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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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-H2^b^ (BALB/c background; *H-2^b^*), C57BL/6, and beige C57BL/6^bg/bg^ mice (all *Slc11a1^s^*), bacterial numbers in spleen and liver remained unchanged during the first 4 weeks of infection, whereas in DBA/2 and congenic BALB/c.DBA/2 (C.D2) mice (both *Slc11a1^r^*) and in (C57BL/6 × DBA/2)F$_1$ mice (*Slc11a1^s/r^*), 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^bg/bg^ 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 Typhimurium 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 Fe^{2+}, Mn^{2+}, or Mg^{2+}), but the mechanism by which parasite replication is blocked has not been completely elucidated (11, 20, 47).

The natural resistance of mice to nontuberculous 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 hepato- 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 *Slc11a1^s^* 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-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/6JOlaHsd (B6), and mutant C57BL/6OlaHsd-*Lyst*$^{bg/bg}$ (B6$^{bg/bg}$) beige mice (four strains expressing the susceptible *Slc11a1*^{s} allele); DBA/2OlaHsd (DBA/2) and BALB.c.DBA/2 (C.D2) mice (two strains expressing the resistant *Slc11a1*^{r} allele) (9); and heterozygous (C57BL/6 × DBA/2)F$_1$ [(B6 × D2)F$_1$] mice were bred at the Pasteur Institute Animal Facilities from breeding couples originally obtained from Harlan Netherlands (BALB/c, C57BL/6, DBA/2, and B6$^{bg/bg}$), from The Netherlands Cancer Institute (BALB.B10), and from E. Skamene (McGill University, Montreal, Canada) (C.D2). All mice were 2 to 3 months old at the time of infection. B6$^{bg/bg}$ mice are spontaneous C57BL/6 mutants for the recessive gene *bg* (a lysosomal trafficking regulator). This mouse strain (*H-2*^{b}) 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-2*^{b} locus from C57BL/10 on a BALB/c background. C.D2-*Slc11a1*^{r} (BALB/c.DBA/2J) mice are BALB/c congenic mice that carry a 30-centimorgan segment of DBA/2 chromosome 1 containing the *Slc11a1*^{r} 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% *n*-decanal (Sigma) in ethanol as a substrate (41). In this assay, only live bacteria are enumerated, because emission of light is dependent on the presence of reduced flavin mononucleotide (FMNH$_2$), a cofactor that is found only in living cells. For statistical analysis (two-way analysis of variance [ANOVA] with Bonferroni posttests), results obtained in relative light units (RLU) were converted to log$_{10}$ values. The CFU/RLU ratio for exponentially growing axenic *M. paratuberculosis* cultures was determined to be 1.2 under our test conditions (5-second delay and 15-second integration time). It is important to note that RLU values are relative and not absolute light units, and the values depend on the sensitivity of the luminometer, the sample and substrate volumes, and the integration time used. However, in comparisons of CFU and RLU data, delta log$_{10}$ values have been found to be nicely correlated (31, 41).

Infection of mice. The luminescent bovine isolate *M. paratuberculosis* S-23 (31) was grown in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase, mycobactin J (Allied Laboratories Inc., Synbiotics Europe; 2 μg/ml), and hygromycin (100 μg/ml) to an optical density between 0.6 and 0.8. The bacteria were centrifuged for 30 min at 2,000 rpm and suspended in phosphate-buffered saline (PBS) to a concentration of 8.5 × 10^6 RLU/ml, and mice were infected intravenously in a lateral tail vein with 0.2 ml (1.7 × 10^6 RLU to 2 × 10^6 CFU) of bacteria.

Counting RLU in organs. At day 1 and weeks 2, 4, 8, and 12 after infection, mice were killed by cervical dislocation; spleens, lungs, and livers were removed aseptically and homogenized by gentle disruption in a loosely fitting Dounce homogenizer. After red cell lysis (to minimize quenching), 1 ml of fresh organ homogenate was centrifuged and resuspended in 1 ml of PBS. The bacterial burden was evaluated by a luminescent assay as previously described (with 0.1 ml *n*-decanal as a substrate) (41). Background RLU values were determined in spleen, liver, and lungs of naïve BALB.B10 mice.

Counting CFU. The number of CFU of luminescent *M. paratuberculosis* was determined by plating serial dilutions in PBS on Middlebrook 7H11–oleic acid-albumin-dextrose-catalase agar supplemented with mycobactin J and with (or without) hygromycin, in order to check for the presence of the pSMT1 plasmid. One hundred-microliter volumes of bacterial culture or organ homogenates were

plated in duplicate. The petri dishes were sealed in plastic bags and incubated at 39°C for 8 weeks before the colonies were counted visually.

Bacterial antigens (Ags). *M. paratuberculosis* ATCC 19698 was grown at 39°C for 4 weeks as a surface pellicle on synthetic Sauton medium supplemented with mycobactin J as described previously (30). The culture filtrate from *M. paratuberculosis* (CF-P) was separated from the bacteria; CF proteins were precipitated with ammonium sulfate and extensively dialyzed against PBS. The CF of *M. bovis* (AN5) (CF-B) was obtained from *M. bovis* cultures grown as surface pellicles for 2 weeks at 37°C on synthetic Sauton medium. Purified protein derivative from *M. bovis* Vallée (PPD-B) was kindly given to us by the late J. Nyabenda (WIV-Pasteur Institute Brussels). PPD was prepared from 6- to 8-week-old cultures of *M. paratuberculosis* strain ATCC 19698 (PPD-P). Recombinant Ag85B from *M. paratuberculosis* was prepared as described previously (30). Briefly, recombinant protein was expressed in Top-10F′ *Escherichia coli* transformed with a plasmid encoding Ag85B from *M. paratuberculosis* with six N-terminal histidines. Recombinant proteins were purified by affinity chromatography on an immobilized nickel-chelate (Ni-nitrilotriacetic acid) column.

Spleen cell gamma interferon (IFN-γ) production. At 4, 8, and 12 weeks after infection, female mice were killed by cervical dislocation; their spleens were removed aseptically and homogenized by gentle disruption in a loosely fitting Dounce homogenizer. Spleens from three or four animals per group were analyzed individually. The spleen cells were washed and suspended to 4 × 10^6 leukocytes/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, 5 × 10^{-5} M 2-mercaptoethanol, and indomethacin (1 μg/ml; Sigma). The cells were cultured in a humidified CO$_2$ incubator in round-bottom 96-well microplates. A volume of 180 μl of cells was added to 20 μl of the respective Ags. CF-B, CF-P, PPD-B, and PPD-P were used at final concentrations of 10 μg/ml and recombinant *E. coli*-derived 85B protein from *M. paratuberculosis* was used at 5 μg/ml in the presence of polymyxin B (5 ng/ml). Culture supernatants from three wells were collected and pooled after 72 h (the peak of cytokine activity) and stored at −20°C until they were tested.

IFN-γ assay. IFN-γ activity was quantified on supernatants using a sandwich enzyme-linked immunosorbent assay with the coating antibody R4-6A2 and the biotinylated detection antibody XMG1.2 (both from Pharmingen). The detection limit was 10 pg/ml. The results were expressed as mean (± standard deviation) pg/ml, calculated from three or four mice per group tested individually.

For statistical analysis, two-way ANOVA with Bonferroni posttests was used to statistically evaluate differences in IFN-γ production from that of naïve mice, by time postinfection, between *M. paratuberculosis* Ag and *M. bovis* Ag or between mouse strains for Ag85B.

RESULTS

Replication of luminescent *M. paratuberculosis* S-23 in mice carrying the *Slc11a1*^{s} (susceptible) or the *Slc11a1*^{r} (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 × 10^6 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 C.D2 congenic mice (both expressing the resistant *Slc11a1*^{r} 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 B6$^{bg/bg}$ mice expressing the susceptible *Slc11a1*^{s} allele showed persistent infection. At week 4 postinfection, *Slc11a1*^{s} mice showed 10- to 100-fold-higher RLU numbers in the liver and spleen than mice expressing the resistant *Slc11a1*^{r} 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 B6$^{bg/bg}$ 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-
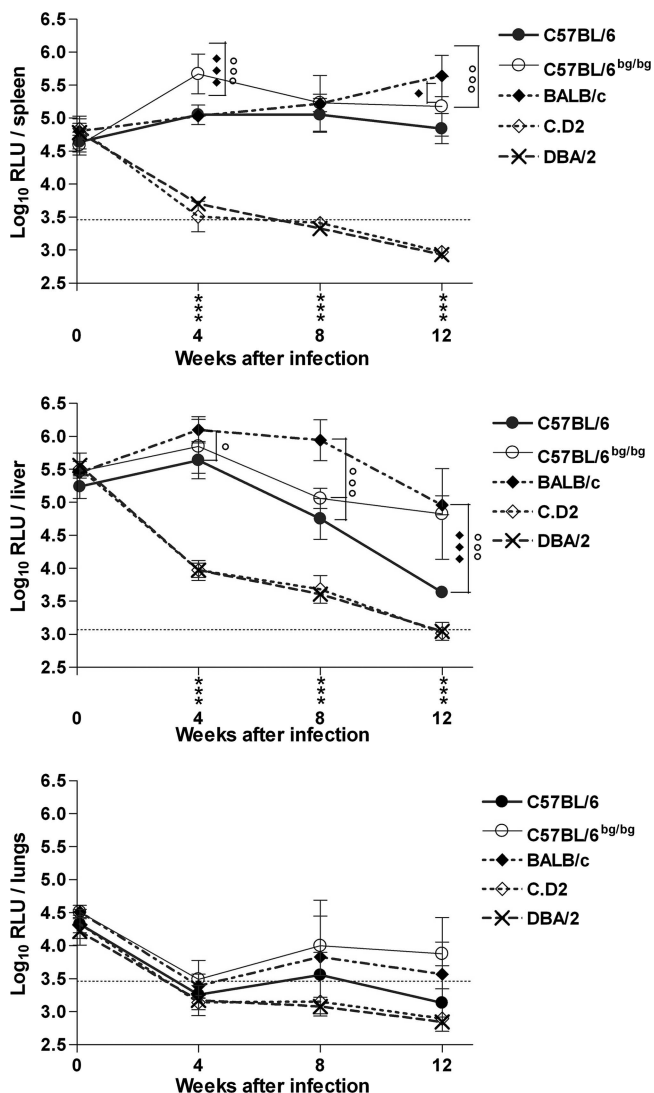
FIG. 1. Susceptibilities of five inbred mouse strains to intravenous infection with luminescent *M. paratuberculosis* S-23. Mice were sacrificed on day 1 and 4, 8, and 12 weeks after infection, and the numbers of mycobacteria in spleen, liver, and lungs were determined by luminometry. The results are reported as $\log_{10}$ RLU/organ (mean ± standard deviation of three to six mice per group tested individually). The detection limits (dotted lines) were determined using organs from noninfected BALB.B10 mice. Statistical analyses were performed using two-way ANOVA with Bonferroni posttests. Comparison between susceptible and resistant mice: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Comparison between BALB/c and C57BL/6 or C57BL/6$^{bg/bg}$ mice: ○, $P < 0.05$; ○○, $P < 0.01$; ○○○, $P < 0.001$. Comparison between C57BL/6 and C57BL/6$^{bg/bg}$ mice: ◆, $P < 0.05$; ◆◆, $P < 0.01$; ◆◆◆, $P < 0.001$.

estingly, mutant beige B6$^{bg/bg}$ mice displayed the same hepatic phenotype as BALB/c mice, and the bacterial loads remained high in the livers of these mice, reported to have increased susceptibility to *M. paratuberculosis* (36). Finally, the bacterial burdens in the lungs decreased very rapidly in both *Slc11a1^s* and *Slca1a1^r* mice during the first month of infection. Interestingly, between week 4 and week 8, bacterial numbers increased again in the lungs of susceptible mice, particularly in beige B6$^{bg/bg}$ mice.

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 10^3 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-2^b* 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^r* 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

TABLE 1. Evolution of *M. paratuberculosis* S-23 numbers in spleen, liver, and lungs as determined by luminometry and classical CFU counting

Organ	Mouse	No. of bacteria[a]					
		4 wk after infection			12 wk after infection		
		Log$_{10}$ RLU	Log$_{10}$ CFU	Log$_{10}$ CFU(h)	Log$_{10}$ RLU	Log$_{10}$ CFU	Log$_{10}$ CFU(h)
Spleen	C57BL/6	5.29 ± 0.44 (5)	5.56 ± 0.58 (5)	5.39 ± 0.66 (5)	4.59 ± 0.21 (5)	5.80 ± 0.11 (5)	5.47 ± 0.13 (5)
	BALB/c	5.16 ± 0.15 (4)	5.63 ± 0.19 (4)	5.66 ± 0.18 (4)	5.23 ± 0.34 (4)	6.20 ± 0.52 (3)	6.02 ± 0.34 (3)
	C.D2	3.88 ± 0.10 (4)	4.70 ± 0.17 (4)	4.58 ± 0.19 (4)	3.52 ± 0.12 (6)	3.97 ± 0.38 (6)	4.02 ± 0.28 (6)
Liver	C57BL/6	5.59 ± 0.06 (5)	6.68 ± 0.09 (5)	6.52 ± 0.10 (5)	3.65 ± 0.22 (5)	5.48 ± 0.25 (5)	5.24 ± 0.25 (5)
	BALB/c	5.87 ± 0.06 (4)	6.62 ± 0.13 (4)	6.38 ± 0.16 (4)	5.33 ± 0.40 (3)	7.07 ± 0.27 (3)	6.50 ± 0.18 (3)
	C.D2	4.40 ± 0.14 (4)	5.58 ± 0.19 (4)	5.42 ± 0.24 (4)	3.22 ± 0.10 (6)	4.80 ± 0.19 (6)	4.60 ± 0.34 (6)
Lungs	C57BL/6	3.68 ± 0.17 (5)	4.01 ± 0.36 (5)	4.07 ± 0.48 (5)	3.06 ± 0.07 (5)	3.76 ± 0.63 (5)	3.34 ± 0.51 (5)
	BALB/c	3.34 ± 0.02 (4)	4.15 ± 0.66 (4)	4.06 ± 0.70 (4)	3.42 ± 0.71 (3)	5.00 ± 0.62 (3)	4.59 ± 0.73 (3)
	C.D2	2.90 ± 0.02 (4)	4.41 ± 0.71 (4)	4.26 ± 0.73 (4)	2.95 ± 0.17 (6)	2.85 ± 0.40 (6)	2.89 ± 0.46 (6)

[a] Organs from individual infected mice were homogenized, and the numbers of bacteria/organ were determined by luminometry or by plating the bacteria on Middlebrook 7H11 agar with [CFU(h)] or without (CFU) hygromycin. The data represent mean (± standard deviation) values for three to six mice (numbers are given in parentheses).

and female mice. There was no statistical difference in bacterial replication between male and female mice, either in resistant (B6 × D2)F$_1$ 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

TABLE 2. Replication of luminescent *M. paratuberculosis* S-23 in male and female BALB.B10 and (B6 × D2)F$_1$ mice

Organ	Time after infection[b]	Colonization[a]			
		BALB.B10		(B6 × D2)F$_1$	
		M	F	M	F
Spleen	d1	5.09 ± 0.13 (5)	4.84 ± 0.18 (4)	5.25 ± 0.14 (3)	5.44 ± 0.08 (5)
	w2	ND	4.65 ± 0.38 (4)	4.32 ± 0.09 (3)	4.27 ± 0.09 (3)
	w4	5.01 ± 0.13 (5)	5.03 ± 0.12 (5)	3.83 ± 0.07 (5)	3.81 ± 0.07 (5)
	w8	4.94 ± 0.23 (6)	5.08 ± 0.30 (4)	3.29 ± 0.06 (5)	3.46 ± 0.17 (5)
	w12	4.81 ± 0.20 (4)	4.80 ± 0.38 (6)	3.64 ± 0.14 (6)	3.54 ± 0.11 (7)
Liver	d1	5.43 ± 0.14 (5)	5.51 ± 0.21 (4)	5.57 ± 0.26 (3)	5.84 ± 0.08 (5)
	w2	ND	5.09 ± 0.12 (4)	3.98 ± 0.13 (3)	4.03 ± 0.10 (3)
	w4	5.95 ± 0.04 (5)	6.09 ± 0.05 (5)	4.23 ± 0.08 (5)	4.24 ± 0.24 (5)
	w8	5.55 ± 0.22 (6)	5.61 ± 0.45 (4)	3.40 ± 0.11 (5)	3.39 ± 0.11 (5)
	w12	5.34 ± 0.55 (4)	4.82 ± 0.58 (6)	3.26 ± 0.07 (6)	3.20 ± 0.03 (6)
Lungs	d1	4.80 ± 0.17 (5)	4.99 ± 0.40 (4)	4.48 ± 0.18 (3)	4.67 ± 0.20 (5)
	w2	ND	3.81 ± 0.10 (4)	3.27 ± 0.03 (3)	3.57 ± 0.16 (3)
	w4	3.26 ± 0.39 (5)	3.07 ± 0.15 (5)	2.92 ± 0.04 (5)	2.93 ± 0.09 (5)
	w8	3.82 ± 0.75 (6)	4.05 ± 0.81 (4)	3.01 ± 0.10 (5)	2.95 ± 0.14 (5)
	w12	3.52 ± 0.69 (4)	3.55 ± 0.73 (6)	2.92 ± 0.07 (6)	2.99 ± 0.20 (6)

[a] Colonization levels of *M. paratuberculosis* S-23 in male (M) and female (F) BALB.B10 and (B6 × D2)F$_1$ 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 log$_{10}$ RLU ± standard deviation from three to six mice (numbers are given in parentheses). ND, not determined.
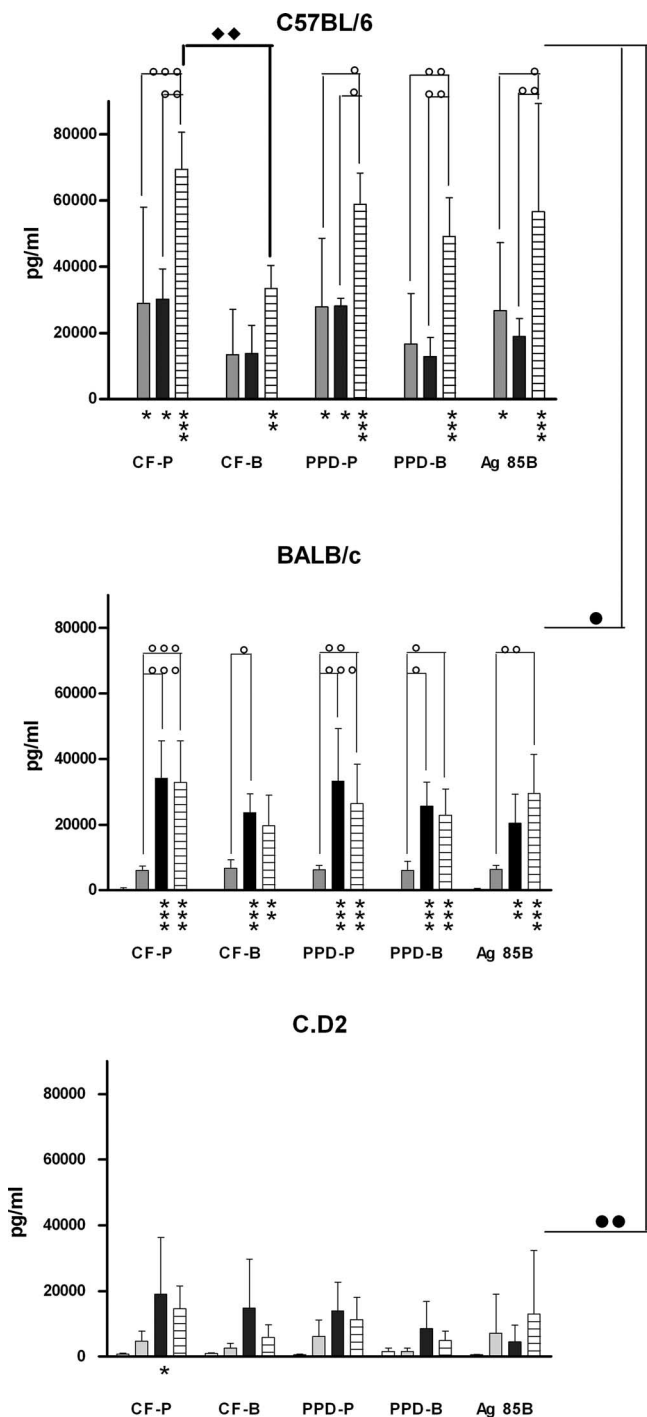[b] d, day; w, week.

FIG. 2. Mycobacterium-specific IFN-γ secretion by splenocytes from *M. 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. 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. 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. paratuberculosis* Ag and *M. bovis* Ag: ◆, $P < 0.05$. Ag85B-specific responses compared between mouse strains: ●, $P < 0.05$; ●●, $P < 0.01$.

weeks 8 and 12 (maximal levels, around 30,000 pg/ml; $P < 0.05$). Differences between *M. paratuberculosis*-specific and *M. 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. 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. paratuberculosis* have been reported in cattle and red deer (14, 21), the role of *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. 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 *Slc11a1* polymorphism exerts a strong genetic influence on the innate susceptibility of mice to intravenous infection with *M. paratuberculosis* S-23, as indicated by a clear difference in bacterial replication in the spleen and liver between *Slc11a1*[s] and *Slc11a1*[r] mice. To our knowledge, this is the first time that the C.D2 congenic mouse strain, expressing the resistant *Slc11a1*[r] allele of DBA/2 origin on a BALB/c background, was used in an *M. 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 *Slc11a1* polymorphism in innate resistance to *M. paratuberculosis*.

NRAMP1, now called 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 (*Slc11a1*[s]) and the other encoding glycine at this position (*Slc11a1*[r]), which confer, respectively, susceptibility or resistance to infection by these pathogens (45). It is still not completely elucidated how 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 Mn^{2+} and Mg^{2+} (20). Induction of different degrees of iron overload by in vivo administration of iron-dextran in *M. avium*-infected mice

seems to indicate that SLC11A1 contributes to macrophage antimicrobial function by excluding Fe^{2+} (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*[r] mice could stimulate antimicrobial activity through generation of hydroxyl 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 *Slc11a1*[s] and *Slc11a1*[r] lungs during the first 4 weeks after intravenous infection. Bacteria were very rapidly eliminated from the lungs of both susceptible and resistant mice, indicating that *Slc11a1*-independent mechanisms are involved in the antimycobacterial defense of pulmonary macrophages. Interestingly, a study of women suffering from *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 is sex and age dependent and restricted to females and the young age group (below 65 years) (18). Recently, a genetic polymorphism in the human *Slc11a1* gene was found to be associated with susceptibility to another mycobacterial pathogen for humans, i.e., *Mycobacterium ulcerans*, which causes Buruli ulcer, a necrotizing skin disease particularly affecting children in Central and West Africa (37).

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*[s] 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 B6[bg/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 B6[bg/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.

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