Increased resistance to mycobacterial infection in the absence of interleukin-10

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SUMMARY

Interleukin-10 (IL-10) down-regulates T helper type 1 cell and macrophage functions. As IL-10 is induced along with tumour necrosis factor (TNF) and IL-12 in mycobacterial infection, we asked whether endogenous IL-10 plays a role in the antimycobacterial response. We demonstrate here that IL-10-deficient mice eliminate *Mycobacterium bovis* Calmette–Guérin bacillus faster than wild-type mice. Granulomas are significantly larger, containing more CD-11b- and CD11c-positive antigen-presenting cells and T cells, and the expression of major histocompatibility complex class II and intracellular adhesion molecule-1 is increased. Macrophages in granulomas of IL-10-deficient mice express high levels of TNF, acid phosphatase and inducible nitric oxide synthase (iNOS). Finally, an increased cutaneous delayed-type hypersensitivity reaction to mycobacterial proteins is further evidence of an augmented cell-mediated immune response. In conclusion, the cell-mediated immunity is enhanced in the absence of IL-10, resulting in a robust granuloma response, which accelerates the clearance of mycobacteria. Therefore, endogenous IL-10 attenuates mycobacterial immunity.

INTRODUCTION

Infection with *Mycobacterium tuberculosis* is a leading global health threat.[1,2] Incomplete understanding of the nature of protective immune-inflammatory responses has hampered the development of effective vaccines and therapies. The cell-mediated immune response plays a key role in host resistance to mycobacterial infection. The cell-mediated immune response is tightly regulated by a fine balance between T helper type 1 (Th1) cytokines [interferon-γ (IFN-γ), interleukin-2 (IL-12), tumour necrosis factor (TNF), IL-18) and Th2 cytokines (IL-4, IL-10, IL-13).[3] Activation of macrophages by IFN-γ derived from T and natural killer cells is essential for the ultimate eradication of *M. tuberculosis* bacilli. Mice with disruption of Th1 cytokine signalling, e.g. IFN-γ and IL-12, are unable to restrict the growth of *M. tuberculosis* and succumb to infection.[4–8] In humans, deficiencies of the IFN-γ and IL-12 receptors[9,10] are also associated with a predisposition to mycobacterial infections. TNF is induced in human and experimental tuberculosis[2] and its role in mycobacterial defence is inferred from neutralization and deletion experiments in mice.[7,11,12]

Besides the dominant Th1 response, mycobacterial infections also induce a Th2 component.[13] IL-4 and IL-10

expression may down-modulate macrophage function and thereby reduce their microbicidal functions.[14] Transgenic expression of IL-10 was demonstrated to antagonize macrophage activity in mycobacterial infection.[15] By contrast, neutralization of IL-10 enhanced host resistance to *M. avium* infection,[13,16] indicating that endogenous IL-10 attenuates the host response. However, based on recent data from IL-10-deficient mice the role of endogenous IL-10 is controversial: Both no effect on *M. bovis* bacillus Calmette–Guérin (BCG)[17] or *M. tuberculosis* infection[18] and increased antimycobacterial immunity to *M. bovis* BCG infection have been reported.[19]

We asked here whether endogenous IL-10 induced by *M. bovis* BCG infection plays a role in the early phase of host resistance. Using IL-10-deficient mice[20] we show that endogenous IL-10 has indeed a regulatory role and attenuates the Th1-type immune response in *M. bovis* infection. In the absence of IL-10, large granulomas are formed containing highly activated macrophages allowing a rapid clearance of bacilli.
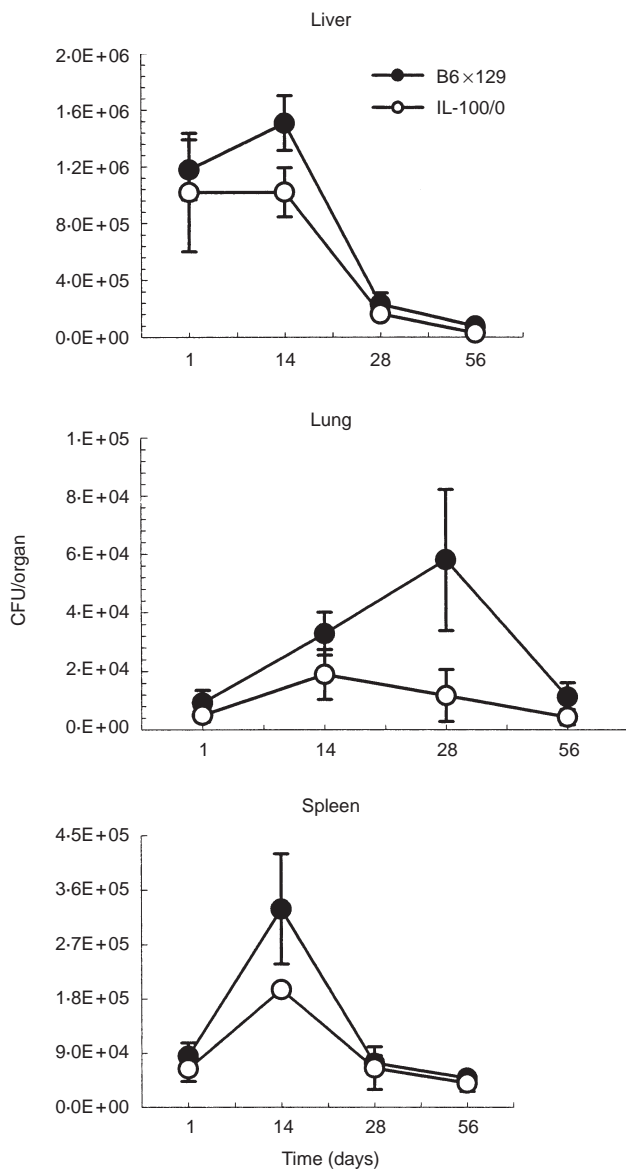
MATERIALS AND METHODS

Animals
Adult, 8–10-week-old female mice of the wild-type strain C57BL6/129, homozygous IL-10-deficient strains,[20] all on a C57BL6/129 genetic background were kept under specific pathogen-free conditions. The genotypes of the mouse populations were confirmed by polymerase chain reaction (PCR) analysis of DNA from tail biopsies.

Figure 1. Bacterial burden in organs of IL-10-deficient mice infected with *M. bovis* BCG.The bacterial load in liver, lung and spleen (CFU) was determined at 1, 14, 28 and 56 days after BCG infection (10^6 BCG intravenously) of IL-10-deficient and wild-type mice. The mean CFU values from four mice are given $\pm$ SD.

Antibodies

The following rat anti-mouse monoclonal antibodies were used for immunohistochemical detection: intracellular adhesion molecule-1 (ICAM-1), F4/80, major histocompatibility complex (MHC) class II antigen, TNF (MP6-XT22), IFN-γ (XMG1), IL-12 (C15·6), CD3, CD4 and CD8, CD11b and CD11c from Pharmingen. Rabbit antibody against inducible nitric oxide synthase (iNOS) was diluted 1:2000 (obtained from Prof. J. Pfeilschifter, Department of Pharmacology, University of Frankfurt, Germany).

Experimental design

Wild-type and IL-10-deficient mice were infected intravenously with 10^6 colony-forming units (CFU) BCG (Pasteur strain). Groups consisted of five mice and the experiments were repeated three times. At 2, 4 and 8 weeks after infection blood was taken by retro-orbital venous puncture, and mice were killed at each time-point. The lung, liver and spleen were weighed. Parts of the organs were either fixed in 4% buffered formalin, or frozen on dry ice and maintained at $-80°$. About 2-μm thick sections of the spleen, liver and lungs were cut and stained with either haematoxylin & eosin or Ziehl–Neelsen for staining the acid-fast bacilli.

Mononuclear cell clusters of more than 15 cells were considered to be granulomas. Two independent observers (NB and BR) counted granulomas and acid-fast bacilli in 25 randomly selected high-power fields (magnification 200 $\times$ and 400 $\times$, respectively) from five liver cross-sections per animal without knowledge of the group. Mean values $\pm$ SD ($n=5$ mice) are given.

Immunohistochemistry

The frozen tissues (liver, spleen and lungs) were cut 6-μm thick on a cryostat, air-dried for storage at $-80°$ and incubated as described.[21] Fixation in acetone (10 min at 4°) was performed just before the immunolabelling. After a rinse in phosphate-buffered saline (PBS) the sections were incubated for 16 hr at 4° with the primary antibodies diluted in PBS. The sections were then washed in PBS and incubated for 30 min at room temperature with rabbit anti-rat serum ABC system (Vector Lab, Burlington), followed by DAB substrate. After rinses in PBS the sections were mounted in Immunomount (Shandon, Pittsburgh, PA).

Colony-forming units

Bacterial loads in the lung, liver and spleen of infected mice were evaluated at 1 day and at 2, 4 and 8 weeks post-infection. Organs were weighed and defined aliquots were homogenized in saline containing 0·04% Tween. Ten-fold serial dilutions of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates containing 10% OADC (Difco, Detroit, MI). Plates were incubated at 37° for 3 weeks and colonies were counted. Data are expressed as CFU per organ ($n=4$ mice per group).

Enzyme-linked immunosorbent assays (ELISAs) for cytokines

TNF, IL-12 and IFN-γ levels in the serum of BCG infected mice at 4 weeks were measured using commercial ELISA kits (R & D Systems, Abingdon, UK).

Detection of acid phosphatase in tissues

Acid phosphatase on frozen tissue sections was performed as reported previously.[22] In order to quantify the enzymatic activity fresh livers and spleens were homogenized in 25 mM Tris-HCl (pH 7·4), 10 mM EDTA and 10 mM EGTA (250 mg of tissue/ml). Crude supernatant was obtained by centrifuging the homogenate at 10 000 ***g*** for 15 min. Acid phosphatase activity was determined by using naphtol AS-Bi phosphate as substrate. In brief, 100 μl of spleen extracts was mixed with 900 μl of reaction buffer: Michaelis buffer containing 0·04% pararosaniline and 0·04% sodium nitrate at pH5 and 0·03% naphtol SA-Bi phosphate. Enzyme activity was determined after 20 min at room temperature at OD 570.

WT   IL-10 KO

(a) HE

(b) ZN
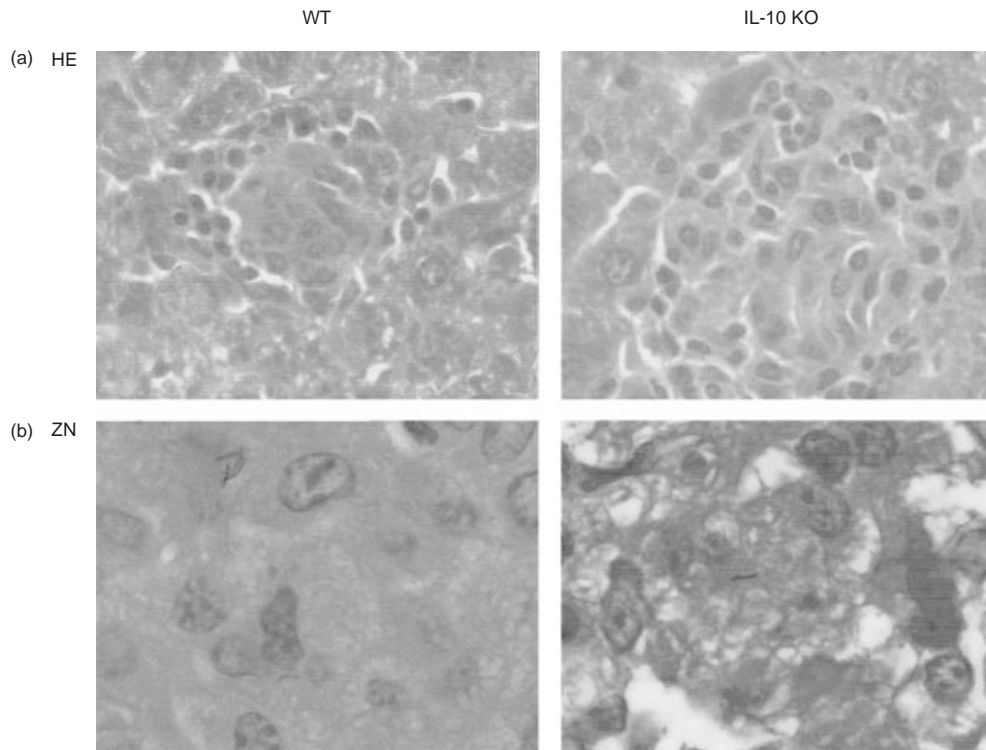
Figure 2. Hepatic granulomas from *M. bovis* BCG-infected wild-type and IL-10-deficient mice. Mice were killed at 2 weeks and the sections were stained with haematoxylin & eosin (a) or with Ziehl–Neelsen (b) to detect the acid-fast bacilli. Magnification × 135.
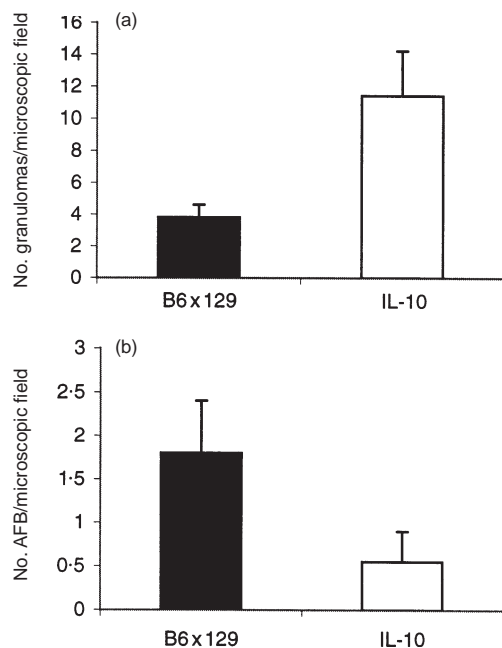
Figure 3. Number of granulomas and of acid-fast bacilli in the liver of wild-type and IL-10-deficient mice 2 weeks post-infection (10^6 BCG intravenously). (a) Number of granulomas: mononuclear cell clusters of more than 15 cells were counted as granulomas; the results are expressed as the mean ± SD ($n = 5$ mice). (b) Number of acid-fast bacilli per granuloma per high-power microscopic fields, expressed as the mean ± SD ($n = 5$ mice).

Delayed-type hypersensitivity (DTH) response

BCG-infected mice were given a subcutaneous injection of 10 µg purified protein derivative (PPD, State Vaccine Institute, Pinelands, South Africa) in the right hind footpad and the swelling was measured at 48 hr using a Mitutoyo micrometer (Bruetsch AG, Zurich, Switzerland). The differences of swelling of the right and left (saline-injected, control) hind footpads were compared.

Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by Student's *t*-test.

RESULTS

Rapid clearance of BCG infection in IL-10-deficient mice

The course of infection of mice injected intravenously with 10^6 CFU BCG was followed over 8 weeks. Wild-type and IL-10-deficient mice were exposed to a comparable inoculum as assessed by mycobacterial colony counts (CFU) per organ at day 1. The mycobacterial organ burden did not increase over the next 2 weeks in liver and lungs, and was only slightly elevated in the spleen of IL-10-deficient mice, while the wild-type mice showed the usual increase of CFU in liver ($P < 0.01$), lung ($P < 0.05$) and spleen ($P < 0.05$) over 2 weeks, and at 4 weeks in lungs ($P < 0.01$) (Fig. 1). The elimination of the mycobacteria was significantly faster over the next 4-week period from the lungs of IL-10-deficient mice as compared to the wild-type controls. In addition, the CFU counts from the liver of IL-10-deficient mice at 56 days were still significantly

(a) WT IL-10 KO
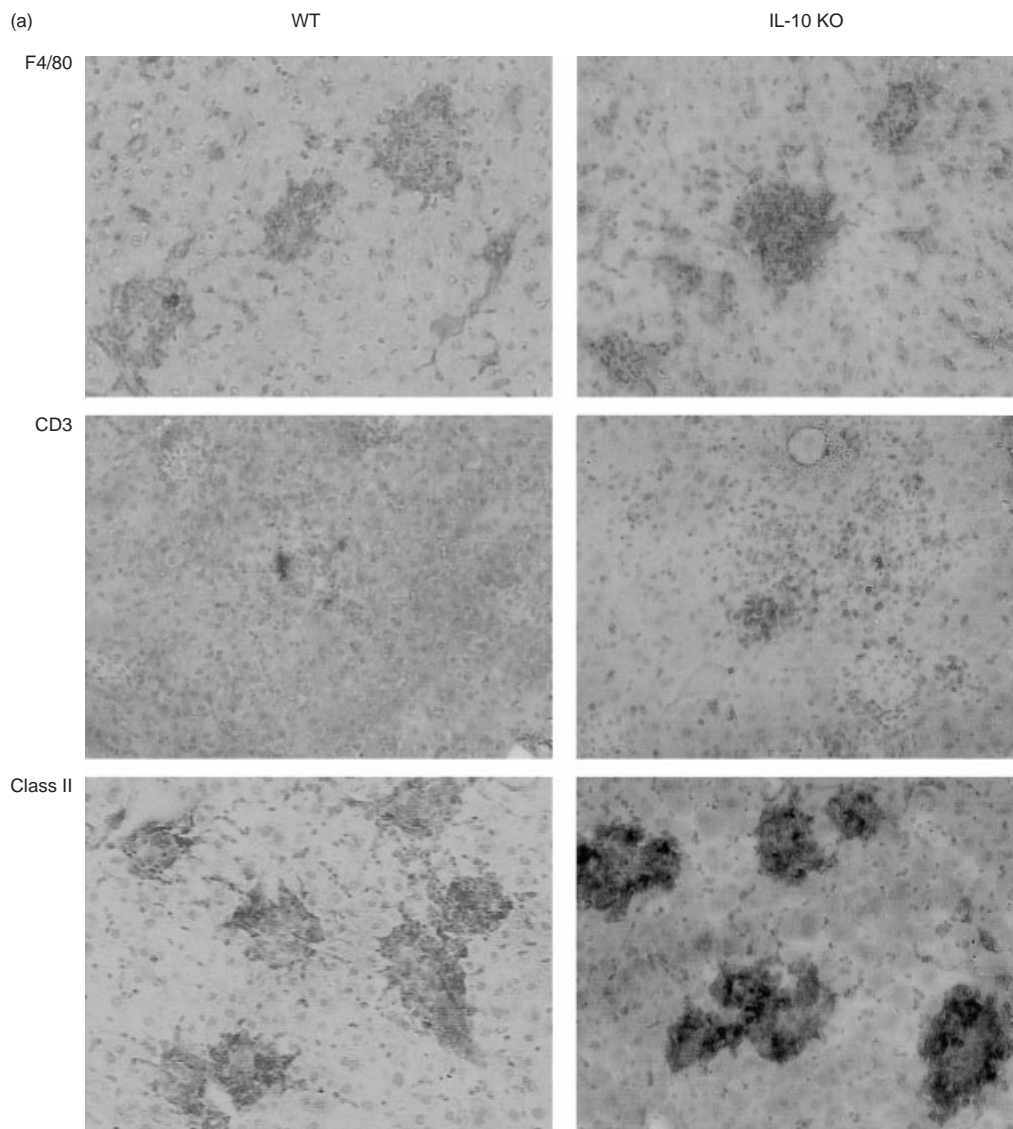
Figure 4. Cellular composition of hepatic granulomas in wild-type and IL-10-deficient mice at 4 weeks post-infection. Immunohistochemistry: expression of (a) F4/80, CD3, MHC class II, and (b) ICAM-1, CD11b and CD11c are distinctly increased in the absence of IL-10. Magnification × 180.

lower ($P < 0.05$) s compared to the wild-type mice (Fig. 1). Therefore, the absence of IL-10 favoured the clearance of mycobacteria, at least during the initial phase of infection. Similar results were obtained at a high inoculum of BCG, at 10^7 CFU. More importantly no mortality was recorded over a 3-month observation period.

These results suggest that endogenous IL-10 might attenuate a Th1 immune response. We therefore asked whether the correlate of cell-mediated immunity, the mycobacterial granuloma formation, is enhanced in the absence of IL-10.

Enhanced granuloma formation in IL-10-deficient mice

We analysed mycobacterial granulomas on microscopic sections of liver, spleen and lung from wild-type and IL-10-deficient mice 2, 4 and 8 weeks after BCG infection (10^6 CFU adminstered by intravenous injection). Typical granulomas were already found at 2 weeks in the liver of wild-type mice. They augmented in number and size over the next 2 weeks and consisted of activated macrophages resembling epithelioid cells and a darker lymphocyte mantle zone. In IL-10-deficient mice the granulomas had a higher cellularity and were increased in size as compared to wild-type mice (Fig. 2a). The bacterial load in tissues was further assessed by Ziehl–Neelsen staining of acid-fast bacilli in liver and spleen. Acid-fast bacilli were almost undetectable in the livers of IL-10-deficient mice at 2 weeks (Fig. 2b).

We further quantified the number of granulomas in liver sections. In the absence of IL-10 more granulomas were found per liver cross-section at 2 weeks (Fig. 3a) as compared to controls ($P < 0.05$). Furthermore, a semiquantitative assessment of acid-fast bacilli per granuloma in tissue sections confirmed significantly fewer mycobacteria ($P < 0.05$) in the liver (Fig. 3b) and lung (not shown) from IL-10-deficient mice. Therefore, a strong granulomatous response in IL-10-deficient mice correlated with a faster clearance of mycobacteria.
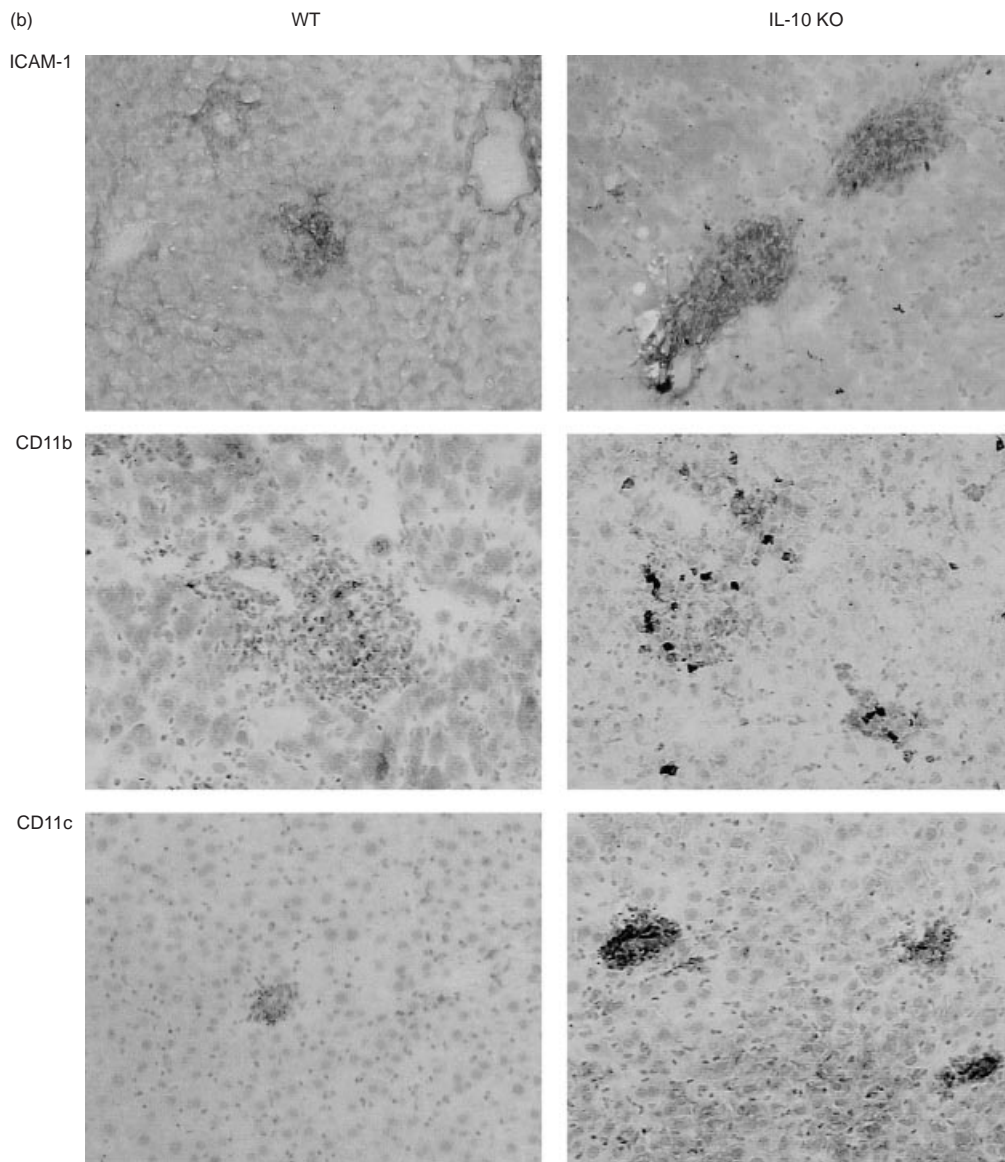
(b) WT IL-10 KO

ICAM-1

CD11b

CD11c

Figure 4. Continued.

Increased cell recruitment and activation of macrophages in granulomas

We then investigated the functionality of the granulomas and asked whether the inflammatory reaction in the liver and lungs of BCG-infected mice is enhanced in the absence of IL-10. Firstly, the recruitment of T cells (both CD4 and CD8 cells) and F4/80-positive macrophages was enhanced in the absence of IL-10 (Fig. 4a). Second, the expression of MHC class II, ICAM-1, CD11c and CD11b was distinctly higher in IL-10-deficient mice in comparison to wild-type controls (Fig. 4b). Although a few neutrophils could be found in the granuloma, neutrophils were not prominent in the granuloma and only slightly increased in the peripheral blood (not shown). Therefore, the increased size of granuloma was due to an augmented recruitment of T cells and macrophages.

We further asked whether macrophages in granulomas showed enhanced activation and determined acid phosphatase activity and iNOS expression. The expression of iNOS by immunostaining and acid phosphatase activity of macrophages in hepatic tissue sections was heightened in the absence of IL-10 (Fig. 5). We further quantified acid phosphatase activity in tissue homogenates, which was increased in the spleen in the absence of IL-10 (Fig. 6). The differences were statistically significant ($P < 0.05$).

These results suggest, that in the absence of endogenous IL-10 macrophages in granulomas have signs of strong activation, explaining the more rapid clearance of mycobacteria.

Enhanced cell-mediated immune response

In order to confirm whether a Th1 response prevailed in IL-10-deficient mice infected with *M. bovis* BCG, we tested the expression of TNF, IL-12 and IFN-γ in mononuclear cells

WT IL-10 KO

APase
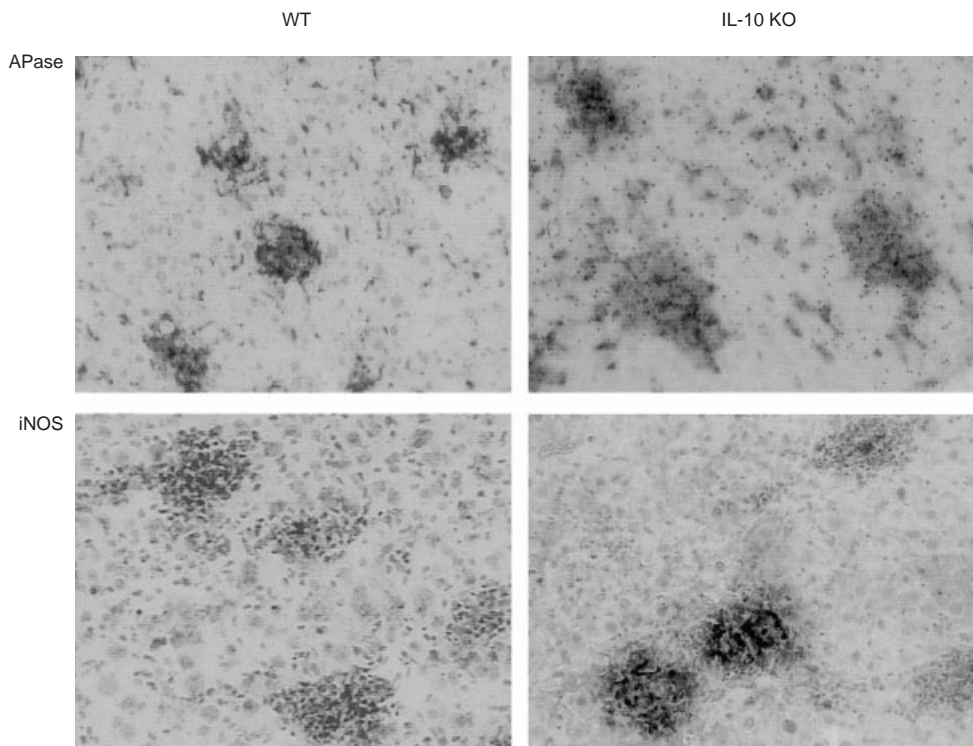
iNOS

Figure 5. Activated macrophages in hepatic granulomas from wild-type and IL-10-deficient mice at 4 weeks post-BCG infection. Distinct up-regulation of acid phosphatase activity and iNOS expression in hepatic granulomas assessed by histochemical reaction and immunostaining, respectively, as described in the Materials and Methods. Magnification × 320.

forming the granuloma by immunostaining. Macrophages reacted strongly with the TNF antibody in IL-10-deficient mice, while the immunoreactivity was very low in wild-type mice (Fig. 7). As specificity control for the anti-TNF antibody tissue from TNF-deficient mice were used which were completely negative (not shown). By contrast, no immunoreactivity was found for IL-12 and IFN-γ in the granuloma. We further measured cytokine level in serum for TNF, IFN-γ and IL-12 levels at 4 weeks after the injection of 10^7 CFU.
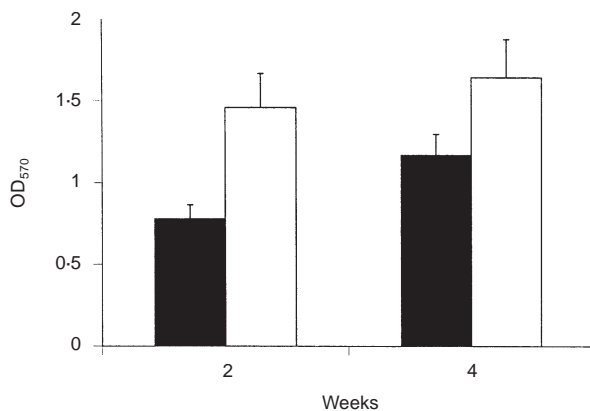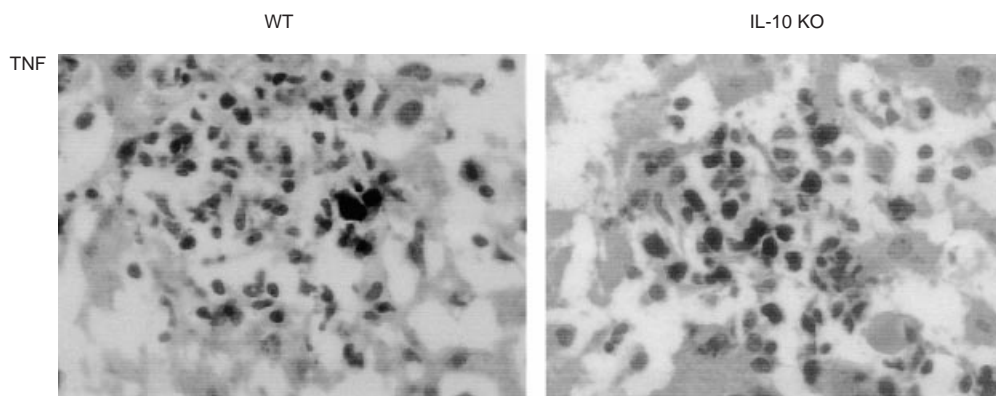
Although the levels were slightly elevated, no significant differences of cytokine serum levels were found in the two experimental groups (data not shown).

Finally, we tested the cutaneous DTH response using PPD as antigen for the challenge. The DTH response in IL-10-deficient mice was significantly ($P < 0.05$) increased as compared to the wild-type controls (Fig. 8). These results suggest that the cell-mediated immune response is enhanced in the absence of IL-10, which is a plausible explanation for the augmented antimycobacterial response.

DISCUSSION

We demonstrate here a vigorous cell-mediated immune response to *M. bovis* BCG infection with a robust granuloma response and enhanced DTH reaction in the absence of IL-10, which favours mycobacterial clearance at least in the initial phase of infection.

Increased mycobacterial clearance from liver and spleen has been demonstrated in *M. bovis* BCG-infected IL-10-deficient mice.[19] In addition, we show a rapid elimination of BCG from the lung, which is usually delayed after intravenous infection. Furthermore, the accelerated mycobacterial clearance was associated with an augmented granuloma response. The enlarged granulomas contained increased numbers of T cells and activated macrophages. While no differences in circulating levels of Th1-type cytokines were found, the activated macrophages expressed large amounts of TNF. Therefore, unrestricted local TNF production may have contributed to the vigorous granuloma response and rapid mycobacterial clearance.

Figure 6. Increased acid phosphatase activity in liver and spleen of IL-10-deficient mice infected with *M. bovis* BCG. IL-10-deficient mice and wild-type mice were infected intravenously with 10^6 BCG bacilli. Fresh spleen was processed for enzymatic analysis at 2 and 4 weeks as described. Data are expressed as the mean ± SD ($n = 4$ mice).
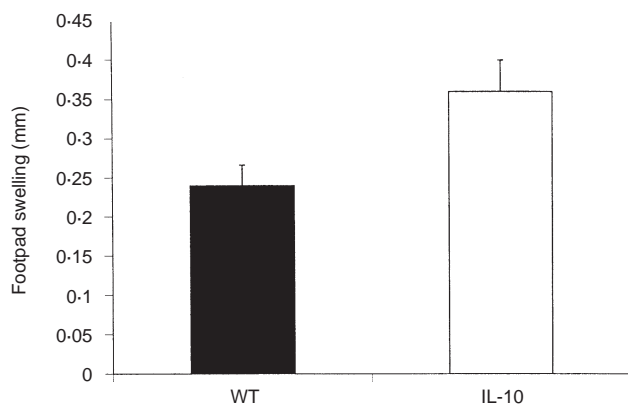
Figure 7. TNF immunoreactivity in mononuclear cells of hepatic granulomas in wild-type and IL-10-deficient mice at 4 weeks post-BCG infection. Immunostaining with TNF antibody of frozen liver sections as described in the Materials and Methods. Magnification × 320.

By contrast, Erb *et al.* reported that IL-10, as well IL-4 or IL-5, is not required for the control in *M. bovis* BCG-infected gene-deficient mice.[17] *In vitro* antigen re-stimulation assays of lymph node lymphocytes from wild-type and IL-10-deficient *M. bovis* BCG-infected mice showed no differences in IFN-γ production.[17,19] These data therefore suggest that T-cell-derived cytokines may not be important for the biological effect. We show here that macrophages from IL-10-deficient mice express increased tissue levels of TNF and iNOS, which most likely account for the increased antimycobacterial response. These data are corroborated by the report of increased pro-inflammatory cytokines, nitric oxide and prostaglandin formation in *in vitro* cultured macrophages from IL-10-deficient mice.[19]

In the present study we extend previous findings by demonstrating significant increased recruitment and activation of mononuclear cells into granulomas, and an enhanced antimycobacterial activity of the granuloma macrophages *in situ*. The augmented granulomatous response is correlated with an increased cutaneous DTH reaction to PPD.

A regulatory role of endogenous IL-10 in mycobacterial infection was expected, since previous experiments using neutralizing antibodies in *M. avium* infection revealed enhanced bacterial clearance. The discrepant results from the previous investigations in IL-10-deficient mice may be related to different strains of mycobacteria, dose and time of analysis, as the enhanced clearance in a resistant mouse strain is only visible in the early phase of infection.[17,18]

An IL-10-regulated Th1-like protective immune response had also been shown in *Chlamydia trachomatis* infection.[23] In the absence of IL-10 the clearance of chlamydia was accelerated, which correlated with enhanced DTH response and increased serum IFN-γ, TNF and IL-12 levels. No systemic release of pro-inflammatory cytokines was found resulting in toxicity in IL-10-deficient mice as reported for parasitic protozoan infections.[23] Even at the high BCG inoculum of 10^7 CFU no mortality was recorded.

Since the absence of IL-10 increased antimycobacterial immunity, it follows that IL-10 overexpression may reduce host resistance. This hypothesis was confirmed by experiments, in which transgenic mice overexpressing IL-10 from the T-cell compartment were infected with *M. bovis* BCG. These mice were unable to control the infection and developed large bacterial burdens.[15] Interestingly, macrophages, but not T-cell functions, were suppressed. Another IL-10 transgenic mouse strain expressing IL-10 in antigen-presenting cells displayed a similar high susceptibility to infection with intracellular pathogens like *Listeria monocytogenes* and *Leishmania major*.[24]

The comparison of the two extreme situations – absence or overexpression of IL-10 – demonstrates opposing effects on mycobacterial infection and suggests that IL-10 indeed has a regulatory role in the control of this intracellular micro-organism.

In conclusion, we demonstrate a rapid and augmented cell-mediated immune response to *M. bovis* BCG infection with the formation of highly mycobactericidal granulomas and reduced bacillary burden in the absence of IL-10, suggesting a regulatory role of IL-10 in mycobacterial infection.

Figure 8. Cutaneous delayed-type hypersensitivity reaction. BCG-infected wild-type and IL-10-deficient mice received an intradermal injection of PPD into the right footpad and the difference of the swelling of the right and left food-pad was measured. Mean values (mm) ± SD ($n = 5$ mice).

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