Cathepsin B-inhibitor promotes the development of Th1 type protective T cells in mice infected with *Leishmania major*

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Abstract : BALB/c mice are genetically susceptible to infection with *Leishmania major* (*L. major*). When such mice infected with *L. major* were treated with specific inhibitors of cathepsin B, a lysosomal cysteine protease that digests exogenous antigenic proteins, the mice acquired resistance against *L. major* infection. T cells from these mice produced large amounts of IFN-γ and low amounts of IL-4 as compared with those of untreated BALB/c mice. In addition, the mice treated with cathepsin B inhibitor produced a high titer of IgG_{2a} specific antibodies and only low titers of IgG_1 and IgE antibodies. This type of response is in contrast with the high specific IgG_1 or IgE antibody responses which are the usual antibody responses in BALB/c mice infected with *L. major*. These findings indicate that cathepsin B may be critically involved in processing antigens of *L. major* to promote exclusively the development of Th2 type CD 4^+ T cell responses. J. Med. Invest. 44 : 33-39, 1997

Key Words : *cathepsin B, antigen-processing, Th1/Th2, experimental leishmaniasis*

INTRODUCTION

T cell activation is initiated by interaction of antigen-specific receptors with antigen related structures on the surface of antigen-presenting cells. Prior to $CD4^+$ T cell activation, antigens must be processed with lysosomal proteases after endocytosis, then these epitopes are presented to $CD4^+$ T cells in association with class II molecules of the major histocompatibility complex (MHC) (1, 2). However, the proteases responsible for the antigen-processing have not been well defined. We developed a specific inhibitor of cathepsin B, the lysosomal cysteine protease, and this inhibitor, CA074, selectively inhibits the activity of cathepsin B *in vivo* as well as *in vitro* (3, 4). Matsunaga *et al*. first reported that CA074 suppresses immune responses, especially the production of antigen-specific IgG_1 or IgE (5, 6). These findings suggest that cysteine proteases in lysosomes play a key role in the functional differentiation of MHC class II restricted $CD4^+$ T cells which promote IgG_1 and IgE antibody production.

Mice resistant to infection with *Leishmania major* (*L. major*) such as DBA/2, C3H, CBA or C57BL/6 strains develop T helper (Th) type 1 $CD4^+$ T cells specific for *L. major* antigens (7, 8). Th1 cells generate interleukin 2 (IL-2) and interferon γ (IFN-γ), and support the production of antigen-specific IgG_{2a} type antibody (9, 10). In contrast, susceptible BALB/c mice develop Th2 $CD4^+$ T cells which generate IL-4 and IL-5, and which support production of specific antibodies of the IgE and IgG_1classes. However, the mechanisms involved in the preferential development of antigen-specific Th1 or Th2 type $CD4^+$ T cells remain to be defined.

In the present study, we examined the influence of the inhibition of cathepsin B activity on murine leishmaniasis to elucidate the participation of lysosomal cysteine proteases in the functional differentiation of Th1 and Th2 cells. We showed that modulation in antigen-processing by a cathepsin B inhibitor suppresses the functional differentiation of Th2 type $CD4^+$ lymphocytes which augment Th1 type immune responses. These findings indicate that lysosomal cathepsin B may be involved in processing antigenic peptides to create a motif that selectively induces Th2 type immune responses.

MATERIALS AND METHODS

Animals : Female BALB/c CrSlc (BALB/c) and DBA/2 CrSlc (DBA/2) were purchased from Japan Shizuoka Laboratory Animal Center (SLC) (Hamamatsu, Japan), and CB-17 scid/scid (SCID) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). The animals were 8-10 weeks of age at the beginning of experimentation.

Parasites : Cultures of *Leishmania major* (MHOM/SU/73/5 ASKH) were provided by Dr. Furuya, Kochi Medical College, Japan. The parasites were propagated in Schneider medium containing 20% fetal bovine serum (GIBCO BRL, Grand Island, NY). Promastigotes were harvested from stationary-phase cultures by centrifugation and washed at least three times in PBS. Parasites were passed at intervals in BALB/c female mice to ensure that virulence was maintained.

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Antigens and Reagents : Antigens were prepared using stationary-phase promastigotes of *L. major*. Soluble leishmanial antigen (SLA) was prepared as described (11) and the protein concentration determined using the Bicinchoninic Acid (BCA) protein assay reagent (Pierce, Rockfold, IL). Cysteine protease inhibitors, E64d and CA074, were synthesized in our laboratory. The specificities of inhibitors were confirmed as described (3,4). In short, E64d is specific for cathepsin B, H and L, whereas CA074 is specific only for cathepsin B. E64d was dissolved in dimethylsulfoxide at a concentration of 10 mg/ml and diluted in PBS at serial concentrations before use. CA074 was dissolved in PBS. E64d or CA074 was administered at doses of 1 and 0.5 mg/mouse/day, respectively. Inhibitors were given 2 hr before inoculation of the parasites and administration of the inhibitors was continued every 12 hr for the indicated periods.

Establishment and assessment of infection : Mice were inoculated with *L. major* subcutaneously in the footpads. For primary infection, 5 x 10^6 promastigotes were injected into the left hind footpad and for rechallenge, the same number of promastigotes were injected into the right hind footpad 60 days after the onset of the first infection. The progression of infection was assessed by monitoring lesion development by measuring the footpad thickness with a vernier caliper (Mitsutoyo, Japan).

In vitro L. major proliferation assay : Promastigotes of *L. major* 1 x 10^6/ml) were cultured in Schneider medium containing 20% fetal bovine serum with or without cysteine protease inhibitor for 72 hr at 25℃. The proliferation of *L. major* was evaluated by counting the parasites every 24 hr.

Measurement of serum immunoglobulin by ELISA : SLA was covalently coated onto 96-well CovaLink NH plates (Nunc, Kastrup, Denmark). After blocking with 0.1% bovine serum albumin in PBS, serum samples serially diluted with PBS were incubated in the plates for 6 hr at 4℃. The results were visualized using alkaline phosphatase-conjugated mAb, anti-mouse IgG (Sigma Chemical Co., St. Louis, MO), anti-mouse IgG_1or IgG_{2a} (Organon Teknika Co., West Chester, PA), or anti-mouse IgE (Southern Biotechnology Associates, Inc., Birmingham, AL) followed by p-nitrophenyl phosphate (Sigma). After terminating the reaction with 3N, NaOH, the optical absorbance at 415 nm was determined on a microplate reader.

Measurement of cytokine production : Cells from popliteal lymph nodes draining the footpad lesions were cultured with SLA (20 μg/ml) for 72 hr. IFN-γ and IL-4 production in the supernatants were measured using ELISA EM-IFNG (IFN-γ) and EM-IL 4 (IL-4) kits (ENDOGEN, Cambridge, MA).

RT-PCR assay : The expression of some cytokines was evaluated by RT-PCR assay. Interleukin 12 (IL-12) and interferon γ inducing factor (IGIF) were measured using peritoneal macrophages. Mice were injected peritoneally with 10% proteose peptone (DIFCO, Detroit, MI) to induce macrophages. Four days later, induced peritoneal macrophages were stimulated with 10 μg/ml lipopolysaccharide (LPS) and 500 U/ml recombinant mouse interferon γ (Boehringer Mannheim, Mannheim, Germany) for the indicated periods. Thereafter, total RNA was extracted with ISOGEN (Wako, Osaka, Japan). The RNA was reverse-transcribed using hexanucleotide random primers (Boehringer Mannheim) in a reaction mixture (Takara Shuzo, Tokyo, Japan), then the cDNA was amplified with Taq DNA polymerase (Takara Shuzo). The thermocycle conditions were 35 cycles of 95℃ for 1 min, 55℃ for 1 min, anda 72℃ for 1 min, for denaturing, annealing and extension, respectively. The primer sequenceswere, 5'-CGTGCTATGGCTGGTGCAAAG-3' and 5'-GAACACATGCCCACTTGCTG-3' for p 40 subunit of IL-12,, 5'-ACTGTACAACCGCAGTAATACGG-3' and 5'-AGTGAACATTACAGATTTATCCC-3' for IGIF, and 5'-ATGGATGACGATATCGCT-3' and 5'-ATGAGGTAGTCTGTCAGGT-3' for β-actin, respectively. Amplified DNAs were resolved by means of 2% agarose gel electrophoresis and stained with ethidium bromide. The early IL-4 was also estimated by RT-PCR assay. Mice were injected with 0.5 ml 2 C 11 culture supernatant including anti-CD 3 monoclonal antibody and spleen cells were taken 1 hr after injection. RNA extraction and reverse-transcription proceeded as described above. The thermocycle conditions were 30 cycles as described above for IL-12 and IGIF. The primer sequences for IL-4 were 5'-CTTCTTTCTCGAATGTACCAGG-3' and 5'-GTTAAAGCATGGTGGCGCAGTAC-3'.

RESULTS

Effect of cysteine protease inhibitor on progression of leishmaniasis

The cysteine protease inhibitor, E64d, has an inhibitory effect on several lysosomal cysteine proteases, cathepsin B, L and H. We examined the effect of E64d on the progression of murine leishmaniasis. Five million promastigotes of *L. major* were subcutaneously inoculated into the left hind footpad and for 6 weeks 1 mg/day (0.5 mg/12 hrs) of E64d was administered intraperitoneally. Footpad swelling was measured to evaluate the progression of infection. As shown in Fig.1, footpad swelling progressed in PBS-treated BALB/c mice. BALB/c mice given E64d after infection did not develop further footpad swelling, suggesting that cysteine protease inhibitor controlled *L. major* infection in susceptible BALB/c mice. In DBA/2 mice which are H-2 compatible with BALB/c mice but resistant to leishmaniasis, E64d did not allow the disease to progress to the extent as in PBS-treated DBA/2 mice.

E64d has a broad inhibitory effect on cysteine proteases such as cathepsin B, L and H. To define the most active component of E64d, it is essential to use a more specific inhibitor. We therefore examined the effect of the inhibitor specific only for cathepsin B, CA074, on murine leishmaniasis. Cathepsin B is a lysosomal cysteine protease that is involved in antigen processing (6, 12). In comparison with PBS-treated BALB/c mice that showed progressive footpad swelling, BALB/c mice treated with CA074 [0.5 mg/day (0.25 mg/12 hrs)] showed resistance against *L. major* infection to a similar degree as BALB/c

mice given E64d (Fig.2 a). Thus, cathepsin B is specifically involved with acquisition of resistance against *L. major* infection. Therefore, we used CA074 in all of the remaining experiments.

No direct influence of the cysteine protease inhibitor on L. major protozoa

The cysteine protease inhibitor could have been cytotoxic against *L. major* protozoa. To exclude this possibility, we examined the direct influence of CA074 on the *in vitro* proliferation of *L. major* promastigotes. As shown in Fig.3 a, *L. major* proliferated comparably regardless of presence or absence of CA074.

We then investigated whether or not this inhibitor influenced the *in vivo* growth of these protozoa in SCID mice which are deficient in functional T and B cells. As shown in Fig.3 b, no difference in lesional footpad thickness was observed between PBS-and CA074-treated SCID mice. In addition, the speed of progression and pattern of lesions were similar between protease inhibitor-treated or untreated nude mice (data not shown). Thus, cathepsin B-specific inhibitor did not directly influence the infectivity of *L. major*, suggesting that the resistance against *L. major* infection by treatment with cathepsin B-specific inhibitor was acquired through an immunologic mechanism lacking in the SCID mice, e.g. T cell-mediated immunity. Furthermore, this resistance was not attenuated in E64d-treated DBA/2 mice which are H-2 compatible with BALB/c mice but resistant to *L. major* infection. These findings indicate that cysteine proteaseSCID inhibitor does not exert suppressive effects on immunity in DBA/2 mice and the suppressive action in BALB/c mice does not depend on the MHC.

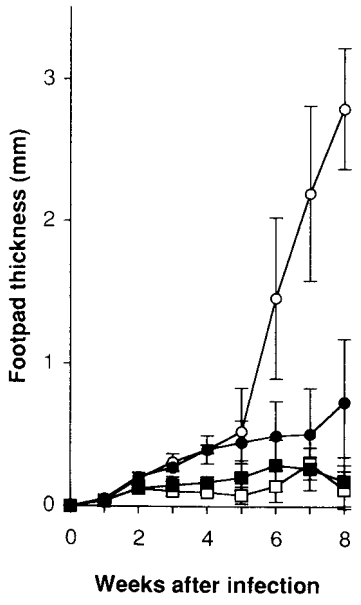

Fig. 1. Progression of infection with Leishmania major in mice treated with cysteine protease inhibitors.

Eight week-old female BALB/c mice (circles) and DBA/2 mice (squares) were inoculated with 5×10^6 *L. major* promastigotes in the left hind footpad. Cysteine protease inhibitor, E64d (1 mg/day (0.5 mg/12 hr) (filled symbols), or PBS as control (open symbols) were then administered for 6 weeks. The progression of infection was evaluated by measuring with calipers the relative lesional swelling at 1 week intervals.

Re-infection with L. major in BALB/c mice treated with CA074

We then investigated the mechanisms of resistance in BALB/c mice treated with cathepsin B-specific inhibitor.

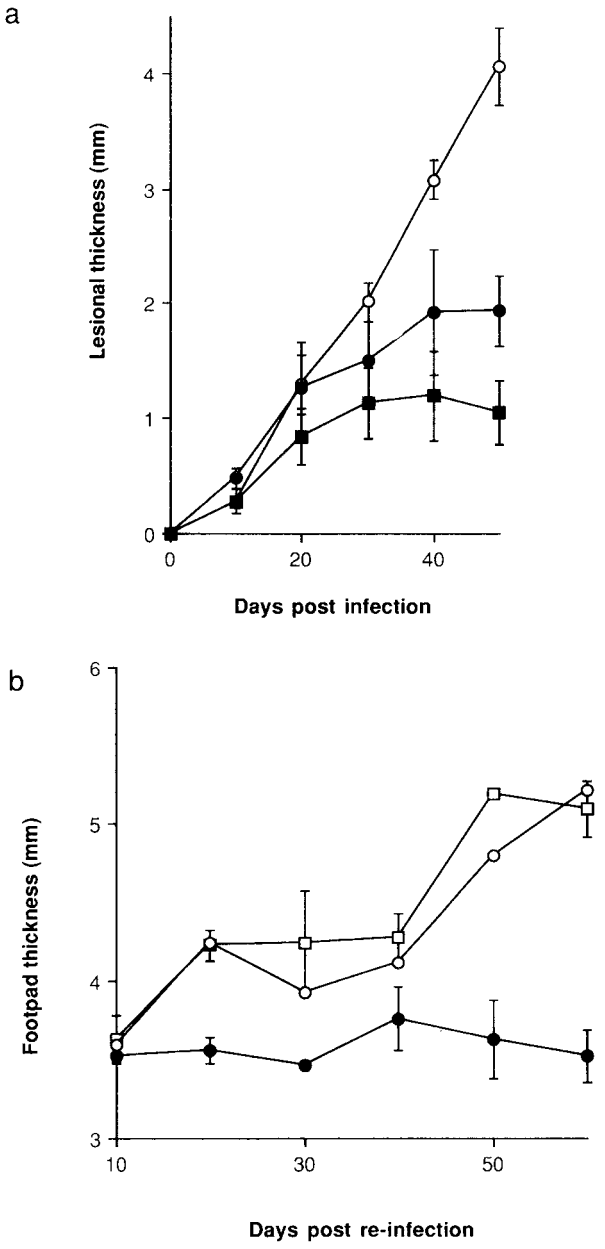

Fig. 2. Cathepsin B-specific inhibitor gives resistance in mice infected with L. major

a. Eight week-old female BALB/c mice were inoculated with 5×10^6 *L. major* promastigotes in the left hind footpad. Cysteine protease inhibitor, E64d [1 mg/day (0.5 mg/12 hr)] (filled circles), CA074 [0.5 mg/day (filled squares) or PBS as control (open circles) were administered for 40 days after inoculation. The progression of infection was evaluated by measuring the relative lesional swelling at 10 day intervals. b. Naive age-matched female BALB/c (open squares), PBS-treated BALB/c (open circles) and CA074-treated mice (closed circles) were inoculated with 5×10^6 promastigotes of *L. major* in the contralateral (right) hind footpad. The progression of infection was evaluated by measuring absolute lesional footpad thickness at 10 day intervals.

Infected BALB/c mice treated with CA074 or PBS were later re-infected via the contralateral footpad 60 days after the onset of primary infection. Re-infected mice were not given further CA074 (Fig.2 b). Swelling at the re-infected footpad prominently progressed in naive and PBS-treated BALB/c mice, whereas CA074-treated BALB/c mice were completely resistant whether or not additional CA074 had been given. Thus, infected BALB/c mice treated with CA074 had acquired protective immunity that was probably mediated by T cells.

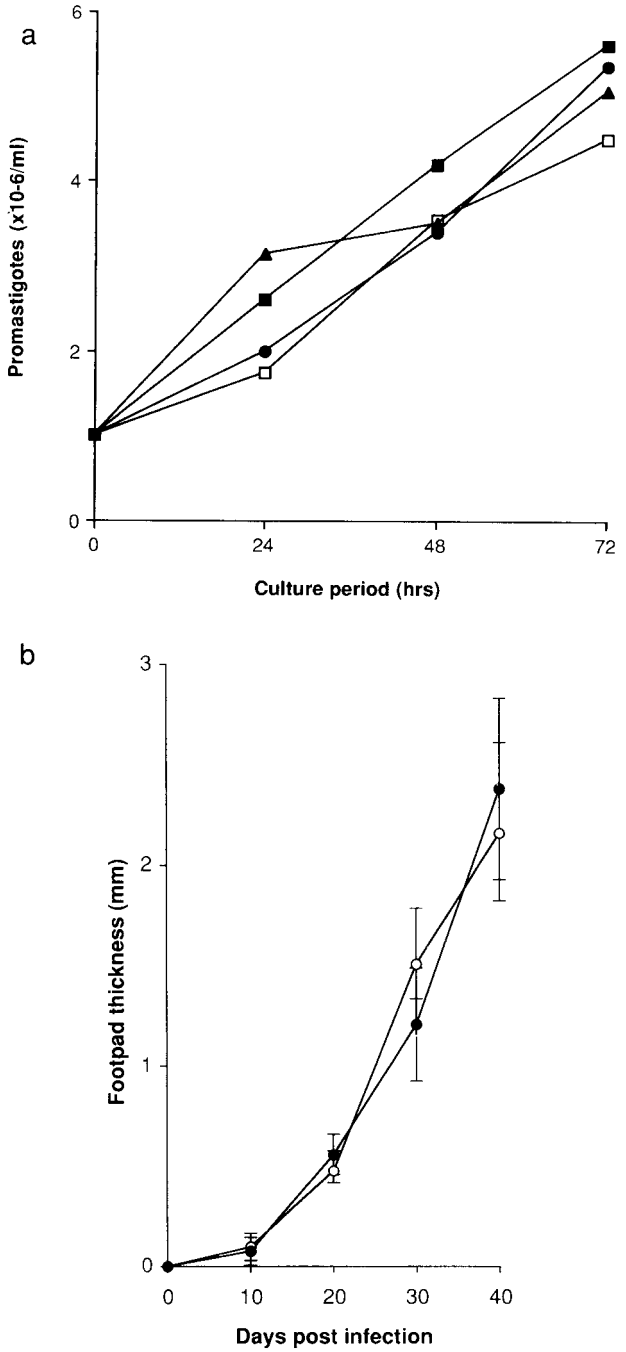

Fig. 3. Direct influence of cathepsin B specific inhibitor on L. major protozoa.

a. Stationary phase of L. *major* promastigotes (1×10^6) were cultured in Schneider medium containing 20% fetal bovine serum with CA 074 (open squares, 0 μg/ml ; filled circles, 1 μg/ml ; filled triangles, 10 μg/ml ; filled squares, 100 μg/ml). The parasites were counted at 24 hr intervals by light microscope. Three individual experiments were performed and the results were the same in each experiment. b. Eight week-old female CB-17 scid/scid mice were inoculated with 5×10^6 *L. major* promastigotes in the left hind footpad. Cysteine protease inhibitor, CA074 [0.5 mg/day (0.25 mg/12 hr)] (filled circles) or PBS as control (open circles) were administered for 40 days after inoculation. The progression of infection was evaluated by measuring the relative lesional swelling at 10 day intervals.

Serum immunoglobulin in CA074-treated BALB/c mice

$CD4^+$ T cells can be classified into two functionally different Th1 and Th2 subsets. Th1 and Th2 individually support immunoglobulin class-switching. The production of IgG_1 and IgE antibodies is supported by Th2 cells, while the response of IgG_{2a} antibodies is supported by Th1 cells. In murine leishmaniasis, Th1 cells are protective T cells that support the production of IgG_{2a} in the resistant mice, while Th2 cells are disease-promoting T cells that support IgG_1 and IgE antibody responses. We examined the proportion of each subclass of *L. major*-specific antibody in the serum to evaluate the immunological status of infected mice (Fig.4). No difference in the amount of *L. major*-specific IgG between PBS-and CA074-treated BALB/c mice was observed. The amount of IgG_{2a} was slightly higher in CA074 than in PBS-treated mice. In contrast, the production of IgG_1 and IgE antibodies promoted by Th2 cells was markedly suppressed in the CA074-treated mice as compared with that in PBS-treated mice, indicating that Th2 function had been inhibited by the cathepsin B specific inhibitor.

The functional phenotype of T cells in CA074-treated BALB/c mice

We examined the functional phenotype of $CD\ 4^+$ T cells in BALB/c mice that acquired resistance against *L. major* infection after treatment with the cathepsin B-specific inhibitor, CA074. Cells from lymph nodes draining the footpad lesions of infected mice were stimulated with soluble *L. major* antigen, and the titers of each cytokine in the culture supernatants was assessed by ELISA (Fig.5).

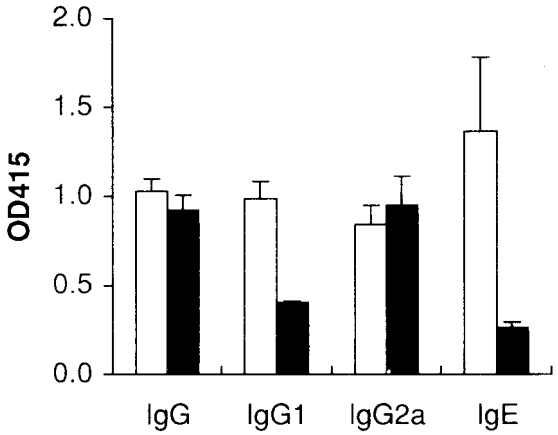

Fig. 4. Isotypes of antibodies specific for L. major antigens in BALB/c mice treated with CA 074.

Sera were obtained from PBS-treated mice (open columns) or CA074-treated mice [0.5 mg/day (0.25 mg/12 hr) for 20 days] (closed columns) mice 20 days after infection. Isotype of the antigen-specific immunoglobulin was assayed by ELISA as described in Material and Methods.

Production of IL-4 or IFN-γ indicates Th2 or Th1 responses, respectively. High levels of IL-4, but not of IFN-γ were produced in PBS-treated mice. In contrast, CA074 increased IFN-γ production and decreased production of IL-4. Thus, the cytokine produced in PBS-treated mice was of the Th2 type, rather than the Th1 type in CA074-treated mice. These findings suggest that Th2 function had been suppressed in BALB/c mice by treatment with the cathepsin B inhibitor so that Th1 function could be sufficiently activated to resolve infection by *L. major*. Thus, inactivation of cathepsin B appears to inhibit the development of Th2 type immune responses that ordinarily develop in BALB/c mice infected with *L. major*, and as a result promotes the development of Th1 cells. In other words, cathepsin B in the lysosomes of antigen-presenting cells processes antigens of *L. major* which acts to promote exclusively the development of Th2 type CD 4⁺ T cells.

No direct influence of cathepsin B-specific inhibitor on factors that promote the development of Th1 type immune responses

Many factors, especially cytokines, regulate the functional differentiation of CD 4⁺ T cells. IL-12, a cytokine produced by APCs, is a powerful mediator that induces Th1 type differentiation (13, 14, 15). Furthermore, IGIF (IFN-γ inducing factor) cloned by Okamura et al. induces IFN-γ production by T cells (16). IL-12 and IGIF are produced by macrophages upon stimulation with LPS plus IFN-γ. We evaluated the influence of CA074 on IL-12 and IGIF production by macrophages using RT-PCR (Fig. 6 a). When the proteose peptone-induced peritoneal macrophages of BALB/c mice were stimulated with LPS plus IFN-γ *in vitro*, IL-12 p40 subunit mRNA expression was induced 6-12 hours following stimulation. This expression profile was not affected by adding CA074 to LPS plus IFN-γ treated cells. CA074 also had no direct influence on the expression of IGIF mRNA. Exposure to IL-4 at an early stage of the immune response promotes Th2 development (17) while extinction of IL-4 effect by anti-IL-4 antibody leads to Th1 development (18). This early IL-4 response is generated by NK 1.1⁺ T cells as described (17, 19). We investigated the influence of CA074 on production of IL-4 in mice that had also been injected with anti-CD3 monoclonal antibody. As shown in Fig.6 b, IL-4 production was not reduced by treatment with CA074. These data indicate that the cathepsin B specific inhibitor, CA074, does not alter appreciably the environment including cytokine factors which promote the development of Th1 cells.

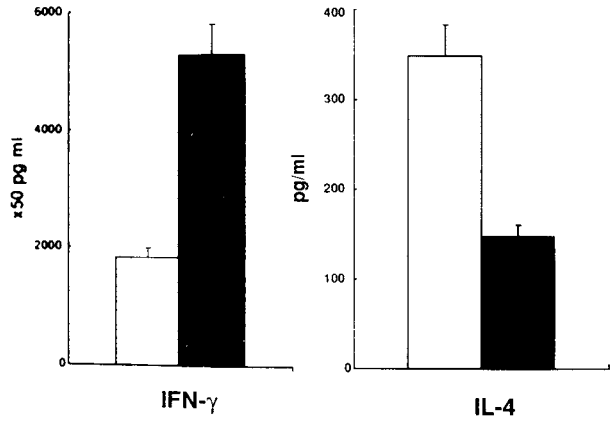

Fig. 5. Cytokine profiles of LN cells in BALB/c mice treated with CA074.

Two million lesional LN cells from PBS-treated mice (open column) and CA074-treated mice [0.5 mg/day (0.25 mg/12 hr) for 20 days] (closed column) 20 days after infection were stimulated with soluble leishmanial antigen (20 μg/ml) for 72 hr. IFN-γ or IL-4 in culture supernatants were assayed using EM-IFNG and EM-IL4 ELISA kits, respectively. Titrations were based on standard curves.

DISCUSSION

We show here that both generic and specific cathepsin B inhibitors interfere with the Th2 type immune response which ordinarily develops in BALB/c mice infected with *Leishmania major* (*L. major*), disease-promoting Th2 type CD 4⁺ T cells that preferentially appear after the infection. When susceptible BALB/c mice infected with this protozoan were treated with a specific inhibitor for cathepsin B, they completely resolved their infection. This acquired resistance was not due to a direct cytotoxic effect on the *L. major* protozoan, but to the switch of Th cell-mediated immune responses from disease-promoting Th2 to protective Th1 type as evaluated by cytokine production, specific antibody production, or antibody responses in the different Ig classes. Thus, cathepsin B

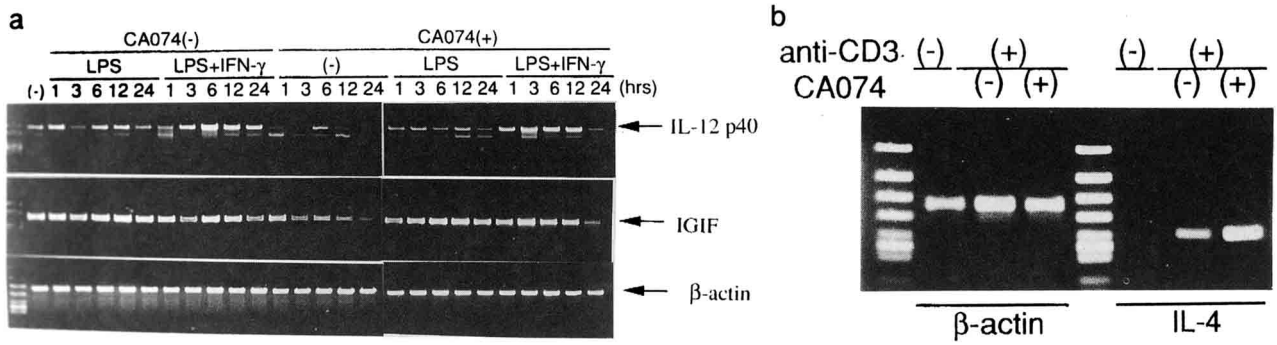

Fig. 6. Cysteine protease inhibitor does not directly affect the cytokine production that regulates Th differentiations.

a. Proteose peptone-induced peritoneal macrophages were stimulated *in vitro* with LPS(10 μg/ml) and rIFN-γ (500 U/ml) and/or CA074 (10 μg/ml) for the indicated periods. Thereafter, cells were harvested and total mRNA was extracted as described in Material and Methods. The mRNA expression of IL-12 p40 subunit and IGIF were evaluated by PCR. b. Eight week-old female BALB/c mice were injected with 0.5 ml 2C11 culture supernatant. One hour after stimulation, total mRNA was extracted from splenocytes and RT-PCR was performed to detect IL-4 mRNA as described in Material and Methods.

inhibitor appears to block the development of the Th2 type immune response which ordinarily develops in BALB/c mice infected with *L. major*, and also promotes the development of Th1 cells.

Many factors, especially cytokines, regulate the functional differentiation of CD 4^+ T cells. Alterations in the cytokine environment changes the Th1/Th2 balance as has been shown by other studies of murine leishmaniasis. Thus, IFN-γ and IL-4 reciprocally counter-regulate the differentiation of Th1 and Th2 cells from their precursor cells (Thp). For example, IFN-γ enhances the development of Th1 but inhibits development of Th2 cells and administration of monoclonal anti-IFN-γ antibodies *in vivo* abrogates the natural resistance of C 3 H/HeN mice to infection with *L. major* (20), whereas IL-4 promotes the opposite effect (18). IL-12 (13, 14, 15) and interferon γ inducing factor (IGIF) (16) secreted by macrophages play key roles in the induction of Th1 responses. From these bases, we examined whether the cathepsin B inhibitor, CA074, directly induces IL-12 or IGIF. This possibility was negated as is shown in Fig.6. IL-4 secreted by NK 1.1^+ T cells promotes the development of Th2 cells (17, 19). We then examined the influence of CA074 on IL-4 production from NK 1.1^+ T cells *in vivo* and found that it had no effect. CD 80 and CD 86 expressed on activated antigen-presenting cells transfer signals that promote the differentiation of CD 4^+ T cells into Th1 and Th2 T cells, respectively (21, 22, 23). Accordingly, we investigated, employing *in vitro* experiments, whether CA074 influences expression of these molecules directly. Here we used LPS-activated B cells but this possibility was also negated (data not shown).

As shown here, the Th2 response was suppressed in BALB/c mice infected with *L. major* and treated with CA074. The Th1 response essential to protective immunity against leishmaniasis became dominant in these animals. A similar effect in mice was induced by cathepsin B inhibitors when so-called IgE-inducible antigens such as ovalbumin (5) or *Nippostrongylus brasiliensis* (data not shown) were used as antigens. Thus, cathepsin B inactivation by its specific inhibitor could be expected to influence immune responses, especially the functional differentiation of CD 4^+ T cells. At present, the detailed molecular mechanisms responsible for cathepsin B inactivation and alteration of phenotype from Th2 to Th1 have not been clarified. However, since cysteine proteases, especially cathepsin B are presumed to be one of the main lysosomal proteases that process exogenous antigens in antigen-presenting cells (6, 12), the presented peptides would be expected to be altered in quality and/or quantity which in turn could affect Th1/Th2 differentiation.

It is important to elucidate in molecular detail the switching mechanisms responsible for T cell development from Th2 to Th1 type immune responses in mice treated with cathepsin B inhibitors in order to understand the mechanism(s) underlying CD 4^+ T cell differentiation and the immune deviation towards IgE production often seen clinically. In addition, experiments using these lysosomal protease inhibitors may shed light upon whether or not some antigenic motifs preferentially direct Th2 or Th1 responses and IgE specific antibody production.

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