The Ag85B protein of *Mycobacterium tuberculosis* may turn a protective immune response induced by Ag85B-DNA vaccine into a potent but non-protective Th$_1$ immune response in mice

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Summary

Clarifying how an initial protective immune response to tuberculosis may later loose its efficacy is essential to understand tuberculosis pathology and to develop novel vaccines. In mice, a primary vaccination with Ag85B-encoding plasmid DNA (DNA-85B) was protective against *Mycobacterium tuberculosis* (MTB) infection and associated with Ag85B-specific CD4$^+$ T cells producing IFN-γ and controlling intramacrophagic MTB growth. Surprisingly, this protection was eliminated by Ag85B protein boosting. Loss of protection was associated with a overwhelming CD4$^+$ T cell proliferation and IFN-γ production in response to Ag85B protein, despite restraint of Th$_1$ response by CD8$^+$ T cell-dependent mechanisms and activation of CD4$^+$ T cell-dependent IL-10 secretion. Importantly, these Ag85B-responding CD4$^+$ T cells lost the ability to produce IFN-γ and control MTB intramacrophagic growth in coculture with MTB-infected macrophages, suggesting that the protein-dependent expansion of non-protective CD4$^+$ T cells determined dilution or loss of the protective Ag85B-specific CD4$^+$ induced by DNA-85B vaccination. These data emphasize the need of exerting some caution in adopting aggressive DNA-priming, protein-booster schedules for MTB vaccines. They also suggest that Ag85B protein secreted during MTB infection could be involved in the instability of protective antituberculosis immune response, and actually concur to disease progression.

Introduction

Tuberculosis (TB) is a chronic respiratory infection that continues to afflict humans especially in the developing countries (World Health Organization report, 2001). For more than 80 years, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine has been the only TB vaccine administered to humans. BCG is efficacious in protecting children from severe forms of TB, but it fails to confer protection against adult TB (Mittal *et al.*, 1996). The lack of protection by BCG vaccines is minimally attributable to a loss of specific memory as shown by the observation that boosting BCG-generated immunity by a second dose of BCG has repeatedly failed to show any beneficial effects (Leung *et al.*, 2001). Therefore, development of a non-fully protective immune response or its instability during *Mycobacterium tuberculosis* (MTB) infection should be included among other factors to explain the lack of BCG protection in adults. Clarifying how an initial protective response to TB looses its efficacy is essential to understand TB pathology and to develop novel vaccines.

Mycobacterial infection in rodents have been largely used as a TB model to test vaccine efficacy in preclinical trials despite evident differences with human TB, especially for the lack of a healthy infected state in these animals. Unfortunately, no vaccines capable of eradicating MTB infection have been found, and none has shown substantial improvement of efficacy over BCG. In BCG-vaccinated mice, bacillary growth stops sooner than in non-vaccinated animals, so that the number of viable pulmonary bacilli present in the stationary phase is substantially reduced (Medina and North, 1999), resulting, however, only in a delay of disease (Doherty *et al.*, 2004). Specific anti-MTB cell-mediated Th$_1$ immune response, although essential in controlling MTB infection in the initial phases (Cooper *et al.*, 1993; Scanga *et al.*, 2000), is not sufficient to halt TB onset, while the continuous activation of host response to the mycobacterial products may even facilitate TB progression (Dannenberg and Collins, 2001). Several mechanisms, often involving mycobacterial constituents, concur to the ability of MTB to escape from the innate and adaptive host immune responses (Palma, 2004).
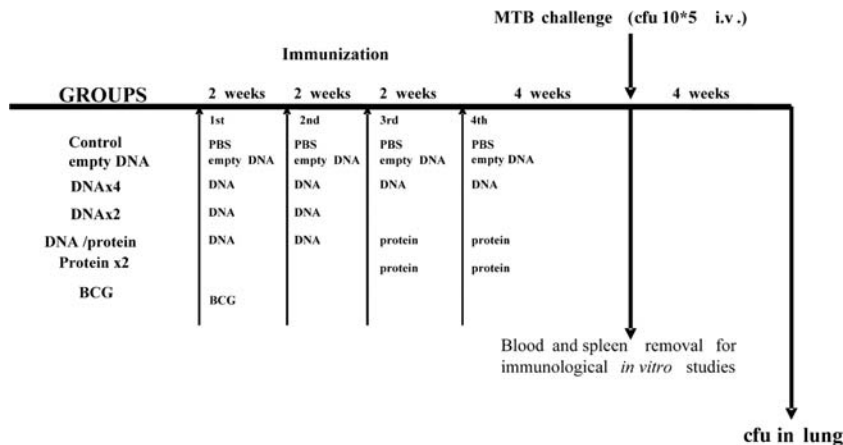
SCHEMATIC DIAGRAM OF EXPERIMENTAL DESIGN

Fig. 1. Schematic diagram of experimental design. C57BL/6 female mice were immunized twice or four times at 2 week intervals, with DNA-85B or Ag85B protein. As a positive control, a single dose of BCG was injected s.c. Four weeks after the last immunization, mice were challenged i.v. with 10^5 cfu of MTB H37Rv or killed to recover blood and spleen. Control = naïve C57BL/6 female mice receiving PBS; empty DNA = plasmid pRC 50 μg/injection i.m.; DNA = plasmid pRC (w/o tPA)-encoding Ag85B (DNA-85B) 50 μg/injection i.m.; protein = rAg85B protein 10 μg/injection s.c.; BCG = BCG Pasteur 10^5 cfu s.c.

Ag85B, a fibronectin-binding protein with mycolyltransferase activity, is the major secretory protein in actively replicating MTB (Belisle *et al.*, 1997). Ag85B is highly immunogenic, as shown by the easy detection of specific humoral and cell-mediated immune responses both in latently and actively infected TB patients (Boesen *et al.*, 1995; Mehra *et al.*, 1996; Smith *et al.*, 2000). In mice, a large proportion of CD4^{+} T cells infiltrating the lung after a MTB challenge, recognize Ag85B (Cooper *et al.*, 1997). For these reasons, Ag85B has been intensely investigated as a promising candidate for subunit TB vaccines. Several studies have shown a significant reduction of the mycobacterial load in the lungs of mice immunized with naked Ag85B-encoding plasmid DNA (DNA-85B) and challenged with virulent MTB (Lozes *et al.*, 1997; Ulmer *et al.*, 1997; Kamath *et al.*, 1999; Feng *et al.*, 2001; Doherty *et al.*, 2004). However, contradictory data on the efficacy of Ag85B protein vaccination have been reported. Vaccination with recombinant Ag85B protein in MPL-DDA or in SAF adjuvant has been unable to induce protection in guinea pig (Horwitz *et al.*, 2000; Olsen *et al.*, 2004), and slight, non-significant or weakly significant reduction in colony-forming unit (cfu) lung following MTB infection have been reported in mice immunized with Ag85B protein in MPL-DDA adjuvant (Olsen *et al.*, 2001; Doherty *et al.*, 2004). Immunization with Ag85B-ESAT-6 fusion protein induced a protective response both in guinea pig and murine TB model, greatly superior to the combination of the two mycobacterial antigens (Olsen *et al.*, 2001; 2004; Doherty *et al.*, 2004). In guinea pig, Ag85B-secretion by recombinant BCG (Horwitz *et al.*, 2000), or boosting with Ag85B protein in AS02A adjuvant after BCG priming (Horwitz *et al.*, 2005) increased the protection generated by BCG vaccination, but in mice Ag85B-secretion by recombinant BCG (Palendira *et al.*, 2005) was not associated with reduction in lung bacterial load. In addition, boosting with Ag85B protein in SBAS2A adjuvant (Tanghe *et al.*, 2001), but not boosting with Ag85B protein in IFA adjuvant (Feng *et al.*, 2001) following DNA-85B priming augmented protective efficacy of DNA-85B vaccination against murine TB.

Moreover, Ag85B-specific CD4^{+} T cells recruited during delayed-type hypersensitivity response to the purified protein derivative (PPD) were not substantially involved in resistance to MTB in memory TB-immune mice (Pais *et al.*, 1998).

For all these reasons we decided to better investigate how the Ag85B protein can modulate a pre-existing and protective immune response induced by DNA-85B vaccination. This was done with the main objective of understanding which mechanisms are involved in the instability of the protective anti-TB immune response.

Results

Boosting with Ag85B protein eliminates the protective effect induced by DNA-85B immunization

Mice receiving different immunogens and control materials, as shown in Fig. 1, were challenged with MTB and 4 weeks after challenge, lungs were explanted and the bacterial load was measured. A significant reduction of the bacterial load was observed in mice receiving two administrations of the DNA-85B vaccine (DNAx2-immunized mice) with respect to unvaccinated naïve control or empty-plasmid DNA recipient mice, indicating protection by the vaccine (Fig. 2A and B). The degree of reduction of the mycobacterial burden was not significantly different from that conferred by BCG, although the immunization with BCG caused the highest nominal reduction of mycobacteria in the lungs. DNAx2-immunized mice receiving two additional boosters of DNA-85B (DNAx4-immunized mice) did not suffer any loss of protection compared with
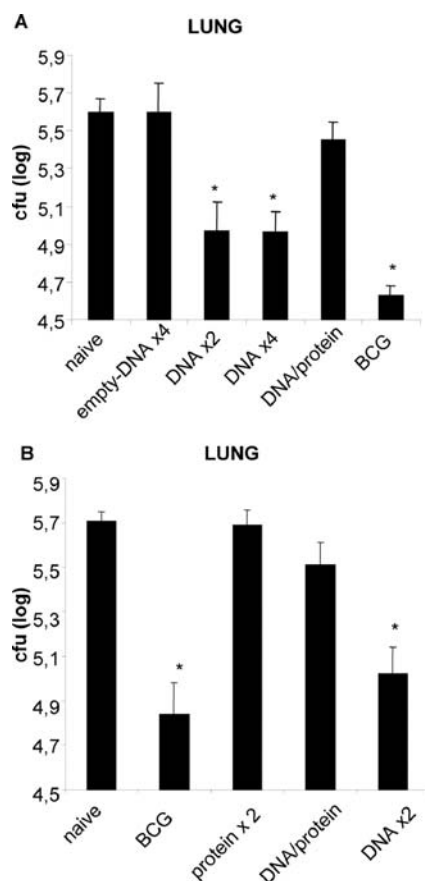
Fig. 2. Protection provided by DNA-85B immunization was lost after boosting with Ag85B protein. Mice (6 mice/group, panel A; 5 mice/group, panel B) were immunized according to Fig. 1, and challenged with MTB H37Rv. Four weeks after infection, the bacteria in the lung were enumerated as described in *Experimental procedures*. In A, data are expressed as mean of the six individual mice ± SE and are representative of one experiment out of two; in B, data are expressed as mean of the five individual mice ± SE. The level of statistical significance for differences between test groups and the control vector were determined by ANOVA test (*significant).

the DNAx2-immunized mice (Fig. 2A). On the contrary, DNAx2-immunized mice boosted twice with recombinant Ag85B protein (DNA/protein-immunized mice) showed a bacterial load not significantly different from control, non-immunized animals indicating that the protection achieved by DNA-85B vaccination was eliminated by Ag85B protein boosting (Fig. 2A and B). Immunization with the Ag85B protein alone did not induce a significant change of the mycobacterial load with respect to the naive animals (Fig. 2B).

Enhanced humoral immune response in DNA/protein-immunized mice

Sera of immunized mice were assayed for specific antibodies (Abs) against Ag85B by ELISA. No anti-Ag85B

Abs were found in the serum of naïve mice while they were found in all mice immunized with Ag85B either as DNA or as protein (Fig. 3). Two injections of Ag85B protein in the absence of adjuvant and without DNA-85B priming were sufficient to induce anti-Ag85B Abs, although exclusively of the IgG_1 subclass, while DNAx2 or DNAx4 immunizations induced mixed responses of IgG_1 and IgG_{2a} subclasses. In DNA/protein-immunized mice, higher Ab levels were seen with an IgG_{2a}/IgG_1 ratio of approximately 2, suggestive of a switch to a predominant Th_1 response.

IFN-γ associated with IL-10 release in Ag85B-specific splenocytes of DNA/protein-immunized mice

In splenocytes of DNAx2- and DNAx4-immunized mice, as well as in mice receiving only Ag85B protein in the absence of adjuvant and without DNA-85B priming, similar cellular anti-Ag85B responses were observed with weak but appreciable cell proliferation, IFN-γ and IL-2 secretion, but no IL-10 secretion (Fig. 4). In contrast, in DNA/protein-immunized mice, a rather remarkable increase in cell proliferation (ranging from five- to 15-fold compared with DNAx2-immunized mice, DNAx4-immunized mice or Ag85B protein only recipients) were observed. Moreover, splenocytes of DNA/protein-immunized mice, differently from splenocytes of DNAx4-immunized mice were still able to proliferate on day 9 of Ag85B protein stimulation, although to a lower extent than on day 6 (usually the optimum for this type of assay), indicating a great expansion of Ag85B-responding cells (cpm of Ag85B-stimulated splenocytes subtracted from cpm of unstimulated cells for DNA/protein-immunized mice: 43 971 ± 5697 and 6660 ± 33 at 6 and 9 days of culture respectively, and for DNAx4-immunized mice: 2854 ± 250 and 243 ± 8 at 6 and 9 days of culture
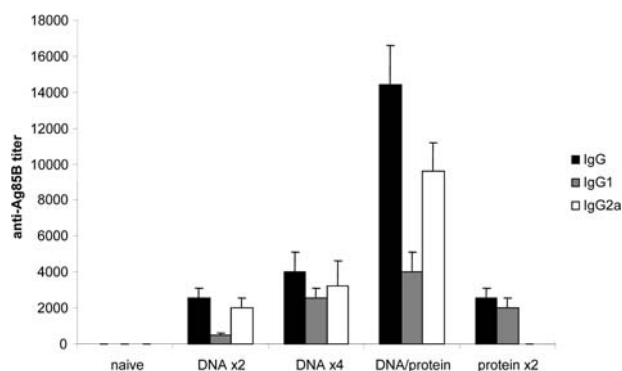

Fig. 3. Ag85B-specific Ab production. Pooled serum samples (7 mice/group) were analysed by ELISA for the presence of anti-Ag85B Abs. The isotype profile of these Abs was characterized using conjugated secondary Abs specific for IgG_1 and IgG_{2a}. Data are plotted as geometric mean ELISA titer ± SEM of three independent experiments.

A

Cell proliferation

B

IFN-γ
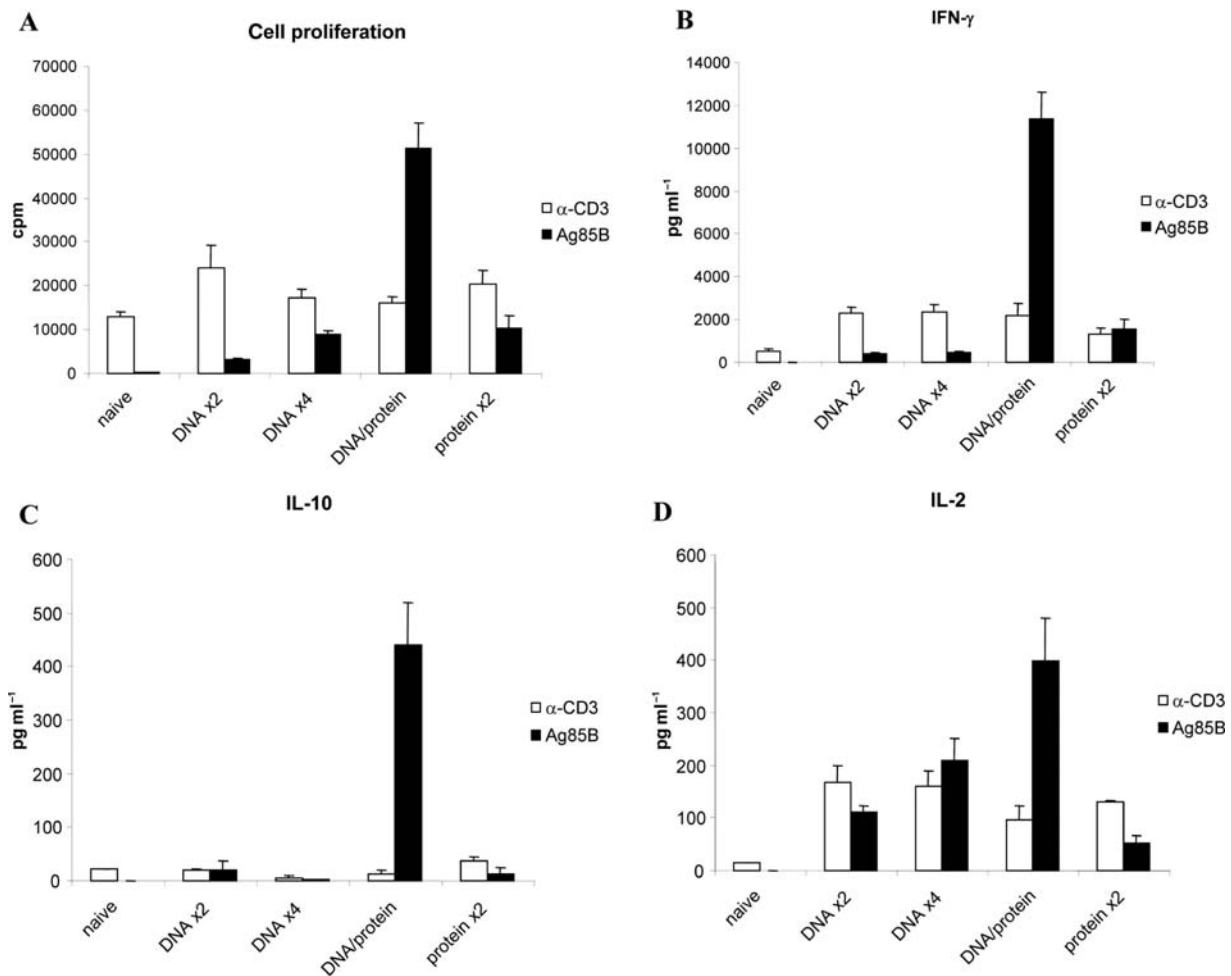
C

IL-10

D

IL-2

Fig. 4. Cell proliferation and cytokine production in response to Ag85B protein. Pooled splenocytes (7 mice/group) of naive unvaccinated mice or vaccinated mice were restimulated with Ag85B protein (5 μg ml⁻¹) or suboptimal concentration of agonistic anti-CD3 Ab (0.05 μg ml⁻¹) for 48 h before testing IFN-γ (B*)*, IL-10 (C) or IL-2 (D) by commercial ELISA kits, or for 5 days before measuring thymidine incorporation in proliferating cells (A). Cell proliferation data are represented as mean cpm (subtracted from the cpm of unstimulated splenocytes) while cytokine data are represented as mean pg ml⁻¹ of five independent experiments. No cytokines were detected in unstimulated splenocytes. Error bars indicate SEM.

respectively). In splenocytes of DNA/protein-immunized mice a strong enhancement of IFN-γ secretion (eight- to 26-fold compared with DNAx2-immunized mice, DNAx4-immunized mice or Ag85B protein only recipients) were also observed. ELISPOT analysis indicated that this overwhelming production of IFN-γ by the splenocytes of DNA/protein-immunized mice, re-stimulated *ex vivo* with recombinant Ag85B protein, was also associated with a strong increase in the number of Ag85B-specific IFN-γ-secreting cells (1640 ± 42 versus 207 ± 2 of IFN-γ-secreting cells per 10⁶ splenocytes of DNA/protein-immunized versus DNAx4-immunized mice respectively; mean ± SD of two independent experiments). IL-2 release was also enhanced (two- to fourfold) with respect to DNA-85B only immunized mice. Interestingly, the excessive IFN-γ release was associated with substantial IL-10 production (Fig. 4). IL-4, TNF-α and IL-12p70 were

not secreted in Ag85-stimulated splenocytes of any of the mice groups (data not shown).

As a antigen-specificity control, stimulation with an agonistic anti-CD3 Ab induced rather comparable lymphocyte proliferation and cytokine secretion in splenocytes of all vaccinated mice (Fig. 4).

The overwhelming antigenic responses in DNA/protein-immunized mice is mediated by CD4⁺ T cells

Depletion of CD4⁺ T cells in splenocytes of DNA/protein-immunized mice almost totally eliminated Ag85B-induced cell proliferation, IFN-γ and IL-10 secretion (Fig. 5). The contribution of CD4⁺ T cells to specific antigenic response was also relevant in DNAx4-immunized mice (Fig. 5). (As the splenocytes of DNAx2-immunized mice behaved
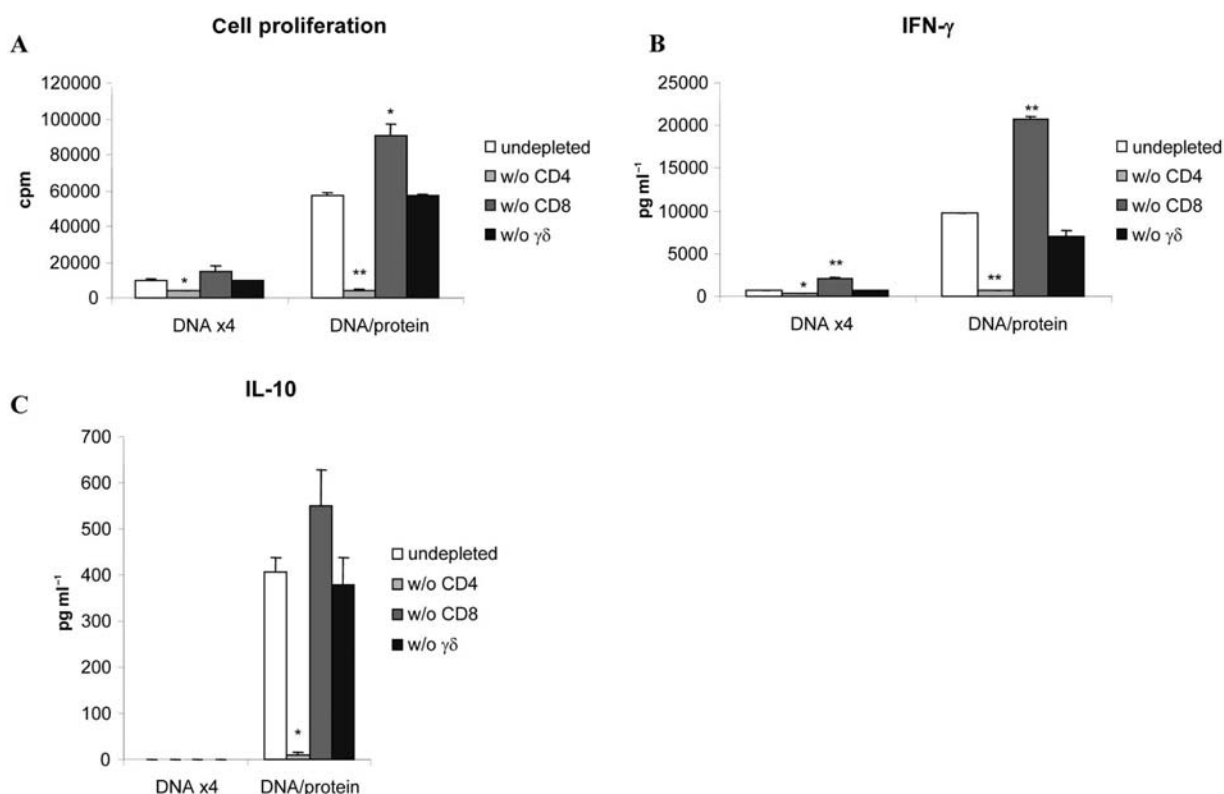
Fig. 5. The contribution of T-cell subsets to Ag85B protein-induced responses. Pooled splenocytes (7 mice/group) of DNAx4-, or DNA/protein-immunized mice were depleted of CD4⁺, CD8⁺ or γδ⁺ T cells by using magnetic beads as described in *Experimental procedures*. Undepleted cells and subsets were restimulated with Ag85B protein (5 μg ml⁻¹) for 48 h before testing the IFN-γ (B) and IL-10 (C) by commercial ELISA kits, or for 5 days before measuring thymidine incorporation in proliferating cells (A). Cell proliferation data are represented as mean cpm (subtracted from the cpm of unstimulated undepleted cells or subsets) while cytokine data are represented as mean pg ml⁻¹ of three independent experiments. No cytokines were detected in unstimulated undepleted cells or subsets. Error bars indicate SEM. The level of statistical significance for differences among undepleted cells and subsets in each group were determined by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

exactly as splenocytes of DNAx4-immunized mice no further data regarding the former group will be shown.)

CD8⁺ T cell-depletion induced a statistically significant increase in IFN-γ release in both vaccinated groups, although particularly elevated in DNA/protein-immunized mice, in which a further enhancement of cell proliferation was also observed (Fig. 5). The increased expression of the activation marker CD137 on CD8⁺ T cells of DNA/protein-immunized mice cultured for 5 days with Ag85B protein (2% ± 1% versus 10% ± 1% unstimulated versus Ag85B-stimulated respectively, $n = 2$; mean ± SD), indicated that, CD8⁺ T cells were also involved in Ag85B protein recall response.

As γδ T cells are involved in the complex immune response mounted against MTB and it is not possible to exclude that peptides in addition to lipoprotein and phosphoantigens may be the natural ligands for these cells, we investigate the role of these cells in Ag85B protein-recall response. Depletion of γδ T cells, however, did not significantly alter Ag85B-induced response of splenocytes of any vaccinated groups (Fig. 5).

Splenocytes of DNA/protein-immunized mice are unable to control MTB growth and release IFN-γ in cocultures with MTB-infected bone marrow-derived macrophages

Bone marrow-derived macrophages (BM-Mφ) were infected with MTB and then cocultured for 8 days with splenocytes of vaccinated mice at 1:2 ratios (MTB-infected macrophage : splenocytes). In the absence of splenocytes, the infected BM-Mφ did not release IFN-γ (Fig. 6A and B). Splenocytes of DNAx4-immunized mice, recognized MTB-infected BM-Mφ and secreted IFN-γ, in an almost exclusively CD4⁺ T cell-dependent manner (Fig. 6A and B). As a result, these splenocytes were able to control intramacrophagic MTB growth (Fig. 6C) with an essential contribution of CD4⁺ T cells (Fig. 6D).

Conversely the splenocytes of DNA/protein-immunized mice completely lost the ability to release IFN-γ when cocultured with MTB-infected BM-Mφ [both at 72 h (data not shown) and 8 days (Fig. 6A)], and were unable to control intramacrophagic mycobacterial growth (Fig. 6C). However, these splenocytes regained an overwhelming
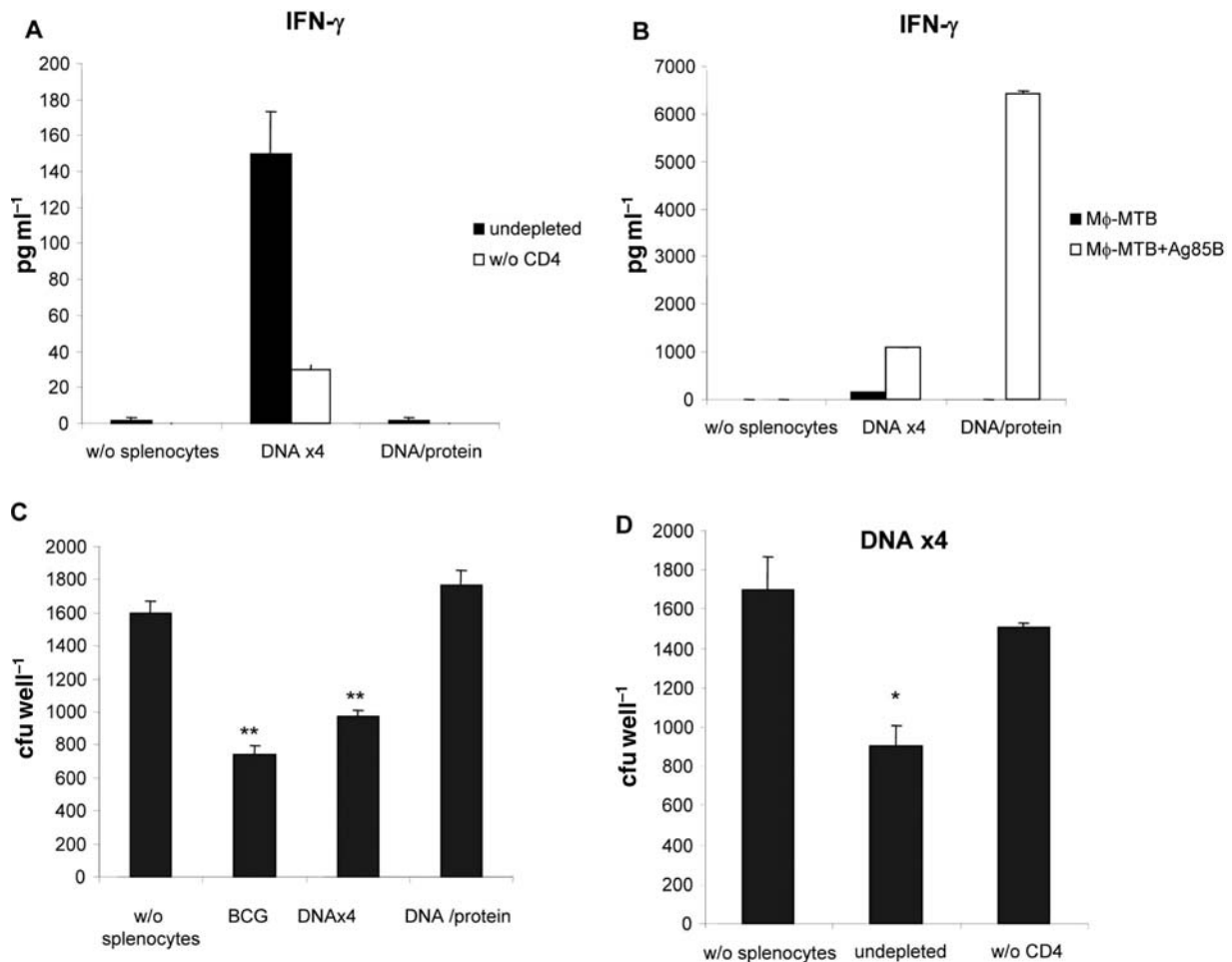
Fig. 6. IFN-γ secretion and MTB growth control by splenocytes of vaccinated mice cocultured with MTB-infected BM-Mφ. BM-Mφ were infected with MTB and then cocultured with pooled splenocytes (7 mice/group) of immunized mice at a 1:2 ratio (MTB-infected BM-Mφ: splenocytes). CD4-depleted splenocyte fractions were prepared by using magnetic beads as described in *Experimental procedures*. In B, cocultures of MTB-infected BM-Mφ and immunized splenocytes were treated or untreated with Ag85B protein (5 μg ml^{-1}). In D, undepleted, or CD4-depleted splenocyte fraction of DNAx4-immunized mice were cocultured with MTB-infected BM-Mφ. After 8 days, IFN-γ was measured in culture supernatants by commercial ELISA kit (A, B) and the bacteria in the cocultures were enumerated as described in *Experimental procedures* (C, D). Data are represented as mean of three independent experiments in A–C; error bars indicate SEM. In D, data are represented as mean ± SE of triplicate determinations of a single experiments. In C and D, the level of statistical significance for cfu differences among MTB-infected BM-Mφ and splenocyte cocultures were determined by Student's *t*-test (*P < 0.05; **P < 0.01).

capacity of producing IFN-γ when Ag85B protein was added to the cocultures (Fig. 6B), indicating that processing and presentation of the exogenous Ag85B protein stimulated a different CD4$^+$ T cell set compared with that required for Ag85B recognition on macrophages infected with live MTB.

Discussion

Understanding why the initially protective immune response to MTB, following infection or vaccination, may be subsequently lost is central to understand the natural history of MTB infection, particularly its latency, and for a rational design of TB vaccines, as well.

In this paper, we demonstrate that a protective Ag85B-specific CD4$^+$ T cell response elicited by a DNA-85B vaccine, may be lost when mice are boosted with Ag85B protein. We also demonstrate that this loss is associated with a overwhelming CD4$^+$ T cell proliferation and IFN-γ production in response to Ag85B protein. However, these Ag85B protein-responding CD4$^+$ T cells lost the ability (preserved in CD4$^+$ T cells of DNA-immunized mice), to produce IFN-γ and control MTB intramacrophagic growth in coculture with MTB-infected macrophages. These data suggest that the Ag85B protein-dependent expansion of non-protective CD4$^+$ T cells determined dilution or loss of the protective Ag85B-specific CD4$^+$ T cells previously induced by DNA-85B vaccination.

Although the mechanisms involved in the expansion of these non-protective Ag85B protein-specific CD4+ T cells are not clearly understood, the high immunogenicity of Ag85B protein, as indicated by specific humoral and cell-mediated responses in mice receiving Ag85B protein in the absence of adjuvant or priming, may be important in the generation of this phenomenon. It could be of interest to investigate whether other secretory MTB antigens can share this particular property of Ag85B protein. However, Ag85B protein antigenic features were not sufficient *per se* to generate the excessive Ag85B protein-specific response, because this appeared exclusively after DNA-85B priming. Therefore, the presence of effector/memory Ag85B-specific CD4+ and CD8+ T cells, along with anamnestic Ag85B presenting dendritic cells (DC) (Chiu *et al.*, 2004), previously induced by DNA-85 vaccination, might have influenced the subsequent response to the exogenous Ag85B protein booster. In addition, the exclusive MHC class II antigen presentation of Ag85B protein was required, in view of the fact that the boosters with DNA, which are currently considered to elicit both MHC class I and class II immune responses (as demonstrated for Ag85A-encoding DNA; Denis *et al.*, 1998), were ineffective in the generation of this phenomenon.

Some studies suggest that vaccination with DC presenting exclusively MHC class II antigens can be indeed deleterious. A florid infiltration of macrophages and CD4+ and CD8+ IFN-γ secreting cells was described in the lungs of mice vaccinated with lung DC cells pulsed *in vitro* with Ag85B protein and challenged with MTB. The potent inflammatory response so generated did not increase protection, but resulted instead in a severe consolidation of the lung tissue (Gonzales-Juarrero *et al.*, 2002). In addition, co-presentation of both class I and class II epitopes on the same DC is required to confer protection against MTB challenge, as shown by immunization with DC pulsed with the related mycobacterial antigen Ag85A (McShane *et al.*, 2002).

The lack of MTB-infected macrophage recognition by Ag85B protein-responding CD4+ T cells of DNA/protein-immunized mice suggests that not all the Ag85B epitopes recognized by the host immune system are efficiently presented by MTB-infected macrophages. It would be interesting to identify which Ag85B epitopes are important in the recognition of MTB-infected macrophages, and which are responsible of non-protective Ag85B protein-specific response. In addition, these data also indicate that the host immune system can react to the various epitopes of the same antigen, generating different responses with different functions. Other mycobacterial antigens seem to behave similarly. Vaccination with a secretory plasmid DNA encoding mycobacterial antigen 85 A stimulates a CD4+ and CD8+ T cell epitopic repertoire broader than that stimulated by MTB infection (Denis *et al.*, 1998). Peripheral blood mononuclear cells (PBMC) from active TB patients secrete IFN-γ in response to both the whole protein and specific MHC class II peptides of the mycobacterial secretory antigen complex ESAT-6, while PBMC from healthy, latently infected individuals produced IFN-γ only in response to the whole ESAT-6 protein. This suggests that in patients with active TB, antigen-specific IFN-γ-secreting CD4+ T subpopulations, absent in healthy individuals, were generated (Vincenti *et al.*, 2003).

The loss of the protective Ag85B-specific CD4+ T cell fraction generated through DNA-85B immunization might have been caused by the IFN-γ released by the highly proliferating, non-protective Ag85B-specific CD4+ T cell fraction, in view of IFN-γ capacity to dampen T cell responses. In fact, during mycobacterial infection, IFN-γ eliminates pathogen-activated CD4+ T cells by inducing apoptosis (Dalton *et al.*, 2000; Florido *et al.*, 2005). This hypothesis is in agreement with the fact that during chronic exposure to antigens in persistent MTB infection, the frequency of IFN-γ-producing effector CD4+ and CD8+ T cells dynamically change (Lazarevic *et al.*, 2005). Furthermore, in human TB, an association between increased apoptosis and IFN-γ secretion in the lesion has been described (Hirsch *et al.*, 2001). On the other hand, that chronic or latent infection may be favoured by a overwhelming or inappropriate cell-mediated immune response, and particularly IFN-γ secretion, is not a unique feature of MTB infection. For instance, persistence of chlamydial infection is clearly associated with IFN-γ capacity to arrest the intracellular developmental stages of these parasites (Sarov *et al.*, 1991). As for MTB, this pro-latency effect of IFN-γ, coexists with the essential role played by this cytokine in controlling the acute infection. Therefore, although IFN-γ is essential in controlling the acute phase of MTB infection (Cooper *et al.*, 1993), the ability to induce a strong IFN-γ response, frequently used, as a parameter to screen TB vaccines, should be carefully re-evaluated. Actually, we found that boosting with Ag85B protein in LTK63 adjuvant following DNA-85B immunization, induced a partial but significant recovery in protection associated with a significant reduction in CD4+ T cell Ag85B-specific IFN-γ secretion and cell proliferation (C. Palma *et al.*, in preparation). These data, in agreement with other data already reported in the literature (Leal *et al.*, 2001), again highlight how generation of an excessive Th$_1$ response, and in particular IFN-γ, could interfere with the development of protective immune responses to TB during vaccination.

As IFN-γ is so crucial in modulating anti-TB response, it was not a surprise to find that mechanisms have been developed to regulate its production. In all vaccinated mice, CD8+ T cell depletion determined a consistent

increase in IFN-γ release and, in DNA/protein-immunized mice, an increase in cell proliferation. In a murine model of *Mycoplasma pulmonis* infection, *in vivo* depletion of CD8⁺ T cells resulted in significantly more severe pulmonary disease due to exacerbation of CD4⁺ Th cell-associated inflammatory lesions (Jones *et al.*, 2002). In addition, the CD4⁺-dependent IL-10 secretion onset in DNA/protein-immunized mice may be an additional self-reduction mechanism to control IFN-γ secretion. In fact, IL-10-producing CD4⁺ T cells are intended to be T reg1 cells developed to reduce Th₁ cell responses (Groux *et al.*, 1997).

It is well known that, immune responses to the pathogens, including MTB, can result in collateral damage to host tissues, and immunoregulatory mechanisms, including induction of regulatory T cells, may be essential to control this immunopathology. Actually, antigen-specific T reg1 cells have been described in TB patients. IL-10-specific Abs increased PPD-specific IFN-γ production by T cells from non-responding TB patients, highlighting the negative control exerted by IL-10 on antigen-induced IFN-γ production (Boussiotis *et al.*, 2000). Persistent tuberculin anergy, characterized by PPD-specific impaired proliferative responses, reduced levels of IL-2, IFN-γ and higher levels of IL-10, have been found in a cohort of pulmonary TB patients from rural Cambodia (Delgado *et al.*, 2002).

Moreover, it is worth to note that the antigen Ag85B, when given both as protein or encoding DNA, preferentially induced a marked Th₁ response being the IL-4 cytokine never detected even in mice receiving Ag85B protein alone and developing an exclusively IgG₁ humoral response often associated with a Th₂ response.

It is tempting to speculate that the Ag85B protein characteristic to switch a pre-existing protective immune response into a non-protective one, described in this TB vaccination model, might take place also during MTB infection in humans. High levels of circulating Ag85B protein have been found in active TB patients (Bentley-Hibbert *et al.*, 1999) and a response to Ag85B protein, mediated by CD4⁺ Th₁-like lymphocytes with IFN-γ release, has been described in TB-infected individuals (Boesen *et al.*, 1995; Mehra *et al.*, 1996; Smith *et al.*, 2000). The response to secreted antigens was markedly higher in patients with active minimal TB than in healthy BCG-vaccinated donors (Boesen *et al.*, 1995). In addition, secretion of IFN-γ was predominant in patients who displayed bilateral pulmonary lesions and lung cavitation (Cardoso *et al.*, 2002; Fortes *et al.*, 2005). Therefore, Ag85B protein, normally secreted during MTB infection, could induce, in a positive feedback loop, selection and expansion of non-protective Ag85B protein-specific CD4⁺ T cells secreting excessive IFN-γ level, thus determining both a progressive decay of the efficacious effector/

memory pathogen-specific T cells, and a consolidation of inflammatory lung lesion. All these factors could contribute to the instability of anti-TB protective immune response and favour TB progression. A parallel could be made with the well-known 'Koch phenomenon' observed when MTB culture filtrate extracts were given to TB patients, causing florid reactions and several deaths in many of the patients with a relatively mild disease (Koch, 1890).

Experimental procedures

Microorganism

MTB H37Rv (ATCC 27294) and BCG were grown at 37°C in Middlebrook 7H9 medium under agitation (120 rpm) up to mid-exponential phase. Aliquot stocks were stored at −70°C until use; the titers of stocks were verified on a regular basis by counting the numbers of cfu on Middlebrook 7H10 agar plates. For the manipulation of plasmids, *E. coli* MC1061 was grown on Luria–Bertani (LB) broth or agar supplemented with ampicillin (100 μg ml⁻¹), as required.

Production of DNA-85B vaccines and recombinant Ag85B protein

Ag85B gene was amplified from H37Rv genomic DNA with sense (5′-AGGATCCTTCTCCCGGCCGGGGCTGCCG-3′) and anti-sense (5′-TGGTACCGCTCAGCCGGCGCCTAACG-3′) primers using a standard PCR procedure. PCR product was cloned in pGEM T-Easy vector (Promega, Madison, WI), sequenced and subcloned in NheI–NotI sites of multigenic expression vector pRC (Papa *et al.*, 1998) for plasmid DNA-85B vaccine. For mature Ag85B protein purification, sense (5′-AGGATCCTTCTCCCGGCCGGGGCTGCCG-3′) and anti-sense (5′-TGGTACCGCTCAGCCGGCGCCTAACG-3′) primers were used for PCR. The product was subcloned in pQE30 (QIAGEN, Hilden, Germany) BamHI-KpnI sites. *E. coli* strain M15 transformed with pQE30–85B was grown in LB broth containing ampicillin (100 μg ml⁻¹) and kanamycin (25 μg ml⁻¹) to the desired OD₆₀₀ and 85B synthesis induced by IPTG (1 mM) for 5 h. Pelleted bacteria were resuspended in Buffer A (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1 μg μl⁻¹ lysozyme), incubated 20 min on ice and sonicated for 10 s, repeated 10 times. His-tagged native Ag85B was purified from lysate contaminants on Ni-NTA agarose columns (Qiagen) and dialysed overnight against a 10 mM TrisHCl, pH 8, 100 mM NaCl solution. Fractions were examined on an SDS-12% polyacrylamide gel. The Ag85B containing fractions were collected, concentrated by ultrafiltration with stirred cell (SC300 Schleicher and Schuell, Dassel, Germany) 10 kDa cut-off membrane, and sterilized by filtration through a 0.22 μm low protein-binding filter. The concentrated material was applied to a Superdex 75 column (Amersham Biosciences, Uppsala, Sweden) to isolate the target protein from other contaminants to a final purity higher than 95%, as determined by Coomassie staining. The LPS content of this preparation was measured by a *Lymulus* amebocyte lysate test and shown to be below 4.3 EU μg⁻¹ of protein. The Ag85B protein obtained was not refolded.

Immunization and mycobacterial infection

C57BL/6 female mice were supplied as specific pathogen-free mice by Harlan (Udine, Italy) and were maintained in specific pathogen-free conditions. Food and water were available *ad libitum*. Animal care and use followed the guidelines of the European Community Council (86/609/EEC). Mice at or between 7 and 8 weeks old were immunized. Fifty micrograms of plasmid, both DNA-85B or control vector, was injected intramuscularly (i.m.) in 50 μl PBS into the hind leg. On the dorsum of the mice, recombinant Ag85B protein (10 μg) was administered subcutaneously (s.c.). Mice were immunized two or four times at 2 week intervals, accordingly to the scheme shown in Fig. 1. As a positive control, a single dose of BCG (10^5 cfu) was injected s.c.

Four weeks after the last boost, mice were challenged intravenously (i.v.) in a lateral tail vein with 10^5 cfu of MTB H37Rv. Infection studies were performed in a biosafety level 3 facility; mice were housed in isolator cages and fed with autoclaved food and water *at libitum*. After 4 weeks, the mice were killed by cervical dislocation and the number of bacteria in lungs was enumerated by homogenizing the tissue and plating 10-fold dilutions, prepared in distilled water, on Middlebrook 7H10 agar. The colonies were counted visually after 21 days of incubation. These studies have been reviewed and approved by Italian Ministry of Health.

Antibody measurement

Serum from immunized mice was collected by retro-orbital bleeding 4 weeks after the last immunization. The levels of total anti-Ag85B IgG Abs were determined by ELISA. Wells were coated with recombinant Ag85B (5 μg ml^{-1}), and several dilutions of mouse serum were incubated for 90 min prior to the addition of goat anti-murine immunoglobulin IgG or IgG$_1$, or IgG$_{2a}$ alkaline phosphate-conjugated Abs (1:2000) (Serotec, Oxford, UK) and then n-nitrophenyl-phosphate (1 mg ml^{-1}) (Sigma Chemical, St Louis, MO). The mean absorbance of naive mouse sera, diluted 1:100, plus three standard deviations was adopted as the cut-off absorbance for determining Ab titers.

Splenocyte preparation and separation of lymphocytes

After 4 weeks from the last immunization, single cell suspensions were prepared from the pooled spleens (7 mice/group), passed through Falcon 2360 cell strainers (BD Discovery Labware), centrifuged, aliquoted and then frozen. From single spleen cell suspension, depletion of CD4$^+$ or CD8a$^+$ T cell populations, were obtained by the magnetically labelled fraction isolated from the specific mouse CD4$^+$ T or CD8a$^+$ T cell isolation kits (Miltenyi Biotec, Auburn, CA), in accordance with the manufacture's instructions. Depletion of γ/δ T cells was obtained by labelling cells with anti-mouse TCR γ/δ (FITC) (BD Biosciences Pharmingen) and operating a positive magnetic selection with anti-FITC Microbeads (Miltenyi Biotec). By FACS analysis, negligible fluorescence was observed in depleted fractions during the labelling of cells with the appropriate monoclonal Ab corresponding to the depleted cell population.

Lymphocyte proliferation

Cells isolated from spleens of naïve or vaccinated mice, either undepleted or CD4-; CD8a-; or γ/δ-depleted, were cultured at

3.5×10^5/250 μl in 96 well flat plates in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM Hepes buffer, 50 μM 2-β-mercaptoethanol, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (complete RPMI, cRPMI). Cells were stimulated with 5 μg ml^{-1} recombinant Ag85B protein or agonistic anti-CD3ε chain Ab (0.05 μg ml^{-1}) (BD Biosciences Pharmingen), for 4, 5 or 8 days, and were then pulsed with [H^3]thymidine (1 μCi well^{-1}) (Perkin Elmer Life and Analytical Sciences, Boston, MA) for an additional 18 h. Incorporation of [H^3]thymidine was measured by β-scintillation counting (Microβ counter, Perkin Elmer), and the incorporation data were expressed as mean cpm in the cultures after 5, 6 or 9 days of stimulation respectively.

Cytokine detection

Culture supernatants were assayed for IFN-γ, IL-2, IL-10, IL-4, TNF-α and IL12p70, by specific quantitative sandwich ELISA Kits (mouse Quantikine, R&D System, Minneapolis, MN), in accordance with the manufacture's instructions. Quantification was made against a standard curve obtained for individual cytokine standards provided by manufacturer.

ELISPOT for IFN-γ-producing cells

The number of IFN-γ-secreting cells in splenocyte preparations was determined by the BD^{TM}ELISPOT mouse IFN-γ ELISPOT Set (BD Biosciences Pharmingen), in accordance with the manufacture's instructions. Briefly, 96 well nitrocellulose plates were coated with 5 μg ml^{-1} of the purified anti-mouse IFN-γ. The cells (2×10^5 well^{-1}), resuspended in cRPMI, were incubated with Ag85B (5 μg ml^{-1}) or media alone. After 24 h at 37°C, cells were removed and the wells were incubated with biotinylated anti-mouse IFN-γ monoclonal Ab, followed by streptavidin-HRP conjugate. The presence of IFN-γ-producing cells was visualized by addition of the AEC substrate (Sigma) and reaction was stopped by washing with water. Spots were counted automatically using ELISPOT reader (A.EL.VIS GmbH, Hannover, Germany).

Flow cytometry

Cells washed with 1% BSA, 0.1% NaN$_3$ in PBS 1× were stained for 20 min with the following monoclonal Abs: anti-mouse CD8a(FITC), anti-mouse CD4(FITC), anti-mouse CD137(R-PE) anti-mouse TCR γ/δ (BD Biosciences Pharmingen). Cells were washed and analysed on a FACScan flow cytometer (Beckton Dickinson Immunocytometry System, San Jose, CA) with the CELL-Quest program after gating on the lymphocyte populations using forward and side scatter. The staining of samples with isotype controls (BD Biosciences Pharmingen) was used as a reference to determine positive and negative populations.

Culture and infection of BM-Mφ with bacteria

Bone marrow-derived macrophages were prepared as previously described (Cowley and Elkins, 2003). Briefly, bone marrow was flushed from femurs of healthy mice with RPMI-1640 and cells were plated at 1×10^6 in 48 well plates in cRPMI with the addition

of 10% L-929-conditioned medium. After 1 day of incubation, the medium was replaced with antibiotic-free cRPMI added with 10% L-929-conditioned medium. The cells were incubated for an additional 7 days, with medium replaced every 2 days. Following the 7 day culture period, the BM-Mϕ cultures (about 3.5×10^5 cells well^{-1}) were infected with MTB, according to the following protocol: bacteria were diluted from frozen stocks in cRPMI and added at a multiplicity of infection of 1:10 (bacterium-to-BM-Mϕ ratio). MTB was co-incubated with BM-Mϕ at 37°C in 5% CO_2 for 3 h and then washed vigorously five times in RPMI 1640 to eliminate extracellular bacteria. Following the last wash (less than 50 cfu were measured at this point in the supernatant culture) splenocytes (undepleted or CD4$^-$ fractions) were added to the culture at a 1:2 ratio (BM-Mϕ-to-splenocyte). For control, infected BM-Mϕ were cultured in cRPMI (500 µl) only. After 8 days of incubation, unless otherwise indicated, culture supernatant was diluted in distilled water and plated on Middlebrook 7H10 agar to evaluate the presence of MTB. The left supernatant was filtered through membranes (pore size 0.22 µm, Millipore, Malsheim, France) and then used for cytokine detection. In the same well, the intracellular MTB was revealed by lysing the BM-Mϕ and splenocytes with distilled water containing 0.1% saponin for 3 min and then plating the culture lysate on Middlebrook 7H10 agar. The MTB colonies were counted visually after 21 days of incubation. Data of total cfu well^{-1} were reported as the sum of both external and intracellular MTB.

Acknowledgements

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