CD14 dependence of TLR4 endocytosis and TRIF signaling displays ligand specificity and is dissociable in endotoxin tolerance

Rajesh Rajaiah[a], Darren J. Perkins[a], Derek D. C. Ireland[b], and Stefanie N. Vogel[a,1]

[a]Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201; and [b]Center for Drug Evaluation and Research, US Food and Drug Administration, Silver Spring, MD 20993

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Dimerization of Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD2) heterodimers is critical for both MyD88- and TIR-domain–containing adapter-inducing IFN-β (TRIF)-mediated signaling pathways. Recently, Zanoni et al. [(2011) *Cell* 147(4):868–880] reported that cluster of differentiation 14 (CD14) is required for LPS-/*Escherichia coli*- induced TLR4 internalization into endosomes and activation of TRIF-mediated signaling in macrophages. We confirmed their findings with LPS but report here that CD14 is not required for receptor endocytosis and downstream signaling mediated by TLR4/MD2 agonistic antibody (UT12) and synthetic small-molecule TLR4 ligands (1Z105) in murine macrophages. CD14 deficiency completely ablated the LPS-induced TBK1/IRF3 signaling axis that mediates production of IFN-β in murine macrophages without affecting MyD88-mediated signaling, including NF-κB, MAPK activation, and TNF-α and IL-6 production. However, neither the MyD88- nor TRIF-signaling pathways and their associated cytokine profiles were altered in the absence of CD14 in UT12- or 1Z105-treated murine macrophages. Eritoran (E5564), a lipid A antagonist that binds the MD2 "pocket," completely blocked LPS- and 1Z105-driven, but not UT12-induced, TLR4 dimerization and endocytosis. Furthermore, TLR4 endocytosis is induced in macrophages tolerized by exposure to either LPS or UT12 and is independent of CD14. These data indicate that TLR4 receptor endocytosis and the TRIF-signaling pathway are dissociable and that TLR4 internalization in macrophages can be induced by UT12, 1Z105, and during endotoxin tolerance in the absence of CD14.

TLR4 endocytosis | agonistic antibody | small-molecule ligands | Eritoran | endotoxin tolerance

Toll-like receptor 4 (TLR4) signaling plays a crucial role in host defense against Gram-negative bacteria by recognizing the outer membrane component, lipopolysaccharide (LPS) (1–3). TLR4 signaling is initiated by transfer of an LPS monomer from LPS binding protein (LBP) to cluster of differentiation 14 (CD14) (GPI-linked or soluble). In turn, CD14 transfers monomeric LPS to myeloid differentiation factor 2 (MD-2), a protein that associates noncovalently with TLR4 (4). Appropriate ligand binding to MD2 results in dimerization of two TLR4/MD2 complexes (4). TLR4 is unique in that it is the only TLR that activates both myeloid differentiation primary response 88 (MyD88) and TIR-domain–containing adapter-inducing IFN-β (TRIF)-dependent signaling pathways (5, 6). MyD88-mediated, TLR4 signaling occurs mainly at plasma membranes and involves IL-1R–associated kinases phosphorylation, association of TNF-receptor–associated factor 6, and downstream signaling that results in NF-κB activation and induction of proinflammatory mediators such as TNF-α and IL-6 (7). In contrast, TRIF-mediated signaling in response to LPS occurs at the endosomal membrane after internalization of the TLR4 that, in turn, activates IFN regulatory factor 3 (IRF3), resulting in production of IFN-β, IP-10, and other IRF-3–dependent genes, as well as delayed NF-κB activation (8). Recent studies have shown that the endocytosis of TLR4 is tightly controlled by several molecules. Rab11a, ARF6, and p120-catenin have been implicated in *Escherichia coli*/LPS-induced TLR4 endocytosis and IRF3 activation (9–11). Zanoni et al. showed that CD14 plays critical roles in translocation of TLR4 into endosomes and in activation of IRF3 that are dependent upon the enzymatic activities of PLCγ2 and Syk (12). However, CD14-independent TLR4 endocytosis and TRIF signaling have not been reported.

UT12 is a monoclonal antibody (MAb) with specificity for the mouse TLR4/MD2 complex and mediates LPS-like signaling (13). It has been shown that UT12 induces endotoxic shock-like symptoms in mice including augmentation of TNF-α and IL-6. Furthermore, UT12 induced long-term tolerance and protection against LPS-induced lethal shock in mice (14). However, the ability of UT12 to induce translocation of TLR4/MD2 into endosomes, as well as its potential for mediating TRIF-dependent signaling, has not been reported. Recently, a group of substituted pyrimido[5,4-b] indoles, synthetic ligands for TLR4 that activate NF-κB that act in a CD14-independent manner, were discovered by high-throughput screening (15). These synthetic ligands induced IL-6 and IP-10 in a TLR4/MD2-dependent, but CD14-independent manner (16). They, too, have not been tested for TLR4 endocytosis and TRIF-dependent intermediates.

In this study, we report, for the first time to our knowledge, CD14-independent translocation of TLR4 to endosomes and TRIF signaling by UT12 and small synthetic TLR4 ligands (1Z105). A TLR4 antagonist, Eritoran, that binds to a deep hydrophobic pocket in MD2 and blocks signaling induced by LPS, UT12, and 1Z105, blocked only TLR4 internalization and dimerization induced by LPS and 1Z105. Despite TLR4/MD2 internalization, endotoxin-tolerized macrophages fail to activate TRIF-mediated signaling. These findings reveal previously unidentified insights into the possible role of CD14 in LPS-mediated TLR4 endocytosis and signaling

Significance

MyD88-dependent signaling is cluster of differentiation 14 (CD14)-dependent only at low LPS concentrations, whereas activation of the TIR-domain–containing adapter-inducing IFN-β (TRIF) pathway requires CD14 at all LPS concentrations, leading to internalization of the Toll-like receptor 4 (TLR4) complex into endosomes whereupon TRIF is recruited. Using alternative TLR4 agonists, or macrophages rendered tolerant to LPS, we dissociate TLR4 complex internalization from CD14 and TRIF-dependent signaling. In response to LPS, CD14 contributes to the formation of a TLR4/MD2 complex dimer that, in turn, promotes endocytosis and IRF3 activation.

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. Email: svogel@som.umaryland.edu.

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and demonstrate that TLR4 endocytosis and signaling are dissociable processes.

Results

UT12-Induced TLR4 Endocytosis and TRIF-Dependent Signaling Are CD14-Independent. Zanoni et al. (12) reported the requirement for CD14 in TLR4 endocytosis and the production of IFN-β by LPS. We compared internalization of TLR4 induced by LPS to that induced by UT12, a MAb directed against a TLR4/MD2 epitope that acts as a TLR4 agonist (13, 17). In WT macrophages, both LPS and UT12 induced TLR4 internalization, whereas in CD14$^{-/-}$ macrophages only UT12 induced TLR4 endocytosis (Fig. S1*A*). An isotype-matched control antibody failed to induce TLR4 internalization in either WT or CD14$^{-/-}$ macrophages (Fig. S1*A*). The effect of UT12 on macrophages from FcR α-chain$^{-/-}$ and γ-chain$^{-/-}$ mice was evaluated to rule out activation of TLR4 internalization and TRIF signaling by FcR-mediated endocytosis. UT12 induced a similar level of TLR4 internalization in the absence of either FcR α- or γ-chains (Fig. S2). UT12-induced TLR4 internalization was time-dependent in both WT and CD14$^{-/-}$ macrophages (Fig. 1 *A* and *B*). LPS- and UT12-induced TLR4 internalization in WT macrophages were similarly time-dependent, but again, LPS, but not UT12, failed to induce TLR4 endocytosis in CD14$^{-/-}$ macrophages (Fig. 1 *A* and *B*).

Activation of signaling molecules required for the MyD88- and/or TRIF-dependent signaling pathways were compared in WT and CD14$^{-/-}$ macrophages stimulated by LPS and UT12. LPS (100 ng/mL) activated the TRIF-signaling intermediates TBK1 and IRF3 in WT macrophages by 30 min; however, LPS failed to activate TBK1 or IRF3 in CD14$^{-/-}$ macrophages, even after 60 min (Fig. 1*C*, *Right*). NF-κB activation by LPS was also defective in CD14$^{-/-}$ macrophages at 30 min (Fig.1*C*, *Left*), but became detectable at 60 min (Fig. 1*C*, *Right*). Similarly, MAPK activation by LPS was defective in CD14$^{-/-}$ macrophage at 30 min, but was increased slightly at 60 min (Fig. 1*C*), consistent with the previously reported role for CD14 in enhancing kinetics of MyD88-dependent gene induction (18). On the other hand, the activation of NF-κB, MAPK, and TBK1/IRF3 by UT12 was CD14-independent both at early and at late time points (Fig. 1*C*).

To measure the effect of CD14 on production of type I IFN and other proinflammatory cytokines induced by LPS or UT12, secreted cytokines/chemokines in culture supernatants of WT and CD14$^{-/-}$ macrophages were analyzed. Both LPS (100 ng/mL) and UT12 (1,000 ng/mL) elevated proinflammatory cytokine levels (e.g., TNF-α and IL-6) in WT and CD14$^{-/-}$ macrophages, although the level of secretion induced by LPS was consistently greater than that induced by UT12 (Fig. 1*D*). In contrast, LPS-induced IFN-β and IP-10 production was significantly blunted in CD14$^{-/-}$ macrophages (Fig. 1*D*) under conditions where UT12-induced IFN-β and IP-10 levels were CD14-independent (Fig. 1*D*).

Zanoni et al. (12) also reported that CD14 was internalized into endosomes with TLR4. Therefore, we investigated the endocytosis of CD14 in LPS- vs. UT12-stimulated WT macrophages. CD14$^{-/-}$ macrophages were used to confirm the specificity of the anti-CD14 antibody. CD14$^{-/-}$ macrophages failed to express CD14 protein whereas WT macrophages strongly expressed CD14 (Fig. 1*E*, *Left*). In contrast to TLR4, surface expression of CD14 was reduced minimally by LPS stimulation at 30 min, but not altered at 90 min (Fig. 1*E*, *Right*). However, UT12 did not induce CD14 internalization at early or late time points (Fig. 1*E*, *Right*). These data clearly suggest that TLR4 and CD14 endocytosis is dissociable.

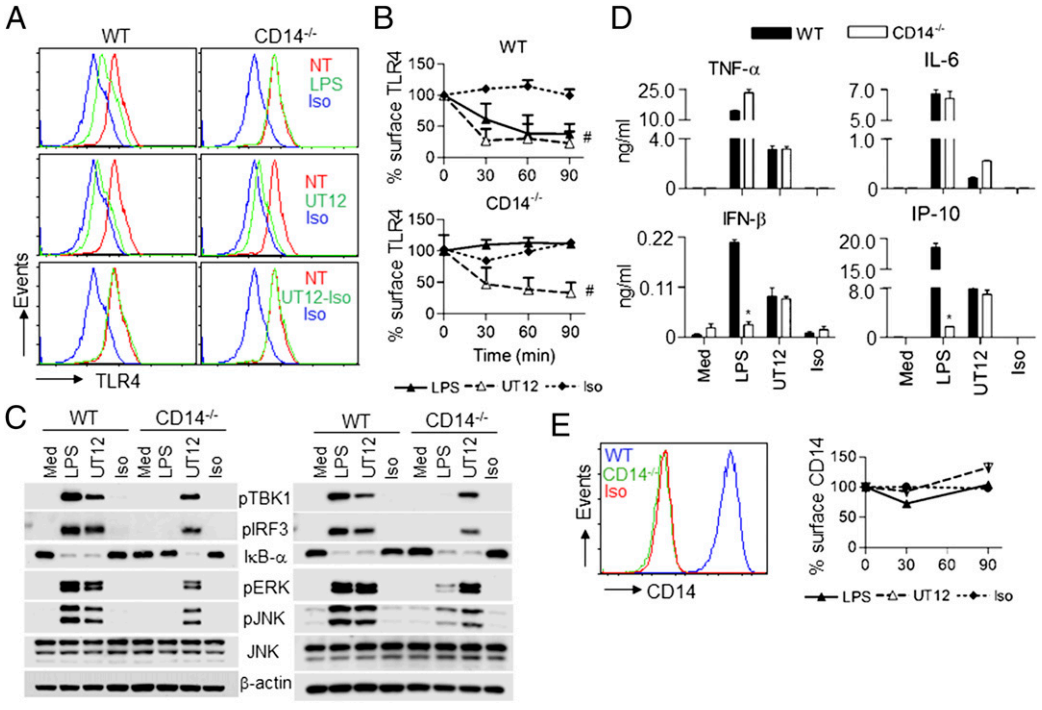

Fig. 1. CD14 is not required for UT12-induced TLR4 endocytosis and downstream signaling in PMs. WT and CD14$^{-/-}$ mouse PMs were medium-treated or treated with LPS (100 ng/mL), UT12 (1,000 ng/mL), and UT12 isotype (1,000 ng/mL) for the indicated times, and TLR4 surface expression was analyzed by flow cytometry (*A* and *B*). A representative histogram was shown after 90 min of treatment (*A*), and TLR4 internalization was quantitated by mean fluorescence intensity (MFI) at each time point (*B*). Total cell lysates from WT and CD14$^{-/-}$ PMs were prepared at 30 min (*A*, *Left*) and 60 min (*A*, *Right*) after treating with LPS, UT12, and UT12-isotype control antibody and activating signaling molecules analyzed by Western blotting (*C*). For cytokine/chemokine secretion, WT and CD14$^{-/-}$ PMs were treated with LPS, UT12, and UT12-isotype control antibody for 16 h, and culture supernatants were analyzed by ELISA (*D*). CD14 expression in WT and CD14$^{-/-}$ PMs was analyzed by flow cytometry (*E*, *Left*). WT PMs were treated with medium only, LPS, UT12, and UT12-isotype control antibody for indicated times, and CD14 surface expression was analyzed by flow cytometry and quantified by MFI (*E*, *Right*). Data represent the mean ± SEM from two to three independent experiments. $^{\#}P < 0.05$, nontreated vs. treated groups; $^{*}P < 0.05$, treated WT vs. treated CD14$^{-/-}$ groups. (NT, not treated; Iso, isotype).

To determine if UT12-induced TLR4 internalization and IRF3 activation involves the same signaling molecules reported for LPS (12), we tested the effect of the Syk and PLC-γ2 inhibitors, piceatannol and U73122, respectively. Both inhibitors blocked LPS- and UT12-induced TLR4 internalization and IRF3 activation similarly (Fig. S3). These data suggest that whereas both Syk and PLC-γ2 are required for UT12-mediated TLR4 internalization and IRF3 activation, CD14 is not.

MyD88 and TRIF Deficiencies Do Not Affect TLR4 Internalization. MyD88 and TRIF are adapter molecules responsible for NF-κB and IRF3 activation, respectively (5–7). As previously reported (12), TLR4 endocytosis induced by LPS was both MyD88- and TRIF-independent, and this was observed for UT12 stimulation as well (Fig. S4 *A* and *C*). Secretion of TNF-α and IL-6 induced by LPS or UT12 was significantly diminished in MyD88$^{-/-}$ macrophages, but IFN-β and IP-10 levels were either equivalent to or slightly less in MyD88$^{-/-}$ compared with WT macrophages (Fig. S4*B*), consistent with the classification of IFN-β and IP-10 as MyD88-independent (8, 19). In TRIF$^{-/-}$ macrophages, both MyD88-dependent (TNF-α and IL-6) and TRIF-dependent (IFN-β and IP-10) cytokines induced by LPS or UT12 were completely inhibited (Fig. S4*D*), consistent with previous reports that TRIF plays an important role in the production of both MyD88- and TRIF-dependent cytokines/chemokines (20, 21).

TRIF-Dependent Cytokine/Chemokine Production by TLR3 Ligand Is CD14-Independent. Because TLR3 is located in endosomes and its ligand, polyinosinic:polycytidylic acid (pI:C), strongly induces IFN-β, we assessed the involvement of CD14 in TLR3 signaling. Several reports have provided evidence for the requirement of CD14 in TLR3-mediated signaling (22, 23). Lee et al. claimed that CD14 physically interacts with pI:C and mediates TLR3 activation (23). pI:C treatment of macrophages did not affect surface expression of TLR4 or CD14 (Fig. S5 *A* and *B*). The production of TNF-α in pI:C-stimulated CD14$^{-/-}$ macrophages was greatly inhibited, in contrast to IL-6, IFN-β, and IP-10, which were minimally or not affected (Fig. S5*C*). Hence, CD14 is not absolutely required for TLR3-mediated, TRIF-dependent cytokines and chemokine secretion.

Synthetic Small-Molecule TLR4 Ligands Induce TLR4 Endocytosis and IRF3 Activation in a CD14-Independent Manner. Recently, Hayashi et al. identified synthetic chemical ligands that activate TLR4 in a CD14-independent, MD2-dependent manner and resulted in the secretion of both MyD88- and TRIF-dependent cytokines/chemokines (16). To extend these findings, we investigated their effects on TLR4 internalization and endocytic signaling. Two agonists, 1Z105, and the less active ligand, 1Z204, dose-dependently induced TLR4 internalization in both WT and CD14$^{-/-}$ macrophages (Fig. S1*B*). An inactive control compound, 1Y88, did not induce TLR4 endocytosis in either WT or CD14$^{-/-}$ macrophages, even at higher concentrations (Fig. 2*A* and Fig. S1*B*). Similar to UT12, 1Z105 (5 μM) potently induced TLR4 endocytosis in a time-dependent fashion. The less active 1Z204 induced TLR4 endocytosis partially at 5 μM (Fig. 2*A*). The activation of signaling events by these compounds was also similar to the effect of UT12 (compare Fig. 2*B* to Fig. 1*C*). Furthermore, cytokine/chemokine production was also CD14-independent (Fig. 2*C*) and correlated with the degree of activation of NF-κB and TBK1/IRF3 axes (Fig. 2*B*).

LPS-induced B7 costimulatory molecules (CD80 and CD86) are TRIF-TRAM–dependent (24, 25). Similar to previously published reports in TRIF-deficient macrophages (24) and TRAM-deficient B220-positive cells (25), LPS-induced up-regulation of CD80 and CD86 was perturbed in CD14$^{-/-}$ macrophages (Fig. S6). However, as expected, both UT12- and 1Z105-induced up-regulation of CD80 and CD86 was not affected in CD14$^{-/-}$ macrophages (Fig. S6). Overall, these data suggest that CD14 is not absolutely required for TLR4 endocytosis and its downstream signaling induced by UT12 and small-molecule TLR4 agonists. To rule out any differences in TLR4 internalization and TRIF signaling

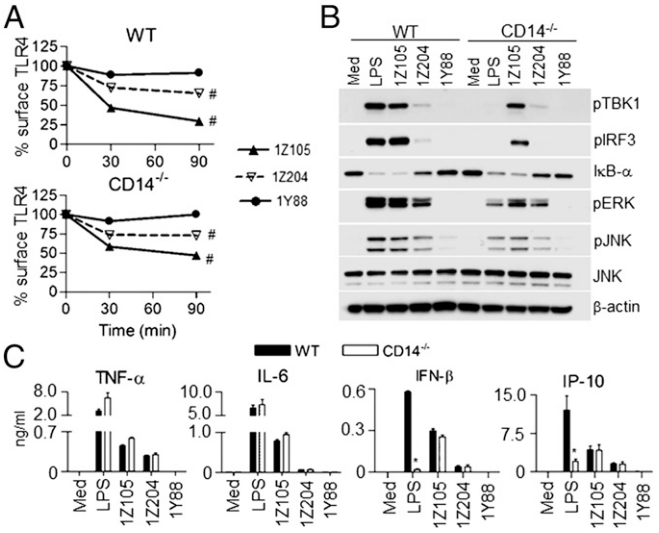

Fig. 2. TLR4 synthetic small-molecule ligands induce receptor endocytosis and related signaling in a CD14-independent manner. WT and CD14$^{-/-}$ PMs were medium-treated or treated with LPS (100 ng/mL) and different synthetic ligands (5 μM) for the indicated times, and TLR4 internalization was analyzed by flow cytometry and quantified by MFI at each time point (*A*) as described in the legend to Fig. 1. Total cell lysates from WT and CD14$^{-/-}$ PMs were prepared 60 min after treating cells with LPS or synthetic ligands, and activation of signaling molecules was analyzed by Western blotting (*B*). For cytokine/chemokine secretion, WT and CD14$^{-/-}$ PMs were treated with LPS and synthetic ligands for 16 h, and culture supernatants were analyzed by ELISA (*C*). Data represent the mean ± SEM from two to three independent experiments. $^{\#}P < 0.05$, non-treated vs. treated groups; $^{*}P < 0.05$, treated WT vs. treated CD14$^{-/-}$ groups.

induced by UT12 and 1Z105 in primary peritoneal macrophages (PMs) vs. bone marrow-derived macrophages (BMDMs), we repeated our studies in BMDMs. BMDMs behaved very similarly to peritoneal macrophages with respect to TLR4 endocytosis, TRIF signaling, and cytokine/chemokine production induced by UT12 and 1Z105 (Fig. S7 *A*–*D*). Surface expression of CD14 was not modulated in BMDM upon stimulation with LPS, UT12, or 1Z105 (Fig. S7*E*), in contrast to the slight decrease in CD14 seen in LPS-stimulated PMs (Fig. 1*E*). Furthermore, we performed colocalization analysis of CD14 to the endosome in WT BMDMs using confocal microscopy. As shown in Fig. S8, CD14 (green) did not colocalize with endosomes (red) when BMDMs were treated with LPS, UT12, or 1Z105.

The dynamin inhibitor, dynasore, prevented LPS-induced TLR4 internalization in WT macrophages (Fig. S9 *A* and *C*, *Left*) as previously reported (8). As expected, LPS did not induce TLR4 internalization in the presence or absence of dynasore in CD14$^{-/-}$ macrophages (Fig. S9 *B* and *C*, *Right*). 1Z105-induced TLR4 internalization was inhibited by dynasore in both WT and CD14$^{-/-}$ macrophages (Fig. S9 *A*–*C*), whereas UT12-induced internalization was not (Fig. S9 *A*–*C*). However, dynasore completely inhibited both 1Z105- and UT12-induced IRF3 activation in both WT and CD14$^{-/-}$ macrophages (Fig. S9*D*), resulting in inhibition of both MyD88-dependent (TNF-α) and TRIF-dependent (IFN-β) cytokines (Fig. S9*E*). This latter observation extends the findings of Kagan et al. who reported complete inhibition of LPS-induced IL-6 (MyD88-dependent) and RANTES (TRIF-dependent) by dynasore in WT macrophages (8). In fact, dynasore enhanced degradation of IκB-α in medium-treated WT and CD14$^{-/-}$ macrophages (Fig. S9*D*) yet did not induce NF-κB–dependent cytokines, suggesting that nuclear translocation of NF-κB did not occur in macrophages treated with dynasore alone.

TLR4 Antagonist Eritoran Inhibits LPS and 1Z105, but Not UT12-Induced TLR4 Internalization. Eritoran (E5564) is a synthetic lipid A analog that binds in the deep hydrophobic pocket of MD2 and

competitively inhibits binding of lipid A to MD2 and thereby inhibits downstream signaling (26). Eritoran was also shown to bind to CD14 and block the transfer of lipid A to MD2 (27). Eritoran blocks LPS-induced MyD88- and TRIF-dependent cytokine production in human and murine macrophages (28). Therefore, we sought to determine if Eritoran would block TLR4 internalization induced by the various TLR4 agonists. Eritoran inhibited LPS- and 1Z105-induced TLR4 internalization in WT macrophages (Fig. 3*A*), consistent with the MD2 dependency of these two agonists. However, Eritoran failed to inhibit UT12-induced TLR4 internalization (Fig. 3*A*), suggesting that the TLR4/MD2 epitope recognized by UT12 is distinct from the MD2 hydrophobic pocket or is not modified by binding of Eritoran to the MD2 hydrophobic pocket. Because 1Z105 induces TLR4 internalization in a CD14-independent manner and was inhibited by Eritoran (Fig. 3*A*), we asked whether Eritoran would inhibit 1Z105-induced TLR4 internalization in $CD14^{-/-}$ macrophages. Eritoran inhibited 1Z105-induced TLR4 internalization in $CD14^{-/-}$ macrophages (Fig. 3*B*), suggesting that Eritoran is acting independently of CD14 despite the fact it is able to bind to CD14. Furthermore, we observed that Eritoran inhibited LPS-, UT12-, and 1Z105-induced activation of TBK1/IRF3, NF-κB, and MAPKs (Fig. 3*C*). R848, a TLR7/8 ligand, was used as control, and Eritoran did not inhibit any signaling induced by this agonist (Fig. 3*C*). Eritoran also inhibited LPS-, UT12-, and 1Z105-induced cytokine/chemokine gene expression (Fig. 3*D*).

To determine if Eritoran interfered in TLR4 dimerization, we compared the ability of LPS, UT12, and 1Z105 to induce TLR4 dimerization in the absence or presence of Eritoran. Eritoran blocked TLR4 dimerization induced by LPS and 1Z105 in TLR4-expressing HEK293T cells (Fig. 3*E*); however, it failed to block TLR4 dimerization induced by UT12 (Fig. 3*E*), consistent with the failure to block UT12-induced TLR4 internalization (Fig. 3*A*). These data clearly indicate that TLR4 dimerization is critical for its internalization induced by different ligands.

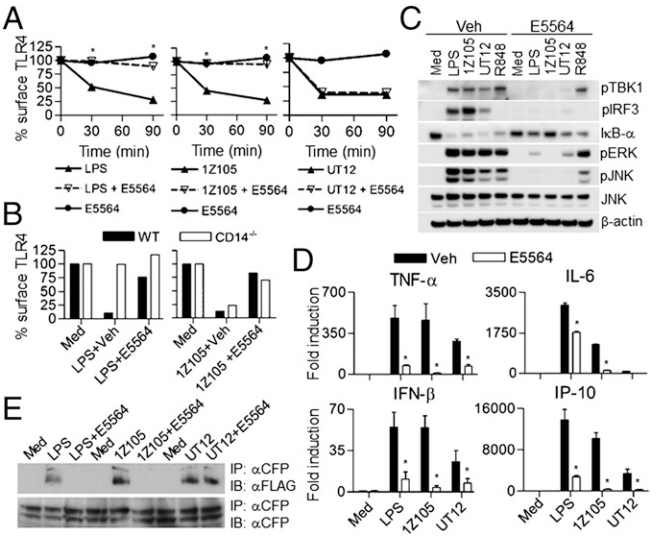

Fig. 3. The TLR4 antagonist E5564 failed to inhibit UT12-induced receptor internalization, but inhibited MyD88- and TRIF-dependent signaling. WT PMs were pretreated with 10 ng/mL E5564 for 60 min and then treated with LPS, UT12, or 1Z105 for the indicated times. TLR4 internalization was analyzed by flow cytometry, and surface expression was quantitated using MFI (*A*). TLR4 internalization induced by 1Z105 was analyzed after 90 min of treatment in $CD14^{-/-}$ PMs in the absence or presence of E5564 as described above (*B*). PMs were stimulated with LPS, 1Z105, UT12, or R848 in the absence or presence of E5564 (10 ng/mL) for 60 min, and total cell lysates were subjected to Western blotting (*C*). PMs were treated with different TLR4 ligands as described above, and RNA was isolated after 1 and 5 h. Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) (*D*). HEK293T cells were treated with different TLR4 ligands in the absence or presence of Eritoran for 30 min, and induction of TLR4 dimerization was analyzed by immunoprecipitation followed by Western analysis (*E*). Data represent the mean ± SEM from two to three independent experiments. $*P < 0.05$, treated without vs. with E5564 groups.

CD14-Independent Endocytosis of TLR4 in Macrophages Rendered Tolerant by LPS and UT12. In mice and macrophages exposed to LPS, a transient period of LPS-hyporesponsiveness ensues, which has been referred to as "endotoxin tolerance," which has been associated with epigenetic changes that result in differential gene expression (29–32). Because the expression of TRIF-dependent genes is strongly "tolerized," we sought to determine the role of TLR4 internalization in this process. Cells were treated overnight with medium (M) only, LPS (L), or UT12 (U) and then restimulated the next day with M, L, or U. We observed decreased surface expression of TLR4 both in nontolerized (M/L, M/U) and tolerized (L/M, L/L, U/U) WT macrophages (Fig. 4*A*, *Left*). In $CD14^{-/-}$ macrophages, however, LPS treatment of mock-tolerized macrophages (M/L) did not reduce the surface expression of TLR4, consistent with our data in Figs. 1 and 2. Interestingly, macrophages rendered tolerant by overnight LPS treatment exhibited significant TLR4 internalization, even in the absence of CD14, and without or with LPS restimulation (Fig. 4*A*, L/M, L/L), although the TLR4 internalization seen in $CD14^{-/-}$ macrophages was somewhat less than that observed in WT macrophages. When UT12 was used as the TLR4 agonist, however, we found that nontolerized (M/U) and tolerized (U/U) macrophages exhibited similar levels of TLR4 endocytosis both in WT and $CD14^{-/-}$ macrophages (Fig. 4*A*). Heterotolerance to LPS or UT12 (U/L or L/U) also induced CD14-independent TLR4 internalization (Fig. 4*B*). Inhibition of LPS- or UT12-induced NF-κB, MAPK, and TBK1/IRF3 signaling was comparable in LPS- or UT12-tolerized WT and $CD14^{-/-}$ macrophages, thus dissociating TLR4 internalization and signaling (Fig. 4*C*). Both TNF-α and IL-6 levels were tolerized to the same extent in WT and $CD14^{-/-}$ macrophages (Fig. 4*D*). Although LPS (M/L) did not induce IFN-β in $CD14^{-/-}$ macrophages (as observed in Fig. 1*D*), production of IFN-β was completely blocked in tolerized macrophages (Fig. 4*D*), despite TLR4 internalization.

Discussion

TLR4 endocytosis and trafficking to the endosomal compartment is important for the regulation of TRIF-mediated signaling induced by LPS (8, 33). This process is tightly regulated by dynamins, clathrin, and associated Rab proteins (9, 34). Kagan and coworkers reported that, upon LPS stimulation, TLR4 is recruited to the endosome from the plasma membrane where it interacts with TRAM and TRIF adaptor molecules, leading to activation of the IRF3 pathway (8). However, the specific mechanism by which TLR4 is transported to the endosome was incompletely defined. The small GTPase ADP ribosylation factor 6 (ARF6) and Rab family of GTPases have been investigated in controlling endocytic transport of receptors (10). Recently, Husebye et al. showed that Rab11a, a small GTPase, regulates recruitment of TLR4 and TRAM to *E. coli* phagosomes and controls both *E. coli*- and LPS-induced IRF3 activation (9). Zanoni and coworkers (12) reported the requirement for membrane-bound CD14 in controlling LPS- and *E. coli*-induced TLR4 endocytosis and TRIF-mediated signaling in macrophages. Furthermore, they reported that TLR4 internalization and IRF3 activation is mediated by PLC-γ2 and Syk (12). Very recently, a requirement for p120-catenin, a prototypic member of subfamily of armadillo repeat domain proteins, has been shown to regulate MyD88-dependent NF-κB and TRIF-dependent IRF3 activation reciprocally. Silencing of p120-catenin diminished LPS-induced TLR4 internalization and IRF3 activation while increasing NF-κB translocation (11).

We and others previously reported that CD14 is required for MyD88-dependent signaling at low, but not high, concentrations of LPS (12, 35). This suggests that CD14 is primarily responsible for the transfer of LPS to MD2, a necessary coreceptor for TLR4, when the concentration of LPS is limiting (36, 37). CD14 dependency for MyD88-dependent signaling is overcome at higher LPS

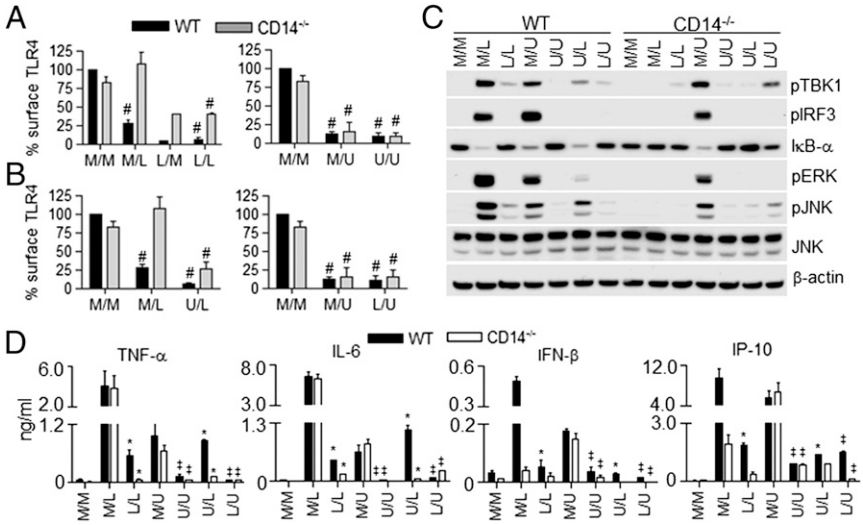

Fig. 4. Endotoxin tolerance induced TLR4 endocytosis in CD14$^{-/-}$ PMs. WT and CD14$^{-/-}$ PMs were tolerized overnight for 18 h with LPS or UT12. Cells were washed to remove endotoxin or UT12 and restimulated with either medium or LPS or UT12 for indicated times. Surface expression of TLR4 (*A* and *B*) was analyzed by flow cytometry as described in Fig. 1. Similarly, WT and CD14$^{-/-}$ PMs were tolerized for 18 h as described above, and total cell lysate was prepared after 30 min of restimulation with medium only, LPS, or UT12; activation of signaling molecules was analyzed by Western blotting (*C*). For cytokine secretion, WT and CD14$^{-/-}$ PMs were tolerized for 18 h as described above and restimulated with LPS or UT12 for 16 h, and culture supernatants were analyzed for cytokine/chemokine level by ELISA (*D*). For each graph, data represent the mean ± SEM from two to three independent experiments. Percentage surface TLR4 in WT and CD14$^{-/-}$ was normalized using medium-treated WT macrophages as 100%. $^{\#}P < 0.05$, medium treated vs. nontolerized and tolerized groups; $^{*}P < 0.05$, M/L (nontolerized) vs. L/L and U/L (tolerized); $^{\ddagger}P < 0.05$, M/U (nontolerized) vs. U/U and L/U (tolerized).

doses, possibly due to a direct interaction of LPS monomers with MD2. In contrast, the CD14 dependency required for TRIF-mediated signaling cannot be overcome by increasing the LPS concentration (12). When LPS or *E. coli* are presented on beads to CD14-deficient dendritic cells, both TLR4 internalization and TRIF-dependent signaling are preserved (12). This implies that, in the case of soluble LPS, CD14 also regulates the trafficking of TLR4 into the endosome where it, in turn, recruits the downstream adapters TRAM and TRIF to the TIR domain of TLR4 dimer.

Our data confirm and significantly extend these findings. TLR4 endocytosis and TRIF-mediated signaling were induced by treatment of macrophages with UT12, a mouse antibody directed against an epitope formed by TLR4/MD2 interaction (13, 14), and small synthetic TLR4 ligands (1Z105 and 1Z204) that bind to MD2 and signal through both MyD88-dependent and TRIF-dependent pathways in the absence of CD14 (16). Although it is possible that the UT12 monoclonal antibody also activates internalization through FcγR-dependent uptake of UT12/TLR4/MD2 immune complexes, UT12 is a mouse IgG3 that has high affinity for FcRn and very low affinity/no affinity toward FcγRI, FcγRIIB, FcγRIII, and FcγRIV (38, 39). For all of these FcγRs, either FcR α- and/or γ-chains are required for activation (40). UT12-induced TLR4 internalization was not altered in macrophages derived from mice deficient in either FcR α- and γ-chains (Fig. S2), ruling out the possibility of FcR involvement in TLR4 internalization. Furthermore, the isotype control antibody for UT12 did not induce TLR4/MD2 internalization.

Moreover, LPS- and 1Z105-, but not UT12-induced TLR4 internalization was blocked by dynasore, and yet, TNF-α and IFN-β levels were completely blocked in UT12-treated macrophages. This suggests that either dynamin is acting further downstream in the TLR4-signaling pathway triggered by UT12 leading to gene expression or that dynasore has an off-target effect that underlies inhibition of MyD88-dependent cytokines. Zanoni et al. previously showed that Syk and PLC-γ2 were key signaling components for TLR4 internalization (12). Consistent with their findings, we found that Syk/PLC-γ2 inhibitors blocked both LPS- and UT12-induced TLR4 internalization, as well as IRF3 phosphorylation. The PLC-γ2 inhibitor partially prevented IκB-α degradation (Fig. S3*E*), supporting the notion that it may also act further downstream and/or have off-target effects on other mediators involved in MyD88-dependent signaling.

More interestingly, macrophages rendered hyporesponsive to LPS or UT12 by a standard "tolerance" regimen (31) retained the ability to internalize TLR4 in a CD14-independent fashion, yet exhibited decreased MyD88- and TRIF-dependent signaling and cytokine production. A correlation between endotoxin tolerance and a transient down-regulation of surface TLR4 after LPS stimulation has been reported by some groups (41), but was completely restored to normal levels at the time of LPS challenge in tolerized cells (30). Regardless, both TLR4 endocytosis by endotoxin tolerance and induction of tolerance are CD14-independent. We reported here that although surface expression TLR4 is endocytosed in endotoxin-tolerized cells, none of the signaling cascades are activated. Thus, the TLR4 endocytosis that occurs during endotoxin tolerance is completely dissociable from TRIF signaling (Fig. S10*D*).

Because CD14 is required for LPS-induced TLR4 delivery to endosomes and is not required for MyD88-dependent signaling at higher LPS concentrations, we propose that CD14 not only assists in LPS transfer to MD2, but perhaps is also is required for stabilizing the TLR4/MD2 complex at the plasma membrane that, in turn, favors complex internalization (Fig. S10*A*). The involvement of CD14 in tight heterodimerization of TLR4/MD2 has been shown recently by Tanimura and coworkers (42). In the case of UT12 and 1Z105, CD14 is required neither for transfer of the ligand nor for internalization of the TLR4/MD2 complex (Fig. S10*A*), possibly due to their innate abilities to bind MD2 directly.

Furthermore, we demonstrated another surprising dissociation between TLR4 endocytosis and TRIF-dependent signaling using Eritoran (Fig. S10*B*), an inactive lipid A analog that binds to a MD2 hydrophobic pocket and competes for LPS binding, thereby blocking both MyD88-dependent and TRIF-dependent signaling. Eritoran treatment failed to induce TLR4 endocytosis, and we confirmed by a TLR4 dimerization assay that this is due to a failure to bring two TLR4/MD2 complexes into an active conformation. The inhibition of LPS- and 1Z105-induced TLR4 endocytosis by Eritoran mediated by preventing the formation of the TLR4/MD2 complex (Fig. S10*C*) confirmed that these molecules compete for the same MD2-binding site. Moreover, our findings that Eritoran

did not inhibit UT12-induced endocytosis, but inhibited UT12-induced signaling, suggests the possibility that the presence of Eritoran in the endosome, along with the UT12-TLR4/MD2 complex, may cause a conformational change within the TIR domains of the TLR4 dimer that prevents its interaction with the adapter, TRIF adapter (Fig. S10*B*).

In summary, we have found that TLR4 endocytosis and TRIF signaling are dissociable. We propose that CD14 may help in the stabilization of TLR4/MD2 complex at plasma membrane that, in turn, leads to endocytosis and TRIF-dependent signaling.

Materials and Methods

Cell Culture.

PMs. C57BL/6J (WT), CD14$^{-/-}$, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice (6–8 wk) were injected with sterile thioglycollate (Remel) as described previously (43). Cells were obtained and treated as described in *SI Materials and Methods*.

BMDMs. BMDMs were derived from C57BL/6 (WT), CD14, FcR α-chain, and γ-chain–deficient mice bone marrows. BMDMs were cultured and treated as described in *SI Materials and Methods*.

Cells stimulation and FACS staining. Cells were stimulated with the indicated concentrations of TLR ligands and RNA was isolated for gene expression studies. Culture supernatants were collected for analyzing cytokine secretion, and cell lysates were prepared for Western analysis. For FACS analysis, PMs and BMDMs (2×10^5) were stimulated with the indicated concentrations of different TLR ligands as described in *SI Materials and Methods*.

Inhibition of TLR4 internalization by dynasore. WT and CD14$^{-/-}$ BMDMs were pretreated with dynasore (80 μM) (Sigma-Aldrich) for 60 min in serum-free culture medium as described in *SI Materials and Methods*.

TLR4 dimerization assay. Four × 10^5 HEK293T cells were plated per well in a six-well tissue culture plate. After 24 h, HEK293T cells were transfected with expression vectors for FLAG-TLR4 (100 ng/well), eCFP-TLR4 (100 ng/well), CD14 (75 ng/well), and MD2 (100 ng/well), and cells were treated as described in *SI Materials and Methods*.

Animal Assurances. All animal studies were carried out with approval from the University of Maryland, Baltimore Institutional Animal Care and Use Committee.

Statistical Analysis. One-way ANOVA with Tukey's Multiple Comparison post hoc test was performed to assess statistical significance (P values <0.05) using GraphPad PRISM 4.0 (GraphPad Software).

Detailed experimental procedures are available in *SI Materials and Methods*.

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