

Subsets of Human Dendritic Cell Precursors Express Different Toll-like Receptors and Respond to Different Microbial Antigens

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Abstract

Toll-like receptors (TLRs) are ancient microbial pattern recognition receptors highly conserved from *Drosophila* to humans. To investigate if subsets of human dendritic cell precursors (pre-DC), including monocytes (pre-DC1), plasmacytoid DC precursors (pre-DC2), and CD11c⁺ immature DCs (imDCs) are developed to recognize different microbes or microbial antigens, we studied their TLR expression and responses to microbial antigens. We demonstrate that whereas monocytes preferentially express TLR 1, 2, 4, 5, and 8, plasmacytoid pre-DC strongly express TLR 7 and 9. In accordance with these TLR expression profiles, monocytes respond to the known microbial ligands for TLR2 (peptidoglycan [PGN], lipoteichoic acid) and TLR4 (lipopolysaccharide), by producing tumor necrosis factor (TNF)- α and interleukin (IL)-6. In contrast, plasmacytoid pre-DCs only respond to the microbial TLR9-ligand, CpG-ODNs (oligodeoxynucleotides [ODNs] containing unmethylated CpG motifs), by producing IFN- α . CD11c⁺ imDCs preferentially express TLR 1, 2, and 3 and respond to TLR 2-ligand PGN by producing large amounts of TNF- α , and to viral double-stranded RNA-like molecule poly I:C, by producing IFN- α and IL-12. The expression of distinct sets of TLRs and the corresponding difference in reactivity to microbial molecules among subsets of pre-DCs and imDCs support the concept that they have developed through distinct evolutionary pathways to recognize different microbial antigens.

Key words: immunity, natural • bacteria • receptors, immunologic • monocytes • cytokines

Introduction

Dendritic cells (DCs) have an outstanding ability to prime naive T cells and to initiate primary T cell-mediated immune responses (1). DCs also dictate the development of T cell-mediated immune responses into either Th1 or Th2 type (2–4). Recent studies generated considerable debate as to how DCs regulate the types of T cell responses (3–7). An evolutionary theory suggests that two DC lineages or subsets can induce Th1 or Th2 responses, respectively, under certain circumstances (2, 4). In mice, the CD8 α^+ DC, but not CD8 α^- DC subset, has the capacity to produce a large amount of IL-12 and induce Th1 versus Th2 responses (5, 6). In humans, CD40 ligand (CD40L)-activated

monocyte-derived DCs (DC1), but not plasmacytoid pre-DC-derived DCs (DC2), produce a large amount of IL-12 and induce Th1 versus Th2 responses (7). However, several studies have demonstrated that a given DC subset can induce either Th1 or Th2 response depending on the types of stimulation and pathogens (8–10). From these studies, an environmental instruction theory was suggested that questioned the existence of distinct DC lineages specialized to the induction of different types of T cell responses (3).

Evolutionary force drove the development of multiple subsets of B and T lymphocytes, which rapidly and efficiently respond to common microbial antigens. Unlike conventional B and T cells, B-1 B cells, γ/δ T cells, and natural killer T cells express restricted and distinct antigen receptors, capable of recognizing common antigens derived from bacteria or damaged host cells (11–14). Thus, if separate DC lineage/subsets with specialized functions really exist, they may express receptors that recognize different

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microbial antigens. Toll-like receptors (TLRs) are ancient receptors that are highly conserved from *Drosophila* to humans and recognize molecular patterns specific to microbial pathogens (15–19). Signaling through TLRs strongly activates DCs to upregulate costimulatory molecules (CD80 and CD86) and to produce proinflammatory cytokines (TNF- α , IL-6, and IL-12) (17–19). We examined the expression of all known human TLRs (TLR1–TLR10) on ex vivo subsets of human blood pre-DCs and immature DCs (imDCs) and their responses to microbial antigens with different pattern structures.

Materials and Methods

Isolation and Culture of Cells. Blood CD11c⁺ imDCs, plasmacytoid pre-DCs, and CD14²⁺CD16⁻ monocytes were isolated from human peripheral blood as described (7, 20). The purity of each cell population was >99%. Monocytes were cultured for 5 d in RPMI 1640 (BioWhittaker) supplemented with 10% FCS (BioWhittaker), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 55 μ M 2-mercaptoethanol, penicillin G, and streptomycin (all from Life Technologies), in the presence of 50 ng/ml GM-CSF (Schering-Plough) and 200 U/ml IL-4 (Schering-Plough). The resulting monocyte-derived imDCs were washed and cultured for 24 h with human CD40L-transfected L cells (irradiated at 5,500 rad) to obtain mature DC1 (7). Plasmacytoid pre-DCs were cultured for 5 d with 10 ng/ml IL-3 (R&D Systems). The resulting plasmacytoid pre-DC-derived imDCs were washed and cultured for 24 h with CD40L-transfected L cells to obtain plasmacytoid pre-DC-derived DCs. To induce

the maturation of imDCs, they were cultured for 24 h with CD40L-transfected L cells. To induce cytokine production, monocytes, CD11c⁺ imDCs, or plasmacytoid pre-DCs were cultured for 24 h at $2 \times 10^4/200 \mu$ l in round-bottom 96-well culture plates in the presence of 10 μ g/ml peptidoglycan (PGN) from *Staphylococcus aureus* (Fluka), 10 μ g/ml of lipoteichoic acid (LTA) from *S. aureus* (Sigma-Aldrich), 10 μ g/ml LPS from *S. minnesota* serotype Re595 (Sigma-Aldrich), 50 μ g/ml poly (I:C) (Sigma-Aldrich), 5 μ M (46 μ g/ml) phosphodiester CpG-oligodeoxynucleotide (ODN) AAC-30 (21, 22). AAC-30 was added at 0, 4, and 16 h to compensate for their degradation by DNase activity in medium.

Reverse Transcription PCR for TLRs. RNA was isolated with the acid guanidinium thiocyanate–phenol–chloroform method (23). Contaminating DNA was removed by digestion with 5 U deoxyribonuclease I (Boehringer) for 30 min at 37°C. Reverse transcription (RT) was performed with random hexamers (Promega) for priming and SuperScriptTMII (Life Technologies). The PCR reaction volume was 50 μ l, containing 0.5 μ M of each primer, 40 nM of each deoxynucleoside triphosphate, and 1.25 U AmpliTaq (PerkinElmer). Primers used are as shown in Table I. A GeneAmp PCR System 9700 (PerkinElmer/Applied Biosystems) was used with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final elongation step of 72°C for 7 min. PCR products were separated on a 3% agarose gel containing ethidium bromide. A 1-kb DNA ladder standard (Life Technologies) was used as a size marker.

Real-Time Quantitative RT-PCR. RNA was isolated with the acid guanidinium thiocyanate–phenol–chloroform method (23). The RT was performed with SuperScriptTMII (Life Technolo-

Table I. Sequences of PCR Primers

RT-PCR primers		
TLR	Forward primers	Reverse primers
1	CGTAAACTGGAAGCTTTGCAAGA	CCTTGGGCCATTCCAAATAAGTCC
2	GGCCAGCAAATTACCTGTGTG	CCAGGTAGGTCTTGGTGTTC
3	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC
4	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGT
5	CATTGTATGCACTGTCACTC	CCACCACCATGATGAGAGCA
6	TAGGTCTCATGACGAAGGAT	GGCCACTGCAAATAACTCCG
7	AGTGTCTAAAGAACCTGG	CTTGGCCTTACAGAAATG
8	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG
9	TTATGGACTTCTGCTGGAGGTGC	CTGCGTTTTGTGGAAGACCA
10	CAATCTAGAGAAGGAAGATGGTTC	GCCCTTATAAACTTGTGAAGGTGT
β -actin	ATCTGGCACCACACCTTCTACAATGAGCTGCG	CGTCATACTCCTGCTTGTGATCCACATCTGC
Real-time PCR primers		
TLR	Forward primers	Reverse primers
2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT
4	CTGCAATGGATCAAGGACCA	TTATCTGAAGGTGTTGCACATTC
7	TTACCTGGATGGAAACCAGCTACT	TCAAGGCTGAGAAGCTGTAAGCTA
9	TGAAGACTTCAGGCCCAACTG	TGCACGGTCACCAGGTTGT

gies). cDNA was analyzed for the expression of TLR genes by the Fluorogenic 5'-nuclease PCR assay (7) using a PerkinElmer ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. Primers for TLRs are shown in Table I. Values are expressed as arbitrary units (relative to ubiquitin $\times 1,000$).

Quantitation of Cytokines by ELISA. ELISA kits from the following companies were used to analyze cytokine production: TNF- α , IL-6 (R&D Systems), IL-12, and IFN- α (Biosource International).

Results

Three populations of cells, monocytes, CD11c⁺ imDCs, and plasmacytoid pre-DCs, were isolated by cell sorting, to a purity >99%. As shown in Fig. 1, monocytes expressed high levels of TLR 1, 2, 4, 5, and 8, low level of TLR6, and undetectable levels of TLR 3, 7, 9, or 10 by RT-PCR. CD11c⁺ imDCs expressed high levels of TLR 1, 2, and 3, low levels of TLR 5, 6, 8, and 10, and undetectable levels of TLR 4, 7, and 9. In marked contrast to monocytes and CD11c⁺ imDCs, plasmacytoid pre-DCs express high levels of TLR 7 and 9 (Fig. 1). They also expressed low levels of TLR 1, 6, and 10, but not TLR 2, 3, 4, 5, or 8. Thus, monocytes and CD11c⁺ imDCs, and plasmacytoid pre-DCs express distinct sets of TLRs.

The expression of TLR 2, 4, 7, and 9 was further analyzed by real-time quantitative RT-PCR, because of their

selective expression on monocytes or plasmacytoid pre-DCs. As shown in Fig. 2, Monocytes express the highest levels of TLR2 and TLR4 of the cell populations examined. The expression of these two receptors is dramatically downregulated upon differentiation of monocytes into imDCs after 5 d of culture with GM-CSF plus IL-4 and is almost lost after further 24 h activation with CD40L. CD11c⁺ imDCs express moderate levels of TLR2 and TLR4, similar to those expressed by imDCs generated in culture (Fig. 2). The expression of TLR2 and TLR4 on CD11c⁺ imDCs is downregulated by CD40L activation. Plasmacytoid pre-DCs do not express TLR2 and TLR4 at any stages of maturation, but express the highest levels of TLR7 and TLR9 (Fig. 2). The expression of TLR7 and TLR9 is progressively down-regulated after differentiation into imDCs with IL-3 and then into mature DCs after CD40L activation. Monocytes and CD11c⁺ imDCs do not express significant levels of TLR7 and TLR9 at any stages of maturation.

Microbial molecules that trigger TLR2, TLR4, and TLR9 signaling have been identified. PGN and LTA from Gram-positive bacteria signal through TLR2 (24–27). LPS from Gram-negative bacteria signals through TLR2 (26). Bacterial DNA containing unmethylated CpG motifs signals through TLR9 (28–30). It is unclear up to now which receptor mediates signaling triggered by poly I:C, a viral double-stranded RNA-like molecule. Based on the TLR-expression data above, we determined whether monocytes, CD11c⁺ imDCs, and plasmacytoid pre-DCs respond to the different microbial TLR-ligands and poly I:C, by producing the proinflammatory cytokines TNF- α , IL-6, IL-12, and IFN- α .

As shown in Fig. 3, the TLR2-ligand, PGN, stimulated monocytes to produce large amounts of TNF- α and IL-6. PGNs stimulate CD11c⁺ imDCs to produce large amounts of TNF- α , and small amounts of IL-6 and IL-12. PGN did not stimulate plasmacytoid pre-DCs to produce any of the cytokines tested. Plasmacytoid pre-DCs died in cultures either with medium or with PGN (data not shown). Similar to PGN, the other TLR2-ligand, LTA, stimulated monocytes to produce large amounts of TNF- α and IL-6. However, unlike PGN, LTA did not stimulate CD11c⁺ imDCs to produce detectable levels of cytokines tested. Plasmacytoid pre-DCs did not respond to LTA.

The TLR4-ligand, LPS, stimulated monocytes to produce large amounts of TNF- α and IL-6. LPS stimulated CD11c⁺ imDCs to produce moderate amounts of IL-6. LPS also stimulated CD11c⁺ imDCs to produce small amounts of IL-12p75 in two out of four donors. LPS did not stimulate plasmacytoid pre-DCs to produce any cytokines tested and cell die in cultures with LPS or medium.

The TLR9-ligand, CpG-ODN AAC-30, selectively stimulated plasmacytoid pre-DCs, but not monocytes and CD11c⁺ imDCs, to produce IFN- α . Interestingly, poly I:C selectively stimulated CD11c⁺ imDCs, but not monocytes and plasmacytoid pre-DCs to produce IFN- α and IL-12p75.

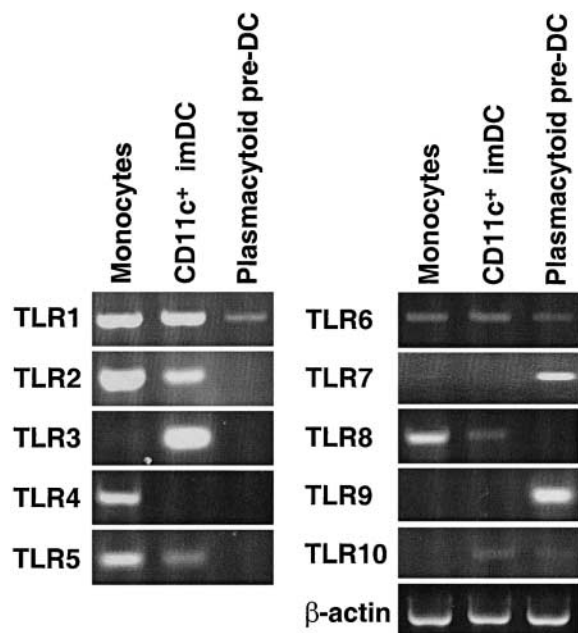


Figure 1. Detection of TLR mRNA by RT-PCR in freshly isolated monocytes, CD11c⁺ imDCs, and plasmacytoid pre-DCs. The three cell populations were isolated by cell sorting to the purity of >99%. cDNAs were amplified for 35 cycles and were separated on a 3% agarose gel containing ethidium bromide. The data shown are representative of three experiments.

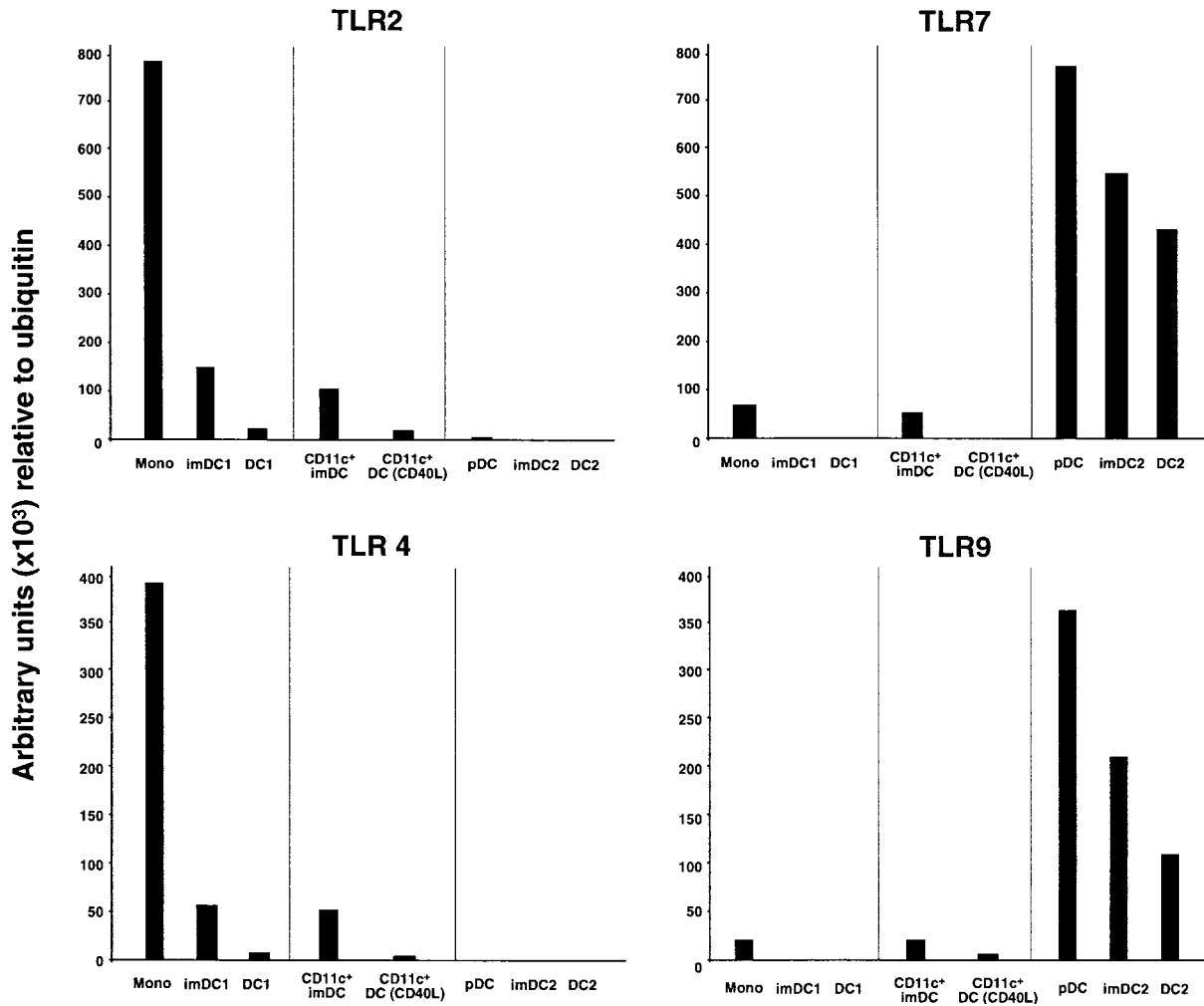


Figure 2. Quantitation of TLR mRNA by real-time quantitative RT-PCR in freshly isolated monocytes (Mono), CD11c⁺ imDCs, and plasmacytoid pre-DCs (pDC), and in immature and mature DCs induced from them. Monocytes were cultured with 50 ng/ml GM-CSF and 200 U/ml IL-4 for 5 d to obtain imDC1 and were further stimulated with CD40L-transfected L cells for 24 h to obtain mDC1. CD11c⁺ imDCs were stimulated with CD40L-transfected L cells for 24 h to obtain mature CD11c⁺ DCs. Plasmacytoid pre-DCs were cultured with 10 ng/ml IL-3 for 5 d to obtain imDC2 and were further stimulated with CD40L-transfected L cells for 24 h to obtain mature DC2. The amounts of mRNA were quantitated by real-time quantitative RT-PCR, and were shown by arbitrary unit relative to the amount of ubiquitin mRNA. The data shown are representative of three experiments.

Discussion

In this study, we demonstrated that monocytes, CD11c⁺ imDCs, and plasmacytoid pre-DCs express distinct sets of TLRs. Monocytes preferentially express TLR 1, 2, 4, 5, and 8, CD11c⁺ imDCs selectively express TLR3, and plasmacytoid pre-DCs strongly express TLR 7 and 9. In accordance with the TLR expression patterns, monocytes respond to microbial molecules known to trigger signaling via TLR2 (PGN, LTA) and TLR4 (LPS), but not via TLR9 (CpG-ODN). By contrast, plasmacytoid pre-DCs, but not monocytes and CD11c⁺ imDCs respond to the TLR9-ligand, CpG-ODN AAC-30. Interestingly, CD11c⁺ imDCs, but not monocytes and plasmacytoid pre-DCs responded to poly I:C. These remarkable differences among monocytes, CD11c⁺ imDCs, and plasmacytoid pre-DCs, in their TLR repertoire expression and responsiveness to microbial antigens, suggests that cells have evolved to recog-

nize different pathogens. This study also sheds some light on how different TLRs instruct DC precursors and imDCs to produce different sets of proinflammatory cytokines and dictate the types of adaptive immune responses. While the expression of TLR2 and TLR4 endorse monocytes with the ability to produce large amounts of proinflammatory cytokines TNF- α and IL-6, the expression of TLR9 endorse plasmacytoid pre-DCs with the ability to produce IFN- α during antibacterial immune responses.

Recent studies on DCs have generated considerable debates as to whether there is a functionally distinct lymphoid DC lineage or subset (1–10). The existence of a lymphoid DC lineage was suggested by the studies showing that DCs can be derived from the earliest T cell precursors within the thymus (31) or from CD19⁺ pro-B cells in cultures (32). In humans, plasmacytoid pre-DCs were suggested to be of

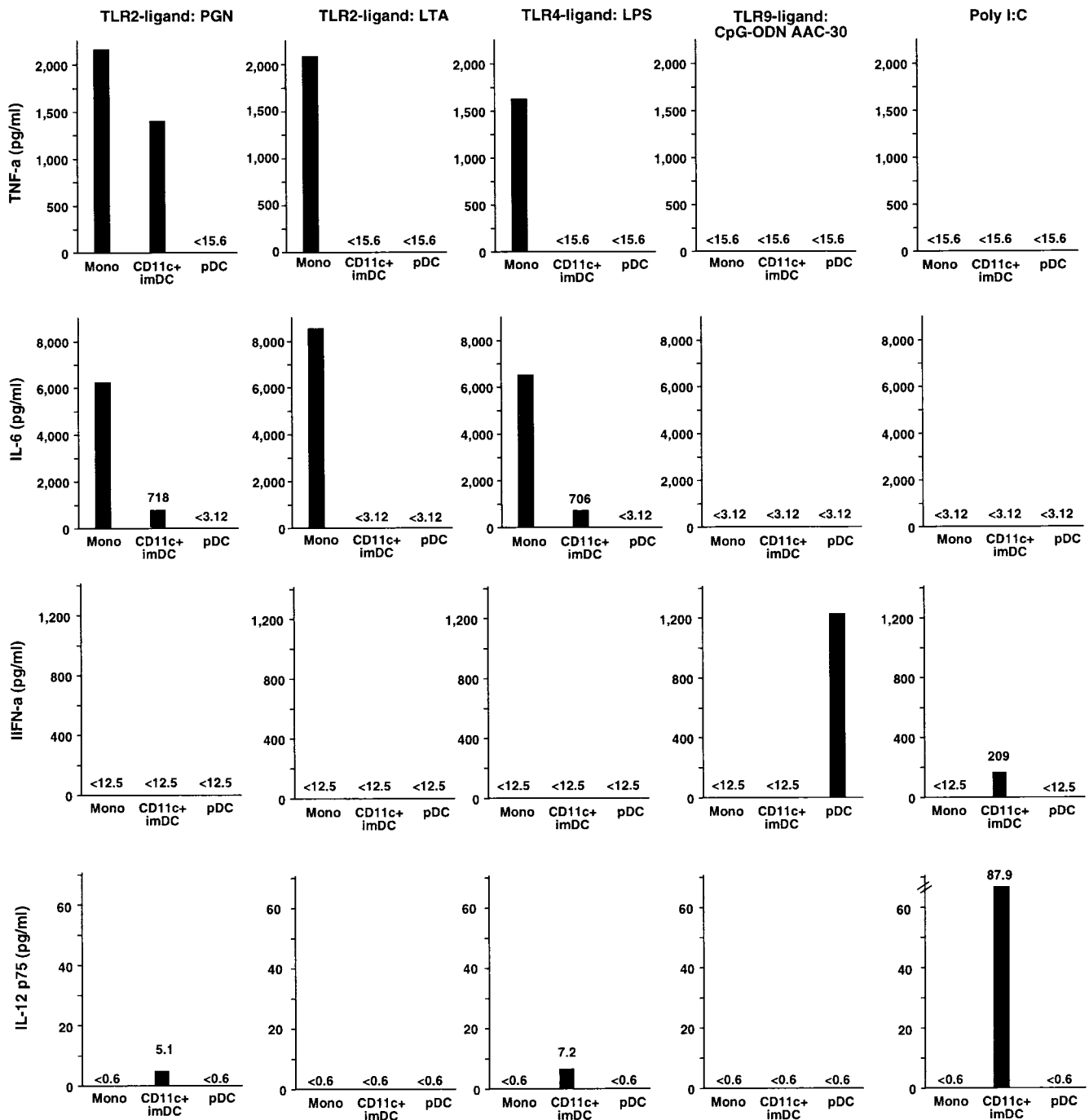


Figure 3. TNF- α , IL-6, IFN- α , and IL-12 production by monocytes (Mono), CD11c⁺ imDCs, and plasmacytoid pre-DCs (pDC) stimulated with microbial molecules that trigger different TLR signaling. The three cell populations were cultured with 10 μ g/ml PGN, 10 μ g/ml LTA, 10 μ g/ml LPS, 50 μ g/ml poly I:C, or with 5 μ M CpG-ODN AAC-30 for 24 h, and the concentrations of cytokines in the supernatants were measured by ELISA. The data shown are representative of four or five experiments.

lymphoid origin, because of their expression of lymphoid but not myeloid makers and their shared developmental pathway with T and B cells (20, 33, 34). The present finding that monocytes and plasmacytoid pre-DCs express distinct sets of TLRs suggests that these two DC precursors may have developed through different evolutionary trails, further supporting the existence of distinct DC lineages.

This study also clarifies the debates on whether plasmacytoid pre-DCs have the ability to produce IL-12 in response to LPS or CD40-ligand, or to produce IFN- α in response to CD40-ligand. Previous studies demonstrated that plasmacytoid pre-DCs did not produce significant amounts of IL-12p75 in response to activation with CD40-ligand, CpG, LPS, or viruses (7, 10, 35). However, studies by

Cella et al. showed that plasmacytoid pre-DCs isolated according to the expression of two immunoglobulin-like receptors (ILT3⁺ILT1⁻) produce large amounts of IL-12p75 in response to LPS (808 pg/ml) and to CD40-ligand (2,100 pg/ml) (36, 37). In addition, Cella et al. showed that ILT3⁺ILT1⁻ plasmacytoid pre-DCs produced IFN- α in response to CD40-ligand (36). These results could not be confirmed by the present and previous studies (7, 10, 35). The finding that plasmacytoid pre-DCs do not express TLR4 and do not respond to LPS in terms of cell survival and cytokine production, suggests that the observed IL-12 production by ILT3⁺ILT1⁻ plasmacytoid pre-DCs induced by LPS or CD40-ligand and their ability to induce TH1 responses may be due the contaminating myeloid DCs or macrophages (36–38). Indeed, ILT3⁺ILT1⁻ plasmacytoid pre-DCs appear to contain 10–30% of cells that do not express high IL-3R α , a key marker for human pre-DC2 (37).

Plasmacytoid pre-DCs have been shown to produce an enormous amount of IFN- α in response to viruses (10, 36, 39, 40), indicating that these cells play a critical role in antiviral innate immunity. The present finding that plasmacytoid pre-DCs express high levels of TLR9 and produce IFN- α in response to TLR9-ligand, bacteria CpG, extend the their function into antibacterial innate immunity.

Although CD11c⁺ imDCs express TLR-2, only PGN, but not LTA, stimulated CD11c⁺ imDCs to produce large amounts of TNF- α . This suggests that TLR2 is the primary receptor for PGN, but not for LTA. Alternatively, TLR2 may form different heterodimers with different TLRs, and CD11c⁺ imDCs only express the partner that allows recognition for PGN, but not for LTA (41, 42). The progressive downregulation of TLRs during pre-DC differentiation into imDCs and mature DCs, as shown here and in previous studies (18, 42), indicates a functional switch of DC lineage from microbial antigen recognition to antigen presentation, thus priming naive T cells and instructing them to differentiate into appropriate effector T cells. Although monocytes have a strong ability to produce TNF- α and IL-6 in response to microbial stimulation, they acquire the ability to produce large amounts of IL-12 and to present antigen to T cells upon differentiation into DCs. While plasmacytoid pre-DCs produce large amounts of IFN- α / β in response to viruses or CpG ODN, they rapidly loss this ability and acquire the ability to present antigen to T cells upon differentiation into DCs.

Recent studies have demonstrated a remarkable functional plasticity of a given DC subset to induce different types of T cell responses depending on the type of invading pathogens (3, 8–10). However, the expression of limited sets of pattern recognition receptors on monocytes and plasmacytoid pre-DCs suggests that neither monocytes nor plasmacytoid pre-DCs can respond to all microbial antigens, and do not have unrestricted functional plasticity. Therefore, the capacity of DCs to regulate the type of T cell-mediated immune responses may ultimately depend on the environmental instruction that they have received, as well as on their evolutionary lineage heritage.

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