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Evaluation of IL-12 in Immunotherapy and Vaccine Design in Experimental *Mycobacterium avium* Infections[1]

Regina A. Silva, Teresa F. Pais, and Rui Appelberg[2]

IL-12 is a pivotal cytokine in the induction of IFN-γ-mediated protective immune responses. We tested the effects of rIL-12 administration to *Mycobacterium avium*-infected mice and found a limited ability to induce protection against the infection; this ability varied according to the mycobacterial strain studied. IL-12 accelerated the expression and production of IFN-γ in both immunocompetent and immunodeficient SCID or CD4-depleted mice. Evidence of NK cell activation was found as well as an enhancement of the ability to adoptively transfer resistance with T cell-enriched spleen cell populations and an increase in inflammatory cell recruitment in the liver. The protective ability of IL-12 was dependent upon the endogenous production of IFN-γ as evaluated by the use of specific neutralizing Abs or IFN-γ gene-disrupted mice. IL-12 potentiated the protective immunity conferred by a subunit vaccine containing *M. avium* culture filtrate proteins and dimethyl dioctadecyl ammonium chloride as an adjuvant. Thus, we show limited immunotherapeutic benefits from IL-12 administration in *M. avium* infections and promising results in its use as a coadjuvant in vaccine design. *The Journal of Immunology,* **1998, 161: 5578–5585.**

Interleukin-12 is a cytokine with a major role in the induction of IFN-γ-dominated immune responses to microbial pathogens. Studies using neutralizing Abs or gene-disrupted animals have underscored the almost absolute requirement for IL-12 in the generation of protective Th1 immunity (1–9). Likewise, the exogenous administration of recombinant cytokine led to protection against different microbes (4, 6, 10–17), including mycobacteria (1, 18–20). In addition, other studies have described the adjuvanticity of IL-12 in vaccines against parasites such as *Leishmania*, where it increased protection (21), and *Schistosoma*, where it decreased pathology (22). On the other hand, the genetic basis of susceptibility to infection was related in some cases to defective IL-12 production (19). Human patients have also been identified that are particularly prone to mycobacterial infections due to defective IL-12 production or signaling (23).

Mycobacterium avium is a facultative intracellular pathogen that frequently infects immunocompromised individuals. Several reports indicate that *M. avium* infection is one of the most common causes of systemic bacterial infection in AIDS patients in the United States and western Europe (24). Most AIDS patients develop disseminated *M. avium* infections when their circulating $CD4^+$ T cell numbers are $<100/mm^3$ (25), suggesting an important role for this T cell subset in the protective immune response against this pathogen. Disseminated *M. avium* infections have also been described in human patients deficient in the IFN-γR (26) in addition to those defective in IL-12 secretion or responsiveness to IL-12.

BALB/c mice are naturally susceptible to *M. avium* infections. However, the growth of certain *M. avium* strains (such as strain 2447) can be arrested after the emergence of protective $CD4^+$ T cells. These cells secrete IFN-γ, which induces bacteriostasis in infected macrophages (27). One of the molecules involved in the modulation of this protective immune response is IL-12. In this murine model of infection, endogenously produced IL-12 acts in an early phase of the infection by *M. avium* 2447, inducing protective T cell-independent mechanisms and, later on, promoting the differentiation of IFN-γ-producing $CD4^+$ T cells (28).

In this study, we evaluated the immunomodulatory effects of the administration of murine rIL-12 (rmIL-12)[3] on the course of *M. avium* infection in BALB/c mice. The effects of the administration of rmIL-12 were analyzed with respect to its role in both the emergence of protective $CD4^+$ T cells and in T cell-independent protective mechanisms. Also, the usefulness of IL-12 as an adjuvant in subunit vaccines against *M. avium* was assessed.

Materials and Methods

Reagents, cytokines, and Abs

Mycobacterial growth media were purchased from Difco (Detroit, MI). RPMI 1640 cell culture medium and FCS were obtained from Life Technologies (Paisley, Scotland). Tween 80 and IFA were purchased from Sigma (St. Louis, MO). Anti-CD4 mAbs were obtained from the hybridoma GK1.5 (TIB 207 cell line; American Type Culture Collection (ATCC), Manassas, VA). The IFN-γ-neutralizing IgG1 was obtained from the hybridoma XMG1.2 (DNAX, Palo Alto, CA), and an irrelevant rat IgG1 against β-galactosidase was purified from hybridoma GL113 (DNAX). Hybridomas were grown in ascites in Harlan Sprague-Dawley nude mice primed with IFA. Abs were purified by affinity chromatography using a protein G-agarose column (Life Technologies). rmIL-12 was a kind gift of the Genetics Institute (Cambridge, MA) and had a specific activity of 3.7×10^6 U/mg.

Mice

Specific pathogen-free BALB/c female mice were purchased from the Gulbenkian Institute for Science (Oeiras, Portugal). Outbred Harlan Sprague-Dawley nude mice were purchased from the Gulbenkian Institute for Science and used to raise ascites from hybridomas. C.B-17 SCID female mice

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[3] Abbreviations used in this paper: rmIL-12, murine rIL-12; PE, phycoerythrin; HPRT, hypoxanthine phosphoribosyltransferase; CFP, culture filtrate protein from *M. avium*; DDA, dimethyl dioctadecyl ammonium chloride.

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were purchased from Bommice (Ry, Denmark), and IFN-gamma gene-disrupted (IFN-$\gamma^{-/-}$) mice were generated by Dalton et al. (29). These mice were kept in sterile housing conditions in cages provided with high-efficiency particulate air filter-bearing caps. All mice were used at 6–8 wk of age.

In some experiments, $CD4^+$ T cell-depleted BALB/c mice were obtained by thymectomy and anti-CD4 mAb administration as described previously (27).

Bacterial infections

M. avium strains 25291 (from ATCC), 2447 (an AIDS isolate obtained from Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium), and 1983 (a low virulence isolate from an HIV-negative patient) were grown in Middlebrook 7H9 medium containing 0.04% Tween 80 at 37°C until the mid-log phase of growth. Bacteria were harvested by centrifugation and resuspended in a small volume of saline containing 0.04% Tween 80. The suspension was briefly sonicated with a Branson sonifier (Danbury, CT) for 15 s at 50 W to disrupt bacterial clumps, diluted, and frozen in aliquots at −70°C until use.

Mice were infected i.v. with 10^8 CFU of *M. avium* 1983 or 10^6 CFU of *M. avium* 2447 or 25291 through a lateral tail vein. Mice were sacrificed by cervical dislocation at different timepoints, and organs were collected, homogenized, serially diluted in a 0.04% Tween 80 solution in distilled water, and plated onto Middlebrook 7H10 agar medium. The plates were incubated for 2 wk at 37°C, and the number of CFU were counted. Data are expressed as the $\log_{10}$ value of the mean number of CFU recovered per organ ± SD of the mean (n = four or five animals). Differences were analyzed by the Student t test using unpaired data.

Cytokine assays

Serum was obtained from blood collected from sacrificed mice after allowing clot formation at 37°C for 30 min followed by an incubation at 4°C for 2 h to allow clot retraction. After centrifugation (10,000 rpm for 20 min at 4°C), serum was collected and frozen at −70°C until use. IFN-γ levels in individual sera were detected and quantified by a two-site sandwich ELISA using anti-IFN-γ-specific affinity-purified mAbs (R4-6A2 as capture and biotinylated AN-18 as detecting) and a standard curve generated with known amounts of rmIFN-γ (Genzyme, Cambridge, CA). The sensitivity of the assay was 80 pg/ml.

Treatment of mice with mAbs and cytokines

To maintain $CD4^+$ T cell depletion in thymectomized animals, 0.2 mg of anti-CD4 mAb (GK1.5) per animal was administered i.p. every 10 days during the experimental infection. For IFN-γ neutralization, 2 mg of XMG1.2 per animal was injected i.p. every 2 wk from the beginning of infection. Control mice received the same amount of purified anti-β-galactosidase mAb of the same isotype (GL113 mAb).

Treatment with rmIL-12 was performed by administrating 0.4 μg of this cytokine per animal i.p. on every other day during 1 mo from the beginning of the infection or during 3 wk starting on day 21 of infection.

Adoptive transfer of spleen cells

Spleens from BALB/c mice that had been infected for 3 or 6 wk with *M. avium* strain 2447 or left uninfected and treated with rmIL-12 or left untreated were aseptically collected and gently teased in RPMI 1640 medium containing 2% FCS (AT medium). Splenic cells were pooled (n = 4), washed, and resuspended in hemolytic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2) for 5–10 min at room temperature. After two washes in AT medium, cells were resuspended in the same medium plus an equal volume of J11d.2 (anti-CD24) and MK-D6 (anti-class II MHC) hybridoma supernatants (total volume of 30 ml) and incubated with rabbit complement (1:30 dilution) for 45 min at 37°C. Clumps of cells and cell debris were discarded, and cells were washed twice in AT medium. Cells were resuspended in AT medium, added to a nylon column containing 1.2 g of wool in a 10-ml syringe barrel, and incubated for 2 h at 37°C. Nonadherent cells were collected by washing the column with 20 ml of warm AT medium. This fraction was washed twice and injected i.v. in 0.5 ml of the same medium to recipient mice that had been irradiated with a Cs source (500 rad/mouse) 24 h earlier. One spleen equivalent was injected per recipient mouse. After 2 h, recipient mice were challenged i.v. with 10^6 CFU of *M. avium* 2447. Mice were killed 30 days later, and viable counts were done on the spleens and livers as described above. The protection achieved was calculated by subtracting the geometric mean of CFU in the organs of mice receiving immune spleen cells from that in mice receiving control spleen cells. The percentage of $CD4^+$ T cells in the final cell suspension varied from 36 to 51%.

Flow cytometry

Single-cell suspensions from the spleens of treated or control infected SCID mice were prepared by teasing a portion of the spleen in medium. For immunofluorescence double staining, 10^6 cells were incubated in microtiter plates for 20 min at 4°C with heat-inactivated rat serum. Cells were washed and subsequently incubated for 30 min at 4°C with FITC-conjugated rat anti-mouse CD90.2/Thy-1.2 (Becton Dickinson, Mountain View, CA) or rat anti-mouse CD11b/Mac1 and R-phycoerythrin (PE)-conjugated hamster anti-mouse CD3ϵ mAbs (PharMingen, San Diego, CA). Cells were washed twice with staining medium (PBS containing 0.1% sodium azide and 5% FCS) and resuspended in staining medium containing propidium iodide to identify and exclude dead cells. Flow cytometric analysis was performed with a FACSort apparatus (Becton Dickinson) equipped with Lysis II software by acquiring 10,000 events.

To analyze the T cell-enriched splenocytes from uninfected and infected donor mice in the adoptive transfer experiments, 10^6 cells from the T cell pools were stained with PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD8 (PharMingen) as described above.

Histology

Portions of the liver or spleen of infected mice were cut from the organs with the aid of a sharp blade, fixed in buffered formaldehyde, and embedded in paraffin. Next, 3-mm-thick sections were cut and stained for acid-fast bacilli by the Ziehl-Neelsen method and either counterstained with hematoxylin or stained with trichrome. Slides were analyzed for differences in the number and size of granuloma structures as well as for the presence or absence of acid-fast rods. Representative fields were photographed with a Nikon microscope (Tokyo, Japan).

RT-PCR

Total RNA from spleen cell suspensions was isolated by guanidinium thiocyanate/phenol/chloroform purification and stored at −70°C until further processing. Total mRNA was reverse transcribed using $p(dT)_{12-18}$ oligonucleotides (Pharmacia Biotech, Uppsala, Sweden) as primers and Superscript reverse transcriptase (Life Technologies) in the presence of 10 U of RNase inhibitor (Promega, Madison, WI) (30). Amplification of cDNA was conducted with *Taq* polymerase (Perkin Elmer-Roche Molecular Systems, Branchburg, NJ) and specific primers for hypoxanthine phosphoribosyltransferase (HPRT) or IFN-γ in a Gene Amp PCR System 9600 (Perkin Elmer) (31) for 30 cycles. The primers used have been described previously (30). Care was taken to analyze the product generated under conditions of linear relation to the concentration of the cDNA. After standardization for HPRT mRNA, the amplification products were run in a 1.5% agarose gel, transferred to nitrocellulose membranes (Hybond N^+; Amersham, Buckinghamshire, U.K.), and hybridized with specific probes labeled with [α-^{32}P]deoxyCTP. Membranes were exposed, and photographic plates were read with the aid of a computer-assisted scanner. Values for the PCR product for IFN-γ were corrected for the amount of HPRT on each sample, taking into account the titration of both the HPRT and IFN-γ cDNA from internal standards. All samples and the titrations were run, blotted, and exposed in parallel to the same plates to ensure a correct comparison of the signals generated. Data are expressed as the mean pixel value of four or five samples from separate mice. Values were analyzed using the Student t test.

Vaccine preparation and immunization studies

M. avium 2447 was grown for 2 wk in Sauton medium supplemented with pyruvate and glucose; culture supernatants were obtained by centrifugation of the bacteria and filtration through 0.45-μm filters. Such culture supernatants were concentrated 100-fold through ultrafiltration using filters with a molecular mass cutoff of 3 kDa. The resulting preparation was then subjected to 80% ammonium sulfate precipitation followed by extensive dialysis against PBS. Therefore, this reagent was named culture filtrate protein from *M. avium* (CFP). To immunize animals, 45 μg of CFP was injected three times s.c. at the base of the tail in separate locations, at weekly intervals. CFP was administered as a solution or was admixed with 0.4 μg of rmIL-12 and/or 250 μg of dimethyl dioctadecyl ammonium chloride (DDA) (Eastman Kodak, Rochester, NY) as adjuvants. The control mice received either PBS alone, rmIL-12 alone, DDA alone, or rmIL-12 in DDA. At 1 mo after the last immunization, mice were challenged i.v. with 10^6 CFU of *M. avium* strain 2447. The mycobacterial loads in the spleen and liver were determined 30 days later as described above.

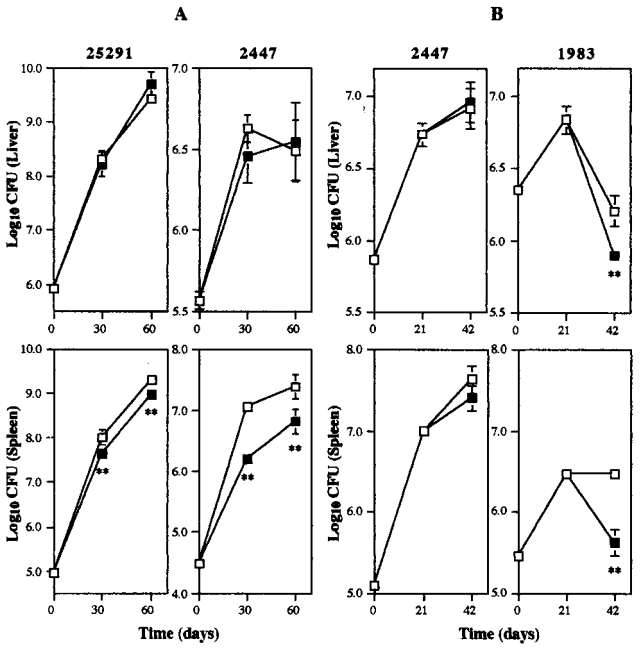

FIGURE 1. Growth of *M. avium* strains in the livers and spleens of BALB/c mice treated i.p. with rmIL-12 (closed symbols) or PBS (open symbols). Mice were treated during the first month of infection (strains 25291 and 2447) (*A*) or for 3 wk after day 21 of infection (strains 2447 and 1983) (*B*). Each timepoint represents the geometric mean of the CFU from four or five animals ± SD. The statistically significant decrease in bacterial growth in treated mice compared with untreated mice is labeled as follows: *, $p < 0.05$; **, $p < 0.01$.

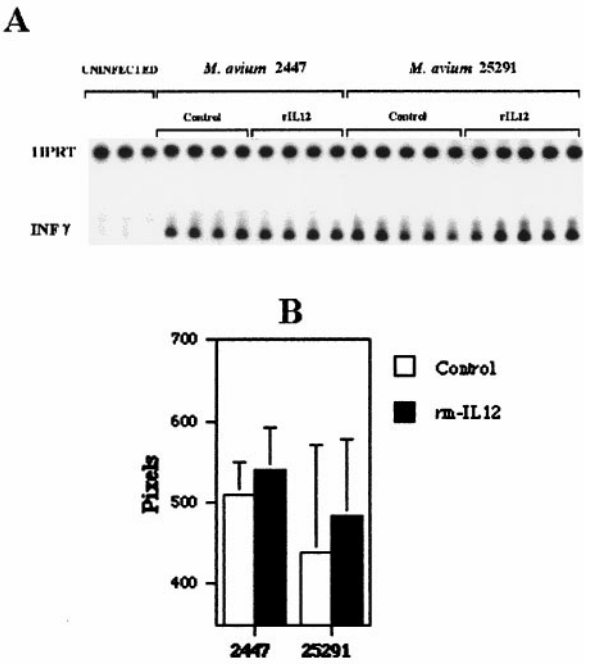

FIGURE 2. RT-PCR analysis of IFN-γ gene expression in splenic cells from BALB/c mice infected with *M. avium* 2447 or 25291 for 1 mo and treated i.p. with rmIL-12 (filled columns) or PBS (open columns) for the entire time period. PCR products were run in an agarose gel, blotted, and hybridized with specific radioactive probes. The filters were exposed to an autoradiography plate, and the latter was scanned (*A*). The intensity of the scanned bands was measured by computer-linked software, standardized for HPRT, and plotted (*B*).

Results

Evaluation of the effects of rmIL-12 administration during M. avium *infection*

BALB/c mice were infected i.v. with 10^6 CFU of either of two strains of *M. avium* of distinct virulence (characterized in Ref. 31) and treated every other day with rmIL-12 (each dose containing 0.4 μg of IL-12/animal) or PBS i.p. during the first month of infection. The growth of *M. avium* was studied in the spleen and liver of the infected animals for 60 days. The results showed that rmIL-12 induced statistically significant protection in the spleens of BALB/c mice infected with strains 25291 and 2447 but had no effect on the mycobacterial growth in the liver (Fig. 1*A*). The protection afforded in the spleen against the most virulent strain 25291 was minimal when compared with the overall growth of the mycobacteria, whereas a 0.9 $\log_{10}$ decrease in mycobacterial numbers was detected in the spleens of rmIL-12-treated mice infected with the low virulence strain 2447 as compared with the PBS-treated control group. After discontinuation of the treatment, differences in splenic mycobacterial loads between the two groups of mice decreased slightly to 0.6 $\log_{10}$, but significantly lower numbers were still observed in the rmIL-12-treated group as compared with the controls. Since our results were different from those reported previously by Kobayashi et al. (19), who described the ability to induce the killing of *M. avium*, we chose to study *M. avium* strain 1983, which has virulence characteristics similar to the strain used in that study. Furthermore, we adapted the therapeutic regimen to the one used by those authors (i.e., 3 wk of cytokine administration starting on day 21 of infection). In addition, we compared the effects of such a regimen on the growth of strain 2447. As shown in Fig. 1*B*, this second IL-12 treatment protocol was not effective in reducing the mycobacterial load during infection by strain 2447. However, we could detect a 0.8 $\log_{10}$ difference in the splenic loads and a very minor although statistically significant decrease in hepatic loads of 0.3 $\log_{10}$ during infection by the low virulence strain 1983. Furthermore, we found killing of mycobacteria in the spleens of mice infected with this latter strain; this killing took place after the administration of the cytokine.

To understand the basis of the protective activity of rmIL-12 during the infection, we analyzed the expression of the protective cytokine IFN-γ. Analysis was performed by RT-PCR of IFN-γ splenic expression in BALB/c mice treated i.p. with rmIL-12 (0.4 μg/animal every other day) or PBS and infected i.v. for 30 days with 10^6 CFU of *M. avium* strains 25291 and 2447. As shown in Fig. 2, treatment with rmIL-12 caused a minor increase in mRNA levels for IFN-γ; however, this increase was not statistically significant. Since the timepoint chosen represents the peak of expression for IFN-γ during *M. avium* infections (25, 31), and as the treatment with rmIL-12 might be accelerating its expression rather than increasing the peak expression of the cytokine, we selected strain 2447 and studied earlier timepoints of infection. Mice were infected as described, and half were treated with rmIL-12 as before. As shown in Fig. 3*A*, protection was induced by rmIL-12 therapy in both the spleen and liver and was already apparent on day 10 of infection. This early protection was associated with higher expression of IFN-γ mRNA on day 10 in rmIL-12-treated animals as compared with the controls (Fig. 3, *B* and *C*). However, such differences disappeared on day 20. The levels of immunoreactive IFN-γ present in the sera of the infected animals correlated well with the RT-PCR studies, in that rmIL-12 treatment induced high levels of IFN-γ on day 10 that decreased by day 20 of infection, such amounts of the cytokine always being higher in the rmIL-12-treated group as compared with the controls (Fig. 3*D*).

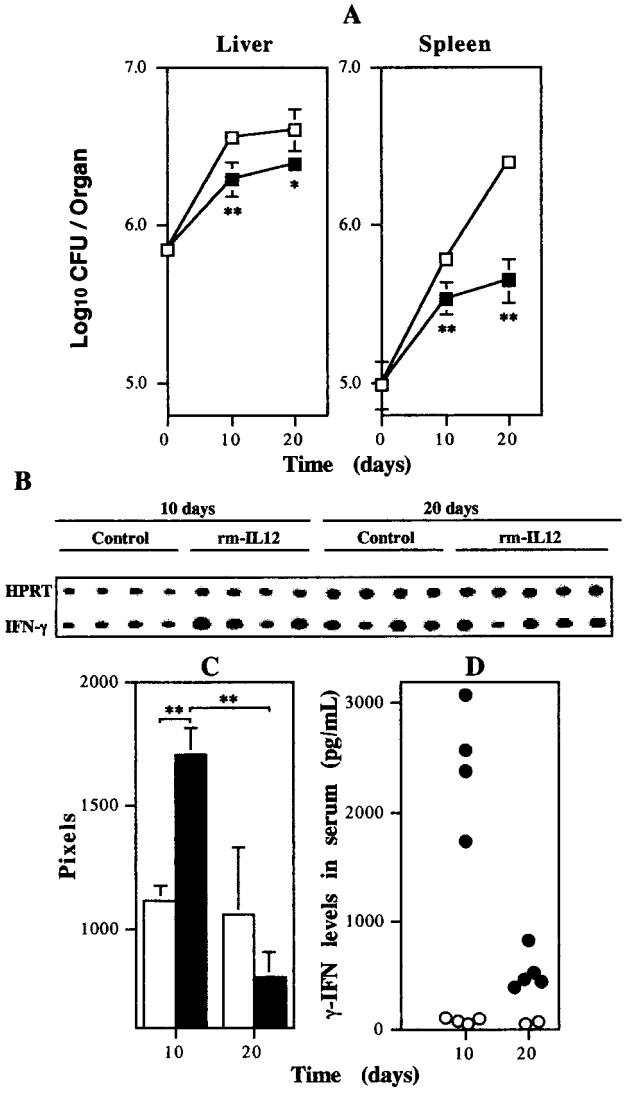

FIGURE 3. The administration of IL-12 leads to an early enhancement of IFN-γ production and improved control of infection. *A*, Growth of *M. avium* strain 2447 in the organs of BALB/c mice treated i.p. with rmIL-12 (closed symbols) or PBS (open symbols) for 20 days. Each timepoint represents the geometric mean of the CFU from four animals ± SD. The statistically significant decrease in bacterial growth in treated mice compared with untreated mice is labeled as follows: ∗, $p < 0.05$; ∗∗, $p < 0.01$. *B*, RT-PCR analysis of HPRT and IFN-γ gene expression in splenic cells from the same mice in a scanning of the blots. *C*, Densitometric analysis of the IFN-γ product after correcting for the expression of HPRT in rmIL-12-treated mice (filled columns) and PBS-treated controls (open columns). *D*, Levels of immunoreactive IFN-γ present in the sera of the same animals, which were treated (closed symbols) or untreated (open symbols) with rmIL-12; each symbol represents the result for an individual mouse.

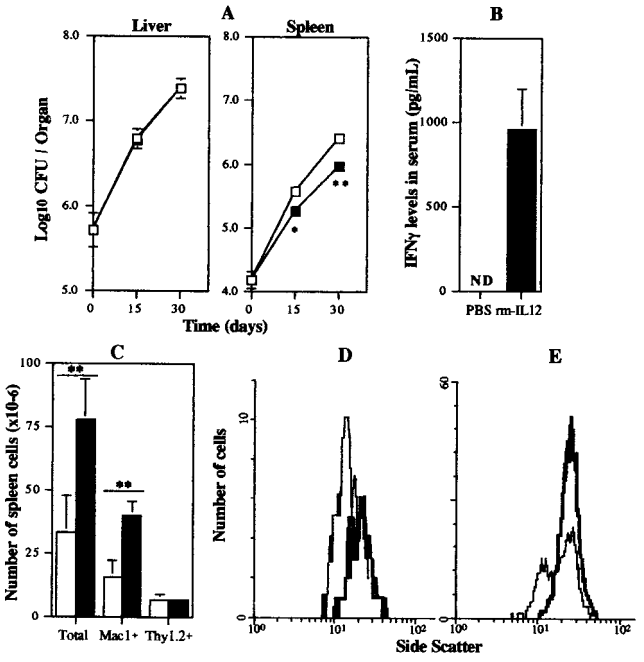

FIGURE 4. IL-12 stimulates innate defense against *M. avium*. *A*, Growth of *M. avium* 2447 in the organs of SCID mice treated i.p. with rmIL-12 (closed symbols) or PBS (open symbols) for 1 mo. Each timepoint represents the geometric mean of the CFU from four animals ± SD. Statistical differences are labeled as follows: ∗, $p < 0.05$; ∗∗, $p < 0.01$. *B*, Levels of immunoreactive IFN-γ present in the sera from the same animals (ND = not detectable by the ELISA technique used). *C*, Total number of cells isolated from the spleens of those animals at day 30 of infection and the number of $Mac1^+$ or of $Thy1.2^+$ cells after the exclusion of dead cells, as evaluated by FACS analysis (open columns represent controls and filled columns represent the rmIL-12-treated group). The side scatter analysis of $CD3^-Thy1.2^+$ (*D*) and $CD3^-Mac1^+$ (*E*) cells was performed after gating on those populations (thin lines represent controls and thick lines represent the rmIL-12-treated group).

Evaluation of the effects of rmIL-12 treatment on T cell-independent immunity

The early enhanced production of IFN-γ could be due either to an acceleration of the T cell responses or to the boosting of innate immunity, namely of those mechanisms involving cells of the NK cell lineage. To evaluate the effects of rmIL-12 administration on T cell-independent immunity, SCID mice were infected i.v. with 10^6 CFU of *M. avium* 2447 for 1 mo; 0.4 μg of rmIL-12 was administered every other day from the beginning of the infection to one group of animals. Fig. 4*A* shows the mycobacterial load in the spleen and liver of the animals infected for 30 days. The administration of rmIL-12 potentiated the protective response in the spleens of SCID mice. However, the magnitude of the reduction in *M. avium* proliferation induced by rmIL-12 treatment was smaller than the one previously observed with immunocompetent BALB/c mice. The sera of rmIL-12-treated SCID mice had detectable levels of immunoreactive IFN-γ; IFN-γ was not detected in the control mice (Fig. 4*B*).

The rmIL-12-treated SCID mice showed a more extensive splenomegaly than control mice at 30 days of infection (data not shown). We characterized the spleen cell population at 30 days of infection by cytofluorometric analysis with anti-CD3 (PE) plus anti-CD90/Thy1.2 (FITC) or anti-CD11b/Mac1 (FITC) staining. A gate on $CD3^-Thy1.2^+$ or $Mac1^+$ cell populations was used to characterize these populations in their complexity (side scatter). These studies showed that rmIL-12 induced an increase in the absolute number of $Mac1^+$ cells but no increase in the $Thy1.2^+$ population (Fig. 4*C*). This cytokine also induced an increase in the granularity of the $CD3^-Thy1.2^+$ and $CD3^-Mac1^+$ cell populations (Fig. 4, *D* and *E*, respectively), which suggests an activation of these cell populations.

Similar studies were performed on thymectomized BALB/c mice treated with anti-CD4 mAbs. Such mice, when given rmIL-12 in a protocol similar to that used in the SCID mice, showed reductions in splenic *M. avium* loads in two independent experiments of 0.3 and 0.7 $\log_{10}$ at day 15 and of 0.4 and 0.7 $\log_{10}$ at day 30 as compared with CD4-depleted mice that did not receive

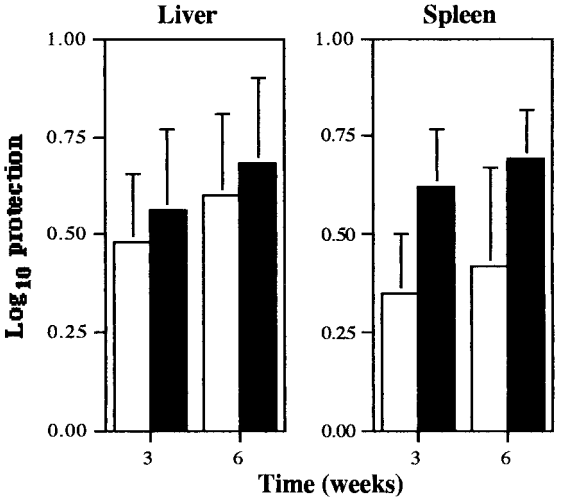

FIGURE 5. Exogenous IL-12 administration accelerates the emergence of T cells able to protect recipient mice against a *M. avium* challenge. The $\log_{10}$ protection afforded by *M. avium*-immune T cells from rmIL-12-treated (filled columns) and nontreated (open columns) animals is shown. Four mice were used per group of either donor or recipient animals. Statistically significant protection ($p < 0.01$) was found with all immune cells. Mice receiving 3 wk immune cells from the IL-12-treated group had significantly fewer viable counts than those receiving the control 3 wk immune cells. The total number of $CD4^+$ T cells adoptively transferred were 22.3×10^6 in controls and 11.4×10^6 in the rmIL-12-treated group for the 3 wk-infected donors and were 19.5×10^6 in controls and 4.3×10^6 in the rmIL-12-treated group for the 6 wk-infected donors.

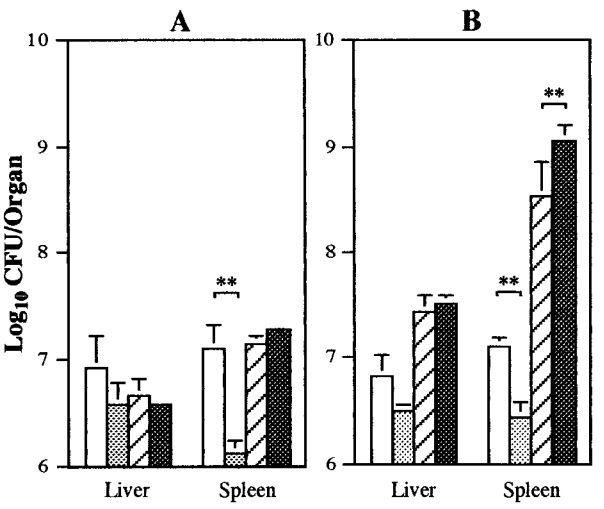

FIGURE 6. The protective effects of IL-12 are dependent upon the endogenous production of IFN-γ. *A*, Growth of *M. avium* 2447 at day 30 of infection in the organs of BALB/c mice treated i.p. with rmIL-12 or PBS for 1 mo and given either IFN-γ-neutralizing Abs or irrelevant Ig. *B*, Growth of *M. avium* 2447 in the organs of IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/-}$ mice infected for 30 days and treated i.p. with rmIL-12 or PBS for 1 mo. Open and striped columns represent PBS-treated mice, whereas stippled columns represent rmIL-12-treated mice. Anti-IFN-γ-treated animals (*A*) or IFN-$\gamma^{-/-}$ mice (*B*) are indicated by striped and heavily stippled columns. Mice that were given irrelevant Ig (*A*) or IFN-$\gamma^{+/-}$ mice (*B*) are indicated by open and lightly stippled columns. Results are shown as geometric means of CFU ± SD. Statistical differences are labeled as follows: **, $p < 0.01$.

the cytokine; this finding indicates that, in this model, the effects of rmIL-12 take place during the first 2 wk of treatment. Furthermore, at day 15 of infection, CD4-depleted mice had the same splenic mycobacterial loads as control thymectomized mice in either the cytokine-treated groups (5.22 ± 0.02 $\log_{10}$ CFU vs 5.24 ± 0.08 $\log_{10}$ CFU) or the groups that did not receive rmIL-12 (5.88 ± 0.02 $\log_{10}$ CFU vs 5.99 ± 0.14 $\log_{10}$ CFU), showing that the early protection at day 15 in this experiment was independent of $CD4^+$ T cells. Later on, however, the protection induced by IL-12 was higher in control compared with CD4-depleted animals. Thus, at day 30 of infection, the protection in the spleens of control mice was 1.1 $\log_{10}$, whereas protection in CD4-depleted animals was 0.7 $\log_{10}$.

Evaluation of the effects of rmIL-12 treatment on T cell-dependent immunity

To test the effects of the rmIL-12 treatment on the T cell populations, we studied the protective activity of T cell-enriched spleen cells from infected animals treated or untreated with the cytokine using a passive cell-transfer assay. A total of 16 donor mice were infected i.v. with 10^6 CFU of *M. avium* 2447 for 3 or 6 wk. Half of the animals were given rmIL-12 (0.4 μg/animal every other day for the entire period), and the other half were treated with PBS. Spleen cells from these immune animals as well as spleen cells from nonimmune controls were collected, enriched for T cells by depletion of Ia^+ cells and nylon wool-adherent cells, and infused into irradiated (500 rad) recipient animals. The latter were then challenged with 10^6 CFU of the same *M. avium* strain, and mycobacterial growth was evaluated 30 days later. We had already determined that the protection afforded by these cells can be abrogated by in vitro lysis of $CD4^+$ T cells by treatment with specific Abs and complement (data not shown). Fig. 5 shows that the injection of rmIL-12 in *M. avium*-infected donor mice accelerated the emergence of a population of T cells that were able to adoptively transfer protection to sublethally irradiated recipient mice.

Study of the protective mechanisms induced by rmIL-12 administration

To test whether the protective effect of rmIL-12 in immunocompetent animals was dependent upon IFN-γ, we compared the effects of rmIL-12 treatment on the growth of *M. avium* in BALB/c mice given neutralizing Abs vs respective controls or in IFN-γ gene-disrupted animals (IFN-$\gamma^{-/-}$) and normal heterozygous controls (IFN-$\gamma^{+/-}$). Mice were infected i.v. with 10^6 CFU of *M. avium* 2447 for 1 mo and treated every other day with either PBS or 0.4 μg of rmIL-12/animal. Fig. 6 shows the mycobacterial loads in spleens and livers of mice infected for 30 days. The protective effects of rmIL-12 in the spleen were dependent upon the endogenous production of IFN-γ. Such effects were particularly evident with the IFN-$\gamma^{-/-}$ mice, but similar results were obtained when immunocompetent BALB/c mice were used and specific neutralizing Abs were given to deplete IFN-γ. Fig. 7 shows the histologic analysis of the livers of these animals. Untreated IFN-$\gamma^{-/-}$ mice had a marked decrease in granuloma formation during infection as compared with control heterozygous mice. Treatment of mice with rmIL-12 increased granuloma formation both in control and IFN-γ-deficient animals. Analysis of the spleens showed invasion of the white pulp by macrophages during infection. Although granulomas were less easily discernible within the lymphoid mass, their number was enhanced by the infusion of rmIL-12 in both immunocompetent and IFN-$\gamma^{-/-}$ mice (data not shown).

Adjuvanticity of rmIL-12 in a subunit M. avium *vaccine preparation*

To evaluate the possible usefulness of IL-12 in the design of antimycobacterial vaccines, we selected secreted proteins from *M.*

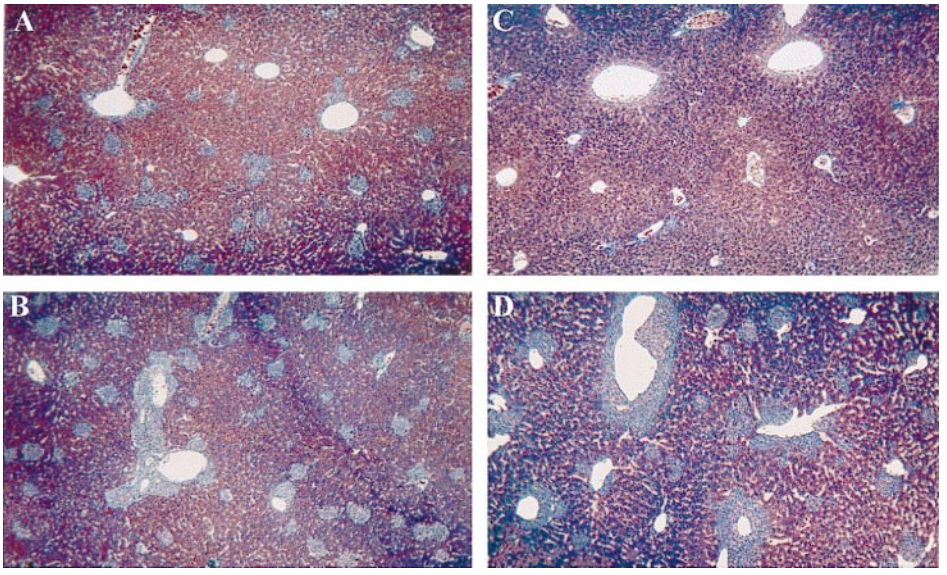

FIGURE 7. Histologic sections of infected livers of IFN-$\gamma^{+/-}$ (*A* and *B*) and IFN-$\gamma^{-/-}$ (*C* and *D*) mice treated with PBS (*A* and *C*) or rmIL-12 (*B* and *D*) after 30 days of infection. Note the paucity of inflammatory foci in untreated IFN-$\gamma^{-/-}$ mice and the increase in inflammatory cell recruitment in both strains of mice after IL-12 treatment. The photomicrographs represent typical views of the livers of groups of four animals.

avium as the Ag source. This choice was based on the previous work of several groups that described the effective use of such preparations in the induction of protective immunity against *Mycobacterium tuberculosis* (32). CFPs were injected either alone or in combination with DDA as an adjuvant (33). rmIL-12 was administered together with CFP or CFP plus DDA to subgroups of animals. Controls for the nonspecific, inflammatory-dependent induction of resistance to infection included the administration of rmIL-12 and DDA, both alone or in combination, as well as the vehicle, PBS. We chose to administer the cytokine with every dose of the vaccine, since preliminary results showed no benefit of a single dose during the first immunization (Ref. 33 and our unpublished observations). As shown in Fig. 8, significant resistance was conferred by the adjuvants alone in this experiment when the challenge took place 1 mo after the last immunization. This was particularly evident in the liver. However, all three preparations containing the Ag, CFP, admixed with DDA, rmIL-12, or both conferred significantly higher protection in the liver than any of the other preparations lacking Ag or the CFP treatment alone, without any adjuvant. In the spleen, in contrast, high levels of protection ($p < 0.01$ toward all other groups) leading to 10-fold lower numbers of mycobacteria were only obtained when CFP was administered in combination with both DDA and rmIL-12.

Discussion

We describe here our assessment of the usefulness of rIL-12 as an immunotherapeutic drug against *M. avium* infections as well as its role as an adjuvant in a subunit vaccine against that pathogen. We used therapeutic regimens that are similar to those used by other researchers and that resulted in beneficial effects without the toxic ones associated with higher doses of IL-12 administration. We found that rIL-12 afforded significant but very limited protection in the spleens of mice infected with either of two strains of *M. avium* of low virulence. Alternatively, the growth of *M. avium* in another target organ, the liver, was not consistently affected by treatment. Also, protection was predominantly afforded by early rather than delayed treatment as tested with one of the strains. However, no valuable protective effects were found during the treatment of an infection by a highly virulent strain of *M. avium*. These results are in tune with our findings and those of others that those latter virulent strains resist the antimycobacterial activity of IFN-γ-activated macrophages (Refs. 34 and 35 and our unpublished observations). Therefore, further activation of those pathways is without success in the control of such infections. The effects of rIL-12 administration to mice infected with *M. avium* have already been studied by Kobayashi et al. (19). They found that IL-12 administered in a regimen very similar to the one used here for strain 1983

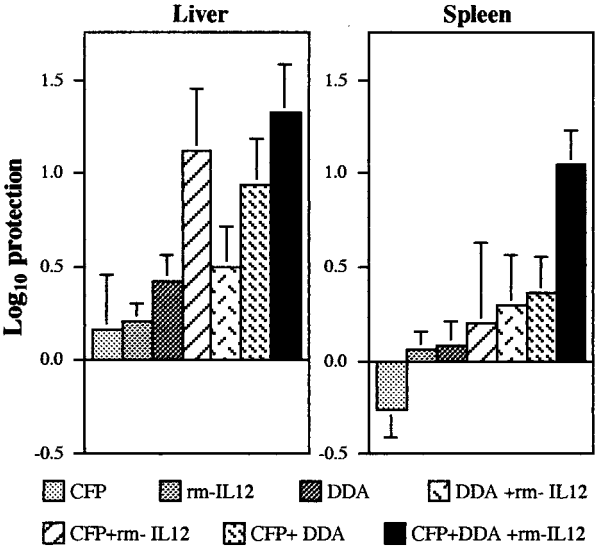

FIGURE 8. Protection afforded by the CFP-based vaccine admixed with either DDA, rmIL-12, or both as well as the respective controls. Mice were injected s.c. three times at weekly intervals with the preparations shown. Animals were challenged i.v. with *M. avium* 2447 at 1 mo after the last immunization, and mycobacterial loads were evaluated 30 days later. Results are shown as $\log_{10}$ protection, which was calculated by subtracting the mean $\log_{10}$ CFU in the treated group from the mean $\log_{10}$ CFU in PBS-treated controls. Therefore, negative values represent exacerbation of the infection by the treatment. In the liver, the three preparations, which contained the Ag (CFP) admixed with DDA ($p < 0.05$ against DDA plus IL-12 and $p < 0.01$ against all other groups not containing CFP or against CFP alone), rmIL-12 ($p < 0.05$ against DDA plus IL-12 and $p < 0.01$ against all other groups not containing CFP or against CFP alone), or both ($p < 0.01$ against all groups not containing CFP or against CFP alone), gave statistically significant higher protection. In the spleen, higher levels of protection were found when CFP was administered in combination with both DDA and rmIL-12 ($p < 0.01$ vs all other groups).

(a strain chosen to have a virulence similar to that of the strain used in their study (36)) led to a killing of the mycobacteria in the spleens of infected animals. We corroborate their observations, but stress that the efficacy of the IL-12 treatment is clearly dependent upon the characteristics of the particular strain of *M. avium* studied. This information may turn out to be of importance if IL-12 is considered for human immunotherapy. We found no clinically apparent toxic effects of prolonged IL-12 therapy, but histologic analysis of the infected livers showed a marked increase in the granulomatous response. This enhancement was partially independent of the infection itself, since we observed marked cellular infiltration and granuloma formation in the livers of uninfected mice treated with similar regimens of rmIL-12 (our unpublished observations). Part of the toxicity of IL-12 was reported to be dependent upon the triggering of TNF-α production (37). Since TNF-α is also involved in resistance to *M. avium* infection (27), the neutralization of this cytokine during IL-12 therapy to decrease the toxicity of therapy would not be warranted. The limited effects of rmIL-12 therapy on the overall growth or killing of mycobacteria have also been documented for other mycobacterial species. In one report, the protection afforded by rmIL-12 against a tuberculous challenge in BALB/c mice was also limited to one $\log_{10}$; however, in that model, such improvement of the control of the infection prolonged the survival of the mice (20). Cooper et al. (1, 18) have also described low levels of protection in an i.v. challenge model of murine tuberculosis. Here, we found that the less virulent the strain of *M. avium*, the bigger the protection conferred by rmIL-12 therapy. Finally, a recent report that appeared after our initial submission of this work shows similar levels of protection after IL-12 therapy of SCID mice infected with *M. avium* (38). In that study, IL-12 was shown to potentiate the efficacy of chemotherapy.

We used strain 2447 to dissect the basis of the protective effect afforded by IL-12. Such protection was dependent both on the induction of innate mechanisms involving the activation of macrophages and NK cells and on the acceleration of the T cell response. Both cell types were responsible for the enhanced early production of IFN-γ that was required for the decrease in mycobacterial proliferation. This was shown using SCID mice or CD4-depleted mice to evidence the innate immunity mechanisms and by performing an adoptive transfer of protection to highlight the role of T cells. Also, the protective effects of IL-12 therapy were higher in immunocompetent than in T cell-deficient animals and were only expressed against strain 2447 when the cytokine was given at a time when T cell-mediated immunity was emerging, rather than when it was already present (i.e., at wk 3 of infection) (Fig. 1, *A* vs *B*). However, the relative importance of either of the protective mechanisms triggered by IL-12 in an immunocompetent mouse was not clear.

In accordance with other studies (20), we found that the protective effect of IL-12 was dependent upon the endogenous production of IFN-γ and was associated with an increased development of granulomatous inflammation in the liver. In the absence of IFN-γ, IL-12 was also able to increase the recruitment of inflammatory cells despite its inability to protect from infection. A similar dependence of the effects of IL-12 on the endogenous production of IFN-γ has also been found in other infectious models (10–12, 14, 16, 17).

More promising to our point of view than the immunotherapeutic potential of rmIL-12 were the findings of the potentiation of the protective immune responses elicited by CFPs by IL-12 coadministration. Although the results are clearly preliminary and more extensive studies should be performed to assess the longevity of the protective immunity generated, it is interesting to note that protection of one $\log_{10}$ or more was found in both the target organs studied only when rmIL-12 was included in the vaccine. It will be important to subject the animals to a more prolonged resting period between immunization and challenge to eliminate the nonspecific protection induced by preparations devoid of Ag, which were studied here to control for the specificity of the vaccine. The limited albeit significant protection afforded by the adjuvant preparations alone in this experiment was most likely due to the accumulation of inflammatory cells at the sites of infection induced by the presence of the adjuvants given only 1 mo before the infection. These cells may be producing cytokines that play a modest protective role on the subsequent infection. Nevertheless, despite the existence of such nonspecific protection, the Ag-specific protection was clearly above the former; most of all, it could only be found in the spleen if rmIL-12 was included in the vaccine. It was curious to note that although the protection afforded by immunotherapy during infection was particularly evident in the spleen, the adjuvant effects of IL-12 were more marked in the liver of the animals. The superiority of the immunotherapy in the spleen can be explained by the later emergence of protection in this organ during infection as compared with the liver. Therefore, treatment with rmIL-12 accelerates protective mechanisms, so that protection appears sooner in the spleen, while protection in the liver may not be further accelerated. This easier recruitment of T cell-mediated protection to the liver as compared with the spleen can also be seen with the vaccinated animals where suboptimal regimens (CFP with only one of the adjuvants) were already effective, whereas the combination of the three components of the vaccine was necessary to boost the protective efficacy of the immunization to act in the spleen.

In summary, we illustrate the variable protection afforded by immunotherapy with rmIL-12 in murine models of *M. avium* infection; this protection depended upon the virulence of the mycobacterial strains being tested. The protection was dependent upon the endogenous production of IFN-γ produced by cells from the NK cell lineage or from protective T cells that emerged more rapidly when the cytokine was provided exogenously. The inability to boost the peak of maximum IFN-γ response was probably the reason for the limited protection afforded by the cytokine. However, the use of rmIL-12 as a coadjuvant in vaccine preparations was a most promising one.

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