

Identification of innate immunity genes and pathways using a comparative genomics approach

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To reveal regulators of innate immunity, we used RNAi assays to monitor the immune response when genes are inhibited in *Caenorhabditis elegans* and mouse macrophages. Genes that altered innate immune responsiveness in *C. elegans* were validated in murine macrophages, resulting in the discovery of 11 genes that regulate the innate immune response in both systems and the subsequent identification of a protein interaction network with a conserved role in innate immunity regulation. We confirmed the role of four of these 11 genes in antimicrobial gene regulation using available mutants in *C. elegans*. Several of these genes (*acy-1*, *tub-2*, and *tbc-1*) also regulate susceptibility to the pathogen *Pseudomonas aeruginosa*. These genes may prove critical to understanding host defense and represent potential therapeutic targets for infectious and immunological diseases.

Caenorhabditis elegans | macrophage

The innate immune system has substantial effects on the regulation of human disease. Innate immunity initiates the action of phagocytic and cytotoxic cells that are the primary short-term means of defense against infection, making a functional innate immune system critical to host defense (1). However, excessive activation of the innate immune response can contribute to many immunological diseases, including asthma, atherosclerosis, and sepsis (2–4). Polymorphisms that are associated with altered susceptibility to many diseases have been identified in innate immunity genes (3), and several genes in innate immune signaling pathways are under investigation as potential therapeutic targets to modulate the inflammatory response (5–9). Thus, the identification of genes that regulate the innate immune response is critical to both the understanding of immunological disease and to the identification of potential targets for treatment of these diseases.

Although many genes that regulate innate immunity have been identified using genetic; biochemical; and, more recently, genomic approaches (10–19), additional genes and gene networks undoubtedly modulate the innate immune response. To discover regulators of the innate immune response to lipopolysaccharide (LPS), we examined immune function in two model systems, *Caenorhabditis elegans* and murine macrophages, after inhibition of candidate genes by RNAi. Both assays used a Gram-negative bacterial stimulus to induce the immune response. In the *C. elegans* assay, we monitored the expression of antimicrobial genes induced by *Escherichia coli* in the nematode; in the mouse macrophage assay, we monitored *E. coli* LPS-induced cytokine production in the cell culture system. We examined all of the genes on *C. elegans* chromosome 1 and 192 candidate genes selected from the literature. The innate immunity genes identified in *C. elegans* were subsequently examined by using siRNA to inhibit the murine homologous genes in macrophages. This approach led to the discovery of 11 genes and a protein interaction network with conserved roles in the regulation of innate immunity. Because the genes and pathways identified in this study exhibit evolutionarily conserved functions

in several model systems, they are likely to be important mediators of the immune response and could be potential therapeutic targets for inflammatory diseases.

Results

RNAi Screen of *C. elegans* Chromosome 1. We demonstrated that *clec-85::gfp* is regulated by multiple immune response pathways in *C. elegans* in the presence of the nonpathogenic bacterium *E. coli* and the pathogen *Pseudomonas aeruginosa* (20). *clec-85* expression is also induced by the pathogen *Serratia marcescens* (21). These observations indicate that *clec-85::gfp* would be a good screening tool to identify regulators of innate immunity. To identify innate immunity genes, we inhibited each gene on *C. elegans* chromosome 1, using an *E. coli* library expressing dsRNA (22), and analyzed changes in *clec-85::gfp* expression in nematodes harboring a *clec-85::gfp* transgene (20). Of the 2,416 chromosome 1 genes examined, we identified 32 that regulated *clec-85::gfp* expression [see Fig. 1 *A* and *B* for examples; see supporting information (SI) Table S1 for the full list].

RNAi Screen of *C. elegans* Orthologs of Candidate Innate Immunity Genes from Other Species. To investigate the function of previously identified candidate innate immunity genes, the expression of the *C. elegans* orthologs of these candidates was inhibited and the effect on *E. coli*-induced *clec-85::gfp* expression was monitored. *E. coli* clones expressing dsRNA for 192 genes derived from a literature review were examined, including 38 orthologs of genes induced by LPS in mouse lung tissue, 39 orthologs of *Drosophila* genes that regulate expression of the antimicrobial gene Dip-tericin, and several positive- and negative-control genes (Table S2) (10, 14). Also examined were an additional 115 genes potentially involved in immune or stress response pathways (Table S2), including members of MAP kinase, TGF- β , and insulin signaling pathways; orthologs of genes known to regulate mammalian innate immunity; and several genes induced by pathogen exposure in *C. elegans* (21, 23–27). We identified 25 genes (distinct from the genes on chromosome 1) that were required for full expression of *clec-85::gfp* (see Fig. 1 *C–G* for examples, see Table S3 for a full list), including the previously identified immune signaling genes *nsy-1* (Fig. 1*H*) and *tir-1* (Table S3) (20, 23, 25, 26).

Although only 4% (5 of 115) of the general stress response pathway genes affected expression of *clec-85*, 13% (5 of 38) of the orthologs of mouse candidates and 36% (14 of 39) of the

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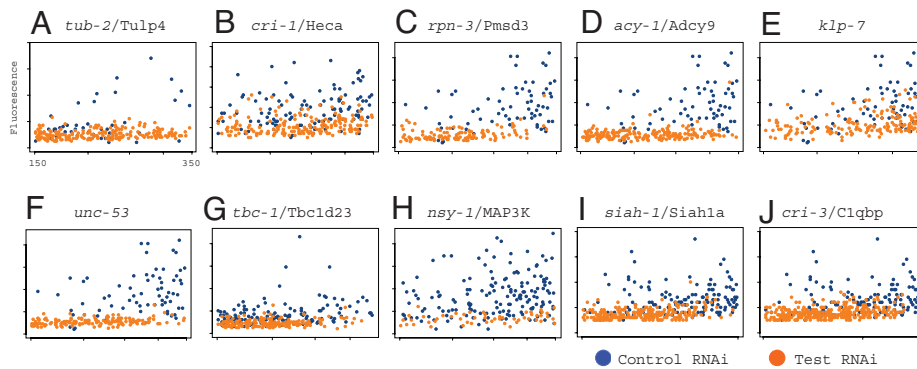


Fig. 1. Identification of genes that regulate *clec-85::gfp* expression. Nematodes harboring the *clec-85::gfp* fusion were treated with the indicated dsRNAs. Depicted are overlay graphs comparing the test RNAi treatments (orange) and a control RNAi treatment (blue); each dot represents a single nematode. The x axis represents the time of flight or length of each animal in COPAS Biosort (Union Biometrica) units, and the y axis represents fluorescence due to the *clec-85::gfp* fusion. Individual graphs are from several different experiments.

orthologs of *Drosophila* candidates affected nematode antimicrobial gene expression (one additional gene was identified whose sequence did not match the predicted sequence in the RNAi library) (Table S3). In contrast, only 1.3% of genes on chromosome 1 affected *clec-85* transcription, suggesting that these candidate genes are highly enriched for potential immune response modulators.

Identification of a Protein Interaction Network That Regulates Innate Immunity. To identify potential protein–protein interactions among our candidate innate immune regulators, we mapped our 57 *C. elegans* gene products (32 from chromosome 1 and 25 from the candidate screen) onto an interactome that contained the *C. elegans* interactome database (28) and several manually added orthologous interactions (29–32). Several interaction networks contained a single protein from our screen; however, one protein interaction network contained four proteins from our screen (TIR-1, Y48G1C.1, KLP-7, and LET-70) that directly interacted with a single protein and several other proteins that interacted indirectly (Fig. 2). The four proteins interacted with a common protein, SIAH-1, an E3 ubiquitin ligase that regulates NF κ B activity (33) (Fig. 2). One of the SIAH-1 interacting proteins, TIR-1, an ortholog of mammalian Sarm, which is a member of the Myd88 family known to be involved in the nematode and mammalian immune response (23, 26, 34).

The SIAH-1 protein interaction network contains several genes that regulate *clec-85* transcription, raising the possibility that other genes in the network might also regulate innate immunity. To test this hypothesis, we inhibited two additional genes in the network: *siah-1*, because it is the central node in the network, and *cri-3*, because it is homologous to C1qbp, which regulates some aspects of mammalian innate immunity (35). Inhibition of both of these genes caused a decrease in the expression of *clec-85::gfp* (Fig. 1 I and J). Thus, in addition to the 57 *C. elegans* genes identified in our chromosome 1 and candidate gene screens, we identified two additional potential innate immune regulators.

Immune Function of Mammalian Orthologs of *C. elegans* Innate Immunity Genes. To examine the role of the 59 *C. elegans* immune response genes in mammalian cells, RNAi was used to inhibit the murine orthologs in a mouse macrophage cell line. Macrophages were transfected with pooled siRNAs (see *Materials and Methods*), stimulated with *E. coli* LPS, and then cytokine production was measured. Inhibition of genes known to be involved in the response to LPS, including the LPS receptor Tlr4 and the signaling adaptors Myd88 and CD14, and IL-6 (1, 36, 37), all

resulted in decreased production of IL-6. In contrast, negative control siRNA treatments had little or no effect on cytokine production (Fig. 3).

Twenty-seven orthologs of the 59 *C. elegans* genes (6 of 32 genes on chromosome 1, 19 of 25 genes in the candidate library, and 2 of 2 of the genes identified in the interactome) were inhibited with siRNA. Inhibition of 11 of these 27 genes caused a significant decrease in IL-6 production (Fig. 3). Several siRNA treatments that affected IL-6 production also affected production of TNF- α (Fig. S1), further demonstrating the role of these genes in modulating the innate immune response.

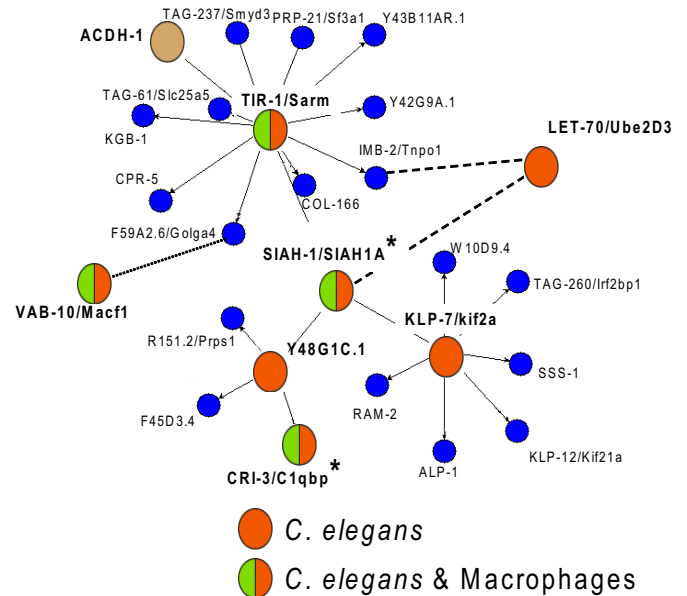


Fig. 2. A protein interaction network with a conserved role in the innate immune response. Depicted is an interactome map derived from <http://vidal.dfci.harvard.edu/interactomedb/i-View/interactomeCurrent.pl> and modified manually. The map depicts *C. elegans* protein–protein interactions (solid lines) derived from yeast 2-hybrid data. The dashed lines depict interactions of the orthologous proteins in *Drosophila* (29, 31, 32); the dotted line depicts an interaction of the orthologous proteins in humans (30). Red circles indicate genes in *C. elegans* that regulate production of *clec-85::gfp*. Circles that are red and green are genes that affect both *clec-85::gfp* production in *C. elegans* and IL-6 production in macrophages. Blue circles indicate genes that have not been tested. The asterisks indicate two genes that were identified in the interaction network that were subsequently tested in *C. elegans* and mouse macrophages and that regulate the innate immune response. The brown circle is a gene that controls susceptibility to *P. aeruginosa* (56).

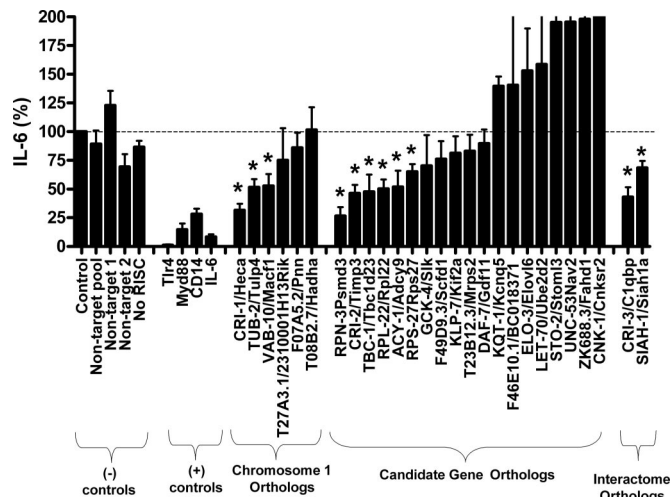


Fig. 3. Orthologs of *C. elegans* candidate innate immunity genes regulate the mammalian innate immune response. The indicated siRNA pools were transfected into a mouse macrophage cell line, the cells were induced with *E. coli* LPS, and IL-6 production was measured. Cytokine production was normalized relative to a negative control siRNA and cell number as described in *Materials and Methods* and is graphed as the percentage of control IL-6 production. Depicted from left to right are five negative controls (four siRNAs that should not target any gene and one that should not associate with the RISC complex), four positive controls (Tlr4, Myd88, CD14, and IL-6), and 27 test genes orthologous to genes that affect antimicrobial production in *C. elegans* (*C. elegans* name listed first, mouse name second). IL-6 values that were significantly different from wild type in the test siRNAs ($P < 0.05$, *t*-test) are indicated with an asterisk.

To verify that the siRNA effect was specific, we tested multiple individual siRNAs for 9 of these 11 genes. We found that at least two individual siRNAs corresponding to each of these genes inhibited IL-6 production (Fig. S2) and that the siRNAs were inhibiting the expression of the cognate endogenous gene (Fig. S3). Because multiple independent siRNAs for each of these

genes induce similar immune phenotypes, our findings demonstrate that 11 genes (3 from *C. elegans* chromosome 1, 6 from the candidate gene library, and 2 from the protein interaction network) (Table 1) regulate the expression of immune response genes (*clec-85* in nematodes and IL-6 and/or TNF α in murine macrophages).

In Vivo Significance of Innate Immune Genes in *C. elegans* Mutants. To examine the *in vivo* significance of these potential innate immune genes, we monitored the production of antimicrobial genes in four available *C. elegans* mutants (*acy-1/Adcy9*, *tub-2/Tulp4*, *tbc-1/Tbc1d23*, and *vab-10/Mac1*) of the 11 genes, using qPCR (20) after exposure to *E. coli*. As observed for known immune response regulators (20), a unique pattern of immune gene expression was observed for each mutant, where deletions both increased and suppressed *E. coli*-induced gene expression (Fig. 4). Mutations in either *acy-1* or *vab-10* affected quite a few genes, whereas mutation of *tub-2* and *tbc-1* had a more subtle effect. These unique antimicrobial gene expression patterns may reflect the complexity of the regulation of the nematode innate immune response.

To further examine the *in vivo* significance of these genes, we tested the response of the four mutant *C. elegans* strains, *acy-1/Adcy9*, *tub-2/Tulp4*, *tbc-1/Tbc1d23*, and *vab-10/Mac1*, to the pathogen *P. aeruginosa*. Nematodes harboring a mutation in *acy-1* (an adenylate cyclase) died more rapidly than wild-type nematodes when exposed to *P. aeruginosa* (Fig. 5A) at 25°C, confirming the requirement for *acy-1* in host defense. In contrast, nematodes harboring mutations in *tub-2*, *tbc-1*, or *vab-10*, did not have altered susceptibility to *P. aeruginosa* at this temperature (data not shown). Kurz *et al.* (38) demonstrated that small differences in resistance to *P. aeruginosa* could be better resolved at 15°C; therefore, we monitored the survival of nematodes harboring mutations in either *tub-2*, *tbc-1*, or *vab-10* in the presence of *P. aeruginosa* at this lower temperature. Nematodes harboring mutations in either *tub-2* or *tbc-1* (but not *vab-10*) were significantly more susceptible to pathogen at this temperature (Fig. 5C and E). To verify that the short lifespan of these mutant nematodes exposed to *P. aeruginosa* was due to an innate

Table 1. Genes with a conserved role in innate immunity regulation

Mouse gene	<i>C. elegans</i> gene*	Macrophage [†]	<i>C. elegans</i> [‡]	<i>Drosophila</i> [§]	Mouse	Other
Heca	<i>cri-1</i> =K07A1.7	IL-6, TNF	<i>clec-85</i>			
Tulp4	<i>tub-2</i> = <i>tag-305</i>	IL-6	<i>clec-85</i> <i>P. aeruginosa</i>		Systemic LPS [¶]	Expression correlates with other immune response genes (57)
Mac1	<i>vab-10</i>	IL-6, TNF	<i>clec-85</i>			Overexpressed in patients infected with Parvovirus B19 (58)
Psm3	<i>rpn-3</i>	IL-6, TNF	<i>clec-85</i>	Diptericin, NF κ B		Binds Traf6, NF κ B (59)
Timp3	<i>cri-2</i> = <i>tag-225</i>	IL-6	<i>clec-85</i>			Regulates cytokines (60,61)
Tbc1d23	<i>tbc-1</i>	IL-6, TNF	<i>clec-85</i> <i>P. aeruginosa</i>		Lung LPS	
Rpl22	<i>rpl-22</i>	IL-6	<i>clec-85</i>	Diptericin		Induced in <i>daf-2</i> (-) (62)
Adcy9	<i>acy-1</i>	IL-6, TNF	<i>clec-85</i> <i>P. aeruginosa</i>	Diptericin		cAMP regulates cytokines (39–42)
Rps27	<i>rps-27</i>	IL-6	<i>clec-85</i>			Induced in <i>daf-2</i> (-) (62)
C1qbp	<i>cri-3</i> = F59A2.3	IL-6, TNF	<i>clec-85</i>			
Siah1a	<i>siah-1</i>	IL-6, TNF	<i>clec-85</i>			Regulates NF κ B (33)

*Because these genes are conserved regulators of innate immunity, we designate the previously unnamed *C. elegans* genes K07A1.7, *tag-225*, and F59A2.3 as *cri-1*, *cri-2*, and *cri-3*, respectively, and rename *tag-305* as *tub-2*, for the second *C. elegans* member of the Tubby family.

[†]siRNA treatment regulates IL-6 and/or TNF- α production as indicated (this work).

[‡]RNAi treatment regulates *clec-85::gfp* expression. *P. aeruginosa* indicates that the mutant is susceptible to pathogen (this work).

[§]Diptericin indicates genes that regulate the LPS-induced production of that antimicrobial in *Drosophila*; Psm3 also regulates LPS-induced NF κ B nuclear translocation in *Drosophila* (14).

[¶]Induced in mouse organs by systemic LPS exposure (I. Yang and D.A.S., unpublished data).

^{||}Induced in mouse lung tissue by LPS exposure (10).

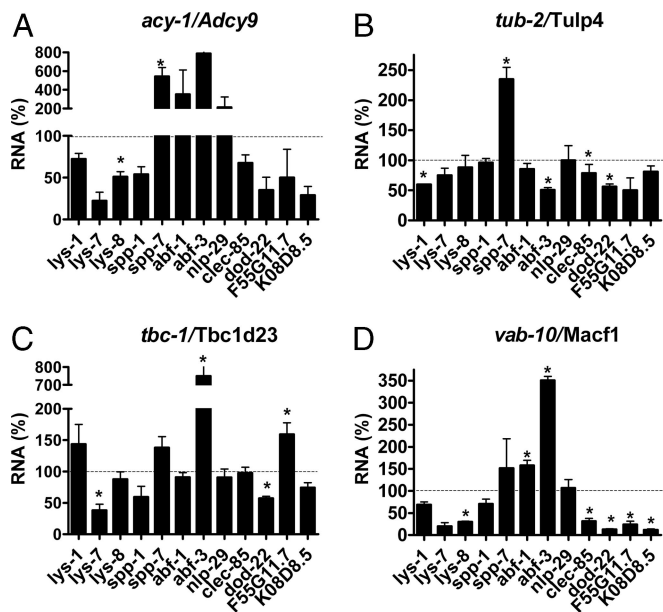


Fig. 4. Mutations in candidate innate immune regulators affect expression of many candidate antimicrobial genes. RNA was prepared from the indicated nematode strains and expression of the indicated antimicrobial genes was measured by using qPCR. Gene expression for each mutant is compared to the wild-type strain. Expression values that were significantly different from wild type ($P < 0.05$, t test) are indicated with an asterisk. Mutant alleles used are: *acy-1*(*md1756*), *tub-2*(*gk417*), *tbc-1*(*tm2282*), and *vab-10*(*e698*).

immune defect and not a general loss of fitness, we examined the survival of *acy-1*, *tub-2*, and *tbc-1* mutant nematodes in the presence of the nonpathogenic bacterium *E. coli*. Nematodes harboring these mutations lived as long or slightly longer than wild-type animals in the presence of *E. coli* (Fig. 2 *B, D*, and *F*), indicating that *acy-1*, *tub-2*, and *tbc-1* likely function in the nematode innate immune response.

Discussion

Using comparative genomics, we identified 11 genes and a protein interaction network that regulate innate immunity. Because these genes regulate multiple immune responses in two different model systems, they are likely to be important mediators/modifiers of the innate immune response. Moreover, these genes could represent potential therapeutic targets for infectious and immunological diseases. We were able to verify the *in vivo* significance of four of these RNAi-identified genes, using available *C. elegans* mutants; all four mutations altered antimicrobial gene expression, and three of the four affected resistance to a common pathogen, *P. aeruginosa*.

The *Drosophila* ortholog of *acy-1/Adcy9*, one of the innate immune genes discovered by using comparative genomics, regulates the production of an antimicrobial protein, Diptericin (14). Likewise, we found that *Adcy9* regulates the production of IL-6 and TNF α in mouse macrophages. This is consistent with previous pharmacological data implicating cAMP in the mammalian cytokine response (39–42). There are nine adenylate cyclases in the mammalian genome; our work identifies *Adcy9* as an important regulator of the innate immune response.

The genes *tub-2/Tulp4* and *tbc-1/Tbc1d23* likewise regulate host defense in the nematode and cytokine production in macrophages. Neither of these genes has been identified as an immune modulator. We also identified seven other genes with conserved innate immune function (Table 1); several of these are likely to also be important innate immune modulators.

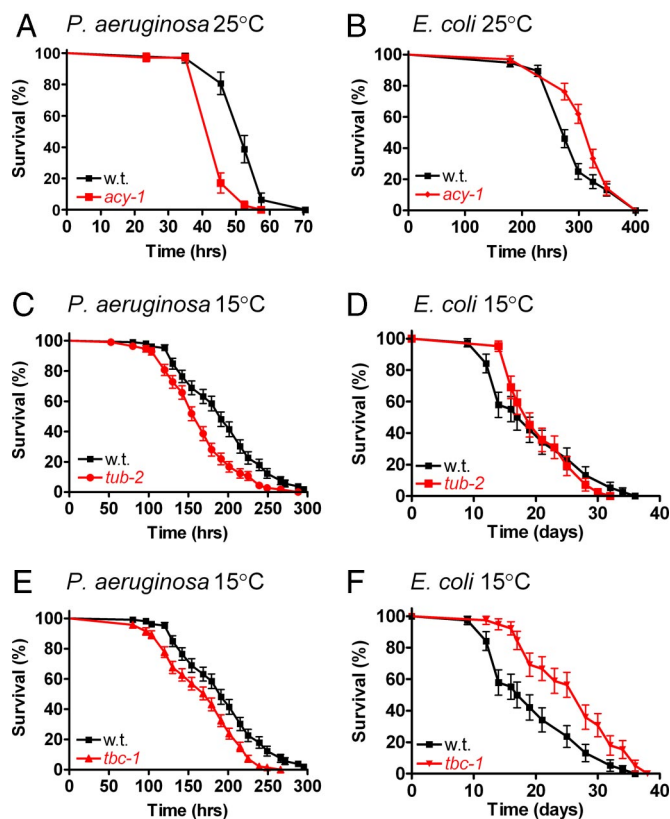


Fig. 5. Identification of genes that regulate *C. elegans* host defense. Depicted are representative survival plots for either wild-type N2 or the mutants *acy-1*(*md1756*), *tub-2*(*gk417*), or *tbc-1*(*tm2282*). The nematode strains were exposed to the indicated bacteria at the indicated temperature. (*A, B, D*, and *F*) Experiments were initiated with 60 nematodes of each strain; a minimum of 36 animals remained through the course of the experiment. (*C* and *E*) Experiments were initiated with 200 nematodes; a minimum of 102 animals remained through the course of the experiment. A statistical comparison of the survival plots indicates that survival of the mutant animals was statistically different from the wild-type N2 strain in *A* ($P < 0.0001$), *B* ($P = 0.0072$), *C* ($P < 0.0001$), *E* ($P < 0.00001$), and *F* ($P = 0.001$) but not in *D* ($P = 0.86$).

Our findings provide further support for the importance of the ubiquitin/26S proteasome pathway in innate immunity (43–45). We identified several genes in the ubiquitin/proteasome pathway, including an E2 ubiquitin ligase (*let-70*) and several E3 ubiquitin ligases (*tag-353*, *C27A12.6*, and *siah-1*). Consistent with a proposed immune signaling role for these ubiquitin ligases, the mammalian ortholog of *tag-353* is thought to associate with the 26S proteasome (46), and *Siah1* has been shown to regulate NF κ B (33). RPN-3/Psmd3, a component of the regulatory subunit of the 26S proteasome (47), affects the innate immune response in *C. elegans*, mouse macrophages (this work), and *Drosophila* (14). Small molecule proteasome inhibitors are undergoing testing for treatment of inflammatory diseases (48–50); our data suggests that siRNA of proteasome subunits may be another potential treatment option.

In mammals, LPS acts through both p38 MAPK and NF κ B pathways. A p38 MAPK pathway also functions in *C. elegans* host defense (25). Although a *C. elegans* ortholog of NF κ B has not been identified (27), our demonstration of the role of the ubiquitin/proteasome pathway in *C. elegans* host defense suggests that some aspects of this innate immune signaling pathway may be conserved in the nematode. It is possible that the ubiquitin/proteasome system could regulate innate immunity in *C. elegans* via a different mechanism; alternatively, the pathway could regulate a protein that is highly diverged from NF κ B.

Our findings also identify several other genes and a protein interaction network that may be important in controlling the innate immune response. The genes and pathways identified by this comparative genetics approach could be important mediators of innate immunity, and therefore may provide a useful set of targets for treatment of many diseases.

Materials and Methods

RNAi of Candidate Antimicrobial Genes in *C. elegans*. RNAi was carried out in liquid culture in 96 well format as described in ref. 20 with specific details outlined in *SI Materials and Methods*. Nematode fluorescence was assayed by using the COPAS Biosort (Union Biometrica), which measures the length (a measure of nematode age) and fluorescence of each nematode in each well, and analyzed as described in ref. 20.

siRNA in Mouse Macrophages. To test the role of genes identified in the *C. elegans* screens on the macrophage immune response, we transfected siRNAs (Dharmacon; pools of four siRNA duplexes per gene) into the mouse macrophage cell line J774A.1 (51). Cells were transfected by using an Amaxa Nucleofector 96-well shuttle, according to the manufacturer's instructions. Transfections were carried out using 2 μ M siRNA and 100,000 cells per well. Cells were maintained in DMEM supplemented with 10% FBS. Twenty-four to 36 h after transfection, ultrapure *E. coli* LPS (List Biological Labs) was added to a final concentration of 20 ng/ml. After 5 h, supernatant was collected, and cytokine production was assayed by using a multiplex cytokine assay kit (Linco) and a Luminex reader (Bio-Rad). Cell viability was monitored, and cell number was

normalized by using fluorescein diacetate (52). Cytokine production was normalized relative to a negative control siRNA (53). siRNAs were initially tested in triplicate; siRNAs that prevented IL-6 production were then assayed at least three more times. RNA was isolated and the level of RNA knockdown monitored by qPCR using the primers listed in Table S4.

Assaying the Effect of Candidate Genes on *C. elegans* Host Defense. qPCR of available *C. elegans* mutants was carried out in three independent biological replicates as described in ref. 20, using wild-type N2 as a control.

Survival of *C. elegans* mutants was monitored at 25°C or 15°C in the presence of *P. aeruginosa* strain PA14 (54), using N2 as a control. Exposures were initiated when the nematodes were in the late L4 stage. Lifespan analysis, using *E. coli* strain OP50, was performed in the presence of the sterilizing agent FUDR (55). Survival analysis was carried out by using Graphpad Prism, Version 4.

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