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IL-10 Underlies Distinct Susceptibility of BALB/c and C57BL/6 Mice to *Mycobacterium avium* Infection and Influences Efficacy of Antibiotic Therapy[1]

Susana Roque,*† Claudia Nobrega,†‡ Rui Appelberg,†‡ and Margarida Correia-Neves[2]*†

Increased production of IL-10 has been frequently associated with augmented susceptibility to infection. However, the correlation between IL-10 activity and susceptibility to mycobacterial infection is still uncertain. Although studies using transgenic mice overexpressing IL-10 consistently showed an increased susceptibility to mycobacterial infection, experimental approaches in which IL-10 activity was reduced or abrogated originated inconclusive data. We show here that this controversy might be due to the mouse strains used in the various experimental procedures. Our results show that BALB/c mice are more susceptible than C57BL/6 to *Mycobacterium avium* infection. This increased susceptibility of BALB/c mice is, to a great extent, due to distinct activity of IL-10 between the two mouse strains. In accordance, reduction of IL-10 activity through the administration of anti-IL-10R mAb, or the absence of IL-10 as studied in IL-10 knockout mice, clearly decreased the susceptibility of BALB/c mice to *M. avium* but had a less obvious effect in C57BL/6 mice. Moreover, abrogation of IL-10 activity in infected BALB/c mice increased the efficacy of antimycobacterial therapy, whereas for the C57BL/6 mice it produced no effect. These observations show that the activity of IL-10 in response to the same mycobacterial stimulus influences not only the susceptibility to infection but also the efficacy of antimycobacterial therapy. This should now be considered in the context of human response to mycobacterial infection, particularly as a possible strategy to improve treatment against infections by mycobacteria. ***The Journal of Immunology,*** **2007, 178: 8028–8035.**

Mice have been extensively used to study the immune response to infection and the availability of numerous inbred strains has allowed the establishment of models of resistance or susceptibility to infection by many different microbial pathogens. A classical example is the use of different mouse strains to analyze *Leishmania major* infection, which led to the in vivo demonstration of the Th1/Th2 paradigm and the relationship between T cell subsets and resistance/susceptibility to this pathogen. In the *L. major* model, C57BL/6 mice are more resistant whereas BALB/c mice are fully susceptible (1). A similar dichotomy between the resistance/susceptibility of these two mouse strains has also been found in experimental infections by *Listeria monocytogenes* (2) and by *Yersinia enterocolitica* (3).

One of the cytokines strongly associated with the increased susceptibility to infection in mice is IL-10 (4–7). This cytokine is essential for dampening the inflammatory response once the infection has been controlled (8) and is produced by different populations of T and B lymphocytes, macrophages, and dendritic cells. The most obvious effect of IL-10 is to inhibit the production of proinflammatory cytokines by different cells of the immunological system (8). The role of IL-10 in determining the host's susceptibility to infection by intracellular pathogens has thus drawn much attention in recent years (8, 9). Studies with *L. monocytogenes*, using either transgenic mice overexpressing IL-10 or the administration of rIL-10 to wild-type mice, have shown that IL-10 activity directly correlates with susceptibility to infection (10, 11). In agreement, blocking IL-10 activity enabled infected mice to control infection with an otherwise lethal dose of *L. monocytogenes* (6). Similarly, increased resistance to infection was also observed for *Leishmania* spp. upon inhibition of IL-10 activity (4, 12, 13). Overall, these studies were concordant and showed that IL-10 increases susceptibility to infection by certain intracellular pathogens (9).

The role of IL-10 in determining susceptibility to mycobacterial infections is still not clear. Whereas transgenic mice overexpressing IL-10 were consistently more susceptible than wild-type mice to infection by *Mycobacterium bovis* bacillus Calmette-Guérin, *Mycobacterium avium*, or *Mycobacterium tuberculosis* (14–16), depletion studies generated less consistent data. Thus, although the use of IL-10- or IL-10R-blocking Abs led to a reduced multiplication of *M. avium* (17–19), the analysis of IL-10 knockout (KO)[3] mice showed that they could be either more resistant to infection or as resistant as wild-type animals (20–24). Among the possible reasons for the discrepancies observed is the genetic background of the different mouse strains used in the various studies. Thus, although studies assessing the role of IL-10 in susceptibility to mycobacteria using blocking Abs used either the C57BL/6 or the BALB/c strains, all studies on IL-10 KO animals were done using

*Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus Gualtar, Braga, Portugal; †Laboratory of Microbiology and Immunology of Infection, Instituto de Biologia Molecular e Celular, University of Porto, Porto, Portugal; and ‡Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Porto, Portugal

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[2] Address correspondence and reprint requests to Dr. Margarida Correia-Neves, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal. E-mail address: mcorreianeves@ecsaude.uminho.pt

[3] Abbreviations used in this paper: KO, knockout; CBA, cytometric bead array; BMDM, bone marrow-derived macrophage.

pure C57BL/6 or mix C57BL/6.129 backgrounds. In fact, mycobacterial infection has been shown to differentially influence IL-10 production in different mouse strains; BALB/c mice present higher secretion than C57BL/6 (25). Results from Turner et al. (16) also showed that CBA/J mice produce more IL-10 within macrophage-rich tuberculosis lesions and exhibit higher mycobacterial loads in the lung when compared with C57BL/6 mice.

The relevance of IL-10 in mycobacterial infections has gained renewed interest given a set of observations in humans that repetitively associated the increased production of IL-10 with increased susceptibility to infection. This has been the case for *M. avium* infection for HIV-coinfected patients (26), for patients with tuberculosis (27–30), and for *Mycobacterium ulcerans*-infected patients (31, 32).

In this study, we show that BALB/c mice are more susceptible than C57BL/6 mice to *M. avium* infections and relate this difference in susceptibility with distinct IL-10 activity. Moreover, the efficacy of antimycobacterial drugs was improved by abrogating IL-10 activity in BALB/c but not in C57BL/6 mice. These findings have implications for the design of adjunctive therapies in mycobacterial infections, namely the possibility of using IL-10-blocking drugs in a subset of patients.

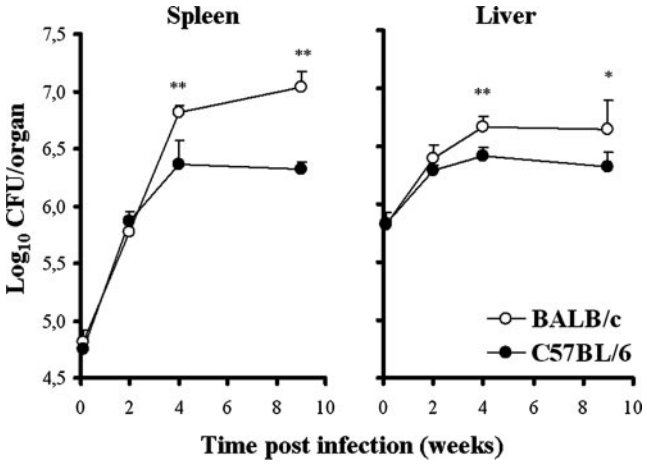

FIGURE 1. BALB/c are more susceptible to *M. avium* infection than C57BL/6 mice. BALB/c and C57BL/6 mice were infected i.v. with 10^6 CFU of *M. avium*. Bacterial load was determined at different time points (1 day, 2, 4, and 9 wk) in spleen and liver. Each point represents the mean ± SD of the CFU from six mice per group from one of six independent experiments.

Materials and Methods

Animals

BALB/c and C57BL/6, 8- to 10-wk-old specific pathogen-free female mice were purchased from Harlan Breeders and Charles River Laboratories. IL-10 KO mice on a C57BL/6 background were purchased from The Jackson Laboratory. IL-10 KO mice on a BALB/c background were bred in our facilities from two breeding pairs provided by Dr. A. O'Garra (National Institute for Medical Research, London, U.K.). All mice were kept in sterile housing conditions under 12-h light cycles and were given chow and tap water or an antibiotic mixture ad libitum. All animal experiments were performed in accordance with national and European guidelines for the care and handling of laboratory animals and have been approved by the National Veterinary Directorate.

Mouse infection and quantification of bacterial load in the organs

Mice were infected i.v., through a lateral tail vein, with 10^6 CFU of *M. avium* strain 2447 (smooth transparent variant, provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). At different time points, mice were sacrificed and the organs (spleen, liver, and lung) were collected, homogenized, serially diluted (0.04% Tween 80 in distilled water), and plated onto Middlebrook 7H10 agar medium. The plates were incubated for 1 wk at 37°C and the number of CFU were counted. When antibiotics were given, their administration was stopped 48 h before sacrifice to avoid the effect of antibiotics on bacterial growth after plating.

In vivo blockage of IL-10 activity

Blockage of IL-10 activity was performed by the administration of an anti-IL-10R mAb, an IgG1 obtained from the rat hybridoma cell line 1B1.2 (a gift from Dr. K. Moore, DNAX, Palo Alto, CA) (33). To block the IL-10R, mice were injected i.p. with 0.5 mg of anti-IL-10R mAb 24 h before or 4 wk after infection (depending on the specific experiment), followed by i.p. injections with 0.2 mg of anti-IL-10R mAb every other day during 4 wk or for the rest of the experimental infection (depending on the specific experiment). Control mice were submitted to the same protocol of administration of Abs, being injected with nonimmune rat IgG (obtained from sera of Lewis rats). Both Abs were purified by affinity chromatography using a protein-G agarose column (Sigma-Aldrich), followed by dialysis against PBS.

Antibiotic regimens

The antimycobacterial therapy was initiated 4 wk after infection, with a mixture of antibiotics consisting of rifampicin (Sigma-Aldrich), clarithromycin (Abbott Laboratories), and ethambutol (Sigma-Aldrich). The mixture of antibiotics was either administered ad libitum in the drinking water or administered by gavage. The mixture administered in the drinking water contained 0.4 mg/ml rifampicin, 1.94 mg/ml clarithromycin, and 0.25 mg/ml ethambutol on sterile tap water (19). Each mouse drank on average 3 ml/day. Chemotherapy against *M. avium* given by gavage consisted of 4.67 mg/ml rifampicin, 22.5 mg/ml clarithromycin, and 2.92 mg/ml ethambutol on sterile tap water. Each mouse was administered 0.3 ml of antibiotics 6 days a week. It should be noted that, on average, all mice received the same dose of antibiotic per week, whether the administration was done by gavage or ad libitum.

Detection of IFN-γ and TNF in serum samples

To obtain serum, mice were anesthetized with isoflurane (Abbott Laboratories) and retro-orbital bleeding was performed before sacrifice. Blood was allowed to clot and the serum was collected after centrifugation and frozen at −80°C until use.

Quantification of IFN-γ in sera was done by a two-side sandwich ELISA with anti-IFN-γ-specific affinity-purified mAbs (R4-6A2 as capture and biotinylated AN-18 as detecting mAbs) and the standard curves were generated with known amounts of IFN-γ (PeproTech). The sensitivity of the assay was 20 pg/ml.

A Cytometric Bead Array (CBA) kit to detect inflammatory cytokines (BD Biosciences) was used to measure TNF in serum samples using a FACSCalibur flow cytometer (BD Biosciences). The sensitivity of the assay for TNF according to the CBA kit specifications was 20 pg/ml.

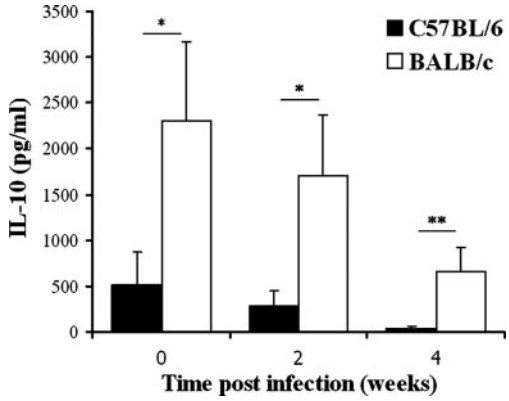

FIGURE 2. Splenocytes from BALB/c mice secrete more IL-10 in vitro than C57BL/6. Splenocytes from BALB/c and C57BL/6 mice noninfected and infected with *M.* avium (for 2 and 4 wk) were stimulated with Con A (5 μg/ml) for 72 h. IL-10 concentration was determined by ELISA in culture supernatants. Data are represented as the mean ± SD of IL-10 concentration from five mice per group from one of two independent experiments.

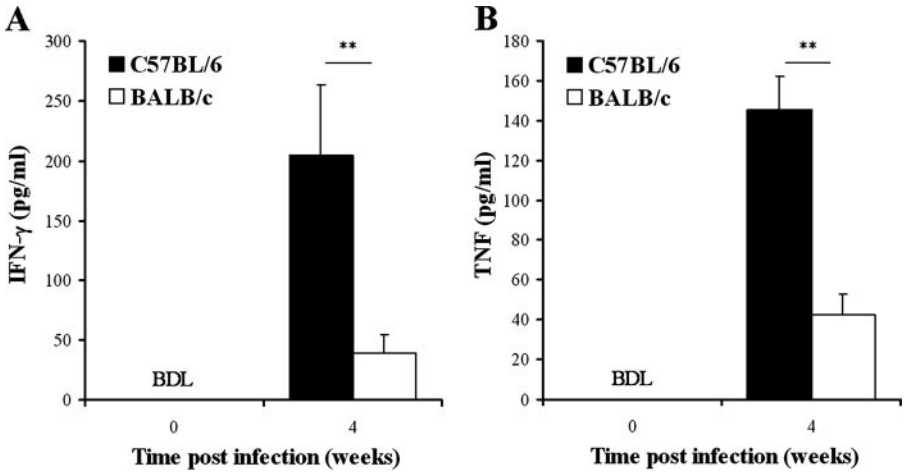

FIGURE 3. *M. avium*-infected C57BL/6 mice show higher IFN-γ and TNF serum levels than BALB/c mice. IFN-γ concentration was determined in the serum by ELISA (*A*) and TNF by CBA (*B*) before and 4 wk after *M. avium* infection of BALB/c and C57BL/6 mice. Data are represented as the mean ± SD of IFN-γ or TNF concentrations from five mice per group from one of two independent experiments. BDL, Below detection level.

In vitro stimulation of splenic cells and IL-10 measurement

Single-cell suspensions were obtained from the spleen of BALB/c and C57BL/6 mice. Erythrocytes were lysed with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$ (pH 7.2)) during 5 min at room temperature. Cells were then distributed into 96-well plates (2.5×10^5 cells/well), and incubated in triplicate in DMEM (Invitrogen Life Technologies), supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies) 2 mM L-glutamine (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 10 mM HEPES (Invitrogen Life Technologies), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen Life Technologies), and either with no further stimulus or with 5 μg/ml Con A (Sigma-Aldrich). Supernatants were collected after 3 days of culture and the concentration of IL-10 measured by ELISA (Duo Set; R&D Systems). The sensitivity of the assay was 32.5 pg/ml.

Flow cytometry

For each immunofluorescence staining for flow cytometry, 5×10^5 cells were used from each individual mouse and incubated with a specific Ab for 20 min at 4°C. Cell surface markers were analyzed using anti-CD25 allophycocyanin, anti-CD25 PE, anti-CD11b PE, anti-CD3 FITC, anti-CD4 FITC (BD Pharmingen), and anti-CD4 PECy5 (BioLegend). All cells were fixed with 2% formaldehyde after staining. The analysis of the cell populations was based on the acquisition of 30,000 events using CellQuest software on a FACSCalibur flow cytometer or a FACSAria cell sorter (BD Biosciences).

For the analysis of Foxp3 intracellular expression, cells were first stained for the surface molecules and, after fixation and permeabilization, were incubated with anti-Foxp3 FITC (eBioscience) based on the manufacturer's recommendations.

For intracellular detection of IL-10, 5×10^5 cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 4 h. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to the culture during the last 2 h of stimulation. Cells were harvested, washed, and stained for the expression of surface molecules. Cells were fixed with 2% formaldehyde and permeabilized with 0.5% saponin. After permeabilization, the FcRs were blocked with Fc-Block (BD Pharmingen) and the cells were stained with anti-IL-10 PE (BD Pharmingen) during 30 min, at room temperature. The analyses of the intracellular stainings were based on the acquisition of 50,000 events. Cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences).

Infection and treatment of bone marrow-derived macrophages (BMDM)

BMDM were prepared from BALB/c, C57BL/6, and IL-10 KO mice on BALB/c background, cultured in 24-well plates, as previously described (34). On day 10 of culture, when the cells had completely differentiated into macrophages, they were infected by adding 10^6 CFU of *M. avium* 2447 to each well containing $\sim 5 \times 10^5$ cells. Macrophages were then incubated for 4 h at 37°C in a 7% CO_2 atmosphere, washed with warm HBSS to remove noninternalized bacteria, and reincubated in DMEM containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 10% L929 cell-conditioned medium. In some wells, macrophages were immediately lysed and the number of viable intracellular bacteria was determined by counting the CFU after plating serial dilutions on Middlebrook 7H10 agar plates. In some experiments, macrophages were treated, just after infection until day 5 postinfection, with daily doses of IFN-γ (1, 10, and 100 U/well). In some experiments, supernatants were collected every day and the IL-10 concentration was determined by ELISA (Duo Set; R&D Systems). The sensitivity of the assay was 32.5 pg/ml. For each condition tested, three culture wells were used.

Statistical analysis

Statistically significant differences between groups were determined using the Student *t* test. Significance was referred like * for $p < 0.05$ and like ** for $p < 0.001$.

Results

BALB/c are more susceptible to M. avium *infection than C57BL/6 mice*

BALB/c and C57BL/6 mice were infected i.v. with a strain of *M. avium* of intermediate virulence (strain 2447 SmT) and the mycobacterial growth was followed for up to 9 wk. As shown in Fig. 1,

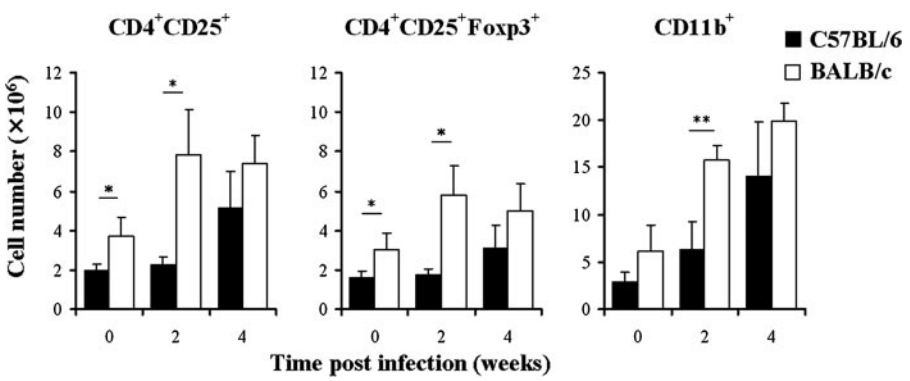

FIGURE 4. Differences in the cell population in the spleen of BALB/c and C57BL/6 *M. avium*-infected mice. Splenocytes from *M. avium*-infected (2 and 4 wk postinfection) and noninfected BALB/c and C57BL/6 mice were stained with Abs specific for CD4 and CD25, CD4, CD25 and Foxp3, and CD11b and analyzed by flow cytometry. Data are represented as the mean ± SD, from five mice per group from one of two independent experiments.

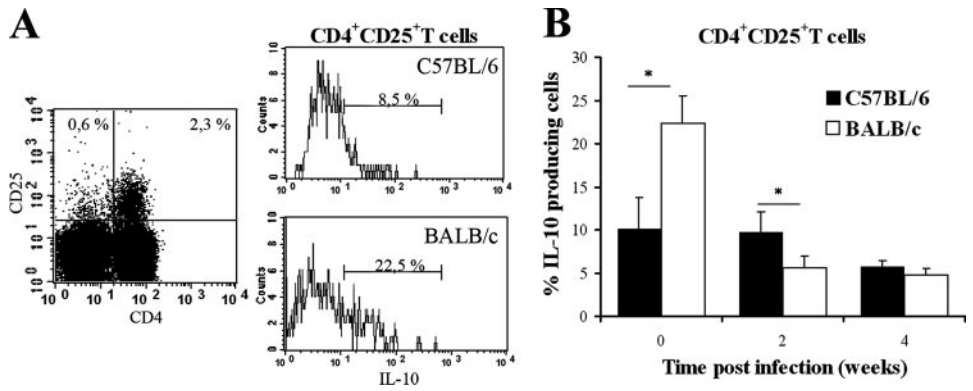

FIGURE 5. BALB/c mice have more $CD4^+CD25^+$ T cells prone to produce IL-10 than C57BL/6. For IL-10 intracellular staining, splenocytes from BALB/c and C57BL/6 mice noninfected and infected with 10^6 CFU of *M. avium*, were placed into culture with PMA and ionomycin in the presence of brefeldin A for 4 h. *A*, Representative dot plot showing CD4 and CD25 expression on stimulated splenocytes from noninfected mice and the histograms showing IL-10 production on $CD4^+CD25^+$ gated cells. *B*, Data are represented as the mean ± SD of the percentage of IL-10-producing cells among the $CD4^+CD25^+$ T lymphocyte from five mice per group from one of two independent experiments.

spleen and liver from BALB/c mice presented higher bacterial loads when compared with C57BL/6 mice. In a total of six experiments performed, this difference was consistently observed from 4 wk after infection onward. Of notice, in two of these six experiments, the increased bacterial load in BALB/c mice was already present 2 wk after infection (data not shown). No consistent differences in bacterial load were observed in the lungs of the two mouse strains (data not shown).

BALB/c mice exhibit an increased ability to produce IL-10 in vitro and decreased serum levels of IFN-γ and TNF when compared with C57BL/6 mice during M. avium *infection*

Given the association between IL-10 production and genetically determined susceptibility to some pathogens, we next studied the production of IL-10 in the two mouse strains after infection with *M. avium*. The amounts of IL-10 produced in vitro by splenocytes from infected and noninfected mice upon stimulation with Con A are shown in Fig. 2. When compared with C57BL/6, BALB/c splenocytes displayed greater ability to secrete IL-10, both before and upon *M. avium* infection, confirming previous results (25). Similar results were observed when the cells were stimulated with PMA and ionomycin (data not shown). Using two distinct techniques, ELISA and CBA, we were unable to quantify IL-10 in the sera from infected and noninfected mice. Because the production of IFN-γ and TNF, key proinflammatory cytokines in the protective immune response against infection by mycobacteria, are down-modulated by IL-10, we measured IFN-γ and TNF in the sera of infected mice. As shown in Fig. 3, C57BL/6 mice presented higher serum levels of IFN-γ and TNF than BALB/c mice at 4 wk of infection.

BALB/c mice have higher numbers of IL-10-producing $CD4^+CD25^+$ splenocytes than C57BL/6 mice

We next investigated which cell populations were responsible for the increased IL-10 production by BALB/c splenocytes. To do so, we first analyzed different cell populations in the spleen from noninfected and infected mice of both strains. We observed no differences in the total number of $CD4^+$ ($CD4^+CD3^+$) and $CD8^+$ ($CD8^+CD3^+$) T cells or of B lymphocytes ($CD19^+$) (data not shown). However, as shown in Fig. 4, the total number of $CD4^+CD25^+$ as well as of $CD4^+CD25^+Foxp3^+$ cells in the spleen was higher in BALB/c than in C57BL/6 mice before and 2 wk after infection (Fig. 4). Moreover, the total number of macrophages ($CD11b^+$) was also superior in the spleen of BALB/c 2 wk postinfection (Fig. 4).

To understand whether these or other cells were responsible for the increased production of IL-10 from BALB/c splenocytes, we performed intracellular staining for IL-10 upon in vitro restimulation of splenocytes from infected and noninfected mice of both strains and analyzed the cells by flow cytometry. We found no strain differences on the percentage of macrophages ($CD11b^+$), B lymphocytes ($CD19^+$), or on the total $CD4^+$ ($CD4^+CD3^+$) and $CD8^+$ ($CD8^+CD3^+$) T lymphocytes (data not shown). In contrast, BALB/c splenocytes from noninfected mice presented a higher percentage of $CD4^+CD25^+$ lymphocytes producing IL-10 when compared with C57BL/6 (Fig. 5). However, this difference disappeared as early as 2 wk postinfection (Fig. 5).

Macrophages from BALB/c mice produce more IL-10 than those from C57BL/6 mice upon in vitro infection with M. avium

Given that following infection, BALB/c mice accumulate more macrophages in the spleen than C57BL/6, and that we were

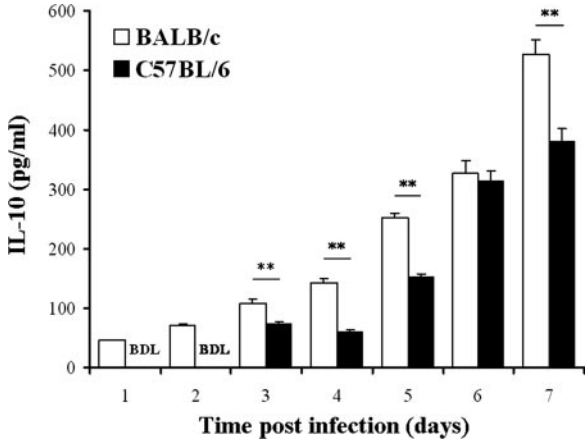

FIGURE 6. BMDM from BALB/c mice produce more IL-10 than those from C57BL/6. BMDM from BALB/c and C57BL/6 mice were infected with *M. avium* (0.5×10^6 cells with 10^6 *M. avium* CFU). At each day upon infection, IL-10 production was measured in the culture supernatants by ELISA. Data are represented as the mean ± SD from three culture wells for each specific condition from one of four independent experiments.

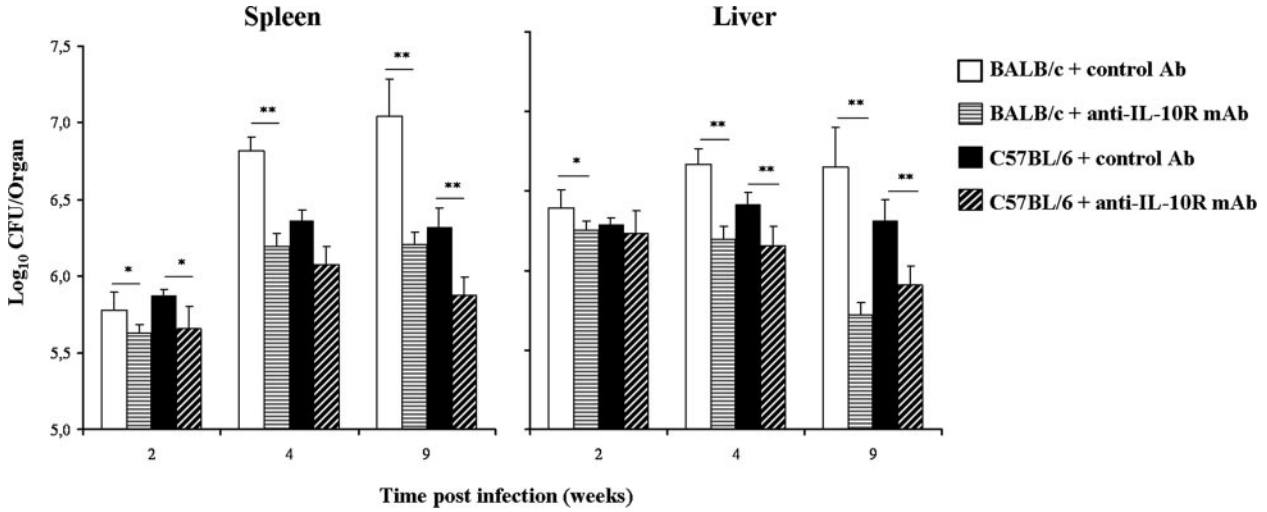

FIGURE 7. IL-10 activity contributes to the distinct susceptibility of BALB/c and C57BL/6 mice to *M. avium* infection. BALB/c and C57BL/6 mice were infected i.v. with 10^6 CFU of *M. avium*. To abrogate IL-10 activity, mice were injected i.p. with 0.5 mg of anti-IL-10R mAb 1 day before infection, followed by injections with 0.2 mg of anti-IL-10R mAb every other day until the end of the experiment. Control mice were submitted to the same protocol of administration, being injected with nonimmune rat IgG. Bacterial load was determined, in spleen and liver, at different time points upon infection. Each bar represents the mean ± SD of the CFU from six mice per group from one of two independent experiments.

unable to clearly assess IL-10 production by $CD11b^+$ cells using flow cytometry upon in vitro splenocyte stimulation, we next studied the production of IL-10 by BMDM. Increased amounts of IL-10 were produced by BALB/c BMDM upon *M. avium* infection when compared with C57BL/6 (Fig. 6). Moreover, IL-10 became detectable by ELISA as early as 1 day

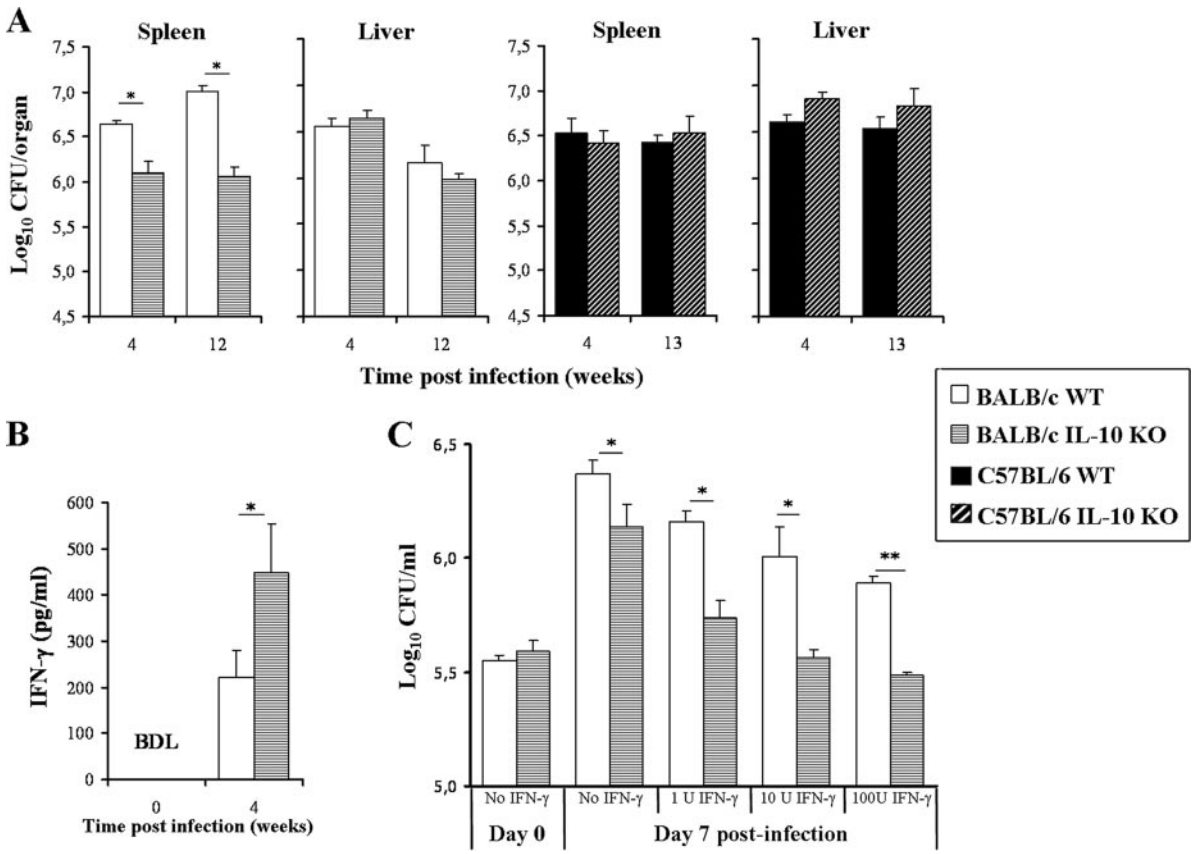

FIGURE 8. Complete absence of IL-10 improves resistance to *M. avium* infection in BALB/c but not in C57BL/6 mice. *A*, IL-10 KO and wild-type mice on the BALB/c background (*left panel*) and on the C57BL/6 background (*right panel*) were infected with *M. avium* i.v. At different time points, the bacterial load was determined in spleen and liver. Each point represents the mean ±SD of the CFU from six mice per group from one of two independent experiments for the BALB/c and four for the C57BL/6 background. *B*, IFN-γ concentration was determined in the serum by ELISA before and 4 wk after *M. avium* infection of BALB/c wild-type and IL-10 KO. Data are represented as the mean ± SD of IFN-γ concentration from five mice per group. BDL, Below detection level. *C*, BMDM from wild-type and IL-10 KO BALB/c mice were infected with *M. avium* (0.5×10^6 cells with 10^6 *M. avium* CFU). Different doses of IFN-γ (1, 10, or 100 U) were given daily. The bacterial load was measured 4 h and 7 days upon infection. Data are represented as the mean ± SD from three culture wells per specific condition from one of four independent experiments.

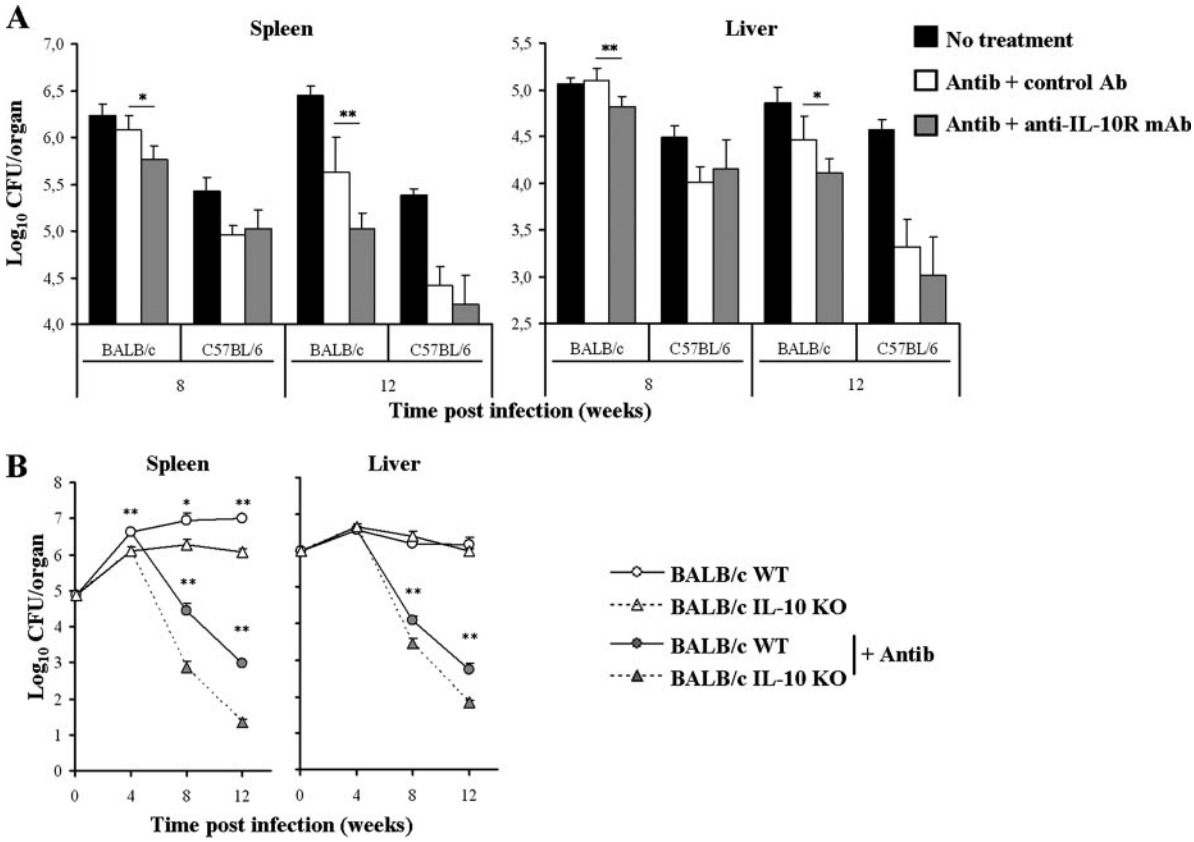

FIGURE 9. Blocking IL-10 activity improves antimycobacterial therapy in BALB/c mice but not in C57BL/6. *A*, To abrogate IL-10 activity in *M. avium*-infected animals, BALB/c and C57BL/6 mice were injected i.p. with 1 mg of anti-IL-10R mAb 4 wk after *M. avium* infection, followed by i.p. injections with 0.2 mg of anti-IL-10R mAb every other day during 4 wk. Control mice were submitted to the same protocol of administration, being injected with nonimmune rat IgG. Antibiotic therapy (Antib) was initiated 4 wk after infection until the end of the experimental period. Control mice were given nonsupplemented water. *B*, IL-10 KO and wild-type mice on the BALB/c background infected with *M. avium* started antibiotic therapy 4 wk after infection. At different time points, bacterial load was determined in spleen and liver. Each point represents the mean ± SD CFU from five to six mice per group from one of two independent experiments.

postinfection in BALB/c BMDM and only 2 days later in C57BL/6 (Fig. 6).

Blocking IL-10 activity attenuates differences in susceptibility to M. avium *between BALB/c and C57BL/6 mice*

We next assessed whether IL-10 activity in vivo underlies the different susceptibility to infection by *M. avium*. We thus used an anti-IL-10R mAb injected during the course of the infection by *M. avium* in both mouse strains. Blocking the activity of IL-10 led to a reduction in the bacterial load in the spleen and liver from both strains (Fig. 7). However, this effect was clearly more pronounced in BALB/c than in C57BL/6 mice (Fig. 7).

To further investigate whether the difference in susceptibility to infection of these two mouse strains depends on IL-10 activity, we next studied the course of *M. avium* infection using IL-10 KO mice in the two genetic backgrounds. As shown in Fig. 8*A*, the complete absence of IL-10 rendered BALB/c mice more resistant to infection (decreased bacterial load in the spleen from 4 wk postinfection onward in two independent experiments), but the same was not observed for C57BL/6 mice. In accordance, 4 wk postinfection serum levels of IFN-γ were higher in the IL-10 KO animals when compared with the BALB/c control mice (Fig. 8*B*). Using cultured BMDM from BALB/c and IL-10 KO mice on a BALB/c background, we found that the growth of *M. avium* inside macrophages was reduced in cells from the latter strain. Administration of IFN-γ to the cells strengthened the difference on the bacterial load between wild-type and IL-10 KO (Fig. 8*C*).

Blocking IL-10 activity improves the efficacy of antimycobacterial therapy in BALB/c but not in C57BL/6 mice

Previous results have shown that blocking IL-10 activity improved the efficacy of antimycobacterial drugs in BALB/c mice during the first 4 wk of treatment (19). Knowing that the different susceptibility of BALB/c and C57BL/6 mice to *M. avium* infection depends, at least partially, on distinct IL-10 activity, we next asked whether the effect of IL-10 activity on the efficacy of the antimycobacterial treatment also varied with the strains of mice. BALB/c and C57BL/6 mice infected with *M. avium* were treated with antibiotics alone or antibiotics plus anti-IL-10R mAb. Fig. 9*A* shows that C57BL/6 mice were less susceptible to infection and that the antimycobacterial drugs were more efficient in mice from this strain. In addition, blocking IL-10 activity improved the efficacy of antimycobacterial drugs in BALB/c mice but had no effect on C57BL/6 mice. Of notice, while this effect was clearly observed in the spleen and liver, it did not influence treatment efficacy in the lung (data not shown).

Finally, we confirmed the relationship between the response to antimycobacterial treatment and IL-10 in BALB/c mice by studying the efficacy of antimycobacterial drugs in IL-10 KO mice. In accordance with a negative relationship between IL-10 levels and treatment outcome, antibiotics were more effective in

BALB/c IL-10 KO mice than in the corresponding wild-type animals (Fig. 9*B*).

Discussion

BALB/c mice have been shown to present increased susceptibility to several intracellular pathogens when compared with other strains such as C57BL/6 mice (2, 3, 35). In this study, we extend these observations to infection by *M. avium* and suggest the basis for such differences. Using BALB/c and C57BL/6 mice, the present work shows that distinct activity of the anti-inflammatory cytokine IL-10 impacts on the course of infection, conditioning not only the susceptibility to mycobacteria but also the success of antimycobacterial therapy. In addition, our data help to clarify the discordant results in the literature on the precise role of IL-10 during the immune response to mycobacterial infections.

Increased production of IL-10 has been clearly associated, in the literature, with decreased resistance to infection with a diverse set of intracellular pathogens (9) but this correlation is less clear in the case of mycobacterial infections in mice. Thus, although blocking studies have shown that endogenously produced IL-10 may exacerbate the infection, many reports where IL-10 KO mice were used failed to substantiate the former observations. However, the latter studies were performed using mutant mice on a pure C57BL/6 or on a mixed C57BL/6.129 background (20–24). We now show that upon *M. avium* infection splenocytes and macrophages from C57BL/6 mice produce less IL-10 than the same cells from BALB/c mice making the former strains less useful in identifying a role for IL-10 than the latter. We believe that the use of different mouse strains in previous studies may underlie the variability of the data generated by different authors. Although in in vitro systems, we clearly showed an increased production of IL-10 by total splenocytes and BMDM from BALB/c when compared with C57BL/6 mice, we were unable to demonstrate distinct levels of IL-10 in the serum of the two mouse strains. Therefore, although our results with in vivo systems clearly showed that IL-10 activity is associated with distinct susceptibility of mice to *M. avium*, we should next investigate the mechanisms underlying such differences. This will help us elucidate the precise involvement of IL-10 in the increased susceptibility of BALB/c mice to infection by *M. avium*.

Also of relevance is the observation that the effect of IL-10 activity abrogation on mycobacterial growth was not exactly the same in the two in vivo models studied: administration of anti-IL-10R Abs in wild-type mice or the use of IL-10 KO mouse strains. Neutralization of IL-10 activity using specific Abs was more efficient in improving resistance to infection in wild-type mice than that observed with IL-10 KO animals. This may be related to compensatory mechanisms developed when a particular immunoregulatory cytokine is congenitally absent, as is the case when IL-10 KO animals are studied. In the context of immune response to infection, we believe the model in which IL-10 activity is blocked in a wild-type background during the course of infection is more informative.

The production of IL-10 upon infection with mycobacteria certainly also depends on factors other than the genetic background. The virulence of the mycobacterial strain used, the infection route, and the infectious dose are also expected to influence the immune response to infection. For example, Wakeham et al. (36) showed that C57BL/6 mice were less susceptible to i.v. infection with bacillus Calmette-Guérin than BALB/c mice, whereas such a difference was not found upon infection with an 8-fold smaller intratracheal inoculum (37). IL-10 seems also to differentially influence the different organs studied within each mouse strain. As we show here in a model of infection by *M. avium*, the spleen is more susceptible to the action of IL-10 than the liver, while the lung seems refractory. This may be related to the nature of the phagocytosing macrophage, i.e., alveolar macrophages, Kupffer cells, or splenic macrophages may respond differently upon mycobacterial infections.

IL-10 is produced by several types of cells of the immune system including the most relevant cells that fight mycobacterial infections: $CD4^+$ T cells and macrophages. Although IL-10 produced by T cells has been initially associated with Th2-type $CD4^+$ T cells, it is now clear that regulatory T cells can also produce great amounts of IL-10 (38) and that even Th1 cells produce this cytokine albeit in minute amounts (39). We found that normal BALB/c have more $CD4^+CD25^+$ T cells than C57BL/6 mice, confirming previous data from another laboratory (40). In addition, BALB/c mice have more $CD4^+CD25^+$ as well as $CD4^+CD25^+Foxp3^+$ or IL-10-secreting $CD4^+CD25^+$ T cells at the moment of infection, suggesting that these cells could be the source of the different amounts of IL-10 produced by splenocytes from infected mice in the two mouse strains. Moreover, the number of macrophages in the spleen at 2 wk postinfection was higher in BALB/c than in C57BL/6 mice and the BALB/c mice BMDM produced more IL-10 upon *M. avium* infection than the ones from C57BL/6 mice. Although these differences disappear as the infection progresses, we propose that they determine the course of the infection at its early onset as it has been shown that the initial response to the pathogen can determine the overall course of the infection (41, 42).

The initial difference on IL-10 production seems to condition not only the host's ability to fight the mycobacterial infection but also the response to antimycobacterial therapy. After 4 wk, although higher in BALB/mice, the bacterial load showed a clear tendency to stabilize in the spleen and liver of both mouse strains. It was at this stage that treatment to block IL-10 activity started. Remarkably, the administration of anti-IL-10R mAb improved antimycobacterial therapy efficacy in BALB/c but not in C57BL/6 mice. Moreover, although the anti-IL-10R mAb was administered exclusively during the first 4 wk of antimycobacterial treatment (from the fourth to the eighth week postinfection), the effect on the efficacy of the chemotherapy was not just observed during this period but was evident for at least another 4 wk after the anti-IL-10R mAb administration was stopped. These observations strongly suggest that reducing IL-10 activity can improve the efficacy of antibiotic therapy in infections by *M. avium*.

The present data may have important implications for research involving mycobacterial infection in humans. A set of reports in the literature associate the increased production of IL-10 with increased susceptibility to tuberculosis (27–30) and to *M. avium* (26). Moreover, distinct IL-10 production has also been associated with different forms of mycobacterial infections. In Buruli ulcer, cells from ulcerative lesions have been shown to express more IL-10 mRNA than the early nodular lesions (32); higher production of IL-10 by blood cells occurs in patients with the most advanced form of the disease (established ulcers) than in patients exclusively with early nodular lesions (31). In addition, *IL-10* gene polymorphisms were associated with distinct susceptibility to infection with *Mycobacterium leprae* (43) and with different forms of tuberculosis (44). In accordance, two recent reports showed that patients with tuberculosis present an increased percentage of regulatory T cells in the blood (45, 46). In light of these recent findings and of our results, it seems clear that the host's IL-10 activity during mycobacterial infections relates to susceptibility/resistance to infection. The evidence that reducing or blocking IL-10 activity

improves antimycobacterial therapy should be considered as a reasonable strategy to increase the efficacy of treatment in a particular group of patients.

Disclosures

The authors have no financial conflict of interest.

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