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Roles of *N*-linked glycans in the recognition of microbial lipopeptides and lipoproteins by TLR2

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Summary

Details of roles of carbohydrates attached to Toll-like receptors (TLRs) in the recognition of pathogen-associated molecular patterns and in the formation of the functional receptor complex still remain unknown. This study was designed to determine whether the glycans linked at Asn114, Asn199, Asn414 and Asn442 residues of TLR2 ectodomain were involved in the recognition of diacylated lipopeptide and lipoprotein. Single and multiple mutants were transfected into HEK293 cells together with a NF- κ B luciferase reporter plasmid. All of these mutants were expressed on the surface. SDS-PAGE of the transfectants demonstrated that these mutants migrated lower than wild-type TLR2 and their molecular masses decreased as the number of mutated Asn residues increased. TLR2^{N114A}, TLR2^{N199A} and TLR2^{N414A} as well as wild-type TLR2 induced NF- κ B activation when stimulated with these ligands, whereas TLR2^{N442A} failed to induce NF- κ B activation. All of triple and quadruple mutants failed to induce NF- κ B activation, but were associated with both wild-type TLR2 and TLR6 in the transfectants. TLR2^{N114A,N199A}, TLR2^{N114A,N414A} and, to a lesser extent, TLR2^{N114A,N442A}, in which two N-linked glycans are speculated to be exposed to the concave surface of TLR2 solenoid, did not only induce NF- κ B activation but also are associated with wild-type TLR2 and TLR6.

These results suggest that the glycan at Asn442 and at least two N-linked glycans speculated to be exposed to the concave surface of TLR2 solenoid are involved in the recognition of ligands by TLR2 and/or in formation or maturation of a functional TLR2 receptor complex.

Introduction

Toll-like receptors (TLRs) play a key role in initiation of innate immune responses against pathogens in mammals and recognize a variety of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses and fungi as well as some host molecules such as heat shock proteins (Takeda *et al.*, 2003). TLRs do not only play key roles in the sensing of microbes in the innate immune system but are also involved in the development of antigen-specific adaptive immunity. They are structurally characterized by leucine-rich repeat (LRR) motifs in the ectodomain and a cytoplasmic Toll/Interleukin-1 receptor signaling domain. It is thought that the ectodomain of TLRs plays essential roles in the recognition of ligands or in the interaction with them. It has recently been reported that TLR9, TLR2 and TLR5 interact directly with CpG-DNA, triacylated lipopeptides (triLPTs) and flagellin, respectively (Meng *et al.*, 2004; Rutz *et al.*, 2004; Mizel *et al.*, 2003). However, the molecular mechanism by which TLRs recognize their ligands is not fully understood. Of all the TLRs so far identified, TLR2 has the broadest specificity. That is, TLR2 recognizes a wide variety of PAMPs such as lipoprotein (LP)/LPT, lipoteichoic acid, lipoarabinomannan, glycosylphosphatidylinositol- anchored protein of the protozoa *Trypanosoma cruzi*, nesserial porin, and yeast zymosan (Takeda *et al.*, 2003).

Carbohydrates attached to proteins have many biological functions (Saxon and Bertozzi, 2001; Lis and Sharon, 1993), although their functions have not been completely elucidated. Several studies have shown that TLRs and their coreceptors are also glycosylated at Asn residues and that N-linked carbohydrates are involved in TLR-mediated signaling and formation of a functional receptor complex (Choe *et al.*, 2005; Weber *et al.*, 2004; Ohnishi *et al.*, 2003; da Silva Correia and Ulevitch, 2002; Ohnishi *et al.*, 2001). Weber *et al.* (2004) have recently demonstrated that human TLR2 undergoes glycosylation at Asn114, Asn199, Asn414 and Asn442. Based on results of site-directed mutagenesis studies, they suggest that N-linked carbohydrates are involved in secretion

of the TLR2 ectodomain when the gene coding the TLR2 ectodomain was transfected into insect cells and human embryonic kidney (HEK) 293 cells. However, it has not been determined whether the N-linked carbohydrates at these Asn residues affect the recognition of PAMPs by TLR2.

We have a great interest in the mechanism by which TLRs recognize PAMPs because we think that elucidation of the mechanisms will provide an insight into how microbial infections are controlled. Recently, we found that TLR2 recognizes both lipid and peptide moieties of the mycoplasmal diacylated LPT (diLPT) [S-(2,3-bisphosphatidylCys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe) (FSL-1) (Okusawa *et al.*, 2004) and that Leu residues at positions 107, 112 and 115 in an LRR motif of TLR2 are involved in the recognition of FSL-1, mycoplasmal LP and *S. aureus* peptidoglycan (PGN) (Fujita *et al.*, 2003).

Therefore, the present study was designed to determine whether the carbohydrates at these Asn residues are involved in the recognition of *M. salivarium* LP (LPm) and FSL-1.

Results

Expression of single and multiple glycosylation mutants in HEK 293 cells

It has recently been demonstrated that Asn114, Asn199, Asn414 and Asn442 residues of human TLR2 ectodomain are glycosylated and that the glycan at Asn442 residue contributes to efficient secretion of the TLR2 ectodomain from Sf9 insect culture cells and HEK 293 cells infected with baculovirus expression constructs of the TLR2 ectodomain (Weber *et al.*, 2004). However, it remains unknown whether these N-linked glycans affect the recognition of ligands by TLR2. In order to determine this, we made single mutants (TLR2^{N114A}, M1; TLR2^{N199A}, M2; TLR2^{N414A}, M3; and TLR2^{N442A}, M4) and multiple mutants (TLR2^{N114A,N199A,N414A,N442A}, M1/2/3/4; M2/3/4; M1/2/3; M1/2/4; M3/4; M2/4; and M2/3) with substitutions of these Asn residues to Ala, and we transfected

them into HEK 293 cells as described in experimental procedures.

First, we examined by flow cytometric analysis whether these glycosylation mutants are expressed on the surfaces of HEK 293 transfectants, because the surface expression is indispensable for the recognition of ligands by TLR2. It was found that single and multiple glycosylation mutants were expressed on the cell surfaces of their transient transfectants as was wild-type TLR2 (TLR2wt), although the expression levels varied (Fig. 1A and 1B). Ratios of mean fluorescence intensities of transfectants stained with an anti-TLR2 monoclonal antibody (mAb) to that of an isotype control are shown in each histogram. Surprisingly, even the quadruple glycosylation mutant M1/2/3/4 with substitutions of all four Asn residues to Ala was expressed on the surfaces of the transfectants (Fig. 1B), suggesting that these mutations do not have effects that are strong enough to inhibit the expression of TLR2 on the cell surface.

Effects of mutations of glycosylation sites on the molecular mass of TLR2

If these four Asn residues of TLR2 are glycosylated, these mutations should cause reduction of the molecular mass of TLR2. Therefore, proteins of HEK 293 transfectants were separated by SDS-PAGE and detected by an anti-TLR2 mAb for single mutants and an anti-V5 mAb for multiple mutants. It was found that each of these mutants migrated lower than TLR2wt and that their molecular masses decreased as the number of mutated Asn residues increased (Fig. 2). These results suggest that these Asn residues of TLR2 are glycosylated, which supports the findings of Weber *et al.* (2004).

Roles of N-linked glycans in the recognition of FSL-1 by TLR2

Takeuchi *et al.* (2001) suggested that TLR2 requires TLR6 as a coreceptor for recognition of

diLPTs. However, we and others have recently demonstrated that FSL-1 and PGN are able to activate NF- κ B reporter activity in HEK 293 transfectants without cotransfection of TLR6 (Fujita *et al.*, 2003 and Mitzuzawa *et al.*, 2001), although these results do not rule out the possibility that endogenous TLR6 is contributed to the activation. Following experiments were therefore carried out to determine whether these glycosylation mutants recognized ligands such as FSL-1 and LPm without cotransfection of TLR6. M1, M2 and M3 as well as TLR2wt induced NF- κ B activation when stimulated with FSL-1 and LPm, but M4 failed to induce NF- κ B activation (Fig. 3). The activation profile of NF- κ B by FSL-1 is slightly different from those by LPm: recognition levels of LPm by M3 were slightly reduced compared with that by TLR2wt (Fig. 3). These subtle differences in the recognition levels might be attributed to differences in the three-dimensional structures or in the affinity of these ligand molecules and the spaces they occupy in the recognition site of TLR2. Judging from the fact that these point mutants and TLR2wt were expressed on the cell surfaces of the transfectants (Fig. 1), these results suggest that the glycan at Asn442 residue is involved in the recognition of these ligands by TLR2.

In order to further determine roles of the glycans at these Asn residues in the recognition, multiple glycosylation mutants were also examined for the recognition of FSL-1 and LPm. M1/2/3/4, M2/3/4, M1/2/3, M3/4, M2/4 and M2/3 failed to induce NF- κ B activation (Fig.4). On the basis of structural representation created Weber *et al.* (2004), all of these mutants possess only one N-linked glycan in the concave surface of TLR2 solenoid. Therefore, we made double mutants, M1/2, M1/3 and M1/4 which possess two N-linked glycans in the concave surface and examined for the recognition of these ligands. It was found that M1/2, M1/3 and, to a lesser extent, M1/4 induced NF- κ B activation (Fig.5).

Taken together, it is considered that at least two N-linked glycans in the concave surface of human TLR2 solenoid are required for the recognition of these ligands by TLR2 and that the glycan at Asn442 plays more important roles in the recognition.

Effects of mutations of glycosylation sites on formation of the TLR2 receptor complex in HEK 293 cells

Although it is well known that TLR2 requires TLR6 as a coreceptor for recognition of diLPTs (Takeuchi *et al.*, 2001). However, we have found that TLR2 is able to recognize FSL-1 without cotransfection of TLR6 in our luciferase reporter system using HEK 293 cells (Fujita *et al.*, 2003), suggesting that a homo-oligomer of TLR2 is also able to activate NF- κ B. Meng *et al.* (2003) have also demonstrated that homo-oligomerization of TLR2 occurs in HEK 293 transfectants to activate NF- κ B. On the basis of the data obtained by RT-PCR, Sandor *et al.* (2003) described that HEK 293 cells constitutively express a cytoplasmic pool of TLR1, whereas others described that there are no mRNAs of TLR1 and TLR6 (Uehori *et al.*, 2003). In addition, Fujita *et al.* (2003) did not detect mRNA of endogenous TLR6 in HEK 293 cells. Thus, it is controversial whether endogenous TLR1 and TLR6 exist in HEK 293 cells. Given that this is the case, the data obtained by Fujita *et al.* (2003) and Meng *et al.* (2003) can not neglect the possibility that endogenous TLR6 of the cells contributed to induction of the activation of NF- κ B.

This study also demonstrated that the glycan at Asn442 and at least two N-linked glycans in the concave surface of human TLR2 are indispensable for the ligand recognition. However, there is a possibility that these mutations do not affect recognition of ligands but affect homo-oligomerization to TLR2 or hetero-oligomerization to TLR6. In order to address this question, experiments were carried out to determine whether each of these mutations affects oligomerization to TLR6wt as well as TLR2wt in HEK 293 cells. Each gene of V5-tagged M1, M2, M3, M4, multiple mutants or TLR2wt was transfected into HEK 293 cells that stably express FLAG-tagged TLR2wt or HA-tagged TLR6wt and then immunoprecipitated with anti-FLAG Ab, followed by detection with anti-V5 or anti-FLAG Ab. It was found that all of these mutants as well as TLR2wt were associated

with FLAG-tagged TLR2wt and HA-tagged TLR6wt (Fig. 6, 7 and 8).

These results suggest that these mutations affect neither homo-oligomerization to TLR2 nor hetero-oligomerization to TLR6, but affect recognition of the ligands. However, they can not rule out the involvement of these mutations in the formation or the maturation of a functional TLR2 receptor complex.

Effects of tunicamycin on the recognition of FSL-1 by TLR2

This study demonstrated that the glycans at Asn199, Asn414 and Asn442 residues were involved in the recognition of FSL-1 and LPm by TLR2. In order to further confirm these findings, HEK 293 cells were treated with tunicamycin, an inhibitor of *N*-linked glycosylation, 5 h after transfection with TLR2wt and NF- κ B reporter genes. It was examined whether the transfectants expressed TLR2 on the cell surface and then induced activation of NF- κ B in response to FSL-1. Tunicamycin treatment had no effect on the surface expression of TLR2 (Fig. 9A) and the activation of NF- κ B by PMA, suggesting that tunicamycin at concentrations used in this study does not affect the viability of the transfectants (Fig. 9B). However, it reduced the NF- κ B activation by FSL-1 in a dose-dependent manner (Fig. 9C).

These results strongly suggest that the glycans at these Asn residues were involved in the recognition of FSL-1 by TLR2 and/or in the formation or the maturation of the functional TLR2 receptor complex.

Discussion

Most of the secreted and membrane-bound proteins are glycosylated. Carbohydrates attached to proteins have many biological functions, such as cell-to-cell communication, signal transduction,

protein folding and stability (Lis and Sharon, 1993; Saxon and Bertozzi, 2001). TLRs are type I transmembrane proteins that play a key role in recognition of microbial invasion and act as a bridge between innate immunity and acquired immunity (Takeda *et al.*, 2003). Analysis of amino acid sequences of all of the TLRs so far identified has shown that TLRs have potential *N*-linked glycosylation sites, and several lines of evidence indicate that carbohydrates attached to TLRs play important roles in recognition of PAMPs and in the formation of a functional receptor complex on the cell surface (Weber *et al.*, 2004; Ohnishi *et al.*, 2001 and 2003; da Silva Correia and Ulevitch, 2002; Choe *et al.*, 2005). The LPS receptor is a multi-protein complex consisting of CD14, TLR4, and MD-2, each of which is known to be glycosylated. Carbohydrates attached to TLR4 and its coreceptors CD14 and MD-2 are involved in their biological functions (da Silva Correia and Ulevitch, 2002). Ohnishi *et al.* (2001 and 2003) demonstrated that human MD-2 undergoes glycosylations at Asn26 and Asn114 and these glycosylations are crucial for TLR4-mediated signal transduction in response to LPS and that TLR4 is able to undergo multiple glycosylations without MD-2 but that the specific glycosylation essential for cell surface expression requires the presence of MD-2. Moreover, da Silva Correia and Ulevitch (2002) found that the double mutant of MD-2 at positions Asn26 and Asn114 failed to support LPS-induced activation of an IL-8 promoter-driven luciferase reporter to induce IL-8 secretion or to activate amino-terminal c-Jun kinase. They therefore suggest that *N*-linked carbohydrates of both MD-2 and TLR4 are essential for maintaining the functional integrity of this receptor. Recently, the three-dimensional structure of the TLR3 ectodomain has been determined at 2.1 Å by X-ray crystallography (Choe *et al.*, 2005). It was shown that the TLR3 ectodomain has a large horseshoe-shaped solenoid assembled from 23 LRRs. Choe *et al.* (2005) predicted on the basis of electron density of crystallography that 8 of 15 potential *N*-linked glycosylation sites are glycosylated. One face of TLR3 is largely masked by carbohydrate and the other is glycosylation-free. They also predict that some of the *N*-linked glycans of other TLRs as well as TLR3 are exposed to the concave surface of the solenoid structure that is thought to

contain the ligand-binding sites of TLRs. Weber *et al.* (2004) have recently demonstrated that TLR2 undergoes glycosylation at Asn114, Asn199, Asn414 and Asn442. On the basis of structural representation created using PyMol (www.pymol.org) based on the Nogo receptor, the crystal structure of which has been determined (He *et al.*, 2003). Weber *et al.* (2004) speculate that the glycans at Asn199, Asn414 and Asn442 are exposed to the concave surface of the solenoid structure. They demonstrate that the glycan at Asn442 contributes to efficient secretion of the TLR2 ectodomain and that monoglycosylated mutants of the TLR2 ectodomain with triple mutants containing N442A fail to be secreted from HEK293 transfectants but that double and triple mutants containing wild type Asn442 are secreted. However, it remains unknown whether these *N*-linked glycans affect the recognition of PAMPs by TLR2.

In order to determine whether the glycans at these sites affect the recognition of PAMPs by TLR2, we made point and multiple mutants with substitutions of these Asn residues to Ala. Substitution of Asn to Asp or Gln may be favored to disrupt the structure of TLR2 to a minimal degree. However, we favored Asn to Ala to avoid charge effects on the structure, because Asp and Gln are anionic and cationic, respectively. We found that the glycan at Asn442 is essential for recognition of FSL-1 and LPm by TLR2. Weber *et al.* (2004) also showed that Asn442 is conserved in TLR2 of 11 mammalian species, including bovine, pig, rat and chicken. Therefore, it is thought that the glycan at Asn442 plays a key role in maintenance of functional structure of the TLR2 receptor complex as well as recognition of ligands by TLR2. In addition, this study demonstrated that M4 with a substitution of Asn442 to Ala was associated with TLR2wt, suggesting that the *N*-linked glycan is not responsible for at least the oligomerization of TLR2 molecule but is responsible for the recognition. However, these results do not rule out the possibility completely that the glycan is involved in the formation or the maturation of the functional TLR2 receptor complex. The activation profile of NF- κ B by FSL-1 is slightly different from those by LPm: recognition levels of LPm by M3 were slightly reduced compared with that by TLR2wt (Fig. 3).

However, a substitution of Asn114 to Ala had no effect on the recognition of these ligands (M1 in Fig. 3), suggesting that the glycan at Asn114 is not responsible for the ligand recognition by TLR2. This speculation might be also supported by the prediction of Weber *et al.* (2004) that Asn114 is solvent-exposed on the convex surface of the TLR2 solenoid. Furthermore, this study demonstrated that the quadruple mutants and triple mutants other than M1/2/4 tested failed to recognize these ligands (Fig. 4). M1/2/4 mutated at three Asn residues other than Asn414 recognized FSL-1 and LPm, although the level of recognition was significantly reduced (Fig. 4). However, M2/3/4 mutated at three Asn residues other than Asn114 and M1/2/3 mutated at three Asn residues other than Asn442 failed to recognize them. This result suggests that the importance of Asn414 in the recognition of FSL-1 and LPm is slightly different from that of Asn114 and Asn199. M1/2/3/4, M2/3/4, M1/2/3, M3/4, M2/4 and M2/3, all of which possess only one N-linked glycan in the concave surface of TLR2 solenoid, failed to induce NF- κ B activation (Fig.4). Therefore we thought that at least two N-linked glycans in the concave surface of TLR2 solenoid are required for the recognition of these ligands by TLR2, because M2 and M3 which possess two N-linked glycans in the concave surface were capable of inducing the activation of NF- κ B (Fig. 3). In order to address this speculation, we made double mutants, M1/2, M1/3 and M1/4 which possess two N-linked glycans in the concave surface and examined for the recognition of these ligands. It was found that M1/2, M1/3 and, to a lesser extent, M1/4 induced NF- κ B activation (Fig.5). The reason why the recognition level by M1/4 is lower than those by others may also imply the importance of N442 in the recognition.

Taken together, the results suggest that at least two N-linked glycans in the concave surface of TLR2 solenoid are required for the recognition of ligands by TLR2 and that the glycan at Asn442 plays a more important role in the recognition than do others. In addition, the glycan at Asn114 is less essential for the recognition than are others. These findings might be supported by the findings and speculation of Weber *et al.* (2004) that the glycan at Asn114 residue is solvent-exposed on the

convex surface of the LRR solenoid of TLR2, whereas the glycans at Asn199, Asn414 and Asn442 residues are located on the concave surface and that Asn442 is conserved in the 11 TLR2 sequences currently known. However, these results do not rule out completely the possibility that these N-linked glycans are involved in the formation or the maturation of a functional receptor complex.

It has been demonstrated that TLR4 contains 9 N-linked glycosylation sites and that TLR4 mutants carrying substitutions in Asn526 or Asn575 failed to be transported to the cell surface (da Silva Correia and Ulevitch, 2002). As stated above, Weber *et al.* (2004) have recently demonstrated that the N-linked carbohydrates of TLR2 were involved in the secretion of the TLR2 ectodomain when the gene coding the TLR2 ectodomain was transfected into insect cells and HEK293 cells. However, the present study demonstrated that single and multiple glycosylation mutants were expressed on the cell surfaces of their transient transfectants as was TLR2wt, although the expression levels varied (Fig. 1). Surprisingly, M1/2/3/4 with substitutions of all four Asn residues to Ala was expressed on the surfaces of the transfectants (Fig. 1). Therefore, our results appear to be in contrast to the findings of Weber *et al.* (2004). This might be explained by the fact that they used HEK 293 cells transfected with the gene coding the TLR2 ectodomain lacking of transmembrane and intracellular domains of TLR2. The results obtained by this study suggest that these mutations at Asn114, Asn199, Asn414 and Asn442 residues do not have effects strong enough to inhibit the expression of TLR2 on the cell surface.

TLR2 requires TLR1 and TLR6 as coreceptors for the recognition of triLPT and diLPT, respectively (Takeda *et al.*, 2003). However, it has been shown that TLR2 transmits a signal by homo-oligomer formation in response to TLR2 agonists (Yang *et al.*, 1999). As stated above, Meng *et al.* (2003) have suggested that homo-oligomerization of human TLR2 is required for the activation of NF- κ B. We have also demonstrated that TLR2 transfected into HEK 293 cells is able to activate NF- κ B without cotransfection of TLR6 in response to FSL-1 (Fujita *et al.*, 2003). However, Zhang *et al.* (2002) have demonstrated that the homodimer of TLR2 fails to induce

activation of NF- κ B. Thus, it has been controversial whether homo-oligomerization of TLR2 is required for the activation of NF- κ B. This study clearly demonstrated that human TLR2 was able to activate NF- κ B by formation of a homo-oligomer. This finding may be supported by the recent findings that the signal by diLPTs is transmitted in a TLR6-independent manner and that both lipid and peptide portions of diLPT contribute to the specificity of recognition by TLR2 heteromer (Buwitt-Beckmann *et al.*, 2005).

Experimental procedures

Cells, Abs and reagents

HEK 293 cells obtained from ATCC (CRL-1573) were maintained in DMEM (Gibco BRL) supplemented with 10% (vol/vol) FBS, penicillin G (100 units/ml) and streptomycin (100 μ g/ml). Tunicamycin and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich. FITC- conjugated goat anti-mouse IgG and peroxidase-conjugated anti-rabbit IgG Abs were purchased from Jackson ImmunoResearch Laboratories. Mouse IgG1 as an isotype control was purchased from BD Biosciences. Anti-TLR2 mAb (IMG-319) was purchased from Biocarta. Anti-V5 mAb and anti-FLAG Ab were purchased from Invitrogen and Sigma -Aldrich, respectively. HA-tagged human TLR6 and a polyclonal Ab to HA were obtained from InvivoGen and Santa Cruz Biotechnology, respectively.

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Preparation of mycoplasmal LP (LPm)

LPm was prepared from *Mycoplasma salivarium* ATCC 23064 by Triton X-114 phase separation according to the method described previously (Shibata *et al.*, 1998). Briefly, mycoplasmal cells were treated with Triton X-114 to extract LPm. LPm in the Triton X-114 phase was precipitated by methanol and used for stimulation after being suspended in sterile PBS by light sonication. Protein concentration was determined by the method described previously (Shibata *et al.*, 1998).

Synthesis of FSL-1

FSL-1 was synthesized according to the method described previously (Shibata *et al.*, 2000). Briefly, the side chain-protected Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe was built up with an automated peptide synthesizer, model 433 (Applied Biosystems). Fmoc-S-(2,3-bispalmitoyloxypropyl)-cysteine (Novabiochem) was manually coupled to the peptide-resin by using a solvent system of 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH₂Cl₂-DMF. The Fmoc and resin were removed from LPT by trifluoroacetic acid. LPTs were purified by preparative HPLC with a reverse-phase C18 column (30 x 250 mm). The purity of FSL-1 was confirmed by analytical HPLC with a reverse-phase C18 column (4.6 x 150 mm) to be 98%. FSL-1 was dissolved in PBS.

Gene cloning

The cDNA of human TLR2 was obtained by RT-PCR of RNA isolated from THP-1 cells. The cDNA of TLR2 was cloned into a pEF6/V5-His TOPO vector (hereafter referred to as pEF/TLR2) (Invitrogen). The point mutants of the human TLR2 gene (TLR2^{N114A}, M1; TLR2^{N199A}, M2; TLR2^{N414A}, M3; and TLR2^{N442A}, M4) were produced by a QuickChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with the pEF/TLR2

construct. The following primers (and their reverse complements) were used for mutagenesis (mutated codons in bold, changed bases in italics): N114A, aat-tac-tta-tct-**GCG**-tta-tcg-tct-tcc-tgg-ttc-aag; N199A, ttg-aag-tca-att-cag-**GCC**-gta-agt-cat-ctg-atc; N414A, ctc-act-ctg-aaa-**GCC**-ttg-act-aac-att-gat-atc-agt; and N442A, gaa-aag-atg-aaa-tat-ttg-**GCC**-tta-tcc-agc-aca-cga. By using these primer sets, multiple mutants such as mutants with four, three and two Asn sites were also made. The abbreviations for multiple mutants are as follows: M1/2/3/4, TLR2^{N114A,N199A,N414A,N442A}; M2/3/4, TLR2^{N199A,N414A,N442A}; M1/2/4, TLR2^{N114A,N199A,N442A}; M1/2/3, TLR2^{N114A,N199A,N414A}; M2/3, TLR2^{N199A,N414A}; M2/4, TLR2^{N199A,N442A}; M3/4, TLR2^{N414A,N442A}; M1/2, TLR2^{N114A,N199A}; M1/3, TLR2^{N114A,N414A} and M1/4, TLR2^{N114A,N442A}. All of these mutations were confirmed by the DNA sequence analysis.

NF-κB reporter assay

HEK 293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 Transfection Reagent (Roche Molecular Biochemicals) with 30 ng of an NF-κB reporter plasmid (pNF-κB-Luc, Stratagene) and 3.5 ng of a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK, Promega) together with 166.5 ng of each gene of single and multiple glycosylation mutants and TLR2wt genes. Twenty-four h after transfection, the cells were stimulated for 6 h with FSL-1 or LPM in the absence of FBS, and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Surface expression of TLR2wt and its glycosylation mutants in HEK293 transfectants

In order to assess the surface expression of TLR2 and its glycosylation mutants in HEK293 cells by flow cytometry, HEK 293 cells grown in 6-cm dishes were transfected with 3 µg each of TLR2^{N114A} (M1), TLR2^{N199A} (M2) TLR2^{N414A} (M3), TLR2^{N442A} (M4), TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N199A,N414A,N442A} (M2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N114A,N199A,N414A} (M1/2/3), TLR2^{N199A,N414A} (M2/3), TLR2^{N199A,N442A} (M2/4), TLR2^{N414A,N442A} (M3/4) and TLR2wt. After 48-h incubation, the cells were removed from the plastic dishes by PBS containing 5 mM EDTA. The cells were incubated at 4 °C for 1 h with or without isotype-matched mouse IgG or anti-TLR2 mAb (IMG-319) and then with FITC-conjugated anti-mouse IgG. The surface expression was measured using a flow cytometer FACS Calibur (BD Biosciences). Ratios of mean fluorescence intensities of transfectants stained with an anti-TLR2 mAb to that of an isotype control were determined based on the data obtained by the cytometer.

SDS-PAGE of TLR2wt and its glycosylation mutants

In order to assess reduction of molecular mass of TLR2 by mutation, HEK 293 cells grown in 6-cm dishes were transfected with 3 µg each of TLR2wt and its mutated genes. After 48-h incubation, the cells were washed twice with ice-cold PBS and then lysed by 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol and 50 mM DTT (an SDS sample buffer) in the presence of inhibitor cocktails of proteases (Roche) and boiled for 10 min. The lysates were centrifuged at 14,000 rpm for 10 min, and the resulting supernatants containing cytosolic and membrane proteins were collected. Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C overnight with a polyclonal anti-TLR2 Ab prepared in our laboratory (Fujita *et al.*, 2003) for single mutants, followed by horseradish peroxidase-conjugated anti-rabbit IgG Ab and anti-V5 mAb for multiple mutants, followed by horseradish peroxidase-conjugated anti-mouse IgG

Ab. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Pharmacia).

Association of glycosylation mutants to TLR2^{wt}

HEK 293 cells that stably express FLAG-tagged TLR2^{wt} were established in our laboratory. The transfectants grown in 10-cm dishes were transfected with 14 µg each of V5-tagged M1, M2, M3, M4, M1/2/3/4, M1/2/3, M1/2/4, M2/3/4, M1/2, M1/3, M1/4 and TLR2^{wt} genes. After 48-h incubation, the cells were lysed in a lysis buffer (pH 7.5) containing 0.4% Triton X-100, 0.3% NP-40, 20 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 12.5 mM β-glycerophosphate and protease inhibitor cocktail (Roche). The lysates were incubated at 4 °C overnight with anti-FLAG mAb and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C for 1 h with anti-FLAG mAb or anti-V5 mAb, and then with horseradish peroxidase-conjugated anti-mouse IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Pharmacia).

Effects of tunicamycin, an inhibitor of N-linked glycosylation, on the recognition of FSL-1 by TLR2^{wt}

HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected by Fugene 6 Transfection Reagent (Roche) with 30 ng of an NF-κB reporter plasmid (pNF-κB-Luc, Stratagene) and 3.5 ng of a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK,

Promega) together with 166.5 ng of TLR2wt gene. Five h after transfection, the cells were incubated for 19 h with various concentrations of tunicamycin. Then, the cells were stimulated for 6 h with FSL-1 or PMA in the absence of FBS, and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions.

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Figure legends

Fig. 1. Surface expression of single and multiple glycosylation mutants in HEK 293 cells

HEK 293 cells were transfected with each of single mutants (A), TLR2^{N114A} (M1), TLR2^{N199A} (M2) TLR2^{N414A} (M3) and TLR2^{N442A} (M4) or each of multiple mutants (B), TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N114A,N199A,N414A} (M1/2/3), TLR2^{N114A,N199A} (M1/2), TLR2^{N199A,N414A} (M2/3), TLR2^{N199A,N442A} (M2/4) and TLR2wt genes. After a 48-h incubation, the transfectants were removed from the plate and then incubated at 4 °C for 1 h with or without isotype-matched mouse IgG or anti-TLR2 mAb (IMG-319) and then with FITC-conjugated anti-mouse IgG. The surface expression was measured using a flow cytometer, FACS Calibur (BD Biosciences). Ratios of mean fluorescence intensities of transfectants stained with an anti-TLR2 mAb to that of an isotype control are shown in each histogram.

Fig. 2. SDS-PAGE of proteins of HEK 293 cells transfected with each gene of single and multiple glycosylation mutants

HEK 293 cells were transfected with each of TLR2^{N114A} (M1), TLR2^{N199A} (M2) TLR2^{N414A} (M3) and TLR2^{N442A} (M4), and TLR2wt genes, or with each gene of V5-tagged TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N199A,N414A,N442A} (M2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N199A,N442A} (M2/4), TLR2^{N414A,N442A} (M3/4) and TLR2wt genes. After a 48-h incubation, the transfectants were lysed in SDS sample buffer, and proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was reacted with polyclonal anti-TLR2 Ab for single mutants and with anti-V5 mAb for multiple mutants. See text for details.

Fig. 3. Recognition of FSL-1 and LPm by single glycosylation mutants

HEK 293 cells (1×10^5) were plated in 24-well plates and transfected transiently with each of TLR2^{N114A} (M1), TLR2^{N199A} (M2) TLR2^{N414A} (M3), TLR2^{N442A} (M4) and TLR2wt genes together with an NF- κ B reporter plasmid and *Renilla* luciferase control reporter plasmid. Cells were stimulated at 37 °C for 6 h with various concentrations of FSL-1 and LPm. Results, expressed as the means $\pm$ SD of triplicate wells, are representative of three separate experiments. See text for details. Upper cartoons show TLR2 solenoid with N-linked glycans on the basis of the model of Weber *et al.* (2004).

Fig. 4. Recognition of FSL-1 and LPm by multiple glycosylation mutants

HEK 293 cells (1×10^5) were plated in 24-well plates and transfected transiently with each of TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N199A,N414A,N442A} (M2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N114A,N199A,N414A} (M1/2/3), TLR2^{N199A,N414A} (M2/3), TLR2^{N199A,N442A} (M2/4), TLR2^{N414A,N442A} (M3/4) and TLR2wt genes together with an NF- κ B reporter plasmid and *Renilla* luciferase control reporter plasmid. Cells were stimulated at 37 °C for 6 h with various concentrations of FSL-1 and LPm. Results, expressed as means $\pm$ SD of triplicate wells, are representative of three separate experiments. See text for details. Upper cartoons show TLR2 solenoid with N-linked glycans on the basis of the model of Weber *et al.* (2004).

Fig. 5. Recognition of FSL-1 and LPm by double glycosylation mutants which expose two N-linked glycans in the concave surface of putative TLR2 solenoid structure

HEK 293 cells (1×10^5) were plated in 24-well plates and transfected transiently with each of TLR2^{N114A,N199A} (M1/2), TLR2^{N199A,N414A} (M1/3), TLR2^{N114A,N442A} (M1/4) and TLR2wt genes together with an NF- κ B reporter plasmid and *Renilla* luciferase control reporter plasmid. Cells were stimulated at 37 °C for 6 h with various concentrations of FSL-1 and LPm. Results, expressed as means $\pm$ SD of triplicate wells, are representative of three separate experiments. See text for details.

Upper cartoons show TLR2 solenoid with N-linked glycans on the basis of the model of Weber *et al.* (2004)

Fig. 6. Association of single glycosylation mutants with TLR2wt in HEK 293 transfectants

HEK 293 cells that stably express FLAG-tagged TLR2wt were established in our laboratory. The stable transfectants were transfected with each of V5-tagged TLR2^{N114A} (M1), TLR2^{N199A} (M2) TLR2^{N414A} (M3), TLR2^{N442A} (M4) and TLR2wt genes. After a 48-h incubation, the cells were lysed in a lysis buffer. The lysates were incubated at 4 °C overnight with anti-FLAG mAb and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C for 1 h with anti-FLAG mAb or anti-V5 mAb, followed by horseradish peroxidase-conjugated anti-mouse IgG Ab. See text for details.

Fig. 7. Association of multiple glycosylation mutants with TLR2wt in HEK293 transfectants

HEK293 cells that stably express FLAG-tagged TLR2wt were transfected with each of V5-tagged TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N199A,N414A,N442A} (M2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N114A,N199A,N414A} (M1/2/3), TLR2^{N199A,N414A} (M2/3), TLR2^{N199A,N442A} (M2/4), TLR2^{N414A,N442A} (M3/4) and TLR2wt genes. After a 48-h incubation, the cells were lysed in a lysis buffer. The lysates were incubated at 4 °C overnight with anti-FLAG mAb and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C for 1 h with anti-FLAG mAb or anti-V5 mAb, followed by horseradish peroxidase-conjugated anti-mouse IgG Ab. See text for details.

Fig. 8. Association of glycosylation mutants with TLR6wt in HEK293 transfectants

HEK293 cells that stably express HA-tagged TLR6wt were established in our laboratory. The stable transfectants were transiently transfected with each of V5-tagged TLR2^{N114A,N199A,N414A,N442A} (M1/2/3/4), TLR2^{N199A,N414A,N442A} (M2/3/4), TLR2^{N114A,N199A,N442A} (M1/2/4), TLR2^{N114A,N199A,N414A} (M1/2/3), TLR2^{N199A,N414A} (M2/3), TLR2^{N199A,N442A} (M2/4), TLR2^{N414A,N442A} (M3/4) and TLR2wt genes. After 48-h incubation, the cells were lysed in a lysis buffer. The lysates were incubated at 4 °C overnight with anti-FLAG mAb and then precipitated with Protein G Sepharose beads. Proteins included in the precipitates were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 °C for 1 h with anti-HA mAb or anti-V5 mAb, followed by horseradish peroxidase-conjugated anti-mouse IgG Ab. See text for details.

Fig. 9. Effects of tunicamycin, an inhibitor of N-linked glycosylation, on surface expression of TLR2 and the recognition of FSL-1 by TLR2wt

HEK293 cells were plated at 1×10^5 cells per well in 24-well plates on the day before transfection. The cells were transiently transfected with an NF- κ B reporter plasmid and a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter together with 166.5 ng of TLR2wt gene. Five h after transfection, various amounts of tunicamycin were added to the cell culture and then incubated for another 19 h. The transfectants were incubated at 4 °C for 1 h with or without isotype-matched mouse IgG or anti-TLR2 mAb (IMG-319) and then with FITC-conjugated anti-mouse IgG. The surface expression (A) was measured using a flow cytometer, FACS Calibur (BD Biosciences). Ratios of mean fluorescence intensities of transfectants stained with anti-TLR2 mAb to that of an isotype control are shown in each histogram. After 19-h incubation, the cells were stimulated for 6 h with PMA (B upper) or FSL-1 (C lower) and then luciferase activity was measured using a Dual-Luciferase reporter assay

system. See text for details.

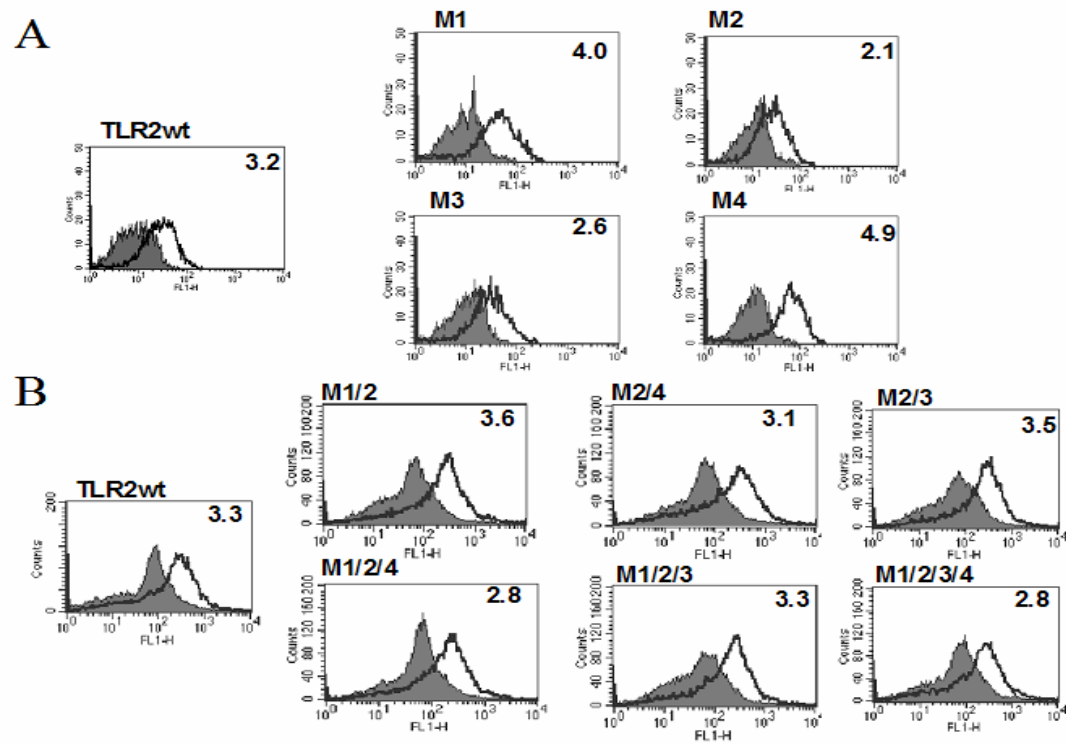


Fig. 1

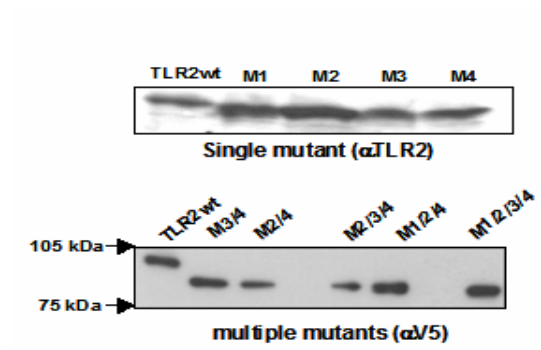


Fig.2

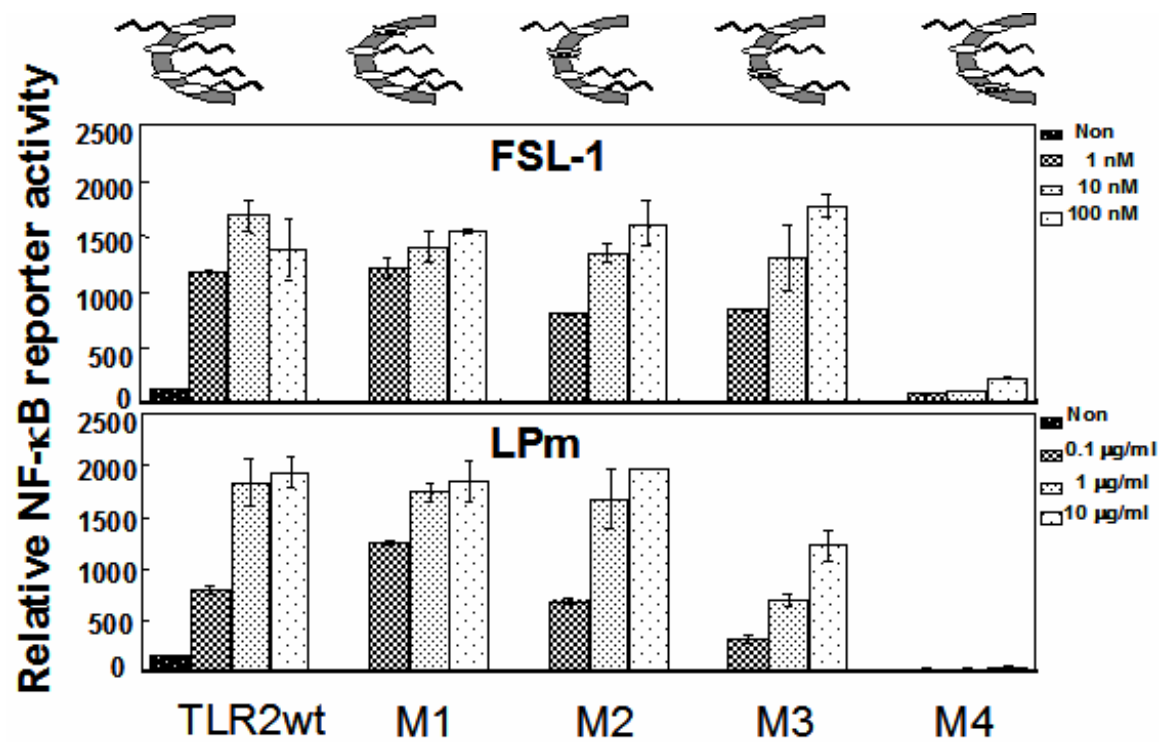


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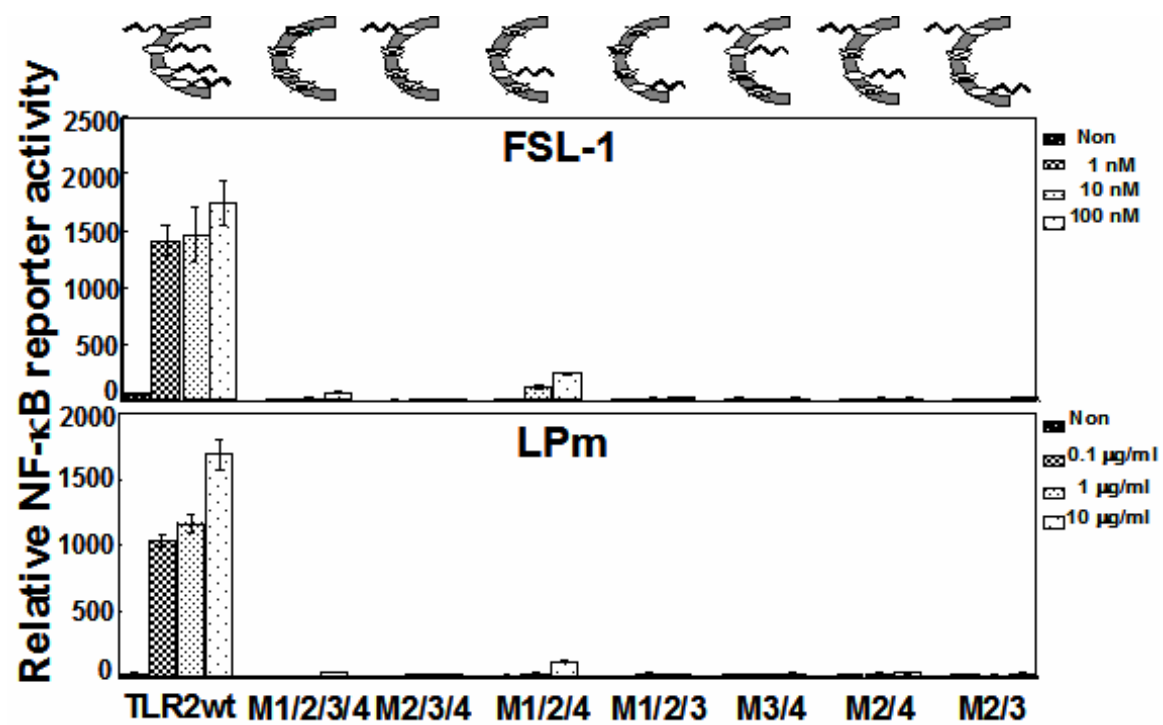


Fig.4

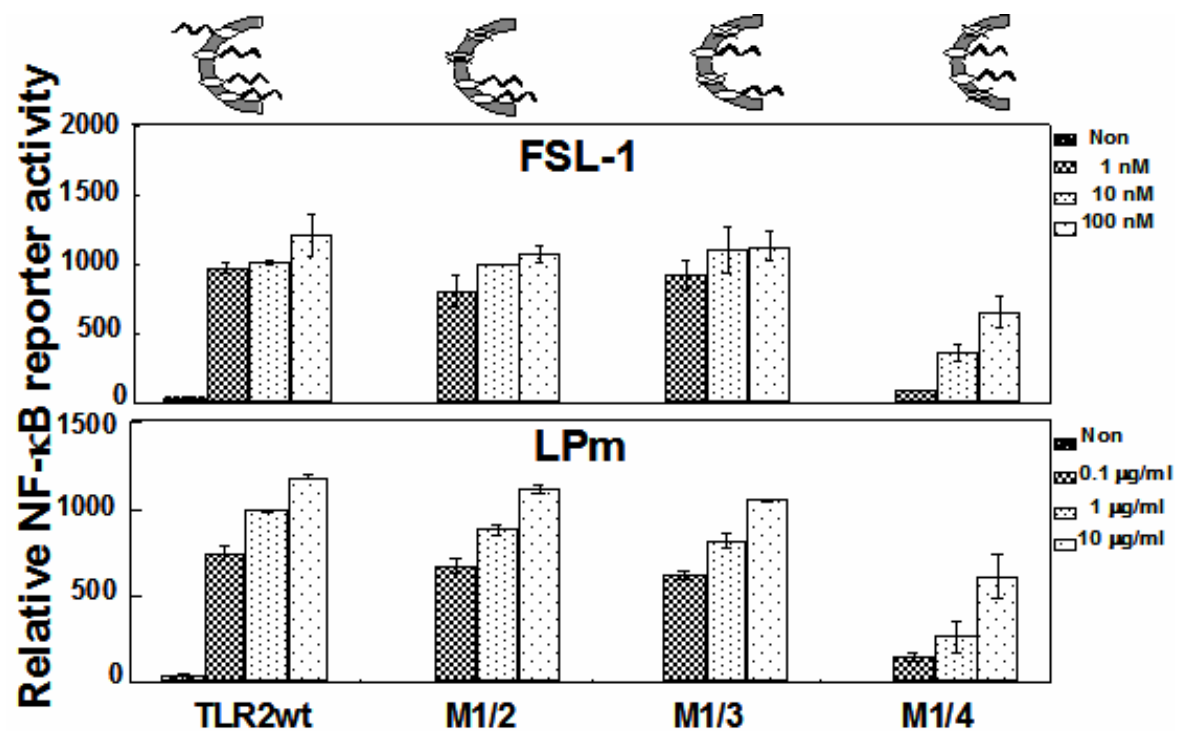


Fig.5

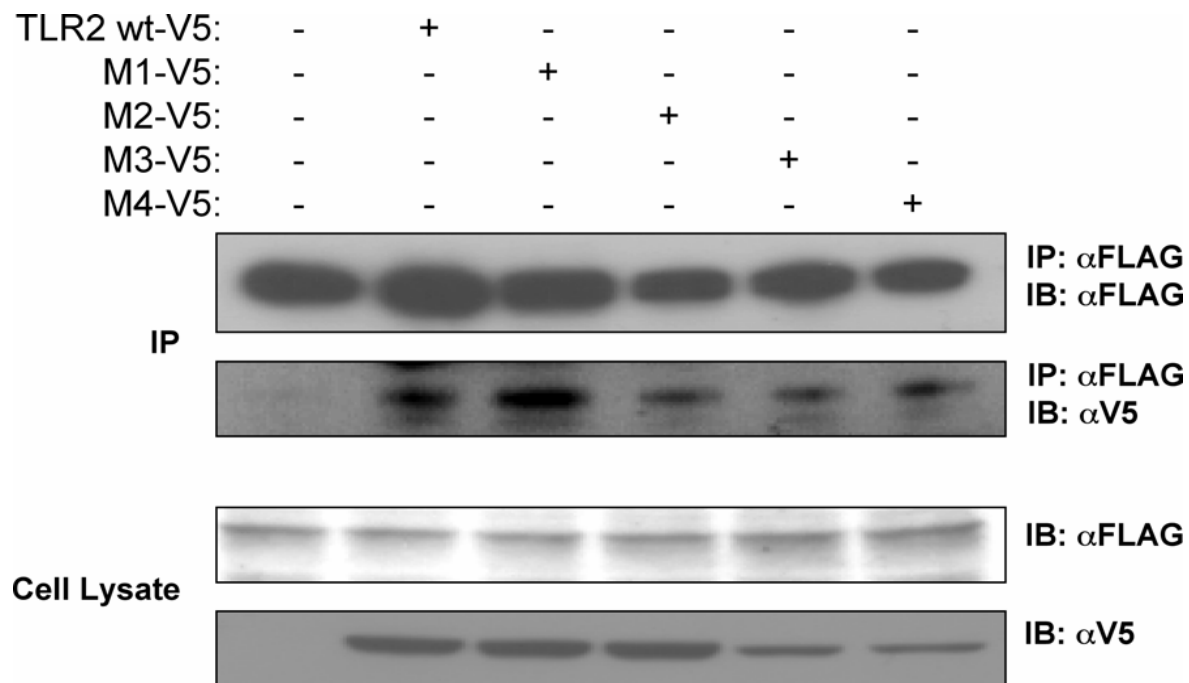


Fig. 6

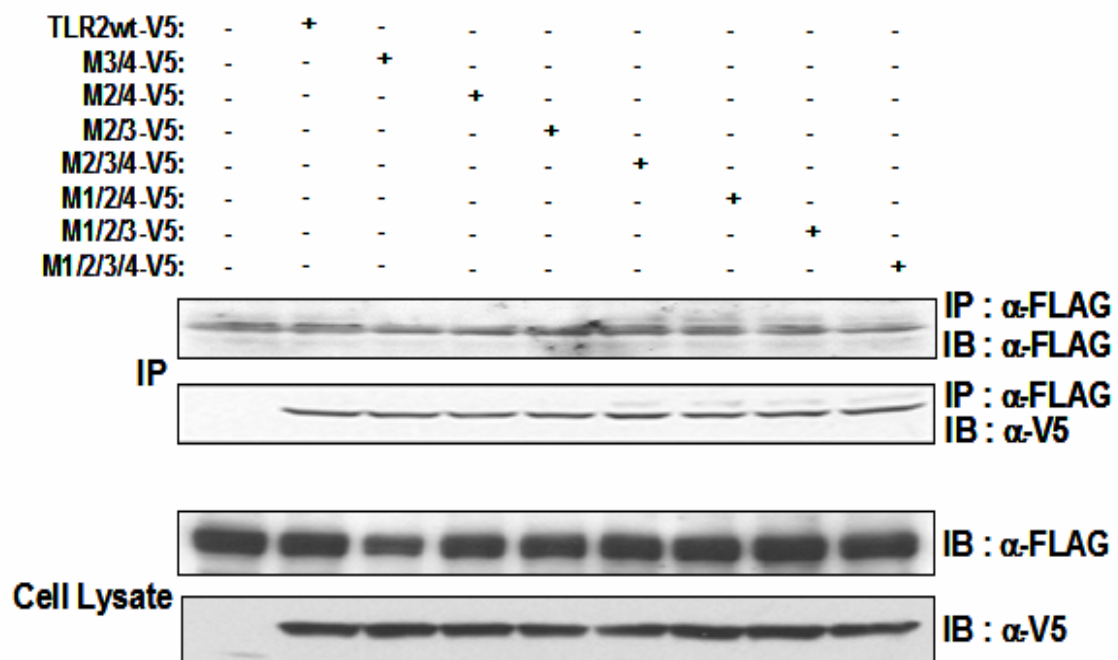


Fig.7

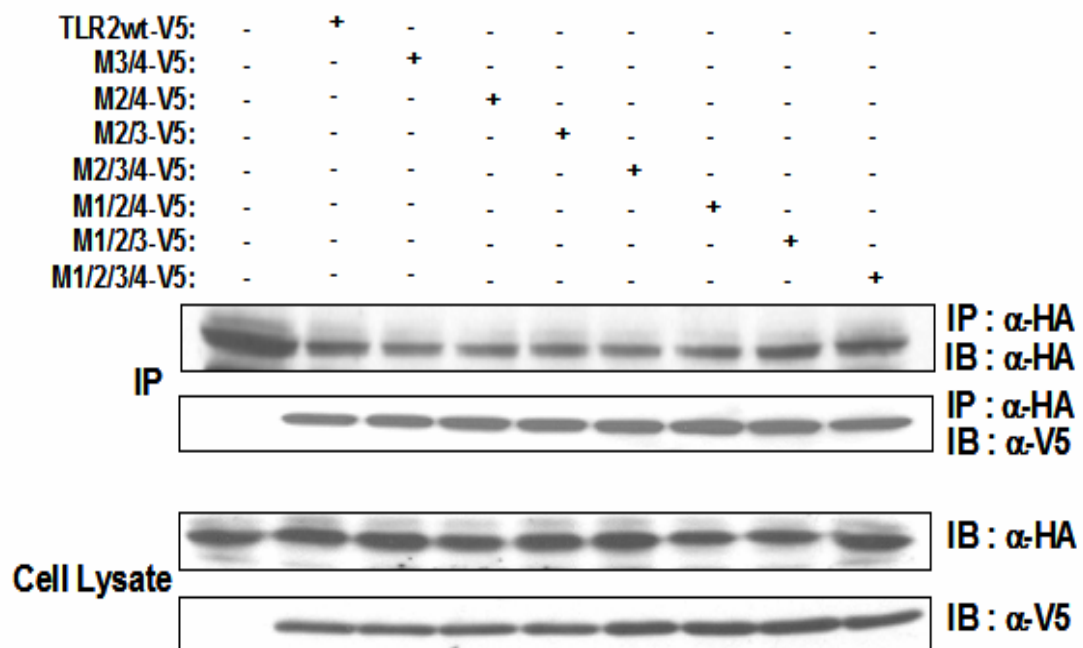


Fig.8

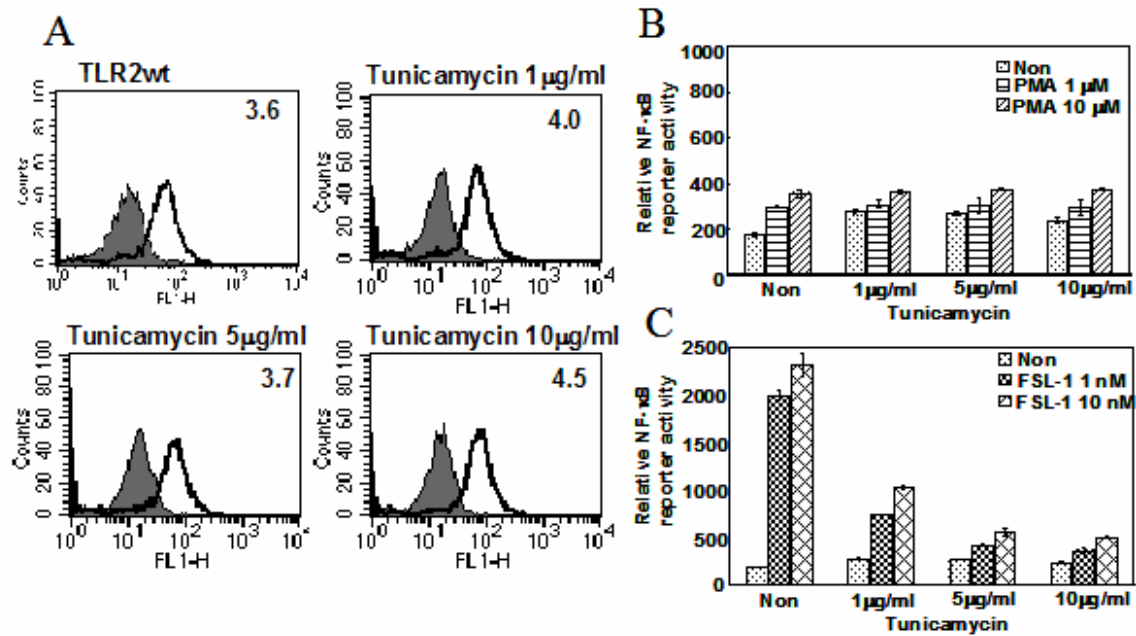


Fig. 9