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TLR4-mediated pro-inflammatory dendritic cell differentiation in humans requires the combined action of MyD88 and TRIF

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Abstract

TLR4 ligation can activate both the MyD88 and the Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) signaling route. Whereas MyD88 is generally recognized as a universal adaptor for pro-inflammatory responses, TRIF is mainly thought to contribute to specific type I IFN responses. Here, we investigated the contribution of both MyD88 and TRIF to TLR4-mediated pro-inflammatory dendritic cell (DC) differentiation in human. Pro-inflammatory cytokine induction was strongly decreased in monophosphoryl lipid A- and LPS-matured monocyte-derived DCs when either MyD88 or TRIF were down-regulated by small interfering RNA electroporation. Induction of co-stimulatory molecule expression was entirely dependent on the TRIF pathway. Our results demonstrate that in human DCs the TRIF pathway is important for overall pro-inflammatory DC differentiation via TLR4 by mediating co-stimulation and playing a non-redundant role in pro-inflammatory cytokine induction.

Keywords

MPLA, LPS, siRNA, maturation, TLR4

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Introduction

Dendritic cells (DCs) are professional APCs that reside in peripheral tissues and function as sensors for various danger signals. These danger signals include PAMPs, which are recognized by PRRs. One class of PRRs is the TLR family. There are 10 human and 12 murine TLRs described, each of which is triggered by a distinct set of PAMPs. TLRs are expressed on the plasma membrane or in endosomal compartments, and can be divided into different sets based on their specific adaptor molecules, which propagate TLR signaling. TLR3, which recognizes double-stranded RNA, signals via the TIR-domain-containing adapter-inducing IFN-β (TRIF) adaptor molecule, while all other TLRs signal via the MyD88 adaptor after ligation with their ligand.[1–3] The only TLR that can use both adaptor molecules is TLR4.

TLR4 is expressed at the plasma membrane. After ligation it is internalized via endocytosis and induces signaling.[4,5] At the plasma membrane TLR4 associates with MyD88 adaptor like (Mal) and MyD88, which induces signaling via the MyD88-dependent pathway, leading to recruitment of IL-1 receptor-associated kinases, phosphorylation of the MAPKs ERK, JNK and p38, and activation of transcription factors activator protein 1 and NF-κB.[6] Signaling via the MyD88-dependent pathway leads to up-regulation of pro-inflammatory cytokines like IL-6, IL-12 and TNF-α.[7–11] After endocytosis of TLR4, Mal and MyD88 dissociate from TLR4, and TLR4 associates with TRIF-related adaptor molecule and TRIF.[5,12] Signal

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transduction via the MyD88-independent and TRIF-dependent pathway causes phosphorylation of IFN regulatory factor 3 and delayed nuclear translocation of NF-κB.[13,14] Signaling via the TRIF pathway eventually leads to secretion of chemokines, such as RANTES and IP-10,[10,14] induction of type I IFNs[6,15,16] and up-regulation of co-stimulatory molecules.[17,18]

The role of MyD88 in TLR4-mediated induction of pro-inflammatory cytokines is clearly established.[10,19–21] It is, however, still a matter of debate whether, upon TLR4 activation, the TRIF signaling pathway contributes to induction of pro-inflammatory cytokines in general or is limited to the antiviral type I IFNs. For example, production of TNF-α has been attributed to the MyD88 pathway independently of the TRIF pathway,[10] while others have described pro-inflammatory cytokine production as being dependent on the TRIF pathway,[18] or both TRIF and MyD88 pathways.[17,22] For APCs of human origin even less is known about the roles of TRIF or MyD88 in the induction of cytokines and co-stimulatory molecules upon TLR4 stimulation. Most research into the roles of TRIF and MyD88 in TLR4 signaling has been performed on human cell lines transfected with TLR4, MD2, CD14 and reporter genes. In this study we investigated the role of the TRIF and MyD88 pathways in TLR4-stimulated primary monocyte-derived DCs by specific down-regulation of either MyD88 or TRIF by small interfering RNA (siRNA). We show that after TLR4 stimulation with different TLR4 ligands, both the MyD88 and the TRIF pathway are essential for pro-inflammatory DC differentiation. In contrast, the TLR4 induced up-regulation of co-stimulatory molecules on DCs is only dependent on the TRIF pathway.

Materials and methods

Generation of monocyte-derived DCs

Monocytes were isolated from fresh aphaeresis material of healthy volunteers (Sanquin Blood Supply, Amsterdam, the Netherlands) upon informed consent by using the Elutra cell separation system (Gambro, Lakewood, CO, USA). The purity of monocytes was confirmed by flow cytometry. Monocytes were cultured at a concentration of 1×10^6 cells/ml in 20 ml Cellgro DC serum-free culture medium supplemented with granulocyte–macrophage CSF (1000 IU/ml), IL-4 (800 IU/ml) (Cellgenix, Freiburg, Germany), and penicillin (100 U/ml) and streptomycin (100 μg/ml), (Invitrogen, Breda, the Netherlands) in a 80 cm^2 cell culture flask (Nunc, Roskilde, Denmark). After 6 d of culture immature DCs (imDCs) were harvested for further experiments. ImDCs were matured with 2.5 μg/ml monophosphoryl lipid A (MPLA) from *Salmonella enterica sv*. Minnesota Re595, 20 μg/ml pI:C (Sigma Aldrich, Steinheim, Germany), 5 μg/ml R848 or 50 ng/ml LPS from *Escherichia coli* O111:B4 (Invivogen, San Diego, CA, USA) in the presence or absence of 1000 IU/ml IFN-γ (Immukine, Boehringer Ingelheim, Alkmaar, the Netherlands). In case DCs were stimulated with LPS, 1% FCS was added to Cellgro medium. imDCs were cultured at a concentration of 2×10^5 DC/well in a 48-well plate (Nunc). For each experiment, maturation of DCs after TLR stimulation or without stimulation was assayed by flow cytometry (data not shown). Culture supernatants were harvested 24–48 h after addition of stimuli. Expression of cell surface molecules was determined after 48 h.

Down-regulation of TRIF or MyD88

TRIF or MyD88 was knocked down by electroporation with ON-TARGET plus SMARTpool control siRNA, or specific siRNA targeting either MyD88 or TRIF, (Thermo Scientific, Lafayette, CO, USA). Per pulse, 1×10^6–5×10^6 imDCs were electroporated with 3 μg siRNA/1×10^6 cells in 200 μl Cellgro culture medium in 4-mm cuvettes (BioRad, Carlsbad, CA, USA) using the BioRad Gene Pulser Xcell (250 V, 150 μF) (BioRad). After electroporation, DCs were re-suspended in culture supernatant and allowed to rest for 2 d before stimulation.

Flow cytometry

Expression of cell surface molecules was determined using a Beckton Dickinson FACS CANTO II (BD Biosciences, San Jose, CA, USA) and analyzed with FACS DIVA software (BD Biosciences). For analysis of expression of co-stimulatory molecules DCs were washed with PBA (PBS supplemented with 0.5% BSA) and incubated with 50 μl mAb or the appropriate isotype control in PBA and 3 mg/ml human γ-globulin in the dark, at 4°C, for 30 min. Abs used were as follows: FITC-conjugated IgG1 isotype control and phycoerythrin (PE)-conjugated IgG2a isotype control [purchased from Sanquin Reagents (Amsterdam, the Netherlands)] and APC-conjugated IgG1 isotype control, anti-human leukocyte antigen (HLA)-DR-PE, anti-CD83-APC, anti-CD40-FITC, anti-CD80-FITC, anti-CD86-APC (BD Biosciences). Cells were washed twice and re-suspended in PBA. DAPI (Sigma-Aldrich) was added immediately before analysis to stain for cell viability and to exclude dead cells from analysis.

Cytokine production

Production of IL-12p70, IL-6, TNF-α and RANTES was determined by ELISA. For detection of TNF-α and IL-6 PeliKine-compact ELISA kit (Sanquin Reagents) was used. For the detection of IL-12p70 a

combination of BT-21 mAb (Diaclone, Besançon, France) and C8.6 (BD Biosciences) was used in an ELISA. For detection of RANTES a DuoSet ELISA kit (R&D systems, Minneapolis, MN, USA) was used.

RT-qPCR

Total RNA was extracted from 1×10^6 cells by peqGOLD Trifast (Peqlab Biotechnologie, Erlangen, Germany). Glycoblue (Invitrogen) was added as a carrier, and total RNA was isolated according to the manufacturer's instructions. RNA concentration was quantified, and single-strand cDNA was synthesized using random hexamers (Invitrogen) and a SuperScript II RNAse H-reverse transcriptase kit (Invitrogen). Gene expression was determined by real-time qPCR using the Applied Biosystems StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers and probes used were as follows: 18 s (quantitect primer assay; Qiagen, Hilden, Germany); MyD88 (quantitect primer assay; Qiagen), TRIF primer sequence—forward primer GCAGCCCCGGAT CCCT, reverse primer TGTCCCCTACCCATTCAC TGTT; probe: FAM-CTTGGGCAGCTCCTGCA GAACCTG-TAMRA, (Eurogentec, Liège, Belgium). Results were normalized to internal control 18 s and expressed relative to control electroporated DCs.

Statistical analysis

Data are expressed as mean ± SEM. A paired *t*-test was performed to compare cytokine levels and values were compared with non-targeting control siRNA (siCTRL). To compare induction of co-stimulatory molecules a two-way ANOVA was used. Analyses were performed using GraphPad Prism 5.01 software (GraphPad, Dan Diego, CA, USA).

Results

TRIF and MyD88 are down-regulated after electroporation with specific siRNA

To determine the role of the TRIF and MyD88 pathways in human DCs after TLR4 stimulation, either TRIF or MyD88 was down-regulated in imDCs by electroporation with specific siRNA. imDCs were electroporated with siCTRL or siRNA targeting either MyD88 (siMyD88) or TRIF (siTRIF) and were allowed to rest. Two d after electroporation, expression of TRIF or MyD88 was determined at the mRNA level by RT-qPCR. Electroporation with siRNA targeting TRIF led to 50% down-regulation of TRIF and had no effect on the expression of MyD88, while treatment with siRNA targeting MyD88 led to 70% down-regulation of MyD88 mRNA and did not affect TRIF expression (Figure 1A).

To confirm specificity of MyD88 and TRIF down-regulation, DCs were stimulated with TLR3 ligand poly I:C (pI:C), which exclusively signals via the TRIF-mediated pathway, or TLR7/8 ligand R848, which exclusively signals via MyD88. IFN-γ was added to optimize DC maturation and cytokine production. TRIF down-regulation strongly inhibited pI:C-induced production of IL-6, IL-12 and TNF-α. In contrast, down-regulation of MyD88 did not affect production of TNF-α and IL-12, but, surprisingly, production of IL-6 was slightly reduced (Figure 1B). In R848-stimulated DCs down-regulation of MyD88 significantly inhibited IL-6, IL-12 and TNF-α production, while down-regulation of TRIF did not affect IL-6, IL-12 or TNF-α production (Figure 1C). Thus, down-regulation of MyD88 or TRIF can be successfully achieved in human primary DCs and specifically affects MyD88- or TRIF-dependent signaling cascades.

Although it has been described that siRNAs can activate TLR7 and thereby induce IFN-α production via MyD88,[23] we do not expect that electroporation with siRNA confounded the results presented herein, as the stimulation of TLR7 is detected in plasmacytoid DCs (pDCs), but not in myeloid DCs.[23] Furthermore, TLR7 is not expressed on monocyte-derived DCs.[24] In addition, the 5'-GUCCUUCAA-3' sequence described to be responsible for activation of the pDCs is not present in the siRNAs used.[23] Besides, in the absence or TLR ligands, after electroporation with the siRNAs no production of cytokines or up-regulation of co-stimulatory molecules was observed (data not shown).

MPLA/IFN-γ-induced up-regulation of cell surface molecules is mediated by the TRIF pathway, but not the MyD88 pathway

As shown previously, stimulation of imDCs with MPLA/IFN-γ leads to up-regulation of HLA-DR, co-stimulatory molecules and DC maturation marker CD83 (Figure 2A).[25,26] To determine which pathway is important in up-regulation of CD83 and co-stimulatory molecules after MPLA/IFN-γ stimulation either TRIF or MyD88 were specifically down-regulated by electroporation. Two d after stimulation cell surface molecule expression was determined using flow cytometry. Down-regulation of MyD88 did not affect induction of HLA-DR, co-stimulatory molecules and CD83 (Figure 2B, C). Down-regulation of TRIF strongly inhibited up-regulation of the DC-maturation marker CD83. In addition, HLA-DR and CD80 were significantly lower and there was a tendency towards lower expression of CD40 and CD86 in TRIF siRNA-treated DCs (Figures 2B, C). Thus, signaling via the TRIF-dependent pathway is involved in the phenotypic maturation of DCs upon TLR4 stimulation.

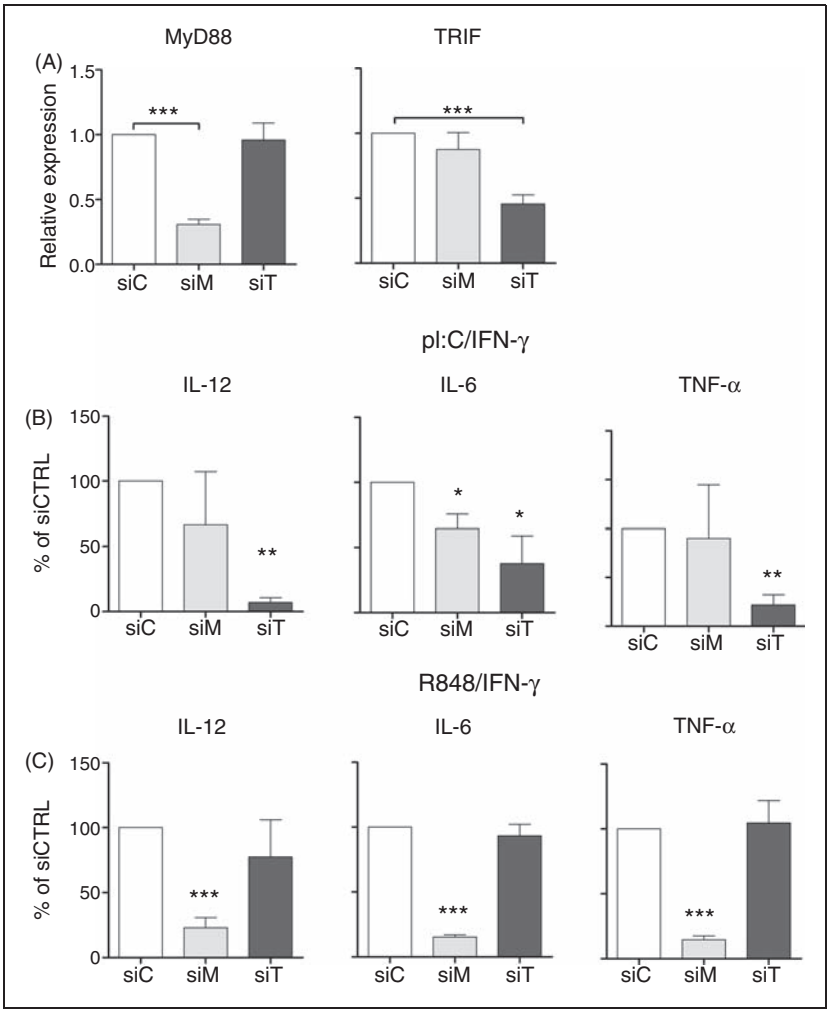

Figure 1. Expression of MyD88 or TRIF is down-regulated after electroporation with specific siRNA. imDCs were electroporated with siCTRL, siMyD88 or siTRIF. (A) Forty-eight h after electroporation mRNA expression of MyD88 ($n = 10$ from eight different donors) or TRIF ($n = 8$ from eight different donors) was determined by RT-qPCR. A paired Students *t*-test was performed for statistical analysis, ***$P < 0.001$. (B, C) Cytokine production by DCs in which MyD88 or TRIF has been down-regulated. Forty-eight h after electroporation of imDCs with siRNA, DCs were stimulated with TLR7/8 ligand R848 or TLR3 ligand pI:C. Production of cytokines was determined by ELISA of culture supernatant. (B) DCs stimulated with pI:C/IFN-γ; cytokine production levels are expressed as percentage of CTRL siRNA-treated cells (IL-6: 224–44,950 pg/ml, mean 12,121 pg/ml; TNF-α: 139–57,550 pg/ml, mean 12,063 pg/ml) and are depicted as mean ± SEM. White bars: siCTRL; light gray bars: siMyD88; dark gray bars: siTRIF ($n = 5$ from five different donors). (C) DCs stimulated with R848/IFN-γ, cytokine production levels are expressed as percentage of CTRL siRNA-treated cells (IL-12: 39–2028 pg/ml, mean 569 pg/ml; IL-6 (9108–105,904 pg/ml, mean 43,517 pg/ml; TNF-α (1252–20,235 pg/ml, mean 7301 pg/ml) and are expressed mean ± SEM. White bars: control-treated DCs, light gray bars: siMyD88 ($n = 8$ from eight different donors) dark gray bars: siTRIF ($n = 4$, from four different donors). A paired *t*-test was performed for statistical analysis; values were compared to siCTRL, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. SiC: non-targeting scrambled siRNA; siM: siMyD88; siT: sTRIF.

Expression of both MyD88 and TRIF are necessary for optimal cytokine secretion after MPLA/IFN-γ stimulation

Next, we determined the role of the MyD88- or TRIF-dependent pathway in cytokine production after TLR4 activation by MPLA, both in presence and absence of IFN-γ. Pro-inflammatory cytokine production has been attributed to MyD88-dependent signaling,[7,13,9,10,11] and the production of type I IFNs and IFN-inducible chemokines, for example RANTES, to TRIF-dependent signaling.[10,14] Two d prior to stimulation with MPLA DCs were electroporated with siRNA targeting either TRIF or MyD88. Compared with the control conditions, down-regulation of MyD88 inhibited production of all pro-inflammatory cytokines induced by MPLA/IFN-γ. Production of RANTES was not affected (Figure 3A). In case only MPLA was used as stimulation, the production of pro-inflammatory IL-6 and TNF-α were also decreased; production of IL-12 was below the detection limit when stimulated in the absence of IFN-γ. Production of the chemokine

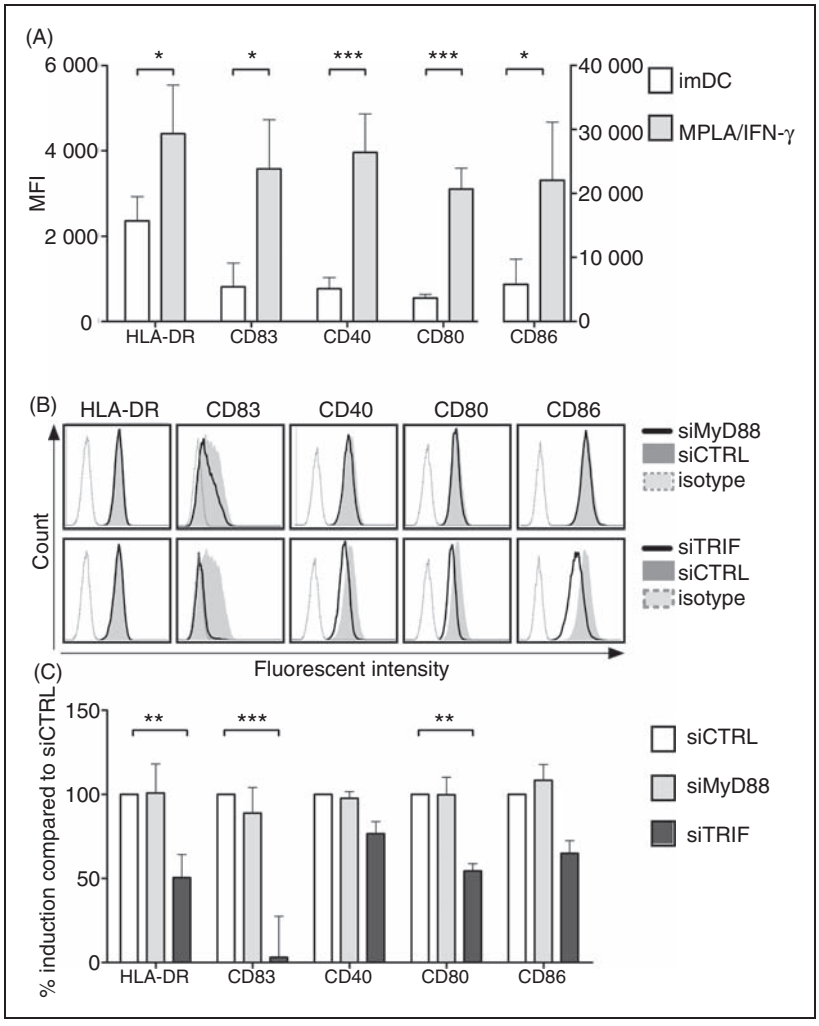

Figure 2. The TRIF-dependent pathway is necessary for induction of expression of co-stimulatory molecules after stimulation with MPLA/IFN-γ. (A) imDCs were stimulated with MPLA/IFN-γ. Forty-eight h after maturation, DCs were harvested and co-stimulatory molecule expression was determined by flow cytometry. White bars: imDCs; light gray bars: MPLA/IFN-γ-matured DCs. For statistical analysis a two-way ANOVA was performed ($n = 11$ from nine different donors), $^{*}P < 0.05$, $^{***}P < 0.001$. (B, C) imDCs were electroporated with siCTRL, siMyD88 or siTRIF. Two d after electroporation imDCs were stimulated with MPLA/IFN-γ. Forty-eight h after maturation DCs were harvested and co-stimulatory molecule expression was determined by flow cytometry. (B, upper graphs) Expression of co-stimulatory molecules on DCs electroporated with siRNA targeting MyD88 (black line, siMyD88) or control siRNA (dark gray area, siCTRL), light gray area: isotype control. (B, lower graphs) Expression of co-stimulatory molecules on DCs electroporated with siRNA targeting TRIF (black line, siTRIF) or control siRNA (dark gray area, siCTRL) light gray area: isotype control. Graphs represent data from a representative experiment out of 14. (C) Induction of co-stimulatory molecule expression by MPLA IFN-γ compared with imDCs on siRNA-treated DCs is shown relative to induction of siCTRL DCs. White bars: siCTRL; light gray bars: siMyD88 ($n = 14$ from 11 different donors); dark gray bars: siTRIF ($n = 8$ from eight different donors). For statistical analysis a two-way ANOVA was performed, $^{**}P < 0.01$, $^{***}P < 0.001$. SiCTRL: non-targeting scrambled siRNA.

RANTES was not significantly lowered by down-regulation of MyD88 (Figure 3B). Down-regulation of TRIF inhibited expression of all pro-inflammatory cytokines, as well as expression of RANTES upon MPLA/IFN-γ stimulation (Figure 3A), when only MPLA was used production of IL-6, TNF-α and RANTES were also decreased (Figure 3B). Overall, these data show that expression of both MyD88 and TRIF is necessary for optimal pro-inflammatory cytokine secretion in response to MPLA. Production of RANTES is only dependent on the TRIF pathway.

LPS-mediated pro-inflammatory cytokine production also requires the joint action of MyD88 and TRIF

MyD88 and TRIF are both necessary for optimal cytokine secretion in response to MPLA. To determine whether this is a general phenomenon for TLR4-stimulated cytokine production or dependent on the specific TLR4 ligand used, we also stimulated DCs with LPS. Knockdown of both MyD88 and TRIF led to decreased levels of IL-6, IL-12 and TNF-α (Figure 4). RANTES was decreased upon TRIF down-regulation,

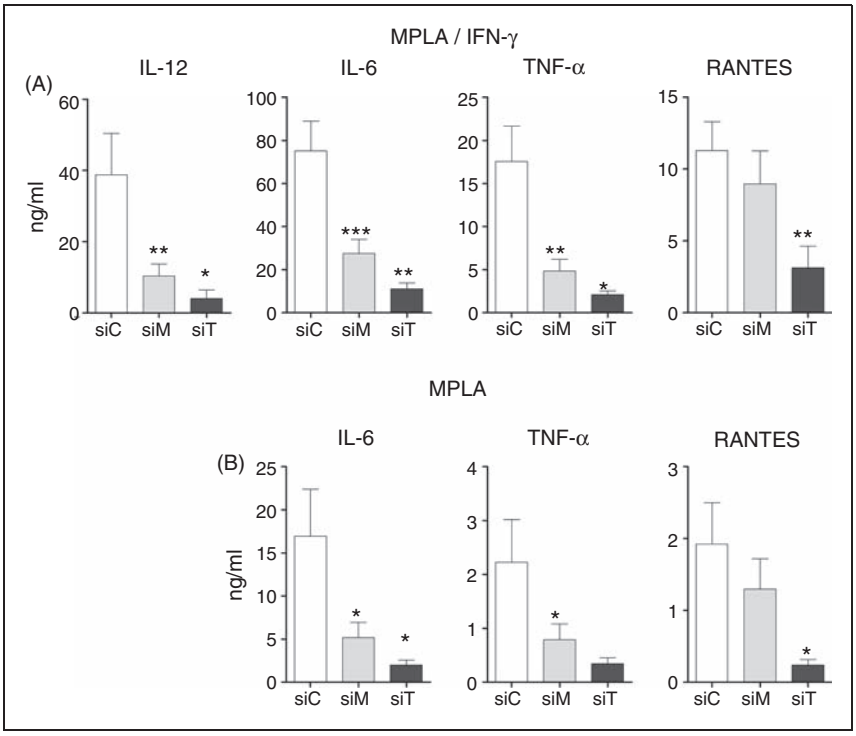

Figure 3. Expression of MyD88 and TRIF are necessary for optimal cytokine production after MPLA/IFN-γ stimulation. imDCs were electroporated with siCTRL, siMyD88 or siTRIF. Two d after electroporation imDCs were stimulated with MPLA or MPLA/IFN-γ. Concentration of cytokines or chemokine RANTES was determined in supernatants harvested 24 h after stimulation with MPLA or MPLA/IFN-γ. (A) Production of cytokines by MPLA/IFN-γ-stimulated DCs. White bars: siCTRL; light gray bars siMyD88 ($n = 17$ from 14 different donors); dark gray bars: siTRIF ($n = 8$ from eight different donors). (B) Production of cytokines by DCs stimulated with MPLA alone. White bars: siCTRL; light gray bars siMyD88 ($n = 11$ from nine different donors); dark gray bars siTRIF ($n = 10$ from eight different donors). A paired *t*-test was performed for statistical analysis, values were compared with the siCTRL condition $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. SiC: non-targeting scrambled siRNA; siM: siMyD88; siT: sTRIF.

while MyD88 down-regulation only slightly decreased RANTES expression (Figure 4). Thus, MyD88 and TRIF are both involved in TLR4 induced pro-inflammatory cytokine production in human DCs irrespective of the TLR4 ligand used.

Discussion

TLR4 is the only TLR that can signal via the MyD88 as well as the TRIF pathway. TLR4 signaling via MyD88 leads to pro-inflammatory cytokine production.[7,9–11, 13] Although TLR4-stimulated TRIF signaling is mainly associated with type I IFN responses,[10,15] there are a few studies in mice that also indicate involvement of TRIF signaling in the production of pro-inflammatory cytokines.[17,18,22] The lack of clarity in the role of the TRIF-mediated pathway in TLR4-mediated pro-inflammatory cytokine production may be explained by the fact that not all studies investigated the role of both the TRIF and the MyD88 pathway in the induction of cytokines, but only one of these routes. While this provides useful data on the role of one signaling route, it does not elucidate whether the other TLR4 signaling route might be equally important for the induction of these cytokines. Moreover, differences between studies may be owing to the fact that in some studies cytokine induction was studied on mRNA and in others at the protein level.[27,28] Finally, the structure of the actual TLR4 agonist may skew TLR4 signaling in the direction of the TRIF- or the MyD88-dependent route.[7,29,30]

Most research on TLR4 in human cells has been performed using cell lines transfected with TLR4, MD-2 and CD14. This study addresses, for the first time, the role of TRIF and MyD88 signaling pathways on TLR4 stimulation in primary human cells by specific down-regulation of MyD88 or TRIF in DCs. Our data help dissect the regulation of TLR4-induced cytokine production in human primary cells. Our data, which were obtained in human DCs, indicate that phenotypic maturation, for example up-regulation of CD83 and co-stimulatory molecules, was dependent solely on the TRIF pathway, confirming previous studies in murine macrophages.[17,18] MyD88 down-regulation strongly inhibited TLR4 mediated pro-inflammatory cytokine production, while hardly affecting RANTES levels. Interestingly, TLR4-mediated TRIF signaling also plays an important role in pro-inflammatory cytokine

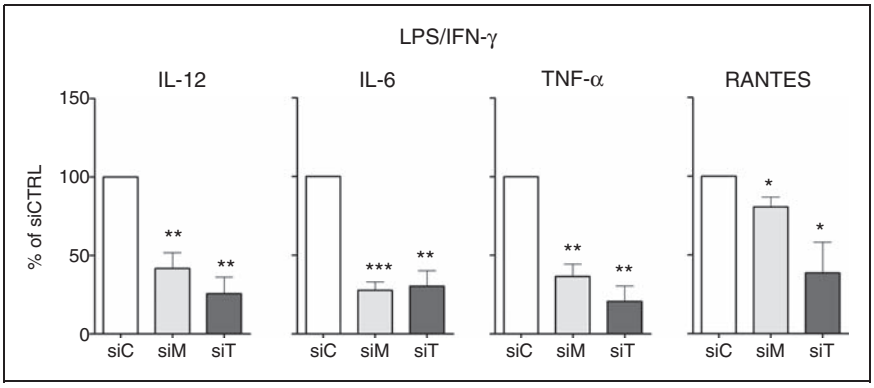

Figure 4. MyD88 and TRIF are both necessary for optimal cytokine production after LPS stimulation. imDCs were electroporated with siCTRL, siMyD88 or siTRIF. Two d after electroporation imDCs were stimulated with LPS and IFN-γ in the presence of 1% FCS. Forty-eight h after maturation culture supernatants were harvested and cytokine levels were determined by ELISA. Cytokine secretion of DCs stimulated with LPS/IFN-γ; cytokine production levels are expressed as percentage of CTRL siRNA-treated cells (IL-12: 0.6–121.0 ng/ml, mean 25.6 ng/ml; IL-6: 40.8–215.5 ng/ml, mean 104.3 ng/ml; TNF-α: 7.3–61.6 ng/ml, mean 32.4 ng/ml; RANTES 1.8–75.3 ng/ml, mean 40.8 ng/ml) and are depicted as mean ± SEM. White bars: siCTRL; light gray bars siMyD88 ($n = 6$ from six different donors); dark gray bars siTRIF ($n = 5$ from five different donors). A paired *t*-test was performed for statistical analysis, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. SiC: non-targeting scrambled siRNA; siM: siMyD88; siT: sTRIF.

production, as besides inhibition of RANTES production, down-regulation of TRIF with specific siRNA induced a strong inhibition of pro-inflammatory cytokine production upon stimulation by MPLA or LPS. This is in contrast to data obtained in THP-1[29] or U937 cell lines,[31] where production of TNF-α was not decreased after down-regulation of TRIF. Unfortunately, no other pro-inflammatory cytokines besides TNF-α were determined in these studies,[29,31] limiting the comparison of the effect of TRIF between our studies in primary monocyte-derived DCs with these studies in human-derived cell lines. Altogether, these data illustrate the value of using primary cells compared with immortalized cell lines for studying TLR4 adaptor usages in humans.

The combination of different MyD88-dependent and TRIF-dependent TLR ligands was previously shown to be synergistic for induction of pro-inflammatory cytokines.[32–34] As TLR4 has both adaptor molecules, TLR4 activation was suggested and demonstrated to be a synergistic event on its own in mice.[15,22] In humans, however, data were conflicting. Our data now show that MyD88 and TRIF also act in synergy upon TLR4 ligation in humans. The non-redundant functions of MyD88 and TRIF signaling by TLR4 ligands may explain why coupling of TLR4 to both the TRIF and MyD88 signaling pathways is strongly evolutionary conserved.[35]

Thus, the ability of TLR4 to signal synergistically via both pathways leads to induction of co-stimulatory markers and production of pro-inflammatory cytokines. Signaling via TLR3 can only use the TRIF adaptor and induces an antiviral response, characterized by production of type-I IFNs.[6,10,15] MyD88-coupled TLRs have a more versatile profile, characterized by production of pro-inflammatory cytokines.[6] Synergy between TRIF-coupled TLR3 and MyD88-coupled TLRs can induce a stronger pro-inflammatory response.[32–34] Only TLR4, with the intrinsic ability to induce signaling via both the MyD88 and the TRIF pathway leads to a strong Th1 response by induction of a maximal pro-inflammatory cytokine response accompanied by maximal up-regulation of co-stimulatory molecules.

In conclusion, for TLR4-mediated responses in human DCs, besides the MyD88 pathway, the TRIF pathway is essential for overall DC maturation by inducing co-stimulation and playing a non-redundant role in pro-inflammatory cytokine production.

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Conflict of interest

The authors do not have any potential conflicts of interest to declare.

References

1. Akira S and Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; 4: 499–511.
2. Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, et al. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 2003; 424: 743–748.
3. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K and Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 2002; 169: 6668–6672.

4. Tanimura N, Saitoh S, Matsumoto F, Akashi-Takamura S and Miyake K. Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochem Biophys Res Commun* 2008; 368: 94–99.
5. Kagan JC, Su T, Horng T, Chow A, Akira S and Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* 2008; 9: 361–368.
6. Kawai T and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010; 11: 373–384.
7. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR and Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 2007; 316: 1628–1632.
8. Kaisho T, Takeuchi O, Kawai T, Hoshino K and Akira S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 2001; 166: 5688–5694.
9. Akira S, Uematsu S and Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783–801.
10. Hirotani T, Yamamoto M, Kumagai Y, Uematsu S, Kawase I, Takeuchi O and Akira S. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. *Biochem Biophys Res Commun* 2005; 328: 383–392.
11. Horng T, Barton GM and Medzhitov R. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* 2001; 2: 835–841.
12. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, et al. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* 2003; 4: 1144–1150.
13. Kaisho T, Akira S, Kawai T, Takeuchi O, Fujita T, Inoue J, et al. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol* 2001; 22: 78–83.
14. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* 2001; 167: 5887–5894.
15. Shen H, Tesar BM, Walker WE and Goldstein DR. Dual signaling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. *J Immunol* 2008; 181: 1849–1858.
16. Weighardt H, Jusek G, Mages J, Lang R, Hoebe K, Beutler B and Holzmann B. Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *Eur J Immunol* 2004; 34: 558–564.
17. Hoebe K, Janssen EM, Kim SO, Alexopoulou L, Flavell RA, Han J and Beutler B. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat Immunol* 2003; 4: 1223–1229.
18. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; 301: 640–643.
19. Horng T, Barton GM, Flavell RA and Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 2002; 420: 329–333.
20. Kawai T, Adachi O, Ogawa T, Takeda K and Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999; 11: 115–122.
21. Zeisel MB, Druet VA, Sibilia J, Klein JP, Quesniaux V and Wachsmann D. Cross talk between MyD88 and focal adhesion kinase pathways. *J Immunol* 2005; 174: 7393–7397.
22. Krummen M, Balkow S, Shen L, Heinz S, Loquai C, Probst HC and Grabbe S. Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. *J Leukoc Biol* 2010; 88: 189–199.
23. Hornung V, Guenthner-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, et al. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 2005; 11: 263–270.
24. Kokkinopoulos I, Jordan WJ and Ritter MA. Toll-like receptor mRNA expression patterns in human dendritic cells and monocytes. *Mol Immunol* 2005; 42: 957–968.
25. ten Brinke A, van Schijndel G, Visser R, de Gruijl TD, Zwaginga JJ and van Ham SM. Monophosphoryl lipid A plus IFNgamma maturation of dendritic cells induces antigen-specific CD8+ cytotoxic T cells with high cytolytic potential. *Cancer Immunol Immunother* 2010; 59: 1185–1195.
26. ten Brinke A, Karsten ML, Dieker MC, Zwaginga JJ and van Ham SM. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine* 2007; 25: 7145–7152.
27. Gais P, Tiedje C, Altmayr F, Gaestel M, Weighardt H and Holzmann B. TRIF signaling stimulates translation of TNF-alpha mRNA via prolonged activation of MK2. *J Immunol* 2010; 184: 5842–5848.
28. Wang L, Trebicka E, Fu Y, Waggoner L, Akira S, Fitzgerald KA, et al. Regulation of lipopolysaccharide-induced translation of tumor necrosis factor-alpha by the toll-like receptor 4 adaptor protein TRAM. *J Innate Immun* 2011; 3: 437–446.
29. Bowen WS, Minns LA, Johnson DA, Mitchell TC, Hutton MM and Evans JT. Selective TRIF-dependent signaling by a synthetic toll-like receptor 4 agonist. *Sci Signal* 2012; 5: ra13.
30. Zughaier SM, Zimmer SM, Datta A, Carlson RW and Stephens DS. Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun* 2005; 73: 2940–2950.
31. Tachado SD, Li X, Bole M, Swan K, Anandaiah A, Patel NR and Koziel H. MyD88-dependent TLR4 signaling is selectively impaired in alveolar macrophages from asymptomatic HIV+ persons. *Blood* 2010; 115: 3606–3615.
32. Lee MS and Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu Rev Biochem* 2007; 76: 447–480.
33. Makela SM, Strengell M, Pietila TE, Osterlund P and Julkunen I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. *J Leukoc Biol* 2009; 85: 664–672.
34. Ilievski V and Hirsch E. Synergy between viral and bacterial toll-like receptors leads to amplification of inflammatory responses and preterm labor in the mouse. *Biol Reprod* 2010; 83: 767–773.
35. Netea MG, Wijmenga C and O'Neill LA. Genetic variation in Toll-like receptors and disease susceptibility. *Nat Immunol* 2012; 13: 535–542.