THE JOURNAL OF IMMUNOLOGY

B Cell-Deficient Mice Are Highly Resistant to *Leishmania donovani* Infection, but Develop Neutrophil-Mediated Tissue Pathology

Sara C. Smelt, Sara E. J. Cotterell, Christian R. Engwerda and Paul M. Kaye

J Immunol 2000; 164:3681-3688; ;
http://www.jimmunol.org/content/164/7/3681

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

B Cell-Deficient Mice Are Highly Resistant to *Leishmania donovani* Infection, but Develop Neutrophil-Mediated Tissue Pathology[1]

Sara C. Smelt,[2] Sara E. J. Cotterell, Christian R. Engwerda, and Paul M. Kaye[3]

Resolution of *Leishmania* infection is T cell-dependent, and B lymphocytes have been considered to play a minimal role in host defense. In this study, the contribution of B lymphocytes to the response against *Leishmania donovani* was investigated using genetically modified IgM transmembrane domain (μMT) mutant mice, which lack mature B lymphocytes. When compared with wild-type mice, μMT mice cleared parasites more rapidly from the liver, and infection failed to establish in the spleen. The rapid clearance of parasites in μMT mice was associated with accelerated and more extensive hepatic granuloma formation compared with wild-type mice. However, the liver of infected μMT mice also showed signs of destructive pathology, associated with the presence of increased numbers of neutrophils. The role of neutrophils in controlling parasite growth in the viscera was determined by depletion with the mAb RB6-8C5. This treatment led to a dramatic enhancement of parasite growth in both the liver and spleen of μMT and wild-type mice. As assessed by transfer of both normal and chronic-infection serum, Ig protects μMT mice from destructive hepatic pathology, but minimally alters their resistance compared with wild-type mice. However, adoptive transfer of $CD4^+$ and $CD8^+$ T cells into recombinase activating gene 1 ($RAG1^{-/-}$) recipients, suggested that T cell function was not altered by maturation in a B cell-deficient environment. Taken together, these data suggest an inhibitory role for B lymphocytes in resistance to *L. donovani* unrelated to the presence or absence of Ig. However, Ig protects μMT mice from the exaggerated pathology that occurs during infection. ***The Journal of Immunology,*** **2000, 164: 3681–3688.**

Visceral leishmaniasis (VL)[4] results from infection with the intracellular protozoan parasites *Leishmania donovani* and *Leishmania infantum* (*chagasi*). These species quickly metastasize to the visceral organs, and establish a systemic infection. In subclinical infection, parasites are contained by the development of a relatively benign, and predominantly mononuclear, granulomatous tissue response in the liver. This hepatic response is also seen in most common inbred strains of mice (1–3). In contrast, clinical VL is associated with gross splenic and hepatic pathology, fever, cachexia, and immunosuppression. Polyclonal B cell activation and hypergammaglobulinaemia invariably produce large amounts of parasite-specific and nonspecific Abs and autoantibodies, particularly of the IgM and IgG isotypes (4). We and others (5, 6) have shown that during VL in mice, the pathological changes seen in the spleen in many respects closely resemble those seen in clinical VL. In particular, there is significant loss of follicular dendritic cells, and subsequent loss of germinal centers (5), both features which may contribute to aberrant B cell function in late stages of disease. To date, however, there has been no formal evaluation of the role of B cells in the progression of VL.

The role of B cells in leishmaniasis has been addressed in models of cutaneous leishmaniasis. Continual administration of anti-IgM Abs, which causes B cell depletion, enhanced resistance to *Leishmania tropica* and *Leishmania mexicana* in BALB/c mice (7). *BALB.xid* mice, which lack B-1 B cells and have a marked reduction in B-2 B cell number, also exhibit enhanced resistance to *Leishmania major* infection (8). More recently, it has been shown that cotransfer of B cells converts T cell-reconstituted *L. major*-resistant, C.B-17 *scid* mice into a susceptible phenotype (9). Similarly, administration of IL-7, a B cell hematopoietic factor, was shown to markedly increase B cell number and exacerbate *L. major* infection (10). In contrast to these data, in a study using gene-targeted mice, no evidence was found for a contribution of B lymphocytes to the development of polarized Th responses to *L. major* in either genetically resistant or susceptible mice (11).

In the present study, B cell-deficient IgM transmembrane domain (μMT) mutant mice were used to investigate the role of B lymphocytes in murine VL caused by infection with *L. donovani*. Our data indicate that 1) in the genetic absence of B cells, mice show enhanced resistance to *L. donovani*, but with associated destructive hepatic pathology; 2) neutrophils play a key role in host resistance to *L. donovani* in both B cell-deficient and wild-type mice; and 3) in B cell-deficient mice, serum transfer protects against exaggerated pathology without altering the heightened level of resistance seen in these mice.

Materials and Methods

Animals and parasites

Female μMT and C57BL/6 mice were used at 6 to 8 wk of age. C57BL/6 (*Nramp1*^{s} (12, 13)) mice were purchased from Bantin & Kingman (Hull, U.K.). C57BL/6 recombinase activating gene 1 ($RAG1^{-/-}$) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME), and

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London United Kingdom

Received for publication July 19, 1999. Accepted for publication January 31, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the British Medical Research Council and the Wellcome Trust. S.C.S. was in receipt of an Medical Research Council Postgraduate Studentship. S.E.J.C. is a recipient of a Wellcome Trust Prize Studentship.

[2] Current address: IMM6, Department of Neuropharmacology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

[3] Address correspondence and reprint requests to Dr. P. M. Kaye, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. E-mail address: paul.kaye@lshtm.ac.uk.

[4] Abbreviations used in this paper: VL, visceral leishmaniasis; KC, Kupffer cell; LDU, Leishman Donovan units; μMT, IgM transmembrane domain; $RAG1^{-/-}$, recombinase activating gene 1; p.i., postinfection.

0022-1767/00/$02.00

4μMT mice (14) were originally obtained from Bantin & Kingman. These strains were bred at the London School of Hygiene and Tropical Medicine under barrier conditions. μMT mice were backcrossed six generations on the C57BL/6 background. The Ethiopian LV9 strain of *L. donovani* was used in these experiments and was maintained by passage in Syrian hamsters and purified as described elsewhere (5). Mice were infected i.v. via the lateral tail vein with 2×10^7 amastigotes in 200 μl of RPMI 1640. The course of visceral infection was determined by examining methanol-fixed, Giemsa-stained imprints of the cut spleen and liver, and quantitating organ parasite burdens as Leishman-Donovan units (LDU) using the formula: LDU = [(number of parasites/1000 host nuclei) $\times$ organ weight in milligrams (mg)]. Induction of hepato-splenomegaly was assessed using liver and spleen indices, calculated using the formula: Organ index = (organ weight in mg/total body weight in mg) $\times$ 100.

Adoptive transfer of T cells

Mice were killed by cervical dislocation and spleens mechanically disrupted by passing through a nylon sieve. Erythrocytes were lysed by treatment with Tris-buffered ammonium chloride (0.747% (w/v) NH_4Cl and 0.017 M Tris, pH 7.5). Cells were then washed with MACS buffer (PBS, pH 7.2, with 0.01% (w/v) NaN_3, 1% (w/v) BSA, and 5 mM EDTA), and incubated with a mixture of anti-CD4 and anti-CD8 magnetic beads (5 μl each/10^7 cells; Miltenyi Biotec , Surrey, U.K.) for 15 min at 12°C. After washing and resuspension at 10^8 cells/ml, cells were positively selected on a MACS column (Miltenyi Biotec). Purity of cells was assessed by FACS using anti-B220-FITC (clone RA3-6B2; PharMingen, San Diego, CA), anti-CD4-PE (clone H129.19) and anti-CD8-FITC (clone 53-6.7) (Sigma, Poole, U.K.). T lymphocyte populations selected for both CD4 and CD8 expression contained <1% $CD4^-$ and $CD8^-$ cells. Unfractionated spleen cells were also analyzed by flow cytometry, as above. A total of 1×10^5 or 1×10^4 of the mixed population of $CD4^+$ and $CD8^+$ T cells, derived from either naive μMT or C57BL/6 mice, were transferred to C57BL/6 $RAG1^{-/-}$ mice in 200 μl RPMI 1640. All mice were infected with *L. donovani* 1 day after transfer.

Adoptive transfer of serum

Serum was obtained by cardiac puncture from naive C57BL/6 and from C57BL/6 mice infected for 56 days with *L. donovani*. Before transfer, serum was centrifuged at 100,000 $\times$ *g* for 30 min at 4°C in a Beckman (Fullerton, CA) TL-100 ultracentrifuge. Two hundred microliters of normal serum or chronic-infection serum was given i.p. to μMT mice at 4 h and again at 8 days postinfection (p.i.). Serum transfer was delayed until 4 h p.i. to allow for clearance of amastigotes from the circulation (15) and to obviate any immediate opsonic effects of antileishmanial Ab (16).

Neutrophil depletion

Monoclonal rat anti-mouse granulocyte (RB6-8C5; Ref. 17), a generous gift from Dr. R. Coffman (DNAX, Palo Alto, CA) and Dr. S. Reiner (University of Chicago, Chicago, IL), was purified from cell culture supernatant by affinity chromatography using a protein G column. RB6-8C5 mAb (0.2 mg) or control rat IgG (Sigma) was administered i.p. at day -1 of infection and every third day thereafter. The efficacy of depletion was monitored by flow cytometric analysis of spleen cells. Control naive μMT, C57BL/6, and BALB/c mice were also treated with RB6-8C5 and observed daily over the time course of infections. Neutrophil depletion did not lead to any deterioration in health status in naive mice. However, we did note a slight reduction in the frequency of peripheral (splenic) $CD8^+$ T cells in both naive and infected RB6-8C5-treated mice (data not shown).

Immunohistology

Neutrophils were identified by staining with RB6-8C5 using the Vectastain Elite immunoperoxidase system (Vector Laboratories, Peterborough, U.K.). Briefly, livers were collected from mice killed by cervical dislocation, immediately embedded in OCT compound (Raymond Lamb, London, U.K.), and snap frozen in isopentane/liquid nitrogen. Sections (6 μm) were cut with a cryostat and were fixed for 2 min in acetone, air dried, and stored at -20°C until stained. Frozen sections were fixed for 8 min in acetone and washed for 20 min in PBS (10 mM NaH_2PO_4, pH 7.5, and 0.9% (w/v) saline) immediately before staining. Sections were additionally blocked with avidin for 15 min, and then with biotin for 15 min followed by 1.5% (v/v) rabbit serum diluted in PBS for 30 min. Excess serum was blotted from sections that were then incubated with primary Ab, diluted in PBS, for 30 min. RB6-8C5 and control rat IgG were used at 2.5 μg/ml. Abs were detected by incubation with biotinylated rabbit-anti-rat IgG (mouse adsorbed; 1:100 (v/v) dilution), and this was subsequently detected with avidin biotinylated-HRP complexes. Sections were developed for peroxidase activity in 3,3-diaminobenzidine tetrahydrochloride (DAB) developing substrate. Sections were counterstained for 1 min with Harris hematoxylin, dehydrated and mounted in DePeX (BDH, Poole, U.K.).

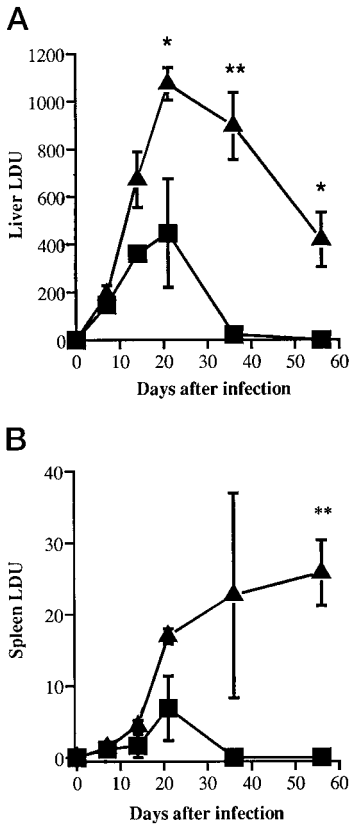

FIGURE 1. μMT mice are resistant to *L. donovani* infection. μMT (■) and C57BL/6 (▲) mice were infected with 2×10^7 *L. donovani* amastigotes, and parasites loads were determined in the liver (*A*) and spleen (*B*) at the times indicated. Data represent mean LDU $\pm$ SE (n = 3 mice) and are representative of three independent experiments with similar results. Significance levels comparing μMT and C57BL/6 mice are indicated; *, $p < 0.05$; **, $p < 0.005$.

Granuloma counting

Liver sections from infected mice were stained with hematoxylin and eosin and the degree of the granulomatous responses assessed in two ways: 1) granuloma density was determined from ~150 fields of view per mouse liver (×63 magnification; n = 2–3 mice/group) and 2) the degree of maturation of granulomas was scored around infected Kupffer cells (KC), as described elsewhere (18).

Statistical analysis

Data were analyzed using either the nonpaired Students *t* test, Peasons linear correlation, or χ^2 as appropriate, using the graphics package Fig P (Biosoft, Cambridge, U.K.).

Results

B cell deficient mice are highly resistant to L. donovani *infection*

Comparisons of the course of *L. donovani* infection in age- and sex-matched μMT and C57BL/6 mice revealed that μMT mice were highly resistant to infection (Fig. 1). Wild-type C57BL/6 mice displayed organ-specific control of *L. donovani* infection, as previously reported in BALB/c mice (5, 19). Hence, liver parasite loads began to resolve 21–28 days after infection, whereas parasites persisted indefinitely in the spleen, albeit at lower levels than in the liver. Not only were peak parasite loads in both organs

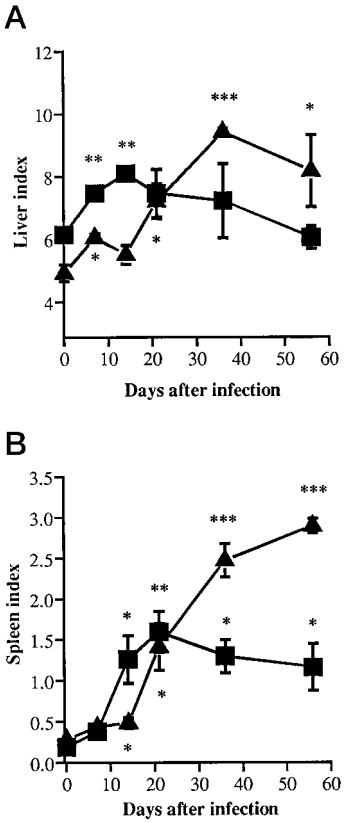

FIGURE 2. Hepato-splenomegaly in μMT and C57BL/6 mice infected with *L. donovani*. Hepatomegaly (*A*) and splenomegaly (*B*) were calculated as described in *Materials and Methods* at the indicated times after infection of μMT (■) and C57BL/6 (▲) mice. Data represent mean organ index ± SE ($n = 3$) and are representative of three independent experiments with similar results. Significance levels, comparing hepatomegaly or splenomegaly at each time point to naive controls, are indicated; ∗, $p < 0.05$; ∗∗, $p < 0.005$; ∗∗∗, $p < 0.001$.

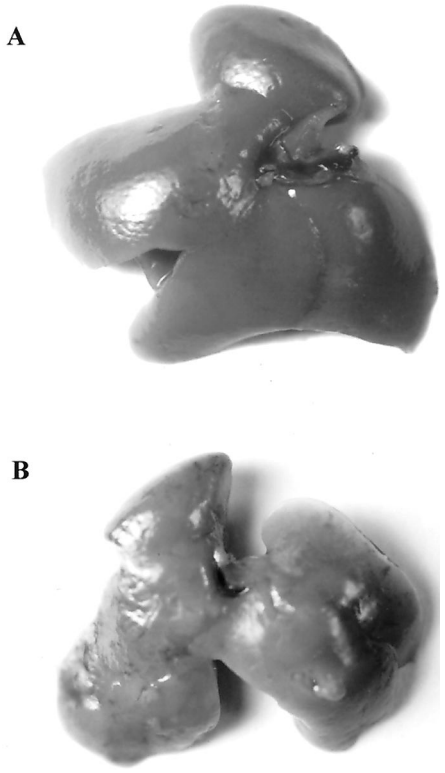

FIGURE 3. Liver necrosis accompanies *L. donovani* infection in μMT mice. Photographs show a representative liver from C57BL/6 (*A*) and μMT (*B*) mice removed at day 14 p.i. with *L. donovani*. Similar overt gross pathology in μMT mice was evident when examined from day 14 to day 21 p.i.

significantly lower in μMT mice, (Fig. 1; $p < 0.05$ at day 28 for liver; $p < 0.005$ at day 56 for spleen) but the long-term outcome of infection was dramatically altered. Hepatic infection in the μMT mouse was reduced to below the limits of detection by impression smears within 8 wk of infection. Furthermore, μMT mice did not develop persistent infection in the spleen (Fig. 1). Hence, B cell-deficient μMT mice are highly resistant to primary infection with *L. donovani*.

Hepato-splenomegaly is a notable feature of *L. donovani* infection. In both C57BL/6 and μMT mice, hepatomegaly was transient but displayed important differences in kinetics (Fig. 2). In C57BL/6 mice, hepatomegaly was greatest following peak parasite load (1.91 ± 0.25-fold increase compared with naive at day 36 p.i.; $p < 0.001$) and at the time when T cell-dependent immune mechanisms started to reduce parasite numbers. In contrast, peak hepatomegaly in μMT mice preceded peak parasite burden (1.31 ± 0.34-fold increase compared with naive at day 14 p.i.; $p < 0.002$), returning to control levels as parasites were cleared from the tissue. However, overt liver pathology was also noted in μMT mice at this time (see Fig. 3 and below). Splenomegaly was a dominant feature of late infection in C57BL/6 mice (10.22 ± 0.31-fold increase compared with naive at day 56 p.i.; $p < 0.0001$; Fig. 2), reflecting the lack of clearance of parasites from this organ. In contrast, although μMT mice demonstrated more rapid onset of splenomegaly (6.61 ± 1.52-fold vs a 1.71 ± 0.23-fold increase in C57BL/6 mice at day 14 p.i.; $p < 0.05$), this was self-limiting in μMT mice, again commensurate with the reduction in parasite load in this organ.

μMT mice exhibit enhanced granuloma formation and maturation in response to L. donovani *infection*

L. donovani infection causes a structured hepatic tissue response in most mouse strains, in which infiltrating monocytes, lymphocytes, and neutrophils aggregate around infected KC to form granulomas (1, 2). The kinetics of this response in wild-type and μMT mice was very different however, as shown in Fig. 4*A*. At day 14 p.i., the livers of μMT mice contained more than three times the number of granulomas, compared with wild-type mice ($p < 0.001$). Furthermore, unlike in wild-type mice where granulomas persisted throughout the period of parasite clearance, the granulomas of μMT mice were transient and these structures rapidly disappeared from the tissue as parasites were eliminated.

Maturation of the tissue response was also assessed by enumerating the degree of inflammation around individual infected KC. At day 14 p.i., inflammatory foci (immature granulomas) had developed around ~60% of infected KC (Fig. 4*B*). In contrast, 90% of infected KC in μMT mice had elicited a local inflammatory response by this time point. Two additional features of the response in μMT mice were also evident from this analysis. First, μMT mice were found to contain many "empty" granulomas at day 14 p.i. (excluded from the analysis shown in Fig. 4*B*). These most likely represented granulomas in which parasites had already been eliminated, and were only rarely observed in C57BL/6 mice at this time. Second, although parasites within some granulomas were being cleared by day 14 p.i., few granulomas had reached a mature stage of development (defined by an organized cuffing of

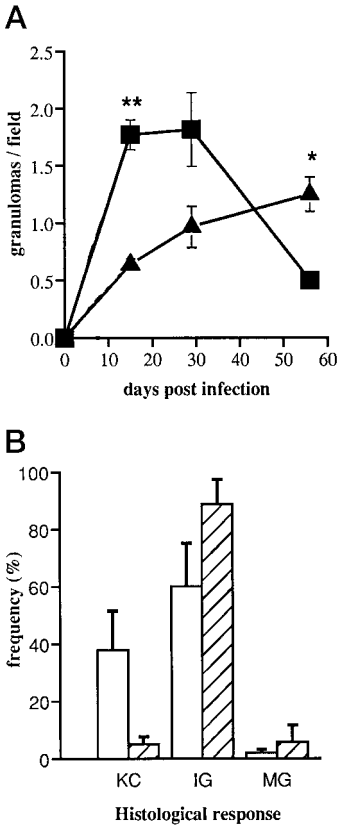

FIGURE 4. Enhanced granuloma development following *L. donovani* infection in μMT mice. *A*, Granuloma density in the liver of μMT (■) and C57BL/6 (▲) mice was determined at the times indicated by examination of hematoxylin-stained cryosections. Data represent mean number of granulomas per ×63 field ± SE (n = 3 mice) an are representative of three independent experiments with similar results. Significance levels comparing μMT and C57BL/6 mice are indicated; *, $p < 0.005$; **, $p < 0.001$. *B*, The frequency of KC showing either no sign of local inflammation (KC), inflammation without organization (immature granuloma; IG), or being the focus of a mature granuloma (MG) were scored in cryosections of day 14 infected livers of C57BL/6 (□) and μMT (▨) mice. Data were obtained by examining ~150 KC per section (n = 3 mice) and are representative of two independent experiments. The difference in distribution of tissue responses between strains was significant by χ^2 analysis, $p < 0.001$.

infiltrating cells). Hence, in the absence of B cells, there are marked changes in the intensity and kinetics of the hepatic granulomatous response to *L. donovani.*

Increased presence of neutrophils in the liver and spleen of μMT mice following infection with L. donovani

Between day 14 and day 21 p.i., the livers of μMT mice exhibited signs of gross destructive pathology (Fig. 3). They were pale in color, suggesting occlusion of blood vessels, and large areas of necrosis were frequent in hematoxylin and eosin-stained sections (data not shown). Neutrophils, stained with mAb RB6-8C5, were commonly associated with these areas of tissue damage, suggesting the latter was the result of an excessive inflammatory response. In contrast, liver necrosis was not observed in infected C57BL/6 mice at any time p.i. Although mononuclear cells were still abundant in the granulomas of μMT mice, neutrophils were also readily detected, suggesting the presence of continued active inflammation within these granulomas (Fig. 5). In contrast, neutrophils were scarce in the granulomas of C57BL/6 mice infected with *L. donovani* (Fig. 5 and Refs. 2 and 20).

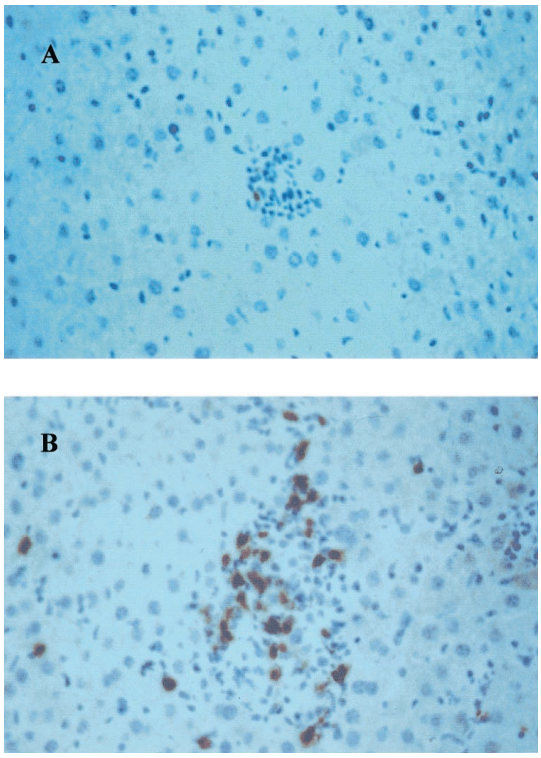

FIGURE 5. Continued active inflammation in the granulomas of μMT mice. Granulomas from day 14 p.i. C57BL/6 (*A*) and μMT (*B*) mice were stained using immunoperoxidase (brown) with the neutrophil-specific mAb RB6-8C5. Sections were counterstained using hematoxylin. Magnification, ×40.

An organized tissue response is not observed in the spleen of *L. donovani*-infected mice (5, 6). Therefore, we assessed the levels of neutrophils in this organ by flow cytometry. In C57BL/6 mice, neutrophils (mid-level FSC/high SSC, F7/4$^+$,and RB6-8C5$^+$) represented <5% of the total spleen cell population at day 14 p.i. In infected μMT mice, neutrophils were clearly abundant and represented 30% of the total population (data not shown). Rapid parasite clearance in μMT mice is therefore characterized by the presence of elevated neutrophil numbers in both organs, and in the liver these neutrophils also appeared to be associated with areas of tissue destruction.

Neutrophil depletion leads to enhanced parasite growth in B cell deficient and wild-type mice

The increased presence of neutrophils in the liver granulomas of μMT mice and their presence near regions of hepatic necrosis suggested that these cells may contribute both to the pathological level of inflammation, as well as the accelerated clearance of parasites observed in μMT mice. To address these questions, μMT and C57BL/6 mice were treated with the neutrophil-depleting mAb RB6-8C5 (21, 22). Neutrophil depletion over the first 2 wk of infection had a surprising and dramatic effect on parasite development in both strains of mice (Table I). Parasite burden was increased 6-fold in the spleen and liver of C57BL/6 mice, resulting in parasite burdens in the latter organ that are not approached even at the peak of disease progression in untreated mice (see Fig. 1). RB6-8C5 treatment also increased parasite loads in μMT mice, although to a greater relative extent than seen in C57BL/6 mice. This may reflect a more critical role for neutrophils in μMT mice or their greater relative abundance.

Table I. *Effect of neutrophil depletion on parasite burden in μMT and C57BL/6 mice*

Group	Day 14 Parasite Load (mean LDU ± SEM)[a]	
	Spleen	Liver
C57BL/6 + IgG	6 ± 3	815 ± 78
C57BL/6 + RB6	36 ± 10*	4960 ± 839*
μMT + IgG	1 ± 1	621 ± 27
μMT + RB6	11 ± 4*	4408 ± 772*

[a] Mice ($n = 4$) were injected with control IgG or RB6-8C5 as described in *Materials and Methods*, and parasites were determined in impression smears 14 days after infection with 2×10^7 amastigotes. Data represent parasites load, calculated as LDU, for one of two experiments which yielded similar results.

*, $p < 0.01$ vs. control Ig.

To determine whether the influence of neutrophils on the course of *L. donovani* infection was limited to mice on the C57 background, we also performed depletion studies in BALB/c mice, in which the immune response to *L. donovani* has been most extensively characterized. BALB/c mice injected with mAb RB6-8C5, exhibited a dramatic increase in day 14 parasite load in both the spleen (9 ± 2 vs 149 ± 4 in control and treated mice, respectively) and in the liver (1191 ± 126 vs 3644 ± 558). Hence, neutrophils appear to play a critical role in the early control of *L. donovani* infection in mice. Furthermore, this neutrophil response is exaggerated in the absence of B cells, and may contribute to gross destructive pathology.

Serum transfer abrogates gross pathology, but minimally affects the resistance of μMT mice to L. donovani *infection*

Other researchers (23, 24) have previously implicated serum Ig and/or immune complexes in the regulation of anti-inflammatory cytokine responses. Therefore, we evaluated whether the lack of serum Ig, a consequence of the B cell deficiency in μMT mice, had any immunoregulatory consequences in our infection model.

To address this question, μMT mice were infected with *L. donovani* and at 4 h p.i. (a time when >95% of parasites were cleared from the circulation (15)); we then adoptively transferred 200 μl of serum derived from naive C57BL/6 mice or mice infected for 56 days with *L. donovani*. Serum transfer was repeated at day 8 p.i., and mice were sacrificed at day 16 p.i. The results of this experiment were both striking and unexpected. Transfer of either normal or chronic-infection serum had minimal effect on the resistance of μMT mice to infection with *L. donovani* (Fig. 6). Furthermore, immunohistological analysis using RB6-8C5 demonstrated that the levels of active inflammation (i.e., neutrophil infiltration) within the granulomas of control and serum-treated μMT mice was similar (data not shown). However, serum transfer almost totally inhibited the gross destructive pathology observed in the livers of μMT mice. Whereas 5/5 control μMT mice showed clear outward signs of liver necrosis (as illustrated in Fig. 3*B*), this was seen in only 1/5 μMT mice receiving normal mouse serum and in 0/5 mice receiving chronic-infection serum. All other serum recipients had livers with normal gross appearance. Hematoxylin and eosin staining of tissue section also revealed few or no signs of necrosis in serum recipients (data not shown). Hence, serum transfer distinguishes the mechanisms involved in the development of tissue damage and enhanced host resistance in μMT mice.

T cells from μMT and C57BL/6 mice have equivalent anti-leishmanial activity

Although serum reconstitution protects μMT mice from tissue damage, it had minimal effect on the enhanced levels of resistance

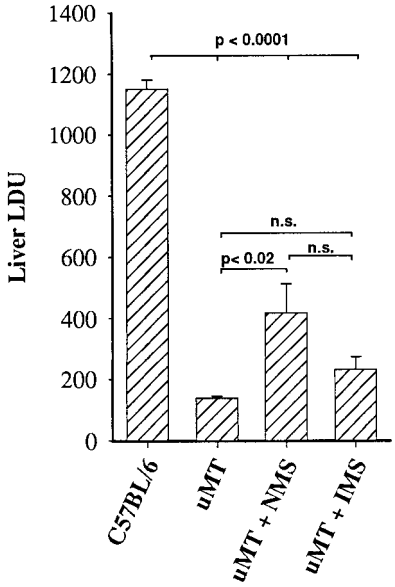

FIGURE 6. Serum transfer has minimal effect on anti-leishmanial activity in μMT mice. Parasite burden was determined at day 16 p.i. in normal C57BL/6 mice, μMT mice, or μMT mice receiving either normal (NMS) or chronic-infection serum (IMS). Data were analyzed by students *t* test ($n = 5$ mice/group), and significance levels are shown on the figure.

of μMT compared with wild-type mice. We therefore sought other explanations for the heightened resistance of μMT mice to *L. donovani*. As lack of B cells has been reported to have effects on the function of T cells in other systems, we wished to determine whether T cells in μMT mice and C57BL/6 mice differed in their ability to protect against *L. donovani* infection. Previous studies have illustrated the importance of both $CD4^+$ and $CD8^+$ T cells for immunity to *L. donovani* in the liver (3). Hence, we copurified both populations directly by MACS using a combination of anti-CD4 and anti-CD8 mAbs. Purified T cells from C57BL/6 mice were 74% $CD4^+$ and 28% $CD8^+$, whereas those isolated from μMT mice were 76% $CD4^+$ and 23% $CD8^+$. These mixed populations of T cells (10^4 or 10^5) were adoptively transferred to C57BL/6 background $RAG1^{-/-}$ mice 1 day before infection with *L. donovani.* On day 28 p.i., mice were sacrificed and each mouse analyzed individually for parasite load and the degree of $CD4^+$ and $CD8^+$ T cell reconstitution (Fig. 7). The data clearly demonstrate that control of *L. donovani* in this transfer model was correlated with $CD4^+$ ($p < 0.002$ and $p < 0.05$ for μMT and C57BL/6 donors, respectively) rather than $CD8^+$ T cell number in reconstituted mice. More importantly, there was no difference in the ability of $CD4^+$ or $CD8^+$ T cells from C57BL/6 and μMT mice to transfer protection to $RAG1^{-/-}$ mice. Hence, the development of T cells in a B cell-deficient environment does not result in any quantitative alterations in their ability to transfer protection against *L. donovani* infection.

Discussion

The data presented in this study demonstrate that B cell-deficient μMT mice are highly resistant to *L. donovani* infection. This is associated with a granulomatous response with evidence of continued active inflammation. Depletion of neutrophils demonstrated an essential role for this cell population in the host response to *L. donovani*, both in μMT mice and, surprisingly, in wild-type C57BL/6 and BALB/c mice. In contrast to wild-type mice, μMT mice also exhibited gross hepatic destruction, a process that could

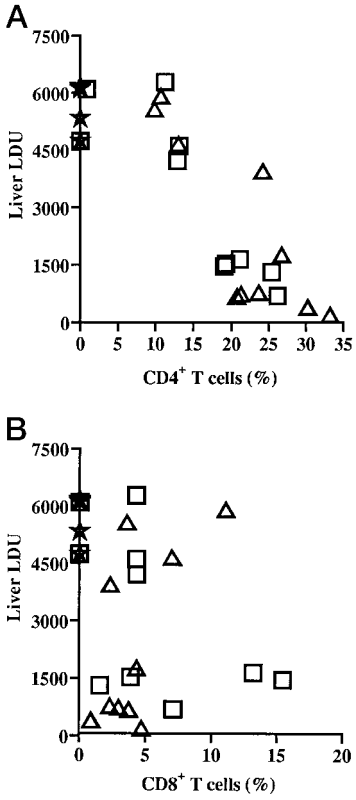

FIGURE 7. T cells from μMT and C57BL/6 mice are equally efficient at transferring resistance to *L. donovani*. $CD4^+$ and $CD8^+$ T cells derived from μMT (□) or C57BL/6 (△) mice were cotransferred to $RAG1^{-/-}$ mice 1 day before infection with *L. donovani*. Control $RAG1^{-/-}$ mice received no cells (⋆). At day 28 p.i., parasite load was determined from impression smears and the frequency of $CD4^+$ and $CD8^+$ T cells in the spleen determined by flow cytometry. Data represent hepatic parasite load (LDU) for individual mice, plotted against either $CD4^+$ (*A*) or $CD8^+$ (*B*) T cell frequency. A significant correlation was observed for $CD4^+$ cells derived from either μMT ($r = -0.86$, $p < 0.002$) or C57BL/6 ($r = -0.84$, $p < 0.005$) donors, whereas no significant correlations were seen for $CD8^+$ cells of either strain.

be inhibited by serum transfer. However, transfer of serum minimally affected resistance to *L. donovani*, allowing us to dissociate tissue destructive processes from antimicrobial effector function in μMT mice.

The rapid curing response of μMT mice following *L. donovani* infection is striking in a number of respects. First, the rapid clearance of parasites in the liver of μMT mice extends both the rate of cure and the degree of pathology beyond those previously defined using MHC congenic mice on the C57 background (25). This is associated with a rapid granulomatous response, whose cellular composition is neutrophil-rich relative to wild-type mice. This suggests the continuation of a process of active inflammation in these granulomas, though this does not culminate in necrosis at these sites. These granulomas effectively eliminate parasites, but without the maturation associated with parasite clearance in wild-type mice. Indeed, unlike the response in C57BL/6 mice and other strains (this manuscript and Refs. 2 and 12), the granulomas in μMT mice are transient, their numbers declining rapidly after parasite clearance. Second, μMT mice are able to effectively control parasites within the spleen, and fail to develop long-lasting splenomegaly and persistent parasite load characteristic of wild-type C57BL/6 mice and BALB/c mice (this manuscript and Ref. 5). The lack of persistence of parasites in the spleen of μMT mice illustrates that immunological effector mechanisms are able to achieve effective control of parasites in this organ, and that these are uncovered in the absence of B cells.

Neutrophil depletion led to dramatic increases in parasite growth compared with that seen in untreated controls, in both μMT and also in C57BL/6 and BALB/c mice. Although the relative increase in parasite burden was slightly greater in μMT mice compared with wild-type mice, this did not appear to fully reflect the observed differences in the numbers of neutrophils within the granulomas of these mice. A possible explanation for this discrepancy is that parasite density in the infected organ becomes a limiting factor. This contention is supported by the observation that in mice with very low levels of infection (liver LDU < 100), the effect of neutrophil depletion is significantly more marked in μMT mice than in C57BL/6 mice (S. C. Smelt, unpublished observations). Nevertheless, these data clearly indicate that in normal mice, as well as in B cell-deficient mice, neutrophils play a vital, but hitherto unrecognized role in the control of *L. donovani* infection.

The direct demonstration of a role for neutrophils in the control of *L. donovani* infection was unexpected, particularly in wild-type C57BL/6 and BALB/c mice. A small number of neutrophils can be detected early after infection infiltrating the liver parenchyma and subsequently within the granuloma (Fig. 5 and Refs. 2 and 19); nevertheless, the granuloma is usually predominantly mononuclear in composition. Clearly, even the limited numbers of neutrophils seen in the tissue are able to make a substantial contribution to host resistance. A balanced neutrophil response may, at the expense of maximal anti-leishmanial activity, represent a mechanism for protecting against excess pathology. In this regard, we have recently shown that anti-CTLA-4 treatment of mice also enhances granuloma formation and parasite clearance. However, unlike the situation seen in μMT mice, the response of anti-CTLA-4-treated mice remains predominantly mononuclear and progresses rapidly to full maturation (18). Furthermore, the enhanced clearance of parasites induced by anti-CTLA-4 does not result in any overt liver destruction. Comparison of these two experimental manipulations of the host response to *L. donovani* supports the notion that successful elimination of pathogens and the containment of pathology requires an appropriately balanced cellular response.

Recent data suggest at least one mechanism by which neutrophils may contribute to the control of *L. donovani* infection. Studies with gene targeted gp91 ($phox^{-/-}$) and $NOS2^{-/-}$ mice, have demonstrated that while late stages of disease resolution are controlled exclusively by reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI) play an important role in the early regulation of parasite multiplication and mononuclear cell recruitment (20). Although these ROI have been assumed to derive from monocytes or macrophages, our data would suggest that neutrophils may make an important, if not exclusive, contribution to ROI production. The production of ROI by neutrophils is regulated by TNF-α, and we have already shown that TNF-α producing cells are recruited into the liver within 3 days of infection with *L. donovani* (19). In contrast, infected KC fail to make TNF-α at this early stage, and have also been shown to be relatively poor producers of ROI (26). Thus, depletion of neutrophils may limit this early effector response and promote early parasite multiplication in KC. However, the increase in parasite loads we have observed in neutrophil-depleted mice are significantly greater than that seen in $phox^{-/-}$ mice, suggesting other roles for neutrophils in the early response. These may include direct parasite killing or the liberation of cytokines which directly or indirectly amplify the host response. Neutrophils are capable of producing a number of cytokines, including TNF-α, IL-1α L-1β, macrophage-inflammatory protein-2,

transforming growth factor-β1, macrophage-inflammatory protein-1α, IL-10, and IL-12. (27–30). Many of these cytokines/chemokines have already been shown to play important roles in *L. donovani* infection (1, 19, 31–33). Further work involving neutrophil depletion in cytokine gene targeted mice will be required to clarify this issue. Unfortunately, effective neutrophil elimination for greater than 14 days was not possible, due to increasingly rapid replenishment of the neutrophil pool (S. C. Smelt, unpublished observations), and thus it remains to be determined whether in the absence of neutrophils, mice retain the capacity to eventually resolve their infections. The latter might be predicted by a switch to a dependency on macrophage-derived RNI within the granulomas (20).

Although there have been a number of immunological aberrations reported in μMT mice, to our knowledge this is the first report of an exaggeration in neutrophil function. IL-10 has been reported as a major regulator of neutrophil function, and B cells have been reported to produce IL-10 in a number of infectious disease settings (34–36). We have detected IL-10 mRNA in B cells isolated from infected C57BL/6 and BALB/c mice (S. C. Smelt, C. R. Engwerda, and P. M. Kaye, unpublished data), but as this occurs relatively late in infection, we believe it unlikely that B cell-derived IL-10 contributes to the negative regulation of neutrophil function early in infection. Attempts to revert the phenotype of μMT mice by the adoptive transfer of B cells from C57BL/6 mice have been inconclusive, as like others (Ref. 37; D. van Essen and D. Gray, unpublished observations), we have only been able to achieve reconstitution levels of $<$10%. At this level, we have not observed any significant differences in parasite burdens or pathology between unreconstituted and B cell-reconstituted μMT mice (data not shown).

In contrast to our inability to modulate the response of μMT mice by B cell transfer, transfer of either normal or chronic-infection serum has a profound but highly selective effect in these mice. Serum transfer has minimal or no effect on parasite load in μMT mice indicating that the enhanced resistance of μMT mice is independent of the presence or absence of either normal or parasite specific Ig. However, μMT mice reconstituted with either normal or chronic-infection serum showed no signs of hepatic destruction. These data suggest that the presence of Igs, and possibly immune complexes (38) result in protection from tissue destructive processes. These data also highlight that the mechanisms controlling destructive pathology are independent of those which regulate the heightened resistance of μMT mice to *L. donovani* infection. Ig has also been shown to be required for the down-modulation of the schistosome egg granuloma, and in this model too, there is a dissociation between the impact on pathology and host resistance (23). However, whereas in schistosomiasis pathology remains focal around deposited eggs, such an association between the site of tissue destruction and the presence of the triggering stimulus is less easy to confirm in the case of *L. donovani* infection. Importantly, *L. donovani* infection of μMT mice now provides an additional tool for future studies into the mechanisms which control these two distinct host processes.

Although dysregulation of the neutrophil response is the most evident phenotype in μMT mice infected with *L. donovani*, our data do not formally rule out other more subtle influences on effector function. Indeed, the maintenance of a highly resistant phenotype even after serum transfer and the elimination of pathology suggests this is the case. The impact of B cells on the development and function of T cells has been extensively analyzed, though often with conflicting results. In *L. major* infections, the presence or absence of B cells fails to influence the commitment to Th 1 or Th 2 development (11). Similarly, immunization with acetylcholine receptor in CFA produces identical responses in wild-type and μMT mice (39), as was the case in a murine model for allergic asthma (40). In contrast, there have been numerous examples where the development of T cell responses following infection is modulated by the absence of B cells. μMT mice are defective in switching from a Th1 to a Th2 response following *Plasmodium chabaudi chabaudi* infection (41). Similarly, after infection with *Chlamydia*, μMT mice fail to mount a significant delayed-type hypersensitivity response and have higher mortality rates than their wild-type counterparts (42). Furthermore, μMT mice demonstrate more rapid graft rejection (43), enhanced primary LCMV-specific CTL responses at high viral loads (38), and a higher death rate among activated $CD8^+$ cells and LCMV-specific $CD8^+$ CTL memory cells after virus infection (44). In our hands, T cells derived from either strain have equal protective efficacy on a cell per cell basis when transferred into $RAG1^{-/-}$ mice. These data confirm that T cells originating from an environment with or without B cells can be primed in the absence of B cells and can develop the immune phenotype required for efficient clearance of *L. donovani*. These data do not argue for or against a subsequent role of B cells in Ag presentation or cytokine regulation of T cell immunity. In addition, the striking rapidity with which hepatic granulomas are lost from the tissue after parasite clearance may suggest a role for B cells in T cell persistence within granulomas. Studies are in progress to address these important issues.

In summary, this study demonstrates that μMT mice are highly resistant to *L. donovani* infection, but suffer from excessive destructive pathology. These two features of infection in μMT mice can be dissociated by the transfer of serum Ab, which limits pathology without compromising host resistance. Furthermore, we have shown that neutrophils are an essential component of host resistance in wild-type mice, and that their role is exaggerated in the absence of B cells. These data suggest a hitherto unrecognized negative association between B cells and neutrophils in the host response to infection.

Acknowledgments

We thank Drs. S. Reiner and R. Coffman for the RB6-8C5 hybridoma and Tracy Holmes for assistance in preparing the manuscript.

References

1. Cotterell, S. E., C. R. Engwerda, and P. M. Kaye. 1999. *Leishmania donovani* infection initiates T cell-independent chemokine responses, which are subsequently amplified in a T cell-dependent manner. *Eur. J. Immunol. 29:203.*
2. McElrath, M. J., H. W. Murray, and Z. A. Cohn. 1988. The dynamics of granuloma formation in experimental visceral leishmaniasis. *J. Exp. Med. 167:1927.*
3. Stern, J. J., M. J. Oca, B. Y. Rubin, S. L. Anderson, and H. W. Murray. 1988. Role of $L3T4^+$ and LyT^-2^+ cells in experimental visceral leishmaniasis. *J. Immunol. 140:3971.*
4. Galvao-Castro, B., J. A. Sa Ferreira, K. F. Marzochi, M. C. Marzochi, S. G. Coutinho, and P. H. Lambert. 1984. Polyclonal B cell activation, circulating immune complexes and autoimmunity in human American visceral leishmaniasis. *Clin. Exp. Immunol. 56:58.*
5. Smelt, S. C., C. R. Engwerda, M. McCrossen, and P. M. Kaye. 1997. Destruction of follicular dendritic cells during chronic visceral leishmaniasis. *J. Immunol. 158:3813.*
6. Saha, B., H. Nanda-Roy, A. Pakrashi, R. N. Chakrabarti, and S. Roy. 1991. Immunobiological studies on experimental visceral leishmaniasis. I. Changes in lymphoid organs and their possible role in pathogenesis. *Eur. J. Immunol. 21: 577.*
7. Sacks, D. L., P. A. Scott, R. Asofsky, and F. A. Sher. 1984. Cutaneous leishmaniasis in anti-IgM-treated mice: enhanced resistance due to functional depletion of a B cell-dependent T cell involved in the suppressor pathway. *J. Immunol. 132:2072.*
8. Hoerauf, A., W. Solbach, M. Lohoff, and M. Rollinghoff. 1994. The Xid defect determines an improved clinical course of murine leishmaniasis in susceptible mice. *Int. Immunol. 6:1117.*
9. Hoerauf, A., M. Rollinghoff, and W. Solbach. 1996. Co-transfer of B cells converts resistance into susceptibility in T cell-reconstituted, *Leishmania* major-resistant C.B-17 *scid* mice by a non-cognate mechanism. *Int. Immunol. 8:1569.*

10. Hoerauf, A., W. Solbach, M. Rollinghoff, and A. Gessner. 1995. Effect of IL-7 treatment on *Leishmania* major-infected BALB.Xid mice: enhanced lymphopoiesis with sustained lack of B1 cells and clinical aggravation of disease. *Int. Immunol. 7:1879.*
11. Brown, D. R., and S. L. Reiner. 1999. Polarized helper-T-cell responses against *Leishmania* major in the absence of B cells. *Infect. Immun. 67:266.*
12. Bradley, D. J., and J. Kirkley. 1977. Regulation of *Leishmania* populations within the host. I. The variable course of *Leishmania donovani* infections in mice. *Clin. Exp. Immunol. 30:119.*
13. Vidal, S. M., D. Malo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. *Cell 73:469.*
14. Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky. 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature 350:423.*
15. Gorak, P. M., C. R. Engwerda, and P. M. Kaye. 1998. Dendritic cells, but not macrophages, produce IL-12 immediately following *Leishmania donovani* infection. *Eur. J. Immunol. 28:687.*
16. Peters, C., T. Aebischer, Y. D. Stierhof, M. Fuchs, and P. Overath. 1995. The role of macrophage receptors in adhesion and uptake of *Leishmania mexicana* amastigotes. *J. Cell Sci. 108:3715.*
17. Fleming, T. J., M. L. Fleming, and T. R. Malek. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol. 151:2399.*
18. Murphy, M. L., S. E. Cotterell, P. M. Gorak, C. R. Engwerda, and P. M. Kaye. 1998. Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, *Leishmania donovani*. *J. Immunol. 161:4153.*
19. Engwerda, C. R., S. C. Smelt, and P. M. Kaye. 1996. An in vivo analysis of cytokine production during *Leishmania donovani* infection in *scid* mice. *Exp. Parasitol. 84:195.*
20. Murray, H. W., and C. F. Nathan. 1999. Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. *J. Exp. Med. 189:741.*
21. Czuprynski, C. J., J. F. Brown, N. Maroushek, R. D. Wagner, and H. Steinberg. 1994. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to *Listeria* monocytogenes infection. *J. Immunol. 152:1836.*
22. Sayles, P. C., and L. L. Johnson. 1996. Exacerbation of toxoplasmosis in neutrophil-depleted mice. *Nat. Immun. 15:249.*
23. Jankovic, D., A. W. Cheever, M. C. Kullberg, T. A. Wynn, G. Yap, P. Caspar, F. A. Lewis, R. Clynes, J. V. Ravetch, and A. Sher. 1998. $CD4^+$ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J. Exp. Med. 187:619.*
24. Sutterwala, F. S., G. J. Noel, P. Salgame, and D. M. Mosser. 1998. Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type I. *J. Exp. Med. 188:217.*
25. Blackwell, J. M. 1983. *Leishmania donovani* infection in heterozygous and recombinant H-2 haplotype mice. *Immunogenetics 18:101.*
26. Lepay, D. A., C. F. Nathan, R. M. Steinman, H. W. Murray, and Z. A. Cohn. 1985. Murine Kupffer cells: mononuclear phagocytes deficient in the generation of reactive oxygen intermediates. *J. Exp. Med. 161:1079.*
27. Lloyd, A. R., and J. J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol. Today 13:169.*
28. Cassatella, M. A. 1995. The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today 16:21.*
29. Romani, L., A. Mencacci, E. Cenci, G. Del Sero, F. Bistoni, and P. Puccetti. 1997. An immunoregulatory role for neutrophils in $CD4^+$ T helper subset selection in mice with candidiasis. *J. Immunol. 158:2356.*
30. Romani, L., A. Mencacci, E. Cenci, R. Spaccapelo, G. Del Sero, I. Nicoletti, G. Trinchieri, F. Bistoni, and P. Puccetti. 1997. Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice. *J. Immunol. 158:5349.*
31. Engwerda, C. R., M. L. Murphy, S. E. Cotterell, S. C. Smelt, and P. M. Kaye. 1998. Neutralization of IL-12 demonstrates the existence of discrete organ- specific phases in the control of *Leishmania donovani*. *Eur. J. Immunol. 28:669.*
32. Tumang, M. C., C. Keogh, L. L. Moldawer, D. C. Helfgott, R. Teitelbaum, J. Hariprashad, and H. W. Murray. 1994. Role and effect of TNF-α in experimental visceral leishmaniasis. *J. Immunol. 153:768.*
33. Wilson, M. E., B. M. Young, B. L. Davidson, K. A. Mente, and S. E. McGowan. 1998. The importance of TGF-β in murine visceral leishmaniasis. *J. Immunol. 161:6148.*
34. Velupillai, P., W. E. Secor, A. M. Horauf, and D. A. Harn. 1997. B-1 cell ($CD5^+B220^+$) outgrowth in murine schistosomiasis is genetically restricted and is largely due to activation by polylactosamine sugars. *J. Immunol. 158:338.*
35. Reed, S. G., C. E. Brownell, D. M. Russo, J. S. Silva, K. H. Grabstein, and P. J. Morrissey. 1994. IL-10 mediates susceptibility to *Trypanosoma cruzi* infection. *J. Immunol. 153:3135.*
36. Palanivel, V., C. Posey, A. M. Horauf, W. Solbach, W. F. Piessens, and D. A. Harn. 1996. B-cell outgrowth and ligand-specific production of IL-10 correlate with Th2 dominance in certain parasitic diseases. *Exp. Parasitol. 84:168.*
37. von der Weid, T., N. Honarvar, and J. Langhorne. 1996. Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *J. Immunol. 156:2510.*
38. Brundler, M. A., P. Aichele, M. Bachmann, D. Kitamura, K. Rajewsky, and R. M. Zinkernagel. 1996. Immunity to viruses in B cell-deficient mice: influence of antibodies on virus persistence and on T cell memory. *Eur. J. Immunol. 26: 2257.*
39. Li, H., F. D. Shi, B. He, M. Bakheit, B. Wahren, A. Berglof, K. Sandstedt, and H. Link. 1998. Experimental autoimmune myasthenia gravis induction in B cell-deficient mice. *Int. Immunol. 10:1359.*
40. Corry, D. B., G. Grunig, H. Hadeiba, V. P. Kurup, M. L. Warnock, D. Sheppard, D. M. Rennick, and R. M. Locksley. 1998. Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol. Med. 4:344.*
41. Langhorne, J., C. Cross, E. Seixas, C. Li, and T. von der Weid. 1998. A role for B cells in the development of T cell helper function in a malaria infection in mice. *Proc. Natl. Acad. Sci. USA 95:1730.*
42. Yang, X., and R. C. Brunham. 1998. Gene knockout B cell-deficient mice demonstrate that B cells play an important role in the initiation of T cell responses to *Chlamydia trachomatis* (mouse pneumonitis) lung infection. *J. Immunol. 161: 1439.*
43. Epstein, M. M., F. Di Rosa, D. Jankovic, A. Sher, and P. Matzinger. 1995. Successful T cell priming in B cell-deficient mice. *J. Exp. Med. 182:915.*
44. Asano, M. S., and R. Ahmed. 1996. CD8 T cell memory in B cell-deficient mice. *J. Exp. Med. 183:2165.*