DOI: 10.1534/genetics.107.075523

Molecular Genetic Analysis of Two Loci (*Ity2* and *Ity3*) Involved in the Host Response to Infection With Salmonella Typhimurium Using Congenic Mice and Expression Profiling

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Manuscript received May 4, 2007
Accepted for publication July 27, 2007

ABSTRACT

Numerous genes have been identified to date that contribute to the host response to systemic Salmonella Typhimurium infection in mice. We have previously identified two loci, *Ity2* and *Ity3*, that control survival to Salmonella infection in the wild-derived inbred MOLF/Ei mouse using a (C57BL/6J × MOLF/Ei)F_2cross. We validated the existence of these two loci by creating congenic mice carrying each quantitative trait locus (QTL) in isolation. Subcongenic mice generated for each locus allowed us to define the critical intervals underlying *Ity2* and *Ity3*. Furthermore, expression profiling was carried out with the aim of identifying differentially expressed genes within the critical intervals as potential candidate genes. Genomewide expression arrays were used to interrogate expression differences in the *Ity2* congenics, leading to the identification of a new candidate gene (*Havcr2*, hepatitis A virus cellular receptor 2). Interval-specific oligonucleotide arrays were created for *Ity3*, identifying one potential candidate gene (*Chi3l1*, chitinase 3-like 1) to be pursued further. The combination of the use of congenics in QTL confirmation and fine mapping and in the identification of candidate genes by expression profiling has been successful and represents a step toward quantitative gene(s) identification.

SALMONELLA enterica serovar Typhimurium (Salmonella Typhimurium) is a gram-negative intracellular bacterium that is responsible for a gastrointestinal illness known as salmonellosis in humans and a typhoid-like systemic disease in mice. Typhoid fever, caused by the host-specific Salmonella Typhi in humans, is a generalized systemic enteric fever, characterized by headache, nausea, abdominal pain, and diarrhea or constipation with case fatality of 16% without appropriate antibiotic treatment (Ohl and Miller 2001). Susceptibility to such infectious diseases is considered to be a complex trait involving numerous genetic and environmental factors, the interactions of which determine the ultimate outcome of infection. Only a few genes that control the host response to Salmonella infection have been identified in humans and include *IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, and *STAT1* (Casanova and Abel 2004). However, the understanding of immunity to Salmonella infection in humans has progressed considerably through the use of mouse models of infection.

A wide range of susceptibilities to intravenous infection with Salmonella Typhimurium have been reported among various laboratory mouse strains (Roy and Malo 2002). The commonly used inbred mouse strain C57BL/6J is known to succumb to intravenous Salmonella Typhimurium infection within 5 days postinfection due to a single point mutation within *Slc11a1* [previously known as *Nramp1* and *Ity* (for immunity to Typhimurium)], a gene having a major impact in controlling the replication of Salmonella Typhimurium within the macrophage (Vidal *et al.* 1995). Another particular strain of interest is the wild-derived inbred mouse MOLF/Ei, which is extremely susceptible to infection despite harboring functional alleles at *Slc11a1* and at another known Salmonella susceptibility locus, *Toll-like receptor 4* (*Tlr4*). Using an F_2 panel of (C57BL/6J × MOLF/Ei) mice, three quantitative trait loci (QTL) linked to the host response to Salmonella Typhimurium infection were identified: *Slc11a1* (*Ity*), *Ity2*, and *Ity3* with respective LOD scores of 18.8, 7.0, and 5.0. The *Ity2* and *Ity3* QTL were only detected in the presence of a *Slc11a1* wild type (at least one MOLF/Ei allele) background. The MOLF/Ei allele has a protective effect at *Ity2* on chromosome 11 under an additive mode of inheritance. MOLF/Ei alleles also contribute to susceptibility at *Ity3* on distal chromosome 1 manifesting the phenotype under a recessive mode of inheritance (Sebastiani *et al.* 1998).

Gene identification using QTL analysis is a challenging task and is based on establishing, as precisely as

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possible, the chromosomal position of the QTL and identifying the genes that are located in the target region. Genes are then selected according to function, sequence, and mouse strain distribution of the QTL alleles. The creation of congenic and subcongenic strains is commonly used in the validation and in the fine mapping of QTL underlying complex traits in mice. Through repeated marker-assisted backcrossing, a congenic mouse is generated such that it carries the QTL interval from the donor strain on a homogenous background representing the recipient strain. The generation of congenics allows the assessment of the effect of a unique QTL on the disease phenotype (Rogner and Avner 2003). This congenic approach has proven fruitful in the genetic dissection of diseases such as seizure susceptibility (Ferraro *et al.* 2004), systemic lupus erythematosus (Haywood *et al.* 2004; Subramanian *et al.* 2005), and type 1 diabetes (Lyons *et al.* 2000). A combination of this classical approach to fine mapping with genomewide expression profiling techniques has been used successfully to prioritize candidate genes (Rozzo *et al.* 2001; Gu *et al.* 2002; Mcbride *et al.* 2003; Klein *et al.* 2004; Johannesson *et al.* 2005; De Buhr *et al.* 2006). In this article, we refine the map of the large *Ity2* and *Ity3* intervals and identify candidate genes combining two genetic dissection tools, the use of congenic strain mapping and expression profiling (genomewide and QTL interval-specific oligonucleotide arrays).

MATERIALS AND METHODS

Mice: The parental mouse strains, MOLF/Ei and C57BL/6J, were purchased from the Jackson Laboratory at 4–6 weeks of age. The congenic mice were backcrossed for 10 generations selecting mice carrying the MOLF/Ei allele at *D1Mcg4* (*Ity*) and between the markers *D11Mit84* and *Mpo* (*Ity2*), resulting in the generation of B6.MOLF-*Ity/Ity2* congenic mice. Two distinct congenic strains were created additionally as a result of recombinants selected within the *Ity2* interval during the intercrossing of N10 mice to homozygosity (B6.MOLF-*Ity/Ity2.RecD*, B6.MOLF-*Ity/Ity2.RecI*) (Figure 2A). The construction of *Ity3* congenic mice has been described previously (Sancho-Shimizu and Malo 2006). The *Ity3* congenic and recombinant subcongenic strains (B6.MOLF-*Ity/Ity3*, B6.MOLF-*Ity/Ity.RecA*, B6.MOLF-*Ity/Ity3.RecB*, B6.MOLF-*Ity/Ity3.RecC*, B6.MOLF-*Ity/Ity3.RecE*) used in the present study differ from the previously published strains in that they have been backcrossed for over 10 generations. The recombinant subcongenic strains were obtained by breeding recombinant chromosomes generated during the intercrossing of the N10 B6.MOLF-*Ity/Ity3* strains to homozygosity. All animal procedures were performed in accordance with regulations of the Canadian Council of Animal Care.

Genotyping of congenic mice: At every generation, *Ity2* mice were genotyped using microsatellite markers *D1Mcg4*, *D11Mit84*, *D11Mit109*, *D11Mit20*, *D11Mit141*, *D11Mit142*, *D11Mit112*, *D11Mit193*, *D11Mit219*, *D11Mit39*, as well as an SSLP marker for *Nos2* (due to an additional proline in MOLF/Ei) and an SSCP marker for *Mpo*. *Nos2* primers, 5′-gtatggtgtgaggttatagagatt-3′ and 5′-gtcatgcaaaatctctccactgcc-3′ and *Mpo* primers, 5′-gctcac tcttgcagatgtgttgac-3′ and 5′-tcgccacaggcaagacctagaccc-3′ were used for PCR amplification of genomic DNA. Animals heterozygous for the target region of chromosome 11 were further bred to obtain the next generation of mice. Additional markers—*D1Mit3*, *D1Mit5*, *D1Mit318*, *D1Mit213*, *D1Mit46*, *D1Mit134*, *D1Mit216*, *D1Mit135*, *D1Mit415*, *D1Mit515*, *D1Mit367*, *D1Mit218*, *D1Mit193*, *D1Mit403*, *D1Mit17*, *D11Mit110*, *D11Mit174*, *D11Mit347*, *D11Mit208*, *D11Mit26*, *D11Mit156*, *D11Mit113*, *D11Mit5*, *D11Mit30*, *D11Mit7*, *D11Mit91*, *D11Mit8*, *D11Mit354*, *D11Mit326*, *D11Mit178*, and *D11Mit70*—were used to fine map the congenic boundaries. *Ity3* mice were genotyped using *D1Mit3*, *D1Mit5*, *D1Mcg4*, *D1Mit216*, *D1Mit218*, *D1Mit193*, *D1Mit63*, and *D1Mit17* for PCR amplification at every generation. Additional restriction digests specific for *Slc11a1* and *Ncf2* were also carried out. *Slc11a1* primers 5′-agctatttgggttcctgact-3′ and 5′-ggtcaaagccattatggtaa-3′ specific for the G169D point mutation were used to amplify genomic DNA, followed by a restriction digest using HpyCH4III. The C57BL/6J allele (D169) produces two fragments of 453 bp and 81 bp, whereas the MOLF/Ei allele (G169) is uncut with a fragment of 534 bp. *Ncf2* primers 5′-atgccttacatgctcaaggtg-3′ and 5′-catgctttcttcggacaggagcagaagc-3′ specific for a point mutation (R394Q) were used to amplify genomic DNA. Digestion with *Bsa*HI restriction enzyme was carried out resulting in two products of 278 bp and 41 bp for C57BL/6J allele (R394) and one for the MOLF/Ei allele (Q394) of 319 bp. The digested products were resolved on a 2% agarose gel.

Sequencing: *Slfn1*, *Slfn3*, and *Slfn4* genes were sequenced using spleen cDNA isolated from B6.MOLF-*Ity/Ity2.RecG*, B6.MOLF-*Ity/Ity2.RecD*, and MOLF/Ei strains to further refine the boundaries of the congenic intervals. The entire coding sequence was sequenced for *Slfn1* and primers used include 5′-actgtagctcatccctcaaa-3′, 5′-caattctttgcttcaaaacc-3′, 5′-acgggggatatttgtttatt-3′, 5′-tgttacgaaaagcaagaggt-3′. The coding region of *Slfn3* and *Slfn4* were partially sequenced using the following primers for *Slfn3*, 5′-gcctatgaggagacattctg-3′ and 5′-tttcaacgagagctttttct-3′; and *Slfn4*, 5′-tgtctgcgtttttaaatgtg-3′ and 5′-aaatggcagaacctttgtta-3′.

Infection and survival analysis: Mice between the ages of 6 and 16 weeks were infected intravenously with ~1000 CFUs of Salmonella Typhimurium strain Keller through the caudal vein (Bihl *et al.* 2001; Sancho-Shimizu and Malo 2006). Mice were monitored daily, survival recorded, and moribund animals sacrificed by carbon dioxide asphyxiation. Kaplan-Meier survival analysis was used for statistical analysis.

Linkage analysis: Genetic linkage was carried out using MapManagerQTX on 232 (C57BL/6J × MOLF/Ei)F_2 mice using eight additional markers on chromosome 11: *Il12b* (5′-ttc atgtgctcgtggcctgatcca-3′ and 5′-gtacccttctaaagaaggccctgg-3′), *D11Mit22*, *D11Mit164*, *D11Mit156*, *D11Mit30*, *Nos2*, *Mpo*, and *D11Mit41*.

Bacterial load enumeration: Infected mice were sacrificed on days 3 and 7 in addition to noninfected control mice. Their spleens and livers were aseptically removed and placed in tubes with 0.9% saline solution. The tissues were homogenized and plated on trypticase soy agar plates in duplicate using at least three serial dilutions. Plates were incubated at 37° overnight and the colonies enumerated the following day.

Whole-genome expression profiling: Total RNA was isolated from the spleens of uninfected controls and infected B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity2.RecD* mice on days 3 and 7 postinfection using Trizol reagent (Invitrogen, Carlsbad, CA). RNA from three males and three females was pooled per strain per time point for the first experiment and one male and one female pooled per strain per time point for the second experiment. The quality of the RNA was verified using BioAnalyzer. Then 10 μg of each sample was used to hybridize to the Affymetrix Mouse Chip 430v.2.0 (Affymetrix, Santa Clara, CA). Labeling of the probe, hybridization, and scanning of the microarrays were done at the McGill and Genome Quebec

Innovation Center (Montreal) as previously described (Caron *et al.* 2006).

Construction of *Ity3* interval-specific 70-mer oligonucleotide microarray: Seventy-mer oligonucleotide probes were designed by Scienion AG (Berlin) for all transcripts possessing an accession ID and mapping to the *Ity3* interval (Ensembl v19.30.1 and Celera) delineated by *D1Mit135* and *D1Mit63*, resulting in a total of 375 transcripts. In addition to these probes, also added were probes for six other genes of interest during infection (*Slc11a1*, *Il1b*, *Il6*, *Nos2*, *Mpo*, *Tlr2*), four probes representing housekeeping genes (*18srRNA*, *Hprt*, *Tbp*, *Gapdh*), and two genes as negative controls for infection on the basis of other array experiments carried out in our laboratory (*Actg2*, *Vcam1*). The 70-mer oligos were synthesized for all transcript probes by MetaBion GmbH (Martinsried, Germany). Finally, hybridization controls, two positive (herring sperm DNA, 10 mg/ml; Cot1DNA, 1 mg/ml) and three negative controls (water, 3× SSC, 14× SSC), were added to the panel. Lyophilized oligos were resuspended in 3× SSC and spotted at a concentration of 70 μM on Sigmascreen microarray slides (Sigma, St. Louis) using the Virtek Chipwriter model SDDC2 to print oligonucleotides. The assembled *Ity3* array contained a total of 2304 spots. Each transcript probe was spotted in triplicate (1152 spots), the housekeeping genes and positive hybridization controls were spotted 24 times (144 spots), and the remaining 1008 spots were represented by the negative controls. The quality of the *Ity3* array was verified after each batch of prints using the SpotQC kit (Integrated DNA Technologies, Coralville, IA).

Interval-specific expression profiling: Total RNA from spleens of uninfected and infected B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ and B6.MOLF-*Ity/Ity3* mice (at the fifth generation of backcross) was isolated as described above. Following RNA extraction, residual DNA was removed using the DNAse-I kit from Ambion and subsequent RNAeasy on-column digestion following the manufacturer's instructions (QIAGEN, Mississauga, Canada). The quality of RNA was confirmed by denaturing gel electrophoresis (formaldehyde). Microarray hybridization was performed as previously described (Charlet *et al.* 2005). In brief, 5–20 μg of total RNA extracted from control and infected mice was labeled with Cy3 or Cy5 dUTP by reverse transcriptase (Amersham Biosciences, Piscataway, NJ). Labeled cDNA was applied to a post-processed array, covered with a glass slip, and placed into a hybridization chamber overnight at 42°. Arrays were placed into 37° 1× SSC, 0.2% SDS to remove the cover glass, then washed in 1× SSC, 0.2% SDS for 15 min; 0.1× SSC, 0.2% SDS for 15 min; and 0.1× SSC for 15 min. Hybridized arrays were scanned with ScanArray 5000XL and hybridization results were quantified with ScanArray software (Perkin-Elmer, Freemont, CA). For each hybridization, one RNA sample from an individual B6.MOLF-*Ity/Ity3* $^{MOLF/B6}$ was hybridized against one RNA sample from an individual B6.MOLF-*Ity/Iy3* mouse at each time point per tissue. A single reciprocal Cy-dye swap (Cy3/Cy5 and Cy5/Cy3) experiment was minimally carried out for all hybridizations, resulting in at least a single replicate for each experiment and totaling 11 arrays studied.

Microarray data analysis: For whole-genome microarrays, the expression values were generated by probe-level analyses using the robust multi-array analysis procedure (Irizarry *et al.* 2003). For *Ity3* arrays, analysis was performed as previously described (Charlet *et al.* 2005). All spots flagged as misrepresentative by ScanArray (array artifacts, etc.) were analytically ignored. Subtracting total spot intensity minus the surrounding background produced a corrected spot intensity. Negative corrected spot intensities were set to +1. Intensity ratios (Cy3/Cy5 or Cy5/Cy3) were determined using corrected spot intensities and log10 transformed. Fold change is calculated from a normalized log-ratio of that gene. Values for each gene were obtained in triplicate for each array (inherent to array design) and averaged. Only genes with fold changes of two or greater in replicate hybridization experiments are reported.

Real-time PCR: Expression of *Slfn1*, *Slfn4*, *Slfn5*, *Butr1*, *Cyfip2*, *Hacvr2*, *Itk*, *Sqstm1*, and *Tgtp* in B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity2.RecD*, as well as *Chi3l1*, *Cdc73 Sft2d2*, and *Niban* in B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ was determined by real-time PCR using the Chromo4 Real Time PCR system (MJ Research). cDNAs were obtained from reverse transcription of infected and control spleen RNAs. The cDNAs were amplified using the following primers: *Slfn1* 5′-gggaacg tgctcagtaga-3′ and 5′-cctgcatttagaatcagca-3′, *Slfn4* 5′-aggtttac cacagaggaatg-3′ and 5′-tctggagagcatatcacctt-3′, *Slfn5* 5′-ggcct ctcggatgatagaaa-3′ and 5′-ggtcttgctgcagggtgt-3′, *Butr1* 5′-caga gaaggacactggattc-3′ and 5′-tgtgactgtacatcttgacca-3′, *Cyfip2* 5′-gctttgacctgtttgacttc-3′ and 5′-gtcagccatcttcttcagag-3′, *Hacvr2* 5′-ctggtgaccctccataataa-3′ and 5′-tctgatcgtttctccagagt-3′, *Itk* 5′-tag cagcaagtcagatgtgt-3′ and 5′-ctgatatcttccacgacctc-3′, *Sqstm1* 5′-ctc taggcattgaggttgac-3′ and 5′-ttggctgagtgttactcttg-3′, *Tgtp* 5′-taa agacgttccctaagagg-3′ and 5′-ctctgtatggtagaagctcag-3′, *Cdc73* 5′-aaacgtcacttggatagacc-3′ and tcaagtgggatttatgcttt-3′, *Chi3l1* 5′-tgaagtacatcgactctcca-3′ and 5′-tttgacactctccttgtcct-3′, *Sft2d2* 5′-cgtggtatagcctttcctat-3′ and 5′-ctggcagtgtgttatgcaag-3′, *Niban* 5′-tgtatgagatcctcctcgat-3′ and 5′-gccatgttgtcttcaaataag-3′. *Tbp* was used as a housekeeping gene control and amplified using the primers 5′-cccttgtacccttcaccaat-3′ and 5′-acagccaagattcac ggtag-3′ using the same cycling conditions. The cycling conditions were 95° for 30 sec, 55° for 30 sec, 72° for 30 sec, 80° for 2 sec for a total of 40 cycles. Stratagenes's Brilliant SYBR Green QPCR Master mix was used for the PCR reactions. All samples were run in duplicate along with a standard curve of four 10-fold serial dilutions of template cDNA. The expression data are expressed in relative fold-change units using uninfected B6.MOLF-*Ity/Ity2* or B6.MOLF-*Ity/Ity3* as the referent according to the following $2^{-\Delta\Delta Ct}$ equation $2^{-[(\text{Gene of interest treatment Ct} - \textit{Tbp} \text{ treatment Ct}) - (\text{Gene of interest reference Ct} - \textit{Tbp} \text{ reference Ct})]}$ (Livak and Schmittgen 2001). The level of significance was assessed using the Student's *t*-test ($P < 0.05$).

RESULTS

***Ity2* and *Ity3* linkage analysis:** We have previously reported the existence of two QTL, *Ity2* on chromosome 11 and *Ity3* on distal chromosome 1, affecting the host response of MOLF/Ei mice to infection with Salmonella Typhimurium. The MOLF/Ei allele at *Ity2* confers resistance to infection in an additive fashion, accounting for 10% of the phenotypic variance. The MOLF/Ei allele at *Ity3* was found to contribute to the susceptibility of MOLF/Ei mice recessively and explained 7% of the phenotypic variance (Sebastiani *et al.* 1998). To confirm the location of these QTL, we have added additional progeny as well as extra chromosome 11-specific markers to the existing (C57BL/6J × MOLF/Ei)F_2 panel used in the initial linkage analysis.

For the chromosome 11 QTL harboring *Ity2*, linkage was reanalyzed using eight novel markers (*Il12b*, *D11Mit22*, *D11Mit164*, *D11Mit156*, *Inos*, *Mpo*, and *D11Mit41*) on a total of 232 (C57BL/6J × MOLF/Ei)F_2 mice, including an additional 41 mice and the original set of 191 mice. The *Ity2* locus yielded a significant peak LOD score of 7.8 at *Nos2* under a model of free regression, with a 2-LOD support interval (99% C.I.) spanning *D11Mit112* to *Mpo*

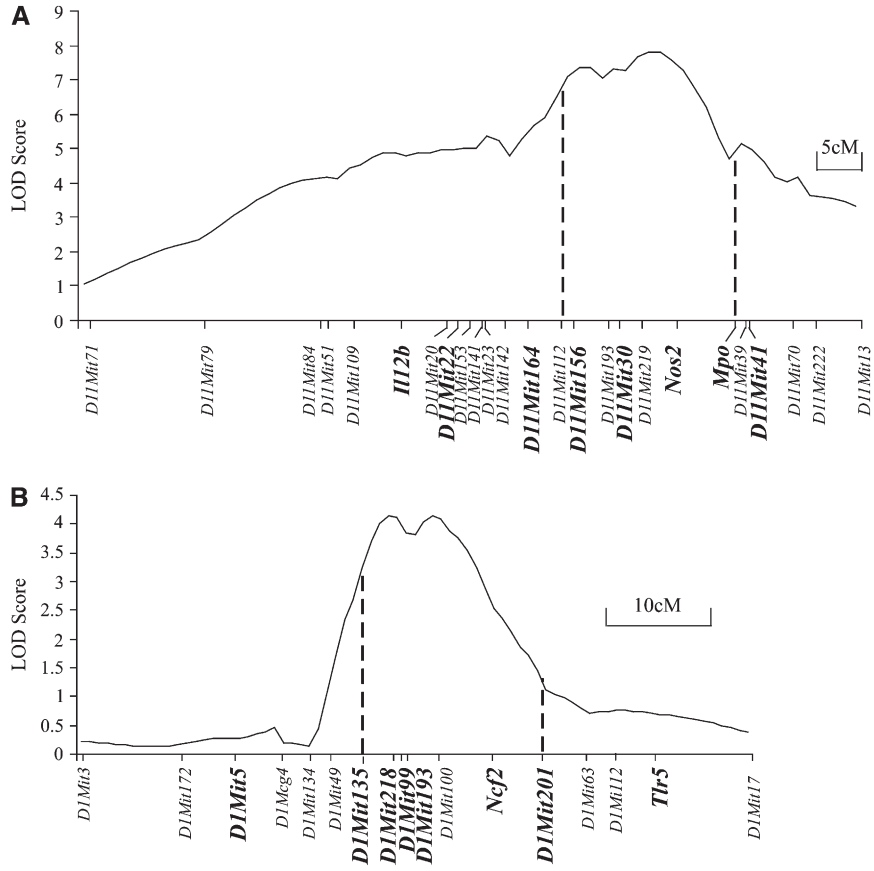


FIGURE 1.—LOD score plots of the *Ity2* and *Ity3* regions in (C57BL/6J × MOLF/Ei)F_2 cross (A and B). The markers in bold are the additional markers used in the linkage analysis of *Ity2* and *Ity3* (C57BL/6J × MOLF/Ei)F_2 cross as assessed by MapManagerQTX (A). The LOD score plot for *Ity3* in (C57BL/6J × MOLF/Ei)F_2 cross is taken from SANCHO-SHIMIZU *et al.* (2006). Dashed lines indicate the boundaries of the 2-LOD support interval of the respective QTL regions.

(Figure 1A). The addition of eight markers and additional F_2 mice led to an increase in the peak LOD score from 7.0 to 7.8 and to a minor repositioning of the relatively large *Ity2* interval as compared to the initial analysis, placing the peak slightly more distally.

Similarly, the *Ity3* QTL on distal chromosome 1 was re-evaluated using eight additional markers, *D1Mit5*, *D1Mit135*, *D1Mit218*, *D1Mit99*, *D1Mit193*, *D1Mit 201*, *Ncf2*, and *Tlr5*, and 41 additional mice, as previously described (SANCHO-SHIMIZU and MALO 2006). The peak LOD score of 4.1 was obtained at *D1Mit218* and *D1Mit100*, encompassing approximately the same 2-LOD support interval from *D1Mit135* to *D1Mit201* as detected in the initial analysis (*D1Mit135* to *D1Mit63*) (Figure 1B) (SEBASTIANI *et al.* 1998).

Generation of congenic mice: Congenic mice were created for *Ity2* and *Ity3* by producing F_1 hybrids between C57BL/6J and MOLF/Ei, followed by at least 10 successive backcross generations to the C57BL/6J parental strain. The target *Ity/Ity2* and *Ity/Ity3* segments were maintained using marker-assisted genotyping. Homozygous founders were established by brother-sister matings of N10 mice. Due to the impact of *Slc11a1* (*Ity*) on the detection of *Ity2* and *Ity3*, congenic B6.MOLF-*Ity* were created by transferring the wild-type allele at *Slc11a1* originating from the MOLF/Ei mice onto a C57BL/6J genetic background, who naturally carry the mutant form of this well-characterized Salmonella susceptibility gene (VIDAL *et al.* 1993). The largest *Ity2* interval transferred spans from *D11Mit110* to *D11Mit91*, a 39.0-Mb interval and the largest *Ity3* interval is 62.0 Mb in size located from *D1Mit218* to *D1Mit17*. Both regions extend well beyond the 2-LOD support interval to ensure that all genetic elements contributing to the QTL-associated phenotype would be transferred to the resulting congenic strains. Moreover, the presence of numerous immunologically relevant genes that map within the relatively large *Ity2* and *Ity3* intervals suggest the possibility that more than one gene may be involved in the disease phenotype underlying the QTL interval.

The recombinant congenics were generated at the same time as the B6.MOLF-*Ity/Ity2* and B6.MOLF-*Ity/Ity3* congenics by selecting mice sharing recombinant chromosomes at the N10 generation, which were further bred to homozygosity through brother–sister matings. This led to the establishment of two recombinant B6.MOLF-*Ity/Ity2* strains (*RecD* and *RecI*) and four recombinant B6.MOLF-*Ity/Ity3* strains (*RecA*, *RecB*, *RecC*, and *RecE*) shown in Figure 2, A and B.

Phenotypic characterization of *Ity2* congenic strains: All congenic strains were infected with Salmonella Typhimurium intravenously and their survival noted. As expected, the C57BL/6J mice were the most susceptible to infection and the transferal of a wild-type MOLF/Ei allele at *Slc11a1* (B6.MOLF-*Ity*) improved resistance to infection significantly (Figure 3A) (SANCHO-SHIMIZU

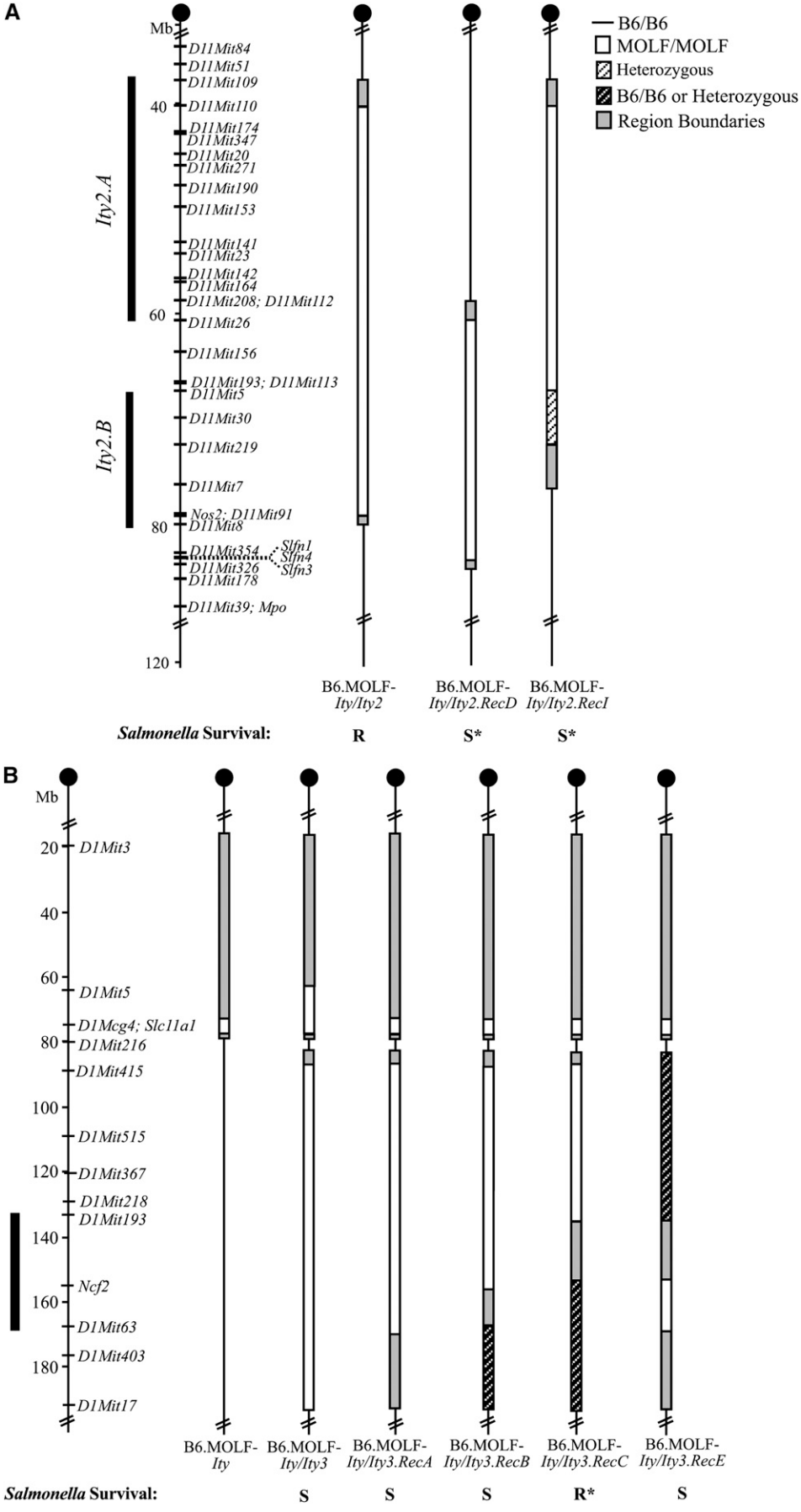


FIGURE 2.—Fine mapping of *Ity2* and *Ity3* loci. A schematic of the *Ity2* congenics on chromosome 11 (A) and the *Ity3* congenics on chromosome 1 (B). The open box represents regions of MOLF/Ei homozygosity (MOLF/MOLF), the solid line regions of C57BL/6J homozygosity (B6/B6), and the hatched segment on white background, heterozygous intervals. Congenics, including individuals with intervals of C57BL/6J homozygosity and heterozygosity (B6/B6 or heterozygous), are indicated by hatched segments on black background. Unresolved intervals at the boundaries of congenic fragments, where the genotypes have not been fixed or remain undetermined, are indicated by shaded boxes. All *Ity2* congenic mice also carry the MOLF/Ei congenic fragment, *Ity*, on chromosome 1. The bold line by the chromosomes indicates the restricted interval defined by phenotyping the congenic mice. The relative susceptibility of each congenic strain is indicated at the bottom of each chromosome as compared to the B6.MOLF-*Ity* referent (R, more resistant; S, more susceptible). An asterisk by the R or S indicates that the survival does not differ from the B6.MOLF-*Ity* referent strain.

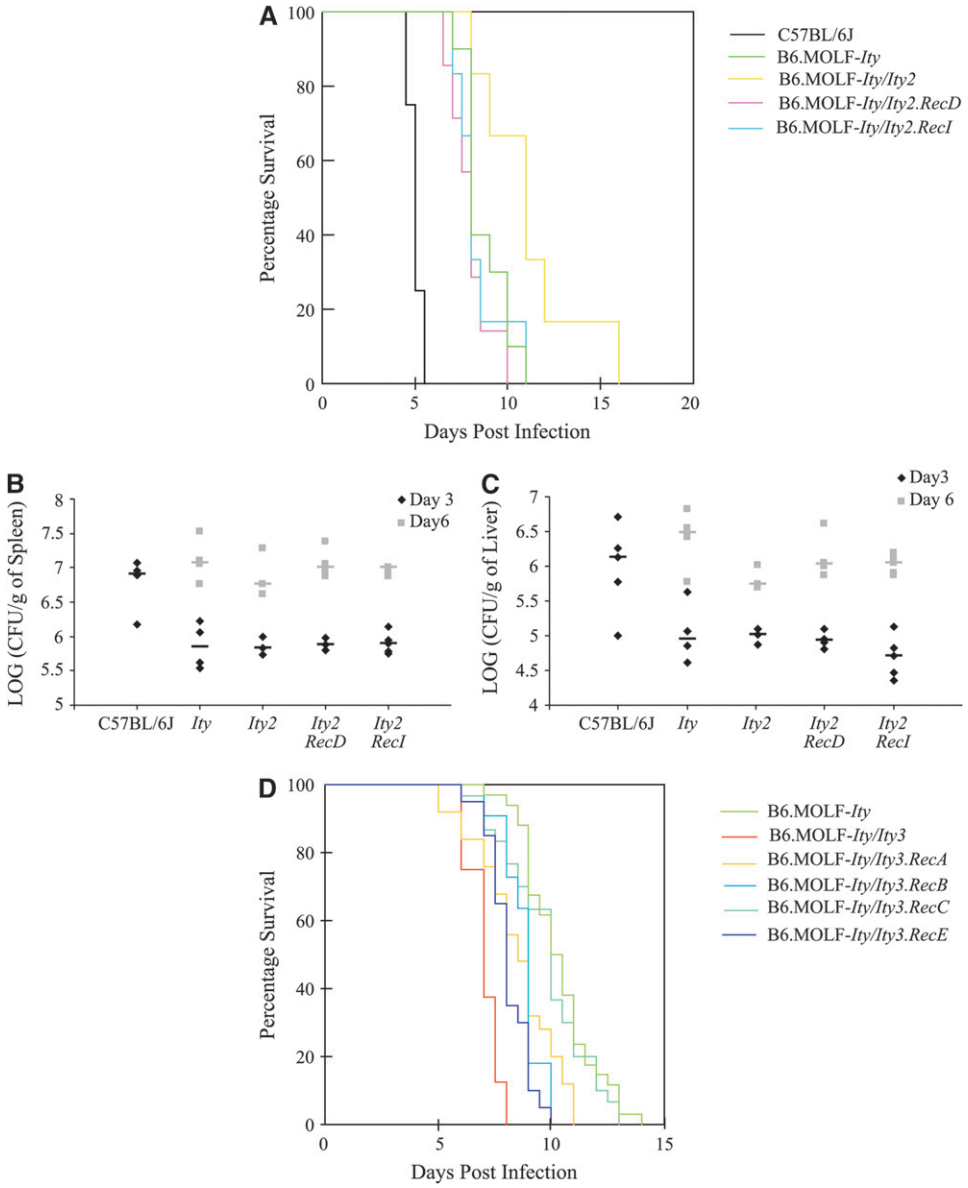


FIGURE 3.—Phenotypic characterization of the *Ity2* and *Ity3* congenic mice. Survival curves for *Ity2* congenic mice are shown in A. One representative survival experiment of seven is shown here. There were at least six mice in each group of mice tested. Bacterial load in the spleen (B) and the liver (C) of the *Ity2* congenics are represented as log (CFU/g of organ). Groups of three to four mice were used for CFU enumeration for each time point; median for each group is shown as a bar. Survival of *Ity3* congenic mice is shown in D. Samples from three independent experiments using at least eight mice per group were pooled and represented here.

and MALO 2006). Survival analysis revealed that only the B6.MOLF-*Ity/Ity2* congenic improved survival time over the control B6.MOLF-*Ity* mice (Figure 3A and Table 1). The remaining *Ity2* congenic strains, B6.MOLF-*Ity/Ity2.RecD* and B6.MOLF-*Ity/Ity2.RecI*, did not differ significantly from the B6.MOLF-*Ity* controls, suggesting that the portion of *Ity2* that these mice carry does not contribute to improved resistance.

In an attempt to further sub-phenotype the *Ity2* congenic mice, the bacterial load in the spleen and liver was determined at various time points upon infection. C57BL/6J mice had ~10-fold higher bacterial loads in the spleen and liver at day 3 compared to the congenic mice and no C57BL/6J mice survived after day 5. This observation is attributable to the fact that these mice carry a nonfunctional mutation in *Slc11a1*, as previously reported (VIDAL *et al.* 1995; SANCHO-SHIMIZU and MALO 2006). No significant difference was observed between any of the *Ity2* congenics and the B6.MOLF-*Ity* controls at all time points (Figure 3, B and C), strongly suggesting that the impact of *Ity2* on survival to infection is not related to the level of bacterial load in these target organs.

Consistent with previously published results, survival of B6.MOLF-*Ity/Ity3* mice was significantly reduced in comparison to the B6.MOLF-*Ity* control (Figure 3D and Table 2). Data presented here represent B6.MOLF-*Ity/Ity3* mice that have been backcrossed for over 10 generations as opposed to previously published data that tested B6.MOLF-*Ity/Ity3* mice intercrossed after 5 generations (SANCHO-SHIMIZU and MALO 2006). The survival analysis represents three independent experiments that have been pooled since no significant difference was observed in survival among the B6.MOLF-*Ity* mice in all

TABLE 1

***Ity2* matrix of pairwise comparison probabilities using the logrank test (Breslow-Gehan) of Kaplan-Meier survival curves**

	C57BL/6J	*Ity*	*Ity/Ity2*	*RecD*	*RecI*
C57BL/6J	1.000				
Ity	<0.001	1.000			
Ity/Ity2	0.002	0.036	1.000		
RecD	0.001	0.193	0.012	1.000	
RecI	0.002	0.488	0.03	0.616	1.000

experiments (mean survival time, MST = 10.7 ± 1.3; MST = 10.1 ± 0.4; and MST = 9.8 ± 0.3 days). B6.MOLF-*Ity/Ity3.RecC* did not differ significantly from the B6.MOLF-*Ity* controls, suggesting that this portion of chromosome 1 did not contribute to the susceptibility phenotype, whereas all other *Ity3* recombinant congenics were more susceptible to infection. On the basis of these analyses, the B6.MOLF-*Ity/Ity3* congenics were more susceptible than *RecA*, *RecB*, *RecC*, and *RecE* (Table 2). In fact, B6.MOLF-*Ity/Ity3.RecA*, *RecB*, and *RecE* have an intermediate phenotype as they were significantly different from B6.MOLF-*Ity* controls as well from B6.MOLF-*Ity/Ity3* mice. However, the congenic harboring the largest MOLF interval, B6.MOLF-*Ity/Ity3*, was the most susceptible to Salmonella infection, suggesting the involvement of more than one gene within the *Ity3* interval.

Refinement of *Ity2* and *Ity3* critical intervals: The centromeric boundary of the congenic *Ity2* interval in B6.MOLF-*Ity/Ity2* was resolved to a 2.3-Mb region between *D11Mit110* and *D11Mit109*. The distal end was delimited by a 790-kb region located between *D11Mit91* and *D11Mit8*. The *Ity2 RecD* interval was resolved proximally by a 1.9-Mb region between *D11Mit112* and *D11Mit26* and distally by a 600-kb interval delimited by *Slfn3* and *D11Mit326*. On the basis of the survival analysis of the *Ity2* congenics, we were able to further restrict the large interval to two more defined regions (Figure 2A, *Ity2.A* and *Ity2.B*), which are both necessary but not sufficient in the resistance phenotype. The two intervals (*Ity2.A* and *Ity2.B*) are present in the resistant strain B6.MOLF-*Ity/Ity2* and one of the two regions absent in the susceptible strains B6.MOLF-*Ity/Ity2.RecD* (missing *Ity2.A*) and B6.MOLF-*Ity/Ity3.RecI* (missing *Ity2.B*). The proximal region, *Ity2.A*, is flanked by *D11Mit109* and *D11Mit26*, an interval spanning ~23 Mb, encoding for 331 genes according to the latest update of the Ensembl genome browser (Ensembl build 43). The distal region, *Ity2.B*, is flanked by *D11Mit5* and *D11Mit8*, an interval of ~13 Mb composed of 364 genes (Ensembl build 43). Even though the survival data seem to point out these two particular regions as critical areas of interest, the region between these two intervals cannot be disregarded in any future analysis and may harbor additional genes affecting the phenotype.

TABLE 2

***Ity3* matrix of pairwise comparison probabilities using the logrank test (Breslow-Gehan) of Kaplan-Meier survival curves**

	Ity	*Ity/Ity3*	*RecA*	*RecB*	*RecC*	*RecE*
Ity	1.000					
Ity/Ity3	<0.001	1.000				
RecA	<0.001	0.01	1.000			
RecB	0.001	<0.001	0.699	1.000		
RecC	0.295	<0.001	0.017	0.054	1.000	
RecE	<0.001	0.002	0.316	0.066	<0.001	1.000

The B6.MOLF-*Ity/Ity3* congenics carry the *Ity3* interval spanning 103 Mb between *D1Mit415* and *D1Mit17*, with a proximal boundary between *D1Mit216* and *D1Mit415* (Figure 2B). The *Ity* interval in B6.MOLF-*Ity/Ity3* mice spans *D1Mcg4* to *D1Mit5* with region boundaries extending proximally to *D1Mit3* and distally to *D1Mit216*. The *Ity* interval in the B6.MOLF-*Ity* and B6.MOLF-*Ity/Ity3* recombinant strains (*RecA*, *RecB*, *RecC*, and *RecE*) are defined by MOLF/Ei alleles at *D1Mcg4* with region boundaries spanning *D1Mcg4* to *D1Mit3* and *D1Mcg4* to *D1Mit216*. The survival analysis of the recessively inherited susceptibility locus *Ity3* suggests that the region of 44.8 Mb between *D1Mit415* and *D1Mit193* in B6.MOLF-*Ity/Ity3.RecC* strain does not contribute to the susceptibility of the mice and could be eliminated as harboring genes for *Ity3* susceptibility. The remaining *Ity3* strains were all variably susceptible to infection, suggesting they all carry intervals necessary for the manifestation of the phenotype. The smallest interval can be deduced from the overlap between the *Ity3 RecB* and *RecE* strains, surrounding *Ncf2* and delineated by *D1Mit193* and *D1Mit63* representing the critical interval (Figure 2B). Within this 34-Mb critical interval, there are a total of 322 genes according to Ensembl build 43.

***Ity2* interval-specific expression profiling:** We next applied transcriptional profiling to help the identification of candidate genes underlying *Ity2* and *Ity3*. Disease QTL have previously been shown to be linked to the heritability of variation in gene expression of positional candidate genes (Aitman *et al.* 1999; Hubner *et al.* 2005). Expression profiles of spleens from B6.MOLF-*Ity/Ity2* (resistant) and B6.MOLF-*Ity/Ity2.RecD* (susceptible) mice were interrogated using the Affymetrix Mouse 430v2.0 chip at days 0, 3, and 7 of infection (Table 3, Figure 4A). Genes that differed by twofold and mapped to chromosome 11 were the primary focus of analysis. Differentially regulated genes were clustered within two chromosomal regions (*D11Mit110–D11Mit26* and *Nos2–D11Mit178*) of different parental origin in the two congenic strains (Figure 4A). The proximal region delineated by *D11Mit110* and *D11Mit26* corresponds to the critical minimal *Ity2.A* interval, although the distal

region (*Nos2–D11Mit178*) does not. No genes located within the *Ity2.B* interval were differentially regulated under the conditions specified.

Among those differentially regulated, there were a total of five genes that were differentially expressed at all time points (Table 3, Figure 4A). Three genes, *Slfn8*, *Crlf3*, and *Ccdc16*, were consistently expressed at higher levels in the resistant B6.MOLF-*Ity/Ity2* strain and two genes, *Zfp207* and *Sqstm1*, had lower expression at all time points. Of these consistently differentially regulated genes, only *Sqstm1* (sequestosome 1) was mapped to the critical minimal *Ity2.A* interval. *Sqstm1* is involved in the polyubiquitination of TRAF6 (TNF receptor-associated factor 6) and in the regulation of NF-κB signaling (Babu *et al.* 2005). Many of the pathways that lead to the upregulation of pro-inflammatory cytokine expression are dependent on proper NF-κB signaling, suggesting a role for *Sqstm1* in the immune response.

In control mice (day 0), there were a total of 12 chromosome 11-specific genes that were differentially regulated (Table 3). Of the 12 genes, 7 were located within the minimal critical *Ity2.A* interval and included in addition to *Sqstm1*, *Zfp62* (zinc-finger protein 62), *Itk* (IL-2 inducible T-cell kinase), *Cyfip2* (cytoplasmic FMR1 interacting protein 2), and three ESTs. The gene *Itk* is clearly involved in T-cell activation (Au-Yeung *et al.* 2006) and *Cyfip2* may also play a role in T-cell regulation, on the basis of recent work showing that high expression of *CYFIP2* in multiple sclerosis patients is associated with increased T-cell adhesion (Mayne *et al.* 2004).

At day 3, we detected an increase in the number of differentially regulated genes on chromosome 11 for a total of 22 genes, of which 14 had lower expression and 8 genes had higher expression levels in B6.MOLF-*Ity/Ity2* mice. Ten genes were located within the minimal *Ity2.A* interval, including several genes involved in the regulation of transcription (*Ublcp1*, *Ankrd43*, *Hist3h2ba*) and genes with potential relevance to the immune response, *Hmmr* (hyaluronan mediated motility receptor), *Tgtp* (T-cell specific GTPase), and *Butr1* (butyrophilin related 1). *Hmmr* is a receptor involved in cell motility and in various kinase signaling cascades, including the ERK1 kinase (Turley *et al.* 2002), *Tgtp* is implicated in anti-viral responses (Carlow *et al.* 1998), and *Butr1* according to UniProtKB/Swiss-Prot (http://ca.expasy.org/sprot/) is predicted to be a member of the immunoglobulin gene family.

At day 7, there were 5 transcripts with reduced expression and 12 transcripts with increased expression in B6.MOLF-*Ity/Ity2* congenics, of which 7 were located within the minimal *Ity2.A* interval. We observed increased expression of two additional transcripts involved in T-cell activation, *Tgtp* and *Havcr2*, a gene involved in dampening T_H1 immune responses and immunological tolerance (Carlow *et al.* 1998; Sanchez-Fueyo *et al.* 2003).

For all time points, the genes with lower expression levels were almost exclusively found in the proximal portion of the minimal *Ity2.A* interval and high transcriptional activity was observed at the distal limit of the *Ity2* QTL interval, outside of the critical interval. This distal region contains members of the Schlafen family of genes (*Slfn1*, *Slfn4*, *Slfn5*, *Slfn8*, and *Slfn10*) that were expressed at higher levels at different time points during infection in the resistant B6.MOLF-*Ity/Ity2* mice. *Slfn1*, and more recently *Slfn8*, has been demonstrated to be involved in the negative regulation of peripheral T-cell growth (Schwarz *et al.* 1998; Geserick *et al.* 2004). The differential regulation observed here may represent either epigenetic effects on gene expression due to rearrangement in the chromatin structure at the limit of the congenic interval or downstream *cis* effects due to the *Ity2* QTL gene(s).

Validation of *Ity2* microarray data: In general, alleles of MOLF/Ei origin present lower levels of expression for several genes located within the *Ity2* QTL interval. We have measured mRNA levels of specific transcripts by quantitative PCR (QPCR) in the congenic strains to validate the observed differential expression in congenic mice and to determine if the interstrain differences in gene expression may be caused by the high genetic diversity known to be present between MOLF/Ei and C57BL/6J mice. We initially focused our validation on the *Slfn* family of genes that were consistently expressed at higher levels in the resistant B6.MOLF-*Ity/Ity2* mice. We first sequenced the target region for *Slfn1* and found numerous sequence variants between MOLF/Ei and C57BL/6J alleles that could have interfered with probe binding, accounting for the apparent differential expression observed using the microarray. Primers were then carefully designed to ensure that MOLF/Ei and C57BL/6J alleles were appropriately amplified by QPCR for all genes tested. We confirmed an expression difference for *Slfn1*, *Slfn4*, and *Slfn5* (Figure 5). The expression of all genes was significantly increased in B6.MOLF-*Ity/Ity2* during infection at day 3. Consistent with microarray results, *Slf5* expression was higher in B6.MOLF-*Ity/Ity2* mice on day 3, as was *Slfn4* on day 7 but also on day 3. *Slfn1* results did not correlate with microarray data due to the numerous sequence variants within the Affymetrix probe.

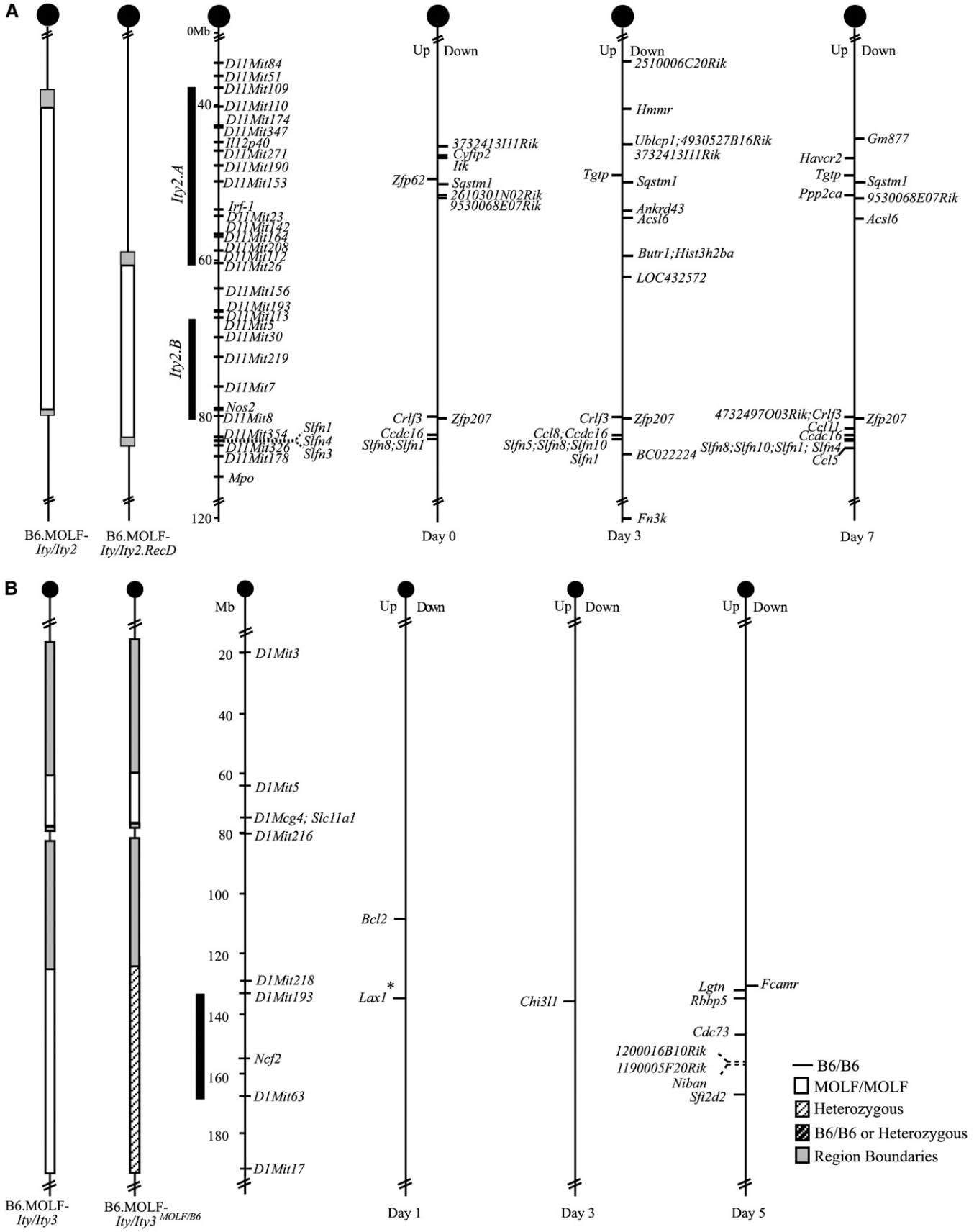
Six positional candidate genes (*Sqstm1*, *Itk*, *Cyfip2*, *Butr1*, *Tgtp*, and *Havcr2*) have been selected on the basis of their differential levels of expression and on their potential role in the host immune response for QPCR analyses. QPCR of *Cypfip2*, *Itk*, *Butr1*, *Tgtp*, and *Sqstm1* expression revealed results that were inconsistent with the microarray data (data not shown). Interestingly, four of these genes, with the exception of *Tgtp*, were all found to be expressed at lower levels in the MOLF-derived allele represented in the B6.MOLF-*Ity/Ity2* congenic, suggesting that the probes for these genes may contain sequence variants that affect hybridization as observed in the *Slfn* genes. All genes tested were either induced or downregulated upon infection. QPCR validation

TABLE 3

***Ity2* whole genome array list of differentially regulated genes on chromosome 11**

Up/down[a]	Affymetrix ID	Gene name	Position (Mb)[b]	Fold change	Function
			Day 0		
Up	1451655_at	*Slfn8*	82.8	10.8	T cell development
	1438235_at	*Crlf3*	79.9	8.0	Cytokine receptor
	1418612_at	*Slfn1*	83.1	5.6	T cell development
	1450942_at	*Ccdc16*	82.6	3.9	Cell cycle
	1451730_at	*Zfp62*	49.1	2.2	Skeletal muscle
Down	1423546_at	*Zfp207*	80.2	−3.3	Metal binding nuclear protein
	1440076_at	*Sqstm1*	50.0	−3.3	Regulates NF-κB signaling
	1443435_at	*3732413I11Rik*	44.4	−3.3	Ubiquitin conjugation
	1456836_at	*Itk*	46.2	−2.5	T cell development
	1430177_at	*2610301N02Rik*	51.8	−2.5	Unknown
	1427108_at	*9530068E07Rik*	52.2	−2.0	Unknown
	1449273_at	*Cyfip2*	46.0	−2.0	Fragile X/T cell adhesion
			Day 3		
Up	1451655_at	*Slfn8*	82.8	15.6	T cell development
	1438235_at	*Crlf3*	79.9	7.1	Cytokine receptor
	1450942_at	*Ccdc16*	82.6	5.5	Cell cycle
	1418612_at	*Slfn1*	82.9	5.1	T cell development
	1425728_at	*Tgtp*	48.7	3.4	T cell GTPase
	1419684_at	*Ccl8*	81.9	2.3	Chemotaxis
	1458458_at, 1456288_at	*Slfn5*	82.8	2.2, 2.0	T cell development
	1444350_at	*Slfn10*	82.8	2.0	T cell development
Down	1451257_at	*Acsl6*	54.1	−3.3	Fatty acid metabolism
	1445845_at	*Ublcp1*	44.3	−3.3	RNA polymerase II CTD
	1423546_at	*Zfp207*	80.2	−3.3	Metal binding nuclear protein
	1429871_at, 1427541_x_at	*Hmmr*	40.5	−3.3, −2.5	Cell motility/ERK kinase
	1429606_at	*4930527B16Rik*	44.3	−2.5	Unknown
	1436998_at	*Ankrd43*	53.3	−2.5	DNA binding
	1418311_at	*Fn3k*	121.3	−2.5	Fructosamine kinase
	1419074_at	*2510006C20Rik*	30.9	−2.5	Unknown
	1421264_at	*Butr1*	58.7	−2.5	Immunoglobulin-like
	1449482_at	*Hist3h2ba*	58.8	−2.5	Histone/nucleosome
	1440076_at	*Sqstm1*	50.0	−2.5	Regulates NF-κB signaling
	1458440_at	*LOC432572*	62.0	−2.5	Unknown
	1443435_at	*3732413I11Rik*	44.4	−2.0	Ubiquitin conjugation
	1425704_at	*BC022224*	84.6	−2.0	Short chain dehydrogenase/reductase
			Day 7		
Up	1451655_at	*Slfn8*	82.8	10.2	T cell development
	1418612_at	*Slfn1*	82.9	4.5	T cell development
	1425728_at	*Tgtp*	48.7	3.8	T cell GTPase
	1438235_at	*Crlf3*	79.9	3.8	Cytokine receptor
	1418126_at	*Ccl5*	83.3	3.0	T cell/macrophage chemokine
	1450942_at	*Ccdc16*	82.6	3.0	Cell cycle
	1417789_at	*Ccl11*	81.9	2.4	Eosinophil chemokine
	1451584_at	*Havcr2*	46.3	2.3	Macrophage/TH1 response
	1444875_at	*Ppp2ca*	51.9	2.1	Kinase
	1424501_at	*4732497O03Rik*	79.8	2.0	RNA processing
	1444350_at	*Slfn10*	82.8	2.0	T cell development
	1427102_at	*Slfn4*	83.0	2.0	T cell development
Down	1440076_at	*Sqstm1*	50.0	−3.3	Regulates NF-κB signaling
	1436789_at	*Ccnjl*	43.4	−2.0	Cyclin/cell cycle
	1423546_at	*Zfp207*	80.2	−2.0	Metal binding nuclear protein
	1427108_at	*9530068E07Rik*	52.2	−2.0	Unknown
	1451257_at	*Acsl6*	54.1	−2.0	Fatty acid metabolism

[a] Up- or downregulated in B6.MOLF-*Ity/Ity2* compared to B6.MOLF-*Ity/Ity2.RecD*.
[b] Physical map positions based on Ensembl build 39.


A
0Mb
D11Mit84
D11Mit51
D11Mit109
40
D11Mit110
D11Mit174
D11Mit347
Il12p40
D11Mit271
D11Mit190
D11Mit153
Irf-1
D11Mit23
D11Mit142
D11Mit164
D11Mit208
D11Mit112
60
D11Mit26
D11Mit156
D11Mit193
D11Mit113
D11Mit5
D11Mit30
D11Mit219
D11Mit7
Nos2
80
D11Mit8
D11Mit354
D11Mit326
D11Mit178
Mpo
120
Ity2.A
Ity2.B
Slfn1
Slfn4
Slfn3
B6.MOLF-Ity/Ity2
B6.MOLF-Ity/Ity2.RecD
Up Down
Zfp62
3732413I11Rik
Cyfip2
Itk
Sqstm1
2610301N02Rik
9530068E07Rik
Crlf3
Zfp207
Ccdc16
Slfn8;Slfn1
Day 0
2510006C20Rik
Hmmr
Ublcp1;4930527B16Rik
3732413I11Rik
Tgtp
Sqstm1
Ankrd43
Acsl6
Butr1;Hist3h2ba
LOC432572
Ccl8;Ccdc16
Slfn5;Slfn8;Slfn10
Slfn1
BC022224
Fn3k
Day 3
Gm877
Havcr2
Tgtp
Sqstm1
Ppp2ca
9530068E07Rik
Acsl6
4732497O03Rik;Crlf3
Ccl11
Ccdc16
Slfn8;Slfn10;Slfn1; Slfn4
Ccl5
Day 7
B
Mb
20
D1Mit3
40
60
D1Mit5
D1Mcg4; Slc11a1
80
D1Mit216
100
120
D1Mit218
D1Mit193
140
Ncf2
160
D1Mit63
180
D1Mit17
B6.MOLF-Ity/Ity3
B6.MOLF-Ity/Ity3 MOLF/B6
Bcl2
*
Lax1
Day 1
Chi3l1
Day 3
Lgtn
Fcamr
Rbbp5
Cdc73
1200016B10Rik
1190005F20Rik
Niban
Sft2d2
Day 5
B6/B6
MOLF/MOLF
Heterozygous
B6/B6 or Heterozygous
Region Boundaries

was confirmed for *Havcr2* demonstrating a difference in the regulation of this gene upon infection (Figure 6A). In B6.MOLF-*Ity/Ity2* mice there was an upregulation during the course of infection whereas this gene was not induced upon infection in B6.MOLF-*Ity/Ity2.RecD* mice.

***Ity3* interval-specific expression profiling:** To investigate expression differences in the *Ity3* interval, we employed a slightly different approach by creating a custom oligonucleotide array including probes representing genes that map within the *Ity3* interval. The assembled array included 375 genes mapping between the markers *D1Mit135* and *D1Mit63* (based on Ensembl build v19.30.1), which corresponds to the region approximating the 2-LOD support interval. The quality of the array hybridization experiments was confirmed by positive hybridization signals emitted by the positive controls (*18srRNA*, *Hprt*, *Tbp*, *Gapdh*, herring sperm DNA, cot1 DNA). Spleen RNA from B6.MOLF-*Ity/Ity3* (N5) and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ (N5) uninfected (day 0), day 1, day 3, and day 5 Salmonella Typhimurium-infected mice was hybridized to the arrays for analysis. The recessively inherited susceptibility phenotype that the *Ity3* locus confers has previously been established using B6.MOLF-*Ity/Ity3* and B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mice such that B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ were more resistant to infection, as were the B6.MOLF-*Ity* mice shown in Figure 3D (Sancho-Shimizu and Malo 2006). Probes that were differentially regulated by twofold in dye swap experiments were considered for further analysis.

No genes were found to be differentially regulated at day 0; however, two genes, *Bcl2* (B-cell leukemia/lymphoma 2) and *Lax1* (lymphocyte transmembrane adaptor 1) (Table 4 and Figure 4B), had higher expression levels in the susceptible B6.MOLF-*Ity/Ity3* mice 1 day after infection with Salmonella Typhimurium. *Bcl2* is involved in suppressing apoptosis and it has been implicated in apoptosis associated with infections; however, this gene was found outside the critical candidate region for *Ity3* (Kroemer 1997; Rios-Barrera *et al.* 2006). *Lax1* has yet to be mapped definitively on the Ensembl mouse genome browser build 43; however, it is located on human 1p32.1, placing it on mouse chromosome 1 around 135.5 Mb within the *Ity3* minimal interval, making it an excellent candidate gene on the basis of its role in lymphocyte signaling (Zhu *et al.* 2002).

Only one gene, chitinase 3-like 1 (*Chi3l1*), had higher expression levels in the B6.MOLF-*Ity/Ity3* mice on day 3. *Chi3l1* is located within the critical *Ity3* interval and has been shown recently to mediate bacterial adhesion and invasion in intestinal epithelial cells and to influence the outcome of oral Salmonella Typhimurium infections in mice, making it an interesting candidate gene to pursue (Mizoguchi 2006).

Seven genes had increased expression and one gene had lower expression on day 5 of infection in B6.MOLF-*Ity/Ity3* mice as detected by array experiments. The only gene to have lower expression in B6.MOLF-*Ity/Ity3* mice was *Fcamr* (FcαμR or Fc receptor, IgA, IgM, high affinity), the Fc receptor responsible for binding to IgM and IgA; however, it was found to be outside the critical interval (Shibuya *et al.* 2000). Of the seven genes with higher expression, six mapped within the critical *Ity3* interval identified through fine mapping and include *Rbbp5* (retinoblastoma-binding protein 5), *Cdc73* (also known as *Hprt2*), *Sft2d2* (SFT2 domain containing 2), *Niban*, *1200016B10Rik*, and *1190005F20Rik* (Table 2 and Figure 4B). Among these genes, only *Sft2d2* presented with a relevant putative function in Salmonella infection, as it is predicted to be involved in the retrograde vesicle transport by similarity to the yeast Sft2p protein (Conchon *et al.* 1999).

Validation of *Ity3* microarray data: On the basis of position and function, four differentially regulated genes, *Chi3l1*, *Cdc73*, *Sft2d2*, and *Niban*, were further interrogated by QPCR. *Cdc73* and *Niban* genes were constitutively expressed and not affected by infection whereas *Sft2d2* was downregulated and *Chi3l1* induced upon infection (data not shown and Figure 6B). Of those tested, only *Chi3l1* was confirmed by QPCR, showing elevated expression levels in B6.MOLF-*Ity/Ity3* spleens throughout infection but only significantly differing from B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$ mRNA levels on day 5, unlike the array data which found a difference at day 3 (Figure 6B). The discrepancy in these results may be due to the difference in the sensitivities of the two expression assays used, arrays *vs.* QPCR.

DISCUSSION

Ity2 and *Ity3* are two QTL implicated in survival to systemic Salmonella Typhimurium infection that have been identified in the wild-derived inbred mouse MOLF/Ei (Sebastiani *et al.* 1998).We have used congenic mouse strains in combination with expression profiling to prioritize candidate genes for each QTL, an approach that has been used successfully in finding disease-causing genes in QTL mapping studies by other groups (Rozzo *et al.* 2001; Gu *et al.* 2002; Mcbride *et al.* 2003; Klein *et al.* 2004; Johannesson *et al.* 2005; De Buhr *et al.* 2006). A weakness of the expression profiling approach to keep in mind, however, is that the causative effect underlying quantitative traits, such as survival to Salmonella Typhimurium infection, may not be due to differential gene

Figure 4.—Location of differentially expressed genes on chromosome 11, *Ity2* (A), and on chromosome 1, *Ity3* (B) during infection. Genes found to be upregulated in the resistant B6.MOLF-*Ity/Ity2* in A or B6.MOLF-*Ity/Ity3* strain in B are on the left of the chromosome and downregulated genes on the right. The asterisk indicates that the position of *Lax1* is approximated on the basis of the fact that it is syntenic to human 1q32.

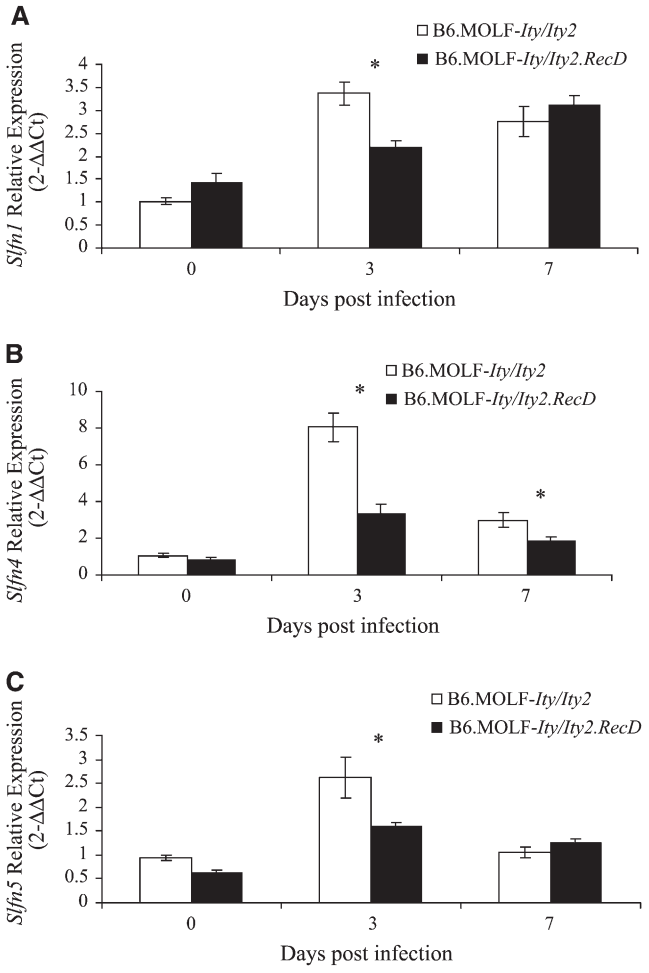


FIGURE 5.—Real-time PCR validation of the *Slfn* cluster of genes during infection. Expression was determined using uninfected B6.MOLF-*Ity/Ity2* mice as the referent. All values are expressed as $2^{-\Delta\Delta Ct}$ units, where the referent is set to 1 and the housekeeping gene used was *Tbp*. Statistical significance was assessed using the Student's *t*-test and is indicated by an asterisk ($P < 0.05$). *Slfn1* (A), *Slfn4* (B), and *Slfn5* (C) are shown here.

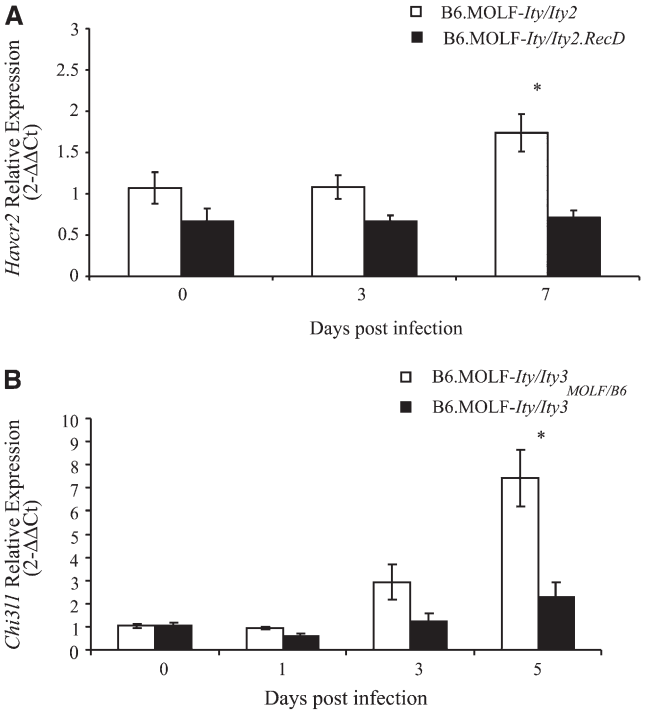


FIGURE 6.—Real-time PCR validation of genes confirmed as differentially regulated in *Ity2* (A) and *Ity3* (B). Expression was determined using uninfected B6.MOLF-*Ity/Ity2* or B6.MOLF-*Ity/Ity3* mice as the referent. All values are expressed as $2^{-\Delta\Delta Ct}$ units, where the referent is set to 1 and the housekeeping gene used was *Tbp*. Statistical significance was assessed using the Student's *t*-test and is indicated by an asterisk ($P < 0.05$). *Ity2* candidate *Havcr2* (A) and *Ity3* candidate *Chi3l1* (B) are shown here.

expression and this approach may be associated with the potential risk of not identifying the disease gene(s). In fact, several important Salmonella-susceptibility loci including *Slc11a1* and *Tlr4* are not regulated at the transcript levels (VIDAL *et al.* 1993; QURESHI *et al.* 1999). On the other hand, differential expression of a candidate gene does not necessarily imply causation. An excellent example of this situation is provided by the gene *Tlr5* in Salmonella susceptibility of MOLF/Ei mice. *Tlr5* was clearly shown to be downregulated in the liver of MOLF/Ei mice; however, *in vitro* and *in vivo* functional analyses clearly showed that *Tlr5* was not the gene underlying the *Ity3* locus (SEBASTIANI *et al.* 2000; ANGERS *et al.* 2006). Despite these limitations, the use of congenic mice in microarray analyses remains extremely valuable in the prioritization of candidate genes for future analysis.

We have confirmed the genetic effect of *Ity2* on resistance to infection and *Ity3* on susceptibility to infection using congenic and subcongenic mouse strains. These mouse strains were also very useful to map *Ity2* and *Ity3* more precisely and in defining the minimal critical region to search for candidate genes. Congenics carrying the chromosome 11 *Ity2* interval as well as *Ity3* on distal chromosome 1 were created in the context of a protective allele at *Slc11a1* within *Ity* (SANCHO-SHIMIZU and MALO 2006). B6.MOLF-*Ity/Ity2* congenics showed improved survival as compared to the B6.MOLF-*Ity* controls, consistent with the prediction from linkage analyses, in which the MOLF/Ei allele at *Ity2* conferred protection and was inherited additively (SEBASTIANI *et al.* 1998). The two subcongenics, B6.MOLF-*Ity/Ity2.RecD* and B6.MOLF-*Ity/Ity2RecI*, enabled us to identify the minimal intervals necessary for resistance, *Ity2.A* and *Ity2.B*, suggesting the involvement of at least two loci. *Ity2.A* covers a 22-Mb proximal portion spanning *D11Mit109* to *D11Mit26* containing 331 genes, and *Ity2.B* consists of a 13-Mb distal interval from *D11Mit5* to *D11Mit8* including 364 genes.

B6.MOLF-*Ity/Ity3* and subcongenics were used to further delineate the critical *Ity3* interval to 34 Mb composed of 320 genes. The region boundaries marked by *D1Mit415* and *D1Mit216*, as well as region boundaries surrounding the *Ity* interval, will be resolved further in future experiments, as their contribution to the susceptibility

TABLE 4

***Ity3* interval specific oligonucleotide array list of differentially regulated genes on chromosome 1 in spleen**

Up/down[a]	Accession ID	Gene name	Position (Mb)[b]	Fold change (average ± SEM)	Function
			Day 1		
Up	NM_172842	*Lax1*	[c]	2.7 ± 0.7	Regulates TCR/BCR signaling
	NM_009741	*Bcl2*	108.4	2.3 ± 0.2	Suppresses apoptosis
			Day 3		
Up	NM_007695	*Chi3l1*	136.0	2.4 ± 0.3	Bacterial adhesion/invasion
			Day 5		
Up	NM_145512	*Sft2d2*	167.0	2.4 ± 0.2	Retrograde vesicle transport
	NM_022018	*Niban*	153.3	2.3 ± 0.2	Unknown
	NM_02876	*1190005F20Rik*	153.2	2.3 ± 0.3	N(2),N(2)-dimethylguanosine tRNA methyltransferase
	NM_025819	*1200016B10Rik*	153.1	2.2 ± 0.1	Unknown
	NM_145991	*Cdc73*	145.4	2.2 ± 0.4	Tumor suppressor; cell cycle regulation
	NM_010709	*Lgtn*	133.0	2.1 ± 0.2	Trafficking receptor for phosphoglycoproteins
	NM_172517	*Rbbp5*	134.3	2.1 ± 0.4	Binds retinoblastoma protein
Down	NM_144960	*Fcamr*	132.6	−3.7 ± 1.1	Fc receptor (IgA, IgM)

[a] Up- or downregulated in B6.MOLF-*Ity/Ity3* compared to B6.MOLF-*Ity/Ity3*$^{MOLF/B6}$.
[b] Physical map positions based on Ensembl build 39.
[c] Physical map position is unknown on the basis of Ensembl build 39; however, it is syntenic to human 1q32.1, corresponding to a region around 135 Mb on mouse chromosome 1.

of B6.MOLF-*Ity/Ity3* mice could not be determined. In spite of this, a clear and consistent phenotype was observed in the *Ity3* congenic strains tested (B6.MOLF-*Ity/Ity3*, *Rec.A*, *Rec.B*, *Rec.E*), suggesting that the homozygous MOLF/Ei interval that they carry is responsible for the observed susceptibility.

Faced with a large number of candidate genes within the *Ity2* and *Ity3* intervals and the laborious and time-consuming creation of high resolution congenic mapping, we have used genomewide expression (*Ity2*) and QTL-specific arrays (*Ity3*) to obtain a list of candidate genes for each QTL. The advantage of microarrays lies partly in its unbiased approach to identify differentially expressed genes, at times leading to the discovery of genes that may not have been previously considered as candidates. For *Ity2*, the primary focus was to identify genes dysregulated in the QTL interval with the aim of identifying pathways that may be implicated in the phenotype. We noted first a bias of differentially regulated transcripts that appear as clusters on chromosomes 1 and 11 (supplemental Tables 1–3 at http://www.genetics.org/supplemental/). These happen to correspond to the chromosomes that carry the congenic intervals. This is probably due to the introduction of a MOLF/Ei genomic interval and may represent epigenetic effects on transcription. The mere introduction of a portion of the chromosome may affect the chromatin structure, *e.g.*, affecting the transcription of a number of genes. Resolving expression differences due to the different congenic fragments in the context of Salmonella infection can be complicated by these extraneous influences.

Within the chromosome 11 interval, we have identified two clusters of differentially regulated genes, a cluster of genes around *Ity2.A* and another just outside the distal interval *Ity2.B*. We observed an overall trend that the differentially regulated genes in the proximal interval were almost always expressed at higher levels in the spleens of the resistant B6.MOLF-*Ity/Ity2* mice, whereas those that were at the distal region had lower expression levels. An example of differentially regulated genes in the distal region is the *Slfn* family of genes. Upon sequencing of *Slfn1*, numerous sequence variants were identified (21 SNPs/1-kb coding sequence), suggesting that difference in intensity signal is probably due to poor probe hybridization in the B6.MOLF-*Ity/Ity2.RecD* mice rather than low transcript levels. Although some expression differences were confirmed upon infection (Figure 5), it is unlikely that these genes are the primary genes underlying *Ity2* as they map outside the target congenic interval. The differential regulation may be due to the allelic differences in the genes, since the susceptible B6.MOLF-*Ity/Ity2.RecD* mice carry the MOLF/Ei allele in this interval, or may be due to epigenetic effects as discussed above.

The candidates of the *Ity2* locus would be those that are differentially regulated in the proximal interval coincident with *Ity2.A* since the B6.MOLF-*Ity/Ity2* mice carry the resistant MOLF/Ei allele in this interval as compared to the susceptible B6.MOLF-*Ity/Ity2.RecD* mice that harbor the C57BL/6J allele. We have identified six such genes in the spleen. Among these genes, the differential expression was confirmed for *Havcr2* (Figure 6A). On day 7, *Havcr2*

(also known as *Tim3*) was found to be upregulated in the resistant congenic B6.MOLF-*Ity/Ity2*. *Havcr2* is specifically expressed on T_H1 cells and thought to negatively regulate T_H1 responses and has been linked to asthma susceptibility (Meyers *et al.* 2005). This may indicate that the resistant B6.MOLF-*Ity/Ity2* mice are more efficient at controlling the inflammatory response than their susceptible counterparts, suggesting a crucial role in the response to infection and leading to their prolonged survival. Hence, *Havcr2* distinguishes itself as promising candidate for *Ity2*.

Many of the genes that were differentially expressed were involved in T cell activities, which is most probably due to tissue bias (Table 3). The apparent lack of differentially expressed genes in the *Ity2.B* interval may suggest that it may contain gene(s) with a protein defect that may in turn affect the expression of the genes within the *Ity2.A* interval. This may be a proposed mechanism by which these two critical intervals interact and are hence both necessary for the resistance phenotype observed in the B6.MOLF-*Ity/Ity2* mice.

Custom oligonucleotide arrays were constructed for the *Ity3* interval and led to the identification of four new candidates, *Chi3l1*, *Cdc73*, *Sft2d2*, *and Niban*, on the basis of position, expression, and potential function in the host response to Salmonella infection. These four genes had higher expression levels in the susceptible B6.MOLF-*Ity/Ity3* mice; however, only one, *Chi3l1*, was validated by QPCR. *Chi3l1* appears to be a very promising candidate on the basis of its function to enhance bacterial invasion and adherence in intestinal epithelial cells. In fact, the administration of *Chi3l1*-neutralizing antibodies prior to oral Salmonella Typhimurium infection was protective in mice (Mizoguchi 2006). Moreover, overexpression of this gene *in vitro* resulted in the presence of greater numbers of intracellular Salmonella Typhimurium (Mizoguchi 2006). Therefore, the upregulation of *Chi3l1* may be consistent with increased susceptibility to infection as observed in B6.MOLF-*Ity/Ity3* mice.

In this study, we have restricted the *Ity2* and *Ity3* intervals and proposed a list of candidate genes to be investigated further. One differentially regulated gene, *Havcr2* in *Ity2*, and another such gene, *Chi3l1* in *Ity3*, have been identified as potential candidates for these respective loci. The wild-derived inbred mouse MOLF/Ei has been separated by over 1 MY of evolution from the classical strains such as C57BL/6J, within which time they have accumulated numerous sequence variants, on the order of 1 SNP/100 bp (Abe *et al.* 2004; Ideraabdullah *et al.* 2004). The evolutionary divergence between the two strains is well illustrated in this study as reflected by the high sequence variation between C57BL/6J and MOLF/Ei alleles at specific genes, such as the *Slfn*'s, which has the potential of affecting interpretation of array studies. Other than sequence variation, the possibility to affect transcriptional regulation on the basis of chromatin structure or due to genetic background effects is also demonstrated through the cluster of differentially regulated genes found distal to the *Ity2.B* interval. *Ity3* was found to account for only 7% of the phenotypic variance, suggesting strongly that Salmonella susceptibility in MOLF/Ei mice results from polygenic inheritance in which the accumulation of weak effects at several different loci and interaction between loci explained the disease phenotype. The use of larger F_2 populations and the creation of novel informative crosses will be necessary to reveal other loci that determine susceptibility to Salmonella infection in this wild-derived mouse. Nevertheless, the new genetic tools developed in these studies will contribute to further resolve the intricacies of Salmonella pathogenesis in the MOLF/Ei strain, contributing to our understanding of their extreme susceptibility to infection.

We thank Béatrice Kenol, Kyoko Yuki, and Catherine Paré for their technical assistance, Makeda Semret for helpful discussions in the creation of the *Ity3* custom oligonucleotide arrays, and Fiona MacIntosh for her help in the printing of the arrays as well as in several hybridization experiments. This project was supported by research grants to D.M. from the Howard Hughes Medical Institute and the Canadian Institute for Health Research. D.M. is a McGill William Dawson Scholar. V.S-S. was supported by studentships from the McGill University Health Center Research Institute and by the McGill University Faculty of Medicine.

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Communicating editor: L. Siracusa