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Endogenously Produced IL-12 Is Required for the Induction of Protective T Cells During *Mycobacterium avium* Infections in Mice[1]

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Immunity to *Mycobacterium avium* depends on the induction of protective $CD4^+$ T cells. In mice, *M. avium* induces a Th1 response leading to protective immunity dependent on IFN-γ and TNF. In this study, we analyzed whether endogenously produced IL-12 was involved in the generation of such protective T cells. We found that the neutralization of IL-12 with the administration of specific mAbs throughout the course of the infection led to the inability of BALB/c mice to control the infection by *M. avium* strain 2447. On the contrary, the late neutralization of IL-12, with the administration of the mAb starting only at the third week of infection, did not affect the growth of *M. avium*. The neutralization of IL-12 blocked the induction of protective T cells detected upon adoptive transfer to sublethally irradiated recipient mice. The neutralization of IL-12 in the recipient mice did not affect the protective activity of immune cells, showing that IL-12 is involved mainly in the induction, and not the expression, of acquired cell-mediated immunity. IL-12 was also shown to be required for a T cell-independent pathway of resistance present in T cell-deficient severe combined immunodeficient (SCID) mice. Finally, animals whose IL-12 was blocked expressed heightened levels of IL-4 and IL-10 message and reduced expression of IFN-γ as compared with control mice. *The Journal of Immunology,* 1995, 155: 2013–2019.

Mycobacterium avium is a facultative intracellular mycobacterium that is a major opportunistic infectious agent in immunocompromised humans, such as those suffering from AIDS. This mycobacterial species exhibits variable virulence when evaluated in the mouse model of infection (1). Virulence is associated with colonial morphotypic variation, as well as with unknown interstrain variations (1). Resistance of mice to *M. avium* is mediated by different cellular mechanisms that include innate macrophage functions encoded by the *Bcg* gene (2), the ability of the macrophage to secrete TNF-α early in infection (3),[4] the activity of NK cells and their ability to secrete IFN-γ and other cytokines (4–6), and the activity of protective $CD4^+$ T cells acquired later during infection (6–8). Strains of *M. avium* with intermediate virulence are, initially, partially controlled by innate mechanisms of resistance that slow down their growth and, later, prevented from proliferating by the immune response dependent on the $CD4^+$ T cells (6).

Cytokines are mediators of both the expression of immunity, e.g., by activating the macrophage, and of the induction of immune cells, not only the T cells, but also NK cells. Cytokines involved in the expression of immunity to *M. avium* include IFN-γ and TNF-α (6). We recently showed that IL-6 was involved in the induction of protective T cells during *M. avium* infection of BALB/c animals (9). Another cytokine that has been shown to be important in the induction of protective T cell immunity, as well as in stimulating innate resistance mediated by NK cells, is IL-12. Thus, IL-12 is necessary for the induction of protective immunity during infections by *Listeria monocytogenes* (10, 11), *Toxoplasma gondii* (12, 13), *Candida albicans* (14), and *Leishmania major* (15–17). IL-12 acts on precursor T cells by promoting their differentiation into a Th1 phenotype (18) and promoting secretion of IFN-γ (11, 12, 18–20). IL-12 also acts on the NK cells by increasing their proliferation and their capacity to secrete cytokines (11, 12, 19). In view of the involvement of T and NK cells

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in resistance to *M. avium* and the presence of a Th1-like response in *M. avium*-infected mice (21), we decided to evaluate the role of IL-12 produced during the mycobacterial infection of BALB/c mice in the induction of protective mechanisms of resistance to infection.

Materials and Methods

Animals

BALB/c female mice were purchased from Gulbenkian Institute for Science (Oeiras, Portugal) and used when they were 8 to 12 wk old. C.B-17 SCID[5] mice were purchased from Bommice (Ry, Denmark) and kept in sterile housing conditions in cages provided with high efficiency particulate air filter-bearing caps. SCID mice were screened for leakiness (presence of lymphocytes). Outbred nude mice, strain HSD, were purchased from Gulbenkian Institute for Science and used to raise ascites from hybridomas.

Reagents and Ab

Bacteriologic medium was from Difco (Detroit, MI), and tissue culture medium was from Life Technologies (Paisley, UK). The hybridomas secreting anti-IL-12 rat IgG1 were cell lines C15.1 and C15.6; the hybridoma GL113 was used to produce an irrelevant rat IgG1 against β-galactosidase. mAbs were isolated from the ascites of the corresponding hybridomas grown in CFA-primed HSD nude mice by passing them through a protein G-agarose column (Life Technologies).

Infections

M. avium strain 2447 is an AIDS isolate with intermediate virulence for mice, as previously shown (1), that induces a protective T cell response involving the activity of IFN-γ (6, 9). Inocula of the *M. avium* strain were prepared from cultures of the bacteria in Middlebrook 7H9 broth containing 0.04% Tween 80 (Sigma Chemical Co., St. Louis, MO). Mice were infected i.v. with 10^6 CFU of *M. avium* by injecting 0.2 ml of the bacterial suspension through one of the lateral veins of the tail. At different time intervals, mice were killed by cervical dislocation, and the organs were collected aseptically and homogenized in a 0.04% solution of Tween 80 in water. Viable counts were determined after plating serial dilutions in Middlebrook 7H10 agar medium and incubating the plates for 2 wk at 37°C. mAbs were injected i.p. in infected mice, starting either at day 0 of infection or at day 21 of infection. Mice received 2 mg of either anti-IL-12 mAbs (C15.1 and C15.6, 1 mg of each) or 2 mg of isotype control in 0.5 ml PBS.

Adoptive transfer of spleen cells

Pools of four spleens from uninfected (control) or *M. avium*-infected BALB/c mice were collected aseptically and teased gently in RPMI 1640 medium containing 2% FCS (AT medium). Cells were washed once in AT medium and suspended for 5 to 10 min in a hemolytic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2) at room temperature. Cells were washed twice in AT medium and resuspended in 30 ml of the supernatants of the hybridomas J11d and MK-D6 (1:1). J11d mAb reacts with B cells, erythrocytes, and granulocytes (cell line TIB 183 from American Type Culture Collection (ATCC), Rockville, MD), and the MK-D6 mAb recognizes I-A (cell line HB 3 from ATCC). These Abs will react with most B cells, granulocytes, and macrophages. Rabbit complement from Serotec (Oxford, UK) was added, and the suspension was incubated for 45 min at 37°C. Debris were removed and the cells washed twice in AT medium. Cells were suspended in AT medium and overlaid onto a nylon wool column (1.2 g of nylon wool in a 10-ml column), where they were incubated for 2 h at 37°C. Nonadherent cells were collected by washing the column with 20 ml of warm AT medium. Cells were checked in the first trials for phenotype in a FACSort (Becton Dickinson, San Jose, CA) and found to be more than 95% $CD3^+$. T cell-enriched splenocytes from uninfected and infected mice were transfused to mice that had been irradiated with a Cs source (500 rad/mouse) 24 h earlier and infected with 10^6 CFU of *M. avium* 2447 2 h earlier. Mice were killed 30 days later, and viable counts were done on the spleens and livers, as decribed above.

Previous studies had shown that the adoptive transfer of NK cell-rich populations did not protect mice in this 30-day assay.

Reverse-transcription PCR

Total RNA from spleen cell suspensions was isolated and reverse-transcribed, as previously described (6). cDNA was amplified by using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Emeryville, CA) and primers specific for the hypoxanthine phosphorybosyltransferase (HPRT) message (22). After standardization of all samples for the same HPRT expression level, amplification was performed with primers for IFN-γ, IL-4, and IL-10 (22). The PCR products were run in an agarose gel, transferred into a nitrocellulose membrane, and hybridized with specific probes labeled with [γ-^{32}P]ATP.

Histology

Small pieces of the spleen or the liver were cut from the organs with the aid of a sharp blade, fixed in buffered Formalin, and embedded in paraffin. Three-micrometer sections were stained with either hematoxylin-eosin or with carbol-fuchsin to stain acid-fast rods. Representative fields were photographed in a Nikon microscope.

Statistical analysis

Each CFU value presented represents the geometric mean of CFU of four animals with the respective SDs. Pairs of data were compared by using Student's *t*-test.

Results

To evaluate the participation of IL-12 in the immunity to *M. avium*, we used a combination of two mAbs (C15.1 and C15.6) to neutralize the activity of endogenously produced IL-12 during the course of an experimental infection with an AIDS-derived strain of *M. avium* with an intermediate virulence for mice, and that was shown previously to induce protective T cells in mice (6). BALB/c animals were infected i.v. with 10^6 CFU of *M. avium* 2447 and given either 2 mg of anti-IL-12 mAbs or 2 mg of the isotype control mAb, on the same day of infection and every 2 wk thereafter. The two anti-IL-12 mAbs, at similar doses, have been shown previously to be effective in in vivo neutralization of IL-12 (23). The spleens and the livers of four animals were collected at 90 days of infection and processed for histologic analysis. Infected control mice showed extensive infiltration of the white pulp of the spleen by macrophages (Fig. 1*A*), where acid-fast bacilli were occasionally seen (Fig. 1*C*). Mice whose IL-12 was inhibited showed even more marked macrophage infiltration, completely disrupting the white pulp (Fig. 1*B*), and the acid-fast bacteria were present in high numbers (Fig. 1*D*). In the liver, granulomas were similar in size and structure between control and anti-IL-12-treated mice, although some lesser degree in coalescence of the granuloma cells in anti-IL-12-treated animals was seen as compared with controls (Fig. 1, *E* and *F*, respectively). The number of acid-fast bacteria was again higher in the lesions of anti-IL-12-treated mice as compared with the controls.

[5] Abbreviations used in this paper: SCID, severe combined immunodeficient; AT, adoptive transfer; HPRT, hypoxanthine phosphorybosyltransferase.

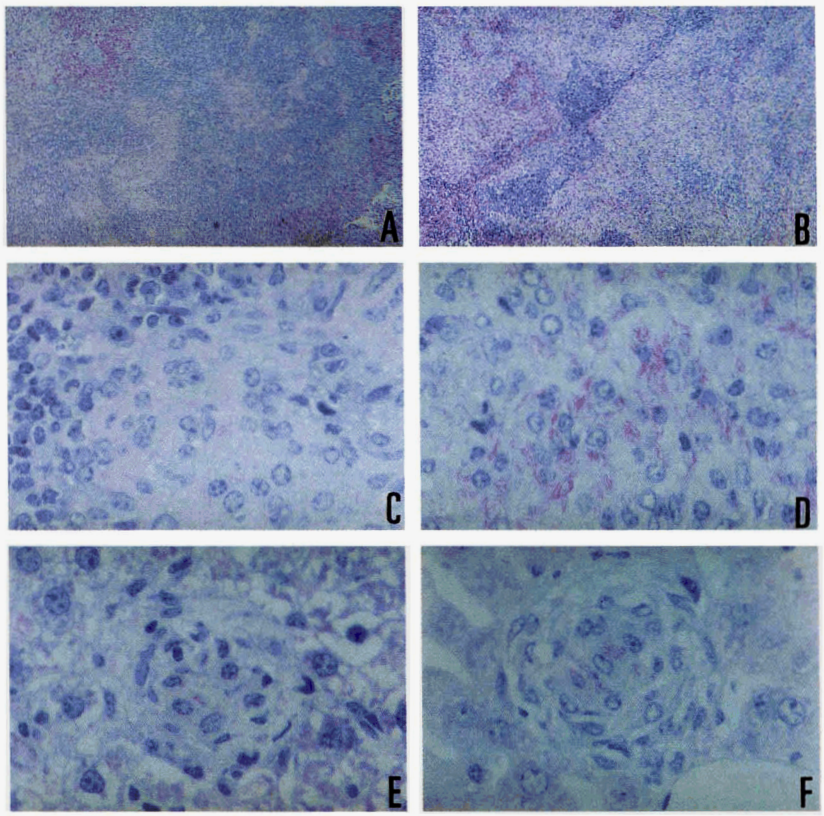

FIGURE 1. Histologic preparations of *M. avium*-infected spleens (*A–D*) and livers (*E* and *F*) at 3 mo of i.v. infection with 10^6 CFU of *M. avium* 2447, of BALB/c mice treated with an isotype control Ab (*A, C,* and *E*) or anti-IL-12 every 2 wk throughout the whole infection (*B, D,* and *F*). *A* and *B,* Low-power view of the spleen of infected mice, showing macrophage infiltration of the white pulp (hematoxylin-eosin staining). *C* and *D,* High-power view of the spleen, showing acid-fast bacilli. *E* and *F,* High-power view of the hepatic granulomas, showing acid-fast rods. Each photograph represents a typical view of the entire section and for all mice studied (four per group).

To have a precise estimate of the increase in mycobacterial proliferation, we then performed viable counts on the spleens and livers of infected mice at different time points. Two protocols were tested, namely one that evaluated the involvement of IL-12 in the early as well as the late phases of infection, and a second one in which the need for the late presence of IL-12 for protective immunity was tested.

Groups of BALB/c mice were infected with 10^6 CFU of strain 2447 and given either 2 mg of anti-IL-12 mAbs or 2 mg of the isotype control mAb, on the day of infection and every 2 wk thereafter. The continued neutralization of IL-12 throughout the course of the infection led to progressive proliferation of *M. avium*, preventing the appearance of mycobacteriostasis in both the spleens and livers of infected animals (Fig. 2). Differences in bacterial load reached two orders of magnitude, and were significant from day 15 onward in the liver and from day 30 onward in the spleen.

A second group of mice was infected with the same dose of *M. avium* 2447, but the administration of mAbs was delayed until the third week of infection. When mice were given anti-IL-12 from day 21 onward (2 mg, every 2 wk), there were minimal differences in bacterial proliferation (Fig. 3).

The previous results are consistent with a role of IL-12 in the induction of protective T cells, but not in the expression of their protective capacities. Thus, we tested whether neutralization of IL-12 in donor mice would abolish the induction of protective T cells detectable upon adoptive transfer of spleen cells to sublethally irradiated recipient mice. As shown in Figure 4, T cell-enriched spleen cell populations from infected mice conferred protection in recipient mice challenged with the homologous

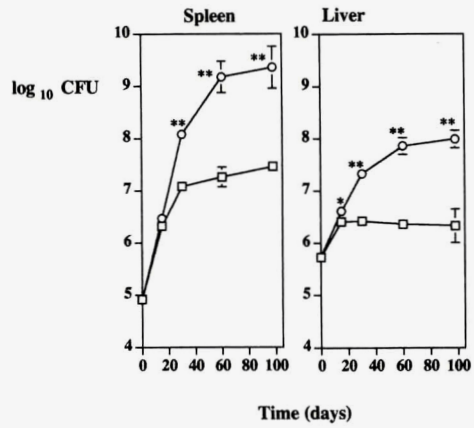

FIGURE 2. Growth of *M. avium* 2447 in the spleen and liver of BALB/c mice treated with an isotype control mAb (squares) or with anti-IL-12 mAbs (circles) every other week from day 0 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

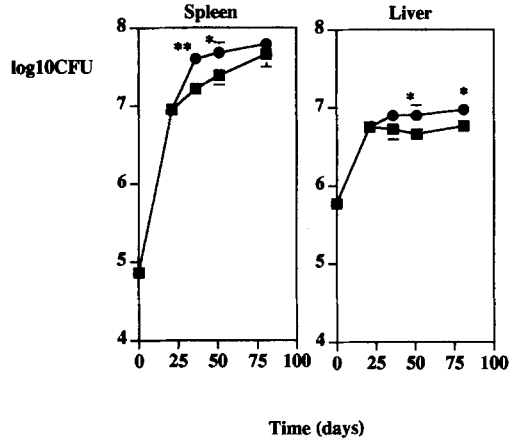

FIGURE 3. Growth of *M. avium* 2447 in the spleen and liver of BALB/c mice treated with an isotype control mAb (squares) or with anti-IL-12 mAbs (circles) every other week from day 21 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

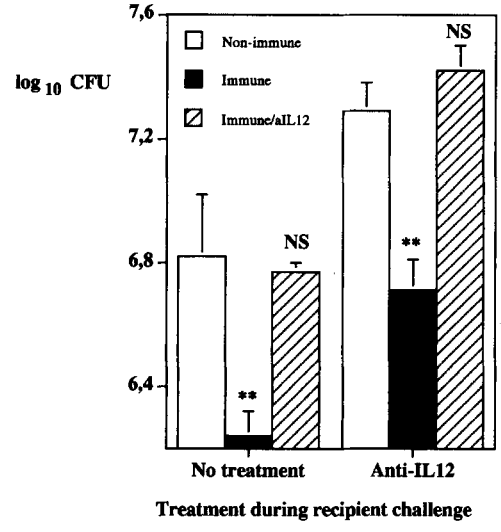

FIGURE 4. Number of viable *M. avium* 2447 in the liver, 30 days after infection of sublethally irradiated BALB/c mice given nonimmune T cells (open bars), T cells from mice infected for 30 days with *M. avium* 2447 (immune T cells; closed bars), or T cells from similarly infected mice, but treated throughout the infection of the donor animals with anti-IL-12 mAbs (striped bars). The recipient mice were either nontreated (*left*) or given anti-IL-12 mAbs at days 0 and 15 of challenge. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

mycobacteria. The neutralization of IL-12 during the 30-day infection period of the donor mice completely inhibited the capacity of these cells to transfer protection (Fig. 4). The neutralization of IL-12 in recipient mice did not affect the protective ability of immune cells (Fig. 4*B*),

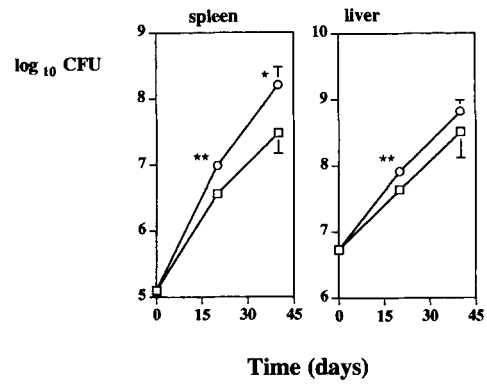

FIGURE 5. Growth of *M. avium* 2447 in the spleen and liver of SCID mice treated with an isotype control mAb (circles) or with anti-IL-12 mAbs (squares) every other week from day 0 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

showing that IL-12 was not involved in the expression of immunity.

To test whether IL-12 acts on NK cells, conferring some early protection to *M. avium*, we neutralized IL-12 in SCID mice infected i.v. with *M. avium* 2447. As shown in Figure 5, neutralization of IL-12 exacerbated the infection in the spleen and, to a lesser extent, in the liver of SCID animals.

Finally, because IL-12 is involved in the induction of the differentiation of T cells into a Th1 pathway (18), and because *M. avium* infections induce a Th1-type of response in mice (21), we analyzed the cytokine expression in treated animals as compared with control animals. RNA from spleen cells from BALB/c mice infected for 30 days with 10^6 CFU of *M. avium* was isolated and reverse-transcribed, followed by PCR amplification for HPRT, IFN-γ, IL-4, and IL-10. This time point was chosen because it had been shown to represent the peak expression of the cytokines expressed in response to this infection (6, 21). The PCR product was run in an agarose gel, and a Southern blot was performed by using a radioactively labeled probe. The autoradiographs were scanned, and the intensity of each band was determined after scanning each plate. Results are shown as the original blots, as well as in graphs plotting the band intensity in pixels (cpm). As shown in Figure 6, *A* and *B*, neutralization of IL-12 led to a marked reduction in IFN-γ expression as compared with control animals. On the other hand, the message for the two type 2 cytokines, IL-4 and IL-10, was increased in mice whose IL-12 had been neutralized from the beginning of the infection as compared with control animals (Fig. 6, *C* and *D*). Differences in cytokine expression were significant statistically for IFN-γ and IL-4 ($p < 0.01$ and $p < 0.05$, respectively).

In vivo neutralization studies were performed once for each situation because of the high expenditure of Abs.

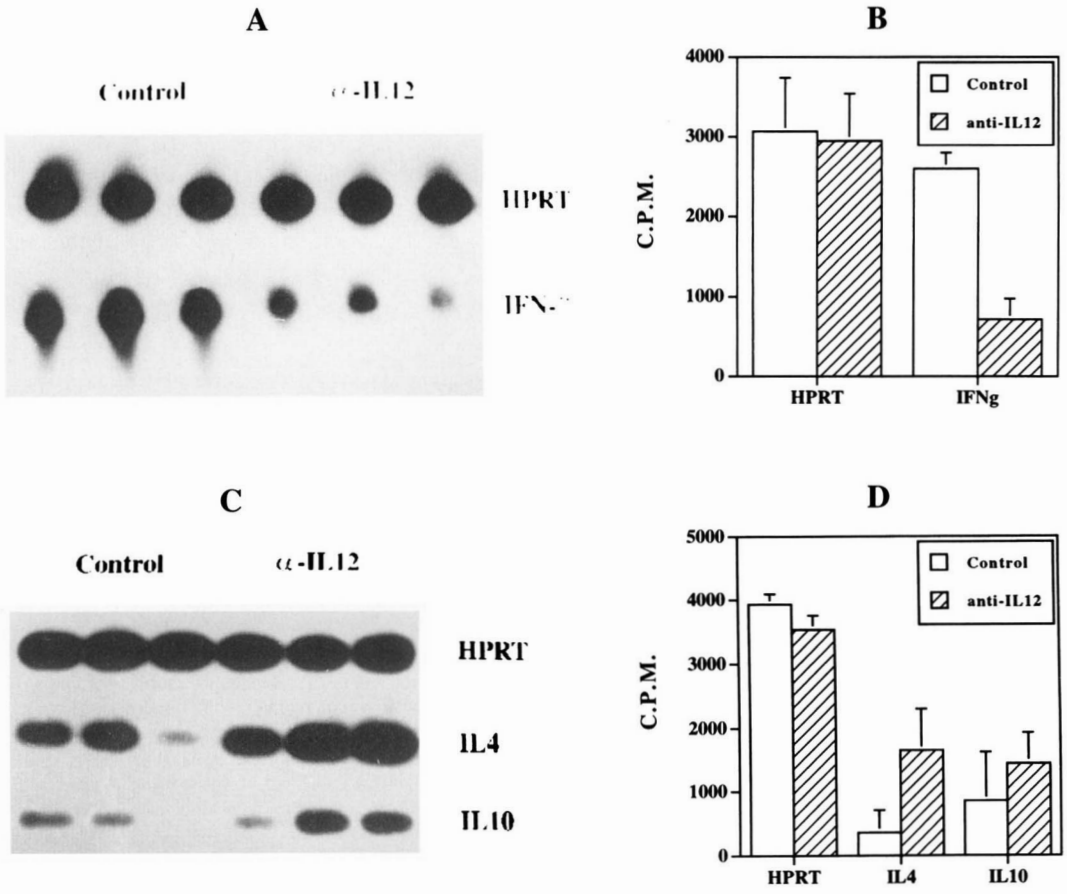

FIGURE 6. Expression of IFN-γ (*A* and *B*) and IL-4 and IL-10 (*C* and *D*) in spleen cells from uninfected and *M. avium* 2447-infected mice. PCR products were run in an agarose gel, transferred to nitrocellulose, and blotted with specific probes. The filters were exposed to an autoradiography plate, and the latter was scanned (*A* and *C*). From the autoradiographs, the intensity of the bands was calculated by using a computer-linked scanner, and plotted (*B* and *D*). The expression of HPRT was also processed in parallel to assure standardization of the samples.

Some experiments, however, confirmed the results from previous experiments. Thus, during the adoptive transfer experiments, the effects of IL-12 neutralization on mice receiving nonimmune cells (Fig. 4) confirmed the data shown in Figure 2. Similarly, the enhancement in *M. avium* growth seen in anti-IL-12-treated SCID mice confirmed the early increase in bacterial load seen in immunocompetent mice.

Discussion

We have presented in this work evidence for the pivotal role of IL-12 in the induction of protective responses to *M. avium* in BALB/c mice. This cytokine appeared to have its main role early in the infection to allow the differentiation of protective T cells, although, at least in the SCID animals, IL-12 might promote the activity of other protective cell types, most likely the NK cells. These results are consistent with the notion that IL-12 is one of the major cytokines involved in the differentiation of T cells, namely those with a Th1 phenotype (18). Indeed, we found that *M. avium* of intermediate virulence induces a predominant Th1 type of response in mice (6, 21).

Once protective T cells had been generated, IL-12 did not seem to be necessary any longer for the expression of acquired immunity. Thus, in our experiments, either the late neutralization of IL-12 during a primary infection or the neutralization of that cytokine in mice receiving immune cells by adoptive transfer did not affect the expression of acquired resistance. In the adoptive transfer experiments, the protection afforded by 30-day immune T cells is rather limited. We have seen that maximal protection is achieved at later time points of infection of donor animals. We limited our studies to 1 mo of immunization to prevent loss of the activity of anti-IL-12 treatments. Thus, protection is limited, but still it can be blocked completely by IL-12 neutralization of donor mice. The fact that IL-12 neutralization in recipient mice increases bacterial growth in parallel in all groups means that at 1 mo of challenge, the endogenous protective mechanisms of recipient mice

(NK cells, T cells, or others dependent on IL-12) are already at work. The protection conferred by immune T cells is measured in addition to those protective responses of the recipient animals.

Although the major role of IL-12 in the experimental *M. avium* infection that we analyzed in this study seemed associated with an effect on the T cell response, a role in the induction of T cell-independent immunity was evident in SCID animals. In this case, NK cells may be the target cells involved in the small degree of protection seen in SCID mice. This is in contrast with what has been found during *T. gondii* infections, in which the major effect of IL-12 was postulated to be on NK cells (12). An important role of IL-12 in NK cell-dependent antimicrobial mechanisms was also observed in *Listeria* infections (10).

IL-12 may be involved in preventing a Th2 response during experimental leishmanial infections in BALB/c mice, and favor the balance toward a Th1 response (15–17). Such activity of IL-12 was dependent on NK cells that could mediate such Th switch through the secretion of IFN-γ (17). In our data, we found that IL-12 neutralization led to an increase in IL-4 and IL-10 expression, suggesting that the absence of a Th2 response observed during experimental *M. avium* infections (21) may be associated with the ability of this microorganism to induce the secretion of IL-12. IL-12 is also able to increase directly IFN-γ secretion by NK and T cells (24). The reduction in IFN-γ production during *M. avium* infections in anti-IL-12-treated animals as compared with controls may thus reflect a lack in the IL-12 stimulation, as well as an emergence of an inhibitory Th2 response. However, the fact that T cells from anti-IL-12-treated animals did not exacerbate the infection in recipient animals suggests that it is the lack of an IFN-γ-secreting T cell population, rather than the activity of a counterprotective Th2 population, that determines susceptibility to *M. avium*. We postulate that IL-12 is an absolute requirement during *M. avium* infections for the induction of a protective IFN-γ secretion by specific T cells.

The potential use of IL-12 for the prophylaxis or treatment of mycobacterial infections is not easily predictable. In *L. major* infections of susceptible hosts, the protective effect of rIL-12 was present only if the cytokine was administered early in the infection, at a time when a commitment to a particular Th cell developmental pathway was occurring (16). However, in a distinct setting, rIL-12 may be effective given early or later during the infection, such as in the case of the experimental *Leishmania donovani* infections (25). Conversely, even though necessary for protection when produced endogenously, IL-12 might be ineffective when given exogenously, such as in the case of *C. albicans* infections (14). Some *M. avium* strains with high virulence fail to induce protective responses (26). In those cases, such failure is caused by an absent Th1 response, but not associated with a Th2 response (21). In a recent and very comprehensive study on the role of IL-12 in the immunity to *Mycobacterium tuberculosis*, Cooper and colleagues (27) found a modest impact of either endogenously produced or exogenously administered IL-12 on the course of the infection in a mouse model. Furthermore, the beneficial effects of rIL-12 therapy were accompanied by significant toxicity. Whether IL-12 administration might protect mice from infections with highly virulent *M. avium* strains would be interesting to study.

Acknowledgments

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