INFECTION AND IMMUNITY, Nov. 2009, p. 4794–4805 Vol. 77, No. 11
0019-9567/09/$12.00 doi:10.1128/IAI.01546-08

Restriction of *Legionella pneumophila* Replication in Macrophages Requires Concerted Action of the Transcriptional Regulators Irf1 and Irf8 and Nod-Like Receptors Naip5 and Nlrc4▽†

Anne Fortier,[1,2] Karine Doiron,[1,2] Maya Saleh,[1,2] Sergio Grinstein,[3] and Philippe Gros[1,2]*

Department of Biochemistry[1] and Center for the Study of Host Resistance,[2] McGill University, Montreal, Canada, and Department of Cell Biology, Hospital for Sick Children, Toronto, Canada[3]

Received 21 December 2008/Returned for modification 12 February 2009/Accepted 19 August 2009

The unique permissiveness of A/J mouse macrophages for replication of *Legionella pneumophila* is caused by a deficiency in the Nod-like receptor (NLR) protein and intracellular sensor for *L. pneumophila* flagellin (*Naip5*). The signaling pathways and proteins activated by Naip5 sensing in macrophages were investigated. Transcript profiling of macrophages from susceptible A/J mice and from resistant A/J mice harboring a transgenic wild-type copy of *Naip5* at 4 h following *L. pneumophila* infection suggested that two members of the Irf transcriptional regulator family, Irf1 and Irf8, are regulated in response to Naip5 sensing of *L. pneumophila*. We show that macrophages having defective alleles of either *Irf1* (*Irf1*$^{-/-}$) or its heterodimerization partner gene *Irf8* (*Irf8*^{R294C}) become permissive for *L. pneumophila* replication, indicating that both the Irf1 and Irf8 proteins are essential for macrophage defense against *L. pneumophila*. Moreover, macrophages doubly heterozygous (*Naip5*$^{AJ/WT}$ *Irf8*$^{R294C/WT}$ or *Nlrc4*$^{-/+}$ *Irf8*$^{R294C/WT}$) for combined loss-of-function mutations in *Irf8* and in either *Naip5* or *Nlrc4* are highly susceptible to *L. pneumophila*, indicating that there is a strong genetic interaction between *Irf8* and the NLR protein family in the macrophage response to *L. pneumophila*. *Legionella*-containing phagosomes (LCPs) formed in permissive *Irf1*$^{-/-}$ or *Irf8*^{R294C} macrophages behave like LCPs formed in *Naip5*-insufficient and *Nlrc4*-deficient macrophages which fail to acidify. These results suggest that, in addition to *Naip5* and *Nlrc4*, *Irf1* and *Irf8* play a critical role in the early response of macrophages to infection with *L. pneumophila*, including antagonizing the ability of *L. pneumophila* to block phagosome acidification. They also suggest that flagellin sensing by the NLR proteins Naip5 and Nlrc4 may be coupled to Irf1-Irf8-mediated transcriptional activation of key effector genes essential for macrophage resistance to *L. pneumophila* infection.

Legionella pneumophila is an intracellular gram-negative bacterium that is ubiquitous in aquatic environment. Following inhalation of *L. pneumophila*-contaminated water droplets, *Legionella* may replicate inside human alveolar macrophages and cause a severe form of pneumonia called Legionnaires' disease (14, 29) or a less severe flu-like disease, Pontiac fever (20). In permissive macrophages, *Legionella* interferes with normal phagosome maturation and survives in a phagosome that neither acidifies nor fuses with lysosomes for several hours (4, 19, 39, 42). On the other hand, a *Legionella*-containing phagosome (LCP) rapidly acquires markers of the endoplasmic reticulum, such as calnexin and glucose-6-phosphatase, and becomes studded with ribosomes (1, 9, 38, 46). This remodeling requires the Dot/Icm type IV secretion system that injects over 30 bacterial protein effectors into the cytosol of host cells during infection (2, 33, 34, 40, 53). These effectors are thought to manipulate host cell functions, enabling establishment of the replicative organelle and promoting intracellular survival of the pathogen.

In contrast to human macrophages, mouse macrophages are generally nonpermissive for intracellular replication of *L. pneumophila*, with the notable exception of the A/J mouse strain (55, 56). Susceptibility of murine macrophages to *L. pneumophila* ex vivo is caused by a single locus on chromosome 13, *Lgn1* (7, 16). In vivo complementation studies with A/J mice transgenic for the *Lgn1* region (8), in vitro gene-silencing experiments with macrophages (54), and recent studies with a null mutant (22) have established that *Naip5* is the gene underlying the *L. pneumophila* susceptibility effect at *Lgn1*. Naip5 belongs to the Nod-like receptor (NLR) family, a group of cytoplasmic proteins that act as intracellular sensors of pathogen-associated molecular patterns (PAMPs) and that represent a first line of defense in innate immunity (10, 27, 47). NLR proteins (over 20 NLR proteins have been described for humans) have a modular structure with (i) a conserved leucine-rich repeat (LRR) responsible for ligand recognition, (ii) a nucleotide-binding domain (NBD) mediating protein oligomerization, and (iii) a signaling-interaction domain that is specific for each NLR subfamily and that includes a caspase-recruitment domain (CARD), a pyrin domain, or a baculovirus inhibitor of apoptosis repeat domain. Activation of NLR proteins by PAMPs causes inflammatory, microbicidal, and cell death responses in macrophages that are mediated by NF-κB, mitogen-activated protein kinase, or caspase-1 (41). Stimulation of Nlrc4, Nlrp1b, and Nlrp3 by their ligand causes assembly of an inflammasome, an intracellular protein complex that leads to activation of proinflammatory caspase-1 and caspase-5 and secretion of proinflammatory cytokines (26, 35, 48).

* Corresponding author. Mailing address: Department of Biochemistry, McGill University, McIntyre Medical Building, 3655 Promenade Sir William Osler, Room 907, Montréal, Québec, Canada H3G 1Y6. Phone: (514) 398-7291. Fax: (514) 398-2603. E-mail: philippe.gros@mcgill.ca.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 31 August 2009.

Genetic and biochemical data have shown that both Naip5 (baculovirus inhibitor of apoptosis repeat domain, NBD, and LRR) and Nlrc4 (CARD, NBD, and LRR) are essential for innate defense against *L. pneumophila* and *Salmonella*, acting as sensors for flagellin species produced by these bacteria (1, 11, 24, 30, 37). Likewise, the human orthologs hNAIP and hNLRC4 were recently shown to be required for inhibition of intracellular *Legionella* replication (52). Flagellin-induced activation of caspase-1 is abolished in *Salmonella*- and *Pseudomonas*-infected macrophages lacking either Nlrc4 (1, 11, 13, 30, 31) or the NLR adaptor protein ASC (CARD and pyrin domain) (13, 24, 44). In addition, ASC has been shown to associate with Nlrc4 via CARD-CARD domain interaction in vitro (15). This suggests a model in which flagellin engagement of the LRR domain of Nlrc4 induces formation of an inflammasome consisting of Nlrc4, ASC, and caspase-1, leading to activation of the latter, interleukin-1β (IL-1β) processing, and pyroptosis (13, 31, 43).

Parallel study of Naip5 activation of caspase-1 in response to *L. pneumophila* flagellin has painted a more complex picture. First, *L. pneumophila*-induced cell death is impaired in *Naip5*-insufficient macrophages (37). Second, *Naip5*-insufficient macrophages show reduced caspase-1 activation in response to *L. pneumophila* infection, as measured by cleavage of synthetic substrates and IL-1β production (37, 57). Third, caspase-1-deficient macrophages show some increase in permissiveness for *L. pneumophila* replication (1, 57). Finally, cytosolic flagellin appears to activate caspase-1 in a Naip5-dependent fashion (32, 37), and 35 amino acids of the carboxyl terminus of flagellin is sufficient to trigger Naip5-dependent inflammasome activation (22). These observations suggest that Naip5 is required for restriction of *L. pneumophila* replication in macrophages through inflammasome-mediated activation of caspase-1 in response to flagellin. On the other hand, processing of caspase-1 in response to *L. pneumophila* infection has been demonstrated in macrophages carrying the A/J hypomorphic allele of *Naip5* (B6. Chr13-A/J) but not in *Nlrc4*-deficient macrophages (21, 22) or in macrophages from *Naip5*-null mice (knockout allele) (22). These studies indicate that mutations in the A/J allele of *Naip5* do not abolish its caspase-1 activation function, while they do impair its capacity to restrict *L. pneumophila* replication, suggesting that there is partitioning between these two *Naip5*-associated functions. In addition, ASC is necessary for *L. pneumophila*-induced caspase-1 activation but is dispensable for bacterial restriction (3, 57). Finally, studies of the maturation of LCPs in *Naip5*-deficient macrophages (6, 9) and in *Nlrc4*-deficient macrophages (1) have shown that Naip5 and Nlrc4 sensing of *L. pneumophila* products occurs very rapidly following phagocytosis (within 1 h).

These results suggest that Naip5 may participate in some signaling pathways (in addition to caspase-1) that play important roles in the early macrophage response and overall defense against *L. pneumophila*. In the present study, we used transcript profiling of *Naip5*-insufficient and *Naip5*-sufficient macrophages to obtain insight into the genes and pathways that are regulated in a *Naip5*-dependent fashion in response to *L. pneumophila* infection. Our results show that the transcriptional regulators Irf1 and Irf8 are rapidly induced in macrophages following infection. In addition, inactivation mutations in either *Irf1* or *Irf8* abrogate resistance to *L. pneumophila*. Finally, studies with macrophages doubly heterozygous for mutations in *Irf8* and *Naip5* or *Nlrc4* demonstrated that there is a strong genetic interaction between members of the Irf and NLR families in the anti-*Legionella* defense of macrophages. This suggests that Naip5-Nlrc4 signaling in response to *Legionella* flagellin is closely linked to Irf-dependent transcription of genes encoding proteins essential for restriction of *L. pneumophila* growth in macrophages.

MATERIALS AND METHODS

Mice. A/J, C57BL/6J (B6), and *Irf1*$^{-/-}$ mutant mice (with a B6 background) were purchased from Jackson ImmunoResearch Laboratories. Transgenic mice that express a *Naip5* resistance allele from the B6 strain with the genetically susceptible A/J background have been described previously (8). These A/J mice correspond to an N8 generation intercross carrying (BAC$^+$) or not carrying (BAC$^-$) a B6-derived BAC clone harboring a wild-type *Naip5* resistance allele. BXH-2 mice were obtained from N. Copeland and N. Jenkins (National Cancer Institute, Frederick, MD) and were maintained as a breeding colony at McGill University. The *IL-12*$^{-/-}$ mutant mice were provided by M. M Stevenson (McGill University, Montreal, Canada). The *Nlrc4*$^{-/-}$ mutant mice were kindly provided by Millennium Pharmaceuticals, Inc., and R. A. Flavell (Yale University). All mice were maintained and handled according to guidelines of the Canadian Council on Animal Care.

Macrophages. Bone marrow-derived macrophages (BMDMs) were isolated from femurs of 12- to 16-week-old mice and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% heat-inactivated fetal bovine serum (HI-FBS), 20% L-cell-conditioned medium (LCCM), 100 U/ml penicillin, and 100 μg/ml streptomycin in bacteriological grade dishes (Fischer) at 37°C in a humidified atmosphere containing 5% CO_2. Seven days later, cells were harvested by gentle washing of the monolayer with phosphate-buffered saline containing citrate. Cells were plated in 150-mm tissue culture-grade plastic plates (15×10^6 cells per plate; Corning) in DMEM containing 10% HI-FBS, 10% LCCM, and 100 μg/ml of thymidine (Sigma) without antibiotics. Macrophages were cultured for an additional 24 h prior to use.

Bacterial strains and infection of macrophages. *L. pneumophila* Philadelphia-1 strain Lp02, a thymidine auxotroph derivative of strain Lp01, was a kind gift from Craig Roy (Yale University School of Medicine, New Haven, CT). The *dotA* mutant was a kind gift from Howard Shuman (Columbia University, New York, NY). An *flaA* deletion (corresponding to nucleotides 1478105 to 1479574 of the Lp01 genome) was generated in strain Lp02 by use of the allelic exchange vector pSR47S kindly provided by Russell Vance (Harvard Medical School, Boston, MA). The Lp02, *dotA*, and Δ*flaA* strains were cultured to stationary phase in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) (Sigma)-buffered yeast extract broth supplemented with 100 μg/ml of thymidine and used to infect BMDMs. BMDMs were exposed to *L. pneumophila* at a multiplicity of infection of 10:1 for 1 h at 37°C, and then cells were washed with warm DMEM and incubated for the period of time indicated below in DMEM supplemented with 10% HI-FBS, 10% LCCM, and 100 μg/ml of thymidine. Bacterial replication is expressed as the log increase in the number of CFU determined by lysis of macrophages with distilled water and plating of the cell lysates onto BCYE agar plates.

RNA isolation. Total cellular RNA was extracted from BMDMs using a commercial reagent (TRIzol; Invitrogen) according to the manufacturer's recommendations. Macrophages were harvested in 2 ml (final volume) of TRIzol reagent. The samples were incubated for 5 min at 20°C, which was followed by chloroform extraction. The aqueous phase was removed, and nucleic acids were precipitated with isopropanol. Pellets were washed with 75% ethanol and dissolved in RNase-free water treated with 0.1% diethlypyrocarbamate. The integrity of each of the RNA preparations was verified by electrophoresis on 1% formaldehyde-containing agarose gels. In some instances, further RNA purification was performed using RNeasy columns (Qiagen) and 100 μg of total RNA according to the manufacturer's recommendations.

Transcription profiling. Mouse 15k v.4 cDNA spotted arrays were generated and hybridized by the UHN Microarray Facility (Toronto, Ontario, Canada) and analyzed as previously described. Individual spots from 16-bit TIFF digitized images were quantified using the QuantArray software (Perkin Elmer). Raw data generated by QuantArray were normalized using the GeneSpring software package (Silicon Genetics) and the Lowess scatter smoothing algorithm. We analyzed six hybridizations consisting of dye swap hybridizations of three biological replicates for BAC$^-$ versus BAC$^+$. Genes with reproducible changes in transcript

abundance were identified using the "one-class" algorithm in Significance Analysis of Microarrays (SAM). SAM assigns a score to each gene on the basis of the change in expression relative to the standard deviation of repeated measurements for that gene. Genes showing significant differences in expression were chosen using a false discovery rate of <0.05% and were further culled by keeping only the genes with a relative variation of >1.5. Results were visualized using the GeneSpring software.

Semiquantitative RT-PCR. For semiquantitative reverse transcription (RT)-PCR, independent RNA samples ($n = 3$) from the same experimental group were pooled, and 3 μg was converted to cDNA with reverse transcriptase (Moloney murine leukemia virus reverse transcriptase; Invitrogen) in a 20-μl RT reaction mixture, as previously described (25). One microliter of the RT reaction mixture was used for *Taq* DNA polymerase (Invitrogen)-mediated PCR amplification; the cycling parameters included an initial denaturation step (3 min at 94°C), followed by 16 to 20 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C and a final elongation step consisting of 7 min at 72°C. Amplicons were resolved in 1% agarose gels analyzed under UV light and were transferred to GeneScreen Plus membranes (Dupont, NEN Research Products). PCR primers were designed using the reported gene sequences. After transfer, DNA was UV cross-linked and prehybridized for at least 4 h at 65°C in a solution containing 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 1 M NaCl with 200 μg ml^{-1} of salmon sperm DNA. Hybridization was then performed overnight at 65°C with an [α-^{32}P]dATP-labeled specific DNA fragment (100,000 cpm/ml of buffer) corresponding to each target gene. After incubation, the membrane was washed twice with 2× SSC-0.1% SDS (15 min per wash, 42°C), once with 2× SSC-0.5% SDS (30 min, 65°C), and once with 0.5× SSC-0.5% SDS (30 min, 65°C) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Northern blot analysis. Total RNA (15 μg) was separated by electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde and blotted onto a GeneScreen membrane (Perkin Elmer). *Irf1* expression was monitored by hybridization to a gene-specific probe labeled with [α-^{32}P]dATP using Klenow fragment DNA polymerase. The membrane was hybridized at 65°C in a solution containing 10% dextran sulfate, 2× SSC, 1% SDS, and 200 μg/ml salmon sperm DNA for 16 h. The membrane was washed twice at 65°C for 15 min in 2× SSC-0.1% SDS and then for 30 min with 2× SSC-0.5% SDS and for 30 min with 0.5× SSC-0.5% SDS. The membrane was stripped by boiling it in 10 mM Tris-HCl, 1 mM EDTA, 1% SDS before rehybridization. The signal was quantified using a phosphorimager.

Fluorescent labeling of *Legionella*. Bacteria were grown as described above. Before infection, 2×10^8 bacteria were transferred to a microcentrifuge tube, pelleted, and suspended in 1 ml of a fluorescein isothiocyanate solution (FITC) (0.5 mg/ml; Sigma) in 100 mM $NaHCO_3$ (pH 8). Bacteria were incubated for 20 min at room temperature, washed three times in 1 ml of $NaHCO_3$ (pH 8), and resuspended in DMEM.

Measurement of phagosomal pH. Measurements of phagosomal pH were obtained by fluorescence ratio imaging. BMDMs were plated on a coverslip and were infected the next day with fluorescein-treated *L. pneumophila*. The labeled bacteria were added to cells at a multiplicity of infection of 10:1, which was followed by centrifugation for 5 min at 4°C and incubation for 15 min at 37°C. Extracellular bacteria were removed by three washes with DMEM. The remaining nonphagocytosed bacteria were labeled with rat anti-*Legionella* antibody (1:100) for 5 min, followed by Cy3-conjugated anti-rat immunoglobulin G (1:100) for 5 min on ice. Phagosomes were allowed to mature for 1 h at 37°C, and then cells were placed in a chamber on the stage of a Zeiss microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a ×100 oil immersion objective. A Sutter filter wheel was used to alternately position the two excitation filters (440 and 490 nm) in front of a xenon lamp. Image acquisition was controlled by MetaMorph software (Universal Imaging Corp., West Chester, PA). Calibration of the ratio of fluorescence to pH was performed in situ for each experiment by equilibrating the cells in isotonic K^+-rich medium buffered to various pH values (between 4.5 and 7.5) in the presence of the K^+/H^+ ionophore nigericin (5 μM) as described previously (17). Calibration curves were constructed by plotting the extracellular pH, which is assumed to be identical to the internal pH, versus the corresponding fluorescence ratio.

RESULTS

***L. pneumophila* infection of BMDMs causes upregulation of *Irf1* and *Irf8* expression.** In the present study, we set out to identify genes, proteins, and biochemical pathways that are important for preventing intracellular replication of *L. pneumophila* in macrophages and whose activity may be under the control of early signaling by the Naip5 protein. In these experiments, we used BMDMs obtained from A/J (*Lgn1^s*) transgenic mice harboring (BAC^+, resistant) or not harboring (BAC^-, permissive) a recombinant BAC clone expressing the resistant *Naip5* allele from strain B6 (*Lgn1^r*). During the first 4 h following infection, both macrophage populations can restrict *Legionella* replication. However, by 8 h, the effect of the *Naip5* gene can be detected, as there is active *L. pneumophila* replication in BAC^- macrophages, while BAC^+ macrophages can suppress bacterial replication. The difference in the number of CFU continues to increase over the next 72 h (Fig. 1A).

To look for genes and pathways regulated by *L. pneumophila* infection in a *Naip5*-dependent or -independent fashion, we carried out transcriptional profiling with cDNA microarrays, comparing RNA populations of infected BMDMs from BAC^+ and BAC^- mice. In this experiment, BAC^- macrophages allowed 3.2-fold replication of *L. pneumophila* over 24 h, whereas for BAC^+ transgenic macrophages there was a 1.8-fold decrease in the number of CFU over the same period (Fig. 1B). At 4 h, the numbers of CFU recovered from the BAC^+ and BAC^- groups were similar (Fig. 1B), and therefore differences in gene expression profiles detected at that time point were more likely to reflect the direct effect of the *Naip5* gene and not secondary effects associated with differences in the intracellular bacterial loads. Transcript profiles were obtained for BAC^- and BAC^+ BMDMs either prior to infection or 4 h following infection. Using the "one-class" algorithm in the SAM application ($P < 0.05$, as determined by a *t* test; cutoff, 1.5-fold change; false discovery rate, 0.5%) (Fig. 1C), we detected 138 transcripts significantly regulated by infection in BMDMs from both the BAC^+ and BAC^- groups following infection with *L. pneumophila* (Table 1; see Table S1 in the supplemental material for the complete list). In addition, we detected 17 transcripts that were differentially expressed only in BAC^+ cells in response to infection and 49 transcripts specific to BAC^- cells (Table 1). For the genes significantly regulated at 4 h postinfection only in BAC^- cells, only in BAC^+ cells, or in both BAC^- and BAC^+ cells, gene ontology analysis identified transcripts associated with signal transduction, immune response, and transcription (Table 1). As the cDNA microarrays used here contained only 15,000 transcripts, the number of genes identified in each class is likely to be an underestimate.

A semiquantitative RT-PCR approach was used to validate the differential expression of several genes detected by transcript profiling. In these experiments, we prioritized genes potentially implicated in immune functions. The *Gapdh* and β-*Actin* mRNA levels were not modulated in response to infection and were used as an internal control for normalization. Robust upregulation of *Gro1*, *NFκBiα*, *Mlp*, and *Irf1* in response to *L. pneumophila* infection was verified (Fig. 2A). In a time course experiment, maximal *Irf1* induction was observed as early as 1 h postinfection (data not shown). We further confirmed infection-dependent induction of *Irf1* and of its heterodimerization partner, *Irf8*, by Northern blot analysis (Fig. 2B). Interestingly, we noted that induction of *Irf1* and *Irf8* mRNA expression in macrophages infected with *L. pneumophila* was somewhat greater in *Naip5*-sufficient (BAC^+) macrophages than in *Naip5*-insufficient (BAC^-) macrophages (Fig. 2B). These results suggest that the transcriptional regulators

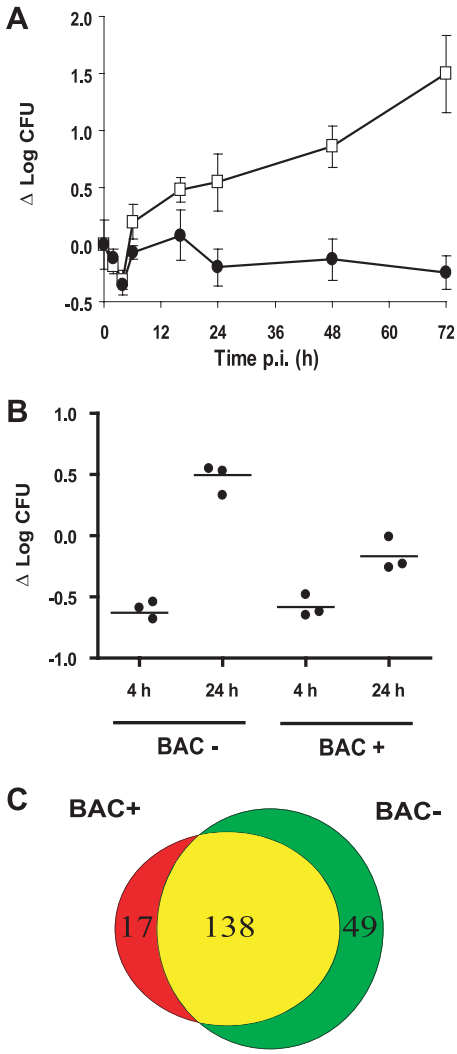

FIG. 1. Differentially expressed transcripts in BMDMs of Naip5 transgenic mice (BAC^+) and of nontransgenic littermates (BAC^-) in response to *L. pneumophila* infection. (A) *L. pneumophila* infection of BMDMs from A/J ($Lgn1^s$) transgenic mice harboring (BAC^+, resistant) (filled circles) or not harboring (BAC^-, permissive) (open squares) the BAC transgene from B6 mice ($Lgn1^r$). Bacterial replication is expressed as the fold increase in the number of CFU (Δ Log CFU). p.i., postinfection. (B) BMDMs from resistant (BAC^+) or susceptible (BAC^-) mice were infected with *L. pneumophila* Lp02, and replication was evaluated at 4 and 24 h postinfection. Total cellular RNA was isolated from BMDMs both prior to and 4 h after *L. pneumophila* infection and was converted to labeled cDNAs, which were hybridized in pairs to 15k spotted cDNA microarrays. (C) Transcripts significantly modulated in response to *L. pneumophila* (SAM analysis, false discovery rate, <0.05%) in BAC^+ (red) and BAC^- (green) cells or in both cell types (yellow), as shown by a Venn diagram.

Irf1 and Irf8 may be part of the signaling program downstream of *Naip5*.

Irf1 and Irf8 are required to restrict intracellular replication of *L. pneumophila* in macrophages. Irf1 is a transcriptional regulator and a member of the interferon response factor (IRF) family, a group of nine proteins that play a critical role in cellular responses to bacterial and viral pathogens by activating transcription of certain gene families in response to early signaling by different classes of interferon. Notably, Irf3 is required for *L. pneumophila*-induced beta interferon (IFN-β) expression and for control of intracellular replication of *L. pneumophila* in lung epithelial cells (36). However, the role of other IRF family members in macrophage defense against *L. pneumophila* infection has not been studied previously. Irf1 heterodimerizes with Irf8 to stimulate transcription of IFN-γ-responsive genes that have an interferon-stimulated response element sequence in their regulatory regions, including *IL-12p40* and *iNOS*. In vivo, this leads to activation of $CD4^+$ T cells and NK cells for production of IFN-γ, thereby amplifying the initial signal and contributing to Th1 polarization of the early T-cell response (45).

To address the role of Irf1 and Irf8 in macrophage defense against *L. pneumophila*, we monitored replication of *L. pneumophila* in macrophages from mice having a nonfunctional allele at each locus. The $Irf1^{-/-}$ mutant used was generated

TABLE 1. Annotated genes regulated during *L. pneumophila* infection[a]

Gene	Ratio of expression at 4 h following infection to expression before infection		Description
	BAC^-	BAC^+	
Significant in BAC^+			
Zfp36	1.3	3.3[b]	Zinc finger protein 36
Irf1	1.4	2.5[b]	Interferon regulatory factor 1
Kif9	1.1	1.6[b]	Kinesin 9
Significant in both BAC^+ and BAC^-			
Gro1	30.1[b]	27.0[b]	GRO1 oncogene
Mlp	3.8[b]	3.2[b]	MARCKS-like protein
Ppp2cb	2.4[b]	2.1[b]	Protein phosphatase 2a, catalytic subunit beta
H3f3b	4.7[b]	4.1[b]	H3 histone, family 3B
Nfkbia	3.8[b]	3.2[b]	Nuclear factor of kappa light chain inhibitor, alpha
Egr1	2.3[b]	2.3[b]	Early growth response 1
Cbx5	2.5[b]	2.1[b]	Chromobox homolog 5
Clecsf9	7.6[b]	7.3[b]	C-type lectin, superfamily member 9
Ier3	2.6[b]	2.4[b]	Immediate early response 3
Pex1	2.2[b]	1.9[b]	Peroxisome biogenesis factor 1
Mak3p	2.1[b]	1.9[b]	Mak3p homolog
Ccnl	2.3[b]	1.8[b]	Cyclin L
Snrpd1	2.0[b]	1.7[b]	Small nuclear ribonucleoprotein D1
Top1	1.9[b]	1.7[b]	Topoisomerase I
Significant in BAC^-			
Ern1	2.7[b]	1.1	Endoplasmic reticulum-to-nucleus signaling 1
Hist4	1.9[b]	1.4	Histone cluster 4
Mrpl52	2.1[b]	1.3	Mitochondrial ribosomal protein L52
Nfkb1	2.0[b]	1.4	Nuclear factor of kappa light polypeptide in B-cells 1

[a] Genes regulated at least 1.5-fold under least one of the genetic conditions are included.

[b] Gene with significant change upon infection (*t* test; Benjamini-Hochberg false discovery rate, <0.05%).

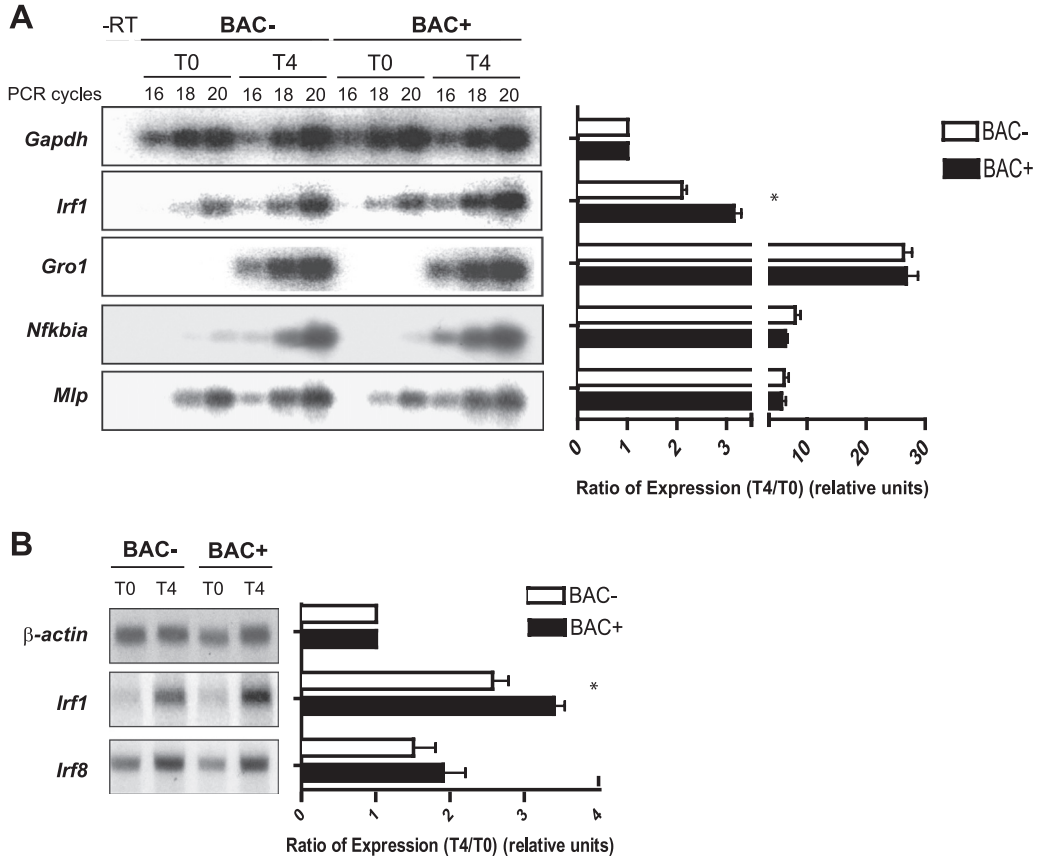

FIG. 2. Analysis of gene expression by semiquantitative RT-PCR and quantitative Northern blotting. BMDMs were infected with *L. pneumophila*, and RNA was harvested either prior to infection (T0) or 4 h following infection (T4). (A) Pooled RNA samples ($n = 3$) were converted to cDNA, and individual transcripts were amplified by PCR using different numbers of PCR cycles, as indicated. The reaction products were separated by electrophoresis and analyzed by Southern blotting with gene-specific probes (indicated on the left). The hybridization signal was quantified with a phosphorimager, and the ratio of expression (T4/T0) for each gene in BAC^- and BAC^+ cells was calculated following standardization using *Gapdh* as an internal control (right panel). (B) *Irf1* and *Irf8* expression in *Naip5*-sufficient (BAC^+) or -insufficient (BAC^-) BMDMs either prior to *L. pneumophila* infection or 4 h following *L. pneumophila* infection was assessed by Northern blotting. RNA (15 μg) was separated by gel electrophoresis, transferred to a hybridization membrane, and hybridized to a ^{32}P-labeled *Irf1* or *Irf8* cDNA probe (lower panels). The data are averages of three independent experiments. The signal was quantified with a phosphorimager and standardized using β-*Actin* as an internal control (upper panel). An expression ratio of 1 indicates no modulation by infection. ∗, $P < 0.05$, as determined by Student's *t* test.

using an otherwise *Legionella*-resistant B6 background (*Lgn1*^{r}). Likewise, BXH-2 is a recombinant inbred mouse strain derived from C3H/HeJ (*Lgn1*^{r}) and B6 (*Lgn1*^{r}) that has a loss-of-function mutation (R294C) in *Irf8* that we have reported previously (50). BMDMs from *Irf1*- and *Irf8*-deficient mice were infected in vitro with *L. pneumophila* Lp02, and 72 h later the total numbers of CFU were determined. Control BMDMs from permissive A/J and BAC^- controls supported a 1.5-log increase in the number of CFU, while resistant controls (B6, BAC^+) could completely suppress *L. pneumophila* replication over the same period (Fig. 3). *Irf1*$^{-/-}$ macrophages seemed to be partially impaired in the ability to control *L. pneumophila* replication (0.5-log increase), while *Irf8*^{R294C} mutant macrophages were completely susceptible to infection (Fig. 3). These results demonstrate that Irf1 and Irf8 are required for macrophage defense against *L. pneumophila*.

Increased susceptibility of *Irf1*$^{-/-}$ and *Irf8*^{R294C} macrophages to *L. pneumophila* is IL-12 independent, is not due to a generalized bactericidal defect, and can be reversed by IFN-γ treatment. Macrophages infected with intracellular parasites,

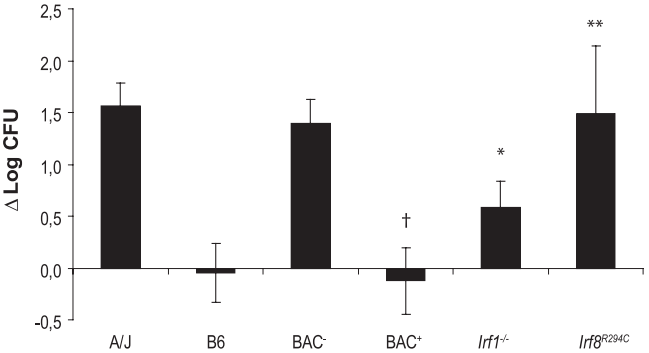

FIG. 3. Intracellular replication of wild-type *L. pneumophila* Lp02 in BMDMs from A/J, B6, BAC^-, BAC^+, *Irf1*$^{-/-}$, and *Irf8*^{R294C} mice. Bacterial replication (monitored at 72 h postinfection) is expressed as the log-transformed increase in the number of CFU (ΔLog CFU). †, $P < 0.01$ compared to BAC^-, as determined by Student's *t* test; ∗, $P < 0.05$ compared to B6, as determined by Student's *t* test; ∗∗, $P < 0.01$ compared to B6, as determined by Student's *t* test.

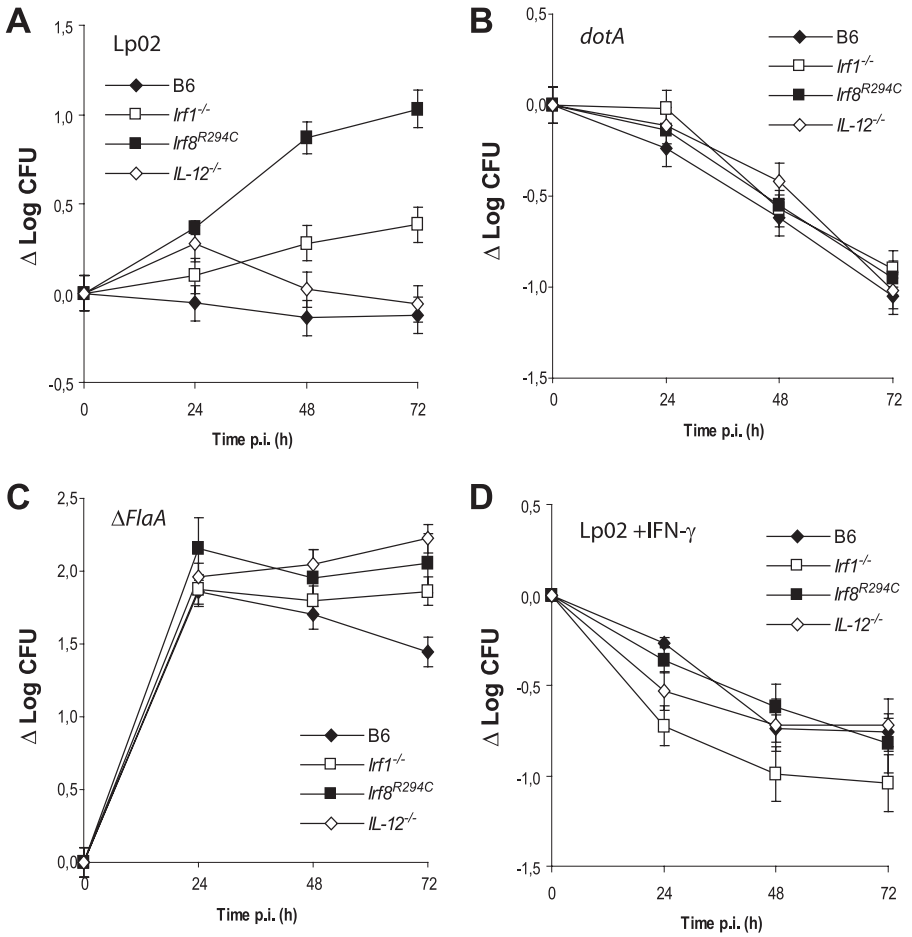

FIG. 4. Time course of infection with *L. pneumophila* Lp02 in BMDMs from B6, *Irf1*$^{-/-}$, *Irf8*^{R294C}, and *IL-12*$^{-/-}$ mice. BMDMs were infected with wild-type *L. pneumophila* Lp02 in the absence (A) or presence (D) of IFN-γ (50 U/ml) 24 h prior to infection, with a type IV secretion system *L. pneumophila* mutant (*dotA*) (B), or with a flagellin-deficient *L. pneumophila* mutant (Δ*flaA*) (C). The data are means ± standard deviations of three independent experiments, each done in duplicate. p.i., postinfection.

including *L. pneumophila*, respond by producing IL-12. An intact IFN-γ–IL-12 cytokine loop is required for expression of a number of protective responses, including expression of effector molecules and bactericidal enzymes synthesized by macrophages. In addition, IL-12 is a major transcriptional target of Irf1 and Irf8 (28). Therefore, we assessed the possibility that *Irf1*$^{-/-}$ and *Irf8*^{R294C} macrophages have increased susceptibility due to a deficiency in IL-12 production by examining the intracellular replication of *L. pneumophila* in macrophages from B6 mice lacking an *IL-12p40* gene (*IL-12p40*$^{-/-}$). Macrophages from *IL-12p40*$^{-/-}$ mice were as resistant to *L. pneumophila* infection as macrophages from B6 controls at 72 h postinfection (Fig. 4A). The specificity of the increased permissiveness of macrophages lacking *Irf1* or *Irf8* for intracellular replication of *L. pneumophila* was validated with two additional controls. *Irf1*$^{-/-}$ and *Irf8*^{R294C} macrophages did not suffer from general impairment of bacteriostatic and bactericidal functions, as both populations could block replication of an avirulent *dotA* mutant defective in the type IV secretion system (Fig. 4B). On the other hand, complete (B6, BAC^{+}) or partial (*Irf1*$^{-/-}$) inhibition of *L. pneumophila* replication was dependent on recognition of bacterial flagellin since macrophages with wild-type and *Irf*-deficient genotypes are equally susceptible to infection with an *L. pneumophila* flagellin (Δ*flaA*) mutant (Fig. 4C). Finally, pretreatment of macrophages from mice with all genotypes with IFN-γ completely abolished intracellular replication of *L. pneumophila* (Fig. 4D). Together, these results indicate that Irf1 and Irf8 are required for the intrinsic resistance of macrophages to intracellular replication of *L. pneumophila*. However, the protective effect of Irf1 and Irf8 in macrophages ex vivo is IL-12p40 independent and can be corrected by exposure to IFN-γ.

Macrophages from *Naip5*$^{S/R}$ *Irf8*$^{R294C/wt}$ and *Nlrc4*$^{-/+}$ *Irf8*$^{R294C/wt}$ doubly heterozygous mice are permissive for *L. pneumophila* replication. We further investigated a possible link between flagellin-dependent Naip5 signaling, inflammasome activation, and stimulation of Irf-dependent gene transcription. It has been proposed that the response to *L. pneumophila* flagellin involves activation of a Naip5-Nlrc4-containing inflammasome, and loss-of-function mutations in Naip5 or Nlrc4 abrogate macrophage resistance to *L. pneumophila* infection (1, 32, 37). Therefore, we set out to test possible genetic interactions between *Irf8* and either *Naip5* or *Nlrc4* by creating macrophages partially deficient in both pathways and

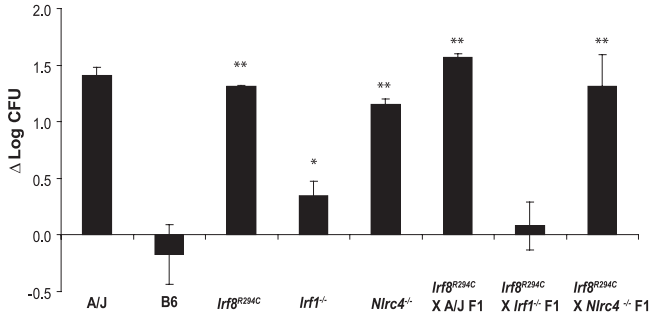

FIG. 5. Intracellular replication of wild-type *L. pneumophila* Lp02 in BMDMs from A/J, B6, $Irf8^{R294C}$, $Irf1^{-/-}$, $Nlrc4^{-/-}$, $(Irf8^{R294C} \times A/J)F_1$, $(Irf8^{R294C} \times Irf1^{-/-})F_1$, and $(Irf8^{R294C} \times Nlrc4^{-/-})F_1$ mice. Bacterial replication (monitored at 72 h postinfection) is expressed as the log-transformed increase in the number of CFU (ΔLog CFU). *, $P < 0.05$ compared to B6, as determined by Student's *t* test; **, $P < 0.01$ compared to B6, as determined by Student's *t* test. p.i., postinfection.

testing the effects of the partial impairment on resistance to *L. pneumophila* infection. For this, we isolated BMDMs from (i) $(A/J \times BXH\text{-}2)F_1$ mice that were doubly heterozygous for loss of function at *Naip5* and *Irf8* ($Naip5^{S/R}$ $Irf8^{R294C/wt}$) and (ii) $(Nlrc4^{-/-} \times BXH\text{-}2)F_1$ mice that were doubly heterozygous for loss of function at *Nlrc4* and *Irf8* ($Nlrc4^{-/+}$ $Irf8^{R294C/wt}$). The *Nlrc4* mutation was created in an otherwise *L. pneumophila*-resistant B6 background (11). BMDMs from these mice were infected in vitro with *L. pneumophila* Lp02, and bacterial replication was monitored 72 h postinfection (Fig. 5). In these experiments, we also tested BMDMs from additional control groups of singly heterozygous animals ($Irf8^{R294C/wt}$ and $Nlrc4^{-/+}$) which were found to be resistant to infection (data not shown). In addition, we assayed BMDMs from $(BXH\text{-}2 \times Irf1^{-/-})F_1$ mice that were doubly heterozygous for *Irf1* and *Irf8* mutations ($Irf8^{R294C/wt}$ $Irf1^{-/+}$). These macrophages were found to be nonpermissive for *L. pneumophila* replication (Fig. 5), indicating that a combined partial loss of function at *Irf1* and *Irf8* is not sufficient to cause susceptibility. By contrast, BMDMs doubly heterozygous ($Naip5^{S/R}$ $Irf8^{R294C/wt}$ or $Nlrc4^{-/+}$ $Irf8^{R294C/wt}$) for combined loss-of-function mutations in *Irf8* and in either *Naip5* or *Nlrc4* were found to be highly susceptible to *L. pneumophila* (Fig. 5). The degree of susceptibility of these double heterozygotes was as great as that of BMDMs from mice homozygous for either *Irf8* (BXH-2), *Naip5* (A/J), or *Nlrc4* mutations (Fig. 5 and data not shown). These results indicate that there is strong genetic interaction between *Irf8* and *Naip5* or *Nlrc4* that restricts *L. pneumophila* replication in macrophages. They suggest a model in which sensing of flagellin and activation of the inflammasome via Naip5 and Nlrc4 either are Irf8 dependent or cause downstream Irf8-dependent transcriptional activation of additional genes and pathways critical for defense against *L pneumophila*.

Naip5, Nlrc4, Irf1, and Irf8 are required for rapid LCP acidification. We previously showed that in wild-type B6 macrophages, Naip5 antagonizes the ability of *L. pneumophila* to modulate phagosome maturation toward an endoplasmic reticulum-derived replicative organelle, causing instead phagosome maturation of a fully acidified phagolysosome within 1 h following infection (9). A similar effect of Nlrc4 on maturation of an LCP was also demonstrated (within 2 h after infection) and was shown to be flagellin dependent and cell death independent (1). We investigated whether the permissiveness for *L. pneumophila* replication detected in $Irf1^{-/-}$ and in $Irf8^{R294C}$ mutant macrophages had effects on phagosome maturation similar to those observed in *Naip5*-insufficient (A/J) and *Nlrc4*-deficient macrophages. For this, we used live-cell microscopy to monitor in real time acidification of LCPs formed in macrophages isolated from BAC^-, BAC^+, B6, $Nlrc4^{-/-}$, $Irf1^{-/-}$, and $Irf8^{R294C}$ mice. Macrophages were infected with *L. pneumophila* Lp02 labeled by covalent attachment of fluorescein, a pH-sensitive ratiometric dye (pK_a 6.4), using a protocol that we have described previously (17). In these experiments, *L. pneumophila* infection was synchronized using a 5-min centrifugation step, followed by 15 min of incubation at 37°C to allow phagocytosis. Nonphagocytosed bacteria were removed by washing. In addition, to ascertain that only internalized bacteria were quantified, possible residual extracellular bacteria were labeled with an anti-*Legionella* antibody, followed by a Cy3-conjugated secondary antibody. Phagosome maturation was allowed to take place for 1 h following phagocytosis.

Figure 6A shows the results of a representative experiment performed with B6 wild-type macrophages. The upper left panel shows a phase-contrast microscopy image (differential interfering contrast) of macrophages interacting with two live *L. pneumophila* cells (as shown by the FITC image), one of which is intracellular and the other of which is extracellular (positive for Cy3 dye staining). As shown in the fluorescence ratio image in the bottom right panel, the intracellular bacterium is in an acidic environment (determined using the pseudocolor calibration scale on the right), while the other bacterium is exposed to extracellular medium which has a near-neutral pH. Calibration of the ratio of fluorescence to pH was performed in situ for each experiment by equilibrating the cells in isotonic K^+-rich medium buffered to various pH values (between pH 4.5 and 7.5) in the presence of the K^+/H^+ ionophore nigericin (5 μM) as described previously (17).

The pH of phagosomes containing *L. pneumophila* Lp02 formed in macrophages from wild-type B6 and *Naip5*-sufficient BAC^+ mice was significantly more acidic than that of *L. pneumophila* phagosomes formed in *Naip5*-insufficient BAC^-, $Nlrc4^{-/-}$, $Irf1^{-/-}$, and $Irf8^{R294C}$ mutant macrophages (pH 5.4 ± 0.1 and pH 6.2 ± 0.2, respectively) (Fig. 6B). The phagosomal pH in all cell types was indistinguishable after addition of the vacuolar H^+ ATPase inhibitor nigericin (not shown). Importantly, the attenuated phagosomal acidification seen in *Naip5*-insufficient, *Nlrc4*- or *Irf1*-deficient, or $Irf8^{R294C}$ mutant macrophages was specific for *L. pneumophila* wild-type strain Lp02, as phagosomes containing avirulent *dotA* bacteria acidified normally (Fig. 6B); i.e., they acidified to an extent comparable to that observed in phagosomes formed in B6 and BAC^+ macrophages. This confirms that the phagosomal acidification mechanism is fully competent in all cell types and that the cells differ only in their responsiveness to virulent wild-type *L. pneumophila*. In addition, this response is flagellin dependent, as shown by the lack of acidification of *flagellin*-deficient mutant (Δ*fla*) LCPs (Fig. 6B). In conclusion, the pattern of the phagosomal acidification defect caused by nonfunctional Irf8 is similar to and concomitant with the pattern caused by Naip5 insufficiency (Fig. 6C). These results suggest that Irf8 expres-

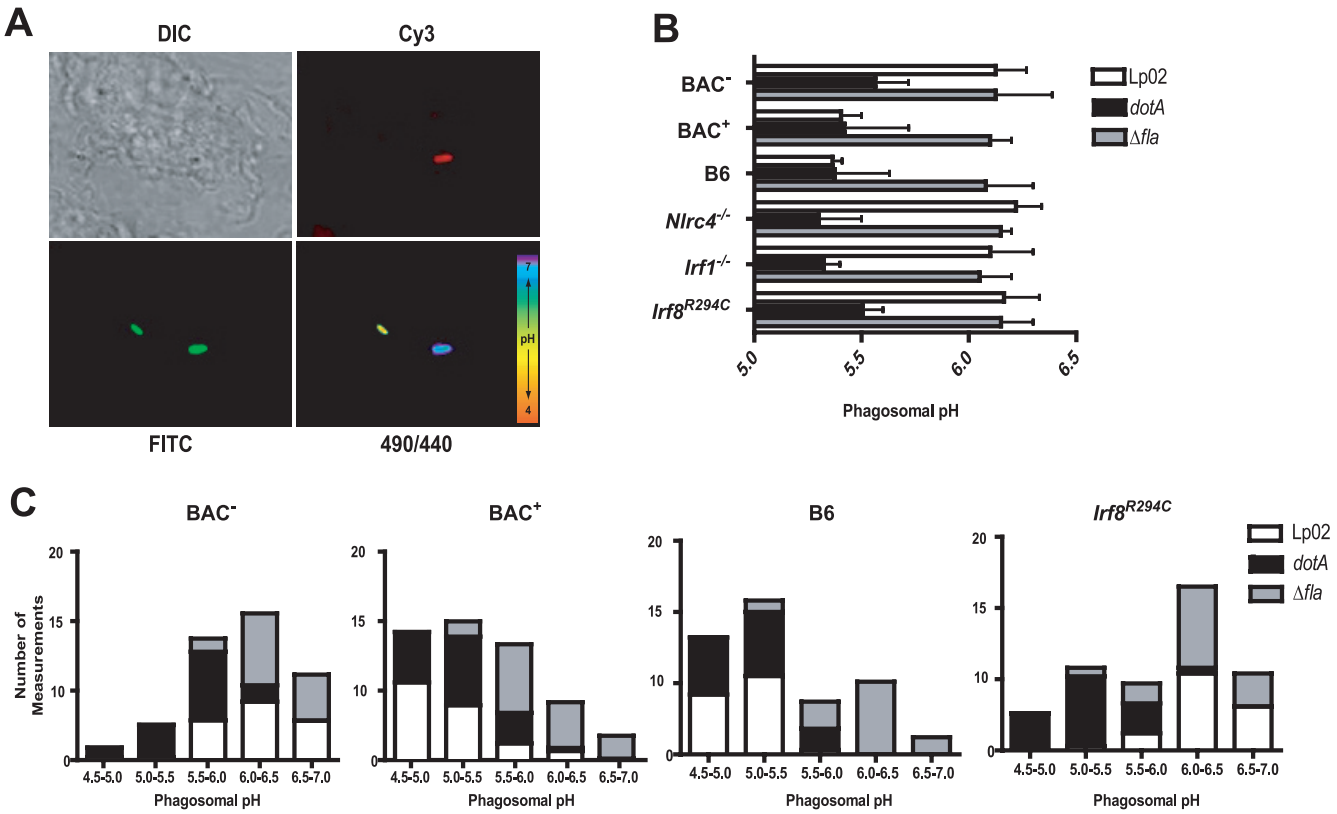

FIG. 6. pH of wild-type, *dotA* mutant, and flagellin-deficient phagosomes containing *L. pneumophila* formed in BAC^-, BAC^+, B6, $Nlrc4^{-/-}$, $Irf1^{-/-}$, and $Irf8^{R294C}$ macrophages. BMDMs were infected with fluorescein-treated *L. pneumophila*, the remaining extracellular bacteria were labeled with a Cy3-conjugated antibody, and phagosomes were allowed to mature for 1 h. (A) Live cells were mounted in thermoregulated chambers and visualized using phase-contrast (differential interfering contrast [DIC]) microscopy. Internalized bacteria were identified by the presence of FITC labeling and the absence of Cy3 labeling. The fluorescence was measured using alternating excitation at 440 and 490 nm, and the fluorescence ratio (490 nm/440 nm) was used for measurement of the pH in the vicinity of the bacterial surface. A pseudocolor pH scale was obtained using a standard curve for known pH solutions for each sample. (B) pH values representative of 100 individual phagosome measurements. (C) Frequency histograms comparing the pH in *Naip5*-insufficient (BAC^-) and *Naip5*-sufficient (BAC^+) macrophages and in *Irf8* mutant ($Irf8^{R294C}$) and *Irf8* wild-type (B6) macrophages. The data are the means of 25 representative individual determinations.

sion, either constitutive or inducible following rapid flagellin- and Naip5-dependent signaling, is required to antagonize the ability of *L. pneumophila* to modulate phagosome maturation.

DISCUSSION

In vivo complementation data, silencing studies, and recent experiments with a knockout mouse mutant have shown that permissiveness of A/J macrophages for infection with *L. pneumophila* is caused by a partial loss of function (hypomorphic allele) of the *Naip5* gene (8, 22, 54). Naip5 is a member of the NLR family of intracellular sensors of PAMPs. More recently, it was observed that loss of function for another NLR protein, Nlrc4, also causes susceptibility to infection with *L. pneumophila* (1, 57). Upon engagement with their ligands, namely bacterial flagellin, anthrax lethal toxin, and ATP-uric acid crystals, Nlrc4, Nlrp1b, and Nlrp3, respectively, have been shown to assemble to form a so-called inflammasome (12), an inflammatory caspase-activator complex composed of an NLR protein platform (Nlrp1b, Nlrp3, or Nlrc4), an inflammatory caspase (caspase-1 or caspase-5), and an adaptor molecule (ASC). A model has been proposed (32, 37, 57) in which recognition of flagellin by Naip5 and Nlrc4 results in inflammasome assembly, caspase-1 activation, and ultimately inhibition of intracellular *L. pneumophila* replication. This model is based on the following observations: (i) macrophages deficient in *Naip5* or *Nlrc4* cannot restrict intracellular replication of *L. pneumophila* (1, 8, 22, 54); (ii) gene silencing of hNAIP or hNLRC4 in human cells leads to enhanced bacterial growth, and overexpression of both molecules strongly reduces *Legionella* replication (52); (iii) in *L. pneumophila*-infected macrophages, caspase-1 activation, IL-1β secretion, and cell death are abolished in the absence of *Naip5* or *Nlrc4* (11, 22, 37); (iv) *flagellin* gene-deficient *L. pneumophila* (Δ*flaA*) can replicate in otherwise resistant macrophages (32, 37); and (v) Naip5 physically interacts in vitro with Nlrc4 (57). Thus, flagellin derived from intracellular *L. pneumophila* would be detected by Naip5 and/or Nlrc4, which would heterodimerize through NBD interaction. Nlrc4 would recruit and activate caspase-1 through CARD-CARD interaction. Then activated caspase-1 may cleave proinflammatory cytokines (IL-1β and IL-18) into their active forms (Fig. 7) and also induce apoptotic cell death in infected macrophages.

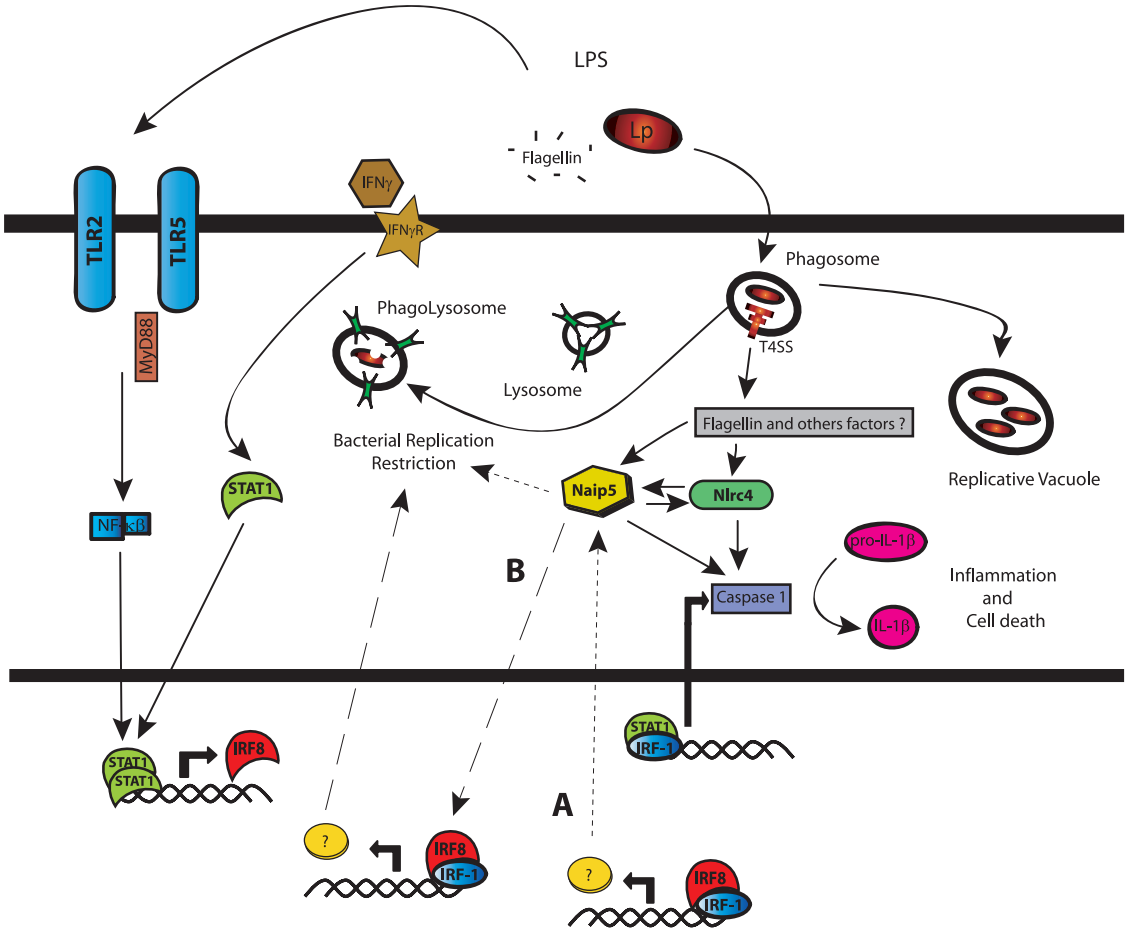

FIG. 7. Model of the role of Irf1, Irf8, and NLR family members in the host response to *Legionella* in macrophages. In addition to Naip5 and Nlrc4, Irf1 and Irf8 are essential for complete restriction of *L. pneumophila* replication in mouse macrophages by regulating either constitutive transcription of a key component of the Naip5-Nlrc4 pathway (A) or an inducible transcription activity following signaling by the Naip5-Nlrc4 pathway (B). Abbreviations: LPS, lipopolysaccharide; Lp, *L. pneumophila*; MyD88, myeloid differentiation primary response gene 88; IFNγR, IFN-γ receptor; T4SS, type IV secretion system; TLR, Toll-like receptor; Naip5, neuronal anti-apoptotic inhibitor protein 5; Nlrc4, Nod-like receptor, CARD domain-containing 4; STAT, signal transducer and activator of transcription.

On the other hand, parallel studies have suggested that Naip5 may play supplementary roles in very early events following phagocytosis of *L. pneumophila* by macrophages (in addition to caspase-1 activation). Indeed, the Naip5 and Nlrc4 status (either wild type or mutant) has dramatic consequences for the early maturation of *L. pneumophila*-containing phagosomes in macrophages, with differential recruitment of the lysosomal markers cathepsin D and Lamp1 and the endoplasmic reticulum markers BAP31 and calnexin being noticed as early as 1 to 2 h postinfection (1, 6, 9). With this in mind, we set out to identify genes, proteins, and biochemical pathways that are important for preventing intracellular replication of *L. pneumophila* in macrophages and whose activity may be influenced by early *Naip5* signaling. Transcript profiling was used to identify genes differentially expressed in *Naip5*-insufficient and *Naip5*-sufficient macrophages 4 h following infection with *L. pneumophila*. *Irf1* was found to be upregulated by *L. pneumophila* infection in macrophages, and the degree of induction was further modulated by the *Naip5* status (Fig. 1 and 2). Further, we demonstrated that loss-of-function mutations in either *Irf1* or its coactivator and heterodimerization partner *Irf8* cause increased susceptibility to *L. pneumophila* infection (Fig. 3). This susceptibility occurs in the context of functional *Naip5* signaling of otherwise resistant B6 macrophages. To ascertain that *Legionella* susceptibility in BXH-2 mice is specifically linked to the R294C mutant *Irf8* variant and not to additional effects of the mixed C3H/HeJ-B6 genetic background of the strain, we first verified the resistance phenotype of both parental strains (see Fig. S3 in the supplemental material) and then we produced a (BXH-2 × A/J)F_2 offspring, and F_2 mice homozygous for the wild-type or mutant *Irf8* allele were identified by genotyping. Scrambling of genetic background effects in these F_2 mice allowed us to verify the effect of *Irf8* alleles on the response to *Legionella*. Macrophages from F_2 mice homozygous for the $Irf8^{R294C}$ mutant allele were found to be significantly more permissive for *Legionella* replication than macrophages from F_2 mice carrying the wild-type *Irf8* allele (see Fig. S1 in the supplemental material). Together, these results demonstrate that sensing and signaling by the NLR protein Naip5 and transcriptional activation by Irf1-Irf8 are both absolutely required to prevent intracellular replication of *L. pneumophila* in macrophages.

Irf1 and Irf8 are members of the IRF family of transcriptional regulators that play a critical role in innate immunity. Irf8 acts as a coactivator with Irf1 to stimulate transcription of IFN-γ-responsive genes that have an interferon-stimulated response element sequence in their regulatory regions, including *IL-12p40*, but it can also act as a corepressor with Irf2 to antagonize Irf1-dependent transcriptional activation. Furthermore, Irf8 can further heterodimerize with PU.1 and other Ets proteins to activate transcription of genes containing IFN-γ activation site or Ets/IRF composite element promoter elements, including *Igκ*, *p67phox*, *p91phox*, *CD20*, *IL-1*, *Tlr4*, and genes encoding members of the macrophage scavenger receptor family (45). Therefore, (i) the rapid induction of *Irf1* and *Irf8* mRNA expression in response to *L. pneumophila* infection and (ii) the increased susceptibility of $Irf1^{-/-}$ and $Irf8^{R294C}$ mutant macrophages suggest a model in which immediate early activation of Irf1-Irf8-dependent transcription in response to phagocytosis of *L. pneumophila* by macrophages (including flagellin sensing by Naip5) is absolutely essential to restrict intracellular replication of this bacterium. Although this represents our favored model, one must also consider the fact that Irf1 and Irf8 are known to play critical roles in maturation of several myeloid lineages, including NK cells, dendritic cells, and macrophages (45). However, several lines of evidence argue against a generalized defect in the antimicrobial defenses of $Irf1^{-/-}$ and $Irf8^{R294C}$ macrophages to account for increased susceptibility of these cells to *L. pneumophila* infection, for the following reasons: (i) BXH-2 mice ($Irf8^{R294C}$) have numbers of F4/80-positive macrophages in their spleens comparable to the numbers in B6 controls (data not shown), (ii) $Irf1^{-/-}$ and $Irf8^{R294C}$ macrophages can efficiently kill avirulent *dotA L. pneumophila* mutants (Fig. 4), (iii) $Irf8^{R294C}$ mutant BXH-2 mice can control early replication of *Mycobacterium bovis* BCG in vivo more efficiently that the $Nramp1^{G169D}$ mutant mouse strain (49), and (iv) mice having a null mutation in *Irf8* become susceptible to infection with certain viruses while they remain resistant to infection with other viruses (18). Nevertheless, it is still possible that Irf1 and Irf8 are required for expression of a macrophage protein(s) that may be required for Naip5 signaling and/or for restriction of intracellular of *Legionella* replication.

What is the functional relationship between Naip5-Nlrc4 sensing of *L. pneumophila* leading to inflammasome activation, Irf1-Irf8 transcriptional activation, and macrophage defenses against *L. pneumophila*? One possibility is that both pathways are essential and function in parallel, possibly responding to distinct subsets of *L. pneumophila* stimuli, where Irf1 and Irf8 are required for the transcription of key components of these pathways (Fig. 7A). This may involve *flagellin*-independent signaling by Toll-like receptor family members or other receptors to activate IRF family members (as observed in *Naip5*-insufficient mice) and *flagellin*-dependent activation of the Naip5-Nlrc4 inflammasome to activate the caspase-1 cascade. Irf1, Irf5, and Irf7 have been identified as key effectors in Toll-like receptor signaling. Irf3 and Irf7 are also involved in cascade events from RIG-I/MDA5, a cytosolic pathogen receptor (45). We have observed that induction of caspase-1 mRNA expression in response to *Legionella* infection in macrophages is not dependent on either Naip5 or Irf1 and Irf8 (see Fig. S2 in the supplemental material). In addition, we observed similar expression of caspase-1 at the protein level in $Irf1^{-/-}$, $Irf8^{R294C}$, and B6 BMDMs (see Fig. S4A in the supplemental material), and caspase-1 activation following infection by *L. pneumophila* seems not to be impaired in $Irf1^{-/-}$ or $Irf8^{R294C}$ BMDMs (see Fig. S4B in the supplemental material). Although $caspase\text{-}1^{-/-}$ macrophages have been shown to be more permissive for *Legionella* replication than wild-type macrophages (57), caspase-1 activation observed in A/J and $ASC^{-/-}$ macrophages does not seem to be sufficient for *Legionella* restriction (3, 21, 22, 57). An additional and exciting possibility is that Irf1 and Irf8 represent immediate-early downstream mediators of Naip5-Nlrc4 inflammasome signaling, acting to amplify the transcriptional response of macrophages to *L. pneumophila* infection (Fig. 7B). This scenario is supported by different key observations. First, macrophages that are doubly heterozygous ($Naip5^{S/R}$ $Irf8^{R294C/wt}$ or $Nlrc4^{-/+}$ $Irf8^{R294C/wt}$) for combined loss-of-function mutations in *Irf8* and in either *Naip5* or *Nlrc4* are highly susceptible to *L. pneumophila*. Although this is not absolute proof, such a strong genetic interaction between *Irf8* and NLR family members is compatible with the hypothesis that both proteins are part of the same signaling pathway in macrophages following phagocytosis of *L. pneumophila* (Fig. 6). Second, we observed that homozygosity for loss of function of *Irf1*, *Irf8*, *Naip5*, and *Nlrc4* is phenotypically expressed as a rapid defect in acidification of the *L. pneumophila*-containing phagosome, taking place within 1 h following phagocytosis. This concordance in the temporal and subcellular expression of the defect is also in agreement with the hypothesis that both sets of proteins are parts of the same pathways (Fig. 5). Finally, we observed that activation of Irf1 and Irf8 transcriptional targets in macrophages (including *IL-12p40* and *iNOS*) following *L. pneumophila* infection is dependent on flagellin, the key ligand of Naip5 and Nlrc4, and is not detected when macrophages are infected with flagellin-deficient *L. pneumophila* mutants (data not shown).

Together, these results suggest that there is a link between *L. pneumophila* sensing by the NLR proteins Naip5 and Nlrc4 and Irf1-Irf8 transcriptional activation of a number of effector genes that play a critical role in the macrophage response to *L. pneumophila* (Fig. 7). These results are in agreement with a recent study showing that there must be cooperation between cytokine signaling (tumor necrosis factor alpha and type I interferon) and Naip5-Nlrc4 signaling for *L. pneumophila* restriction by macrophages (5). It is noteworthy that both tumor necrosis factor alpha and type I interferon signaling have been found to be regulated by Irf1 and/or Irf8 (45, 51). Although there are many potential transcriptional targets of Irf1 and Irf8 that are known to be critical for the macrophage antimicrobial arsenal, an obvious and attractive set of targets to explain the *Naip5*-dependent effect on modulation of *L. pneumophila* phagosome maturation in macrophages is the family of p47 GTPases (23). The genes encoding these proteins, which include IRG-47, LRG-47, TGTP, IGTP, IIGP1, and GTP 1, are interferon-responsive genes that are recruited to the nascent phagosomes. IRG-47 and IGTP are regulated by Irf1 very rapidly (1 to 4 h) and are known to play an important role in maturation of phagosomes into phagolysosomes, a process impaired in *L. pneumophila* phagosomes formed in *Naip5*- and *Nlrc4*-deficient macrophages (1, 6, 9).

ACKNOWLEDGMENTS

P.G. is a distinguished scientist of the Canadian Institutes of Health Research and a James McGill Professor of Biochemistry. A.F. was supported by a fellowship from the Canadian Institutes of Health Research.

REFERENCES

1. **Amer, A., L. Franchi, T. D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez.** 2006. Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf. J. Biol. Chem. **281:**35217–35223.
2. **Berger, K. H., J. J. Merriam, and R. R. Isberg.** 1994. Altered intracellular targeting properties associated with mutations in the Legionella pneumophila dotA gene. Mol. Microbiol. **14:**809–822.
3. **Case, C. L., S. Shin, and C. R. Roy.** 2009. Asc and Ipaf inflammasomes direct distinct pathways for caspase-1 activation in response to *Legionella pneumophila*. Infect. Immun. **77:**1981–1991.
4. **Clemens, D. L., and M. A. Horwitz.** 1995. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J. Exp. Med. **181:**257–270.
5. **Coers, J., R. E. Vance, M. F. Fontana, and W. F. Dietrich.** 2007. Restriction of Legionella pneumophila growth in macrophages requires the concerted action of cytokine and Naip5/Ipaf signalling pathways. Cell. Microbiol. **9:**2344–2357.
6. **Derre, I., and R. R. Isberg.** 2004. Macrophages from mice with the restrictive Lgn1 allele exhibit multifactorial resistance to *Legionella pneumophila*. Infect. Immun. **72:**6221–6229.
7. **Diez, E., M. C. Beckers, E. Ernst, C. J. DiDonato, L. R. Simard, C. Morissette, F. Gervais, S. I. Yoshida, and P. Gros.** 1997. Genetic and physical mapping of the mouse host resistance locus Lgn1. Mamm. Genome **8:**682–685.
8. **Diez, E., S. H. Lee, S. Gauthier, Z. Yaraghi, M. Tremblay, S. Vidal, and P. Gros.** 2003. Birc1e is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila. Nat. Genet. **33:**55–60.
9. **Fortier, A., C. de Chastellier, S. Balor, and P. Gros.** 2007. Birc1e/Naip5 rapidly antagonizes modulation of phagosome maturation by Legionella pneumophila. Cell. Microbiol. **9:**910–923.
10. **Fortier, A., E. Diez, and P. Gros.** 2005. Naip5/Birc1e and susceptibility to Legionella pneumophila. Trends Microbiol. **13:**328–335.
11. **Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant, and G. Nunez.** 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. Nat. Immunol. **7:**576–582.
12. **Franchi, L., J. H. Park, M. H. Shaw, N. Marina-Garcia, G. Chen, Y. G. Kim, and G. Nunez.** 2008. Intracellular NOD-like receptors in innate immunity, infection and disease. Cell. Microbiol. **10:**1–8.
13. **Franchi, L., J. Stoolman, T. D. Kanneganti, A. Verma, R. Ramphal, and G. Nunez.** 2007. Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. Eur. J. Immunol. **37:**3030–3039.
14. **Fraser, D. W., T. R. Tsai, W. Orenstein, W. E. Parkin, H. J. Beecham, R. G. Sharrar, J. Harris, G. F. Mallison, S. M. Martin, J. E. McDade, C. C. Shepard, and P. S. Brachman.** 1977. Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med. **297:**1189–1197.
15. **Geddes, B. J., L. Wang, W. J. Huang, M. Lavellee, G. A. Manji, M. Brown, M. Jurman, J. Cao, J. Morgenstern, S. Merriam, M. A. Glucksmann, P. S. DiStefano, and J. Bertin.** 2001. Human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. Biochem. Biophys. Res. Commun. **284:**77–82.
16. **Growney, J. D., and W. F. Dietrich.** 2000. High-resolution genetic and physical map of the Lgn1 interval in C57BL/6J implicates Naip2 or Naip5 in Legionella pneumophila pathogenesis. Genome Res. **10:**1158–1171.
17. **Hackam, D. J., O. D. Rotstein, W. Zhang, S. Gruenheid, P. Gros, and S. Grinstein.** 1998. Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. J. Exp. Med. **188:**351–364.
18. **Holtschke, T., J. Lohler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, K. P. Knobeloch, L. Gabriele, J. F. Waring, M. F. Bachmann, R. M. Zinkernagel, H. C. Morse III, K. Ozato, and I. Horak.** 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell **87:**307–317.
19. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:**2108–2126.
20. **Kaufmann, A. F., J. E. McDade, C. M. Patton, J. V. Bennett, P. Skaliy, J. C. Feeley, D. C. Anderson, M. E. Potter, V. F. Newhouse, M. B. Gregg, and P. S. Brachman.** 1981. Pontiac fever: isolation of the etiologic agent (Legionella pneumophilia) and demonstration of its mode of transmission. Am. J. Epidemiol. **114:**337–347.
21. **Lamkanfi, M., A. Amer, T. D. Kanneganti, R. Munoz-Planillo, G. Chen, P. Vandenabeele, A. Fortier, P. Gros, and G. Nunez.** 2007. The Nod-like receptor family member Naip5/Birc1e restricts Legionella pneumophila growth independently of caspase-1 activation. J. Immunol. **178:**8022–8027.
22. **Lightfield, K. L., J. Persson, S. W. Brubaker, C. E. Witte, J. von Moltke, E. A. Dunipace, T. Henry, Y. H. Sun, D. Cado, W. F. Dietrich, D. M. Monack, R. M. Tsolis, and R. E. Vance.** 2008. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. Nat. Immunol. **9:**1171–1178.
23. **MacMicking, J. D.** 2005. Immune control of phagosomal bacteria by p47 GTPases. Curr. Opin. Microbiol. **8:**74–82.
24. **Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson, and V. M. Dixit.** 2004. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature **430:**213–218.
25. **Marquis, J. F., A. Nantel, R. LaCourse, L. Ryan, R. J. North, and P. Gros.** 2008. Fibrotic response as a distinguishing feature of resistance and susceptibility to pulmonary infection with *Mycobacterium tuberculosis* in mice. Infect. Immun. **76:**78–88.
26. **Martinon, F., and J. Tschopp.** 2007. Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ. **14:**10–22.
27. **Martinon, F., and J. Tschopp.** 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell **117:**561–574.
28. **Masumi, A., S. Tamaoki, I. M. Wang, K. Ozato, and K. Komuro.** 2002. IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages. FEBS Lett. **531:**348–353.
29. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, and W. R. Dowdle.** 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. **297:**1197–1203.
30. **Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller, and A. Aderem.** 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nat. Immunol. **7:**569–575.
31. **Miao, E. A., R. K. Ernst, M. Dors, D. P. Mao, and A. Aderem.** 2008. Pseudomonas aeruginosa activates caspase 1 through Ipaf. Proc. Natl. Acad. Sci. USA **105:**2562–2567.
32. **Molofsky, A. B., B. G. Byrne, N. N. Whitfield, C. A. Madigan, E. T. Fuse, K. Tateda, and M. S. Swanson.** 2006. Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection. J. Exp. Med. **203:**1093–1104.
33. **Ninio, S., and C. R. Roy.** 2007. Effector proteins translocated by Legionella pneumophila: strength in numbers. Trends Microbiol. **15:**372–380.
34. **Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karaliuskas, R. H. Duerr, J. P. Achkar, S. R. Brant, T. M. Bayless, B. S. Kirschner, S. B. Hanauer, G. Nunez, and J. H. Cho.** 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature **411:**603–606.
35. **Ogura, Y., F. S. Sutterwala, and R. A. Flavell.** 2006. The inflammasome: first line of the immune response to cell stress. Cell **126:**659–662.
36. **Opitz, B., M. Vinzing, V. van Laak, B. Schmeck, G. Heine, S. Gunther, R. Preissner, H. Slevogt, P. D. N′Guessan, J. Eitel, T. Goldmann, A. Flieger, N. Suttorp, and S. Hippenstiel.** 2006. Legionella pneumophila induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J. Biol. Chem. **281:**36173–36179.
37. **Ren, T., D. S. Zamboni, C. R. Roy, W. F. Dietrich, and R. E. Vance.** 2006. Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog. **2:**e18.
38. **Robinson, C. G., and C. R. Roy.** 2006. Attachment and fusion of endoplasmic reticulum with vacuoles containing Legionella pneumophila. Cell. Microbiol. **8:**793–805.
39. **Roy, C. R., K. H. Berger, and R. R. Isberg.** 1998. Legionella pneumophila DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. **28:**663–674.
40. **Segal, G., and H. A. Shuman.** 1998. How is the intracellular fate of the Legionella pneumophila phagosome determined? Trends Microbiol. **6:**253–255.
41. **Sirard, J. C., C. Vignal, R. Dessein, and M. Chamaillard.** 2007. Nod-like receptors: cytosolic watchdogs for immunity against pathogens. PLoS Pathog. **3:**e152.
42. **Sturgill-Koszycki, S., and M. S. Swanson.** 2000. Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles. J. Exp. Med. **192:**1261–1272.
43. **Sutterwala, F. S., and R. A. Flavell.** 2009. NLRC4/IPAF: a CARD carrying member of the NLR family. Clin. Immunol. **130:**2–6.
44. **Suzuki, T., L. Franchi, C. Toma, H. Ashida, M. Ogawa, Y. Yoshikawa, H. Mimuro, N. Inohara, C. Sasakawa, and G. Nunez.** 2007. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. PLoS Pathog. **3:**e111.
45. **Tamura, T., H. Yanai, D. Savitsky, and T. Taniguchi.** 2008. The IRF family transcription factors in immunity and oncogenesis. Annu. Rev. Immunol. **26:**535–584.
46. **Tilney, L. G., O. S. Harb, P. S. Connelly, C. G. Robinson, and C. R. Roy.** 2001. How the parasitic bacterium Legionella pneumophila modifies its

phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J. Cell Sci. **114:**4637–4650.
47. **Ting, J. P., R. C. Lovering, E. S. Alnemri, J. Bertin, J. M. Boss, B. K. Davis, R. A. Flavell, S. E. Girardin, A. Godzik, J. A. Harton, H. M. Hoffman, J. P. Hugot, N. Inohara, A. Mackenzie, L. J. Maltais, G. Nunez, Y. Ogura, L. A. Otten, D. Philpott, J. C. Reed, W. Reith, S. Schreiber, V. Steimle, and P. A. Ward.** 2008. The NLR gene family: a standard nomenclature. Immunity **28:**285–287.
48. **Tschopp, J., F. Martinon, and K. Burns.** 2003. NALPs: a novel protein family involved in inflammation. Nat. Rev. Mol. Cell Biol. **4:**95–104.
49. **Turcotte, K., S. Gauthier, L. M. Mitsos, C. Shustik, N. G. Copeland, N. A. Jenkins, J. C. Fournet, P. Jolicoeur, and P. Gros.** 2004. Genetic control of myeloproliferation in BXH-2 mice. Blood **103:**2343–2350.
50. **Turcotte, K., S. Gauthier, A. Tuite, A. Mullick, D. Malo, and P. Gros.** 2005. A mutation in the Icsbp1 gene causes susceptibility to infection and a chronic myeloid leukemia-like syndrome in BXH-2 mice. J. Exp. Med. **201:**881–890.
51. **Vila-del Sol, V., C. Punzon, and M. Fresno.** 2008. IFN-gamma-induced TNF-alpha expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages. J. Immunol. **181:**4461–4470.
52. **Vinzing, M., J. Eitel, J. Lippmann, A. C. Hocke, J. Zahlten, H. Slevogt, D. N′Guessan, P., S. Gunther, B. Schmeck, S. Hippenstiel, A. Flieger, N. Suttorp, and B. Opitz.** 2008. NAIP and Ipaf control Legionella pneumophila replication in human cells. J. Immunol. **180:**6808–6815.
53. **Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg.** 1998. Conjugative transfer by the virulence system of Legionella pneumophila. Science **279:**873–876.
54. **Wright, E. K., S. A. Goodart, J. D. Growney, V. Hadinoto, M. G. Endrizzi, E. M. Long, K. Sadigh, A. L. Abney, I. Bernstein-Hanley, and W. F. Dietrich.** 2003. Naip5 affects host susceptibility to the intracellular pathogen Legionella pneumophila. Curr. Biol. **13:**27–36.
55. **Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman.** 1988. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J. mice. Infect. Immun. **56:**370–375.
56. **Yoshida, S., Y. Goto, Y. Mizuguchi, K. Nomoto, and E. Skamene.** 1991. Genetic control of natural resistance in mouse macrophages regulating intracellular *Legionella pneumophila* multiplication in vitro. Infect. Immun. **59:**428–432.
57. **Zamboni, D. S., K. S. Kobayashi, T. Kohlsdorf, Y. Ogura, E. M. Long, R. E. Vance, K. Kuida, S. Mariathasan, V. M. Dixit, R. A. Flavell, W. F. Dietrich, and C. R. Roy.** 2006. The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection. Nat. Immunol. **7:**318–325.

Editor: J. L. Flynn