Pyruvate kinase deficiency confers susceptibility to *Salmonella typhimurium* infection in mice

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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.

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Abbreviations used: BMDM, BM-derived macrophages; LOD, logarithm of odds; Nramp, Natural resistance-associated macrophage protein; PHZ, phenylhydrazine; PK, pyruvate kinase; RBC, red blood cell; RCS, recombinant congenic strain; RES, reticuloendothelial system; Tlr, Toll-like receptor.

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 host response to *Salmonella* infection is also controlled by genetic factors. In humans, patients with mutations leading to sickle cell anemia (12), chronic granulomatous disease (13), or the syndrome of Mendelian susceptibility to mycobacterial disease (14) are more susceptible to infection with non–host-specific *Salmonella*.

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
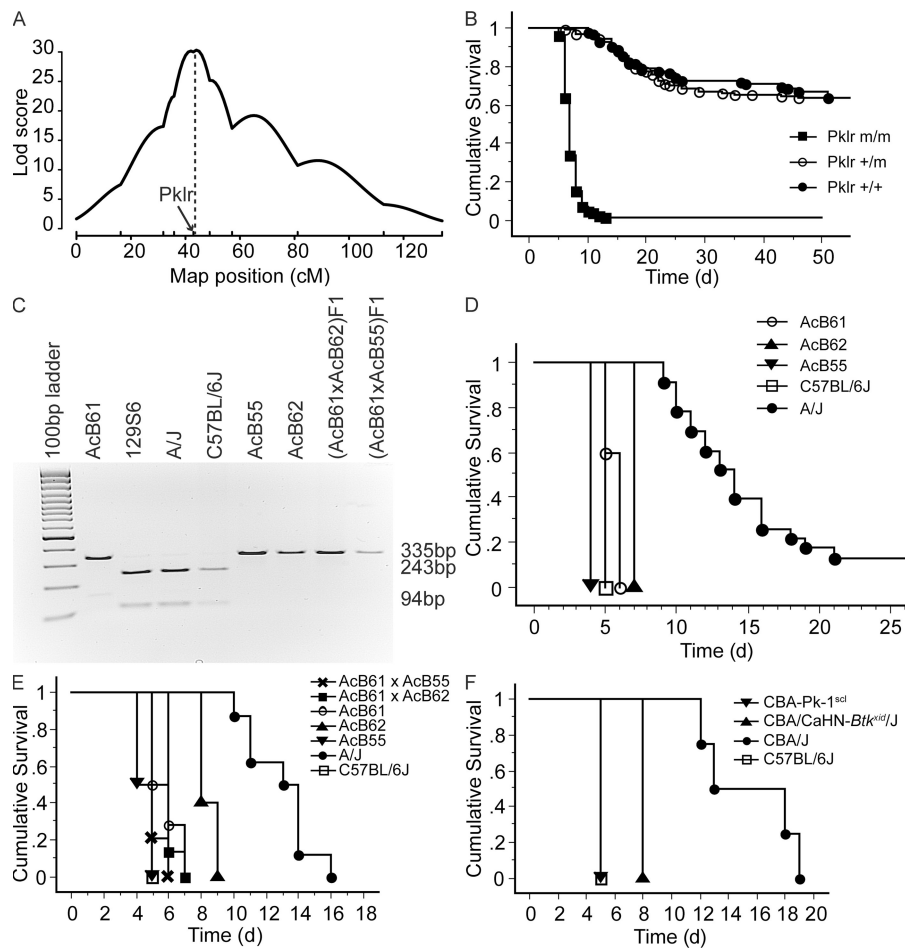

Figure 1. Candidacy of *Pklr* as the gene underlying *Ity4*. (A) Interval mapping under a nonparametric model showing the LOD score trace of chromosome 3 for the survival phenotype in 247 (AcB61 × 129S6)F2 mice. *Pklr* maps directly under the peak LOD score, as shown on the graph. The positions of the 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 A/J and C57BL/6J). (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-1slc, and controls. As is the case for the mutant RCSs, the CBA-Pk-1slc is more susceptible to *Salmonella* compared with wild-type controls (*n* = 5 for CBA-Pk-1slc; *n* = 4 for CBA/CaHN-*Btk*xid/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 nonfunctional *Tlr4* allele show a 1,000-fold reduction in LD_{50} (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 *Nramp1*, we hypothesized that additional genes would influence the outcome of infection and segregate in the RCSs. We showed that *Nramp1* 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 *Nramp1*. 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 chro-

mosome 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 rise of the peak 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 nonresolved 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 time 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 *Nramp1*-resistant RCSs have showed such susceptibility (30). The AcB55 strain was even more
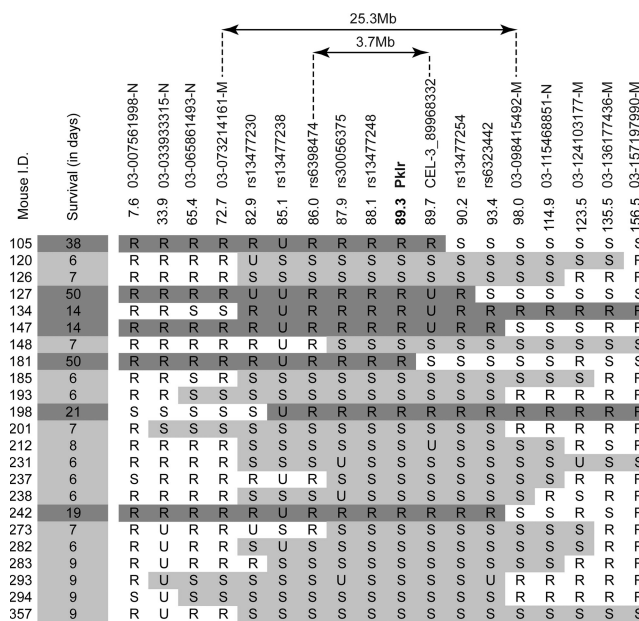

Figure 2. Fine mapping of the *Pklr* region. The *Ity4* support interval was reduced from 25.3 to 3.7 Mb by selected genotyping of the non-resolved recombinants in the *Pklr* region. "R" represents mice carrying at least one resistant allele, i.e., 129S6 homozygous or heterozygous mice. "S" represents the mice carrying susceptible alleles, i.e., AcB61 homozygous. "U" indicates an unknown genotype. The list and physical position (Mb) of the markers are shown at the top of the graph. Dark gray areas represent the mice, showing a resistant phenotype and their associated resistant haplotype around *Pklr*. Light gray areas represent the mice showing a susceptible phenotype and their associated susceptible haplotype around *Pklr*.
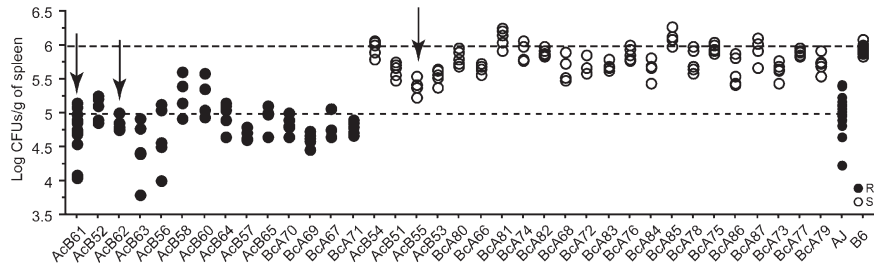
Figure 3. Splenic bacterial load after infection with BCG in RCSs. Mice were infected intravenously with BCG, and the splenic bacterial load was determined 21 d after infection. The splenic bacterial load is shown for each individual mouse tested in each RCS and for the parental strains. The effect of *Nramp1* is clearly visible, as all the strains carrying a resistant (R) allele at *Nramp1* have lower bacterial load compared with the strains carrying a susceptible (S) allele. The arrows point to the three RCSs known to carry the *Pklr* mutation. No adverse effect of the mutated *Pklr* allele is seen in this model.

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 *Nramp1* 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-1slc/Pk-1slc; hereafter CBA-Pk-1slc) (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-1slc 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-Btkxid/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 *Birc1e* 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 *Nramp1* 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 *Nramp1* (G169; Fig. 4), whereas replication of *S. typhimurium* is observed in Raw 264.7 macrophages carrying the mutant *Nramp1* allele (D169). Also, no obvious differences in the quality or quantity of cells recruited to the spleen and liver were observed on histopathologic
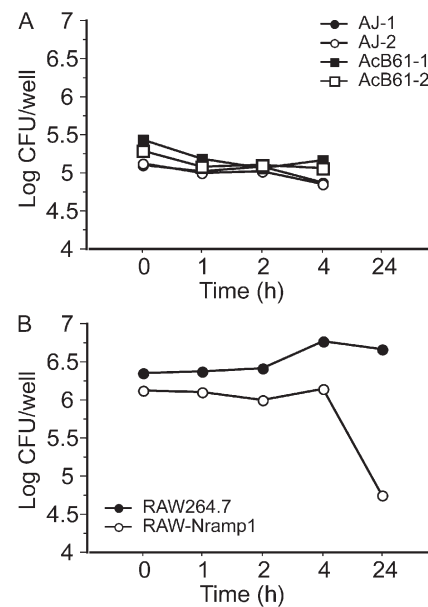

Figure 4. In vitro infection of BMDMs and RAW cells with *S. typhimurium.* The bacterial loads per well in A/J and AcB61 BMDMs (A) or RAW cells (B) after infection with *S. typhimurium* are shown. The *Pklr* mutation does not adversely affect the in vitro capacity of BMDMs to control the bacterial proliferation. On the contrary, RAW264.7 cells, which carry a functional mutation in *Nramp1*, cannot control the bacterial replication compared with RAW cells transfected with a functional *Nramp1* allele (RAW-Nramp1).
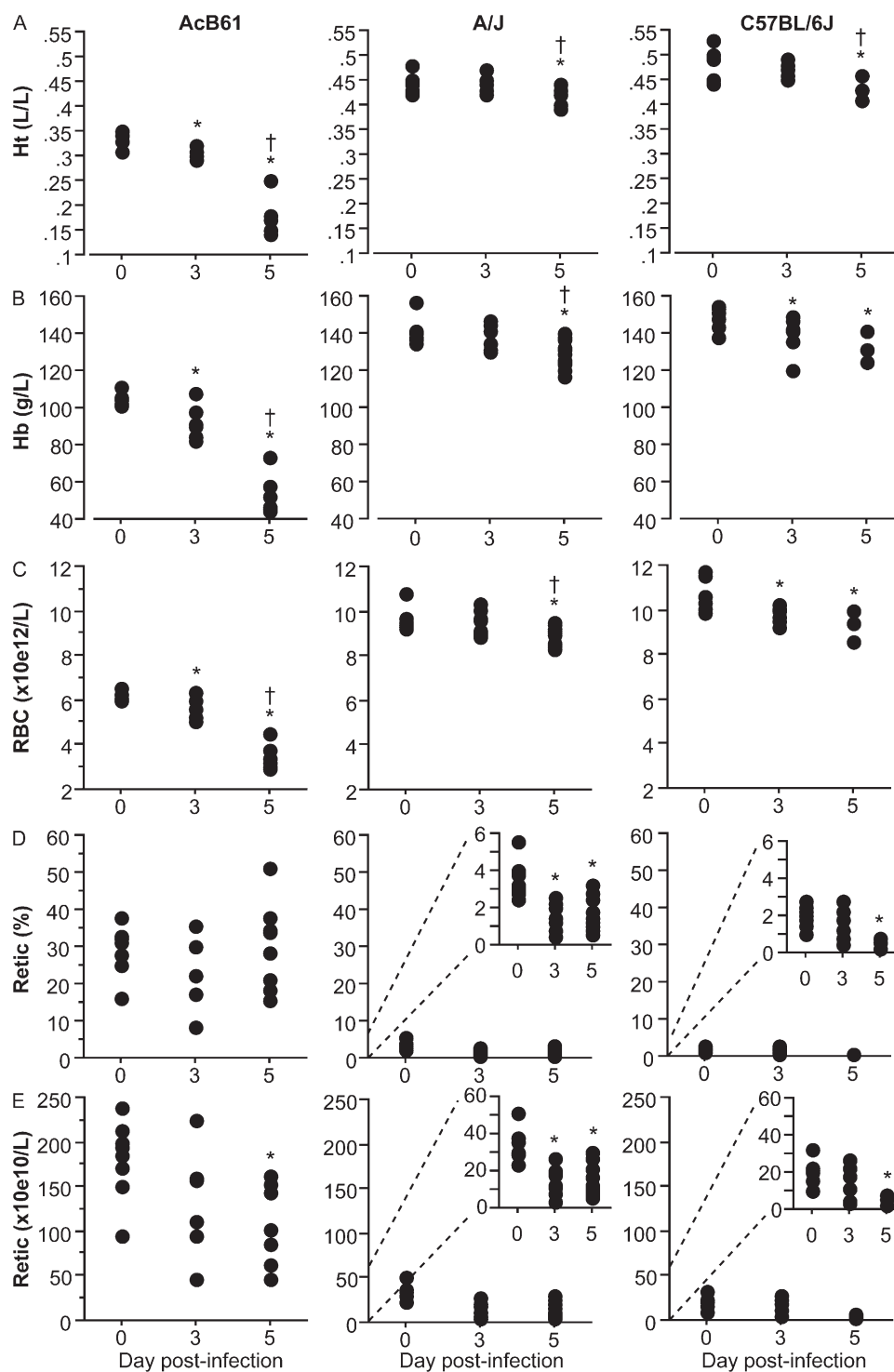
Figure 5.   RBC parameters in AcB61, A/J, and C57BL/6J mice before (day 0) and after (days 3 and 5) infection with *S. typhimurium*.
The values for each individual mouse are shown. The AcB61 mice show a constitutive anemia with reticulocytosis. During *Salmonella* infection, the anemia in AcB61 mice 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 *S. typhimurium* 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
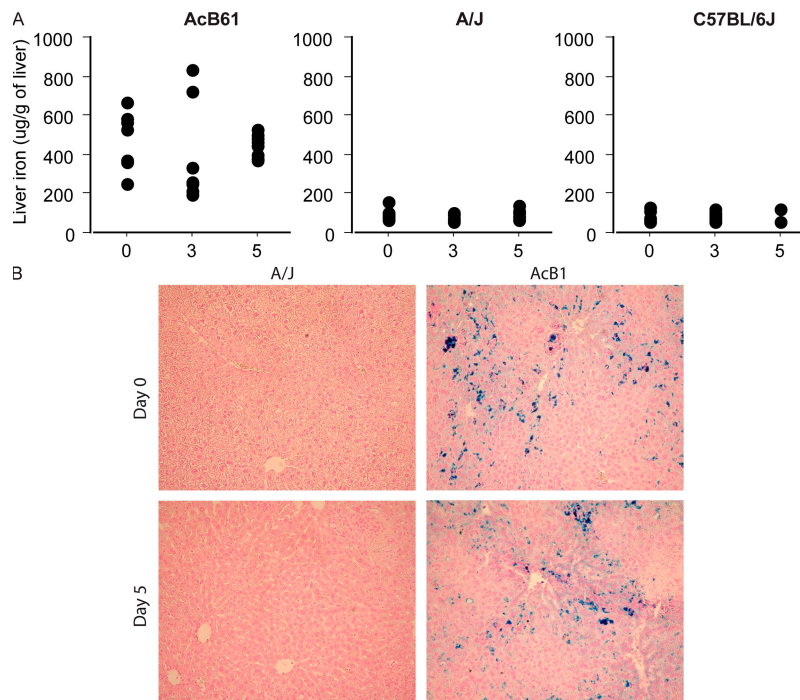

Figure 6. **Iron content in the liver of mice during *Salmonella* infection.** (A) Liver iron is increased in AcB61 mice compared with the two parental strains. (B) Perl's staining. Increased amounts of iron are detected in AcB61 compared with A/J in noninfected mice and in mice at 5 d after infection.

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 ∼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 seemed 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
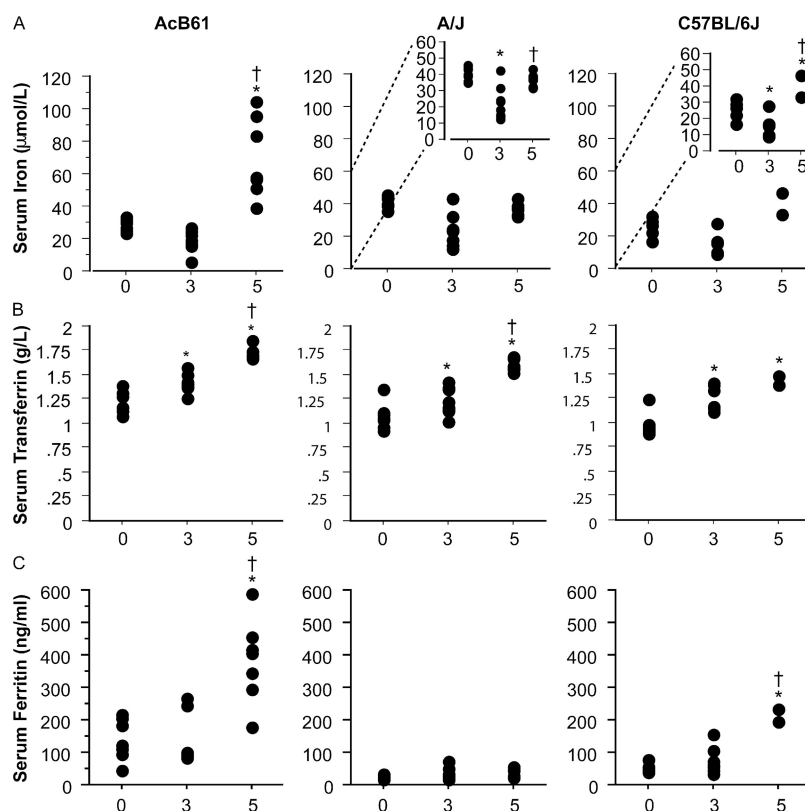

Figure 7.   Iron parameters in AcB61, A/J, and C57BL/6J mice before (day 0) and after (days 3 and 5) infection with *S. typhimurium*. The values for each individual mouse are shown. (A) During *Salmonella* infection, serum iron tends to decrease on day 3 and to increase on day 5. (B) Serum transferrin 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 AcB1 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.
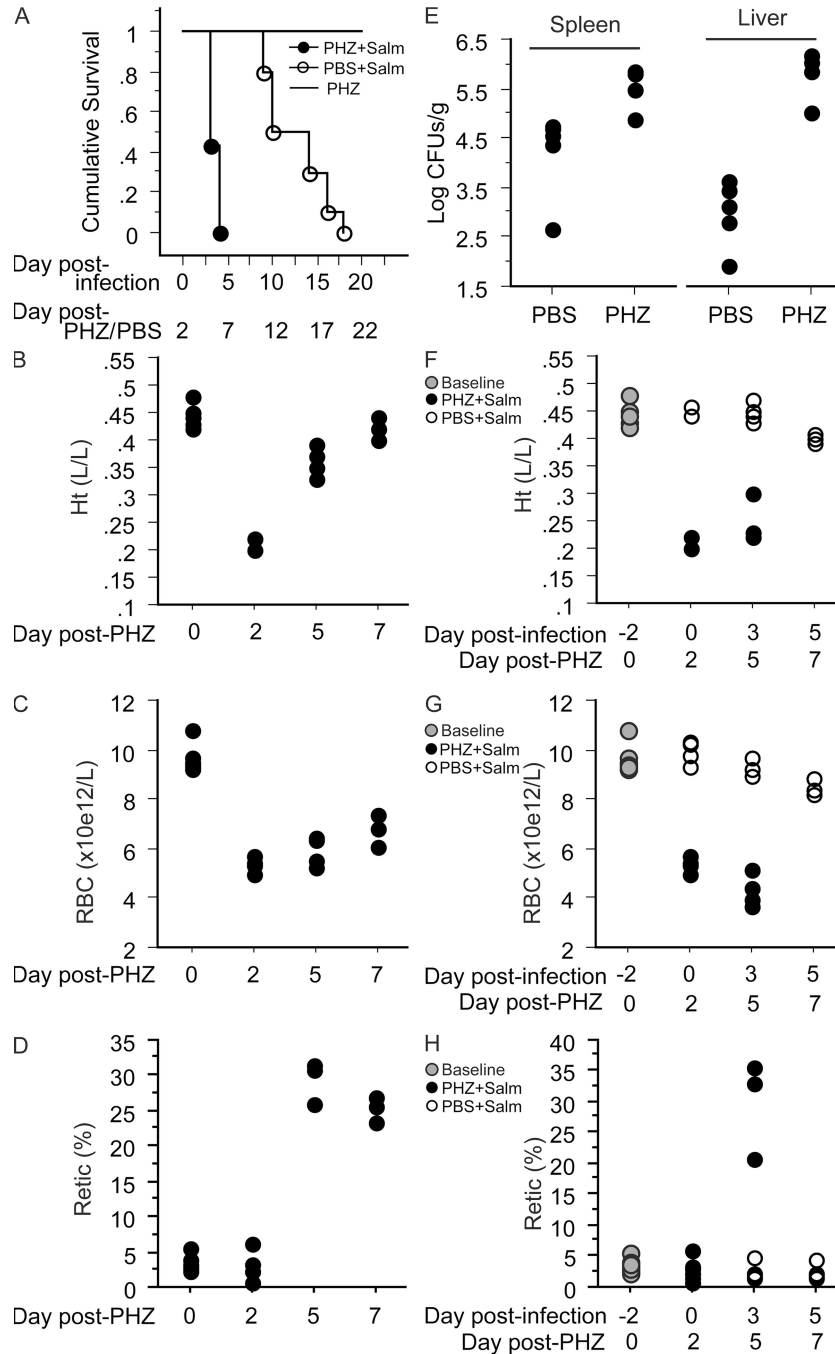

Figure 8. Survival, bacterial load, and RBC parameters in A/J mice injected with PHZ before *Salmonella* infection. Mice were injected intraperitoneally with PHZ or PBS 2 d before infection with *S. typhimurium*. (A) Cumulative survival in A/J mice injected with PHZ only (PHZ; *n* = 4), PHZ and *Salmonella* (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
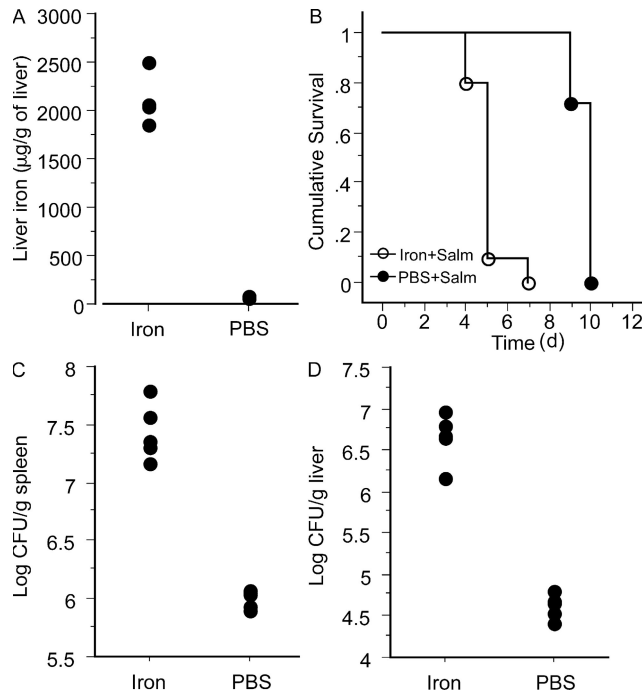

Figure 9. Survival after *S. typhimurium* infection in iron–overloaded mice. A/J mice were injected intraperitoneally with iron dextran or PBS 3 times a week for 3 wk before infection with *S. typhimurium*. (A) Repeated iron injections resulted in increased liver iron (Iron; $n = 4$) compared with control mice (PBS; $n = 2$). (B) Iron-overloaded mice (Iron+Salm; $n = 10$) showed decreased survival after *Salmonella* infection compared with control mice (PBS+Salm; $n = 7$). Iron-overloaded mice (Iron) also had an increased bacterial load in their spleen (C) and liver (D) at day 3 after infection compared with control mice (PBS; $n = 5$ for both groups).

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-1[slc], 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, 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 sepsis–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 AcB55 mice were generated from A/J and C57BL/6J 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 animal facility. A/J, C57BL/6J, CBA/J, and CBA/CaHN-*Btk^{xid}*/J mice were purchased from The Jackson Laboratory, whereas the 129S6/SvEvTac (129S6) mice were purchased from Taconic. (AcB61 × 129S6)F2 (30), (AcB61 × AcB62)F1, and (AcB61 × AcB55)F1 mice were generated at the MGHRI animal facility, and the CBA-Pk-1^{slc} 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 ∼10³ 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 CO_2 asphyxiation. Bacterial loads in the spleen and liver were determined by plating of serial dilutions of organ homogenates on trypticase soy agar. The survival data for the (AcB61 × 129S6)F2 mice were described elsewhere (30). All mice were aged between 2 and 6 mo at the time of infection.

Genotyping. DNA was extracted from biopsy samples of mice tails using overnight digestion in lysis buffer and proteinase K, followed by a 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 *Sfa*N1 restriction enzyme digestion and resolved on ethidium bromide–stained 1.5% agarose gel. The *Pklr* mutation disrupts the *Sfa*N1 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 2×10^4 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 petris (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 petris (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 1×10^6/ml and 1.5×10^6/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 CO_2 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 Médicine Vétérinaire of the 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 CO_2 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 intraperitoneally 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 intraperitoneal 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.

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