MAJOR ARTICLE

Role of T Cells in Innate and Adaptive Immunity against Murine *Burkholderia pseudomallei* Infection

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Antigen-specific T cells are important sources of interferon (IFN)–γ for acquired immunity to intracellular pathogens, but they can also produce IFN-γ directly via a "bystander" activation pathway in response to proinflammatory cytokines. We investigated the in vivo role of cytokine- versus antigen-mediated T cell activation in resistance to the pathogenic bacterium *Burkholderia pseudomallei*. IFN-γ, interleukin (IL)–12, and IL-18 were essential for initial bacterial control in infected mice. *B. pseudomallei* infection rapidly generated a potent IFN-γ response from natural killer (NK) cells, NK T cells, conventional T cells, and other cell types within 16 h after infection, in an IL-12– and IL-18–dependent manner. However, early T cell– and NK cell–derived IFN-γ responses were functionally redundant in cell depletion studies, with IFN-γ produced by other cell types, such as major histocompatibility complex class II^{int} F4/80^{+} macrophages being sufficient for initial resistance. In contrast, *B. pseudomallei*–specific CD4^{+} T cells played an important role during the later stage of infection. Thus, the T cell response to primary *B. pseudomallei* infection is biphasic, an early cytokine-induced phase in which T cells appear to be functionally redundant for initial bacterial clearance, followed by a later antigen-induced phase in which *B. pseudomallei*–specific T cells, in particular CD4^{+} T cells, are important for host resistance.

Burkholderia pseudomallei, the causative agent of melioidosis, is a gram-negative bacterium that is endemic in areas of Southeast Asia and northern Australia [1]. Clinical manifestations vary from acute infection to chronic localized pathologic symptoms to latent infection that can reactivate decades later [2]. There is no vaccine, and mortality in acute cases can exceed 40%, with 10%–15% of survivors relapsing despite prolonged treatment [2]. Although serologic evidence of exposure to *B. pseudomallei* is found in the majority of children living in areas where the organism is endemic [3], it is not known what immune mechanisms or defects confer resistance versus susceptibility to active disease. *B. pseudomallei* is classified as a class B potential agent for biological warfare and terrorism. A better understanding of immune responses to *B. pseudomallei* is needed for the generation of a novel vaccine or immunotherapeutic approaches for melioidosis.

B. pseudomallei is a facultative intracellular pathogen that, like *Listeria monocytogenes*, resides in the host cell cytosol after lysis of the phagosome [4, 5]. Individuals with severe melioidosis have elevated concentrations of many serum cytokines, such as interferon (IFN)–γ, interleukin (IL)–12, and IL-18 [6], and restimulation of peripheral blood mononuclear cells from recovering patients generates an antigen-specific IFN-γ immune response to *B. pseudomallei* [7]. We and others have developed mouse models to study the immunological mechanisms of protection against *B. pseudomallei* [4, 8–10]. Using cytokine neutralizing monoclonal anti-

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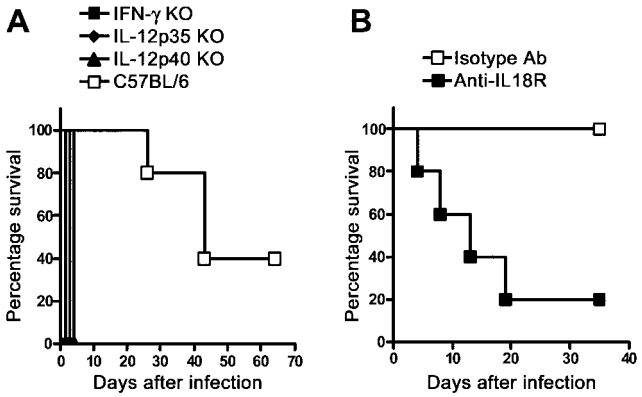

Figure 1. Susceptibility of 57BL/6 mice deficient in interferon (IFN)–γ interleukin (IL)–12p35, IL-12p40, or IL-18 to *Burkholderia pseudomallei* infection. The data depict the survival of *(A)* various gene knockout (KO) mice or *(B)* mice treated with anti–IL-18 receptor (R) antibody (Ab) vs. isotype control Ab–treated C57BL/6 mice, ($n = 5$–6 mice/group), infected intraperitoneally with 1×10^6 cfu of *B. pseudomallei* strain 576 per mouse. Data are representative of at least 2 separate experiments with similar results.

bodies (MAbs), we have shown that IFN-γ is essential for resistance to *B. pseudomallei* [9]. These clinical and experimental observations indicates that *B. pseudomallei* is a potent activator of cell-mediated immunity; but, to date, the in vivo source(s) of IFN-γ and the role of T cells in resistance to infection have not been defined.

In other models of primary infection, multiple cell types produce IFN-γ [11–14]. NK cells, NK T cells, and macrophages can contribute to early resistance through IFN-γ production [15–17]. Antigen-specific T cells play well-documented roles in IFN-γ–dependent protection against intracellular pathogens [18–20]. However, there is growing evidence that conventional T cell receptor (TCR) α/β^+ $CD4^+$ and $CD8^+$ T cells can also produce IFN-γ in the absence of cognate antigen in response to IL-12 and IL-18 [21–25]. However, the relative importance in vivo of these 2 pathways of T cell activation has not previously been examined in any model of infection. We previously demonstrated that *B. pseudomallei* and *L. monocytogenes* stimulate T cells (and NK cells) to produce IFN-γ in an IL-12– and IL-18–dependent manner in vitro [23]. We investigated cytokine-mediated T cell (and NK cell) production of IFN-γ in vivo after *B. pseudomallei* infection and determined its importance for the initial control of bacterial growth. In addition, we tested the hypothesis that antigen-specific T cells may be detected later during infection and whether they contribute to resistance against primary melioidosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. pseudomallei* strain 576, isolated from a patient with melioidosis in Thailand, was obtained from Ty Pitt (Health Protection Agency, London, UK) [26–28]. Bacteria were cultured in tryptone soy (TS) broth or TS agar. Bacteria were grown statically for 24–48 h at 37°C, collected by centrifugation, washed in PBS, and frozen at −80°C in PBS that contained 30% glycerol. Dead *B. pseudomallei* strain 576 organisms were prepared by γ-irradiation (6500 Gy) of 30% glycerol stocks. Once they were confirmed as nonviable by plating, bacteria were washed, resuspended in RPMI 1640 medium (Sigma), and stored at −80°C. All procedures using live bacteria were performed under Advisory Committee on Dangerous Pathogens category 3 containment.

Mice. Female 8–10-week-old C57BL/6 (B6), B6 IFN-$\gamma^{-/-}$, B6 IL-12p35$^{-/-}$, B6 IL-12p40$^{-/-}$, B6 μMT, and B6 *rag1*$^{-/-}$ mice, bred at the London School of Hygiene and Tropical Medicine (LSHTM), were housed under specific pathogen–free conditions, with free access to food and water. Mouse experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee.

Antibodies and in vivo cell depletion. Anti-CD4 (YTS191) and anti-CD8 (YTS169) MAbs and isotype control Mac-5 antibodies were obtained from Roman Lukaszewski (Defence Science and Technology Laboratory, Salisbury, UK). Mice were administered 500 μg of MAb intraperitoneally (ip) 4 days before infection and 250 μg 1 day before infection. Depletion was maintained by further administration of 250 μg of MAb every 3 days after infection. NK cells were depleted by the intravenous (iv) injection of 25–30 μL of rabbit anti-asialoGM1 polyclonal antibody per mouse (endotoxin levels, 380 ng/mL; Cedarlane Labs) 1 day before infection. The efficiency of depletions in the spleen at the time of infection and time points thereafter was >99% for $CD4^+$ T cells with YTS191, >97% for $CD8^+$ cells with YTS169, and >98% for NK cells with anti-asialoGM1, as verified by flow-cytometric analysis of splenocytes with noncompeting anti-CD4 MAb RM4-5, anti-CD8 MAb 53–6.7, and anti-NK1.1 (BD Biosciences). Macrophages were depleted by iv administration of clodronate liposomes. The efficiency of splenic $F4/80^+$ macrophage depletion was >99% at day 3 after treatment and >90% at day 7 after treatment. [29, 30]. Clodronate was a gift from Roche Diagnostics. Clodronate liposomes were prepared as described elsewhere [30]. The MAbs anti–β-galactosidase (isotype control, GL117), anti–IL-12 (C17.8; provided by Helena Helmby, Department of Infectious and Tropical Diseases, LSHTM, and originally obtained from G. Trinchieri, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland [31]), and anti–IL-18 receptor (R) (TC30-28E3; provided by Anne O'Garra, National Institute for Medical Research, London, UK, and originally produced at DNAX Research Institute, Palo Alto, CA [32]), were administered (1 mg) ip 6 h before infection.

Infection of mice. Bacteria were thawed, diluted in PBS,

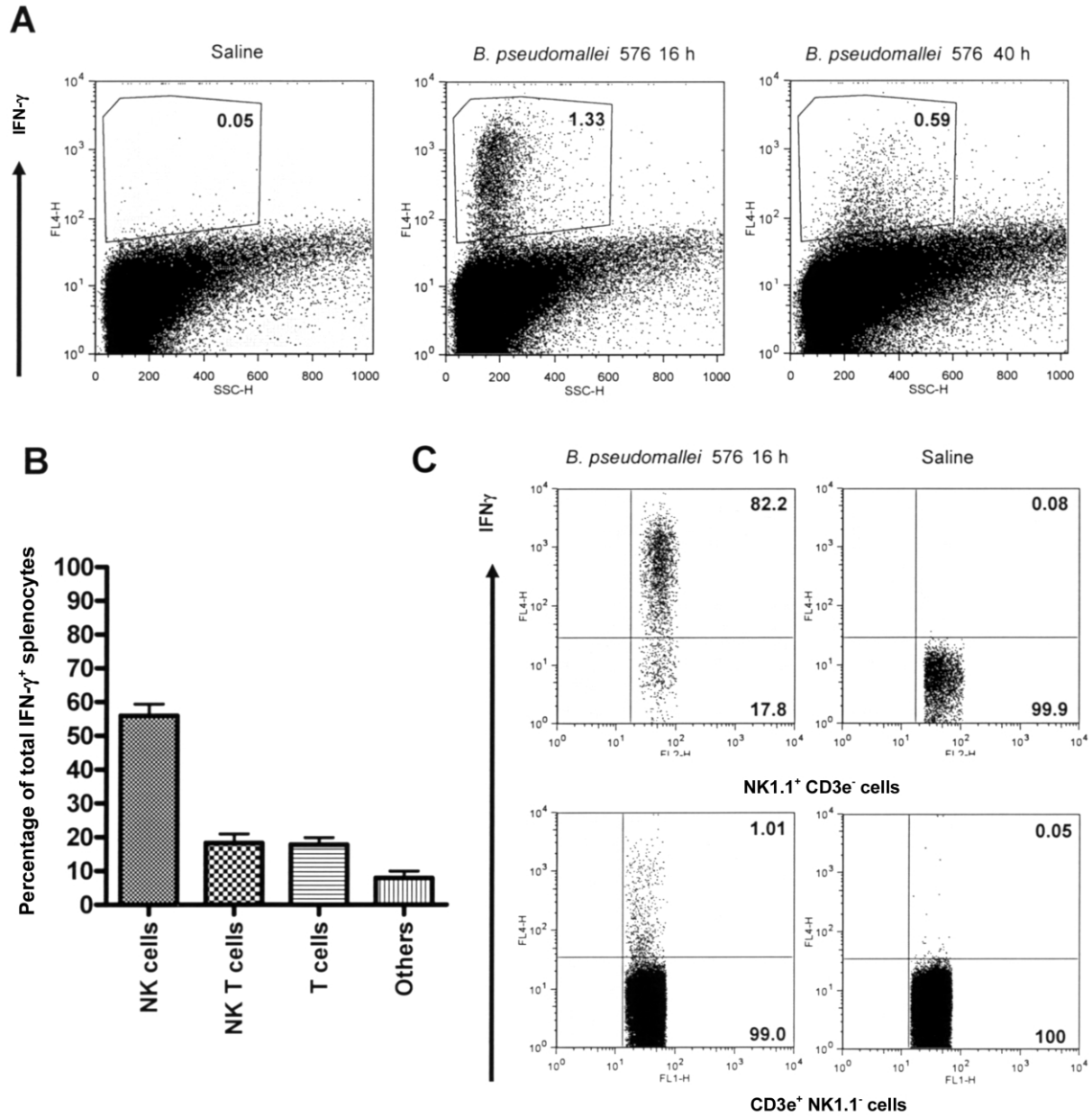

Figure 2. Induction of an early, transient, splenic interferon (IFN)–γ response from multiple cell types after *Burkholderia pseudomallei* infection. C57BL/6 mice ($n = 5$) were injected intraperitoneally with saline or 1×10^7 cfu of *B. pseudomallei* strain 576. *A,* IFN-γ production at 16 or 40 h after infection in splenocytes, analyzed directly ex vivo by intracellular cytokine staining. *B,* IFN-γ–producing splenocytes phenotyped by flow-cytometric analysis 16 h after infection: NK cells (CD3ε^-NK1.1$^+$), NK T cells (CD3ε^+NK1.1$^+$), T cells (CD3ε^+ NK1.1$^-$), and others (CD3ε^- NK1.1$^-$). *C,* Proportion of splenic NK cells (CD3ε^-NK1.1$^+$) and T cells (CD3ε^+ NK1.1$^-$) making up the total IFN-γ response at 16 h after infection. Nos. in each quadrant indicate the percentage of gated cells in that quadrant. Data are representative of at least 5 independent experiments.

and administered ip (0.2 mL). For each infection, the inoculum was plated onto TS agar plates to confirm the inoculation dose.

Determination of organ bacterial burden. Spleens were aseptically removed and homogenized in sterile PBS or RPMI 1640 (Sigma) by passing them through 70-μm cell strainers, using a syringe plunger. Dilutions of tissue homogenates were plated onto TS agar and incubated at 37°C; colonies were enumerated after 24 h.

Preparation and stimulation of murine splenocytes in vitro. Spleens were removed aseptically, and splenocyte suspensions were produced by passing them through sterile 70-μm cell strainers. Erythrocytes were lysed, and cells were washed and

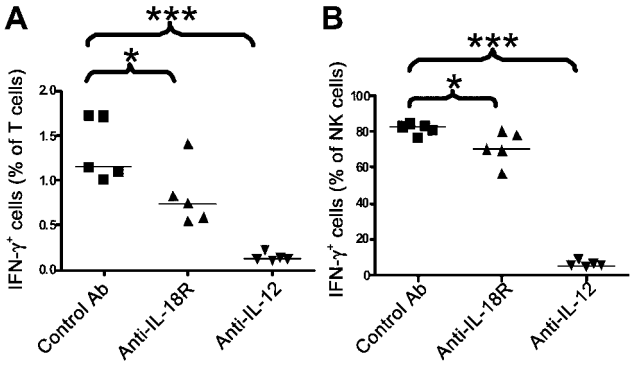

Figure 3. Interleukin (IL)–12– and IL-18–dependent early interferon (IFN)–γ production by T cells and NK cells during *Burkholderia pseudomallei* infection. C57BL/6 mice ($n = 5$ mice/group) were treated with anti–IL-12 antibodies (Abs), anti–IL-18-receptor (R) Abs, or isotype control Abs 1 day before intraperitoneal injection with 1×10^7 cfu of *B. pseudomallei* strain 576 per mouse. At 16 h after infection, spleens were removed, and T cells ($CD3\varepsilon^+NK1.1^-$) *(A)* and NK cells ($CD3\varepsilon^-NK1.1^+$) *(B)* from individual mice were analyzed for IFN-γ production by intracellular cytokine staining. Graphs indicate the percentage of each cell type producing IFN-γ. Horizontal lines indicate median percentages per group. $^*P < .05$; $^{***}P < .0001$. Data are representative of 2 independent experiments.

resuspended in RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (FCS), 10 mmol/L L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol. Cells were plated in U-bottom 96-well plates (2.5×10^6 cells/mL), restimulated, and incubated for 18–24 h at 37°C in 5% CO_2.

Flow-cytometric analysis for cell-surface marker and intracellular IFN-γ staining. Cells intended for intracellular IFN-γ staining were treated with brefeldin A (10 μg/mL; Sigma) for 3 h. Cells were washed in 1% FCS-PBS, and nonspecific antibody binding was blocked with anti-CD16/32 (1μg/mL, 2.4G2; BD Biosciences). MAbs used for cell-surface staining were fluorescein isothiocyanate (FITC)–anti-CD4 (RM4-5), FITC–anti-CD8 (53–6.7), FITC–anti-7/4, FITC–anti-CD11b (M1/70.15) (Caltag Laboratories), phycoerythrin (PE)–anti-NK1.1 (PK136), PE–anti-CD49b (DX5), PE–anti-Gr1 (RB6-8C5), PE–anti-F4/80, FITC-0 and peridinin-chlorophyll-protein–anti-CD3ε (145-2C11), and allophycocyanin (APC)–anti-CD11c (HL3) (BD Biosciences). Cells were stained with antibodies, washed twice, and fixed for 20 min in 2% paraformaldehyde. Cells were permeabilized in 0.1% saponin–1% FCS-PBS, incubated with APC– or PE–anti–IFN-γ (0.5 μg/tube) (XMG1.2; BD Biosciences), washed twice, and fixed overnight in 2% paraformaldehyde. Cells were analyzed using a FACScalibur instrument with CellQuest software (version 3.3; BD Biosciences) under category 3 aerosol biocontainment.

Statistical analysis. Survival curves were compared using log rank Kaplan-Meier tests. Student's *t* test was used for all other statistical tests. $P < .05$ was considered to be statistically significant.

RESULTS

Necessity of IFN-γ, IL-12, and IL-18 for protection against primary* B. pseudomallei *infection. C57BL/6 mice are relatively resistant to *B. pseudomallei* ip infection [4, 8], which results in rapid phagocytosis and transport of bacteria to the spleen [33, 34], so this was chosen as an appropriate route for the assessment of resistance to *B. pseudomallei* infection. Infection with 1×10^6 cfu of *B. pseudomallei* strain 576 per mouse did not result in any deaths within the first 20 days of infection. C57BL/6 mice cleared most bacteria from the spleen within the first few days of an ip infection, but they ultimately died and had abscesses that contained *B. pseudomallei* in multiple organs. The natural resistance of C57BL/6 mice made them a suitable model for the study of mechanisms of host resistance to primary *B. pseudomallei* infection.

To identify host factors controlling initial resistance, C57BL/6 or isogenic IFN-$\gamma^{-/-}$, IL-12p35$^{-/-}$, or IL-12p40$^{-/-}$ mice were infected with *B. pseudomallei* and monitored for survival. Wild-type mice died starting 30 days after infection, whereas IFN-$\gamma^{-/-}$, IL-12p35$^{-/-}$, and IL-12p40$^{-/-}$ mice all died within the first 4 days of infection (figure 1*A*). To address the importance of IL-18 in resistance, C57BL/6 mice were treated with anti–IL-18R antibodies or with an isotype control antibody before infection. Blockade of the IL-18R rendered C57BL/6 mice more susceptible to *B. pseudomallei* infection than mice given isotype-matched control antibodies ($P < .01$); anti–IL-18R–treated mice

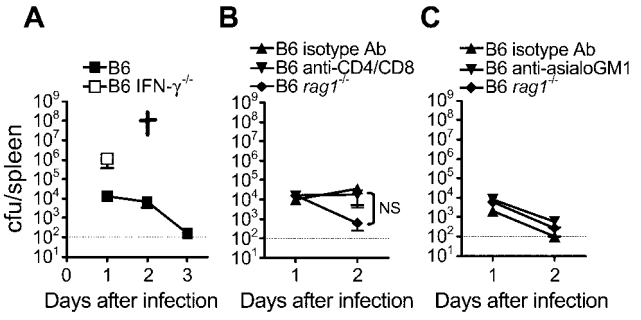

Figure 4. Increased bacterial organ loads early after infection with *Burkholderia pseudomallei* in mice deficient in interferon (IFN)–γ but not in mice deficient in either T or NK cells. The data depict splenic bacterial burdens in mice ($n = 5$ mice/group) deficient in IFN-γ *(A),* $CD4^+$/ $CD8^+$ T cells *(B),* NK cells (treated with anti-asialoGM1) *(C),* or B and T cells (*rag1*$^{-/-}$) *(B* and *C)* after intraperitoneal infection with 1×10^6 cfu of *B. pseudomallei* strain 576. The cross in panel A illustrates that these mice all died of infection on day 2, before splenic bacterial burdens could be determined in this particular experiment. The dotted line in each graph illustrates the detection limit of 100 cfu/spleen. Data are representative of 2 independent experiments. Ab, antibody; NS, not statistically significant.

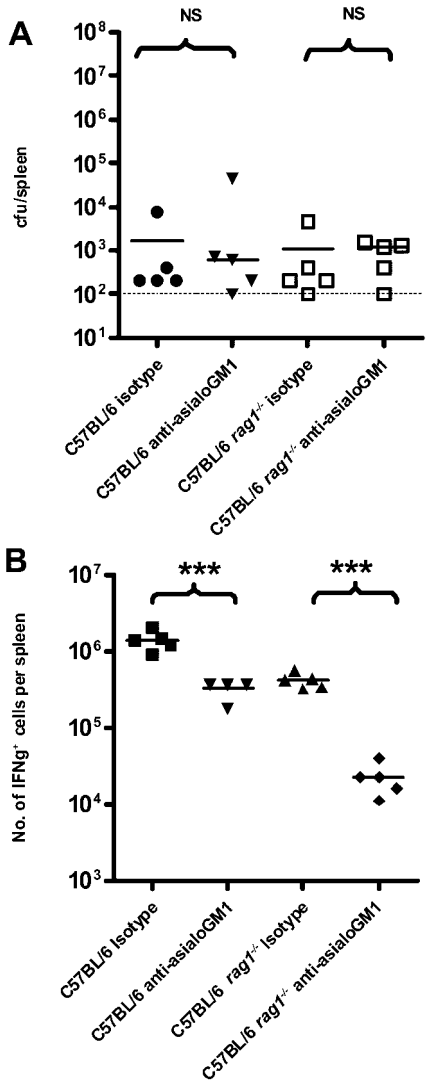

Figure 5. Initial control of *Burkholderia pseudomallei* in the spleen in the absence of both T and NK cell–derived interferon (IFN)–γ. *A,* Splenic bacterial burdens 2 days after intraperitoneal (ip) infection with 1×10^6 cfu of *B. pseudomallei* strain 576 per mouse in individual C57BL/6 and isogenic *rag1*$^{-/-}$ mice treated with control serum or depleted of NK cells with anti-asialoGM1 ($n = 5$ mice/group). *B,* No. of IFN-γ–producing splenocytes from individual mice ($n = 5$ mice/group) 16 h after ip infection with 1×10^7 cfu of *B. pseudomallei* strain 576 per mouse. Horizontal lines in each group indicate median values. ***$P < .0001$. Data are representative of 2 independent experiments NS, not statistically significant.

died starting 3 days after infection, with a median survival time (MST) of 13 days, compared with >40 days for isotype antibody–treated mice (figure 1*B*). Together, these results demonstrate that IFN-γ and the IFN-γ–inducing cytokines IL-12 and IL-18 are essential for initial resistance to *B. pseudomallei.*

Rapid IL-12– and IL-18–dependent T cell– and NK cell–derived IFN-γ responses in vivo induced by* B. pseudomallei *infection. To investigate the cellular source(s) of the protective IFN-γ response, splenocytes were harvested from saline-treated or *B. pseudomallei*–infected mice, incubated in brefeldin A (in the absence of any in vitro stimulation), and assayed for IFN-γ production by flow-cytometric analysis. Control cells from mice injected with saline exhibited negligible levels of IFN-γ production at all time points (figure 2*A*). Splenocytes from mice infected with 1×10^7 cfu/mouse for 16 h displayed strong IFN-γ production; ~1.3% of recovered splenocytes produced IFN-γ (figure 2*A*). Splenic IFN-γ responses after infection with 1×10^6 cfu/mouse were qualitatively identical but of a lower magnitude than responses to infection with 1×10^7 cfu/mouse (data not shown). The magnitude of the IFN-γ response at 16 h after infection was markedly reduced by 40 h after infection (figure 2*A*). Flow-cytometric analysis at 16 h after infection indicated that the majority of IFN-γ–producing cells were NK cells (CD3ε$^-$/NK1.1$^+$), with additional contributions from T cells (CD3ε$^+$/NK1.1$^-$), NK T cells (CD3ε$^+$/NK1.1$^+$), and CD3ε$^-$/NK1.1$^-$ cells (figure 2*B*). Approximately 1% of T cells and ~80% of NK cells produced IFN-γ at 16 h after infection (figure 2*C*).

To investigate the dependency of in vivo IFN-γ responses on IL-12 and IL-18, C57BL/6 mice were treated with anti–IL-12 or anti–IL-18R MAbs before infection. Intracellular cytokine staining at 16 h after infection revealed that the splenic T cell IFN-γ response was reduced by 91% after IL-12 neutralization ($P < .0001$) and by 35% after IL-18R blockade ($P < .05$) (figure 3*A*). Similarly, a 93% ($P < .0001$) and 16% ($P < .05$) reduction in the NK cell–derived IFN-γ response occurred with IL-12 and IL-18R blockade, respectively (figure 3*B*). Thus, *B. pseudomallei* infection induces a rapid, transient, splenic IFN-γ response in vivo that is primarily derived from NK cells and T cells and is strongly IL-12 dependent but weakly IL-18 dependent.

Functional redundancy of T cell– and NK cell–derived IFN-γ for initial control of* B. pseudomallei *infection. To define the contribution of each IFN-γ–producing cell type to resistance, we studied bacterial clearance from the spleens of mice after the depletion of IFN-γ, T cell subsets, or NK cells. C57BL/6 mice had 1×10^4 bacteria in their spleens at day 1 after infection; this decreased to 100 bacteria by day 3 (figure 4*A*). As was predicted by the in vivo survival data (figure 1), IFN-γ$^{-/-}$ mice were unable to control bacterial replication, having 100-fold more bacteria in their spleens by day 1, and all of them died of infection by day 2 (figure 4*A*).

In contrast, T and B cell–deficient *rag1*$^{-/-}$ mice, which had been depleted of both CD4$^+$ (>99% depletion) and CD8$^+$ (>97% depletion) T cells by MAbs, and NK cell–depleted (with anti-asialoGM1; >98% depletion) mice were as proficient as C57BL/6 mice at controlling bacterial growth in the spleen (figure 4*B* and 4*C*). Surprisingly, the combined depletion of both T cells (>97% depletion) and NK cells (>99% depletion by anti-asialoGM1 treatment of *rag1*$^{-/-}$ mice) also had no effect on the efficiency of bacterial clearance at 48 h after infection (figure

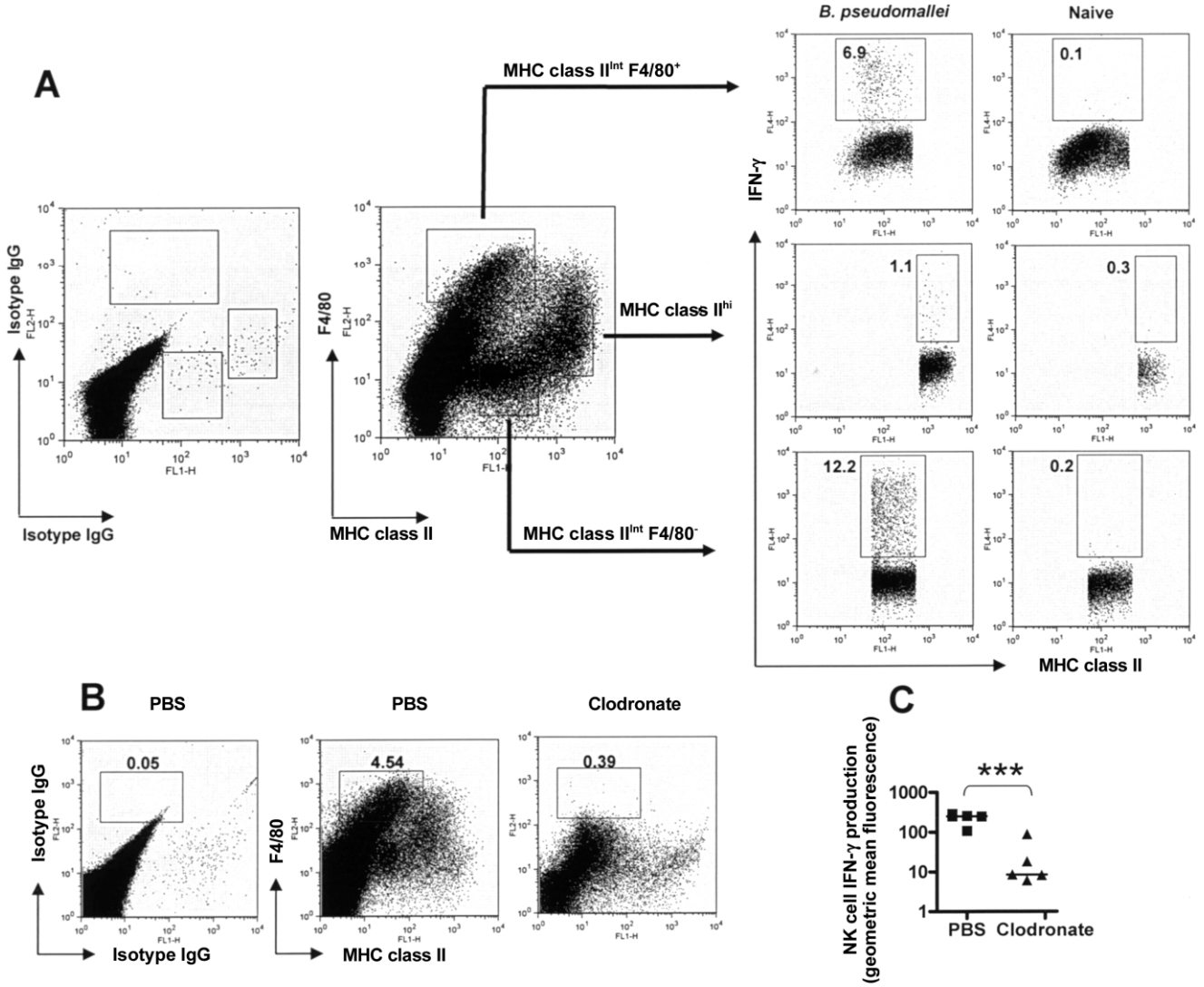

Figure 6. Major histocompatibility complex (MHC) class II^{int}, clodronate-sensitive macrophages as an in vivo source of early interferon (IFN)–γ. *A,* Spleen cells from *rag1*$^{-/-}$ mice ($n = 5$ mice/group), infected intraperitoneally for 16 h with 1×10^7 cfu of *Burkholderia pseudomallei* strain 576 per mouse, analyzed by flow-cytometric analysis for F4/80 and MHC class II expression. *B,* Splenic F4/80$^+$ cells analyzed by flow-cytometric analysis for susceptibility to depletion by clodronate-containing liposomes in uninfected *rag1*$^{-/-}$ mice ($n = 5$) treated with saline or clodronate-containing liposome 7 days before infection. *C,* Saline and clodronate-treated mice ($n = 5$), infected as described in panel A, and their NK cell–derived IFN-γ responses 16 h later. Data are representative of 2 independent experiments showing similar results.

5*A*). The determination of IFN-γ responses at 16 h after infection revealed a 76% reduction in total IFN-γ–producing splenocytes after NK cell depletion in C57BL/6 mice (figure 5*B*), which is consistent with the frequency of these cells determined by direct assay in figure 2*B*. NK cell depletion of *rag1*$^{-/-}$ mice reduced the IFN-γ response by 95% (figure 5*B*). Therefore, although IFN-γ production is essential for preventing rapid death, there is extensive redundancy in the source of this cytokine, and as little as 5% of this response is sufficient to provide initial control of *B. pseudomallei* replication in vivo.

***Macrophage production of IFN-γ after infection with* B. pseudomallei.** Despite the elimination of both NK cells and T cells, anti-asialoGM1–treated *rag1*$^{-/-}$ mice expressed low but detectable numbers of NK1.1$^-$ IFN-γ–producing cells (figure 5*B* and data not shown), which suggests that IFN-γ production by nonlymphoid cells might compensate for the loss of T cells and NK cells in these mice. Further phenotyping of these splenocytes from *B. pseudomallei*–infected *rag1*$^{-/-}$ mice identified major histocompatibility complex (MHC) class II^{int} F4/80$^+$ cells and MHC class II^{int} F4/80$^-$ cells as 2 further sources of IFN-γ (figure 6*A*). In contrast, we observed no IFN-γ production by MHC class II^{hi} dendritic cells (DCs) (figure 6*A*) or any cells expressing CD11c, CD11b, or Gr1 (data not shown). The treatment of *rag1*$^{-/-}$ mice with clodronate-containing liposomes 7

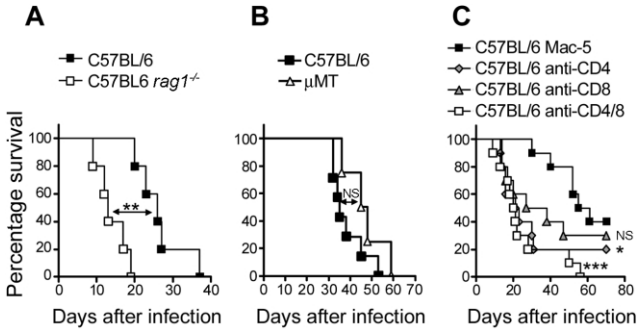

Figure 7. Increased susceptibility to *Burkholderia pseudomallei* later in primary infection in mice deficient in T cells but not B cells. C57BL/6 mice and C57BL/6 *rag1*$^{-/-}$ mice *(A)* or C57BL/6 mice and C57BL/6 μMT mice *(B)* were infected intraperitoneally with 1×10^6 cfu of *B. pseudomallei* strain 576 per mouse and monitored for survival. *C,* C57BL/6 mice ($n = 10$ mice/group), treated with anti-CD4$^+$– and/or anti-CD8$^+$–depleting antibodies or the isotype antibody (Mac-5), infected with 5×10^5 cfu/mouse of *B. pseudomallei* strain 576, and monitored for subsequent survival. *$P < .05$; **$P < .001$; and ***$P < .0001$ vs. Mac-5. Experiments were performed twice with similar results (minimum of 5 mice/group). NS, not statistically significant.

days before infection (which generated spleens lacking marginal zone and red pulp macrophages but not DCs; data not shown) eliminated >90% of F4/80$^+$ cells, which suggests that MHC class II^{int} F4/80$^+$ cells are phagocytic (figure 6*B*). Furthermore, clodronate treatment 7 days before infection reduced by 89% the amount of IFN-γ produced by individual NK cells 16 h after infection; this was measured as a decrease in the geometric mean $\pm$ SD IFN-γ fluorescence from 229.4 $\pm$ 31.4 in control mice to 25.8 $\pm$ 15.7 in clodronate-treated mice (figure 6*C*). Taken together, these data suggest that *B. pseudomallei* infection stimulates macrophages to produce IFN-γ in vivo and that, in the absence of macrophages, the efficiency of IFN-γ production by NK cells is substantially reduced.

Protective role of T cells during the later phase of* B. pseudomallei *infection. Although T cells were dispensable for the initial control of *B. pseudomallei,* we investigated their role during later stages of infection. *rag1*$^{-/-}$ mice, which lack B and T cells, died of infection more rapidly (MST, 13 days) than did wild-type mice (MST, 26 days; $P = .002$) (figure 7*A*). In contrast, μMT mice, which lack B cells, were as susceptible as wild-type mice, which indicates that B cells are not essential for primary resistance (figure 7*B*). To compare the contributions of CD4$^+$ and CD8$^+$ T cell subsets in this protection, mice were depleted of CD4$^+$ T cells (CD4$^-$) with >99% efficiency, CD8$^+$ T cells (CD8$^-$) with >97% efficiency, or both (CD4$^-$/8$^-$) before infection and for 50 days after infection (figure 7*C*). The MST was 58 days for control antibody–treated mice, 22 days for CD4$^-$ mice ($P = .0373$), and 20.5 days for CD4$^-$/8$^-$ mice ($P = .0004$). Although CD8$^-$ mice had a shorter MST than did control mice (58 vs. 32.5 days), this was not statistically significant ($P = .1996$). Thus, T cells contribute to resistance against *B. pseudomallei* during the later stages of infection, with CD4$^+$ T cells, rather than CD8$^+$ T cells, playing the dominant role under these conditions.

To test whether infection with *B. pseudomallei* primes antigen-specific T cells, splenocytes from C57BL/6 mice, obtained 10 days after infection, were restimulated in vitro with killed *B. pseudomallei* (1 bacterium/10 splenocytes) and analyzed for IFN-γ production. An IFN-γ response to dead bacteria was observed in splenocytes from infected but not uninfected mice, with CD4$^+$ and CD8$^+$ T cells producing IFN-γ (figure 8). The majority of this IFN-γ response was inhibited by the addition of cyclosporin A (figure 8), which blocks TCR-mediated but not cytokine receptor–mediated T cell activation [35]. Taken

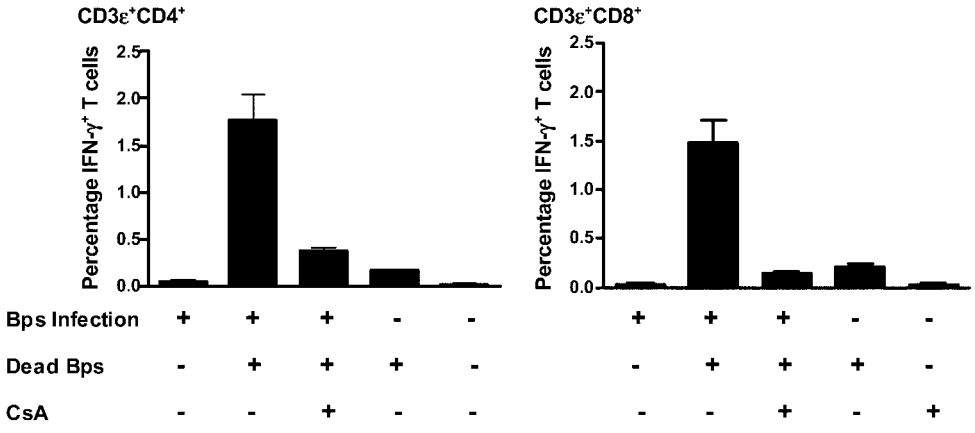

Figure 8. Detection of *Burkholderia pseudomallei* (Bps)–specific T cells in the spleens of infected mice. C57BL/6 mice ($n = 5$) were infected intraperitoneally with 1×10^6 cfu of Bps strain 576 per mouse. Control mice ($n = 5$) were injected with pyrogen-free saline. Ten days, later spleen cells from infected and control mice were stimulated in vitro overnight with dead *B. pseudomallei* strain 576 in the presence or absence of cyclosporin A (CsA). Interferon (IFN)–γ production by CD3ε^+CD4$^+$ and CD3ε^+CD8$^+$ T cells was assessed by intracellular cytokine staining. Data indicate the mean response from 5 individual mice $\pm$ 1 SE and are representative of 2 independent experiments showing similar results.

together, these data indicate that primary infection with *B. pseudomallei* primes populations of antigen-specific $CD4^+$ and $CD8^+$ T cells and suggests that $CD4^+$ T cells, in particular, play an important role in protection against infection.

DISCUSSION

We used a mouse model of infection with *B. pseudomallei* to study the role of T cells and IFN-γ in protection against primary infection. The results presented here on gene-knockout mice are consistent with those of our previous antibody depletion–based studies in confirming the absolute requirement for IFN-γ within the first 24 h of infection for the control of bacterial replication [9]. In addition, the results of our studies of IL-12p40$^{-/-}$ mice, which lack functional IL-12 and IL-23, suggested that either or both of these cytokines is essential for host resistance. The equivalent susceptibility of IL-12p35$^{-/-}$ mice confirms that IL-12 is essential for early host resistance. Using anti–IL-18R–blocking antibodies, we also demonstrated that IL-18 plays an important role in primary resistance to *B. pseudomallei* infection. Thus, innate immunity against *B. pseudomallei* shares many features with IFN-γ–mediated resistance to other intracellular bacteria, including *Salmonella* species and *L. monocytogenes* [32, 36, 37].

The rapid in vivo impact of depleting either IFN-γ per se or IFN-γ–inducing cytokines correlated with the presence of IFN-γ–producing spleen cells in infected mice within 16 h of exposure. It is likely that the magnitude of the early splenic IFN-γ response to *B. pseudomallei* infection, which was detected directly ex vivo without the need for in vitro stimulation, was dependent not only on the dose but also on the bacterial strain used, given that different *B. pseudomallei* strains, which vary in virulence, also vary in the magnitude of cytokine responses they elicit [38]. Early during infection, the dominant source of IFN-γ was NK cells, with additional contributions from T cells, NK T cells, and macrophages. In each case, this was strictly dependent in vivo on the cytokines IL-12 and, to a much lesser extent, IL-18. The phenomenon of multicellular sources of early IFN-γ has also been reported for *Salmonella* species [39], although the cell types responsible differed from those we observed with *B. pseudomallei,* which perhaps reflects variations in experimental design.

Remarkably, depletion of 95% of the early IFN-γ response (by removal of both T and NK cell populations) did not hinder initial bacterial control. Significant redundancy therefore exists between the various cellular sources of innate IFN-γ, and the minimum threshold of IFN-γ needed for initial bacterial clearance can be attained even in mice deficient in both T and NK cells. These findings clearly show the in vivo importance of other cell types, such as MHC class II^{int} $F4/80^+$ macrophages, which may compensate for the loss of T and NK cells as initial sources of IFN-γ during *B. pseudomallei* infection. This is consistent with other reports of macrophage-derived IFN-γ providing early protection against infection with *Listeria* and *Chlamydia* species [15, 16, 40]. MHC class II^{int} $F4/80^-$ cells also produced IFN-γ during infection, but the precise lineage of these cells remains unknown. In contrast, $Gr1^{hi}$ neutrophils and MHC class II^{hi} $CD11c^+$ DCs did not produce IFN-γ under these conditions. Interestingly, clodronate treatment also severely reduced IFN-γ production by NK cells, which suggests that macrophages (but not DCs) play a dual role in the early IFN-γ response to *B. pseudomallei* infection by acting as a source of IFN-γ and as an indirect inducer of IFN-γ production by other cell types, presumably through the production of IL-12 and IL-18.

We have previously shown in vitro that dead *B. pseudomallei* organisms induce IFN-γ secretion by splenic NK cells and α/β TCR^+ T cells. The T cell response was both IL-12 and IL-18 dependent and occurred within 12 h after exposure of previously uninfected spleen cells to the pathogen [23]; these findings were mirrored by those of the present vivo studies. We and others proposed that IFN-γ derived from this cytokine-mediated bystander T cell response could contribute to innate resistance against intracellular pathogens [23, 25, 41]. Indeed, the potential protective effects of such cells were seen when they were adoptively transferred into IFN-$\gamma^{-/-}$ recipients [41]. The data presented here, of infection in immunocompetent wild-type (rather than transgenic or knockout) mice, suggest that bystander T cell activation does, indeed, occur in vivo. However, prior depletion of these cells had no effect on initial control of bacterial growth. Therefore, bystander T cell activation, at least for primary melioidosis, is not obligatory for host survival. It is possible, however, that, in other models of infection, bystander T cell–derived IFN-γ could constitute a greater proportion of the total IFN-γ response and may not be compensated for by other cell types.

In contrast to the functional redundancy of bystander T cell responses, we found that antigen-specific T cell responses to *B. pseudomallei* clearly contributed to resistance against *B. pseudomallei* during the later phase of infection. We believe that their protective role is directed toward macrophage activation rather than toward B cell help, given that B cell–deficient μMT mice had MSTs that were equivalent to those of wild-type control mice. Although antibody can clearly be protective against *B. pseudomallei* infection [42, 43], our data demonstrate that it is not essential for primary resistance.

Considerable effort is now being focused on the generation of vaccination and immunotherapeutic approaches to reduce the incidence of melioidosis in countries where this infection is endemic and to protect against potential bioterrorism exposure. Mouse models of melioidosis will be critical for the determination of appropriate vaccination strategies, antigen discovery, and preclinical testing of candidate vaccines. The data presented here define, for the first time (to our knowledge),

the role of T cell–mediated immunity in this model. Our results suggest that safe and effective subunit vaccines against *B. pseudomallei* should target the generation of IFN-γ–secreting T cells for optimal protection against this important disease.

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