in May-Grunwald-Giemsa stained BALF. Furthermore, eosinophils were distinguished as weakly autofluorescent cells and alveolar macrophages as large and spontaneously autofluorescent cells. Eosinophils were gated (Figure 2a), and mean fluorescence intensity (MFI) of the CCR3 and VLA-4 receptor staining was estimated.

Asthmatic eosinophils showed high expression of CCR3 and VLA-4. Infection with *H. polygyrus* coincided with decreased expression of CCR3 (*P* = 0.02; calculated from MFI of separate BALF samples) but not VLA-4 on eosinophils that migrated to the airways of asthmatic mice (Figure 2b,c).

Heligmosomoides polygyrus infection inhibited the eotaxin-triggered chemotaxis of eosinophils

To test whether the reduction of CCR3 expression evoked by *H. polygyrus* infection had a functional effect, an eosinophil chemotaxis assay was performed. A significant reduction of eosinophil migration towards eotaxin between the two groups of mice was observed; 71.4% $\pm$ 13.8% of BALF eosinophils from asthmatic noninfected mice were chemoattracted by the chemokine, while only 42% $\pm$ 11.5% eosinophils from *H. polygyrus* infected asthmatic mice (*P* = 0.01).

Changes in eotaxin concentration

Eotaxin was measured *ex vivo* in BALF. Asthmatic mice infected with the nematode had lower levels of this chemokine than noninfected animals subjected to ova sensitization (Figure 3). Production of eotaxin was also detected in *in vitro* culture of cells from mediastinal lymph nodes upon ova re-stimulation. Again, the concentration of the chemokine was lower in *H. polygyrus* infected ova treated mice (data not shown).

Differences in the cytokines response

IL-5, IL-4, IL-10, TNF- α and IL-6 production by splenocytes cultured *in vitro* after ova stimulation was lower in the group of mice that were treated with ova and infected with *H. polygyrus*. ova-specific MCP-1 production was not affected upon *H. polygyrus* infection (Figure 4). Splenocytes from asthmatic mice after re-stimulation with ova *in vitro* produced also measurable amounts of IFN- γ (584.4 $\pm$ 127.2 pg/mL). Decreased production of IFN- γ was observed in worm infected asthmatic mice (240.2 $\pm$ 50.7 pg/mL; *P* = 0.006).

The production by splenocytes of IL-5, IL-4, TNF- α and IL-10 following *H. polygyrus* antigen stimulation was diminished in mice treated with ova and infected with *H. polygyrus* with comparison to *H. polygyrus* – only infected mice (Figure 4). Neither stimulation of splenocytes with ova nor with *H. polygyrus* antigen led to production of IL-12.

Similar changes in the production of cytokines between studied groups of animals were true also for mediastinal lymph node cells stimulated with ova or *H. polygyrus* antigen (data not shown).

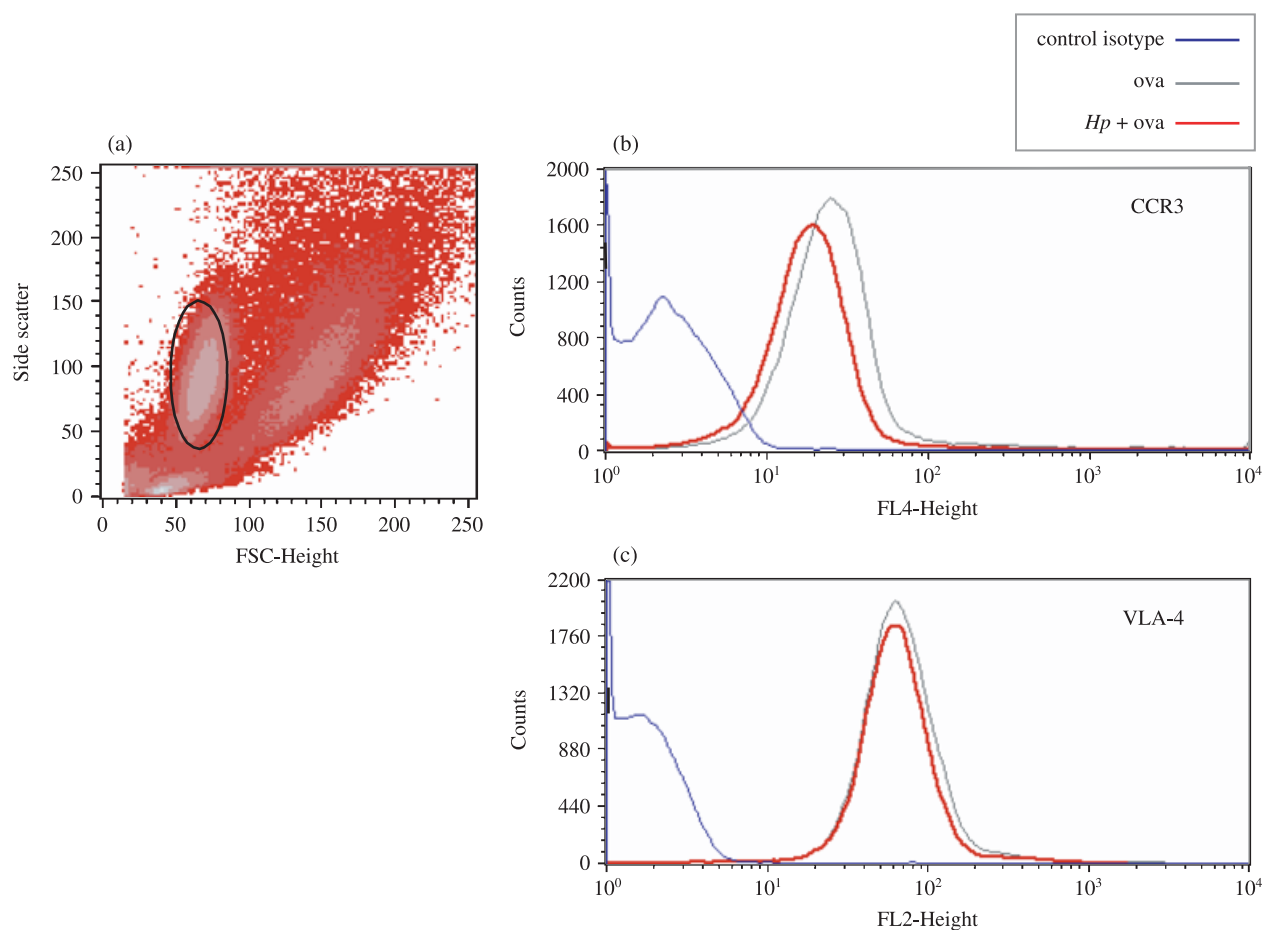


Figure 2 CCR3 and VLA-4 expression on eosinophils in BALF: (a) shows representative dot plots of FSC vs. SSC of 100 000 mixed cells from BALF of mice treated with ova. Eosinophils were separated by FSC and SSC side scatters and lack of autofluorescence; (b) shows expression of CCR3 on eosinophils from mice treated with ova that were worm-free (ova) and from mice treated with ova and infected with *H. polygyrus* (*Hp* + ova); (c) shows expression of VLA-4 on eosinophils from BALFs obtained from ova and *Hp* + ova groups.

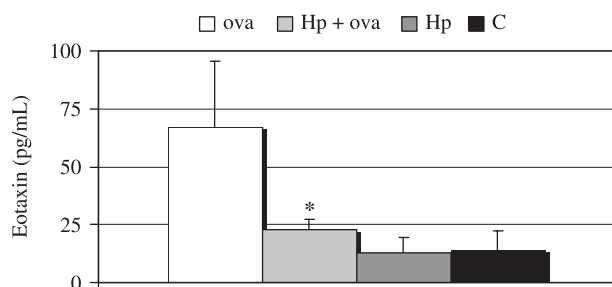


Figure 3 Effect of *H. polygyrus* infection on concentration of eotaxin in BALF. **P* < 0.05, compared to values obtained in ova-only treated mice.

DISCUSSION

The results presented in this report support the recent finding that *H. polygyrus* infection suppresses allergen-induced airway

eosinophilia (24). The immunoregulatory network in the periphery evoked by chronic nematode infection could act to attenuate the allergic reactions in the lung. In our studies, we observed a reduction in the total number of cells present in the airways, especially eosinophils and this coincided with a significantly decreased level of eotaxin in BALF and was consistent with decreased chemokine production after *in vitro* allergen re-stimulation of mediastinal cells. Interestingly, when compared to noninfected littermates, lung eosinophils from helminth-infected mice had decreased expression of CCR3 receptor as well as impaired eotaxin-triggered chemotaxis. Additionally, IL-4, IL-5, IL-10, IFN- γ and IL-6 as well as TNF- α production by ova-stimulated spleen lymphocytes were negatively influenced by helminth infection. However, no changes in the spleen T cell composition of these two groups of mice were observed as the percentage of CD4+, CD8+ and CD4+, CD25+ was not affected (data not shown).

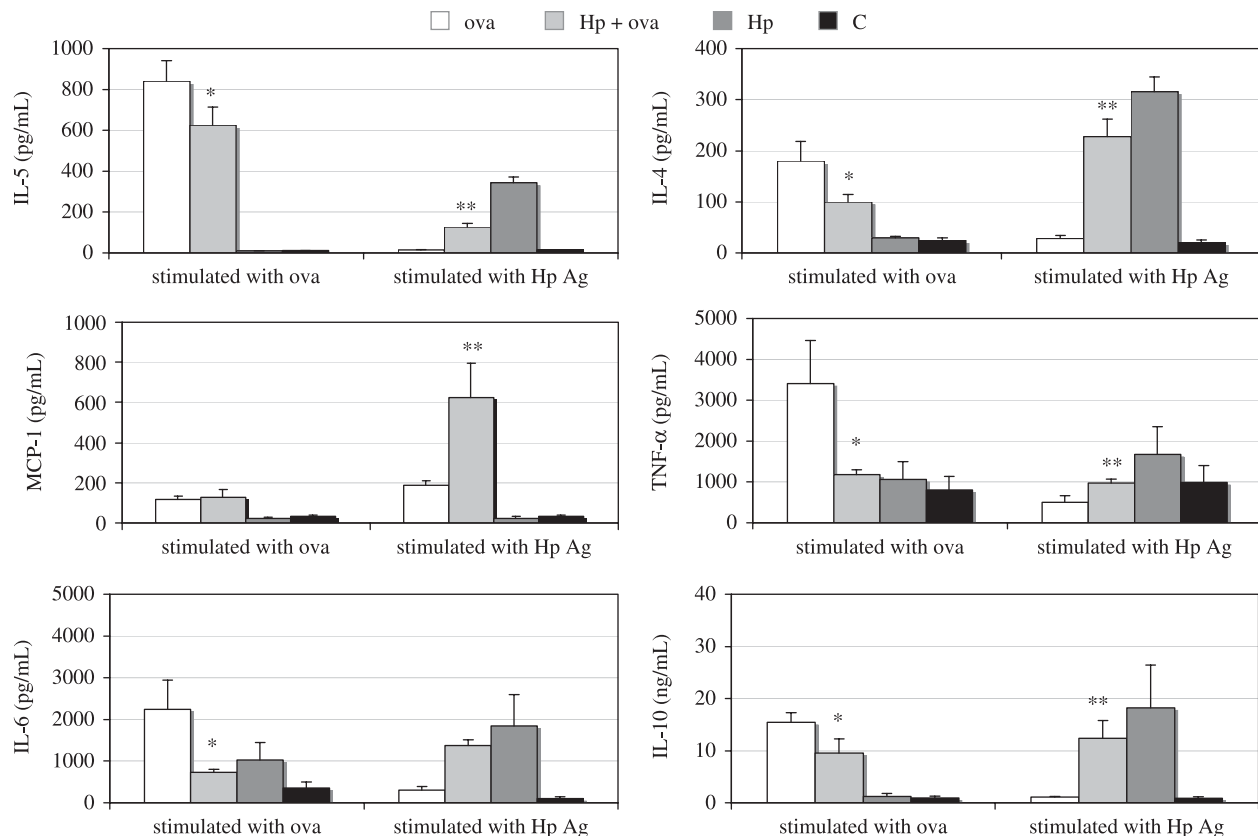


Figure 4 Effect of *H. polygyrus* infection on cytokine production. Spleens were isolated from ova-treated mice that did not receive *H. polygyrus* (ova) or from mice colonized with *H. polygyrus* and treated with ova (Hp + ova). * $P < 0.05$, compared to values obtained in ova-only treated mice and ** $P < 0.05$ compared to *H. polygyrus*-only treated mice.

The main aim of our studies was to check whether the decreased influx of eosinophils into the mice airways was due to the reduced expression of receptors essential for attracting eosinophils to the site of inflammation. We were able to show that *H. polygyrus* infection selectively down-regulated CCR3 expression on lung eosinophils and reduced chemotaxis of these cells toward eotaxin *in vitro*. As the levels of eotaxin, a chemokine which exclusively binds to CCR3 was lowered too, these results suggest that the nematode utilized specific mechanisms to hinder the eosinophilic response. The CCR3 receptor is involved in eosinophil differentiation and is up-regulated by Th2 cytokines in CD34+ progenitor cells (25). We conclude, therefore that the impaired Th2 cytokines response in the group of asthmatic mice infected with the nematode provides a weaker signal for high CCR3 expression. The current finding that helminth infection modulates the expression of CCR3 receptor might be of special interest and to our knowledge no report of such nematode action has been published. This receptor due to its wide expression on cell types prominent in allergies, that is, Th2 cells, eosinophils, mast cells and

basophils is proposed to be a target for therapy against allergic asthma, allergic rhinitis and atopic dermatitis (26). Potential down-regulation of CCR3 density on T cells by *H. polygyrus* infection might influence the general Th2 type of immune response and therefore attenuate murine allergic pulmonary inflammation.

The other explanation for the decreased concentration of eotaxin and decreased CCR3 expression on eosinophils might be a direct action of nematode products. Many helminths have evolved specific molecules to prevent the recruitment and activation of adequate types of proinflammatory cells (27,28). Also in our model, *H. polygyrus* might theoretically release eotaxin cleavage factor and CCR3 receptor antagonist that would reduce its biological function and as a consequence lead to the suppression of allergy.

The concentration of chemokine MCP-1 following ova stimulation was not affected by nematode infection. This cytokine is responsible for chemotaxis of monocytes/macrophages lineage. Macrophages and infiltrating monocytes comprise one of the largest populations of cells within the airways of asthmatic patients with allergic asthma and might

contribute to its pathological features (29). In our studies the percentage of macrophages found in BALF of helminth infected and noninfected mice did not differ, which suggests that alveolar macrophages are not the cell population influenced by *H. polygyrus* infection and additionally support our hypothesis of selective action of the nematode towards eosinophils. Furthermore, filarial parasites of humans secrete a homologue of the human cytokine macrophage migration inhibitory factor (MIF) that was shown to, depending on the assay conditions, inhibit migration and chemotaxis on human peripheral blood-derived monocytes/macrophages (30). However, studies of Pennock *et al.* (31) failed to isolate MIF from *H. polygyrus* homogenate. These data together with the present studies suggest that *H. polygyrus* has not evolved mechanisms to down-modulate the response of host macrophages. *Heligmosomoides polygyrus*, similar to other parasitic nematodes, possesses the ability to induce alternatively activated macrophages (32). This leucocytes population inhibits Ag-specific CD4+ T cell proliferative response during filarial infection (33). Thus, in the present disease model, alternatively activated macrophages might be implicated in the control of eosinophils-mediated lung pathology.

We found also that helminth infection down-regulated not only typical pro-allergic Th2 responses but also the production of cytokines associated with Th1 arm of the immunity. This contradicts the recent report that showed chronic infection with *H. polygyrus* did not influence Th1 ova-specific responses and did not inhibit ova-specific delayed type hypersensitivity responses in the periphery (34). This discrepancy could depend on the quite different time course of infection and sensitization used in the two studies. However, our results are in accordance with the work of Chen *et al.* (35) and Elliott *et al.* (36) who showed that the parasite down-regulated IFN- γ production and thus enhanced susceptibility to *Citrobacter rodentium* infection in mice or attenuated piroxam-induced colitis in IL-10 deficient mice, respectively. IL-10 is an immunosuppressive cytokine and has been shown to reduce the allergic reaction by multiple mechanisms (37,38). However, in the case of helminth-mediated suppression of allergy, the evidence for IL-10 participation in the process is inconsistent (24,39,40). In our studies, spleen cells stimulated with ova did not produce more of this cytokine upon helminth infection, and the percentage of regulatory T lymphocytes in spleen co-expressing CD4+ and CD25+ receptors was similar in the groups of allergic mice and allergic mice that were infected with the nematode. This might suggest that IL-10 and regulatory T lymphocytes in the case of *H. polygyrus* are not the primary modulators of the allergic response and implicate the existence of an alternative mechanism or mechanisms.

Suppression of allergy by helminths was suggested to evolve as a consequence of limited capacity of immune

system for responsiveness (41). Thus, through competition or division of effort, the allergic response would be reduced when co-existing with the nematode infection. This was reflected in our studies as splenocytes of asthmatic mice infected with *H. polygyrus* produced weaker cytokine responses than cells from asthmatic-only animals upon stimulation with ova. Simultaneously, splenocytes from asthmatic mice infected with *H. polygyrus* and stimulated with *H. polygyrus* antigen gave weaker cytokine responses than mice infected only with *H. polygyrus*. A second possible explanation is that intestine dwelling nematodes might attract the eosinophils to the gut and thus reduce number of these leucocytes in the airways. However, we showed that during chronic *H. polygyrus* infection, the number of eosinophils in the intestine is reduced below the control level. It is possible that eosinophils are attracted to the gut but they undergo nematode-induced apoptosis as is in the case with other nematodes (42).

To summarize, we estimated that *H. polygyrus* infection reduced eosinophil responses in the airways of asthmatic BALB/c mice through the down-regulation of eotaxin concentration, CCR3 expression on eosinophils and chemotactic activity of these cells toward eotaxin. We postulate that attenuation of allergic inflammation during chronic nematode infection could result from a dichotomy of the immune response in the face of co-existing disease challenge.

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