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Increase of tuberculous infection in the organs of B cell-deficient mice

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SUMMARY

Protective immunity against infection with *Mycobacterium tuberculosis* is imparted by T cells rather than antibodies, but B cells can play a role as antigen-presenting cells and in granuloma formation. We re-evaluated the role of B cells in the course of tuberculous infection in μ-chain knock-out (Ig^-) mice. Surprisingly, the organs of *M. tuberculosis*-infected Ig^- mice were found to have three- to eight-fold elevated counts of viable bacilli compared with normal littermates at 3–6 weeks post-infection. Splenic interferon-gamma responses to whole antigen were unimpaired, whilst proliferation to certain mycobacterial peptides was found to be diminished. However, bacille Calmette–Guérin (BCG) vaccination significantly reduced the infection in Ig^- mice. The mechanisms by which B cells can influence primary tuberculous infection need further study.

Keywords tuberculosis B cells antibodies mouse infection

INTRODUCTION

It is generally accepted that T cell-mediated immunity provides protection to mice infected with *Mycobacterium tuberculosis*, whereas passively injected antibodies are not effective [1–3]. These results have led to the conclusion that antibodies and, by inference, B cells, play no obvious beneficial role in immunity to tuberculosis. Despite this prevailing opinion, the following experimental and clinical data indicated, at least indirectly, a possible regulatory function of B cells: (i) Igh genes influence the magnitude of *M. bovis* bacille Calmette–Guérin (BCG) granuloma formation [4,5]; (ii) passive transfer of antibodies promoted the multiplication of BCG in infected mice [6]; (iii) in tuberculosis patients, serum antibody levels against the 38-kD antigen of *M. tuberculosis* associated with severe forms of tuberculosis, whilst those against the 16-kD antigen associated with milder disease [7]. Furthermore, the beneficial role of antibodies to lipoarabinomannan (LAM) was proposed on the grounds that localized disease in children associated with higher antibody levels than those in disseminated disease [8]. One explanation for these data could be that B cells, through their capacity to function as antigen-presenting cells (APC) [9], also play a regulatory role for *M. tuberculosis*-specific T cells [10,11].

The generation of B cell-deficient knock-out mice (μMT) [12] has now opened the possibility of re-evaluating the role of antibodies and B cells in the course of tuberculous infection. These mice were generated by disruption of one of the IgM μ-chain transmembrane region exons and therefore possess no mature B cells and are unable to produce antibodies. Our results show that infection of these mice with virulent *M. tuberculosis* led to significant increases in bacillary loads and suggest that B cells can exert beneficial effects on the host responses to tuberculous infection.

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MATERIALS AND METHODS

Mice

B cell-deficient mice (μMT; [12]) were kindly donated by Dr K. Rajewsky (Institute of Genetics, Cologne, Germany) and Dr M. S. Neuberger (Laboratory of Molecular Biology, Cambridge, UK). They were bred in the local Biological Services Unit by pairing homozygous (−/−) males with heterozygous females (−/+). Animals were typed by sandwich ELISA measuring serum IgM (antisera from Southern Biotechnology, Birmingham, AL). Female μMT (Ig^-) and littermate control mice (Ig^+), 8–12 weeks old, were used in the experiments.

Bacteria and antigens

Mycobacterium tuberculosis H37Rv and *M. bovis* BCG (Pasteur) were grown as a suspension culture in Middlebrook's 7H9 culture medium and stored in liquid nitrogen until required. A soluble extract from *M. tuberculosis* H37Rv (MTSE) was prepared as described earlier [8]. The recombinant 38-kD protein [13] was obtained through the WHO protein bank (Braunschweig, Germany); concanavalin A (Con A) from Sigma (Poole, UK). Synthetic peptides were produced by standard automated solid-phase

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synthesis for Fmoc amino acid pentafluorophenyl esters as described earlier [14].

Infection and enumeration of colony-forming units
Live *M. tuberculosis* H37Rv (10^6/mouse in 0·1 ml) were injected intravenously. Spleens were removed after an additional period of 3–6 weeks, homogenized, serial dilutions prepared in PBS and plated onto 7H11 agar plates. Colony-forming units (CFU) were counted after 2–3 weeks. In some experiments, aliquots of spleen cells were cultured in the presence of antigens to study cytokine production *in vitro* (see below).

Lymphokine production in vitro *and cytokine ELISA*
To determine *in vitro* interferon-gamma (IFN-γ) and IL-4 production, 5×10^6 spleen cells were cultured in complete culture medium (RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamicin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol) in 24-well plates in the presence of MTSE (25 μg/ml), recombinant *M. tuberculosis* 38-kD protein (25 μg/ml), synthetic peptides (50 μg/ml) or Con A (6 μg/ml). Supernatants were harvested after 72 h of culture and tested by ELISA for IFN-γ and IL-4 content. IFN-γ was measured by sandwich ELISA using the rat anti-murine IFN-γ MoAbs R46A2 to capture and biotinylated XMG1.2 for detection (both from Pharmingen, San Diego, CA). IL-4 was measured by sandwich ELISA with the rat anti-murine IL-4 MoAbs 11B11 to capture and biotinylated BVD6-24G2 to detect (both from Pharmingen). The assays were then developed with avidin-peroxidase (Sigma) and the results expressed as pg/ml by reference to a standard curve derived from recombinant IFN-γ or IL-4 (Pharmingen). IL-2 was determined with the cytokine-dependent cell line HT-2 in a standard assay in supernatants harvested after 24 h of culture [9].

RESULTS

Infection of B cell-deficient mice with M. tuberculosis *H37Rv*
Groups of μMT mice (Ig$^-$) and littermate controls (Ig$^+$) were infected intravenously with *M. tuberculosis* H37Rv. Three to 6 weeks post-infection, infected organs were removed and bacterial CFU established. In three separate experiments, splenic bacterial counts at 3, 4 and 6 weeks post-infection were found to be about three- to eight-fold higher in B cell-deficient μMT mice compared with littermate mice with normal B cell competence ($P < 0{\cdot}01$–$0{\cdot}001$) (Fig. 1, experiments I–III). CFU in livers and lungs of μMT mice killed 6 weeks post-infection were also found to be significantly higher in relation to normal mice. However, the pronounced increase in bacterial organ loads in μMT mice did not lead to higher mortality over an 18-week post-infection period (data not shown). Similarly, infection with the vaccine strain *M. bovis* BCG (Pasteur) resulted also in significantly higher CFU in μMT mice compared with littermate controls (Fig. 1, experiment IV). Nevertheless, vaccination with live BCG protected the μMT mice against subsequent challenge with virulent *M. tuberculosis* H37Rv (Fig. 2), indicating that T cell memory responses were not impaired.

T cell responses in infected B cell-deficient mice
To assess whether B cell-deficient mice had an associated impairment of T cell-mediated immunity, we determined *in vitro* IFN-γ and IL-4 production by antigen-stimulated lymphocytes from infected animals. Spleen cells harvested 4 weeks post-infection

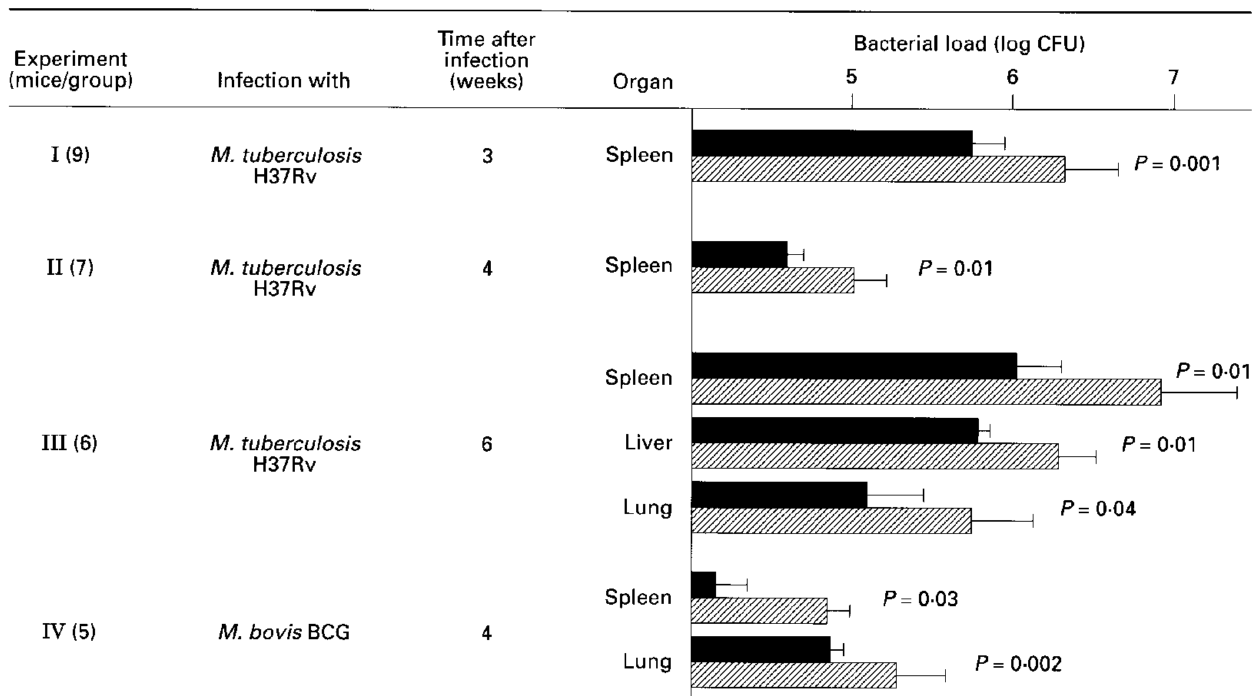

Fig. 1. Viable bacterial counts in the organs of infected mice. Groups of μMT Ig$^-$ (▨) and Ig$^+$ (■) littermate mice were infected intravenously with 10^6 *Mycobacterium tuberculosis* H37Rv (experiments I–III) or *M. bovis* bacille Calmette–Guérin (BCG) (experiment IV). Mean bacterial colony counts (CFU) values were compared by Student's *t*-test.

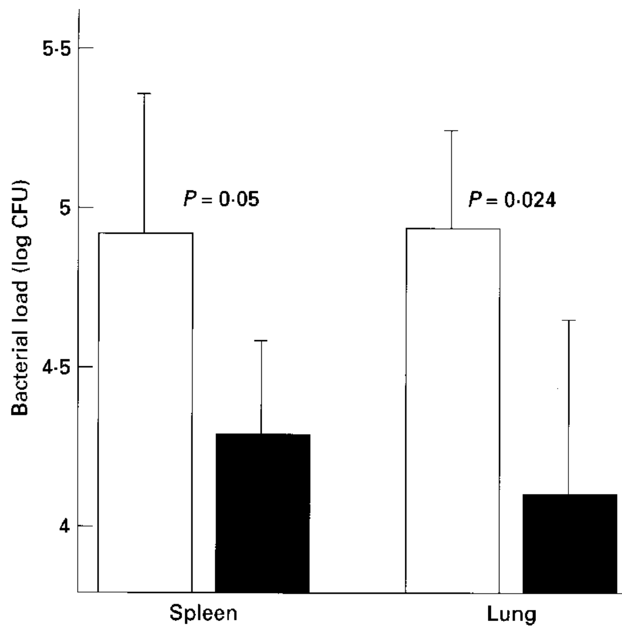

Fig. 2. Effectiveness of *Mycobacterium bovis* bacille Calmette–Guérin (BCG) vaccination in Ig$^-$ mice. One group ($n = 5$) of Ig$^-$ mice was vaccinated by s.c. inoculation of 5×10^4 *M. bovis* BCG, 4 weeks before *M. tuberculosis* H37Rv challenge and CFU assay as described in Fig. 1. □, Not vaccinated; ■, vaccinated.

were cultured in the presence of a soluble extract of *M. tuberculosis* (MTSE) or with the purified recombinant 38-kD antigen of *M. tuberculosis* [13,15,16] and culture supernatants were tested for IFN-γ and IL-4 contents by ELISA. Con A- and antigen-induced IFN-γ levels did not differ significantly between μMT Ig$^-$ mice and Ig$^+$ littermate controls (Fig. 3), indicating that the higher bacterial loads in homozygous μMT mice were not due to impaired production of this important cytokine. In contrast, we noted that Con A-induced IL-4 levels were significantly reduced in homozygous μMT mice ($P = 0{\cdot}01$, Fig. 3), whereas antigen-stimulated IL-4 levels were below the sensitivity level of our assay (100–120 pg/ml) both in μMT mice and littermate controls (Fig. 3).

Having established that no overall impairment of *M. tuberculosis*-specific T cell responses existed in μMT mice, we analysed next if differences existed between normal and B cell-deficient mice in the fine specificity of their recognition of T cell epitopes from the prominent *M. tuberculosis* 38-kD protein using a set of 19 synthetic peptides derived from the sequence of this protein [15]. The results of peptide-induced IL-2 production showed that splenic T cells from *M. tuberculosis*-infected μMT mice were unable to respond to stimulation with three peptides (residues 250–269, 260–279 and 325–343), which were stimulatory in control animals. However, four different epitopic peptides (residues 44–63, 65–83, 123–143, 240–259) were recognized in both μMT and littermate control mice (Fig. 4a). This pattern of epitope recognition was similar in mice which were vaccinated with BCG and then challenged with *M. tuberculosis* (Fig. 4b).

DISCUSSION

Our principle finding of enhanced bacterial organ counts in μMT mice suggested that B cells play a definite role in the containment of murine tuberculous infection. However, despite an up to eight times higher bacterial load, the absence of humoral immunity did not constitute a lethal defect, such as found in MHC class I or IFN-γ knock-out mice [17–19].

We considered several mechanisms by which B cells could influence the course of *M. tuberculosis* infection. First, B cells, due to their function as APC [9], could influence the specificity and phenotype of a protective CD4$^+$ T cell response. B cells take up antigen via their antigen-specific immunoglobulin receptors and present processed epitopes to T cells [10]. This antigen-specific and MHC class II-restricted interaction may determine which of the possible T cell determinants will be ultimately presented and thus modulate the specificity of the anti-mycobacterial T cell responses [20–22]. Indeed, we found that at least some mycobacterial epitopes were not recognized in either *M. tuberculosis*-infected or BCG-vaccinated and *M. tuberculosis*-challenged μMT mice (Fig. 4).

Second, B cells can bias the phenotype of T cell responses toward a Th2 profile [11,23,24], resulting in the secretion of IL-4, which could inhibit the protective IFN-γ response and

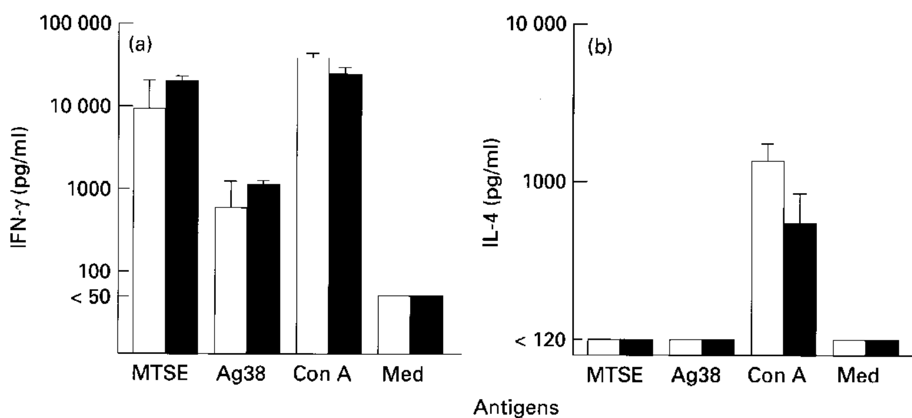

Fig. 3. Lymphokine production *in vitro*. Spleen cells from Ig$^-$ μMT mice (■) and Ig$^+$ littermate controls (□) were harvested 4 weeks after *Mycobacterium tuberculosis* H37Rv infection and cultured for 3 days (see Materials and Methods). Cytokine levels in culture supernatants were tested by ELISA. Mean values ± s.d. from individual mice (five mice/group). (a) IFN-γ. (b) IL-4. (CFU counts: see experiment II in Fig. 1.)

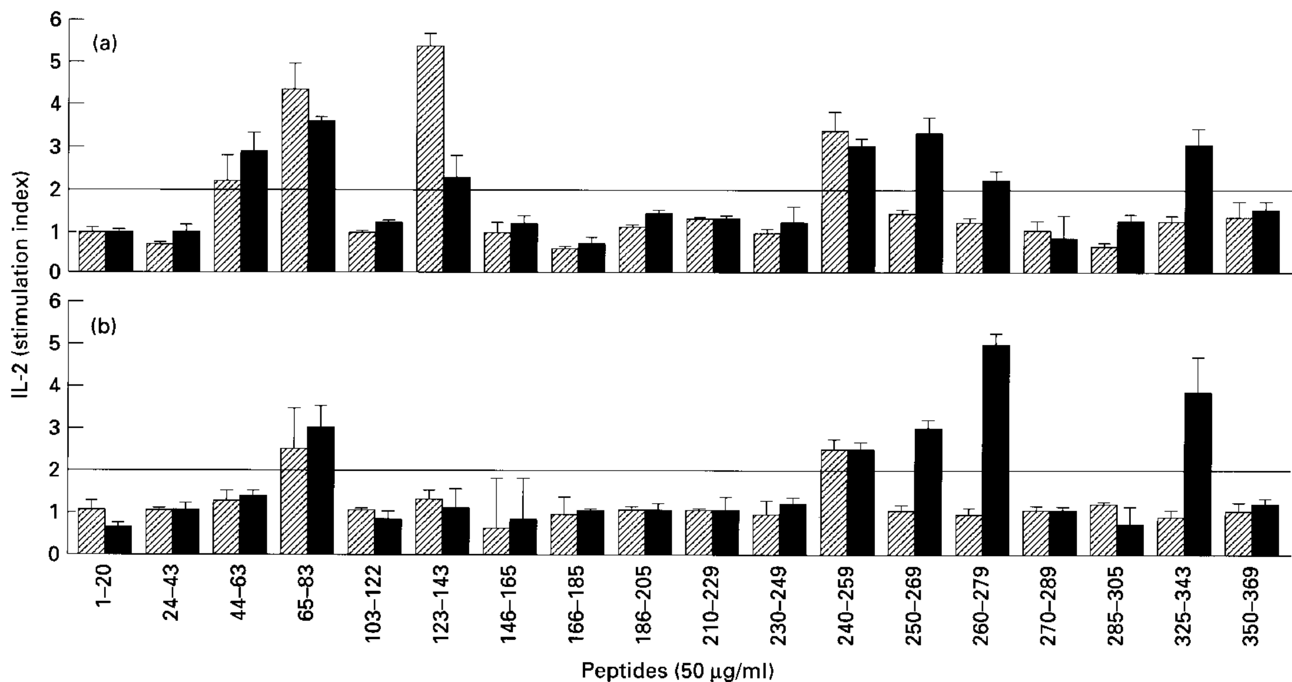

Fig. 4. T cell recognition of selected peptide epitopes of the 38-kD antigen. (a) Peptide-induced IL-2 production *in vitro* by splenocytes from *Mycobacterium tuberculosis*-infected mice. (b) Peptide-induced IL-2 production *in vitro* by splenocytes from *M. bovis* bacille Calmette–Guérin (BCG) vaccinated mice, which were subseqeuently challenged with *M. tuberculosis* H37Rv (see Fig. 2). Splenocytes from four mice/ group were pooled and incubated with synthetic peptides at 50 μg/ml. Mean stimulation indices ± s.d. of triplicate cultures (stimulation indices = IL-2 production in cultures with peptide/IL-2 production without peptide). ■, Ig^+; ▨, Ig^-.

perhaps enhance the pathogenic DTH reactions (reviewed in [25]). However, we found the IFN-γ response to mycobacterial antigens unimpaired. Although antigen-stimulated IL-4 levels were below detection, it is possible that higher concentrations of this cytokine are produced at sites of active disease and involved in granuloma formation. This interpretation is supported by our finding of diminished IL-4 response to Con A stimulation by spleen cells from B cell-deficient animals.

Third, association between antibody levels of certain specificity and clinical features [7,8] is also indicative of a possible role of antibodies in the development of tuberculosis in man. Antibodies could neutralize soluble mycobacterial components like LAM, which has been shown to suppress T cell responses *in vitro* [26]. Alternatively, opsonizing antibodies could also facilitate bacterial uptake and influence dissemination of the infection. In this clinical context it is also pertinent to quote data on tuberculosis in patients with primary hypogammaglobulinaemia: a longitudinal 10-year study in the UK suggested that patients with hypogammaglobulinaemia are more likely to contract tuberculosis than normal people [27].

In conclusion, we demonstrated that B cell-deficient mice infected with virulent tubercle bacilli had significantly higher bacterial loads. Possible explanations for these findings have been based on the T cell regulatory function of antigen-presenting B cells, whilst we cannot rule out other B cell functions or a direct effect of antibodies. Irrespective of the underlying mechanisms, the basic observation may help to advance our understanding of the complex cellular interactions during tuberculous infection.

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