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Regulation of Toll-Like Receptors in Human Monocytes and Dendritic Cells[1]

Alberto Visintin,* Alessandra Mazzoni,* Jessica H. Spitzer,* David H. Wyllie,† Steven K. Dower,† and David M. Segal[2]*

A number of pathogens induce immature dendritic cells (iDC) to migrate to lymphoid organs where, as mature DC (mDC), they serve as efficient APC. We hypothesized that pathogen recognition by iDC is mediated by Toll-like receptors (TLRs), and asked which TLRs are expressed during the progression of monocytes to mDC. We first measured mRNA levels for TLRs 1–5 and MD2 (a protein required for TLR4 function) by Northern analysis. For most TLRs, message expression decreased severalfold as monocytes differentiated into iDC, but opposing this trend, TLR3 and MD2 showed marked increases during iDC formation. When iDC were induced to mature with LPS or TNF-α, expression of most TLRs transiently increased and then nearly disappeared. Stimulation of iDC, but not mDC, with LPS resulted in the activation of IL-1 receptor-associated kinase, an early component in the TLR signaling pathway, strongly suggesting that LPS signals through a TLR. Surface expression of TLRs 1 and 4, as measured by mAb binding, was very low, corresponding to a few thousand molecules per cell in monocytes, and a few hundred or less in iDC. We conclude that TLRs are expressed in iDC and are involved in responses to at least one pathogen-derived substance, LPS. If TLR4 is solely responsible for LPS signaling in humans, as it is in mice, then its extremely low surface expression implies that it is a very efficient signal transducer in iDC. ***The Journal of Immunology,*** **2001, 166: 249–255.**

The transport of Ag from sites of inflammation in peripheral tissues to lymphoid organs and its presentation to naive and memory T cells is mediated by dendritic cells (DC)[3] (1, 2). In the periphery, DC are highly endocytic and, therefore, well adapted for the capture of Ag, but are poor APC. These immature DC (iDC) respond to LPS and other pathogen-derived substances, inflammatory cytokines, and CD40 ligand by processing Ag (3), migrating to lymph nodes, and differentiating into highly efficient APC, known as mature DC (mDC). iDC are derived from a number of cell types (4), one of which, the monocyte, differentiates into iDC in vitro when cultured with GM-CSF and IL-4 (5). The differentiation of monocytes into iDC is accompanied by a change in surface phenotype, most notably losses in CD14 and CD64, and a gain in CD1a expression. The maturation of iDC requires the activation of NF-κB (6) and results in the up-regulation of HLA-DR, CD83, B7-1, B7-2, and CD40, and the production of cytokines such as IL-12 and TNF-α. By inducing maturation, receptors for LPS and other pathogen-associated molecular patterns (PAMPs) on iDC play pivotal roles in the development of adaptive responses to Ags.

The innate recognition of PAMPs is mediated by genomically encoded pattern recognition receptors (7, 8). Recently, the *Drosophila* receptor, Toll, was shown to be essential for protective immunity to fungal infections in flies (9), and since then a number of immunologically relevant homologs of Toll have been discovered in organisms as disparate as plants, insects, and mammals (8, 10–13). In humans, six Toll-like receptor (TLR) homologs have been published to date (14–18), and at least four others have been identified (12). All are type I integral membrane receptors with extracellular leucine rich regions and intracellular portions that are homologous to the signaling domain of the IL-1R. The extracellular domain of human TLR4 associates with a second protein, MD2, which is required for optimal LPS-induced signaling (19). In transfection experiments, human TLRs 2 and 4 recruit and activate IL-1 receptor-associated kinase (IRAK) in response to a variety of PAMPs, including the Gram-negative bacterial toxin, LPS, resulting in downstream activation of NFκB and c-Jun NH_2-terminal kinase, and secretion of IL-8 and IL-12 (20–32). In mice, gene knockout studies indicate that TLR4, but not TLR2, is required for LPS responsiveness, whereas TLR2 is essential for responses to several Gram-positive PAMPs (33, 34). The functions of other TLRs have yet to be defined.

To understand the roles TLRs play in mammalian immunity, it is essential to define their expression patterns in normal cells and tissues. This is particularly important in DC, where PAMP recognition drives their maturation. Therefore, we asked whether DC express TLRs, and how TLR expression patterns change as monocytes differentiate into iDC and mature into mDC. In this report we followed mRNA expression for TLRs 1–5 and MD2 by Northern analysis and compared these results with surface expression using mAbs against TLRs 1 and 4. We observed a characteristic pattern of TLR and MD2 expression at each stage of DC differentiation, and found that LPS induced striking, cell-specific changes in the expression of TLRs in both iDC and monocytes. Moreover, we demonstrate that LPS induces IRAK activation and TNF-α secretion in iDC, which express most TLRs, but not in mDC, which lack TLR expression, suggesting that TLRs play an important role in iDC activation and maturation.

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[3] Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; mDC, mature DC; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; IRAK, IL-1 receptor-associated kinase.

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Table I. *Properties of TLR probes*

	Forward Primer[a]	Reverse Primer	Probe Length[b]	Residues (accession no.)[c]
TLR1	CAATTCAGTTT CCCACCCATCA G (23)	GCCACGTTTG CTCTTTTCCTT G (22)	802	1497–2298 (HSU88540)
TLR2	TATCGTCTTCC TGGTTCAAGCC (22)	AACAGAGCACA GCACATGCCA GAC(24)	1452	473–1925 (HSU88878)
TRL3	GGTAACGATTC CTTTGCTTGGC TTC (25)	TAGTGGCTTGA CAGCTCAGGG ATG (24)	1157	941-2097 (HSU88879)
TLR4	TCCCTCCAGGT TCTTGATTACA GTC (25)	TGCTCAGAAAC TGCCAGGTCT G (22)	652	1736–2387 (HSU93091)
TLR5	CACGGAAGGT TGTGATGAAGA GG (23)	CAGCCATCTCT AAGGAAGTGT CTGC (25)	426	2578–3003 (AF051151)

[a] Primers used to generate probes are reported in 5′ orientation; primer length is given in parentheses.
[b] Length of TLR-specific portions of cDNA probes in base pairs. Probes contain an additional 18 bp from the vector.
[c] Regions of TLR message recognized by the probes based upon the numbering schemes used in the indicated GENBANK entry (accession number in parentheses).

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Materials and Methods

Dendritic cells

Immature human DC were generated according to Sallusto et al. (5). Briefly, elutriated monocytes from healthy National Institutes of Health Blood Bank donors were cultured in DC medium (complete medium (RPMI 1640 containing 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 55 μM 2-ME) supplemented with 50 ng/ml recombinant human GM-CSF and 34.5 ng/ml recombinant human IL-4 (PeproTech, Rocky Hill, NJ)), for 6–8 days. To induce maturation, iDC were treated in DC medium with 100 ng/ml LPS (*Escherichia coli* serotype 026:B6; Sigma, St. Louis, MO) unless stated otherwise, in which case they were treated with 20 ng/ml recombinant human TNF-α (PeproTech) for the indicated periods of time.

Flow cytometry

Cell surface staining was performed using the following anti-human mAbs from PharMingen (San Diego, CA): anti-CD3^{PE}; anti-CD14^{PE}; anti-CD19^{FITC}; anti-CD80^{FITC}; anti-CD86^{FITC}; anti-HLA-DR^{FITC}; anti-CD1a^{PE}; and anti-CD83^{PE}. The FITC- labeled anti-CD64 mAb (32.2) was a gift from Dr. Michael Fanger (Dartmouth Medical School, Hanover, NH). The GD2.F4 mAb (mouse IgG1) was raised against the extracellular domain of human TLR1 and was shown to be specific for TLR1 in transfection experiments (43). The mAb against TLR4, HTA1216 (mouse IgG1), has been described previously (35) and was a gift of Dr. Kensuke Miyake (Saga Medical School, Saga, Japan). The binding of both anti-TLR mAbs was detected using a FITC-labeled goat anti-mouse secondary reagent (Boehringer Mannheim, Indianapolis, IN) and was compared with cells treated with an isotype-matched nonbinding control mAb (MOPC 300) and with the mIgG1 anti-CD44 mAb, NIH44.1 (36). The NIH44.1 mAb was also radioiodinated and used to determine the number of molecules bound per cell at saturation (37). Staining was performed in the presence of 100 μg/ml nonimmune human IgG to block nonspecific binding to FcγR. Ten thousand cells were acquired for each sample, and dead cells were gated out based on their light scatter properties. DC preparations always contained <5% lymphocytes based on light scatter and staining for B and T cell markers. Cells from the same donor were used when comparisons were made between monocytes, iDC, and mDC.

Mixed lymphocyte reaction

DC were washed, irradiated (30 Gy), and added as stimulator cells to 96-well plates containing 1×10^5 responder cells per well. Responder cells were allogeneic T cells (>95% CD3$^+$) purified from PBMC by negative selection using a mixture of mouse anti-human-CD14, -CD19, and -CD16 Abs (PharMingen) and sheep anti-mouse IgG-coated Dynabeads (Dynal, Oslo, Norway). After a 3-day stimulation, cells were pulsed with 5 μCi/ml of [^{3}H]thymidine (NEN, Boston, MA) for 16 h, then harvested, and incorporation was measured by scintillation counting. Data are expressed as cpm (mean ± SD) of triplicate cultures.

Probes

Specific probes for TLRs 1–5 were generated by RT-PCR using total RNA from PBL obtained from normal donors and the primer pairs indicated in Table I. Probe lengths and sequences are also indicated in Table I. The β-actin and GAPDH probes were generated using primers from Promega (Madison, WI). Amplified products were cloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and the identity of each insert was confirmed by automated DNA sequencing. Inserts were removed by *Eco*RI digestion, gel purified, and random primed using [α-^{32}P]dCTP. TLRs 1–5 are only distantly related, and as expected, do not cross-hybridize as indicated by the distinct size of each message seen in the Northern blots. However, TLRs 1 and 6 (accession number, AB020807) are highly homologous isoforms, and our TLR1 probe would likely cross-hybridize with TLR6 (17). The size of the TLR6 message is unknown (17), and if it is the same as TLR1, our TLR1 Northern results could include contributions from the TLR6 isoform. The full-length cDNAs of hMD2 and hCD14 were gifts from Dr. Kensuke Miyake and Dr. Brian Seed (Massachusetts General Hospital, Boston, MA), respectively.

RNA preparation and Northern analysis

Total RNA from $\sim 10^7$ cells/point was prepared using Trizol extraction (Life Technologies, Gaithersburg, MD). Ten micrograms of total RNA from each sample was then analyzed using standard Northern blotting procedures (hybridization was for at least 16 h at 42°C in 10 ml Hybrisol I (Intergen, Purchase, NY) containing 25 ng probe). Each blot shown in this paper is representative of data obtained from three separate donors. In some cases membranes were probed twice; after hybridization with the first probe, membranes were stripped by adding boiling 1% SDS and then hybridized with the second probe. Equal loading was confirmed by ethidium bromide staining of RNA in the original gel before transfer. Where shown, each ethidium bromide gel represents one of several used in data analysis. Transcript sizes and banding patterns correlated with previously reported data (16, 38).

TNF-α secretion

iDC or mDC (5×10^5; matured for 24 h in LPS and rested for 2 h in DC medium) were treated with 100 ng/ml of *E. coli* LPS (055:B5; Sigma) or 50 ng/ml PMA + 1 μg/ml ionomycin in 200 μl DC medium for 5 h. Duplicate 50-μl samples of supernatants were assayed for TNF-α content using a human TNF-α ELISA kit (Endogen, Woburn, MA), which has a limit of detection of 20 pg/ml.

IRAK assay

iDC or mDC (5×10^6 cells per point, mDC were matured for 20 h in LPS and rested for 2 h in DC medium) were stimulated with 1 μg/ml of LPS (45 min) or 10 ng/ml of IL-1β (10 min). Where indicated, cells were preincubated for 10 min with 5 μg of an anti IL-1β mAb (R&D Systems, Minneapolis, MN). Cells were then lysed in 1 ml of lysis buffer (0.4% Nonidet

P-40, 60 nM *n*-octyl-β-D-glucopyranoside, 137 mM NaCl, 2 mM EDTA, 50 mM NaF, 10% glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 μg/ml each of leupeptin and aprotinin (Sigma)). Lysates were centrifuged for 10 min at 12,000 $\times$ *g*, 4°C and IRAK was immunoprecipitated for 12 h at 4°C from 700 μl of supernatant using 20 μl of packed protein A-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) coupled with 2 μg of anti IRAK pAb (Upstate Biotechnology, Waltham, MA). The beads were washed three times in lysis buffer, twice in kinase buffer (20 mM Tris pH 7.5, 20 mM NaCl, 1 mM EDTA, 3% glycerol, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM PMSF) and then incubated for 40 min in 25 μl of kinase buffer supplemented with 1.5 μg of myelin basic protein (Sigma) and 10 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) at 37°C. Samples were boiled for 4 min in SDS-PAGE loading buffer containing 2 mM DTT and resolved on a 12% SDS-PAGE gel, after which the gel was dried and subjected to autoradiography. To measure total IRAK protein, lysates (20 μl) were resolved by 10% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and Western blotted with the anti IRAK pAb (1:1000 in PBS, 3% BSA, and 0.1% Tween 20) used for the immunoprecipitations. Specific IRAK bands were detected using a HRP-labeled goat anti-rabbit pAb and the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Results

TLR message expression during iDC differentiation

To obtain DC for TLR analysis, monocytes were cultured with GM-CSF and IL-4, and by day 6 a homogeneous population of iDC was recovered based upon expression of defining surface markers including high amounts of CD1a, low levels of CD86, and practically no CD14 or CD64 (Fig. 1*A*). In parallel, cells were examined for TLR message by Northern analysis. Fig. 1*B* shows that fresh monocytes express high levels of TLRs 1, 2, 4, and 5, all

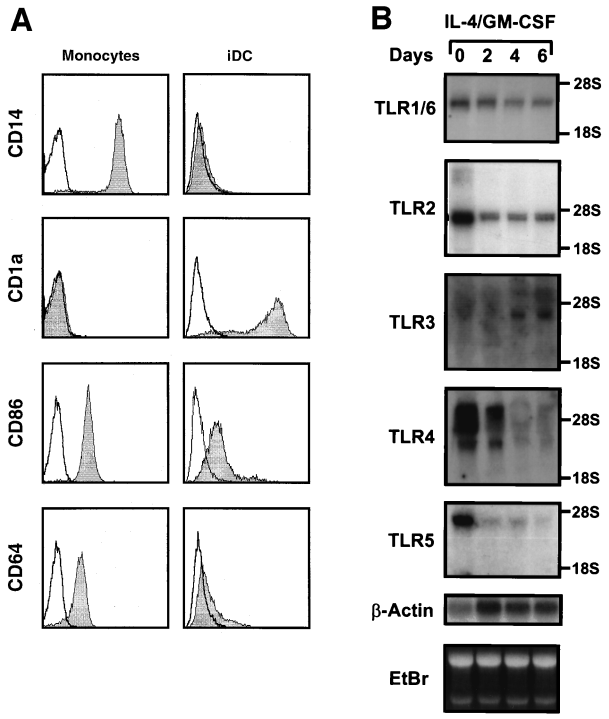

FIGURE 1. TLR expression changes as monocytes differentiate into iDC. *A*, Phenotypic characterization of monocytes and iDC (6 days of culture) by FACS analysis. Shaded histograms indicate cells stained for the indicated marker, open histograms represent unstained controls. *B*, TLR message levels by Northern analysis. Total RNA from fresh monocytes (0 days) or monocytes cultured in DC medium (GM-CSF + IL-4) for 2, 4, or 6 days was analyzed using TLR-specific probes or a β-actin control probe. The ethidium bromide (EtBr)-stained gel (*bottom*) is representative of several gels used to compose this figure, and indicates relative amounts of RNA loaded per lane.

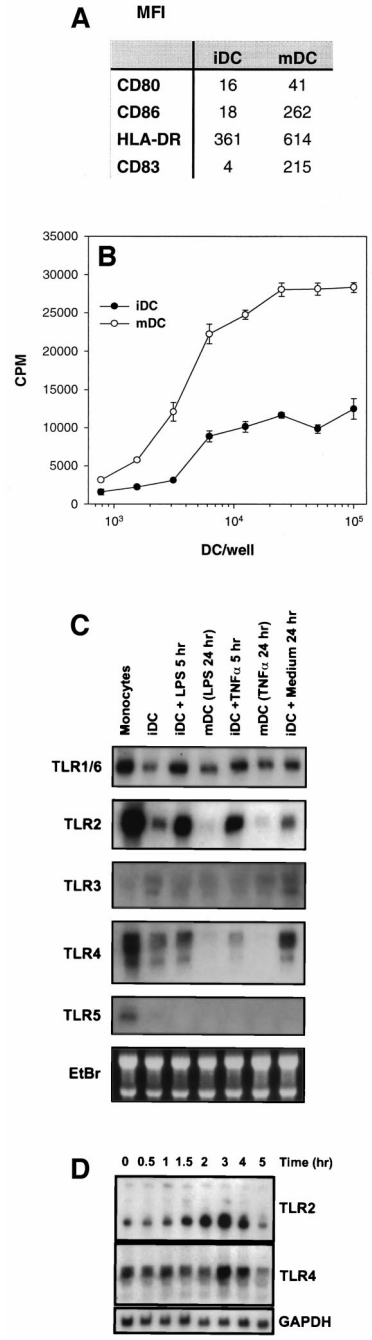

FIGURE 2. TLR expression during DC maturation. *A*, Modulation of DC surface markers following LPS-induced differentiation. DC were left untreated or were stimulated in DC medium with LPS for 24 h, stained for the indicated markers, and analyzed by FACS. Numbers represent the mean fluorescence intensities of the whole population minus the background fluorescence. *B*, Enhanced APC function of mDC. DC, either untreated (iDC, ●) or stimulated in DC medium with LPS for 24 h (mDC, ○) were irradiated and added to 1 $\times$ 10^5 responder allogeneic T cells. [^{3}H]thymidine incorporation was measured after 3 days. Background T cell proliferation was <500 cpm. *C*, Northern analysis of TLR expression in monocytes, iDC, and mDC. As indicated, mDC were generated by incubating iDC for 24 h with either LPS or TNF-α. Also shown is TLR expression in iDC after 5-h treatment with either LPS or TNF-α. A control in which iDC were incubated in medium alone for 24 h is indicated in the right most lane. Within each panel, cells were derived from the same donor in the same experiment, and each panel is representative of at least three experiments. One representative ethidium bromide gel is shown at the bottom. *D*, Early kinetics of TLR 2 and 4 expression. iDC were stimulated for the indicated periods of time with 100 ng/ml LPS, and RNA was probed for TLRs 2 and 4; GAPDH served as a control for RNA integrity. All data are from the same donor.

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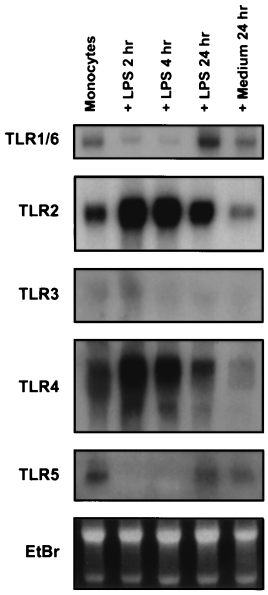

FIGURE 3. Effects of LPS on TLR expression in monocytes. Fresh monocytes (*left lane*) were treated for the indicated times with 100 ng/ml LPS or medium, and tested for TLR expression by Northern analysis as in Fig. 2*C*.

of which decreased as monocytes differentiated into iDC. Changes were apparent as early as the second day of culture, and were complete at day 4, by which time low levels of TLRs 1, 2, and 4 were observed, whereas TLR5 was barely detectable. Because TLR6, an isoform of TLR1, would also be detected by our TLR1 probe, we hereafter refer to bands recognized by this probe as TLR1/6. The only TLR to increase during iDC formation was TLR3, which was undetectable in monocytes but clearly discernable as a band in iDC. β-actin, which was used as a control for RNA integrity, increased during iDC formation, indicating that TLR down-regulation in iDC was not an artifact of message degradation.

TLR message changes during DC maturation

Next, TLR message expression was examined in mDC. DC maturation is induced by PAMPs such as LPS by inflammatory cytokines, such as TNF-α, and by CD40 ligand. FACS and functional analyses (Fig. 2, *A* and *B* and data not shown) confirmed that LPS and TNF-α did induce DC maturation as indicated by marked increases in CD80, CD83, CD86, and HLA-DR and by enhanced Ag-presenting capacity. Northern analyses revealed that all TLRs with the exception of TLR1/6 were strongly repressed in mDC (Fig. 2*C*, iDC + LPS 24 h or iDC + TNF-α 24 h), whereas TLR1/6 was expressed at levels comparable to those seen in iDC. However, to reach the low levels of TLR expression seen in mDC, the DC passed through an intermediate with increased levels of TLRs 1/6, 2, and 4 (Fig. 2*C*, 5-h treatment with LPS or TNF-α). We then focused on the early time points of expression of TLRs 2 and 4, putative signaling receptors for LPS, following stimulation with LPS. The pattern shown in Fig. 2*D* reveals a coordinated up-regulation of the two TLRs that peaked at 3 h, and declined rapidly thereafter. Thus, TLR expression defines an intermediate in the DC maturation pathway in which some TLRs are expressed at relatively high levels, before their down-regulation in mDC.

To determine whether the regulation of TLR expression differs in monocytes and iDC, we stimulated monocytes for 2, 4, and 24 h with LPS and examined their RNA by Northern analysis. Fig. 3 shows that LPS induced changes in TLR expression patterns in monocytes that were quite distinct from those seen in iDC (compare with Fig. 2). Monocytes, in contrast to iDC, did not respond to prolonged (24-h) LPS treatment by down-regulating TLR message. This effect was particularly apparent in TLRs 1/6, 2, 4, and 5, which were highly expressed in monocytes stimulated for 24 h but not in mDC. The effects of short-term (2- or 4-h) LPS stimulation fell into two patterns. In the first pattern, seen in TLRs 1/6 and 5, LPS induced a dramatic decrease in message as early as 2 h, followed by a return to either pretreatment or higher levels by 24 h. In the second pattern, seen in TLRs 2 and 4, rapid increases in message were followed by a return to near prestimulation levels. Interestingly, TLR1/6 changed from the first pattern in monocytes, to the second pattern in iDC. These results show that levels of TLR expression are regulated in a cell type-specific manner and independently, except for TLRs 2 and 4, which were coordinately expressed in all cells tested.

Surface expression of TLRs 1 and 4

To correlate message levels with surface protein expression, we stained cells with mAbs against TLRs 1 and 4. FACS analysis (Fig. 4, *A* and *B*) showed that TLR1 is easily detected on monocyte cell surfaces, but that the level of surface expression drops by ~10-fold in iDC and another 2-fold in mDC, where it is just barely detectable. TLR4 was present on monocytes at easily detectable levels but was too low to detect by FACS in iDC and mDC. The numbers of molecules of TLRs expressed on monocytes were estimated by comparing the fluorescent signal from an anti-CD44 mAb with those from the mAbs against TLRs 1 and 4. In these experiments, all mAbs were mouse IgG1, and the same secondary reagent was used in staining. Using radioiodinated anti-CD44, we estimated that $\sim 1.5 \times 10^5$ mAb molecules bound per cell at saturation (corresponding to twice as many CD44 molecules, if the mAb bound divalently; Ref. 37). It is apparent in Table II that surface expression of both TLRs 1 and 4 is extremely low compared with CD44 (an adhesion molecule), corresponding to a few thousand molecules per cell on monocytes, and a few hundred molecules or less on iDC. Moreover, surface expression of both

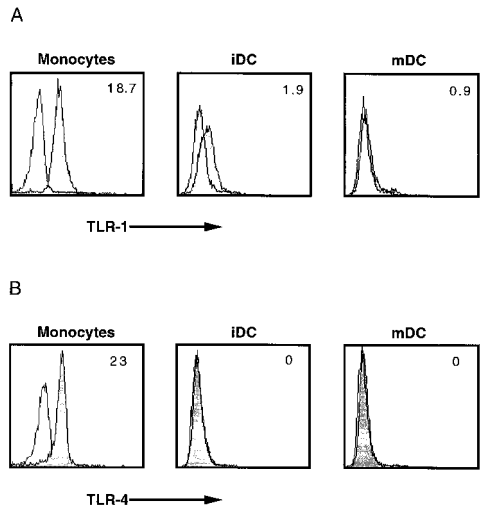

FIGURE 4. Cell surface expression of TLR1 (*A*) and TLR4 (*B*). Fresh human monocytes, iDC, and mDC were labeled with the mAbs GD2.F4 (anti-hTLR1) and HTA1216 (anti-TLR4), and stained with a FITC-labeled secondary Ab (shaded histograms). Open histograms represent cells labeled with an irrelevant isotype-matched Ab. Numbers in upper right hand corners indicate the mean fluorescence intensities of the anti-TLRs mAbs minus the background.

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Table II. *Quantitative estimation of TLR1 and 4 surface expression*

		Average ΔMFI[a]	*n*	Mol/Cell[b]	Range
TLR1	Monocytes	14.2	15	2100	0–5400
	iDC	1.6	6	230	30–440
	mDC	1.2	3	180	130–210
TLR4	Monocytes	8.8	6	1300	400–3200
	iDC	<0.5	4	<75	NM

[a] Cells were labeled with saturating amounts of anti-TLR mlgG1 mAbs and stained with a FITC-labeled secondary Ab. Avg ΔMFI, average mean fluorescent intensities of *n* samples of cells labeled with anti-TLR mAbs minus MFIs from cells treated with an irrelevant mlgG1 (MOPC 300).

[b] The binding of radioiodinated NIH44.1 (mlgG1) to CD44 on monocytes was used to relate ΔMFI to Mol/Cell. At saturation, 1.5×10^5 molecules of NIH44.1 bound per cell by Scatchard analysis, corresponding to a ΔMFI of 990, or 150 Mol/Cell/ΔMFI. Numbers of anti-TLR mAb bound per cell (Mol/Cell) were calculated by multiplying coresponding ΔMFI values by 150. Range indicates the lowest and highest numbers of mAb molecules bound per cell in the *n* samples. NM, not measurable.

TLRs 1 and 4 showed high donor variability. For example, monocytes from one donor expressed ~400 TLR4 molecules/cell, whereas another expressed 3200, with the remaining donors distributed more or less evenly in between. Monocytes from two donors failed to stain with the anti-TLR1 mAb, and the remaining 13 ranged from 400 to 5400 molecules/cell (Table II).

Activation of iDC and mDC with LPS

We next asked whether TLRs might play a role in LPS-induced signal transduction in iDC. TLR4, which is essential for LPS signaling in mice, requires a second protein, MD2, for optimal LPS responses, so we first probed monocytes, iDC, and mDC for its expression (Fig. 5). Interestingly, MD2 is expressed at significantly higher levels in iDC than in monocytes, leading to an inversion of the MD2/TLR4 ratio in these two cell types. For comparison, CD14 message is totally lacking in iDC (Fig. 5) as expected from its lack of surface expression (Fig. 1*A*). CD14 is known to be required for LPS responsiveness (39), and in our studies is supplied in soluble form from the serum. Thus, although surface expression of TLR4 is exceptionally low, all components known to be required for a functional TLR4 are present in iDC. Because iDC lose expression of TLRs 2, 3, 4, and MD2 during maturation, we would predict that if any of these is essential for LPS signaling, then mDC should not respond to LPS. We tested this by measuring TNF-α secretion. As shown in Fig. 6*A*, LPS triggered a robust TNF-α response in iDC but failed to induce TNF-α secretion in mDC, even though mDC were capable of responding to PMA plus ionomycin. A second prediction of TLR signaling is that LPS should induce IRAK activation in iDC but not mDC. IRAK is the first kinase to be activated by TLRs, IL-1R, and IL-18R, but is not known to be activated by any other receptor. Fig. 6*B* shows that LPS does in fact induce IRAK activation in iDC, but not in mDC as measured in a kinase assay, although both cell types express similar amounts of IRAK as seen in the anti-IRAK Western blot. Activation was not due to a secondary effect of IL-1 induction, because a neutralizing anti-IL-1 mAb failed to block LPS-induced activation of IRAK, and similar results were obtained with an anti-IL-18 mAb (data not shown). Thus, our data strongly suggest that LPS activates iDC through one or more TLRs. However, we have not yet been able to determine precisely which TLRs are involved because none of the anti-TLR Abs available to us blocks LPS signaling in iDC (data not shown).

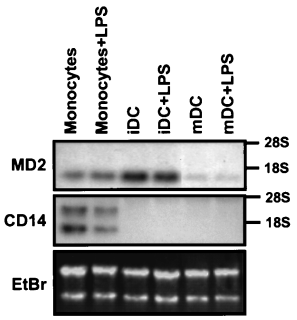

FIGURE 5. MD2 and CD14 expression. Monocytes, iDC, and mDC RNA were probed for MD2 and CD14 expression by Northern analysis. Where indicated, cells were treated for 5 h with 100 ng/ml LPS before RNA extraction.

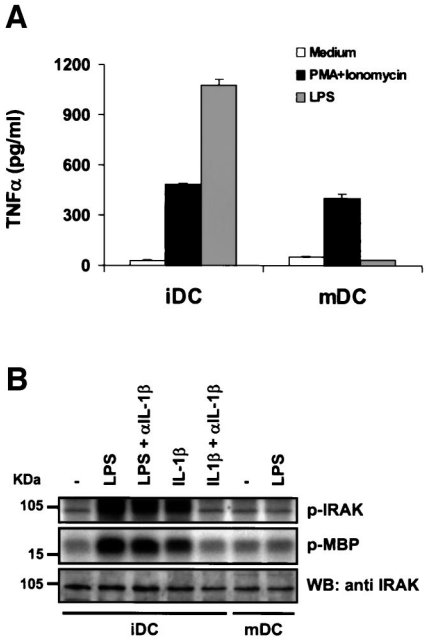

FIGURE 6. *A*, LPS induces TNF-α secretion in iDC but not mDC. Cells were incubated for 5 h in medium alone (open columns), medium containing PMA + ionomycin (filled columns), or medium containing LPS (gray columns), and supernatants were analyzed for secreted TNF-α. Error bars represent SDs of duplicate samples. This experiment has been repeated once with similar results. *B*, LPS induces IRAK activation in iDC but not mDC. iDC and mDC were treated with either LPS or IL-1β with or without a 10-min preincubation with neutralizing anti-IL-1β mAb. Cells were lysed and IRAK activation was determined using an in vitro kinase assay. The figure shows autophosphorylation of IRAK (p-IRAK), transphosphorylation of myelin basic protein (p-MBP), and total IRAK (WB: anti-IRAK), determined by Western blotting whole cell lysates. This experiment was performed on iDC from three different donors with similar results.

Discussion

The innate recognition of PAMPs by DCs is a critical step in the generation of acquired immune responses. TLRs belong to a large family of membrane proteins with cytoplasmic signaling domains and, at least for TLRs 2 and 4, extracellular domains capable of recognizing PAMPs. In this report we have followed the expression of several TLRs as monocytes differentiate into iDC and then mDC, and provide a comprehensive study of TLR expression in normal human monocytes and DC. It is clear from our data that at the level of mRNA, monocytes express substantial amounts of most of the TLRs studied here, and that the levels of expression decrease markedly during iDC formation. Nevertheless, significant amounts of message for several TLRs are present in iDC. The distinctive increase in TLR3 expression in iDC, as compared with

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monocytes, suggests that this TLR may play a special role in DC function and, conversely, the striking decrease in TLR5 implies that it lacks relevance in these cells. As iDC mature into mDC, they lose expression for all TLRs except TLR1/6, which correlates with a loss of responsiveness to LPS as assessed by TNF-α production and IRAK activation. After submission of this manuscript, another paper examining TLR message expression was published by Muzio et al. (38). Their results are in general agreement with ours, except for TLR5, which they find expressed in DC at levels even higher than in monocytes. The reason for this discrepancy is not known, but may be due to differences in the methods of DC preparation.

At the level of surface expression, TLRs 1 and 4, the two TLRs for which we had mAbs, were expressed in low numbers on monocytes and, as expected from the Northern analyses, even lower numbers on iDC. TLR1 protein is barely detectable by FACS on iDC and mDC, whereas TLR4 cannot be detected, but may be present in very small amounts on iDC because these cells make TLR4 message. Du et al. (40) previously reported that mouse macrophage lines express amounts of TLR4 protein that are so low that they are limiting for LPS responsiveness, meaning that increases in TLR4 expression resulted in increases in LPS responsiveness. Therefore, the low expression of TLRs 1 and 4 that we observed in normal cells is likely to be an important aspect of TLR function because under limiting conditions, cellular responses to PAMPs could be stringently regulated by controlling the amounts of TLR protein produced. Moreover, the exceptionally high variability in TLR surface expression that we observed among normal donors might indicate a high variability in the way different individuals respond to PAMPs. However, because monocytes exhibit relatively homogeneous distributions of TLRs 1 and 4, it is unlikely that subpopulations of differentially reactive cells exist within an individual. Another factor that could control TLR4 function is MD2, a molecule that associates with the extracellular portion of TR4 and is required for maximal TLR4 function (19). The fact that the MD2/TLR4 ratios are inverted in monocytes and iDC suggests that MD2 might be limiting for the LPS response in monocytes, whereas TLR4 would be limiting in iDC. However, there might be other ramifications of the relatively high MD2 expression in iDC, for example, MD2 might be secreted from iDC, it might bind to other iDC-specific TLRs (e.g., TLR3), or it might compensate in some way for the lack of CD14 surface expression in iDC.

We have observed that LPS stimulation regulates the expression of all five TLRs in monocytes and iDC, but the TLRs are regulated differently in the two cell types. Two TLRs, 2 and 4, are required for responses to a number of bacterial products, and it is of interest that these two TLRs are coordinately regulated in both monocytes and iDC, perhaps reflecting their similar functions. Although it has not been proven conclusively, a consensus is emerging that TLR4 is the sole signal-transducing receptor for LPS in the human as it is in mice, and that TLR2 is a receptor for several other bacterial PAMPs (41). If this is true, then one important question arising from our results is whether TLR4 is expressed on iDC in sufficient amounts to account for the LPS response. We estimate that iDC express at most 150 TLR4 molecules per cell, and expression could be considerably less, even zero. In the case of the IL-1R, which shares homology with the TLRs in the cytoplasmic signaling domains, 10 or fewer ligated receptors can induce a response (42), thus providing a precedent for signaling by extremely low numbers of receptors in a closely related system. Alternatively, iDC may not express TLR4 molecules on their surfaces at all, and either LPS or a bacterial contaminant may have activated TLR2 or a different TLR in our experiments. A third possibility is that TLRs are expressed primarily in intracellular compartments, and function by interacting with internalized PAMPs. We are currently investigating these possibilities. Regardless of TLR specificity, the observations that LPS activates IRAK, and that the ability of LPS to trigger iDC function parallels TLR expression, provide strong evidence that PAMPs signal through TLR-dependent pathways in iDC. By inducing iDC maturation, these TLR-dependent signals could play a pivotal role in the development of adaptive responses to pathogens.

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