0022-1767/93/15110-5425$02.00/0
The Journal of Immunology

Vol 151, 5425–5430, No. 10, November 15, 1993
Printed in U.S.A.

IL-10 Neutralization Augments Mouse Resistance to Systemic *Mycobacterium avium* Infections[1]

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Abstract. In this contribution, we examined the involvement of the cytokine IL-10 in the progression of experimental murine *Mycobacterium avium* infections in susceptible BALB/c mice. Addition of anti-IL-10 antibodies in the supernatants of peritoneal macrophages infected with virulent *M. avium* resulted in a significantly enhanced mycobacteriostatic activity of macrophages. In BALB/c mice infected with the B101 or B102 virulent *M. avium* strains, examination of the cytokine release profile in splenocytes from infected mice showed that infection was associated with an initial copious release of both IFN-γ and IL-10. IL-10 production increased as the infection progressed, whereas IFN-γ levels diminished. Infected mice were given repeated infusions of a rat mAb against mouse IL-10 or rat IgM. Examination of IgM serum levels in anti-IL-10-treated mice (infected or not) showed that depletion of endogenous IL-10 resulted in much decreased IgM levels. Results showed that infusions of large dosages of the monoclonal anti-IL-10 resulted in a very significantly diminished bacterial growth in the spleens. These findings indicate that IL-10 may have a negative impact on resistance to *M. avium* infections, due, at least in part, to decreased macrophage activity. *Journal of Immunology,* 1993, 151: 5425.

Infections with the opportunistic pathogen *Mycobacterium avium* intracellulare constitute an important health problem for immunosuppressed patients, especially in subjects with AIDS (1, 2). Such infections constitute an important problem, because many strains of *M. avium* are highly resistant to first-line antituberculous drugs; the patients involved often require the administration of four to five drugs in combinations that can be quite toxic (2).

Histologic evidence suggests that virulent *M. avium* infect and replicate within cells of the mononuclear phagocyte series (1, 2). Data obtained in human and mouse cells suggest that the intramacrophage growth of virulent strains of *M. avium* is very difficult to modulate positively (3, 4). IFN-γ is the main lymphokine that has macrophage-activating activity against many microbes, but it does not universally enhance bacteriostatic activity against virulent *M. avium* strains (4). Moreover, growth of *M. avium* occurs in humans with AIDS, despite evidence of macrophage activation in the blood and infected organs of these patients as well as cytokine release (5). This raises the question as to whether some immune processes are actually promoting mycobacterial growth.

IL-10 is a key cytokine that is secreted primarily by cells of the macrophage lineage and also by cells of the TH2 subset (6 and references therein). Among its recently discovered functions include mast cell activation (7), macrophage deactivation (8), and blocking of IFN-γ release by TH1 cells as well as activation of B cell subsets (9). The exact role of IL-10 in microbial infections has not been fully elucidated. It is clear, however, that IL-10 is synthesized in tissues of susceptible mice infected with intracellular pathogens (10), and also that IL-10 diminishes the microbicidal activity of macrophages (11).

In this study, we examined the contribution of IL-10 in experimental *M. avium* infections.

Materials and Methods

Mice

Female BALB/c mice (Charles River Inc., St. Constant, Canada) weighing 18 to 20 g were used. They were fed Purina Chow and acidified tap water ad libitum.

Received for publication March 19, 1993. Accepted for publication August 7, 1993.

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[1] This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada, Health and Welfare Canada (NHRDP), the Quebec Lung Association, and the Fonds de la recherche en santé du Québec (FRSQ). M.D. is an FRSQ scholar.

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Peritoneal macrophage-*M. avium* interaction

This in vitro system was recently described in detail elsewhere (12). Briefly, resident peritoneal macrophages were obtained by lavage of the peritoneal cavity. Two $\times 10^6$ macrophages/ml were resuspended in Neumann-Tytell medium (GIBCO, Grand Island, NY) supplemented with 4 mM glutamine and 5×10^5 M 2-ME (Sigma, St. Louis). Cells were plated at 100 μl/well in 96-well microtiter plates (Linbro, McLean, VA). Plates were incubated for 2 h at 37°C/5% CO_2, and nonadherent cells removed by washings. Medium was then replaced with complete medium containing 10^6 CFU of *M. avium*. After 6 h, wells were washed extensively in warm HBSS. Plates were incubated for indicated periods at 37°C/5% CO_2. Cells were lysed by adding 0.2 ml of medium supplemented with 0.2% saponin. Lysates were plated on 7H10 agar (Difco, Detroit, MI) as described elsewhere (12). Supernatants were sampled at intervals, and plating of extracellular medium showed the presence of less than 5% of the *M. avium* found in the cell monolayers. In selected experiments, 50 μg of anti-IL-10 mAb or control rat IgM were added to the medium 6 h before infection and daily afterward.

In vivo infections

Mice were infected with 10^5 CFU of *M. avium* B101 or B102, both virulent strains (13). Strains were maintained in vitro as described elsewhere (12). The infection was assessed by plating spleen homogenates on 7H10 agar, as described elsewhere (13). Endogenous IL-10 was depleted by injecting 2 mg weekly of the SXC.1 antibody in PBS (14), beginning 1 wk before infection. Control mice received the same dose of purified rat IgM antibodies (Immunocorp, Montreal) or boiled SXC.1 antibodies.

Anti-IL-10

SXC.1 and SXC.2 hybridomas secreting rat IgM anti-mouse IL-10 were obtained from DNAX Institute (Palo Alto, CA). The antibodies were obtained from serum-free hybridoma supernatants, purified by two sequential ammonium sulfate (35%) precipitation steps and affinity chromatography. Resuspended antibody contained 310 EU endotoxin/ml. Pure rat IgM was free of detectable endotoxin.

IL-10 measurements

SXC.1 anti-IL-10 (5 μg/ml) was coated overnight on Immulon-2 plates (Costar, Toronto). After washing, supernatants or standard IL-10 were applied. Captured IL-10 was detected using a biotinylated SXC.2 anti-IL-10 and avidin-conjugated horseradish peroxidase (Pierce Chemicals, Bedford, IL) and 1 mg/ml of 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonate] (Sigma) as a substrate. This procedure was adapted from Gazzinelli et al. (15).

IFN-γ measurements

IFN-levels were determined by ELISA. Briefly, plates were coated with anti-IFN-γ R46A2 (ATCC HB 170; 5 μg/ml), supernatants or standard added, and bound IFN-γ revealed by adding 0.5 μg/ml of biotin-labeled XMG1.2, a rat IgG1 anti-mouse IFN-γ antibody (16), and a conjugate of streptavidin-horseradish peroxidase (Pierce). This procedure was adapted from Muralidhar et al. (17). Results for both IL-10 and IFN-γ are expressed in ng/ml in reference to standards provided by DNAX Institute for IL-10 and the Biological Response Modifiers Program (Frederick, MD) for IFN-γ.

Splenocyte supernatants

Splenocytes were removed and a single-cell suspension prepared. RBC were lysed with NH_4Cl. Cells were incubated in 1.0 ml of RPM1 1640 medium with 10% FCS and antibiotics at 5×10^5 cells/well in 24-well plates (Linbro). Con A (5 μg/ml, Sigma) or *M. avium*-sonicated Ag (15 μg/ml) were used for stimulation. *M. avium* was suspended at 2 mg/ml in isotonic PBS with BSA (1 mg/ml) then vigorously sonicated (Heat Systems/Ultrasonics), centrifuged, filtered (0.22 μm Millipore filter), and stored at −20°C. After 24 h, supernatants were removed and frozen at −70°C before cytokine measurements.

IgM measurements

Sera IgM levels were assessed by the radial immunodiffusion technique (18). Antisera and control IgM were obtained from Sigma.

Determination of anti-IL-10 activity in the sera of treated mice

Spleen cells from normal animals were obtained and adjusted at 5×10^6 leukocytes/ml in RPMI 1640 (GIBCO) with antibiotics, 5% FCS, and Con A (5 μg/ml) in the presence of increasing concentrations of IL-10 and test sera. Supernatants were obtained at 24 h and IFN-γ levels were measured by ELISA (see above). This CSIF[3] assay assessed IL-10 bioactivity and its inhibition by sera from treated mice.

Statistical analysis

Differences between groups were analyzed by Student's *t*-test or by analysis of variance.

[3] Abbreviations used in this paper: CSIF, cytokine synthesis inhibitory factor.

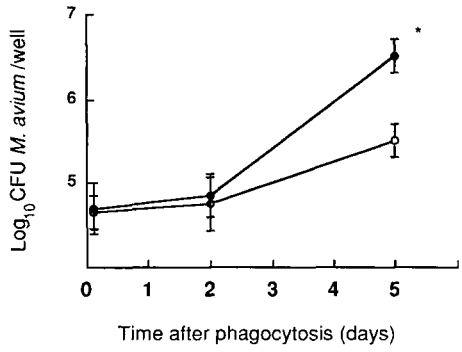

FIGURE 1. Endogenous macrophage IL-10 augments intramacrophage growth of *M. avium*. Macrophages from BALB/c mice were infected with *M. avium* B102 and anti-IL-10 antibodies (○) or rat IgM (●) added at 5 μg/ml daily. Results are means ± SE of five determinations from three separate experiments, $^{*}p < 0.01$, Student's *t*-test.

Results

IL-10 Increases macrophage susceptibility to *M. avium*

In a first set of experiments, we examined the ability of anti-IL-10 antibodies to modulate the mycobactericidal/mycobacteriostatic activity of resident peritoneal macrophages (Fig. 1). Examination of cell supernatants from *M. avium*-infected macrophages showed that after 5 days of infection, supernatants contained 42 ± 15 ng/ml of IL-10, whereas uninfected macrophages released 3 ± 1 ng/ml of IL-10 (both data are means ± SE of 4 experiments with triplicate determinations). This establishes that *M. avium* infection induces secretion of IL-10, well above control values. Figure 1 shows that including anti-IL-10 antibodies (5 μg daily) in the supernatants resulted in significantly diminished *M. avium* growth in macrophages (Fig. 1). The difference in mycobacterial growth was 0.8 to 1.0 $\log_{10}$ CFU, and was reproducible in three separate experiments. At all times sampled, macrophage numbers and cell viability were determined by trypan blue exclusion; these parameters were similar for all infected cells, whether or not they were treated with anti-IL-10.

IFN-γ and IL-10 release by splenocytes from *M. avium*-infected mice

Next, we examined the ability of spleen cells to release IFN-γ and IL-10 in the course of *M. avium* B102 infections (Fig. 2). Splenocytes were stimulated with 5 μg/ml of Con A or with *M. avium* Ag, and supernatants were sampled and frozen before cytokine analysis. Data obtained showed that *M. avium* infection resulted in a transient increase in Con A-driven IFN-γ production, peaking at day 30 and a significant decrease until day 75, when IFN-γ levels were lower than day 0 values ($p \leq 0.05$). Conversely, IL-10

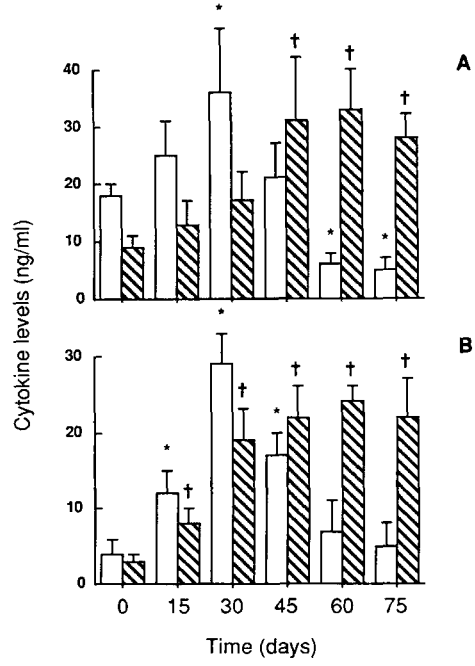

FIGURE 2. IL-10 (□) and IFN-γ (□) activity in stimulated spleen cell supernatants from mice infected with *M. avium* B102. Data are from cells stimulated with Con A (5 μg; *A*) or from *M. avium* sonicates (*B*). Data from three separate experiments are shown; each point represents pooled spleen cells from six mice. $^{*\dagger}p < 0.05$ vs day 0 IFN-γ and IL-10 values (analysis of variance).

values increased steadily during infections (Fig. 2*A*). Figure 2*B* shows splenocyte release of IFN-γ and IL-10 in response to *M. avium* Ag; IFN-γ release augmented until day 30, after which they returned to control values, whereas IL-10 levels steadily increased.

Depletion of endogenous IL-10 leads to decreased serum IgM levels

Next, we examined levels of serum IgM in mice given anti-IL-10 or a control rat IgM, infected or not with *M. avium*. The rationale for this was the recently described depletion of IgM levels after anti-IL-10 treatment (14).

Figure 3 shows that a 5-wk treatment with anti-IL-10 antibodies resulted in a significant decrease in serum IgM levels, both in uninfected mice and in mice infected with *M. avium*. These data suggest that endogenous IL-10 was successfully depleted. In separate experiments, we determined the presence of high levels of anti-IL-10 activity by the CSIF assay (see Materials and Methods) in the sera of treated mice at 75 days, whereas mice that received rat IgM had no such activity (data not shown).

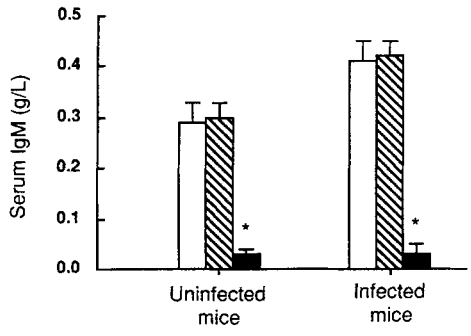

FIGURE 3. Sera IgM levels in the experimental groups. Mice were left uninfected or were infected with 10^5 CFU *M. avium* B102 for 75 days. □, untreated; ▨, treated with rat IgM; ■, treated with SXC.1. Data are means ± SE of results from eight mice. *$p < 0.01$ vs two other groups (analysis of variance).

Depletion of endogenous IL-10 leads to decreased *M. avium* growth in the spleens

In a last set of experiments, we examined the ability of anti-IL-10 antibodies to modulate *M. avium* infections in vivo (Fig. 4). Data obtained showed that neutralizing endogenous IL-10 led to a significant reduction in *M. avium* B101 growth in the spleens, with a 1.5 $\log_{10}$ reduction in CFU at day 100 (Fig. 4*A*). Similarly, B102 growth was very significantly reduced by IL-10 neutralization (3 $\log_{10}$ reduction in spleen CFU at day 10; Fig. 4B). Examination of sera of *M. avium* B102-infected mice at day 100 showed that anti-IL-10-treated mice had 8 ± 4 ng/ml of IFN-γ (mean ± SE, $n = 8$) whereas infected mice given control rat IgM had 1.2 ± 0.6 ng/ml (mean ± SE, $n = 7$) ($p \leq 0.001$, *t*-test). Repeated injections of boiled SXC.1 antibodies failed to modify *M. avium* infections (data not shown).

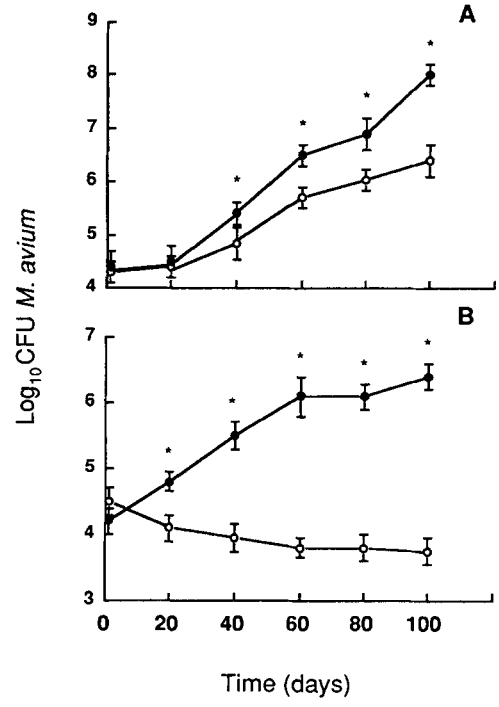

FIGURE 4. *A*, Endogenous IL-10 increases *M. avium* B101 growth in mice. Mice received 10^5 CFU of *M. avium* B101 by the i.v. route and repeated i.p. infusions of anti-IL-10 antibodies (○) or rat IgM (●). Each point is the mean ± SE of six mice. *$p < 0.05$ (Student's *t*-test). Repeated twice with identical results. *B*, Endogenous IL-10 increases *M. avium* B102 growth in mice. Mice received 10^5 CFU of *M. avium* B102 by the i.v. route and repeated i.p. infusions of anti-IL-10 antibodies (○) or rat IgM (●). Each point is the mean ± SE of five mice. *$p < 0.05$ (Student's *t*-test). Repeated twice with identical results.

Discussion

This contribution provides evidence that IL-10, a cytokine produced by activated macrophages and TH2 lymphocytes, may play a negative role in the progression of *M. avium* infections. IL-10 has been shown to exert a number of pleiotropic effects on cells of the immune system (6). IL-10 inhibits macrophage-dependent synthesis of cytokines by TH1 cells and acts as growth factor for mast cells and B cell subsets (6, and references therein). It also down-regulates macrophage production of cytokines such as TNF-α and IL-1 (19) as well as macrophage release of toxic nitrogen reactive intermediates (20).

Endogenous IL-10 production by mouse macrophages was shown to diminish macrophage effector function against *M. avium* (Fig. 1). Recent data has shown that IL-10 could block macrophage killing of *Trypanosoma*, *Toxoplasma*, and *Schistosoma mansonii* (10, 20). However, in these microbicidal mechanisms, IL-10 blocked IFN-γ-mediated killing and the importance of endogenous IL-10 was not examined. Increase of macrophage susceptibility by depletion of endogenous IL-10 by macrophages may have resulted from an increased synthesis of reactive molecules (H_2O_2, NO) that kill intracellular bacteria, or via an increased monokine synthesis that amplified these antimicrobial systems. *M. avium* infection in vitro determined a large release of IL-10 by infected cells. It remains to be determined which microbicidal mechanism(s) was enhanced via IL-10 depletion.

The SXC.1 administration resulted in greatly diminished IgM levels in the sera of control and infected mice, as demonstrated recently (14). The sera of mice infused with anti-IL-10 contained significant amounts of anti-IL-10 activity, based on neutralization of IL-10 bioactivity in a CSIF assay. Also, examination of sera from anti-IL-10 and control mice showed the presence of appreciable amounts of IFN-γ in the sera of anti-IL-10-treated mice, despite a much larger

bacterial load in control mice. These findings suggest a successful IL-10 neutralization. A negative role for this cytokine in *M. avium* infections was clearly shown in vivo. It is of some interest to note that high levels of IL-10 were generated by spleen cells in a mouse model of AIDS (15). Also, recent data suggest that B cells from AIDS patients constitutively secrete high levels of IL-10 (21). This enhanced IL-10 releasability may be partly associated with the increased susceptibility of AIDS patients to systemic *M. avium* infections, as well as infections with other pathogens.

M. avium infection was associated with a transient increase in splenocyte IFN-γ production, which diminished as infection progressed (day 100), whereas mitogen- and Ag-driven IL-10 levels progressively increased. These data suggest a process whereby TH1 or TH0 subsets of cells are activated in the early stages of the disease, followed by copious IL-10 release, which may be linked to a high level of macrophage activation. It has been shown that IFN-γ and IL-10 block each other's release (22). IL-10 production associated with susceptibility to an infectious agent has already been described for *Trypanosoma cruzi* (10). This argues for an association between TH1-protective mechanisms and TH2-induced susceptibility, similar to findings in infections with *Leishmania* and *Listeria* (23, 24). However, in contrast to findings with these microbes, we have been unable to increase the resistance of *M. avium*-infected mice with large doses of in vivo anti-IL4 antibodies (unpublished data). Also, IL-4 may also act as a co-factor to enhance mouse macrophage mycobacteriostatic activity (13). However, many cells other than $CD4^+$ lymphocytes release IL-10 and IFN-γ, most notably macrophages and NK cells, respectively. The complete picture of host resistance to such a complex pathogen as *M. avium* is likely to be complicated, because cytokines have so many biologic activities (25). For example, the growth of some strains of *M. avium* in the organs of T cell-deprived mice is relatively similar to their growth in the organs of immunocompetent mice (26, 27).

While this manuscript was being revised, Bermudez and Champsi (28) reported that injection of a rabbit polyclonal antiserum against IL-10 rendered beige mice more susceptible to dissemination of an intestinal infection with a strain of *M. avium* (4 wk after inoculation). No control was provided to assess the success of the IL-10 neutralization procedure. Our results confirm and considerably expand these observations in a distinct experimental model; we demonstrate a role for endogenous IL-10 in macrophage resistance to *M. avium* nitrocellulare, and provide the growth kinetics of a systemic infection in mice treated with a rat mAb to IL-10, a reagent potentially more specific than rabbit serum.

Other cytokines generated in the course of *M. avium* infections favor bacterial growth, notably transforming growth factor-β, which enhances in vivo and in vitro growth of virulent strains of *M. avium* (13). In vitro studies have shown that IL-10 and transforming growth factor-β synergize with each other to block macrophage antimicrobial activity (11). Thus, *M. avium* infections are characterized by the recruitment of a cytokine profile that augments immunosuppression; interestingly, these same cytokines are generated in copious amounts in AIDS patients (21, 29). It is possible that antagonists of disease-promoting cytokines may become useful in enhancing host resistance.

Acknowledgments

We thank E. Shavers for technical assistance.

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