# Dampened TLR2–mediated Inflammatory Signaling in Bats

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## Abstract

**Bats are considered natural hosts for numerous viruses. Their ability to carry viruses that cause severe diseases or even death in other mammals without falling ill themselves has attracted widespread research attention. Toll-like receptor 2 forms heterodimers with Toll-like receptor 1 or Toll-like receptor 6 on cell membranes, recognizing specific pathogen-associated molecular patterns and playing a key role in innate immune responses. Previous studies have shown that moderate Toll-like receptor 2–mediated immune signals aid in pathogen clearance, while excessive or inappropriate Toll-like receptor 2–mediated immune signals can cause self-damage. In this study, we observed that *TLR2*, unlike *TLR1* or *TLR6*, has undergone relaxed selection in bats compared with other mammals, indicating a reduced functional constraint on *TLR2* specifically in bats. Indeed, our cell-based functional assays demonstrated that the ability of Toll-like receptor 2 to bind with Toll-like receptor 1 or Toll-like receptor 6 was significantly reduced in bats, leading to dampened inflammatory signaling. We identified mutations unique to bats that were responsible for this observation. Additionally, we found that mutations at residues 375 and 376 of Toll-like receptor 2 in the common ancestor of bats also resulted in reduced inflammatory response, suggesting that this reduction occurred early in bat evolution. Together, our study reveals that the Toll-like receptor 2–mediated inflammatory response has been specifically dampened in bats, which may be one of the reasons why they could harbor many viruses without falling ill.**

***Key words:*** **bats, immunity, TLR2, evolution, inflammation.**

## Introduction

Bats (order Chiroptera) comprise the second-largest mammalian group in the world and are unique as the only mammals capable of true and sustained flight (Hao et al. 2024). Bats have been identified as natural hosts for various viruses, including Marburg virus, Hendra virus, and Nipah virus (Letko et al. 2020; Weber et al. 2023). Polymerase chain reaction (PCR) and serological evidence further suggest that bats may also serve as natural hosts for the Ebola virus (Weber et al. 2023). Furthermore, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which triggered a global pandemic at the end of 2019, is suspected to have originated in bats (Zhou et al. 2020). It is noteworthy that many viruses, which can cause severe diseases and even death in other animals, typically do not induce apparent illness in bats (Shi 2010; Wynne and Wang 2013). Previous studies have indicated that certain bat-specific features prevent bats from inducing excessive immune and inflammatory responses following viral infections (Hayman 2019), such as a unique mutation at amino acid position 358 in the STING protein, a complete loss of the *Pyhin* gene family, and the absence of NLRP1 in Old World fruit bats (Ahn et al. 2016; Xie et al. 2018; Tian et al. 2023).

Toll-like receptors (TLRs) are a family of pathogen-associated receptors with the ability to recognize a diverse array of pathogen-associated molecular patterns (PAMPs; Lim and Staudt 2013). This nomenclature was originated from the first identification of the Toll protein in the fruit fly *Drosophila melanogaster* (Hashimoto et al. 1988). Members of the TLR family share a common protein structure, encompassing an ectodomain with leucine-rich repeats (LRRs) for the PAMP recognition, a transmembrane domain, and an intracellular Toll-IL-1 receptor domain for downstream signal transduction (Kang and Lee 2011; Lim and Staudt 2013). To date, TLRs have been widely identified in vertebrates and are known for their important roles in innate immunity (Leulier and Lemaitre 2008).

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The TLR family contains varying members across different animal species, such as 10 members in humans and 12 in mice (Anderson et al. 1985; Cook et al. 2004; Beutler et al. 2006). Each TLR family member can detect a specific class of PAMPs (Cook et al. 2004; Beutler et al. 2006; Lim and Staudt 2013). Specifically, TLR2 can form a heterodimer with either TLR1 or TLR6 to recognize lipoproteins, TLR3 recognizes double-stranded RNA, TLR4 recognizes lipopolysaccharide, TLR5 recognizes flagellin, TLR7 and TLR8 recognize single-stranded RNA, and TLR9 recognizes DNA (Takeda and Akira 2004; Lim and Staudt 2013). Upon activation by ligands, TLRs recruit a specific set of adaptor proteins, such as MyD88 and TRIF, initiating a cascade of downstream signaling events culminating in the secretion of inflammatory cytokines, type I interferon, chemokines, and antimicrobial peptides (Kaisho and Akira 2006). The importance of TLRs goes beyond their classical roles in host defense. Recent research has unveiled intricate connections between TLR activation and various physiological processes, ranging from tissue development to the maintenance of homeostasis (Smith et al. 1997; Kielian et al. 2002; Warden et al. 2019; Burgueño and Abreu 2020). Additionally, investigations into the modulation of TLR signaling have opened avenues for therapeutic interventions, with potential applications in the treatment of inflammatory disorders and infectious diseases (Xun et al. 2021; Squillace and Salvemini 2022).

Previous studies on bat TLRs were relatively limited and primarily centered on TLR3, 7, 8, and 9, which are TLRs that recognize nucleic acids (Escalera-Zamudio et al. 2015; Schad and Voigt 2016; Jiang et al. 2017). This focus may be attributed to the widely acknowledged associations between these receptors and viral infections. However, it is crucial to acknowledge that other TLRs also play important roles in antiviral immunity. For instance, TLR1, 2, and 6 actively contribute to the recognition of herpes simplex virus, SARS-CoV-2, and respiratory syncytial virus (Kurt-Jones et al. 2004; Murawski et al. 2009; Zheng et al. 2021). Our recent study also demonstrated lineage-specific mutations at sites in MyD88 in Old World fruit bats, and these sites are involved in its binding with TLR2, resulting in diminished expression of downstream inflammatory factors upon ligand stimulation in these bats (Tian et al. 2023). In this study, we observed that *TLR2* in bats evolves at a faster rate compared with other mammals, particularly along the common ancestral lineage of all bats. Furthermore, through functional experiments, we discovered that bat TLR2 exhibits a dampened function in its ability to heterodimerization with TLR1 or TLR6, leading to a dampened inflammatory response, which may be attributed to the occurrence of lineage-specific mutations at sites involved in heterodimerization in bat TLR2. Two adjacent residues in TLR2, 375 and 376, which are involved in heterodimerization, simultaneously mutated in the common ancestor of bats. Introducing these two mutations into human TLR2 significantly weakened its binding affinity with both TLR1 and TLR6. These findings reveal the unique evolutionary trajectory of TLR2 in bats compared with other mammals.

## Results

### TLR2 Is Under Relaxed Selection in Bats

To test the possibility of differential selection pressure on *TLR2*, *TLR1*, and *TLR6* between bats and other mammals, we estimated the nonsyonymous and synonymous substitution rate ratios ($\omega$) using PAML (Yang 2007). In a dataset comprising 26 bat species spanning 9 families and 52 nonbat mammal species from 24 families across 9 orders (Fig. 1a; supplementary tables S1 and S2 and dataset S1 to S3, Supplementary Material online), we found that *TLR2* in bats has a significantly higher $\omega$ than in other mammals ($P = 5.89 \times 10^{-8}$), indicating that bat *TLR2* has undergone accelerated evolution, reflecting either relaxed or positive selection. Notably, the common ancestor of bats showed a much higher $\omega$ for *TLR2* than other mammals ($\omega = 1.69$, $P = 8.18 \times 10^{-4}$), though not significantly different from 1 ($P = 0.301$; supplementary table S2, Supplementary Material online). The results estimated by the RELAX program (Wertheim et al. 2015) support that selective pressure on *TLR2* was relaxed in bats compared with other mammals, with $k = 0.73$ (likelihood ratio test, $P = 1.195 \times 10^{-4}$; supplementary table S3, Supplementary Material online). By contrast, we did not observe a higher $\omega$ in bats (or their common ancestor) relative to other mammals for *TLR1* and *TLR6*, respectively (supplementary table S2, Supplementary Material online). Together, these findings suggest that *TLR2* has experienced relaxed selection in bats compared with other mammals, indicating a reduced functional constraint on *TLR2* specifically in bats.

### TLR2-Mediated Inflammatory Signaling Is Dampened in Bats

Next, we investigated the response to agonists of human, black flying fox (*Pteropus alecto*), and Brazilian free-tailed bat (*Tadarida brasiliensis*) TLR2 when cotransfected with their respective TLR1 or TLR6. Compared with the transfection of TLR2 alone, cotransfection of human TLR2 with either TLR1 or TLR6 resulted in a significantly higher expression of interleukin (IL) 8 and tumor necrosis factor α (TNF α) upon stimulation with their corresponding agonists (Fig. 1b; supplementary table S5, Supplementary Material online). However, no significant difference was observed in the cells of both bat species (Fig. 1c and d; supplementary fig. S1, supplementary table S5, Supplementary Material online). Colocalization imaging indicates that TLR1, TLR2, and TLR6 of both bat species are localized on the cell membrane, and there is a good colocalization of TLR2 with both TLR1 and TLR6, which is similar to that in humans (supplementary fig. S2, Supplementary Material online). Using a coimmunoprecipitation (Co-IP) approach, we found that, upon agonist stimulations, human TLR2 showed apparent interactions



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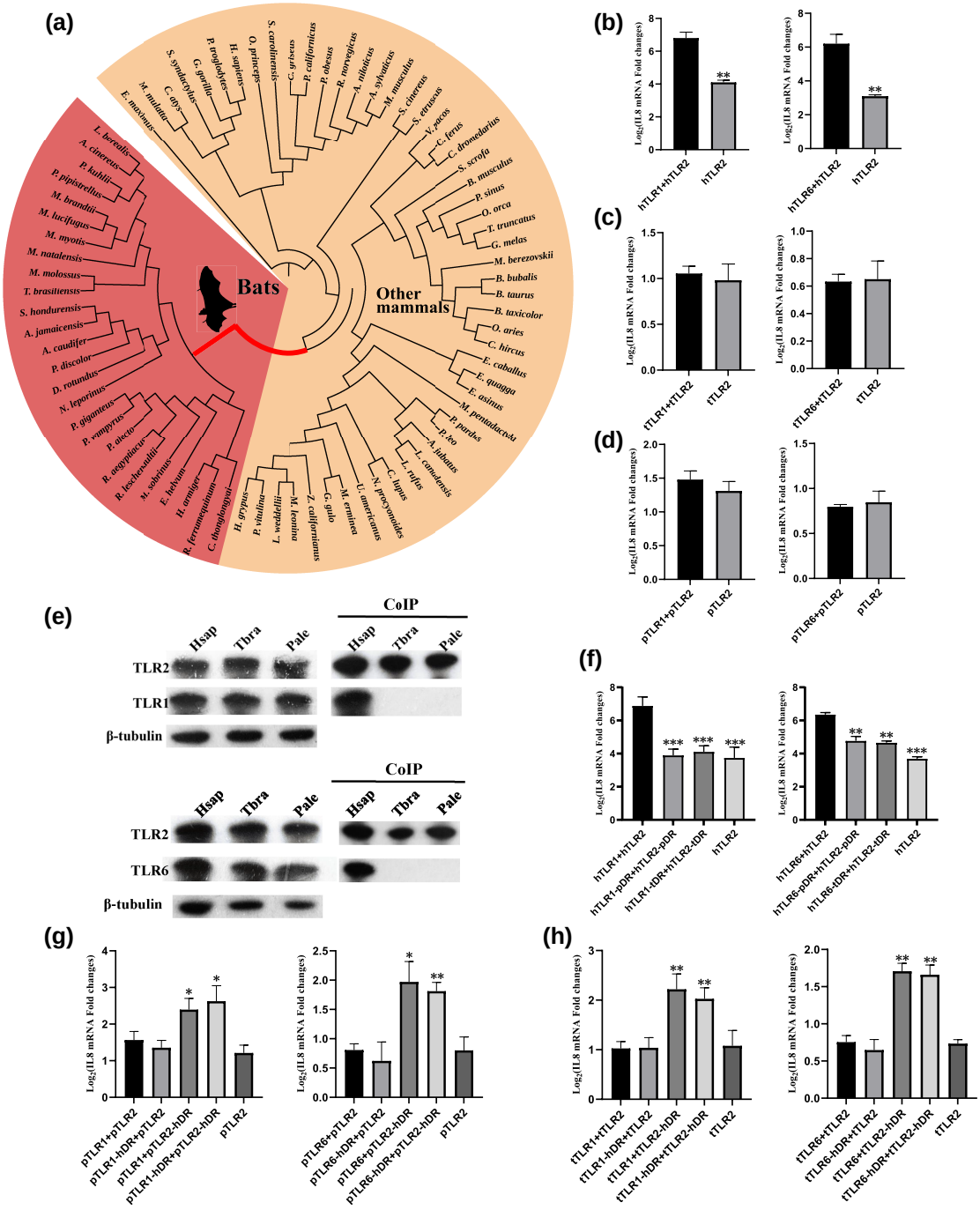


**Fig. 1.** Dampened ability of bats' TLR2-TLR1 and TLR2-TLR6 heterodimers to induce downstream inflammatory signaling is attributed to the reduced binding affinity between TLR2 and TLR1 or TLR6. a) A phylogenetic tree depicting the species used in this study. The bat icon was taken from www.svgrepo.com. The bold line with the bat icon above represents the common ancestral branch of bats. b to d) Expression levels of IL8 were measured after transfecting PEAKrapid, PaKi, and Tb 1 lu cells with TLR2 from *H. sapiens* (h), *P. alecto* (p), and *T. brasiliensis* (t) alone or cotransfected them with their respective TLR1 or TLR6, followed by stimulation with Pam3CSK4 or FSL-1 (Student's *t* test, $^{**}0.001 \leq P \leq 0.01$). e) Total lysates and Co-IP were obtained from cells cotransfected with TLR2-HA and either TLR1-Flag or TLR6-Flag. Hsap, *H. sapiens*; Tbra, *T. brasiliensis*, Pale, *P. alecto*. f) The DRs of TLR1, TLR2, and TLR6 from bats were introduced into their corresponding human molecules. Various plasmid combinations were transfected, and the relative expression levels of inflammatory factors were quantified following stimulation. The significance of each dataset was determined by comparison with the positive control, as shown by the first bar in each graph (Student's *t* test, $^{***}P < 0.001$, $^{**}0.001 \leq P < 0.01$). g to h) The DRs of human TLR1, TLR2, and TLR6 were introduced into their corresponding molecules from bats. Various plasmid combinations were transfected, and the relative expression levels of inflammatory factors were quantified following stimulation. The significance of each dataset was determined by comparison with the positive control, as shown by the first bat in each graph (Student's *t* test, $^{**}0.001 \leq P < 0.01$, $^{*}0.01 \leq P < 0.05$). Bars without asterisks (*) indicate no significant difference.

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with both TLR1 and TLR6, but the TLR2 of both bat species did not pull down TLR1 and TLR6 under the same conditions (Fig. 1e).

To rule out the possibility of our results being influenced by species specificity of agonists and to determine which among TLR1, TLR2, and TLR6 primarily compromises binding affinity, we exchanged the regions responsible for dimerization (dimerization region [DR]) among these three molecules between humans and the two bat species, through constructing various chimeric molecules (supplementary fig. S3, Supplementary Material online). Results revealed that inserting bat DRs into human TLR1, TLR2, and TLR6 significantly decreased the expression of inflammatory factors after Pam3CSK4 (a TLR2/1 agonist) or fibroblast stimulating lipopeptide 1 (i.e. FSL-1, a TLR2/6 agonist) stimulation (Fig. 1f; supplementary figs. S4 and S5, Supplementary Material online). Through cotransfection with different plasmid combinations, we found that while replacing the DRs of TLR1 and TLR6 has minimal impact, substituting the DR of TLR2 notably reduced the expression level of inflammatory factors (supplementary figs. S5 and S6 and table S5, Supplementary Material online). After inserting the human DR into the TLR2 of both bat species, there was a significant increase in the expression level of inflammatory factors upon stimulation, while inserting the human DR into bat TLR1 or TLR6 showed no apparent impact (Fig. 1g and h; supplementary figs. S7 and S8 and table S5, Supplementary Material online). These results indicate that bats may have significantly dampened or even lost the ability to form TLR2-TLR1 and TLR2-TLR6 heterodimers, resulting in a decreased capacity to mediate the expression of inflammatory factors after stimulation. The alterations in the DR of TLR2 appear to be the primary cause.

## TLR2 Mutations Unique to Bats Lead to Dampened Inflammatory Signaling

The dampened binding ability of TLR2 with TLR1 or TLR6 specifically in bats indicates the possible occurrence of bat-specific mutations at sites involved in their interaction. Thus, we compared the differences in the sites involved in heterodimerization with TLR1 or TLR6 in TLR2, as identified in previous studies, between bats and other mammals (Jin et al. 2007; Kang et al. 2009). Many of these sites exhibited lineage-specific amino acid changes in bats, especially those involved in ionic interactions (Fig. 2a; supplementary fig. S9, Supplementary Material online). Specifically, residues 321, 347, 369, 374, 375, 378, and 404, involved in ionic interactions, exhibit different amino acid changes in bats compared with other mammals. Meanwhile, residues 373 and 376, involved in hydrophobic interaction or hydrogen bonding, also show bat-specific differences (Fig. 2a; supplementary fig. S9, Supplementary Material online). Notably, the two adjacent residues, 375 and 376, in bats exhibit amino acid changes that are completely different from those in other mammals (Fig. 2a).

Since most of the species-specific mutations occur in the sites involved in ionic interactions in bat TLR2, we conducted the analysis of alanine scanning mutagenesis on these sites in human TLR2 to investigate the individual impact of single mutations. These results indicate that the hTLR2-K347A (i.e. human TLR2 with the K347A mutation) and hTLR2-E375A variants significantly reduced the expression level of inflammatory factors mediated by TLR2-TLR1 (Fig. 2b; supplementary table S5, Supplementary Material online). However, the mutations of individual sites to alanine did not have a significant impact on the expression level of inflammatory factors mediated by TLR6-TLR2 upon being stimulated by FSL-1 (Fig. 2c; supplementary table S5, Supplementary Material online). Subsequently, we introduced mutations at these sites from the black flying fox (*P. alecto*) into human TLR2 and obtained results consistent with our analysis of alanine scanning mutagenesis (Fig. 2d and e; supplementary table S5, Supplementary Material online). Additionally, we mutated residues involved in hydrophobic interactions and hydrogen bonding, which harbor bat-specific mutations in human TLR2. The results showed that mutating residue 376 led to a significant decrease in the expression of TLR2-TLR1–mediated inflammatory factors, while having no apparent effect on TLR2-TLR6 (Fig. 2f and g; supplementary table S5, Supplementary Material online). However, the expression level of inflammatory factors in bat cells did not show a significant change upon stimulation with agonists when a specific residue was mutated in bat TLR2 (supplementary figs. S10 to S12, supplementary table S5, Supplementary Material online). Together, the sites in bat TLR2 involved in the heterodimerization of TLR1 and TLR6 exhibit bat-specific mutations. These mutations may reduce the binding affinity of bat TLR2 with TLR1 and TLR6, leading to dampened inflammatory signaling.

## Dampened Inflammatory Signaling Occurred in the Common Ancestor of Bats

Residues 375 and 376 in TLR2 are the only two residues that differ completely across all bat species compared with nonbat mammals. Residue 375 is strictly Glu (E) in nonbat mammals but consistently Asn (N) in all bats (Fig. 3a). In bats, residue 376 is Phe (F), Ser (S), or Leu (L), which have not been observed in other mammals (Fig. 3a). Although residue 376 is Thr (T) in certain nonbat mammals, previous studies have shown that Y376T mutation in human TLR2 did not affect the activation level of TLR2/1 (Fig. 3a; Koymans et al. 2018; Su et al. 2019). By reconstructing the TLR2 amino acid sequence of the common ancestor of bats, we found that residues 375 and 376 in the common ancestor of bats had mutated to Asn (N) and Leu (L), respectively. Compared with other mammals, the amino acids at the two sites are unique in the common ancestor of bats, whereas those at other sites involved in heterodimerization are also found in other mammals. Applying homology-based structural modeling, we



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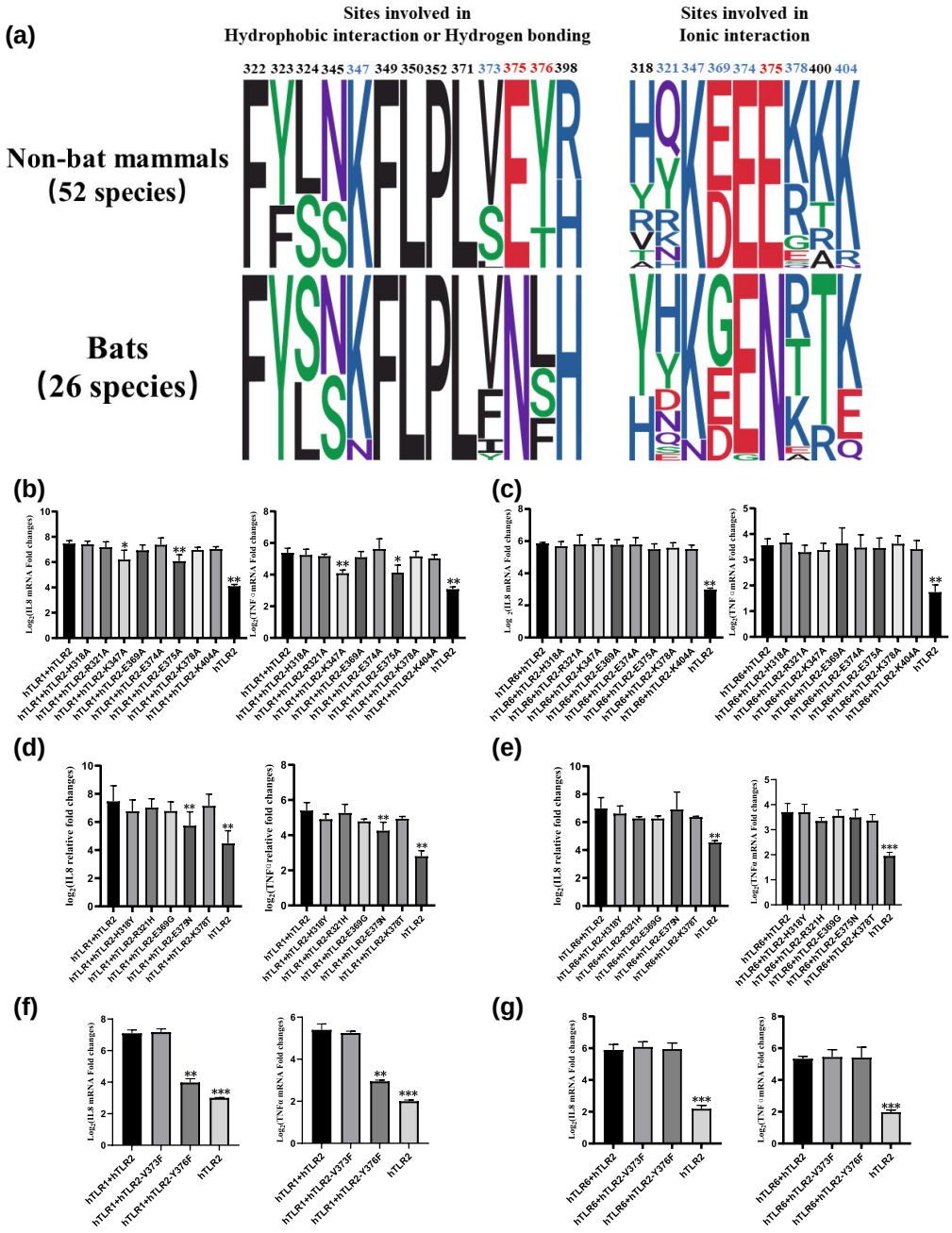


**Fig. 2.** Bat-specific mutations in TLR2 reduce the expression levels of IL8 mediated by TLR2-TLR1 and TLR2-TLR6. a) Logo plots showing sequence conservation and variation. Residues 321, 347, 369, 373, 374, 378, and 404 are unique in some bat species, while residues 375 and 376 differ from those in nonbat mammals across all bat species. b and c) Relative expression levels of inflammatory factors were measured following stimulation, after mutating the sites involved in ionic interactions in human TLR2 to alanine (i.e. a). The significance of each dataset was determined by comparison with the positive control, as shown by the first bar in each graph (Student's *t* test, $^{**}0.001 \leq P < 0.01$, $^{*}0.01 \leq P < 0.05$). Bars without asterisks (*) indicate no significant difference. d and e) Mutating the sites involved in ionic interactions in human TLR2 to the corresponding sites found in bats (*P. alecto*). The significance of each dataset was determined by comparison with the positive control, as shown by the first bar in each graph (Student's *t* test, $^{***}P < 0.001$, $^{**}0.001 \leq P < 0.01$). f and g) Mutating the sites involved in hydrophobic interactions or hydrogen bonding in human TLR2 to their corresponding sites in bats (*P. alecto*). The significance of each dataset was determined by comparison with the positive control, as shown by the first bar in each graph (Student's *t* test, $^{***}P < 0.001$, $^{**}0.001 \leq P < 0.01$).

predicted that simultaneous mutations at these two sites in human TLR2 to NL will severely impair its interaction with TLR6 (Fig. 3b). To validate this prediction, we mutated the two sites in human TLR2 to NF (i.e. Asn and Phe), NS (i.e. Asn and Ser), or NL (i.e. Asn and Leu), resembling those in bats, and cotransfected them with TLR1 or TLR6 (Fig. 3a). When these TLR2 mutants are cotransfected with TLR1 or TLR6, the expression levels



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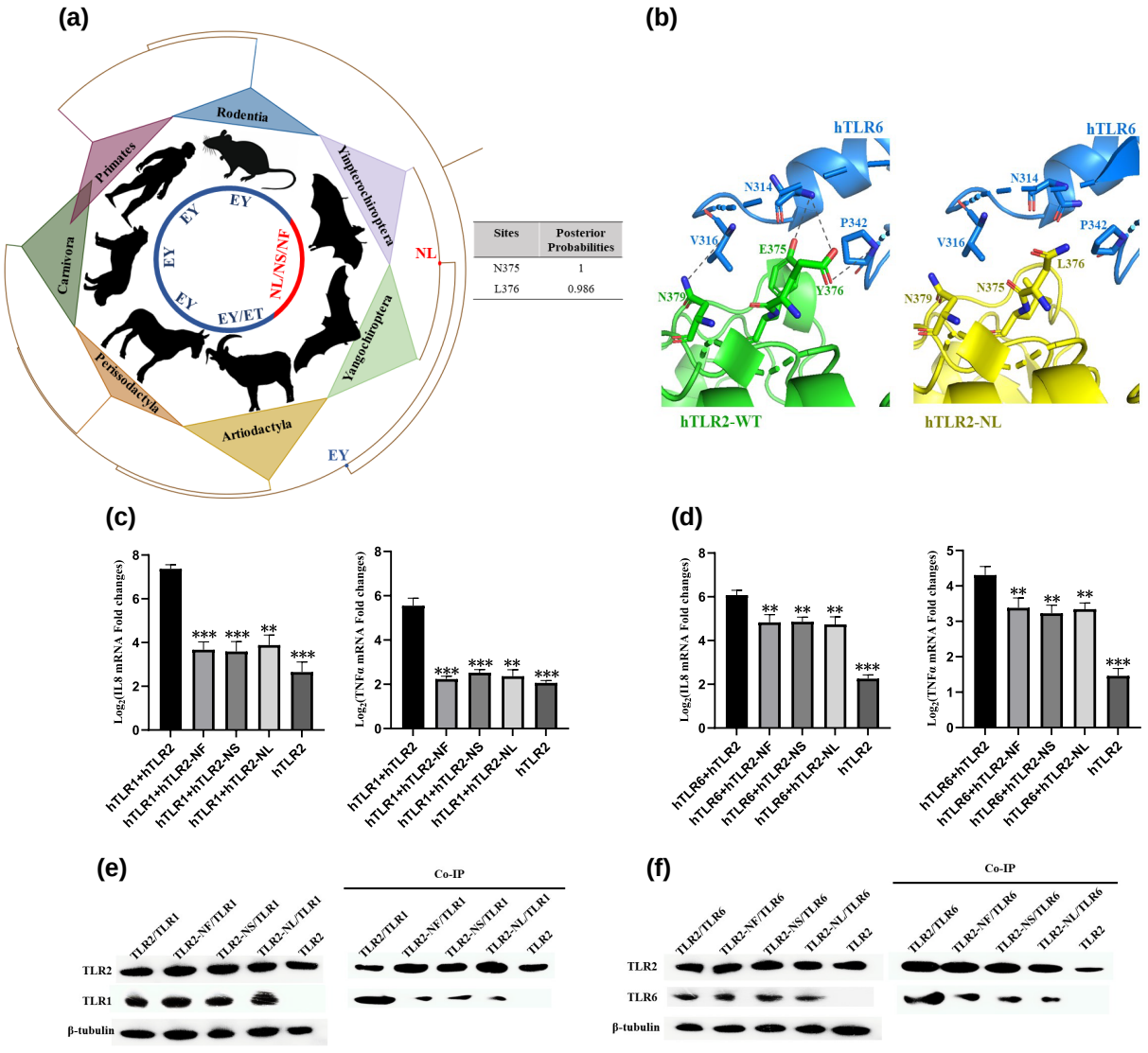


**Fig. 3.** Mutations at sites 375 and 376 in the common ancestor of bats impair binding affinity of TLR2 to TLR1 and TLR6. a) A phylogenetic tree illustrating the amino acids at positions 375 and 376 of TLR2 across various mammalian lineages. In extant bats, these positions are NL/NS/NF, whereas they were NL in the common ancestor of all bats. The seven animal icons were adapted from www.svgrepo.com. b) Homology modeling predicts the interaction between human TLR6 and both wild-type human TLR2 and human TLR2 variant carrying the bat-specific mutations at residues 375/376 (NL). Dashed lines represent hydrogen bonds. c and d) Relative changes in inflammatory factor expression poststimulation after introducing various bat-specific mutations at sites 375 and 376 into human TLR2 and cotransfecting these mutants with either TLR1 or TLR6. The significance of each dataset was determined by comparison with the positive control, as shown by the first bar in each graph (Student's *t* test, $^{***}P < 0.001$, $^{**}0.001 \leq P < 0.01$). e and f) Total lysates and Co-IP were collected from cells cotransfected with TLR2-HA or its mutant variants carrying bat-specific mutations at residues 375 and 376, along with TLR1 or TLR6.

of inflammatory factors were significantly decreased compared with the wild-type TLR2 (Fig. 3c and d; supplementary table S5, Supplementary Material online). The results of the Co-IP assay showed that the human TLR2 carrying bat-specific mutations appeared to have pulled down less TLR1 and TLR6 compared with the human wild-type TLR2 (Fig. 3e and f). By contrast, the single mutation hTLR2-E375N or hTLR2-Y376F significantly attenuated the expression of inflammatory factors mediated by TLR2-TLR1, but had no significant impact on TLR2-TLR6 (Fig. 2). These findings suggest that dual mutations at sites 375 and 376, compared with the mutation at site 375 or 376 alone, significantly reduce the interaction between TLR2 and TLR6. However, after mutating these two sites in TLR2 in both bat species to EY (i.e. Glu and Tyr), as found in humans no significant changes were observed in the expression levels of inflammatory factors following stimulation (supplementary figs. S13 and S14, Supplementary Material online). In summary, the dual mutations at residues 375 and 376 in TLR2 in the common ancestor of bats significantly reduced its binding affinity with TLR1 and TLR6, leading to a dampened inflammatory signaling. This evolutionary event occurred early in bat evolution, leading to a distinct TLR2-mediated inflammatory pathway in



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bats. This may have significantly set them apart from other mammals.

## Discussion

In this work, we conducted comparative analyses of molecular evolution, protein structure homology modeling, co-IP, and cell-based stimulation experiments to investigate whether TLR2 exhibits an evolutionary strategy and functional changes in bats distinct from other mammals. Our findings reveal that TLR2 in bats generally exhibits a faster evolutionary rate, especially in the common ancestor of bats, indicative of relaxed selection, compared with that in other mammals, while TLR1 and TLR6 do not show this pattern (Fig. 1a; supplementary tables S2 and S3, Supplementary Material online). Our study aligns with previous studies, which have suggested that TLRs in bats have generally undergone purifying selection, with some evolving faster than those in other mammals (Escalera-Zamudio et al. 2015; Schad and Voigt 2016; Jiang et al. 2017). Therefore, we hypothesized that TLR2-mediated inflammatory pathways in bats may exhibit unique characteristics compared with other mammals. Consistent with our hypothesis, the expression levels of inflammatory factors upon stimulation in cells co-transfected with both bat TLR2 and either TLR1 or TLR6 did not significantly differ from those transfected with TLR2 alone (Fig. 1b to d). This could be attributed to the impaired binding ability between bat TLR2 and TLR1 or TLR6 (Fig. 1e). We then demonstrated that the reduced ability to form heterodimers primarily stems from specific changes in the DR of bat TLR2 by swapping the DRs of human and bat TLR2 (Fig. 1f to h). TLR2-TLR1 and TLR2-TLR6 heterodimers are thought to be preformed on the cell surface for ligand-specific recognition and signaling in other mammals (Oliveira-Nascimento et al. 2012). Our results suggest that although bat TLR1, TLR2, and TLR6, like other mammals, are membrane localized, they do not preform heterodimers as observed in other species.

We investigated the sites previously identified as involved in the heterodimerization of TLR2-TLR1 and TLR2-TLR6, aiming to understand the genetic mechanisms underlying the dampened binding affinity of bat TLR2 with TLR1 and TLR6. An earlier study on other TLRs in bats has found that all positively selected sites are restricted to the LRR domain, which is involved in pathogen recognition (Jiang et al. 2017). Upon comparing the sites involved in the heterodimerization of TLR2 between bats and other mammals, we found many bat-specific amino acid changes (Fig. 2a). Introducing bat-specific mutations into human TLR2, we found that mutations at residues 347, 375, and 376 significantly affected the expression of inflammatory factors mediated by TLR2-TLR1, while no sites had a significant impact on TLR2-TLR6–mediated expression. Residue 375 was found for the first time in this study to have a single-point mutation that is enough to significantly impact the binding affinity of TLR2-TLR1 in humans, while residues 347 and 376 have been reported to affect TLR2-mediated inflammatory signaling (Koymans et al. 2018; Sahasrabudhe et al. 2018; Su et al. 2019). TLR2's K347 forms hydrogen bonds with TLR1's T361 and T363 (Jin et al. 2007) and participates in the inhibition of TLR2-TLR1 activation by pectin (Sahasrabudhe et al. 2018). These studies indicate a dual role for K347 in both the heterodimerization and activation processes of TLR2-TLR1. Residue 376 participates in the formation of the hydrophobic core in both TLR2-TLR1 and TLR2-TLR6, along with binding to some molecules, including agonists and inhibitors (Jin et al. 2007; Kang et al. 2009; Koymans et al. 2018; Su et al. 2019). Previous studies have indicated that the Y376T mutation does not notably impact the intensity of agonist stimulation for TLR2-TLR1, but Y376A does (Koymans et al. 2018; Su et al. 2019). This disparity may have resulted from differences in amino acid properties, protein docking conformation, or experimental methods. In TLR2-TLR1 and TLR2-TLR6 heterodimers, ionic bonds surround the hydrophobic core on the interface, supporting hydrophobic interactions. TLR2-TLR6 possesses a larger hydrophobic core than TLR2-TLR1, resulting in stronger binding affinity (Jin et al. 2007; Kang et al. 2009; supplementary fig. S15, Supplementary Material online). This may explain why single-point mutations at any sites are not enough to significantly impact TLR2-TLR6–mediated inflammatory responses but do affect TLR2-TLR1 (Fig. 2). Among all the sites involved in the heterodimerization of TLR2, only residues 375 and 376 have unique amino acids in bats that are completely different from those in other mammals, and these two sites underwent mutations in the common ancestor of bats. We found that simultaneous mutating of these two adjacent sites in human TLR2 reduced the binding affinity of TLR2 to TLR1 and TLR6, leading to a decrease in the expression levels of inflammatory factors mediated by TLR2-TLR1 and TLR2-TLR6. These results indicate that the dual mutations in the common ancestor of bats played a crucial role in forming the bat-specific TLR2-mediated inflammatory pathway. Whether it is a single-point mutation at any site or simultaneous mutations at residues 375 and 376, neither seems to completely inhibit the function of TLR2-TLR1 and TLR2-TLR6 (Fig. 2). We speculate that this is because mutations at individual sites, which alone do not significantly affect the function of TLR2-TLR1 and TLR2-TLR6, may have additive or synergistic effects that reduce the binding affinity of bat TLR2 with both TLR1 and TLR6. We also introduced human TLR2 residues involved in heterodimerization into bat TLR2 and measured the expression levels of IL-8 and TNFα after adding agonists. Neither single nor double mutations significantly increased the expression of these inflammatory factors (supplementary figs. S10 to S14, Supplementary Material online). These results suggest that single or double mutations are insufficient to restore the dimerization function of bat TLR2. Compared with human TLR2, bat TLR2 has accumulated many mutations in its DR, making one or two residue changes inadequate to compensate for the functional defects. The results of the DR swap experiments

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indicate that, with enough compensatory mutations, it may still be possible to restore the ability of bat TLR2 to form heterodimers (Fig. 1f to h).

While we found that the TLR2-mediated inflammatory response has been specifically dampened in bats, caveats remain. Our analysis included the full-length coding sequences (CDS) of TLR1, TLR2, and TLR6 in 26 bat species. Although these species span 9 families and capture some bat diversity, the dataset remains limited, especially given that the order Chiroptera includes over 1,400 species. Certain families, such as Vespertilionidae and Pteropodidae, are well represented, while others, like Rhinolophidae and Hipposideridae, are represented by only one species, and some families, such as Emballonuridae, are entirely absent. This uneven representation may affect our understanding of the evolutionary history and molecular mechanisms underlying bat TLR2-mediated inflammatory pathways. As sequencing efforts expand and genome data quality improves, we anticipate a more comprehensive understanding of this area. Additionally, our cell-based assays require further validation through organoids and animal models, which provide a more physiological context for studying bat immunity.

In summary, our study elucidates the unique evolutionary strategy of bat TLR2. Bat TLR2 exhibits reduced binding affinity with TLR1 and TLR6 due to bat-specific mutations, leading to dampened inflammatory signaling. These characteristics may enable bats to better serve as natural reservoirs for viruses, thereby preventing excessive immune damage to their own tissues. As we uncover the uniqueness of TLR2 evolution in bats, further studies could explore additional TLRs and their interplay within the bat immune system. Overall, investigating the broader implications of TLR changes, alongside other immune adaptations in bats, may provide valuable insights into developing therapeutic strategies for managing immune responses and preventing diseases in humans. Our work contributes to a deeper understanding of the nuanced immune mechanisms in bats, offering potential avenues for future research and therapeutic interventions.

## Materials and Methods

### Sequence Acquisition and Evolutionary Analysis

We obtained full-length CDS of TLR1, TLR2, and TLR6 from 78 mammalian species. These sequences were sourced from GenBank or predicted from published genomes. The procedure of prediction was as follows: initially, the amino acid sequences of human TLR1, TLR2, or TLR6 were employed as the query sequences for tBLASTn (Gertz et al. 2006) to determine the location of the corresponding homologous gene in other mammals' genomes. Subsequently, GENEWISE software (Birney et al. 2004) was used to precisely predict gene structures for obtaining full-length CDS. We aligned all CDS of TLR1, TLR2, or TLR6 using the MUSCLE program (v.3.8.31; Kumar et al. 2016). In addition, we estimated the ratio of nonsyonymous and synonymous substitution rates ($\omega$) using the Codeml program implemented in the PAML package (Yang 2007). We used the RELAX program in the HyPhy package to analyze selective strength (Wertheim et al. 2015).

### Cell Culture and Plasmid Construction

Human PEAKRapid, bat *P. alecto* kidney-derived PaKi cells, and bat *T. brasiliensis* lung epithelial cell lines (Tb 1 lu) from our laboratory (Yan et al. 2021) were cultured in Dulbecco's modified Eagle's medium (Gibco Inc.) supplemented with 10% of fetal bovine serum (Wisent Inc.), 2.0 mM L-glutamine, 110 mg/L sodium pyruvate, and 4.5 g/L D-glucose. Cells were cultured at 37 °C; in 5% $CO_2$ with the regular passages every 2 d. The plasmids of pcDNA3.1-hTLR2-HA (HA, hemagglutinin tag), pcDNA3.1-pTLR2-HA, pcDNA3.1-tTLR2-HA, pcDNA3.1-hTLR1-Flag, pcDNA3.1-pTLR1-Flag, pcDNA3.1-tTLR1-Flag, pcDNA3.1-hTLR6-Flag, pcDNA3.1-pTLR6-Flag, pcDNA3.1-tTLR6-Flag, pcDNA3.1-Flag-pMyD88, and pcDNA3.1-Flag-tMyD88 were commercially synthesized. The empty plasmid was from our laboratory (Tian et al. 2023). The various point mutation plasmids used in our study were obtained through site-directed mutagenesis. The chimeric-mutation plasmids used in our study were generated through overlap extension PCR, involving the exchange of the DRs responsible for human and bat TLR1, TLR2, and TLR6. Specifically, the DRs are human TLR1 spanning from the 310th to the 385th amino acid, TLR2 from the 318th to 404th amino acid, and TLR6 from the 311st to the 390th amino acid, as well as the corresponding regions in the two bat TLRs (Jin et al. 2007; Kang et al. 2009).

### Immunofluorescence Analysis of TLRs Expression and Localization

PEAKRapid, PaKi cells, and Tb 1 lu cells were transferred to six-well plates 1 d prior to the experiment. Upon reaching 70% to 80% confluence the next day, pcDNA3.1-TLR2-HA was cotransfected with pcDNA3.1-TLR1-Flag or pcDNA3.1-TLR6-Flag through Lipofectamine 2000 (Thermo Fisher Scientific). After 6 h, cells were cultured with fresh culture medium. After 48 h of culture, we discarded the medium and fixed cells with a 1:1 mixture of methanol and acetone at −20 °C for 1 h. Cells were washed three times with PBS (i.e. Phosphate-Buffered Saline) and then incubated in PBS containing 3% bovine serum albumin at 37 °C for 1 h. Following three PBS washes, cells were incubated with a 1:300 dilution of rabbit anti-HA polyclonal antibody at 37 °C for 2 h and then incubated with a 1:1,000 dilution of mouse anti-Flag monoclonal antibody at 37 °C for 2 h. Secondary antibodies targeted with Cy5 or DyLight 488 were incubated for 1 h at 37 °C.

### Co-IP Assay

We conducted a Co-IP assay using the IP kit equipped with protein A + G magnetic beads (Beyotime Biotechnology, Shanghai, China). Initially, PEAKRapid, PaKi cells, and Tb 1 lu cells were cotransfected with pcDNA3.1-TLR2-HA along with either pcDNA3.1-TLR1-Flag or pcDNA3.1-



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TLR6-Flag. Second, cells cotransfected with TLR2 and TLR1 were stimulated with 1 μg/mL Pam3CSK4, while those cotransfected with TLR2 and TLR6 were stimulated with 3 μg/mL FSL-1, both for 4 h. Following stimulation, cell lysates were prepared by lysing cells in lysis buffer containing protease inhibitor and centrifuging at 12,876 × *g* under 4 °C for 5 min. The supernatant was then collected and incubated with 12μL of protein A + G magnetic beads bound to anti-HA antibody. After gently inverting overnight at 4 °C, the beads were washed five times with lysis buffer and then collected by magnetic separation. Finally, samples were harvested after incubation in 1× SDS (i.e. Sodium Dodecyl Sulfate) loading buffer at 100 °C for 10 min. Electrophoresis was performed to separate proteins in a polyacrylamide gel with a concentration gradient of 4% to 12%. The separated protein bands were then transferred onto a polyvinylidene fluoride membrane by electrophoresis. Membranes were blocked with 5% fetal bovine serum (Wisent Inc.) for 1 h at 37 °C. Primary antibodies were incubated at 37 °C for 1 h, followed by incubation with secondary antibodies labeled with horseradish peroxidase for 1 h at 37 °C.

### Quantitative Real-Time PCR

PEAKRapid, PaKi cells, and Tb 1 lu cells were transferred to 24-well plates 1 day before the following experiment. Upon cells reaching 70% to 80% confluence the next day, human TLR2 along with either TLR1 or TLR6 was transfected into PEAKRapid cells at a 1:1 ratio using Lipofectamine 2000. Bat TLR1 or TLR6, TLR2, and MyD88 were cotransfected into bat cells at a 1:1:2 ratio. After 48 h of culture, 1 μg/mL Pam3CSK4 was added into the medium of cells transfected with TLR1 and TLR2 and 3 μg/mL FSL-1 to TLR6 and TLR2-transfected cell medium. After 24 h of stimulation, total RNA extraction was carried out using EASY spin kits (Aidlab Biotechnologies Co. Ltd.) according to the manufacturer's protocols. cDNA synthesis was performed using 1 μg total RNA with a reverse transcription system from Novoprotein Scientific Co. Ltd. Expression levels of *IL8* and *TNFα* were standardized by comparing them with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Quantitative real-time PCR (qRT-PCR) was performed on the ABI7500 Real-Time PCR Detection System (Applied Biosystems) employing NovoStart SYBR qPCR SuperMix Plus (Novoprotein Scientific, Shanghai, China), as per the manufacturer's guidelines (supplementary table S3, Supplementary Material online). Each qRT-PCR measurement was replicated three to five times. The fold change in the expression level was calculated using the $2^{-\triangle\triangle Ct}$ method. Data analysis was conducted using GraphPad Prism software 7.0 (GraphPad, USA), with statistical significance set at $P < 0.05$.

### Homology-Based Structural Modeling

The region of TLR2 involved in dimerization with mutations at sites 375 and 376 in humans was predicted by iterative threading assembly refinement (I-TASSER) v.5.1 using the wild-type human TLR2 as a template (Jin et al. 2007; Yang and Zhang 2015). For human TLR6, the DR was predicted based on the crystal structure of mouse TLR6 reported previously (Kang et al. 2009). The docking between wild-type and mutant TLR2 with TLR6 initially utilized homology-based docking by HDOCK (Yan et al. 2017), followed by refinement using RossettaDock (v.4.0; Lyskov and Gray 2008; Yan et al. 2017) for fine-tuning. All types of noncovalent interactions were analyzed using RING v.3.0 (Clementel et al. 2022). Structural alignment and visualization were implemented in PyMOL (v.2.5.2) (DeLano 2002).

## Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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## Author Contributions

H.Z. conceived and designed the study. J.Z., X.Z., and C.H. performed the experiments. J.Z. and S.T. conducted the computational analysis. J.Z. and H.Z. wrote the manuscript.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Data Availability

All data in this study are included in the article and/or Supplementary Material online.

## References

Ahn M, Cui J, Irving AT, Wang LF. Unique loss of the *Pyhin* gene family in bats amongst mammals: implications for inflammasome sensing. Sci Rep. 2016:**6**(1):21722. https://doi.org/10.1038/srep21722.

Anderson KV, Jürgens G, Nüsslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell. 1985:**42**(3):779–789. https://doi.org/10.1016/0092-8674(85)90274-0.

Beutler B, Jiang Z, Georgel P, Crozat K, Croker B, Rutschmann S, Du X, Hoebe K. Genetic analysis of host resistance: toll-like receptor signaling and immunity at large. Annu Rev Immunol. 2006:**24**(1):353–389. https://doi.org/10.1146/annurev.immunol.24.021605.090552.

Birney E, Clamp M, Durbin R. Genewise and genomewise. Genome Res. 2004:**14**(5):988–995. https://doi.org/10.1101/gr.1865504.



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Burgueño JF, Abreu MT. Epithelial Toll-like receptors and their role in gut homeostasis and disease. Nat Rev Gastroenterol Hepatol. 2020:**17**(5):263–278. https://doi.org/10.1038/s41575-019-0261-4.

Clementel D, Del Conte A, Monzon AM, Camagni GF, Minervini G, Piovesan D, Tosatto SCE. RING 3.0: fast generation of probabilistic residue interaction networks from structural ensembles. Nucleic Acids Res. 2022:**50**(W1):W651–w656. https://doi.org/10.1093/nar/gkac365.

Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. Nat Immunol. 2004:**5**(10):975–979. https://doi.org/10.1038/ni1116.

DeLano W. The PyMol molecular graphics system. 2002. http://www.pymol.org (Accessed on January 15, 2023).

Escalera-Zamudio M, Zepeda-Mendoza ML, Loza-Rubio E, Rojas-Anaya E, Méndez-Ojeda ML, Arias CF, Greenwood AD. The evolution of bat nucleic acid-sensing toll-like receptors. Mol Ecol. 2015:**24**(23):5899–5909. https://doi.org/10.1111/mec.13431.

Gertz EM, Yu YK, Agarwala R, Schäffer AA, Altschul SF. Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. BMC Biol. 2006:**4**(1):41. https://doi.org/10.1186/1741-7007-4-41.

Hao X, Lu Q, Zhao H. A molecular phylogeny for all 21 families within Chiroptera (bats). Integr Zool. 2024:**19**(5):989–998. https://doi.org/10.1111/1749-4877.12772.

Hashimoto C, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell. 1988:**52**(2):269–279. https://doi.org/10.1016/0092-8674(88)90516-8.

Hayman DTS. Bat tolerance to viral infections. Nat. Microbio. 2019:**4**(5):728–729. https://doi.org/10.1038/s41564-019-0430-9.

Jiang H, Li J, Li L, Zhang X, Yuan L, Chen J. Selective evolution of Toll-like receptors 3, 7, 8, and 9 in bats. Immunogenetics. 2017:**69**(4):271–285. https://doi.org/10.1007/s00251-016-0966-2.

Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007:**130**(6):1071–1082. https://doi.org/10.1016/j.cell.2007.09.008.

Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol. 2006:**117**(5):979–987. https://doi.org/10.1016/j.jaci.2006.02.023.

Kang JY, Lee JO. Structural biology of the Toll-like receptor family. Annu Rev Biochem. 2011:**80**(1):917–941. https://doi.org/10.1146/annurev-biochem-052909-141507.

Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S, Han SH, Lee H, Paik SG, Lee JO. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity. 2009:**31**(6):873–884. https://doi.org/10.1016/j.immuni.2009.09.018.

Kielian T, Mayes P, Kielian M. Characterization of microglial responses to *Staphylococcus aureus*: effects on cytokine, costimulatory molecule, and Toll-like receptor expression. J Neuroimmunol. 2002:**130**(1-2):86–99. https://doi.org/10.1016/S0165-5728(02)00216-3.

Koymans KJ, Feitsma LJ, Bisschop A, Huizinga EG, van Strijp JAG, de Haas CJC, McCarthy AJ. Molecular basis determining species specificity for TLR2 inhibition by staphylococcal superantigen-like protein 3 (SSL3). Vet Res. 2018:**49**(1):115. https://doi.org/10.1186/s13567-018-0609-8.

Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016:**33**(7):1870–1874. https://doi.org/10.1093/molbev/msw054.

Kurt-Jones EA, Chan M, Zhou S, Wang J, Reed G, Bronson R, Arnold MM, Knipe DM, Finberg RW. Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. Proc Natl Acad Sci U S A. 2004:**101**(5):1315–1320. https://doi.org/10.1073/pnas.0308057100.

Letko M, Seifert SN, Olival KJ, Plowright RK, Munster VJ. Bat-borne virus diversity, spillover and emergence. Nat Rev Microbiol. 2020:**18**(8):461–471. https://doi.org/10.1038/s41579-020-0394-z.

Leulier F, Lemaitre B. Toll-like receptors–taking an evolutionary approach. Nat Rev Genet. 2008:**9**(3):165–178. https://doi.org/10.1038/nrg2303.

Lim KH, Staudt LM. Toll-like receptor signaling. Cold Spring Harb Perspect Biol. 2013:**5**(1):a011247. https://doi.org/10.1101/cshperspect.a011247.

Lyskov S, Gray JJ. The RosettaDock server for local protein-protein docking. Nucleic Acids Res. 2008:**36**(Web Server):W233–W238. https://doi.org/10.1093/nar/gkn216.

Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, Tripp RA, Kurt-Jones EA, Finberg RW. Respiratory syncytial virus activates innate immunity through toll-like receptor 2. J Virol. 2009:**83**(3):1492–1500. https://doi.org/10.1128/JVI.00671-08.

Oliveira-Nascimento L, Massari P, Wetzler LM. The role of TLR2 in infection and immunity. Front Immunol. 2012:**3**:79. https://doi.org/10.3389/fimmu.2012.00079.

Sahasrabudhe NM, Beukema M, Tian L, Troost B, Scholte J, Bruininx E, Bruggeman G, van den Berg M, Scheurink A, Schols HA, et al. Dietary fiber pectin directly blocks toll-like receptor 2-1 and prevents doxorubicin-induced ileitis. Front Immunol. 2018:**9**:383. https://doi.org/10.3389/fimmu.2018.00383.

Schad J, Voigt CC. Adaptive evolution of virus-sensing toll-like receptor 8 in bats. Immunogenetics. 2016:**68**(10):783–795. https://doi.org/10.1007/s00251-016-0940-z.

Shi Z. Bat and virus. Protein Cell. 2010:**1**(2):109–114. https://doi.org/10.1007/s13238-010-0029-7.

Smith NP, Nelson MR, Moore D, Gazzard BG. Cerebral abscesses in a patient with AIDS caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Int J STD AIDS. 1997:**8**(7):459–460. https://doi.org/10.1258/0956462971920352.

Squillace S, Salvemini D. Toll-like receptor-mediated neuroinflammation: relevance for cognitive dysfunctions. Trends Pharmacol Sci. 2022:**43**(9):726–739. https://doi.org/10.1016/j.tips.2022.05.004.

Su L, Wang Y, Wang J, Mifune Y, Morin MD, Jones BT, Moresco EMY, Boger DL, Beutler B, Zhang H. Structural basis of TLR2/TLR1 activation by the synthetic agonist diprovocim. J Med Chem. 2019:**62**(6):2938–2949. https://doi.org/10.1021/acs.jmedchem.8b01583.

Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004:**16**(1):3–9. https://doi.org/10.1016/j.smim.2003.10.003.

Tian S, Zeng J, Jiao H, Zhang D, Zhang L, Lei CQ, Rossiter SJ, Zhao H. Comparative analyses of bat genomes identify distinct evolution of immunity in old world fruit bats. Sci Adv. 2023:**9**(18):eadd0141. https://doi.org/10.1126/sciadv.add0141.

Warden AS, Azzam M, DaCosta A, Mason S, Blednov YA, Messing RO, Mayfield RD, Harris RA. Toll-like receptor 3 activation increases voluntary alcohol intake in C57BL/6J male mice. Brain Behav Immun. 2019:**77**:55–65. https://doi.org/10.1016/j.bbi.2018.12.004.

Weber N, Nagy M, Markotter W, Schaer J, Puechmaille SJ, Sutton J, Dávalos LM, Dusabe MC, Ejotre I, Fenton MB, et al. Robust evidence for bats as reservoir hosts is lacking in most African virus studies: a review and call to optimize sampling and conserve bats. Biol Lett. 2023:**19**(11):20230358. https://doi.org/10.1098/rsbl.2023.0358.

Wertheim JO, Murrell B, Smith MD, Pond SLK, Scheffler K. RELAX: detecting relaxed selection in a phylogenetic framework. Mol Biol Evol. 2015:**32**(3):820–832. https://doi.org/10.1093/molbev/msu400.

Wynne JW, Wang LF. Bats and viruses: friend or foe? PLoS Pathog. 2013:**9**(10):e1003651. https://doi.org/10.1371/journal.ppat.1003651.

Xie JZ, Li Y, Shen XR, Goh G, Zhu Y, Cui J, Wang LF, Shi ZL, Zhou P. Dampened STING-dependent interferon activation in bats. Cell Host Microbe. 2018:**23**(3):297–301.e4. https://doi.org/10.1016/j.chom.2018.01.006.

Xun Y, Yang H, Kaminska B, You H. Toll-like receptors and toll-like receptor-targeted immunotherapy against glioma. J Hematol Oncol. 2021:**14**(1):176. https://doi.org/10.1186/s13045-021-01191-2.

Downloaded from https://academic.oup.com/mbe/article/42/1/msae253/7921912 by Wuhan University Library user on 10 January 2025

Yan H, Jiao H, Liu Q, Zhang Z, Xiong Q, Wang BJ, Wang X, Guo M, Wang LF, Lan K, et al. ACE2 receptor usage reveals variation in susceptibility to SARS-CoV and SARS-CoV-2 infection among bat species. Nat Ecol Evol. 2021:**5**(5):600–608. https://doi.org/10.1038/s41559-021-01407-1.

Yan Y, Zhang D, Zhou P, Li B, Huang SY. HDOCK: a web server for protein-protein and protein-DNA/RNA docking based on a hybrid strategy. Nucleic Acids Res. 2017:**45**(W1):W365–W373. https://doi.org/10.1093/nar/gkx407.

Yang J, Zhang Y. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res. 2015:**43**(W1): W174–W181. https://doi.org/10.1093/nar/gkv342.

Yang Z. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol. 2007:**24**(8):1586–1591. https://doi.org/10.1093/molbev/msm088.

Zheng M, Karki R, Williams EP, Yang D, Fitzpatrick E, Vogel P, Jonsson CB, Kanneganti TD. TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. Nat Immunol. 2021:**22**(7):829–838. https://doi.org/10.1038/s41590-021-00937-x.

Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020:**579**(7798): 270–273. https://doi.org/10.1038/s41586-020-2012-7.

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