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Dual Signaling of MyD88 and TRIF Is Critical for Maximal TLR4-Induced Dendritic Cell Maturation

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TLR4 is a unique TLR because downstream signaling occurs via two separate pathways, as follows: MyD88 and Toll IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF). In this study, we compared and contrasted the interplay of these pathways between murine dendritic cells (DCs) and macrophages during LPS stimulation. During TLR4 activation, neither pathway on its own was critical for up-regulation of costimulatory molecules in DCs, whereas the up-regulation of costimulatory molecules was largely TRIF dependent in macrophages. LPS-induced secreted factors, of which type I IFNs were one of the active components, played a larger role in promoting the up-regulation of costimulatory molecules in macrophages than DCs. In both cell types, MyD88 and TRIF pathways together accounted for the inflammatory response to LPS activation. Furthermore, signaling of both adaptors allowed maximal T cell priming by LPS-matured DCs, with MyD88 playing a larger role than TRIF. In sum, in our experimental systems, TRIF signaling plays a more important role in LPS-induced macrophage activation than in DC activation. *The Journal of Immunology*, 2008, 181: 1849–1858.

A djuvants are compounds that enhance the immune response to an Ag, and hence, increase the effectiveness of vaccines. Until recently, the mechanisms of adjuvant action were unknown (1). The discovery of TLRs and their microbial ligands provided an explanation for the immune-boosting properties of adjuvants (2–4) because several adjuvants contain microbial products that activate TLRs. Activation of these receptors on APCs induces an innate immune response that initiates adaptive immunity in certain scenarios. Furthermore, several reports have demonstrated that TLR agonists can act as effective adjuvants for host defense against pathogens and cancer (5–9). This has resulted in an intense interest in developing TLR agonists as adjuvants for clinical vaccines (10–13). A complete understanding of the mechanisms by which TLR agonists augment immune responses will allow us to take a logical approach in designing improved vaccines for clinical application.

Dendritic cells (DCs) are the most potent APCs, and TLR activation on DCs is an important pathway linking the innate and adaptive immune systems (14). TLR stimulation induces phenotypic changes in DCs, specifically the up-regulation of costimulatory molecules (UCM; e.g., CD40, CD80, and CD86) and the production of proinflammatory cytokines (e.g., IL-6 and TNF-α). These changes have been termed DC maturation, and prior reports have indicated that such changes may explain the ability of TLR-based adjuvants to augment adaptive immunity (1, 4). Importantly, the use of TLR-matured DCs may be an effective therapeutic strategy to treat cancer and provide protection against pathogens (15).

LPS, a TLR4 agonist, is contained in several adjuvants that are highly effective in both experimental and clinical settings (5, 11, 16, 17). TLR4 is unique, as follows: in contrast to the other TLRs, downstream signaling can occur via two independent pathways (4, 18). The first pathway depends on the MyD88 signal adaptor protein. This MyD88-dependent pathway is critical for the production of several proinflammatory cytokines, e.g., IL-6 and TNF-α. In contrast, MyD88 signaling is not required for UCM in response to TLR4 activation (19). This finding led to the discovery of a second, MyD88-independent pathway downstream of TLR4, which depends on the Toll IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF) signal adaptor protein (20, 21). A prior study indicated that the TRIF pathway was essential for UCM during LPS activation (22). In this report, UCM was shown to be a consequence of the type I IFN receptor pathway. However, this study predominantly focused on macrophages. Other studies have investigated the type I IFN signaling pathway within different DC subsets and found that the ability of plasmacytoid DCs (pDCs) to up-regulate CD40 in response to LPS was impaired in the absence of type I IFN signaling, and that conventional DCs required both MyD88 and type I IFNs or STAT1, a transcription factor that is critical for type I and type II IFN signaling, for maximal CD40 up-regulation during LPS activation (23–25). Other reports also found that STAT1 was only partially required for LPS-induced UCM on macrophages (26). In sum, we do not fully understand whether the mechanisms involved in macrophage activation in response to LPS stimulation are similar to those that occur within DCs.

In the current study, we examined the roles of the MyD88 and TRIF pathways in TLR4-induced activation in both macrophages and DCs. We used both in vitro and in vivo methods and...
two forms of TRIF mutant mice. We found, in agreement with a prior report (22), that the UCM is largely abrogated in thioglycolate-elicited peritoneal macrophages (TGC-PECs) when TRIF is absent. In contrast, we demonstrated that UCM on DCs occurs without TRIF signaling. We provide evidence that secreted factors, of which the type I IFNs are active components, play a more important role in the UCM in macrophages than DCs. Maximal UCM and T cell priming by LPS-activated DCs required both MyD88 and TRIF pathways. Hence, our results provide new information that the MyD88- and TRIF-dependent pathways, which were previously considered parallel signaling pathways, act synergistically within DCs to maximize DC maturation and T cell priming. This result may have important implications for future vaccine strategies.
Materials and Methods

Mice and reagents

B6.129/SvJ-MyD88<sup>fl/fl</sup> (denoted as MyD88<sup>−/−</sup>) mice were a gift from S. Akira (Osaka University, Osaka, Japan) and were backcrossed 10 times onto the C57BL/6 background. C57BL/6 WT, TLR4<sup>−/−</sup>, Myd88<sup>−/−</sup>, and Trif<sup>−/−</sup> mice were purchased from The Jackson Laboratory. B6;129 mice were purchased from Taconic Farms. B6;129 cells/mice manifested a similar phenotype to C57BL/6 counterparts in our assays (data not shown). All mice were kept in a specific pathogen-free condition. The Yale University Institutional Animal Care and Use Committee approved use of animals in this study.

*Escherichia coli* 0111:B4 (smooth LPS) was obtained from InvivoGen. LPS from Salmonella minnesota R595 (smooth LPS) and lipid A (containing a phosphorylated N-acetyl-glucosamine dimer) from *S. minnesota* R595 were obtained from Alexis and used for in vitro assays at 100 ng/ml or at the indicated dose. For in vivo LPS administration, mice were injected i.p., with LPS at 100 µg/mouse. Neutralizing goat IgGs directed against mouse IFN-α/β R1 were obtained from R&D Systems. Isotype control Ab was obtained from Sigma-Aldrich. The Abs were used at a final concentration of 5 µg/ml. For experiments with DCs, the Ab was added in combination with LPS for 6 h, whereas for macrophages it was added for 24 h. This is based on the time to peak UCM in DCs (Fig. 1D) and macrophages (Fig. 2B).

Cell preparation and MLR

Splenocytes were harvested and RBC were lysed by incubation with ammonium chloride solution. T cells were then purified via negative selection (>95% purity) using EasySep T cell enrichment kit (StemCell Technologies). Irradiated (28 Gy) allogeneic splenocytes or bone marrow-derived DCs (BMDCs) were used as APCs. BMDCs were prepared and cultured in vitro in the presence of GM-CSF, as per our previously published work (27). After 4 days of culture, the BMDCs were harvested and stimulated with 100 ng/ml LPS. Irradiated allogeneic BMDCs (1 × 10<sup>5</sup>, 3 × 10<sup>5</sup>, 1 × 10<sup>5</sup>) or whole splenocytes (1 × 10<sup>5</sup>) were cultured with T cells (1 × 10<sup>4</sup>) per well for 3 days in 96-well plates in complete Bruf’s medium (Invitrogen). At this point, [*]<sup>H</sup>thymidine was added to the wells and DNA was harvested and analyzed by a scintillation counter as an indicator of cell proliferation (PerkinElmer Life Science). Control wells included the following: T effector cells without APCs, medium only, and APCs only. Thiglycolate-elicited peritoneal cells were prepared as previously published work (28). Peritoneal cells were harvested 4 days after i.p. injection of TGC-PECs. The peritoneal washout cells were incubated to adhere on plastic 24-well plates for 2 h. Nonadherent cells were subsequently removed, and the remaining adherent cells were used as TGC-PECs. The resulting cells were >90% CD11b<sup>+</sup>.

For the experiments in which conditioned medium was used, BMDCs and TGC-PECs of each phenotype were stimulated with LPS in vitro. The conditioned medium was collected 24 h later and added to TLR4<sup>−/−</sup> BMDCs or TGC-PECs. At this point, the supernatants from BMDCs and TGC-PECs contained similar concentrations of cytokines (see Fig. 4, A and B). Expression of CD40 and CD86 on the TLR4<sup>−/−</sup> cells cultured with the conditioned medium was assessed via flow cytometry after 6 h of culture for DCs and 24-h culture for TGC-PECs. This is based on the time to peak UCM in DCs (Fig. 1D) and macrophages (Fig. 2B). Controls were TLR4<sup>−/−</sup> cells cultured with PBS.

Analysis of costimulatory molecule expression

Fluorescently labeled mAbs against CD8, CD11b, CD11c, CD40, CD80, CD86, and PDCA-1 were obtained from eBioscience, BD Biosciences, and Miltenyi Biotec. The staining procedure was performed as per our previously published work (27). Fluorescence was assessed via a FACSCalibur flow cytometer (BD Immunocytometry Systems), and data

FIGURE 2. LPS-induced UCM on peritoneal macrophages is mostly dependent on TRIF signaling in vitro. A, TGC-PECs from C57BL/6 WT, TLR4<sup>−/−</sup>, Myd88<sup>−/−</sup>, and Trif<sup>−/−</sup> mice were stimulated with PBS (shade histogram) or rough LPS (open histogram). MFI is shown adjacent each histogram (gray, PBS; black, LPS). B, Time course of the change of MFI between PBS- and LPS-treated cells for each genotype during LPS activation of TGC-PECs. C, TGC-PECs from C57BL/6 WT, TLR4<sup>−/−</sup>, Myd88<sup>−/−</sup>, Trif<sup>−/−</sup>, and IRF-3<sup>−/−</sup> mice were stimulated with smooth or rough LPS (100 ng/ml) for 24 h, and CD40 and CD86 expression was examined. The data are representative of three independent experiments. The change in MFI between PBS-treated and LPS-activated cells for each genotype is shown.
analyzed using FlowJo software (Tree Star). We determined that >90% of CD11c<sup>+</sup> PDCA-1<sup>+</sup> splenic cells were B220<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>+</sup> pDCs (data not shown).

ELISA

Concentration of IL-6 and IFN-β in serum of LPS-injected mice or IL-6, IL-10, IL-12p40, and TNF-α in the culture supernatant of LPS-stimulated BMDCs or TGC-PECs was measured by ELISA kits from BD Biosciences and PBL Biomedical Labs, according to manufacturer’s instructions.

Statistical analysis

Repeated measures were evaluated with a two-way ANOVA. Comparison of means was analyzed by Student’s t test. Significance was evaluated at a p value < 0.05. Statistical analysis was performed on GraphPad Prism Software.

Results

LPS-induced UCM on DCs occurred without TRIF or MyD88 signaling in vitro

We first stimulated wild-type (WT), Myd88<sup>−/−</sup>, and TRIF-deficient (Trif<sup>−/−</sup>) murine BMDCs with LPS in vitro. We found that Trif<sup>−/−</sup> BMDCs can up-regulate both CD40 and CD86 after 6 h of LPS stimulation, although the response was partly reduced compared with WT and Myd88<sup>−/−</sup> BMDCs (Fig. 1A). We obtained similar results when we used BMDCs derived from mice where the TRIF gene was specifically deleted (TRIF<sup>−/−</sup>; Fig. 1A) (20), and this effect persisted across a range of LPS doses (Fig. 1B). As expected, TLR4<sup>−/−</sup> BMDCs did not up-regulate costimulatory molecules in response to LPS (Fig. 1, A and B). We obtained similar results when we stimulated BMDCs with lipid A, the biologically active moiety of LPS (Fig. 1C). Furthermore, we observed similar results when we stimulated cells with either rough or smooth forms of LPS, and we noted that IRF-3<sup>−/−</sup> DCs manifested a similar phenotype to TRIF-deficient cells (Fig. 1C).

Hence, our results suggest that TRIF signaling is not essential for UCM on DCs in response to LPS activation in vitro.

Next, we investigated the importance of TRIF and MyD88 signaling over a time course. We found that WT, Myd88<sup>−/−</sup>, and Trif<sup>−/−</sup> BMDCs all up-regulated costimulatory molecules during a 24-h time course of LPS treatment, whereas TLR4<sup>−/−</sup>
BMDCs failed to do so (Fig. 1D). We found that Trif<sup>−/−</sup> BMDCs had reduced UCM relative to WT BMDCs across the time course, although the kinetics of the response was similar for CD40, CD80, and CD86 (Fig. 1D). By 24 h post-LPS stimulation, Tri<sup>−/−</sup> BMDCs manifested similar CD86 up-regulation compared with TLR4<sup>−/−</sup> DCs (Fig. 1D). In Myd88<sup>−/−</sup> BMDCs, the up-regulation of CD86 was not impaired during LPS activation; in fact, by 24 h, Myd88<sup>−/−</sup> DCs manifested a superior ability to up-regulate CD86 compared with WT DCs (Fig. 1D). In contrast, Myd88<sup>−/−</sup> BMDCs manifested an impaired ability to up-regulate CD40 and CD80 6 h post-LPS activation compared with WT DCs. However, the levels of CD40 and CD86 were comparable in Myd88<sup>−/−</sup> and WT DCs by 24 h post-LPS activation (Fig. 1D). Thus, we found that both TRIF and MyD88 are involved, but neither alone is critical for UCM on DCs in response to LPS stimulation. Furthermore, these data suggest that MyD88 and TRIF have distinct roles in up-regulating different costimulatory molecules during LPS activation. TRIF signaling is important for maximal and sustained up-regulation of CD86 after LPS activation, whereas MyD88 plays no role in this effect. Additionally, MyD88 and TRIF may synergize to rapidly up-regulate CD40 and CD80 during LPS activation.

UCM on thioglycollate-elicited peritoneal macrophages during LPS activation is largely TRIF dependent

Our results obtained with DCs appear to be in contrast with a prior report that examined the role of TRIF signaling in the UCM during LPS activation (22). However, in this study, the predominant cell type used was TGC-PECs rather than DCs. Hence, we examined the role of TRIF and MyD88 signaling in the UCM in this cell type during LPS activation. We found that the UCM was almost completely abrogated in Tri<sup>−/−</sup> cells after 24 h of LPS stimulation, whereas Myd88<sup>−/−</sup> macrophages manifested a similar phenotype to WT LPS-activated macrophages (Fig. 2A). During time course experiments, we noted a small degree of UCM in the Tri<sup>−/−</sup> macrophages during the early phase of LPS activation (Fig. 2B). Finally, we obtained similar results when we compared smooth and rough LPS and found that IRF-3<sup>−/−</sup> macrophages demonstrated a similar phenotype to Tri<sup>−/−</sup> macrophages (Fig. 2C). These results indicate that the importance of TRIF signaling for UCM during LPS stimulation is different between DCs and macrophages. Furthermore, the data indicate that both smooth and rough forms of LPS induce similar responses in our assays. Because of this, smooth LPS is used for the remainder of the manuscript.

LPS-induced UCM on DCs occurred without TRIF or MyD88 signaling in vivo

We next examined whether TRIF or MyD88 signaling was critical for UCM after LPS activation in vivo. We injected mice with LPS or PBS, then isolated their spleen cells 3 h later and assessed the UCM on splenic DCs. Consistent with our in vitro studies, LPS induced up-regulation of CD40 and CD86 on WT, Myd88<sup>−/−</sup>, and TRIF-deficient CD11c<sup>+</sup> DCs (Fig. 3A). As expected, LPS failed to induce UCM in TLR4<sup>−/−</sup> DC11c<sup>+</sup> DCs (Fig. 3A). Additionally, we found that UCM occurred without TRIF or MyD88 signaling across a time course (Fig. 3B). In agreement with our in vitro results, MyD88 signaling on its own was not important for CD86 up-regulation, whereas TRIF signaling played a role. However, in CD40 and CD80 up-regulation, both MyD88 and TRIF signaling were important, indicating synergy between these pathways (Fig. 3B). Lastly, we assessed the UCM within the major DC subsets, pDCs, lymphoid DCs (CD8<sup>+</sup>, CD11b<sup>−</sup>), and myeloid DCs (CD8<sup>−</sup>, CD11b<sup>+</sup>). TRIF signaling was important for LPS-induced CD86 up-regulation on all DC subsets (Fig. 3C). TRIF signaling was also involved in LPS-induced CD40 up-regulation on pDCs and lymphoid DCs (Fig. 3C). However, in all of these cell types, TRIF-deficient DCs were able to UCM to some degree after in vivo LPS administration, whereas TLR4<sup>−/−</sup> cells failed to respond (Fig. 3C). MyD88 signaling was important for LPS-induced CD40 up-regulation on pDCs and lymphoid and myeloid DCs. In sum,

FIGURE 4. Impact of TRIF or MyD88 signaling on proinflammatory cytokines in BMDCs and TGC-PECs. A. C57BL/6 WT, TLR4<sup>−/−</sup>, Myd88<sup>−/−</sup>, and Tri<sup>−/−</sup> BMDCs were stimulated with LPS. Both Myd88<sup>−/−</sup> and Tri<sup>−/−</sup> BMDCs manifested a reduced production of IL-6, TNF-α, IL-12p40 vs WT BMDCs (Myd88<sup>−/−</sup>; p < 0.0001 vs WT, Tri<sup>−/−</sup>; p < 0.009 vs WT, for each of these cytokines). However, the response in Myd88<sup>−/−</sup> BMDCs was inferior to Tri<sup>−/−</sup> BMDCs (p < 0.02, for each cytokine). With IFN-β, both Myd88<sup>−/−</sup> and Tri<sup>−/−</sup> BMDCs manifested an inferior response vs WT (p = 0.001, Myd88<sup>−/−</sup> vs WT; p = 0.0003, Tri<sup>−/−</sup> vs WT), but Tri<sup>−/−</sup> BMDCs manifested an inferior response vs Myd88<sup>−/−</sup> BMDCs (p = 0.009, Myd88<sup>−/−</sup> vs Tri<sup>−/−</sup>). For LPS-induced IL-10 production, Myd88<sup>−/−</sup> BMDCs manifested an inferior response vs WT (p < 0.0001). IL-10 responses of Tri<sup>−/−</sup> BMDCs were similar to WT BMDCs. B. Both Myd88<sup>−/−</sup> and Tri<sup>−/−</sup> TGC-PECs manifested reduced production of IL-6 and TNF-α vs WT TGC-PECs in response to LPS (Myd88<sup>−/−</sup> and Tri<sup>−/−</sup>; p < 0.0001 vs WT for each cytokine). No significant differences were noted between Myd88<sup>−/−</sup> and Tri<sup>−/−</sup> TGC-PEC cells. Both Myd88<sup>−/−</sup> and Tri<sup>−/−</sup> TGC-PECs manifested a reduced production of IL-6 and TNF-α vs WT TGC-PECs (Myd88<sup>−/−</sup> and Tri<sup>−/−</sup>; p < 0.0001 vs WT for each cytokine). However, the response in Myd88<sup>−/−</sup> response was inferior to the Myd88<sup>−/−</sup> response (p = 0.01). C. C57BL/6 WT, TLR4<sup>−/−</sup>, Myd88<sup>−/−</sup>, and Tri<sup>−/−</sup> mice were injected with 100 μg of LPS (or PBS, as indicated). Serum IL-6 production was reduced in both LPS-treated Myd88<sup>−/−</sup> (p = 0.0001) and Tri<sup>−/−</sup> mice (p = 0.009) vs WT counterparts. Myd88<sup>−/−</sup> mice manifested an inferior response vs Tri<sup>−/−</sup> mice (p = 0.002). Serum IFN-β production was reduced in LPS-treated Tri<sup>−/−</sup> mice (p = 0.005), but was not impaired in LPS-treated Myd88<sup>−/−</sup> mice (p = 0.3) vs WT counterparts. Results are representative of three independent experiments with a total of six mice/group.
both MyD88 and TRIF are involved for UCM in DCs in response to in vivo LPS administration. However, neither pathway on its own is essential for either response.

**Differential importance of TRIF and MyD88 in LPS-induced cytokine production between BMDCs and TGC-PECs**

We next examined the importance of TRIF signaling in the production of proinflammatory cytokines by DCs in our assays. In agreement with prior work, Myd88−/− BMDCs manifested a severely impaired ability to produce IL-6, TNF-α, and IL-12p40 in response to LPS (Fig. 4A) (29). Without TRIF signaling, the production of these cytokines was reduced 3-fold (Fig. 4A). In contrast, TRIF signaling was required for IFN-γ production in response to LPS activation in agreement with a prior report (21), whereas without MyD88 signaling IFN-γ levels were reduced 5-fold (Fig. 4A). Additionally, we found that MyD88 signaling was more important than TRIF signaling for LPS-induced IL-10 production (Fig. 4A). However, IL-10−/− BMDCs and macrophages were able to up-regulate costimulatory molecules to a similar degree as WT cells (data not shown).

We next examined the inflammatory responses produced by LPS-activated peritoneal macrophages. We found that in contrast to BMDCs, both TNF-α and IL-6 production were completely abrogated in the absence of either MyD88 or TRIF (Fig. 4B). TRIF-deficient cells manifested a completely abrogated IFN-γ response, whereas Myd88−/− cells produced this cytokine, but at lower levels than the response noted with WT cells (Fig. 4B). These results indicate that there are some differences between macrophages and DCs in LPS-induced proinflammatory responses.

When LPS was administered in vivo, we demonstrated that systemic IL-6 production was completely MyD88 dependent, whereas without TRIF signaling IL-6 production was reduced 3-fold (Fig. 4C). In contrast, we noted that systemic IFN-γ production was completely TRIF dependent, whereas without MyD88 signaling the responses were similar to WT. In sum, MyD88 and TRIF signaling pathways play distinct roles in proinflammatory cytokine production after TLR4 activation between macrophages and DCs. In DCs, MyD88 is critical for LPS-induced IL-6, IL-12p40, and TNF-α production, whereas TRIF plays a more important role in IFN-γ production.

**LPS-induced secreted factors play a larger role in the UCM in peritoneal macrophages as compared with BMDCs**

We next examined the importance of secreted factors in the UCM during LPS activation of BMDCs and TGC-PECs. We harvested conditioned medium from BMDCs or TGC-PECs after stimulation with LPS for 24 h and used this medium to culture TLR4−/− cells for 6 h for DCs and 24 h for TGC-PECs (in A, the TLR4−/− cells were BMDCs; in B, they were TGC-PECs). These time points were chosen based on the time to peak UCM during LPS stimulation in DCs (Fig. 1D) and TGC-PECs (Fig. 2B). After this point, expression of CD40 and CD86 was assessed. *p < 0.01. The graphs show the change in MFI in TLR4−/− cells cultured in harvested medium (for each genotype shown on x-axis) vs TLR4−/− cells cultured in PBS. C and D, BMDCs and TGC-PECs were stimulated with LPS in the presence of isotype control or IFN-α/β-neutralizing Ab, as detailed in Materials and Methods. Expression of CD40 and CD86 was subsequently measured. *p < 0.01. The data are representative of three independent experiments with similar results.
Conditioned medium harvested from LPS-activated WT BMDCs induced the UCM in TLR4−/− BMDCs to a small degree (~10–15 arbitrary unit increase in mean fluorescence intensity (MFI)) (Fig. 5A). In contrast, conditioned medium from LPS-activated WT macrophages induced a larger degree of UCM in TLR4−/− macrophages (~30 MFI arbitrary unit increase, a 3-fold larger increase than the corresponding experiment in BMDCs) (Fig. 5, A and B). TLR4−/− cells cultured in either medium originating from LPS-activated Myd88−/− or Trifps2A→B2 cells induced a smaller degree of UCM compared with medium harvested from WT cells (Fig. 5, A and B). When TLR4−/− BMDCs were cultured in medium harvested from LPS-activated WT BMDCs or TGC-PECs, we found that TLR4−/− BMDCs were equally able to UCM regardless of whether the conditioned medium originated from DCs or TGC-PECs (~10–15 arbitrary unit increase in MFI induced by either medium, data not shown). In sum, these data indicate that secreted factors play a larger role for LPS-induced UCM in TGC-PECs than in BMDCs.

We next examined whether neutralizing the type I IFNs would inhibit the UCM in either LPS-activated BMDCs or peritoneal macrophages, because prior work has indicated that the type I IFNs are sufficient to induce UCM in macrophages (22). The addition of a type I IFN receptor antagonist impaired the ability of WT BMDCs and TGC-PECs to up-regulate CD86, although type I IFN receptor inhibition had a greater effect in impairing CD86 up-regulation in macrophages than in DCs (Fig. 5, C and D). For CD40 up-regulation, the effect of type I IFN receptor inhibition was similar between BMDCs and macrophages. Type I IFN receptor antagonism had no effect on TRIF-deficient cells, whereas the UCM in LPS-activated

**FIGURE 6.** LPS increased the T cell-priming capabilities of DCs in the absence of TRIF signaling in vitro. C57BL/6 WT, TLR4−/−, Myd88−/−, and Trifps2A→B2 BMDCs were stimulated with LPS. Experiments were performed after either 3 h of stimulation (A) or 24 h of stimulation (B). After each time point, the BMDCs were washed, irradiated, and then cultured with purified allogeneic BALB/c T cells. The graphs display the change in T cell proliferation (thymidine incorporation) and IL-2 production between PBS- and LPS-activated DCs for each experimental group. Data are representative of three independent experiments with similar results. A, LPS-activated Myd88−/− BMDCs induced inferior IL-2 production (p = 0.02) and T cell proliferation (p = 0.01) vs WT BMDCs. Trifps2A→B2 BMDCs manifested a similar phenotype to WT DCs. The augmentation of IL-2 induced by LPS-activated Myd88−/− BMDCs was similar to LPS-activated TLR4−/− BMDCs, although LPS-activated Myd88−/− BMDCs induced a higher level of T cell proliferation vs LPS-activated TLR4−/− BMDCs (p = 0.03). B, LPS-activated Myd88−/− BMDCs and Trifps2A→B2 BMDCs induced reduced T cell IL-2 responses compared with WT BMDCs at 24 h. These responses were greater than those induced by TLR4−/− LPS-activated BMDCs (IL-2; Myd88−/−, p = 0.04; Trifps2A→B2, p = 0.0002). LPS-stimulated Myd88−/− BMDCs induced similar enhancement of T cell proliferation compared with those induced by LPS-activated TLR4−/− BMDCs, whereas LPS-induced Trifps2A→B2 BMDCs did enhance T cell proliferation (p = 0.009) vs LPS-activated TLR4−/− DCs.

**FIGURE 7.** LPS treatment in vivo augmented ability of APCs to prime T cells independently of TRIF signaling. C57BL/6 WT, Myd88−/−, TLR4−/−, and Trifps2A→B2 mice were injected with 100 μg of LPS or PBS. Splenocytes were harvested from these mice at 0, 4.5, and 9 h; irradiated; and cultured with allogeneic BALB/c T cells. A, The graph shows the change in T cell proliferation (thymidine incorporation) induced between PBS- and LPS-activated APCs of each genotype. Proliferation induced by Myd88−/− splenocytes was inferior to that induced by WT cells (p = 0.004) and Trifps2A→B2 cells (p = 0.0001). B, The graph shows the change in IL-2 production between PBS- and LPS-activated APCs for each genotype. Both LPS-stimulated WT and Trifps2A→B2 splenocytes induced the production of IL-2 by allogeneic T cells as compared with PBS-stimulated cells. This effect was abrogated in Myd88−/− and TLR4−/− splenocytes. The results are representative of two independent experiments with a total of six mice/group. PBS; LPS.
Myd88\(^{-/-}\) cells was impaired by this receptor antagonist. This is most likely due to the fact that LPS-induced type I IFN production is completely abrogated in TRIF-deficient cells (Fig. 4, A and B), whereas the type I IFN response is only partly abrogated in Myd88\(^{-/-}\)/H11002 cells. The amount that the type I IFN receptor antagonist inhibited the UCM in WT DCs or macrophages in response to LPS activation was generally similar to the amount of UCM induced on TLR4\(^{-/-}\)/H11002 DCs or macrophages cultured in the presence of the conditioned medium from LPS-activated WT cells (Fig. 5, compare A and B with C and D). For example, type I IFN receptor inhibition reduced CD86 up-regulation on WT TGC-PECs by 30 MFI arbitrary units, whereas conditioned medium from WT LPS-activated macrophages increased CD86 on TLR4\(^{-/-}\) macrophages by \(-35\) MFI arbitrary units. This indicates that the type I IFNs are major active components of the secreted factors that induce UCM during LPS stimulation in both DCs and macrophages.

LPS-activated TRIF-deficient BMDCs induced superior T cell priming compared with MyD88\(^{-/-}\) BMDCs

LPS-induced maturation of DCs allows these cells to maximally prime T cells (3, 18). We next compared the importance of MyD88 and TRIF signaling in this effect. C57BL/6 (H2\(^{b}\)) BMDCs were stimulated with LPS for 3 or 24 h, washed, irradiated, and cultured with purified allogeneic BALB/c T cells. T cell proliferation (thymidine incorporation) and IL-2 production were measured. The change between PBS- and LPS-activated DCs for each experimental group was plotted. Data are representative of three independent experiments with similar results.
T cell priming in this assay. MyD88 plays a more critical role in this process, especially following a brief period of LPS stimulation.

**TRIF is not required for augmenting the ability of in vivo LPS-stimulated APCs to enhance T cell priming**

We next examined the role MyD88 and TRIF signaling in the ability of TLR4-activated APCs to enhance T cell priming using in vivo approaches. Hence, mice were injected i.p. with LPS or PBS, and spleen cells were harvested at the indicated time points. We then measured the activity of these irradiated cells (C57BL/6, H2b) to stimulate allogeneic (BALB/c, H2d) T cells. We found that spleen cells from LPS-treated Trif−/− mice and LPS-treated WT mice were equally able to augment T cell priming, and these responses were superior to the augmentation of priming induced by LPS-activated Myd88−/− BMDCs (Fig. 7). In all of the aforementioned groups, the response was superior to spleen cells from LPS-treated TLR4−/− mice (Fig. 7). These data demonstrate that MyD88 signaling within APCs during in vivo TLR4 activation is important for enhancing T cell priming, whereas TRIF is dispensable for this process.

The combination of MyD88 and TRIF is critical for DC maturation in response to LPS and subsequent T cell priming

Because some of our experiments indicated that synergy exists between the MyD88 and TRIF pathways during TLR4 activation, we conducted experiments with DCs or TGC-PECs that were deficient in both MyD88 and TRIF. Myd88/Trif DKO BMDCs or BMDCs (Fig. 8, A–C). Furthermore, LPS-activated DKO or TLR4−/− BMDCs failed to augment T cell priming in the MLR in contrast to WT LPS-activated BMDCs (Fig. 8, D and E). These data demonstrate that in our assays the MyD88 and TRIF signaling pathways together fully account for UCM in LPS-activated DCs and macrophages and augmentation of T cell priming by LPS-activated DCs.

**Discussion**

In our study, we compared the roles of MyD88 and TRIF in LPS-induced activation between BMDCs and peritoneal macrophages. In DCs, we found that neither MyD88 nor TRIF was critical for LPS-induced UCM (Figs. 1 and 3). In contrast, in macrophages, LPS-induced UCM was largely TRIF dependent (Fig. 2). LPS-induced TRIF-dependent, secreted factors played a more important role in UCM in macrophages than in DCs, especially for CD86 up-regulation (Fig. 5). We found that LPS-induced production of proinflammatory cytokines, IL-6, IL-12p40, and TNF-α by DCs, was completely abrogated without Myd88 signaling in agreement with a prior study (19), whereas this response was generally reduced 2- to 4-fold without TRIF signaling (Fig. 4A). However, in DCs, TRIF was critical for the production of IFN-β after LPS activation with MyD88 playing a less important role (Fig. 4A). In macrophages, we found that either MyD88 or TRIF was critical for the production of IL-6 and TNF-α, but only TRIF signaling was absolutely critical for IFN-β production (Fig. 4B). Hence, our study has demonstrated a differential requirement for TRIF signaling in LPS activation of DCs vs macrophages, with TRIF playing a larger role in the latter cell type than the former.

Our results indicate that the type I IFNs are major active components of the secreted factors that induce UCM during LPS activation, which agrees with prior work (22). This is suggested by our results that demonstrated that the degree of inhibition of UCM during LPS activation by type I receptor antagonism was similar to the induction of UCM induced by conditioned medium from LPS-activated cells (Fig. 5). Furthermore, type I IFNs are most likely important components of the secreted factors released in response to LPS, via both the MyD88- and TRIF-dependent pathways, which promote UCM. This is supported by our data that both TRIF- and MyD88-deficient cells (DCs or macrophages) manifested an impaired ability to produce IFN-β in response to LPS activation (Fig. 4, A and B). Our study has not ruled out that other secreted factors may also play a role in UCM during LPS stimulation. Because TLR4−/− BMDCs were equally able to UCM when cultured in conditioned medium harvested from either LPS-activated WT BMDCs or WT TGC-PECs, our study implies TGC-PECs are more susceptible than BMDCs to LPS-induced UCM mediated by secreted factors. Moreover, cell-intrinsic mechanisms are also involved in LPS-induced UCM, and our work suggests that these mechanisms are more important in DCs than macrophages. Future work will be required to examine the nature of such mechanisms, and the mechanisms by which macrophages are more susceptible than DCs to LPS-induced UCM mediated by secreted factors.

Functionally, we found that MyD88 signaling within DCs played a more important role than TRIF in enhancing adaptive T cell responses, although the maximal ability of LPS-matured DCs to prime adaptive T cell responses was dependent on both MyD88 and TRIF. Both the production of proinflammatory cytokines and UCM on DCs are important for priming adaptive T cell responses. Our results suggest that inflammatory cytokines contribute to a larger degree in this process than the UCM because the effects of LPS in increasing a DC’s ability to prime T cells were largely diminished when stimulated by allogeneic LPS-activated Myd88−/− DCs. In these DCs, the production of proinflammatory cytokines, IL-6, IL-12, and TNF-α, was completely abrogated, but the UCM was only impaired to a minor degree during LPS activation in comparison with WT DCs. In LPS-activated TRIF-deficient DCs, both the production of these inflammatory cytokines and the UCM were only partially impaired. However, these DCs manifested a superior ability to augment T cell priming compared with Myd88−/− DCs. Hence, these results suggest that efforts to boost immune responses in susceptible populations should focus on strategies to augment the inflammatory milieu induced by DCs.

A previous study has demonstrated that Myd88 signaling in DCs is not required for UCM during LPS activation (19). This led to the concept that a Myd88-independent pathway downstream of TLR4 must exist. Subsequent studies revealed the presence of several signal adaptors downstream of TLR4, including TIR domain-containing adaptor protein (TIRAP) and TRIF (30). Prior studies demonstrated that several characteristics of the Myd88-independent pathway were due to signaling via TRIF (20, 21). In particular, a prior report showed that LPS-induced UCM and the production of TNF-α were completely abrogated in TRIF-deficient macrophages (22). Our report is in general agreement with this prior study, although we did find that TRIF-deficient macrophages were able to UCM to a small degree during LPS activation after 6 h (Fig. 2B). The difference between our report and the previous study is that the latter did not assess the response at this early phase of LPS activation, but only after 24 h of stimulation. After 24 h of LPS stimulation, both our study and the prior report demonstrate that UCM is almost entirely TRIF dependent. In contrast to these findings, our study found that TRIF signaling was less important for UCM and for the production of IL-6, IL-10, IL-12p40, and TNF-α in DCs upon LPS activation. Our work indicates that in DCs, both Myd88 and TRIF are required for LPS-induced UCM.

In conclusion, we have determined that Myd88 and TRIF participate together in transducing signals downstream of TLR4 in
DCs. In macrophages, there is a greater dependency on TRIF signaling for LPS-induced UCM. These results imply that maximal immune-boosting properties of TLR4-based DC immunotherapy for cancer and infectious diseases may require dual signaling via MyD88 and TRIF.

Disclosures
The authors have no financial conflict of interest.

References


