The Journal of Immunology

γδ-T Cells Are Critical for Survival and Early Proinflammatory Cytokine Gene Expression During Murine *Klebsiella* Pneumonia

Thomas A. Moore, Bethany B. Moore, Michael W. Newstead and Theodore J. Standiford

J Immunol 2000; 165:2643-2650; ;
doi: 10.4049/jimmunol.165.5.2643
http://www.jimmunol.org/content/165/5/2643

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

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

γδ-T Cells Are Critical for Survival and Early Proinflammatory Cytokine Gene Expression During Murine *Klebsiella* Pneumonia[1]

Thomas A. Moore,[2] Bethany B. Moore, Michael W. Newstead, and Theodore J. Standiford

Although cells of the innate inflammatory response, such as macrophages and neutrophils, have been extensively studied in the arena of Gram-negative bacterial pneumonia, a role for T cells remains unknown. To study the role of specific T cell populations in bacterial pneumonia, mice deleted of their TCR β- and/or δ-chain were intratracheally inoculated with *Klebsiella pneumoniae*. γδ T cell knockout mice displayed increased mortality at both early and late time points. In contrast, mice specifically lacking only αβ-T cells were no more susceptible than wild-type mice. Pulmonary bacterial clearance in γδ-T cell knockout mice was unimpaired. Interestingly, these mice displayed increased peripheral blood dissemination. Rapid up-regulation of IFN-γ and TNF-α gene expression, critical during bacterial infections, was markedly impaired in lung and liver tissue from γδ-T cell-deficient mice 24 h postinfection. The increased peripheral blood bacterial dissemination correlated with impaired hepatic bacterial clearance following pulmonary infection and increased hepatic injury as measured by plasma aspartate aminotransferase activity. Combined, these data suggest that mice lacking γδ-T cells have an impaired ability to resolve disseminated bacterial infections subsequent to the initial pulmonary infection. These data indicate that γδ-T cells comprise a critical component of the acute inflammatory response toward extracellular Gram-negative bacterial infections and are vital for the early production of the proinflammatory cytokines IFN-γ and TNF-α. *The Journal of Immunology*, 2000, 165: 2643–2650.

Klebsiella pneumoniae is a leading cause of morbidity and mortality in community-acquired and nosocomial bacterial pneumonia (1–3). The primary host defense mechanism within the lung during bacterial pneumonia is the rapid clearance of the invading bacteria from the respiratory tract (reviewed in Ref. 4). Although the lung microenvironment contains several distinct leukocyte populations, the first line of defense during pulmonary infection is the alveolar macrophage. In response to pulmonary challenge with bacteria, alveolar macrophages secrete a variety of cytokines and chemokines capable of recruiting and activating blood neutrophils and monocytes into the pulmonary microenvironment. The host innate immune cells and cytokines involved in their activation/recruitment have been extensively studied (reviewed in Refs. 5 and 6); however, less is known about the role of T cells during Gram-negative bacterial pneumonia.

An emerging field of interest is the interaction between cells of the innate and acquired immune response during pathogenic insult (7–10). T cells expressing the αβ-TCR complex comprise the majority of peripheral T cells (~90%), whereas γδ-TCR-expressing cells are in the minority (~10%). αβ-T cell-mediated immunity has been defined in recent years according to the profile of cytokines produced and the corresponding immune response generated (11, 12). Th1 cells produce IL-2, IL-12, and IFN-γ but not IL-4, IL-5, or IL-10. Th1 responses result in cell-mediated immunity such as delayed-type hypersensitivity and macrophage activation. In contrast, Th2 T cells produce IL-4, IL-5, and IL-10 but not IL-2, IL-12, or IFN-γ. The resultant immune response promotes humoral immune responses. Interestingly, recent studies have indicated that γδ-T cell clones can also be segregated into "Th1" or "Th2" classifications, with a bias toward production of Th1 cytokines (13, 14).

Although αβ-T cells have been shown to be important in a variety of infection models (15–18), less is known about the role of γδ-T cells during infection. The role of γδ-T cells during infection appears to vary depending on the pathogenic model studied. A protective role for γδ-T cells has been shown in several models, particularly in the setting of intracellular pathogens such as *Toxoplasma* (19) and *Listeria* infection (20, 21). Conversely, mice deficient in γδ-T cells have increased resistance to i.p. infection with *Salmonella choleraesuis* (22).

To study the role of specific T cell populations in Gram-negative bacterial pneumonia, mice deleted of their TCR β- and/or δ-chain by homologous recombination were intratracheally inoculated with *K. pneumoniae*. Mice specifically lacking γδ-T cells have increased susceptibility to pulmonary bacterial challenge when compared with αβ-T cell knockout and wild-type mice. γδ-T cell knockout mice have significantly impaired early expression of pulmonary and hepatic IFN-γ and TNF-α mRNA following *K. pneumoniae* infection, increased peripheral blood bacterial dissemination, and increased hepatic bacterial burden subsequent to the initial pulmonary infection. These data detail a heretofore unrecognized critical role for γδ-T cells during acute, extracellular bacterial infections and further support the concept that γδ-T cells bridge host innate and acquired immune responses.

Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109

Received for publication January 11, 2000. Accepted for publication June 7, 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 research was supported in part by a research grant from the American Lung Association (to T.A.M.) and Grants HL57243, HL58200, and P50HL60289 (to T.J.S.) from the National Institutes of Health. T.A.M. is a Parker B. Francis Fellow in Pulmonary Research and an Edward Livingston Trudeau Fellow of the American Lung Association.

[2] Address correspondence and reprint requests to Dr. Thomas A. Moore, University of Michigan Medical Center, Division of Pulmonary and Critical Care Medicine, 6301 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0642. E-mail address: tmoore@umich.edu

0022-1767/00/$02.00

Materials and Methods

Animals

C57BL/6J-Tcrb (αβ-T cell deficient), C57BL/6J-Tcrd (γδ-T cell deficient), C57BL/6J-TcrbTcrd (αβ/γδ-T cell deficient), and C57BL/6J wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions within the animal care facility at the University of Michigan (Unit for Laboratory Animal Medicine) until the day of sacrifice.

K. pneumoniae *inoculation*

K. pneumoniae strain 43816 serotype 2 (American Type Culture Collection, Manassas, VA) was grown in trypic soy broth (Difco, Detroit, MI) overnight at 37°C. Bacterial concentration was determined by measuring the amount of absorbance at 600 nm and compared with a predetermined standard curve. Bacteria were then diluted to the desired concentration for intratracheal inoculation. Mice were anesthetized with pentobarbital (diluted 1:7 in saline). The trachea was exposed, and 30 μl inoculum or saline was administered via a sterile 26-gauge needle. An aliquot of the inoculated *K. pneumoniae* suspension was serially diluted onto blood agar plates to determine actual dose of intratracheally injected bacteria.

Whole lung or liver homogenization for CFU and myeloperoxidase (MPO)[3] analyses

At designated time points, the mice were euthanized by inhalation of CO_2. The lungs or liver were perfused with 1–2 ml PBS/5 mM EDTA and removed for analyses as previously described (23). Briefly, organs were homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK) in 1 ml PBS/complete protease inhibitor mixture (Boehringer Mannheim Biochemical, Chicago, IL). For organ CFU determination, a small aliquot of tissue homogenate was serially diluted and plated on blood agar plates, incubated at 37°C, and colonies counted.

Lung MPO activity, as an indirect measurement of total neutrophil numbers, was quantitated by a method as described previously (23). Briefly, 100 μl lung homogenate was mixed with 100 μl MPO homogenization buffer (0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA) and vortexed. The mixture was sonicated and centrifuged at 12,000 × *g* for 15 min. The supernatant was then mixed 1:15 with assay buffer and read at 490 nm. MPO units were calculated as the change in absorbance over time.

Peripheral blood CFU analyses

For determination of peripheral blood dissemination, heparinized blood was collected by cardiac puncture at the indicated time points. Serial dilutions were plated onto blood agar plates, incubated at 37°C, and colonies counted.

Total lung and liver leukocyte isolation

Total lung and liver leukocytes were isolated as previously described (24). Briefly, tissue was minced with scissors to a fine slurry in 15 ml/lung digestion buffer (RPMI 1640/5% FCS/1 mg/ml collagenase (Boehringer Mannheim Biochemical)/30 μg/ml DNase (Sigma, St. Louis, MO). Tissue slurries were enzymatically digested for 30 min at 37°C. Any undigested fragments were further dispersed by drawing the solution up and down through the bore of a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and spun (3000 rpm) through a 20% Percoll gradiant to enrich for leukocytes before further analysis. Liver leukocytes were prepared following the same procedure as for lung leukocytes with the following modification: cells were spun at a lower speed (1500 rpm) through a 35% Percoll gradient. Cell counts and viability were determined using trypan blue exclusion counting on a hemacytometer. Cytospin slides were prepared and stained with a modified Wright-Giemsa stain.

Multiparameter flow cytometric analyses

Total lung and liver leukocytes were isolated as described above. For analyses of T cell subsets, isolated leukocytes were stained with biotinylated anti-γδ-TCR or anti-αβ-TCR and anti-CD4-FITC plus anti-CD8-FITC. TCR expression was detected by the addition of streptavidin-PE (all reagents from PharMingen, San Diego, CA, unless otherwise noted). In addition, cells were stained with anti-CD45-Tricolor (Caltag Laboratories, South San Francisco, CA), allowing discrimination of leukocytes from nonleukocytes and thus eliminating any nonspecific binding of T cell surface markers on nonleukocytes. T cell subsets were analyzed by first gating on CD45-positive "lymphocyte sized" leukocytes, then examined for FL1 and FL2 fluorescence expression. Cells were collected on a FACScan or FACScalibur cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). Analyses of data were performed using the CellQuest software package. Percent positive cells indicated in histogram plots represent the percentage of positive cells back-calculated to total leukocytes.

Isolation and RT-PCR amplification of whole lung mRNA

Whole lung or liver (2 lobes) was harvested at the indicated time points, immediately "snap frozen" in liquid nitrogen, then stored at −70°C for further analyses. Total cellular RNA from the frozen tissue was isolated by homogenizing in 3 ml TRIzol Reagent (Life Technologies, Gaithersburg, MD) following the TRIzol protocol. Total RNA was determined by spectrometric analysis at 260 nm wavelength. IFN-γ, TNF-α, and β-actin mRNA expression was determined by RT-PCR using the Access RT-PCR system kit from Promega (Madison, WI) following the manufacturer's protocol. The following primer pairs (all primers 5′ → 3′) were used for specific mRNA amplification: mouse (m) IFN-γ sense, GGC TGT TTC TGG CTG TTA CTG CCA CG; mIFN-γ antisense, GAC AAT CTC TTC CCC ACC CCG AAT CAG; mTNF-α sense, CCT GTA GCC CAC GTC GTA GC; mTNF-α antisense, AGC AAT GAC TCC AAA GTA GAC C; mβ-actin sense, CTT CTA CAA TGA GCT GCG TGT G; mβ-actin antisense, GAT TCC ATA CCC AAG AAG GAA GG. cDNA products were detected on a 2% agarose gel containing ethidium bromide and bands visualized and photographed using UV transillumination.

Southern hybridization analyses

RT-PCR agarose gels were transferred in 0.4N NaOH onto Zetaprobe membrane (Bio-Rad, Richmond, CA). The following antisense internal probes specific for amplified cDNA products were used: mIFN-γ, GAG ATA ATC TGG CTC TGC AGG; mTNF-α, GCT CAG CCA CTC CAG CTG CTC C; mβ-actin, GCC TGG ATG GCT ACG TAC ATG GC. Southern filter hybridization was performed by incubating membranes with ^{32}P end-labeled oligonucleotide internal probes diluted in hybridization buffer (6× SSC, 0.5% SDS, 5× Denhardts) for 2–3 h. Membranes were then washed twice with 2× SSC/0.1% SDS buffer followed by two washes with 0.1× SSC/0.1% SDS buffer. Membranes were exposed to autoradiographic film and developed after adequate exposure time. A digital picture of each autoradiograph was taken and band intensities analyzed using NIH Image public domain software (developed at the Research Services Branch of the National Institute of Mental Health; available for download at http://rsb.info.nih.gov/nih-image). Specific IFN-γ and TNF-α band intensities were normalized to β-actin to account for differences in total RNA loading in each sample.

Plasma aspartate aminotransferase (AST) analyses

Plasma levels of AST, as an indication of hepatic cellular injury, was determined on plasma samples collected 2 days post *K. pneumoniae* inoculation. AST activity was quantitated by the Clinical Chemistry Laboratory at the University of Michigan Medical Center using an automated spectrophotometric assay.

Statistical analyses

Statistical significance was determined using the unpaired, two-tailed Alternate Welsh *t* test and nonparametric Mann-Whitney test. Calculations were performed using Instat for Macintosh (GraphPad Software, San Diego, CA). Statistical analyses of survival curves were performed by the logrank test using the Prism software program (GraphPad Software).

Results

Mice specifically lacking γδ-T cells are more susceptible to K. pneumoniae*-induced mortality*

The importance of T cells during acute Gram-negative bacterial pneumonia was examined using mice completely lacking all subsets of mature T cells as a result of TCR β- and δ-chain deletions. Intratracheal inoculation of 5×10^3 *K. pneumoniae* into C57BL/6 control mice induced mortality within 3–4 days postinfection and resulted in an overall mortality rate of 40–50% by day 10. Interestingly, C57BL/6J-TcrbTcrd (αβ/γδ-T cell-deficient) mice inoculated with the same dose of *K. pneumoniae* displayed increased mortality at both early and late time points (Fig. 1A, $p < 0.05$), indicating a protective role for T cells from *Klebsiella*-induced mortality.

[3] Abbreviations used in this paper: MPO, myeloperoxidase; AST, aspartate aminotransferase; m, mouse.

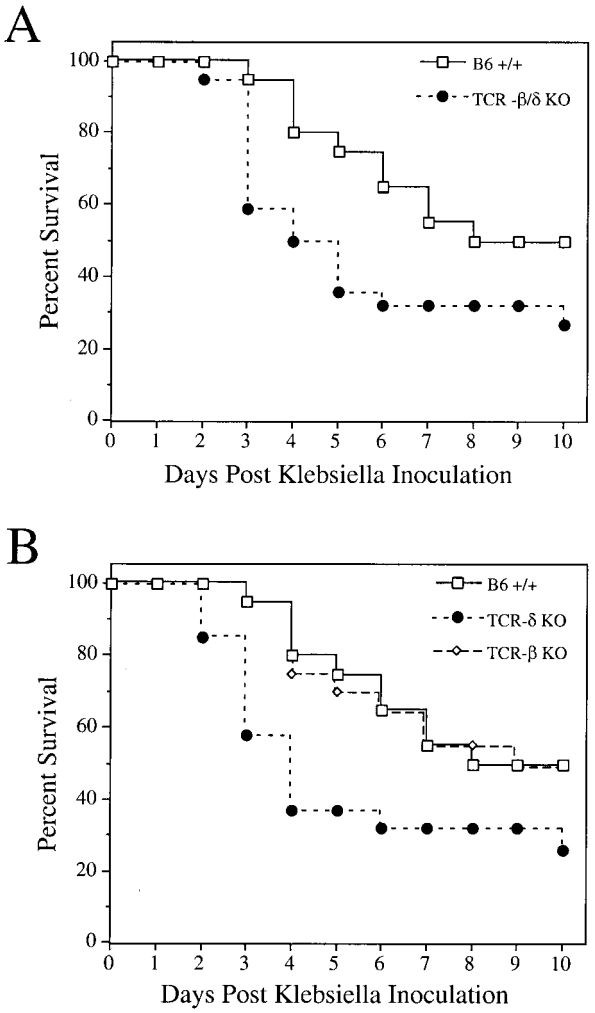

FIGURE 1. Increased mortality in T cell knockout mice following *K. pneumoniae* inoculation is due to the specific absence of γδ-T cells. The indicated animal groups were intratracheally inoculated with 5×10^3 *K. pneumoniae* and mortality was observed over the course of 10 days. *A*, C57BL/6J-TcrbTcrd (αβ/γδ-T cell knockout), and C57BL/6J wild-type mice were infected as described. Note that mice completely lacking T cells displayed increased mortality at both early and late time points ($p < 0.05$, logrank test). *B*, To determine which T cell subset was critical for survival following *Klebsiella* infection, C57BL/6J-Tcrb (αβ-T cell knockout) and C57BL/6J-Tcrd (γδ-T cell knockout) mice were inoculated as described. Mice specifically lacking γδ-T cells displayed increased mortality that was indistinguishable from that seen in mice lacking both T cell subsets ($p < 0.05$, logrank test). In contrast, mice lacking αβ-T cells were no more susceptible to infection than C57BL/6 control mice. Data were generated from two to three independent experiments with a total of 20–22 mice.

To determine whether the specific absence of αβ-T cells or γδ-T cells was responsible for this observed increase in mortality, C57BL/6J-Tcrb (αβ-T cell knockout) and C57BL/6J-Tcrd (γδ-T cell knockout) were infected with *K. pneumoniae*. Interestingly, the increased sensitivity of T cell knockout mice was due to the absence of γδ-T cells rather than αβ-T cells (Fig. 1*B*, $p < .05$). In contrast, αβ-T cell knockout mice were no more susceptible to *K. pneumoniae* induced mortality than their wild-type control littermates.

γδ-T cells represent a small subset of total pulmonary leukocytes

γδ-T cells have been shown to constitute a minority subset of the total T cell population in most peripheral lymphoid organs; however, less is known about γδ-T cell distribution within the lung. To determine the frequency of γδ-T cells, total lung leukocytes were harvested from C57BL/6J wild-type mice by enzymatic dissociation and subjected to multiparameter flow cytometric analyses. Fewer than 1% of lung leukocytes expressed the γδ-TCR (Fig. 2). Essentially all of these pulmonary γδ-T cells lacked CD4 and CD8 expression (data not shown). In contrast, αβ-T cells represent 15–20% of total lung leukocytes, with >90% of these expressing CD4 or CD8 (data not shown). Interestingly, γδ-T cells (percentage and total) were increased in lungs of αβ-T cell knockout mice, representing 4–5% of total lung leukocytes. As expected, these animals were devoid of any αβ-TCR-expressing cells.

γδ-T cell knockout mice have unimpaired pulmonary bacterial clearance but display elevated peripheral blood dissemination

The most plausible explanation for the increased mortality of γδ-T cell knockout mice following intratracheal inoculation of *K. pneumoniae* would be impaired clearance of bacteria from the pulmonary airspace. To address this, lung bacterial burden in γδ-T cell knockout and αβ-T cell knockout mice was examined 1 and 2 days postintratracheal infection. Bacterial counts increased to a similar degree in lungs of αβ-T cell knockout, γδ-T cell knockout, and wild-type mice by 1-day postinoculation (data not shown). Pulmonary bacterial numbers continued to increase in all three groups of animals by day 2, but again with no significant differences between the T cell subset-deficient animals and wild-type mice (Fig. 3*A*). Furthermore, neutrophil recruitment, as measured by lung MPO activity, was similar between γδ-T cell knockout, αβ-T cell knockout, and wild-type mice at these time points, indicating that pulmonary neutrophil influx could occur in the absence of γδ-T cells or αβ-T cells (data not shown).

A consequence of *Klebsiella* pneumonia is the rapid dissemination of bacteria to the bloodstream. Within 1 day following intratracheal inoculation, bacteria were detectable in the peripheral blood of both T cell subset knockout groups as well as wild-type mice. However, there were no observed differences in dissemination between these groups of mice (data not shown). However, peripheral blood bacterial counts were significantly increased ($p < 0.02$) in γδ-T cell knockout mice when compared with mice lacking αβ-T cells by 2 days postinoculation. (Fig. 3*B*). Although not quite reaching the level of statistical significance, the trend in all individual experiments was for γδ-T cell knockout mice to have elevated blood bacterial counts when compared with B6 wild-type mice.

γδ-T cell knockout mice have impaired early expression of IFN-γ and TNF-α mRNA in lung following K. pneumoniae *infection*

IFN-γ and TNF-α have been shown to be critically important in resolution of pulmonary bacterial infections. To determine whether impaired localized expression of these two cytokines could contribute to the increased peripheral blood bacterial burden seen in γδ-T cell knockout mice, IFN-γ and TNF-α mRNA expression in lung and liver was examined by RT-PCR. IFN-γ mRNA was rapidly induced in the lungs of both B6 wild-type mice and αβ-T cell knockout mice within 1 day of infection. Most interestingly, γδ-T cell knockout mice had a 5-fold reduction in IFN-γ mRNA when compared with B6 mice and a 7-fold reduction vs αβ-T cell knockout mice (Fig. 4, *A* and *C*). This impaired IFN-γ production by γδ-T cell knockout mice was transient; by day 2, IFN-γ mRNA levels had increased to that seen in infected B6 control mice. TNF-α mRNA production was similarly impaired in γδ-T cell knockout mice 1 day postinfection, albeit less dramatically than seen with IFN-γ (2-fold reduction vs B6; 3-fold vs αβ-T cell knockout mice; Fig. 4, *B* and *C*). As with IFN-γ, this reduction in

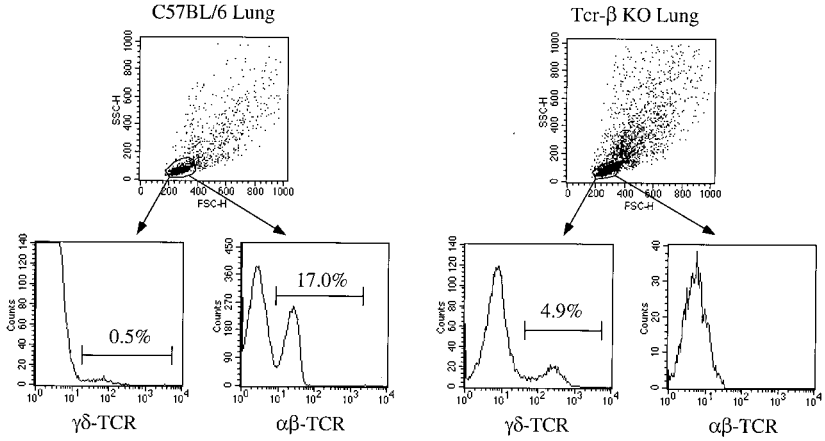

FIGURE 2. γδ T cells comprise a small subset of total lung leukocytes. Total lung leukocytes were obtained by enzymatic digestion and stained for multiparameter flow cytometric analyses as described in *Materials and Methods*. TCR expression was analyzed on lymphocyte sized cells as determined by forward and side light scatter characteristics. Lymphocyte-sized cells represent 45–50% of total lung cells in C57BL/6 mice and 40–45% of lung cells in αβ-T cell-deficient mice. Cell percentages were then back-calculated to percentage in total lung leukocytes. γδ T cells comprise <1% of total lung leukocytes in C57BL/6 mice, whereas αβ-T cells represent 15–20% of lung cells. Interestingly, γδ-T cells (percentage and total) are increased in lungs of αβ-T cell knockout mice; representing 4–5% of total lung leukocytes. Data are representative of two to three independent flow cytometric analyses.

TNF-α was restricted to the first day of infection; by day 2, levels had increased to that seen in control mice. Mice lacking αβ-T cells were unimpaired in their production of pulmonary IFN-γ and TNF-α mRNA 1 day postinfection. By day 2, these mice expressed increased cytokine message when compared with wild-type mice.

γδ T cell knockout mice have increased hepatic cellular injury following K. pneumoniae *infection*

Data thus far indicate that γδ-T cell knockout mice had increased peripheral blood bacterial dissemination in conjunction with impaired early expression of IFN-γ and TNF-α. To determine whether this increased blood bacterial burden may lead to increased hepatic injury, plasma AST levels were measured as an indication of hepatic cellular injury. All three groups of mice, γδ-T cell knockout, αβ-T cell knockout, and wild-type mice had increased plasma AST levels 2 days postintratracheal inoculation of bacteria when compared with saline-injected control mice ($p < 0.05$). Of interest, γδ-T cell knockout mice had significantly elevated AST levels when compared with αβ-T cell knockout mice (Fig. 5, $p < 0.05$).

γδ T cell knockout mice display elevated liver bacterial burden following intratracheal inoculation with K. pneumoniae

The increased peripheral blood bacterial burden seen in γδ-T cell knockout mice suggested an impaired ability of these mice to resolve the disseminated bacterial infection. As blood borne bacteria are predominantly cleared within the liver (25–27), we analyzed total liver bacterial burden 2 days following intratracheal inoculation of *K. pneumoniae*. Total liver bacterial burden was significantly increased in γδ-T cell knockout mice when compared with αβ-T cell knockout mice (Fig. 6, $p < 0.01$).

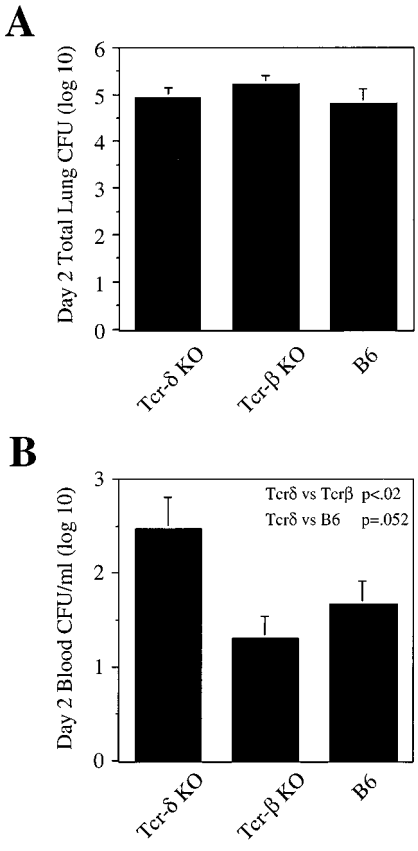

FIGURE 3. γδ-T cell knockout mice have unimpaired pulmonary bacterial clearance but display elevated peripheral blood dissemination. Animals were inoculated with *K. pneumoniae* as described, and bacterial burden in lung and blood was determined on day 2 postinfection. *A*, Bacterial counts increased to a similar degree in lungs of both αβ-T cell knockout and γδ-T cell knockout mice 2 days postinoculation when compared with B6 wild-type mice. *B*, Peripheral blood bacterial counts were statistically increased ($p < 0.02$) in γδ-T cell knockout mice when compared with mice lacking αβ-T cells 2 days postinoculation. Although not reaching the level of statistical significance ($p = 0.052$), the trend in all individual experiments was for γδ-T cell knockout mice to have elevated blood bacterial counts when compared with B6 wild-type mice. Data were generated from three to four independent experiments with a total of 30–40 mice. Statistical significance was determined using the unpaired, two-tailed Alternate Welsh *t* test and nonparametric Mann–Whitney test.

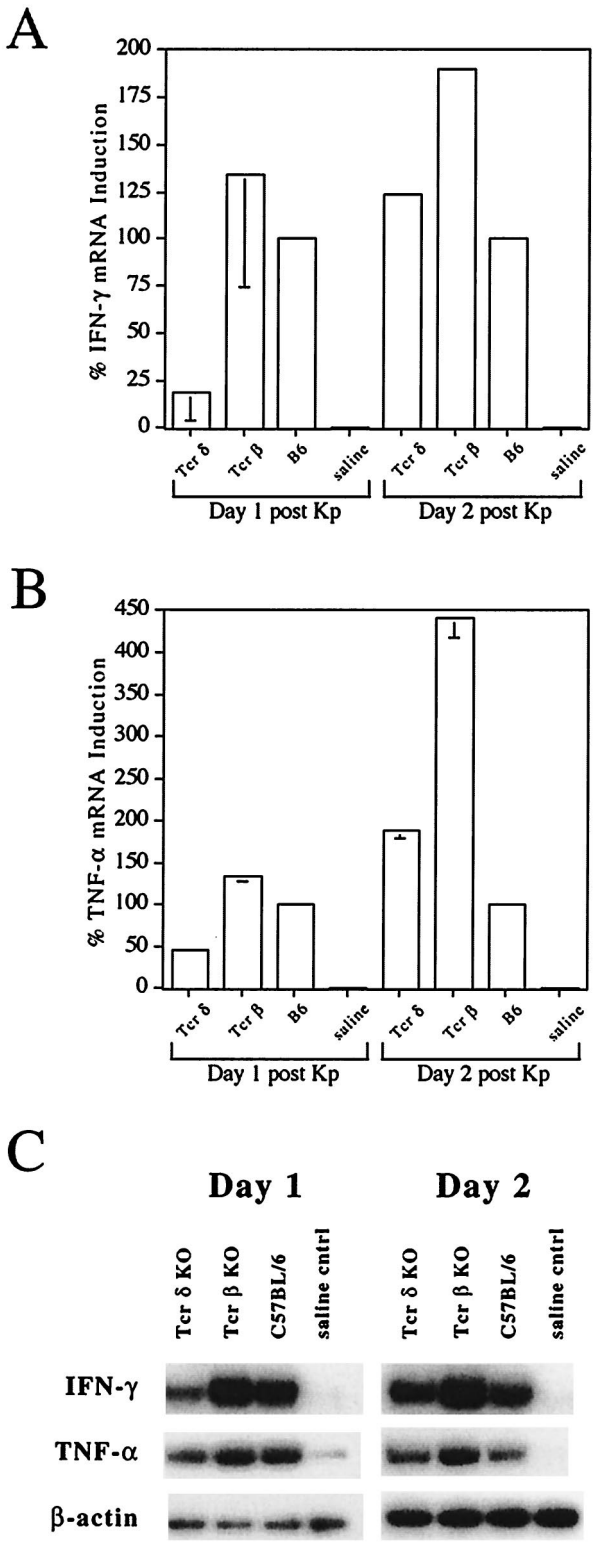

FIGURE 4. γδ-T cell knockout mice have impaired pulmonary IFN-γ and TNF-α mRNA expression 24 h following *K. pneumoniae* infection. RT-PCR analyses of pulmonary IFN-γ and TNF-α induction following *K. pneumoniae* inoculation was performed as described in *Materials and Methods*. Specific IFN-γ and TNF-α band intensities were normalized to β-actin to account for differences in total RNA loading in each sample. IFN-γ and TNF-α mRNA induction in C57BL/6-infected mice was set at 100% to allow for comparison of the relative induction of these cytokines in inoculated γδ-T and αβ-T cell-deficient mice. *A*, IFN-γ mRNA induction following bacterial inoculation. Most interestingly, 1 day following infection, γδ-T cell knockout mice had a 5-fold reduction in IFN-γ mRNA when compared with B6 mice and a 7-fold reduction vs αβ-T cell knockout mice. *B*, TNF-α mRNA induction following bacterial inoculation. TNF-α mRNA production was similarly impaired in γδ-T cell knockout mice 1 day postinfection, albeit less dramatically than seen with IFN-γ (2-fold reduction vs B6; 3-fold vs αβ-T cell knockout mice). *C*, Representative RT-PCR/Southern blot analysis of IFN-γ and TNF-α mRNA expression. Data were determined from two independent experiments with three animals per group at each time point. RNA from individual mice within a group was pooled for subsequent RT-PCR analyses.

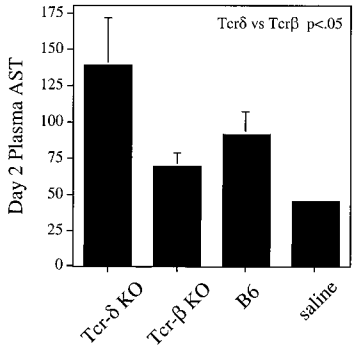

FIGURE 5. γδ-T cell knockout mice have elevated hepatic cellular injury following *K. pneumoniae* infection. Plasma AST levels were measured as an indication of hepatic cellular injury 2 days following intratracheal inoculation of *K. pneumoniae*. Of interest, γδ-T cell knockout mice had significantly elevated AST levels when compared with αβ-T cell-deficient mice ($p < 0.05$). All three groups of mice, γδ-T cell knockout, αβ-T cell knockout, and wild-type mice, had increased plasma AST levels 2 days postintratracheal inoculation of bacteria when compared with saline-injected control mice ($p < 0.05$). AST levels were determined from three independent experiments with a total of 26 mice per group. Statistical significance was determined using the unpaired, two-tailed Alternate Welsh *t* test and nonparametric Mann-Whitney test.

γδ-T cell knockout mice have impaired early expression of IFN-γ and TNF-α mRNA in liver following K. pneumoniae *infection*

The increased hepatic bacterial burden seen in γδ-T cell knockout mice suggests an impaired liver response to Gram-negative bacteria. We examined hepatic expression of IFN-γ and TNF-α mRNA to determine whether production was impaired. Like the lung, liver IFN-γ and TNF-α mRNA levels 24 h post infection were markedly impaired in γδ-T cell knockout mice when compared with both αβ-T cell knockout and wild-type mice (Fig. 7). Interestingly, reduction in TNF-α mRNA was more pronounced in liver than in lung whereas the reduction in IFN-γ mRNA was similar in both organs. As with the lung, impaired liver TNF-α and IFN-γ mRNA expression was transient, with cytokine mRNA levels returning to that seen in αβ-T cell knockout and wild-type mice by day 2 (data not shown).

γδ T cells represent a small subset of total liver leukocytes

γδ T cell knockout mice appear to have an impaired ability to resolve *K. pneumoniae* infection once dissemination beyond the primary pulmonary site of infection has occurred. To determine the frequency of γδ-T cells in the liver, cells were isolated and examined for TCR expression. Similar to the lung, γδ-T cells represented a small fraction of total liver leukocytes in C57BL/6 mice. Liver γδ T cell numbers were increased in αβ-T cell knockout mice, although not as dramatically as seen in lung leukocytes (Fig. 8).

Discussion

Innate host responses during bacterial pneumonia have been well characterized. However, little is known about the role of T cells

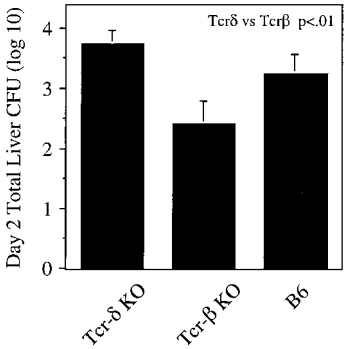

FIGURE 6. γδ-T cell knockout mice display elevated liver bacterial burden following intratracheal inoculation with *K. pneumoniae*. Animals were inoculated with *K. pneumoniae* as described and bacterial burden in liver was determined on day 2 postinfection. Total liver bacterial burden was significantly increased in γδ-T cell-deficient mice when compared with αβ-T cell-deficient mice ($p < 0.01$). Data were generated from three independent experiments with a total of 23 mice. Statistical significance was determined using the unpaired, two-tailed Alternate Welsh *t* test and nonparametric Mann-Whitney test.

during pulmonary infections, and in particular, how T cells may interact or regulate the innate host inflammatory response. To definitively address this issue, we used mice rendered genetically deficient in specific T cell subsets by deletion of the β- and/or δ-chain of the TCR complex. Mice lacking both T cell subsets were found to exhibit increased mortality at both early and late time points following intratracheal inoculation of *K. pneumoniae*. Of interest, this enhanced susceptibility was exclusively due to the specific absence of the minority γδ-T cells rather than the majority αβ-T cell population. The absence of γδ-T cells resulted in increased peripheral blood bacterial dissemination 2 days postinfection, which correlated with increased hepatic bacterial burden and cellular injury. Rapid expression of the proinflammatory cytokines IFN-γ and TNF-α has been shown to be critical for an effective host inflammatory response in several bacterial pneumonia models (6, 28, 29). We observed that mice lacking γδ-T cells had pronounced defects in IFN-γ and TNF-α mRNA expression 1 day postinfection, particularly in the liver. Combined, these data indicate that the absence of γδ-T cells results in decreased pulmonary and hepatic IFN-γ and TNF-α production immediately following intratracheal inoculation of *K. pneumoniae*. This decreased proinflammatory cytokine production likely contributes to increased blood and liver bacterial burden, resulting in increased liver damage.

Our data clearly indicate a critical role for γδ-T cells in effective host responses to Gram-negative bacterial pneumonia. The profound effects seen in the absence of a cell population comprising <1% of lung or liver leukocytes are intriguing. Production of the proinflammatory cytokines TNF-α and IFN-γ, known to be critical during bacterial pneumonia, was severely impaired immediately following infection in mice lacking γδ-T cells. The absolute number of lung γδ-T cells remained constant during the first 3 days of infection (data not shown), suggesting that resident rather than recruited γδ-T cells are necessary for early TNF-α and IFN-γ mRNA expression. However, it is worth noting that there was not an absolute absence of either cytokine within the first 24 h following infection, and, by day 2 postinfection, cytokine production was normal. This suggests that cells other than γδ-T cells, such as NK cells or αβ-T cells, likely produce these cytokines albeit with slower kinetics. This is supported by the recent observation that γδ-T cells responded more strongly than αβ-T cells to systemic bacterial infections or LPS stimulation (30).

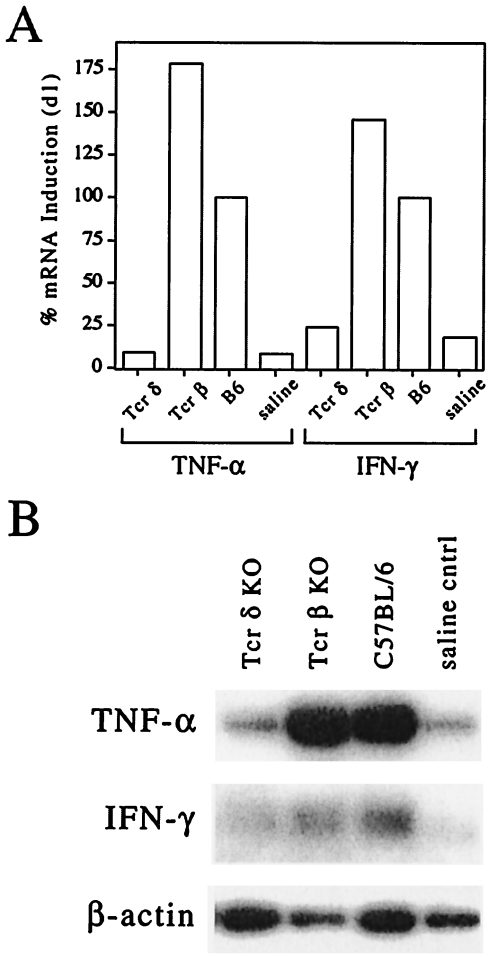

FIGURE 7. γδ-T cell knockout mice have impaired hepatic IFN-γ and TNF-α mRNA expression 24 h following intratracheal *K. pneumoniae* infection. Representative RT-PCR analyses of liver IFN-γ and TNF-α induction following *K. pneumoniae* inoculation was performed as described in *Materials and Methods*. Specific IFN-γ and TNF-α band intensities were normalized to β-actin to account for differences in total RNA loading in each sample. IFN-γ and TNF-α mRNA induction in C57BL/6 infected mice was set at 100% to allow for comparison of the relative induction of these cytokines in inoculated γδ-T and αβ-T cell-deficient mice. *A*, Liver TNF-α and IFN-γ mRNA induction 24 h postinfection. *B*, Representative RT-PCR/Southern blot analysis of IFN-γ and TNF-α mRNA expression used for determination of percent induction.

The finding of increased *Klebsiella*-induced mortality in γδ-T cell knockout mice in the absence of increased lung bacterial burden was unexpected. In addition, pulmonary neutrophil recruitment was unimpaired in γδ-T cell knockout mice. These findings are in contrast to a recent study of nocardial pneumonia in γδ-T cell knockout mice, where it was reported that these animals had unimpeded bacterial growth that correlated with a paucity of inflammatory neutrophil recruitment (31). The observed lack of differences in pulmonary bacterial clearance is particularly surprising given the delay in TNF-α and IFN-γ mRNA expression in the lung. These two cytokines in particular have been shown to be critical for effective lung anti-bacterial host defenses (6, 28, 29). Combined, these data suggest that γδ-T cell knockout mice are not succumbing to overwhelming pulmonary bacterial infection.

Despite similar pulmonary bacterial burdens in γδ-T and αβ-T cell knockout mice, a significantly greater bacterial burden was observed in the blood of mice lacking γδ-T cells. There are several possible explanations for this increase in blood bacterial burden in

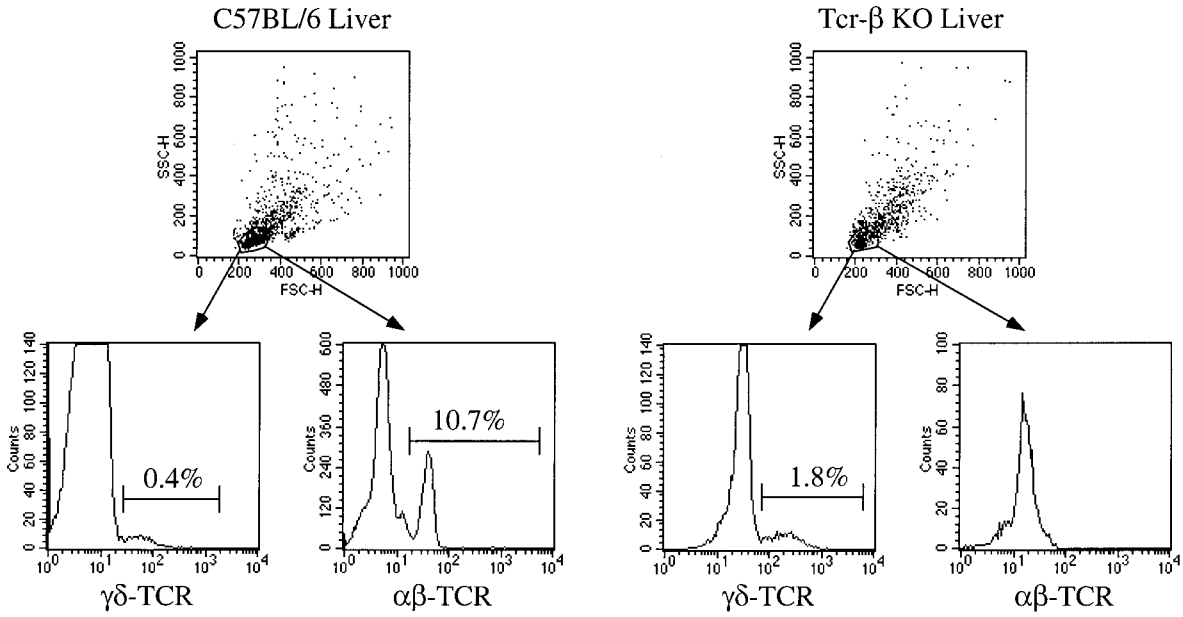

FIGURE 8. γδ-T cells comprise a small subset of total liver leukocytes. T cell subsets were analyzed as described in Fig. 2. Lymphocyte-sized cells represent 45–50% of liver cells in C57BL/6 mice and 30–35% of liver cells in αβ-T cell-deficient mice. As in lung, γδ-T cells represent a small fraction of total liver leukocytes in C57BL/6 mice. γδ T cells are increased in livers of αβ-T cell knockout mice, although not as dramatically as seen in lung leukocytes. Data are representative of two to three independent flow cytometric analyses.

the absence of elevated pulmonary bacterial counts. One possibility is increased pulmonary architectural damage in γδ-T cell knockout mice following bacterial infection leading to increased bacterial "leakage" into the peripheral blood. However, histological examination of lung sections obtained 1 or 2 days postinfection revealed no overt differences between γδ-T cell knockout mice and wild-type mice or αβ-T cell knockout mice (data not shown). A second possibility we favor is that bacterial seeding of the blood occurs at a similar rate in both γδ-T cell knockout and αβ-T cell knockout mice. However, mice lacking γδ-T cells may have an impaired ability to clear bacteria from the blood stream, resulting in increased bacterial burden and mortality.

The observation of increased liver bacterial numbers and cellular injury supports the hypothesis that mice lacking γδ-T cells have an altered or impaired response to Gram-negative bacteremia, resulting in increased bacterial growth in liver and/or blood. It is interesting to note that early induction of hepatic proinflammatory cytokines in response to blood-borne bacteria is markedly impaired in γδ-T cell deficient mice, further supporting an impaired host inflammatory response. Studies examining the role of proinflammatory cytokines, in particular TNF-α, in sepsis or endotoxemia models indicate that a dampened proinflammatory response results in less end-organ injury, including hepatic injury (32–34). However, these models generally use a bolus injection of bacteria or LPS and thus likely reflect a different pathogenic challenge than seen in our model of continual bacterial "leakage" from the primary pulmonary site of infection into peripheral blood. Another possible explanation for increased hepatic injury is that the presence of γδ-T cells may protect against hepatic injury, and that the absence of these cells predisposes the liver to injury in the setting of sepsis. Experimental evidence to dispute this possibility was seen in a model of *S. choleraesuis* bacteremia where the absence of γδ-T cells protected mice from hepatic cellular injury (35). A final and more likely explanation for increased liver injury may simply be a reflection of the increased bacterial burden seen in the blood liver of γδ-T cell knockout mice, resulting in increased endotoxin exposure and enhanced LPS-induced hepatic injury (36).

Mice lacking αβ-T cells, a major component of the T cell compartment in both lung and liver, appear to have compensated in part for the lack of αβ-T cells by increasing the number of γδ-T cells in the lymphoid organs. Although not reaching the level of statistical significance, the consistent trend was for αβ-T cell knockout mice to have decreased blood and liver bacterial numbers, less hepatic injury, and increased proinflammatory cytokine mRNA induction than their corresponding infected wild-type littermates. Combined, these data support our hypothesis that γδ-T cells are a critical component of the host immune response to Gram-negative bacterial infections. It is worth noting that increased numbers of γδ-T cells in the lungs of αβ-T cell knockout mice did not result in lower pulmonary bacterial numbers, in contrast to the trend for improved systemic responses by these mice. However, with these observations in mind, the increased number of γδ-T cells in αβ-T cell knockout mice did not result in enhanced survival when compared with wild-type infected mice. These data suggest that our intratracheal inoculation model of *Klebsiella* pneumonia results in two potentially different types of infections, localized and systemic, and that γδ-T cells may play different roles in these two types of infections. To better study the role of γδ-T cells in disseminated, systemic bacterial infections, we have begun studies characterizing host responses to i.v. inoculation *K. pneumoniae*.

The decrease in early IFN-γ production in γδ-T cell knockout mice suggests that γδ-T cells themselves are the cellular source of IFN-γ. However, it is possible that γδ-T cells stimulate production of IFN-γ, from NK cells for example, so that their absence would result in decreased IFN-γ production. Indeed, this scenario has been shown in *Listeria*-infected animals (20). Rapid IFN-γ production, necessary for clearance of *Listeria*, has been shown to be NK cell-derived. However, in the absence of γδ-T cells, there is a marked decrease in NK cell production of IFN-γ. Although we cannot exclude this scenario in our model, we favor the possibility that γδ-T cells themselves are the cellular source of IFN-γ. Mice rendered genetically deficient in IFN-γ production display

increased mortality following intratracheal *K. pneumoniae* inoculation when compared with their IFN-γ competent littermates (data not shown). Interestingly, the survival curves for IFN-γ knockout and γδ-T cell knockout mice were essentially identical, suggesting that IFN-γ production is γδ-T cell-derived. Additionally, in vivo NK cell depletion by anti-NK1.1 Ab treatment had no detrimental effect on survival (data not shown). One would predict that if the IFN-γ was NK cell-derived rather than γδ-T cell-derived, then NK cell-depleted mice would display an increased susceptibility to pulmonary bacterial challenge similar to γδ-T cell knockout and IFN-γ knockout mice. As optimal TNF-α production has been shown to be IFN-γ-dependent, the absence of γδ-T cell-derived IFN-γ could explain the reduction in TNF-α. Current experiments are ongoing to determine the exact cellular source(s) of IFN-γ and TNF-α.

Other studies have suggested a link between γδ-T cells and the production of TNF-α and IFN-γ. Macrophages from γδ-T cell knockout mice display impaired TNF-α production when stimulated with LPS in vitro (37). Preincubation of these macrophages with wild-type γδ-T cells restored LPS-induced TNF-α production. This priming activity of γδ-T cells was partially inhibited by anti-IFN-γ Abs, suggesting that γδ-T cell derived IFN-γ was required for optimal TNF-α secretion by macrophages challenged with LPS in vitro (and possibly Gram-negative bacteria in vivo). Similar findings were seen in an in vivo model of *S. choleraesuis* sepsis (22). In a model of listerosis, IFN-γ production by NK cells was shown to be markedly reduced in γδ-T cell-deficient animals (20). Spleen cells harvested from infected γδ-T cell-deficient animals were transiently impaired in their TNF-α production following in vitro stimulation, having decreased production on day 1 postinfection but not by day 4.

Taken together, these data indicate that of the three major IFN-γ-producing populations (γδ-T cells, αβ-T cells, NK cells), only mice deficient in γδ-T cells exhibit increased mortality to *K. pneumonia*. The markedly decreased expression of IFN-γ and TNF-α mRNA seen in *K. pneumoniae*-infected γδ-T cell knockout mice suggests that γδ-T cells, although comprising a small percentage of lung and liver leukocytes, are critically important for production of proinflammatory cytokines vital for the resolution of Gram-negative bacterial infections. Furthermore, our data suggest that γδ-T cell knockout mice succumb due to an impaired ability to clear disseminated bacteria from the bloodstream and liver rather than due to an inability to clear the organism from the primary site of infection in the lung. Most importantly, our data indicate that γδ-T cells comprise a critical component of the acute inflammatory response toward extracellular Gram-negative bacterial infections and are vital for the early production of the proinflammatory cytokines IFN-γ and TNF-α.

Acknowledgments

We thank Drs. Gary Huffnagle and Borna Mehrad for informative discussions throughout the course of this work.

References

1. Sahm, D. F., M. K. Marsilio, and G. Piazza. 1999. Antimicrobial resistance in key bloodstream bacterial isolates: electronic surveillance with the Surveillance Network Database-USA. *Clin. Infect. Dis. 29:259.*
2. Burwen, D. R., S. N. Banerjee, and R. P. Gaynes. 1994. Ceftazidime resistance among selected nosocomial Gram-negative bacilli in the United States: National Nosocomial Infections Surveillance System. *J. Infect. Dis. 170:1622.*
3. Podschun, R., and U. Ullmann. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin. Microbiol. Rev. 11:589.*
4. Lipscomb, M. F., D. E. Bice, C. R. Lyons, M. R. Schuyler, and D. Wilkes. 1995. The regulation of pulmonary immunity. *Adv. Immunol. 59:369.*
5. Standiford, T. J. 1997. Cytokines and pulmonary host defenses. *Curr. Opin. Pulm. Med. 3:81.*
6. Moore, T. A., and T. J. Standiford. 1998. The role of cytokines in bacterial pneumonia: an inflammatory balancing act. *Proc. Assoc. Am. Physicians 110:297.*
7. Mak, T. W., and D. A. Ferrick. 1998. The γδ T-cell bridge: linking innate and acquired immunity. *Nat. Med. 4:764.*
8. Born, W., C. Cady, J. Jones-Carson, A. Mukasa, M. Lahn, and R. O'Brien. 1999. Immunoregulatory functions of γδ T cells. *Adv. Immunol. 71:77.*
9. Boismenu, R., and W. L. Havran. 1997. An innate view of gamma delta T cells. *Curr. Opin. Immunol. 9:57.*
10. Kaufmann, S. H. 1996. γδ and other unconventional T lymphocytes: what do they see and what do they do? *Proc. Natl. Acad. Sci. USA 93:2272.*
11. Mosmann, T. R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today 17:138.*
12. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
13. Duhindan, N., A. J. Farley, S. Humphreys, C. Parker, B. Rossiter, and C. G. Brooks. 1997. Patterns of lymphokine secretion amongst mouse γδ T cell clones. *Eur. J. Immunol. 27:1704.*
14. Wen, L., D. F. Barber, W. Pao, F. S. Wong, M. J. Owen, and A. Hayday. 1998. Primary γδ cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J. Immunol. 160:1965.*
15. Kaufmann, S. H. 1993. Immunity to intracellular bacteria. *Annu. Rev. Immunol. 11:129.*
16. Susa, M., B. Ticac, T. Rukavina, M. Doric, and R. Marre. 1998. *Legionella pneumophila* infection in intratracheally inoculated T cell- depleted or -nondepleted A/J mice. *J. Immunol. 160:316.*
17. White, D. W., R. L. Wilson, and J. T. Harty. 1996. $CD8^+$ T cells in intracellular bacterial infections of mice. *Res. Immunol. 147:519.*
18. Dunkley, M. L., R. L. Clancy, and A. W. Cripps. 1994. A role for CD4+ T cells from orally immunized rats in enhanced clearance of *Pseudomonas aeruginosa* from the lung. *Immunology 83:362.*
19. Kasper, L. H., T. Matsuura, S. Fonseka, J. Arruda, J. Y. Channon, and I. A. Khan. 1996. Induction of gammadelta T cells during acute murine infection with *Toxoplasma gondii. J. Immunol. 157:5521.*
20. Ladel, C. H., C. Blum, and S. H. Kaufmann. 1996. Control of natural killer cell-mediated innate resistance against the intracellular pathogen *Listeria monocytogenes* by γδ T lymphocytes. *Infect. Immun. 64:1744.*
21. Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. Kaufmann. 1993. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. *Nature 365:53.*
22. Emoto, M., H. Nishimura, T. Sakai, K. Hiromatsu, H. Gomi, S. Itohara, and Y. Yoshikai. 1995. Mice deficient in γδ T cells are resistant to lethal infection with *Salmonella choleraesuis. Infect. Immun. 63:3736.*
23. Greenberger, M. J., R. M. Strieter, S. L. Kunkel, J. M. Danforth, R. E. Goodman, and T. J. Standiford. 1995. Neutralization of IL-10 increases survival in a murine model of *Klebsiella pneumonia. J. Immunol. 155:722.*
24. Huffnagle, G. B., M. F. Lipscomb, J. A. Lovchik, K. A. Hoag, and N. E. Street. 1994. The role of $CD4^+$ and $CD8^+$ T cells in the protective inflammatory response to a pulmonary cryptococcal infection. *J. Leukocyte Biol. 55:35.*
25. Gregory, S. H., L. K. Barczynski, and E. J. Wing. 1992. Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections. *J. Leukocyte Biol. 51:421.*
26. Klein, A., M. Zhadkewich, J. Margolick, J. Winkelstein, and G. Bulkley. 1994. Quantitative discrimination of hepatic reticuloendothelial clearance and phagocytic killing. *J. Leukocyte Biol. 55:248.*
27. Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1996. Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *J. Immunol. 157:2514.*
28. Laichalk, L. L., S. L. Kunkel, R. M. Strieter, J. M. Danforth, M. B. Bailie, and T. J. Standiford. 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella pneumonia. Infect. Immun. 64:5211.*
29. Rubins, J. B., and C. Pomeroy. 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect. Immun. 65:2975.*
30. Lahn, M., H. Kalataradi, P. Mittelstadt, E. Pflum, M. Vollmer, C. Cady, A. Mukasa, A. T. Vella, D. Ikle, R. Harbeck, R. O'Brien, and W. Born. 1998. Early preferential stimulation of γδ T cells by TNF-α. *J. Immunol. 160:5221.*
31. King, D. P., D. M. Hyde, K. A. Jackson, D. M. Novosad, T. N. Ellis, L. Putney, M. Y. Stovall, L. S. Van Winkle, B. L. Beaman, and D. A. Ferrick. 1999. Cutting edge: protective response to pulmonary injury requires γδ T lymphocytes. *J. Immunol. 162:5033.*
32. Standiford, T. J., R. M. Strieter, N. W. Lukacs, and S. L. Kunkel. 1995. Neutralization of IL-10 increases lethality in endotoxemia: cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. *J. Immunol. 155:2222.*
33. Walley, K. R., N. W. Lukacs, T. J. Standiford, R. M. Strieter, and S. L. Kunkel. 1996. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. *Infect. Immun. 64:4733.*
34. Marchant, A., C. Bruyns, P. Vandenabeele, M. Ducarme, C. Gerard, A. Delvaux, D. De Groote, D. Abramowicz, T. Velu, and M. Goldman. 1994. Interleukin-10 controls interferon-γ and tumor necrosis factor production during experimental endotoxemia. *Eur. J. Immunol. 24:1167.*
35. Ishigami, M., H. Nishimura, K. Yoshioka, S. Kakumu, and Y. Yoshikai. 1999. The role of intrahepatic γδ-T cells for liver injury induced by *Salmonella* infection in mouse. *Microbiol. Immunol. 43:461.*
36. Nolan, J. P. 1981. Endotoxin, reticuloendothelial function, and liver injury. *Hepatology 1:458.*
37. Nishimura, H., M. Emoto, K. Hiromatsu, S. Yamamoto, K. Matsuura, H. Gomi, T. Ikeda, S. Itohara, and Y. Yoshikai. 1995. The role of γδ T cells in priming macrophages to produce tumor necrosis factor-α. *Eur. J. Immunol. 25:1465.*