

Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in *Mycobacterium tuberculosis* infection

Diana Di Liberto,¹ Massimo Locati,^{2,3} Nadia Caccamo,¹ Annunciata Vecchi,² Serena Meraviglia,¹ Alfredo Salerno,¹ Guido Sireci,¹ Manuela Nebuloni,⁴ Neus Caceres,⁵ Pere-Joan Cardona,⁵ Francesco Dieli,¹ and Alberto Mantovani^{2,3}

¹Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, 90134 Palermo, Italy

²Istituto Clinico Humanitas IRCCS, 20089 Rozzano, Italy

³Istituto di Patologia Generale and ⁴Pathology Unit, L. Sacco Institute of Medical Sciences, University of Milan, 20133 Milan, Italy

⁵Unitat de Tuberculosi Experimental, Department of Microbiology, Fundació Institut per a la Investigació en Ciències de la Salut Germans Trias i Pujol and Universitat Autònoma de Barcelona, 08916 Badalona, Spain

D6 is a decoy and scavenger receptor for inflammatory CC chemokines. D6-deficient mice were rapidly killed by intranasal administration of low doses of *Mycobacterium tuberculosis*. The death of D6^{-/-} mice was associated with a dramatic local and systemic inflammatory response with levels of *M. tuberculosis* colony-forming units similar to control D6-proficient mice. D6-deficient mice showed an increased numbers of mononuclear cells (macrophages, dendritic cells, and CD4 and CD8 T lymphocytes) infiltrating inflamed tissues and lymph nodes, as well as abnormal increased concentrations of CC chemokines (CCL2, CCL3, CCL4, and CCL5) and proinflammatory cytokines (tumor necrosis factor α , interleukin 1 β , and interferon γ) in bronchoalveolar lavage and serum. High levels of inflammatory cytokines in D6^{-/-} infected mice were associated with liver and kidney damage, resulting in both liver and renal failure. Blocking inflammatory CC chemokines with a cocktail of antibodies reversed the inflammatory phenotype of D6^{-/-} mice but led to less controlled growth of *M. tuberculosis*. Thus, the D6 decoy receptor plays a key role in setting the balance between antimicrobial resistance, immune activation, and inflammation in *M. tuberculosis* infection.

CORRESPONDENCE

Francesco Dieli:
dieli@unipa.it

Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAL, bronchoalveolar lavage; BCG, bacille Calmette-Guérin; BUN, blood urea nitrogen; i.n., intranasal; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted.

The formation of granulomas at the site of mycobacterial infection is an essential component of host immunity for controlling infection. This process is dependent on the activation of mycobacteria-reactive T lymphocytes (1), particularly IFN- γ -secreting CD4 and CD8 T cells (2, 3). Granuloma formation, however, is a complex process that requires not only the activation of lymphocytes, but also their recruitment with monocytes to the site of the infection, migration into the tissues, and juxtaposition around mycobacteria-infected macrophages (4). This colocalization facilitates the activation of bactericidal mechanisms in infected macrophages by T cell-derived cytokines (1). Some mycobacte-

ria, however, survive within macrophages, and persistent antigenic stimulation perpetuates the process, leading to chronic granuloma formation characterized by dense accumulations of infected macrophages, epithelioid cells, and T lymphocytes (5). These granulomas contain the mycobacterial infection and prevent dissemination to other organs, but they are also responsible for lung immunopathology, as the granulomas displace and destroy parenchymal tissue (6). One of the major roles of the granuloma is to localize and contain not only the bacteria but also the inflammatory response to the bacteria itself.

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Indeed, if immune cells are not tightly controlled within the lungs, this could lead to excess inflammation. Thus, rigorous control of the organization of granulomas is likely necessary to prevent immunopathology. In most cases, after the repair of an inflamed or damaged tissue, inflammation subsides and the tissue returns to its homeostatic norm (7). However, if the resolution phase of inflammation is ineffective, chronic inflammatory pathologies may develop (7, 8). Failure to resolve ongoing inflammation is an invariable key feature of pathologies, which are typically characterized by the high-level expression of inflammatory cytokines and chemokines (9). Therefore, understanding the resolution phase of transient inflammatory responses will probably yield insights into some of these chronic inflammatory pathologies.

TNF- α and the related cytokine lymphotoxin- α (10–13) are potent proinflammatory cytokines with a wide range of activities in both the inflammatory and immune responses, and they play an essential role in host resistance against infection with *Mycobacterium tuberculosis* and other mycobacteria (10–12). TNF- α -deficient mice infected by aerosol with *M. tuberculosis* develop normal T cell responses to mycobacterial antigens, but because of the failure of granuloma formation in the infected organs they are profoundly susceptible to the infection, succumbing with extensive necrosis in the lungs and infected organs (12). Other than TNF- α , the role of other soluble mediators in regulating granuloma formation and persistence is poorly understood. Chemokines and their receptors are involved in cell migration and are logical candidates for a role in granuloma formation, although their expression has been studied to a limited degree in *M. tuberculosis* infection (for review see reference 14). In general, the production of chemokines at a certain level may be a factor in preventing cell movement out of the granuloma.

Recently, studies of the D6 chemokine receptor (for review see reference 9) have provided novel insights regarding the mechanism of chemokine removal from inflamed sites. D6 is structurally similar to the other chemokine receptors, and is most homologous to CCR4 and CCR5. However, several properties set D6 apart from other CCRs. First, D6 is extremely promiscuous, recognizing 15 chemokines. Interestingly, all D6 ligands are inflammatory CC chemokines, whereas constitutive CC chemokines, as well as chemokines of other subfamilies, are not recognized. Thus, D6 is a promiscuous receptor with selectivity for inflammatory CC chemokines. Second, the D6 expression pattern is unusual, with the predominant expression sites being trophoblasts in the placenta and lymphatic endothelial cells in the skin, gut, and lung. Third, and most crucially, D6 does not signal in response to the binding of any of its ligands. Conversely, the only apparent function of D6 is to convey the entire ligand–receptor complex intracellularly, target the chemokine for degradation, and recycle the receptor to the membrane. This observation, together with the ability of D6 to bind most inflammatory CC chemokines, has resulted in the proposal that D6 functions as a “scavenging” or “decoy” receptor (9, 15),

and has suggested a role for D6 in controlling the resolution of CC chemokine–driven inflammatory responses. Accordingly, D6^{-/-} mice display exaggerated inflammatory responses after skin application of phorbol esters (15) and subcutaneous injection of complete Freund’s adjuvant (16). In this last model, D6^{-/-} mice developed granulomas characterized by angiogenesis, extensive leukocyte infiltration, and prominent areas of necrosis more rapidly and severely than WT counterparts. However, whether D6 plays a similar role in inflammatory responses caused by infectious microorganisms, or has a role in the induction of a protective response to infectious agents, is presently unknown. To test this possibility, we have studied the course of *M. tuberculosis* infection in D6^{-/-} mice.

RESULTS

Expression of the D6 receptor during

M. tuberculosis infection

Preliminarily, we investigated the expression of the D6 receptor during *M. tuberculosis* infection. In human lungs and lymph nodes from a patient with pulmonary tuberculosis, D6 expression was observed in lymphatic endothelial cells and only occasionally in scattered leukocytes (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070608/DC1>). In the mouse, qualitative RT-PCR experiments indicated that D6 transcripts in the liver, spleen, and lungs do not undergo significant changes at 4, 8, and 12 wk after infection with *M. tuberculosis* (Fig. S2). These data suggest that D6 is mainly expressed in lymphatic endothelial cells, in agreement with previous reports (17), and that its expression does not change appreciably during *M. tuberculosis* infection.

M. tuberculosis infection in D6^{-/-} mice

To determine whether the D6 receptor plays a role in the control of *M. tuberculosis* infection, WT and D6^{-/-} mice were infected via the intranasal (i.n.) route with 2,000 CFU. This dose did not cause mortality in C57BL/6 mice in our study (Fig. 1 A). However, *M. tuberculosis* infection in D6^{-/-} mice resulted in increased mortality, with ~20% of mice succumbing by week 8, 50% of mice succumbing by week 12, and 100% of mice succumbing at week 16, at which time the experiments were terminated (Fig. 1 A). The phenotype of D6^{-/-} was dramatic and was found highly reproducible over a period of 3 yr in two additional experiments performed on a total of 17 mice per group. Despite the exaggerated susceptibility of D6^{-/-} mice to *M. tuberculosis* infection, there was no difference between the two groups of mice in bacterial loads, as determined by CFU counts in the lung, liver, and spleen at 2, 4, 8, and 12 wk after infection (Fig. 1 B).

Of note, CFU data at 4, 8, and 12 wk after *M. tuberculosis* infection were rechecked in two additional independent experiments with three mice per group performed over a period of 2 yr, confirming the results reported in Fig. 1 B. We then examined by histology the effect of D6 deficiency on the inflammatory response in the lungs, liver, and spleen. Although in WT mice a moderate cellular infiltration was

seen at 12 wk after infection and most of the lung airspace remained intact, an exaggerated response was evident in surviving $D6^{-/-}$ mice (see Fig. 5 A). Histological analysis also

showed a diffuse liver necrosis in $D6^{-/-}$ mice when compared with WT mice (Fig. 1 C). In agreement with the histology results, at 12 wk after *M. tuberculosis* infection, serum

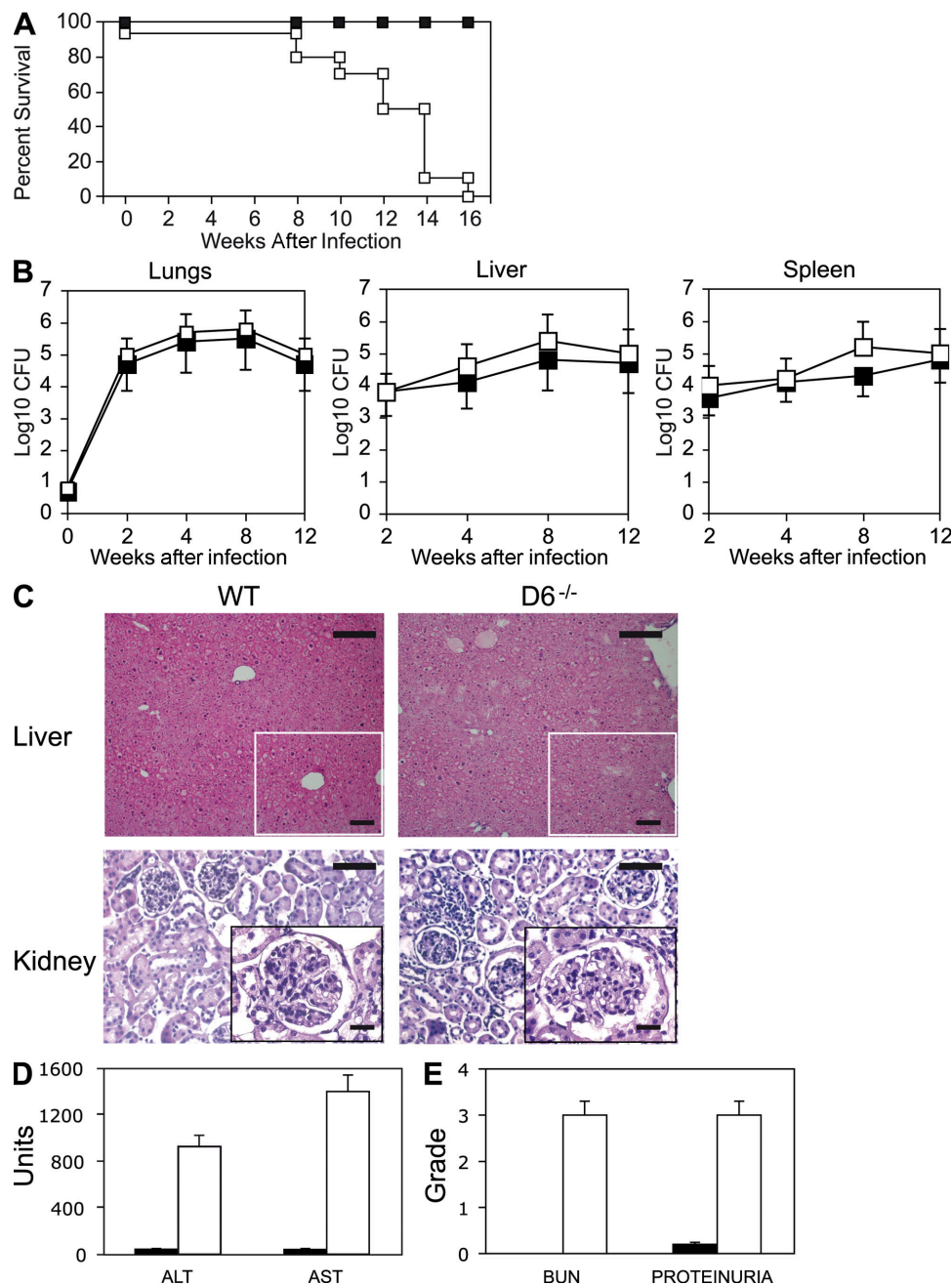


Figure 1. *M. tuberculosis* infection in $D6^{-/-}$ mice. (A) Survival curve of WT (closed squares) and $D6^{-/-}$ (open squares) mice infected i.n. with 2×10^3 CFU *M. tuberculosis* ($n = 10$). Data indicate the percentage of surviving mice over time. The difference in the survival rates of WT and $D6^{-/-}$ mice was statistically significant ($P < 0.001$). (B) The course of *M. tuberculosis* infection in WT (closed squares) and $D6^{-/-}$ (open squares) mice infected i.n. with 2×10^3 CFU *M. tuberculosis* and followed against time in the lungs, liver, and spleen. Data shown are the mean bacterial counts \pm SD. Differences between WT and $D6^{-/-}$ mice were not statistically significant at any time point. (C) Liver and kidney sections from WT and $D6^{-/-}$ mice at 12 wk after infection with *M. tuberculosis*. Hematoxylin and eosin staining is shown. Bars, 250 μ m. Insets show magnified structures. Original magnification was 100 \times for the main images and 400 \times for the insets. (D) Transaminase (ALT and AST) levels are shown. (E) BUN and proteinuria values are shown. Similar results were obtained in five different experiments (shaded bars, WT mice; open bars, $D6^{-/-}$ mice). Differences between WT and $D6^{-/-}$ mice were highly statistically significant ($P < 0.001$). Data are the mean \pm the SD.

transaminases were abnormally increased in $D6^{-/-}$ compared with WT mice (Fig. 1 D). Areas of focal necrosis and mononuclear cell infiltrates were also evident in the kidneys (Fig. 1 C), and consistent with this, at 12 wk after *M. tuberculosis* infection renal function was also severely compromised in $D6^{-/-}$ mice, which had significantly higher blood urea nitrogen (BUN) levels and frequency of severe proteinuria as compared with WT mice (Fig. 1 E). Of note, although *M. tuberculosis* growth occurred in the liver (Fig. 1 B), we failed to detect *M. tuberculosis* CFU in kidneys of both WT and $D6^{-/-}$ mice over a 12-wk period (not depicted). Thus, despite the successful control of the growth of the infecting mycobacteria, $D6$ deficiency led to increased mortality associated with lung inflammation and combined renal and liver failure.

Leukocyte infiltration in tissues during *M. tuberculosis* infection in $D6^{-/-}$ mice

We examined the cellular infiltrate in the lungs and liver of $D6^{-/-}$ mice after *M. tuberculosis* infection. At predetermined time points, organs were removed, the total number of cells was calculated, and flow cytometric analysis was performed to determine cell populations (Fig. 2). Numbers of DCs (F4/80⁻ and CD11c⁺) in the lungs (Fig. 2 A) were not significantly different between $D6^{-/-}$ and WT mice. The numbers of macrophages and CD4 and CD8 lymphocytes were significantly higher in $D6^{-/-}$ mice at all time points after infection. Approximately 45% of CD4 and CD8 T cells infiltrating the lungs of *M. tuberculosis*-infected $D6^{-/-}$ mice at 8 and 12 wk after infection expressed activation markers (CD25^{high}, CD44^{high}, and CD62L^{low}), whereas only 30% of CD4 and

35% of CD8 T cells expressed the same activation markers in WT mice, although differences were not statistically significant (unpublished data). Similar findings were also detected in the liver, although the differences between $D6^{-/-}$ and WT mice were lower than in the lungs and generally reached significance at 8 and 12 wk after infection (Fig. 2 B).

Cell migration to lymph nodes

Because of the finding that the lungs of $D6^{-/-}$ mice contained more macrophages and T lymphocytes than the lungs of WT mice, we asked whether a greater number of these cells migrated from the lungs to the draining lymph nodes. To investigate this possibility, flow cytometric analysis was performed on the mediastinal lymph nodes of $D6^{-/-}$ and WT mice after *M. tuberculosis* infection. As shown in Fig. 2 C, the number of macrophages showed a significant increase in the lymph nodes of $D6^{-/-}$ mice at 4 wk after infection. At 4 and 8 but not 12 wk after infection, there were also statistically significant differences in the total number of DCs in the lymph nodes of $D6^{-/-}$ mice. The numbers of CD4 and CD8 T lymphocytes were significantly higher in the lymph nodes of $D6^{-/-}$ mice at 4 and 8 wk after infection, but although the latter constantly increased, the former peaked at week 4 and then decreased. The increased migration of DCs and T lymphocytes to the lymph nodes was expected to result in increased numbers of primed T lymphocytes, followed by migration to the lungs. ELISPOT analysis confirmed this hypothesis, showing that significantly more CD4 and CD8 T lymphocytes from the lungs of $D6^{-/-}$ mice were primed to produce IFN- γ when stimulated by bacille Calmette-Guérin (BCG)-infected DCs (Fig. 3).

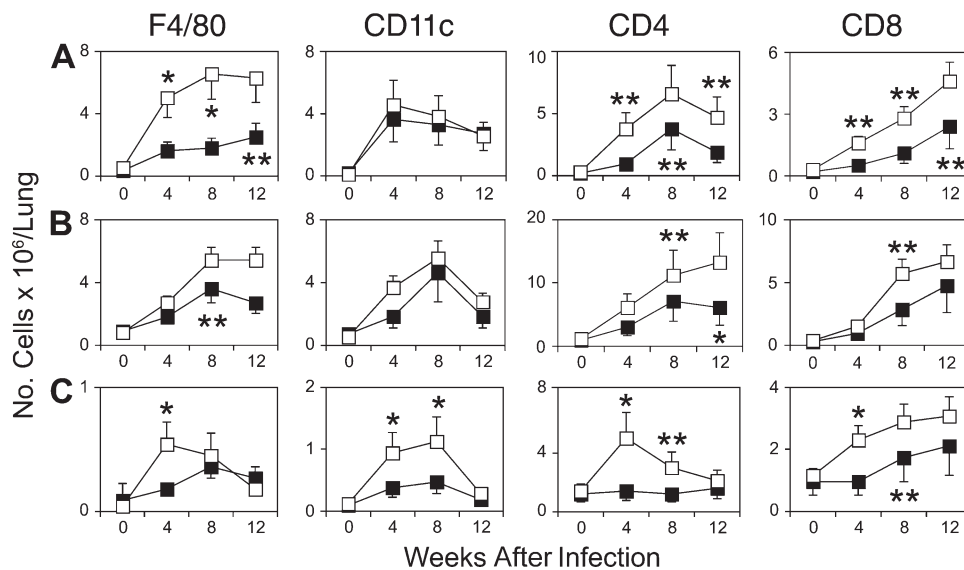


Figure 2. Leukocyte recruitment to tissues and lymph nodes after infection with *M. tuberculosis*. Leukocytes were isolated from the lungs (A), livers (B), and mediastinal lymph nodes (C) of WT (closed squares) and $D6^{-/-}$ (open squares) mice and analyzed by flow cytometry at different times after infection with *M. tuberculosis*. The cells were stained with fluorescent antibodies to F4/80 (macrophages), CD11c (bright; DCs), CD4, and CD8. Shown are the mean cell numbers \pm SD for each cell type. Similar results were obtained in five different experiments. *, $P < 0.001$; **, $P < 0.01$.

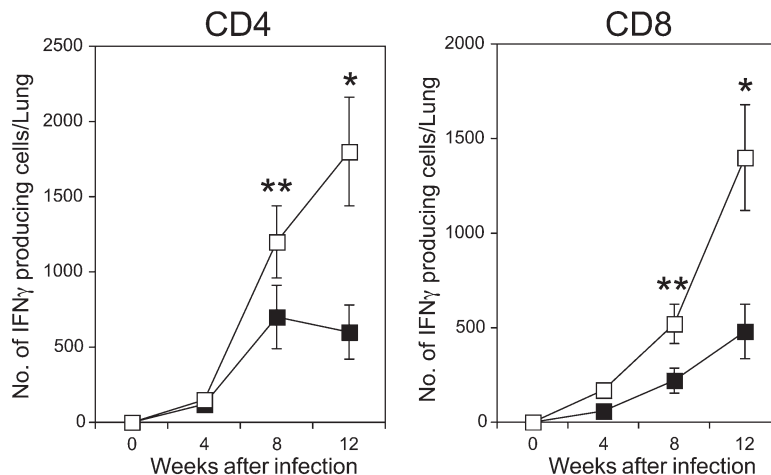


Figure 3. IFN- γ -producing CD4 and CD8 T cells in the lungs after infection with *M. tuberculosis*. Lung leukocytes were isolated from WT (closed squares) and D6^{-/-} (open squares) mice at the indicated time points after infection and were restimulated in vitro with irradiated BCG-infected WT DCs for 36–40 h at 37°C. The numbers of IFN- γ -producing CD4 and CD8 T cells were quantified by ELISPOT at the indicated weeks. The mean numbers \pm SD of IFN- γ -producing CD4 and CD8 T cells from WT and D6^{-/-} mice at each time point are shown. Similar results were obtained in five different experiments. *, P < 0.005; **, P < 0.01.

Chemokine and cytokine levels in D6^{-/-} mice

Previous studies have shown that exacerbated inflammation in mice lacking the D6 receptor is associated with higher levels of chemokines in blood and tissues (15, 16, 18). We therefore examined chemokine levels in the bronchoalveolar lavage (BAL) and serum of *M. tuberculosis*-infected mice. As shown in Fig. 4 A, the concentrations of all tested chemokines were significantly higher in the BAL of D6^{-/-} than of WT mice at 12 wk after infection. Similarly, concentrations of CCL2/monocyte chemoattractant protein 1 (MCP-1), CCL3/macrophage inflammatory protein 1 α (MIP-1 α), CCL4/MIP-1 β ,

and CCL5/regulated on activation, normal T cell expressed and secreted (RANTES) were also significantly increased in the serum of D6^{-/-} mice at 12 wk after infection (Fig. 4 B). Control of *M. tuberculosis* infection depends on the activation of macrophages by IFN- γ and TNF- α , leading to the induction of inducible NO synthase and the production of reactive nitrogen intermediates such as NO. TNF- α is reported to be essential for the early expression of mRNA encoding CC and CXC chemokines, the initial recruitment of CD4 T lymphocytes, and the formation and maintenance of the granulomas (12, 19, 20). To determine whether these components of the

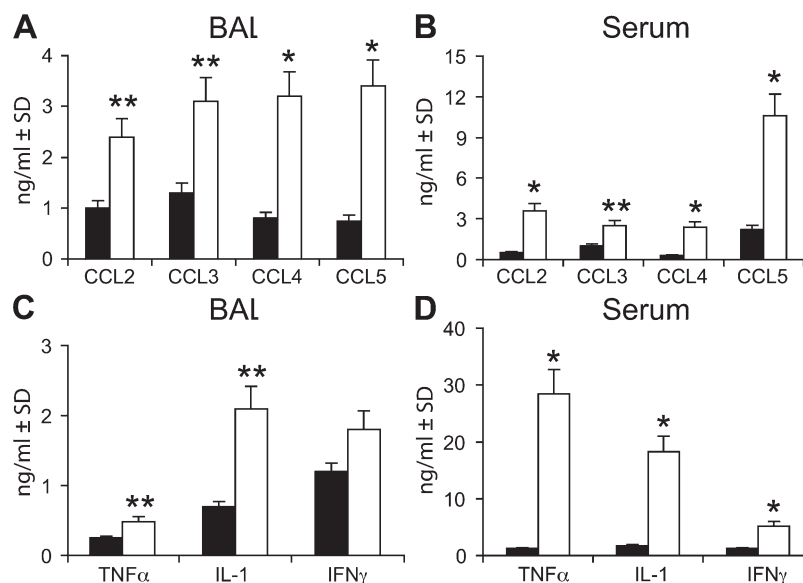


Figure 4. Chemokine and cytokine levels in the BAL and serum of *M. tuberculosis*-infected WT and D6^{-/-} mice. WT (shaded bars) and D6^{-/-} (open bars) mice were infected i.n. with *M. tuberculosis*, and BAL or sera were obtained 12 wk later. The levels of CC chemokines (A and B) and cytokines (C and D) were measured by ELISA. Shown are the mean values \pm SD. Similar results were obtained in three different experiments. *, P < 0.001; **, P < 0.01.

Table I. Effects of treatment with anti-CC chemokine antibodies on the survival of D6^{-/-} mice to *M. tuberculosis* infection

Mice	Treatment	Weeks after infection		
		8	12	16
WT	Nil	0/10	0/10	0/10
D6 ^{-/-}	Nil	3/10	5/10	9/10
D6 ^{-/-}	Control antibody	2/10	4/10	9/10
D6 ^{-/-}	Anti-CCL2/MCP-1	3/10	3/10	8/10
D6 ^{-/-}	Anti-CCL3/MIP-1 α	4/10	6/10	9/10
D6 ^{-/-}	Anti-CCL4/MIP-1 β	2/10	4/10	8/10
D6 ^{-/-}	Anti-CCL5/RANTES	2/10	4/10	9/10
D6 ^{-/-}	Mix antibody	0/10*	1/10*	3/10**

WT mice and D6^{-/-} mice either untreated or treated with monoclonal antibodies to single individual CC chemokines, or with a cocktail of anti-CC chemokines (mix antibody) or irrelevant control antibodies, were infected with *M. tuberculosis*. Mice were scored for survival over time. Data shown are the numbers of deaths out of the number of treated mice ($n = 10$ per group). *, $P < 0.001$; and **, $P < 0.01$ when compared with values in D6^{-/-} mice either untreated (Nil) or treated with control antibody.

antimycobacterial immune response were affected by D6 deficiency, ELISA was performed on the BAL and serum of *M. tuberculosis*-infected mice at 8 (unpublished data) and 12 wk after infection. Concentrations of TNF- α , IL-1 β , and IFN- γ were significantly higher in the BAL (Fig. 4 C) and serum (Fig. 4 D) of *M. tuberculosis*-infected D6^{-/-} mice at all tested time points, as compared with infected WT mice. Thus, *M. tuberculosis* infection in the presence of D6 deficiency causes a local and systemic inflammatory response characterized by increased levels of proinflammatory chemokines and cytokines.

Neutralization of chemokines in D6^{-/-} mice in vivo reverses pathology but increases susceptibility to infection

The results in the previous paragraph clearly show that *M. tuberculosis* infection in the absence of the D6 receptor is characterized by an exaggerated inflammatory response and increased mortality, despite the successful control of bacterial growth. To investigate whether the highly increased levels of chemokines observed in D6^{-/-} mice were involved in the pathogenesis, we attempted to block inflammatory chemokines in vivo. For this purpose, mice were treated with a mixture of antibodies to the CC chemokines CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES, as previously described (15, 18), or with antibodies to each individual chemokine. On the same days, control mice received i.p. injections of an equivalent amount of irrelevant antibodies. Mice were injected weekly with *M. tuberculosis*, starting from the third week after infection. Following this treatment schedule, CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES production in the serum of D6^{-/-} mice was consistently reduced (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20070608/DC1>), even below levels detected in WT mice, thus demonstrat-

ing the efficacy of the blocking. Conversely, D6^{-/-} mice treated with control antibody had very high levels of serum chemokines, as did untreated D6^{-/-} mice. TNF- α , IL-1 β , and IFN- γ levels in serum were also consistently reduced by treatment with antibodies to CC chemokines (Table S1), and cellular influx to the infected tissues significantly decreased (unpublished data). Additionally, both renal and liver function appeared improved by this treatment, as suggested by the low levels of serum transaminases and BUN, and by the low proteinuria detected in these mice (Table S1). Overall, the most impressive consequence of the neutralization of CC chemokines in D6^{-/-} mice was a significantly prolonged survival, as demonstrated by the only 10% mortality at 12 wk and 30% mortality at 16 wk after infection with *M. tuberculosis* (Table I). However, in spite of the ability of chemokine-blocking antibodies to reduce the overall inflammatory response (Fig. 5 A), the ability of D6^{-/-} mice treated in this manner to control the growth of *M. tuberculosis* in the lungs was impaired, with higher CFU counts found at 8 and 12 wk (Fig. 5 B). This result therefore indicates that the D6 receptor plays a crucial role in *M. tuberculosis* infection, and in the balance between immunopathology and protective immune responses.

DISCUSSION

A better understanding of the immunological mechanisms of pathogenesis and protection is of essential importance for the design of novel vaccines and immunotherapies against tuberculosis. It has been proposed that the protective response to *M. tuberculosis* infection requires CD4 and CD8 lymphocytes, the Th1-type cytokines IFN- γ and TNF- α , and activated macrophages (for review see reference 21). The cooperation between the cells and cytokines requires close interaction, which is achieved after migration and granuloma formation in the lungs. The hallmark of infection in the lung is granuloma formation, consisting of clusters of macrophages, lymphocytes, and DCs, which physically contains the mycobacteria and creates a microenvironment for immune cell interaction, limiting *M. tuberculosis* growth and dissemination. Chemokines are potent leukocyte activators and chemoattractants aiding granuloma formation and thought to be critical for the immune response to *M. tuberculosis* (14). In vitro experiments demonstrated that *M. tuberculosis* infection of bone marrow-derived mouse macrophages results in the expression of TNF- α , as well as several chemokines, including ligands for the chemokine receptors CXCR3, CCR5, and CCR2. Neutralization of TNF- α by using antibody or TNFR1-deficient macrophages demonstrated that expression of certain chemokines (CXCL9/monokine induced by IFN- γ , CXCL10/IFN-inducible protein 10, CXCL11/IFN-inducible T cell α chemoattractant, CCL5/RANTES, and CCL2/MCP-1) after *M. tuberculosis* infection was dependent, at least in part, on TNF- α . However, the lack of TNF- α did not completely abrogate chemokine expression, indicating that there are other factors, induced as a result of infection, that stimulate chemokine production (22).

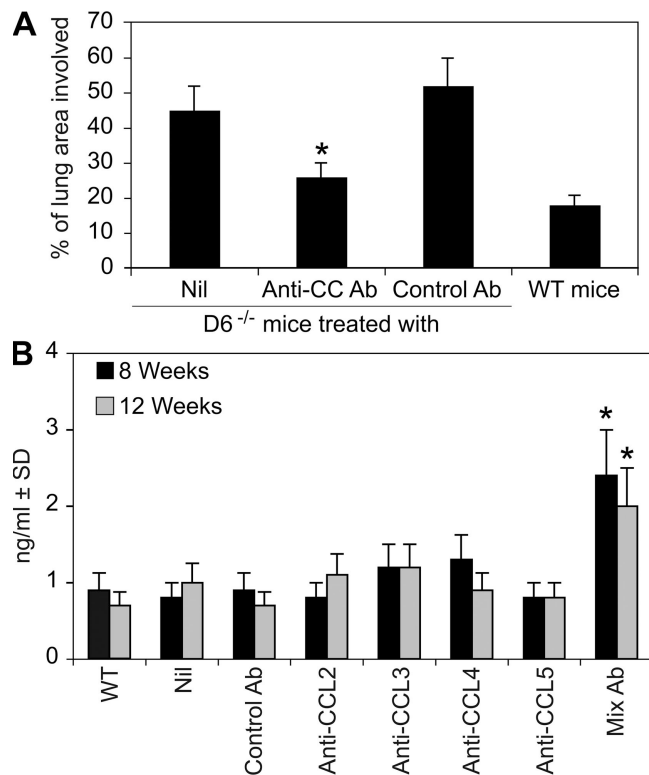


Figure 5. *M. tuberculosis* growth in $D6^{-/-}$ mice treated with anti-CC chemokine antibodies. (A) WT mice and $D6^{-/-}$ mice either untreated (Nil), or treated with a cocktail of anti-CC chemokine (Anti-CC Ab) or irrelevant control antibodies (Control Ab), were infected with *M. tuberculosis*. Granulomatous infiltration in the lungs of *M. tuberculosis*-infected mice was assessed by analyzing two lung lobes from each mouse. Data are expressed as the percentage \pm SD of the lung area involved, calculated by dividing the granuloma-involved area by the total tissue area. A representative of two different experiments is shown. (B) The course of *M. tuberculosis* infection followed against time in lungs. WT mice and $D6^{-/-}$ mice either untreated (Nil), or treated with antibodies against individual chemokines, with a cocktail of anti-CC chemokine (Mix Ab), or with irrelevant control antibodies (Control Ab), were infected with *M. tuberculosis*. Data shown are the mean bacterial counts \pm SD. *, $P < 0.01$ when compared with CFU values in $D6^{-/-}$ mice either untreated (Nil) or treated with control antibody.

In mouse models, gene expression of CXC and CC chemokines has been detected in the lungs after *M. tuberculosis* infection (22–24). CXCR3-deficient mice (25) have an impaired granuloma formation after aerosol infection with *M. tuberculosis*, although this effect is transient, occurring at the early stages of infection. CCR2-deficient mice are extraordinarily susceptible to moderate- or high-dose *M. tuberculosis* administered i.v. (26), and susceptibility is dose-dependent (27). Conversely, CCR5 (28) may not be essential to the development of a protective response to *M. tuberculosis* infection. CCL2/MCP-1-deficient mice did not demonstrate an increased susceptibility to *M. tuberculosis* infection, but whether cell infiltration or histology was affected in these mice was not reported (29, 30). However, transgenic mice overexpressing CCL2/MCP-1 were more susceptible to tuberculosis (31).

The action of chemokines is tightly controlled by decoy receptors (9), which internalize and target chemokines for intracellular degradation, thus avoiding excessive inflammatory responses and the consequent tissue damage. D6 is a decoy receptor that recognizes most inflammatory CC chemokines, implying that it may play a key role in resolving CC chemokine-driven inflammatory responses. The results reported in this paper confirm this possibility in a mouse model of *M. tuberculosis* infection through the mucosal (i.n.) route and show that $D6^{-/-}$ mice have an exaggerated inflammatory response leading to death, despite their ability to efficiently control bacterial load. Upon i.n. delivery of low-dose *M. tuberculosis*, 20% of mice succumbed at week 8, 50% of mice succumbed at week 12, and 100% of mice succumbed at week 16, despite the successful control of the growth of *M. tuberculosis* bacilli. The phenotype of $D6^{-/-}$ was dramatic and was found highly reproducible over a period of 3 yr in two additional experiments performed on a total of 17 mice per group. Moreover, CFU data at 4, 8, and 12 wk after *M. tuberculosis* infection were found highly reproducible in two additional independent experiments performed over a period of 2 yr. Mortality in $D6^{-/-}$ mice coincided with an overwhelming local and systemic inflammatory response that mainly compromised liver and kidney functions in the host, characterized by increased and sustained numbers of macrophages, DCs, and CD4 and CD8 T lymphocytes, and increased production of inflammatory CC chemokines and cytokines (TNF- α , IL-1 β , and IFN- γ). However, we failed to detect *M. tuberculosis* CFU in kidneys of both WT and $D6^{-/-}$ mice over a 12-wk period. This might reflect either *M. tuberculosis* growth below the CFU detection limit or the development of kidney damage in the absence of kidney infection. A similar increase in macrophage, DC, and T cell numbers was detected in the mediastinal lymph nodes, suggesting the possibility that more T lymphocytes were being primed and migrating to the lungs. Such increased priming likely accounts for the substantially increased numbers of T lymphocytes migrating to the lungs of $D6^{-/-}$ mice after *M. tuberculosis* infection, as confirmed by ELISPOT analysis showing that significantly more CD4 and CD8 T lymphocytes from the lungs of $D6^{-/-}$ mice were primed to produce IFN- γ when stimulated in vitro by BCG-infected DCs. The finding that $D6^{-/-}$ mice had more inflammation is intriguing and convincingly supports an immunoregulatory role for the D6 receptor. Recent data in other systems have shown that $D6^{-/-}$ mice display exaggerated responses to inflammatory stimuli, resulting in an aggressive cutaneous inflammatory pathology after the application of phorbol esters (15). In another study (16), $D6^{-/-}$ mice displayed an exaggerated response to the subcutaneous injection of complete Freund's adjuvant. Unfortunately, none of these studies has been able to define a mechanism for the heightened immune response in the $D6^{-/-}$ mice. Although there are several different possibilities for the increased inflammatory response in *M. tuberculosis*-infected $D6^{-/-}$ mice, the one we favor is that lack of the D6 receptor fails to remove CC chemokines from many different

organs and tissues, determining their increased concentration. Such increased expression/circulation of CC chemokines may, in turn, lead to signaling through their receptors and could result in the influx of immune cells and abundant, uncontrolled production of proinflammatory cytokines, which ultimately mediate tissue damage. However, the increased expression may also have been a reaction to the numbers of cells in infected organs. Whatever the case, the concentrations of CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES were found to be highly increased in the BAL and serum of *M. tuberculosis*-infected D6^{-/-} mice and sustained over time; moreover, blocking all four of them in D6^{-/-} mice in vivo (CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES) by repeated injection of a cocktail of neutralizing antibodies significantly reduced cell recruitment, production of proinflammatory CC chemokines and cytokines, liver and kidney damage, and, most importantly, the mortality of D6^{-/-} mice. However, despite controlling inflammatory responses, these mice became more susceptible to infection with *M. tuberculosis*, as demonstrated by higher CFU counts in the lungs at 8 and 12 wk after infection. The data reported in Table I clearly show that none of the neutralized chemokines alone accounts for the reduced inflammatory response and mortality, and for the increased susceptibility to infection.

CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES are all ligands of CCR5, which can attract and activate macrophages and Th1 lymphocytes. However studies in CCR5^{-/-} mice have found that although CCR5 plays a role in the migration of DCs to and from lymph nodes (28, 32), it is not indispensable for granuloma formation and immune protection against *M. tuberculosis* infection (32). CCL5/RANTES has been associated with the generation of type 1 cytokine-producing granulomas (33). CCR2-deficient mice are extraordinarily susceptible to moderate- or high-dose *M. tuberculosis* administered i.v. but not to low aerosol doses (27), and CCL2/MCP-1-deficient mice do not demonstrate an increased susceptibility to *M. tuberculosis* infection (29). However, there is redundancy in the chemokine system. Specific to this study, the chemokines CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES are ligands of CCR5, but CCL3/MIP-1 α and CCL5/RANTES can also signal through CCR1 and CCR3. This finding makes it difficult to dissect the precise roles of individual chemokines and their receptors. In this study, enhancing the type 1 T cell response or the ability of T lymphocytes to enter the lungs over the WT level does not have an obvious beneficial effect on the control of infection and inflammation, indicating that there are additional factors that must be induced or enhanced to increase the ability of the host to eliminate *M. tuberculosis* infection.

In summary, our study suggests an important role for the D6 receptor in immune regulation. D6 controls chemokine accumulation and activity, migration, and possibly, the maintenance of DCs and T cells in infected organs, as well as the production of inflammatory cytokines. Therefore D6, through its ability to avoid an excess of chemokines in the

inflamed tissue, may favor resolution of chronic inflammatory responses and overall provide a fine mechanism for the control of the balance between protective immune responses and immunopathology.

MATERIALS AND METHODS

Mice. D6^{-/-} mice were generated as previously described (15). Homogeneous populations were established by backcrossing heterozygous mice to C57BL/6 mice for more than eight generations. The resultant heterozygous mice were bred to obtain homozygotes. WT C57BL/6J and D6^{-/-} mice were bred in a specific pathogen-free/viral antibody-free barrier facility and obtained from Charles River Laboratories. 8–14-wk-old male and female mice were used in accordance with institutional guidelines in compliance with national (D.L. N.116, Gazzetta Ufficiale della Repubblica Italiana, supplement 40, 18-2-1992) and international law and policies (European Economic Community Council, 1987, Directive 86/609, Official Journal of European Communities L 358,1; and Institute of Laboratory Animal Resources, Committee on Life Sciences, National Research Council, 1996, Guide for the Care and Use of Laboratory Animals). Each experimental group consisted of 7–10 mice. All infected mice were maintained under germ-free conditions and were routinely monitored for mouse pathogens. Experiments were performed in specific pathogen-free facilities.

Chemicals and reagents. All chemicals were purchased from Sigma-Aldrich, unless otherwise noted. Middlebrook 7H9 liquid medium and 7H10 agar were obtained from Difco Laboratories. Antibodies used in flow cytometry were obtained from BD Bioscience or R&D Systems.

***M. tuberculosis* and infection of mice.** Mice were infected via the i.n. route with *M. tuberculosis* H37Rv (2×10^3 CFU in 20 μ l). This resulted in reproducible delivery of 50–100 viable CFU *M. tuberculosis*, as confirmed by CFU determination on the lungs of infected mice 1 d after infection. In fact, in a total of 10 mice per group from four independent experiments, day 1 *M. tuberculosis* CFU ranged from 50 to 100 (with only one exception in one WT mouse of 110 CFU), and in any case no statistical significant differences between day 1 CFU in WT (72.5 ± 21) and D6^{-/-} (75 ± 19) mice were detected ($P = 0.9658$ by the Student's *t* test). Moreover, using the i.n. infection route, we failed to detect *M. tuberculosis* CFU in nasal tissues in two different experiments. The tissue bacillary load was quantified by plating serial dilutions of the lung, liver, and spleen homogenates into 7H10 agar, as described previously (34).

Flow cytometric analysis of tissue cells. To determine cellular infiltrate, the lungs, liver, and mediastinal lymph nodes were removed at different weeks after infection and digested in the presence of 200 U/ml of collagenase, and mononuclear cell suspensions were obtained through Lympholyte M (Cederlane Laboratories) gradient centrifugation. The viability of cells, as determined by Trypan blue exclusion, was >90%. A single-cell suspension was prepared by pushing the tissue through a cell strainer. In some experiments, lung mononuclear cells were enriched in T cells by passage through a nylon wool column, and CD4 and CD8 T cells were then sorted by anti-CD4 or -CD8 immunomagnetic beads (Miltenyi Biotec), according to the manufacturer's instructions (34). The cells were incubated for 24 h at 37°C in complete medium to allow cells and beads to dissociate. Single-cell suspension were counted. The samples were triple stained with fluorochrome-conjugated anti-CD3, -CD4, -CD8, -CD44, -CD62L, -CD25, -CD11c, and -F4/80, in FACS buffer (0.1% Na azide, 0.1% BSA, and 20% mouse serum). Purified or PE-, FITC-, CyChrome-, or allophycocyanin-conjugated isotype control Igs were used as controls. After washes, the cells were fixed in 4% paraformaldehyde for 1 h and collected on a FACSCalibur (BD Biosciences). Analysis was performed on CellQuest software (BD Biosciences).

ELISPOT analysis for IFN- γ . The ELISPOT method (35) was used to detect IFN- γ secretion by individual CD4 or CD8 T cells from infected

mice, after stimulation with BCG-infected DCs in vitro prepared as previously described (36). In brief, ELISPOT plates (BD Biosciences) were coated with IFN- γ capture antibody overnight at 4°C. The capture antibody was discarded, and the plates were washed and blocked with complete media for 2 h at room temperature. 150,000 purified CD4 or CD8 T cells per well were cultured with irradiated BCG-infected WT DCs for 36–40 h at 37°C. The cells were discarded, and plates were washed with deionized water and PBS/Tween 20. Secondary biotinylated antibody was added for 2 h and incubated at room temperature, followed by washing with PBS/Tween 20. Streptavidin-alkaline phosphatase was added to the plates for 1 h, followed by washing and development of a color reaction using the AEC substrate reagent kit (BD Biosciences). The reaction was stopped when spots developed by running the plate under water. The spots were enumerated using an ELISPOT reader (Bioreader 3000; BioSys).

ELISA for cytokines and chemokines. BAL was obtained by flushing 2 ml PBS into the lungs of killed mice (37). The concentrations of chemokines (CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL11/eotaxin, and CCL22/MDC) and cytokines (TNF- α and IFN- γ) were quantified by ELISA in BAL and sera using commercially available kits (R&D Systems).

Determination of serum transaminases, BUN, and proteinuria. Serum alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) were measured by the standard photometric method using an automatic analyzer (type 7350; Hitachi Chemical Diagnostics) and a commercial kit (Sigma-Aldrich) adapted to small-sample volumes, as previously described (38). The normal ranges of ALT and AST were obtained testing sera from 10 mice, age- and sex-matched with animals used in experimental groups (38). Proteinuria was measured on a 0–4+ scale using a colorimetric assay strip (38) for albumin (Albustix; Bayer): 0, absent; 1+, ≤ 30 mg/100 ml (mild); 2+, 100 mg/100 ml (moderate); 3+, 300 mg/100 ml (severe); and 4+, $>2,000$ mg/100 ml (severe). BUN levels were measured by impregnating Azostix strips (Bayer) with a drop of fresh blood (38) and using the following scale: 1+, 5–15 mg/100 ml (normal); 2+, 15–26 mg/100 ml (mild); and 3+, ≥ 30 mg/100 ml (severe).

Neutralization of chemokines in vivo. To block inflammatory chemokines, mice were treated with a mixture of goat antibodies to the CC chemokines CCL2/MCP-1, CCL3/MIP-1 α , and CCL4/MIP-1 β , and a monoclonal antibody to CCL5/RANTES purchased lyophilized from R&D Systems, resuspended in sterile PBS, and mixed as previously described (16, 18). Mice were given i.p. injections of 200 μ l of the mixture, equivalent to 100 μ g of each antibody. Alternatively, mice were treated with 400 μ g of individual antibodies specific for a single CC chemokine. On the same days, control mice received i.p. injections equivalent to 400 μ g of irrelevant antibodies (normal goat IgG; Sigma-Aldrich) in 200 μ l PBS. Mice were injected weekly, starting from the third week after infection with *M. tuberculosis*.

Histology and morphometry. Tissue samples for histological studies were fixed in 10% normal buffered formalin, followed by paraffin embedding. 5–6- μ m sections were stained with hematoxylin and eosin. For histometry, 5- μ m-thick sections from each specimen were stained with hematoxylin and eosin and photographed at 6 \times using a stereoscopic zoom microscope (SMZ800; Nikon) and a digital camera (Coolpix 990; Nikon). Sections of at least eight lung lobes were studied in each case. A sequence of appropriate software programs were used (Scion Image [Scion Corporation]; Photoshop 5.0 [Adobe Systems Inc.]) to determine the area of each single lesion and the total tissue area on photomicrographs at each time point. Measurements were done blindly by two independent pathologists (P.J. Cardona and N. Caceres). Data are percentages calculated by dividing the granuloma-involved area by the global tissue area and multiplying by 100.

Statistics. The double-tailed Student's *t* test was used to compare the significance of differences between groups. Kaplan-Meier analysis was used to

determine the statistical significance of the differences in survival times of WT and D6 $^{-/-}$ mice. The significance of differences of log₁₀ CFU counts between groups was determined using one-way analysis of variance (ANOVA), with Scheffé tests for post-ANOVA individual comparisons. *P* < 0.05 was considered significant.

Online supplemental material. Fig. S1 shows lung and draining lymph nodes sections from *M. tuberculosis* patients stained for D6 and the macrophage marker CD68. Fig. S2 shows expression levels of the D6 transcript in mouse tissues during *M. tuberculosis* infection. Table S1 reports the effects of treatment of D6 $^{-/-}$ mice with anti-CC chemokine antibodies. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070608/DC1>.

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