Infectious diseases remain a major cause of death worldwide, especially in children and young adults, with most of the burden falling on the populations of the poorest countries (www.who.int/infectious-disease-report). With emerging and reemerging pathogens, the globalization of exchanges between countries, and the constant threat of antimicrobial resistance (1), it becomes increasingly clear that a better understanding of the host–pathogen interactions in vivo is needed. One approach to this problem is the study of the genetic determinants of the host response to infection, which frequently leads to a better understanding of disease pathogenesis. The outcome of an encounter between hosts and pathogens results from the battle of two genomes, in addition to environmental factors and the fitness of the two protagonists. Although microbial organisms have and continue to acquire virulence factors enabling them to thrive in a particular niche (2, 3), the host genomes have accumulated polymorphisms that, at times, confer resistance or susceptibility to specific pathogens (4–6). The importance of the host genetic makeup in the response to infection was illustrated in a seminal study by Sorensen et al. (7), where it was shown that the relative risk of death from infectious diseases for an adoptee was significantly increased when their biological parent had died of infectious disease before age 50. Additional evidence for a major role of genetic factors comes from twin studies (8), linkage and association studies (9, 10), and numerous studies of specific gene defects conferring susceptibility or resistance to individual pathogens (5, 11).

The mouse response to acute Salmonella typhimurium infection is complex, and it is under the influence of several genes, as well as environmental factors. In a previous study, we identified two novel Salmonella susceptibility loci, Ity4 and Ity5, in a (AcB61 × 129S6)F2 cross. The peak logarithm of odds score associated with Ity4 maps to the region of the liver and red blood cell (RBC)–specific pyruvate kinase (Pklr) gene, which was previously shown to be mutated in AcB61. During Plasmodium chabaudi infection, the Pklr mutation protects the mice against this parasite, as indicated by improved survival and lower peak parasitemia. Given that RBC defects have previously been associated with resistance to malaria and susceptibility to Salmonella, we hypothesized that Pklr is the gene underlying Ity4 and that it confers susceptibility to acute S. typhimurium infection in mice. Using a fine mapping approach combined with complementation studies, comparative studies, and functional analysis, we show that Pklr is the gene underlying Ity4 and that it confers susceptibility to acute S. typhimurium infection in mice through its effect on the RBC turnover and iron metabolism.

Pyruvate kinase deficiency confers susceptibility to Salmonella typhimurium infection in mice

Marie-France Roy,1,2 Noémie Riendeau,2 Christian Bédard,5 Pierre Héléie,5 Gundula Min-Oo,2,3 Karine Turcotte,2,3 Philippe Gros,2,3 François Canonne-Hergaux,6 and Danielle Malo1,2,4

1 Department of Human Genetics, 2 Center for the Study of Host Resistance, 3 Department of Biochemistry, 4 Department of Medicine, McGill University Health Center, Montréal, Québec, H3G 1A4, Canada 5 Département de Pathologie et de Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, J2S 2M2, Canada 6 Institut National de la Santé et de la Recherche Médicale, U656, Fer et Synthèse d’Hème, Génétique, Physiologie et Pathologie, Faculté de Médecine Xavier Bichat, Paris, 75870, France

The mouse response to acute Salmonella typhimurium infection is complex, and it is under the influence of several genes, as well as environmental factors. In a previous study, we identified two novel Salmonella susceptibility loci, Ity4 and Ity5, in a (AcB61 × 129S6)F2 cross. The peak logarithm of odds score associated with Ity4 maps to the region of the liver and red blood cell (RBC)–specific pyruvate kinase (Pklr) gene, which was previously shown to be mutated in AcB61. During Plasmodium chabaudi infection, the Pklr mutation protects the mice against this parasite, as indicated by improved survival and lower peak parasitemia. Given that RBC defects have previously been associated with resistance to malaria and susceptibility to Salmonella, we hypothesized that Pklr is the gene underlying Ity4 and that it confers susceptibility to acute S. typhimurium infection in mice. Using a fine mapping approach combined with complementation studies, comparative studies, and functional analysis, we show that Pklr is the gene underlying Ity4 and that it confers susceptibility to acute S. typhimurium infection in mice through its effect on the RBC turnover and iron metabolism.
Additionally, particular MHC haplotypes are associated with increased risk of typhoid fever (caused by the human-specific serovars Typhi and Paratyphi) (15). There are most likely several additional polymorphisms that have an impact on the host response to Salmonella infection, but their identification in human populations is hindered by the difficulties and complexities associated with human-based studies. In this context, the mouse model of typhoid fever is used to identify additional genetic factors that control the host response to Gram-negative, intracellular pathogens, factors that can later be studied in humans or relevant veterinary species.

Infection of mice with *Salmonella enterica* serovar Typhimurium (hereafter *Salmonella typhimurium*), either orally or parenterally, results in the localization and replication of the bacteria in the spleen and liver, thereby mimicking human typhoid fever (16). Using this model, several genes were identified as having a strong impact on the mouse susceptibility to acute *S. typhimurium* infection (17), including *Natural resistance-associated macrophage protein 1* (*Nramp1*; also known as *Slc11a1*) and *Toll-like receptor 4* (*Tlr4*). *Nramp1* is a transmembrane protein involved in the transport of divalent cations (18, 19). After phagocytosis, it is recruited to the membrane of the phagolysosome (20), where it is believed to deprive the bacteria from essential divalent cations, including iron. Mice carrying a nonfunctional allele at *Nramp1* are extremely susceptible to *Salmonella* infection and succumb to infection with $<10^8$ bacteria.

Additionally, particular MHC haplotypes are associated with increased risk of typhoid fever (caused by the human-specific serovars Typhi and Paratyphi) (15). There are most likely several additional polymorphisms that have an impact on the host response to Salmonella infection, but their identification in human populations is hindered by the difficulties and complexities associated with human-based studies. In this context, the mouse model of typhoid fever is used to identify additional genetic factors that control the host response to Gram-negative, intracellular pathogens, factors that can later be studied in humans or relevant veterinary species.

Infection of mice with *Salmonella enterica* serovar Typhimurium (hereafter *Salmonella typhimurium*), either orally or parenterally, results in the localization and replication of the bacteria in the spleen and liver, thereby mimicking human typhoid fever (16). Using this model, several genes were identified as having a strong impact on the mouse susceptibility to acute *S. typhimurium* infection (17), including *Natural resistance-associated macrophage protein 1* (*Nramp1*; also known as *Slc11a1*) and *Toll-like receptor 4* (*Tlr4*). *Nramp1* is a transmembrane protein involved in the transport of divalent cations (18, 19). After phagocytosis, it is recruited to the membrane of the phagolysosome (20), where it is believed to deprive the bacteria from essential divalent cations, including iron. Mice carrying a nonfunctional allele at *Nramp1* are extremely susceptible to *Salmonella* infection and succumb to infection with $<10^8$ bacteria.

Figure 1. Candidacy of *Pklr* as the gene underlying *Ity4*. (A) Interval mapping under a nonparametric model showing the LOD score trace of chromosome 3 for the survival phenotype in 247 (AcB61 × 129S6)F2 mice. *Pklr* maps directly under the peak LOD score, as shown on the graph. The positions of the typed markers are shown as small vertical bars above the x axis. (B) Survival after *Salmonella* infection in 306 F2 mice according to their genotype at the *Pklr* mutation (m/m, *Pklr* mutant mice; +/+ , wild type mice; +/m, heterozygous mice). Homozygous *Pklr* mutant mice are extremely susceptible to *Salmonella*. (C) *Pklr* genotyping through *Sfa* N1 restriction digestion. Homozygote *Pklr* mutant mice show a single 335-bp band, whereas homozygous wild-type mice present two bands. The AcB61, AcB55, AcB62, and F1 strains are homozygous for the *Pklr* mutation. (D) Cumulative survival after *Salmonella* infection. The three RCSs known to carry a mutated *Pklr* gene show a concordant phenotype ($n = 5$ for the RCSs; $n = 20$ for AcB61 and AcB62; $n = 6$ for AcB55). (E) Survival after *Salmonella* infection in F1 mice derived from (AcB61 × AcB62) and (AcB61 × AcB55) crosses. No complementation is observed ($n = 15$ for the F1s and AcB61; $n = 5$ for AcB62; $n = 6$ for AcB55). (F) Survival after *S. typhimurium* infection in another *Pklr* mutant mouse strain, CBA-Pk-1<sup>1</sup>, and controls. As is the case for the mutant RCSs, the CBA-Pk-1<sup>1</sup> is more susceptible to *Salmonella* compared with wild-type controls ($n = 5$ for CBA-Pk-1<sup>1</sup>, $n = 4$ for CBA/CaHN-Btk<sup>exv</sup>/J and CBA/J; $n = 2$ for C57BL/6J).
organisms (21–24). Tlr4 is the pattern recognition receptor for LPS, and it is responsible for most of the mouse response after infection with Salmonella (25, 26). Mice carrying a non-functional Tlr4 allele show a 1,000-fold reduction in LD50 (27, 28), whereas increasing the number of Tlr4 from 0 to 1, 2, and 3 copies of the gene brings an incremental protective effect from death after S. typhimurium infection (29).

Hoping to expand our understanding of the genetic determinants of the host response to Salmonella infection using the mouse model of typhoid fever, we recently undertook a systematic screening of a set of 36 A/J and C57BL/6J recombinant congenic strains (AcB/BcA RCSs) for their response to acute Salmonella infection (30). Although we knew beforehand that the parental strains (A/J and C57BL/6J) differ in their susceptibility to Salmonella infection, mainly because C57BL/6J carries a point mutation in Ntamp1, we hypothesized that additional genes would influence the outcome of infection and segregate in the RCSs. We showed that Ntamp1 alone is, indeed, not sufficient to explain the phenotypic variance among the RCSs, and that additional genes influence the response to Salmonella. In particular, we have identified the strains AcB61 and AcB62 as extremely susceptible to S. typhimurium, despite the observation that they carry a functional allele at Ntamp1. Interval mapping performed on a (129S6/SvEvTac [or 129S6] × AcB61)F2 cross revealed a major locus (Immunity to typhimurium locus 4 [Ity4]) influencing the survival phenotype at position 44cM on mouse chromosome 3, with a logarithm of odds (LOD) score of 28.8. The AcB61 strain was previously found to carry a spontaneous mutation in the liver and red blood cell (RBC)–specific pyruvate kinase gene Pklr (31). Pklr-deficient mice present a constitutive hemolytic anemia with reticulocytosis and splenomegaly (32), and they are more resistant to Plasmodium chabaudi infection (31). Because the position of Pklr is exactly under our major Ity4 peak on chromosome 3, we present the evaluation of its candidacy as the gene underlying the susceptibility of the AcB61 strain to Salmonella and characterize the phenotypic expression of the Pklr deficiency during infection. Additionally, we further characterize the effect of Salmonella infection on the mouse erythroid response and iron metabolism. The results presented in this paper show that Pklr is the gene underlying Ity4 and that it influences susceptibility to Salmonella infection through its effect on RBC turnover and iron homeostasis.

RESULTS

Evaluation of the candidacy of Pklr as the gene underlying Ity4

In a previous study, we mapped a Salmonella susceptibility locus to mouse chromosome 3 in a F2 cross between the extremely susceptible AcB61 strain and the totally resistant 129S6 strain (30). This locus, named Ity4, maps to position 44cM with a LOD score of 28.8, explaining 42% of the phenotypic variance. The AcB61 strain was previously shown to carry a point mutation in the Pklr gene, rendering it resistant to P. chabaudi (31). Because Pklr maps directly under our chromosome 3 peak LOD score (Fig. 1 A), we evaluated its candidacy as the gene underlying Ity4.

We first genotyped 306 (AcB61 × 129S6)F2 mice for the known Pklr mutation and examined their survival after S. typhimurium infection according to their genotype at Pklr. Fig. 1 B shows a strong correlation between the mouse genotype at Pklr and survival after Salmonella infection. Mice homozygous for the AcB61 mutated allele have very little chance of surviving the infection, whereas mice carrying at least one 129S6 allele are much more resistant, indicating that Pklr or a gene tightly linked to Pklr confers susceptibility to Salmonella. Repeating the interval mapping, this time including the Pklr genotypes in the analysis, led to a peak in the LOD score from 28.8 (30) to 30.3 (Fig. 1 A). A fine mapping approach targeted to the (AcB61 × 129S6)F2 mice that presented non-resolved recombination around Pklr, allowed us to decrease the support interval from a 25.3-Mb region to a region of 3.7-Mb just surrounding Pklr (Fig. 2). Examination of the survival after Salmonella infection for the three RCSs known to carry a mutated Pklr allele (AcB61, AcB62, and AcB55; reference [31] and Fig. 1 C) showed a concordant phenotype with all three strains being extremely susceptible (Fig. 1 D), whereas none of the other Ntamp1-resistant RCSs have shown such susceptibility (30). The AcB55 strain was even more
susceptible than the C57BL/6J parent, with all mice dying on day 4. We attribute this extreme susceptibility to Salmonella to the observation that the AcB55 strain carries mutated alleles at both N ramp 1 and Pklr. We also observed a lack of complementation in F1 mice derived from crosses between AcB61 and AcB62, and between AcB61 and AcB55, indicating that the same gene is responsible for their susceptibility to Salmonella (Fig. 1, C and E). Because these three strains are known to carry the same point mutation in Pklr, it is likely that this gene is responsible for their susceptibility to S. typhimurium infection. Finally, a Pklr mutation has also been reported in a colony of CBA/N mice from Japan (CBA/N-Pk-1 slc/Pk-1 slc; hereafter CBA-Pk-1 slc) (33), these mice have a phenotype similar to the AcB61 mice with constitutive hemolytic anemia and reticulocytosis (34) and increased survival to infection with P. chabaudi AS (35). When CBA-Pk-1 slc mice were infected with S. typhimurium, they also showed a markedly increased susceptibility in terms of survival compared with the wild-type CBA/J, or even the CBA/CaHN-Btk xid/J, which carry the xid mutation (36, 37) (Fig. 1 F), confirming the role of the Pklr mutation in mouse susceptibility to acute Salmonella infection. Collectively, these results indicate that Pklr is very likely the gene underlying the susceptibility of the AcB61 mice to Salmonella.

The increased susceptibility of the AcB61 mice caused by the Pklr mutation appears quite specific for Salmonella infection. These mice have indeed been tested for susceptibility to infection with several pathogens. The AcB61 and its parental strain, A/J, have been shown to be susceptible to the intracellular pathogen Legionella pneumophila (38), which is caused by a mutation within the Bir1e gene located on chromosome 13 (39). The AcB61 mice have also been shown to be resistant to malaria because of the Pklr mutation (31) and to BCG because of the presence of a N ramp 1 wild-type allele (Fig. 3). In addition, the AcB61 and parental A/J mice carry a naturally occurring mutation within the complement hemolytic factor 5 (chromosome 2) that causes C5 deficiency and may render them highly susceptible to other pathogens, including Listeria monocytogenes, Candida albicans (40), and Staphylococcus aureus. Therefore, the AcB61 strain carries several host resistance/susceptibility genes that are specific for different pathogens. These loci were, however, inherited from the parental A/J strain, and only the Pklr mutation is a novel spontaneous mutation that occurred in the development of the AcB61 RCS.

Finally, the susceptibility of the AcB61 mice does not appear to be caused by an intrinsic defect in phagocyte function or recruitment. Indeed, in vitro experiments showed that the capacity of AcB61 BM-derived macrophages (BMDMs) to control bacterial load is comparable to that of its parental strain A/J, and to that of RAW 264.7 macrophages transfected with a wild-type allele at N ramp 1 (G169; Fig. 4), whereas replication of S. typhimurium is observed in Raw 264.7 macrophages carrying the mutant N ramp 1 allele (D169). Also, no obvious differences in the quality or quantity of cells recruited to the spleen and liver were observed on histopathologic
Figure 5. RBC parameters in AcB61, A/J, and C57BL/6J mice before (day 0) and after (days 3 and 5) infection with *S. typhimurium*. The values for each individual mouse are shown. The AcB61 mice show a constitutive anemia with reticulocytosis. During *Salmonella* infection, the anemia in AcB61 mice rapidly worsens, whereas the parental strains, A/J and C57BL/6J, are only beginning to develop a mild anemia (A-C). The erythropoietic response appears blunted by *Salmonella* infection in all three groups (E). $n = 8$ for each group and each time point, except for AcB61 on day 3 ($n = 6$), A/J on day 5 ($n = 12$), and C57BL/6J on day 5 ($n = 3$; most mice being dead early on day 5). *, significantly different ($P < 0.05$) from day 0. †, significantly different ($P < 0.05$) from day 3. ANOVA with Fisher’s protected least significant difference post-hoc test done in StatView 5.0.
examination of A/J and AcB61 mice 5 d after infection (unpublished data).

Salmonella-induced anemia

Having identified Pklr as the gene underlying Ity4, we became interested in understanding how a RBC defect confers susceptibility to *S. typhimurium*. We have previously shown (29) that mice on a C57BL/10 background develop a severe anemia during the course of *Salmonella* infection. Knowing that the AcB61 mice present a constitutive anemia, we hypothesized that the added anemia-inducing stimuli of the *Salmonella* infection would lead to the rapid development of an even more severe anemia in these mice, contributing to their early demise. To test this hypothesis, and also to shed light on the pathophysiology of *Salmonella*-induced anemia in mice, we studied the RBC parameters before and during *Salmonella* infection in AcB61, A/J, and C57BL/6J mice. As expected, the AcB61 mice present a constitutive anemia with reticulocytosis (Fig. 5). During *Salmonella* infection, the anemia rapidly worsens in AcB61 mice to reach critically low levels on day 5 (Fig. 5, A–C). By comparison, the RBC parameters showed only a slight decrease in the A/J and C57BL/6J parental strains. The reticulocyte response appeared blunted by *Salmonella* infection in all three groups (decreasing reticulocytes; Fig. 5 E), which probably contributes to the development of anemia during infection. The sustained percentage of reticulocytes (Fig. 5 D) in AcB61 mice despite the worsening anemia and decreasing absolute reticulocyte count may indicate increased resistance of younger Pklr mutant erythrocytes to phagocytosis by activated macrophages during *Salmonella* infection. These results suggest that *Salmonella*-induced anemia results from a slightly decreased erythropoiesis response in the face of increased clearance of aging RBCs. Additionally, these findings show that *Salmonella* infection rapidly worsens the anemia in AcB61 mice, and that the severity of the anemia could contribute to the early demise of these mice. In the face of severe sepsis, the low hematocrit seen in the AcB61 (the mean ± the SEM = 16.6 ± 1.3%) certainly interferes with the capacity of the body to maintain oxygen delivery to vital organs (41).

The development of anemia during infection, referred to as anemia of inflammation, is well described, and it may be explained by several mechanisms, including changes in iron homeostasis, decreased proliferation of erythroid progenitors, reduced erythropoietin production, and decreased RBC life span (42). To gain a better understanding of the mechanisms underlying the development of anemia in our mice, we first evaluated the erythropoiesis response in the BM of A/J, C57BL/6J, and AcB61 mice before and 5 d after *Salmonella* infection (unpublished data). No noticeable differences were detected between the different groups in regard to the myeloid/erythroid ratios before or during infection, suggesting that the compensatory response to the decreased RBC life span in AcB61 occurs mainly in the spleen and liver, as previously described (32) and as seen during histopathologic examination (unpublished data). Interestingly, on day 5, *Salmonella*-induced pathologies were noted in the three strains studied. The myeloid/erythroid ratio was increased with numerous

![Figure 6](image-url)
segmented granulocytes. Additionally, variable numbers of foci characterized by fibrin deposition, more or less degenerated neutrophils, and necrotic cells were found. These foci were sometimes angiocentric and/or associated with thrombosed vessels. In one mouse, bacteria were visible in the center of one of these foci. These findings illustrate the attempt of the mice to produce increased numbers of myeloid cells in response to acute sepsis and indicate that *Salmonella* is capable of reaching the BM.

**Iron homeostasis during *Salmonella* infection**

The crucial role of iron in the regulation of RBC synthesis led us to investigate its metabolism in our three strains of mice before and during infection with *S. typhimurium*. We first measured the level of iron within the liver of our mice. As shown in Fig. 6 A, the level of iron in the liver of AcB61 mice is increased by \( \sim 5 \) times compared with A/J or C57BL/6J, suggesting that the rapid turnover of RBC in these mice somehow leads to accumulation of iron in the liver. Intracellular iron staining of sections of the liver (Fig. 6 B) and spleen (not depicted) confirmed that there is increased iron store for AcB61 compared with C57BL/6J (not depicted) and A/J. Microscopic observations (not depicted) suggest iron accumulation in both Kupffer cells and hepatocytes with some signs of erythrophagocytosis in the Kupffer cells. In the time points studied, however, we could not detect an effect of acute *Salmonella* infection on the level of hepatic iron.

We further measured the levels of iron, transferrin, and ferritin in the serum of our mice, before and during *Salmonella* infection (Fig. 7). Serum iron tended to decrease 3 d after infection in all 3 groups (significant in A/J and C57BL/6J), followed by a significant rise on day 5, especially in AcB61 and C57BL/6J (Fig. 7 A). The circulating transferrin increased during infection in all three groups and it was usually higher in AcB61 (Fig. 7 B). Finally, the levels of serum ferritin increased during infection in AcB61 and C57BL/6J, and they were higher in AcB61 at all time points (Fig. 7 C), most likely reflecting the increased iron load in these mice.

Collectively, these results indicate that *Salmonella* infection impacts on the iron metabolism, as indicated by the changes in serum iron, serum transferrin, and serum ferritin. Additionally, these results suggest that hepatic iron overload

---

**Figure 7.** Iron parameters in AcB61, A/J, and C57BL/6J mice before (day 0) and after (days 3 and 5) infection with *S. typhimurium*. The values for each individual mouse are shown. (A) During *Salmonella* infection, serum iron tends to decrease on day 3 and to increase on day 5. (B) Serum transferrin is generally increased in AcB61 compared with the two parental strains, and it increases during *Salmonella* infection in all strains. (C) Serum ferritin is markedly increased in AcB61 compared with the two parental strains and it increases during infection in the most susceptible strains, AcB61 and C57BL/6J. \( n = 8 \) for each group and each time point, except for AcB61 at day 3 and 5 (\( n = 7 \)), C57BL/6J at day 3 (\( n = 7 \)), and C57BL/6J at day 5 (\( n = 2 \), most mice being dead early on day 5). *, significantly different \( P < 0.05 \) from day 0. †, significantly different \( P < 0.05 \) from day 3. ANOVA with Fisher’s protected least significant difference post-hoc test was performed in StatView 5.0.
in Pklr-deficient AcB61 mice, as indicated by increased liver iron and increased ferritin (compared with A/J and C57Bl/6J), may contribute to their increased susceptibility to *Salmonella* by providing increased iron access to the invading intracellular pathogen. We therefore hypothesize that the susceptibility of the AcB61 mice to acute *S. typhimurium* infection is caused by the rapidly worsening anemia and the increased iron stores, two direct consequences of the PK deficiency.

Figure 8. Survival, bacterial load, and RBC parameters in A/J mice injected with PHZ before *Salmonella* infection. Mice were injected intraperitoneally with PHZ or PBS 2 d before infection with *S. typhimurium*. (A) Cumulative survival in A/J mice injected with PHZ only (PHZ; n = 4), PHZ and *Salmonella* (PHZ+Salm; n = 14), or PBS and *Salmonella* (PBS+Salm; n = 10). (B–D) PHZ injection in A/J mice leads to the rapid development of anemia, followed by a regenerative response (n = 8 on day 0; n = 4 on day 3 and 5; n = 3 on day 7). (E) Higher *Salmonella* CFUs are found in the spleen and liver of PHZ-treated mice (n = 4) compared with PBS-treated mice (n = 5). (F–H) Mice injected with PHZ before *Salmonella* infection showed a severe anemia at day 3 after infection and appear unable to recover from the PHZ-induced anemia compared with mice that received PHZ only (B and C vs. F and G), despite a seemingly adequate reticulocyte response (D vs. H; n = 4 for all groups and time points; n = 8 for baseline).
Salmonella infection in mice rendered anemic through injection of phenylhydrazine (PHZ)

To investigate the impact of anemia on the mouse response to Salmonella infection, we induced acute hemolytic anemia in A/J mice, a strain closely related to AcB61, using PHZ. PHZ, which is a potent oxidizing agent that causes a transient but severe hemolytic anemia with reticulocytosis, was injected intraperitoneally 2 d before Salmonella infection. The sole injection of PHZ was well tolerated by the mice, with 100% survival (Fig. 8 A) and no clinically visible adverse effects. 2 d after PHZ injection, the mice were severely anemic and by day 5, they showed a vigorous reticulocytosis response with improving anemia (Fig. 8, B–D). Mice infected with Salmonella 2 d after PHZ injection showed a dramatic increase in mortality compared with the controls with all mice dying on days 3 or 4 (Fig. 8 A). The decreased survival correlated with increased bacterial load in the spleen and liver of the PHZ-treated mice (Fig. 8 E). The Salmonella infection appeared to compromise the capacity of the A/J mice to recover from the PHZ-induced anemia, despite a seemingly adequate reticulocyte response (Fig. 8, B–D vs. F–H), indicating again that Salmonella infection most likely increases the removal of mature RBCs from the circulation. These findings suggest that severe hemolytic anemia, as seen in AcB61 or PHZ-treated A/J mice, contributes to susceptibility to Salmonella infection, although the very rapid death of the A/J mice injected with both PHZ and Salmonella may be caused by additional effects of the PHZ and accompanying acute hemolytic crisis.

Salmonella infection in iron-overloaded mice

We were then interested in testing our hypothesis that the iron overload seen in the AcB61 mice is detrimental to the host in the face of Salmonella infection. Therefore, we injected A/J mice with iron dextran 3 times a week for 3 wk before infection with Salmonella. The sole injection of iron was well tolerated by the mice, which showed no adverse effect from the repeated intraperitoneal iron injections. Measurements of the liver iron after 3 wk of iron injections confirmed that the mice were, indeed, iron overloaded (Fig. 9 A). After Salmonella infection, the iron-overloaded mice showed much increased susceptibility to Salmonella infection in terms of survival (Fig. 9 B), and increased splenic (Fig. 9 C) and hepatic (Fig. 9 D) bacterial load compared with control mice injected with PBS. These results are in agreement with previous reports of increased susceptibility of mice to Salmonella after iron overload (43, 44) and confirm that the susceptibility of the AcB61 mice to Salmonella is, at least partially, caused by their increased iron load.

DISCUSSION

In this study, we investigated the candidacy of Pklr, which is the gene encoding for the liver and RBC-specific pyruvate kinase, as a candidate for Ity4, which is a S. typhimurium susceptibility loci previously identified by us in a (AcB61 × 129S6)F2 cross (30). Using a fine mapping approach, we were able to reduce the interval of Ity4 from a 25.3-Mb region to a region of 3.7 Mb just surrounding Pklr. Although this region still contains close to 100 genes, the phenotypic correlation between the 3 Pklr mutant RCSs and the CBA-Pk-1slc, the lack of complementation in F1 crosses derived from these strains, and the functional data discussed in the following paragraphs strongly support Pklr as the gene underlying Ity4.

The Pklr mutation investigated in this study was first identified in the AcB61 and AcB55 RCSs as a gene conferring resistance to P. chabaudi infection (31). PK is an essential enzyme for glycolysis in RBCs. Erythrocytes deficient in PK have decreased ATP, increased glycolytic intermediates such as 2,3-diphosphoglycerate, and a shortened life span (45). As a result, humans or animals with PK deficiency show a constitutive hemolytic anemia with reticulocytosis. The mutation found in some AcB/BcA RCSs arose during the breeding of the RCS and is not present in the parental strains (31). It is associated with an isoleucine to asparagine substitution at amino acid position 90 resulting in a loss of function of the PK. When infected with P. chabaudi, PK-deficient mice were more resistant than wild-type mice, as indicated by lower peak parasitemia and increased survival.

Although it may first appear unexpected for a RBC defect to confer both resistance to malaria and susceptibility to Salmonella infection, we induced acute hemolytic anemia in A/J mice, a strain closely related to AcB61, using PHZ. PHZ, which is a potent oxidizing agent that causes a transient but severe hemolytic anemia with reticulocytosis, was injected intraperitoneally 2 d before Salmonella infection. The sole injection of PHZ was well tolerated by the mice, with 100% survival (Fig. 8 A) and no clinically visible adverse effects. 2 d after PHZ injection, the mice were severely anemic and by day 5, they showed a vigorous reticulocytosis response with improving anemia (Fig. 8, B–D). Mice infected with Salmonella 2 d after PHZ injection showed a dramatic increase in mortality compared with the controls with all mice dying on days 3 or 4 (Fig. 8 A). The decreased survival correlated with increased bacterial load in the spleen and liver of the PHZ-treated mice (Fig. 8 E). The Salmonella infection appeared to compromise the capacity of the A/J mice to recover from the PHZ-induced anemia, despite a seemingly adequate reticulocyte response (Fig. 8, B–D vs. F–H), indicating again that Salmonella infection most likely increases the removal of mature RBCs from the circulation. These findings suggest that severe hemolytic anemia, as seen in AcB61 or PHZ-treated A/J mice, contributes to susceptibility to Salmonella infection, although the very rapid death of the A/J mice injected with both PHZ and Salmonella may be caused by additional effects of the PHZ and accompanying acute hemolytic crisis.

Salmonella infection in iron-overloaded mice

We were then interested in testing our hypothesis that the iron overload seen in the AcB61 mice is detrimental to the host in the face of Salmonella infection. Therefore, we injected A/J mice with iron dextran 3 times a week for 3 wk before infection with Salmonella. The sole injection of iron was well tolerated by the mice, which showed no adverse effect from the repeated intraperitoneal iron injections. Measurements of the liver iron after 3 wk of iron injections confirmed that the mice were, indeed, iron overloaded (Fig. 9 A). After Salmonella infection, the iron-overloaded mice showed much increased susceptibility to Salmonella infection in terms of survival (Fig. 9 B), and increased splenic (Fig. 9 C) and hepatic (Fig. 9 D) bacterial load compared with control mice injected with PBS. These results are in agreement with previous reports of increased susceptibility of mice to Salmonella after iron overload (43, 44) and confirm that the susceptibility of the AcB61 mice to Salmonella is, at least partially, caused by their increased iron load.

DISCUSSION

In this study, we investigated the candidacy of Pklr, which is the gene encoding for the liver and RBC-specific pyruvate kinase, as a candidate for Ity4, which is a S. typhimurium susceptibility loci previously identified by us in a (AcB61 × 129S6)F2 cross (30). Using a fine mapping approach, we were able to reduce the interval of Ity4 from a 25.3-Mb region to
Salmonella infection, this association is not unheard of. In fact, several hemoglobinopathies and RBC enzymatic defects are known to confer protection to malaria in human populations (46), while at the same time conferring susceptibility to various pathogens, including Salmonella (12, 47). A similar observation can be made in mice, where, for instance, β-thalassemia is not only associated with increased resistance to malaria (46), but also with susceptibility to Salmonella (48). The reasons underlying the increased susceptibility to infection in patients with RBC defects are not completely understood, but may be related to the severity of the anemia, phagocyte dysfunction, or iron overload (47). Our experiments indicate that these mechanisms are also implicated in the increased susceptibility of the AcB61 mice to acute S. typhimurium infection.

Adequate tissue oxygen delivery is essential for life. Whole-body oxygenation is determined by the arterial content in oxygen (which is dependant in part on hemoglobin concentration), the cardiac output, and the oxygen extraction ratio at the organ level (41). During severe sepsis, such as during acute systemic Salmonella infection in mice, the normal physiologic mechanisms allowing adequate delivery of oxygen are compromised as a direct consequence of the systemic inflammatory response (49). Although the AcB61 mice seem perfectly adapted to live with their anemia in a normal situation, it is conceivable that the rapid decrease of their hematocrit to critically low levels during Salmonella infection superimposed with the sepis-induced compromised cardiovascular function is detrimental to their survival. In this regard, we have shown that mice of a similar genetic background rendered anemic through PHZ injection are also more susceptible to Salmonella infection. However, a study published in 1967 showed that anemia induced by acute bleeding (hematocrit lowered to 30%, a level much higher than what is seen in AcB61 5 d after infection) did not increase the susceptibility of mice to Salmonella in contrast to hemolytic anemia induced by PHZ or anti-mouse erythrocyte antibodies (50), suggesting that the actual hemolytic nature of the anemia may be more important in increasing the susceptibility of mice to Salmonella than the anemia itself. The hemolytic anemias of our models are most likely mainly extravascular, and therefore are associated with phagocytosis of altered, but still intact, RBCs by the reticuloendothelial system (RES). Microscopic observations of liver sections after Perl’s staining in AcB61 mice suggest some evidences of erythrophagocytosis that likely contributed to PK-deficient RBC removal, development of anemia, and iron accumulation. This erythrophagocytosis may by itself lead to increased susceptibility to Salmonella because it has been shown that erythrophagocytosis, by itself, diminishes the capacity of macrophages to kill Salmonella in vitro (51, 52).

In addition to the adverse effect of the hemolytic anemia, the iron overload found in the AcB61 mice also contributes to their increased susceptibility to Salmonella. Iron is an essential nutrient to both the host and the pathogen and, during infection, they compete for this essential metal (53). The normal host is a very hostile environment for microorganisms, which require 10^{11} to 10^{12} higher concentration of free iron than what is usually available within the host. Because pathogenic microorganisms have evolved specialized mechanisms to acquire iron during infection, it is conceivable that any situation leading to iron overload may favor the multiplication of invading organisms. Indeed, iron overload in humans has been linked to increased susceptibility to some pathogens, including Mycobacterium tuberculosis (54), HIV (55), fungal organisms (56), and L. monocytogenes (57). We showed that A/J mice that were rendered iron overloaded through repeated administration of iron are markedly impaired in their resistance to Salmonella infection. These results are in agreement with previous studies, which have shown that iron overload increases (43, 44), whereas iron deficiency decreases, the susceptibility of mice to Salmonella infection (58). The impact of iron overload on host susceptibility to Salmonella infection may be explained by the role of iron as a growth factor for Salmonella and also by the role of iron in cell-mediated immunity. In fact, in vitro studies have shown that although Salmonella-infected macrophages usually increase their export of iron, resulting in decreased availability of iron for bacterial growth, iron supplementation will impair their production of TNFα and NO, resulting in increased bacterial proliferation (59). Additionally, acute iron depletion in mice through the administration of deferoxamine, which is an iron chelator capable of binding both intra- and extracellular iron, increased their susceptibility to Salmonella because of impaired NADPH-dependent respiratory burst (60). Finally, iron overload was shown to alter the development of TH1 response during Candida albicans infection in mice (61). Our results and the findings in the aforementioned studies indicate that a fine balance of iron is needed for optimal host defense against Salmonella and other pathogens.

During the course of our experiments, we have also investigated the mechanisms underlying the development of anemia in mice during Salmonella infection. We have previously found that mice infected with S. typhimurium develop anemia, which worsens throughout the course of infection (29). We show that the development of anemia in mice during Salmonella infection is associated with decreased erythropoiesis, as indicated by the decreasing reticulocyte count found in A/J, AcB61, and C57BL/6J. During infection, circulating proinflammatory cytokines are believed to contribute to a decreased erythropoiesis response by a direct inhibition of the proliferation of the progenitor cell, decreased erythropoietin secretion, and decreased sensitivity to the effect of erythropoietin (42). The decreased availability of iron, which is caused by sequestration in the RES, may also contribute to the decreased erythropoiesis (62). The relatively short half-life of RBC in normal mice (60 d in mice compared with 120 d in human) probably contributed to the rapid development of anemia in mice during infection. Finally, the increased phagocytosis of mature RBCs by the activated macrophages of the RES is certainly also involved in the development of anemia during Salmonella infection. This phenomenon was especially evident in AcB61 mice, which presented a sustained
percentage of reticulocytes throughout infection in the face of worsening anemia and decreasing absolute reticulocyte counts, and in PHZ-treated, Salmonella-infected mice, which showed a persisting anemia despite an adequate reticulocyte response. Collectively, these findings indicate that the pathogenesis of anemia during Salmonella infection includes both decreased erythropoiesis and increased RBC removal from the circulation.

In conclusion, we have shown that Pklr is the gene underlying Ity4. As seen with other RBC pathologies, the PK deficiency in mice is both protective against malaria and detrimental in regards to acute infection with S. typhimurium. The increased susceptibility to acute S. typhimurium infection in PK-deficient AcB61 mice is related to their severe hemolytic anemia during infection and their iron overload. Finally, mouse infection with S. typhimurium induces anemia through decreased proliferation of RBC progenitors and increased destruction of erythrocytes.

MATERIALS AND METHODS

Animals used. All animal experiments were performed under conditions specified by the Canadian Council on Animal Care, and the animal use protocol was approved by the McGill University Animal Care Committee. AcB61, AcB62, and AcB65 mice were generated from A/J and C57BL/6/j mice through reciprocal double backcrosses, followed by inbreeding for several generations (38). These mice were either purchased from Emerillon Therapeutics, or bred by us at the Montreal General Hospital Research Institute. A/J, C57BL/6J, CBA/J, and CBA/CaHN-Therapeutics, or bred by us at the Montreal General Hospital Research Institute in Montreal, Canada. AcB61, AcB62, and AcB65 mice were generated at The Jackson Laboratory, whereas the 129S6/SvEvTac (129S6) mice were purchased from Taconic. (AcB61 × 129S6)F2 (30), (AcB61 × AcB62)F1, and (AcB61 × AcB65)F1 mice were generated at the MGHRI animal facility, and the CBA-Pk-/- mice were originally obtained from H. Asai (Japan SLC Animal Facility, Shizuoka, Japan) (33) and are now maintained at McGill University (35).

In vivo Salmonella infection. The Salmonella infections were performed as previously described (29). In brief, S. typhimurium strain Keller was grown in trypticase soy broth, and each mouse was infected intravenously with ~10^6 CFUs diluted in 200 μl of 0.9% saline. The infectious dose was verified by plating of serial dilutions on trypticase soy agar. For survival analysis, the mice were monitored twice daily, and moribund animals were killed by CO2 asphyxiation. Bacterial loads in the spleen and liver were determined by serial dilutions of the lysates plated on trypticase soy agar. For complete blood counts, mice were killed by CO2 asphyxiation at various time points, and blood was collected by cardiac puncture and allowed to clot at room temperature for 2 h. Serum was harvested after centrifugation and kept at −80°C for future analysis. Liver sections were harvested, snap frozen in liquid nitrogen, and stored at −80°C for liver iron determination. Liver iron, serum iron, transferrin, and ferritin were measured at the Laboratory of Biochemistry at the Institut Fédératif de Recherche 02, CHU Bichat-Claude Bernard (Paris, France). Intracellular iron staining of sections of liver using Perl's solution was performed at the Plateau de Morphologie, Anatomie-Cytologie Pathologiques IFR02.

Genotyping. DNA was extracted by biopsy samples of mice tails using overnight digestion in lys buffer and proteinase K, followed by chloroform extraction. Genotyping for the Pklr mutation was performed using restriction enzyme digestion, as previously described (31). In brief, Pklr exon 2–specific primers surrounding the Pklr mutations were used to amplify genomic DNA. The PCR products were subjected to SfaN1 restriction enzyme digestion and resolved on ethidium bromide–stained 1.5% agarose gel. The Pklr mutation disrupts the SfaN1 restriction site. Fine mapping around Pklr was performed by sequencing of PCR-amplified DNA fragments surrounding known single-nucleotide polymorphisms. The sequencing was performed at McGill University and Genome Quebec Innovation Center (Montreal, Canada).

Genetic analysis. The genetic analysis for the survival phenotype in (AcB61 × 129S6)F2 mice was reported elsewhere (30). We repeated the one-locus interval mapping for the survival phenotype of the (AcB61 × 129S6)F2 mice in R/qtl (63) under a nonparametric model, this time including the direct genotyping data for the Pklr mutation.

Mycobacterium bovis (BCG) infection. M. bovis (BCG; strain Montreal) was passed in vitro and prepared for in vivo infections, as previously described (64). In brief, a single-cell suspension free of aggregates and containing 2 × 10^8 CFU in 0.2 ml of sterile PBS was used to inoculate mice by the intravenous route. At 3 wk after infection, the extent of M. bovis replication was determined by plating serial dilutions of spleen homogenates on Dubos solid agar and by counting the number of CFUs 21 d after incubation of plates at 37°C, as previously described (64).

In vitro infection of BMDMs and RAW 264.7. BMDMs were isolated from femurs of 10–20-wk-old male A/J and AcB61 mice. BMDMs were plated on polystyrene tissue culture petri dishes (Primaria; Becton Dickinson) at a density of 1.0 × 10^6/ml in growth media (RPMI 1640) containing 2 mM L-glutamine (Invitrogen), 10% FBS (HyClone), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen). Nonadherent cells were transferred after 24 h at 37°C and 5% carbon dioxide to fresh polystyrene petri (Thermo Fisher Scientific). Cultures were grown for 6 d with 15% (vol/vol) L-929 cell-conditioned medium as a source of M-CSF. The cell lines RAW 264.7 and RAW 264.7 expressing a functional Nramp1 protein were maintained in DME, as previously described (65). BMDMs and RAW 264.7 cells were harvested and plated on 24–well plates (Primaria) at a density of 1 × 10^5/ml and 1.5 × 10^5/ml, respectively, in RPMI 1640 media containing 2 mM L-glutamine and 10% FBS 24 h before infection. S. typhimurium (strain Keller) was added to the cells at multiplicities of infection of 10:1. After a period of 45 min, RPMI 1640 containing 100 μg/ml gentamycin was added for a period of 1 h. The medium was subsequently replaced by RPMI 1640 containing 10 μg/ml gentamycin (time point 0). Cell monolayers were lysed at different time points with 1% Triton X-100 diluted in PBS, and bacterial counts were established by serial dilutions of the lysates plated on trypticase soy agar.

Hematology, histology, and iron studies. For complete blood counts, mice were killed by CO2 asphyxiation at various time points, and blood was collected by cardiac puncture and immediately transferred to pediatric 200 μl EDTA tubes. Analyses were performed at the Faculté de Médecine Vétérinaire de l’Université de Montréal. Sections of the liver, the spleen, the femur, and the sternum were collected for histologic examination, which was also done at the Faculté de Médecine Vétérinaire. For iron studies, mice were killed by CO2 asphyxiation at various time points, and blood was collected by cardiac puncture and allowed to clot at room temperature for 2 h. Serum was harvested after centrifugation and kept at −80°C for future analysis. Liver sections were harvested, snap frozen in liquid nitrogen, and stored at −80°C for liver iron determination. Liver iron, serum iron, transferrin, and ferritin were measured at the Laboratory of Biochemistry at the Institut Fédératif de Recherche 02, CHU Bichat-Claude Bernard (Paris, France). Intracellular iron staining of sections of liver using Perl’s solution was performed at the Plateau de Morphologie, Anatomie-Cytologie Pathologiques IFR02.

PHZ-induced hemolytic anemia. A/J mice were intraarterially injected with ~150 mg/kg of PHZ hydrochloride diluted in 100 μl of PBS (66) 2 d before infection with Salmonella. Control mice received the same volume of PBS, intraperitoneally. The course of the hemolytic anemia and survival was followed in noninfected and Salmonella-infected mice. Liver and spleen CFUs were measured as previously described (29) in mice infected with Salmonella 3 d after the PHZ injection. The bacterial load was measured 2.5 d after infection.

Iron overload. Iron overload was induced in A/J mice by intraarterial injection of iron dextran (Sigma-Aldrich) 3 times a week for 3 wk (for a total of 8 injections) at a dosage of 1.2 mg per mouse diluted in 100 μl of PBS (61), before infection with Salmonella. Control mice were injected intraperitoneally with the same volume of PBS. After infection, survival time was recorded, and the splenic and hepatic bacterial loads were determined at 3 d after infection.
The authors wish to acknowledge the contribution of Rosalie Wilkinson, Line Larivière, and Line Laroche for technical assistance; Olivier Thibaudau and Nicolas Sorhaindo for their contribution to the iron studies; Mayes Naccache, Loïc Guilot, and Albert Descoteaux for their help with BMDM experiments; and Etienne Richer for assistance during the infection of the mice. The RCS mice and genotypes were provided by Emerillon Therapeutics and funded in part by Genome Quebec.

This work was supported by a grant from the Canadian Genetic Disease Network. M.F. Roy was the recipient of a Canadian Institutes of Health Research fellowship. D. Malo is a William Dawson Scholar.

The authors have no conflicting financial interests.

Submitted: 13 December 2006
Accepted: 16 October 2007

REFERENCES


2960

PYRUVATE KINASE DEFICIENCY AND SALMONELLA TYPHIMURIUM | Roy et al.


