Protection against allergic airway inflammation during the chronic and acute phases of *Trichinella spiralis* infection

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Summary

Background Modulation of the host immune response by helminths has been reported to be essential for parasite survival and also to benefit the host by suppressing inflammatory diseases such as allergies. We have previously shown that excretory-secretory products of *Trichinella spiralis* muscle larvae have immunomodulatory properties and induce *in vitro* the expansion of $CD4^+CD25^+FOXP3^+$ Treg cells in a TGF-β-dependent manner.

Objective We aimed at determining the effect of the acute (intestinal) and the chronic (muscle) phase of *T. spiralis* infection on experimental allergic airway inflammation
(EAAI) to Ovalbumin (OVA) and the involvement of Treg cells.

Methods The chronic phase was established before OVA-sensitization/challenge and the acute phase at two-time points, before and after OVA-sensitization. Mice were infected with 400 *T. spiralis* larvae and after euthanasia different pathological features of EAAI were measured. Adoptive transfer of $CD4^+$ T cells from *Trichinella* infected mice to OVA sensitized/challenged recipients was also performed.

Results We found that the chronic as well as the acute phase of *Trichinella* infection sup-press EAAI as indicated by reduction in airway inflammation, OVA-specific IgE levels in sera, Th2-cytokine production and eosinophils in bronchoalveolar lavage. This protective effect was found to be stronger during the chronic phase and to be associated with increased numbers of splenic $CD4^+CD25^+FOXP3^+$ Treg cells with suppressive activity. Adoptive transfer of splenic $CD4^+$ T cells from chronically infected mice with elevated numbers of Treg cells resulted in partial protection against EAAI.

Conclusions and Clinical Relevance These results demonstrate that the protective effect of *T. spiralis* on EAAI increases as infection progresses from the acute to the chronic phase. Here, Treg cells may play an essential role in the suppression of EAAI. Elucidating the mechanisms and molecular helminth structures responsible for this regulatory process is relevant to develop alternative tools for preventing or treating allergic asthma.

Keywords allergy, experimental allergic airway inflammation, helminths, immunomodula-tion, Tregs, *Trichinella spiralis*

Introduction

Helminthic infections and allergies are characterized by a dominant Th2-type of immune response that mediates high levels of circulating IgE antibodies and eosinophilia. Even though in general a common type of immune responses is induced, an inverse correlation between certain helminth infections and allergy has been observed [1].

A series of epidemiological findings indicate that infection with helminths such as *Schistosoma spp*., *Necator americanus* and *Brugia malayi* is associated with

reduction of allergic manifestations in humans [2–5]. Using murine models it has also been shown that certain helminths can suppress experimental allergic airway inflammation (EAAI) and that regulatory T (Treg) cells play an essential role in this process [6–8]. In addition, helminth-induced alternatively activated macrophages [9] and regulatory B cells [10, 11] have also been reported to down modulate immunopathologies. Creating an immunosuppressive network appears to be a strategy of helminths to survive in their host and its effect can be extended to third-party antigens such as allergens.

Different factors, however, may influence whether helminth infections protect or exacerbate allergic manifestations. These factors include the helminth species and whether humans are definitive or accidental hosts [12]. We have previously shown that infection with *Toxocara* canis, a roundworm of dogs that can also infect humans exacerbates EAAI [13]. As humans are accidental hosts for *Toxocara* infections, we were interested in determining the effect on allergic manifestations by a helminth that not only infects humans but also mice and completes its life cycle in a single host (definitive host). For this reason, we chose *Trichinella spiralis* for this study. After ingestion of *T. spiralis* infected meat the larvae are released in the stomach and migrate to the small intestine, where they mature into adult worms. Female worms release within a week after infection, newborn larvae that rapidly disseminate throughout the host, and eventually enter skeletal muscles to remain there for many years [14]. Trichinellosis is characterized by two phases; the acute enteral (intestinal) phase that begins few days after ingestion of infected meat and the chronic (muscle) phase that occurs weeks after infection. We have shown that the excretory-secretory (ES) products of this helminth's muscle larvae have immunomodulatory properties as indicated by its suppressive effect on dendritic cell maturation *in vitro* [15]. In addition, using splenocytes derived from OVA (ovalbumin)-TCR transgenic D011.10 mice, we showed that *T. spiralis* ES products (TspES) induce *in vitro* the expansion of $CD4^+CD25^+FOXP3^+$ Treg cells in a TGF-β-dependent manner [16]. Studies by others have shown that IL-10 and TGF-β control the level of inflammation induced by *T. spiralis* especially during the chronic phase of infection [17]. Modulation of the immune response by *T. spiralis*, protecting the host against other immunopathologies such as experimental autoimmune encephalomyelitis and experimental colitis has been demonstrated [18, 19]. In this study, we aimed at determining the effect of the acute vs. the chronic phase of *T. spiralis* infection on EAAI to OVA and the involvement of Treg cells. Here, the acute phase was established at two time points, before and after OVA-sensitization.

Our data show that the chronic as well as the acute phase of *Trichinella* infection protect against EAAI. This protective effect was found to be stronger as the infection progressed to the chronic phase and to be associated with increased numbers of $CD4^+CD25^+FOXP3^+$ T cells in the spleen. The Treg cells induced after *T. spiralis* infection were shown to have suppressive activity and adoptive transfer of $CD4^+$ T cells from the spleen of chronic infected mice containing elevated numbers of Treg cells resulted in partial protection against EAAI. All together these findings indicate that *T. spiralis* suppresses OVA-induced EAAI which increases as infection progresses. Furthermore, Treg cells may play an essential role in this suppression.

Materials and methods

Animals and parasites

Six- to 10-week-old BALB/c female mice (Harlan, Zeist, the Netherlands) were housed under specific pathogen-free conditions at the animal care facility of the National Institute for Public Health and the Environment (RIVM). All animal experiments were approved by the Committee on Animal Experimentation of the RIVM (permit numbers 201100247, 201100193 and 201100119).

Infection with Trichinella spiralis *and induction of experimental allergic airway inflammation*

Mice were either infected with *T. spiralis* muscle larvae, treated (sensitized/challenged) with OVA to induce EAAI or the combination of both. Infection was performed by oral administration of 400 *T. spiralis* larvae per mouse in 500 μL sterile phosphate-buffered saline (PBS) using a syringe fitted with a blunt needle. The EAAI was induced by sensitizing/challenging mice with OVA as previously described by Smits et al. [10]. Briefly, mice were first sensitized on days 0 and 5 by intraperitoneal (i.p.) administration of 10 mg OVA (grade V, Sigma-Aldrich, Steinheim, Germany) adsorbed onto 2.25 mg alum (aluminium hydroxide; Pierce, Thermo Scientific, Rockford, IL, USA) in 100 μL PBS (OVA/Alum). To induce allergic airway inflammation, OVA-challenges were given on days 15, 16 and 17 days after sensitization by exposing the mice for 30 min to OVA/PBS aerosol (1%v/v) that was generated by a nebulizer (Ultra-NebTM2000; DeVilbiss, Langen, Germany). Control uninfected mice received alum i.p. and only PBS during aerosol challenge. The effect of established chronic or acute phases of *T. spiralis* infection on EAAI was investigated by infecting mice 25 days (− 25 days + OVA) or 5 days (− 5 days + OVA), respectively, before OVA-treatment (sensitization/challenge). To determine the effect of the acute phase of infection on the onset of EAAI, mice were infected 12 days (+ 12 days + OVA)

after OVA-sensitization (Fig. 1), which was three days before the OVA-challenge and 5 days before mice were killed. Three other groups of mice were infected with *T. spiralis* (without OVA-treatment) at the same time points described above and are referred as − 25 days , − 5 days and + 12 days. In total, there were 8 groups of 8 mice each: One uninfected PBS/alum-treated control group, one OVA-treated group, three *T. spiralis*-infected (− 25 days, − 5 days and + 12 days) groups and three groups that received both infection and OVA-treatment (− 25 days + OVA, − 5 days + OVA and + 12 days + OVA). Mice from all groups were killed on day 18 as indicated in Fig. 1. It is important to mention that as *T. spiralis* establishes persistent infection, the infected animals from group − 25 days, − 5 days and + 12 days treated or not with OVA were by the time they were killed, 43, 25 and 5 days infected. To verify that mice were infected with *T. spiralis*, adult parasites and larvae were counted in the intestines and carcasses of the infected mice. Carcasses were digested by overnight incubation with 4.3% hydrochloric acid and 0.9% pepsin in PBS at 37°C to obtain the larvae [20]. Intestines were incubated for 2–4 h with PBS containing 0.85% sodium chloride at 37°C to release adult worms [21]. Adults and larvae where counted by light microscopy. After 43 and 25 days of *T. spiralis* infection, larvae were only present in the carcass and no adults were present in the intestine of infected mice indicating the establishment of the muscle (chronic) phase of infection. After 5 days of infection, only adult worms were observed in the intestine, while no muscle larvae were found in the carcass indicating the establishment of the intestinal (acute) phase of infection.

Analysis of bronchoalveolar lavage cells

The bronchoalveolar lavage (BAL) fluid was harvested after mice were killed by injecting 1 mL PBS with a syringe through the tracheal tube into the airways and carefully recovering the fluid by aspiration with the same syringe. The BAL fluid was centrifuged at 400 × g, at 4°C, for 10 min. The supernatant was stored at −20°C for cytokine determination and the cell pellet was re-suspended in 1 mL PBS. Total cell numbers were determined by mixing 0.5 mL of the cell suspension with 9.5 mL Isoton II (Beckman Coulter BV, Mijdrecht, the Netherlands) and counted using a cell counter (Beckman Coulter). Cytospins using the cell suspension were made, and differential cell counts were performed after staining with May–Grünwald–Giemsa. At least 400 cells per slide were counted by light microscopy.

Histology of the lungs

After harvesting the BAL fluid, the lungs were perfused via the right cardiac ventricle by injection of 10 mL PBS and thereafter removed. The right lung was fixed with 10% neutral-buffered formalin and embedded in paraffin (Kendall; Tyco Healthcare, Boston, MA, USA). Transverse sections of 3 μm were stained with haematoxylin–eosin and pathological changes (peribronchiolar inflammation, perivascular inflammation, hypertrophy and hyperplasia of goblet cells) were blindly scored by board certified veterinary pathologists using a semiquantitative scoring system from absent (0), minimal (1), slight (2), moderate (3) to marked (4). Sections were also stained with periodic acid schiff reagent to identify goblet cells and determine mucus production.

Total IgE and OVA-specific IgE

Total serum IgE antibodies were measured by using the OptEIATM Set according to the manufacturer's recommendations (BD Pharmingen, San Diego, CA, USA). Determination of OVA-specific IgE levels was done using the OVA-IgE ELISA kit according to the manufacturer's recommendations (AbD serotec, Oxford, UK).

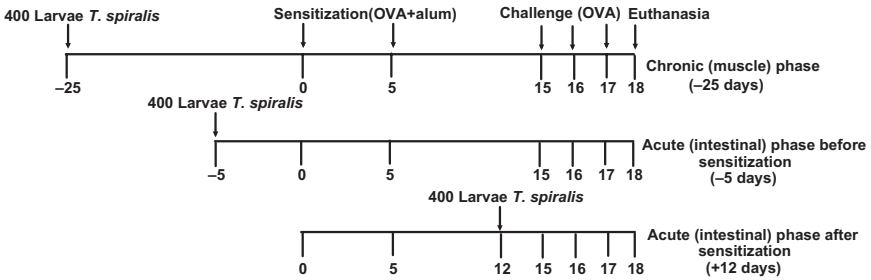

Fig. 1. Schematic representation of the experimental design. The chronic phase of *Trichinella* infection was established 25 days (− 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (− 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. OVA-sensitization was done on days 0 and 5 and OVA-challenges on days 15, 16 and 17 followed by euthanasia on day 18.

Flow cytometric analysis

Splenocytes were stained for 30 min at 4°C with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen, Carlsbad, CA, USA). Next, cells were pre-incubated with Fc receptor blocking antibodies (purified CD16/CD32) (BD Pharmingen) for 10 min at 4°C. Anti-CD4-APCH7 and anti-CD25-PerCPCy5.5 (BD Pharmingen) were added to the cells and incubated for 30 min at 4°C. For intracellular staining, fixation and permeabilization of the cells were performed according to the manufacturer's recommendation (eBioscience, Frankfurt, Germany). Then, blocking antibodies were added for 10 min followed by addition of anti-FOXP3-APC (Bioscience, San Diego, CA, USA) for 30 min at 4°C. Cells (5 x 10^5 events) were acquired and analysed on a FACSCanto II (BD Biosciences, Erembodegem, Belgium). Data were analysed using the FlowJo software (version 7.6, Tree Star Inc, Ashland, OR,USA).

In vitro *suppression assay*

Suppression assays were carried out as previously described [16]. Briefly, 5 x 10^4 $CD4^+CD25^+$ T cells isolated from 4 pooled spleens per group were cultured in 96-well round-bottom plates (Corning) in complete RPMI-1640 medium (Invitrogen) with 5 x 10^4 carboxyfluorescein succinimidyl ester (CFSE)-labelled effector T cells ($CD4^+CD25^-$) from naïve BALB/c mice. As antigen presenting cells, naïve bone marrow-derived dendritic cells (BM-DC) (1 x 10^5) were added in the presence of 2.5 μg/mL Concanavalin-A (Con-A) (Sigma-Aldrich, St. Louis, MO, USA) during 4 days. The BM-DC was collected from killed naive mice by flushing femurs and tibiae with PBS (Invitrogen) as previously described [14]. These cells were re-suspended at 2.5 × 10^5 cells/mL in complete RPMI-1640 medium containing 1% penicillin/streptomycin, l% glutamine (Gibco-Invitrogen), 50 μM β-mercaptoethanol (Invitrogen) and 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, CA, USA). Granulocyte–macrophage colony-stimulating factor (GM-CSF ; Cytocen, Utrecht, the Netherlands) at 20 ng/mL was added on days 0, 2 and 4. On day 7, cells were ready to be used. Isolation of $CD4^+CD25^+$ T cells from spleen cells was done by using the Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, $CD4^+$ T cells were purified by depletion of non-$CD4^+$ cells with negative selection. From purified $CD4^+$ T Cells, $CD25^+$ T cells were isolated by positive selection of $CD4^+CD25^+$ regulatory T cells using $CD25^+$ magnetic microbeads. The purity of the $CD4^+CD25^+$ Treg cell and $CD4^+CD25^-$ T effector cell population was typically 90% and 85% respectively. Effector T cells derived from the spleen of naïve BALB/c mice were also isolated by using the Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH). The effector $CD4^+CD25^-$ cells were labelled at 10^6 cells/mL in PBS containing CFSE at a concentration of 2 μM for 10 min at 37°C. Cells were washed and added to the suppression assays. Proliferation of effector cells was measured by flow cytometry using a FACSCanto II (BD Biosciences). The percentage of cell division was calculated using the FlowJo software (Tree Star Inc.).

Cytokine determination

The concentration of IL-2, IL-4, IL-5, IL-10, IL-17 and IFN-γ in BAL fluid and culture supernatants from lung-draining mediastinal lymph node cells were measured on the Luminex 100 (Luminex, Austin, TX, USA) using a Bio-Plex assay (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

Adoptive transfer of $CD4^+$ T cells

Spleens from mice chronically infected (43 days) with *T. spiralis* were aseptically collected and $CD4^+$ T cells were isolated using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec GmBH) according to manufacturer's instructions. $CD4^+$ T cells from uninfected mice were isolated using the same protocol. The purity of the isolated $CD4^+$ T cells was > 80% based on flow cytometry. For adoptive transfer, isolated $CD4^+$ T cells were first washed in PBS, and 5 × 10^6 cells were injected into the tail vein of naive mice. Adoptive transfer for each recipient mouse was performed one day before each OVA-challenge.

Culture of lymph node cells

Freshly removed lung-draining lymph nodes were pooled from 4 mice per group. These lymph nodes were dispersed by pressing them through a cell strainer using a plunger. Erythrocytes were removed by resuspending the lymph nodes cells in 4 mL RBC lysis buffer (Biolegend, San Diego, CA, USA) and incubating for 5 minutes on ice. The lymph nodes cells were washed and re-suspended in RPMI-1640 containing 1% penicillin/streptomycin, l% glutamine (Gibco-Invitrogen), 50 μM β-mercaptoethanol (Invitrogen), 10% fetal bovine serum (FBS) (Gibco-Invitrogen). Cells were cultured in triplicate in 96-well round-bottomed plates (Corning) at 5 × 10^5 cells/well in complete RPMI-1640 with or without OVA. After 5 days, supernatants were collected and stored at − 20°C for cytokine analysis.

Statistical analyses

One-way analysis of variance (ANOVA) was performed by the Bonferroni's Multiple Comparison test to analyse differences in means between different groups. For anal-

ysis of the histological scores of the lungs, the Kruskal–Wallis-Dunn's Multiple Comparison test was used. Analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Chronic phase of T. spiralis *infection reduces OVA-IgE serum levels in EAAI*

To determine the effect of *Trichinella* infection on EAAI, total IgE and OVA-specific IgE levels were measured in sera taken from all mice at the time they were killed. Fig. 2A shows that the levels of total IgE in OVA-treated mice were higher than those in control mice. *Trichinella* infection resulted for all three group of animals (− 25 days, + 5 days and + 12 days) in increased levels of total IgE, which were higher than that of the OVA-treated animals but not significantly different from that of mice that were both infected and OVA-treated. Interestingly, the level of OVA-specific IgE was significantly lower in the group of mice that were chronically infected and OVA-treated (− 25 days + OVA) compared to mice that were OVA-treated only. Acute *Trichinella* infection either 5 days (− 5 days + OVA) before or 12 days (+ 12 days + OVA) after OVA-sensitization did not affect the levels of OVA-specific IgE.

Acute and chronic phases of T. spiralis *infection before OVA sensitization reduces BAL-Th2 cytokine in EAAI*

To determine the effect of *T. spiralis* infection on EAAI, local cytokines in BAL fluid were measured at day 18 after OVA-treatment. The different cytokines measured in BAL of infected animals only were not significantly different from those of the control group. In contrast, BAL from the OVA-treated mice showed significantly higher levels of IL-2, IL-4, IL-5 and IL-10 (Fig. 3) compared with the control group. The concentrations of these cytokines were significantly lower in the mice infected either 25 (− 25 days + OVA) or 5 (− 5 days + OVA) days before OVA sensitization compared with the OVA-treated mice. No significant changes in the levels of these cytokines were observed in BAL of animals infected 12 (+ 12 days + OVA) days after OVA sensitization. The levels of the cytokines IL-13, IL-17, IFN-γ and TGF-β were not significantly different among the different groups (data not shown).

Acute and chronic phases of T. spiralis *infection reduces airway eosinophilia and lung pathology in EAAI*

Differential counting of cells in BAL revealed that infection with *T. spiralis* either 25 days or 5 days before as well as 12 days after OVA sensitization resulted in a significant decrease in the number of eosinophils in BAL compared with mice that were OVA-treated only (Fig. 4). Other cells (macrophages, neutrophils and lymphocytes) were also counted; however, their numbers were not significantly different compared with the OVA-treated animals (data not shown). Figure 5A shows that no lung tissue inflammation (upper row) and production of mucus (lower row) were found in the *Trichinella* infected mice or in the uninfected PBS/alum-treated control animals. In contrast, mice

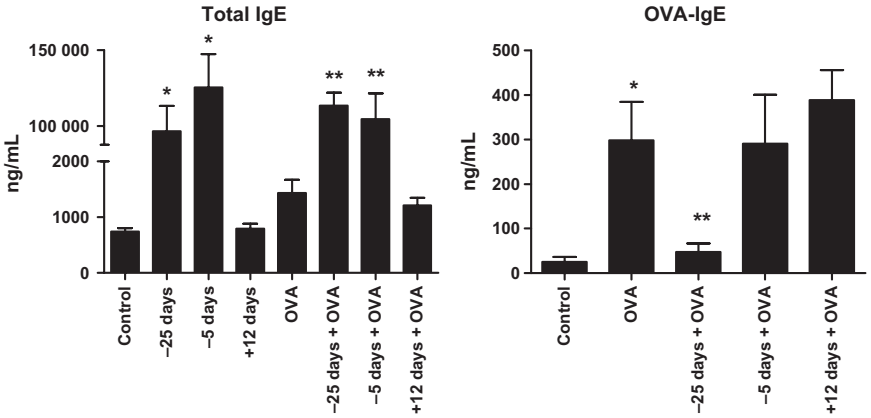

Fig. 2. Effect of *Trichinella spiralis* infection on OVA-IgE serum levels in experimental allergic airway inflammation. The chronic phase of *Trichinella* infection was established 25 days (− 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (− 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. On day 18, blood was collected and total IgE or OVA-specific IgE levels in serum were determined by means of ELISA. OVA-specific and total IgE levels for the different groups (8 mice/group) are represented as mean (ng/mL ± SE). Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from control group. **Significantly different ($P < 0.05$) from OVA group.

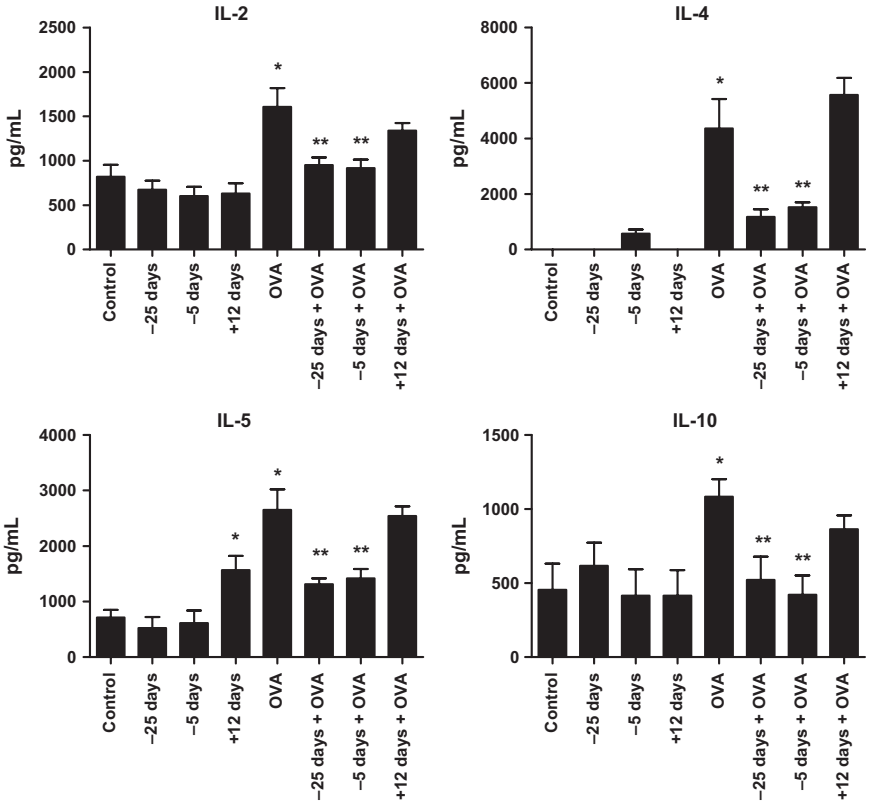

Fig. 3. Effect of *Trichinella spiralis* infection on bronchoalveolar lavage (BAL)-cytokines in experimental allergic airway inflammation. The chronic phase of *Trichinella* infection was established 25 days (– 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (– 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. On day 18, BAL was collected and the cytokine levels measured using a multiplex bead-based assay. Cytokine levels for the different groups (8 mice/group) are represented as mean (pg/mL ± SE). Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from control group. **Significantly different ($P < 0.05$) from OVA group.

treated with OVA showed multifocal cellular infiltrates around bronchiole and blood vessels. These peribronchiolar and perivascular cell infiltration were composed of many eosinophilic and neutrophilic granulocytes as well as lymphocytes (Fig. 5B, upper row). Furthermore, there were increased numbers of mucin-containing goblet cells present at the respiratory surface of bronchi and bronchiole, indicating an increase in mucus production (Fig. 5B, lower row). In mice infected 25 days (– 25 days + OVA) before OVA-sensitization, tissue inflammation after OVA-challenge was greatly reduced with significantly less peribronchiolar and perivascular cellular infiltration compared with OVA-treated mice (Fig. 5C). This effect was also observed in mice infected 5 days (– 5 days + OVA) before OVA-sensitization however less compared with mice that were infected 25 days before OVA-sensitization. A decrease in pulmonary inflammation for mice that were infected 12 days (+ 12 days + OVA) after OVA-sensitization was also observed; however, it was not significantly different compared with the group of uninfected OVA-treated mice (Fig. 5).

Acute and chronic phases of T. spiralis *infection induce functional regulatory T cells in EAAI*

To determine whether *Trichinella* infection induce functional Treg cells, we analysed the expression of CD4, CD25 and FOXP3 on splenocytes of infected mice. Flow cytometric analysis revealed that the proportion of $CD4^+25^+FOXP3^+$ T cells in $CD4^+$ gated splenocyte populations was significantly elevated in chronically infected (– 25 days) mice compared with the control group. The number of these cells in the other two groups of infected PBS/alum-treated mice (– 5 days and + 12 days) were not significantly different from the control group (Fig. 6A). Interestingly, the proportion of Treg cells in splenocytes of all infected OVA-treated mice was significantly higher compared with the OVA-treated mice only. The proportion of Treg cells in splenocytes of mice infected 25 days (– 25 days + OVA) before OVA-sensitization was the highest (28%) compared with the other OVA-treated/infected groups: 18% (– 5 days + OVA) and 16% (+ 12 days + OVA). To determine whether the

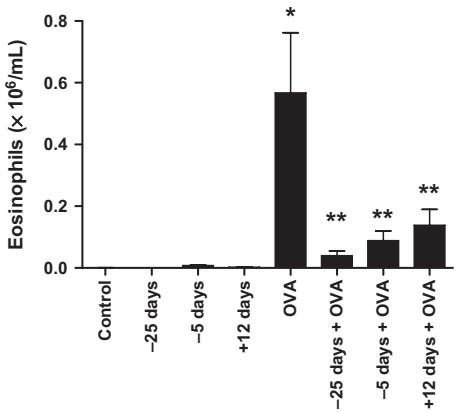

Fig. 4. Effect of *Trichinella spiralis* infection on bronchoalveolar lavage (BAL)-eosinophils in experimental allergic airway inflammation. The chronic phase of *Trichinella* infection was established 25 days (– 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (– 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. BAL fluid was collected for eosinophil count. Data for the different groups (8 mice/group) are presented as bars indicating mean values (cells/mL ± SE). Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from group control. **Significantly different ($P < 0.05$) from group OVA.

$CD4^+CD25^+$ Treg cells induced during infection are functional Treg cells in terms of suppressive capacity, functional assays were performed. For this purpose, $CD4^+CD25^+$ cells were purified from the spleens of control, OVA-treated and infected OVA-treated mice and incubated with CFSE-labelled effector cells (EC: $CD4^+CD25^-$) and DC in the presence or absence of ConA. Figure 6B shows a decrease in the effector cell proliferation from 89.9%, when these cells where incubated with Treg cells from control mice, to 67.5% when effector cells were incubated with Treg cells from mice infected 25 days before OVA-sensitization.

Adoptive transfer of $CD4^+$ T cells from T. spiralis *infected mice reduces EAAI*

To determine whether $CD4^+$ T cells from *T. spiralis* chronically infected mice containing high proportion of Treg cells, protect against EAAI, adoptive T cell transfer was performed. For this purpose, $CD4^+$ T cells isolated from the spleens of chronically *T. spiralis* infected or uninfected mice were transferred to OVA-treated mice one day before each OVA-challenge. Figure 7(A and B) shows that the numbers of eosinophils and IL-5 levels in BAL were significantly reduced in OVA-treated mice that received $CD4^+$ T infected cells from chronically infected animals, compared with OVA-treated mice and OVA-treated mice that received $CD4^+$ T cells from uninfected animals. The levels of all other tested cytokines in BAL were not significantly different between these groups of animals (data not shown). Neither the levels of total IgE nor of OVA-IgE in sera of the OVA-treated recipient animals were affected by the $CD4^+$ T cell transfer (data not shown). Histological examination of lungs (Fig. 7C, upper row) indicated that transfer of $CD4^+$ T cells from infected mice to OVA-treated mice partially reduced cell infiltration around the bronchiole and vessels. The mucus production was also reduced in OVA-treated mice that received $CD4^+$ T cells from infected mice (Fig. 7C, lower row). This result is summarized in the Fig. 7D, which shows that peribronchiolar cellular infiltration was reduced in OVA-treated mice that received $CD4^+$ T cells from infected mice. Perivascular cellular infiltration was also lower in mice that received $CD4^+$ T cells from infected mice, but it was not significantly different from mice that received $CD4^+$ T cells from uninfected mice.

Adoptive transfer of $CD4^+$ T cells from T. spiralis *infected mice suppresses OVA-specific cytokine production in lung draining lymph nodes in EAAI*

To determine the effect of the adoptive $CD4^+$ T cell transfer from *Trichinella* infected to OVA-treated mice on cytokine production, lung-draining lymph nodes cells from the different groups were incubated with or without OVA for 5 days. Figure 8 shows that the levels of IL-4, IL-13 and IL-10 measured in the culture supernatants resulted as expected in increased levels of these cytokines in response to OVA. Interestingly, these cytokines were reduced to the background levels of the control mice when $CD4^+$ T cell from spleens of *T. spiralis* chronically infected mice were given. No significant changes in cytokine levels were observed when mice received $CD4^+$ T cells from uninfected mice. It is noteworthy to mention that for the OVA-treated animals the levels of these cytokines in medium only were higher compared to the control uninfected PBS/alum treated mice. These increased background levels are probably due to the OVA sensitization and challenge treatment.

Discussion

Studies with certain helminths have shown that infection with these parasites can protect against allergic diseases. The inverse association between helminth infection and allergy often involves regulatory T (Treg) cells, which has given a new mechanistic basis to the *hygiene hypothesis* based on their role in dampening both Th1 and Th2 effectors responses. Several

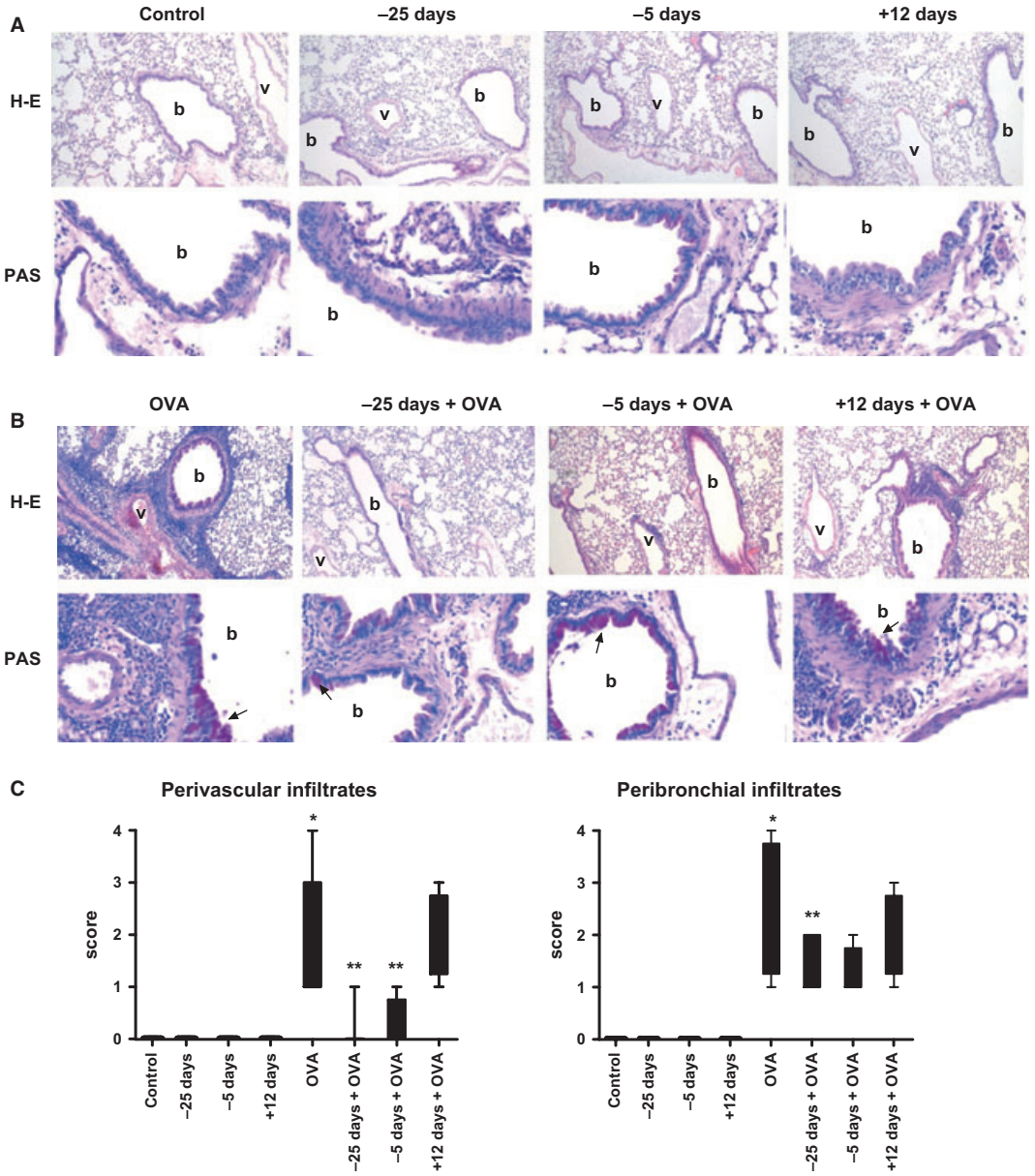

Fig. 5. Effect of *Trichinella spiralis* infection on pulmonary inflammation in experimental allergic airway inflammation. The chronic phase of *Trichinella* infection was established 25 days (– 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (– 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. Lungs were fixed with formaldehyde and sections were cut and stained with H–E (A) and periodic acid schiff (B). Arrows indicate the mucin-filled cells (bright-fuchsia) and the bronchiole (b) and vessels (v) are shown. These figures represent the average of the histological scores. (C) The perivascular and peribronchial infiltrates in histological sections of the lung were blindly scored using a semiquantitative scale from absent (0), minimal (1), slight (2), moderate (3) to marked (4). Data for the different groups (8 mice/group) are presented as box and whiskers indicating the minimum and maximum values. Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from group control. **Significantly different ($P < 0.05$) from group OVA.

epidemiological studies support this inverse association between helminth infections and allergic disease in humans [2, 12, 22], and using murine models several groups have shown that certain helminths can reduce allergic responses [6, 8, 10, 23, 24].

In this study, mice infected with *T. spiralis* at three different time points were sensitized and challenged with OVA allergen to determine the effect of the different phases of infection on EAAI. We observed that events occurring during the muscle or chronic phase of *T. spiralis* infection protect against EAAI. Infected mice showed significantly reduced levels of OVA-specific IgE in serum, decreased levels of Th2 cytokines and low numbers of eosinophils in BAL. Histological analysis of

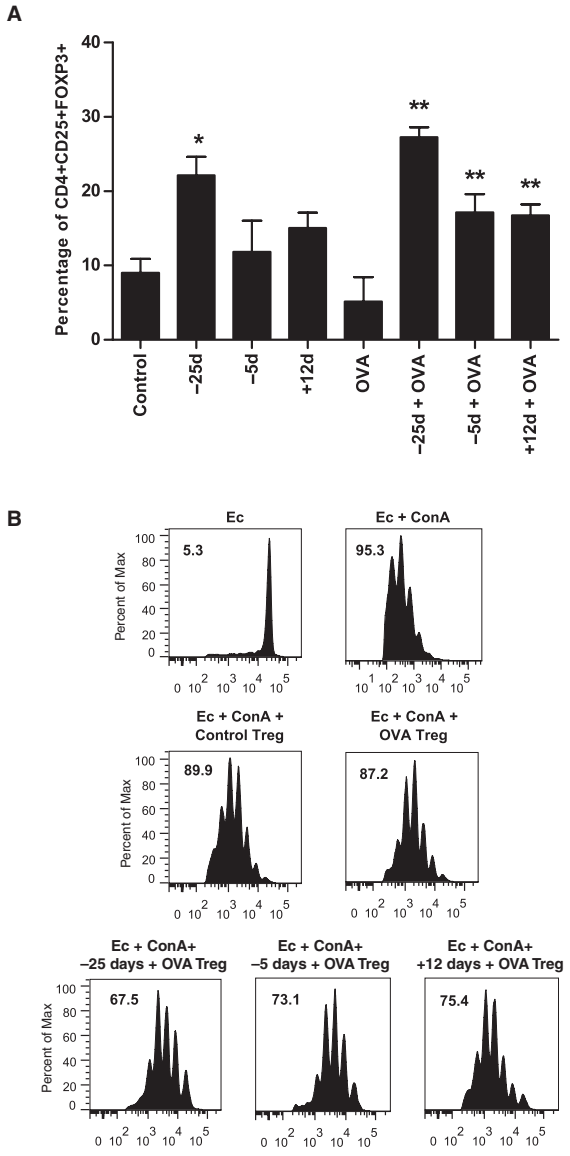

Fig. 6. Effect of *Trichinella spiralis* infection on the induction of functional Tregs in experimental allergic airway inflammation. The chronic phase of *Trichinella* infection was established 25 days (− 25 days + OVA) before OVA-sensitization/challenge. The acute phase was established at two time points, 5 days (− 5 days + OVA) before and 12 days (+ 12 days + OVA) after OVA-sensitization. Mice from the control group were uninfected phosphate-buffered saline/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. Isolated splenocytes were stained and analysed by flow cytometry. (A) Percentage of $CD4^+CD25^+$ cells expressing $FOXP3^+$ in $CD4^+$ gated cells. Data for the different groups (8 mice/group) are represented as mean (% ± SE). Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from group control. **Significantly different ($P < 0.05$) from group OVA. (B) Proliferative response of effector $CD4^+$ $CD25^-$ CFSE-labelled T cells (Ec) cultured for 4 days with DC and ConA with or without $CD4^+$ $CD25^+$ Treg cells isolated from splenocytes derived from the different groups. Histograms represent the percentages of the cells that have divided. Proliferation was measured by flow cytometry. Numbers inside the histograms indicate the percentage of dividing cells.

the lungs indicated that mice in the chronic phase of infection had significantly lower OVA-induced peribronchiolitis and perivasculitis compared to mice that were OVA-treated only. Protection against experimental airway inflammation in mice chronically infected with other helminths has also been reported. For instance, *Schistosoma mansoni* infected BALB/c mice were protected against EAAI induced by OVA as indicated by reduction of eosinophils in BAL, Th2 cytokine production, OVA-specific IgE levels and reduction of the number of inflammatory cells in lungs. The authors also showed that the critical role of $CD4^+CD25^+FOXP3^+$ regulatory T cells was independent of IL-10 [7]. A study by Dittrich et al. investigated the effect of chronic infection with the filarial parasite *Litomosoides sigmodontis* on the mouse OVA-induced EAAI model. Infection with this filarial parasite suppressed all pathological features of the allergic model. Additionally, significantly increased numbers of Treg cells were observed in spleen and mediastinal lymph nodes in infected and sensitized mice compared with sensitized controls [8]. Similarly, Liu et al. showed that *Schistosoma japonicun* chronic infection suppressed airway eosinophilia, mucus production, OVA-specific IgE responses and production of IL-4 and IL-5 [25]. In a study that also used the OVA-induced EAAI model, Smit et al. (2007) showed that *S. mansoni* infection protects against EAAI only during the chronic but not the acute phase of infection [10].

Our findings indicate that protection was most prominent during the chronic or muscle phase of *T. spiralis* infection, which was already established before sensitization and aerosol challenge with OVA. The acute or intestinal phase of infection established before OVA-sensitization/challenge had also a protective effect, although to a lesser extent. It is important to mention that by the time these animals received the OVA-challenge, mice were at a chronic phase of infection, which was perhaps the reason for the partial effect on EAAI. Since humans can be sensitized to aeroallergens early in life and before consumption of *Trichinella* infected meat, we included a group of mice that were *Trichinella* infected after OVA-sensitization and shortly before OVA-challenge. By the time, the OVA-challenge was given these mice were in the intestinal or acute phase of infection. Here, partial protection against EAAI was also observed, which was restricted to significant decrease in the levels of eosinophils in BAL and partial reduction of pulmonary inflammation. Unfortunately, it is not possible to study the effect of a chronic *Trichinella* infection after allergen sensitization since the period of time between OVA-sensitization and challenge in this EAAI model is too short. The observed difference between the effect of *T. spiralis* and *S. mansoni* acute infection on EAAI may be due to their different life

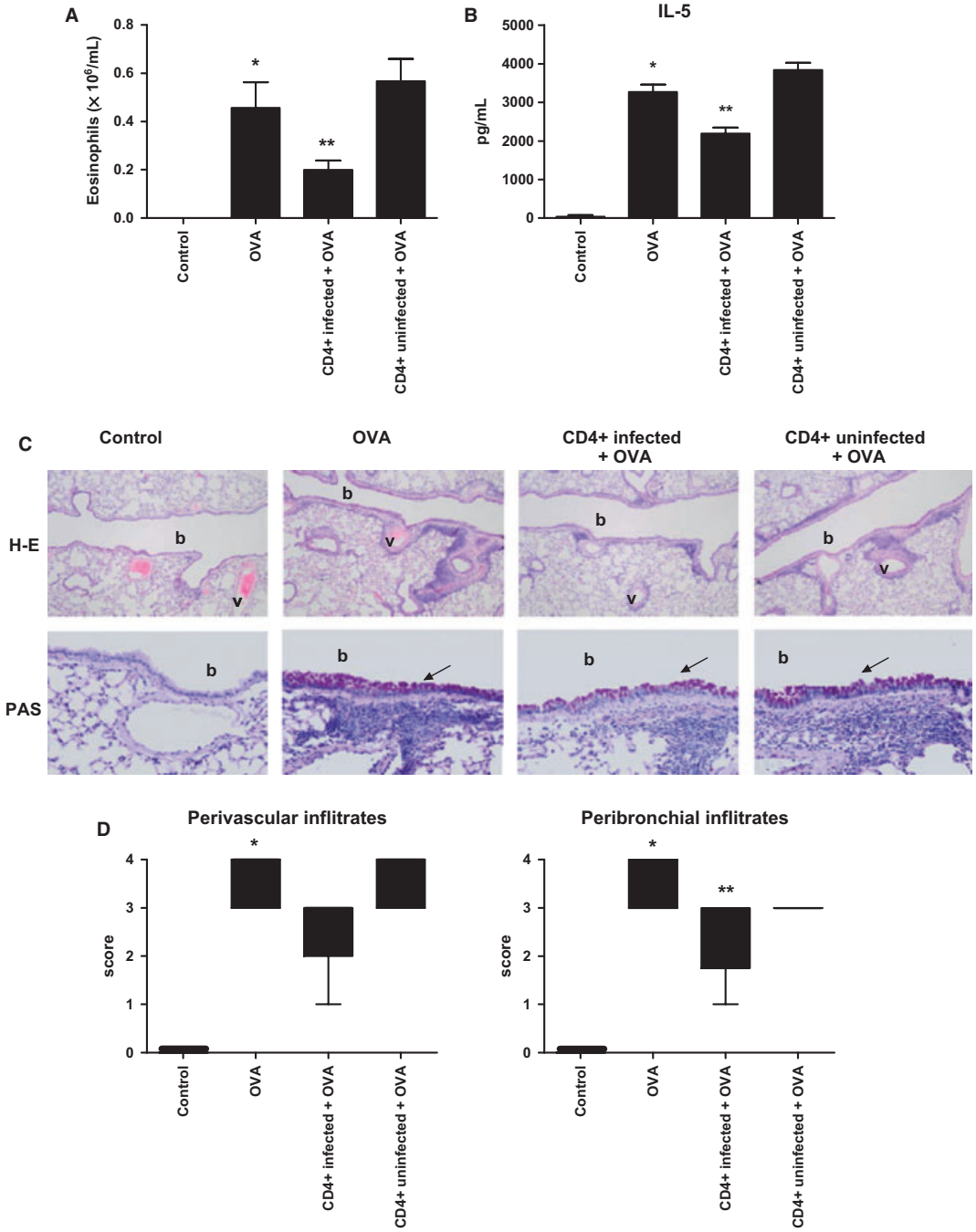

Fig. 7. Effect of adoptive transfer of $CD4^+$ T cells from *Trichinella spiralis* infected mice on IL-5 levels in bronchoalveolar lavage (BAL) and pulmonary inflammation in experimental allergic airway inflammation. Isolated $CD4^+$ T cells from either chronic *T. spiralis* infected (– 25 days) or uninfected mice were transferred to OVA sensitized mice one day before each challenge. (A) After euthanasia, BAL fluid was collected for eosinophil cell counting. (B) IL-5 levels in BAL were measured using a multiplex bead-based assay. (C) Lungs were fixed with formaldehyde and sections were cut and stained with H–E and periodic acid schiff. Bronchiole (b) and vessels (v) are shown. Arrows indicate the mucin-filled cells (bright-fuchsia). The figures represent the average of the histological scores (D). The perivascular and peribronchial infiltrates in histological sections of the lung were blindly scored as mentioned in the legend of Fig. 5. Data are presented as box and whiskers indicating the minimum and maximum values for the different groups (8 mice/group). Data shown are pooled from two independent experiments. Significantly different ($P < 0.05$) from group control. **Significantly different ($P < 0.05$) from the OVA group. The $CD4^+$ infected + OVA group refers to the OVA-treated mice that received $CD4^+$ from infected mice. The $CD4^+$ uninfected + OVA group are the OVA-treated mice that received $CD4^+$ from uninfected mice.

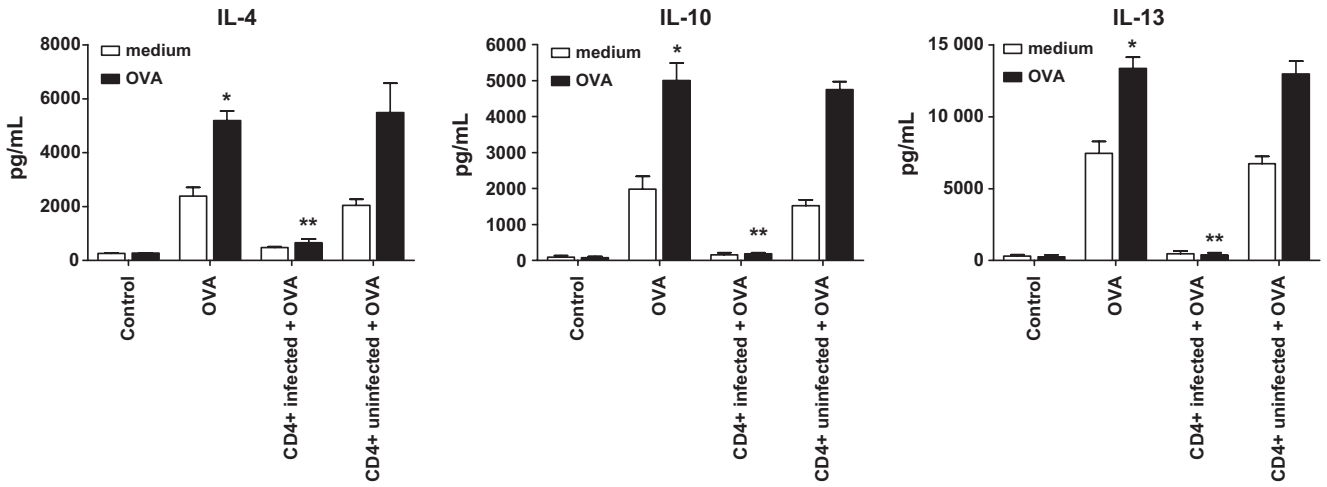

Fig. 8. Effect of adoptive transfer of $CD4^+$ T cells from spleen of chronically *Trichinella spiralis* infected mice on OVA-specific cytokine production. Isolated $CD4^+$ T cells from either chronic *T. spiralis* infected or uninfected mice were transferred to OVA-sensitized mice one day before each OVA-challenge. Lung-draining lymph nodes cells from these mice were cultured with or without OVA for 5 days. Cytokine levels in the culture supernatants were measured using a multiplex bead-based assay. Results for the different groups (8 mice/group) are represented as mean values (pg/mL ± SE). Data shown are pooled from two independent experiments. *Significantly different ($P < 0.05$) from control group. **Significantly different ($P < 0.05$) from OVA group. The $CD4^+$ infected + OVA group refers to the OVA-treated mice that received $CD4^+$ from infected mice. The $CD4^+$ uninfected + OVA group are the OVA-treated mice that received $CD4^+$ from uninfected mice.

cycles and immunopathology induced. Acute schistosomiasis is characterized by a systemic hypersensitivity reaction against the migrating schistosomula and eggs. Once in the blood capillaries, the schistosomula pass via the blood flow to the lungs and continues to the liver where they mature, pair and initiate egg deposition [26, 27]. These schistosomula can stay in the pulmonary capillaries from 3 to 16 days, and there is evidence that during acute schistosomiasis individuals can suffer from lung eosinophilia, and pulmonary symptoms such as cough and shortness of breath [22]. Although *Trichinella* infection has also a migratory phase, where the newborn larvae pass through the lung microvascular system on its way to the skeletal muscle, this is a rapid process in which the larvae are usually not trapped in the lungs [28]. We did not observe any pulmonary inflammation in the mice that received *T. spiralis* infection only. Furthermore as suggested by Furze et al. [29], *T. spiralis* larvae are particularly susceptible to immune attacks, and immunomodulatory mechanisms induced by adult worms in the early (acute) phase of *T. spiralis* infection might protect the future larvae development. The surface and ES products from the adult parasite might act in multiple ways, provoking significant changes in the gut microenvironment, which can have broad effects on the immune system, both within and beyond the gut as has been shown by other parasites [30]. One of these strategies might be the induction of Treg cells that have been found by us and others [29] to be enhanced also during the acute phase of infection.

Protection of C57/BL mice against EAAI by chronic *T. spiralis* infection has been recently reported by Park et al. [31]. In this study, we show that also an acute phase of *T. spiralis* infection protects against EAAI and in addition we report on the possible role of Treg cells in this process. Helminth induced Treg cells have been shown to contribute to immune suppression allowing the parasite to escape from host protective immune responses. Studies in humans have shown that individuals with chronic helminth infections develop prominent anti-inflammatory networks that reduce antigen-specific immune responses to both the helminth and unrelated antigens [32]. Using mouse models, it has been demonstrated that $CD4^+CD25^+$ T cells induced by *Heligmosomoides polygyrus* suppressed allergic responses to OVA or Der P 1 in mice [6]. In our study, we observed increased number of $CD4^+CD25^+FOXP3^+$ cells in spleen of *T. spiralis* infected mice, particularly during the chronic phase of infection. This cell population was also found to be functional in suppression assays *in vitro*. Although the proportion of Treg cells in the spleen from the group infected 5 days before OVA-sensitization/challenge (− 5 days + OVA) is comparable with that of the group infected 12 days after OVA-sensitization (+ 12 days + OVA), the suppression of EAAI in the latter group was less. Here, timing may play an important role determining the overall effect of Treg cells on EAAI since mice from the group − 5 days + OVA were killed 25 days after infection, whereas mice from the group + 12 days + OVA were killed 5 days after infection. Whether at 5 days after

infection the proportion of Treg cells that accumulate in lungs and draining lymph nodes is low and therefore they do not efficiently suppress EAAI remains to be investigated.

Here, we also show that transfer of $CD4^+$ T cells isolated from chronically infected mice containing higher proportions of suppressive $CD25^+FOXP3^+$ cells to OVA-treated mice conferred partial protection against EAAI. Our findings concur with those of Wilson et al., who showed suppression of airway eosinophilia in OVA-treated BALB/c mice after the transfer of $CD4^+$T cells isolated from mesenteric lymph nodes from infected mice with *H. polygyrus* [6]. Our results indicate that administration of $CD4^+$ T cells from chronically *Trichinella* infected mice containing high numbers of Treg cells just one day before each OVA-challenge is sufficient for these cells to efficiently migrate into lymph nodes, and strongly suppress OVA-specific cytokine production. Studies by others have already shown that Treg cells can migrate into inflammatory sites in various inflammatory diseases and suppress peripheral effector T cell function where the various expression patterns of chemokine receptors and integrins contribute to Treg cell trafficking [33]. Our results show that Treg cells might be the cellular mediators responsible for the protective effect of *T. spiralis* infection; however, other cell populations such as regulatory B cells, alternatively activated macrophages and even DC carrying parasite antigens might also be involved in immunosuppression [9–11, 34].

In conclusion, infection with *T. spiralis* confers protection against EAAI. This parasite induces a regulatory network that is probably orchestrated by secreted molecules as well as somatic antigens from the adult and larval stages of the parasite. The magnitude of this protective effect increases as infection progresses from the intestinal or acute phase to the muscle or chronic phase of infection. During infection, the immunological balance inclines towards a regulatory response where the Treg cells may play an important role. Future studies should focus on elucidating the mechanisms and molecular helminth structures responsible for inducing this regulatory process to develop alternative tools for preventing or treating allergic asthma.

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Conflict of interests: All authors declare no conflict of interest.

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