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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: 1555–1564.
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 pg/μg 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 ml magnesium hydroxide (Inject Alum; Pierce) in 0.2 ml PBS on days 0 and 14. Airway challenge was performed with in-transplant 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 slides were analyzed via light microscopy and documented using a Leica DFC8000 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 μg/ml OVA in RPMI 1640 supplemented with 10% FCS, 1 mM l-glutamine, 100 U/ml penicillin G, and 100 μg/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). Detection of OVA-specific IgE, ELISA plates were coated with 10 μg/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; 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 against mouse IgG1 and mouse IgG2 (both Rockland Immunochemicals) and nitrophenylphosphate (Sigma-Aldrich) as phosphatase substrate. To calculate Ab amounts, we used purified mouse IgG1, IgG1, and IgG2a (all BD Biosciences) standards. To determine total IgE levels, we coated plates with 4 μg/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, adaptive 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 × 10⁶ 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-CD3ε (clone 145-2C11; Miltenyi Biotec), anti-CD19 (clone eBio1D3; eBioscience), anti-CD11b (clone M1/70; 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 μg/ml anti-mouse FcyRIII/II (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 × 10⁷ purified macrophages or B cells were resuspended in 0.1 ml PBS and i.v. transferred on day 24 in OVA-sensitized mice before intranodal provocation. In the DSS colitis model, AvCystatin- or control-treated macrophages (2 × 10⁶ 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, BALF, fluid, lung, and peribronchial lymph nodes (PLNs) 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 μg/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 (ProTecTech) for 7 d. DCs were harvested and pretreated with 1 μg/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 μg/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, macrophages 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 cell death ligands, we preincubated macrophages with various combinations of the ARG-1 inhibitor BFC (Calbiochem, Merck, Germany) or anti–PD-L1 and anti–PD-L2 Abs (eBioscience, Germany) for 1 h and then added to DC/T cell 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 μg/ml streptomycin. After 48 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 μg/ml 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 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 MIH5; 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–ICAM1–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 isolation kit and innuPRIDeal 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 Isomerize A by using the 2^[-DDCt] 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 ± 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-primed 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-primed 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 individual groups were tested to be significant for *p < 0.05, **p < 0.01, or ***p < 0.001.

**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 decreased 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 ~25–30% 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-Mreg 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
architecture (Fig. 4). Strikingly, an i.v. transfer of AvCystatin-Mreg gave no and a day 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 mLNs, 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-a, 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-a 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 following 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-
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, mfxa, 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. 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+...
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 immunomodulatory effects, 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 atomic 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 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 ± 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.
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-a, 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).
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, FcγRIII, and FcγRII, 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 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).
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 phenotype, 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.

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Disclosures

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References
