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Successful Therapy of Lethal Murine Visceral Leishmaniasis with Cystatin Involves Up-Regulation of Nitric Oxide and a Favorable T Cell Response[1]

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The virulence of *Leishmania donovani* in mammals depends at least in part on cysteine proteases because they play a key role in CD4$^+$ T cell differentiation. A 6-fold increase in NO production was observed with 0.5 μM chicken cystatin, a natural cysteine protease inhibitor, in IFN-γ-activated macrophages. In a 45-day BALB/c mouse model of visceral leishmaniasis, complete elimination of spleen parasite burden was achieved by cystatin in synergistic activation with a suboptimal dose of IFN-γ. In contrast to the case with promastigotes, cystatin and IFN-γ inhibited the growth of amastigotes in macrophages. Although in vitro cystatin treatment of macrophages did not induce any NO generation, significantly enhanced amounts of NO were generated by macrophages of cystatin-treated animals. Their splenocytes secreted soluble factors required for the induction of NO biosynthesis, and the increased NO production was paralleled by a concomitant increase in antileishmanial activity. Moreover, splenocyte supernatants treated with anti-IFN-γ or anti-TNF-α Abs suppressed inducible NO generation, whereas i.v. administration of these anticytokine Abs along with combined therapy reversed protection against infection. mRNA expression and flow cytometric analysis of infected spleen cells suggested that cystatin and IFN-γ treatment, in addition to greatly reducing parasite numbers, resulted in reduced levels of IL-4 but increased levels of IL-12 and inducible NO synthase. Not only was this treatment curative when administered 15 days postinfection, but it also imparted resistance to reinfection. These studies provide a promising alternative for protection against leishmaniasis with a switch of CD4$^+$ differentiation from Th2 to Th1, indicative of long-term resistance. ***The Journal of Immunology*****, 2001, 166: 4020–4028.**

Infection with the flagellated protozoan *Leishmania* is a major health problem with significant morbidity and mortality in the tropics and subtropics. Over 350 million people live in areas where the disease is common, and large epidemics affecting hundreds of thousands have occurred as recently as 1991 (1). *Leishmania donovani*, the etiological agent for the severe visceral form of leishmaniasis known as kala azar in humans, multiplies in the phagolysosomes of macrophages of the infected host. At present, there is no satisfactory, widely available vaccine against leishmaniasis, and chemotherapy remains the major medical mode of managing the disease. However, the existing drugs used against leishmaniasis, such as antimonials, pentamidine, and amphotericin B, are highly toxic, have serious side effects, and elicit drug resistance (2). Compounding these problems is the fact that many countries and regions where the disease is endemic are economically poor. Limitations of this nature have undoubtedly necessitated the current drive to develop and produce effective therapy against all forms of leishmanial infection, particularly the fatal visceral form.

An attractive target for new therapy is a family of cathepsin L-like and cathepsin B-like cysteine proteases, found in all species of *Leishmania* examined and required for parasite growth and virulence (3, 4). Elimination of cathepsin L-like gene families by homologous recombination resulted in loss of virulence in highly susceptible BALB/c mice (3, 4), whereas deletion of the cathepsin B-like gene led to reduced survival of parasites in macrophages (3, 5). Therefore, it was thought worthwhile to investigate the possible role of cystatin, a natural cysteine protease inhibitor, in modulating *L. donovani* infection. However, one major complicating factor in chemotherapeutic treatment is the depressed immune functions exhibited by the victims of disseminated leishmaniasis. Appropriate T cell-mediated responses are of primary importance in an effective host defense in visceral leishmaniasis (6). There is also a correlation between host control over parasite replication and the activation of Th1-type effector cells that produce the macrophage-activating cytokines IFN-γ and IL-2 (7). Both in humans (8, 9) and in experimental animal models (10, 11), *L. donovani* infection is accompanied by parasite-specific immune depression mediated by T cells and macrophages, thereby preventing spontaneous cure and the development of protective immunity. Moreover, nonhealing infections in susceptible strains of mice such as BALB/c are accompanied by the preferential expansion of IL-4-producing Th2-type cells (12). Therefore, immunostimulation of the infected host is an effective strategy for circumventing immunosuppression. IFN-γ could be used as an immunopotentiator for augmenting the capacity of macrophages to eliminate *Leishmania* infection. Moreover, the safety of parenteral human rIFN-γ has been demonstrated for various diseases, including leprosy, cancer, and AIDS (13–15). However, IFN-γ treatment alone is not sufficient to promote a Th1 response and/or suppress in vivo activation of Th2 cells because even continued IFN-γ therapy has little effect on the eventual outcome of a *Leishmania major* infection in BALB/c mice (16).

Studies to date suggest that the parasite cysteine proteases may themselves help to ensure a Th2-like response in BALB/c mice

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that leads to parasite proliferation (17). Thus, inhibition of such cysteine proteases might slow or even prevent parasite proliferation and allow the host immune system to function effectively and confer protective immunity by effecting a switch in $CD4^+$ T cell differentiation from Th2 to Th1 (18). Tight binding and reversible natural inhibitors that belong to the cystatin superfamily regulate cysteine proteases. Experiments have revealed that chicken cystatin, the best-characterized inhibitor of cysteine proteases (19, 20), stimulated NO (3) production by IFN-γ-activated macrophages (21). Because NO is the key effector molecule for antileishmanial activity, we tested the capacity of cystatin to elicit a Th1-mediated adaptive response and to prevent and treat infections with *L. donovani*, a lethal Th2-mediated disease, in BALB/c mice. Our investigation was aimed toward elucidating the dual role of cystatin in suppressing the functional differentiation of Th2-type $CD4^+$ T cells and in turn augmenting Th1 response, together with the ability to up-regulate NO, the latter property being totally unrelated to the former. Here we present data demonstrating that cystatin can synergize with subthreshold concentrations of IFN-γ in inducing Th2-Th1 conversion and generation of NO, resulting in abrogation of parasite infection.

Materials and Methods

Parasites and Ag

L. donovani strain AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with kala azar (22). The strain was maintained in BALB/c mice by i.v. passage every 6 wk. *L. donovani* promastigotes for use in experiments were obtained by allowing isolated splenic amastigotes to transform in parasite growth medium for 72 h at 22°C. The growth medium consisted of medium 199 (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) FCS. Soluble leishmanial Ag (SLA)[3] was prepared from promastigotes by freeze-thawing the cell suspension (5×10^9 cells/ml in 100 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 50 μg/ml leupeptin, and 1.6 mM PMSF) three to five times followed by sonication for 5×45 s at 20 kilocycles/min in an ice bath. The contents were centrifuged at $10{,}000 \times g$ for 20 min, and the supernatant was dialyzed, filtered, and stored at −70°C. It was used at a concentration of 20 μg/ml.

Macrophages

Macrophages were collected by peritoneal lavage from mice (BALB/c; 20–25 g) given i.p. injection of 0.5 ml 4% thioglycolate broth 5 days before harvest and were used as described earlier (23). The culture medium consisted of RPMI 1640 supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. A total of >90% of the cell preparation was identified as macrophages by microscopic observation, and the macrophages were routinely found to be >95% viable by trypan blue exclusion.

Splenocyte culture

Spleens were aseptically removed and teased into single-cell suspensions in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (50 μM), L-glutamine (2 μM), HEPES (10 μM), and 10% (v/v) FCS (24). RBCs were removed by lysis with 0.83% (w/v) NH_4Cl. The remaining cells were washed twice with culture medium, and the viable mononuclear cell number was determined by counting trypan blue-unstained cells in a hemocytometer. Splenocyte suspensions (1×10^6 cells/ml) were dispensed into 35-mm tissue culture plates and incubated at 37°C in 5% CO_2 for 48 h. Cells were harvested for RT-PCR and flow cytometric analysis. Culture supernatants were removed and frozen at −20°C until further use.

In vitro L. donovani *proliferation assay*

Promastigotes of *L. donovani* were cultured in medium 199 containing 10% (v/v) FCS with or without chicken cystatin (egg white, $E^{1\%}_{280} = 8.7$; Sigma, St. Louis, MO) and IFN-γ for 72 h at 22°C. The proliferation of promastigotes was evaluated by counting them every 24 h in a hemocytometer.

[3] Abbreviations used in this paper: SLA, soluble leishmanial Ag; iNOS, inducible NO synthase; HPRT, hypoxanthine phosphoribosyl transferase; NMMA, N^G-monomethyl-L-arginine; LDU, Leishman-Donovan units.

In vitro assay of L. donovani *growth in macrophages*

Promastigotes were used to infect cultures of adherent macrophages on glass cover slips (18 mm^2; 5×10^5 macrophages/cover slip) in 0.5 ml of RPMI 1640/10% FCS at a ratio of 10 parasites/macrophage. Infection was allowed to proceed for 4 h, unphagocytosed parasites were removed by washing with medium, and cells were resuspended in RPMI 1640/10% FCS with or without chicken cystatin and IFN-γ, along with each component added alone, for 48 h at 37°C. Cells were then fixed in methanol and stained with Giemsa stain for determination of intracellular parasite numbers. The mean percentages of survival in treated cultures were calculated on the basis of considering the number of *Leishmania* in untreated cultures as 100%.

Determination of NO concentration

NO, quantified by the accumulation of nitrite in the culture medium, was measured according to the method of Ding et al. (25). Briefly, 100 μl of culture supernatants was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at room temperature for 10 min. Absorbance at 540 nm was then measured. Sodium nitrite ($NaNO_2$) diluted in culture medium was used as a standard.

Establishment and assessment of infection

Mice were inoculated with *L. donovani* AG83 through the tail vein. Initially, 10^7 promastigotes/mouse were injected, and for reinfection the same number of promastigotes was injected 60 days after the first infection. At 1 and 15 days after inoculation of parasites, cystatin, either alone or in combination with a suboptimal dose of IFN-γ (10^4 U), was injected into the tail vein in various doses for 4 consecutive days. Forty-five days after the start of infection, animals were sacrificed, and their spleens were weighed. Multiple spleen impression smears were prepared and stained with Giemsa stain. Spleen parasite burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of amastigotes per 1000 nucleated cells × spleen weight (grams) (26).

RT-PCR analysis of cytokine mRNA

RT-PCR was performed to determine the cytokine profile of mRNA for IL-12 p40, IL-4, inducible NO synthase (iNOS), and hypoxanthine phosphoribosyltransferase (HPRT). Reverse transcription of 1 μg of RNA was performed according to the manufacturer's protocol for the Superscript One-Step RT-PCR system (Life Technologies). The primers for all these genes have been published (27). After the appropriate number of PCR cycles, the amplified cDNA was separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Flow cytometric analysis

Mice were infected with *L. donovani* (10^7 parasites/mouse) and treated with cystatin plus IFN-γ 15 days after infection. For intracellular IL-4 staining, splenocytes were isolated 45 days postinfection, plated aseptically (1×10^6 cells/ml), and stimulated with SLA (20 μg/ml) for 48 h, whereas for IL-12 p40 staining, purified splenic macrophages were stimulated likewise. Cells were incubated with monensin (Sigma) (2 μM) for 4 h, washed in PBS containing 0.1% NaN_3/1% FCS at 4°C, and fixed with paraformaldehyde. They were then permeabilized with saponin and treated with PE-conjugated anti-mouse IL-12 p40 and IL-4 mAbs. Cells were analyzed on a FACSCalibur cytofluorometer using the CellQuest software (BD Biosciences, San Jose, CA). The area of positivity was determined using an isotype-matched mAb.

Statistical analysis

The significance of the data was evaluated by the two-tailed *t* test.

Results

Up-regulation of nitrite production in mouse peritoneal macrophages by cystatin and IFN-γ

To determine whether chicken cystatin, a natural inhibitor of cysteine proteases, could modulate the infection of macrophages by *L. donovani* and NO synthesis, macrophages were subjected to treatment with various agents (Fig. 1). Neither cystatin (0.5 μM) nor IFN-γ (100 U/ml) when added alone could induce marked production of nitrite after 48 h of incubation (1.51 ± 0.23 nmol/10^6 cells and 3.32 ± 0.35 nmol/10^6 cells, respectively). However, the

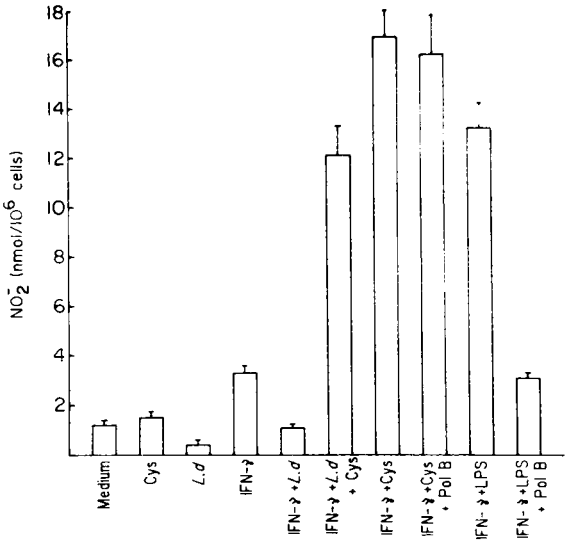

FIGURE 1. NO production by mouse peritoneal macrophages subjected to treatment with various agents. Macrophages (10^6/ml) were incubated for 48 h in culture medium with cystatin (5×10^{-7} M), *L. donovani* (macrophage:parasite, 1:10), IFN-γ (100 U/ml), IFN-γ plus *L. donovani*, IFN-γ plus cystatin plus *L. donovani*, IFN-γ plus cystatin, IFN-γ plus cystatin plus polymyxin B preincubation (10 U/ml for 2 h), IFN-γ + LPS (1 μg/ml), and IFN-γ plus LPS plus polymyxin B preincubation. Data represent the mean ± SD of three experiments.

nitrite level was significantly increased (6-fold) when cystatin was added simultaneously with IFN-γ, as compared with IFN-γ alone. Moreover, although *L. donovani* infection caused a suppression of NO production in IFN-γ-activated macrophages (1.12 ± 0.17 nmol/10^6 cells), combined treatment of infected macrophages with cystatin and IFN-γ for 48 h produced 12.18 ± 1.24 nmol NO_2^-/10^6 cells. The up-regulation of NO by cystatin in IFN-γ-stimulated macrophages was not due to the presence of LPS as a contaminant because preincubation of cystatin with polymyxin B, an LPS inhibitor, did not alter NO production. However, the NO-inducing effect in the control experiment, consisting of LPS preincubated with polymyxin B, was completely abolished.

Effect of cystatin and IFN-γ on the progression of leishmaniasis

Because cystatin together with IFN-γ can up-regulate NO, the effector molecule responsible for antileishmanial activity, it was thought worthwhile to evaluate the efficacy of the combination chemotherapy in a BALB/c mouse model of visceral leishmaniasis. BALB/c mice (6 wk old, ~20 g) were infected i.v. with *L. donovani* AG83 promastigotes as described in *Materials and Methods*. The infection was allowed to proceed for 45 days, during which spleen weight increased from 100.46 ± 11.17 mg to 1650.16 ± 139.92 mg. Two types of drug treatment schedule were chosen, one at the onset of infection and the other at established infection. IFN-γ or cystatin or a combination of both were administered i.v. daily for 4 consecutive days beginning 1 day after infection (onset of infection) and 15 days after infection (established infection). Various cystatin dosages were used (from 0.1 to 10 mg/kg/day), with a constant dose of 10^4 U of IFN-γ per mouse. Three i.p. injections of $>10^5$ U IFN-γ alone, given every other day, halted the visceral replication of *L. donovani* (7); however, treatment with four injections of 10^4 U produced only modest inhibition and no killing (7). Therefore, a dose of 10^4 U IFN-γ was selected to use in combination with cystatin. All animals were sacrificed 45 days after inoculation, and the degree of leishmanicidal potency of cystatin or IFN-γ or both was assessed in terms of spleen weight and splenic amastigote burden. The combination therapy with cystatin and IFN-γ was found to be much more potent than either component in terms of 100% parasite suppression. In the case of both onset of infection and established infection, a dose of 5 mg/kg/day of cystatin together with 10^4 U IFN-γ per mouse given for 4 days greatly reduced and possibly eliminated all parasites from the spleen, with subsequent reduction in its weight to nearly normal levels (Fig. 2). Absence of parasites in the spleen was further confirmed by culturing spleen specimens in transformation medium at 22°C for 96 h. Cystatin or IFN-γ alone at the same dose had little effect.

Effect of cystatin and IFN-γ on the growth of promastigotes

To ascertain the possibility that cystatin along with IFN-γ might exert a direct cytotoxic effect on *L. donovani*, the influence of the combination regimen on the in vitro proliferation of *L. donovani* promastigotes was assessed (Fig. 3). *L. donovani* proliferated comparably regardless of the presence or absence of cystatin and IFN-γ in the growth medium.

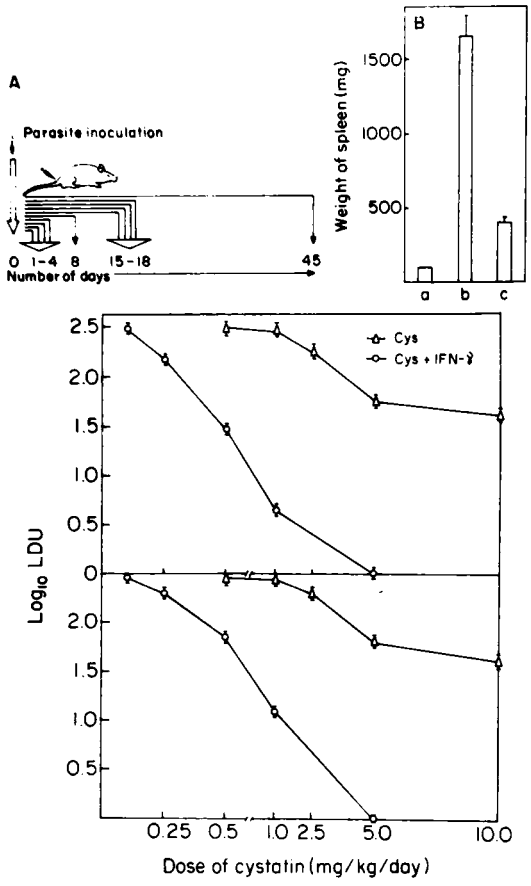

FIGURE 2. Schematic representation of experimental protocol for treatment of *L. donovani*-infected BALB/c mice. Mice were infected with 1×10^7 *L. donovani* promastigotes. *A*, Days 1–4, treatment at onset of infection; day 8, first appearance of amastigotes in the spleen; days 15–18, treatment at established infection; day 45, sacrifice. *B*, Reduction in the weight of spleen after treatment with IFN-γ (10^4 U) and cystatin (5 mg/kg/day). *a*, Normal untreated; *b*, infected untreated; and *c*, infected treated. *C* and *D*, Suppression of spleen parasites by cystatin and cystatin plus IFN-γ. Cystatin at the indicated doses was administered daily for 4 consecutive days, 1 day (*C*) and 15 days (*D*) after infection. The $\log_{10}$ LDU of infected controls was 2.49 ± 0.04 in *C* and 2.48 ± 0.03 in *D*, whereas those of the IFN-γ (10^4 U)-treated group were 2.29 ± 0.04 and 2.41 ± 0.02 in *C* and *D*, respectively. All data are mean $\log_{10}$ LDU ± SD of five mice.

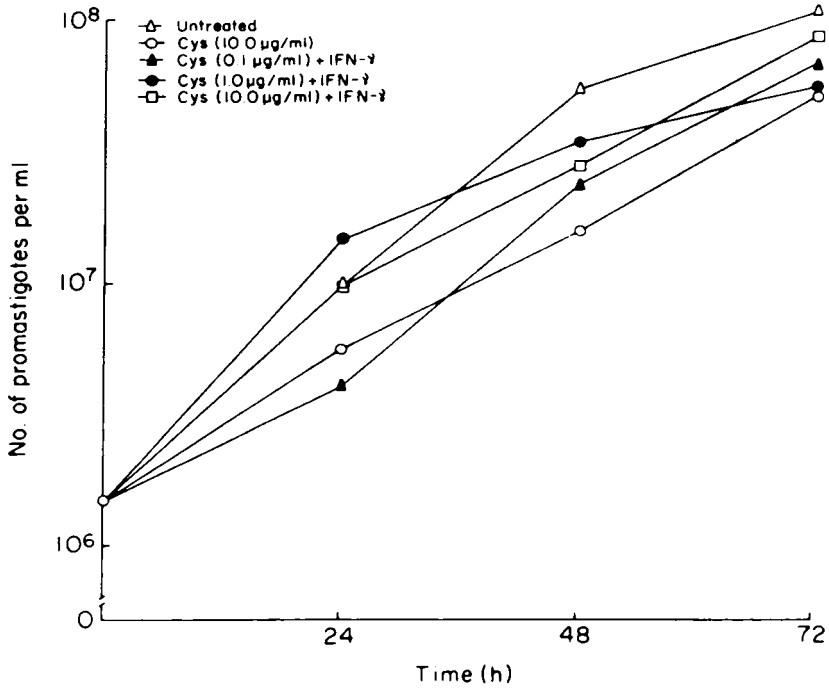

FIGURE 3. Effect of cystatin and IFN-γ on growth of *L. donovani* promastigotes. Varying concentrations of cystatin were used, whereas the concentration of IFN-γ was fixed at 100 U/ml. *L. donovani* promastigotes were cultured in medium 199 containing 10% FCS and were counted at 24-h intervals using a light microscope.

Effect of combined therapy on amastigote proliferation

To assess the effects of combination treatment on *L. donovani* amastigotes, the inhibition of amastigote multiplication within macrophages by IFN-γ and cystatin was compared with that of either component given alone. *Leishmania*-infected cultures were treated with cystatin or IFN-γ or both for 48 h at 37°C in macrophage culture medium. Controls were placed in medium alone. After drug treatment, cells were washed and placed in drug-free medium for an additional 20 h. They were then stained, and viable parasites per macrophage were counted. It is difficult to differentiate viable from nonviable amastigotes immediately after treatment. Therefore, a 20-h interval between drug treatment and staining was chosen to allow for the disposal of dead parasites. Combination IFN-γ and cystatin treatment was most effective with an IC_{50} of 4.3 μg/ml for cystatin, whereas cystatin alone had no inhibitory effect (Fig. 4*A*). To test whether reactive NO is involved in the growth inhibition of amastigotes, we used the specific NO synthase inhibitor, N^G-monomethyl-L-arginine (NMMA). Complete reversal of antileishmanial effect was observed upon the addition of NMMA (Fig. 4*A*). Moreover, NO_2^- release by macrophages treated with a suboptimal dose of IFN-γ (100 U/ml) progressively increased with increasing cystatin concentration until 1 μM, when it reached a maximum level (Fig. 4*B*). In contrast, cystatin when added to macrophage culture alone was unable to induce any NO production. There was no toxic effect from cystatin on macrophages in vitro at the highest concentration used for treatment. Therefore, these results suggest that increased antileishmanial activity after combination treatment with IFN-γ and cystatin may be correlated with increased production of NO.

Effect of in vivo administration of cystatin on NO up-regulation

Because cystatin was unable to induce any NO production in peritoneal macrophages, we set about to determine whether the generation of NO by macrophages isolated from cystatin-treated mice also corroborated their in vitro effect. Peritoneal macrophages isolated from BALB/c mice given i.v. injections of cystatin produced significantly higher levels of NO_2^- (13.02 ± 1.56 nmol/10^6 cells) compared with control untreated counterparts (1.20 ± 0.18 nmol/

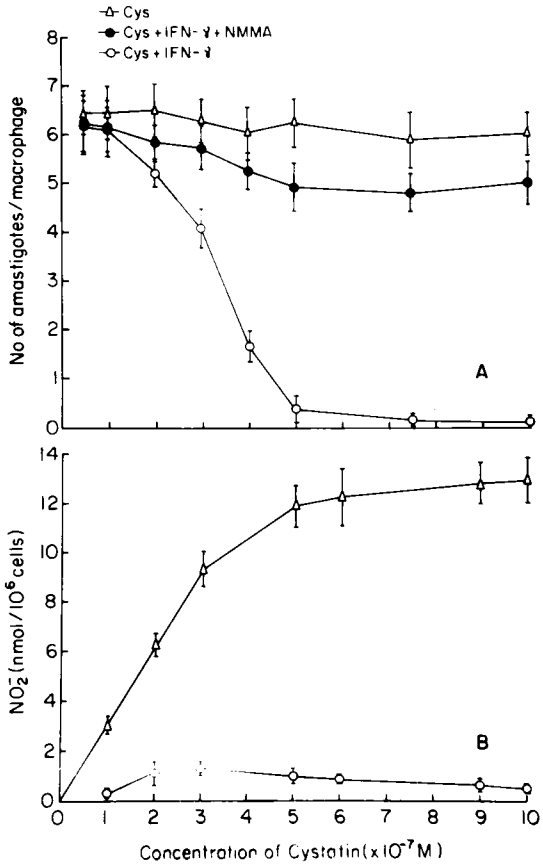

FIGURE 4. Effect of cystatin and IFN-γ on amastigote proliferation and NO production in mouse peritoneal macrophages. *A*, Infected macrophages were treated with various concentrations of cystatin either alone or in combination with IFN-γ (100 U/ml) for 48 h at 37°C. In another set of experiments, NMMA (2.5 μM) was used along with cystatin and IFN-γ. Infected control and IFN-γ-treated cultures contained 7.14 ± 0.67 and 6.38 ± 0.52 amastigotes/macrophage, respectively. *B*, Dose-response curves of various concentrations of cystatin for the release of NO_2^- by macrophages treated with or without IFN-γ (100 U/ml). Data are mean ± SD of three experiments.

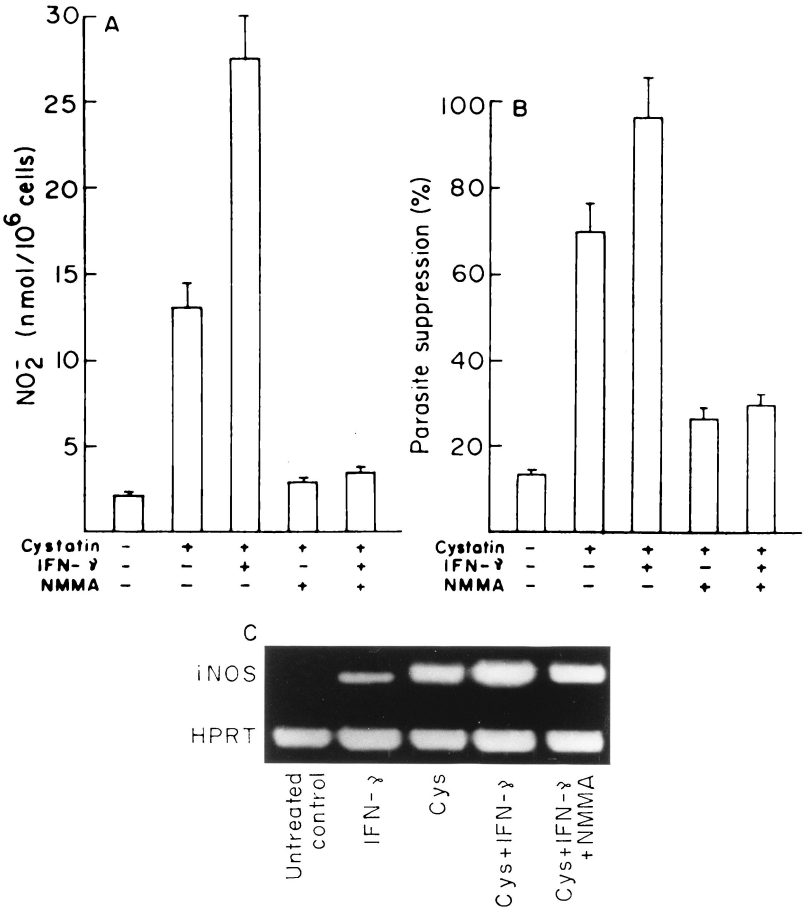

FIGURE 5. Generation of NO (*A*) and intracellular proliferation of *L. donovani* (*B*) in peritoneal macrophages after treatment of mice with cystatin or IFN-γ or both. Mice received i.v. injections of either cystatin (5 mg/kg/day) or IFN-γ (10^4 U/mouse) or both for 4 consecutive days. Peritoneal macrophages were harvested 10 h after the last injection and infected with *L. donovani*. After a 4-h infection and a 20-h incubation period, the percentage of parasite suppression and the amount of NO_2^- in the medium, which serves as a measure of NO, were determined. The nature of iNOS expression was also determined by RT-PCR of its mRNA transcript (*C*). In the case of inhibition experiments, the NO inhibitor, NMMA (5 mM), was coinjected with cystatin and was also present in the culture medium of macrophages. Values are the means of three experiments; error bars indicate the SDs.

10^6 cells; $p < 0.001$; Fig. 5*A*). These cells also inhibited the replication of *L. donovani* (Fig. 5*B*). NMMA, the NO synthase inhibitor, was found to reverse the stimulatory effect of cystatin. Moreover, macrophages isolated from BALB/c mice given i.v. administration of 5 mg/kg/day cystatin and 10^4 U IFN-γ produced much higher levels of NO_2^- (27.50 ± 2.47 nmol/10^6 cells) compared with their in vitro counterparts (12.18 ± 1.24 nmol/10^6 cells; $p < 0.001$; Fig. 5*A*). The level of nitrite produced by various regimens was reflected in the expression of iNOS mRNA, which, after isolation of total RNA, was subjected to RT-PCR analysis as described in *Materials and Methods* (Fig. 5*C*).

The role of cytokines in the in vivo activation by cystatin

To look into the mechanism of regulation of NO in the in vivo situation, spleen cells were isolated from mice given i.v. injections of cystatin. It was found that the generation of NO by peritoneal macrophages from control untreated mice was increased after culturing them in supernatants of splenocytes obtained from cystatin-administered animals (12.10 ± 1.10 compared with 1.20 ± 0.18 nmol/10^6 cells; Fig. 6). The increase in NO production was paralleled by an increase in the antileishmanial activity of these cells (Fig. 6). Splenocyte supernatant from mice treated with both cystatin and IFN-γ had a considerably stronger effect on NO production by virgin macrophages (data not shown). In an effort to determine the involvement of specific cytokines in the in vivo activation of macrophages by cystatin, peritoneal macrophages from untreated mice were cultured in splenocyte supernatants obtained from either cystatin-treated or PBS-treated mice in the presence or absence of mAbs against various cytokines. It was found that the addition of either anti-IFN-γ or anti-TNF-α greatly reduced the ex vivo NO_2^- production as well as the antileishmanial activity (Fig. 6). No detectable levels of nitrite were found in splenocyte supernatants. To further ascertain the roles of IFN-γ and TNF-α in vivo, mice were i.v. administered 5 mg/kg/day of cystatin alone or in combination with 200 μg of anti-IFN-γ, anti-TNF-α, or control hamster IgG at the onset of infection. Spleen parasite burden was then determined 45 days after infection (Table I). Anticytokine mAbs reactive against either IFN-γ or TNF-α greatly reduced cystatin-mediated protection, whereas the parasite load in mice treated with cystatin plus control Abs was similar to that in mice treated with cystatin alone. As an additional control, neutralizing mAbs to other cytokines, IL-1α, IL-1β, and IL-6, were also examined. However, these cytokines were ineffective in stimulating NO production by macrophages in culture (Fig. 6), and administration of mAbs against these cytokines had little effect on cystatin protection.

Cytokine production in treated mice

To gain an insight into the levels of various cytokines and iNOS after combination chemotherapy, we examined the flow cytometric pattern as well as the mRNA expression for a Th1 cytokine (e.g., IL-12), a Th2 cytokine (e.g., IL-4), and iNOS, which catalyzes the generation of NO from L-arginine and mediates the leishmanicidal activity of treated macrophages. Because both the treatment protocols (onset of infection as well as established infection) for combined therapy resulted in almost complete suppression of spleen parasite burden, we selected the therapeutic treatment of IFN-γ and cystatin (15 days after injection) for the measurement of cytokines. RT-PCR analysis of cytokine mRNA levels confirmed that susceptible BALB/c mice treated with a combined dose of IFN-γ

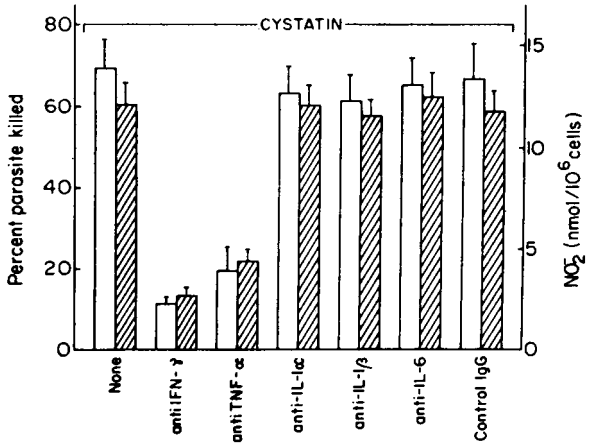

FIGURE 6. Effect of anticytokine mAbs on NO production and intracellular parasite suppression in peritoneal macrophages grown in splenocyte supernatant from cystatin-treated mice. Peritoneal macrophages harvested from untreated mice (5×10^5 cells/18 mm^2 cover slips) were grown for 24 h in supernatants of splenocytes isolated from mice treated with cystatin (four i.v. injections, 5 mg/kg/day). Macrophages were cultured in splenocyte supernatants either alone or in the presence of 20 μg of each of the anticytokine mAbs and were infected with *L. donovani*. After a 4-h infection and a 20-h incubation period, the percentage of parasite suppression (□) and the amount of NO_2^- in the medium (▨) were determined. The number of amastigotes per macrophage and the amount of NO_2^- generated in infected controls cultured in splenocyte supernatants of PBS-treated mice were 4.53 ± 0.10 and 1.26 ± 0.18 nmol/10^6 cells, respectively. Values are means of three experiments; error bars indicate SDs.

and cystatin could reverse an established Th2 response into a dominant Th1 response (Fig. 7*A*). Thus, *Leishmania*-infected cells from mice treated with IFN-γ and cystatin contained more IL-12 p40 mRNA and less IL-4 mRNA than those from infected untreated controls. Mice treated with either component alone at that dose exhibited little amounts of IL-12 p40 mRNA, although administration of cystatin alone showed some increase over untreated ones. Similarly, mice treated with both IFN-γ and cystatin also had low levels of parasite-specific IgG1, a Th2-associated isotype, but higher levels of parasite-specific IgG2a (data not shown). In addition, the iNOS mRNA expression, which was very low in the spleen cells of *L. donovani*-infected mice, was significantly induced by the combined therapy of IFN-γ and cystatin.

The effect of combination chemotherapy on the production of IL-12 p40 and IL-4 was determined by flow cytometric analysis of spleen cells isolated from infected BALB/c mice in the presence or absence of cystatin and IFN-γ. For this, splenocytes and purified macrophages were separately stimulated in vitro with SLA and permeabilized, and a one-color flow cytometry for IL-12 p40 and IL-4, respectively, was performed. Results reveal that a higher frequency of cells from mice subjected to combination chemotherapy (9.13%; Fig. 7*Be*) produced IL-12 p40 compared with untreated ones, in which the intracellular IL-4 status in $CD4^+$ T cells was 14.24% (Fig. 7*Bh*). Moreover, mice treated with cystatin produced more IL-12 p40 and less IL-4 than those treated with a suboptimal dose of IFN-γ. These results suggest that Th2 functions have been effectively suppressed in BALB/c mice by combination chemotherapy so that Th1 function could be activated sufficiently to resolve infection with *L. donovani.*

Reinfection with L. donovani *in BALB/c mice treated with cystatin and IFN-γ*

To further ascertain that the combination regimen has conferred long-standing immunity, infected BALB/c mice treated with cystatin and IFN-γ were later reinfected i.v. 60 days after primary infection. Spleen parasite burden in the reinfected animals progressed prominently in PBS-treated BALB/c mice, whereas cystatin- and IFN-γ-treated mice were largely resistant, as observed up to 7 wk (Fig. 8). Thus, infected BALB/c mice subjected to a combination chemotherapy with cystatin (5 mg/kg/day) and a suboptimal dose of IFN-γ (10^4 U/mouse) acquired protective immunity.

Discussion

Papain family cysteine proteases occur in abundance in trypanosomes and *Leishmania* and are believed to play key roles in parasite-host interactions including establishment of infection (28). The present study has demonstrated that BALB/c mice with fatal visceral leishmaniasis can be clinically cured of the disease by chicken cystatin, a natural cysteine protease inhibitor, in synergy with IFN-γ. The impetus for this combination chemotherapy was the earlier observation that cystatin could up-regulate NO release from IFN-γ-activated macrophages (21). NO produced by cytokine-activated macrophages during parasite infections is known to play a central role in the control of parasite killing (29). That the mice treated with cystatin and a suboptimal dose of IFN-γ were indeed cured was shown by the reversion of spleen size to near-normal levels and the complete suppression of spleen parasite burden. Moreover, this therapy was effective in mice with ongoing infections in which a nonprotective Th2 response had been established. After treatment and the resulting resolution of parasitism, the cytokine profile in these mice indicated a switch to a protective Th1 pattern. Treatment with either component alone had very little effect. The superior efficacy of the combination treatment in eliminating intracellular amastigotes of *L. donovani* in both an in vitro macrophage model and an in vivo mouse model of visceral leishmaniasis demonstrated the effectiveness of this approach. Neither the components nor the dosage used proved toxic to macrophages, as evidenced by their viability (trypan blue exclusion) and the release of lactate dehydrogenase from cells (data not shown). During the experimental period, all the animals remained healthy, without any apparent weight loss.

Increased microbicidal activity of cystatin- and IFN-γ-activated macrophages is achieved by a nitrogen-dependent mechanism. Enhanced NO generation resulted upon incubating cystatin with IFN-γ-activated mouse peritoneal macrophages in vitro, and the leishmanicidal activity acquired correlated with the induction of NO production. It may be mentioned that Engel et al. (30) showed a

Table I. *Effect of anticytokine mAbs on parasite suppression in infected mice*

Treatment Group[a]	Spleen Parasite Burden (LDU)	% Parasite Suppression
L. donovani-infected mice treated with:		
PBS alone	306.9 ± 31.0 (4)[b]	0
Cystatin	52.0 ± 6.3 (3)	83.1
Cys + anti-IFN-γ	187.1 ± 16.7* (3)	39.0
Cys + anti-TNF-α	152.0 ± 19.9* (3)	48.1
Cys + anti-IL-1α	63.8 ± 5.4 (3)	79.2
Cys + anti-IL-1β	61.4 ± 8.4 (3)	80.0
Cys + anti-IL-6	74.1 ± 5.9 (3)	75.9
Cys + Control IgG	64.6 ± 7.0 (3)	78.9

[a] Infected mice were administered i.v. cystatin (5 mg/kg/day) for 4 consecutive days 1 day after infection. On day 4, mice were injected with 200 μg of mAbs to IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, or hamster IgG (control Ab). Spleen parasite burden was determined 45 days after infection and expressed as LDU.

[b] Numbers in parentheses represent the number of mice.

*, $p < 0.001$ compared with cystatin treatment groups.

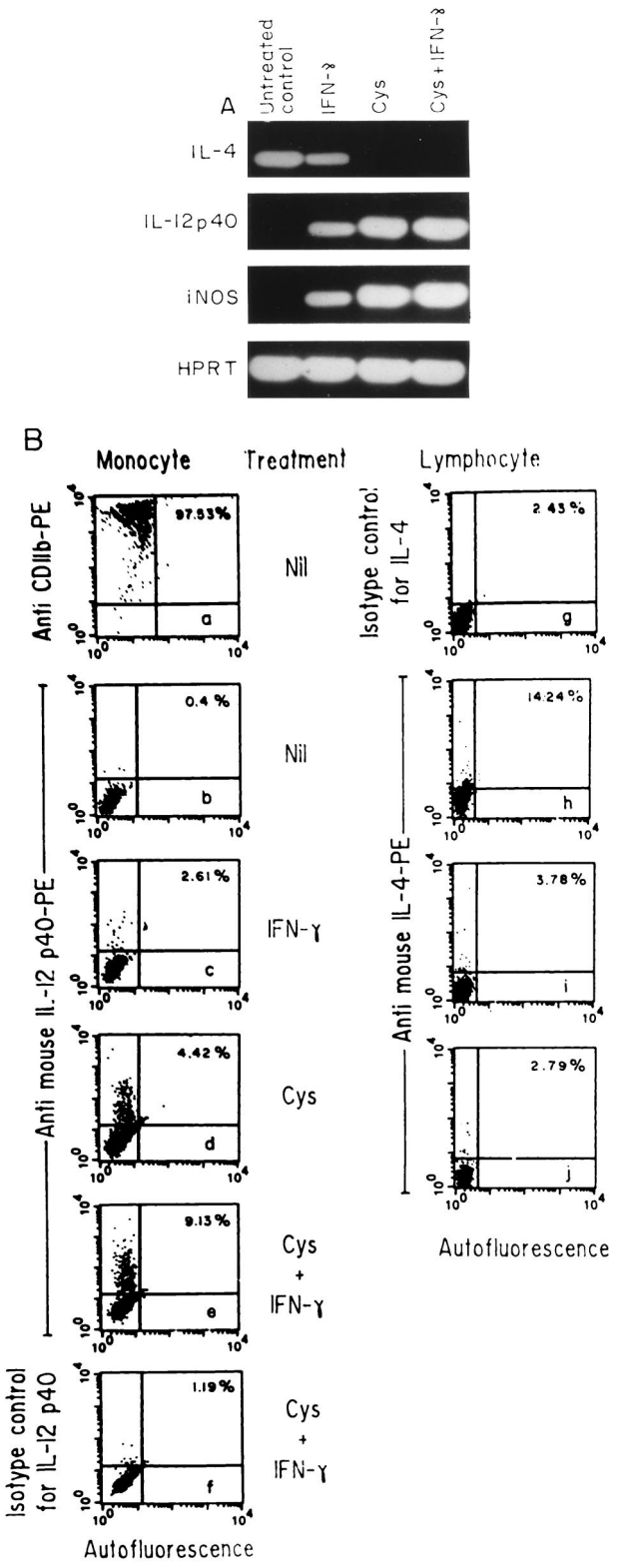

FIGURE 7. Induction of Th1-phenotype in *L. donovani*-infected mice subjected to combination chemotherapy as analyzed by (*A*) RT-PCR and (*B*) flow cytometry. *A*, Expression of IL-4, IL-12, iNOS, and HPRT mRNA by spleen cells of infected mice treated i.v. with either cystatin or IFN-γ or both. RT-PCR products were visualized by ethidium bromide staining. RNA samples were obtained from three mice in each group. Results are representative of three separate samples. HPRT expression levels were used as controls for RNA content and integrity. *B*, Expression of intracellular IL-12 p40 and IL-4 by splenic macrophages and lymphocytes after in vitro stimulation with SLA. Cells were permeabilized and stained with anti-mouse IL-12 p40-PE, IL-4-PE, and isotype-matched control rat Ab and were analyzed by flow cytometry.

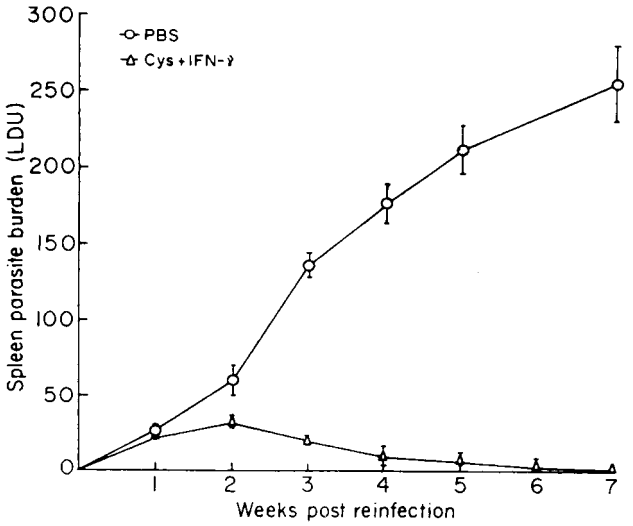

FIGURE 8. Course of reinfection with *L. donovani* in mice previously treated with curative doses of IFN-γ and cystatin. Naive age-matched BALB/c mice and cured mice (IFN-γ plus cystatin-treated) were i.v. administered with 1×10^7 promastigotes of *L. donovani* through the tail vein. The progression of infection was monitored by determining the spleen parasite burden, expressed as LDU, up to 7 wk after reinfection.

parasiticidal effect of synthetic cysteine protease inhibitors on intracellular *Trypanosoma cruzi* by inactivation of cruzain, a major protease of the parasite. The reason cystatin may induce an increase in NO synthesis from activated macrophages remains unclear. However, it is known that the biological effect of cystatin as a synergic NO inducer is not related to the inhibition of cysteine protease activity because the irreversible and structurally unrelated cysteine protease inhibitor E64 did not induce any increase in nitrite level (21). Also, from the structural standpoint, the observation that saturation of cystatin-inhibitory sites by reduced-alkylated papain did not interfere with cystatin-induced NO release from activated macrophages suggests the noninvolvement of inhibitory sites in the process (21). Although our in vitro studies demonstrated the inability of cystatin alone to induce NO synthesis in macrophages, our in vivo studies argue against this. Thus, cystatin given in vivo induced NO synthesis in peritoneal macrophages, along with an enhancement of inhibition of parasite growth. This suggests that NO generation by macrophages may be an indirect effect of cystatin activation requiring the cooperation of macrophages and other cells of the immune system. The ability of cystatin to induce NO in synergy with IFN-γ in vitro is suggestive of the compensatory role of IFN-γ for the effector molecules of immune system cells. Therefore, protection against infections with *L. donovani* induced by i.v. administration of cystatin and IFN-γ correlated with the development of activated macrophages secreting NO. Moreover, strong evidence for the participation of both IFN-γ and TNF-α in cystatin regulation of NO production and protection against leishmaniasis in vivo was suggested by the observation that anti-IFN-γ or anti-TNF-α mAbs could effectively block the significant increase in NO production by normal untreated macrophages when activated with the splenocyte supernatant from cystatin-treated mice. Further, the in vivo administration of anti-IFN-γ or anti-TNF-α mAbs could block the induction of cystatin-mediated protection against *L. donovani* infection. The immunologic stimulus for the production of cytotoxic levels of NO in vitro by murine macrophages is the synergistic effect of IFN-γ

and exogenous TNF or microbes and microbial products to stimulate endogenous release of TNF-α by macrophages (31–34). Exogenous agent-stimulated TNF acts in an autocrine fashion to amplify the actual synthesis and release of NO by IFN-γ-primed cells (33, 35). The data obtained with anti-IFN-γ and anti-TNF-α mAbs in cystatin-treated mice suggest that a similar synergism exists in vivo. It is likely that the in vivo administration of cystatin stimulates TNF synthesis, which in turn triggers IFN-γ production by spleen lymphocytes. Therefore, IFN-γ and TNF-α are the principal agents in macrophage activation by cystatin in vivo, and the protection afforded by combined treatment against experimental visceral leishmaniasis ultimately depended on the physiologic generation of NO. Indeed, cystatin has been shown very recently to cause production of increased amounts of TNF-α in IFN-γ-primed macrophages (36). Because LPS alone can induce NO production, we examined the effect of the LPS inhibitor polymyxin B and confirmed that the LPS contamination of cystatin used was insufficient to induce NO production from macrophages.

Several studies have emphasized the importance of Th1 cytokines in host defense mechanisms against infection caused by various microbial pathogens (37). Some insight as to how therapy with IFN-γ and cystatin influences the production of various cytokines and macrophage NO was gained by examination of mRNA levels and flow cytometric analysis of spleen cells 45 days after infection. Transcript levels of IL-4 were reduced in mice given combined therapy, whereas those for iNOS and IL-12 p40 were significantly elevated. Flow cytometric analysis of cells secreting cytokines also corroborated the mRNA transcript results. Because IL-4 can suppress both NO and IL-12 p40 production, it is possible that a reduction in the IL-4 level after combined treatment may assist IFN-γ in promoting both macrophage NO and IL-12 production. In contrast, IL-12 induces IFN-γ production and cytotoxic activity by NK and T cells (38) and can initiate the differentiation of Th1 cells from naive T cells (39–41). IL-12 enhances IFN-γ production by Th1 clones (42) and promotes the proliferation of Th1 but not Th2 cells (39). However, because IFN-γ can prime macrophages to produce IL-12 p40 (43), it is possible that the administration of exogenous IFN-γ as done in this study acts as a positive stimulus for enhanced IL-12 production, which in turn may promote both higher IFN-γ production and Th1 cell development.

Taken together, the findings in this report support the view that a complex series of cytokines and cell-mediated interactions contributes to the host's innate response to visceral leishmaniasis. The stimulatory capacity of cystatin may also confound studies involving site-specific targeting for enhanced macrophage activation. The absence of any host cell or animal toxicity at therapeutic doses suggests that the parasites are more susceptible to inhibitor, perhaps because of the redundancy of cysteine proteases in mammalian cells vs the parasite. It may be mentioned that administration of a cathepsin B-specific inhibitor to highly susceptible BALB/c mice resulted in a switch from the usual inefficient Th2 cytokine response to a Th1 response that cleared the *L. major* infection (18). In contrast, the administration of vinyl sulfone inhibitor, an irreversible cysteine protease inhibitor, to a mouse model of cutaneous leishmaniasis did not result in a switch from Th2 to Th1 cytokines. It exerted its antileishmanial effect by inhibiting parasite replication (44). In the present study, cure as well as resistance acquired by susceptible BALB/c mice due to combination chemotherapy were attributed to two mechanisms: 1) the direct action of cystatin for the induction of the NO that kills the parasite; and 2) the switch of $CD4^+$ T cell-mediated immune responses from the disease-promoting Th2 to the protective Th1 type. The switching of an established inappropriate Th response to an appropriate one has implications not only for the treatment of nonhealing leishmaniasis but also for the treatment of other chronic infectious diseases.

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