

## $\gamma\delta$ T Cells Regulate the Early Inflammatory Response to *Bordetella pertussis* Infection in the Murine Respiratory Tract

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**The role of  $\gamma\delta$  T cells in the regulation of pulmonary inflammation following *Bordetella pertussis* infection was investigated. Using a well-characterized murine aerosol challenge model, inflammatory events in mice with targeted disruption of the T-cell receptor  $\delta$ -chain gene ( $\gamma\delta$  TCR<sup>-/-</sup> mice) were compared with those in wild-type animals. Early following challenge with *B. pertussis*,  $\gamma\delta$  TCR<sup>-/-</sup> mice exhibited greater pulmonary inflammation, as measured by intra-alveolar albumin leakage and lesion histomorphometry, yet had lower contemporaneous bacterial lung loads. The larger numbers of neutrophils and macrophages and the greater concentration of the neutrophil marker myeloperoxidase in bronchoalveolar lavage fluid from  $\gamma\delta$  TCR<sup>-/-</sup> mice at this time suggested that differences in lung injury were mediated through increased leukocyte trafficking into infected alveoli. Furthermore, flow cytometric analysis found the pattern of recruitment of natural killer (NK) and NK receptor<sup>+</sup> T cells into airspaces differed between the two mouse types over the same time period. Taken together, these findings suggest a regulatory influence for  $\gamma\delta$  T cells over the early pulmonary inflammatory response to bacterial infection. The absence of  $\gamma\delta$  T cells also influenced the subsequent adaptive immune response to specific bacterial components, as evidenced by a shift from a Th1 to a Th2 type response against the *B. pertussis* virulence factor filamentous hemagglutinin in  $\gamma\delta$  TCR<sup>-/-</sup> mice. The findings are relevant to the study of conditions such as neonatal *B. pertussis* infection and acute respiratory distress syndrome where  $\gamma\delta$  T cell dysfunction has been implicated in the inflammatory process.**

Early interactions between host defense cells and bacterial pathogens are critical in determining the nature and extent of inflammation and the subsequent resolution of infection. Previous studies have suggested that  $\gamma\delta$  T cells have an important role in regulating the initial inflammatory response to microbial invasion and, in so doing, prevent excessive tissue injury (15, 24, 12, 43, 44, 47). Experimental evidence suggests  $\gamma\delta$  T cells execute their regulatory function through influencing the movement and function of key inflammatory effector cells such as neutrophils (15, 11), macrophages (12, 47), and NK cells (27, 49). T cells expressing the  $\gamma\delta$  receptor develop early in ontogeny and are disproportionately abundant within epithelial surfaces, including those in the lung (18). Their intraepithelial distribution and capacity to recognize nonprotein antigens, sometimes in a non-major histocompatibility complex-restricted fashion, has led to their consideration as part of the first line of the host defense. Furthermore, the common overlap of pathogen-encoded  $\gamma\delta$  cell antigens with molecules expressed by stressed host cells has led to the view that  $\gamma\delta$  T-cell responses are driven more by nonspecific markers of cell injury than by the overt presence of microbial or other foreign antigens (18).

The early activation of  $\gamma\delta$  T lymphocytes can lead to gamma interferon (IFN- $\gamma$ ) production that is instrumental in the up-regulation of both macrophage and natural killer (NK) cell functions central to early antibacterial protection prior to the  $\alpha\beta$  T-cell response (18). IFN- $\gamma$  also influences the downstream acquisition of a Th1 phenotype by  $\alpha\beta$  T cells (25, 45).  $\gamma\delta$  T cells

thus bridge the innate and acquired immune response by providing initial protection of epithelia and tissues from invasion and injury in instances where  $\alpha\beta$  T cells are not yet operational and then down-regulating the antigen-specific adaptive immune response after the danger has passed to minimize potential immune-mediated injury (43). Experimental data suggest that this T-cell population exerts its influence through chemokine and cytokine production that in turn regulates the movement and activation of neutrophils, macrophages, NK cells, and NK receptor-positive (NKR<sup>+</sup>) T cells (15, 24, 12, 43, 47). NK cells are part of the innate immune response, effecting cytolysis and releasing inflammatory cytokines (48, 35). NKR<sup>+</sup> T cells, typically defined as NK1.1<sup>+</sup>  $\alpha\beta$ TCR<sup>+</sup> cells in mice, are a novel lymphoid lineage distinct from NK cells (41). On stimulation, this T lymphocyte subtype can rapidly secrete large amounts of both Th1 (IFN- $\gamma$  and tumor necrosis factor beta) (8) and Th2 (interleukin-4 [IL-4] and IL-5) (52) cytokines in a nonspecific manner prior to the development of the acquired immune response and are a proposed link between the innate and adaptive immune responses, in a manner analogous to  $\gamma\delta$  T cells (2, 16). Roles for NK cells and NKR<sup>+</sup> T cells in airway inflammation have recently been described (21, 23, 50). However, the distinction between  $\gamma\delta$  and NKR<sup>+</sup> T cells is often indistinct, and their relative functions during immune responses are unclear. Consistent with a regulatory role for  $\gamma\delta$  T cells, in vivo and in vitro production of the proinflammatory cytokines IL-6, IL-12, and IFN- $\gamma$  are increased in  $\gamma\delta$  TCR<sup>-/-</sup> mice during the innate, early-phase host response to *Listeria monocytogenes* infection (43), while other studies suggest  $\gamma\delta$  T cells influence macrophage and neutrophil trafficking and activation through production of CCL3/MIP-1 $\alpha$  (47) and IL-10 (19), respectively.

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$\gamma\delta$  T cells can modulate either the severity of disease or the protective immune response in murine models of tuberculosis, malaria, and herpes simplex virus type 1 encephalitis (45). In humans, large expansion of the  $\gamma\delta$  T-cell population during infection with *L. monocytogenes*, *Mycobacterium tuberculosis*, *Brucella melitensis*, and *Ehrlichia chaffeensis* suggest their importance in the host response (45). Of particular interest in the context of pulmonary injury is a recent study implicating temporary functional suppression of  $\gamma\delta$  T cells in the uncontrolled inflammation associated with acute respiratory distress syndrome (ARDS) triggered by sepsis (19).

*Bordetella pertussis* is a gram-negative bacterium that causes whooping cough, a respiratory disease that results in infant morbidity and mortality. The murine *B. pertussis* respiratory challenge model is an established, well-characterized experimental system particularly useful in the study of bacterium-mediated pulmonary inflammation (22, 32, 33). Although little is known about the role of  $\gamma\delta$  T cells during *B. pertussis* infection, a role for  $\gamma\delta$  T cells in the response of infected children has been suggested by the migration of circulating  $\gamma\delta$  T cells to the airways (3), and murine studies have reported airway  $\gamma\delta$  T cells following exposure to *B. pertussis* (30).

We investigated the role of  $\gamma\delta$  T cells in *B. pertussis* infection by comparing the degree of pulmonary inflammation following infection of mice with targeted disruption of the T-cell receptor  $\delta$ -chain gene ( $\gamma\delta$  TCR<sup>-/-</sup>) and conventional C57BL/6 (wild type [WT]) mice. Measures of inflammation included bronchoalveolar lavage fluid (BALF) albumin and myeloperoxidase (MPO) levels, leukocyte cytology, and flow cytometric analysis in addition to lung lesion morphometry.  $\gamma\delta$  TCR<sup>-/-</sup> mice exhibited greater degrees of pulmonary inflammation mediated through increased neutrophil exudation. This increased response accounted for the apparent paradox of finding smaller bacterial loads early postinfection in the lungs of mice lacking  $\gamma\delta$  T cells.  $\gamma\delta$  TCR<sup>-/-</sup> mice also had a significantly greater Th2 response to the *B. pertussis* virulence factor filamentous hemagglutinin (FHA), not evident in controls, suggesting that these cells influence the quality of adaptive immunity. Taken together, these findings suggest  $\gamma\delta$  T cells play a nonredundant role in the initial pulmonary inflammatory response to bacterial infection and also influence the induction of cell-mediated immunity to specific bacterial antigens.

#### MATERIALS AND METHODS

**Mice.** Male and female C57BL/6  $\gamma\delta$  T-cell receptor knockout ( $\gamma\delta$  TCR<sup>-/-</sup>) (Jackson Laboratory, Bar Harbor, Maine) and C57BL/6 WT (Harlan Co., United Kingdom) mice were at least 8 weeks old at the initiation of experiments and were maintained in a specific-pathogen-free biosafety facility with free access to water and standard mouse rations. Animals were maintained under the guidelines of the Irish Department of Health and the research ethics committee of the National University of Ireland, Maynooth.

**Bacterial inoculum and aerosol infection.** *Bordetella pertussis* (strain W28) was grown under agitation conditions at 36°C in Stainer-Scholte liquid medium. Bacteria from a 48-h culture were resuspended at a concentration of approximately  $2 \times 10^{10}$  CFU per ml in physiological saline containing 1% casein. The challenge inoculum was administered as an aerosol over a period of 12 min with a nebulizer directed into an aerosol chamber containing groups of 20 to 24 mice. All experiments were performed at least twice.

**Enumeration of pulmonary bacterial loads.** Infected mice were euthanized at fixed time points after experimental challenge (four mice per time point), and the lungs were aseptically removed and homogenized in 1 ml of sterile physiological saline with 1% casein on ice. One hundred microliters of undiluted homogenate or of serially diluted homogenate from individual lungs was spotted in triplicate

onto each of three Bordet-Gengou agar plates, and the number of CFU was estimated after 5 days of incubation. Results are reported as the mean number of *B. pertussis* CFU for individual lungs from the four mice sacrificed at each time point. Results are plotted on a logarithmic scale, with a limit of detection of approximately  $0.3 \log_{10}$  CFU per lung.

**Bronchoalveolar lavage fluid collection and analysis.** To assess the nature and severity of the pulmonary inflammatory response to aerosol *B. pertussis* challenge, BALF samples were obtained from euthanized mice by repeated administration and aspiration of 0.5-ml volumes of phosphate-buffered saline (PBS) via cannulation of the trachea. A total of 5 ml of sample was obtained from each mouse group at each time point through pooling of samples from six animals. Following aseptic erythrolysis by osmotic disruption, cells from the lavage fluid were recovered by centrifugation at  $300 \times g$  for 7 min and resuspended in PBS. Total leukocytes were counted, and cytospin preparations were stained with Giemsa to determine the differential cell count at each time point. The means and standard errors (SE) of the means of the absolute numbers of macrophages, neutrophils, and lymphocytes were determined following counting of 600 cells. The cell-free supernatant was analyzed for albumin and MPO content. Albumin levels were measured using a murine albumin enzyme-linked immunosorbent assay (ELISA) kit (mouse albumin ELISA kit; Universal Biologicals Ltd., Cambridge, United Kingdom). The detection limits were between 7.8 and 500 ng/ml. To measure MPO activity, supernatant samples were incubated in a 50 mM potassium phosphate buffer containing H<sub>2</sub>O<sub>2</sub> (1.5 mol/liter) in the presence of *o*-dianisidine dihydrochloride (167  $\mu$ g/ml; Sigma Aldrich). The enzymatic activity was determined spectrophotometrically by measuring the change of absorbance at 460 nm over 3 min using a 96-well plate reader (46). For flow cytometry, BALF samples ( $10^5$  cells) were incubated in 2% normal rabbit serum at 0°C for 20 min and normal mouse serum at 0°C for 15 min. Samples were then incubated in the dark on ice for 20 min with 100  $\mu$ l of appropriately diluted Armenian hamster anti-mouse  $\gamma\delta$  TCR antibody conjugated to fluorescein isothiocyanate, Armenian hamster anti-mouse NK1.1 antibody conjugated to phycoerythrin, or Armenian hamster anti-mouse CD3 antibody conjugated with peridinin chlorophyll  $\alpha$  protein (all sourced from PharMingen, San Diego, CA). Appropriate isotype control antibodies were employed in parallel samples. After labeling, cells were washed twice with PBS, and the relative fluorescence intensities were determined on a 4-decade log scale by flow cytometric analysis using FACSCalibur instrumentation (Becton Dickinson, Mountain View, CA). Data were analyzed using CellQuest software (Becton Dickinson).

**Histopathology and lesion morphometry.** To assess pulmonary inflammation, entire mouse lungs were fixed by immersion in 10% formalin, and following paraffin embedding, 3- $\mu$ m longitudinal sections of each of the five lobes were cut, stained with hematoxylin and eosin, and examined (8 mice per group per time point). The areas of inflammatory lesions in each lung section were measured using computer-aided histomorphometry (Palmrobo software, version 1.2.3; Palm microlaser technologies AG Ltd., Bernried, Germany), and a mean lesion area was calculated for each mouse type at each time point. Assessments of histopathology and morphometry were performed without prior knowledge of sample identity.

**Measurement of *B. pertussis*-specific immune responses.** The concentration of *B. pertussis*-specific immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgG3 in collected sera from WT and  $\gamma\delta$  TCR<sup>-/-</sup> mice 43 days postchallenge were measured by ELISA as previously described (28). Briefly, plates were coated with heat-inactivated *B. pertussis* or with the *B. pertussis* virulence factor FHA (1  $\mu$ g/ml). After blocking and the addition of serum samples, alkaline phosphatase-labeled rat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (PharMingen, San Diego, CA) were used to detect *B. pertussis*-specific antibody subclasses. Antibody levels were expressed as the mean endpoint titers calculated by regression of the straight part of the curve of optical density versus serum dilution to a cutoff of two standard deviations above background control values.

Spleen cells from infected WT and  $\gamma\delta$  TCR<sup>-/-</sup> mice 43 days after challenge ( $n = 4$  per group per experiment, repeated at least twice) were resuspended at  $2 \times 10^6$  per ml in RPMI 1640 medium and tested for in vitro proliferation to *B. pertussis* antigens as previously described (28). Briefly, cells were cultured for 4 days with heat-inactivated *B. pertussis* ( $10^4$  CFU per ml equivalent), inactivated pertussis toxin (5  $\mu$ g/ml), FHA (5  $\mu$ g/ml), pertactin (5  $\mu$ g/ml), concanavalin A (5  $\mu$ g/ml, positive control), or medium alone (negative control). For the final 4 h of culture, [<sup>3</sup>H]thymidine was added and the results expressed as the mean cpm of thymidine incorporation ( $\pm$ SE). Spleen cells from infected WT and  $\gamma\delta$  TCR<sup>-/-</sup> mice 43 days after challenge were also cultured with *B. pertussis* antigens as described for the proliferation assay. At the 72-h time point, culture supernatants were assessed for the presence of IL-4, IL-5 and IFN- $\gamma$  by ELISA (PharMingen) and calculated by comparison with known cytokine standards as previously described

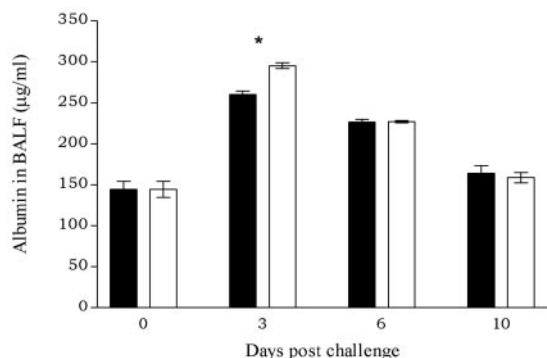


FIG. 1. Lung vascular permeability in  $\gamma\delta$  TCR<sup>-/-</sup> (open bar) compared to WT (solid bar) mice following aerosol *B. pertussis* challenge. Mouse albumin levels were measured in BALF as an index of vascular leakage. Results are means  $\pm$  SE from 6 mice per group. \*,  $P < 0.01$  (compared to the corresponding value for WT mice).

(26). Although the kinetics of cytokine production varies, the 72-h time point has previously been shown acceptable for detection of these cytokines (1).

**Statistical analysis.** Statistical analysis was performed with GraphPad PRISM 3 software. Analysis of variance (one and two way) with Bonferroni's multiple-comparison test was used to assess the significance of differences between the two groups.  $P$  values of  $<0.05$  were considered significant. Student's  $t$  test was used to determine significance between groups in antibody subclass and cytokine assays.  $P$  values of  $<0.05$  were considered significant.

## RESULTS

**$\gamma\delta$  T cells reduce the extent of early-stage *Bordetella pertussis*-mediated pulmonary inflammation through effects on neutrophil trafficking.** To determine the potential regulatory role of  $\gamma\delta$  T cells in the inflammatory response to aerosol *Bordetella pertussis* challenge, the degree of pulmonary inflammation was compared between  $\gamma\delta$  TCR<sup>-/-</sup> and WT mice at early time points postinfection. Quantitation of inflammation was achieved through measurement of albumin leakage into BALF and through computer-aided lesion histomorphometry. BALF albumin levels were higher ( $P < 0.01$ ) and the mean area of foci of lung inflammation larger ( $P < 0.05$ ) in  $\gamma\delta$  TCR<sup>-/-</sup> than in WT mice at 3 and 6 days postinfection, respectively (Fig. 1 and 2). In both mouse phenotypes at 3, 6, and 10 days postchallenge, pulmonary inflammation was characterized by multifocal aggregates of admixed intact and degenerate neutrophils and macrophages, frequently adjacent to small bronchioles. At day 6, the larger areas of inflammation in  $\gamma\delta$  TCR<sup>-/-</sup> mice contained greater numbers of degenerate neutrophils (Fig. 3). From 10 days, increased numbers of macrophages and lymphocytes and reduced numbers of neutrophils were features of the inflammatory response with occasional marginally located fibroblasts noted in both strains of mice.

Further investigation of the inflammatory effector mechanisms influenced by  $\gamma\delta$  T cells was carried out through quantitation of MPO levels and of leukocyte numbers in BALF. Increased BALF MPO at 6 days in the  $\gamma\delta$  TCR<sup>-/-</sup> compared to the WT mice ( $P < 0.05$ ) (Fig. 4) and the detection of greater numbers of neutrophils in BALF at both 3 and 6 days postinfection ( $P < 0.01$ , respectively) (Table 1) corresponded to greater neutrophil exudation into the alveolar spaces in the absence of  $\gamma\delta$  T cells. Measurements of macrophage numbers

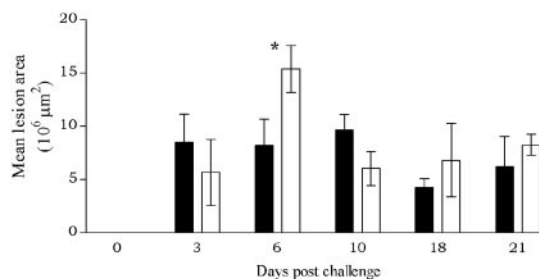


FIG. 2. Pulmonary inflammation was more extensive in  $\gamma\delta$  TCR<sup>-/-</sup> (open bar) compared to WT (solid bar) mice at day 6 following aerosol *B. pertussis* challenge. Computer-aided histomorphometry was used to quantify the mean surface area of inflammatory lesions in both mouse types. Results are means  $\pm$  SE from 8 mice per group. \*,  $P < 0.05$  (compared to the corresponding value for WT mice).

in BALF also indicated increased extravasation of these leukocytes in  $\gamma\delta$  TCR<sup>-/-</sup> compared to WT mice at 6 days postchallenge ( $P < 0.01$ ). Taken together, these findings demonstrate that, in the absence of  $\gamma\delta$  T cells, neutrophil and macrophage exudation into airspaces is enhanced, resulting in more extensive pulmonary inflammation.

**$\gamma\delta$  TCR<sup>-/-</sup> mice have reduced pulmonary *Bordetella pertussis* load in the early phase of infection.** One explanation for the increased inflammation in the  $\gamma\delta$  TCR<sup>-/-</sup> mice could have been increased bacterial carriage in these animals at early time points postchallenge. The relationship between the inflammatory response and the pulmonary bacterial load was assessed by comparing patterns of clearance of *B. pertussis* from the lungs of the  $\gamma\delta$  TCR<sup>-/-</sup> and WT mice. Mice lacking  $\gamma\delta$  T cells with enhanced early inflammatory injury had significantly reduced lung bacterial counts at 3 days ( $P < 0.01$ ) and 6 days ( $P < 0.01$ ) postinfection compared to WT controls (Fig. 5). Thus, the increased inflammatory damage seen in this model cannot be accounted for by increased bacterial carriage. The fact that bacterial clearance rates were similar in both mouse types from day 10 suggested that  $\gamma\delta$  T cells have an influence on the inflammatory response involved in bacterial killing in only the early phase following infection.

**$\gamma\delta$  T cells participate in the early host response to aerosol *B. pertussis* challenge and influence the pulmonary recruitment of NK cells and of NKR<sup>+</sup> T and CD3<sup>+</sup> T cells.** We examined the kinetics of  $\gamma\delta$  T cell, NK cell, NKR<sup>+</sup> CD3<sup>+</sup> T cell, and NKR<sup>-</sup> CD3<sup>+</sup> T cell recruitment into the pulmonary airspaces in response to *B. pertussis* infection through flow cytometric analysis of BALF. As expected,  $\gamma\delta$  T cells could not be detected in  $\gamma\delta$  TCR<sup>-/-</sup> mice, whereas infection of WT mice resulted in an influx of  $\gamma\delta$  T cells that persisted over 3, 6, and 10 days postinfection (Table 2), indicating that this cell type responds rapidly to *B. pertussis* infection. Recruitment of NK cells, of NKR<sup>+</sup> CD3<sup>+</sup> T cells, and of NKR<sup>-</sup> CD3<sup>+</sup> T cells into airspaces differed between the two mouse groups over this time period. Increased numbers of NK cells were noted in BALF from  $\gamma\delta$  TCR<sup>-/-</sup> mice compared to WT mice at 3 ( $P < 0.05$ ), 6 ( $P < 0.01$ ), and 10 ( $P < 0.01$ ) days postinfection. Increased numbers of NKR<sup>-</sup> CD3<sup>+</sup> T cells were also detected in the  $\gamma\delta$  TCR<sup>-/-</sup> mice at day 3 ( $P < 0.01$ ). Numbers of NKR<sup>+</sup> CD3<sup>+</sup> T cells were reduced at day 6 ( $P < 0.01$ ) and 10 ( $P < 0.01$ ) in lavage samples from  $\gamma\delta$  TCR<sup>-/-</sup> mice. Taken together, these



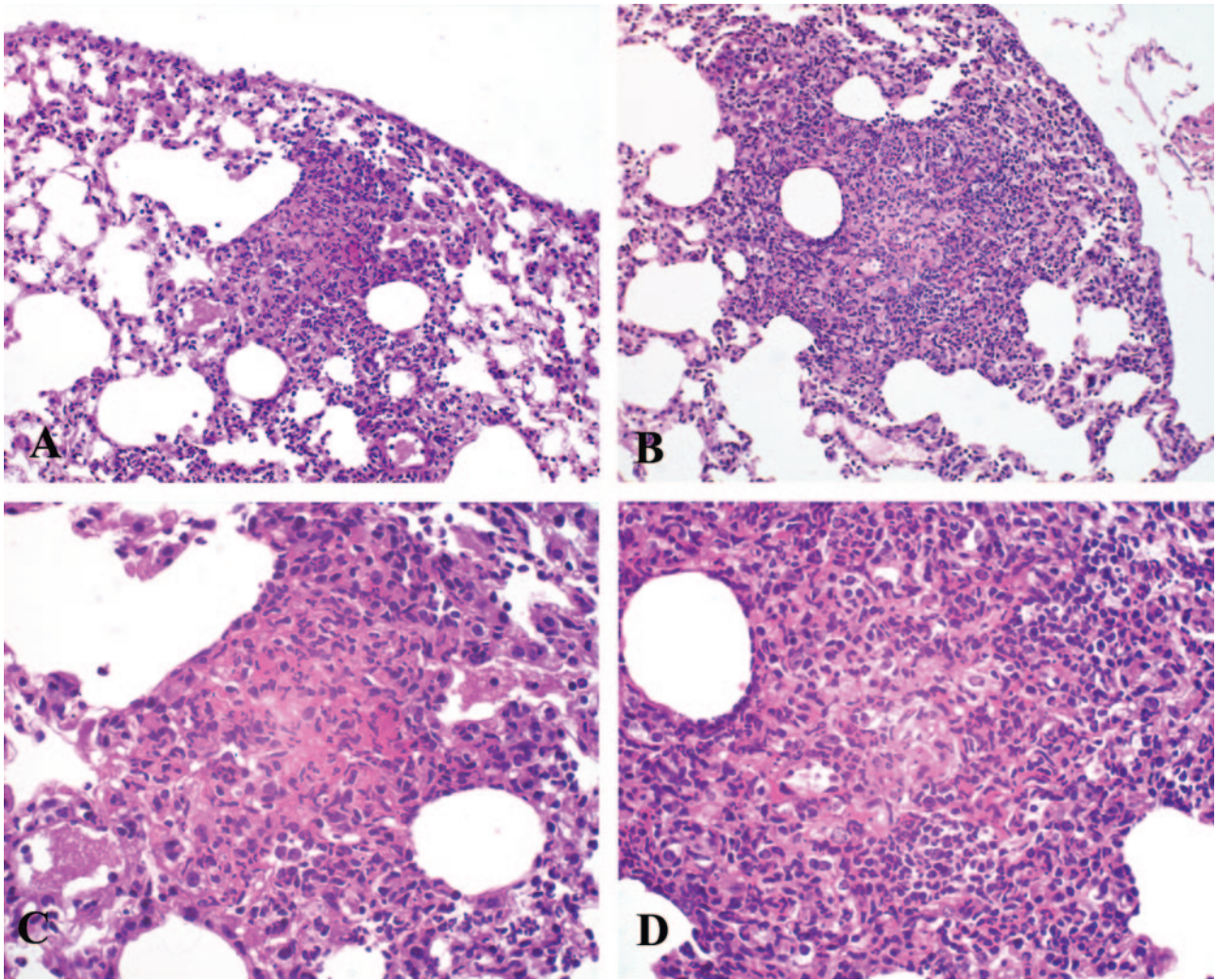


FIG. 3. Photomicrographs of lung sections illustrating quantitative differences in the inflammation in  $\gamma\delta$  TCR<sup>-/-</sup> and WT mice at day 6 following *B. pertussis* infection. Mice were aerosol challenged, giving an initial pulmonary load of approximately  $4.5 \times 10^{10}$  CFU per lung. Focal infiltrates of admixed intact and degenerate neutrophils, macrophages, and lymphocytes efface alveolar architecture in WT (A and C) and  $\gamma\delta$  TCR<sup>-/-</sup> (B and D) mice. Staining was done with hematoxylin and eosin. Magnification,  $\times 200$  (A and B);  $\times 400$  (C and D).

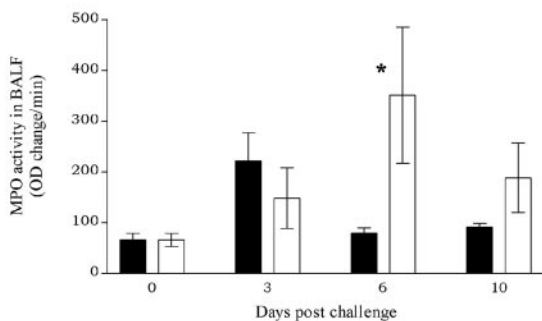


FIG. 4. Neutrophil accumulation in airspaces of  $\gamma\delta$  TCR<sup>-/-</sup> (open bar) compared to WT (solid bar) mice following aerosol *B. pertussis* challenge. MPO levels were measured in BALF as an index of neutrophil influx into airspaces. Results are means  $\pm$  SE from 6 mice per group. \*,  $P < 0.05$  (compared to the corresponding value for WT mice); OD, optical density.

differences in responses in the two mouse types indicate that  $\gamma\delta$  T cells have a critical role in the coordination of the early immune response in the airways.

**$\gamma\delta$  T cells influence the quality of the adaptive immune response to *B. pertussis*.** In addition to the coordination of the early host response to airway pathogens, it was likely that  $\gamma\delta$  T cells would also influence the development of the subsequent adaptive immune response. The influence of  $\gamma\delta$  T cells on both the humoral and cellular components of the adaptive response to *B. pertussis* challenge was therefore assessed by the measurement of *B. pertussis*- or FHA-specific antibody subclasses in mouse sera or *B. pertussis* induction of T-cell responses in spleens obtained 43 days postinfection, at which point bacteria had been cleared from the lungs. While the overall response in both  $\gamma\delta$  TCR<sup>-/-</sup> mice and WT controls was Th1 in character, dominated by IgG2a (Fig. 6A) and IFN- $\gamma$  production (Fig. 7D), as previously reported (32), an important variation was observed.  $\gamma\delta$  TCR<sup>-/-</sup> mice had significantly increased levels of FHA-specific IgG1 and reduced IgG2a (Fig. 6B) compared to

TABLE 1. Cytological examination of leukocytes present in BALF from *B. pertussis*-infected  $\gamma\delta$  TCR<sup>-/-</sup> mice or controls<sup>a</sup>

Day postchallenge and mouse type	Total leukocytes (10 <sup>4</sup> cells per ml)	Neutrophils (10 <sup>4</sup> cells per ml) (%)	Macrophages (10 <sup>4</sup> cells per ml) (%)	Lymphocytes (10 <sup>4</sup> cells per ml) (%)
Unexposed controls				
WT	4.4 ± 11.8	0.3 ± 0.7 (6.3)	4.0 ± 10.8 (91.7)	0.1 ± 0.3 (2.1)
$\gamma\delta$ TCR <sup>-/-</sup>	4.5 ± 11.9	0.2 ± 0.6 (5.0)	4.2 ± 11.1 (93.3)	0.1 ± 0.2 (1.8)
3				
WT	62.5 ± 11.5	26.9 ± 5.0 (43.1)	16.0 ± 2.9 (25.6)	19.6 ± 3.6 (31.4)
$\gamma\delta$ TCR <sup>-/-</sup>	94.7 ± 14.4	40.4 ± 6.1* (42.6)	22.2 ± 3.4 (23.5)	32.1 ± 4.9 (33.9)
6				
WT	214.5 ± 23.8	96.5 ± 10.7 (45.0)	51.2 ± 5.7 (23.9)	66.8 ± 7.4 (31.1)
$\gamma\delta$ TCR <sup>-/-</sup>	289.0 ± 52.0	143.9 ± 25.9* (49.8)	69.7 ± 12.6* (24.1)	75.4 ± 13.6 (26.1)
10				
WT	274.5 ± 57.6	133.8 ± 28.1 (48.8)	70.2 ± 14.7 (25.6)	70.4 ± 14.8 (25.7)
$\gamma\delta$ TCR <sup>-/-</sup>	258.1 ± 39.6	128.6 ± 19.7 (49.8)	36.9 ± 5.7 (14.3)	92.7 ± 14.2 (35.9)

<sup>a</sup> Leukocyte composition of BALF samples following aerosol challenge of  $\gamma\delta$  TCR<sup>-/-</sup> and WT mice with *B. pertussis* or from unexposed controls. A total of 5 ml of lavage sample was obtained from each mouse group at each time point through pooling of samples from six animals. Cells were recovered by centrifugation and counted by hemocytometer. Cytospin preparations were stained with Giemsa to determine the differential cell count at each time point. The means ± SE of the absolute numbers of neutrophils, macrophages, and lymphocytes were determined following counting of 600 cells. \*, *P* < 0.01 (compared to the corresponding value for WT mice).

WT mice (*P* < 0.01) and had a significantly greater IL-4 response to FHA (*P* < 0.01) than WT animals (Fig. 7B). This finding suggests that  $\gamma\delta$  T cells play a role in directing the quality of adaptive immunity and influence the induction of cell-mediated immunity to specific antigens.

## DISCUSSION

This study examined the role of  $\gamma\delta$  T cells in the early host response to *B. pertussis*, an important respiratory pathogen. We show that in the first days of infection, prior to the development of adaptive immunity,  $\gamma\delta$  T cells play a nonredundant role in influencing pulmonary inflammation. Specifically, in their absence, the influx of neutrophils, macrophages, NK cells, and NKR<sup>+</sup> T cells into airways is altered and pulmonary inflammation, as measured by histomorphometry, BALF albumin

and MPO levels, is increased. Intriguingly, these early events also have longer term influences on the quality of adaptive immunity in that the pattern of cytokine and antibody subclass production was altered to the bacterial virulence factor FHA, suggesting that the early encounter between  $\gamma\delta$  T cells and pathogen shapes subsequent immunity.

The apparent paradox of finding decreased pulmonary bacterial loads in mice missing  $\gamma\delta$  T cells, a key component of the host first line of defense is also accounted for by the increased early inflammatory response in  $\gamma\delta$  TCR<sup>-/-</sup> animals, but as our lung histomorphometry and BALF albumin data clearly illustrate, this reduction in bacterial load is at the expense of increased pulmonary injury. It is important to consider that the small BALF albumin increase in  $\gamma\delta$  TCR<sup>-/-</sup> mice at day 3, although statistically significant, may not have biological significance if interpreted in isolation. However, as part of a range of inflammatory parameters including histopathology, fluorescence-activated cell sorting, BALF cytology, and MPO measurements, the BALF albumin increase contributes to the evidence pointing to increased inflammation.

BALF cytology and MPO measurement show that enhanced neutrophil trafficking into and activation within airspaces accounts for the early, accentuated inflammatory response in  $\gamma\delta$  TCR<sup>-/-</sup> mice. The larger amounts of reactive oxygen metabolites and lysosomal enzymes that result from increased neutrophil migration and activation are likely to account for the increased parenchymal injury seen in the  $\gamma\delta$  TCR<sup>-/-</sup> animals. The absence of  $\gamma\delta$  T cells also results in enhanced macrophage extravasation to the interstitium and alveoli. This event could have further contributed to pulmonary injury, given that  $\gamma\delta$  T cells can prevent macrophage-mediated tissue damage by lysing activated macrophages (12). Alternatively, this increased macrophage influx could reflect a compensatory response to remove the increased numbers of neutrophils and debris in the airspaces of  $\gamma\delta$  TCR<sup>-/-</sup> mice. Taken together, the data support evidence from previous studies that  $\gamma\delta$  T cells play a nonredundant, integrated role in early immune interactions. In

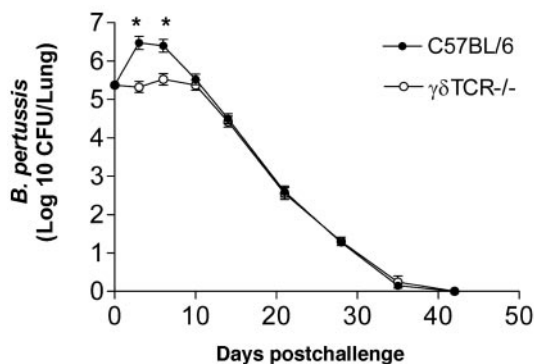


FIG. 5. The early pulmonary bacterial load is reduced in  $\gamma\delta$  TCR<sup>-/-</sup> mice following aerosol challenge with *B. pertussis*.  $\gamma\delta$  TCR<sup>-/-</sup> and WT mice were challenged by aerosol inoculation with *B. pertussis*. The pulmonary bacterial load was monitored at intervals after challenge by performing viable counts on lungs. Results are mean ± SE CFU counts performed on individual lungs in triplicate for 4 mice per group at each time point. \*, *P* < 0.01 (compared to the corresponding values for WT mice).

TABLE 2. Flow cytometric analysis of cells present in BALF from *B. pertussis*-infected  $\gamma\delta$  TCR<sup>-/-</sup> mice or controls<sup>a</sup>

Day postchallenge and mouse type	$\gamma\delta$ T cells (10 <sup>4</sup> cells per ml)	NK cells (10 <sup>4</sup> cells per ml)	T cells (10 <sup>4</sup> cells per ml)	NK T cells (10 <sup>4</sup> cells per ml)
Unexposed controls				
WT	<0.01 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	<0.01 ± 0.00
$\gamma\delta$ TCR <sup>-/-</sup>	0.00 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	<0.01 ± 0.00
3				
WT	4.26 ± 0.20	20.35 ± 0.96	30.69 ± 1.63	3.81 ± 0.15
$\gamma\delta$ TCR <sup>-/-</sup>	0.00 ± 0.00	29.86 ± 1.61**	41.84 ± 2.85*	3.75 ± 0.08
6				
WT	2.10 ± 0.10	4.00 ± 0.21	7.49 ± 0.40	13.44 ± 1.63
$\gamma\delta$ TCR <sup>-/-</sup>	0.00 ± 0.00	5.84 ± 0.40*	4.15 ± 0.28*	8.13 ± 2.85*
10				
WT	3.40 ± 0.16	41.31 ± 2.21	86.56 ± 5.11	4.05 ± 2.60
$\gamma\delta$ TCR <sup>-/-</sup>	0.00 ± 0.00	81.06 ± 5.71*	83.14 ± 3.96	2.67 ± 0.18*

<sup>a</sup> Numbers of  $\gamma\delta$  T cells, NKR<sup>+</sup> CD3<sup>-</sup> (NK) cells, NKR<sup>-</sup> CD3<sup>+</sup> cells (T cells), and NKR<sup>+</sup> CD3<sup>+</sup> cells (NKR<sup>+</sup> T cells) in BALF from  $\gamma\delta$  TCR<sup>-/-</sup> or WT mice following aerosol *B. pertussis* challenge or from unexposed controls. Cells were labeled with monoclonal antibodies directed against  $\gamma\delta$  TCR, NK1.1, and CD3 and analyzed by flow cytometry. Increased numbers of NK cells and of NKR<sup>-</sup> CD3<sup>+</sup> T cells were noted in BALF from  $\gamma\delta$  TCR<sup>-/-</sup> mice compared to WT mice at days 3, 6, and 10 and at day 3, respectively. Reduced numbers of NKR<sup>-</sup> CD3<sup>+</sup> T cells and of NKR<sup>+</sup> CD3<sup>+</sup> T cells were detected in the  $\gamma\delta$  TCR<sup>-/-</sup> mice at day 6 and at days 6 and 10, respectively. Results are means ± SE from six mice per group. \*,  $P < 0.01$ ; \*\*,  $P < 0.05$  (compared to the corresponding value for WT mice).

their absence, potentially harmful inflammatory responses mediated by neutrophils (11, 15, 34, 43, 44) and macrophages (12, 47) are enhanced (4). It must also be considered that BALF cytology may underestimate the extent of macrophage extravasation in response to infection, as many macrophages remain in the interstitial space rather than migrating directly into alveoli in a manner similar to neutrophils (42).

In previous studies using  $\gamma\delta$ -T-cell-depleted mice, the more extensive hepatic and pulmonary neutrophil infiltration observed following *L. monocytogenes* (15) and *M. tuberculosis* (11) infection, respectively, suggested  $\gamma\delta$  T cells influence neutrophil migration. In line with current findings, intraperitoneal inoculation of  $\gamma\delta$ -T-cell-depleted mice with *L. monocytogenes* by Fu et al. (15) resulted in more extensive, neutrophil-rich lesions than controls within 10 days postinfection. Furthermore, these  $\gamma\delta$ -T-cell-depleted mice exhibited reduced bacterial loads. It is likely that  $\gamma\delta$  T cells form part of an integrated

signaling network that result in complex interactions with a multitude of other host immune and stromal cells. This may be affected by  $\gamma\delta$ -T-cell production of a range of chemokines and cytokines, including CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , CCL5/RANTES, lymphotactin, IFN- $\gamma$ , IL-6, IL-10, or IL-12, that influence leukocyte trafficking and activation (14, 17, 19, 20, 29, 38, 43, 47). While this study has shown that  $\gamma\delta$  TCR<sup>-/-</sup> limit pulmonary injury, the precise mechanism operating here awaits further study. In particular, it is known that specific subpopulations of  $\gamma\delta$  T cells such as V $\delta$ 1 T cells perform highly specific and often opposing functions in different tissues and during different infections (7). The murine respiratory challenge model of *B. pertussis* infection would be an ideal system to study the function of these subpopulations in a clinically relevant model. It is also likely that the absence of  $\gamma\delta$  T cells results in other changes in the integrated functions of the immune response. For example,  $\gamma\delta$  T cells are important in

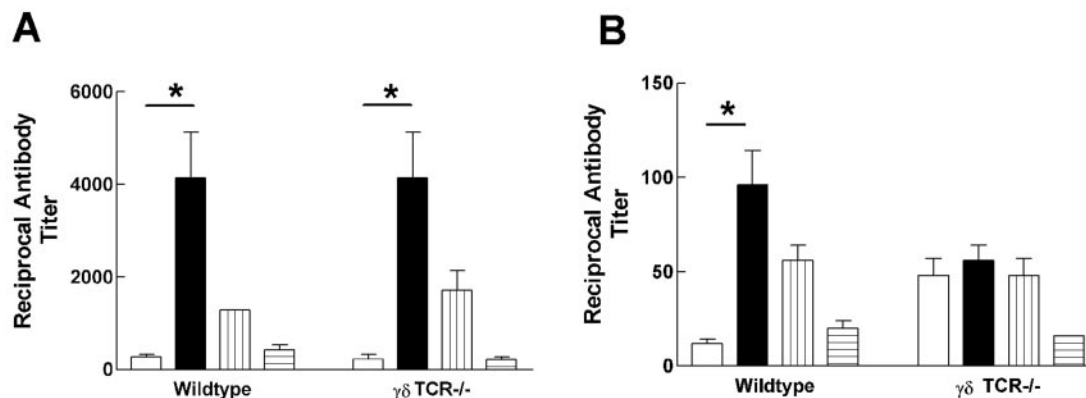


FIG. 6. Antibody responses in *B. pertussis*-infected WT or  $\gamma\delta$  TCR<sup>-/-</sup> mice against inactivated whole bacteria (A) or the *B. pertussis* virulence factor FHA (B). Increased IgG1 and reduced IgG2a was noted in  $\gamma\delta$  TCR<sup>-/-</sup> compared to WT mice. Subclasses of *B. pertussis*- or FHA-specific IgG were measured from serum sampled 43 days after aerosol challenge of  $\gamma\delta$  TCR<sup>-/-</sup> or WT mice. IgG1 (open bar), IgG2a (solid bar), IgG2b (vertical stripes), and IgG3 (horizontal stripes) were measured by ELISA. Results are means ± SE of ELISA titers from 4 mice per group measured in quadruplicate. \*,  $P < 0.01$  (comparing the IgG1 and IgG2a responses).



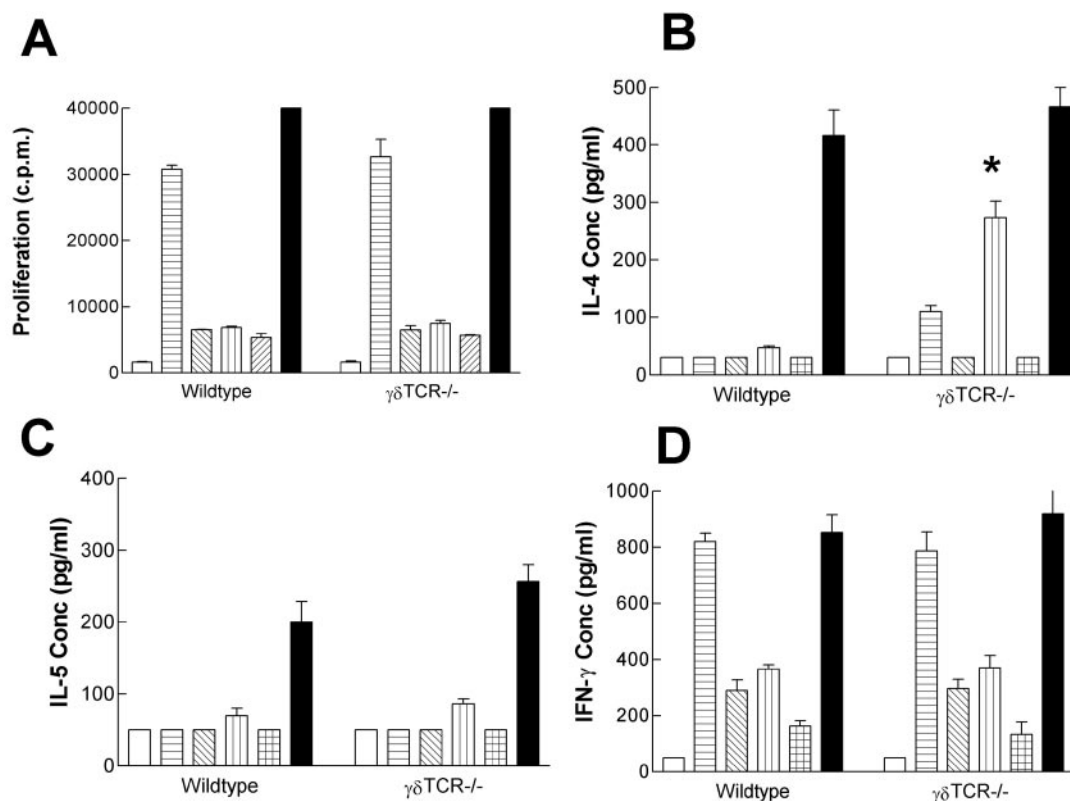


FIG. 7. Enhanced IL-4 production by splenocytes from  $\gamma\delta$  TCR<sup>-/-</sup> mice in response to the *B. pertussis* virulence factor FHA, compared to WT mice. Proliferation (A), IL-4 (B), IL-5 (C), and IFN- $\gamma$  (D) responses to either medium control (open bar), heat-inactivated *B. pertussis* ( $10^4$  CFU per ml equivalent) (horizontal stripes), inactivated pertussis toxin (diagonal stripes), FHA (vertical stripes), pertactin (hatching), or concanavalin A positive control (solid bar) at  $5 \mu\text{g/ml}$  are shown. Results are the means  $\pm$  SE for 4 mice per group, assayed individually in triplicate. \*,  $P < 0.01$  (compared to the corresponding value for WT mice); Conc, concentration.

homeostasis and the modulation of inflammation (6); then the lack of these functions throughout the development of these mice may lead to differential responses from various other cells that are involved in resolving inflammation.

A clearer understanding of the particular influence of  $\gamma\delta$  T cells on the trafficking of neutrophils may have broader significance in whooping cough and in the pathogenesis of conditions such as ARDS. In the nonimmunized neonate, *B. pertussis* infection has a very high mortality and is characterized by elevated neutrophil counts and by necrotizing pulmonary inflammation (40) but no reported bacterial dissemination. Interestingly, this is a period in which there is a switch from a predominant V $\delta$ 1 to a V $\delta$ 2 population in humans (10). However, recent work has shown that V $\delta$ 1 T cells require dendritic or accessory cells to recognize antigen (9, 26), and it is well documented that dendritic cell populations in the neonate are quantitatively and qualitatively impaired (36, 37). Thus, the susceptibility of human neonates to *B. pertussis* and the associated high morbidity and mortality may be associated with a period when  $\gamma\delta$  T cell responses are suboptimal. Our observation of increased neutrophil-mediated inflammation in  $\gamma\delta$  TCR<sup>-/-</sup> mice is consistent with that theory. Similarly, the implication that a temporary suppression of  $\gamma\delta$ -T-cell activity in the lung results in the uncontrolled inflammation associated with ARDS is relevant to the findings of the current study. Previous work has suggested that pulmonary  $\gamma\delta$  T cells were

essential to control inflammation through the anti-inflammatory impact of IL-10 on neutrophils (19).

The current study also demonstrates that the absence of  $\gamma\delta$  T cells alters pulmonary recruitment of particular lymphocyte subtypes. Flow cytometry of BALF indicated differences in the movement of NK, NKR<sup>+</sup> T, and NKR<sup>-</sup> T cells into airways in the absence of  $\gamma\delta$  T cells. NKR<sup>+</sup> T cells and  $\gamma\delta$  T cells are often erroneously considered to perform identical functions, serving merely to amplify adaptive immunity or provide endogenous danger signals. However, this study and others indicate that both  $\gamma\delta$  and NKR<sup>+</sup> T cells perform nonredundant functions in immunity. This is seen in the striking contrast between the present study and a previous study (5). Using a similar approach, that study demonstrated that deletion of NKR<sup>+</sup> cells resulted in a disseminating and lethal *B. pertussis* infection in mice, reminiscent of that seen in IFN- $\gamma$  knockout mice (28). In contrast, we found that absence of  $\gamma\delta$  T cells did not result in disseminated infection, atypical extrapulmonary pathology, or increased mortality (data not shown). Taken together, these data indicate that, in the murine model of *B. pertussis* infection, the principle function of NKR<sup>+</sup> T cells is to provide IFN- $\gamma$  early in the host response to infection to prevent bacterial dissemination, whereas  $\gamma\delta$  T cells appear to have a greater influence on inflammatory cell trafficking.

The observation that the cytokine response to *B. pertussis* antigens is dominated by IFN- $\gamma$  in both WT and  $\gamma\delta$  TCR<sup>-/-</sup>

mice suggests that  $\gamma\delta$  T cells are not essential for the development of a Th1 response. However, this study suggests that these cells do influence the quality of adaptive immunity.  $\gamma\delta$  TCR<sup>-/-</sup> mice had a significantly greater Th2 response to the *B. pertussis* virulence factor FHA, not evident in WT controls. This supports findings of a previous study that demonstrated a role for  $\gamma\delta$  T cells in antigen presentation and IL-12 production, suggesting this cell population may contribute to the Th1 response evoked by infection (39). However, the observed IL-4-dominated response to FHA seen in this study may reflect a loss of a  $\gamma\delta$  T cell-dendritic cell interaction involving FHA. This would support previous findings suggesting that such an interaction results in IL-10 production (13, 31, 51).

The findings of this study suggest that, although less than 3% of the pulmonary leukocyte population in mice (19),  $\gamma\delta$  T cells have a critical, nonredundant role in the initial inflammatory response to a gram-negative bacterial infection. Current evidence suggests that their function includes the prevention of exaggerated, harmful inflammatory host responses through their influence over the movement of key inflammatory effector cells and the regulation of the host response to bacterial antigens. Such findings are relevant to the study of conditions such as ARDS where temporary suppression of  $\gamma\delta$ -T-cell function is thought to contribute to increased pulmonary inflammation in response to sepsis (19).

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