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Nramp1 Is Not a Major Determinant in the Control of *Brucella melitensis* Infection in Mice

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***Brucella,* the causative agent of brucellosis in animals and humans, can survive and proliferate within macrophages. Macrophages mediate mouse resistance to various pathogens through the expression of the *Nramp1* gene. The role of this gene in the control of *Brucella* infection was investigated. When BALB/c mice (*Nramp1*^{s}) and C.CB congenic mice (*Nramp1*^{r}) were infected with *Brucella melitensis,* the number of *Brucella* organisms per spleen was significantly larger in the C.CB mice than in the BALB/c mice during the first week postinfection (p.i.). This *Nramp1*-linked susceptibility to *Brucella* was temporary, since similar numbers of *Brucella* were recovered from the two strains of mice 2 weeks p.i. The effect of *Nramp1* expression occurred within splenocytes intracellularly infected by *Brucella*. However, there was no difference between in vitro replication rates of *Brucella* in macrophages isolated from the two strains of mice infected in vivo or in *Nramp1* RAW264 transfectants. In mice, infection with *Brucella* induced an inflammatory response, resulting in splenomegaly and recruitment of phagocytes in the spleen, which was amplified in C.CB mice. Reverse transcription-PCR (RT-PCR), performed 5 days p.i., showed that inducible nitric oxide synthase, tumor necrosis factor alpha (TNF-α), interleukin-12 p40 (IL-12p40), gamma interferon (IFN-γ), and IL-10 mRNAs were similarly induced in spleens of the two strains. In contrast, the mRNA of KC, a C-X-C chemokine, was induced only in infected C.CB mice at this time. This pattern of mRNA expression was maintained at 14 days p.i., with IFN-γ and IL-12p40 mRNAs being more intensively induced in the infected C.CB mice, but TNF-α mRNA was no longer induced. The higher recruitment of neutrophils observed in the spleens of infected C.CB mice could explain the temporary susceptibility of C.CB mice to *B. melitensis* infection. In contrast to infections with *Salmonella*, *Leishmania,* and *Mycobacterium,* the expression of the *Nramp1* gene appears to be of limited importance for the natural resistance of mice to *Brucella*.**

Brucella is a gram-negative facultative intracellular bacterium that survives and replicates in host macrophages. *Brucella melitensis* preferentially infects small ruminants and is the most pathogenic *Brucella* species for human beings (15). As with other facultative intracellular pathogenic bacteria, the clearance of *Brucella* infection depends on both cell-mediated immunity and humoral responses (31, 34, 42). Natural resistance to *Brucella* infection has been reported in the past in swine and more recently in cattle (1). In cattle, natural resistance to *B. abortus*, based on recovery after a challenge during pregnancy, is heritable, and genetic analysis data from classical breeding studies are consistent with a multigenic control of the resistant phenotype. *B. abortus* infection of bovine blood monocyte-derived macrophages or mammary macrophages in vitro has demonstrated that macrophages from a resistant phenotype were better able to control the intracellular replication of *Brucella* (23, 36). These macrophages were also observed to have a similar activity towards *Mycobacterium bovis* BCG and *Salmonella enterica* serovar Dublin (37). The striking link between resistance to brucellosis and macrophage function in both cattle and mice supports the hypothesis that a bovine homolog of the murine *Nramp1* (natural resistance-associated macrophage protein 1) gene is a major candidate gene for determining resistance to brucellosis. The murine *Nramp1* gene encodes an integral phosphoglycoprotein membrane protein, with 12 putative transmembrane domains and a conserved transport motif (43), which is expressed in the macrophages of reticuloendothelial organs (19, 40). The *Nramp1* gene plays an important role early in the macrophage activation pathway and hence in the innate immunity of the host. It has many pleiotropic effects on macrophage function (5). The Nramp1 protein belongs to the Nramp family of membrane metal transporters found in animals, yeasts, and plants (11). The Nramp protein is highly conserved in prokaryotes and eucaryotes, and genes encoding Nramp homologs have been identified in pathogenic bacteria (12). Nramp1 is a divalent cation (Fe^{2+}, Zn^{2+}, and Mn^{2+}) transporter and is localized in the late endosomal and lysosomal compartments. The exact way in which it acts as a transporter is not known, but it can produce a flux of divalent cations in either direction against a proton gradient (6). In the mouse, a point mutation in the coding region of the gene *Nramp1* results in a single amino acid change from Gly to Asp at position 169 (43). The effect of this mutation results in a susceptibility phenotype of mice in the early phases of infection with *S. enterica* serovar. *Typhimurium*, *Leishmania donovani*, or various species of *Mycobacterium* (44).

The bovine homolog of the murine *Nramp1* gene (known as

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bovine *NRAMP1*) has been cloned, and the cDNA has been sequenced (16). Bovine NRAMP1 is expressed primarily in the macrophages of the reticuloendothelial system. An in vitro model for the expression of resistance- and susceptibility-associated alleles of bovine *NRAMP1* as stable transgenes in the murine RAW264.7 macrophage cell line was developed recently (3). The point mutation within the coding region of murine *Nramp1* that distinguishes between resistant and susceptible alleles of the mouse *Bcg* gene (43) has never been documented in cattle or the other mammalian species in which the *Nramp1* homolog has been sequenced. Inbred mouse strains differ in their ability to control *B. abortus* infection: C57BL/10 mice more effectively control the initial replication of the virulent *B. abortus* strain 2308 than do BALB/c mice, and they clear the infection within 16 weeks whereas BALB/c mice remain infected (24). A recent study using macrophages from these two strains of mice did not find any difference in the ability of these macrophages to control the intracellular growth of *B. abortus* strain 2308 (39). Both resistant C57BL/10 and susceptible BALB/c mouse strains are homozygous for the *Nramp1*^{s} allele, and susceptibility to *B. abortus* infection has been reported in the CBA mouse strain, which is homozygous for the *Nramp1*^{r} allele (24). Since the genetic control of *B. abortus* infection is known to be a multigenic trait, we compared the ability of two strains of mice, BALB/c (*Nramp1*^{s}) and the congenic C.CB mouse (*Nramp1*^{r}), to control *B. melitensis* infection in order to study the effect of the *Nramp1* gene in the context of a common genetic BALB/c background. The effect of *Nramp1* expression on the intracellular survival of *B. melitensis* in macrophages and on the inflammatory response induced by *B. melitensis* infection was also investigated.

MATERIALS AND METHODS

Mice. Eight-week-old female BALB/c (*Nramp1*^{s}/*Nramp1*^{s}) and C.CB (*Nramp1*^{r}/*Nramp1*^{r}) mice were obtained from our animal facility and were randomized 1 week before inoculation. C.CB mice are the congenic *Nramp1*^{r} counterparts of BALB/c mice that have been produced and bred in our laboratory (27). BALB/c and C.CB mice were genotyped using PCR-restriction fragment length polymorphism specific for the point mutation associated with the susceptibility allele of the *Nramp1* gene. The size of the CBA chromosome 1 segment transferred by repeated backcrosses to BALB/c mice has been evaluated, by microsatellite typing, to be between 11.3 and 21.8 centimorgans (cM) (I. Lantier and F. Lantier, personal communication).

Bacteria and infection. Mice were infected by the intraperitoneal (i.p.) route with 10^4 CFU of the *B. melitensis* H38S virulent strain. This dose induces splenic infection that persists for at least 10 weeks (8). This strain was grown for 24 h on Trypticase soy agar (Life Technologies, Cergy Pontoise, France) supplemented with 0.1% (wt/vol) yeast extract (Difco, Detroit, Mich.) (TSA-YE), harvested in buffered saline solution, adjusted spectrophotometrically at 600 nm, and diluted to 5×10^4 CFU ml^{-1}. Viable counts were determined retrospectively by counting on TSA-YE plates. Eight infected mice from the BALB/c or C.CB strains were killed by cervical dislocation on days 2, 4, 6, 14, 21, and 28 postinfection (p.i.). Their spleens and livers were harvested, weighed, and frozen at −20°C. After the tissues were homogenized in BSS, bacterial counts were performed on TSA-YE plates. To normalize the distribution of individual counts for statistical analysis, the number of CFU per organ was transformed as $\log_{10}$ (CFU $\log_{10}$/CFU), as previously reported (7).

Gentamicin protection assay. Infected BALB/c and C.CB mice were killed on days 5 and 14 p.i. The spleens were removed, and individual cell suspensions (four mice per strain per time point) were prepared by the conventional method of pressing the tissues through nylon gauze. Erythrocytes were lysed by adding 0.155 M NH_4Cl to the cell pellet, and the cells were washed and finally adjusted to 10^7 cells per ml of gentamicin-free minimum essential medium (MEM) (Life Technologies) supplemented with 5% fetal calf serum (FCS) (Life Technologies). A 1-ml volume of each cell suspension was collected, the cells were lysed in 0.1% Triton X-100 and the viable cells were counted by plating serial dilutions on TSA-YE. The cell suspensions were then centrifuged and resuspended at 10^7 cells per ml of MEM–5% FCS supplemented with gentamicin (200 μg ml^{-1}) (Sigma, St. Louis, Mo.) to kill any extracellular or adherent *Brucella* organisms. The cells were incubated with stirring at room temperature for 60 min. They were washed twice with gentamicin-free medium and adjusted to 10^7 cells per ml of gentamicin-free medium, and counts of the viable *Brucella* organisms were done again on 1 ml of this cell suspension plated on TSA-YE. The counts were expressed as $\log_{10}$ CFU per spleen with or without incubation with gentamicin.

Intracellular survival assay from macrophages infected in vivo by *B. melitensis*. BALB/c and C.CB mice were infected i.p., as described above, with 10^4 CFU of H38S strain and killed 3 days p.i. Peritoneal exudate cells (PEC) were harvested by washing the peritoneal cavity with 3 ml of MEM-FCS. PEC from three mice per strain were pooled, centrifuged at 600 × *g* for 10 min, and then resuspended in RPMI 1640 medium plus 20 mM HEPES (Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 100 μg of gentamicin ml^{-1} to kill any extracellular *Brucella* organisms. The cells were adjusted to 5×10^5 cells ml^{-1}, seeded in 24-well plates (1 ml per well), and incubated at 37°C under 5% CO_2. At various time intervals, the viable counts of intracellular *Brucella* organisms were determined after the cells were washed with gentamicin-free medium, and then the cells were lysed with 0.1% Triton X-100. The results are expressed as mean of $\log_{10}$ CFU per well ± standard deviation from three wells at each time point.

In vitro intracellular survival assay with *Nramp1* RAW 264.7 transfectants. RAW264.7 transfectant cell lines R21 (*Nramp1* deficient) and R37 (*Nramp1* expressing) were provided by C. H. Barton and cultured as described previously (2). Stable *Nramp1* expression was confirmed in R37 but not R21 cell lines by Western blotting using anti-*Nramp1* antibodies (C. H. Barton, personal communication). Cells were grown without geneticin and seeded at 3×10^5 cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS in 24-well plates and allowed to adhere for 10 h at 37°C under 5% CO_2. The cells were then exposed overnight to recombinant murine gamma interferon (IFN-γ) (10U/ml) (19301T [Pharmingen, San Diego, Calif.]). The inoculum of *B. melitensis* H38S was prepared from a 24-h culture on a TSA-YE slope. Bacteria were collected, washed, and resuspended in DMEM–10% FCS to 6×10^7 CFU ml^{-1}. The exact titer of the infectious inoculum was determined by plating on TSA-YE plates. For infection, the cells were incubated with 250 μl of bacteria suspension (corresponding to a multiplicity of infection of 50:1). The plates were centrifuged (3 min at 1,500 × *g*) to optimize the contact between the cells and bacteria and incubated for a further 60 min at 37°C with 5% CO_2. The cells were then washed thoroughly with MEM-FCS to remove any nonadherent bacteria and then reincubated for a further 60 min with 1 ml of complete RPMI medium supplemented with gentamicin (100 μg ml^{-1}) to kill any remaining extracellular bacteria. At this time (T0) and then at 5, 24, and 48 h p.i., the culture supernatant from three wells was removed and the cells were washed with gentamicin-free medium and lysed with 0.1% Triton X-100. Lysates were rapidly diluted in BSS and plated on TSA-YE. The results are expressed as mean $\log_{10}$ CFU per well ± standard deviation from three wells at each time point.

Cell preparation, MAbs, and flow cytometry analysis. Spleen cells were individually prepared from infected and control (uninfected) BALB/c and C.CB mice as described above for the gentamicin protection assay. Cells were finally adjusted to 3×10^7 ml^{-1} in MEM-FCS with gentamicin (100 μg ml^{-1}). Rat anti-mouse Ly-6G (Gr-1) (RB6-8C5; working dilution, 1:200 [Pharmingen]) and anti-murine pan macrophages (BM8; working dilution, 1:50 [Bachem, Voisins-le-Bretonneux, France]) were used to analyze the neutrophils (18) and major subpopulations of resident tissue macrophages (29), respectively. *R*-Phycoerythrin (RPE)-conjugated goat anti-rat immunoglobulin G (IgG) F(ab′)$_2$ was used as secondary antibody (STAR73; working dilution, 1:50 [Serotec, Kidlington, United Kingdom]). An irrelevant rat IgG2a (MCA1212 [Serotec]) was used as the primary monoclonal antibody (MAb) for a negative control. All antibodies were diluted in 10 mM phosphate-buffered saline (pH 7.4) containing 0.2% (wt/vol) bovine serum albumin (PBS-B) either alone or with 10% mouse serum for RPE anti-rat Ig. For labeling, cells (3×10^6) were incubated at 4°C for 20 min with 50 μl of antibody (primary or secondary) at the appropriate concentration, washed three times with PBS-B, and fixed with 0.5% paraformaldehyde in PBS. The cells were analyzed on a FACStar^{Plus} instrument (Becton Dickinson, San-Jose, Calif.). Two types of gating were performed with forward and side scatter: one for the leukocyte population and the other for phagocyte population. The proportion of nonspecific binding of the secondary antibody was determined using the leukocyte gate and deduced from the proportion of positive cells. Data were expressed as the number of positive cells per spleen by multiplying the proportion of positive cells by the total number of splenocytes per mouse. The

proportion of positive cells was also analyzed by gating the phagocyte population, including neutrophils and macrophages.

Analysis of mRNA expression by RT-PCR. BALB/c and C.CB mice of infected or control groups were killed on days 5 and 14 p.i. Splenocytes from four mice per group were isolated and pooled. Total RNA from 3×10^7 cells was then extracted from each sample by using Trizol (Life Technologies) as described by the manufacturer. The reverse transcription (RT) reaction was then performed at 42°C for 90 min on 20 μg of total RNA, using the Moloney murine leukemia virus reverse transcriptase (Life Technologies) and oligo(dT) (12–18 oligo-dT [Life Technologies]) in the presence of 1 μCi of [^{32}P]dCTP (ICN, Orsay, France), to measure the amount of cDNA synthesized (21). A 1-ng portion (0.2 ng for β_2-microglobulin) of each cDNA was amplified using 2.5 U of Gold Star polymerase (Eurogentec, Seraing, Belgium) and 1 μM specific primers. The specific primers for β_2-microglobulin, interleukin-2 (IL-2), IL-4 (14), tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase iNOS (21) and the lengths of the amplicons are described elsewhere. The amplicons were defined as follows: IFN-γ (5′-primer, 5′-GCT CTG AGA-CAA-TGA-ACG-CT-3′; 3′-primer, 5′-AAA-GAG-ATA-ATC-TGG-CTC-TGC-3′; 226 bp), IL-12p40 (5′-primer, 5′-CGT-GCT-CAT-GGC-TGG-TGC-AAA-G-3′; 3′-primer, 5′-GC C-CAA-GAA-CTT-GCA-GAT-GAA-G-3′; 316 bp), IL-10 (5′-primer, 5′-ATG-CAG-GAC-TTT-AAG-GGT-TAC-TTG-3′; 3′-primer, 5′-TAG-ACA-CCT-TG G-TCT-TGG-AGC-TTA-3′; 224 bp), IL-18 (5′-primer, 5′-ACT-GTA-CAA-CC G-CAG-TAA-TAC-GG-3′; 3′-primer, 5′-AGT-GAA-CAT-TAC-AGA-TTT-A TC-CC-3′; 437 bp), MIP1-α (5′-primer, 5′-GTC-TTC-TCA-GCG-CCA-TAT-GG-3′; 3′-primer, 5′-GGC-ATT-CAG-TTC-CAG-GTC-AG-3′; 225 bp), and KC (5′-primer, 5′-TTT-GGA-CAA-TTT-TCT-GAA-CC-3′; 3′-primer, 5′-GAT-TC A-CCT-CAA-GAA-CAT-CC-3′; 123 bp).

cDNAs were amplified by repeated cycles at 95°C for 20 s, at the appropriate annealing temperature for 45 s and at 75°C for 45 s. PCR amplifications were performed during the exponential phase of amplification, with a nonsaturating number of cycles (15 to 35) being used for each cytokine. Amplification of β_2-microglobulin (17 cycles) was used as a control. PCR products were run on 1.2% agarose gels supplemented with ethidium bromide, and their sizes were evaluated using molecular size standards (123-bp ladder [Life Technologies]). For each cytokine, the relative expression of the mRNA was determined in infected and control BALB/c and C.CB mice by a semiquantitative analysis. PCR products from a given set of samples were analyzed for the cytokine and β_2-microglobulin genes. The amounts of PCR products obtained in exponential phases of amplification were quantified by measuring the intensity of fluorescence of amplicons by densitometric analysis (Kodak Image station 440 [NEN Life Science Products, Paris, France]). The ratio of the cytokine amplification product to the β_2-microglobulin amplification product, which is representative of the relative expression of the cytokine mRNA in the sample, was then calculated (28). For a given cytokine, the samples were compared, and gene expression was considered to be significantly different if the calculated values varied by a factor of at least 1.5.

Statistics. Analysis of variance (two-factor ANOVA) was performed on data followed by Student-Newman-Keuls or Scheffe tests for orthogonal analysis. These tests were done using Superanova (Abacus Concepts, Berkeley, Calif.).

RESULTS

***Nramp1* expression confers temporary susceptibility to *B. melitensis* infection in mice.** The kinetics of infection was analyzed in BALB/c (*Nramp1*^{s}) mice and C.CB congenic (*Nramp1*^{r}) mice inoculated i.p. with 10^4 CFU of *B. melitensis.* In both groups of mice, the numbers of *Brucella* organisms isolated from spleens increased during the first week p.i. and then decreased, to remain constant over the month following infection without any clearance of *Brucella.* Surprisingly, within this time, the number of *Brucella* organisms isolated from the spleen was significantly larger in C.CB mice than in BALB/c mice ($P < 0.005$). The number of *Brucella* organisms per spleen was actually larger in C.CB mice than in BALB/c mice only for the first week p.i. ($P < 0.005$); the numbers were similar for the two strains of mice thereafter (Fig. 1). A similar pattern was observed for the liver, where the kinetics of *Brucella* persistence closely matched the findings for the spleen ($r = 0.8669$; df = 44; $P < 0.0001$).

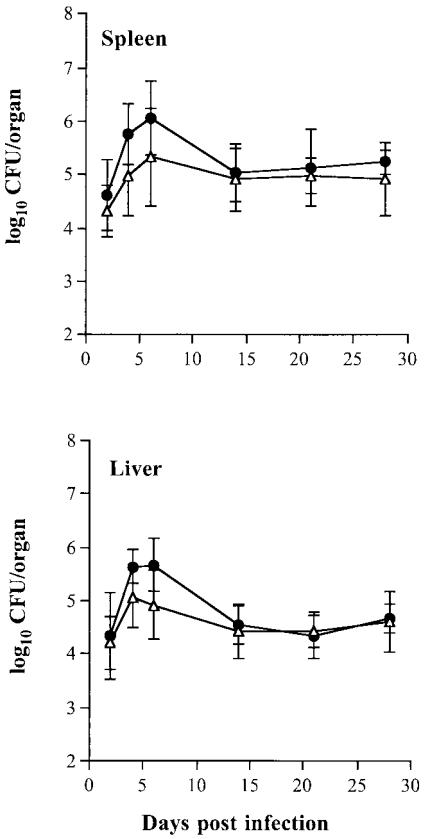

FIG. 1. Growth of *B. melitensis* in spleens and livers of BALB/c (*Nramp1*^{s}) (Δ) and congenic C.CB (*Nramp1*^{r}) (●) mice. Mice ($n = 8$ mice per time point) were inoculated i.p. with 10^4 CFU of *Brucella.* Results are expressed as means ± SD. The number of *Brucella* organisms per spleen was significantly larger in C.CB mice than in BALB/c mice during the first week p.i. ($P < 0.005$).

***Nramp1* expression contributes to the intracellular localization of *B. melitensis* in the spleen.** To elucidate the relationship between the number of *Brucella* organisms isolated per organ and the in vivo localization of the organisms, we enumerated *Brucella* organisms associated with the splenocytes of mice in a gentamicin protection assay. Measurements were performed 5 and 14 days p.i. in BALB/c and C.CB mice. On day 5 p.i., we found that the mouse strain had a very significant effect ($P < 0.001$) on the number of *Brucella* organisms isolated from the splenocyte suspension, independent of gentamicin treatment: a larger number of *Brucella* was isolated from C.CB splenocytes than from an equivalent number of BALB/c splenocytes, regardless of whether ($P < 0.01$) the cells had been treated in vitro with gentamicin. On day 14 p.i., this difference was no longer observed (Fig. 2).

Control of intracellular infection with *B. melitensis* ex vivo and in vitro is not dependent on *Nramp1* expression. The *Nramp1* gene product is known to control the intracellular replication of various pathogens in macrophages. To examine further the role of *Nramp1* on *Brucella* survival and replication in macrophages, we compared the intracellular behavior of *Brucella* in PEC isolated from infected BALB/c and C.CB mice, which were then cultured in vitro (known as ex vivo). The

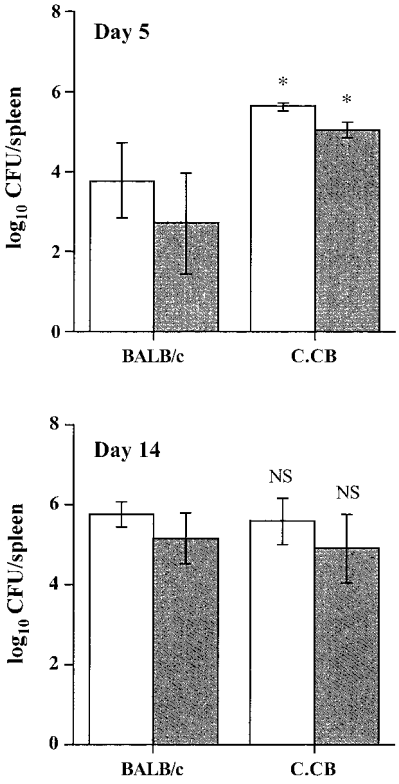

FIG. 2. Isolation of *B. melitensis* associated with splenocytes from infected BALB/c and C.CB mice. Splenocytes were isolated on day 5 or 14 after i.p. inoculation of mice with *B. melitensis.* The number of *Brucella* organisms per spleen was counted before (open bars) and after (shaded bars) in vitro incubation with gentamicin. Results are expressed as means ± SD ($n = 4$ mice per time point). *, statistically different from BALB/c mice ($P < 0.01$); NS, not significant

ex-vivo intracellular survival of *Brucella* in PEC collected 3 days after i.p. infection was analyzed for 72 h. The *Nramp1* expression had no significant effect on the intracellular replication of *Brucella* in PEC at any time. Moreover, no difference was shown between control antisense *Nramp1* transfectants (R21 cell line) and *Nramp1* transfectants (R37) infected in vitro by *B. melitensis* (data not shown). These findings demonstrated that *Nramp1* expression has no significant effect on the in vitro intracellular replication of *B. melitensis* in murine macrophages.

Effect of *Nramp1* expression on the inflammatory response induced by *B. melitensis* infection. In the absence of any direct effect of *Nramp1* expression on the intracellular replication of *B. melitensis* in isolated macrophages infected in vivo or in vitro, we analyzed the pleiotropic effect of *Nramp1* on the inflammatory response in the spleen. Indeed, spleen weight increased steadily after infection with *B. melitensis* (on average from 100 to 600 mg) in both mouse strains. However, between 14 and 28 days p.i., splenomegaly was significantly greater in C.CB mice than in BALB/c mice ($P < 0.01$) (Fig. 3). An increase in liver weight was also noticed and was associated with significant greater hepatomegaly in the C.CB mice ($P < 0.05$).

We therefore examined and compared cytokine gene expression in splenocytes of infected and uninfected BALB/c and C.CB mice. RT-PCR analyses performed 5 days p.i. showed that TNF-α, IL-12p40, IFN-γ, iNOS, and IL-10 mRNAs were expressed in the splenocytes of infected mice but not in those of the control mice. In contrast, no significant induction of expression of MIP-1α, IL-18, IL-2, and IL-4 transcripts (ratio of amplification, < 1.5) could be demonstrated, with similar levels of mRNAs being observed in BALB/c and C.CB mice regardless of whether they were infected (Fig. 4A). A semiquantitative analysis showed that the mRNA expression of TNF-α, IL-12p40, iNOS, and IL-10 was not significantly different in the two strains of mice but that the level of IFN-γ mRNAs was two to three times higher in infected C.CB mice than in infected BALB/c mice when their relative expression of mRNAs was compared. In contrast, the transcript for the chemokine KC was specifically amplified in the infected C.CB mice alone. A similar pattern of cytokine mRNAs was observed when spleens were analyzed 14 days p.i., except for TNF-α mRNAs, which no longer displayed induction. Nevertheless, the relative expression of mRNAs for each cytokine, representative of four mice for each of the different groups, showed that the mRNAs were more strongly expressed in infected C.CB mice than in infected BALB/c mice: KC (sev-

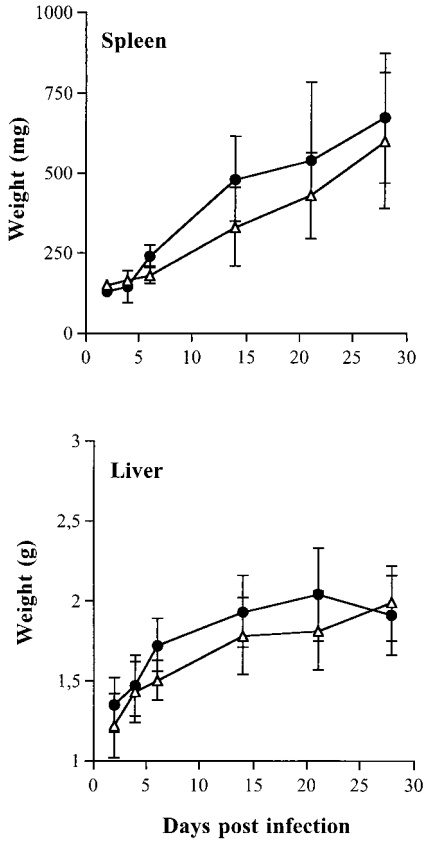

FIG. 3. Kinetics of spleen and liver weights after *B. melitensis* infection of BALB/c (Δ) and C.CB (●) mice. Results are expressed as means ± SD ($n = 8$ mice per time point). The increase in spleen and liver weights in the C.CB mice was significantly greater than that in the BALB/c mice ($P < 0.01$ and $P < 0.05$ for spleen and liver, respectively).

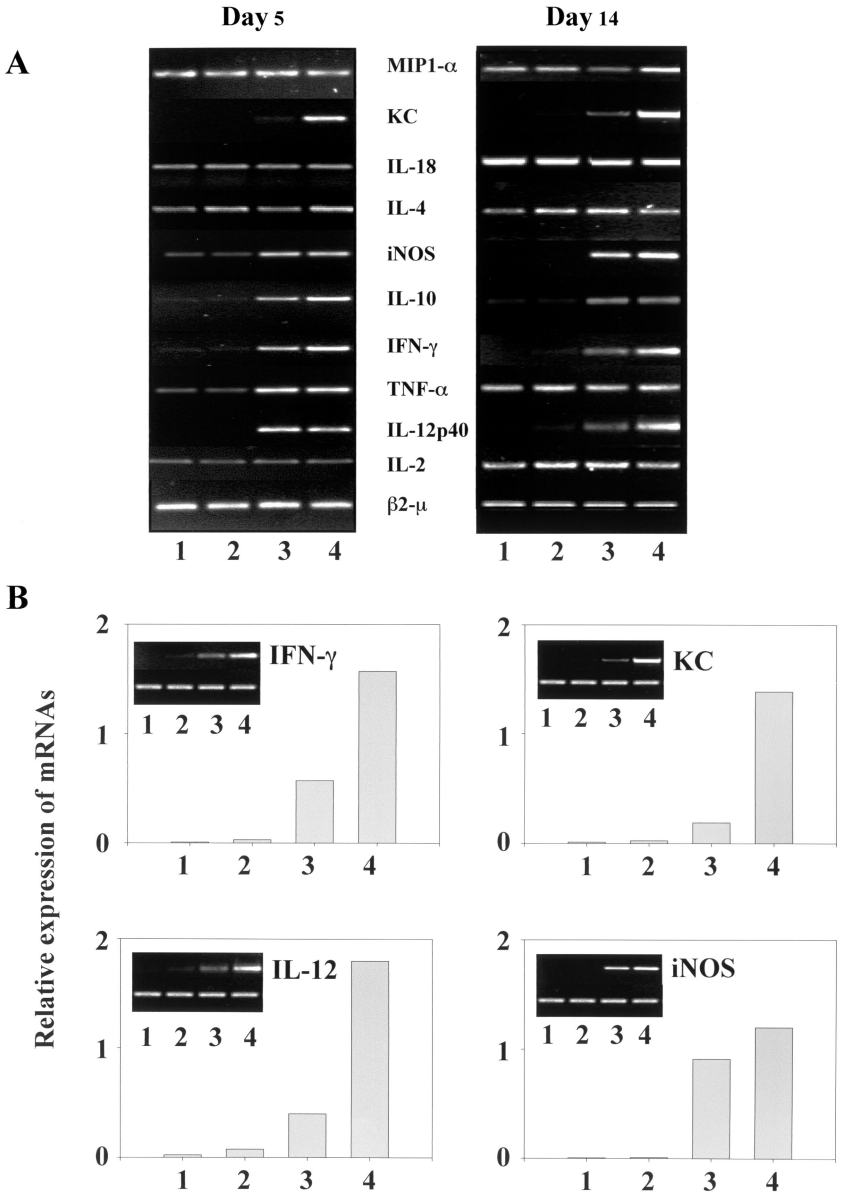

FIG. 4. Cytokine mRNAs expression in spleens of *B. melitensis*-infected or uninfected BALB/c and C.CB mice. (A) The gene expression of different cytokines was analyzed by RT-PCR performed on mRNAs of infected BALB/c (lanes 3) and C.CB (lanes 4) mice and their respective controls (lanes 1 and 2). (B) Four mice per group were sacrificed at 5 and 14 days p.i., and spleen cells were isolated and pooled for mRNA preparation. For each cytokine, the relative expression of mRNAs was calculated from the relative intensity of the amplification product, determined by densitometric analysis, with respect to that of the housekeeping gene, β_2-microglobulin. Values for the expression of KC, IL-12, IFN-γ, and iNOS mRNAs 14 days p.i. are shown.

enfold increase), IL-12p40 (fivefold increase), and IFN-γ (two- to threefold increase) (Fig. 4B).

Finally, we analyzed the kinetics of phagocyte recruitment in spleens from BALB/c and C.CB mice on days 5 and 14 p.i. The number of neutrophils and macrophages was measured by flow cytometry, using RB6-8C5 and BM8 markers, respectively, and positive cells being analyzed by gating focused on leukocytes. In both strains of mice, the numbers of both neutrophils (RB6-8C5$^+$ cells) and macrophages (BM8$^+$ cells) had increased significantly on days 5 and 14 p.i., independent of the mouse strain ($p < 0.005$) (Fig. 5). On day 14 p.i., there were six and seven times as many neutrophils and macrophages, respectively, in the spleens of infected C.CB mice than in the spleens of the control mice. The numbers of neutrophils and macrophages were only two and three times greater in the spleens of infected BALB/c than in those of the controls at this time. Nevertheless, the proportion of RB6-8C5$^+$ cells, defined by gating focused on phagocytes, being significantly higher ($P < 0.05$) in control BALB/c mice (32.68% ± 8.53%) than in control C.CB mice (23.30% ± 3.40%), the absolute numbers of neutrophils or macrophages per spleen did not appear significantly different in infected BALB/c and C.CB mice (Fig. 5).

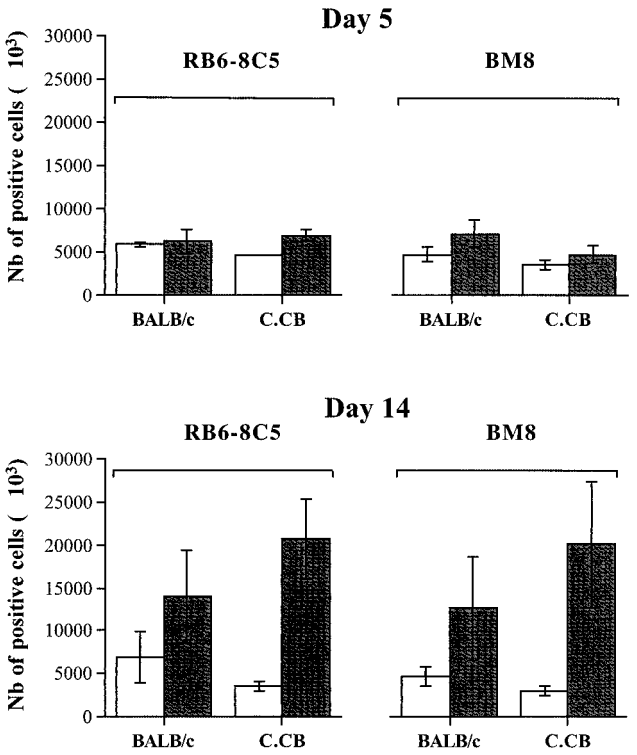

FIG. 5. Fluorescence-activated cell sorter analysis of RB6-8C5 and BM8 marker expression in splenocytes from BALB/c and C.CB mice after infection by *B. melitensis*. Control and infected mice were sacrificed 5 or 14 days p.i. Spleen cells were labeled with MAbs and fluorescence-conjugated secondary antibody, and positive cells were analyzed in total gated leukocytes. Results are expressed as means ± SD of the number of positive cells per spleen ($n = 4$). Differences in the numbers of RB6-8C5$^+$ and BM8$^+$ cells were not statistically significant for infected BALB/c and C.CB mice.

These findings clearly demonstrate that the splenomegaly induced by *Brucella* infection was associated with the recruitment of both neutrophils and macrophages to the spleen and that the expression of *Nramp1* influences this recruitment.

DISCUSSION

Differences in the resistance or susceptibility of mice to infection with *B. abortus* have been recognized to be multifactorial (24). In this study, we have investigated the possible contribution of the *Nramp1* gene to the control of *B. melitensis* infection by analyzing the infection of C.CB mice (*Nramp1^r*), which are congenic counterparts of BALB/c mice (*Nramp1^s*). Surprisingly, our data showed that during the first week, C.CB mice were more susceptible than BALB/c mice to *B. melitensis* infection in terms of the larger numbers of *Brucella* organisms isolated from the spleens and livers of mice. However, the susceptibility to *B. melitensis* infection associated with *Nramp1* expression was transient, since similar numbers of *Brucella* organisms were recovered from the two strains of mice 2 weeks p.i. This finding is not in agreement with results of other studies that assign a definite role to the *Nramp1* gene in natural resistance to unrelated pathogens, such as *Salmonella, Leishmania*, and some strains of *Mycobacterium.* Nevertheless, the effect of the expression of *Nramp1* on the outcome of infectious diseases seems to depend on the pathogen concerned. The expression of this gene does indeed seem to be of limited importance in the resistance of 129sv mice (*Nramp1^r*) to virulent *M. tuberculosis* (32) and does not play any role in the susceptibility of mice to *Chlamydia* (33). Furthermore, a recent study reported a link between the expression of the *Nramp1* gene and the susceptibility of mice to *Francisella tularensis* during the early phases of infection (25), as confirmed in this study. Our data show that expression of the *Nramp1* gene had a positive but temporary impact on the survival of *B. melitensis* in mice.

Brucella organisms survive and replicate in host macrophages, where the *Nramp1* gene is expressed. Experiments were therefore performed to investigate the relationship between the intracellular localization of *Brucella* and the different patterns of infection observed in BALB/c and C.CB mice during the early phases of infection. The number of *Brucella* organisms associated with cells isolated from infected spleens was still larger in C.CB than in BALB/c mice after treatment with gentamicin, an antibiotic able to kill extracellular *Brucella.* These findings confirmed data obtained using homogenized spleens and demonstrated that the early effect on infection triggered by the *Nramp1* gene occurred in intracellular *Brucella* organisms.

The in vitro replication of *Brucella* was similar in macrophages isolated from the two strains of mice infected in vivo and in *Nramp1* RAW264.7 transfectants. This excludes any direct involvement of the *Nramp1* gene in the intracellular replication of *Brucella* in macrophages. Such a discrepancy between in vivo and in vitro data has been reported for C57BL/10 and BALB/c mice, strains which are resistant and susceptible, respectively, to *B. abortus* infection. The macrophages of these mice show no difference in their ability to control *Brucella* replication (39). In contrast to *Nramp1* RAW264.7 transfectants, which inhibit the intracellular replication of *S. enterica* serovar Typhimurium (20), stable transgenes of the resistance-associated allele of the bovine *NRAMP1* gene in RAW264.7 cells control the in vitro replication of *B. abortus* but not of *S. enterica* serovar Dublin (3). These apparently conflicting data suggest that (i) the polymorphism of the resistance-associated allele of bovine *NRAMP1* gene affects the process of intracellular replication of *Brucella* but not of *Salmonella;* (ii) the point mutation in the coding region of the murine *Nramp1* gene, resulting in a single amino acid substitution of Gly by Asp at position 169, does not affect the in vitro intracellular replication of *B. melitensis* in macrophages; and (iii) genes other than *Nramp1* are involved in the natural resistance of mice to *Brucella.* Nevertheless, transgenic mice could provide a useful model for studying the specific effects of the expression of the resistance-associated allele of the bovine *NRAMP1* gene on the outcome of *Brucella* infection.

Several studies have reported a link between the antibacterial activity of the murine *Nramp1* gene and the phagolysosomal pathway and, more precisely, with phagosomal acidification (4, 22). The ability of *Brucella* to survive in acidic phagosomes and the requirement for early acidification of this compartment for their survival within macrophages (35) could explain why the expression of the *Nramp1* gene does not affect

the intramacrophage development of *Brucella.* One explanation could be that *Brucella* exploits the ion transport function of *Nramp1* to its own advantage, as was suggested recently for *F. tularensis* (26). Indeed, numerous genera of bacteria express genes encoding the bacterial ortholog of the eukaryotic Nramp protein, known as MntH (from the proton-dependent manganese transporter) (12). A homolog of the *MntH* gene of *Agrobacterium tumefaciens* is present in the genome of *B. melitensis* (13) and displays 68% nucleotide identity (GenBank accession number AE009498). This suggests that a divalent cation transporter homolog of the Nramp1 family could be expressed in *Brucella* and that this could compete for metal ions within the intraphagosomal vesicles of macrophages.

In the absence of any direct effect of *Nramp1* expression on the in vitro intramacrophage replication of *B. melitensis*, we analyzed in vivo data by considering the pleiotropic effects of the *Nramp1* gene on the inflammatory response and macrophage activation pathways (5). The infection of BALB/c and C.CB mice resulted in an inflammatory response characterized by splenomegaly, which began 5 days p.i., and in the influx of neutrophils (RB6-8C5$^+$ cells) and macrophages (BM8$^+$ cells) into the spleen. This response was amplified in C.CB mice, in line with the higher bacterial load. In both strains of mice, in accordance with the onset of splenomegaly observed on day 5 p.i., the induction of TNF-α, iNOS, IL-12p40, and IFN-γ, but not of IL-2 and IL-4 mRNAs, was typical of the inflammatory and Th1 cytokine responses required to impair *Brucella* infection (30). The induction of IL-10 mRNAs was probably a consequence of the inflammatory response, since IL-10 is required to control this response. This pattern of cytokine expression was maintained during the second week of infection, except for TNF-α, which was no longer expressed. IFN-γ and IL-12p40 mRNAs were more intensively induced in the infected C.CB mice than in the infected BALB/c mice. Despite the early enhanced susceptibility to *B. melitensis* infection of the C.CB mice, they develop an amplified Th1 cytokine response, which fits in with the greater capacity of macrophages carrying the *Nramp1^r* allele to promote the Th1 cytokine response in response to *Leishmania* infection than of macrophages carrying the *Nramp1^s* allele (41). In addition to Th1 cytokines, a dramatic difference was observed in the expression of the C-X-C chemokine KC, which was induced (on day 5 p.i.) in the C.CB mice only. This differential induction of the KC chemokine in infected *Nramp1^r* mice is not surprising, since its expression is known to be controlled by the *Nramp1* gene (38). KC is a potent inducer of neutrophil migration (9); therefore, its production could potentiate the recruitment of neutrophils, as observed in infected C.CB mice. This recruitment could be amplified by neutrophils themselves since high-level expression of Nramp1 has been recently reported for these cells (10). The polymorphism of other genes beside the *Nramp1* gene, transferred in congenic C.CB mice, like IL-8RB (http://www.informatics.jax.org), could also participate in the recruitment of neutrophils. We detected a higher recruitment of neutrophils in the spleens of infected C.CB mice than in those of BALB/c mice. Therefore, the temporary susceptibility of C.CB mice to *B. melitensis* infection could be related to a greater recruitment of phagocytic cells. Since no direct effect of *Nramp1* on the intramacrophage replication of *Brucella* organisms was observed, this suggests that the increased inflammatory response in spleens of C.CB mice did not correlate with the increased control of splenic *B. melitensis.* However, the increased inflammatory response in spleens of C.CB mice, maintained for 3 weeks p.i., could also be a response to the increased bacterial load in these spleens; phagocytic cells represent both host and effector cells in *Brucella* infection. In any case, the subsequent development of a pronounced compensatory Th1 response by C.CB mice counteracts and stabilizes the infection in the spleen. The inflammatory response and Th1 response stabilize the number of *Brucella* organisms in the spleen at a similar level in the two strains of mice, but in both cases they do not lead to the clearance of the infection.

In conclusion, *Nramp1* gene expression confers only a temporary advantage for *B. melitensis* survival in mice and is of limited importance for the natural resistance of mice to *Brucella.*

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