Genetic Resistance of Mice to Mycobacterium paratuberculosis Is Influenced by Slc11a1 at the Early but Not at the Late Stage of Infection

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Received 16 August 2007/Returned for modification 10 October 2007/Accepted 9 February 2008

We have recently described the development of a luminescent Mycobacterium paratuberculosis strain of bovine origin expressing the luxAB genes of Vibrio harveyi. With this luminescent isolate, fastidious and costly enumeration of CFU by plating them on agar can be replaced by easy and rapid luminometry. Here, we have reevaluated the effect of Slc11a1 (formerly Nramp1) polymorphism on susceptibility to M. paratuberculosis, using this luminometric method. A series of inbred mouse strains were infected intravenously with luminescent M. paratuberculosis S-23 and monitored for bacterial replication in spleen, liver, and lungs for 12 weeks. The results indicate that, as for Mycobacterium avium subsp. avium, innate resistance to infection is genetically controlled by Slc11a1. In BALB/c, congenic BALB.B10.H2b (BALB/c background; H-2b), C57BL/6, and beige C57BL/6/6hc/hb mice (all Slc11a1t), bacterial numbers in spleen and liver remained unchanged during the first 4 weeks of infection, whereas in DBA/2 and congenic BALB/c.BDA/2 (C.D2) mice (both Slc11a1l) and in (C57BL/6 × DBA/2)F1 mice (Slc11a1t), the bacterial numbers had decreased more than 10-fold at 4 weeks postinfection in both male and female mice. At later time points, additional differences in bacterial replication were observed between the susceptible mouse strains, particularly in the liver. Whereas bacterial numbers in the liver gradually decreased more than 100-fold in C57BL/6 mice between week 4 and week 12, bacterial numbers were stable in livers from BALB/c and beige C57BL/6/6hc/hb mice during this period. Mycobacterium-specific gamma interferon responses developed earlier and to a higher magnitude in C57BL/6 mice than in BALB/c mice and were lowest in resistant C.D2 mice.

The natural resistance of mice to the intracellular pathogens Leishmania donovani and Salmonella enterica serovar Typhi-murium and to Mycobacterium bovis BCG vaccine is controlled by the natural resistance-associated macrophage protein 1 (NRAMP1), now called SLC11A1 (34). The SLC11A1 gene is expressed in late endosomes of macrophages derived from spleen and liver, in which it regulates antimicrobial activity (12). Several studies have demonstrated that the SLC11A1 protein is involved directly or indirectly in the maturation process of the phagosome by transport of bivalent cations (such as Fe2+, Mn2+, or Mg2+), but the mechanism by which parasite replication is blocked has not been completely elucidated (11, 20, 47).

The natural resistance of mice to nontuberculosis mycobacterial infections caused by Mycobacterium simiae, Mycobacterium intracellulare, and Mycobacterium avium subsp. avium is also controlled by Slc11a1 (1, 27). The role of Slc11a1 in susceptibility to Mycobacterium paratuberculosis, the etiological agent of bovine paratuberculosis, or Johne’s disease, is less clear and has been studied mostly in the context of Crohn’s disease. The association between M. paratuberculosis and Crohn’s disease has been questioned for a long time, but recent improvements in isolation and genomic techniques seem to suggest a stronger association of M. paratuberculosis as either a causative agent or an opportunistic infection of Crohn’s disease patients (6, 7, 26). Three studies have examined Slc11a1 polymorphisms in patients with inflammatory bowel disease (15, 33, 38). From these reports, it can be concluded that the etiology of Crohn’s disease is the result of a complex interplay of genetic, infectious, and immunologic factors, and (as for studies of AIDS patients with pulmonary M. avium complex infection) these observations suggest that Slc11a1 is one, but only one, determinant of genetic susceptibility (39).

Mice are generally considered to be resistant to M. paratuberculosis and unsuitable for the study of this intestinal pathogen of cattle, goats, sheep, and wild ruminants (13). Some authors have reported on genetic variations in the susceptibility of mice to M. paratuberculosis infection, but in these studies, bacterial replication was analyzed by measuring hepatopo- or splenomegaly (10, 40), not by actual enumeration of bacteria. The last technique is seriously hampered by the fact that M. paratuberculosis, a slowly growing mycobacterial species, requires 6 to 8 weeks of culture before colonies can be counted visually. Chiodini and Buergelt compared three susceptible Slc11a1l mouse strains (BALB/c, C57BL/6, and C57BL/10) using the LINDA strain isolated from a Crohn’s disease patient (8). This study indicated that the reduction in bacterial burden was associated with the development of caseous necrotic lesions. Veazey et al. analyzed actual CFU counts in M. paratuberculosis and...
paratuberculosis-infected C57BL/6 and C3H mice, which express the susceptible and resistant Slc11a1 alleles, respectively, but which also differ at numerous other loci (43, 44).

We have recently reported on the construction of a luminescent M. paratuberculosis isolate that expresses the luxAB genes of Vibrio harveyi introduced by transformation with the shuttle plasmid pSMT1 (31). With this luminescent isolate, fastidious enumeration of CFU can be replaced by easy and inexpensive luminometry (31). Here, we have used this luminescent M. paratuberculosis isolate to reevaluate the role of Slc11a1 in the susceptibilities of a series of inbred mouse strains to intravenous M. paratuberculosis infection.

MATERIALS AND METHODS

Mice. BALB/cOlaHsd (BALB/c), BALB.B10-H2 b (BALB.B10), C57BL/6OlaHsd (B6), and mutant C57BL6OlaHsd-Lystb/- (B6b/-) beige mice (four strains expressing the susceptible Slc11a1 allele); DBA/2OlaHsd (DBA/2) and BALBc/DBA2 (CD2) mice (two strains expressing the resistant Slc11a1 allele) (9); and heterozygous (C57BL6 × DBA2)/J, (B6 × DB2J), mice bred at the Pasteur Institute Animal Facilities from breeding couples originally obtained from Harlan Netherlands (BALB/c, C57BL/6, DBA/2, and B6b/-), from The Netherlands Cancer Institute (BALB.B10), and from E. Skamene (McGill University, Montreal, Canada) (CD2). All mice were 2 to 3 months old at the time of infection. B6b/- mice are spontaneous C57BL/6 mutants for the recessive gene bg (a lysosomal trafficking regulator). This mouse strain (H-2b) presents phenotypic manifestations resembling Chediak-Higashi syndrome in humans, and the mouse beige gene is actually a homolog of the human CHS1 gene (35). Beige mice have defects in blood clotting, reduced chemotactic and bactericidal activity of granulocytes, and abnormal giant lysosomal granules. These mice also have a severe deficiency in natural killer cell lytic activity. BALB.B10 mice are major histocompatibility complex congenic mice with an H-2b locus from C57BL/10 on a BALB/c background. CD2-Slec11a1R (BALB/c × DBA/2) mice are BALB/c congenic mice that carry a 30-centimorgan segment of DBA/2 chromosome 1 containing the Slec11a1 allele (28).

Luminescence assay. The number of bioluminescent bacteria was determined using a bioluminescence assay with a Lumat LB 9507 luminometer (Berthold Technologies) and 1%

RESULTS

Replication of luminescent M. paratuberculosis S-23 in mice carrying the Slc11a1<sup>R</sup> (susceptible) or the Slc11a1<sup>S</sup> (resistant) allele. In order to determine whether susceptibility to M. paratuberculosis infection was influenced by the Slc11a1 gene, five inbred mouse strains were infected intravenously with 1.7 × 10<sup>6</sup> RLU of luminescent M. paratuberculosis S-23 and monitored for 12 weeks for replication in spleen, lungs, and liver. As shown in Fig. 1, DBA/2 and CD2 congenic mice (both expressing the resistant Slc11a1<sup>S</sup> allele) eliminated M. paratuberculosis very rapidly (the values came down to the detection cutoff values 8 to 12 weeks after infection), whereas BALB/c, C57BL/6, and beige B6b/- mice expressing the susceptible Slc11a1<sup>R</sup> allele showed persistent infection. At week 4 postinfection, Slc11a1<sup>R</sup> mice showed 10- to 100-fold-higher RLU numbers in the liver and spleen than mice expressing the resistant Slc11a1<sup>R</sup> allele (P < 0.001). Monitoring infection for another 8 weeks demonstrated further differences between the three strains displaying the susceptible phenotype. In the spleens of BALB/c mice, bacterial numbers increased slightly until week 12, whereas in B6 and mutant B6b/- mice, splenic bacterial numbers remained stable. In the livers of B6 mice, bacterial numbers gradually declined by week 12 to levels almost comparable to the levels in resistant mice. In contrast, the bacterial loads were stable in livers from BALB/c mice. Inter-
In order to verify that these differences in resistance observed in luminometry corresponded to actual differences in numbers of bacteria, a limited set of organ homogenates was also plated on Middlebrook 7H11 agar with and without hygromycin (a resistance marker on the pSMT1 plasmid), and the actual number of CFU was determined. As shown in Table 1, the results obtained by luminometry closely matched those obtained by CFU plating. At 4 weeks after infection, 10-fold-higher CFU counts were detected in spleens and livers from susceptible B6 and BALB/c mice than in spleens and livers from resistant C.D2 mice. At week 12 postinfection, the differences were even more dramatic, and CFU counts in livers were about 100-fold lower in C.D2 than in BALB/c mice, confirming the difference observed in RLU (5.33 log_{10} versus 3.22 log_{10}). At week 4, the CFU/RLU ratios in spleens were 1.9 and 2.95 in susceptible B6 and BALB/c mice and 6.6 in resistant C.D2 mice. In the liver, the respective CFU/RLU ratios were 12.3, 5.62, and 15.1, and in the lungs, they were 2.13, 6.4, and 32.3 at this early time point. These ratios reflected the observed resistance pattern, with high ratios being caused by impaired bacterial fitness (resulting in decreased metabolism and light emission of the bacteria). The CFU/RLU ratios at week 12 showed a similar trend, but this comparison at late time points has to be made with more caution because of some loss of the pSMT1 plasmid in vivo in susceptible animals (31). Thus, CFU counts in the presence or absence of the resistance marker hygromycin were comparable in the three organ homogenates of resistant C.D2 mice at week 12, whereas in livers from susceptible BALB/c mice, there was a 3.7-fold difference in this ratio. The luminescence assay of duplicate samples showed very reproducible results with a maximal variation of 10%, whereas results from CFU plating presented up to 25% intra-assay variation. Also, the range of detection for luminometry was much broader (between 10^6 and 10^7 RLU) than for CFU counting (10 to 100 CFU/petri dish), requiring multiple organ dilutions.

Thus, using both luminometry and CFU plating, we demonstrated that susceptibility to intravenous _M. paratuberculosis_ infection is influenced by the mouse genotype. Moreover, results in C.D2 mice indicated that the Slc11a1 gene is very likely responsible for the resistant phenotype, although the role of another gene on the 30-centimorgan segment of DBA/2 chromosome 1 cannot be formally excluded.

**Replication of luminescent _M. paratuberculosis_ S-23 in male and female BALB.B10 mice and (C57BL/6 × DBA/2)F1 mice.**

As shown in Table 2, major histocompatibility complex congenic BALB.B10 mice (_H-2b_ haplotype on a BALB/c background) displayed the susceptible phenotype, with stable RLU counts in spleen between day 1 and week 4 postinfection and increased RLU counts in liver during that same period. Heterozygous (_B6 × D2)_F1 mice demonstrated a dramatic reduction in bacterial numbers (a 20- to 60-fold decrease, depending on the organ, between day 1 and week 4), indicating that the _Slc11a1_ resistant allele was dominant in its effect on susceptibility to _M. paratuberculosis_ (Table 2). This dramatic reduction in RLU counts in resistant mice was observed as early as 2 weeks postinfection, highlighting the role of innate immunity in this resistance. Since Johne’s disease in cattle does not show any preference for cows or bulls (25), we also compared genetic susceptibilities to luminescent _M. paratuberculosis_ in male and female BALB.B10 mice and (C57BL/6 × DBA/2)F1 mice.
and female mice. There was no statistical difference in bacterial replication between male and female mice, either in resistant (B6 × D2)F1 or in susceptible BALB.B10 mice (Table 2) (P > 0.05). Susceptibilities were also similar in male and female B6 and BALB/c mice with the susceptible phenotype and in DBA/2 mice with the resistant phenotype (data not shown).

**Mycobacterium-specific cell-mediated immune response following experimental *M. paratuberculosis* infection in susceptible and resistant mice.** In order to analyze whether the cell-mediated immune response could be correlated with differences in susceptibility, production of the Th1-type cytokine IFN-γ was analyzed in spleen cell cultures from two *M. paratuberculosis*-infected susceptible mouse strains (B6 and BALB/c) and from one *M. paratuberculosis*-resistant strain (C.D2). As shown in Fig. 2, very strong mycobacterium-specific IFN-γ production could be detected in B6 mice as early as 1 month after infection in response to CF and PPD from *M. paratuberculosis* (about 30,000 pg/ml; P < 0.05). *M. paratuberculosis*-specific responses were higher than responses against CF and PPD from *M. bovis*, with statistical significance at 12 weeks postinfection (P < 0.01). Confirming previous findings (30), IFN-γ responses induced with recombinant Ag85B from *M. paratuberculosis* were also very high in B6 mice at 4 weeks postinfection (P < 0.05). As was also observed after intravenous *M. bovis* BCG vaccination (17), IFN-γ responses in BALB/c mice were about fivefold lower than in B6 mice (about 6,000 pg/ml) at week 4 postinfection and reached statistical significance at 12 weeks postinfection (P < 0.05 and P < 0.01, respectively). In B6 mice, IFN-γ levels did not change between week 4 and week 8 but increased again at week 12 up to maximal levels of between 60,000 and 70,000 pg/ml (P < 0.05). In BALB/c mice, IFN-γ responses reached a plateau between

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**TABLE 2. Replication of luminescent *M. paratuberculosis* S-23 in male and female BALB.B10 and (B6 × D2)F1 mice**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after infection</th>
<th>Colonizationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>BALB.B10</td>
<td>(B6 × D2)F1</td>
</tr>
<tr>
<td>Spleen</td>
<td>d1</td>
<td>4.33 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w2</td>
<td>4.69 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w4</td>
<td>4.54 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w8</td>
<td>4.56 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w12</td>
<td>4.57 ± 0.15 (5)</td>
</tr>
<tr>
<td>Liver</td>
<td>d1</td>
<td>4.54 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w2</td>
<td>4.69 ± 0.15 (5)</td>
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<tr>
<td></td>
<td>w4</td>
<td>4.54 ± 0.15 (5)</td>
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<td></td>
<td>w8</td>
<td>4.56 ± 0.15 (5)</td>
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<tr>
<td></td>
<td>w12</td>
<td>4.57 ± 0.15 (5)</td>
</tr>
<tr>
<td>Lungs</td>
<td>d1</td>
<td>4.54 ± 0.15 (5)</td>
</tr>
<tr>
<td></td>
<td>w2</td>
<td>4.69 ± 0.15 (5)</td>
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<td>w4</td>
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<td>w8</td>
<td>4.56 ± 0.15 (5)</td>
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<td></td>
<td>w12</td>
<td>4.57 ± 0.15 (5)</td>
</tr>
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a Colonization levels of *M. paratuberculosis* S-23 in male (M) and female (F) BALB.B10 and (B6 × D2)F1 mice in spleen, liver, and lungs, as quantified on day 1 and 2, 4, 8, and 12 weeks after intravenous infection. The data represent mean Log10 RLU ± standard deviation from three to six mice (numbers are given in parentheses). ND, not determined.

b d, day; w, week.
weeks 8 and 12 (maximal levels, around 30,000 pg/ml; $P < 0.05$). Differences between $M. \text{paratuberculosis}$-specific and $M. \text{bovis}$-specific responses remained weak in infected BALB/c mice. Finally, C.D2 mice demonstrated the lowest (albeit still substantial) IFN-γ response of the three mouse strains tested, and this was probably a reflection of the lack of $M. \text{paratuberculosis}$ replication in the mouse strain. Maximal levels of about 10,000 pg/ml could be detected following stimulation with CF-P at 8 weeks postinfection ($P < 0.05$). IFN-γ responses by unstimulated cells and by stimulated cells from naïve, uninfected mice were low (between 100 and 1,000 pg/ml) and were slightly higher in C.D2 mice than in BALB/c and C57BL/6 mice.

**DISCUSSION**

Although some genetic variations in susceptibility to $M. \text{paratuberculosis}$ have been reported in cattle and red deer (14, 21), the role of $\text{Slc11a1}$ polymorphisms in Johne’s disease is not very clear. In sheep, a large study of two fine-wool Merino flocks highly infected with $M. \text{paratuberculosis}$ demonstrated associations of particular polymorphisms in the gene with susceptibility or resistance to infection (29). In other animal species, the situation seems more controversial. Here, we have demonstrated by a luminometric method that $\text{Slc11a1}$ polymorphism exerts a strong genetic influence on the innate susceptibility of mice to intravenous infection with $M. \text{paratuberculosis}$ S-23, as indicated by a clear difference in bacterial replication in the spleen and liver between $\text{Slc11a1s}$ and $\text{Slc11a1r}$ mice. To our knowledge, this is the first time that the C.D2 congenic mouse strain, expressing the resistant $\text{Slc11a1r}$ allele of DBA/2 origin on a BALB/c background, was used in an $M. \text{paratuberculosis}$ study. It is clear that the intravenous infection route used in these experiments is not the natural route used by this enteric pathogen, and oral-infection experiments are needed to definitively demonstrate the role of $\text{Slc11a1}$ polymorphism in innate resistance to $M. \text{paratuberculosis}$.

$\text{NRAMP1}$, now called $\text{SLC11A1}$, was identified as a major, innate resistance component of host antimicrobial activity against a number of intracellular pathogens. This integral membrane protein, present in both prokaryotes and eukaryotes, is highly conserved, suggesting that it plays a basic physiological role, as proven by its conservation throughout evolution. In mice, this gene presents two allelic forms, one encoding an aspartic acid at position 169 ($\text{Slc11a1s}$) and the other encoding glycine at this position ($\text{Slc11a1r}$), which confer, respectively, susceptibility or resistance to infection by these pathogens (45). It is still not completely elucidated how $\text{SLC11A1}$ controls the replication of intracellular parasites. Some studies have suggested a direct involvement in the transport of iron or other bivalent cations, such as $\text{Mn}^{2+}$ and $\text{Mg}^{2+}$ (20). Induction of different degrees of iron overload by in vivo administration of iron-dextran in $M. \text{avium}$-infected mice between $M. \text{paratuberculosis}$ Ag and $M. \text{bovis}$ Ag: $\bullet$, $P < 0.05$. Ag85B-specific responses compared between mouse strains: $\bullet$, $P < 0.05$; $\bullet\bullet$, $P < 0.01$. 

**FIG. 2.** Mycobacterium-specific IFN-γ secretion by splenocytes from $M. \text{paratuberculosis}$-infected C57BL/6, BALB/c, and C.D2 mice. IFN-γ production was measured in spleen cell culture supernatants from C57BL/6, BALB/c, and C.D2 mice before and 4 weeks (gray bars), 8 weeks (black bars), and 12 weeks (hatched bars) after infection with $M. \text{paratuberculosis}$ S-23. The cells were stimulated for 72 h with CF-P or CF-B (10 µg/ml), PPD-P or PPD-B (10 µg/ml), or recombinant Ag85B (5 µg/ml) from $M. \text{paratuberculosis}$. Shown are means plus standard deviations of three or four mice tested individually. Statistical analyses were performed using two-way ANOVA with Bonferroni posttests. Comparison to naïve mice: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Comparison to previous time point: ○, $P < 0.05$; ⋄, $P < 0.01$; ⌊⌊⌋, $P < 0.001$. Comparison between $M. \text{paratuberculosis}$ Ag and $M. \text{bovis}$ Ag: $\bullet$, $P < 0.05$. Ag85B-specific responses compared between mouse strains: $\bullet$, $P < 0.05$; $\bullet\bullet$, $P < 0.01$. 

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seems to indicate that SLC11A1 contributes to macrophage antimicrobial function by excluding Fe^{3+} (essential for the pathogen) from the phagosomal vacuole through H^{+}-coupled transport (11). Also, in vitro studies with M. avium showed that the addition of small quantities of iron to resident macrophages from Slc11a1^-/- mice could stimulate antimicrobial activity through generation of hydroxy radicals and stabilization of SLC11A1 mRNA (47).

The murine Slc11a1 gene is expressed in macrophages from spleen and liver but not from lungs (46). This could explain why in our experiments M. paratuberculosis infection was controlled to the same extent in spleen and liver but not from lungs (46). This could explain mutations in nucleotide oligomerization domain 2 (Nod2), in M. avium suggested by Saunders and Cheers for beige mice infected with M. avium-M. intracellulare pulmonary disease also failed to find a correlation with Slc11a1 polymorphisms (16).

Slc11a1 polymorphism does not play a role in the susceptibility of mice (3, 23), red deer (21), or cattle (3, 4) to virulent M. bovis or M. tuberculosis, although in humans, certain Slc11a1 alleles are risk factors for tuberculosis (4, 5, 22), possibly through regulation of interleukin-10 production (2). The situation is complex, and the effect of Slc11a1 polymorphism on tuberculosis susceptibility seems to be restricted to particular ethnic groups, e.g., Asian subjects (19), and the association seems to indicate that SLC11A1 contributes to macrophage antimicrobial function by excluding Fe^{3+} (essential for the pathogen) from the phagosomal vacuole through H^{+}-coupled transport (11). Also, in vitro studies with M. avium showed that the addition of small quantities of iron to resident macrophages from Slc11a1^-/- mice could stimulate antimicrobial activity through generation of hydroxy radicals and stabilization of SLC11A1 mRNA (47).

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Whereas initial resistance to M. paratuberculosis was clearly controlled by Slc11a1, at later stages, additional factors influence bacterial replication in mice expressing the susceptible Slc11a1^-/- allele. Thus, BALB/c mice controlled infection less efficiently than B6 mice in spleen, in lungs, and particularly in liver. Differences in the magnitudes of the early mycobacterium-specific Th1 (IFN-γ) response observed in these two mouse strains in the spleen may play a role, but certainly, other factors are also involved. Indeed, Ag-specific IFN-γ production was significantly lower in resistant C.D2 mice than in susceptible BALB/c and B6 mice. Also, beige B6bg/bg mice (presenting macrophage lysosomal defects and deficient lytic natural killer cell activity) were more susceptible than mice of the parental B6 strain, particularly with respect to control of the infection in the liver and lungs, although acquired immunity levels, reflected by mycobacterium-specific spleen cell IFN-γ responses, were comparable in B6 mice and in these mutant B6bg/bg mice (30). This suggests that attraction of neutrophils and natural killer cells to the liver could be an important control mechanism following intravenous M. paratuberculosis infection, as suggested by Saunders and Cheers for beige mice infected with M. avium (32). In this respect, it is interesting to note that mutations in nucleotide oligomerization domain 2 (Nod2), involved in signaling of proinflammatory chemokines, have been reported in 15% of patients suffering from Crohn’s disease (24). A defect in neutrophil recruitment caused by a decreased interleukin-8 response to muramyl dipeptide has also been described in Crohn’s disease (42). Clearly, more work is needed to characterize the precise immune mechanisms involved at later stages of M. paratuberculosis infection in mice, particularly with respect to proinflammatory chemokine production.

ACKNOWLEDGMENTS

This work was partially supported by grants from the FWO-Vlaanderen (G.0376.05). V. Rosseels is supported by the Belgian Science Policy (Ylief). V. Roupie is a FRIA bursary recipient. We also acknowledge support from the USDA Animal Health Project (NEB 14-108 to R.G.B.). We are grateful to F. Jurion and P.-Y. Adnet for excellent technical assistance. We also thank E. Skamene (McGill University, Montreal, Canada) for giving us breeding couples of the congenic C.D2 mouse strain. Finally, we thank Erik Jongert (WIV- Pasteur Institute) for helping us with the statistical analyses.

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Editor: J. L. Flynn