

A type I interferon autocrine–paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells

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Dendritic cells (DC) produce interleukin-12 (IL-12) in response to Toll-like receptor (TLR) activation. Two major TLR signaling pathways participate in the response to pathogens: the nuclear factor- κ B (NF- κ B)-dependent pathway leading to inflammatory cytokine secretion including IL-12 and the interferon (IFN)-dependent pathway inducing type I IFN and IFN-regulated genes. Here we show that the two pathways cooperate and are likely both necessary for inducing an optimal response to pathogens. R-848/Resiquimod (TLR7 ligand in the mouse and TLR7/8 ligand in human) synergized with poly(I:C) (TLR3 ligand) or lipopolysaccharide (LPS; TLR4 ligand) in inducing high levels of bioactive IL-12p70 secretion and IFN- β mRNA accumulation by mouse bone marrow-derived DC (BM-DC). Strikingly, IL-12p70 but not IL-12p40 secretion was strongly reduced in BM-DC from STAT1^{-/-} and IFNAR^{-/-} mice. STAT1 tyrosine-phosphorylation, IL-12p35, and IFN- β mRNA accumulation were strongly inhibited in IFNAR^{-/-} BM-DC activated with the TLR ligand combinations. Similar observation were obtained in human TLR8-expressing monocyte-derived DC (moDC) using neutralizing anti-IFNAR2 antibodies, although results also pointed to a possible involvement of IFN- λ 1 (also known as IL-29). This suggests that TLR engagement on DC induces endogenous IFNs that further synergize with the NF- κ B pathway for optimal IL-12p70 secretion. Moreover, analysis of interferon regulatory factors (IRF) regulation in moDC suggests a role for IRF7/8 in mediating IRF3-independent type I IFN and possibly IL-12p35 synthesis in response to TLR7/8.

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Abbreviations used: CHX, cycloheximide; IRF-3, IFN regulatory factor 3; LRR, leucine-rich repeats; moDC, monocyte-derived DC; NF- κ B, nuclear factor- κ B; pDC, plasmacytoid DC; TICAM, TIR-containing adaptor molecule; TIR, Toll/IL-1 receptor; TLR, toll-like receptor; TRIF, TIR-containing adaptor inducing IFN- β .

DCs are BM-derived APCs that play an important role in bridging innate resistance and adaptive immunity (1). Immature DCs reside in peripheral tissues where during infection they release soluble mediators (cytokines, chemokines, and interferons) participating in the innate inflammatory responses. After capture of pathogen-derived antigens and activation by pathogens, DCs migrate from tissues via the lymph to lymphoid organs where they become mature DCs and prime naive T cells to initiate acquired immune responses.

DCs recognize motifs that are conserved between large classes of microbial pathogens and bind to germline-encoded receptors (2). Among

these receptors, the family of Toll-like receptors (TLR) is composed of at least 11 members that are differentially expressed among inflammatory cells (3, 4). TLR are type 1 transmembrane proteins characterized by the presence of an extracellular domain containing leucine-rich repeats (LRR) involved in ligand recognition and a cytoplasmic Toll/IL-1 receptor (TIR) domain similar to that of the IL-1R family and involved in activation of signal transduction (5, 6). Each member of the TLR family recognizes molecular structures of the different classes of pathogens and triggers a specific cellular activation program through the recruitment of cytoplasmic adaptors to their TIR domain (5–7). Two major independent but complementary pathways in TLR signaling are classically considered: (a) the nuclear factor- κ B (NF- κ B) activation through recruitment of the adaptor MyD88 upon TLR2,

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4, 7, 8, and 9 activation or TIR-containing adaptor inducing IFN- β (TRIF)-TIR-containing adaptor molecule (TICAM)-1 upon TLR3 activation, leading to the production of inflammatory cytokines such as IL-1 β , IL-6, TNF- α , and IL-12, and (b) the IFN regulatory factor 3 (IRF3) activation through TRIF-TICAM-1 recruitment to TLR3 and TLR4 leading to type I IFN production and subsequently to induction of IFN-responsive genes such as antiviral genes and the CXC-chemokine IP-10/CXCL10 (6, 8). Additionally, recent observations have identified IRF7 as an important effector in TLR7-, 8-, and 9-mediated type I IFN production (9, 10).

Several populations of DCs have been characterized. Myeloid DCs have been described both in humans and in the mouse and produce IL-12 upon TLR engagement (3, 11, 12). They express TLR2, TLR3, and TLR4, in both species, whereas TLR7 is only expressed in the mouse myeloid DCs and TLR8 in the human ones. The plasmacytoid DC (pDC), alternatively named natural type I IFN producer, have also been identified in both species and are the main producers of type I IFN in response to viruses, although in mouse they can also produce IL-12 (13, 14). TLR7 and TLR9 are the main receptors expressed on pDCs involved in virus and bacteria recognition and type I IFN production through a strictly MyD88-dependent signaling pathway (15).

IL-12, a cytokine that augments the cytolytic activity and induces IFN- γ production and proliferation in NK and T cells, is a potent inducer of Th1 responses and is considered as a bridge between innate resistance and adaptive immunity (12, 16–18). The bioactive form of IL-12 is the heterodimeric IL-12p70 composed of the p35 and the p40 chains encoded by separate genes. Free IL-12p40 is often produced in large excess over the IL-12p70 heterodimer. The initial production of IL-12 occurs rapidly and is independent of IFN- γ and of signals from T cells (19, 20), however, production of IL-12p70 is facilitated by stimulation through CD40-ligand (21) and IFN- γ priming (22). The *in vitro* models for myeloid DCs classically used to study secretion of IL-12p70 are BM-derived DCs (BM-DCs) in mouse and monocyte-derived DCs (moDCs) in human. In the mouse system, IFN- γ priming is usually required in order to observe significant IL-12p70 secretion in response to TLR stimulation (22, 23). In contrast, human moDCs produce IL-12p70 in response to single TLR stimulation (12).

Although large amount of type I IFN, mainly IFN- α , are produced by pDCs after exposure to viruses (14, 24), most cells including myeloid DCs can produce IFN- β (25, 26). Because IFN- γ is a potent enhancer of IL-12p70 secretion and because IFN- γ and type I IFN receptors activate a common signaling pathway through STAT1 phosphorylation (25, 27), we investigated whether endogenous type I IFN play a role in IL-12p70 production. Herein, using BM-DCs from type I IFN receptor deficient (IFNAR $^{-/-}$) or STAT1 $^{-/-}$ mice or human moDCs cultured in the presence of anti-IFNAR blocking antibodies, we demonstrated that IL-12p70 production in response to TLR activation was dependent on production of endogenous type I IFN that regulated IL-12p35 mRNA accumulation.

RESULTS

R-848 synergizes with LPS or poly(I:C) in inducing IL-12p70 secretion by mouse BM-DCs

We analyzed whether combinations of ligands for different TLR could induce IL-12p70 secretion by mouse BM-DCs in the absence of exogenous IFN. As previously reported, high levels of IL-12p40 were induced after activation with LPS or R-848 (28) and less efficiently with poly(I:C) (Fig. 1 A). In contrast, when used alone, these ligands induced only very low levels of IL-12p70 (Fig. 1 B). However, the combination of R-848 with LPS- or poly(I:C)-induced secretion of IL-12p70 at levels comparable to that induced by LPS in the presence of exogenous IFN- γ (Fig. 1 B). Moreover, neutralization of IL-10 by anti-IL-10R antibody increased the production of IL-12p70 by BM-DCs activated by combinations of R-848 with either LPS or poly(I:C) (Fig. 1 B). Production of IL-12p40 was also increased by these combinations and further up-regulated by neutralizing IL-10 (Fig. 1 A). No synergy for IL-12p70 production was observed

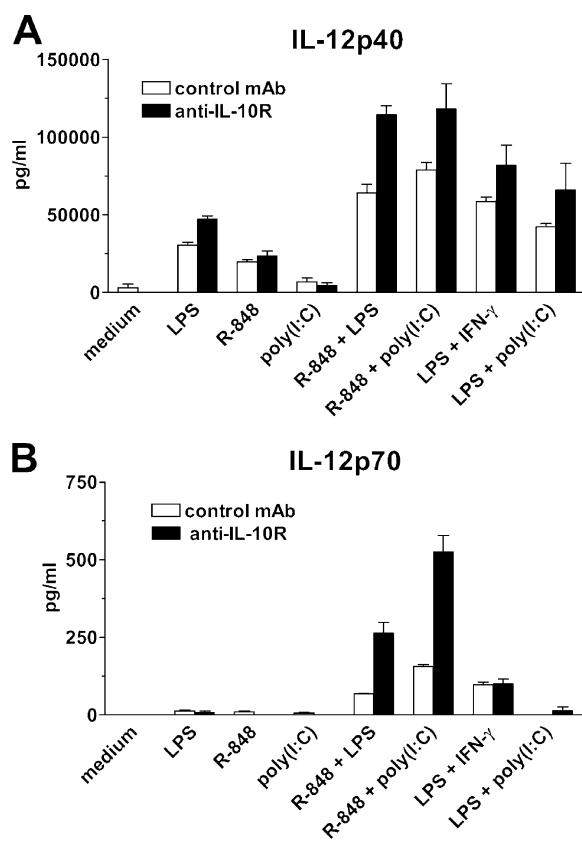


Figure 1. R-848 synergizes with LPS or poly(I:C) in inducing IL-12p70 secretion by mouse BM-DCs without IFN- γ priming.

BM-DCs from wild-type mice (129Sv) were activated with LPS (25 ng/ml), R-848 (10 μ M), or poly(I:C) (25 μ g/ml) alone or in combination, or with LPS plus IFN- γ (20 ng/ml) in the presence of 10 μ g/ml anti-IL10 receptor mAb (anti-IL-10R) or rat IgG1 control mAb. After 24 h, supernatants were collected and (A) IL-12p40 and (B) IL-12p70 were measured using specific ELISA. Data are expressed as mean \pm SD of culture triplicates and are representative of four experiments.

with PGN plus R-848, PGN plus LPS (unpublished data) or LPS plus poly(I:C) combinations with or without anti-IL-10R antibody (Fig. 1). To favor IL-12 secretion, all further experiments with mouse BM-DCs were performed in the presence of anti-IL-10R antibodies.

STAT1 is required for optimal IL-12p70 production

STAT1 is an essential component of the signaling cascade of both type I and type II IFN receptors (25). Because IFN γ increased IL-12 through STAT1 phosphorylation and because we also observed in BM-DCs STAT1 phosphorylation in response to TLR activation (see Fig. 4), we analyzed the capacity of BM-DCs derived from STAT1 $^{-/-}$ mice to produce IL-

12 in response to TLR ligand combinations. No differences in phenotype were observed by flow cytometry analysis of BM-DCs from wild-type or STAT1 $^{-/-}$ mice (Fig. 2 A) and they produced similar amounts of IL-6 upon activation (Fig. 2 B). Unlike IL-6, secretion of the IFN-dependent CXCL10 chemokine (29) was almost completely abrogated in BM-DCs from STAT1 $^{-/-}$ mice (Fig. 2 C). Interestingly, IL-12p70 secretion was dramatically decreased in BM-DCs from STAT1 $^{-/-}$ mice activated with combinations of R-848 with LPS or poly(I:C) (Fig. 2 D), whereas production of IL-12p40 (Fig. 2 E) was either not affected for the TLR alone or only weakly decreased for the selected ligand combinations. These results indicated that IL-12p70, but not IL-12p40 secretion was strongly dependent on a STAT1-dependent pathway.

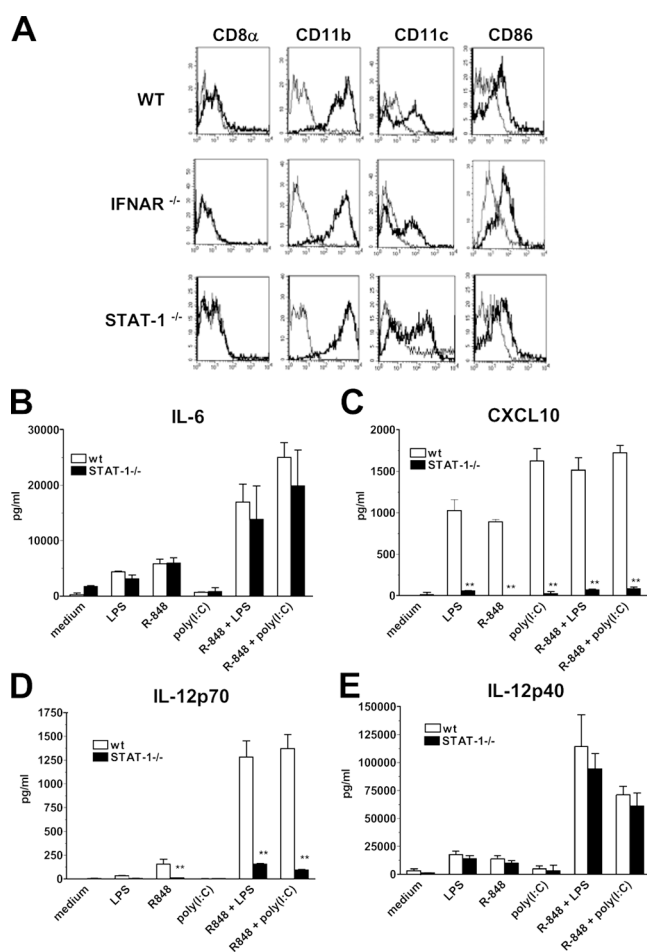


Figure 2. STAT1 is required for optimal IL-12p70 production by BM-DCs. (A) Similar phenotype of BM-DCs derived from wild-type (WT), STAT1 $^{-/-}$, and IFNAR $^{-/-}$ mice. Expression of CD8 α , CD11b, CD11c, and CD86 was analyzed by flow cytometry on BM-DCs derived from the indicated mice. (B–E) BM-DCs from wild-type or STAT1 $^{-/-}$ mice were activated for 24 h with different TLR ligands and their combinations in the presence of anti-IL-10R mAb as described in Fig. 1. Concentrations of (B) IL-6, (C) CXCL10, (D) IL-12p70, and (E) IL-12p40 were quantified in supernatants by specific ELISA. Data are expressed as mean \pm SD of culture triplicates and are representative of three experiments. Statistical significance is indicated (**P < 0.01).

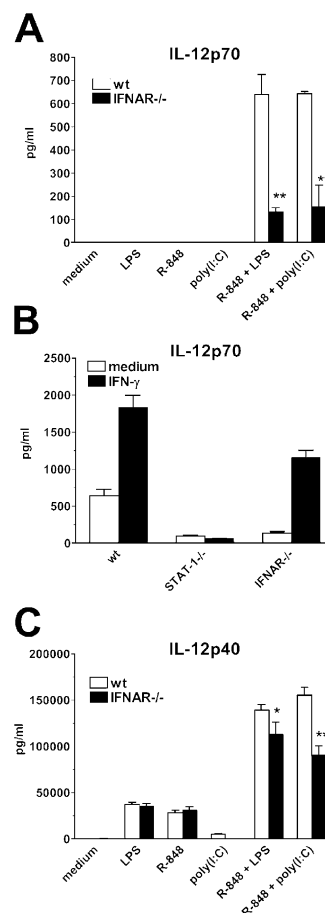


Figure 3. Type I IFNs are required for optimal IL-12p70 production by mouse BM-DCs. (A and C) BM-DCs from wild-type (wt) or IFNAR $^{-/-}$ mice were activated with the TLR ligands indicated and their combinations in the presence of anti-IL-10R mAb as described in Fig. 1. (B) BM-DCs from wild-type, STAT1 $^{-/-}$, or IFNAR $^{-/-}$ mice were activated with the combination R-848 plus LPS and with or without 20 ng/ml IFN- γ . Supernatants were collected after 24 h and (A and B) IL-12p70 and (C) IL-12p40 were measured using specific ELISA. Data are expressed as mean \pm SD of culture triplicates and are representative of three experiments. Statistical significance comparing results from each deficient mice to wild-type mice is indicated (*P < 0.05; **P < 0.01).

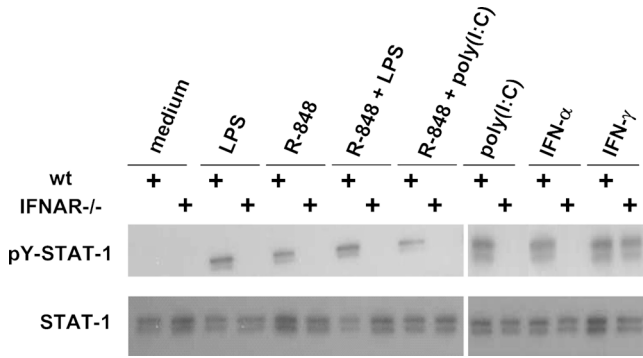


Figure 4. Tyrosine phosphorylation of STAT1 by TLR ligands and their combinations requires a type I IFN pathway. BM-DCs from wild-type or IFNAR1^{-/-} mice were activated with IFN- α (1,000 U/ml), IFN- γ (20 ng/ml), or different TLR ligands at concentration described in Fig. 1. After 2 h, stimulation was stopped with cold PBS and BM-DCs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-phosphotyrosine STAT1 (pY-STAT1) or anti-STAT1 antibodies and analyzed as described in Materials and methods. Results are representative of three experiments.

Type I IFN are required for optimal IL-12p70 secretion

To determine whether the role of STAT1 in IL-12p70 secretion was linked to endogenous IFN production, we performed similar experiments using IFN- $\alpha\beta$ receptor 1-defi-

cient mice (IFNAR^{-/-}). As shown in Fig. 3 A, a strong reduction (>75% decrease) of IL-12p70 production induced by the selected TLR ligand combinations was observed with BM-DCs from IFNAR^{-/-} mice. As expected, BM-DCs from IFNAR^{-/-}, but not from STAT1^{-/-} mice, could respond to exogenous IFN- γ as evidenced by augmentation of the IL-12p70 production in response to R-848 and LPS (Fig. 3 B). Unlike IL-12p70, IL-12p40 secretion was only slightly affected by the absence of functional type I IFN receptor (Fig. 3 C). Moreover, no significant inhibition of IL-6 but strong inhibition of CXCL10 production was observed with IFNAR^{-/-} BM-DCs (unpublished data). These data showed that BM-DCs from IFNAR^{-/-} mice behaved essentially as cells derived from STAT1^{-/-} mice. Indeed, R-848, poly(I:C), LPS and their combinations induced tyrosine-phosphorylation of STAT1 in BM-DCs from wild-type mice but not in BM-DCs derived from IFNAR^{-/-} mice (Fig. 4). Altogether this suggested that endogenous type I IFNs were involved in the observed IL-12p70 secretion in response to the combination of R-848 with either LPS or poly(I:C).

Accumulation of IL-12p35 and type I IFN mRNA is strongly reduced in BM-DCs from IFNAR^{-/-} mice

Real-time PCR for IL-12p35 and IL-12p40 mRNA were performed with BM-DCs from wild-type and IFNAR^{-/-}

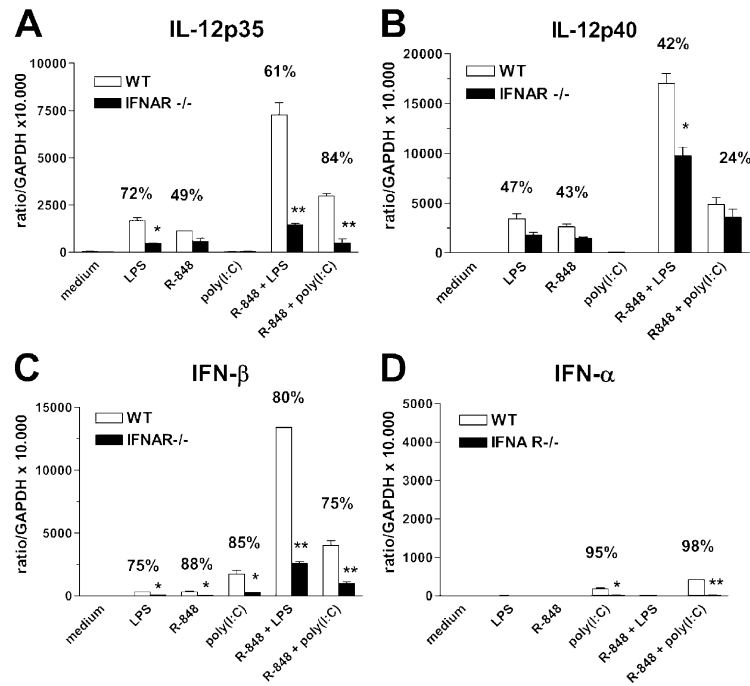


Figure 5. Induction of IL-12p35, IL-12p40, IFN- β , and IFN- α mRNA in TLR-stimulated wild-type and IFNAR1^{-/-} BM-DCs. BM-DCs from wild-type or IFNAR1^{-/-} mice were activated with TLR ligands indicated in the presence of anti-IL10R antibodies as described in Fig. 1. After 4.5 h, stimulations were stopped with cold PBS and mRNA was extracted and reverse transcribed. Real-time PCR was performed using primers spe-

cific for mouse (A) IL-12p35, (B) IL-12p40, (C) IFN- β , or (D) IFN- α (all genes) genes on cDNA normalized with GAPDH. Data are expressed as -fold induction of the gene of interest over GAPDH calculated with the following formula $(1.8^{(CT_{GAPDH} - CT_{gene})})$. Percentages of inhibition are indicated. Statistical significance is indicated (*P < 0.05; **P < 0.01). Results are representative of three experiments.

mice. As shown in Fig. 5 (A and B), IL-12 p35 and p40 mRNA were induced in BM-DCs from wild-type mice after a 4.5-h activation by LPS or R-848 and at higher levels by the combination of R-848 with either LPS or poly(I:C). The induction of IL-12p35 mRNA accumulation was strongly decreased in BM-DCs from IFNAR^{-/-} mice (50–80% decrease; Fig. 5 A), although that of IL-12p40 was decreased to a lower degree (20–40% decrease; Fig. 5 B). Thus, TLR-induced IL-12p35 mRNA accumulation was more dependent on type I IFN than that of IL-12p40 mRNA, consistent with the stronger decrease of IL-12p70 than IL-12p40 secretion by blocking type I IFN signaling.

Real-time PCR for type I IFN showed marginal induction of IFN- β mRNA by treatment with LPS and R-848 and strong up-regulation by poly(I:C) and the combinations of R-848 plus either LPS or poly(I:C) (Fig. 5 C). However, IFN- β mRNA accumulation was strongly reduced in IFNAR^{-/-} BM-DCs in all conditions (Fig. 5 C). In contrast, using primers hybridizing to all IFN- α genes, very low IFN- α mRNA accumulation was detected only in response to poly(I:C) and R-848 plus poly(I:C) combination in wild-type BM-DCs, but again not in IFNAR^{-/-} BM-DCs (Fig. 5 D). This strong inhibition of IFN- α/β induction was most likely a consequence of the absence of an IFN-dependent amplification loop for type I IFN in BM-DCs from IFNAR^{-/-} mice (30).

IL-12 secretion, STAT1 phosphorylation, and IRF1 induction in human moDCs in response to TLR ligands

Like mouse BM-DCs, human moDCs secreted very high levels of IL-12 when activated with R-848 and LPS (31) or poly(I:C) combinations (Fig. 6, A and B). However, as moDCs produced relatively high levels of IL-12p70 upon single TLR ligand stimulation (12; Fig. 6 A), all further analysis were performed using single TLR activation. LPS and R-848 but not poly(I:C) also promoted strong secretion of IL-12p40 (Fig. 6 B). Activation with PGN (Fig. 6, A and B) or other TLR2 ligands like PAM3Cys and zymosan (unpublished data) did not promote IL-12p70 secretion while inducing IL-12p40. Tyrosine phosphorylation of STAT1 was observed upon LPS, R-848, and poly(I:C) activation, with R-848 inducing a rapid STAT1 phosphorylation already detectable after a 1-h stimulation (Fig. 6 C). Interestingly, PGN was the only TLR activator that did not induce tyrosine phosphorylation of STAT1 and IL-12p70 secretion. In contrast, induction of IRF1, an IFN-inducible transcription factor (32), was observed whatever TLR ligand used including PGN (Fig. 6 D). Inhibition of protein synthesis with cycloheximide (CHX) almost completely inhibited STAT1 tyrosine phosphorylation in response to R-848 and poly(I:C) and partially that induced in response to LPS (Fig. 6 D). As expected, the presence of CHX, did not affect the STAT1 tyrosine phosphorylation directly induced by a mixture of IFN- α and IFN- β (Fig. 6 D), but inhibited the synthesis of IRF1 protein in response to IFN- α/β and the different TLR ligands (Fig. 6 D).

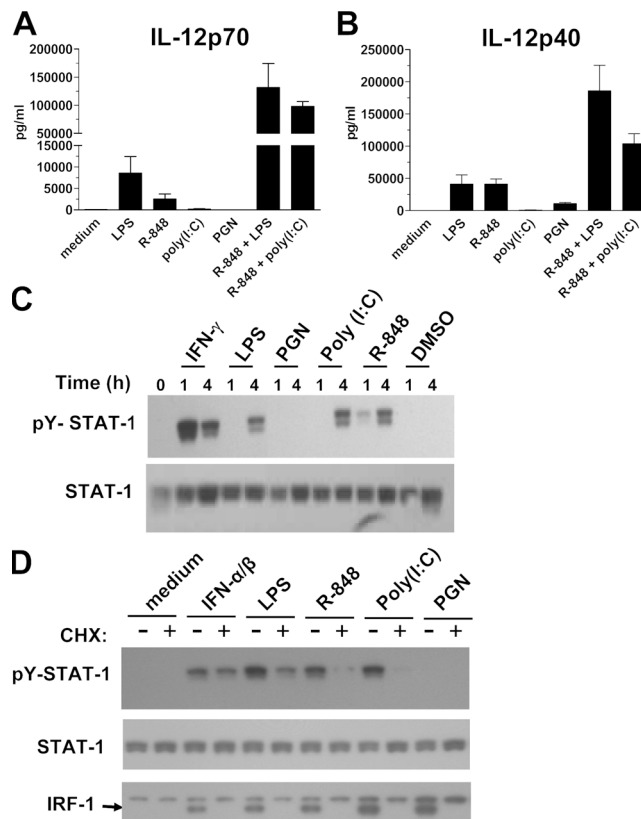


Figure 6. TLR-induced IL-12p70 secretion by human moDCs is correlated with the capacity of the TLR ligand to induce STAT1 tyrosine phosphorylation. (A and B) moDCs were activated for 24 h with or without optimal concentrations of LPS, R-848, poly(I:C), PGN, or their combinations. (A) IL-12p70 and (B) IL-12p40 were measured in supernatants using specific ELISA. Data are expressed as mean \pm SD of six experiments performed with different donors. (C) moDCs were cultured with or without 30 ng/ml IFN- γ , LPS, PGN, poly(I:C), R-848, or DMSO (1/1,000 final dilution for R-848 control). (D) moDCs were activated with or without the TLR ligands as in C for 1 or 4 h (C) or 2 h (D), or a mixture of IFN- α and IFN- β (500 U/ml each) in presence or not of CHX (25 μ g/ml) added 15 min before activation. Activations were stopped with cold PBS, moDCs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-pY-STAT1, anti-STAT1, or anti-IRF1 antibodies and analyzed as described in Materials and methods. Results are representative of three independent experiments.

Type I IFN participate in IL-12p70 secretion by human moDCs in response to R-848 and LPS

Addition of a neutralizing anti-human IFNAR2 antibody partially blocked IL-12p70 secretion by moDCs in response to LPS and R-848 (Fig. 7 A). The levels of IL-12p70 induced by poly(I:C) were generally too low to conclude on a significant effect of anti-IFNAR2 mAb (unpublished data). No effect of anti-IFNAR2 mAb was observed on IL-12p40 production after LPS or R-848 activation (Fig. 7 B). Addition of exogenous type II or type I IFN (IFN- α or - β) during LPS or R-848 activation up-regulated IL-12p70 secretion by moDCs (Fig. 7 C) but did not modify IL-12p40 production (unpublished data). Secretion of CXCL10 in re-

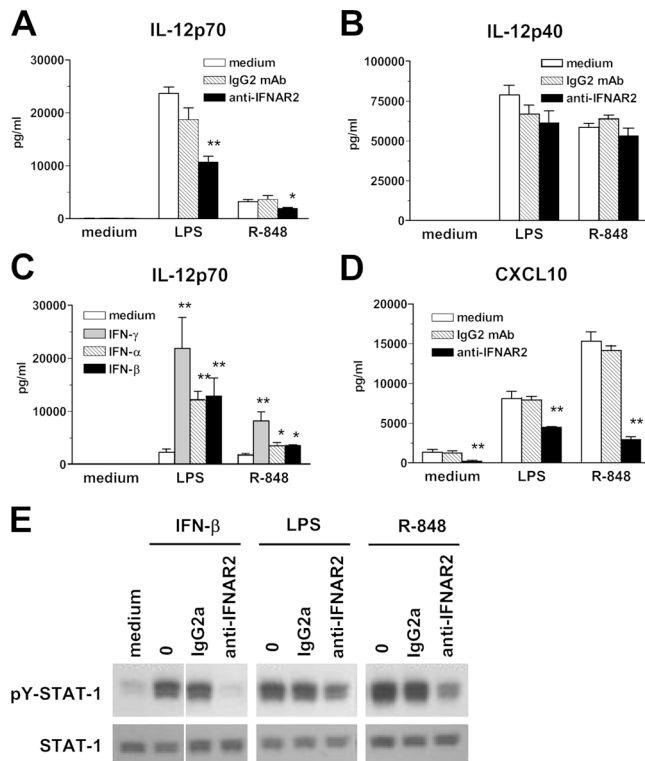


Figure 7. Neutralizing type I IFN partially blocks LPS- and R-848-induced IL-12p70 secretion and STAT1 tyrosine phosphorylation in human moDCs. (A, B, and D) moDCs were activated for 24 h with or without LPS or R-848 in the presence or the absence of 30 μ g/ml neutralizing anti-IFNAR2 antibodies (CD118) or IgG2a control mAb. Concentrations of (A) IL-12p70, (B) IL-12p40, and (D) CXCL10 were quantified by specific ELISA in supernatants. Statistical significance of results with anti-IFNAR2 mAb compared with control mAb is indicated (* $P < 0.05$; ** $P < 0.01$). (C) moDCs were activated with LPS or R-848 with or without exogenous IFN- α , IFN- β , or IFN- γ and IL-12p70 was quantified by ELISA after a 24-h incubation. Data are expressed as mean \pm SD of culture triplicates and are representative of three experiments with different donors. Statistical significance is indicated (* $P < 0.05$; ** $P < 0.01$). (E) moDCs were pretreated for 30 min with or without 30 μ g/ml neutralizing anti-IFNAR2 antibodies or IgG2a control mAb and then activated for 2 h with or without LPS, R-848, or 1,000 U/ml IFN- β . Analysis of pY-STAT1 in moDCs lysates was performed by Western blot analysis as described in Fig. 6. Results are representative of three independent experiments.

response to LPS or R-848 was only partially inhibited by the anti-IFNAR2 antibody (Fig. 7 D). Furthermore, this antibody also only partially inhibited LPS- and R-848-induced STAT1 phosphorylation in moDCs, whereas it totally neutralized that induced by 1,000 U/ml of IFN- β (Fig. 7 E) or IFN- α (unpublished data). This suggests that this antibody either could not achieved complete neutralization of the type I IFN receptor in TLR ligand-activated moDCs or that other mechanisms were implicated in both STAT1 tyrosine phosphorylation and induction of IFN-dependent molecules such as CXCL10 in human moDCs.

Analysis of type I IFN expression by real-time PCR indicated that IFN- β mRNA was induced in moDCs after

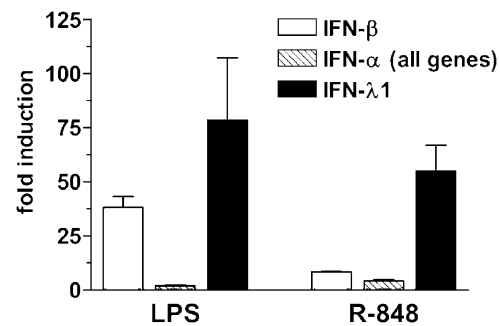


Figure 8. Induction of IFN- β , IFN- α , and IFN- λ 1 (also known as IL-29) mRNA in TLR-stimulated human moDCs. moDCs were activated for 4 h with or without LPS or R-848. mRNA were extracted and reverse transcribed. Real-time PCR was performed using primers specific for human IFN- β , IFN- α all genes, or IFN- λ 1 (also known as IL-29) with cDNA normalized to GAPDH expression. Expression of the gene of interest was quantified by applying the formula $1.8^{(CT\ GAPDH - CT\ gene\ of\ interest)}$. Data are expressed as the -fold induction of the gene of interest in the different activation conditions compared with medium and are representative of four independent experiments.

LPS and R-848 activation (Fig. 8). In contrast, IFN- α mRNA (all genes) was only very weakly induced by LPS or R-848 (Fig. 8). Confirming real-time PCR, IFN- α production was not detected at the protein level in supernatants of activated-moDCs, although IFN- β could be readily detected in response to LPS (unpublished data). Interestingly, IFN- λ 1 (IL-29) mRNA was found to be induced by LPS and R-848 in moDCs (Fig. 8), indicating that IFN other than type I IFNs, that does not use the type I IFN receptor 1 and receptor 2 chains but signals through STAT1 activation (27, 33, 34) might be involved in STAT1 phosphorylation and possibly in IL-12p70 secretion in moDCs.

Unlike LPS and poly(I:C), R-848 did not activate IRF3 in moDCs, but induced IRF7 and IRF8

To address whether LPS and R-848 used a common signaling pathway for inducing IFN in moDCs, we analyzed the activation status of IRF3 that has been described as the major transcription factor involved in IFN- β induction after its phosphorylation and translocation within the nucleus (35, 36). However, unlike LPS and poly(I:C), R-848 did not induce nuclear translocation of IRF3 in moDCs (unpublished data). Furthermore, serine-phosphorylated IRF3 was detected only in nuclear extracts of moDCs activated by LPS or poly(I:C) but not in cells stimulated with R-848 (Fig. 9 A). Thus, these data suggest that R-848 may induce type I IFN in moDCs through an IRF3-independent pathway. The expression of other IRFs involved in type I IFN gene regulation (30) was then analyzed by real-time PCR. In accordance with a previous study (37), we detected a weak constitutive expression of IRF4, IRF5, and IRF7 mRNA in resting moDCs. However, IRF7, but not IRF4 and IRF5, mRNA was found to accumulate in moDCs activated with LPS, poly(I:C) or R-848 (Fig. 9 B). Finally, IRF8 expression was also increased after LPS and R-848 but not poly(I:C) activation in moDCs (Fig. 9 B).

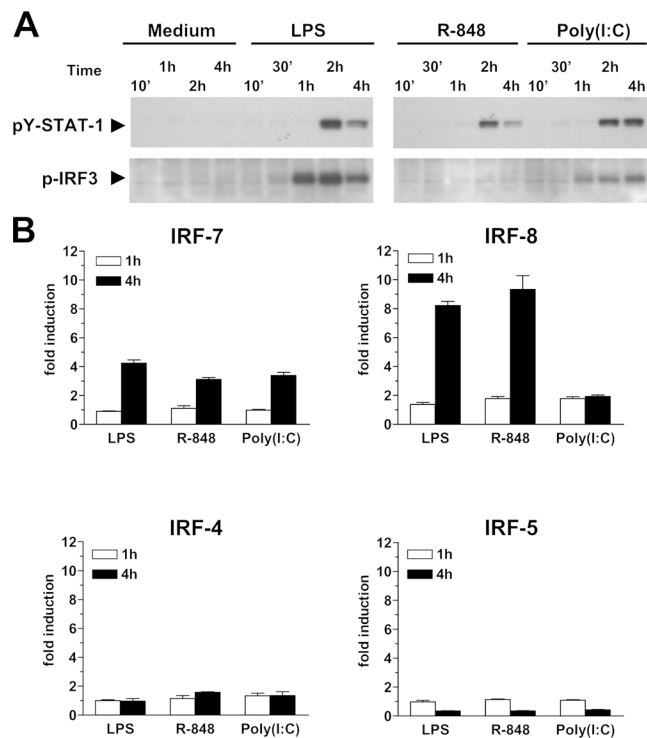


Figure 9. (A) LPS and poly(I:C) but not R-848, induce nuclear translocation of phosphorylated IRF3 in moDCs. moDCs were activated with or without the indicated TLR ligands. At the time indicated, stimulations were stopped with cold PBS and nuclei were isolated as described in Materials and methods. After SDS-PAGE of nuclear extracts and Western blot, membranes were incubated with anti-pY-STAT1 or anti-phospho-IRF3 (p-IRF3) antibodies and analyzed as described in Materials and methods. Results are representative of three independent experiments. (B) Expression of IRF7, IRF8, IRF4, and IRF5 mRNA in TLR-stimulated human moDCs. moDCs were activated for 1 or 4 h with or without LPS, poly(I:C) or R-848. mRNA were extracted and reverse transcribed. Real-time PCR was performed using primers specific for the human IRF indicated with cDNA normalized to GAPDH expression. Expression of the gene of interest was quantified by applying the formula $1.8^{(CT\text{ GAPDH} - CT\text{ gene of interest})}$. Data are expressed as the -fold induction of the gene of interest in the different activation conditions compared with medium and are representative of three independent experiments.

DISCUSSION

In the present study we demonstrate that an autocrine loop of type I IFN is required for bioactive IL-12p70 secretion by myeloid DCs. This conclusion is supported by the following observations: (a) both in humans and mice, all TLR ligands tested or their combinations that lead to IL-12p70 production also induced STAT1 tyrosine phosphorylation; (b) TLR2 ligands that did not trigger STAT1 phosphorylation failed to stimulate IL-12p70 secretion; (c) optimal IL-12p70 production correlated with the strongest type I IFN induction; (d) BM-DCs from STAT1^{-/-} mice did not produce IL-12p70; (e) BM-DCs from IFNAR^{-/-} mice did not phosphorylate STAT1 and secreted neither IL-12p70 nor synthesized IL-12p35 mRNA; and (f) in human moDCs, STAT1 phosphorylation and IL-12p70 production were altered by

neutralizing anti-IFNAR2 antibodies whereas IL-12p70 secretion was amplified by addition of exogenous type I IFN.

Induction of type I IFN has been demonstrated to play an important role in the cascade of gene expression after TLR signaling (26, 38, 39). In particular, TLR3, 4, 7, and 9, but not TLR2 activation can induce synthesis and secretion of IFN- β by monocytes-macrophages and DCs that secondarily acts through an autocrine-paracrine loop to induce the expression of IFN-regulated genes (29, 40–42). In agreement with previous studies on TLR3 and TLR4 (42–44), we show that STAT1 tyrosine phosphorylation observed in BM-DCs in response to R-848 and to R-848/LPS or R-848/poly(I:C) combinations is dependent on type I IFN receptor signaling.

The requirement for type I IFN in IL-12p70 production reported herein was not previously described mainly because most investigations in mice used IFN- γ priming for IL-12 production thus masking the requirement for endogenous type I IFN. Moreover, most previous studies with macrophages or DCs from IFNAR^{-/-} or STAT1^{-/-} mice have only analyzed the expression of the p40 gene that is less dependent on endogenous IFN synthesis and signaling (45, 46). Our observation is consistent with previous studies in humans showing that exogenous type I IFN enhances IL-12p70 production (47, 48). Other studies in mice and humans reported that type I IFN blocks IL-12 production but most studies have analyzed IL-12p40 or used nonspecific bioassays for IL-12 p70 (49–54), and applied type I IFN concentration far higher compared with the low levels endogenously detected in our systems. Thus, this discrepancy is likely to be related in part to differential effects of high versus low dose or long lasting versus short exposure to type I IFN as previously reported (55, 56). Nevertheless, these observations show that upon in vivo viral infection IL-12 can be produced in the absence of type I IFN possibly as a consequence of contribution of other types of IFN (IFN- γ , IFN- λ). In this context, and in agreement with a recent report (57), we observed that LPS- and R-848-activated moDCs can produce IFN- λ 1 that may contribute to STAT1 phosphorylation (33, 34) and IL-12p70 production.

The role of STAT1 phosphorylation in IL-12p70 production may shed new light on the in vivo deficiency observed in STAT1^{-/-} mice or in STAT1-deficient human patients (58, 59). In particular, an abrogated type I IFN loop may also result in an impaired IL-12p70 production participating in the deficiency in TH-1 immune responses observed in these deficiencies.

IL-12 is an inflammatory cytokine whose production is dependent on NF- κ B activation (12) and our study shows that the TLR signaling pathway leading to type I IFN production cooperates with the NF- κ B-dependent pathway to trigger bioactive IL-12p70 production. Accordingly, TLR2 ligands that do not activate the IFN-dependent pathway only induce IL-12p40 production in a MyD88- and NF- κ B-dependent manner. Importantly our results point to a role of type I IFN in the accumulation of IL-12p35 transcripts.

IRF1 and NF- κ B/c-Rel have been shown to play major roles in the transcriptional activation of the IL-12p35 gene (60, 61). We observed IRF1 induction in response to TLR ligands suggesting that endogenous type I IFN may trigger IRF1 production required for transcription induction of IL-12p35. However, other signaling pathways such as TLR2 can also lead to IRF1 induction without triggering of IL-12p70, suggesting the cooperation between several transcription factors for IL-12p35 induction. Of interest, Liu et al. (62) recently reported that ICSBP/IRF8 acts in synergy with IRF1 to activate IL-12p35 mRNA transcription through binding to an ICSBP-response element in the human IL-12p35 promoter. The sequence of this binding element does not match the conventional IRFE and ISRE sequences, or the newly described subset of ISRE (ETS/IRF response element, EIRE) that can interact with ICSBP, IRF4, and PU.1 (63). It has also been recently shown that TLR7 and probably TLR9 activation forms a complex with MyD88, TRAF6, and IRF7 that results in IRF7 activation (9, 10). We observed up-regulation of IRF8 and IRF7, but not of IRF4 or IRF5 in response to LPS, R-848, or poly(I:C) activation. Thus, it is likely that induction/activation of IRF1 and IRF8 or possibly IRF7 by TLR7 and TLR8 and the role of IFN and STAT1 in their induction may be important for optimal IL-12p35 expression.

We show a selective synergistic activity between R-848 and either LPS or poly(I:C) for IFN α / β and IL-12p70 production in mouse BM-DCs and in human moDCs. This synergy may be dependent on the cooperative recruitment of MyD88 by TLR7 in the mouse and by TLR8 in human moDCs and TRIF by TLR4 and TLR3. The absence of synergy between LPS and poly(I:C) may be related to the common use of TRIF and possible competition for this adaptor. Alternatively, induction/activation of transcription factors of the IRF family are most likely candidates to explain the synergy between TLR4 or TLR3 and TLR7/8 activation for type I IFN and possibly IL-12 up-regulation. IRF3 is constitutively expressed by myeloid DCs (37) and activated through TLR3 and TLR4 engagement but not by TLR8 in human moDCs and by TLR7 in the mouse (10). Thus, the synergy between TLR3/4 and TLR7/8 might be due to the IRF3-dependent synthesis of IFN- β through TLR3/4 activation that further acts in an autocrine/paracrine feedback loop to induce IRF7 synthesis. IRF7 can be then activated by TLR7 or TLR8 engagement (in mouse BM-DCs or human moDCs, respectively) to promote optimal type I IFN and possibly IL-12p35 synthesis. It would be important to validate this hypothesis to assess the status of IRF7 phosphorylation upon TLR7/8 only versus TLR7/8 and TLR3/4 engagement.

Natural ligands for mTLR7 and hTLR8 are ssRNA rich in uracil and guanosin found in different viruses and also RNA from host cells (64–66). It is therefore possible that during viral infections the release of viral RNA and/or host RNA from endocytosed infected cells may simultaneously engage both TLR3 and TLR7 or TLR8 within endosomal

compartments of DCs. Moreover, several reports have shown TLR4-mediated responses to certain viruses (67–72) and TLR4 endogenous ligands (such as HSP60) might be induced during viral infection. In bacterial and parasitic infections production of type I IFN in addition to TLR-induced proinflammatory cytokines has also been shown (73, 74). Thus, physiological conditions engaging simultaneously TLR7/8 and other MyD88-signaling TLR with either TLR3/4 or other pathways of type I IFN production may represent optimal responses to certain infections. Similarly, during certain specific infections leading to both pDCs and myeloid DCs activation, a paracrine loop of type I IFN from pDCs may amplify IL-12 production from myeloid DCs. The simultaneous and synergic activation of both pathways in vivo is probably indicative of a serious productive infection and may be needed to signal a full-fledged innate and adaptive response, whereas the simple presence of individual TLR ligands may only results in a limited and transient inflammatory response. It was recently suggested that the synergistic stimulation of innate responses in mice by dsRNA and CpG DNA (75) could be exploited therapeutically for activating antitumor resistance.

MATERIALS AND METHODS

Hematopoietic factors, cytokines, reagents

Mouse cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine (Life Technologies), 50 μ M β -mercaptoethanol (Sigma-Aldrich), and 100 μ g/ml gentamycin (Schering-Plough). Recombinant TNF- α and GM-CSF (R&D Systems) were used at a final concentration of 100 U/ml and 10 ng/ml, respectively. Rat anti-mouse IL-10 receptor antibodies (clone 1B1.3A) were used at a concentration of 10 μ g/ml (DNAX).

Human cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 100 μ g/ml gentamycin. Hematopoietic factors and cytokines were recombinant human proteins. GM-CSF, IL-4, IFN- α 2b (Schering-Plough Research Institute), IFN- γ , and IFN- β (R&D Systems) were used at optimal concentration of 100 ng/ml, 10 ng/ml, 1,000 U/ml, 20 ng/ml, and 1,000 U/ml, respectively. Mouse anti-human IFNAR2 antibodies (CD118; PBL Biomedical Laboratories) and mouse anti-human IL-10 receptor (3c5.2b) antibodies (DNAX) were used at the concentration of 30 and 10 μ g/ml, respectively.

BM-DCs or moDCs were activated with 25 ng/ml *Escherichia coli* LPS (Sigma-Aldrich), 10 μ M R-848 (synthesized at Schering-Plough Research Institute), 10 μ g/ml PGN from *Staphylococcus aureus* (Sigma-Aldrich), or 25 μ g/ml poly(I:C; InvivoGen). All cytokines and TLR ligands, except LPS, were shown to be endotoxin-free, as determined by a Limulus-Amebocyte Assay (BioWhittaker).

Mice

Female 129Sv, IFNAR $^{-/-}$ (129Sv background), and STAT1 $^{-/-}$ (129Sv background) mice, 6–10 wk old, were purchased from B&K Universal or Taconic, bred at the Charles River animal facility, and maintained in our facilities under standard conditions. Procedures involving animals and their care were conducted in conformity with European Economic Community Council Directive 86/609, OJL 358,1, December 12, 1987.

Generation of DCs from human monocytes or from mice BM progenitors

Mouse BM-derived DCs. Mice were killed by CO₂ inhalation. BM cells were flushed out of the bones with cold PBS-FCS-EDTA. For cell depletions, cells were incubated for 30 min at 4°C with a mixture of the mAbs:

Table I. Primers used for PCR amplification

	Forward	Reverse
Human		
CD4 promoter	5'-TTCCACACTGGGCCACCTAT	5'-TTGTGGGCTTACCACTGCTG
IFN- β	5'-AGCTGAAGCAGTTCAGAAG	5'-AGTCTCATTCCAGCCAGTGC
IFN- α (all genes)	5'-GTGAGGAAATACTCCAAAGAATCAC	5'-TCTCATGATTCTGCTCTGACAA
IFN- λ 1/IL-29	5'-GTGGTGCTGGTGACTTTGG	5'-CTCCTGTGGTGACAGAGATTG
IRF4	5'-GAGCCAAGCATAAGGTCTGC	5'-TCAGCTCCTTCACGAGGATT
IRF5	5'-CGGACTGATGTGGAGATGTG	5'-CTCTCCTTCTGGCCCAAAT
IRF7	5'-TACCATCTACCTGGGCTTCG	5'-AGGGTTCAGCTTCACAG
IRF8	5'-GCTGATCAAGGAGCCTTCTG	5'-ACCAGTCTGGAAGGAGCTGA
Mouse		
CD4 promoter	5'-GGCACTAGTACCACCATTTGTG	5'-AGGAACGATTGATGCCTTGC
IFN- β	5'-GCACCTGGGTGGAATGAGACT	5'-AGTGGAGAGCAGTTGAGGACA
IFN- α (all genes)	5'-TCTGATGCAGCAGGTGGG	5'-AGGGCTCTCCAGACTTCTGCTCTG
IL-12p35	5'-CACCTTGCCTCCTAAACC	5'-CACCTGGCAGGTCCAGAGA
IL-12p40	5'-ACAGCACCAGCTTCTCATCAG	5'-TCTCAAAGGCTTCATCTGCAA
GAPDH	5'-TGCACCACCAACTGCTTAG	5'-GGATGCAGGGATGATGTTG

anti-CD3 molecular complex (17A2), anti-CD8 β (53-5.8), anti-CD11b (M1/70), anti-CD19 (1D3), anti-Ly6G/C (RB6-8C5), and antierythrocyte (TER119; all antibodies from BD Biosciences). Cells and goat anti-rat IgG-coated Dynabeads (Dyna) were mixed under agitation for 15 min at 4°C. Beads and attached cells were removed with a Dynal magnet. Depleted cells were cultured in 24-well culture plates at 2×10^5 cells/ml in the presence of GM-CSF and TNF- α . At day 3, 50% of the medium was removed and fresh medium containing GM-CSF plus TNF- α was added for the next 2 d. Flow cytometry analysis was performed on a FACScalibur flow cytometer (BD Biosciences) with rat anti-mouse CD8 α (53-6.7), anti-CD11b (M1/70), CD86 (GL1), and hamster anti-CD11c (HL3; BD Biosciences).

Human moDCs. PBMC were purified from human peripheral blood by Ficoll-Hypaque centrifugation. Monocytes were purified by adherence after preparation of PBMC followed by a 52% Percoll gradient and differentiated into moDCs after 5 d in the presence of GM-CSF and IL-4 (76).

Quantification of cytokine production

BM-DCs or moDCs were cultured at 10^5 cells/well in 200 μ l in 96-well flat-bottom culture plates and stimulated with LPS, R-848, poly(I:C), PGN, or combinations of ligands for 24 h. Culture supernatants were collected and IL-12p70, IL-12p40, IL-6, and IP-10 levels were determined by specific ELISA kits from R&D Systems for mouse and BD Biosciences for human cytokines.

Analysis of STAT1 and IRF3 phosphorylation

Cells were collected, washed once in RPMI 1% FCS, starved overnight in RPMI 1% FCS, and activated for different times with different activators. Stimulation was stopped by addition of cold PBS. Cells were washed and lysed in GBL lysis buffer on ice for 30 min. Then, lysates were centrifuged and phosphatase and protease inhibitors were added (phosphatase inhibitor cocktail set I, protease inhibitor cocktail set III; Calbiochem-Novabiochem). 1 vol of $2 \times$ SB (Novex) containing Bond Breaker TCEP solution (Pierce Chemical Co.) was added to lysates. In some experiments, nuclei were isolated from moDCs using the CelLytic nuclear extraction kit (Sigma-Aldrich). Lysates were boiled for 2 min at 95°C before running on a SDS-PAGE Tris-glycine (4-20%) gel. Transfer was at 85 V for 90 min in transfer buffer (Novex) containing 20% of methanol using PVDF membrane transfer (Immobilon-P; Millipore). Membrane was blocked with PBS 0.2% tween 20 (Sigma-Aldrich), 3% BSA (Sigma-Aldrich) for 45 min. Membranes were incubated overnight at 4°C with antiphosphotyrosine STAT1 (Cell Signaling), anti-STAT1 (Cell

Signaling), anti-IRF1 (Santa Cruz Biotechnology) or antiphosphoserine396 IRF3 (77) antibodies at dilutions of 1/1,000, 1/1,000, 1/1,000, and 1/10,000, respectively, in blocking buffer (PBS/BSA/Tween 20). Membranes were washed three times in PBS/Tween20 and incubated with goat anti-rabbit secondary HRP-conjugated antibody (Jackson ImmunoResearch Laboratories) at 1/5,000 dilution. After three washes, membranes were incubated with ECL (Amersham Biosciences) for 1 min in the dark at room temperature and developed in using Biomax MR-1 film (Eastman Kodak Co.).

RNA extraction, cDNA reverse transcriptase, and real-time RT-PCR analysis

Cells were lysed and total RNA was extracted by RNeasy kit (Qiagen). Genomic contamination was excluded by amplification (quantitative PCR, Light Cycler; Bio-Rad Laboratories) of the nontranscribed part of the human or mouse CD4 promoter (Table I; human probe: CACTGGACACAAT-TGCCCTCAGG), mouse probe: 5'-TGGTTGAAAGATGGCTACAGAGAAGAGG). First-strand cDNAs were prepared as previously described (78). Synthesis of cDNAs was controlled and normalized by performing real-time PCR amplification of the housekeeping gene GAPDH for both human and mouse. Real-time PCR were performed using SYBR green Master Mix (Applied Biosystem), primers described in Table I (400 nM final) and 25 ng of cDNA. Amplification of genes of interest and GAPDH were always performed on the same plate (thermo-fast 96-wells, semi skirled; ABgene, Epsom) with a final volume of 25 μ l. Cycle conditions were one cycle for 9 min at 94°C and then 94°C for 15 s, 60°C for 1 min for 50 cycles, followed by one cycle at 94°C for 3 min. Expression of the gene of interest was quantified by applying the formula $1.8^{(CT_{GAPDH} - CT_{gene\ of\ interest})}$.

Statistical analysis

Experimental results were analyzed for statistical significance (Student's *t* test or a paired *t* test) using SigmaStat Software package. Statistical significance is indicated in the figures (**P* < 0.05; ***P* < 0.01).

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