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Differential involvement of IFN-β in Toll-like receptor-stimulated dendritic cell activation

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Abstract

Toll-like receptor (TLR) can activate dendritic cells (DC) through common signaling pathways requiring a cytoplasmic adapter, MyD88. However, the signaling is differentially regulated among TLR family members. TLR4 can activate MyD88-deficient bone marrow-derived DC (BMDC), and lead to induction of IFN-inducible genes and up-regulation of co-stimulatory molecules such as CD40, implying that the MyD88-independent signaling pathway functions downstream of TLR4. Because these effects can also be induced by type I IFN, we have analyzed whether type I IFN is involved in TLR4-induced responses. In response to lipopolysaccharide (LPS), IFN-β gene expression was augmented in both wild-type and MyD88-deficient BMDC. Expression of all IFN-inducible genes except immune-responsive gene 1 (IRG1) was abolished and CD40 up-regulation was decreased in LPS-stimulated BMDC lacking either IFN-α/β receptor (IFN-α/βR) or signal transducer and activator of transcription 1 (STAT-1). Similar to the LPS response, TLR9 signaling can also induce expression of IFN-β and IFN-inducible genes, and up-regulation of CD40. However, all these effects were MyD88 dependent. Thus, in TLR4 signaling, IFN-β expression can be induced either by the MyD88-dependent or -independent pathway, whereas, in TLR9 signaling, it is dependent on MyD88. In CpG DNA-stimulated DC, expression of IFN-inducible genes except IRG1 was dependent on type I IFN signaling as in LPS-stimulated DC. However, in contrast to TLR4 signaling, TLR9 signaling requires type I IFN signaling for CD40 up-regulation. Taken together, this study demonstrates differential involvement of type I IFN in TLR4- and TLR9-induced effects on DC.

Introduction

Toll-like receptors (TLR) are a group of transmembrane proteins expressed mainly on antigen-presenting cells (APC) such as dendritic cells (DC) or macrophages. TLR recognize pathogen-derived products and mediate a variety of signals not only for inducing pro-inflammatory cytokines, but also for up-regulating co-stimulatory molecules. These effects function as immune adjuvants to establish the host defense mechanism (1).

The mammalian TLR family consists of 10 members (2,3). They possess common intracytoplasmic regions, which are structurally similar to IL-1 receptor (IL-1R) family members. The region is called as the Toll/IL-1 receptor homology (TIR) domain and is involved in signaling pathways that are common among TLR. A cytoplasmic adapter, MyD88, associates with TLR and IL-1R through its TIR domain. Then this chain of signaling cascades can lead to activation of NF-κB or mitogen-activated protein kinases (MAPK) (4–6). MyD88-deficient ($MyD88^{-/-}$) cells cannot produce any amounts of IL-12 or tumor necrosis factor (TNF)-α in response to ligands for TLR including TLR2, TLR4 and TLR9, indicating the essential roles of MyD88 in TLR-induced cytokine production (7–9).

However, TLR signaling is also differentially regulated in a TLR-specific manner. By analyzing $MyD88^{-/-}$ mice, TLR4 signaling was found to be unique in that it can activate the

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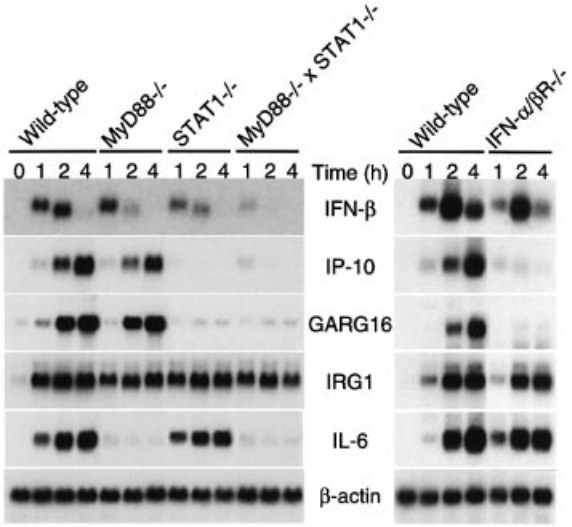

Fig. 1. Expression of IFN-β and IFN-inducible genes in LPS-stimulated DC. BMDC from wild-type and mutant mice were stimulated with 100 ng/ml LPS for the indicated periods. Total RNA (5 μg) were electrophoresed, transferred and hybridized with probes for IFN-β, IP-10, GARG16, IRG1, IL-6 and β-actin.

MyD88-independent signaling pathway (7). This pathway then leads to activation of NF-κB, MAPK cascade and IFN-regulatory factor (IRF)-3 (10), and brings about enhanced expression of a set of IFN-inducible genes (10) or DC maturation (11). Another TIR domain containing adapter, TIRAP/Mal, is possibly involved in the pathway, because it can associate exclusively with TLR4 (12,13). However, it remains unknown whether TLR4 signaling directly induces these biological effects.

Type I IFN consist of >10 members of IFN-α and a single form of IFN-β, and are produced upon viral infection in a variety of cells including DC. They can activate NK cell activity, up-regulate MHC class I expression, induce cytokines or chemokines such as IFN-inducible protein 10 (IP-10) and lead to DC maturation, thereby bringing about effective antiviral defense (14). In this study, we have evaluated how type I IFN contribute to LPS-induced effects. We have found that IFN-β, induced in a MyD88-independent manner, are involved in LPS-induced effects, including induction of the IFN-inducible genes. The genes include not only a T_h1 cell-recruiting chemokine, IP-10, gene but also glucocorticoid attenuated response gene 16 (GARG16) or immune-responsive gene 1 (IRG1) whose products have not been functionally characterized yet (15,16). Expression of similar sets of genes including the IFN-β gene was also enhanced through TLR9 signaling. However, unlike TLR4, all of these effects were dependent on MyD88. Thus, IFN-β is differentially involved in biological responses through TLR4 and TLR9.

Methods

Animals

C57BL/6 mice were used as wild-type mice and purchased from CLEA Japan (Tokyo, Japan). IFNα/βR$^{-/-}$ mice were purchased from B & K Universal (Hull, UK) (17). The signal transducer and activator of transcription 1 (STAT-1)$^{-/-}$ mice were kindly provided by Dr Robert D. Schreiber (Washington University, St Louis, MO) (18). MyD88$^{-/-}$, TLR2$^{-/-}$ and TLR9$^{-/-}$ mice were described previously (19–21), and maintained in Osaka University mice facility. MyD88$^{-/-}$ STAT-1$^{-/-}$ mice were generated by crossing MyD88$^{+/-}$ STAT-1$^{+/-}$ mice.

Reagents

LPS derived from *Escherichia coli* O55:B5 was purchased from Sigma (St Louis, MO). Phosphorothioate-stabilized oligodeoxynucleotides containing unmethylated CpG motifs (21) were purchased from Hokkaido System Science (Sapporo, Japan). Mycoplasmal lipopeptide macrophage-activating lipopeptide-2 (MALP-2) was kindly provided by Dr P. F. Mühlradt (Gesellschaft für Biotechnologische Forsechung, Braunscheweig, Germany) (22). IFN-β was purchased from PBL Biomedical (New Brunswick, NJ).

Northern blot analysis

Bone marrow (BM)-derived DC were prepared according to the method of Inaba *et al.* (23). BM cells were obtained from the mice and plated at 1×10^6 cells/ml in 24-well plates with 10% (v/v) FCS/RPMI 1640 containing 10 ng/ml murine granulocyte macrophage colony stimulating factor (GM-CSF; Genzyme Techne, Minneapolis, MN). Every 2 days, non-adherent cells were discarded and the remaining cells were fed with fresh medium containing 10 ng/ml murine GM-CSF. At day 6, loosely adherent cells were harvested by gentle pipeting and cultured for a further 2 days. At day 8, non-adherent cells were harvested and stimulated without stimuli or with 100 ng/ml LPS, 0.1 μM CpG DNA or 30 ng/ml MALP-2 for indicated periods. Total RNA was extracted using an RNeasy kit (Qiagen, Tokyo, Japan), electrophoresed, transferred to a nylon membrane and hybridized with cDNA probes as described previously (10).

Flow cytometric analysis

Cells were first incubated with anti-CD16/32 (2.4G2; BD Biosciences, Mountain View, CA) to minimize non-specific binding of antibody to FcR. They were further stained with biotinylated anti-CD40 mAb (3/23; BD Biosciences) for 20 min at 4°C, washed and subsequently developed with streptavidin–phycoerythrin (BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences) with CellQuest software (BD Biosciences).

EMSA

The nuclear extracts of BMDC were prepared and incubated with specific probes for the IFN-stimulated regulatory element (ISRE; 5′-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3′) or NF-κB (5′-ATCAGGGACTTTCCGCTGGGGACTTTCC-3′) DNA-binding sites (7,10). Then the extracts were electrophoresed and visualized by autoradiography.

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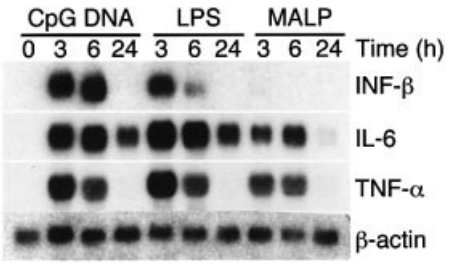

Fig. 2. IFN-β gene up-regulation in TLR9-, TLR4- or TLR2-stimulated wild-type DC. Wild-type DC were stimulated with 0.1 μM CpG DNA, 100 ng/ml LPS or 30 ng/ml MALP-2 for the indicated periods. Total RNA (5 μg) was subjected to Northern blot analysis using probes for IFN-β, IL-6, TNF-α and β-actin.

Results and discussion

IFN-β induction by LPS is independent of MyD88

According to ELISA analysis, LPS could not induce either IFN-α or IFN-γ production from wild-type DC (data not shown). However, IFN-β gene expression was augmented in LPS-stimulated wild-type DC (Figs 1 and 2). The induction was detected at 1 h after LPS stimulation, reached a maximum level at 2–3 h and then declined thereafter. LPS can induce IFN-β, but not IFN-α, gene expression in wild-type peritoneal macrophages (24,25). Thus, like macrophages, DC could enhance IFN-β, but not IFN-α, production in response to LPS.

Gene induction of cytokines including IL-6 was abolished in MyD88$^{-/-}$ DC (Fig. 1) (7). We have analyzed whether LPS-induced IFN-β gene expression is retained in MyD88$^{-/-}$ DC. MyD88$^{-/-}$ DC showed significant, although reduced, elevation of IFN-β gene expression in response to LPS (Fig. 1). The elevation was dependent on TLR4, because LPS-stimulated TLR4$^{-/-}$ DC showed no IFN-β gene expression (data not shown). These results indicate that IFN-β gene can be induced by TLR4 signaling in a MyD88-independent manner.

IFN-β induction is involved in TLR4-mediated up-regulation of IP-10 and GARG16 genes

As in MyD88$^{-/-}$ macrophages (10), MyD88$^{-/-}$ DC manifested up-regulation of IFN-inducible genes including IP-10 and GARG16 in response to LPS (Fig. 1). Furthermore, IRG1 gene expression was also enhanced in MyD88$^{-/-}$ DC. The IRG1 gene, originally identified as a LPS-inducible gene (16), was also up-regulated by IFN-β (data not shown).

We have next examined whether expression of these genes by LPS is enhanced directly through TLR4 signaling or secondarily induced through the function of IFN-β. Type I IFN, IFN-α and IFN-β, bind to their common receptor, IFN-α/βR. Deletion of IFN-α/βR can lead to refractoriness to type I IFN (17). Therefore, we have analyzed effects of LPS on IFN-α/βR$^{-/-}$ DC. LPS could induce expression not only of IL-6 or TNF-α (data not shown), but also of IFN-β in IFN-α/βR$^{-/-}$ DC. However, the mutant DC could not enhance expression of IP-10 or GARG16 in response to LPS (Fig. 1).

STAT-1 is a critical signal transducer for type I IFN (18). Similar to IFN-α/βR$^{-/-}$ DC, STAT-1$^{-/-}$ DC retained LPS-induced up-regulation of IFN-β or other cytokine genes, but did not manifest any increased expression of IP-10 or GARG16 (Fig. 1). These results indicate that IFN-β signaling is essential for the TLR4-induced up-regulation of IP-10 and GARG16 genes. As type I IFN signaling activates expression of the target gene through the ISRE site (26), our results are consistent with the previous finding that the ISRE site in the IP-10 gene promoter is essential for LPS-induced IP-10 gene expression (27).

TLR4 signaling can induce IRG1 gene expression in a MyD88- and IFN-β-independent manner

In contrast to IP-10 and GARG16 genes, the IRG1 gene (16) was induced in LPS-stimulated IFN-α/βR$^{-/-}$ and STAT-1$^{-/-}$ DC (Fig. 1), indicating that IRG1 gene induction through TLR4 signaling is independent of IFN-β signaling. Toshchakov *et al.* described that IP-10 and MCP-5 genes are up-regulated in LPS-stimulated macrophages in an IFN-β-dependent manner (28). Thus, IRG1 gene expression is regulated distinctly from other IFN-inducible genes.

Kinetic analysis of the gene expression also supports differential involvement of IFN-β in the expression of IFN-inducible genes. IP-10 and GARG16 expression reached a peak at 4 h after addition of LPS, whereas IFN-β and IRG1 expression was already enhanced within 1 h. Thus, both IFN-β and IRG1 genes is considered to be directly induced by TLR4 signaling. However, their regulatory mechanism is clearly different. Notably, IFN-β gene expression by LPS was slightly impaired in MyD88$^{-/-}$ or STAT-1$^{-/-}$ DC and severely decreased in MyD88$^{-/-}$STAT-1$^{-/-}$ DC (Fig. 1), indicating that IFN-β gene induction requires either the MyD88-dependent pathway or positive feedback mechanism by IFN-β itself. In contrast, IRG1 gene induction by LPS was unaltered in MyD88$^{-/-}$, STAT-1$^{-/-}$ and MyD88$^{-/-}$ STAT-1$^{-/-}$ DC, indicating that IRG1 gene expression is independent of MyD88 and IFN-β signaling. At present, little is known how IRG1 gene expression is regulated. Further studies are necessary to clarify how TLR4 signaling activates IRG1 expression.

TLR9 signaling can induce IFN-β gene expression in a MyD88-dependent manner

Unlike TLR4, TLR2 cannot induce up-regulation of IFN-inducible genes including IP-10 and GARG16 [(10) and data not shown]. A TLR2 ligand, MALP-2, induced IL-6 and TNF-α gene expression, but did not induce IFN-β gene expression in wild-type DC (Fig. 2). The results are in accordance with the involvement of IFN-β in mediating up-regulation of IFN-inducible genes.

We have next tested effects of a TLR9 ligand, CpG DNA, on DC. CpG DNA in this and a previous study (21) could not induce IFN-α production (data not shown), but could induce IFN-β gene up-regulation (Figs 2 and 3). The peak of the expression levels was reached at 4–6 h after stimulation. This induction kinetics is delayed compared with that of TLR4 signaling (Figs 1 and 3). Unlike TLR4 signaling, TLR9 signaling could not induce IFN-β gene expression in MyD88$^{-/-}$ DC. Thus, CpG DNA can induce IFN-β gene expression in a MyD88-dependent manner.

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Critical roles of IFN-β in TLR9-mediated augmentation of IFN-inducible genes

We have analyzed expression of IFN-inducible genes, IP-10, GARG16 and IRG1, in CpG DNA-stimulated DC. As in LPS-stimulated wild-type DC, expression of these genes was also augmented in CpG DNA-stimulated wild-type DC (Fig. 3). Expression of all of these genes as well as the IFN-β gene was almost abolished in MyD88$^{-/-}$ DC. In order to assess whether IFN-β contributes to CpG DNA-induced up-regulation of IFN-inducible genes, we have investigated effects of CpG DNA on IFN-α/βR$^{-/-}$ DC (Fig. 3). In response to CpG DNA, the mutant DC retained IFN-β and IRG1 induction, but did not augment expression of the IP-10 or GARG16 genes. The results suggest that up-regulation of IFN-inducible genes by CpG DNA depends largely on the action of IFN-β.

IP-10 or GARG16 gene up-regulation was detected at 4 h after stimulation of wild-type DC. The kinetics of gene induction was later than that of the IFN-β gene. This seems consistent with IFN-β-induced up-regulation of these genes. Meanwhile, IRG1 gene up-regulation was the same as that of IFN-β. These data demonstrate that TLR9 signaling, like TLR4 signaling, can induce IP-10 and GARG16 in an IFN-β-dependent manner, and can lead to IRG1 expression in an IFN-β-independent manner.

The IFN-β promoter carries two critical *cis*-acting elements, NF-κB and ISRE sites, both of which are essential for IFN-β gene expression upon viral infection (29–32). We have analyzed whether CpG DNA can induce DNA binding activities to NF-κB or ISRE sites in CpG DNA-stimulated MyD88$^{-/-}$ DC (Fig. 4). In response to CpG DNA, both binding activities were induced in wild-type DC. However, these activities were not observed at all in CpG DNA-stimulated MyD88$^{-/-}$ DC. Meanwhile, MyD88$^{-/-}$ DC increased their DNA binding activities to NF-κB and ISRE (data not shown) sites in response to LPS (Fig. 4) (7). Thus, NF-κB and ISRE site binding activities induced by TLR9 signaling were dependent on MyD88.

TLR-induced regulation of CD40 expression

TLR4 signaling can augment surface expression of CD40 in a MyD88-independent manner (11). Therefore, we have further investigated whether IFN-β is also involved in CD40 up-regulation. IFN-β could enhance CD40 expression of both wild-type and MyD88$^{-/-}$ DC (Fig. 5). As expected, IFN-β could not up-regulate CD40 in either IFN-α/βR$^{-/-}$ or STAT-1$^{-/-}$ DC.

When stimulated with LPS, IFN-α/βR$^{-/-}$ DC showed increased expression of CD40 (Fig. 6A). However, the expression level was lower than that of LPS-stimulated wild-type DC. In addition, STAT-1$^{-/-}$ DC and MyD88$^{-/-}$ DC also showed reduced levels of CD40 expression in response to LPS (Fig. 6B). Notably, the expression of CD40 was further

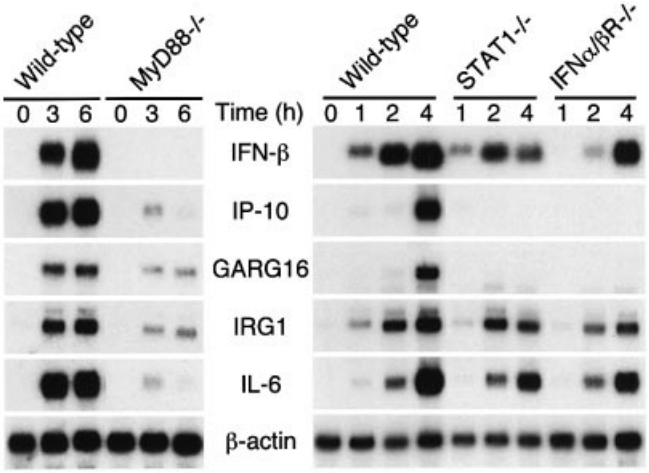

Fig. 3. Expression of IFN-β and IFN-inducible genes in CpG DNA-stimulated DC. BMDC from wild-type and mutant mice were stimulated with 0.1 μM CpG DNA for the indicated periods. Total RNA (5 μg) were electrophoresed, transferred and hybridized with probes for IFN-β, IP-10, GARG16, IRG1, IL-6 and β-actin.

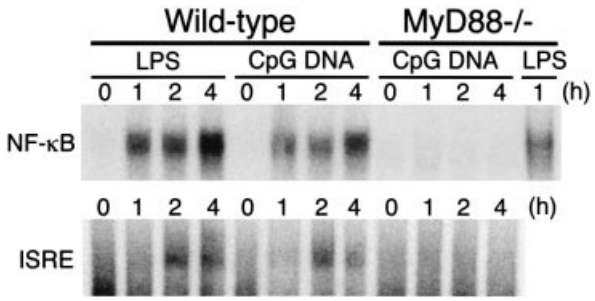

Fig. 4. EMSA of the nuclear extract from LPS or CpG DNA-stimulated DC. BMDC from wild-type and MyD88$^{-/-}$ mice were stimulated with 1 μg/ml LPS or 1 μM CpG DNA for the indicated periods. NF-κB or ISRE DNA binding activity in the nuclear extract was analyzed by EMSA.

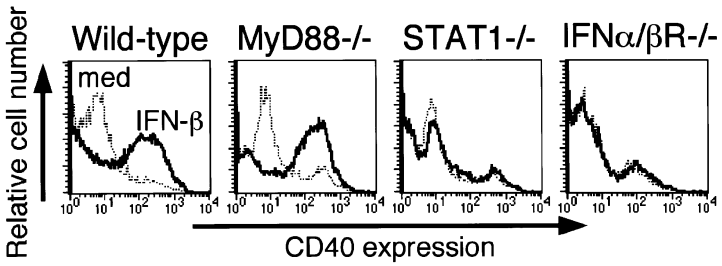

Fig. 5. CD40 expression in IFN-β-stimulated DC. DC from wild-type or mutant mice were stimulated with 1000 U/ml IFN-β for 24 h and analyzed by flow cytometry.

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decreased in MyD88$^{-/-}$ STAT-1$^{-/-}$ DC. Thus, optimal CD40 induction by LPS requires both MyD88 and IFN-β signaling.

Next, we have assessed involvement of IFN-β in CpG DNA-induced CD40 up-regulation. CpG DNA can induce CD40 expression on DC in a TLR9-dependent manner (Fig. 6D). IFN-α/βR$^{-/-}$ DC showed detectable, but diminished, up-regulation of CD40 in response to CpG DNA (Fig. 6C). In addition, STAT-1$^{-/-}$ DC also manifested severely reduced expression of CD40 in response to CpG DNA (Fig. 6D). Thus, IFN-β and its signaling are thought to be crucially involved in CpG DNA-induced CD40 up-regulation.

In this study, we have analyzed how IFN-β is involved in TLR-mediated effects on DC. TLR4 signaling can activate MyD88$^{-/-}$ DC to express a set of IFN-inducible genes. Mutant DC with defective type I IFN signaling have lost their expression (Fig. 1). Furthermore, the IFN-β gene can be induced by LPS in a MyD88-independent manner (Fig. 1). Therefore, it can be reasonably assumed that IFN-β is responsible for LPS-induced expression of IFN-inducible genes. It is also noteworthy that IRG1 is unique among IFN-inducible genes in that its gene induction by TLR4 is independent of type I IFN. Thus, IFN-β and IRG1 are direct

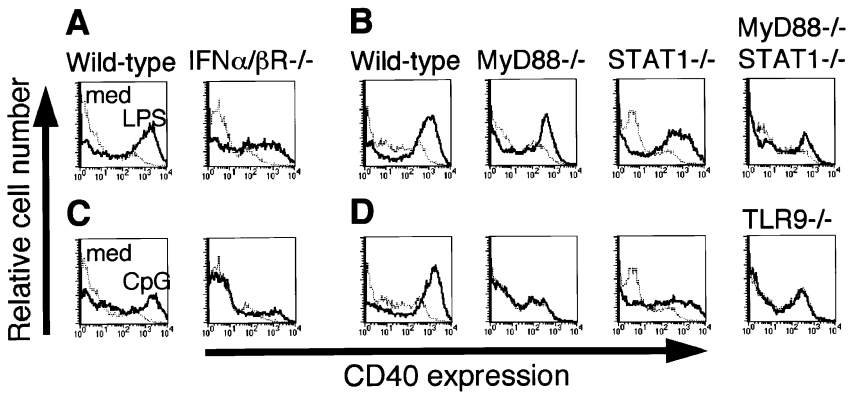

Fig. 6. CD40 expression of LPS (A and B)- or CpG DNA (C and D)-stimulated DC. DC from wild-type or mutant mice were stimulated with 100 ng/ml LPS or 0.1 μM CpG DNA for 24 h and analyzed by flow cytometry.

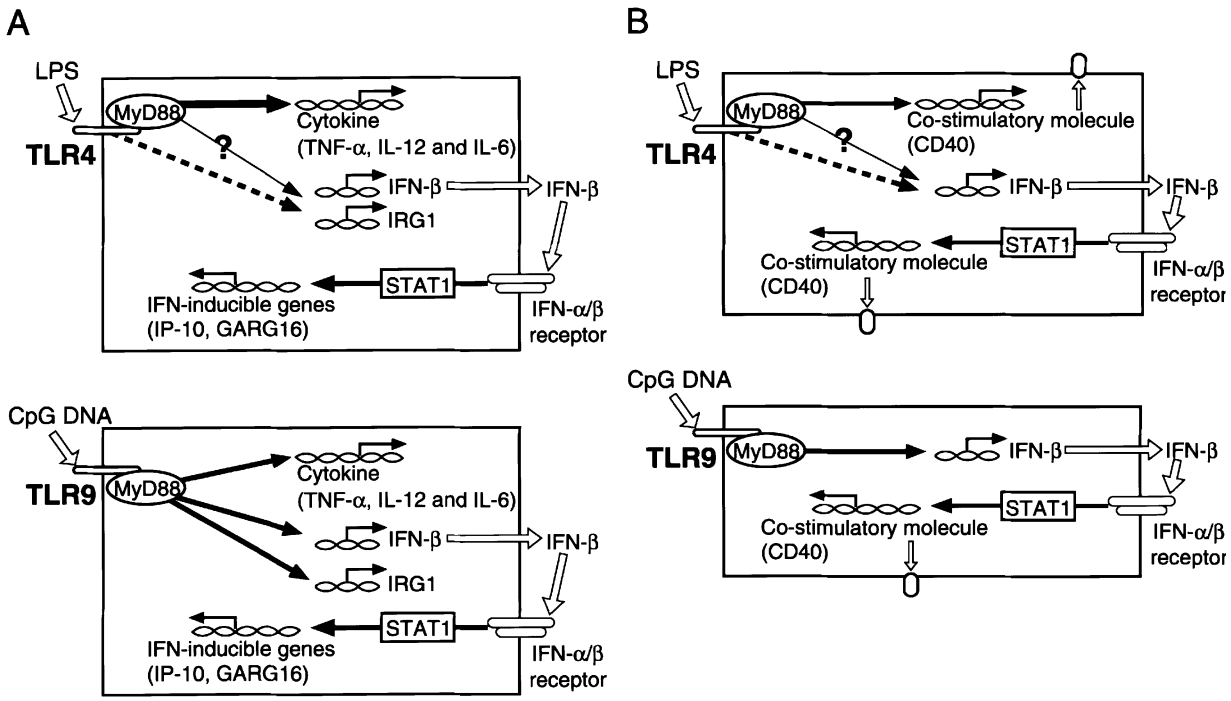

Fig. 7. Differential involvement of IFN-β in TLR4- and TLR9-induced effects. (A) IFN-β is critical for IFN-inducible gene expression induced by TLR4 or TLR9. IFN-β can be induced in a MyD88-independent and -dependent manner through TLR4 and TLR9 respectively. However, in either case, IFN-β is critical for expression of IFN-inducible genes. (B) CD40 up-regulation by TLR9 signaling is more dependent on IFN-β than that by TLR4 signaling. Mutant DC with defective type I IFN signaling retained more CD40 expression in TLR4-stimulated DC than in TLR9-stimulated DC (Fig. 6A–D). Thus, IFN-β is differentially involved in TLR4- and TLR9-induced CD40 up-regulation. Broken lines indicate the MyD88-independent pathway in TLR4 signaling.

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targets of the TLR4-mediated MyD88-independent pathway (Fig. 7A). IRF-3 activation, required for IFN-β gene expression (26,33), seems to be located along the pathway. However, it remains unknown how TLR4 signaling can lead to IRF3 activation. Another TLR4 adapter, TIRAP/Mal, is possibly involved in it, but further studies are necessary to clarify the MyD88-independent signaling pathway.

TLR9 signaling can induce similar sets of type I IFN-inducible genes to those induced by TLR4 signaling (Fig. 3). TLR9 signaling can induce IFN-β in a MyD88-dependent manner, and IFN-β and its signaling are responsible for the expression of IFN-inducible genes such as IP-10 or GARG16 (Fig. 7A). It remains unknown how TLR9 signaling can activate IFN-β gene expression without activating the MyD88-independent pathway. Taken together, IFN-β, induced in an either MyD88-independent or -dependent manner, is critically involved in enhanced expression of IFN-inducible genes through TLR4 or TLR9 (Fig. 7A).

LPS can induce DC maturation without MyD88, characterized by up-regulation of surface CD40 expression (11). Mutant DC with defective type I IFN signaling showed reduced levels of CD40 expression in response to LPS, indicating the involvement of type I IFN on CD40 up-regulation. LPS-stimulated MyD88$^{-/-}$ DC also exhibited slightly decreased expression of CD40 as compared with LPS-stimulated wild-type DC. Thus, MyD88-dependent and IFN-β-induced effects coordinately contribute to optimal CD40 induction by LPS (Fig. 7B). In contrast, TLR9-mediated CD40 up-regulation is largely dependent on type I IFN production (Figs 6C and D, and 7B). Thus, in terms of CD40 up-regulation, type I IFN is more critical in TLR9- than in TLR4-mediated effects on BMDC.

Type I IFN can be induced by not only viral but also bacterial infection (34). Because this phenomenon can be attributed to TLR signaling, it should be critical for manipulating host defense mechanisms to make it clear how TLR induce type I IFN production and how type I IFN is involved in TLR-induced biological effects. The present study can provide critical information for this goal.

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Abbreviations

APC	antigen-presenting cell
BM	bone marrow
DC	dendritic cell
GARG16	glucocorticoid attenuated response gene 16
GM-CSF	granulocyte macrophage colony stimulating factor
IFN-α/βR	IFN-α/β receptor
IL-1R	IL-1 receptor
IP-10	IFN-inducible protein 10
IRF	IFN-regulatory factor
IRG1	immune-responsive gene 1
ISRE	IFN-stimulated regulatory element
LPS	lipopolysaccharide
MALP-2	macrophage-activating lipopeptide-2
MAPK	mitogen-activated protein kinase
STAT-1	signal transducer and activator of transcription 1
TIR	Toll/IL-1 receptor homology
TLR	Toll-like receptor
TNF	tumor necrosis factor

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