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Activation of Toll-Like Receptor 2 on Human Dendritic Cells Triggers Induction of IL-12, But Not IL-10

Sybille Thoma-Uszynski,∗ Sylvia M. Kiertscher,† Maria Teresa Ochoa,∗ Deborah A. Bouis,¶ Michael V. Norgard,†† Kensuke Miyake,† Paul J. Godowski,** Michael D. Roth,† and Robert L. Modlin2§*

Mammalian Toll-like receptors (TLRs) are required for cell activation by bacterial lipoproteins (bLP) and LPS. Stimulation of monocytes with bLP and LPS results in a TLR-dependent induction of immunomodulatory genes leading to the production of pro-inflammatory cytokines. In this paper, we compared the expression and response of TLRs on monocytes and dendritic cells (DC). TLR2, but not TLR4, was detected on peripheral blood monocytes and DC, in lymphoid tissue CD1a+ DC as well as on in vitro monocyte-derived DC. Upon stimulation with bLP or LPS, monocytes produced IL-12 and IL-10 at similar levels, whereas monocyte-derived DC produced comparable levels of IL-12, but little IL-10. Greater than 90% of the bLP-induced production of IL-12 was blocked by anti-TLR2 mAb. Thus, DC express TLR2 and activation of this receptor by bLP provides an innate mechanism by which microbial pathogens preferentially activate cell-mediated immunity. The Journal of Immunology, 2000, 165: 3804–3810.

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3 Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; bLP, bacterial lipoproteins; M. tb., Mycobacterium tuberculosis; TRITC, tetramethylrhodamine isothiocyanate.

Cell activation and signaling via mammalian Toll-like receptors (TLRs) represents an innate immune response that has been conserved from Drosophila to humans (1). A variety of microbial ligands have been shown to activate immune responses via mammalian TLRs. In particular, bacterial lipoproteins (bLP) and LPS have been demonstrated to activate cells in a TLR-dependent manner leading to the activation of NF-κB and induction of NF-κB-controlled genes including pro-inflammatory cytokines, costimulatory molecules, and apoptotic signals (2–8). The differentiation of monocytes into dendritic cells (DC) has a critical impact on the immune response. During differentiation, DC up-regulate the expression of MHC class I, MHC class II, and costimulatory molecules, and thus increase their efficiency as APC. After exposure to microbial agents, DC respond by producing immunostimulatory cytokines, including IL-12, and further up-regulating the expression of MHC and costimulatory molecules. This microbial-induced DC maturation provides crucial elements required for selective activation of T cells along a Th1 pathway (9–12). Previously, we demonstrated that activation of primary human monocytes with bLP or LPS was blocked by an anti-TLR2 mAb (8). In this study, we characterized the phenotype and function of TLRs on human monocytes and DC.

Materials and Methods

Reagents

The following microbial ligands were used: LPS (Salmonella typhosa) was purchased from Sigma (St. Louis, MO), the synthetic bacterial lipopeptide PamCysSerLys (PamCysSerLys), was obtained from Boehringer Mannheim (Indianapolis, IN), the 19kDa lipoprotein of Mycobacterium tuberculosis (M. tb.) was obtained from John Belisle (Colorado State University, Fort Collins, CO) as previously detailed (8). Bacterial lipopeptides Treponema pallidum (Tp47), Borrelia burgdorferi (OspA), and M. tb. (19kDa) were synthesized as described (13). TLR expression was evaluated using a mAb to TLR2 (clone 2392, IgG1; Ref. 5) and TLR4 (clone HTA125, IgG2a; Ref. 14). The specificity of the anti-TLR2 Ab has been previously described (Ref. 7; http://www.science.mcg.org/content/data/1040361.shtml).

Isolation of human monocytes and culture of macrophages and DC

PBMC were isolated from fresh blood by Ficoll centrifugation. A total of 1.5 × 10^6 cells/ml in RPMI 1640 supplemented with 0.1 mM sodium pyruvate, 2 mM penicillin, 50 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 1% pooled human serum (Omega Scientific, Tarzana, CA) were incubated in tissue culture flasks for 1 h at 37°C for adherence. For derivation of DC, adherent cells were cultured in complete RPMI 1640/10% human serum in the presence of 800 U/ml GM-CSF and 500 U/ml IL-4 for up to 7 days as previously described (15).

Flow cytometry

After various time points of culture, DC were harvested and stained for cell surface expression of TLR2, TLR4, and CD14. Cells were labeled with the primary mAb followed by a goat anti-mouse FITC-conjugated secondary Ab (Caltag, Burlingame, CA). Nonspecific Fc-receptor binding was blocked using normal human serum. In some experiments, cells were costained with mAb against CD14 (clone TU4, IgG2a), HLA-DR (clone TU36, IgG2b), MHC I (HLA-A, -B, -C; clone w6/32, IgG2a), CD40 (clone 5C3, IgG1), CD80 (clone L370.4, IgG1), CD86 (clone IT2.2, IgG2b), CD83 (clone HB15e, IgG1), or CD11c (clone 3.9, IgG1). Expression of TLR2 and TLR4 on DC present in fresh PBMC was evaluated by a similar approach. PBMC stained with primary mAb as detailed above, were incubated with allophycocyanin-conjugated goat anti-
mouse secondary mAb (Caltag) and then counterstained with an Ab mixture containing FITC-conjugated mAb against CD3, CD14, CD16, CD19, CD20, and CD56 (anti-lineage mixture 1), an anti-HLA-DR-PE-conjugate, and an anti-CD11c-PerCP-conjugate (all from Becton Dickinson, Franklin Lakes, NJ). DC were identified as cells that expressed high levels of HLA-DR and CD11c in the absence of lineage-specific markers.

**Immunofluorescence labeling and confocal microscopy**

Reactive tonsil samples from healthy individuals were obtained from the Human Tissue Research Center at University of California, Los Angeles. Samples were embedded in OCT medium (Ames, Elkhart, IN), snap frozen in liquid nitrogen, cut to cryostat sections of 3–4 μm thickness, and fixed in acetone.

Double immunofluorescence was performed by serially incubating sections with mouse anti-human mAb to CD1a (clone NA1/34, IgG2a), followed by incubation with isotype-specific FITC-conjugated goat anti-mouse IgG2a (Caltag), then anti-TLR2 (clone 2392, IgG1) followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL). Controls included staining with isotype-matched irrelevant Abs. Slides were examined with a Leica-TCS-SP inverted confocal laser scanning microscope (Deerfield, IL) equipped with krypton and argon lasers. Sections were excited with 488 nm blue light for FITC and 568 nm yellow light for TRITC. Fluorescent images were recorded sequentially through separate optical detectors with a 500- to 550-nm band pass setting for FITC and 590-nm long-pass setting for TRITC, respectively. Pairs of images were superimposed for colocalization analysis.

**Cytokine ELISA**

Cells (1 × 10^5) were plated in duplicate wells in 96-well flat-bottom plates in the absence or presence of anti-TLR2 blocking mAb or isotype control before the addition of LPS or bLP. After 21 h of coincubation, supernatants were harvested and assayed for cytokine content using commercially available ELISA for IL-12p40 (clones AO8E6E5 and A25C4B6) and IL-10 (clones 945A5D11 and 945A5A10; Biosource, Camarillo, CA). For measurement of IL-12p70, TNF-α, and IL-6, cytoscreen kits were used (Biosource).

**Results**

**TLR2 is expressed on monocytes and monocyte-derived DC**

We investigated the expression of TLR2 in cells of the monocyte lineage. DC were derived from monocyte precursors isolated by plastic adherence and treated with 800 U/ml GM-CSF and 500 U/ml IL-4. FACS analysis showed expression of TLR2 on both monocytes and DC at different stages of differentiation (Fig. 1A). The level of TLR2 expression on DC decreased, but did not disappear in culture. After 7 days, cultures contained characteristic immature monocyte-derived DC that were CD3^−, CD83^−, CD14^−, but expressed high levels of MHC I and MHC II in combination with CD86, CD40, and CD11c (Fig. 1B). TLR2 was also present on fully mature DC, obtained after treatment of d7 monocyte-derived DC with TNF-α for 48 h (data not shown). In contrast to TLR2, TLR4 protein was variably detected on monocytes (usually absent or present at very low levels) and not present on monocyte-derived DC (data not shown) or peripheral blood DC (Fig. 2A).

**TLR2 is expressed in vivo on circulating peripheral blood and lymphoid tissue DC**

Different subsets of human DC have been defined depending on the precursor used to generate them, or their localization in the

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**FIGURE 1.** A, Expression of TLR2 and CD14 during differentiation of monocyte-derived DC. Monocytes were isolated from fresh total PBMC by adherence to plastic and assessed for cell surface expression of TLR2 and CD14 (d0). Adherent monocytes were cultured for different periods of time (d3, d5, d7) in the presence of 800 U/ml GM-CSF and 500 U/ml IL-4 and analyzed for expression of TLR2 and CD14. Results represent one of four experiments. B, Phenotype of monocyte-derived DC on day 7. Cultures contained characteristic immature monocyte-derived DC that were CD3^−, CD83^−, CD14^−, but expressed high levels of MHC I and MHC II in combination with CD86, CD40, and CD11c.
body. To extend our results obtained with DC generated in vitro from monocytes, we applied four-color flow cytometry to characterize TLR2 expression on circulating human peripheral blood DC. Freshly isolated PBMC were stained with a mixture of lineage-specific mAb to exclude T cells, B cells, NK cells, and macrophages from further analysis and the lineage-negative population was evaluated for expression of HLA-DR and CD11c. Expression of TLR2 on the lineage-HLA-DR+/CD11c+ population was then evaluated using a specific mAb and a secondary allophycocyanine-conjugated goat-anti-mouse mAb. Results are representative for a total of two experiments. B, CD1a+DC in lymphoid tissue express TLR2. Confocal laser microscopy images after double immunofluorescence labeling of CD1a (green) and TLR2 (red). The merged image shows colocalization of CD1a+/TLR2+ double-positive cells (yellow).

FIGURE 2. A, Peripheral blood DC express TLR2, but not TLR4. PBMC were stained with an anti-lineage-mAb mixture (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56) and the negative population was evaluated for expression of HLA-DR and CD11c. Expression of TLR2 on the lineage-HLA-DR+/CD11c+ population was then evaluated using a specific mAb and a secondary allophycocyanine-conjugated goat-anti-mouse mAb. Results are representative for a total of two experiments. B, CD1a+DC in lymphoid tissue express TLR2. Confocal laser microscopy images after double immunofluorescence labeling of CD1a (green) and TLR2 (red). The merged image shows colocalization of CD1a+/TLR2+ double-positive cells (yellow).

Monocyte-derived DC respond to bLP by production of IL-12 and via TLR2 activation

To investigate the biological relevance of the finding that DC express TLR2 on their cell surface we evaluated the capacity of monocyte-derived DC to produce IL-12 after stimulation with bLP or LPS. A titration of the 19-kDa lipoprotein was performed using human monocytes (Fig. 3A). After 21 h of incubation, the IL-12p40 release into the supernatant was determined. Subsequent experiments were performed using 1 µg/ml lipoprotein or lipopeptide, because this represented a suboptimal dose for inhibition experiments. The synthetic lipopeptides Tp47, OspA, and 19 kDa were also found to activate monocyte-derived DC to release IL-12p40 (Fig. 3B). Deacylated forms of Tp47, OspA, and 19 kDa had little inducing activity, indicating that the lipid moiety of the microbial ligand is essential to mediate cell activation.

Monocyte-derived DC were coincubated with the 19-kDa lipoprotein of M. tb. or the synthetic lipopeptide PAM₃CysSerLys₄ or LPS. Stimulation of monocyte-derived DC by 19-kDa lipoprotein, PAM₃CysSerLys₄, or LPS resulted in the production of IL-12p40 (Fig. 3, C–E). We next investigated whether the ability of microbial ligands to induce IL-12 production in human DC was dependent on TLR2. The anti-TLR2 mAb used for flow cytometry has also been shown to block TLR2 activation and signaling (8). Before stimulation with 19-kDa bLP, the synthetic lipopeptide PAM₃CysSerLys₄ or LPS, monocyte-derived DC were incubated in the presence of the TLR2 blocking mAb or an isotype control (IgG1). Supernatants were collected after 21 h of stimulation, and the amount of IL-12 released was determined by ELISA. The anti-TLR2 mAb blocked IL-12 production induced by the 19-kDa bLP by greater than 90% and by the synthetic lipopeptide by 70% (Fig. 3, C–E). In contrast, the isotype control had no effect. The response to LPS was only partially inhibited in the presence of the anti-TLR2 blocking mAb.

The bioactive form of IL-12 is a p70 heterodimer composed of p40 and p35 subunits. Because IL-12p70 is secreted at lower levels than IL-12p40, we performed experiments in the presence of IFN-γ, which is known to enhance IL-12 production (16). For measurement of IL-12p70, monocyte-derived DC were stimulated with bLP or lipopeptides in the presence of 100 U/ml IFN-γ. We found that bLP or synthetic lipopeptides induced the secretion of IL-12p70 in these DC (Fig. 4). In these experiments, the levels of IL-12p70 were ~100-fold lower than IL-12p40, a ratio consistent with other published studies (17, 18).

Monocyte-derived DC respond to synthetic bLP by production of TNF-α and IL-6

To extend the functional study on monocyte-derived DC, we analyzed the supernatant after stimulation with synthetic lipopeptides for two additional proinflammatory cytokines. DC activation with synthetic lipopeptides resulted in release of TNF-α and IL-6 (Fig. 5).

The anti-inflammatory cytokine IL-10 is differentially induced in monocytes, but not monocyte-derived DC after stimulation with lipoproteins

Because IL-12 and IL-10 have distinct effects in Th1-type immune responses, we compared the levels of IL-12 and IL-10 in monocytes and monocyte-derived DC after stimulation with LPS and bLP. After stimulation with the microbial ligands for 21 h, the cytokine content was determined by ELISA. Monocytes produced both IL-12 and IL-10 after TLR activation. However, in contrast, monocyte-derived DC produced comparable amounts of IL-12, but little IL-10 (Fig. 6, A–C). The ability of lipoproteins to stimulate...
IL-10 was also dependent on TLR2, because anti-TLR2 strongly inhibited the ability of 19 kDa to release IL-10 from monocyte-derived DC (Fig. 6D). Hence, our data indicate that DC preferentially produced IL-12 vs IL-10 after activation of TLRs.

Discussion
The innate immune system has dual roles in host defense, providing a direct and immediate response against microbial invaders and an instructive role, influencing the nature of adaptive immunity. This instructive role of the innate immune system is served in part by DC which, upon encounter with microbial inflammatory stimuli, acquire the ability to migrate to regional lymph nodes and mature into potent professional APCs (9, 10). Equally important, activated DC released cytokines that can direct the adaptive T cell response toward either a Th1 or Th2 pattern (19). Therefore, we studied the ability of DC to respond to microbial ligands, the role of TLRs in this response and the profile of cytokines released by this interaction. Our results demonstrate expression of TLR2 on both circulating blood DC and on DC generated in vitro from monocytes, the TLR2-dependent activation of monocyte-derived DC by bLP and LPS and the preferential induction of IL-12 over IL-10 following this activation. Our data define TLR2-dependent DC activation as a mechanism that bridges innate and adaptive immune responses to microbial pathogens.

For over 100 million years, Drosophila has maintained a host defense system in which microbial ligands activate Toll family members to induce immunologic responses that eliminate the foreign invader (20). The host defense system of Drosophila has been conserved throughout evolution to be retained by the mammalian immune system (1). Of the mammalian TLR family members, TLR2 and TLR4 have been studied in the greatest detail. Both human TLR2 and TLR4 are expressed on lymphoid tissue, with TLR2 expressed on CD14+ monocytes (2, 3, 5). Using anti-TLR2 mAb and immunofluorescence, we confirmed the expression of TLR2 on peripheral blood monocytes and demonstrated the expression of TLR2 on monocyte-derived DC. TLR2 was also detected on peripheral blood HLA-DR+CD11c+ DC and lymphoid tissue CD1a+ DC. Although TLR4 was variably detected on peripheral blood monocytes, it was not detected on DC, despite a recent study showing TLR4 mRNA in human DC (21). It is possible that the anti-TLR4 mAb is of lower affinity than the anti-TLR2 mAb, or that the number of molecules of TLR4 expressed on cells is generally lower than TLR2. In this paper, we conclude that TLR2 on human DC enables their response to microbial ligands.

Several groups, including ours, have shown that bLP and preparations of Gram-positive bacteria mediate cellular signaling via TLR2 (7, 8, 22, 23). In particular, lipoproteins from M tuberculosis, Borrelia burgdorferi, and Treponema pallidum activated monocyte cytokine release via TLR2 (6, 8). Using TLR2-deficient mice, two studies demonstrated that cellular activation by cell wall preparations of Gram-positive bacteria or lipopeptides required TLR2 (24,
We now provide evidence that the ability of bLP to activate human DC was dependent on TLR2, because the production of IL-12 could be blocked by 90% by an anti-TLR2 mAb. These data suggest the expression of TLR2 by DC confers their ability to respond to bLP and by the production of IL-12 enhances cell-mediated immunity to microbial infection.

Studies on LPS signaling point to a major role for TLR4 in mediating activation in mice (26–28). However, in humans, the concept of ligand specificity for distinct TLR family members is less clear, particularly for LPS. Two of the initial studies reported TLR2 as a signaling receptor for LPS using transfected cell lines (3, 4), and further evidence points to the possibility that TLR2 can mediate the response to LPS in humans (5, 8). Using primary human cells, LPS activation of human monocytes could be blocked with anti-TLR2 mAb (5, 8). In the present study, human DC were found to express TLR2, but not TLR4, and that the response of monocyte-derived DC to LPS could be partially blocked by an anti-TLR2 mAb. It is interesting to note that in Drosophila, there is evidence that distinct Toll proteins mediate responses to different microbial ligands. Toll itself is primarily involved in host defense against fungi, whereas the homologous protein 18-wheeler participates in antibacterial responses. There is also overlap such that Toll has some role in antibacterial responses and 18-wheeler in antifungal immunity (29). By analogy, it is possible that there is overlap in the specificity of mammalian TLR2 and TLR4 for LPS. A second possibility is based on the finding that TLR proteins form homodimers (5) and reportedly heterodimers. In this case, it may be possible that a TLR2-containing complex may be blocked by the anti-TLR2 mAb, even though TLR4 is the critical mediator of the response to LPS. A third possibility is that another TLR family member shares the ability to signal after activation with LPS. Until now, the mammalian TLR family contains many members (30, 31), only two of which, TLR2 and TLR4, have been studied in detail. Finally, many LPS preparations contain a mixture of ligands that stimulate both TLR2 and TLR4; however, further purification of LPS or the use of synthetic lipid A reveals that TLR4 is the primary receptor involved in cell activation (32).

For many pathogens, the outcome of the immune response to the infection depends on the pattern of cytokines produced by T cells, a central part of the adaptive immune response. The T cell pattern is directed, in part, by the balance of cytokine produced by cells of the innate immune system. Cells of the monocyte/macrophage lineage participate in the process by secreting the cross-regulatory cytokines IL-12 and IL-10. IL-12 is a powerful signal for the generation of Th1 cytokine responses, whereas IL-10 can inhibit both the release of IL-12 and the effect of IL-12 on T cells, thus downregulating Th1 responses. In this paper, we found that bLP induce both IL-12 and IL-10 from human monocytes.

In contrast to the ability of bLP and LPS to induce both IL-12 and IL-10 from monocytes, we provide striking evidence that these microbial ligands stimulate IL-12 vs IL-10 in DC as compared with monocytes. Cross-linking of CD40 on monocytes induces both IL-12 and IL-10 from human monocytes.

In order to investigate the ability of bLP and LPS to induce both IL-12 and IL-10 from monocytes, we provide striking evidence that these microbial ligands stimulate IL-12 but no or little IL-10 from human monocyte-derived DC. DC cells are known to produce IL-12 after stimulation with LPS or ligation with CD40 or interaction with MHC II (12, 18, 33). After stimulation with LPS or microbial extracts of Toxoplasma gondii, DC produce rapidly IL-12 and are attracted to T cell areas in the spleen (10). A recent study describes mycoplasm as another pathogen involved in DC activation, resulting in release of IL-12, TNF-α, and IL-6 (34). Similar to our finding that bLP and LPS differentially induce IL-12 vs IL-10 in DC as compared with monocytes, cross-linking of CD40 on monocytes induces both IL-12 and IL-10, yet on monocyte-derived DC induces IL-12 but not IL-10 (18, 33, 35). Therefore, it would appear that activation of DC by various cell surface receptors, including...
mediated immunity is necessary. notherapy in microbial infections and other diseases in which cell-
The release of IL-12 and to generate a Th1 cytokine response. Such
develop strategies to target these microbial ligands to DC to induce
in a TLR2-dependent manner. Therefore, it should be possible to
and the ability of microbial lipoproteins to induce IL-12 from DC
tern for cells of the monocyte lineage (17).
provides an alternative mechanism for regulating the cytokine pat-
gulate monocyte IL-12 release but down-regulate IL-10 secretion
by ELISA.
CD40 or TLR2 provides a mechanism to selectively generate a
Th1 cytokine response. Similarly, the ability of IFN-γ to up-regu-
late monocyte IL-12 release but down-regulate IL-10 secretion
provides an alternative mechanism for regulating the cytokine pat-
tern for cells of the monocyte lineage (17).
Our study provides evidence for the presence of TLR2 on DC
and the ability of microbial lipoproteins to induce IL-12 from DC
in a TLR2-dependent manner. Therefore, it should be possible to
develop strategies to target these microbial ligands to DC to induce
the release of IL-12 and to generate a Th1 cytokine response. Such
an approach could prove useful as immunoregulation or immu-
nontherapy in microbial infections and other diseases in which cell-
mediated immunity is necessary.

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FIGURE 6. Monocytes and monocyte-
derived DC produce IL-12 upon activation
with bLP whereas IL-10 is differentially
induced only in monocytes. Cytokines in
nonstimulated wells were below the level
of detection (<10 pg/ml). Fresh isolated
monocytes and monocyte-derived DC on
d7 were stimulated with either 19kDa li-
poprotein of M. tb. (A), synthetic lipope-
tide PAM3CysSerLys4 (B), or LPS (C),
and the supernatants were analyzed for IL-
12p40 and IL-10 release after 21 h of cul-
ture. The results shown are from one rep-
resentative experiment from a total of three
experiments. D. The ability of 19 kDa to
release IL-10 from monocyte-derived DC
was dependent on TLR2. Monocyte-de-
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