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

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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 p47 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.

**Chlamydia** 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 factor 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 for尔斯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 *Ctgq-3* (22, 23) as a dominant Mendelian trait containing a cluster of three p47 GTPases (*Irgb10, Igtp*, and *Igtp2* (*Gpfl* 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 knockout 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 quantitate 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*)) were purchased from The Jackson Laboratory. Seven- to 8-wk-old male or female BXD strains were bred and maintained at our facility, which is fully accredited by the Association for Accreditation and Assessment of Laboratory Animal Care International (24). Breeder pairs of *Igtp* knockout mice were backcrossed to a C57BL/6J background (25) were provided by Dr. M. Starnbach (Harvard Medical School, Boston, MA) and bred and maintained in our facility. The Animal Care and Use Committee at University of Tennessee Health Science Center (Memphis, TN) approved all animal studies.

**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 enumerating 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.

Chlamydia 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 infected with 500 IFU of *C. psittaci* and then challenged with 106 IFU 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) injected i.m. 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.

**In vitro peritoneal macrophage infection**

Peritoneal macrophages from C57BL/6J and DBA/2J mice were elicited by 5% protease peptone and plated at 5 × 103 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/dispass (catalog no. 10269638001; Thermo Fisher Scientific, Waltham, MA)
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 \textit{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 $\geq$ 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.
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 × 10^4 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 RNA silencing: r(GAACGUUUCCAGAAAGAAA)dTdT (sense) and r(UUU...

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 the location of 3,600 informative markers. At a single chromosomal level, interval mapping evaluates potential QTL at regular intervals and estimates the significance at each location with a graphical representation of the likelihood odds ratio (LOD) (27). A permutation test 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 10^4 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 10^4 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 BM8, product no. MF48005; Caltag Laboratories) conjugated with allophycocyanin (product no. RM2905; Caltag Laboratories) at 1 μg/10^6 cells; Ly6G-PE (clone 1A8, catalog no. 551461; BD Biosciences) at 0.5 μg/10^6 cells; CD3-Alexa 488 (clone 145-2c11, catalog no. 557666; BD Biosciences) at 0.5 μg/10^6 cells; and CD11b-PE-Cy7 (clone M1/70, catalog no. 553141; BD Biosciences) at 0.5 μg/10^6 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 CytetSet kit, catalog no.
both strains of mice were plated and preincubated with IFN-γ/H9253 of infection. siRNA then infected with C57BL/6J and DBA2/J mice were pretreated with interferon gamma (0 and 20 ng/ml) medium for 24 h before infection with C. psittaci. Results are representative of two experiments.

FIGURE 5. Igpp2 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 Igpp2 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.

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: CMC1063; BioSource International and Cxcl2 (mouse MIP-2 CytoSet kit, catalog no. CMC2453; BioSource International) were assessed according to company protocol.

Genotyping

DNA was extracted from snipped 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: 5ʹ-CCTGGTGTCAGCGGAAGCCT-3ʹ (forward primer), 5ʹ-GTTGAAAGGAAGCTGACCCTGTT-3ʹ (reverse primer), and 5ʹ-TGGACTGAGGCTTCCTCCAGAACC-3ʹ (probe); Iigp2: 5ʹ-AAGACATCTCAGACATCTGCAAGA-3ʹ (forward primer), and 5ʹ-CCTTTACAGTCGGCACA-3ʹ (probe). Probes were labeled with the FAM reporter fluorochrome and the TAMRA quencher.

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:
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. 18-8814; eBioscience). SuperSignal West Pico chemiluminescent substrate (Pierce) was used as a substrate. 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 Biotechnology); Irgb10, goat anti-rabbit-HRP (catalog sc-2004; Santa Cruz Biotechnology). The following secondary Abs were used: 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. sc-1616; Santa Cruz Biotechnology); IL12a at 1:1000 (catalog no. 610880; BD Biosciences); GTPI (M-14) at 1/500 (catalog no. 01-6500; Cell Signaling); IL6 and IGF (catalog no. sc-11088; Santa Cruz Biotechnology); and actin (I-19) at 1:1000 (catalog no. 16-1000; Sigma-Aldrich). The proportion of Irgb10 that was hybridized to a mouse genome 2.0 array chip (Affymetrix) was highly up regulated in DBA/2J mice.

Microarray analysis

Table I. Differential microarray analysis

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene No.</th>
<th>Enrichment</th>
<th>p Value</th>
</tr>
</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>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>15</td>
<td>R = 3.20</td>
<td>6.96E-05</td>
</tr>
<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>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>11</td>
<td>R = 4.45</td>
<td>3.11E-05</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>10</td>
<td>R = 2.98</td>
<td>1.90E-03</td>
</tr>
<tr>
<td>Hemopoietic cell lineage</td>
<td>9</td>
<td>R = 3.17</td>
<td>2.00E-03</td>
</tr>
<tr>
<td>TLR signaling pathway</td>
<td>9</td>
<td>R = 2.74</td>
<td>5.50E-03</td>
</tr>
<tr>
<td>Ag processing and presentation</td>
<td>9</td>
<td>R = 3.81</td>
<td>5.38E-04</td>
</tr>
</tbody>
</table>

*Microarray analysis of mRNA collected from peritoneal lavage 3 days postinfection and normalized 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 1/500 (catalog no. 610880; BD Biosciences); GTPI (M-14) at 1/500 (catalog no. sc-11088; Santa Cruz Biotechnology); IL12a at 1:1000 (catalog no. 610880; BD Biosciences); GTPI (M-14) at 1/500 (catalog no. 01-6500; Cell Signaling); IL6 and IGF (catalog no. sc-11088; Santa Cruz Biotechnology); and actin (I-19) at 1:1000 (catalog no. 16-1000; Sigma-Aldrich). The proportion of Irgb10 that was hybridized to a mouse genome 2.0 array chip (Affymetrix) was highly up regulated in DBA/2J mice.

Microarray analysis

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>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2J</td>
<td></td>
<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>
</tr>
<tr>
<td>Ila</td>
<td>16.9</td>
<td>1.44E-03</td>
<td>Interleukin 1α</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>10.4</td>
<td>7.01E-03</td>
<td>Chemokine (C-X-C motif) ligand 2</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>9.8</td>
<td>1.64E-02</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
</tr>
<tr>
<td>Il1rn</td>
<td>8.3</td>
<td>1.44E-03</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>Il1f9</td>
<td>6.2</td>
<td>1.90E-02</td>
<td>Interleukin 1 family, member 9</td>
</tr>
<tr>
<td>Il12a</td>
<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-C motif) ligand 3</td>
</tr>
<tr>
<td>Tnf</td>
<td>4.2</td>
<td>3.77E-03</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Il1b</td>
<td>3.7</td>
<td>6.24E-03</td>
<td>Interleukin 1 β</td>
</tr>
<tr>
<td>Xcl1</td>
<td>3.3</td>
<td>4.95E-02</td>
<td>Chemokine (C motif) ligand 1</td>
</tr>
<tr>
<td>Ls</td>
<td>3.1</td>
<td>1.97E-02</td>
<td>Lymphotoxin B</td>
</tr>
<tr>
<td>Il6</td>
<td>2.6</td>
<td>3.21E-02</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>Csf3</td>
<td>2.5</td>
<td>5.96E-03</td>
<td>Colony stimulating factor 3 (granulocyte)</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bnip1</td>
<td>4.7</td>
<td>2.16E-03</td>
<td>Bone morphogenetic protein 1</td>
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<td>Irok4</td>
<td>3.6</td>
<td>7.17E-02</td>
<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.
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 Ctrq-3 (Fig. 2, A and B) (22, 23). This region encodes 18 genes, including a cluster of three p47 GTPases, Irgb10, Igtp, and Iigp2 (Fig. 2C). The genetic differences at Ctrq-3 accounted for 83% of the overall phenotypic difference (trait variance) defined as survival upon chlamydial challenge. The previously described Ctrq-1 and 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 Irgb10 and 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 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 Igtp or 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 Irgb10 in C57BL/6 mice vs DBA/2J. Also, C57BL/6J was found to preferentially express a 47-kDa protein product for 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 Ctrq-3 locus using microsatellite markers divided the locus into a proximal Ctrq-3 containing the Irgb10 and Igtp DBA/2J allele and a distal Ctrq-3 containing the C57BL/6J allele.
inhibition was observed when host cells were activated with ex- 
harvested 48 h postinfection, but a 1–2 log difference in growth 
macrophages infected with a multiplicity of infection of 1 and 
chlamydial load in unstimulated cell culture samples of peritoneal 
Inhibition was seen in IFN-
C57BL/6J mice (Fig. 5). In contrast, little or no chlamydial growth 
infection was observed when host cells were activated with ex- 
ogenous IFN-γ at the time of infection (Fig. 4C). Chlamydial 
growth inhibition was not reversed by addition of the inducible NO 
synthase inhibitor (N(G)-monomethyl-L-arginine (1-NNMA)) (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 de- 
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 C57BL/6J-derived 
fibroblasts and 67.2% in DBA/2J-derived fibroblasts treated with 
IFN-γ. Inhibition of Iigp2 resulted in partial restoration of chla- 
moidal 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 pheno- 
typic 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 pa- 
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 C57BL/6J and 
DBA/2J mice. A gene ontology analysis comprising a set of 
~1000 transcripts with >2.5-fold difference in expression reve- 
aled potential downstream pathways reflecting the primary ge- 
genetic differences (Table I). Transcripts for proinflammatory cyto-
kin and chemokine (Cxcl1 (KC), Cxcl2 (Mip2), and Cxcl11) 
genes were found to be up-regulated >10-fold in susceptible 
DBA2J mice (Table II). Known upstream regulators of inflamma- 	ory cytokine release (stimulation of pattern recognition receptors 
such as TLRs and up-regulation of MyD88) were increased in re- 
sistant (C57BL/6J) vs susceptible (DBA/2J) mice, indicating al- 
ternative pathway usage between the two strains. Furthermore, >2 
log up-regulation of genes involved in differentiation and prolif-
eration of macrophages (ifit204 and ifit203) and >10-fold up-reg- 
eration of NK cell activating genes was seen in resistant (C57BL/ 
6J) vs susceptible (DBA/2J) mice. These results suggested that 

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

Inflammatory responses were characterized by histology and cy-
tology. 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

FIGURE 7. Interruption of neutrophil recruitment reverses the suscepti-
ble phenotype. BALB/cJ and Cxcr2 knockout mice were infected i.p. with 
C. psittaci (10⁴ IFU). A, Weight changes are recorded over time. *, All 
BALB/cJ mice died after 10 days (n = 3). B, Peritoneal lavage collected 6 
days postinfection was analyzed by flow cytometry (n = 3). Top, Cxcr2 
knockout mice demonstrated an absence of neutrophils, which were abun-
dant in BALB/cJ mice (p = 0.01). Bottom, The majority of cells in the 
peritoneal cavity of Cxcr2 knockout mice were macrophages. C, Chlamy-
dial load in the peritoneal cavity of Cxcr2 knockout mice and BALB/cJ mice 
were similar (n = 3; p = 0.19).

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<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>−</sup> cells) 
to the site of infection, compared with C57BL/6J mice, which 
recruited predominantly macrophages (CD11b<sup>+</sup>Ly6G<sup>−</sup>F4/80<sup>+</sup> cells) (Fig. 6B). This was confirmed by the production of Cxcl1 and Cxcl2 in the peritoneal cavity by ELISA in only the sus- 
ceptible 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 neutro-
phils to the site of infection and survived challenge with C. psittaci
FIGURE 8. Igtp knockout mice are susceptible to Chlamydia. A. Igtp knockouts, C57BL/6J and 129S1/SvImJ strains, were infected i.p. with C. psittaci and observed for weight changes. All C57BL/6J mice (+) survived and all Igtp knockouts (**) and 129S1/SvImJ mice (****) died (n = 3). B. Cross section of the spleen taken from Igtp knockout mouse on day 7 postinfection shows that the surface is coated with fibrin and inflammatory cells. C. Microsatellite marker analysis of genes taken from C57BL/6J (B6), Igtp knockout (ko), and 129S1/SvImJ (S1) demonstrates a predicted shift for markers D11Zbh1, 13, 12, and 2, demonstrating that Igtp knockout mice share a common genotype with 129S1/SvImJ mice for this critical interval.

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. QTL 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 these 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 catalyzes 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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