The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2002

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J Immunol (2002) 168 (2): 554–561.
https://doi.org/10.4049/jimmunol.168.2.554

Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines

Kol A. Zarember and Paul J. Godowski[1]

Members of the Toll-like receptor (TLR) family mediate dorsoventral patterning and cellular adhesion in insects as well as immune responses to microbial products in both insects and mammals. TLRs are characterized by extracellular leucine-rich repeat domains and an intracellular signaling domain that shares homology with cytoplasmic sequences of the mammalian IL-1 receptor and plant disease resistance genes. Ten human TLRs have been cloned as well as RP105, a protein similar to TLR4 but lacking the intracellular signaling domain. However, only five TLRs have described functions as receptors for bacterial products (e.g., LPS, lipoproteins). To identify potential sites of action, we used quantitative real-time RT-PCR to examine systematically the expression of mRNAs encoding all known human TLRs, RP105, and several other proteins important in TLR functions (e.g., MD-1, MD-2, CD14, MyD88). Most tissues tested expressed at least one TLR, and several expressed all (spleen, peripheral blood leukocytes). Analysis of TLR expression in fractionated primary human leukocytes ($CD4^+$, $CD8^+$, $CD19^+$, monocytes, and granulocytes) indicates that professional phagocytes express the greatest variety of TLR mRNAs although several TLRs appear more restricted to B cells, suggesting additional roles for TLRs in adaptive immunity. Monocyte-like THP-1 cells regulate TLR mRNA levels in response to a variety of stimuli including phorbol esters, LPS, bacterial lipoproteins, live bacteria, and cytokines. Furthermore, addition of *Escherichia coli* to human blood ex vivo caused distinct changes in TLR expression, suggesting that important roles exist for these receptors in the establishment and resolution of infections and inflammation. *The Journal of Immunology,* **2002, 168: 554–561.**

The highly specific adaptive immune system requires days to weeks to refine Igs and cell-mediated immune recognition systems to eliminate invading pathogens. In contrast, genome-encoded innate immune systems target structurally conserved pathogen-associated microbial products (PAMPs),[2] thereby allowing immediate and in most cases sufficient responses to limit or eradicate invading microbes. At least 10 human Toll-like receptors (TLRs) have been cloned (1–5), and some have been shown to mediate cellular responses to PAMPs (6). TLR2 has been reported to signal the presence of bacterial lipoproteins (7) and lipoteichoic acids (8). A polymorphism in TLR2 in humans has been associated with increased susceptibility to infection with *Staphylococcus aureus* (9); TLR2 knockout mice are similarly susceptible (10) and demonstrate the role of TLR2 in responses to bacterial lipoproteins (10). TLR4 is a signal-transducing receptor for LPS in cultured cells, and TLR4 mutant mice are hyporesponsive to LPS (11), as are humans with a polymorphic allele of TLR4 (12). TLR4 forms a complex with MD2, a secreted leucine-rich repeat domain-containing protein that is required for surface expression and LPS-regulated activation of TLR4 (13, 14). In addition to sensing LPS, TLR4 mediates signals initiated by exogenous compounds (e.g., Taxol) (15), respiratory syncytial virus protein F (16), and several endogenous proteins that may be present during inflammation such as heat shock protein 60 (17) and the proinflammatory extra domain A of fibronectin (18). A TLR-related protein, called RP105, was identified in B cells and acts as both an LPS sensor and a regulator of B cell proliferation (19). Like TLR4, RP105 requires an MD2-related protein, MD1, for its surface expression (20, 21). TLR9 detects unmethylated CpG motifs that occur in microbial DNA and act as immune activators (22). TLR5 is a signaling mediator of bacterial flagellin (23, 24) and may play a role in resistance to *Salmonella* infection (25). Cotransfection of different TLRs may lead to either enhancement or inhibition of recognition of particular PAMPs (26, 27), suggesting that cellular responses to PAMPs are dependent on the total repertoire of TLRs displayed on a cell, necessary cofactors, and the levels of each protein present.

Activation of most TLRs leads to recruitment of MyD88, a homolog of *Drosophila* Tube that contains an intracellular signaling domain and a death domain (28). MyD88 interacts with IL-1 receptor-associated kinase, leading to initiation of a signal transduction cascade culminating in nuclear translocation of NF-κB family members and altered gene expression. In addition to MyD88-mediated signaling, TLR2 has been shown to interact with the Rac1 GTPase, thereby initiating a signaling cascade that also results in NF-κB translocation and possibly other nontranscriptional cellular responses (29). Chimeras with intracellular signaling domains of TLRs coupled to the extracellular domain of CD4 demonstrate that many TLRs can cause NF-κB transcriptional activation (1, 4).

Department of Molecular Biology, Genentech Inc., South San Francisco, CA 94080

Received for publication June 7, 2001. Accepted for publication November 7, 2001.

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[1] Address correspondence and reprint requests to Dr. Paul J. Godowski, Department of Molecular Biology, Genentech, Inc., Mailstop 37, One DNA Way, South San Francisco, CA 94080. E-mail address: ski@gene.com

[2] Abbreviations used in this paper: PAMP, pathogen-associated microbial product; TLR, Toll-like receptor; BLP, bacterial lipopeptide; sBLP, synthetic bacterial lipopeptide.

0022-1767/02/$02.00

To explore settings in which human TLRs may play a role, we surveyed the expression of all known TLR mRNAs in normal human tissues and in cells activated by microbial or inflammatory compounds. The results suggest that dynamic regulation of TLR expression may be an important consideration in the elucidation of TLR biology.

Materials and Methods

Mammalian and bacterial cells

The monocytic leukemia cell line THP-1 was grown in RPMI 1640 with 10% FCS, 10 mM HEPES, 2 mM glutamine, penicillin, and streptomycin (100 U/ml each) at 37°C in a 5% CO_2 atmosphere. *Escherichia coli* (ATCC 4157) and *S. aureus* (ATCC 12598) were stored at −80°C as DMSO stocks and grown in Luria broth. Log phase subcultures were grown at 37°C, washed in PBS, and quantitated turbidometrically.

Stimuli

THP-1 cells were differentiated with 10 ng PMA/ml for 12–18 h. LPS was purchased from List Biological Laboratories (Campbell, CA) and re-extracted to remove contaminating endotoxin proteins as described (30). Stimulation of cells was performed in growth medium supplemented with 10% FCS as a source for LPS-binding protein. Synthetic bacterial lipopeptide (sBLP; *N*-α-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-L-cysteine-L-serine-L-lysine$_4$), was a gift from A. Zychlinsky (New York University School of Medicine, New York, NY). Cytokines were purchased from Calbiochem (La Jolla, CA) or Oncogene Research Products (San Diego, CA).

Taqman

Commercially available cDNA pools (Clontech Laboratories, Palo Alto, CA) were prepared by the manufacturer from DNase-treated poly(A)-selected RNA purified from tissues of healthy adults (Table I). PCR using primers designed to amplify a 943-bp TNF-β product from genomic templates and a 610-bp TNF-β product from cDNA did not detect any 943-bp products after 40 cycles. Pools of cDNA were normalized simultaneously to four separate housekeeping genes (α-tubulin, β-actin, G3PDH, and phospholipase A_2) by the manufacturer. RNA used in Figs. 3–5 were prepared from cells using a Qiagen (Valencia, CA) Mini or Midi RNA-easy kit and random-primed cDNA prepared using the Clontech reverse transcription kit as per manufacturer's instructions. DNase treatment was included for isolation of RNA from primary cells. Taqman probe/primer combinations used in these studies were identified using the Primer Express software package (Applied Biosystems, Foster City, CA) and are presented in Table II. All primers were stored at a concentration of 10 OD/ml, and 0.05 μl of each forward and reverse primer as well as 0.1 μl of the probe were used per 25-μl reaction. Taqman PCR was performed in 25-μl volumes using AmpliTaq Gold polymerase and universal reaction buffer with 5.5 mM $MgCl_2$ (Applied Biosystems). Threshold cycle numbers (*Ct*) were determined with Sequence Detector Software (version 1.6; Applied Biosystems) and transformed using the Δ*Ct* or ΔΔ*Ct* methods as described by the manufacturer using *GAPDH* or *RPL19* as the calibrator genes where indicated.

Table I. *Sources of RNA used for cDNA analysis*

Tissue	Source	Age (yr)
PBL[a]	550 M/F	18–40
Heart	8 M/F	25–59
Liver	1 M	41
Spleen	5 M/F	44–70
Ovary	5 F	30–60
Lung	2 F	24–32
Kidney	8 M/F	24–55
Testis	25 M	28–64
Thymus	9 M/F	18–32
Placenta	7 F	22–35
Skeletal muscle	27 M/F	20–60
Colon	20 M/F	17–76
Brain	2 M	43 and 55
Small intestine	11 M/F	15–60
Prostate	20 M	20–58
Pancreas	20 M/F	25–59
Leukocytes		
$CD4^+$	20 M/F	25–50
$CD8^+$	33 M/F	24–51
$CD14^+$	36 M/F	25–54
$CD19^+$	34 M/F	24–54

[a] PBL, peripheral blood leukocytes.

Purification of peripheral blood leukocytes

Human venous blood was obtained after informed consent and was anticoagulated with citrate. Blood was placed in sterile 50-ml polypropylene tubes and incubated for 6 h either with buffer alone or 1×10^5 *E. coli*/ml at 37°C in a humidified 5% CO_2 atmosphere with intermittent mixing. Blood was then mixed with 2 volumes of ice cold HBSS(−), overlaid on Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged for 40 min at 400 × *g* at 20°C. Mononuclear cells were aspirated from the interface and washed in HBSS(−) supplemented with 0.5% human serum albumin. The RBC/granulocyte phase was subjected to isotonic RBC lysis using 10 volumes of 123 mM NH_4Cl, 8 mM $KHCO_3$, and 25 μM EDTA. After centrifugation, granulocytes were resuspended in lysis buffer followed with 4 volumes of HBSS(−) for a final wash. Washed mononuclear cells were further subjected to positive selection using anti-CD14-conjugated paramagnetic beads and purified using the AutoMACs system per manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

Immunoprecipitation of TLRs from THP1 cells

THP-1 cells were treated as described above and then extracted in lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 25 mM HEPES, pH 7.2) supplemented with 50 μg/ml PMSF for 1 h at 4°C, and then centrifuged for 10 min at top speed in an Eppendorf microfuge (Beckman Coulter, Fullerton, CA). Supernatants were incubated rotating at 4°C with 5 μg mouse mAbs against human TLR1 (mAb 2736) or TLR4 (mAb 2723) per 500 μl detergent extract with 25 μl protein A/G beads (Pierce, Rockford, IL). Beads were washed three times with lysis buffer and then boiled in 6× SDS-PAGE sample buffer, and the eluted proteins were resolved on a Novex 4–12% SDS-PAGE gel and silver stained using the SilverSNAP kit (Pierce).

Results

TLRs and associated molecules in adult tissues

Distribution of human TLR mRNA in adult tissues was assayed by Taqman, a real-time fluorescence PCR technique that provides sensitive detection and quantitative enumeration of sequences over at least 7 orders of magnitude of starting number. Human tissue cDNA pools were prenormalized by the vendor (Clontech) to four housekeeping genes and shown to be devoid of contaminating genomic DNA as described in *Materials and Methods*. To facilitate comparison between tissues, all samples were normalized to the expression of the particular TLR in spleen (spleen = 1). As shown in Fig. 1, tissues involved in immune function (e.g., spleen, peripheral blood leukocytes) displayed the most diverse repertoire of TLR mRNAs, as did those expected to encounter microbes (e.g., lung, small intestine, colon). TLR3 and TLR5 were expressed ubiquitously, whereas RP105 and TLR10 were more restricted.

Comparison of TLR expression in fractionated peripheral blood leukocytes

Because peripheral blood cells expressed high levels of TLR mRNAs compared with other tissues, we next examined TLR expression in purified subpopulations containing >95% pure $CD4^+$, $CD8^+$, $CD14^+$, or $CD19^+$ leukocytes. The cDNAs were prenormalized as above, and the data were normalized to the expression of the target sequence in the $CD14^+$ population. Although expression of MyD88 was similar in all cell types (±2.5-fold of the average), relative expression of specific TLR mRNAs varied up to 3 orders of magnitude (Fig. 2). Several TLRs (e.g., TLR9, TLR10, and RP105) were expressed at highest levels in $CD19^+$ B cells,

Table II. *Oligonucleotide primers and probes used for Taqman analysis*[a]

cDNA	Forward	Probe	Reverse
TLR1	CAGTGTCTGGTACACGCATGGT	TGCCCATCCAAAATTAGCCCGTTCC	TTTCAAAAACCGTGTCTGTTAAGAGA
TLR2	GGCCAGCAAATTACCTGTGTG	TCCATCCCATGTGCGTGGCC	AGGCGGACATCCTGAACCT
TLR3	CCTGGTTTGTTAATTGGATTAACGA	ACCCATACCAACATCCCTGAGCTGTCAA	TGAGGTGGAGTGTTGCAAAGG
TLR4	CCAGTGAGGATGATGCCAGAAT	TGTCTGCCTCGCGCCTGGC	GCCATGGCTGGGATCAGAGT
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAGGGCAGGTGCTTATCTGACCTTAACA	CCAACCACCACCATGATGAG
TLR6	GAAGAAGAACAACCCTTTAGGATAGC	TGCAACATCATGACCAAAGACAAAGAACCT	AGGCAAACAAAATGGAAGCTT
TLR7	TTAACCTGGATGGAAACCAGCTA	AGAGATACCGCAGGGCCTCCCG	TCAAGGCTGAGAAGCTGTAAGCTA
TLR8	TGTTATGACAGCAGAGGGTGATG	TGACCCCAGCCACAACGACACT	GAGTTGAAAAAGGAGGTTATAGGATAAATC
TLR9	GGACCTCTGGTACTGCTTCCA	ACGATGCCTTCGTGGTCTTCGACAAA	AAGCTCGTTGTACACCCAGTCT
TLR10	GGAGGCTGAGGCAGGAGAAT	CTTGAACCCGGGAGGTGGCAGTT	GTGGCTCGATCTCAGCTCACT
RP105	CTGGACTGCACTTGCTCGAATAT	CACACGTGGTCTCCTCCGAGCTT	CACAGGAAAGCTTGACATCAGATAG
MYD88	GAGCGTTTCGATGCCTTCAT	TGCTATTGCCCCAGCGACATCCA	CGGATCATCTCCTGCACAAA
MD1	TAGCTCTCATGTCTCAAGGCTCAT	CCCATCTGTGAGGCGGCTCTGC	ATCTGCTCTCCTTTCCTTCTTCC
MD2	ATTCCAAGGAGAGATTTAAAGCAATT	TCAATCTCTATATAACTGTCAACACCATGAATCTTCCAAA	CAGATCCTCGGCAAATAACTTCTT
CD14	CGCTCCGAGATGCATGTG	TCCAGCGCCTGAACTCCCTCA	TTGGCTGGCAGTCCTTTAGG
GAPDH	GAAGGTGAAGGTCGGAGTC	CAAGCTTCCCGTTCTCAGCC	GAAGATGGTGATGGGATTTC

[a] The software package Primer Express (Applied Biosystems) was used to identify appropriate Taqman primer sets. All forward and reverse probes were chosen to have a melting temperature of ~60°C, and the probes to have a melting temperature of ~70°C. Probes were selected that had a greater number of cytosine than of guanine nucleotides. Amplification primers were further restricted to those containing at least 60% A/T in the 3′ ends. All probes were labeled on the 5′ end with 6-carboxyfluorescein (FAM) as a reporter and on the 3′ end with 6-carboxytetramethylrhodamine (TAMRA) as the quenching dye with the exception of the GAPDH probe which was labeled on the 5′ end with 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE). All sequences are presented in the 5′→3′ direction.

whereas others (e.g., TLR2, TLR4, and TLR8) were expressed maximally in $CD14^+$ mononuclear cells. These findings have been reproduced independently using RNA prepared from leukocytes purified by magnetic immune selection (data not shown.)

Regulation of TLR mRNAs by purified PAMPs and cytokines

Because $CD14^+$ cells expressed the widest variety of TLRs at high levels and are thought to play primary roles in the initial phases of the innate immune response, we initially focused on identifying agents that alter the expression of TLR mRNA in the monocyte-like leukemic THP-1 cell line.

Differentiation of THP-1 by treatment with PMA increases their sensitivity to LPS by several orders of magnitude as measured in an assay of TNF production (data not shown). To determine whether enhanced sensitivity to LPS could be due to changes in TLR expression, we analyzed by Taqman the expression of TLRs and associated molecules in undifferentiated and PMA-treated THP-1 cells (Fig. 3*A*). In addition to significant up-regulation of the LPS receptor TLR4, RP105 was also significantly up-regulated in these cells as were the mRNAs for CD14, MD2, and MyD88 and other TLRs not known to be involved in LPS signaling (e.g., TLR1, TLR6, TLR7, TLR8). Treatment of THP-1 cells with LPS altered expression of several TLR mRNAs in both the presence and the absence of PMA differentiation. For example, LPS treatment increased mRNA levels of TLR 1–8 but not TLR9 or TLR10 mRNAs. Interestingly, the 90-fold PMA-induced increase in RP105 expression was abolished by treatment with LPS. MyD88 mRNA was up-regulated by LPS and by PMA, presumably to accommodate the increased amounts of TLR proteins synthesized in these cells. Fig. 3*B* demonstrates that for TLR1 and TLR4, protein expression correlates with mRNA expression shown in Fig. 3*A*.

The effects of both PMA and LPS on regulation of TLR message levels may be due either to direct effects or to autocrine stimulation by secreted factors such as cytokines. To test this possibility, we treated PMA-differentiated THP-1 cells with several different cytokines and performed Taqman analysis after 6 h of treatment. As shown in Fig. 3*C*, all agents tested had measurable effects on the expression of TLR mRNAs. TLR8 expression, although moderately responsive to all stimuli tested, increased >40-fold with IFN-γ treatment. IL-6, which also increased expression of all TLRs, increased TLR7 expression >12-fold. Thus, a variety of cytokines induced by microbial products such as LPS may also alter TLR expression. Remarkably, specific agonists of TLR2 and TLR4 (sBLP and LPS, respectively) differentially altered the expression of other TLRs. For example, whereas TLR3 is up-regulated by LPS, it is repressed by sBLP. Also, whereas bacterial lipopeptide down-regulated RP105 by 2-fold, LPS down-regulated RP105 mRNA by 100-fold, suggesting that the consequences of activation of different TLRs are distinct.

To identify changes in TLR expression in response to a more physiological stimulus, we coincubated PMA-differentiated THP-1 cells with live bacteria. As shown in Fig. 4, both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria altered expression of several TLRs. Although TLR2, -5, -7, -8, and -10 appeared to be similarly affected, expression of TLR3 was more responsive to Gram-positive bacteria (7-fold) than to Gram-negative bacteria (~2-fold). RP105 was down-regulated >30-fold by *E. coli*, whereas *S. aureus* caused <10-fold down-regulation.

Changes in TLR expression levels in response to microbial challenge of primary leukocytes

Innate immune responses in vivo are orchestrated through the activation of a variety of cellular and humoral systems that may have

	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	MD1	MD2	RP105	MyD88
brain	0.005	0.013	0.020	0.088	0.122	n.d.	0.143	n.d.	0.133	0.025	0.007	0.003	n.d.	0.019
colon	0.012	0.022	0.305	0.112	0.255	0.012	0.149	0.020	0.088	0.023	0.003	0.013	0.004	0.219
heart	0.004	0.014	0.094	0.082	0.255	0.002	0.020	0.002	0.037	0.001	0.001	0.014	n.d.	0.023
ovary	0.057	0.253	0.482	0.190	12.701	0.026	0.281	0.010	0.446	0.034	0.040	0.541	0.007	0.137
kidney	0.023	0.030	0.624	0.046	1.075	0.006	0.028	0.001	0.046	0.001	0.001	0.007	n.d.	0.114
PBL	2.391	4.548	0.049	1.348	2.263	1.472	1.026	2.781	0.305	0.074	0.341	0.447	0.004	1.459
Liver	0.014	0.093	0.165	0.028	0.286	0.008	0.008	0.020	0.025	0.001	0.004	0.014	0.000	0.154
prostate	0.020	0.098	0.618	0.058	2.147	0.055	0.187	n.d.	0.144	0.012	0.006	0.018	0.000	0.254
lung	0.775	0.890	1.656	0.475	2.749	0.257	1.170	1.320	0.187	0.028	0.298	0.242	0.013	0.911
s. intestine	0.014	0.050	0.542	0.111	0.992	n.d.	0.132	n.d.	0.153	0.026	0.006	0.060	0.022	0.164
pancreas	0.198	0.253	1.627	0.075	2.256	0.016	0.177	0.037	0.109	0.028	0.017	0.062	0.000	0.301
spleen	1.016	1.006	1.042	1.036	1.002	1.012	1.017	0.811	1.023	1.010	1.004	1.039	1.017	1.016
placenta	0.162	0.197	4.996	0.233	1.750	0.050	1.784	0.696	0.054	0.007	0.059	0.276	0.018	0.305
testis	0.019	0.050	1.605	0.043	1.369	0.006	0.144	0.142	0.078	0.003	0.003	0.034	0.000	0.241
sk. muscle	0.000	0.001	0.002	0.003	0.009	n.d.	n.d.	0.005	0.021	0.000	0.000	0.000	n.d.	0.001
thymus	0.060	0.053	0.074	0.056	0.541	0.047	0.251	0.014	0.289	0.058	0.018	0.030	0.004	0.373

FIGURE 1. Real-time quantitative PCR (Taqman) analysis of expression of TLRs in human tissues. Taqman was performed on prenormalized cDNAs derived from poly(A)-selected DNase-treated RNAs purified from pools of healthy human tissues described in Table I. Reactions were performed in three to six wells, and the results are expressed relative to expression levels of each transcript in spleen. n.d., Not detected (or detected $<$50% wells); s., small; sk., skeletal; PBL, peripheral blood leukocytes.

both positive and negative effects on cellular activation. To test whether the changes seen in TLR expression in simple in vitro settings were mirrored in more complex ex vivo settings, human venous blood was anticoagulated with citrate and incubated with 1×10^5 *E. coli* per ml for 6 h. RNA was isolated from monocytes and granulocytes and analyzed for TLR expression. As shown in Fig. 5*A*, mRNA expression levels for TLR2, TLR3, and MyD88 were similar whereas granulocytes express more TLR1, -4, -6, and -8–10. In contrast, monocytes express more TLR5 and almost 60 times more TLR7 than granulocytes. The expression of RP105 and its chaperone MD1 is $>$100-fold higher in monocytes than in granulocytes, whereas expression of TLR4 and its chaperone MD2 are 5- to 10-fold lower in monocytes than in granulocytes.

Treatment of whole blood with *E. coli* resulted in significant changes in the expression of several TLRs and associated molecules. TLR1, TLR6, and TLR9 were down-regulated in both cell types, whereas TLR3, TLR7 and MyD88 were up-regulated. Both TLR8 and TLR10 were up-regulated in monocytes but down-regulated in granulocytes. Microbe-induced differential regulation of TLR mRNA by phagocytes in whole blood suggests active modification of cellular responsiveness of these cells during the course of infection.

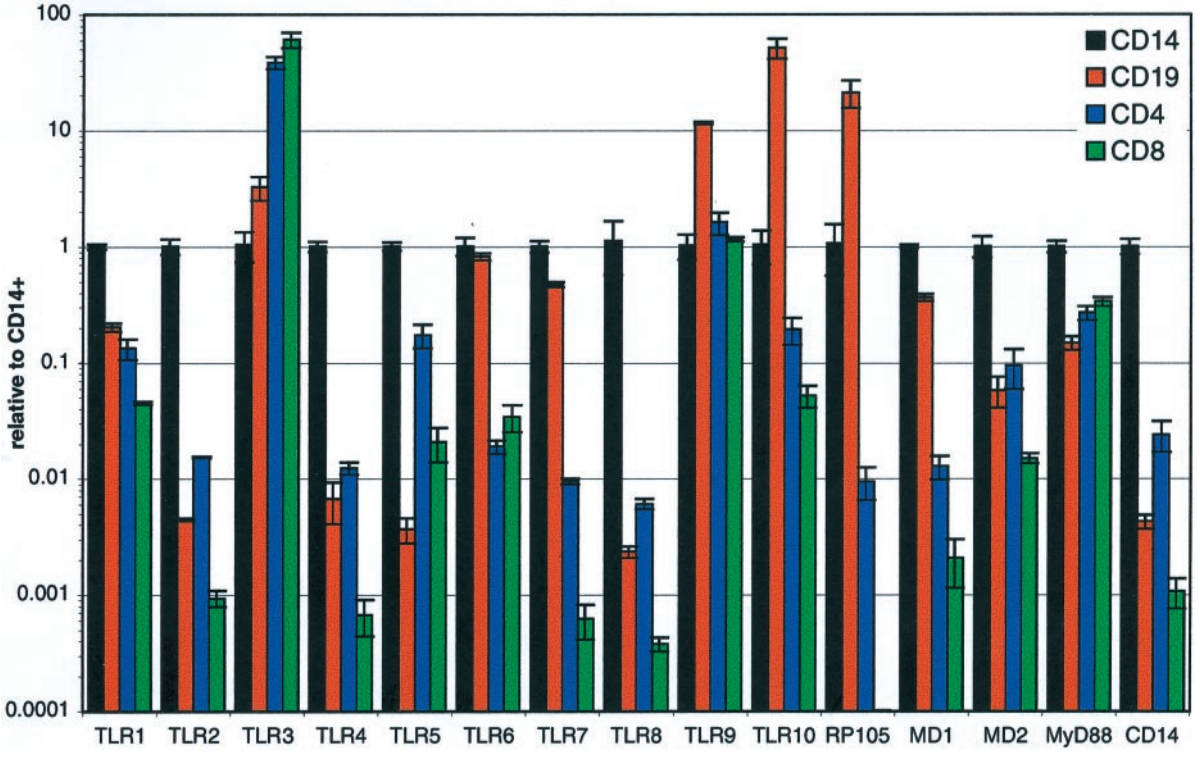
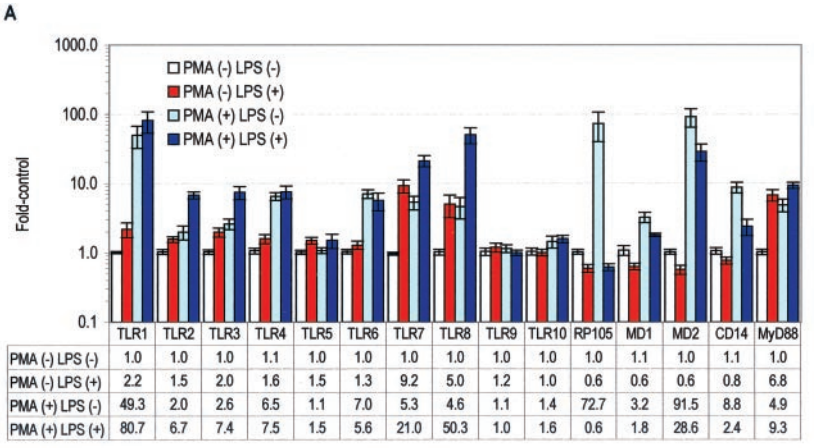
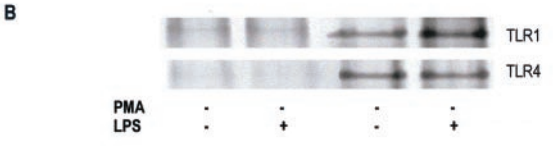


FIGURE 2. Analysis of TLR expression in leukocyte subpopulations. Taqman was performed on prenormalized cDNAs prepared from $CD14^+$ monocytes (36 donors), $CD19^+$ B cells (34 donors), $CD4^+$ T helper/inducer cells (20 donors), and $CD8^+$ T suppressor/cytotoxic cells (33 donors). All cDNAs were assayed in triplicate; results are expressed relative to expression in $CD14^+$ monocytes $\pm$ SD.



	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	RP105	MD1	MD2	CD14	MyD88
PMA (-) LPS (-)	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0
PMA (-) LPS (+)	2.2	1.5	2.0	1.6	1.5	1.3	9.2	5.0	1.2	1.0	0.6	0.6	0.6	0.8	6.8
PMA (+) LPS (-)	49.3	2.0	2.6	6.5	1.1	7.0	5.3	4.6	1.1	1.4	72.7	3.2	91.5	8.8	4.9
PMA (+) LPS (+)	80.7	6.7	7.4	7.5	1.5	5.6	21.0	50.3	1.0	1.6	0.6	1.8	28.6	2.4	9.3



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	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	RP105	MD1	MD2	CD14	MyD88
IL-1B	0.81	1.47	0.73	0.84	1.77	0.62	**3.19**	**2.89**	1.36	1.01	0.53	1.05	0.40	0.58	0.86
IL-6	**3.07**	**2.65**	2.09	1.89	**3.60**	2.50	**12.59**	**4.05**	1.90	2.04	**3.11**	**2.55**	**3.87**	**13.85**	**2.58**
IL-10	1.03	1.22	1.10	1.03	**2.52**	0.73	**2.59**	**4.78**	0.82	0.66	1.07	1.06	0.82	**2.61**	0.81
TNFα	1.11	**2.63**	1.21	0.74	**2.71**	1.08	**4.69**	**2.65**	1.69	1.16	0.62	1.40	1.03	0.53	1.34
IFNγ	1.75	1.98	0.67	1.96	0.54	1.14	**3.59**	**42.05**	0.80	1.21	0.39	0.76	1.89	**5.38**	1.99
sBLP	0.95	1.38	0.39	0.62	**2.81**	0.50	1.56	**8.76**	1.00	0.74	0.47	0.65	0.37	0.73	0.79
LPS	1.64	**3.40**	**2.87**	1.16	1.40	0.80	**3.97**	**10.90**	0.86	1.10	**0.01**	0.56	0.31	0.27	1.92

FIGURE 3. TLR mRNA expression is responsive to inflammatory stimuli in THP1 cells. *A*, THP-1 cells were treated at 1–2 × 10^6/ml in growth medium with 10 ng/ml PMA for 16–20 h, followed by treatment with *E. coli* J5 LPS at 10–100 ng/ml for 6 h. Taqman analysis was performed in triplicate, and results were normalized to GAPDH expression and expressed relative to untreated control cells. Data represent the mean of two to three independent experiments ± SEM. *B*, THP-1 cells were treated as indicated with PMA or LPS as in *A* and then extracted and immunoprecipitated as described in *Materials and Methods* with Abs to TLR1 or TLR4. The resulting immunoprecipitates were resolved by SDS-PAGE and detected by silver stain. *C*, PMA-differentiated THP-1 cells were treated with IL-1β, IL-10, TNF-α, and IFN-γ at concentrations of 20 ng/ml and IL-6, LPS, and sBLP at 10 ng/ml. Shaded squares indicate an up-regulation of >2.5-fold, and the only transcript down-regulated >2.5-fold (RP105) is shown in bold. Data are relative to untreated PMA-differentiated controls and are normalized to GAPDH expression.

Discussion

Mammalian TLRs play a central role in innate immunity by mediating recognition of pathogen-associated microbial patterns. Human polymorphisms in TLR2 and TLR4 are associated, respectively, with increased susceptibility to *S. aureus* infections (9) and LPS hyporesponsiveness (12) underscoring the importance of intact PAMP recognition systems in human health. In addition to immune functions, invertebrate TLRs have been shown to act during development and in cell to cell interactions (31–33). Further complexity in understanding TLR biology arises from the recognition that some TLRs act as coreceptors (e.g., TLR1, TLR6) with other TLRs (e.g., TLR2) and can promote or inhibit cellular responsiveness to activating ligands (26, 34). To identify settings both within the immune system and throughout the body in which TLRs may regulate important biological processes, we initiated a study of the expression of human TLRs.

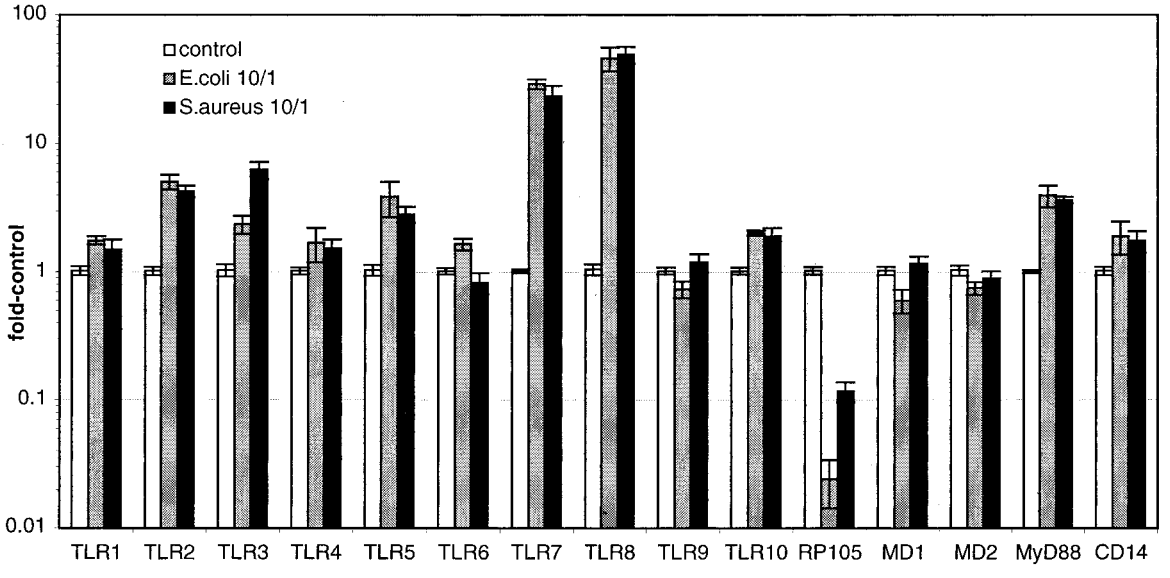
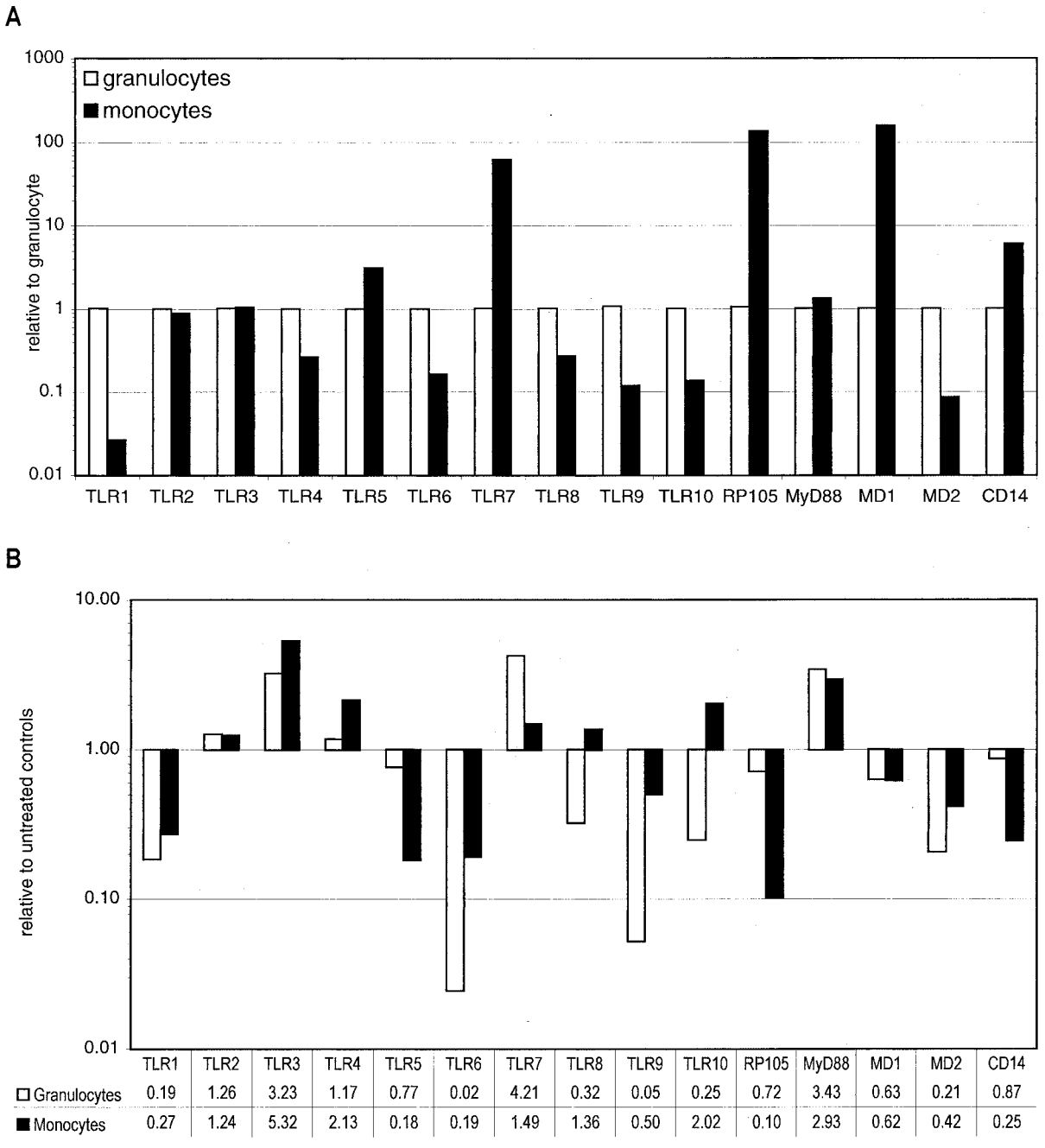


FIGURE 4. Treatment of THP-1 cells with live bacteria results in changes in TLR mRNA expression. THP-1 cells were cultured in the absence or presence of 10 bacterial cells per THP-1 cell. After 6 h, RNA was isolated and analyzed as in Fig. 3. Data represent the mean ± SEM of at least two independent experiments.



	TLR1	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10	RP105	MyD88	MD1	MD2	CD14
Granulocytes	0.19	1.26	3.23	1.17	0.77	0.02	4.21	0.32	0.05	0.25	0.72	3.43	0.63	0.21	0.87
Monocytes	0.27	1.24	5.32	2.13	0.18	0.19	1.49	1.36	0.50	2.02	0.10	2.93	0.62	0.42	0.25

FIGURE 5. Incubation of whole human blood with *E. coli* alters the TLR mRNA expression level in professional phagocytes. Citrate-anticoagulated human venous blood was incubated with or without *E. coli* at 10^5/ml in a humidified CO_2 incubator. After 6 h, phagocyte populations were purified as described in *Materials and Methods* and subjected to Taqman analysis for TLR expression. *A,* Expression of TLRs in monocytes relative to expression in granulocytes (50 ng cDNA/25 μl reaction). *B,* Changes in expression of TLRs in monocyte and granulocyte subpopulations of *E. coli*-treated blood expressed relative to untreated controls. Data represent the mean of two to four independent experiments using different donors and are normalized to expression of RPL19.

Consistent with their roles in immune surveillance, TLR mRNAs are expressed at higher levels in tissues exposed to the external environment such as lung and the gastrointestinal tract as well as in immunologically important settings such as peripheral blood leukocytes and spleen (Fig. 1). TLR3 and TLR5, which share significant sequence homology, are both expressed broadly at levels comparable with those in spleen (perhaps suggesting a general role in different tissues) and are both regulated by bacteria and their products (Figs. 4 and 5). Although TLR5 has been shown to mediate a signal in response to bacterial flagellin (23, 24), no function has yet been associated with TLR3. Human intestinal epithelial cells constitutively express TLR3 and decrease its expression in Crohn's disease but not in ulcerative colitis (35). The demonstration that TLR3 is expressed in epithelia and in monocytes, granulocytes, T cells, and B cells differs from a report by Muzio et al. (36) who detected expression only in monocyte-derived ex vivo-differentiated dendritic cells.

The pattern of TLR expression in different peripheral blood leukocyte populations implies specific roles in each population. Although $CD14^+$ cells expressed less TLR3, TLR9, and TLR10 than other cells, they expressed the highest levels of TLR2, TLR4, TLR5, and TLR8, and levels of TLR1, TLR6, and TLR7 were

comparable to those of $CD19^+$ cells. Similar to RP105, which was previously reported to be a B cell surface protein (37), TLR9 and TLR10 are expressed in B cells at levels at least 50 times greater than in other blood cells. TLR1, which has been reported to enhance TLR2 signaling in response to some agonists (27) and inhibit responses to others (26), was expressed at roughly similar levels in all leukocyte populations tested and also in lung and pancreas. MyD88 expression was also similar in all isolated cell populations tested and was detectable throughout the body including in nonmyeloid tissues. Although MyD88 also serves as a signaling intermediate in other pathways (e.g., IL-1R), its broad expression is suggestive of general importance of TLR function in most cells. Although mRNA expression data cannot exclude the possibility that small amounts of TLRs expression seen in some cells types may be functionally important, other data suggest that TLR mRNA levels correlate with their function, i.e., cell lines expressing relatively little TLR4 as detected by Taqman (e.g., HEK 293, not shown) are hyporesponsive to LPS (38). However, because TLR mRNAs are strongly responsive to a variety of stimuli (see below), cellular responsiveness to a given TLR agonist may differ drastically on prior activation by other TLR agonists.

To characterize determinants of TLR mRNA expression in a well-controlled in vitro setting, we selected the THP-1 acute monocytic leukemia cell line for our studies. In the presence of PMA, these cells differentiate toward a more monocyte-like phenotype (39) and coordinately up-regulate many TLRs (Fig. 3). For example, PMA increases TLR1 mRNA expression 50-fold, whereas TLR-4, -6, -7, and -8 expression increased between 5- and 7-fold. The TLR chaperones MD1 and MD2 were also PMA responsive, with MD-2 mRNA increasing at least 90-fold. In addition to being required for TLR4 LPS responsiveness (13), MD2 also increases TLR2 responsiveness to TLR2-specific agonists (40). Strikingly, the expression of RP105, which was reported to be a B cell marker (37) and to mediate LPS-signaling in B cells (19), increased >70-fold during differentiation, leading us to hypothesize that RP105 is also important in monocyte function. Both MyD88 and CD14 expression increased significantly and with increased expression of TLR4 and possibly RP105, likely contribute to the strong increase in sensitivity of THP-1 cells to LPS during maturation. Endotoxin protein-depleted LPS prepared as described (30) further increased the expression of many TLR mRNAs. TLR8, which was up-regulated 5-fold in response to PMA alone, was further up-regulated by LPS to a final expression level 50 times that of untreated undifferentiated THP-1 cells. RP105 was strongly suppressed on LPS stimulation (~100-fold) perhaps contributing to the LPS-induced hyposensitivity of monocytic cells to secondary LPS stimulation.

A hallmark of cellular responses to activation of innate immune systems is the release of cytokines such as TNF-α, IL-1β, and IFN-γ. Interestingly, several TLR mRNAs were specifically up-regulated by particular cytokines in PMA-differentiated THP-1 cells. For example, IL-6 increased TLR7 expression by >10-fold, whereas IFN-γ increased TLR8 expression by >40-fold. Thus, differential production of cytokines in particular pathological contexts may impact the spectrum of TLR-mediated responses of which cells are capable in that setting. Although up-regulation of MyD88 by IL-6 was previously reported (41), a variety of stimuli (e.g., PMA, LPS) increased its expression possibly to accommodate the increased expression of TLRs. Several studies found differential regulation of TLR2 and TLR4 in response to microbial products and some cytokines (42–45). Interestingly, although activation of mouse monocytes with LPS causes little change in the total level of TLR4 mRNA, the abundance of a splice variant mRNA encoding a secreted antagonist form is strongly increased (46).

Treatment of PMA-differentiated THP-1 cells with specific agonists of TLR2 (sBLP) or of TLR4 (endotoxin protein-depleted LPS) caused different changes in the expression of other TLR mRNAs. Although both up-regulated TLR8 to a similar extent, TLR3 was up-regulated 3-fold by LPS and repressed 2.5-fold by BLP. RP105 was down-regulated 100-fold in response to LPS but only 2-fold by BLP. Thus, in humans, like *Drosophila*, activation of different PAMP receptors specifically alters host transcriptional responses presumably to better contend with the particular activating pathogen. Flies defective in Toll signaling are unable to up-regulate antifungal cytotoxic peptides and succumb to fungal infection (47), whereas flies with defects in another TLR family member, termed 18-wheeler, are unable to appropriately sense bacterial pathogens and fail to produce antibacterial peptides (48). The simplest interpretation of the observations in THP-1 cells is that activation of different mammalian TLRs likewise results in specific transcription factor activation and responses. A recent study has also demonstrated some differences in gene expression due to activation by selective TLR2 and TLR4 agonists (49). Interestingly, a likely agonist of TLR9, *Shigella* plasmid DNA, causes a down-regulation of human antimicrobial peptides in monocytes (50). Furthermore, activation of TLR2 by bacterial lipoproteins leads to apoptosis in a variety of host cells, notably monocytes (7, 51). Thus, some microbes may use TLR receptors to down-regulate innate immune systems during infection.

In vitro experiments with single cell types in simple laboratory medium rarely replicate the complex networks of interactions between humoral and cellular agents active in settings like human blood. We therefore examined steady state levels of TLR mRNA in different cell populations after incubation in human blood ex vivo with *E. coli*. Relative to unstimulated monocytes, granulocytes express higher levels of most TLR mRNAs with the exception of TLR7, which is expressed 60-fold higher in monocytes than in granulocytes. We also found that granulocytes express >100-fold less RP105 and MD1 than monocytes, again suggesting that these proteins play a role in monocyte function. Similar to results seen in vitro with THP-1 cells, primary monocytes also down-regulate their RP105 in response to *E. coli*. Interestingly, two TLRs with significant down-regulation in response to *E. coli* are TLR1 and TLR6, both of which have been shown to alter the ligand specificity of other TLRs (26, 27). Although classically considered to be terminally differentiated, granulocytes express several NF-κB/Rel proteins that undergo nuclear translocation on activation (52), leading to cellular changes including alterations in the expression of TLRs in response to bacterial stimulation. The expression of TLRs in granulocytes, the most abundant professional phagocyte, underscores further the essential biological role of the TLRs in first line innate immune defenses in vivo.

Note Added in Proof. L. Alexopoulou et al. (53) recently demonstrated that TLR3 is a receptor for viral double-stranded RNA.

Acknowledgments

We appreciate the excellent technical assistance of the Genentech Oligonucleotide Synthesis group, Cloning group, and Sequencing group as well as the Bioinformatics Department. We thank Gwen Jewel for phlebotomy and Jerrold Weiss, Lynn Stoll, Austin Gurney, Melanie Mark, Menno van Lookeren, Ruey-Bing Yang, Sanjeev Satyal, Kerstin Schmidt, and Mario Navas for helpful discussions.

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