Modulation of Human T Cell Responses and Macrophage Functions by Onchocystatin, a Secreted Protein of the Filarial Nematode *Onchocerca volvulus*

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*J Immunol* 2001; 167:3207-3215; doi: 10.4049/jimmunol.167.6.3207

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Modulation of Human T Cell Responses and Macrophage Functions by Onchocystatin, a Secreted Protein of the Filarial Nematode *Onchocerca volvulus*¹


Immune responses of individuals infected with filarial nematodes are characterized by a marked cellular hyporesponsiveness and a shift of the cytokine balance toward a Th2/Th3 response. This modulation of cellular immune responses is considered as an important mechanism to avoid inflammatory immune responses that could eliminate the parasites. We investigated the immunomodulatory potential of a secreted cysteine protease inhibitor (onchocystatin) of the human pathogenic filaria *Onchocerca volvulus*. Recombinant onchocystatin (rOv17), a biologically active cysteine protease inhibitor that inhibited among others the human cysteine proteases cathepsins L and S, suppressed the polyclonally stimulated and the Ag-driven proliferation of human PBMC. Stimulated as well as unstimulated PBMC in the presence of rOv17 produced significantly more IL-10, which was paralleled in some situations by a decrease of IL-12p40 and preceded by an increase of TNF-α. At the same time, rOv17 reduced the expression of HLA-DR proteins and of the costimulatory molecule CD86 on human monocytes. Neutralization of IL-10 by specific Abs restored the expression of HLA-DR and CD86, whereas the proliferative block remained unaffected. Depletion of monocytes from the PBMC reversed the rOv17-induced cellular hyporeactivity, indicating monocytes to be the target cells of immunomodulation. Therefore, onchocystatin has the potential to contribute to a state of cellular hyporesponsiveness and is a possible pathogenicity factor essential for the persistence of *O. volvulus* within its human host. The Journal of Immunology, 2001, 167: 3207–3215.

*Onchocerca volvulus* is the causative agent of river blindness, a vector-transmitted nematode infection that affects ~20 million persons in tropical regions of Africa and Latin America. The parasites persist in their hosts for 10 years or longer (1, 2), and in the majority of infected persons onchocerciasis is associated with markedly reduced cellular immune reactions and polarization of T cell responses toward a Th2 and Th3 type (3–5). It is believed that these alterations are a key element in the persistence of the worms within their hosts. The down-regulation of inflammatory host immune reactions is suggested to be induced by secreted products of the nematodes (6, 7).

Despite the probable importance of this immunomodulation for the balance of the host-parasite relationships, there is little information on pathogenicity factors accounting for it. Several studies demonstrated that phosphorylcholine, a lipid component bound to proteins of filariae, inhibits murine B and T cell proliferative responses by interference with signaling cascades leading to cellular activation (8). Phosphorylcholine was also shown to increase the IL-10 production of B1 cells (9) and could thus contribute to the polarization of Th cell responses. Homologues of various cytokines, such as macrophage migration-inhibitory factor (10) or tgh-2 (11, 12) released by the filarial nematode *Brugia malayi*, appears to mimic host cytokines and to interfere with the host’s cytokine network. Furthermore, a secreted protein, the cysteine protease inhibitor (cystatin) of the rodent filaria *Acanthocheilonemasthfragia*, was shown to down-regulate proliferative T cell responses and to up-regulate the production of IL-10 by splenocytes in vitro by hitherto unknown mechanisms (13). In addition, *B. malayi* cystatin inhibits the Ag presentation of human B lymphocyte lines (14). These examples show that nematode components have the potential to interfere with regulatory host immune responses very much like certain pathogenicity factors described from bacterial and protozoan infections (15, 16).

Filarial cystatin of *O. volvulus* (onchocystatin) was described by Lustigman et al. (17, 18), who suggested a role in the regulation of parasite cysteine proteases during molting or hatching of the worms. Such an activity is compatible with the described function of cystatins, which are tight-binding natural protease inhibitors. They represent important regulators of proteolytic processes catalyzed by cysteine proteases (19). Among others, the cystatins have important functions in the regulation of proteases relevant in immune responses. Thus, the endogenous inhibitor cystatin C influences the fate of newly synthesized peptide-MHC class II complexes by regulating the activity of cathepsin S, a cysteine protease that is essential in li-chain degradation in B cells and dendritic cells (20–22). In addition, human cystatin C has been described to inhibit the phagocytic function of monocytes and granulocytes (23, 24), and it has been shown that various members of the cystatin superfamily up-regulate the inducible NO production of murine macrophages (25).

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Received for publication May 8, 2001. Accepted for publication July 24, 2001.

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³This study was supported by a grant from Deutsche Forschungsgemeinschaft (to S.H.).

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These functions of cystatins imply that the role of filarial cystatin is not necessarily restricted to the molting process of the nematodes. This is also underlined by the fact that *A. viteae* cystatin is secreted by nematode stages that do not molt, i.e., the male and female worms and blood microfilariae (13). Indeed, our initial data, obtained in a murine spleen cell model system, show *A. viteae* cystatin to have several immunomodulatory activities, e.g., up-regulation of IL-10 and of NO production by murine spleen cells (13). These data encouraged us to study the immunomodulatory role of filarial cystatin in a homologous system. Using a combination of human peripheral blood cells and recombinant onchocystatin, we could establish that filarial cystatin is a potential pathogenic factor of *O. volvulus* in humans.

Materials and Methods

Cloning and expression of recombinant proteins

The cDNA of the mature 17-kDa onchocystatin (Ov17),\(^4\) was cloned by PCR from a Uni-ZAP XR cDNA library of infective larvae of *O. volvulus* (provided by S. Williams, Smith College, Northampton, MA). The cDNA was amplified by PCR with degenerate primers derived from the internal sequence of onchocystatin. Forward primer, gttcagttgcaaggagcc; reverse primer, tcatacttcttttgttccc. PCR amplification yielded a 423-bp fragment which was cloned into a TOverhang vector (pGEM-T Easy Vector System; Promega, Madison, WI). The recombinant protein was termed rOv17 (Fig. 1A). The cDNA was subcloned into the EcoRI site of an expression vector yielding polypeptides with a six-histidine tag (pET System; Novagen, Madison, WI). The recombinant plasmid was transformed into BL21 (DE3) or BL21 (DE3) pLyS-competent Escherichia coli and screened for expression by SDS-PAGE. rOv17 was purified under nondenaturing conditions by affinity chromatography using Ni-NTA resin (Qiagen, Hilden, Germany) and dialyzed twice against PBS. The control protein, recombinant 33-kDa onchocystatin (rOv33), an O. volvulus protein with homologies to aspartic protease inhibitors (26), was expressed and purified under identical conditions.

Because rOv17 and rOv33 were expressed in *E. coli*, the endotoxin contamination was evaluated by quantitative, chromogenic *Limulus* avene
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lase assay (BioWhittaker, Walkersville, MD). The endotoxin concentration of rOv17 was determined to be 0.04–0.13 pg LPS/0.01 μM protein. The control rOv33 showed endotoxin concentrations of 1.0–2.0 pg/0.01 μM protein.

Human cathepsin inhibition assays

The inhibitory activity of rOv17 toward human cathepsins was characterized by K\(_I\) determination. Human cathepsin B was purchased from Sigma (Deisenhofen, Germany), human cathepsin L was purchased from Calbiochem (La Jolla, CA), and human cathepsin S was supplied by Dr. 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 rOv17 using the fluorogenic substrates Z-Arg-Arg-AMC (40 μM; Bachem, Heidelberg, Germany) for cathepsin B, Z-Phe-Arg-AMC (5 μM; Bachem) for cathepsin L, and Z-Val-Val-Arg-AMC (40 μM; Bachem) for cathepsin S (27, 28). The K\(_I\) values were calculated using the program GraphPad Prism.

Quantification of secreted Ov17 by ELISA

Five female *O. volvulus* worms isolated from patients suffering from *Onchocerca volvulus* infections were cultured individually for 96 h in 1 ml RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The culture supernatants were collected after 24 h. ELISA plates were coated with 50 μl of the culture supernatant of a 24-h culture, mixed with 25 μl 0.1 M carbonate buffer (pH 9.5) and in parallel with graded concentrations of rOv17. A 1:5000 dilution of a monospecific rabbit serum against rOv17 was reacted with the proteins. The amount of bound Abs was quantified with a peroxidase-labeled goat anti-rabbit Ab diluted 1/10,000 (Dianova, Hamburg, Germany). Values obtained with the preimmune serum were subtracted.

Ag-driven and polyclonally stimulated proliferation of human PBMC

The peripheral mononuclear cell fraction of healthy donors was isolated from citrated venous blood by density gradient sedimentation using Ficoll-Hyphaque (Pharmacia Biotech Products, Freiburg, Germany). Cells were resuspended in very low endotoxin RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS (Biochrom). PBMC (3.5 × 10\(^5\)/well) were cultured in 96-well flat-bottom plates at 37°C and stimulated with 10 IU/ml purified protein derivative (PPD; Chiron Behring, Marburg, Germany), 10 μg/ml PHA (Sigma), or immobilized anti-CD3 Ab (4 μg/ml in a final volume of 50 μl for 2 h; Orthoclone-Okt3; gift from Janssen-Cliag, Neuss, Germany). Recombinant proteins were added at a concentration of 0.1–0.5 μM. Additionally, human PBMC were cultured with LPS (100 pg/ml; E.coli 0127:B8, Sigma) for 72 h. PPD-stimulated PBMC were cultured for 96 h, and polyclonally stimulated PBMC were cultured for 72 h at 37°C.

Proliferation was quantified by [\(^{3}H\)thymidine incorporation during the last 20 h of incubation. For neutralization studies, anti-rOv17 rabbit immune serum (1 μl/well), a rabbit control serum against sporozoites of *Eimeria tenella* (1 μl/well), anti-IL-10 Ab (5 μg/ml, clone CBRS1; gift from Prof. Dr. H. D. Volk, Charité Medical School, Berlin, Germany), anti-TNF-α Ab (5 μg/ml; clone 1825.121; R&D Systems, Wiesbaden-Nordenstadt, Germany), and a mouse IgG1 isotype control Ab (5 μg/ml; R&D Systems) were used. All experiments were performed in triplicate. Viability of the cells in presence of recombinant proteins were controlled by trypan blue exclusion.

Depletion of monocytes (CD14\(^+\) cells) and isolation of purified monocytes from human PBMC

CD14\(^+\) cells were depleted from human PBMC using CD14 MicroBeads and vs of columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of CD14\(^+\) cells was done according to the manufacturers' instructions and resulted in the enrichment of CD14\(^+\) cells of 98–99% as determined by FACS analysis using FITC-labeled anti-human CD14 Ab (10 pg/ml; UCHM-1; Dianova). Furthermore, CD14\(^+\) depletion was estimated by measuring TNF-α production. CD14\(^−\)-depleted cells were stimulated with LPS (5 μg/ml) and TNF-α was quantified after 6 h in the culture supernatant. CD14\(^−\)-depleted cells produced on the average 92 pg/ml TNF-α in comparison with 2308 pg/ml TNF-α produced by nondepleted PBMC.

Purified monocytes were isolated from MNC by counterflow centrifugation using a Beckmann JE-6B elutriator (Beckmann Instruments, Munich, Germany). The monocyte fraction collected consisted of <95% monocytes, as determined by modified Randolph staining and by FACS analysis after staining with anti-CD14 Ab (10 μg/ml; Dianova).

Quantification of cytokines in the cell culture supernatant

Cytokine production was determined in the culture supernatants of unstimulated and stimulated PBMC as well as in purified monocyte cultures cocultured with 0.5 μM recombinant proteins. Culture supernatants were collected after 6, 24, and 48 h of incubation. Cytokines were quantified by ELISA according to the manufacturer’s instructions (TNF-α, IL-2, IL-12p70: OptEIA; BD PharMingen; Hamburg, Germany; IL-10 and IFN-γ: DuoSet, R&D Systems). The assays were performed in triplicates.

Expression of monocyte surface molecules

Unstimulated human PBMC (8 × 10\(^6\)/well) were cultured for 72 h at 37°C in 48-well flat-bottom plates with different concentrations of rOv17 and rOv33 (10–100 nm). The expression of monocyte surface molecules was determined in a final volume of 200 μl using the following Abs for staining: R-PE -labeled anti-human HLA-DR Ab (0.48 μg/ml; clone L243; Becton Dickinson, Heidelberg, Germany); FITC-labeled anti-human CD40 (1 μg/ml; clone 5C3; PharMingen); FITC-labeled anti-human CD80 (1 μg/ml; clone 1825.121; R&D Systems), and PE-labeled anti-human CD86 (1 μg/ml; clone 2331; PharMingen). FITC-labeled mouse IgG1 Ab (1 μg/ml; clone 7D4; Coulter Immunotech, Hamburg, Germany) and PE-labeled mouse IgG2a Ab (4 μg/ml; clone X 39; Becton Dickinson) were used as isotype controls.

FACS analyses of 3 × 10\(^5\) PBMC/measurement were done by using a FACSscan instrument (Becton Dickinson). The monocyte population was identified by its light scatter properties and in addition by staining of CD14 with PE-cyanin 5-labeled anti-human CD14 Ab (0.1 μg/ml; clone RMO52; Coulter Immunotech).

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\(^3\) S. Hartmann, A. Schinenmeyer, B. Sonnenburg, B. Vray, and R. Lucius. 2001. Cystatins of filarial nematodes up-regulate the nitric oxide production of IFN-γ-activated murine macrophages through the production of TNF-α and IL-10. Submitted for publication.

\(^4\) Abbreviations used in this paper: Ov17, 17-kDa onchocystatin; rOv17, recombinant onchocystatin; Ov33, 33-kDa O. volvulus protein; rOv33, recombinant Ov33; PPD, purified protein derivative; E/S, excretory/secretory; TLR, Toll-like receptors.
Statistical analysis

Statistical analysis of T cell proliferation data and expression of the cell surface molecules were performed with the Student t test. Cytokine data were analyzed with the Wilcoxon test. Data were presented as means ± SEM.

Results

Production of rOv17 and inhibition of human cathepsins B, L, and S by rOv17

To investigate the immunomodulatory potential of onchocystatin, a full length mature recombinant protein (rOv17) with an N-terminal six-histidine tag was produced in E. coli. Affinity-purified rOv17 was tested for its capacity to inhibit the activity of the human cysteine proteases cathepsins B, L, and S. These proteases are involved in the proteolytic processing of Ag and in Ig chain degradation (29, 30). Ki determinations showed that rOv17 strongly inhibited the activity of human cathepsin L (K_i = 0.038 nM) and cathepsin S (K_i = 0.033 nM), whereas the activity of human cathepsin B was less efficiently inhibited by rOv17 (K_i = 494 nM). Therefore, onchocystatin is an inhibitor of immunologically relevant human cysteine proteases. Consequently, the inhibitor could theoretically interfere with Ag processing and Ig chain degradation of APC. The inhibitory domains of the parasite protein are likely to correspond to the structures of human cystatin; as a sequence alignment shows the typical evolutionary conserved motifs of cysteine protease inhibitors, which bind and inhibit the protease. The N-terminal inhibitory domain reaches from aa 51–53, the central domain from aa 98–102, and the C-terminal domain from aa 153–154. In contrast, most of the other regions vary considerably between onchocystatin and human cystatins, such that the overall amino acid identity of the proteins was comparatively low with values between 18 and 22%. As a particular element, onchocystatin contains a stretch of amino acids (aa 37–48) which is lacking in the human cystatins. This stretch does not show any homologies to known sequences.

Quantification of Ov17 secretion by female O. volvulus worms

The cDNA of onchocystatin encodes for a hydrophobic leader sequence which is typical for secreted proteins. Therefore, we evaluated whether onchocystatin is secreted by O. volvulus worms in vitro. To this end, the quantity of onchocystatin in the culture supernatants of intact individual female worms was determined by a calibrated ELISA. A monospecific rabbit serum raised against recombinant Ov17 detected a release of 10–40 ng onchocystatin per female worm in 24 h, suggesting that onchocystatin has the potential to interfere with the host immune system in vivo.

Inhibition of polyclonally stimulated and Ag-driven proliferation of human PBMC by rOv17

As one model for polyclonally stimulated T cell responses, we studied the effect of rOv17 on the PHA-induced T cell proliferation. rOv17 inhibited the cellular proliferation in a dose-dependent manner (12–42% inhibition with concentrations between 0.1 and 0.5 μM rOv17), whereas the control protein rOv33 had significantly less activity. The most effective concentration of 0.5 μM was subsequently used to analyze the antiproliferative activity of rOv17 toward PBMC of 10 healthy blood donors. In this concentration, rOv17 inhibited the proliferative response of human PBMC by 39% (p < 0.001; Fig. 1a), whereas the control protein rOv33 induced only a slight inhibition of proliferation (difference between rOv17 and rOv33, p > 0.001). In a further set of experiments, we used immobilized anti-CD3 Ab to stimulate PBMC of 10 blood donors. Again, 0.5 μM rOv17 resulted in a mean inhibition of the anti-CD3 Ab-stimulated proliferation of human PBMC by 42% (p = 0.002; Fig. 1b), whereas the control protein rOv33 had a significantly weaker effect. The specificity of the inhibitory effect of rOv17 was determined using monospecific rabbit Abs against rOv17. Addition of rabbit anti-Ov17 serum to the cultures completely abolished the rOv17-inhibited induction of PHA-stimulated proliferation, whereas a rabbit control serum had no effect (Fig. 1c).

The influence of rOv17 on the Ag-driven proliferation of human PBMC was assessed by stimulation of PBMC of three well-responding healthy blood donors with PPD. Addition of 0.5 μM rOv17 resulted in a significant reduction of the Ag-driven proliferation of the PBMC by a mean of 52% (Fig. 1d). In contrast, an 0.5 μM concentration of the control protein rOv33 did not inhibit the PPD-induced Ag-driven T cell proliferation.

The inhibition of both Ag-driven and polyclonally stimulated T cell proliferative responses suggests that rOv17 interferes with an immune mechanism that is involved in both types of cellular proliferation.

Cytokine production of unstimulated and stimulated PBMC in presence of rOv17

To test whether the rOv17-induced suppression of T cell proliferation is due to cytokines produced by PBMC, we quantified cytokines that mediate activation and regulation of T cell proliferation, such as IL-2, IL-4, TNF-α, IFN-γ, IL-10, and IL-12p40. The amounts of these cytokines in the presence or absence of rOv17 were determined in the culture supernatants of anti-CD3-stimulated PBMC of 10 donors after 48 h. The tests revealed that rOv17 induced a significant increase in IL-10 production (p = 0.002) and a significant decrease in IL-12p40 production (p = 0.025) as well as a diminished IFN-γ production which was, however, not significant (Fig. 2). The control protein rOv33 had significantly weaker effects on these cytokines. The production of IL-2, IL-4, and TNF-α by stimulated PBMC was not significantly affected by rOv17 or rOv33 (data not shown).

In parallel, the amounts of IL-10, IL-12p40, and TNF-α in the presence or absence of rOv17 were determined in the culture supernatants of unstimulated PBMC of five individuals after 6, 24, and 48 h. The tests revealed that onchocystatin induced an initial increase of the proinflammatory cytokine TNF-α at 6 h (p > 0.001). This peak in TNF-α production was followed by a significant increase of the inhibitory cytokine IL-10 (p > 0.001) at 24 h (Fig. 3, a and b) which was also elevated at 48 h. The effect of the control protein rOv33 on both cytokines was significantly lower (Fig. 3). No significant production of IL-12p40 was observed by unstimulated PBMC by rOv17 (data not shown). Thus, the increase of the IL-10 production was the most prominent effect in the cytokine pattern of stimulated and of unstimulated human PBMC exposed to rOv17.

Because IL-10 is mainly produced by monocytes, we exposed purified monocytes to rOv17 and rOv33 and quantified the amount of IL-10 in the culture supernatant. Again, rOv17 induced a significant production of IL-10 at a concentration of 5 nM (medians of three experiments: rOv17, 168 pg/ml, p > 0.05; rOv33, 0 pg/ml). These data demonstrate a direct effect of rOv17 on monocytes and suggest that the measured increase of IL-10 production is due to monocytes.

Expression of monocyte surface molecules in presence of rOv17

Both the Ag-driven and the polyclonally stimulated proliferations of PBMC require costimulatory signals for full T cell activation. Because important costimulatory molecules are expressed by monocytes, we examined the influence of various concentrations of rOv17 on the expression of the costimulatory surface markers of
monocytes CD40, CD80, and CD86. The analysis of CD86 on human monocytes of PBMC of four blood donors revealed that rOv17 reduced the expression of CD86 by 37% ($p < 0.018$) at a concentration of 10 nM (Fig. 4a). An increase in the rOv17 concentration did not significantly enhance this effect. The control protein rOv33 did not significantly affect the CD86 expression (Fig. 4a). The expression of the two other surface markers, CD40 and CD80, which were poorly expressed on monocytes, remained unchanged in the presence of rOv17 (data not shown).

Ag-driven T cell stimulation is also dependent on presentation of Ags in association with MHC II molecules of APC. To assess whether the inhibition of Ag-driven T cell responses by rOv17 acts by interference with the Ag presentation, we quantified the HLA-DR surface molecules of human monocytes of PBMC of four blood donors. In the presence of 10 nM rOv17, the expression of HLA-DR was significantly reduced by 57.9% ($p < 0.0014$), whereas an increase of the concentration to 50 nM rOv17 enhanced this effect (72% reduction in comparison with cells without addition of rOv17; $p < 0.0015$; Fig. 4b). In contrast, the control protein rOv33 did not affect HLA-DR expression (Fig. 4b). Similar to the experiments with PBMC, rOv17 reduced the expression of HLA-DR and, less pronounced, of CD86 on purified monocytes (median reduction, 66% for HLA-DR and 40% for CD86).

Neutralization studies using anti-IL-10 and TNF-α Abs

IL-10 exerts suppressive and deactivating effects on T cells and macrophages, alters the pattern of cytokine production of T cells, and induces changes in the expression of monocyte surface molecules (31–33). To test the role of IL-10 for the observed alterations, we exposed PBMC to rOv17 and neutralized IL-10 by addition of anti-IL-10 Abs. The experiment revealed that addition of anti-IL-10 Abs did not restore the inhibition of anti-CD3-induced proliferation of PBMC ($n = 4$; Fig. 5). This suggests that IL-10 is not a major component of the rOv17-induced inhibition of T cell proliferation.

Because the expression of CD86 and HLA-DR on monocytes was described to be negatively regulated by IL-10, we tested whether IL-10 was the cause of the down-regulation in our situation. Neutralization of IL-10 not only restored the diminished expression of CD86 on human monocytes ($n = 3$) by rOv17 but led to expression of 3-fold higher CD86 levels than those of the medium controls (Fig. 6a). In addition, neutralization of IL-10 restored the diminished expression of HLA-DR on monocytes ($n = 3$) to 72–93% of the control levels (Fig. 6b). Therefore, IL-10 is responsible for the down-regulated expression of CD86 and HLA-DR on human monocytes in this scenario.

Because TNF-α may stimulate T cells and monocytes, triggering among others the production of IL-10 (34), this proinflammatory cytokine could contribute to the effects exerted by rOv17. Neutralization of TNF-α by addition of anti-TNF-α Abs did not restore the diminished cellular proliferation (data not shown). However, addition of anti-TNF-α Abs led to a reduction by 31.2% of the rOv17-induced IL-10 production of unstimulated human PBMC (data not shown), showing that TNF-α accounts...
for a part of the increase in IL-10. Therefore, one could expect that this cytokine indirectly acts on the levels of CD86 and HLA-DR.

Together, these data suggest that the proliferative block induced by cystatin acts independently of TNF-α and IL-10, whereas the expression of CD86 and HLA-DR is influenced by IL-10.

**Determination of the target cell of onchocystatin within the PBMC**

The fact that purified monocytes are IL-10 producers in the presence of rOv17 as well as the observed changes in monocyte surface molecules induced by rOv17 suggested that these cells are involved in the rOv17-induced immunomodulation. To study their role, PBMC depleted of monocytes and complete PBMC were compared with regard to the rOv17-induced inhibition of proliferation. Monocytes were depleted from PBMC by CD14 MicroBeads, resulting in a reduction of CD14 cells to 1–2% of the total PBMC population. Monocyte-depleted PBMC (n = 4) were normalized for the number of lymphocytes, stimulated with PHA, and cocultured with rOv17 and the control protein rOv33, respectively. The PHA-stimulated proliferation of complete PBMC were inhibited by 37% in the presence of 0.5 μM rOv17, whereas the proliferation of monocyte-depleted PBMC was not suppressed in presence of rOv17 (Fig. 7). The control protein rOv33 had a slight, but significantly weaker inhibitory effect on unmanipulated PBMC, which was completely abolished in monocyte-depleted PBMC cultures. These data show that the inhibition of proliferation of PHA-stimulated human PBMC induced by rOv17 is mediated by monocytes.

**Discussion**

This report describes immunomodulatory activities of onchocystatin, a secreted protein of the filaria *O. volvulus*. We show that recombinant onchocystatin inhibits Ag-driven and polyclonally induced T cell-proliferative responses of human PBMC, induces changes of their cytokine expression, and down-regulates the expression of CD86 as well as HLA-DR. The data suggest that onchocystatin could act as a pathogenicity factor of *O. volvulus*, with properties similar to certain bacterial modulins (15, 35), which
modulate host immune responses and enhance the survival and reproduction of the bacteria. Filarial parasites persist within their vertebrate hosts for extended time spans, e.g. up to 14 years as shown for *O. volvulus* (2). This survival within an immunocompetent host might be due to a modulation of the host’s immune system exerted by excretory/secretory (E/S) products of the parasites. Indeed, E/S products of filariae suppressed proliferative lymphocyte responses (6, 8, 13, 36), but despite the possible importance for the understanding of the pathogenicity mechanisms only few components of E/S products were thus far characterized on the molecular level. One of these components is the cystatin protein of the filaria *A. viteae*, an E/S Ag that inhibits proliferative responses and induces changes of the cytokine pattern of murine spleen cells, as shown in an earlier publication (13).

To establish the relevance of this possible pathogenicity factor for a human pathogenic filarial infection and to enlarge our knowledge of the underlying mechanisms, we characterized the immunomodulatory potential of onchocystatin using a recombinant, biologically active cystatin (rOv17) which comprised the full sequence of the mature inhibitor. Models of the protease-inhibitor interaction suggest that three conserved domains of the cystatins mediate the inhibition of papain-like cysteine proteases (37). The N-terminal conserved inhibitory domain has been shown to have a predominant role because it blocks the active site cleft of the protease (38–40), whereas the inhibitory potential of the two other domains is dependent on the respective protease (41, 42). Given that onchocystatin contains all three conserved domains, it is not surprising that rOv17 strongly inhibits human cysteine proteases like cathepsins S and L. These cathepsins have essential functions in the processing of Ags and in Ag presentation. Cathepsin L degrades Ag, and it mediates the final proteolytic steps in Ii chain degradation in thymus epithelial cells (29, 43). Cathepsin S processes Ag and is essential in the completion of Ii chain degradation in dendritic cells and B cells (20, 22). Therefore, it is tempting to speculate that onchocystatin acts through inhibition of immunologically relevant host proteases. Fluorescein-labeled rOv17 is taken up by macrophages (our unpublished data) and could thus reach the cellular compartments where Ag processing and the subsequent steps of Ag presentation by MHC II molecules take place.

The strong inhibition of the Ag-driven T cell proliferation suggests that the target molecule in this situation is a cysteine protease with a role in immune processes. Indeed, synthetic as well as microbial cysteine protease inhibitors have been shown to modulate Ag-specific cellular responses. Katunuma et al. (44) describe a marked suppression of proliferation of primed splenocytes to hepatitis B virus Ag in the presence of two cathepsin B inhibitors, E64 and CA-074. Furthermore, another study by Vidard et al. (45) demonstrates that the cysteine protease inhibitor leupeptin influences the generation of immunogenic OVA peptides, which leads to modulation of immune responses. The target proteases of the above mentioned cysteine protease inhibitors remain to be determined. Moreover, it has been shown that cysteine proteases are responsible for invariant chain degradation in APCs and that the ratio of cystatin C to these cathepsins determines the fate of newly synthesized peptide-MHC class II molecules as well as the differentiation of Th cell subclasses (21, 43). Therefore, cysteine protease inhibitors have the capacity to modulate immune responses, and filarial parasites may use this mechanism to interfere with immune responses of their hosts.

However, our data show that onchocystatin down-regulates not only the Ag-driven cellular proliferation but also the polyclonally
induced T cell proliferation. Using PHA and anti-CD3 Ab as stimuli of human PBMC, we showed that onchocystatin also inhibits T cell proliferation independent of Ag processing and presentation. Polyclonally induced and Ag-driven T cell proliferation have in common that they require cytokines and coreceptors for full T cell activation (46). Our results indicate that the cellular hyporeactivity induced by onchocystatin is accompanied by a modulation of the cytokine production and by alterations of the expression of monocyte surface molecules. One main characteristic was the up-regulation of the IL-10 production of PBMC in the presence of rOv17. IL-10 is a cytokine that shows immunosuppressive effects due to its ability to suppress the production of IL-12 and IFN-γ by PBMC and increased the expression of CD86 and HLA-DR by monocytes. High levels of IL-10 production by Ag-stimulated or by unstimulated PBMC have also been described in lymphatic filariasis. Mahanty et al. (47) demonstrated increased IL-10 values that coincided with T cell hyporeactivity in lymphatic filariasis patients. Similarly, experimental studies in mice infected with Brugia pahangi revealed that parasite-reactive Th1 cells are suppressed in vivo by a mechanism that involves IL-10 and the resident APC (48). Another study investigating cellular hyporeactivity of O. volvulus-infected people has shown that both IL-10 and TGF-β mediate cellular hyporeactivity (5). An up-regulation of IL-10 coincident with cellular hyporesponsiveness has also been described for urinary schistosomiasis (49) as well as for Toxoplasma gondii infection in mice (50). Thus, the observed induction of IL-10 by onchocystatin could be an essential feature in the host-parasite interaction, the increased levels of IL-10 leading among others to a polarization of host T cell responses toward Th2/Th3 response, which is characteristic for filarial infections (5, 51, 52). However, in our experimental set-up, IL-10 does clearly not account for the inhibition of proliferative PBMC responses, because neutralization of IL-10 by an anti-IL-10 Ab reversed the production of IFN-γ by PBMC and increased the expression of CD86 and HLA-DR by monocytes. High levels of IL-10 production by Ag-stimulated or by unstimulated PBMC have also been described in lymphatic filariasis. Mahanty et al. (47) demonstrated increased IL-10 values that coincided with T cell hyporeactivity in lymphatic filariasis patients. Similarly, experimental studies in mice infected with Brugia pahangi revealed that parasite-reactive Th1 cells are suppressed in vivo by a mechanism that involves IL-10 and the resident APC (48). Another study investigating cellular hyporeactivity of O. volvulus-infected people has shown that both IL-10 and TGF-β mediate cellular hyporeactivity (5). An up-regulation of IL-10 coincident with cellular hyporesponsiveness has also been described for urinary schistosomiasis (49) as well as for Toxoplasma gondii infection in mice (50). Thus, the observed induction of IL-10 by onchocystatin could be an essential feature in the host-parasite interaction, the increased levels of IL-10 leading among others to a polarization of host T cell responses toward Th2/Th3 response, which is characteristic for filarial infections (5, 51, 52). However, in our experimental set-up, IL-10 does clearly not account for the inhibition of proliferative PBMC responses, because neutralization of IL-10 did not abrogate the inhibition of proliferative responses. Other, IL-10-independent mechanisms, like the contact-dependent macrophage-mediated cell cycle arrest exerted by filarial parasites (53), could account for this effect.

Furthermore, this study determines that the target cells of rOv17-induced down-regulation of proliferation are monocytes. Depletion of monocytes from the PBMC reversed the inhibitory effects on the cellular proliferation induced by onchocystatin. Recently, macrophages were shown to be important regulatory cells by which filariae inhibit cellular proliferation (53). Our study is in line with these findings. In addition, this report characterizes for the first time a single filarial protein that is responsible for the modulation of macrophage functions observed in infections with filariae. Modulation of monocyte functions is not restricted to filarial cystatin but seems to be an intrinsic feature of these proteins. Leung-Tack et al. (24) reports that human cystatin C, a protein constitutively secreted by mononuclear phagocytes (54, 55), interferes with the phagocytic function and the oxidative burst of monocytes via a bioactive peptide that is released during the interaction of the inhibitor with a cysteine protease. Furthermore, it

![Figure 6](http://www.jimmunol.org/) Expression of CD86 and HLA-DR on human monocytes in the presence of recombinant onchocystatin and anti-IL-10 Ab. PBMC of four blood donors were incubated with various concentrations of rOv17 or the control protein rOv33 and 5 μg/ml anti-IL-10 Ab. Expression of CD86 (a) and HLA-DR (b) on monocytes was determined by FACScan. Data are presented in percent expression in comparison with unstimulated control cells. The monocyte population was identified by its light scatter properties and by CD14 staining.

![Figure 7](http://www.jimmunol.org/) T cell proliferation of monocyte-depleted PBMC in the presence of recombinant onchocystatin. Monocyte-depleted PBMC and complete PBMC of four blood donors were stimulated with PHA in the presence of 0.5 μM rOv17 or of the control protein rOv33. Proliferation in presence of PHA corresponds to 100% proliferation.
has been shown that members of the three subgroups of the cystatin superfamily up-regulate the inducible NO production of murine macrophages (25). It is interesting that NO is a potent inhibitor of murine T cell proliferation (56). Moreover, it has been shown that cystatin C limits acute inflammatory reactions due to cysteine proteases released during inflammation. This is compatible with the fact that cystatin C is down-regulated in chronic inflammatory reactions that are dominated by mononuclear phagocytes and lead to tissue pathology (54). Therefore, cystatins can be considered regulatory proteins, which can modulate monocyte activities in addition to its protease-inhibitory function. From this perspective, it is conceivable that filariae interfere with regulatory functions of host macrophages by releasing cystatin.

The receptor on the target cell by which rOv17 mediates the immunomodulations is thus far unclear. The early increase of TNF-α with a subsequent decrease and rising of the IL-10 production by PBMC after contact with rOv17, as well as the modulation of monocyte surface molecules, is reminiscent to the reaction of cells to bacterial endotoxins LPS (57, 58). Our controls show that the observed effects are not due to LPS, because the rOv33 control protein, which contained higher levels of LPS than rOv17, did not induce similar effects. In addition, 10-fold higher quantities of free LPS than contained in rOv17 preparations did not induce the changes observed for rOv17. Because it is not LPS that leads to changes in the cross-regulation of T cell responses, with a resulting predominance of Th2/Th3-like responses. Furthermore, the inhibition of T cell proliferative responses by rOv17, which is independent of IL-10, could account for the cellular hyporesponsiveness observed in filarial infections. It is likely that other mechanisms, e.g., the release of phosphorylcholine-containing proteins (8) and of cytokine homologues (10, 11), contribute to immunomodulation exerted by filarial E/S products. Further studies will have to determine the in vivo role of these molecules and focus on the identification of the cell ligand and the molecular mechanisms of modulation by Onchocerca volvulus antigen.

References


