An Evolutionarily Conserved Innate Immunity Protein Interaction Network

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Background: Innate immunity affects infectious and inflammatory diseases.
Results: Using RNAi and proteomic data, we identified a novel evolutionarily conserved protein network that modulates innate immunity.
Conclusion: Studies using mutant C. elegans and mice demonstrate the utility of this network for disease investigation.
Significance: This innate immunity network provides a novel set of targets for future innate immunity disease studies.

The innate immune response plays a critical role in fighting infection; however, innate immunity also can affect the pathogenesis of a variety of diseases, including sepsis, asthma, cancer, and atherosclerosis. To identify novel regulators of innate immunity, we performed comparative genomics RNA interference screens in the nematode Caenorhabditis elegans and mouse macrophages. These screens have uncovered many candidate regulators of the response to lipopolysaccharide (LPS), several of which interact physically in multiple species to form an innate immunity protein interaction network. This protein interaction network contains several proteins in the canonical LPS-responsive TLR4 pathway as well as many novel interacting proteins. Using RNAi and overexpression studies, we show that almost every gene in this network can modulate the innate immune response in mouse cell lines. We validate the importance of this network in innate immunity regulation in vivo using available mutants in C. elegans and mice.

The innate immune response plays a critical role in fighting infection (1) but can also affect the pathogenesis of numerous diseases with an inflammatory component (2–5). Evidence for the importance of innate immunity in host defense in humans comes from the identification of polymorphisms in innate immunity signaling genes that render those individuals highly susceptible to infection (6). The innate immune response can also affect the risk of many diseases with an inflammatory component ranging from atherosclerosis to arthritis to cancer to sepsis (3, 7–10). Thus, it is critical that the innate immune response be properly modulated, active when needed to fight infection, and inactive when not needed to prevent other diseases. Thus, genes that transduce innate immune signals have become targets for the development of therapeutics for numerous indications (11), and polymorphisms in those genes that affect disease risk could be used to develop diagnostic tests for personalized medicine (3).

We have performed comparative genomics RNAi screens in Caenorhabditis elegans and mouse macrophages to identify novel, conserved regulators of innate immunity (12). The nematode C. elegans lacks an adaptive immune response, but it does have an innate immune response that involves production of antimicrobial genes to fight infection. Most but not all C. elegans pathogens infect the nematode digestive tract and induce the increased expression of antimicrobial genes in intestinal epithelial cells (13, 14). Production of these presumed antimicrobials is controlled by many conserved innate immunity signaling genes (15–23). The pattern of antimicrobial genes induced by different pathogens is specific to each pathogen (15, 18, 23–27), suggesting that C. elegans can distinguish between pathogens, although the mechanisms of how it does so are still unclear. Macrophages are key phagocytic innate immune cell that affect many diseases (28). Our hypothesis is that by identifying orthologous genes that affect innate immunity in multiple species, suggesting evolutionary conservation of their function, we can expect that these genes will likewise affect innate immunity and disease in humans.

Using RNAi screens in these simple model systems, we have identified many candidate regulators of innate immunity (12, 29–31). To sort through these candidates and determine their possible function, we examined our candidates for potential protein-protein interactions using a C. elegans protein interaction database (12). This led to the discovery of a network of proteins that included several of our candidate innate immunity regulators. Here, we expand the analysis of this innate immunity protein interaction network to include interactions in multiple species (C. elegans, Drosophila, and humans). Using
RNAi, we show that almost every gene in this network modulates the response to lipopolysaccharide (LPS) in mouse macrophage cell lines. We then validated the importance of this network in vivo using available mutations in C. elegans and mice. Thus, this novel innate immunity protein interaction network should provide a valuable resource for further innate immunity and inflammatory disease studies.

**EXPERIMENTAL PROCEDURES**

**RNAi in Mouse Macrophage Cell Lines—**RNAi was performed largely as described previously (12). In brief, pools of four siRNA duplexes or individual siRNA duplexes (Dharmacon) were transfected into either of two mouse macrophage cell lines (J774A.1 or RAW264.7) using the Amaxa nucleofector 96-well shuttle according to the manufacturer's instructions. Negative control siRNAs used were either a pool of four siRNA duplexes that do not target any mouse gene (Dharmacon nontargeting pool 1) or a single siRNA duplex not targeting any gene (Dharmacon nontargeting siRNA 1). Cells were then plated at either 100,000 cells/well in a 96-well format for ELISAs or 250,000 cells per well in a 6-well format for qPCR studies. 24 h after plating, the cells were exposed to the indicated pathogen-associated molecular patterns (PAMPs) for 6 h. *Escherichia coli* O111:B4 LPS was from List Biological Laboratories; PAM3CSK4 was from Invivogen. The LPS dose of 20 ng/ml was chosen for RNAi experiments as this dose gave an essentially complete response without overwhelming the system. Six-hour exposures were used as this would capture rapidly induced cytokines such as TNFα and cytokines induced later such as IL-6 (32). Following the exposures, cytokine production was monitored by ELISA (R&D Systems) or 250,000 cells per well in a 6-well format for qPCR.

Viability of the cells was monitored by staining cells with fluorescein diacetate and measuring resultant fluorescence on a plate reader as described (33). For qPCR studies, RLT buffer was added directly to the cells, and RNA was purified using the RNeasy kit (Qiagen). qPCR was then performed using the QuantiTect SYBR Green RT-PCR assay kit (Qiagen) and an ABI 7900 Real Time thermocycler. Primer sequences used for qPCR were listed in supplemental Table 1. Expression levels were normalized using primers for β-actin.

In separate experiments, phagocytosis was monitored using cells subjected to RNAi as described above. Phagocytosis of FITC-labeled *E. coli* particles was measured using the Vybrant phagocytosis assay kit (Molecular Probes) as described (34).

**C. elegans Survival Assays—**C. elegans survival assays were conducted largely as described previously (35). In brief, animals in the late L4 stage were exposed to either pathogenic *Pseudomonas aeruginosa* strain PA14 (36) or nonpathogenic *E. coli* strain OP50 at 25 °C on standard nematode growth medium (37). The sole exception to this was the temperature-sensitive repo-1 mutant, which was allowed to develop at the permissive temperature (15 °C) and which was subsequently exposed to PA14 at the restrictive temperature of 26 °C at the young adult stage. Life span analysis using *E. coli* was performed in the presence of the sterilizing agent 5-fluoro-2′-deoxyuridine (38). For the survival assays in the presence of heat-killed *E. coli*, bacteria were incubated at 65 °C for 3 h, concentrated 10-fold, and plated on nematode growth medium plates (37) supplemented with 50 μg/ml ampicillin. Strains used were wild type N2, FX05176 *klp-12(tm5176)* IV, VC767 sat-18(gk334) I, FX01968 *siah-1(tm1968)* IV, VC812 tag-260(ok1339) V, and repo-1(ok430ts) IV. The repo-1 strain was outcrossed four times; the *klp-12* and *siah-1* strains were outcrossed twice. The *set-18* and *tag-260* strains were not reported to be outcrossed. The presence of all deletions was verified by PCR on genomic DNA. Deletion mutations have also been isolated in *klp-7*/Kif2a and *ant-1.1*/Slc25a5; both homozygous mutant strains were reported to be lethal or arrest during development and were therefore not tested in pathogen assays. Occupancy of the bacterial lawn and pharyngeal pumping rates of repo-1 mutant animals were monitored 18 h after shifting the young adults onto *P. aeruginosa* bacteria.

**Generation of Macrophages Overexpressing Macf1—**For the *Macf1* overexpression experiments, a plasmid containing the *Macf1* cDNA cloned downstream of the CMV promoter (39) was co-transfected with plasmids containing NFkB-AP1-luc (a 133-bp derivative of the IL-8 promoter driving firefly luciferase expression (40)) and SV40-rluc (normalization control from Promega) into RAW264.7 cells using FuGENE-HD (Roche Applied Science) according to the manufacturer's instructions. 24 h after transfection, cells were exposed to LPS for 6 h, and luciferase activity was measured using the Dual-Luciferase reporter assay kit (Promega). Firefly luciferase activity was normalized relative to *Renilla* luciferase activity. As a control, a plasmid driving expression of chloramphenicol acetyltransferase (CAT) using the CMV promoter (pCDNA3.1/CAT, Invitrogen) was transfected in place of *Macf1* in some experiments.

**Generation and Phenotyping of Bone Marrow-derived Macrophages (BMDM) with Decreased Macf1 Expression—**Generation of the *Macf1* knockout strains was performed as described (41). *Macf1*+/+ mice were crossed with B6.129-Lyzscre/cre mice (JAX) (42). The Lyz promoter drives cre expression and thus deletion of *Macf1* in the myeloid lineage in this strain. BMDM lacking *Macf1* (*Macf1*−/−, Lyzscre/cre) were compared with control BMDM expressing *Macf1* (*Macf1*+/+, Lyzscre/cre); both groups of mice were siblings derived from *Macf1*+/+, Lyzscre/cre × *Macf1*+/+ mice mating.

BMDM were generated as described (34). In brief, femur and tibia marrow was harvested, filtered, and plated in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), penicillin and streptomycin (Fisher), and 20 ng/ml mouse M-CSF (R&D Systems). After 6 days, nonadherent stem cells were washed away, and adherent cells were collected by trypsinization for further experiments. Plating was performed similarly to the RNAi experiments described above. The extent of bone marrow stem cells differentiating into macrophages was similar between *Macf1*+/+ mice and their wild type siblings (determined by F4/80 staining, wild type BMDM 76 ± 6% F4/80+, 21 ± 3 mean fluorescence intensity; *Macf1*+/+ BMDM 83 ±

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2The abbreviations used are: qPCR, quantitative PCR; PAMP, pathogen-associated molecular pattern; BMDM, bone marrow-derived macrophage.
3% F4/80^+, 21 ± 3 mean fluorescence intensity, n = 4, p = 0.98, both strains were homozygous for Lys2^{cre/cre}).

**Statistical Analyses**—All data are from a minimum of three biological replicates. Statistical analyses for ELISAs and qPCRs were performed in GraphPad Prism 5 using unpaired t tests to determine significance (p < 0.05). Analysis of C. elegans survival data also was performed using Graphpad Prism 5. We previously presented siRNA data for Kif2a siRNA (12); in this prior study, Kif2a siRNA’s effect on the LPS response did not reach statistical significance, largely due to one outlier in the data (mean = 78% of control, n = 8, p = 0.197). The data in this study are statistically significant (mean 52%, n = 7, p = 0.003), and both data sets when considered together are also significant (mean 66%, n = 15, p = 0.0028).

**RESULTS**

**Identification of an Innate Immunity Protein Interaction Network**—Using comparative genomics RNAi screens in C. elegans and mouse macrophages, we previously identified a protein interaction network that includes several proteins that modulate the innate immune response (supplemental Fig. 1) (12). RNAi-mediated inhibition of several genes in this network either decreased expression of putative antimicrobial genes in C. elegans or production of LPS-induced inflammatory cytokines in murine macrophages or both (12). This protein interaction network was initially identified based largely on published interactions identified using yeast two-hybrid assays on C. elegans proteins (43). This proteomic approach has been extensively validated and has a roughly 80% confirmation rate using secondary binding assays (43–46). To further define the members of this protein interaction network, we searched multiple protein-protein interaction databases to identify all published protein-protein interactions in this network in C. elegans (43, 47), Drosophila (48, 49), or humans (46, 50–54), focusing on homologous proteins in each species (gene homologies in supplemental Table 2). These database searches were used to refine the network and were followed by a comprehensive literature review of each network protein in all three species. The final version of the innate immunity protein interaction network is depicted in Fig. 1; for simplicity, only proteins present in mammals are depicted in Fig. 1, even though this network depicts interactions identified in multiple species. The complete list of protein-protein interactions in Fig. 1 are listed in Table 1. All these interactions are bona fide protein-protein interactions (not computational predictions), and many have been identified multiple times in multiple species using several different binding assays.

In addition to many novel candidate proteins, this network contains several proteins in the canonical LPS response pathway. The canonical LPS response pathway in this network includes a MyD88 family member, TRAF6, IκBα, and two IκBα regulators, the E3 ubiquitin ligase SCFP^H^-TCF and the E2 ubiquitin-conjugating enzyme UBE2d2 (55, 56). We previously showed that RNAi-mediated inhibition of three genes in this network (Siah1, Macf1, and Ube2d2) affected production of putative C. elegans antimicrobial genes, and RNAi-mediated inhibition of two network genes (Siah1a and Macf1) affected LPS-induced cytokine production in mouse macrophages (12).

Although several genes in this network are in the canonical pathway for the LPS response, most genes in this network have not previously been implicated in innate immunity regulation and have not been tested by us or others for an effect on the response to LPS.

**Most Genes in This Network Modulate LPS-induced Cytokine Production in Mouse Macrophage Cell Lines**—To test the function of the remaining genes in this network, we used RNAi to inhibit 10 genes as follows: nine additional novel candidates as well as the known TLR4 signaling gene MyD88 as a control. Pools of four siRNA duplexes targeting each of these genes were transfected into the J774A.1 mouse macrophage cell line; the cells were stimulated with LPS, and then cytokine production was monitored by ELISA. As a positive control, we showed that inhibition of the LPS receptor TLR4 strongly inhibited IL-6 production (Fig. 2A). Inhibition of seven of the 10 network genes led to a statistically significant decrease in LPS-induced IL-6 production (Fig. 2A) without affecting cell viability (Fig. 2B). qPCR analysis demonstrated that these siRNA treatments were inhibiting expression of the corresponding endogenous gene (Fig. 2C).

*Sf3a1 and Golga4 Regulate LPS-induced Cytokine Secretion*—We performed several further RNAi experiments with the two novel genes that exhibited the strongest phenotypes, *Sf3a1* and *Golga4*. First, we demonstrated that multiple individual siRNA duplexes targeting each of these two genes induced similar phenotypes, a decrease in LPS-induced IL-6 production (Fig. 3). Multiple individual duplexes exhibit the same phenotype, suggesting that the effect on cytokine production is due to inhibition of the corresponding endogenous gene. We also monitored the produc-
Innate Immunity Network

TABLE 1

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<th>Interaction/species</th>
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<td>IkBo-SCFβ-TrCP</td>
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<td>Many</td>
<td>Many 117 and references therein</td>
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</table>

\(^a\) The abbreviations used are as follows: Co-IP = co-immunoprecipitation; Y2H = yeast two-hybrid assay; Affin chrom = affinity chromatography; In vitro ubiq = in vitro ubiquitination assay; TAP = tandem affinity purification, and E2 ubiq = provides ubiquitin for E3 ubiquitin ligase in biochemical assay.

\(^b\) tir-1 is the sole C. elegans Myd88 family member. Although tir-1 is most homologous to human Sarm, it functions most like MyD88 in that it is required for resistance to Gram-positive, Gram-negative, and fungal pathogens and functions upstream of the p38 MAPK pathway (15, 16, 118, 119).

FIGURE 2. Most genes in the innate immunity protein interaction network affect LPS-induced IL-6 production in the J774A.1 mouse macrophage cell line. A, pools of four siRNA duplexes per gene were transfected into the mouse macrophage cell line J774A.1.; cells were stimulated with 20 ng/ml LPS for 6 h, and IL-6 production was monitored by ELISA on cell supernatants. IL-6 production was normalized relative to a control pool of siRNA duplexes (CT1, Dharmacon nontargeting siRNA pool). CT2 is a second negative control (Dharmacon nontargeting siRNA 1). TLR4, the LPS receptor, is presented as a positive control. Two genes in this network (Macf1 and Siah1a) were inhibited previously; the data for these two genes from this prior publication (12) is presented at the end of the panel. B depicts the effects on viability of the indicated siRNA treatments normalized so that viability of control siRNA was equal to 1, C, depicts the results of qPCR, which was used to monitor RNA knockdown of the indicated genes. Asterisks indicate siRNA treatments that induced IL-6 levels that were statistically different from the control (p < 0.05). No viability measurements (B) were statistically significantly different from control.

As an additional test of these two genes, we used RNAi to inhibit them in a second mouse macrophage cell line, RAW264.7 cells. As a control for the RNAi, we found that RNAi-mediated inhibition of genes known to be required for LPS-induced IL-6 production (the LPS receptor TLR4, the TLR signaling adaptor MyD88, and IL-6 itself) led to a strong inhibition of IL-6 production, whereas RNAi-mediated inhibition of several other cytokines and chemokines (TNFα, IL-10, and RANTES) and found that inhibition of Sf3a1 and Golga4 decreased their production as well (supplemental Fig. 2), indicating that these two genes have a more general effect on innate immunity.
the network play an important role in regulating innate immunity. Although no viable mutation was available, SF3A1 and SF3A2 (along with SF3A3) form the Sf3a complex. SF3a interacts with the U2 small nuclear ribonucleoprotein, which in turn interacts with the pre-mRNA branch point near the 3' splice site in pre-mRNA (59) and facilitates mRNA splicing in conjunction with the rest of the spliceosome (60–63). We first verified that inhibition of Sf3a2, like inhibition of Sf3a1, diminished LPS-induced IL-6 production in mouse macrophages (Fig. 4B), that this inhibition decreased Sf3a2 RNA levels (Fig. 4C), and that viability was not affected by Sf3a2 inhibition (data not shown). The C. elegans repo-1/Sf3a2 allele is a temperature-sensitive allele that may be neomorphic: it is weakly semi-dominant at the restrictive temperature of 26 °C. 3 We also obtained four additional available C. elegans mutant strains corresponding to genes in the innate immunity protein interaction network: klp-12(tm1576), set-18(gk334), siah-1(tm1968), and tag-260(ok1339), orthologs of Kif21a, Smyd3, Siah1, and Irf2bp1, respectively. The klp-12/Kif21a, set-18/Smyd3, siah-1/Siah1, and tag-260/Irf2bp1 alleles are all deletion alleles that should be nulls. All of these mutants other than Kif21a correspond to genes that exhibited a phenotype in the macrophage siRNA assay. These five mutant lines were exposed to the nematode and human pathogen P. aeruginosa strain PA14 (36, 64, 65), and survival was monitored when compared with the wild type strain N2 (37).

set-18/Smyd3 mutant nematodes survived a slightly shorter length of time than wild type nematodes when exposed to P. aeruginosa strain PA14 (Fig. 5A), set-18/Smyd3 mutant animals also lived slightly shorter lengths of time when grown in the presence of the nonpathogenic E. coli strain OP50, the standard laboratory C. elegans food source (Fig. 5B), raising the possibility that set-18/Smyd3 could be regulating either host defense or general fitness. Live E. coli has been reported to be very slightly pathogenic to C. elegans under some conditions (66, 67). We therefore monitored the survival of C. elegans strains grown in the presence of heat-killed E. coli, and we found that the life span of set-18/Smyd3 mutant animals was indistinguishable from that of the wild type strain under these conditions (Fig. 5C). Thus, the set-18/Smyd3 mutant animals exhibited a moderate host defense defect in the presence of both P. aeruginosa and live E. coli.

Nematodes harboring mutations in either klp-12/Kif21a or tag-260/Irf2bp1 survived slightly longer in the presence of pathogenic P. aeruginosa but not nonpathogenic E. coli (Fig. 5, D–G), demonstrating that these strains were moderately resistant to pathogens. To monitor the effect of the temperature-sensitive repo-1/Sf3a2 mutation on host defense, nematodes were allowed to develop at the permissive temperature (15 °C) in the presence of E. coli and were shifted to the restrictive temperature (26 °C) and plates containing P. aeruginosa as young adults. repo-1/Sf3a2 mutant animals also survived longer in the presence of pathogenic bacteria (Fig. 5H); this resistance to pathogen was all the more striking as the mutant nematodes appeared visibly unhealthy and had a diminished life span in the presence of nonpathogenic E. coli (Fig. 5G). The pathogen resistance of repo-1 mutant animals was likely not due to avoidance of the pathogen; 65% of repo-1 mutant animals (n = 57) remained within the pathogenic bacterial lawn compared with

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59% of wild type N2 animals \((n = 69)\). Similarly, the pathogen resistance of repo-1 mutant animals also was likely not due to failure to ingest the pathogen as repo-1 mutant nematodes exhibited normal pharyngeal pumping rates in the presence of \textit{P. aeruginosa} \((202 \pm 10 \text{ pumps/min repo-1 and 204} \pm 10 \text{ pumps/min wild type N2, expressed as mean } \pm \text{ S.E., } n = 15)\).

Finally, siah-1/Siah1 mutant animals did not display altered sensitivity to \textit{P. aeruginosa} exposure (Fig. 5f). Thus, four of the five innate immunity network mutants tested in \textit{C. elegans} displayed altered nematode host defense.

\textbf{Macf1 Regulates Cytokine Production in Vivo in Mouse Macrophages—RNAi-mediated inhibition of Macf1 in the J774A.1 mouse macrophage cell line decreased LPS-induced IL-6 production (Fig. 2A) \((12)\). To further explore the role of Macf1 in the regulation of the LPS response, we used a similar approach to inhibit Macf1 in a second immortalized mouse cell line.}
macrophage cell line, RAW264.7. Surprisingly, we observed that inhibition of Macf1 in this second cell line induced the opposite phenotype, an increase in cytokine production (Fig. 6A). To further explore the role of Macf1, we overexpressed Macf1 in the RAW264.7 macrophage cell line and monitored the expression of an NFκB-AP1-luciferase reporter (a 133-bp derivative of the IL-8 promoter (40)) using SV40-rluc (Promega) as a normalization control, and we found that overexpression of Macf1 but not chloramphenicol acetyltransferase (CAT) diminished the response to LPS (Fig. 6B), which confirmed the RNAi phenotype in the RAW264.7 macrophages. These opposing results in different immortalized cell lines underlined the importance of investigating the effect of these genes in vivo.

To determine the function of Macf1 in vivo, we generated BMDM-deficient in Macf1 expression. To do so, we crossed conditional Macf1flox/flox (41) mice with Lyzs-cre mice (JAX) (42), which drives deletion of Macf1 in the myeloid lineage. We generated BMDM from Macf1flox/flox;Lyzs-cre mice and control sibling Macf1+/-;Lyzs-cre mice and found that Macf1 expression was decreased to 33.0 ± 5.1% of wild type levels in the BMDM from the floxed mice.

BMDM with decreased Macf1 expression exhibited increased LPS-induced IL-6 production (Fig. 6C), confirming the Macf1 RNAi data in the RAW264.7 cells. In addition to affecting the response to the TLR4 agonist LPS, Macf1 also affected the response to the TLR2/1 agonist PAM3CSK4 (68) as BMDM with decreased Macf1 expression exhibited increased PAM3CSK4-induced IL-6 production as well (Fig. 6D).

**DISCUSSION**

In this age of systems biology, with high throughput RNAi screens, microarrays, proteomics, and other approaches, it is becoming increasingly important to sift through large datasets to define genes and pathways of interest (69). Many studies have used microarrays or RNAi to identify candidate genes for a phenotype of interest followed by some type of network or interactome analysis to sort through the data. For example, Li et al. (70) use a combination of protein interaction studies and RNAi to identify a network that regulates type I interferon production, and Amit et al. (71) use similar strategies to understand the transcriptional network underlying mammalian pathogen responses. Several investigators are also using screens in simpler model systems such as C. elegans or Drosophila to investigate innate immunity, and some of these studies report validation of these model systems data in mammalian cells using RNAi (see for example Refs. 22, 72).

We have performed candidate-based and genomic RNAi screens to identify regulators of the innate immune response to LPS (12) and have incorporated a comparative genomics approach into these studies to facilitate the analysis and strengthen our conclusions. Such cross-species studies offer the opportunity to identify networks not easily identifiable in individual species datasets (73). Additionally, the use of multiple RNAi screens could overcome the reported high false-positive rate in mammalian RNAi screens due to off-target affects (74). Finally, the availability of cheap, rapidly obtainable mutants in model organisms like C. elegans (75) allows for rapid testing of in vitro results using in vivo disease models.

Exposure of C. elegans to different pathogenic and nonpathogenic bacteria and fungi induces the expression of different subsets of antimicrobial genes (15, 18, 23–27). This specificity indicates that C. elegans can discriminate between different pathogens, even between different Gram-negative bacteria, suggesting that a response to a single PAMP such as LPS cannot explain the complete spectrum of the innate immune response in C. elegans. There is evidence for both PAMP and damage-associated molecular pattern-mediated innate immune activation in C. elegans, although the relative contribution of each and the specific details are still unclear. Evidence for the PAMP model comes from Aballay et al. (76) who show that intact Salmonella enterica LPS is required for a robust nematode innate immune response and from Vigneshkumar et al. (77) who show that P. aeruginosa LPS can alter C. elegans antimicrobial gene expression. Evidence for a damage-associated molecular pattern response comes from two studies (78, 79) that show that pathogen-mediated alterations in translation can affect the nematode innate immune response. Thus, it is still unclear what aspects of pathogen recognition are conserved between mammals and nematodes, and thus it remains to be determined whether the genes we identified that function in innate immunity in both models exhibit conservation of overall mechanism or if they function differently.

Using a combination of comparative genomics RNAi screens and protein interaction analysis, we have identified a small network of proteins almost all of whom modulate the innate...
**Innate Immunity Network**

Immune response to LPS in mouse macrophage cell lines. The utility of this network for the identification of important innate immune regulators was evidenced by a follow up analysis on the two novel genes whose inhibition generated the largest effects, *Sf3a1* and *Golga4*. Both *Sf3a1* and *Golga4* are required for robust LPS-induced IL-6 secretion, because RNAi-mediated inhibition of these genes strongly diminished IL-6 release from macrophages in two different mouse macrophage cell lines. However, the two genes may affect different aspects of the innate immune response. *Sf3a1* and its interacting protein *Sf3a2* were required for production of IL-6 protein and IL-6 RNA. Other reports show that RNAi-mediated knockdown of *Sf3a* subunits can affect cell survival in HeLa cells (80); the difference in our data may be due to incomplete (but still very strong) knockdown in macrophages or other cell type-specific differences. The importance of the *Sf3a* complex in innate immunity regulation is further evidenced by the strong effect of the temperature-sensitive *Sf3a2* mutation on *C. elegans* resistance to pathogenic bacteria, which is all the more striking given that this mutation has the opposite effect on overall cell health. Because inhibition of two different mRNA splicing regulators in this complex diminished the LPS response, we infer that the *Sf3a* complex is regulating the alternative splicing of a critical innate immunity regulator. Many TLR genes are reported to be alternatively spliced in response to immune cell activation (81). For example, *TNFa*, *c-fos*, *TLR4*, *MyD88*, and *NFKB* have been reported to be differentially spliced in response to LPS or other stimulation (82–86); these alternate splice forms can have very different functions. The interaction of *MyD88* family members with *Sf3a1* raises the possibility that *MyD88* could alter *Sf3a1* activity and thus mRNA splicing of an innate immunity regulator gene(s).

In contrast, although inhibition of *Golga4* strongly decreased the amount of IL-6 protein secreted, we found that *Golga4* inhibition had a more moderate effect on IL-6 RNA accumulation. This is consistent with the reported requirement for GOLGA4 in TNFa and IL-10 secretion. GOLGA4 is required for TNFa and IL-10 transport through post-Golgi vesicles (87, 88); because IL-6 and TNFa traffic through a similar initial pathway of secretion (89–91), it is likely that GOLGA4 affects the LPS-induced secretion of both cytokines. Our IL-6 RNA results suggest that GOLGA4 may also have a small effect on signaling as well. The expression of many cytokine trafficking genes is increased in response to LPS stimulation (91), and the reported interaction of a MyD88 family member with GOLGA4 could be a direct protein-protein interaction that facilitates cytokine secretion as well.

In addition to these two genes, we validated several other network genes in *vitro* using available knock-out nematodes or mouse macrophages. In general, mutants with innate immune defects in *C. elegans* are susceptible to pathogens, although there are conditions under which the nematode innate immune response can have a negative impact on survival and thus mutants could enhance pathogen resistance (92).

**tag-260/Irf2bp1** mutant nematodes were moderately resistant to pathogen. The fact that IRF2BP1 could affect innate immunity is not surprising given the fact that IRF2BP1 is reported to be a co-repressor for IRF2 (93), which can affect the LPS response in macrophages (94–96). *klp-12/Klf21a* mutant nematodes were also moderately resistant to pathogen. The kinesin KIF21A is a plus-end-directed microtubule motor (97) that has not been implicated in innate immunity previously. *set-18/Smyd3* mutant nematodes were slightly susceptible to pathogenic bacteria but not heat-killed *E. coli*; SMYD3 is a histone methyltransferase that affects transcription (98, 99) and could therefore have many conceivable effects on innate immunity. Although the nematode *siah-1/Siah1* mutation did not affect survival of the presence of *P. aeruginosa*, we note that there are many other pathogens that infect nematodes that we have not tested. Moreover, overexpression of *Siah1* in mammalian cells has been reported to stimulate *NFkB* activity (100), consistent with our macrophage RNAi data.

We also demonstrated that deletion of another network gene, *Macf1*, from mouse macrophages led to increased PAMP-induced IL-6 production. Unlike *Sf3a1* and *Golga4*, which exhibited similar phenotypes when inhibited in either of two mouse macrophage cell lines, *Macf1* exhibited different effects in these lines, and the effect in RAW264.7 cells but not J774A.1 cells phenocopied the in *vitro* effect. We are uncertain of precisely why the two immortalized lines differ, but one possibility is that because MACF1 affects subcellular trafficking (101–105), perhaps it has both positive and negative effects on different aspects of innate immunity, and differential knockdown could cause different effects. Regardless, it does reinforce the importance of in *vitro* follow-up studies to validate in *vivo* RNAi data.

Roughly one-third of *C. elegans* genes that regulated antimicrobial production in our nematode RNAi screen affected production of LPS-induced cytokine production in mouse macrophages (12). Moreover, 83% (10/12) of the genes in this protein network regulated LPS-induced cytokine production, and 5 of 6 of these network genes affected innate immunity in *vitro*. In contrast, inhibition of only 2 out of 100 other candidate genes (identified using computational approaches examining PAMP-induced gene expression data in the literature) led to an altered LPS-induced cytokine response. 4 Although our comparative genomics and proteomics approach is identifying novel innate immunity regulators at high efficiency, it remains to be determined whether the mechanisms by which these genes act are conserved between species.

In summary, we have identified a network of orthologous interacting proteins in *C. elegans*, *Drosophila*, and mammals, demonstrated that most proteins in this network regulate the response to the TLR4 agonist LPS in *vitro*, demonstrated that the two genes in this network that exhibited the strongest RNAi-induced phenotypes (*Sf3a1* and *Golga4*) do so through different mechanisms, and validated five of these candidates in *vivo* using knock-out nematodes and mice. Thus, this novel evolutionarily conserved protein interaction network will provide very fertile ground for future investigation. Future studies will involve determining the mechanisms by which these genes act and determining what role they play in different diseases that are affected by innate immunity.

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4 S. Alper, unpublished data.
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