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A Toll-Spätzle Pathway in the Tobacco Hornworm, *Manduca sexta*

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Abstract

Insects synthesize a battery of antimicrobial peptides (AMPs) and expression of AMP genes is regulated by the Toll and Imd (immune deficiency) pathways in *Drosophila melanogaster*. *Drosophila* Toll pathway is activated after Spätzle (Spz) is cleaved by Spätzle processing enzyme (SPE) to release the active C-terminal C106 domain (*DmSpz*-C106), which then binds to the Toll receptor to initiate the signaling pathway and regulate expression of AMP genes such as *drosomycin*. Toll and Spz genes have been identified in other insects, but interaction between Toll and Spz and direct evidence for a Toll-Spz pathway in other insect species have not been demonstrated. Our aim is to investigate a Toll-Spz pathway in *Manduca sexta*, and compare *M. sexta* and *D. melanogaster* Toll-Spz pathways. Co-immunoprecipitation (Co-IP) assays showed that *MsToll*^{ecto} (the ecto-domain of *M. sexta* Toll) could interact with *MsSpz*-C108 (the active C-terminal C108 domain of *M. sexta* Spz) but not with full-length *MsSpz*, and *DmToll*^{ecto} could interact with *DmSpz*-C106 but not *DmSpz*, suggesting that Toll receptor only binds to the active C-terminal domain of Spz. Co-expression of *MsToll*-*MsSpz*-C108, but not *MsToll*-*MsSpz*, could up-regulate expression of *drosomycin* gene in *Drosophila* S2 cells, indicating that *MsToll*-*MsSpz*-C108 complex can activate the Toll signaling pathway. *In vivo* assays showed that activation of AMP genes, including *cecropin*, *attacin*, *moricin* and *lebobcin*, in *M. sexta* larvae by purified recombinant *MsSpz*-C108 could be blocked by pre-injection of antibody to *MsToll*, further confirming a Toll-Spz pathway in *M. sexta*, a lepidopteran insect.

Keywords

Toll; Spätzle; antimicrobial peptide; *drosomycin*; *moricin*; antibody-blocking

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1. Introduction

The innate and adaptive immune systems are two major branches of the defense system in multicellular organisms. Innate immunity is the first defensive line that controls initial steps of immune responses. It can also profoundly impact the establishment of adaptive immune responses (Medzhitov and Janeway, 2000; Medzhitov and Janeway, 1997). In innate immune responses, a group of germline-encoded pattern recognition receptors (PRRs) can recognize and bind to conserved pathogen-associated molecular patterns (PAMPs) present on the invading microorganisms, such as bacteria and fungi, but not on the host cells (Janeway, 1989; Medzhitov and Janeway, 1997). The innate immune system is composed of humoral and cellular components. Cellular immune responses mainly include blood cells (hemocytes)-mediated responses such as nodule formation, phagocytosis and melanotic encapsulation, whereas synthesis of antimicrobial peptides (AMPs) and activation of the prophenoloxidase system are major components of humoral immune responses (Kanost et al., 2004; Rao et al., 2010). Expression of AMP genes in *Drosophila melanogaster* is regulated by the Toll and immune deficiency (Imd) pathways (Choe et al., 2002; De Gregorio et al., 2002; Lemaitre et al., 1995; Lemaitre et al., 1996; Ramet et al., 2002). *Drosophila* Toll pathway is activated by Gram-positive bacteria and fungi, resulting in systemic production of AMPs (Aggarwal and Silverman, 2008; Hetru and Hoffmann, 2009). Moreover, the Toll signaling pathway and other pathways are involved in controlling hemocyte proliferation and density (Sorrentino et al., 2004; Zettervall et al., 2004), as well as melanization (Bettencourt et al., 2004).

The Toll pathway was initially identified in early *Drosophila* embryonic development, the dorsal – ventral (DV) patterning of the embryo, and the dorsal group of genes includes *Toll*, *tube*, *pelle*, *cactus*, the NF- κ B homolog *dorsal*, and seven genes up-stream of the *Toll* (Belvin and Anderson, 1996; Morisato and Anderson, 1995). Tolls and Toll-like receptors (TLRs) have been identified in many animal species, including mammals (Shinkai et al., 2006; Takeuchi and Akira, 2010), chicken (Fukui et al., 2001), fish (Tsujita et al., 2004), insects (Ao et al., 2008; Christophides et al., 2002; Evans et al., 2006; Imamura and Yamakawa, 2002; Kanzok et al., 2004; Luna et al., 2002; Yamagata et al., 1994), shrimp (Yang et al., 2007), and sponge (Wiens et al., 2007). However, mammalian TLRs function as pattern recognition receptors but do not have a role in development (Kimbrell and Beutler, 2001), whereas the *Drosophila* Toll pathway is involved in both immunity (Lemaitre et al., 1996) and developmental processes (Belvin and Anderson, 1996; Halfon et al., 1995; Qiu et al., 1998).

Activation of the *Drosophila* Toll pathway is preceded by activation of Spätzle (Spz), the Toll receptor ligand (Morisato and Anderson, 1994; Schneider et al., 1994). Under non-signaling conditions, a predominantly hydrophobic C-terminal domain of Spz is masked by a prodomain of Spz. Embryonic dorsal-ventral patterning, Gram-positive bacterial and fungal cell wall components and virulence factors can activate Spz (Valanne et al., 2011). Spz is processed into its active C-terminal C-106 domain in a process that involves activation of a cascade of serine proteinases. Proteolysis of Spz causes a conformational change, which exposes determinants of C-106 domain that are critical for binding to the Toll receptor (Arnot et al., 2010). Two Spz-C106 dimers bind to two Toll receptors and the binding triggers a conformational change in the Toll receptors to form stable dimers (Hu et al., 2004). The dimeric Toll complexes interact with an adaptor protein MyD88 via intracellular TIR (Toll-interleukin 1 resistance) domains (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). Tube and kinase Pelle are recruited by MyD88 to form a MyD88-Tube-Pelle heterotrimeric complex through their death domain (DD)-mediated interactions (Moncrieffe et al., 2008; Sun et al., 2002; Xiao et al., 1999). Intracellular signaling leads to phosphorylation and degradation of Cactus and release of

Dorsal-related immunity factor (Dif) and/or Dorsal, which translocate to the nucleus and activate transcription of AMP genes (Imler and Hoffmann, 2001; Wu and Anderson, 1998).

The Toll signaling pathway has been well studied in *D. melanogaster*, but less characterized in other insect species. Although *Toll* and *Spz* genes have been identified in other insects, including *Anopheles gambiae* (Christophides et al., 2002; Luna et al., 2002), *Aedes aegypti* (Kanzok et al., 2004), *Apis mellifera* (Evans et al., 2006), *Bombyx mori* (Imamura and Yamakawa, 2002; Wang et al., 2007), and *Manduca sexta* (An et al., 2010; Ao et al., 2008), interaction between a Toll receptor and a Spz from the same insect species other than *D. melanogaster* has not been demonstrated. We previously discovered a Toll receptor from *M. sexta* (Ao et al., 2008), and *Spz-1* gene has also been identified (An et al., 2010). *M. sexta* Spz-1A (*MsSpz*) was cleaved and activated by proteinase HP8 to release the active C-terminal domain *MsSpz*-C108 (An et al., 2010). Injection of *MsSpz*-C108 into *M. sexta* larvae can up-regulate several AMP genes (An et al., 2010), suggesting that there is a Toll pathway in *M. sexta*. In this study, we showed direct interaction between *M. sexta* Toll (*MsToll*) and *MsSpz*-C108 and further confirmed a Toll-Spz pathway in *M. sexta* by both *in vitro* and *in vivo* assays. We established stable *Drosophila* S2 cell lines expressing *M. sexta* and *D. melanogaster* Tolls (*MsToll* and *DmToll*) and their ecto-domains (*MsToll*^{ecto} and *DmToll*^{ecto}), Spz proteins (*MsSpz* and *DmSpz*) and their active C-terminal domains (*MsSpz*-C108 and *DmSpz*-C106). Co-immunoprecipitation (Co-IP) assays showed that *MsToll*^{ecto} and *DmToll*^{ecto} could interact with *MsSpz*-C108 and *DmSpz*-C106, but not *MsSpz* and *DmSpz*, respectively. Co-expression of *MsToll*-*MsSpz*-C108 and *DmToll*-*DmSpz*-C106 in S2 cells could up-regulate *drosomycin* but not *diptericycin* gene. Activation of AMP genes, including *cecropin-6*, *attacin-1*, *attacin-2*, *moracin* and *lebocin*, by recombinant *MsSpz*-C108, *Staphylococcus aureus* and *Escherichia coli* peptidoglycans in *M. sexta* larvae could be blocked by pre-injection of antibody to *MsToll*. Our results demonstrated a Toll-Spz pathway in *M. sexta*, a lepidopteran insect.

2. Materials and Methods

2.1 Insect rearing and cell line

M. sexta eggs were originally purchased from Carolina Biological Supplies (Burlington, NC, USA). Larvae were reared on an artificial diet at 25°C (Dunn and Drake, 1983), and the fifth instar larvae were used for the experiments. *D. melanogaster* Schneider S2 cells were purchased from American Type Culture Collection (ATCC).

2.2 Construction of recombinant pMT/BiP/V5-His A expression vectors

cDNA fragments encoding *MsToll* (residues 13–963), *MsToll*^{ecto} (residues 13–718), *MsTIR* (residues 766–963), *MsSpz* (residues 20–295), *MsSpz*-C108 (residues 188–295), *DmToll* (residues 28–1097), *DmToll*^{ecto} (residues 28–805), *DmTIR* (residues 858–1097), *DmSpz* (residues 26–244), and *DmSpz*-C106 (residues 139–244) were amplified by PCR using forward and reverse primers listed in Table S1. Forward primers for *MsSpz*, *MsSpz*-C108, *DmSpz* and *DmSpz*-C106 contain codons for an in-frame Flag sequence and a *Kpn* I site, while reverse primers contain a stop codon followed by a *Pme* I site. Forward primers for *MsToll*, *MsToll*^{ecto}, *MsTIR*, *DmToll*, *DmToll*^{ecto} and *DmTIR* contain a *Kpn* I site, while reverse primers contain an *Apa* I site. PCR reactions were performed with the following conditions: 94°C for 3 min, 35 cycles of 94°C for 30s, Tm-5°C for 30s, 72°C for 30s to 4min, followed by a final extension at 72°C for 10min. The PCR products were recovered by agarose gel electrophoresis-Wizard® SV Gel and PCR Clean-Up System (A9285, Promega) and subcloned into T-Easy vectors (A1360, Promega). Plasmid DNAs in T-vectors were purified using PureYield™ Plasmid Miniprep System (A1222, Promega) according to the manufacturer's instruction and digested with *Kpn* I/*Pme* I or *Kpn* I/*Apa* I,

DNA fragments were recovered and inserted into *Kpn*I/*Pme*I or *Kpn*I/*Apa*I digested pMT/BiP/V5-His A vector (V413020, Invitrogen) using T4 DNA ligase (M0202L, NEB). Recombinant expression vectors were then purified and sequenced by an Applied Biosystems 3730 DNA Analyzer in the DNA Sequencing and Genotyping Facility at University of Missouri – Kansas City, and used to generate stable S2 cell lines.

2.3 Cell culture and establishment of stable S2 cell lines

D. melanogaster Schneider S2 cells were maintained at 27°C in Insect Cell Culture Media (SH30610.02, Hyclone), supplemented with 10% heat-inactivated fetal bovine serum (#10082063, Invitrogen) containing 1% penicillin-streptomycin solution (G6784, Sigma-Aldrich). For DNA transfection, cells were seeded overnight in serum-free medium (SH30278.01, Hyclone). GenCarrier-1™ transfection reagent (#31-00110, Epoch Biolabs) was used for transient transfection based on the manufacturer's instructions. Cells in culture dishes or plates were grown to 70% confluence prior to transfection. DES®-Inducible/Secreted Kit with pCoBlast (K5130-01, Invitrogen) was used to construct stable S2 cell lines. To select stable S2 cells expressing recombinant proteins, pCoBlast (Invitrogen) was cotransfected with recombinant pMT/BiP/V5-His A vectors. After 48h transfection, S2 cells were centrifuged and re-suspended in complete growth medium containing 25µg/ml Blasticidine S hydrochloride (No.15205, Sigma-Aldrich). Resistant colonies appeared 1 week later.

2.4 Western blot analysis and immunoprecipitation (Co-IP) assay

For Western blot analysis, copper sulfate (final concentration of 250µM) was added to the stable S2 cell lines (2×10^6 cells/well) in 6-well plates, and protein expression was induced for 48h. Cell culture medium (2 ml each) was collected, stable S2 cells were homogenized in 400 µl lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5mM PMSF, protease inhibitor cocktail (P8340, Sigma-Aldrich)]. The cell homogenates were incubated on ice for 15 min and sonicated briefly several times, and then centrifuged at 15,000 g for 15 min at 4°C. The supernatants were collected as cell extracts for Western blot analysis. The cell culture media (10µl each from 2 ml total) and cell extracts (10µl each from 400 µl total, equivalent to $\sim 5 \times 10^4$ cells) were separated on 10%, 12%, or 15% SDS-PAGE and proteins were transferred to nitrocellulose membranes (162-0097, Bio-Rad). The membrane was blocked with 5% BSA in Tris-buffered saline (25mM Tris-HCl, pH7.6, 150 mM NaCl) containing 0.05% Tween-20 (TBS-T) at room temperature for at least 3h and then incubated overnight with primary antibody at 4°C in 5% BSA in TBS-T with gentle rocking. Then, the membrane was washed four times with TBS-T and incubated with secondary antibody in 5% BSA in TBS-T for 2h at room temperature. After washing four times with TBS-T (10min each time), the signal was developed by using ECL Chemiluminescence Detection Kit (RPN2134, GE Healthcare) or alkaline phosphatase (AP)-conjugate color development Kit (#170-6432, Bio-Rad). Anti-Flag M2 antibody (F-1804, Sigma-Aldrich, 1:5000 dilution) and anti-V5 antibody (V-8012, Sigma-Aldrich, 1:5000 dilution) were used as primary antibodies, horseradish peroxidase-conjugate anti-mouse antibody (SC-2005, Santa Cruz Biotechnology, 1:10,000) was used as secondary antibody for chemiluminescence, and alkaline phosphatase-conjugate anti-mouse antibody (A4312, Sigma-Aldrich, 1:10,000) was used as secondary antibody for color development.

Immunoprecipitation (Co-IP) assay was performed by using 300µl of cell extract, which is equivalent to approximately 10^6 cells, or equivalent cell culture medium containing recombinant proteins. The cell extracts or cell culture media were pre-cleared for 30min with 30µl Protein G Sepharose (50% slurry, No.17-0618-01, GE Healthcare) in a total volume of 500µl. After centrifugation, the supernatant was incubated with anti-Flag M2 or anti-V5 antibody (final concentration of 10µg/ml) at 4°C for 10h with gentle rocking. Then,

30µl Protein G Sepharose (50% slurry) in lysis buffer was added to the protein-antibody mixture and incubated at 4°C overnight with gentle rocking. The Sepharose beads containing immunoprecipitated proteins were collected after centrifugation, washed three times with lysis buffer, re-suspended in 50µl of 1×SDS sample buffer, boiled at 95°C for 5 min, and used for subsequent immunoblotting analysis.

2.5 Purification of recombinant *MsSpz* and *MsSpz-C108*

To purify recombinant proteins, stable S2 cells expressing *MsSpz* or *MsSpz-C108* in 75-cm² flasks were induced for protein expression after addition of copper sulfate (final concentration of 250µM). Cell culture medium was collected continuously for 10 days starting at 24h after protein expression by collecting culture medium every day and re-suspending the cells with fresh medium. To purify recombinant proteins, cell culture medium was combined, cell debris was removed by centrifugation at 1000 g for 10 min at room temperature, and cell-free medium was incubated overnight at 4°C with 500µl of Anti-Flag M2 agarose beads (A2220, Sigma-Aldrich) equilibrated with initial buffer (50mM Tris-HCl, 150mM NaCl, pH7.4). Anti-Flag M2 agarose beads were then packed into a column (1.5cm × 1cm) and cell-free medium was loaded to the column several times at a flow rate of 0.05ml/min. Then, the column was washed with the initial buffer until A280 of the effluent was near zero. The bound proteins were sequentially eluted with one ml aliquots of the elution buffer (0.1M glycine-HCl, pH 3.5, 1% Triton X-100) into vials containing 100 µl of 1M Tris-base, pH 8.0. Fractions were analyzed by 12% or 15% SDS-PAGE. Fractions containing recombinant *MsSpz* or *MsSpz-C108* were de-salted with D-salt™ Excellulose™ GF-5 desalting column (#1851850, Pierce) pre-equilibrated with H₂O, and fractions containing recombinant proteins were pooled and concentrated.

2.6 Cleavage of recombinant *MsSpz* by *M. sexta* larval plasma

To test whether *MsSpz* can be activated by *M. sexta* larval plasma, induced cell-free plasma was collected from immunized *M. sexta* larvae that were injected with a mixture of heat-killed yeast (*Saccharomyces cerevisiae*, 1.7×10⁷ cells), dry *Micrococcus luteus* (33 µg) and heat-killed *Escherichia coli* XL-1 blue (3.3×10⁷ cells) at 24h post-injection, and the control plasma was collected from naïve larvae. Purified *MsSpz* (50µl of 100ng/µl, 5 µg) was incubated with 10µl induced or control plasma in a total of 75µl at room temperature for 2h. Then 25µl 4×SDS loading buffer was added to the reaction mixture. The reaction mixture was heated to 95°C for 5 min and aliquots (10µl, an equivalent of 1µl original plasma and 0.5 µg recombinant protein) were analyzed by 15% SDS-PAGE and immunoblotting using mouse monoclonal anti-Flag M2 antibody (F-1804, Sigma-Aldrich, 1:5000 dilution) or rabbit polyclonal anti-*MsSpz-C108* antibody (a gift from Dr. Michael Kanost, Kansas State University) (1:1000 dilution) as primary antibody.

2.7 Dual-Luciferase Reporter Assay

For dual-luciferase reporter assays, S2 cells were plated in 96-well culture plates (10⁴ cells/well) overnight in serum-free medium. These S2 cells were then transiently cotransfected with recombinant pMT/BiP/V5-His A expression plasmid (0.3µg), pGL3B, pGL3B-*drosomycin*, pGL3B-*dipteracin* or pGL3B-*attacin* firefly luciferase reporter plasmid (0.15µg) (Rao et al., 2011), and renilla luciferase reporter plasmid (0.015µg) (as an internal standard) (pRL-TK, Promega). After overnight transfection, serum-free medium was replaced with complete growth medium containing 250µM copper sulfate (final concentration) for protein expression, and firefly luciferase and renilla luciferase activities were measured at 48h after protein expression using the Dual-Luciferase Reporter Assay System (E1980, Promega) in the GloMax® Multi Microplate Luminometer (Promega). Relative luciferase activity (RLA) was obtained as the ratio of firefly luciferase activity to renilla luciferase activity. RLA from S2 cells cotransfected with empty pMT/BiP/V5-His A and pGL3B (empty reporter vector)

plasmids was used as the calibrator. These experiments were repeated at least three times (three independent biological samples, or three independent cell cultures), and a representative set of data was used to make figures.

2.8 Real-time PCR analysis

S2 cells were plated in 6-well culture plates (10^5 cells/well) overnight in serum-free medium, and transiently transfected with recombinant pMT/BiP/V5-His A expression vectors (3 μ g each vector). After overnight transfection, serum-free medium was replaced with complete growth medium containing 250 μ M copper sulfate to induce expression of recombinant proteins. After protein expression for 48h, total RNAs were extracted from these S2 cells using TRIzol[®] Reagent (T9424, Sigma-Aldrich) according to the manufacturer's instructions. Residual genomic DNA was digested by RQ1 RNase-free DNase (M6101, Promega). cDNA was prepared from 1 μ g total RNA in a 25 μ l reaction using moloney murine leukemia virus (M-MLV) reverse transcriptase (M1701, Promega) with an anchor-oligo(dT)₁₈ primer following the manufacturer's instructions. Each cDNA sample (diluted 1:50) was used as template for quantitative real-time PCR analysis. The *Drosophila* ribosomal protein 49 (*rp49*) gene was used as an internal standard to normalize the amount of RNA template. The primer pairs (Table S1) were designed based on the sequences of *rp49*, *drosomycin* and *dipthericin*. The real-time PCR was performed in 20 μ l reactions containing 10 μ l 2 $\times$ SYBR[®] GreenER[™] qPCR SuperMix Universal (No. 204141, Qiagen), 4 μ l H₂O, 4 μ l diluted cDNA template, and 1 μ l (10pmol) each of the forward and reverse primers. Real-time PCR program was 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15s, 60°C for 1 min and the dissociation curve analysis. Data from three replicas of each sample were analyzed by the ABI 7500 SDS software (Applied Biosystems) using a comparative method ($2^{-\Delta\Delta CT}$). The baseline was set automatically by the software to maintain the consistency. cDNA sample from S2 cells transfected with empty pMT/BiP/V5-His A plasmid was used as the calibrator. The expression levels of *drosomycin* and *dipthericin* transcripts in other cDNA samples were calculated by the $2^{-\Delta\Delta CT}$ method, which stands for the n-fold difference in relative expression to the calibrator. All the data were presented as relative mRNA expression. These experiments were repeated at least three times.

2.9 Injection of *MsSpz* and *MsSpz-C108* into *M. sexta* larvae

Day 1 fifth instar *M. sexta* naïve larvae were injected with water (as a negative control), purified recombinant *MsSpz* (3 μ g/larva), or *MsSpz-C108* (1 μ g/larva). Twenty hours later, fat body and hemocyte samples were collected, total RNA was isolated with TRIzol[®] Reagent (Sigma-Aldrich), and cDNA was prepared with ImProm-II reverse transcriptase (Promega) as described above. Each cDNA sample (diluted 1:50) was used as template for real-time PCR analysis. *M. sexta* ribosomal protein S3 (*rpS3*) gene was used as an internal standard to normalize the amount of RNA template. AMP genes, including *cecropin-6* (AY232302.1), *attacin-1* (DQ072728.1), *attacin-2* (AY232304.1), *lebocin-b* and *lebocin-c* (GU563901.1 and GU563900.1), *moricin* (AY232301.1) and lysozyme (S71028.1) were detected with primer pairs (10 pmol each) listed in Table S1. cDNA sample from naïve larvae was used as the calibrator. The expression levels of AMP genes from other samples were calculated by the $2^{-\Delta\Delta CT}$ method. All the data were presented as relative mRNA expression. These experiments were repeated at least three times.

2.10 Injection of antibody to *MsToll* into *M. sexta* larvae

To test whether *MsSpz-C108* binds to *MsToll* in *M. sexta* larvae to stimulate expression of AMP genes, day 1 fifth instar *M. sexta* naïve larvae were pre-injected with purified IgG to the ecto-domain of *MsToll* (Toll Ab, 5 μ g/larva) or IgG from pre-immune rabbit serum (Control Ab, 5 μ g/larva), and these larvae were then injected with water, purified

recombinant *MsSpz* (3 µg/larva), *MsSpz*-C108 (1 µg/larva), TLRgrade peptidoglycan from *Staphylococcus aureus* (PG-SA) or *Escherichia coli* (PG-K12) (1 µg/larva) (Invivogen), or without second injection (control) at 1h after pre-injection of antibody. Twenty hours later, fat body and hemocyte samples were collected for quantitative real-time PCR analysis. Total RNA and cDNA samples were prepared as described above. *M. sexta* ribosomal protein S3 (*rpS3*) gene was used as an internal standard to normalize the amount of RNA template. Expression of *cecropin-6*, *attacin-1*, *lebocin-b/c*, *moricin* and lysozyme genes were determined by real-time PCR as described above. These experiments were repeated at least three times.

2.11 Data analysis

One representative set of data was used to make figures using the Graphpad Prism software, and the significance of difference was determined by an unpaired t-test or by one way ANOVA followed by a Tukey's multiple comparison test with the Graphpad InStat software (GraphPad, San Diego, CA).

3. Results

3.1 Expression of recombinant *M. sexta* and *D. melanogaster* Toll and Spz proteins in S2 cells

The Toll-Spz signaling pathway has been well understood in *D. melanogaster*, but is not well characterized in other insect species. In *M. sexta*, *Toll* and *Spz-1* genes have been identified (An et al., 2010; Ao et al., 2008). In order to investigate a Toll-Spz pathway in *M. sexta* and compare *M. sexta* and *D. melanogaster* Toll pathways in S2 cells, we established stable S2 cell lines expressing Toll receptors (*MsToll* and *DmToll*) and their TIR (*MsTIR* and *DmTIR*) and ecto-domains (*MsToll^{ecto}* and *DmToll^{ecto}*), as well as Spz proteins (*MsSpz* and *DmSpz*) and their active C-terminal domains (*MsSpz*-C108 and *DmSpz*-C106). Immunoblotting results showed that recombinant *D. melanogaster* and *M. sexta* Spz proteins and their active C-terminal domains were detected in both cell culture media and cell lysates (Fig. 1A and B). For the active C-terminal domains of Spz, a single protein band was detected in the cells and the cell culture media (Fig. 1A). For the full-length Spz proteins, multiple protein bands were detected (Fig. 1B), suggesting differential post-translational modifications of Spz proteins, and different modified forms of Spz were present in the cell culture media and cells. For the Toll receptors, *M. sexta* Toll (*MsToll*), *MsToll^{ecto}* (the ecto-domain) and *MsTIR* (Fig. 1C and E), as well as *D. melanogaster* Toll (*DmToll*) and *DmTIR* (Fig. 1E and F) were detected only in S2 cells but not in cell culture media. However, *DmToll^{ecto}* (the ecto-domain of *DmToll*) was detected as multiple protein bands in S2 cells and a single protein band in the cell culture medium (Fig. 1D), also suggesting differential post-translational modifications of *DmToll^{ecto}*. One of the *DmToll^{ecto}* protein bands was just above the 80kDa marker (Fig. 1D, lane 2), which may be a cleavage product because the calculated mass of *DmToll^{ecto}* is 95.8 kDa. For *DmToll*, cleavage products with sizes slightly larger than *DmTIR* were also detected (Fig. 1F, lane 4), suggesting that *DmToll* may be cleaved in the ecto-domain at a site close to the transmembrane domain.

3.2 Recombinant *M. sexta* Spz is activated by induced larval plasma

To investigate activation of *M. sexta* Spz, recombinant *MsSpz* and *MsSpz*-C108 were purified from stable S2 cell lines by antibody affinity chromatography (Fig. 2A and B). Both recombinant *MsSpz* and *MsSpz*-C108 contain a Flag-tag at the N-terminus, and they were recognized by anti-Flag antibody (Fig. 2B). To determine whether recombinant *MsSpz* can be activated by proteinases in *M. sexta* larval plasma, purified *MsSpz* was treated with induced *M. sexta* cell-free plasma at room temperature for 2h, and the cleavage products were detected by monoclonal anti-Flag (Fig. 2C) or polyclonal anti-*MsSpz*-C108 (Fig. 2D)

antibody. Purified *MsSpz* (calculated mass of 34.1 kDa) and *MsSpz*-C108 (calculated mass of 14.6 kDa) could be recognized by anti-Flag and anti-*MsSpz*-C108 antibodies (Fig. 2C and D, lanes 2 and 4, respectively). After treating with induced *M. sexta* larval plasma, *MsSpz* band disappeared and a major band at ~20 kDa was recognized by anti-Flag antibody (Fig. 2C, lane 3, arrowhead), which corresponded to the N-terminal fragment of *MsSpz* since the Flag-tag was at the N-terminus, and a cleavage product at ~12kDa was recognized by antibody to *MsSpz*-C108 (Fig. 2D, lane 3, arrowhead), which corresponded to the C-terminal *MsSpz*-C108. A control experiment using naïve plasma showed that very little *MsSpz* was cleaved (data not shown). These results suggest that *MsSpz* can be activated by proteinases in the hemolymph of *M. sexta* larvae, and these proteinases may also be induced by microorganisms. The cleavage *MsSpz*-C108 was smaller than the recombinant *MsSpz*-C108 (Fig. 2D, comparing lanes 3 and 4) since recombinant protein contained a Flag-tag at the N-terminus. In the plasma sample alone, endogenous *MsSpz* or *MsSpz*-C108 was not detected by anti-*MsSpz*-C108 antibody (Fig. 2D, lane 1), probably due to low concentration of Spz protein in plasma (An et al., 2010). But a band at ~23kDa in the plasma sample was recognized by anti-Flag antibody (Fig. 2C, lane 1, asterisk), and several bands were recognized by anti-*MsSpz*-C108 antibody (Fig. 2D, lane 1), indicating non-specific recognition of plasma proteins by antibodies.

3.3 *MsToll*^{ecto} interacts with *MsSpz*-C108 but not with *MsSpz*

In *D. melanogaster*, after Spz is activated by proteolysis, the C-terminal active domain (*DmSpz*-C106) is released from prodomain and recognized by the Toll receptor to initiate intracellular signaling pathway (Arnot et al., 2010; Hu et al., 2004; Morisato and Anderson, 1994; Schneider et al., 1994; Valanne et al., 2011). To determine whether *MsSpz* or *MsSpz*-C108 can bind to *MsToll* receptor, we over-expressed the ecto-domain of *MsToll* (*MsToll*^{ecto}) with a V5-His-tag to the C-terminus, and *MsSpz* and *MsSpz*-C108 with a Flag-tag to the N-terminus in S2 cells (Fig. 1). Co-immunoprecipitation (Co-IP) assays were performed by mixing individual cell lysates or using co-expression cell lysates. For *MsSpz*, both cell lysate and cell culture medium were used because different modified forms of *MsSpz* were present in culture medium and cells (Fig. 1B). Our results showed that when cell lysates containing *MsSpz*-C108 and *MsToll*^{ecto} were mixed, Flag antibody could pull down both *MsSpz*-C108-Flag and *MsToll*^{ecto}-V5 (Fig. 3A and B, lane 4), whereas V5 antibody could precipitate both *MsToll*^{ecto}-V5 and *MsSpz*-C108-Flag (Fig. 3C and D, lane 4). Similar results were obtained when cells co-expressing *MsToll*^{ecto}-V5 and *MsSpz*-C108-Flag were used for the Co-IP assay (Fig. 3A–D, lane 5). When cell lysates and culture media containing *MsSpz* and *MsToll*^{ecto} were mixed, or cells co-expressing *MsToll*^{ecto} and *MsSpz* were used for the Co-IP assays, Flag antibody only pulled down *MsSpz*-Flag but not *MsToll*^{ecto}-V5 (Fig. 3E and F, lanes 5–8), while V5 antibody could precipitate only *MsToll*^{ecto}-V5 but not *MsSpz*-Flag (Fig. 3G and H, lanes 5–8). These results suggest that *MsToll*^{ecto} can interact with *MsSpz*-C108 but not with the full-length *MsSpz*. We also performed Co-IP assays of *DmToll*^{ecto} with *DmSpz*-C106 or *DmSpz*, and the results showed that *DmToll*^{ecto} could interact with *DmSpz*-C106 but not with *DmSpz* (Fig. S1). Together, these results indicate that Toll receptor only binds to active form Spz.

3.4 Co-expression of *MsToll* with *MsSpz*-C108 but not *MsSpz* in S2 cells can activate *drosomycin* gene

In *D. melanogaster*, the Toll-Spz pathway activates NF- κ B factors Dorsal and Dif to induce expression of *drosomycin* gene, while the Imd pathway activates NF- κ B factor Relish to induce *diptericin* gene expression (Tanji and Ip, 2005). To investigate whether *MsToll*-*MsSpz*-C108 can activate expression of *drosomycin* or *diptericin* gene, *Drosophila* S2 cell lines expressing or co-expressing *MsToll* and *MsSpz*-C108 were applied because no *M. sexta* cell line is available. We first over-expressed the TIR domains of *MsToll* and *DmToll*

to determine whether *MsToll* can activate antimicrobial peptide (AMP) genes in S2 cells. Dual luciferase activity assay showed that over-expression of *DmTIR* and *MsTIR* could activate *drosomycin* promoter significantly, but did not activate *diptericin* or *attacin* promoter (Fig. S2), indicating that *MsToll* can activate *drosomycin* gene in S2 cells.

We next over-expressed individual *D. melanogaster* and *M. sexta* Toll (Toll and Toll^{ecto}) and Spz (Spz and the active C-terminal domain) proteins or co-expressed different combination of Toll and Spz proteins in S2 cells, and then determined activation of *drosomycin* or *diptericin* reporter gene by dual luciferase reporter assays. As a positive control, co-expression of *DmToll-DmSpz-C106*, but not *DmToll-DmSpz*, significantly increased relative luciferase activity of *drosomycin* reporter (~40-fold), but did not activate *diptericin* reporter (Fig. 4). Similarly, co-expression of *MsToll-MsSpz-C108*, but not *MsToll-MsSpz*, also activated *drosomycin* reporter to a significantly higher level (~25-fold) than the control, although the level activated by *MsToll-MsSpz-C108* was slightly lower than that activated by *DmToll-DmSpz-C106*. Over-expression of *DmSpz-C106* alone also activated *drosomycin* reporter to a significantly higher level than the control. However, over-expression of Toll, Toll^{ecto} and Spz proteins or co-expression of other combinations of Toll and Spz, particularly *DmToll-MsSpz-C108* and *MsToll-DmSpz-C106*, did not activate *drosomycin* reporter (Fig. 4), suggesting that only a correct pair of Toll-Spz (*DmToll-DmSpz-C106* or *MsToll-MsSpz-C108*) can activate the Toll signaling pathway.

To confirm the dual luciferase results, we also performed real-time PCR in S2 cells over-expressing or co-expressing Toll and Spz proteins (Fig. 5). Over-expression of *DmSpz-C106* alone significantly increased *drosomycin* mRNA level (~4-fold) compared to the control. Co-expression of *MsToll-MsSpz-C108* and *DmToll-DmSpz-C106* increased *drosomycin* transcript levels to significantly higher levels (14- and 18-fold, respectively) than over-expression of *DmSpz-C106* alone (Fig. 5A). However, over-expression or co-expression of these proteins did not significantly change the *diptericin* mRNA levels compared to the control (Fig. 5B). These results are consistent with those of dual luciferase assays (Fig. 4), and further confirm that *MsToll-MsSpz-C108* can activate *drosomycin* but not *diptericin* gene in S2 cells.

3.5 The Toll-Spz pathway regulates expression of AMP genes in *M. sexta* larvae

Several antimicrobial peptide (AMP) genes, including *cecropin-6*, *attacin-1*, *attacin-2*, *morcin*, *gloverin*, *lebocin-b* and *lebocin-c*, and lysozyme have been identified in *M. sexta* (Kanost et al., 2004; Rao et al., 2012). It has been reported that injection of *MsSpz-C108* but not *MsSpz* into *M. sexta* larvae can activate some AMP genes (An et al., 2010). To further confirm regulation of AMP genes in *M. sexta* larvae, purified recombinant *MsSpz*, *MsSpz-C108*, or water (the control) was injected into day 1 fifth instar *M. sexta* naïve larvae, and expression of AMP genes in hemocytes and fat body was determined. Quantitative real-time PCR results showed that water injection did not activate AMP genes in hemocytes and fat body compared to the naïve larvae, while injection of *MsSpz* activated AMP genes to low levels compared to the naïve larvae or the water injection control, probably due to activation of some *MsSpz* by hemolymph proteinases (Fig. 6). However, injection of *MsSpz-C108* activated all AMP genes (except lysozyme) in hemocytes and fat body to significantly higher levels than the control (water-injection) and naïve larvae (Fig. 6). These results suggest that *M. sexta* AMP genes can be regulated by the Toll-Spz pathway. Lysozyme was activated by *MsSpz* and *MsSpz-C108* to a similarly low level, which was still significantly higher than that of the naïve larvae or the water-injection control. Lysozyme mRNA level was significantly lower than that of any other AMP genes.

3.6 Activation of AMP genes in *M. sexta* larvae by *MsSpz*-C108 is blocked by antibody to *MsToll*

In *Drosophila*, Gram-positive Lys-type peptidoglycan (PG) activates the Toll pathway, while Gram-negative *meso*-diaminopimelic acid (DAP)-type PG activates the Imd pathway (Gottar et al., 2002; Leulier et al., 2003; Michel et al., 2001). In *M. sexta*, both Lys-type and DAP-type PGs can activate expression of *M. sexta* AMP genes (Rao and Yu, 2010). To further confirm a Toll-Spz pathway in *M. sexta* and to test whether activation of AMP genes by Lys-type and DAP-type peptidoglycans in *M. sexta* is regulated by the Toll and/or Imd pathways, an antibody blocking assay was performed since attempts to silence *MsToll* gene by RNAi failed. *M. sexta* larvae were first injected with purified IgG to the ecto-domain of *MsToll* or control IgG from pre-bleed serum, then injected with water, recombinant *MsSpz* or *MsSpz*-C108, *S. aureus* PG (PG-SA, Lys-type PG), *E. coli* PG (PG-K12, DAP-type PG), or without a second injection (control), and induced expression of AMP genes in hemocytes and fat body was determined.

Real-time PCR results showed that in the control IgG pre-injected larvae, injection of water did not activate AMP genes, while injection of *MsSpz* could activate AMP genes to low levels in both hemocytes and fat body (Fig. 7), probably due to activation of some *MsSpz* in the hemolymph. But injection of *MsSpz*-C108 and PG-SA activated all the AMP genes (except lysozyme) to significantly higher levels in hemocytes and fat body compared to the injection of water (Fig. 7), and injection of PG-K12 also activated some AMP genes (e.g. *moricin* in hemocytes and fat body, and *lebocin-b/c* in fat body) to significantly higher levels than the water injection (Fig. 7D, H and I). Among *MsSpz*-C108, PG-SA and PG-K12, *MsSpz*-C108 activated almost all AMP genes to significantly higher levels than PG-SA and PG-K12 did, and PG-SA activated most AMP genes to significantly higher levels than PG-K12 did, but PG-K12 activated *moricin* in hemocytes (Fig. 7D) and *lebocin-b/c* in fat body (Fig. 7H) to significantly higher levels than PG-SA did.

In the *MsToll* IgG pre-injected larvae, activation of AMP genes (except lysozyme) by *MsSpz*-C108, PG-SA and PG-K12 in hemocytes and fat body was all [except for *lebocin-b/c* in hemocyte (Fig. 7C)] significantly suppressed compared to those of the control IgG pre-injected larvae (Fig. 7). These results suggest that *MsSpz*-C108, PG-SA and PG-K12 may all activate AMP genes in *M. sexta* larvae via the Toll-Spz pathway, and binding of *MsToll* IgG to *MsToll* blocks *MsSpz*-C108 from binding to *MsToll* and thus suppresses activation of the downstream AMP genes. Although overall activation level of lysozyme by *MsSpz*-C108, PG-SA and PG-K12 was significantly lower than any of the other AMP genes, PG-K12 activated lysozyme to significantly higher levels than *MsSpz*-C108 and PG-SA did, and pre-injection of *MsToll* antibody did not block PG-K12-activated expression of lysozyme in hemocytes and fat body (Fig. 7E and J). In addition, pre-injection of *MsToll* antibody stimulated *lebocin-b/c* expression in hemocytes activated by PG-K12 (Fig. 7C), which was different from the suppression pattern of *lebocin-b/c* in fat body after PG-K12 activation (Fig. 7H). These results suggest that regulation of lysozyme in fat body and hemocytes and *lebocin-b/c* in hemocytes may not be regulated by the Toll-Spz pathway.

4. Discussion

Invertebrates, such as insects, mainly rely on innate immunity to fight against pathogens. Induced expression of antimicrobial peptide (AMP) genes is an important defense mechanism in insect innate immunity (Ashida M, 1998; Hancock and Scott, 2000; Imler and Bulet, 2005; Lemaitre and Hoffmann, 2007; Williams, 2007). AMP gene expression is regulated by signal transduction pathways, such as the Toll and Imd pathways in *D. melanogaster* (Choe et al., 2002; De Gregorio et al., 2002; Lemaitre et al., 1995; Lemaitre et al., 1996; Ramet et al., 2002). *Drosophila* Toll-Spz signaling pathway controls dorsal-ventral

patterning in the embryonic development and also retains a common function in stimulating expression of AMP genes. Although *Toll* and *Spz* genes have been identified in different insect species (An et al., 2010; Ao et al., 2008; Christophides et al., 2002; Evans et al., 2006; Imamura and Yamakawa, 2002; Kanzok et al., 2004; Luna et al., 2002; Wang et al., 2007), the role of Toll-Spz pathway in regulating AMP gene expression in other insect species has not been well studied. Progress in understanding the Toll-Spz pathway that operates in the innate immune system requires more investigation of molecular and biochemical functions of Toll and Spz in diverse taxa. In this study, we have identified a Toll-Spz signaling pathway in a lepidopteran insect, *M. sexta*.

In *M. sexta*, Toll and Spz have been identified (An et al., 2010; Ao et al., 2008), but interaction between *M. sexta* Toll and Spz and direct evidence for a Toll-Spz pathway have not been demonstrated. In *D. melanogaster*, it has been shown that proteolytic processing of *DmSpz* releases the active *DmSpz*-C106, which is required for interaction with *DmToll* (Arnot et al., 2010). The binding of two active *DmSpz*-C106 dimers to one *DmToll* receptor and the formation of Toll-Spz heterodimers are also essential (Hu et al., 2004). We showed by co-immunoprecipitation (Co-IP) assay that *MsToll*^{ecto} could interact with *MsSpz*-C108, but not with the full-length *MsSpz* (Fig. 3), a result consistent with that of *DmToll*^{ecto} and *DmSpz*-C106 (Fig. S1), suggesting that proteolytic activation of *MsSpz* is required for interaction of active *MsSpz*-C108 with *MsToll*. We also showed that *MsSpz* could be processed to active *MsSpz*-C108 by proteinases in the hemolymph of *M. sexta* larvae (Fig. 2C and D), and these hemolymph proteinases are also induced by microorganisms. One interesting result is that *DmToll*^{ecto} was differentially post-translationally modified in S2 cells (Fig. 1D) and different modified forms of *DmToll*^{ecto} could all interact with *DmSpz*-C106 (Fig. S1), and cleavage products of *DmToll* was also observed (Fig. 1F, lane 4). These results suggest that *Drosophila* Toll pathway may also be regulated by proteinases that can cleave *DmToll*.

To test whether *MsToll*-*MsSpz*-C108 complex can activate AMP genes, both *in vitro* experiments in *Drosophila* S2 cells (since no *M. sexta* cell line is available) and *in vivo* experiments in *M. sexta* larvae were performed. Over-expression of *MsTIR* and *DmTIR* in S2 cells both could activate *drosomycin* (a target gene of the Toll pathway) but not *dipterizin* (a target gene of the Imd pathway) (Fig. S2), suggesting that *MsToll* can activate the Toll pathway in S2 cells. Co-expression of *MsToll*-*MsSpz*-C108 and *DmToll*-*DmSpz*-C106 could activate *drosomycin* to similarly high levels (Figs. 4 and 5), but did not activate *dipterizin*, further confirming that *MsToll*-*MsSpz*-C108 complex can activate the Toll signaling pathway in S2 cells. It has been shown that *Bombyx mori* Spz can activate AMP genes in *M. sexta* larvae (Wang et al., 2007), but we showed that co-expression of *MsToll*-*DmSpz*-C106 or *DmToll*-*MsSpz*-C108 did not activate *drosomycin* (Figs. 4 and 5). *MsSpz* is 44% and 23% identical to *B. mori* and *D. melanogaster* Spz-1, respectively, together these results suggest that Toll and Spz binding may be specific and only the correct pair of Toll-Spz can activate the Toll pathway. *D. melanogaster* contains nine *Toll* and six *Spz* genes; it would be interesting to know whether other Toll-Spz pairs can trigger signaling pathways in *D. melanogaster*.

In *D. melanogaster*, expression of AMP genes is regulated by the Toll and immune deficiency (Imd) pathways (De Gregorio et al., 2002; Lemaitre et al., 1995; Lemaitre et al., 1996). The Toll pathway is activated by fungi and Lys-type peptidoglycan (PG) of Gram-positive bacteria via peptidoglycan recognition protein (PGRP)-SA, PGRP-SD and Gram-negative binding protein 1 (GNBP1) (Bischoff et al., 2004; Gobert et al., 2003; Michel et al., 2001), while the Imd pathway is activated by *meso*-diaminopimelic acid (DAP)-type PG of Gram-negative bacteria and some *Bacilli* species via PGRP-LC (Choe et al., 2002; Gobert et al., 2003; Gottar et al., 2002; Kaneko et al., 2004). In *M. sexta*, several AMP genes,

including *cecropin*, *attacin*, *moricin*, *lebocin* and *gloverin*, as well as lysozyme have been identified (Kanost et al., 2004; Rao et al., 2012), and they can be activated by different bacterial cell wall components (Rao and Yu, 2010). Among these *M. sexta* AMP genes, *cecropin* and *attacin* are common AMP genes found in most insect species, but *moricin*, *lebocin* and *gloverin* are only found in lepidopteran species (Axen et al., 1997; Chowdhury et al., 1995; Hara and Yamakawa, 1995; Kanost et al., 2004). It is not clear whether *M. sexta* AMP genes, particularly lepidoptera-specific *moricin*, *lebocin* and *gloverin*, are regulated by the Toll and/or Imd pathways. Injection of *MsSpz*-C108 activated AMP genes (*cecropin*, *attacin*, *lebocin* and *moricin*) in *M. sexta* larvae to significantly higher levels than the control (water-injection) (Fig. 6), suggesting that these *M. sexta* AMP genes are regulated by the Toll-Spz pathway. *MsSpz*-C108 activated lysozyme mRNA expression to much lower level compared to other AMP genes (Fig. 6), but lysozyme protein is highly induced by *MsSpz*-C108 in hemolymph (An et al., 2010).

In *Drosophila*, though Lys-type and DAP-type PGs can activate the Toll and Imd pathways, respectively, but PGRP-SD can bind to DAP-type PG and may be responsible for activation of the Toll pathway by Gram-negative bacteria (Leone et al., 2008), and *Anopheles gambiae* PGRP-LC is responsible for activation of AMP genes stimulated by *S. aureus* but not by *E. coli* (Meister et al., 2009). In *M. sexta*, it is not clear whether Lys-type and DAP-type PGs can activate the Toll-Spz and/or Imd pathways. We tried to silence *MsToll* by RNA interference (RNAi) in order to further confirm the Toll-Spz pathway and activation of AMP genes by *S. aureus* and *E. coli* PGs in *M. sexta* larvae, but all our attempts using siRNA and dsRNA failed. We then used antibody to the ecto-domain of *MsToll* to block *MsToll* in *M. sexta* larvae from binding to the injected *MsSpz*-C108. Our antibody blocking assay showed that activation of AMP genes in both hemocytes and fat body of *M. sexta* larvae by *MsSpz*-C108 and *S. aureus* PG (PG-SA, Lys-type PG) was significantly inhibited when larvae were pre-injected with antibody to *MsToll* but not the control antibody (Fig. 7). These results further confirm that *MsToll*-*MsSpz*-C108 can form a complex in *M. sexta* larvae to mediate the Toll-Spz signaling pathway and regulate AMP genes expression. *E. coli* PG (PG-K12, DAP-type PG) activated expression of some AMP genes (*moricin* in hemocytes and fat body, and *lebocin*-b/c in fat body) was also suppressed by pre-injection of antibody to *MsToll*. These results suggest that both the Lys-type PG-SA and DAP-type PG-K12 can activate the Toll-Spz pathway in *M. sexta*, but PG-K12 is a weaker elicitor than PG-SA in stimulation of the Toll-Spz pathway. Expression of *lebocin*-b/c in hemocytes was stimulated after *MsToll* was blocked by antibody, suggesting that *lebocin*-b/c expression in hemocytes is not regulated by the Toll-Spz pathway. It is not clear why expression of *lebocin*-b/c in hemocytes and fat body is regulated differently. Expression of *gloverin* in hemocytes and fat body was also regulated in a similar pattern like *lebocin*-b/c (Xu X-X, Zhong X and Yu X-Q, unpublished results). This may be related to expression pattern of *M. sexta Spz*, as it is expressed and induced in hemocytes but not induced in fat body (An et al., 2010). Activation of lysozyme by *MsSpz*-C108, PG-SA and PG-K12 was always lower than that of any other *M. sexta* AMP genes, and the activation was not blocked by pre-injection of *MsToll* antibody. In addition, PG-K12 is a stronger elicitor than *MsSpz*-C108 or PG-SA in activation of lysozyme. Thus, lysozyme may also not be regulated by the Toll-Spz pathway. Expression of *lebocin*-b/c in hemocytes and lysozyme in both hemocytes and fat body may be regulated by other signaling pathways such as the Imd pathway since Rel genes similar to *Drosophila* Relish have been identified in *M. sexta* (Rao X-J and Yu X-Q, unpublished results).

In summary, we used a biochemical assay to show that *MsToll*^{ecto} and *DmToll*^{ecto} could interact with *MsSpz*-C108 and *DmSpz*-C106, respectively, but not with full-length Spz (Figs. 3 and S1), used *in vitro* assays to show that *MsToll*-*MsSpz*-C108 and *DmToll*-*DmSpz*-C106 complexes could activate *drosomycin* but not *dipteracin* gene in S2 cells (Figs.

4 and 5), used *in vivo* assays to show that activation of *M. sexta* AMP genes by *MsSpz*-C108 was significantly inhibited by pre-injection of antibody to *MsToll* (Fig. 7). Our results together demonstrated a Toll-Spz signaling pathway in a lepidopteran insect, *M. sexta*. This study may help better understand signaling pathways in lepidopteran insects, and the origin and evolution of animal innate immune signaling pathways.

>*M. sexta* Toll interacts with *MsSpz*-C108 (the active C-terminal domain of Spz) but not with full-length *MsSpz*. >Co-expression of *MsToll* with *MsSpz*-C108 but not *MsSpz* activates *drosomycin* in *Drosophila* S2 cells. >Activation of AMP genes in *M. sexta* larvae by *MsSpz*-C108 is blocked by antibody to *MsToll*. > Activation of *M. sexta* AMP genes by Lys-type and DAP-type peptidoglycans is blocked by antibody to *MsToll*. >Both *in vitro* and *in vivo* results demonstrate a Toll-Spz pathway in *M. sexta*, a lepidopteran insect.

Supplementary Material

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Acknowledgments

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Fig. 1. Expression of recombinant *M. sexta* and *D. melanogaster* Toll and Spätzle proteins in S2 cells

M. sexta and *D. melanogaster* Toll (*MsToll* and *DmToll*) and their TIR (*MsTIR* and *DmTIR*) and ecto-domains (*MsToll^{ecto}* and *DmToll^{ecto}*), Spätzle proteins (*MsSpz* and *DmSpz*) and their active C-terminal domains (*MsC108* and *DmC106* for *MsSpz*-C108 and *DmSpz*-C106, respectively) were expressed in *Drosophila* S2 cells, and recombinant proteins in cell culture media (10 μ l each from 2ml total) and cell lysates (10 μ l each from 400 μ l total) were identified by immunoblotting using anti-Flag M2 antibody or anti-V5 antibody as the primary antibody, alkaline phosphatase-conjugate anti-mouse antibody as the secondary antibody with alkaline phosphatase (AP) conjugate color development kit. A: *MsSpz*-C108 (lanes 1 and 2) and *DmSpz*-C106 (lanes 3 and 4); B: *MsSpz* (lanes 1 and 2) and *DmSpz* (lanes 3 and 4); C: *MsToll^{ecto}* (lanes 1 and 2) and *MsToll* (lanes 3 and 4); D: *DmToll^{ecto}*; E: *MsTIR* (lanes 1 and 2) and *DmTIR* (lanes 3 and 4); F: *DmTIR* (lanes 1 and 2) and *DmToll* (lanes 3 and 4). Lanes 1 and 3: culture media; lanes 2 and 4: cell lysates. The

calculated masses of these recombinant proteins are: *MsSpz*-C108 (14.6 kDa), *DmSpz*-C106 (14.1 kDa), *MsSpz* (34.1 kDa), *DmSpz* (27.2 kDa), *MsToll^{ecto}* (81.9 kDa), *MsToll* (109.4 kDa), *DmToll^{ecto}* (95.8 kDa), *DmToll* (128.5 kDa), *MsTIR* (26.2 kDa), and *DmTIR* (30.8 kDa).

Fig. 2. Activation of recombinant *M. sexta* Spätzle by induced larval plasma

Recombinant *M. sexta* Spätzle (*MsSpz*) and its active C-terminal domain (*MsC108* for *MsSpz*-C108) were purified by antibody affinity chromatography and analyzed by SDS-PAGE (A) (1 μ g *MsSpz* and 1.5 μ g *MsSpz*-C108) and Western blotting (B) (0.5 μ g each protein) using anti-Flag antibody. Purified recombinant *MsSpz* was activated by induced cell-free plasma collected at 24h after larvae were injected with a mixture of *S. cerevisiae*, *M. luteus* and *E. coli*, and cleavage products were analyzed by Western blotting using anti-Flag (C) or anti-*MsSpz*-C108 (D) as primary antibody, alkaline phosphatase-conjugate anti-mouse antibody as the secondary antibody. Panels A and B: lanes 1 and 2 were *MsSpz* and *MsC108* (*MsSpz*-C108), respectively. Panels C and D: lane 1, induced plasma (1 μ l); lane 2, purified recombinant *MsSpz* (0.5 μ g); lane 3, induced plasma (1 μ l) + purified *MsSpz* (0.5 μ g); lane 4, purified recombinant *MsC108* (*MsSpz*-C108) (0.5 μ g). Arrowhead indicates the N-terminal cleavage product of *MsSpz* (C, lane 3) or C-terminal cleavage product of *MsSpz* (D, lane 3), while arrow indicates purified recombinant *MsC108* (*MsSpz*-C108) (C and D, lane 4).

Fig. 3. *M. sexta* Toll^{ecto} (the ecto-domain) interacts with *MsSpz-C108* but not with *MsSpz*
 The ecto-domain of *M. sexta* Toll (*MsToll^{ecto}*), Spätzle-1A (*MsSpz*) and the active C-terminal domain of *MsSpz* (*MsSpz-C108*) were expressed or co-expressed in *Drosophila* S2 cells for 48h. Proteins in both culture media and cell lysates were used for co-immunoprecipitation (Co-IP) assays as described in the Materials and Methods. *MsToll^{ecto}* contained a V5-tag, while *MsSpz* and *MsSpz-C108* contained a Flag-tag. Monoclonal antibody (anti-FLAG or anti-V5) was added to combined cell lysates, combined culture media with cell lysates, co-expression culture media or cell lysates, and immunoprecipitated (IP) proteins or Co-IP proteins were detected by immunoblotting using anti-Flag or anti-V5 antibody as the primary antibody, horseradish peroxidase-conjugate anti-mouse antibody as the secondary antibody with the ECL chemiluminescence detection kit (A–D), or alkaline phosphatase-conjugate anti-mouse antibody as the secondary antibody with alkaline phosphatase (AP) conjugate color development kit (E–F). Lanes 1–3 from A–D and lanes 1–4 from E–F were cell lysates or culture media alone (protein inputs). In the figure, M is for culture media, C for cell lysates, Co-M for culture media from co-expression, and Co-C for cell lysates from co-expression.

Fig. 4. Co-expression of *MsToll* with *MsSpz*-C108 but not *MsSpz* activates *drosomycin* promoter reporter in S2 cells

S2 cells were transiently co-transfected with recombinant expression plasmid(s) (expressing *MsToll*, *MsToll*^{ecto}, *DmToll*, *DmToll*^{ecto}, *MsSpz*, *MsC108* (*MsSpz*-C108), *DmSpz* or *DmC106* (*DmSpz*-C106), or co-expressing any combinations of Toll and Spz) and a promoter reporter (pGL3B, pGL3B-*drosomycin* or pGL3B-*diptericin*). These cells were induced for protein expression for 48h, and relative luciferase activity (RLA) in these cells was measured by dual-luciferase assay. Activity in cells co-transfected with empty pMT/BiP/V5-His A and pGL3B plasmids was set as 1 (the calibrator). Bars represent the mean of three individual measurements $\pm$ SEM. Identical letters are not significant difference ($p > 0.05$), while different letters indicate significant difference among groups ($p < 0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test.

Fig. 5. Co-expression of *MsToll* with *MsSpz*-C108 but not *MsSpz* activates endogenous *drosomycin* gene in S2 cells

S2 cells were transiently transfected with recombinant expression plasmid(s) (expressing *MsToll*, *MsToll*^{ecto}, *DmToll*, *DmToll*^{ecto}, *MsSpz*, *MsC108* (*MsSpz*-C108), *DmSpz* or *DmC106* (*DmSpz*-C106), or co-expressing any combinations of Toll and Spz), and then induced for protein expression for 48h. Total RNAs were prepared from these cells, and expression of endogenous *drosomycin* (A) and *dipteracin* (B) genes was determined by real-time PCR. Ribosomal protein 49 (*rps49*) gene was used as an internal control. Bars represent the mean of three individual measurements $\pm$ SEM. Identical letters are not significant difference ($p > 0.05$), while different letters indicate significant difference among groups ($p < 0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test.

Fig. 6. Activation of AMP genes by *MsSpz*-C108 in *M. sexta* larvae

Day 1 fifth instar *M. sexta* naïve larvae were injected with purified recombinant *MsSpz* (3 µg/larva), *MsSpz*-C108 (1 µg/larva), or water (control), or left untreated (naïve), hemocytes and fat body were then collected at 20h post-injection for preparation of total RNAs.

Expression of AMP genes, including *cecropin-6*, *attacin-1*, *attacin-2*, *lebocin-b/c* (primers were for the common regions of the two mRNAs), *moricin* and lysozyme, in hemocytes (A) and fat body (B) was determined by real-time PCR. Ribosomal protein S3 (*rpS3*) gene was used as an internal control. The bars represent the mean of three individual measurements ± SEM. Relative expression of each AMP gene in naïve larvae was set as 1. Identical letters are not significant difference ($p > 0.05$), while different letters indicate significant difference ($p < 0.05$) among different treatments for each AMP gene determined by one way ANOVA followed by a Tukey's multiple comparison test.

Fig. 7. Activation of AMP genes in *M. sexta* larvae by *MsSpz*-C108 is blocked by pre-injection of antibody to *MsToll*

Day 1 fifth instar *M. sexta* naïve larvae were pre-injected with purified IgG to the ecto-domain of *MsToll* (Toll Ab, 5 µg/larva) or IgG from pre-bleed serum (control Ab, 5 µg/larva). These larvae were then injected with purified recombinant *MsSpz* (3 µg/larva), *MsSpz*-C108 (1 µg/larva), PG-SA (1 µg/larva), PG-K12 (1 µg/larva), or without second injection (control) at 1h after pre-injection of antibody. Hemocytes and fat body were collected at 20h after second injection for preparation of total RNAs. Expression of AMP genes (*cecropin-6*, *attacin-1*, *lebocin-b/c* and *moricin*) in hemocytes (A–E) and fat body (F–J) was determined by real-time PCR. Ribosomal protein S3 (*rpS3*) gene was used as an internal control. The bars represent the mean of three individual measurements ± SEM. Relative expression of each AMP gene after pre-injection of antibody but without second injection (control) was set as 1. Asterisks indicate significant difference (p < 0.05) between Toll and Control antibody pre-injections for each AMP gene determined by an unpaired t-test.