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

Regina A. Silva, Teresa F. Pais, and Rui Appelberg

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/mm³ (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 × 10⁶ 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. CB-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.
were purchased from Bomninci (Ry, Denmark), and IFN-gamma gene-

disrupted (IFN-γ−/−) 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 ob-

tained 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 centrifuga-

tion, washed in staining medium containing 0.04% Tween 80, and sus-

pended in staining medium containing propidium iodide to identify and 

exclude dead cells. Flow cytometric analysis was performed with a FACSsort 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, 106 cells from the T cell 

pools were stained with PE-conjugated anti-mouse CD4 and FITC-conju-

gated 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 embed-

ded in paraffin. Next, 3-μm-thickness 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 differ-

ences in the number and size of granuloma structures as well as for the 

presence or absence of acid-fast rods. Representative fields were pho-

tographed 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 

processing. Total mRNA was reverse transcribed using p(dT)12–18 oligo-

nucleotides (Pharmacia Biotech, Uppsala, Sweden) as primers and Super-

script first-strand synthesis kit (Life Technologies) with 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 phosphori-

bosyltransferase (HPRT) or IFN-γ in a Gene Amp PCR System 9600 (Per-

kin 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 stan-

dardization 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-32P]dCTP. Membranes were exposed, and photo-

diagram plates were read with the aid of a computer-assisted scanner. Val-

ues 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 super-


nenatants were concentrated 100-fold through ultrafiltration using filters 

with a molecular mass cutoff of 3 kDa. The resulting preparation was then 

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.

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 immunohistochemistry double staining, 106 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-phycocerythrin (PE)-conjugated ham-

ster anti-mouse CD3e 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 FACSsort 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, 106 cells from the T cell 

pools were stained with PE-conjugated anti-mouse CD4 and FITC-conju-

gated anti-mouse CD8 (PharMingen) as described above.
BALB/c mice were infected i.v. with $10^6$ CFU of either of two infection $M. avium$

Evaluation of the effects of rmIL-12 administration during

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 $\mu$g 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. 1A). 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 shown in Fig. 1A, 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 $\mu$g/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. 3A, 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. 3B 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. 3D).
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 10⁶ 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. 4A 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. 4B).

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⁺ cells 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. 4C). 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 log₁₀ at day 15 and of 0.4 and 0.7 log₁₀ at day 30 as compared with CD4-depleted mice that did not receive...
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 5.54 ± 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 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* 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. Later on, 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$_{10}$.

**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-γ$^{-/-}$) and normal heterozygous controls (IFN-γ$^{+/-}$). Mice were infected i.v. with 10$^6$ CFU of *M. avium* 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-γ$^{-/-}$ 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-γ$^{-/-}$ 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-γ$^{-/-}$ mice (data not shown).

**Adjutivancy 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.
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.
(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 corroborated 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<sub>10</sub>; 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<sub>10</sub> 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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