

The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection

Dario S Zamboni¹, Koichi S Kobayashi^{2,3}, Tiana Kohlsdorf⁴, Yasunori Ogura³, E Michelle Long⁵, Russell E Vance⁵, Keisuke Kuida⁶, Sanjeev Mariathasan⁷, Vishva M Dixit⁷, Richard A Flavell³, William F Dietrich⁵ & Craig R Roy¹

Baculovirus inhibitor of apoptosis repeat-containing 1 (Birc1) proteins have homology to several germline-encoded receptors of the innate immune system. However, their function in immune surveillance is not clear. Here we describe a Birc1e-dependent signaling pathway that restricted replication of the intracellular pathogen *Legionella pneumophila* in mouse macrophages. Translocation of bacterial products into host-cell cytosol was essential for Birc1e-mediated control of bacterial replication. Caspase-1 was required for Birc1e-dependent antibacterial responses *ex vivo* in macrophages and in a mouse model of Legionnaires' disease. The interleukin 1 β converting enzyme–protease-activating factor was necessary for *L. pneumophila* growth restriction, but interleukin 1 β was not required. These results establish Birc1e as a nucleotide-binding oligomerization–leucine-rich repeat protein involved in the detection and control of intracellular *L. pneumophila*.

Mutations affecting members of the baculovirus inhibitor of apoptosis repeat (BIR)–containing 1 (Birc1; also called Naip) family of proteins are associated with a severe form of spinal muscular atrophy in humans¹ and with enhanced susceptibility to bacterial infection in mice². Despite genetic data indicating that Birc1 proteins have important biological functions, it is unclear how these proteins operate. Structural predictions based on amino acid similarity suggest that Birc1 proteins contain three adjacent N-terminal BIR domains, which are protein-interaction modules that typically bind to members of the caspase family of cysteine proteases³. Birc1 proteins also contain a centrally located nucleotide-binding oligomerization domain (Nod) and a C-terminal leucine-rich repeat (LRR) domain, suggesting similarity to members of the Nod-LRR protein family (also called NACHT and CATERPILLAR). The Nod-LRR family includes several proteins that activate innate immune signaling pathways in response to microbial stimulation^{4–7}. Although structurally similar to other Nod-LRR proteins, Birc1 proteins are not established as being involved in the immune response to microbial infection, and signaling pathways 'downstream' of Birc1 proteins have not been elucidated.

The Gram-negative bacterium *Legionella pneumophila* is the causative agent of a severe bacterial pneumonia known as Legionnaires'

disease. Essential for pathogenesis is the ability of *L. pneumophila* to replicate in macrophages. Vacuoles containing *L. pneumophila* avoid fusion with lysosomes after macrophage uptake and are remodeled into unique endoplasmic reticulum–derived organelles that permit bacterial replication⁸. The A/J mouse is the 'preferred' murine host for *L. pneumophila* infection studies because A/J macrophages permit more bacterial replication than do C57BL/6 macrophages⁹. *Lgn1*, an autosomal recessive locus, is responsible for mouse strain–specific variations in susceptibility to *L. pneumophila* infection^{10,11}. Notably, the *Lgn1* locus maps to *Birc1e* on mouse chromosome 13 (refs. 12,13), indicating involvement of Birc1e in a pathway that controls *L. pneumophila* growth in mouse macrophages.

There are amino acid differences at 14 positions in the C57BL/6 versus A/J Birc1e proteins¹³. In addition, A/J macrophages have lower expression of Birc1e², and RNA silencing of *Birc1e* renders macrophages more permissive for intracellular replication of *L. pneumophila*¹³. Differences in sequence and/or expression might influence the permissiveness of strain-specific Birc1e proteins. Isolated BIR domains from human Birc1 inhibit cell death caused by ectopic production of caspase-1 in HEK293 cells¹⁴, suggesting that caspase-1-dependent activities can provide 'readouts' for Birc1-mediated responses. Here we have adapted this HEK293 cell assay

¹Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06536, USA. ²Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA. ³Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510, USA. ⁴Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut 06520, USA. ⁵Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115, USA. ⁶Vertex Pharmaceuticals, Cambridge, Massachusetts 02139, USA. ⁷Molecular Oncology Department, Genentech, South San Francisco, California 94080, USA. Correspondence should be addressed to C.R.R. (craig.roy@yale.edu).

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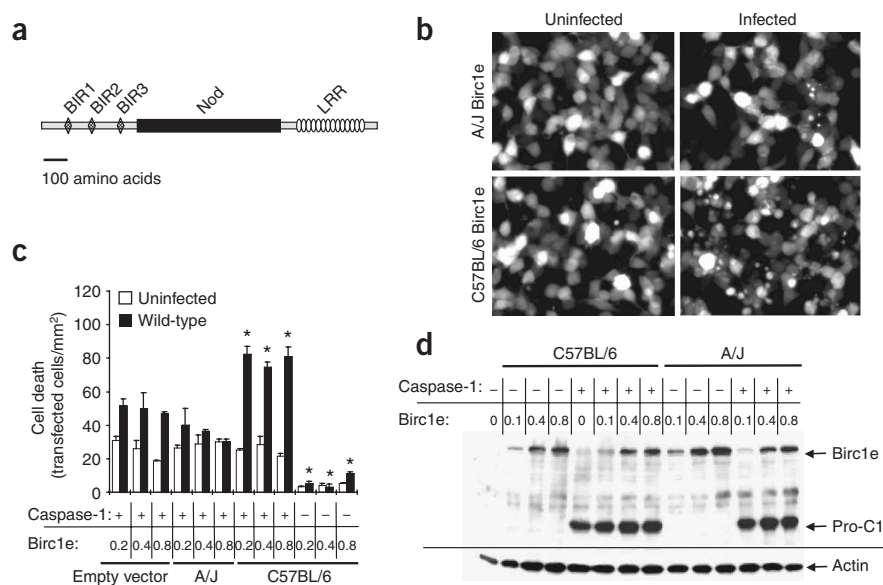


Figure 1 Birc1e-mediated caspase-1-dependent cell death of HEK293 cells. (a) Mouse Birc1e. (b) Fluorescent micrographs of RFP in uninfected (left) and infected (right) HEK293 cells cotransfected with plasmids encoding Fc γ R1I, RFP, caspase-1 and A/J or C57BL/6 Birc1e (left margins). Original magnification, $\times 100$. (c) Death of HEK293 cells cotransfected with plasmids encoding Fc γ R1I, RFP and/or caspase-1 and A/J or C57BL/6 Birc1e or the empty vector pcDNA3 (below graph; numbers after Birc1e indicate the amount of plasmid DNA (μ g) transfected into the cells). Vertical axis indicates HEK293 cells showing morphological signs of cell death. *, $P < 0.01$, versus the empty-vector control (ANOVA). (d) Immunoblot analysis of HEK293 cells left untransfected or transfected with plasmids encoding caspase-1 and C57BL/6 or A/J Birc1e. Arrows (left margin) indicate Birc1e, pro-caspase-1 (Pro-C1) and actin; above lanes, numbers after Birc1e indicate the amount of plasmid DNA (μ g) transfected into the cells. Data are representative of five (b), three (c) or four (d) experiments; error bars indicate s.d. (c).

to identify functional differences between the A/J and C57BL/6 Birc1e proteins to identify Birc1e as a Nod-LRR protein capable of responding to *L. pneumophila* infection and to identify other components of the Birc1e signaling pathway.

RESULTS

Birc1e-mediated regulation of caspase-1

We cotransfected cells with plasmids encoding red fluorescent protein (RFP), Fc immunoglobulin G receptor 2 (Fc γ R1I), mouse caspase-1 and either A/J or C57BL/6 Birc1e and infected the transfected cells with immunoglobulin-opsinized bacteria (Fig. 1). Robust intracellular replication of wild-type *L. pneumophila* in HEK293 cells results in cell death. To prevent host cell death by caspase-1-independent pathways, we used a thymidine auxotroph of *L. pneumophila* that traffics properly but has defective intracellular replication. Infection with *L. pneumophila* resulted in fragmentation and membrane blebbing of HEK293 cells expressing C57BL/6 Birc1e but not those expressing A/J Birc1e (Fig. 1b). This cell death was dependent on cotransfection of caspase-1 (Fig. 1c). Thus, *L. pneumophila* infection results in caspase-1-dependent death of cells expressing B57BL/6, but not A/J, Birc1e. Immunoblot analysis demonstrated similar amounts of C57BL/6 and A/J Birc1e in transfected HEK293 cells (Fig. 1d). This result suggests a lack of function intrinsic to the A/J Birc1e protein.

Bacterial products activate Birc1e

Nod proteins function as sensors of microbial products that gain access to the host cytosol^{4,5,7}. Although *L. pneumophila* resides in a membrane-bound vacuole, a specialized type IV secretion system

encoded by *dot* and *icm* (Dot-Icm) transports bacterial products into the host cytosol^{15,16}. To determine if Birc1e responds to bacterial products delivered into the cytosol, we analyzed caspase-1 activation induced by a strain of *L. pneumophila* lacking a functional Dot-Icm transporter (*dotA*). Wild-type but not *dotA* *L. pneumophila* induced death of HEK293 cells expressing C57BL/6 but not A/J Birc1e (Fig. 2a). The frequency of cell death increased in proportion to the multiplicity of infection (MOI) and was dependent on caspase-1. Therefore, induction of the Birc1e and caspase-1-dependent cell death pathway required the delivery of bacterial molecules into host cytosol.

Domains required for Birc1e activation

To identify domains in Birc1e required for signaling, we assessed *L. pneumophila*-induced death of cells expressing truncated versions of C57BL/6 or A/J Birc1e. Deletion of the C57BL/6 Birc1e LRR domain increased caspase-1-dependent cell death of uninfected but not infected HEK293 cells compared with that of HEK293 cells lacking Birc1e (Fig. 2b). This result suggests that deletion of the LRR domain generated a Birc1e protein that no longer responded to bacterial infection and had a low constitutive activity and would be consistent with published studies of other Nod-LRR family members¹⁷.

L. pneumophila infection did not induce caspase-1-mediated death in cells expressing C57BL/6 Birc1e proteins lacking the ATP-GTP-binding site motif (p-loop) in the nucleotide-binding region or the three N-terminal BIR domains (Fig. 2b). Therefore, both BIR domain interactions and Nod functions were required for Birc1e-mediated responses to *L. pneumophila* infection. Specifically, the BIR1 and BIR2 but not BIR3 domains were essential for *L. pneumophila*-induced caspase-1-dependent cell death. These results are consistent with a scenario in which the LRR domain regulates Birc1e activation, which uses both the Nod and BIR domains.

Birc1e-mediated regulation of endogenous caspase-1

The caspase-1-mediated death of HEK293 cells provided a convenient assay for investigating Birc1e signaling in response to bacterial infection and suggested possible involvement of Birc1e in regulating caspase-1. If Birc1e-mediated regulation of caspase-1 represents a pathway of physiological importance, endogenous caspase-1 activation in C57BL/6 and A/J macrophages might vary in response to *L. pneumophila* infection. To assess macrophage endogenous caspase-1 activation in response to *L. pneumophila* infection, we stained mouse bone marrow-derived macrophages with a fluorescent dye that binds with high affinity to the active form of caspase-1 (5-carboxyfluorescein-YVAD (FAM-YVAD))¹⁸. After infection by *L. pneumophila*, a larger proportion of C57BL/6 than A/J macrophages stained positive for active caspase-1 (Fig. 3). The high intensity of caspase-1 activation in C57BL/6 macrophages was not due to higher uptake of *L. pneumophila*, as we recovered equal numbers of bacteria from C57BL/6 and A/J macrophages (data not shown). These results

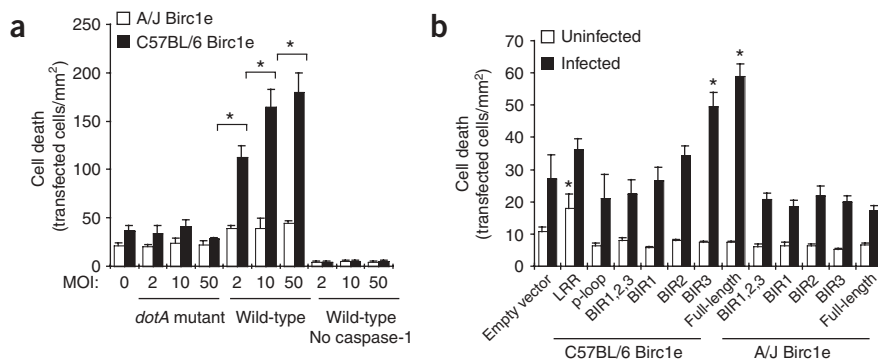


Figure 2 Dot-Icm-dependent Birc1e response in HEK293 cells. **(a)** Death of HEK293 cells cotransfected with plasmids encoding FcγRII, RFP, caspase-1 and A/J or C57BL/6 Birc1e and infected with either wild-type *L. pneumophila* or *L. pneumophila* lacking a functional Dot-Icm secretion system (*dotA* mutant). 'Wild-type No caspase-1' indicates cells that were not transfected with plasmid encoding caspase-1 and were infected with wild-type *L. pneumophila*. *, $P < 0.01$ (*t*-test). **(b)** Death of HEK293 cells cotransfected with plasmids encoding FcγRII, RFP, caspase-1 and A/J or C57BL/6 Birc1e mutants (horizontal axis). Cells were left uninfected or were infected with wild-type *L. pneumophila*. *, $P < 0.01$, versus the empty-vector control (ANOVA). Vertical axes indicate HEK293 cells showing morphological signs of cell death. Data are representative of two **(a)** or four **(b)** experiments; error bars indicate s.d.

suggest that *L. pneumophila*-induced caspase-1 activation is impaired in A/J macrophages. Very few infected macrophages lacking caspase-1 (*Casp1*^{-/-}) demonstrated FAM-YVAD staining after *L. pneumophila* infection, indicating that FAM-YVAD staining required caspase-1 (Fig. 3b). The low-intensity staining detected in infected *Casp1*^{-/-} macrophages might represent weak cross-reactivity between FAM-YVAD and the related caspase-11 and caspase-12. Few C57BL/6 macrophages infected with *dotA* *L. pneumophila* demonstrated FAM-YVAD staining. This result confirmed the Dot-Icm dependence of *L. pneumophila*-induced caspase-1 activation.

To investigate whether the Birc1e protein was the main determinant responsible for the differences noted in caspase-1 activation in A/J and C57BL/6 mouse macrophages, we assessed *L. pneumophila* growth and caspase-1 activation in macrophages derived from C57BL/6 × A/J F₂ mice. We generated bone marrow macrophages from a single litter consisting of seven mice heterozygous for the dominant C57BL/6 *Birc1e* and recessive A/J *Birc1e* alleles and four mice homozygous for the recessive A/J *Birc1e* allele. We noted high correlation between caspase-1 activation and restriction of *L. pneumophila* growth ($r = 0.74$; Fig. 4a). A large proportion of macrophages derived from heterozygous mice stained positive for active caspase-1 and were able to restrict *L. pneumophila* growth. A lower proportion of macrophages derived from mice homozygous for the recessive *Birc1e* allele stained positive for active caspase-1 and were able to restrict *L. pneumophila* growth. Finally, we measured caspase-1

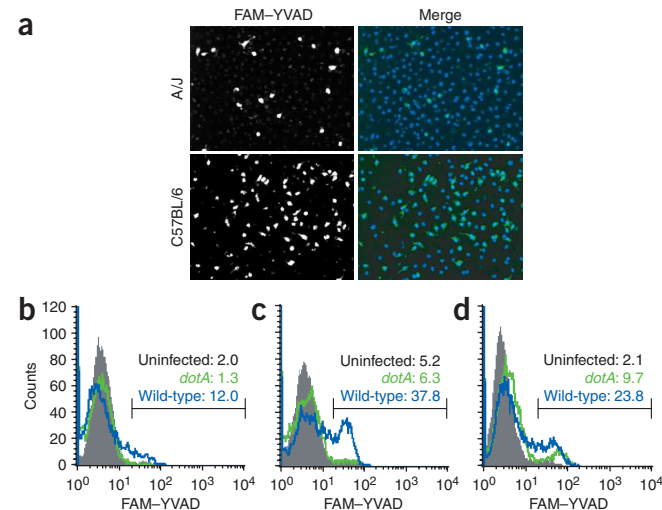
Figure 3 Impaired *L. pneumophila*-induced caspase-1 activation in A/J macrophages. **(a)** Fluorescent micrographs of bone marrow-derived macrophages from A/J (top) or C57BL/6 (bottom) mice infected with wild-type *L. pneumophila*. Left, FAM-YVAD staining of active caspase-1; right, merged images of FAM-YVAD (green) and DAPI staining of cell nuclei (blue). Original magnification, ×100. **(b-d)** FAM-YVAD staining of *Casp1*^{-/-} **(b)**, C57BL/6 **(c)** and A/J **(d)** bone marrow-derived macrophages infected with wild-type (blue) or *dotA* (green) *L. pneumophila*. Gray, uninfected macrophages. Numbers above bracketed lines indicate the percent of cells positive for FAM-YVAD staining. Data are representative of four **(a)** or eight **(b-d)** experiments.

activation in *L. pneumophila*-infected macrophages derived from B6.A-Chr13^{A/J} consomic mice. The B6.A-Chr13^{A/J} consomic mouse is a C57BL/6 mouse homozygous for A/J chromosome 13, which contains the permissive *Birc1e* allele. The intensity of caspase-1 activation in *L. pneumophila*-infected B6.A-Chr13^{A/J} macrophages was lower than that in infected C57BL/6 macrophages (Fig. 4b). Thus, the endogenous *Birc1e* allele encodes a protein capable of inducing caspase-1 activation in response to *L. pneumophila* infection.

We also used interleukin 1β (IL-1β) as a 'readout' of caspase-1 activation in macrophages. The 'pro-IL-1β' protein is produced by macrophages after stimulation of Toll-like receptors; however, secretion of the active cytokine requires conversion of 'pro-IL-1β' to mature IL-1β. This cleavage is mediated by active caspase-1 (ref. 19). C57BL/6 macrophages showed an increase in IL-1β secretion after infection with wild-type *L. pneumophila* but not after infection with *dotA* *L. pneumophila* (Fig. 4c). This result was consistent with the Dot-Icm dependency of *L. pneumophila*-induced caspase-1 activation. After *L. pneumophila* infection, C57BL/6 macrophages secreted much more IL-1β than did A/J macrophages (Fig. 4c). This result confirmed that *L. pneumophila*-induced caspase-1 activation was attenuated in A/J macrophages.

Birc1e activation by *L. pneumophila* mutants

To determine whether intracellular bacterial growth and targeting of bacteria to an endoplasmic reticulum-derived compartment are essential for Birc1e activation, we measured IL-1β secretion and caspase-1 activation by mutant strains of *L. pneumophila*. The *L. pneumophila* *thyA* mutant is a thymidine auxotroph that can traffic to the endoplasmic reticulum but cannot replicate because of the low amount of thymidine in the vacuole²⁰. The *L. pneumophila* *thyA* mutant induced more IL-1β production and caspase-1 activation than did *L. pneumophila* *dotA* in C57BL/6 macrophages (Fig. 5a,b). The *L. pneumophila* *icmS* and *icmW* mutants have a functional



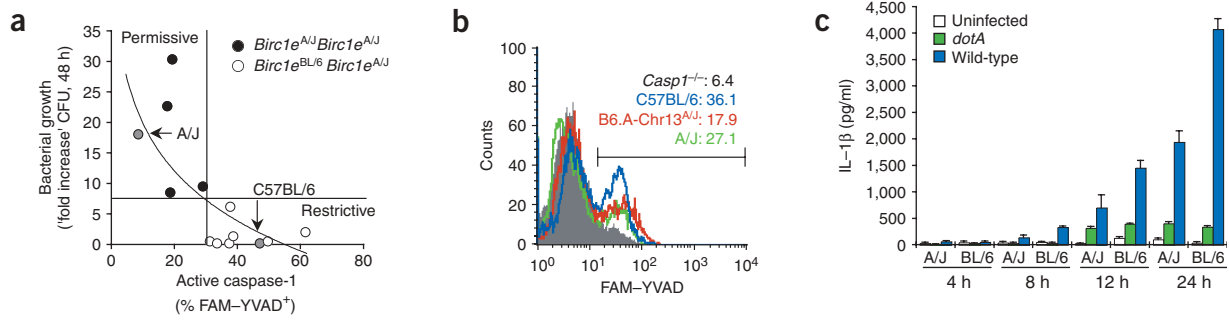


Figure 4 *Birc1e* regulation of *L. pneumophila*-induced activation in macrophages. (a) Bacterial growth and caspase-1 activation in macrophages from *C57BL/6* × *A/J* F_2 mice expressing two copies of the *A/J* *Birc1e* allele (*Birc1e^{A/J}Birc1e^{A/J}*) or one copy each of the *C57BL/6* and *A/J* *Birc1e* alleles (*Birc1e^{BL/6}Birc1e^{A/J}*), assessed 48 h after *L. pneumophila* infection. Each individual circle represents data obtained from macrophages from a single mouse. Arrows indicate control macrophages from *A/J* and *C57BL/6* mice. (b) Caspase-1 activation in macrophages derived from a *Casp1^{-/-}* mouse (gray), a *C57BL/6* mouse (blue), an *A/J* mouse (green) and a *B6.A-Chr13^{A/J}* consomic mouse (red), assessed 8 h after infection with *L. pneumophila*. Numbers above bracketed lines indicate the percent of cells positive for FAM-YVAD staining. (c) IL-1 β production by *A/J* and *C57BL/6* macrophages after infection with wild-type or *dotA* *L. pneumophila* or no bacteria. Data in c are the average values obtained from sampling three independent wells containing 2.5×10^5 macrophages \pm s.d. Data are representative of three (a), two (b) or five (c) experiments.

Dot-Icm system but are unable to replicate in macrophages because of their localization in phagosomes, which rapidly fuse with lysosomes after internalization^{21,22}. Although they are unable to avoid phagosome-lysosome fusion, the *icmS* and *icmW* mutants induced considerable caspase-1 activation and IL-1 β production in *C57BL/6* macrophages but not in *A/J* or *B6.A-Chr13^{A/J}* macrophages (Fig. 5a,c,d). These data demonstrate that *Birc1e* activation requires the translocation of bacterial products into the host cytosol but not endoplasmic reticulum residence or intracellular replication of *L. pneumophila*.

Birc1e-mediated restriction of *L. pneumophila* growth

To assess whether caspase-1 function is important for *L. pneumophila* growth restriction, we used cell-permeable compounds that covalently bind to and inhibit the proteolytic activity of caspases. *C57BL/6* macrophages treated with either a 'pan-caspase' inhibitor (Z-VAD) or an inhibitor that acts preferentially on caspase-1 (Z-YVAD) were significantly more permissive for *L. pneumophila* growth than were control macrophages treated with vehicle (Fig. 6a). Neither caspase inhibitor augmented *L. pneumophila* growth in *A/J* macrophages (Fig. 6b). An inhibitor specific for caspase-3 and caspase-7 (Z-DEVD) had no effect on *L. pneumophila* growth in either *C57BL/6* or *A/J* macrophages (Fig. 6a,b). These results suggest that *Birc1e* regulation of caspase-1 is important for *L. pneumophila* growth restriction.

We used macrophages lacking either caspase-1 (*Casp1^{-/-}*) or caspase-3 (*Casp3^{-/-}*) to further assess the function of caspases in *L. pneumophila* growth restriction. *Casp1^{-/-}* *C57BL/6* macrophages were significantly more permissive for *L. pneumophila* growth than were *Casp1^{+/-}* and *Casp1^{+/+}* macrophages (Fig. 6c and Supplementary Fig. 1 online). Absence of caspase-1 did not enhance the growth of *L. pneumophila* in macrophages homozygous for the permissive *A/J* *Birc1e* allele (Fig. 6d). Thus, the epistatic interaction between the genes encod-

ing *Birc1e* and caspase-1 demonstrates complementary gene action and confirms that caspase-1 is important for *Birc1e*-mediated restriction of *L. pneumophila* growth in mouse macrophages. Caspase-3 deficiency had no apparent effect on *L. pneumophila* growth in either *C57BL/6* or *A/J* macrophages (Fig. 6c,d).

Because Legionnaires' disease is caused by replication of *L. pneumophila* in alveolar macrophages, we assessed the susceptibility of *Casp1^{-/-}* mice to pulmonary infection. Consistent with *ex vivo* studies in macrophages, the *in vivo* growth of *L. pneumophila* in *Casp1^{-/-}* mice homozygous for the restrictive *C57BL/6* *Birc1e* allele was similar to the growth noted in *Casp1^{+/+}* mice homozygous for the permissive *A/J* *Birc1e* allele (Table 1). In contrast, *Casp1^{+/+}* *C57BL/6* mice restricted *in vivo* growth of *L. pneumophila* (Table 1). These findings confirm the *in vivo* relevance of *Birc1e*-mediated caspase-1 activation.

Adapter proteins involved in *Birc1e*-mediated responses

The proteins Asc (encoded by *Pycard*) and Ipaf (encoded by *Card12*) are adaptors that regulate caspase-1 activation²³. To determine if Asc or Ipaf was required for *Birc1e*-mediated restriction of *L. pneumophila*

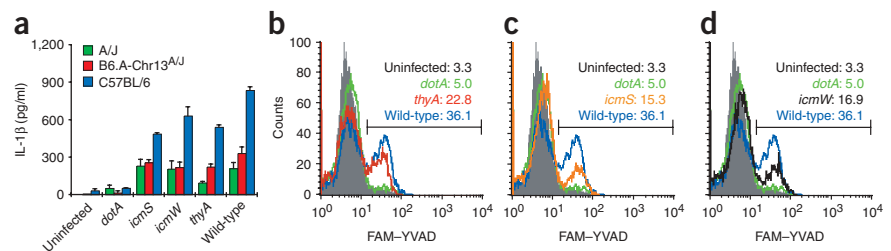


Figure 5 *Birc1e* activation is independent of *L. pneumophila* replication and trafficking. (a) IL-1 β production by *A/J*, *C57BL/6* and *B6.A-Chr13^{A/J}* macrophages 12 h after infection by *L. pneumophila* (strains, horizontal axis). Data are the average values obtained from sampling three independent wells containing 2.5×10^5 macrophages \pm s.d. and are representative of two experiments. (b-d) Caspase-1 activation in *C57BL/6* macrophages 8 h after infection with *thyA* (b), *icmS* (c) or *icmW* (d) *L. pneumophila* (red). Uninfected macrophages (gray) and macrophages infected with wild-type (blue) and *dotA* (green) *L. pneumophila* are presented for comparison. Numbers above bracketed lines indicate the percent of cells positive for FAM-YVAD staining. Each panel (b-d) has identical traces for cells left uninfected or infected with wild-type or *dotA* *L. pneumophila* obtained in parallel with the traces in each panel for the mutants *thyA* (b), *icmS* (c) or *icmW* (d). Data are representative of two experiments.

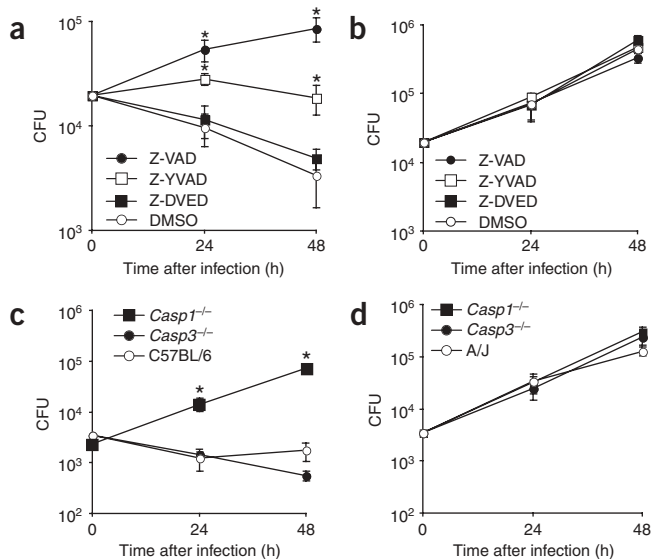


Figure 6 Caspase-1 dependency of *L. pneumophila* growth restriction in macrophages. (a,b) Bacterial growth in C57BL/6 (a) and A/J (b) macrophages after infection (time, horizontal axes). Macrophages were treated with 50 μ M of the 'pan-caspase' inhibitor Z-VAD-FMK, the caspase-1 inhibitor Z-YVAD-FMK, the caspase-3 inhibitor Z-DEVD-FMK or dimethylsulfoxide alone (DMSO) 45 min before infection with *L. pneumophila*. *, $P < 0.05$ (*t*-test). (c,d) Bacterial growth in *Casp1*^{-/-}, *Casp3*^{-/-} and C57BL/6 (c) or A/J (d) macrophages expressing two copies of the C57BL/6 *Birc1e* allele (c) or two copies of the A/J *Birc1e* allele (d), after infection with *L. pneumophila*. *, $P < 0.05$ (*t*-test). CFU, colony-forming units. Data are the average values obtained from sampling three independent wells containing 2.5×10^5 macrophages \pm s.d. and are representative of five experiments.

growth, we measured caspase-1 activation, IL-1 β secretion and bacterial growth in *L. pneumophila*-infected C57BL/6, Asc-deficient or Ipaf-deficient macrophages. *L. pneumophila* growth in *Card12*^{-/-} macrophages was similar to growth in *Casp1*^{-/-} macrophages, suggesting that Ipaf was important for Birc1e-mediated bacterial growth restriction (Fig. 7a). In contrast, *L. pneumophila* growth was similar in *Pycard*^{-/-} macrophages and C57BL/6 macrophages, suggesting that Asc is dispensable for control of *L. pneumophila* replication (Fig. 7a). Secretion of IL-1 β by Ipaf-deficient macrophages was attenuated but still detectable, supporting the idea of involvement of Ipaf in *L. pneumophila*-induced caspase-1 activation (Fig. 7b). IL-1 β secretion by Asc-deficient macrophages was undetectable, suggesting that Asc was required for caspase-1-dependent processing of IL-1 β . The defect in IL-1 β secretion was not due to a global defect in caspase-1

Figure 7 Adapter proteins involved in Birc1e-mediated restriction of *L. pneumophila* growth. (a) Bacterial growth in Ipaf-deficient (*Card12*^{-/-}), caspase-1-deficient (*Casp1*^{-/-}), Asc-deficient (*Pycard*^{-/-}) and WT C57BL/6 macrophages 48 h after infection with *L. pneumophila*. *, $P < 0.05$ (*t*-test). (b) IL-1 β production by C57BL/6, Ipaf-deficient, caspase-1-deficient and Asc-deficient macrophages infected with wild-type or *dotA* *L. pneumophila*. (c,d) Caspase-1 activation in Asc-deficient macrophages (c; green) and Ipaf-deficient macrophages (d; red) 8 h after infection with *L. pneumophila*. C57BL/6 macrophages (blue) and caspase-1-deficient macrophages (gray) macrophages are presented for comparison. Numbers above bracketed lines indicate the percent of cells positive for FAM-YVAD staining. Data are the average values obtained from sampling three independent wells containing 2.5×10^5 macrophages \pm s.d. (a,b) and are representative of three (a,b) or two (b,c) experiments.

Table 1 Birc1e- and caspase-1-mediated restriction of *L. pneumophila* growth in vivo

Mice	Average CFU/lung (log 10 ⁶)	'Fold increase' in CFU (48 h/4 h)	<i>n</i>
<i>Casp1</i> ^{-/-} (<i>Birc1e</i> ^{BL/6} <i>Birc1e</i> ^{BL/6})	2.3 \pm 0.8	2.3	6
C57BL/6 (<i>Birc1e</i> ^{BL/6} <i>Birc1e</i> ^{BL/6})	1.2 \pm 0.4*	1.2	6
A/J \times C57BL/6 F ₂ (<i>Birc1e</i> ^{A/J} <i>Birc1e</i> ^{A/J})	2.3 \pm 1.4**	2.3	7

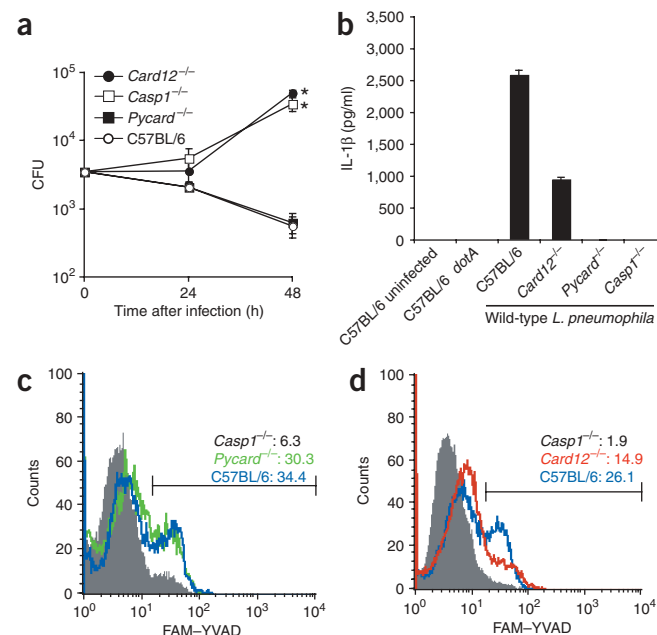
Mice were infected with 1×10^6 *L. pneumophila* by intranasal inoculation and *L. pneumophila* in the lungs were counted 48 h after infection. *n*, number of mice. *, $P = 0.034$, versus *Casp1*^{-/-} (*t*-test); **, $P = 0.950$, versus *Casp1*^{-/-} (*t*-test). Data are representative of two experiments.

activation in Asc-deficient macrophages, as the amount of active caspase-1 was similar in Asc-deficient and C57BL/6 macrophages (Fig. 7c). This result was consistent with the restriction of bacterial replication noted in Asc-deficient macrophages. These data suggested that IL-1 β secretion is not required for Birc1e-mediated restriction of *L. pneumophila* growth in macrophages. The amount of caspase-1 activation in infected Ipaf-deficient macrophages was lower than that in infected C57BL/6 macrophages (Fig. 7d) and similar to that in infected A/J macrophages, suggesting that the Ipaf-deficient phenotype was similar to the phenotype of macrophages lacking a functional Birc1e protein. Coimmunoprecipitation studies showed a physical association between Ipaf and Birc1e (Supplementary Fig. 2 online). These results suggest that Birc1e and Ipaf-restrict *L. pneumophila* replication by a caspase-1-dependent cell-autonomous process that requires neither Asc function nor IL-1 β secretion.

DISCUSSION

To prevent infection caused by intracellular bacteria, innate immune responses control the activities of multiple germline-encoded receptors. Here we have demonstrated that the Birc1e protein regulated caspase-1 activation in response to infection of host cells by *L. pneumophila*. This result establishes Birc1e as an important component of the innate immune system and demonstrates the importance of Birc1e in regulating the host response to *L. pneumophila* infection.

There are several possible mechanisms by which Birc1e-mediated control over caspase-1 activation could restrict *L. pneumophila* growth.



C57BL/6 macrophages die more rapidly than A/J macrophages after *L. pneumophila* infection²⁴. Caspase-1-mediated macrophage death is induced by infection with multiple pathogens, most having specialized secretion systems^{25,26}. Thus, our data are consistent with control of *L. pneumophila* growth restriction in C57BL/6 macrophages (at least in part) by a caspase-1-dependent cell death pathway. In addition to its controlling cell death, we cannot rule out the possibility that active caspase-1 may also restrict *L. pneumophila* replication by proteolytically degrading factors in the host cytosol that are important for intracellular growth of vacuolar pathogens. Identifying the substrates cleaved by active caspase-1 after *L. pneumophila* infection will reveal the molecular events regulating restriction of bacterial growth.

Our data have shown that caspase-3 is not important during *L. pneumophila* infection of macrophages. Those data are in contrast to published studies in which results obtained using caspase inhibitors suggested an important function for caspase-3 in evasion of lysosome fusion and intracellular replication by *L. pneumophila*²⁷. Hypothetical involvement of Birc1e in the regulation of caspase-3 during *L. pneumophila* infection was a likely possibility, as the BIR domains of human Birc1 can inhibit caspase-3 activation²⁸. Although our data do not rule out the possibility that Birc1e also regulates caspase-3 function during infection, it is apparent from these studies that the control of Birc1e over caspase-1 function has a greater effect on macrophage permissiveness than does any possible Birc1e regulation of caspase-3 function. In addition, these data suggest that caspase-3 is not essential for *L. pneumophila* replication in macrophages.

We noted that in addition to Birc1e and caspase-1, the Ipaf adapter was required for *L. pneumophila* growth restriction in C57BL/6 macrophages. Ipaf is also a member of the Nod-LRR protein family¹⁷. However, unlike Birc1e, Ipaf contains an N-terminal caspase-recruitment domain but lacks BIR domains. This caspase-recruitment domain can interact with the caspase-recruitment domain of caspase-1 (ref. 17). Similar to A/J macrophages producing a nonfunctional Birc1e protein, Ipaf-deficient macrophages were permissive for *L. pneumophila* growth and secreted less IL-1 β . In agreement with published studies documenting physical interaction between the Nods of human Ipaf and Birc1 (ref. 29), we documented here a physical association between mouse Birc1e and Ipaf.

The pathway used for caspase-1-dependent processing and secretion of IL-1 β was distinct from the pathway used for caspase-1-dependent inhibition of *L. pneumophila* growth. Asc-deficient macrophages did not secrete IL-1 β after *L. pneumophila* infection but controlled *L. pneumophila* replication and activated caspase-1. Those data suggest that Ipaf and Asc have separate and distinct functions in controlling innate immune responses to *L. pneumophila*. Asc is essential for caspase-1-dependent processing and secretion of IL-1 β , whereas Ipaf is required for caspase-1-dependent restriction of intracellular *L. pneumophila*. We propose a model in which Birc1e and Ipaf are important components of the signaling pathway that controls caspase-1-dependent responses to intracellular pathogens⁴⁻⁷. In this model, microbial infection activates Birc1e and induces the assembly of an 'inflammasome' complex containing Ipaf, caspase-1 and any caspase-1-cleaved substrates involved in restricting the intracellular growth of *L. pneumophila*.

Our data have shown that Birc1e activation by *L. pneumophila* requires the Dot-Icm secretion system, yet the microbial ligand detected by this Nod-LRR protein remains unidentified. It is possible that Birc1e is responding to a common bacterial determinant, such as a cell-wall component, that gains access to the host cytosol through a channel created in the host membrane by bacterial secretion systems. Alternatively, Birc1e might respond directly to a protein substrate of the Dot-Icm system or to perturbations in host cell functions resulting

from the activities of bacterial effector proteins translocated into host cells by the Dot-Icm system. Proteins translocated into host cells by the Dot-Icm system might alter the normal functioning of host organelles such as mitochondria and the endoplasmic reticulum and induce forms of cell stress that can occur even in the absence of microbial infection. This second model would allow involvement of Birc1 proteins in the regulation of cell survival pathways in response to noninfectious disease processes. Assays used here to measure Birc1e-dependent responses to *L. pneumophila* should facilitate the identification and characterization of the molecules that activate this family of Nod-LRR proteins, which will lead to a more complete understanding of the cellular functions controlled by Birc1 protein family members.

METHODS

Bacteria. *L. pneumophila* serogroup 1 was used in this study. HEK293 cells were infected with strains CR24 (*thyA*) or CR25 (Δ *dotA* and *thyA*), which are thymidine auxotrophs derived from strain Lp01 (ref. 20). Macrophages were infected with Lp01 (wild-type) or the isogenic mutant strains CR393 (Δ *icmS*), CR157 (Δ *icmW*) or CR58 (Δ *dotA*)²¹. Mice were infected with CR1326 (F2111), a virulent clinical *L. pneumophila* serogroup 1 strain that has been described³⁰. *L. pneumophila* were grown for 48 h on charcoal yeast extract agar (1% yeast extract, 1% *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), pH 6.9, 3.3 mM L-cysteine, 0.33 mM Fe(NO₃), 1.5% bacto agar and 0.2% activated charcoal). For *in vivo* infection studies, *L. pneumophila* were grown to mid-exponential phase (optical density of about 1.0 at 600 nm) in liquid ACES-buffered yeast extract broth (1% yeast extract, 1% ACES, pH 6.9, 3.3 mM L-cysteine and 0.33 mM Fe(NO₃)).

Plasmids and transfection studies. *Casp1* cDNA was generated from RNA isolated from A/J macrophages using the OneStep RT-PCR kit (Qiagen). *Casp1* cDNA was amplified with the forward primer 5'-TTAGGATCCGC CACCATGGCTGTGAGGGCAAAGAGGAAG-3' and the reverse primer 5'-GTTGCGGCCGCTTAATGTCCCGGAAGAGGTAGAAAC-3', and the resulting PCR product was cloned directly into pcDNA3.1/Hygro(+) (Invitrogen) after digestion with *Bam*HI and *Not*I. The plasmids encoding RFP and Fc γ RII have been described^{31,32}. A/J and C57BL/6 *Birc1e* cDNAs were generated from RNA isolated from macrophages and were cloned into pcDNA3 after digestion with *Hind*III and *Bam*HI (C57BL/6) or *Bam*HI and *Not*I (A/J), as described¹³. The amino acids deleted from the Birc1e variants used in **Figure 2** were as follows: Δ LRR, 1062–1402; Δ ploop, 464–487; Δ BIR1, 97–124; Δ BIR2, 197–224; Δ BIR3, 315–342; Δ BIR1,2,3, 97–124, 197–224 and 315–342. All Birc1 constructs were cloned into pcDNA3.

HEK293 cells were plated at a density of 3×10^4 cells/well in 24-well plates and were cultured for 16 h in α -MEM supplemented with 1 mM L-glutamine and 10% FBS. Transfections were done with the Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. The amount of plasmid DNA used for each transfection was 200 ng for plasmids encoding Fc γ RII, RFP and caspase-1, and 400 ng for plasmids encoding Birc1e. Where necessary, salmon sperm carrier DNA was added to each sample so that the total amount of DNA in each transfection was maintained at 1 μ g.

Fluorescence microscopy was used to quantify the RFP⁺ cells showing changes in cytoplasmic morphology. To confirm that scores assigned based on cytoplasmic morphology gave an accurate assessment of cell death, cells were stained in parallel (according to the manufacturer's instructions) with fluorescein isothiocyanate–Annexin V (Sigma).

Fc γ RII⁺ cells were infected as described³² with immunoglobulin G–opsonized bacteria. Unless otherwise noted, an MOI of 10 *L. pneumophila* per host cell was used. To limit cell death caused by intracellular bacterial replication, thymidine was not added to the tissue culture medium after infection.

Immunoblot analysis. Transfected cells were lysed with a radioimmunoprecipitation assay lysis buffer kit (Santa Cruz Biotechnology). Lysates were separated by SDS-PAGE, and proteins were transferred (Semidry Transfer Cell; Bio-Rad) at 15 V for 30 min to Immobilon P membranes (Millipore) in transfer buffer (50 mM Tris, 40 mM glycine and 10% methanol). Membranes

were blocked for 1 h at 25 °C in Blotto (PBS, 5% nonfat dry milk and 0.1% Tween-20). Membranes were stained with primary antibody for 3 h, were washed in Blotto and were incubated for 1 h at 25 °C with horseradish peroxidase-conjugated goat antibody to mouse (anti-mouse) or goat anti-rabbit (1:3,000 dilution; Zymed). Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer) was used for antibody detection. The primary antibodies were rabbit anti-caspase-1 (Santa Cruz Biotechnology), monoclonal mouse anti-Myc (clone 9E10; American Type Culture Collection) and rabbit anti-actin (Sigma). For immunoprecipitation, HEK293 cells grown in six-well plates were transfected with plasmids encoding Myc-tagged Birc1e, hemagglutinin-tagged Ipaf and hemagglutinin-tagged Nod1. Then, 24 h after infection, HEK293 cells were lysed in radioimmunoprecipitation assay buffer supplemented with a complete protease inhibitor 'cocktail' (Roche). Lysates were incubated for 16 h with monoclonal antibody 9E10 (anti-Myc) and proteins were precipitated with protein G agarose beads (Sigma). Immune complexes and cellular lysates were separated by SDS-PAGE and proteins were identified by immunoblot analysis. Monoclonal antibody HA.11 (Covance) was used for the identification of hemagglutinin-tagged proteins.

Mice. *A/J*, C57BL/6 and B6.A-Chr13^{A/J} mice were purchased from Jackson Laboratories. *Casp1*^{-/-}, *Casp3*^{-/-}, ASC-deficient and Ipaf-deficient mice have been described^{19,23,33}. Asc-deficient mice used were backcrossed to C57BL/6 mice for eight generations. *Casp1*^{-/-} and *Casp3*^{-/-} mice were crossed with *A/J* mice to generate heterozygous F₁ progeny. F₁ progeny were intercrossed to generate F₂ progeny. Genotyping of F₂ progeny was done by PCR with genomic DNA obtained from tail tissue as template. D13mit148 forward (5'-TGCTTGTGCTCATGCATACA-3'), D13mit148 (reverse 5'-AAAGGAAGGTGGCAAGTAATAGG-3'), D13mit128 forward (5'-TTCTAAATATGCATATGACTGCC-3') and D13mit128 reverse (5'-TATGTCATATAACCATTTTCAGCATAGA-3') were used for *Birc1e* genotyping. ICE5P (5'-GAGACATATAAGGGAGAAGGG-3'), ICE3P (5'-ATGGCACACCACAGATATCGG-3') and NEO3 (5'-TGCTAAAGCGCATGCTCCAGACTG-3') were used for *Casp1* genotyping. CPP32KO3 (5'-GCAGTGAGAATGTGCATAAA-3'), CPP32KO5 (5'-GGGAAACCAACAGTAGTCAGT-3') and METPHIL (5'-TGCTAAAGCGCATGCTCCAGA-3') were used for *Casp3* genotyping.

Macrophage infection and endogenous caspase-1 staining. Bone marrow-derived macrophages were cultured as described³⁴. Macrophage monolayers were infected with *L. pneumophila* that was grown to early stationary phase on charcoal yeast extract agar plates. For studies in which active caspase-1 staining was assessed by flow cytometry, 1 × 10⁶ macrophages in non-tissue-culture-treated six-well plates were infected for 8 h with *L. pneumophila* at an MOI of 20. Before being stained, macrophages were removed with cold PBS. Macrophages were stained for 1 h with FAM-YVAD-fluoromethylketone ((FAM-YVAD-FMK; Immunochemistry Technologies) as recommended by the manufacturer. Data were acquired on a FACSCalibur (Becton Dickinson) and were analyzed with CellQuest software (Becton Dickinson).

For experiments in which active caspase-1 staining was assessed with fluorescence microscopy, 2.5 × 10⁵ macrophages on glass coverslips were infected 8 h before caspase staining (FAM-YVAD-FMK). Cultures were then washed extensively, fixed for 1 h with 3.5% formaldehyde solution, stained for 15 min with 3.5 μM DAPI (4',6-diamino-2-phenylindole), washed and then mounted in Gel/Mount (Biomed). For quantification of IL-1β secretion, macrophages in 24-well plates (2.5 × 10⁵ cells/well) were infected with *L. pneumophila* at an MOI of 10. IL-1β in the supernatant was measured at various times with a mouse IL-1β enzyme-linked immunosorbent assay kit (BD OptEIA) according to the manufacturer's instructions.

For measurement of intracellular growth of *L. pneumophila*, macrophages were added to 24-well plates at a density of 2.5 × 10⁵ cells per well. For growth curves with caspase inhibitors, *L. pneumophila* bacteria were added to each well at an MOI of 0.1. For all other growth curves *L. pneumophila* bacteria were added to each well at an MOI of 0.012. Macrophages were lysed in sterile H₂O at various times and the cell lysates were combined with the tissue culture supernatant from that well, ensuring that all bacteria in the well would be counted. Serial dilutions from each well were plated on charcoal yeast extract agar plates. Colony-forming units were assessed by counting of bacterial colonies present after 96 h of incubation at 37 °C.

Endogenous caspases were blocked with the 'pan-caspase' inhibitor Z-VAD-FMK (Sigma), the caspase-1-specific inhibitor Z-YVAD-FMK (Calbiochem) and the caspase-3- and caspase-7-specific inhibitor Z-DEVD-FMK (Calbiochem). Macrophage cultures were treated with inhibitors at a final concentration of 50 μM or an equivalent volume of dimethyl sulfoxide (control) 45 min before infection, and inhibitors were maintained during the course of infection.

In vivo infection studies. *In vivo* infection studies used 6- to 8-week-old mice. Mice were anesthetized by subcutaneous injection of 0.2 ml of a solution containing ketamine (12 mg/ml) and xylazine (1.2 mg/ml). Mice were infected intranasally with 40 μl of a suspension containing 2.5 × 10⁷ *L. pneumophila*/ml. Then, 4 h or 48 h after infection, lungs were explanted and placed in sterile tubes containing 10 ml of distilled H₂O. Lungs were homogenized for 30 s with a PowerGen 125 homogenizer (Fisher Scientific). Dilutions of the lung lysate were plated on charcoal yeast extract agar for determination colony-forming units. Live animal experiments were approved by the Institutional Animal Care and Use Committee (protocol 2004-07847) of Yale University (New Haven, Connecticut).

Statistical analysis. All experiments were done multiple times independently and yielded similar results. Each figure contains data from a single experiment done in triplicate and the data points represent the average ± s.d. Probability (*P*) values were calculated with the *t*-test and analysis of variance (ANOVA) and were considered significant at a value of 0.05 or less. The Bonferroni correction was applied for multiple comparisons.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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