

ORIGINAL ARTICLE

Gastrointestinal nematode infection interferes with experimental allergic airway inflammation but not atopic dermatitis

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Summary

Background Some helminth infections are negatively associated with the prevalence of allergic disorders, arguing for a modulation of allergic reactions by the parasites, depending on the worm species, intensity and phase of infection and the type of disease.

Objective The aim of this study was to analyse the influence of a chronic infection with the gastrointestinal nematode *Heligmosomoides polygyrus*, in a murine model of allergic airway disease and of atopic dermatitis (AD), respectively.

Methods Mice were infected with *H. polygyrus* and systemically sensitized with the model allergen ovalbumin. Subsequently, the animals were challenged with the allergen either via the airways for induction of airway disease, or via skin patches for induction of dermatitis.

Results Mice concomitantly infected with *H. polygyrus* showed diminished eosinophil and lymphocyte recruitment into the lungs and decreased allergen-specific IgE levels when compared with sensitized and airway challenged controls. In addition, animals showed a trend towards reduced airway hyper-reactivity. In contrast, no significant differences in the severity of eczematous skin lesions were observed between infected and control animals in the AD model. Although *H. polygyrus* infection reduced CD8⁺ and CD4⁺ T-cell infiltration into the skin and production of allergen-specific IgE, mast cell recruitment was significantly increased in worm-infected mice in the dermatitis model. The worm infection was associated with significantly elevated numbers of Foxp3⁺ regulatory T cells (Treg) in peribronchial lymph nodes in *H. polygyrus*-infected sensitized and airway challenged mice. In contrast, Treg cells were basically absent in eczematous skin and their number was not increased in skin-draining lymph nodes of mice with experimental dermatitis.

Conclusion Infection with the gastrointestinal nematode used in our study leads to significant inhibition of mucosa-associated but not cutaneous allergic reactions, pointing to a site specificity of the immunomodulation exerted by helminths. This finding might be an important aspect for future considerations of helminths for treatment of allergic diseases.

Keywords allergic hyper-reactivity, atopic dermatitis, IL-10, intestinal nematode, regulatory T cell
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Introduction

The increased prevalence of allergic disorders like asthma, rhinitis and atopic dermatitis (AD) in the last 10–15 years is best explained by the modified hygiene hypothesis

stating that dysregulation of the immune system due to decreased stimulation of regulatory immune responses results in skewing of the immune response against harmless environmental antigens like pollen, animal proteins or house dust mites towards increased Th2 cytokine and IgE production [1–7].

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Recent reports describe a negative correlation between infections with certain parasitic worms and the prevalence of allergic disorders in humans [8–11]. Similarly, infections with gut nematodes, male schistosomes and other helminths resulted in reduced airway or gastrointestinal inflammation and inhibition of airway hyper-reactivity (AHR) in animal models of allergic asthma, anaphylaxis and food allergy [12–18]. However, other reports indicate an enhancing effect of helminth infections on allergies, such as an aggravation of allergic airway inflammation in humans infected with *Ascaris lumbricoides* or *Anisakis simplex* [19, 20].

It is very likely that these divergent effects of helminths on allergic diseases result from differences in the species of the nematode, the kinetics of infection [21] or even the type of allergic disease studied. Whereas bronchial asthma is a chronic disease with continuously strong Th2 inflammatory responses in the lung mucosa leading to recruitment of inflammatory cells and subsequently to pathological changes of lung tissues [22], the concept for the pathogenesis of AD seems less clear. AD is described as a chronic inflammatory disease of the skin associated with cutaneous hyper-reactivity [23]. The current concept of the pathophysiology of AD assumes a systemic Th2 response in addition to a biphasic T-cell response in the skin, with Th2 cells in acute and Th1 cells in the chronic phase of the disease [24].

In this study, we therefore aimed to delineate whether protection via infection with the murine gastrointestinal nematode *Heligmosomoides polygyrus* is restricted to mucosal sites of inflammation as in the lung, or is also effective in cutaneous tissues. For this purpose, we compared the impact of an *H. polygyrus* infection in two models of allergic diseases, a Th2-dependent model of mucosal airway inflammation (experimental asthma) and a mixed Th1/Th2-dependent model of cutaneous inflammation (experimental dermatitis). Here we report significant differences in the immunomodulation exerted by the intestinal nematode in these two models, pointing to the tissue specificity of the modulatory effect of the parasite infection.

Material and methods

Animals

Female, 6–10-week-old, BALB/c mice were obtained from Harlan-Winkelmann (Borchen, Germany). All experimental procedures were performed in compliance with protocols approved by the local State Office of Health and Social Affairs. *H. polygyrus* was maintained by serial passage in BALB/c mice. Infective larvae were obtained from fecal cultures.

Murine asthma model

Mice were sensitized twice (day 0 and day 14) intraperitoneally (i.p.) with 20 µg ovalbumin (OVA) (grade VI, Sigma-Aldrich, Taufkirchen, Germany) emulsified in 2 mg of aluminium hydroxide (Imject® Alum, Pierce, Rockford, IL, USA) as an adjuvant in a total volume of 200 µL. On days 28 and 29, mice were intranasally (i.n.) challenged with 50 µg OVA in 50 µL phosphate-buffered saline (PBS). Naïve control animals were sham-sensitized with aluminium hydroxide in PBS and i.n. challenged with PBS instead of OVA.

Airway responsiveness was measured via whole-body plethysmography on day 31 in unrestrained mice after a challenge with increasing doses of methacholine (Sigma-Aldrich) as described elsewhere [25, 26].

At day 31 mice were killed and lung, spleen, lymph nodes and blood samples were collected for further analysis. Bronchoalveolar lavage (BAL) fluid was harvested and BAL cells were stained with DiffQuick (Fisher Diagnostic Scientific, Schwerte, Germany) and differentiated by morphological criteria (counts of 200 cells under light microscopy in a blinded fashion), as described previously [27]. Supernatants were stored at –20 °C for subsequent cytokines analysis.

Murine dermatitis model (atopic eczema)

Mice were sensitized i.p. on days 1, 14 and 21 with 10 µg OVA (Sigma-Aldrich) adsorbed to 10 µg aluminium hydroxide (Imject® Alum, Pierce) in 100 µL PBS or with PBS and alum as control. Epicutaneous (e.c.) allergen application was performed as described previously [28]. Each mouse had a total of three 1-week exposures to the same abdominal skin site. Naïve animals were treated e.c. with PBS/alum. At day 70, mice were killed and skin biopsies, spleen, lymph nodes and blood samples were collected for further analysis.

To evaluate the severity of OVA-induced eczematous skin lesions, we used a standardized clinical skin score (CSS) based on clinical features of human AD [29], which considers the typical skin features of AD, like erythema, oedema/papules, oozing/crusts, dryness and extension [30]. For total CCS, the marked lesions that were visible on the patched skin area were graded as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) by two independent persons. The total score was taken as the index of dermatitis severity [28].

Nematode infection

One hundred and fifty *H. polygyrus* L3 per mouse were applied orally at the time of the first allergen sensitization (Figs 1a and b). Controls for effects of nematode infection were *H. polygyrus*-infected sham-sensitized animals.

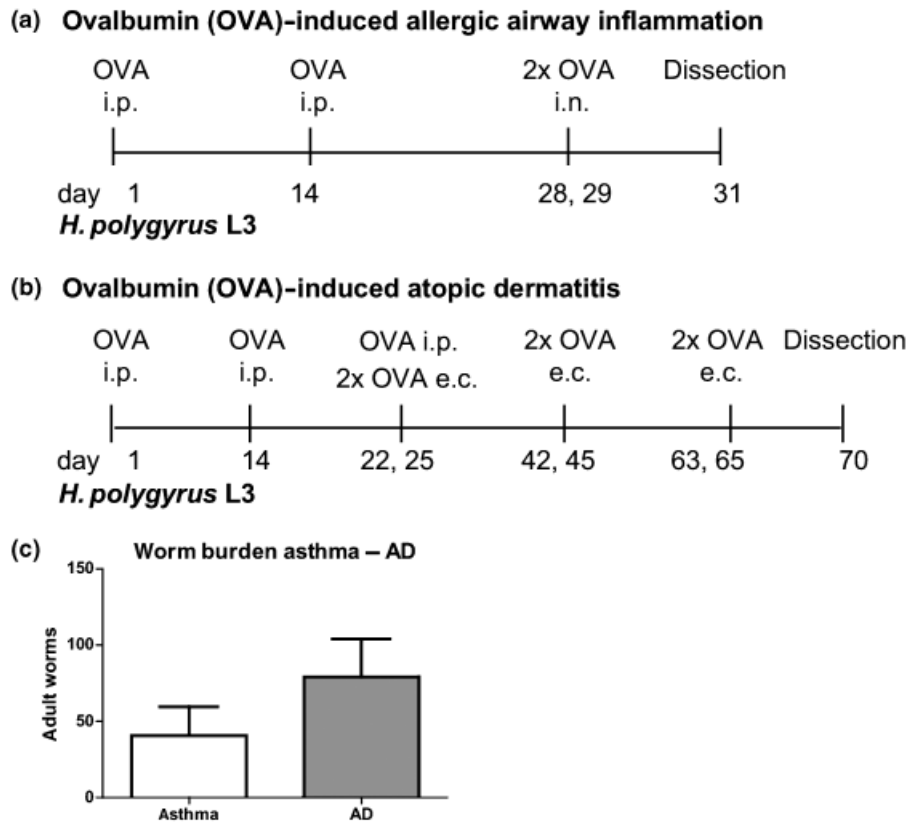


Fig. 1. Ovalbumin (OVA)-induced models of murine allergic airway inflammation (a) and murine atopic dermatitis (b) and worm infection (*Heligmosomoides polygyrus* L3). i.p., intraperitoneal application; i.n. intranasal application; e.c., epicutaneous application. (c) Number of adult worms in both allergy models at the time-point of dissection. Representative data of two experiments with five to six animals per group are shown. Data are shown as means with SE.

Persistence was monitored throughout the experiments by determination of fecal egg output. At the end of the experiments, adult worm burden in the intestine was determined.

Preparation of *Heligmosomoides polygyrus* adult worm antigen

Soluble worm antigen was prepared from adult worms kept in culture in RPMI medium containing 100 U/mL penicillin and 100 µg/mL streptomycin for 24 h. Worm material was homogenized and sonicated (1 min, 60 W) on ice in PBS (pH 7.4). The homogenate was centrifuged (20 min, 20 000 g, 4 °C) and the supernatant was passed through a 0.4 µm filter (Schleicher & Schuell, Dassel, Germany) for sterilization. Antigen extracts were stored at –80 °C.

Immunoglobulin – ELISA

Levels of total IgE and OVA-specific IgE, IgG1 and IgG2a were measured in sera by ELISA as described previously [18]. In brief, sandwich ELISA was performed using

anti-Ig-antibody as the capture and biotinylated anti-Ig-antibody as the detection antibody. After incubation with streptavidin peroxidase, the reaction of the substrate TMB was photometrically detected at 460 nm. To determine antibody concentrations, commercial IgE standard or standardized OVA-specific sera were used. Allergen-specific IgE, IgG1 and IgG2a were measured accordingly but biotinylated allergen (3 µg/mL biotinylated ovalbumin) was used as the detection molecule.

Cytokine analysis

Splenocytes and lymph node cells were isolated and cultured (5×10^5 cells/well) in RPMI 1640 for 72 h in the presence of 50 µg/mL ovalbumin, 10 µg Hp antigen or 2.5 µg ConA, respectively. RPMI was supplemented with penicillin, streptomycin, L-glutamin and 5% FCS (Hyclone). Cell culture supernatants were stored at –20 °C until analysis of cytokines by ELISA, according to the manufacturer's instructions (IL-4, IL-5, IL-10, IFN-γ, TNF-α, IL-12p40, ELISA BD OptEIA; TGF-β R&D).

RNA preparation, RT-PCR and real-time PCR

Total RNA was extracted from 30 mg frozen skin samples using the RNeasy mini kit (QIAGEN, Hilden, Germany). 1–2 µg of total RNA were transcribed into cDNA using TaqMan[®] reverse transcription reagents according to the manufacturer's guidelines. Real-time PCR was performed to analyse the expression level of the cytokines TGF-β, IL-10, IL-4, IFN-γ and the transcription factor Foxp3 in skin tissue using the 7300 real-time PCR system (Applied Biosystems, Heidelberg, Germany) and TaqMan reagents (TGF-β (Mm00441729_g1), Foxp3 (Mm00475156_m1), IL-4 (Mm00445259_m1), IL-10 (Mm00439616_m1), IFN-γ (Mm00801778_m1) primer and probe, housekeeping gene GAPDH [glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1), Applied Biosystems]. PCR conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Expression relative to the endogenous GAPDH control was determined using the $\Delta\Delta C_t$ method as described elsewhere [31].

Analysis of regulatory T cells by FACS

Regulatory T cells (Tregs) in peribronchial, mesenteric and skin-draining (inguinal plus axillary) lymph nodes were characterized by expression of the surface markers CD4, CD25 and CD103 [32], and the transcription factor Foxp3. Cells were washed in cold PBS/0.2% BSA and stained 15 min on ice with anti-CD4-FITC (BD Biosciences, clone RM4-5, Heidelberg, Germany), anti-CD25-APC (BD Biosciences, clone PC61), anti-CD103-biotin (a gift from Alexander Scheffold, DRFZ, Berlin, Germany, clone M290) and streptavidin-PE-Cy7 (BD Biosciences). Foxp3-staining was performed using the PE-anti-mouse Foxp3 staining kit purchased from eBioscience (San Diego, USA, clone FJK-16s) according to the manufacturer's instructions. Nonspecific surface binding of antibodies was prevented by addition of anti-mouse FcγR (clone 2.4G2, gift from Alexander Scheffold, DRFZ). Nonspecific intracellular binding during staining of Foxp3 was inhibited by blocking with whole rat IgG (Jackson Laboratories, Cambridgeshire, UK). FACS analysis was performed on LSR II (BD Biosciences) and analysed with FlowJo software (Treestar, Ashland, OR, USA).

Histological analysis

Skin samples were embedded in Tissue Tek OCT compound (Jung, Nußloch, Germany). Frozen tissue blocks were cut into 5 µm sections and fixed in acetone. For histological analysis, sections were stained with haematoxylin for 1 min. The thickness of the epidermis and the dermis was determined by Axiovisions measuring tools on the Axio-plan light microscope (Zeiss, Berlin, Germany) at ×100

magnification. Ten measurements were obtained per mouse and expressed in micrometres.

Immunohistochemistry

Sections of 4 µm were prepared as described above, blocked with 10% normal goat serum (Dako, Hamburg, Germany) and incubated with rat anti-mouse CD4 (clone RM4-5) and anti-mouse CD8 (clone 53-6.7), followed by incubation with biotinylated goat anti-rat IgG. CD11c-positive cells were detected using purified hamster anti-mouse antibody (HL3) and a biotinylated anti-hamster IgG cocktail (all BD Pharmingen, Heidelberg, Germany). To rule out unspecific binding of antibodies, negative controls without primary and/or secondary antibodies were performed, respectively. Signals were detected by Chem-Mate[®] alkaline phosphatase/red detection kit (Dako) and by haematoxylin counter staining. To determine infiltrating mast cells, 5 µm skin sections were stained for 1 h with o-toluidine blue or Giemsa, respectively. Positive-stained cells were counted in 10 high-power fields (HPFs) at ×200 magnification and expressed as cells per HPF. Foxp3 and major basic protein (MBP, antibody kindly provided by Dr Jamie Lee, Mayo Clinic Scottsdale, AZ, USA) staining for the detection of Tregs and eosinophils in the skin was performed as described previously [33–36].

Statistical analysis

Data were compared by non-parametric analysis (SPSS software, Munich, Germany and Prism software) using the Mann–Whitney *U*-test for independent groups. *P*-values <0.05 were considered as statistically significant.

Results

Infection with Heligmosomoides polygyrus ameliorates allergic airway inflammation but not the development of atopic dermatitis

To study the role of a gastrointestinal nematode infection in allergen-mediated sensitization and disease, we used two different murine models: ovalbumin (OVA)-induced airway and skin inflammation. In both approaches, BALB/c mice were infected with *H. polygyrus* simultaneous to the first intraperitoneal sensitization with the model allergen OVA using alum as an adjuvant (Figs 1a and b), resulting in a chronic infection state and equal numbers of adult worms in Hp-infected groups at the time-point of dissection in both models (Fig. 1c). Mice were challenged with OVA either via the airways or via the skin by OVA-saturated skin patches (Figs 1a and b).

Both OVA sensitized and airway challenged mice (OVA group) and *H. polygyrus*-infected, sensitized and

challenged mice (OVA/Hp group) showed significantly increased total cell numbers in BALF in comparison with naïve mice (OVA $P=0.0095$; OVA/Hp $P=0.02$). However, OVA/Hp mice had lower total cell numbers in BALF than the OVA control group ($P=0.01$). Differentiation of total cells clearly showed that the increase in the OVA group was mainly due to a significant increase in eosinophils (OVA vs. naïve $P=0.0139$). In BALF of OVA/Hp animals, significantly less eosinophils than in OVA animals (OVA vs. OVA/Hp $P=0.03$) were detected, although still elevated in comparison with naïve mice ($P=0.019$, Fig. 2a). This was also true regarding lymphocytes in BALF, which were found to be significantly reduced in OVA/Hp animals compared with OVA animals ($P=0.0022$), although still increased when compared with naïve animals ($P=0.0131$). However, interestingly, the number of macrophages showed a strong trend of increase (OVA vs. OVA/Hp $P=0.06$) and regarding the percentage of macrophages of total BALF cells we found a significant increase in OVA/ Hp mice compared with OVA mice ($P=0.04$, data not shown).

In contrast, helminth infection without sensitization and airway challenges did not increase BAL cell numbers in comparison with the naïve group (data not shown). Measurements of AHR of OVA/Hp-mice revealed a trend towards a down-regulation (Fig. 2b). These data show that the infection with *H. polygyrus* has an inhibitory influence on some parameters of allergic airway disease and are in accordance to the data obtained by Kitagaki et al. (2006) and Wilson et al. (2005) [12, 13].

In parallel, we analysed the influence of intestinal worm infection on a non-mucosal allergic reaction in a murine model of allergen-induced AD. Interestingly, the CSSs of eczematous mice (OVA group) were not at all improved by the concomitant infection with *H. polygyrus* (Fig. 2c), but rather showed a tendency towards aggravation [mean skin scores: 3.0 ± 2 in sham-sensitized controls; 7.4 ± 1 in the OVA group; 8.4 ± 1 in the OVA/Hp group, (naïve vs. OVA $P=0.0008$; naïve vs. OVA/Hp $P=0.0001$)]. The thickness of the epidermis of atopic lesions was significantly elevated in both OVA and OVA/Hp groups compared with sham-treated mice (52 and $61 \mu\text{m}$ vs. $35 \mu\text{m}$; $P=0.001$; Fig. 2d) and even significantly thicker in the OVA/Hp group (OVA vs. OVA/Hp $P=0.0026$) as observed in histological sections of eczematous regions. In contrast, no effect on the epidermis could be determined in helminth-infected mice without allergic sensitization and dermal challenge (data not shown). Skin sections stained for CD4⁺ and CD8⁺ lymphocytes showed a significantly increased infiltration of both cell populations in OVA mice in comparison with naïve mice ($P<0.0001$; $P=0.0005$), which was significantly down-modulated in OVA/Hp mice (OVA vs. OVA/Hp $P=0.0012$, $P=0.005$; naïve vs. OVA/Hp $P=0.0001$, $P=0.0667$; Figs 2e and f). However, in contrast to the decrease in lymphocytes, the inflamed skin of OVA/Hp

mice contained significantly more mast cells in comparison with the skin of the other groups (OVA vs. OVA/Hp $P=0.0022$; naïve vs. OVA/Hp $P=0.0003$; Figs 2g and i). Regarding eosinophils, all groups of mice either treated or untreated displayed similar numbers in patched skin (Fig. 2h), arguing for only a minor role of eosinophils in this model of AD.

These data indicated that *H. polygyrus* infection induced significant changes in cell recruitment into the inflamed skin, but failed to inhibit the development of experimental AD in this model. It is possible that the persistence of eczema is owing to the increase in mast cell recruitment in OVA/Hp mice, leading to aggravation of allergen-induced dermatitis.

Infection with Heligmosomoides polygyrus diminishes allergen-specific antibody responses in both disease models

Analyses of allergen-specific and total IgE responses of mice in both disease models revealed that immunization with OVA induced significant levels of OVA-specific IgE, total IgE and OVA-specific IgG1 as compared with the control groups. In both allergy models, infection with *H. polygyrus* decreased the levels of allergen-specific IgE ($P=0.002$ asthma; $P=0.0079$ AD) as compared with OVA animals (Figs 3a and d; naïve vs. OVA: $P=0.0139$ asthma, $P=0.0357$ AD; naïve vs. OVA/Hp: $P=0.00139$ asthma, $P=0.0357$ AD). In contrast, the total IgE levels were significantly increased (OVA vs. OVA/Hp: $P=0.005$ asthma, $P=0.0159$ AD; naïve vs. OVA/Hp: $P=0.0095$ asthma, $P=0.0159$ AD; naïve vs. OVA: $P=0.0095$ asthma, $P=0.0286$ AD) in the OVA/Hp group (Figs 3b and e). Furthermore, sera of OVA/Hp-treated mice showed a significant decrease of allergen-specific IgG1 ($P=0.026$ asthma, $P=0.0043$ AD) as compared with the OVA group (Figs 3c and f; naïve vs. OVA/Hp: $P=0.0139$ asthma, $P=0.0357$ AD; naïve vs. OVA: $P=0.0139$ asthma, $P=0.0238$ AD). Allergen-specific IgG2a remained unaltered in the helminth-infected mice (data not shown).

These data show that in both allergy models, a concomitant infection with the gastrointestinal nematode significantly decreased serum levels of allergen-specific IgE, although total IgE remained high.

Alterations of cytokine patterns induced by Heligmosomoides polygyrus infection in both disease models

To assess the mechanism of Hp-induced immune modulation in the two disease models we analysed the cytokine production. Stimulation of splenocytes with allergen revealed reduced levels of IL-4 (naïve vs. OVA $P=0.0286$; naïve vs. OVA/Hp $P=0.0635$; OVA vs. OVA/Hp $P=0.0159$; Fig. 4a) and IL-10 production (OVA vs. OVA/Hp

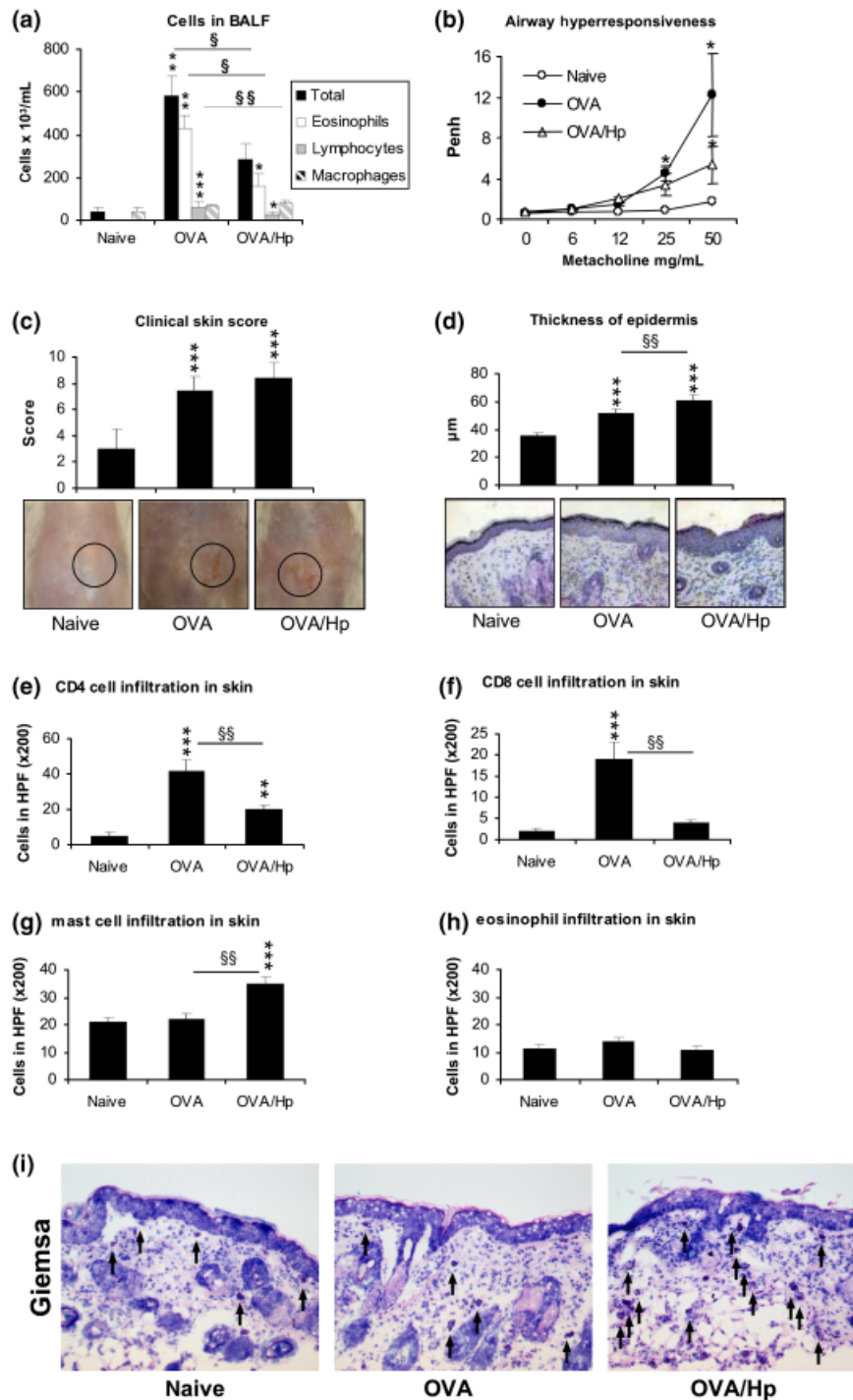


Fig. 2. Effects of the nematode infection on the asthma (a,b) and atopic dermatitis (AD) (c–i) model. (a) Total cells, eosinophils, lymphocytes and macrophages in bronchoalveolar lavage fluid (BALF), (b) airway hyperresponsiveness to increasing metacholine concentrations indicated as Penh (pause enhanced) values, (c) severity of skin eczema indicated as a clinical skin score and representative photos, (d) thickness of epidermis in micrometre (μm) and representative haematoxylin stainings, (e) CD4⁺ T cells, (f) CD8⁺ T cells, (g) mast cells and (h) eosinophils in patched skin sections; (e–h) cells in high-power field, 200-fold magnification. (i) Skin sections of the AD model stained for mast cells by Giemsa staining (mast cells indicated by arrows). Naive: animals sensitized and challenged with PBS; OVA: mice sensitized and challenged with ovalbumin; OVA/Hp: mice sensitized and challenged with ovalbumin and concomitantly infected with *Heligmosomoides polygyrus*. Data are shown as means with SE. Representative data of two independent experiments with five to six animals per group. Significance to naive: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$; significance between OVA and OVA/Hp: § $P < 0.05$, §§ $P < 0.005$.

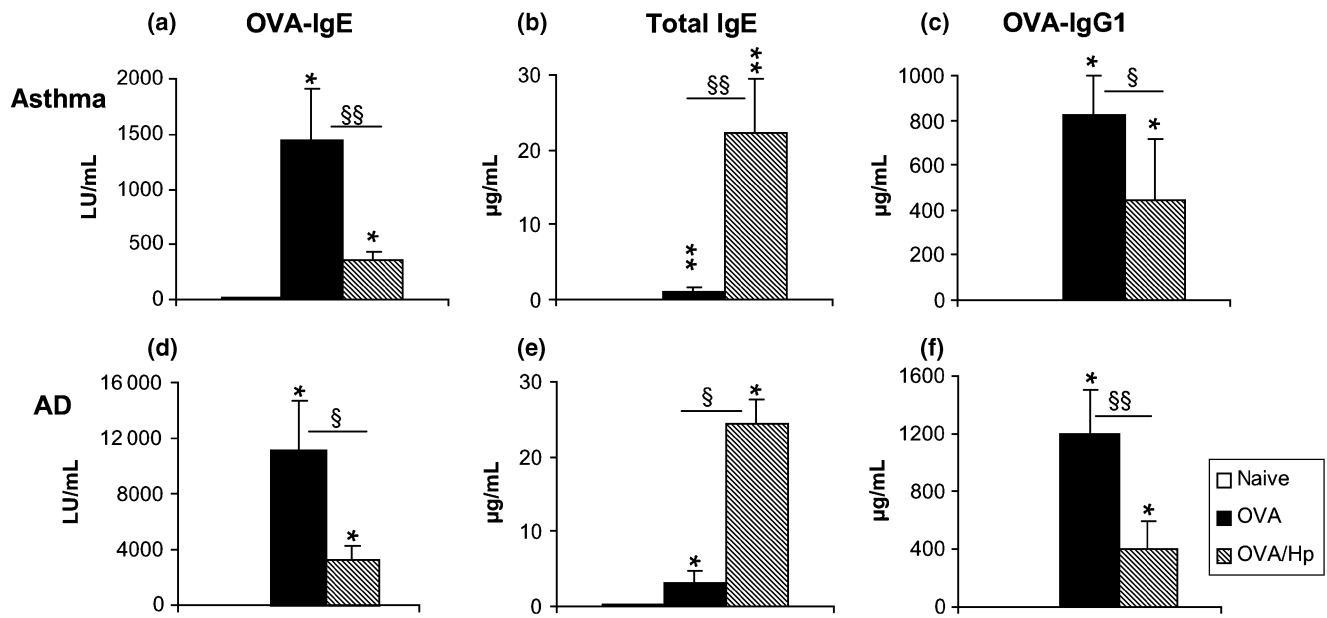


Fig. 3. Evaluation of serum antibodies in the asthma and the dermatitis (AD) model. (a–c) Asthma model: (a) OVA-specific IgE in lab units (LU), (b) total IgE, (c) OVA-specific IgG1. (d–f) Atopic dermatitis model (AD): (d) OVA-specific IgE in lab units (LU), (e) total IgE and (f) OVA-specific IgG1. Naïve: animals sensitized and challenged with PBS; OVA: mice sensitized and challenged with ovalbumin; OVA/Hp: mice sensitized and challenged with ovalbumin and concomitantly infected with *Heligmosomoides polygyrus*. Data are shown as means with SE. Representative data of two independent experiments with five to six animals per group. Significance to naïve: * $P < 0.05$, ** $P < 0.005$; significance between OVA and OVA/Hp: § $P < 0.05$, §§ $P < 0.005$.

$P = 0.0049$; Fig. 4c) in the OVA/Hp group in comparison with the OVA group in the asthma model (naïve vs. OVA or OVA/Hp $P = 0.0238$). No differences were found with regard to IL-5 (Fig. 4b). In contrast, in the AD model, the analyses revealed that OVA-specific cytokine production by spleen cells of AD animals did not differ between OVA and OVA/Hp mice, regarding the cytokines IL-4, IL-5 and IL-10 (Figs 4g–i). However, the OVA and OVA/Hp mice did show a significant increase in OVA-specific IL-4, IL-5 and IL-10 in comparison with naïve animals (naïve vs. OVA/Hp: IL-4: $P = 0.0057$, IL-5: $P = 0.0057$, IL-10: $P = 0.0025$; naïve vs. OVA: IL-4: $P = 0.115$, IL-5: $P = 0.0119$, IL-10: $P = 0.0317$). In contrast, stimulation of spleen cells of OVA/Hp animals with parasite antigen led to secretion of high amounts of IL-4 (naïve vs. OVA/Hp: $P = 0.0281$ asthma, $P = 0.0029$ AD; OVA vs. OVA/Hp: $P = 0.065$ asthma, $P = 0.0019$ AD), IL-5 (OVA vs. OVA/Hp: $P = 0.0043$ asthma, $P = 0.004$ AD; naïve vs. OVA/Hp: $P = 0.0357$ asthma, $P = 0.0033$ AD) and IL-10 (OVA vs. OVA/Hp: $P = 0.002$ asthma, $P = 0.0098$ AD; naïve vs. OVA/Hp: $P = 0.023$ asthma, $P = 0.0029$ AD), in both models, which were not observed in uninfected animals (Figs 4d–f, j–l). However, the levels of these cytokines remained lower in the AD than in the asthma model.

Measurements of IL-4, IL-5, IL-10 and TGF- β in the BAL fluid of mice, the site of inflammation, did not reveal a significant effect of *H. polygyrus* infection (data not shown). Cytokine mRNA measured in the inflamed skin

via real-time PCR showed a trend of up-regulation of IL-4 and IL-10 mRNA and down-regulation of TGF- β mRNA in the OVA/Hp group (data not shown).

Together, these data show that the alterations of systemic and local patterns of cytokines induced by the nematode infection were relatively similar in both disease models. However, the parasite-specific cytokine production was clearly Th2 biased and interestingly IL-10 was significantly higher in the asthma model as compared with the AD model.

Regulatory T cells are induced in mesenteric and peribronchial but not in skin-draining lymph nodes of Heligmosomoides polygyrus-infected mice

To analyse the role of Tregs in the differential outcome of disease, we determined CD4⁺ Treg frequencies according to the expression of the surface markers CD25⁺ and integrin α_E (CD103) β_7 in lymph nodes from the site of allergic inflammation and of infection. The expression of CD103 distinguishes Tregs with an inflammation-seeking phenotype [effector/memory-like (e/m) Tregs] from naïve-like naturally occurring Tregs only expressing CD25⁺ [32]. In the asthma model, analysis of peribronchial (PBLN) and mesenteric lymph node cells (MLN) revealed a significant increase in e/m Treg cells ($P = 0.0159$; $P = 0.02$) in OVA/Hp mice compared with the OVA group (Figs 5a–c; PBLN naïve vs. OVA $P = 0.036$; naïve vs. OVA/

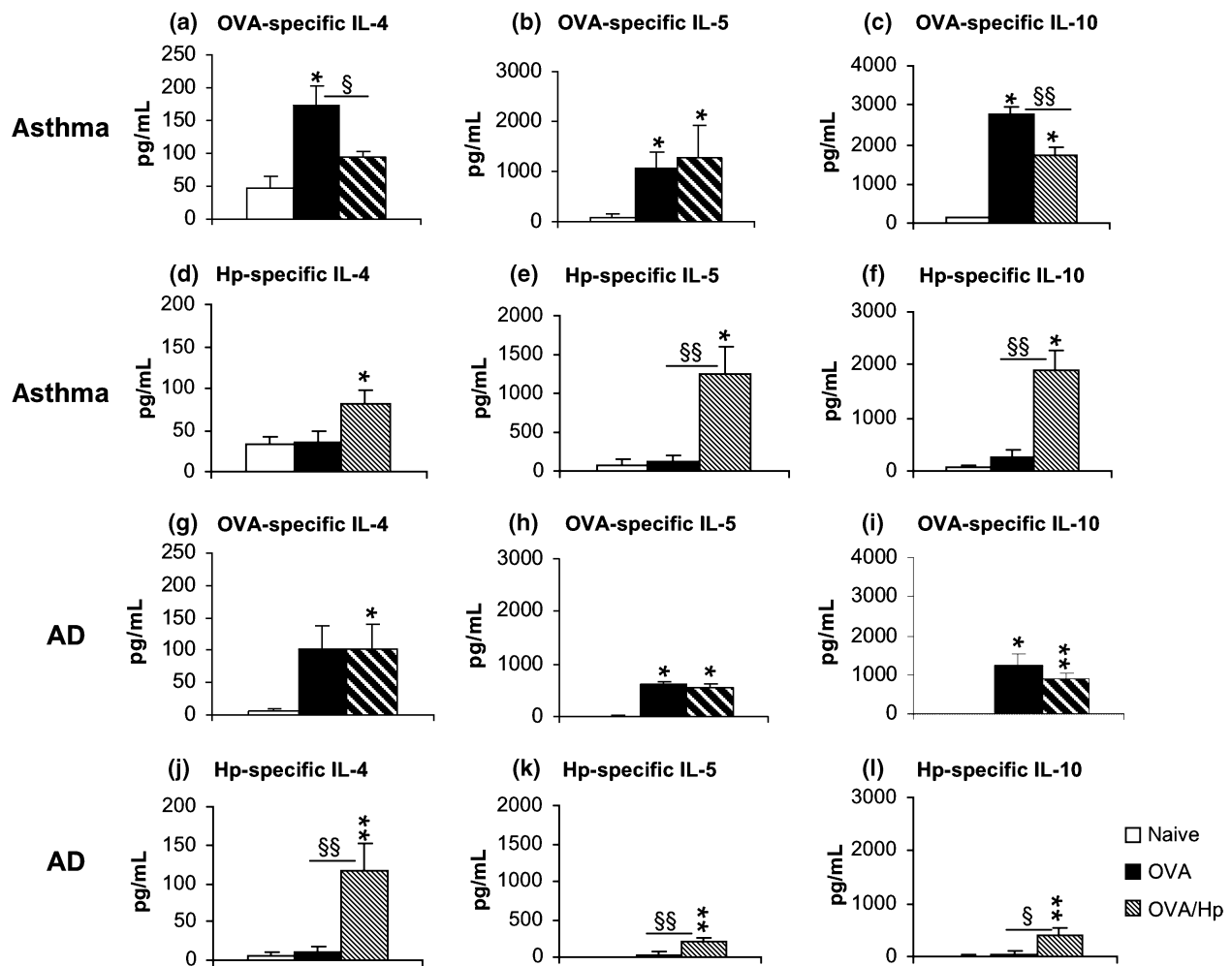


Fig. 4. Cytokine analyses of splenocyte cultures stimulated with ovalbumin (OVA) or nematode antigen (Hp) in the asthma (a–f) and the atopic dermatitis (AD) (g–l) model. (a, g) OVA-specific IL-4; (b, h) OVA-specific IL-5; (c, i) OVA-specific IL-10; (d, j) *Heligmosomoides polygyrus* (Hp)-specific IL-4; (e, k) Hp-specific IL-5; and (f, l) Hp-specific IL-10. Naive: mice sensitized and challenged with PBS; OVA: mice sensitized and challenged with ovalbumin; OVA/Hp: mice sensitized and challenged with ovalbumin and concomitantly infected with *H. polygyrus*. Data are shown as means with SE. Representative data of two independent experiments with five to six animals per group. Significance to naïve: * $P < 0.05$, ** $P < 0.005$; significance between OVA and OVA/Hp: § $P < 0.05$, §§ $P < 0.005$.

Hp $P = 0.029$; MLN naïve vs. OVA $P = 0.13$; naïve vs. OVA/Hp $P = 0.0286$). More than 95% of the so-defined Treg cells were confirmed to be Foxp3 positive (Fig. 5g). Hence, a significant increase of Treg cells in OVA/Hp mice did not only occur in mesenteric lymph nodes draining the site of infection, but was also found in lymph nodes draining the site of inflammation (peribronchial lymph nodes).

Regarding the numbers of CD4⁺CD25⁺CD103⁺ e/m Tregs in the AD model, we found a significant increase of Treg numbers in the mesenteric lymph node cells of OVA/Hp-treated animals, as compared with the OVA group ($P = 0.0043$; Figs 5d and f; naïve vs. OVA $P = 0.0476$; naïve vs. OVA/Hp $P = 0.0357$). However, in the skin-draining lymph node cells, Treg numbers of the OVA/Hp group were not elevated as compared with the OVA group (Figs 5d and e). Furthermore, histological sections of the

inflamed skin stained for Foxp3-expressing cells revealed a strong reduction of Foxp3⁺ cells in OVA-treated mice and their virtual absence in OVA/Hp mice (Fig. 5i). These results could be confirmed by an analysis of Foxp3 mRNA by real-time PCR with material of skin lesions (OVA vs. OVA/Hp $P < 0.05$, Fig. 5h). Hence, the data indicate that Tregs might play an important role in the amelioration of asthma but not dermatitis by intestinal helminths.

Discussion

Our study provides first evidence that parasite-induced immune modulation by gastrointestinal (GI) nematodes may be restricted to mucosa-associated tissues, whereas skin-associated diseases seem to remain mostly unaffected. Thus, in our study, a beneficial effect of the GI

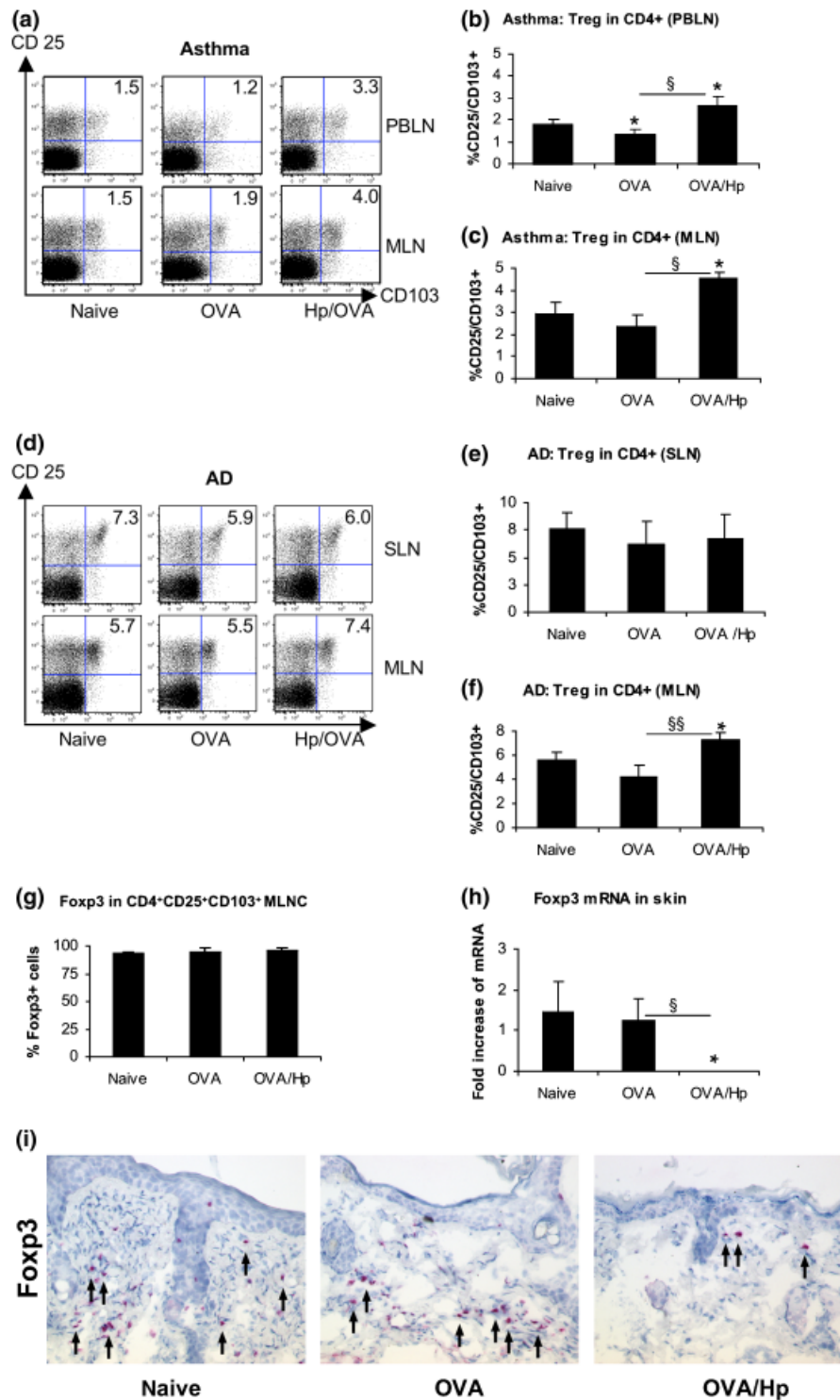


Fig. 5. Analysis of regulatory T cells (Treg) in the asthma and atopic dermatitis (AD) model. (a) Representative dot plots of flow cytometry analysis of CD4⁺CD25⁺CD103⁺ Tregs in peribronchial lymph node cells (PBLN) and mesenteric lymph node cells (MLN) in the asthma model, (b) mean values of Tregs in PBLN, (c) mean values of Tregs in MLN, (d) representative dot plots of flow cytometry analysis of CD4⁺CD25⁺CD103⁺ Tregs in skin-draining lymph node cells (SLN) and MLNs in the AD model, (e) mean values of Tregs in SLN, (f) mean values of Tregs in MLN, (g) representative mean values of Foxp3 expression in CD4⁺CD25⁺CD103⁺ Tregs in MLNs, (h) fold increase of Foxp3 mRNA compared with the naïve group in patched skin tissue samples of the AD model, (i) skin sections of the AD model stained for Foxp3-positive cells (red dots, Tregs indicated by arrows). Pictures of representative stainings are shown. Naïve: animals sensitized and challenged with PBS; OVA: mice sensitized and challenged with ovalbumin; OVA/Hp: mice sensitized and challenged with ovalbumin and concomitantly infected with *H. polygyrus*. Data are shown as means with SE. Representative data of two independent experiments with six to five animals per group. Significance to naïve: * $P < 0.05$; significance between OVA and OVA/Hp: § $P < 0.05$, §§ $P < 0.005$.

nematode *H. polygyrus* was only observed in an experimental model of airway inflammation, but not in a murine model of allergen-induced dermatitis.

Infecting mice with *H. polygyrus* at the time-point of first sensitization interfered with the allergic airway disease via several mechanisms. First, the recruitment of inflammatory eosinophils into the lungs was significantly reduced, thereby probably preventing the release of effector molecules that lead to tissue damage and development of AHR [37–39]. Furthermore, lymphocyte numbers in Hp-infected OVA-sensitized animals were significantly reduced, indicating reduced inflammation due to less Th2-related cytokine release and cell destruction in comparison with sensitized but uninfected mice [40]. Apart from this, interestingly, the percentage of macrophages was significantly increased in OVA/Hp mice compared with OVA mice. Macrophages were reported to play a pivotal role in the anti-inflammatory effect of a nematode immunomodulator and of a nematode infection in murine models of asthma and colitis [41, 42] and might also play a role in our setting. More importantly, worm-infected mice in the asthma model had increased numbers of Tregs. Such cells were described to reduce allergic airway disease by down-regulation of Th2-cytokines and allergen-specific IgE production after transfer to allergic mice [12, 13, 18].

In the model of AD, we detected decreased numbers of CD4 and CD8 T cells in skin lesions of OVA/Hp treated mice, concomitant with an influx of mast cells into the eczema in OVA/Hp animals. Decrease in lymphocytes would be expected to lead to reduced allergic skin inflammation in the chronic phase [43]; however, monitoring of clinical parameters such as the CSS and the thickness of epidermis rather showed aggravation than amelioration in OVA/Hp mice. Regarding this, it is quite likely that enhanced recruitment of mast cells may account for the aggravation of eczema in OVA/Hp mice due to increased activation and mediator release [44]. Eosinophils might also have been responsible for increased inflammation with decreased lymphocyte numbers [45]; however, the number of eosinophils was not altered in any of the investigated groups. Taken together, the recruitment of cells differed significantly in the two allergy models and may reasonably have accounted for the different outcomes of disease progression.

Second, *H. polygyrus* infection significantly decreased the levels of allergen-specific IgE in both allergy models, probably resulting in less efficient sensitization of mast cells and basophils [46]. The reason for this decrease might be a diminished IgE production, e.g. as a result of increased local production of IL-10. Alternatively, in the case of the dermatitis model, where mast cell numbers were massively increased in the inflamed skin, absorption of free serum IgE by the mast cells might have contributed to a reduction in the levels of allergen specific IgE. Thus,

OVA-specific serum IgE might have decreased due to different reasons in both models.

Third, the influence of *H. polygyrus* infection on allergen-specific cytokine production in both models was found to be quite similar, although the levels of cytokines measured in both models were different. In addition, in both disease models, *H. polygyrus* infection resulted in an overall parasite-specific Th2 milieu as detected by *H. polygyrus*-specific systemic induction of IL-4, IL-5 and IL-10. One mediator responsible for the down-regulation of asthma might be the anti-inflammatory cytokine IL-10. It has been shown that systemic production of IL-10 can interfere with the development of airway hyperresponsiveness and allergic inflammation [47, 48]. The importance of IL-10 in helminth-driven modulation of allergic responses has already been shown in reports of infection with *H. polygyrus* or the blood fluke *Schistosoma mansoni* [13, 15]. However, in AD IL-10 seems to enhance the disease: increased local IL-10 levels were found in AD skin lesions [49], and likewise antisense RNA against IL-10 was shown to improve eczema [50]. Such divergent roles of IL-10 in atopic immune responses may also explain the different outcomes of the GI nematode infection in the two models presented here. Thereby, increased systemic IL-10 production due to the helminth infection might have accounted for disease aggravation instead of amelioration in the AD model.

Several studies provide convincing evidence for a role of Tregs in helminth-induced amelioration of allergic airway disease [12, 13, 18, 51]. In accordance with data of a previous study on the role of Tregs in *H. polygyrus* infection [36], the present study shows that the frequency of e/m Tregs increased significantly in the mesenteric lymph nodes draining the site of helminth infection in both models of allergy. This Treg subset is characterized by the expression of the integrin α_E (CD103) β_7 and was shown to exhibit a particularly high suppressive capacity [32], especially in the context of *H. polygyrus* infection [36]. Such activated e/m Treg cells efficiently control inflammation in models of colitis, delayed-type hypersensitivity and arthritis, and migrate preferentially to inflamed tissue [52].

Interestingly, we detected a significant increase of e/m Tregs only in the peribronchial lymph nodes draining the lung in the asthma model, but not in the skin draining lymph nodes in the dermatitis model. The induction of organ-selective Treg homing strongly depends on the site of priming [53]. Hence, it is tempting to speculate that e/m Tregs induced or expanded by a concomitant nematode infection within the mesenteric lymph nodes also migrated to other inflamed mucosal tissues (and the relevant draining lymph nodes), and thereby interfered with allergic disorders of the asthmatic type. In contrast, parasite-induced Treg cells primed in mucosa-associated lymphoid organs might have failed to enter other compartments,

such as the skin, due to the lack of respective homing receptors for the entry of such sites. We hypothesize that Tregs induced or activated in the context of the nematode infection express a homing receptor pattern beneficial for the entry to the inflamed mucosal site and thereby impair the further development of airway inflammation. This may also explain why AD patients infected with *Trichuris trichiura* (strictly enteric life cycle) rather suffer from aggravation of the disease [54], and why infection with *Enterobius vermicularis* (strictly enteric life cycle) but not with *A. lumbricoides* (life cycle with migratory phase through host) was associated with an increased risk of AD [55].

In conclusion, our data suggest that infection with the GI nematode *H. polygyrus* leads to amelioration of allergic airway inflammation but does not diminish AD. A lack of Tregs at the site of inflammation in the AD model and boost of Treg numbers in the draining lymph nodes in the asthma model point to the importance of Tregs in helminth immunomodulation as indicated by several other studies [12, 13, 18]. The production of the regulatory cytokine IL-10 might lead to reduced inflammation in the lung, but may even enhance inflammation in atopic eczema. Hence, our data show clear differences in immune cell recruitment and cytokine production in response to nematode infection in the different disease models. These variations may explain the seeming contradictions between reports on the effects on worm infections on allergic and inflammatory diseases, and should be considered in the design of eventual clinical studies aiming at the treatment of allergic diseases.

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