THE JOURNAL OF IMMUNOLOGY

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J Immunol 2006; 176:7438-7446; ;
doi: 10.4049/jimmunol.176.12.7438
http://www.jimmunol.org/content/176/12/7438

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

T Cell-Independent, TLR-Induced IL-12p70 Production in Primary Human Monocytes[1]

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IL-12p70 is a key cytokine for the induction of Th1 immune responses. IL-12p70 production in myeloid cells is thought to be strictly controlled by T cell help. In this work we demonstrate that primary human monocytes can produce IL-12p70 in the absence of T cell help. We show that human monocytes express TLR4 and TLR8 but lack TLR3 and TLR7 even after preincubation with type I IFN. Simultaneous stimulation of TLR4 and TLR8 induced IL-12p70 in primary human monocytes. IL-12p70 production in peripheral blood myeloid dendritic cells required combined stimulation of TLR7/8 ligands together with TLR4 or with TLR3 ligands. In the presence of T cell-derived IL-4, but not IFN-γ, stimulation with TLR7/8 ligands was sufficient to stimulate IL-12p70 production. In monocytes, type I IFN was required but not sufficient to costimulate IL-12p70 induction by TLR8 ligation. Furthermore, TLR8 ligation inhibited LPS-induced IL-10 in monocytes, and LPS alone gained the ability to stimulate IL-12p70 in monocytes when the IL-10 receptor was blocked. Together, these results demonstrate that monocytes are licensed to synthesize IL-12p70 through type I IFN provided via the Toll/IL-1R domain-containing adaptor inducing IFN-β pathway and the inhibition of IL-10, both provided by combined stimulation with TLR4 and TLR8 ligands, triggering a potent Th1 response before T cell help is established. *The Journal of Immunology,* **2006, 176: 7438–7446.**

Monocytes circulate in the blood stream (mainly $CD14^+CD16^-$ monocytes) and migrate to inflammatory sites (mainly $CD14^+CD16^+$ monocytes) (1). They contribute to immune responses in two ways: 1) in the presence of maturation stimuli, monocytes differentiate into dendritic cells (DCs)[4] and thereby specialize in Ag presentation; and 2) because of their high frequency (4–6% of blood leukocytes), monocytes also represent a major source of cytokines that modulate innate and adaptive immune responses. An excessive stimulation of these cells is associated with a high local or systemic toxicity as has been described in rheumatoid arthritis and sepsis (2, 3), highlighting the importance of studying the exact mechanisms leading to monocyte activation and subsequent cytokine secretion.

IL-12 is secreted by innate immune cells and has been shown to directly and indirectly regulate T and B cell responses. Its main function is the induction of IFN-γ in both T cells and NK cells, thereby supporting a Th1 response (4–6). IL-12 synergizes with polyclonal T cell activators (5, 7, 8) and indirectly regulates B cell Ig isotype switching through its direct effects on T cell cytokine expression (9).

IL-12 is mainly secreted by cells of the monocyte/macrophage lineage; minor sources of IL-12 are B cells and plasmacytoid DCs (PDCs) receiving T cell help (10–12). IL-12 consists of a p35 subunit and a p40 subunit that form the biologically active heterodimer IL-12p70. Although IL-12p35 mRNA is ubiquitously expressed, inflammatory cells display only low levels of p35 mRNA (13). Secretion of p35 protein is even more strictly controlled and occurs only after heterodimerization with IL-12p40. Because the p40 subunit is abundantly expressed, it has been proposed that the heterodimerization itself may be regulated by posttranscriptional mechanisms (13–16). This implies that IL-12p70 release is limited to well-defined biological settings. In view of the key function of biologically active IL-12, it is of special interest to study the biological mechanisms leading to its secretion. To date, primary monocytes have been shown to produce IL-12p70 after stimulation with inactivated whole Gram-positive bacteria and in response to LPS with Th cell costimulation through IFN-γ or CD40L (17–20).

TLRs are a subgroup of pattern recognition receptors (PRRs) characterized by two main structural elements: 1) the leucine-rich repeats in the N-terminal domain that are involved in the recognition and binding of the ligand; and 2) the C-terminal Toll/IL-1R domain that is crucial for downstream signal transduction (21). As PRRs, TLRs recognize specific molecular motifs present in microbial cell walls or nucleic acids. LPS (endotoxin) that is expressed on the cell walls of Gram-negative bacteria is the ligand for TLR4. TLR2 ligands comprise a variety of lipoproteins expressed in bacterial cell walls. RNA molecules represent the natural ligands for TLR7 and TLR8 (22–25). Two main TLR signaling pathways are known to date. The first, common to all TLRs, is signaling through the scaffold adaptor molecule MyD88, and the second, only described for TLR3 and TLR4, is through activation of Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (21, 26–28). Both pathways lead to NF-κB and MAPK activation. Signaling

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Received for publication October 5, 2005. Accepted for publication April 6, 2006.

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[1] This study was supported by Deutsche Forschungsgemeinschaft Grants DI 898/1-1 (to I.B.-D.) and Sonderforschungsbereich 571, DFG HA 2780/4-1, and the Dr. Mildred Scheel Stiftung Grant 10-2074 (to G.H.). This work is part of the thesis of S.I.R. at the University of Munich, Munich, Germany.

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[4] Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; BHK, baby hamster kidney; MALP-2, macrophage-activating lipopeptide-2; MDC, myeloid DC; MoDC, monocyte-derived DC; PRR, pattern recognition receptor; rh, recombinant human; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β.

0022-1767/06/$02.00

through TRIF by TLR3 and TLR4 differs in the time frame of NF-κB activation, IFN regulatory factor 3 phosphorylation, and subsequent IFN-β synthesis. Information about the synergistic or additive effects of parallel stimulation of different TLRs is limited. To date, the synergies of TLR2 with dectin-1, TLR4 with Nod1/2, DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) with TLR4, adenosine A_{2A} receptor with TLR2, TLR4, TLR7, and TLR8, and TLR4 with TLR7/8 have been described in different cell types (29–34). These studies indicate that synergies of different PRRs may represent subtle means for regulating cell type-specific responses in innate immune defense.

In the present study we hypothesized that such synergies may license primary human monocytes to produce IL-12p70. We found that among a number of different TLR agonists only the combination of a TLR8 ligand with a TLR4 ligand can license primary monocytes to produce IL-12p70. The TLR4-TLR8 synergism involves the induction of type I IFN and a dose-dependent inhibition of TLR4-induced IL-10 secretion.

Materials and Methods

Isolation and culture of monocytes and myeloid DCs (MDCs)

Human PBMCs were prepared by Ficoll-Hypaque density gradient centrifugation (Biochrome) of heparinized blood from healthy volunteers (aged 23–47). Monocytes and MDCs were isolated by MACS. Untouched monocytes were prepared in a step-by-step protocol by depleting PDCs with anti-BDCA-4 microbeads, T cells with anti-CD3 microbeads, B cells with anti-CD19 microbeads, MDCs with anti-BDCA-1 microbeads, and NK cells with anti-CD16 and anti-CD56 microbeads. Monocytes were identified as $CD14^+$, $HLA\text{-}DR^+$, $CD11c^+$, $CD4^{low}$, $CD16^-$, $CD123^-$, $CD3^-$, $CD8^-$, $CD56^-$, and $CD34^-$ cells. The purity of isolated monocytes ($CD11c^+HLA\text{-}DR^+CD14^+$) was 96% ± SEM (2%). If only MDCs, but not monocytes, were isolated from PBMCs, MDCs were positively selected with anti-BDCA-1 microbeads after depletion of PDCs with anti-BDCA-4, monocytes with anti-CD14, and B cells with anti-CD19 microbeads. All microbeads were purchased from Miltenyi Biotec. MDC purity was 97 ± 2%. Cells were cultured in RPMI 1640 (Biochrom) supplemented with 2% autologous serum, 10 mM HEPES (from Sigma-Aldrich), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories) in 96-well round-bottom plates at a concentration of 2.5×10^5 monocytes/0.2 ml/well and 5×10^4 MDCs/0.2 ml/well, respectively. Supplemented medium (except autologous serum which was added separately) was tested as being free of endotoxin (lack of TNF-α induction in monocytes) (35). PBMCs were cultured at 0.4×10^6 cells/0.2 ml/well under the same conditions.

Generation of monocyte-derived DCs (MoDCs)

Monocyte-derived DCs were generated from monocytes isolated from PBMCs by adherence to plastic. PBMCs were prepared as described above and resuspended in a density of 5×10^6 cells/ml in DC medium. DC medium was RPMI 1640 (Biochrom) supplemented with 2% (v/v) pooled human AB serum (BioWhittaker), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Twenty milliliters of the cell suspension were filled into one 750-ml cell culture flask and incubated at 37°C and 5% CO_2. After 1 h, nonadherent cells were removed, and remaining cells were washed twice with 0.9% NaCl solution. Adherent cells were then resuspended in fresh DC medium, harvested with a cell scraper after 24 h, washed, and purity analyzed by flow cytometry (95–98% $CD14^+$). Monocytes were cultured at 5×10^5 cells/ml in DC medium supplemented with 1000 U/ml recombinant human (rh) GM-CSF (Immunex) and 500 U/ml rhIL-4 (Promega), 500 U/ml rhIFN-α, or rhIFN-β (PeproTech), respectively. On day 5, an aliquot of the immature MoDCs was analyzed by flow cytometry. MoDCs generated with GM-CSF/IL-4 were $CD14^-$, $CD80^{low}$, $CD83^-$, and $CD86^-$. Cells cultured with GM-CSF/IFN-α were $CD14^{low}$, $CD80^{low}$, $CD83^-$, and $CD86^-$. Day 5 MoDCs were resuspended at 5×10^5 cells/ml in flat-bottom, 96-well plates.

Stimulation of cells

All TLR ligands were titrated to define optimal stimulating conditions in the different cell types analyzed in the study. Cells were stimulated with LPS from *Escherichia coli* (0.1 and 1 μg/ml; Sigma-Aldrich), R848 (0.25, 0.5, 2.5, and 5 μg/ml; InvivoGen), Pam_3CSK_4 (50, 200, and 1000 ng/ml; InvivoGen), macrophage-activating lipopeptide-2 (MALP-2; 2.5, 5, and 1000 ng/ml; Alexis Biochemicals), poly(I:C) (100 μg/ml; Sigma-Aldrich), and loxoribine (500 μM; Sigma-Aldrich). All TLR ligands were resuspended in endotoxin-free water (aqua ad injectabilia) except LPS, which was resuspended in medium. Poly(I:C) was subjected to ≥10 precipitations and washes in ethanol to remove endotoxin. The stepwise purification process was documented by a gradual loss of activity to induce TNF-α in PBMCs. The final preparation of poly(I:C) used throughout the study did not induce intracellular TNF-α in monocytes (highly sensitive endotoxin assay) (35) or detectable levels of TNF-α in PBMCs but still activated TLR3-expressing MDCs.

For CD40 ligation, baby hamster kidney (BHK)-CD40L and BHK-pTCF (neomycin-resistant, mock-transfected control cell line) were provided by H. Engelmann (University of Munich, Munich, Germany) and UV irradiated at 0.75 J/cm^2. IFN-α (100 and 1000 U/ml as indicated), IFN-β (100 U/ml) (both from Strathmann Biotech), and IFN-γ (1000 U/ml; Roche) were added simultaneously or 1 h before monocyte or MDC stimulation. For the blockade of IL-10, monocytes were preincubated with Fc block (Miltenyi Biotech) for 10 min, washed, resuspended and then preincubated with anti-IL-10 and anti-IL-10R neutralizing mAbs (mouse anti-human IL-10, catalog no. MAB217, and IL-10R-α, catalog no. MAB274; R&D Systems) at a final concentration of 10 μg/ml for 1 h before stimulation. For IFN-αR blocking experiments, monocytes were incubated with Fc block and then preincubated with either neutralizing anti-IFN-αR mAb (mouse anti-human IFN αβ receptor chain 2, catalog no. 21385-1; PBL Biomedical Laboratories) or non-neutralizing anti-IFN-αR Ab (rabbit anti-human IFN αβ receptor chain 2, catalog no. 31385-1; PBL Biomedical Laboratories) at 20 μg/ml for 1 h before stimulation. MoDCs were stimulated with 2.5 μg/ml R848 or 500 μM loxoribine with or without simultaneous addition of LPS (0.1 μg/ml).

Detection of cytokines

Monocyte supernatants were harvested 24 h after stimulation unless indicated otherwise. Supernatants from MDCs were harvested after 24 h, and supernatants from MoDCs were analyzed after 48 h. IL-12p70, IL-12p40, IL-10, IFN-γ, and TNF-α concentrations in the cell supernatants were measured by ELISA. All ELISA Ab pairs were purchased from BD Biosciences, and the protocols were performed according to the manufacturer's recommendations. The IFN-α ELISA was purchased from Bender MedSystems.

Flow cytometry

Flow cytometric data were obtained on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). Staining was performed following standard procedures. The mAbs used anti-CD14 (FITC), anti-HLA-DR (PerCP), anti-CD11c (allophycocyanin), anti-CD4 (PE), anti-CD8 (allophycocyanin), anti-CD80 (PE), anti-CD86 (allophycocyanin), anti-CD83 (FITC), anti-CD123 (PE), anti-CD3 (FITC), anti-CD3 (allophycocyanin), anti-CD56 (PE), anti-CD16(FITC), anti-CD34 (allophycocyanin), anti-CD19 (PE), and TNF-α (PE) (all from BD Pharmingen), as well as anti-BDCA-2 (FITC) from Miltenyi Biotec.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

For RNA preparation cells were washed with 0.9% NaCl, and cell pellets were lysed in 300 μl of lysis buffer from the MagnaPure LC mRNA isolation kit I supplemented with 1% DTT (Roche) and frozen at −80°C until further handling. Preparation of mRNA was performed with the MagnaPure-LC device using the mRNA-I standard protocol. An aliquot of 8.2 μl of RNA was reverse-transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT) as primer (First Strand cDNA synthesis kit; Roche) according to the manufacturer's protocol in a thermocycler. The reaction mix was diluted to a final volume of 0.5 ml and stored at −20° C until PCR analysis.

Parameter-specific primer sets optimized for the LightCycler (Roche) were developed and purchased from SEARCH-LC. The PCR was performed with the LightCycler FastStart DNA SYBR Green I kit (Roche) according to the protocol provided in the parameter-specific kits. The transcript numbers were normalized according to the expression of cyclophilin B and β-actin per microliter of cDNA.

Statistical analysis

Data are depicted as mean ± SEM. Statistical significance of differences was determined by paired two-tailed Student's *t* test using Microsoft Excel software. Statistically significant differences are indicated with * for $p < 0.05$ and ** for $p < 0.005$.

Results

Primary human CD14+CD16⁻ monocytes express functional TLR4 and TLR8 but not TLR7

To study IL-12p70 production in human primary monocytes we isolated untouched resting ($CD14^+CD16^-$) monocytes as described in *Materials and Methods* and schematically explained in Fig. 1. The purity achieved by this negative selection method was 96 ± 2% (Fig. 1), with no $CD16^+$ monocytes detectable in the cell preparation. Of note, in stimulated monocyte preparations IFN-γ was below the detection limit, confirming the absence of T cells and NK cells. All experiments were conducted with autologous serum to avoid endotoxin exposure of monocytes.

To address expression and function of TLRs and Nods, we first analyzed their mRNA expression as shown in Fig. 2*A*. Monocytes expressed low levels of TLR8, intermediate levels of Nod-2, high levels of TLR2 and TLR4, and no Nod-1 or TLR3 mRNA. Type I IFN has previously been shown to enhance mRNA de novo synthesis of TLR7 in human B cells (36). In contrast with B cells, in monocytes we found that neither recombinant IFN-β (Fig. 2*A*) nor IFN-α (data not shown) failed to up-regulate TLR7 or any other PRRs studied, including TLR4. In contrast, *MyD88*, a well-known IFN-inducible gene, was up-regulated and thus served as a positive control for IFN-I stimulation.

Next, human monocytes were stimulated with the TLR7/8 agonist R848, the TLR7 agonist loxoribine, and the TLR4 agonist LPS. After 24 h the cell surface expression levels of HLA-DR and CD80 were quantified (Fig. 2*B*). Despite the low expression of TLR8 mRNA, R848 potently activated human monocytes comparable to the way LPS did. In contrast, loxoribine failed to induce the expression of HLA-DR and CD80. In B cells, TLR7 sensitivity is known to be strongly enhanced by the addition of IFN-α. We stimulated monocytes in the presence and absence of IFN-α. In contrast to B cells, type I IFN did not enhance the sensitivity of human monocytes toward R848 and loxoribine (Fig. 2*B*). For loxoribine, expression of HLA-DR and CD80 slightly below the medium control may be due to a TLR-independent toxic effect of loxoribine. Similar to HLA-DR and CD80, TNF-α and IL-12p40 in the monocyte supernatants were not altered by costimulation with IFN-I (data not shown). Together, these data indicated that TLR8 and TLR4, but not TLR7, are functional in primary human monocytes.

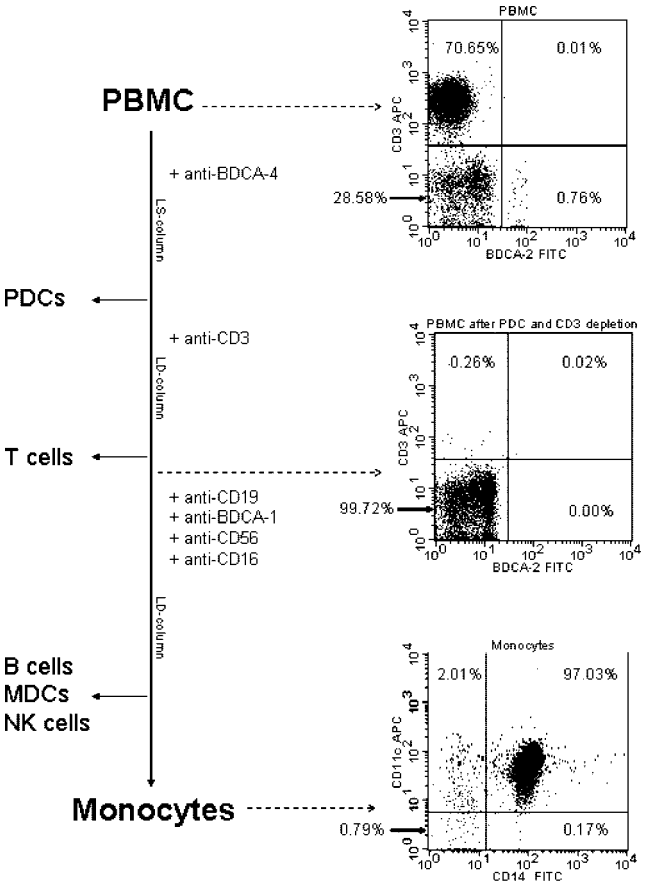

FIGURE 1. Isolation of monocytes from human PBMC. Untouched monocytes were isolated by the initial depletion of PDCs and T cells with Ab-coated anti-BDCA-4 and anti-CD3 microbeads and the subsequent depletion of B cells, NK cells, and MDCs with anti-CD19, anti-CD56, anti-CD16, and anti-BDCA-1 microbeads. The monocytes obtained by this method were $CD14^+CD16^-$ and had a purity of 96 ± 2%.

TLR8 and TLR4 synergize to induce IL-12p70 production in human monocytes

Recent data suggest that TLR7/8 and TLR4 synergize in the activation of murine and human dendritic cells (33, 34). Because monocytes express functional TLR8 but not TLR7, monocytes were used as an experimental system to study a possible synergy between TLR8 and TLR4. R848 in monocytes represents a selective TLR8 stimulus because functional TLR7 is absent.

We found that only the combination of TLR4 and TLR8 agonists induced significant IL-12p70 production (Fig. 3), whereas TNF-α and IL-12p40 were also produced in response to single TLR ligation. Consistent with the absence of functional TLR7 on primary monocytes, the selective TLR7 ligand loxoribine failed to induce TNF-α and IL-12p40 as well as IL-12p70 production when combined with LPS (Fig. 4). Together, these data indicated that IL-12p70 production in primary human monocytes is under the combined control of TLR8 and TLR4.

Failure of TLR2 ligands to synergize with R848 or LPS for IL-12p70 production in human monocytes

Because TLR4 activation by LPS is confined to Gram-negative pathogens, we wanted to know whether the pathogen-associated molecular patterns typically associated with Gram-positive pathogens would also induce IL-12p70 production as seen with LPS. We stimulated human monocytes with the TLR2-activating bacterial lipopeptides (MALP-2 and Pam_3CSK_4) alone or in combination with R848 or LPS.

Monocytes produced TNF-α and IL-12p40 in response to MALP-2, Pam_3CSK_4, R848, and LPS (Fig. 4). The TLR3 ligand poly(I:C) failed to induce TNF-α and IL-12p40 as expected because of a lack of TLR3 mRNA expression (data not shown). No synergistic TNF-α or IL-12p40 induction was observed for TLR2 ligands in combination with either LPS or R848 (Fig. 4). None of the combinations of TLR2 ligands with R848 or LPS induced IL-12p70 secretion. Furthermore, the Nod2 ligand muramyl dipeptide did not synergize with R848 (data not shown). As expected, IL-12p70 secretion was also seen for the combination of R848 with the Th cell stimulus CD40L (Fig. 4), but not when R848 was combined with IFN-γ (data not shown).

TLR-induced IL-12p70 production in BDCA-1⁺ MDCs

Next, we studied TLR expression and IL-12p70 induction in peripheral blood MDCs. MDCs were found to express TLR2, TLR3, TLR4, and TLR8 (Fig. 5*A*). Furthermore, TLR7 was up-regulated when MDCs were incubated for 3 h with IFN-α (Fig. 5*A*). The TLR7/8 ligand R848 induced IL-12p70 production only in combination with the TLR4 ligand LPS or the TLR3 ligand poly(I:C),

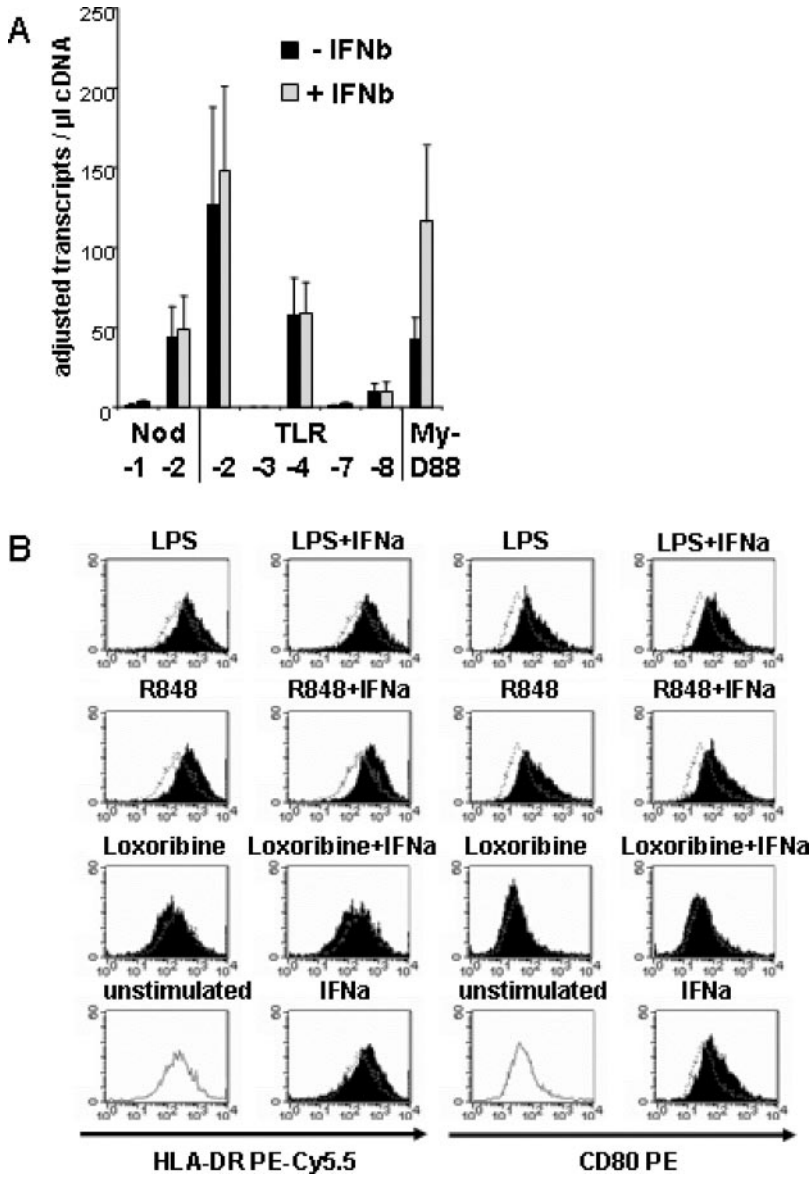

FIGURE 2. Influence of type I IFN on TLR mRNA expression and the influence of TLR-induced cell surface expression of activation markers on human monocytes. *A*, PRR mRNA expression. Purified monocytes (0.5×10^6 cells) were cultured in medium with 2% autologous serum in the presence and absence of IFN-β (IFNb) (100 U/ml) for 3 h before mRNA extraction. mRNA expression of PRRs was determined by quantitative real-time RT-PCR. IFN-β-inducible MyD88 mRNA expression served as a positive control for IFN-β stimulation. Results are presented as mean transcript numbers of adjusted transcripts per microliter of cDNA ± SEM of five individual donors. *B*, Cell surface expression of activation markers after TLR4, TLR7, and TLR8 stimulation in the presence and absence of IFN-α. Monocytes (1.25×10^6/ml) were incubated with LPS (1 μg/ml), R848 (2.5 μg/ml), or loxoribine (0.5 mM) in the presence and absence of IFN-α (100 U/ml). HLA-DR and CD80 surface expression were measured after 24 h by FACS analysis. Unfilled histograms represent the unstimulated control. One representative experiment of at least three is shown.

but not with the TLR2 ligands PAM_3CSK_4 and MALP-2 (Fig. 5*B*). For the TLR7 ligand loxoribine, IL-12p70 production was only found when TLR7 expression was up-regulated by IFN-α and when the TLR7 ligand loxoribine was combined with LPS (Fig. 5*B*). Of note, the TLR3 ligand poly(I:C) alone was sufficient to induce IL-12p70 in MDCs (Fig. 5*B*), whereas poly(I:C) was not functional in isolated monocytes lacking TLR3 (TNF-α production in monocytes (pg/ml) with poly(I:C), 0; LPS at 1 μg/ml: 3961; mean of four independent experiments). Together, these data indicate that, in contrast to monocytes, MDCs express functional TLR3 and, in the presence of IFN-α, start to express functional TLR7; furthermore, the data show that in both monocytes and MDCs the TLR7/8 ligand R848 alone was not sufficient to induce IL-12p70. The requirement for combined stimulation with R848 and LPS for IL-12p70 induction was confirmed in mixed cell populations (PBMC) containing both monocytes and MDCs (IL-12p70 production in PBMC (pg/ml) with R848, 0; LPS at 100 ng/ml, 0; LPS at 1 μg/ml, 0; R848 plus LPS at 100 ng/ml, 54; R848 plus LPS at 1 μg/ml, 76). Similar to isolated monocytes and MDCs, for TNF-α no synergy was found in PBMC (TNF-α in PBMC (pg/ml) with R848, 3537; LPS at 100 ng/ml, 987; LPS at 1 μg/ml, 1328; R848 plus LPS at 100 ng/ml, 2827; R848 plus LPS at 1 μg/ml, 2579; mean of four independent experiments). It is important to note that in PBMC IL-12p70 was induced by R848 alone when cells were incubated in medium containing FCS instead of autologous serum (autologous serum used throughout this study) (I. Bekeredjian-Ding, unpublished observation). FCS, but not autologous serum, leads to nonspecific costimulation of immune cells.

IL-12p70 production in MDCs generated in GM-CSF and IL-4 or GM-CSF and IFN-I

The comparison of MoDCs generated with GM-CSF plus IL-4 or GM-CSF plus IFN-α or IFN-β revealed a striking difference (Fig. 6). Although MoDCs generated in the presence of GM-CSF and IL-4 (Th cell-derived cytokine) readily secreted IL-12p70 in response to R848 or LPS alone, MoDCs generated in the presence of GM-CSF and type I IFN (data for GM-CSF plus IFN-β not shown), similarly to monocytes and peripheral blood MDCs, required combined stimulation with R848 and LPS. Interestingly, the selective TLR7 agonist loxoribine induced IL-12p70 release when combined with LPS in both MoDC cell types, indicating the presence of functional TLR7 in MoDCs (Fig. 6).

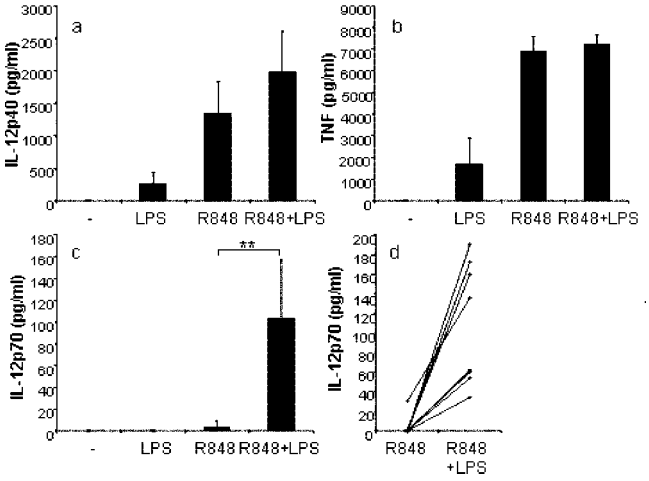

FIGURE 3. R848- and LPS-induced cytokine expression on monocytes. Purified monocytes (1.25×10^5/ml) were stimulated with LPS (1 μg/ml), R848 (2.5 μg/ml), or LPS (1 μg/ml) plus R848 (2.5 μg/ml) for 24 h. IL-12p40 (*a*), TNF-α (*b*), and IL-12p70 (*c*) concentrations in the supernatants were measured by ELISA. Data from nine independent experiments are shown as means ± SEM. Analysis of IL-12p70 secretion revealed a significant donor variability, but IL-12p70 production after R848 stimulation alone was detectable in only one donor as shown in *d*. **, $p = 0.001$.

Type I IFN is required for IL-12p70 production in monocytes

Recently, it has been postulated that autocrine IFN-β secretion is crucial for IL-12p70 production from murine bone marrow-derived dendritic cells (33). To assess the role of type I IFN in monocytes, monocytes were stimulated with R848 or LPS in the presence or absence of exogenous type I IFN. We found that priming or simultaneous incubation with recombinant IFN-I did not enhance TNF-α, IL-12p40, or IL-12p70 production in response to R848 or LPS (data not shown).

Subsequently, we examined monocyte-derived IL-12p70 secretion in monocytes stimulated with LPS plus R848 in the presence of IFN-αR-blockade. As shown in Fig. 7, treatment of monocytes with neutralizing anti-IFN-αR Ab abolished the IL-12p70 release. These data indicated that, in monocytes, autocrine type I IFN production is required for IL-12p70 secretion upon synergistic activation with LPS and R848 but that it is not sufficient to costimulate IL-12p70 production in response to single TLR-mediated stimulation as shown above.

R848 inhibits LPS-induced IL-10 production in monocytes

To further define the mechanisms responsible for the synergy of R848 and LPS leading to IL-12p70 production, we analyzed the mRNA expression in human monocytes stimulated with R848 or LPS. We found that both R848 and LPS up-regulated TLR4, MyD88, TRIF, and IP-10 expression (mRNA expression, adjusted transcripts per microliter of cDNA ± SEM: unstimulated control TLR4, 60 ± 5; MyD88, 46 ± 10; TRIF, 33 ± 2; IP-10, 13 ± 5; LPS TLR4, 139 ± 25; MyD88, 78 ± 20; TRIF, 85 ± 16; IP-10, 310 ± 139; R848 TLR4, 110 ± 10; MyD88, 85 ± 10; TRIF, 146 ± 21; IP-10, 561 ± 193). Although up-regulation of TLR4, MyD88, and TRIF mRNA may contribute to synergistic IL-12p70 induction, the functional contribution of increased mRNA levels to the observed effects is uncertain. Another candidate mechanism is the regulation of IL-10, which is well known to suppress IL-12 production, especially IL-12p70. We found that, despite higher TNF-α and IL-12p40 induction, R848 induced much less IL-10 than LPS (Fig. 8). Furthermore, we observed a dose-dependent inhibition of LPS-induced IL-10 production by R848 (Fig. 8, *A* and *B*). This effect was dependent on simultaneous stimulation with LPS and R848, because inhibition of LPS-induced IL-10 production was much lower when R848 was added 1 or 4 h after LPS (data not shown).

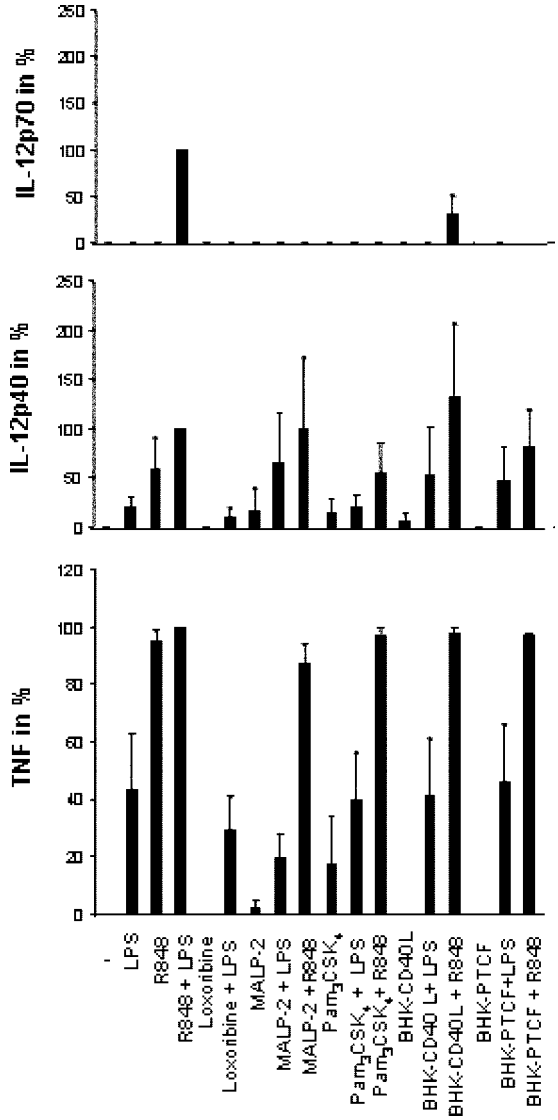

FIGURE 4. Cytokine production of PRR-stimulated monocytes. Purified monocytes (1.25×10^6/ml) were stimulated for 24 h with the PRR ligands indicated below. Cytokine secretion was determined in the supernatants by ELISA. The means ± SEM of four independent experiments are summarized. Cells were incubated with LPS (1 μg/ml), R848 (2.5 μg/ml), LPS plus R848, loxoribine (0.5 mM), loxoribine plus LPS, or with MALP-2 (1 μg/ml), Pam_3CSK_4 (1 μg/ml), or UV-irradiated BHK-CD40L or BHK-pTCF cells alone or in combination with LPS or R848. Values of individual donors are normalized because of high donor variability with R848 plus LPS representing 100% of cytokine secretion. One hundred percent corresponds to 56 ± 54 pg/ml for IL-12p70 (*top panel*), 1869 ± 804 pg/ml for IL-12p40 (*middle panel*), and 9686 ± 916 pg/ml for TNF-α (*bottom panel*).

Blocking of IL-10 enables single TLR-mediated induction of IL-12p70 in monocytes

To confirm a key role for IL-10, we performed IL-10 blocking experiments. Monocytes were treated with anti-IL-10 and anti-IL-10R Abs 1 h before TLR stimulation. We found that when functional IL-10 was blocked, both R848 and LPS alone were able to induce IL-12p70; furthermore, synergistic IL-12p70 induction by

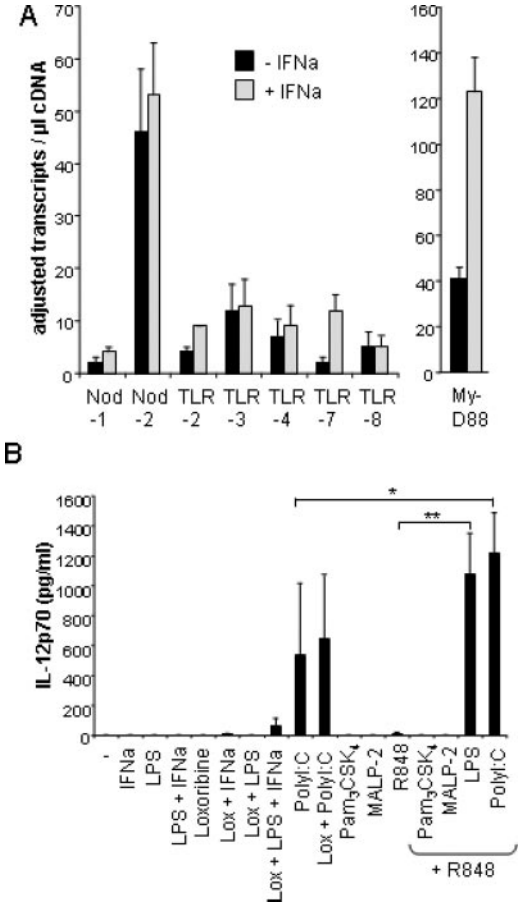

FIGURE 5. PRR expression and IL-12p70 production in myeloid dendritic cells. MDCs were positively isolated using anti-BDCA-1 microbeads as described above. *A*, Analysis of PRR mRNA expression. Purified MDCs (2.5×10^5 cells) were incubated with (▨) or without (■) IFN-α (IFNa; 100 U/ml) for 3 h before isolation of mRNA. PRR mRNA expression was measured by quantitative real-time RT-PCR. Results of four independent experiments are shown as mean transcript numbers of adjusted transcripts per microliter of cDNA ± SEM. *B*, IL-12p70 production after stimulation with TLR ligands. Purified MDCs (2.5×10^5/ml) were stimulated with LPS (1 μg/ml), loxoribine (Lox; 0.5 mM), IFN-α (IFNa; 1000 U/ml), R848 (2.5 μg/ml), Pam_3CSK_4 (1 μg/ml), MALP-2 (1 μg/ml), and poly(I:C) (100 μg/ml) or combinations of these substances as indicated. After 24 h the concentration of IL-12p70 in the supernatants was determined by ELISA. Results are presented as means ± SEM from five individual donors. *, $p = 0.01$; **, $p = 0.0005$.

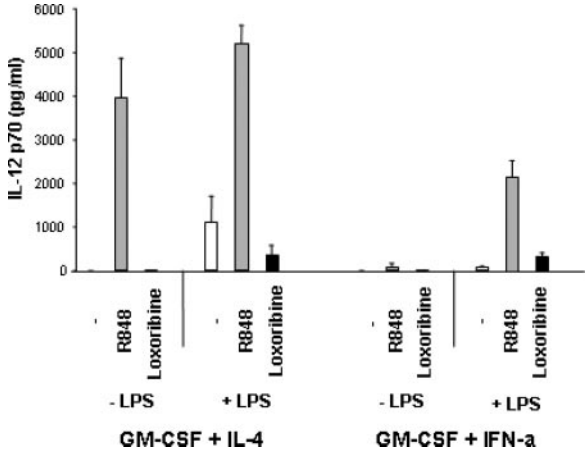

FIGURE 6. IL-12p70 production in MoDCs. MoDCs generated with GM-CSF plus IL-4 (*left panel*) and GM-CSF plus IFN-α (*right panel*) were compared after stimulation with R848 (2.5 μg/ml) or loxoribine (0.5 mM) in the presence (+) or absence (−) of LPS (0.1 μg/ml). Means ± SEM of four independent experiments are shown.

R848 and LPS was further enhanced (Fig. 8*C*). Based on these results, we conclude that IL-12p70 secretion from LPS- and R848-stimulated human monocytes is prevented by autocrine release of IL-10 and that R848 supports LPS-induced IL-12p70 production by reducing LPS-induced IL-10 secretion.

Discussion

In the literature a number of stimuli have been described that enhance both IL-12p35 and IL-12p40 secretion (14, 15, 37–39), and it is well established that T cell-derived cytokines license myeloid cells for the secretion of the bioactive heterodimer of IL-12p35 and IL-12p40, IL-12p70. Human monocytes have been shown to produce IL-12p70 upon microbial stimulation in the presence of T cell help mediated through either cell-cell interaction via CD40-CD40L or by costimulation with T cell-derived IFN-γ. To our knowledge, it has not been demonstrated that IL-12p70 production in human monocytes can occur in the absence of T cell help or factors derived from activated NK cells. In this study we demonstrate for the first time that primary human monocytes, upon appropriate microbial stimulation, can become a considerable source of IL-12p70 production in the absence of T cell help. We demonstrate that, for monocytes, the only appropriate combination of microbial stimuli that licenses monocytes to produce IL-12p70 is simultaneous stimulation with TLR4 and TLR8 ligands.

There have been reports showing that whole bacteria have the potential to induce IL-12p70 production in monocytes (18, 19). The exact mechanism leading to IL-12p70 release after stimulation with these microbes is, to date, unclear. The data presented in this study suggest that the secretion of IL-12p70 from human monocytes in the absence of T cell help is controlled by ligation of TLR4 in combination with TLR8. This double requirement may

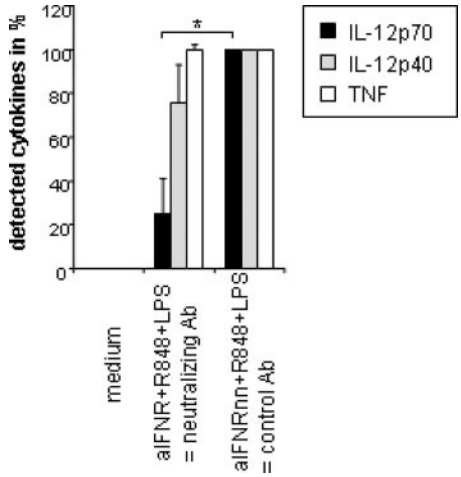

FIGURE 7. Effect of IFNαR blockade on monocyte-derived IL-12p70 secretion. Monocytes (1.25×10^6/ml) were preincubated with a neutralizing IFN-αR Ab (aIFNR) or a non-neutralizing IFN-αR Ab (aIFNRnn) in the concentration of 20 μg/ml for 1 h before addition of R848 in the final concentration of 2.5 μg/ml and LPS at 1 μg/ml. Results from three experiments are summarized and shown as means ± SEM. Data are normalized, with 100% representing the cytokine concentration after stimulation with R848 plus LPS in the presence of the non-neutralizing IFN-αR Ab. One hundred percent corresponds to 80 ± 67 pg/ml IL-12p70, 2549 ± 337 pg/ml IL-12p40, and 7147 ± 797 pg/ml for TNF-α. *, $p = 0.025$ for IL-12p70.

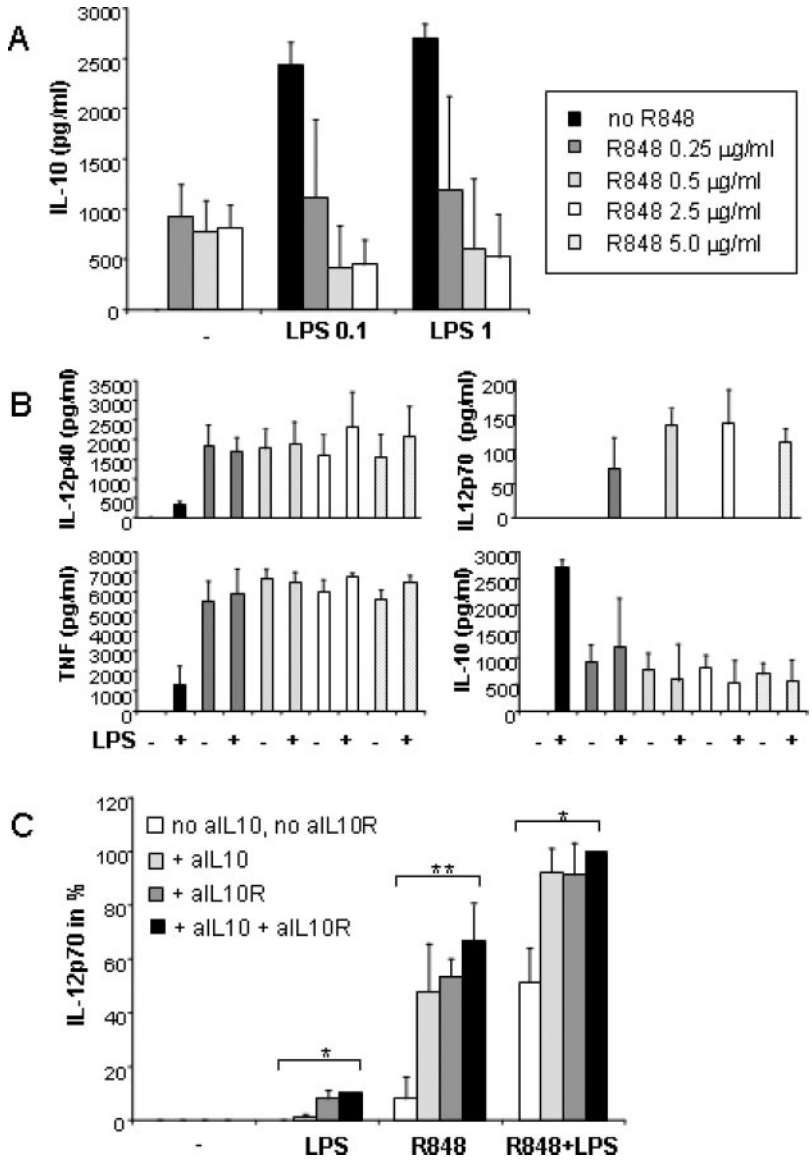

FIGURE 8. Role of IL-10 for IL-12p70 production in human monocytes. Monocytes (1.25×10^6/ml) were incubated with LPS, R848, LPS plus R848, or medium alone in the concentrations indicated in the diagrams. Cytokine concentrations in the supernatants after 24 h of stimulation were measured by ELISA. *A*, IL-10 secretion. Data from three independent experiments are summarized as means ± SEM. *B*, Comparison of monocyte IL-12p40 (*upper left*), TNF-α (*lower left*), IL-12p70 (*upper right*), and IL-10 (*lower right*) secretion. The means ± SEM of three independent experiments are shown. *C*, Effect of IL-10- or IL-10R-blockade on IL-12p70 production. Fc receptors of freshly isolated monocytes were blocked, and monocytes (1.25×10^6/ml) were subsequently preincubated with Abs against human IL-10 (aIL10; 10 μg/ml) or IL-10R (aIL10R; 10 μg/ml) for 1 h before stimulation with R848 (0.5 μg/ml) and LPS (1 μg/ml). The means ± SEM of four independent experiments are shown. The data are normalized to R848 plus LPS plus anti-IL-10 plus anti-IL-10R-stimulated IL-12p70 concentrations representing 100% (1019 pg/ml ± 387). *, $p = 0.012$ for LPS ± (anti-IL-10 plus anti-IL-10R) and 0.008 for LPS plus R848 ± (anti-IL-10 plus anti-IL-10R), respectively. **, $p = 0.0005$ for R848 ± (anti-IL-10 plus anti-IL-10R).

represent a safeguard mechanism preventing inappropriate secretion of this potentially harmful key Th1 cytokine in the early phase of an infection when T cell help is not yet established.

At first sight, the amount of IL-12p70 released by primary human monocytes seems relatively low compared with MDC that produce ~40-fold higher amounts of IL-12p70 on a per-cell basis. However, peripheral blood contains ~20 times more monocytes than MDC, and, therefore, the total amount of IL-12p70 produced by monocytes is significant.

Our results obtained in monocytes and primary blood MDCs differed from those obtained in MoDCs generated in the presence of GM-CSF and IL-4 in which single TLR stimulation was sufficient for IL-12p70 induction. Because IL-4 belongs to the T cell-derived cytokines costimulating IL-12p70 secretion (17, 40), the presence of IL-4 in MoDC cultures may account for their low stimulatory threshold for IL-12p70 production. Consequently, MoDCs generated in the presence of GM-CSF and IL-4 may not represent an adequate experimental system for studying the induction of IL-12p70 in the physiological myeloid cell compartment.

The dual requirement for a TLR4 ligand and a TLR8 ligand for IL-12p70 production in monocytes in our study highlights the requirement to perform studies on IL-12p70 under endotoxin-free conditions. Other reports that describe IL-12p70 production in response to single TLR stimulation may need to be revisited under this aspect. In this context it is important to note that the primary human monocyte is one of the most LPS-sensitive cell types known. Therefore, we used autologous serum from the same donor and not FCS in our experimental setting throughout the study. Even the FCS that tested as endotoxin-negative in the standard *Limulus* amebocyte lysate assay in our hands still induces TNF-α in PBMC (I. Bekeredjian-Ding, unpublished observation). As a consequence, PBMC were found to produce IL-12p70 upon stimulation with R848 alone when FCS was used instead of autologous serum (I. Bekeredjian-Ding, unpublished observation).

Interestingly, combined stimulation via TLR8 and TLR4 may also play a role for the activation of myeloid cells by certain viruses. For example, the respiratory syncytial virus has been shown to activate immune cells through a TLR4-dependent mechanism and was shown to induce IL-12p70 production in human myeloid cells (41). Other viruses may induce IL-12p70 via dual engagement of TLR3 and TLR7 or TLR8 (42, 43). According to our results, this will not occur in monocytes lacking TLR3 but is expected in MDCs expressing TLR3.

In contrast with the human primary monocytes that exclusively express TLR8, MDCs express both TLR7 and TLR8. To date, it remains unclear whether TLR7 or TLR8 is involved in synergistic activation of MDCs via LPS and R848 (33, 34, 44). In this work we demonstrate for the first time that the single ligation of TLR8 independent of TLR7 is sufficient for cooperation with TLR4. An additional advantage of analyzing TLR requirements in monocytes is the virtual absence of IL-12p70 upon stimulation with single TLR ligands, whereas a higher background of IL-12 production upon single TLR ligand stimulation obscures this effect in MoDCs.

In contrast with mice, CpG DNA does not support priming of Th cell responses in primates (45). One could speculate that this defect in priming Th cell responses is due to the failure of CpG DNA to directly induce IL-12p70 in human myeloid cells. In the human immune system, TLR9, the receptor for CpG DNA, is expressed in B cells and PDCs, but not in cells of the myeloid lineage (10, 46). B cells and PDCs only produce IL-12 upon simultaneous stimulation with CpG DNA and Th cell-derived CD40L (10, 12). Our results now demonstrate that murine myeloid cells are not the only cells capable of T cell-independent IL-12p70 production upon appropriate microbial stimulation, but human myeloid cells can also do this if they receive combined stimulation via TLR4 and TLR3 together with TLR7 or TLR8. Further studies need to examine whether there are conditions under which stimulation with TLR7/8 alone is sufficient to elicit IL-12p70 production in human myeloid cells. Our preliminary results show that, in the human immune system, recognition of certain formulations of microbial RNA, the natural ligand for TLR8, may serve this purpose.

Because the TLR4 ligand LPS is only expressed by Gram-negative bacteria, the question arises of how whole Gram-positive bacteria are capable of triggering IL-12p70 production. We addressed this issue by testing synthetic TLR2 ligands that are mainly expressed in cell walls of Gram-positive bacteria. To our surprise, the combination of TLR2 ligands with either R848 or LPS failed to induce IL-12p70 in human monocytes. To explain IL-12p70 induction by Gram-positive bacteria, one could speculate that whole bacteria, by providing microbial molecules at higher density, represent a stronger stimulus for TLR2, or that additional receptors are involved that may not yet have been identified.

There are a number of mechanisms, including TGF-β and IL-10, that inhibit IL-12 production (47–49). Furthermore, there is evidence that prostaglandin E_2 and other $G_{\alpha s}$-linked receptors interfere with IL-12 secretion (50, 51). Our results confirm a key role for IL-10 in inhibiting TLR-induced IL-12p70 production. Neutralization of IL-10 enhanced IL-12p70 production in all conditions tested. In this context, it is noteworthy that IL-10 inhibits genes activated through the TLR-induced TRIF-dependent pathway (52–54). In our hands, R848 supports LPS-induced IL-12p70 by decreasing LPS-stimulated IL-10 production.

Most viruses and many bacterial strains have been shown to induce type I IFN production. A recent study performed in IFN-αR knockout mice provided evidence for the requirement of IFN-β priming and STAT1 activation for subsequent IL-12p70 secretion in murine bone marrow-derived DCs (33). Furthermore, a report from Hermann et al. (18) and two other studies (53, 54) claimed that IL-10 inhibits LPS-induced IFN-β production. Based on these studies, we speculated that the costimulation of R848 with recombinant type I IFN could substitute for LPS-induced autocrine release of IFN-β in our experimental setting. However, we found that although the addition of type I IFN was insufficient to support TLR4- or TLR8-induced IL-12p70 production in our hands, blocking of the IFN-αR abolished IL-12p70 secretion in monocytes upon combined stimulation with TLR4 and TLR8. These findings indicate that type I IFN is required but not sufficient for IL-12p70 release by TLR4 and TLR8.

Autoimmune diseases are known to be triggered by bacterial and viral infections. We postulate that the induction of IL-12p70 through synergistic activation via TLR4 and TLR8 as seen in the present study could contribute to autoimmunity. The pathogenic mechanisms leading to autoimmune disease may involve elevated serum concentrations of type I IFNs, insufficient clearance of apoptotic particles, and recognition of endogenous RNA. During an infection of a predisposed individual, the presence of LPS in the serum may initiate the shift to a Th1 response by synergizing with endogenous RNA and subsequently triggering the release of the potentially harmful Th1 cytokine IL-12p70 from monocytes and MDCs.

In conclusion, combined activation of myeloid cells via TLR4 and TLR8 offers the following advantages over single TLR stimulation: 1) the key Th1 cytokine IL-12p70 is induced; 2) the ratio of IL-12p70 to proinflammatory cytokines is increased in favor of IL-12p70 and a Th1 response; and 3) a reduction in the dose needed to support strong Th1 responses will lead to reduced systemic toxicities as compared with single TLR ligands. Combined TLR4 and TLR8-mediated stimulation may lead to improved strategies for immunotherapy of cancer, viral infection, and allergies.

Disclosures

The authors have no financial conflict of interest.

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