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# Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells<sup>1</sup>

Marta Muzio,<sup>2,\*</sup> Daniela Bosisio,<sup>\*</sup> Nadia Polentarutti,<sup>\*</sup> Giovanna D'amico,<sup>\*</sup> Antonella Stoppacciaro,<sup>†</sup> Roberta Mancinelli,<sup>\*</sup> Cornelis van't Veer,<sup>‡</sup> Giselle Penton-Rol,<sup>\*</sup> Luigi P. Ruco,<sup>†</sup> Paola Allavena,<sup>\*</sup> and Alberto Mantovani<sup>\*§</sup>

Members of the Toll-like receptor (TLR) family probably play a fundamental role in pathogen recognition and activation of innate immunity. The present study used a systematic approach to analyze how different human leukocyte populations express specific transcripts for the first five characterized TLR family members. TLR1 was expressed in all leukocytes examined, including monocytes, polymorphonuclear leukocytes, T and B cells, and NK cells. In contrast TLR2, TLR4, and TLR5 were expressed in myelomonocytic elements. Exposure to bacterial products, such as LPS or lipoarabinomannan, or to proinflammatory cytokines increased TLR4 expression in monocytes and polymorphonuclear leukocytes, whereas IL-10 blocked this effect. TLR3 was only expressed in human dendritic cells (DC) wherein maturation induced by bacterial products or cytokines was associated with reduced expression. TLR3 mRNA expression was detected by in situ hybridization in DC and lymph nodes. These results demonstrate that TLR1 through TLR5 mRNAs are differentially expressed and regulated in human leukocytes. In particular, expression of TLR3 transcripts is restricted to DC that are the only elements which express the full TLR repertoire. These data suggest that TLR can be classified based on expression pattern as ubiquitous (TLR1), restricted (TLR2, TLR4, and TLR5 in myelomonocytic cells), and specific (TLR3 in DC) molecules. *The Journal of Immunology*, 2000, 164: 5998–6004.

The innate immune system recognizes pathogens by means of certain conserved structural features of the microbes such as LPS; the targets of recognition represent molecular patterns, also called pathogen-associated molecular patterns, rather than particular structures (1). This evolutionary strategy of the host both tends to prevent the generation of microbial escape mutants and allows a limited number of germline-encoded receptors to recognize a great variety of molecular structures associated with pathogens. The innate immune recognition is mediated by a structurally diverse set of receptors that belong to several distinct protein families. Among them are humoral proteins circulating in the plasma, endocytic receptors expressed on the cell surface, and signaling receptors that can be expressed either on the cell surface or intracellularly. The best characterized interaction is between LPS and LPS-binding protein (LBP).<sup>3</sup> The LBP/LPS complex is then shuttled to the monocyte/macrophage-specific surface receptor CD14. Alternatively, the LBP-LPS complex can be recognized

by a soluble version of CD14 that subsequently activates nonmyeloid cells (2, 3). Regardless, CD14 likely acts to present LPS to a distinct transmembrane receptor, namely, a member of the Toll-like receptor (TLR) family (4, 5, 6). TLR4 has been genetically identified as an essential and nonredundant component of the LPS receptor signaling complex that controls innate immune responses in vivo (7, 8, 9). Other groups have recently suggested that another member of the TLR family, namely, TLR2, can provide LPS responsiveness to insensitive cell lines (10, 11); in fact, it has been recently shown that TLR2 can restore the responsiveness of cells to peptidoglycans and lipoteichoic acids (12). Recent evidence, based on the analysis of TLR4-deficient mice, demonstrates that TLR4 may be more specifically involved for LPS signaling (13). On the other hand, study of TLR2-deficient mice show that TLR2 specifically confers responsiveness to several Gram-positive bacterial cell walls as to *Staphylococcus aureus*, peptidoglycans, and zymosan (13, 14).

TLR4 activates the MyD88 signaling pathway, initially identified for the IL-1R (15, 16). Indeed, LPS activates the MyD88/IRAK signaling cascade in monocytic and in endothelial cells (17). Available information suggests that a stereotyped signaling response is activated by different TLR family members.

Despite the assumption that TLR mediate innate immune response, no data are available regarding their expression pattern in immunocompetent cells. There are many members of the TLR family; six have been characterized (4, 5, 6, 18), and other partial cDNA sequences are deposited in the databases. Their number may reflect specialized functions, redundancy, and/or differential expression and roles in different cell types. The present study was designed to carefully characterize the pattern of expression of the first five TLR mRNAs. The results obtained demonstrate differential expression and regulation of TLR and suggest a novel classification of these molecules.

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<sup>3</sup> Abbreviations used in this paper: LBP, LPS-binding protein; TLR, Toll-like receptor; DC, dendritic cell; PMN, polymorphonuclear leukocyte.

## Materials and Methods

### Cell culture reagents and stimuli

The cell culture medium routinely used was RPMI 1640 with 2 mM glutamine and 10% FCS (complete medium). All reagents contained <0.125 endotoxin units/ml of endotoxin as checked by *Limulus* amoebocyte lysate assay (Microbiological Associates, Walkersville, MD). LPS (*Escherichia coli* 005:B5; Difco, Detroit, MI) was used at 10 ng/ml. Human recombinant TNF- $\alpha$  (BASF/Knoll, Ludwigshafen, Germany) was used at 500 U/ml. Human recombinant IL-1 $\beta$  was a kind gift from Dr. J. E. Sims (Immunex, Seattle, WA). Human recombinant IL-10 was from Shering Plough (Kenilworth, NJ). Human recombinant IFN was purchased from Boehringer Mannheim (Marburg, Germany). Lipoarabinomannans were a kind gift from Dr. Belisle (Colorado State University, Fort Collins, CO). Mannose-capped lipoarabinomannan is isolated from *Mycobacterium tuberculosis* strain H<sub>37</sub>Rv. Noncapped lipoarabinomannan (AraLAM) is isolated from a rapid growing *Mycobacterium* spp. Phosphatidylinositol mannoside is isolated from *M. bovis* strain bacillus Calmette-Guérin. Actinomycin D and cycloheximide were purchased from Sigma (St. Louis, MO).

Circulating human monocytes, polymorphonuclear cells (PMN), lymphocytes, and NK cells were separated from blood of normal donors (>95% pure as assessed by morphology) by Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation as described in detail elsewhere (19). Dendritic cells (DC) were obtained and matured in vitro as described previously (20). Th1 and Th2 cells were a kind gift from Dr. D. D'Ambrosio (Roche Milano Ricerche, Italy). Large and small B lymphocytes were a kind gift from Dr. J. Golay (Mario Negri Institute). NK cells were kindly provided by Dr. Carla Paganin (Mario Negri Institute). After the appropriate treatment, cells were examined for TLR mRNA as detailed below.

### Northern blot analysis

Total RNA was isolated by the guanidine isothiocyanate method with minor modifications. Eight micrograms of total RNA was analyzed by electrophoresis through 1% agarose/formaldehyde gels, followed by Northern blot transfer to Gene Screen Plus membranes (New England Nuclear, Boston, MA). The plasmids containing human TLR cDNAs were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol; Amersham, Buckinghamshire, U.K.). Membranes were pretreated and hybridized in 50% formamide (Merck, Rahway, NJ) with 10% dextran sulfate (Sigma) and washed twice with 2 $\times$  SSC (1 $\times$  SSC, 0.15 M NaCl, and 0.015 M sodium citrate) and 1% SDS (Merck) at 60°C for 30 min, and finally washed twice with 0.1 $\times$  SSC at room temperature for 30 min. Membranes were exposed for 4–48 h at –80°C with intensifying screens. RNA transfer to membranes was checked by UV irradiation, as shown in each figure.

### Plasmids

TLR1 and TLR3 plasmids were obtained from Immunex. TLR4 plasmid has been described previously (14). A partial TLR2 cDNA was obtained by RT-PCR and subcloned into pCRII vector (Invitrogen, San Diego, CA). A partial TLR5 cDNA containing plasmid was obtained by Research Genetics (Huntsville, AL; dbEST Image clone no. 277229 3').

### In situ hybridization

TLR3 cDNA fragment (540 bp insert after *EcoR* V digestion) and control probes IL-1 were labeled with biotin-dCTP using random primers methods (Renaissance; NEN Life Science, Boston, MA). Five-micrometer cryostat sections from lymph nodes and monocyte-derived DC cytomers were fixed with 4% buffered paraformaldehyde, dehydrated in ethanol, rehydrated in 1 $\times$  PBS and 50 mM MgCl<sub>2</sub>, washed in 0.1 M Tris-HCl (pH 7.5) and 0.1 M glycine, and acetylated in 2 $\times$  SSC, 0.1 M triethanolamine, and 0.5% acetic anhydride (pH 8), and dehydrated in ethanol. Dry sections were prehybridized for 1 h at 37°C with 60% formamide, 4 $\times$  SSC, 500 mg/ml ssDNA, and hybridized overnight at 60°C with 20 pg/slide/probes, 4 $\times$ SSC, 10 mM DTT, 5 $\times$  Denhardt's solution, 200  $\mu$ g/ml salmon sperm DNA, and 10% w/v dextran sulfate. Unbound and specifically bound probes were removed by washes with 2 $\times$  SSC for 20 min at room temperature and with 1% SSC and 0.01 SDS for 15 min at 60°C. The slides were then dried and sequentially incubated with streptavidin-FITC, mouse anti-FITC, biotinylated anti-mouse streptavidin-FITC, and finally with anti-FITC-HRP (Dako, Glostrup, Denmark). All of the incubations lasted for 30 min and were followed by a 10-min wash with cold TBS (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.01% Tween 20) on ice in the dark. After a final wash, the reactions were developed with 3-amino-9-ethylcarbazole, rinsed in water, and counterstained with hematoxylin. Nonspecific bound probe was controlled by previous digestion with 100  $\mu$ g/ml ribonuclease A and 10 U/ml ribonuclease T (Sigma, Poole, U.K.). Our study was made on activated tumor draining lymph nodes.

## Results

### Expression pattern of TLR transcripts

Based on their sequence similarity, five human cDNAs have been identified and grouped into the same gene family, namely, the TLR family (4–6). Standard Northern blot analysis has been previously performed to detect specific transcripts for TLR1 to TLR5 in human tissues. In contrast, no data are currently available regarding the expression pattern of TLR mRNA in fresh human leukocyte populations (5).

We have systematically screened different human cell types to detect specific TLR transcripts. We separated fresh human monocytes, T lymphocytes, NK cells, and PMN from peripheral blood of healthy donors. B cells were prepared from tonsils and Th1 or Th2 cells are in vitro-derived lines from human lymphocytes. Mature DC were derived in vitro from monocyte precursors (see *Materials and Methods*).

To determine whether cells activation could somehow regulate TLR mRNA levels, the cells were also treated with different stimuli. Monocytes and PMN were activated by adding LPS to the cell culture medium; T lymphocytes were treated with PHA to trigger stimulation.

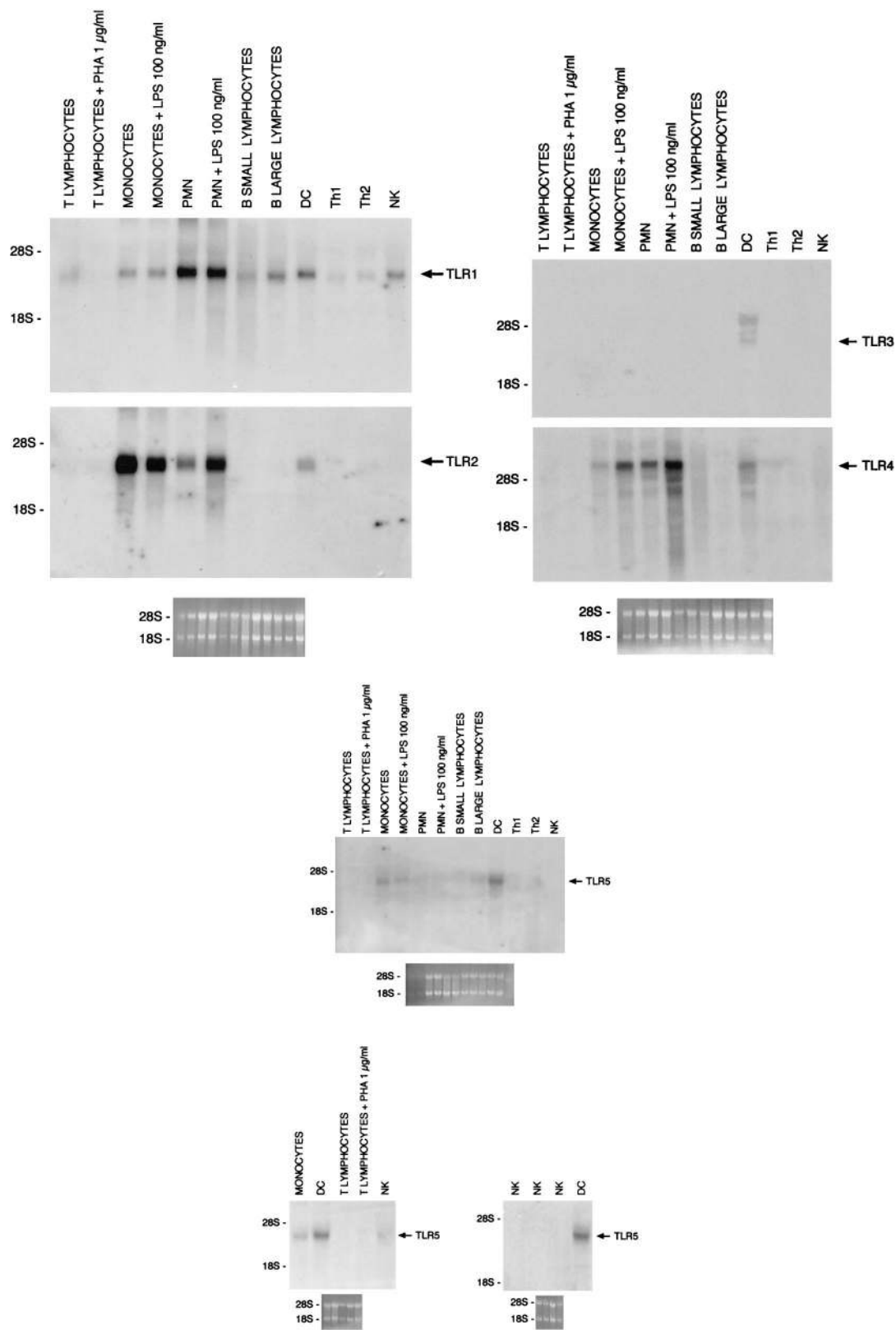
Total RNA was extracted from the cells and analyzed with Northern blot to detect specific TLR transcripts. As shown in Fig. 1, TLR1 mRNA is ubiquitously expressed. In contrast, TLR2 to TLR5 show a restricted pattern of expression; in particular TLR2, TLR4, and TLR5 are present in monocytes, PMN, and DC. To note, TLR3 is exclusively expressed by DC, but absent in all of the other leukocytes analyzed. Preliminary observations suggested that TLR4 mRNA expression can be up-regulated by LPS treatment of the cells. (Fig. 1).

### Selective regulation of TLR4 and TLR2 in monocytes and PMN

TLR2 and TLR4 have been suggested to be involved in LPS signaling. LPS as well as other pro- and anti-inflammatory signals has been shown to regulate expression of signaling components of the IL-1R and the decoy receptor (21). It was therefore of interest to assess how LPS as well as other prototypic pro- and anti-inflammatory molecules affected expression of the myeloid-restricted TLR2 and TLR4 in monocytes.

Untreated monocytes express appreciable levels of TLR4 and TLR2 transcripts in the absence of deliberate stimulation. Treatment with bacterial LPS for 3 h, significantly augmented in a dose-dependent manner TLR4 mRNA. As low as 0.1 ng/ml LPS was sufficient to increase TLR4 expression (Fig. 2A). In contrast, up to 100 ng/ml failed to regulate TLR2 expression (Fig. 3). Induction of augmented expression of TLR4 was blocked by the transcription inhibitor actinomycin D and by the protein synthesis inhibitor cycloheximide, supporting that LPS acts at different levels of regulation (Fig. 2A). On the other hand, LPS treatment of the cells induced TLR2 mRNA levels in PMN but not in monocytes (six different donors; Fig. 1 and data not shown).

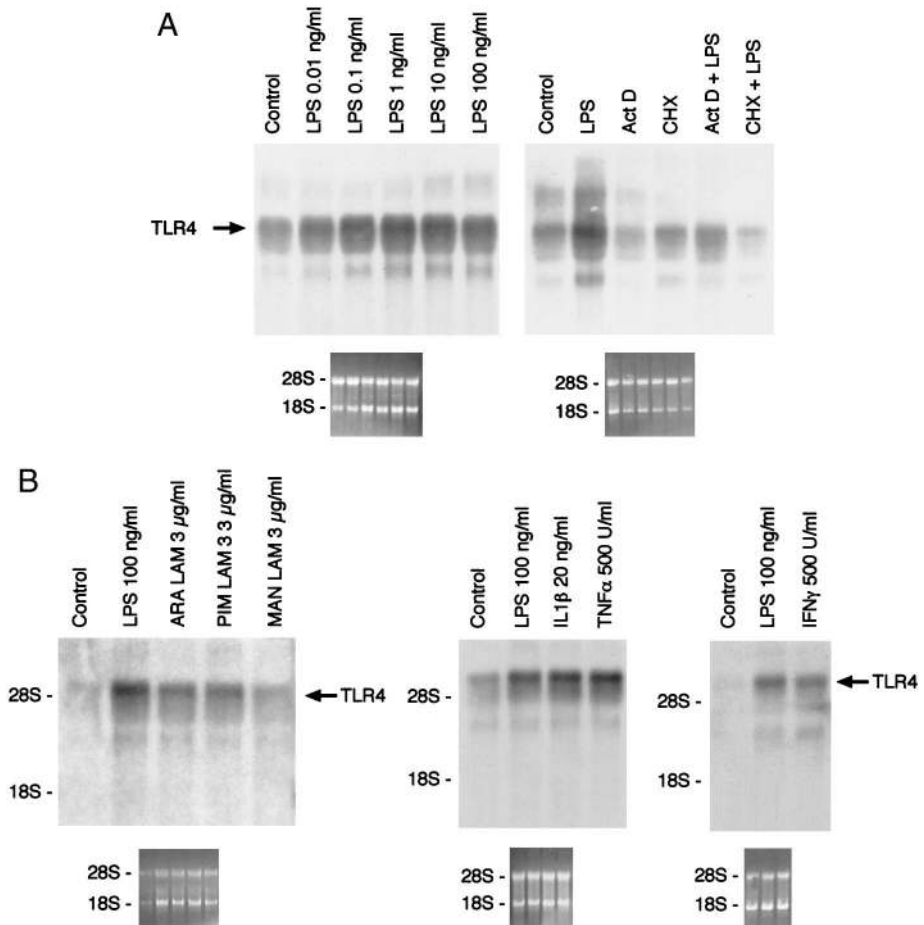
We further focused on TLR4 and analyzed whether additional bacterial components or primary inflammatory cytokines regulated its transcript levels. As shown in Fig. 2B, AraLAM from *Mycobacterium* significantly augmented TLR4 transcript levels. Furthermore, proinflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ , all induced TLR4 transcription. Qualitatively similar data were obtained for monocytes and PMN (Fig. 2B and data not shown, respectively). We next analyzed whether anti-inflammatory cytokines could revert this effect. Fresh human monocytes were incubated with IL-10 or LPS or simultaneously with both stimuli. As evident from Fig. 3, as low as 20 ng/ml IL-10 completely blocked LPS-activated



**FIGURE 1.** TLR expression in immunocompetent cells. Fresh human leukocyte subpopulations were separated and cultured in vitro in the absence or presence of the indicated stimuli for 3 h. After incubation, total RNA was extracted and Northern blot analysis was performed. Specific TLR transcripts are indicated by an arrow. The lower part of the panel shows the ethidium bromide staining after RNA transfer to the membrane. The results shown here are representative of two (TLR1 and TLR2), three (TLR3 and TLR4), or four (TLR5) independent experiments with similar qualitative results.



**FIGURE 2.** Bacterial components and proinflammatory cytokines augment TLR4 transcripts. Peripheral blood monocytes (A) or PMN (B) were separated from the blood of healthy donors and incubated with the indicated stimuli for 3 h. After incubation, total RNA was extracted and Northern blot analysis for TLR4 transcripts was performed. ActD indicates the transcription inhibitor. ActD, actinomycin D; CHX, cycloheximide. Noncapped lipoarabinomannan (AraLAM), phosphatidylinositol mannoside (PimLAM), and mannose-capped lipoarabinomannan (ManLAM) are *Mycobacterium* components (see *Materials and Methods*). The lower part of the panel shows the ethidium bromide staining after RNA transfer to the membrane. The results shown here are representative of two to three independent experiments with similar qualitative results.



TLR4 induction. In contrast, the levels of TLR2 transcript in monocytes remained unchanged in response to LPS and/or IL-10.

All in all, these observations suggest that TLR4 (in monocytes and PMN) and TLR2 (in PMN) can be regulated at sites of infection or inflammation either directly by bacterial components or indirectly by primary cytokines. In contrast, the anti-inflammatory cytokine IL-10 inhibits the effect of LPS on TLR4, but not TLR2 transcripts.

#### *TLR3 is exclusively expressed by DC*

DC are a heterogeneous system of leukocytes highly specialized in the priming of T cell-dependent immune responses. The hallmark of DC is the ability to capture pathogens and Ags of various origin, to process and present antigenic peptides, and to migrate through tissues to reach secondary lymphoid organs, where the stimulation of naive T cells takes place. Upon exposure to immune or inflammatory signals, DC undergo functional maturation and re-enter the circulatory system to home to the T cell areas of lymphoid organs. Given their central role in the switching from innate to acquired immune responses, we analyzed the expression pattern of TLRs in mature human DC vs precursor monocytes.

After culture in the presence of GM-CSF, IL-4, or IL-13 for 7 days, precursor monocytes differentiate into DC. Upon an additional exposure to inflammatory signals (such as TNF- $\alpha$ , IL-1 $\beta$ , or LPS), they undergo functional maturation (20). As shown in Figs. 1 and 4, differentiated DC express detectable levels of all of the TLR analyzed. Importantly, TLR3 was exclusively expressed by DC but absent in precursor monocytes. Moreover, the expression

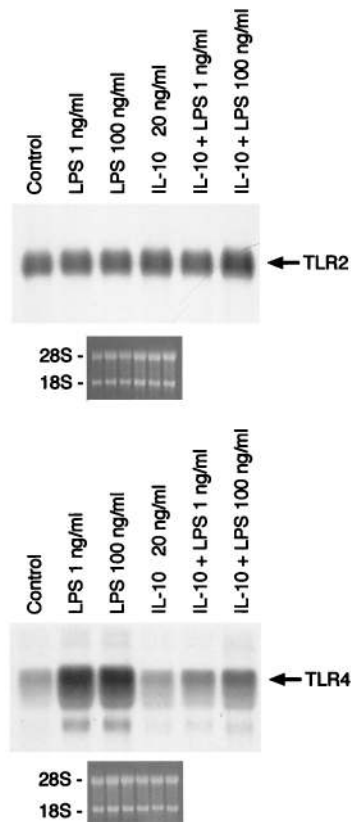
of TLR3 dramatically increased during differentiation of the cells in vitro. Finally, when DC were treated with inflammatory signals to fully mature them, TLR3 expression significantly decreased while TLR4 expression augmented (Fig. 4B); this may represent a regulatory mechanism after DC have encountered pathogens.

TLR3 mRNA expression in human DC was also investigated using in situ hybridization. In the experiments reported in Fig. 5, A and B, monocyte-derived DC were hybridized for TLR3 and by way of comparison for IL-1 $\beta$ . It was found that >95% of the cells had a strong cytoplasmic signal for both mRNAs. The study was extended to sections of human tissues. Langerhans type cells were mostly negative for TLR3 (data not shown). However, in the T cell-dependent areas of lymph nodes, several interdigitating reticulum cells with DC characteristics were positive (Fig. 5, C and D).

The distribution of TLR3-positive cells in the lymph nodes is remarkably similar to that of DC of the paracortex, as shown by CD1a staining of a sequential section (Fig. 5E) (22).

## Discussion

Despite the assumption that at least some TLR family members mediate innate immune response, very little information was available regarding their expression pattern in immunocompetent cells and no functional data are available for TLR other than TLR2 and TLR4. The existence of many of them may reflect specialized functions, redundancy, and/or differential expression and roles in different cell types. Herein, we have characterized the pattern of mRNA expression of the first five TLR.

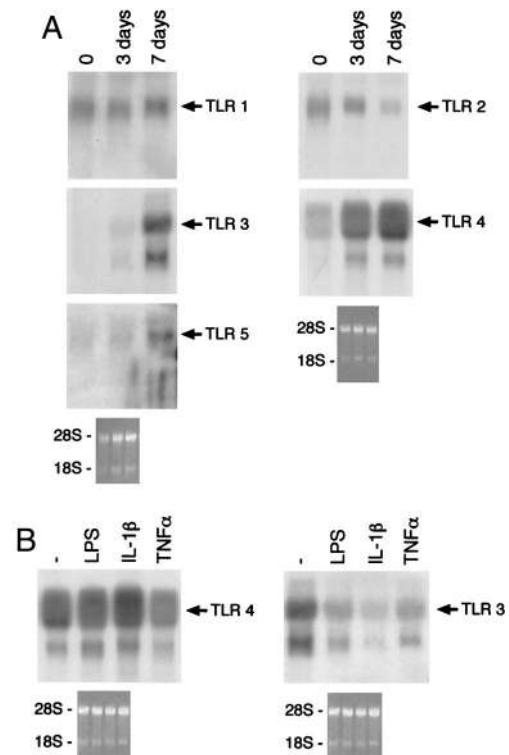


**FIGURE 3.** LPS and IL-10 divergently regulate TLR4 but not TLR2 mRNA levels. Monocytes were separated from blood of healthy donors and treated with LPS with or without simultaneous addition of hrIL-10. After 3 h, total RNA was extracted and Northern blot analysis was performed. Specific TLR2 and TLR4 transcripts are indicated by an arrow. The lower part of the panel shows the ethidium bromide staining after RNA transfer to the membrane. The results shown here are representative of two independent experiments with similar qualitative results.

We separated fresh human monocytes, NK cells, PMN, B cells, T lymphocytes, Th1 or Th2 lymphocytes, and monocyte-derived DC. Total RNA was extracted from the cells and analyzed by Northern blot to detect specific TLR transcripts. To note, TLR1, TLR2, and TLR4 probes allowed a signal detection on the filter only after a few hours of autoradiography. On the other hand, TLR3 and TLR5 probes required at least an overnight exposure of the filter to evidence a specific transcript, suggesting that distinct TLR transcripts may be produced at different levels; however, the levels of receptor expression will also depend on the stability of the protein so that availability of specific Abs will permit a definitive quantitative analysis of TLR expression in different cell types.

The results presented here show that the first characterized five TLR family members show differential expression and regulation of their specific transcripts in human leukocyte populations. TLR1 is expressed in all subsets examined. No significant regulation of its expression was observed, except for the down-regulation of specific transcripts in T cells after exposure to PHA. TLR2, TLR4, and TLR5 were only present in myelomonocytic cells and are undetectable in lymphoid subsets, resting or activated. In one of six different donors, TLR5 messenger was barely detectable in NK cells.

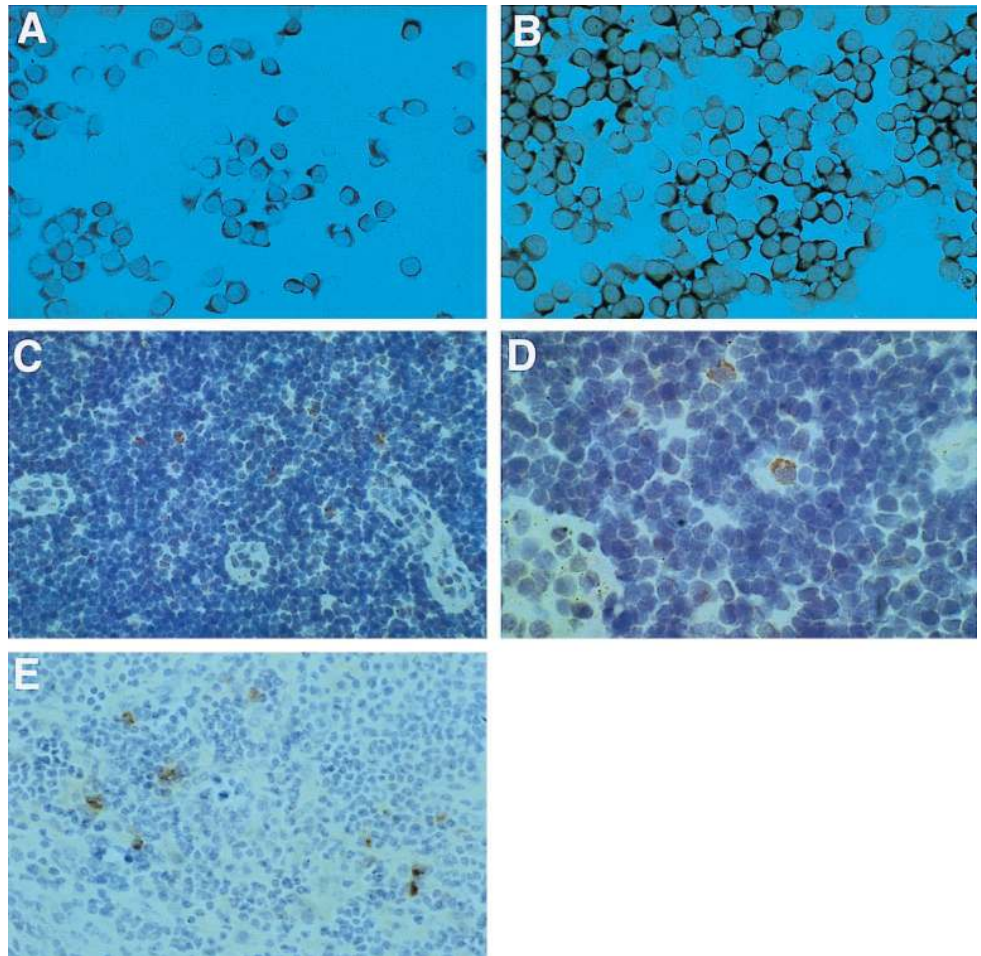
When regulation was examined, TLR4 was found to be increased by bacterial products and primary proinflammatory cytokines. Exposure to bacterial products, such as LPS or lipopolysaccharide,



**FIGURE 4.** TLR3 is exclusively expressed by monocyte-derived DC. DC were derived from monocytes after in vitro culture for 7 days (see *Materials and Methods*). Total RNA was extracted from cells at different stages of differentiation and analyzed for TLR expression: precursor monocytes (indicated as 0), 3-day cultured monocytes (indicated as 3 days), and 7-day cultured differentiated DC (indicated as 7 days) are shown in A. After differentiation, DC were induced to maturation with TNF- $\alpha$ , IL-1 $\beta$ , or LPS treatment, and total RNA was extracted. Northern blot analysis was performed to detect specific TLR3 and TLR4 transcripts (B). The lower parts of A and B show the ethidium bromide staining after RNA transfer to the membrane. The results shown here are representative of three independent experiments with similar qualitative results.

mannan, or to proinflammatory cytokines, increased TLR4 expression in monocytes and PMN, whereas IL-10 blocked this effect. In contrast, TLR2 was unaffected by these pro- and anti-inflammatory signals in monocytes but it was augmented in PMN. All in all, these observations suggest that TLR4 (in monocytes and PMN) and TLR2 (in PMN) expression can be regulated at sites of infection or inflammation, either directly by bacterial components or indirectly by primary cytokines. It should be noted that TLR4 is a component of the receptor complex for Gram-negative bacteria (13); on the other hand, TLR2 may be more specifically involved in the signaling receptor for Gram-positive bacteria (13, 14). Intriguingly, our data show that the levels of expression of TLR4 and TLR2 are differentially regulated in monocytes, supporting the hypothesis that eventual responsiveness of the cells to distinct bacterial components may be modulated by external stimuli. The present findings with LPS and TLR4 confirm and extend our own preliminary data (16). It has been previously reported that TLR4 expression is inhibited by LPS in a mouse cell line (7). It is unclear whether this divergence reflects species or cell differences. Given the structural and functional relation of TLR with IL-1 receptors, it is of interest that pro- and anti-inflammatory signals have been shown to have reciprocal and divergent effects on signaling components of the IL-1 receptor complex and on the decoy receptor (21).

**FIGURE 5.** In situ hybridization: expression of TLR3 mRNA in monocyte-derived DC and lymphatic tissues. TLR3 mRNA was expressed by the majority of the 7-day cultured monocyte-derived DC (see *Materials and Methods*). *A* and *B*, TLR3 and IL-1 $\beta$  expression, respectively. In lymph nodes, TLR3-positive cells were scattered in the paracortical areas (*C*) and show the morphology of classical DC (*D*). These same DC are also positive for CD1a (*E*). In situ hybridization with biotin-labeled probes, development with streptavidin-FITC, and anti-FITC-AP, and B-CIP in dark blue, no counterstain (monocyte-derived DC; *A* and *B*), or anti-FITC-HRP and 3-amino-9-ethylcarbazole in red, counterstain in hematoxylin (lymph node; *C–E*). Original magnifications: *A* and *B*,  $\times 600$ ; *C* and *E*,  $\times 400$ ; and *D*,  $\times 1000$ .



TLR3 transcripts were selectively expressed in human DC both in vitro and in vivo. As assessed by in situ hybridization, most Langerhans cells in the skin did not express TLR3; on the other hand, TLR3 expressing DC were clearly detectable in the T cell areas of lymph nodes. DC are heterogeneous in terms of ontogeny, marker phenotype, and function (23). In particular, the monocyte-derived DC used for the present in vitro studies clearly differ from Langerhans cells in many respects, including lack of Birbeck granules, chemokine receptor expression, and expression of the mannose receptor (23, 24). The expression of TLR3 in DC of different origin and function will need to be investigated in detail.

Interestingly, in vitro experiments showed that TLR3 expression was inhibited upon exposure to LPS or proinflammatory cytokines that induce functional maturation. Therefore, DC are unique in that they express TLR3 and have the whole repertoire of five characterized TLRs. This full repertoire may reflect the unique role of DC in sensing pathogens and causing transition from innate to specific immunity.

Collectively, these data suggest that it may be useful to classify TLR based on their mRNA expression pattern as ubiquitous (TLR1), restricted, (TLR2, TLR4, and TLR5), and specific (TLR3) molecules.

## Acknowledgments

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