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The relative impact of bacterial virulence and host genetic background on cytokine expression during *Mycobacterium avium* infection of mice

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SUMMARY

Resistance to *Mycobacterium avium* depends on both genetically encoded macrophage functions and acquired T-cell immunity. Cytokines may play a role in either type of resistance. We studied the expression of interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) in naturally susceptible BALB/c (*Bcg*^{s}) and naturally resistant C.D2 (*Bcg*^{r}) congenic mice infected with two strains of *M. avium* (one highly virulent and another of low virulence). We observed that cytokine expression patterns correlated better with the virulence of the micro-organism than with the genetic background of the host. The control of the infection by the low virulence strain in either mouse strain was associated with an increased expression of IFN-γ and IL-2. Only *Bcg*^{s} mice infected with a virulent strain of *M. avium* were unable to restrict bacterial growth. An increased expression of IL-4, early during infection, was detected in the course of the latter infection but played no role in determining the susceptibility to infection. Neutralization of IFN-γ or IL-2 with specific monoclonal antibodies led to an exacerbation of the infection in *Bcg*^{r} mice by the two strains of *M. avium* and in *Bcg*^{s} mice infected with the low virulence strain of *M. avium*.

INTRODUCTION

Natural resistance to infection by intracellular parasites such as *Leishmania donovani*, *Salmonella typhimurium* and different mycobacterial species is controlled partially by a dominant autosomic gene encoded on mouse chromosome 1, called *Lsh*, *Ity* or *Bcg* according to the parasite.[1–3] This gene is present in two allelic forms in inbred strains of mice, *Bcg*^{r} encoding resistance and *Bcg*^{s} susceptibility.[1,3] The *Bcg* gene is thought to act through some as yet undetermined macrophage function(s).[1–3] Recently, a candidate gene has been cloned and shown to code for a membrane protein homologous to transporter proteins.[4] In the mouse, resistance or susceptibility to *Mycobacterium avium* infection is also controlled in the early phase by the expression of the *Bcg* gene,[5,6] even though the mechanisms underlying macrophage susceptibility or resistance to *M. avium* are still unknown and, probably, multifactorial.[6] The *Bcg* gene can affect the expression of Ia and antigen presentation.[1,3] It is thus possible that this gene may not only be directly responsible for mechanisms of killing, but may also modulate the specific immune response to the infection. Resistance to *M. avium* by *Bcg*^{r} mice is not affected by T-cell depletion[7] but, on the other hand, *Bcg*^{s} animals acquire CD4$^+$ T-cell mediated resistance to strains of *M. avium* of intermediate virulence.[7–9] Highly virulent *M. avium* strains fail to induce protective T cells, and proliferate progressively in naturally susceptible mice.[7,10]

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Differential cytokine production during an immune response can play an important role in regulating the outcome of an infection, as has been clearly demonstrated in an experimental model of cutaneous leishmaniasis.[11] In the mouse, CD4$^+$ T cells that produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) but little or no IL-4 and IL-5 (T-helper type 1 cells; Th1) induce the activation of macrophages to kill intracellular parasites, delayed-type hypersensitivity, and production of IgG2a, but not of IgG1 and IgE.[12,13] In contrast, responses by CD4$^+$ T cells that predominantly produce IL-4 and IL-5 (Th2 cells) result in the generation of IgG1- and IgE-secreting B cells and eosinophilia.[12,13] In human leprosy, a close association between susceptibility (lepromatous leprosy) and a Th2 response, and resistance (tuberculoid leprosy) and a Th1 response, has been reported.[14] In contrast, in an experimental infection by *M. tuberculosis*, the Th2 response appearing during the chronic phase of the infection was not associated with susceptibility.[15] Different levels of cytokine production by the host may be associated with different abilities of *M. avium* to replicate intracellularly. Thus, we compared the pattern of cytokine production in BALB/c (*Bcg*^{s}) and C.D2 (BALB/c.*Bcg*^{r} congenic strain) mice infected with a highly virulent strain (25291) and a less virulent strain (2447) of *M. avium*.

MATERIALS AND METHODS

Mice

Specific pathogen-free BALB/c (*Bcg*^{s}) mice were purchased from the Gulbenkian Institute (Oeiras, Portugal). C.D2 (congenic BALB/c.*Bcg*^{r}) mice[2] were supplied by Dr E.

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Skamene, and bred in our facilities. The animals were kept under standard hygiene conditions, and used at the age of 8–10 weeks.

Infection

Mice were infected intravenously (i.v.) with 10^6 colony-forming units (CFU) of *M. avium* 25291 (from the American Type Culture Collection; ATCC, Rockville, MD) or *M. avium* 2447 (an AIDS isolate; obtained from Dr F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). At 3, 15, 30 and 60 days after infection, mice were killed by ether anaesthesia and the spleens were removed to make cell suspensions. From these, RNA was subsequently isolated. To monitor *in vivo* bacterial proliferation, mice were infected as above and at different time points they were killed by cervical dislocation. The organs were collected aseptically and grinded in tissue homogenizers, serially diluted in a 0·04% Tween-80 solution in distilled water, and plated onto 7H10 agar medium. The plates were incubated for 2 weeks at 37° and the number of colonies counted.

RNA extraction

Cells (10^6) from single spleens were lysed with 1 ml of 4 M guanidinium thiocyanate. They were layered over 1 ml of 5·7 M CsCl and centrifuged 80 000 ***g*** for 22 hr at 20° with a TLA-100 rotor in a Beckman ultracentrifuge (Nyon, Switzerland). After removal of the supernatant, the RNA pellets were washed with 70% ethanol, and dissolved with diethylpyrocarbonate-treated distilled water (DEPC-dH_2O). RNA was precipitated once at −70° for 3 hr with 1/10 volumes of 3 M sodium acetate (pH 5·2) and 2·5 volumes of cold absolute ethanol. The RNA pellets were dried and redissolved in DEPC-dH_2O. All RNA samples were stored at −80° until assayed. The quality of RNA was checked by formaldehyde agarose gel electrophoresis.

Detection of cytokine gene expression by polymerase chain reaction (PCR)

Cytokine gene expression in total spleen cells was studied using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, St Quentin, Yvelines, France). mRNA of each sample were first reverse-transcribed (RT) into cDNAs, which in turn were subjected to PCR amplification using specific primers for individual cytokines. In brief, first-strand DNA was synthesized in a final volume of 20 μl with the following components: 2 μl total RNA, 2·5 μM oligo d(T) 12–18 mer primers, 5 mM $MgCl_2$, 10 mM Tris–HCl buffer, pH 8·3, 50 mM KCl, 1 mM dNTP, and 10 U RNase inhibitor. The mixture was incubated at 42° for 30 min, followed by 5 min at 99° and flash cooling to 4°. The cDNA preparations were stored at −80° until PCR. PCR was performed using the GeneAmp PCR system 9600 (Perkin Elmer Cetus). cDNA (5 μl) was mixed with 2 mM $MgCl_2$, 10 mM Tris–HCl buffer, pH 8·3, 50 mM KCl, 1 μl of 10 OD primers and 1·25 U AmpliTaq DNA polymerase, in a final volume of 50 μl. Amplification was repeated for 30 cycles. Each cycle consisted of 10 seconds at 91° for denaturation, 25 seconds at 59° for annealing, and 25 seconds at 72° for extension. The final extension lasted 4 min at 72° in all instances. Negative controls for PCR consisted of: (1) samples in which the reverse transcriptase was omitted to detect any contamination by previously amplified cDNA; and (2) reagent control in which RNA was replaced by DEPC-dH_2O.

Semi-quantitative PCR

Total spleen cell RNA from individual mice and total RNA from the HDK1 (Thl) and D10 (Th2) T-cell clones was extracted and reverse transcribed, as described elsewhere.[16,17] cDNA samples were concomitantly amplified by PCR using specific sets of primers for a house keeping gene, hypoxanthine phosphoribosyltransferase (HPRT). Dot-blots of the products were hybridized with a specific [γ-^{32}P]-ATP-labelled probe, internal to the amplified HPRT gene product. Autoradiographies were quantified in a MASTERScan (BIONIS-CSPI, Richebourg, France) and samples were adjusted to similar levels of HPRT mRNA, according to the standard curve derived from known dilutions of HDK1 or D10 cDNA samples.

After adjustment for HPRT levels, standards and experimental cDNA samples were amplified for IL-2, IL-4 and IFN-γ sequences, with primers synthesized at the Pasteur Institute (Paris, France), spanning intervening sequences in the gene, as described elsewhere.[16,17] The resulting PCR products were dot blotted and hybridized with lymphokine-specific [γ-^{32}P]ATP-labelled internal probes, in parallel with a titration of the standard HDK1 or D10 products run in each membrane for every experiment. Units of interleukin gene expression in experimental samples relative to pg of input RNA were then calculated after quantification of these final dot blots from the linear part of the standard curves. Details about primer and probe design, as well as about optimal cycle number for the PCR amplifications and the principles for standardization of the experimental samples, are elsewhere.[16,17]

Reagents and antibodies

Cytokine-neutralizing monoclonal antibodies (mAb) were obtained from hybridomas XMG1·2 (anti-IFN-γ IgG1; a kind gift from Dr P. Vieira, DNAX, Palo Alto, California), 11-B-11 (anti-IL4 IgG1; a kind gift from Dr R. Coffman, DNAX) and S4B6 (anti-IL-2 IgG; ATCC). Hybridomas were grown in ascites in HSD nude mice primed with incomplete Freund's adjuvant (IFA). Antibodies were purified using the Econo-Pac Serum IgG purification chromatography column (Bio-Rad, Richmond, CA).

In vivo *cytokine depletion studies*

For IL-2 depletion, mice were treated intraperitoneally (i.p) with 2 mg of the S4B6 antibody on days 0 and 15 after infection. For IFN-γ depletion, mice were treated i.p. with 2 mg of XMG1.2 on days 0, 15, 30 and 45 of infection. Previous experiments have shown that the administration of xenogeneic or syngeneic immunoglobulin does not affect the course of the infection compared to phosphate-buffered saline (PBS)-treated mice (A. G. Castro, unpublished observations).[8,9] Here, controls received the same volume of PBS as the antibody preparations.

Statistical analysis

Data are shown as means. Where appropriate, standard deviations (SD) are plotted. Data were compared using the Student's *t*-test.

RESULTS

The expression of three cytokines (IFN-γ, IL-2 and IL-4) was analysed in spleen cells from uninfected or *M. avium*-infected

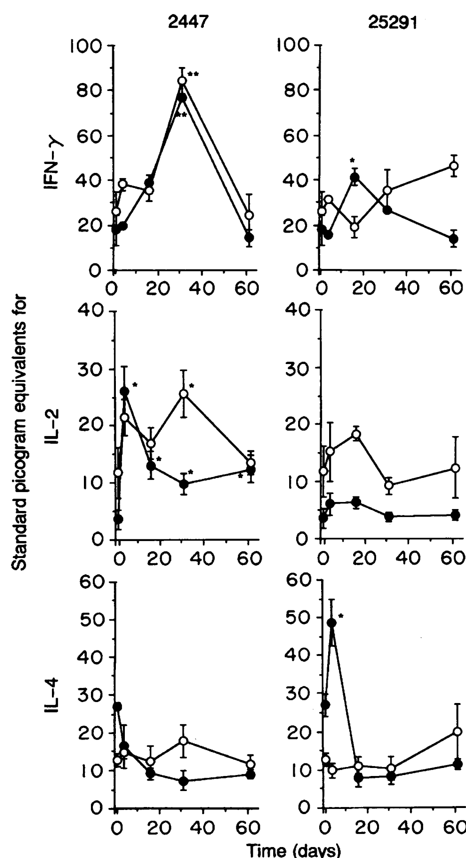

Figure 1. Semi-quantitative analysis of cytokine gene expression by RT-PCR in spleen cells of BALB/c (*Bcg*^{s}, closed symbols) and C.D2 (*Bcg*^{r}, open symbols) mice during infection with 10^6 CFU of either strain 2447 or strain 25291 of *M. avium*. Data are presented as arbitrary units corresponding to picograms of input RNA from standard Th1 or Th2 cell lines giving the same dot blot hybridization signal after standardization for HPRT gene expression. Each point represents the mean value for three mice and the bars represent the SD of the mean. A statistically significant increase in expression compared to uninfected mice (time 0) is labelled * for $P < 0.05$ and ** for $P < 0.01$.

mice after standardization of the message for similar HPRT expression levels. The levels of expression of these cytokines before infection (time point 0 in the graphs) differed between the two mouse strains. Naturally resistant animals had slightly higher baseline expression levels of IFN-γ and IL-2 (even though not statistically significant) than naturally susceptible mice (Fig. 1). On the other hand, naturally susceptible mice showed a higher expression of IL-4 than naturally resistant animals ($P < 0.01$; Fig. 1).

Induction of cytokine gene expression in mice infected by the less virulent *M. avium*

BALB/c and C.D2 mice infected with *M. avium* 2447 were killed at days 3, 15, 30 and 60 after infection. Analysis of cytokine mRNA levels in whole spleen cells by semi-quantitative RT-PCR is shown in Fig. 1. Mice infected with the less virulent *M. avium* 2447 showed, throughout the infection, enhanced levels of IL-2 and a peak in the expression of IFN-γ on day 30, irrespective of the mouse strain studied (Fig. 1). Although IL-2 expression at day 30 of infection was higher in C.D2 animals than in BALB/c mice ($P < 0.01$), there was no difference in peak IFN-γ expression between the two mouse strains. This less virulent *M. avium* strain was not able to induce an IL-4 response and, indeed, the expression of this cytokine was reduced below basal levels in BALB/c mice ($P < 0.01$ from day 15 onwards; Fig. 1).

Induction of cytokine gene transcription in mice infected by the highly virulent *M. avium*

The susceptible BALB/c mice infected with the highly virulent *M. avium* 25291 exhibited, early during infection, a transient increase in the expression of IL-4 with no changes in the basal expression of IL-2 (Fig. 1). The levels of expression of IFN-γ were enhanced to a small degree, peaking at day 15 of infection (Fig. 1). The levels of cytokine expression in C.D2 mice were different from those in BALB/c mice: a higher expression of IFN-γ at day 60 ($P < 0.01$) and of IL-2 throughout the whole experimental period ($P < 0.05$ at day 15 and $P < 0.01$ at day 30; Fig. 1). However, IL-2 expression in C.D2 mice did not increase significantly during infection compared to the unifected animals.

Comparing the induction of cytokines by the two strains of mycobacteria, it was apparent that the infection by the less virulent *M. avium* (strain 2447) led to higher levels of expression of IFN-γ and IL-2 than the infection with the highly virulent 25291 strain. This was found in both BALB/c and C.D2 mice and, indeed, the levels of cytokine expression were mostly dependent on the virulence of the mycobacteria than on the host that was studied.

Anti-IFN-γ antibodies increase the susceptibility of C.D2 mice to *M. avium* infection

We have already shown[2] that the neutralization of IFN-γ in BALB/c mice infected with *M. avium* 2447 exacerbated the infection. As C.D2 mice also presented an increase in the expression of IFN-γ (Fig. 1) we evaluated the role played by this cytokine in the restriction of *M. avium* growth observed in these naturally resistant mice. We treated mice infected with either strain of *M. avium* with anti-IFN-γ neutralizing antibodies. The treatment with anti-IFN-γ from the beginning of the infection until day 45 led to an increase in the bacterial loads, in both the liver and the spleen, of C.D2 mice infected with either *M. avium* 25291 or 2447 compared to untreated animals (Fig. 2).

Neutralization of IL-4 does not affect the course of infection of the virulent strains of *M. avium* in susceptible mice

As we observed an early peak of IL-4 expression in BALB/c mice infected with strain 25291, we decided to evaluate the role played by this cytokine in the subsequent course of the infection. BALB/c mice were infected with 10^6 CFU of *M. avium* 25291 and given one dose of the 11-B-11 antibody on the same day of the infection. As shown in Fig. 3, there was no effect on the bacterial proliferation after *in vivo* neutralization of IL-4.

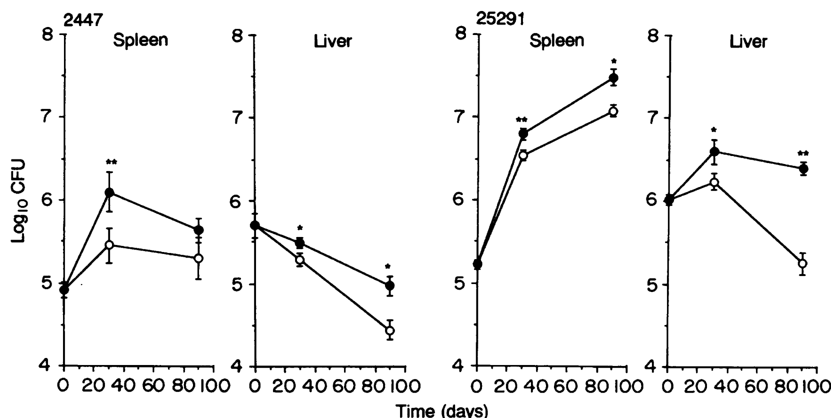

Figure 2. Proliferation of *M. avium* strains 2447 and 25291 in the spleen and liver of C.D2 (*Bcg*^{r}) mice inoculated with 10^6 CFU of either bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IFN-γ monoclonal antibodies (2 mg/mouse at days 0, 15, 30 and 45 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. A statistically significant increase in bacterial growth in treated compared to untreated mice is labelled * for $P < 0.05$ and ** for $P < 0.01$.

Anti-IL-2 antibody has minor effects on the resistance to *M. avium* 2447

Protective T cells arise in infected BALB/c mice during the first month of infection by *M. avium* 2447.[7–9] Here we saw that IL-2 was induced in both mouse strains by this *M. avium* strain (Fig. 1). To evaluate the role played by IL-2 in the induction of acquired immunity, we neutralized this cytokine during the first month of infection by *M. avium* 2447 in either BALB/c or C.D2 mice. Mice infected with 10^6 CFU *M. avium* 2447, received either no treatment or 2 mg of S4B6 anti-IL-2 antibody on days 0 and 15 of the infection. IL-2 neutralization during the early phase of the infection led to a small albeit statistically significant increase in the bacterial load in the spleen compared to the control animals (Fig. 4).

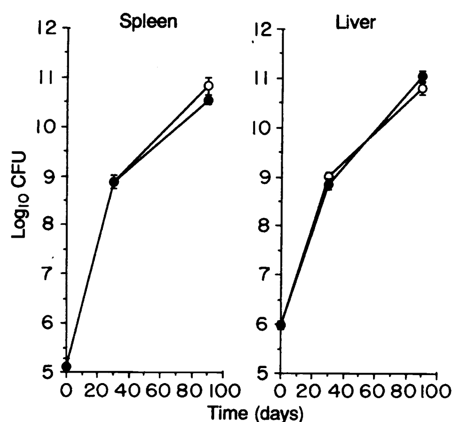

Figure 3. Proliferation of *M. avium* strain 25291 in the spleen and liver of BALB/c (*Bcg*^{s}) mice inoculated with 10^6 CFU of the bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IL-4 monoclonal antibodies (2 mg/mouse at day 0 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. No statistically significant differences were found between treated and untreated groups.

DISCUSSION

Innate resistance to a micro-organism is often defined according to the fate of the infection by that microbe in different animal strains. Such is the case of the macrophage-mediated innate mechanisms of resistance encoded by the *Bcg/Ity/Lsh* gene. Regarding the resistance to mycobacteria, such a definition is just a relative one, i.e. the degree of resistance depends on the mycobacterial species and even on the strain of a particular species. Thus, naturally resistant strains of mice are not able to restrict the growth of all mycobacteria; conversely, not all mycobacteria can grow in the naturally susceptible animals. For instance, naturally resistant mice still allow the growth of *M. lepraemurium* although such growth is higher in the naturally susceptible animals.[18] On the other hand, naturally resistant mice do not allow the proliferation of a low dose of bacillus Calmette–Guérin (BCG) Montreal, whereas such an inoculum proliferates in naturally susceptible animals.[1,3] Finally, not all strains of BCG are affected by the differential expression of the two allelic forms of the *Bcg* gene.[19] We have shown here that the resistance to *M. avium*, encoded by the *Bcg* gene,[5,6,20] is variable according to the strain *M. avium* of the bacterium, and is not an absolute trait. Thus, for a given strain, C.D2 mice were always more resistant than BALB/c animals. However, given the differences in virulence of the two strains of *M. avium* studied here, the growth rates were not only determined by the host's genetic background but also by the bacterial virulence. Naturally resistant mice could exert an almost complete bacteriostasis on the less virulent *M. avium* strain. However, the growth of the highly virulent *M. avium* strain in those naturally resistant mice was closely similar to the growth of the less virulent strain in naturally susceptible animals after the inoculation of similar numbers of viable organisms. Furthermore, differences in resistance were found in different organs, livers being better equiped to restrict *M. avium* growth than the spleens. Having defined the experimental model, it was important to determine how genetic resistance/susceptibility might affect the expression of particular cytokines and, on the other hand, how resistance was itself dependent on certain cytokines.

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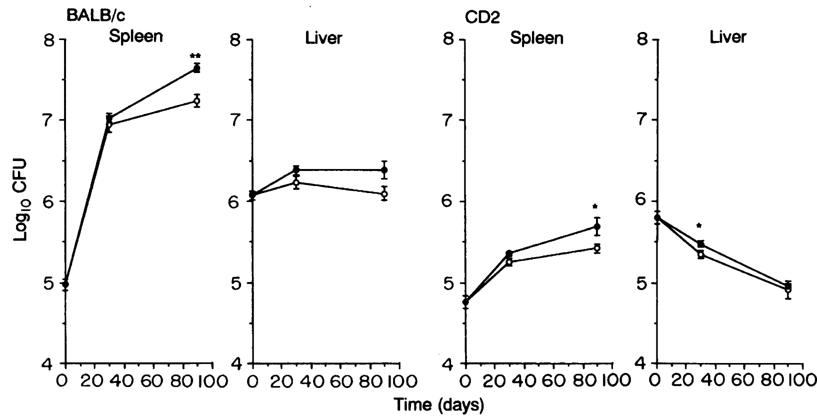

Figure 4. Proliferation of *M. avium* strain 2447 in the spleen and liver of BALB/c (*Bcg*^{s}) and C.D2 (*Bcg*^{r}) mice inoculated with 10^6 CFU of the bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IL-2 monoclonal antibodies (2 mg/mouse at days 0 and 15 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. A statistically significant increase in bacterial growth in treated compared to untreated mice is labelled * for $P < 0.05$ and ** for $P < 0.01$.

Bcg^r mice have been shown to exert a T-cell independent bacteriostasis on *M. avium* growth,[7] which is most probably associated with an unknown macrophage function(s). Although T cells were found not to be necessary for the control of a highly virulent strain of *M. avium* in *Bcg*^{r} animals,[7,21] it is still unknown whether cytokines produced during an infection of a naturally resistant mouse are necessary for the control of the infection, because cell types other than T cells have been shown to produce protective cytokines during infection by different micro-organisms, including *M. avium.*[9] In the *Bcg*^{s} mice, the initial growth of *M. avium* is not as well controlled as in the resistant mice,[7,21] although there are cytokine-dependent mechanisms that induce some degree of early bacteriostasis.[9] However, these *Bcg*^{s} mice may acquire T-cell dependent immunity, leading to bacteriostasis of *M. avium.*[7–9,21] On the other hand, there are *M. avium* strains that somehow avoid the induction of protective T cells and grow in an uncontrolled manner in those naturally susceptible animals.[9,10] Our present results add some new information to this already complex picture of *M. avium*–host interactions. We studied the cytokine expression during the infection by *M. avium* in this model by looking at the influences of the genetic background of the host and the virulence of the infecting micro-organism, and we have shown that the expression of different cytokines varies according to the type of interaction between *M. avium* and the host.

Cytokine expression during the four types of infection (BALB/c or C.D2 mice infected with either *M. avium* 2447 or 25291) correlated better with the virulence characteristics of the infecting mycobacteria than with the host genetic background. The less virulent strain of *M. avium* induced, in both mouse strains, a higher expression of both IFN-γ and IL-2. While macrophages from BALB/c mice were permissive to the early proliferation of *M. avium* 2447, $CD4^+$ T cells were able to activate them to restrict its growth.[7,9] This late control of the infection in BALB/c mice was associated with a clear Th1-type immune response with significant expression of IL-2 and IFN-γ but not of IL-4. We have previously shown that $CD4^+$ T cells producing IFN-γ were important in the acquisition of bacteriostasis of *M. avium* 2447.[7] Here we suggest that these $CD4^+$ T cells have a Th1 phenotype.

Naturally susceptible BALB/c mice are unable to control the growth of the highly virulent *M. avium* 25291.[7,10] In BALB/c mice infected with this mycobacterial strain, there was no induction of protective T cells.[9] Here, we found that these mice do not develop a Th1 response and that they show a transient and early expression of IL-4. This IL-4 production, even though associated with susceptibility to infection, was not responsible for the lack of control of the infection since neutralization of the cytokine did not affect the progression of the bacterial proliferation. We interpret these results as an association between susceptibility and a lack of induction of a protective Th1 response rather than a causal relationship between a Th2 response and susceptibility to *M. avium.* Furthermore, the early production of IL-4 may not be associated with a T-cell response because of its early nature and a lack of a continued production of this cytokine later in the infection. The cell type responsible for such IL-4 expression is not known, although cells other than T cells have been shown to produce IL-4, namely mast cells.[22] Thus, in contrast with other infections by intracellular pathogens, susceptibility does not seem to be mediated by a Th2 response.

Naturally resistant C.D2 mice were able to restrict, to a certain extent, the proliferation of the virulent strain of *M. avium.* The growth curve for this infection was similar in terms of magnitude and kinetics to the one observed in BALB/c mice infected with the less virulent mycobacterium. The resistance of C.D2 mice to *M. avium* 25291 was associated with a minor IL-2 and IFN-γ response. The neutralization of IFN-γ in this infection led, however, to enhanced proliferation of the infectious organism. These results suggest that, despite a genetically determined higher anti-mycobacterial activity of the macrophages, the highly virulent strain of *M. avium* is still able to proliferate in the initial period of the infection. This bacterial growth is then responsible for the activation of immune cells capable of producing IFN-γ that will induce bacteriostasis or killing. The IFN-γ was not necessarily produced by T cells in the case of the naturally resistant mice,

where T-cell depletion does not affect resistance to *M. avium*. In this regard, it has been shown for other micro-organisms[23,24] that stimulation of *Bcg*^{r} immune cells by bacteria induces IFN-γ to higher degrees than that observed with *Bcg*^{s} cells. In that system, natural killer (NK) cells were the cells secreting the IFN-γ and their action was regulated by adherent cells, which were the ones that expressed the function(s) encoded by the *Bcg* gene.[23,24]

Finally, C.D2 mice were able to restrict the growth of *M. avium* 2447 from the early time points of infection. Although this indicates a major role of the *Bcg*-encoded macrophage anti-mycobacterial activity, we showed here a role for IFN-γ in restriction of growth of this *M. avium* strain. Since T cells do not seem necessary for the overall restriction of growth,[7] we postulate that other cells such as NK cells may be producing that cytokine. Despite the fact that T cells were not needed for the control of this infection and that protective T cells were not detectable in this infection,[3] we found a cytokine response of the same magnitude as the one observed in BALB/c mice infected with the same strain of *M. avium* and characteristic of a Th1 type of response.

Unexpectedly, the neutralization of IL-2 did not have a major effect on the restriction of *M. avium* growth. The immune response to *M. avium* is rather indolent, as is the course of the infection and, therefore, the need for high levels of T-cell stimulating cytokines is probably not observed. Other cytokines may also complement the IL-2 deficiency. We have shown a major role for IL-6 in the induction of protective T cells in *M. avium* infections of BALB/c mice,[8] and preliminary results show a major role for IL-12 in that respect (A. G. Castro, R. A Silva and R. Appelberg, unpublished data).

In summary, we have depicted here the complex interactions between *M. avium* and a murine host showing that the innate resistance encoded by the *Bcg* gene is not an absolute trait. Furthermore, we have found that the influence played by that gene and its pleiotropic effects on cytokine expression are minor compared to the role played by the virulence characteristics of the micro-organism. The differences in virulence of the micro-organism led to different requirements regarding the need for protective T cells acting upon the innate resistance or susceptibility encoded by the *Bcg* gene. Finally, the failure to generate a protective T-cell response was not due to the emergence of a Th2 response but, rather, to the lack of induction of a Th1 response.

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