

Analogous Cytokine Responses to *Burkholderia pseudomallei* Strains Contrasting in Virulence Correlate with Partial Cross-Protection in Immunized Mice

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Cytokine mRNA levels were assessed in *Burkholderia pseudomallei*-susceptible BALB/c mice and *B. pseudomallei*-resistant C57BL/6 mice following administration of a sublethal dose of less virulent (LV) *B. pseudomallei*, a candidate immunogen tested for protection against a highly virulent (HV) challenge. Compared on the basis of the bacterial loads, the cytokine patterns induced by HV and LV *B. pseudomallei* were similar, involving gamma interferon, interleukin-10, and other cytokines. Partial cross-protection between *B. pseudomallei* strains is shown to be associated with cytokine profiles involving both type 1 and type 2 cytokines.

Melioidosis is a potentially acute fulminating disease of humans and animals caused by the gram-negative intracellular bacterium *Burkholderia pseudomallei* (36). Infection occurs by subcutaneous inoculation of contaminated soil or surface water and can result in latent infection or a diverse range of clinical presentations (6). As recently reported by Currie et al. (8), recrudescence of disease may occur years after initial exposure, which suggests that the immune responses induced may not be effective for clearance of the organism. Differences in host and pathogen virulence factors are known to be important in determining disease severity (33). Gamma interferon (IFN- γ) plays a critical role in innate host resistance during primary infection (26), but mechanisms of adaptive immunity have not been widely studied. BALB/c mice are susceptible to infection with highly virulent (HV) *B. pseudomallei*, whereas C57BL/6 mice are relatively resistant (17). Cytokine responses following an HV *B. pseudomallei* challenge (34) are unlike those seen in other models of intracellular infection, and resistance and susceptibility do not correlate with divergent cytokine profiles commonly associated with development of distinct T helper cell subsets. Instead, early cytokine profiles are similar in BALB/c and C57BL/6 mice, with the only demonstrated difference being in the magnitude of the cytokine response (34). Responses involving a similar range of cytokines have been reported in patients (4, 11, 31).

Characterization of less virulent (LV) strains of *B. pseudomallei* (12, 13, 35) allows us to determine their potential as candidate immunogens for induction of adaptive immunity against HV *B. pseudomallei*. In models of infection with other intracellular pathogens (3, 5, 22, 27, 28, 29), cytokine responses are dependent upon the level of virulence of the challenge strain. The present study investigated the influence of *B. pseudomallei* virulence on disease progression and cytokine

responses in melioidosis. Cytokine profiling of the spleen and liver was carried out by reverse transcription-PCR, and bacterial growth in the blood, liver, and spleen was determined. Immunization experiments were performed by using LV *B. pseudomallei* as a candidate immunogen for protection against an HV challenge to analyze the relationship between cytokine responses and immunity to *B. pseudomallei*.

The *B. pseudomallei* strains used were NCTC 13178 (HV) and NCTC 13179 (LV). The virulence of the strains has been described previously (33). *B. pseudomallei* strain ATCC 23343 was used as a reference strain in immunization experiments. For cytokine studies, C57BL/6 and BALB/c mice (8 to 16 weeks old) were administered 25 CFU of either HV or LV *B. pseudomallei* in 200 μ l of phosphate-buffered saline (PBS) intravenously (i.v.). At various times following inoculation, five mice per group were euthanized with CO₂ and the bacterial load in the blood was determined (17). The liver and spleen were excised, and one half was used to determine the bacterial load (17) while the other half was stored at -80°C until RNA extraction. A second model of primary infection was established with a larger inoculum (6×10^4 CFU) of LV *B. pseudomallei*. This model allowed a comparison of HV and LV *B. pseudomallei* at equivalent bacterial loads. RNA was extracted from the liver with TRIZOL reagent (Life Technologies) (7, 34) and from the spleen with SV total RNA isolation spin columns (Promega). DNase treatment, RNA quantification, reverse transcription, and PCR with cytokine-specific primers were performed as previously described (34). For lipopolysaccharide-induced CXC chemokine (LIX), primer sequences were designed with OLIGO v. 5 software (24) and PCR parameters were optimized with a plasmid containing LIX cDNA, which was transformed into competent *Escherichia coli* strain JM109 as previously described (14, 25). The primers 5' TCC AGC TCG CCA TTC A 3' (sense) and 5' TCC GCT TAG CTT TCT TTT TG 3' (antisense) were designed to amplify a 319-bp LIX product. LIX PCR products were gel purified with a QIAQUICK gel extraction kit (Qiagen) and sequenced with Big Dye Terminator kit (Perkin-Elmer) on an

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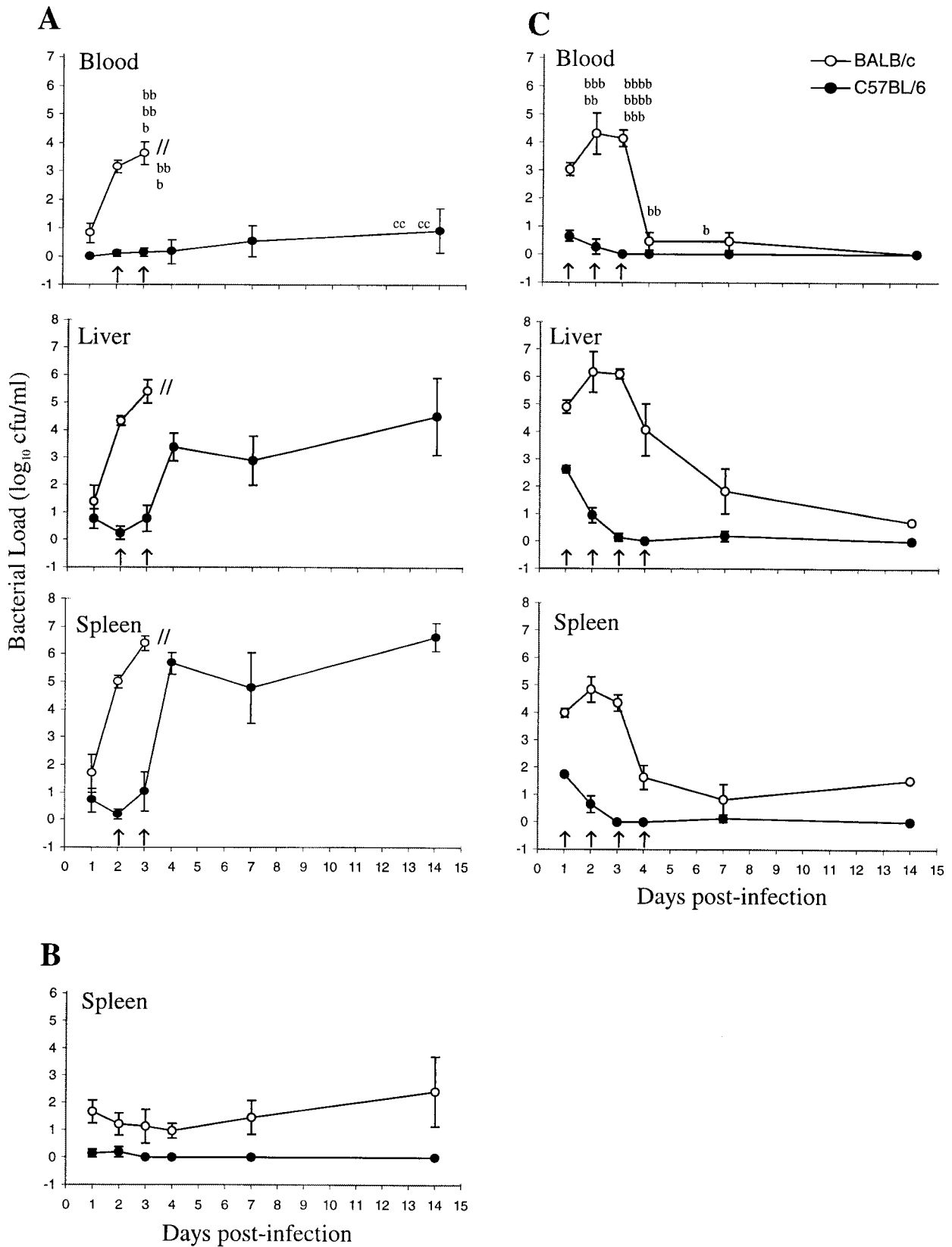


FIG. 1. Growth of HV *B. pseudomallei* NCTC 13178 (A) and LV *B. pseudomallei* NCTC 13179 (25-CFU [B] and 6×10^4 -CFU [C] challenges) in BALB/c and C57BL/6 mice. Results are shown as the mean bacterial load (\log_{10} CFU per milliliter) of five mice \pm the standard error of the mean. Significant differences ($P < 0.01$) are indicated by arrows. b, BALB/c mouse death; c, C57BL/6 mouse death.

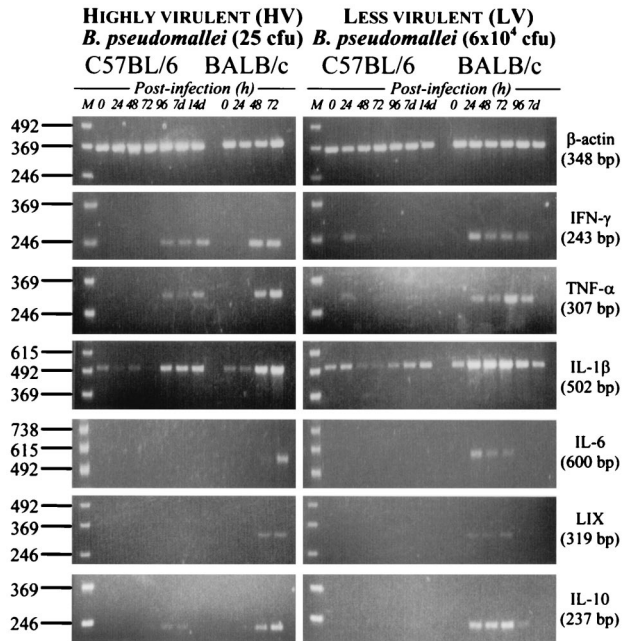


FIG. 2. Production of cytokine mRNA in livers of BALB/c and C57BL/6 mice infected i.v. with either 25 CFU of HV *B. pseudomallei* NCTC 13178 (a) or 6×10^4 CFU of LV *B. pseudomallei* NCTC 13179 (b). The values on the left are the molecular sizes of the markers in lanes M in kilodaltons.

ABI 310 sequencer (Perkin-Elmer). Sequences were checked for homology with previously described gene sequences for LIX (30). The PCR was repeated twice for three separately prepared cDNA samples.

For immunization experiments, groups of 10 mice were administered either PBS (nonimmunized) or 0.1 50% lethal dose (LD_{50}) of LV *B. pseudomallei* NCTC 13179 or reference strain ATCC 23343 (33). Two weeks later, mice were challenged with one of three different doses of HV *B. pseudomallei* NCTC 13178 (10 LD_{50} s, 1 LD_{50} , or 0.1 LD_{50}). Additional control groups received PBS (negative controls). Another series of five mice were immunized and challenged as already described and subsequently used to determine the bacterial load in the spleen at 72 h after a secondary challenge. Statistical analysis of bacterial load data was performed with a two-tailed Student *t* test for independent samples. Data are expressed as the mean \pm the standard error of the mean. Mortalities in immunized versus nonimmunized groups were analyzed by the Mann-Whitney U test. *P* values of <0.01 were considered significant.

HV *B. pseudomallei* replicated exponentially in the blood, livers, and spleens of BALB/c mice until host death at 72 h (Fig. 1A). LV *B. pseudomallei* (25 CFU) was effectively contained in all of the organs (Fig. 1B; data not shown). In mice administered 6×10^4 CFU, exponential growth of LV *B. pseudomallei* occurred in BALB/c mice until 72 h, in contrast to C57BL/6 mice, which reduced the challenge inoculum (Fig.

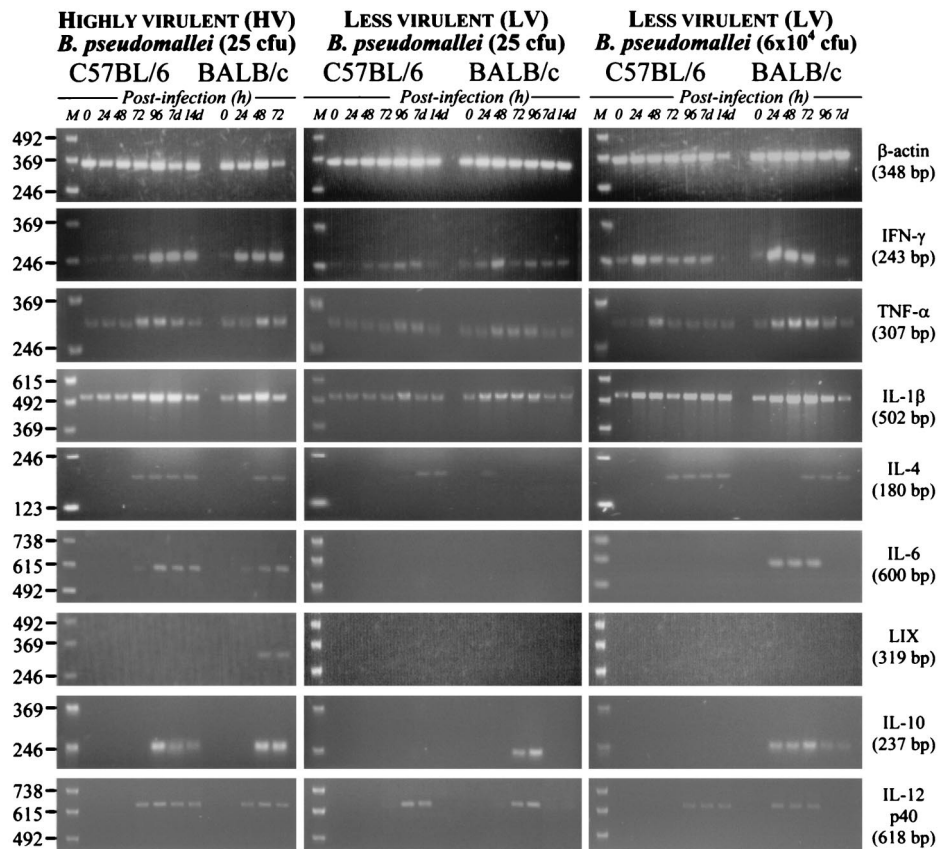


FIG. 3. Production of cytokine mRNA in spleens of BALB/c and C57BL/6 mice infected i.v. with either 25 CFU of HV *B. pseudomallei* NCTC 13178 (a), 25 CFU of LV *B. pseudomallei* NCTC 13179 (b), or 6×10^4 CFU of *B. pseudomallei* NCTC 13179 (c). The values on the left are the molecular sizes of the markers in lanes M in kilodaltons.

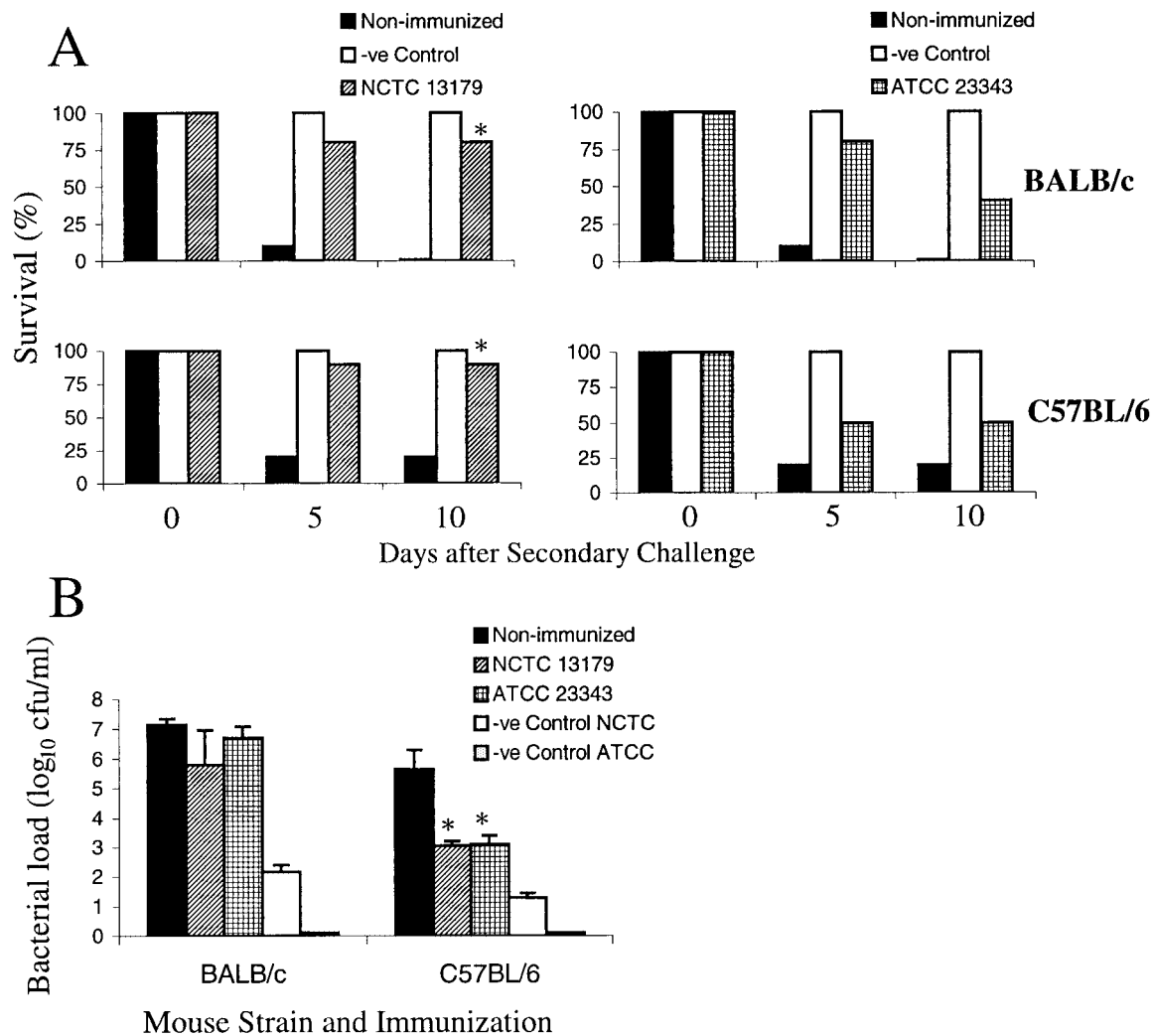


FIG. 4. LV *B. pseudomallei* NCTC 13179 and reference strain ATCC 23343 confer partial protection against HV *B. pseudomallei* NCTC 13178 (10-LD₅₀ challenge data are shown). Significant differences in mortality (A) between mice immunized with LV *B. pseudomallei* NCTC 13179 and nonimmunized controls are indicated (*, $P = 0.0001$). The bacterial loads in the spleens of mice immunized with either LV *B. pseudomallei* NCTC 13179 or ATCC 23343 and rechallenged with HV *B. pseudomallei* demonstrate partial protection rather than sterilizing immunity (B). Although the bacterial loads of immunized C57BL/6 mice were, on average, lighter than those of nonimmunized controls, these differences were not statistically significant (*, $P = 0.084$ for NCTC 13179 and $P = 0.05$ for ATCC 23343).

1C). BALB/c mice that survived the early phase of infection rapidly reduced the bacterial load (Fig. 1C). The reduction in the bacterial load of BALB/c mice that survived the high-level challenge with LV *B. pseudomallei* suggests the potential for the development of an appropriate immune response in these innately susceptible mice. Due to progressive deaths of BALB/c mice, the day 14 data (Fig. 1C) represent one mouse.

Cytokine responses in the liver were most pronounced in mice challenged with either HV *B. pseudomallei* or the larger dose of LV *B. pseudomallei* (Fig. 2). Responses in the liver involved IFN- γ , tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), IL-6, and IL-10 (Fig. 2) and correlated with the bacterial load (Fig. 1). In mice administered 25 CFU of LV *B. pseudomallei*, absence of a detectable cytokine responses in the liver was associated with a light bacterial load (data not shown). This indicates a requirement for a substantial *B.*

pseudomallei load in the liver before induction of a local cytokine responses. LIX mRNA was detected in the livers of BALB/c, but not C57BL/6, mice during infection (Fig. 2). No mRNA for IL-2 or IL-4 was detected in the livers of mice following a challenge with *B. pseudomallei* (data not shown).

In the spleen, cytokine responses involved IFN- γ , TNF- α , IL-1 β , IL-4, and IL-12 (p40 results are shown, but similar results were obtained with p35) and also correlated with the bacterial load (Fig. 1). LIX mRNA was detected in the spleens of BALB/c, but not C57BL/6, mice infected with HV *B. pseudomallei* (Fig. 3). The TNF- α and IL-12 responses in the spleens of C57BL/6 mice infected with 6×10^4 CFU of LV *B. pseudomallei* (Fig. 3) did not correlate with the bacterial load (Fig. 1). Instead, mRNAs for TNF- α , IL-12, and IL-4 in these mice were not detected until 48 to 96 h (Fig. 3), when the bacterial load was $<10^1$ CFU/ml (Fig. 1). IL-4 also demon-

strated delayed induction kinetics in the spleens of BALB/c mice challenged with 6×10^4 CFU of LV *B. pseudomallei* (Fig. 3). IL-2 mRNA was not detected in the spleens of mice of either strain (data not shown). Twenty-five CFU of LV *B. pseudomallei* induced cytokine responses in the spleen that were similar but less pronounced than those induced by HV *B. pseudomallei*. The association between the cytokine mRNAs and the bacterial loads in the spleens of these mice did not follow the same trend as that observed with HV *B. pseudomallei*. For example, responses in BALB/c mice at 48 to 96 h coincided with bacterial loads of $\leq 10^2$ CFU/ml. Equivalent bacterial loads in the liver at 48 to 96 h were not associated with cytokine responses (data not shown). In contrast to BALB/c mice, C57BL/6 mice infected with 25 CFU of LV *B. pseudomallei* demonstrated no increase in splenic IL-10 mRNA (Fig. 3). Preferential expression of IL-10 in BALB/c mice suggests a possible role for this cytokine in innate susceptibility to *B. pseudomallei* infection.

Partial immunoprotection was demonstrated in mice immunized with either NCTC 13179 or reference strain ATCC 23343 (Fig. 4A). Immunized mice did not develop symptoms of disease prior to a secondary challenge, despite the recovery of viable *B. pseudomallei* from the majority of the animals at day 14. Significantly better protection was obtained with NCTC 13179 ($P = 0.0001$) than with ATCC 23343 ($P = 0.143$). Bacterial load data from immunized mice (Fig. 4B) confirmed partial, rather than sterilizing, immunity. Bacterial loads at 72 h after a secondary challenge of mice immunized with either NCTC 13179 or ATCC 23343 were not significantly different from those of nonvaccinated controls (Fig. 4B). These studies, however, cannot discriminate which strain is the one cultured after a secondary challenge, and hence, it is possible that an HV challenge could change host immunity so that increased replication of LV *B. pseudomallei* may occur.

This study demonstrates an overall similarity between the cytokine profiles induced by *B. pseudomallei* strains with contrasting levels of virulence when compared on the basis of bacterial loads. These observations are in contrast to reports on other intracellular pathogens (2, 5, 15, 21, 27, 29). This study also demonstrates an association between these responses and partial cross-protection of mice immunized with LV *B. pseudomallei* and challenged with HV *B. pseudomallei*. Early IFN- γ and IL-12 responses in the spleens of immunized mice, combined with low-level persistence of viable LV *B. pseudomallei*, were considered potentially beneficial for the stimulation of a more effective host response for adaptive immunity against HV *B. pseudomallei*. Although immunized mice demonstrated significantly better survival after a subsequent challenge with HV *B. pseudomallei* than did nonimmunized animals, the protection was not sterilizing, as evidenced by the recovery of high numbers of bacteria from the spleen. Also, even though the cytokine patterns induced by HV and LV *B. pseudomallei* are similar, the magnitude of the response to LV *B. pseudomallei* in the spleen does not simply reflect the bacterial load within the tissue, as predicted on the basis of a model of HV *B. pseudomallei* infection (34). Although the lack of IL-2 mRNA in the spleens of mice during *B. pseudomallei* infection cannot be explained, these observations are consistent with recent reports on other intracellular pathogens (10, 18, 20).

High levels of IL-8 have been demonstrated in patients presenting with acute melioidosis (11). A related chemokine in mice is LIX (23, 30, 37). Detection of LIX in BALB/c, but not C57BL/6, mice suggests that LIX may play a role in innate susceptibility to *B. pseudomallei* infection. To our knowledge, there have been only two previous reports of LIX responses in mice following bacterial infection (19, 32). Lauw et al. (16) demonstrated a limited number of neutrophil-chemoattractant chemokines other than IL-8 in patients infected with *B. pseudomallei*. Hence, neutrophil-chemoattractant chemokines other than LIX (e.g., ENA-78) may play an important role in the immune response to *B. pseudomallei* infection. Barnes et al. (1) recently demonstrated diverse chemokine responses in mice infected with *B. pseudomallei*. The ability of *B. pseudomallei* to survive and replicate within neutrophils makes the potential role of neutrophil-chemoattractant chemokines in melioidosis particularly interesting (9, 36).

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