Increased neutrophil influx but no impairment of protective immunity to tuberculosis in mice lacking the CD44 molecule

Andre Kipnis,*,[1] Randall J. Basaraba,* Joanne Turner,[†] and Ian M. Orme*

*Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins; and
[†]Department of Internal Medicine, Center for Microbial Interface Biology, The Ohio State University, Columbus

Abstract: Up-regulation of expression of the cell-surface marker CD44 is a major characteristic of T lymphocytes responding in the lungs of mice infected with *Mycobacterium tuberculosis*. These lymphocytes express an activated/memory phenotype as seen by their high expression of the CD44 molecule and low expression of CD62L and CD45RB cell-surface molecules. Based on increasing evidence that the CD44 molecule participates in several aspects of the inflammatory response, we evaluated its role in the response to infection with *M. tuberculosis* using gene-disrupted mice. In this report, we show that CD44 expression is not necessary for the proper trafficking of protective cells to the lungs of mice infected with *M. tuberculosis* or the direct expression of protective immunity leading to control and containment of the bacterial load in this organ. However, although there were no differences in the bacterial load or migration of activated T lymphocytes to the inflamed lung, the absence of the CD44 molecule resulted in a substantially increased accumulation of neutrophils in the lung. These data indicate that loss of CD44 expression does not alter expression of T helper cell type 1 immunity to tuberculosis in the lungs but has major effects on the overall cellular composition of the immunopathological response. *J. Leukoc. Biol.* 74: 992–997; 2003.

Key Words: *mouse · tuberculosis infection · lung · cell recruitment · lung inflammation*

INTRODUCTION

Mice infected by aerosol exposure to *Mycobacterium tuberculosis* gradually develop severe granulomatous inflammation in the lungs. The granuloma formation is dependent on the secretion of cytokines such as tumor necrosis factor α (TNF-α) [1] and CC chemokine ligand 2 [2, 3], as well as the expression of appropriate adhesion molecules by endothelial cells and circulating leukocytes. The role of granuloma formation encompasses innate and acquired immunity, the latter involving the specific influx of protective T cells and macrophages to prevent or limit dissemination of the infection from the lungs [4, 5]. Successful containment of the infection requires the influx of T cells and mononuclear phagocytes from the blood [6], leading to activation of the latter by interferon-γ (IFN-γ) [7, 8].

Migration of T cells into sites of inflammation where immunity must be expressed is an integral and crucial component of cell-mediated immunity. CD44 is an adhesion receptor involved in multiple functions including tissue remodeling and T cell extravasation [9]. More recently, evidence has emerged showing an important role for CD44 in resolving inflammation in the lungs and thus preventing lung-tissue damage. If CD44 is absent as a result of targeted gene disruption [CD44-knockout (KO) mice], then injection of an inflammatory material into the lungs causes massive inflammation, which can be fatal [10]. It would be anticipated, therefore, that pulmonary infection of CD44-KO mice with *M. tuberculosis* would cause uncontrolled inflammation as well as a reduction in the number of T cells capable of entering the lungs.

To address this, wild-type and CD44-KO mice were exposed to a low-dose aerosol infection with virulent *M. tuberculosis*. As the infection progressed, the alveolar spaces and lung tissues showed abnormally high numbers of neutrophils entering these areas, but despite this, no differences were seen in the bacterial load or in the T cell influx over this period of time. These data indicate that although the loss of CD44 on leukocytes results in a severe pyogenic form of granulomatous inflammation in the lungs during tuberculosis infection, its absence did not affect the generation, arrival, or expression of protective immunity. These data thus confirm the important role of CD44 in controlling and limiting lung inflammation, at least in the context of granulocyte influx, but show that CD44 is not an essential molecule needed for T cell entry into the lungs.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type and CD44 gene-disrupted mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free conditions and were used between 8 and 10 weeks of age.

[1] Correspondence: Department of Microbiology, Immunology and Pathology, Colorado State University, 200 West Lake St., 1682 Campus Delivery, Fort Collins, CO 80523-1682. E-mail: akipnis@lamar.colostate.edu

Received June 30, 2003; revised August 1, 2003; accepted August 4, 2003; doi: 10.1189/jlb.0603301.

Bacterial strains

M. tuberculosis strain Erdman, originally obtained from Trudeau Institute (Saranac Lake, NY), was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and then was frozen in aliquots at −70°C until needed.

Bacterial infections

Mice were infected using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN), such that ~100 bacteria were deposited in the lungs of each animal. The number of viable bacteria in target organs was determined at specific time points by plating serial dilutions of partial organ homogenates on nutrient Middlebrook 7H11 agar and counting colonies after 21 days of incubation at 37°C.

Bronchoalveolar lavages (BAL)

The thoracic cavity was opened, the trachea was exposed and cannulated, and the lungs were washed with 1 ml ice-cold phosphate-buffered saline (PBS) by gentle instillation. Cells were washed, resuspended in Dulbecco's minimal essential medium (DMEM; Cellgro, Herndon, VA) containing 10 mM HEPES (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Sigma-Aldrich), and 1% MEM nonessential amino acids (100×; Sigma-Aldrich), supplemented with 10% heat-inactivated endotoxin low fetal calf serum (Atlas Biologicals, Fort Collins, CO), cytospun onto glass slides, and stained with hematoxylin and eosin.

Single-cell suspension preparation

Lungs from infected and age-matched control animals were perfused through the heart with cold PBS containing 30 U/mL heparin (Sigma-Aldrich). After removal, the lungs were sectioned in ice-cold media using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0.7 mg/ml; Sigma-Aldrich) and DNase (30 μg/ml; Sigma-Aldrich) at 37°C for 30 min. A single-cell suspension from digested lung tissue was obtained by passing the organs gently through a 70-μm nylon cell strainer. Red blood cells were lysed using Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$). Cells were resuspended in DMEM with supplements, counted, stained with fluorescent antibodies, and analyzed by flow cytometry.

Flow cytometry

A single-cell suspension was prepared as described above and resuspended in deficient RPMI (dRPMI) containing 0.1% azide. Cells were incubated for at least 1 h with dRPMI containing azide and stained with specific antibody for 30 min at 4°C in the dark. Cells were stained with antibodies (25 μg/ml) recognizing CD4 (peridinin chlorophyll protein-labeled clone GK1.5), CD8 (allophycocyanin-labeled clone 53-6.7), and CD69 [fluorescein isothiocyanate (FITC)-labeled clone H1.2F3]. Control cells labeled with isotype antibodies were also prepared.

For intracellular IFN-γ staining, cells were stimulated with 0.1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51) in the presence of monensin for 4 h at 37°C, 5% CO_2. Cells were stained with antibodies for cell-surface molecules, as described above, before permeabilization with BD Cytofix/Cytoperm (BD PharMingen, San Diego, CA), according to the manufacturer's instructions. FITC anti-IFN-γ (clone XMG1.2) or Ig G1 isotype-control antibody was incubated with the cells for a further 30 min, washed twice, and resuspended in dRPMI with azide before analysis. All antibodies were purchased from BD PharMingen. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA) dual laser flow cytometer with excitation at 488 nm and 633 nm, and data were analyzed using CellQuest software (Becton Dickinson). Lymphocytes were gated based on their forward- and side-scatter (FSC and SSC, respectively) characteristics. Ten thousand CD4- or CD8-positive lymphocytes were then analyzed.

Histology

At specific time points postinfection, the right caudal lung lobe was perfused with 10% formaldehyde-buffered saline. Tissues were sectioned and stained with hematoxylin and eosin. A veterinary pathologist analyzed sections without prior knowledge of the sample identification.

Statistical analysis

Student's *t*-test was used for comparisons of means, and values of $P < 0.05$ were considered statistically significant.

RESULTS

Mice lacking CD44 are equally resistant to pulmonary tuberculosis

To determine if mice lacking the CD44 molecule were more susceptible to *M. tuberculosis*, control, and CD44-KO, mice were exposed to ~100 bacteria by aerosol (**Fig. 1**). The bacterial load in the lung and spleen was monitored for over 65 days, and it was found that mice lacking the CD44 molecule were able to control the bacterial growth in the lung (Fig. 1a) and spleen (Fig. 1b) equally well as compared with wild-type mice.

Cellular recruitment of activated $CD69^+$ T cells to the lung of infected mice was not impaired in CD44-deficient mice

Activated T cells entering the lung were identified by their expression of the C-type lectin CD69 [11]. As shown in **Figure 2**, $CD69^+$, $CD4^+$, and $CD8^+$ T cells accumulated in the lungs of CD44-KO mice in equal amounts to those observed in the wild-type mice, indicating that lack of the CD44 receptor did not influence the expression of CD69.

To determine if effector T cells, which express high levels of CD44 [12, 13], were altered in the CD44-KO mice, isolated lung cells were triggered ex vivo with anti-CD3 and anti-CD28 antibodies together with an inhibitor of the Golgi apparatus. As shown in **Figure 3**, the number of T cells that stained positive for IFN-γ peaked at 25 days postinfection in conjunction with the initial control of the bacterial load (Fig. 1a). Following this, the number of IFN-γ^+ T cells in the lungs decreased through day 65. These data show that the absence of the CD44 receptor did not alter the ability of IFN-γ-positive lymphocytes to accumulate in the lung during infection.

Substantially increased neutrophil influx into the alveolar spaces and lung tissues of infected, CD44-deficient mice

Representative sections of lung from wild-type and CD44-KO mice were evaluated histologically for lesion burden and distribution of inflammatory cell infiltrates. At 10 days postinfection, there were no visible lesions in wild-type or CD44-KO mice. At day 20 postinfection, the lesion burden was similar between the two groups; however, the distribution of inflammatory cells differed. In the CD44-KO mice, the lesions consisted of equal proportions of epithelioid macrophages and lymphocytes; however, neutrophils were prominent within alveoli and small conducting airways compared with the wild-type controls (**Fig. 4**). This cellular distribution was seen cytologically as well, as neutrophils in BAL fluid from CD44-KO mice were the predominant cell type compared with wild-type mice (**Fig. 5** and **Table 1**). Lesions in the CD44-KO mice were more loosely organized than those from the wild-type group, which had discrete accumulations of perivascular and peribronchiolar aggregates of lymphocytes, some of which involved bronchiolar lumina (Fig. 4). Individual

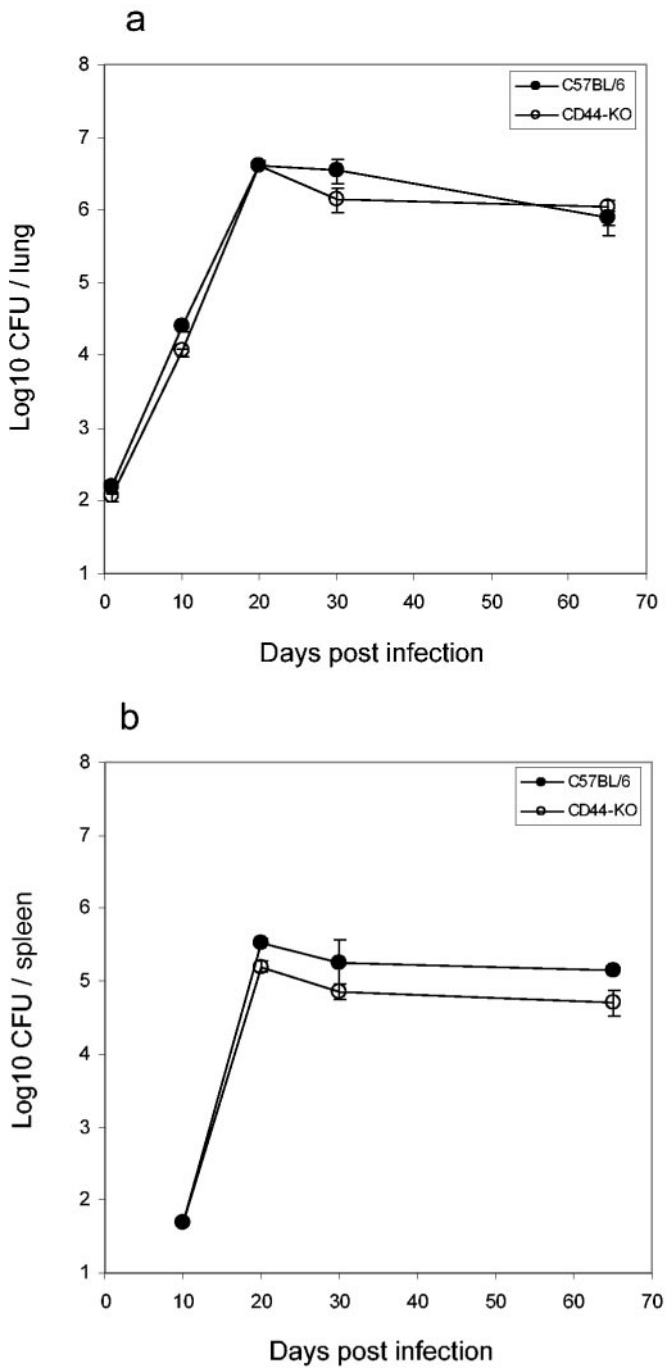

Fig. 1. Mice lacking the gene for CD44 have similar *M. tuberculosis* bacterial loads in the lung and spleen compared with C57BL/6 mice. Wild-type (●) and CD44 gene-disrupted (○) mice were infected by the aerogenic route, and lungs (a) or spleen (b) were evaluated for bacterial growth over a period of time. Data points represent the mean (±SEM) of values from four mice and are representative of two independent experiments. CFU, Colony-forming units.

necrotic cells were scattered throughout the lesions in equal proportions between the two groups. At day 30 postinfection, the lesions were similar in size and cellular make-up; however, focal lesions in the CD44-KO mice began to coalesce. At day 65 postinfection, significant histological differences were seen between the two groups. Lesions within the CD44-KO mice were more severe and were predominately composed of epithelioid macrophages with lymphoid aggregates and significant numbers of neutrophils as seen at day 20 (Table 1 and data not shown). Despite the significant lesion burden, necrosis was not a significant feature but was greater than the wild-type group. Additionally, inflammatory cell infiltrates extended into the small conducting airway lumina similar to that seen in the lungs at 20 days postinfection.

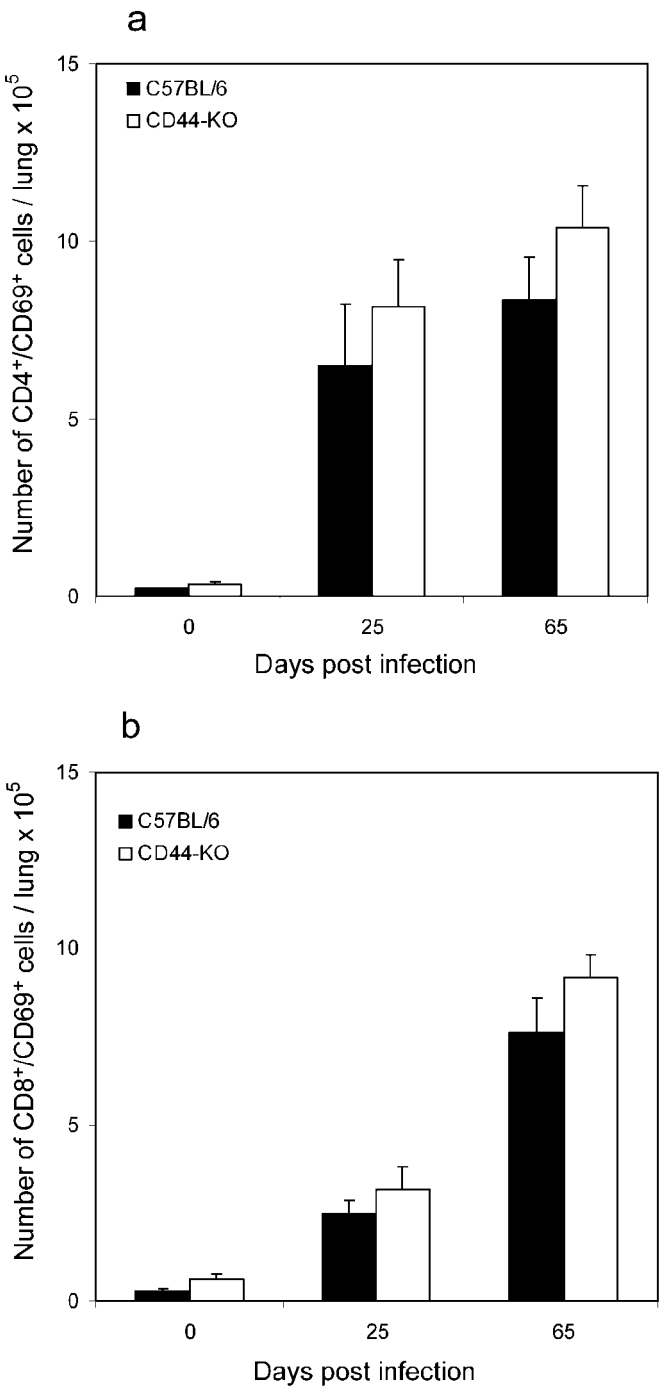

Fig. 2. Wild-type (solid bars) and CD44-KO (open bars) mice are equally able to recruit activated T cells to the lung following aerosol infection. Following infection, lung cells were stained for their expression of the early activation marker CD69, and their relative numbers were determined by gating lymphocytes according to their size (FSC) and granularity (SSC) and for staining with anti-CD4 (a) or anti-CD8 (b) fluorescent antibodies. Day 0 represents data from noninfected mice of matched age. Data represent the mean of five mice ± SEM and are representative of two independent experiments.

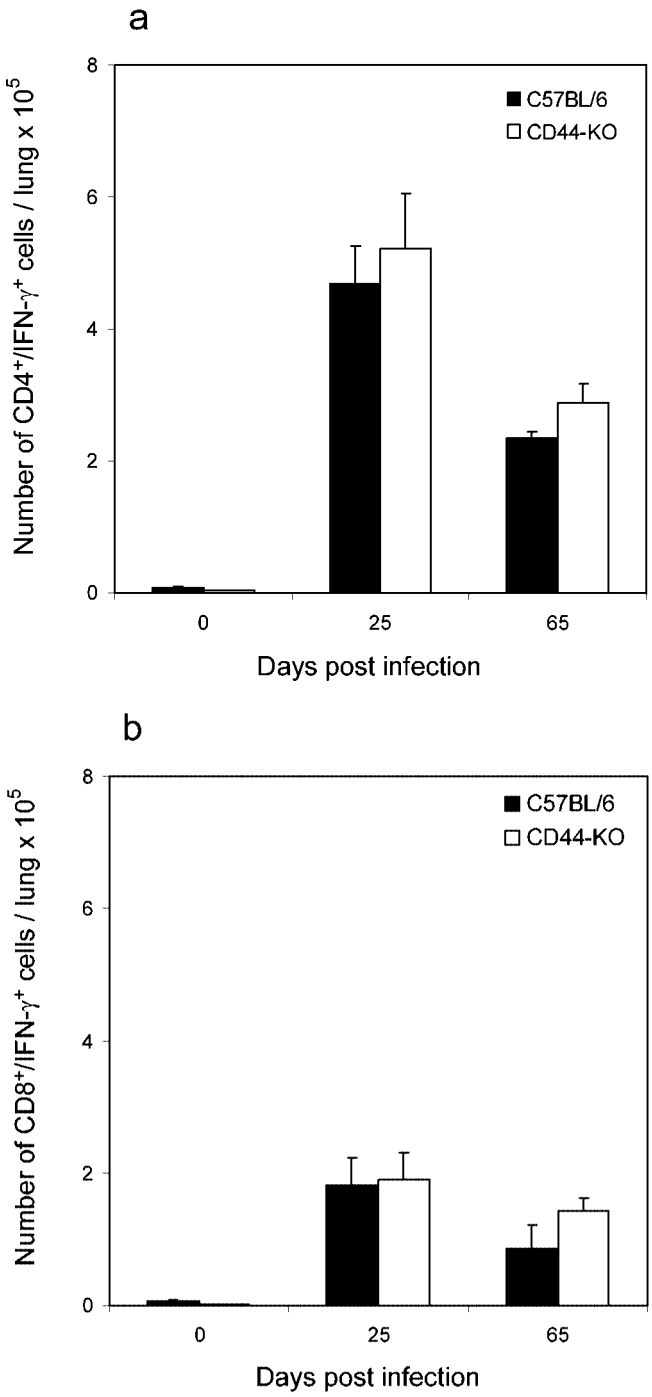

Fig. 3. The number of CD4 (a) and CD8 (b) T lymphocytes that produced IFN-γ in the lungs was determined. Infected and noninfected mice were analyzed during the course of infection for the ability of their cells in the lung to produce IFN-γ. Lung-cell preparations from wild-type (solid bars) and CD44-KO (open bars) mice were stimulated with anti-CD3 and anti-CD28, and the number of CD4 and CD8 T lymphocytes was determined. Day 0 represents data from noninfected mice of matched age. Data represent the mean of five mice ± SEM and are representative of two independent experiments.

DISCUSSION

The results of this study show that following an aerosol infection of mice with *M. tuberculosis*, the inflammatory response in the lung consisting of the accumulation of macrophages and

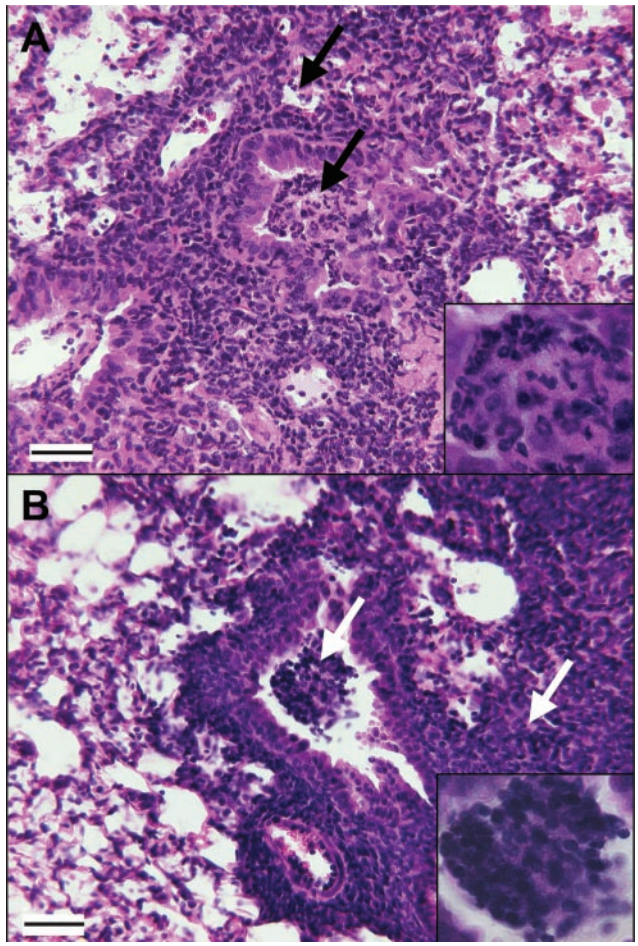

Fig. 4. Light photomicrograph of a lung from CD44-KO (A) and wild-type (B) mice after aerosol infection with *M. tuberculosis*. In the CD44-KO mice, neutrophils are the predominant, inflammatory cell type in bronchiole (inset) and alveolar lumina (black arrows), with fewer loosely organized peribronchiolar and perivascular lymphocytes 20 days postinfection. In wild-type mice, lymphocytes are in dense peribronchiolar and perivascular aggregates and occasionally within bronchiolar lumina (inset) 20 days postinfection (white arrows). Size bars are 100 μm.

lymphocytes proceeded normally despite the absence of expression of the CD44 molecule, a major adhesion C-lectin molecule. In such mice, lymphocytes were able to migrate to the sites of inflamed tissue, resulting in control and containment of bacterial growth in the gene-disrupted mice at the same levels as the wild-type animals. In contrast to the mononuclear cell response, the absence of expression of CD44

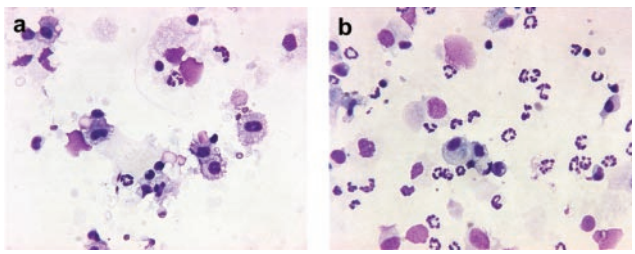

Fig. 5. BAL were performed following aerosol infection. A representative slide is shown from wild-type (a) and CD44-deficient (b) mice 20 days postinfection.

TABLE 1. Cellular Composition of the BAL Fluid from Wild-Type and CD44-KO Mice Following Infection with *M. tuberculosis*

	Wild-type mice			CD44-KO mice		
Days postinfection[a]	0	20	65	0	20	65
Cell types[b]						
Macrophages	98.2	74.4	78	93.5	62.7	74.7
Lymphocytes	1	18.3	19.5	4.8	12.5	9
Neutrophils	0.8	7.3	2.5	1.7	24.8*	16.3*

[a] Day 0 represents uninfected mice. [b] Two hundred cells were counted from each mouse BAL, and cell types are expressed as percentages. Data represent mean percentages of four mice for each time point and are representative of two independent experiments. *$P < 0.05$ compared with control.

resulted in a dramatically increased accumulation of neutrophils into the lungs and alveolar spaces, an event that did not however appear to interfere with the ability of the CD44-deficient mice to control the infection.

These observations are consistent with those of others that CD44 is needed to control inflammation in the lungs, at least in the context of granulocyte influx. It has been shown that during an inflammatory response, CD44-KO mice accumulate active forms of hyaluronan, the ligand for CD44, which is induced by the inflammatory cytokines TNF-α and interleukin-1β [14, 15], but removal of this ligand can be impaired in the absence of CD44 [10]. Additionally, the removal of apoptotic cells from the inflammatory site, a protective mechanism to avoid cell lysis and subsequent release of immunogenic and/or toxic contents, seems to be defective in the absence of CD44. Other factors that may account for the increased inflammatory response in the CD44-KO mice are prolonged inflammatory gene expression as well as reduced levels of active transforming growth factor-β [10].

During a low-dose aerosol infection with *M. tuberculosis*, a mononuclear cell inflammatory response slowly takes place with several stages of pathology observed [16]. However, the major difference seen between the wild-type and CD44-KO mice in the current study was not within the above cells but consisted instead of a substantially increased neutrophil influx in the lung parenchyma and alveolar spaces. This excessive number of neutrophils could be explained by an increased influx and/or by a decreased clearance rate. Recent studies have in fact shown that the expression of CD44 on the surface of neutrophils acts as a negative regulator for their migration across endothelial barriers [17], and in the absence of CD44, neutrophils can migrate faster than those in wild-type mice [18], resulting in their accumulation in the inflamed tissues. Such an observation would certainly explain our results here.

In this regard, we have shown a pyogenic response in the lungs of mice lacking γδ T cells, and we have previously speculated that these cells may play a role in controlling and reducing the influx of neutrophils [19]. It is reasonable to speculate therefore that CD44-KO mice are deficient in promoting the adequate attraction of γδ T cells to the lungs, which in turn, contributes to the increase in neutrophilic influx. In fact, our data may support this idea, as we have observed a higher percentage of γδ T cells in wild-type mice than in CD44-KO mice (data not shown).

In contrast to our findings, Leemans et al. [20] have recently reported that CD44-deficient mice are more susceptible to infection with *M. tuberculosis*, with higher bacterial loads in the lung and liver, and with a concomitant defect of the CD44-KO mice in terms of attracting macrophages and IFN-γ-producing T cells. The difference in our study seems to reside in the bacterial dose of infection, in that we used a low dose of ~100 bacteria deposited into the lungs, whereas Leemans et al. [20] used a dose 1000× higher than ours delivered intranasally. That report did not identify the actual, initial bacterial load obtained by that procedure, but it could be the case that it was higher than ours. It has been proposed elsewhere [21] that in a low-dose infection with *M. tuberculosis*, a minimal immune response is generated that is sufficient to control the infection, and if the bacterial load is much higher, deficiencies not otherwise interfering with this response now become important. Despite this, however, both studies observed the florid neutrophil influx, and this pyogenic granulomatous response was almost certainly responsible for the eventual mortality observed in the studies by Leemans et al. [20].

In summary, our observations show that following a low-dose aerosol infection with *M. tuberculosis*, a protective immune response proceeds normally in the absence of CD44 expression, and trafficking of T cells to the lungs is necessary for such protection appearing unaltered. However, the absence of CD44 expression resulted in significantly increased neutrophil accumulation in the lungs without compromising the control of bacterial growth.

ACKNOWLEDGMENTS

This work was supported by NIH Grants AI-40488 and AI-45707 and by a grant to A. K. from the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil. The authors are grateful to Ana Paula J. Kipnis for assistance in BAL analyses.

REFERENCES

1. Bean, A. G., Roach, D. R., Briscoe, H., France, M. P., Korner, H., Sedgwick, J. D., Britton, W. J. (1999) Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J. Immunol.* **162,** 3504–3511.
2. Peters, W., Scott, H. M., Chambers, H. F., Flynn, J. L., Charo, I. F., Ernst, J. D. (2001) Chemokine receptor 2 serves an early and essential role in resistance to *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA* **98,** 7958–7963.
3. Kipnis, A., Basaraba, R. J., Orme, I. M., Cooper, A. M. (2003) Role of chemokine CCL2 in the protective response to early murine pulmonary tuberculosis. *Immunology* **109,** 547–551.
4. Saunders, B. M., Cooper, A. M. (2000) Restraining mycobacteria: role of granulomas in mycobacterial infections. *Immunol. Cell Biol.* **78,** 334–341.
5. Johnson, C. M., Cooper, A. M., Frank, A. A., Orme, I. M. (1998) Adequate expression of protective immunity in the absence of granuloma formation in *Mycobacterium tuberculosis*-infected mice with a disruption in the intracellular adhesion molecule 1 gene. *Infect. Immun.* **66,** 1666–1670.
6. Cooper, A. M., Flynn, J. L. (1995) The protective immune response to *Mycobacterium tuberculosis. Curr. Opin. Immunol.* **7,** 512–516.

7. Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., Orme, I. M. (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* **178,** 2243–2247.
8. Flynn, J. L., Chan, J., Triebold, K. J., Dalton, D. K., Stewart, T. A., Bloom, B. R. (1993) An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* **178,** 2249–2254.
9. Ponta, H., Sherman, L., Herrlich, P. A. (2003) CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.* **4,** 33–45.
10. Teder, P., Vandivier, R. W., Jiang, D., Liang, J., Cohn, L., Pure, E., Henson, P. M., Noble, P. W. (2002) Resolution of lung inflammation by CD44. *Science* **296,** 155–158.
11. Cosulich, M. E., Rubartelli, A., Risso, A., Cozzolino, F., Bargellesi, A. (1987) Functional characterization of an antigen involved in an early step of T-cell activation. *Proc. Natl. Acad. Sci. USA* **84,** 4205–4209.
12. Serbina, N. V., Flynn, J. L. (1999) Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infect. Immun.* **67,** 3980–3988.
13. Feng, C. G., Bean, A. G., Hooi, H., Briscoe, H., Britton, W. J. (1999) Increase in gamma interferon-secreting CD8(+), as well as CD4(+), T cells in lungs following aerosol infection with *Mycobacterium tuberculosis*. *Infect. Immun.* **67,** 3242–3247.
14. Maiti, A., Maki, G., Johnson, P. (1998) TNF-alpha induction of CD44-mediated leukocyte adhesion by sulfation. *Science* **282,** 941–943.
15. Mohamadzadeh, M., DeGrendele, H., Arizpe, H., Estess, P., Siegelman, M. (1998) Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J. Clin. Invest.* **101,** 97–108.
16. Rhoades, E. R., Frank, A. A., Orme, I. M. (1997) Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent *Mycobacterium tuberculosis*. *Tuber. Lung Dis.* **78,** 57–66.
17. Si-Tahar, M., Sitaraman, S., Shibahara, T., Madara, J. L. (2001) Negative regulation of epithelium-neutrophil interactions via activation of CD44. *Am. J. Physiol. Cell Physiol.* **280,** C423–C432.
18. Wang, Q., Teder, P., Judd, N. P., Noble, P. W., Doerschuk, C. M. (2002) CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice. *Am. J. Pathol.* **161,** 2219–2228.
19. D'Souza, C. D., Cooper, A. M., Frank, A. A., Mazzaccaro, R. J., Bloom, B. R., Orme, I. M. (1997) An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to *Mycobacterium tuberculosis*. *J. Immunol.* **158,** 1217–1221.
20. Leemans, J. C., Florquin, S., Heikens, M., Pals, S. T., van der Neut, R., Van Der Poll, T. (2003) CD44 is a macrophage binding site for Mycobacterium tuberculosis that mediates macrophage recruitment and protective immunity against tuberculosis. *J. Clin. Invest.* **111,** 681–689.
21. Scott, H. M., Flynn, J. L. (2002) *Mycobacterium tuberculosis* in chemokine receptor 2-deficient mice: influence of dose on disease progression. *Infect. Immun.* **70,** 5946–5954.