The Toll-like receptor 4 (TLR4)/myeloid differentiation factor-2 (MD-2) complex is essential for LPS recognition and induces innate immune responses against Gram-negative bacteria. As activation of TLR4/MD-2 is also critical for the induction of adaptive immune responses, TLR4/MD-2 agonists have been developed as vaccine adjuvants, but their efficacy has not yet been ascertained. Here, we demonstrate that a funiculosin variant, FNC-RED, and FNC-RED and FNC derivatives, FNC-RED-P01 and FNC-P01, respectively, act as agonists for both murine and human TLR4/MD-2. FNC-RED induced nuclear factor-κB (NF-κB) activation via murine TLR4/MD-2, whereas FNC had no TLR4/MD-2 stimulatory activity. Bioassay analysis revealed that FNC-RED binds to murine TLR4/MD-2 but not murine radioprotective 105 (RP105)/myeloid differentiation factor-1 (MD-1), another LPS sensor. FNC-RED induced CD14-independent expressions of pro-inflammatory cytokines and co-stimulatory molecules in murine macrophages and dendritic cells. In contrast, FNC-RED stimulation was reduced in CD14-dependent LPS responses, including dimerization and internalization of TLR4/MD-2. FNC-RED-P01 and FNC-P01, respectively, activated human TLR4/MD-2, unlike FNC-RED. Finally, computational analysis revealed that this species-specific activation by FNC-RED and FNC-RED-P01 resulted from differences in electrostatic surface potentials between murine and human TLR4/MD-2. We conclude that FNC-RED and its synthetic derivative represent a novel category of murine and human TLR4/MD-2 agonist.

This work was supported by Hokuriku Life Science Cluster, MEXT Regional Innovation Strategy Support Program (to K.T.); Hokuriku Innovation Cluster for Health Science, MEXT Regional Innovation Cluster Program, Toyama/Ishikawa Region (to K.T.); Grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) KAKENHI JP16K19596 (to N.O.), JP16K19532 (to Y.W.), and JP15K07960 (to H.H.); NEXT Program (LR025 to Y.F.); and JST, PRESTO (JPMJPR13M1 to Y.N.). This work supported in part by the Platform Project for Supporting in Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from the Japan Agency for Medical Research and Development (to T.H.). Our laboratory (Dept. of Immunobiology and Pharmacological Genetics, University of Toyama) has Grant/Research support from Teika Pharmaceutical Co., Ltd. N. O., T. Kato, S. F., and K. T. are employees of Teika Pharmaceutical Co., Ltd.

This article contains supplemental Figs. S1–S13 and Table S1.

1 Both authors contributed equally to this study.

2 To whom correspondence may be addressed: Dept. of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, 2630 Sugitani, Toyama-shi, Toyama 930-0194, Japan. Tel.: 81-76-434-7673; Fax: 81-76-434-5009; E-mail: ynagai@med.u-toyama.ac.jp.

3 To whom correspondence may be addressed: Dept. of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, 2630 Sugitani, Toyama-shi, Toyama 930-0194, Japan. Tel.: 81-76-434-7673; Fax: 81-76-434-5009; E-mail: takatsuki@med.u-toyama.ac.jp.
pathogen products, and they are characterized by extracellular leucine-rich repeat motifs and intracellular Toll/IL-1 receptor (TIR) domains (1). TLR4 was the first TLR to be identified, and its extracellular domain forms a heterodimeric complex with myeloid differentiation factor-2 (MD-2) (2). Neither Tlr4\(^{-/-}\) nor Ly96\(^{-/-}\) (MD-2\(^{-/-}\)) mice respond to LPS (3, 4), so both members of the TLR4/MD-2 complex are essential for LPS responses. Crystal structure analysis revealed that MD-2 has a large hydrophobic cavity that could accommodate lipid A, a bioactive moiety of LPS (5). LPS binding to MD-2 induces homodimerization of a 1:1 TLR4/MD-2 complex, in a tail-to-tail orientation with two TLR4 molecules (6). Formation of the LPS-TLR4/MD-2 complexes then causes the recruitment of intracellular adaptor proteins, TIR domain-containing adaptor protein, and myeloid differentiation primary response gene 88 (MyD88) (7). MyD88 leads to early activation of MAPKs and the transcriptional factor nuclear factor-κB (NF-κB) to induce inflammatory cytokine genes such as IL-6 and TNF-α (1). TLR4 signals via another adaptor protein TIR domain-containing adaptor-inducing IFN-β (TRIF) to NF-κB and interferon regulatory factor (IRF)1 and IRF3 transcriptional factors (8).

Upon ligand recognition, TLRs not only initiate the production of pro-inflammatory cytokines, but they also activate antigen-presenting cells (APC) to modulate adaptive immune responses against pathogens (8). Therefore, TLR agonists have been the focus of attempts to develop new-generation vaccines. Many preclinical and clinical studies have used purified TLR agonists to enhance adaptive immune responses during vaccination (9). Among these agonists, monophosphoryl lipid A (MPL), an artificially detoxified derivative of lipid A, is one of the most used adjuvants in human licensed vaccines (10). Previous studies revealed that MPL is much weaker than lipid A in inducing TLR4/MD-2 dimerization and MyD88-dependent immune responses (11, 12). Nonetheless, MPL can induce stronger T-helper cell type 1 responses than classical adjuvants such as aluminum salt precipitates (13). This ability is due to a signaling bias toward the TRIF pathway, resulting in the safe stimulation of adaptive immune responses without excessive production of inflammatory cytokines (14). Based on these characteristics, lipid A analogs represent attractive candidates for adjuvants, and other lipid A mimetics are under development (15–19).

TLRs also recognize endogenous ligands called danger-associated molecular patterns (DAMPs). Intriguingly, DAMPs can enhance adjuvanticity when co-immunized with inactivated vaccines or synergize with exogenous TLR ligands to induce efficient cellular immune responses (20, 21). Additionally, a chemical compound with no structural similarity to lipid A engages the TLR4/MD-2 complex and triggers pro-inflammatory responses (22). These facts suggest that non-lipid A ligands of natural origin may activate TLR4/MD-2.

We have now screened synthetic and natural products to discover new non-endotoxin-derived TLR4/MD-2 agonists. Stimulatory activity was detected as NF-κB activation in Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 by flow cytometry. Here, we identify a funiculosin (FNC) variant, FNC-RED, as a novel ligand for murine TLR4/MD-2 through this screen. Furthermore, we synthesized derivatives with a phosphatase group that activated human TLR4/MD-2. Responses induced by FNC-RED and derivatives were then investigated in vitro on murine and human immune cells to explore structure-function relationships.

Results

Reduced compound of funiculosin induces NF-κB activation via murine TLR4/MD-2

To identify non-endotoxin-derived TLR4/MD-2 agonists, we prepared Ba/F3 transfectant cells expressing murine TLR4/MD-2, murine CD14, and an NF-κB-GFP reporter construct. Using this, we could screen thousands of compounds in a relatively short time and easily detect TLR4/MD-2-induced NF-κB activation by flow cytometry (23). Among 1,320 compounds and natural products from a commercially available compound library and pharmaceutical companies (see “Experimental procedures”) screened for their abilities to activate NF-κB, we identified only one sample, termed TIK-139, that was as active as lipid A and taxol (supplemental Fig. 1A). TIK-139 was obtained from reduced products of FNC extracted from the soil bacterium *Penicillium funiculosum* (24, 25). HPLC analysis revealed that at least five compounds were contained in TIK-139 (supplemental Fig. 1B). We fractionated these compounds and tested their abilities in Ba/F3 transfectant cells. Among five fractions, fraction 4 induced the strongest ability to activate NF-κB (supplemental Fig. 1C). The MTS510 mAb specifically recognizes the murine TLR4/MD-2 complex (26). NF-κB activation induced by fraction 4 was completely blocked by this mAb (supplemental Fig. 1D), although it only partially inhibited lipid A- or taxol-induced NF-κB activation (supplemental Fig. 1D).

We then investigated the structure of the active compound contained in fraction 4. The fraction was separated by HPLC, and an acetylated sample was analyzed by high-resolution MS (ESI-QTOF MS) and NMR (supplemental Fig. 2, A–C). With this indirect strategy, the chemical structure was determined (Fig. 1A). Because the active molecule was a reduced compound of FNC, we termed it FNC-RED. It was composed of a hydrophilic region, an acidic hydroxy group, and an alkyl chain, which are structurally and chemically analogous to lipid A (supplemental Fig. 3).

We then synthesized FNC-RED from FNC (see “Experimental procedures”). The NF-κB activation potential of the synthesized FNC-RED was similar to that of fraction 4 in Ba/F3 transfectant cells (Fig. 1B), whereas FNC had no such ability (Fig. 1C). Upon FNC-RED stimulation, the levels of NF-κB-GFP were increased in a dose-dependent manner (Fig. 1B). We confirmed that FNC-RED induced NF-κB activation via TLR4/MD-2

bone marrow-derived macrophage; DAMP, damage-associated molecular pattern; EDC, N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide; FNC, funiculosin; FNC-RED, reduced compound of FNC; LBPl, LPS-binding protein; MPL, monophosphoryl lipid A; NF-κB, nuclear factor-κB; NHS, N-hydroxysuccinimide; qRT-PCR, quantitative RT-PCR; rLBP, recombinant mouse LBP; RU, resonance unit; SPR, surface plasmon resonance; TIR, Toll/IL-1 receptor; TRIF, TIR domain-containing adaptor-inducing IFN-β; PDB, Protein Data Bank; Ct. Ab, control antibody; MFI, mean fluorescence intensity; PE, phycoerythrin; Ab, antibody.
MD-2 using the MTS510 mAb (Fig. 1B). Polymyxin B has been used to detect and clear LPS contamination (27). Polymyxin B treatment clearly suppressed lipid A-induced NF-κB activation (Fig. 1D). In contrast, FNC-RED-induced NF-κB activation was not affected by polymyxin B treatment, suggesting that neither lipid A nor LPS contaminated the FNC-RED preparation.

To examine whether FNC-RED binds to murine TLR4/MD-2, we performed surface plasmon resonance (SPR) using a Biacore instrument. The murine TLR4 protein forms a complex with murine MD-2 in solution (28). Furthermore, in the presence of LPS, this 1:1 TLR4/MD-2 complex forms a dimeric structure of the LPS-binding 2:2 TLR4/MD-2 complex in solution and crystal (28). The binding of FNC-RED or FNC to murine TLR4/MD-2 was detected (Fig. 1E). Although the binding rates and affinities were unable to be determined, we observed differences in SPR sensorgrams between FNC-RED and FNC. The dissociation rate for the binding of FNC-RED to murine TLR4/MD-2 was 0.038 s⁻¹, similar to that of LPS to murine TLR4 or MD-2 alone, as reported previously (29). In contrast, the dissociation rate for the binding of FNC to murine TLR4/MD-2 was 0.961 s⁻¹, 25-fold higher than that of FNC-RED (Fig. 1E). This suggests that FNC binds to TLR4/MD-2, but its binding might be unstable. A TLR4 homologue, radio-protective 105 (RP105), forms a complex with myeloid differentiation factor-1 (MD-1) (30, 31). RP105- or MD-1-deficient B cells were hyporesponsive to LPS (32, 33), suggesting that the RP105/MD-1 complex cooperates with the TLR4/MD-2 complex in LPS responses. The resonance unit (RU) values for the bindings of FNC-RED and FNC to murine RP105/MD-1 were much lower than those for murine TLR4/MD-2 (supplemental Fig. 4). These results clearly demonstrate that FNC-RED is a novel agonist of murine TLR4/MD-2.

To explore a structure-based mechanism for the binding and stimulatory effect of FNC-RED, the radius of gyration (Rg) of FNC and FNC-RED was calculated by taking into account their conformational change (1,000 conformations) in solution phase. FNC had Rg values of 4.8 Å at the highest frequency (supplemental Fig. 5A). As shown in a representative structure of FNC with this Rg value, the pyran ring contributed to make an intramolecular hydrogen bond with the acidic hydroxy group (supplemental Fig. 5B). In contrast, FNC-RED had Rg values of more than 5.2 Å (supplemental Fig. 5, A and C). Rg values of less than 4.4 Å were also observed in FNC-RED (supplemental Fig. 5, A and D). These findings indicate that lack of the pyran ring leads to the acquisition of mobility. Therefore, the alkyl chain of FNC-RED may have enhanced its accessibility to the hydrophobic cavity of MD-2, as is well known in lipid A whose acyl chains are deeply inserted into the cavity (5, 6).

**FNC-RED induces expression of pro-inflammatory cytokines via MyD88 and TRIF pathways in murine macrophages**

TLR4 activation induces expression of pro-inflammatory cytokines and type 1 IFN (1). Therefore, we examined whether FNC-RED had this activity and required MD-2 for responses in bone marrow-derived macrophages (BMDMs). A high concentration of FNC (100 µg/ml) decreased cell viability of BMDMs, whereas FNC-RED had no such adverse effect (supplemental Fig. 6). Expression levels of TNF-α mRNA were increased by lipid A, MPL, or FNC-RED stimulation within 4 h in BMDMs from WT mice (Fig. 2A, left), and these responses did not occur with BMDMs from TLR4- or MD-2-deficient mice (Fig. 2B, left). We obtained similar results with IL-12p40 mRNA expression (Fig. 2B, right). Lipid A or MPL stimulation increased expression levels of IFN-β mRNA within 2 h (Fig. 2A, right). However, we found no significant increase in IFN-β mRNA in FNC-RED-stimulated WT BMDMs (Fig. 2A, right).

We also evaluated the requirements for MyD88 and TRIF in FNC-RED-induced responses. Lipid A- or MPL-induced TNF-α and IL-12p40 mRNA expressions were impaired in MyD88- or TRIF-deficient (Ticam1<sup>−/−</sup>) BMDMs compared with those from WT mice (Fig. 2C). Additionally, TRIF-deficient BMDMs showed better TNF-α expression than those lacking MyD88. Thus, FNC-RED activates the TLR4/MD-2 complex on murine macrophages and its treatment increases expression levels of pro-inflammatory cytokines via MyD88- and TRIF-dependent pathways. However, FNC-RED has no significant stimulatory effect on IFN-β expression.

**FNC-RED induces adaptive immune responses in murine dendritic cells**

Adaptive immune responses, including CD86 up-regulation and IL-12p40 expression, are key reactions on APCs, such as dendritic cells (34, 35). Therefore, we investigated the activity of FNC-RED on adaptive immune responses. Up-regulation of CD86, a co-stimulatory molecule for T cells, and MHC class II, is induced by TLR4/MD-2 stimulation on the cell surface via the TRIF pathway (11, 36). Lipid A or MPL stimulation induced CD86 and MHC class II up-regulation on murine bone marrow-derived conventional dendritic cells (BM-cDCs) from WT mice (Fig. 3A and supplemental Fig. 7). FNC-RED stimulation...
also induced up-regulation of CD86 and MHC class II, but high concentrations were required compared with lipid A and MPL. These responses were slightly attenuated in MyD88-deficient BM-cDCs (Fig. 3A and supplemental Fig. 7). In contrast, TRIF-deficient BM-cDCs were greatly impaired in these responses induced by not only lipid A or MPL but also FNC-RED stimulation (Fig. 3A and supplemental Fig. 7). Additionally, FNC-RED as well as MPL increased expression levels of TNF-α and IL-12p40 were measured by qRT-PCR. Data are shown as means ± S.D. §, p < 0.001 versus WT. C, BMDMs from WT, Myd88−/−, or Traf3−/− mice were stimulated with lipid A (1 μg/ml), MPL (1 μg/ml), or FNC-RED (50 μg/ml) for 4 h. Total RNA was extracted, and expression levels of TNF-α and IL-12p40 were measured by qRT-PCR. Data are shown as means ± S.D. §, p < 0.001 versus WT. Similar results were obtained in three independent experiments.

**FNC-RED activates NF-κB but not MAPK pathway**

NF-κB and MAPKs are key molecules for TLR4-induced pro-inflammatory cytokine expression (1). We then investigated whether FNC-RED stimulation activates these pathways. An IκB kinase inhibitor BAY11-7082 and a MAPK inhibitor PD98059 significantly inhibited lipid A-induced IL-12p40 production in BM-cDCs from WT mice (Fig. 4A, left). In contrast, FNC-RED-induced IL-12p40 production was suppressed by BAY11-7082 but not PD98059 (Fig. 4A, right), suggesting that MAPKs may not be involved in FNC-RED-stimulated signaling pathways. We confirmed that FNC-RED activates NF-κB pathway by detecting IκBα degradation in WT BMDMs (Fig. 4, B and C). In contrast, FNC-RED stimulation did not induce Erk1/2 phosphorylation (Fig. 4, B and C). Thus, lipid A and FNC-RED stimulations may utilize different signaling pathways to induce pro-inflammatory cytokine production.

**FNC-RED-induced NF-κB activation does not require LBP or soluble/membranous CD14**

LBP is a soluble protein mainly produced by the liver and augments LPS responses (37, 38). CD14 exists on cell mem-
branes and in serum in membranous and soluble forms, respectively (39). CD14 has a key role in sensitizing cells to LPS by delivering it to the TLR4/MD-2 complex (40). To determine the role of LBP and CD14 for FNC-RED responses, we first examined whether FBS was required for FNC-RED-induced NF-κB activation, because FBS contains bovine LBP and the soluble form of bovine CD14. In contrast to lipid A stimulation, low concentrations of FNC-RED (0.1 or 0.5 µg/ml) induced NF-κB activation in the absence but not in the presence of FBS (supplemental Fig. 8A). This NF-κB activation was significantly blocked by MTS510 mAb (supplemental Fig. 8B). Additionally, high concentrations of BSA blocked FNC-RED-induced NF-κB activation in the absence of FBS (supplemental Fig. 9). These results indicate that albumin or some factors in FBS block FNC-RED-induced NF-κB activation.

We then treated Ba/F3 transfectant cells with a recombinant mouse LBP (rmLBP) or recombinant mouse CD14 (rmCD14) in the absence of FBS. The rmLBP and rmCD14 augmented NF-κB activation induced by lipid A in a dose-dependent manner (Fig. 5A). In contrast, these recombinant proteins had no effect on FNC-RED-induced NF-κB activation. We also examined whether an anti-mouse CD14 mAb (clone 4C1) blocked FNC-RED-induced NF-κB activation in the presence of FBS on Ba/F3 transfectant cells. The CD14 mAb blocked NF-κB activation induced by a low dose of lipid A (0.3 ng/ml) (Fig. 5B). However, this was not seen in the FNC-RED-stimulated cells (Fig. 5B), suggesting that membranous CD14 may be dispensable for FNC-RED-induced NF-κB activation. These results suggest that LBP and soluble/membranous CD14 may not be involved in FNC-RED-induced responses.

Figure 3. FNC-RED induces adaptive immune responses in mouse dendritic cells. A, BM-cDCs from WT, Myd88−/−, or Ticam1−/− mice were stimulated with the indicated concentrations of lipid A, MPL, or FNC-RED for 20 h. The cells were harvested, and CD86 expression was analyzed by flow cytometry. Open and filled histograms depict those stained with isotype-matched antibody or anti-CD86 antibody, respectively. Percentages of CD86-positive cells were depicted in each histogram. B, BM-cDCs from WT mice were stimulated with lipid A (1 µg/ml), MPL (1 µg/ml), or FNC-RED (50 µg/ml) for 2 h. Total RNA was extracted, and expression levels of IL-12p40, TNF-α, and IFN-β were measured by qRT-PCR. Data are shown as means ± S.D. #, p < 0.01; §, p < 0.001 versus medium (Med.). Similar results were obtained in three independent experiments.
FNC-RED stimulation is impaired in the dimerization and internalization of TLR4/MD-2

Membranous CD14 has a key role in the formation of tight dimerization of TLR4/MD-2 to initiate MyD88-dependent pro-inflammatory cytokine expression and the internalization of TLR4/MD-2 toward endosome, where TRIF signaling induces type 1 IFN production (11, 41). To confirm that membranous CD14 is dispensable for FNC-RED-induced responses (Fig. 5B), we studied the formation of TLR4/MD-2 dimerization using Ba/F3 cells expressing murine TLR4-GFP, murine TLR4-FLAG, murine MD-2-FLAG, and murine CD14 (42). After lipid A stimulation, TLR4-FLAG was co-precipitated with TLR4-GFP, suggesting that lipid A-induced TLR4/MD-2 dimerization (Fig. 6A). In contrast, FNC-RED did not have this effect.

Then we focused on the internalization of TLR4/MD-2. After lipid A stimulation, TLR4/MD-2 rapidly internalized (Fig. 6B), and this response persisted for 18 h in Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 (data not shown). However, FNC-RED had a defect in inducing TLR4/MD-2 internalization even at 18 h of stimulation (Fig. 6B and data not shown). Similar results were obtained when BM-cDCs were stimulated with lipid A or FNC-RED (Fig. 6C). To confirm the importance of membranous CD14 in TLR4/MD-2 internalization, we stimulated CD14-deficient BM-cDCs with lipid A or FNC-RED. To eliminate the effect of soluble bovine CD14, we set up the cultures without FBS. CD14-dependent TLR4/MD-2 internalization was observed when stimulated with lipid A at a longer interval (18 h) (Fig. 6D). In contrast, CD14 dependence was not seen in FNC-RED-stimulated BM-cDCs. This was con-
Funiculosin variants activate the TLR4/MD-2 complex

A

FBS (-):

| Lipid A (ng/ml) | FNC-RED (µg/ml) |
|----------------|-----------------|
| Medium         | 0.1             |
| 90             | 154             |
| 104            | 157             |
| 322            | 857             |
| 100            | 962             |
| 10             | 154             |
| 104            | 857             |
| 623            | 929             |
| 98             | 194             |
| 1156           | 911             |
| 91             | 194             |
| 245            | 111             |
| 104            | 161             |
| 199            | 911             |
| 113            | 194             |
| 693            | 183             |
| 104            | 857             |
| 199            | 910             |
| 104            | 154             |

B

FBS (+):

| Lipid A (ng/ml) | FNC-RED (µg/ml) |
|----------------|-----------------|
| 0.3            | 0.5             |
| 285            | 72              |
| 280            | 71              |
| 676            | 270             |
| 723            | 272             |
| 0.3            | 5.0             |
| 285            | 72              |
| 157            | 270             |
| 676            | 230             |
| 723            | 272             |

---

rmLBP (ng/ml) | rmCD14 (ng/ml) | NF-κB-GFP
---|---|---

---

Medium | Lipid A or FNC-RED | + rmLBP or rmCD14
---|---|---

---

Ct. IgG2b | αmCD14 Ab
---|---

---

% of Max (count)
Funiculosin variants activate the TLR4/MD-2 complex

sistent with the result that the anti-CD14 mAb did not inhibit FNC-RED-induced NF-κB activation (Fig. 5B). These results demonstrate that FNC-RED does not induce membranous CD14-dependent TLR4/MD-2 activation, including its tight dimerization and internalization.

Derivatives of FNC-RED and FNC activate human TLR4/MD-2

Our experiments then focused on potential activity for FNC-RED on human TLR4/MD-2. To explore this, we stimulated Ba/F3 cells expressing human TLR4/MD-2 and human CD14 with either lipid A or FNC-RED. Although lipid A stimulation induced NF-κB activation in Ba/F3 transfectant cells, such a response was not observed with FNC-RED even at a high concentration (50 μg/ml) (supplemental Fig. 10).

To increase affinity of FNC-RED with human TLR4/MD-2, we synthesized two derivatives, which have a phosphate group in FNC-RED and FNC, designated FNC-RED-P01 and FNC-P01, respectively (Fig. 7A). FNC-RED-P01 and FNC-P01 showed weak NF-κB activation in Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 (Fig. 7B). Likewise, FNC-RED-P01 and FNC-P01 induced low levels of TNF-α expression in BMDMs compared with FNC-RED (supplemental Fig. 11A, left). However, these derivatives did not induce IFN-β expression in BMDMs (supplemental Fig. 11A, right).

Intriguingly, both derivatives acquired potency for NF-κB activation in Ba/F3 transfectant cells expressing human TLR4/MD-2 and human CD14 (Fig. 7B). To examine whether these activities were dependent on human TLR4/MD-2, we stimulated naive Ba/F3 cells expressing NF-κB-GFP reporter construct alone, with them, and with other stimuli. Because naive Ba/F3 cells express murine TLR2 (data not shown), a TLR2 ligand Pam3CSK4 induced NF-κB activation (supplemental Fig. 11B). In contrast, lipid A, FNC-RED, and the derivatives did not induce this response, suggesting that the derivatives activate NF-κB via human TLR4/MD-2. Because FNC-RED-P01 had a much more potent activity than FNC-P01 (Fig. 7B), the former was utilized in the following experiments. Because polymyxin B treatment suppressed lipid A-induced but not FNC-RED-P01-induced NF-κB activation in Ba/F3 transfectant cells, lipid A or LPS was unlikely to contaminate the FNC-RED-P01 preparation (supplemental Fig. 11C). In contrast to FNC-RED, FNC-RED-P01-induced NF-κB activation was not affected by the addition of FBS (supplemental Fig. 11D).

Next, we examined whether FNC-RED-P01 activated human macrophages. Expression levels of TNF-α and monocyte chemoattractant protein-1 (MCP-1) mRNA were increased by lipid A stimulation in human THP-1 macrophages (Fig. 7C, left and middle). FNC-RED-P01 stimulation increased expression levels of these genes in THP-1 cells in a dose-dependent manner. These responses did not occur in FNC-RED-stimulated THP-1 cells. As FNC-RED did not induce IFN-β expression in BMDMs (Fig. 2A), FNC-RED-P01 had no stimulatory effect on IFN-β expression in THP-1 cells (Fig. 7C, right). Taken together, FNC-RED-P01 induces innate immune responses via human TLR4/MD-2.

Docking of FNC-RED and FNC-RED-P01 to murine or human TLR4/MD-2

To elucidate a structure-based mechanism for the species-specific activation of TLR4/MD-2 by FNC-RED and FNC-RED-P01, we performed a computational analysis. Because up to four FNC-RED molecules can be accommodated in the hydrophobic cavity of MD-2 (supplemental Fig. 12), we utilized lipid IVα, which has four acyl chains, as a reference for our docking study of FNC-RED and FNC-RED-P01. The data suggest that the differences in electrostatic surface potentials between murine and human TLR4/MD-2 may be important for the species-specific discrimination of FNC-RED and FNC-RED-P01 (supplemental Fig. 13, A–D). Additionally, the binding affinities of FNC-RED and FNC-RED-P01 for murine or human TLR4/MD-2 were calculated as the gain of free energy (ΔΔG, FNC-RED-human TLR4/MD-2 as reference (ΔΔG = 0 kcal/mol)) (supplemental Fig. 13E). The differences between FNC-RED and FNC-RED-P01 in the binding affinity for human or murine TLR4/MD-2 were consistent with our findings in their stimulatory effects and the docking models.

Discussion

Recent advances in our understanding of innate immunity now permit a rational approach for designing and selecting compounds with stimulatory properties. In this study, we identified a reduced compound of FNC, termed FNC-RED, as a specific agonist for the murine TLR4/MD-2 complex. In contrast to lipid A-induced TLR4/MD-2 activation (Fig. 8A), FNC-RED induces CD14-independent TNF-α production and CD86 up-regulation but failed to activate CD14-dependent tight dimerization and internalization of TLR4/MD-2 (Fig. 8B). These features reduce excessive innate immune responses, including IFN-β expression, but augment CD86 and MHC class II expression. Although FNC-RED-P01, a derivative of FNC-RED, induced weak NF-κB activation in Ba/F3 expressing murine TLR4/MD-2 and murine CD14, it potently activated human THP-1 macrophages compared with mouse BMDMs. FNC-RED-P01 induced TNF-α and MCP-1 mRNA expression but not IFN-β mRNA expression in THP-1 macrophages.
Funiculosin variants activate the TLR4/MD-2 complex

### A

| Lipid A (μg/ml) | 0.03 | 1.0 | 50 |
|-----------------|------|-----|----|
| IB: anti-FLAG   |      |     |    |
| IB: anti-GFP    |      |     |    |
| IP: anti-GFP    |      |     |    |

### B

| Lipid A (μg/ml) | 0.1  | 1.0  |
|-----------------|------|------|
| FNC-RED (μg/ml) | 1.0  | 10   |

% of Max (count)

| TLR4/MD-2 |
|-----------|
| Isotype   |
| αTLR4/MD-2 Ab |

### C

**BM-cDC**

FBS (+):

| Lipid A (μg/ml) | 0.1  | 1.0  |
|-----------------|------|------|
| FNC-RED (μg/ml) | 5.0  | 50   |

**BM-cDC**

FBS (-):

| Lipid A (0.1 μg/ml) | 1 h  | 18 h |
|----------------------|------|------|
| FNC-RED (1 μg/ml)    | 1 h  | 18 h |

% of Max (count)

| TLR4/MD-2 |
|-----------|
| Isotype   |
| αTLR4/MD-2 Ab |

---

*J. Biol. Chem.* (2017) 292(37) 15378–15394
Funiculosin variants activate the TLR4/MD-2 complex

Therefore, our study revealed a novel category of murine and human TLR4 agonists. In this study, we utilized Ba/F3 transfectant cells to screen TLR4/MD-2 agonists. Through this cell-based assay, we can easily detect activation of the NF-κB pathway by flow cytometry. Moreover, the screening time is significantly less compared with other assays, including ELISA, Western blotting, etc. This new approach allowed us to identify a rare class of bioactive compounds that activate murine and human TLR4/MD-2.

FNC was first identified as an antibiotic (24, 25) and effects on innate immunity were not suspected. We infer from this study that FNC-RED and its derivatives are novel agonists for TLR4/MD-2. FNC was not developed for clinical use as an antibiotic due to its mitochondrial toxicity by inhibiting cytochrome bc complex (43). We found that FNC-RED showed lower cytotoxicity compared with FNC in BMDMs (supplemental Fig. 6). In addition, cytotoxicity of FNC-RED-P01 was not detected at a concentration of 10 μg/ml in THP-1 macrophages (data not shown). These facts suggest that reduction of or addition of a phosphate group to FNC can lower its cytotoxicity.

LBP and CD14 are key accessory molecules that make efficient transfer of LPS to TLR4/MD-2 and increase the sensitivity of LPS detection (44). Recent studies have identified the LPS-binding sites of CD14 and the key charged residues in LBP and CD14 for LPS transfer (45–47). In addition, CD14 mediates LPS-induced endocytosis of TLR4, which induces TRIF-dependent responses in endosomes (41). We found that these molecules are not required for FNC-RED-induced murine TLR4/MD-2 activation. This may also be the case with FNC-RED-P01, because it has no effect on type I IFN expression (Fig. 7C). Thus, it is likely that FNC-RED and FNC-RED-P01 bind to neither LBP nor CD14, whereas these molecules form a high-affinity complex with LPS (44). Intriguingly, Neoseptin-3 also does not require CD14 for its stimulatory effect on TLR4 signaling (22).

Intriguingly, FNC-RED stimulation does not activate MAPK signaling (Fig. 4, A and B). A previous paper reported that CD14 increased LPS-induced phosphorylation of MAPKs in endothelial cells (48). Furthermore, LPS-induced phosphorylation of MAPKs but not NF-κB in macrophages was dependent on CD14 (49). Therefore, NF-κB and MAPK signaling under TLR4/MD-2 can be dissociated by CD14. As CD14 is not involved in FNC-RED-mediated TLR4/MD-2 activation, this may explain why FNC-RED does not activate MAPKs.

Our data indicate that some factors in serum inhibit FNC-RED-induced murine TLR4/MD-2 activation. It has been reported that LPS binds to various serum proteins, including albumin and hemoglobin (50–52). We found that BSA inhibited FNC-RED-induced NF-κB activation in the serum-free culture (supplemental Fig. 9), indicating that albumin binds to FNC-RED. Notably, the designing of FNC-RED-P01 resolved this problem (supplemental Fig. 11D). This suggests that a hydroxy group in pyridine ring may allow FNC-RED to bind albumin.

There is much evidence for a critical role of TLR4 in the induction of non-infectious chronic inflammation by recognizing DAMPs (53). In addition, it was reported that TLR4 signaling caused influenza infection to be lethal (54), and a synthetic TLR4 antagonist protected this lethality (55). Therefore, our structure-function and computational analyses may be useful for the synthesis of a new TLR4 antagonist that blocks TLR4-associated inflammation. Furthermore, our screening system should be applicable to searches for new TLR4 antagonists.

In conclusion, we have identified novel TLR4/MD-2 agonists, and we dissected mechanisms through which FNC-RED and FNC-RED-P01 can activate the receptor.

Experimental procedures

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and were used at 8–12 weeks of age. C57BL/6, Tlr4−/− (3), Ly96−/− (4), Myd88−/− (56), Ticam1−/− (36), and Cd14−/− (41) mice were maintained in microisolator cages under specific pathogen-free conditions and maintained in the animal facility of University of Toyama and the Institute of Medical Science, the University of Tokyo. The experimental protocols were approved by the Animal Studies Committees at University of Toyama (Approval No. A2016MED-9) and the Institute of Medical Science and the University of Tokyo (Approval No. A13-20), and all experiments were performed according to the guidelines for the care and treatment of experimental animals at University of Toyama and the University of Tokyo.

Reagents

Tested compounds were obtained from ICCB Known Bioactive Library (Funakoshi, Tokyo, Japan) and kindly provided from pharmaceutical companies. Lipid A from Escherichia coli F583 (Rd mutant), taxol from Taxus brevifolia, and BSA were purchased from Sigma. MPL from Salmonella minnesota R595, Pam3CSK4, polymyxin B, and BAY11-7082 were purchased from InvivoGen (San Diego, CA). PD98059 was purchased from Merck Millipore (Darmstadt, Germany). FBS was purchased from GE Healthcare. Recombinant mouse LBP and recombinant murine LBP-FLAG, murine LBP-GFP, murine CD14-FLAG, murine CD14-GFP, murine CD14-FLAG, and murine CD14 were stimulated with medium alone, LPS (0.03 or 1 μg/ml), or FNC-RED (50 μg/ml) for 60 min. The cultured cells were then subjected to immunoprecipitation with anti-GFP and immunoblotting (IB) with anti-FLAG or anti-GFP as described under the "Experimental procedures." B. Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 were stimulated with medium (Mde) alone or the indicated concentration of lipid A or FNC-RED in the presence of FBS (10% v/v) for 1 h. The cells were harvested and stained with control antibodies Ct. Ab or anti-mouse TLR4/MD-2 mAb (clone MTS510). 

Open and filled histograms depict those stained with Ct. Ab and MTS510, respectively. Percentages of TLR4/MD-2-positive cells are depicted in each histogram. C. BM-cDCs from WT and Cd14−/− mice were stimulated with medium alone or indicated concentration of lipid A or FNC-RED in the absence of FBS for 1 or 18 h. The cells were harvested and stained with Ct. Ab or MTS510. Open and filled histograms depict those stained with Ct. Ab and MTS510, respectively. Percentages of TLR4/MD-2-positive cells were depicted in each histogram. All data are representative of at least three independent experiments.

Figure 6. FNC-RED stimulation is impaired in CD14-dependent TLR4/MD-2 activation. A. Ba/F3 cells expressing murine TLR4-FLAG, murine TLR4-GFP, murine CD14-FLAG, and murine CD14 were stimulated with medium alone, LPS (0.03 or 1 μg/ml), or FNC-RED (50 μg/ml) for 60 min. The cultured cells were then subjected to immunoprecipitation with anti-GFP and immunoblotting (IB) with anti-FLAG or anti-GFP as described under the "Experimental procedures." B. Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 were stimulated with medium (Mde) alone or the indicated concentration of lipid A or FNC-RED in the presence of FBS (10% v/v) for 1 h. The cells were harvested and stained with control antibodies Ct. Ab or anti-mouse TLR4/MD-2 mAb (clone MTS510). 

Open and filled histograms depict those stained with Ct. Ab and MTS510, respectively. Percentages of TLR4/MD-2-positive cells are depicted in each histogram. C. BM-cDCs from WT and Cd14−/− mice were stimulated with medium alone or indicated concentration of lipid A or FNC-RED in the absence of FBS for 1 or 18 h. The cells were harvested and stained with Ct. Ab or MTS510. Open and filled histograms depict those stained with Ct. Ab and MTS510, respectively. Percentages of TLR4/MD-2-positive cells were depicted in each histogram. All data are representative of at least three independent experiments.
binant mouse CD14 were purchased from R&D Systems (Minneapolis, MN). TIK-139 was provided from Teika Pharmaceutical Co., Ltd. (Toyama, Japan). To obtain TIK-139, an extract from *P. funiculosum* was catalytically reduced in the presence of a palladium-carbon catalyst. Funiculosin was provided from Novartis (Basel, Switzerland) to synthesize FNC-RED.

**Cell culture**

Ba/F3 cells, an IL-3-dependent murine pro-B cell line, were cultured in RPMI 1640 medium containing 100 mM 2-mercaptoethanol and 1 ng/ml recombinant mouse IL-3 (R&D Systems). Ba/F3 cells expressing murine TLR4, MD-2, and CD14 were cultured.

**Figure 7.** Derivative of FNC-RED with a phosphate group activates human TLR4/MD-2. *A*, chemical structures of FNC-RED-P01 and FNC-P01. *B*, Ba/F3 cells expressing murine TLR4/MD-2 and murine CD14 (mTLR4/mMD-2/mCD14) or human TLR4/MD-2 and human CD14 (hTLR4/hMD-2/hCD14) were stimulated with the indicated concentration of FNC-RED-P01 or FNC-P01 for 18 h. The cells were harvested, and GFP expression was monitored by flow cytometry. Open and filled histograms depict those cultured with medium alone and FNC-RED-P01 or FNC-P01, respectively. Black and gray values depict MFI of GFP expression in cultured cells stimulated with medium alone and FNC-RED-P01 or FNC-P01, respectively. *C*, THP-1 cells were cultured as described under “Experimental procedures.” Total RNA was extracted, and expression levels of TNF-α, MCP-1, and IFN-β were measured by qRT-PCR. Data are shown as means ± S.D. N.S., not significant. $p < 0.001$ versus medium (Med.). Similar results were obtained in three independent experiments.
CD14, and NF-κB-GFP were established by electroporation, as described previously (23). BaF/3 cells expressing TLR4F (the C terminus of TLR4 was tagged with the FLAG epitope), TLR4G (the C terminus of TLR4 was tagged with the GFP epitope), MD-2, and CD14 were described previously (42).

Bone marrow cells were plated at 1 x 10^6 cells/ml in 24-well plates with FBS (10% v/v) in RPMI 1640 medium supplemented with 10 ng/ml recombinant murine GM-CSF (R&D Systems) as described (57). At day 6, loosely adherent cells were collected by gentle pipetting and used as BM-cDCs. For macrophages, bone marrow cells from C57BL/6 mice were plated in 10-cm bacteriological plastic plates with FBS (10% v/v) in RPMI 1640 medium supplemented with 100 ng/ml recombinant murine macrophage colony-stimulating factor (M-CSF) (R & D Systems). At

**Figure 8. Schematic models of lipid A- or FNC-RED-stimulated TLR4/MD-2 activation.**

A, lipid A first binds to LBP and is transferred to the TLR4/MD-2 complex via either CD14 (membranous or soluble CD14)-dependent or CD14-independent manner. In CD14-dependent manner, lipid A induces tight dimerization of TLR4/MD-2 at the plasma membrane leading to TNF-α production via MyD88-dependent pathway. Furthermore, membranous CD14 (mCD14) is required for the internalization of TLR4/MD-2 to endosomes, where TRIF induces IFN-β production. In CD14-independent manner, TLR4/MD-2 is weakly dimerized by lipid A stimulation but can initiate MyD88- and TRIF-dependent pathways, leading to TNF-α production and CD86 up-regulation, respectively. B, FNC-RED stimulation induces CD14-independent but not CD14-dependent TLR4/MD-2 activation. Because CD14 or LBP is not involved in FNC-RED-stimulated activation, FNC-RED does not induce tight dimerization and internalization of TLR4/MD-2. Although the dimerization of TLR4/MD-2 is not detected in FNC-RED-stimulated Ba/F3 transfectant cells by immunoprecipitation assay, it may induce weak dimerization of TLR4/MD-2 at the plasma membrane to promote TNF-α production and CD86 up-regulation.
Funiculosin variants activate the TLR4/MD-2 complex

day 7, adherent cells were harvested and used as BMDMs. All cells were incubated at 37°C in a humidified 5% CO2 incubator.

THP-1 was purchased from DS Pharma Biomedical (Osaka, Japan). THP-1 cells were treated with phorbol myristate acetate (500 ng/ml) for 48 h and then washed with PBS twice. The cells were further cultured for 12 h and then stimulated with the indicated concentrations of lipid A, FNC-RED, or FNC-RED-P01 for 4 h.

**Antibodies and flow cytometry**

Purified anti-mouse CD14 (clone 4C1), purified rat IgG2b, and rat IgG1 were purchased from BD Biosciences. Purified anti-mouse LBP (clone 749405) was purchased from R&D Systems. Purified anti-mouse TLR4/MD-2 (clone MTS510) was purchased from BioLegend (San Diego, CA).

PE-conjugated anti-mouse TLR4/MD-2 (clone MTS510) was purchased from eBioscience (San Diego, CA). The following antibodies were purchased from BioLegend: PE-conjugated anti-mouse CD86 (clone GL-1) and FITC-conjugated anti-mouse CD14 (clone M5/114.15.2).

**Preparation of FNC-RED**

To a stirred solution of funiculosin (25.0 mg, 50.9 μmol) in THF (400 μl), water (40 μl), and acetic acid (20 μl) was added palladium(OH)2/carbon (15.0 mg, palladium 20%, wetted with ~50% water), and the mixture was stirred under H2 (3 MPa) for 6 days at room temperature. After the reaction, palladium catalyst was removed by membrane filtration. The filtrate, after addition of toluene, was concentrated in vacuo. The residue was purified by HPLC to give FNC-RED (12.0 mg, 24.9 μmol). Column was Cosmosil 5C18-AR-300, 10 x 250 mm (Nacalai Tesque, Kyoto, Japan); mobile phase was as follows: A = H2O and B = CH3CN (B concentration, 0–30 min at 70–100%, 30–55 min at 100%); flow rate was as follows: 3.5 ml/min; detection 220 nm; retention time (FNC-RED) was 27.3 min. A full description of the syntheses of FNC-RED-P01 and FNC-RED-P01 will be published elsewhere.

**Western blotting**

Cells were washed and lysed for 60 min in iced lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, protease inhibitor mixture (Nacalai Tesque), and phosphatase inhibitor mixture (Nacalai Tesque). Lysates were subjected to SDS-PAGE and Western blotting analysis. The following antibodies for immunoblotting were purchased from Cell Signaling (Beverly, MA): anti-1xBa, anti-ERK1/2, anti-p-ERK1/2, and goat anti-rabbit IgG horseradish peroxidase. Anti-actin antibody was purchased from Sigma. Donkey anti-mouse IgG horseradish peroxidase was purchased from Chemicon International Inc. (Temecula, CA). The reactive bands were visualized by ECL Prime or ECL Select (GE Healthcare).

**Immunoprecipitation and immunoprobing**

To examine homodimerization of TLR4, Ba/F3 cells expressing murine TLR4-FLAG, murine TLR4-GFP, murine MD-2-FLAG, and murine CD14 were incubated with lipid A or FNC-RED. After 60 min, the cells were washed and lysed with lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor mixture (Nacalai Tesque). After 30 min of incubation on ice, nuclei were removed by centrifugation. Beads coupled with a rabbit anti-GFP (Invitrogen) were added to cell lysates and rotated for 2 h at 4°C. Beads were washed with lysis buffer, and bound proteins were subjected to SDS-PAGE and Western blotting with mouse anti-FLAG (Sigma) or rabbit anti-GFP (MBL, Nagoya, Japan), followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit.
Funiculosin variants activate the TLR4/MD-2 complex

antibody (Cell Signaling). The reactive bands were visualized by ECL Prime (GE Healthcare).

Cell viability assay

Cell viability assay was conducted by using a Cell Titer 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI).

Conformational analysis of ligand flexibility

Conformational samplings of FNC and FNC-RED were performed using 100-ns molecular dynamics simulations using program Desmond version 4.7 (59). The OPLS3 force field was used for the simulations. Initial model structures were placed into a TIP3P water molecule solvated with 0.15M NaCl occupying up to 50 Å from lipid molecules. After minimization and relaxation of model, the production of molecular dynamics phase was performed for 100 ns in the isothermal-isobaric (NPT) ensemble at 300 K and 1 bar using the Langevin dynamics. Long-range electrostatic interactions were computed. After simulation, 1,000 conformations were extracted from trajectory with 0.1-ns interval. Conformational flexibility of ligands was analyzed with the $R_g$ for all conformations calculated by MOE (Chemical Computing Group, Inc., Montreal, Canada).

Computational modeling of ligand-bound model of human and murine TLR4/MD-2

Our approach for docking of FNC-RED and FNC-RED-P01 to the lipid A-binding sites of human and murine TLR4/MD-2 utilized four main steps as follows: (i) molecular modeling of FNC-RED-P01 bound to human TLR4/MD-2 model by docking with the positional and hydrogen-bonding constraints; (ii) molecular modeling of FNC-RED-human TLR4/MD-2, FNC-RED-P01-murine TLR4/MD-2, and FNC-RED-murine TLR4/MD-2 complexes based on the FNC-RED-P01-human TLR4/MD-2 model; (iii) minimization of all models by molecular dynamics simulations; and (iv) estimation of ligand-binding free energy. For a small molecule, ionization and energy minimization were performed by the OPLS3 force field in the LigPrep Script in the Maestro (Schrodinger LLC, New York). These minimized structures were employed as input structures for docking simulations. Initial atomic coordinates of human and murine TLR4/MD-2 were derived from the crystal structures of lipid A-human TLR4/MD-2 complex (PDB code 3fxi) (6) and lipid IVa-murine TLR4/MD-2 complex (PDB code 3vq1) (28), respectively. First, we utilized the computational model of lipid IVa-human TLR4/MD-2 complex for docking of FNC-RED-P01 because the lipid IVa scaffold is more suitable for reference position of the docking of FNC-RED-P01 than lipid A. To incorporate the lipid IVa onto human TLR4/MD-2, the lipid IVa-murine MD-2 complex was superposed to human MD-2. The human TLR4/MD-2 structure with lipid IVa was refined for docking simulations using the Protein Preparation Wizard Script within Maestro. Docking simulations were performed using the Glide (61, 62) SP docking program (Schrodinger LLC) with hydrogen bonding constraints and positional restraint to acyl chains of lipid IVa. Hydrogen bonding constraints among the side chain charged groups of Lys-341, Lys-362 of human TLR4, Lys-388 of human TLR4*, and Lys-122 of human MD-2 were introduced because these charged residues are not conserved in the murine TLR4/MD-2 sequences. Up to 100 docking poses of FNC-RED-P01 were generated in a grid box defined by each acyl chain position (R2, R3, R2’, and R3’) of lipid IVa. In this work, docking of FNC-RED-P01 based on R2’ acyl chain position of lipid IVa was omitted from candidates due to the lack of probable poses. After the docking simulations were completed, three representative poses of FNC-RED-P01 from R2, R3, and R3’ position on the lipid A-binding site were selected by docking score and manual inspection using Maestro. Molecular modeling of FNC-RED-P01 murine TLR4/MD-2 was modeled from FNC-RED-P01 human TLR4/MD-2 model by superposition of the murine TLR4/MD-2 structure. FNC-RED-bound models for human and murine TLR4/MD-2 were generated from each FNC-RED-P01-bound model by the deletion of the phosphate group of FNC-RED-P01. All initial complex models were subjected to 1-ns molecular dynamics-based energy minimization using program Desmond version 4.7 (59), giving rise to the final three-dimensional model. The OPLS3 force field was used for the simulations. Initial model structures were placed into a TIP3P water molecules solvated with 0.15 M NaCl occupying up to 10 Å from protein molecules. After minimization and relaxation of model, the production of molecular dynamics phase was performed for 1 ns in the isothermal-isobaric (NPT) ensemble at 300 K and 1 bar using the Langevin dynamics. Long-range electrostatic interactions were computed. Finally, ligand-binding free energy of the minimized models was calculated using the MM-GBSA (Schrodinger LLC).

Statistical analysis

Statistical significance was evaluated by one-way analysis of variance followed by post-hoc Tukey test. $p < 0.05$ was considered statistically significant.

Author contributions—Y. N. conceived and designed the experiments. N. O. conducted the experiments, collected data, and analyzed data. H. H. and Y. W. conducted the experiments. K. Mizote, A. S., Y. F., and K. F. contributed the NMR and Mass analysis, and the synthesis of FNC-RED, FNC-RED-P01, and FNC-P01. R. F., N. T., Y. M., and K. Miyake contributed to establish the Ba/F3 transfectant cells, and provided Ly96⁻/⁻ mice and Cd14⁻/⁻ mice. S. A-T. contributed to construct the Ba/F3 transfectant cells. T. Y-M., U. O., and T. Kato, S. F., and T. Kimura contributed to screen TIK-139. T. H. contributed the Biacore analysis. T. Kato, S. F., and T. Kimura contributed to screen TIK-139. T. H. contributed the computational analysis. Y. N. and N. O. interpreted data and wrote the manuscript. K. T. was involved in project planning and supervision. All authors reviewed the manuscript.

Acknowledgments—We thank all members of our laboratories at the University of Toyama and Toyama Prefectural Institute for Pharmaceutical Research for helpful discussions. We are grateful to Dr. Shizuo Akira (JFRerc, Osaka University, Osaka, Japan) for providing us Tlr4⁻/⁻, Mydd88⁻/⁻, and Ticam1⁻/⁻ mice. We thank Novartis for providing us funiculosin. We also thank Dr. Paul W. Kincaide (Oklahoma Medical Research Foundation, Oklahoma City, OK) for critical review of the manuscript. We sincerely thank Toyama Prefecture for supporting our laboratory.
References

1. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384
2. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J. Exp. Med. 189, 1777–1782
3. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. 162, 3749–3752
4. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. Nat. Immunol. 3, 667–677
5. Ohno, U., Bukase, K., Miyake, K., and Satow, Y. (2007) Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. Science 316, 1632–1634
6. Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009) The structural basis of lipopolysaccharide recognition by the TLR4/MD-2 complex. Nature 458, 1191–1195
7. Kagan, J. C., and Medzhitov, R. (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 125, 943–955
8. Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. Nature 449, 819–826
9. Duthie, M. S., Windish, H. P., Fox, C. B., and Reed, S. G. (2011) Use of synthetic TLR4 agonist protects mice from pneumonic tularemia. Proc. Natl. Acad. Sci. U.S.A. 113, E884–E893
10. Honda, K., Nagai, Y., Matsunaga, T., Saijoh, S., Akashi-Takamura, S., Hayashi, H., Fujii, I., Miyake, K., Muraguchi, A., and Takatsu, K. (2012) Glycyrrhizin and isoliquiritigenin suppress the LPS sensor toll-like receptor 4/MD-2 complex signaling in a different manner. J. Leukoc Biol. 91, 967–976
11. Ando, K., Suzuki, S., Saiki, T., Tamura, G., and Arima, K. (1969) Fusicocin, a new antibiotic. I. Isolation, biological and chemical properties (studies on antiviral and antitumor antibiotics). 8. J. Antibiot. 22, 189–194
12. Ando, K., Matsuura, I., Nawata, Y., Endo, H., Sasaki, H., Okyotomi, T., Saeki, T., and Tamura, G. (1978) Fusicocin, a new antibiotic. II. Structural elucidation and antifungal activity. J. Antibiot. 31, 533–538
13. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., and Miyake, K. (2000) Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4–MD2 complex on mouse peritoneal macrophages. J. Immunol. 164, 3471–3475
14. Cooperstock, M. S. (1974) Inactivation of endotoxin by polymyxin B. Annticocb. Agents Chemother. 6, 422–425
15. Ohno, U., Bukase, K., Miyake, K., and Shimizu, T. (2012) Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. Proc. Natl. Acad. Sci. U.S.A. 109, 7421–7426
16. Shin, H. J., Lee, H., Park, J. D., Hyun, H. C., Sohn, H. O., Lee, D. W., and Kim, Y. S. (2007) Kinetics of binding of LPS to recombinant CD14, TLR4, and MD-2 proteins. Mol. Cells 24, 119–124
17. Miyake, K., Yamashita, Y., Hitoshi, Y., Takatsu, K., and Kimoto, M. (1994) Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD molecule: unresponsiveness of X-linked immunodeficient B cells. J. Exp. Med. 180, 1217–1224
18. Miyake, K., Shimazu, R., Kondo, J., Niki, T., Akashi, S., Ogata, H., Yamashita, Y., Miura, Y., and Kimoto, M. (1998) Mouse MD-1, a molecule that is physically associated with RP105 and positively regulates its expression. J. Immunol. 161, 1348–1353
19. Ogata, H., Su, I., Miyake, K., Nagai, Y., Akashi, S., Mecklenbräuker, I., Rajewsky, K., Kimoto, M., and Tarakhovsky, A. (2000) The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. J. Exp. Med. 192, 23–29
20. Nagai, Y., Shimazu, R., Ogata, H., Akashi, S., Sudo, K., Yamasaki, H., Hayashi, S., Iwakura, Y., Kimoto, M., and Miyake, K. (2002) Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. Blood 99, 1699–1705
21. Banchereau, J., and Steinman, R. M. (1998) Dendritic cells and the control of immunity. Nature 392, 245–252
22. Descigno, M., Winzler, C., Delia, D., Mutini, C., Lutz, M., and Ricciardi-Castagnoli, P. (1997) Dendritic cell maturation is required for initiation of the immune response. J. Leukoc Biol. 61, 415–421
23. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) Role of adaptor TRIF in the MyD88-dependent toll-like receptor signaling pathway. Science 301, 640–643
24. Fenton, M. J., and Golenbock, D. T. (1998) LPS-binding proteins and receptors. J. Leukoc Biol. 64, 25–32
25. Wurfl, M. M., Monks, B. G., Ingalls, R. R., Dedrick, R. L., Delude, R., Zhou, D., Lamping, N., Schumann, R. R., Thieringer, R., Fenton, M. J., Wright, S. D., and Golenbock, D. (1997) Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS
Funiculosin variants activate the TLR4/MD-2 complex

responses ex vivo, whereas in vivo responses remain intact. J. Exp. Med. 186, 2051–2056.

39. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433.

40. Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kosugi, A., and Miyake, K. (2003) Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. J. Exp. Med. 198, 1035–1042.

41. Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F., and Kagan, J. C. (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. Cell 147, 868–880.

42. Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Kobayashi, M., Konno, K., Matsumoto, F., Fukase, K., Kusumoto, S., Nagai, Y., Kusumoto, Y., Kosugi, A., and Miyake, K. (2004) Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int. Immunol. 16, 961–969.

43. Brasseur, G., and Brivet-Chevillotte, P. (1994) Specificities of the two core N inhibitors of mitochondrial bc1 complex, antimycin and funiculosin: strong involvement of cytochrome b-asparagine-208 in funiculosin binding. FEBS Lett. 354, 23–29.

44. Hallman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994) Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. 179, 269–277.

45. Ryu, J. K., Kim, S. J., Rah, S. H., Kang, J. I., Jung, H. E., Lee, D., Lee, H. K., Lee, J. O., Park, B. S., Yoon, T. Y., and Kim, H. M. (2017) Reconstruction of LPS transfer cascade reveals structural determinants within LBP, CD14, and TLR4-MD2 for efficient LPS recognition and transfer. Immunity 46, 38–50.

46. Kim, J. I., Lee, C. J., Jin, M. S., Lee, C. H., Paik, S. G., Lee, H., and Lee, J. O. (2005) Crystal structure of CD14 and its implications for lipopolysaccharide signaling. J. Biol. Chem. 280, 11347–11351.

47. Kelley, S. L., Luik, T., Nair, S. K., and Tapping, R. I. (2013) The crystal structure of human soluble CD14 reveals a bent solenoid with a hydrophobic amino-terminal pocket. J. Immunol. 190, 1304–1311.

48. Arditi, M., Zhou, J., Torres, M., Durden, D. L., Stins, M., and Kim, K. S. (1995) Lipopolysaccharide stimulates the tyrosine phosphorylation of mitogen-activated protein kinases p44, p42, and p41 in vascular endothelial cells in a soluble CD14-dependent manner. Role of protein tyrosine phosphorylation in lipopolysaccharide-induced stimulation of endothelial cells. J. Immunol. 155, 3994–4003.

49. Perera, P. Y., Mayadas, T. N., Takeuchi, O., Akira, S., Zaks-Zilberman, M., Goyert, S. M., and Vogel, S. N. (2001) CD11b/CD18 acts in concert with CD14 and toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression. J. Immunol. 166, 574–581.

50. Jürgens, G., Müller, M., Gariel, P., Koch, M. H., Nakakubo, H., Blume, A., and Brandenburg, K. (2002) Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A. J. Endotoxin Res. 8, 115–126.

51. Esparza, G. A., Teghanemt, A., Zhang, D., Gioannini, T. L., and Weiss, J. P. (2012) Endotoxin-albumin complexes transfer endotoxin monomers to MD-2 resulting in activation of TLR4. Innate Immun. 18, 478–491.

52. Kaca, W., Roth, R. I., and Levin, J. (1994) Hemoglobin, a newly recognized lipopolysaccharide (LPS)-binding protein that enhances LPS biological activity. J. Biol. Chem. 269, 25078–25084.

53. Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. (2010) The sterile inflammatory response. Annu. Rev. Immunol. 28, 321–342.

54. Niu, Q. M., Shiyy, K., Teijaro, J. R., Farber, D. L., Netzel-Arnett, S., Antalis, T. M., Fasano, A., and Vogel, S. N. (2010) Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors in vitro and in vivo. Mucosal Immunol. 3, 29–39.

55. Shirey, K. A., Lai, W., Scott, A. J., Lipsky, M., Mistry, P., Pletnev, L. M., Karp, C. L., McAlees, J., Gioannini, T. L., Weiss, J., Chen, W. H., Ernst, R. K., Rossignol, D. P., Gussovsky, F., Blanco, J. C., and Vogel, S. N. (2013) The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature 497, 498–502.

56. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115–122.

57. Inaba, K., Inaba, M., Romanı, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176, 1693–1702.

58. Ohoto, U., Miyake, K., and Shimizu, T. (2011) Crystal structures of mouse and human RP105/MD-1 complexes reveal unique dimer organization of the toll-like receptor family. J. Mol. Biol. 413, 815–825.

59. Bowers, K. J., Chow, E., Xu, H., Dör, R. O., Eastwood, M. P., Gregersen, B. A., Klepeis, J. L., Kolossvary, I., Moraes, M. A., Sacerdotti, F. D., Salmon, J. K., Shan, Y., and Shaw, D. E. (2006) In Proceedings of the 2006 ACM/IEEE Conference on Supercomputing (SC06), Tampa, FL (Horner-Miller, J. K., Shan, Y., and Shaw, D. E. (2006) in Proceedings of the 2006 ACM/IEEE Conference on Supercomputing (SC06), Tampa, FL (Horner-Miller, B., ed) 115–122.

60. Saxty, G. M., Adzhigirey, M., Day, T., Annabimoju, R., and Sherman, W. (2013) Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. J. Comput. Aid Mol. Des. 27, 221–234.

61. Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., Perry, J. K., Shaw, D. E., Francis, P., and Shenkin, P. S. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J. Med. Chem. 47, 1739–1749.

62. Halgren, T. A., Murphy, R. B., Friesner, R. A., Beard, H. S., Frye, L. L., Pollard, W. T., and Banks, J. L. (2004) Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J. Med. Chem. 47, 1750–1759.