Cytokine Gene Expression in Innately Susceptible BALB/c Mice and Relatively Resistant C57BL/6 Mice during Infection with Virulent *Burkholderia pseudomallei*

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Production of cytokines including gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) is an important early-stage host response following infection with intracellular pathogens. Development of immunity to these pathogens is determined to a large extent by the timing and relative level of expression of the cytokines. Numerous studies have shown that early cytokine responses involving interleukin-12 (IL-12) and IFN-γ are important for resistance to intracellular pathogens, whereas responses involving IL-4 and IL-10 increase host susceptibility. These often-indistinct early cytokine responses influence the differentiation of naïve CD4$^+$ T helper cells, which later develop into what have commonly been termed Th1- and Th2-type cells. The characterization of CD4$^+$ T-helper-cell responses as Th1 or Th2 type is based largely on the cytokine profiles during the specific phase and has been used in recent years to account for the innate resistance and susceptibility of different inbred strains of mice to several intracellular pathogens. Studies investigating cytokine production in terms of CD4$^+$ T-helper-cell polarization in *Burkholderia pseudomallei* infection have not been undertaken. In this study, we used semiquantitative reverse transcription-PCR to assess induction of cytokine mRNA in liver and spleen of *B. pseudomallei*-susceptible BALB/c and relatively resistant C57BL/6 mice following infection with virulent *B. pseudomallei*. The levels of mRNA for IFN-γ, TNF-α, IL-1β, IL-6, IL-10, and IL-12 increased in both BALB/c and C57BL/6 mice 24 to 36 h after infection. A comparison of BALB/c and C57BL/6 responses revealed the relative levels of expression of mRNA for several of these cytokines, including IFN-γ, were greater in BALB/c mice, suggesting a role for endotoxic shock and cytokine-mediated immunopathology in the development of acute melioidosis. Early induction of mRNA for the cytokines classically associated with development of Th1- and Th2-type responses was absent or minimal, and induction levels in both strains of mice were similar. During the specific phase, cytokine mRNA profiles occurred as a combination of Th1- and Th2-type patterns. Collectively, these results demonstrate that cytokine mRNA responses in BALB/c and C57BL/6 mice following infection with virulent *B. pseudomallei* do not develop as polarized Th1- or Th2-type profiles. Considering the role of TNF-α and IFN-γ in the processes of endotoxic shock, these results also indicate that selected cytokines, while important for resistance to *B. pseudomallei* infection, are also potential contributors to immunopathology and the development of acute fulminating disease.

Melioidosis describes a diverse range of glanders-like clinical presentations resulting from infection with the bacterium *Burkholderia pseudomallei*. Melioidosis affects both humans and animals and was first described in Rangoon in 1911 (52). The most commonly recognized regions of endemicity are Thailand and northern Australia (16, 44). Active cases of melioidosis have also been reported in many areas where the disease is not endemic, including Africa, France, Sri Lanka, and Turkey (33). *B. pseudomallei* is an opportunistic gram-negative bacterium which exists in the environment as a saprophyte (44, 46). The organism is widely distributed in the soil and surface water of regions of endemicity, including Malaysia (46), Singapore (48), Thailand (16), and northern Australia (3). As a result, there is a high rate of infection in communities that have frequent contact with soil and surface water (16, 24). Examples include rice-farming communities in Thailand, Aboriginal communities in Australia, and Papua New Guinea/ Torres Strait Islander populations. Disease becomes particularly prevalent during periods of high rainfall, when it is

believed that the organism is physically leached from the soil, temporarily creating a more widespread distribution (24, 46). Infection is thought to occur via ingestion, inhalation, or subcutaneous inoculation of contaminated soil or surface water (24, 33). The potential for transmission of *B. pseudomallei* via insect vectors including the mosquito *Aedes aegypti*, which is common in some areas of endemicity, and the rat flea *Xenopsylia cheopsis* has been described (39). However, there is little supporting evidence for natural vector-borne transmission of *B. pseudomallei*. Unlike other soil-borne bacteria, such as *Clostridium botulinum* and *Clostridium tetani*, for which one factor (the exotoxin) is solely responsible for disease (21), *B. pseudomallei* has a broad range of virulence determinants that are likely to influence pathogenesis and clinical presentation (44). These include various exotoxins, endotoxin, malleobactin (*B. pseudomallei* siderophore), flagella, an antiphagocytic capsule, and fimbriae or pili (44, 53). The specific role of each of these virulence determinants in the pathogenesis of melioidosis remains unclear.

There is a wide spectrum of clinical presentations resulting from infection with *B. pseudomallei*. Classically, three general categories of disease are recognized: acute, subacute, and chronic (33, 44). Symptoms depend partly on where lesions are situated in the body and may include subcutaneous abscesses,

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pneumonitis, and septic arthritis (33, 44). Diagnosis is confounded by the lack of cardinal symptoms, the possible abrupt onset of clinical signs, and the potential involvement of virtually any organ in the body. This has led to melioidosis being termed "the great imitator" of all infectious diseases (33, 44). Infection may also occur asymptomatically (33, 44). The range of clinical presentations and the potential for asymptomatic infection presumably reflect differences in the route of inoculation, inoculum size, virulence of the infecting strain, and immune competence and genetic predisposition of the host. Acute septicemic melioidosis is the most severe form of disease (11, 33, 44). It is associated with a high mortality rate, often despite intensive antibiotic therapy (11, 12, 36). Acute septicemic melioidosis remains a significant cause of death due to gram-negative sepsis in some areas of endemicity (11, 12). At the opposite end of the spectrum of clinical presentations is the chronic form of disease. Chronic infection can be manifested as a localized infection in almost any organ of the body (36, 44). Similar to asymptomatic infection, chronic infection may persist for many years (12, 44). Both chronic and asymptomatic infection may intensify to an acute form of disease, particularly when the host is immunocompromised (12, 44). The potential for relapse of disease following extended periods of antibiotic therapy has also been recognized (12). Risk factors for disease include alcoholism, renal dysfunction, and diabetes mellitus (12, 33, 36). The ability of *B. pseudomallei* to lie dormant in the host for prolonged periods has been associated with an ability to survive inside phagocytes including macrophages (25, 29). This has led to the recent description of *B. pseudomallei* as a facultative intracellular bacterium (29).

In the past two decades there have been many studies on the epidemiology (3, 16), bacteriology (44), and antibiotic susceptibility (15) of *B. pseudomallei*. There have been relatively few studies on the immunopathogenesis of melioidosis, in part because of the lack of a suitably defined animal model. As a result, the interactions between *B. pseudomallei* and the host, with particular reference to the immunopathogenesis of acute versus chronic infection, remain poorly characterized. BALB/c and C57BL/6 mice have been used extensively to investigate the immunopathogenesis of several intracellular infections including listeriosis (13), leishmaniasis (23, 42), yersiniosis (4, 7), and mycobacterial infection (2, 8). In many of these BALB/c-C57BL/6 models, BALB/c mice are innately susceptible to infection whereas C57BL/6 mice are relatively resistant. Previous studies in our laboratory have demonstrated that BALB/c mice are also highly susceptible to infection with virulent *B. pseudomallei*, while C57BL/6 mice are relatively resistant (24, 32). Following intravenous (i.v.) infection of BALB/c mice with as few as 37 CFU of virulent *B. pseudomallei*, substantial bacterial growth occurs in liver and spleen, followed by an overwhelming bacteremia to which the mice succumb within 72 to 96 h (32). C57BL/6 mice do not develop bacteremia; however, bacterial growth in liver and spleen that eventually leads to a fatal outcome demonstrates incomplete resistance (32). The BALB/c-C57BL/6 model is considered to represent an excellent model for the acute and chronic forms of human melioidosis (24, 32). Recent studies by Hoppe et al. have confirmed these observations (25). The availability of this model provides an opportunity to investigate the immunopathogenesis of melioidosis in vivo and, in particular, the mechanisms that lead to the development of either acute or chronic disease.

Cytokines have been extensively studied and characterized in recent years. It is now known that selected cytokines are critical immunoregulatory determinants of disease pathogenesis and progression. Studies on a broad range of intracellular pathogens including *Listeria* (19, 27), *Leishmania* (23, 42, 43),

and *Yersinia* (4, 7) spp. and mycobacteria (8) have demonstrated that gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and several interleukins (IL) are crucial in determining the level of innate host resistance. These cytokines, which may be produced within minutes of infection (19, 51a), regulate the antimicrobial activity of macrophages and influence the interactions between macrophages and lymphocytes that have been shown to be important for effective anti-*B. pseudomallei* activity (50). These studies and others (34, 40) have resulted in the general classification of cytokine responses following intracellular infection as either Th1 or Th2 type, based on the activity of CD4⁺ T helper cells and the cytokine profiles induced. In this classification, the Th1-type cytokine profile typically involves IFN-γ, IL-2, and TNF-β, resulting in increased macrophage activation and subsequent host resistance to intracellular infection. On the other hand, a preferential Th2-type cytokine response involves IL-5, IL-6, IL-10, and IL-13, expression of which decreases macrophage activation, leading to increased host susceptibility. While divergent Th1- and Th2-type cytokine profiles typically coincide with development of T-cell specificity 4 to 5 days postinfection, it is the cytokines present during the early stages of infection that influence and ultimately determine the differentiation pathway of naïve CD4⁺ T helper cells (34, 42, 43). Early IL-12 is known to induce a predominantly Th1-type cellular response (23, 27, 30, 42, 43). In contrast, early IL-4 has been associated with increased Th2-type cell development (23, 27, 30, 42, 43). Distinct induction of polarized Th1- and Th2-type cytokine profiles was first demonstrated by Mosmann et al. in vitro (34) and later by Scott in vivo using a murine model of *Leishmania major* infection (43). The two periods of immune activation following infection, both of which are ultimately aimed at activating macrophages for killing intracellular pathogens, have been referred to as the T-cell-independent pathway (early) and the T-cell-dependent pathway (late) (2, 5, 5a). The importance of early cytokine production by natural killer (NK) cells, macrophages, and other cells such as mast cells in the former pathway has been demonstrated (5, 5a, 27, 42). It is now known that the combination of cytokines produced in the early nonspecific T-cell-independent phase, which influences the differentiation pathway of T lymphocytes in the later specific T-cell-dependent phase, is perhaps the most crucial factor that dictates the level of innate host resistance to intracellular pathogens.

Innate resistance of mouse strains such as C57BL/6 mice to numerous intracellular pathogens has been associated with increased activity of Th1-type cells and induction of a strong Th1-type cytokine response (4, 8, 23, 42). In contrast, the innate susceptibility of BALB/c mice to numerous intracellular pathogens has been linked to hypoproduction of IFN-γ and a preferential Th2-type cytokine response involving increased activity of Th2-type cells. This is because Th1-type cytokines typically stimulate cell-mediated immunity, which is important for resistance to intracellular pathogens (4, 27). Cytokines produced in the Th2-type profile are usually regarded as anti-inflammatory and are involved in humoral immunity, which is considered important for resistance to extracellular pathogens (4, 27, 42). Since the concept of CD4⁺ T-helper-cell subsets was first introduced, distinct induction of Th1- and Th2-type cytokine profiles has been demonstrated in numerous models of intracellular infection (23, 27, 42, 43). However, many studies have since described induction of nondistinct (i.e., nonpolarized Th1- and Th2-type) cytokine profiles following intracellular infection (7, 37). Recent reviews on cytokine responses and intracellular pathogens have suggested that, in terms of cytokine responses, exceptions to the polarized Th1- and Th2-

type patterns are more common than the classical paradigm predicts (1, 30). A comprehensive study investigating cytokine induction in melioidosis has not previously been undertaken.

In the present study, we used the highly specific and sensitive technique of reverse transcription-PCR (RT-PCR) to assess cytokine gene expression in BALB/c and C57BL/6 mice following infection with a virulent strain of *B. pseudomallei*. Specifically, we compared the levels of induction of mRNA for IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12 in liver and spleen of mice following infection. The cytokines assessed covered both classical Th1- and Th2-type cytokines as well as a range of proinflammatory and anti-inflammatory cytokines.

MATERIALS AND METHODS

Animals. Male and female C57BL/6 and BALB/c mice (8 to 16 weeks of age; purchased from James Cook University Small Animal Breeding Unit, Townsville, Queensland, Australia) were used. Mice were maintained in a positive-pressure environment at 23°C and were fed a pelleted protein-enriched diet, and water was provided ad libitum. Experiments were conducted according to National Health and Medical Research Council guidelines.

Bacteria. The virulent strain of *B. pseudomallei* used in this study was isolated from clinical material from a fatal case of pneumonia at the Townsville General Hospital. The isolate was cultured on Ashdown agar and was identified by colonial morphology and API 20NE (bioMeriéux, La Balme, France). The bacteria were grown in brain heart infusion broth (Oxoid, Hampshire, England) at 37°C for 18 h and were stored at −80°C. The number of viable bacteria was determined by plating serial dilutions of the suspension onto Ashdown agar and counting the colonies after 24 to 48 h of incubation at 37°C. This strain of *B. pseudomallei* has previously been characterized in terms of virulence, with a 10-day 50% lethal dose of <10 CFU in BALB/c mice (32).

Infection of mice and preparation of organs. Mice were inoculated i.v. with 2.5×10^2 CFU of virulent *B. pseudomallei* in 200 μl of phosphate-buffered saline (PBS) via the lateral tail vein. Control mice received PBS only. At 0, 24, 36, 48, and 72 h following infection, three mice were euthanized and the livers and spleens were aseptically excised. Samples were also prepared from C57BL/6 mice at 96 h, 7 days, and 14 days. Samples were immediately wrapped in aluminum foil, submerged in liquid nitrogen to prevent RNA degradation, and stored at −80°C until use.

RNA extraction and RT. Total RNA was extracted using Trizol reagent (Gibco-BRL, Life Technologies), in accordance with the manufacturer's instructions and chloroform-isoamyl alcohol purification based on methods previously described (14, 51). Ground-glass Dounce tissue homogenizers (Quantum Scientific) were used. Following precipitation with isopropanol and subsequent ethanol washes, the resultant RNA pellets were resuspended in diethyl pyrocarbonate-treated water. RNase-free DNase (Promega) was used to remove genomic DNA based on methods described by Dilworth and McCarrey (18). Total RNA was quantified using spectrophotometry at 260 nm, and the quality of the RNA was determined by the ratio of optical density at 260 nm to that at 280 nm. Ratios of greater than 1.8 were considered acceptable. Three micrograms of total RNA was reverse transcribed in a total volume of 20 μl containing 0.5 μg of oligo(dT)$_{15}$ (Promega) as the primer, 1× First Strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl$_2$; Gibco-BRL, Life Technologies), 10 mM dithiothreitol (Gibco-BRL, Life Technologies), 500 μM deoxynucleoside triphosphate (dNTP) mixture (Promega), and 200 U of Superscript II reverse transcriptase (Gibco-BRL, Life Technologies) based on methods previously described (7, 19, 22a, 51). This included an initial step of incubation at 42°C for 2 min without Superscript II, followed by the addition of Superscript II and incubation at 42°C for 50 min and then at 95°C for 4 min. After RT, 1.5 U of RNase H (Promega) was added and the mixture was incubated at 37°C for 20 min to remove any residual RNA from the reaction product. Heating cycles for RT-PCR were performed in an automated DNA thermal cycler (Bresatec; MJ Research Inc.; PTC-100). The resultant cDNA was stored at −20°C until PCR amplification.

PCR procedure. The final PCR mixture contained 2 μl of cDNA, 1× PCR buffer (1 mM Tris-HCl [pH 9.0], 5 mM KCl, 0.01% Triton X-100 [Promega]), 1 to 4.5 mM MgCl$_2$ (Promega), 200 μM dNTP mixture (Promega), 1 U of *Taq* DNA polymerase (Promega), 1 μM concentrations of (each) sense and antisense primers, and sterile water to 100 μl. Cytokine-specific primers were synthesized by Gibco-BRL, Life Technologies, according to sequence designs previously described (7, 19, 35, 40) (Table 1). The optimal MgCl$_2$ concentration for each primer pair were determined experimentally. The thermal cycling parameters were as follows: 95°C for 2 min, followed by 30 cycles each of denaturation at 94°C for 50 s, annealing of primer and fragment at 60°C for 50 s, and primer extension at 72°C for 1 min. A final extension of 72°C for 4 min was included. In preliminary experiments, amplification for 30 cycles was shown to lie in the linear portion of the curve for the amount of PCR product produced. PCR products were visualized by UV light after electrophoresis through a 2.0 to 2.5% agarose gel containing 0.5 μg of ethidium bromide/ml. As the DNA size marker, a 123-bp ladder (Gibco-BRL, Life Technologies) was used. Analysis was performed using

TABLE 1. Cytokine-specific primer pair sequences used in PCR

Cytokine	Oligonucleotide sequence (5′–3′)[a]	Size (bp)[b]	Reference
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C TAA AAC GCA GCT CAG TAA CAG TCC G	348	35
IFN-γ	AGC GGC TGA CTG AAC TCA GAT TGT AG GTC ACA GTT TTC AGC TGT ATA GGG	243	35
TNF-α	GGC AGG TCT ACT TTG GAG TCA TTG C ACA TTC GAG GCT CCA GTG AAT TCG G	307	35
IL-1β	TCA TGG GAT GAT GAT GAT AAC CTG CT CCC ATA CTT TAG GAA GAC ACG GAT T	502	19
IL-2	TGA TGG ACC TAC AGG AGC TCC TGA G GAG TCA AAT CCA GAA CAT GCC GCA G	167	35
IL-4	CGA AGA ACA CCA CAG AGA GTG AGC T GAC TCA TTC ATG GTG CAG CTT ATC G	180	35
IL-6	CTG GTG ACA ACC ACG GCC TTC CCT A ATG CTT AGG CAT AAC GCA CTA GGT T	600	19
IL-10	ACC TGG TAG AAG TGA TGC CCC AGG CA CTA TGC AGT TGA TGA AGA TGT CAA A	237	7
IL-12(p35)	GCA AGA GAC ACA GTC CTG GG TGC ATC AGC TCA TCG ATG GC	618	40
IL-12(p40)	GAG GTG GAC TGG ACT CCC GA CAA GTT CTT GGG CGG GTC TG	618	40

[a] For each primer pair, the sense sequence is shown above the antisense sequence.
[b] Predicted size of the PCR product.

a GELDOC 1000 system and Molecular Analyst software. PCR using primers for β-actin was performed on each individual sample as an internal positive-control standard. As a negative control, PCR omitting cDNA (water as the substitute) was run concurrently. PCR using primers for β-actin was also performed using non-reverse-transcribed total RNA for each sample to confirm that amplification was based solely on cDNA. PCR-assisted mRNA amplification was repeated twice for three separately prepared cDNA samples. Results shown are representative of triplicate cDNA samples.

RESULTS

Cytokine mRNA levels for IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12 in liver of BALB/c and C57BL/6 mice following infection with virulent *B. pseudomallei* are shown in Fig. 1. Results for spleen are shown in Fig. 2. The top panel of each figure shows bright, size-specific bands (348 bp) of constant intensity for the anticipated β-actin-specific cDNA product. The β-actin gene is expressed at a relatively constant level in cells and is commonly used in semiquantitative RT-PCR systems to assess the relative efficiency of each individual PCR. These results confirm the semiquantitative nature of the method and allow comparison of the kinetics of cytokine mRNA production over time. The absence of PCR product from PCR using primers for β-actin and water instead of cDNA, which were concurrently processed with each PCR run, ensured a lack of contaminating exogenous DNA. Since DNA polymerase amplifies both cDNA and genomic DNA with equal efficiencies, the absence of PCR product from PCR using non-reverse-transcribed total RNA and primers for β-actin confirmed that the amplification was based solely on cDNA (18).

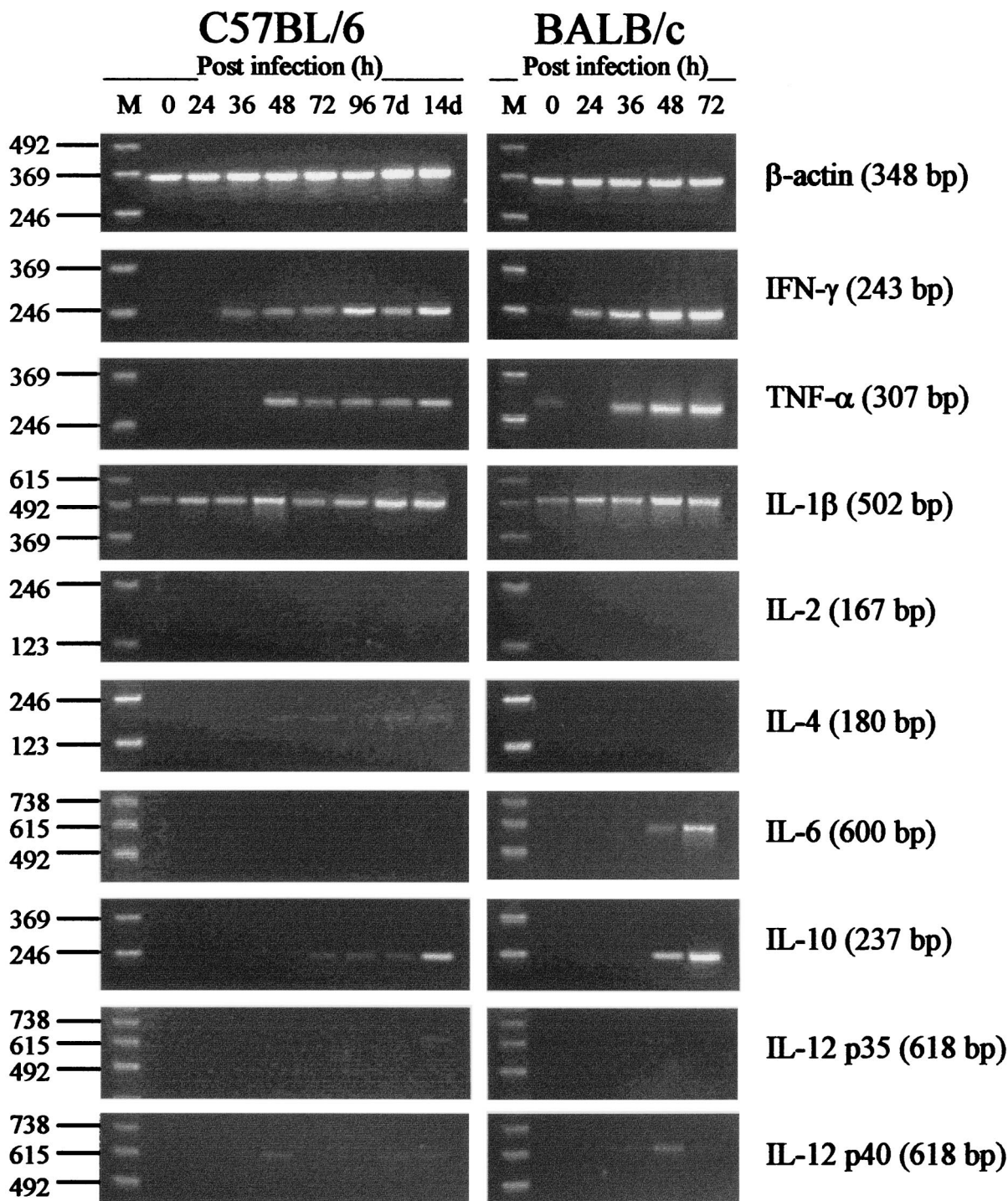In liver, baseline levels of mRNA for the cytokines assayed

FIG. 1. Cytokine mRNA responses in liver of C57BL/6 and BALB/c mice infected i.v. with 2.5×10^2 CFU of virulent *B. pseudomallei*. At various times after infection (0 to 96 h and 7 and 14 days), liver was excised and total RNA was extracted. Genomic DNA was removed, and cDNA was subjected to PCR using cytokine-specific primers. Molecular weight markers (M) are shown in the left lane of each gel. Data are representative of three mice at each time point.

were minimal. The only cytokine mRNA detected in uninfected mice was low levels of mRNA for IL-1β and, in BALB/c mice, very low levels of mRNA for TNF-α. Increases in the amounts of mRNA for several cytokines were detected in liver of both BALB/c and C57BL/6 mice 24 to 36 h after infection. In BALB/c mice, production of large amounts of mRNA was

observed for both IFN-γ and TNF-α. Induction of mRNA for these cytokines was rapid, beginning at 24 h after infection and peaking at 48 to 72 h, prior to host death. Increases in the amounts of mRNA for IL-1β and IL-6 were also observed in BALB/c mice 36 to 48 h after infection. Among the other cytokines assayed, only an increase in IL-10 mRNA at 48 to
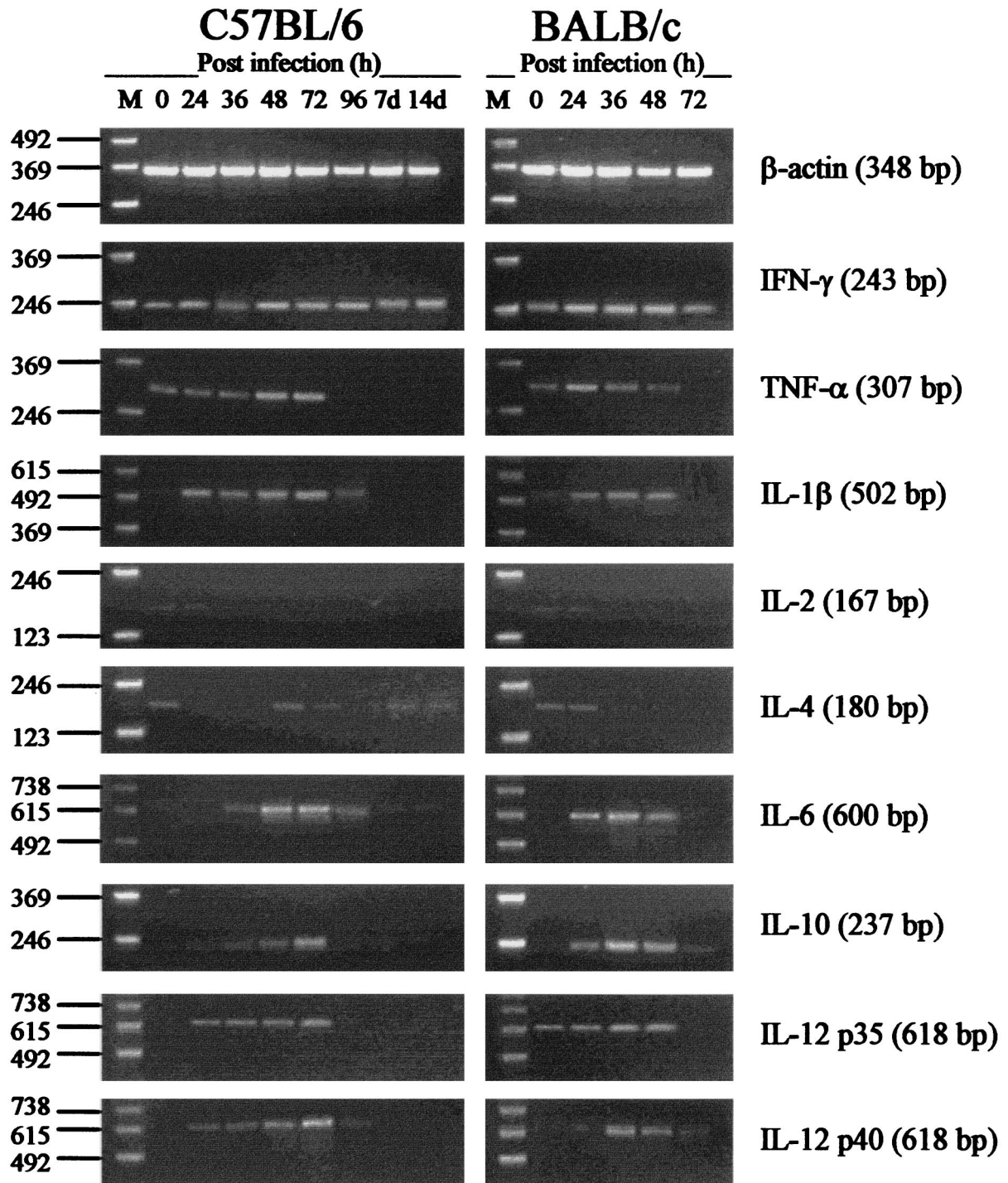
FIG. 2. Cytokine mRNA responses in spleen of C57BL/6 and BALB/c mice infected i.v. with 2.5×10^2 CFU of virulent *B. pseudomallei*. At various times after infection (0 to 96 h and 7 and 14 days), spleen was excised and total RNA was extracted. Genomic DNA was removed, and cDNA was subjected to PCR using cytokine-specific primers. Molecular weight markers (M) are shown in the left lane of each gel. Data are representative of three mice at each time point.

72 h in BALB/c mice was detected. No increase in the amount of mRNA for IL-12, IL-4, or IL-2 was detected in BALB/c mice. In liver of C57BL/6 mice, increases in the amounts of mRNA for IFN-γ, TNF-α, and IL-1β were observed 48 to 72 h after infection, though the increases were less marked than BALB/c responses. Low levels of mRNA for IL-10 were also

detected during the later stages of infection in C57BL/6 mice. No mRNA for the other cytokines assayed was detected in liver of C57BL/6 mice at any time point.

In spleen, low baseline levels of mRNA for IFN-γ and TNF-α were detected in both BALB/c and C57BL/6 mice. Very low baseline levels of mRNA for IL-1β and IL-4 were

also detected in both strains of mice. Detectable levels of mRNA for IL-12p35 were observed in spleen of uninfected BALB/c mice. Increases in mRNA for IFN-γ were observed in spleen of both BALB/c and C57BL/6 mice 24 to 36 h following infection. These increases peaked at 24 to 48 h in BALB/c mice and at 48 to 72 h in C57BL/6 mice. In C57BL/6 mice, a moderate increase in the level of mRNA for IFN-γ persisted in spleen for the duration of the assay period (14 days). Minor-to-moderate increases in the levels of mRNA for TNF-α, IL-1β, IL-6, IL-12, and IL-10 were detected 24 to 48 h after infection in both mouse strains. The majority of these mRNA increases peaked at 24 to 48 h in BALB/c mice and at 48 to 72 h in C57BL/6 mice. The levels of mRNA for most of these cytokines were minimal or undetectable at 96 h and thereafter. No increase in the level of mRNA for IL-2 was detected in spleen of either BALB/c or C57BL/6 mice at any time point. Low levels of mRNA for IL-4 were detected in C57BL/6 mice during the later stages of infection.

DISCUSSION

B. pseudomallei is able to survive and multiply within mammalian cells (25, 29). However, the role of intracellular parasitism in the immunopathogenesis of melioidosis remains unknown. In recent years, BALB/c-C57BL/6 murine models have been used extensively to investigate the immunopathogenesis of several intracellular infections, including listeriosis (13), leishmaniasis (23, 42), yersiniosis (4, 7), and mycobacterial infection (2, 8). These studies and others (19, 27, 34, 40, 43) have demonstrated that production of selected cytokines and development of distinct Th1- and Th2-type cytokine profiles are critical in determining the pathway of disease progression and the level of innate host resistance to intracellular pathogens. More-extensive studies on a broader range of intracellular pathogens have revealed that cytokine cascades induced by intracellular organisms are highly specific for the infecting agent and often do not develop as classical polarized Th1- and Th2-type patterns (1, 7, 30, 37). As is the case for numerous other intracellular pathogens, BALB/c mice have been shown to be highly susceptible to infection with virulent *B. pseudomallei* while C57BL/6 mice are relatively resistant (24, 32). The BALB/c-C57BL/6 murine model has been partially characterized in terms of the level of innate host resistance to virulent *B. pseudomallei* and is becoming increasingly popular as a means of investigating the pathogenesis of melioidosis in vivo (24, 25, 32, 51). The purpose of this study was to investigate the cytokine cascades induced by virulent *B. pseudomallei* in BALB/c and C57BL/6 mice to determine the role of early cytokine production and CD4$^+$ T-helper-cell polarization in determining the pathway of disease progression in this model. We conducted a comprehensive PCR-assisted mRNA analysis of cytokine induction in liver and spleen of BALB/c and C57BL/6 mice in both the early and later stages of infection with virulent *B. pseudomallei*. We assessed a broad range of cytokines, IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12, to determine the roles of both classical Th1- and Th2-type cytokines and a range of proinflammatory and anti-inflammatory cytokines in the development of acute versus chronic murine melioidosis.

Preliminary studies in our laboratory correlated high levels of mRNA for the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in liver during the early stages of infection with development of acute disease in BALB/c mice (51). These studies demonstrated minor increases in the levels of mRNA for TNF-α and IL-1β in C57BL/6 mice. The results of the present study confirm the association between high levels of

mRNA for TNF-α, IL-1β, and IL-6 in liver and development of acute *B. pseudomallei* infection in BALB/c mice (Fig. 1). This study also demonstrates moderate increases in the levels of mRNA for TNF-α and IL-1β in liver during the later stages of infection in C57BL/6 mice (Fig. 1). Similar trends in the production of IFN-γ were observed in liver, where mRNA for IFN-γ was detected at much higher levels in BALB/c mice in the early stages of infection than in C57BL/6 mice (Fig. 1). The moderate increase in the level of mRNA for IFN-γ that was detected during the early stages of infection in C57BL/6 mice persisted for the duration of the assay period (14 days; Fig. 1). Several studies have demonstrated a genetic predisposition of BALB/c and C57BL/6 mice to be low- and high-IFN-γ responders, respectively (8, 17, 28). The general classification of BALB/c and C57BL/6 mice as low- and high-IFN-γ responders has been used in recent years to account for the innate susceptibility and resistance of these mouse strains to numerous intracellular pathogens (4, 7, 8, 17). The results of the present study demonstrate that this is not the case for *B. pseudomallei* infection. Instead, this study indicates that production of IFN-γ, TNF-α, and other cytokines in melioidosis represents a critical balance between responses that are important for host resistance and responses that can contribute heavily to immunopathology, depending on the timing and relative level of expression of cytokines. While sufficient cytokine responses involving IFN-γ appear to be important for resistance to *B. pseudomallei* (C57BL/6 mice; Fig. 1), hyperproduction of selected cytokines is associated with development of acute fulminating disease (BALB/c mice; Fig. 1).

In this study, cytokine mRNA responses in spleen were less marked than responses in liver. Increases in the levels of mRNA for IFN-γ, TNF-α, IL-1β, and IL-6 were observed in spleen of both BALB/c and C57BL/6 mice during the early stages of infection (Fig. 2). The levels of induction of mRNA for these cytokines in spleen of both BALB/c and C57BL/6 mice were similar, although peak levels generally occurred earlier in BALB/c mice (24 to 36 h) than in C57BL/6 mice (48 to 72 h; Fig. 2). The results of the present study confirm production of mRNA for IL-6 in liver of BALB/c mice but not C57BL/6 mice (Fig. 1). The potential contribution of hepatocytes to IL-6 production in melioidosis is an interesting possibility, particularly when considered in terms of the potential intracellular interactions between *B. pseudomallei* and the host cell. Increased levels of mRNA for IL-6 were observed in spleen of both mouse strains (Fig. 2). These results indicate that the importance of IL-6 as a synergistic factor in determining the progression of acute disease in BALB/c mice, as suggested in a previous study (51), may be limited. However, it is important to consider the amount of IL-6 produced in toto. The results of the present study suggest that this is greater in BALB/c mice. Hence, the potential contribution of IL-6 to the development of acute disease in BALB/c mice cannot be completely excluded.

IL-12, produced by macrophages after interaction with bacteria or parasites, is a heterodimer consisting of the subunits p35 (35 kDa) and p40 (40 kDa) (4a). Although p35 and p40 are encoded by separate genes and are independently regulated, cells must express both subunits to generate the bioactive form of IL-12 (4a). Minor increases in the levels of mRNA for IL-12 (both p35 and p40 subunits) were observed in spleen of both BALB/c and C57BL/6 mice during the early stages of infection with virulent *B. pseudomallei* (Fig. 2). Because these responses were minimal and similar in both strains of mice, it is reasonable to suggest a limited role for IL-12 in determining the pathway of disease progression in this model. However, reduction of IL-12 bioactivity by monoclonal antibodies in a recent

study on murine melioidosis by Santanirand et al. (41a) resulted in increased host susceptibility to *B. pseudomallei* infection, suggesting an important role for IL-12 in melioidosis. It should be noted, however, that these studies were based on the use of a dissimilar murine model and used a strain of *B. pseudomallei* different from the one used in this study. Furthermore, the authors did not assess cytokine responses per se. The potency of IL-12 and the known complexity in the regulation of cytokine genes, particularly for IL-12 (4a), must be considered when interpreting results of cytokine transcriptional studies.

Moderate increases in the levels of mRNA for IL-10 were observed in spleen of both BALB/c and C57BL/6 mice during the early stages of infection (Fig. 2). As observed with other cytokines assayed, levels of mRNA for IL-10 peaked in BALB/c mice earlier than in C57BL/6 mice (Fig. 2). Increased levels of mRNA for IL-10 were also detected in liver of both mouse strains during the later stages of infection (Fig. 1). Because IL-10 is a potent anti-inflammatory cytokine, it is possible that IL-10 has an important suppressive immunoregulatory role in the early stages of *B. pseudomallei* infection. Among its many activities, IL-10 inhibits the generation of nitrogen radicals in macrophages and suppresses proinflammatory cytokine production (37, 45). In view of the results of the present study, it may be argued that the earlier appearance of IL-10 in spleen of BALB/c mice could lead to a preferential Th2-type cytokine response, diminished cell-mediated immunity, and increased host susceptibility. However, concurrent up-regulation of IL-12, IFN-γ, and TNF-α combined with a lack of IL-4 during the same period argues against the development of classical polarized Th1- and Th2-type profiles. Simultaneous increases in the levels of mRNA for IL-10 and IFN-γ, as observed in this study, are consistent with recent reports on cytokine responses to other intracellular pathogens (7, 19, 37). Such studies have suggested a potential beneficial role of IL-10 for increased host resistance during intracellular infection, despite the known anti-inflammatory actions of this cytokine (45). A cytokine pattern involving high levels of IL-10 and IFN-γ without an increase in IL-2 or IL-4 has recently been coined the Tr1-type profile (22, 30). While the results of the present study do appear to "fit" this pattern more closely than the classical polarized Th1- and Th2-type profiles, we suggest that such a classification could serve to oversimplify the complex nature of T-cell responses occurring in vivo. Description of other prominent cytokine patterns and T-cell subsets (e.g., Th3 profile [reviewed in reference 30]; Th0 cells [reviewed in reference 29a]) adds to the difficulty in interpreting cytokine profiles that are not polarized, particularly when considering such a broad range of individual cytokines. As recently suggested by Kelso, T-cell cytokine profiles following infection most likely form a continuous spectrum in both levels and combinations, in which polarized Th1- and Th2-type profiles merely represent possible extreme poles of the spectrum (29a, 30).

It is important to consider the role of virulence in the determination of cytokine profiles (31, 38). In this study, we used a highly virulent strain of *B. pseudomallei*, as determined by the 50% lethal dose in BALB/c mice (32). Other studies have demonstrated that cytokine production in vivo is regulated to a large extent by the virulence of the infecting strain of pathogen (31, 38). Highly virulent *Listeria monocytogenes* and nonvirulent *L. monocytogenes* invoke different cytokine profiles (31, 38). There is increasing evidence to suggest that particular bacterial virulence factors such as exotoxins are potent modulators of cytokine responses. Examples include pneumolysin of *Streptococcus pneumoniae* (26) and listeriolysin O of *L. mono-*

cytogenes (31). Virulence factors such as these may help to explain the link between strain virulence and induction of distinct cytokine profiles. The role of virulence in determining cytokine profiles in melioidosis has yet to be investigated.

In terms of cytokine responses in patients infected with *B. pseudomallei*, few studies have been conducted. Initial studies by Brown et al. correlated high levels of urinary neopterin and serum IFN-γ with acute clinical presentation and fatal outcome in melioidosis (9, 10). These studies suggested that severe melioidosis involves impairment of specific T-cell-mediated immunity and induction of nonspecific and ineffective T-cell responses generated predominantly by CD4+ T cells. It is interesting to consider these implications in relation to the known inhibitory actions of the Tr1-type cytokine profile on the generation of specific T-cell-mediated immunity (22). A potential role for TNF-α in the development of acute melioidosis was later demonstrated by Suputtamongkol et al. (47). The description of IL-6 as a potential predictor of mortality in *B. pseudomallei* sepsis by Friedland et al. (20) is another demonstration of the importance of proinflammatory cytokine hyperproduction in melioidosis. While these studies have investigated production of cytokines on a limited basis, the present study represents the first comprehensive analysis of the role of a broad range of cytokines in melioidosis. The results of this study demonstrate that the trends in cytokine production occurring in the BALB/c-C57BL/6 murine model parallel the few known cytokine responses occurring in human melioidosis. High levels of mRNA for IFN-γ, TNF-α, and IL-6 coincide with the development of acute disease in BALB/c mice. In C57BL/6 mice, moderate increases in the levels of mRNA for these cytokines correlate with development of chronic disease. Response trends observed in BALB/c mice are similar to those reported in patients acutely infected with *B. pseudomallei* and demonstrate the importance of cytokine hyperproduction in acute melioidosis. The cytokine responses observed in C57BL/6 mice demonstrate the importance of production of selected cytokines including IFN-γ at sufficient levels in chronic melioidosis. These findings are in accordance with those of Santanirand et al. (41, 41a), who recently demonstrated an important role for IFN-γ in resistance to *B. pseudomallei* in a murine model. However, the reduction of cytokine bioactivity by monoclonal antibodies in previous studies could provide little insight into the actual immune mechanisms (responses) operating in acute versus chronic melioidosis. Finally, the results of the present study demonstrate that early cytokine responses in the BALB/c-C57BL/6 murine model following infection with virulent *B. pseudomallei* do not develop as polarized Th1- and Th2-type profiles. These results are consistent with recent reviews on the complex nature of cytokine responses following infection with intracellular pathogens. While not unexpected, the results of the present study are inconsistent with preliminary suggestions by others of skewed Th1- and Th2-type profiles in melioidosis (25). It is important to note however, that the previous implication of CD4+ T-helper-cell switching in melioidosis was based on antibody isotype responses and not cytokine profiles per se (25). Furthermore, the previous study used a strain of *B. pseudomallei* of lower virulence than the strain used in this study, which may influence Th1- and Th2-type cytokine profiles.

In several animal models of endotoxemia, monoclonal antibodies to specific cytokines have been used to reduce cytokine-mediated immunopathology (6, 49). Similar approaches for control of cytokine hyperproduction in BALB/c mice during infection with virulent *B. pseudomallei* may prove efficacious. However, because an increase in the transcriptional activity of cytokine genes does not necessarily translate into release of

functional cytokine gene products, investigation into the bioactivity and levels of specific cytokines in serum in the BALB/c-C57BL/6 murine model is required prior to any attempts of immunomodulation. The use of immunotherapy for melioidosis as an adjunct to antibiotic therapy, particularly for the acute septicemic form of disease, remains a possibility.

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