

Supplementary Material: Contains Figures S1-S8, Table S1, Table S2

Figure S1. Phylogenetic analysis of vertebrate TLRs and arthropod Tolls. Alignment of Toll receptors from several arthropods and TLRs from several vertebrates. Arthropod species are: *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bemisia tabaci*, *Bombyx mori*, *Camponotus floridanus*, *Culex quinquefasciatus*, *Danaus plexippus*, *Drosophila melanogaster*, *Operophtera brumata*, *Parasteatoda tepidariorum*, *Penaeus vannamei*, *Procambarus clarkii*, *Trichonephila clavipes*, *Helicoverpa armigera*, *Ostrinia furnacalis*, *Plutella xylostella*. Vertebrate species were: *Bos taurus*, *Canis lupus familiaris*, *Felis catus*, *Gallus gallus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*. Specific gene ID numbers are listed in Table S1. Vertebrate TLRs are highlighted in yellow, arthropod Toll1-8 members are highlighted in green, while Toll9 members are highlighted in pink.

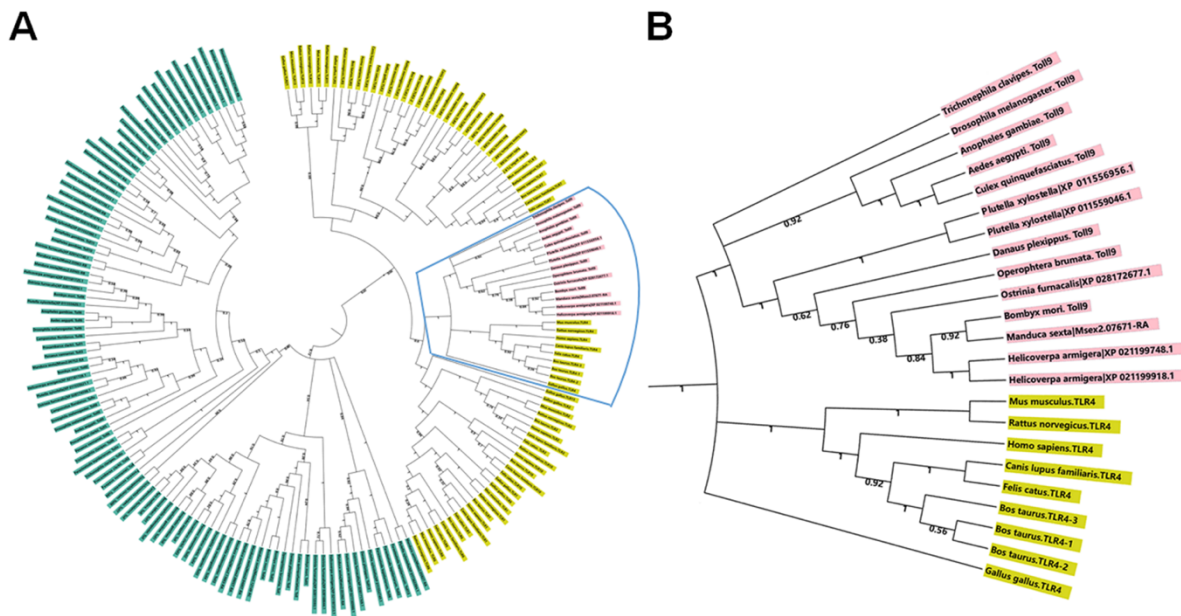


Figure S2. Three-dimensional model structures for *B. mori* Toll ectodomains. Model structures were generated using SWISS-MODEL and PyMOL. *Drosophila* Toll (Toll-1) served as the template for all members except BmToll9 where *Homo sapiens* TLR4 was used as the template. CF and NF: the cysteine cluster at the C-terminus and N-terminus of LRRs, respectively.

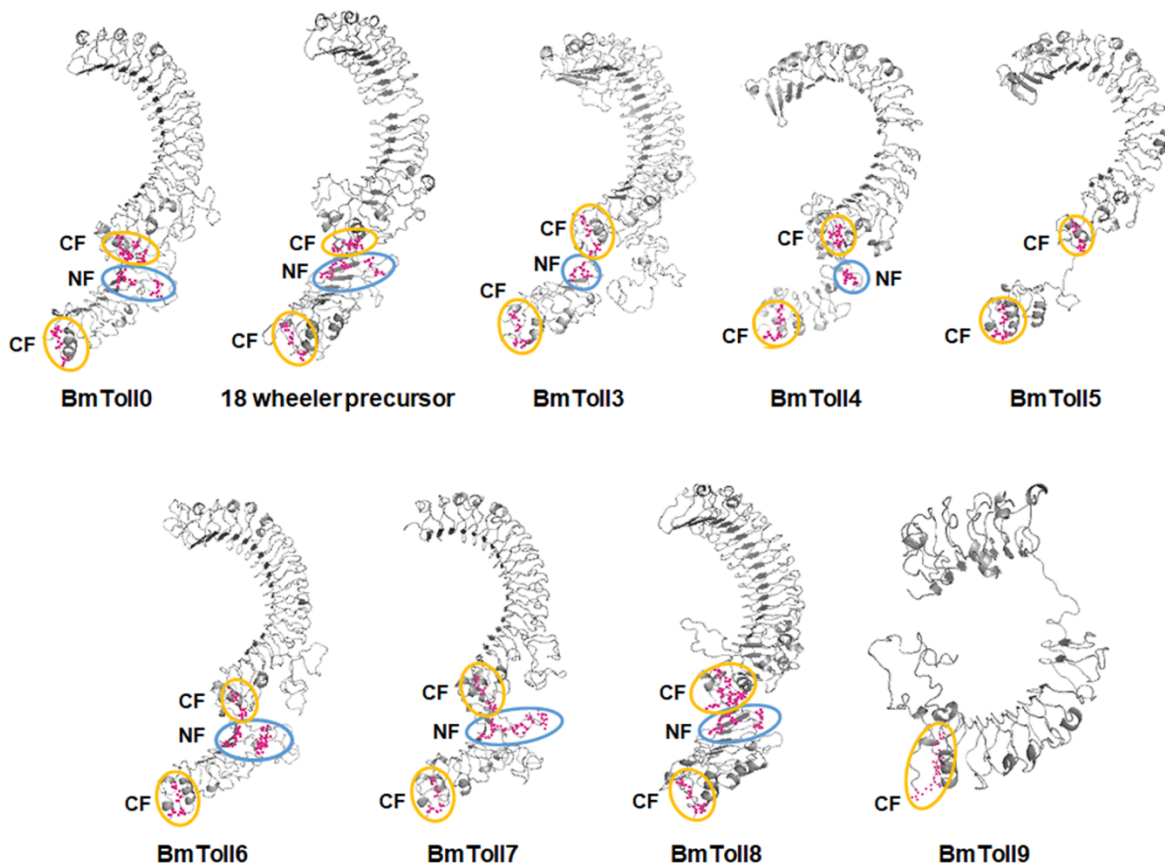


Figure S3. Expression of BmToll9 and BmMLs in larval stage fat body, hemocytes and midgut. (A) RT-PCR assay showing that BmToll9 mRNA is detected in the fat body (Fb), hemocytes (Hc) and midgut (Mg) of day 3 fifth instar *B. mori* larvae that were not immune challenged. Mass markers are indicated to the left. (B) Immunoblot probed with a rabbit polyclonal BmToll9^{ecto} antibody showing that BmToll9 protein is also detected in the absence of immune challenge. Tubulin visualized by an anti-tubulin antibody served as a loading control, while molecular mass markers (M) are shown to the left. (C) Confocal images showing BmToll9 on the surface of fat body, hemocyte, and midgut cells. BmToll9 was detected using a FITC-labeled anti-BmToll9^{ecto} antibody while nuclei were stained with DAPI. Scale bars for fat body and hemocyte images are 5 μm while scale bar for midgut cells is 20 μm . (D) Immunoblots probed with specific rabbit polyclonal antibodies showing that BmMD-2A, BmMD-2B, and BmML-1 are detected in the fat body (Fb), hemocytes (Hc), midgut (Mg) and plasma of non-immune challenged day 3 fifth instar *B. mori* larvae. Molecular mass markers are indicated to the left.

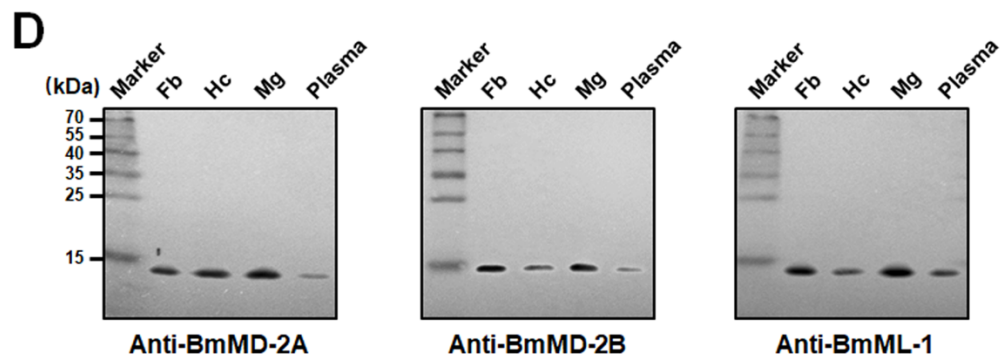
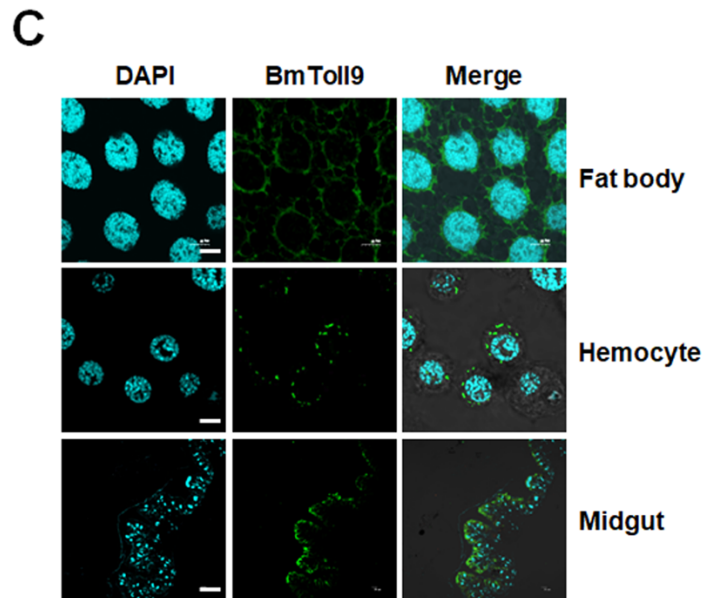
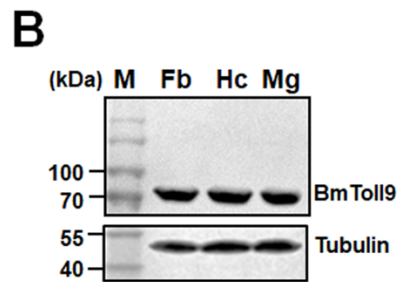
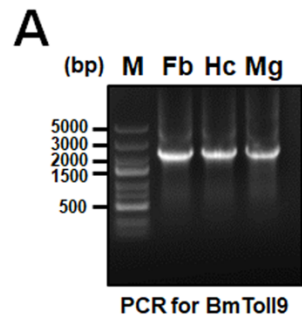


Figure S4. Confocal microscopy images showing that BmToll9 colocalizes with BmMD-2A and BmMD-2B on the surface of fat body, hemocyte, and midgut cells. Fat body, hemocytes and midgut were collected from day 3 fifth instar *B. mori* and processed as described in the Materials and Methods. Native BmToll9 was detected using FITC-labeled anti-BmToll9^{ecto}, while BmMD-2A (A) or BmMD-2B (B) were detected using anti- BmMD-2A or anti- BmMD-2B and an Alexa Fluor® 568 (red fluorescence) goat anti-rabbit secondary antibody. Cell nuclei were stained using DAPI. Scale bars for hemocytes and fat body cells are 5 μ m, while scale bar for midgut cells is 20 μ m.

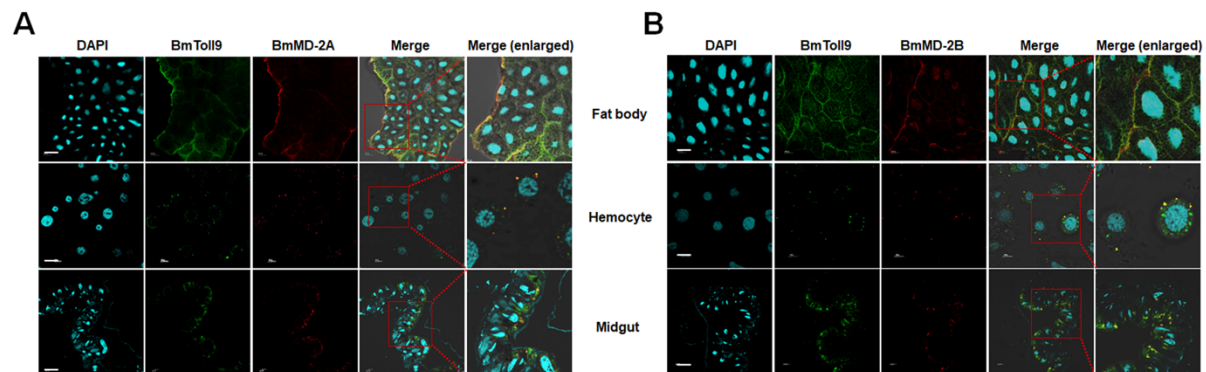


Figure S5. Expression of BmToll9 and BmML proteins in BmN and S2 cells. (A) RT-PCR assay showing that BmMD-2A, BmMD-2B and BmML-1 but not BmToll9 mRNAs are constitutively expressed in BmN cells. (B) Immunoblots probed with specific rabbit polyclonal antibodies showing that BmMD-2A, BmMD-2B and BmML-1 are detected in BmN cells and BmN cell-conditioned medium but not in S2 cells or S2 cell-conditioned medium. (C) Expression of recombinant GFP, BmToll9 and BmToll9^{ecto} in BmN cells. BmN cells were transfected with pIEx-4-GFP-V5, pIEx-4-BmToll9-V5 or pIEx-4-BmToll9^{ecto}-V5 followed by detection of recombinant proteins in cell lysates or the medium at 48 h post-transfection by immunoblotting using mouse monoclonal anti-V5 or rabbit anti-BmToll9^{ecto}. (D) Expression of recombinant BmML proteins and MD-2 in BmN cells. BmN cells were transfected with pIEx-4-BmMD-2A-His, pIEx-4-BmMD-2B-His, pIEx-4-BmML-1-His or pIEx-4-MD-2-His followed by detection of recombinant proteins in cell lysates or the medium at 48 h post-transfection by immunoblotting using a mouse monoclonal anti-His antibody (*upper blots*). BmML proteins in the cell culture medium were also detected by immunoblotting using rabbit polyclonal anti-BmMD-2A, anti-BmMD-2B, or anti-BmML-1 antibody (*lower blots*). (E, F) Expression of recombinant BmToll9, BmMD-2A and BmMD-2B in S2 cells. S2 cells were transfected with the same plasmids used to transfect BmN cells but were then selected to stably express BmToll9-V5, BmMD-2A-His or BmMD-2B-His. Recombinant BmToll9-V5 was detected in cell lysates by anti-V5 or anti-BmToll9^{ecto} while BmMD-2A-His or BmMD-2B-His were detected in cell lysates and the medium by anti-His (F). Molecular mass markers are indicated to the left.

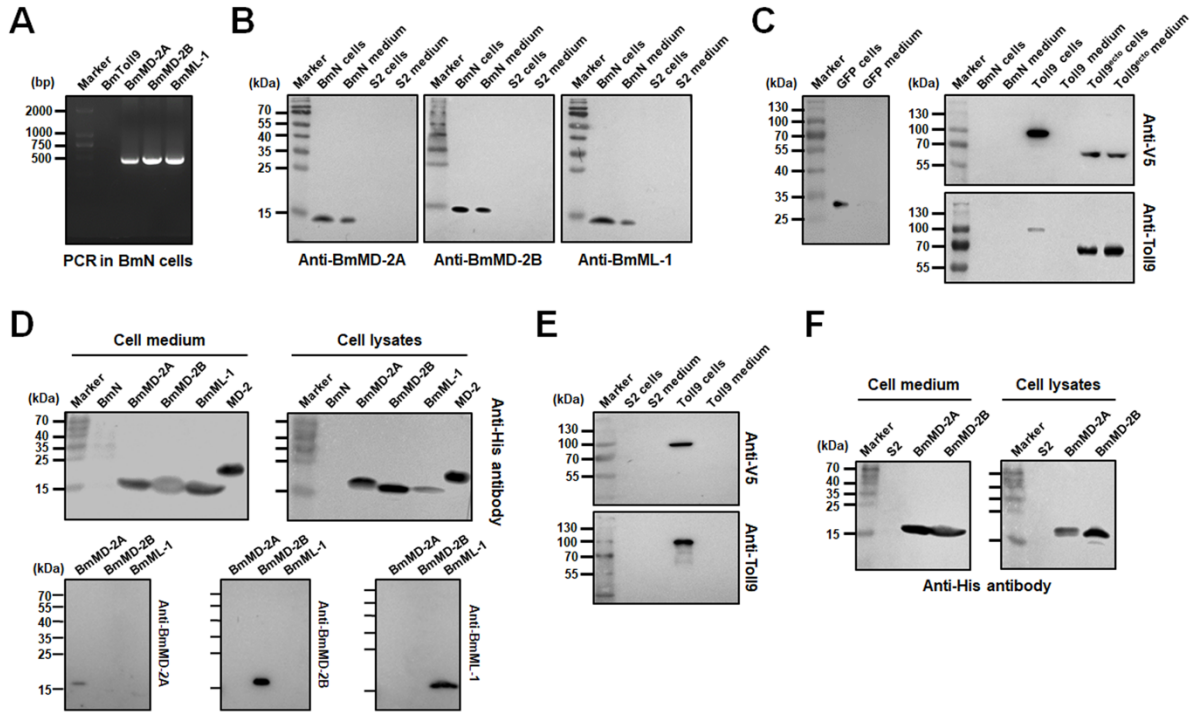


Figure S6. BmMD-2A and BmMD-2B do not synergistically interact with BmToll9 to activate AMP gene promoter activity. Mean relative luciferase activity \pm SEM in extracts prepared from S2 cells that were co-transfected with pIEx-4-BmToll9-V5 or pIEx-4-GFP-V5 plus pGL3B-*moricin*. Dual luciferase assays were then conducted using extracts prepared from cells to which no factors were added, LPS only was added, LPS plus BmMD-2A-His or BmMD-2B-His were added, or LPS plus BmMD-2A-His and BmMD-2B-His were added for 12 h. Three biological replicates were generated per treatment. For each graph, different letters above bars indicate treatments significantly differed from one another ($p < 0.05$; ANOVA followed by a post hoc Tukey's HSD test).

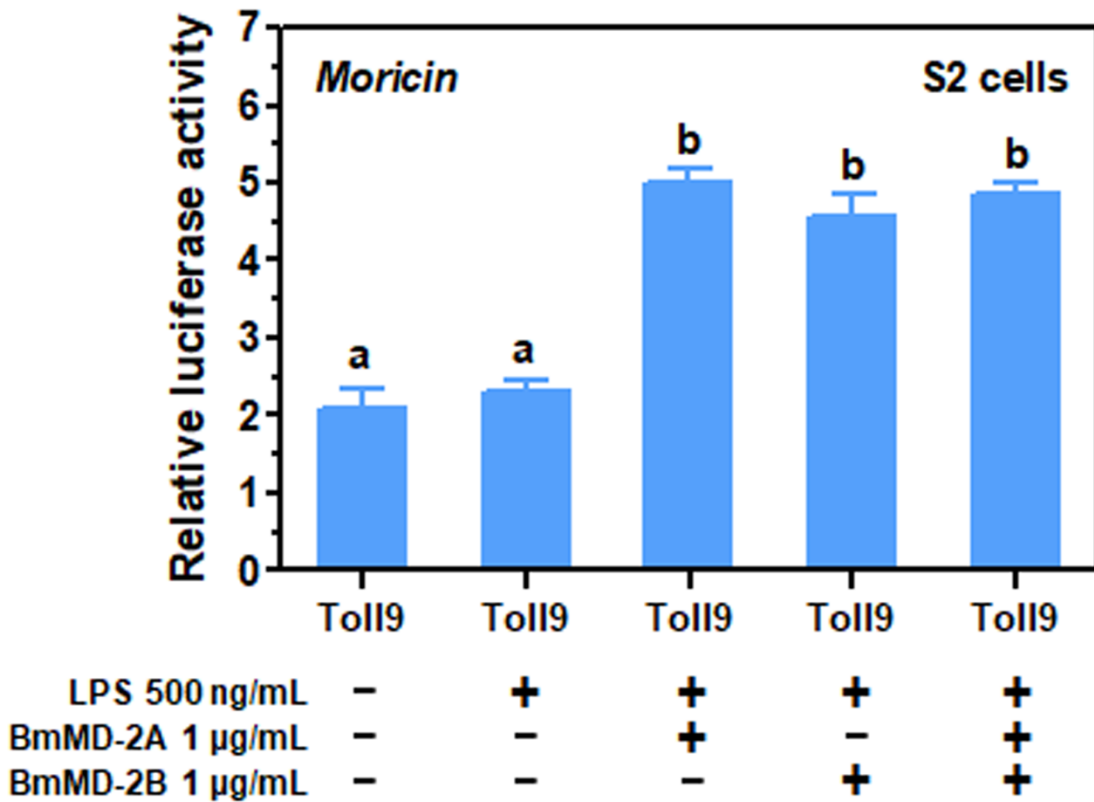


Figure S7. Expression of recombinant proteins and LPS sensitivity of RAW264.7 cells. (A) Immunoblot showing that RAW264.7 cells transfected with pcDNA3.1-GFP-HA, pcDNA3.1-BmToll9-HA, or pcDNA3.1-BmToll9-TLR4 express GFP-HA, BmToll9-HA and BmToll9-TLR4-HA. Each recombinant protein was detected using a mouse monoclonal anti-HA antibody. Molecular mass markers are indicated to the left. (B) Light microscopy images of RAW264.7 cells showing morphological changes induced by LPS. RAW264.7 cells were treated with or without LPS (100 ng/mL) for 24 h. Normal RAW264.7 cells are round, while LPS-activated cells changed morphology obviously with pseudopodia. Identical changes in morphology were observed in both non-transfected cells and cells transfected with pcDNA3.1-GFP-HA, pcDNA3.1-BmToll9-HA, or pcDNA3.1-BmToll9-TLR4.

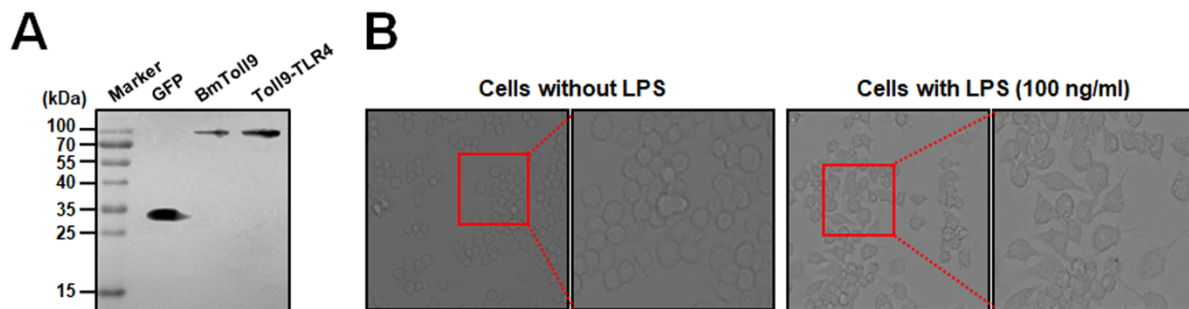


Figure S8. Expression of recombinant proteins in BMDM cells. BMDM (bone marrow derived macrophage) cells were collected from TLR4^{-/-} mice and examined by light microscopy (A) or flow cytometry against CD11b/F480 (B) after no stimulation or stimulation with M-CSF (50 ng/ml) for 48 h (A) or sorted in a flow cytometer (B). (C) Immunoblot showing that activated BMDM cells express TLR4-HA, BmToll9-HA, or BmToll9-TLR4-HA after adenovirus transduction. Each recombinant protein was detected using a mouse monoclonal anti-HA antibody. Molecular mass markers are indicated to the left.

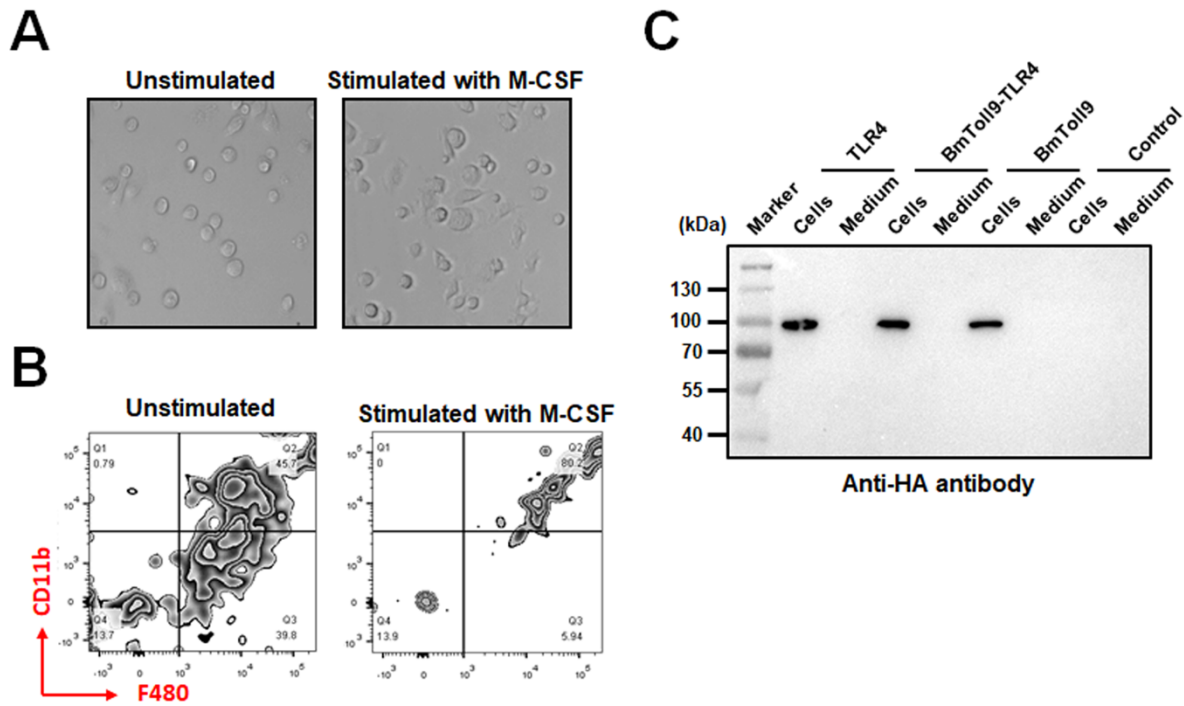


Table S1. NCBI gene accession numbers for all TLRs/Tolls in the phylogenetic tree

Species	Sequence name	NCBI accession number
<i>Bos taurus</i>	TLR1	NP_001039969.1
<i>Bos taurus</i>	TLR2	NP_776622.1
<i>Bos taurus</i>	TLR3-1	NP_001008664.1
<i>Bos taurus</i>	TLR3-2	XP_010818544.2
<i>Bos taurus</i>	TLR4-1	NP_776623.5
<i>Bos taurus</i>	TLR4-2	XP_005210643.1
<i>Bos taurus</i>	TLR4-3	XP_024851214.1
<i>Bos taurus</i>	TLR5	NP_001035591.1
<i>Bos taurus</i>	TLR6	NP_001001159.1
<i>Bos taurus</i>	TLR7	NP_001028933.1
<i>Bos taurus</i>	TLR8	NP_001029109.1
<i>Bos taurus</i>	TLR9	NP_898904.1
<i>Bos taurus</i>	TLR10	NP_001070386.1
<i>Canis lupus familiaris</i>	TLR1	XP_005618321.1
<i>Canis lupus familiaris</i>	TLR2	NP_001005264.2
<i>Canis lupus familiaris</i>	TLR3	XP_005630025.1
<i>Canis lupus familiaris</i>	TLR4	NP_001002950.2
<i>Canis lupus familiaris</i>	TLR5	NP_001184105.1
<i>Canis lupus familiaris</i>	TLR6	XP_022272951.1
<i>Canis lupus familiaris</i>	TLR7	NP_001041589.1
<i>Canis lupus familiaris</i>	TLR8	XP_003435496.1
<i>Canis lupus familiaris</i>	TLR9	NP_001002998.1
<i>Canis lupus familiaris</i>	TLR10	NP_001166598.1
<i>Felis catus</i>	TLR1	XP_003985489.3
<i>Felis catus</i>	TLR2	XP_003984979.1
<i>Felis catus</i>	TLR3	NP_001073298.1
<i>Felis catus</i>	TLR4	NP_001009223.1
<i>Felis catus</i>	TLR5	XP_011289108.2
<i>Felis catus</i>	TLR6	XP_003985516.1
<i>Felis catus</i>	TLR7	NP_001073602.1
<i>Felis catus</i>	TLR8	XP_019679113.1
<i>Felis catus</i>	TLR9	NP_001009285.1
<i>Felis catus</i>	TLR10	XP_006931230.1
<i>Gallus gallus</i>	TLR1	BAD67422.1
<i>Gallus gallus</i>	TLR2	NP_989609.1
<i>Gallus gallus</i>	TLR3	NP_001011691.3

<i>Gallus gallus</i>	TLR4	NP_001025864.1
<i>Gallus gallus</i>	TLR5	NP_001019757.1
<i>Gallus gallus</i>	TLR6	NP_001075178.3
<i>Gallus gallus</i>	TLR7	NP_001011688.1
<i>Gallus gallus</i>	TLR15	NP_001032924.1
<i>Homo sapiens</i>	TLR1	AAV85640.1
<i>Homo sapiens</i>	TLR2	AAC34133.1
<i>Homo sapiens</i>	TLR3	ABC86908.1
<i>Homo sapiens</i>	TLR4	AAV82270.1
<i>Homo sapiens</i>	TLR5	AAZ17468.1
<i>Homo sapiens</i>	TLR6	ABW37063.1
<i>Homo sapiens</i>	TLR7	AAZ99026.1
<i>Homo sapiens</i>	TLR8	AAQ88663.1
<i>Homo sapiens</i>	TLR9	AAZ95519.1
<i>Homo sapiens</i>	TLR10	AAV78485.1
<i>Mus musculus</i>	TLR1	NP_001263374.1
<i>Mus musculus</i>	TLR2	NP_036035.3
<i>Mus musculus</i>	TLR3	NP_001344245.1
<i>Mus musculus</i>	TLR4	NP_067272.1
<i>Mus musculus</i>	TLR6	AAH55366.1
<i>Mus musculus</i>	TLR7	AGX25544.1
<i>Mus musculus</i>	TLR8	AAK62677.1
<i>Mus musculus</i>	TLR11	NP_991388.2
<i>Mus musculus</i>	TLR13	NP_991389.1
<i>Rattus norvegicus</i>	TLR1	NP_001165591.1
<i>Rattus norvegicus</i>	TLR2	NP_942064.1
<i>Rattus norvegicus</i>	TLR3	NP_942086.1
<i>Rattus norvegicus</i>	TLR4	NP_062051.1
<i>Rattus norvegicus</i>	TLR5	NP_001139300.1
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<i>Rattus norvegicus</i>	TLR11	NP_001138251.2
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<i>Rattus norvegicus</i>	TLR13	XP_006227480.1
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<i>Aedes aegypti</i>	Toll7	XP_001655730.1
<i>Aedes aegypti</i>	Toll9	EAT34302.2
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<i>Anopheles gambiae</i>	Toll11	XP_309461.4
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<i>Bemisia tabaci</i>	Toll1	ASK86670.2
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<i>Helicoverpa armigera</i>		XP_021181700.1
<i>Helicoverpa armigera</i>		XP_021197313.1
<i>Helicoverpa armigera</i>		XP_021190980.1
<i>Helicoverpa armigera</i>		XP_021199748.1
<i>Helicoverpa armigera</i>		XP_021199918.1
<i>Manduca sexta</i>		Msex2.03963-RB
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<i>Manduca sexta</i>		Msex2.03960-RB
<i>Manduca sexta</i>		Msex2.07405-RB
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<i>Manduca sexta</i>	Msex2.13883-RA
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<i>Plutella xylostella</i>	XP_011565206.1
<i>Plutella xylostella</i>	XP_011554740.1
<i>Plutella xylostella</i>	XP_011562284.1
<i>Plutella xylostella</i>	XP_011556956.1
<i>Plutella xylostella</i>	XP_011559046.1

Table S2. Primers used in this study

Primer name	Primer sequence (5' → 3')	Size (bp)
For PCR		
BmToll9	F: ATGATATTGAAGATCATTAAG R: TCAAGCTAAAGATACGTTTTCC	2268
BmMD-2A	F: ATGTTGTTTTTCATCAC R: TTAGACCAGTTTAGCG	438
BmMD-2B	F: ATGGCTCTTACTCTTG R: CTAAACCAGCCTGACATTC	465
BmML-1	F: ATGTTTGAAACGAGCGC R: TTAAGAAATCTTAACAGG	465
For qRT-PCR		
Moricin	F: CGCTCCAGCAAAAATACCTA R: TACCGACTGCCTTTCCTACA	156
Cecropin B	F: CTATCCTTCGTCTTCGCTCT R: ATGTTCTGCCCATTTTTTC	104
Attacin 1	F: CAGTGAACCTCGGATGGAACC R: CCGTGCCCGTTTACATTGTC	160
Gloverin 2	F: AAGTTTACGGACCTTCTGATTACG R: AGTGCCAAAGACCTTGCCCTC	119
rp49	F: CAGGCGGTTCAAGGGTCAATAC R: TGCTGGGCTCTTCCACGA	213
For HpQE60		
BmToll9 ^{ecto}	F: GAAACGAGGAAAACAC R: TAACTATTTAGACCAGC	600
BmMD-2A	F: AAATTCTTCAAGGATTGCG R: GACCAGTTTAGCGTTTATAAG	387
BmML-1	F: ACAAACGTGAGACAATG R: AGAAATCTTAACAGGTAC	408
BmMD-2B	F: GAATTCAACGTTGTCAC R: AACCAGCCTGACATTC	408
For pIEx-4		
BmToll9-V5	F: ATGATATTGAAGATCATTAAGCTATTAATAATTCTTTCTGCT CTTCATCGCATCAGCGGAAACG R: TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAG	2310

GCTTACCAGCTAAAGATACGTTTTTC

	F: ATGATATTGAAGATCATTAAAGCTATTA AAAATTCTTTCTGC TCTTCATCGCATCAGCGGAAACG	
BmToll9 ^{ecto} -V5	R: TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAG GCTTACC TTTGTACTGATTTTTTAATC	1695
	F: ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTG CCCATCCTGGTCGAGCTGG	
GFP-V5	R: TTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAG GCTTACC CTTGTACAGCTCGTCCA	762
	F: ATGTTGTTTTTCATCACTGC	
BmMD-2A-His	R: GACCAGTTTAGCGTTTATAAG	435
	F: ATGGCTCTTACTCTTG	
BmMD-2B-His	R: AACCAGCCTGACATTCG	462
	F: ATGTTTGAAACGAGCGC	
BmML-1-His	R: AGAAATCTTAACAGGTAC	462
	F: ATGTTGCCATTTATTCTC	
MD-2-His	R: ATTGACATCACGGCGGTG	480
	F: ATGATATTGAAGATCATTAAAGCTATTA AAAATTCTTTC OR: CTCGCCCTTGCTCACCATAGCTAAAGATACGTTTTTC OF: GAAAACGTATCTTTAGCTATGGTGAGCAAGGGCGAG	
BmToll9-GFP	R: CTTGTACAGCTCGTCCATGCCGAGAGTGATCC	2985
	F: ATGTTGTTTTTCATCACTGCGGGCGGTGCTCTTGGCCAG	
BmMD-2A-RFP	OR: CCTCGCCCTTGCTCACCATGACCAGTTTAGCGTTTATAAG OF: CTTATAAACGCTAAACTGGTCATGGTGAGCAAGGGCGAGG	1146
	R: CTACTTGTACAGCTCGTCCATGCCGCCGGTGGAGTGG	
	F: ATGGCTCTTACTCTTGGCTGTTATTCGCTGCATTTTC	
BmMD-2B-RFP	OR: CCTCGCCCTTGCTCACCATAACCAGCCTGACATTCG OF: CGAATGTCAGGCTGGTTATGGTGAGCAAGGGCGAGG	1173
	R: CTACTTGTACAGCTCGTCCATGCCGCCGGTGGAGTGG	
For pGL3B		
	F: AATAGTATTA AATTTTTAATATATCTC	
Moricin	R: TGCCACAATAAAAACAAAG	1000
	F: ATGCGGTATTTACTTTGC	
Cecropin B	R: AATGTAATTAATTCAGACTTG	1500
	F: GTTATTTTATTACCGTG	
Attacin 1	R: CTTGATCTGTGTCGTATTAC	1000
BmToll9-TLR4		2250

cloning	F: ATGATATTGAAGATCATTAAGCTATTA AAAATTCTTTCTGC OR: CCACAATCACACTGACCACTTTG TACTGAT TTTTAATCATC OF: GATGATTAAAAATCAGTACAAAGTGGTCAGTGTGATTGTGG R: GGTCCAAGTTGCCGTTTCTTGTTCTTCCTCTGCTGTTTGC	
For pcDNA3.1		
BmToll9- TLR4-HA	F: ATGATATTGAAGATCATTAAGCTATTA AAAATTCTTTCTGC R: TTAAGCGTAGTCTGGGACGTCGTATGGGTAGGTCCAAGTT GCCGTTTC	2280
BmToll9-HA	F: ATGATATTGAAGATCATTAAGCTATTA AAAATTCTTTCTGC R: TTAAGCGTAGTCTGGGACGTCGTATGGGTAAAGCTAAAGAT ACGTTTTTC	2295
TLR4-HA	F: ATGATGCCTCCCTGGCTCCTGGCTAGGACTCTGATCATGGC R: TTAAGCGTAGTCTGGGACGTCGTATGGGTAGGTCCAAGTT GCCGTTTC	2535
BmMD-2B- Flag	F: ATGGCTCTTTACTCTTGGCTGTTATTCGCTGCATTTCTCG R: TTACTTATCGTCGTCATCCTTGTAATCAACCAGCCTGACAT TC	489
BmMD-2A- Flag	F: ATGTTGTTTTTCATCACTGCGGCGGTGCTCTTGGCCAGTG R: TTACTTATCGTCGTCATCCTTGTAATCGACCAGTTTAGCGT TT	462

Note: F for forward primer, R for reverse primer, OF for overlapping forward primer, and OR for overlapping reverse primer.