A Genomic and Proteomic Analysis of Activation of the Human Neutrophil by Lipopolysaccharide and its Mediation by p38 Mitogen-Activated Protein Kinase

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SUMMARY

Bacterial lipopolysaccharide evokes several functional responses in the neutrophil that contribute to innate immunity. While certain responses, such as adhesion and synthesis of tumor necrosis factor-α, are inhibited by pretreatment with an inhibitor of p38 mitogen-activated protein kinase, others, such as actin assembly, are unaffected. The aim of the present study was to investigate the changes in neutrophil gene transcription and protein expression following lipopolysaccharide exposure and to establish their dependence on p38 signaling. Microarray analysis indicated expression of 13% of the 7070 Affymetrix gene set in nonstimulated neutrophils, and LPS-upregulation of 100 distinct genes, including cytokines and chemokines, signaling molecules, and regulators of transcription. Proteomic analysis yielded a separate list of upregulated modulators of inflammation, signaling molecules, and cytoskeletal proteins. Poor concordance between mRNA transcript and protein expression changes was noted. Pretreatment with the p38 inhibitor SB203580 attenuated 23% of LPS-regulated genes and 18% of LPS-regulated proteins by ≥40%. This study indicates that p38 plays a selective role in regulation of neutrophil transcripts and proteins following lipopolysaccharide exposure, clarifies that several of the effects of lipopolysaccharide are post-transcriptional and post-translational, and identifies several proteins not previously reported to be involved in the innate immune response.
INTRODUCTION

Lipopolysaccharide (LPS), a component of the outer cell wall of Gram-negative bacteria, evokes a variety of functional responses in the human neutrophil (PMN) after binding to a plasma membrane receptor complex that involves the Toll-like receptors (TLRs) (1-5). These "immediate" functional responses, including actin assembly, adhesion, activation of nuclear factor-kappa B (NF-κB), and priming for an enhanced secretory response and for release of reactive oxygen intermediates, appear to be central both to the innate immune response and to the pathogenesis of several inflammatory human diseases, including sepsis and the acute respiratory distress syndrome (6). p38 mitogen-activated protein kinase (p38 MAPk) has been shown to mediate LPS-induced PMN adhesion, NF-κB activation, and TNF-α and IL-8 translation and release (7), and its blockade attenuates LPS-induced PMN accumulation in the airspace (8). However, other cascades almost certainly lead to downstream effectors of the LPS signal; for example, actin assembly appears to be p38 MAPk-independent (9). An improved understanding of the transcriptional and translational responses of the neutrophil to LPS and the modulation of these responses by p38 MAPk might carry pathogenetic and therapeutic implications.

Historically, it has been believed that the downstream PMN transcriptional response to LPS is static and that PMN functional responses to LPS that depend on de novo protein synthesis are primarily limited to the release of cytokines (10). However, recent studies indicate a robust transcriptional response (11). To date, most studies have relied upon and reported a short list of functional assays of the LPS-exposed PMN; therefore, no exhaustive investigation of either the transcriptional response or protein synthetic repertoire of the PMN has been reported. While several techniques have been used to evaluate transcripts, the screening of global changes in mRNA by microarray analysis has only recently become possible. In this way, thousands of genes can be screened in an unbiased fashion for transcript
abundance. Such genomic screens in mammalian cells have previously been applied to define altered expression profiles in response to agonists (12), drug action (13), and during cell cycle progression (14).

While DNA microarray technology is expected to provide insight into the response of the human PMN to LPS (15), inhibition of LPS-stimulated IL-1 and TNF-α production by p38 MAPk inhibitors in THP-1 cells (16), and of TNF-α synthesis in human PMNs (9), occurs at a translational level and would therefore not be detected by DNA microarrays. Furthermore, in other systems, such as yeast and human liver, mRNA and protein levels show poor correlation (17,18). Proteomics is a complementary tool for assessing global changes in cellular protein expression, thereby providing additional insight into cellular signal regulation. A proteomic approach has proven useful in different systems for dissecting signal transduction cascades and describing their output (19,20) and has even recently been used to detect novel upstream messengers involved in LPS signal transduction (21). We have applied DNA microarrays and proteomics to define and compare transcriptional and post-transcriptional alterations in the LPS-exposed PMN and to establish the dependence of these alterations on p38 MAPk signaling.

EXPERIMENTAL PROCEDURES

Materials — Endotoxin-free reagents and plastics were used in all experiments. Aprotinin, leupeptin, AEBSF, E-64, pepstatin, and bestatin protease inhibitors, spermine HCl, and α-cyano-4-hydroxycinnamic acid (CHCA) were all purchased from Sigma Chemical (St. Louis, MO). SB203580, a p38 MAPk inhibitor, was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). For 2D-PAGE, rehydration buffer, equilibration buffers, vertical electrophoresis solutions, and 10% homogeneous polyacrylamide slab gels were purchased from Genomic Solutions, Inc. (GSI; Ann Arbor, MI). Sequencing grade porcine trypsin was purchased from Promega (Madison, WI).
**LPS incubation** — PMNs were isolated by the plasma Percoll method (22), a technique which yields less than 5% monocytic contamination, and resuspended at a concentration of 15.4 x 10^6/ml in RPMI-1640 culture medium (BioWhittaker, Walkersville, Maryland, USA) supplemented with 10 mM HEPES (pH 7.6) and 1% heat-inactivated platelet-poor plasma. After addition of 100 ng/ml *E. coli* 0111:B4 LPS (List Biological), incubation was carried out with continuous rotation (4 hours, 37°C) both in the presence and absence of SB203580. Both Affymetrix analysis and proteomic analysis utilized 75 x 10^6 cells. For microarray analysis, non-stimulated and 4 hour-treated PMNs were collected from three separate donors. A more detailed time course following LPS exposure was performed using polymerase chain reaction. For proteomic analysis, LPS incubations from separate donors (n=6) were performed and then analyzed individually. Control and post-LPS-incubation PMNs were washed (0.34 M sucrose/1 mM EDTA/10 mM Tris) and then lysed in a modified rehydration buffer (GSI, Ann Arbor, MI) supplemented with 2 M thiouria, 50 mM dithiothreitol (DTT), 22.5 mM spermine HCl, and a cocktail of six protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM AEBSF, 5 µM E-64, 1 uM pepstatin, 10 uM bestatin). DNA was pelleted by centrifugation at 250,000g for 60 minutes (23).

**Affymetrix oligonucleotide array** — Five µg total RNA was isolated with Trizol (GIBCO) and RNeasy columns (Qiagen), and subsequently labeled with biotin as described by Affymetrix. Briefly, first strand synthesis was accomplished with Superscript II reverse transcriptase (GIBCO) using a T7-oligo(dT)₅₄ primer for 1 hour at 42°C, followed by second strand synthesis using *E. coli* DNA polymerase I and RNase H (GIBCO) at 16°C for 2 hours. dsDNA was used as a template for in vitro transcription with T7 RNA polymerase in the presence of biotin-labeled UTP and CTP using the BioArray High Yield RNA Transcript Labeling Kit (Enzo). Fifteen µg cRNA was fragmented and used for hybridization to Affymetrix HuGene 6800FL Genechips. Each sample was hybridized initially
using a Test2 Genechip to test for sample degradation and full-length in vitro translation. Data were analyzed using Affymetrix Genechip software. Results from three separate donors were analyzed.

Reverse transcription and polymerase chain reaction (RT-PCR) — cDNA was prepared by reverse transcription using 2 µg total RNA, derived from 20 x 10^6 cells treated as indicated. Polymerase chain reactions were performed using specific primers for Mx-1, TNF-α, MCP-1, p65, S100A4, and GAPDH.

2-D PAGE — The protein concentration of the lysates was measured as described by Bradford et al. (24). Poor isoelectric focusing (IEF) results were encountered unless the polycationic spermine was diluted (data not shown); therefore, lysates were diluted with rehydration buffer (GSI; Ann Arbor, MI) to achieve a final spermine concentration of 6 mM. Equal protein loads (1.5 mg) of control and LPS-stimulated neutrophils were used to rehydrate IEF gels overnight (18 cm, pH 3-10 nonlinear Immobiline DryStrip IEF gels; Amersham Pharmacia Biotech; Piscataway, NJ). IEF was performed at 20°C to 100 kVh (Phaser; GSI; Ann Arbor, MI) under mineral oil, followed by two 10 minute sodium dodecyl sulfate (SDS) equilibration steps (DTT- and then iodoacetamide-containing equilibration buffers; GSI, Ann Arbor, MI), and then by vertical electrophoresis on 10% homogeneous polyacrylamide slab gels (GSI; Ann Arbor, MI) at 500V. Protein spots were visualized by agitation in colloidal Coomassie Brilliant Blue G-250 (25) (16 hours), followed by destaining in deionized water (20 hours). In separate experiments, control and LPS-stimulated PMN lysates from three donors were pooled and then analyzed by 2-D PAGE using overlapping narrow isoelectric point (pI) ranges (18 cm, pH 5.0-6.0, pH 5.5-6.7, pH 6-11; Amersham Pharmacia Biotech; Piscataway, NJ). Identical IEF and vertical electrophoresis parameters were used for all gels.

Image analysis of 2-D gels — Colloidal Coomassie-stained gels were digitized using a Powerlook II (UMAX Data Systems, Inc., Taiwan) flatbed scanner with 8-bit dynamic range and 150 dpi resolution.
BioImage (GSI; Ann Arbor, MI) 2D-Analyzer software was used to locate, quantitate, and match protein spots on the control and LPS gel images. Analysis was performed by assigning 50 common anchor spots between paired images; the remaining spots were compared by a constellation matching algorithm. All data were then carefully reviewed by the operator to account for any discrepancies. Protein loading between control and experimental gels may have varied because of inconsistencies in rehydration of the different IEF gel strips; therefore, gel images were normalized so that the sum of the integrated intensities of all matched spots on paired gels was made equal. Control and LPS-stimulated gel images from individual donor experiments were matched to generate composite images; composite images were then matched into a master composite image in order to track the LPS response of protein spots among different donors (26). Only those spots that were common (image-matched) to all original twelve (pH 3.0-10.0) gels were considered for further analysis. For these spots, the LPS-induced change in integrated intensity in the six experiments was subjected to statistical analysis with a two-tailed student's t-test, and those spots with p<0.05 were identified by peptide mass fingerprinting (described below). For the narrow range (pH 5.0-6.0; 5.5-6.7; 6-11) 2-D PAGE experiments using pooled donors, only those spots with concordant regulation exceeding 1.5-fold, or which appeared de novo in the LPS gel, in two repeat experiments were further analyzed.

In-gel tryptic digestion — In-gel digestion of protein spots was performed with sequencing grade porcine modified trypsin using the method of Hellman et al. (27). Tryptic peptides were then extracted (50 µl of 50% acetonitrile/5% trifluoroacetic acid, 2 hours), the supernatant taken to dryness in a vacuum centrifuge, and then redissolved in trifluoroacetic acid (20 µl, 0.5%). Peptides were then purified and concentrated using ZipTipC18 pipette tips (Millipore; Bedford, MA).

MALDI-TOFMS — Analyses were performed on an Applied Biosystems matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) Voyager-DE PRO mass spectrometer (Framingham,
MA) operated in delayed extraction mode. Samples (0.5 µl) were spotted onto a sample plate to which matrix (0.5 µl of 10 mg/ml CHCA) was added. The sample-matrix mixture was dried at room temperature and then analyzed in reflector mode. CHCA was also spotted alone as a negative control. Spectra were the sum of 100 laser shots, and those peaks with a signal to noise ratio of greater than 3:1 were selected for database searching. Spectra were internally calibrated using autolytic trypsin peptides (m/z 842.51, 2211.10).

Database searching algorithm — The monoisotopic masses for each protonated peptide were: a) entered into the program MS-Fit (http://prospector.ucsf.edu) for searches against the Swiss-Prot, NCBI, and GenPept databases, and b) entered into Mascot (http://matrixscience.com), an algorithm testing statistical significance of peptide mass fingerprinting identifications. For MS-Fit searches, masses derived from trypsin, CHCA, keratin, and Coomassie Brilliant Blue G-250 were excluded. Search parameters included a maximum allowed peptide mass error of 0.1 Da (0.8 Da in the few instances in which linear mode was used), consideration of one incomplete cleavage per peptide, pI range of 3.0 to 10.0, and MW range of 1 kD to 200 kD. Accepted modifications included carbamidomethylation of cysteine residues (from iodoacetamide exposure following IEF) (28), and methionine oxidation, a common modification occurring during SDS-PAGE (29). Protein identifications were assigned when three criteria were met: 1) statistical significance (p<0.05) of the match when tested by Mascot (http://matrixscience.com); 2) >20% sequence coverage by the tryptic peptides; and 3) concordance (+/-15%) with the molecular weight (MW) and pI of the parent 2-D PAGE protein spot. The following special exceptions were considered: a) protein identifications not fulfilling criterion 2 were still assigned if criteria 1 and 3 were fulfilled and no other Homo sapiens proteins with peptide mass match p values <0.05 were identified by Mascot; b) if criterion 3 was not fulfilled (lower than expected MW), a cleavage product of the identified protein was inferred and the cumulative MW of the tryptic peptides was compared with that of the 2D-PAGE spot to ensure that it was not exceeded; c) if criterion 3 was
not fulfilled (isolated discordance between theoretical and observed pI), post-translational modification of an unrecovered peptide was inferred; and d) if two or more Homo sapiens protein assignments with >4 mutually exclusive matching peptides were identified, a protein mixture in the 2-D PAGE spot was inferred and further analysis halted (quantitative conclusions regarding the individual protein constituents could not be drawn).

RESULTS

*Genes differentially expressed in LPS-stimulated neutrophils* — Human PMNs were a) left untreated and b) incubated in the presence of 100 ng/ml LPS for 4 hours. As a control to confirm that the PMNs were quiescent at baseline and that LPS resulted in normal stimulation, mRNA was isolated, cDNA was prepared, and PCR for TNF-α was performed. Little TNF-α expression was seen in non-stimulated cells, whereas LPS treatment led to an increase in expression in each of the donors subsequently used for microarray analysis (data not shown). No macrophage-colony stimulating factor (M-CSF) receptor was detected by oligonucleotide microarray analysis, confirming no significant monocytic contamination.

Human PMNs express a limited repertoire of mRNA transcripts at baseline, but respond to LPS with differential expression of genes in many families. Considering only those genes present by microarray analysis in all three donors, unstimulated PMNs expressed 13.0% (923 of 7070 genes) of the Affymetrix gene set. Gene classes represented at baseline include metabolic enzymes, structural proteins, receptors, signaling proteins, and transcription factors. By comparison, human monocytes expressed ~40% and human fibroblasts ~35% of the represented genes (data not shown). By the criterion of a >3-fold
increase in expression in all three donors on Affymetrix oligonucleotide array analysis, exposure of PMNs to LPS for 4 hours resulted in the upregulation of 100 genes (Table I).

Genes from several different functional classes were induced in PMNs following LPS exposure. Of interest, a number of transcriptional regulators were induced, including transcription factors of the NF-κB family. The transcriptional NF-κB complex has previously been implicated in the regulation of the genes induced by LPS (11). The genes for several cytokines and chemokines were also found to be upregulated. These include TNF-α, IL-1β, IL-6, MCP-1, MIP-3α, and MIP-1β (Table I). To confirm results from the microarray analysis, PCR was performed. PCR analysis on selected genes indicates that the time course for changes can be rapid or delayed, but parallel the changes found in the array at the 4 h time point (data not shown). Other up-regulated genes included those for metabolic enzymes, immune response molecules, kinases, phosphatases, signaling molecules, adhesion and cytoskeletal components, interferon-stimulated genes, and those with unknown or miscellaneous function (Table I).

LPS stimulation of PMN also resulted in the downregulation of 56 genes (Table II). Downregulated genes were identified as transcriptional regulators, protein and lipid kinases and phosphatases, structural molecules, and signaling molecules. Genes for metabolic proteins were also evident, as were several uncharacterized genes.

2-D PAGE and image analysis — In contrast to the limited number of transcripts found at baseline, PMNs were found to express a large number and variety of proteins in the nonstimulated state (Figure 1A and 1C, Tables III-V). Reproducible protein expression patterns were found on the pH 3.0-10.0 gels, and the majority of proteins fell in the pH 5.0-7.0 range (Figure 1A). The basic region (pH>7.0) consistently exhibited poor resolution, precluding meaningful image analysis and further workup (data not shown). Depending on the spot-finding parameters (minimum spot intensity, filter width) selected on the image analysis software, spot-by-spot manual editing was found to be necessary in order to avoid
over- and underdetected spots; moreover, further manual editing was performed to screen for unmatched and mismatched spots following matching of paired control and LPS-stimulated gels. After spot editing, ~1200 well-resolved spots were evident on each pH 3.0-10.0 gel. In an attempt to improve resolution of the pI range bearing the greatest number of well-resolved spots, overlapping narrow pH range gels (pH 5.0-6.0; 5.5-6.7; 6-11) were also run. Of interest, a similar number of well-resolved spots (~1200) were detected on the narrow pH range gels (Figure 1C, 1D). Assuming a detection limit for Coomassie of 15 ng (0.25 pmol, or $1.5 \times 10^{11}$ molecules, for a 60 kDa protein), and a protein load per gel corresponding to $75 \times 10^6$ PMNs, we estimate a detection limit on our gels of 2000 molecules/cell for a 60 kDa protein. As investigators have suggested in other cell lines with the use of high-resolution 2D-PAGE methods (30), we estimate that >10,000 proteins are expressed in the resting PMN.

Human PMNs respond to LPS with the differential expression of a large number of proteins. In the six individual pH 3.0-10.0 experiments, the number of protein spots that increased in integrated intensity by at least 50% following LPS exposure was 185, 122, 104, 104, 96, and 131, respectively. The number of protein spots that decreased by at least 50% following LPS exposure was 72, 151, 102, 98, 128, and 97, respectively. While gel-to-gel regional variability in resolution was expected to account for individual spots not being well visualized on particular gels, only those spots that were matched to all twelve original gels were analyzed further. Overall, the number of spots matched to all twelve original gels was 125. The number of spots that were both matched to all twelve original gels and that increased by at least 50% in integrated intensity in the individual experiments following LPS exposure was 46, 13, 17, 27, 22, and 20, respectively. The number of spots that were matched to all twelve gels and that decreased by at least 50% was 6, 22, 17, 22, 34, and 28, respectively. The LPS-induced change in integrated intensity of the 125 spots that were matched to all twelve original gels was subjected to statistical analysis with a two-tailed student’s t-test and those spots with statistically significant ($p<0.05$) regulation among the six experiments were identified by peptide mass fingerprinting (Table III).
Identification of LPS-regulated proteins — On the pH 3.0-10.0 gels, several proteins were consistently upregulated (Table III), including regulators of inflammation (annexin III) and signaling molecules (Rab GDP dissociation inhibitor β). Several actin fragments were seen to be consistently upregulated in the six experiments following LPS exposure (Table III). Of interest, the proteasome β chain was also consistently upregulated. Downregulated proteins included other signaling molecules, such as Rho GTPase activating protein 1.

On the pH 5.0-6.0 and 5.5-6.7 gels, several proteins were found to increase by greater than 1.5-fold following LPS exposure (Table IV, V), including cytoskeletal proteins, such as moesin, nonmuscle myosin heavy chain, and a putative phosphorylated form of nonmuscle myosin heavy chain, and signaling molecules, such as protein phosphatase 1 and PO4-stathmin. The putative phosphorylated form of nonmuscle myosin heavy chain (spot #1101) was positioned 0.03 pH unit more acidic than the unmodified protein (spot #1102) (Figure 1D), and was distinguished by a tryptic peptide (m/z 1366.74) not present in the unmodified protein, consistent with phosphorylation of serine 685. Serine 685 is predicted by NetPhos 2.0 Prediction Server (http://www.cbs.dtu.dk/services/NetPhos/ (31)) to be a high-probability phosphorylation residue, and by ScanProsite (http://www.expasy.ch/tools/scnpsite.html) to be a substrate for protein kinase C. The tryptic phosphopeptide identified in PO4-stathmin, extending from residue 15 to 27 (1468.7 Da), is consistent with phosphorylation of either serine 16, a known substrate for Ca2+/calmodulin (CaM)-dependent kinases (32), or serine 25, a known substrate for p38δ and ERK (Figure 2A) (33). Assuming that no other multiply phosphorylated stathmin species have escaped detection, analysis of the integrated intensities of the PO4-stathmin and stathmin spots indicates that the percentage of the PO4 form of total cellular stathmin has increased from 11% to 38% with LPS stimulation (Figure 2B). This is similar to a previous report of an increase from <10% to 35-40% of the Ser25 phosphorylated form in Jurkat cells stimulated with anti-CD3 (34).
Effect of SB203580 on LPS-stimulated gene expression — Gene expression analysis of PMNs stimulated with LPS indicated that the majority of genes induced by LPS were unaffected by prior treatment of PMN with SB203580. Of the 100 genes upregulated by LPS, the upregulation of 23 was inhibited by greater than 40% (Table VI). The majority of these genes affected by SB203580 were inhibited by less than 60%, whereas only 6 were inhibited by greater than 80%, all of which represent previously identified interferon-stimulated genes. Induction of cytokine genes by LPS, with the exception of IL-6, was generally unaffected by SB203580.

Effect of SB203580 on LPS-stimulated protein expression — Similar to the effect of SB203580 on LPS-stimulated gene expression, little effect of SB203580 was seen on expression levels for the majority of LPS-regulated proteins (Table VII). Two exceptions are annexin III and α-enolase, for which LPS-stimulated expression was attenuated in the presence of the p38 MAPk inhibitor.

Comparison of microarray and proteomics results — Of the LPS-regulated proteins identified by peptide mass fingerprinting for which probes were present on the oligonucleotide microarray, poor concordance was found at the mRNA level (Table VIII). For 13 LPS-upregulated proteins, 2 corresponding mRNA transcripts were upregulated, 1 downregulated, 5 unchanged, and 5 were not detected by the Affymetrix chip. For 5 downregulated proteins, 3 corresponding transcripts were downregulated, 1 unchanged, and 1 not detected. Varying patterns of LPS-regulation emerge for those candidates detected at both the transcript and protein level. Proteasome β chain was upregulated at both the transcript and protein level (Table VIII), with no notable effect of SB203580 on expression at either level. Similarly, CAP1, RhoGAP 1, and ficolin 1 were downregulated at both the mRNA transcript and protein level (Table VIII), with no notable effect of SB203580. Annexin III was downregulated at the
transcript level and upregulated at the protein level, with an inhibitory effect of SB203580 seen only at the protein level (Table VIII).

DISCUSSION

Interaction of bacterial LPS with the human PMN represents a model system for studying the activation and output of the innate immune system during infection and inflammation. A recent publication describes the gene expression changes of a cultured monocytic cell line after infection by the Gram-positive bacteria *Listeria monocytogenes* (35). The cell wall components of Gram-positive bacteria, like Gram-negative-derived LPS (i.e., from *E. coli*), are known to signal through TLRs (36,37). Importantly, many of the expression changes found in LPS-stimulated PMNs in the present study were also described in the bacteria-exposed monocytic cells, indicating that many of the gene expression changes seen in bacterial infection are likely mediated by TLRs (38,39), and that the LPS model system accurately reflects exposure of immune cells to infection. Nevertheless, the reliance upon DNA microarrays alone affords insight only upon the transcriptional response without corroboration at the protein level. In the present study, application of both DNA microarray and proteomics technology to our model system provides unique insight upon both the cellular biology of the activated PMN and the responsiveness and regulation of its transcriptional and translational machinery. As will be discussed below, our study identifies, in particular, novel aspects of the LPS-stimulated PMNs transcriptional regulation, activity in the innate immune response, signaling, cytoskeletal reorganization, and priming for granule release.

In the present study, the increase in NF-κB transcript abundance (Table I) detected by the microarrays corroborates the findings of other studies of PMNs and monocytes (40) and indicates a mechanism for the responsiveness and scope of the PMNs transcriptional machinery following LPS exposure. NF-κB,
recently described to be activated by LPS through the TLR/MyD88/IRAK/IKK pathway (1,4), is the only transcriptional complex reported to be induced by LPS in the PMN. However, because the transcriptional NF-κB complex has been implicated in the regulation of only a portion of the genes induced by LPS in this study (data not shown), the importance of alternative transcriptional regulators in the PMN is clear. Of interest, several other known and putative transcriptional regulators with less well defined functions were also upregulated in the present study, including PLAGL2, a putative zinc-finger protein, XBP-1, MTF-1, Ets-2, B-ATF, and DIF-2. On the other hand, LPS-downregulated genes include ATF-2 (a known target of p38), NFATC4, TOB-1, NF-E2, MXI-1 and LYL-1. Although the exact role of these gene products in regulating cell function is unknown, these data indicate that the range of transcriptional responses in the LPS-stimulated PMN is much broader than previously suggested, and that the signaling capabilities of the PMN in the immune response are thereby likely extended in scope and specificity.

As expected from the literature, the genes for several cytokines and chemokines, including IL-1β, IL-6, and MIP-1β were found to be upregulated (Table I). On the other hand, the notable absence of upregulated cytokines in the proteomics experiments reflects their removal in the post-LPS-incubation wash performed prior to lysis for 2D-PAGE. Upregulation of these inflammatory mediators is well documented in PMNs exposed to LPS, and in animal models of LPS-induced sepsis syndrome and acute respiratory distress syndrome, a PMN-mediated illness (41,42). Several genes in this family were upregulated that have not, to our knowledge, been described in LPS-stimulated cells, including MCP-1, GRO3, IL-10RA, and HM-74, an orphan G protein-coupled receptor with homology to chemokine receptors. The downregulation of TRAIL, the lymphotoxin b receptor, and IL8RB were also observed. The modulation of genes involved in cytokine signaling, including the adapter molecules TRAF1 (LPS and TNF receptor signaling) and A20 (TNF receptor signaling), as well as several kinases and phosphatases may indicate a change in cytokine responsiveness after LPS treatment. Relevant in this
regard from the proteomics data are the upregulation of protein phosphatase 1, which has been shown to
regulate PMN NADPH oxidase activation and translocation (43,44) as well as to regulate LPS-induced
NF-κB activation (45), the downregulation of Rho-GAP1, which has been shown to regulate NADPH
oxidase activity in the PMN (46), as well as upregulation of PO₄-stathmin (Table IV), a phosphoprotein
postulated to function as a relayer and integrator of multiple signal transduction pathways (34). Several
non-cytokine, non-chemokine genes involved in the immune response were also upregulated, including
the complement pathway members C3, C3AR1, and PFC; the protease inhibitors ELANH2 (elastase
inhibitor), SLP1, PI-3 and PI-9; and the acute phase protein orosomucoid. LPS-regulation of C3AR1
and orosomucoid expression has not previously been reported. In the proteomics experiments, the
downregulation of ficolin-1 (Table III), a collectin-like cell surface protein reported to activate the
complement system and to mediate adhesion and phagocytosis in monocytes, but not previously
reported in granulocytes (47), may represent negative modulation of the innate immune response. The
finding that genes other than cytokines and chemokines are regulated by the PMN in response to LPS
indicates that the PMN plays a more sophisticated role in host-defense and immunity than previously
thought.

Treatment of the PMN with LPS lead to the induction of a set of genes associated with the anti-viral
Type I interferons, IFNα/β. This induction occurs independently of the release of IFN or another
unidentified soluble factor.² Furthermore, the set of genes expressed is smaller than that induced by
IFNα/β, as described by Der et al.(12). This may be due to differences in the scope of the signaling
systems activated by LPS and IFNα/β, or the time course of analysis of genes in the LPS-stimulated
PMN. The implication that LPS treatment of PMN allows PMN to express anti-viral activity is
currently being tested. Of interest was the finding that induction of interferon-stimulated genes was
blocked by pretreatment of PMNs with SB203580. Work from our laboratory has indicated that STAT
activation does not occur in response to LPS in PMNs.² In addition, interferon-regulatory factor 3, a
known regulator of interferon-stimulated gene transcription, is not a direct target of p38 kinase.\textsuperscript{2} Therefore, gene expression analysis of LPS-stimulated PMNs has uncovered a previously uncharacterized signal transduction system that is sensitive to inhibition of p38 MAPk.

Knowledge of the genes downregulated by LPS permits the development of further hypotheses addressing PMN function in the face of infection. Strikingly, several downregulated genes and gene products are structural in nature (e.g., paxillin, actinin, calponin 2) (Tables II, V). A known consequence to the PMN of LPS exposure is decreased motility (48). Upregulation of genes for adhesion molecules (ICAM-1, CD44, ALCAM, and TSG-6), and downregulation of genes for structural proteins, indicates a genetic basis for this observation. Downregulation of two genes implicated in cytoskeletal regulation, Pix-\(\alpha\) and RhoB, was also observed. The calcium-binding protein S100A4, down-regulated in LPS-treated PMNs (Table II), has been implicated in cell motility and metastasis (49). Decreased motility may be beneficial in sustaining the inflammatory response at sites of infection. In addition, LPS treatment results in an inhibition of apoptosis (50). Therefore, the longer residence time of the PMN at sites of infection is consistent with the long term genetically coded changes seen in these gene profiling experiments, and indicates that the changes in gene expression are functionally relevant to host-defense and immunity.

By providing information on post-translational modification, the proteomics data may provide further insights into the cytoskeletal remodeling effects of LPS upon the PMN. We contend that the actin fragments identified (Table III) are unlikely to represent technical artifacts. Rather, their specificity (identical MW/pI among different experiments), statistically significant upregulation by LPS, as well as the use of a lysis buffer containing chaotropes and multiple protease inhibitors, argue instead that these fragments are physiologic consequences of LPS exposure in the human PMN. More specifically, the upregulation of these fragments following LPS exposure (Table III) suggests that LPS may activate an actin-cleaving enzyme, which, in turn, remolds the cytoskeleton. Intriguing in this vein, calpain has
recently been reported to play an important role in cell migration and cytoskeletal organization of fibroblasts (51). The possibility that LPS may induce calpain activation, and that calpain activation may regulate cytoskeletal reorganization and motility, is currently under investigation. An alternative possibility is that actin cleavage is a marker of neutrophil apoptosis (52).

Other LPS-regulated proteins may play important roles in cytoskeletal reorganization. The upregulation of protein tyrosine kinase 9-like (A6-related protein) may modulate LPS-induced actin polymerization, as it bears a high degree of homology to twinfilin (A6), an actin monomer-binding protein that localizes to sites of rapid filament assembly in cells and is believed to regulate actin filament turnover (53). In turn, LPS-induced downregulation of Rho-GTPase activating protein 1 (Table III) may regulate twinfilin (and protein tyrosine kinase 9-like) activity, as twinfilin has been shown to colocalize with Rac1 and Cdc42, and to be regulated by active Rac1 in NIH 3T3 cells (53). Activation of Rho proteins may be facilitated by LPS-upregulation of moesin (Table V), because moesin reportedly induces the dissociation of Rho from GDI (54). Rac1 may, in turn, promote activation of the actin filament-nucleating Arp2/3 complex through interactions with WASP (Wiskott-Aldrich Syndrome Protein) family proteins (55), and, interestingly, is also postulated to regulate the dynamics of both the actin and microtubule cytoskeletons via phosphorylation of stathmin (Table IV) (56). Calponin H2 is an actin-binding protein not previously reported in PMNs that is postulated to play a role in cytoskeletal organization (57). Its downregulation by LPS (Table V) likely modulates LPS-induced cytoskeletal reorganization. The upregulation of nonmuscle myosin heavy chain and a putative phosphorylated form of myosin heavy chain (putative protein kinase C substrate by prediction rules) in the LPS-exposed PMN (Table IV) is of uncertain significance; myosin has been implicated in multiple functions in the PMN, including locomotion, fluid pinocytosis, and phagocytosis (58). Of interest, however, S100A4 (downregulated, Table II) has been reported to regulate cytoskeletal dynamics by inhibiting protein kinase C-mediated phosphorylation of nonmuscle myosin heavy chain (59).
LPS induction of stathmin phosphorylation (Table IV, Figure 2) may represent another mechanism by which the cytoskeleton is remodeled. Stathmin is a phosphoprotein reportedly involved in both signal transduction and in regulation of the microtubulin filament network; furthermore, phosphorylation of stathmin has been reported to modulate its tubulin-binding avidity (60). Inferences can be made about both the phosphorylation site on PO4-stathmin and the responsible kinase induced by LPS. Four phosphorylation sites in stathmin have been well described: Ser16, Ser25, Ser38, and Ser63 (32,33). Ser16 has been reported as a substrate for Ca2+/calmodulin (CaM)-dependent kinases (32), and Ser25 as primarily a substrate for p38 and ERK (33), with p34^cdc2 also active but bearing a 5-fold preference for Ser38 (34). As stated above, the phosphopeptide identified in PO4-stathmin, extending from residue 15 to 27 (1468.7 Da), is consistent with phosphorylation of either Ser16 or Ser25 (Figure 2). While both p38δ and p38α MAPk isoforms are expressed in the human PMN, LPS has been shown selectively to activate the p38α isoform in human PMNs (9). The p38α isoform, however, has been shown to be relatively inactive at Ser25; in fact, p38δ is approximately 100-fold more active at Ser25 and selective p38α inhibitors do not inhibit the stress-activated phosphorylation of stathmin in 293 cells (33). Further support for the lack of involvement of p38 signaling in phosphorylation of stathmin in our system is the apparent lack of effect of SB203580 (a selective p38α and p38β inhibitor) on LPS-induced expression of PO4-stathmin (Table IV). As p34^cdc2 is relatively inactive at Ser25 (34), we conclude that the phosphorylation site is likely to be Ser16, a reported substrate of CaM-dependent kinase. While CaM kinases have previously been implicated in gene activation in LPS-exposed myelomonocytic HD11 cells (61), stathmin signaling has not, to our knowledge, been previously reported in either PMNs or lipopolysaccharide signal transduction.

Cytoskeletal reorganization, a well-described regulator of granule release (62), may underlie LPS-induced priming for PMN granule release, but several LPS-regulated proteins may provide more specific clues. LPS exposure led to increased levels of grancalcin, a calcium-binding protein previously
detected in PMNs and shown to translocate to granules and plasma membrane in the presence of physiologic concentrations of calcium (63). Similarly, annexin III, a calcium-binding protein highly expressed in PMN granule membranes and implicated in calcium-mediated secretion (64), and in granule fusion (65), was also found to be upregulated. Exocytosis of granule contents may also be facilitated by LPS-upregulation of Rab GDP dissociation inhibitor (Table III), which has been proposed to recycle Rab after vesicle fusion by extracting it from the membrane and loading it onto newly formed transport intermediates (66).

Parallel use of DNA microarrays and proteomics affords a powerful strategy for comparison of corresponding mRNA transcripts and proteins, thereby affording new insight upon the mechanisms by which the cell regulates its signaling responses to the external environment. Of interest, a poor correlation was found between corresponding transcripts and proteins (Table VIII), as reported in other systems (17,18). The finding in some cases of unchanged transcript abundance in the face of regulated protein levels indicates post-transcriptional modulation following LPS exposure. The finding of undetected transcripts in the face of regulated levels of the corresponding proteins may indicate previous transcription of these genes in an earlier state of the myeloid maturation of the PMN, producing stable protein species that have undergone post-translational alteration following LPS exposure. The use of SB203580, a p38 inhibitor, adds further insights into the mechanisms of LPS regulation. At the level of mRNA expression, SB203580 inhibited 23% of LPS-stimulated genes by ≥40% and 11% of genes by ≥60%; therefore, p38 plays a specific role in gene regulation in the PMN. In particular, proteasome β chain was upregulated at both the mRNA transcript and protein level (Table VIII), with no notable effect of SB203580 on expression at either level, consistent with a non-p38-mediated pathway of primary transcriptional upregulation induced by LPS. Similarly, CAP1, RhoGAP 1, and ficolin 1 were downregulated at both the mRNA transcript and protein level (Table VIII), with no notable effect of SB203580, consistent with a non-p38-mediated pathway of primary transcriptional downregulation.
Interestingly, annexin III was downregulated at the transcript level and upregulated at the protein level, with an inhibitory effect of SB203580 seen only at the protein level (Table VII), consistent with a p38-mediated post-transcriptional upregulation induced by LPS.

Limitations of the present study should be noted. Gene expression analysis by cDNA microarrays does not distinguish between transcriptional regulation and mRNA stabilization; similarly, 2-D PAGE proteomics by itself does not distinguish among transcriptional, translational, or post-translational regulation of protein abundance. Transcript detection by microarray technology is limited to the probes included; protein identification by 2-D PAGE proteomics is limited to well-resolved regions of the gel, may perform less well with hydrophobic and high molecular weight proteins, and tends to select for more abundant protein species (30). Harvesting of the LPS-incubated PMNs at 4 hours may have prevented detection of earlier, transient changes, and may have thereby introduced artefactual transcript-protein discordance. Furthermore, the post-LPS incubation, pre-2D-PAGE cell washes would be expected to remove secreted proteins from further analysis, with uncertain effects on detected protein abundance depending on such factors as the degree of de novo synthesis and extent of degranulation/exocytosis. As protein-binding of Coomassie blue has a limited dynamic range and is typically not linear throughout the range of detection, image analysis of Coomassie-stained protein spots should be considered semi-quantitative. For some protein spots, the apparent magnitude of regulation by LPS may have been blunted by the spot approaching staining saturation in the control gel. By limiting our analysis to those protein spots common to all twelve pH 3.0-10.0 2-D gels, we likely excluded some LPS-regulated proteins that happened to be either poorly resolved on a subset of the gels or unmatched by the image analysis software. By further limiting the analysis to those matched spots on the pH 3.0-10.0 gels for which a two-tailed t-test demonstrated p<0.05, the list of regulated proteins was likely also limited by statistical power. In addition to those regulated proteins listed in Table III, three others were upregulated and three downregulated with p<0.09 (data not shown).
Limiting our reported results to those changes that met statistical significance among the donors carries further important implications. We have encountered a two-order of magnitude range of response in unselected donor LPS-induced PMN functions, such as TNF-α and superoxide anion release (data not shown). The sources of this physiological heterogeneity remain uncertain, but may possibly include such factors as natural mutations of the LPS receptor component, TLR4 (67). By selecting for LPS effects common to all donors, we may not have characterized the range of genomic and proteomic heterogeneity present in the population, and thereby may have focused on only a narrow portion of a broader biological response to LPS. We contend that this reductionist approach is valid as it would be expected to enrich for biologically integral responses of the PMN to LPS. Nevertheless, correlation of genomic and proteomic profiles with functional phenotypes of the PMN may bear important diagnostic and therapeutic implications, and will be pursued in future studies.

Widespread regulation of numerous non-cytokine/chemokine genes and proteins in the LPS-stimulated human PMN is a novel finding. These data indicate that, despite a narrow scope of gene expression in the nonstimulated state, the terminally differentiated, short-lived PMN likely plays a role in the innate immune response that is far more sophisticated and dynamic than the simple release of preformed inflammatory mediators. While gene expression appears to be an important mechanism by which PMNs respond acutely to infection, mRNA transcript/protein concordance is limited, and post-transcriptional (and post-translational) modifications also play an important role. The alteration of multiple transcriptional regulators, G-protein regulators, PO4-stathmin, and protein phosphatase 1 indicates that one of the responses to LPS exposure is to modify subsequent signaling events by bacterial components or by other cytokines and chemokines. Finally, the finding that p38 MAP kinase mediates LPS-regulation of a limited subset of transcripts and proteins underlines the continuing need to define signal transduction cascades in the neutrophil.
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FOOTNOTES

1 The abbreviations used are: DTT, dithiothreitol; ELISA, enzyme-linked immunoabsorbent assay; IEF, isoelectric focusing; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MAPk, mitogen-activated protein kinase; MW, molecular weight; NF-κB, nuclear factor-kappa B; pI, isoelectric point; PMN, neutrophil (polymorphonuclear leukocyte); TLR, Toll-like Receptor; TNF, tumor necrosis factor; CHCA, α-cyano-4-hydroxycinnamic acid; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

2 Manuscript in preparation.
FIGURE LEGENDS:

Figure 1. 2-D PAGE of LPS-exposed human PMNs. A,B  Colloidal Coomassie blue-stained pH 3.0-10.0 2-D PAGE gels (A, control; B, LPS-exposed), with upregulated (solid arrows) and down-regulated (hatched arrows) proteins indicated. These results are representative of six separate experiments. C,D  Colloidal Coomassie blue-stained pH 5.0-6.0 2-D PAGE gels (C, control; D, LPS-exposed), with upregulated (solid arrows), new (solid arrow, open arrowhead), and down-regulated (hatched arrows) proteins indicated. LPS-exposed PMNs from three blood donors were pooled.

Figure 2. A, The predicted sequence of the tryptic phosphopeptide in PO₄-stathmin (1468.72 Da). The peptide mass measured by MALDI-TOFMS and the predicted mass differed by 14 ppm. As indicated, two possible alternate phosphorylation sites are possible: serine 16 and serine 25. B, PO₄-stathmin and stathmin were identified on the control and LPS-exposed pH 5.0-6.0 gels. Consistent with phosphorylation, the PO₄-stathmin spot was distinguished by a peptide of mass 1468.72 Da (i.e., 80 Da greater than the peptide of 1388.72 Da seen in the stathmin spot). Assuming that no other multiply phosphorylated stathmin species have escaped detection, analysis of the integrated intensities of the PO₄-stathmin and stathmin spots indicates that the percentage of the PO₄ form of total cellular stathmin has increased from 11% to 38% with LPS stimulation. The decrease in integrated intensity for stathmin was equal in amount to the increase in PO₄-stathmin following LPS exposure.
Table I.
Human neutrophil genes induced after 4 hours of LPS exposure.

| Description                                | Genbank™ Accession | Fold Change (X) |
|--------------------------------------------|--------------------|-----------------|
| **Transcriptional Regulation**             |                    |                 |
| Pleiomorphic adenoma gene-like 2           | D83784             | 16.8            |
| NFKB2                                      | S76638             | 12.3            |
| NFKBIE                                     | U91616             | 11.5            |
| p65                                        | L19067             | 8.4             |
| BCL3                                       | U05681             | 7.7             |
| X-box binding protein 1                    | M31627             | 7.5             |
| Metal-regulatory transcription factor 1    | X78710             | 7.4             |
| Ets-2                                      | J04102             | 7.4             |
| c-Rel                                      | X75042             | 6.2             |
| NFKB1                                      | M58603             | 5.8             |
| Basic leucine zipper transcription factor, ATF-like | U15460 | 4.7             |
| IKB                                        | M69043             | 3.8             |
| MAX dimerization protein                   | L06895             | 3.6             |
| DIF2                                       | S81914             | 3.1             |
| **Cytokines and receptors**                |                    |                 |
| MCP-1                                      | M69203             | 78.7            |
| MIP-1b                                     | M72885             | 48.8            |
| Alpha-helix coiled-coil rod homolog        | AF014958           | 20.8            |
| IL-1b                                      | X04500             | 17.6            |
| GRO3 (beta)                                | M57731             | 17.3            |
| TNFa                                       | X02910             | 14.5            |
| MIP-3a                                     | U64197             | 8.1             |
| IL10RA                                     | U00672             | 7.3             |
| IL-6                                       | Y00081             | 6.3             |
| GROa                                       | X54489             | 4               |
| HM74                                        | D10923             | 3.8             |
| **Immune response**                        |                    |                 |
| Orosomucoid                                 | X02544             | 20.2            |
| Complement component C3                    | K02765             | 12.8            |
| Protease inhibitor 9                       | U71364             | 9.5             |
| Complement component 3a receptor 1         | U28488             | 6.1             |
| Protease inhibitor 3                       | L10343             | 4.9             |
| SLP1/antileukoprotease                     | X04470             | 4.7             |
| ELANH2/elastase inhibitor                  | M93056             | 4.6             |
| CD58                                       | Y00636             | 3.8             |
| Complement component PFC                   | M83652             | 3.5             |
| Kinases                  |          |          |
|-------------------------|----------|----------|
| CNK/FNK/PLK-like        | U56998   | 16.2     |
| Cot                     | D14497   | 11.9     |
| Pim-2                   | U77735   | 9.5      |
| LIMK2                   | D45906   | 4.3      |

| Phosphatases            |          |          |
|-------------------------|----------|----------|
| PAC-1/DUSP2             | L11329   | 11.8     |
| DUSP5                   | U15932   | 5.3      |
| PHA1                    | U73477   | 3.4      |

| Signaling Molecules     |          |          |
|-------------------------|----------|----------|
| TNFAIP1/A20             | M59465   | 10       |
| TRAF1                   | U19261   | 6.2      |
| RanBP2                  | D42063   | 5.6      |
| GNA15                   | M63904   | 5.2      |
| PTAFR                   | D10202   | 3.9      |

| Adhesion and cytoskeleton |          |          |
|---------------------------|----------|----------|
| ICAM1                     | M24283   | 22.4     |
| CEACAM1 (Bilary glycoprotein) | X16354 | 6.3      |
| LIMS1                     | U09284   | 6.1      |
| SNL/actin bundling protein | U03057 | 5.9      |
| Galectin-1/LGALS1         | M57710   | 4.7      |
| MEMD/ALCAM                | U30999   | 4.2      |
| CD44                      | HG2981-HT3125 | 3.9 |
| TSG-6                     | M31165   | 3.7      |

| Metabolic                |          |          |
|--------------------------|----------|----------|
| GTP cyclohydrolase I     | U19523   | 13.5     |
| NDUVF2/ubiquinone reductase | M22538 | 8.6      |
| PSMA6/(proteosome iota)  | X59417   | 8.4      |
| UDP-galactose transporter (SLC35A2) | D84454 | 7.3      |
| PLAU (urokinase)         | X02419   | 6.4      |
| KYNU/L-kynurenine hydrolase | U57721 | 5.5      |
| AMPD3                    | D12775   | 5        |
| P4HA1/prolyl 4-hydroxylase | M24486 | 4.7      |
| Gamma-glutamylcysteine synthetase | L35546 | 4.5      |
| ATP6D                    | J05682   | 4.2      |
| ATP6S1                   | D16469   | 4        |
| Glycerol kinase          | X68285   | 3.6      |
| FAC1L1                   | L09229   | 3.5      |
| AK3                      | X60673   | 3.3      |
### Interferon-inducible

| Gene Name                  | Accession Number | Fold Change |
|---------------------------|------------------|-------------|
| ISG15                     | M13755           | 22.5        |
| Mx1                       | M33882           | 19.4        |
| IFI56                     | M24594           | 12.1        |
| INDO                      | M34455           | 5.2         |
| GBP1                      | M55542           | 4.3         |
| PRKR                      | U50648           | 3.7         |
| IFIT4                     | U52513           | 3.6         |
| IFI54                     | M14660           | 3.5         |
| IFI58                     | U34605           | 3.5         |
| IFP35                     | U72882           | 3           |

### Other

| Gene Name                  | Accession Number | Fold Change |
|---------------------------|------------------|-------------|
| Gos2                      | M72885           | 48.8        |
| MIHC/IAP1                 | U37546           | 7.2         |
| KIAA0105                  | D14661           | 5.1         |
| KIAA0118                  | D42087           | 5           |
| SNAP23                    | U55936           | 5           |
| CASP5                     | U28015           | 4.8         |
| KIAA0113                  | D30755           | 4.8         |
| KIAA0255                  | D87444           | 4.7         |
| Hepatoma-derived GF       | D16431           | 4.7         |
| PTGS2                     | D28235           | 4.6         |
| CD48                      | M37766           | 4.3         |
| UNC119 homolog            | U40998           | 4.2         |
| KIAA0151                  | D63485           | 3.9         |
| KIAA0151                  | D63485           | 3.9         |
| Rab1b                     | XM035660         | 3.8         |
| Annexin VII               | J04543           | 3.7         |
| KIAA0110                  | D14811           | 3.7         |
| Adrenomedullin            | D14874           | 3.7         |
| AIM1                      | U83115           | 3.6         |
| KIAA0250                  | D87437           | 3.2         |
| P5-1                      | L06175           | 3.2         |
| Scavenger receptor expressed by endothelial cells | D63483         | 3.2         |
| VHL                       | L15409           | 3.1         |
Table II.
Human neutrophil genes repressed (>4-fold) after 4 hours of LPS exposure.

| Description                                      | Genbank™ Accession | Fold Change (X) |
|--------------------------------------------------|--------------------|-----------------|
| **Kinases**                                      |                    |                 |
| CAMK, II, gamma                                  | U50360             | -4              |
| Diacylglycerol kinase, delta                     | D63479             | -4.2            |
| PRKCL2/PRK2 protein kinase C-like 2              | U33052             | -4.3            |
| MAPKAPK3                                         | U09578             | -6.3            |
| Protein Kinase Ht31, cAMP-dependent              | HG2167-HT2237      | -8              |
| CAMK II                                          | L07044             | -9.8            |
| **Transporters**                                 |                    |                 |
| SLC25A5/solute carrier family 25, member 5       | J02683             | -4.2            |
| SLC19A1; folate transporter                      | U17566             | -4.4            |
| SLC2A3; facilitated glucose transporter          | M20681             | -5              |
| **Metabolic**                                    |                    |                 |
| Carbonic anhydrase IV                            | L10955             | -4.4            |
| RNase A family, k6                               | U64998             | -4.5            |
| Glycogen phosphorylase; liver                    | M14636             | -4.6            |
| Inositol polyphosphate-5-phosphatase             | U57650             | -4.6            |
| Inositol 1,3,4-trisphosphate 5/6-kinase          | U51336             | -4.7            |
| Transketolase                                    | L12711             | -4.8            |
| Protein phosphatase 4, reg. subunit 1 (clone 23840) | U79267              | -4.9            |
| Cytidine deaminase                               | L27943             | -5.4            |
| MGAT1                                            | M55621             | -5.4            |
| HMOX1                                            | X06985             | -5.4            |
| MAN2A2                                           | L28821             | -5.8            |
| Glycogenin (Also Represents: U31525)             | HG4334-HT4604      | -5.9            |
| **Structural**                                   |                    |                 |
| Fibrinogen-like protein (pT49 protein)           | Z36531             | -4.2            |
| H2AFZ                                            | M37583             | -4.7            |
| Paxillin                                         | U14588             | -4.9            |
| Lamin B R                                       | L25931             | -5.9            |
| Dynamin 2                                        | L36983             | -6.2            |
| Actinin 1                                        | M95178             | -6.7            |
| Alpha-tubulin                                    | X01703             | -10             |
| Tubulin, Alpha 1, Isoform 44                     | HG2259-HT2348      | -15             |
| **Transcriptional Regulators**                   |                    |                 |
| Lymphoblastic leukemia derived sequence 1        | M22638             | -4.4            |
| Receptors                      |                  |     |
|--------------------------------|------------------|-----|
| Nuclear factor erythroid 2 isoform f | S77763           | -6  |
| Transducer of ERBB2, 1         | D38305           | -6.9|
| NFATC4                        | L41067           | -7.8|
| ATF-2 (CRE-Bpa)                | L05515           | -9.6|
| Lymphotoxin beta receptor      | L04270           | -4.4|
| Folate receptor 3 (gamma)      | U08471           | -5  |
|                               | U11875           | -5.3|
| Signaling                      |                  |     |
| Pix-alpha; cool-2 (KIAA0006)   | D25304           | -4.5|
| ARHB/RhoB                     | M12174           | -4.5|
| TNFSF10; TRAIL                | U37518           | -6.6|
| Ca²⁺ binding                  |                  |     |
| ANX1                          | L19605           | -4.3|
| S100A4                        | M80563           | -4.8|
| ANX1                          | X05908           | -4.8|
| Other                          |                  |     |
| Proteolipid protein 2         | L09604           | -4.9|
| Protein Phosphatase 1, alpha catalytic subunit | HG1614-HT1614 | -5  |
| TIMP2                         | M32304           | -5.1|
| KIAA0199                      | D83782           | -5.2|
| Lipin 2 (KIAA0249)            | D87436           | -5.6|
| LRMP (Jaw1)                   | U10485           | -5.8|
| CUGBP2                        | U69546           | -6.9|
| Clone 23933                   | U79273           | -7  |
| PECAM1                        | L34657           | -8  |
| Delta sleep inducing peptide  | Z50781           | -8.7|
| DiGeorge synd. critical region gene 2 (KIAA0163) | D79985 | -9 |
| SELPLG; CD162; selectin P ligand | U25956       | -32 |
Table III.
Analysis of pI 3.0-10.0 2-D PAGE gels. Mean fold-change in expression level among six PMN donors is reported. The change in expression for the proteins listed was statistically significant (p<0.05) by a two-tailed student's t-test.

| Identification [spot #] | Swiss-Prot Accession # | Estimated MW/pI | Theoretical MW/pI | Peptides Matched / Submitted (%) | Protein Covered (%) | Mean Change (X) |
|-------------------------|------------------------|-----------------|-------------------|-----------------------------------|---------------------|-----------------|
| **Upregulated**         |                        |                 |                   |                                   |                     |                 |
| Proteasome β chain [646] | P28070                | 27/5.7          | 29.2/5.72         | 9/12 (75%)                       | 36%                 | 1.51            |
| Annexin III [550]      | P12429                | 31/5.7          | 36.4/5.6          | 14/18 (78%)                      | 42%                 | 1.37            |
| Actin fragment [544]    | P02570                | 32/5.5          | (41.7/5.29)       | 13/15 (87%)                      | (34%)               | 1.74            |
| Actin fragment [591]    | P02570                | 30/5.4          | (41.7/5.29)       | 14/18 (78%)                      | (29%)               | 1.60            |
| α-enolase [380]         | P06733                | 41/5.7          | 47.2/7.01         | 9/10 (90%)                       | 24%                 | 1.65            |
| Rab GDP dissociation inhibitor β [289] | P50395    | 50/6.1          | 50.7/6.11         | 10/11 (91%)                      | 25%                 | 1.24            |
| Glutathione S-transferase P [648] | P09211 | 23/5.5          | 23.4/5.43         | 6/8 (75%)                        | 41%                 | 1.54            |
| Pre-B-cell colony enhancing factor [1152] | P43490 | 53/7.0          | 55.5/6.69         | 12/16 (75%)                      | 25%                 | 1.29            |
| **Downregulated**       |                        |                 |                   |                                   |                     |                 |
| Adenylyl cyclase-associated protein 1 [256] | Q01518  | 55/7.3          | 51.7/8.07         | 16/22 (73%)                      | 34%                 | 0.53            |
| Rho-GAP 1 [283]         | Q07960                | 50/5.8          | 50.4/5.85         | 7/9 (78%)                        | 22%                 | 0.67            |
| Ficolin 1 [511]         | O00602                | 33/6.5          | 35/6.39           | 10/12 (83%)                      | 25%                 | 0.74            |

1The theoretical pI and MW of native actin are indicated. Protein coverage indicates coverage of native actin.
Table IV.
Analysis of pH 5.0-6.0 2-D PAGE gels. Results are from pooled samples for control (n=3) and LPS-exposed (n=3) PMNs from human donors. Expression of the reported proteins was altered >1.5-fold following LPS exposure in two repeat experiments. “New” designates proteins seen in the LPS gel in two repeat experiments but not detectable in the corresponding control gels.

| Identification [spot #] | Swiss-Prot Accession # | Estimated MW/pI | Theoretical MW/pI | Peptides Matched / Submitted (%) | Protein Covered (%) | Fold Change (X) |
|-------------------------|------------------------|-----------------|------------------|----------------------------------|---------------------|----------------|
| **Upregulated**          |                        |                 |                  |                                  |                     |                |
| Protein tyrosine kinase 9-like [468] | Q9Y3F51 | 34/5.81 | 39.5/6.37 | 10/14 (71%) | 34% | 1.8 |
| Protein phosphatase 1, catalytic subunit, β isoform [378] | P37140 | 38/5.73 | 37.2/5.84 | 7/10 (70%) | 22% | 2.0 |
| PO4-stathmin [577] | P169492 | 18/5.36 | 17.3/5.76 | 9/12 (75%) | 42% | 2.15 |
| Nonmuscle myosin heavy chain [1102] | 1890363 | 145/5.32 | 145/5.23 | 20/21 (95%) | 17% | New |
| Putative PO4-nonmuscle myosin heavy chain [1101]4 | 1890362,3 | 145/5.29 | 145/5.23 | 14/16 (87%) | 13% | New |
| Leukocyte elastase inhibitor [318] | P30740 | 42/5.71 | 42.7/5.9 | 9/13 (69%) | 22% | 2.4 |
| Grancalcin [1004] | P28676 | 24/5.36 | 24.0/5.02 | 7/10 (70%) | 31% | New |
| **Downregulated**        |                        |                 |                  |                                  |                     |                |
| Adenosylhomocysteinase [324] | P23526 | 48/5.82 | 47.7/6.04 | 7/9 (78%) | 14% | 0.4 |
| PEST phosphatase interacting protein homolog [234]7 | 41001626 | 48/5.30 | 47.6/5.35 | 11/13 (85%) | 30% | 0.5 |

1TrEMBL accession number.
2Accession number and theoretical pI and MW for the unmodified protein are indicated.
3NCBI accession number.
4See text for explanation.
5Among three experiments, the ratio of PO4-stathmin expression increase following LPS exposure in the presence of SB203580 divided by that in the absence of SB203580 was 0.93.
6Genpept accession number.
7This search was performed using average masses measured by linear mode MALDI-TOFMS. Mascot search (http://www.matrixscience.com) was not possible as software does not link to Genpept database.
Table V. 
Analysis of pI 5.5-6.7 2-D PAGE gels. Results are from pooled samples for control (n=3) and LPS-exposed (n=3) PMNs from human donors. Expression of the reported proteins was altered >1.5-fold following LPS exposure in two repeat experiments.

| Identification [spot #] | Swiss-Prot Accession # | Estimated MW/pl | Theoretical MW/pl | Peptides Matched/Submitted (%) | Protein Covered (%) | Fold Change (X) |
|-------------------------|------------------------|-----------------|-------------------|-------------------------------|---------------------|-----------------|
| **Upregulated**         |                        |                 |                   |                               |                     |                 |
| Transaldolase [475]     | P37837                 | 38/5.95         | 37.5/6.36         | 13/17 (76%)                   | 33%                 | 2.5             |
| Isocitrate dehydrogenase [431] | O75874               | 46/6.25         | 46.7/6.35         | 7/7 (100%)                    | 13%                 | 2.3             |
| Moesin [201]            | P26038                 | 61/6.09         | 67.8/6.07         | 11/13 (85%)                   | 17%                 | 2.1             |
| α-enolase [459]         | P06733                 | 43/5.64         | 47.2/7.01         | 7/10 (70%)                    | 17%                 | 3.8             |
| **Downregulated**       |                        |                 |                   |                               |                     |                 |
| Calponin H2 [240]       | Q99439                 | 34/6.65         | 33.7/6.94         | 10/11 (90%)                   | 27%                 | 0.5             |
Table VI.
Effect of SB203580 on LPS-stimulated gene expression. Genes are reported for which the SB203580/control expression ratio is ≤ 0.60.

| Gene name   | Fold Change Ratio (SB203580/Control) | Fold Change (X) in Absence of SB203580 |
|-------------|--------------------------------------|----------------------------------------|
| ISG15       | 0.09                                 | 22.5                                   |
| HCR         | 0.38                                 | 20.8                                   |
| Mx-1        | 0                                    | 19.4                                   |
| IFI56       | 0                                    | 12.1                                   |
| PI-9        | 0.57                                 | 9.5                                    |
| Ets-2       | 0.59                                 | 7.4                                    |
| IL-6        | 0.45                                 | 6.3                                    |
| Rel         | 0.50                                 | 6.2                                    |
| LIMS1       | 0.58                                 | 6.1                                    |
| C3AR1       | 0.49                                 | 6.1                                    |
| INDO        | 0.35                                 | 5.2                                    |
| KIAA0105    | 0.41                                 | 5.1                                    |
| SNAP23      | 0.58                                 | 5.0                                    |
| SLPI        | 0.58                                 | 4.7                                    |
| ELNAH2      | 0.49                                 | 4.6                                    |
| HM-74       | 0.57                                 | 3.8                                    |
| PKR         | 0                                    | 3.7                                    |
| MAD         | 0.21                                 | 3.6                                    |
| IFIT4       | 0.12                                 | 3.6                                    |
| Glycerol kinase | 0                             | 3.6                                    |
| IFI54       | 0                                    | 3.5                                    |
| IFI58       | 0.39                                 | 3.5                                    |
| IPF35       | 0.46                                 | 3.0                                    |
| Protein name                          | Fold Change Ratio | Fold Change (X) in Absence of SB203580 |
|--------------------------------------|-------------------|---------------------------------------|
|                                      | (SB203580/Control) | SB203580                              |
| **Upregulated**                      |                   |                                       |
| Proteasome β chain                   | 0.8               | 1.51                                  |
| Annexin III                         | 0.6               | 1.37                                  |
| Actin fragment [544]                 | 0.8               | 1.74                                  |
| Actin fragment [591]                 | 0.8               | 1.60                                  |
| α-enolase                           | 0.6               | 1.65                                  |
| Rab GDP dissociation inhibitor β     | 1.1               | 1.24                                  |
| Glutathione S-transferase P          | 1.2               | 1.54                                  |
| Pre-B-cell colony enhancing factor   | 1.2               | 1.29                                  |
| **Downregulated**                    |                   |                                       |
| Adenylyl cyclase-assoc. protein 1    | 1.3               | 0.53                                  |
| Rho-GAP 1                            | 0.8               | 0.67                                  |
| Ficolin 1                            | 1.0               | 0.74                                  |
Table VIII.
LPS-regulated proteins for which a probe was present on the Affymetrix chip. A comparison of corresponding protein and mRNA transcript changes following LPS exposure is shown. NC designates no measurable change.

| Protein                                             | Protein Fold Change (X) | mRNA Fold Change (X) |
|-----------------------------------------------------|-------------------------|----------------------|
| **Upregulated**                                     |                         |                      |
| Proteasome β chain                                  | 1.5                     | 1.9 ↑                |
| Leukocyte elastase inhibitor                        | 2.4                     | 4.6 ↑                |
| Rab GDI β                                           | 1.24                    | NC                   |
| Grancalcin                                          | New                     | NC                   |
| Transaldolase                                       | 2.5                     | NC                   |
| Moesin                                              | 2.1                     | NC                   |
| Nonmuscle myosin heavy chain                        | New                     | NC                   |
| Glutathione S-transferase P                         | 1.54                    | Absent               |
| Pre-B cell enhancing factor                         | 1.29                    | Absent               |
| Isocitrate dehydrogenase                            | 2.3                     | Absent               |
| PO4-stathmin                                        | 2.1                     | Absent (stathmin)    |
| Protein phosphatase 1, β catalytic subunit          | 2                       | Absent               |
| Annexin III                                         | 3.1                     | 3.1 ↓                |
| **Downregulated**                                   |                         |                      |
| Adenylyl cyclase-associated protein 1               | 1.9                     | 2.1 ↓                |
| Rho-GAP 1                                            | 1.5                     | 2.7 ↓                |
| Ficolin 1                                            | 1.4                     | 1.7 ↓                |
| Adenosylhomocysteinase                              | 2.5                     | Absent               |
| Calponin H2                                         | 2                       | NC                   |
Figure 1.
Figure 2.

A

\[ \text{ASGQAFELILSPR} \]

\[ 15 \quad 27 \]

B

Control

PO4-stathmin \quad \text{Stathmin}

LPS-exposed

PO4-stathmin \quad \text{Stathmin}
A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase
Michael B. Fessler, Kenneth C. Malcolm, Mark W. Duncan and G. Scott Worthen

J. Biol. Chem. published online April 9, 2002

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