A Toll Receptor and a Cytokine, Toll5A and Spz1C, Are Involved in Toll Antifungal Immune Signaling in the Mosquito Aedes aegypti

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The fungal-specific immune response in the mosquito Aedes aegypti involves the Toll immune pathway transduced through REL1, a homologue of the NF-κB transcription factor Drosophila Dorsal. The Toll receptor and its ligand, Spätzle (Spz), link extracellular immune signals to the Toll intracellular transduction pathway. Five homologues to the Drosophila Toll (Toll1) receptor (Toll1A, Toll1B, Toll5A, Toll5B, and Toll4) and three homologues to the Drosophila cytokine Spätzle (Spz1A, 1B, and 1C) were identified from genomic and cDNA sequence data bases. Toll1A, Toll5A, Toll5B, and Spz1A were specifically induced in the mosquito fat body following fungal challenge. This transcriptional up-regulation was mediated by REL1. Spz1C was constitutively expressed in the mosquito fat body, whereas Spz1B and Toll4 were primarily expressed in ovarian tissues of female mosquitoes. The transcripts of Toll1B were induced in the mosquito fat body following fungal challenge. This transcriptional up-regulation was mediated by REL1. Spz1C was constitutively expressed in the mosquito fat body, whereas Spz1B and Toll4 were primarily expressed in ovarian tissues of female mosquitoes. The transcripts of Toll1B were induced in the mosquito fat body following fungal challenge. This transcriptional up-regulation was mediated by REL1. Spz1C was constitutively expressed in the mosquito fat body, whereas Spz1B and Toll4 were primarily expressed in ovarian tissues of female mosquitoes. The transcripts of Toll1B were induced in the mosquito fat body following fungal challenge. This transcriptional up-regulation was mediated by REL1. Spz1C was constitutively expressed in the mosquito fat body, whereas Spz1B and Toll4 were primarily expressed in ovarian tissues of female mosquitoes. The transcripts of Toll1B were induced in the mosquito fat body following fungal challenge. This transcriptional up-regulation was mediated by REL1. Spz1C was constitutively expressed in the mosquito fat body, whereas Spz1B and Toll4 were primarily expressed in ovarian tissues of female mosquitoes.

Mosquitoes are vectors of numerous human diseases, including malaria, which is responsible for over two million deaths per year (1, 2). Other mosquito-borne diseases, such as dengue fever (3), the West Nile encephalitis virus (4), and lymphatic filariasis (5), are also the cause of morbidity and mortality despite enormous control efforts. Innate immunity plays a pivotal role in the interaction between a pathogen and its vector; therefore, studies on vector immunity and its interaction with pathogens are essential for future development of novel control strategies against these vector-borne diseases.

In Drosophila, the Toll (Toll1) receptor was characterized genetically as a key component in dorsoventral patterning in embryogenesis (6, 7) and later shown also to signal for antifungal and anti-Gram (+) bacterial immune responses (8). A complex of signaling adaptors assembles around the TIR (Toll-Interleukin-1-Resistance) domain of Toll (9). Drosophila MyD88 (DmMyD88) connects Toll and kinase pelle, a homologue of mammalian IL-1R-associated kinases. Another adaptor molecule, Tube, interacts with both DmMyD88 and pelle. The immune signaling by Toll receptor activates two closely related NF-κB proteins, Dif (Dorsal-related immunity factor) in adults and Dorsal and/or Dif in larvae. Cactus, a Drosophila member of the IκB family of NF-κB inhibitors, retains Dif and/or Dorsal in inactive forms in the cytoplasm. Toll signaling activates the phosphorylation of Cactus, resulting in ubiquitination and proteasome-dependent degradation of Cactus. Dif and/or Dorsal then translocate to the nucleus, where they activate the transcription of target genes.

Both Drosophila Toll and mammalian Toll-like receptors are activated upon microbial challenge. Toll-like receptors have been shown to be directly stimulated by microbial molecules, whereas the activation of Drosophila Toll requires a cytokine Spätzle (Spz/Spz1), a member of the cysteine knot family of growth factors. Proteolytic processing of the secreted polypeptide Spz is necessary to activate Drosophila Toll immune pathway (10). Recently, the direct interaction between Toll and Spz has been described (11, 12), showing that the signal-dependent processing of the extracellular protein Spz and then the binding of the cleaved form (C-106) to Toll receptor are required for the activation of this immune pathway. There are five additional Spz homologues (Spz2–6) in the Drosophila genome; all contain a neutrophin-like cysteine knot domain (13). They may encode ligands for other members of the Drosophila Toll family, but their involvement in development or immunity has not yet been characterized. In Anopheles gambiae, six Spz homologues (Spz1–6) as a 1:1 orthologue of Drosophila Spz have been identified from the published genomic sequence (14).

In Drosophila, the Toll family of receptors comprises nine members, Toll and Toll-2 to Toll-9 (15). Although the dual function of Toll in immunity and development is apparent, the functions of Toll-2 to Toll-9 are currently under investigation.

Received for publication, September 18, 2006, and in revised form, October 24, 2006 Published, JBC Papers in Press, October 26, 2006, DOI 10.1074/jbc.M608912200

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3 The abbreviations used are: TIR, Toll-interleukin-1-resistance domain; Dif, Dorsal-related immunity factor; Spz, Spätzle; dsRNA, double-stranded RNA; RT-PCR, reverse transcription PCR; Vg, vitellogenic.

**This work was supported by National Institutes of Health Grant 1 RO1 AI059492. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) DQ437573 and DQ437574.
Toll-5 and -9 have been shown to activate the Drosomycin promoter in tissue culture cells (15, 16, 17). However, in vivo significance of these Toll receptors in immunity is still unclear. The other Toll receptors neither activate transcription of the genes encoding antimicrobial peptides nor interact with DmMyd88 (18). Most Tolls are highly expressed during embryogenesis and metamorphosis (15).

In mosquitoes, the expansion of Toll (Toll1) has been observed. Four genes from An. Gambiae, TOLL 1A, 1B, 5A, and 5B, form an orthologous group with the fruit fly Toll and Toll-5 (Tehao) (14). In Aedes aegypti, three homologous genes of Drosophila Toll (Toll-1) have been identified and named as AeToll1A, AeToll1B, and AeToll5 (19, 20). We renamed these Aedes Tolls as Toll1A, Toll1B, and Toll5A for the nomenclature convenience. In cell culture studies, both Toll1A and Toll5A were shown to activate the expression of a firefly luciferase gene under the control of Drosomycin promoter (20).

The Toll receptor and its ligand Spz link the extracellular immune signals to the Toll intracellular signal transduction pathway in Drosophila. Recently, by way of transgenic and RNA interference studies of REL1 (a homologue of Drosophila Dorsal), we characterized the mosquito Toll immune pathway as the antifungal immune response and the specific activation of Aedes Serpin-27A (Spn27A) (21, 22). Aedes Serpin27A is a homologue of Drosophila Spn27A and Anopheles Serpin-2, which have been shown to be key modulators of the Pro-phenoloxidase cascade (23, 24) and specifically activated by fungal challenge (21). We used these tools to define which specific Toll receptor and cytokine Spz were directly involved in the Toll immune pathway. Here, we present evidence that in Ae. aegypti Spz1C and Toll5A function as major mediators of this pathway.

**MATERIALS AND METHODS**

**Experimental Insects—**UGAL/Rockefeller, the wild-type strain of the Ae. aegypti mosquitoes, and two strains of transgenic mosquitoes, DE1L (gain-of-function of REL1) and iREL1 (loss-of-function of REL1) (22), were maintained in laboratory culture as described elsewhere (25). Adult mosquitoes were provided with water and a 10% sucrose solution. All dissections were performed in Ae. aegypti physiological saline (26).

**Data Base Searches and Phylogeny—**The cDNA and the predicted amino acid sequences were obtained by the TBLASTN search against nucleotide sequences published in the TIGR Ae. aegypti gene index (The TIGR Gene Index Databases, www.tigr.org/tdb/tgi, The Institute for Genomic Research, Rockville, MD). Ae. aegypti genomic sequence data base (7.63X sequence assembly by Aedes aegypti Sequencing Project, Broad Institute of Harvard & MIT (www.broad.mit.edu) and The Institute for Genomic Research (www.tigr.org)) deposited in the Broad Institute was queried by TBLASTN with Drosophila Toll and Spz. All hits were examined manually for the conservation of common domain structures of Toll receptor and Spz families. The TIR domains of various Toll receptors and neutrophin-like cysteine knot domains of Spzs were aligned using ClustalW, manually adjusted, and then subjected to phylogenetic analysis.

**Septic Injury, Natural Infection, and Survival Experiments—**Septic injuries were performed by pricking mosquitoes in the rear part of the abdomen with an acupuncture needle (0.20 × 25 mm) dipped into bacterial culture (stationary phase of bacteria in Luria Bertani broth; OD ~ 2.0) or a fungal spore suspension (~5 × 10^7 viable spores/ml) of B. bassiana strain GHA. The viable spore number was calculated by spreading the suspension onto Sabouraud dextrose-agar plates. Survival experiments were carried out under the same conditions. Following each dsRNA injection after 4–5 days recovery, groups of 20–25 Ae. aegypti female mosquitoes were challenged by a spore suspension. To test survival rate after natural infection, anesthetized mosquitoes were manually shaken for 10 s in a Petri dish containing B. bassiana spores.

**Infections and Northern Hybridization Analysis—**For the pathogen-specific study, 2- or 3-day-old adult Ae. aegypti females were injected with a stationary phase culture of Enterobacter cloacae, Micrococcus luteus, or a spore suspension of B. bassiana. For the stage-specific study, adult males and females, fourth instar larvae, and pupae were collected with and/or without the challenge of fungal spores. For the tissue-specific study, fat bodies, ovaries, and midguts were dissected from naïve mosquitoes and mosquitoes 1 day after fungal challenge. The Vg-ΔREL1-A transgenic and wild-type UGAL mosquitoes were fed at 2–3 days after emerging and collected at 24 h post-blood meal. The Vg-IRel1 transgenic and wild-type UGAL mosquitoes were forced to lay eggs at 3 days after the second blood feeding and then challenged with B. bassiana spores the next day. The mosquitoes were collected 1 day after challenge. For RNA interference experiments, the mosquitoes 5 days after RNA interference treatment were challenged with fungal spore and then collected 2 days later. Total RNA was prepared using TRIzol (Invitrogen). Samples of 5 μg of total RNA were separated on a formaldehyde gel, blotted, and hybridized with the corresponding DNA probe.

**Reverse Transcription and PCR—**Reverse transcription was carried out using an Omniscript reverse transcriptase kit (Qiagen) in a 20-μl reaction mixture containing oligo(dT) primers and 2 μg of total RNA at 37°C for 1 h. PCR was performed using Platinum High Fidelity Supermix (Invitrogen). The protocol for amplifying the cDNA product of Toll5B was 35 cycles of 95°C for 30 s and then 50°C for 30 s, followed by 72°C for 45 s.

**Synthesis and Microinjection of dsRNA—**Templates for the synthesis of dsRNA were generated using RT-PCR and incorporating a T7 phage promoter sequence into both sense and antisense primers. RT-PCR was performed using the Titan one-step RT-PCR kit (Roche Applied Science) with samples of 0.2 μg of total RNA as templates to generate a 400- to 600-bp gene-specific cDNA fragment. Tubes containing RNA and RNase inhibitor (1 u/μl; Roche Applied Science) were incubated for 30 min at 50°C for the RT reaction. Amplification conditions included rapid heating to 94°C for 2 min followed by 30 to 35 cycles of 55°C for 30 s, 72°C for 1 min, and 94°C for 30 s. PCR primers as follows (each primer contains T7 sequence at 5′): T7-Toll1A-Forward, 5′-T7-AATACGACTCATA-CTAGG; T7-Toll1A-Reverse, 5′-T7-CATCGTACGCTTGTGATAGTTGA- ACTGC-3′; T7-Toll1L-Reverse, 5′-T7-CATCGTACGCTTGAGT-LTCCGACGAAG-3′; T7-Toll1B-Forward, 5′-T7-CATCGTACGCTTGAGT-LTCCGACGAAG-3′; T7-Toll5A-Forward, 5′-T7-AACAATCTAAAACATAGCATC-ACCACATCCTTACTTGCAGACATG-3′; T7-Toll5B-Forward, 5′-T7-CTG-
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ATCACCTTTCGTCGCTGATGG-3', T7-Toll5B-Reverse, 5'-T7-ATGTGCCATGGCATATCTGAC-3'; T7-Toll4-Forward, 5'-T7-CTGGACAAGTCCGAGTCC-3', T7-Toll4-Reverse, 5'-T7-CATCGGTCACAAAACCTCTGACC-3'; T7-Spz1A-Forward, 5'-T7-CTGACACTAACTATGGCAG-3', T7-Spz1A-Reverse, 5'-T7-TGTCCTCCTGCCTTCGTCGCTGATGG-3'; T7-Spz1B-Forward, 5'-T7-CCTGTGTAAGCAGACAGAC-3', T7-Spz1B-Reverse, 5'-T7-CAGAAGATCCTGCTGATG-3'; T7-Spz1C-Forward, 5'-T7-AACTCTTACTTATGGTTGTCGTT-3', T7-Spz1C-Reverse, 5'-T7-CACATTACAGCAGCTTGGAC-3'.

Synthesis of dsRNA was accomplished by simultaneous transcription of both strands of template DNA using the MEGAscript kit (Ambion). The plasmid LITMUS 28iMal containing a nonfunctional portion of the Escherichia coli male gene that encodes maltose-binding protein was used to generate control dsRNA. After RNA synthesis, the samples were treated by means of phenol/chloroform extraction and ethanol precipitation. dsRNA. After RNA synthesis, the samples were treated by means of phenol/chloroform extraction and ethanol precipitation.

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**RESULTS**

Diversity Expansion of Toll and Spz in Ae. aegypti—Four genes from *An. gambiae* (TOLL 1A, 1B, 5A and 5B) and three from *Ae. aegypti* (Toll1A, Toll1B, and Toll5A) have been suggested to form an orthologous group with the fruit fly Toll (Toll-1) and Toll-5 (Tehao) (14, 19). An additional search using TBLASTN against the Ae. aegypti genome sequence revealed two more genomic loci encoding possible orthologues of *Drosophila* Toll and Toll-5. The open reading frames from these two loci were predicted using FGENESH+ (27) (sun1.softberry.com) by comparing ~100 kb of the flanking genomic DNA sequence of a possible *Ae. aegypti* orthologue region with the amino acid sequence of *Drosophila* Toll, and these new Toll homologues were named Toll5B and Toll4, respectively, based on the phylogenetic relationship with *Drosophila* and *Anopheles* Tolls (Fig. 1A). The partial cDNA sequences of these Toll genes have been deposited in the GenBank™ data base (accession number DQ437573 for Toll5B, DQ437574 for Toll4). Toll-related receptors are characterized by TIR, a 150-amino acid intra-cytoplasmic domain, which has been found in members of the interleukin-1 receptor family and plant disease resistance genes (28). Although the presence of C-terminal extension distinguished the Toll1 group (*Drosophila* Toll, *Anopheles* Toll1A and Toll1B, *Aedes* Toll1A and Toll1B) from Toll5 group (*Drosophila* Toll-5, *Anopheles* Toll5A and Toll5B, *Aedes* Toll5A) (19, 24), our phylogenetic studies with whole published amino acid sequences (data not shown) or TIR domains (Fig. 1A) of Toll receptors indicated that these five *Aedes* Tolls (Toll1A, Toll1B, Toll5A, Toll5B, and Toll4) and four *Anopheles* Tolls (TOLL1A, TOLL1B, TOLL5A, and TOLL5B) formed a phylogenetic cluster with *Drosophila* Toll and Toll-5 (Fig. 1A), which are known to function in *Drosophila* immunity. Interestingly, eight of the nine mosquito Tolls (all except *Aedes* Toll4) were grouped together with 90% bootstrap values, suggesting that these mosquito Toll proteins have evolved from a common ancestor.

The TBLASTN search against nucleotide sequences published in the TIGR *Ae. aegypti* gene index indicated four sequences (TC36481, TC47492, TC39244, and TC47353) encoding each deduced protein similar to *Drosophila* Spz. In addition, we identified four more genomic loci encoding genes homologous to *Drosophila* Spz2, Spz4, Spz5, and Spz6 from the currently published genomic sequences of *Ae. aegypti*. In *An. gambiae*, six Spz homologues (Spz1–6) have been identified in the genomic sequence (19); however, we could not use *Anopheles* Spz1 sequences in multiple alignment and phylogenetic analyses shown in Fig. 1, B and C, because the partial sequence published as *Anopheles* Spz1 is, as yet, missing the neutrophin-like cysteine knot domain (so-called C-106 region in *Drosophila* Spz) common to various Spz proteins. The phylogenetic studies using aligned C-106 of each Spz homologue suggest that three of them (TC36481, TC47492, and TC39244) belong to an orthologue group with *Drosophila* Spz, whereas five others form a 1:1 orthologue group with both *Drosophila* and *Anopheles* Spz2 to Spz6 (Fig. 1, B and C). *Aedes* Spz6 sequences were aligned with internal deletion of 73 amino acids (72 amino acids in *Drosophila* Spz6) based on the alignment of *Drosophila* Spz homologues (10).

The Tissue-specific Expression Profiles of Tolls and Spzs in *Ae. aegypti*—In *Drosophila*, the Toll pathway mediates immune responses specific to fungal and Gram (+) bacterial infections. The Toll (Toll-1) is the only identified Toll receptor involved in this pathway. We have chosen to limit our analysis to *Ae. aegypti* Tolls (Toll1A, 1B, 5A, 5B, and Toll4), which were grouped with *Drosophila* Toll (Fig. 1A). To understand possible respective functions of each *Ae. aegypti* Toll and Spz in immunity and/or embryogenesis, we first focused on the characterization of tissue-specific expression of the five Tolls and three Spz homologues (Spz1A, 1B, and 1C) with or without fungal challenge (Fig. 2). In addition, we tested gene expression during ovarian and embryonic development (Fig. 3). The transcript sizes for Toll1A or Toll5A were 4–5 kb. A 3- to 4-kb band was observed by means of Northern analysis of Toll1B or Toll4. The transcript sizes for Spz1A, Spz1B, and Spz1C were ~1.5, ~0.9, and ~1.2 kb, respectively.

Of the five *Aedes* Tolls, the transcripts of Toll5A, Toll1A, and Toll5B were inducible by fungal infection in the mosquito fat body (Figs. 2 and 3), suggesting their involvement in mosquito immunity. Among these three Tolls, Toll5A was the most strongly activated in fat bodies and also inducible in ovarian tissues in an expression pattern very similar to that of Spn27A (Fig. 2), which was shown to be regulated by REL1 transcription factor (21, 22). Toll1A expression was weakly inducible in fat bodies, whereas strong non-inducible expression of Toll1A was observed in ovarian tissues (Fig. 2A). The expression of Toll5B was not detected using Northern analyses, but RT-PCR amplification of Toll5B mRNA suggested its inducible expression in the mosquito fat bodies (Fig. 2B). Toll4 was mainly expressed in ovarian tissues, although it was weakly expressed in a non-inducible manner in fat bodies (Fig. 2A). The expression of...
**Toll1B** was not detected either in fat bodies or ovarian tissues using Northern analyses or RT-PCR (Fig. 3A).

The gene expression profiles of three *Aedes* homologues of *Drosophila* Spz were quite distinct from each other. Spz1A was expressed in fat bodies and ovarian tissues in an inducible manner by fungal challenge, whereas the expressions of Spz1B and Spz1C were non-inducible (Fig. 2A). Interestingly, the expression of Spz1B was only observed in the ovary and that of Spz1C mainly in the fat body (Fig. 2A). The expressions of all tested Tolls and Spzs were not observed in the midgut (Fig. 2A). We tested these Tolls and Spzs expression patterns during mosquito ovary and egg development (Fig. 3). Toll5A, Toll1A, Toll4, and Spz1B were expressed in pre-, vitellogenic, and postvitellogenic ovaries but not in eggs, suggesting their maternal origin. On the contrary, the expression of Toll1B was detected only in 1-day-old eggs and not during any other developmental stage (Fig. 3). These results suggest that Toll1B likely plays a role exclusively in embryonic development and that it is likely of zygotic origin.

**The Stage-specific and Pathogen-specific Expression Profiles of Tolls and Spzs in *Ae. aegypti***—Northern blot analyses were performed to examine the expression profiles of Tolls and Spzs in fourth instar larvae and in female and adult male mosquitoes after fungal challenge (Fig. 4A). Only naïve pupae were sampled because septic injury was lethal to mosquitoes at the pupal stage. The expressions of Toll5A and Toll1A were elevated after fungal challenge in larvae and in female and male mosquitoes (Fig. 4A). RT-PCR amplification of Toll5B mRNA showed inducible expression of Toll5B in larvae and in female and male mosquitoes after fungal infection (Fig. 4B). The expression of Spz1A and Spn27A was elevated in adult mosquitoes, but not in larvae, suggesting that there are different mechanisms of Toll immune activation between larvae and adult mosquitoes. Toll4 and Spz1C were constitutively expressed independently of fun-
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Expression of Spz1B was only detected in female mosquitoes (Fig. 4A), in accord with its strict ovary-specific expression (Fig. 2A).

Next, we tested the expression profiles of the inducible immune genes (Toll5A, Toll1A, and Spz1A) after infection with different types of pathogens, Gram-negative bacteria (E. coli), Gram-positive bacteria (Micrococcus luteus), and fungal spores (B. bassiana) (Fig. 4C). The expression levels were elevated independently of the type of infection, although they were more specifically elicited by fungal infection (Fig. 4C). We could not determine a specific activation of Toll5B after bacterial challenge (data not shown). The expression of Toll1B, Toll4, Spz1B, and Spz1C was not inducible by any type of septic injury (Fig. 3A and data not shown).

Transcriptional Activation of Aedes Tolls by REL1 Transcription Factor—Autoregulation of pathway components by Drosophila Toll pathway has been reported for Spz, Toll, pelle, Cactus, and Dif (29). The fungal-specific activation profiles of both Toll5A and Toll1A were reminiscent of those of Aedes Spn27A (21) and Spz1A (Fig. 4C), which have been shown to be regulated by the Aedes Toll/REL1 immune pathway (21, 22). To understand the role of the Toll/REL1 immune pathway in regulating the expression of both Toll genes, we utilized transgenic mosquitoes with an altered Toll/REL1 pathway. To generate the REL1 gain-of-function phenotype with overexpressed REL1 isoforms, the Vg-DREL1-A transgenic mosquitoes were fed on blood and examined 24 h later (22). To generate the REL1 loss-of-function phenotype with knockdown REL1 transcripts, the Vg-iREL1 transgenic mosquitoes were forced to lay eggs 3 days after the second blood feeding (22). The expression of both Toll5A and Toll1A was fully activated in the Vg-DREL1-A transgenic mosquitoes without any septic injury but was not in the Vg-iREL1 transgenic mosquitoes after infection with B. bassiana spores (Fig. 5, A and B). RT-PCR analyses of Toll5B in transgenic mosquitoes also showed that the gene was activated by REL1 transcription factor (Fig. 5, C and D), confirming that the expression of these three Aedes Tolls is regulated by the Toll/REL1 pathway.

Toll Immune Signaling by Spz1C and Toll5A in Ae. aegypti—To understand possible roles of each Aedes Toll in Toll/REL1 immune signaling, we tested the expression of mosquito...
immune genes after treatment with dsRNAs complementary to the three Tolls, Toll5A, Toll1A, and Toll5B. When each dsRNA was introduced into the mosquito, the mRNA level of a corresponding transcript clearly decreased, showing the effective knock down by RNA interference (Fig. 6, A and B). The mRNA levels of Aedes Spn27A and Spz1A significantly declined in REL1A and Toll5A dsRNA-treated mosquitoes (Fig. 6A), suggesting that Toll5A is a major Toll receptor involved in the Toll/REL1 immune pathway. In addition, we found a clear reduction of expression levels of both Toll1A and Toll5B in both REL1 and Toll5A dsRNA-treated mosquitoes (Fig. 6A). In Toll1A dsRNA-treated mosquitoes, we were able to find a subtle decrease of mosquito immune genes (Fig. 6A). No clear difference in immune gene expression was found in MalE or Toll5B dsRNA-treated mosquitoes (Fig. 6A). In the independent experiment, the Toll4 dsRNA treatment did not affect immune gene expression in Ae. aegypti (data not shown).

Drosophil a Spz (Spz1), a cytokine, directly binds to Drosophila Toll (Toll1) activating the Toll immune pathway (11, 12). We investigated possible roles of each Aedes Spz homologue in the mosquito Toll/REL1 immune pathway by means of RNA interference-mediated-specific knock downs. The mRNA level decreased appreciably after introduction of each dsRNA (Spz1A, Spz1B, and Spz1C, respectively), showing the effective knock down by RNA interference (Fig. 6C). We observed a manifest decline of Aedes Spn27A and Toll5A gene expression only in Spz1C dsRNA-treated mosquitoes (Fig. 6C), suggesting that Spz1C is involved in the activation of the Toll/REL1 immune pathway. However, we could not find any effect on the Spz1A expression level in Spz1C dsRNA-treated mosquitoes (Fig. 6C). Expression levels of Spn27A or Toll5A were not changed in Spz1A or Spz1B dsRNA-treated mosquitoes (Fig. 6C).
To address whether Toll5A and Spz1C were affected during the REL2-mediated immune response (30, 31), we compared the expression of Spn27A and Defensin A (DefA) (Fig. 7) after treatments with dsRNAs complementary to Toll5A, Spz1C, REL1, and REL2 and respective challenges with B. bassiana and E. cloacae (Fig. 7, A and B). The expression of Spn27A was not affected by REL2 dsRNA treatment (Fig. 7A). In contrast, DefA expression was only impaired after REL2 dsRNA treatment and the subsequent challenge with E. cloacae (Fig. 7B). These results showed that Toll5A and Spz1C are specific components of the Toll/REL1 pathway in Ae. aegypti.

**Toll Antifungal Immune Response by Toll5A and Spz1C in Ae. aegypti**—When Toll5A or Spz1C dsRNA-treated mosquitoes were challenged by septic injury with B. bassiana spores, they were significantly sensitive to the fungal infection, similar to the REL1 dsRNA-treated mosquitoes (Fig. 8, A and B). In experiments in which the mosquitoes were naturally infected by B. bassiana spores, the survival tests after Toll5A or Spz1C dsRNA treatment resulted in a partial increase of fungal susceptibility (Fig. 8C). The dsRNA treatment of Toll1A (Fig. 8A) and Spz1A (Fig. 8B) did not affect fungal susceptibility of Ae. aegypti. These results indicate that Toll5A and Spz1C are likely the specific Toll receptor and cytokine, respectively, involved in the Toll/REL1 systemic immune pathway of Ae. aegypti.

**DISCUSSION**

The *Drosophila* Toll receptor (Toll/Toll-1) and a cytokine, Spz (Spz1), have been identified as key signaling components of the *Drosophila* Toll immune pathway (32). Our searches for the mosquito homologues of *Drosophila* Toll and Spz and their phylogenetic analyses indicate that the diversity of Toll receptors and cytokines has expanded in the mosquito *Ae. aegypti*, suggesting possible new aspects of the Toll immune pathway in mosquito immunity.

An insect fat body is the major organ of systemic immune response upon microbial infection. Our previous studies involving fat body-specific transgenic alterations of the Toll/REL1 pathway were carried out in *Ae. aegypti*, producing immune-independent gain-of-function and loss-of-function phenotypes and have confirmed a central role of REL1 in the Toll pathway (21, 22). In this work, we addressed questions regarding the fat body specificity of the initial signaling molecules, the Toll receptor and its ligand Spz. The dsRNA knock down of Toll5A and Spz1C resulted in increased fungal susceptibility and reduced Spn27A gene expression, which are two representative phenotypes of the mosquito Toll/REL1 immune pathway. Thus, our results strongly suggest that, among diversified Tolls and Spzs in *Aedes* mosquitoes, both Toll5A and Spz1C have a major role in the fat body-specific activation of the Toll/REL1 immune pathway.

The observed phenotypes further indicate that Spz1C may be a direct ligand of Toll5A and their interaction triggers the Toll immune signal transduction to the REL1/Cactus cassette. However, the role of Spz1A in Toll signaling remains unclear. The transcript level of Spz1A was affected in Toll5A dsRNA-treated mosquitoes, but not in those of Spz1C. Experiments with the gain-of-function or loss-of-function
transgenic mosquitoes of REL1 have shown that Spz1A gene expression is regulated by REL1/Cactus cassette (22). Taken together, these data suggest activation of Spz1A occurs via the Spz1C-independent mechanism through Toll5/REL1/Cactus signaling. Mammalian toll-like receptors induce multiple inflammatory cytokines, including interferons, that express antiviral, antiangiogenic, and immunoregulatory properties (33). Our data presented here suggest that Spz1A is a mosquito cytokine that is induced by mosquito Toll immune signaling. Its role in mosquito immunity requires further investigation.

The expression patterns of Tolls in Ae. aegypti propose a clue as to their functions. Toll genes have distinct expression patterns in Drosophila embryos, and these complex tissue-specific patterns of expression suggest a role in embryonic development for most Tolls in Drosophila (34). Similarly, the expression of Toll1B and Toll4 was independent of immune challenge and mainly expressed in the tissues involved in mosquito development. In particular, Toll1B is only expressed at the early stage of laid eggs, suggesting its role in embryonic development. Although Toll1A, Toll5A, and Toll5B were expressed in the ovary, they were also induced by fungal challenge in the mosquito fat body, suggesting roles in mosquito immunity. In cell culture studies, Toll1A and Toll5A were shown to activate the expression of a firefly luciferase gene under the control of a Drosomycin promoter (20). As previously described, the expression of Toll1A and Toll5B is dependent on the Toll/REL1 immune pathway, suggesting a possible role for these Toll receptors in mosquito immunity. Indeed, treatment with Toll1A dsRNA reduced the constitutive level of Toll5A transcripts when the test was done without fungal challenge (data not shown). Moreover, in experiments where the mosquitoes were naturally infected by B. bassiana spores, the survival tests after Toll5A or Spz1C dsRNA treatment resulted in only partial increase of the fungal susceptibility when compared with that after REL1 dsRNA treatment (Fig. 8C). These results indicate that, in addition to Toll5A, Toll1A and/or Toll5B could play roles in the activation of the antifungal Toll/REL1 immune pathway. The specific immune functions of Toll1A and Toll5B require further investigation.

Acknowledgments—We thank Kanwal S. Alvarez and Aileen M. McAinsh for editing the manuscript.

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