Structure and dynamics of Toll immunoreceptor activation in the mosquito Aedes aegypti

Aedes aegypti has evolved to become an efficient vector for arboviruses but the mechanisms of host-pathogen tolerance are unknown. Immunoreceptor Toll and its ligand Spaetzle have undergone duplication which may allow neo-functionalization and adaptation. Here we present cryo-EM structures and biophysical characterisation of low affinity Toll5A complexes that display transient but specific interactions with Spaetzle1C, forming asymmetric complexes, with only one ligand clearly resolved. Loop structures of Spaetzle1C and Toll5A intercalate, temporarily bridging the receptor C-termini to promote signalling. By contrast unbound receptors form head-to-head homodimers that keep the juxtamembrane regions far apart in an inactive conformation. Interestingly the transcriptional signature of Spaetzle1C differs from other Spaetzle cytokines and controls genes involved in innate immunity, metabolism and tissue regeneration. Taken together our results explain how upregulation of Spaetzle1C in the midgut and Toll5A in the salivary gland shape the concomitant immune response.

Insect Toll receptors perform critical functions in both embryogenesis and innate immunity. They are part of an ancient defence system that also includes the immunodeficiency (IMD) pathway and are conserved in vertebrates as the Toll-like receptors. Tolls and TLRs have a modular structure with an ectodomain made up of leucine-rich repeats (LRRs) and associated capping structures, a single transmembrane helix and a cytosolic Toll/Interleukin 1 receptor (TIR) domains. Most studies of innate immune function in insects have focussed on the fruit fly Drosophila melanogaster. The Drosophila genome encodes 9 Toll receptors that have diverse roles in development and other areas of cellular regulation as well as in immunity. Immune function is mediated mainly by the Toll receptor 1 (Toll1), while Toll6 and Toll7 are expressed in the nervous system and have equivalent functions to vertebrate neurotrophin receptors.

Toll receptors are activated by a complementary family of six cytokine-like molecules called Spaetzle (Spz). Spz1 proteins are secreted as larger dimeric precursors consisting of a natively unstructured pro-domain, which is proteolytically cleaved upon activation and a C-terminal cystine-knot fold similar to that found in human neurotrophins, such as nerve growth factor. Lys-type peptidoglycan from Gram-positive bacteria is detected by recognition proteins PGRP-SA and GNBP1 and these complexes activate a cascade of serine proteases. The terminal proteinase, Spaetzle processing enzyme (SPE), has trypsin-like specificity and cleaves the Spz precursor specifically at the junction between the pro-domain and cystine-knot, which remain associated by non-covalent interactions. In contrast Spz2 (also called neurotrophin-1, NT1) and Spz5 (NT2) are cleaved during secretion by furin-like proteases and Spz5 is secreted in a mature form without its pro-domain. Growing evidence suggests...
promiscuity in ligand binding, with Toll1 and Toll7 recognising Spz1, Spz2 and Spz5 and, more controversially, vesicular stomatitis virus (VSV) virions\(^{10-12}\), while Toll6 senses Spz2, Spz5 and possibly dsRNA\(^{13-14}\).

Mosquitoes and fruit flies are both dipterans but they diverged in evolution about 260 million years ago\(^{13,16}\). Comparative genomic analysis of Aedes aegypti, Anopheles gambiae and Drosophila melanogaster reveals that most genes involved in innate immunity are conserved in the mosquito, including the Toll and Spaetzle families\(^{17}\). Nevertheless, the Toll family has undergone significant diversification with the loss of orthologs in mosquitoes (no Toll2 or Toll3 in A. aegypti for example) and species-specific expansion of two more (Toll10 and Toll11). The underlying driving force of gene duplication is likely interconnected arms-race between pathogens and insects. In Drosophila Toll mediates most immune functions, while in Aedes two gene reduplications have occurred to produce an orthologous group of four closely related receptors Toll1A, Toll1B, Toll5A and Toll5B. The phylogeny of insect Tolls and vertebrate TLRs the latter appear to have evolved from a duplication and shows the importance of revisiting Toll signalling in hematophagous insects that impact human health.

**Results**

**Cryo-EM structures of Toll5A and Spz1C**

Recombinant Toll5A ectodomain was mixed with a 3-fold molar excess of Spz1C and the resulting complex was then purified by size exclusion chromatography immediately prior to grid preparation as described in Methods. Three oligomeric states of the receptor were observed at near-atomic resolutions, suggesting that Toll5A is highly dynamic in the presence of Spz1C. Homodimers of Toll5A at 3.4 Å maximum resolution had 85,810 particles, 2:1 heterodimers of Toll5A bound to Spz1C at 4.23 Å had 40,153 particles, and a 3:1 heterotrimer at 3.74 Å had 42,866 particles (Fig. 1 and Supplementary Fig. 1). While all three types of particles spread randomly across the grid, according to their large Euler angular distribution, they display significant variations in local resolutions, likely a consequence of their stoichiometric and conformational heterogeneity. Overall, the N-terminal LRR domains of each receptor chain were more resolved than their C-terminal moieties with the latter partially truncated upon density modification (Supplementary Fig. 2). Similarly, two ligands are observed but only one is well resolved, while the density of the second one is partial and seems to be restricted to the cys-knot core of the molecule. Although there is space to accommodate a second ligand without any steric clashes, the lack of resolution in this area does not allow visualisation. The heterotrimer complex is therefore treated as a 2:1 complex. The vacated ligand binding site is occupied by another receptor chain in the 3:1 heterotrimer, illustrating the transition between homo- and heterodimerisation (Supplementary Fig. 1). The three Toll5A molecules observed are referred to as chain A (teal), chain B (dark blue) and chain C (powder blue) in superimposable conformations in all three structures (RMSD below 2 Å). The Spz1C homodimer is composed of chain D (for distal, in yellow) and chain P (for proximal, in orange), attached to Toll5A chain A at the concave side and to chain B at its “back” or convex side. The structure of Toll5A homodimers (chains A and B) was solved at the highest overall resolutions (3.4 – 4.4 Å), with a head-to-head arrangement, maintaining the C-terminal juxtamembrane regions far apart (Fig. 1a and Supplementary Table 1). The Cα atoms of Cys-783 at the C-termini of each receptor chain are separated by 207 Å. Such a structural arrangement – if sterically possible when the receptor is expressed on the same cell – would ensure that Toll5A is locked in an inactive state preventing TIR domain association and signalling. If receptors are situated on neighbouring cells, such an orientation might be relevant to cell adhesion.

The overall structure of Toll5A is comparable to DmToll with a conserved number of leucine-rich repeats in both the N- and C-terminal domains, and conserved cysteine-rich cap structures surrounding these domains. The sequences of Toll5A and DmToll are 30 % identical and 50 % similar. In particular, the extended N-terminal cap formed by two hairpins and a parallel β-sheet, which is involved in Spz binding, is conserved in A. aegypti Toll5A but not in Drosophila Toll-5, also known as Tehao, which remains an orphan receptor\(^2\).

Superposition of Toll5A and DmToll structures reveals that the diameter of the N-terminal LRR solenoid of Toll5A is 5–10 Å greater compared to the Drosophila Toll1 receptor, with an inner diameter of about 50 Å and an outer diameter of 90 Å and an overall RMSD of 3 Å (Supplementary Fig. 3). Toll5A has nine predicted glycosylation sites, 6 of which were observed (Asn-linked glycans visible at positions 151, 194, 481, 521, 634 and 687) and found to restrict access to its surface. In
contrast, DmToll has thirteen sites, which are not conserved with Toll5A, except for Asn-481 on the ascending flank of LRR13. Glycans restrict protein-protein contacts on the concave surface and the flanks of the receptor.

Toll5A forms head-head symmetric homodimers with LRRNT1 cap residues between Thr-34 and Tyr-79 binding the concave surface in the vicinity of Tyr-202 (LRR4) to Asn-419 (LRR13). For instance, Tyr-56 is hydrogen bonded to Tyr 226 for each chain (Fig. 2). The buried surface area is about 1000 Å². Interestingly, receptor-receptor contacts in Toll5A differ from those observed in Drosophila Toll structures25,33, which involve the N-terminal capping region and the hinge region between LRR domains. The Toll5A homodimer interface overlaps with the Spz1C ligand binding site, which suggests a direct competition between LRR domains. The Toll5A homodimer interface overlaps with the Spz1C ligand binding site, which suggests a direct competition between receptor-receptor and receptor-ligand interactions. Moreover, the presence of unliganded receptor in the Toll5A-Spz1C sample suggests that ligand binding is reversible.

Spz1C binding breaks receptor symmetry

We observed a ligated heterodimer of Toll5A with a stoichiometry of two receptor ectodomains to one Spz1C ligand (2:1 complex) at a lower resolution (4.2–8.4 Å). Spz1C is a covalent dimer stabilised by two intermolecular disulphide bonds instead of one in Drosophila (Supplementary Fig. 3). The central disulphide bond between Cys-94 is conserved in DmSpz, while Spz1C has an additional intermolecular bond between Cys-59 located basally in an area directly involved in binding to the N-terminal concave surface of the receptor. Proximal Cys-59 is located within hydrogen bonding distance of the hydroxyl group of Toll5A Tyr-226 in LRR5 (Fig. 2). The concave interface formed by the N-terminus up to LRR7 buries only -890 Å² of accessible surface area, which represents less than half that observed in the Drosophila complex32,34. Drosophila Spz possesses a five-residue insertion (Asn-59-Gln-62) in the vicinity of mosquito Cys-59, which expands its interface (Supplementary Fig. 3). Nonetheless, Spz1C adopts an asymmetric binding mode, reminiscent of that found in Drosophila despite the lack of sequence conservation (Supplementary Fig. 4), in which chain P contributes about 610 Å² and chain D, 280 Å² to the concave binding site. The interactions of Spz1C chain P at the concave site involves mostly residues from the first and third β-strand of Spz1C (Gln-14 and Leu-16, and Tyr-71, respectively) with the LRRNT1 of Toll5A (Tyr-79, Tyr-65 and His-54, respectively). Hydrogen bonds occur between His-15 and His-54, and Tyr-71 and Tyr-56, which contribute to the asymmetric binding mode seen in the complex.

The overall structure of Spz1C is however, very similar to DmSpz, if the flexible Trp-loops between residues His-15 and Gln-40, and the β-wings between residues Tyr-71 and Val-89, are omitted. Indeed, the R.M.S.D of superimposed Cys-knot domains is about 2.2 Å over 53 Cα atoms in the absence of these flexible regions25,33. Interestingly, in the mosquito Toll5A-Spz1C complex, these loops define asymmetric contacts at the dimerization interface with the “back” of chain B (Fig. 2). Chain D of Spz1C binds extensively to the dimerization interface with over 1,300 Å² of its accessible surface area, while chain
P contributes only 180 Å², confined to a loop that protrudes from the convex side of LRR14. Remarkably, the integrity of the LRR14 loop seems to determine the dynamic interactions between Toll5A and Spz1C, while its cleavage stabilizes the complex (Supplementary Figs. 6 and 7). This property is reminiscent of the Z-loop of nucleic-sensing TLRs22,35–37. We will therefore refer to it as the Z-loop with conserved Asn and Asp residues suitable for Asparagine endopeptidase (AEP) processing38,39, while noting the absence of a cathepsin site in Toll5A.

In the crystal structure of refolded DmSpz, the β-wings (residues 75–93) are symmetric in both protomers, displaced from the Cys-knot framework by 90° and participating in crystal packing34. By contrast in our cryo-EM structure, the β-wings adopt a very different conformation contacting the Z-loop in a pincer-like grasp (Fig. 2c–e and

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**Fig. 2 | Ligand-induced asymmetry.** a Close-up top view of the homodimer interface with N-terminal receptor-receptor interactions at LRRNT1-LRR14 and accessible Z-loops. b Zoomed in side view Spz1C concave binding to LRRNT1-LRR7. c Spz1C convex binding at the dimerization interface. Spz1C mediates extensive contacts between β-wings and the Toll5A Z-loop located between LRR14-LRR15 (Asn455-Asp465), with the distal Trp-loop stabilizing the hinge region between LRR domains. d Receptor-receptor contacts persist within the asymmetric heterocomplex. e Asymmetry in Spz1C. The proximal Trp-loop adopts a closed helical conformation with a buried Trp-31 that caps the primary receptor LRRNT1, both β-wings and the Z-loop, while the distal Trp-loop is extended and deviates from the core of the molecule, contributing to the asymmetry of the heterodimer complex.
Supplementary Fig. 6A). Molecular surface analysis of the receptor and the ligand suggests that the electrostatic charge distribution and shape complementarity are suitable for receptor-ligand coupling upon conformational selection (Supplementary Fig. 5). The dimerization interface of 1480 Å² is one and a half times the area of the concave binding surface, suggesting a higher affinity binding compared to the concave interface. While the absence of particles with a stoichiometry of 1:1 may indicate a sequential mechanism of assembly in nonequilibrium unsaturated complexes.

Interestingly, Spz1C possesses highly asymmetric Trp-loops. The distal one is extended and participates in dimerization with Trp-31E stacking against Phe-610F at the junction between LRR domains. The other loop adopts a short helical structure, which buries Trp-31F in a hydrophobic network that includes receptor N-terminal residue Phe-60O (Fig. 2). It is possible to model Spz1C with both protomers in a hydrophobic network that includes receptor N-terminal residue Phe-610F at the junction between LRR domains. The other loop adopts a short helical structure, which buries Trp-31F in a hydrophobic network that includes receptor N-terminal residue Phe-60O (Fig. 2). It is possible to model Spz1C with both protomers in a hydrophobic network that includes receptor N-terminal residue Phe-610F at the junction between LRR domains. The other loop adopts a short helical structure, which buries Trp-31F in a hydrophobic network that includes receptor N-terminal residue Phe-60O (Fig. 2).

Toll5A ectodomain competes with ligand binding
A third particle was observed with a stoichiometry of 3 receptors and 1 ligand (Supplementary Figs. 1 and 2). It is a hybrid of the homodimer (Toll5A chains A and C) and the 2:1 Toll5A-Spz1C heterodimer (Toll5A chains A and B) leading to a trimer of A, B and C chains, with Spz1C bound to the back of chain B and the concave side of chain A without any further contacts with chain C. Some minor differences are observed in main-chain and side-chains positions, which might be due to flexibility in both receptor and ligand molecules and the intermediate resolution of the heterotrimer map at 3.7–4.7 Å, compared to the homo- and heterodimer. While binding of a first Spz1C ligand shifts the receptor to adopt an asymmetric dimer configuration, the second binding site remains predominantly associated to another receptor chain.

Receptor specificity is achieved with low ligand binding affinity
The interaction between Spz1C, and the ectodomain of Toll5A was characterised using a range of biophysical techniques. We used surface plasmon resonance to measure the kinetics of association and dissociation between mosquito and Drosophila Toll and Spz proteins (Fig. 3A and Supplementary Fig. 8) and found that Toll5A only binds Spz1C. Surprisingly, A. aegypti Spz2A did not bind TollIA, despite being the closest structural homologues of DmToll and DmSpz1 in the mosquito. Spz2A did not bind Toll5A, and neither did DmSpz1. Furthermore, Spz1C did not bind TollIA or DmToll, suggesting receptor-ligand specificity. By contrast, Toll5A and Spz2C interact with a Kₘ value of ~2 μM, whereas Drosophila Toll binds Spz with a much higher affinity of 30–80 nM, consistent with previous studies. Hence, the Toll5A-Spz1C complex is species- and paralogue specific despite being of low affinity.

Ligand binding triggers a conformational change within Toll5A dimers in solution
We then used SEC-MALS experiments in the presence and absence of Spz1C to characterise the stoichiometry of the receptor and its complex. This technique revealed a concentration-dependent shift in the stoichiometry of the receptor, from a monomer to a homodimer in the absence of ligand when the concentration was increased from 20 to 50 μM prior to size-exclusion chromatography (Fig. 3). In the presence of 20 μM Toll5A saturated with Spz1C, a complex formed that was polydisperse but at 50 μM Toll5A saturated with Spz1C, the mixture appeared monodisperse with a mass corresponding to 206 kDa. SEC-MALS indicates a mass of 175 kDa for the unbound receptor dimer so the 206 kDa form is consistent with a 2:1 complex of Toll5A and a disulphide-linked Spz1C dimer (26 kDa).

We then explored this 2:1 complex further using SEC-SAXS at 50 μM and compared it to the receptor homodimer at the same concentration and in the same buffer. SAXS analysis allows rapid assessment of structural changes in response to ligand binding and can quantitatively characterize flexible molecules. The Guinier plot demonstrates the aggregate-free state of the receptor and its complex (Supplementary Fig. 9). As one of the few structural techniques amenable to dynamical systems, SAXS analysis suggests that the 2:1 complex is less flexible than the homodimer, despite its slightly larger dimensions (Supplementary Table 2). Hence, the receptor likely undergoes conformational changes upon ligand binding.

Toll5A-Spz1C complexes undergo dynamic exchange
The linearity of the Guinier plot (Supplementary Fig. 9) does not ensure the ideality of the sample, hence further direct methods were used in solution and under native conditions. We used Analytical Ultracentrifugation (AUC) to determine relative concentrations, sedimentation coefficients, molecular weight and shape (frictional ratio) of Toll5A and Toll5A-Spz1C complexes (Supplementary Fig. 10). These experiments reveal that Toll5A ectodomain alone is in equilibrium between monomers (≤ 3 μM) and dimers that prevail at concentrations ≥ 30 μM (Fig. 4A). The concentration-dependent stoichiometry differs from SEC-MALS, for which monomeric receptor was observed at 20 μM, most likely as a consequence of sample dilution or the effect of the matrix on protein-protein interactions during the size-exclusion chromatography step that precedes MALS.

The shape of the AUC curves indicates that receptor-receptor interactions undergo slow exchanges, defined by discrete peaks at 5.6S, and 7.3S, respectively. In contrast, the presence of Spz1C at equimolar concentration causes the formation of Toll5A-Spz1C complexes with sedimentation coefficients ranging between 5.5 and 8.6 S (Fig. 4B). Toll5A forms heterogeneous complexes with its ligand, which may include a 1:1 complex at 6.7S, as well as 7.3S (homodimers) and 7.85 species (2:1 heterodimers) in different conformational states. However, in the presence of excess Spz1C, better resolved molecular species sediment as two distinct populations of 6.7S and 8.5S. These complexes are reminiscent of the 1:1 and 2:2 complexes formed by DmToll and Spz in similar experimental conditions.

To determine whether these conformers are present for mosquito proteins at more physiological concentrations of ligand, we measured the composition of Toll5A-Spz1C mixtures at nanomolar concentrations using mass photometry (Fig. 4C, D). Our experimental setting was able to measure accurately molecules and complexes above 60 kDa, while free Spz1C was below the threshold, which resulted in erroneous mass determination. At 25 nM Toll5A in the presence of 25 nM Spz1C,
Toll5A is predominantly monomeric but a 2:1 complex is detected along with a species at 48 kDa, corresponding most likely to Spz1C. By contrast, at 50 nM Toll5 in the presence of 50 nM Spz1C, 1:1 ligated monomer and 2:2 heterodimer complexes appear in addition to Toll5A monomer and 2:1 complex. Hence, mass photometry detects stoichiometric conversions in non-equilibrium conditions. Toll5A homo-dimer is not detected consistent with the AUC data that suggests a KD value for this interaction in the µM range. None of these techniques detected the heterotrimer, which is therefore most likely a side effect of cryo-EM’s capacity of visualising transition intermediates.

Spz1C drives the production of antimicrobial peptides

In order to assay the activity of Spz1C, we stimulated the Aedes aegypti cell line Aag2, which constitutively expresses Toll5A (Supplementary Fig. 11), with either full-length Spz1C proprotein or processed forms and Gram-negative bacteria for IMD pathway stimulation, as a positive control for a potent innate immune response. RT-qPCR was used to measure the induction of a range of antimicrobial peptides. Aag2 cells do not up-regulate antimicrobial peptides (AMP) upon stimulation with pro-Spz1C. In contrast, processed Spz1C potently stimulated the production of several antimicrobial peptides, including Defensin 1, Cecropin A, Glycine-rich repeat protein (GRRP) holotricin and Attacin B, (Fig.5A). Using RT-PCR, we found all members of the Spz family to be constitutively expressed in Aag2 cells, except Spz2 (Supplementary Fig. 11). Given that Aag2 cells were shown to be unresponsive to microbial stimulation of the Toll pathway41,42, our result confirms full Toll signalling capacity when provided with the active ligand. Aag2 cells may therefore be defective either in microbial sensing or within the protease cascade activating Spz.

Upon activation, we found that Spz1C signalling overlaps but is distinct to that induced by a soluble extract of heat inactivated Gram-negative bacteria (GNB), which in addition activates the production of Gambicin, a general-purpose AMP. Neither GNB or Spz1C triggers the production of Vago, which is regulated by the RNA interference pathway, or Diptericin, an AMP potently induced by GNB in Drosophila via the IMD pathway43.

Next, we assayed production of GRRP holotricin, an AMP that is strongly induced by Spz1C, in a dose-response experiment (Fig.5b). Activation of holotricin expression occurs over a wide range of concentrations with an EC50 at sub-nanomolar concentrations. Furthermore, Spz1C signalling displays the same hallmarks of negative cooperativity observed in the Drosophila pathway, with 10% to 90% maximal signalling requiring an increase in ligand concentration of about 600-fold.

Interestingly, we found that different Spz paralogues have different signalling activities, as suggested by the capacity of Aedes aegypti Spz5 to potently activate gambicin (GAM) (Fig. 5c). While Drosophila Spz1 is able to stimulate GAM moderately in Aag2 cells, this property is not shared by Spz1C. In Drosophila, Spz5 is recognised by Toll, Toll6 and Toll710,11. Given that Aag2 cells express other members relating to the Toll family of receptors (Supplementary Fig. 11), it is conceivable that one of them triggers GAM activation reflecting its ligand specificity as opposed to the documented promiscuity of the Drosophila system.
Spz1C regulates genes involved in immunity and homeostasis

We have used RNASeq to define the transcriptomic signature linked to Spz1C and compare it to activation of the immune system by Gram-negative bacteria and purified DAP-PGN, which in Drosophila activates the IMD signalling pathway. As shown in Fig. 6 there are 85 genes regulated by Spz1C, of which 83 are up-regulated and only 2 are down-regulated. These include GRRP holotricin, confirming its role as a marker of Spz1C, as well as other molecules linked to complement and immunity. Many genes encoding Clip Serine proteases, Serpins and Gram-negative Binding Protein (GNBP) are upregulated. In Drosophila these proteins function in peptidoglycan recognition and proteolytic cascades that activate Spz.

The Patched 1 (Ptc1) 7-TM receptor is strongly induced with potential implications for tissue regeneration44. The ligand for Ptc1 is Hedgehog, a morphogen involved in embryonic segmentation. This signalling pathway also plays a crucial role in maintaining adult tissue homeostasis. It is not known whether Ptc1 functions in insect immunity. Another upregulated gene encodes a putative ecdysone inducible protein ortholog L2 and is homologous to Drosophila Imaginal morphogenesis protein-Late 2 (IMPL2)45, which is involved in the regulation of metabolism, growth, reproduction and lifespan. Two genes are down-regulated: Fatty Acyl-CoA Reductase (FAR) and the prostaglandin EP4 receptor. Interestingly, eicosanoid biosynthesis is controlled by the Toll pathway in some insects and also human PGE2 triggers a negative feedback loop, in which TLR4 signalling is restricted.

Discussion

In this study we show that the haematophagous mosquito A. aegypti has a duplicated Toll ligand, Spz1C, apparently unique to the Aedes genus (Supplementary bioinformatic analysis and Supplementary Fig. 12), that specifically activates the Toll5A paralogue with low affinity. We provide direct evidence of their interaction using purified proteins and show that ligand binding may be restricted by receptor self-association in a dose-dependent manner, resulting in conformational intermediates that we visualise by single particle cryo-EM. Functionally, Spz1C and Toll5A are known sensors of fungal infections in the fat body of adult mosquitoes26. Interestingly, anti-dengue activity has been assigned to the entomopathogenic fungus Beauveria bassiana29, and to the Toll pathway27,28, without elucidating the potential roles of individual Toll receptors or their respective ligands.

Here we provide evidence of a robust Spz1C response in Aag2 cells that differs from its Drosophila orthologue and mosquito paralogue. We also note that duplicated Toll and Spz are more divergent in sequence than 1-to-1 orthologues, with Spz1 the only duplicated cytokine within its family, while duplication occurred for members of the Toll family including Toll1, Toll5 and Toll9 and expansion of the Toll6/7/8 family with two additional members, Toll10 and Toll11 with potential anti-plasmodial properties46. Aedes aegypti diverged from Anopheles gambiae, which is the main vector for malaria, about 217 Myr ago. Aedes aegypti efficiently vectors flaviviruses such as Dengue, as evident from the large number of nonretroviral integrated
RNA viruses present in the A. aegypti but not the A. gambiae genome. Interestingly, the Asian tiger mosquito A. albopictus, which diverged from A. aegypti more recently, further expanded SpzIC genes. In contrast, more divergent species from the Aedes genus, Anopheles and Culex genera, which are not (or less) competent to vector these viruses, do not possess this paralog. However, a direct role of SpzIC in vectorial competence enabling flaviviruses, such as DENV, to exist symbiotically at high titre in mosquitoes remains to be established.

Our biophysical characterisation shows that SpzIC binds specifically to Toll5A with micromolar affinity compared to the nanomolar binding of DmSpz1 by DmToll1. DmSpz1 is also able to bind promiscuously to Toll6 and Toll7 and so it may be that low affinity binding confers signalling specificity for Toll5A. Consistent with this idea the Aedes Spz5 paralog but not SpzIC activates the AMP gambicin in Aag2 cells as strongly as bacterial extracts while DmSpz1 induces a partial activation (Fig. 5c). Thus A. aegypti has evolved a tiered Toll mediated immune system compared to Drosophila where Toll1 alone fulfills most immune functions.

Low affinity ligand binding and its ramifications in terms of signalling has been extensively characterised for mammalian cytokine receptors for interferons and interleukin ligands\(^{37-44}\). Such cytokines display pleiotropic effects while inducing a spectrum of redundant and yet distinct cellular functions. Receptor-ligand association and dissociation rates (\(k_{on}\) and \(k_{off}\)) have been found to be key in determining signalling outcomes. On-rates determine the amount of STAT transcription factor activation upon controlling the number of ligand-receptor complexes formed at the plasma membrane. In contrast, off-rates correlate to the kinetics of STAT activation depending on the half-life of ligand-receptor complexes. Alternatively, cell surface abundance of cytokine receptors plays a major role in triggering different transcriptional programs and cell fates, through obeying the mass action law and titrating cytokine concentrations. Our hypothesis in the case of Aedes Toll5A and SpzIC is that cell fate will be regimented in two ways depending on receptor and ligand concentrations: (i) ligand concentration changes the intensity of the signal (increased SpzIC for increased antimicrobial peptide production as illustrated in

**Fig. 5** Cleaved but not proprotein Spaetzle1C activates antimicrobial peptide production. Spz1C activates a set of antimicrobial peptides (a), over a large range of concentrations (b), and in an isoform-specific manner (c). Expression of antimicrobial peptides in Aag2 cells at 12 hours after stimulation with heat-inactivated extract from Gram-negative bacteria (abbreviated GNB or Spz1C (proS1C and S1C: microbial peptides in Aag2 cells at 12 hours after stimulation with heat-inactivated E.coli) conditioning buffer was used as a negative control. Data are presented as box plot with whiskers min to max values. (n = 12 biologically independent experiments for each concentration).

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Fig. 5); and (ii) increased receptor density might promote inhibitory self-association leading to immune quiescence.

The structures we present provide a plausible molecular explanation for negatively cooperative signalling. Binding of the first Spz1C homodimer to form the asymmetric complex can occur transiently at relatively low ligand concentrations but the 2:2 form would require saturation, and hence only form at saturating ligand concentrations. There are two classical theoretical models of sequential binding that lead to negatively cooperative receptor signalling as initially proposed by Koshland50,51. Binding of the first monomeric ligand to a dimeric receptor partially activates signalling but full activation requires the less favourable binding of the second ligand monomer. In the second model, the binding of two monomeric ligands to form a 2:2 complex is required for signal transduction. The two models lead to somewhat different theoretical stimulus-response curves and our experiments appear to fit better with Model 1 for the production of antimicrobial peptides (Fig. 5). If the ligand is limiting there is an ultrasensitive response with a pronounced threshold, more reminiscent of positive than negative cooperativity52. In our cell culture assays, Spz1C is likely not limiting, however this phenomenon may be relevant for development and homeostasis controlled by Spz in the whole organism.

The activation profile of Spz1C differs from Drosophila Spz1 and Aedes Spz5, despite their shared cystine-knot fold, having a transcriptomic profile that indicates functions that go beyond immunity. Spz1C specifically activates the production of Hedgehog receptor, insect insulin-binding protein L2 and genes involved in reprogramming fatty acid metabolism. If Spz1C and Toll5A were both upregulated in the salivary glands, and achieved the double ligated state as suggested by Bonizzoni et al.53, it is also conceivable that Spz1C and its receptor could be involved in viral tolerance by promoting lipid metabolism and homeostasis. The mechanism of action may differ in the midgut where Spz1C expression is induced to limit viral invasion, an immunological role31. By contrast in the salivary gland30, where Toll5A up-regulation may increase avidity for the ligand, a double ligated and hence fully saturated receptor complex might lead to a signal that differs from low density single-ligated receptors. Hence, future studies need to tackle the signalling capacities of 2:1 and 2:2 complexes by detecting the recruitment of MyD88 first, and if so, by checking if different sets of genes are controlled by both type of complexes. Fluorescence microscopy experiments will be best suited to detect such complexes along the presence of unliganded receptor homodimers to distinguish between these models.

Remarkably and despite a significantly lower affinity, Spz1C is as potent in stimulating mosquito Aag2 cells as DmSpz is at stimulating Drosophila S2 cells. An alternative explanation is that cleavage of the Z-loop promotes stable ligand binding and thus processing might account for the discrepancy between protein affinity and cellular potency of Spz1C. On the other hand, Spz1C binding prevents cleavage of Toll5A’s Z-loop, a finding that is not consistent with the Z-loop impeding ligand binding. Additionally, we expressed Toll5A in insect cells without detecting such processing, which likely rules out its spontaneous occurrence. However, Drosophila Toll can be cleaved at an equivalent residue Asp 458, and forms a stable dimer with the ectodomain remaining intact5. Taken together, we conclude that this
region is critical for promoting ligand-induced dimerization while the physiological relevance of its processing requires further examination. Of note, the nucleic-acid binding TLRs that are activated by endoproteolytic cleavage display positive cooperativity44.

In light of the above it is likely that there will be a degree of synergy between Toll/SA signalling and the vectorial capacity of Aedes aegypti. Our study sheds new light on Toll signalling, while raising fundamental questions. Do Toll receptors undergo endoproteolytic processing to regulate their activity? Which oligomeric forms occur in vivo and what are their respective signalling outputs? More importantly, can Spz1C signalling be exploited to fight mosquito-borne diseases? If so, the structural data in hand can guide future transmission-blocking strategies.

**Methods**

**Bioinformatic analysis**

Mosquito Toll and Spz sequences were retrieved via BLAST searches in Uniprot46 and Vectorbase47. Sequence alignments were performed using Muscle48. Homology modelling was carried out using Modeller version 9.9. Alignments were visualised in Jalview49 and 3D-models in PyMol (Molecular Graphics System) and Chimera50.

**DNA constructs**

*Aedes aegypti* genomic DNA from 5 male and 5 female mosquitoes was a kind gift from Dr Emilie Pondeville (University of Glasgow, UK). Constructs were either derived from genomic or synthetic DNA upon codon optimisation to improve the protein production yields for TollIA (Vectorbase identifier AAE026297), TollIS (AAE007619), Spz1A (UniProt accession code Q17P53), Spz1C (UniProt accession code Q16J57; Vectorbase identifier AAE013433) and SpzX (AAE013434). Constructs were cloned into baculovirus transfer vector pFastBac within BamHI and NotI, and into pMT-V5/His-A within KpnI and NotI (ThermoFisher).

**Cell culture**

Aag2 cells were a kind gift from Prof Alain Kohl (University of Glasgow, UK), and were maintained in Schneider’s Insect Medium with L-glutamine and sodium bicarbonate (Merck KGaA, Darmstadt, Germany) supplemented with 10% (v/v) foetal bovine serum (FBS - Sigma-Aldrich, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 28 °C in a humidified atmosphere without CO2.

SF9 cells (ThermoFisher) used for baculovirus preparation were maintained in Insect- XPRESS™ Protein-free Insect Cell Medium with L-Glutamine (Lonza) at 28 °C under agitation. S2 cells used for stable insect expression were a gift from Prof Jean-Luc Imler (University of Strasbourg, France). They were maintained in the same medium at 28 °C – 218) and the Cystine-knot domain (Spz1C residues 219–320) after establishing the domain boundaries using limited trypsin proteolysis.

Typically, 4 litres SF9 at 2 million cells per ml were infected at a MOI= 2.0 and cultured under agitation at 19 °C instead of 27 °C for optimal expression over 5 days. The supernatant was harvested after removing cells by centrifugation at 2000 g for 10 min and filtered on a Sartobran P sterile capsule of 0.45 μm (Sartorius). The buffer was then exchanged to buffer A (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA) and concentrated to 500 ml using a Centramate tangential flow filtration system ( Pall). It was loaded on a Strep-Tactin®XT Superflow® resin (IBA) following manufacturer recommendations. The resin was equilibrated in 10 column volumes of buffer A prior to use and subsequently washed with 10 column volumes of the same buffer to remove non-specifically bound proteins. Strep-tagged protein was eluted in buffer A containing 50 mM biotin. Peak fractions were pooled and purified by anion-exchange on a 5 ml Hitrap Q (Cytiva, formerly GE Healthcare Life science) in a NaCl gradient from 50 mM to 1 M, 50 mM Tris-HCl, pH 7.5, followed by size exclusion chromatography on a Superdex 75 10–300 GL column (Cytiva) at 0.5 ml/min in buffer C. 50 mM NaCl, 50 mM Tris-HCl, pH 7.5. Fractions were analysed by Coomassie-stained SDS-PAGE. Protein concentrations were quantified by absorption at 280 nm. A typical yield was 3 mg of purified protein per litre of cell culture. *Drosophila* Spz production has been described elsewhere51.

**Toll ectodomain preparation.** A stable Schneider 2 cell line containing pMT-V5/His-A *Aedes aegypti* TollIA (AAE026297) ectodomain (residues 1–835) and TollIS (AAE007619) ectodomain (residues 1–789), a C-terminal TEV-cleavable Protein A fusion and a Flag-tag was induced with copper sulfate 0.5 mM at 3 million cells/ml. Cells were cultured at 27 °C under agitation and harvested after 3–4 days. Culture supernatant were filtered, buffer-exchanged and concentrated to 0.5 L in 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.05% Tween 20. Protein A Flag tagged proteins were isolated with IgG Sepharose® 6 Fast Flow (Cytiva), incubated with TEV protease at a 1:10 (w/w) ratio at 4 °C overnight in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5 (buffer D). TEV-cleaved proteins lacking Protein A Flag tag were collected in the flow through upon washing in buffer D. Protein A eluted in 0.1 M Na Acetate pH 3.4. Fractions of interest were pooled and further purified by anion-exchange (as above) and size exclusion chromatography on a Superdex 200 10–300 GL column (Cytiva) at 0.5 ml/min in buffer C.

**Surface plasmon resonance**

SPR experiments were performed on a Biacore T200 instrument with dextran-based Sensor Chip CMS (Cytiva) in 100 mM NaCl, 20 mM HEPES pH 7 running buffer and a flowrate of 30 μl/min. The chips were activated by 1-Ethyl-3-(3-dimethyaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide, and Spz1A, Spz1C and DmSpz proproteins and cyste-knot ligands were immobilized by amine coupling at pH 4.5. Sensorgrams were recorded and corrected by subtraction of control signal from an empty flow cell. Purified ectodomain analytes of TollIA, TollIS and DmToll were injected at concentrations between 0.1 and 7.5 μM in ten-fold dilution series. Kinetic analysis was performed by fitting sensorgrams to a two-state reaction model.

**Analytical ultracentrifugation**

Analytical ultracentrifugation experiments were performed on an Optima XL-A/1 (Beckman Coulter) centrifuge equipped with a four-hole titanium rotor, double-sector centrepieces, and an interference optical system for data acquisition. Sedimentation velocity runs were performed at 50,000 rpm with 3-min intervals between scans for a total of 190 scans at 20 °C. The sample volume was 400 μL. Data were analysed using Sedfit software version 16.1c52. The partial specific volumes, buffer density, and viscosity were estimated using SEDNTERP software (2012-08-23 version)53.

**SEC-MALS**

Size exclusion chromatography-coupled multi-angle light scattering (SEC-MALS) was used to analyse protein monodispersities and molecular weights. SEC was performed using an Akta Purifier (GE Healthcare) and a Superose 6 10/300 GL column (GE Healthcare) in 50 mM Tris-HCl pH 7.5, 50 mM NaCl. For each measurement, 50 μl of protein at a given concentration was injected and gel filtrated at a flow rate of 0.5 ml/minute. Light scattering was recorded using a miniDAWN TREOS instrument (Wyatt Technology). Protein concentration in each elution peak was determined using differential refractive index (dRI).
The data were analysed using the ASTRA 6.2 software (Wyatt Technology).

SEC-SAXS
SAXS measurements were performed at Diamond Light Source (Oxfordshire, UK), beamline 21 (B21) at a wavelength 0.89–1.3 Å with a sample to detector distance of 3.7 m and a Rigaku R-Axis IIe (Rigaku) detector, covering a momentum transfer of 0.0007-0.34 Å⁻¹.

RNA sequencing and RT-qPCR
Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) following manufacturer recommendations. Contaminating DNA was removed using TURBO DNA-free Kit (ThermoFisher Scientific, USA). The quantity and quality of RNA was checked using a NanoDrop spectrophotometer (ThermoFisher) and Bioanalyzer for RNA sequencing samples. Library preparation was performed with TruSeq Stranded mRNA Library Prep Kit (Illumina). Sample sequencing was performed on an Illumina NextSeq 500 using mRNA derived from different conditions (Mock Aag2 cells and stimulated with 100 nM of SpzIC, GNB and PNG). The reads obtained by RNA-seq were analysed using the cufflinks RNA-seq workflow available at Aedes aegypti genome on VectorBase (www.vectorbase.org). All graphic representations were made using Cufmeltedb package (v3.10) on R. RT-qPCR was conducted in a Rotor-Gene Q system (Qiagen) for over 40 cycles with an annealing temperature of 60 °C, with each well containing 2 μl RNA (10 ng/μl), 0.8 μl 10 μM specific primers (final concentration, 300 nM), 5.4 μl H2O, 1 μl 2X Luna WarmStart RT Enzyme Mix (NEB) and 10 μl 2X Luna Universal One-step Reaction mix (NEB). Samples were measured in triplicates. Assessment of the expression of each target gene was based on relative quantification (RQ) using the comparative critical threshold (CT) value method. The RQ of a specific gene was evaluated in each reaction by normalization to the CT obtained for endogenous control gene elongation factor 1 alpha (eEF1a). Three independent infection experiments were conducted. The primers for quantitative RT-PCR used in this study are presented (Supplementary Table 3). Data were analysed by t-test, Mann-Whitney, Kruskal Wallis or ANOVA test depending on the application conditions and P value of <0.05 was considered significant on R software.

Quantification and statistical analysis
Quantification and statistical analyses pertain to the analysis of cryo-EM and RNAseq data are integral parts of algorithms and software used.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Sequences are available from Vectorbase for Toll1A under the identifier: AAEI013434; Toll5A: AAEI013433; and SpzX: AAEI013434. Sample sequencing data are integral parts of algorithms and software used.

The RNA-seq data for this study are publicly available through the European Nucleotide Archive under accession number PRJEB50861. SAXS data were deposited at the Small Angle Scattering database with accession numbers SASDKX8 for Toll5A alone (https://www.sasbdb.org/data/SASDKX8) and, SASDKY8 for Toll5A with SpzIC (https://www.sasbdb.org/data/SASDKY8), respectively. The cryo-EM 3D maps corresponding to the homodimer, the ligated heterodimer and heterotrimer were deposited in EMDB database with accession codes EMD-11984; EMD-11982 and EMD-11983, respectively. The corresponding
atomic models were deposited in PDB with accession codes 7B1D, 7B1B and 7B1C, respectively. There are no restrictions on data availability. Source data are provided with this paper.

References
1. Gay, N. J., Symmons, M. F., Gangloff, M. & Bryant, C. E. Assembly and localization of Toll-like receptor signalling complexes. Nat. Rev. Immunol. 14, 546–558 (2014).
2. Hoffmann, J. A. The immune response of Drosophila. Nature 426, 33–38 (2003).
3. McIlroy, G. et al. SI-Toll-6 and Toll-7 function as neurotrophin family members. Dev. Comp. Immunol. 76, 11881–11886 (2008).
4. Wang, L. et al. Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity. Proc. Natl. Acad. Sci. USA 105, 11881–11886 (2008).
5. Jang, I. H. et al. A Spätzle-processing enzyme required for Toll signaling activation in drosophila innate immunity. Dev. Cell 10, 45–55 (2006).
6. Foldi, I. et al. Three-tier regulation of cell number plasticity by neurotrophins and Tolls in Drosophila. J. Cell Biol. 216, 1421–1438 (2017).
7. Chowdhury, M. et al. Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in Drosophila. J. Biol. Chem. 294, 10172–10181 (2019).
8. Shelly, S., Lukanova, N., Bambina, S., Berman, A. & Cherry, S. Crystallization of Spätzle, a cystine-knot protein involved in embryonic development and innate immunity in Drosophila melanogaster. Acta Crystallogr. Sect. F–Struct. Biol. Cryst. Commun. 64, 707–710 (2008).
9. Arnot, C. J., Gay, N. J. & Gangloff, M. Molecular mechanism that induces activation of Spätzle, the ligand for the Drosophila toll receptor. J. Biol. Chem. 285, 19502–19509 (2010).
10. Wang, L. et al. Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity. Proc. Natl. Acad. Sci. USA 105, 11881–11886 (2008).
11. Jang, I. H. et al. A Spätzle-processing enzyme required for Toll signaling activation in drosophila innate immunity. Dev. Cell 10, 45–55 (2006).
12. Foldi, I. et al. Three-tier regulation of cell number plasticity by neurotrophins and Tolls in Drosophila. J. Cell Biol. 216, 1421–1438 (2017).
13. Chowdhury, M. et al. Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in Drosophila. J. Biol. Chem. 294, 10172–10181 (2019).
14. Nonaka, S. et al. Characterization of Spz5 as a novel ligand for Drosophila Toll-1 receptor. Biochem. Biophys. Res. Commun. https://doi.org/10.1016/j.bbrc.2018.10.096. (2018).
15. Shelly, S., Lukanova, N., Bambina, S., Berman, A. & Cherry, S. Autophagy Is an Essential Component of Drosophila Immunity against Vesicular Stomatitis Virus. Immunity 30, 588–589 (2009).
16. Lamiable, O. et al. Analysis of the contribution of hemocytes and 7B1C, respectively. There are no restrictions on data availability. Source data are provided with this paper.

References
1. Gay, N. J., Symmons, M. F., Gangloff, M. & Bryant, C. E. Assembly and localization of Toll-like receptor signalling complexes. Nat. Rev. Immunol. 14, 546–558 (2014).
2. Hoffmann, J. A. The immune response of Drosophila. Nature 426, 33–38 (2003).
3. McIlroy, G. et al. SI-Toll-6 and Toll-7 function as neurotrophin family members. Dev. Comp. Immunol. 76, 11881–11886 (2008).
4. Wang, L. et al. Peptidoglycan recognition protein-SD provides versatility of receptor formation in Drosophila immunity. Proc. Natl. Acad. Sci. USA 105, 11881–11886 (2008).
5. Jang, I. H. et al. A Spätzle-processing enzyme required for Toll signaling activation in drosophila innate immunity. Dev. Cell 10, 45–55 (2006).
6. Foldi, I. et al. Three-tier regulation of cell number plasticity by neurotrophins and Tolls in Drosophila. J. Cell Biol. 216, 1421–1438 (2017).
7. Chowdhury, M. et al. Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in Drosophila. J. Biol. Chem. 294, 10172–10181 (2019).
8. Shelly, S., Lukanova, N., Bambina, S., Berman, A. & Cherry, S. Autophagy Is an Essential Component of Drosophila Immunity against Vesicular Stomatitis Virus. Immunity 30, 588–589 (2009).
9. Lamiable, O. et al. Analysis of the contribution of hemocytes and autophagy to Drosophila antiviral immunity. J. Virol. JVI.00238–JVI.00238 (2016).
10. Angleró-Rodríguez, Y. I., Tikhe, C. V., Kang, S. & Dimopoulos, G. Aedes aegypti Toll pathway is induced through dsRNA sensing in endosomes. Dev. Comp. Immunol. 122, 104138 (2021).
11. Nene, V. et al. Genome sequence of Aedes aegypti, a major arbovirus vector. Science 316, 1716–1723 (2007).
12. Sin, G. et al. Genome sequence of the Asian tiger mosquito, aedes albopictus, reveals insights into its biology, genetics, and evolution. Proc. Natl. Acad. Sci. USA https://doi.org/10.1073/pnas.1516410112. (2015).
13. Christopoulou, M. et al. Immunity-related genes and gene families in Anopheles gambiae. Science 298, 159–165 (2002).
14. Gay, N. J. & Gangloff, M. Structure and function of Toll receptors and their ligands. Annu. Rev. Biochem. 76, 141–165 (2007).
15. Gay, N. J., Gangloff, M. & Weber, A. N. Toll-like receptors as molecular switches. Nat. Rev. Immunol. 6, 693–698 (2006).
16. Kang, J. Y. & Lee, J. O. Structural biology of the toll-like receptor family. Annu. Rev. Biochem. 80, 917–941 (2011).
17. Liu, L. et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 320, 379–381 (2008).
41. Zhang, R. et al. Regulation of antimicrobial peptides in Aedes aegypti Aag2 Cells. Front. Cell. Infect. Microbiol.  https://doi.org/10.3389/fcimb.2017.00022. (2017).
42. Russell, T. A., Ayaz, A., Davidson, A. D., Fernandez-Sesma, A. & Maringer, K. Imd pathway-specific immune assays reveal NF-κB stimulation by viral RNA PAMPs in Aedes aegypti Aag2 cells. bioRxiv https://doi.org/10.1101/2020.06.30.179879. (2020).
43. Tanji, T., Hu, X., Weber, A. N. R. & Ip, Y. T. Toll and Imd Pathways Synergistically Activate an Innate Immune Response in Drosophila melanogaster. Mol. Cell. Biol. 27, 4578–4588 (2007).
44. Lum, L. & Beachy, P. A. The hedgehog response network: Sensors, switches, and routers. Science https://doi.org/10.1126/science.1093820. (2004).
45. Roed, N. K. et al. Structures of insect Imp-L2 suggest an alternative strategy for regulating the bioavailability of insulin-like hormones. Nat. Commun.  https://doi.org/10.1038/s41467-018-06192-3. (2018).
46. Redmond, S. N. et al. Association mapping by pooled sequencing and RNAseq analysis reveals genetic differences between panels of African populations. PLoS Genet. 13, e1006456. (2017).
47. Koshland, D. E. The structural basis of negative cooperativity: thresholds and ultrasensitivity from negative ensemble velocity ultracentrifugation and Lamm equation modeling. Biophys. J.  https://doi.org/10.1016/S0006-3495(00)76713-0. (2000).
48. Levitzki, A. & Koshland, D. E. Negative cooperativity in regulatory enzymes. Nat. Rev. Mol. Cell Biol. 3, 733–744. (2002).
49. Levin, D., Harari, D. & Schreiber, G. Stochastic Receptor Expression and Synergy Determine Cell Fate upon Interferon Treatment. Mol. Cell. Biol. 31, 3252–3266 (2011).
50. Levička, T., Koushidi, E. & Koshland, D. E. Negative cooperativity in regulatory enzymes. Proc. Natl. Acad. Sci. USA 62, 1121–1128 (1969).
51. Koshland, D. E. The structural basis of negative cooperativity: Receptors and enzymes. Curr. Opin. Struct. Biol. 6, 757–761 (1996).
52. Button, M. & Koshland, D. E. The structural basis of negative cooperativity: Receptors and enzymes. Curr. Opin. Struct. Biol. 6, 757–761 (1996).
53. Bonizzi, M. et al. Complex Modulation of the Aedes aegypti Transcriptome in Response to Dengue Virus Infection. PLoS One 7, e50512. (2012).
54. Leonard, J. N. et al. The TLR3 signaling complex forms by cooperative receptor dimerization. Proc. Natl Acad. Sci. USA 103, 258–263 (2008).
55. Bateman, A. et al. UniProt: The universal protein knowledgebase in 2021. Nucleic Acids Res. 49, D480–D489 (2021).
56. Giraldo-Calderón, G. I. et al. VectorBase: An updated Bioinformatics Resource for invertebrate vectors and other organisms related with human diseases. Nucleic Acids Res. 43, D707–13 (2015).
57. Edgar, R. C. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics https://doi.org/10.1186/1471-2105-14-31 (2013).
58. Webb, B. & Sali, A. Comparative Protein Structure Modeling Using MODELLER. Curr. Protoc. Bioinform. 47, 5.6.1–5.6.32. (2014).
59. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. & Barton, G. J. Jalview Version 2 A multiple sequence alignment editor and analysis workbench. Bioinformatics https://doi.org/10.1093/bioinformatics/btp333. (2009).
60. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).
61. Gangloff, M. et al. Structural insight into the mechanism of activation of the toll receptor by the dimeric ligand Spätzle. J. Biol. Chem. 283, 14629–14635 (2008).
62. Schuck, P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. Biophys. J. https://doi.org/10.1016/S0006-3495(00)76713-0. (2000).
63. Laue TM, Shah BD, Ridgeway TM, P. S. Computer-aided interpretation of analytical sedimentation data for proteins. Analytical Ultra-centrifugation in Biochemistry and Polymer Science. (Royal Society of Chemistry, Cambridge, UK, 1992).
64. Cowieson, N. P. et al. Beamline B21: High-throughput small-angle X-ray scattering at Diamond Light Source. J. Synchrotron Radiat. 27, (2020).
65. Franke, D. et al. ATLAS 2.8: A comprehensive data analysis suite for small-angle scattering from macromolecular solutions. J. Appl. Crystallogr. https://doi.org/10.1107/S1600577717007786. (2017).
66. Svergun, D. I. Determination of the regularization parameter in indirect-transform methods using perceptual criteria. J. Appl. Crystallogr. https://doi.org/10.1107/S002188989201663. (1992).
67. Tegunov, D. & Cramer, P. Real-time cryo-electron microscopy data preprocessing with Warp. Nat. Methods https://doi.org/10.1038/s41592-019-0580-y. (2019).
68. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. CryoS-PARC: Algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods https://doi.org/10.1038/nmeth. 4169. (2017).
69. Terwilliger, T. C., Ludtke, S. J., Read, R. J., Adams, P. D. & Afonine, P. V. Improvement of cryo-EM maps by density modification. bioRxiv https://doi.org/10.1101/845032. (2019).
70. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. Sect. D Biol. Crystallogr. https://doi.org/10.1107/S0907444910007493. (2010).
71. Kidmose, R. T. et al. Namdinator - Automatic molecular dynamics flexible fitting of structural models into cryo-EM and cryo-lography experimental maps. IUCrJ https://doi.org/10.1107/S2052525219007619. (2019).
72. Afonine, P. V. et al. Real-space refinement in PHENIX for cryo-EM and cryo-crystallography. Acta Crystallogr. Sect. D Struct. Biol. https://doi.org/10.1107/S0907444919006551. (2018).
73. Afonine, P. V. et al. New tools for the analysis and validation of cryo-EM maps and atomic models. Acta Crystallogr. Sect. D Struct. Biol. https://doi.org/10.1107/S0907444919006551. (2018).

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Author contributions
M.G. and Y.S. designed biochemical experiments. M.G, S.G.S and Y.S. performed AUC experiments and analysed SEC-SAXS data. M.G. performed model building and structure refinement with the help of M.C.M. M.J.M. carried out MP experiments and analysed SEC-SAXS data. M.G. analysed structural and functional data and wrote the manuscript with N.J.G.

Competing interests
The authors declare no competing interests.
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