CD44 is a Macrophage Binding Site for *Mycobacterium tuberculosis* that Mediates Macrophage Recruitment and Protective Immunity against Pulmonary Tuberculosis

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ABSTRACT

Cell migration and phagocytosis are both important for controlling *Mycobacterium tuberculosis* infection and are critically dependent on the reorganization of the cytoskeleton. Since CD44 is an adhesion molecule involved in inflammatory responses and connected to the actin cytoskeleton we investigated the role of CD44 in both these processes. Macrophage recruitment into *M. tuberculosis*-infected lungs and delayed-type hypersensitivity sites was impaired in CD44 deficient (CD44⁻/⁻) mice. In addition, the number of T lymphocytes and the concentration of the protective key cytokine interferon-γ were reduced in the lungs of infected CD44⁻/⁻ mice. The production of interferon-γ by splenocytes of CD44⁺/⁻ mice was profoundly increased upon antigen-specific stimulation. Flow cytometry analysis revealed that soluble CD44 can directly bind to virulent *M. tuberculosis*. Mycobacteria also interacted with macrophage-associated CD44, as reflected by a reduced binding and internalization of bacilli by CD44⁻/⁻ macrophages. CD44⁻/⁻ mice displayed a decreased survival and an enhanced mycobacterial outgrowth in lungs and liver during pulmonary tuberculosis. Together, we identified CD44 as a new macrophage binding site for *M. tuberculosis* that mediates mycobacterial phagocytosis, macrophage recruitment and protective immunity against pulmonary tuberculosis.
Mycobacterium tuberculosis is one of the most threatening microorganisms causing more deaths annually than any other single human pathogen (1). Each year, over 8 million new cases of tuberculosis and 2 million deaths from this disease occur worldwide (2, 3). The increasing incidence of tuberculosis over the last decade has increased the need to define host factors that control resistance to tuberculosis.

Effective host defence against M. tuberculosis is primarily dependent on the interplay between macrophages (Mφs), T cells and dendritic cells (4, 5). This interaction requires migration and activation of leukocytes which is dependent on the ability of cells to adhere to each other or to the extracellular matrix via adhesion molecules. CD44 is a member of the hyaluronate receptor family of cell adhesion molecules that has been shown to play a selective role in controlling lymphocyte migration (6, 7). CD44 is expressed on hematopoietic cells and is linked to cytoskeletal elements like hyaluronic acid, collagen, fibronectin, and osteopontin (8). It is necessary for extravasation of activated T cells into inflammatory sites (6), but it is not required for normal leukocyte circulation (7). In vitro experiments suggest that CD44 is also involved in cytoskeleton-dependent phagocytosis of heat-killed Staphylococcus aureus by polymorphonuclear cells (PMNs) (9).

Among mammals, CD44 is a highly conserved receptor (10), which suggests that CD44 is under strong evolutionary pressure, and as such is an important molecule. Data on the in vivo role of CD44 during inflammatory responses are however limited. CD44 may play a role in the pathogenesis of inflammatory disorders, as indicated by studies on arthritis (11-14), contact hypersensitivity (7), and autoimmune encephalomyelitis (15), presumably by contributing to the migration of activated leukocytes to sites of inflammation. Although these studies reveal a role for CD44 during inflammatory responses, little is known about the in vivo role during infections with pathogenic microorganisms. A single study, using an inactivating antibody directed against CD44 suggested that this adhesion molecule is not needed for resistance against oral infection with Toxoplasma gondii (16). A potential role for CD44 in the immune response to M. tuberculosis is suggested by the observation that CD44high-expressing T cells (memory T cells) accumulate in the lungs of mice during infection with this pathogen (17-19).

In the present study we determined the role of CD44 in M. tuberculosis leukocyte migration and phagocytosis and investigated the significance of our findings in resistance against pulmonary tuberculosis. For this, we infected CD44−/− or CD44+/+ mice
intranasally with a virulent strain of *M. tuberculosis*. Our results show that CD44 plays an important role in the recruitment of Mφs, and is a binding site on Mφs for uptake of *M. tuberculosis*, that mediates protective immunity against lung tuberculosis in vivo. As studies in mice without functional α₄ or α₄β₇ integrin, P-selectin, ICAM-1 or complement receptor 3 (18, 20-22) have shown that these adhesion molecules are not involved in clearance of mycobacteria, the present study identifies a unique function for CD44 in resistance against mycobacterial infection.

**MATERIAL & METHODS**

*Mice*

Specified pathogen-free 8 to 10 week old male and female CD44 deficient (CD44⁻) mice on a C57BL/6 background (23) were a kind gift of Dr. A. Berns, (Netherlands Cancer Institute, Amsterdam, The Netherlands). Wild type (CD44⁺/⁺) C57Bl/6 mice were purchased from Iffa Credo, L'Arbresle, France. Animals were maintained in biosafety level 3 facilities. In all experiments, sex and age matched controls were used. The Animal Care and Use Committee of the University of Amsterdam, The Netherlands, approved all experiments.

*Experimental infection*

A virulent laboratory strain of *M. tuberculosis* H37Rv was grown for 4 days in liquid Dubos medium containing 0.01% Tween 80. A replicate culture was incubated at 37°C, harvested at mid-log phase and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed twice with sterile 0.9% NaCl. Tuberculosis was induced as described previously (24, 25). Briefly, mice were anaesthetized by inhalation with isoflurane (Abbott Laboratories Ltd., Kent, U.K.) and infected intranasally with 1×10⁵ live *M. tuberculosis* H37Rv bacilli in 50 μl saline, as determined by viable counts on 7H11 Middlebrook agar plates. Bacterial counts recovered from lungs one day postinfection (p.i.) were shown previously to be similar to the number of bacteria in the inoculum (25).

*Lung cell differentiation*

Pulmonary cell suspension was obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and resuspended in medium.
Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution, and the remaining cells were washed twice with RPMI. Total leukocytes in pulmonary cell suspensions were counted by using a hemacytometer and TÜRK's solution (Merck, Gibbstown, N.J.). The percentages of Mφs, polymorphonuclear cells (PMNs) and lymphocytes were determined using cyto spin preparations stained with modified Giemsa stain (Diff-Quick, Baxter, McGraw Perk, IL).

**FACS analysis**
Pulmonary cell suspensions obtained from infected mice were analyzed by FACS (Becton Dickinson, Franklin Lakes, Nj) as described previously (25). Cells were brought to a concentration of 4x10⁶ cells/ml FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN₃ and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labeled Abs against CD3 (anti-CD3-phycoerythrin), CD4 (anti-CD4-CyChrome), CD8 (anti-CD8-FITC, anti-CD8-PerCP). All Abs were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA). To correct for aspecific staining an appropriate control antibody (rat IgG₂, Pharmingen) was used. Cells were fixed with 2% paraformaldehyde, and surface molecules were analyzed by gating the CD3⁺ population. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining.

**Histology and immunohistochemistry**
Lungs were removed 2 or 5 weeks after inoculation with *M. tuberculosis*, fixed for 24h in 4% paraformaldehyde in PBS for 24 hours and embedded in paraffin. For the delayed hypersensitivity response experiment footpads were removed from antigen challenged mice following immunization with heat-killed *M. tuberculosis*. After formalin fixation, specimens were decalcified in formic acid and embedded in paraffin. Hematoxylin-eosin stained slides were coded and semi-quantitatively scored for inflammatory infiltrates and lung granuloma formation by a pathologist. The presence of CD44⁺ cells, granulocytes and macrophages were demonstrated by immunohistochemistry. Slides were deparaffinized and endogenous peroxidase activity was quenched by a solution of methanol/0.03% H₂O₂. For CD44 staining, slides were then treated with 10 mM sodium citrate solution (pH 6.0) for 10 minutes at 98°C in a microwave oven, incubated with rat anti-mouse CD44 IgG1 (KM114, Pharmingen), followed by a further incubation with rabbit anti-rat-biotine (Dako, Glostrup, Denmark), and streptavidin-ABC solution.
CD44 MEDIATES PHAGOCYTOSIS AND MIGRATION

(Dako). For color development, 1% H₂O₂ and 3,3-di-amino-benzidine tetrachloride (DAB, Sigma, St Louis, MO) in Tris-HCl was used. The sections were mounted in glycerin gelatin without counter staining and analyzed. For granulocyte and macrophage staining, slides were digested with respectively a solution of 0.25% pepsine (Sigma, St. Louis, MO) in 0.01M HCl, or 0.1% trypsin (Sigma). Sections were then incubated in 10% normal goat serum (Dako) and respectively exposed to FITC-labeled anti-mouse Ly-6G mAb (Pharmingen) or rat anti-mouse F4/80 IgG2b mAb (Serotec, Oxford, England). For staining of granulocytes, slides were incubated with rabbit anti-FITC antibody (Dako) followed by a further incubation with a biotinylated swine anti-rabbit antibody (Dako). For macrophage staining, slides were incubated with goat anti-rat-HRP (Southern Biotechnology associates, AL, USA). Slides were finally incubated in a streptavidin-ABC solution (Dako) and developed using 1% H₂O₂ and DAB (Sigma) in Tris-HCl. The slides were counterstained with hematoxylin.

Splenocyte stimulation

Single cell suspensions were obtained by crushing spleens through a 40 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4), and the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium). Cells were suspended in medium (RPMI 1640, 10% FCS, 1% antibiotic-antimycotic (GibcoBRL, Life Technologies, Rockville, MD)), seeded in 96-well round bottom culture plates at a cell density of 1×10⁶ cells per well in triplicate, and stimulated with 20 μg/ml purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark). For stimulation of splenocytes of uninfected CD44⁺/⁺ and CD44⁻/⁻ mice, cells were stimulated with 10 μg/ml Staphylococcal enterotoxin B (SEB, Sigma), 7 μg/ml SEA (Sigma) or coated anti-CD3 (clone 145-2C11, home-made) and 1 μg/ml anti-CD28 (clone 37.51, Pharmingen) Abs. Supernatants were harvested after a 48-h incubation at 37°C in 5% CO₂, and cytokine levels were analyzed by ELISA.

Cytokine measurements

IFN-γ, IL-2, IL-4 were measured in lung homogenates, and spleen cell supernatants by specific ELISA’s using matched Ab pairs according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).
Binding and phagocytosis of *M. tuberculosis* by CD44<sup>++</sup> and CD44<sup>-/-</sup> macrophages

Mφs were isolated from CD44<sup>++</sup> and CD44<sup>-/-</sup> mice by washing the peritoneal cavity with RPMI 1640 supplemented with 10% FCS. Collected cells were allowed to adhere to 96 wells tissue culture plates (10<sup>5</sup> cells) for 1 hour at 37°C, after which nonadherent cells were removed by rinsing the cell monolayer with medium. More than 95% of the cells were Mφ as identified by cytospin preparations stained with modified Giemsa stain. Binding and phagocytosis of *M. tuberculosis* was determined as described previously (22, 26). Briefly, Mφ monolayers were infected with *M. tuberculosis* in RPMI 1640 and 10% FCS at a bacteria/Mφ ratio (multiplicity of infection, MOI) of 10, and then incubated for 2 h at 37°C, after which supernatants were aspirated. Each well was washed three times with RPMI 1640 and 10% FCS to remove the remaining non-adherent mycobacteria. For the assessment of mycobacterial load, cells remaining attached to the tissue culture wells were incubated with sterile distilled H<sub>2</sub>O with 0.1% dodecyl sulfate sodium salt (Merck, Darmstadt, Germany) for 10 min at 25°C. After a serial 10-fold dilution of the cell lysate, the number of mycobacteria that were associated with the Mφs was determined by plating on Middlebrook 7H11, and colony forming units (CFUs) were enumerated after 21 days. To assess whether bacilli were phagocytosed, we infected Mφs for 2 and 17 h at a MOI of 2 after which cells were washed three times with PBS, fixed with 2% paraformaldehyde, and removed from the plate. Cytospins were made and stained with Ziehl-Neelsen stain for acid-fast bacilli and counterstained with 0.1% methylene blue stain. At least 100–200 cells were counted on each cytospin. The number of intracellular bacilli in each Mφ was counted. Cell viability of Mφ cultures was assessed from duplicate wells by trypan blue exclusion, and appeared similar in both groups.

**Binding of CD44 to *M. tuberculosis***

*M. tuberculosis* H37Rv bacteria (2×10<sup>5</sup>) were incubated for 30 min at 37°C with 1 ng human soluble CD44 standard protein (Bender MedSystems, Vienna, Austria) or with diluent in octuplicate. Subsequently, mycobacteria were centrifuged for 15 min at 2000g and incubated with an FITC-labeled mouse anti-human CD44 std Ab (IgG1, clone SFF304, Bender MedSystems) for 30 min on ice. After incubation, mycobacteria were centrifuged, washed with FACSbuffer, and fixed with 2% paraformaldehyde. CD44 binding was analyzed on a FACSscan (Becton Dickinson, Franklin Lakes, NJ).
Enumeration of bacteria

Groups of eight mice per time point were sacrificed 2 or 5 wk postinfection (p.i.), and lungs, liver and spleen were removed aseptically. Organs were homogenized with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 volumes of sterile 0.9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after a 21-day incubation period at 37°C. For cytokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150mM NaCl, 15mM Tris, 1mM MgCl₂ pH 7.4, 1mM CaCl₂, 1% Triton, 100μg/ml pepstatin A, leupeptin and aprotinin), and incubated on ice for 30 min. Supernatants were sterilized using a 0.22 μm filter (Corning Incorporated, Corning, NY) and frozen at -20°C until assays were performed.

Delayed-type hypersensitivity (DTH) response to PPD

To measure DTH responses, we examined the swelling responses of footpads in mice. Briefly, CD44⁺/⁺ and CD44⁻/⁻ mice (n=5) were immunized intradermally at the base of the tail with 0.1 mg of heat killed M. tuberculosis H37Ra (Difco Laboratories, Detroit, USA) in 0.1 ml of mineral oil (Sigma). Twelve days after immunization, mice were challenged with 40 μg PPD in saline into one hind footpad and in the other with saline alone. Measurements of footpad thickness were performed with a Mitutoyo model 7326 engineer's micrometer (Mitutoyo MTI Corporation, Aurora, Ill.) before and 24 h after the PPD challenge. The increase in footpad thickness was calculated as the difference in swelling between the 0 and 24h measurements. Specific DTH reactivity was calculated as the difference between the swelling of the PPD-injected footpads and the swelling of the saline-injected footpads.

Statistical analysis

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests. For comparison of survival curves Kaplan-Meier analysis with a log rank test was used. Values of P < 0.05 were considered statistically significant.
RESULTS

Accumulation of CD44 \(^+\) cells in the pulmonary compartment during M. tuberculosis infection

Previous studies have documented that CD44\(^{high}\) cells accumulate in mice lungs during \textit{M. tuberculosis} infection (17-19). To verify this observation in our model we studied the expression of CD44 in lung tissue of wild-type mice infected i.n. with \textit{M. tuberculosis}. Immunohistochemical staining of CD44 revealed a strong increase in the number of CD44\(^+\) cells in lung tissue of mice with \textit{M. tuberculosis} (Fig. 1A versus 1B).

![Figure 1](image)

**Figure 1** Expression of CD44 in lung tissue of CD44\(^{+/+}\) mice after intranasal infection with \(1 \times 10^5\) \textit{M. tuberculosis}. Representative view of the lung of a non-infected mouse showing only a few CD44\(^+\) passenger leukocytes (A) compared to the lung of a mouse 5 weeks after \textit{M. tuberculosis} infection showing a prominent influx of CD44\(^+\) leukocytes (B). Magnification x50. CD44 immunostaining

\textit{CD44 promotes the recruitment of M\(\phi\)s and lymphocytes to infected lungs}

To investigate whether CD44 is involved in leukocyte recruitment, we assessed the numbers and phenotypes of pulmonary leukocytes from CD44\(^{+/+}\) and CD44\(^{-/-}\) mice during tuberculosis (Table I). For this, we i.n. infected 8 mice per group with \(1 \times 10^5\) CFU \textit{M. tuberculosis} and sacrificed them after 2 or 5 wk. While the absolute number of leukocytes in lungs of CD44\(^{+/+}\) and CD44\(^{-/-}\) mice was similar 2 wk p.i., CD44\(^{-/-}\) mice had almost 50\% less M\(\phi\)s in their pulmonary compartment than CD44\(^{+/+}\) mice \((P<0.05)\). At 5 wk p.i., lungs of CD44\(^{-/-}\) mice contained more leukocytes than lungs of CD44\(^{+/+}\) mice \((P<0.05)\). At this time point the percentage of M\(\phi\)s was similar in both mouse strains. The percentage of pulmonary lymphocytes of CD44\(^{-/-}\) mice was however significantly reduced compared to CD44\(^{+/+}\) mice 5 wk p.i. \((P<0.05)\).
Subtyping of lymphocytes at 2 wk showed that the percentages of CD4+ and CD8+ T cells was similar in both groups (data not shown). Five wk p.i. CD44−/− mice displayed a lower percentage of CD3/CD4+ cells (59 ± 2 versus 64 ± 0.4), whereas the percentage of CD3/CD8+ T cells was similar in both strains (data not shown). The percentage of PMNs in the lungs of CD44−/− mice was increased compared to CD44+/+ mice at both time points (P<0.05).

Table I Effect of CD44 deficiency on cellular composition of total lung cells during tuberculosis

<table>
<thead>
<tr>
<th></th>
<th>Cells x10^6/ml</th>
<th>% MPs</th>
<th>% PMNs</th>
<th>% Lymphocytes</th>
</tr>
</thead>
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<tr>
<td>2 wk postinfection</td>
<td>CD44+/+</td>
<td>870 ± 383</td>
<td>27.0 ± 3.1</td>
<td>47.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>CD44−/−</td>
<td>877 ± 273</td>
<td>12.5 ± 2.1*</td>
<td>66.1 ± 0.7*</td>
</tr>
<tr>
<td>5 wk postinfection</td>
<td>CD44+/+</td>
<td>2754 ± 655</td>
<td>30.3 ± 2.5</td>
<td>32.0 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>CD44−/−</td>
<td>3710 ± 415*</td>
<td>29.5 ± 2.1*</td>
<td>45.9 ± 2.9*</td>
</tr>
</tbody>
</table>

* Leukocytes in lungs of CD44+/+ and CD44−/− mice infected for 2 and 5 wk with M. tuberculosis. Cells from eight mice per group were counted and differentiated on cytospin preparations stained with modified Giemsa stain. *P < 0.05 CD44−/− mice vs CD44+/+ mice.

Reduced granuloma formation in CD44−/− mice

We next investigated the contribution of CD44 to the histopathology of lungs from M. tuberculosis-infected mice. At 2 weeks after infection, lungs of CD44+/+ mice displayed sharply demarcated granulomas generally located around small bronchi and vessels. These granulomas were comprised of lymphocytes, and MPs (Fig. 2A). On the contrary, CD44−/− mice were unable to form well-shaped granulomas in reaction to M. tuberculosis, but displayed enlarged and disorganized lesions, containing predominantly PMNs (Fig. 2B). At 5 weeks after infection, the inflammation became more diffuse in CD44+/+ and CD44−/− mice. Whereas the infiltrate in lungs from CD44+/+ mice was still predominantly composed of lymphocytes and MPs (Fig. 2C), PMNs were more dominant in lungs of CD44−/− mice (Fig. 2D). Moreover, edema and pleuritis were more pronounced in CD44−/− than in CD44+/+ mice.

Accumulation of type 1 cells in spleens of CD44−/− mice

Because CD44 has been implicated in migration of leukocytes to sites of inflammation we determined the number of cells in the spleen during tuberculosis in CD44+/− and CD44+/+ mice. At 2 and 5 wk p.i., 35% and 54% more cells were recovered from spleens of CD44−/− mice than from spleens of CD44+/+ mice (P<0.05) (Fig. 3A). To obtain insight into the functional properties of splenocytes, we assessed their capacity to
produce type 1 (IFNγ) and type 2 (IL-4) cytokines upon antigen specific stimulation with PPD. Intriguingly, splenocytes of CD44−/− mice secreted 12 (P<0.05) and 7.5 (P<0.05) times more of the protective IFNγ at respectively 2 and 5 wk p.i. than splenocytes of CD44+/+ mice (Fig. 3B). IL-4 was undetectable in all samples. To exclude that the enhanced IFNγ production of CD44−/− splenocytes was constitutively present, we stimulated splenocytes from 5 uninfected CD44−/− and CD44+/+ mice with four different stimulators (αCD3/28, SEB, SEA, and PPD), and found that CD44 deficiency per se did not influence IFNγ release by naïve splenocytes (data not shown).

**Figure 2** Representative histopathological sections of lungs of CD44+/+ (A) and CD44−/− (B) mice after inoculation with *M. tuberculosis*. A. Characteristic small granuloma in the lung of a CD44+/+ mouse 2 weeks after *M. tuberculosis* infection (magnification x 50, H&E staining). B. In contrast, CD44−/− mice were unable to form well-shaped granulomas and lungs displayed a diffuse inflammatory infiltrate largely composed of PMNs (magnification x 50, H&E staining). C. At 5 weeks after infection, a diffuse almost confluent inflammation was observed in the lungs of CD44+/+ mice. Lymphocytes and MPs represented the majority of leukocytes (magnification x 100, H&E staining). D. At the same time point, lungs of CD44−/− mice showed a diffuse granulocytic inflammatory infiltrate (original magnification x 100, H&E staining).

**Reduced cytokine concentrations in lungs of infected CD44−/− mice**

To obtain insight into the influence of CD44 in local type 1 and type 2 cytokine concentrations during tuberculosis we measured IFNγ, IL-2 (type 1) and IL-4 (type 2) in lung homogenates at 2 and 5 wk p.i. At both time points, the pulmonary levels of these cytokines were lower in CD44−/− than in CD44+/+ mice (Table II).
**Table II** Effect of CD44 deficiency on *M. tuberculosis*-mediated induction of type 1 cytokines (IFNγ, IL-2) and type 2 cytokine (IL-4) in lungs

<table>
<thead>
<tr>
<th></th>
<th>IFNγ (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
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<tr>
<td>2 wk postinfection</td>
<td></td>
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</tr>
<tr>
<td>CD44+/+</td>
<td>165.0 ± 10.0</td>
<td>488.9 ± 37</td>
<td>3699 ± 100</td>
</tr>
<tr>
<td>CD44+/−</td>
<td>110.0 ± 6.8  *</td>
<td>329.7 ± 28.8 *</td>
<td>2859 ± 216 *</td>
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<tr>
<td>5 wk postinfection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44+/+</td>
<td>212.5 ± 16.4</td>
<td>681.0 ± 46.7</td>
<td>2115 ± 145</td>
</tr>
<tr>
<td>CD44+/−</td>
<td>168.6 ± 17.7</td>
<td>541.0 ± 23.5 *</td>
<td>1418 ± 165 *</td>
</tr>
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</table>

*Cytokines were measured in lung homogenates of CD44+/+ and CD44+/− mice 2 and 5 wk p.i. Data represent the mean and SEM of eight mice. *P < 0.05.*
CD44 binds to M. tuberculosis

The role of CD44 in mediating mycobacterial association was further investigated by incubating human recombinant CD44std with live M. tuberculosis. Flow cytometry analysis of surface binding of tubercle bacilli to CD44 demonstrated a clear shift of fluorescence intensity (Fig. 5). An almost 3 times higher fluorescence intensity was observed in M. tuberculosis incubated with CD44std and subsequently stained with anti-CD44-FITC (mean channel fluorescence (MCF) of 94 ± 8) compared with mycobacteria treated with only anti-CD44-FITC (MCF of 34 ± 14) (Fig. 5, P<0.05). In addition, the majority of mycobacteria stained negative when incubated with anti-CD44std-FITC (mean 15.4 ± 4.8%), while 50.6 ± 1.9% of M. tuberculosis incubated with recombinant CD44std became FITC positive, indicating direct binding of CD44 to mycobacteria (P<0.05).
Increased bacterial load in lungs, and liver of CD44−/− mice
We next investigated the impact of CD44 deficiency on controlling mycobacterial growth. As shown in Fig. 6, from 2 wk to 5 wk, *M. tuberculosis* grew more slowly in CD44+/+ mice than in CD44−/− mice. As a consequence, CD44−/− mice had 2.6 and 7-fold more mycobacteria in lungs than CD44+/+ mice did at respectively 2 wk p.i. (P<0.05) and 5 wk p.i. (P<0.05). In addition, livers of CD44−/− mice contained more mycobacteria than livers of CD44+/+ mice (P<0.05, 5 wk). In contrast, the numbers of *M. tuberculosis* CFU recovered from spleens were similar in both mouse strains at each time point.

Reduced survival in *M. tuberculosis*-infected CD44−/− mice
To study whether CD44 deficiency also influenced survival, CD44−/− and CD44+/+ mice (n=10 per group) were i.n. infected with *M. tuberculosis* and survival was monitored for 7 months. Compared to CD44+/+ mice, CD44−/− mice showed a significant reduction in survival (Fig. 7). At 210 days p.i. survival of CD44−/− mice was 60%, whereas all CD44+/+ mice remained alive during this observation period (P<0.05). Thus, differences in bacterial loads in lungs and liver were paralleled by differences in survival, supporting a protective role for CD44 in the immune response to *M. tuberculosis*.
CD44 is important for mononuclear cell recruitment to DTH sites

The recruitment of leukocytes into inflamed areas is critical for the development of DTH responses. To study the role of CD44 in leukocyte migration more extensively we determined DTH responses in CD44\(^{-/-}\) and CD44\(^{+/+}\) mice. Mice were immunized and subsequently challenged in one footpad with PPD, after which the swelling of footpads was measured. Both CD44\(^{-/-}\) and CD44\(^{+/+}\) mice showed significant footpad thickening following the challenge. Surprisingly, swelling responses in CD44\(^{-/-}\) mice were twice as high as in CD44\(^{+/+}\) mice (Fig. 8A). Histological analysis revealed a pronounced edema in the footpads of CD44\(^{-/-}\) mice accompanied by a dense and diffuse inflammatory infiltrate (Fig. 8E) predominantly composed of PMNs (Fig. 8F). The footpads of CD44\(^{-/-}\) mice, however, showed the classical histological picture of a DTH reaction with a quite well-defined inflammatory infiltrate limited to the subcutis with slight edema (Fig. 8B) which was primarily composed of mononuclear cells (Fig. 8D).

**DISCUSSION**

Cell migration and phagocytosis are both critically dependent on cytoskeletal rearrangements and are important for resistance against tuberculosis. Since CD44 is an adhesion molecule involved in inflammatory processes and linked to the actin cytoskeleton, we investigated the role of CD44 in both these processes during pulmonary tuberculosis. The deficiency of CD44 led to a profound defect in the early recruitment of M\(\phi\)s, and to a more modest reduction in the influx of lymphocytes to the pulmonary compartment. In addition, CD44 was identified as a site on M\(\phi\)s that is important for binding and subsequent uptake of *M. tuberculosis*. We further demonstrated that CD44 plays a role in the protective immune response to pulmonary tuberculosis *in vivo*, as indicated by a reduced survival and an enhanced mycobacterial outgrowth in lungs and livers of CD44\(^{-/-}\) mice.

The present study demonstrated a strong reduction of M\(\phi\) numbers in lungs of CD44\(^{-/-}\) mice early in the infection, indicating that CD44 promotes M\(\phi\) recruitment to the site of mycobacterial infection. In accordance with this finding, an earlier study
Figure 8 A. DTH response in footpads of mice. CD44+/+ (□) and CD44−/− (■) mice were immunized with heat-killed M. tuberculosis and challenged in one hind footpad with PPD and in the other one with saline. Footpad swelling was measured 0, and 24 hours after the Ag challenge and calculated as described in the method section. Data are shown as mean ± SEM of 5 mice. *P < 0.05 versus control.

B. Representative view of the footpad of a CD44+/+ mice 48 hours after a DTH reaction showing a classical picture of a DTH reaction: a demarcated inflammatory infiltrate with slight edema (H&E staining, original magnification x 50). E. The histological analysis of the footpad of CD44−/− demonstrated a dense and diffuse inflammatory infiltrate together with a pronounced edema. Immunohistochemical detection of PMNs (C, F) and mononuclear cells (D, G) in DTH footpads showed that the inflammatory infiltrate of CD44−/− mice was mostly composed of PMNs (F, original magnification x 100), whereas mononuclear cells were the predominant cell type found in CD44+/+ mice (D, original magnification x 100).
has shown that shedding of CD44 by antibody treatment led to a reduction of mononuclear cell influx into the central nervous system during experimental allergic encephalomyelitis (15). In line, migration of CD44−/− Mφs into atherosclerotic lesions was reduced (28). In contrast, inhibition of binding of CD44 to its main ligand hyaluronate, did not influence influx of monocytes and Mψs 16 h after intraperitoneal administration of SEB (6). This suggests that migration of Mψs is mediated via CD44 but can be independent of hyaluronate. Presumably, other CD44 ligands like fibronectin (29), osteopontin (30) or collagen types I and IV (29, 31) are more important for Mψ migration via CD44. The observation that Mψ numbers were similar in CD44−/+ mice compared to CD44+/+ mice later in the infection may reflect a relative deficiency in Mψ influx since the bacterial load was higher in CD44−/+ mice 5 wk p.i.. CD44 could also be only important in the early phase of migration and/or is compensated for by other adhesion receptors. Apparently, this early phase is important for the outcome of the disease.

CD44−/− macrophages not only demonstrated a reduced migration to the site of infection, they also were found to be less capable of binding and phagocytosing M. tuberculosis. Indeed, we provide for the first time evidence that soluble CD44 directly binds to M. tuberculosis and that CD44 expressed on Mψs supports binding and subsequent phagocytosis of mycobacteria. CD44 has already been shown to be involved in phagocytosis of heat-killed Staphylococcus aureus by human PMNs (9). Furthermore, group A Streptococcus has been demonstrated to bind keratinocyte CD44 to induce cytoskeleton changes that promote tissue invasion of these bacteria (32).

The impaired mycobacterial clearance in CD44−/− mice was also associated with a reduced lymphocyte percentage in lungs late in the infection. The importance of CD44 on lymphocytes during mycobacterial infections is suggested by observations of increased numbers of pulmonary CD44+ T-lymphocytes during lung tuberculosis (17-19, 33) that adoptively transferred protection against M. tuberculosis when obtained from mycobacterial heat-shock protein 65-vaccinated mice (34-36). In line with the present findings CD44 was also involved in the extravasation of activated antigen-specific T cells to the SEB inflamed peritoneal cavity (6). A possible explanation for the fact that CD44−/− mice displayed only modestly reduced lymphocyte numbers may be found in the higher mycobacterial load in the CD44−/− animals as compared to CD44+/+ mice. Furthermore, adoptive transfer experiments of Silva et al. suggest that protection against M. tuberculosis
is IFNγ dependent (36). Consistently, we found lower IFNγ levels in lungs of CD44\(^{-/-}\) infected animals.

Granulomas are well-organized structures composed of aggregated Mφs, lymphocytes and epithelioid cells, known to wall off the infectious site and preventing further spreading (37). We found that mice deficient for CD44 form less well-shaped granulomas than CD44\(^{+/+}\) mice. Accordingly, CD44\(^{-/-}\) mice had more mycobacteria disseminated to their livers that CD44\(^{+/+}\) mice. CD44 has already been shown to mediate cell aggregation via inter-CD44 binding or multivalent HA binding by CD44 on neighboring cells (38). Additionally, HA dependent binding has been demonstrated to cause aggregation of Mφs and lymphocytes (39, 40). Differentiated Mφ-epithelioid cells in granulomas even produce extracellular matrix proteins like osteopontin and fibronectin (41), which are natural ligands for CD44. Apparently, cell aggregation in the development of granulomas is partly regulated by CD44.

The inability of CD44\(^{-/-}\) mononuclear cells to efficiently migrate to inflammatory sites was more extensively studied in footpad DTH responses to PPD. This demonstrated that infiltration of mononuclear cells at the site of antigen challenge was reduced in immunized CD44\(^{-/-}\) mice compared to CD44\(^{+/+}\) mice, whereas the influx of PMNs and swelling were increased. This further supports the concept that CD44 participates in the recruitment of mononuclear cells to sites of inflammation. Strikingly, in both the M. tuberculosis infection experiment and the DTH experiment the number of PMNs in CD44\(^{-/-}\) mice was greatly enhanced compared to CD44\(^{+/+}\) mice, suggesting that the lack of Mφ and lymphocyte influx at the site of inflammation in CD44\(^{-/-}\) mice may lead to enhanced PMN migration in a CD44-independent compensatory response. Another possibility for the PMN influx may be enhanced survival of these cells as a consequence of diminished CD44-mediated apoptosis of neutrophils (42, 43). Obviously, further experimental work is necessary to distinguish between these possibilities.

We found enhanced IFNγ release by PPD-stimulated splenocytes from infected CD44\(^{-/-}\) mice. Generally, there is a predominance of a type 1 cytokine response in the spleen of mice infected with M. tuberculosis early in the infection. Later during infection this type 1 response declines, and by the time a latent infection is established a type 2 response prevails (44), giving rise to speculation that protective type 1 cells migrate from the spleen to the infected lungs (45). We found an increase in type 1 splenocytes of CD44\(^{-/-}\) mice both early and late in the infection. This suggests that migration and/or adhesion of splenocytes to the lung of CD44\(^{-/-}\) mice is impaired, which is in line with the
lower number of lung lymphocytes and reduced IFNγ levels in the lungs of these animals. The similar mycobacterial loads in the spleens of CD44−/− and CD44+/+ mice may reflect a protective function of IFNγ producing splenocytes against *M. tuberculosis* (i.e. the anticipated higher mycobacterial numbers were not found).

The present report provides strong evidence that CD44 exerts protective effects against *M. tuberculosis*. Interestingly, in other studies blocking of adhesion molecules other than CD44 did not influence the clearance of mycobacteria. ICAM-1−/− mice or mice treated with mAb to α4 or α4β7 integrin infected aerogenically with *M. tuberculosis*, displayed mycobacterial growth in lungs that was similar to the growth in their respective control mice (18, 20). In addition, P-selectin and/or ICAM-1−/− mice systemically infected with *M. bovis* also showed an unaltered clearance of mycobacteria (21), and mice deficient for complement receptor 3, a β2 integrin, did not differ from wild-type mice with respect to survival and bacterial burden during *M. tuberculosis* infection (22). Thus the present study identifies a unique function for CD44 as an adhesion molecule in mediating resistance against mycobacterial infection presumably by promoting binding and phagocytosis of *M. tuberculosis* by Mφs and migration of Mφs to the site of infection.

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REFERENCES

CD44 MEDIATES PHAGOCYTOSIS AND MIGRATION


