Comparative Medicine
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Vol 52, No 4
August 2002
Pages 322-327

Characterization of Lymphocyte Subsets in the Bronchiolar Lymph Nodes of BALB/c Mice Infected with Cilia-Associated Respiratory Bacillus

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Cilia-associated respiratory (CAR) bacillus is an unclassified, gram-negative, extracellular bacterium that causes chronic respiratory tract disease in rodents. Infected mice develop microscopic lesions characterized by a primary lymphocytic response followed by macrophage and neutrophilic infiltration. To characterize the lymphocytic subsets that respond to CAR bacillus infection, BALB/c mice were inoculated with 10^5 CAR bacillus bacteria. At seven weeks after inoculation, mice were euthanized and the tracheobronchiolar and hilar lymph nodes were collected and stained for cell surface markers to T cells (CD3, CD4, and CD8), B cells (B220, CD5), natural killer (NK) cells (pan-NK) and intracellular interleukin 10 (IL-10) and interferon-γ (IFN-γ). Flow cytometric analysis of lymph nodes from CAR bacillus-infected mice revealed 11% increase in frequency of B cells ($B220^+$), 12% increase in the frequency of double-negative ($CD4^-CD8^-CD3^+$) T cells, and slight increase in the B-1 subset of B cells ($B220^+CD5^+$). There was no change in the frequency of NK cells. The CAR bacillus-infected mice had an overall decrease in the frequency of T cells. Intracellular cytokine staining revealed distinct populations of T cells producing IL-10 and IFN-γ, and IL-10 production from B cells; NK cells were not a substantial source of IFN-γ. To our knowledge, this is the first characterization of lymphocytic responses and suggestion that B cells and double-negative T cells may be principally responsible for the lesions associated with CAR bacillus infection.

The cilia-associated respiratory (CAR) bacillus is an unclassified, extracellular, gram-negative, gliding bacterium that causes respiratory tract disease in mice and rats (1-6). The CAR bacillus was first identified in a colony of aging rats with chronic respiratory tract disease (1), and was later cultured and characterized (2). The host immune response to the long filamentous bacterium is unusual. The normal immune response to extracellular bacterial pathogens is mediated by alveolar macrophages and recruitment of neutrophils within the first few hours after infection (7). This innate immune response typically is effective at clearing many respiratory tract pathogens and prevents colonization, as documented in mouse pneumonia models of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (8, 9). However, a primary lymphocytic response is seen in mice infected with CAR bacillus. Lesions in mice experimentally infected with CAR bacillus are evident microscopically, beginning at postinoculation day 21 (10), and include mild mucosal hyperplasia and mild peribronchiolar lymphocytic cuffing, without evidence of activated alveolar macrophages or neutrophils. In late stages of disease, neutrophilic infiltrates are present in the bronchiolar lumen, alveolar macrophages accumulate in the alveoli, peribronchiolar lymphoid infiltrates form follicles, and the draining lymph nodes are markedly enlarged.

Susceptible BALB/c mice have a predominant interleukin (IL-) 10 response along with an increase in interferon (IFN)-γ activity (10, 11). Potential cellular sources of IFN-γ and IL-10 include T cells, B cells, natural killer (NK) cells, and macrophages (12, 13). The nature of the inflammatory response associated with CAR bacillus disease suggests that lymphoid cell populations predominate in the host's response. To further characterize this cellular response, BALB/c mice were experimentally infected, and the tracheobronchiolar and hilar lymph nodes were collected at postinoculation week seven to examine the lymphocyte populations by use of flow cytometry.

Materials and Methods

Culturing of the CAR bacillus. A CAR bacillus isolate obtained from a mouse (provided by Dr. Tom Spencer, NIH) was maintained in cell culture on murine 3T3 fibroblasts in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (2, 14). Bacterial pellets were collected from culture flasks by use of differential centrifugation as described (10). Briefly, cellular debris was removed by centrifugation at 900 ×*g* for 10 min, and bacteria were pelleted from the supernate by centrifugation at 20,000 ×*g* for 10 min, then were re-suspended in 1 ml of phosphate-buffered saline (PBS). Bacterial concentration was determined microscopically, using a hemocytometer, and suspensions were diluted to achieve a final inoculum containing

Received: 12/28/01. Revision requested: 2/12/02. Accepted: 4/30/02.
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Supported by Department of Health and Human Services grants RR08624-01 and 5 T32 RR 07004-22 from the National Institutes of Health.
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10^5 CAR bacillus organisms/30 μl of PBS. This concentration has been documented to be an adequate infective dose to induce CAR bacillus disease in BALB/c mice (10). The inoculum was screened by use of polymerase chain reaction (PCR) analysis to ensure that it was free of *Mycoplasma* sp. contamination, as described (10).

Experimental model. Six-week-old female BALB/c mice, free of known pathogens including the respiratory tract pathogens, CAR bacillus, *Mycoplasma pulmonis*, Sendai virus, and pneumonia virus of mice, were obtained from the Frederick Cancer Research and Development Facility (Frederick, Md.). Mice were group housed in polycarbonate microisolator cages (Allentown Caging, Allentown, Pa.) in accordance with the *Guide for the Care and Use of Laboratory Animals* (15), and were allowed ad libitum access to Autoclavable Laboratory Rodent Diet 5010 (Purina Mills, Inc. Richland, Ind.) and tap water. Sentinel mice (VAF/Plus; adult, female, Crl:CD-1 mice from Charles River Laboratories, Inc., Wilmington, Mass.) were monitored at the conclusion of the study and were free of antibodies to CAR bacillus, *M. pulmonis*, Sendai virus, and pneumonia virus of mice, as well as other adventitious pathogens of mice. All experiments were approved by the University of Missouri Institutional Animal Care and Use Committee.

Mice were anesthetized with isoflurane, and a skin incision was made on the ventral surface of the neck to expose the trachea. After intratracheal instillation of 10^5 CAR bacillus in 30 μl of PBS, the skin incision was closed with surgical adhesive (Nexaband, Veterinary Products Laboratories, Phoenix, Ariz.). Control mice were injected with 30 μl of PBS. Two studies were performed: the first comprised eight control and 12 infected mice, the second had 15 control and 10 infected mice.

Mice were euthanized during postinoculation week seven by use of carbon dioxide asphyxiation. Blood samples were obtained by cardiocentesis, and the sera were stored at -70°C. The tracheobronchiolar and the hilar lymph nodes were collected from the mice. The single pair of lymph nodes from each infected mouse was adequate for analysis; however, lymph nodes from three to four control mice had to be pooled to attain sufficient cell numbers for analysis.

Select inoculated mice were screened for antibodies to *M. pulmonis* by use of an ELISA (Research Animal Diagnostic Laboratory, Columbia, Mo.), and for *Mycoplasma* spp. contamination by use of PCR amplification of paraffin-embedded lung sections as described (10). Results of serologic assays and PCR analysis of lung sections from CAR bacillus-infected mice were uniformly negative for contaminating *Mycoplasma* spp.

Histologic examination. A portion of the left lung lobe at the level of the left bronchus was fixed in Omnifix (Ancon Genetics, Inc., Melville, N.Y.) and processed for histologic evaluation. Specimens of lung tissue were embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin to evaluate lesion severity. Another 5-μm section was stained with a Steiner silver stain to demonstrate CAR bacillus colonization (16). Colonization was confirmed in all infected mice.

Lymphocyte isolation and stimulation. Freshly collected lymph nodes were placed in 3 ml of filter-sterilized medium (RPMI 1640 medium with 10% FBS, 15 m*M* HEPES, 200 μg of gentamycin sulfate/ml, pH 7.2). All chemicals and media were obtained from Sigma Chemical Company (St. Louis, Mo.). Cells were released by teasing lymph nodes apart, using a 25-gauge needle. The remaining tissue was pulverized in a petri dish using the flat end of a syringe plunger. The suspension was then passed through an 18-gauge needle several times and expelled through a 0.38-mm mesh screen into a conical centrifuge tube. The suspension was centrifuged at 500 ×*g* for 10 min at 4°C, and the pellet was re-suspended in 10 ml of medium, centrifuged, and re-suspended in 4 ml of medium. The number of viable cells was determined by use of trypan blue dye exclusion, and cells were resuspended in medium to achieve a final concentration of 10^6 viable cells/ml for infected mice and 2×10^5 viable cells/ml for pooled control mice.

Isolated cells were stimulated for four hours at 37°C by addition of phorbol myristate acetate (50 ng/ml of a solution containing 200 μg/ml of ethanol) and ionomycin (500 ng/ml of a solution containing 2.5 mg/ml of DMSO) as described (17). Monensin (GolgiStop, Pharmingen, San Diego, Calif.) was concurrently added during cell stimulation to inhibit cytokine secretion from the Golgi apparatus. Stimulated cells were centrifuged at 500 ×*g* for five min at 4°C, re-suspended in filter-sterilized staining buffer (Dulbecco's phosphate-buffered saline without Mg^{2+} or Ca^{2+}, 1% heat inactivated FBS, 0.09% sodium azide, pH 7.4), and 1.0-ml aliquots containing 10^6 cells (2×10^5 cells for control samples) were placed into 1.5-ml tubes.

Antibody combinations. Three-color flow cytometric analyses, using monoclonal antibodies (mAb) (all antibodies from Pharmingen, San Diego, Calif.), were performed to identify subsets of T and B lymphocytes and intracellular cytokine production. The T cells were identified, using a conjugated anti-CD3 mAb, and T-cell subsets were identified, using conjugated anti-CD4 and CD8 mAb. The B cells were identified, using a conjugated anti-B220 mAb, and the B-1 subset was identified, using a conjugated anti-CD5 mAb. Two-color flow cytometry was used to assess NK cells and IFN-γ production. The antibody combinations are given in Table 1.

Cell surface antigen and intracellular cytokine staining. Cell aliquots were centrifuged at 500 ×*g* for five min at 4°C and resuspended in 50 μl of staining buffer. Cells were stained for surface antigens and intracellular cytokines following the manufacture's instructions (Pharmingen). Cell surface antigens were stained with 0.06 μg of anti-CD3, CD4, CD5, CD8, B220 (0.5 mg/ml), 0.5 μg of pan-NK (0.5 mg/ml), or equivalent amount of irrelevant isotype control antibodies for 30 min at 4°C. Cells were pelleted at 500 ×*g* and washed twice with staining buffer. Cells were then fixed and permeabilized (Cytofix/CytoPerm, Pharmingen) for 20 min at 4°C, and washed twice. Permeabilized cells were incubated for 30 min at 4°C with 1.0 μg of conjugated anti-cytokine mAb (either anti-IL10 and/or anti-IFN-γ) or irrelevant isotype control antibodies for intracellular cytokine staining. Cells were centrifuged, washed twice, and transferred to a polystyrene tube for flow cytometric analysis (FACScan Becton Dickinson Immunoytometry Systems, Palo Alto, Calif.).

Using CELLQUEST software (Becton Dickinson, Franklin Lakes, N.J.), frequency of positive-staining cells was evaluated. Typically 20,000 events were acquired from control mice, and at least 50,000 events were acquired from experimental mice, excluding dead cells and monocytes by use of forward and side scatter gating. Statistical quadrants were set on the basis of a frequency of < 2% in isotype control samples. The data are reported as the averaged percentage of positive-staining cells from individual mice from a single experiment. Similar trends

Table 1. Monoclonal antibody pairs used for flow cytometric analysis

	Antibody[a] combinations		
1	FITC[b] anti-CD3 (17A2)[f]	PE[c] anti-CD4 (GK1.5)	APC[d] anti-CD8 (53-6.7)
2	FITC anti-CD3 (17A2)	PE anti-IL-10 (JES5-16E3)	APC anti-IFN (XMG1.2)
3	FITC anti-B220 (RA3-6B2)	Cy[e] anti-CD5 (53-7.3)	PE anti-IL-10 (JES5-16E3)
4	PE anti-pan-NK (DX5)	APC anti-IFN (XMG1.2)	

[a]All antibodies are rat monoclonal antibodies.
[b]Fluorescein isothyocyanate conjugated.
[c]R-phycoerythrin conjugated.
[d]Allophycocyanin conjugated.
[e]Cy-Chrome conjugated.
[f]Monoclonal antibody designation.

were seen in replicate studies.

Statistical analysis. Data were evaluated, using SigmaStat Statistical Software (SPSS Marketing, San Rafael, Calif.). Differences in cellular phenotypes were analyzed, using Student's *t* test, when data were normally distributed. If the normality test failed, a Mann-Whitney rank sum test was performed. A value of $P < 0.05$ was considered statistically significant.

Results

Lymphocytic response. The lymphocytic responses of mice chronically infected with CAR bacillus were assessed by isolating lymphocytes from regional lymph nodes. At gross necropsy, the tracheobronchiolar and hilar lymph nodes of CAR bacillus-infected mice were markedly enlarged, compared with those of sham-infected control mice. Lymphocytes were isolated and counted. The 17-fold increase in total lymphocytes isolated from CAR bacillus- infected mice was significantly ($P < 0.01$) greater, compared with values for sham-infected controls (Table 2). Consequently, there was an increase in all cell populations.

Analysis of B cells. Lymphocytes from the tracheobronchiolar and hilar lymph nodes were examined by use of flow cytometry for the frequency of B cells, the B-1 subset of B cells, and simultaneous production of IL-10 from each of these B cell populations. The B cells were the most prominent cell type in the lymph nodes of CAR bacillus-infected mice, representing 46.7% of the total cell population. This was an 11.1% increase ($P < 0.03$), compared with values for control mice (Fig. 1, Table 2). The B cells were further evaluated for CD5 expression to identify B-1 cells. The CAR bacillus-infected mice had only a 1.1% increase in B-1 cells ($P > 0.05$, Fig. 1, Table 2). There was an increase in B cells that produced IL-10 from CAR bacillus-infected mice, with a 6.6% frequency, compared with 4.6% for control mice ($P < 0.03$, Fig. 2). However, the frequency of IL-10-producing B-1 cells was 4.1% in infected mice, compared with 5.1% in control mice ($P > 0.05$).

Analysis of NK cells. Numbers of NK cells were evaluated from draining lymph nodes of CAR bacillus-infected mice, and their IFN-γ production was analyzed by use of two-color flow cytometry. The population of NK cells in lymph nodes from CAR bacillus-infected mice remained unchanged, compared with that of control mice (Table 2). Intracellular cytokine staining did not indicate that NK cells were an appreciable source of IFN-γ, as the frequency of NK cells from control and infected mice that were producing IFN-γ was < 1% ($P > 0.05$, data not shown).

Analysis of T cells. The T-cell responses were evaluated from the total lymphocyte population of lymph nodes from CAR bacillus-infected mice, using three-color analysis with CD3, CD4, and CD8 cell surface markers. There was a decrease in the CD4 and CD8 populations of $CD3^+$ cells; however, the CD4-to-CD8 ratio was equivalent in control and infected mice. There was a 12% increase ($P < 0.01$) in the $CD4^-CD8^-$ (double-negative) population of $CD3^+$ cells (Fig. 1, Table 2). The low numbers of double-positive ($CD4^+CD8^+$) $CD3^+$-staining cells likely represents background artifact because double-positive cells do not usually exist in peripheral lymph nodes.

Table 2. Total cellularity and percentage of cells expressing surface marker in lymph nodes of CAR bacillus-infected BALB/c mice[a]

	Control mice (n = 15)	Infected mice (n = 10)	
Total cell numbers	1.0×10^6 (0.5)	17.0×10^6 (1.6)	
	Frequency	Frequency	% Change
T cells			
CD4[b]	65.5 (2.2)	60.9 (1.9)	-4.6
CD8[b]	27.2 (1.0)	22.8 (0.8)[f]	-4.4
DP[b,c]	4.0 (2.0)	2.6 (0.3)	-1.4
DN[b,d]	3.2 (1.1)	15.3 (2.5)[f]	+12.1
B cells			
B220	35.6 (3.6)	46.7 (1.0)[f]	+11.1
CD5[e]	11.8 (1.9)	12.9 (0. 7)	+1.1
NK cells			
Pan-NK	2.5 (0.3)	2.8 (0.2)	+0.03

[a]The data presented represent the mean (SEM) percentage of cells from individual animals of experiment 2. Similar trends were observed in experiment 1.
[b]Represents the frequency of cells expressing the surface marker after back gating on $CD3^+$ cells.
[c]DP = double-positive, expressing CD4 and CD8 surface marker on $CD3^+$ cells.
[d]DN = double-negative, expressing neither CD4 nor CD8 surface marker on $CD3^+$ cells.
[e]Represents the frequency of cells expressing CD5 surface marker after back gating on $B220^+$ cells.
[f]Represents significant difference ($P < 0.03$) between infected and control mice.

The $CD3^+$ cells also were evaluated for cytokine production of IFN-γ and IL-10. Numbers of IFN-γ-producing $CD3^+$ cells were increased ($P < 0.05$) in CAR bacillus-infected mice (Fig. 2). The frequency of IFN-γ producing cells was 8.1%, compared with 5.2% of the cells in control mice. Furthermore, the geometric mean fluorescence intensity was 45.4 for infected mice, compared with 28.8 for control mice, suggesting that cells from infected mice produced more IFN-γ ($P > 0.05$).

Numbers of IL-10-producing $CD3^+$ cells in CAR bacillus-infected mice also were increased ($P < 0.05$). Infected mice had a frequency of 5.1%, compared with 3.7% for controls (Fig. 2). The geometric mean fluorescence intensity was 39.6 and 37.7 for infected and control mice respectively; suggesting that the rate of IL-10 production was unchanged ($P > 0.05$).

Discussion

Mice infected with CAR bacillus mount an immune response evident by increases in serum antibody, local cytokine production, and cellular infiltrates in the pulmonary parenchyma (10, 11). The histologic lesions characteristic of CAR bacillus-in-

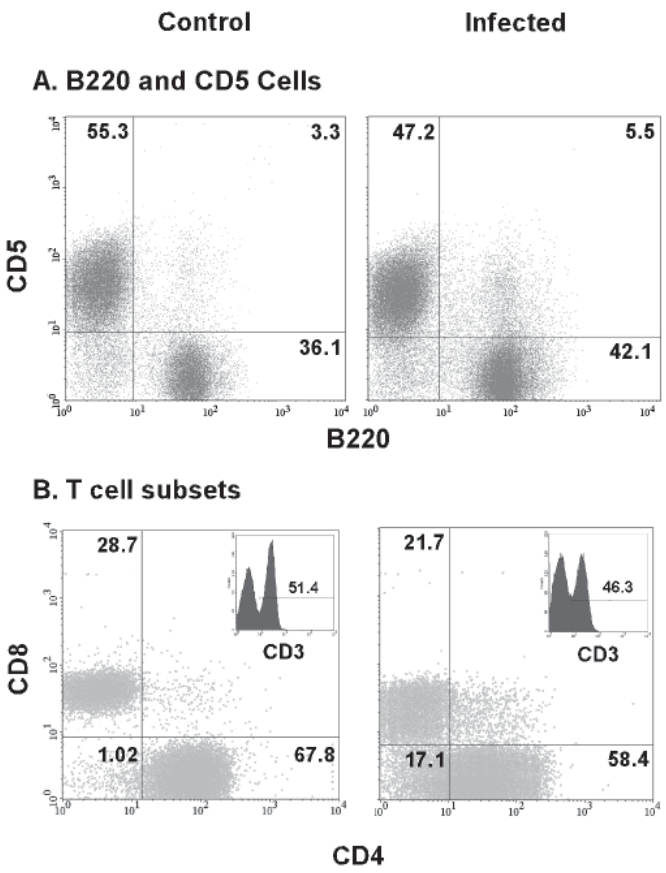

Figure 1. Flow cytometric analysis of B cells (A) and T cells (B). These dot plots are from individual mice representative of lymphocytic subsets observed in mice infected with the cilia-associated respiratory (CAR) bacillus. The frequency of $CD3^+$ cells is shown in the histogram insets in Figure 1B. The CD4 and CD8 populations of those $CD3^+$ cells are represented in the dot plot. Mean values for the frequency of B and T cells are given in Table 2.

duced disease including marked peribronchiolar lymphocytic infiltrates, mucosal hyperplasia, infiltrates of alveolar macrophages and neutrophils, and persistent bacterial colonization; all were observed in mice of this study. In addition, enlargement of the tracheobronchiolar and hilar lymph nodes was evident. The lymphocytic response was evaluated ex vivo, using flow cytometric analysis of lymphocytes isolated from draining lymph nodes.

Our results indicated that, although CAR bacillus-infected mice have an increase in all cellular populations, there is increased frequency of B cells, B-1 cells, and double- negative T cells. We previously reported that immunoglobulin (Ig) M and IgG3 responses in susceptible mice infected with CAR bacillus are markedly increased (10). Furthermore, CAR bacillus-infected mice with B-cell deficiencies develop less severe lesions, with decreased IgM and IgG3 responses (18), suggesting that B cells and the immunoglobulins they produce are critical components of the immunopathogenesis of CAR bacillus-induced disease. Because IgM and IgG3 are associated with T cell-independent (TI) antigen stimulation of B cells (19, 20), we hypothesize that CAR bacillus initiates disease through a TI antigen-dependent mechanism. The findings of the study reported here support this hypothesis in that B cells were found to be the predominant cell type in the lymph nodes of CAR bacillus-infected mice.

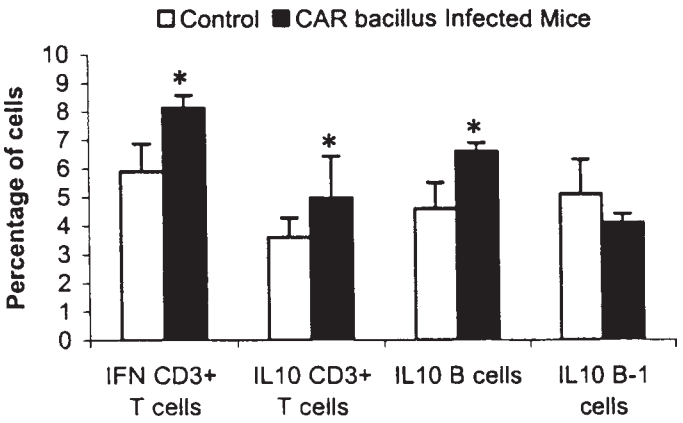

Figure 2. Cytokine production from $CD3^+$ T cells, B cells, and B-1 cells from CAR bacillus-infected mice. Error bars represent the SEM values. *Represents significant ($P < 0.03$) difference between infected and control mice.

The TI antigens are typically polysaccharide antigens that stimulate antibody production from B cells without T-cell help (21). The mucopolysaccharide capsule and lipopolysaccharide of CAR bacillus (22) are potential TI antigens that may be responsible for initiating and maintaining the chronic immune response to CAR bacillus. Further support for this hypothesis can be found in studies of *Mycoplasma pulmonis* infections in mice. *Mycoplasma pulmonis* induces lesions that are virtually identical to those seen in CAR bacillus-infected mice. The *M. pulmonis*-susceptible mice also have increased antibody responses, compared with those in resistant mice (23), and *M. pulmonis*-infected mice with deficiencies in B cells have been observed to develop less severe disease than do their immunocompetent counterparts, with decreased IgM and IgG3 concentrations (24). Collectively, these findings suggest that TI antigen-mediated stimulation of B cells initiates and/or perpetuates CAR bacillus disease. However, further studies are necessary to confirm or refute this hypothesis.

The role of T cells in the development of CAR bacillus disease is uncertain; however, athymic nude mice infected with CAR bacillus have lesions similar to those of immunocompetent mice (18). Similarly, T cell-deficient mice infected with *M. pulmonis* are as susceptible to disease as are immunocompetent controls (25). This suggests that T cells may have a limited role in the development of CAR bacillus-induced disease. The study reported here documented that CAR bacillus-infected mice have decreased frequency of CD4- and CD8-positive T cells, compared with frequency in control mice, with a similar CD4-to-CD8 ratio. These findings support the hypothesis that lung pathologic changes are sequelae to the immune response to a TI antigen.

In this study, we also identified an increase in double-negative $CD3^+$ T cells in CAR bacillus-infected mice. These double-negative cells may represent γδ T cells, which usually are found principally at epithelial borders and recognize a limited repertoire of antigens similar to the function of B-1 cells. They secrete inflammatory and anti-inflammatory cytokines in response to bacterial and host antigens (26, 27), such as that seen in response to CAR bacillus infection (10, 11). Additionally, γδ T cells may be present during the acute phase of infection or in the late stage of infection (28), explaining their presence in the chronic states of the disease during this study. Further studies are

needed to clarify the role of γδ T cells in the pathogenesis of CAR bacillus-induced disease.

We have previously reported that IFN-γ and IL-10 are expressed in CAR bacillus-induced disease, with a dominant IL-10 response (10, 11); however, the sources of these cytokines were not determined. Intracellular cytokine staining suggests that the increase of IFN-γ activity in susceptible mice is at least partially the result of activated $CD3^+$ T cells. IFN-γ production by pro-inflammatory T cells is an appropriate immune response to a bacterial pathogen; however, in one of our previous studies, we documented a 25-fold increase in mRNA production of IL-10 in CAR bacillus-infected mice (11). That IL-10 response probably antagonized IFN-γ production, resulting in susceptibility to infection.

Results of this study documented IL-10 production from $CD3^+$ T cells and B cells, but not appreciable production by B-1 cells, a known source of B cell-derived IL-10 (29). It is unlikely that this IL-10 response accounts for the large increase in mRNA seen in our previous study (11), suggesting that neither T nor B cells are a substantial source of IL-10 in CAR bacillus-induced disease. Since IL-10 can originate from a variety of cell types, including T cells, B cells, macrophages, and epithelial cells (30, 31), it is likely that macrophages are the principal source of this down-regulatory cytokine in CAR bacillus-infected mice.

In conclusion, B cells and double-negative $CD3^+$ T cells are the predominant lymphocyte populations in the lymph nodes of CAR bacillus-infected mice. A portion of the IFN-γ response during CAR bacillus infection originates from T cells, but not NK cells, and portions of the IL-10 response originates from T cells and B cells. However, other cellular sources, such as macrophages, likely contribute to the overwhelming IL-10 response associated with CAR bacillus-induced disease (11). The cellular source of IL-10 and the role of IL-10 in the pathogenesis of CAR bacillus disease will be the subject of future studies.

Acknowledgments

We thank Beth Livingston for technical support, Louise Barnett of the Cell and Immunobiology Core Facility for assistance with flow cytometric analysis, and Kim Mullinax, Laurie Roesel, and the Research Animal Diagnostic and Investigative Laboratory support staff for their assistance.

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