

was bacterially expressed and purified on glutathione agarose, and the purified protein was eluted with glutathione and quantified. Eluted GST-cGASP was added to the MBP-DOR slurry just before addition of in vitro-translated full-length GASP probe.

32. Cells expressing only endogenous GASP and cells overexpressing GFP-cGASP (~40 times the level of endogenous GASP as estimated by immunoblotting) were grown to 80% confluency and treated with 5  $\mu$ M EGF in DMEM for the indicated times or left untreated. Cells were washed with phosphate-buffered saline (PBS), and the EGF receptor was immunoprecipitated with rabbit anti-EGFR-affinity resin (Santa Cruz Biotechnology, Santa Cruz, CA), separated by SDS-PAGE, and immunoblotted with goat antibodies to EGFR (Santa Cruz Biotechnology), followed by horseradish peroxidase (HRP)-conjugated secondary antibody to goat (Jackson ImmunoResearch, Malvern, PA) and development with ECL reagents (Amersham, Piscataway, NJ).

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**Supporting Online Material**

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Materials and Methods

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## Identification of *Bphs*, an Autoimmune Disease Locus, as Histamine Receptor H<sub>1</sub>

Runlin Z. Ma,<sup>1,2</sup> Jianfeng Gao,<sup>3</sup> Nathan D. Meeker,<sup>2</sup> Parley D. Fillmore,<sup>2</sup> Kenneth S. K. Tung,<sup>4</sup> Takeshi Watanabe,<sup>5</sup> James F. Zachary,<sup>2</sup> Halina Offner,<sup>6</sup> Elizabeth P. Blankenhorn,<sup>7</sup> Cory Teuscher<sup>3\*</sup>

*Bphs* controls *Bordetella pertussis* toxin (PTX)-induced vasoactive amine sensitization elicited by histamine (VAASH) and has an established role in autoimmunity. We report that congenic mapping links *Bphs* to the histamine H<sub>1</sub> receptor gene (*Hrh1*/H1R) and that H1R differs at three amino acid residues in VAASH-susceptible and -resistant mice. *Hrh1*<sup>-/-</sup> mice are protected from VAASH, which can be restored by genetic complementation with a susceptible *Bphs*/*Hrh1* allele, and experimental allergic encephalomyelitis and autoimmune orchitis due to immune deviation. Thus, natural alleles of *Hrh1* control both the autoimmune T cell and vascular responses regulated by histamine after PTX sensitization.

PTX is a major virulence factor of *B. pertussis*, the causative agent of Whooping Cough (1). The holotoxin is a hexameric protein that conforms to the  $\alpha\beta$  model of bacterial exotoxins (2). The  $\alpha$  subunit is an adenosine diphosphate (ADP)-ribosyl transferase, which affects signal transduction by ribosy-

lation of the  $\alpha$  subunit of trimeric G<sub>i</sub> proteins, whereas the  $\beta$  oligomer binds cell surface receptors on a variety of mammalian cells (2, 3). PTX, when administered in vivo, elicits a range of responses including disruption of glucose regulation, leukocytosis, adjuvant activity, increased vascular permeability associated with alteration of blood-tissue barrier functions, and sensitization to vasoactive amines (VAAS) (4, 5). The latter two phenotypes are the result of PTX-induced changes in vascular endothelial cells. Inbred strains of mice differ in susceptibility to vasoactive amine challenge after PTX sensitization in that genetically susceptible strains die from hypotensive and hypovolemic shock, whereas resistant strains do not (4). Additionally, the genetic control of susceptibility to lethal shock is vasoactive amine specific (4) and is mediated through a variety of mechanisms (6–9).

Hypersensitivity to histamine after PTX sensitization (VAASH) is controlled by an

autosomal dominant locus known as *Bphs* (10). *Bphs* was one of the first nonmajor histocompatibility complex-linked genes shown to be involved in susceptibility to multiple autoimmune diseases (10). Previously, we mapped *Bphs* on mouse chromosome 6 using backcross populations generated with susceptible SJL/J and resistant C3H/HeJ and CBA/J mice (10). As the first step in positionally cloning *Bphs*, we generated a panel of recombinant, interval-specific congenic lines using marker-assisted selection to introgress the SJL/J *Bphs* allele (*Bphs*<sup>s</sup>) onto the C3H/HeJ background (11). These lines were tested for susceptibility to VAASH (Table 1). The results establish that *Bphs* resides within an interval  $\leq 1$  cM between *D6Mit107* and *D6Mit41*, encompassing *Hrh1*. Additionally, homozygous and heterozygous C3H.SJL-*Bphs* line D mice are as sensitive to VAASH as SJL/J, over a dose range of 6.25 to 100 mg/kg (table S1). This is consistent with dominance and a lack of gene dosage effect at this locus (10).

It is known that the histamine H<sub>1</sub> receptor antagonist mepyramine can block VAASH in rats (12), and, because *Hrh1* resides within the interval encoding *Bphs*, *Hrh1* was a candidate gene for *Bphs*. Therefore, we cloned and sequenced the *Hrh1* alleles from cDNA samples of 14 inbred strains of mice (table S2). With the exception of C3H/HeJ and CBA/J, all mouse strains are susceptible to VAASH. *Hrh1* sequences from susceptible and resistant mouse strains exhibited multiple, single nucleotide polymorphisms. However, among these polymorphisms, only three led to distinct and concordant amino acid changes in the predicted sequence (L263P, M313V, and S331P), all of which distinguish C3H/HeJ and CBA/J from all other strains of mice (fig. S1).

The identity of *Hrh1* as *Bphs* was further verified using C57BL/6-*Hrh1*<sup>-/-</sup> (13) and C57BL/6-*Hrh2*<sup>-/-</sup> (14) mice. *Hrh1*<sup>-/-</sup> mice were completely resistant to VAASH, whereas *Hrh2*<sup>-/-</sup> mice were fully susceptible (Table

<sup>1</sup>Laboratory Animal Center, Institute of Genetics, Chinese Academy of Sciences, Beijing, China 100101.

<sup>2</sup>Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802–6178, USA.

<sup>3</sup>Department of Medicine, University of Vermont, Burlington, VT 05405–0068, USA.

<sup>4</sup>Department of Pathology, University of Virginia, Charlottesville, VA 22908–0001, USA.

<sup>5</sup>Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan.

<sup>6</sup>Department of Neurology, Oregon Health and Science University and Neuroimmunology Research,

Veterans Affairs Medical Center, Portland, OR 97201–3098, USA.

<sup>7</sup>Department of Microbiology and Immunology, MCP Hahnemann University, Philadelphia, PA 19129–1129, USA.

\*To whom correspondence should be addressed. E-mail: cteusche@zoo.uvm.edu

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2). Genetic complementation experiments using F<sub>1</sub> hybrids between *Hrh1*<sup>-/-</sup> and mice bearing a resistant *Bphs* allele (*Bphs*<sup>h</sup>) or a susceptible allele (*Bphs*<sup>s</sup>) were performed. Susceptibility could be restored with a single *Bphs*<sup>s</sup> allele from SJL/J, 129X1/SvJ, or C57BL/6-*Hrh2*<sup>-/-</sup> mice but not with a resistant allele from C3H/HeJ mice (Table 2). Additionally, pretreatment of PTX-sensitized homozygous and heterozygous C3H.SJL-*Bphs* line D mice with histamine H<sub>1</sub> receptor (H1R) selective antagonists before histamine challenge blocked VAASH, whereas H<sub>2</sub> and H<sub>3</sub> selective antagonists did not (table S3). Taken together, our results establish that *Hrh1* is *Bphs* and suggest that one or a combination of the critical amino acids at positions 263, 313, and 331 in the third intracellular loop, associated with signal transduction (15), underlies susceptibility and resistance to VAASH.

Next, we assessed the role of *Bphs/Hrh1* in autoimmune disease. In the first study, (C3H/HeJ × SJL/J) × C3H/HeJ mice were phenotyped for susceptibility to experimental allergic encephalomyelitis (EAE), experimental allergic orchitis (EAO), and VAASH (11). Linkage of VAASH to EAE and EAO susceptibility was detected ( $\chi^2 = 6.42$ ;  $P =$

0.01 for EAE and  $\chi^2 = 5.60$ ;  $P = 0.02$  for EAO). When the populations were combined, the significance of the linkage was greater indicating that *Bphs/Hrh1* alleles behaved in a similar fashion genetically in both autoimmune diseases ( $\chi^2 = 11.49$ ;  $P = 0.00007$ ). These results confirm that *Bphs* is linked to susceptibility to EAE and EAO and are consistent with previous results indicating that *Bphs* is one of several autoimmune disease modifying genes shared between these two models (16).

Because PTX sensitization at the time of immunization induces CD4<sup>+</sup> T cells that are responsible for mediating enhanced and protracted delayed-type hypersensitivity responses (17) by producing extremely high levels of  $\gamma$ -interferon (IFN $\gamma$ ) after antigenic stimulation (18, 19), and because histamine has recently been shown to regulate immune deviation through differential expression of histamine H<sub>1</sub> and H<sub>2</sub> receptors (20), we directly assessed the role of *Hrh1* in susceptibility to both myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (MOG 35-55)-induced EAE and testis antigen-induced EAO. With respect to MOG-induced EAE, the day of disease onset was delayed, and the severity of clinical signs

markedly reduced in C57BL/6-*Hrh1*<sup>-/-</sup> compared with C57BL/6 wild-type mice (Fig. 1A). Similar results were seen with autoimmune orchitis (table S4). These results again indicate that *Hrh1* fulfills the role of *Bphs* in both EAE and EAO.

The examination of a variety of T cell parameters showed that proliferation of draining lymph node (DLN) cells did not differ between wild-type and *Hrh1*<sup>-/-</sup> mice immunized with the MOG 35-55 (Fig. 1B). In contrast, cytokine production by splenocytes differed considerably. IFN $\gamma$  production was markedly decreased (Fig. 2A), whereas interleukin-4 (IL-4) production was increased in *Hrh1*<sup>-/-</sup> mice compared with wild-type mice (Fig. 2B). Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), IL-2, and IL-5 cytokines were measured with no difference in cytokine production detected between the two groups (fig. S2). Additionally, there was no difference in the levels of total immunoglobulin G (IgG), IgG1, or IgG2a antibody to MOG (fig. S3) or in the brain and spinal cord histopathology between *Hrh1*<sup>-/-</sup> and wild-type mice, with the exception that *Hrh1*<sup>-/-</sup> mice tended to have more eosinophilic infiltrates (fig. S4). These results provide a mechanistic basis for the genetic role of *Bphs/Hrh1* in eliciting pathogenic T

**Table 1.** Genotypes of C3H.SJL-*Bphs* congenic lines show colocalization of *Bphs* with *Hrh1*. Animals were backcrossed and recombinant lines were identified and selected between N12 and N24. Mice from each line were sensitized with PTX on day 0 by intraperitoneal (i.p.) injection of 10.0  $\mu$ g crude PTX (lines A to D) or by intravenous (i.v.) injection of 400 ng purified PTX (List Biological Laboratories, Inc.) (lines E to F). Control animals received carrier. Three days later, mice were

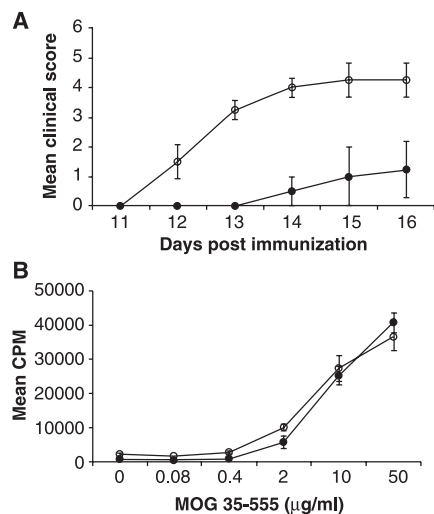
challenged by either i.p. (lines A to D) or i.v. (lines E to F) injection of 50.0 mg/kg histamine in phosphate-buffered saline (dry weight free base). Results are expressed as the number of animals dead at 30 min over the number of animals studied. Marker map locations are in cM according to MGD ([www.informatics.jax.org/searches/linkmap\\_form.shtml](http://www.informatics.jax.org/searches/linkmap_form.shtml)). S, SJL/J-derived alleles at the various marker loci; H, C3H/HeJ alleles; NS, not studied.

Marker	Map location	Line								
		A	B	C	D	E	F	G		
<i>D6Mit237</i>	7.2	H	H	H	H	H	H	H	H	
<i>D6Mit239</i>	15.9	S	H	H	H	H	H	H	H	
<i>Tcrb</i>	20.5	S	H	H	H	H	H	H	H	
<i>D6Mit16</i>	30.5	S	H	H	H	H	H	H	H	
<i>D6Nds3</i>	30.5	S	H	H	H	H	H	H	H	
<i>D6Mit8</i>	35.2	S	S	H	H	H	H	H	H	
<i>D6Nds2</i>	39.5	S	S	S	H	H	H	H	H	
<i>D6Mit65</i>	46.0	S	S	S	H	H	H	H	H	
<i>D6Mit105</i>	45.5	S	S	S	S	H	H	S		
<i>D6Mit36</i>	46.0	S	S	S	S	H	H	S		
<i>D6Mit286</i>	46.5	S	S	S	S	H	H	S		
<i>D6Mit54</i>	48.2	S	S	S	S	H	H	S		
<i>D6Mit107</i>	49.0	S	S	S	S	S	H	H		
<b><i>Hrh1</i></b>	49.0	<b>S</b>	<b>S</b>	<b>S</b>	<b>S</b>	<b>S</b>	<b>H</b>	<b>H</b>		
<i>D6Mit41</i>	50.0	S	S	S	S	S	H	H		
<i>D6Mit366</i>	50.5	S	S	S	S	S	S	H		
<i>D6Mit115</i>	51.0	S	S	S	S	S	S	H		
<i>D6Mit216</i>	58.6	S	S	S	S	S	S	H		
<i>D6Mit218</i>	61.2	H	H	H	H	H	H	H		
<i>D6Mit13</i>	63.6	H	H	H	H	H	H	H		
<i>D6Nds8</i>	63.6	H	H	H	H	H	H	H		
<i>D6Mit15</i>	74.0	H	H	H	H	H	H	H		
Control		0/4	0/4	0/4	0/4	NS <sup>b</sup>	NS	NS		
PTX		25/27	4/4	5/7	6/8	5/5	0/6	0/9		
<i>Bphs</i>		S	S	S	S	S	H	H		

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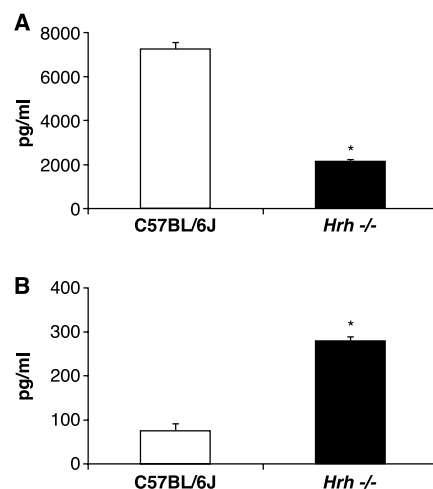
cells during the induction phase of autoimmune disease.

H1R has been reported to be essential



**Fig. 1.** (A) Mean clinical disease course for C57BL/6J (○) and C57BL/6-*Hrh1*<sup>-/-</sup> (●) mice immunized with MOG 35-55. Data are plotted as the mean clinical score ± SD. The Wilcoxon-Gehan test for time to event and the Friedman test for correlated observations indicated that both the onset ( $P = 0.04$ ) and severity of clinical signs ( $P < 0.0001$ ) seen in the C57BL/6-*Hrh1*<sup>-/-</sup> animals was significantly different from C57BL/6J mice. (B) Antigen specific ex vivo T cell proliferative responses for C57BL/6J (○) and C57BL/6-*Hrh1*<sup>-/-</sup> (●) mice immunized with MOG 35-55. Data are plotted as mean counts per minute (CPM) ± SD and were calculated from triplicate wells.

for eliciting optimal T and B cell responses because *Hrh1*<sup>-/-</sup> mice exhibited reduced antigen-specific proliferative responses but still exhibited normal cytokine receptor-mediated signaling and toll receptor-mediated signaling (21). With respect to antigen-specific proliferation, our results differ from the earlier report by Banu and Watanabe. However, this difference is readily attributable to the different immunization protocols used. Subsequently, it was shown



**Fig. 2.** IFN $\gamma$  (A) and IL-4 (B) production by MOG 35-55 stimulated splenocytes from C57BL/6J wild-type and C57BL/6-*Hrh1*<sup>-/-</sup> mice. Determination of statistical significance between groups was done using the Student's  $t$  test. (Asterisk indicates  $P < 0.001$ ).

**Table 2.** Assessment of VAASH in C57BL/6-*Hrh1*<sup>-/-</sup> and C57BL/6-*Hrh2*<sup>-/-</sup> mice and F<sub>1</sub> progeny between C57BL/6-*Hrh1*<sup>-/-</sup> mice and *Bphs*<sup>sl/sl</sup> and *Bphs*<sup>hh/hh</sup> strains. Mice were sensitized with 400 ng purified PTX (List Biological Laboratories, Inc.) by i.v. injection on day 0. On day 3, mice were challenged with histamine (mg dry weight free base) by i.v. injection, and deaths were recorded at 30 min post challenge. The results are expressed as the number of animals dead over the number of animals studied.

Strain or cross	Histamine (mg/kg)	Dead/tested
C57BL/6- <i>Hrh1</i> <sup>-/-</sup>	100.0	0/5
	50.0	0/4
	25.0	0/2
C57BL/6- <i>Hrh2</i> <sup>-/-</sup>	100.0	4/4
	50.0	4/4
	25.0	2/2
<i>(Hrh1</i> <sup>-/-</sup> × C3H/HeJ) F <sub>1</sub>	100.0	0/4
	50.0	0/4
	25.0	0/4
	12.5	0/4
	6.25	0/4
<i>(Hrh1</i> <sup>-/-</sup> × SJL/J) F <sub>1</sub>	100.0	5/5
	50.0	5/5
	25.0	5/6
	12.5	2/4
	6.25	1/4
<i>(Hrh1</i> <sup>-/-</sup> × 129X1/SvJ) F <sub>1</sub>	100.0	4/4
	50.0	4/4
	25.0	4/4
	12.5	1/4
	6.25	0/7
<i>(Hrh1</i> <sup>-/-</sup> × <i>Hrh2</i> <sup>-/-</sup> ) F <sub>1</sub>	100.0	4/4
	50.0	3/3
	25.0	2/2

that histamine acting through H1R enhances T helper-1 cell (T<sub>H</sub>1) responses, whereas both T<sub>H</sub>1 and T<sub>H</sub>2 responses are suppressed by signaling through H2R. H1R is more abundant than H2R on T<sub>H</sub>1 cells. Thus the disruption of *Hrh1* leads to immune deviation of T cell responses characterized by the suppression of IFN $\gamma$  production and an up-regulation in the production of T<sub>H</sub>2-related cytokines (20). These findings are consistent with the role of *Bphs/Hrh1* in MOG-induced EAE, in which the T cell response in *Hrh1*<sup>-/-</sup> mice was strongly T<sub>H</sub>2 biased and associated with less severe disease.

We have identified *Hrh1* as the general autoimmune disease susceptibility locus *Bphs* and have presumably delineated the critical amino acid residues that underlie the coupling of H1R to the Gq/11-second messenger pathway that mediates cell-specific responses (15, 21). To date, H1R has not been modeled with respect to its coupling to second messenger pathways. However, the cytoplasmic amino acids that are phosphorylated and are involved in H1R desensitization by phorbol esters have been identified, and they are distinct from the polymorphic residues identified in this study (22). In endothelial cells, H1R-mediated signaling responses include the ability of histamine to directly cause vasodilation and increased vascular permeability (5) and the release from Weibel Palade (WP) bodies (7) of stored vasoactive factors (IL-8, von Willebrand Factor, P-selectin, endothelin, and CD63) whose synthesis is induced by inflammatory stimuli such as PTX (8, 9). The role of histamine as a secretagogue for the release of stored vasoactive factors from WP bodies (7–9) is consistent with the fact that VAASH persists upward of 60 days (4). Histamine and histamine-releasing agents also have dramatic effects on the blood-testis (23) and blood-brain barriers (24), and, in both EAO and EAE, tissue levels of histamine correlate with the onset of disease (25, 26). It has long been known (27, 28), and recently reaffirmed (29, 30), that H1R selective antagonists significantly inhibit susceptibility to EAE, and we have observed similar results in EAO (table S4). In T cells, signaling through H1R results in Ca<sup>2+</sup> mobilization causing the translocation of nuclear factor of activated T cells (NFAT) to the nucleus and activation of the IL-2 gene (21) and, via an unknown mechanism, immune deviation of T cells (20). Our results demonstrate how the characterization of a monogenic, qualitative intermediate phenotype can result in the identification of a gene that mimics a quantitative trait locus due to its activity in multiple cell types. The identification of *Hrh1* as *Bphs* also represents an example of an autoimmune disease susceptibility gene that func-

# A Conserved p38 MAP Kinase Pathway in *Caenorhabditis elegans* Innate Immunity

Dennis H. Kim,<sup>1\*</sup> Rhonda Feinbaum,<sup>1\*</sup> Geneviève Alloing,<sup>1†</sup>  
 Fred E. Emerson,<sup>1</sup> Danielle A. Garsin,<sup>1</sup> Hideki Inoue,<sup>2</sup>  
 Miho Tanaka-Hino,<sup>2</sup> Naoki Hisamoto,<sup>2</sup> Kunihiro Matsumoto,<sup>2</sup>  
 Man-Wah Tan,<sup>1‡</sup> Frederick M. Ausubel<sup>1§</sup>

A genetic screen for *Caenorhabditis elegans* mutants with enhanced susceptibility to killing by *Pseudomonas aeruginosa* led to the identification of two genes required for pathogen resistance: *sek-1*, which encodes a mitogen-activated protein (MAP) kinase kinase, and *nsy-1*, which encodes a MAP kinase kinase. RNA interference assays and biochemical analysis established that a p38 ortholog, *pmk-1*, functions as the downstream MAP kinase required for pathogen defense. These data suggest that this MAP kinase signaling cassette represents an ancient feature of innate immune responses in evolutionarily diverse species.

The evolutionary conservation in the mechanisms of innate immunity between *Drosophila melanogaster* and mammals suggests that the study of immune function in diverse species may yield key insights into the evolutionary origins and molecular mechanisms of the mammalian innate immune system (1). The development of experimental host-pathogen systems that use invertebrate hosts greatly facilitates the genetic analysis of immune function of a host organism. Here, we report on our initial efforts toward the comprehensive forward genetic analysis of immune function in the nematode, *Caenorhabditis elegans*.

To screen for mutants with enhanced susceptibility to pathogens (Esp), we exposed mutagenized F<sub>2</sub> generation L4 larval stage nematodes to *Pseudomonas aeruginosa* strain PA14 under slow killing assay conditions, which involve an infection-like process requiring live pathogenic bacteria (2). The population of worms was then screened after a period of 16 to 30 hours for dead animals (wild-type animals typically began to die at approximately 34 hours). Under these conditions, hermaphrodite animals died as gravid

adults; thus, putative mutants were recovered by transferring individual dead worms containing their brood to plates seeded with the standard laboratory nematode food source, *Escherichia coli* OP50, whereupon the progeny were recovered. Potential mutants were rescreened by a comparative evaluation of survival on OP50 and pathogen PA14.

We screened an estimated 14,000 haploid genomes and selected for further study two of an initially isolated 10 mutants, *esp-2(ag1)* and *esp-8(ag3)*, that had similar and the most penetrant Esp phenotypes. When animals were exposed to PA14 under standard assay conditions, 100% of *esp-2* animals, 90% of *esp-8*, and 0% of wild-type animals were killed by 31 hours (Fig. 1A). To monitor the course of infection in the *esp-2* and *esp-8* mutant animals, worms were fed green fluorescent protein (GFP)-labeled PA14 (2). Accumulation of PA14 in the intestine (detected as early as 6 hours in *esp-2* and *esp-8* animals after exposure to GFP-labeled PA14) was correlated with the kinetics of killing. After 20 hours on GFP-labeled PA14, *esp-2* and *esp-8* animals had an overwhelming accumulation of PA14 in the anterior portion of their intestines, whereas wild-type animals showed minimal accumulation of GFP-labeled PA14 (Fig. 1D). Of note, the enhanced susceptibility of *esp-2* and *esp-8* mutant animals was not specific to the Gram-negative pathogen, *P. aeruginosa*, because the mutants were also hypersusceptible to the Gram-positive pathogen *Enterococcus faecalis* (Fig. 1C), which also kills *C. elegans* (3).

An implicit concern with the isolation of *C. elegans* mutants with enhanced susceptibility to pathogens is that many loss-of-function mutations might compromise the overall health of the worm, resulting in a secondary increased sensitivity to pathogens. In fact,

tions at critical checkpoints during both the induction and effector phases of two different induced autoimmune diseases.

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## Supporting Online Material

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Materials and Methods

Figs. S1 to S4

Tables S1 to S4

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<sup>1</sup>Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA. <sup>2</sup>Department of Molecular Biology, Graduate School of Science, Nagoya University and CREST, Japan Science and Technology Corporation, Chikusa-ku, Nagoya 464-8602, Japan.

\*These authors contributed equally to this work.

†Present address: Laboratoire de Biologie Vegetale et Microbiologie, Universite de Nice-Sophia Antipolis, Parc Valrose 06108 Nice Cedex 2, France.

‡Present address: Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, Room M337, Stanford, CA 94305–5120, USA.

§To whom correspondence should be addressed. E-mail: ausubel@molbio.mgh.harvard.edu