DmMyD88 controls dorsoventral patterning of the *Drosophila* embryo

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MyD88 is an adapter protein in the signal transduction pathway mediated by interleukin-1 (IL-1) and Toll-like receptors. A *Drosophila* homologue of *MyD88* (*DmMyD88*) was recently shown to be required for the Toll-mediated immune response. In *Drosophila*, the Toll pathway was originally characterized for its role in the dorsoventral patterning of the embryo. We found that, like *Toll*, *DmMyD88* messenger RNA is maternally supplied to the embryo. Here we report the identification of a new mutant allele of *DmMyD88*, which generates a protein lacking the carboxy-terminal extension, normally located downstream of the Toll/IL-1 receptor domain. Homozygous mutant female flies lay dorsalized embryos that are rescued by expression of a transgenic *DmMyD88* complementary DNA. The *DmMyD88* mutation blocks the ventralizing activity of a gain-of-function *Toll* mutation. These results show that *DmMyD88* encodes an essential component of the Toll pathway in dorsoventral pattern formation.

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INTRODUCTION

The Toll pathway was identified in *Drosophila* on the basis of its role in dorsoventral patterning during early embryogenesis. Genetic screens have led to the identification of maternal effect genes involved in the generation, transmission and interpretation of the signals specifying dorsoventral polarity in the embryo (reviewed in Belvin & Anderson, 1996). Loss-of-function mutations in 11 of these genes result in a common maternal-effect lethal phenotype: fertilized eggs laid by homozygous mutant females produce embryos in which all cells adopt the cell fate normally restricted to the cells on the dorsal surface of the embryo, thus resulting in hollow tubes of dorsal cuticle. Loss-of-function mutations in the twelfth gene, *cactus*, result in the opposite (ventralized) phenotype. Genetic and molecular studies are consistent with a model in which a proteolytic cascade activated on the ventral side of the

embryo generates an active ligand for the transmembrane receptor Toll (reviewed in LeMosy *et al.*, 1999). Activated Toll triggers phosphorylation and degradation of the inhibitor Cactus which releases the Rel transcription factor Dorsal, allowing its nuclear translocation. In the nucleus, Dorsal directs the expression of ventral-specific genes, such as *twist*, and represses dorsal-specific ones. Signal transduction from Toll to Cactus requires the proteins Tube and Pelle (Hecht & Anderson, 1993). These two proteins co-localize at the plasma membrane and interact through their death domains (Xiao *et al.*, 1999). Pelle is a serine/threonine kinase that can phosphorylate itself, and also Tube and Toll (Grosshans *et al.*, 1994; Shen & Manley, 1998; Towb *et al.*, 2001). How Pelle signals to Cactus is still unknown.

Components of this pathway between the putative Toll ligand Spätzle and Cactus also have a major role in *Drosophila* adults in the control of fungal and Gram-positive bacterial infections (reviewed in Hoffmann & Reichhart, 2002). The output of this pathway in adults is the nuclear translocation of the Rel protein Dorsal-related immunity factor (Dif), which upregulates the transcription of antimicrobial peptide genes such as *drosomycin* (Rutschmann *et al.*, 2000). The discovery of the critical role of Toll in innate immunity in flies led to the identification of homologous genes in mammals that have been called Toll-like receptors (TLRs) and shown to be required for the recognition of microbial ligands (reviewed in Imler & Hoffmann, 2001). TLRs and receptors of the interleukin-1 (IL-1) family interact with the protein MyD88 to activate the Rel transcription factor NF- κ B, and MyD88 interacts with the Pelle-related kinases of the IRAK family (Muzio *et al.*, 1997; Wesche *et al.*, 1997; Medzhitov *et al.*, 1998; Li *et al.*, 2002). These interactions are mediated by homophilic associations involving two well-defined structural domains of MyD88: the carboxy-terminal Toll/IL-1 receptor (TIR) domain interacts with the cognate domains in the intracytoplasmic tails of the TLRs (Medzhitov *et al.*, 1998), and the amino-terminal death domain mediates interaction with the corresponding domain of IRAK (Muzio *et al.*, 1997). Sequencing of the *Drosophila* genome led to the identification of a molecule related to MyD88 (*DmMyD88*) that interacts physically with Toll and with the kinase Pelle and functions upstream of Tube and Pelle (Horng & Medzhitov, 2001; Sun *et al.*, 2002; Tauszig-Delamasure *et al.*, 2002). *DmMyD88* differs from its mammalian counterpart by the presence of a

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162 amino-acid C-terminal extension following the TIR domain which is encoded by a separate exon. We have previously reported (Tauszig-Delamasure *et al.*, 2002) that flies carrying a transposon inserted at the 5' end of this gene have an impaired response to infection. We now report the identification of another mutant allele of *DmMyD88*, which encodes a protein devoid of its C-terminal extension. Analysis of these mutant flies reveals that *DmMyD88* encodes a component of the dorsoventral pathway in *Drosophila* embryos.

Fig. 1 | The PBc03881 transposon alters the translation of the maternally contributed *DmMyD88* transcript. (A) Structure and cytological position of the *DmMyD88* gene. The white boxes represent exons. The positions of the EP(2)2133 and PBc03881 transposable elements are indicated. At the bottom is shown the cytological map surrounding the *DmMyD88* locus and the extent of the DNA deleted by Df3591 and Df5423. (B) Northern blot analysis of poly(A)⁺ RNA extracted from 0–2-h and 6–18-h embryos. Maternal expression is observed for *Toll* and *DmMyD88*, but not for *18-wheeler* (*18w*), which was used as a control for the staging of the embryos. In this and the following northern blots, *rp49* is used as a loading control. (C) Northern blot analysis of poly(A)⁺ RNA extracted from 0–2-h embryos laid by wild-type (WT), *DmMyD88*^{PBc03881} and *DmMyD88*^{EP(2)2133} homozygous females. (D) Schematic representation (not drawn to scale) of the wild-type (WT) *DmMyD88* protein and the truncated *DmMyD88* protein encoded by *DmMyD88*^{PBc03881} homozygous flies. The chimaeric protein and cDNA sequences at the junction between *DmMyD88* (in bold) and *white* are shown beneath. Exon 4 of *DmMyD88* splices into exon 2 of *white* out of frame, resulting in a premature stop codon.

Table 1 | Viability of maternal and zygotic *DmMyD88* mutants

Genotype	Progeny viability (% hatched larvae)	Zygotic viability, obtained/expected (%)
EP(2)2133/EP(2)2133	82	ND
PBc03881/PBc03881	0	85
PBc03881/Df5423	92	112
PBc03881/Df3591	0	52
PBc03881*/PBc03881*	95	ND
PBc03881/PBc03881; UAS:DmMyD88/V ³² -Gal4	99	ND

In all cases, at least 100 individuals were scored. ND, not determined. The sterility phenotype of homozygous *DmMyD88*^{PBc03881} females and of *DmMyD88*^{PBc03881} females transheterozygous over a deficiency that uncovers *DmMyD88* (Df3591) is completely penetrant. This phenotype is rescued by excision of the transposon (PBc03881*) or by the expression of a *UAS:DmMyD88* transgene. *DmMyD88*^{PBc03881} homozygotes and transheterozygotes over a deficiency that uncovers *DmMyD88* (Df3591) have decreased viability compared with *DmMyD88*^{PBc03881} flies transheterozygous over a deficiency that does not uncover *DmMyD88* (Df5423).

RESULTS AND DISCUSSION

We showed previously that *DmMyD88* is a component of the Toll pathway operating during the immune response to infection in adult flies (Tauszig-Delamasure *et al.*, 2002). In particular, we described the phenotype of flies from the line EP(2)2133 (*DmMyD88*^{EP(2)2133}), which contains a P-element inserted in the non-coding region of the first exon of the *DmMyD88* gene (Fig. 1A). These flies, like *Toll* mutant flies, are highly susceptible to infection by fungi or by Gram-positive bacteria. However, *DmMyD88*^{EP(2)2133} female flies are fertile (Table 1), suggesting either that this insertion does not affect *DmMyD88* expression in the embryo or that *DmMyD88* is dispensable for the establishment of embryonic dorsoventral polarity. We found that *DmMyD88* messenger RNA is present in 0–2-h embryos (Fig. 1B; see also Fig. 4A), before zygotic expression begins, revealing the existence of a maternal contribution for this gene, as is true of the other genes of the Toll pathway (reviewed in Morisato & Anderson, 1995).

The *Drosophila* *DmMyD88* gene is composed of five exons. The fifth exon encodes a C-terminal domain following the essential TIR domain. This extension is not present in mammalian MyD88. A large-scale mutagenesis was performed by mobilizing a *piggyBac* transposable element (see Methods) (S. Thibault, personal communication). By inverse polymerase chain reaction (PCR) and DNA sequencing of *piggyBac* insertion sites, we identified a line containing an insertion in the last intron of *DmMyD88* (PBc03881; Fig. 1A). The level of *DmMyD88* transcript is not affected in preblastoderm embryos from mothers homozygous for the PBc03881 insertion (*DmMyD88*^{PBc03881}) (Fig. 1C) or in *DmMyD88*^{PBc03881} adult flies (data not shown), as opposed to the transcript in embryos from *DmMyD88*^{EP(2)2133} mothers, which is significantly reduced (Fig. 1C; Tauszig-Delamasure *et al.*, 2002). However, reverse-transcriptase-mediated PCR (RT-PCR) experiments revealed that the PBc03881 insertion interferes with the splicing of the *DmMyD88* transcript, resulting in a chimaeric mRNA in which the last exon of *DmMyD88* is replaced by exons 2, 3 and 4 of the *white* gene (data not shown) (see Goodwin *et al.*, 2000). This transcript encodes a truncated protein in which the C-terminal extension is replaced by 39 random amino acids (Fig. 1D).

Fig. 2 | *DmMyD88*^{PBc03881} homozygous adult flies present an immune phenotype. (A) Northern blot analysis of total RNA extracted from flies 48 h after pricking (+) or not (–) with a mixture of Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria. The probes used are indicated at the right. HM, homozygous. Inducible expression of *drosomycin* is strongly reduced in *DmMyD88* mutants. This phenotype is rescued by expression of *DmMyD88* through the UAS/Gal4 system using the *hsp-Gal4* driver. (B) Flies of the indicated genotype were infected naturally with *B. bassiana* spores and incubated at 29 °C. Live flies were counted daily to determine the percentage survival. *DmMyD88*^{PBc03881} homozygotes (open circles) were sensitive to infection. Normal survival was restored by expression of a UAS-*DmMyD88* transgene through the *hsp-Gal4* driver (filled circles). *DmMyD88*^{PBc03881} heterozygotes were used as wild-type control (open squares). (C) Deletion of the C-terminal extension of *DmMyD88* does not affect stability of the protein. S2 cells were transfected with plasmids expressing tagged versions of wild-type (WT) *DmMyD88* or a deletion mutant lacking the C-terminal extension (ΔCTE). At 48 h after transfection, the proteins were analysed by western blotting with an anti-V5 monoclonal antibody. (D) *DmMyD88*, but not the ΔCTE version, activates the *drosomycin* promoter. S2 cells were co-transfected with a *drosomycin*–luciferase reporter plasmid and an expression vector that was empty (–) or expressed wild-type (WT) *DmMyD88* or the ΔCTE deletion mutant. Results are means ± s.d. for three transfections.

We determined whether the *DmMyD88*^{PBc03881} homozygous adults present an immune phenotype similar to that of the previously characterized *DmMyD88*^{EP(2)2133} mutant strain (Tauszig-Delamasure et al., 2002). As shown in Fig. 2A, induction of the *drosomycin* gene in response to infection by a mixture of Gram-positive and Gram-negative bacteria or by fungi is severely reduced in *DmMyD88*^{PBc03881} homozygous flies, and this phenotype can be rescued upon expression of a *DmMyD88* complementary DNA through the UAS/Gal4 system (Brand & Perrimon, 1993). In agreement with these molecular data, *DmMyD88*^{PBc03881} homozygous

flies are highly susceptible to infection by fungi (Fig. 2B) and by Gram-positive bacteria (data not shown). Thus, PBc03881 is a new mutant allele of *DmMyD88*. The deletion of the C-terminal extension, or the addition of 39 random amino acids, might destabilize the protein. Alternatively, the C-terminal extension of *DmMyD88* might be required for the normal function of the protein. To test these possibilities, we constructed plasmids to express epitope-tagged versions of the wild-type protein and a truncated version containing only the death domain and the TIR domain (ΔCTE). After transfection of S2 cells, both proteins could be detected by western blotting, indicating that deletion of the C-terminal extension does not affect the stability of the protein (Fig. 2C). However, only overexpression of wild-type *DmMyD88* led to the induction of a *drosomycin* reporter construct in transfected cells, indicating that the C-terminal extension is important for *DmMyD88* function (Fig. 2D).

Although *DmMyD88*^{PBc03881} homozygous flies develop normally, we noticed that the females were sterile (Table 1). We analysed the cuticles secreted by the embryos laid by *DmMyD88*^{PBc03881} homozygous females and observed that foltz-körpers and ventral denticle belts were absent (compare Fig. 3B with Fig. 3A). These cuticular structures are representative of dorsolateral and ventral cell fates, respectively, and their absence is a characteristic feature of dorsalized embryos. In agreement with this observation, we were not able to detect the nuclear localization of Dorsal on the ventral side of these mutant embryos (compare Fig. 3D with Fig. 3C). In addition, in the same embryos, we did not observe expression of the Dorsal target gene *twist* (compare Fig. 3F with Fig. 3E). These results indicate that *DmMyD88* is required maternally for the establishment of the dorsoventral axis of the embryo. The fact that *DmMyD88*^{EP(2)2133} female flies are fertile implies that the level of maternal *DmMyD88* transcript is not crucial for the developmental function of *DmMyD88*. To confirm that the PBc03881 insertion affects *DmMyD88* function, we undertook several control experiments, summarized in Table 1. First, the sterility of *DmMyD88*^{PBc03881} females could be complemented by the deficiency Df5423 (which does not uncover *DmMyD88*) but not by Df3591 (which uncovers *DmMyD88*) (see Fig. 1A and Tauszig-Delamasure et al., 2002), indicating that it resulted from the insertion of the transposon and not from a second-site mutation. Second, we excised precisely the *piggyBac* element and observed that female fertility was restored. Finally, we were able to rescue the *DmMyD88*^{PBc03881} sterility phenotype by expressing a *DmMyD88* cDNA in the female germline through the UAS/Gal4 system. Taken together, these data demonstrate that the sterility of *DmMyD88*^{PBc03881} homozygous female flies is a consequence of a mutation in *DmMyD88*. To determine whether *DmMyD88* functions in the Toll pathway in the embryo, we used the *Toll*^{10B} allele, which is a dominant gain-of-function allele of *Toll* (Schneider et al., 1991). *Toll*^{10B} females generate ventralized embryos. We found that *Toll*^{10B} females homozygous for *DmMyD88*^{PBc03881} produced dorsalized embryos indistinguishable from those laid by *DmMyD88*^{PBc03881} homozygous females (data not shown). Moreover, embryos from *Toll*^{10B} mothers expressed *twist* both dorsally and ventrally (Fig. 3G; Roth et al., 1989), whereas embryos from *DmMyD88*^{PBc03881}; *Toll*^{10B} double-mutant females did not express *twist* (Fig. 3H), like those from *DmMyD88*^{PBc03881} homozygous females (Fig. 3F). Thus, these data show that *DmMyD88* is a component of the dorsal group of genes in the Toll pathway.

Fig. 3 | *DmMyD88*^{PBc03881} homozygous females lay dorsalized embryos. All embryos are oriented with the anterior end to the left and the dorsal side up. (A, C, E) Wild-type embryos. (B, D, F) Embryos laid by *DmMyD88*^{PBc03881} homozygous females. (G) Embryo laid by *DmMyD88*^{PBc03881}/*CyO*; *Toll*^{10B}/+ female. (H) Embryo laid by *DmMyD88*^{PBc03881}/*DmMyD88*^{PBc03881}; *Toll*^{10B}/+ female. (A, B) Cuticle preparations. The arrowhead points to the denticle belts and the arrow to the foltzkörper, which are absent from the mutant embryos. (C, D) Stage 5 embryos stained with anti-Dorsal (anti-Dl) antibodies. Dorsal is located in the ventral nuclei of wild-type embryos but not in embryos from *DmMyD88*^{PBc03881} mothers. (E–H) Stage 5 embryos stained with anti-Twist (anti-Twi) antibodies. *twist* is expressed ventrally in wild-type embryos (E) and all around in embryos from *Toll*^{10B} mothers (G) but is not expressed in embryos from *DmMyD88*^{PBc03881} homozygous females (F) and from females double mutant for *Toll*^{10B} and *DmMyD88*^{PBc03881} (H).

Zygotes that lack *DmMyD88* function develop to adults, indicating that *DmMyD88* is not absolutely required zygotically. To determine whether homozygous *DmMyD88*^{PBc03881} flies have decreased viability, we crossed females carrying the PBc03881 insertion balanced with the *CyO* balancer to males of the same genotype or to males of the *Df3591* or of the *Df5423* deficiencies balanced with *CyO*. In the progeny of these crosses, we counted the number of flies with straight wings (not *CyO*) and we calculated the percentage of flies obtained compared with those expected. As shown in Table 1, homozygous *DmMyD88*^{PBc03881} flies and transheterozygotes for *DmMyD88*^{PBc03881} over *Df3591* (*DmMyD88*[−]), but not over *Df5423* (*DmMyD88*⁺), have a decreased likelihood of survival. Similar observations have been made in the case of *tube* and *pelle*, although no precise zygotic defect leading to lethality could be associated with mutations in these genes (Hecht & Anderson, 1993).

To address a possible role of *DmMyD88* later in embryogenesis, we determined its pattern of expression by *in situ* hybridization. During germ band retraction, we observed strong expression in the migrating anterior and posterior midgut primordia (Fig. 4B). At stage

Fig. 4 | Embryonic expression of *DmMyD88*. *In situ* hybridization with a *DmMyD88* antisense strand probe. No specific signal was detected with the sense strand probe (data not shown). All embryos are oriented with the anterior end to the left. (A) Lateral view of a stage 2 embryo. *DmMyD88* transcript is maternally deposited in the embryo. (B) Lateral view of a stage 12 embryo. Zygotic expression is first seen in the anterior (arrowhead) and posterior (arrow) midgut invaginations. (C) Dorsal view of a stage 14 embryo. *DmMyD88* is expressed in the salivary glands (arrowhead), in four regions of the endoderm and in the anal plate (arrow). (D) Lateral view of a stage 16 embryo. *DmMyD88* transcript is detected in the salivary glands, in the anterior midgut and in the first (arrow) and third (arrowhead) midgut constrictions.

14, endoderm expression resolved into four bands along the gut and *DmMyD88* expression was also detected in the salivary glands and in the anal plate (Fig. 4C). Later on, at stage 16, weak expression was observed in the most anterior portion of the midgut and in the first and third midgut constrictions (Fig. 4D). Thus *DmMyD88* is not detected in the domains where *Toll* has been shown to have a zygotic function, namely the epidermis and the somatic musculature (Halfon & Keshishian, 1998), suggesting that *Toll* might function independently of *DmMyD88*.

In conclusion, we have uncovered a new function for the gene *DmMyD88*, which is its requirement for the establishment of the dorsoventral axis of *Drosophila* embryos. Our results suggest that the intracytoplasmic Toll pathway is essentially the same in the early embryo and in adult fat body cells, with the notable exception of the transcription factor Dif, which is not required during embryogenesis but has a crucial role in the Toll-mediated response to fungal and Gram-positive bacterial infections (Rutschmann et al., 2000, 2002). *DmMyD88* had not been identified in the screens searching for genes on the second chromosome specifying the dorsoventral pattern. It is possible that these screens were not completely saturating (Schupbach & Wieschaus, 1991) and that other genes that might not have been identified include those encoding the factors that bridge the kinase Pelle to the inhibitor Cactus. Our data also point to the importance of the C-terminal extension following the TIR domain. This extension is not present in the mammalian MyD88 but it is in mosquito MyD88 (Christophides et al., 2002). Further experiments will establish whether it affects the folding of the protein or its subcellular localization, or whether it mediates interaction with other components of the receptor complex.

METHODS

Northern blotting and RT-PCR. RNA was isolated with Trizol reagent (Gibco/BRL) in accordance with the manufacturer's instructions. Poly(A)⁺ RNA was prepared with oligo(dT)-coupled beads (Oligotex; Quiagen) and analysed by northern blotting through standard

procedures by using a probe spanning the TIR domain. RT-PCR was performed as described previously (Dimarcq et al., 1997).

Tissue culture and transfections. S2 cells were purchased from Invitrogen and grown at 23 °C in Schneider's medium (Biomedica) supplemented with 10% FCS. Cells (10^6 ml⁻¹) were transfected in 6-cm diameter dishes by the calcium phosphate precipitation technique with 0.1 µg of reporter plasmid, 0.1 µg of a β-galactosidase expression vector and 1 µg of expression vector. After 48 h, cells were lysed in reporter lysis buffer, and luciferase activity was measured in a luminometer (BCL Book; Promega) immediately after addition of the substrate (luciferin; Promega). β-Galactosidase activity was measured by using *o*-nitrophenyl-β-D-galactoside as a substrate, and the values were used to normalize variability in the efficiency of transfection. For the western blot experiment, cells were transfected with the Effectene transfection reagent (Qiagen). The *DmMyD88* expression plasmid pJL245 has been described previously (Tauszig-Delamasure et al., 2002). A PCR fragment containing nucleotides 1352–1741 of the *DmMyD88* cDNA (GenBank accession number AF422803) fused to sequences encoding the V5 epitope was digested by *Xba*I and *Not*I and used to replace the corresponding fragment of pJL245. The resulting plasmid (pJL289) encodes a truncated version of *DmMyD88* in which the 161 amino acids of the C-terminal extension are replaced by the 14 amino-acid sequence of the V5 epitope. Detection of the proteins by western blotting was performed as described (Tauszig-Delamasure et al., 2002).

Fly crosses. The *piggyBac* insertion studied in this work is a full-length *piggyBac* element containing the mini-white gene *w⁺mC* cloned into the *Hpa*I site (disrupting the transposase open reading frame). The transposase stock used to mobilize it was generated by cloning the *piggyBac* transposase downstream of the constitutive *Drosophila* α-1 tubulin promoter into a *w⁺* P-element (S. Thibault, personal communication). One hundred *DmMyD88^{PBc03881}/CyO*, α-1 tubulin-transposase males were crossed to *SM5/Lobe* females. From the progeny, two individuals with white eyes were obtained and used to establish stocks and to verify the excision of the *piggyBac* by PCR.

The *P{UAS-DmMyD88.JLI}* (*UAS-DmMyD88*) transgene used is described in Tauszig-Delamasure et al. (2002). To rescue the dorsalized *DmMyD88^{PBc03881}* phenotype, two independent *UAS-DmMyD88* lines (one on chromosome II and one on chromosome III) were used. The *V³²-Gal4* driver (Maxton-Küchenmeister et al., 1999) was used to obtain high level of maternal *Gal4* expression. The genotype of the females (obtained by standard crosses) put to lay was *DmMyD88^{PBc03881},V³²-Gal4/DmMyD88^{PBc03881}*, *UAS-DmMyD88/+* or *DmMyD88^{PBc03881}/DmMyD88^{PBc03881}*, *UAS-DmMyD88/V³²-Gal4*. For the rescue of the immune phenotype, we used the *hsp-Gal4* driver. Because of the leakiness of the heat-shock promoter, no heatshock was necessary to obtain rescue of the phenotype.

DmMyD88^{PBc03881}/Lobe;Toll^{10B}/TM3 males were crossed to *DmMyD88^{PBc03881}/CyO;+/+* females to generate *DmMyD88^{PBc03881}/DmMyD88^{PBc03881}; Toll^{10B}/+* and *DmMyD88^{PBc03881}/CyO; Toll^{10B}/+* females.

Cuticle preparation and embryo staining. To prepare cuticles, embryos were collected 24 h after laying, dechorionated in 50% bleach, devitelinized by vortex-mixing in equal volumes of heptane and methanol, rinsed with ethanol and transferred to a slide containing a drop of 1:2 lactic acid:Hoyer's solution (Wieschaus & Nusslein-Volhard, 1986). The slides were incubated overnight at 60 °C.

For immunohistochemistry and *in situ* hybridization, embryos were fixed in 4% formaldehyde. Primary antibodies were used at the following concentrations: rabbit anti-Twist (Roth et al., 1989) at 1:2000 and rabbit anti-Dorsal (Gillespie & Wasserman, 1994) at 1:1000. Detection of the primary antibodies was performed with biotinylated horse anti-rabbit IgG (1:500 dilution) and avidin-horseradish peroxidase (Vectastain Elite Kit; Vector Labs). For *in situ* hybridization, digoxigenin-labelled probes were generated in accordance to the manufacturer's instructions (Roche Diagnostics). Expressed sequence tag GH28482 (<http://flybase.bio.indiana.edu:82/>) containing the *DmMyD88* cDNA cloned in pOT2a was linearized with *Xho*I at the 3' end of the cDNA to produce the sense probe using T7 RNA polymerase, or with *Eco*RI at the 5' end to generate the anti-sense probe using Sp6 RNA polymerase. *In situ* hybridization was performed as described (Bernardoni et al., 1999), except that the signal was revealed with an alkaline-phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics).

Infection experiments. Septic injury was performed by pricking flies with a tungsten needle previously dipped into a concentrated culture of *Escherichia coli* and *Micrococcus luteus*, which induces an immune response. Flies were then kept at 25 °C for 48 h before extraction of their total RNA. For survival experiments, groups of 25 flies were manually shaken for 30 s on a Petri dish containing a sporulating culture of *Beauveria bassiana*. Flies that died in the first 3 h were discarded; surviving flies were kept at 29 °C and scored every 24 h for 8 days.

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