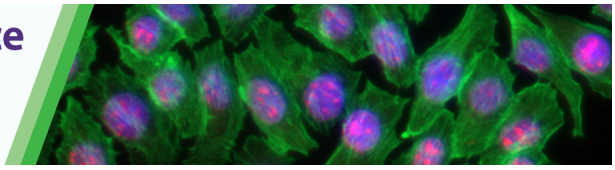

The Journal of Immunology

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A Helminth Immunomodulator Reduces Allergic and Inflammatory Responses by Induction of IL-10-Producing Macrophages[1]

Corinna Schnoeller,* Sebastian Rausch,* Smitha Pillai,* Angela Avagyan,† Bianca M. Wittig,‡ Christoph Loddenkemper,§ Alf Hamann,¶ Eckard Hamelmann,† Richard Lucius,* and Susanne Hartmann[2]*

The coincidence between infections with parasitic worms and the reduced prevalence of allergic disease in humans and in animal models has prompted the search for helminth molecules with antiallergic and antiinflammatory potential. We report herein that filarial cystatin, a secreted protease inhibitor of filarial nematodes, suppresses Th2-related inflammation and the ensuing asthmatic disease in a murine model of OVA-induced allergic airway responsiveness. Treatment with recombinant filarial cystatin inhibited eosinophil recruitment, reduced levels of OVA-specific and total IgE, down-regulated IL-4 production, and suppressed allergic airway hyperreactivity when applied during or after sensitization and before challenge with the allergen. Depletion of macrophages by clodronate-containing liposomes prevented the curative effects and restored the levels of infiltrating cells, IgE, and allergic airway reactivity. Blocking of IL-10 by application of anti-IL-10 receptor Abs restored the reduced number of infiltrating cells and the levels of OVA-specific IgE. In contrast, depletion of regulatory T cells by anti-CD25 Abs had only limited effects. Cystatin also modulated macrophage-mediated inflammation in a murine model of dextran sulfate sodium-induced colitis, leading to reduction of inflammatory infiltrations and epithelial damage. Our data demonstrate that treatment with a single helminth protein can exert the antiallergic effects of helminth infections. ***The Journal of Immunology,*** **2008, 180: 4265–4272.**

Epidemiological studies in humans and experimental data from animal models provide evidence that infections with parasitic worms are negatively associated with allergic and inflammatory immune responses (1–3). However, intensity and chronicity of infection are important factors that are associated with the amelioration of allergic inflammation, whereas acute infections may procure aggravation of allergic responses (4). Down-modulation of allergic inflammation has been explained as a survival strategy of worms, which is initiated to modulate inflammatory host responses directed against them. As a bystander effect, worm-induced immune modulation leads to down-regulation of other immune responses, such as allergic reactions in humans and experimental animals (5, 6) or viral and bacterial infections (7, 8). This effect of helminth infections has been explained by induction of regulatory T (Treg)[3] cells that inhibit recruitment and activation of effector cells (6, 9, 10), by driving the balance between different Ab isotypes (11) and/or T cell populations (12, 13). Thus, helminth infections, although responsible for an overall negative impact on the host's health, bear the intriguing potential to positively modulate pathological inflammatory responses. Evidence for this hypothesis was provided by clinical trials in patients with inflammatory bowel disease, in which the eggs of intestinal worms reduced symptom severity (14, 15).

The immunomodulatory effects of parasitic worms are exerted by secreted compounds, which have been shaped and optimized during coevolution with their vertebrate hosts. We have previously described in vitro effects of the recombinant expressed secreted cysteine protease inhibitors (cystatins) of the filarial nematodes *Acanthocheilonema viteae* and *Onchocerca volvulus*. These worms live in subcutaneous tissues of their hosts and are thus in permanent contact with the host immune system. The described filarial cystatins target mouse as well as human macrophages and induce antiinflammatory conditions (16–18). The effect was specific for cystatins of filarial nematodes, whereas recombinant cystatins of the free-living nematode *Caenorhabditis elegans* did not modulate macrophage functions in vitro (19).

In this study we investigated the influence of cystatin from the filaria *A. viteae* in vivo on airway disease and colitis and show that a single helminth immunomodulator prevents murine allergic airway reactivity and inhibits acute colitis, probably via enhancing IL-10 production by macrophages. Importantly, cystatin seems to interfere with pathways that are common to macrophage- and Th2-mediated immunopathology, suggesting that parasitic nematodes use a surprisingly simple strategy to interfere with inflammation. Hence, this mechanism of immunomodulation by a single parasite compound may be exploited to create new antiinflammatory compounds.

*Department of Molecular Parasitology, Humboldt University; and †Pediatric Pneumology and Immunology, ‡Gastroenterology/Infectiology/Rheumatology, §Pathology/Research Center for ImmunoScience, and ¶Experimental Rheumatology, Charité, Berlin, Germany

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[2] Address correspondence and reprint requests to Dr. Susanne Hartmann, Department of Molecular Parasitology, Humboldt University, Philippstrasse 13, 10115 Berlin, Germany. E-mail address: susanne.hartmann@rz.hu-berlin.de

[3] Abbreviations used in this paper: Treg, regulatory T cell; AHR, airway hyperreactivity; Av17, 17-kDa cystatin of *Acanthocheilonema viteae*; BALF, bronchoalveolar lavage fluid; DHFR, dihydrofolate reductase; DSS, dextran sodium sulfate; EU, endotoxin unit; MCh, methacholine; MLV, multilamellar vesicle; PBLNC, peribronchial lymph node cells.

Materials and Methods

Expression and purification of recombinant A. viteae *cystatin*

The cDNA of *A. viteae* cystatin was cloned and expressed in *Escherichia coli* as described previously (16). The control protein recombinant mouse dihydrofolate reductase (DHFR) was expressed according to the manufacturer's instructions (Qiagen). Recombinant filarial cystatin (Av17) and recombinant DHFR were identically purified by affinity chromatography using a Ni-NTA column and dialyzed against PBS/0.05% Triton X-100. To remove endotoxin contaminations, EndoTrap columns were used according to the manufacturer's instructions (Profos). Final endotoxin concentrations were detected by a *Limulus* amoebocyte lysate test (Cambrex) (endotoxin concentration rAv17: 2.5 pg/μg protein = 0.05 endotoxin units (EU) per application of rAv17; rDHFR: 2.8 pg/μg protein = 0.056 EU per application of rDHFR).

Murine asthma model and measurement of airway hyperreactivity (AHR)

Female BALB/c mice (Harlan Winkelmann) were sensitized twice (day 0 and day 14) i.p. with 20 μg OVA (grade VI, Sigma-Aldrich) emulsified in 2 mg of aluminum hydroxide (Imject Alum, Pierce). On days 28 and 29, mice were challenged intranasally with 50 μg OVA. Airway responsiveness was measured via whole-body plethysmography on day 31 in unrestrained mice after challenge with increasing doses of metacholine (MCh, Sigma-Aldrich) as described elsewhere (20). Recombinant *A. viteae* cystatin (20 μg) or the same amount of control protein (both proteins applied without adjuvant) in PBS was injected i.p. four times in weekly intervals during the sensitization (days 1, 7, 14, and 21), or three times after sensitization (days 21, 23, and 25) before airway allergen challenges. Naive control animals were treated with aluminum hydroxide in PBS and challenged with PBS. All experimental procedures were approved by the animal ethics committee.

Bronchoalveolar lavage (BAL) and staining for mucus production

BAL was harvested, and BAL cells were stained with DiffQuick (Fisher Scientific) and differentiated by morphological criteria (count of 200 cells under light microscopy in a blinded fashion), as previously described (21). Supernatants were stored at −20°C for subsequent analysis of cytokines. Staining for mucus production of the lung with periodic acid-Schiff and hematoxylin was performed as described elsewhere (22).

Serum levels of IgE

Total IgE levels and OVA-specific IgE concentrations in serum were determined by ELISA, as described earlier (23).

Cytokine analysis

Spleen mononuclear cells were isolated by density gradient centrifugation (Lympholyte-M, Cedarlane Laboratories) and cultured (5×10^5 cells/well) in RPMI 1640 for 96 h in the presence of 50 μg/ml OVA, 10 μg/ml Av17, or 10 μg/ml DHFR. RPMI 1640 was supplemented with penicillin, streptomycin, L-glutamin, and 10% FCS (HyClone, Perbio Science). Cell culture supernatants were stored at −20°C until performance of cytokine ELISA, as described (21) (IL-4, IL-5, IL-10 BD OptEIA, BD Biosciences). Real-time PCR to analyze the expression level of TGF-β in lung tissue was performed using the 7300 Real-Time PCR System (Applied Biosystems) and TaqMan reagents (TGF-β primer and probe: Mm 00441729_g1, Applied Biosystems; housekeeping gene GAPDH: Mm 99999915_g1, Applied Biosystems). PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of TGF-β relative to the endogenous GAPDH control was determined using the comparative threshold cycle ($\Delta\Delta C_T$) method, as described earlier (24).

Treg cells in peribronchial lymph node cells (PBLNC)

Treg cells in PBLNC were characterized by expression of the surface markers CD4, CD25, and CD103 (25) and the transcription factor Foxp3. Cells were washed in PBS/0.2% BSA and stained for 15 min with anti-CD4-FITC (BD Biosciences, clone RM4–5), anti-CD25-APC (BD Biosciences, clone PC61), anti-CD103-Bio (gift from A. Scheffold, German Rheumatism Research Center (DRFZ), Berlin, clone M290), and streptavidin-PECy7 (BD Biosciences). Foxp3 staining was performed with the PE-anti-mouse Foxp3 staining kit (eBioscience, clone FJK-16s). Nonspecific surface binding was prevented by addition of anti-mouse FcγR (clone 2.4G2, gift from A. Scheffold). Nonspecific intracellular binding was blocked with whole rat IgG (The Jackson Laboratory). FACS analysis was performed on LSR II (BD Biosciences) and FlowJo software (TreeStar).

Depletion of macrophages and Treg cells and neutralization of IL-10

Macrophages were depleted by intranasal and i.p. application of clodronate liposomes 2 days before challenges (100 μl per application). Clodronate liposomes were prepared as described elsewhere (26). In brief, an emulsion of phosphatidylcholin and cholesterol in chloroform was vacuum evaporated, and multilamellar vesicles emerged by gentle shaking with a clodronate suspension under nitrogen conditions. The vesicles were kept under nitrogen until washing with sterile PBS twice and resuspension in the same buffer. Macrophage depletion was confirmed by flow cytometry or histological cell differentiation. Treg cells were depleted by i.p. application of 100 μg anti-CD25 Abs (clone PC61, gift from A. Scheffold) 5 days before challenges. Treg depletion was confirmed by flow cytometry. Anti-IL-10 receptor Abs (clone 1B1, gift from K. Falk, Max-Delbrück-Center, Berlin) were applied three times i.p., 500 μg each time along with filarial cystatin. An isotype-matched control Ab was used as control (Sigma-Aldrich).

Colitis model

Seven- to 8-wk-old male C57BL/6 mice weighing 18–20 g were fed with sterile drinking water containing 2.5% dextran sodium sulfate (DSS, m.w. 40,000; ICN Biomedicals) for 7 days. rAv17 or rDHFR (20 μg) was injected i.p. four times (days 1, 3, 5, and 7). An additional group received DSS and was sham-treated with the protein application buffer. Appearance of feces and weight change was monitored daily. Animals were sacrificed on day 8 by cervical dislocation. The colon was resected between the ileocecal junction and the proximal rectum and divided into three segments (proximal, middle, and distal). Each segment was fixed in formalin, embedded in paraffin, cut into 4-μm sections, and stained with H&E to assess the degree of inflammation by a pathologist. Experimental procedures were approved by the animal ethics committee.

Statistical analysis

Each experiment was performed with 5–6 animals/group, and the experiments shown are representative of two to three independent experiments unless stated otherwise. Statistical analysis was performed with the two-tailed Mann-Whitney *U* test. Data are presented as means ± SE. Values of $p < 0.05$ were considered to be statistically significant.

Results

Filarial cystatin inhibits the development of allergic airway hyperresponsiveness

To study the effect of filarial cystatin (Av17) on allergen-mediated sensitization and airway disease, we used a mouse model of OVA-induced allergic airway hyperreactivity. BALB/c mice were i.p. injected with *E. coli*-expressed recombinant cystatin from *A. viteae*, during or after sensitization with OVA and subsequently challenged with allergen via the airways. Treatment with four doses of cystatin (20 μg each) during sensitization (preventive model, Fig. 1*A*), but not with the irrelevant *E. coli*-expressed recombinant control protein DHFR, significantly reduced the total numbers of cells ($p < 0.028$) in bronchoalveolar lavage fluid (BALF) to the level of naive mice (Fig. 2*A*). The positive effect of cystatin application was most pronounced for eosinophils ($p < 0.05$) (Fig. 2*A*). Similar results regarding total cell and eosinophil recruitment ($p < 0.02$, $p < 0.028$) were obtained when cystatin was applied three times after sensitization (prechallenge model, Figs. 1*B* and 2*A*). Histological analysis of the lung tissue corroborated these data, showing only background levels of cell infiltration within the lung tissue and a nearly absent mucus production in mice cotreated with OVA/cystatin (Fig. 2*B*). The effect of cystatin on allergen-induced sensitization and airway inflammation was accompanied by a significant reduction ($p < 0.028$) in the development of in vivo airway hyperreactivity (AHR) in treated mice in the preventive (Fig. 2*C*) as well as in the prechallenge model ($p < 0.028$, Fig. 2*D*). Application of Av17 alone showed no effect on AHR (data not shown).

Treatment with cystatin also significantly reduced serum levels of total IgE ($p < 0.0003$, Fig. 2*E*) as well as of OVA-specific IgE ($p < 0.0002$, Fig. 2*F*) when applied in the preventive model, an

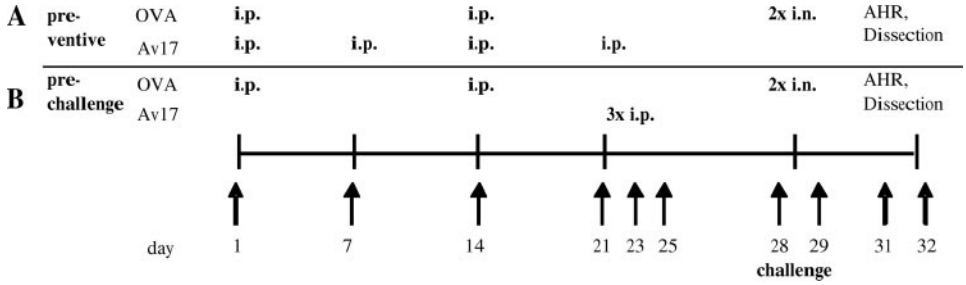

FIGURE 1. Scheme of the preventive (*A*) and the prechallenge (*B*) model of airway hyperreactivity.

effect not seen after injection of the control protein DHFR. This effect was specific to IgE, as serum levels of OVA-specific IgG1 and IgG2a were not significantly altered compared with sensitized and challenged controls (data not shown). The significant inhibition of IgE production was accompanied by a reduced capacity of sera from cystatin/OVA-treated mice to induce degranulation of basophils (data not shown). However, cystatin application in the prechallenge model showed only a trend of a down-regulation of total IgE production, although OVA-IgE levels decreased significantly ($p < 0.02$; Fig. 2, *E* and *F*). Together, these data suggest that cystatin interferes with the recruitment of inflammatory cells and with IgE production, thus inhibiting the main features of allergen-induced alterations in this mouse model both during and after sensitization.

BALF of mice treated with OVA/cystatin contained less IL-4 as compared with BALF of animals treated with OVA only ($p < 0.02$), or with OVA/DHFR ($p < 0.028$) as shown in the preventive and in the prechallenge model (Fig. 2*G*). This effect was systemic, as spleen cells of OVA/cystatin-treated mice also produced significantly less IL-4 in response to allergen stimulation in vitro compared with controls in both models ($p < 0.015$, $p < 0.0004$; Fig. 2*G*). Levels of IL-10 in BALF were similar between the groups, whereas production of IL-10 by spleen cells stimulated with cystatin in vitro was significantly increased in the preventive model ($p < 0.01$, see Fig. 6, *D* and *E*) and even more pronounced in the prechallenge model ($p < 0.002$, see Fig. 6*D*). The levels of IL-5, IL-12, IL-13, and IFN-γ in BALF and culture supernatant of OVA-restimulated spleen cells and levels of TGF-β mRNA of lung tissue, as determined by real-time PCR, were not significantly altered in mice treated with cystatin compared with controls (data not shown), and also proliferation of spleen cells in response to OVA was not significantly altered in cystatin-treated animals (data not shown). These data are compatible with an overall down-regulation of effector functions of the allergic immune reaction by filarial cystatin, resulting in abrogation of allergic sensitization, impaired Th2 immune responses, reduced eosinophilic airway inflammation, and decreased airway hyperreactivity.

Filarial cystatin inhibits acute colitis

To determine whether the effect of cystatin was specific to Th2-mediated immunopathology or whether it also inhibits other types of inflammation, we tested the nematode immunomodulator in a murine model of colitis. Colitis was induced by application of DSS

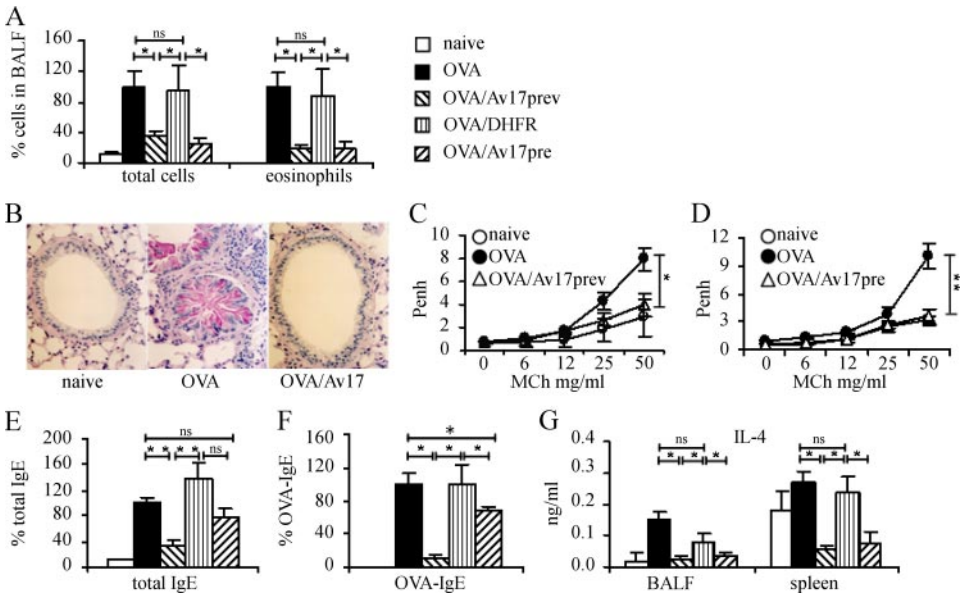

FIGURE 2. Influence of filarial cystatin (Av17) on allergic airway hyperreactivity. *A*, Total cell numbers and eosinophil numbers in the BALF of mice treated with filarial cystatin (Av17) or the recombinant control protein DHFR observed in the preventive and the prechallenge model, respectively. *B*, Representative lung sections of preventive model stained with periodic acid-Schiff and hematoxylin for analysis of mucus production (red, magnification ×400). *C*, Airway hyperreactivity after application of different dosages of methacholine to mice treated with filarial cystatin in the preventive and (*D*) in the prechallenge model. *E*, Total IgE concentration and (*F*) OVA-specific IgE concentrations in sera. *G*, IL-4 levels in BALF and OVA-specific IL-4 production of spleen cells. Naive indicates PBS-treated mice; OVA, OVA-treated mice; OVA/Av17prev, OVA and filarial cystatin (Av17)-treated mice in preventive approach; OVA/DHFR, OVA and DHFR-treated mice; OVA/Av17 pre, OVA and filarial cystatin (Av17)-treated mice in prechallenge mode. Representative data of three individual experiments with 5–6 animals per group; to directly compare data of the prechallenge with the preventive model in one graph, we expressed data in percentages (OVA group is 100%) if necessary; ∗, $p < 0.05$, ∗∗, $p < 0.005$.

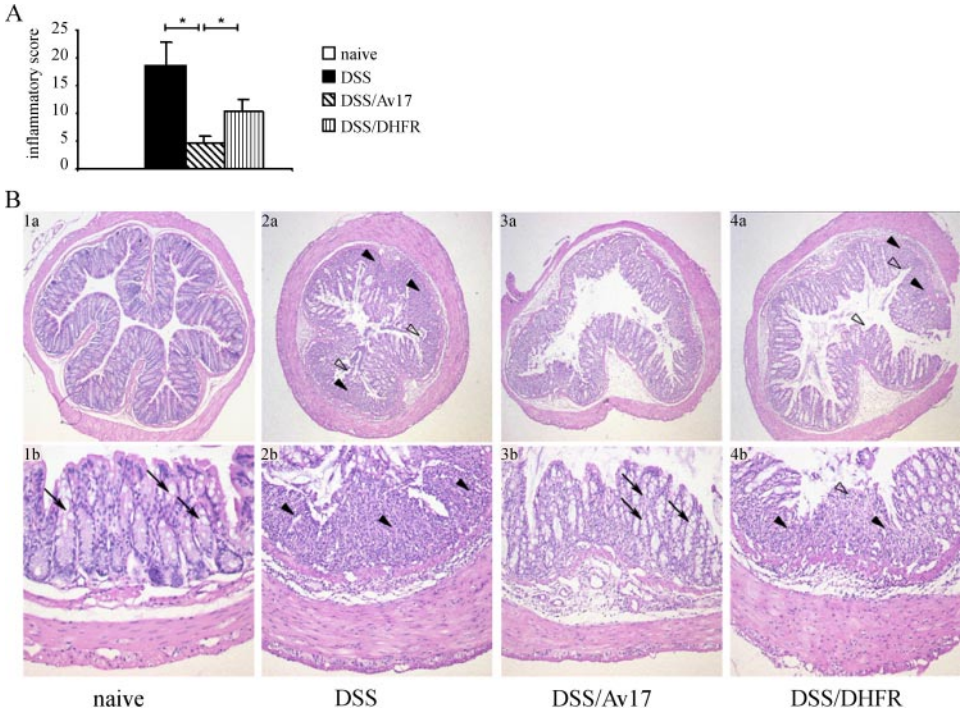

FIGURE 3. Influence of filarial cystatin on DSS-induced colitis. *A*, Inflammatory score of mice treated with DSS in the drinking water and filarial cystatin or the recombinant control protein DHFR. Combined data from two experiments with 4–5 animals per group are shown. *B*, Colon histology: appearance of the colon in a healthy mouse (1), mouse receiving DSS and the protein application buffer (2), mouse receiving DSS and Av17 (3), and control animal treated with DSS and the recombinant control protein DHFR (4). Note extensive epithelial damage with loss of crypts and erosions (open arrow heads), dense inflammatory cell infiltrations (filled arrow heads), goblet cell depletion and thickening of colon wall (*2b* and *4b*) as compared with only focal and superficial erosions associated with less inflammatory cell infiltrations (*3b*). Black arrows indicate normal appearance of crypts with goblet cells. Magnification ×40 (*upper row*, *1a–4a*) and ×200 (*lower row*, *1b–4b*). Representative data of one individual from two experiments with 2–3 animals per group are shown; *, $p < 0.05$.

in the drinking water. Intraperitoneal administration of four 20-μg doses of filarial cystatin revealed a significant reduction ($p < 0.03$) of the inflammatory index (54%) of the colon as compared with sham treatment with DSS/DHFR (Fig. 3, *A* and *B*). DSS-treated animals showed extensive epithelial damage with loss of crypts, erosions, dense inflammatory cell infiltrations, goblet cell depletion, and thickening of the colon wall as compared with focal erosions associated with less inflammatory cell infiltrations in the cystatin-treated mice.

Filarial cystatin targets macrophages

In previous studies, we have shown that filarial cystatin alters macrophage function in vitro. Therefore, we investigated the relevance of macrophages in our mouse model of OVA-induced airway inflammation. We selectively depleted macrophages from OVA/cystatin-treated animals using clodronate containing liposomes (multilamellar vesicles (MLV)) 2 days before airway allergen challenges of sensitized mice. This treatment led to the loss of >95% of macrophages in the BALF and in the peritoneum, as confirmed by FACS staining of F4/80-positive cells negative for CD19 and CD3 (data not shown). Depletion of macrophages had no significant influence on the cell recruitment into the lung within the OVA group (Fig. 4*A*). In contrast, in cystatin-treated animals the depletion of macrophages led to a reversion of the total cell numbers ($p < 0.008$) and eosinophils ($p < 0.002$) in BALF to the level of the OVA-sensitized and challenged control group (Fig. 4*A*). The treatment also partly restored the production of total IgE ($p < 0.05$, data not shown) and of OVA-specific IgE in OVA/cystatin-treated mice ($p < 0.007$; Fig. 4*B*). OVA-specific IL-4 production of restimulated spleen cells was not significantly altered (Fig. 4*C*). However, AHR of macrophage-depleted, OVA/cystatin-treated mice rose ($p < 0.04$) significantly (Fig. 4*D*). Together, these results indicate that macrophages translate the immunomodulatory effects of cystatin by inhibition of airway inflammation, airway hyperreactivity, and IgE production.

Cystatin treatment increases the number of Treg cells

To determine whether cystatin treatment augments the numbers of Treg cells, we analyzed the coexpression of the surface markers CD4, CD25, and CD103 on PBLNC. The proportion of Treg cells was significantly elevated in OVA/cystatin-treated animals as compared with OVA controls (3 vs 1.9%; $p < 0.05$) and to OVA/DHFR controls (3 vs 2.1%; $p < 0.05$; Fig. 5, *A* and *C*). More than 96% of the Treg cells expressed Foxp3 (Fig. 5*B*), a reliable marker for Treg cells. To analyze the role of Treg cells in cystatin-induced immunomodulation we treated sensitized animals with anti-CD25 Abs 5 days before the first airway allergen challenge, which significantly decreased the number of $CD4^+CD25^+CD103^+$ Treg cells to 0.4% in PBLNC (data not shown). In cystatin-treated mice with depleted Treg cells, the levels of total cells as well as eosinophils showed a trend of restoration (Fig. 5*D*). The production of total IgE ($p < 0.002$; data not shown) and OVA-specific IgE ($p < 0.002$; Fig. 5*E*) was significantly restored compared with cystatin-treated animals with nonmanipulated Treg cells. However, no changes were determined for allergen-specific IL-4 production (Fig. 5*F*) and development of AHR in cystatin-treated mice after depletion of Treg cells (not shown). Application of isotype-matched control Abs had no significant effects on all parameters measured. These data indicate that Treg cells are involved in the

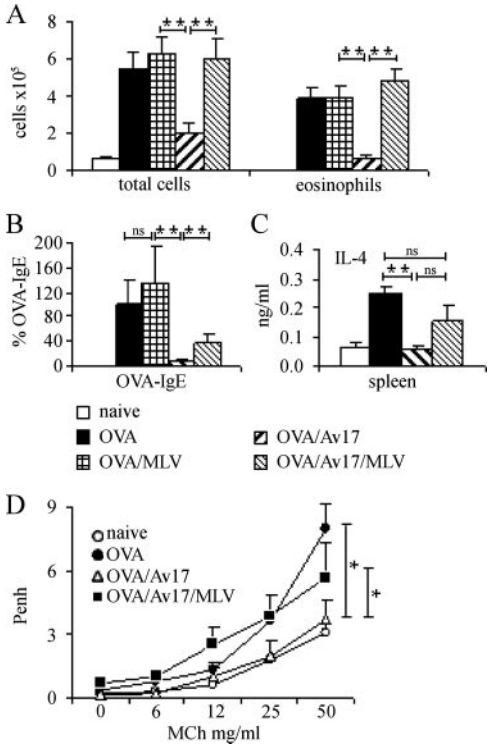

FIGURE 4. Effects of filarial cystatin are dependent on macrophages. Mice were sensitized with OVA and treated with cystatin during the sensitization (preventive model), and macrophages were depleted by clodronate-filled MLV application 2 days before challenges. *A*, Total cell numbers and eosinophil numbers in BALF; *B*, levels of OVA-specific serum IgE; *C*, OVA-specific IL-4 production of spleen cells; *D*, airway hyperreactivity of mice after treatment with different dosages of methacholine. Naive indicates PBS-treated mice; OVA, OVA-treated mice; OVA/MLV, OVA and MLV-treated mice; OVA/Av17, OVA and filarial cystatin (Av17)-treated mice; OVA/Av17/MLV, OVA and Av17-treated mice in which macrophages were depleted by application of MLVs containing clodronate; Penh, pause enhanced. Data are representative for three individual experiments with 4–6 animals per group; to directly compare IgE values, we expressed data in percentages (OVA group is 100%); ∗, $p < 0.05$, ∗∗, $p < 0.005$.

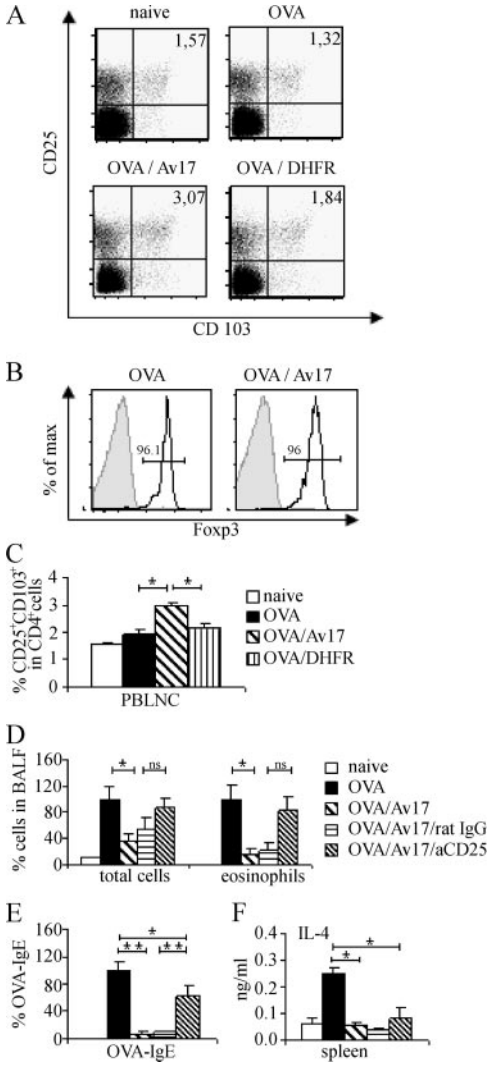

FIGURE 5. Depletion of $CD25^+$ cells partly reversed the immunomodulation exerted by filarial cystatin. Mice were sensitized with OVA and treated with cystatin during the phase of sensitization (preventive model); Treg cells were depleted by application of anti-CD25 Abs (clone PC61) 5 days before challenge. *A*, FACS plots of PBLNC of one representative animal per group; *B*, histogram of $Foxp3^+$ cells in $CD4^+CD25^+CD103^+$ PBLNC; *C*, numbers of Treg cells ($CD4^+CD25^+CD103^+$) in PBLNC; *D*, total cell and eosinophil numbers in BALF; *E*, levels of OVA-specific serum IgE; *F*, OVA-specific IL-4 production of spleen cells. Naive indicates PBS-treated mice; OVA, OVA-treated mice; OVA/Av17, OVA and filarial cystatin (Av17)-treated mice; OVA/Av17/aCD25, OVA and Av17-treated mice in which Treg cells were depleted by application of anti-CD25 Abs; OVA/DHFR, OVA and DHFR-treated mice; OVA/Av17/rat IgG, mice treated with OVA and Av17 plus isotype-matched control Abs; PBLNC, peribronchial lymph node cells. Representative data of three individual experiments with 4–6 animals per group; to directly compare cells in BALF and IgE values, we expressed data in percentages (OVA group is 100%); ∗, $p < 0.05$, ∗∗, $p < 0.005$.

cystatin-induced effects on airway hyperreactivity, albeit to a less prominent degree than are macrophages.

Inhibition of allergic responses by cystatin is dependent on IL-10

Because previous in vitro studies have shown that cystatin induces the production of IL-10 by macrophages and depletion of these cells reverses the effects of cystatin in the asthma model, we hypothesized that IL-10 might be a key mediator of cystatin-induced immunomodulation. We analyzed its influence using antagonizing anti-IL-10 receptor Abs (anti-IL-10R) in the prechallenge model of OVA-induced airway hyperreactivity, as Av17-specific IL-10 production in spleen cells was even more pronounced in the prechallenge approach in comparison to the preventive approach (Fig. 6*D*). Anti-IL-10R was injected three times along with the filarial cystatin after sensitization and before the first allergen airway challenge with OVA. Blocking of the IL-10R in OVA/cystatin-treated animals completely restored total cell numbers in BALF to the degree observed in sensitized and challenged positive control animals ($p < 0.02$) (Fig. 6*A*). This effect of blocking IL-10R was most pronounced for the eosinophilic airway infiltration ($p < 0.02$) (Fig. 6*A*). Similarly, the development of AHR (data not shown) and the production of OVA-specific IgE were increased to the levels observed in OVA-sensitized and OVA-challenged control mice ($p < 0.03$) (Fig. 6*B*). However, the inhibition of allergen-specific IL-4 production in OVA/cystatin-treated animals was not altered after application of anti-IL-10R Abs (Fig. 6*C*). In all cases, application of isotype-matched control Abs had no effects on the cystatin-induced parameters, and application of aIL-10R did not alter the values of the OVA-positive control group (Fig. 6, *A* and *B*). These data indicate that IL-10 is a key cytokine in filarial

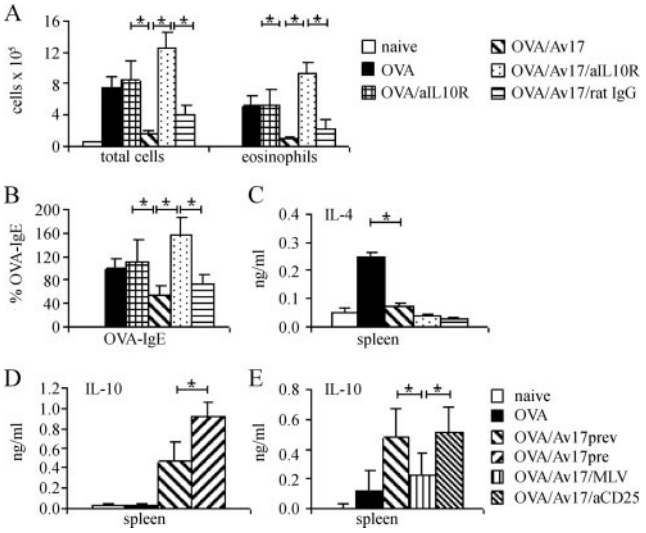

FIGURE 6. Suppression of allergic responses by filarial cystatin is dependent on IL-10. Mice were sensitized with OVA and treated three times with cystatin (20 μg/ml) and with anti-IL-10R Abs (three times 500 μg per animal) after sensitization and before challenge with OVA (prechallenge model). *A*, Total cell numbers and eosinophil numbers; *B*, levels of OVA-specific serum IgE; *C*, OVA-specific IL-4 production of spleen cells; *D*, IL-10 production of spleen cells after stimulation with Av17 in the preventive and prechallenge model; *E*, IL-10 production in response to Av17 stimulation in spleen cells after depletion of macrophages or Tregs (preventive model). Naive indicates PBS-treated mice; OVA, OVA-treated mice; OVA/aIL-10R, mice treated with OVA and anti-IL-10 receptor Abs; OVA/Av17prev, OVA and filarial cystatin (Av17)-treated mice (preventive model); OVA/Av17 pre, OVA and filarial cystatin (Av17)-treated mice (prechallenge model); OVA/Av17/aIL-10R, mice treated with OVA, Av17, and anti-IL-10 receptor Abs (prechallenge model); OVA/Av17/rat IgG, mice treated with OVA, Av17, and isotype-matched control Abs (prechallenge model); OVA/Av17/MLV, OVA and Av17-treated mice in which macrophages were depleted (preventive model); OVA/Av17/aCD25, OVA and Av17-treated mice in which Treg cells were depleted (preventive model). Representative data of two individual experiments with 6 animals per group are shown in *A–C*; representative data of three individual experiments with 4–6 animals are shown for 6*D* and *E*; to directly compare IgE values, we expressed data in percentages (OVA group is 100%); $*$, $p < 0.05$.

cystatin-induced modulation of allergic disease, although IL-10-independent mechanisms, such as the suppression of allergen-specific IL-4, also have to be taken into account.

Because macrophages and Treg cells are both potent sources of IL-10, we asked which of these cells is primarily responsible for the IL-10 production after treatment with filarial cystatin. Cystatin-treated animals showed significantly increased levels of IL-10 in spleen in comparison to OVA-treated animals ($p < 0.002$ prechallenge model, $p < 0.01$ preventive model, $p < 0.024$ comparison of prechallenge to preventive model; Fig. 6, *D* and *E*). However, after depletion of macrophages, IL-10 production was significantly decreased in the OVA/cystatin-treated animals ($p < 0.04$; Fig. 6*E*), whereas such an effect was not observed in the animals depleted of Treg cells. Treg cell-depleted mice actually showed a trend of elevated IL-10 values (Fig. 6*E*). These data highlight the pivotal role of macrophages in the cystatin-induced modulation of allergic airway inflammation and hyperreactivity.

Discussion

Infections with parasitic worms have the potential to suppress allergic and inflammatory immune responses (27, 28). Our study provides evidence that such an effect can be exerted by a single immunomodulatory protein of a parasitic nematode, filarial cystatin (Av17). Application of cystatin in a murine model of OVA-induced airway inflammation and hyperreactivity during or even after allergen sensitization counteracted the Th2 effector mechanisms responsible for allergic airway disease in several ways. First, cystatin treatment significantly reduced the recruitment of inflammatory cells, particularly eosinophils, into the lungs, preventing the release of effector molecules that lead to tissue damage (29, 30). Second, cystatin lowered the production of allergen-specific and total IgE, resulting in less efficient sensitization of mast cells and basophils (31), as determined by decreased degranulation of basophils sensitized with sera of cystatin/OVA-treated mice (unpublished observation). Third, cystatin treatment reduced local production of IL-4 in BALF as well as systemic IL-4 production by spleen cells. This Th2 cytokine plays a major role in the development of allergic reactions by inducing the production of IgE, promoting Th2 responses and enhancing tissue homing of inflammatory effector cells (32). Therefore, we assume that the reduction of IL-4 levels by cystatin contributed to the inhibition of IgE production and the lowering of Th2 responses in our model.

Our data show that IL-10 is a key element in the cystatin-induced immunomodulation, as blocking of IL-10 with an antagonistic anti-IL-10R Ab reversed the effect of cystatin on cell recruitment and production of IgE. Other studies have also shown that systemic production of IL-10 inhibits the development of airway hyperresponsiveness and allergic inflammation (33, 34). IL-10 was reported to modulate Th2 responses by suppression of allergen-specific IgE production and concomitant induction of noninflammatory Ab isotypes, by reduction of proinflammatory cytokines released by mast cells, basophils, and eosinophils, and by indirect interference with Th2-associated phenomena such as mucus production (35, 36). Thus, IL-10 is capable of redirecting pathologic allergic responses by a broad range of suppressive mechanisms. Accordingly, IL-10 levels in allergic patients undergoing successful immunotherapy are increased (37). However, the inhibitory effect of IL-10 is not restricted to Th2 responses, as the cytokine also modulates the maturation of dendritic cells, inhibiting the expression of MHC class II and costimulatory molecules (38). We now propose that up-regulation of IL-10 production by macrophages is the main mechanism of cystatin-induced immunomodulation in our asthma model and is potentially involved in down-regulation of macrophage-initiated immune responses observed in acute DSS colitis.

The importance of IL-10 in cystatin-induced protection against asthma is in accordance with reports that IL-10 modulates allergic immune responses in mice infected with the gut nematode *Heligmosomoides polygyrus* or the blood fluke *Schistosoma mansoni* (39, 40). However, other studies demonstrate that helminth-induced protection against allergic disease is not dependent on IL-10 and they propose a role for TGF-β (6, 41). That we were not able to demonstrate increased expression of TGF-β at the level of mRNA is in agreement with the current notion that parasitic worms down-regulate inflammation using multiple immunological pathways (27). We propose that cystatin-triggered macrophages are the main source of antiinflammatory IL-10, as depletion of macrophages with clodronate liposomes 2 days before airway allergen challenge completely ablated the antiallergic effect of cystatin and led to significantly diminished IL-10 levels in cystatin-treated animals. Treatment with clodronate liposomes (MLV) specifically targets macrophages, while other potentially IL-10-producing cells remain present. Transcription profiling of murine macrophages after exposure to filarial cystatin revealed an up-regulation of IL-10 expression, but no changes in expression of markers for classical or alternative macrophage activation (unpublished

results). Thus, cystatin-treated macrophages resembled type II-activated macrophages (42). Just recently it has been shown that infection with the trematode parasite *S. mansoni* prevents experimental colitis via a macrophage-mediated mechanism (43). We are currently studying the molecular mechanisms of immune modulation by filarial cystatin, such as the signaling pathways involved and the domains of cystatin responsible for the described effects. Our preliminary results indicate that mitogen-activated protein kinases are involved in the cystatin-induced modulation of macrophages, which is in line with recent data showing that inhibitors of mitogen-activated protein kinases may be suitable targets for antiinflammatory therapy of asthma (44). So far it is speculative as to which receptor on macrophages is addressed by Av17. One potential candidate is the scavenger receptor CD36 that is predominantly expressed on macrophages, platelets, and endothelial cells (45). Interestingly, CD36 is the receptor for the cell adhesion protein PfEMP1 of the malaria parasite *Plasmodium falciparum*, and binding of PfEMP1 to CD36 leads to the induction of IL-10 (46). Alternatively, cystatin could bind to the TGF-β receptor II. In this context human cystatin is shown to physically interact with the TGF-β receptor and thereby antagonize TGF-β signaling in cancer cells (47). Furthermore, it would also be feasible that cystatin binds to the FcγR in complex with Abs and thereby generates type II macrophages. Here, crosslinking of FcγR during activation of macrophages is shown to result in abrogation of IL-12 production and strong induction of IL-10 (48).

A second candidate cell for the production of IL-10 is Treg cells, as production of IL-10 by Treg cells has been suggested to play a central role in control of allergic airway disease (49). However, depletion of Treg cells by anti-CD25 treatment did not lead to reduction of IL-10 levels in our experiments, strongly suggesting that Treg cells are not the main source of IL-10 in our setting. Interestingly, in other infections such as toxoplasmosis or schistosomiasis, Th1 cells or B cells are shown to be the relevant IL-10 sources (40, 50). Because our depletion experiments revealed that Treg cells contribute to the lowering of eosinophil numbers and OVA-specific IgE production, we suggest that IL-10-independent Treg cell functions could have a role in the antiallergic effect of cystatin (51, 52). Interestingly, Treg cells have also been described to be essentially involved in the inhibition of allergic responses by infection with the gut nematode *Heligmosomoides polygyrus* (6). In this model, transfer of mesenteric lymph node cells of nematode-infected mice into OVA-sensitized mice suppressed murine allergic responses in vivo.

Taken together, our results show a novel mechanism of immune modulation exerted by filarial cystatin, which is different from other well-described helminth immunomodulators. Among others, a phosphatidylserine with specific chain length expressed by *S. mansoni* eggs engages TLR2 expressed on dendritic cells, which in turn induces IL-10-producing regulatory T cells (53). A secreted glycoprotein of the filarial nematode *A. viteae* inhibited FcεRI-mediated mast cell responses by forming a complex with TLR4, which resulted in the sequestration of protein kinase C-α, a molecule important for mast cell activation (54, 55). We provide evidence that cystatin protects from inflammation in a systemic fashion, as reflected by its effects on remarkably different compartments, such as the lung and the gut. Moreover, the protein inhibits Th2-related as well as macrophage-related inflammation, which stands in stark contrast to the effect of TLR2 and TLR4 agonists of bacterial origin that have recently been shown to downregulate Th2-driven allergic responses in mouse models (56, 57). These components act through stimulation of Th1 responses, as evidenced by elevated production of IFN-γ and other Th1 cytokines, which is clearly not the case for filarial cystatin.

The broad-acting antiinflammatory activity of cystatin, which supposedly protects the worms from inflammation triggered by worm-derived components, might be exploited for the treatment of immune-related diseases, as it selectively mimics the advantageous properties of a parasite infection without entailing its undesired side effects.

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Disclosures

The authors have no financial conflicts of interest.

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