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of January 25, 2015.

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J Immunol published online 14 January 2015
<http://www.jimmunol.org/content/early/2015/01/14/jimmunol.1401217>

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A Novel Regulatory Macrophage Induced by a Helminth Molecule Instructs IL-10 in CD4⁺ T Cells and Protects against Mucosal Inflammation

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Immunomodulation is a common feature of chronic helminth infections and mainly attributed to the secretion of bioactive molecules, which target and modify host immune cells. In this study, we show that the helminth immunomodulator AvCystatin, a cysteine protease inhibitor, induces a novel regulatory macrophage (Mreg; AvCystatin-Mreg), which is sufficient to mitigate major parameters of allergic airway inflammation and colitis in mice. A single adoptive transfer of AvCystatin-Mreg before allergen challenge suppressed allergen-specific IgE levels, the influx of eosinophils into the airways, local and systemic Th2 cytokine levels, and mucus production in lung bronchioles of mice, whereas increasing local and systemic IL-10 production by CD4⁺ T cells. Moreover, a single administration of AvCystatin-Mreg during experimentally induced colitis strikingly reduced intestinal pathology. Phenotyping of AvCystatin-Mreg revealed increased expression of a distinct group of genes including LIGHT, sphingosine kinase 1, CCL1, arginase-1, and costimulatory molecules, CD16/32, ICAM-1, as well as PD-L1 and PD-L2. In cocultures with dendritic cells and CD4⁺ T cells, AvCystatin-Mreg strongly induced the production of IL-10 in a cell-contact-independent manner. Collectively, our data identify a specific suppressive macrophage population induced by a single parasite immunomodulator, which protects against mucosal inflammation. *The Journal of Immunology*, 2015, 194: 000–000.

Chronic immune disorders such as inflammatory bowel disease and asthma constitute a significant health burden on the Western world, and new treatment strategies are needed. Experimental and epidemiological evidence suggests that helminths can prevent deleterious inflammatory immune responses by modulating the host immune response, and thereby exert bystander effects on allergic and autoimmune diseases (1). As a consequence, there is increasing interest in delineating the mechanisms through which helminths elicit immunoregulatory mechanisms, in

particular whether these pathways can be exploited to treat aberrant immune reactions. Administration of both parasite eggs and living adult worms of the *Trichuris* species have been demonstrated to alleviate inflammatory bowel disease in human patients (2, 3). However, although the concept of worm therapy has been described as safe and effective, the application of living parasites still bears the risk for inappropriate side effects. Thus, therapeutic intervention by applying defined helminth-secreted immunomodulators may represent a targeted strategy to treat inflammatory responses; indeed, excretory/secretory components of nematode parasites have been shown to efficiently suppress inflammation in experimental models (reviewed in Refs. 1, 4). Our previous studies showed that cystatin from the filarial roundworm *Acanthocheilonea viteae* (AvCystatin) is a potent immunomodulator (5, 6) that protects mice against OVA-induced airway inflammation in a macrophage- and IL-10-dependent manner (7). AvCystatin exploits host signaling pathways and modulates MAPK p38 and ERK to manipulate macrophage responses, leading to transient production of IL-10 (8). Because the repeated therapeutic application of a helminth molecule exerts the risk for allergic responses or reduced immunomodulatory activity due to Ab responses, we aimed for a therapy based on the application of regulatory cells induced by the helminth compound. In this study, we applied purified macrophages treated with AvCystatin in two models of mucosal diseases, experimental airway inflammation and chemically induced colitis, and evaluated their therapeutic effect, as well as their phenotype, by analyzing the expression of selected activation markers typical for macrophage subpopulations (9, 10). In both disease models, we ascertained AvCystatin-modulated macrophages to be sufficient for suppressing the respective disease, thus highlighting the potential for defined helminth molecules in the treatment of chronic human inflammatory diseases.

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Received for publication May 15, 2014. Accepted for publication December 8, 2014.

This work was supported by the German Research Foundation within the Sonderforschungsbereich 650 and the Heisenberg Program (S.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ARG1, arginase 1; BAL, bronchoalveolar lavage; DC, dendritic cell; DRFZ, Deutsches Rheumaforschungszentrum Berlin; DSS, dextran sodium sulfate; mLN, mesenteric lymph node; MPO, myeloperoxidase; Mreg, regulatory macrophage; PAS, periodic acid-Schiff; PBLN, peribronchial lymph node; PEC, peritoneal exudate cell; sphk-1, sphingosine-kinase-1; Treg, regulatory T cell.

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Materials and Methods

Protein expression and purification

Recombinant AvCystatin and a control protein (SNAP-tag protein, a mutant of the DNA repair protein O(6)-alkylguanine-DNA alkyltransferase) were expressed in *Escherichia coli* and purified by affinity chromatography. Endotoxin levels for purified AvCystatin (0.2–0.7 $\mu\text{g}/\mu\text{g}$ protein) were analyzed using a chromogenic LAL test kit (Lonza). Denatured AvCystatin (heat-inactivated AvCystatin) was used as a control.

Animals and ethics statement

Female BALB/c and C57BL/6 mice aged 6–10 wk were purchased from Charles River. IL-10^{-/-} (BALB/c background) were a kind gift by Dr. Ute Hoffmann (Deutsches Rheumaforschungszentrum Berlin [DRFZ], Berlin, Germany). DO11.10 TGN mice were obtained from the Federal Institute of Risk Assessment in Berlin, Germany. All experiments were approved by the animal ethics committee of the local government (State Office of Health and Social Affairs Berlin, Germany; registration no. G0144/10) and performed in strict accordance with the German law and national guidelines for animal protection.

OVA-induced airway inflammation model

BALB/c mice were sensitized by i.p. injections with 20 μg OVA (Sigma-Aldrich) emulsified in 2 mg aluminum hydroxide (Inject Alum; Pierce) in 0.2 ml PBS on days 0 and 14. Airway challenge was performed with intranasal application of 50 μg OVA per animal on days 28 and 29, and animals were sacrificed on day 31 (Supplemental Fig. 1A). Control mice were sensitized with aluminum hydroxide in PBS and challenged with PBS (healthy control). Blood samples were collected for serum Ab analysis and the trachea flushed with a protease inhibitor mixture (Roche complete Mini protease inhibitor tablets) dissolved in PBS for cytokine detection.

DSS-induced colitis model

Colitis was induced by providing 3% dextran sodium sulfate (m.w. 36,000–50,000; MP Biomedicals, Heidelberg, Germany) dissolved in drinking water to female C57BL/6 mice aged 9–11 wk for 8 d (Supplemental Fig. 1B). Healthy control mice received normal drinking water. Body weight development was surveyed daily. After dissection on day 8, colon length was measured and parts of distal colon fixed in 3.5% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for histological analysis.

Histological analyses

Cytospin slides were prepared from 100 μl bronchoalveolar lavage (BAL) fluid, stained with the Hemacolor staining set for microscopy (Merck), and analyzed in a blinded fashion to determine cell numbers. Lungs were fixed in 3.5% formalin for histological analysis of mucous production (periodic acid-Schiff [PAS] stain) and inflammatory cell infiltration (H&E stain). Stained slices were analyzed via light microscopy and documented using a Leica DFC480 camera and Leica Application Suite Software version 2.8.1. H&E-stained colon sections were used for histopathological scoring. The scoring parameters were as follows: inflammation (0: no inflammation; 1: increased number of inflammatory cells in lamina propria; 2: inflammatory cells extending into the submucosa; 3: transmural inflammatory infiltrates) and tissue damage (0: no mucosal damage; 1: discrete epithelial lesion; 2: erosion or focal ulceration; 3: severe mucosal damage with extended ulcerations extending into bowel wall). Myeloperoxidase-positive (MPO⁺) cells were counted in 10 high-power fields in histological cross sections of the colon applying a polyclonal Ab against MPO⁺ (A0398; Dako) and the labeled streptavidin biotin detection method.

Detection of cytokine and Ab responses

Splenocytes from recipient mice were stimulated for 4 d in 96-well plates with 50 $\mu\text{g}/\text{ml}$ OVA in RPMI 1640 supplemented with 10% FCS, 1 mM L-glutamine, 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin (cRPMI). Supernatants were assessed for IL-4, IL-5, IL-13, IFN- γ , and IL-10 by sandwich ELISA according to the manufacturer's protocol (eBioscience). For detection of OVA-specific IgE, ELISA plates were coated with 10 $\mu\text{g}/\text{ml}$ OVA at 4°C overnight and unspecific binding blocked by 3% BSA in PBS for 1 h. After incubation with mouse serum, HRP-IgE (rat anti-mouse IgE H chain HRP [MCA419P; Serotec]) was used as detection Ab, and specific binding was visualized by application of the HRP-substrate TMB. OVA-specific IgG1 and IgG2 were detected applying AP-conjugated Abs (anti-mouse IgG1 and anti-mouse IgG2a; both Rockland Immunochemicals) and nitrophenylphosphate (Sigma-Aldrich) as phosphatase substrate. To calculate Ab amounts, we used purified mouse IgE, IgG1, and IgG2a (all BD Biosciences) standards. To determine total IgE levels, we

coated plates with 4 $\mu\text{g}/\text{ml}$ IgE capture Ab (purified anti-mouse IgE [R35-72]; BD Biosciences) at 4°C overnight and further treated as described for OVA-specific IgE.

Cell purification, adoptive transfer, and tracking

Donor mice were i.p. treated with 20 μg recombinant AvCystatin or control protein. After 18 h, peritoneal exudate cells (PEC) were isolated and either directly used for cell transfer (3×10^6 cells/mouse) or macrophages, and B cells were purified and used for transfer. Macrophages were purified using autoMACS after labeling PEC with biotinylated Abs anti-CD3e (clone 145-2C11; Miltenyi Biotec), anti-CD19 (clone eBio1D3; eBioscience), anti-CD11c (clone N418; eBioscience), anti-Gr1 (clone RB6-8C5, kind gift of the DRFZ), and anti-CD49b (clone Dx5; eBioscience) and anti-biotin beads (Miltenyi Biotec). Addition of 40 $\mu\text{g}/\text{ml}$ anti-mouse Fc γ RIII/III (clone 2.4G2; DRFZ) was used to avoid nonspecific binding. Purification of peritoneal B cells was performed using anti-CD19 MicroBeads (Miltenyi Biotec). Purity of macrophages (>95%) or B cells (90–95%) was determined by flow cytometry and cytospin analyses. A total of 2×10^6 purified macrophages or B cells were resuspended in 0.1 ml PBS and i.v. transferred on day 24 in OVA-sensitized mice before intranasal provocation. In the DSS colitis model, AvCystatin- or control-treated macrophages (2×10^6 cells) were administered i.v. to mice 1 d after access to DSS. For macrophage tracking, purified macrophages were labeled with CFSE before adoptive transfer. Seven days after transfer, spleen, BAL fluid, lung, and peribronchial lymph nodes (PBLNs) or mesenteric lymph nodes (mLNs) and colon lamina propria were analyzed for CFSE⁺ macrophages by flow cytometry. Lung and colon tissue were digested for 1 h at 37°C in a water bath using collagenase VIII (Sigma Aldrich, Steinheim, Germany). Frequencies of eosinophils in colon lamina propria leukocyte isolates were analyzed by flow cytometry using a PE-labeled anti-Siglec-F Ab (clone E50-2440; BD Biosciences).

IL-10 expression by T cells

CD4⁺ T cells were purified from spleens of OVA-sensitized and -challenged mice 7 d after adoptive transfer of macrophages by using the CD4⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's guidelines. Purified cells (>95% CD4⁺) were stimulated with Con A (2.5 $\mu\text{g}/\text{ml}$) for 48 h, and supernatants were analyzed for IL-10 via ELISA. Total RNA was extracted from remaining cells and reversely transcribed into cDNA to determine IL-10 expression by real-time PCR.

Coculture assays

Dendritic cells (DCs) were generated from bone marrow by culture in presence of 20 ng/ml murine GM-CSF (PeproTech) for 7 d. DCs were harvested and pretreated with 1 $\mu\text{g}/\text{ml}$ OVA peptide 323–339 (GenScript, Piscataway, NJ) for 2 h. CD4⁺ T cells were derived from spleens of DO11.10 TGN mice using the CD4⁺ T cell isolation kit (Miltenyi Biotec). Cocultures were set up with macrophages pretreated and isolated as described earlier in a T/DC/M ratio of 5:1:1. In some experiments, DCs were omitted and T/M cocultures were stimulated with 1 $\mu\text{g}/\text{ml}$ anti-CD3/anti-CD28 Abs (eBioscience). For transwell approaches, macrophages were kept separate from T cells and DCs by inlays with a 1- μm filter membrane (Greiner, Germany). In some experiments, macrophage supernatants instead of macrophages were added. To that end, macrophages were cultured for 18 h and supernatants were subsequently added to cocultures of T cells and DCs. Alternatively, macrophages were pretreated with OVA peptide and kept with T cells in the absence of DCs. After 48 h, supernatants from cocultures were harvested and analyzed for levels of IL-10 by ELISA.

To investigate the mechanism of IL-10 induction in T cells, we pretreated CD4⁺ T cells with various concentrations of the S1PR1 antagonist VPC23019 (Avanti Polar Lipids, Alabaster, AL) for 1 h before incubation with DCs and macrophages. To determine a possible function of ARG-1 and programmed death ligands, we preincubated macrophages with various concentrations of the ARG-1 inhibitor BEC (Calbiochem/Merck, Germany) or anti-PD-L1 and anti-PD-L2 Abs (eBioscience, Germany) for 1 h and then added to DC/T cocultures.

Macrophage characterization

For in vitro characterization of macrophages, PECs were seeded in tissue-culture dishes and incubated at 37°C and 5% CO₂ in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin. After 4 h, nonadherent cells were removed by washing with fresh medium. Remaining macrophages were cultured overnight and stimulated on the following day with either 0.5 μM recombinant AvCystatin or control treatments. Macrophages were washed with Dulbecco PBS after indicated time points and frozen at –80°C in lysis buffer (AnalytikJena). For ex vivo characterization, macrophages were purified from PECs of AvCystatin- or control-

treated animals as described earlier 1, 4, and 18 h after application. Purified macrophages were lysed and frozen at -80°C for later RNA isolation and gene expression analysis. Surface marker expression was determined for CD11b $^{+}$ F4/80 $^{+}$ macrophages 18 h after i.p. application of AvCystatin or control treatment by using anti-MHC-II-FITC (clone M5/114.15.2; eBioscience), anti-CD274-PE (clone MH5; BD Biosciences), anti-CD273-PE (clone TY25; BD Biosciences), anti-CD40-PE (clone 1C10; eBioscience), anti-CD86-biotin (clone GL1; eBioscience), streptavidin-PE-Cy7 (BD Biosciences), anti-CD16/32-allophycocyanin (clone 2.4G2, kind gift from the DRFZ), anti-CD80-PE (clone 16-10A1; eBioscience), and anti-ICAM-1-allophycocyanin (clone KAT1, kind gift from the DRFZ). Cells were analyzed using FACS-Canto cytometer (BD) and FlowJo Software version 8.5.3.

Macrophage RNA isolation, reverse transcription, and real-time PCR

Total RNA isolation and reverse transcription were performed by using the innuPREP RNA kit (AnalytikJena) and the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems) using SYBR Green PCR reagents (FastStart Universal SYBR Green Master [Rox]; Roche) following the manufacturer's guidelines. Relative changes in gene expression were calculated with ABI 7300 SDS Software (Applied Biosystems), and expression levels of transcripts were normalized to the cycle threshold values of the endogenous housekeeping gene Peptidyl Isomerase A by using the $2^{-\Delta\Delta\text{ct}}$ method. The gene-specific primer sequences can be obtained upon request.

Statistical analysis

Statistical analysis and graphical output of data were performed with the two-tailed Mann-Whitney *U* test by using GraphPad Prism, version 5.0a (GraphPad Software), and one-way ANOVA followed by Tukey's multiple comparison test. Data are presented as means $\pm$ SD. Differences between individual groups were tested to be significant for $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

Results

AvCystatin-primed PECs suppress airway inflammation

In previous studies, we demonstrated the importance of macrophages and IL-10 in mediating the immunosuppressive effects of AvCystatin *in vivo* (7). Addressing whether the protective effects of AvCystatin are mediated through modulation of specific immune cell populations, we transferred AvCystatin-treated PECs into animals suffering from OVA-induced airway inflammation. Donor cells were isolated 18 h after i.p. treatment with either AvCystatin or denatured AvCystatin. Application of AvCystatin-treated PECs before intranasal challenge with OVA led to reduced OVA-specific and total IgE levels compared with control treatments. In contrast, OVA-specific IgG1 and IgG2a responses were not altered by transfer of AvCystatin-PEC (Fig. 1A). Local cytokine responses in BAL fluid showed a marked reduction of Th2 cytokines IL-4, IL-5, and IL-13 in animals treated with AvCystatin-PEC. In addition, recipient animals demonstrated significantly increased local IL-10 levels (Fig. 1B). The cytokine profile of splenocytes after allergen-specific restimulation showed a similar pattern with decreased Th2 cytokines and increased IL-10 production (Fig. 1C). IFN- γ was not detectable. Foxp3 $^{+}$ regulatory T cells (Tregs) as a possible source for IL-10 were analyzed in peribronchial lymph nodes and spleens, but found in similar frequencies in all groups (data not shown). Analysis of cells in the BAL fluid revealed a significant reduction in the total number of infiltrating immune cells, predominantly because of an $\sim 50\%$ reduction in eosinophils after transfer of AvCystatin-PECs (Fig. 1D). Histological analysis of lung sections confirmed reduced mucous production in bronchioles and lower airway cell infiltration compared with control treatments (Fig. 1E). Previous *in vitro* studies revealed transient expression of IL-10 in macrophages after AvCystatin stimulation that rapidly decreased to background levels (6), and IL-10 was previously found to be required for suppression of airway inflammation in AvCystatin protein-treated mice (5). To analyze whether PEC-derived IL-10 was necessary for the observed effects, we isolated

AvCystatin-PEC from IL-10-deficient mice and transferred them into recipient mice as described above. Transfer of AvCystatin-primed IL-10 $^{-/-}$ PECs led to a comparable reduction in IgE (Fig. 1A), local and systemic Th2 cytokines (Fig. 1B, 1C), and BAL eosinophils (Fig. 1D). Thus, transfer of AvCystatin-PECs significantly dampened allergen-induced airway inflammation independent of cell-intrinsic IL-10 production.

AvCystatin regulatory macrophages are sufficient to protect mice from airway inflammation

To assess whether macrophages could account for the suppressive effect of the AvCystatin-PEC transfer, we sorted macrophages from the peritoneum of donor mice 18 h after i.p. treatment with AvCystatin. A single transfer of AvCystatin regulatory macrophage (Mreg) before challenge with the allergen was found to be sufficient to suppress allergic inflammation in recipient mice, similar to transfer of whole PECs (Fig. 2). OVA-specific and total IgE Ab responses were significantly suppressed in mice treated with AvCystatin-Mreg when compared with recipients of control-treated macrophages, whereas OVA-specific IgG1 and IgG2a were unaltered (Fig. 2A). IL-4, IL-5, and IL-13 were significantly decreased in BAL fluid and cultures of OVA-stimulated splenocytes, whereas IL-10 levels were significantly increased (Fig. 2B, 2C). Eosinophil infiltration to the lungs was significantly suppressed upon treatment with AvCystatin-Mreg (Fig. 2D), and mucus production and cellular infiltration in bronchioles were decreased in recipients of AvCystatin-Mreg (Fig. 2E). To assess whether AvCystatin-Mreg survived and survey their migratory pattern in recipient animals, we i.v. transferred CFSE-labeled macrophages on day 24 into OVA-sensitized animals before challenge, and their distribution in the lung, BAL fluid, PBLNs, and spleen was assessed in recipient animals on day 31 by flow cytometry. Labeled AvCystatin-Mreg were detected in the spleen and to a lower extent in the PBLNs of recipient mice (Fig. 2F). However, no AvCystatin-Mreg were detectable in lungs or BAL (data not shown).

In contrast with AvCystatin-Mreg, peritoneal B cells, which constituted $\sim 25\text{--}30\%$ of the transferred AvCystatin-PECs in the initial adoptive transfer experiments, did not significantly affect total and OVA-specific Ab production (Supplemental Fig. 2A), local and systemic cytokine responses (Supplemental Fig. 2B, 2C), or cellular infiltration into the alveolar space (Supplemental Fig. 2D). These findings show that adoptively transferred AvCystatin-Mreg are sufficient to protect against allergic airway inflammation, traffic to spleen and lung draining lymph node, and persist in recipients for at least 7 d after transfer.

AvCystatin-Mreg induce IL-10 in CD4 $^{+}$ T cells in vivo

To analyze the cellular origin of IL-10 observed in the BAL of mice receiving AvCystatin-Mreg, we purified CD4 $^{+}$ T cells from the spleen of healthy control mice or OVA-sensitized and -challenged animals treated with AvCystatin-Mreg or control macrophages. T cells from mice treated with AvCystatin-Mreg demonstrated increased expression of *il-10* transcript (Fig. 3A), and increased levels of IL-10 were detectable in culture supernatants after mitogen stimulation (Fig. 3B). Thus, these data show a prominent role of CD4 $^{+}$ T cells as cellular sources of IL-10 *in vivo* after AvCystatin-Mreg transfer.

AvCystatin-Mreg ameliorate pathology in DSS-induced colitis

To assess whether AvCystatin-Mregs mediate their suppression only in the context of pulmonary inflammation or whether they interfere also with inflammation in other mucosal barrier tissues, we tested AvCystatin-Mreg in a model of acute intestinal inflammation, dextran sodium sulfate (DSS)-induced colitis.

Application of 3% DSS in drinking water on 8 consecutive days led to weight loss, shortening of the colon, and destruction of the mucosal

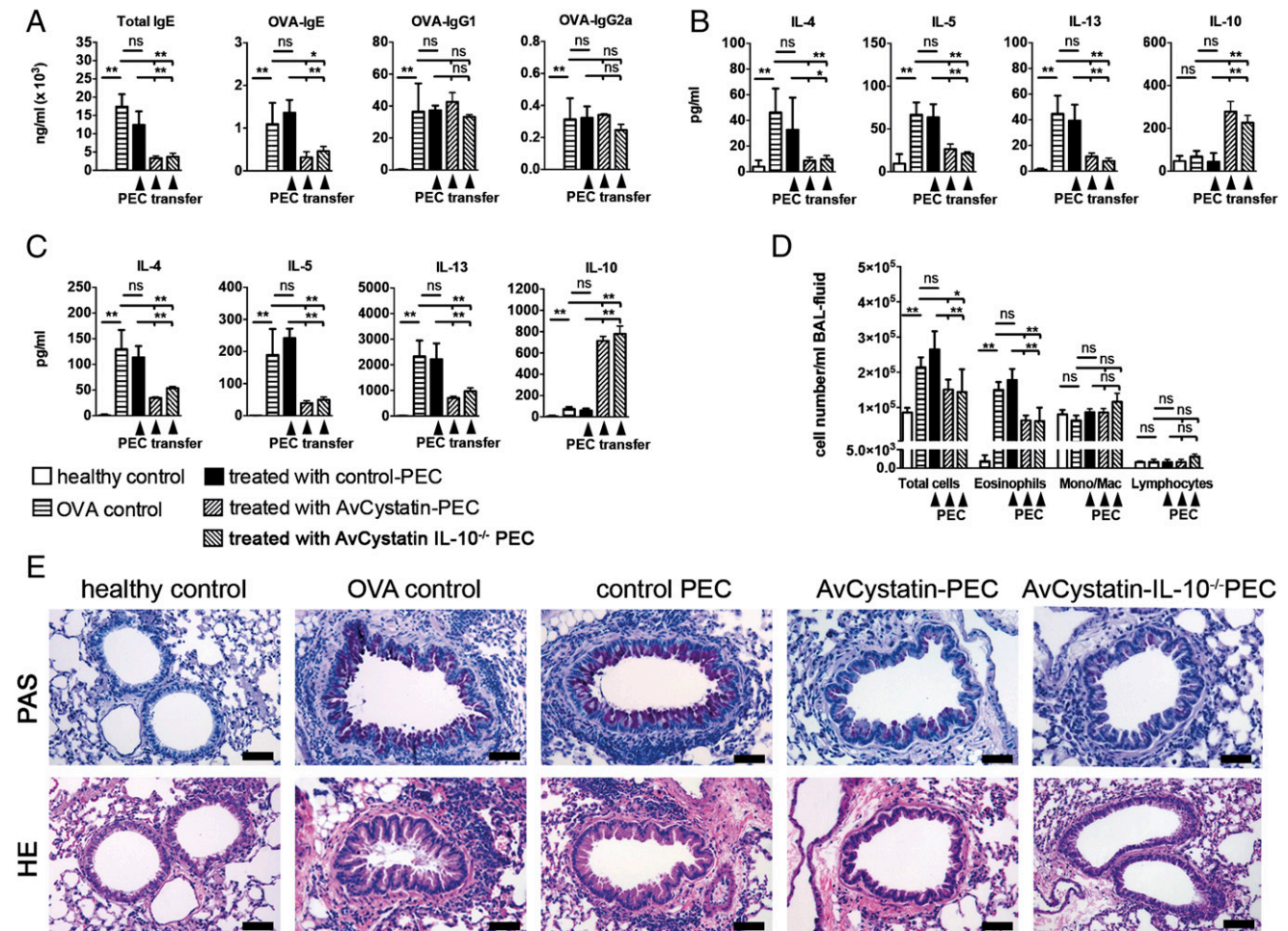


FIGURE 1. Transfer of AvCystatin-PECs protects against airway inflammation. PECs were isolated from wild type and IL-10^{-/-} mice 18 h after i.p. injection of AvCystatin or control protein (denatured AvCystatin) and adoptively transferred into OVA-sensitized mice before allergen challenge on day 24. Animals were analyzed 2 d after intranasal challenge. **(A)** Levels of total and allergen-specific IgE, OVA-IgG1, and OVA-IgG2a in recipient mice. **(B)** Local cytokine response in BAL fluid measured by quantification of IL-4, IL-5, IL-13, and IL-10 by ELISA. **(C)** Systemic cytokine response in culture supernatants of OVA-stimulated splenocytes. **(D)** Cell counts in BAL fluid. **(E)** Representative pictures of lung sections stained with PAS and H&E. Scale bars, 100 μm. Results are presented as mean ± SD and are representative for two independent experiments with five to six animals per group. **p* < 0.05, ***p* < 0.01.

architecture (Fig. 4). Strikingly, an i.v. transfer of AvCystatin-Mreg given on day 1 of DSS application protected mice from experiencing major signs of inflammatory bowel disease such as body weight loss (Fig. 4A) and colon shortening (Fig. 4B). Histopathology was reduced by transfer of AvCystatin-Mreg, but not control macrophages, as seen by a conservation of the normal mucosal architecture (Fig. 4C) and an overall improved histopathological score (Fig. 4D). Furthermore, low numbers of MPO-expressing cells and eosinophils were detectable in colon tissue sections and lamina propria cellular isolates of mice transferred with AvCystatin-Mreg (Fig. 4E, 4F). To track the migration of the transferred macrophages, additional mice received CFSE-labeled AvCystatin-Mreg derived from Ly5.2⁺ congenic donors (Fig. 4G). CFSE⁺ cells were detectable in the spleen, gut-draining mLN, and were found in the highest frequencies in the colon lamina propria (Fig. 4G). In contrast, we were unable to detect CFSE-labeled macrophages in peripheral lymph nodes analyzed as a control site. Hence AvCystatin-Mregs are capable of migrating to the colon of DSS-treated mice and significantly alleviate experimental colitis.

AvCystatin-Mregs show a novel hybrid phenotype independent of autocrine IL-10

To phenotypically characterize immunosuppressive AvCystatin-Mregs, we purified peritoneal macrophages 1, 4, and 18 h after AvCystatin or

control treatment. AvCystatin-Mregs induced early expression of proinflammatory and anti-inflammatory genes *il-12/23p40*, *tnf-α*, and *il-10* (Fig. 5A). In addition, the M1 marker *iNOS* was transiently upregulated at early time points, together with *il-6* (Fig. 5A). At the time point chosen for the transfer studies (18 h), these early markers were downregulated and AvCystatin-Mreg instead demonstrated increased levels of the M2b markers sphingosine kinase-1 (*sphk-1*), *light*, and *ccl1* (Fig. 5A). In parallel, the M2a macrophage marker *arg-1* was upregulated (Fig. 5A), whereas the M2a markers *ym-1* and *relm-α* were not significantly expressed (Fig. 5A). TGF-β transcript levels were not increased in control macrophages or AvCystatin-Mregs (Fig. 5A). To confirm the AvCystatin effect in vitro, we treated peritoneal macrophages with 0.5 μM (9.6 μg/ml) AvCystatin or denatured (control) protein. Real-time PCR analysis of macrophages reflected the in vivo results by showing an early and transient expression of proinflammatory and anti-inflammatory markers followed by expression of *sphk-1*, *light*, and *arg-1* 5–48 h after stimulation (Supplemental Fig. 3).

To further characterize the effect of AvCystatin on macrophages in vivo, we determined the expression of surface markers associated with macrophage function. Flow-cytometric analysis of peritoneal F4/80⁺CD11b⁺ macrophages revealed upregulation of MHC-II, CD40, CD80, CD86, FcγRIII/II, PD-L1, PD-L2, and ICAM-1 in response to AvCystatin, whereas denatured AvCystatin protein had only inter-

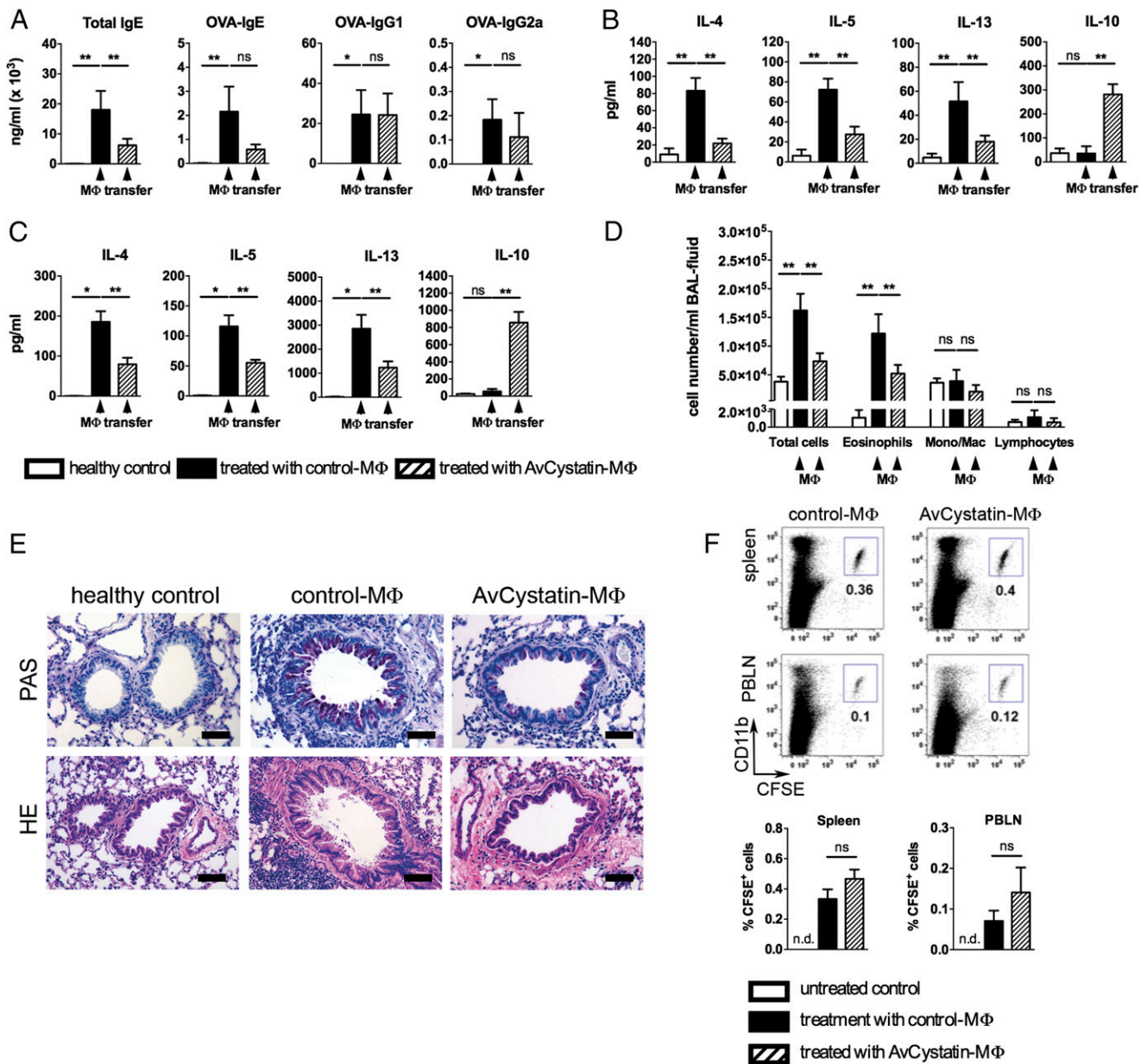


FIGURE 2. Purified AvCystatin-Mregs are sufficient to alleviate allergic inflammation in mice. Macrophages were purified from AvCystatin- or control-treated (denatured AvCystatin) mice 18 h after injection and transferred in OVA-sensitized mice on day 24. Animals were analyzed 2 d after intranasal challenge. (A) Detection of serum Abs in recipient mice. (B) Concentrations of IL-4, IL-5, IL-13, and IL-10 in BAL fluid. (C) Systemic cytokine response in culture supernatants of OVA-stimulated splenocytes. (D) Cell counts in BAL fluid. (E) Representative pictures of lung sections stained with PAS and H&E. Scale bars, 100 μm. (F) Detection of CFSE⁺-labeled control- and AvCystatin-Mregs in spleen and PBLN by flow cytometric analysis depicted as exemplary FACS plots and frequencies of total live cells. Values represent mean ± SD from two independent experiments with five to six mice per group for (A)–(E) and three mice per group for (F). **p* < 0.05, ***p* < 0.01. MΦ, macrophage; n.d., not detected.

mediate effects, suggesting the effect of AvCystatin on macrophages may at least be partially dependent on its activity and additionally to its innate recognition by macrophages in vivo (Fig. 5B).

We previously showed that macrophages produce IL-10 in response to AvCystatin treatment (6). To determine whether an autocrine effect of IL-10 secreted by AvCystatin-Mreg contributed to the phenotypic shift from M1 to M2a/M2b activation, we compared the expression of selected marker genes for macrophages of wild type and IL-10-deficient mice at an early and late time point. In vivo contact with AvCystatin induced early upregulation of *iNOS*, *il12/23p40*, *tnf-α*, and *il-6* in macrophages of wild type and IL-10-deficient mice (Supplemental Fig. 4). Early marker gene expression was downregulated to

background levels 18–20 h after stimulation. Instead, the expression of *sphk-1*, *light*, and *arg-1* was upregulated to a comparable level in wild type and IL-10-deficient macrophages (Supplemental Fig. 4). Thus, AvCystatin-Mreg exhibit a novel combination of M2a/M2b markers, and the shift from an early M1 to an M2a/M2b phenotype in response to AvCystatin contact is independent of autocrine IL-10.

A soluble factor is responsible for IL-10 instruction by AvCystatin-Mreg in T cells

To investigate the necessities for AvCystatin-Mreg-driven IL-10 induction in CD4⁺ T cells, we applied an in vitro assay of bone marrow-derived DCs presenting OVA to DO11.10 TGN CD4⁺

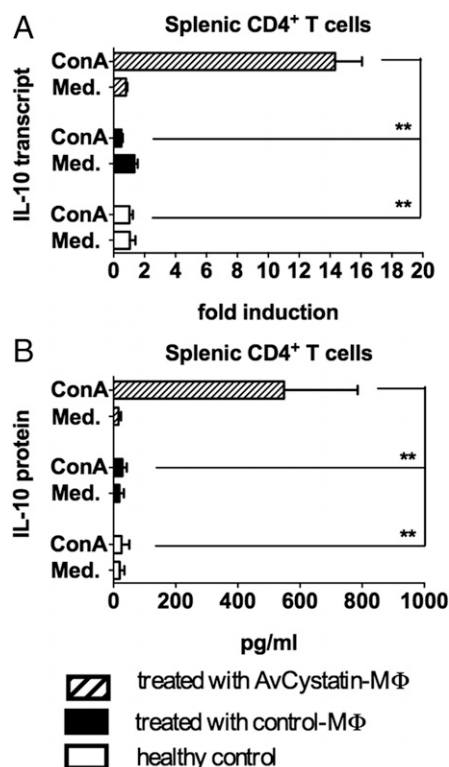


FIGURE 3. AvCystatin-Mregs induce CD4⁺IL-10⁺ T cells in vivo. Splenic CD4⁺ T cells were purified from healthy mice or allergic animals after treatment with AvCystatin-Mreg or control-macrophages (denatured AvCystatin). IL-10 transcript (**A**) and IL-10 protein (**B**) were analyzed after stimulation with Con A. Results (mean $\pm$ SD) are shown for one of two independent experiments with five to six mice per group. Normalized data are expressed as fold induction compared with untreated control mice. ** $p < 0.01$.

T cells in presence or absence of AvCystatin-Mreg and control macrophages, respectively. Indeed, we found that AvCystatin-Mregs, but not control macrophages, specifically induced IL-10 in cocultures (Fig. 6A). SPHK-1, ARG-1, PD-L1, and PD-L2 have formerly been reported to directly or indirectly interfere with CD4⁺ T cell responses (11–13). To address whether these marker genes expressed by AvCystatin-Mreg were involved in the induction of IL-10 by CD4⁺ T cells, we applied chemical inhibitors and blocking Abs in vitro to interfere with the respective factors. Pretreatment of AvCystatin-Mreg with different concentrations of an ARG-1 inhibitor did not affect IL-10 induction (Fig. 6A). Similarly, blocking of the conversion of sphingosine to sphingosine-1-phosphate by the SPHK-1 antagonist VPC23019 did not interfere with IL-10 induction in vitro (Fig. 6B). Blocking of PD1 signaling in T cells by Abs against PD-L1/2 also did not reduce IL-10 production (Fig. 6C).

We next investigated the necessities for the induction of IL-10 in the in vitro system. To assess whether the recognition of the cognate Ag OVA on DCs was a prerequisite, we added AvCystatin-Mregs to cocultures of plain DCs or OVA-presenting DCs and T cells. Only AvCystatin-Mregs in the presence of OVA-presenting DCs induced the production of IL-10 (Fig. 6D). Asking whether Ag-presenting DCs could be replaced by polyclonal stimulation, we stimulated CD4 T cells by anti-CD3/anti-CD28 Abs. Indiscriminate and low levels of IL-10 were detected in the presence of AvCystatin-Mregs or control macrophages (Fig. 6D). To further verify the dependence of IL-10 production on Ag presentation by DC, we cocultured OVA peptide-loaded AvCystatin-Mregs and control macrophages with T cells in the absence of DCs. No IL-10 induction was observed (Fig. 6D). We next investigated whether cell contact between AvCystatin-Mregs and other cells was necessary for the induction of

IL-10, and thus performed transwell assays in which macrophages were kept separate from T cells and DCs. Comparable levels of IL-10 were induced in transwell cultures (Fig. 6E). Finally, the addition of culture supernatants of AvCystatin-Mregs induced similar levels of IL-10 as detected in mixed cultures containing AvCystatin-Mregs (Fig. 6F). Taken together, these findings show that a yet undefined factor secreted by AvCystatin-Mreg instructs IL-10 production in cultures of Ag-specific stimulated CD4⁺ T cells.

Discussion

In this article, we describe a novel type of regulatory macrophage induced by the helminth immunomodulatory protein AvCystatin and demonstrate their ability to efficiently suppress mucosal inflammation in experimental airway inflammation and colitis.

Recent publications indicated the relevance of innate immune cells in immunomodulation by parasitic worms (14–16), but little is known about the target cells exploited by helminth immunomodulatory proteins or the mechanisms conferring suppression of ongoing immune responses. In previous studies, we demonstrated that AvCystatin protein administration suppresses inflammation when applied to mice in models of airway inflammation and gut inflammation (7, 17). In this study, we show that the adoptive transfer of AvCystatin-modulated macrophages is sufficient to recapitulate the anti-inflammatory effects of AvCystatin, in the absence of the compound itself. We thus provide compelling evidence that a therapeutic approach for the treatment of inflammatory disorders based on cells modulated by a single parasitic molecule may be feasible. Such an approach would be attractive because it allows the exploitation of helminth immunomodulatory therapy whereas simultaneously removing the need to administer live helminths or helminth-derived products to patients. Because helminth-secreted molecules can exert immunogenic activity, their use to treat inflammatory diseases in humans might also be disputable in repeated therapies. Cell-based therapies constitute a promising approach in which cells are differentiated into an immunosuppressive or regulatory phenotype and administered into patients. Experimentally, macrophages have been evaluated as good candidates for cell-based therapeutic intervention not only for atopic and autoimmune diseases, but also for the treatment of kidney diseases (18–24). Furthermore, regulatory macrophages have been successfully applied to human transplantation patients in first clinical trials to interfere with overt immune responses (25, 26) and are discussed as a future therapy in solid-organ transplantation and beyond (27).

We previously identified macrophages as target of AvCystatin and IL-10 as central suppressive mediator of the AvCystatin effects in vivo (7). AvCystatin uses MAPK signaling pathways in macrophages leading to the transient expression of IL-10 (8). IL-10 production is considered as a hallmark of regulatory macrophages (28) because it exerts suppression on APCs, eosinophils, mast cells, basophils, and T cells, and thus represents an attractive candidate to control, among others, allergic diseases (29, 30). In this study, we show that the transient cell-intrinsic IL-10 expression in macrophages after exposure to AvCystatin is not required for their anti-inflammatory effects in vivo. We show that a single transfer of AvCystatin-Mreg counteracted the Th2 effector mechanisms responsible for allergic airway inflammation via reduction of recruitment of inflammatory cells into the lungs, reduced allergen-specific and total IgE, and suppressed production of local and systemic IL-4, IL-5, and IL-13. In contrast, local IL-10 levels in the lungs and systemic IL-10 production by CD4⁺ T cells was sharply increased after AvCystatin-Mreg transfer, suggesting that IL-10 controlled allergy-associated Th2 responses.

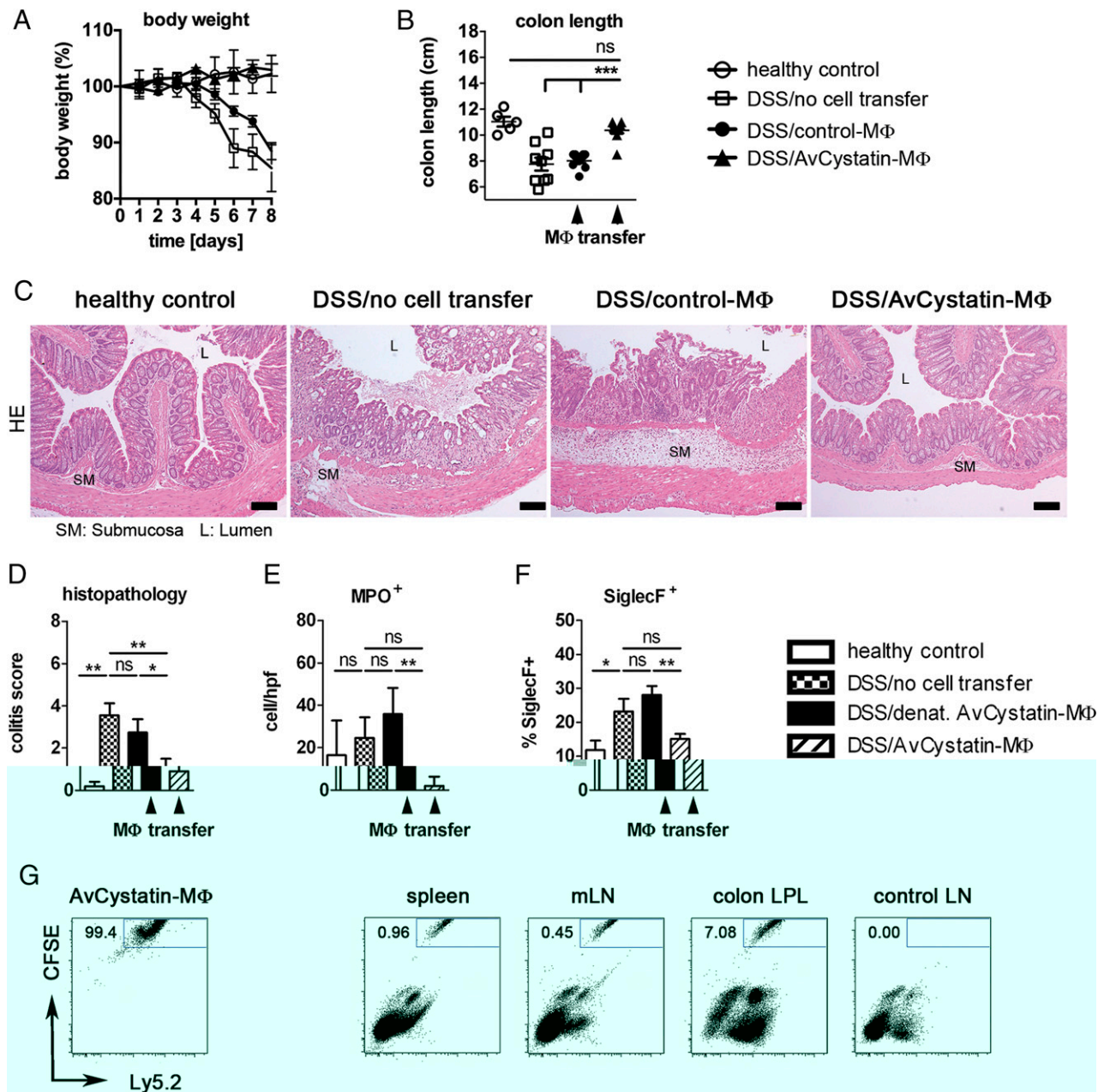


FIGURE 4. AvCystatin-Mreg alleviates DSS-induced colitis. AvCystatin- or control (denatured AvCystatin)-treated macrophages were transferred into recipients 1 d after access to 3% DSS in drinking water. Animals were sacrificed on day 8. **(A)** Body-weight development. **(B)** Colon length on day 8. **(C)** Exemplary pictures of H&E-stained colon cross sections used for histopathological scoring. Scale bars, 100 μ m. **(D)** Histopathological colitis score. **(E)** Quantification of MPO⁺ immune cells in colon cross sections. **(F)** Eosinophil frequencies in live colon lamina propria leukocyte isolates analyzed by flow cytometry for SiglecF⁺ cells. **(G)** Exemplary flow-cytometry plots showing CFSE-labeled Ly5.2⁺ AvCystatin-Mreg as transferred to mice in colitis model (left plot) and traced in spleen, mLN, and colon lamina propria leukocyte isolates. Far right plot depicts peripheral lymph node cells. Pooled data of two independent experiments with five to nine mice per group are presented as mean $\pm$ SD (A–F), and pooled cell isolates from one of two tracking experiments performed with two mice each (G) are shown. * p < 0.05, ** p < 0.01, *** p < 0.001. LPL, lamina propria lymphocytes.

This finding suggests that AvCystatin-Mregs mediate their immunosuppressive effects at least partially through modulation of the Ag-specific cytokine response in CD4⁺ T cells.

Tracking of labeled AvCystatin-Mregs showed that they located to the lymph nodes draining the sites of inflammation as well as the spleen in both disease models. The complete protection against body-weight loss and suppressed colon pathology seen in recipients of a single transfer of AvCystatin-Mregs in the colitis model correlated with high frequencies of AvCystatin-Mregs migrating to the colon and lower numbers of MPO⁺ cells (mostly neutrophils) and eosinophils contributing to tissue damage in the DSS model

(31, 32). Whether AvCystatin-Mregs trafficking to the colon in this model suppressed the production of chemoattractants for innate cells critically involved in mucosal inflammation during DSS-induced colitis remains to be investigated.

To gain insight into the mechanism of AvCystatin-Mreg-mediated immunosuppression, we performed transcriptional profiling of AvCystatin-Mregs. In vitro and in vivo treatment with AvCystatin initially upregulated the expression of the proinflammatory mediators *il-12/23p40*, *tnf- α* , *il-6*, and *iNOS*, characteristic of the M1 macrophage subset. In addition, AvCystatin transiently induced IL-10 expression early after treatment, as previously described (8).

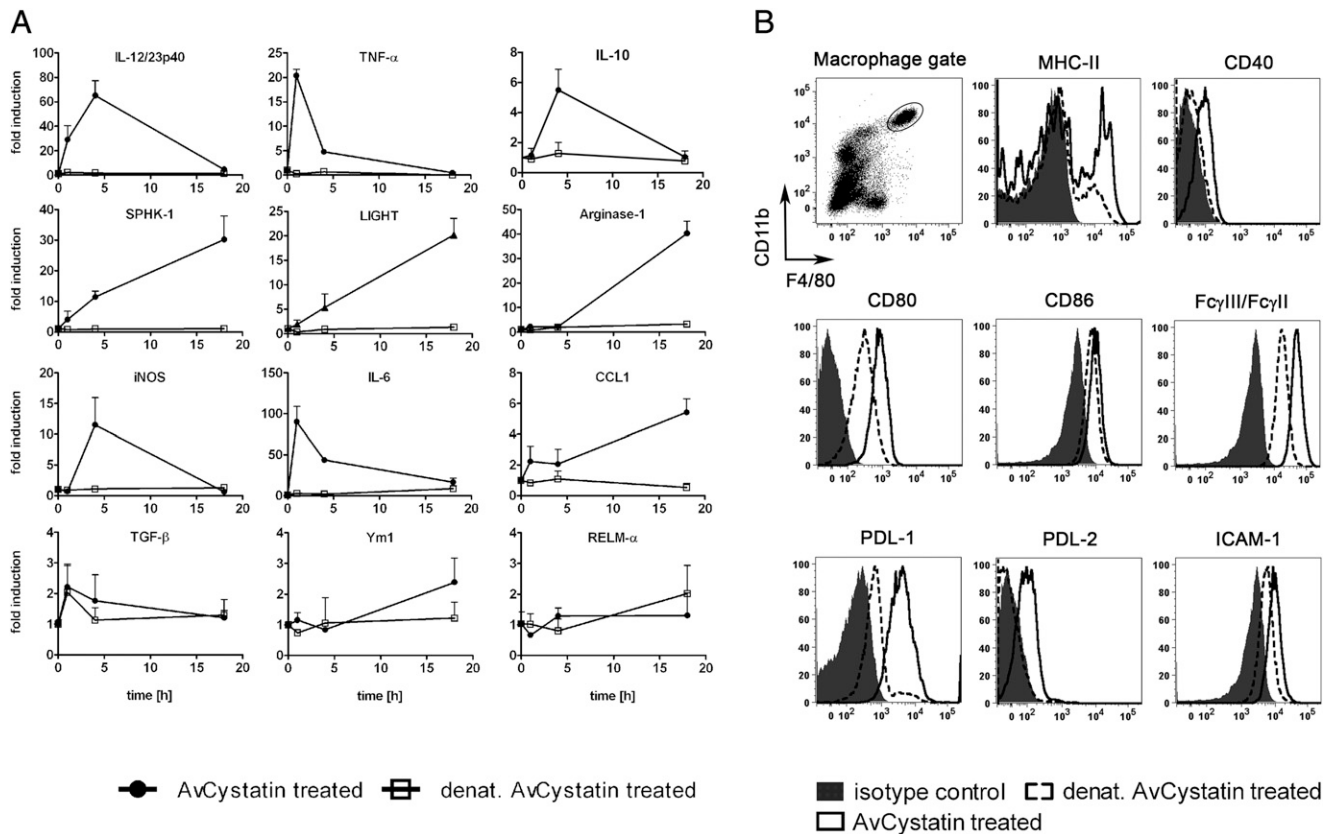


FIGURE 5. Phenotypic characterization of AvCystatin-Mregs. **(A)** Ex vivo transcriptional profiling of macrophages isolated from AvCystatin or control pretreated mice. Results (mean $\pm$ SD) are representative of two independent experiments. Normalized data are expressed as fold induction compared with untreated macrophages. For each group, macrophages from 8–10 animals were pooled and gene expression analyzed in triplicates. **(B)** Surface marker analysis for expression of MHC-II, CD40, CD80, CD86, Fc γ III/Fc γ II (CD16/32), PD-L1, PD-L2, and ICAM-1 by F4/80⁺CD11b⁺ peritoneal macrophages after i.p. treatment of mice with AvCystatin or denatured AvCystatin-control for 18 h. Data are representative of three independent experiments. Cells were pooled from five to six mice per group.

However, at later time points, the macrophage signature was significantly altered and resembled an M2a/M2b hybrid phenotype characterized by the increased expression of *spHK-1*, *light*, *ccl1*, Fc γ RIII, and Fc γ RII, and *arg-1* (33). This hybrid phenotype of AvCystatin-Mreg was stable for at least 7 d in vitro (data not shown).

SPHK-1, ARG-1, and programmed death ligands have been shown to exert regulatory effects on CD4⁺ T cells. SPHK-1 phosphorylates sphingosine to sphingosine 1-phosphate, which, in turn, may induce IL-10 expression in T cells (34), whereas ARG-1 accounts for the suppressed immune response in a variety of chronic infections (35). Blockade of PD-L2 has been recently shown to restore Th2 hyporesponsiveness during chronic nematode infection (36). However, interfering with SPHK-1, ARG-1, and blocking PD-L1/PD-L2 had no effect on IL-10 induction in cocultures of AvCystatin-Mregs, DCs, and T cells. Notably, we could show by transwell approaches and application of macrophage supernatants that AvCystatin-Mregs release a soluble factor inducing IL-10 production in vitro; however, the responsible factor remains to be identified. Because levels of active TGF- β were similar in cultures of AvCystatin-Mregs and control macrophages, as well as in cocultures with DCs and CD4⁺ T cells, we can exclude this candidate as a mediator of the anti-inflammatory capacity of AvCystatin-Mregs (data not shown). Furthermore, the presence of DCs was essential for IL-10 production in cocultures of AvCystatin-Mregs with T cells and could not be substituted by Mregs presenting the cognate Ag to T cells. The precise interplay of Mregs, DCs, and T cells and mechanism of IL-10 induction needs further investigation.

The regulatory potential of distinct macrophage subsets has attracted considerable attention. Alternatively activated macrophage subsets induced by Th2 cytokines, immune complexes, or TGF- β /IL-10 treatment have proven efficacy in suppressing murine chemically induced colitis, experimental autoimmune encephalomyelitis, and kidney diseases (19–23). Our study shows that AvCystatin-Mregs acquire a phenotype characterized by the combined expression of markers assigned previously to the M2a and M2b subsets (34), reflecting the broad spectrum of macrophage activation as recently shown by Xue et al. (37).

Studies assessing the regulatory potential of murine and human regulatory macrophages induced by in vitro treatment with IFN- γ showed that murine Mregs restricted T cell responses in an iNOS-dependent manner and led to prolonged allograft survival upon adoptive transfer into mice receiving a heterotopic heart graft (38). Also, human IFN- γ induced Mregs are capable of suppressing T cell proliferation and eliminating activated T cells in in vitro cocultures, but do so in an IDO-dependent manner (26). Another study by Brem-Exner et al. (18) showed that IFN- γ -induced murine macrophages support Foxp3⁺ Treg enrichment in cocultures with T cells. Whether AvCystatin-induced Mregs specifically eliminate activated T cells in murine disease models remains to be investigated. An iNOS- or Foxp3⁺ Treg-dependent suppression of T cell responses, however, seems unlikely, because the AvCystatin-induced Mregs did not express iNOS at the time point of adoptive transfer (Fig. 5) and local/systemic Treg frequencies were not altered in mice receiving AvCystatin-Mreg transfers (data not shown).

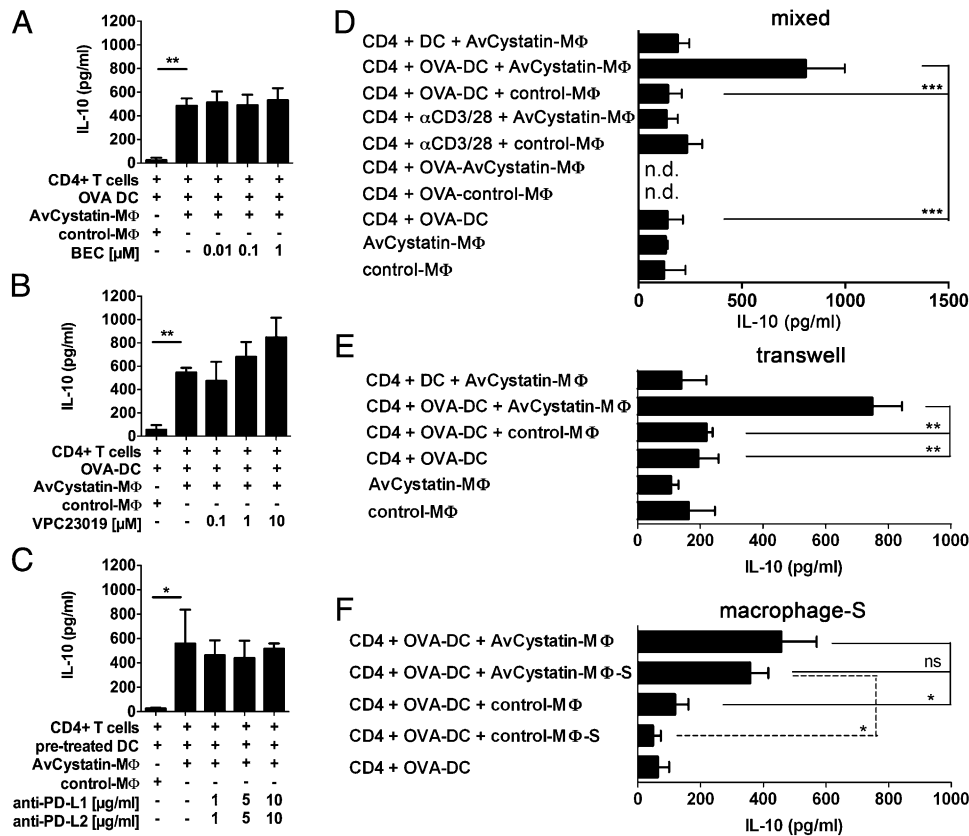


FIGURE 6. Contact-independent IL-10 induction by AvCystatin-Mreg in vitro. (**A–C**) IL-10 levels in cocultures of AvCystatin-Mreg or control macrophages with DO11.10 TGN CD4⁺ T cells stimulated by DCs presenting OVA peptide. Various concentrations of the ARG-1 inhibitor BEC (**A**), S1PR1 antagonist VPC23019 (**B**), and anti-PD-L1/2 Abs (**C**) were added to possibly interfere with IL-10 induction. IL-10 levels were compared for mixed cocultures in presence or absence of DCs or containing macrophages pretreated with OVA peptide, allowing for cell contact between macrophages and T cells (**D**) and transwell approaches separating macrophages from T cells and DCs (**E**). (**F**) Comparison of IL-10 production in mixed cocultures and CD4⁺/DC cocultures supplemented with macrophage supernatants (S). Results are presented as mean $\pm$ SD of culture triplicates and are representative for two independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

Helminth-experienced macrophages have been described previously as capable of inducing IL-10 in T cells and suppressing murine intestinal inflammation. Hunter et al. (19) showed that macrophages are essential for the anticolitic effect of murine tape-worm infections and that adoptive transfers of alternatively activated macrophages generated in vitro recapitulate the protective effects of the helminth infection, linked to an increased IL-10 production by T cells. Atochina et al. (39) showed that macrophages treated with a secreted glycan of the blood fluke *Schistosoma mansoni* adopt an alternatively activated status and are capable of inducing IL-10 in T cells. However, T cells in this study also produced increased levels of IL-13, a feature distinct from T cells in our in vivo and in vitro studies not expressing Th2 cytokines (data not shown). Intestinal macrophages from schistosome-infected mice were shown to confer protection against chemically induced colitis upon adoptive transfer (40), but information about the phenotype of such macrophages is lacking. The described anticolitic effect was independent of the alternative activation of macrophages and, importantly, mouse strain dependent (BALB/c: permissive to protective effect; C57BL/6: refractory to anticolitic effect of macrophage transfer) (40). In this article, we show that AvCystatin-Mregs suppress inflammatory reactions in both mouse strains, specifically airway inflammation in BALB/c mice and DSS colitis in C57BL/6 mice.

In conclusion, AvCystatin induces a specific macrophage phenotype in vitro and in vivo characterized by an early and transient expression of proinflammatory and anti-inflammatory activation markers followed by a conversion toward a suppressive M2a/M2b hybrid phe-

notype, which has not been described before to our knowledge. The phenotypical conversion of AvCystatin-Mregs from M1 activation toward an M2a/M2b signature did not depend on an autocrine IL-10 effect. Importantly, the Mreg characterized in our studies efficiently interferes with distinct inflammatory reactions after adoptive transfer and induces the production of IL-10 by CD4⁺ T cells in vivo, as well as in cultures of Ag-specific stimulated CD4⁺ T in vitro.

Our findings are in line with several studies that demonstrate the longevity of macrophages and their immunosuppressive potential (22, 23, 41, 42). Yet, to our knowledge, this is the first study to characterize a macrophage population induced by a single pathogen-derived immunomodulator that exerts immunosuppressive activities in unrelated inflammatory settings. Further studies have to show whether the immunosuppressive potential of AvCystatin-Mregs can be exploited as a tool to develop new anti-inflammatory therapies.

Acknowledgments

We thank Bettina Sonnenburg and Marion Müller for excellent technical assistance. We further thank the DRFZ (German Arthritis Research Center) for providing Ab reagents.

Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure 1

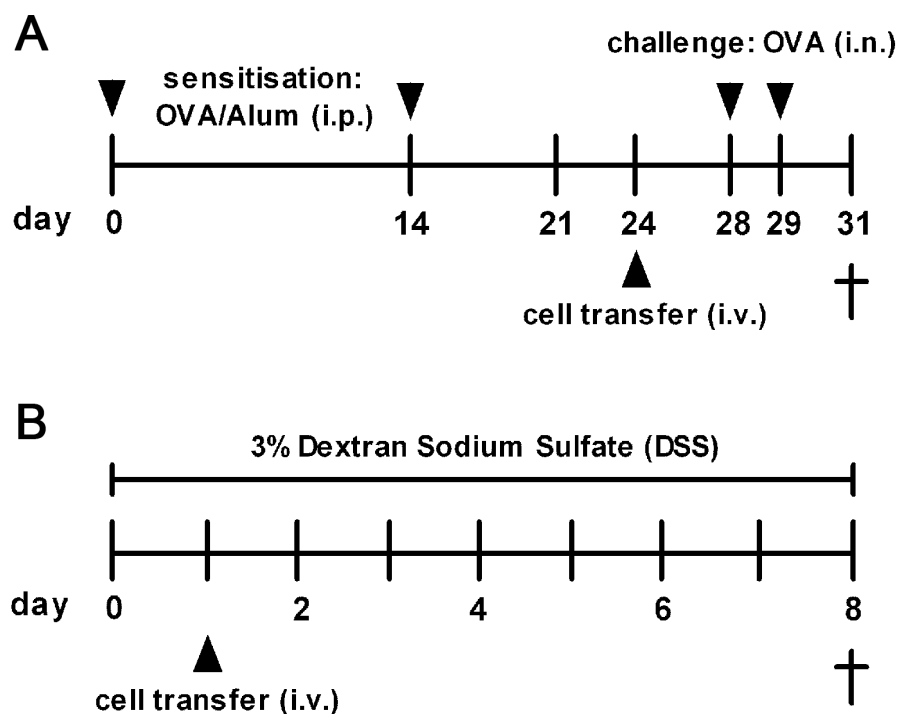


Figure S1. Disease models. (A) Scheme of OVA-induced airway inflammation. Animals were intraperitoneally sensitized with 20 $\mu\text{g}/\text{animal}$ OVA/Alum in PBS on days 0 and 14 and intranasally challenged with 50 $\mu\text{g}/\text{animal}$ OVA on days 28 and 29. (B) Scheme of DSS-induced colitis. Animals were given 3% DSS in drinking water from day 0 to 8.

Supplementary Figure 2

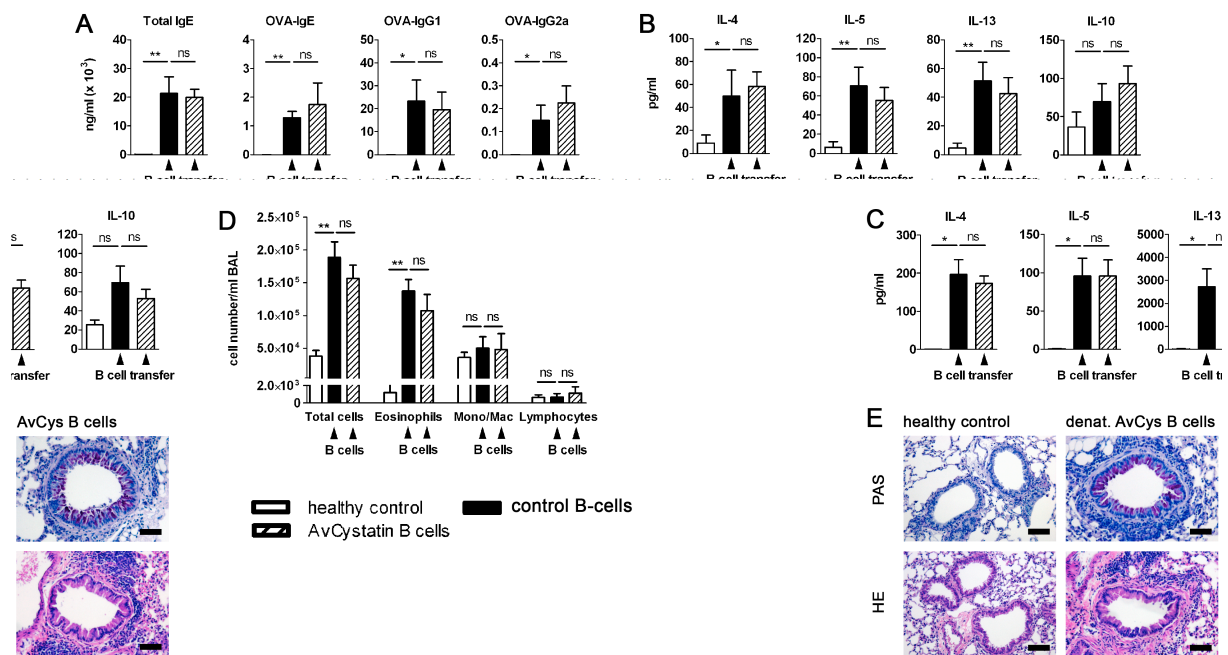


Figure S2. Transfer of AvCystatin-experienced B cells does not protect against airway inflammation. 2×10^6 purified B cells from AvCystatin- or control (denatured AvCystatin)-treated mice were transferred i.v. to ovalbumin-sensitized mice 18 hours post treatment. (A) Total and allergen-specific IgE, OVA-IgG1 and -IgG2a. (B) Th2 cytokine and IL-10 response in BAL-fluid. (C) OVA-specific cytokine response of recipient splenocytes. (D) Analysis of cells in the BAL-fluid. (E) Representative pictures of lung sections stained with periodic acid schiff (PAS) and hematoxylin eosin (HE). Bars, 100 μ m. Results are presented as mean $\pm$ SD for one experiment with 5-6 mice per group. *, $P < 0.05$; **, $P < 0.01$

Supplementary Figure 3

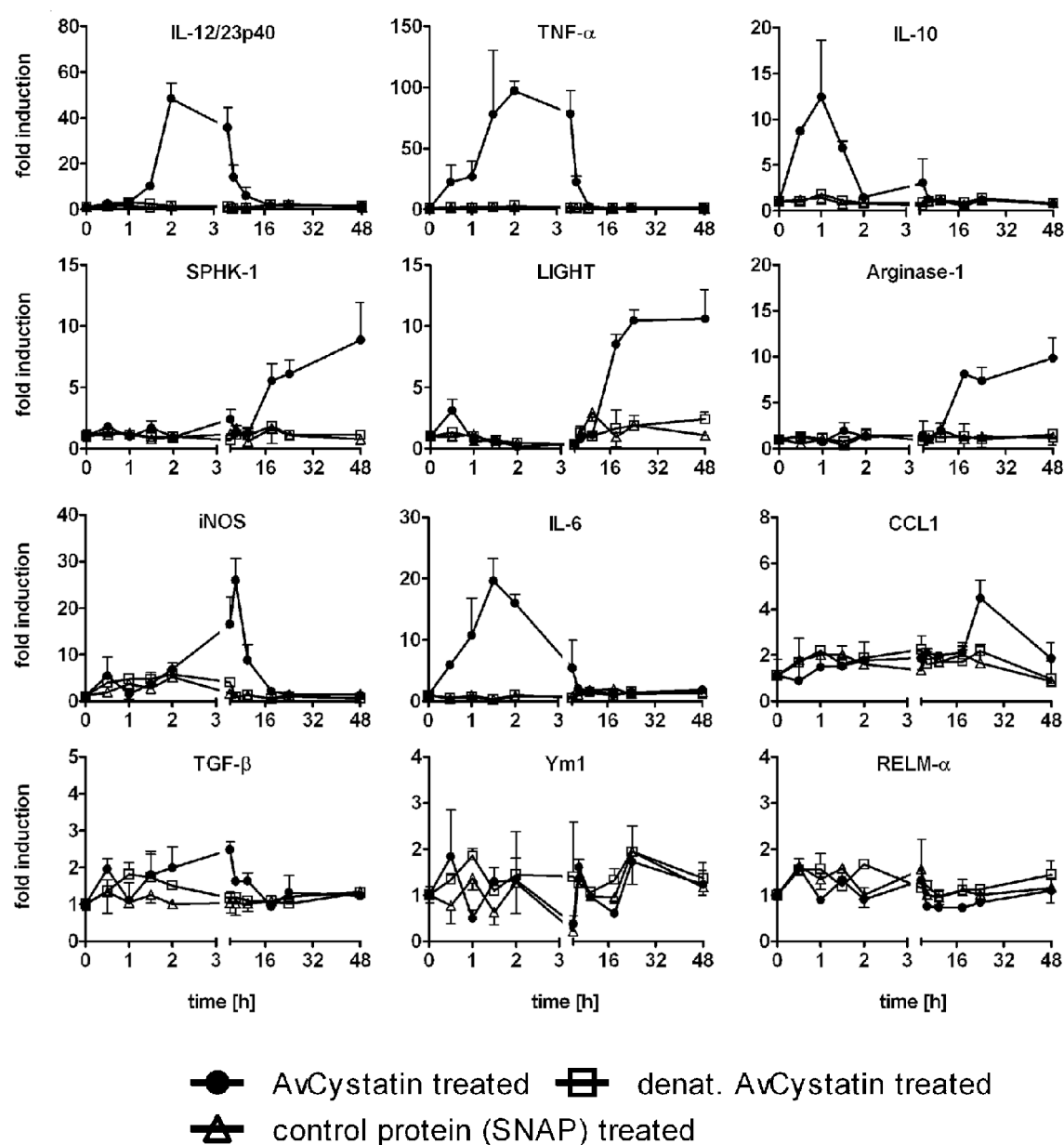


Figure S3. Gene expression of macrophages *in vitro*. (A) Macrophages purified from peritoneal exudate cells of naïve donor mice were treated *in vitro* with AvCystatin, denaturated AvCystatin or a recombinant control protein (SNAP) and analysed for gene expression by real-time PCR for expression of selected macrophage marker genes. Results (mean + SD) are representative of three independent experiments.

Supplementary Figure 4

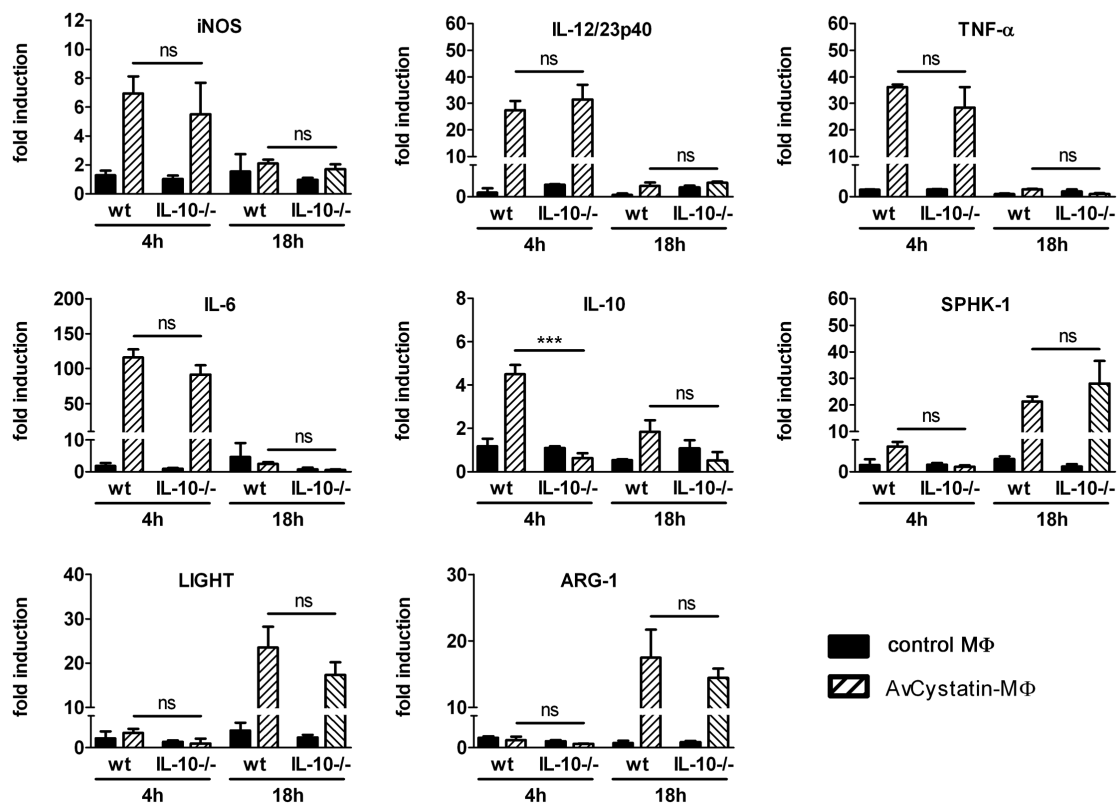


Figure S4. Phenotype of AvCystatin-Mreg is independent of autocrine IL-10. *Ex vivo* transcriptional profiling of macrophages isolated from AvCystatin or control (denatured AvCystatin) treated wild type and IL-10^{-/-} mice. Results (means + SD) are representative of two independent experiments. Normalized data are expressed as fold induction compared to untreated macrophages. For each group, macrophages from 8-10 animals were pooled and gene expression analysed in triplicates.