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The p47 GTPases Iigp2 and Irgb10 Regulate Innate Immunity and Inflammation to Murine Chlamydia psittaci Infection

Isao Miyairi,**†¶ Venkat R. R. Arva Tatireddigari,‡ Olaimatu S. Mahdi,‡ Lorne A. Rose,‡ Robert J. Belland,‡ Lu Lu,‡# Robert W. Williams,**† and Gerald I. Byrne‡†

C57BL/6J mice were 10⁵-fold more resistant to Chlamydia psittaci infection than DBA/2J mice by LD₁₀₀ determinations. Linkage analysis using BXD recombinant inbred strains revealed a single effector locus at a 1.5-Mbp region on chromosome 11 encoding a cluster of three p47 GTPases (Irgb10, Igtp, and Iigp2). Western blots of infected tissue showed that Irgb10 was elevated in resistant mice and one of the two possible Iigp2 protein isoforms was preferentially expressed in susceptible mice. The BXD39 strain, susceptible at Irgb10 and resistant at Iigp2, had an intermediate phenotype implicating the nonredundant role of these 47 GTPases. C57BL/6J and DBA/2J exhibited a difference in IFN-γ-dependent chlamydial control, which was reversible by Iigp2 small interfering RNA knockdown. Microarrays of infected peritoneal lavage revealed >10-fold up-regulation of neutrophil-recruiting chemokines in susceptible mice and >100-fold increase in macrophage differentiation genes in resistant mice, indicating that the susceptibility pattern involves the stimulation of different inflammatory cell-recruiting pathways. Massive neutrophil recruitment was seen in susceptible mice by histology and flow cytometry, and neutrophil chemokine receptor (CXCR2) knockout mice on a susceptible background survived a lethal challenge, confirming that neutrophil recruitment was required for susceptibility. Congenic Igtp knockout mice also susceptible at Irgb10 and Iigp2 on a resistant background recruited neutrophils and succumbed to infection. We conclude that Irgb10 and Iigp2 act together to confer differential susceptibility against murine chlamydial infection. Data indicate that these p47 GTPases have cell-autonomous effects that result in vastly different inflammatory stimulations, leading to either recovery or death. The Journal of Immunology, 2007, 179: 1814–1824.

Chlamydiae are important obligate intracellular pathogens responsible for a wide spectrum of diseases in humans. Chlamydia trachomatis causes acute and chronic ocular and genital tract infections such as trachoma and tubal infertility, respectively. Chlamydia pneumoniae causes acute pulmonary infections and has been implicated in the pathogenesis of atherosclerosis (1). Chlamydia psittaci is a zoonosis that causes severe pulmonary infections and is listed as a category B select agent. The severity of chlamydial infection has been correlated with host genetic factors such as HLA haplotypes, polymorphisms in cytokine genes, and pattern recognition receptors that affect inflammatory responses in humans (2–6), although the full spectra of genetic determinants that influence susceptibility to chlamydial infections remain largely unknown. Identification of key host genetic factors could have a large impact on understanding chlamydial pathogenesis, improved therapeutics, and public health management strategies.

A genome-wide search for susceptibility loci in humans requires the investigation of affected family members, and empirical approaches of associating disease manifestations with population-based genetic polymorphisms are confounded by many variables. One experimental approach that has been successfully used to help focus human population studies and define genetic resistance and susceptibility to specific pathogens is the use of mice as tractable models for human infectious diseases. Ninety-five percent of murine genes have human homologues, and numerous germline contributions have been made by the study of murine models (7). Forward genetics using linkage analysis provide an unbiased strategy to screen for relevant susceptibility genes in vivo. A number of susceptibility loci and several pertinent genes have been identified using these general methods. Examples of identified resistance genes include Mx for influenza A (8), Naip for Legionella (9, 10), and Nrpm1 for Mycobacterium (11).

Inbred mice have been shown to exhibit differences in susceptibility to chlamydial disease. In models of acute genital tract, respiratory, and systemic infections, C57BL/6 mice consistently have less pathogen burden or disease compared with C3H/HeN mice (12–22). Various factors such as Th1-weighted responses, inflammatory cytokine expression, and H2 haplotypes have been correlated with resistance by using a variety of empirical approaches. An unbiased genome-wide search was recently performed by conventional linkage analysis using the F2 generation of inbred crosses to identify genetic loci that controlled chlamydial load and revealed C. trachomatis resistance quantitative trait loci on chromosomes 2, 3, and 11 (Ctrq-1, Ctrq-2, and Ctrq-3, respectively) (22, 23). Further analysis revealed that two members of the...
p47 GTPase family, Irgb10 and Igtp (also known as Irgm3), conferred cell-autonomous control of chlamydial growth in cell culture.

Factors that constitute pathogen control do not necessarily correlate with a disease phenotype. We therefore sought genetic traits that affect survival in an i.p. infection model using a high throughput forward genetic approach and recombinant inbred mouse strains. Recombinant inbred strains are generated by inbreeding F2 progeny for >20 generations by sib mating. In our case, the parental C57BL/6J and DBA/2J strains result in recombinant inbred strains referred to as BXDs. Currently 80 BXD strains, each typed at >3,600 highly informative markers, provide a genetically immortalized resource and a powerful prefabricated tool for genetic mapping (24). In our study, infecting a panel of BXD strains efficiently identified a 1.5-Mbp region of chromosome 11 within Ctrq-3 (22, 23) as a dominant Mendelian trait containing a cluster of three p47 GTPases (Irgb10, Igtp, and Igtp2 (Gpi or Irgm2)). In addition to Irgb10, an Igtp2 protein isoform was implicated in conferring susceptibility. Differences in susceptibility were characterized as a moderate decrease in chlamydial pathogen load, attenuated inflammation, and absolute host survival in C57BL/6J mice compared with DBA/2J mice that succumbed to infection. In vivo challenge using Igtp knock out mice confirmed the involvement of p47 GTPases in innate resistance against Chlamydia and extended the role of these effectors to inflammation induction in modifying the disease phenotype. This study also highlights the use of recombinant inbred mice as a more generally applicable method to study host genetic resistance factor in an accurate, rapid, and cost efficient manner.

Materials and Methods

Experimental outline

Infection models that demonstrate a clear difference in susceptibility to chlamydial challenge were initially screened and then optimized. The i.p. lethal challenge model was ultimately chosen for its clarity and because it was a representative disease model. The BXD recombinant inbred strains were infected and then quantitative trait loci (QTL) analysis was performed to identify the susceptibility locus. Candidate genes were interrogated by genotype, transcriptional, and proteomic analysis for expression differences, which were then verified by the use of mouse strains carrying mutant alleles or RNA silencing. The downstream immunological pathway was investigated by microarray analysis, flow cytometry, histology, and verified using knockout mice.

Mice

Seven- to 8-wk-old male mice (C57BL/6J, DBA/2J, BALB/cJ, C3H/HeN, 129S1/SvImJ, WSB/EiJ, CAST/EiJ, PWD/PhJ, MOLF/EiJ, and C.129S2 (B6)-Il8rbtm1Mwm/J (B6)-129S1/SvImJ, WSB/EiJ, CAST/EiJ, PWD/PhJ, MOLF/EiJ, and C.129S2 (B6)-Il8rbtm1Mwm/J were bred and maintained at our facility, which was investigated by microarray analysis, flow cytometry, histology, and verified using knockout mice.

Chlamydia strain and evaluation of chlamydial load

The C. psittaci 6BC strain was propagated in L929 cells and stored at −80°C. Chlamydial titer was evaluated by infecting L929 cells and enumerated by indirect fluorescence microscopy as previously described (26). Tissues obtained from various organs were homogenized in sucrose phosphate glucose buffer, centrifuged at 1500 rpm, and then the supernatants were collected and kept at −80°C for inclusion forming unit (IFU) assays.

Abbreviations used in this paper: QTL, quantitative trait loci; IFU, inclusion-forming unit; LOD, likelihood odds ratio; siRNA, small interfering RNA.

FIGURE 1. Differences in innate immunity determine susceptibility to Chlamydia in inbred strains of mice. A. C57BL/6J and DBA/2J mice infected i.p. with C. psittaci at doses ranging from 10⁰ to 10⁶ IFU were monitored for survival. Each data point represents at least three mice per group. B. Average days to death for DBA/2J mice according to inoculum size. Mice were assessed twice daily. C. C57BL/6J and DBA/2J mice were immunized with 500 IFU of C. psittaci or mock solution (PBS) i.p. and then challenged 6 wk later with 10⁶ IFU of C. psittaci i.p. All C57BL/6J mice and immunized DBA/2J mice survived in contrast to all mock-immunized DBA/2J mice, which died 8 days postinfection.

Chlamydial infection and determination of phenotype

Various doses of the C. psittaci 6BC strain were suspended in 200 μl of Dulbecco’s PBS for i.p. or i.v. infection or in 20 μl for intranasal infection. DBA/2J and C57BL/6J were immunized by i.m. injection with 500 IFU of viable C. psittaci 6BC and then rechallenged with 10⁶ IFU i.p. 6 wk postimmunization. All mice were observed for at least 14 days to confirm protection against challenge and for signs of recovery.

In vitro peritoneal macrophage infection

Peritoneal macrophages from C57BL/6J and DBA/2J mice were elicited by 5% proteose peptone and plated at 5 × 10⁵ cells/well on a 24-well plate. Cells were infected with C. psittaci at a multiplicity of infection of 1 with or without IFN-γ (20 ng/ml) and collected after 48 h by scraping for an infectivity assay.

Primary fibroblast isolation and growth

The peritoneum was exposed aseptically and dissected out in small fragments then incubated in collagenase/disperse (catalog no. 10269638001;
Roche) for 1 h. The suspension was washed in PBS and centrifuged, suspended in DMEM supplemented with 10% FBS, gentamicin, and vancomycin, and plated onto 6-well plastic plates. Nonadherent debris was washed away after overnight incubation. Cells were grown until confluency and trypsinized and passed for four or five generations before use.

FIGURE 2. Recombinant inbred strains identify a 1.5-Mb susceptibility locus on chromosome 11. Twenty-four BXD strains were i.p. infected with *C. psittaci* (10^4 IFU) and monitored for survival. A, Results were analyzed by WebQTL software (www.genenetwork.org) and interval mapping is shown as a blue line representing the LOD score across the entire mouse genome. A single high peak is present on chromosome 11. Permutation analysis calculated a high level of significance at LOD > 4.8 as represented by the red line. B, A close-up view of chromosome 11 (50–62 Mb) with the peak at the LOD score of 11.3. All data are accessible through the GeneNetwork web site (www.genenetwork.org; trait identification no. 10806). C, A 1.5-Mb QTL on chromosome 11 represented by 18 genes and a cluster of p47 GTPases (*Irgb10*, *Igtp*, and *Iigp2*) at the distal end.
FIGURE 3. Proteomic differences in Irgb10 and Iigp2 determine phenotype. A. Expression levels of Irgb10, Igtp, and Iigp2 normalized to GAPDH in peritoneal lavage specimens from infected C57BL/6J and DBA/2J are shown (n = 3). Significant differences in expression were observed at the time points indicated by an asterisk. However no consistent pattern is observed (+, p = 0.03; **, p = 0.02; Student’s two-tailed t test). B. Western blot analysis of Irgb10, Igtp, Iigp2, and the actin control demonstrates more abundant Irgb10 in resistant C57BL/6J mice and expression of the 47-kDa and 45-kDa Iigp2 isoforms. Susceptible DBA/2J mice preferentially express the 45-kDa isoform. C. BXD39, DBA/2J, and C57BL/6J mice were infected i.p. and weight changes were monitored. All C57BL/6J mice survived; all DBA/2J mice (** died and all BXD39 mice (+ lost weight and became sick but only one of four died. In the BXD39 mouse Irgb10 and Igtp are of DBA/2J origin and the coding exon of Iigp2 is from C57BL/6J.

RNA silencing

RNA silencing was performed per the protocol using a RNA interference (RNAi) human/mouse starter kit (catalog no. 301799; Qiagen). Briefly, primary fibroblasts were plated at a density of 1.5 × 10⁴ cells on a 48-well plate. HiPerFect transfection reagent (Qiagen) and 15 nM small interfering RNA (siRNA) were added and incubated for 6–12 h before infection. HP GenomeWide siRNA (catalog no. SI01075151; Qiagen) was used for genome-wide siRNA under a fluorescent microscope. Transfection efficiency was monitored by Alexa Fluor 488-labeled control siRNA under a fluorescent microscope.

QTL mapping

QTL mapping was performed using web-based complex trait analysis (www.genenetwork.org). A single marker regression across all chromosomes was performed where a hypothetical QTL was evaluated at regular intervals and estimates the significance at each location with a graphical representation of the likelihood odds ratio (LOD) (27). A permutation test establishes genome-wide significance criteria of 5% for the trait. BXD mice were challenged with 10⁴ IFU of C. psittaci and became sick but only one of four died. In the BXD39 mouse Irgb10 and Igtp are of DBA/2J origin and the coding exon of Iigp2 is from C57BL/6J.

significant QTL on chromosome 11. Five additional strains with breakpoints near the QTL were then selected to facilitate fine mapping.

Histology

Mice were infected with 10⁴ IFU of C. psittaci i.p. and euthanized on day 5. Organs were fixed in 10% formalin, embedded in paraffin wax, and stained with H&E. Necropsies of the i.p. infected mice were performed as a courtesy by Dr. W. Hill (Comparative Medicine, University of Tennessee Health Science Center, Memphis, TN) and Dr. K. Boyd (Animal Resource Center, St Jude Children’s Research Hospital, Memphis, TN), and histologies of infected lungs and Igtp knockout mice were performed at the University of Missouri Research Animal Diagnostics Laboratory.

Flow cytometry

Murine peritoneal exudates were collected, blocked with BD Fc block (catalog no. 553141; BD Biosciences), and incubated with three or four fluorochrome-conjugated Abs. Stained cells were analyzed on a BD LSR II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). The following Abs were used: F4/80 (clone BMS, product no. MF48005; Caltag Laboratories) conjugated with allophycocyanin (product no. RM2905; Caltag Laboratories) at 1 μg/10⁶ cells; Ly6G-PE (clone 1A8, catalog no. 551461; BD Biosciences) at 0.5 μg/10⁶ cells; CD3-Alexa 488 (clone 145-2c11, catalog no. 557666; BD Biosciences) at 0.5 μg/10⁶ cells; and CD11b-PE-Cy7 (clone M1/70, catalog no. 552850; BD Biosciences) at 0.5 μg/10⁶ cells. Three individual adult mice per strain were analyzed unless specified otherwise.

ELISA

Cell culture supernatants or peritoneal lavage supernatants were stored at 4°C until assessment. Cxcl1 (mouse KC Cytokine kit, catalog no.
Both strains of mice were plated and preincubated with IFN-γ/H11005 of infection siRNA then infected with C57BL/6J and DBA2/J mice were pretreated with Irgb10 and Iigp2. Partial reversal of IFN-γ induced inhibition is demonstrated in fibroblasts obtained from C57BL/6J mice; reduced chlamydial inhibition is demonstrated in fibroblasts obtained from susceptible DBA/2J mice.

**FIGURE 4.** Modest difference in chlamydial control is seen in vitro and in vivo between resistant and susceptible mice. A, Chlamydial load in the peritoneal lavage 3, 5, and 7 days postinfection (n = 3); *, p = 0.05. B, Chlamydial load in the spleen, lungs, and blood (0.4 ml) taken 6 days postinfection (n = 3–5); ***, p = 0.03. C, Peritoneal macrophages from both strains of mice were plated and preincubated with IFN-γ-containing (0 and 20 ng/ml) medium for 24 h before infection with C. psittaci. Cells were scraped after 40 h for titration of C. psittaci. Results are representative of two experiments, ***, p = 0.002.

**FIGURE 5.** Iigp2 mediates IFN-γ induced inhibition of Chlamydia. Peritoneal fibroblasts obtained from C57BL/6J and DBA2/J mice were pretreated with siRNA then infected with C. psittaci 6BC (multiplicity of infection = 1) with or without IFN-γ. Shown is fluorescent microscopy of infected fibroblasts from C57BL/6J mice dually stained with Hoechst and primary Ab against chlamydial LPS and FITC secondary Ab. Blue indicates nuclei and green indicates chlamydial inclusion. Top left, Control siRNA only. Top right, IFN-γ and control siRNA treated. Bottom left, IFN-γ and Iigp2 siRNA treated. Partial reversal of IFN-γ induced chlamydial inhibition is demonstrated in fibroblasts obtained from susceptible DBA/2J mice; p = 0.03. No significant inhibition is induced by IFN-γ in fibroblasts obtained from susceptible DBA/2J mice.

**Genotyping**

DNA was extracted from snaped rodent tails using the DNeasy tissue kit (Qiagen). Fine mapping of the susceptibility locus was performed by detection of a single sequence-length polymorphism based on microsatellite markers as previously described (23). The following was added per reaction: 18 µl of PCR SuperMix (catalog no. 10572-014; Invitrogen Life Technologies), 3.3 µM forward and reverse primers, and 50 ng of DNA. A standard PCR cycling program was applied at 94°C for 2 min and 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 72°C for 5 min, and then 4°C. The entire reaction volume was run on 4% agarose gel with ethidium bromide.

**Quantitative PCR**

Crude extraction of RNA was performed with TRIzol (catalog no.15596-028; Invitrogen Life Technologies) and contaminating DNA was removed using a TURBO DNA-free kit (catalog no. 1907; Ambion) per the manufacturer’s protocol. Reverse transcription was performed using TaqMan reverse transcription reagents (catalog no. 808-0234; Applied Biosystems). Quantitative PCR was performed on the cDNA using specific primers and probes as follows. Irgb10: 5’-CCTGGTGTCAGCCGAAGCT-3’ (forward primer), 5’-GTTTAGAAGAAGACTGACCCATG-3’ (reverse primer), and 5’-TGGACTCAAGGCTTCTGCCAGAAACC-3’ (probe); Iigp2: 5’-AGACATCTTCAGCATGATGACA-3’ (forward primer), 5’-ACCCTTTCAGCATGATGACA-3’ (reverse primer), and 5’-ATCCCTTAGAGA TCATTCTCAAGTCTGACAA-3’ (probe). Probes were labeled with the FAM reporter fluorochrome and the TAMRA quencher. PCR was performed using Taq DNA polymerase (TaqMan PCR master mix kit; Applied Biosystems). Mouse GAPD (GAPDH) endogenous control (catalog no. 4352932E; Applied Biosystems) was used to normalize data. Amplification conditions consisted of 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C using the ABI Prism 7000 sequence detection system.

**Western blot analysis**

Western blot analysis was performed by standard protocol. Peritoneal lavage fluid was centrifuged and lysed in buffer containing 1% Nonidet P-40, run on a 10% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane at 500 mA for 3 h. The membrane was then blocked in PBS with 0.1% Tween 20 containing 5% skim milk for 1 h. The resulting blot was incubated with the following primary Abs overnight in 4°C:

CMI063; BioSource International) and Cxcl2 (mouse MIP-2 CytoSet kit, catalog no. CMC2453; BioSource International) were assessed according to company protocol.
Table I. Differential microarray analysis

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene No.</th>
<th>Enrichment</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>23</td>
<td>R = 2.90</td>
<td>4.95E-06</td>
</tr>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>15</td>
<td>R = 3.06</td>
<td>1.10E-04</td>
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<td>Cell adhesion molecules</td>
<td>15</td>
<td>R = 3.20</td>
<td>6.96E-05</td>
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<tr>
<td>Type 1 diabetes mellitus</td>
<td>14</td>
<td>R = 8.43</td>
<td>4.41E-10</td>
</tr>
<tr>
<td>NK cell-mediated cytotoxicity</td>
<td>12</td>
<td>R = 3.01</td>
<td>6.32E-04</td>
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<tr>
<td>Complement and coagulation cascades</td>
<td>11</td>
<td>R = 4.45</td>
<td>3.11E-05</td>
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<td>Apoptosis</td>
<td>10</td>
<td>R = 2.98</td>
<td>1.90E-03</td>
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<tr>
<td>Hemopoietic cell lineage</td>
<td>9</td>
<td>R = 3.17</td>
<td>2.00E-03</td>
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<tr>
<td>TLR signaling pathway</td>
<td>9</td>
<td>R = 2.74</td>
<td>5.50E-03</td>
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<tr>
<td>Ag processing and presentation</td>
<td>9</td>
<td>R = 3.81</td>
<td>5.38E-04</td>
</tr>
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</table>

*Microarray analysis of mRNA collected from peritoneal lavage 3 days post-infection and statistically analyzed for differential expression. Differentially regulated genes (≥2.5-fold) were entered into a gene ontology software (Webgestalt: http://bioinfo.vanderbilt.edu/webgestalt) (43) linked to Kyoto Encyclopedia of Genes and Genomics (KEGG) pathways, revealing over-represented pathways such as cytokine-cytokine receptor interaction and Jak-STAT signaling pathways. R is the ratio of enrichment for the KEGG pathway (R = observed gene number/expected gene number), p values indicate the significance of enrichment calculated from the hypergeometric test.*

Irgb10 at 1/4000 (provided by Dr. I. Bernstein-Hanley and Dr. M. Starnbach, Harvard University Medical School, Boston, MA); Igtp at 1/5000 (catalog no. 610880; BD Biosciences); GTPI and actin, goat TrueBlot-HRP anti-goat IgG (catalog no. sc-1616; Santa Cruz Biotechnology); Igtp, goat anti-mouse-HRP (catalog no. 55539; ICN Biotechnologies); Irgb10 at 1/4000 (provided by Dr. I. Bernstein-Hanley and Dr. M. Starnbach, Harvard University Medical School, Boston, MA); Igtp at 1/500 (catalog no. 610880; BD Biosciences); GTPI (M-14) at 1/500 (catalog no. sc-11088; BD Biosciences); GTPI and actin, goat TrueBlot-HRP anti-goat IgG (catalog no. sc-1616; Santa Cruz Biotechnology). The following secondary Abs were used: Irgb10, goat anti-rabbit-HRP (catalog no sc-2004; Santa Cruz Biotechnology); Igtp, goat anti-mouse-HRP (catalog no. 55539; ICN Biotechnicals); GTPI and actin, goat TrueBlot-HRP anti-goat IgG (catalog no.18-8814; eBioscience). SuperSignal West Pico chemiluminescent substrate (catalog no. 34077; Pierce) was used as a substrate.

Microarray analysis

Three micrograms of the total RNA was used and prepared according to the Affymetrix eukaryotic array processing protocol. Amplified labeled aRNA (15 μg) was hybridized to a mouse genome 2.0 array chip (Affymetrix). Image analysis and data quantification were performed by using Affymetrix GeneChip operating software (GCOSver1). Four biological repeats were tested for each strain of mice. Microarray data normalization was performed using the robust multiple array average (Gene Expression Omnibus Repository no. GSM1188240-5). Differential expression was evaluated by Student’s t test and genes that were differentially expressed at ≥2.5-fold were analyzed using Webgestalt gene ontology software (http://bioinfo.vanderbilt.edu/webgestalt) (28).

Table II. Differential expression of genes in the cytokine-cytokine receptor interaction pathway

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Difference</th>
<th>p Value (t Test)</th>
<th>Description</th>
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<tr>
<td>DBA/2J</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cxcl11</td>
<td>17.4</td>
<td>2.22E-03</td>
<td>Chemokine (C-X-C motif) ligand 11</td>
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<td>Il1a</td>
<td>16.9</td>
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<td>Interleukin 1α</td>
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<td>7.01E-03</td>
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<td>Chemokine (C-X-C motif) ligand 1</td>
</tr>
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<td>Il1r</td>
<td>8.3</td>
<td>1.44E-03</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
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<td>1.90E-02</td>
<td>Interleukin 1 family, member 9</td>
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<td>5</td>
<td>7.14E-03</td>
<td>Interleukin 12α</td>
</tr>
<tr>
<td>Ccl3</td>
<td>4.5</td>
<td>1.13E-02</td>
<td>Chemokine (C-X-C motif) ligand 3</td>
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<td>Tnf</td>
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<td>3.77E-03</td>
<td>Tumor necrosis factor</td>
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<td>Il1b</td>
<td>3.7</td>
<td>6.24E-03</td>
<td>Interleukin 1 β</td>
</tr>
<tr>
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<td>4.95E-02</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
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<tr>
<td>Ltb</td>
<td>3.1</td>
<td>1.97E-02</td>
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<td>Il6</td>
<td>2.6</td>
<td>3.21E-02</td>
<td>Interleukin 6</td>
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<td>Csf3</td>
<td>2.5</td>
<td>5.96E-03</td>
<td>Colony stimulating factor 3 (granulocyte)</td>
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<td>Bnap1</td>
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<td>Interleukin-1 receptor-associated kinase 4</td>
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<td>Cxcl4</td>
<td>2.8</td>
<td>6.50E-02</td>
<td>Chemokine (C-X-C motif) ligand 4</td>
</tr>
</tbody>
</table>

*Genes identified in the cytokine-cytokine receptor interaction pathway. Redundant genes were excluded. Average fold difference in transcriptional levels and p value calculated by t test (n = 3). Chemokine genes involved in neutrophil recruitment were highly up regulated in DBA/2J mice.*

*Genes up-regulated in DBA/2J mice.*

*Genes up-regulated in C57BL/6J mice.*

### Results

**Differences in innate immunity determine susceptibility to C. psittaci in inbred mice**

Various existing models of differential susceptibility to chlamydial infection were initially considered. All models of primary chlamydial challenge demonstrated a similar trend in susceptibility regardless of the route of infection or the species of *Chlamydia* used, implicating a common set of resistance genes for C57BL/6J mice (12–22). An i.p. infection model of *C. psittaci* was ultimately chosen for its clarity and ease of application and because it appeared to be representative. It was found that all C57BL/6J mice survived i.p. infection with doses up to 10<sup>6</sup> IFU of *C. psittaci* whereas all DBA/2J mice became systemically ill and died at 7–12 days postinfection with doses as low as 10<sup>5</sup> IFU (Fig. 1 A and B). This provided a clear phenotypic distinction between the strains with small intrastrain variability, key criteria for efficient forward genetic analysis. Assessment of other routes such as intranasal and i.v. infection demonstrated a similar trend in susceptibility but exhibited less tractable readout assays (data not shown). Immunized DBA/2J mice tolerated challenge with >10<sup>3</sup> × LD<sub>100</sub> of *C. psittaci*, demonstrating the integrity of the acquired immune response in DBA/2J mice (Fig. 1C). Therefore, the difference in susceptibility to *Chlamydia* in these mice was most likely attributable to innate responses.

Recombinant inbred strains identify a 1.5-Mb susceptibility interval on chromosome 11

Twenty-four BXD strains of both genders were infected i.p. with *C. psittaci*. Although the majority of mice (all individuals for 21
strains) exhibited survival kinetics similar to those of the parental strains, three strains exhibited an intermediate phenotype such as significant weight loss without death and were scored accordingly. QTL mapping identified an 1.5 Mb region of chromosome 11 with a peak LOD score of 11.3 at 57 Mb (the 2 LOD confidence interval is between 56.5 and 58 Mb), mapping within the previously described \textit{Ctrq-3} (Fig. 2, A and B) (22, 23). This region encodes 18 genes, including a cluster of three p47 GTPases, \textit{Irgb10}, \textit{Igtp}, and \textit{Iigp2} (Fig. 2C). The genetic differences at \textit{Ctrq-3} accounted for 83% of the overall phenotypic difference (trait variance) defined as survival upon chlamydial challenge. The previously described \textit{Ctrq-1} and \textit{Ctrq-2} loci on chromosomes 2 and 3 (22), did not reach a level of significance in our analysis. All data are accessible through the GeneNetwork web site (www.genenetwork.org; trait identification no. 10806).

Proteomic differences in \textit{Irgb10} and \textit{Iigp2} determine phenotype

We initially identified single nucleotide polymorphisms that account for differences in the IFN-stimulated response element, promoters, or coding regions related to the three p47 GTPases using a bioinformatics approach. We expanded our analysis by examining the phenotype and genotype of five additional inbred strains (129S1/SvImJ, WSD/EiJ, PWD/PhJ, MOLF/EiJ, and CAST/EiJ) with predicted differences in this critical region. This revealed a number of single nucleotide polymorphisms in untranslated regions and six missense mutations (data not shown). The common missense mutations in the \textit{Irgb10} gene did not appear to account for alterations in protein structure or conserved motifs such as the G domains that are important for p47 GTPase function. No common missense mutations were identified for \textit{Igtp} or \textit{Iigp2}. Transcriptional levels of the three p47 GTPases in the peritoneal lavage measured over a time course of 0.5–6 days postinfection also failed to show a consistent difference between the two strains (Fig. 3A), indicating that gene expression differences could not distinguish the resistance/susceptibility phenotypes in this infection model. Western blot analysis of proteins extracted from peritoneal lavage 6 days postinfection did, however, demonstrate a relative increase in steady state protein levels for \textit{Irgb10} in C57BL/6J vs DBA/2J. Also, C57BL/6J was found to preferentially express a 47-kDa protein product for \textit{Iigp2} whereas DBA/2J predominantly expressed a 45-kDa isoform (Fig. 3B). The isoform corresponds to a predictable splicing variant that excludes a coding exon.

Strain BXD39 was unique in that these mice demonstrated an intermediate phenotype with significant weight loss for all mice and death in 25% of the infected group (Fig. 3C). Analysis of the BXD39 genotype at the \textit{Ctrq-3} locus using microsatellite markers divided the locus into a proximal \textit{Ctrq-3} containing the \textit{Irgb10} and \textit{Igtp} DBA/2J allele and a distal \textit{Ctrq-3} containing the C57BL/6J allele.
coding exon of *Iigp2*. This result indicated that *Iigp2* also individually contributed to the susceptibility phenotype in addition to the role previously described for *Irgb10* (22, 23).

**Iigp2 has a cell-autonomous role in IFN-γ-mediated chlamydial inhibition**

A maximum 1–2 log fold difference in chlamydial load was observed in peritoneal lavage specimens (3, 5, and 7 days) or spleen, lung, and blood taken 6 days after i.p. infection of resistant and susceptible mice (Fig. 4, A and B). No difference was seen in chlamydial load in unstimulated cell culture samples of peritoneal macrophages infected with a multiplicity of infection of 1 and harvested 48 h postinfection, but a 1–2 log difference in growth inhibition was observed when host cells were activated with exogenous IFN-γ at the time of infection (Fig. 4C). Chlamydial growth inhibition was not reversed by addition of the inducible NO synthase inhibitor (Nω-monomethyl-arginine (1-NMMA)) (data not shown), implicating alternative IFN-γ-induced antimicrobial effects. Because a previous study demonstrated that the genetic differences in p47 GTPases were reflected in cultured embryonic fibroblasts (22, 23), we treated primary peritoneal fibroblasts derived from resistant and susceptible mice with IFN-γ in the presence or absence of siRNA for *Iigp2*. The presence of siRNA inhibited *Iigp2* transcription levels by 62.6% in C57BL6J-derived fibroblasts and 67.2% in DBA/2J-derived fibroblasts treated with IFN-γ. Inhibition of *Iigp2* resulted in partial restoration of chlamydial growth, implicating the cell autonomous role of *Iigp2* in C57BL/6J mice (Fig. 5). In contrast, little or no chlamydial growth inhibition was seen in IFN-γ-treated fibroblasts derived from DBA/2J mice, which strongly implicates *Iigp2* in conferring phenotypic difference in these two strains of mice. This relatively modest difference in chlamydial load in cells from resistant and susceptible mice may not fully explain the 5 log LD100 difference and prompted us to determine whether other innate immune parameters were also affected.

**Transcriptional data reflect differences in cell differentiation and recruitment**

Mouse genome microarray analysis was performed on mRNA collected from the peritoneal exudates of infected C57BL6J and DBA/2J mice. A gene ontology analysis comprising a set of ~1000 transcripts with >2.5-fold difference in expression revealed potential downstream pathways reflecting the primary genetic differences (Table I). Transcripts for proinflammatory cytokine and chemokine (*Cxcl1* (KC), *Cxcl2* (Mip2), and *Cxcl11*) genes were found to be up-regulated >10-fold in susceptible DBA2J mice (Table I). Known upstream regulators of inflammatory cytokine release (stimulation of pattern recognition receptors such as TLRs and up-regulation of *MyD88*) were increased in resistant (C57BL/6J) vs susceptible (DBA/2J) mice, indicating alternative pathway usage between the two strains. Furthermore, >2 log up-regulation of genes involved in differentiation and proliferation of macrophages (ifih204 and ifih203) and >10-fold up-regulation of NK cell activating genes was seen in resistant (C57BL/ 6J) vs susceptible (DBA/2J) mice. These results suggested that vastly different immune pathways were stimulated in resistant vs susceptible mice.

**Susceptible mice recruited significant amounts of activated neutrophils to the peritoneal cavity**

Inflammatory responses were characterized by histology and cytology. Histological examination of the site of infection 5 days after challenge revealed a thick layer of mucopurulent material lining the surface of the peritoneal cavity in DBA/2J but not C57BL/6J mice. The surface of intraabdominal organs exposed to the peritoneal cavity such as the liver and the spleen were covered with this mucopurulent layer and it is represented here by a cross section analysis of the spleen (Fig. 6A). Flow cytometric analysis further demonstrated that DBA/2J mice recruited significantly more integrin-positive neutrophils (CD11b+Ly6G+ F4/80− cells) to the site of infection, compared with C57BL6J mice, which recruited predominantly macrophages (CD11b+Ly6G+ F4/80+ cells) (Fig. 6B). This was confirmed by the production of Cxcl1 and Cxcl2 in the peritoneal cavity by ELISA in only the susceptible DBA/2J mice (Fig. 6C). These data confirm the robust difference in the nature of the inflammatory responses between resistant and susceptible strains suggested by transcriptional analysis.

**Interruption of the chemokine pathway reverses the susceptible phenotype**

When mice with homozygous defects in the chemokine receptor (*Cxcr2* gene) were infected, the animals failed to recruit neutrophils to the site of infection and survived challenge with *C. psittaci*
in contrast to the wild-type controls (BALB/cJ) that died although there was no difference in chlamydial load between the wild-type and knockout mice (Fig. 7). This demonstrates a direct causal relationship between the neutrophil response and death in the susceptible mice. Conversely, Igtp knockout mice backcrossed to the C57BL/6J strain succumbed to infection with kinetics similar to susceptible strains and died with evidence of neutrophil-dominant inflammatory response (Fig. 8, A and B). Analysis of the region that flanks the Igtp gene and also encodes Irgb10 and Iigp2 by single sequence length polymorphism revealed that this region was derived from the 129S1/SvImJ strain, which exhibited a Chlamydia-susceptible phenotype (Fig. 8C). Although this result highlights the limitations inherent in the use of knockout mice generated using embryonic stem cells derived from the 129S6/SvEvTac strain, it also reinforces the involvement of this chromosome region and the role of these p47 GTPases in resistance to Chlamydia.

Discussion

The work reported here provides data to extend the role and repertoire of p47 GTPases in innate immunity to Chlamydia. The Iigp2 gene was implicated in microbial pathogenesis for the first time, and this study highlights how even modest chlamydial growth control by p47 GTPases may profoundly impact the fundamental nature of the inflammatory response and result in a dramatic difference in disease outcome.

The study also illustrates the power of BXD recombinant inbred mouse strains and the GeneNetwork database (www.genetwork.org) to study resistance to Chlamydia and helps define an efficient approach to rapidly map host infectious disease susceptibility genes using an expanded set of recombinant inbred strains. Fine mapping of resistance loci using BXD recombinant inbred strains proved to be as accurate as traditional approaches but is more rapid and cost effective. QTTL data were generated in less than a month by assessing susceptibility to random BXD strains analyzed by WebQTL (GeneNetwork database), identifying a locus on chromosome 11 containing p47 GTPases. p47 GTPases are IFN-γ inducible proteins that are distributed between specific membrane compartments and the cytosol, relocalize to the vacuolar compartments during infection, and are thought to be directly involved in the control of intracellular pathogens (29–31). Twenty-three p47 GTPases have been identified by genomic investigations in mice, six of which have been cloned (32).

In this study we used forward genetic approaches to demonstrate that the chromosomal locus Ctrq-3 (22), which encodes three p47 GTPases (Irgb10, Igtp and Iigp2), accounts for the phenotypic differences in susceptibility against Chlamydia in C57BL/6J and disease prone DBA/2J mice as a single dominant trait. Proteomic differences in Irgb10 and Iigp2, but not Igtp, implicated the former two genes in the pathogenesis of chlamydial infection. These results extend a recent study that demonstrated the involvement of Irgb10 in the differential control of Chlamydia trachomatis systemic infection in C57BL/6J and C3H/HeJ mice (23). The previous study showed differences in the transcriptional regulation of Irgb10 between resistant and susceptible mice, whereas our results did not find transcriptional changes but rather implicated altered p47 GTPase protein expression as a major strain distinction. A direct effect of Iigp2 in chlamydial control was confirmed in cell culture by the reversal of IFN-γ-mediated chlamydial growth inhibition by RNA silencing. These results contrast with those of the study by Nelson et al. (33), who evaluated the role of p47 GTPases in controlling chlamydial growth in murine genital epithelial cell infection by RNA silencing. These authors found that the silencing of Iigp1 (also known as Irga6) but not of Igtp or Iigp2 led to a partial reversal of IFN-γ-mediated inhibition of C. trachomatis serovar L2 growth. However, a recent report suggests that a Iigp1 knockout mouse is resistant to chlamydial infection in vivo (34).

The differences in the chlamydial species (C. trachomatis serovar L2 vs C. psittaci), the route (systemic vs local), and host cell type (epithelial vs macrophages) may be important contributing factors to the discrepancies seen. It is evident, however, that a common susceptibility locus is now identified for two species of Chlamydia that are capable of infecting a variety of host cell types in systemic
models of infection, indicating the critical function of the p47 GTPases. The function of these genes has also been evaluated in fibroblasts. If individual p47 GTPases show tissue and pathogen specificity, then further assessment in epithelial cells and pulmonary or genital tract infections using other chlamydial strains will help establish this corollary. The in vivo antimicrobial effect of Igtp against a number of intracellular pathogens have been reported based on the susceptibility phenotype of the Igtp knockout strain (25, 35, 36). Although the Igtp knockout mice behaved much like a susceptible strain upon chlamydial challenge, we found that these mice have polymorphic p47 GTPases (Irgb10 and Iigp2) in the flanking region derived from the original 129Sv-derived embryonic stem cell line and, therefore, it is not possible to know whether the phenotype is due to a defect in Igtp or the presence of a susceptible Irgb10 or Iigp2 gene.

Numerous studies have implicated the central immunoprotective role of IFN-γ in chlamydial infection for humans and mice (28). However, it is clear that that the major effector mechanism for IFN-γ differs substantially between humans and mice. In humans, IDO, which catabolizes tryptophan and limits intracellular chlamydial growth by starvation for this amino acid, is central (37), whereas in mice IDO does not appear to play a substantial role in the control of chlamydial infection. Several studies now strongly implicate p47 GTPases (33) or inducible NO synthase (38, 39) as murine anti-chlamydial effectors, but evidence that implicates these activities in humans is lacking. Expression of the human immune-related GTPase, Irgm, an ortholog of the murine p47 GTPase Irgm (also known as Irgm1 and Lrg47), demonstrated control of intracellular Mycobacterium growth by autophagy induction in a cell culture transfection study (40). However, the repertoire of immune-related p47 GTPases in humans is limited to three genes, none of which is induced by IFN-γ (32, 40). Mice and humans may deploy their immune resources against vacuolar pathogens in radically different ways (41, 42), which is an important consideration when exploiting mice as a model of human infectious diseases. Therefore, it is important to understand the downstream pathways of primary effectors because they are more likely to be shared at this level.

Most studies of p47 GTPases implicate the direct control of intracellular pathogen growth as their cell autonomous function by either phagosome disruption or induction of autophagy (31, 43). Our observations suggest that p47 GTPases have functions beyond simple quantitative control of intracellular Chlamydia growth. Genome-wide transcriptional data and evaluation of the immune phenotype in C57BL/6j and DBA/2j strains demonstrated that divergent innate immune pathways were stimulated as a result of the primary genetic differences in Irgb10 and Iigp2. In our model the ultimate event that defined death was the recruitment of neutrophils in susceptible mice, which implicates a role of p47 GTPases directly or indirectly in the regulation of inflammation. This finding has added relevance in the context of chlamydial diseases, because in chlamydial pathogenesis the major complications of infection are a result of host inflammatory responses such as tubal or conjunctival scarring in infertility or trachoma, respectively.

The p47 GTPases have been implicated in immune regulatory mechanisms such as Ag presentation (29). Mycobacterial and trypanosome infection in Lrg-47 knockout mice results in lymphopenia or defective hemopoiesis respectively, although the mechanisms are still unknown (44, 45). In our model, although cell autonomous dysfunction in chlamydial control may lead to direct pathogenic consequences, it is also possible that p47 GTPases play a role in regulating downstream events in a manner that would attenuate inflammation, such as induction of apoptosis, chemokine expression, or efficient Ag presentation as suggested by our transcriptional analysis. Establishing the functional consequences of p47 GTPase differences in innate susceptibility will be a key objective for understanding control mechanisms for a variety of important intracellular pathogens.

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Disclosures

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References


