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Helminth Infection with *Litomosoides sigmodontis* Induces Regulatory T Cells and Inhibits Allergic Sensitization, Airway Inflammation, and Hyperreactivity in a Murine Asthma Model[1]

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Numerous epidemiological studies have shown an inverse correlation between helminth infections and the manifestation of atopic diseases, yet the immunological mechanisms governing this phenomenon are indistinct. We therefore investigated the effects of infection with the filarial parasite *Litomosoides sigmodontis* on allergen-induced immune reactions and airway disease in a murine model of asthma. Infection with *L. sigmodontis* suppressed all aspects of the asthmatic phenotype: Ag-specific Ig production, airway reactivity to inhaled methacholine, and pulmonary eosinophilia. Similarly, Ag-specific recall proliferation and overall Th2 cytokine (IL-4, IL-5, and IL-3) production were significantly reduced after *L. sigmodontis* infection. Analysis of splenic mononuclear cells and mediastinal lymph nodes revealed a significant increase in the numbers of T cells with a regulatory phenotype in infected and sensitized mice compared with sensitized controls. Additionally, surface and intracellular staining for TGF-β on splenic CD4$^+$ T cells as well as Ag-specific TGF-β secretion by splenic mononuclear cells was increased in infected and sensitized animals. Administration of Abs blocking TGF-β or depleting regulatory T cells in infected animals before allergen sensitization and challenges reversed the suppressive effect with regard to airway hyperreactivity, but did not affect airway inflammation. Despite the dissociate results of the blocking experiments, these data point toward an induction of regulatory T cells and enhanced secretion of the immunomodulatory cytokine TGF-β as one principle mechanism. In conclusion, our data support the epidemiological evidence and enhance the immunological understanding concerning the impact of helminth infections on atopic diseases thus providing new insights for the development of future studies. ***The Journal of Immunology,*** **2008, 180: 1792–1799.**

Allergic diseases have been increasing during the past four to five decades with allergic asthma now being the most prevalent chronic airway disease in industrialized countries (1). Yet, in developing countries, there is a considerably lower prevalence of allergic diseases (2), and the prevalence rates show clear-cut differences between rural and urban areas within one country regardless of its developmental status (3). Numerous epidemiological studies have sought to identify factors underlying these observations. One of these parameters is childhood infections, which in several studies show a negative association with atopy and development of allergic diseases (4, 5). On the basis of these data, it has been proposed that the lack of intense infections in industrialized countries due to improved hygiene, vaccination, and the use of antibiotics may alter the human immune system, thus increasing the risk for allergic reactions against environmental and food proteins. This so-called "hygiene hypothesis" was first used to explain the development of allergies by a predisposing imbalance with low type 1 (Th1) and heightened type 2 (Th2) immune responses (6). However, recent observations have challenged this explanation. First, Th1 diseases such as type 1 diabetes have also been progressively increasing in the past few decades. Moreover, recent data even support an association between the occurrence of type 1 diabetes and asthma at the population level (7), suggesting a common denominator underlying the increase of both Th1 and Th2 diseases instead of an imbalance between Th1 and Th2 immune responses. Second, the occurrence of helminth infections and allergic diseases, both conditions being accompanied by strong Th2 immune responses, are nearly exclusive (2) or at least negatively associated (8–11). It therefore was proposed that the hygiene hypothesis has to be modified to state that a robust regulatory network induced by a high overall infection rate, regardless of the nature of its immunological skewing, is central to the balance and the prevention of either Th1 and/or Th2 diseases (12, 13). Yet, there is very little data available to explain how helminth

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infections might protect against allergy, and the immunological basis of the regulatory phenomenon is not very well understood. We therefore established a murine model to study the effects of a helminth infection with the filarial species *Litomosoides sigmodontis* on the development of allergic sensitization and airway disease. *L. sigmodontis* is a parasite naturally occurring in the cotton rat (*Sigmodon hispidus*) and was chosen because it is the only filaria known to undergo complete development in fully immunocompetent BALB/c mice (14). We show here for the first time that infection with *L. sigmodontis* before allergen sensitization and airway challenges completely abrogated all aspects of the allergic phenotype, i.e., allergic sensitization, airway inflammation (AI),[4] and airway hyperreactivity (AHR), which was associated with an increase in regulatory T cells (Tregs) and high TGF-β production.

Materials and Methods

Animals

Pathogen-free, female BALB/c mice (Harlan Winkelmann), 6–8 wk of age at the time of filarial infection, were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure (Tecniplast) according to institutional-approved guidelines on OVA-free diets and water ad libitum for all experiments.

L. sigmodontis *infection procedure*

Adult female worms of *L. sigmodontis* were isolated from the pleural and peritoneal cavities of infected cotton rats 30 days postinfection under sterile conditions (15). Five to six worms per recipient were implanted into the peritoneal cavity of anesthetized BALB/c mice. Control mice underwent the same surgical procedure but without implantation of worms (=sham treated). Microfilaremia was determined microscopically on day 38 postimplantation.

Sensitization and challenge protocol

Mice were sensitized by i.p. injections of 20 μg of OVA grade VI (Sigma-Aldrich) in 2 mg of aluminum hydroxide (Pierce) on days 1 and 14, i.e., 11 and 25 days after filarial implantation. Intranasal allergen challenges were performed with 50 μg of OVA grade VI (Sigma-Aldrich) in 50 μl of PBS on days 28 and 29, i.e., 39 and 40 days after filarial implantation (F^+/OVA/OVA). Control animals were sham infected and sham sensitized (F^-/PBS/PBS), sham infected and OVA sensitized (F^-/OVA/OVA), or filarial infected and sham-sensitized (F^+/PBS/PBS).

In vivo cell depletion by Ab treatment

For depletion experiments, *L. sigmodontis*-infected animals received 0.5 mg/mouse anti-CD25 Ab (clone PC61, provided by Dr. H. K. Bottomly, Department of Immunobiology, Yale University School of Medicine, New Haven, CT) i.p. 1 day before the first airway allergen challenge (day 29; F^+/OVA/OVA/anti-CD25, $n = 10$) or anti-TGF-β Ab (R&D Systems) at 50 μg/mouse i.p. 1 h before each airway challenge (day 39 and 40; F^+/OVA/OVA/anti-TGF, $n = 10$). Administration of Abs was controlled by appropriate isotype controls in preliminary experiments where no effects on allergen-induced responses were observed in OVA/OVA groups. We confirmed depletion of $CD25^{high}$ cells by >95% by FACS analysis of spleens the day when OVA sensitization was started. Negative and positive control animals for this set of experiment were prepared as described above: F^+/PBS/PBS, F^-/OVA/OVA, F^+/OVA/OVA; additionally, one group of sham-infected and OVA-sensitized mice was accordingly treated with Abs (F^-/OVA/OVA/anti-CD25, $n = 8$) to control for depletion of Tregs.

In vivo airway reactivity (AR)

On day 42, in vivo lung function measurement was performed by whole-body plethysmography (Buxco Technologies) as previously reported (16). AR was expressed as fold increase (FI) of enhanced pause (Penh) values for each concentration of methacholine relative to baseline (PBS) Penh values.

Bronchoalveolar lavage (BAL)

On day 43, lungs were lavaged twice with 0.8 ml of PBS. Cytospin preparations were stained with DiffQuik (Dade Behring) and differentiated according to standard morphologic criteria by counting 200 cells via light microscopy.

Peritoneal lavage

Peritoneal lavage was performed via abdominal incision and two peritoneal lavages with 2 ml of PBS on day 43. Supernatants and cells were obtained and analyzed as described for BAL. Additionally, cells were stained for FACS analysis.

Immunoglobulin serum levels

On day 43, blood was drawn via the tail vein and serum levels of total, OVA-specific, and filarial-specific Ig were measured by means of ELISA (17, 18). Detection limits were: total IgE, 0.98 ng/ml; OVA-specific IgE, 6.5 light units (LU)/ml; OVA-specific IgG1, 0.65 ng/ml; OVA-specific IgG2a, 1.4 LU/ml. For filaria-specific Abs, a cutoff value (i.e., arithmetic mean of negative control sera plus 2 SDs) was calculated to discriminate between seronegative and seropositive samples.

Cytokine production and proliferation

On day 43, spleen mononuclear cells (MNCs) were isolated and cultured with Con A (*2.5* μg/ml; Sigma-Aldrich) or OVA (50 μg/ml) at 1×10^6 cells/well. Cytokines in supernatants were assessed using commercially available ELISA kits (optEia; BD Pharmingen). Detection limits were: IFN-γ, 7.88 pg/ml; IL-4, 15.3 pg/ml; IL-5, 283 pg/ml; IL-10, 27.9 pg/ml; and TGF-β, 130.8 pg/ml. Proliferative responses of spleen MNCs (0.2×10^6 cells/well) cultured with medium, Con A, or OVA (concentrations as above) were determined after addition of [^{3}H]thymidine (0.5 μCi/200 μl; Amersham Biosciences) for the last 16 h of a 72-h culture period. Data are expressed as FI over incorporation by cells cultured with medium alone. For cytoplasmic cytokine staining, spleen MNCs were cultured in 6-well plates at a density of 1×10^6 cells/ml with PMA (20 ng/ml) and ionomycin (500 ng/ml) for 6 h and brefeldin A was added after 3½ h.

Flow cytometry analysis

Surface molecule expression of CD4, CD8, CD3, CD25, CD69, ICOS, CD45RB, TGF-β (IQ Products), MHC class II, CD11c, CD80, CD86, ICOS ligand (ICOSL) by spleen and peritoneal lavage cells was analyzed by flow cytometry (unless stated otherwise, mAb were provided as a gift from R. A. Kroczek, Robert-Koch-Institut, Berlin, Germany). To prevent unspecific binding of mAb, all samples were preincubated with anti-FcRII/III mAb (2.4G2, 100 μg/ml; American Type Culture Collection) and purified rat IgG (200 μg/ml; Nordic) 10 min before and during staining. Cytoplasmic cytokine staining for IL-4, IL-10, and TGF-β (IQ Products) was performed with the BD GolgiPlug kit (BD Biosciences) according to the manufacturer's instruction. FACS analysis on a minimum of 100,000 live cells was performed on a BD FACSCalibur (BD Biosciences) with exclusion of dead cells via staining with propidium iodide where applicable. Data were analyzed via CellQuest software (BD Biosciences).

Histology

Eosinophils on acetone-fixed cryopreserved lung sections were visualized by means of immunohistochemistry with a rabbit polyclonal anti-mouse eosinophilic major basic protein antiserum (a gift from J. Lee, Mayo Clinic, Scottsdale, AZ) via fluorescence microscopy (19).

Statistics

Unless indicated otherwise, all experiments were performed in three independent sets with $n \geq 5$ mice/group. Data from three independent experiments were combined. Values were compared using the two-tailed Student *t* test.

Results

Infection with L. sigmodontis *induces local and systemic immune responses*

Infection with *L. sigmodontis* was performed with premature female parasites known to exert potent immune modulatory properties (18). In accordance with the literature (14), infection with *L. sigmodontis* induced systemic immune responses as detected by the following parameters on day 43: first, a significant influx of inflammatory cells at the site of infection, the peritoneal cavity,

[4] Abbreviations used in this paper: AI, airway inflammation; AHR, airway hyperactivity, AR, airway reactivity; BAL, bronchoalveolar lavage; mLN, mediastinal lymph node; MNC, mononuclear cell; Treg, regulatory T cell; SS, systemic sensitization; DC, dendritic cell.

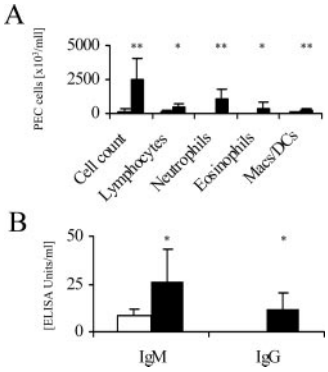

FIGURE 1. Filarial infection induces local and systemic immune responses. *A*, Female BALB/c mice received five premature female filariae via surgical i.p. implantation on day –11(■). Controls were sham-operated animals (□). Peritoneal lavage cells were analyzed on day 32. Macs, Macrophages. *, $p \leq 0.01$; **, $p \leq 0.001$. *B*, Filaria-specific IgM, IgA (data not shown), and IgG serum levels after infection with *L. sigmodontis* (■) compared with sham-treated animals (□). *, $p \leq 0.05$. Data from five independent experiments were pooled, $n > 5$/group.

was observed. Cytospin preparations of peritoneal lavages revealed that this inflammatory influx was predominated by neutrophils accompanied by significant numbers of lymphocytes, eosinophils, and cells of macrophage and dendritic cell (DC)-like morphology (Fig. 1*A*). Second, *L. sigmodontis*-specific IgM and IgG Ab production was detected (Fig. 1*B*). Third, spleen cells of infected animals showed recall proliferation and a characteristic pattern of cytokine secretion similar to data from the literature (20) with elevated levels of both Th1- and Th2-type cytokines in response to stimulation with *L. sigmodontis* Ag (data not shown).

Infection with L. sigmodontis *suppresses allergen-specific Ig production*

To study the effects of a parasitic infection on the development of allergic sensitization, AI and AR, we modified a widely used mouse model (21) In this model, systemic sensitization (SS) and local challenges with the Ag OVA elicited OVA-specific Ig production, pulmonary inflammation dominated by eosinophils, and AHR in positive sham-infected controls (F^-/OVA/OVA).

As expected, infection of BALB/c mice with *L. sigmodontis* worms resulted in enhanced production of total IgE Abs. Yet, when OVA-specific Ig levels in response to allergen sensitization were analyzed, results showed that infection with the parasite before sensitization markedly reduced allergen-specific Ig production (Fig. 2). For the IL-4-dependent Igs IgE and IgG1, this suppression was statistically significant. Additionally, we found a trend toward a reduction of allergen-specific IgG2a production, considered to be a Th1-induced Ig, suggesting that filarial infection had a general effect on allergen-specific Ig production rather than an effect on the Th1/Th2 cytokine balance.

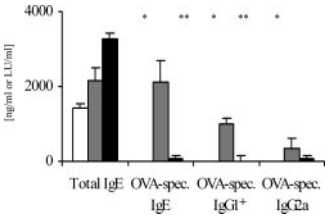

FIGURE 2. Infection with *L. sigmodontis* suppresses allergen-specific Ig production. Female BALB/c mice received filariae on day –11 before sensitization to OVA on days 0 and 14 followed by intranasal OVA challenges on days 28 and 29. Negative controls received PBS. Shown are serum Ig levels from filaria-treated, sham-sensitized (F+/PBS/PBS, □), sham-treated and OVA-sensitized (F^-/OVA/OVA, ▨), and filaria-treated and OVA-sensitized mice (F^+/OVA/OVA, ■). *, $p \leq 0.01$; **, $p \leq 0.0001$; +, $\times 10^2$. Total IgE and OVA-IgG1 are expressed in ng/ml and OVA-IgE and OVA-IgG2a in LU/ml. Data from five independent experiments were pooled, $n > 5$/group.

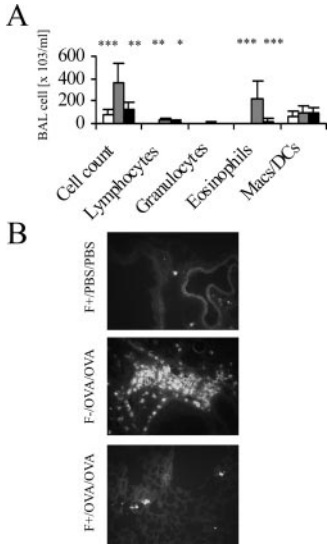

FIGURE 3. Infection with *L. sigmodontis* drastically reduces eosinophilic AI. *A*, Differential cell counts of BAL fluid from negative controls (F^+/PBS/PBS, □), noninfected and OVA-sensitized (F^-/OVA/OVA, ▨), and infected and OVA-sensitized mice (F^+/OVA/OVA, ■). Macs, Macrophages. *, $p \leq 0.05$; **, $p \leq 0.001$; ***, $p \leq 0.0001$. *B*, Staining of whole lung tissue with polyclonal Ab for major basic protein. One representative experiment of five is shown.

Infection with L. sigmodontis *drastically reduces eosinophilic AI*

Analysis of BAL fluid and fluorescent immunohistology demonstrated that AI upon allergen sensitization and airway challenges was markedly suppressed after infection with *L. sigmodontis*: total cell count and BAL composition were reduced to negative control values, and staining of lung tissues with the eosinophil-specific anti-major basic protein Ab confirmed that *L. sigmodontis* infection before allergen sensitization and airway challenges resulted in a near complete abrogation of eosinophil influx into the lungs (Fig. 3). Counting of eosinophils in the subepithelial layer of representative middle-sized airways also revealed significant differences with smaller numbers in infected and sensitized animals compared with controls (data not shown).

Infection with L. sigmodontis *reduces methacholine-induced AHR*

Infection with *L. sigmodontis* not only reduced SS and AI, but also significantly inhibited development of in vivo AR to increasing doses of methacholine. We continuously observed a reduction of ~30% in the infected, sensitized animals compared with sham-treated and OVA-sensitized mice, whereas the latter showed marked increases in AR compared with negative controls (Fig. 4).

Allergen-specific recall proliferation of spleen cells is reduced after infection with L. sigmodontis

Stimulation of spleen MNCs from sham-infected and OVA-sensitized animals (F^-/OVA/OVA) with OVA resulted in enhanced proliferative responses compared with sham-sensitized controls, suggesting a

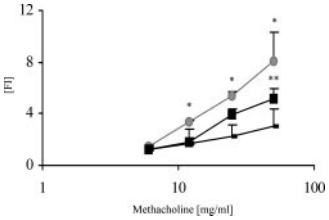

FIGURE 4. Infection with *L. sigmodontis* reduces methacholine-induced AHR. Airway reactivity was analyzed by whole body plethysmography on day 32, 2 days after the last OVA airway challenge. Expressed are the FI in Penh of negative controls (F^+/PBS/PBS, black lines, mean absolute values for 12 animals from three independent experiments: 0.86 + 0.74) vs noninfected and OVA-sensitized (F^-/OVA/OVA, gray circles) vs infected and OVA-sensitized mice (F^+/OVA/OVA, black squares). *, $p \leq 0.05$ compared with negative controls; **, $p \leq 0.05$ in comparing F^-/OVA/OVA to F+/OVA/OVA.

significant recall response. Infection with *L. sigmodontis* before allergen sensitization and airway challenges led to a significant reduction of the allergen-induced proliferation of spleen MNCs. Furthermore, we observed an unspecific suppressive effect on mitogen-induced proliferation after stimulation of spleen MNCs with Con A from sensitized animals infected with *L. sigmodontis* (Fig. 5*A*).

Allergen-specific cytokine production by spleen MNCs is modulated after infection with L. sigmodontis

Infection with *L. sigmodontis* markedly altered recall cytokine secretion by spleen cells of sensitized mice upon stimulation with OVA. The Th2 cytokines IL-4, IL-5 (Fig. 5*B*), and IL-13 (data not shown) were drastically reduced after filarial infection compared with cells from sham-infected and sensitized mice, supporting a pivotal role for these cytokines in the induction of SS, AI, and AR. Interestingly, production of the Th2 cytokine IL-10, which is known to exert regulatory properties and is believed to play an important role in immune modulation by helminth parasites (9, 22), was also down-regulated similarly to other Th2 cytokines.

There was however no concomitant increase in Th1 responses associated with the suppression of Th2 cytokine production. Infection with *L. sigmodontis* induced spontaneous secretion of significant amounts of the Th1 cytokine IFN-γ: spleen cells from *L. sigmodontis*-infected, sham-sensitized animals (F+/PBS/PBS) displayed marked IFN-γ production in response to in vitro stimulation with OVA, an unknown Ag to this experimental group. In contrast, IFN-γ production by spleen cells of sham-infected and nonsensitized animals after stimulation with OVA was below the limit of detection (data not shown). In animals that were sensitized and challenged after filarial infection (F+/OVA/OVA), the amount of IFN-γ production by OVA-stimulated spleen cells was decreased compared with infected and nonsensitized animals (F^+/PBS/PBS), probably the result of a strong Th2 skewing induced by allergen sensitization with alum as an adjuvant. (Fig. 5*B*) Additionally, we detected significantly reduced levels of IL-1β and IL-6, two typical proinflammatory cytokines, in the supernatant of OVA-stimulated spleen cells from *L. sigmodontis*-infected and -sensitized animals compared with sham-infected and -sensitized controls (data not shown). This widespread suppression encompassing different types of cytokines again pointed toward an undirected and generalized immune suppressive effect in sensitized animals upon infection with *L. sigmodontis*.

The only analyzed cytokine that remained elevated in cell supernatants from OVA-sensitized and -challenged animals after infection with *L. sigmodontis* was TGF-β. Infection with *L. sigmodontis* resulted in high spontaneous TGF-β production by spleen cells (F+/PBS/PBS) while spleen cells from sham-infected and

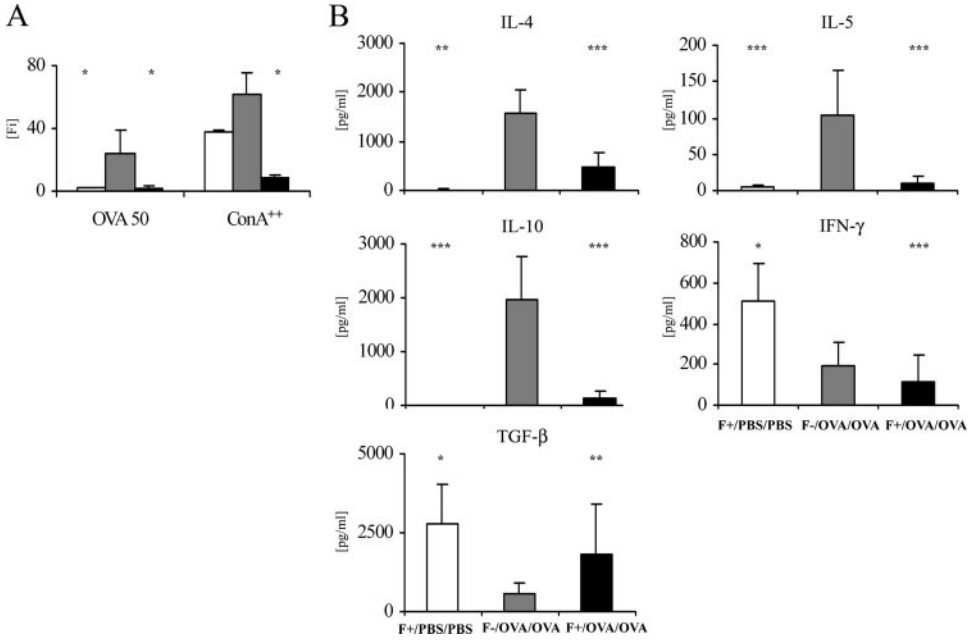

FIGURE 5. Recall proliferation and allergen-induced cytokine secretion by spleen cells are markedly altered by infection with *L. sigmodontis*. *A*, Infection with *L. sigmodontis* (F^+/OVA/OVA, ■) reduced OVA-induced recall proliferation (F^-/OVA/OVA, ▨) to background levels (F^+/PBS/PBS, □). Proliferation induced via Con A was also profoundly reduced upon filarial infection. *B*, Secretion of Th2 cytokines (IL-4, IL-5), Th2/Treg cytokine IL-10, and Treg cytokine TGF-β as well as Th1 cytokine IFN-γ were affected differently by infection with filariae. For assessment of recall proliferation, spleen MNCs were stimulated with OVA (Ag specific stimulation) or with Con A (polyclonal stimulation) and proliferation was measured after 72 h of culture. For assessment of recall cytokine secretion, splenic MNCs were cultured with OVA (Ag-specific recall response) or Con A (polyclonal response, data not shown) for 96 h when supernatants were harvested for ELISA analysis. *, $p \leq 0.05$; **, $p \leq 0.001$; ***, $p \leq 0.0001$; ++, $\times 10^1$. Data are combined from three independent experiments with four to five mice per experiment.

Table I. *Intracytoplasmatic staining reveals a distinct cytokine pattern compared to recall cytokine secretion*[a]

	IL-4 (%)[b]	IFN-γ (%)[b]	IL-10 (%)[b]	TGF-β (%)[b]
F⁻/OVA/OVA	4.65	5.50	0.61	4.25
F⁺/OVA/OVA	7.47	7.24	2.46	11.34
Ratio F⁺:F⁻	1.61	1.32	4.03	2.67

[a] MNCs were isolated from spleens, restimulated with PMA and ionomycin for 6 h, and then fixed, permeabilized, and stained with anti-CD3 Ab and anti-CD8 Ab and Ab detecting respective cytokine as described in *Materials and Methods*. Analysis gates were set on $CD3^+$ lymphocytes, allowing discrimination between $CD3^+CD8^+$ lymphocytes and $CD3^+CD8^-$ (=$CD4^+$) lymphocytes expressing the respective cytokine. One representative set from three sets of experiments with three mice per experiment is shown.

[b] Frequency of CD4 lymphocytes secreting a given cytokine within the $CD3^+$ lymphocyte population.

sham-sensitized animals reproducibly showed TGF-β secretion levels in response to OVA stimulation below detection limits (experimental group not shown). This effect was, in contrast to IFN-γ production, observed regardless of a subsequent sensitization with allergen (in Fig. 5*B*, compare F⁺/PBS/PBS vs F⁺/OVA/OVA for IFN-γ, vs TGF-β). In contrast, sensitization with OVA without infection with *L. sigmodontis* (F⁻/OVA/OVA) resulted in a much lower secretion of TGF-β.

Infection with L. sigmodontis *results in a distinct intracytoplasmatic cytokine secretion pattern compared with recall cytokine secretion after allergen sensitization*

To analyze the effects of filarial infection on the cytokine pattern of individual T cells, we performed intracellular staining on the single-cell level. Although infection with *L. sigmodontis* led to a generalized, apart from TGF-β, suppression of OVA-induced cytokine secretion by the OVA-specific subpopulation of spleen cells, intracellular cytokine staining revealed that this inhibition did not encompass all subsets of T cells. As shown in Table I, when addressing allergen-independent overall cytokine secretion by $CD4^+$ lymphocytes, infection with *L. sigmodontis* increased the numbers of IL-4- and IFN-γ-producing cells. Additionally, both IL-10- and TGF-β-producing $CD4^+$ T cells showed a 3- to 4-fold increase upon infection with *L. sigmodontis*, compared with sensitized and sham-infected animals. This increase of allergen-independent production of IL-4, IL-5, IFN-γ, IL-10, and TGF-β was also detected when spleen cells from *L. sigmodontis*-infected animals were stimulated with mitogen (Con A) and cytokine levels were analyzed via ELISA (data not shown), pointing toward a difference in Ag-specific (recall) and polyclonally-induced cytokine profiles.

Infection with L. sigmodontis *alters local DC phenotype and increases numbers of systemic Tregs*

To further elucidate the mechanism underlying the effects of infection with *L. sigmodontis*, analysis of intraperitoneal cells (the site of *L. sigmodontis* infection) and spleen MNCs (the site of systemic immune reactions) was performed. Flow cytometry analysis of intraperitoneal cells revealed the lymphocytes to be composed mainly of $CD4^+$ and $CD8^+$ T cells in an early state of activation ($CD69^+ICOS^+$). Influx of macrophages and DCs was confirmed by flow cytometry analysis. Interestingly, at the site of filarial infection, the expression of the activation markers CD80 and CD86, but also of ICOSL by intraperitoneal DCs was increased (Fig. 6).

When analyzing spleen MNCs, we were able to observe an increase in the proportion of $CD4^+$ T cells with a regulatory phenotype. This was characterized by the simultaneous expression of

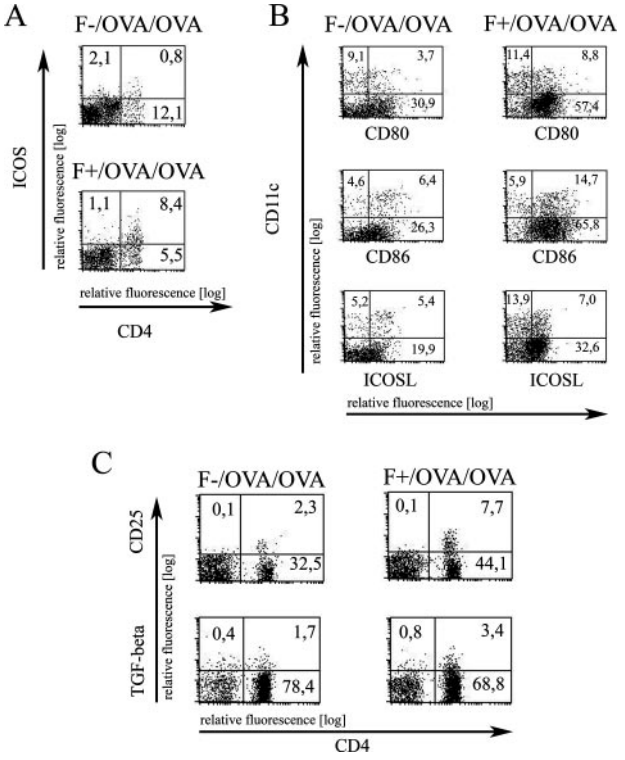

FIGURE 6. Filarial infection induces phenotypical changes of intraperitoneal T cells and DCs and increases systemic Tregs. Infection with *L. sigmodontis* up-regulates ICOS expression on peritoneal exudate T cells (*A*) and maturation markers CD80, CD86, and ICOSL on DCs (*B*). Plots depict percentage of viable lymphocytes that express $ICOS^+$ and $CD4^+$ (*A*) or MHC class II^+/FSC^{high} cells which additionally express CD11c (=DCs) and the respective maturation marker (*B*). Numbers represent percentage of gated cells expressing each combination of Ag. *C*, Infection with *L. sigmodontis* increases the percentage of CD4/CD25/$CD45RB^{low}$ positive lymphocytes (*upper plots*) as well as CD4/TGF-$\beta^{surface}$ positive lymphocytes (*lower plots*) among total splenic lymphocytes. For analysis of T cells expressing CD4/CD25/$CD45RB^{low}$, gates were set on viable lymphocytes and low expression of CD45RB. Shown in the *upper plots* are those cells which additionally express CD25 and CD4. For analysis of TGF-$\beta^{surface}$ expression on $CD4^+$ lymphocytes, gates were set on viable lymphocytes and CD3 expression. Shown above are those cells which additionally express CD4 and TGF-β on the cell surface. Numbers represent percentage of gated cells expressing each combination of Ag. One representative set of three independent sets of experiments with three mice per experiment is shown, mean values for *C* and SDs of the combined data of these experiments being 7.1 ± 0.98 (F⁻/OVA/OVA) and 3.73 ±1.35 (F⁺/OVA/OVA) for Tregs of the CD4/CD25/$CD45RB^{low}$ phenotype, 2.88 ± 0.32 (F⁻/OVA/OVA) and 1.83 ± 0.77 (F⁺/OVA/OVA) for TGF-$\beta^{surface}$ Tregs.

CD4/CD25/$CD45RB^{low}$ (Fig. 6). Additionally, among $CD4^+$ lymphocytes in the spleen, we observed an enhanced proportion of cells expressing TGF-β on the cell surface (Fig. 6). Analyzing the contribution of Tregs to local immune responses, we also found an increase in the Treg marker *Foxp3* content in mediastinal lymph nodes, suggesting an increase of Tregs in this location as well (data now shown).

Suppression of allergic airway disease after infection with L. sigmodontis *is partly abrogated after CD25 or TGF-β depletion*

In a final set of experiments, we asked whether the increase in systemic Tregs expressing TGF-β and the increased secretion of TGF-β after recall stimulation was of functional significance. To this end, we depleted Tregs by >95% one day before the first Ag airway challenge by i.p. Ab administration with anti-CD25 Ab or

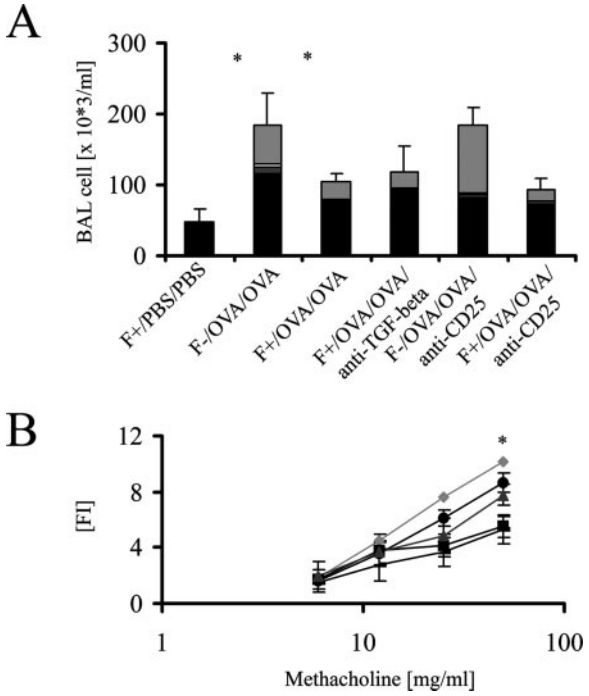

FIGURE 7. Blockade of TGF-β or CD25 fails to affect AI but partially restores methacholine-induced AHR. Abs were administered at the time points indicated in *Materials and Methods*. *A*, Differential cell counts of BAL fluid: expressed are numbers of eosinophils (first block from top, ▨), neutrophils (second block, □), lymphocytes (third block, ▨), and macrophages (fourth block, ■); ∗, $p \leq 0.05$ F^-/OVA/OVA compared with F^+/PBS/PBS and F^+/OVA/OVA, respectively; other values were not significantly different. *B*, AR to methacholine analyzed by whole-body plethysmography as described in *Materials and Methods* on day 32 from negative controls (F^+/PBS/PBS, black lines, $n = 8$, mean absolute values from eight animals from two independent experiments: 0.71 + 0.18) vs noninfected and OVA sensitized (F^-/OVA/OVA, gray diamonds, $n = 8$) vs infected and OVA-sensitized mice (F^+/OVA/OVA, filled squares, $n = 8$) vs infected and OVA-sensitized and anti-TGF-β-treated (F^+/OVA/OVA/anti-TGF-β, filled circles, $n = 10$) or anti-CD25-treated (F^+/OVA/OVA/anti-CD25, filled triangles, $n = 10$), respectively. ∗, $p \leq 0.05$ for F^-/OVA/OVA compared with F^+/PBS/PBS and F^+/OVA/OVA, respectively; statistically significant differences ($p < 0.05$) were also observed for F^+/OVA/OVA compared with F^+/OVA/OVA/anti-TGF-β and F^+/OVA/OVA/anti-CD25, respectively.

depleted TGF-β by Ab administration 1 h before each of the two Ag airway challenges. This led to a partial restoration of airway hyperresponsiveness: animals treated with anti-CD25 Ab or anti-TGF-β (F^+/OVA/OVA/anti-CD25 or F^+/OVA/OVA/anti-TGF-β) showed significantly increased airway hyperresponsiveness, compared with the untreated filaria-infected control (F^+/OVA/OVA) ($p = 0.016$ and. $p = 0.025$, respectively, for AR to 50 mg of methacholine). However, airway responsiveness in the Ab-treated groups was still lower than in the positive control group (F^-/OVA/OVA) (Fig. 7*B*). Furthermore, we were not able to observe the effects of treatment with anti-CD25 and/or anti-TGF-β on restoration of total BAL cell counts (Fig. 7*A*) or OVA-specific Ab production (data not shown). Treatment of sensitized, sham-infected mice with Ab (F^-/OVA/OVA/anti-CD25) did not show significant alterations with regard to AI or AHR compared with the untreated control group (F^-/OVA/OVA) nor did treatment with isotype controls in F^+/OVA/OVA groups (data not shown).

Discussion

Helminth infections, despite inducing Th2-polarized immune responses are negatively associated with the development of allergic diseases (8–11). We analyzed the effects of filarial infection on the development of an allergen-induced sensitization and airway disease in a murine asthma model to dissect the immunological mechanism governing this paradox. We demonstrate here that infection with the filarial parasite *L. sigmodontis* before allergen sensitization and airway challenges suppressed all aspects of the asthmatic phenotype, including specific IgE production, AI, and development of AHR. This effect was associated with the induction of Tregs and high levels of TGF-β production.

So far only few studies have addressed the question how parasites modulate allergic immune responses in appropriate in vivo models (23–31). One common denominator in all of the cited studies, including our own results, is the suppression of Th2 responses to allergen as a consequence of parasitic infection. Wilson et al. (29) recently published the most pivotal data to this respect. They have been able to demonstrate that infection with a different parasite *Heligsomoides polygyrus* suppresses AI associated with a decrease in IL-5 and eotaxin levels in the BAL. Through elegant transfer experiments they were able to show that it is the increase in Tregs seen in infected animals that confers suppression of the allergic phenotype albeit not associated with direct action of the immunosuppressive cytokine IL-10 (30).

Our own observations, using a different parasite but a very similar asthma model as the one used by Wilson et al. (29), are quite similar to those of Wilson et al. in many respects. We also observed suppression of AI, associated with a decrease in Th2 cytokines upon Ag-specific restimulation. Additionally, in our hands, we observed a suppressive effect of the parasite infection on Ag-specific Ig production and development of AHR. Similar to the findings of Wilson et al (29) also, we observed an increase in the numbers of Tregs. Extending these observations though, we observed an increase in the number of T cells expressing TGF-β on their surface and associated an increase in Ag-specific TGF-β secretion upon restimulation of splenic MNCs. Depletion experiments, however, only partially confirmed our hypothesis that Tregs and TGF-β are the major cell type/mediators responsible for the suppressive effect on AHR, AI, and SS. By depleting Tregs or TGF-β, we did observe a significant effect on AHR, but AI and SS were not altered by this treatment. Thus, the question remains which cells types/mediators are responsible for the effects on these hallmarks of the asthmatic phenotype.

One possible explanation might be that, as it has been described many times before, AHR and AI/SS are regulated independently of each other with other cell types/mediators involved in the suppression of the latter. The fact that our data differ from the findings of Wilson et al. (29) is not surprising given the fact that a different parasite system was used and other mediators/regulatory cell types have already been identified in other parasite systems (27).

We have not formally tested the possibility that IL-10 might be another suppressive mediator involved. Given the fact that IL-10 was down-regulated in an Ag-specific-manner in parasite-infected animals in our initial experiments, this possibility initially seemed to be of lesser likelihood. Our findings regarding IL-10, known to be the prototype of a regulatory cytokine or even considered to be a marker for Tregs (32), underscore the controversy regarding the role of IL-10 in allergic immune reactions, where some studies indicate that IL-10 promotes rather than inhibits atopic symptoms (33–35). When taken together, however, our data and the data of Wilson et al. (29) might provide an explanation for this discrepancy: we showed that in contrast to a diminished Ag-specific IL-10 production of splenic MNCs (Fig. 5), allergen-independent IL-10 secretion induced by restimulation with PMA and ionomycin, as detected by intracellular cytokine staining (Table I), and protein

secretion upon Con A stimulation (data not shown) was up-regulated after infection with *L. sigmodontis*. Infection with *L. sigmodontis* leads to a characteristic time course of cytokine secretion by spleen cells, initially characterized by a Th2-skewed pattern. At the time point we analyzed our animals, however, IL-10 became the prevailing cytokine with other Th2 cytokines vanishing (20). The allergen-independent up-regulation of IL-10 might therefore constitute IL-10 secretion by parasite-driven (-specific) T cells similar to observations made by Wilson et al. (29) when they looked at *H. polygyrus*-stimulated cytokine secretion. This suggests that different regulatory phenomena might underlie the phenomenon of parasite-induced suppression of inflammatory responses: allergen-specific secretion of TGF-β, which according to our results might regulate AHR, and allergen-independent (parasite-driven) IL-10 (and TGF-β) secretion. Given the interdependency of IL-10 and TGF-β (36–39), these two effects might depend on one another to convey the suppressive effects of *L. sigmodontis* on AI and SS. Statistically significant restoration of AI and SS might only be observed when both mediators are blocked, a question we did not address. Interestingly Wilson et al. (29) also show that lack of IL-10 production by the transferred regulatory cells does not affect their regulatory capacity. This still does not rule out a role for IL-10 in suppressing the allergic phenotype since the increase in IL-10 secretion might be due to cells other than the transferred cells secreting the IL-10, possibly in response to a signal by the Tregs.

The changes in the cytokine pattern after infection with *L. sigmodontis* were associated with the increase of two Treg populations ($CD4^+CD25^+CD45RB^{low}$ and $CD4^+TGF\text{-}\beta_{surface)}$ described in different disease models, among them allergic airway diseases (30, 40, 41). Preliminary FoxP3 mRNA analysis of mediastinal lymph nodes also showed an increase of this marker for Tregs (data not shown) similar to the observations of Wilson et al. (29). A role for Tregs in conveying immunosuppression in a *L. sigmodontis* infection model has been shown (42) and Wilson et al. (29) have proven Tregs to be responsible for the suppression of the allergic AI in their *H. polygyrus* model. Our own experiments showed partial restoration of AHR through depletion of Tregs; however, restoration of AI and SS was not observed after depletion of Tregs in infected animals before airway allergen challenges. Our results raise the question whether other regulatory cell types might be involved in the immune suppression induced by this parasite. In this line, both Mangan et al. (26) and Smits et al. (30) have delineated the interesting possibility that a subtype of B cells might also be able to convey parasite-induced immunosuppression in certain disease models, an hypothesis we did not test in our system.

Clearly, our results show a change in DC phenotype at the site of infection (Fig. 6), possibly a crucial aspect in the search of the pathways involved in the suppressive effects. Since the phenotype of a DC presenting a given Ag is essential for the direction of the subsequent immune response (43, 44), it seems possible that the up-regulation of the inducible costimulatory T cell molecule ICOS and its ligand on DCs, ICOSL or other alterations in the DC phenotype that we did not analyze, confers an altered T cell priming. Accordingly, Akbari et al. (45) have shown the interaction of ICOS-ICOSL to be necessary for the induction of T cells that prevented the development of AHR after mucosal tolerance induction. At this time point, our studies cannot fully answer whether infection with *L. sigmodontis* affects only the priming of T cells (most likely to occur in the spleen, the draining site of the peritoneal infection with the parasite) or rather effector functions of the various cell types involved in the local compartment at the time of airway allergen challenge. Given the effects of the anti-CD25 depletion before allergen challenge, it seems possible that $CD25^+$ Tregs suppress airway responsiveness at the effector phase. However, AI and SS might be regulated at a different time point because they were not affected by CD25 depletion before challenge, aspects which would need to be addressed through depletion studies at different time points.

The study we present shows the effects of parasite infection on all aspects of asthmatic airway disease. Wilson et al. (29) provide exhaustive evidence for a role for Tregs in suppressing allergic AI; however, they do not find changes in IgE to be associated with this suppression and they do not address the influence of helminth infection on AHR. Given our results with regard to the regulation of airway responsiveness, AI, and SS and the numerous studies that show these three hallmarks of allergic airway disease to be regulated independently (46, 47), we believe it is important to address further immunological questions regarding the effects of parasite infections in a model where, similar to epidemiological findings in humans, all of these aspects have been affected to understand the underlying suppressive mechanisms. Our findings may therefore help to interpret epidemiological data from human studies (8–10, 48) and add to the understanding of the immunology of parasites in general. We realize that shortcomings of a model that relies on systemic rather than inhalational priming, yet we believe that our results provide important starting points when taking studies on parasite-induced immunosuppression of allergic diseases to the next level. Carefully designed human (and further animal) studies on the relationship of allergic diseases and parasitic compounds are needed to shed light on the mechanisms used by parasites for immunomodulation and to correctly assess their therapeutic potential.

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