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Toll-Like Receptor (TLR)2 and TLR4 in Human Peripheral Blood Granulocytes: A Critical Role for Monocytes in Leukocyte Lipopolysaccharide Responses

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Leukocyte responsiveness to LPS is dependent upon CD14 and receptors of the Toll-like receptor (TLR) family. Neutrophils respond to LPS, but conflicting data exist regarding LPS responses of eosinophils and basophils, and expression of TLRs at the protein level in these granulocyte lineages has not been fully described. We examined the expression of TLR2, TLR4, and CD14 and found that monocytes expressed relatively high levels of cell surface TLR2, TLR4, and CD14, while neutrophils also expressed all three molecules, but at low levels. In contrast, basophils expressed TLR2 and TLR4 but not CD14, while eosinophils expressed none of these proteins. Tested in a range of functional assays including L-selectin shedding, CD11b up-regulation, IL-8 mRNA generation, and cell survival, neutrophils responded to LPS, but eosinophils and basophils did not. In contrast to previous data, we found, using monocyte depletion by negative magnetic selection, that neutrophil responses to LPS were heavily dependent upon the presence of a very low level of monocytes, and neutrophil survival induced by LPS at 22 h was monocyte dependent. We conclude that LPS has little role in the regulation of peripheral blood eosinophil and basophil function, and that, even in neutrophils, monocytes orchestrate many previously observed leukocyte LPS response patterns. The Journal of Immunology, 2002, 168: 4701–4710.

Exposure to bacterial LPS, a normal part of our environment, is probably the most frequent stimulus of the innate immune system, and one of the most profound. Recently, the central components of the LPS receptor have been identified. LPS responses require CD14 (1), in association with the accessory protein MD-2 (2, 3) and the serum protein LPS-binding protein, to present LPS to Toll-like receptor (TLR) (4). Intracellular signaling is mediated by TLR4 homodimerization through pathways predominantly involving MyD88 and MyD88-adapter-like/Toll-IL-1R domain-containing adapter protein (4, 5), and the IL-1R-associated kinases, which ultimately activate NF-κB and mitogen-activated protein kinases in a manner similar to that of IL-1 (6–8). The related receptor TLR2, originally reported as a receptor for LPS (9), is probably principally involved in response to microbial lipoproteins that often contaminate commercial LPS preparations (10), although CD14 and MD-2 can facilitate repurified LPS signaling via this receptor (11). Signaling via TLR2 is more complex, and is at least partly dependent upon heterodimerization of this receptor with either TLR1 or TLR6 (12–14).

Dependent upon dose and route of exposure, LPS can cause or be associated with septic shock, the exacerbation of allergic inflammation (e.g., in asthma) (15, 16), and immune deviation from Th2 phenotypes to Th1 phenotypes (17). At a cellular level, the multiple functions of LPS and bacterial lipoproteins include the priming of responses to inflammatory mediators (18–20), cell activation (1), proliferation (21), and both the inhibition (22) and induction of apoptosis (23). However, there is still uncertainty about which peripheral blood leukocyte types respond to LPS.

The responses of monocytes to LPS include induction of cytokine synthesis (24), with concomitant effects on the survival, proliferation, and immune deviation of other cell types. Neutrophils are the other primary leukocyte type involved in protection of the host from bacterial invasion and have long been held to be sensitive to LPS, resulting in modulation of adhesion molecule expression, cytokine generation, and cell life span (1, 18, 22, 25). However, gradient-based cell preparation techniques almost invariably leave a low level of monocyte contamination of neutrophil preparations. Eosinophils have been recently reported to be LPS responsive and to express CD14 protein and TLR2 and TLR4 mRNAs (26), but a contradictory report found them to be CD14 negative and their apparent LPS responsiveness to be dependent upon the presence of monocytes (27). Basophils have been shown to be LPS responsive, but again in cell suspensions not fully depleted of CD14+ monocytes (28, 29), and their patterns of TLR expression are wholly unknown. Therefore, we set out to investigate whether patterns of TLR mRNA and protein expression on these cell types correlated with patterns with LPS responsiveness in standard gradient-purified preparations and after further purification by negative selection to remove contaminating monocytes.

Materials and Methods

Reagents

General laboratory reagents were from Sigma (Poole, U.K.). LPS from Escherichia coli serotype 0111:B4 was from Sigma. Repurified LPS (10) was a generous gift from Dr. S. Vogel (Uniformed Services University of Health Sciences, Bethesda, MD). Synthetic bacterial lipopeptide Pam3CysSerLys4 was from EMC Microcollections (Tübingen, Germany).
PE-conjugated anti-TLR4 mAb (clone HTA125, isotype IgG2a), PE-conjugated anti-TLR2 mAb (clone TL2.1, isotype IgG2a), PE-conjugated anti-CD11b and FITC-conjugated anti-L-selectin, and isotype controls were from eBioscience (San Diego, CA). Cytokines and chemokines were from PeproTech (London, U.K.). FCS and PBS were from Life Technologies (Paisley, U.K.). All experiments were performed using a lot of FCS with known extremely low endotoxin levels (0.371 ng/ml, contributing <0.01 ng/ml endotoxin when used at 2% in our assay buffers). HotStar Taq and mini-RNasey purification kits were purchased from Qiagen (Crawley, U.K.), dNTPs were purchased from Hybaid (Ashford, U.K.), Moloney murine leukemia virus H² reverse transcriptase (RT) and RNAsin from Promega (Southampton, U.K.). PCR primer pairs and real-time probes were designed using MacVector software (Accelrys, Cambridge, U.K.). PCR primer pairs were based on areas of TLRs showing least homology to each other and were purchased from Sigma Genosys (Cambridge, U.K.) and MWG Biotech (Ebersberg, Germany). ice-cold-time PCR primers and dual-labeled oligonucleotide probes were from MWG Biotech.

Cell preparation
Peripheral venous blood was taken with informed consent from normal volunteers in accordance with a protocol approved by the South Sheffield Research Ethics Committee. Blood was anticoagulated with trisodium citrate, plasma, and platelets removed by centrifugation, and following dextran sedimentation PBMC were separated from granulocytes by density over a plasma/Percoll (Amersham Pharmacia, St. Albans, U.K.) gradient as described (30), using a method developed to produce nonactivated leukocytes suitable for study of LPS responses (30, 31). In some experiments, leukocyte populations were further purified by negative magnetic selection (32). Ab mixtures were purchased from StemCell Technologies (Vancouver, Canada). Eosinophils and neutrophils were purified from PBMC using a mixture containing Abs to CD2, CD14, CD16, CD19, CD56, and glycoporphin A. Basophils were purified from PBMC by a mixture containing Abs to CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56, and glycoporphin A. Monocyte depletion of granulocyte or PBMC preparations was achieved using an anti-CD14 custom mixture, or a custom mixture containing Abs to CD3, CD14, CD16, CD19, and glycoporphin A, all incubated in PBS without Ca²⁺/Mg²⁺ (2% FCS, and 10 mM HEPES (pH 7.3–7.4)) with the relevant Abs and magnetic colloid according to manufacturer’s instructions (at room temperature for all purifications except eosinophils, which were incubated with Ab mixtures on ice), applied to a sterile column containing metal mesh and eluted from the column in buffer containing 1 mM EDTA (32). Purified cells were washed into the appropriate assay buffer and counted using a hemocytometer.

Modulation of cell surface marker expression
Leukocytes were resuspended at 5 × 10⁶ cells/ml in assay buffer (Dulbecco’s modified PBS containing Ca²⁺/Mg²⁺, 2% FCS, 10 mM HEPES, and 0.18% glucose (pH 7.3–7.4)) and stimulated for 1 h at 37°C in 50-µl aliquots with buffer or agonists. Control samples treated with buffer alone provided baseline levels. Following stimulation, all samples were washed with ice-cold FACS buffer (PBS without Ca²⁺/Mg²⁺, 10 mM HEPES, and 0.25% BSA (pH 7.3–7.4)), pelleted by centrifugation (1000 × g for 2 min at 4°C), and stained with the relevant Abs (see Flow cytometry). L-selectin expression and CD11b expression levels were all quantified as the percentage of basal values using the geometric mean of their fluorescence, except for neutrophil L-selectin expression, where cells formed a bimodal distribution of high and low L-selectin expression. Thus, for neutrophils, L-selectin levels were quantified in terms of percentage of cells showing high expression before and after stimulation.

Flow cytometry
Leukocytes were resuspended at 5 × 10⁶ cells/ml in FACS buffer (see Modulation of cell surface marker expression) and 50-µl aliquots stained with appropriate Abs and matched isotype controls by incubation on ice for 30 min, washed in ice-cold FACS buffer, pelleted by centrifugation (1000 × g for 4°C), and resuspended in PBS as follows: PE-anti-CD11b and FITC-anti-L-selectin, 1/25; PE-anti-TLR2, PE-anti-CD11b, and PE-anti-CD14, 1/10; FITC-anti-HLA-DR (Sigma, 1/50; PE-anti-CD123, 1/50; and biotin-anti-CD123, 0.3 µg/ml. To minimize nonspecific binding of anti-TLR mAbs, incubation was conducted in the presence of 50 µg/ml mouse IgG (Sigma). To separate neutrophils and eosinophils in mixed granulocyte preparations, cells were double-stained with anti-CD4 stuck (Serotec, Oxford, U.K.). To separate basophils in mixed PBMC populations, cells were stained with anti-HLA-DR FITC and anti-CD123-biotin concurrently with the other primary Abs, washed once, and stained withallophycocyanin-streptavidin (0.15 µg/ml; eBioscience). Flow cytometry was performed using a dual-laser FACSCalibur (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences) with appropriate single-stained samples for setting of compensation. To investigate TLR expression in whole blood, 100-µl aliquots of freshly sampled blood (anticoagulated with trisodium citrate) were incubated with PE-conjugated anti-TLR mAbs and isotype controls in the presence of 50 µg/ml mouse IgG and anti-VLA-4 FITC as above for 30 min on ice. Samples were washed by the addition of 1 ml of FACS buffer, pelleted by centrifugation (1000 × g for 2 min), and resuspended in FACS buffer, the rBC were lysed, and leukocytes were fixed using OptiTyse B (Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. OptiTyse B separates eosinophils and neutrophils on forward light scatter (FSC)/side light scatter (SSC) plots (33). Eosinophils and neutrophils were defined according to FSC/SSC gating combining with VLA-4 staining, and monocytes were defined by FSC/SSC gating.

Neutrophil survival
Granulocyte preparations were depleted of monocytes by CD14-negative selection under aseptic conditions. Aliquots of cells (2.5 × 10⁶ cells/ml, 100-µl aliquots) were cultured in Falcon Flexiwell plates (BD Biosciences) with buffer or stimuli (LPS, in the presence or absence of autologous PBMC at either 1.5 × 10⁶ or 1.5 × 10⁵ cells/ml) in RPMI 1640, 10% FCS, penicillin, and streptomycin at 37°C in 5% CO₂, as described previously (34, 35). After each time point replicates were pooled, washed in ice-cold FACS buffer, and stained with anti-TLR Abs as described above, and cell viability was determined by vital dye staining in accordance with established techniques (36, 37) using ToPro-3 (1/100,000 dilution; Molecular Probes, Eugene, OR), an alternative to propidium iodide whose fluorescence is detectable in the FL-4 channel (38) (pilot data not shown) demonstrated that To-Pro-3⁺ cells were all annexin V positive, consistent with their identity as a late apoptotic population (36, 37)). Granulocyte apoptosis was quantified by morphology on cytospins as described (34, 35).

RT-PCR
RNA was purified from aliquots of cells (≥5 × 10⁶ cells) using RNeasy kits according to the manufacturer’s instructions. Contaminating genomic DNA was removed using DNAfree (Ambion, Huntington, U.K.), and cDNA was prepared from ≥2 µg total RNA using an RNaseH⁻ Moloney murine leukemia virus enzyme. RT-PCR of cDNA and non-RT controls was performed using HotStar Taq according to the manufacturer’s instructions on 35 cycles for a Hybrid PCR Express (Hybaid), with the following primer pairs at their appropriate optimal melting temperature as determined by MacVector software: TLR2 forward primer (5’-GGTCATCATA TAAGCCTCCTC-3’) and reverse primer (5’-AGTCACGTGTTGGAATTG ACCAT-3’); TLR4 forward primer (5’-CAGCTTGTCTCAGATTGAATTG ACCAT-3’) and reverse primer (5’-AGCCTGCTAGACATCAGTTGAG GGAACC-3’); CD14 forward primer (5’-GCTGGCCCGTGTATGAAGAAAGA 3’); and reverse primer (5’-GGTCCCTGGTACGGTCTCCT-3’); and MD-2 forward primer (5’-GTCAGAAGACGTAGTTGTCGTT-3’) and reverse primer (5’-CCGTTTGAAGATCCTGGTG-3’). PCR products were analyzed by 1–2% agarose gel electrophoresis.

Real-time PCR
To quantify IL-8 mRNA generation, cDNA samples and their non-RT controls were analyzed by real-time quantitative PCR. Purified leukocyte populations (≥5 × 10⁶ cells/ml, 50-µl aliquots) were stimulated with LPS or control stimuli for 1 h at 37°C in parallel with the experiments above investigating L-selectin shedding/CD11b up-regulation. A total of 1 µl of cDNA or non-RT control (in duplicate) was amplified in 25 µl using HotStar Taq in the presence of 3 mM Mg²⁺ in an ABI 7700 thermal cycler (PE Applied Biosystems, Foster City, CA), and fluorescence was monitored at each cycle. Cycle parameters were 95°C for 15 min to activate Taq followed by 40 cycles of 94°C for 15 s, 58°C for 15 s, and 72°C for 30 s (34, 35). Probes (final concentration 500 nM) were labeled with 5’FAM and 3’TAMRA, and control DNA stocks were stored in single-use aliquots. In each plate, target levels were quantified against a standard curve created from serial dilutions of a genomic-DNA-free THP-1 relative unit and normalized to a similarly quantitated GAPDH cDNA level, to control for loading and reverse transcription efficiencies. Thus, relative IL-8 units were expressed in dimensionless units, calculated according to the following

\[ \text{Relative IL-8 units} = \frac{\text{Sample fluorescence}}{\text{Threshold cycle (Ct) of the standard curve}} \times \frac{\text{Standard DNA concentration}}{\text{Sample DNA concentration}} \times \frac{1}{10^{-\Delta \text{Ct}}} \]
Fig. 1. TLR and CD14 expression on neutrophils, eosinophils, basophils, and monocytes. Neutrophils and eosinophils in mixed granulocyte preparations, purified basophils, and monocytes in mixed PBMC populations were stained with isotype control Abs or Abs to TLR2 (IgG2a), TLR4, and CD14 (IgG1) as described. Neutrophils and eosinophils were separated by FSC/SSC gating and VLA-4 double staining, and mean data were statistically significant. Eosinophils showed a fluorescence as a percentage of CD14 levels (Fig. 2), but these were observed in every donor, and eosinophils, and neutrophils in non-CD14-depleted granulocyte preparations were stained in whole blood showed very low but significant expression (26), we also investigated expression of these receptors by RT-PCR and flow cytometry. To investigate granulocyte expression of TLR2, TLR4, and CD14 at the protein level and compare it with expression in LPS-responsive monocytes, we stained monocytes in mixed PBMC populations (separated by FSC/SSC gating), eosinophils, and neutrophils in non-CD14-depleted granulocyte preparations (separated by VLA-4 staining), and basophils in both mixed PBMC (separated by HLA-DR and CD123 staining) and purified populations. Illustrative histograms and mean data are shown in Fig. 1. Monocytes consistently expressed the highest levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils with all these Abs resulted in only very small shifts in the levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils and eosinophils with expression in LPS-responsive monocytes, we stained monocytes, due to their known autofluorescence (33), and at the protein level were consistently negative in all donors for expression of TLR2, TLR4, and CD14. In contrast, in both mixed PBMC populations and in purified cell preparations (n = 3 for each), basophils consistently expressed low levels of TLR2 and TLR4, but no CD14 protein. In comparison, we examined TLR and CD14 expression on neutrophils, eosinophils, and monocytes in whole blood (Fig. 2). Once again, eosinophils showed no detectable TLR or CD14 expression. Monocytes expressed TLR2, TLR4, and CD14 in whole blood with an identical pattern to that seen in purified PBMC populations, although with lower mean fluences than were seen in purified cells. Calculating monocyte TLR fluorescence as a percentage of CD14 levels (Fig. 2F), we found that the ratios of TLR2:CD14 and TLR4:CD14 were identical between monocytes stained in whole blood and monocytes stained in purified PBMC preparations, suggesting that the lower fluences in whole blood represented reduced sensitivity rather than upregulation of surface markers during purification. Consistent with this, neutrophils stained in whole blood showed very low but significant levels of surface TLR2 and CD14 staining, but no detectable TLR4.

Because eosinophils have been described to express TLR2 and TLR4 mRNA (although without investigation of protein expression) (26), we also investigated expression of these receptors by RT-PCR and flow cytometry. To investigate granulocyte expression of TLR2, TLR4, and CD14 at the protein level and compare it with expression in LPS-responsive monocytes, we stained monocytes in mixed PBMC populations (separated by FSC/SSC gating), eosinophils, and neutrophils in non-CD14-depleted granulocyte preparations (separated by VLA-4 staining), and basophils in both mixed PBMC (separated by HLA-DR and CD123 staining) and purified populations. Illustrative histograms and mean data are shown in Fig. 1. Monocytes consistently expressed the highest levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils with all these Abs resulted in only very small shifts in the levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils and eosinophils with expression in LPS-responsive monocytes, we stained monocytes, due to their known autofluorescence (33), and at the protein level were consistently negative in all donors for expression of TLR2, TLR4, and CD14. In contrast, in both mixed PBMC populations and in purified cell preparations (n = 3 for each), basophils consistently expressed low levels of TLR2 and TLR4, but no CD14 protein. In comparison, we examined TLR and CD14 expression on neutrophils, eosinophils, and monocytes in whole blood (Fig. 2). Once again, eosinophils showed no detectable TLR or CD14 expression. Monocytes expressed TLR2, TLR4, and CD14 in whole blood with an identical pattern to that seen in purified PBMC populations, although with lower mean fluences than were seen in purified cells. Calculating monocyte TLR fluorescence as a percentage of CD14 levels (Fig. 2F), we found that the ratios of TLR2:CD14 and TLR4:CD14 were identical between monocytes stained in whole blood and monocytes stained in purified PBMC preparations, suggesting that the lower fluences in whole blood represented reduced sensitivity rather than upregulation of surface markers during purification. Consistent with this, neutrophils stained in whole blood showed very low but significant levels of surface TLR2 and CD14 staining, but no detectable TLR4.

Because eosinophils have been described to express TLR2 and TLR4 mRNA (although without investigation of protein expression) (26), we also investigated expression of these receptors by

Statistics

Comparison of two groups was performed using the Student t test, and comparison of more than two data sets was performed using ANOVA and Tukey’s post-test, using the Prism 3.0 program (GraphPad, San Diego, CA).

Results

We investigated TLR expression in leukocyte populations using RT-PCR and flow cytometry. To investigate granulocyte expression of TLR2, TLR4, and CD14 at the protein level and compare it with expression in LPS-responsive monocytes, we stained monocytes in mixed PBMC populations (separated by FSC/SSC gating), eosinophils, and neutrophils in non-CD14-depleted granulocyte preparations (separated by VLA-4 staining), and basophils in both mixed PBMC (separated by HLA-DR and CD123 staining) and purified populations. Illustrative histograms and mean data are shown in Fig. 1. Monocytes consistently expressed the highest levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils with all these Abs resulted in only very small shifts in the levels of TLR2, TLR4, and CD14 proteins. The staining of neutrophils and eosinophils with expression in LPS-responsive monocytes, we stained monocytes, due to their known autofluorescence (33), and at the protein level were consistently negative in all donors for expression of TLR2, TLR4, and CD14. In contrast, in both mixed PBMC populations and in purified cell preparations (n = 3 for each), basophils consistently expressed low levels of TLR2 and TLR4, but no CD14 protein. In comparison, we examined TLR and CD14 expression on neutrophils, eosinophils, and monocytes in whole blood (Fig. 2). Once again, eosinophils showed no detectable TLR or CD14 expression. Monocytes expressed TLR2, TLR4, and CD14 in whole blood with an identical pattern to that seen in purified PBMC populations, although with lower mean fluences than were seen in purified cells. Calculating monocyte TLR fluorescence as a percentage of CD14 levels (Fig. 2F), we found that the ratios of TLR2:CD14 and TLR4:CD14 were identical between monocytes stained in whole blood and monocytes stained in purified PBMC preparations, suggesting that the lower fluences in whole blood represented reduced sensitivity rather than upregulation of surface markers during purification. Consistent with this, neutrophils stained in whole blood showed very low but significant levels of surface TLR2 and CD14 staining, but no detectable TLR4.

Because eosinophils have been described to express TLR2 and TLR4 mRNA (although without investigation of protein expression) (26), we also investigated expression of these receptors by
FIGURE 2. TLR expression in whole blood. Neutrophils, eosinophils, and monocytes in whole blood were stained with isotype control Abs or Abs to TLR2 (IgG2a), TLR4 (IgG2a), and CD14 (IgG1) as described. Eosinophils, neutrophils, and monocytes formed discrete FSC/SSC populations after fixation and lysis with Optilyse B (A). B, VLA-4 staining of a broad granulocyte gate, encompassing the eosinophil and neutrophil FSC/SSC regions, showing VLA-4⁺ and VLA-4⁻ populations. Eosinophils were defined as cells appearing in the eosinophil FSC/SSC gate and showing VLA4⁺ staining, neutrophils were defined as cells appearing in the neutrophil FSC/SSC gate and showing VLA-4⁻ staining, and monocytes were defined according to the FSC/SSC plot. C–E, Mean neutrophil, eosinophil, and monocyte staining, respectively, by the mAbs (n = 4). F, The level of monocyte TLR2 and TLR4 staining as a percentage of CD14 staining for monocytes stained in gradient-purified PBMC preparations (filled bars) and monocytes stained in whole blood (open bars). Significant increases in mean fluorescence compared with control are indicated: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

RT-PCR. Purified eosinophil preparations contained low numbers of neutrophils and lymphocytes, which were quantified by cytospins. Results of RT-PCR on serial dilutions of eosinophil cDNA were compared with RT-PCR of serial dilutions of contaminating cell cDNAs and correlated with the cytospin counts. We found that purified eosinophils expressed mRNA for TLR4 in three of three samples thus analyzed and expressed MD-2 and CD14 in two of three samples. RT-PCR for TLR2 yielded positive signals, but these were also positive in the dilutions of cDNA from contaminating cells, thus could not be shown to be specifically eosinophil derived. Basophils showed a different pattern of mRNA expression from eosinophils. Levels of RNA in these samples were very low, but RT-PCR showed positive bands of expected size at 35 cycles for TLR2, TLR4, and MD-2 in two of three samples and expression of CD14 in one of three samples.

To correlate patterns of TLR expression with leukocyte LPS responses, leukocytes were purified by preparative techniques resulting in nonactivated, LPS-responsive cells (31–33). Purification of granulocytes by plasma/Percoll gradients resulted in preparations typically containing >97% granulocytes, with a mean monocyte-neutrophil ratio of 1.526 (Table I). Pilot experiments showed that 2% FCS was required for LPS responsiveness of neutrophils in accordance with published data (1). Fig. 3, A–D, shows that neutrophils in these populations responded to stimulation with either LPS or fMLP by shedding L-selectin and up-regulating CD11b. The presence of a fixed concentration of 0.1 nM fMLP together with the varying concentrations of LPS resulted in additive modulation of cell surface markers. In additional experiments, granulocyte preparations were depleted of monocytes by CD14 negative selection, using a CD14 Ab (MEM-15) that does not block LPS-induced responses (Ref. 39 and data not shown). Although neutrophils express low levels of CD14, this was not sufficient to cause their retention in the negative selection column. The resulting populations showed a significant reduction in monocyte contamination with a monocyte-neutrophil ratio of ~1:3000 (Table I). CD14-depleted neutrophils retained their basal levels of L-selectin and their responsiveness to fMLP (Fig. 3, E and G). However, LPS was an order of magnitude less potent at inducing shedding of L-selectin (Fig. 3F) and was less efficacious in the up-regulation of CD11b in monocyte-depleted neutrophils compared with nondepleted cells (Fig. 3G).

In contrast, Fig. 4 shows that eosinophils in granulocyte preparations, or when highly purified by negative magnetic selection (typically >97% pure and at least 92% pure (Table I)), shed L-selectin in response to fMLP and IL-5 but not in response to LPS, whether alone or combined with a fixed low (0.1 nM) concentration of fMLP. In mixed cell suspensions and in keeping with published data (40), the eosinophil-stimulating chemokine eotaxin failed to significantly modulate eosinophil L-selectin expression but was effective in purified cells.

Eosinophils did not express TLR2, TLR4, or CD14, and did not respond to LPS. However, basophils expressed TLR2 and TLR4. Therefore, we investigated basophil responses to LPS in mixed and highly pure cell preparations. Fig. 5A shows that basophils in mixed PBMC populations responded to stimulation with fMLP and IL-3 by up-regulating CD11b expression, but basophils in

Table I. Purity of leukocyte populations*

<table>
<thead>
<tr>
<th>Cell Preparation</th>
<th>% By FACS Granulocyte</th>
<th>% By FACS Lymphocyte</th>
<th>% By FACS Monocyte</th>
<th>% By Cytoosp Neutrophil</th>
<th>% By Cytoosp Eosinophil</th>
<th>% By Cytoosp PBMC</th>
<th>By FACS Granulocyte-monocyte ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>95.97 ± 1.54</td>
<td>2.54 ± 0.80</td>
<td>0.22 ± 0.05</td>
<td>92.75 ± 0.95</td>
<td>5.5 ± 1.1</td>
<td>1.72 ± 0.34</td>
<td>526 ± 80, range</td>
</tr>
<tr>
<td>CD14-depleted</td>
<td>96.94 ± 0.92</td>
<td>3.03 ± 0.91</td>
<td>0.04 ± 0.011</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>213–707, n = 6</td>
</tr>
<tr>
<td>neutrophils</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 4</td>
<td>n = 4</td>
<td>n = 4</td>
<td>2968 ± 717, range</td>
</tr>
<tr>
<td>Purified eosinophils</td>
<td>97.78 ± 0.2</td>
<td>1.99 ± 0.16</td>
<td>0.24 ± 0.064</td>
<td>1.61 ± 1.21</td>
<td>96.43 ± 1.18</td>
<td>1.96 ± 0.33</td>
<td>587 ± 135, range</td>
</tr>
<tr>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
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<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
<td>211–1086, n = 6</td>
</tr>
</tbody>
</table>

* Purities of leukocyte cell preparations estimated by flow cytometry (FSC/SSC gating) and differential cytospin counts. Data are shown ± SEM; **, significant difference in estimated monocyte counts between standard and CD14-depleted neutrophil preparations, p < 0.01.
Therefore, we used quantitative real-time PCR to investigate NL-8 mRNA generation in response to LPS in the populations of neutrophils and purified eosinophils, in parallel samples from those experiments investigating changes in L-selectin/CD11b expression. Purified basophils were also included in these analyses but yielded insufficient mRNA for quantification of IL-8 levels. Fig. 6 shows that neutrophils, both in standard cell preparations and in those from which monocytes have been removed, up-regulated IL-8 mRNA expression in response to LPS. Eosinophil preparations also showed an apparently similar fold increase in IL-8 mRNA levels in response to LPS stimulation, but on a background of 100 times lower IL-8:GAPDH mRNA levels (Fig. 6).

We investigated whether LPS modulated TLR expression in neutrophils, using CD14-depleted populations to exclude alterations of TLR expression resulting from monocyte-derived cytokines. Fig. 7, A–D, shows that culture in the absence of added stimuli induced up-regulation of TLR2 expression at 4 and 22 h on neutrophils (with no differences observed between nonapoptotic and apoptotic cells in TLR2 expression at these time points; data not shown), without any change in TLR4 expression. Coculture with LPS at 100 or 1000 ng/ml prevented the up-regulation of TLR2 expression seen after 4 h of culture in every experiment, as illustrated in Fig. 7B (although these data failed to achieve significance when expressed as changes in mean fluorescence; Fig. 7E), but did not affect the TLR2 up-regulation seen at 22 h. TLR4 expression was unaffected by culture in any condition for any time point tested (Fig. 7D and data not shown).

LPS is widely regarded as a key regulator of neutrophil survival with a profound antiapoptotic effect (22, 25). However, our data...
suggested that neutrophil LPS responses were regulated by only low-level TLR expression and were partially dependent upon low levels of monocytes. We investigated regulation of neutrophil apoptosis and found that in the monocyte-depleted neutrophil populations LPS prevented the low level of neutrophil apoptosis occurring spontaneously after 4 h of culture (Fig. 8A). However, in the 22-h cultures, LPS alone had no significant effect in preserving cell viability and protecting from apoptosis as measured by ToPro-3 staining and cytospin morphology, respectively. In parallel samples, we added low numbers of monocytes by coincubation of neutrophils with monocytes in mixed cell suspensions with minimal purification, to investigate the effect of low-level monocyte contamination and to determine whether purification techniques affected responsiveness. We compared these responses to those of leukocytes in purified populations where CD14+ cells had been depleted.

Neutrophils in standard gradient-purified preparations responded to LPS at 4 h but not 22 h, in keeping with the results above (n = 3; data not shown).

**Discussion**

We have investigated the protein expression patterns of the LPS receptor components in human peripheral blood leukocytes and correlated these with response patterns to LPS. We performed these experiments in mixed cell suspensions with minimal purification, to investigate the effect of low-level monocyte contamination and to determine whether purification techniques affected responsiveness. We compared these responses to those of leukocytes in purified preparations where CD14+ cells had been depleted.

Neutrophils in standard gradient-purified preparations responded to LPS in a serum-dependent manner, with rapid shedding of L-selectin, up-regulation of CD11b, and induction of IL-8 mRNA generation, in keeping with data published by other groups (1, 18, 19, 41). In additional experiments, we depleted neutrophils of residual monocytes by CD14 negative selection (using a mAb that did not affect CD14 function). This resulted in a decrease in numbers of cells in an estimated monocyte FSC/SSC gate, which, although containing only few events, correlated with PBMC numbers as measured by cyto spins. We observed that these cells continued to make IL-8 mRNA in response to LPS stimulation, but, in contrast, responses to LPS as measured by L-selectin shedding and CD11b up-regulation were significantly reduced (while responses to fMLP were unimpaired). These data suggest that neutrophil signaling in response to LPS was unimpaired, but that the adhesion molecule changes were amplified by the presence of monocytes, probably through the LPS-induced secretion of other monocyte-derived proinflammatory mediators. To investigate neutrophil LPS responses in more detail, we studied the regulation of life span in...
CD14-depleted neutrophil preparations, and in some samples added back monocytes at two different concentrations. In monocyte-depleted neutrophil preparations, LPS prevented the low levels of apoptosis seen after culture for 4 h. Culture of these cells for 22 h in medium alone resulted in a population of which most showed apoptotic morphology and approximately one-third had become nonviable. In contrast to the findings at 4 h, the presence of LPS throughout the 22-h culture did not significantly affect neutrophil apoptosis rates or viability, nor did addition of monocytes (mean monocyte:neutrophil ratio, 1:125 at low density) when cultured in medium alone. Strikingly, when LPS was present in the neutrophil/monocyte coculture, there was an almost complete abrogation of neutrophil apoptosis and preservation of cell viability. The ability of LPS-stimulated monocytes to promote neutrophil survival is not in itself surprising, because stimulated monocytes

**FIGURE 7.** Neutrophil TLR2 but not TLR4 expression is regulated by cell culture and cytokines. Monocyte-depleted neutrophil preparations were cultured for 4 or 22 h as described in the presence and absence of LPS (measured in nanograms per milliliter), and TLR expression was determined by flow cytometry. Data are displayed for viable cells, gated by ToPro-3 staining. A. The staining of isotype control (thin solid line), TLR4 (dotted line), and TLR2 (thick solid line) at baseline. After 4 h of culture, TLR2 expression is up-regulated (B), unless 100 ng/ml LPS is present, while after 22 h TLR2 expression is up-regulated further. Data shown in D are the mean ± SEM changes in Ab binding over time in cells incubated in medium alone (filled bars, t = 0 h; hatched bars, t = 4 h; open bars, t = 22 h). Data shown in E are the mean ± SEM effect on TLR2 expression after 4 h in the presence of varying concentrations of LPS (measured in nanograms per milliliter). TLR2 expression after a 22-h culture was not altered by LPS (data not shown). Data are the mean of four experiments, with significant up-regulation of TLR2 expression indicated: ***, p < 0.01.

**FIGURE 8.** Neutrophil survival in culture shows significant monocyte dependence. Monocyte-depleted neutrophil preparations from the same experiments as shown in Fig. 7 were cultured for 4 or 22 h as described in the presence and absence of LPS (measured in nanograms per milliliter). In some samples PBMC populations were added at low density (LD) or high density (HD), with medium or 100 ng/ml LPS. Neutrophil cell death was quantified by determination of the percentage showing apoptotic morphology by light microscopy, and the percentage remaining viable by staining with a DNA-binding dye. A. The effect of LPS (measured in nanograms per milliliter) on neutrophil apoptosis after 4 h of culture. B. The effect of LPS (measured in nanograms per milliliter) on neutrophil apoptosis after 22 h of culture, either alone or in the presence of monocytes in PBMC populations. Viability in the same samples was also quantified by FACS (C). Data are the mean of n = 3–4 ± SEM. Significant differences between neutrophils cultured in medium alone and those cultured with stimuli in A, between those cultured with medium at 22 h and those cultured with stimuli in B, and between those cultured in medium alone at 22 h and both the 0-h baseline sample and those cultured with stimuli in C are indicated: ***, p < 0.01.
make survival factors such as IL-1 and GM-CSF (24, 25). However, the failure of LPS to prolong neutrophil survival in the 22-h cultures in the absence of added monocytes suggests that previously observed responses of neutrophil to LPS may in part have been dependent upon low levels of monocyte contamination (typical contamination levels of 1–3% PBMC in isolated neutrophil preparations would result in a final proportion of ~0.1–0.3% monocytes). We also subsequently studied neutrophils that had been depleted of monocytes using a non-CD14-selecting Ab mixture, and found again that these cells showed reduced apoptosis in response to LPS at 4 h, but not at 22 h, demonstrating that the results above were not due to the CD14-mediated depletion of a more responsive neutrophil subset. Previously, enhanced neutrophil survival following LPS stimulation has been attributed to autocrine IL-1 release, although these experiments were performed without complete removal of monocytes (45). Our data show that neutrophils can respond to LPS with IL-8 mRNA generation and delayed apoptosis at early time points, but this antiapoptotic effect is lost at later time points where neutrophils become dependent upon other cells for survival factors.

Neutrophil TLR/CD14 expression patterns were consistent with the observed LPS responses. Patterns of TLR/CD14 expression for neutrophils, eosinophils, and monocytes were similar in whole blood and purified cells, suggesting that the preparative techniques used had not resulted in modulation of TLR expression, and although we cannot exclude the possibility that cell preparation modified LPS responsiveness it appears to be relatively unlikely. Interestingly, in cultured neutrophils the expression of TLR2, but not TLR4, was regulated. TLR2 expression was up-regulated in cultured neutrophils, an effect prevented by coincubation with LPS at early time points. Our data are in keeping with a previous study that showed TLR2 expression on neutrophils was down-regulated by LPS exposure at early but not late time points (46), although, in contrast to our data, this study found that prolonged (20-h) culture in medium alone caused a small decrease in TLR2 expression. These differences between our data and those of Flo et al. (46) are unlikely to be due to the lack of monocyte depletion in the latter study, as when we added monocytes back to the culture the basal expression of TLR2 was not altered. Furthermore, at the mRNA level in polymorphonuclear leukocytes expression of both TLR2 and TLR4 is up-regulated within 3 h of LPS stimulation (47), although we observed changes only in protein expression in TLR2, suggesting that regulation of TLR2 (46) and TLR4 expression at the protein and mRNA levels is different and potentially complex. In monocytes and macrophages, stimulation by LPS or related ligands results in TLR2 and/or TLR4 mRNA up-regulation (47–50). One recent study described up-regulation of functional TLR4 by LPS in monocytic-differentiated HL-60 cells but not in granulocytic-differentiated cells (51), and Flo et al. (46) showed that regulation of TLR2 protein expression by cytokines and LPS was different between granulocytes and monocytes, demonstrating lineage-specific patterns of regulation of expression and function.

TLR4 is the major receptor involved in responses to the majority of LPS species (7), with TLR2 involved predominantly in responses to bacterial lipopeptides, including those contained in commercial LPS preparations such as those we used in this study (10, 14, 52–54). Because TLR2 is expressed at apparently higher levels on neutrophils than is TLR4, and because its expression is regulated by culture and LPS exposure while that of TLR4 is not, it would be tempting to speculate that TLR2 is the dominant receptor on neutrophils accounting for responses to commercial LPS preparations through lipopeptide contaminants. We have also found that the selective TLR2 ligand synthetic bacterial lipopeptide can induce L-selectin shedding and CD11b up-regulation in purified neutrophils (data not shown). However, like the IL-1R (55), TLR4 is functional at extremely low receptor copy number (a few hundred receptors per cell in immature dendritic cells (56)), and we have found that repurified LPS, which signals exclusively via TLR4 (10), is a potent stimulator of neutrophil CD11b and L-selectin responses (efficacious at <10 ng/ml) and an effective inhibitor of neutrophil apoptosis after 4 h of culture, showing that the low levels of TLR4 on neutrophils are functional (data not shown).

Our study is the first to examine eosinophil TLR protein expression, and we found that peripheral blood eosinophils did not express TLR2, TLR4, or CD14, although at the mRNA level there was evidence for TLR4 expression. These data add to recent but conflicting reports of the LPS responsiveness of eosinophils. One of these studies showed that eosinophils expressed TLR2 and TLR4 mRNA and low levels of CD14 protein, and demonstrated LPS responsiveness in assays of cytokine generation (26). However, this study did not deplete eosinophil populations of CD14+ cells. A second study examined eosinophil apoptosis rates in response to LPS and found, by comparing CD14-depleted and non-CD14-depleted cell populations (analogous to our data obtained in neutrophils above), that prevention of eosinophil apoptosis by LPS was monocyte dependent (27). This latter group also showed that eosinophils did not express CD14. We found no evidence of eosinophil LPS responsiveness in assays of L-selectin shedding and CD11b up-regulation, whether in mixed populations or in those that had been purified by a mixture of mAbs including CD14 depletion with a nonblocking CD14 mAb. In the purified eosinophil preparations we observed very low levels of IL-8 mRNA generation in response to LPS, but these were at levels consistent with the very low neutrophil contamination of the purified eosinophil preparations, suggesting that the IL-8 mRNA response probably originated from neutrophils. Thus, our data are in agreement with Meerschaert et al. (27), and we find no evidence that peripheral blood eosinophils are responsive to LPS.

Our data suggest a similar story for the basophil. Only a few studies have examined the responses of basophils to LPS, most of which have shown that in mixed cell suspensions LPS primes or enhances basophil histamine release (20, 28). However, one study showed that purified basophils did not make IL-8 mRNA when stimulated with LPS, but did make IL-8 mRNA in response to control stimuli (43). L-selectin shedding and CD11b up-regulation are inducible in the basophil and correlate with histamine release induced by a variety of secretagogues (57). We found that circulating basophils showed no response to LPS in these assays, although control stimuli were effective. At the protein level, basophils did not express all the components of the LPS receptor, because they expressed TLR2 and TLR4 at levels similar to or greater than those in neutrophils, but not CD14. However, lack of membrane CD14 is not necessarily a bar to LPS responsiveness (58), and soluble CD14 is present in FCS as used in all our assays (59), which has been shown to enable LPS responses in some, but not all, CD14-negative cells (60, 61). Soluble CD14 is effectively delivered to sites of allergic inflammation (62), and the presence of TLR2 and TLR4 on the basophil suggests that LPS responsiveness in this cell type may be inducible at sites of inflammation. Due to a lack of reagents, we were unable to investigate expression of the LPS coreceptor MD-2 in basophils, but it is also possible that a lack of MD-2 contributes to their nonresponsiveness to LPS.

Signaling via TLRs may modulate many aspects of inflammatory responses. TLR4 signals in response to the endogenous proteins heat shock protein-60 and fibrinogen (63, 64) and to exogenous nonbacterial stimuli such as respiratory syncytial virus (65). Allergic inflammatory responses may be significantly modified by
infective stimuli (15, 16, 66, 67). Our data suggest that the mono- 
cyte is a key orchestrator of LPS responsiveness and that, even for
neutrophils, its contribution to observed LPS responses is highly
significant.

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