USING GENETIC ANALYSIS AND THE MODEL ORGANISM *CAENORHABDITIS ELEGANS* TO IDENTIFY BACTERIAL VIRULENCE FACTORS AND INNATE IMMUNE DEFENSES AGAINST PATHOGENS

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

An estimated twenty-five percent of the fifty-seven million annual deaths worldwide can be directly attributed to infectious disease. Mammals contain both adaptive and innate immune systems to deal with invading pathogens. The genetic model organism *Caenorhabditis elegans* lacks an adaptive immune system, which makes it a powerful model organism to study the innate immune system without the added complexity of an adaptive immune system. Multiple human pathogens can cause lethal infections in *C. elegans* and several *C. elegans* innate immune pathways have been identified that are conserved with mammals and protect the nematode from infection. The goal of this work was to identify novel bacterial virulence factors and innate immune defenses against pathogens by using the genetic model organism *C. elegans*. We established *C. elegans* as a model for *Yersinia pestis* infection and used this model to identify novel bacterial virulence factors that were also important for virulence in a mammalian model of infection. Previous studies demonstrated that *C. elegans* can identify bacterial pathogens using sensory neurons and activate an avoidance response that requires components of G-protein signaling pathways. We screened forty *C. elegans* strains containing mutations in chemosensory G-protein coupled receptors for altered survival on pathogen and identified *npr-1* to be required for full *C. elegans* defense against

pathogens:. We found that activation of the NPR-1 nervous circuit enhances host susceptibility to microbial infection while inhibition of the circuit boosts innate immunity. This data provides the first evidence that innate immunity in *C. elegans* is directly linked to the nervous system and establishes the nematode as a novel system to study neuroimmunology. From this work, we have identified *Y. pestis* virulence-related genes and *C. elegans* innate immune effector genes required for innate immunity to human bacterial pathogens.

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1. Introduction

1.1 Infection and immunity

An estimated 25% of the 57 million annual deaths worldwide can be directly attributed to infectious disease (Morens, Folkers et al. 2004). Mammals contain both adaptive and innate immune systems to deal with invading pathogens. The adaptive immune system includes specialized immune cells, such as T- and B-cells, that respond in a manner specific for the invading organism and generate memory for future defense against that organism. Innate immune defenses comprise nonspecific mechanisms used by metazoan to prevent microbial infections. Activation of the innate immune system upon pathogen recognition results in a rapid and definitive microbicidal response to invading microorganisms. The genetic model organism *Caenorhabditis elegans* does not have an adaptive immune system, which makes it a powerful model organism to study the innate immune system independent of the influences of an adaptive immune system.

1.2 *Caenorhabditis elegans*

C. elegans is a free-living nematode that is found in temperate soil environments and feeds on bacteria as a food source. Physiologically, *C. elegans* is unsegmented and bilaterally symmetrical, has a fluid-filled pseudocoelomic cavity and is protected by a tough cuticle on the exterior of the animal consisting mainly of collagen. The internal

anatomy of the worm includes a mouth, pharynx, intestine and anus. Over ninety-nine percent of the *C. elegans* population is the hermaphrodite sex, which has two ovaries, two gonad arms and a single uterus. Males make up only 0.05% of the population, and have a single-lobed gonad, vas deferens, and a specialized tail used for mating.

The genome of *C. elegans* has five pairs of autosomes and one pair of sex chromosomes. Sex in *C. elegans* is based on an X0 sex-determination system. Hermaphrodite *C. elegans* have a matched pair of sex chromosomes (XX); the rare males have only one sex chromosome (X0).

C. elegans hermaphrodites can either self-fertilize or mate with males. After the hermaphrodite nematode lays eggs, it takes approximately 3.5 days at 20°C to complete the life cycle and reach adulthood. After hatching, *C. elegans* has four larval stages (L1-L4). In the absence of food, *C. elegans* can enter an alternative third larval stage called the dauer state; during this stage the nematodes are stress-resistant and long-lived. Individual nematodes have an identical pattern of cell lineage and exact number of cells for a total of 959 cells in the adult hermaphrodite and 1031 in the adult male (Sulston and Horvitz 1977). Additionally, 131 cells are eliminated by programmed cell death in the hermaphrodite during development. Once reaching adulthood, the nematode will lay approximately 300 eggs in four days, and then has a life span of approximately 2-3 weeks.

1.3 Advantages of *C. elegans* as a model organism

There are several advantages to working with *C. elegans* as a model organism. The nematode has simple growth conditions and a rapid generation time. Nematode stocks can be frozen and later revived, making it easy to maintain lines of mutants. Over 3000 mutant nematode stocks are available from the *Caenorhabditis* Genetics Centre (CGC) to academic researchers. As mentioned previously, *C. elegans* hermaphrodites can either self-fertilize or mate with males. Self-fertilization results in a clonal population of genetically identical offspring, while mating can be used as a tool to generate strains carrying multiple mutations.

The *C. elegans* genome was the first multicellular organism to be completely sequenced. The published sequence is approximately 100 million base pairs long and contains approximately 20,000 genes (1998). This sequence facilitated the development of a useful genetic tool available for use in *C. elegans*: RNA interference (RNAi). By feeding the nematodes *E. coli* expressing double stranded RNA (dsRNA) complementary to the sequence of the gene of interest or injecting the dsRNA directly into the worm, the function of the gene is silenced (Fire, Xu et al. 1998; Timmons and Fire 1998). Using this technique, the function of individual *C. elegans* genes can be identified without the time-consuming effort of generating a mutant strain. Libraries of bacterial clones expressing dsRNA that individually target each gene in the *C. elegans*

genome have been generated, allowing researchers to do reverse genetic screens for a phenotype of interest (Bargmann 2001).

These manipulations give us a powerful *in vivo* genetic system to study biological processes. Sydney Brenner is credited with establishing the nematode as a model system to study molecular and developmental biology through the use of genetic techniques. In his original paper, Brenner describes techniques for handling *C. elegans* in the laboratory and uses ethyl methanesulphonate (EMS) to induce mutations and identify an array of mutants with behavioral and growth phenotypes (Brenner 1974). Some examples of biological processes modeled using worms include embryogenesis (Vanderslice and Hirsh 1976; Deppe, Schierenberg et al. 1978; Labouesse and Mango 1999), sex determination (Hodgkin and Brenner 1977; Goodwin and Ellis 2002), cell cycle (Horvitz and Sulston 1980; Koreth and van den Heuvel 2005), larval development (Cassada and Russell 1975; Byerly, Cassada et al. 1976; Ambros 2000), programmed cell death (Sulston and Horvitz 1977; Kinchen and Hengartner 2005), insulin signaling (Kimura, Tissenbaum et al. 1997), aging (Kenyon, Chang et al. 1993), organogenesis (Mango 2007) and neurobiology (Sattelle and Buckingham 2006).

Neurobiology is one of the most studied fields using *C. elegans* as a genetic model. In comparison to the genetic model system *Drosophila melanogaster*, which has around 10^5 neurons, the *C. elegans* nervous system has 302 neurons. Moreover, connections between all the neurons have been established. Most neurons are not

required for viability or reproduction, therefore, whole animal behavioral phenotypes can be seen by genetic or laser ablation of neurons. Neural pathways have been identified for certain behaviors, including chemotaxis, thermotaxis, mechanotransduction, and male mating behavior (Mori 1999; Hobert 2003; Lipton, Kleemann et al. 2004; Syntichaki and Tavernarakis 2004; Bianchi 2007).

1.4 Bacterial infection of *C. elegans*

It is only recently that *C. elegans* was developed as a useful model to study innate immunity and resistance to infection. In its natural environment, the nematode feeds on a wide range of bacteria, some innocuous and some potentially pathogenic. Conserved innate immune responses can be studied by identifying the mechanisms used by the nematode to defend against pathogenic microbes.

From a host-pathogen perspective, an assay for virulence involves replacing the nematodes normal laboratory food of non-pathogenic *Escherichia coli* strain OP50 with the pathogen under investigation. A wide variety of microbes, including both Gram-negative and Gram-positive bacteria as well as fungal strains, are pathogens of the nematode. The range of infection mechanisms is as wide as the diversity of the pathogens. *Salmonella enterica* and *Serratia marcescens* infect via a persistent intestinal colonization that causes lethality within a few days (Aballay, Yorgey et al. 2000; Labrousse, Chauvet et al. 2000; Kurz, Chauvet et al. 2003). *Pseudomonas aeruginosa* can either colonize the intestine or produce a toxin which mediates rapid death within a few

hours, depending on the medium on which it is grown (Tan, Mahajan-Miklos et al. 1999). *Microbacterium nematophilium* colonizes the anus of the animal, causing a swelling that is debilitating but not lethal (Hodgkin, Kuwabara et al. 2000). Certain strains of *Yersinia* can form a biofilm that surrounds the nematode, causing a quick lethality that was attributed to nematode starvation (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003). The use of *C. elegans* as a model for host-pathogen interactions has been extensively reviewed in (Nicholas and Hodgkin 2004) and (Sifri, Begun et al. 2005).

1.5 *C. elegans* defenses against pathogens

1.5.1 Physical defenses of the nematode

The nematode has both mechanical and humoral innate immune defenses against infection by pathogen. Physically, a multi-layered cuticle surrounds the nematode, leaving only the three openings prone to infection: the mouth, anus and vulval opening. *C. elegans* feeds on bacteria, and the mouth is the most common route of infection. The first barrier that the pathogen encounters is the grinder, a tri-lobed structure made of cuticle, located in the pharynx, which physically breaks down bacteria. It has been shown that nematodes lacking a functional grinder are hypersusceptible to *S. enterica* infection (Labrousse, Chauvet et al. 2000). Living bacteria that are able to pass through the grinder into the intestine are faced with a number of putative anti-microbial factors. The intestinal cells are known to specifically express

certain lectins and proteases. The nematode also contains a number of lysozymes, which we can predict to be an important part of the immune response in the intestine. Continued research is required to determine which genes are constitutively expressed to aid in digestion and which are induced to protect against bacterial infection; and further, which pathways are involved in the induction of anti-bacterial factors. (Nicholas and Hodgkin 2004; Kim and Ausubel 2005)

1.5.2 Known innate immune genetic pathways

1.5.2.1 CED genes/ Programmed cell death pathway

An important feature of a variety of mammalian–pathogen interactions is programmed cell death (PCD) of both immune and somatic cells. Key components of the mammalian cell death machinery were initially identified for their homologs in *C. elegans* (Hedgecock, Sulston et al. 1983; Horvitz, Shaham et al. 1994). In *C. elegans* PCD, EGL-1, which is similar to mammalian cell death activators, activates the PCD cascade by inhibiting CED-9, the *C. elegans* homolog of the mammalian cell death inhibitor Bcl-2. CED-9 normally inhibits PCD by sequestering the CED-4 apoptotic ATPase and the CED-3 caspase in an inactive ternary complex called the apoptosome (Hengartner, Ellis et al. 1992; Hengartner 1997; Conradt and Horvitz 1998). Previous work in our laboratory has shown that loss-of-function mutation in *egl-1*, *ced-4* or *ced-3* or a gain-of-

function mutation in *ced-9* results in increased susceptibility to *S. enterica* -mediated killing, implicating this pathway in immune defense (Aballay and Ausubel 2001).

1.5.2.2 TOL-1/ Toll-like receptor pathway

One class of transmembrane receptors identified as important in mammals for host innate immunity and pathogen recognition are the Toll-like receptors (TLRs). The *C. elegans* genome appears to encode a single TLR, TOL-1, as well as single copies of TRF-1, PIK-1 and IKB-1, homologues of the mammalian downstream signal transduction components TNF receptor-associated factor 1 (TRAF1), interleukin 1 receptor associated kinase (IRAK) and inhibitor of κB (IκB), respectively (Pujol, Link et al. 2001). Work from our laboratory has demonstrated that loss of function mutation in *tol-1*, *trf-1* and *ikb-1* results in increased susceptibility to *S. enterica* -mediated killing and a dramatic increase in *S. enterica* invasion of the pharyngeal tissue compared to wild-type nematodes, however epistasis studies using double mutants found that TRF-1, but not IKB-1, might be required for the effects of TOL-1 in immunity and that there might be other downstream components that regulate TOL-1-mediated immunity in a redundant manner (Tenor and Aballay 2008).

1.5.2.3 DBL-1/ TGF-β pathway

The DBL-1/ TGF-β pathway was first identified in the worm for its role in body size regulation and in male tail formation (Patterson 2000) and then later identified for

its role in innate immunity (Mallo, Kurz et al. 2002). The gene *dbl-1* encodes a TGF-β-like ligand. Extracellular DBL-1 binds the heterodimeric DAF-4/SMA-6 receptor, which phosphorylates the SMA-2/SMA-3/SMA-4 SMAD complex. The phosphorylated SMAD complex can then enter the nucleus of the cell to control gene expression. Loss-of-function mutants in these genes are hypersensitive to infection. Upregulation of certain innate immune effector molecules in response to pathogen is dependent on the presence of DBL-1 (Mochii, Yoshida et al. 1999; Mallo, Kurz et al. 2002)

1.5.2.4 DAF-2/ Insulin-like signaling pathway

The DAF-2/DAF-16 pathway, also known as the insulin-like signaling pathway, has been well characterized for its role in C. elegans aging; *daf-2* mutants have a longer life span and also resist infection by both Gram-negative and Gram-positive bacterial pathogens (Garsin, Villanueva et al. 2003). In the presence of an agonist ligand, such as the insulin-like peptide DAF-28, the DAF-2 receptor is activated. This activates the phosphatidylinositol-3 OH kinase AGE-1, which catalyses the conversion of phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3). PIP3 then binds the complex AKT-1/AKT-2 and exposes two phosphorylation sites, which are phosphorylated by the kinase PDK-1. This activates AKT-1 to phosphorylate the transcription factor DAF-16 and causes it to be sequestered in the cytoplasm. In the presence of an antagonist ligand such as INS-1, (or in a *daf-2* loss-of-function mutant), the pathway is not active, DAF-16 is not phosphorylated and translocates into the

nucleus, where it is known to enhance expression of stress response and antimicrobial genes, including lysozymes and saposins (Murphy, McCarroll et al. 2003). Both the DAF-2 loss of function mutant and the DAF-16 overexpression mutant are highly resistant to infection by pathogens (Garsin, Villanueva et al. 2003).

1.5.2.5 HSF-1/ Heat shock pathway

One hallmark of mammalian innate immune defense against pathogens is inflammation associated with fever. Increased temperature promotes expression of heat-shock (HS) proteins (HSPs) that are found in high levels in almost all inflammatory diseases (van Eden, van der Zee et al. 2005). Our laboratory has demonstrated that heat shock of the nematodes significantly increases immunity to bacterial pathogens through a mechanism that requires the heat shock transcription factor HSF-1 and associated HSP chaperone proteins (Singh and Aballay 2006). It was shown that HSF-1 interacts with the DAF-2/DAF-16 insulin-like signaling pathway described above; HSF-1-regulated proteins are effectors of the DAF-2/DAF-16 pathway required for pathogen resistance (Singh and Aballay 2006).

1.5.2.6 PMK-1/ MAP kinase pathway

The PMK-1 pathway is homologous to the mammalian p38 MAP kinase pathway. The MAP3K gene *nsy-1* and the MAP2K gene *sek-1* were found in a genetic screen for worms hyper-susceptible to *P. aeruginosa*(Kim, Feinbaum et al. 2002). These

two genes were found to act in a pathway upstream of the MAP kinase gene *pmk-1*; RNAi against this gene also results in increased susceptibility to infection (Kim, Feinbaum et al. 2002). This pathway has also been implicated in immunity to *S. enterica* and the *Bacillus thuringiensis* pore-foming toxin Cry5B (Kim, Feinbaum et al. 2002; Aballay, Drenkard et al. 2003; Huffman, Abrami et al. 2004). The adaptor protein TIR-1 has been shown to be upstream of this kinase cascade, yet a receptor has not been identified for recognition of pathogen (Couillault, Pujol et al. 2004). TIR-1, NSY-1 and SEK-1 likely form a complex, since they can be co-immunoprecipitated when co-expressed in cell culture (Chuang and Bargmann 2005). This pathway also regulates the expression of innate immune response genes (Troemel, Chu et al. 2006).

1.6 Introduction to thesis work

The goal of this work is to identify and characterize both novel bacterial virulence factors and innate immune defenses against pathogens by using the genetically tractable model organism *C. elegans*. Detailed methodologies for this thesis are described in Chapter 7.

Y. pestis is a Gram-negative bacterium and the causative agent of the black plague. Previous work had established that *C. elegans* could be used as a model to study the *Y. pestis* biofilm formed within the flea vector that transmits the infection to humans (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003; Tan and Darby 2004). Our work identified a strain of *Y. pestis* that killed *C. elegans* in a biofilm-independent manner,

which we predicted to be more homologous to *Y. pestis* infection in mammals. We then characterized this infection and investigate the role of known *Y. pestis* virulence factors in infection of *C. elegans*. Finally, we discovered novel virulence factors using *C. elegans* and demonstrated that they are also important for *Y. pestis* infection in mammals by using an intranasal mouse model of pneumonic plague developed by our lab and others. (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008)

In comparison to the intranasal mouse model of plague, the two more common modes of *Y. pestis* infection in mouse are the subcutaneous and intravenous routes. These models of plague are highly lethal with extremely low LD_{50}s. When selecting a model to assess the virulence of the attenuated mutants identified using *C. elegans*, we were concerned that sensitive differences in survival would be unseen in these rapidly virulent models of plague. After establishing the intranasal mouse model of plague, we sought to use this model to evaluate a point of controversy in the *Y. pestis* field where the subcutaneous and intravenous models of infection had generated ambigious results (Elvin, Williamson et al. 2004; Mecsas, Franklin et al. 2004).

Approximately 10% of Europeans are protected against HIV-1 infection because they carry a mutant allele of the human chemokine receptor CCR5 (*CCR5-Δ32*). It was proposed that since the origin of this allele occurred in the Middle Ages, long before the AIDS pandemic, it could possibly confer resistance to another highly lethal pandemic rampant at that time: the black plague. When mice were infected with *Y. pestis* using the

intravenous or subcutaneous routes significant differences in survival were not observed (Mecsas, Franklin et al. 2004). On the other hand, peritoneal macrophages isolated from CCR5-deficient mice showed significantly reduced uptake of *Y. pestis* in vitro (Elvin, Williamson et al. 2004), suggesting the potential for an altered immune response.

We decided to use the intranasal mouse model of *Y. pestis* infection to assess morbidity and mortality of CCR5-/- mice during plague infection. We found that lack of CCR5 does not enhance host resistance to the intranasal mouse model of plague. However, CCR5-/- mice exhibited higher levels of circulating RANTES and MIP-1α, CCR5 ligands that mediate Natural Killer (NK) cell migration, than those exhibited by wild-type mice at the baseline and throughout the course of *Y. pestis* infection. These results imply that plague was not a selective factor in the emergence of the non-functional *CCR5-Δ32* allele in humans; yet our data does suggest a previously unknown role for NK cell migration in *Y. pestis* infection. RANTES and MIP-1α mediate NK cell migration through both CCR5 and the chemokine receptor CCR2, suggesting that CCR2 function might be compensating for the lack of CCR5 in plague defense. (Styer, Click et al. 2007)

CCR5 and CCR2 are members of the chemosensory G-protein coupled receptor class. Genes in this class are known to play a role in a wide variety of physiological activities, including odorant sensation, neurotransmission and immunity (Pierce,

Premont et al. 2002; Rozengurt 2007). A previous study demonstrated that expression of human CCR5 in *C. elegans* sensory neurons allowed the nematode to sense the CCR5 ligand MIP-1α (Teng, Dekkers et al. 2006), suggesting that *C. elegans* uses at least part of the same downstream machinery as mammals to transduce GPCR signals. *C. elegans* contains over 1,300 putative chemoreceptory GPCR genes, approximately 7% of the genome, but only a limited number have a known role in chemosensation and related behaviors (Bergamasco and Bazzicalupo 2006). In order to analyze the role of these genes in *C. elegans* innate immunity, we screened forty *C. elegans* strains containing mutations in GPCRs for an altered survival phenotype and identified mutants with enhanced susceptibility and enhanced resistance to pathogen infection.

Interestingly, a large proportion of these genes were known to be exclusively expressed in the *C. elegans* nervous system. *C. elegans* has been long used as a model system to study neurobiology (Sattelle and Buckingham 2006). *C. elegans* neurons are known to express numerous secreted peptides of the TGF-β family, the insulin family and neuropeptide families (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996; Li, Nelson et al. 1999; Nathoo, Moeller et al. 2001; Pierce, Costa et al. 2001; Li, Armstrong et al. 2004). These secreted factors have the potential to act in an endocrine fashion to affect both neuronal and non-neuronal cells throughout the animal. Ablation of different neurons is known to affect adult lifespan in the nematode. Since the insulin-like pathway not only controls lifespan but also immunity it is possible that some of the

neuroendocrine pathways described above also regulate innate immunity. The compromise between simplicity and genetic tractability makes the nematode a prime model system in which to study neural regulation of the innate immune system.

We found that loss-of function mutants in *npr-1* are highly susceptible to various pathogens. This gene is also known to be involved in aggregation and bordering behavior on a lawn of bacteria, appropriate oxygen response and locomotion in the presence of food. *npr-1* is expressed in several neurons and is required for nematode to defend itself against multiple different pathogens. We determined that the same neural circuit involved in the *npr-1* behavioral phenotype is also involved in the *npr-1* immune phenotype We further identified *C. elegans* immune effector genes expressed in tissues in contact with pathogen that are down-regulated in *npr-1* mutant nematodes. This data provides the first evidence that innate immunity in *C. elegans* is directly linked to the nervous system and establishes the nematode as a novel system to study neuroimmunology.

2. Development of *C. elegans* as a model system to study *Yersinia pestis* infection

2.1 Introduction

2.1.1 *Yersinia pestis*

Yersinia pestis is a Gram-negative bacillus responsible for three forms of plague: bubonic, pneumonic and septicemic plague. All three forms have been responsible for epidemics with high mortality rates throughout history, perhaps the most famous being the Black Death, which killed approximately one-third of the European population from 1347 to 1353. (Perry and Fetherston 1997; Gage and Kosoy 2005)

Three biovars of *Y. pestis* are known: Biovar Antiqua, which is thought to correspond to the Plague of Justinian in 541; Biovar Medievalis, which is thought to correspond to the Black Death; and Biovar Orientalis, which is thought to correspond to the Third Pandemic, which started in China in 1855 and was still considered an active pandemic until 1959 (Guiyoule, Grimont et al. 1994). *Y. pestis* was found to be the causative agent of plague during the Third Pandemic in 1894 by Alexandre Yersin, a bacteriologist from the Pasteur Institute.

Plague is generally a zoonotic infection contracted by humans either through direct contact with an infected animal or transmitted by a flea that has fed on an infected host. The most common host is the black rat. The rat flea *Xenopsylla cheopsis* bites the

infected rat and *Y. pestis* proliferates within the gut of the flea, forming a biofilm that blocks digestion. When the infected flea bites another host, *Y. pestis* is regurgitated into the bloodstream of the new host to cause the plague infection. (Oyston and Isherwood 2005)

Y. pestis infection in humans is most commonly seen as bubonic plague, where *Y. pestis* spreads through the lymphatic system to the lymph nodes, which swell to form the characteristic 'buboes'. When the lymphatic system drains into the blood, *Y. pestis* spreads throughout the body, causing a highly lethal septicemia. If infection spreads into the lungs, a highly contagious pneumonic plague can be spread from person to person. Initial symptoms of pneumonic plague are difficult to distinguish from other respiratory pneumonias. Without proper diagnosis and treatment, the infection can be fatal in one to six days with 50–90% mortality. (Perry and Fetherston 1997)

The strain used in this work is *Y. pestis* strain KIM, which is of the Biovar Medievalis. The 4.6Mb chromosome of strain KIM was fully sequenced in 2002; it contains a chromosomally encoded pathogenicity island called the *pgm* locus (102Kb) (Deng, Burland et al. 2002). *Y. pestis* also contains three plasmids: pCD1 (70Kb), pPCP1 (9.6Kb) and pMT1 (100Kb). The pCD1 plasmid encodes a type III secretion system (TTSS), the Ysc-Yop system, which is required for full virulence in mammalian systems (Heesemann and Laufs 1983; Heesemann, Algermissen et al. 1984; Hodgkin, Kuwabara et al. 2000). The pPCP1 plasmid encodes the plasminogen activator protease, which is

important for dissemination of *Y. pestis* after subcutaneous injection into mammalian hosts (Wren 2003). The pMT1 plasmid encodes the F1 capsular antigen, which seems to inhibit bacterial adhesion to epithelial cells (Liu, Chen et al. 2006), as well as murine toxin, which enhances survival of the organism in the flea midgut (Hinnebusch, Rudolph et al. 2002).

2.1.2 Previous work done on *Y. pestis* using *C. elegans*

Recently, it was reported that some *Yersinia* species kill *C. elegans* by a mechanism that correlates with the formation of a biofilm in the head of the animals. It was proposed that biofilm production, which requires the *hmsHFRS* operon, prevents food intake and causes death by starvation (Darby, Hsu et al. 2002). To identify other virulence factors that are required for the biofilm-mediated killing of *Yersinia pseudotuberculosis*, the virulence phenotype of 39 transposon insertion strains was studied in *C. elegans.* Seven mutants in unknown genes and in genes that encode factors involved in haemin storage and lipopolysaccharide biosynthesis were identified (Joshua, Karlyshev et al. 2003). A later report indicates that biofilm formation depends on a continuous exposure of the nematodes to *Yersinia*, a result confirmed by our laboratory (Tan and Darby 2004).

2.1.3 A biofilm-independent model of *Y. pestis* infection in *C. elegans*

Although previous work had established that *C. elegans* could be used as a model to study the *Y. pestis* biofilm formed within the flea vector that transmits the infection to humans (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003; Tan and Darby 2004), we sought to establish a *C. elegans* model of internal *Y. pestis* infection that could potentially be more relevant to mammalian pathogenesis. Our work identifies a strain of *Y. pestis* that kills *C. elegans* in a biofilm-independent manner. We then characterize this infection and investigate the role of known *Y. pestis* virulence factors in infection of *C. elegans*. Finally, we discover novel virulence factors using *C. elegans* and demonstrate that they are also important for *Y. pestis* infection in mammals by using a mouse model of plague developed by our lab and others. (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008)

2.2 Results

2.2.1 *Y. pestis* kills *C. elegans* in a biofilm-independent manner

We sought to develop a *Y. pestis*–*C. elegans* pathogenesis model to aid in the identification and characterization of virulence factors that are not necessarily related to biofilm formation but important for mammalian pathogenesis. We postulated that *Y. pestis* strains deficient in biofilm formation could enter the *C. elegans* intestine and kill the animals by a mechanism that is different from blocking food intake. The commonly studied *Y. pestis* strain KIM5 lacks the *pgm* pathogenicity locus, which contains the

hmsHFRS genes required for biofilm formation. We found that *Y. pestis* KIM5 is not capable of forming a biofilm on the surface of the animals and that the intestinal lumen of the nematodes is distended and full of bacteria after 48 hours of infection with *Y. pestis* KIM5 expressing the *Aequiorea victoria* green fluorescent protein (GFP)(Figure 1A). In contrast, nematodes fed *Escherichia coli* expressing GFP have minimal distension of the lumen (Figure 1B).

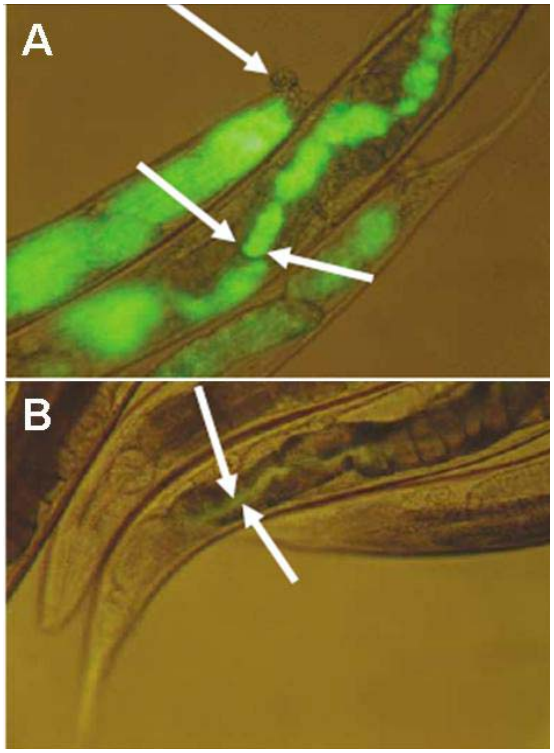

Figure 1: *Y. pestis* KIM5 causes an internal infection in *C. elegans*

C. elegans N2 young adults were exposed to *Y. pestis* KIM5 expressing GFP (A) or *E. coli* DH5α expressing green fluorescent protein (GFP) (B) for 48 hours, transferred to *E. coli* OP50 lawns and then visualized using a Leica MZ FLIII fluorescence stereomicroscope. The margins of the intestinal lumen of single nematodes are indicated with arrows. An arrow also points to the swollen tail of an animal infected with *Y. pestis*.

In each panel, two arrows show the width of the intestinal lumen; in panel A, an additional arrow points to the characteristic swollen tail phenotype seen in nematodes infected with *Y. pestis* KIM5. To our knowledge, the presence of a swollen tail has not been previously reported in *C. elegans* infected with other human pathogens, but it is very similar to that seen in animals that are infected with a specific *C. elegans* pathogen, *Microbacterium nematophilum.* These bacteria adhere to the anal region of the nematodes and induce localized swelling of the underlying hypodermal tissue(Hodgkin, Kuwabara et al. 2000).

When survival was tracked over time, the nematodes died more quickly when fed *Y. pestis* KIM5 strain than *E. coli* OP50, the usual food source for growing *C. elegans* in the laboratory (Figure 2). The time required for 50% of the nematodes to die (time to death 50; TD_{50}) when exposed to *Y. pestis* KIM5 at 25°C was calculated in five independent experiments and was determined to be 3.63±0.27 days, whereas the TD50 was 9.54±1.67 days when the nematodes were exposed to *E. coli* OP50.

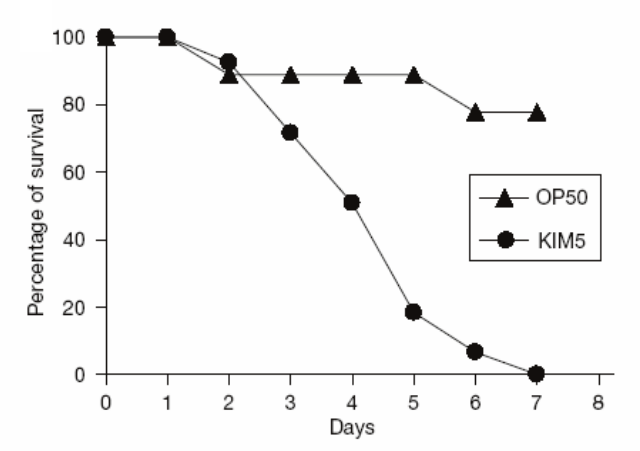

Figure 2: Survival of *C. elegans* exposed to *Y. pestis* KIM5

Groups of 40 *C. elegans* N2 (wild type) young adult animals were exposed to control *E. coli* OP50 or *Y. pestis* KIM5 (P<0.001) and scored for survival over time.

2.2.2 Characterization of the *Y. pestis* infection of *C. elegans*

Previous work has shown that certain human pathogens can resist the mechanical and humoral stresses of the *C. elegans* intestinal environment and persistently colonize the gut (Aballay, Yorgey et al. 2000; Labrousse, Chauvet et al. 2000; Garsin, Sifri et al. 2001; Kurz, Chauvet et al. 2003). To determine whether *Y. pestis* KIM5 was capable of persistently colonizing the *C. elegans* intestine, nematodes were fed on *Y. pestis* KIM5/GFP for 24 h and then transferred to non-labelled *E. coli*. Figure 3 shows the presence of *Y. pestis*/GFP in representative animals 24 h (panel A) or 48 h (panel B) after the animals were transferred to *E. coli*, whereas *E. coli*/GFP was not observed in a representative animal 24 h after the animal was transferred to *E. coli* (panel C).

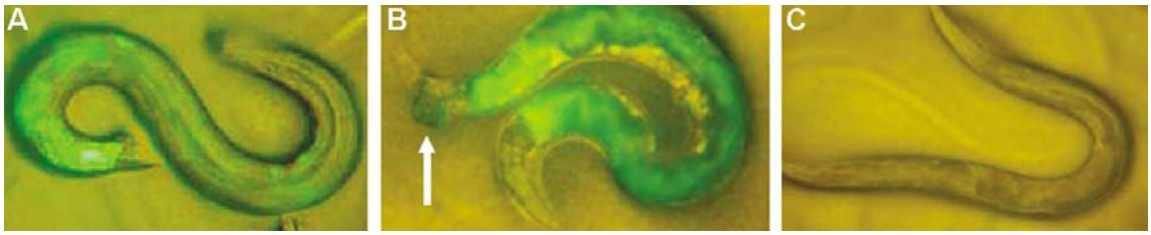

Figure 3: *Y. pestis* persistently colonizes the *C. elegans* intestine

C. elegans N2 young adult animals were exposed to *Y. pestis* KIM5/green fluorescent protein (GFP) (A,B) or *E. coli* DH5α/GFP (C) for 24 h, transferred to *E. coli* OP50 for 24 h (A,C) or 48 h (B), and then visualized using a Leica MZ FLIII fluorescence stereomicroscope. An arrow points to the swollen tail of an animal infected with *Y. pestis* (B).

We also tested whether the persistent colonization of the *C. elegans* intestine by *Y. pestis* correlated with the killing of the animals. Using the transfer experiment described above, we showed that nematodes exposed to *Y. pestis* KIM5 for 24 h died at the same rate as when they were continuously fed on *Y. pestis*, whereas those transferred after an infection time of only 6 h were rescued from a lethal infection (Figure 4). These results indicate that an initial infection period of 24 h is sufficient for *Y. pestis* to establish a persistent, lethal colonization of the *C. elegans* intestine.

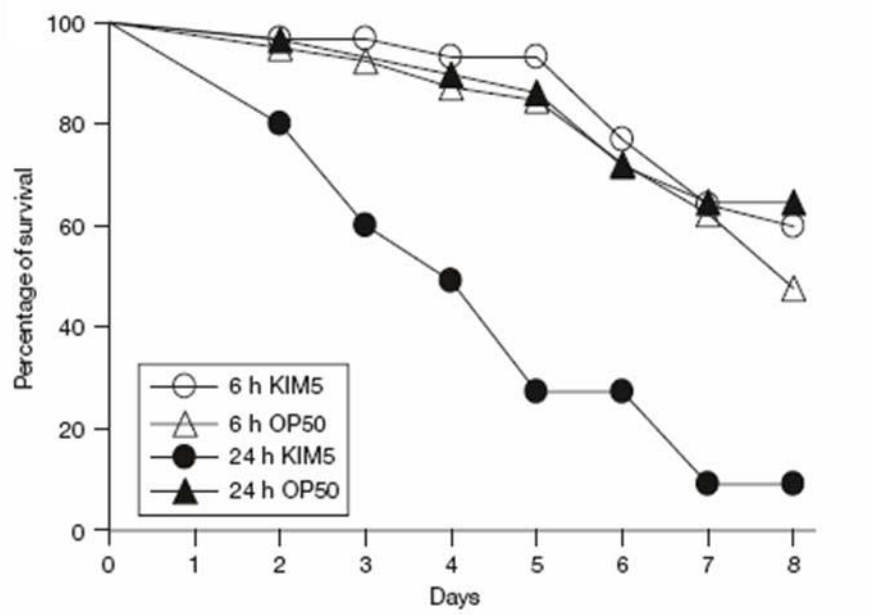

Figure 4: Survival of *C. elegans* following temporary exposure to *Y. pestis*

Groups of 40 *C. elegans* N2 young adult animals were exposed for 6 h to *Y. pestis* KIM5 ($P<0.90$) or *E. coli* OP50 ($P<0.32$), or for 24 h to *Y. pestis* KIM5 ($P<0.001$) or *E. coli* OP50, and then transferred to *E. coli* OP50 and scored for survival over time.

2.2.3 The role of known *Y. pestis* virulence factors in the killing of *C. elegans*

2.2.3.1 *Y. pestis* virulence plasmids pCD1 and pPCP1

As multiple *Y. pestis* virulence determinants are encoded on virulence plasmids, we decided to study the role of the plasmids pCD1 and pPCP1 in the biofilm-independent *Y. pestis*:*C. elegans* infection model. The pCD1 plasmid encodes a type III secretion system (TTSS) required for full virulence in mammalian systems, although it is not sufficient to cause disease (Heesemann and Laufs 1983; Heesemann, Algermissen et al. 1984; Hodgkin, Kuwabara et al. 2000). The pPCP1 plasmid encodes the plasminogen activator protease, which is important for dissemination of *Y. pestis* after subcutaneous

injection into mammalian hosts (Wren 2003). To determine the importance of these plasmids in virulence in *C. elegans*, we compared the susceptibility of the nematodes to *pgm- Y. pestis* strains cured of one or both plasmids (Table 1).

Table 1: Genotype of *Y. pestis* strains

Strain	Description
KIM5	pCD1+ pPCP+
KIM6	pCD1- pPCP+
KIM8	pCD1+ pPCP-
KIM10	pCD1- pPCP-

Figure 5 shows that strains lacking one or two virulence plasmids were equally virulent as *Y. pestis* KIM5, which carries both virulence plasmids. Although the TTSS is required for full virulence in a mammalian host, its components are not expressed at the range of temperatures in which *C. elegans* can be grown. Therefore it is not surprising that these results show that virulence factors encoded in pCD1 and pPCP1 are not required for *Y. pestis* virulence in *C. elegans.*

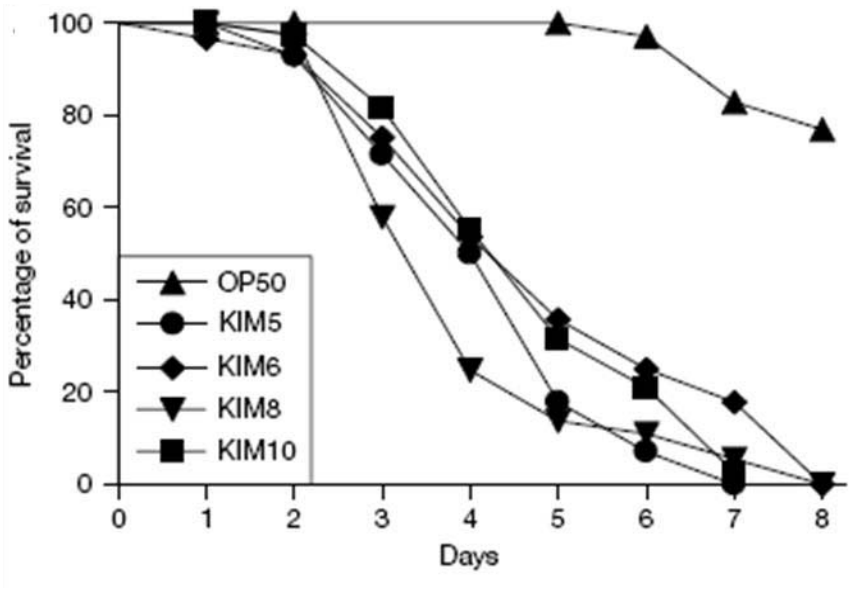

Figure 5: Role of *Y. pestis* virulence plasmids in virulence in *C. elegans*

Groups of 40 *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5 (pCD1+, pPCP1+), *Y. pestis* KIM6 (pCD1−, pPCP1+; $P<0.0885$), *Y. pestis* KIM8 (pCD1+, pPCP1−; $P<0.5202$), *Y. pestis* KIM10 (pCD1−, pPCP1−; $P<0.1388$) or *E. coli* OP50 ($P<0.001$) and scored for survival over time.

2.2.3.2 Putative virulence genes *ompT*, *y3857* and *yapH*

In addition to plasmid-encoded virulence factors, several chromosomally encoded virulence factors have been identified in *Y. pestis*, including genes encoding a potential second TTSS (Revell and Miller 2001). As shown in Figure 6, at least three *Y. pestis* KIM5 deletion mutants in chromosomally encoded genes presumably related to virulence (*ompT*, *y3857* and *yapH*) showed reduced virulence in nematodes. *ompT* encodes a member of the omptin family of outer-membrane proteases, which have been implicated in the virulence of *E. coli*, *S. enterica*, *Shigella flexneri* and *Y. pestis* (Kukkonen and Korhonen 2004). *y*3857 encodes a putative exported metalloprotease that shows

significant amino-acid sequence similarity to virulence-related metalloproteases from *Yersinia ruckeri*, *Photorhabdus luminescens*, *Serratia marcescens* and *Proteus mirabilis* (Walker, Moghaddame-Jafari et al. 1999; Fernandez, Secades et al. 2002; Bowen, Rocheleau et al. 2003). The *yapH* gene encodes a putative autotransporter adhesion protein; the importance of this family of proteins in bacterial attachment to the surface of host cells and protection from complement and antimicrobial peptides has been studied in several species (Roggenkamp, Ackermann et al. 2003; Rose, Meyer et al. 2003; Wehrl, Brinkmann et al. 2004).

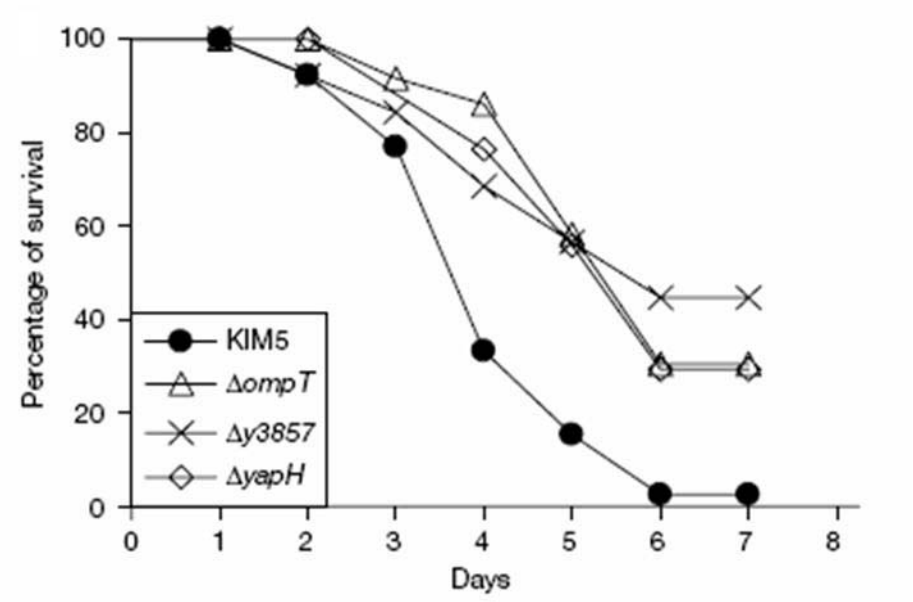

Figure 6: Role of known virulence genes in *Y. pestis* virulence in *C. elegans*

Groups of 40 *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5 or *Y. pestis* KIM5 strains with deletion mutations in the genes *ompT* ($P<0.001$), *y3857* ($P<0.001$) and *yapH* ($P<0.001$) and scored for survival over time.

2.2.3.3 The Ail family of outer membrane proteins

Another gene family of interest in *Y. pestis* virulence is the *ail* family of outer membrane proteins. Members of the Ail/Lom family, which include Ail of *Y. enterocolitica* and *Y. pseudotuberculosis*, Rck and PagC of *S. enterica*, and OmpX of *E. coli*, are outer membrane proteins that share significant amino acid sequence similarity and identity and are predicted to have similar membrane topologies (Miller, Bliska et al. 1990; Pulkkinen and Miller 1991; Heffernan, Wu et al. 1994; Mecsas, Welch et al. 1995; Yang, Merriam et al. 1996; Vogt and Schulz 1999). Although members of this family of proteins exhibit diverse functions, several of them function to protect bacteria from complement-mediated lysis. Previous analyses of the *Y. pestis* genome identified four genes (*y1324*, *y1682*, *y2034*, and *y2446*) which were predicted to encode Ail/Lom family proteins (Parkhill, Wren et al. 2001; Deng, Burland et al. 2002). Our collaborators at the University of Miami demonstrated that the *Y. pestis* gene *y1324*, but none of the other three putative *ail* genes, is required for resistance to complement-mediated killing at both 26°C and 37°C (Figure 7).

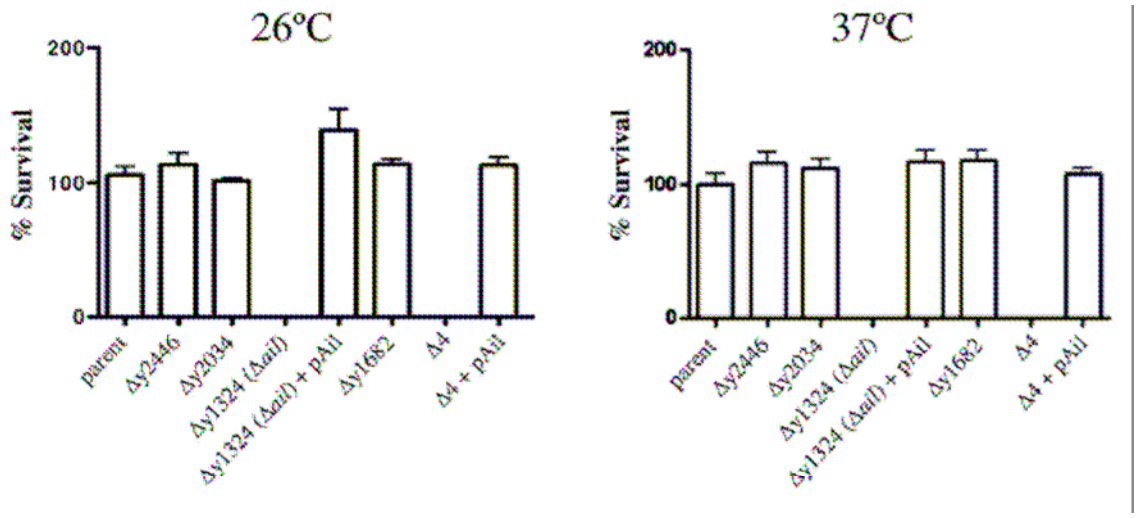

Figure 7: *Y. pestis* gene *y1324* is required for serum resistance

Y. pestis wild-type and mutant strains were grown overnight at 26 or 37°C (~5 x 10^6 bacteria) were incubated with 80% normal human serum (NHS) or heat inactivated serum (HIS) for 1 h at 37°C. Aliquots were diluted and plated on TBA plates at 30°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h x 100. This experiment was performed by Sara Schesser Bartra in Greg Plano's laboratory at the University of Miami.

The protein encoded by *y1324* was designated Ail, and was found to be expressed at high levels at both 26°C and 37°C (Figure 8), while the other putative members of the *Y. pestis* Ail/Lom family were only expressed at 37°C.

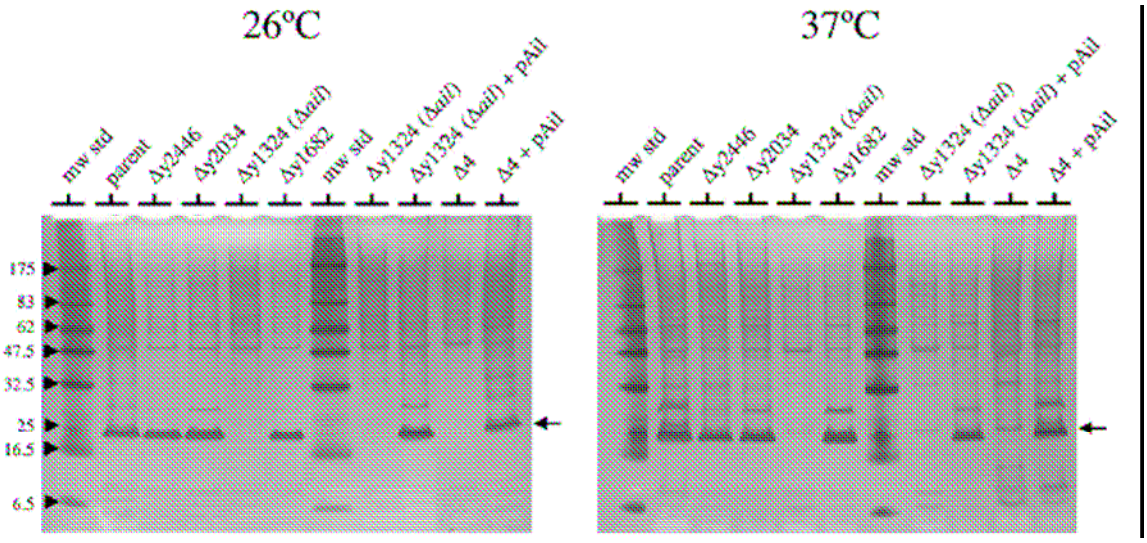

Figure 8: *Y. pestis* Ail is expressed at both 26°C and 37°C

Silver-stained SDS-PAGE gel of outer membrane proteins isolated from the parent and mutant strains of *Y. pestis*. The location of the Ail (y1324) protein is indicated by an arrow. The positions of molecular mass standards (in kilodaltons) are indicated on the left. The strains used were *Y. pestis* strains KIM8 (parent), KIM8Δ*y2446*, KIM8Δ*y2034*, KIM8Δ*y1324* (Δ*ail*), KIM8 Δ*y1324* (Δ*ail*)/pAil, KIM8Δ*y1682*, KIM8Δ*4*, and KIM8Δ*4*/pAil. mw std, molecular mass standards. This experiment was performed by Sara Schesser Bartra in Greg Plano's laboratory at the University of Miami.

To determine if any of the *Y. pestis* Ail/Lom family proteins play a role in the biofilm-independent infection of *C. elegans*, we exposed N2 young adult nematodes to the parent strain *Y. pestis* KIM8 and to strains containing a deletion in one of each of the putative Ail/Lom family genes and a strain with all four genes deleted. When the worms were fed *Y. pestis* strains specifically deficient in expression of *y1682*, *y2034* or *y2446*, lethality was similar to wild-type. In contrast, worms exposed to the Δ*ail* strain or to the strain with all four ail-like genes deleted (Δ4) showed significantly increased survival ($P<0.0001$). Providing the *ail* gene in *trans* (pAil) to the Δ*ail* or Δ4 strain restored the

ability of these strains to efficiently kill *C. elegans*. These results indicate that the *Y. pestis* Ail protein plays an important role in the biofilm-independent killing of *C. elegans* (Figure 9).

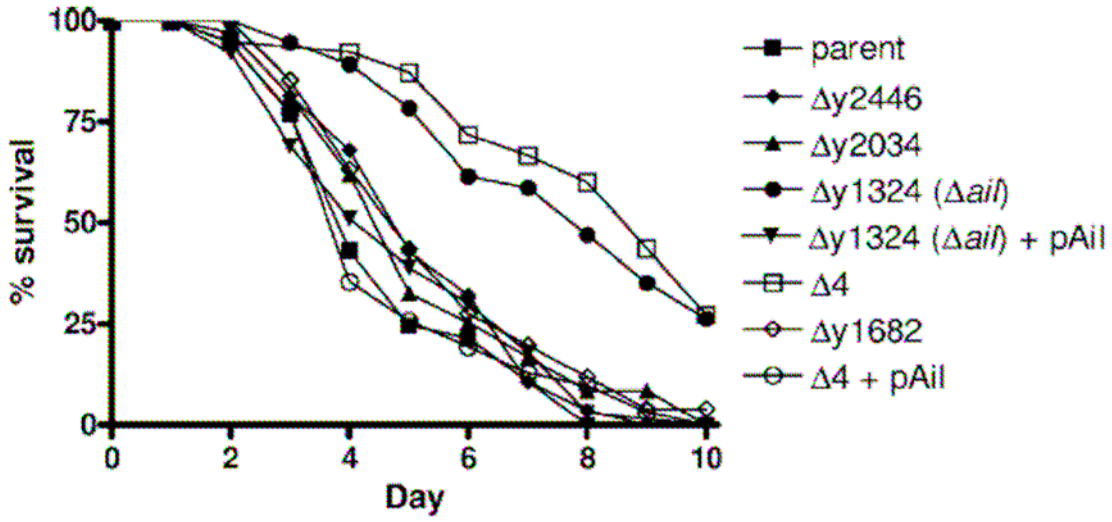

Figure 9: Role of the *ail* family genes in *Y. pestis* virulence in *C. elegans*

Groups of 40 *C. elegans* N2 young adults were exposed to *Y. pestis* KIM8 (parent), KIM8Δ*y2446*, KIM8Δ*y2034*, KIM8Δ*y1324* (Δ*ail*), KIM8Δ*y1324* (Δ*ail*)/pAil, KIM8Δ*y1682*, KIM8Δ*4*, and KIM8Δ*4*/pAil and scored for survival over time.

2.2.4 Identification of novel *Y. pestis* virulence factors using *C. elegans*

To identify novel *Y. pestis* virulence factors, a transposon mutagenesis library was generated using the Epicentre EZ-TN kit to transform competent *Y. pestis* KIM5 cells. A total of 984 *Y. pestis* mutants were isolated. To establish that the kanamycin resistant colonies contained the EZ::TN transposon and that the transposon had integrated randomly into the *Y. pestis* genome, DNA from eleven random clones was digested and probed for the KAN-2 cassette using a DIG labeled probe detectable by

southern blot. Single bands of different length were seen, confirming that the library contained mutants with random, single insertions (Figure 10).

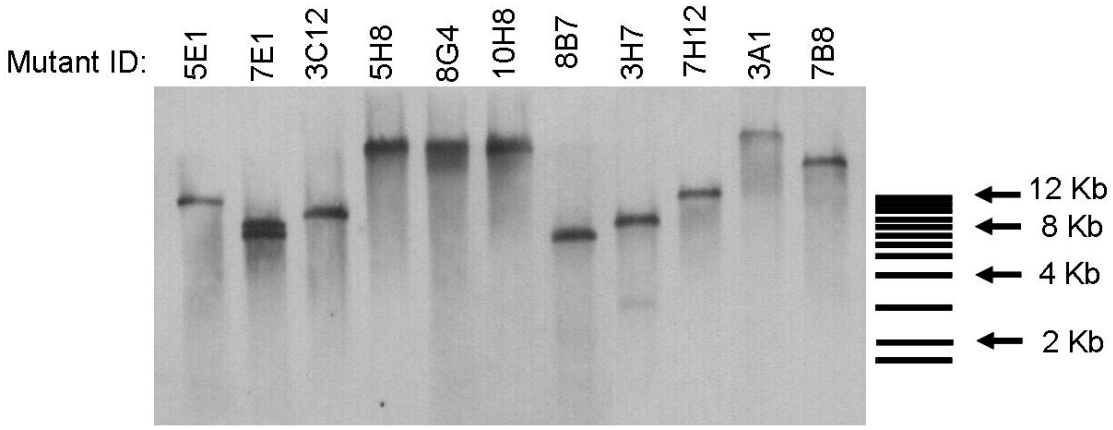

Figure 10: The *Y. pestis* Tn library contains single, random mutations

DNA blot analysis showed that each of the mutant strains identified contained a single EZ::TN insertion. Total genomic DNA isolated from *Y. pestis* KIM5 mutants was isolated using the DNeasy Tissue Kit (Qiagen). Genomic DNA was digested with EcoRI restriction endonuclease overnight at 37°C and then run on a 0.7% agarose gel. A DIG labelled Kan-2 probe was generated with the PCR DIG probe synthesis kit (Roche Diagnostics) under the recommended PCR conditions. Hybridization followed by immunological detection of DNA was performed according to the digoxigenin (DIG) system user's guide (Roche Diagnostics).

To identify novel *Y. pestis* virulence factors, the library was screened as described previously (Tenor, McCormick et al. 2004) for mutants that failed to kill or showed attenuated killing of *C. elegans*. Six out of 984 insertion mutants were identified on the basis of their slower killing of *C. elegans* than the killing by wild-type *Y. pestis* KIM5 (Table 2); survival curves for the least virulent three candidates are shown in Figure 11.

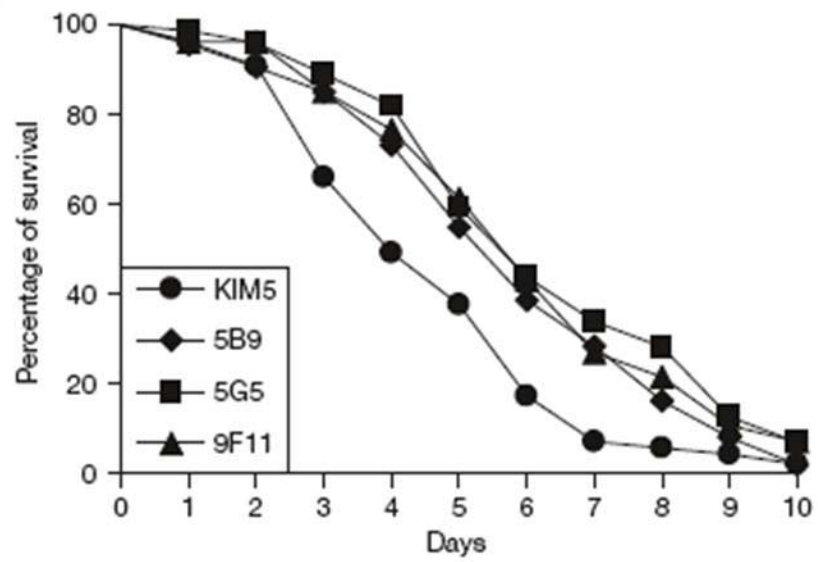

Figure 11: Survival of selected *Y. pestis* Tn mutants in *C. elegans*

Groups of 80 *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5, *Y. pestis* 5B9 ($P<0.0096$), *Y. pestis* 5G5 ($P<0.0001$) or *Y. pestis* 9F11 ($P<0.0006$) and scored for survival over time.

The molecular characterization of the isolated strains indicates that all the mutations are in genes that have previously not been implicated in *Y. pestis* virulence, yet three of the insertions are in genes related to virulence in other species (Table 2).

Table 2: *Y. pestis* insertion mutants isolated based on attenuated *C. elegans* killing

Strain	Gene	Gene product description	Virulence phenotype in mouse[1]	Significance[a]	*N*[a,2]
5B9	*y0340*	Similar to putative exported protein of *Salmonella enterica*	Attenuated	*P*=0.0004	23
5G5	*y1021*[3]	Cytochrome o ubiquinol oxidase subunit II	Attenuated	*P*=0.0001	23
5F1	*y2663*	Putative membrane protein	Wild type	*P*=1.000	23
6B4	*y3913*[c]	Putative RNase R exoribonuclease	ND	ND	ND
8B7	*y0941*[c]	Putative membrane protein; part of an adhesin system	ND	ND	ND
9F11	*y4018*	Unknown	Wild type	*P*=0.3055	8

[1] ND, not determined

[2] N, number of animals analyzed

[3] Genes related to virulence in other species

2.2.5 Assessment of novel *Y. pestis* virulence factors in a mouse model of plague

To determine whether *Y. pestis* virulence-related genes identified using *C. elegans* have a role in mammalian pathogenesis, we developed an intranasal mouse model of *Y. pestis* pathogenesis to study the virulence phenotypes of four isolated mutants. We found that the medial lethal dose (MLD or LD_{50}) of *Y. pestis* KIM5 is about 10^5 colony forming units (CFUs) of *Y. pestis* KIM5. After intranasal inoculation of *Y. pestis* KIM5, bacteria are present in the lungs at relatively low numbers for several days, but the lung infection is often cleared by days 4–6. Histology on day 3 after intranasal inoculation shows micro-abscesses in the spleen (Figure 12A) and liver (Figure 12B). These abscesses enlarge, leading to the complete destruction of the spleen architecture in some mice by days 6 (Figure 12C). The liver maintains its normal architecture through day 8, though it has distinct microabcesses throughout (Figure 12D).

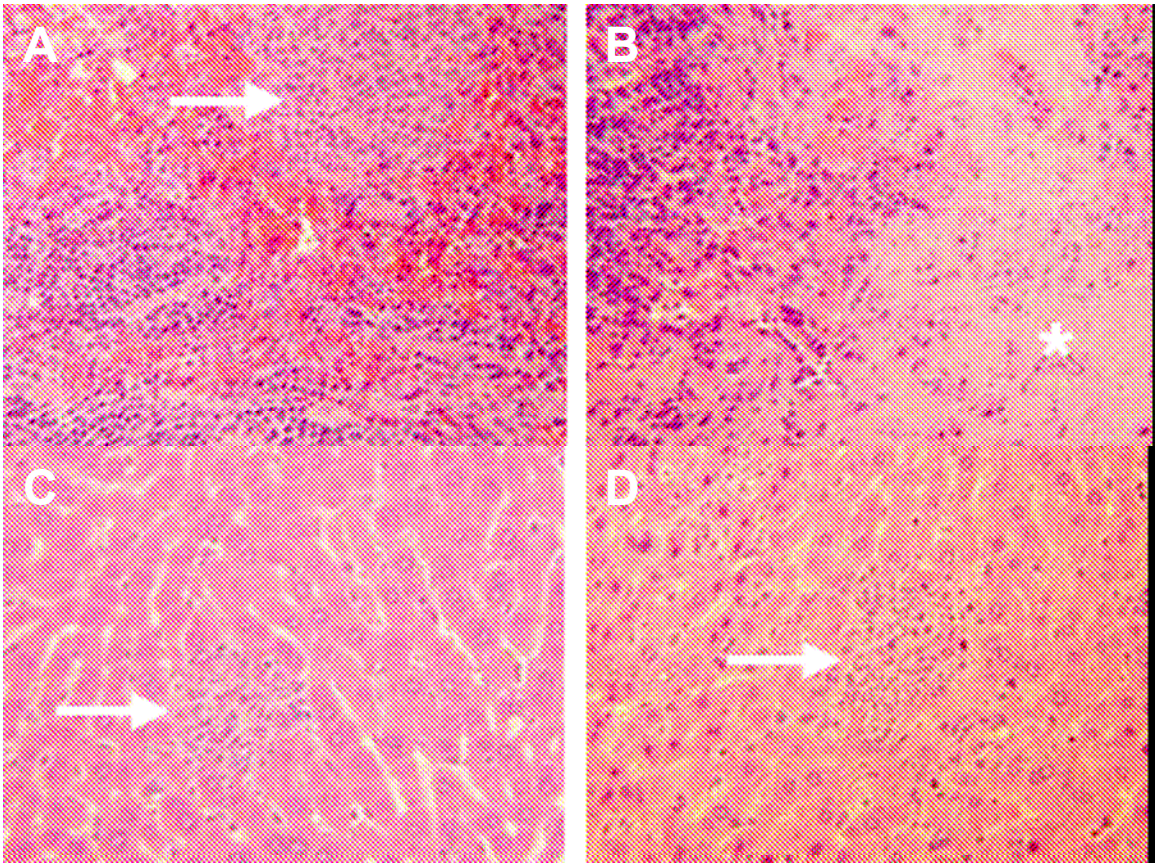

Figure 12: Morphology of mouse spleen and liver during *Y. pestis* infection

Murine spleen (A,B) and liver (C,D) are shown at day 3 (A,C) and at the time of killing for humane end points on day 6 (B) or day 8 (D). Circumscribed micro-abscesses (arrows) develop at early time points in the red pulp of the spleen (A) and the liver parenchyma (C), with preservation of the overall architecture and minimal or no surrounding inflammation. At the time of killing, the red pulp of the spleen (B) is largely replaced by necrotic tissue with nuclear debris (asterisk). Splenic rupture is common (not shown), and, in some cases, the spleen cannot be identified as a distinct organ at necropsy. In contrast, the liver (D) retains its normal architecture, with distinct micro-abscesses (arrows) and a general inflammatory response. This experiment was performed by Greg Hopkins in Richard Frothingham's laboratory at Duke University.

Although the route of infection is the lungs, this model is more representative of septicemic plague in humans than of pneumonic plague. Figure 13 shows the virulence

phenotypes of two mutants identified using the *C. elegans* model that also have reduced virulence in the mouse model of *Y. pestis* pathogenesis. The virulence phenotype of *Y. pestis* 9F11 is provided as an example of a mutant in a gene that is required for full virulence in *C. elegans* but not in mammals. As a control, mice were inoculated with *Y. pestis* KIM10, which lacks both the pCD and pPCP plasmids that are necessary for virulence in the mammalian model. This indicates that the intranasal inoculation of bacteria alone does not cause lethality.

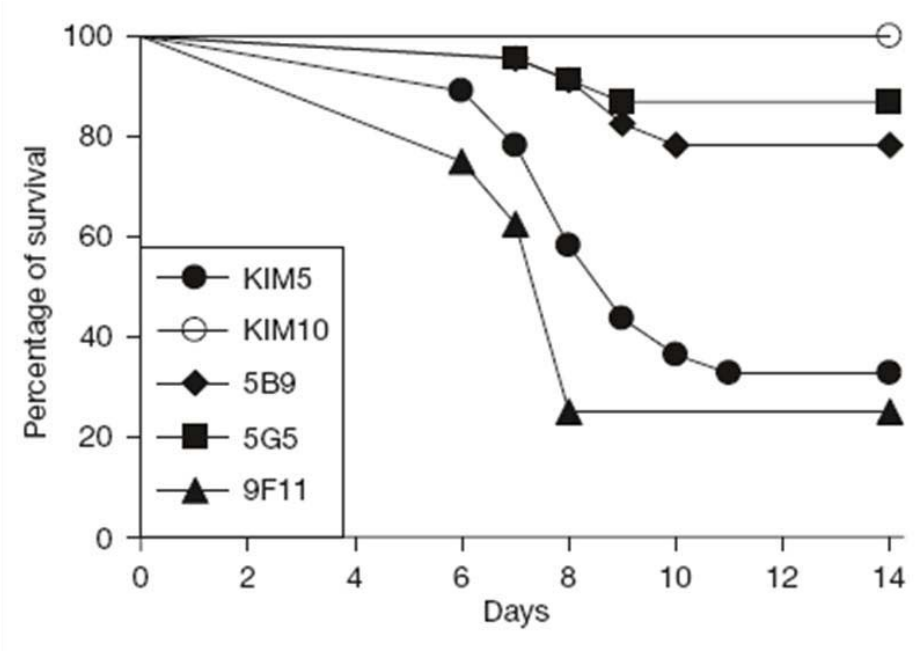

Figure 13: Mutants identified using *C. elegans* have reduced virulence in mice

Female BALB/c mice (12-week-old) were inoculated intranasally with *Y. pestis* KIM5 (n=55), *Y. pestis* KIM10 (n=8, P<0.0001), *Y. pestis* 5B9 (n=23; P<0.0004), *Y. pestis* 5G5 (n=23; P<0.0001) or *Y. pestis* 9F11 (n=8; P<0.3055) mutant strains (10^6 CFU/mouse) and scored for survival over time. After 14 days, surviving animals were euthanized.

2.3 Discussion

Previous laboratories had published data using *C. elegans* as a model organism to study the biofilm formed by *Y. pestis* in the gut of the flea host (Darby, Hsu et al. 2002; Joshua, Karlyshev et al. 2003; Tan and Darby 2004). By identifying a strain of *Y. pestis* unable to form a biofilm, we developed *C. elegans* as a model to study internal infection of *Y. pestis*, a model of virulence more relevant to mammalian pathogenesis. We tested the validity of this model by investigating the role of known virulence factors in *Y. pestis* virulence in the *C. elegans* host.

One drawback of using *C. elegans* to model bacterial pathogenesis is that the nematode is grown at temperatures of 25°C and below. It is well established that exposure to temperatures of around 37°C in mammalian hosts results in the upregulation of a range of *Y. pestis* virulence factors. As expected, the known *Y. pestis* virulence plasmids and the genes encoded on them were found to be dispensable in the *C. elegans* model of *Y. pestis* infection.

However, other *Yersinia* virulence factors, such as invasin, and the *Y. enterocolitica* chromosomal YSA TTSS are preferentially expressed at 26°C (Revell and Miller 2001). In addition, *Y. pestis* virulence factors that are required for colonization of the flea are optimally expressed at lower temperatures; the plasmid pMT1-encoded Ymt protein, which has phospholipase D activity, is preferentially expressed at 26°C and has an important role in enabling infection of the flea (Hinnebusch, Rudolph et al. 2002).

However, soon after being injected by the flea, *Y. pestis* has to deal with mammalian defenses. Therefore, the bacterium probably has to express proteins at 25°C that are required for defense against mammalian innate defenses. For example, *Y. pestis* is constitutively serum resistant at 25°C and 37°C, whereas, in the enteropathogenic *Yersiniae*, serum resistance is expressed only at 37°C. Thus, although temperature is a limitation, we theorized that *C. elegans* could be useful for studying virulence factors that are optimally expressed at 25°C or constitutively expressed at both 25°C and 37°C. It is possible that these factors are crucial for *Y. pestis* survival in the human host in the very beginning stages of infection and might also be targets for novel vaccine development.

Interestingly, a protein identified as *Y. pestis* Ail was necessary for full *Y. pestis* virulence in the biofilm-independent *C. elegans* infection model. Ail is also required for resistance to complement in serum from several mammalian hosts (Bartra, Styer et al. 2008), suggesting that the *C. elegans* gut contains defenses similar to human complement. However, the *C. elegans* genome appears to encode only a limited number of putative complement components (Nonaka and Yoshizaki 2004), such as factor H, a regulator of the alternative pathway (Krushkal, Kemper et al. 1998). This suggests that *Y. pestis* Ail may provide protection against other antibacterial immune effectors or has alternative functions important for virulence in *C. elegans*. For example, *Y. enterocolitica* Ail protein functions as an adhesin/invasin, in addition to its role in serum resistance (Miller and Falkow 1988). However, no role in attachment to, or invasion of, eukaryotic cells was

associated with the *Y. pseudotuberculosis* Ail protein (Yang, Merriam et al. 1996). Future work is needed to determine the role of *Y. pestis* Ail in defense against *C. elegans* immune effectors.

After using reverse genetics to identify the role of known virulence genes in *Y. pestis* virulence in the biofilm-independent *C. elegans* model, we sought to identify novel *Y. pestis* virulence factors using a forward genetics screen. We generated a *Y. pestis* mutant library by selecting clones containing a randomly inserted kanamycin transposon cassette. We then identified six mutants with reduced virulence in the *C. elegans* model. Out of four mutants tested in an intranasal mouse model of plague developed by our lab and others, two had significantly reduced virulence; the genes associated with these two mutants are *y1021*and *y0340*. Y1021 is a cytochrome o ubiquinol oxidase subunit, other proteins of this type have been related to virulence in other pathogens, including *Francisella tularemia* (Weiss, Brotcke et al. 2007), *E .coli* (Motley, Morrow et al. 2004; Snyder, Haugen et al. 2004) and multiple serovars of *Salmonella enterica* (Turner, Barber et al. 2003). A role for Y0340 in virulence was previously unknown; the protein contains a domain of unknown function (DUF943) which classifies it as an enterobacterial putative membrane protein similar to other putative membrane proteins from *E. coli* and *Salmonella typhi* (Marchler-Bauer, Anderson et al. 2007). Further work is needed to identify the particular role these genes play in *Y. pestis* virulence.

In spite of the long evolutionary distance and differences in growth temperatures between nematodes and mammals, the results reported here show that *C. elegans* can be used as an alternative host to study *Y. pestis* virulence factors that are required for pathogenesis in mammalian systems. Virulence factors expressed at low temperatures may be required for pathogen survival in response to innate immunity pathways conserved in nematodes, fleas and mammals. (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008)

3. The role of CCR5 in the intranasal mouse model of plague

3.1 Introduction

3.1.1 CCR5

CCR5 is a seven membrane-spanning chemokine receptor found primarily on the surface of mammalian immune cells, where it appears to act as a pathogen associated molecule pattern receptor (Floto, MacAry et al. 2006). CCR5 was identified as a key receptor for HIV-1 entry into to T lymphocytes via a fusion-mediated event (Alkhatib, Combadiere et al. 1996; Choe, Farzan et al. 1996; Deng, Liu et al. 1996; Doranz, Rucker et al. 1996). A *CCR5-Δ32* allele confers protection against HIV-1 infection, and is present in an average of 10% of humans of European origin. The *CCR5-Δ32* deletion in humans results in the deletion of the second extracellular loop of CCR5, causing an earlier stop codon. Thus, the resulting truncated CCR5 molecule, lacking the final three transmembrane domains and the adjacent loops, is retained within the cell. Without CCR5 on the cell surface, the HIV envelope protein does not bind and the virus does not enter the cell. There appears to be strong positive selection in humans, with no negative consequence to *CCR5* deletion homozygosity in either humans or mice (Dean, Carrington et al. 1996; Huang, Paxton et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996; Carrington, Dean et al. 1999).

A recent finding indicates that the frequency of the *CCR5-Δ32* allele in DNA samples from Bronze Age skeletons is comparable to that in modern German DNA samples (Hummel, Schmidt et al. 2005), suggesting that during human evolution the deletion may have conferred resistance to microbial infections prevalent at different times. The evidence of continuous positive selection for the high frequency of the variant has been attributed to resistance to HIV, smallpox, and plague infections.

3.1.2 Previous work on CCR5 and *Y. pestis*

Tests for protection against infection of CCR5-deficient mice with *Yersinia pestis*, the agent for bubonic plague, yielded ambiguous results. When *Y. pestis* was intravenously inoculated, neither significant differences in survival nor differences in bacterial load in the caecum or Peyer's patches between CCR5+/+ and CCR5−/− mice were observed (Mecsas, Franklin et al. 2004). On the other hand, peritoneal macrophages isolated from CCR5-deficient mice showed significantly reduced uptake of *Y. pestis* in vitro (Elvin, Williamson et al. 2004), suggesting the potential for an altered immune response, although subcutaneous infection of these mice yielded no significant protection against lethality in comparison to CCR5+/+ mice. *Y. pestis* is extremely virulent in both the subcutaneous and intravenous models of plague; the infection circumvents the innate immune system and causes a rapid lethality. We hypothesized that since the intranasal mouse model of plague has a slower disease progression,

sensitive differences in survival between CCR5+/+ and CCR5−/− mice caused by a deficient innate immune response could be more easily detected.

3.1.3 CCR5 and the intranasal mouse model of plague

To assess the role of CCR5 in the intranasal mouse model of plague, we infected CCR5+/+ and CCR5−/− mice with *Y. pestis* and tracked morbidity and mortality, as well as serum cytokine levels. Although a lack of CCR5 played no protective role in *Y. pestis* infection, we found that infected CCR5−/− mice exhibit higher levels of circulating RANTES and MIP-1α, two CCR5 ligands known to mediate Natural Killer (NK) cell migration, suggesting a previously unknown role for NK cell migration in immunity to *Y. pestis* infection.

3.2 Results

3.2.1 Morbidity and mortality during *Y. pestis* infection

To study the role of CCR5 in immunity to *Y. pestis* infection, we used the intranasal mouse model of infection we developed (Styer, Hopkins et al. 2005). CCR5−/− and CCR5+/+ C57Bl/6 mice were infected with 8×10^5 CFU of *Y. pestis*. As shown in Figure 14, overall survival of CCR5−/− mice is not significantly different from that of CCR5+/+ control mice, indicating that lack of CCR5 does not protect from the intranasal challenge with *Y. pestis*. Survival in CCR5 KO mice did not differ significantly from wild-type B6 mice (46% versus 73%, $P = 0.246$; difference = −27%, 95% confidence

interval −62% to 8%). The confidence interval excludes a major resistance phenotype in the CCR5−/− mice, but does not exclude the possibility the CCR5−/− mice are more susceptible than wild-type to this intranasal plague challenge. There was a trend toward decreased survival time in the CCR5−/− mice ($P = 0.108$).

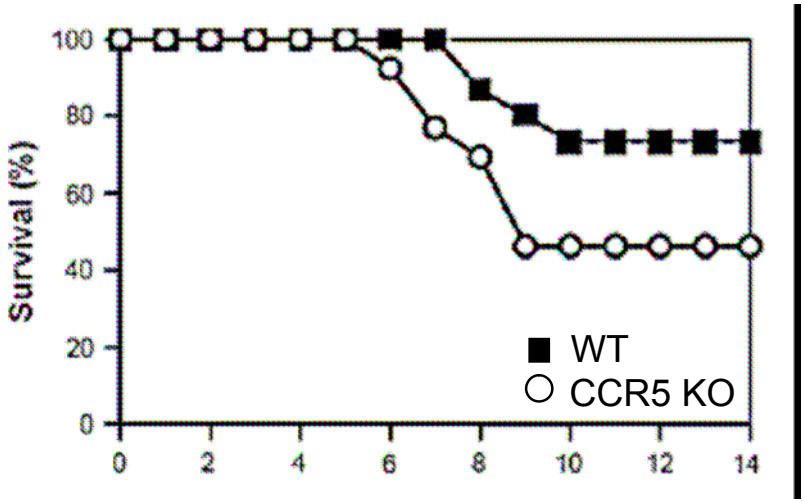

Figure 14: Survival of CCR5 -/- mice infected with *Y. pestis*

Seven to eight week-old female CCR5−/− C57Bl/6 ($n = 13$, open circles) and CCR5+/+ C57Bl/6 ($n = 15$, closed squares) mice were inoculated intranasally with *Y. pestis* KIM5 (8×10^5 CFU/mouse) and survival was monitored for 14 days. No significant differences were observed either in overall survival ($P = 0.246$, Fisher' exact test) or in time of survival ($P = 0.108$, Mann–Whitney rank sum test) between the two groups.

In order to more closely analyze the effects of infection on the health of the mice, morbidity was determined quantitatively by tracking weight change which is commonly used as an objective measure of illness. CCR5−/− and CCR5+/+ mice were weighed daily for 14 days after infection and the average percentage of weight loss for all animals in each group was calculated (Figure 15). Infected mice began to lose weight soon after the

infection, on average losing 15–20% of their weight by 6 days post infection. By 14 days after infection, surviving CCR5−/− and CCR5+/+ mice had regained most of their initial weight. There was no significant difference in weight loss on any day from day 1 to day 6 ($P = 0.619$ on day 6, the primary weight endpoint day). After day 6, weight comparisons were not done due to survivor bias.

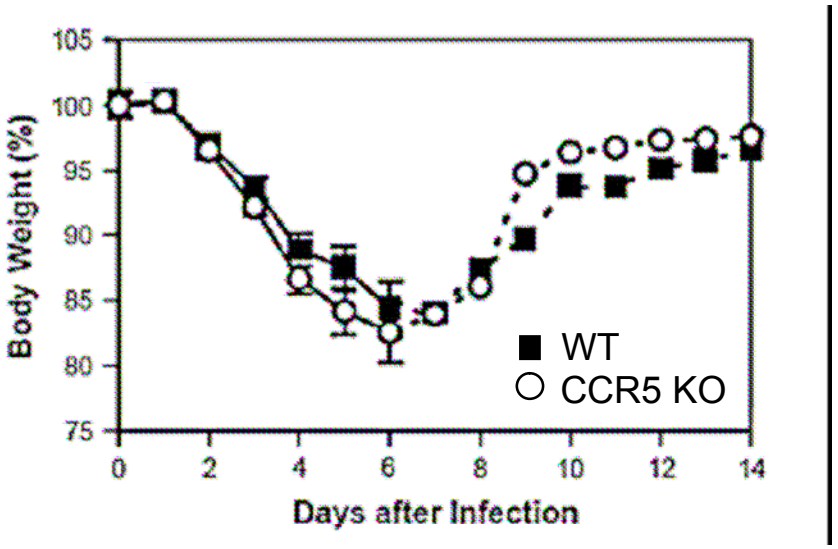

Figure 15: Morbidity of CCR5 -/- mice infected with *Y. pestis*

Seven to eight week-old female CCR5−/− C57Bl/6 ($n = 13$, open circles) and CCR5+/+ C57Bl/6 ($n = 15$, closed squares) mice were inoculated intranasally with *Y. pestis* KIM5 (8×10^5 CFU/mouse). Morbidity, in terms of average relative body weight, was monitored for 14 days. Weight difference at day 6, prior to onset of survivor bias, was not significant ($P = 0.619$, *t*-test).

3.2.2 Altered chemokine and cytokine responses to *Y. pestis* infection

Chemokines and cytokines play a key role in the control of bacterial infections by activating immune cells and organizing their migration. Thus, we measured the levels of RANTES and MIP-1α, which are known to signal through CCR5, as well as the levels of

additional chemokines and cytokines in serum of wild-type and CCR5−/− animals at various times during infection with *Y. pestis*. Chemokine and cytokine profiles in serum were studied as a readout of systemic inflammation because *Y. pestis* KIM5 is present in the lungs at relatively low numbers and the lung infection is often cleared and followed by a systemic spread of the bacteria into liver and spleen (Styer, Hopkins et al. 2005).

Analysis of serum by Luminex assay revealed higher serum concentrations of chemokines and cytokines in wild-type C57Bl/6 animals in response to infection when compared to the uninfected baseline levels in the same animals. Overall, the levels of chemokines and cytokines begin increasing 48 h postinfection and they significantly increased 72 h postinfection. The analysis of serum chemokines and cytokines also indicates that infected CCR5−/− mice exhibit higher levels of circulating RANTES (Figure 16A) and MIP-1α (Figure 16B) than those exhibited by wild-type mice at the baseline and throughout the course of *Y. pestis* infection. Since RANTES and MIP-1α are CCR5 ligands, their higher levels in CCR5−/− animals than in wild-type mice may be due to an attempt to compensate for the absence of CCR5 signaling. No significant differences between wild-type and CCR5−/− animals were observed in response to infection for chemokines IP10 or KC or various cytokines tested, including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, TNF-α, and IFN-γ.

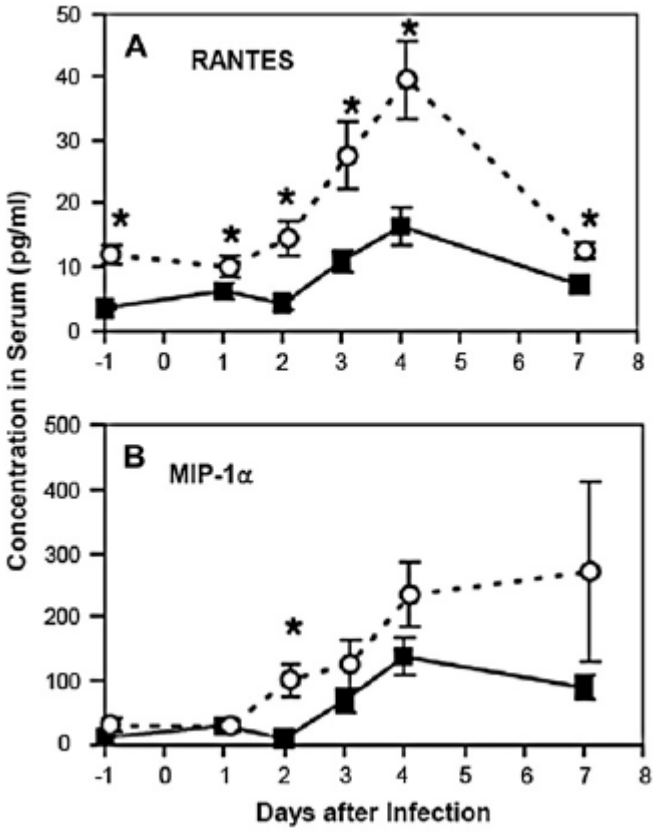

Figure 16: Altered cytokine responses in CCR5 -/- mice in response to *Y. pestis*

Serum was drawn from all mice 5 days prior to and 7 days after infection with *Y. pestis*. Additional serum was obtained from half the mice on alternate days during the first 4 days after infection. Chemokine and cytokine levels were analyzed by Luminex assay with Lindco reagents. Shown are the average chemokine concentrations in picograms per milliliter over time for RANTES and MIP-1α in the serum of infected CCR5−/− (open circles) and CCR5+/+ (closed squares) C57Bl/6 mice. Error bars, SEM. Statistically significant differences between the strains, indicated by asterisks, were observed in RANTES and MIP-1α ($P < 0.05$, Wilcoxon rank sum test). This experiment was performed by Eva Click in Richard Frothingham's laboratory at Duke University.

3.3 Discussion

In humans, CCR5 is a chemokine receptor located on the surface of immune cells, where it appears to act as a pathogen associated molecule pattern receptor. A *CCR5-Δ32* allele mediates resistance to HIV infection in about 10% of the European population; there appears to be no negative consequence to CCR5 deletion homozygosity in either humans or mice. Interestingly, a recent finding indicated that the frequency of the this allele in DNA samples from Bronze Age skeletons is comparable to that in modern German DNA samples (94), suggesting that strong selection for this allele occurred prior to the current HIV pandemic. It was proposed that the *CCR5-Δ32* allele could have been selected for in the population because it provided protection against another lethal epidemic: plague.

Previous work examining the role of CCR5 in immunity against plague had ambiguous results. CCR5 knock-out mice had similar similar survival when exposed to *Y. pestis* through either the intravenous or subcutaneous routes of infection (45, 46). However, peritoneal macrophages from CCR5-deficient mice had significantly reduced uptake of *Y. pestis* in vitro (45), an important step for *Y. pestis* dissemination in the host. As *Y. pestis* is extremely virulent in both the subcutaneous and intravenous models of plague: the LD_{50} inoculation for these two methods is 2 and 10^2 colony forming units (CFUs), respectively. In comparison, the LD_{50} for the intranasal mouse model of plague is 10^6 CFUs. We hypothesized that by tracking both morbidity and mortality in a less

virulent model of plague, we would be more likely to detect sensitive differences in survival between CCR5+/+ and CCR5−/− mice.

We determined that CCR5+/+ and CCR5−/− mice are equally susceptible to *Y. pestis* infection through the intranasal route of exposure in terms of morbidity and mortality. These experiments provide the first evidence indicating that the CCR5 deletion does not provide protection against *Y. pestis* using intranasal inoculation. The confidence interval around the survival rates observed in this study (46% for CCR5−/− mice versus 73% for wild-type, $P = 0.246$; difference = −27%, 95% confidence interval −62% to 8%) excludes any significant survival advantage. Although we cannot rule out the possibility that CCR5 has a beneficial effect in the host response to *Y. pestis*, the survival analysis rules out the possibility of a protective role for the lack of CCR5 in this model. These results support previous plague studies using different routes that have also found no benefit to the lack of CCR5. Taken together, these results suggest that plague was not a selective factor in the emergence of the non-functional *CCR5-Δ32* allele in humans.

We also tracked chemokine levels over time during infection of both CCR5+/+ and CCR5-/- mice throughout infection. Interestingly, the chemokines with elevated levels in the CCR5 deletion strain in response to infection, RANTES and MIP-1α, are chemoattractants that mediate Natural Killer (NK) cell migration. The migration of immune cells, including NK cells, is a key process to control bacterial infections that

requires finely tuned mechanisms of control. The higher levels of these chemokines observed during *Y. pestis* infection in CCR5 animals compared to those of wild-type animals open the possibility that the lack of CCR5-mediated responses may be compensated by the elevated levels of chemokines which can eventually activate additional CCR receptors to maintain an appropriate NK cell response to *Y. pestis* infection. Since no significant differences were observed in response to infection for chemokines IP10 and KC (GRO-α) or the various cytokines tested, including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, TNF-α, and IFN-γ, we do not believe that inflammation plays a detrimental role in CCR5−/− animals.

Previous studies have found that NK cell migration is inhibited by the *Y. pestis* virulence factor YopM and inbred mice strains that maintain a robust level of NK cells are more resistant to plague infection (Kerschen, Cohen et al. 2004; Congleton, Wulff et al. 2006). Additionally, CCR5−/− mice were previously shown to have deficiencies in NK cell recruitment when infected with *Toxoplasma gondii* (Khan, Thomas et al. 2006). Our chemokine data supports a role for CCR5-mediated recruitment NK cells in immunity to *Y. pestis* infection, though further experiments will be required to address the role of NK cells in immunity to *Y. pestis* and the mechanisms that elicit their activation and migration. RANTES and MIP-1α mediate NK cell migration through both CCR5 and the chemokine receptor CCR2, suggesting that CCR2 function might be compensating for the lack of CCR5 in plague defense.

CCR5 and CCR2 are both members of the chemosensory G-protein coupled receptor (GPCR) class. Genes encoding GPCRs represent the largest class of genes in the *C. elegans* genome; GPCRs also represent the largest transmembrane receptor super-family in humans, mice, and fruit flies (Pierce, Premont et al. 2002). Genes in this class are known to play a role in a wide variety of physiological activities, including odorant sensation, neurotransmission and immunity (Pierce, Premont et al. 2002; Rozengurt 2007). A previous study demonstrated that expression of human CCR5 in *C. elegans* sensory neurons allowed the nematode to sense the CCR5 ligand MIP-1α (Teng, Dekkers et al. 2006), suggesting that *C. elegans* uses at least part of the same downstream machinery as mammals to transduce GPCR signals.

4. *C. elegans* chemosensory GPCRs are involved in immunity to pathogens

4.1 Introduction

4.1.2 *C. elegans* GPCRs

C. elegans contains over 1,300 putative chemosensory GPCR genes but only a limited number have a known role in chemosensation and related behaviors (Bergamasco and Bazzicalupo 2006). As a soil-dwelling organism, the nematode is constantly exposed to different microbial species, some potentially pathogenic. We hypothesized that *C. elegans* uses GPCRs to sense pathogens and regulate the innate immune response. Interestingly, GPCR genes for which the foci of expression in the nematode are known are predominantly expressed in the nervous system. We decided to investigate the role of *C. elegans* GPCR genes expressed in the nervous system in immune defense against pathogen and explore the interactions between the nervous system and the innate immune system in the nematode.

4.1.1 Regulation of immunity by the nervous system in mammals

The nervous system is uniquely designed to partner effectively with the immune system at the site of infection. Just as it controls several vital functions, the nervous system in humans participates in a feedback loop that tightly regulates innate immune responses. Humoral mediators of the immune system slowly diffuse to create a

concentration gradient for an effective immune response at the site of infection while the nervous system is constantly present in distinct locations and can respond within seconds to varied stimuli, stimulating the immune response. The nervous system receives inputs from infected local sites, integrates them to coordinate appropriate responses, and creates memory that should increase chances for surviving a second attack. (Tracey 2002; Andersson 2005; Sternberg 2006)

The major connection between the nervous system and the immune system in mammals is the sympathetic nervous system. Both primary and secondary lymphoid organs are innervated by the sympathetic nervous system. These nerve fibers contain an abundant amount of one of the most studied links between the immune system and the nervous system: Neuropeptide Y (NPY)(Romano, Felten et al. 1991). The receptors for NPY are GPCRs expressed on several types of immune cells, including macrophages, peripheral blood mononuclear cells, T- and B- cells and dendritic cells. Through these receptors, NPY is known to regulate varied immune activities including phagocytosis and the production of reactive oxygen species, chemotaxis and proliferation of lymphocytes, and the production of antibodies and cytokines (Bedoui, von Horsten et al. 2007).

4.1.2 Control of biological processes by the nervous system in *C. elegans*

The powerful genetic approaches available to *C. elegans* research have been used to address central questions concerning the functions of the nervous system (Sattelle and

Buckingham 2006). With its 302 neurons and 56 glial cells, which represent 37% of all somatic cells in a hermaphrodite, the nervous system is perhaps the most complex organ of *C. elegans*. Ablation of different neurons has demonstrated that sensory neurons regulate a variety of physiological processes, including dauer formation and adult lifespan (Bargmann and Horvitz 1991; Schackwitz, Inoue et al. 1996; Alcedo and Kenyon 2004; Bishop and Guarente 2007). In addition, *C. elegans* neurons are known to express numerous secreted peptides of the TGF beta family, the insulin family, and neuropeptide families (Ren, Lim et al. 1996; Schackwitz, Inoue et al. 1996; Li, Nelson et al. 1999; Nathoo, Moeller et al. 2001; Pierce, Costa et al. 2001; Li, Kennedy et al. 2003). This myriad of secreted factors has the potential to act at a distance to modulate various physiological processes by regulating the function of neuronal and non-neuronal cells throughout the animal. Since the insulin-like pathway not only controls lifespan but also immunity it is possible that some of the neuroendocrine pathways described above also regulate innate immunity. The compromise between simplicity and genetic tractability makes the nematode a prime model system in which to study neural regulation of the innate immune system.

4.1.3 Avoidance behavior of *C. elegans* in response to pathogen

Like other free-living nematodes, *C. elegans* lives in the soil where it is in contact with soil-borne microbes, including human microbial pathogens (Chang, Berg et al. 1960; Walters and Holcomb 1967; Wasilewska and Webster 1975; Caldwell, Anderson et

al. 2003; Kenney, Anderson et al. 2006), and has evolved mechanisms to recognize different pathogens and to respond by activating the expression of genes related to innate immune responses conserved across metazoans (Mallo, Kurz et al. 2002; Kerry, Tekippe et al. 2006; O'Rourke, Baban et al. 2006; Shapira, Hamlin et al. 2006; Troemel, Chu et al. 2006; Wong, Bazopoulou et al. 2007). *C. elegans* also has the ability to avoid pathogenic bacteria such as *Bacillus thuringiensis* (Schulenburg and Muller 2004; Hasshoff, Bohnisch et al. 2007), *M.nematophilum* (Yook and Hodgkin 2007), *Photorhabdus luminescens* (Sicard, Hering et al. 2007), *P. aeruginosa* (Zhang, Lu et al. 2005; Beale, Li et al. 2006; Laws, Atkins et al. 2006) and *S. marcescens* (Pujol, Link et al. 2001; Zhang, Lu et al. 2005; Pradel, Zhang et al. 2007) through a process that requires specific genes and neurons. Additionally, it was demonstrated that *C. elegans* can learn aversive behavior to *P. aeruginosa* using olfactory sensing (Zhang, Lu et al. 2005). It is currently unknown how the nematode can sense pathogenic bacteria, though mutants in the G_i-like protein ODR-3 and the G-protein receptor kinase GRK-2 were incapable of *S. marcescens* lawn avoidance (Pradel, Zhang et al. 2007). These results suggest that G-protein coupled receptors may participate in nervous circuits that receive inputs from either pathogens or infected sites and integrate them to coordinate appropriate defense responses.

4.1.4 Investigating *C. elegans* as a model for neuroimmunology

We hypothesized that *C. elegans* uses GPCRs to sense bacterial pathogens and activate avoidance behaviors and the innate immune response; therefore, mutants in

receptors involved in sensing bacterial pathogens would have altered survival on pathogen. Forty *C. elegans* strains carrying mutations in chemosensory GPCRs were available through the Caenorhabditis Genome Center and screened for altered survival on the human opportunistic pathogen *P. aeruginosa*. *P. aeruginosa* was the original human pathogen used when *C. elegans* was first developed as a model for innate immunity and host-pathogen interactions; the strain used in these studies is *P. aeruginosa* PA14, a clinical isolate that has been described previously (Mahajan-Miklos, Tan et al. 1999; Tan, Mahajan-Miklos et al. 1999). It was used to screen the mutants for an immune phenotype due to its well-characterized pathogenesis in the model and swift lethality compared to other human pathogens that are also pathogens of *C. elegans* (Sifri, Begun et al. 2005). Eight GPCR genes were identified for their role in immunity to *P. aeruginosa*.

4.2 Results

4.2.1 GPCRs are involved in *C. elegans* innate immunity

To study the role of GPCRs in the regulation of the innate immune response, we first studied the susceptibility of forty *C. elegans* strains carrying mutations in chemosensory GPCRs to the human opportunistic pathogen *P. aeruginosa* (Table 3).

Table 3: GPCRs are involved in innate immunity to *P. aeruginosa*

Strain	Gene	Description	Closest human homolog[1]	Expression Pattern	TD50 Mean±SEM, N	P-value	PA14 phenotype[2]
N2	---	---	---	---	57.20 ± 5.748 N=5	---	WT
RB799	*C25G6.5*	Putative GPCR	Prolactin-releasing peptide receptor	AIA, AIY, PVQ	68.00 ± 5.115 N=4	0.2147	WT
RB1284	*C30F12.6*	Putative GPCR	Thyrotropin-releasing hormone receptor	pharynx, intestine	68.33 ± 5.925 N=3	0.2528	WT
RB1289	*C43C3.2*	Putative GPCR	Melanin-concentrating hormone receptor 1	unknown	68.00 ± 7.000 N=2	0.3427	WT
RB1288	*C48C5.1*	Putative GPCR	Neuromedin U receptor 2	unknown	81.50 ± 9.500 N=2	0.0753	ERP
RB1423	*C49A9.7*	Putative GPCR	Substance P receptor	unknown	70.00 ± 7.572 N=3	0.2244	WT
RB1321	***C56G3.1***	**Putative GPCR**	**Isoform B of Somatostatin receptor**	**unknown**	**78.33 ± 1.764 N=3**	**0.0347**	**ERP**
RB1162	*cfz-2*	Frizzled family of membrane receptors	Frizzled-8 precursor	pharyngeal neurons	51.25 ± 4.820 N=4	0.4689	ESP
RB665	*dop-1*	D1-like dopamine receptor	D(1B) dopamine receptor	head support cells, RIS, AVM, ALM, ALN, PLN, PVQ, PLM, PHC, ALM, AUA, RIB, RIM	68.75 ± 3.945 N=4	0.1616	WT
LX702	*dop-2*	D2-like dopamine receptor	Isoform 3 of D(2) dopamine receptor	RIA, SIA, SIB, RID, PDA	55.00 ± 3.606 N=3	0.7951	WT
BZ873	*dop-3*	D2-like dopamine receptor	Isoform 2 of D(2) dopamine receptor	neurons of the head, ventral cord and tail, GABAergic neurons	39.50 ± 0.500 N=2	0.1252	ESP

[1] Best BLASTP matches to longest protein product (www.wormbase.org)

[2] ERP: enhanced resistance to *P. aeruginosa*; ESP: enhanced susceptibility to *P. aeruginosa*; Strains were considered to be significantly ERP or ESP when TD_{50} was significantly different from wild-type using Student's exact t-test (bold). Additional strains are designated ERP or ESP due to significant differences ($p<0.0001$) in survival compared to wild-type in two independent experiments using PRISM to apply a logrank test.

Table 3: GPCRs are involved in innate immunity to *P. aeruginosa,* as continued.

Strain	Gene	Description	Closest human homolog[a]	Expression Pattern	TD50 Mean±SEM, N	P-value	PA14 phenotype[b]
RB761	*F35G8.1*	Putative GPCR	Isoform 2 of Neuropeptide FF receptor 2	unknown	60.00 ± 6.245 N=3	0.7642	WT
RB509	*gnrr-1*	GoNadotropin-Releasing hormone Receptor	Isoform 1 of Gonadotropin-releasing hormone receptor	unknown	74.33 ± 6.173 N=3	0.1023	WT
RB1349	***F57H12.4***	**Putative GPCR**	**Isoform 1A of Growth hormone secretagogue receptor type 1**	**unknown**	**89.33 ± 3.283 N=3**	**0.0071**	**ERP**
RB896	*gar-1*	G-protein-linked acetylcholine receptor	Muscarinic acetylcholine receptor M1	ciliated head neurons, PVM	72.00 ± 4.000 N=3	0.1212	WT
RB756	*gar-2*	G-protein-linked acetylcholine receptor	Muscarinic acetylcholine receptor M2	sensory, ventral cord neurons, HSN	64.00 ± 5.033 N=3	0.4542	WT
JD217	*gar-3*	G-protein-linked acetylcholine receptor	Muscarinic acetylcholine receptor M1	pharyngeal muscle, I3, extrapharyngeal neurons	75.50 ± 7.500 N=2	0.1389	WT
VC158	*lat-2*	Latrophilin receptor	Uncharacterized protein LPHN2	g1 gland cells, arcade cells	56.50 ± 3.279 N=4	0.9245	WT
DA609	***npr-1***	**G-protein coupled neuropeptide receptor**	**Isoform 2 of Neuropeptide FF receptor 2**	**AQR, ASE, ASG, ASH, URX, IL2L/R OLQ, AUA, SAAD, RMG, SMBD, M3, VD, DD PQR, PHA, PHB, RIV, RIG, SDQ**	**37.80 ± 4.067 N=5**	**0.0246**	**ESP**
XA3702	*npr-2*	G-protein coupled neuropeptide receptor	Isoform 2 of Neuropeptide FF receptor 2	unknown	62.67 ± 2.186 N=3	0.5111	WT
CX3410	*odr-10*	Odorant receptor	Olfactory receptor 5B17	AWA	62.00 ± 2.517 N=3	0.5648	WT
RB1141	*R13H7.2*	Putative GPCR	Neuromedin U receptor 2	Intestine, head neurons	64.67 ± 2.028 N=3	0.3757	WT

Table 3: GPCRs are involved in innate immunity to *P. aeruginosa,* as continued.

Strain	Gene	Description	Closest human homolog[a]	Expression Pattern	TD50 Mean±SEM, N	P-value	PA14 phenotype[b]
DA1814	*ser-1*	Serotonin/octopamine receptor	5-hydroxytryptamine 2A receptor	RMH, RMF, RMD, pharyngeal muscles	63.00 ± 8.505 N=3	0.578	WT
RB1622	*ser-3*	Serotonin/octopamine receptor	Isoform 2 of Alpha-1A adrenergic receptor	Head, tail neurons	64.25 ± 4.956 N=4	0.3983	WT
AQ866	*ser-4*	Serotonin/octopamine receptor	5-hydroxytryptamine receptor 1B	PVT, RIB, DVA, RIS, DVC	59.00 ± 2.517 N=3	0.8269	WT
DA2100	*ser-7*	Serotonin/octopamine receptor	Isoform D of 5-hydroxytryptamine receptor 7	Pharyngeal neurons MC, M4, I2, I3, M5, M3, I4, I6 and M2	61.00 ± 2.000 N=3	0.6435	WT
CB5414	*srd-1*	Serpentine Receptor, class D	Melanin-concentrating hormone receptor 2	ASI	58.00 ± 2.082 N=3	0.9218	WT
VC459	*srd-2*	Serpentine Receptor, class D	G protein-coupled receptor MRGX1	unknown	62.67 ± 6.839 N=3	0.572	WT
RB1526	*srd-44*	Serpentine Receptor, class D	DRG kappa 1 splice variant KOR 1A	unknown	59.00 ± 6.494 N=4	0.8413	WT
RB1419	*srw-140*	Serpentine Receptor, class W	Isoform 1A of Growth hormone secretagogue receptor type 1	unknown	58.67 ± 8.192 N=3	0.885	WT
RB1306	***str-182***	**7-transmembrane olfactory receptor**	**none**	**unknown**	**83.33 ± 7.860 N=3**	**0.0342**	**ERP**
VC342	*str-31*	7-transmembrane olfactory receptor	Frizzled-8 precursor	unknown	64.25 ± 6.945 N=4	0.4556	WT
RB785	*T02E9.3*	Putative GPCR	Isoform 2 of D(2) dopamine receptor	Head, tail neurons	57.00 ± 2.646 N=3	0.9806	WT
VC125	*tag-126.*	Tyramine receptor	beta-1-adrenergic receptor	Head, tail neurons, vulva	68.67 ± 0.333 N=3	0.1854	WT
VC224	*tag-24*	Biogenic amine receptor	Alpha-2A adrenergic receptor	Head, tail neurons	76.67 ± 6.839 N=3	0.0774	ERP

Table 3: GPCRs are involved in innate immunity to *P. aeruginosa,* as continued.

Strain	Gene	Description	Closest human homolog[a]	Expression Pattern	TD50 Mean±SEM, N	P-value	PA14 phenotype[b]
VC270	*tag-49*	Putative GPCR	Neuromedin-K receptor	Intestine, renal gland cells, nervous system	67.67 ± 5.840 N=3	0.2778	WT
VC273	*tag-89*	Putative GPCR	Thyrotropin-releasing hormone receptor	unknown	70.00 ± 3.937 N=4	0.1264	WT
RB1365	*uvt-6*	Vitellogenin-linked GPCR	Somatostatin receptor type 3	Head,tail neurons, ventral nerve cord, anal depressor cell, VM1	53.33 ± 7.311 N=3	0.6933	WT
RB1393	*Y58G8A.4*	Putative GPCR	Prolactin-releasing peptide receptor	unknown	59.00 ± 4.619 N=3	0.8369	WT
RB1405	*Y59H11AL.1*	Putative GPCR	Substance-K receptor	unknown	62.33 ± 1.202 N=3	0.5305	WT

Out of the forty mutants studied, three mutants exhibited significantly enhanced resistance to *P. aeruginosa* and one mutant exhibited significantly enhanced susceptibility to *P. aeruginosa* when comparing time to death of 50% of animals (TD_{50}) of the mutant strain to the TD_{50} of wild-type nematodes for data points corresponding to at least three independent experiments using Students exact *t*-test, significance denoted by asterisks (Figure 17).

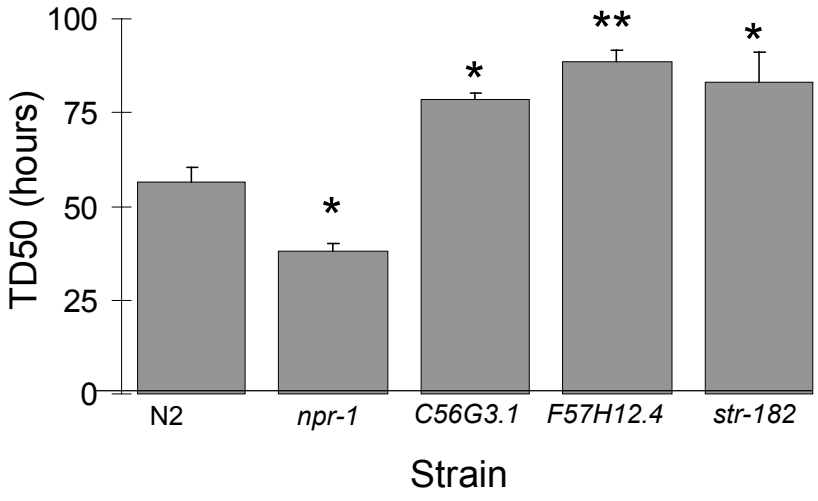

Figure 17: TD_{50}s of *C. elegans* GPCR mutants on *P. aeruginosa*

C. elegans strains carrying mutations in chemosensory GPCRs were screened for altered survival on *P. aeruginosa. C56G3.1(ok1439)*(P=0.0347), *F57H12.4 (ok1504)* (P=0.0071), and *str-182(ok1419)* (P=0.0342) had enhanced resistance to *P. aeruginosa* and *npr-1(ad609)* (P=0.0246) had enhanced susceptibility to *P. aeruginosa.* Shown is the time required for 50% of the nematodes to die (TD_{50}) as mean +/- SEM corresponding to at least three independent experiments, each of which used at least 40 adult nematodes per strain.

Additional strains were designated ERP or ESP due to significant differences ($p<0.0001$) in survival compared to wild-type in two independent experiments using

PRISM to apply a logrank test. Figure 18 shows the survival over time of GPCR mutants with enhanced resistance to *P. aeruginosa*.

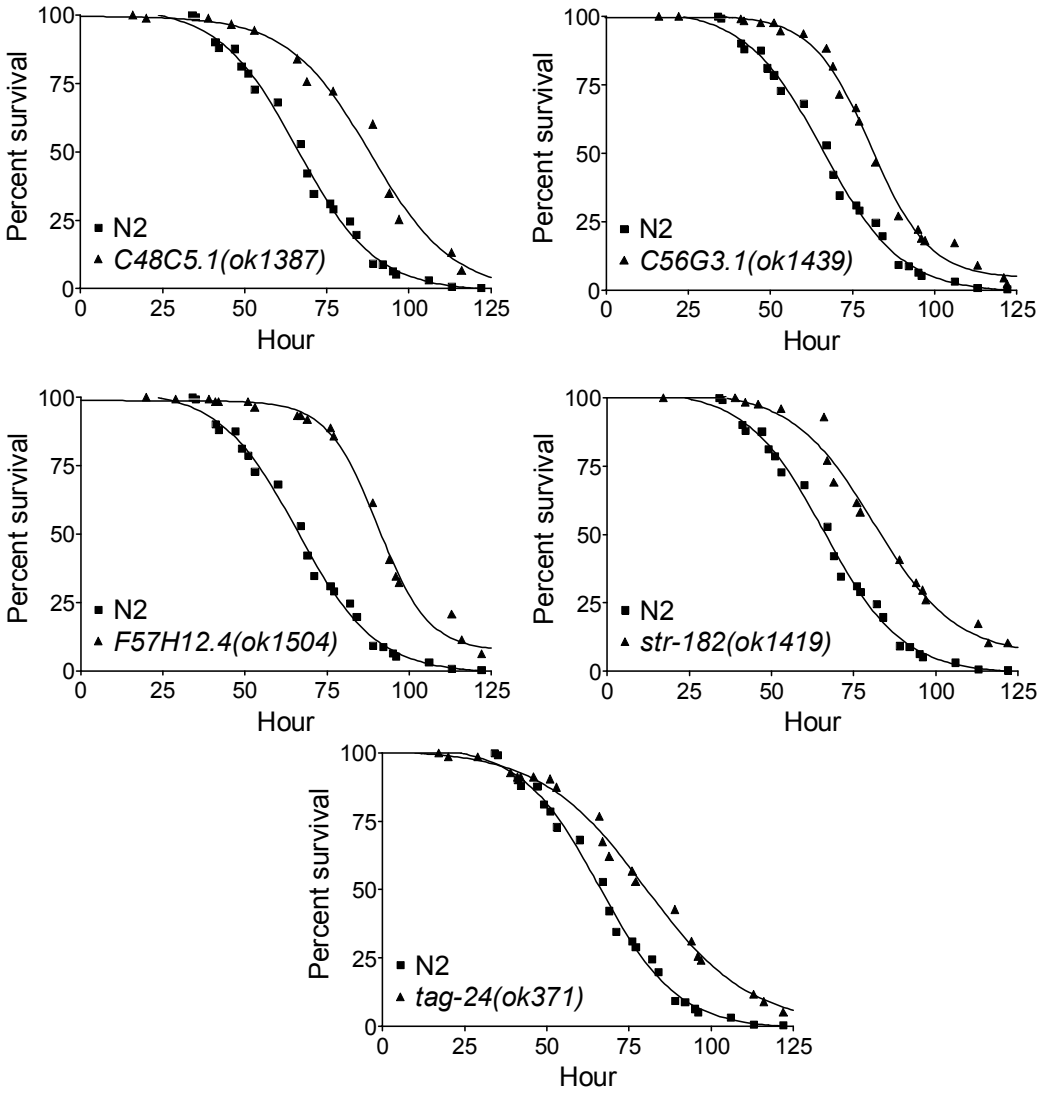

Figure 18: GPCR mutants with enhanced resistance to *P. aeruginosa*

Wild-type N2, *C48C5.1(ok137)* (P=0.0001), *C56G3.1(ok1439)* (P=0.0001), *F57H12.4(ok1504)* (P=0.0001), *str-182(ok1419)* (P=0.0001) and *tag-24(ok371)* (P=0.0001) nematodes were exposed to *P. aeruginosa* and scored for survival over time. The graphs represent the combined results of at least two independent experiments, N≥40 adult nematodes per strain.

Three strains have enhanced susceptibility to *P. aeruginosa* infection; two of these are shown here (Figure 19). The third strain contains a mutation in *npr-1*, which encodes a GPCR related to mammalian neuropeptide Y receptors; we selected this gene for further study in the next chapter.

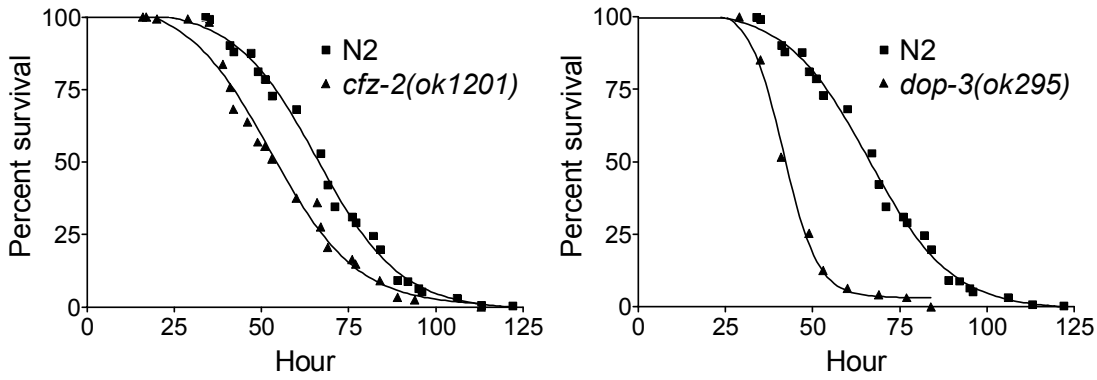

Figure 19: GPCR mutants with enhanced susceptibility to *P. aeruginosa*

Wild-type N2, *cfz-2(ok1201)* (P=0.0001), and *dop-3(ok295)* (P=0.0001) nematodes were exposed to *P. aeruginosa* and scored for survival over time. The graphs represent the combined results of at least two independent experiments, N≥40 adult nematodes per strain

4.3 Discussion

C. elegans genes encoding putative chemosensory GPCRs are the largest gene class in the genome, but only a limited number have a known role in chemosensation and behavior (Bergamasco and Bazzicalupo 2006). We hypothesized that the nematode uses GPCRs to sense bacterial pathogens and activate the innate immune response against pathogens. To study the role of GPCRs in the regulation of innate immune

responses, we determined the susceptibility phenotype of *C. elegans* strains carrying mutations in chemosensory GPCRs exposed to *P. aeruginosa.*

Out of the forty mutants studied, three mutants exhibited significantly enhanced resistance to *P. aeruginosa* and one mutant exhibited significantly enhanced susceptibility to *P. aeruginosa* when comparing time to death of 50% of animals (TD_{50}) of the mutant strain to the TD_{50} of wild-type nematodes. An additional four strains were identified as having a significant survival phenotype when survival curves were assessed using PRISM to apply a logrank test. The function of most of these genes is currently unknown, though seven out of the eight strains we identified for their role in innate immunity have a homologous human receptor with at least 40% similarity. This suggests that our work in *C. elegans* could provide useful information about neural receptors involved in the innate immune response in humans.

Genes encoding GPCRs were found to be predominantly expressed in the nematode nervous system (Table 3). In order to further analyze the role of the nervous system in innate immune defenses, we selected the *npr-1* gene for additional study. NPR-1 is involved in a known neural circuit that integrates behavioral responses to environmental oxygen, food and other animals; we have discovered here that the *npr-1* gene is required for immune defense against *P. aeruginosa*.

5. Innate immunity in *C. elegans* is regulated by neurons expressing NPR-1

5.1 Introduction

5.1.1 NPR-1

npr-1 is expressed in twenty different neurons, including the sensory neurons AQR, ASE, ASG, ASH, URX, PQR, PHA and PHB, and is involved in a number of *C. elegans* behavioral phenotypes (Coates and de Bono 2002). In nature, *npr-1* is found in *C. elegans* wild isolates as one of two alleles that differ in a single amino acid at position 215. The Bristol N2 strain denoted wild-type in the *C. elegans* field contains valine at position 215 (*npr-1* 215V) and is termed "solitary"; these animals are evenly distributed across a bacterial lawn (Figure 20A). Strains that contain phenylalanine at position 215 (*npr-1* 215F), such as the German strain RC301, are termed "social"; these animals aggregate into groups, also known as clumping, and show a preference for the thickest part of a bacterial lawn, also known as bordering (Figure 20B).

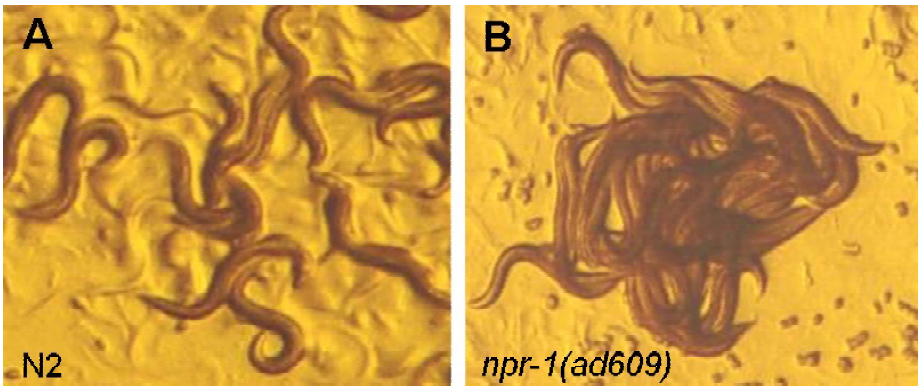

Figure 20: Characteristic aggregation phenotype of *npr-1(lf)* mutants

(A) *C. elegans* wild-type N2 animals and (B) *npr-1(ad609)* mutants were propagated at 20°C as hermaphrodites on modified NG agar plates seeded with *E. coli* strain OP50 and then visualized using a Leica MZ FLIII stereomicroscope. The characteristic clump of *npr-1(ad609)* nematodes shown here is at the edge of the bacterial lawn.

5.1.2 The NPR-1 nervous circuit controls aggregation behavior

Genetic studies have dissected a chemosensory circuit that requires *npr-1* activity to coordinate oxygen preference and aggregation (Coates and de Bono 2002; de Bono, Tobin et al. 2002; Gray, Karow et al. 2004; Cheung, Cohen et al. 2005; Chang, Chronis et al. 2006; Rogers, Persson et al. 2006)(Figure 21). Aggregation and bordering of *npr-1(ad609)* nematodes depend on *gcy-35* and *tax-2* or *tax-4* genes (de Bono and Bargmann 1998; Cheung, Arellano-Carbajal et al. 2004; Cheung, Cohen et al. 2005). GCY-35 is a soluble guanylyl cyclase (sGC) which is involved in *C. elegans* response to oxygen in the environment through a cGMP pathway involving TAX-2 and TAX-4, two subunits of a

cGMP-gated-ion-channel (de Bono and Bargmann 1998; Cheung, Arellano-Carbajal et al. 2004; Cheung, Cohen et al. 2005).

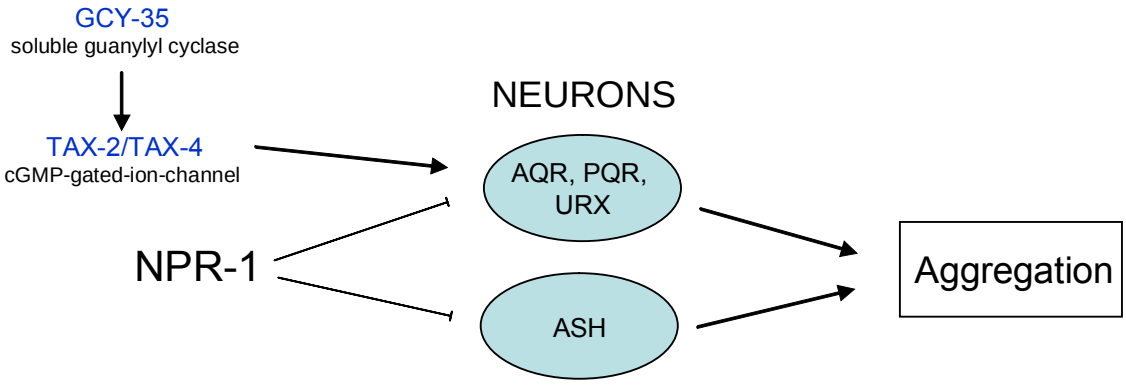

Figure 21: The NPR-1 nervous circuit controls aggregation behavior

Activation of the TAX-2/TAX-4 cGMP-gated ion channel via the GCY-35 guanalyl cyclase in the AQR, PQR and URX body cavity neurons activates social feeding behaviour. This activation can be inhibited by NPR-1 activity in ASH, AQR, PQR, URX and other neurons.

5.1.3 NPR-1 is involved in neural control of innate immunity

Here we examine the role of the NPR-1 nervous circuit in immune defense. We found that activation of the NPR-1 nervous circuit enhances host susceptibility to microbial infection while inhibition of the circuit boosts innate immunity. To provide additional insight into the function of the NPR-1 nervous circuit, we also performed microarray analyses on animals with altered neural functions due to mutation in *npr-1*. The microarray analysis of genes downregulated in *npr-1* mutant nematodes shows an enrichment in genes that are upregulated by *P. aeruginosa* infection in wild-type animals, expressed in the intestine, and/or have already been linked to *C. elegans* innate immune pathways. Interestingly, we found that genes misregulated in *npr-1* mutants are enriched in genes that are similarly misregulated in immunocompromised animals due to deficient PMK-1/P38 MAPK signaling. From this work, we are able to provide insight into the neural mechanisms that regulate innate immunity by taking advantage of the simple and well studied nervous and innate immune systems of *C. elegans.*

5.2 Results

5.2.1 *npr-1* mutants are susceptible to infection

The *npr-1* gene encodes a GPCR related to mammalian neuropeptide Y receptors (de Bono and Bargmann 1998). Through our screen of *C. elegans* GPCRs, we found that the mutant strain *npr-1(ad609)* was highly susceptible to *P. aeruginosa* infection (Figure 22).

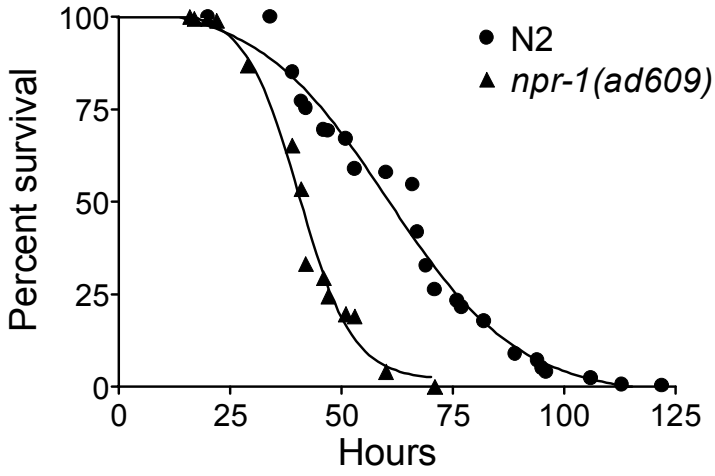

Figure 22: *npr-1* mutant nematodes are hypersusceptible to *P. aeruginosa*

Wild-type N2 and *npr-1(ad609)* (P=0.0001) nematodes were exposed to *P. aeruginosa* and scored for survival over time. The graph represents combined results of four independent experiments, N≥40 adult nematodes per strain.

In order to determine whether the enhanced susceptibility to *P. aeruginosa* exhibited by *npr-1(ad609)* animals was due to a reduction in lifespan or a deficient response to potentially pathogenic bacteria, *npr-1(ad609)* nematodes were fed heat-killed *P. aeruginosa* on plates supplemented with ampicillin. No difference in survival was seen

between *npr-1(ad609)* and wild-type nematodes under these conditions, suggesting that NPR-1 is required for proper immune response to live, pathogenic bacteria and that does not affect the lifespan of the animals (Figure 23).

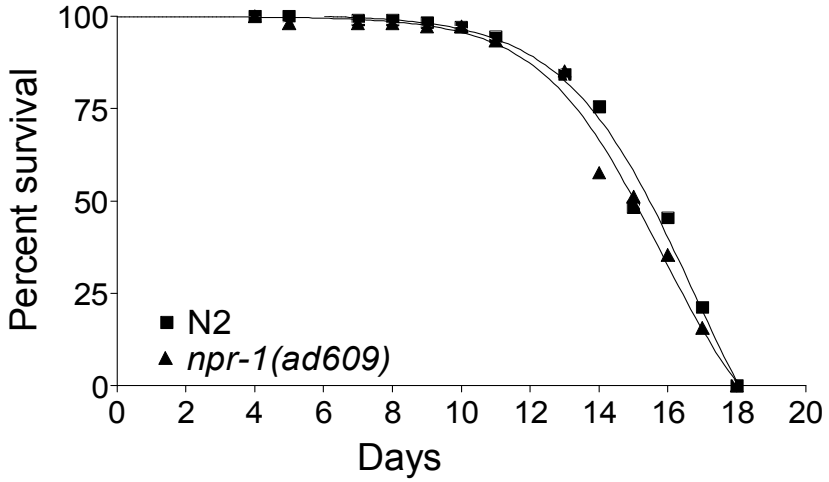

Figure 23: Survival of *C. elegans* on heat-killed *P. aeruginosa*

Wild-type N2 and *npr-1(ad609)* (P=0.1411) nematodes were exposed to heat-killed *P. aeruginosa* and scored for survival over time. The graph represents the combined results of two independent experiments, N=100 adult nematodes per strain.

We confirmed that NPR-1 is required for *C. elegans* defense to *P. aeruginosa* by exposing five additional *npr-1* mutants to the pathogen and comparing their survival to that of wild-type animals (Figure 24). The enhanced susceptibility to *P. aeruginosa* of *npr-1(ky13)*, *npr-1(n1353*, *npr-1(ur89)*, and *npr-1(g320)* confirms that NPR-1 is required for the defense response to this pathogen. Furthermore, these results make it unlikely that the enhanced susceptibility of *npr-1* animals is caused by secondary mutations or the effect of a particular allele on a process unrelated to NPR-1 function. Interestingly, while the

German wild isolate RC301, which contains the *npr-1(g320) allele* (de Bono and Bargmann 1998), is not significantly susceptible to *P. aeruginosa* compared to wild type, the *npr-1(g320) allele* confers susceptibility to *P. aeruginosa* in an N2 background (Figure 24). These results suggest that the German isolate may have evolved a mechanism to compensate for the increased susceptibility to pathogens due to reduced NPR-1 activity.

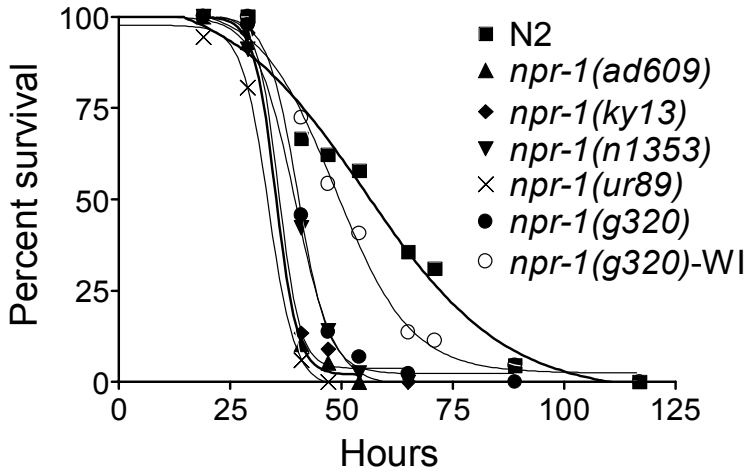

Figure 24: The *npr-1* susceptibility phenotype is not allele specific

Wild-type N2 , *npr-1(ad609)* (P=0.0001), *npr-1(ky13)* (P=0.0001), *npr-1(n1353)* (P=0.0001), *npr-1(ur89)* (P=0.0001), *npr-1(g320)* (P=0.0001), and the wild isolate (WI) *npr-1(g320)* (P=0.0922) were exposed to *P. aeruginosa* and scored for survival over time. Shown is a representative assay of at least 3 independent experiments, N=48 adult nematodes per strain.

To determine whether the immune deficiency due to mutation in the *npr-1* gene is specific for *P. aeruginosa* infection, we exposed *npr-1(ad609)* nematodes to *S. enterica* and *E. faecalis*, two human pathogens known to kill *C. elegans* (Aballay, Yorgey et al. 2000; Labrousse, Chauvet et al. 2000; Garsin, Sifri et al. 2001). As shown in Figure 25,

npr-1(ad609) nematodes exhibited enhanced susceptibility to these pathogens, suggesting that NPR-1 is required for immune responses to pathogens in general.

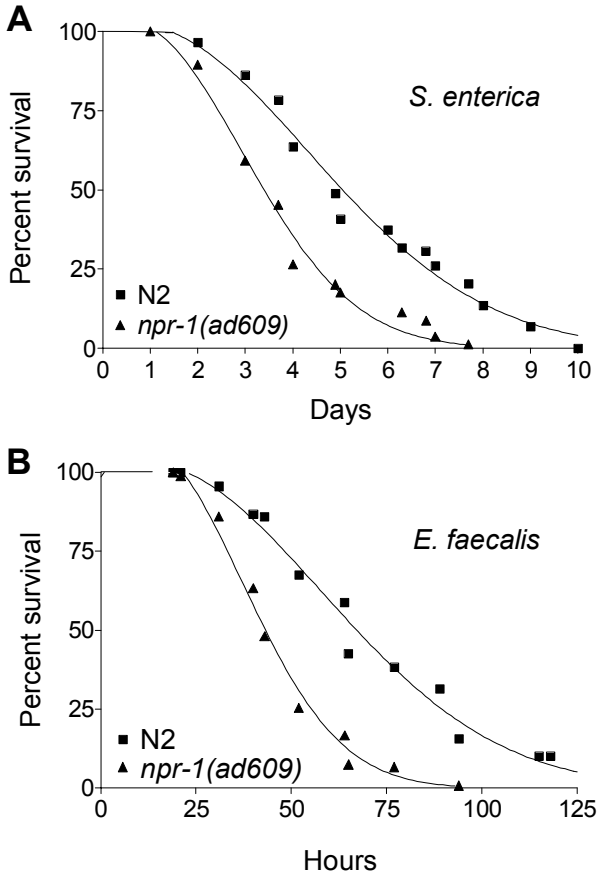

Figure 25: *npr-1* mutant nematodes are hypersusceptible to other pathogens

(A) Wild-type N2 and *npr-1(ad609)* (P=0.0001) nematodes were exposed to *S. enterica* and scored for survival over time. (B) Wild-type N2 and *npr-1(ad609)* (P=0.0001) nematodes were exposed to *E. faecalis* and scored for survival over time. The graph represents combined results of two independent experiments, N≥40 adult nematodes per strain.

5.2.2 Role of the *npr-1* behavioral phenotype in the immune deficiency

NPR-1 is involved in a neural circuit that integrates behavioral responses to environmental oxygen, food, and other animals. In nature, NPR-1 is found in two allelic forms that differ in a single amino acid at position 215, NPR-1(215V) and NPR-1(215F) (de Bono and Bargmann 1998). The NPR1(215V) allele, which is found the in standard laboratory strain, has high activity whereas the NPR-1(215F) allele has low activity (Coates and de Bono 2002; Rogers, Reale et al. 2003). Wild-type or *npr-1(215V)* animals exhibit strong aerotaxis responses in the absence of *E. coli* which is the food source for *C. elegans* in the laboratory. Moreover, while wild-type or *npr-1(215V)* animals fail to avoid high oxygen in the presence of *E. coli*, *npr-1(215F)* and *npr-1(lf)* animals have strong hyperoxia avoidance in the absence or presence of *E. coli* (Gray, Karow et al. 2004). As nematode aggregation into feeding groups decreases local oxygen, *npr-1(215F)* and *npr-1(lf)* form clumps of nematodes when the animals are grown at densities high enough to allow this behavioral response. In addition, *npr-1(215F)* and *npr-1(lf)* show a preference for the thickest part of a bacterial lawn, the region in which oxygen levels are the lowest (Coates and de Bono 2002).

One potential explanation for the reduced life-span of nematodes grown on bacterial pathogens seen in *npr-1(lf)* nematodes is that the clumping affects nematode susceptibility to pathogen infection in a non-specific fashion. However, the animal

density in the assays where the susceptibility to pathogens is tested is not sufficient to elicit clumping, making this possibility unlikely (Figure 26B).

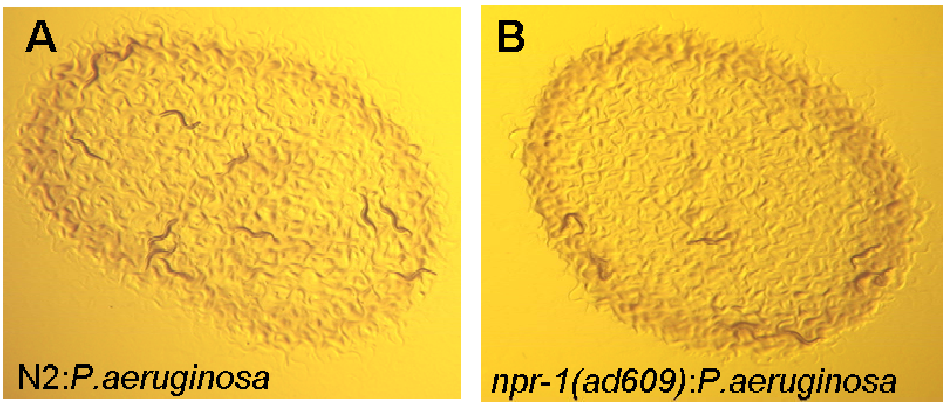

Figure 26: Nematodes exposed to *P. aeruginosa* under standard killing conditions

(A) Twelve wild-type N2 and (B) twelve *npr-1(ad609)* nematodes were exposed to *P. aeruginosa* for 24 hours under standard killing assay conditions and visualized using a Leica MZ FLIII stereomicroscope. Under these conditions, *npr-1(ad609)* nematodes do not form characteristic clumps of the strain.

Even though *npr-1(ad609)* nematodes do not exhibit a clumping behavior under the conditions used to study *P. aeruginosa*-mediated killing, they still exhibit a preference for the thickest part of the lawn compared to wild-type nematodes (Figure 26). Since *P. aeruginosa* elicits avoidance behavior in wild-type animals, but not in *npr-1(ad609)* animals whose hyperoxia avoidance is stronger than *P. aeruginosa* avoidance, we studied the role of avoidance in *C. elegans* susceptibility to *P. aeruginosa*. As shown in Figure 27, wild-type animals grown on plates completely covered by *P. aeruginosa* died at a higher rate than animals grown on plates containing a small lawn of *P. aeruginosa* in the center of the plate.

Conversely, *npr-1(ad609)* animals, whose preference for the border of bacterial lawns overwrites *P. aeruginosa* avoidance, are equally susceptible when grown on full or center lawns. *npr-1(ad609)* animals also exhibit an enhanced susceptibility to *P. aeruginosa* compared to wild type when the infections are performed in plates containing full lawns (Figure 27A). These results indicate that avoidance is part of *C. elegans* defense response and that the increased susceptibility to *P. aeruginosa* of *npr-1(ad609)* animals is due in part to lack of avoidance to this specific pathogen.

To confirm that the enhanced susceptibility of *npr-1(ad609)* nematodes is in part due to lack of *P. aeruginosa* avoidance, animals were grown at 21% oxygen were compared to those grown at 8% oxygen at 23°C. Under 8% oxygen, *npr-1(ad609)* animals do not exhibit a preference for the bacterial border and avoid the *P. aeruginosa* lawn. As shown in Figure 28, *npr-1(ad609)* animals are more resistant to *P. aeruginosa*-mediated killing at 8% oxygen than at 21% oxygen. This result, together with the demonstration that *npr-1(ad609)* animals are still more susceptible to *P. aeruginosa*-mediated killing than wild-type animals grown at 8% oxygen (Figure 27B), indicate that animals lacking NPR-1 are more susceptible to *P. aeruginosa* due to lack of both avoidance and additional immune responses. The increased susceptibility of *npr-1(ad609)* to *S. enterica*, a pathogen that does not elicit an avoidance behavior (Tenor and Aballay 2008), is consistent with a role of NPR-1 in the regulation of general immune responses that are independent of pathogen avoidance.

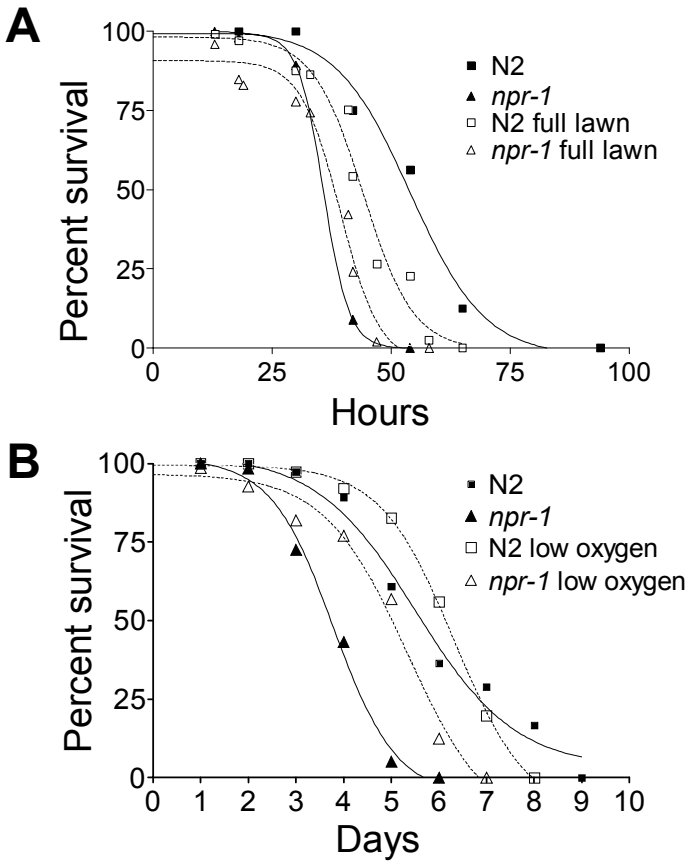

Figure 27: Avoidance behavior is required for *C. elegans* immunity to *P. aeruginosa*

(A) Wild-type N2 and *npr-1(ad609)* nematodes were exposed to either a full lawn or a center lawn of *P. aeruginosa* on a 3.5 cm in diameter plate and scored for survival over time. Under both conditions *npr-1(ad609)* animals were more susceptible to *P. aeruginosa*-mediated killing (P=0.0001). Wild-type animals on full lawns were more susceptible to *P. aeruginosa*-mediated killing than animals on center lawns (P=0.0001); *npr-1(ad609)* animals were similarly susceptible (P=0.07). The graph represents combined results of three independent experiments, N≥40 adult nematodes per strain. (B) Wild-type N2 and *npr-1(ad609)* nematodes were exposed to *P. aeruginosa* at either 21% or 8% oxygen and scored for survival over time. Under both conditions *npr-1(ad609)* animals were more susceptible to *P. aeruginosa*-mediated killing than wild-type (P=0.0001). *npr-1(ad609)* animals at 21% oxygen were more susceptible to *P. aeruginosa*-mediated killing than animals at 8% oxygen (P=0.0001); wild-type animals were similarly susceptible (P=0.95). The graph represents combined results of two independent experiments, N=40 adult nematodes per strain.

5.2.3 Aggregation suppressors rescue the *npr-1* immune deficiency

Genetic studies have dissected a chemosensory circuit that requires *npr-1* activity to coordinate oxygen preference and aggregation (Coates and de Bono 2002; de Bono, Tobin et al. 2002; Gray, Karow et al. 2004; Cheung, Cohen et al. 2005; Chang, Chronis et al. 2006; Rogers, Persson et al. 2006). Aggregation and bordering of *npr-1(ad609)* nematodes depend on *gcy-35* and *tax-2* or *tax-4* genes (de Bono and Bargmann 1998; Cheung, Arellano-Carbajal et al. 2004; Cheung, Cohen et al. 2005). GCY-35 is a soluble guanylyl cyclase (sGC) which is involved in *C. elegans* response to oxygen in the environment through a cGMP pathway involving TAX-2 and TAX-4, two subunits of a cGMP-gated-ion-channel (de Bono and Bargmann 1998; Cheung, Arellano-Carbajal et al. 2004; Cheung, Cohen et al. 2005). A drop in oxygen levels causes activation of this pathway and depolarizes the AQR, PQR, and URX neurons where these genes are expressed in and also reduces aggregation and bordering of *npr-1(ad609)* nematodes (Cheung, Cohen et al. 2005). To determine whether the genes involved in the NPR-1 neural circuit regulate innate immune response, we studied the pathogen susceptibility of *npr-1(ad609)* animals carrying loss-of-function mutations *gcy-35, tax-2,* and *tax-4.* As shown in Figure 28, the enhanced susceptibility to *P. aeruginosa* of *npr-1(ad609)* animals was rescued by mutations in *gcy-35, tax-2,* or *tax-4*.

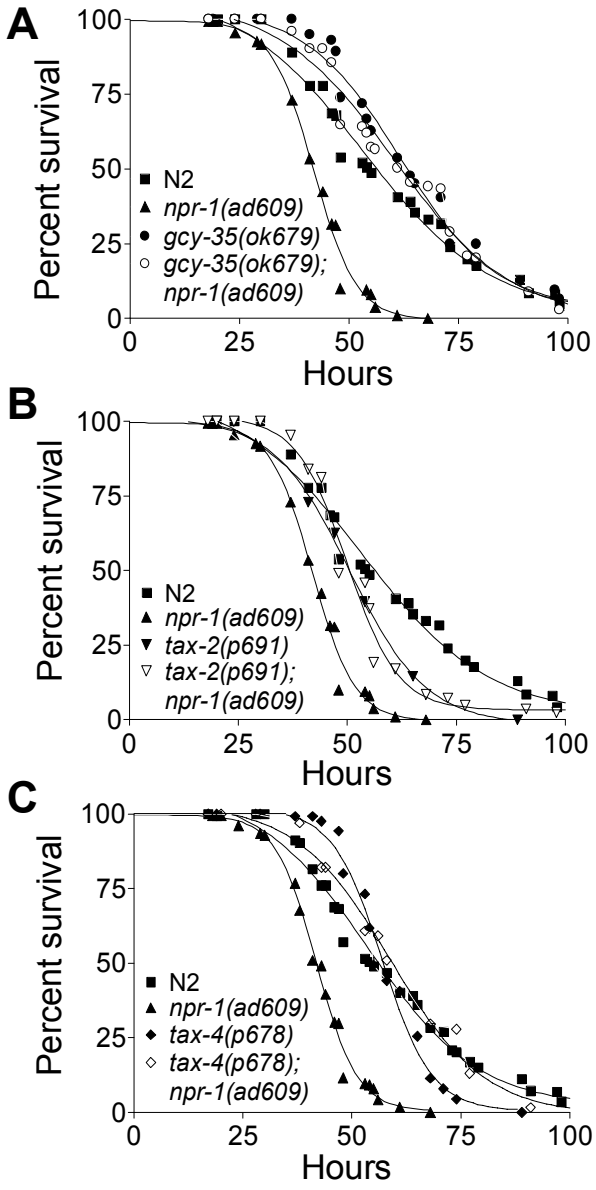

Figure 28: Mutations in *gcy-35*, *tax-2* or *tax-4* rescues the *npr-1* immune defect

(A) Wild-type N2, *npr-1(ad609)* (P=0.0001), *gcy-35(ok769)* (P=0.0125), and *gcy-35(ok769);npr-1(ad609)* (P=0.0639) were exposed to *P. aeruginosa*. (B) Wild-type N2, *npr-1(ad609)* (P=0.0001), *tax-2(p691)* (P=0.0930), *tax-2(p691);npr-1(ad609)* (P=0.0031) were exposed to *P. aeruginosa*. (C) Wild-type N2, *npr-1(ad609)* (P=0.0001), *tax-4(p678)* (P=0.1673), *tax-4(p678);npr-1(ad609)* (P=0.3611), were exposed to *P. aeruginosa*. The graphs represent combined results of at least three independent experiments, N≥40 adult nematodes per strain.

NPR-1 is expressed in at least twenty different neurons, including the sensory neurons AQR, PQR, URX, and ASH, where it suppresses depolarization (Coates and de Bono 2002). To determine whether the enhanced susceptibility of *npr-1(ad609)* nematodes was due to the hyperactivity of neurons within the known NPR-1 neural circuit, we studied the susceptibility to *P. aeruginosa*-mediated killing of *npr-1(ad609)* animals expressing wild-type *npr-1* in URX, AQR, PQR, and ASH neurons. As shown in Figure 29, expression of *npr-1* in these neurons partially rescues the enhanced susceptibility to *P. aeruginosa* of *npr-1(ad609)* animals, indicating that URX, AQR, PQR, ASH, and additional neurons may be part of a neural circuit that suppresses innate immunity.

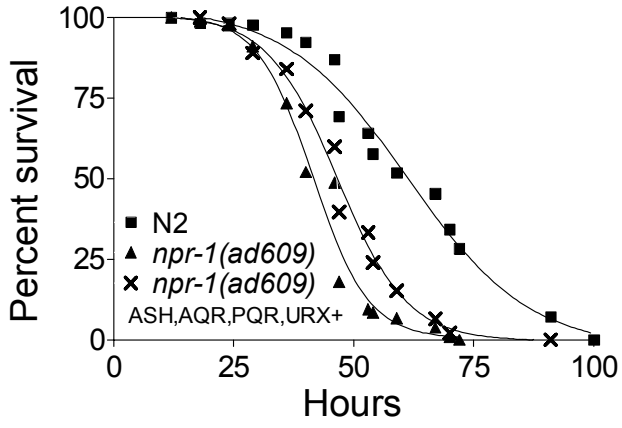

Figure 29: Reconstitution of NPR-1 in specific neurons partially rescues immunity

Wild-type N2, *npr-1(ad609)* (P=0.0001), and the *npr-1* rescue strain *npr-1(ad609) kyEx1823*[*gpa-3::npr-1*, *gcy-32::npr-1*] (P=0.0001), were exposed to *P. aeruginosa*. The rescue strain was also significantly different from *npr-1(ad609)* (P=0.0009). The graph represents combined results of at least three independent experiments, N≥40 adult nematodes per strain.

To confirm that AQR, PQR, and URX neurons may be part of a neural network that inhibits innate immunity, we studied the susceptibility to *P. aeruginosa* of a strain in which these neurons were genetically ablated by expressing the cell-death activator gene *egl-1* under the control of the *gcy-36* promoter, which is specific for AQR, PQR, and URX expression (Chang, Chronis et al. 2006). The strain lacking AQR, PQR and URX neurons exhibited a significantly increased survival on *P. aeruginosa* (Figure 30), indicating that AQR, PQR and URX neurons downmodulate innate immunity.

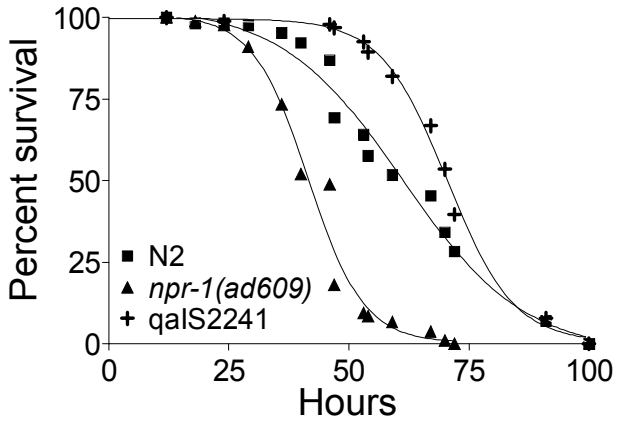

Figure 30: Genetic ablation of AQR, PQR and URX neurons enhances immunity

Wild-type N2, *npr-1(ad609)* (P=0.0001), and *qaIS2241,* a strain which lacks AQR, PQR, and URX neurons, (P=0.0007) were exposed to *P. aeruginosa.* The graph represents combined results of at least three independent experiments, N≥40 adult nematodes per strain.

Taken together, these results indicate that genes and cells involved in the NPR-1 neural circuit modulate innate immune responses. Activation of the NPR-1 nervous circuit enhances host susceptibility to microbial infection while inhibition of the circuit boosts innate immunity.

5.2.4 Candidate immunity genes have altered expression in *npr-1* mutants

As in mammals, peristalsis, low pH, and antimicrobial substances prevent microbial colonization of the *C. elegans* intestine. In addition, accumulating evidence indicates that different genetic pathways regulate the expression of *C. elegans* genes that are markers of immune response (Mallo, Kurz et al. 2002; Kerry, Tekippe et al. 2006; O'Rourke, Baban et al. 2006; Shapira, Hamlin et al. 2006; Troemel, Chu et al. 2006; Wong, Bazopoulou et al. 2007). To provide insight into the immune function of the NPR-1 nervous circuit, we utilized gene expression microarrays to find clusters of genes upregulated or downregulated in *npr-1(ad609)* animals mutants relative to wild-type animals grown on live *P. aeruginosa.* We identified 22 genes upregulated by NPR-1(Table 4) and 16 genes downregulated by NPR-1(Table 5). Interestingly, a large proportion of the genes upregulated by NPR-1 had at least one of three features: they are upregulated by *P. aeruginosa* infection in wild-type animals, expressed in the intestine, and/or have already been linked to known *C. elegans* innate immune pathways (Table 4). Further analysis revealed that five of the genes most highly downregulated by NPR-1 are found in a cluster on chromosome V that appears to have been duplicated further downstream on that chromosome (Table 5). Of these five genes, three are also known to be downregulated by the *C. elegans* p38 MAP kinase, PMK-1, which also plays a crucial role in innate immunity (Kim, Feinbaum et al. 2002; Aballay, Drenkard et al. 2003; Huffman, Abrami et al. 2004; Kim, Liberati et al. 2004; Troemel, Chu et al. 2006).

Table 4: Genes upregulated by NPR-1

Gene	Description	Intestine enriched[1]	↑ on PA14[2]	Known immune pathway[3]	Microarray mean ± SEM[4]	qRT-PCR mean ± SEM[d]	*p-value*
dct-17	germline tumor affecting		Y	daf-16	0.176±0.023	0.5411±0.0505	0.0003
F15D4.5	similarity to synaptonemal complex protein				0.303±0.029	ND	
dod-21	lifespan abnormal(RNAi)			daf-16	0.3385±0.0325	0.4256±0.0775	0.0007
F36F12.8	zinc finger protein				0.3575±0.0115	0.6271±0.1184	0.0346
F46F2.3	none	Y		dbl-1	0.364±0.054	ND	
F13H8.3	Predicted inosine-uridine nucleoside hydrolase				0.3845±0.0595	ND	
gst-24	glutathione S-transferase	Y	Y		0.3945±0.0285	ND	
stdh-2	steroid dehydrogenase, lifespan abnormal(RNAi)		Y		0.3995±0.0805	0.4456±0.0713	0.0015
T10D4.6	none				0.4065±0.0505	ND	
Y69A2AR.25	similarity to human neurogenic locus notch				0.4135±0.0345	ND	
gst-20	glutathione S-transferase				0.4165±0.0405	ND	
T24B8.5	similarity to roundworm mucin MUC-5		Y	daf-16, pmk-1	0.426±0.028	ND	
T28F2.2	slow growth, decreased brood size(RNAi)	Y			0.4275±0.0045	ND	
F36G9.12	predicted transcription factor activity		Y		0.436±0.027	ND	
col-101	cuticle collagen				0.467±0.013	ND	
C14C6.5	none	Y	Y	pmk-1	0.468±0.008	ND	
clec-85	c-type lectin	Y	Y	dbl-1, pmk-1	0.6025±0.0995	0.5661±0.0549	0.0042
dod-24	CUB like region, lifespan abnormal(RNAi)		Y	daf-16	0.7555±0.0185	0.5546±0.0201	0.0001
lec-11	galectin family, binds sugar in vitro	Y	Y		0.7655±0.0875	0.6171±0.0401	0.0024
abf-1	antibacterial factor	Y	Y		0.8015±0.0225	0.682±0.0157	0.0001
lys-8	putative lysozyme, lifespan abnormal(RNAi)	Y	Y	daf-16,dbl-1, pmk-1	0.832±0.003	0.7331±0.0648	0.0092
lys-2	putative lysozyme	Y	Y	pmk-1	0.851±0.044	0.5561±0.0257	0.0004

[1] Genes enriched for expression in the intestine

[2] Genes upregulated in response to *P. aeruginosa* infection

[3] Genes previously linked to known innate immune pathways

[4] Expression level relative to wild-type

Table 5: Genes downregulated by NPR-1

Gene	Description	Known immune pathway[1]	Microarray mean ± SEM[2]	qRT-PCR mean ± SEM[b]	qRT-PCR *p-value*
Y19D10A.7 *F56A4.9*[3]	receptor with similiarity to human insulin-like growth factor 1 precursor	pmk-1	14.295±0.845	13.84±2.208	0.0021
Y19D10A.4 *C01B4.7*[c]	Permease of the major facilitator superfamily, similar to human sialin	pmk-1	4.305±0.1677	ND	
Y19D10A.16 *C01B4.6*[c]	similar to human Aldose 1-epimerase	pmk-1	4.272±0.4356	5.941±0.3604	0.0001
nspb-1-5	Nematode Specific Peptide family, group B		4.246±1.256	ND	
F43C11.3	none		3.6555±0.4285	ND	
nlp-25	neuropeptide-like protein		3.5235±0.6755	ND	
C42D4.3	Fibronectin		3.4845±0.1205	ND	
Y19D10A.5 *C01B4.8*[c]	Permease of the major facilitator superfamily, similar to human sialin		3.4±0.106	ND	
Y19D10A.11 *F56A4.12*[c]	Permease of the major facilitator superfamily, similar to human sialin		3.171±0.263	ND	
nspa-9	Nematode Specific Peptide family, group A		2.9805±0.5015	ND	
grl-21	Grl domain,intercellular signalling		2.9605±0.0925	ND	
col-97	cuticle collagen		2.9565±0.4775	ND	
col-39	cuticle collagen		2.9505±0.5555	ND	
ZK180.5	Similar to human Diacylglycerol kinase kappa		2.883±0.17	ND	
col-62	cuticle collagen		2.5785±0.2495	ND	
clec-72	C-type lectin		2.51±0.439	ND	

[1] Genes previously linked to known innate immune pathways

[2] Expression level relative to wild-type

[3] Five gene cluster duplicated on chromosome V

Overall, the microarray analysis indicate that genes misregulated in animals deficient in NPR-1 function are enriched in genes that are similarly misregulated in animals deficient in PMK-1 function, suggesting that at least part of the PMK-1 pathway may be suppressed by the NPR-1 nervous circuit . Inhibition of *pmk-1* gene expression by RNAi in *npr-1(ad609)* nematodes results in increased susceptibility (Figure 31), indicating that while the NPR-1 mediated immune pathway has overlapping targets with the PMK-1 mediated immune pathway, the relationship between the two is synergistic.

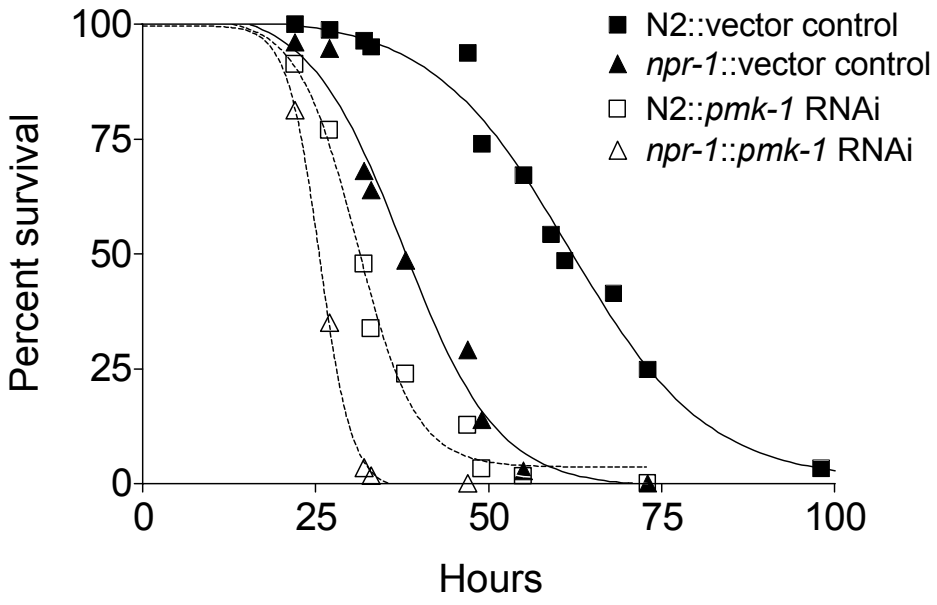

Figure 31: NPR-1 and PMK-1 act synergistically

Wild-type and *npr-1(ad609)* nematodes grown on *E. coli* carrying a vector control or on *E. coli* expressing *pmk-1* double-stranded RNA were exposed to *P. aeruginosa*. Significant differences were found when wild-type was compared to *npr-1(ad609)* ($P<0.0001$), when *pmk-1*(RNAi) was compared to *npr-1(ad609);pmk-1*(RNAi) ($P<0.0001$), and when *npr-1(ad609)* was compared to *npr-1(ad609);pmk-1*(RNAi) ($P<0.0001$). The graph represents combined results of two independent experiments, $N \geq 40$ adult nematodes per strain.

To obtain an insight into the mechanism by which *gcy-35* mutation rescues the enhanced susceptibility to *P. aeruginosa* of *npr-1(ad609)* animals (Figure 28), we used quantitative RT-PCR (qRT-PCR) to compare the expression levels of selected genes of *npr-1(ad609)* to that of *npr-1(ad609);gcy-35(ok769)* animals. As shown in Figure 32, *gcy-35* mutation in *npr-1(ad609)* animals rescues the altered expression of 5 out of 12 genes tested that are markers of *C. elegans* immune response. These results indicate that the NPR-1 nervous circuit modulates the expression of immune-related genes, many of which are known to be expressed in tissues in direct contact with pathogens during infection.

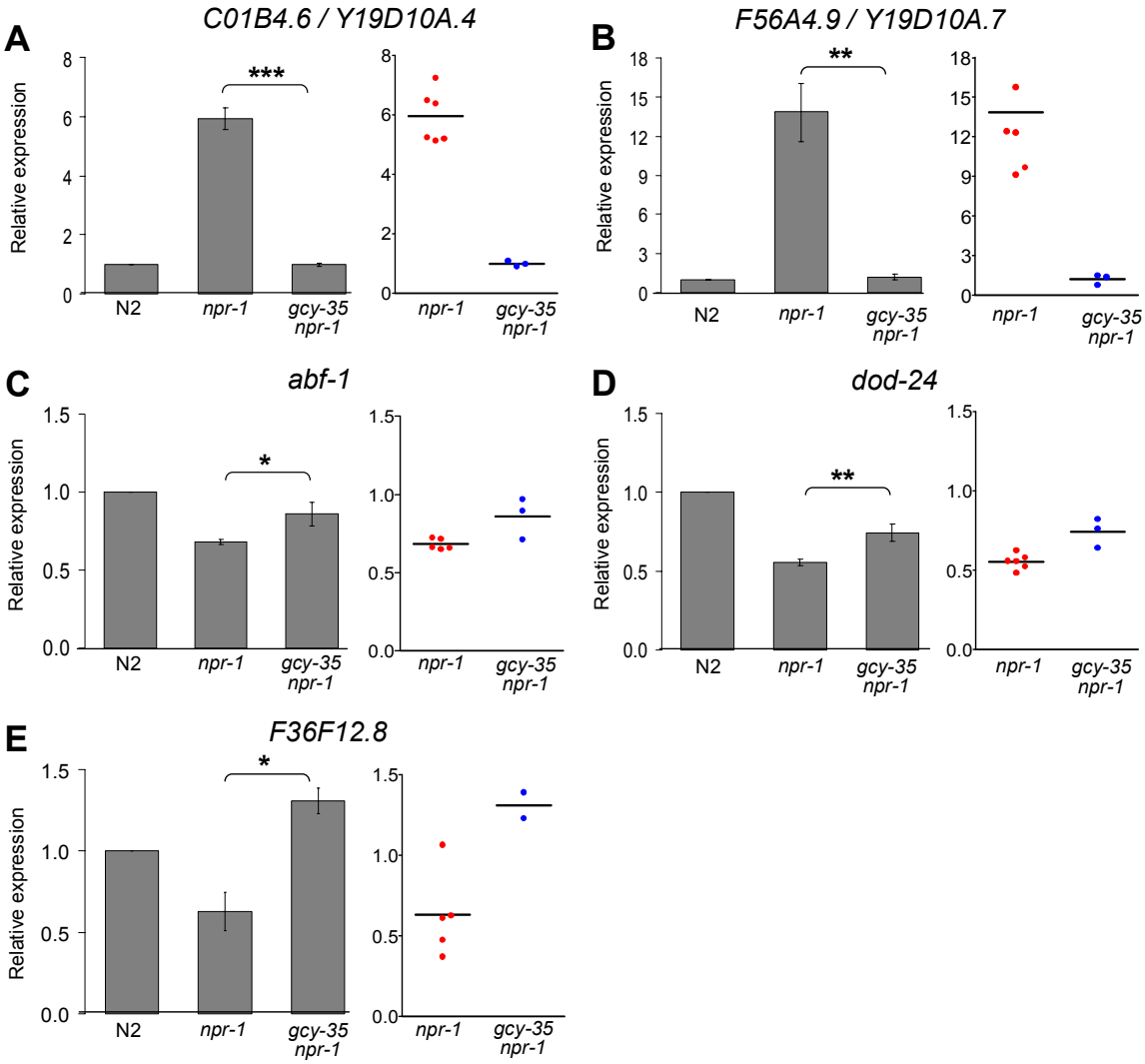

Figure 32: The NPR-1 neural circuit regulates expression of immunity genes

(A-F) Quantitative reverse transcription–PCR analysis of *C01B4.6/Y19D10A.4, F56A4.9/Y19D10A.7, abf-1, dod-24,* and *F36F12.8* expression in *npr-1(ad609)* and *gcy-35(ok769);npr-1(ad609)* nematodes relative to wild-type nematodes exposed to *P. aeruginosa*. Data were analyzed by normalization to pan-actin *(act-1,-3,-4)* and relative quantification using the comparative cycle threshold method. Student's exact *t*-test indicates differences among the groups are significantly different; bar graphs correspond to mean ± SEM. Point graphs correspond to gene quantification in independent isolations of *npr-1(ad609)*(N=6) and *gcy-35(ok769);npr-1(ad609)*(N=3).

5.6 Discussion

Uncontrolled activation of the innate immune system is know to have deleterious effects which can lead to infection and cancer, as well as conditions such as Crohn's disease, rheumatoid arthritis, atherosclerosis, diabetes, and Alzheimer's disease. A wealth of data (reviewed in (Tracey 2002; Andersson 2005; Sternberg 2006)) indicates that the nervous system receives inputs from infected local sites and integrates them to coordinate appropriate responses. However, since the regulation of innate immunity by the nervous system remains understudied, the precise mechanisms by which the two systems influence each other are still unclear. In this study, we demonstrate that the nervous system of *C. elegans* controls organismal defense responses using a nervous circuit that requires the function of a GPCR, a GMP-gated ion channel, and a soluble guanylate cyclase. The simplicity and genetic tractability of *C. elegans* offers a new system in which to study the neural mechanisms that integrate and coordinate appropriate immune responses.

The NPR-1 nervous circuit coordinates responses to oxygen in addition to innate immunity. Our results indicate that the strong preference of *npr-1(ad609)* nematodes for low oxygen conditions found at the border of the bacterial lawn overwrites the natural *C. elegans* avoidance behavior in response to *P. aeruginosa*. Previous work with *S. marcescens* identified the bacterial product serrawettin W2 as a factor that elicits lawn avoidance to that particular pathogen, yet *S. marcescens* mutants unable to produce this

factor were equally virulent in *C. elegans*, indicating that behavioral avoidance in *C. elegans* could be uncoupled from the innate immune response (Pradel, Zhang et al. 2007). Here we demonstrate that nematode avoidance behavior in response to *P. aeruginosa* is required for survival of wild-type nematodes and that defective avoidance behavior is partially responsible for the increased susceptibility of *npr-1(ad609)* nematodes. However, *npr-1(ad609)* nematodes are also susceptible to *P. aeruginosa* under conditions independent of the avoidance response, suggesting that NPR-1 integrates avoidance behavior with the innate immune response to defend against *P. aeruginosa* infection.

Previous studies dissected a chemosensory circuit that requires *npr-1* activity to coordinate oxygen preference and aggregation (Coates and de Bono 2002; de Bono, Tobin et al. 2002; Gray, Karow et al. 2004; Cheung, Cohen et al. 2005; Chang, Chronis et al. 2006; Rogers, Persson et al. 2006). Aggregation and bordering of *npr-1(ad609)* nematodes depends on *gcy-35* and *tax-2* or *tax-4* genes (de Bono and Bargmann 1998; Cheung, Arellano-Carbajal et al. 2004; Cheung, Cohen et al. 2005). We have found here that the same nervous circuit involved in oxygen preference and aggregation is involved in innate immunity; activation of the NPR-1 nervous circuit enhances host susceptibility to microbial infection while inhibition of the circuit boosts innate immunity (Figure 33).

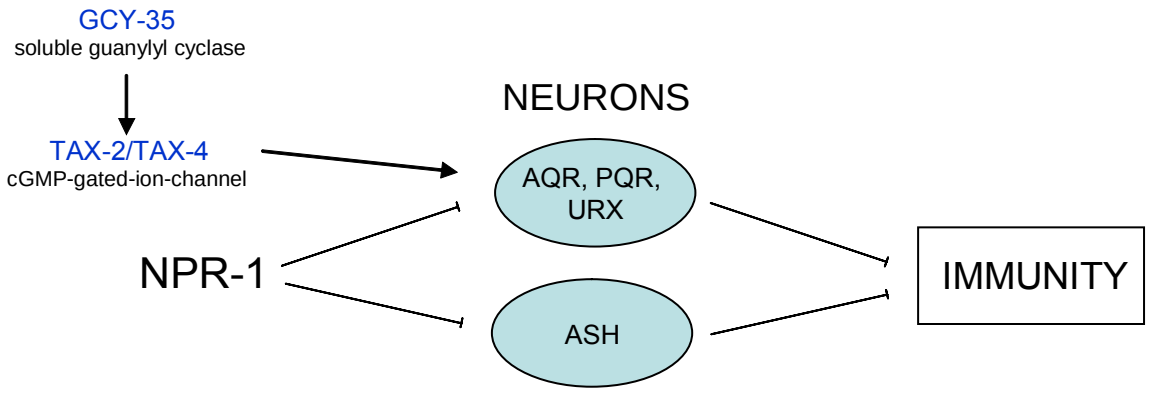

Figure 33: Model for regulation of immunity through the NPR-1 nervous circuit

Model of the neural control of innate immunity in *C. elegans*: NPR-1 suppresses depolarization of ASH, AQR, PQR and URX neurons that inhibit innate immunity, while GCY-35, TAX-2, and TAX-4 are required for the activation of AQR, PQR and URX neurons.

To provide insight into the mechanism by which the NPR-1 nervous circuit regulates innate immunity, we performed a genome-wide microarray analysis to identify genes misregulated in *npr-1* mutants. Upon infection by *P. aeruginosa*, *C. elegans* upregulates genes encoding putative anti-bacterial factors, lysozymes, saposins, and lectin genes (Shapira, Hamlin et al. 2006; Troemel, Chu et al. 2006; Alper, McBride et al. 2007), the majority of which are expressed in the intestine, the site of infection within the nematode. Our analysis of genes differentially expressed in *npr-1(lf)* relatively to wild-type animals exposed to *P. aeruginosa* indicate that about 20% of the genes missregulated in *npr-1(lf)* animals are similarly missregulated in *pmk-1(lf)* animals. The overlap between genes regulated by the NPR-1 nervous circuit and the PMK-1 innate immune pathway indicate that the nervous system regulates the PMK-1 pathway. To further

investigate the nature of the interaction between the NPR-1 nervous circuit and the PMK-1 pathway, we investigated the susceptibility to pathogens of animals deficient in both *npr-1* and *pmk-1* activity. The genetic analysis demonstrates that *npr-1* and *pmk-1* act synergistically, indicating that certain PMK-1-mediated immune responses are not regulated by the NPR-1 nervous circuit, perhaps through tissue-specific mechanisms.

These results demonstrate that the nervous system is involved in innate immunity to pathogens in *C. elegans*, thus providing a novel genetic model organism in which to study neuroimmunology.

6. Discussion

6.1 Discussion of results

Over the past 10 years, *C. elegans* has been developed as a model for host-pathogen interactions. Multiple human pathogens are also pathogens of *C. elegans* and conserved innate responses defend the nematode from infection. The goal of this work was to identify novel bacterial virulence factors and innate immune defenses against pathogens by taking advantage of the genetically tractable model organism *C. elegans*.

The initial studies describing *C. elegans* as a host for bacterial infection used the opportunistic pathogen *P. aeruginosa*, followed shortly thereafter by *S. enterica* (Aballay, Yorgey et al. 2000; Labrousse, Chauvet et al. 2000; Kurz, Chauvet et al. 2003). It is now known that a wide variety of pathogens, including Gram-negative and Gram-postive bacteria as well as fungal pathogens, are able to infect *C. elegans* with a lethality ranging from hours to days (Sifri, Begun et al. 2005). The simple growth conditions and rapid lifespan of *C. elegans* allows for easy, rapid screening of bacterial mutants using survival assays.

We sought to establish a *C. elegans* model of *Y. pestis* infection in order to rapidly identify novel bacterial virulence factors. Previous studies demonstrated that species of *Yersinia* infected *C. elegans* by forming a bioilm on the exterior of the nematode (Darby,

Hsu et al. 2002). We found that *Y. pestis* strain KIM5, which lacks the HMS genes required for biofilm formation, was able cause a persistent, internal infection, which we predicted to be more relevant to mammalian infection studies (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008).

We validated the utility of the model system by exposing the nematodes to *Y. pestis* strains containing mutations in known or predicted virulence genes. One gene particularly important for *Y. pestis* infection in *C. elegans* is *y1324*, which was identified due to its homology to *ail* genes in other species. Other members of this family include Ail in *Y. enterocolitica* and *Y. pseudotuberculosis*, Rck and PagC of *S. enterica*, and OmpX of *E. coli* (Miller, Bliska et al. 1990; Pulkkinen and Miller 1991; Heffernan, Wu et al. 1994; Mecsas, Welch et al. 1995; Yang, Merriam et al. 1996; Vogt and Schulz 1999). Several of members of this family have been found to protect bacteria from complement-mediated lysis. We find here that *Y. pestis* Ail protects the bacterium from complement-mediated lysis and is required for virulence in *C. elegans*. (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008)

To identify novel *Y. pestis* virulence factors, we generated a library of bacterial clones containing random, single transposon insertion mutations. Using *C. elegans* survival assays, we selected candidate mutants with attenuated virulence and tested these strains in a mammalian model of *Y. pestis* infection. This work demonstrates that the simple and rapid *C. elegans* survival assay can be used to identify novel *Y. pestis*

virulence factors that are also important for *Y. pestis* infection in mammals (Styer, Hopkins et al. 2005; Bartra, Styer et al. 2008).

After using *C. elegans* as a model to study bacterial virulence determinants, we decided to look at host factors involved in immunity against *Y. pestis* infection. We chose to investigate the role of one gene in particular with a controversial role in immunity to plague. CCR5 is a seven membrane-spanning chemokine receptor found primarily on the surface of immune cells, where it appears to act as a pathogen associated molecule pattern receptor (Floto, MacAry et al. 2006). Approximately 10% of Europeans are protected against HIV-1 infection because they carry a mutant allele of the human chemokine receptor CCR5 (*CCR5-Δ32*). Since the origin of this allele occurred in the Middle Ages, long before the AIDS pandemic, it was proposed that this allele was selected for because it conferred resistance to another highly lethal pandemic rampant at that time, the black plague. Previous tests for protection against *Y. pestis* lethality using intravenous and subcutaneous mouse models of infection demonstrated no significant increase in immunity, yet peritoneal macrophages isolated from CCR5-deficient mice showed significantly reduced uptake of *Y. pestis*, a necessary step for dissemination in the host (Elvin, Williamson et al. 2004; Mecsas, Franklin et al. 2004).

To assess the role of CCR5 in the intranasal mouse model of plague, we infected CCR5+/+ and CCR5−/− mice with *Y. pestis* and tracked morbidity and mortality, as well as serum cytokine levels. We found that while the lack of CCR5 does not enhance host

resistance to *Y. pestis*, CCR5-/- mice exhibited higher levels of circulating RANTES and MIP-1α, CCR5 ligands that mediate Natural Killer (NK) cell migration, than those exhibited by wild-type mice at the baseline and throughout the course of *Y. pestis* infection. These results imply that plague was not a selective factor in the emergence of the non-functional *CCR5-Δ32* allele in humans; yet our data does suggest a previously unknown role for NK cell migration in *Y. pestis* infection. (Styer, Click et al. 2007)

The CCR5 chemokine receptor is a member of the chemosensory G-protein coupled receptor (GPCR) class. Genes encoding GPCRs represent the largest class of transmembrane receptor super-family genes in humans, mice, and fruit flies as well as *C. elegans* (Pierce, Premont et al. 2002), yet only a limited number of the *C. elegans* genes have a known role in chemosensation (Bergamasco and Bazzicalupo 2006).

C. elegans has the ability to avoid pathogenic bacteria (Zhang, Lu et al. 2005; Beale, Li et al. 2006; Laws, Atkins et al. 2006) through a process that requires specific genes and neurons. Although the receptors used by the nematode to directly sense pathogenic bacteria are unknown, mutants in G-protein signal transduction cascade components were incapable of avoidance behavior in response to the pathogen *S. marcescens* (Pradel, Zhang et al. 2007), suggesting a role for GPCRs in pathogen identification. Interestingly, GPCR genes for which the foci of expression in the nematode are known are predominantly expressed in the nervous system. We decided to investigate the role of *C. elegans* GPCR genes in immune defense and explore the

interactions between the nervous system and the innate immune system in the nematode.

We screened forty *C. elegans* strains containing mutations in chemosensory GPCRs for altered survival on pathogen and identified one gene in particular, *npr-1,*to be required for full *C. elegans* defense against pathogens:. We found that the NPR-1 nervous circuit enhances host susceptibility to microbial infection; inhibition of this circuit by NPR-1 boosts innate immunity.

To identify genes with altered regulation in *npr-1* mutant nematodes during infection, we performed a full genome microarray analysis. Interestingly, genes misregulated in *npr-1* mutants are enriched with genes similarly misregulated in immunocompromised animals with deficient PMK-1/P38 MAPK signaling. Our work on the NPR-1 nervous circuit provides the first evidence that innate immunity in *C. elegans* is directly linked to the nervous system.

6.2 Future directions

This work began by establishing *C. elegans* as a host for a novel mechanism of *Y. pestis* infection and expanded into the development of *C. elegans* as a model for neuroimmunology research. The breadth of this research provides a wide variety of avenues to explore.

Using *C. elegans* as a host, we identified three *Y. pestis* genes previously unknown for their role in *Y. pestis* virulence. Future work is necessary to characterize

the particular role these genes have in pathogenesis. In particular, the reduced virulence of *Y. pestis 5B9* is interesting because the product of the *y0340* gene encodes a member of an uncharacterized family of putative exported proteins that are found exclusively in several pathogens in the family Enterobacteriaceae. *Y. pestis* KIM5 encodes seven individual paralogs of the *y0340* gene product in its chromosome. In fact, *y0340* is the last gene in an operon encoding three of the seven Y0340 paralogs. Y0340 is presumably exported, though further work is required to determine whether Y0340 is fully secreted or anchored to the outer membrane of the bacterial cell. Secreted Y0340 might interfere with host innate immune defenses or membrane bound Y0340 could act similarly to *Y. pestis* Ail to defend the pathogen against complement-like effectors.

Our work investigating the role of CCR5 in the host response to *Y. pestis* infection suggests that NK cell migration is required for defense against plague. This is consistent with previous studies have found that NK cell migration is inhibited by the *Y. pestis* virulence factor YopM, inbred mice strains that maintain a robust level of NK cells are more resistant to plague infection, and that CCR5−/− mice have deficiencies in NK cell recruitment when infected with the protozoan parasite *Toxoplasma gondii* (Kerschen, Cohen et al. 2004; Khan, Thomas et al. 2006). Although our data ruled out a protective role for CCR5 deletion in survival in an intranasal model of plague, it did not exclude a beneficial role for a functional CCR5 receptor in defense against *Y. pestis.* Further studies are necessary to determine whether CCR5-/- mice are significantly more

susceptible to *Y. pestis* infection. During *T. gondii* infection, CCR5 controls recruitment of NK cells to the liver and spleen (Khan, Thomas et al. 2006). Additional experiments are necessary to determine whether CCR5 controls recruitment of NK cells into these tissues during plague infection. RANTES and MIP-1α mediate NK cell migration through both CCR5 and also the chemokine receptor CCR2, suggesting that CCR2 function might be compensating for the lack of CCR5 in plague defense and a double mutant in these two genes would have enhanced susceptibility to *Y. pestis*.

Chemosensory GPCRs such as CCR5 are known for their role in immunity in mammals (Pierce, Premont et al. 2002; Rozengurt 2007). We have shown here that chemosensory GPCRs are also involved in the innate immune response to pathogens in *C. elegans*. These genes are mainly expressed in the nervous system in nematodes and although the previously identified innate immunity gene TOL-1 is known to be expressed in the nervous system (Tenor and Aballay 2008), this work represents the first direct link between specific neurons and the innate immune system in the nematode and establishes *C. elegans* as a model for the field of neuroimmunology.

Further studies are necessary to establish parallels between mammalian and *C. elegans* neuroimmunology. Immediately following mammalian infection, the peripheral nervous system acts to assist the innate immune system in host defense by activating inflammation. This inflammation then signals the sympathetic and parasympathetic nervous systems to produce anti-inflammatory effector molecules to moderate host

responses. We have identified here a nervous circuit that inhibits immunity in *C. elegans*; it will be interesting to determine whether an alternative nervous circuit balances this activity by enhancing innate immunity. (Tracey 2002; Andersson 2005; Sternberg 2006)

Previous studies have demonstrated that putative immune effector molecules such as the anti-bacterial factor related (*abf*) genes, lysozyme (*lys*) genes, saposin-like protein family (*spp*) genes, and sugar binding galectin and C-type lectin genes (*lec*, *clec*) are generally expressed in tissues exposed to pathogen (Alper, McBride et al. 2007). We have shown here that expression of some of these factors is dependent on the presence of NPR-1, but the question remains how the signal is transduced from the nervous system to the site of infection. Several chemical neurotransmitters are found in *C. elegans* that are important for mammalian neural transmission, including acetylcholine, glutamate, γ-aminobutyric acid (GABA), and four amines: octopamine, tyramine, dopamine and serotonin (Brownlee, Holden-Dye et al. 2000; Jorgensen 2005; Chase and Koelle 2007; Rand 2007). Since the mutant in the *dop-3* dopamine receptor is also susceptible to infection (Figure 19), this suggests that dopamine could be important for transmission of the immune signal. In addition, there are 41 genes encoding peptides that could function in neural transmission: twenty FMRFamide-like peptide genes (*flp*) and twenty-one neuropeptide-like protein (*nlp*) genes (Li, Nelson et al. 1999). Further study is needed to identify the mechanism by which the signal is transmitted by the nervous system to the site of infection.

In this work, we have used genetic tools and the nematode *C. elegans* to analyze bacterial virulence factors and host innate immune defenses. In this relatively new field of study, future efforts will provide greater understanding of *C. elegans* as a model for host-pathogen interactions and the extent to which studies using the genetically tractable nematode are applicable to host-pathogen interactions in human infectious disease.

6.3 Concluding remarks

Using a *C. elegans* pathogenesis system to study host-pathogen interactions, we were able to easily and rapidly identify novel bacterial virulence factors that were also important for virulence in mammals. Further work is necessary to classify the role of these factors in *Y. pestis* virulence. On the other side of the host-pathogen equation, we studied the *C. elegans* innate immune response to infection. We discovered that the nematode nervous system plays a significant role in regulating innate immune responses, thus establishing the nematode as a novel system to study neuroimmunology. Since *C. elegans* contains similar neurotransmitters as humans, it will be interesting to identify the neuroendocrine pathways downstream of the receptors identified here and to determine the relevance of these findings in mammalian neuroimmunology.

7. Materials and Methods

7.1 Development of *C. elegans* as a model system to study *Y. pestis* infection

7.1.1 Bacterial strains

E. coli OP50 (Brenner 1974), *Y. pestis pgm*− strains KIM5, KIM6, KIM8 and KIM10 (Une and Brubaker 1984) and *Y. pestis pgm*+ strain KIM6+ (Perry, Pendrak et al. 1990) have been described previously. *Y. pestis* KIM5Δ*ompT*, *Y. pestis* KIM5Δ*yapH*, *Y. pestis* KIM5Δ*y3857*, *Y. pestis* KIM8Δ*y2446*, *Y. pestis* KIM8Δ*y2034*, *Y. pestis* KIM8Δ*y1324* (Δ*ail*), *Y. pestis* KIM8Δ*y1324* (Δ*ail*)/pAil, *Y. pestis* KIM8Δ*y1682*, *Y. pestis* KIM8Δ4, and *Y. pestis* KIM8Δ4/pAil were gifts from the Plano laboratory (U. Miami). For serum survival experiments *Y. pestis* strains were grown at 6, 26, or 37°C in HIB in the presence of 2.5 mM calcium chloride.

7.1.2 *C. elegans* killing assay

C. elegans wild-type N2 animals were maintained as hermaphrodites at 20ºC, grown on modified NG agar plates and fed with *E. coli* strain OP50 as described (Brenner 1974). Bacterial cultures were grown in Luria-Bertani (LB) broth with appropriate antibiotics at 37°C. Bacterial lawns used for *C. elegans* killing assays were prepared by spreading 20 μl of an overnight culture of the bacterial strains on modified NG agar medium (0.35% instead of 0.25% peptone) in 3.5 cm diameter plates. Plates

were incubated at 25ºC for 12 hrs before seeding them with nematodes. For the killing assay, animals were placed on a bacterial lawn and the plates were incubated at 25ºC. N2 animals were transferred daily to fresh plates during the reproductive period and thereafter they were transferred approximately every 5 days. Worm mortality was scored over time, and a worm was considered dead when it failed to respond to touch.

7.1.3 Serum survival assay

Normal human serum (NHS) was pooled from at least five healthy volunteers. Animal sera were purchased from Valley Biomedical Products and Services, Inc. (Winchester, VA) or Sigma (St. Louis, MO). Heat-inactivated serum was used as a control in all experiments. Harvested and washed *Y. pestis* or *E. coli* was diluted in phosphate-buffered saline (PBS) (pH 7.4) to obtain an optical density at 620 nm (OD_{620}) of 0.2 (~1 x 10^8 bacteria/ml). Fifty microliters of the diluted bacteria (~5 x 10^6 bacteria) was added to 200 μl of NHS, normal goat serum (NGS), normal sheep serum (NSS), normal rabbit serum (NRS), normal mouse serum (NMS), normal guinea pig serum (GPS), heat-inactivated serum (HIS), or PBS (final concentration of serum, 80%). The samples were incubated at 37°C with shaking for 1 h. The number of surviving bacteria (CFU) in each sample was determined by dilution and plating on TBA plates at 30°C for 1 or 2 days. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h x 100.

7.1.4 Outer membrane preparation/ SDS-PAGE

Outer membranes preparations were obtained essentially as described by Biedzka-Sarek et al. (Biedzka-Sarek, Venho et al. 2005). Bacteria were grown overnight in HIB at 26 or 37°C. Bacterial cultures (5 ml) were harvested by centrifugation at 8,000 x *g* for 10 min at 4°C. Pellets were resuspended in 2 ml of 10 mM Tris-HCl-5 mM $MgCl_2$ (pH 8.0) (buffer A), and the bacteria were lysed by two passages through an ice-cold French pressure cell. Unlysed cells and large debris were removed by centrifugation as described above. Bacterial membranes were collected by ultracentrifugation at 100,000 x *g* for 1 h at 4°C. Membrane pellets were resuspended in buffer A containing 2% Triton X-100. The Triton X-100-resistant outer membrane protein fraction was pelleted by ultracentrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Volumes of cellular fractions corresponding to equal numbers of bacteria or equal amounts of protein were mixed 1:1 (vol/vol) with 2x electrophoresis sample buffer and analyzed by SDS-PAGE essentially as previously described (Plano and Straley 1995) and silver stained.

7.1.5 Screen for bacterial mutants deficient in *C. elegans* killing

Y. pestis KIM5 mutant libraries were generated using the EZ::TN transposon according to the manufacturer's instructions (www.epicentre.com/transposomics.htm). Individual transposant strains were grown overnight at 25ºC in 200 µl LB liquid broth in 96-well microtiter plates. Bacterial lawns used for *C. elegans* killing assays were

prepared by spreading 10 μl of the bacterial transposant strains on modified NG agar medium (0.35% instead of 0.25% peptone) in 24 well plates. Plates were incubated overnight at 25°C before seeding 10 *glp-4* worms per well and they were examined daily. The use of *glp-4* for these studies has been described before (Tenor, McCormick et al. 2004). Putative nonpathogenic or attenuated mutants identified in the preliminary screen were retested and subjected to multiple *C. elegans* killing assays using N2 animals to determine the kinetics of *C.elegans* mortality. The transposon insertions were individually transduced into wild-type *Y. pestis* KIM5 by phage P22 transduction. All isolated mutants grew at the same rate as the wild-type strain in rich LB or NG media, indicating that the attenuated pathogenicity phenotypes observed were not simply a result of growth defects.

7.1.6 Molecular analysis of insertion mutants

DNA blot analysis showed that each of the mutant strains identified contained a single EZ::TN insertion. Total genomic DNA isolated from *Y. pestis* KIM5 mutants was isolated using the DNeasy Tissue Kit (Qiagen). Genomic DNA was digested with EcoRI restriction endonuclease overnight at 37°C and then run on a 0.7% agarose gel. A DIG labelled Kan-2 probe was generated with the PCR DIG probe synthesis kit (Roche Diagnostics) under the recommended PCR conditions. Hybridization followed by immunological detection of DNA was performed according to the digoxigenin (DIG) system user's guide (Roche Diagnostics). Sequences flanking the 3′ transposon ends of

the Tn*phoA* mutants were amplified using arbitrarily-primed PCR (O'Toole and Kolter 1998) and the PCR products ranging from 200 to 400 bp were sequenced. The sequence information was compared with the databases at the National Center for Biotechnology Information.

7.1.7 Mouse model of *Y. pestis* pathogenesis

To establish an intranasal mouse model of virulence, we first determined the fifty percent lethal dose (LD50) for *Y. pestis* KIM5. Tenfold serial dilution (10^2 to 10^6) of bacteria in sterile phosphate-buffered saline (PBS) was inoculated into ten-twelve week old BALB/C mice. One milliliter of overnight cultures grown at 25oC in LB broth with shaking was pelleted using centrifugation for five minutes at 5,000rpm. The pellets were resuspended and washed three times with PBS. Using the OD_{600} measurement of the washed culture, cfu per milliliter were calculated and then verified through serial dilutions and plating. Ten-twelve week old BALB/C mice were anesthetized using an isofluorine chamber and then inoculated intranasally with 25ul per nares then monitored daily for a period of 2 weeks. Twenty animals were used in three independent experiments to determine the LD_{50} of *Y. pestis* KIM5 to be approximately 10^5 CFU.

For comparative mortality studies, the same methods for preparing inoculum were used. An estimated inoculum of 1 X 10^6 cfu /50 ul was used in all cases. Ten-twelve week old BALB/C mice were anesthetized using an isofluorine chamber and then

inoculated intranasally with 25ul per nares then monitored daily for a period of 2 weeks. To further characterize the disease progression, CFU counts were obtained for selected organs. The same methods for preparing inoculum were used. An estimated inoculum of 1 X 10^6 cfu /50 ul was used in all cases. Ten-twelve week old BALB/C mice were anesthetized using an isofluorine chamber and then inoculated intranasally with 25ul per nares. Ten animals were sacrificed in two independent experiments on day four and six after inoculation. Lung, liver and spleen were homogenized and plated on LB media, supplemented with antibiotic when necessary. Colony counts were made after incubation for 2 days at 25°C.

7.2 The role of CCR5 in the intranasal mouse model of plague

7.2.1 Bacterial strain

Y. pestis pgm− strain KIM5, was grown in BHI medium supplemented with 100 μM $FeCl_2$ (EMD Chemicals) at 150 rpm, 26 °C. Log phase culture was washed and resuspended in PBS at approximately 2 × 10^7 bacteria/ml. Inoculum concentration was verified by counting colony-forming units (CFU) of plated dilutions.

7.2.2 Mouse infection model

Eight-week old CCR5+/+ and CCR5−/− mice (C57Bl/6, Jackson Labs, *n* = 15 and 13, respectively) were infected intranasally with 8 × 10^5 CFU *Y. pestis* KIM5 and subsequently monitored for weight loss and other clinical signs of disease. Blood

samples (50–100 μl) were obtained from all mice 5 days prior to and 7 days after infection. In addition, half the mice in each group were bled on alternate days through day 4 post infection. Serum was collected by centrifugation of mouse blood and stored frozen at −80 °C until analyzed. Mice were euthanized for humane reasons when moribund or sacrificed after 21 days.

7.2.3 Serum cytokine/chemokine analysis

Cytokine/chemokine analysis of 15 μl of serum was performed using a 22-plex premixed kit and protocol from Linco Research, supplemented with a final 15 min incubation in 4% paraformaldehyde for decontamination. Data were collected on a BioRad Bioplex System using Software 4.0.

7.2.4 Statistical analysis

Survival was compared by the Fisher exact test. Survival times were compared by the Mann–Whitney rank sum test. Weights were compared by the *t*-test on each day after infection. The primary weight outcome was defined by the last day on which at least 80% of both groups were alive. Concentrations of cytokines and chemokines were compared at multiple time points by the Wilcoxon rank sum test. All tests were two-tailed.

7.3 *C. elegans* chemosensory GPCRs are involved in immunity to pathogens

7.3.1 Bacterial Strains

The following bacterial strains were used: *E. coli* OP50 (Brenner 1974) and *P. aeruginosa* PA14 (Tan, Mahajan-Miklos et al. 1999). Cultures were grown in Luria–Bertani (LB) broth at 37°C.

7.3.2 Nematode Strains

The following *C. elegans* strains were cultured under standard conditions (Brenner 1974) and fed *E. coli* OP50. Wild-type animals were *C. elegans* Bristol strain N2. Other strains used in this work include: RB799 *C25G6.5(ok594)* X, RB1284 *C30F12.6(ok1381)* I, RB1289 *C43C3.2(ok1388)* X, RB1288 *C48C5.1(ok1387)* X, RB1423 *C49A9.7(ok1620)* IV, RB1321 *C56G3.1(ok1439)* X, RB1162 *cfz-2(ok1201)* V, RB665 *dop-1(ok398)* X, LX702 *dop-2(vs105)* V, BZ873 *dop-3(ok295)* X. RB761 *F35G8.1(ok527)* X, RB509 *F54D7.3(ok238)* I, RB1349 *F57H12.4(ok1504)* IV, RB896 *gar-1(ok755)* X, RB756 *gar-2(ok520)* III, JD217 *gar-3(vu78)* V, VC158 *lat-2(ok301)* II, DA609 *npr-1(ad609)* X, XA3702 *npr-2(ok419)* IV CX3410 *odr-10(ky225)* X, RB1141 *R13H7.2(ok1167)* IV, DA1814 *ser-1(ok345)* X, OH313 *ser-2(pk1357)* X, RB1622 *ser-3(ok1995)* I, AQ866 *ser-4(ok512)* III, DA2100 *ser-7(tm1325)* X, CB5414 *srd-1(eh1)* II, VC459 *srd-2(ok638)* II, RB1526 *srd-44(ok1831)* X, RB1419 *srw-140(ok1616)* V, RB1306 *str-182(ok1419)* V, VC342 *str-31(ok442)* V, RB785 *T02E9.3(ok568)* V, VC125 *tag-126(ok325)* X,

VC224 *tag-24(ok371)* X, VC270 *tag-49(ok381)* IV, VC273 *tag-89(ok514)* IV, RB1365 *uvt-6(ok1541)* X, RB1393 *Y58G8A.4(ok1583)* V, RB1405 *Y59H11AL.1(ok1598)* IV.

7.3.3 *C. elegans* killing assay

C. elegans wild-type N2 animals and mutants were maintained as hermaphrodites at 15°C, grown on modified NG agar plates and fed with *E. coli* strain OP50 as described (Brenner 1974). Bacterial lawns used for *C. elegans* killing assays were prepared by placing a 10-20 μl drop of an overnight culture grown at 37°C of *P. aeruginosa* on modified NGM agar (0.35% instead of 0.25% peptone) on plates 3.5 cm in diameter. Plates were incubated at 25°C for at least 12h before nematode infection. At least ten synchronized gravid adult *C. elegans* wild-type N2 animals and mutants were placed on at least four replicate plates containing pathogen and scored for survival over time. Animals were considered dead when they failed to respond to touch. Live animals were transferred daily to fresh plates until there ceased to be progeny on the plate. Killing assays were performed at 25°C.

7.3.4 Statistical analysis

Animal survival was plotted as a non-linear regression curve using the PRISM (version 4.00) computer program. Survival curves are considered significantly different than the control when P values are <0.05. Prism uses the product limit or Kaplan-Meier method to calculate survival fractions and the logrank test, which is equivalent to the Mantel-

Heanszel test, to compare survival curves. The time for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated using Prism software (version 4.00) using a non-linear regression analysis of survival proportions utilizing the equation: Y=Bottom + (Top-Bottom)/(1 + 10 $^{(LogEC_{50}-X)*HillSlope)}$), where Top is set at 100, Bottom is set at 0, X is the time in days and Y is the percentage of nematodes alive at time X. In this instance, TD_{50} is equivalent to EC_{50}.

7.4 Innate immunity in *C. elegans* is regulated by neurons expressing NPR-1

7.4.1 Bacterial Strains

The following bacterial strains were used: *E. coli* OP50 (Brenner 1974), *S. enterica* serovar *typhimurium* SL1344 (Wray and Sojka 1978), *E. faecalis* OG1RF (Murray, Singh et al. 1993), and *P. aeruginosa* PA14 (Tan, Mahajan-Miklos et al. 1999). *E. coli*, *P. aeruginosa*, and *S. enterica* cultures were grown in Luria–Bertani (LB) broth at 37°C. *E. faecalis* liquid cultures were grown in brain–heart infusion broth at 37°C.

7.4.2 Nematode Strains

The following *C. elegans* strains were cultured under standard conditions (Brenner 1974)and fed *E. coli* OP50. Wild-type animals were *C. elegans* Bristol strain N2. Other strains used in this work include: DA609 *npr-1 (ad609)* X, RB1321 *C56G3.1 (ok1439)* X, RB1349 *F57H12.4 (ok1504)* IV, VC224 *tag-24 (ok371)* X, CX4148 *npr-1(ky13)* X, DA508 *npr-1(n1353)* X, DA650 *npr-1(g320)* X, IM222 *npr-1(ur89)* X, RC301 *npr-1(g320)*X, CX6448

gcy-35 (ok769) I, CX7157 *gcy-35 (ok769)* I; *npr-1 (ad609)* X, PR691, *tax-2(p691)* I, AX826, *tax-2(p691)* I; *npr-1(ad609)* X, PR678, *tax-4(p678)* III, CX4819, *tax-4(p678)* III; *npr-1(ad609)* X, CX9147 *npr-1(ad609)* X; *kyEx1823* [*gpa-3::npr-1*, *gcy-32::npr-1*], CX7102 *lin-15 (n765) qaIs2241 [gcy-36::egl-1, gcy-35::gfp, lin-15(+)]* X.

7.4.3 *C. elegans* Killing Assay

7.4.3.1 Standard

C. elegans wild-type N2 animals and mutants were maintained as hermaphrodites at 15°C, grown on modified NG agar plates and fed with *E. coli* strain OP50 as described (Brenner 1974). Bacterial lawns used for *C. elegans* killing assays were prepared by placing a 10-20 μl drop of an overnight culture grown at 37°C of the bacterial strains (*E. coli, S. enterica, P. aeruginosa*) on modified NGM agar (0.35% instead of 0.25% peptone) on plates 3.5 cm in diameter. *E. faecalis* overnight cultures were grown at 37°C and plated on brain–heart infusion agar supplemented with 50 μg/ml gentamicin on plates 3.5 cm in diameter. Plates were incubated at 25°C (*E. coli, S. enterica, P. aeruginosa*) or 37°C (*E. faecalis, P. aeruginosa*) for at least 12h before nematode infection. At least ten synchronized gravid adult *C. elegans* wild-type N2 animals and mutants were placed on at least four replicate plates containing pathogen and scored for survival over time. Animals were considered dead when they failed to respond to touch. Live animals were

transferred daily to fresh plates until there ceased to be progeny on the plate. Killing assays were performed at 25°C, unless otherwise stated.

7.4.3.2 Full lawn

Full lawn plates used for *C. elegans* killing assays were prepared by spreading a 20 μl drop of an overnight culture grown at 37°C of *P. aeruginosa* on the complete surface of modified NGM agar on plates 3.5 cm in diameter. Plates were incubated at 25°C for at least 12h before nematode infection. At least ten synchronized gravid adult *C. elegans* wild-type N2 animals and mutants were placed on at least four replicate plates containing pathogen and scored for survival over time.

7.4.3.3 Low Oxygen

C. elegans wild-type N2 animals and *npr-1(ad609)* mutants were grown under 21% oxygen and 8% oxygen at room temperature (~23°C). *P. aeruginosa* infection plates were prepared as described above. At least ten synchronized gravid adult *C. elegans* wild-type N2 animals and mutants were placed on at least four replicate plates containing pathogen and scored for survival over time.

7.4.4 *C. elegans* survival on killed *P. aeruginosa*

Synchronized L4 larvae were transferred to a lawn of heat-killed *P.aeruginosa* on a plate of modified NGM containing 100 μg/ml 5-fluorodeoxyuridine (FUdR) and 50 μg/ml ampicillin. FUdR is an inhibitor of DNA synthesis that blocks the development of

progeny. The assay was performed at 25°C. Animals were scored at the indicated times and considered dead upon failure to respond to touch. Animals missing from the agar plate were censored on day of loss.

7.4.5 Microarray

Gravid adult wild-type and *npr-1(ad609)* nematodes were lysed using a solution of sodium hydroxide and bleach, washed, and the eggs were synchronized for 22 hours in S basal liquid medium at room temperature. Synchronized L1 animals were placed onto NGM plates seeded with *E. coli* OP50 and grown until L4 (36 hours at 25°C). The L4 animals were exposed to *P. aeruginosa* for 4 hours at 25°C and then harvested by washing the plates with M9 buffer. RNA extracted using Trizol reagent for two independent isolations. cDNA was generated and hybridized to Affymetrix C. elegans Genome Array following the manufacturer's instructions at the Duke Microarray Facility. Detailed protocols are available on the Duke Microarray Facility Web site (http://microarray.genome.duke.edu). GeneSpring Software 7.0 (Agilent Technologies) was used to perform normalizations and fold change analysis.

7.4.6 Quantitative real-time PCR

Gravid adult wild-type, *npr-1(ad609),* and *gcy-35(ok769)/npr-1(ad609)* nematodes were lysed using a solution of sodium hydroxide and bleach, washed, and the eggs were synchronized for 22 hours in S basal liquid medium at room temperature. Synchronized

L1 animals were placed onto NGM plates seeded with *E. coli* OP50 and grown until L4 (36 hours at 25°C). The L4 animals were exposed to *P. aeruginosa* for 4 hours at 25°C and then harvested. The animals were collected by washing the plates with M9 buffer, and RNA extracted using Trizol reagent. Genomic DNA was removed by treating the RNA samples with DNase using the DNA-free kit according to manufacturer's instruction (Ambion). qRT-PCR was conducted using the Applied Biosystems Taqman One-Step Real-time PCR protocol using SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems 7900HT real-time PCR machine in 96 well plate format. Fifty nanograms of RNA were used for real-time PCR. Twenty-five microliter reactions were set-up and performed as outlined by the manufacturer (Applied Biosystems). Gene expression for six independent isolations of *npr-1(ad609)* nematodes and three independent isolations of *gcy-35(ok769)/npr-1(ad609)* nematodes were compared to wild type using the comparative Ct method after normalization to *act-1,-3,-4* (pan-actin) (Shapira, Hamlin et al. 2006). Primer sequences are available upon request.

7.4.7 Statistical Analysis

Animal survival was plotted as a non-linear regression curve using the PRISM (version 4.00) computer program. Survival curves are considered significantly different than the control when P values are <0.05. Prism uses the product limit or Kaplan-Meier method to calculate survival fractions and the logrank test, which is equivalent to the Mantel-Heanszel test, to compare survival curves. Differences in qRT-PCR expression

levels between *npr-1(ad609)* nematodes and wild-type were determined using one-sample *t* tests. Differences in qRT-PCR expression levels between *npr-1(ad609)* and *gcy-35(ok769)/npr-1(ad609)* nematodes were determined using the Student's exact *t* test.

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