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Core fucose is critical for CD14-dependent Toll-like receptor 4 signaling

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Abstract

Core fucosylation, a posttranslational modification of *N*-glycans, modifies several growth factor receptors and impacts on their ligand binding affinity. Core-fucose-deficient mice generated by ablating the α1,6 fucosyltransferase enzyme, Fut8, exhibit severe pulmonary emphysema, partly due to impaired macrophage function, similar to aged *Toll-like receptor 4* (*Tlr4*)–deficient mice. We therefore suspect that a lack of core fucose affects the TLR4-dependent signaling pathway. Indeed, upon lipopolysaccharide stimulation, *Fut8*-deficient mouse embryonic fibroblasts (MEFs) produced similar levels of interleukin-6 but markedly reduced levels of interferon-β (IFN-β) compared with wild-type MEFs. Lectin blot analysis of the TLR4 signaling complex revealed that core fucosylation was specifically found on CD14. Even though similar levels of TLR4/myeloid differentiation factor 2 (MD2) activation and dimerization were observed in *Fut8*-deficient cells after lipopolysaccharide stimulation, internalization of TLR4 and CD14 was significantly impaired. Given that internalized TLR4/MD2 induces IFN-β production, impaired IFN-β production in *Fut8*-deficient cells is ascribed to impaired TLR4/MD2 internalization. These data show for the first time that glycosylation critically regulates TLR4 signaling.

Key words: CD14, core fucose, endocytosis, IFN-β, TLR4

Introduction

Core fucose, α1,6-linked fucosylation of the innermost *N*-acetylglucosamine residue of N-linked glycan, is transferred by the fucosyltransferase, Fut8. Even though most glycosyltransferase-deficient mice exhibit limited phenotypes (Orr et al. 2013), embryonic lethality has been observed in *Fut8*-deficient mice in the C57BL/6 parental background (Wang et al. 2005). This indicates a critical role for core fucose. Indeed, ligand binding affinity of transforming growth factor-β1 receptor (Wang et al. 2005), epidermal growth factor receptor (Wang et al. 2006) and integrin α3β1 (Zhao et al. 2006) is significantly reduced in the absence of core fucose, leading to impaired downstream signaling. Core fucosylation of the T-cell receptor is

also required for its proper signaling activity (Fujii et al. 2016). *Fut8*-deficient mice in the ICR genetic background survive but exhibit schizophrenia-like abnormalities (Fukuda et al. 2011). Furthermore, deletion of core fucose from immunoglobulin G1 (IgG1) markedly improves its binding affinity to Fcγ receptor IIIa, leading to a more than 50-fold increase in antibody-dependent cell-mediated cytotoxicity (Shinkawa et al. 2003; Okazaki et al. 2004).

Accumulating data show the up-regulation of *Fut8* mRNA or core fucosylated proteins in several malignant tumors (Block et al. 2005; Hutchinson et al. 1991; Muinelo-Romay et al. 2008; Takahashi et al. 2000), and a pathological role of core fucose has also been extensively studied. Core fucosylation of E-cadherin affects cell-cell adhesion of cancer cells (Osumi et al. 2009) and knockdown of *Fut8* inhibits metastasis of lung cancers (Chen et al. 2013) and epithelial-mesenchymal transition (Lin et al. 2011), which is often activated during cancer invasion and metastasis (Mani et al. 2008; Thiery 2002).

Under a mixed C57BL/6 and 129SvJ strain background, 30% of *Fut8*-deficient mice survive but exhibit an emphysema-like phenotype (Gao et al. 2012; Wang et al. 2005). Interestingly, TLR4 deficiency also causes pulmonary emphysema in aged mice (Zhang et al. 2006). Moreover, TLR4 receptor complexes are composed of heavily N-glycosylated proteins; the numbers of potential N-glycosylation sites of TLR4, myeloid differentiation factor 2 (MD2) and CD14 are 9, 2 and 4, respectively (da Silva Correia and Ulevitch 2002; Meng et al. 2008). These observations raise the possibility that a lack of core fucose can affect TLR4-dependent signaling. TLR4, a member of the TLR family, is expressed in myeloid lineage cells and some nonimmune cells, such as endothelial cells, and plays an instrumental role in innate immune responses against microbial pathogens (Kawai and Akira 2006; Plociennikowska et al. 2015). Since the discovery that TLR4 is the receptor of lipopolysaccharide (LPS), a component of Gram-negative bacteria, and that TLR4 induces the production of pro-inflammatory mediators (Beutler et al. 2001; Poltorak et al. 1998), extensive studies have identified several key molecules in the TLR4 complex. First, TLR4 needs to associate with myeloid MD2 to be delivered to the plasma membrane to receive LPS (Nagai et al. 2002). Second, binding of LPS facilitates the dimerization of the TLR4/MD2 complex (Park et al. 2009), leading to the recruitment of two pairs of adaptor proteins, toll-interleukin 1 receptor (TIR) domain containing adaptor protein/myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF)/TRIF-related adaptor molecule. Recent evidence shows that TLR4 mediates two distinctive signaling pathways: the "MyD88-dependent" and "TRIF-dependent" pathways (Kagan et al. 2008; Kawai and Akira 2010; Tanimura et al. 2008). MyD88 recruits interleukin-1 receptor-associated kinases, and these multimolecular complexes trigger a signaling cascade, leading to early-phase activation of nuclear factor κB (NFκB) and mitogen-activated protein (MAP) kinases that controls the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Meanwhile, TRIF activates the interferon regulatory factor 3 (IRF3) transcription factor, leading to the expression of type I interferons (IFN) and IFN-inducible chemokines, such as IL-10 and regulated on activation, normal T cell expressed and secreted (RANTES); late-phase activation of NFκB and MAP kinases then follows. The MyD88-dependent pathway occurs at the plasma membrane. Internalization of TLR4 is necessary for the TRIF-dependent pathway (Zanoni et al. 2011). Therefore, availability of the TLR4 signaling complex at either the plasma membrane or endosomes determines the production level of pro-inflammatory cytokines and IFN-β. CD14, originally identified as an LPS binding molecule (Wright et al. 1990), has been recently shown to be required for TLR4 endocytosis, thereby critically regulating the TRIF-dependent pathway (Zanoni et al. 2011).

In this study, we found that core fucosylation is critical for endocytosis of CD14 and TLR4 and that the TRIF-dependent TLR4 signaling pathway is significantly impaired in the absence of core fucose.

Results

TRIF-dependent TLR4 signaling is impaired in Fut8$^{-/-}$ cells

We first examined whether or not the absence of core fucose could affect the TLR4 signaling pathway. *Fut8*-deficient mice exhibit embryonic lethality (Wang et al. 2005), and MEFs have been used to elucidate the mechanism of TLR4 activation (Kurt-Jones et al. 2004); therefore, we decided to use MEFs derived from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ embryos. To determine LPS responsiveness, we first stimulated MEFs with LPS and performed enzyme-linked immunosorbent assay (ELISA)-based measurement of the production of type I IFN and a pro-inflammatory cytokine, IL-6, to observe the TRIF-dependent and MyD88-dependent pathways, respectively. There was no difference in IL-6 secretion between *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells when stimulated with LPS (Figure 1A). In contrast to *Fut8*$^{+/+}$ MEFs, which produce IFN-β after LPS stimulation, *Fut8*$^{-/-}$ MEFs produced undetectable levels of IFN-β, indicating impairment of TRIF-dependent TLR4 signaling in the absence of core fucose. We also measured *IL-6* and *IFN-β* transcripts following LPS stimulation in the two types of cells and found a marked reduction of *IFN-β* mRNA in *Fut8*$^{-/-}$ cells (Figure 1B).

The MyD88-dependent pathway activates NF-κB via the phosphorylation of inhibitory κB (IκB) proteins. The TRIF-dependent pathway leads to IRF3 activation and *IFN-β* transcription. We therefore assayed IκBα and IRF3 in these cells following LPS stimulation (Kawai et al. 2001). We found similar level of IκBα phosphorylation in the two cell types, while phosphorylation of IRF3 in *Fut8*$^{-/-}$ cells was markedly reduced (Figure 1C), confirming our finding that *Fut8* deficiency leads to the impairment of TRIF-dependent TLR4 signaling.

CD14 is core fucosylated

To reveal the presence of core fucose in the *N*-glycans on TLR4 and its related molecules, we performed glycosidase treatment and a series of lectin blot analyses. TLR4-FLAG, MD2-Myc and CD14 were each expressed in wild-type human embryonic kidney (HEK) 293 T cells or in *FUT8*-deficient cells, as a negative control. First, peptide: *N*-glycanase (PNGase) F treatment caused a clear mobility shift of TLR4-FLAG, MD2-Myc and CD14 (Figure 2A), indicating that all of these proteins were modified with *N*-glycans. Next, these proteins were probed with several types of lectins. *Aleuria aurantia* lectin (AAL), which preferentially recognizes core fucose, detected CD14 in *FUT8*$^{+/+}$ but not in *FUT8*$^{-/-}$ HEK293T cells, indicating that selective core fucosylation specifically occurs on CD14 (Figure 2B). CD14 was also reactive with wheat germ agglutinin (WGA), which reacts with sialylated complex-type *N*-glycans. Since neither AAL nor WGA reacted with TLR4 and MD2, these both seem to have oligomannose-type *N*-glycans. None of the glycoproteins tested reacted with *Ulex europaeus* agglutinin-I (UEA-I), which detects ABO blood-type fucosylated glycans. Just under the CD14 signal, anti-CD14 IgG, used for immunoprecipitation, was detected with the AAL and WGA lectins. These results suggest that the lack of

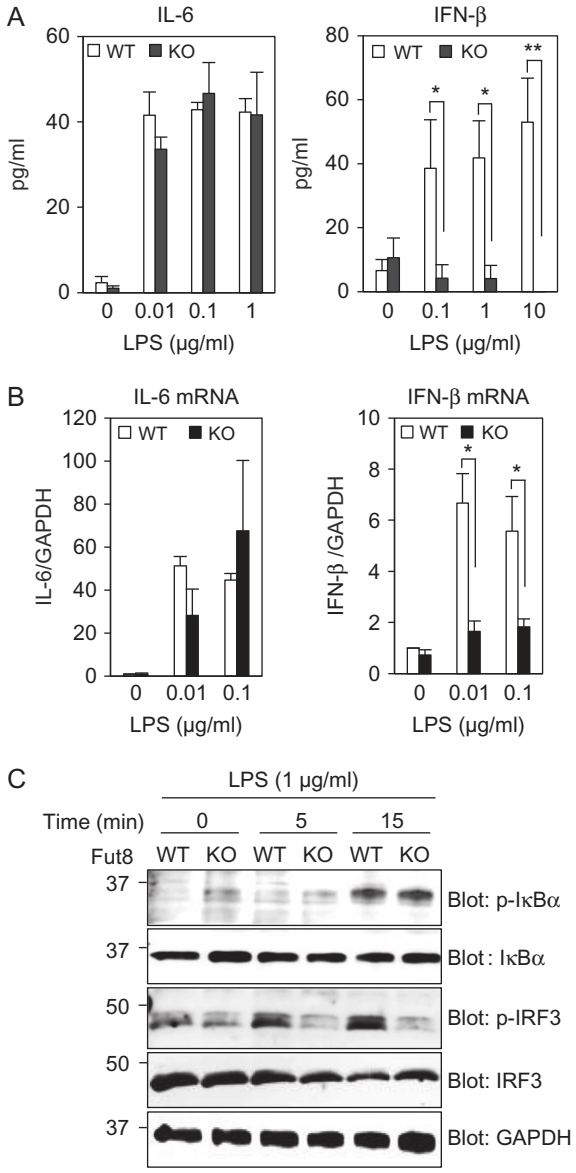

Fig. 1. Loss of core fucose led to impaired IFN-β production. (**A**) *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were treated with 0.01–10 μg/mL LPS for 16–20 h, and the levels of IL-6 and IFN-β in the culture media were measured by ELISA. *$P < 0.05$; **$P < 0.01$. $n = 4$. (**B**) *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were treated with 0.01–0.1 μg/mL LPS for 2 h, and the levels of *IL-6*, *IFN-β* and *GAPDH* mRNA were measured by real-time qPCR. The relative expression levels to *GAPDH* are shown as mean ± standard error of the mean (SEM) of three independent experiments. (**C**) *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were treated with 1 μg/mL LPS for 0, 5 or 15 min, then the cell lysates were analyzed by western blotting to detect IRF3, its phosphorylated form, or GAPDH (representative results of three repeats are shown). WT, wild-type; KO, knock-out, error bars, SEM.

core fucose on CD14 could affect TLR4 complex formation or reactivity to LPS, leading to alteration of TLR4 signaling.

TLR4/MD2 complex formation overcomes the absence of core fucose

We next investigated the underlying mechanism of impaired TRIF-dependent TLR4 signaling in the absence of core fucose. MD2 is essential for TLR4 to respond to LPS (Nagai et al. 2002). We first assessed whether TLR4/MD2 complex formation was affected by the absence of core fucose. We used two distinct anti-TLR4 antibodies: Sa15-21 detects all TLR4, while MTS510 specifically recognizes the TLR4/MD2 complex (Figure 3A) (Akashi et al. 2003; Akashi-Takamura et al. 2006). For both antibodies, cell-surface and total TLR4 expression were measured using intact and membrane-permeable conditions for flow cytometric analysis. Analysis with Sa15-21 showed that cell-surface TLR4 levels were 27.3 ± 2.0% lower in *Fut8*$^{-/-}$ cells than in *Fut8*$^{+/+}$ MEFs, while total TLR4 levels were similar between two cell types (Figure 3B). This result indicates that core fucosylation partly affects the transport of TLR4 to the cell surface. In contrast, analysis with MTS510 showed that both cell-surface and total levels of the TLR4/MD2 complex were similar between these cells (Figure 3C). Taking into consideration a recent report that MD2 is dispensable for TLR4 localization at the cell surface (Wakabayashi et al. 2006), a significant proportion of the TLR4 present at the cell surface could be expected to be free from MD2, and therefore detectable with Sa15-21 but not MTS510. Hence, our results indicate that the abundance of MD2-free TLR4 at the cell surface is reduced in the absence of core fucose. Moreover, membrane-permeabilization increased the intensity of Sa15-21 but not MTS510 staining, indicating that MD2-free TLR4 is also present in the intracellular compartment.

Next, TLR4/MD2 complex formation in *FUT8*$^{-/-}$ HEK293T cells was biochemically compared with their parental *FUT8*$^{+/+}$ cells. To do this, both TLR4-FLAG and MD2-Myc were co-expressed in the two cell types. We found that TLR4-FLAG co-pulled down comparable levels of MD2 in the two cell types (Figure 3D). In the immunoprecipitates with anti-FLAG, 25 kDa signals were seen in all lanes stained with anti-Myc; these signals were considered to be artifacts arising from staining of the light chain of the IgG used for immunoprecipitation. These results confirmed that TLR4/MD2 complex formation is not affected by the absence of core fucose, even though transport of TLR4 to the cell surface is partly impaired.

Lack of core fucose does not impair the LPS evoked dimerization of TLR4/MD2 complex

Next, to compare the binding or incorporation of LPS between *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells, we measured the association of fluorescein isothiocyanate (FITC)-labeled LPS in these cells by flow cytometry analysis. To judge the TLR4-specific LPS binding, *TLR4*$^{-/-}$ cells were also analyzed. Thirty minutes after the addition of 5 μg/mL FITC-LPS, similarly higher levels of LPS interaction were observed in *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells, as compared with *TLR4*$^{-/-}$ cells (Figure 4A). The binding/incorporation of FITC-LPS increased over 30 min in all cell types (Figure 4B) (Kondo et al. 2013). LPS binding to both cell types was also confirmed by subsequent dimerization of the TLR4/MD2 complex, which is critical for TLR4 activation to transduce downstream signals (Jin and Lee 2008). To see the effect of core fucose on LPS-stimulated TLR4 dimerization, TLR4-FLAG, TLR4-green fluorescent protein (GFP) and MD2-Myc were expressed together in *FUT8*$^{+/+}$ or *FUT8*$^{-/-}$ HEK293T cells. Without LPS stimulation, TLR4 dimerization was negligible in both cell types. After LPS stimulation, the TLR4-FLAG/TLR4-GFP complex was observed by co-immunoprecipitation, indicating that LPS treatment led to comparable levels of TLR4 dimerization in *FUT8*-deficient cells and control cells (Figure 4C). Taken together, these results indicate that LPS-evoked TLR4 signaling complex formation was not affected by the absence of core fucose.

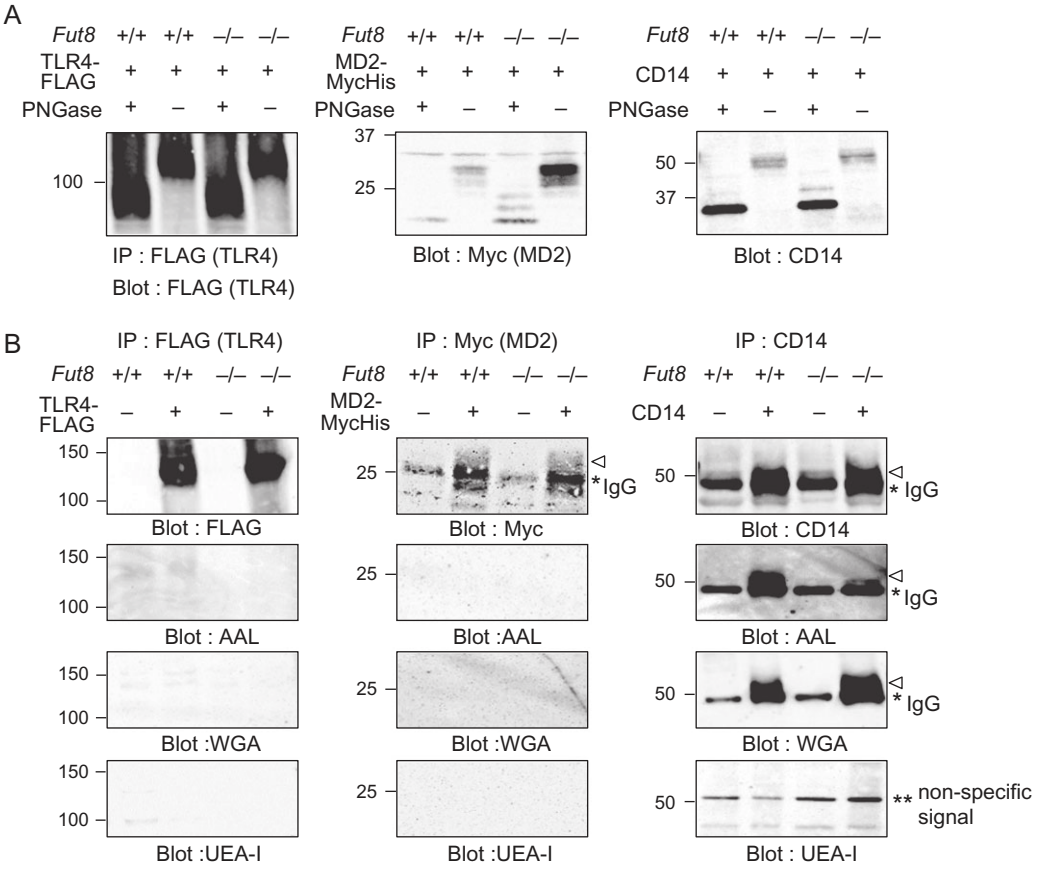

Fig. 2. (**A**) Cell lysates from *FUT8*$^{+/+}$ or *FUT8*$^{-/-}$ HEK293T cells, over-expressing either TLR4-FLAG (Left), MD2-Myc (Middle) or CD14 (Right), were treated with PNGase, and analyzed by western blotting. TLR4-FLAG was enriched by immunoprecipitation with anti-FLAG. (**B**) TLR4 and CD14 are core fucosylated. Cell lysates from *FUT8*$^{+/+}$ or *FUT8*$^{-/-}$ HEK293T cells, over-expressing either TLR4-FLAG (Left), MD2-Myc (Middle) or CD14 (Right), were used for immunoprecipitation with anti-FLAG, Myc or CD14 antibody, respectively. Immunoprecipitated proteins, shown by arrowheads, were then detected with the same antibodies, or with fucose-specific AAL lectin. *IgG heavy or light chain. IgG light chain is derived from the antibody used for immunoprecipitation. **Nonspecific signal. IP, immunoprecipitation; WB, western blot.

LPS-evoked loss of cell-surface TLR4 and CD14 is attenuated in Fut8$^{-/-}$ cells

Because CD14 is core fucosylated, a lack of core fucose could affect the regulatory activity of CD14 on TLR4 signaling. CD14 has recently been shown to be essential for LPS-induced TLR4 endocytosis (Zanoni et al. 2011). Therefore, intact and membrane-permeable conditions were used in flow cytometric analyses to measure cell-surface and total CD14 expression, respectively. We first observed that levels of cell-surface CD14 were reduced by 24.4 ± 3.2% in *Fut8*$^{-/-}$ MEFs, compared with those in *Fut8*$^{+/+}$ MEFs, while the levels of total CD14 were similar in the two cell types (Figure 5A). This result indicates impaired CD14 transport to the cell surface in *Fut8*$^{-/-}$ cells. Next, the Sa15-21 and MTS510 antibodies were again used to characterize the cell-surface dynamics of the TLR4 signaling complex before and after LPS stimulation in these cells. It should be noted that MTS510 recognizes the TLR4/MD2 complex, but LPS binding to this complex leads to a conformational change that impairs MTS510 binding (Akashi et al. 2003). We first observed that Sa15-21 binding was reduced in *Fut8*$^{+/+}$ cells upon LPS stimulation, indicating a loss of cell-surface TLR4. In contrast, the reduction of Sa15-21 binding was not observed in *Fut8*$^{-/-}$ cells (Figure 5B and C). This result indicates that the lack of core fucose impairs the loss of cell-surface TLR4 upon LPS stimulation. In both cell types, MTS510 binding was decreased after LPS stimulation, confirming that LPS binding to TLR4/MD2 occurs and interferes with MTS510 binding. The higher levels of the TLR4/MD2 complex observed in *Fut8*$^{-/-}$ cells may be a result of attenuated loss of cell-surface TLR4 causing higher levels of cell-surface TLR4 residency. Furthermore, LPS stimulation led to the reduction of surface CD14 expression in *Fut8*$^{+/+}$ cells, while such a reduction was absent in *Fut8*$^{-/-}$ cells. Taken together, these results suggest that a lack of core-fucose results in impaired endocytosis of CD14 and TLR4, leading to impaired CD14-dependent TLR4 signaling. Alternatively, a lack of core fucose might reduce the cell-surface levels of CD14 and TLR4 by affecting their intracellular transport, turnover and CD14 shedding, also resulting in altered TLR4 signaling. Since similar transferrin uptake was observed in *Fut8*$^{-/-}$ cells and *Fut8*$^{+/+}$ cells (Figure 5D), the machinery generally controlling endocytosis was not impaired by *Fut8* deficiency.

Discussion

How glycosylation of the TLR4 complex affects its signaling is poorly understood because most crystal structure studies of the related molecules have been conducted after enzymatic removal of *N*-glycans. In several limited studies, the role of *N*-glycans in the TLR4 complex has been investigated mainly by mutagenesis approaches to remove

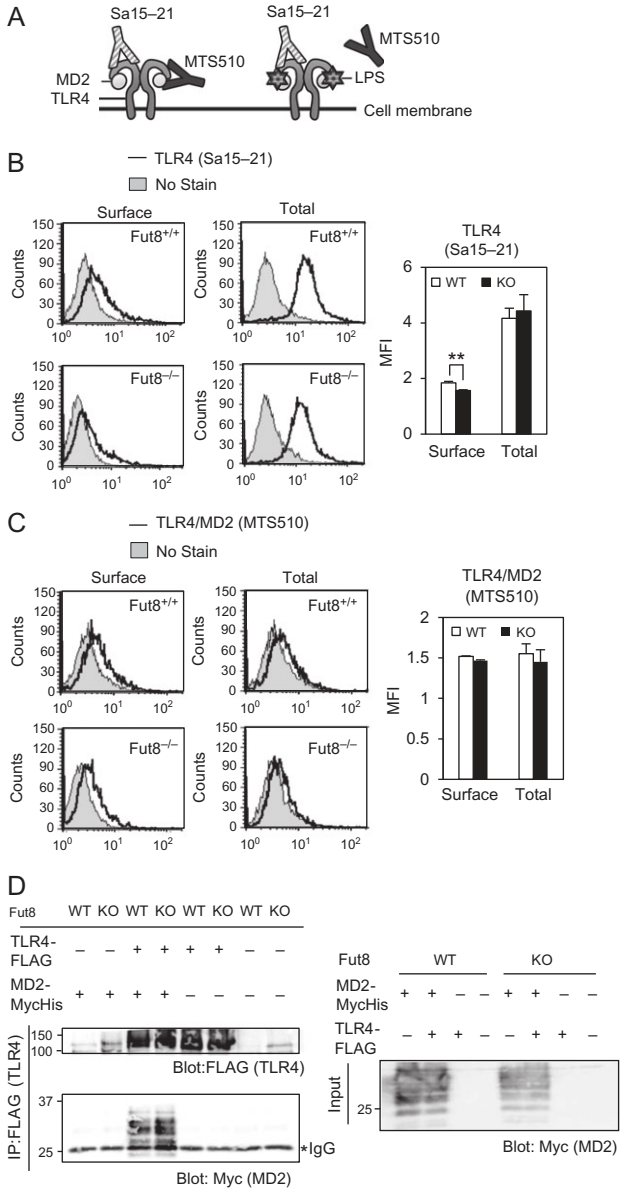

Fig. 3. TLR4/MD2 complex was expressed on the cell surface in the absence of core fucose. (**A**) Two anti-TLR4 antibodies were used in this study. Sa15-21 detects all TLR4. MTS510 specifically recognizes the TLR4/MD2 complex, and only in the absence of LPS binding. (**B**,**C**) Cell-surface and total levels of TLR4 or TLR4/MD2 complex on *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were assessed by flow cytometry using the anti-TLR4 antibodies, Sa15-21 (**B**) and MTS510 (**C**). In the right panels, expression levels per cell are shown as the mean fluorescence intensity (MFI) relative to unstained controls ± SEM of three independent experiments (error bars). (**D**) Lysates of *FUT8*$^{+/+}$ or *FUT8*$^{-/-}$ HEK293T cells, over-expressing mouse TLR4 and MD2, were used for immunoprecipitation with anti-FLAG antibody. TLR4-FLAG and its co-immunoprecipitated MD2 were analyzed by western blotting. *IgG light chain, derived from the antibody for immunoprecipitation.

N-glycosylation sites. Removal of N-glycosylation sites from MD2 does not affect its cell-surface residency but results in impaired TLR4 responsiveness to LPS, while several TLR4 N-glycosylation mutants fail to reach the cell surface (da Silva Correia and Ulevitch 2002). Alanine replacement of a particular N-glycosylation site on CD14 greatly reduces its secretion (Meng et al. 2008). These studies suggest that N-glycosylation of TLR4, MD2 and CD14 could regulate their functionality. In this study, we show that core fucose critically regulates CD14-dependent TLR4 signaling. The reduction of cell-surface TLR4 and CD14 upon LPS stimulation was attenuated in the *Fut8*$^{-/-}$ cells, strongly suggesting impaired endocytosis. Core fucosylation is generally indispensable for intracellular transport and secretion of glycoproteins. For instance, fucosylated α-fetoprotein (Taketa et al. 1993) and haptoglobin (Okuyama et al. 2006) in plasma are early detection markers for hepatocellular carcinoma and pancreatic cancer, respectively, but their nonfucosylated forms are also abundantly found in plasma. A glycosylation-deficient extracellular superoxide dismutase mutant fails to be secreted, but core fucosylation is not critical for secretion (Ota et al. 2016). We speculate that the lack of core fucose on CD14 causes inadequate association with the TLR4/MD2 complex, leading to impaired endocytosis. In order to clarify the general role of core fucosylation in the TLR4 signaling pathway, we compared the production of IL-6 and IFN-β upon LPS stimulation between *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ splenic monocytes, and found almost no difference. Furthermore, as compared with wild-type mouse macrophage RAW 264 cells, the mutant *Fut8*$^{-/-}$ cells produced comparable levels of cytokines upon LPS stimulation. Therefore, the regulatory role of core fucose could be cell-type specific. One of the possible explanations of core fucose-independent TLR4 signaling in macrophages is that a low level of Fut8 expression was observed in macrophages and monocytes. Considering recent reports regarding the important contribution of core fucose to endothelial functionality (Wang et al. 2009) and the importance of the TRIF-dependent TLR4 signaling pathway in endothelial cells (Lloyd-Jones et al. 2008), core fucosylation could possibly regulate the endothelial response to LPS. Even though serum soluble CD14 is considered to be a marker for macrophage activation and a mediator of bacterial LPS action (Lien et al. 1998), an important role of membrane-bound CD14 in the endothelial response to LPS is now recognized (Lloyd-Jones et al. 2008).

Biomarker studies in various cancers, such as hepatocellular carcinoma (Jia et al. 2009; Nakagawa et al. 2006; Yin et al. 2015) and pancreatic cancer (Tan et al. 2015) have identified about 300 serum glycoproteins to be core fucosylated. However, limited information is available for membrane-bound glycoproteins modified with core fucose. In this study, we show that CD14 has core fucose, which is dispensable for LPS-evoked TLR4/MD2 dimerization but critical for subsequent internalization of TLR4 and CD14 (Figure 6). Although the sensitivity of anti-TLR4 and CD14 antibodies may differ, the AAL lectin blots consistently showed a strong CD14 signal, suggesting a higher level of core fucosylation in CD14. Because the internalization of transferrin appears to be normal in *Fut8*$^{-/-}$ cells, it seems unlikely that the absence of core fucose has a general effect on the endocytosis machinery. There are several possible underlying mechanisms to explain how the lack of core fucose impairs the endocytosis of TLR4 and CD14. A recent report showed that the extracellular region of CD14 is important for LPS responsiveness (Matsumiya et al. 2007). It is also known that core fucosylation affects protein conformation. For instance, removal of core fucose alters the conformation in limited regions of IgG1 to enhance IgG1 binding to Fcγ receptor IIIa, resulting in enhanced antibody-dependent cell-mediated cytotoxicity (Matsumura et al. 2007). It is conceivable that core fucosylation could define the optimum interaction between CD14 and the active TLR4/MD2 dimer. How glycosylphosphatidylinositol-anchored proteins, such as CD14, regulate membrane-bound receptor endocytosis is poorly understood, but is an important question to be clarified.

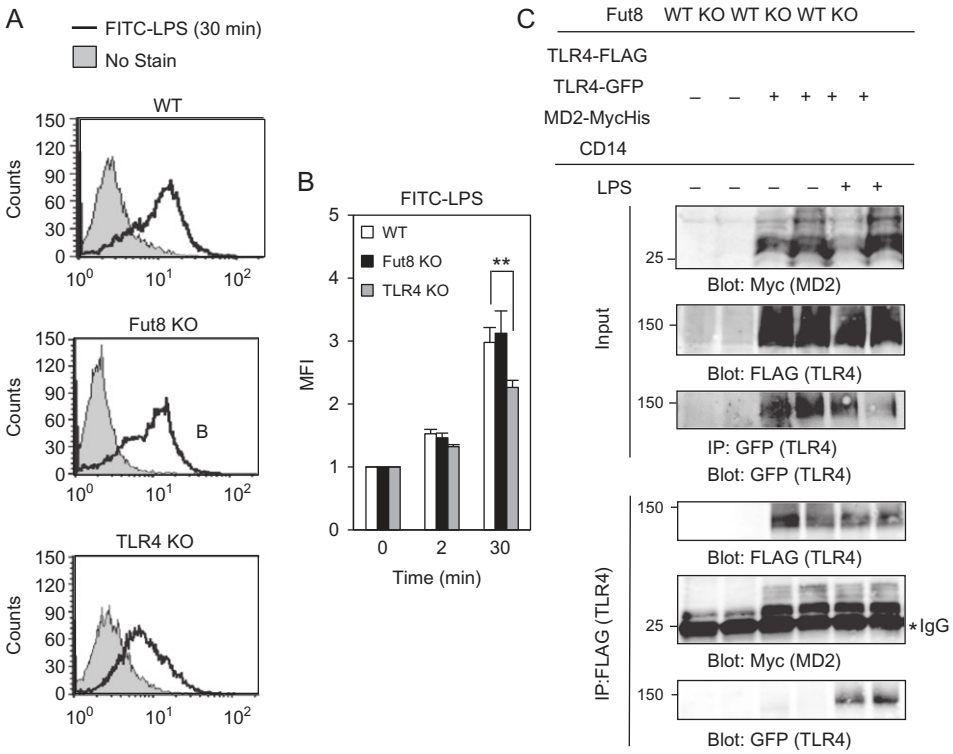

Fig. 4. Loss of core fucosylation did not affect TLR4 binding to LPS or subsequent TLR4/MD2 dimerization. (**A**) *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were stimulated with FITC-conjugated LPS (5 μg/mL). Representative histograms of cell-surface FITC at 30 min after stimulation are shown, assessed by flow cytometry. (**B**) *Fut8*$^{+/+}$, *Fut8*$^{-/-}$ or *TLR4*$^{-/-}$ MEFs were stimulated with FITC-conjugated LPS (5 μg/mL) for 0, 2 or 30 min. Cell-surface FITC levels are then shown as the mean fluorescence intensity (MFI) relative to cells without stimulation ± SEM of three independent experiments (error bars). (**C**) *FUT8*$^{+/+}$ or *FUT8*$^{-/-}$ HEK293T cells were co-transfected with mouse TLR4-FLAG, TLR4-GFP, MD2-MycHis or CD14 vectors. After treatment with or without LPS (1 μg/mL) for 20 min, the cell lysates were used for immunoprecipitation with anti-FLAG (M2) antibody. TLR4-FLAG and its co-precipitated proteins were analyzed by western blotting using anti-FLAG (for TLR4-FLAG), anti-GFP (for TLR4-GFP) or anti-Myc (for MD2-Myc) antibodies. To detect TLR4-GFP in the cell lysates (input), anti-GFP antibody was use for immunoprecipitation. *IgG light chain, derived from the antibody for immunoprecipitation. The 27 kDa IgG signal overlaps with the MD2 signal.

Accumulating evidence shows that glycosylation affects the intracellular localization of functional glycoproteins (Kitazume et al. 2010; Kizuka et al. 2015; Pinho and Reis 2015). Our study sheds light on the overlooked regulatory role of glycosylation on microbe-induced receptor trafficking.

Materials and methods

Materials

LPS (*Escherichia coli*, 055:B5) and FITC-conjugated LPS were from Sigma-Aldrich. Protein molecular weight standards were from Bio-Rad, and all other chemicals from Sigma-Aldrich or Wako Chemicals. Protein concentration was determined with a BCA protein assay (Thermo Fisher). Commercially available antibodies used were as follows: mouse monoclonal anti-Flag (M2, Sigma-Aldrich), anti-GFP (mFX75, Wako Chemicals or 9F9.F9, abcam), anti-CD14 (4B4F12, abcam or Sa2-8, eBioscience), anti-c-Myc (9E10, Invitrogen), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Chemicon), rabbit monoclonal anti-IRF3 (D83B9, Cell Signaling), anti-phospho-IRF3 (4D4G, Cell Signaling), R-phycoerythrin-conjugated rat monoclonal anti-mouse TLR4/MD2 complex (MTS510, eBioscience), anti-mouse TLR4 (Sa15-21, BioLegend), rat IgG2a (R2A04, Invitrogen) and rat IgG2aκ (RTK2758, BioLegend) isotype controls and their FITC-conjugates, and FITC-conjugated rat anti-mouse CD14 (Sa14-2, BioLegend). Biotinylated WGA, AAL and UEA-I lectins were from J-OIL MILLS.

Cell culture

Wild-type and *FUT8*$^{-/-}$ HEK293T cells were kindly provided by Dr. Jianguo Gu (Tohoku Pharmaceutical University) (Gu et al. 2015). MEFs derived from wild-type and *Fut8*$^{-/-}$ mice (C57BL/6 genetic background) were established as described in a previous study (Wang et al. 2005). HEK293T cells and MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin and streptomycin at 37°C in a 5% CO_2-humidified atmosphere.

Constructs and transfection

Full-length mouse MD2 from pMXp (Akashi et al. 2003) was cloned into pcDNA3.1 (Invitrogen) to be tagged with the MycHis epitope at the C-terminus, (MD2-MycHis). Full-length mouse TLR4 from pMXp was cloned into C-terminal p3XFLAG-CMV14 (Sigma) or pAcGFP-N3 (Clontech) to be tagged with the three tandem Flag epitopes (TLR4-FLAG) or GFP at the C-terminus (TLR4-GFP), respectively. Mouse CD14 was cloned into pcDNA3.1. HEK293T cells, grown in 10-cm tissue culture dishes, were transfected with each plasmid (1–6 μg) using polyethyleneimine (Polysciences) (Boussif et al. 1995). After 36 h, cells were collected for further analysis.

Real-time quantitative polymerase chain reactions

Total RNA was isolated from the cells using RNAiso Plus (TaKaRa), and 0.5 μg of the RNA was reverse-transcribed with ReverTra Ace qPCR RT Master mix with gDNA Remover (TOYOBO) according

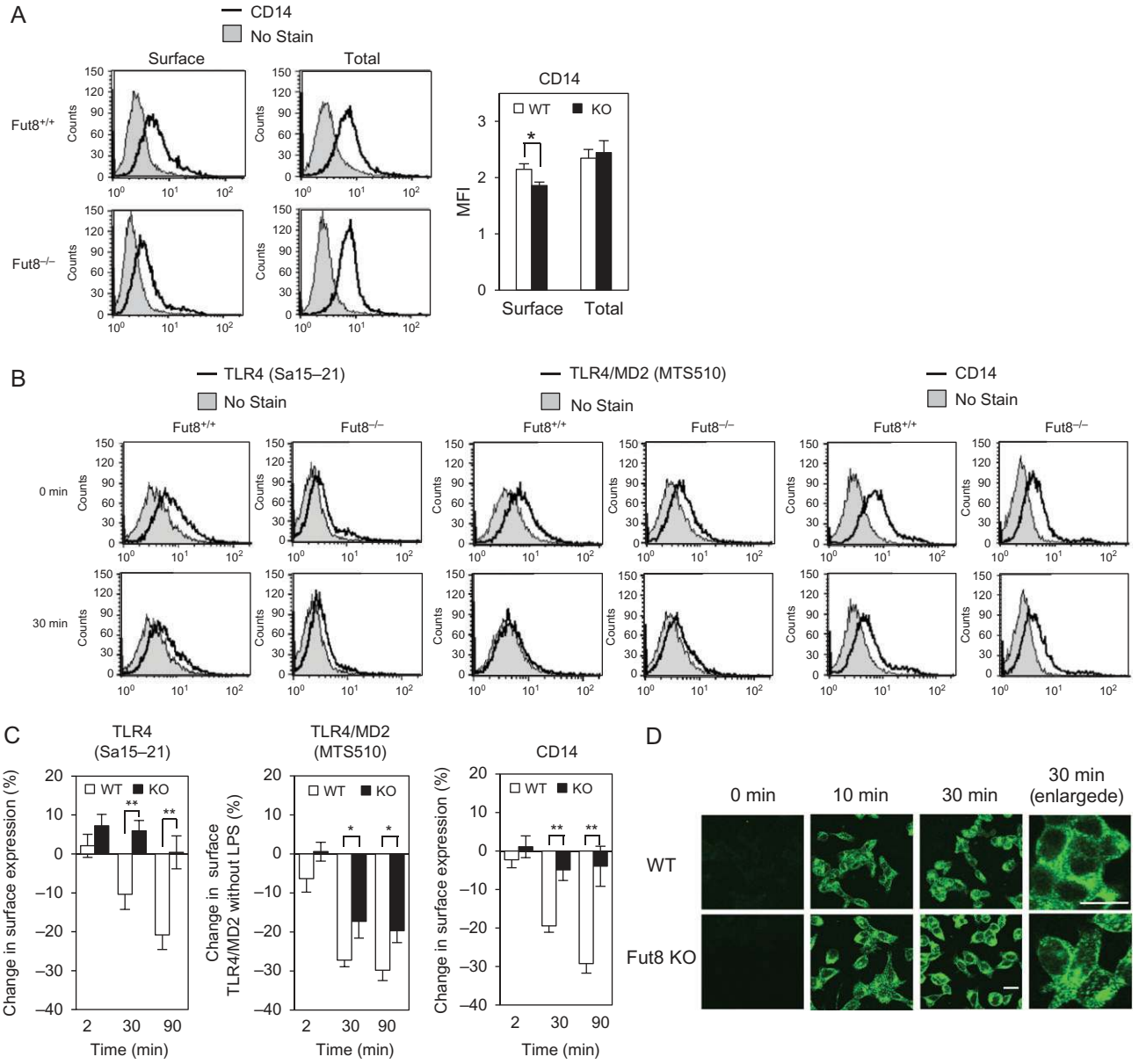

Fig. 5. Core fucose is required for LPS-induced internalization of TLR4 and CD14. (**A**) Surface and total levels of CD14 on *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were assessed by flow cytometry and shown as the mean fluorescence intensity (MFI) relative to unstained control ± SEM of three independent experiments (error bars). (**B**,**C**) After stimulation with LPS (5 μg/mL) for 0, 2, 30 or 90 min, cell-surface levels of TLR4 itself (Sa15-21), TLR4/MD2 complex without LPS (MTS510) or CD14 on *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs were assessed by flow cytometry. (**B**) Representative histograms after stimulation with LPS for 0 and 30 min are shown. (**C**) The mean fluorescence intensity (MFI) relative to cells without stimulation is shown as the mean ± SEM of three independent experiments (error bars). *$P < 0.05$; **$P < 0.01$. (**D**) At 0, 10 or 30 min after addition of FITC-labeled transferrin (10 μg/mL) to *Fut8*$^{+/+}$ or *Fut8*$^{-/-}$ MEFs, surface-exposed transferrin was removed and washed out. The cells were then fixed with and processed for immunofluorescence analysis. Green, transferrin; scale bar, 20 μm; WT, wild-type; KO, knock-out. This figure is available in black and white in print and in color at *Glycobiology* online.

to the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was carried out with an ABI 7300 (Applied Biosystems) instrument using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the appropriate specific primers (Tong et al. 2012). The expression levels of the target gene were measured in duplicate and normalized to the corresponding *GAPDH* expression levels.

Immunoprecipitation

For analysis of LPS-dependent signaling or TLR4 complex formation, cells were stimulated with LPS (1 μg/mL) for 0–20 min. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), and lysed with buffer containing 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl (pH 7.4), 1% Triton X-100 and a protease inhibitor cocktail (Roche). The cell lysates (1.5–2 mg of protein) were incubated with the antibodies (2.5 μg), which were preincubated with 20 μl of protein G Sepharose 4 Fast Flow beads (GE Healthcare) for 1 h at 4°C. After incubation for 1 h, the beads were washed three times with the lysis buffer, and the bound proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer. For detection of TLR4 dimer, the antibodies were preincubated with Dynabeads Protein G (Novex) for 1 h at 4°C.

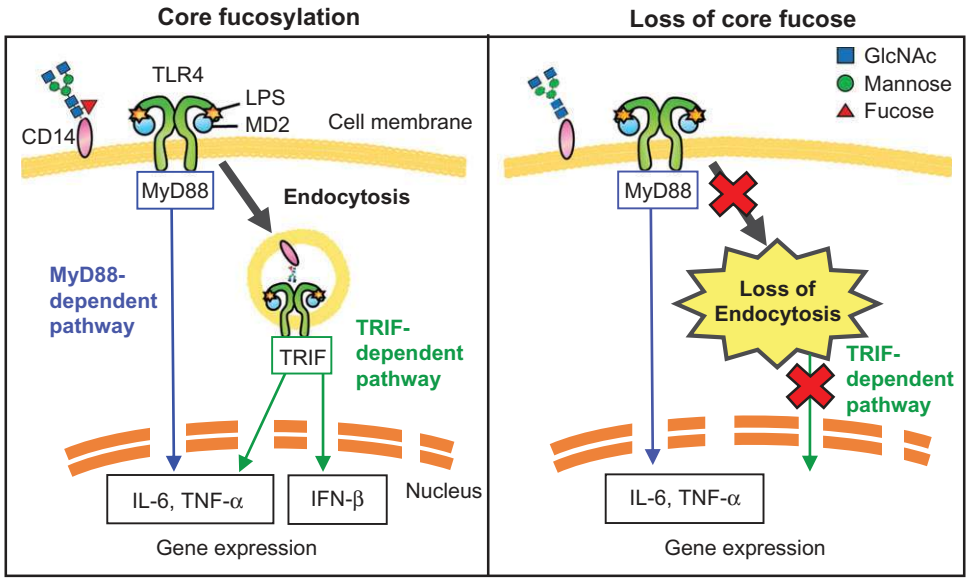

Fig. 6. Core fucose is critical for endocytosis of CD14-dependent TLR4/MD2 complex. LPS stimulation causes dimerization of TLR4/MD2 complex, which mediates MyD88-dependent and TRIF-dependent signal transduction. The MyD88-dependent pathway controls the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, while the TRIF-dependent pathway, which controls production of type I interferons, requires endocytosis of TLR4 complex. CD14 is heavily core fucosylated. In the absence of core fucose in fibroblasts, both TLR4 and CD14 fail to be internalized, and impaired TRIF-dependent signaling is observed. This figure is available in black and white in print and in color at *Glycobiology* online.

Western and lectin blotting

Cell lysates (10–80 μg of protein) or immunoprecipitates were separated using 10% or 5–20% SDS-polyacrylamide gel electrophoresis, respectively, and transferred to nitrocellulose membranes. After incubation with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with primary antibody, followed by horse radish peroxidase-conjugated secondary antibody. For lectin blotting, nitrocellulose membranes were washed with Tris-buffered saline containing 0.1% Tween-20 for 30 min at room temperature. The membranes were then incubated with biotinylated lectins (1:1000) followed by incubation with horse radish peroxidase-avidin (VECTASTAIN ABC Standard Kit). The intensities of the protein signals were quantified using an ImageQuant LAS-4000mini instrument (GE Healthcare). The signals were detected with ECL Prime (Amersham) for TLR4/MD2 complexes and IRF3, and with ECL Pro (Perkin Elmer) for the TLR4 dimer. Cell lysates (10–20 μg of protein) for CD14 and MD2 or immunoprecipitates for TLR4 were heated with denaturation buffer (20 mM Tris–HCl (pH 8.5), 0.2% SDS,1% 2-mercaptoethanol) for 3 min at 100°C, and incubated with PNGase F (Roche) in 20 mM Tris–HCl (pH 8.5) and 1% NP-40 for 16 h (CD14 and MD2) or 3 h (TLR4) at 37°C.

Flow cytometric analysis

After stimulation with LPS (5 μg/mL) for 0–90 min, MEFs were collected and suspended as single cell suspensions in fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin and 0.1% NaN_3 in PBS). The cells were incubated with 0.2–0.5 μg/mL R-phycoerythrin-conjugated anti-TLR4/MD2 (MTS510), anti-TLR4 (Sa15-21) or FITC-conjugated anti-CD14 (Sa14-2) for 1 h at 4°C. To determine the binding or incorporation of LPS, the MEFs were incubated with FITC-conjugated LPS (5 μg/mL) for 0–90 min. Cells were analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences) (Kondo et al. 2013). To measure total CD14 levels, MEFs were permeabilized using a BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences) as previously reported (Ohtsubo et al. 2005). In brief, MEFs were collected in chilled 2 mM EDTA in PBS, and washed with chilled 2% fetal bovine serum in PBS. After permeabilization for 20 min at 4°C, cells were incubated with FITC-conjugated anti-CD14 (Sa14-2) for 30 min at 4°C in BD Perm/Wash ™ buffer, and resuspended in FACS buffer for analysis by flow cytometry.

Transferrin uptake

Uptake of transferrin was performed as previously described (Lakshminarayan et al. 2014). MEF cells were incubated with 10 μg/mL transferrin–Alexa488 (Thermo Fisher Scientific). After incubation for 0–30 min at 37°C, cell-surface-exposed transferrin was removed by acid washes with ice-cold 0.5 M glycine at pH 2.2. The cells were then washed in PBS, fixed with 4% paraformaldehyde in PBS for 30 min and washed again with PBS. The samples were mounted in CC/Mount (Diagnostic BioSystems) and photographed with an FV1000-D laser scanning confocal microscope (Olympus).

Enzyme-linked immunosorbent assay

MEFs were plated into 6-well tissue culture plates (3×10^5 cells/well) one day before LPS stimulation. After the cells were stimulated with various concentrations of LPS for 16–20 h, the culture media were collected to measure the levels of IL-6 and IFN-β by ELISA, according to the manufacturers' instructions (Diaclone for IL-6, PBL for IFN-β).

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Conflict of interest statement

None declared.

Authors' contributions

JI, SKo, Ski and NT designed the experiments and wrote the manuscript. JI and SKo performed most of the experiments. RF supported cell biology experiments. HK and YK designed molecular biology experiments. TS, SA-T, KM and EM interpreted the data.

Abbreviations

AAL, Aleuria aurantia lectin; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; Fut8, α1,6 fucosyltransferase enzyme; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK, human embryonic kidney; IFN, interferon; IgG1, immunoglobulin G1; IL-6, interleukin-6; IRF3, interferon regulatory factor 3; IκB, inhibitory κB; LPS, lipopolysaccharide; MD2, myeloid differentiation factor 2; MEFs, mouse embryonic fibroblasts; MyD88, differentiation primary response gene 88; NFκB, nuclear factor κB, PBS, phosphate-buffered saline; PNGase, peptide: N-glycanase; qPCR, quantitative polymerase chain reaction; RANTES, regulated on activation, normal T cell expressed and secreted; SDS, sodium dodecyl sulfate; SEM, standard error of mean; TIR toll-interleukin 1 receptor; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α, TRIF, TIR-domain-containing adapter-inducing interferon-β.

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