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Icsbp1/IRF-8 Is Required for Innate and Adaptive Immune Responses against Intracellular Pathogens

Karine Turcotte, * Susan Gauthier, * Danielle Malo, †*,†† Mifong Tam, † Mary M. Stevenson, † and Philippe Gros*†


The chronic myeloid leukemia syndrome of the BXH-2 mouse strain (Mus musculus) is caused by a recessive mutation (R294C) in the transcriptional regulator Icsbp1/IRF-8. In trans activation assays using an IL-12p40 gene reporter construct introduced in RAW 264.7 mouse macrophages, we show that the Icsbp1C294 isoform behaves as a partial loss-of-function. The Icsbp1C294 hypomorph allele appears to have a threshold effect on IL-12 production, with pleiotropic consequences on resistance to different types of infections in vivo. Despite the presence of a resistance Nramp1G169 allele, BXH-2 mice (Icsbp1C294) show impaired control of Mycobacterium bovis (bacille Calmette-Guérin) multiplication both early and late during infection, with uncontrolled replication linked to inability to form granulomas in infected liver and spleen. Studies in informative (BXH-2 × BALB/c)F2 mice show that homozygosity for Icsbp1C294 causes susceptibility to Salmonella enterica serovar Typhimurium to a level comparable to that seen for mice lacking functional Nramp1 or TLR4. Finally, impaired Icsbp1C294 function is associated with the following: 1) increased replication of the Plasmodium chabaudi AS malarial parasite during the first burst of blood parasitemia, and 2) recurring waves of high blood parasitemia late during infection. These results show that Icsbp1 is required for orchestrating early innate responses and also long-term immune protection against unrelated intracellular pathogens. The Journal of Immunology, 2007, 179: 2467–2476.

Nramp1 (Scl11a1) plays a critical role in macrophage defense against intracellular pathogens. In mice, mutations at Nramp1 cause susceptibility to infection with phylogenetically unrelated intracellular pathogens, including Mycobacterium bovis (bacille Calmette-Guérin (BCG)) and Salmonella enterica serovar Typhimurium (Salmonella Typhimurium) (1–4). In humans, polymorphic variants near or within the NRAMP1 gene are associated with differential susceptibility to tuberculosis and leprosy in areas of endemic diseases (5–7). Nramp1/Scl11a1 gene codes for a membrane phosphoglycoprotein formed of 12 transmembrane domains expressed in the lysosomal compartment of mononuclear phagocytes and in tertiary granules of neutrophils (8, 9). It functions as a pH-dependent divalent cation (e.g., Mn2+, Fe2+, and Zn2+) efflux pump at the phagosomal membrane (10), acting as a biological chelator for metals essential for intracellular replication of pathogens contained within phagosomes (10, 11).

There are two Nramp1 allelic variants in mice that are associated with resistance (dominant, Nramp1G169) or susceptibility (recessive, Nramp1D169) to infections. The G169D mutant variant is unstable and is rapidly degraded in macrophages, and behaves as a complete loss-of-function (12). In permissive animals (Nramp1D169), infected i.v. with M. bovis (BCG), there is rapid bacterial replication in the spleen that peaks at 3 wk, followed by a curative phase associated with immune response, granuloma formation, and bacterial clearance. By contrast, there is little, if any, bacterial replication in the spleen and liver of resistant mice (Nramp1G169) during the same period (13).

In inbred mouse strains, there is a strict correlation between Nramp1 alleles and susceptibility (D169) and resistance (G169) to M. bovis (BCG) infection, with the notable exception of the BXH-2 strain. BXH-2 is a recombinant inbred strain derived from C57BL/6J (Nramp1D169) and C3H/HeJ (Nramp1G169) inbred strains (14) that is susceptible to M. bovis (BCG) infection despite being homozygote for C3H-derived resistance Nramp1G169 allele (3). The susceptibility phenotype of BXH-2 is variable with spleen bacterial counts 3 wk postinfection being 5- to 100-fold superior to those seen in the C3H/HeJ parent (3, 15). In addition, BXH-2 mice develop by 1 year of age a progressive and fatal myeloid leukemia by a two-step mutagenesis process, as follows: the first step is an inherited recessive mutation that causes early myeloproliferation of a Mac1- /GR1+ granulocyte precursor detectable within a few weeks after birth; the second late stage oncogenic event is by insertional mutagenesis of a replication-competent B-tropic ecotropic murine leukemia virus, which is itself generated by recombination between two integrated copies of N-tropic ecotropic murine leukemia virus designated Emv1 and Emv2 (16–18).

We have previously shown that the first mutagenetic event leading to myeloproliferation is inherited as a single recessive gene mapping to chromosome 8, and designated Myls. Homozygosity for the BXH-2 allele at the Myls locus results in abnormalities in spleen, lymph nodes, and bone marrow in the form of a massive infiltration of immature Mac1- /GR1+ granulocytes (19).
**Materials and Methods**

**Animals**

Inbred mouse strains C57BL/6J (B6), C3H/HeJ (C3H), A/J, and BALB/cJ mice were purchased from The Jackson Laboratory. A breeding stock of several inbred strains, and segregating wild-type and mutant alleles, was obtained from N. Jenkins and N. Copeland (National Cancer Institute, Frederick, MD) was subsequently described (23). Six-week-old male and female mice were purchased from The Jackson Laboratory. A breeding stock of several inbred strains, and segregating wild-type and mutant alleles, was obtained from N. Jenkins and N. Copeland (National Cancer Institute, Frederick, MD) was subsequently described (23). Six-week-old male and female mice were purchased from The Jackson Laboratory.

**Infection with Salmonella Typhimurium**

BXH-2, BALB/cJ, A/J, C3H/HeJ, and (BALB/cJ × BXH-2)F1 mice were phenotyped for susceptibility to Salmonella Typhimurium infection, as we have previously described (25). One milliliter of a frozen culture of S. Typhimurium strain Keller (originally obtained from H. Robson, Royal Victoria Hospital, Montreal, Canada) was used to seed 100 ml of tryptose soy broth (OD600 nm of 0.1–0.2). Inocula were prepared by diluting the S. Typhimurium culture to concentrations of 0.5 × 10^5 to 1.0 × 10^6 CFU per 200 ml of PBS, and 0.2 ml of this suspension was used to infect mice by i.v. injection in the caudal tail vein. Survival of the animals was monitored several times per day, and moribund animals or animals showing obvious signs of distress were euthanized.

**Infection with P. chabaudi AS**

An lactate dehydrogenase virus-free isolate of P. chabaudi AS, originally obtained from D. Walliker (University of Edinburgh, Edinburgh, Scotland), was maintained by weekly passage in C57BL/6J mice by i.p. infection with 10^5 parasitized RBC (PRBC). For experimental infections, C57BL/6J, C3H/HeJ, and BXH-2 mice were infected i.p. with 10^5 PRBC suspended in 1 ml of pyrogen-free saline. Following infection (days 4–54), the percentage of PRBC was determined daily on duplicate thin blood smears stained with Diff-Quick (American Scientific Products), as described (26). Death was recorded daily, and moribund animals were sacrificed.

**Infection with M. bovis (BCG)**

M. bovis (BCG, strain Montreal) was passaged in vitro and prepared for in vivo infections, as we have previously described (2). Briefly, a single-cell suspension of 10^7 CFU in 0.2 ml of sterile PBS was used to inoculate mice by the i.v. route (caudal tail vein). At 1, 3, 5, and 8 wk postinfection, mice were euthanized and weighed, and the spleen and liver were removed aseptically. The extent of M. bovis (BCG) replication was determined by plating serial dilutions of spleen and liver homogenates on Dubos solid agar, and by counting the number of CFUs 21 days following incubation of plates at 37°C, as previously described (27).

**In vitro production of IL-12 by splenocytes**

C57BL/6J and BXH-2 mice were euthanized under anesthesia, and spleens were removed aseptically. Spleen cell suspensions were obtained by gently disrupting the tissue by passing through a 70-μm mesh in 3 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen Life Technologies), 10 mM HEPES, and 5 mM 2-ME. Nucleated spleen cells were then cultured following the lysis of erythrocytes with hemolytic lysis solution, as previously described (28). Splenocytes were seeded in tissue culture vessels at 5 × 10^6 cells/ml, following the incubation with given amounts of INF-γ and CpG (5′-TCCATGACGTTCCGTAGGT-3′) for 24 h at 37°C.
Tissue culture supernatants were collected and assayed for IL-12p40 production using a two-site sandwich ELISA method, as we have previously described (29).

RNA expression studies

Total cellular RNA was extracted from spleen and liver using a commercial reagent, and following the manufacturer recommended instructions (TRIZol; Invitrogen Life Technologies). Tissues were frozen on dry ice and homogenized by mechanical disruption using a Polytron (Brinkmann Instruments) in TRIZol reagent. The samples were incubated for 5 min at 20°C, followed by chloroform extraction. The aqueous phase was removed, and nucleic acids were precipitated with isopropanol. Pellets were washed with 75% ethanol and dissolved in RNase-free water treated with 0.1% diethylpyrocarbamate. For cDNA synthesis, total RNA (2 μg) was converted to cDNA in a 20 μl reaction containing 1 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies), dNTPs (500 μM each), and oligo(dT) primers (5 μM); First Strand Buffer; 10 mM DTT; and 1 μl of RNAGuard (Amersham), and the reaction was allowed to proceed for 50 min at 37°C. The reaction was inactivated by 15-min incubation at 70°C. Expression of individual genes was tested by standard PCR amplification of reverse-transcription products (RT-PCR) using total oligo(dT)-primed cDNA from spleen. The reaction mixture contained 0.5 μM each gene-specific oligo, 2 μl of cDNA (1/10 dilution), 10 μl of SYBR Green reaction mix (Invitrogen Life Technologies), and 1.25 mM MgCl2, and PCR was conducted in a LightCycler 2.0 System (Roche). PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide (0.1% final), and photographed under UV (15).

Histology

Spleen and livers were harvested from C57BL/6J, C3H/HeJ, and BXH-2 animals infected with M. bovis (BCG) and were fixed in 4% buffered formaldehyde, embedded in paraffin, and sectioned, as we have previously described (19). Sections (5 μM) were stained with H&E for evaluation of pathologic changes, including appearance of granuloma.

Cytokine detection

Determination of 32 cytokines, chemokines, and growth factors in serum from experimental mice was conducted using RayBio Mouse Cytokine Ab Array II membranes (RayBiotech). Incubations, washes, and exposure to films were conducted, according to instructions from the manufacturer. Briefly, the arrays were blocked at 4°C overnight before being incubated (20°C for 2 h) with 700 μl (BCG infection) or 900 μl (P. chabaudi infection) of pooled mouse serum. The membranes were washed and incubated with biotin-conjugated primary Ab and HRP-conjugated streptavidin, according to the instructions of the manufacturer.

Plasmid construction

A 425-bp portion of the IL-12p40 gene promoter region containing ISRE and other regulatory sequences (30) was PCR amplified from C57BL/6J genomic DNA using oligonucleotide primers 5′-CCGCTCGAGTTCTCTCTGTCGCTCTACAACCC3′ and 5′-GGAGATCTCTAGTGTTCTCTCGCTGGCTTT3′, followed by cloning into plasmid vector pGL3, which contains a luciferase reporter gene (Promega). A full-length cDNA corresponding to the mouse IRF-1 gene was modified by an in-frame addition of an antigenic cMyc epitope tag at the C terminus of the protein. Full-length mouse Icsbp1 cDNAs from C57BL/6J (wild type, Icsbp1p294) and from BXH-2/TyJ (Icsbp1p294M) were modified by the in-frame addition of the antigenic HA epitope tag at the C terminus of the protein. Modified IRF-1 and Icsbp1 cDNAs were cloned into expression plasmid pcDNA3, which uses CMV promoter/enhancer sequences to drive high-level expression of recombinant cDNAs. Large-scale preparations of expression and reporter plasmids were obtained by banding on CsCl density gradients and used for transient transfection assays.

Cell culture, transfections, luciferase assays, and Western blots

RAW 264.7 (RAW) cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Life Technologies). RAW cells seeded in a 24-well tissue culture dish (1 x 10⁵/well) were transfected using Lipofectamine PLUS (Invitrogen Life Technologies), with DNA mixtures consisting of 400 ng of luciferase pGL3 reporter construct with variable amounts of expression vectors (up to 100 ng), and according to an experimental protocol provided by the supplier of Lipofectamine reagent (Invitrogen Life Technologies). pcDNA3 empty vector DNA was used to equalize all transfection reactions to the same amount of DNA. Twenty-four hours following transfection, cells were assayed for luciferase activity using the dual luciferase assay reporter system (Promega). A full-length cDNA corresponding to the mouse IRF-1 gene was modified by an in-frame addition of an antigenic cMyc epitope tag at the C terminus of the protein. Full-length mouse Icsbp1 cDNAs from C57BL/6J (wild type, Icsbp1p294) and from BXH-2/TyJ (Icsbp1p294M) were modified by the in-frame addition of the antigenic HA epitope tag at the C terminus of the protein. Modified IRF-1 and Icsbp1 cDNAs were cloned into expression plasmid pcDNA3, which uses CMV promoter/enhancer sequences to drive high-level expression of recombinant cDNAs. Large-scale preparations of expression and reporter plasmids were obtained by banding on CsCl density gradients and used for transient transfection assays.

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FIGURE 1. Transcriptional activation of the murine IL-12p40 promoter by IRF-1 in association with Icsbp1p294 or Icsbp1p294M protein isoforms. A, Schematic representation of the Icsbp1 protein showing the DNA binding domain, the IAD, and the position of the R294C mutation from BXH-2. B, RAW cells were transfected with expression plasmids containing IRF-1-cMyc (100 ng), a firefly luciferase reporter construct containing the IL-12p40 promoter (400 ng), a Renilla luciferase internal control (100 ng), and two different DNA preparations of pcDNA3 expression vectors containing either the wild-type Icsbp1p294-HA or the BXH-2 mutant Icsbp1p294M-HA variant or the empty vector (100 ng). Luciferase activity was detected and quantified, as described in Materials and Methods. Each line represents the average of four transfected wells ± SD of the mean. C, RAW cells were transiently transfected with indicated amounts of pcDNA3 expression constructs containing either the wild-type Icsbp1p294-HA or the BXH-2 mutant Icsbp1p294M-HA cDNAs, along with the IRF-1-cMyc construct (100 ng), the IL-12p40 reporter, and the Renilla luciferase internal control (see above). Each line represents the average of four transfected wells ± SD on the mean. Expression of wild-type and mutant HA-tagged Icsbp1 protein isoforms in transfected RAW macrophages was monitored by Immunoblotting using a mouse anti-HA mAb. Actin expression was measured to ascertain similar protein loading on gel and similar transfer to blotting membrane.
system (Promega). Whole cell extracts were separated on 8% SDS-polyacrylamide gel and transferred by electroblotting on 0.45-μm polyvinylidene difluoride membranes (Millipore). Similar loading of cell lysate and equal protein transfer to membrane were verified by staining with Ponceau S red (Sigma-Aldrich). The blots were incubated with mouse anti-HA mAb (1:500; Covance Research Products) in TBST (10 mM Tris-HCl (pH 8), 150 mM NaCl, 0.05% Tween 20) plus 5% skim milk (16 h at 4°C), followed by washing and incubation with a goat anti-mouse secondary Ab conjugated to HRP (1:20,000; Jackson ImmunoResearch Laboratories). Polyvinylidene difluoride membranes were stripped and incubated with the polyclonal goat anti-actin (I-19) Ab (1:1,000; Santa Cruz Biotecnology) in TBST plus 5% skim milk (16 h at 4°C), followed by washing and incubation with a donkey anti-goat secondary Ab-conjugated HRP (1:7,500; Santa Cruz Biotecnology). Chemiluminescence was used for the detection of immune complexes on the immunoblot (SuperSignal West Pico; Santa Cruz Biotecnology). RT-PCR and gel electrophoresis. Expression of IL-12p40 transcripts was monitored by RT-PCR and gel electrophoresis. D. Expression of IL-12p40 in serum of M. bovis (BCG)-infected C57BL/6J, C3H/HeJ, and BXH-2/TyJ mice. Groups of BXH-2 (resistant Nramp1G169; mutant IcsbpR294) and parental controls C57BL/6J (B6; susceptible Nramp1D169, wild-type IcsbpH294), and C3H/HeJ (C3H; resistant Nramp1G169, wild-type IcsbpH294) mice (n = 4–6) were injected i.v. with 2 × 10^4 M. bovis (BCG)/mouse (arrow), and the number of CFUs (logCFU ± SEM) was determined in spleen (A) and liver (B) at 1, 3, 5, and 8 wk postinfection. C. RNA was isolated from infected spleens, and expression of IL-12p40, IL-12p35, and β-actin transcripts was monitored by RT-PCR and gel electrophoresis. E. Expression of IL-12p40 in serum of M. bovis (BCG)-infected B6, C3H, and BXH-2 mice. Splenocytes harvested from B6 (Icsbp1R294) and BXH-2 (Icsbp1C294) mice were stimulated in vitro with various amounts of IFN-γ and CpG, as indicated. Endogenous IL-12p40 production was measured by ELISA in culture supernatants 24 h after the induction. Each bar represents the average of two independent measurements.
plasmid or with 10 ng of Icsbp1R294 plasmid, and where the corresponding proteins were either readily detectable or undetectable, respectively (Fig. 1C). These results indicate that the R294C mutation constitutes a partial loss-of-function (hypomorph allele), affecting both transcriptional activity of Icsbp1 and protein expression, stability, or processing.

The activity of the Icsbp1R294 and Icsbp1C294 variants was evaluated in vivo by monitoring production of IL-12p40 protein by C57BL/6J (B6; susceptible,Nramp1D169; wild-type, Icsbp1R294), and C3H/HeJ (C3H; resistant, Nramp1G169; wild type, Icsbp1R294) mice were injected i.v. with 2 × 10⁶ M. bovis (BCG), and the number of CFUs (logCFU ± SEM) and the number of granulomas (number per field at ×200 magnification, 18 fields counted) were determined at 3 and 8 wk in spleen (A) and liver (B). Representative images of H&E-stained sections at ×10 (C–E; I–K) and ×400 original magnification (F–H; L–N). Granulomas are identified by arrows.

FIGURE 4. Effect of Icsbp1R294C mutation on granuloma formation in M. bovis (BCG)-infected mice. Groups of BXH-2 (resistant Nramp1G169; mutant Icsbp1C294) and parental controls C57BL/6J (B6; susceptible, Nramp1D169; wild-type, Icsbp1R294), and C3H/HeJ (C3H; resistant, Nramp1G169; wild type, Icsbp1R294) mice were injected i.v. with 2 × 10⁶ M. bovis (BCG), and the number of CFUs (logCFU ± SEM) and the number of granulomas (number per field at ×200 magnification, 18 fields counted) were determined at 3 and 8 wk in spleen (A) and liver (B). Representative images of H&E-stained sections at ×10 (C–E; I–K) and ×400 original magnification (F–H; L–N). Granulomas are identified by arrows.
mutant *Icsbp1<sup>C294</sup>* allele increased spleen bacterial load 3 wk after infection (15). The *Icsbp1<sup>C294</sup>* allele effect was most visible in otherwise resistant F<sub>2</sub> mice homozygote or heterozygote for the dominant protective *Nramp1<sup>G169</sup>* allele. To further explore the effect of *Icsbp1<sup>C294</sup>* on host defenses against *M. bovis* (BCG) infection, groups of C57BL/6J (permissive *Nramp1<sup>D169</sup>*, wild-type *Icsbp1<sup>W294</sup>*, C3H/HeJ (nonpermissive *Nramp1<sup>169</sup>*), wild-type *Icsbp1<sup>B294</sup>*), and BXH-2 (nonpermissive *Nramp1<sup>G169</sup>*), mutant *Icsbp1<sup>B294</sup>* were infected i.v. with 2 × 10<sup>4</sup> *M. bovis* (BCG), and the extent of microbial replication was determined in the spleen and the liver at weeks 1, 3, 5, and 8 postinfection (Fig. 3). In resistant C3H controls, little, if any, bacterial replication was observed in the spleen over the course of the experiment (Fig. 3A). In contrast, there was active microbial replication in the spleen of C57BL/6J mice that peaked at 3 wk postinfection, followed by clearance of the bacterial load up to 8 wk; the differential growth of *M. bovis* (BCG) in the spleen of B6 and C3H mice is determined by *Nramp1* alleles (Fig. 3A). By contrast, BXH-2 mice were permissive to *M. bovis* (BCG), despite a resistance *Nramp1<sup>G169</sup>* allele, and were unable to control splenic bacterial replication, which continued for up to 8 wk postinfection (Fig. 3A). Likewise, although both C3H and B6 were able to clear the infection from liver over 8 wk, BXH-2 were unable to do so and displayed persistent infection in this organ (Fig. 3B). In addition and as expected from previous results (Figs. 1 and 2), C3H and B6 mice showed robust IL-12p40 production that was detectable in the serum at 3 wk postinfection and that persisted to 8 wk in B6 (Fig. 3D); however, there was no such IL-12p40 production in BXH-2 mice at any time postinfection (Fig. 3, C and D). These results indicate that the *Icsbp1*<sup>B294</sup> mutation impairs innate (early) and acquired (late) immune response to mycobacterial infection in BXH-2.

The physiological basis for impaired response to *M. bovis* (BCG) infection was investigated. A key protective mechanism in host response to infection with mycobacteria is the formation of granulomas in infected tissues. These structures typically formed by the clustering of large phagocytic cells positive for acid fast bacilli surrounded by lymphocytes at the periphery limit the spread of mycobacterial infection (31). The effect of *Icsbp1*<sup>B294</sup> mutation on the type and number of granulomas formed in response to *M. bovis* (BCG) infection was investigated in spleen (Fig. 4A) and liver (Fig. 4B) of C57BL/6J, C3H/HeJ, and BXH-2 at 3 and 8 wk postinfection. In C57BL/6J and C3H mice controls, there was a good correlation between the extent of bacterial replication and the number of granulomas formed. In B6 mice, transient bacterial replication associated with the susceptibility *Nramp1<sup>D169</sup>* allele was concomitant to robust granulomatous response in both spleen and liver that peaked at 3 wk and persisted at 8 wk. In resistant C3H (*Nramp1<sup>G169</sup>*) mice, bacterial loads were paralleled by low numbers (spleen) or absence of detectable granulomas (liver) in infected organs. By contrast, in BXH-2 (*Icsbp1<sup>C294</sup>*), there was only a very modest granulomatous response in spleen and a complete absence of such response in liver at both 3 and 8 wk postinfection, despite continuing *M. bovis* (BCG) replication in these organs. Histological examination of granulomas formed in the three mouse strains revealed additional differences. Granulomas formed in BXH-2 tended to be of smaller size and less well organized than granulomas formed in C57BL/6J and C3H/HeJ controls, this difference being most obvious at the early 3-wk time point (Fig. 4, F–H).

These results show that the *Icsbp1<sup>C294</sup>* mutation overrides the protective advantage that resistance alleles at *Nramp1* provide against *M. bovis* (BCG) infection. They also indicate that failure to control mycobacterial replication in both the early and late phases of infection in BXH-2 is associated with inability to mount adequate granulomatous response in infected organs.

Effect of *Icsbp1<sup>B294</sup>* on host response to infection with *Salmonella Typhimurium*

To further explore the relationship between *Icsbp1* and *Nramp1* in host resistance to intracellular pathogens, we tested the effect of the *Icsbp1<sup>B294</sup>* mutation on the outcome of infection with *Salmonella Typhimurium*. In inbred mouse strains, two major loci, *Nramp1* and *Tlr4*, are known to control *Salmonella* Typhimurium populations in the host, including extent of survival following acute infection (32). Mouse strains such as C57BL/6J and BALB/cJ harbor a *Nramp1<sup>D169</sup>* susceptibility allele (recessive) and rapidly succumb to *Salmonella* Typhimurium infection, whereas strains such as A/J, DBA/2, 129S6/SvEvTac, and CBA/J that have a resistance *Nramp1<sup>G169</sup>* allele (dominant) that restricts bacterial replication in liver and spleen can survive longer to the infection. In addition, C3H/HeJ mice carry a recessive mutation (*P712H*) in the *Tlr4* gene that renders them susceptible to *Salmonella* Typhimurium infection, whereas strains such as A/J, BALB/cJ (*Tlr4<sup>P712</sup>*), and C57BL/6J (*Tlr4<sup>H712</sup>*) harbor a *Nramp1<sup>D169</sup>* susceptibility allele (dominant) that restricts bacterial replication in liver and spleen to survive longer to the infection. The number of mice bearing each haplotype is indicated in parentheses (n).

FIGURE 5. Effect of *Icsbp1<sup>B294</sup>* mutation on survival of *Salmonella Typhimurium*-infected mice. A, Groups of BXH-2 (mutant *Icsbp1<sup>B294</sup>*; resistant *Nramp1<sup>G169</sup>*; mutant *Tlr4<sup>H712</sup>*), C57BL/6J (wild-type *Icsbp1<sup>W294</sup>*; susceptible *Nramp1<sup>D169</sup>*; wild-type *Tlr4<sup>P712</sup>*), and C3H/HeJ (wild-type *Icsbp1<sup>W294</sup>*; resistant *Nramp1<sup>D169</sup>*; mutant *Tlr4<sup>H712</sup>*), BALB/cJ (wild-type *Icsbp1<sup>W294</sup>*; susceptible *Nramp1<sup>D169</sup>*; wild-type *Tlr4<sup>P712</sup>*), and A/J (wild-type *Icsbp1<sup>W294</sup>*; resistant *Nramp1<sup>D169</sup>*; resistant *Tlr4<sup>P712</sup>*). Control mice were infected with 1 × 10<sup>8</sup> *Salmonella* Typhimurium, and survival was monitored during 28 days. B, (BALB/c × BXH-2)F<sub>2</sub> mice bearing at least one wild-type (dominant) resistance allele for both *Tlr4* and *Nramp1* loci were similarly infected, and survival is shown for subgroups of *F<sub>2</sub>* according to their haplotype combination for wild-type (*Icsbp1<sup>B294</sup>* or mutant (*Icsbp1<sup>C294</sup>* alleles. The number of mice bearing each haplotype is indicated in parentheses (n).
segregate was produced between BXH-2 (mutant Icsbp\textsuperscript{C294}, wild-type Nramp1\textsuperscript{G169}, mutant Tlr4\textsuperscript{H712}) and BALB/c (wild-type Icsbp\textsuperscript{R294}, mutant Nramp1\textsuperscript{D169}, wild-type Tlr4\textsuperscript{P712}). (BALB/c\text/H11003 BXH-2)\textsubscript{F2} mice were genotyped for Nramp1, Tlr4, and Icsbp1 (see Materials and Methods), and animals bearing at least one copy of wild-type dominant alleles for both Nramp1 and Tlr4 loci were selected and infected with 1\text/H11003 10\textsuperscript{3} live Salmonella. The effect of wild-type (Icsbp\textsuperscript{R294}) or mutant (Icsbp\textsuperscript{C294}) alleles on survival to infection was evaluated (Fig. 5B). Homozygosity for mutant Icsbp\textsuperscript{C294} allele was associated with increased mortality in F\textsubscript{2} mice, with a mean survival time (7.0 \pm 1.2 days) similar to that of C3H/HeJ (7.0 \pm 0 days), C57BL/6J (5 \pm 0 days), and BALB/cJ (6 \pm 0 days) susceptible controls (Fig. 5A). F\textsubscript{2} mice heterozygous (Icsbp\textsuperscript{R294C294}) or homozygous (Icsbp\textsuperscript{R294R294}) for wild-type Icsbp\textsuperscript{R294} showed significantly prolonged survival time compared with Icsbp\textsuperscript{C294} mutant littermates (log rank test; \(p\) value 0.0002).

These results show that the Icsbp\textsuperscript{C294} mutation can overcome the protective advantage that resistance alleles at Nramp1 and Tlr4 provide against acute infection with Salmonella Typhimurium.

**FIGURE 6.** Effect of Icsbp\textsuperscript{R294C} mutation on the course of infection with *P. chabaudi* AS. A, Groups of C57BL/6J (B6), C3H/HeJ (C3H), and BXH-2 female mice were injected i.p. with 1 \times 10\textsuperscript{6} *P. chabaudi* AS PRBC, and blood-stage replication of the parasite (% infected RBCs) was monitored between days 4 and 54 postinfection (Materials and Methods). Each point represents the mean of the number of PRBC per group of infected mice (\(n = 8\) \pm SEM). B, Course of blood stage parasitemia in eight individual female BXH-2 mice (each line represents a single mouse) showing recurrent waves of parasite replication. C, The expression of several cytokines and chemokines in serum of *P. chabaudi* AS-infected mice (pooled serum from three mice) was determined using RayBio mouse array II membranes (Materials and Methods). IL-12p40, p40 subunit of the cytokines IL-12 and IL-23; STNF RI, soluble receptor of TNF-\(\alpha\); TIMP-1, tissue inhibitor of metalloproteinases-1; TARC, thymus and activation-regulated chemokine.
chabaudi, with peak parasitemia reaching ~50% at day 7 following i.p. infection with 1 × 10⁶ PRBC (Fig. 6A). In contrast, resistant C57BL/6J mice sustain comparatively lower levels of blood parasitemia (~25% PRBC), which peaks later in infection (day 8–9). C3H/HeJ and C57BL/6J clear the infection by day 19, and do not sustain sequelae from the primary infection (Fig. 6A). In this model, BXH-2 mice displayed an intermediate level of parasitemia at the peak of infection (~40% PRBC), which was observed at day 8. Interestingly, following a recovery period, blood parasitemia rose again in BXH-2 mice in what appeared to be successive waves of parasitemia. Although detectable in averaged data from groups of BXH-2 (Fig. 6A), this unique recrudescence is most easily observed by examining parasitemia values from individual BXH-2 mice (Fig. 6B). Although several BXH-2 animals succumbed at or soon after the peak parasitemia, others completely recovered only to see parasitemia rise again to high levels (~50–60%). Up to three such asynchronous waves of recurring parasitemia could be seen during the 40-day observation period following recovery from the initial peak of infection (Fig. 6B).

Recurrence of parasite replication in BXH-2 mice following recovery from initial bursts of parasitemia suggests that the Icsbp1C294 mutation impairs expression of long-term protective mechanisms against the malarial parasite.

To further probe the molecular basis for the loss of immunity against P. chabaudi AS in BXH-2, we compared cytokine/chemokine responses in sera of the three mouse strains in the early stages (days 2 and 5) of infection using commercially available cytokine Ab arrays (see Materials and Methods) (Fig. 6C). BXH-2 mice showed an overall depressed cytokine response early in the infection compared with resistant C57BL/6J and permissive C3H/HeJ controls. This was most obvious for IL-12p40, the soluble receptor of TNF-α, and to a lesser degree IFN-γ, thymus and activation-regulated chemokine, tissue inhibitor of metalloproteinases-1, and MCP-1. These observations suggest a pleiotropic effect of the Icsbp1C294 mutation on the inflammatory cytokine response during malaria infection.

Discussion

We have previously shown that BXH-2 mice carry a mutation in the Icsbp1/IRF-8 gene (15). Homozygosity for this mutant allele has two major effects in vivo. It causes an expansion of the granulocytic compartment, which provides a replicative niche for a B-tropic ecotropic leukemia virus that generates clonal leukemic cells by insertional mutagenesis. It also appears to impair natural defenses against M. bovis (BCG) infection (15).

The Icsbp1R294C mutation found in BXH-2 maps in the IAD of the protein, a protein domain supporting heterodimerization of Icsbp1 with other IRF family members for activation or repression of target genes. R294 maps in a region of the IAD that has been studied in other IRF family members, including the close homologue IRF-4 (also known as Pip) (37). Multiple sequence alignments, secondary structure predictions, and mutagenesis experiments have been used to identify functionally important residues of the IAD of IRF-4. In IRF-4, α-helices at positions 300–314 and 325–335 bear a net positive charge that appears important for surface contacts between IRF-4 and its interacting partner PU.1. R294 in Icsbp1 corresponds to residue R333 in IRF-4, and thus maps within this helical domain. R333 has not been mutated in IRF-4, but conserved R328 within the same α-helix has been studied. The conservative mutation R328K was shown to decrease ternary complex formation with PU.1 and DNA binding by ~50%, whereas opposite nonconservative R328E and neutral R328Q substitutions completely disrupted ternary complex formation (38). An induced mutation at position 289 (R289E) in Icsbp1, corresponding to R328 in IRF-4, was previously shown to disrupt the interaction of Icsbp1 with IRF-2 and PU.1 (39). Together these results suggest that R294C affects a protein domain critical for interaction with other IRF family members, and a possible molecular basis for the reduced activity detected in this study for Icsbp1C294 in trans activation assay. However, we cannot formally exclude the possibility that it is not the loss of arginine at position 294, but rather the gain of a cysteine at this position that may underlie altered function of the Icsbp1C294 variant. The sulfhydryl side chain of Cys294 may participate in novel intra- or intermolecular disulfide bridges that may alter either the structure of Icsbp1 or the stability of heterodimers formed with other transcriptional activators. Although such a mechanism is not compatible with the recessive behavior of the Icsbp1R294C allele, the characterization of additional mutations at R294 will be required to clarify this point.

Results in Fig. 1 show that the Icsbp1C294 is a functionally hypomorphic allele with a pleiotropic effect on the Icsbp1 protein, which includes reduced protein stability/expressivity and reduced trans activation potential toward the IL-12p40 gene promoter. In contrast, when the ability of Icsbp1C294-expressing BXH-2 splenocytes to produce the IL-12p40 protein in response to IFN-γ and CpG was tested ex vivo, the Icsbp1C294 variant behaved as a complete loss-of-function (Fig. 2). Several possibilities can be put forward to reconcile these two observations. The first is that the transcriptional activity of Icsbp1C294 measured in our assay may be overestimated, possibly due to the large amount of protein produced in transfected cells. Second, the mutation may have different effects on heterodimerization with multiple IRF proteins, and thus, different overall effects on transcriptional regulation of individual gene targets. Third, the hypomorphic nature of Icsbp1C294 may have a threshold effect on myelopoiesis and IL-12 protein production. This situation would result in severe reduction of early production of type I cytokines in response to inflammatory or infectious stimuli. We favor the latter explanation because the phenotypic expression of the mutant Icsbp1C294 allele of BXH-2 in vivo, with respect to both myeloproliferation (19) and susceptibility to infection with M. bovis (BCG) (Fig. 3) or Toxoplasma gondii (40), is inherited as a fully recessive trait.

Previous studies in mice bearing a null mutation at Icsbp1 (Icsbp1−/−) have highlighted the role of Icsbp1 in host defenses against certain bacterial (41, 42, 43), parasitic (44, 45), and viral infections (45, 46). In the present study, we used BXH-2 in additional, well-studied models of infection to explore the role of Icsbp1 in innate and acquired immune defenses. In the case of M. bovis (BCG), innate defenses play a critical role in controlling growth of M. bovis (BCG) in spleen and liver during infection, and an intact Nramp1 protein is essential for macrophages to restrict intracellular replication during this period (47). In contrast, clearance of infection in Nramp1-defective permissive animals requires granuloma formation and robust T cell-mediated immune response in infected organs (13). In this study, we observe that Icsbp1 plays an important role in both phases. Early during infection (0–21 days), altered Icsbp1 function (Icsbp1C294) in BXH-2 overcomes the genetic advantage of a wild-type Nramp1G169 allele in these mice. Although BXH-2 mice show CFU counts at 3 wk lower than those seen in Nramp1-defective (Nramp1G169) C57BL/6J controls, they clearly support more replication than C3H controls that bear wild-type alleles at Nramp1 and Icsbp1 (Fig. 3). These results highlight the role of both genes in early response to mycobacterial infections. The effect of Icsbp1 mutation on Nramp1 could be explained in two mutually nonexclusive manners. First, Nramp1 transcription in phagocytes has been shown to be mediated by Icsbp1 in association with Miz-1 and PU.1-interacting partners, and Icsbp1−/− null mice express low levels of Nramp1 mRNA (48). However, Nramp1 expression was found similar in an in vitro experimental design using IFN-γ- and CpG-induced peritoneal and splenic macrophage-enriched cell populations.
from C57BL/6J, C3H/HeJ, and BXH-2 strains, suggesting that the Icsbp1C294 mutation in BXH-2 does not seem to affect Nramp1 expression (data not shown). Second, reduced activity of Icsbp1C294 causes reduction of the type 1 cytokine IL-12 production, which is known to be critical to control mycobacterial infection (49). During the second, curative phase of the infection, BXH-2 mice apparently failed to mount an effective T cell-mediated immune response, with M. bovis (BCG) continuing to replicate in the spleen and liver for up to 8 wk postinfection. Strikingly, continuous microbial replication in BXH-2 was associated with the inability to form granulomas in infected organs, compared with C57BL/6 mice that have a bacterial load at 3 wk superior to BXH-2, but that can nevertheless clear the infection by 8 wk (Figs. 3 and 4). Lack of granulomatous response in infected BXH-2 was linked to absence of type 1 cytokine production in the serum, most notably IL-12p40 (Fig. 3). IL-12 was previously shown to be essential in host response to mycobacteria in mice (49–51) and in human patients (5). IL-12p40 gene codes for the p40 subunit of the cytokines IL-12 and IL-23, which are both known to be major inducers of IFN-γ production by T and NK cells. IFN-γ induces macrophages to activate their bactericidal activities, among which the production of NO, and consequently the destruction of the bacilli, as previously illustrated in IL-12p40 and IFN-γ mutant mice. Indeed, defective granuloma formation has been previously reported in IL-12p40−/− (49) and in IFN-γ−/− mutant mice (52) infected with avirulent (M. bovis BCG) or virulent mycobacteria (M. tuberculosis) (50). Taken together, these results highlight the importance of Icsbp1 in orchestrating innate and acquired immune responses during mycobacterial infections in humans.

Innate resistance to infection with another intracellular pathogen, S. Typhimurium, requires intact Nramp1 and Tlr4 function, and elimination of either genes in inbred mouse strains BALB/c (Nramp1D1000; impaired macrophage microbicidal function) and C3H/HeJ (Tlr4H712; impaired macrophage activation and lack of Th1 effector response) causes susceptibility, as measured by increased mortality following acute infection (32). In this study, we observed (Fig. 5) that impaired Icsbp1 function in Icsbp1C294/C294 homozygotes significantly reduced survival times in (BALB/c × BXH-2)F1 bearing functional allelic combinations at Nramp1 and Tlr4. Again, the effect could be explained in part by reduced expression of protective type 1 cytokines, including IL-12 (53), or more directly through reduced transcriptional activity of the Icsbp1C294 protein isoform. Indeed, the promoter of the Tlr4 gene contains a composite IFN response factor/Ets motif bound by Pu.1 and Icsbp1 (54).

Finally, we observed that BXH-2 mice show an increased susceptibility to malaria in a blood-stage model caused by P. chabaudi AS. In this model, early inflammatory response during rising parasitemia is protective and requires secretion of Th1 cytokines IL-12, IFN-γ, IL-6, and TNF-α by spleen cells of the myeloid lineage, including macrophages, dendritic cells, and NK cells. This inflammatory response is progressively dampened by expression of regulatory cytokines TGF-β/IL-10, and the balance between the two responses is critical. Clearance of the infection and long-term protection requires both T cell- and B cell-mediated immune responses (35). In this study, we observed increased blood parasitemia at the peak of infection in Icsbp1-deficient BXH-2 mice (Fig. 6), which was concomitant to reduced expression of IL-12p40 and IFN-γ early during infection. This is in agreement with published data showing that deletion of the IL-12p40 gene causes increased parasitemia at the peak of Plasmodium infection (55, 56). Macrophages from IL-12p40−/− mice were shown to secrete higher levels of TGF-β, which down-regulates NO and consequently restricts their ability to kill intracellular parasites (57). Therefore, Icsbp1 function is required for early control of blood-stage replication of the malarial parasite.

In BXH-2, we further observed waves of recurrent parasitemia in animals surviving the initial burst of blood-stage parasite replication. In individual mice, we noted up to three waves of recurring parasitemia, suggesting that although BXH-2 mice can respond and eliminate bursts of parasitemia, impaired Icsbp1 function results in failure to mount adequate memory response to prevent their recurrence. Waves of recurring parasitemia could emerge from small remaining numbers of infected RBCs (incomplete clearance of infection), or from replication of parasites sheltered in reticuloendothelial organs. The parasite could also be able to switch antigenic variants, and take advantage of a delayed elimination by the immune system impaired due to absence of IL-12 and IFN-γ. Similar waves of recurring Plasmodium parasitemia have been observed in IL-12p40−/− mice (55, 56), and this has been attributed to lower levels of Th1-dependent IgG2a and IgG3 and higher level of Th2-dependent IgG1 than wild-type mice during the infection (55). Likewise, mice defective in GM-CSF show recrudescence parasitemia following malaria infection; in this case, the impaired host response has been associated with reduced numbers of mature macrophages in these mutants (58).

Together, our studies in BXH-2 mice clearly demonstrate that Icsbp1 plays a key role in early innate host response to a range of bacterial and parasitic pathogens. Furthermore, they show that intact Icsbp1 is required for the development of long-term protective immune responses. The pleiotropic effect of Icsbp1C294 on host defenses is best explained by a threshold effect of a hypomorph allele on IL-12 production. These results raise the possibility that parallel mutations in the Icsbp1 gene may be associated with mycobacterial infections in humans. In particular, we suggest that Icsbp1 may be a good candidate for two human conditions, as follows: 1) the so-called “BCG-osis” observed in patients developing disseminated infection following BCG vaccination, and 2) the Mendelian susceptibility to mycobacterial diseases (MSMD), which is a broader syndrome that involves infection with weakly virulent mycobacterial species (including environmental mycobacteria), with occasional concomitant infection with nontyphoidal or typhoidal species of Salmonella. Previous studies of MSMD patients have identified independent mutations in the IFN-γR1, STAT1, and NF-κB essential modulator (IκB kinase γ), and interestingly in the IL-12 p40 and IL-12 Rβ1 genes (59, 60). The unique infection susceptibility phenotype of BXH-2 mice reported in this study for M. bovis (BCG) and Salmonella, together with the noted absence of IL-12p40 production by BXH-2 phenotype, identify Icsbp1 as a strong candidate for MSMD.

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Disclosures
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