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Acute Immune Response to *Mycobacterium massiliense* in C57BL/6 and BALB/c Mice▽

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Mycobacterium massiliense **is an environmental opportunistic pathogen that has been associated with soft tissue infection after minor surgery. We studied the acute immune response of C57BL/6 and BALB/c mice infected intravenously with 10^6 CFU of an *M. massiliense* strain isolated from a nosocomial infection in Brazil. The results presented here show that *M. massiliense* is virulent and pathogenic to both C57BL/6 and BALB/c mice, inducing a granulomatous inflammatory reaction that involves the activation of macrophages, dendritic cells, and natural killer cells induced by gamma interferon and interleukin-17 (IL-17) in C57BL/6 mice and by IL-12 in BALB/c mice.**

Mycobacteria that do not belong to the complex *Mycobacterium tuberculosis* are known as nontuberculous mycobacteria (NTM) or atypical mycobacteria. NTM are ubiquitous microorganisms found worldwide in soil and water (3, 23, 38). These environmental mycobacteria are considered emerging and environmental opportunistic pathogens (6, 23).

Mycobacterium massiliense is an environmental nonphotochromogenic, rapidly growing *Mycobacterium* strain that has been associated with soft-tissue infection after minor surgery or intramuscular injection (3, 5, 17, 22, 26, 46) and with pulmonary infection due to diseases, such as cystic fibrosis (29, 41). This species differs only slightly from *Mycobacterium abscessus*, sharing a 99.6% sequence identity of their 16S rRNA genes; genetic differences can be observed by comparative sequence analysis of the *rpoB* and *hsp65* genes (1, 25, 42). Infections with these agents tend to respond poorly to macrolide-based chemotherapy (3), even though the organisms are susceptible to clarithromycin (15, 44, 47).

M. massiliense infection mainly affects immunocompetent individuals and occasionally is associated with disseminated disease (8). An outbreak of *M. massiliense* occurred in Goiania, Brazil, where 30 individuals were infected after undergoing knee joint and laparoscopic surgery (5). Despite the fact that the infected individuals were from different hospitals, a unique *M. massiliense* strain was identified and characterized by pulsed-field gel electrophoresis.

Disease pathogenesis involves host-pathogen interactions that directly affect parasite clearance. Typically, when environmental bacteria are passively introduced into the host, rapid bacterial clearance occurs due to an efficient innate immune response (30). Nonetheless, accidental infections with *M. massiliense* have been described as having a chronic evolution and, in some cases, the disease is disseminated irrespective of the host's immune status. Such findings raise the possibility that this species is more virulent and/or pathogenic than other environmental mycobacteria, such as *M. chelonae* and *M. abscessus*.

Recently, a murine model of *M. abscessus* infection was described, and isogenic mice were shown to be good models to address the immune response of the host (34, 39). In the present study, we analyzed the immune response of C57BL/6 and BALB/c mice infected with a clinical isolate of *M. massiliense* obtained from the recent outbreak in Goiania, Brazil. We show here that *M. massiliense* is virulent and pathogenic to both C57BL/6 and BALB/c mice, inducing a granulomatous inflammatory reaction that involves the activation of macrophages, dendritic cells (DCs), and natural killer (NK) cells induced mainly by gamma interferon (IFN-γ) and interleukin-17 (IL-17) in C57BL/6 mice and by IL-12 in BALB/c mice.

MATERIALS AND METHODS

Clinical isolation of *M. massiliense*. One isolate of *M. massiliense*, GO2, originally obtained from an outbreak that occurred in Goiania, Brazil (5), was maintained in Löwenstein-Jensen medium (20) and inoculated into 1 liter of 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) with orbital agitation for 96 h at 36°C. The bacilli were collected by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in PBS–0.05% Tween. The microorganisms were then dissociated with low-power sonication and titrated in 7H11 agar plates. Aliquots were frozen at −80°C until used. Upon defrosting, all aliquots were retitrated to confirm the concentration of CFU.

Animals. C57BL/6 and BALB/c females, 4 to 8 weeks of age and weighing between 18 and 22 g, were obtained from the animal facilities of the Instituto de Patologia Tropical e Saúde Pública. The animals were maintained in specific-pathogen-free-conditions. Mice were housed three to four animals per cage and allowed unlimited food and water. The results presented here represent one of three independent experiments. For each experiment, 25 mice from each lineage

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TABLE 1. Nucleotide sequences of primers used in the RT-PCR

Cytokine	Primer sequence (5′–3′)	
	Forward	Reverse
IL-12	CCACTGGAACTACACA AGAACG	GCACAGGGTCAT CATCAAAG
IL-17	AACATGAGTCCAGGG AGAGC	GCTGAGCTTTGA GGGATGAT
TNF-α	ATGGCCTCCCTCTCAT CAGT	CACTTGGTGGTT TGCTACGA
IL-10	TCAGCCAGGTGAAGA CTTTCT	TCATTTCCGATA AGGCTTGG
IL-4	GCAACGAAGAACACC ACAGA	CTGCAGCTCCAT GAGAACAC
IFN-γ	CAAGTGGCATAGATG TGGAAG	TGGCTCTGCAGG ATTTTCAT
β-actin[a]	GACGGCCAGGTCATC ACTAT	ATGCCACAGGAT TCCATACC

[a] Housekeeping reporter gene.

strain were used. The Ethical Committee of Universidade Federal de Goiás approved all experimental procedures.

Experimental infection. After optimizing the infection dose, mice were intravenously infected with ~10^6 CFU. All inocula were plated in 7H11 agar plates. To evaluate their immune responses, the animals were euthanized with CO_2 at 1, 3, 7, 14, or 30 days postinfection. The lungs, spleens, and livers were aseptically removed and used to perform CFU counts, cellular cultures, and flow cytometric analyses.

CFU determination. Bacterial load was determined as previously described (24). Briefly, organs were aseptically removed and homogenized in 2 ml of PBS. For each organ, the suspension was serially diluted 10-fold, and 0.1 ml of each appropriate dilution was cultured in triplicate by plating onto 7H11 agar supplemented with OADC. The colonies were enumerated after 8 days of incubation at 35°C.

Cytokine mRNA quantification. Total cellular RNA was isolated from spleens. Briefly, tissues were homogenized in TRIzol reagent (Invitrogen) and processed according to the manufacturer's instructions. Quantification of total RNA obtained from the tissues was done spectrophotometrically, and 2 μg of purified RNA was used to generate cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Quantitative real-time PCR was performed using 360 ng of cDNA in a LightCycler 1.0 (Roche Diagnostics) using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics).

The sequences of the primer pairs used for each PCR are listed in Table 1.

Melting-curve analysis was used to check the quality of the PCR products. Each result was normalized to the expression of the housekeeping gene β-actin. Quantification of gene expression was performed by using the Pfaffl method (36).

Cell isolation and culture. Single-cell suspensions from liver and spleen were prepared by pressing the organs through a 70-μm-pore-size mesh (cell strainer; BD Falcon) with a sterile syringe plunger. Cells were then resuspended in complete medium (CM) composed of RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 20 mM HEPES, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids (Sigma), and penicillin and streptomycin at a density of 10^6 cells/ml in 96-well plates, followed by incubation with monensin (3 μM) for 6 h at 37°C in an atmosphere of 5% CO_2 for further analysis by flow cytometry.

Flow cytometric analysis. For fluorescence-activated cell sorting (FACS) analysis, the cells were treated with Golgi stop solution (monensin; BD Pharmingen), incubated for 6 h, and then collected for analysis. To perform surface and intracellular staining, the cells were transferred to a 96-well plate and treated with PBS supplemented with 0.01% sodium azide for 20 min. After centrifugation (900 × *g* for 5 min), an antibody solution consisting of anti-CD11b-PE, anti-CD11c-APC, anti-CD14-PerCP (all from eBioscience), anti-CD62L-APC, and anti-NKG2A-FITC (all from BD Pharmingen, San Jose, CA) was added to the cells, and the plates were incubated for 18 min at 4°C. The cells were then washed twice with PBS supplemented with 0.01% sodium azide and treated with PermFix (BD Pharmingen) for 18 min. Then, after a wash with PermWash (BD Pharmingen), a solution of anti-TNF-α-FITC (BD Pharmingen) monoclonal antibody diluted in PermWash was added to the cells, and the plates were incubated in the dark for 15 min. After a wash with PermWash and PBS supplemented with 0.01% sodium azide, the cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer. A minimum of 50,000 events were acquired per sample, and the data were analyzed using DIVA software (Becton Dickinson).

Measurement of intracellular nitric oxide. Spleen and liver cells were cultured for 48 h in CM at 37°C in an atmosphere of 5% CO_2 in preparation for nitric oxide (NO). Intracellular NO levels were measured with Griess reagent as previously described (19).

Histopathology. Organs were fixed for 24 h in 3.7% formaldehyde, transferred to 70% ethanol for 24 to 48 h, and then embedded in paraffin. Sections (5 μm thick) were cut on a rotary microtome, stretched in a water bath, mounted on glass slides, and stained with hematoxylin and eosin (H&E) or by the Fite's acid-fast staining method. An experienced pathologist blinded to the nature of the slides examined and performed qualitative analysis with one of of three slides prepared.

Statistical analysis. Means and standard deviations (SD) were calculated for all results. The analysis of variance (ANOVA) test was used to compare differences between the groups. The Student *t* test was performed for continuous variables. A P value of <0.05 was considered significant.

RESULTS

***M. massiliense* intravenous infection.** Mice were intravenously infected with *M. massiliense* and, at different times after exposure, the lungs, spleens, and livers were analyzed for bacterial load. C57BL/6 and BALB/c mice exhibited an increased bacterial load during the first days of infection in all organs analyzed. However, 14 days postinfection, BALB/c mice presented higher bacterial loads than C57BL/6 mice ($P < 0.05$, Fig. 1). Infection with $<10^5$ bacilli/mouse CFU did not lead to an increase in bacterial load during the study period, and the bacterial load was almost cleared before the establishment of the specific immune response (~30 days, insets in Fig. 1). Based on these results, we performed further analysis of the immune response using 10^6 bacilli/mouse.

To further characterize the pathogenesis of the *M. massiliense* isolate, the lungs, spleens, and livers of infected mice were evaluated microscopically. The liver presented microgranulomatous lesions starting on the first day of infection and these lesions persisted and increased during the duration of the study. These lesions were mainly comprised of mononuclear cell infiltrates (Fig. 2). The number of mononuclear cell infiltrates increased equally between C57BL/6 and BALB/c mice; however, the hepatic lesions in the livers of C57BL/6 mice started to present central necrosis 14 days postinfection (Fig. 2F and H).

Hepatosplenomegaly was observed in all infected animals mainly at 14 and 30 days postinfection. Small, circular, white lesions were observed on the surface of the livers of infected C57BL/6 mice. No visible macroscopic lesions were observed in the lungs of C57BL/6 or BALB/c mice (Fig. 3A and B, 30 days postinfection). At 30 days postinfection, both mouse strains presented condensed granulomatous infiltrates in their livers (Fig. 3C and D).

At 30 days postinfection, the lungs of infected BALB/c mice presented scattered lesions, while those of infected C57BL/6 mice presented small and concise granulomatous lesions (Fig. 3E and F). No visible lesions were observed in the spleen, although bacilli were detected in this organ by Fite's acid-fast staining method (data not shown).

Early production of NO by spleen and liver cells is induced by *M. massiliense* infection. To characterize the innate immune response elicited by *M. massiliense*, spleen and liver cells were

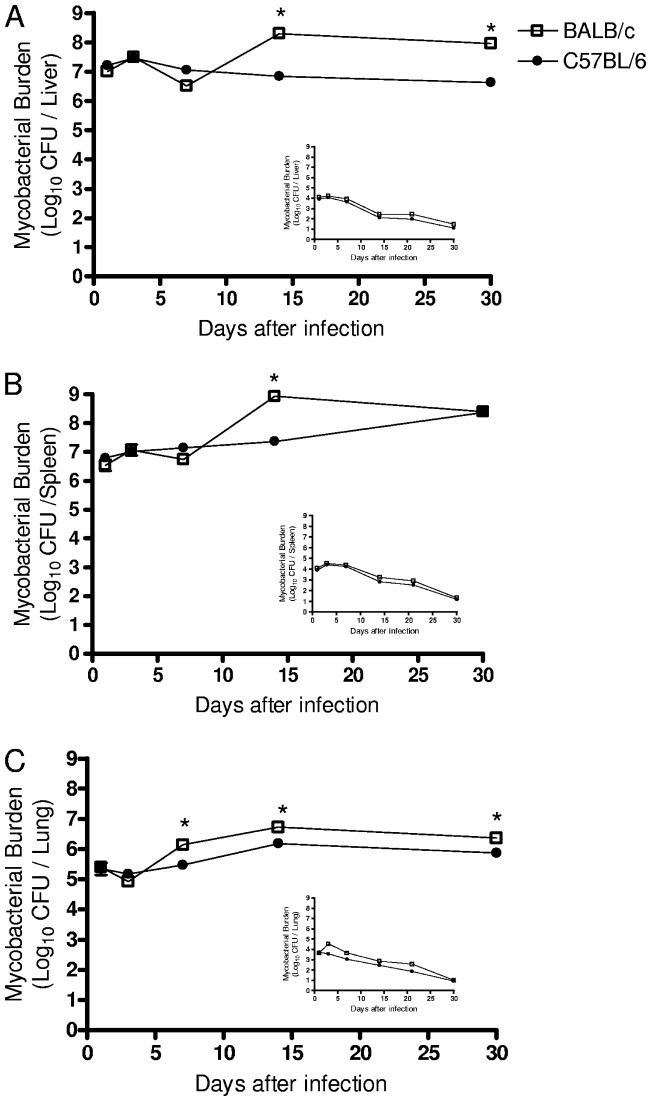


FIG. 1. The *M. massiliense* bacterial load is maintained during infection. C57BL/6 and BALB/c mice were injected intravenously with 10^6 CFU of *M. massiliense*, and the bacterial load of their livers, spleens, and lungs (A, B, and C, respectively) were determined at 1, 3, 7, 14, and 30 days postinfection. The data show the means ± the SD bacterial load of five mice per group per time point. The results represent one of three independent experiments. The inset graphs represent infection with 10^4 CFU of *M. massiliense* for the same organs. *, $P < 0.05$.

cultured ex vivo and their spontaneous production of NO was measured after 48 h of culture (Fig. 4). When comparing uninfected versus infected animals, we found that the levels of NO were doubled and tripled in both liver and spleen cells at 7 and 14 days postinfection, respectively (Fig. 4).

Differential cytokine gene expression between C57BL/6 and BALB/c mice infected with *M. massiliense*. To further understand the mechanism of the immune response against *M. massiliense* infection, we analyzed and compared the levels of cytokine mRNA in the spleens of infected versus uninfected animals. We observed a relatively discrete increase in the levels of tumor necrosis factor alpha (TNF-α) mRNA in both strains in infected mice. In contrast, the levels of IL-17 in the spleen were fourfold higher in C57BL/6 mice 7 days postinfection than in uninfected mice (Fig. 5).

We also analyzed the levels of Th1- and Th2-inducing cytokines. Our results demonstrated that infected C57BL/6 mice exhibited a steady increase in the levels of both IFN-γ and IL-4 at 7 days postinfection. When comparing the increase in the levels of both of these cytokines (IFN-γ and IL-4), we found that IFN-γ had a much more pronounced increase in infected C57BL/6 mice (Fig. 5). Infected BALB/c mice exhibited only a discrete increase in most of the cytokines analyzed in the spleen during the acute phase of infection, whereas the relative levels of IL-12 were higher in this strain than in infected C57BL/6 mice throughout the duration of the experiment ($P < 0.05$). At 14 days postinfection, BALB/c mice expressed fivefold more mRNA for IL-12 than did infected C57BL/6 mice (Fig. 5, $P < 0.05$). The expression of IL-10 mRNA in spleen cells from infected animals presented a pattern similar to that observed for the expression of TNF-α mRNA; only infected C57BL/6 infected mice exhibited a significant increase in the levels of IL-10 mRNA 7 days postinfection with *M. massiliense* infection.

Macrophages and DCs accumulate in the liver and spleen after infection with *M. massiliense*. Macrophages, defined as $CD14^+$ cells, were quantified at 7 and 14 days postinfection in the spleen and livers (Fig. 6A1). Intravenous infection with *M. massiliense* induced a significant increase in the percentage of total spleen TNF-α^+ cells in both C57BL/6 and BALB/c mice at 7 and 14 days postinfection (Fig. 6A3 and B). Activated macrophages ($CD14^+$ $CD11b^+$, Fig. 6A1), nonactivated DCs ($CD11c^+$ $CD11b^-$, Fig. 6A2), and activated DCs ($CD11c^+$ $CD11b^+$, Fig. 6A2) in the spleens of infected mice produced significantly higher levels of TNF-α compared to uninfected mice (Fig. 6C).

In addition, *M. massiliense* infection in both C57BL/6 and BALB/c mice led to an increased accumulation of activated macrophages in the liver and spleen (Fig. 6D and E). However, macrophages in the liver were found to be more activated than those in the spleen at 7 days postinfection in BALB/c mice and at 7 and 14 days postinfection in C57BL/6 mice.

Moreover, an increased accumulation of activated DCs was observed in the livers and spleens of BALB/c mice at 7 and 14 days postinfection. In C57BL/6 mice, the frequency of DCs was increased in the liver at 7 and 14 days postinfection and in the spleen at 14 days postinfection (Fig. 6F).

NK cells accumulate in the spleen after *M. massiliense* infection. To determine whether *M. massiliense* infection affects the activity of NK cells, we evaluated tissue-resident NK cells by analyzing their cell surface expression of NK2G-AC by flow cytometry (Fig. 7). Regardless of the mouse strain, *M. massiliense* infection induced a pronounced increase and activation of NK cells in the spleen at 14 days postinfection (Fig. 7, $NK2G\text{-}AC^+$, $CD62L^-$ cells, $P < 0.05$). In addition, the percentage of total NK cells in the livers and spleens of infected animals increased threefold by day 14 postinfection.

DISCUSSION

We present here the first detailed study of the acute immune response against *M. massiliense* in C57BL/6 and BALB/c mice.

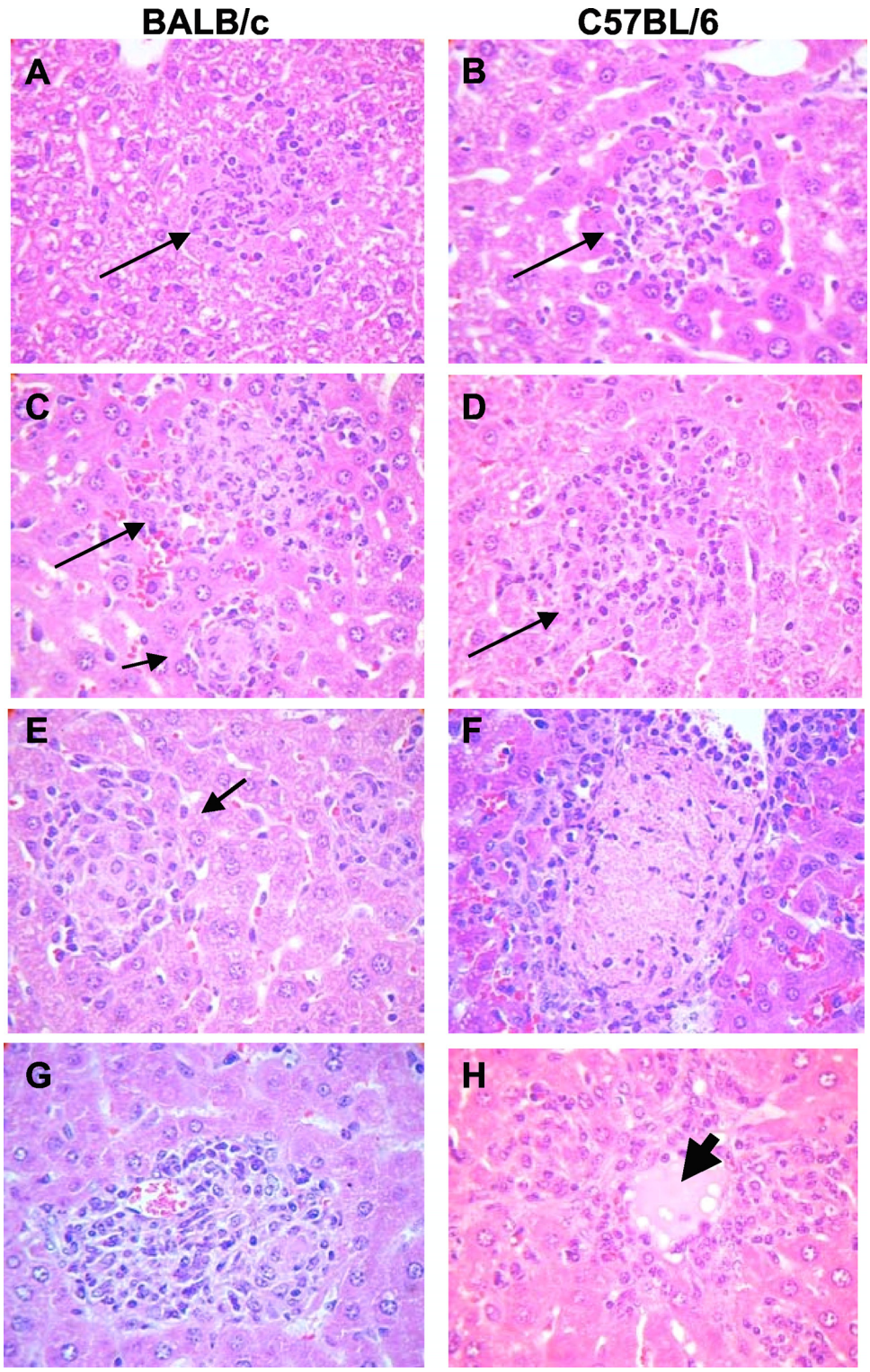


FIG. 2. Development of early granulomatous lesions in the livers of *M. massiliense*-infected mice. Histological findings in the livers of C57BL/6 and BALB/c mice infected with *M. massiliense*. Liver sections were stained with H&E and examined at 200× magnification. Inflammatory cell infiltrates (arrows) are shown for days 1 (A and B), 3 (C and D), 7 (E and F), and 14 (G and H) postinfection. The heavy arrow in panel H indicates necrosis observed in the liver of C57BL/6 mice 14 days postinfection.

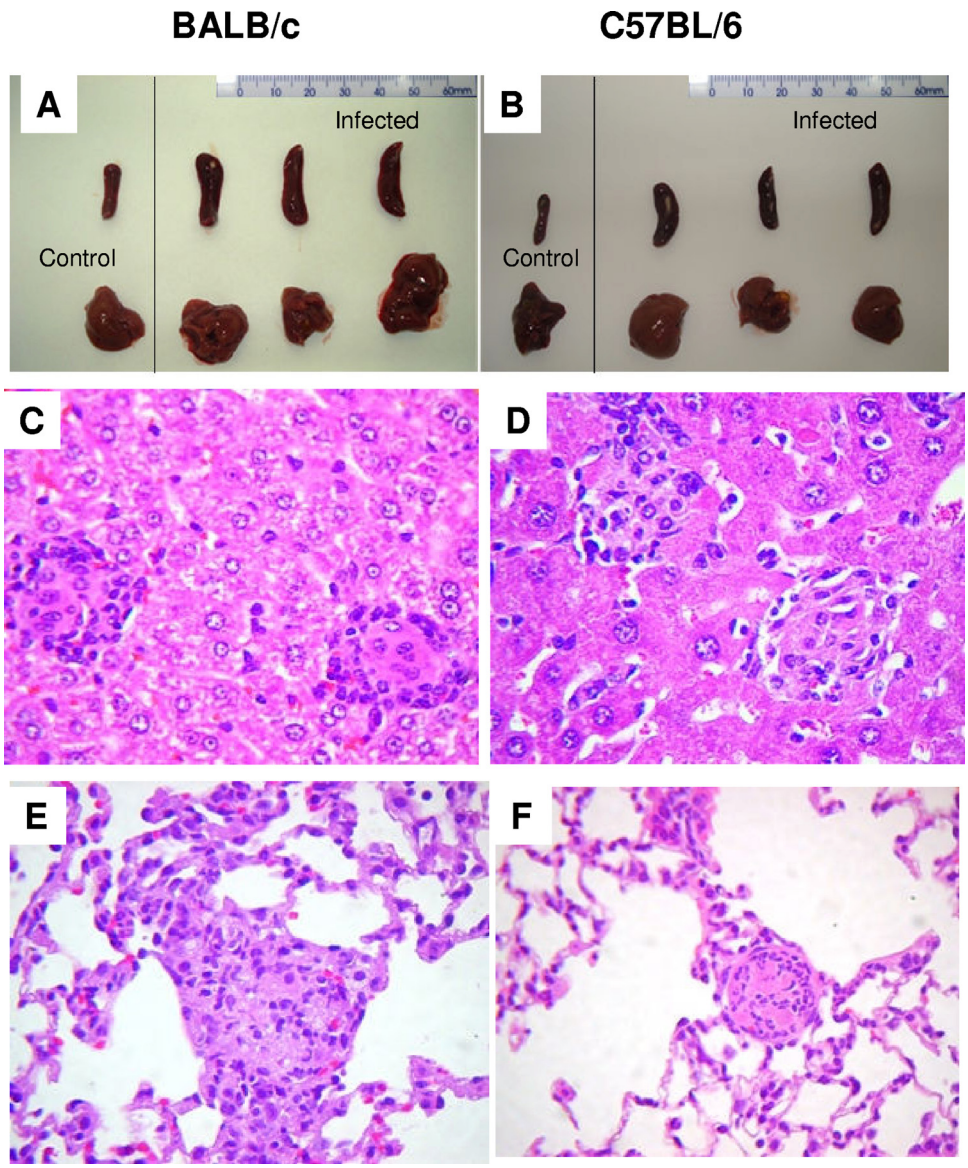


FIG. 3. *M. massiliense* infection compromises the lungs, spleens, and livers of infected animals. Macroscopic and histological findings observed in C57BL/6 and BALB/c mice infected with *M. massiliense*. (A) Comparison of the spleen and liver of a control uninfected mouse (left) and an infected BALB/c mouse. (B) Comparison of the spleen and liver of a control uninfected mouse (left) and an infected C57BL/6 mouse. Lung and liver sections were stained with H&E and examined at 400× magnification (C to F). (C and D) Liver granulomatous reactions at 30 days postinfection in C57BL/6 and BALB/c mice, respectively. (E and F) Granulomatous lesions in the lungs of C57BL/6 and BALB/c mice, respectively.

M. massiliense was pathogenic upon intravenous infection of the mice with 10^6 bacilli, resulting in bacterial growth and granulomatous lesions in their lungs, spleens and livers.

We chose to evaluate *M. massiliense* infection in C57BL/6 and BALB/c mice because these isogenic mouse strains are commonly used to study mycobacteria infection (35). Our results indicate that *M. massiliense* has a pathogenicity similar to that of other NTM. In contrast to what is observed during the infection of mice with *M. tuberculosis* (9, 24, 34), *M. massiliense* reaches a log growth phase in only 15 days, the period after which the bacteria reach a steady growth phase. At 90 days postinfection, $\sim 10^4$ bacteria are still present in the livers and spleens of infected mice (data not shown). As previously reported in the literature for mycobacteria (48), C57BL/6 mice control bacterial loads better than BALB/c mice when infected with *M. massiliense*. This statistically significant difference in the ability of the mice to control bacterial load does not reflect their resistance to infection because both mouse strains were not able to clear the infection by mycobacteria.

Although C57BL/6 mice infected with NTM, such as *M. abscessus* via an aerosol route (10^2 bacilli/mouse) or by an intravenous route (10^7 CFU/mouse), can efficiently clear the pathogen 30 days after infection (4, 34, 39), intravenous infection with 10^6 CFU of *M. massiliense* results in the persistence of the pathogen, with concentration levels reaching $\sim 10^7$ CFU in the liver, spleen, and lungs during a similar 30-day time period (Fig. 1).

Our results demonstrate that *M. massiliense* induces severe

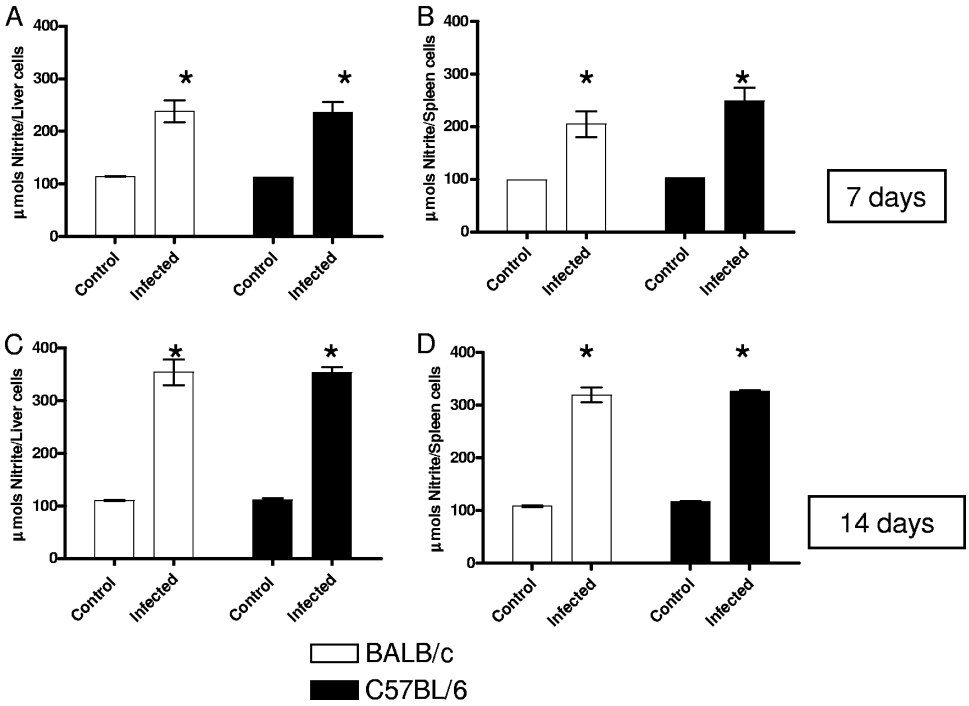


FIG. 4. Evidence that NO is induced by the immune response against *M. massiliense* in C57BL/6 and BALB/c mice. NO production by liver (A and C) and spleen (B and D) cells at 7 and 14 days postinfection intravenously with 10^6 CFU of *M. massiliense*. The results are presented as total micromoles produced per 10^6 cells during 48 h of culture. The data shown are from one of three independent experiments. *, $P < 0.05$.

hepatosplenomegaly (Fig. 3) that could be the result of hemolysis or inflammation. Analysis of peripheral blood cells of infected animals revealed that hemolysis was not occurring. However, we did observe a significant increase in the total number of cells in the liver and spleen as a result of numerous focal granulomatous lesions, which were observed mainly in the liver (data not shown). Our results demonstrate that *M. massiliense* can persist and induce an immune inflammatory response, indicating that this microorganism is pathogenic to mice as it is to humans (Fig. 1, 2, and 3).

It is interesting that the granulomatous lesions in the livers of infected C57BL/6 and BALB/c mice exhibited different patterns. Infected BALB/c mice presented mononuclear and neutrophilic inflammatory lesions in the liver, which evolved to concise microgranulomatous lesions at 30 days postinfection infection. In contrast, the livers of infected C57BL/6 mice always presented mononuclear cell infiltrates with central necrosis at 14 days postinfection (Fig. 2 and 3). In the absence of reported studies with which to compare our results on murine infection with *M. massiliense*, a parallel can be made to infections with other NTM and *M. tuberculosis* microorganisms. Infection of BALB/c mice with mycobacteria induces an early neutrophilic inflammatory response; however, only when C57BL/6 are deficient in IFN-γ can necrotic lesions be observed upon infection with virulent *M. tuberculosis* (23).

Induction of necrosis can be correlated with pathogen virulence as well as with an excessive immune response (37). It is well established that C57BL/6 mice are efficient Th1 responders and that this is mainly due to their rapid response to intracellular pathogens, a process that is principally mediated by the production of NO and TNF-α. Infection of either C57BL/6 or BALB/c mice with *M. massiliense* can induce the production of NO by spleen and liver cells ex vivo. Nonetheless, at 7 days postinfection, C57BL/6 mice exhibited a considerable increase in the levels of iNOS mRNA in the spleen compared to infected BALB/c mice (data not shown). The importance of NO production and the clearance of bacilli is emphasized by the finding that deletion of the NOS2 gene increases the susceptibility of mice to *M. tuberculosis* infection (13). Mycobacteria have been shown to employ several mechanisms for the induction of NO production; for example, the 19-kDa lipoprotein can activate macrophages via TLR2 and thus induce the synthesis of iNOS and consequent production of NO (7, 28, 33). The precise mechanisms by which *M. massiliense* induce the production of NO may be similar to those used by other strains of mycobacteria, but further studies are needed to confirm this possibility.

The early production of cytokines during an infection orchestrates the specific immune response that will be elicited by the pathogen (39). We clearly demonstrated that *M. massiliense* infection in C57BL/6 mice induces the upregulation of cytokine mRNA in the spleen cells, leading to the production of IFN-γ, IL-17, and IL-4 production at 7 days postinfection. BALB/c mice, on the other hand, exhibited a massive upregulation of IL-12 mRNA expression at 14 days postinfection (Fig. 5). Thus, infection of C57BL/6 mice with *M. massiliense* appears to elicit an immune response earlier than in BALB/c mice.

Taken together, our results suggest that production of pro- and anti-inflammatory cytokines leads to the recruitment and organization of mononuclear cells in infected C57BL/6, allowing them to control their bacterial burden in the different

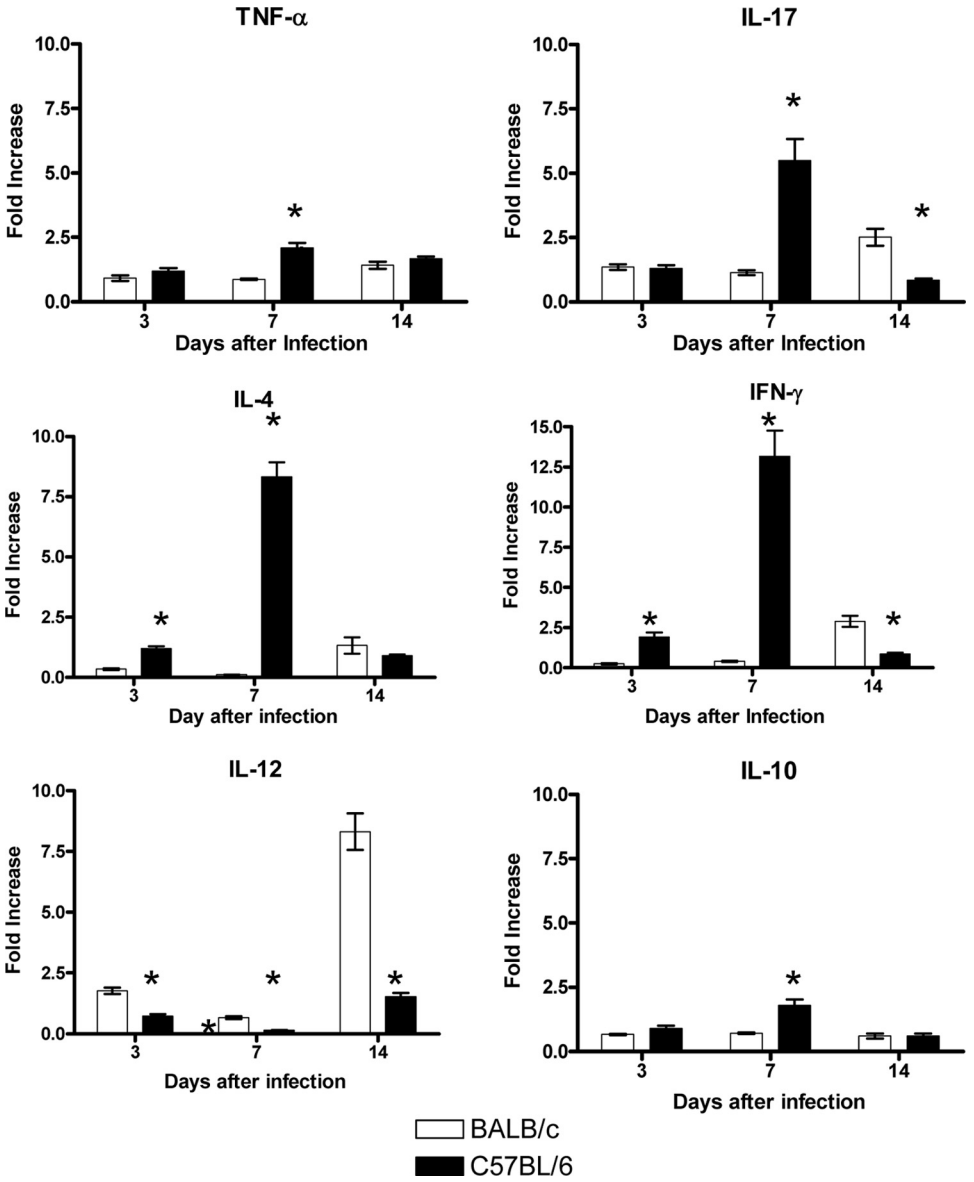
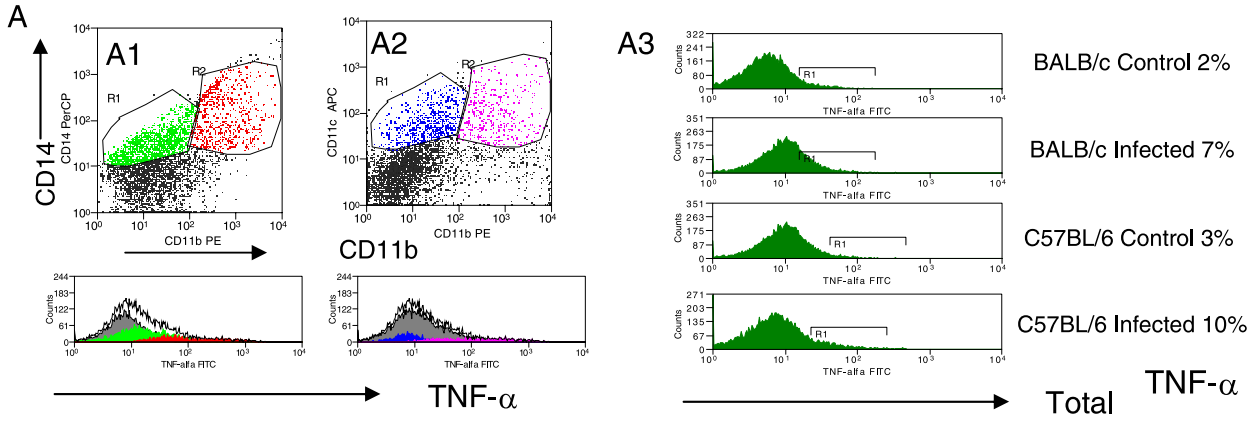


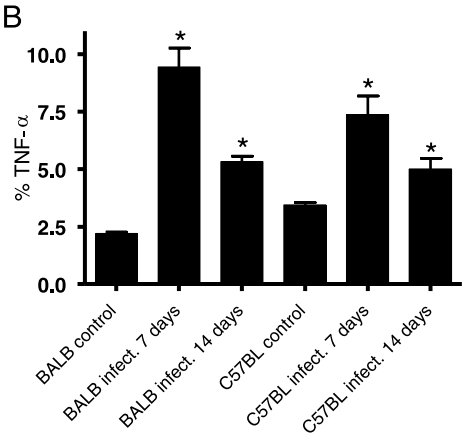
FIG. 5. Modulation of host cytokine gene expression in the spleens of C57BL/6 and BALB/c mice in response to *M. massiliense* infection. mRNA was extracted from the spleens of infected mice on days 3, 7, and 14 postinfection. The levels of mRNA were quantified by real-time PCR using gene specific primers for inflammatory cytokine genes. *, $P < 0.05$ for C57BL/6 versus BALB/c cytokine transcript levels.

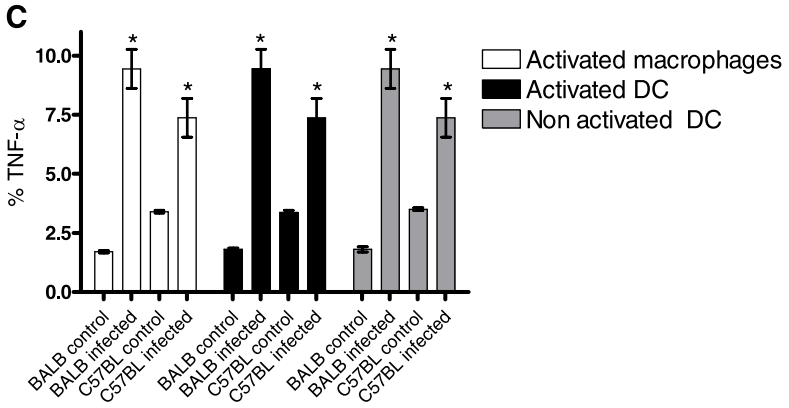
organs analyzed. In contrast, infected BALB/c mice exhibited mononuclear cell and neutrophil infiltration in the liver with poor cytokine production at 7 days postinfection, which may reflect their decreased ability to control bacterial growth.

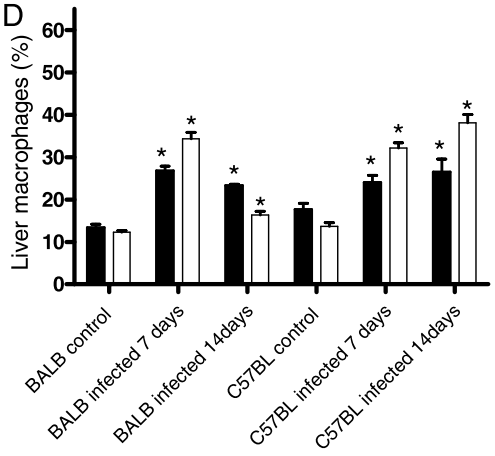
Although few studies have characterized the immune response to experimental NTM infections, the response elicited by these agents is similar to that observed in tuberculosis, with a prominent Th1 or cellular immune response (10, 34, 39). In the present study, we observed that the levels of Th1-associated cytokines, such as IFN-γ and IL-12, were increased in both mouse strains at different levels, whereas, with the exception of IL-4 at 7 days postinfection, Th2 cytokines were produced at much lower levels in C57BL/6 mice (Fig. 5). Th1 cytokines are potent macrophage activators; consequently, they are crucial for the control of bacterial growth. When bacterial levels reached a plateau in infected mice, macrophages, DCs, and NK cells were increased and activated in the different organs analyzed (Fig. 6 and 7). This finding supports the idea that the immune response elicited by *M. massiliense* is similar to that induced by *M. tuberculosis* (24), which is mediated by activated macrophages, DCs, and NK cells.

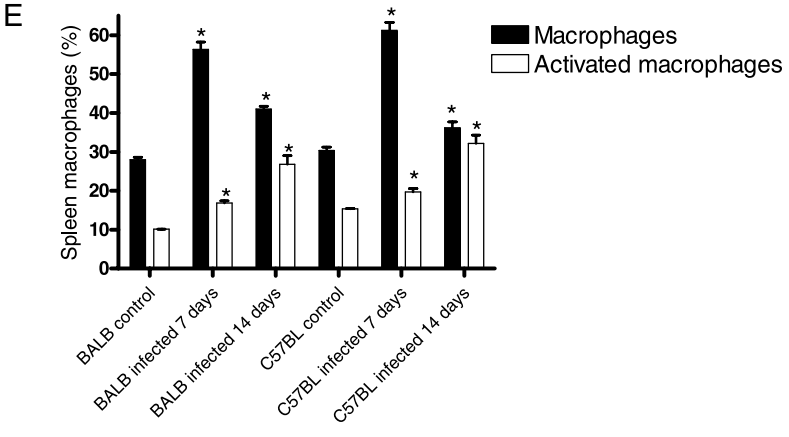
Whether the production of IL-10 is associated with susceptibility to *M. tuberculosis* infection is still a matter of debate. Some studies have shown that IL-10 production is correlated to the reactivation of *M. tuberculosis* infection in C57BL/6 infected mice, whereas other studies have clearly shown that infected BALB/c and BALB.xid mice produce higher levels of IL-10 without affecting disease evolution or reactivation (2, 23, 24, 43). The discrepancies in these studies raise the possibility that the role of IL-10 in mediating *M. tuberculosis* infection

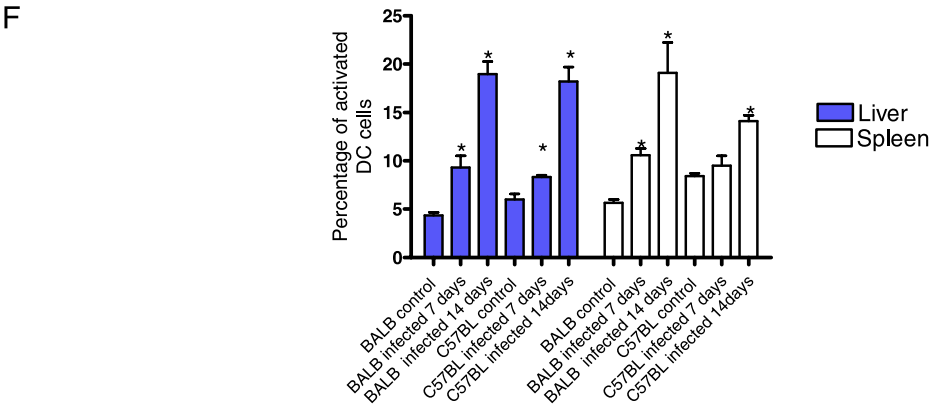












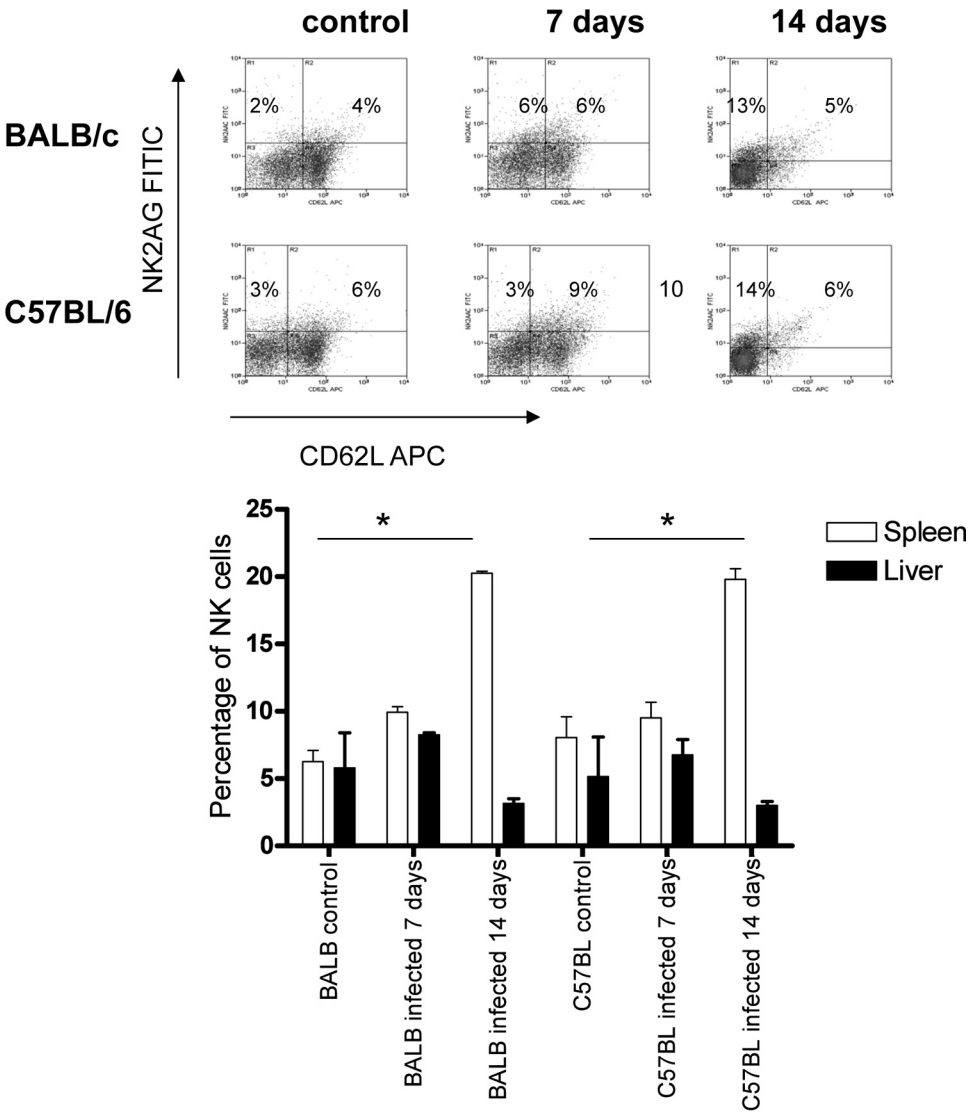


FIG. 7. Increase of NK cells in response to *M. massiliense* infection in C57BL/6 and BALB/c mice. Animals were inoculated intravenously with 10^6 CFU of *M. massiliense*, and spleen and liver NK2AG$^+$ and CD62L$^+$ cells were analyzed by flow cytometry on days 7 and 14 postinfection. Experiments were repeated three times. Each dot plot represents one of four mice analyzed. The graph shows the means ± the SD percentage of cells obtained from four mice in one experiment. *, $P < 0.05$.

may be strain dependent. Recent evidence, however, implicates IL-10 in the modulation of inflammatory lesions, thereby preventing tissue destruction (11).

During *M. tuberculosis* infection in mice, macrophages and DCs play different roles. Macrophages secrete proinflammatory cytokines and initiate the granulomatous inflammatory response, whereas DCs are primarily involved in the induction of specific *M. tuberculosis* T-cell responses (18). DCs also produce IL-12 and chemokines, among other molecules, which directly induce macrophage activation (14, 21, 40). Macrophages and DCs actively participate in the immune response against *M. massiliense* because upon infection they produce TNF-α, an important proinflammatory cytokine that is involved in macrophage and neutrophil recruitment/activation

FIG. 6. Macrophages and DCs are activated in response to *M. massiliense* infection. Macrophages, characterized by expression of CD14 (A1), and DCs, characterized by expression of CD11c (A2), were evaluated for their activation status (CD11b) and expression of TNF-α (A3 and B). The percentage of TNF-α$^+$ macrophages and DCs in the spleens of infected mice was quantified according to the activation status of each cell population (C). The number of macrophages, according to their activation status, in the livers and spleens of infected mice was determined (D and E, respectively). (F) Proportion of DCs in the liver and spleen during infection.

(28) and in the containment of bacteria by granuloma formation (31). Our findings that activated ($CD11b^+$) macrophages and DCs producing TNF-α can be observed within the granulomatous lesions in the livers of infected animals led us to infer that early activation of macrophages and DCs is responsible for the formation of the concise granulomatous inflammatory reaction observed 30 days postinfection. This finding is in agreement with the idea that TNF-α is the major cytokine orchestrating the induction of a mononuclear inflammatory response that culminates in granuloma formation (11, 27, 28, 31).

Infection with *M. massiliense* induced the upregulation of IFN-γ and IL-17 mRNA in C57BL/6 mice at 7 days postinfection in the spleen. Although these mice exhibited higher levels of IL-17 mRNA, they did not present a neutrophilic inflammatory reaction. Several reports suggest that IL-17 is responsible for neutrophil recruitment and activation (32), whereas others demonstrate a role for Th1 cells in the regulation of IL-17 production (12). The balance between IL-17 and IFN-γ production appears to be responsible for granuloma formation as well as for bacterial clearance (12, 45).

The fact that infected C57BL/6 mice had higher levels of IFN-γ than IL-17 suggests that it is sufficient for the activation of macrophages and DCs ($CD11b^+$ TNF-α^+ cells). The intriguing finding that infected BALB/c mice exhibited lower IL-17 mRNA levels but still presented a heterogeneous population of neutrophils and macrophages in the inflammatory lesions warrants further investigation. In addition, infected BALB/c mice presented higher bacterial loads in the liver, spleen, and lungs than infected C57BL/6 at 14 days postinfection, which was only changed in the spleens of BALB/c mice later during infection (30 days, Fig. 1). The higher bacterial load observed in BALB/c mice at 14 days postinfection was accompanied by increased expression of IL-12; whether one was the cause or effect of the other needs to be further investigated.

Our results clearly show that activated NK cells accumulate in the liver and spleen during the early phase of *M. massiliense* infection. Whether this increase in NK cell frequency has a role in the immune response induced by *M. massiliense* infection requires further investigation (i.e., using IL-15 KO mice). Our previous studies on *M. tuberculosis* infection have shown that NK cells can produce IFN-γ early in the response; however, NK cells are not crucial for the induction of Th1-mediated immune responses or the control of bacterial load (16, 24).

The results presented here demonstrate that *M. massiliense* is pathogenic for both C57BL/6 and BALB/c mice, inducing a granulomatous inflammatory reaction that involves the activation of macrophages, DCs, and NK cells induced by IFN-γ and IL-17 in C57BL/6 and IL-12 in BALB/c mice.

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