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A nematode immunomodulator suppresses grass pollen-specific allergic responses by controlling excessive Th2 inflammation

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ABSTRACT

Helminth parasites modulate the immune system by complex mechanisms to ensure persistence in the host. Released immunomodulatory parasite components lead to a beneficial environment for the parasite by targeting different host cells and in parallel to a modulation of unrelated inflammatory responses in the host, such as allergy. The aim of this study was to investigate the effect of the potent helminth immunomodulator, filarial cystatin, in a murine model of airway inflammation and hyperreactivity induced by a clinically relevant aeroallergen (timothy grass (*Phleum pratense*) pollen) and on the function of peripheral blood mononuclear cells (PBMCs) from timothy grass pollen allergic patients. BALB/c mice were systemically sensitised with a recombinant major allergen of timothy grass pollen (rPhl p 5b) and then challenged with timothy grass pollen extract (GPE) via the airways. Filarial cystatin was applied i.p. during the sensitisation phase. Airway hyperresponsiveness to methacholine challenges, inflammation of airways, inflammatory cell recruitment, cytokine production and lung histopathology were investigated. In a translational approach, PBMCs from allergic subjects and healthy controls were treated in vitro with cystatin prior to stimulation with GPE. Administration of filarial cystatin suppressed rPhl p 5b-induced allergen-specific Th2-responses and airway inflammation, inhibited local recruitment of eosinophils, reduced levels of allergen-specific IgE and down-regulated IL-5 and IL-13 in the bronchoalveolar lavage (BAL). Ex vivo restimulation with cystatin of spleen cells from cystatin-treated mice induced the production of IL-10, while cystatin inhibited allergen-specific IL-5 and IL-13 levels. Human PBMCs from timothy grass pollen allergic patients displayed a shift towards a Th1 response after treatment with cystatin. These results show that filarial cystatin ameliorates allergic inflammation and disease in a clinically relevant model of allergy. This data indicate that filarial cystatin has a modulatory effect on grass pollen-specific responses warranting further investigation of potential preventive and therapeutic options in the treatment of allergies.

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1. Introduction

Helminth infections have been almost completely eradicated in western countries over the past 50 years while the prevalence of allergic diseases has dramatically increased. One possible explanation of these phenomena is the hygiene hypothesis (Strachan, 1989; Yazdanbakhsh et al., 2002), which states that improved public health, use of antibiotics, medications and vaccines that reduce the occurrence of viral, bacterial or helminth infections early in life in return contribute to a higher susceptibility of immune dysregulation and a higher prevalence of allergic and autoimmune disorders. Moreover, epidemiological studies observed lower levels of immunopathological diseases such as Th1-related autoimmune diseases or aberrant Th2-related diseases e.g. asthma or allergic rhinitis in helminth-infected populations (Fallon and Mangan, 2007; Patel et al., 2008; Cooke, 2009; Smits et al., 2010).

Many studies in animals and humans have shown that helminth infections primarily induce a Th2 immune response involving the cytokines, IL-4, IL-5, IL-9, IL-10 and IL-13, and immunoglobulin IgE, whereas the immune system is subsequently suppressed during the chronic phase of the infection (Maizels and Yazdanbakhsh, 2003). To down-modulate host protective Th2-type immune responses, helminths induce different regulatory cell types that

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actively suppress effector cells (Metwali et al., 2006; Grainger et al., 2010; Wilson et al., 2010), produce the anti-inflammatory cytokines, IL-10 and TGF-β (Dewals et al., 2010; Hubner et al., 2012), and create a balance between different types of antibodies (Adjobimey and Hoerauf, 2010), allowing parasite persistence within the host (Maizels and Yazdanbakhsh, 2003). Interestingly, the most promising strategy for treatment of allergic diseases is the suppression of overwhelming Th2 immunity and the induction of regulatory mechanisms. Accordingly, it would be intriguing to apply helminth infections or helminth-derived products for prevention and intervention to down-regulate bystander Th2-type immune disorders (Fallon and Mangan, 2007; Holgate and Polosa, 2008; Harnett and Harnett, 2010).

Among the best-characterised secreted helminth molecules, cysteine protease inhibitors (cystatins) are considered to be a major factor in interfering with the host's immune system or essential enzymes (Maizels et al., 2001; Hartmann and Lucius, 2003; Hewitson et al., 2009). Previous studies reported the regulatory effect of cystatins from the filarial nematodes, *Onchocerca volvulus* (Ov17) and *Acanthocheilonema viteae* (AvCystatin, Av17) (Hartmann et al., 1997; Schönemeyer et al., 2001). AvCystatin was shown to be a parasite-specific modulator of macrophages and to induce IL-10 production by splenocytes while inhibiting T cell proliferation (Hartmann et al., 1997; Schierack et al., 2003). In addition, it was shown that AvCystatin reduced allergic and inflammatory responses in vivo, in a mouse model of airway hyperreactivity (AHR) induced by the model allergen, ovalbumin (OVA). In this model depletion of macrophages, as well as blocking of IL-10, prevented the curative effects and restored allergic airway inflammation (Schnoeller et al., 2008). Moreover, AvCystatin injected into mice induced macrophages expressing IL-10 by exploiting the host's signalling pathways (Klotz et al., 2011a,b).

Although the OVA-induced mouse model is most commonly used, it bears certain limitations such as the quite possible induction of tolerance and therefore alleviation of airway inflammation and the difficulties in extending the findings to clinically relevant allergic diseases with different etiologies (Wiedermann et al., 1999; Schramm et al., 2004; van Hove et al., 2007; Fuchs and Braun, 2008). We therefore extended the previous findings and used a clinically relevant allergen. Grass pollen allergens are the most prevalent allergens in causing allergic disorders and approximately 40% of allergic patients are sensitised to grass pollen, whereby timothy grass (*Phleum pratense*) pollen constitutes the most common allergens in Europe with harmful effects during the high season (April to October) (Andersson and Lidholm, 2003). Timothy grass' pollen major allergen group V isoform b (Phl p 5b) is responsible for more than 90% of the allergic activity in grass pollen sensitised patients (Petersen et al., 1993; Flicker et al., 2000).

In this study the influence of filarial cystatin (AvCystatin) on airway inflammation and hyperreactivity induced by a clinically relevant human aeroallergen was investigated. Furthermore, a "proof-of-principle" study was used to examine whether AvCystatin also modulates allergen-specific responses of peripheral blood mononuclear cells (PBMCs) from allergic patients.

2. Materials and methods

2.1. Protein expression of recombinant cystatin

The cDNA of AvCystatin and a control protein (dihydrofolate reductase, DHFR) were cloned in pET28a and expressed in *Escherichia coli* as previously described (Hartmann et al., 1997). Recombinant AvCystatin (rAvCystatin) and recombinant DHFR (rDHFR) were purified by affinity chromatography using a Ni–NTA column and dialysed against PBS/0.05% Triton X-100. Endotoxin-free rAvCystatin was generated using EndoTrap columns according to the manufacturer's instructions (Profos, Cell Sciences, Canton, MA, USA). Final endotoxin concentrations were detected by a *Limulus* amoebocyte lysate test (Cambrex, Walkersville, MD, USA). The average endotoxin concentration was 1.5 lipopolysaccharide (LPS) pg/μg of protein, 0.03 endotoxin units (EU) per administration. Protein concentrations were measured with the bicinchoninic acid test using a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) according to the manufacturer's instructions.

2.2. Grass pollen-induced sensitisation and inflammation

Female BALB/c mice, 8 weeks old (Charles River, Sulzfeld, Germany) were sensitised three times (days 1, 14 and 21) i.p. with 5 μg of recombinant major allergen group V isoform b (rPhl p 5b) (GenBank Acc. No. Q40963, a gift from Allergopharma, Reinbek, Germany) emulsified in 2 mg of aluminium hydroxide (Alum) (Imject Alum, Pierce, ThermoFisher Scientific). Subsequently, on days 28 and 29, mice were anaesthetised by an i.p. injection of ketamine/rompun (Bayer; Bela-Pharm, Berlin, Germany) and challenged intranasally (i.n.) by the application of 25 μl of timothy grass pollen extract (GPE) containing 9 μg of rPhl p 5b per nostril. Sensitised mice were treated i.p. with 20 μg of AvCystatin or the control protein, rDHFR (a recombinant protein control to AvCystatin) on four occasions, on days 1, 7, 14 and 21 (during the sensitization phase) (Fig. 1). Naive controls were injected with Alum in PBS and mock-challenged using 25 μl of PBS per nostril. The Berlin Office for Health and Social Policy Germany, approved all experimental procedures.

2.3. Measurement of AHR

AHR was determined on day 31 by whole body plethysmography in unrestrained animals by provocation with increasing doses of methacholine (MCh) (Sigma–Aldrich, Germany) as described elsewhere (Hamelmann et al., 1997b). Baseline was established in response to PBS inhalation. The mean values of the enhanced pause (Penh) for every MCh concentration were calculated. Index Penh values of individual animals (Penh value of MCh minus baseline) were analysed as mean values for each group.

2.4. Bronchoalveolar lavage (BAL)

One day after AHR measurements (day 32), mice were sacrificed, blood samples collected and BAL fluid harvested by flushing the lung airways via the trachea twice with 0.8 ml of ice-cold PBS containing a complete protease inhibitor cocktail (Roche, Mannheim, Germany). The tubes were weighted and BAL fluid from the first lavage was centrifuged (10 min at 340*g*, 4 °C), supernatants were collected and stored at −80 °C for subsequent analysis of cytokines. BAL cells were recovered and combined from both lavages, resuspended in PBS and counted (total cell number). A 20 μl aliquot was stained with Kimura stain and mast cells (MCs) were counted via light microscopy in a blinded fashion. One hundred microliters of the cell suspension using a cytospin centrifuge was spun down for staining and differentiation (ThermoShandon Cytospin 4, Frankfurt, Germany). Dried glass slides with cytospinned cells were stained with DiffQuick (Fisher Scientific, Germany) according to the manufacturer's instructions. The numbers of eosinophils, lymphocytes and macrophages were differentiated by morphological criteria (counts of 200 cells/slide under light microscopy in a blinded fashion) as previously described (Blumchen et al., 2006).

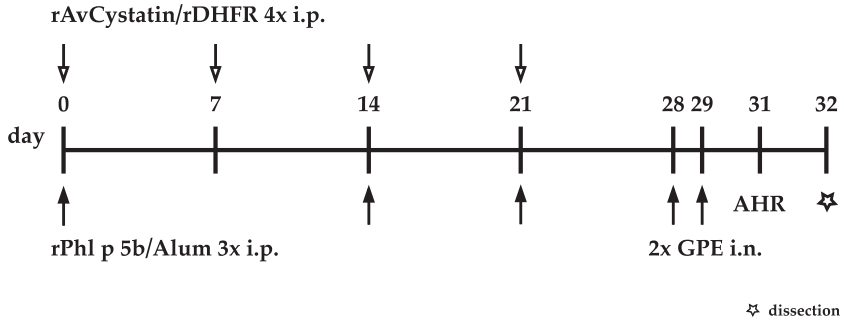


Fig. 1. Scheme of i.p. treatment with AvCystatin in the rPhl p 5b-induced model of allergic asthma. Animals were treated with AvCystatin or the control protein recombinant dihydrofolate reductase (rDHFR) four times at weekly intervals during the sensitisation phase with rPhl p 5b/Alum and challenged twice intranasally (i.n.) by the application of 25 μl of grass pollen extract (GPE) per nostril. Control asthmatic animals were sensitised and challenged with allergen and treated with PBS. Measurement of airway hyperreactivity (AHR) was performed on day 31 and the dissection performed at day 32.

2.5. Antibody levels and mMCP-1 in serum

Total IgE and allergen-specific IgE, IgG1 and IgG2a serum levels were determined by sandwich ELISA as previously described (Dittrich et al., 2008).

Sera were assayed for mouse mast cell protease-1 (mMCP-1) by a specific ELISA Ready-Set-Go kit (eBioscience, CA, USA) according to the manufacturer's instructions.

2.6. Histological analysis

Lungs were fixed in 3.7% phosphate-buffered formalin, embedded in paraffin wax and 2 μm sections were cut and stained using standard histological protocols with H&E for histomorphological evaluation, with periodic acid-Schiff reagent (PAS) for carbohydrates detecting goblet cells and toluidine blue for MCs. Sections were evaluated via light microscopy.

2.7. Cytokine analysis

Spleens and peribronchial lymph nodes (PBLNs) of the different groups of mice were passaged under sterile conditions through 70 μm nylon mesh. Obtained lymphocytes were resuspended in RPMI-1640 culture medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FCS (HyClone, Perbio Science, Bonn, Germany) and cultured (3.5×10^6 cells/ml) for 96 h, 37 °C, 5% CO_2 in the presence of 5 μg/ml of rPhl p 5b and/or 10 μg/ml of AvCystatin. Cell culture supernatants were harvested at day 3 and concentrations of IL-4, IL-5, IL-10, IL-13 and IFN-γ were analysed using cytokine ELISA kits (BD Biosciences, Heidelberg, Germany or eBioscience) according to the manufacturer's instructions.

2.8. Allergic and healthy subjects

Three allergic patients (mean age 33 years old; two males and one female) with clinical symptoms and histories of grass pollen allergy and two healthy controls (mean age 35 years old; two females) were examined outside of the grass pollen season and verified for allergen-specific IgE to timothy grass (*P. pratense*). All asthmatic patients had allergen-specific IgE to grasses (6.8–9.8 kAU/l, class 3 ImmunoCAP, Phadia AB, Uppsala, Sweden), while the non-allergic healthy controls showed no history of allergic symptoms nor timothy grass allergen-specific IgE in serum. On entering the study, subjects were free of oral steroids or immunotherapy for at least 3 months. Blood samples (up to 40 ml) from allergic patients and healthy controls were collected by the study nurse at a time outside of the pollen season. All patients gave their written informed consent. The study was approved by the local Ethics Committee (University Hospital Charité, Berlin, Germany) and monitored by the study doctor.

2.9. PBMCs

Blood was collected by a certified study nurse into tubes containing anticoagulant (Lithium Heparin) (BD, Heidelberg, Germany) and centrifuged at 700*g* at room temperature (RT) for 5 min to isolate the patient's plasma, which was further centrifuged at 1,400*g* to use as cell-free autologous serum for culture medium. The cell fraction was diluted with an equal volume of RPMI-1640 medium, carefully layered and centrifuged (800*g*, RT, 20 min) over a Lymphoprep® density gradient (Axis-Shield PoC AS, Oslo, Norway). After centrifugation the mononuclear cells were removed from the interface, diluted with RPMI-1640 medium and washed once at 600*g*, 4 °C, 15 min and twice for 15 min at 250*g*, 4 °C. Isolated and washed PBMCs were resuspended at 2×10^6 cells/ml in RPMI-1640 culture medium containing 5% patient autologous serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 0.1% β-mercaptoethanol. In all experiments viability of isolated PBMCs was over 95%.

2.10. Proliferation assay and PBMC culture

Freshly isolated human PBMCs were resuspended in PBS at 10×10^6 cells/ml and stained with carboxyfluorescein succinimidyl ester (CFSE) at a final concentration of 4 μM (eBioscience). CFSE-labelled cells were washed, counted and resuspended in complete medium at 1×10^6 cells/ml and incubated at 37 °C, 5% CO_2 for 3 days (for supernatants) or for 7 days (for surface marker and intracellular cytokine staining) in 24-well plates. PBMCs from allergic and healthy controls were stimulated with: GPE (5 μg/ml) only, AvCystatin (2.5 μg/ml) 2 h before addition of GPE (5 μg/ml) or with AvCystatin (2.5 μg/ml) and GPE (5 μg/ml) at the same time. The AvCystatin and GPE concentrations as well as the time of stimulation were experimentally determined beforehand (data not shown).

2.11. Flow cytometry

Single cell suspensions of murine lymphocytes were labelled with e450-conjugated anti-mouse CD4, Alexa700-conjugated anti-mouse CD25 and PE-conjugated anti-mouse Foxp3 according to the manufacturer's instructions (eBioscience).

CFSE-stained PBMCs were analysed by flow cytometry on day 7. Briefly, 1×10^6 cells/well were incubated with 50 ng/ml of phorbol-12-myristate-13-acetate (PMA) and 1 μg/ml of Ionomycin

(Sigma–Aldrich) for 5 h at 37 °C, 5% CO_2 and for the last 4 h Brefeldin A (5 μg/ml; Sigma–Aldrich) was added. Cells were prepared for intracellular analysis according to the BD Cytofix/Cytoperm Kit protocol (BD Biosciences) with modifications and stained with the following antibodies: Alexa Fluor 405-conjugated anti-human CD4 (to circumvent PMA-induced loss in surface CD4 on human T cells) (Acres et al., 1986; Bigby et al., 1990), Alexa Fluor 430-conjugated anti-human IFN-γ (all as a gift from DRFZ (German Rheumatism Research Centre, Berlin, Germany). Stained samples were acquired using FACSCanto or LSR II flow cytometers (BD Biosciences) and analysed using FlowJo software (Tree Star, OR, USA).

2.12. Statistical analysis

The GraphPad Prism software utilising the unpaired Student's *t*-test and one-way ANOVA was used. Data are presented as means ± SEM $P < 0.05$ was considered to be significant.

3. Results

3.1. Filarial cystatin reduces AHR and decreases airway inflammation in the lungs

For the establishment of allergic AHR and inflammation, animals were sensitised three times i.p. with rPhl p 5b and subsequently challenged twice i.n. with GPE. Treatment with AvCystatin or the control protein, rDHFR, was performed i.p. on four occasions during the sensitisation phase (Fig. 1). Development of AHR to increasing doses of MCh was significantly elevated in asthmatic controls compared with naive controls ($P = 0.003$) (Fig. 2A). In contrast, animals treated with AvCystatin showed significantly decreased Penh values compared with asthmatic controls ($P = 0.02$) (Fig. 2A), while a group treated with rDHFR showed no improvement. Analysis of the BAL fluid showed significantly reduced numbers of total cells ($P = 0.01$), eosinophils ($P = 0.002$) and MCs ($P = 0.03$) in animals treated with AvCystatin compared with asthmatic controls (Fig. 2B). Treatment with the control protein rDHFR did not cause significant changes in cell composition of the BAL fluid compared with asthmatic animals (Fig. 2B).

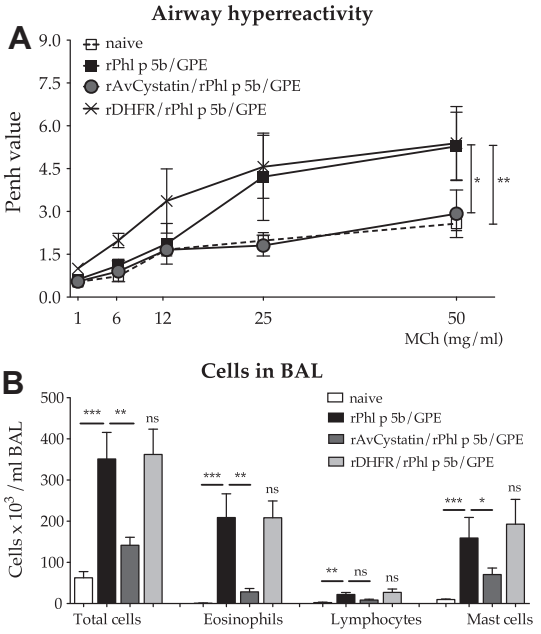


Fig. 2. Treatment with AvCystatin suppresses airway hyperreactivity (AHR) and inflammatory cell influx into the lungs. (A) AHR was measured at day 31 by whole body plethysmography in response to inhaled methacholine (MCh). AHR is displayed by pause enhanced (Penh) values. (B) Influx of cells into the bronchoalveolar lavage (BAL) fluid was determined by differential-morphological analysis. The mean values ± SEM are shown for five to six mice per group; representative data of one from three independent experiments. Comparison was with asthmatic controls. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ are considered statistically significant; ns, not significant. GPE, grass pollen extract; rDHFR, recombinant dihydrofolate reductase.

Analysis of histological sections of the lungs of AvCystatin-treated mice showed down-regulated local inflammation compared with disease control groups (Fig. 3). AvCystatin-mediated suppression was associated with reduced eosinophilia around bronchioles and small vessels as observed in H&E stained lung tissue (Fig. 3C). Moreover, reduced goblet cell hyperplasia was observed in PAS-stained lung sections compared with asthmatic mice (Fig. 3F, G). As MCs play an important role in the development of allergic asthma during acute reactions and contribute to lung remodelling and inflammation during the later stages of inflammation (reviewed in Metz et al., 2007), the lung tissue was stained with toluidine blue to detect connective tissue MCs residing in lungs. As expected, asthmatic animals showed increased local infiltration of MCs into the tissue compared with naive animals (8.5 ± 4.6 versus 5.7 ± 2.2 MCs/10 high power fields (hpf)). In contrast, AvCystatin-treated animals exhibited no mastocytosis in lung tissues and were similar to naive animals in this aspect (4.5 ± 2.1 MCs/10 hpf) whereas rDHFR treatment showed no suppressive effect (Fig. 3I–L). Additionally, analysis of sera revealed that AvCystatin reduced levels of MC-protease ($P = 0.018$) (Fig. 4C). Hence, filarial cystatin ameliorated local inflammatory responses induced by the grass pollen allergen on several levels.

3.2. Filarial cystatin alters local production of cytokines

In order to understand the effect of AvCystatin-mediated inhibition on the local immune responses against allergens, the levels of cytokines were measured ex vivo in the BAL fluid. Treatment with AvCystatin significantly suppressed allergen-specific Th2 production of IL-5 ($P = 0.005$) and IL-13 ($P = 0.02$) compared with asthmatic animals (Fig. 4A). Levels of IL-4 were not significantly increased in the BAL fluids of asthmatic animals. Interestingly, although IL-10 levels did not correlate with disease status in the BAL fluid there was a trend towards increased IL-10 production in AvCystatin-treated animals compared with allergic controls or animals treated with the control protein, rDHFR (Fig. 4A). In addition, treatment with AvCystatin led to significant suppression of allergen-specific Th2 responses (IL-4, IL-5 and IL-13; $P = 0.04$, $P = 0.009$, $P = 0.03$, respectively) detected in the supernatants of lung-draining PBLN cells restimulated in vitro with the allergen, rPhl p 5b (Fig. 4B).

3.3. Treatment with filarial cystatin down-regulates systemic responses

To understand whether AvCystatin also interfered with allergen-specific responses at a systemic level, the production of Th2-associated cytokines by splenocytes and serum antibody levels were determined. In addition to alleviation of rPhl p 5b-induced allergic asthma in local tissues, AvCystatin significantly down-regulated systemic production of grass pollen-specific Th2-type cytokines such as IL-4, IL-5 and IL-13 ($P = 0.004$, $P < 0.001$ and $P = 0.02$, respectively) detected in spleen cell cultures after restimulation with rPhl p 5b while IFN-γ was not increased (Fig. 4D). High levels of rPhl p 5b-specific IL-10 were detected in asthmatic animals ($P < 0.001$) compared with naive animals, however the levels were significantly decreased after treatment with AvCystatin ($P < 0.001$) in contrast to mice treated with the control protein, rDHFR

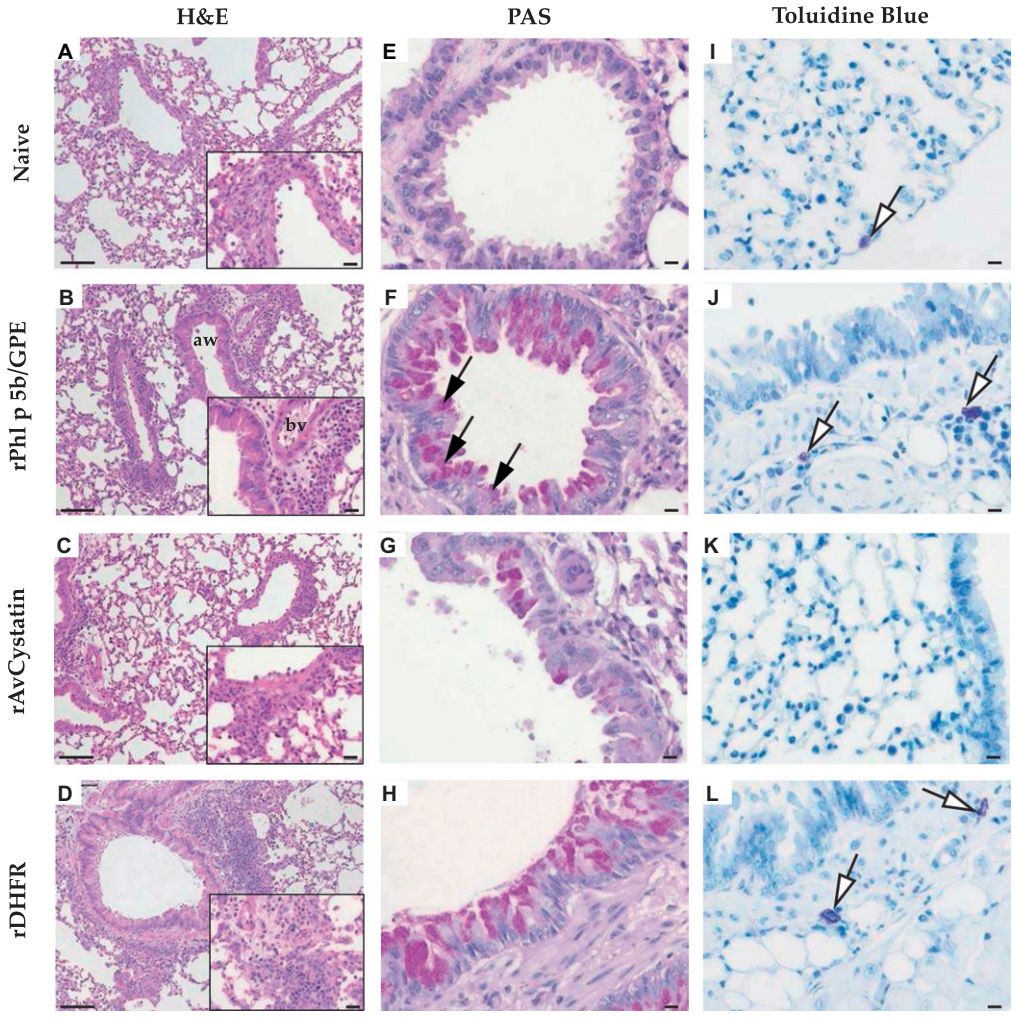


Fig. 3. Administration of AvCystatin suppresses inflammation in the lungs. Representative lung sections were stained with H&E (A–D) for detection of eosinophils. (E–H) Staining with periodic acid-Schiff reagent (PAS) for quantification of goblet cells, indicated by black arrows. (I–L) Staining with Toluidine Blue for resident connective tissue mast cells (MCs), indicated by white arrows. Quantification of the data is in the text (Section 3.1). Naive, naive control; rPhl p 5b/grass pollen extract (GPE), asthmatic; rAvCystatin, AvCystatin-treated; rDHFR, recombinant dihydrofolate reductase-treated animals; aw, airways; bv, blood vessel. Original magnification: H&E 100× (inset 400×); PAS 400×, Toluidine Blue: 400×; scale bars (A–D main images) = 100 μm; scale bars (A–D insets, E–L) = 20 μm.

(Fig. 4D). As IL-4 and IL-13 are involved in isotype class switching and/or IgE production, serum antibody levels were analysed in all groups. Treatment with AvCystatin significantly decreased production of total IgE and rPhl p 5b-specific IgE and IgG1 ($P = 0.004$, $P = 0.01$ and $P = 0.03$, respectively) (Fig. 4E–G). No significant changes in rPhl p 5b-specific IgG2a levels were observed after AvCystatin administration compared with asthmatic controls ($P = 0.66$) (data not shown). Thus, systemic administration of filarial cystatin was effective in down-regulating allergen-specific cytokine and antibody production at the systemic level.

3.4. Frequencies of $CD4^+CD25^+Foxp3^+$ T cells and increased levels of AvCystatin-specific IL-10

Regulatory cells play an important role in down-modulation of allergy and asthma. Therefore isolated splenocytes were analysed ex vivo for the expression of regulatory T cell (Treg) markers including CD25 and Foxp3. Elevated total numbers of $CD4^+CD25^+$-$Foxp3^+$ T cells were detected in spleens from AvCystatin-treated animals compared with asthmatic controls (Fig. 5B). However, the frequencies of $CD25^+Foxp3^+$ cells in the $CD4^+$ compartment of the spleen were not significantly changed in the AvCystatin-treated group compared with the asthmatic control animals (Fig. 5A), indicating that AvCystatin does not lead to a preferential expression or recruitment of Tregs.

Although systemic grass pollen-specific IL-10 was reduced by AvCystatin treatment (Fig. 4D), restimulation of splenocytes with AvCystatin resulted in significant production of IL-10 in animals treated with AvCystatin during sensitisation ($P = 0.01$) (Fig. 5C), in line with increased levels of IL-10 in the BAL fluid in the AvCystatin-treated group (Fig. 4A). Interestingly, ex vivo responses to AvCystatin by splenocytes isolated from AvCystatin-treated animals did not lead to a significant increase in any other cytokine examined (Fig. 5D). Thus, these data indicate that filarial cystatin specifically induces the expression of IL-10 in the murine system.

3.5. Filarial cystatin modulates allergen-specific responses of human PBMCs

In order to assess whether AvCystatin is able to exert its immunomodulatory effect, not only in the murine model but also in the human system, the effect of AvCystatin was tested on PBMCs isolated from clinically characterised, timothy grass (*P. pratense*)

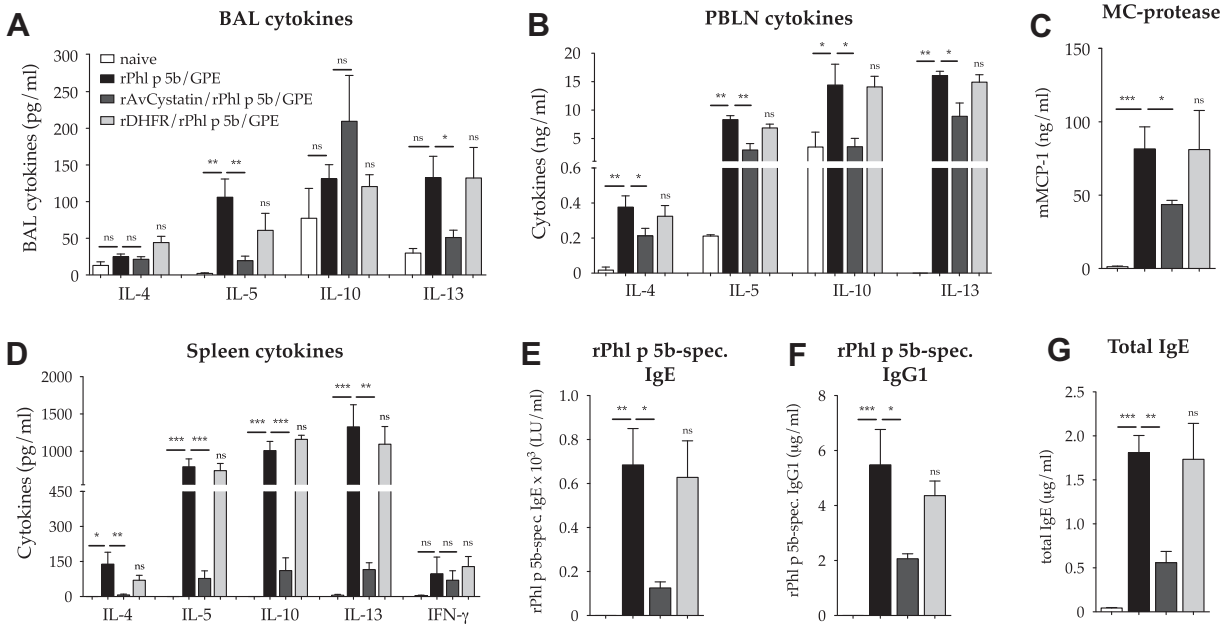


Fig. 4. Treatment with AvCystatin interferes with local and systemic responses. (A) Cytokines detected in the bronchoalveolar lavage (BAL) fluid, (B) cytokine allergen-specific responses in peribronchial lymph nodes (PBLNs), (C) mast cell-protease (mMCP-1) detected in serum, (D) cytokines in spleen cell cultures restimulated in vitro with rPhl p 5b, (E) allergen-specific IgE, (F) IgG1, (G) total IgE antibody levels detected in the serum. The mean values ± SEM are shown for five to six mice per group; representative data from one of three independent experiments. Comparison was with allergic controls. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ are considered statistically significant; ns, not significant.

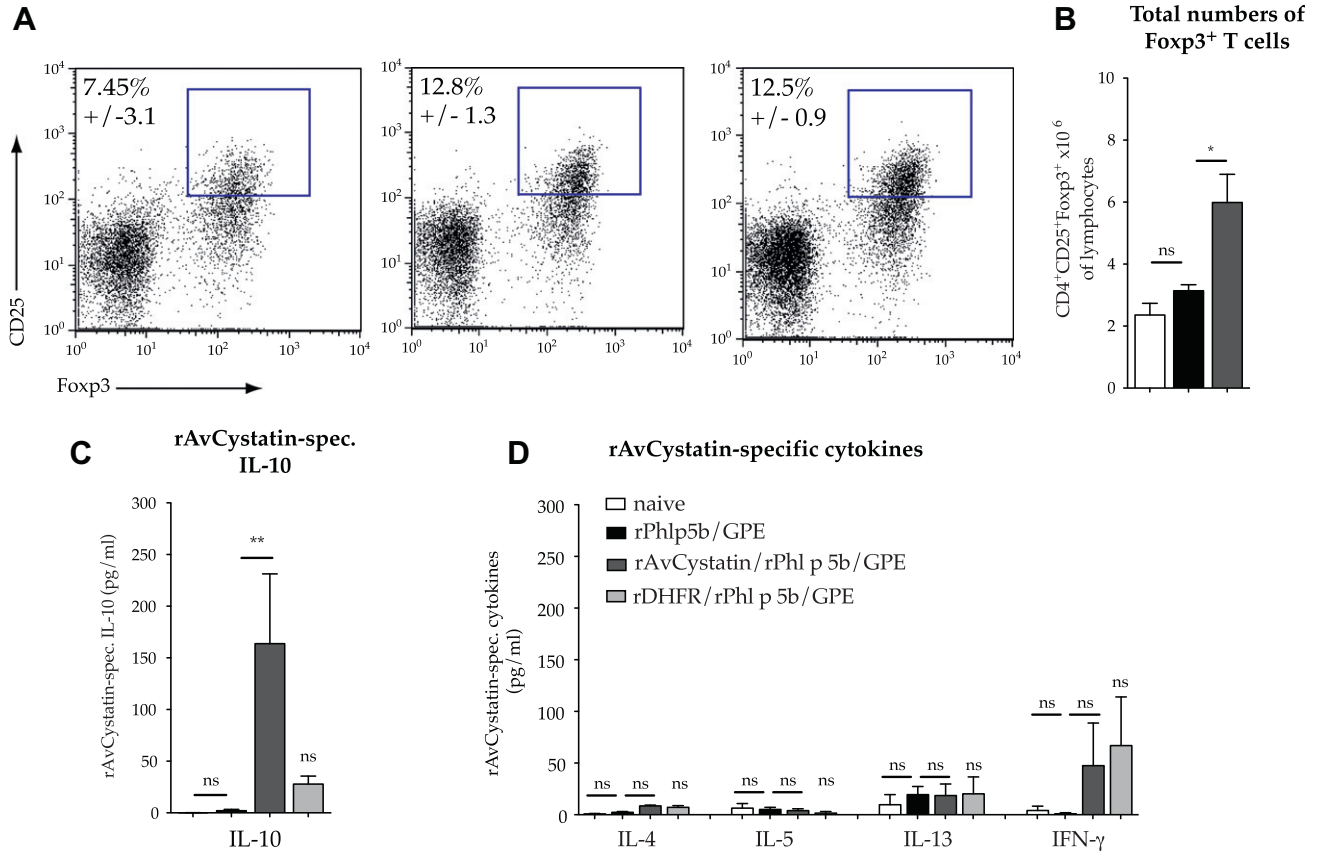


Fig. 5. AvCystatin induces selective IL-10 expression in systemic responses. (A) Frequency of $CD25^{+}Foxp3^{+}$ T cells of $CD4^{+}$ cells. Shown are representative contour plots from flow cytometric analysis (FACS) of one animal per group ± SEM. (B) Total cell numbers of $CD4^{+}CD25^{+}Foxp3^{+}$ T cells from lymphocytes. AvCystatin-specific (C) IL-10, (D) IL-4, IL-5, IL-13 and IFN-γ production by spleen cells. The mean values ± SEM are shown for five to six mice per group; representative data from one of three independent experiments. Comparison was with allergic controls; $^{*}P < 0.05$; $^{**}P < 0.01$ are considered statistically significant; ns, not significant.

pollen allergic patients in comparison with healthy controls, in the context of GPE-specific restimulation.

As an initial approach, an in vitro system outside of the pollen season was established in order to determine the optimal kinetics of allergen and AvCystatin stimulation. The most potent proliferation of human PBMCs was observed at day 7 with a concentration of 5 μg/ml of GPE as assessed by differences in frequencies of $CFSE^{low}$ lymphocytes between three grass allergic subjects and two healthy controls (9.6% ± 2.68 and 3.52% ± 0.21, respectively). A dose of 2.5 μg/ml of AvCystatin was found to have a maximum effect for modulating human PBMC responses to GPE as well as the timing of AvCystatin exposure was revealed to be critical for its efficacy. The above-established culture conditions were used to further analyse the efficacy of AvCystatin in modulating specific responses to grass pollen in allergic patients (Fig. 6).

GPE restimulation of PBMCs from allergic patients resulted in a specific response characterised by production of high levels of IL-13, which were not detected in healthy control cells restimulated with GPE (Fig. 6A). Administration of AvCystatin (2.5 μg/ml) 2 h prior to GPE stimulation resulted in significantly suppressed IL-13 ($P = 0.004$) (Fig. 6A) and increased IFN-γ production ($P = 0.01$) by restimulated PBMCs from allergic subjects (Fig. 6B). Incubation with AvCystatin also led to a non-significant increase in IL-10 production by PBMCs compared with GPE-stimulation alone ($P = 0.49$) (Fig. 6C). In contrast, PBMCs from healthy controls did not produce IL-13 or IFN-γ after GPE or AvCystatin stimulation, although low levels of IL-10 were induced after stimulation with GPE (Fig. 6A–C). Interestingly, the frequency of allergen-specific $CD4^+IFN\text{-}\gamma^+$ T cells was increased after incubation with AvCystatin prior to GPE stimulation in comparison with GPE-only stimulated cells (Fig. 6D). Importantly, incubation with AvCystatin alone did not induce proliferation of cells (data not shown). This suggests that the administration of AvCystatin prior to allergen stimulation modulated the in vitro responses of PBMCs from allergic patients by altering the cytokine profile of specific effector cells.

4. Discussion

Previously the ability of the potent helminth modulator, AvCystatin, to suppress Th2-related inflammation in a murine model of OVA-induced allergic AHR was demonstrated (Schnoeller et al., 2008). In this study the aim was to further assess the immunomodulatory capacities of AvCystatin in a more clinically relevant context. Thus, a murine model and human cells characterised by allergic responses against timothy grass pollen were utilised. Herein, we demonstrate that AvCystatin interferes with the sensitisation phase with a grass pollen allergen and prevents the development of airway inflammation and hyperresponsiveness after allergen challenge.

Allergic airway inflammation was induced by the clinically relevant grass pollen aeroallergen, rPhl p 5b, associated with the production of Th2-specific cytokines that contribute to multiple features of asthma such as eosinophilia, IgE production, goblet cell metaplasia, AHR, smooth muscle remodelling and overall Th2 induction and maintenance (Dent et al., 1990; Hamelmann et al., 1997a; Hogan et al., 1997).

Administration of AvCystatin led to drastically reduced recruitment of inflammatory cells into the lungs and interfered with local and systemic production of allergen-specific Th2-cytokines (such as IL-4, IL-5 and IL-13) and specific-IgE, which correlated with decreased AHR.

Furthermore, AvCystatin suppressed local MC recruitment and reduced levels of MCP, therefore showing the potential to also interfere with MC activation. This presents the possibility that

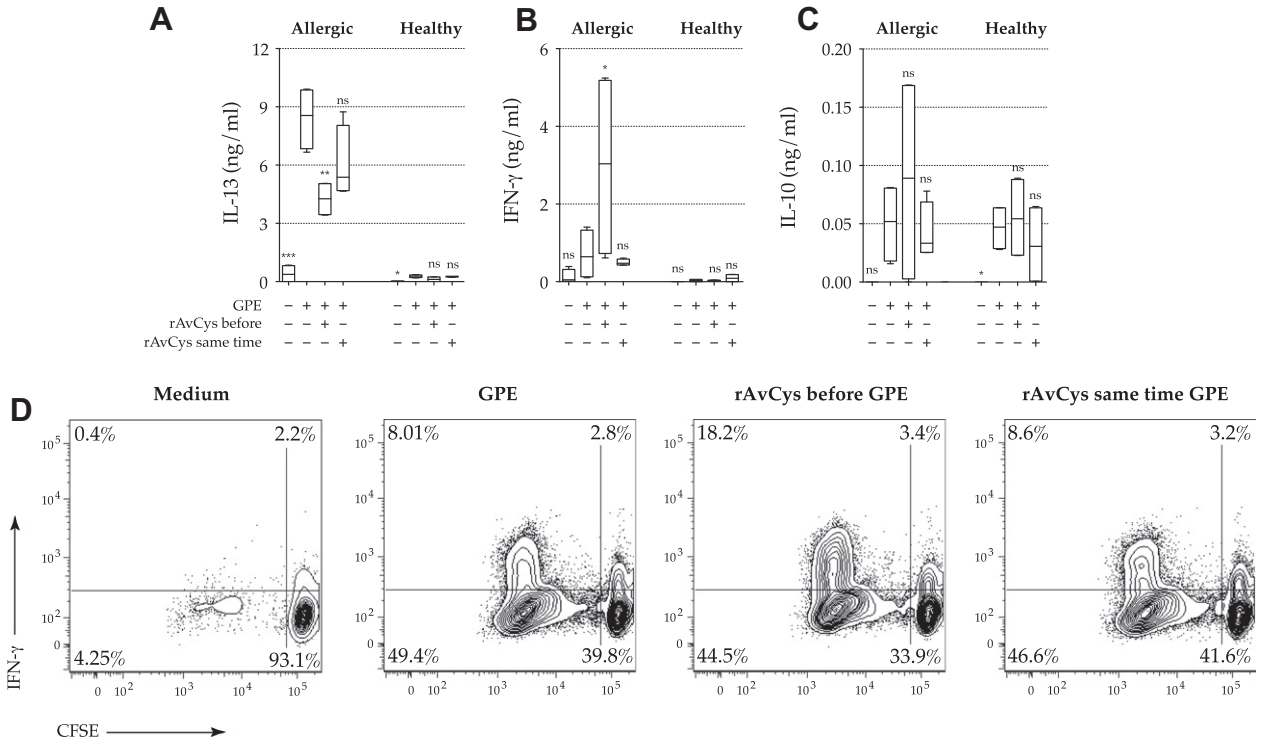


Fig. 6. AvCystatin applied prior to stimulation with grass pollen extract (GPE) reduced production of allergen-specific IL-13 and increased levels of IFN-γ. Levels of (A) IL-13, (B) IFN-γ and (C) IL-10 in supernatants from allergic subjects and healthy controls. The mean values ± SEM from each group are shown. Comparison was with GPE-stimulation of allergic and healthy controls, respectively. (D) Dot plots from flow cytometric analysis (FACS) with frequencies of $CD4^+CFSE^-IFN\text{-}\gamma^+$ T cells gated on $CD4^+$ T cells from allergic patients restimulated with the medium control, GPE stimulation, AvCys stimulation before GPE stimulation and AvCys stimulation at the same time as GPE-stimulation. The mean values from the group as the percentage of $CD4^+$ live T cells. Allergic: $n = 3$; healthy controls: $n = 2$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ are considered statistically significant; ns, not significant.

suppression of Th2 responses and subsequent IgE class switching by AvCystatin resulted in reduced IgE binding to the high-affinity receptor, FcεRI, on MCs and thus reduced allergen-triggered MC degranulation. Accordingly, the filarial nematode protein, ES-62, has been shown to reduce FcεRI-mediated responses of human and murine MCs (Melendez et al., 2007). However, whether MCs play a critical role in the induction of chronic asthma in the clinically relevant timothy grass pollen-induced model is unclear. Related animal models that used systemic sensitisation with model allergens, such as OVA, mixed with Alum (Takeda et al., 1997; Williams and Galli, 2000) did not require MCs for the induction of allergic inflammation and disease, whereas development of disease in mice exposed to repetitive i.n. antigen challenges in the absence of Alum was shown to be MC-dependent (Nakae et al., 2007).

Interestingly, administration of AvCystatin led to significantly increased cystatin-specific IL-10 production in the spleen. This is in line with a previous study on the OVA-induced allergy model where AvCystatin effectively ameliorated allergic disease in an IL-10-dependent manner (Schnoeller et al., 2008). Indeed it was recently shown that AvCystatin, upon uptake by macrophages, induces phosphorylation of the mitogen-activated protein kinase signalling pathways, ERK1/2 and p38, and IL-10 production (Klotz et al., 2011a,b). Taken together the data suggest that IL-10 may be a key player in the immunomodulatory capacity of AvCystatin in both models of asthma.

AvCystatin is derived from a parasitic worm and others showed that infections with helminths such as *Schistosoma mansoni* (Mangan et al., 2006) or *Heligmosomoides polygyrus* (Bashir et al., 2002; Kitagaki et al., 2006) decreased allergen-induced inflammation, which correlated with increased levels of IL-10 and, very often, elevated numbers of $CD4^+CD25^+Foxp3^+$ T cells. In the present study the source of IL-10 was not identified, although in previous studies of concurrent experimental helminth infections and allergy-related disorders IL-10 was shown to be produced by various cell sources (Mangan et al., 2004; Amu et al., 2010). In particular, data from this laboratory have shown that not macrophages but $CD4^+$ $Foxp3^-$ T cells may be the prominent source of IL-10 in response to AvCystatin (Ziegler et al., unpublished data). Only a few individual helminth-derived proteins have yet been described to have immunomodulatory capacities in allergy or models of allergic diseases. Interestingly, most of those act via IL-10-, TGF-β-, IFN-γ- and/or $CD4^+CD25^+$ T cell-dependent mechanisms (reviewed in Daniłowicz-Luebert et al., 2011).

Although AvCystatin induced high levels of IL-10, asthmatic but not AvCystatin-treated mice were also capable of producing high levels of IL-10 in response to the grass pollen allergen. Thus, it is not yet clear whether IL-10 directly mediates the effects of AvCystatin and if so, how AvCystatin-specific IL-10 mediates its outcome when high levels of grass pollen-specific IL-10 are also detected in allergic disease controls. One possibility is that the source of AvCystatin-induced IL-10 is critical in treated animals, although it is not known which cells produce IL-10. One possibility is that induction of IL-10 is preferentially favoured in Tregs or alternative cell sources in AvCystatin-treated mice. The exact source of IL-10 shall be investigated in further studies.

In addition to showing an effect of AvCystatin in a murine model with a clinically relevant allergen, the current work has demonstrated the efficacy of AvCystatin as a potent immunomodulator of allergic immune responses in human PBMCs. Pre-incubation with AvCystatin before allergen stimulation led to a significant decrease in grass pollen-specific IL-13 by PBMCs from allergic patients and resulted in increased levels of IL-10 and a significant production of IFN-γ by allergen-specific $CD4^+$ T cells. Furthermore, increased production of IFN-γ by responding allergic $CD4^+$ T cells was confirmed via flow cytometry. In line with these observations, Park et al. (2012) recently reported that stimulation with the recombinant macrophage migration inhibitory factor (MIF) homologue of *Anisakis simplex* (r*As*-MIF) increased levels of IFN-γ and IL-10, while decreasing Th2-cytokines in PBMCs from atopic asthmatic patients but not from patients with non-atopic asthma (Park et al., 2012). However, in that study patients' PBMCs were only stimulated polyclonally with mitogen and not in an allergen-specific manner as in the present study.

With regard to allergy treatment, many studies have reported that IFN-γ seems to be beneficial and is increased in patients after successful allergen-specific immunotherapy (Larche et al., 2006). Immunotherapy in atopic patients induces allergen-specific Th1 cells that suppress the effector function of allergen-specific Th2 cells and alter antibody production (Ebner et al., 1997; Akdis and Akdis, 2011). IFN-γ is known to be present in healthy subjects but not in atopic patients when assessing levels of this cytokine produced in vivo (Hough et al., 1996). Leonard et al. (1997) proposed that the difference between atopy and asthma is not related to high production of Th2 cytokines (i.e., IL-4), but may be inversely correlated with levels of IFN-γ in response to antigen from house dust mite (HDM) stimulation of allergic PBMCs (Leonard et al., 1997). Similarly another study demonstrated a selective redirection of ragweed-specific Th2 responses towards Th1 responses with a significant increase in IFN-γ production (Simons et al., 2004). However, the exact role of IFN-γ in regulating allergic disease remains controversial. Nevertheless, there are also numerous studies reporting a significant negative correlation between levels of Th2-associated cytokines and IFN-γ as a promising allergy treatment in the human system (Pochard et al., 2002). Defective production of IFN-γ after in vitro restimulation of PBMCs constitutes a general feature of atopic disease (Machura et al., 2010) and is associated with less severe asthma symptoms and lung function reductions (Message et al., 2008).

IFN-γ can directly inhibit the production of Th2 cytokines in a bystander manner and thus, in this fashion may mediate decreases in Th2 associated changes that lead to allergic disease including the production of allergen-specific IgE (Pène et al., 1988; Romagnani, 1990; Parronchi et al., 1992). Moreover, it is known that in an allergic state certain proportions of Th1 and Th2-type cytokines are produced by allergen-specific T cell clones. It has been proposed that the overall imbalance in the Th2 to Th1 cytokine ratios rather than the absolute amounts of individual cytokines is responsible for the development and maintenance of allergy (Tournoy et al., 2002; Romagnani, 2004; Ngoc et al., 2005; Zhang et al., 2011).

In conclusion, these findings show that AvCystatin has the capacity to down-modulate allergic responses induced by a clinically relevant allergen in a murine model of asthma while inducing expression of AvCystatin-specific IL-10. Furthermore, AvCystatin was shown that also has the ability to modulate allergic responses in human PBMCs, thus indicating possible clinical and therapeutic implications. A better understanding of the mechanisms through which AvCystatin mediates its effect in mice and humans, together with extended trials with human material, will be beneficial in determining the efficacy and therapeutic potential in the future.

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