

# Cystatins of filarial nematodes up-regulate the nitric oxide production of interferon- $\gamma$ -activated murine macrophages

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## SUMMARY

*Cystatins of two filarial nematodes were studied with regard to their capacity to up-regulate the production of nitric oxide (NO) in vitro, and the effects were analysed. Recombinant cystatin of the human pathogenic filaria Onchocerca volvulus and of the rodent filaria Acanthocheilonema viteae significantly enhanced the NO production of interferon (IFN)- $\gamma$ -activated macrophages of BALB/c and C3H/HeJ mice. Truncated cystatins lacking the N-terminal protease inhibitory active site, and showing marginal protease inhibitory activity, up-regulated the NO production to the same extent as the full-length proteins, indicating that the effect on the NO production is independent of cysteine protease inhibition. NO did not contribute to the suppression of proliferative T cell responses exerted by filarial cystatins, as shown in other studies, since NO synthase inhibitors did not restore proliferative responses. The up-regulation of NO production induced by filarial cystatins was partly dependent on the production of interleukin-10 and tumour necrosis factor- $\alpha$ , since depletion of both cytokines by antibodies led to a diminution of the enhanced NO production by 22–48%. Our data suggest that filarial cystatins are potent triggers of the production of NO, a mediator which was shown to have a role as an effector molecule against filarial worms in vitro and in vivo.*

**Keywords** *filariae, cysteine protease inhibitor, cystatin, inducible nitric oxide synthase, interleukin-10, tumour necrosis factor- $\alpha$*

## INTRODUCTION

Filarial infections are characterized by marked cellular hyporesponsiveness of their hosts, an immune evasion mechanism which is considered to contribute to the longevity of the worms, with life spans of more than 10 years (1). Recombinant filarial cystatins of the human pathogenic filaria *Onchocerca volvulus* and of the rodent filaria *Acanthocheilonema viteae* were shown to down-regulate host immune responses (2,3). Both proteins interfere with proliferation of T cells induced by unspecific or antigen specific stimuli, up-regulate the production of tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-10 by human peripheral blood mononuclear cells (PBMC) and murine spleen cells, and down-regulate costimulatory signals on human monocytes (3). The underlying mechanisms leading to these reactions are hitherto poorly understood.

Cystatins are natural and specific inhibitors of the proteolytic activity of the widely distributed cysteine endo- and exoproteases. Based on the amino acid sequence, the cystatin superfamily can be assigned to three major families (4). The filarial cystatins, which are secreted by all parasite stages dwelling the mammalian host, show amino acid homologies to family 2 of the cystatin superfamily. Although the overall homology is quite low, the inhibitory and binding domains of the parasite molecules show the typical conserved motifs of cysteine protease inhibitors. Interestingly, members of all three cystatin families were shown to up-regulate the release of nitric oxide (NO) from interferon (IFN)- $\gamma$  activated macrophages (5). Therefore, we were interested to study whether filarial cystatins also exert an effect on the inducible NO production of murine macrophages. NO, a short living molecule with diverse biological roles was, amongst others, shown to be a major defence molecule of immune cells with effects on protozoans and helminth parasites. On the other hand, NO has been shown to induce a strong inhibition of lymphocyte proliferation *in vitro* and to regulate cytokine gene expression in various cell

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types (6–8). NO is synthesized by macrophages from L-arginine by NO-synthase (NOS) (9,10). The inducible NOS isoform (iNOS or NOS2) is induced by IFN- $\gamma$  and leads to rapid production of large amounts of NO (11).

NO has been identified as a major effector molecule produced by activated macrophages (12). For parasitic protozoa, NO production by macrophages usually results in cytoostasis, reduced growth and inhibited invasiveness of parasites (13) and was shown to regulate the innate immune response to protozoan parasites (14). The up-regulation of NO by application of chicken cystatin was potent enough to cure mice from fatal visceral leishmaniasis (15). In filarial infections, NO released by activated macrophages was described to exert cytotoxic effects on worms *in vitro*. Adult worms and microfilariae of *Brugia malayi*, *Onchocerca lienalis* and *Litomosoides sigmodontis* were shown to be substantially affected by NO released from IFN- $\gamma$  activated macrophages (16–18), although this effect could not be shown *in vivo* (18). Treatment of immunocompetent, non-permissive mice with an inhibitor of NO synthase abrogated resistance to *B. malayi* (19), suggesting a major role for NO as antifilarial effector molecule. However, the same group has recently shown that NO is not an obligate requirement for the elimination of *B. malayi* in mice (20). In contrast, NO in parasitic infections has also been associated with suppression of T cell proliferation (21–23), suggesting that NO might have opposite effects in different situations. Regardless of the role of NO in filarial infections, the trigger leading to NO production was so far not known. In the present study, we investigated the effect of two recombinant filarial cystatins on the NO production of murine macrophages, established that filarial cystatins are potent inducers of NO and examined the role of NO in the immunomodulation exerted by these proteins.

## MATERIALS AND METHODS

### Expression of rAv17 and rOv17, the truncated filarial cystatins and control proteins

The cDNAs of *A. viteae*-cystatin (Av17) (2) and of *O. volvulus*-cystatin (Ov17) (3,24) without the sequence encoding for the signal peptide were amplified by polymerase chain reaction (PCR) using primers (Ov17: forward primer: 5'-GTTTCAGTTGCAAGGAGCC-3', reverse primer: 5'-TCATACTTCTTTTGTTC-3'; Av17: forward primer: 5'-GTTTTGGTGCCTGTGAA-3', reverse primer: 5'-Tcacactgatgagactac-3') derived from the full-length sequences. In parallel, cDNAs of a truncated form of Av17, lacking 23 N-terminal AA and of Ov17, lacking 33 N-terminal AA, were produced by amplification with primers (Ov17: forward primer: 5'-TGGGAAGATCGCGATCCA-

3': Av17: forward primer 5'-TGGCAGGAACGCAATCCG-3'). Both truncated proteins were designed to start downstream from of the N-terminal active site. The PCR fragments were cloned into a T-overhang vector (pGEM-T, Easy Vector Systems; Promega, Madison, WI, USA) and further subcloned into the *EcoRI* site of an expression vector yielding polypeptides with a leader of 6 histidines (pET-28 System; Novagen, Madison, WI, USA). Similarly, the cDNAs of two control proteins [a 33-kDa protein of *O. volvulus* (Ov33) (25), mouse dehydrofolate-reductase (DHFR)] were subcloned into the same expression vector. The plasmids were transformed into competent *Escherichia coli* BL21 cells. Screening of transformants for expression was carried out by analysis of bacterial protein after induction with isopropylgalactoside. The cell pellet of a 1.5-ml culture was resuspended in 50  $\mu$ l sample buffer and analysed by SDS-PAGE, followed by staining with Coomassie blue. Recombinant Av17 (rAv17), recombinant Ov17 (rOv17), the truncated recombinant proteins (trAv17, trOv17) and recombinant DHFR (rDHFR) were purified from the *E. coli* lysate by affinity chromatography on a Ni<sup>2+</sup>-NTA resin under non-denaturing conditions according to the manufacturer's instructions (Novagen). The affinity purified fractions were pooled and dialysed twice against phosphate-buffered saline (PBS) for 48 h. Recombinant Ov33 (rOv33) was purified from the *E. coli* lysate under denaturing conditions and subsequently dialysed against PBS, pH 7.4.

### Cysteine protease inhibitor assay

The inhibitory activity of rOv17, rAv17, trOv17 and trAv17 (0.1–5 nM) were tested in a colourimetric enzyme assay as described by Anastasi *et al.* (26). The activity of papain (20  $\mu$ l of an 15-nM solution; Sigma, München, Germany) was assayed with the substrate Z-Phe-Arg-NMec (20  $\mu$ M, Bachem, Bubendorf, Switzerland).

### Quantification of nitrite in the culture supernatant of macrophages

Resident peritoneal macrophages were harvested by washing the peritoneal cavity of male BALB/c, C3H/HeJ or C3H/HeN mice (Charles River, Sulzfeld, Germany) with chilled Hank's balanced salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS, Gibco/BRL, Eggenstein, Germany). Cells were pooled, depleted of erythrocytes by NH<sub>4</sub>Cl lysis, washed and resuspended in complete RPMI-1640 medium (Gibco/BRL) supplemented with 5% fetal calf serum (FCS), penicillin (100 U/ml, Gibco/BRL), streptomycin (100  $\mu$ g/ml, Gibco/BRL) and 2 mM L-glutamine (Gibco/BRL). Cells were counted in a trypan blue solution (Gibco/BRL), plated

in 96-well, flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) at a density of  $2 \times 10^5$  cells per well and allowed to adhere to the microtitre plate for 2 h at standard cell culture conditions. Non-adherent cells were removed by washing with complete RPMI. The macrophages were activated with recombinant murine (rm) IFN- $\gamma$  (100 U/ml, Reptech, NJ, USA). The activated cells were incubated for 24 h in the presence of rAv17, rOv17, trAv17, trOv17 or the control proteins rDHFR or rOv33 in a final volume of 300  $\mu$ l. Each protein was used at a concentration of 3.3  $\mu$ g/ml. Anti-mouse TNF- $\alpha$  mAb (clone MP6-XT3, Becton Dickinson, Heidelberg, Germany), anti-mouse IL-10 mAb (clone JES5-2A5, Becton Dickinson) as well as the isotype matched controls (clone A95-1 and clone R3-34, Becton Dickinson) were added at concentrations of 1–6  $\mu$ g/ml. NO production was assayed by measuring nitrite, its stable degradation product, by the Griess reaction. The Griess reaction was prepared according to Green *et al.* (27). Briefly, 100  $\mu$ l aliquots of the cell culture supernatant were mixed with an equal volume of Griess reagent [1% sulfanilamide (Sigma), 0.1% naphthylethylene diamine dihydrochloride (Sigma), 2%  $H_3PO_4$  (Merck, Darmstadt, Germany)] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated microplate reader (Dynatech MR7000).  $NO_2^-$  was quantified using 100  $\mu$ l  $NaNO_2^-$  of different concentrations as a standard. The specificity of NO production was examined by addition of 0.5 mM  $N^G$ -monomethyl-L-arginine (L-NMMA; Sigma) and 125  $\mu$ M L-N<sup>6</sup>-(1 iminoethyl)-lysine hydrochloride (L-NIL; Alexis, San Diego, CA, USA). The endotoxin concentration of the *E. coli* derived proteins was quantified using the limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). Addition of polymixin B (10 U/ml or 100 U/ml, respectively; Gibco/BRL) served to test for LPS-induced effects on macrophages.

Experiments were performed in triplicate and data are presented as mean  $\pm$  SD are presented. Each experiment shown is representative of at least three experiments.

### T cell proliferation assay

Spleens of male BALB/c mice were removed aseptically, minced and passed through a stainless steel mesh to obtain single cell suspensions. The spleen cells were depleted of erythrocytes using  $NH_4Cl$  lysis, washed and resuspended in complete RPMI.  $3.5 \times 10^5$  spleen cells per well were plated in 96-well, flat-bottom culture tissue plates and lymphocyte proliferation was induced with concanavalin A (ConA, 2  $\mu$ g/ml, Sigma) or phytohemagglutinin (PHA, 120  $\mu$ g/ml, Sigma). Concentrations of 2.5  $\mu$ g/ml and 5  $\mu$ g/ml of rAv17, rOv17, rDHFR and rOv33 were added to the mitogen-stimulated spleen cells. The NO synthase inhibitors, L-NMMA and L-

NIL were added in concentrations of 0.5 mM and 125  $\mu$ M, respectively. The final volume of each well was 200  $\mu$ l and experiments were performed in triplicate. Synthesis of DNA was estimated by incorporation of  $^3H$ -thymidine (1  $\mu$ Ci/well; Amersham Pharmacia Biotech, Ghent, Belgium) which was added during the last 20 h of a 72-h culture. Experiments were performed in triplicate. A single experiment shown is at least representative of two other experiments.

### Statistical analysis

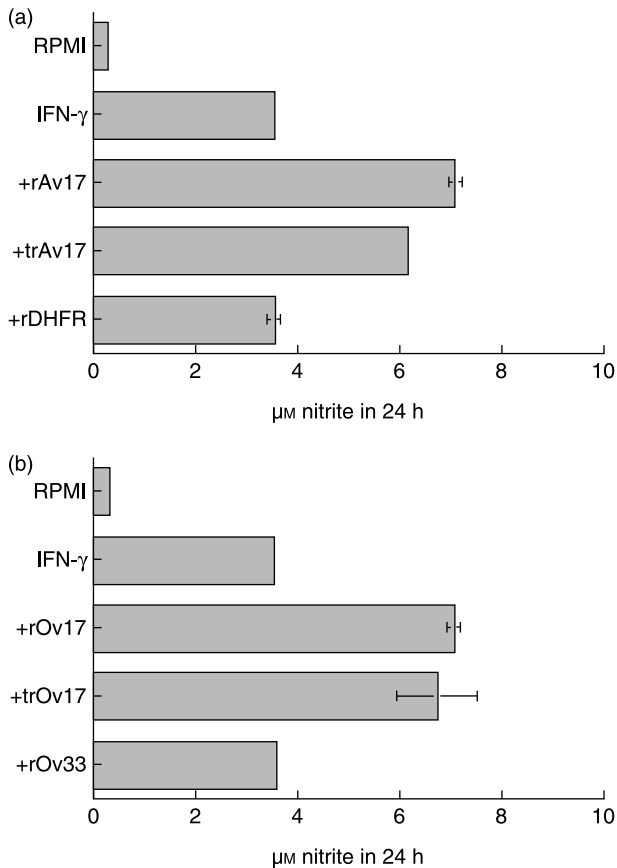
Statistical analysis of NO production and T cell proliferation data were performed using the Wilcoxin test.

## RESULTS

### NO-production of macrophages in the presence of the filarial cystatins

Peritoneal macrophages of BALB/c mice were stimulated with IFN- $\gamma$  (100 U/ml) for 24 h and the NO-production was measured by quantification of the stable degradation product nitrite in the culture supernatant. The 24-h culture supernatant of IFN- $\gamma$ -activated macrophages of BALB/c mice contained  $3.5 \pm 0.05$   $\mu$ M nitrite, whereas no nitrite was detected in the supernatants of nonactivated cells. The addition of rAv17 or rOv17 (3.3  $\mu$ g/ml) to IFN- $\gamma$ -activated macrophages significantly enhanced the nitrite concentration in the culture supernatants (rAv17:  $7.0 \pm 0.12$   $\mu$ M,  $P = 0.05$ ; rOv17:  $7.0 \pm 0.17$   $\mu$ M,  $P = 0.05$ ). In contrast, addition of the same amounts of control proteins had no effect on the inducible NO production of activated macrophages (rDHFR:  $3.5 \pm 0.12$   $\mu$ M nitrite; rOv33:  $3.6 \pm 0.05$   $\mu$ M nitrite) (Figure 1). Titration of the recombinant cystatins showed that 3.3  $\mu$ g/ml was an effective concentration. However, a dose dependent enhancement of the NO production was observed in the presence of 0.1–6  $\mu$ g/ml recombinant filarial cystatin but the differences in NO up-regulation between 1 and 6  $\mu$ g/ml were only marginal (data not shown). Incubation of unstimulated murine macrophages with both filarial cystatins did not up-regulate the NO production.

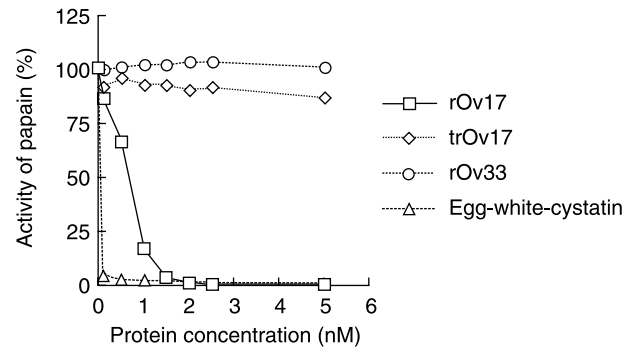
To investigate whether the NO up-regulation in the presence of filarial cystatin was related to the biological activity of the proteins as cysteine protease inhibitors, we attempted to produce biologically inactive truncated recombinant proteins (trAv17, trOv17) lacking the N-terminal protease inhibitory domain. A concentration of 1 nM rOv17 reduced the enzymatic activity of papain by 83%. In contrast, trOv17 (1 nM) led to an inhibition of papain by 9% only (Figure 2). Similarly, rAv17 (1 nM) reduced the enzymatic activity of papain by 94% while trAv17 (1 nM) inhibited the papain activity by 16% (data not shown). 1 nM of the positive



**Figure 1** NO production of IFN- $\gamma$ -activated murine macrophages. Peritoneal macrophages were activated with IFN- $\gamma$  for 24 h and incubated with a constant amount (3.3  $\mu$ g/ml) of rAv17, trAv17, rOv17, trOv17 or a recombinant control protein (rDHFR, rOv33). The NO production of macrophages was determined in the culture supernatant by measuring nitrite. (a) Results obtained with the full-length *A. viteae*-cystatin (rAv17), the biologically inactive truncated *A. viteae*-cystatin (trAv17) and the control protein rDHFR. (b) Results obtained with *O. volvulus*-cystatin (rOv17), the biologically inactive truncated *O. volvulus*-cystatin (trOv17) and the *O. volvulus* control protein rOv33. Data shown are means  $\pm$  SD of triplicate determinations.

control egg white cystatin inhibited papain activity by 98%. Determination of the  $K_i$  value of trOv17 using human cathepsin L and S showed 300–900-fold less inhibitory activity of the truncated molecule in comparison to the full-length molecule (data not shown). Thus, recombinant full-length filarial cystatins are biologically active protease inhibitors compared to the truncated proteins which inhibit papain only marginally. These experiments confirm that the *N*-terminal inhibitory domain is essential for the biological activity of filarial cystatins.

Addition of the recombinant truncated proteins in NO stimulation assays significantly increased the NO production of stimulated macrophages (trAv17:  $6.1 \pm 0.03$   $\mu$ M,



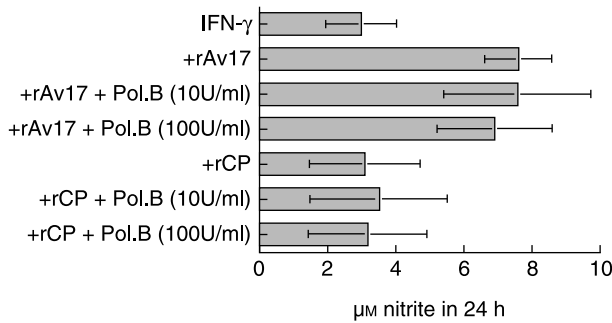
**Figure 2** Cysteine protease inhibitory activity of recombinant *O. volvulus*-cystatin. Various amounts of recombinant *O. volvulus*-cystatin (rOv17) and the truncated molecule (trOv17) or the control proteins (rOv33, egg-white-cystatin) were incubated with 0.32 nM papain. The activity of papain in the presence of *O. volvulus*-cystatin and the controls was determined. Incubation of papain with the substrate and without addition of the recombinant proteins or controls corresponds to 100% activity of the enzyme.

$P = 0.05$  in comparison to the control protein rDHFR; trOv17:  $6.7 \pm 0.8$   $\mu$ M,  $P = 0.05$  in comparison to the control protein rOv33) to a level similar to the that obtained with the full-length proteins (Figure 1). These results clearly show that the *N*-terminal inhibitory domain of the cystatins is not involved in the up-regulation of the inducible NO production of IFN- $\gamma$ -activated macrophages.

To substantiate these results, we tested peptides which represent the evolutionary conserved inhibitory sites of cystatins in the NO assay. The peptides QVVAG and LLGG, as well as the control peptides KPPR and QAPN, did not modify the NO production of activated macrophages (data not shown). In addition, a tripeptide derivative (*Z*-Leu-Val-diazomethylketone) and the synthetic inhibitor E64 (*L*-*trans*-epoxysuccinyl-leu-*agmatine*), which are both irreversible inhibitors of cysteine proteases, did not show an effect on the NO production of murine macrophages (data not shown). These data indicate that the protease inhibitory function of the cystatins is not involved in the up-regulation of NO production by macrophages.

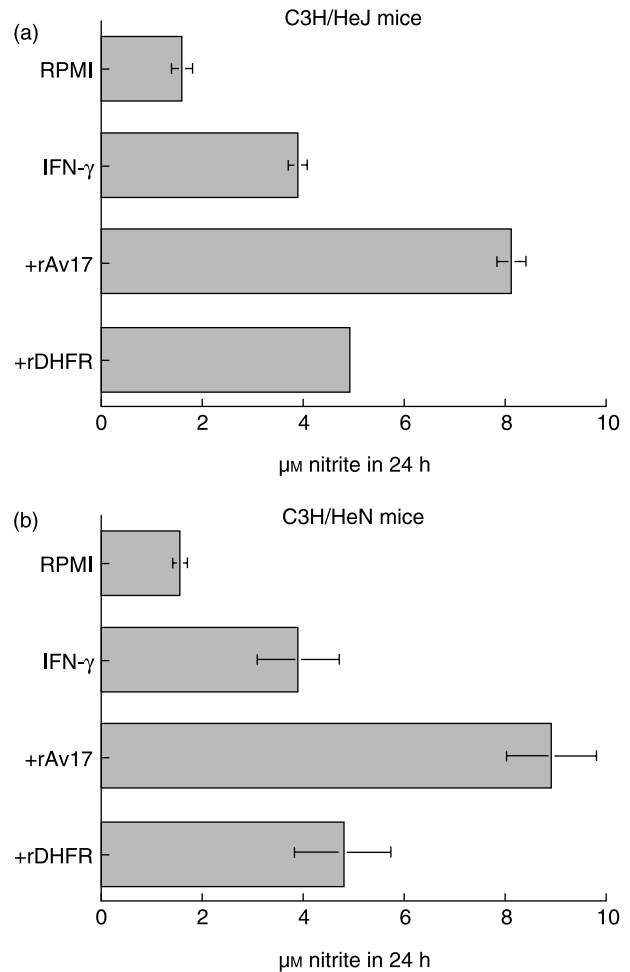
### Exclusion of LPS as cause of NO up-regulation

Bacterial lipopolysaccharide (LPS), a component which can induce NO-production in macrophages, is frequently present in *E. coli*-expressed proteins as a contaminant. We attempted to exclude LPS as a cause of NO-production by several approaches. First, we used two recombinant control proteins which had been expressed and purified with an identical protocol. Quantification of LPS using the limulus amoebocyte lysate assay revealed LPS concentrations between 2 and 5 ng/ml for the filarial cystatins as well as the



**Figure 3** Influence of polymyxin B on NO production of IFN- $\gamma$ -activated murine macrophages. Peritoneal macrophages were activated with IFN- $\gamma$  for 24 h and incubated with a constant amount of rAv17 (3.3  $\mu$ g/ml) or a recombinant control protein (rCP) with or without polymyxin B (10 U/ml, 100 U/ml). The NO production of macrophages was determined in the culture supernatant by measuring nitrite. Data shown are means  $\pm$  SD of triplicate determinations.

control proteins (rDHFR, rOv33). Therefore, the significant differences in NO-production cannot be attributed to high levels of LPS in the rAv17 or rOv17 preparations. Second, addition of the LPS inhibitor polymyxin B (10 U/ml and 100 U/ml) to the cultures did not result in a substantial reduction of the rAv17-induced NO production by macrophages. Exposure of activated macrophages to 3.3  $\mu$ g/ml rAv17 led to a significant production of  $7.6 \pm 1.0$   $\mu$ M ( $P = 0.05$ ) nitrite compared to the control protein ( $3.1 \pm 1.6$  nitrite) (Figure 3). Addition of 10 U/ml polymyxin B did not change the rAv17 values (rAv17:  $7.6 \pm 2.4$   $\mu$ M nitrite) which was significantly different ( $P = 0.1$ ) from the addition of polymyxin B to the control protein (Figure 3). Also, addition of 100 U/ml polymyxin B did not markedly change the nitrite production in the presence of rAv17 ( $6.9 \pm 1.7$   $\mu$ M nitrite) which was significantly different ( $P = 0.05$ ) from the addition of 100 U/ml polymyxin B to the control protein (Figure 3). This results underline that the measured increase of NO-production by filarial cystatins is not due to LPS. Third, we used peritoneal macrophages from C3H/HeJ mice, which poorly respond to LPS (28), in NO stimulation assays. The 24-h culture supernatants of IFN- $\gamma$ -activated macrophages from C3H/HeJ mice contained  $3.9 \pm 0.18$   $\mu$ M nitrite, and addition of rAv17 increased the nitrite concentration to  $8.1 \pm 0.3$   $\mu$ M (Figure 4a). The rAv17 induced increase of NO was significantly different ( $P = 0.05$ ) compared to the recombinant control protein, rDHFR, which did not significantly alter the NO production of macrophages of C3H/HeJ mice ( $4.9 \pm 0.06$   $\mu$ M). Activated macrophages of the LPS-receptive C3H/HeN mice produced  $3.9 \pm 0.8$   $\mu$ M in 24 h. Addition of rAv17 led to an increased production of nitrite ( $8.9 \pm 0.9$   $\mu$ M), which was significantly different from the NO production in the presence of the



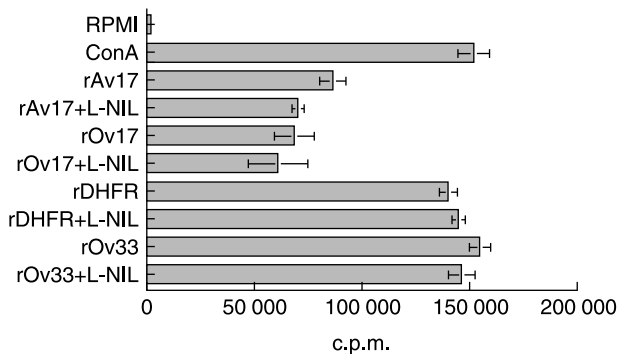
**Figure 4** NO production of IFN- $\gamma$ -activated macrophages from LPS low-responder and LPS-receptive mice. (a) Results obtained with CeH/HeJ mice. (b) Results obtained with C3H/HeN mice. Peritoneal macrophages were activated with IFN- $\gamma$  for 24 h and incubated with a constant amount (3.3  $\mu$ g/ml) of rAv17 or the recombinant control protein (rDHFR). The NO production of macrophages was determined in the culture supernatant by measuring nitrite. Data shown are means  $\pm$  SD of triplicate determinations.

control protein rDHFR ( $4.8 \pm 0.9$   $\mu$ M nitrite,  $P = 0.05$ ) (Figure 4b).

Taken together, these data indicate that the observed up-regulation of the NO production by activated macrophages is not due to LPS contamination of the *E. coli*-expressed filarial cystatins.

#### NO-production induced by filarial cystatins does not suppress T cell proliferation

Previous studies have shown that both filarial cystatins inhibit the proliferation of murine spleen cells and human



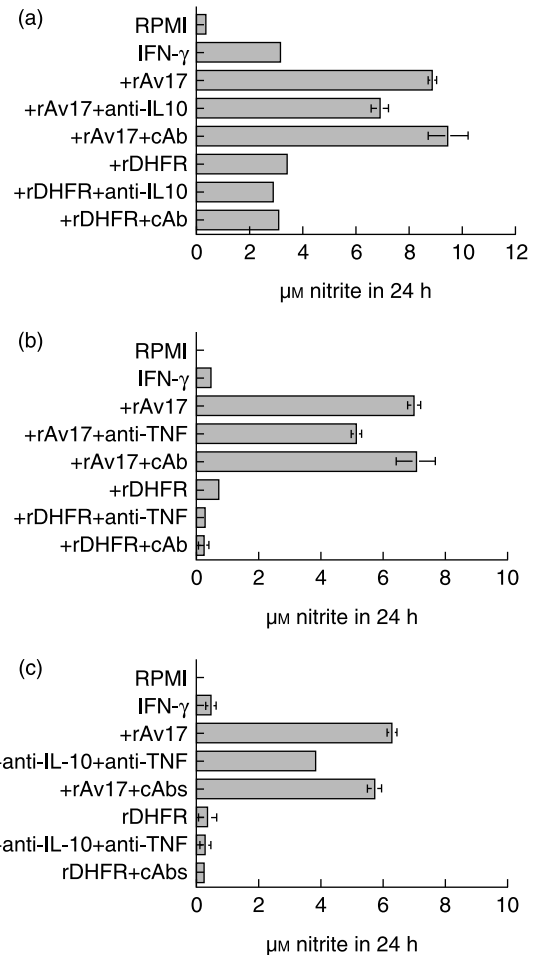
**Figure 5** ConA-stimulated T cell proliferation of BALB/c spleen cells. Cellular proliferation was measured in the presence of a constant amount of rAv17, rOv17 (2.5 µg/ml) or the control proteins (rDHFR, rOv33), with or without the iNOS inhibitor L-NIL (125 µM). Proliferation was quantified by incorporation of <sup>3</sup>H-thymidine. Data shown are means ± SD of triplicate determinations.

PBMC (2,3). Because this effect can theoretically be caused by NO, we studied the relationship between cystatin-induced NO up-regulation of macrophages and T cell hyporeactivity by addition of the iNOS inhibitor L-NIL. As expected, the proliferation of ConA stimulated spleen cells of BALB/c-mice (151451 ± 7399 c.p.m.) was suppressed by addition of 2.5 µg/ml of rAv17 or rOv17 by 44% (85562 ± 6052 c.p.m.,  $P = 0.05$ ) or by 55% (67641 ± 9856 c.p.m.,  $P = 0.05$ ), respectively, but was not suppressed by addition of the control proteins rDHFR or rOv33 (Figure 5). Addition of the iNOS inhibitor L-NIL to the cultures did not reverse the T cell hyporeactivity in the presence of the filarial cystatins (Figure 5). Higher concentrations of rAv17 and rOv17 (5 µg/ml) increased the suppression of the ConA-stimulated proliferation of BALB/c mice by 73% and 76%, respectively. Again, the presence of the iNOS inhibitor L-Nil did not restore the T cell reactivity (rAv17 + L-Nil 69% reduction of cellular proliferation, rOv17 + L-Nil 84% reduction of cellular proliferation).

In another set of experiments, ConA- and PHA-stimulated T cell proliferation in the presence of another specific iNOS inhibitor, L-NMMA, was examined. Again, both filarial cystatins suppressed T cell proliferation significantly, but the T cell proliferation was not restored by addition of L-NMMA (data not shown). These data suggest that the T cell hyporeactivity of polyclonally stimulated spleen cells induced by filarial cystatins is not due to NO.

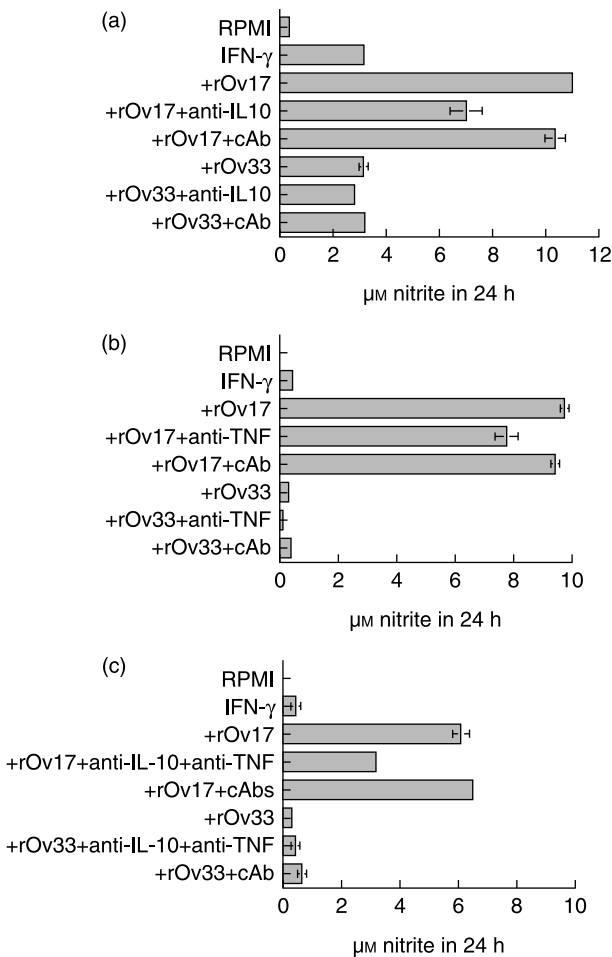
#### The NO up-regulation induced by filarial cystatins is partly mediated by IL-10 and TNF-α

Both recombinant filarial cystatins, rAv17 and rOv17, up-regulate the spontaneous and the mitogen-stimulated



**Figure 6** Influence of anti-IL-10 and anti-TNF-α antibodies on the NO production of IFN-γ-activated murine macrophages in the presence of *A. viteae*-cystatin. Peritoneal macrophages were activated with IFN-γ for 24 h and incubated with a constant amount (3.3 µg/ml) of rAv17 or a recombinant control protein (rDHFR). Anti-IL-10 antibodies, as well as anti-TNF-α antibodies and isotype matched control antibodies, were added at a concentration of 1 µg/ml. The NO production of macrophages was determined in the culture supernatant by measuring nitrite. (a) Results obtained in the presence of anti-IL-10 antibodies. (b) Results obtained in the presence of anti-TNF-α antibodies. (c) Results obtained in the presence of anti-IL-10 and anti-TNF-α antibodies. Data shown are means ± SD of triplicate determinations.

production of IL-10 and TNF-α by PBMC (2,3). To assess, whether these cytokines are involved in the up-regulation of the NO production, the cytokines were depleted by addition of antibodies to the cultures. Addition of anti-IL-10 antibodies (1 µg/ml) to IFN-γ stimulated macrophages diminished the up-regulation of NO in the presence of rAv17 from  $8.8 \pm 0.14$  µM nitrite to  $6.9 \pm 0.29$  µM nitrite (reduction by 22%,  $P = 0.05$ ) compared to an isotype matched control antibody (Figure 6). Up-regulation of NO in the presence of



**Figure 7** Influence of anti-IL-10 and anti-TNF- $\alpha$  antibodies on the NO production of IFN- $\gamma$ -activated murine macrophages in the presence of *O. volvulus*-cystatin. Peritoneal macrophages were activated with IFN- $\gamma$  for 24 h and incubated with a constant amount (3.3  $\mu$ g/ml) of rOv17 or a recombinant control protein (rOv33). Anti-IL-10 antibodies, as well as anti-TNF- $\alpha$  antibodies and isotype matched control antibodies, were added at a concentration of 1  $\mu$ g/ml each. The NO production of macrophages was determined in the culture supernatant by measuring nitrite. (a) Results obtained in the presence of anti-IL-10 antibodies. (b) Results obtained in the presence of anti-TNF- $\alpha$  antibodies. (c) Results obtained in the presence of anti-IL-10 and anti-TNF- $\alpha$  antibodies. Data shown are means  $\pm$  SD of triplicate determinations.

rOv17 was reduced from  $11.0 \pm 0.1$   $\mu$ M nitrite to  $7.0 \pm 0.6$   $\mu$ M nitrite (reduction by 36%,  $P = 0.05$ ) (Figure 7) in the presence of anti-IL-10 antibodies. Addition of anti-TNF- $\alpha$  antibodies (1  $\mu$ g/ml) to the activated macrophages diminished the rOv17-induced NO production from  $6.9 \pm 0.19$   $\mu$ M nitrite to  $5.0 \pm 0.18$   $\mu$ M nitrite (reduction by 29%,  $P = 0.05$ ) compared to an isotype matched control antibody (Figure 6). Up-regulation of NO in the presence of rOv17 was reduced from  $9.7 \mu\text{M} \pm 0.17$  nitrite) to

$7.7 \pm 0.39$   $\mu$ M nitrite (reduction by 24%,  $P = 0.05$ ) (Figure 7) in the presence of anti-TNF- $\alpha$  antibodies.

The simultaneous addition of anti-IL-10 and anti-TNF- $\alpha$  antibodies led to a reduction of the rOv17-induced NO up-regulation from  $6.2 \pm 0.12$   $\mu$ M nitrite to  $3.8 \pm 0.21$   $\mu$ M nitrite (reduction by 39%,  $P = 0.05$ ). The rOv17-induced NO up-regulation was reduced from  $6.1 \pm 0.27$   $\mu$ M nitrite to  $3.2 \pm 0.10$   $\mu$ M nitrite (reduction by 48%,  $P = 0.05$ ) (Figures 6 and 7) in the presence of anti-IL-10 and anti-TNF- $\alpha$  antibodies. These results suggest that IL-10 and TNF- $\alpha$  contribute to the up-regulation of NO-production induced by filarial cystatins.

## DISCUSSION

This study demonstrates that cystatin of the human pathogenic filaria *O. volvulus* and of the rodent filaria *A. viteae* significantly enhance the NO production of IFN- $\gamma$ -activated peritoneal macrophages. Previous studies have shown that other members of the cystatin superfamily (i.e. chicken cystatin, human stefin B and rat T-kininogen) up-regulate the NO production of IFN- $\gamma$ -activated murine macrophages (5). This effect is restricted to natural cysteine protease inhibitors as we have shown that synthetic inhibitors, E 64 and Z-Leu-Val-diazomethylketone failed to increase the NO production. Thus, the filarial cystatins share with other members of the cystatin superfamily the potential to up-regulate the NO production of IFN- $\gamma$ -activated macrophages.

To examine whether the conserved inhibitory active centres (29) of filarial cystatins are involved in the immunomodulatory process, both filarial cystatins were expressed as truncated proteins which lacked the *N*-terminal active site. These truncated proteins did not inhibit the activity of the cysteine protease papain, but up-regulated the NO production to the same extent as the full-length proteins. Furthermore, peptides representing the *N*-terminal active centres did not have an effect on the inducible NO production of macrophages. The NO induction by truncated cystatins is compatible with the observation of Verdote *et al.* (5) who showed that cystatins which are inactivated by complexation with a protease still induce NO. These findings suggest that the protease inhibitory sites of the cystatins are not involved in the induction of NO production by activated macrophages.

As the up-regulation of NO by filarial cystatins is not due to inhibition of a protease, we hypothesized that the recombinant proteins might modulate the expression of cytokines involved in iNOS expression. The inducible NO synthase of macrophages is stimulated primarily by the Th1 cytokine IFN- $\gamma$  (30). However, IL-2 and TNF- $\alpha$  added in combination with IFN- $\gamma$  show a marked cooperative effect on the induction of both iNOS mRNA and enzyme activity (31). Interestingly, Th2 cytokines, such as IL-10, also up-regulate

NO production when added in combination with other stimuli, such as IFN- $\gamma$ , TNF- $\alpha$  or LPS (32–34). The full-length filarial cystatins was shown to strongly up-regulate the production of TNF- $\alpha$  as well as IL-10 (2,3). Therefore, we evaluated the role of TNF- $\alpha$  and IL-10 in the NO up-regulation mediated by filarial cystatins. The addition of anti-TNF- $\alpha$  and anti-IL-10 antibodies to the cultures partly reversed (22–48%) the NO up-regulating effect. Similar observations have been made by Verdot *et al.* (35) who demonstrated that chicken cystatin stimulates NO release from IFN- $\gamma$  activated mouse macrophages via the synthesis of TNF- $\alpha$  and IL-10. A role for TNF- $\alpha$  in the up-regulation of NO by cystatin has also recently been demonstrated by Das *et al.* (15). The authors demonstrated that anti-TNF- $\alpha$  antibodies could effectively block the increase in NO production by macrophages which had been activated with splenocyte supernatant from cystatin-treated mice. Therefore, TNF- $\alpha$  and IL-10 seem to be involved in the up-regulation of NO induced by filarial cystatins as these proteins stimulate the production of TNF- $\alpha$  and IL-10 by a hitherto unknown mechanism leading to the up-regulation of NO production by IFN- $\gamma$ -activated macrophages.

High levels of IL-10 are a characteristic feature found in filarial infections. Interestingly, it was demonstrated that increased IL-10 production of antigen-stimulated or unstimulated PBMC of lymphatic filariasis patients coincides with T cell hyporeactivity (36,37). Similarly, experimental studies in mice infected with *Brugia pahangi* revealed that parasite-reactive Th1 cells are suppressed *in vivo* by a mechanism which involves IL-10 and the resident antigen-presenting cells (38). In this context, it is interesting to note that the proinflammatory cytokine TNF- $\alpha$  triggers, amongst others, the production of IL-10 (39).

Previous studies have shown that filarial cystatins interfere with cellular immune responses of the host. Both filarial cystatins inhibited the proliferation of mouse spleen cells and human PBMC after polyclonal or antigen-specific stimulation (2,3). Therefore, we assessed whether NO is a critical element in this inhibition of cellular proliferation. For this purpose, mitogen-stimulated spleen cells in the presence of both filarial cystatins were coincubated with two specific iNOS inhibitors (L-NMMA and L-NIL). The results shown in this study confirm that the filarial cystatins induce a suppression of proliferation of murine spleen cells. However, this suppression of the polyclonally stimulated cellular proliferation could not be reversed by addition of iNOS inhibitors. Thus, the suppression of T cell proliferation induced by filarial cystatins was, at least in our experimental set up, not mediated by NO, which is a conclusion that is consistent with other published data. Studies with the lymphatic filaria *Brugia malayi* showed that adherent peritoneal exudate cells (PEC) from mice implanted with adult worms or with the

infective larval stage (L3) of *B. malayi* inhibited T cell proliferation but iNOS inhibitors had no effect on the suppression caused by PEC (40,41). Similarly, studies with mice infected with lactate dehydrogenase elevating virus demonstrated that NO production of macrophages was not responsible for T cell suppression, although spleen macrophages exhibited a six- to 10-fold increased NO production compared to macrophages from uninfected mice (42).

In contrast, several studies with parasite models revealed that NO production by murine macrophages was associated with suppression of T cell reactions during infection. In a murine model of filariasis, BALB/c mice infected with *B. pahangi* had a defective Ag-specific T cell proliferation which was dependent on inducible NO activity (43). Similarly, suppressive peritoneal macrophages isolated from mice infected with *Trypanosoma brucei* exhibited transcriptional up-regulation of iNOS and released significant amounts of NO. The inhibition of NO synthesis completely blocked suppressor cell activity and resulted in recovery of infected cells from immunosuppression (21,44). Abrahamson & Coffman (45) showed that the suppression of proliferative responses in *T. cruzi*-infected mice largely resulted from increased NO production by macrophages. Moreover, the production of NO in hamsters with progressive visceral leishmaniasis was found to be causative for the down-regulation of lymphoproliferative responses (23). Furthermore, the suppression of splenic proliferative responses observed in chronic *Echinococcus multilocularis* infections was dependent on the NO production by macrophages from infected mice (22). These data suggest that, in certain settings, NO production leads to inhibition of T cell reactivity, which can be interpreted as an immune evasion mechanism.

The up-regulation of the inducible NO production by filarial cystatins is dependent on the activation by IFN- $\gamma$  and is concomitant with an inhibition of T cell proliferation. This coincidence raises the question whether an IFN- $\gamma$  dependent mechanism of immune depression is operative in filariasis. Several studies indicate that such mechanisms exist. Splenocytes from *B. pahangi* mf-infected mice produce high levels of IFN- $\gamma$  upon *in vitro* restimulation while the infection leads to suppressed proliferative responses in the spleen. The use of IFN- $\gamma$  receptor deficient mice showed that signalling via the IFN- $\gamma$  receptor is essential in induction of NO as well as the subsequent suppression of proliferative responses (43). Furthermore, PEC from mice implanted with *B. malayi* mf down-regulated the proliferation of a conalbumin-specific T-cell clone in an NO-dependent manner (40). These studies imply a role of an IFN- $\gamma$  dependent mechanism of cellular suppression in filariasis in systems using mf, and not adult worms or L3. However, the IFN- $\gamma$ -dependent NO-up-regulation induced by cystatins demonstrated in this study does not lead to suppression of

mitogen-stimulated proliferative T cell responses. We cannot exclude the existence of a cystatin-induced role of NO on T cell proliferation which is operative in a natural situation of T cell stimulation where cytokines have a more important role.

To further elucidate the *in vivo* role of the filarial cystatin-induced NO production, we investigated the influence of NO on the motility of *A. viteae*-worms. Microfilariae were strongly inhibited in their motility in the presence of *A. viteae*-cystatin and IFN- $\gamma$  stimulated macrophages which correlated with the production of nitrite in the culture supernatant (unpublished observation). In contrast, adult stages and infective larvae showed no cytostatic effect in the presence of *A. viteae*-cystatin. These data coincide with findings of others which describe a susceptibility of microfilariae of *O. lienalis* and *B. malayi* to NO (16,17) and suggest a role of filarial cystatin in the regulation of microfilariae density *in vivo*.

NO is as an antifilarial effector molecule (16–19) and is associated with suppression of the Ag-specific T cell proliferation in a murine model of filariasis (40,43). This study identifies filarial cystatin as a possible trigger of NO-production and might therefore help to elucidate the complex host–parasite interactions leading to resistance or immune suppression in filarial infections.

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