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**Irf3 Polymorphism Alters Induction of Interferon Beta in Response to Listeria monocytogenes Infection**

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Genetic makeup of the host plays a significant role in the course and outcome of infection. Inbred strains of mice display a wide range of sensitivities to *Listeria monocytogenes* infection and thus serve as a good model for analysis of the effect of genetic polymorphism. The outcome of *L. monocytogenes* infection in mice is influenced by the ability of this bacterium to induce expression of interferon beta mRNA, encoded in mouse by the *Ifnb1* (interferon beta 1, fibroblast) gene. Mouse strains that lack components of the IFNβ signaling pathway are substantially more resistant to infection. We found that macrophages from the B6J substrain of the common C57BL/6 inbred strain of mice are impaired in their ability to induce *Ifnb1* expression in response to bacterial and viral infections. We mapped the locus that controls differential expression of *Ifnb1* to a region on Chromosome 7 that includes interferon regulatory factor 3 (*Irf3*), which encodes a transcription factor responsible for early induction of *Ifnb1* expression. In C57BL/6ByJ mice, *Irf3* mRNA was inefficiently spliced, with a significant proportion of the transcripts retaining intron 5. Analysis of the *Irf3* locus identified a single base-pair polymorphism and revealed that intron 5 of *Irf3* is spliced by the atypical U12-type spliceosome. We found that the polymorphism disrupts a U12-type branchpoint and has a profound effect on the efficiency of splicing of *Irf3*. We demonstrate that a naturally occurring change in the splicing control element has a dramatic effect on the resistance to *L. monocytogenes* infection. Thus, the C57BL/6ByJ mouse strain serves as an example of how a mammalian host can counter bacterial virulence strategies by introducing subtle alteration of noncoding sequences.

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**Introduction**

Bacterial pathogens utilize a wide range of approaches to down-modulate or subvert host immune responses. *L. monocytogenes* is an intracellular pathogen that, following invasion of the host cell, is capable of escaping the host phagolysosomes and replicating in the cytoplasm. Within the cytoplasm, the bacterial DNA is thought to be recognized by an unknown host receptor, activating a signaling cascade that rapidly induces *Ifnb1* expression [1]. This signaling cascade relies on TANK-binding kinase 1 (TBK1)-mediated phosphorylation of IRF3, a transcription factor that, following dimerization and translocation to the nucleus, induces expression of *Ifnb1* [2–4].

In a murine model of infection, activation of host IFNβ signaling is an important *L. monocytogenes* virulence strategy. Mouse lines that lack components of the IFNβ signaling pathway (*Ifnb1, Ifnar1*) are significantly more resistant to *L. monocytogenes* infection [2,4–6]. A similar protective effect of an *Irf3* knockout suggests that Toll-like receptor (TLR)-independent induction of IFNβ is detrimental to control of listeriosis [7]. Several independent observations suggested that IFNβ signaling sensitizes lymphocytes for cell death, leading to an increase in sensitivity to *L. monocytogenes* [8]. *L. monocytogenes* activates such proapoptotic genes as *Trail* (*Tnffs10*), *Pkr* (*Eif2ak2*), and *Daxx* in spleen and bone marrow macrophages of wild-type, but not *Ifnar*-deficient mice [4]. This is consistent with the observation that *Trail* knockout mice are more resistant to *L. monocytogenes* infection [9]. It has also been noted that following infection, mice lacking components of the IFNβ signaling machinery have higher total numbers of macrophages. This could be due to the ability of Type I interferon signaling to accelerate cell death of *L. monocytogenes*-infected macrophages [3].

Inbred mouse strains are extensively used as a model system to study host immune response throughout the course of *L. monocytogenes* infection. In addition, common stains display a wide range of sensitivities to intravenous infection with *L. monocytogenes* [10]. Our initial analysis of genetic determinants affecting susceptibility to *L. monocytogenes* infection...
infection was carried out using a pair of differentially susceptible inbred mouse strains: BALB/cByJ and C57BL/6ByJ [11]. These strains were selected based on the ancestry of the 13-member CXB Recombinant Inbred (RI) Panel, which serves as a useful tool for mapping single gene traits [12]. While our study identified two major genetic loci that controlled differential sensitivity to L. monocytogenes infection, it was clear that there were additional genetic factors that we did not detect due to the limited size of our cross. Here, we report identification and characterization of one of these additional factors, a polymorphism in the C57BL/6ByJ inbred mouse strain that affects expression of Ifnb1 and results in increased resistance to L. monocytogenes infection. Our data demonstrate that a single base-pair polymorphism in intron 5 of Ifn3 reveals an important role for splicing in control of IFNβ induction and innate immune function.

Results

C57BL/6ByJ Strain-Specific Defect in Induction of Ifnb1 Expression

Our analysis of L. monocytogenes infection of macrophages derived from bone marrow (i.e., bone marrow macrophages; BMMs) of BALB/cByJ and C57BL/6ByJ strains revealed strain-specific differences in infection-induced cell death. Across a range of time points and infectious doses, BALB/cByJ BMMs had consistently higher cell death than BMMs from C57BL/6ByJ mice (Figure 1A and 1B). Interestingly, 18 h following infection with L. monocytogenes, there were also significant (p < 0.001) differences in death of BMMs from J and ByJ substrains of the common C57BL6 lineage (Figure 1C). The observed differences in cell death could be due to small differences in replication of bacteria in infected BMMs (Figure 1D). However, recent studies that have demonstrated a role for IFNβ signaling in the outcome of L. monocytogenes infection have also suggested that it plays a role in the survival of macrophages [2]. Therefore, we chose to test if there are mouse strain-specific differences in IFNβ signaling, and we analyzed the course of Ifnb1 mRNA induction in L.

Intracellular L. monocytogenes is thought to induce Ifnb1 expression by activating an as-of-yet unidentified cytoplasmic receptor that initiates signaling through the TBK1 and inhibitor of kappaB kinase epsilon (IKBKE) kinases [1]. TBK1 and IKBKE also participate in transducing signals from various TLRs in response to viral and bacterial infections [14]. Therefore, we tested if the defect in C57BL/6ByJ mice was in a L. monocytogenes-specific component of the TBK1/IKBKE signaling pathway or in a component shared with other pathways. Treatment of C57BL6ByJ BMMs with...
lipopolysaccharide or poly I:poly C, which induce Ifnb1 expression through TLR4 and TLR3, respectively, failed to induce Ifnb1 mRNA at the same levels as observed in C57BL/6J BMMs (Figure 2B and 2C). On the other hand, C57BL/6ByJ BMMs treated with 200 hemagglutinating units (HU) of Sendai virus, which induces Ifnb1 expression through a RIG-I/MAVS–dependent pathway [15], had levels of Ifnb1 mRNA comparable to those observed in C57BL/6J BMMs at the later stages of infection but nevertheless had a noticeable delay at the earlier stages (Figure 2D). These observations indicate that the defect in Ifnb1 induction in C57BL/6ByJ mice is likely to lie in a shared component of the signaling pathway. However, our initial analysis Tbk1, Ikbke, and Irf3 failed to identify differences in the coding sequence or overall expression levels of these mRNAs in C57BL/6ByJ versus C57BL/6J mice (unpublished data).

Mapping of the Ifnb1 Induction Trait

As mentioned above, our original choice of mouse strain for genetic analysis was based on the availability of the CXB RI mapping panel. However, analysis of the transcriptional response to L. monocytogenes infection in macrophages from all 13 CXB strains revealed no differences in induction of Ifnb1 (unpublished data), precluding the use of the panel for mapping. Because C57BL/6ByJ mice carry a recessive mutation, we therefore chose a backcross as our mapping strategy to identify the locus in the C57BL/6ByJ mouse genome that harbors the mutation preventing induction of Ifnb1. C57BL/6ByJ and C57BL/6J mice have virtually no polymorphisms that can be used to monitor allelic segregation in a cross. On the other hand, BALB/cByJ mice are similar to C57BL/6J mice in their induction of Ifnb1, and we therefore chose C57BL/6ByJ and BALB/cByJ as parental strains for our cross. We backcrossed F1 male progeny of C57BL/6ByJ and BALB/cByJ mice to C57BL/6ByJ females and used the resulting 54 C(B.C)N2 progeny to construct a genetic map with 56 microsatellite markers evenly distributed throughout the mouse genome [16]. To generate phenotypic data, we first used real time reverse transcriptase PCR (RT-PCR) to analyze the dynamics of L. monocytogenes-induced Ifnb1 expression in BMMs isolated from 43 backcrossed mice (unpublished data). We then used transformed real time RT-PCR Ct values representing the levels of Ifnb1 mRNA at the 4-h time point directly as a quantitative trait. Mapping of this trait using MapManger QTX identified a peak likelihood ratio statistic score of 35.1 (logarithm of the odds [LOD] = 7.6) at the D7Mit229 marker [17] (Figure S3). Using the MapManger QTX built-in permutation test function, we established that the identified linkage is highly significant with an experimental p-value less
then $10^{-4}$. QTL support interval was approximated as 1.5 LOD drop-off from the peak score [18] and extended from D7Mit27 to D7Mit158 markers, spanning empirical genetic distance of 6.9 cM (0 cM MGI) and a physical fragment of 2.35 Mb. The D7Mit229 marker is located on mouse Chromosome 7 adjacent to Irf3 [16], identifying Irf3 as the primary candidate gene.

**Irf3 mRNA in C57BL/6ByJ Strain Is Not Efficiently Spliced**

Interestingly, our initial analysis of C57BL/6J and C57BL/6ByJ substrains did not find any differences in overall Irf3 mRNA levels or polymorphisms in the coding region of Irf3 (unpublished data). However, analysis of the structure of Irf3 mRNA using a series of overlapping primers revealed differences in the Irf3 transcripts between the two substrains.

As expected, in C57BL/6J mice the majority of Irf3 transcripts were completely spliced, whereas in C57BL/6ByJ mice, splicing of Irf3 was not complete and the majority of transcripts retained intron 5 (Figure 4A and quantified in Figure 4B). Retention of intron 5 introduces a premature stop codon at amino acid 243, rather than producing the full-length 419 amino-acid protein. To test if the observed differences in splicing had functional significance, we analyzed activation of IRF3 in BMMs by monitoring the formation of IRF3 dimers following bacterial (*L. monocytogenes*) or viral (Sendai virus) infection. We found that untreated BMMs from C57BL/6ByJ mice had significantly lower levels of IRF3 protein than untreated BMMs from C57BL/6J mice (Figure 4C and 4D). Moreover, following a 2-h infection with *L. monocytogenes* or Sendai virus, there were no detectable IRF3 dimers in C57BL/6ByJ BMMs, although IRF3 dimers were readily detectable in C57BL/6J BMMs. Nevertheless, we observed that Sendai virus–infected C57BL/6ByJ BMMs are capable of inducing *Ifnb1* expression (see Figure 2D). This is consistent with earlier observations that Irf3–deficient cells rely on IRF7 to have a normal interferon response to several viral infections [19,20]. Interestingly, our polyclonal antibodies failed to detect a truncated form of IRF3 even in the presence of proteasome inhibitor (MG132), suggesting that the unspliced form of Irf3 might not be efficiently translated (Figure 4C). Overall, these results show that C57BL/6ByJ BMMs have dramatically lower amounts of functional IRF3 protein, and in conjunction with the existing Irf3 knock-out data [2,4], explain the increased resistance of C57BL/6ByJ mice to *L. monocytogenes* infection (see Figure 3).
A to T Polymorphism Impairs Slicing of Irf3 Intron 5 in C57BL/6ByJ Strain

Sequencing of the entire 7.2-kb genomic region of Irf3 [21], including 1 kb of upstream and downstream sequences, revealed a single A to T polymorphism in the middle of intron 5 in C57BL/6ByJ mice (Figure 5). To establish if this polymorphism altered the splicing efficiency of the intron, we monitored splicing using both cell culture–based and in vitro approaches. For cell culture–based experiments, we derived minigene constructs containing the complete intron 5 (from either C57BL/6J or C57BL/6ByJ) flanked by exons 5 and 6, and expressed them under the control of the heterologous CMV promoter. In order to rule out the possibility that C57BL/6ByJ mice carry additional mutations that affect splicing, we first tested our constructs in a C57BL/6ByJ fibroblast-like cell line (Y5). Following transfection into Y5 cells, the efficiency of splicing of intron 5 was monitored by real time RT-PCR using primers specific to the vector and exon 5–6 junctions, and the total amount of RNA expressed from each construct was measured using primers specific to the exon fragment, which is identical in both constructs (see Figure 6A schematic). When normalized for the total amount of expressed RNA, there was significantly more spliced product generated from the C57BL/6J construct than from the C57BL/6ByJ construct (Figure 6A). This result indicates that in C57BL/6ByJ cells, C57BL/6J Irf3 intron 5 is spliced more efficiently than the C57BL/6ByJ version of the intron.

The effect of the A to T substitution on splicing efficiency of intron 5 was further confirmed using an in vitro splicing assay, in which a uniformly radioactively labeled Irf3 pre-mRNA containing intron 5 flanked by 50 bp of exon 5 and exon 6 was incubated in HeLa nuclear extract. As expected, the C57BL/6J-derived Irf3 pre-mRNA substrate was spliced efficiently, as evidenced by the appearance of both intermediate and fully spliced products (Figure 6B). By contrast, there was no detectable splicing of the C57BL/6ByJ-derived Irf3 pre-mRNA substrate even when incubated for 60 min in the splicing reaction mixture. These results confirmed that the A to T substitution had a direct effect on efficiency of Irf3 splicing.

Splicing of Murine Irf3 Intron 5 Relies on U12 Splicesome

Pre-mRNA splicing occurs in a ribonucleoprotein complex called the spliceosome [22]. Splicing is initiated through recognition of several intron-defining splicing signals, including the 5′ and 3′ splice sites and the branchpoint, which is usually located near the 3′ end of the intron. Introns can be classified into two categories: U2-type introns, which comprise the major class of introns, and U12-type introns. U2-type introns are characterized by the presence of a conserved
GU dinucleotide at the 5' end of the intron and a conserved AG dinucleotide at the 3' end, whereas U12-type introns can harbor AT–AC, AT–AG, and GU–AG dinucleotides at their 5' and 3' boundaries, respectively. Furthermore, on U12-type introns, the 5' and 3' splice sites and branchpoint are highly conserved and differ from those of the conventional U2-type introns [23,24], and the characteristic polypyrimidine tract is typically absent [25].

Because the A to T polymorphism is located within an intron and affects splicing efficiency, we hypothesized that it might alter the function of a splicing signal. The polymorphism is located 46 bp upstream of the 3' splice site, within a region where the branchpoint is typically found. The 5' boundary of the murine Irf3 intron 5 matches the U2-type intron GTRAGT consensus sequence (Figure 5) [23,24]. However, the region surrounding the polymorphism more closely resembled the U12 branchpoint consensus (TCCTTA-ACy) than the U2 branchpoint consensus (YURAY). To determine whether intron 5 of the Irf3 gene was a U2-type or U12-type intron, we monitored splicing of the wild-type C57BL/6J-derived Irf3 pre-mRNA substrate following inactivation of U2 or U12 snRNA by oligonucleotide-directed
RNase H cleavage. The in vitro splicing assay in Figure 6C shows that splicing of the C57BL/6J-derived Ifnb1 transcript occurred following inactivation of U2 snRNA but not following inactivation of U12 snRNA. As expected, splicing of the control U2-type intron–containing adenovirus major late (Ad ML) pre-mRNA substrate was fully dependent on the presence of U2 snRNA. These observations indicate that splicing of intron 5 of the Ifnb1 gene relies on the U12-dependent mechanism.

**Ifnb1 Intron 5 A to T Polymorphism Affects Induction of Ifnb1 Expression**

Ifnb1 transcripts that retain intron 5 are detected in both C57BL/6ByJ and C57BL/6J strains (see Figure 4A). Therefore, it appears that even in the C57BL/6J strain, splicing of intron 5 is somewhat inefficient, whereas in the C57BL/6ByJ strain, intron 5 splicing is substantially impaired. To test if the observed phenotypic differences in induction of Ifnb1 protein expression in the two mouse strains are due to differences in splicing efficiency of Ifnb1 intron 5, we performed complementation experiments. To achieve this, we transfected BMMs from Ifnb1 knockout mice with full-length in vitro transcribed Ifnb1 mRNA species harboring either the C57BL/6J or C57BL/6ByJ version of intron 5. Fully spliced Ifnb1 mRNA was used as a positive control, and mRNA containing a partial deletion of IRF domain (AXma) was used as a negative control. Previous experiments had shown that introduction of single-stranded RNA into the cell cytosol leads to Ifnb1-dependent induction of Ifnb1 expression [26–28] (O. G., unpublished data). Therefore, transfection of intron 5–containing Ifnb1 mRNAs into BMMs that lack Ifnb1 should lead to a level of Ifnb1 induction that is proportional to the splicing efficiency of intron 5. We measured the levels of Ifnb1 mRNA by real time RT-PCR 19 h following transfection of BMMs, and found that there was ~5-fold more (p < 0.01) Ifnb1 mRNA expressed in BMMs transfected with the C57BL/6J-derived Ifnb1 mRNA than in BMMs transfected with C57BL/6ByJ-derived Ifnb1 mRNA (Figure 7). Following a 4-h infection with L. monocytogenes, BMMs transfected with either mRNA showed further induction of Ifnb1 expression. Nevertheless, the C57BL/6J-derived Ifnb1 mRNA induced significantly (p < 0.01) higher amounts of Ifnb1 mRNA than the C57BL/6ByJ-derived version. As expected, BMMs transfected with Ifnb1 mRNA lacking the IRF domain induced only low levels of Ifnb1 mRNA expression. Because the Ifnb1 knockout mice used in this experiment are on the C57BL/6J background [19], this experiment also ruled out the possibility that impaired splicing of Ifnb1 intron 5 could be due to a linked C57BL/6ByJ polymorphism. Therefore, we conclude that decreased splicing efficiency of Ifnb1 intron 5 is directly responsible for the reduction in Ifnb1 expression observed in C57BL/6ByJ mice.

**Discussion**

Previous studies have demonstrated an important role of IFNβ signaling in host defense against L. monocytogenes infection. L. monocytogenes evolved to take advantage of the host signaling pathways and is capable of inducing Ifnb1 expression in order to down-modulate the antibacterial host defense. Here, we show that at least one inbred strain of mice can resist this pathogen’s tactic by carrying a single nucleotide polymorphism that changes the efficiency of splicing of its Ifnb1 transcription factor. While this naturally occurring polymorphism does not eliminate IRF3 activity, the resulting reduction in IRF3 protein levels is sufficient to confer 10-fold higher resistance to L. monocytogenes infection. Considering that complete loss of IRF3 function is detrimental to immune defense, and Ifnb1 knockout mice are more sensitive to encephalomyocarditis infection [19], it would be interesting to determine if the level of IRF3 in C57BL/6ByJ mice is sufficient to maintain protection against viral infections. Nevertheless, our finding indicates that genetic changes in noncoding regions of the host genome is one of the mechanisms that can be used to fine tune the effectiveness of host defenses against infections.

It has been suggested that in the process of evolution, U12-type introns are either lost or undergo subtype switching (from AT–AC to GT–AG) and are eventually converted to U2-type introns [25]. Our data provide additional support for this hypothesis. Although splicing of IRF3 intron 5 is dependent on the U12 spliceosome, the splice donor site is a typical U2 site. Even more interesting is the fact that the region of the human and rhesus intron 5 that is homologous to the putative murine U12 branchpoint site contains a G in place of the T that is found in rodents (Figure 5, inset box). This substitution places a purine residue in front of a putative branchpoint adenosine, thus creating a perfect match (inset box) to the U2 YURAY branchpoint consensus. Therefore, intron 5 of the murine Ifnb1 gene might represent one of the final steps in the conversion of a U12-type intron to a U2-type intron.

The amount of IRF3 available in the cell is tightly controlled and overproduction of IRF3 is lethal to BMMs.
Figure 6. Mouse Strain-Specific Differences in *Irf3* Intron 5 Splicing

(A) Relative levels of the spliced to unspliced forms of an *Irf3* intron 5–containing minigene expressed in C57BL/6ByJ fibroblast-like Y5 cells 12 h following transfection. Levels are normalized to the total amount of expressed RNA. The location of the primers used in the analysis is shown in the schematic diagram. The efficiency of splicing of intron 5 was monitored using primers specific to the vector and exon 5–6 junctions (top pair), whereas the total amount of RNA expressed from each construct was measured using primers specific to the exon fragment (bottom pair).

(B) In vitro splicing of the C57BL/6J or C57BL/6ByJ minigene pre-mRNA was analyzed at 0, 15, 30, and 60 min following addition of the substrate to the nuclear extract. Identities of the spliced products are shown in the left.

(C) In vitro splicing of the C57BL/6J minigene pre-mRNA was analyzed at 0, 30, and 60 min following inactivation of U2 or U12 snRNA by oligonucleotide directed RNase H cleavage. As a control, splicing of the U2-type intron–containing adenovirus major late (Ad ML) pre-mRNA substrate was analyzed. doi:10.1371/journal.pgen.0030152.g006
Intron 5, or Deleted for IRF Domain (Dxma).

Irf3 mRNA expression was measured by RT-PCR either 19 h (T19) following transfection or after 15 h transfection plus 4 h infection with L. monocytogenes. A representative of three independent experiments is shown. doi:10.1371/journal.pgen.0030152.g007

Figure 7. Levels of Ifnb1 mRNA Expression in C57BL/6ByJ BMMs following Transfection of Irf3 mRNAs That Are Fully Spliced, Contain Intron 5, or Deleted for IRF Domain (ΔXma).

Ifnb1 mRNA expression was measured by RT-RT-PCR either 19 h (T19) following transfection or after 15 h transfection plus 4 h infection with L. monocytogenes (T15 + 4h LM). A representative of three independent experiments is shown.

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Recent evidence indicates that splicing of U12-type introns could be a rate-limiting step in gene expression [31]. Our analysis suggests that this may also be the case for intron 5 in the murine Irf3 gene. Macrophages from common strains, such as C57BL/6J and BALB/cByJ, have two populations of Irf3 transcripts: a major, fully spliced species and a minor species that retains intron 5 (see Figure 4A). The presence of multiple Genbank (i.e., BC082274, BC003233; http://www.ncbi.nlm.nih.gov.Genbank/index.html) and EST entries of Irf3 mRNAs that retain intron 5 further supports this hypothesis. Therefore, the rate of intron 5 splicing could control the amount of Irf3 available in the cell. Interestingly, it has been shown that activity of human IRF3 is also regulated at the level of splicing [32]. However, in contrast to rodents Irf3, regulation of human IRF3 involves alternative intron 1 splice acceptor sites that can produce an active or a dominant negative, Irf3α, form of IRF3 [33]. Because it appears that by converting to a U2 intron, human IRF3 intron 5 lost its rate-limiting function, it is intriguing to contemplate that this led to emergence of an alternative splicing control mechanism for human Irf3.

Our choice of mouse strains for genetic analysis of susceptibility to L. monocytogenes infection was based on the existence of the ByJ-based CBX RI mapping panel. The C57BL/6By substrain has been used to generate at least seven of the 13 CBX RI strains, but none of the CBX strains appear to have a defect in induction of Ifnb1. To resolve this discrepancy, we sequenced Irf3 intron 5 in all C57BL/6By-derived CBX strains. None of the sequenced Irf3 introns contained the mutation found in the C57BL/6ByJ strain (unpublished data). Therefore, it appears that the A to T mutation rose in the C57BL/6ByJ background only recently, after the generation of the CBX RI strains. It is possible that the return of D. W. Bailey’s substrains to the Production Department of Jackson Laboratories in 1974 could have created a bottleneck that fixed the mutation in the current C57BL/6ByJ population.

Splicing of mRNA is a critical step in protein expression, and in humans, genetic polymorphisms that produce aberrant or alternate splicing products have been associated with a wide range of diseases [34]. We used genetic analysis of the mouse model system to provide definitive evidence of the important role of splicing in control of infection. We found that a mouse strain-specific defect in induction of Ifnb1 is due to a single nucleotide polymorphism in intron 5 of Irf3. Our analysis of this polymorphism revealed that splicing is a critical step in the control of Irf3 expression and, as a result, in the course and outcome of L. monocytogenes infection. While intron 5 of murine Irf3 has features of both U2 and U12 introns, we provide evidence that its splicing is dependent on the U12 spliceosome. Therefore, it appears that in rodents the U12 spliceosome can use U2 splice sites. This suggests that the spectrum of U12-type introns present in mammalian genomes could be wider than previously thought. Finally, our comparison of rodent and primate Irf3 genomic sequences also revealed the intriguing possibility that we have identified an intermediate step in the process of conversion from a U12- to U2-type intron.

Materials and Methods

Animals. Six-to-twelve-week-old animals were used in all experiments. BALB/cByJ, C57BL/6J, and C57BL/6ByJ mice were obtained from Jackson Laboratories (http://www.jax.org/). B/ByJ.C N2 mice were created by backcrossing B/ByJ.C F1 males to C57BL/6ByJ females. All mouse strains were bred and maintained under specific pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School. All experiments involving live animals were carried out in accordance with the guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institute Animal Care and Use Committee.

In vivo infections. Pre-titered TSB-glycerol stocks of L. monocytogenes strain 10403S were stored at −80°C. Prior to infection, 1-ml bacterial aliquots were recovered for 1 h at 37°C in 9 ml of TSB (BD Biosciences, http://www.bdbiosciences.com/), washed, and resuspended to the desired cfu in PBS. Mice were injected with a defined dose of L. monocytogenes strain 10403S in 0.4 ml of PBS. At defined time points, infected animals were killed by CO2 asphyxiation. Livers and spleens of infected animals were aseptically harvested, weighed, and homogenized in 0.02% Triton X-100. Aliquots of serial 5-fold dilutions in sterile water were plated in duplicate on TSB agar (BD Biosciences) plates containing 10 μg/ml streptomycin. After overnight incubation, the number of bacteria per milligram of tissue was determined by counting colonies at the appropriate dilution.

Generation of bone marrow macrophages. BMMs were generated by differentiating bone marrow cells in a complete BM medium (DMEM, 10% heat-inactivated FCS (Invitrogen, http://www.invitrogen.com/), 100 μM penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 10% L929 fibroblast-conditioned medium as a source of M-CSF) for 6 d in 10-cm Petri dishes (VWR, http://www.vwr.com/).

Cell lines. Fibroblast-like YF5 and macrophage-like YM14 cell lines were generated by immortalization of C57BL/6ByJ bone marrow cells as previously described [35,36]. Clones were selected based on morphology.

Ex vivo experiments. Differentiated BMMs were detached from the Petri plates by incubation in cold PBS, washed, resuspended in BM medium without antibiotics and used to seed multwell dishes at 1 × 104 cells/cm². Following overnight incubation the medium was replaced with DMEM containing the agent used to stimulate BMM. For L. monocytogenes, after 1 h the medium was replaced with BM medium containing 10 μg/ml gentamicin (Fisher) to remove extracellular bacteria. Lipopolysaccharide (Sigma) was added at 1 μg/ml, poly I:C (Sigma) at 25 μg/ml and Sendai Virus (generous gift of Kate Fitzgerald, University of Massachusetts) at 200 U/ml. At defined
timepoints BMMs were lysed in TRIzol (Invitrogen) and RNA was isolated according to manufacturer’s protocol.

**BMM cell death assay.** 5 x 10^5 BMMs were seeded in 96-well tissue culture plate and following overnight incubation the cells were infected with a defined multiplicity of infection (MOI) of *L. monocytogenes*. After 1 h incubation, non-adherent cell culture medium was replaced with fresh complete RPMI 10% FCS containing 10 μg/ml of actinomycin D (MP Biomedicals, http://www.mpbiotech.com/). At defined time points the supernatants were collected and the remaining cells were lysed for 20 min at room temperature in 50 μl/well of complete medium plus 1μl of cell lysis solution from CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega, http://www.promega.com/). Lactose dehydrogenase (LDH) assay for supernatants and cell lysates was performed according to the manufacturer’s protocol. Fluorescence was recorded at 560/590 nm using a Synergy HT microplate reader (Bio-Tek, http://www.bioteck.com/). Relative concentration of LDH in supernatants was calculated by the equation: 100% × (LDHsup/LDHcell).

**Real time PCR.** Relative mRNA levels were quantified by real time RT-PCR on an ABI 7500 instrument (http://www.appliedbiosystems.de/) utilizing SYBR Green chemistry (ABI SYBR master mix) and primers used to detect specific mRNAs are described in Table S1. Ribosomal protein S17 (RpS17) and actin mRNA were used as a housekeeping gene to quantify the relative amounts of mRNA in each experiment. Each experiment included at least two biological and three experimental replicates.

**Genetic mapping.** BMMs from 46 B1/Bj(C.B1Bj) N2 mice were infected in duplicate with *L. monocytogenes* strain 10403S at MOI = 5. Four hours following infection, total RNA was isolated and used for real time RT-PCR analysis of *Ifnb1* mRNA induction. *Ifnb1* Ct values from duplicate samples were adjusted for variation in total RNA concentration using *RpS17* Ct values, transformed by subtracting the average C57BL/6ByJ parental value, and used as direct trait values for mapping using MapManager software. Under ideal conditions, such transformed Ct values can be viewed as log5 of the fold difference in *Ifnb1* expression compared to the C57BL/6ByJ parent. A genetic map was constructed using 56 microsatellite markers [16]. Experimental p-value for linkage was evaluated using a built-in permutation test and was found to be less than 10^-4.

**IRF3 immunoblotting.** BMMs were infected with *L. monocytogenes* (MOI = 5) or Sendai Virus (600 HU) for 4 h. Cells were lysed in the presence of protease inhibitors (Roche, http://www.roche.com/) and following centrifugation, supernatant aliquots containing 20 μg of protein were loaded per lane of a native polyacrylamide gel. IRF3 was visualized using a rabbit anti-IRF3 antibody (Invitrogen). For total IRF3 protein analysis, lysates were obtained from infected in vitro transcribed *Irf3* mRNA was not detectable in livers and spleens of animals infected with 1 x 10^7 *L. monocytogenes* until the 24-h time point. Forty-eight hours after infection, spleens of C57BL/6J animals had 6-fold higher levels of *Ifnb1* mRNA than C57BL/6ByJ animals.

**RNA transfection.** The cDNAs of fully spliced *Irf3*, *Ifb3* lacking internal XmnI fragment, and strain-specific *Ifb3* species retaining intron 5 were amplified with IRF3F-1 and IRF3dT32SspIR oligonucleotides (Table S1). IRF3dT32SspIR was designed to introduce poly(A) at the 3' end of *Ifb3* coding sequences. Amplified *Ifb3* fragments were cloned in front of T7 promoter of pCR2.1 vector (Invitrogen). To generate *Ihb3* mRNAs, 1 μg of respective SspI linearized plasmids was used as templates in in vitro transcription reactions (Maxiscript T7; Ambion, http://www.ambion.com/). One microgram of DNase treated, repurification RNA was used for nucleofection of 1 to 1.5 x 10^6 BMMs in 100 μl of complete mouse macrophage nucleofector solution (82 μl mouse macrophage nucleofector solution, 18 μl supplement (IRF3dR)) or the exon 5–6 junction (IRF3Ex5/6R5). For in vitro splicing assays, minigene *Ihb3* pre-mRNA templates were PCR-amplified from pNT5j and pNT3Bj using a T7-containing primer, purified, and transcribed in vitro using 17 polymerase in the presence of [alpha-32P]UTP. In vitro splicing reactions were performed essentially as described previously [37], except that 90% HeLa nuclear extract was used. Spliced products were resolved on 12% denaturing polyacrylamide gels (19:1) in 8 M urea in Tris-Borate-EDTA buffer, and visualized using a Fujifilm FLA-500 phosphorimager (http://www.fujifilm.com/). U2 and U12 snRNAs were inactivated by RNase H-directed cleavage as described previously [38] using DNA oligonucleotides complementary to nucleotides 27–49 of the U2 snRNA or to nucleotides 11–28 of the U12 snRNA.

**Genes.** All genes mentioned in the text and their corresponding National Center for Biotechnology Information (NCBI) GeneID (http://www.ncbi.nlm.nih.gov/sites/entrez) and Ensembl (http://www.ensembl.org/) identifiers are described in Table S2.

**Figure S1.** Levels of *Ifnb1* mRNA in Tissues of Infected Animals. *Ifnb1* mRNA was not detectable in livers and spleens of animals infected with 1 x 10^7 *L. monocytogenes* until the 24-h time point. Forty-eight hours after infection, spleens of C57BL/6J animals had 6-fold higher levels of *Ifnb1* mRNA than C57BL/6ByJ animals. Found at doi:10.1371/journal.pgen.0030152.sg001 (238 KB EPS).

**Figure S2.** Survival of C57BL/6J and C57BL/6ByJ Mice following Intravenous Infection with 1 x 10^6 cfu *L. monocytogenes* Strain 10403S. The majority of C57BL/6ByJ mice survive for more than 10 d following infection. Found at doi:10.1371/journal.pgen.0030152.sg002 (72 KB EPS).

**Figure S3.** MapManager QTX Chromosome 7 Interval Mapping Results. Likelihood ratio statistic scores represent thresholds of suggestive, significant, and highly significant linkages, respectively. Found at doi:10.1371/journal.pgen.0030152.sg003 (206 KB EPS).

**Figure S4.** High Levels of Spliced *Ihb3* mRNA Induce Death of BMMs. Survival of C57BL/6J *Ihb3*–/– BMMs following 24-h transfection of in vitro transcribed *Ihb3* mRNAs was monitored by the release of the cytosolic enzyme LDH into the supernatant. Found at doi:10.1371/journal.pgen.0030152.sg004 (240 KB EPS).

**Table S1.** Sequences of Primers Used in This Study. Found at doi:10.1371/journal.pgen.0030152.t001 (19 KB PDF).

**Table S2.** NCBI GeneID and Ensembl Identifiers for Genes Mentioned in the Text. Found at doi:10.1371/journal.pgen.0030152.t002 (27 KB PDF).

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