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Parasite-Specific Immunomodulatory Functions of Filarial Cystatin

Peter Schierack,[1,2] Richard Lucius,[1] Bettina Sonnenburg,[1] Klaus Schilling,[3] and Susanne Hartmann[1]*

Department of Molecular Parasitology, Humboldt University,[1] and Institute of Microbiology and Animal Epidemic Diseases, Freie Universität Berlin,[2] 10115 Berlin, and Institute of Biochemistry, University of Jena, 07740 Jena,[3] Germany

Cystatins of parasitic nematodes are well-described pathogenicity factors which contribute to downregulation of T-cell proliferation of their hosts and induce anti-inflammatory cytokine responses. We compared the immunomodulatory effects of two cystatins of the filarial nematodes *Onchocerca volvulus* and *Acanthocheilonema viteae* with two homologous proteins of the free-living nematode *Caenorhabditis elegans*. Like filarial cystatins, the *C. elegans* cystatins (rCysele1 and rCysele2) possessed domains relevant for inhibition of papain-like proteases and were biologically active inhibitors of human cathepsins B, L, and S. However, the inhibition of cathepsin B by *C. elegans* cystatin was much stronger. *C. elegans* cystatins lacked a domain involved in inhibition of legumain-like proteases that was present in *O. volvulus* cystatin. Filarial cystatins suppressed the proliferation of human peripheral blood mononuclear cells (PBMC) and murine spleen cells, while the *C. elegans* cystatins had this effect to a much lesser extent. Whereas filarial cystatins markedly increased the production of interleukin (IL)-10, *C. elegans* cystatins increased the production of IL-12 and gamma interferon (IFN-γ) by human PBMC. The cystatins of both the filariae and *C. elegans* induced an upregulation of inducible nitric oxide by IFN-γ-stimulated murine macrophages. These data suggest that filarial cystatins but not the *C. elegans* cystatins downregulate proliferative responses of host cells due to characteristics which might reflect an adaptation of filariae to their parasitic life style.

Endoparasitic nematodes are specifically adapted to a life within immunocompetent hosts, where they are continuously exposed to immune effector mechanisms. Their survival depends in part on the evasion of harmful immune responses or the modulation of the immune system. It is thought that secreted immunomodulatory molecules of parasitic nematodes are pathogenicity factors with a key role in balancing the host-parasite relationship (1, 40). This raises the question of whether secreted immunomodulators of parasitic nematodes have characteristics distinct from the molecules of their free-living relatives.

The tissue-dwelling filarial nematodes can be considered extremely well adapted parasites, because they live in direct contact with their host's immune system and nevertheless have life spans of 10 years and more (37). This long persistence is ascribed to modulation of the immune system, which leads to inhibition of inflammatory responses that could eliminate the parasites (23). One characteristic of humans infected with filariae is a distinct cellular hyporeactivity and a polarization of the immune response towards a Th2 type (9, 39). These traits can be explained by the recruitment of alternatively activated macrophages which block cellular proliferation by cell-to-cell contact (19) and the release of recently described immunomodulatory components by the worms.

We and others have shown that a cysteine protease inhibitor (cystatin) downregulates T-cell proliferation and alters macrophage functions (16, 25, 36, 42), and a serine protease inhibitor (serpin) was reported to impair functions of granulocytes (49).

Filariae were also shown to produce a functionally active macrophage migration-inhibitory factor (35) as well as a transforming growth factor beta (TGF-β) homologue (10). Furthermore, phosphorylcholine, a phospholipid component associated with several filarial proteins, inhibits cellular proliferation by interfering with signal transduction chains that lead to cellular activation (15). Comparison of such parasite-derived immunomodulators with molecules of free-living nematodes should provide a clue to whether the capacity of these components to interact with the immune system represents a specific adaptation to the parasitic life style.

In this study we chose the secreted filarial immunomodulator cystatin as a model protein for a comparison with homologous proteins of the free-living nematode *Caenorhabditis elegans*. Filarial cystatin accounts for a major proportion of the immunosuppressive activity of secreted filarial proteins (16) and therefore has to be considered a major pathogenicity factor of filariae. Cystatins are natural, tight-binding cysteine protease inhibitors and represent important regulators of proteolytic processes. Many cystatins possess two distinct inhibitory functions, the inhibition of papain-like proteases (family 1) and of legumain-like proteases (family 13). Several immunomodulatory properties of common cystatins have been described. Human cystatin C inhibits the phagocytic function of monocytes and granulocytes (18), and various members of the cystatin superfamily upregulate the inducible nitric oxide production of murine macrophages (44, 45). In addition, cystatins have the potential to modulate immune responses, as they interfere with steps in the antigen-processing pathway in major histocompatibility complex (MHC) class II antigen presentation (27, 33, 47).

Filarial cystatin is clearly involved in parasite-induced immunomodulation, as it induces cellular hyporeactivity of T

* Corresponding author. Mailing address: Department of Molecular Parasitology, Institute of Biology, Philippstr. 13, 10115 Berlin, Germany. Phone: 0049 30 2093/6450. Fax: 0049 30 2093/6051. E-mail: susanne.hartmann@rz.hu-berlin.de.

cells, modulates the production of cytokines, and downregulates essential costimulatory molecules on macrophages (16, 42). A filarial cystatin was shown to interfere with antigen presentation by inhibiting multiple cysteine protease activities found in endosomes/lysosomes of B cells (25). In vivo experiments revealed that application of filarial cystatin via osmotic pumps results in upregulated production of tumor necrosis factor alpha (TNF-α) and a diminished antigen-specific proliferation of spleen cells in mice (36). Recently, the cystatin of the intestinal nematode *Nippostrongylus brasiliensis* was also shown to be involved in immune evasion by modulating antigen processing in antigen-presenting cells (5).

Our results reveal distinct differences between cystatins of filarial nematodes and *C. elegans* in their interference with cellular immune responses in vitro. While filarial cystatins induce a marked suppression of proliferative T-cell responses and production of Th2-like cytokines, the *C. elegans* cystatins suppress cellular proliferation to a lesser extent and lead to Th1-like cytokine responses. This pattern suggests that the cystatins of parasitic nematodes evolved in a manner that allows very specific modulation of host responses.

MATERIALS AND METHODS

***C. elegans* culture and preparation of total RNA from *C. elegans*.** *C. elegans* worms were cultured on NGM (nematode growth medium) plates seeded with *Escherichia coli* OP50 and kept at room temperature. The *C. elegans* mixed culture was harvested, and bacteria were removed by washing three times with M9 buffer (0.02 M KH_2PO_4, 0.035 M Na_2HPO_4, 0.085 M NaCl, 1 mM $MgSO_4$). RNA was isolated by guanidinium thiocyanate (Tristar; AGS, Heidelberg, Germany). mRNA was transcribed into cDNA by reverse transcription-PCR (Moloney murine leukemia virus; Promega, Mannheim, Germany).

Cloning and expression of recombinant proteins. The cDNA of the mature cystatin proteins, without the signal sequence, were amplified by PCR with degenerate primers derived from internal sequences of *C. elegans* chromosome III, genes K08B4.6 and R01B10.1, showing similarities to cystatin domains (48). The Cysele1 forward primer was CAAATTGCTGGTGGATTG; the Cysele1 reverse primer was AATTTTTTCATCAGGTTTCAC; the Cysele2 forward primer was GGTATGATGACTGGAGGA; and the Cysele2 reverse primer was GAACTGCTCGTCAGCGGT. PCR amplification yielded a 360-bp fragment (rCysele1) and a 372-bp fragment (rCysele2), which were cloned into a T overhang vector (pGEM-T Easy Vector System; Promega, Madison, Wis.). The *Not*I-restricted fragments from the pGEM vector were unidirectionally subcloned into the *Not*I site of the expression vector pET28a, yielding polypeptides with a six-histidine tag (pET System; Novagen, Madison, Wis.). The recombinant plasmids were transformed into competent *E. coli* BL21(DE3) and screened for expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

rCysele1 and rCysele2 were purified under nondenaturing conditions by affinity chromatography with Ni-nitrilotriacetic acid resin (Qiagen, Hilden, Germany) and dialyzed four times against phosphate-buffered saline. The recombinant filarial cystatins Ov17 (rOv17) (42), a cystatin of the parasitic nematode *O. volvulus*, and recombinant Av17 (rAv17) (16), a cystatin of the rodent filaria *A. viteae*, were expressed and purified under identical conditions. The control protein, recombinant Ov33 (rOv33) (20), an *O. volvulus* protein with homologies to an aspartic protease inhibitor, was expressed and purified under identical conditions. This control protein was chosen in view of its closely related function but different structure.

Because rCysele1, rCysele2, rOv17, rAv17, and rOv33 were expressed in *E. coli*, endotoxin contamination was evaluated by the quantitative, chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.). The endotoxin concentration of different batches of rCysele1 was determined to be 0.3 to 4 ng/of protein per ml, and that of rCysele2 was 0.4 to 5.5 ng of protein per ml. rOv17 and rAv17 and the control protein rOv33 showed endotoxin concentrations of 0.5 to 5 ng of protein per ml. In addition, we used cystatin preparations with added polymyxin B, and we used CeH/HeJ mice to compare with C3H/HeN mice in the experiments.

Human cathepsin inhibition assays. The inhibitory activity of rCysele1 and rCysele2 to human cathepsins was characterized by K_i value determinations.

Human cathepsin B was purchased from Sigma (Deisenhofen, Germany), human cathepsin L was from Calbiochem (Schwalbach, Germany), and human cathepsin S was kindly provided by B. Wiederanders (Institute of Biochemistry, University of Jena, Jena, Germany). K_i values were determined by measuring the activity of human cathepsin B (1.2 nM), cathepsin L (0.1 nM), or cathepsin S (0.1 nM) in the presence of various concentrations of rCysele1 and rCysele2 with the fluorogenic substrates benzyloxycarbonyl (Z)-Arg-Arg-aminoethylcoumarine (AMC) (10 μM; Bachem, Heidelberg, Germany) for cathepsin B, Z-Phe-Arg-AMC (10 μM; Bachem) for cathepsin L, and Z-Val-Val-Arg-AMC (10 μM; Bachem) for cathepsin S (3). The K_i values were calculated with the program GraphPadPrism.

Antigen-driven and polyclonally stimulated proliferation of human PBMC. The peripheral blood mononuclear cell (PBMC) fraction of healthy donors was isolated from citrate-treated venous blood by density gradient sedimentation with Ficoll-Hypaque (Pharmacia Biotech Products, Freiburg, Germany). Cells were resuspended in RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10% fetal bovine serum (Biochrom). Then 3.5×10^5 PBMC/well were cultured in 96-well flat-bottomed plates at 37°C and stimulated with either 10 IU of purified protein derivative (PPD) (Chiron Behring & Co, Marburg, Germany) together with IFN-α (125 U/ml) (46) or anti-CD3 antibody (4 μg/ml) (Orthoclone-Okt3, kind gift from Janssen-Cliag, Neuss, Germany). Recombinant proteins were added at a concentration of 0.5 μM. PPD-stimulated PBMC were cultured for 96 h and polyclonally stimulated for 72 h at 37°C. Proliferation was quantified by [^{3}H]thymidine incorporation (1 μCi/well; Amersham, Buckinghamshire, United Kingdom) during the last 20 h of incubation. All experiments were performed in triplicate. Viability of the cells in the presence of recombinant proteins was controlled by trypan blue exclusion.

Quantification of cytokines in cell culture supernatant. Cytokine production was determined in the culture supernatants of unstimulated and stimulated PBMC cocultured with 0.5 μM recombinant proteins. Culture supernatants were collected after 24 h and 48 h of incubation. Cytokines were quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions (OptEIA; Pharmingen, Hamburg, Germany). The assays were performed in triplicate.

Nitric oxide production of murine peritoneal macrophages. BALB/c mice were killed by ether inhalation. Peritoneal macrophages were harvested by flushing the peritoneal cavity several times with RPMI. Erythrocytes were lysed with lysis buffer. Murine peritoneal macrophages were washed three times with RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10% fetal bovine serum (Biochrom) and cultured at a density of 2×10^5 cells/well in 96-well flat-bottomed plates. Murine peritoneal macrophages were allowed to adhere for 2 h at 37°C. Medium and nonadherent cells were removed by washing with RPMI. Murine peritoneal macrophages were stimulated by IFN-γ (100 U/ml) in a final volume of 300 μl. Purified recombinant proteins were added at a concentration of 0.05 to 0.5 μM. After 24 h, 100 μl of supernatant was removed and analyzed by Griess reaction (11). The absorbance was determined at 550 nm. All experiments were performed in triplicate. Arithmetic mean values ± standard deviation are shown.

Antigen-driven and polyclonally stimulated proliferation of murine spleen cells. Spleen cells of naive BALB/c and ovalbumin receptor transgenic mice (30) (kindly provided by T. Kamradt, Deutsches Rheuma-Forschungszentrum, Berlin, Germany) were cultured in 96-well flat-bottomed plates at a density of 3.5×10^5 cells/well, and proliferation was stimulated with 2 μg of concanavalin A per ml (Sigma, Deisenhofen, Germany) or 40 μM ovalbumin (Sigma). Proliferation of cells was quantified after 72 h by incorporation of [^{3}H]thymidine (1 μCi/well; Amersham, Buckinghamshire, United Kingdom) added to the cultures during the last 20 h of incubation. Purified recombinant proteins were added at a concentration of 0.25 to 0.5 μM to the spleen cells. Viability of T cells in the presence of the recombinant proteins was controlled by trypan blue exclusion. All experiments were performed in triplicate. Arithmetic mean values ± standard deviation are shown.

Statistical analysis. Statistical analysis of T-cell proliferation and cytokine data was performed with the Mann-Whitney U test. Nitric oxide data were analyzed with the Wilcoxon rank test. Data are presented as means and standard deviations.

RESULTS

Sequence similarities of nematode cystatins and protease-inhibitory activity of Cysele1 and Cysele2. Sequence alignment of filarial cystatins with Cysele1 and Cysele2 showed that the

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              10        20        30        40        50        60        70        80
              .         .         .         .         .         .         .         .
Av17          -----------------VLVRCEEPANMESEVQAPNLLGGFTWQERNPEEKEIQDLLPKVLIKLNQLSNVEYHLMPIKLLKVSSQVVAGLRYKM
Ov17          VQLQGAKSARAKNPSKMESKTGENFTQDRPVLLGG--WEDRDPKDEEILELLPSILMKVNEQSNDEYHLMPIKLLKVSSQVVAGVKYKM
Cysele1       ---------------------------QIAGGLSDVNASEYTGAAWNSVPEIN----SKNNGQNYMVPIKVVKAQVQVVAGTNTVL
Cysele2       ---------------------------GMMTGGSVEQDASQKEYSDKAWKAVKGINDQASNNGPYYYAPIKVTKASTQVVAGISTKL
human cystatin C  ----------------------SSPGKPPRLVGGPMDASVEEEG--VRRALDFAVGEYNKASNDMYHSRALQVVRARKQIVAGVNYFL
                                        **             . .    .  .      . .   . .      *.***        .
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              110       120       130       140       150       160       170
              .         .         .         .         .         .         .
Av17          EIQVAQSFTECKK---SSGEEVNLKTCKRLEGH---PDQIITLEAWENSWENFLQVKILEKKEVLSSV--
Ov17          DVQVAR--SQCKK---SSNEKVDLTKCKKLEGH---PEKVMTLEVWEIPWENFMRVEILGTKEV------
Cysele1       EVLVGE--STCPRQGSVQASQVTAANCPLKSGG---KRELYKVSIWEIPWENFKQTKAEKIRGVKPDEKI
Cysele2       EVLVGE--SNCKK-GELQAHEITSSNCQIKDGG---SRALYQVTIWEIPWENFEQFTVEKIRDVTADEQF
human cystatin C  DVELGR--TTCTK------TQPNLDNCPFHDQPHLKRKAFCSFQIYAVPWQGTMTLSKSTCQDA------
                       ...  .       . * .             *             *
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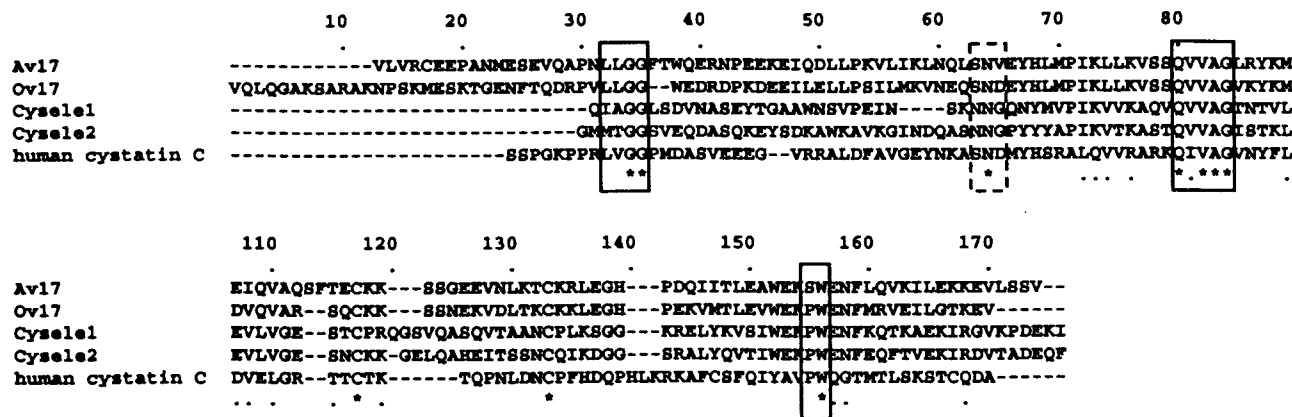
FIG. 1. Alignment of amino acid sequences of *C. elegans* cystatin 1 (Cysele1), *C. elegans* cystatin 2 (Cysele2), *A. viteae* cystatin (Av17), onchocystatin (Ov17), and human cystatin C. The first amino acid of each sequence is the proposed N terminus of the mature protein. Similar amino acids are marked by dots, and identical amino acids are marked by asterisks. The evolutionarily conserved papain binding sites are boxed. The conserved asparaginyl endopeptidase (AEP) inhibitory domain is boxed with a dashed line. The sequence data are available from GenBank under the following accession numbers: Av17, L43053; Ov17, M37105; Cysele1, AJ310669; Cysele2, AJ310670; and human cystatin C, AAA52164.

three typical conserved motifs of cysteine protease inhibitors that bind and inhibit proteases of the papain family were present in all nematode cystatins (Fig. 1). However, the N-terminal active domain of both *C. elegans* cystatins showed only the essential amino acid glycine, whereas the two amino acids in front of the glycine were different (Fig. 1). In contrast, most of the other regions varied considerably between the filarial cystatins and *C. elegans* cystatins as well as between *C. elegans* cystatins and human cystatin C. Interestingly, a second active domain, SND, inhibiting legumain-like proteases, enzymes known to be involved in MHC class II antigen processing (2, 24), was lacking in the *C. elegans* cystatins. However, the corresponding sites were found in mammalian cystatin C as well as in *O. volvulus* and *Brugia malayi* cystatins. Another difference between the filarial cystatins and the *C. elegans* cystatins was the presence of an N-terminal amino acid extension in the filarial cystatins. Theses stretches did not show any homologies to known sequences.

To compare the immunomodulatory properties of filarial cystatins with the properties of Cysele1 and Cysele2, full-length mature recombinant proteins (rCysele1 and rCysele2) with an N-terminal six-histidine tag were produced in *E. coli*. rCysele1 and rCysele2 were tested for their capacity to inhibit the activity of papain-like proteases (family 1 proteases) like the human cysteine proteases cathepsin B, S, and L. K_i determinations showed that rCysele1 and rCysele2 were potent inhibitors of human cathepsin B, L, and S (Table 1). In comparison, recombinant *O. volvulus* cystatin also strongly inhibited the

activity of cathepsins S and L, whereas the activity of human cathepsin B was less efficiently inhibited by *O. volvulus* cystatin (Table 1) (42). The interaction of filarial cystatins and *C. elegans* cystatins with human cysteine proteases, which have essential functions in cellular processes, especially in macrophages, indicated that inhibitors of *C. elegans* and of parasitic nematodes have a similar potential to interfere with the activity of host proteases. The only significant difference between the filarial cystatins and the *C. elegans* cystatin was observed for cathepsin B, which was significantly more strongly inhibited by the *C. elegans* cystatins (Table 1).

Polyclonal proliferation of murine spleen cells and human PBMC. To compare the effects of filarial cystatins and *C. elegans* cystatins on polyclonal T-cell proliferation, we examined the reaction of human PBMC stimulated with anti-CD3 antibodies, rOv17, the cystatin of the human pathogenic filaria *Onchocerca volvulus*, and rCysele1 and rCysele2. rOv17 inhibited the anti-CD3-stimulated proliferative responses by 43.6% ($P = 0.001$, Fig. 2), while the effect of rCysele1 and rCysele2 was much lower (15.1% and 21.6% inhibition, respectively), similar to that of the recombinant control protein rOv33, which also slightly inhibited cellular proliferation (12.1% inhibition). These data suggest that the *C. elegans* cystatins did not induce cellular hyporeactivity, very much in contrast to the filarial cystatins.

In a parallel approach, we studied the reaction of concanavalin A-stimulated murine spleen cells to rAv17, the cystatin of the rodent filaria *A. viteae*, in comparison to rCysele1 and rCysele2. While rAv17 significantly suppressed cellular proliferation by 63.5% ($P = 0.001$), rCysele1 and rCysele2 had no effect on the polyclonally stimulated proliferation of BALB/c spleen cells (Fig. 3). The data from the murine system supported the above results obtained with human cells.

Antigen-stimulated proliferation of human PBMC and murine spleen cells. To compare the effects of nematode cystatins on antigen-driven T-cell proliferation, we used purified protein derivative (PPD) of *Mycobacterium tuberculosis* to stimulate human PBMC. While *O. volvulus* cystatin induced an inhibi-

TABLE 1. K_i values of nematode cystatins with human cathepsins B, L, and S as cysteine proteases

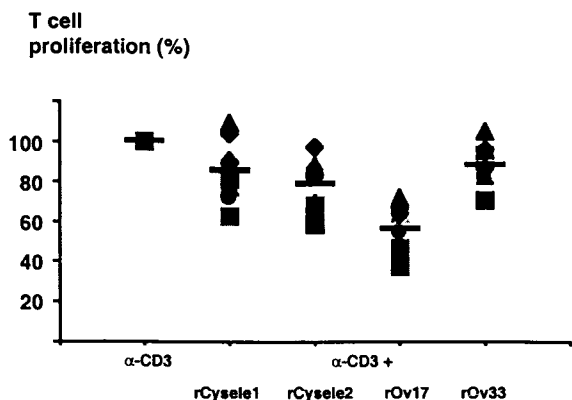| Cathepsin	K_i (nM)		
	rCysele1	rCysele2	rOv17
B	38.88 ± 4.06	1.18 ± 0.9	494.7 ± 154.9
L	0.0143 ± 0.001	0.036 ± 0.004	0.038 ± 0.004
S	0.1214 ± 0.017	0.18 ± 0.014	0.033 ± 0.003

T cell proliferation (%)

FIG. 2. T-cell proliferation of human PBMC in the presence of recombinant *C. elegans* cystatins. Anti-CD3 antibody-stimulated proliferation of PBMC from 10 healthy human donors in the presence of 0.5 μM rCysele1, rCysele2, or rOv17 or of the control protein rOv33. Proliferation of PBMC in the presence of anti-CD3 antibody was set at 100%.

tion of 72% of the PPD-stimulated proliferation of human PBMC, rCysele1 as well as rCysele2 did not exert a significant effect on the PPD-induced proliferation of human PBMC (Fig. 4). The recombinant *O. volvulus* control protein rOv33 also did not show a significant effect on the PPD-induced proliferation of the same blood samples.

In parallel, we studied a murine model of cellular proliferation with spleen cells of ovalbumin receptor transgenic mice stimulated with 40 μM ovalbumin. In the presence of rAv17 (0.25 μM), we found an inhibition of cellular proliferation of 68.4% ($P = 0.01$). In contrast, rCysele1 and rCysele2 as well as the control protein rOv33 had no significant effects on the antigen-driven cellular proliferation of the murine spleen cells (Fig. 5).

These results demonstrate that the filarial cystatins rOv17 and rAv17 interfere with the antigen-driven cellular proliferation of human PBMC and mouse spleen cells, as we have shown in previous studies, while the cystatins of *C. elegans* did not share this capacity.

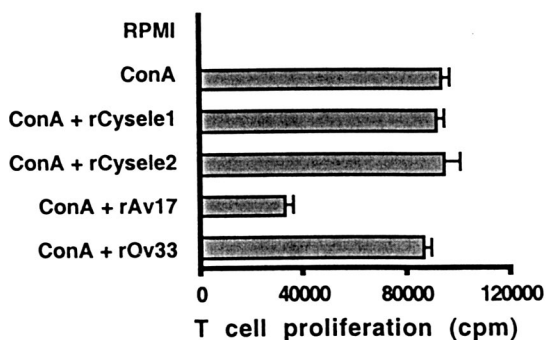**Cytokine production of human PBMC in presence of**

FIG. 3. Mitogen-induced murine T-cell proliferation in the presence of recombinant nematode cystatins. Concanavalin A (ConA)-stimulated proliferation of BALB/c spleen cells in the presence of a constant amount (0.25 μM) of rCysele1, rCysele2, rAv17, or rOv33. The data shown were obtained with 3.5×10^5 spleen cells from one representative mouse.

rCysele1 and rCysele2. One possible mechanism by which the filarial cystatins mediate the suppression of T cells is interference with cytokine expression by immune cells. Previous studies have shown that the most prominent effect on cytokine production in the presence of the filarial cystatins was an upregulation of IL-10. In contrast, IL-12 and IFN-γ production was decreased in the presence of rOv17 in comparison to the control protein (42). We comparatively analyzed whether this pattern of cytokine production was also induced by *C. elegans* cystatin.

Tests with anti-CD3-stimulated human PBMC showed that rOv17 induced a significant upregulation of IL-10. PBMC produced in the presence of rOv17, a mean of 2.8 ng of IL-10/ml ($P = 0.001$), while the IL-10 production by PBMC in the presence of the *C. elegans* cystatins reached the level of the control protein (1.5 ng/ml) (Fig. 6). The differences in IL-10 production between rOv17 and rCysele1 and rOv17 as well as rCysele2 were significant ($P = 0.05$).

The IL-12 production of anti-CD3-stimulated human PBMC was not significantly altered by the presence of rOv17, while rCysele1 and rCysele2 significantly ($P = 0.001$) enhanced the release of IL-12 by about 4.3-fold in comparison to the anti-CD3 stimulation (Fig. 6). The IL-12 levels measured in the presence of the control protein were even higher than those induced by rCysele1 and rCysele2. The IFN-γ production showed a trend to be downregulated in the presence of the *O. volvulus* cystatin, while rCysele1 and rCysele2 had a tendency to upregulate the production of IFN-γ (Fig. 6), similar to the control protein. The differences between the IFN-γ levels of the cystatins compared to the control protein were not statistically significant. However, differences in the IFN-γ levels between rOv17 and rCysele1 as well as rCysele2 were significant ($P = 0.05$).

In parallel, we determined the amounts of IL-10 and IL-12p40 in culture supernatant of unstimulated PBMC of six individuals after 24 h. The test revealed that both *C. elegans* cystatins had no effect on the production of IL-10 and IL-12p40 of unstimulated cells and therefore reacted like the control protein rOv33. In contrast, rOv17 induced a significant increase of the cytokine IL-10 in unstimulated cells ($P = 0.001$, Fig. 6) but had no influence on the IL-12p40 production in unstimulated cells.

These data show that the filarial cystatins increased IL-10 production significantly better than the *C. elegans* cystatin. In contrast, *C. elegans* cystatins enhanced the production of the Th1 cytokines IL-12 and IFN-γ, which was not the case for the parasite cystatins.

Production of inducible nitric oxide by nematode cystatins. Members of the cystatin superfamily, e.g., chicken cystatin, have been described to cause an upregulation of the inducible nitric oxide production of IFN-γ-activated macrophages. (44), and the same applies to *A. viteae* and *O. volvulus* cystatins (17). Therefore, we tested whether *C. elegans* cystatins also modulate the nitric oxide production of IFN-γ-activated macrophages. Peritoneal macrophages of BALB/c mice were stimulated with IFN-γ for 24 h, and nitric oxide production was measured by the stable degradation product nitrite in the culture supernatant. The culture supernatant of IFN-γ-activated macrophages of BALB/c mice contained 2 μM ± 0.2 μM nitrite, whereas no nitrite was detected in nonactivated cells.
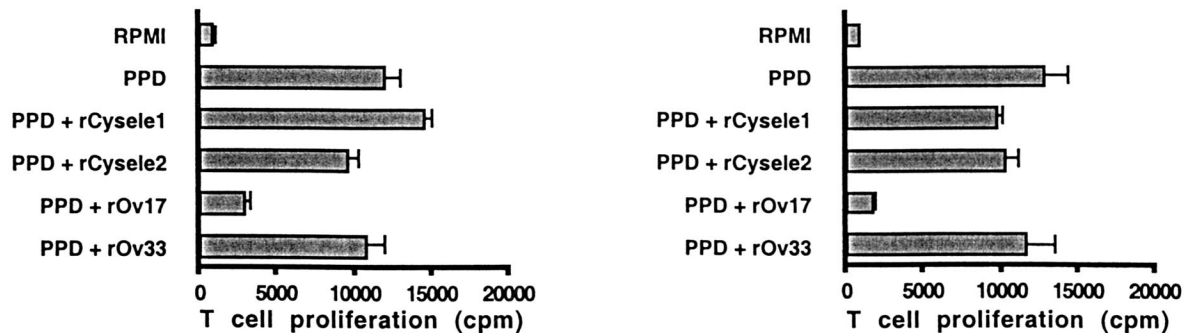
FIG. 4. Antigen-driven proliferation of human PBMC in the presence of recombinant nematode cystatins. PPD-stimulated proliferation of PBMC of two representative blood donors in the presence of 0.5 μM rCysele1, rCysele2, or rOv17 or of the control protein rOv33. The data shown were obtained with 3.5×10^5 cells from two representative blood donors.

The addition of 0.5 μM rCysele1 led to an increase in nitric oxide production up to 8.5 μM ± 0.66 μM nitrite ($P = 0.001$), while 0.5 μM rCysele2 led to an increased production of nitric oxide of 4 μM ± 0.39 μM nitrite ($P = 0.001$, Fig. 7). The filarial cystatin rOv17 stimulated nitric oxide production to 5.4 μM ± 0.32 μM in the presence of the molecule at 0.5 μM ($P = 0.001$), while the recombinant filarial control protein (rOv33) had no effect on the inducible nitric oxide production of macrophages.

These data show that the capacity to upregulate the inducible nitric oxide production is a common feature of nematode cystatins, irrespective of the life style of the worms.

DISCUSSION

This study compares the immunomodulatory activity of cystatins of parasitic filarial nematodes and of the free-living nematode *C. elegans*. One of the major characteristics of filarial cystatins shown here is their capacity to interfere with proliferation of host T cells. In contrast, the *C. elegans* cystatins Cysele1 and Cysele2 used in our experiments had this effect to a much lesser extent. Neither the antigen-driven proliferation
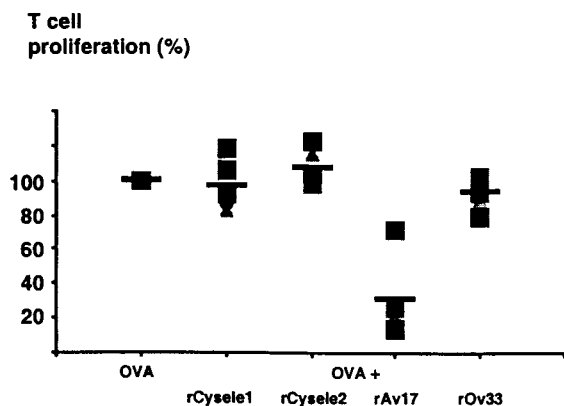

FIG. 5. Antigen-driven proliferation of murine spleen cells in the presence of recombinant nematode cystatins. Ovalbumin (OVA)-stimulated proliferation of spleen cells of ovalbumin receptor transgenic mice in the presence of a constant amount (0.25 μM) of rCysele1, rCysele2, rAv17, or rOv33. The data shown were obtained with spleen cells from five individual mice.

of ovalbumin-stimulated spleen cells from ovalbumin receptor transgenic mice nor the mitogen-stimulated proliferation of mice were affected by *C. elegans* cystatins compared to the cystatin of the rodent filaria *A. viteae*. This lack of immunomodulatory capacity was also found when human PBMC stimulated with anti-CD3 antibodies or with PPD were exposed to cystatins of *C. elegans*. In contrast, the cystatin of the human-pathogenic filaria *O. volvulus* strongly interfered with the proliferation of human PBMC.

These data on filarial cystatins support the results of earlier studies. *A. viteae* cystatin inhibits murine T-cell proliferation, which coincides with the upregulation of IL-10 (16). *O. volvulus* cystatin suppresses polyclonally stimulated as well as antigen-driven cellular proliferation of human PBMC, accompanied by downregulation of MHC class II protein and the costimulatory molecule CD86 on monocytes (42). Effects in line with these data were described for *Brugia malayi* cystatin, which inhibits class II MHC-restricted antigen processing (25). Moreover, application of *Litomosoides sigmodontis* cystatin to experimental animals reduced the antigen-specific cellular reactivity of murine spleen cells ex vivo (36).

In an attempt to identify differences between the nematode cystatins that could account for the observed differential immunomodulatory properties, we comparatively characterized filarial cystatins and *C. elegans* cystatins by several approaches. Our K_i value determinations of nematode cystatins performed with the human cathepsins B, L, and S may reflect differences in the amino acid sequences. Differences in the N-terminal active domain of cystatins have been shown to change their inhibitory capacity (4, 13, 14). While the K_i values of *O. volvulus* cystatin and *C. elegans* cystatin for cathepsins L and S were in the same range, cathepsin B was significantly better inhibited by *C. elegans* cystatins than by *O. volvulus* cystatin. This is of particular interest because cathepsin B was described to play a role in the destructive degradation of antigenic fragments during the process of antigen presentation (7, 27). It has been shown that inhibition of cathepsin B resulted in enhancement rather than inhibition of presentation of specific antigens (7, 26). This suggests that efficient cathepsin B inhibitors like Cysele1 and Cysele2 might protect epitopes from proteolytic destruction and would thus support antigen presentation and antigen-specific proliferation of T cells.

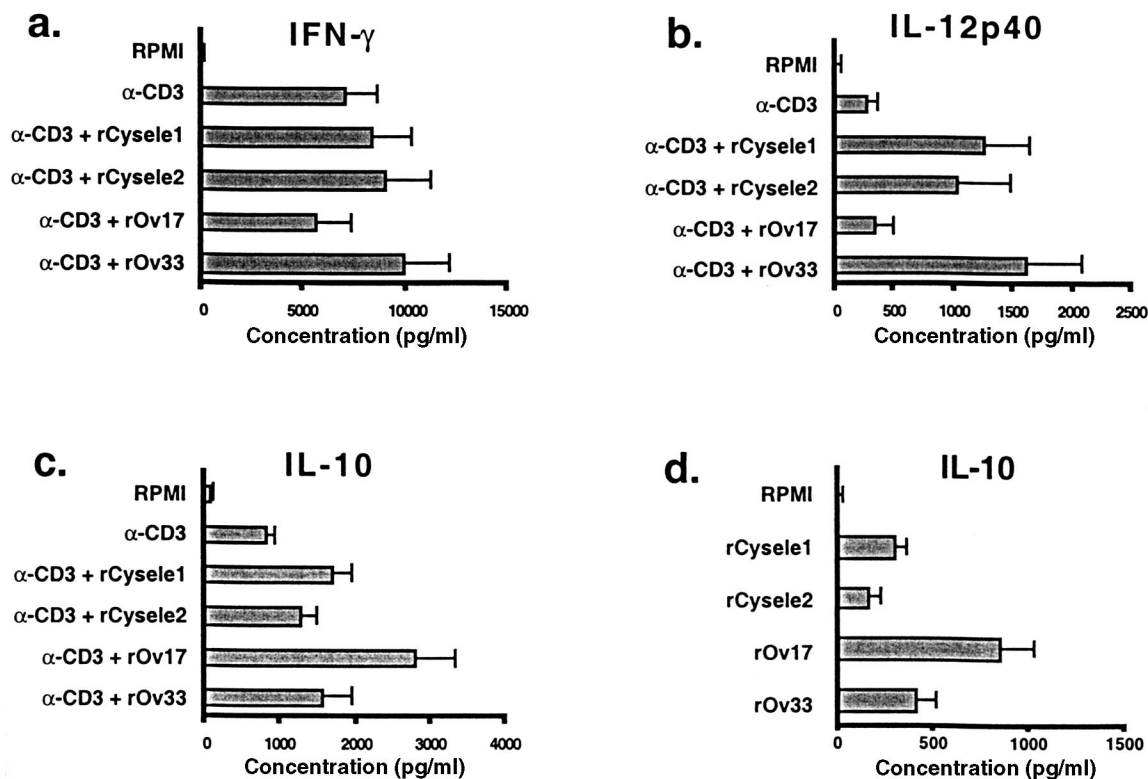Apart from antigen presentation, protease inhibitors of the

FIG. 6. Production of IL-10, IL-12, and IFN-γ by human PBMC in the presence of recombinant nematode cystatins. Anti-CD3 antibody-stimulated production of IL-10 (a), IL-12 p40 (b), and IFN-γ (c) by PBMC from eight healthy donors after 48 h. (d) Spontaneous production of IL-10 in the blood of six donors after 24 h. The concentration of rCysele1, rCysele2, rOv17, and the control protein rOv33 was 0.5 μM.

papain-like proteases (family 1) also have other important functions. In experimental murine leishmaniasis, it was shown that treatment of mice with a synthetic cathepsin B inhibitor induced a switch of CD4$^+$ T-cell differentiation from Th2 to Th1. CA074, a specific inhibitor of cathepsin B, appeared to block the development of the Th2 response that usually develops in BALB/c mice infected with *Leishmania major* and, in turn, promoted the development of Th1 cells (21). Further studies with site-directed mutagenesis will show whether the increased inhibition of cathepsin B by *C. elegans* cystatins is related to the observed amino acid differences in the N-terminal inhibitory domain. However, cathepsins L and S also have essential functions in processing and presentation of antigens (31, 32, 33, 41), and our experiments show that they are similarly inhibited by nematode cystatins.

Our evaluation of the cytokine production of human PBMC in the presence of *O. volvulus* cystatin confirms earlier studies on the effect of filarial cystatins on murine cells and human cells (16, 42). The comparison with *C. elegans* cystatins revealed a prominent difference. Human PBMC exposed to *O. volvulus* cystatin induced TNF-α, followed by a strong production of IL-10. This IL-10 burst would support Th2-like cytokine responses (29). In contrast, both *C. elegans* cystatins upregulated the production of the Th1 cytokines IL-12 and IFN-γ, after an initial release of TNF-α. In this regard, the *C. elegans* cystatins behave very much like chicken cystatin, which increases the levels of IL-12 and reduces the levels of IL-4,

leading to a switch of CD4$^+$ differentiation from Th2 to Th1, as shown in a study of experimental leishmaniasis (6).

The molecular basis for this differential induction of cytokines is currently unclear. The induction of TNF-α by all nematode cystatins is compatible with a binding of proteins to toll-like receptors of human PBMC, a class of receptors which have been described to mediate responses to microbial ligands (43). An upregulation of TNF-α followed by an upregulation of IL-10 is a characteristic pattern of events evoked by contact of macrophages with bacterial lipopolysaccharide and related substances (28, 38). However, an influence of lipopolysaccharide has been excluded in this and previous studies by careful controls. We therefore assume that cystatins have the inherent property to induce TNF-α and IL-10. The degree to which IL-10 is produced might be determined by the other cytokines produced at the same time as a response to cystatin. The relative lack of IL-10 upregulation by *C. elegans* cystatin might be due partly to the parallel increased production of the Th1 cytokines IL-12 and IFN-γ.

The theoretical consequences of the IL-10 burst after contact with filarial cystatins coincide well with our observations. IL-10 is a cytokine which causes immunosuppressive effects, leading to reduced T-cell proliferation (8). A combination of IL-10 and TGF-β produced by regulatory T cells is well known to account for immunological tolerance to food antigens (12) and for cellular unresponsiveness in filarial infections (9). Such a combined production of both cytokines did not occur in our
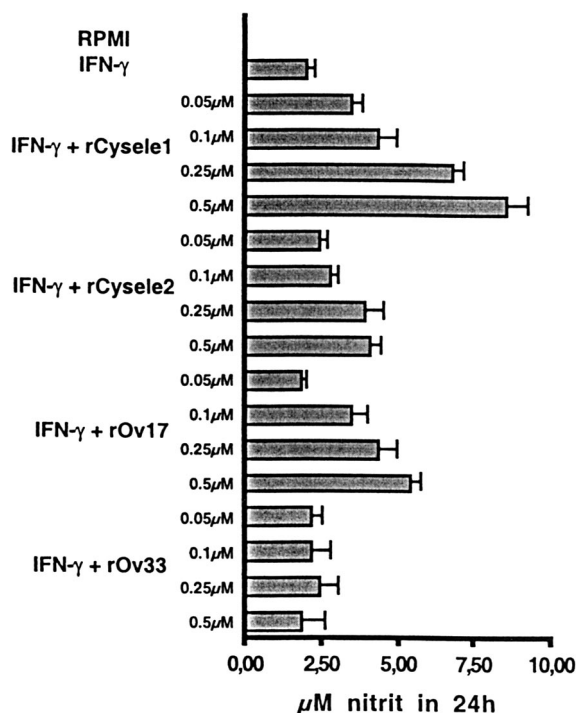
FIG. 7. Nitric oxide production by IFN-γ-activated murine macrophages in the presence of recombinant nematode cystatins. Peritoneal macrophages from BALB/c mice were activated with IFN-γ for 24 h and incubated with various concentrations of rCysele1, rCysele2, rOv17, and rOv33. The nitric oxide production of macrophages was determined in the culture supernatant by measuring nitrite. Data shown are means ± standard deviation of triplicate determinations.

context, since the TGF-β responses of human PBMC were not altered after contact with nematode cystatins (our unpublished data). However, IL-10 on its own might have modulatory effects, since cellular hyporeactivity in filarial infections has been associated with this cytokine (22, 34). Therefore, an increased capacity to induce IL-10 might be an adaptation to the parasitic life style.

The enhancement of the inducible nitric oxide production of IFN-γ-stimulated murine macrophages by all tested nematode cystatins confirms earlier studies on filarial cystatins (17). This upregulation of inducible nitric oxide can be considered a common quality of cystatins, as chicken cystatin, human stefin B, and rat kininogen upregulate the nitric oxide production of IFN-γ-activated murine macrophages (44). Interestingly, the upregulation of nitric oxide by cystatin is considered not to be due to its biological activity as a protease inhibitor, as related synthetic protease inhibitors did not cause this effect (17, 44). The induction of nitric oxide by cystatins was suggested to be at least partly due to a modulation of the cytokine production exerted by these molecules (17, 45).

In conclusion, our comparison with *C. elegans* cystatins reveals that cystatins of the parasitic filarial nematodes seem to exert their effect by at least two distinct mechanisms that act in concert, the inhibition of proteases and the induction of cytokines. The low inhibition by *O. volvulus* cystatin of cathepsin B together with a strong inhibition of cathepsins L and S is prone to inhibit antigen processing and presentation. In contrast, the

strong inhibition of cathepsin B by the *C. elegans* cystatins will support antigen presentation and at the same time lead to Th1-like responses. These tendencies would be reinforced by the cytokine responses, in that the filarial cystatins induce mainly IL-10, which inhibits antigen presentation and favors the development of Th2-like responses. In contrast, the *C. elegans* cystatins reinforce the tendency towards a Th1-like response by inducing IL-12 and IFN-γ. This intricate interplay between protease inhibition and induction of cytokine response supposedly leads to the induction of anti-inflammatory responses and contributes to the persistence of filariae in the host.

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