Journal of Cellular Biochemistry 89:848–861 (2003)

Gene Expression in Human Neutrophils During Activation and Priming by Bacterial Lipopolysaccharide

Yasuhiro Tsukahara,[1] Zheng Lian,[1] Xueqing Zhang,[2] Constance Whitney,[2] Yuval Kluger,[3] David Tuck,[4] Shigeru Yamaga,[1] Yasuhiro Nakayama,[1] Sherman M. Weissman,[1] and Peter E. Newburger[2]*

[1]Department of Genetics, Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT
[2]Department of Pediatrics and Cancer Center, University of Massachusetts Medical School, Worcester, MA
[3]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT
[4]Center for Medical Informatics, Yale University School of Medicine, New Haven, CT

Abstract Circulating neutrophils play a key role both in the systemic inflammatory response and in complications of bacterial infection such as septic shock and septic multiple organ dysfunction syndrome. We have analyzed gene expression patterns in human neutrophils stimulated by *E. coli* lipopolysaccharide (LPS), with or without prior exposure to LPS, using differential display and oligonucleotide chip techniques. We identified 307 genes that were activated or repressed after treatment with LPS at 10 ng/ml and 385 genes after LPS at 100 ng/ml, compared with untreated neutrophils. The two sets included many transcription factors, cytokines, chemokines, interleukins, and surface antigens, as well as members of the toll-like receptor, Rel/NF-κB, and immune mediator gene families. Time course analysis showed that the early and late neutrophil responses to LPS share some common mechanisms, but many changes in gene expression are transient or late to develop. Neutrophils also showed a priming response to LPS, in which 97 genes significantly changed expression on re-exposure to lower dose LPS and were analyzed by unsupervised hierarchical clustering. These findings indicate that the neutrophil is a transcriptionally active cell responsive to environmental stimuli and capable of a complex series of both early and late changes in gene expression. Supplementary material for this article can be found on the Journal of Cellular Biochemistry website (http://jws-edci.interscience.wiley.com:8998/jpages/0730-2312/suppmat/89/v89.page.html). J. Cell. Biochem. 89: 848–861, 2003. © 2003 Wiley-Liss, Inc.

Key words: adhesion; chemokine; granulocyte; interleukin; microarray; myeloid; NF-kappaB; toll-like receptor; tumor necrosis factor

Sepsis syndrome, including septic shock and multiple-organ dysfunction syndrome, is the leading cause of death in intensive care units with a mortality rate of 20–50% [Bone et al., 1997] and the incidence of sepsis continues to increase, according to the Centers for Disease Control and Prevention (http://www.eurekalert.org/pub_releases/2002-05/euhs-so051502.php). Present theories of sepsis syndrome pathophysiology propose that it derives from a systemic inflammatory reaction with massive cytokine release from various types of cells such as macrophages, endothelial cells, and neutrophils. However analyses of data from septic patients show that a complex mixture of proinflammatory and anti-inflammatory cytokines may be present. Therefore, the precise pathogenesis of the sepsis syndrome remains unclear.

Circulating neutrophils play a key role both in the systemic inflammatory response and in complications of bacterial infection such as septic shock and septic multiple organ dysfunction syndrome. Bacterial products such as formyl peptides and lipopolysaccharide (LPS) as well as cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-8, and granulocyte–macrophage colony stimulating factor

Yasuhiro Tsukahara and Zheng Lian contributed equally to this research.

Grant sponsor: U.S. National Institutes of Health; Grant number: R01-DK54369; Grant sponsor: The Arthritis Foundation; Grant sponsor: The John H. Pierce Pediatric Cancer Research Fund.

*Correspondence to: Peter E. Newburger, University of Massachusetts Medical School, Cancer Center LRB 404, Worcester, MA 01655.
E-mail: peter.newburger@umassmed.edu

Received 26 February 2003; Accepted 27 February 2003
DOI 10.1002/jcb.10526

© 2003 Wiley-Liss, Inc.

can prime or activate neutrophils to release reactive oxygen intermediates and inflammatory cytokines which may cause tissue injury and septic shock [Dianzani et al., 2001; Mollapour et al., 2001; Neufert et al., 2001].

The life span of circulating mature neutrophils is 6–10 h. After this time, the cells migrate into tissue or undergo apoptosis that minimizes the release of lytic enzymes and proinflammatory molecules. Previous studies have shown that LPS prolongs neutrophil survival by inhibiting apoptosis and primes the neutrophil oxidative burst; both effects may contribute to tissue injury [Condliffe et al., 1998; Sweeney et al., 1998]. On the other hand, tolerance to LPS induced by repeated pretreatment with LPS has been reported to reduce spontaneous activation and adhesion of circulating neutrophils [Barroso-Aranda et al., 1994; Sweeney et al., 1998]. In addition, endotoxin tolerance appeared to decrease the release of IL-1 and IL-8 from neutrophils [Marie et al., 1998; McCall et al., 1993]. Thus, LPS elicits varying functional responses in neutrophils, depending upon the dosage and schedule of exposure.

Analysis of the pattern of the functional changes of neutrophils stimulated with LPS can be useful for investigation of the pathogenesis of sepsis. In a previous study using a differential display method, we reported a comprehensive study of changes in mRNA expression patterns in human neutrophils following exposure to opsonized *E. coli* [Subrahmanyam et al., 2001]. The results indicated that active regulation of gene expression plays a major role in the neutrophil's contribution to cellular inflammatory responses. In the present study, we have analyzed gene expression patterns in human neutrophils stimulated by *E. coli* LPS, with or without prior priming by LPS, and have compared the data with those from neutrophils exposed to *E. coli* in order to gain insight into the primed state of neutrophils and evaluate the relative effects of LPS and whole bacteria.

MATERIALS AND METHODS

Reagents

RPMI 1640 and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD); LPS (*E. coli* serotype 026:B6) and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO); Lymphoprep from Mycomed (Oslo, Sweden); RNA wiz from Ambion (Austin, TX), cytochrome *c* from Boeringer Mannheim (Mannheim, Germany); and restriction enzymes (*Bgl*II, *Sac*I, *Apa*I) from New England Biolabs, Inc. (Beverly, MA). All reagents (except LPS), serum, buffers, and media were free of LPS (<0.01 ng/ml by limulus amoebocyte lysate assay; Sigma).

Preparation of Human Neutrophils

Neutrophils were isolated from freshly drawn venous blood of healthy male volunteers (30–40 years old) using dextran sedimentation, centrifugation through ficoll-Hypaque (Lymphoprep), and very brief hypotonic lysis of erythrocytes as previously described [Subrahmanyam et al., 1999]. Morphologically, the neutrophil preparations were more than 99% pure except for the presence of 1–3% eosinophils. Monocyte content was $<0.5\%$ by light microscopy.

Incubation of Neutrophils With LPS

Neutrophils (2.5×10^6 cells/ml) were incubated by shaking at 37°C in RPMI 1640 with 10% heat-inactivated fetal bovine serum in the following experimental groups (Fig. 1). Group 1 (normal control): neutrophils were incubated without LPS exposure for 2 h. Group 2 (continuous exposure to LPS): neutrophils were incubated with LPS (100 ng/ml) for 2 h. Group 3 (LPS priming): neutrophils were incubated with LPS (100 ng/ml) for 30 min, then LPS was washed out, and the cells incubated for 1 h and then exposed to LPS (10 ng/ml) for 30 min. Group 4 (priming control): neutrophils were incubated with LPS (100 ng/ml) for 30 min. Group 5 (priming control): neutrophils were incubated for 90 min and then exposed to LPS (10 ng/ml) for 30 min. Group 6 (priming control): neutrophils were incubated with LPS (100 ng/ml) for 30 min, then LPS was washed out, and the cells incubated for 90 min, group 7: neutrophils were incubated without LPS for 90 min, then with LPS (100 ng/ml) for 30 min, group 8: neutrophils were incubated with LPS (100 ng/ml) for 120 min, group 9: neutrophils were incubated with LPS (100 ng/ml) for 30 min, washed out with medium, and incubated for 90 min, then re-exposed to LPS (10 ng/ml) for additional 30 min.

In addition, the differential display experiments also included group 10 (not shown in

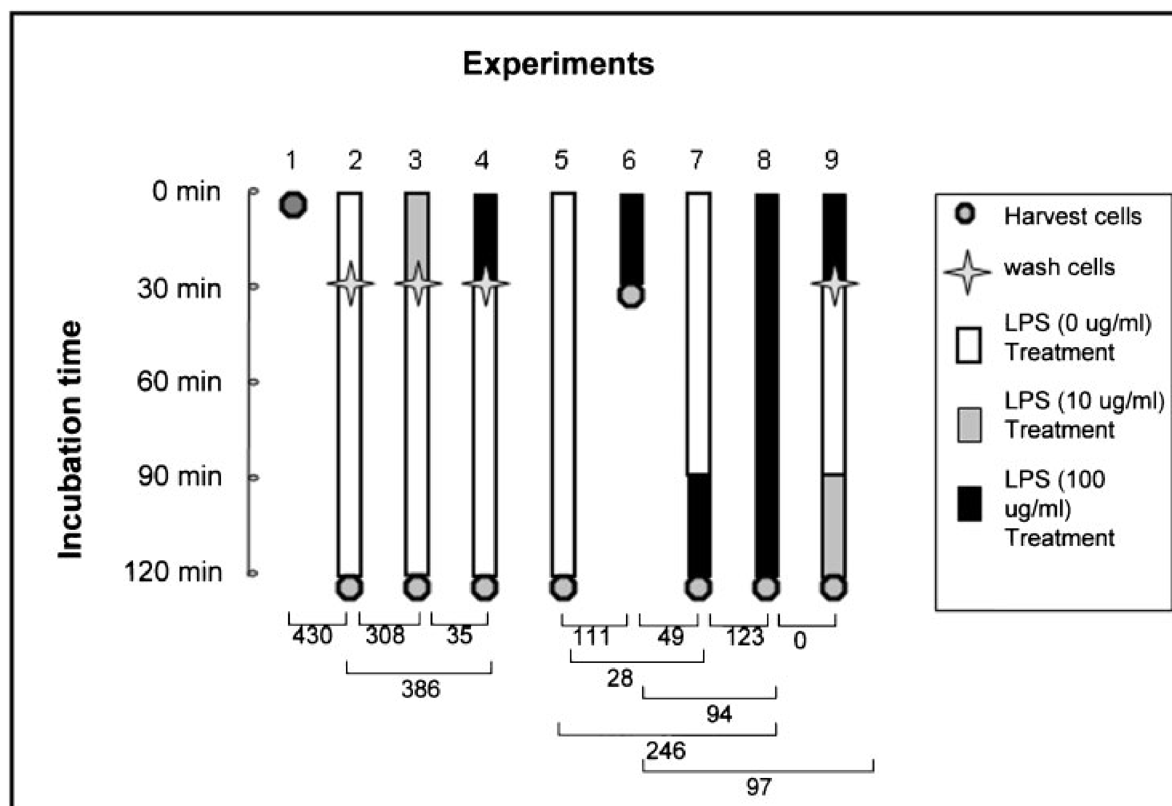

Fig. 1. Experimental design. Graphical representation of six experimental conditions: group 1, time 0 control-neutrophils harvested at time 0 without incubation; group 2, incubation/wash control-neutrophils were incubated without LPS exposure for 2 h and washed at 30 min. Group 3, neutrophils were incubated with LPS (10 ng/ml for 30 min, then LPS was washed out and the cells incubated for 90 min; group 4, neutrophils were incubated with LPS (100 ng/ml) for 30 min, then LPS was washed out, and the cells incubated for 90 min; group 5, incubation control-neutrophils were incubated without LPS exposure for 2 h; group 6, neutrophils were incubated with LPS (100 ng/ml) for 30 min. Group 7, neutrophils were incubated for 90 min and then exposed to LPS (100 ng/ml) for 30 min; group 8, neutrophils were incubated with LPS (100 ng/ml) for 2 h; group 9, neutrophils were incubated with LPS (100 ng/ml) for 30 min, then LPS was washed out, and the cells incubated for 1 h and then exposed to LPS (10 ng/ml) for 30 min. The **bottom** of the figure shows the numbers of differentially expressed genes in comparisons of the indicated pairs of experiments.

figure): neutrophils were incubated for 90 min and then exposed to LPS (10 ng/ml) for 30 min.

RNA Isolation and Differential Display

Total cellular RNA was isolated from neutrophils using RNAwiz reagent (Ambion) or guanidine HCl as previously described [Subrahmanyam et al., 1999]. Three to six micrograms of total RNA was used for cDNA synthesis of each experimental group. The cDNA first strand was synthesized using a T-7 Sal-Oligo d(T) primer as described previously [Subrahmanyam et al., 2001]. The double-stranded cDNA was digested with one of three different restriction enzymes (*Apa*I, *Bgl*II, and *Sac*I) and ligated to Y-shaped adaptors with a complementary overhang. DNA fragments were then amplified by PCR with gamma-^{32}P-labeled primer as described previously [Subrahmanyam et al., 2001]. PCR products were separated on a sequencing gel of 6% polyacrylamide. The gel was dried and exposed to X-ray film. Differential display gel bands whose maximum intensity changes exceeded or equaled $\Delta 2$, on a scale of 1+ to 8+, were recorded as significantly changed and the bands recovered by PCR and sequenced. Most of the band intensities were quantified by eye and confirmed by a second investigator; a subset of bands were quantified by Phosphoimager. We have previously confirmed the correlation between band intensities by eye and by Phosphoimager [Subrahmanyam et al., 2001].

Classification and Analysis of DNA Fragments

Sequencing data from differential display analyses were searched against nr and dbest databases of the National Center for Biotechnology Information (NCBI) by the Basic Local Alignment Search Tool (BLAST) and were classified as known genes, ESTs, genomic sequences, or novel genes as described [Subrahmanyam et al., 2001]. Known genes from differential display were clustered into 27 functional groups. Information such as function, subcellular location, family and superfamily classification, map position, gene name(s), etc., was recorded in a database using Access (Microsoft, Redmond, WA).

We used LOCUS link ID as a unique key to known genes and used the terms listed as gene symbol and gene name to identify the gene. Public gene database searches were completed in August 2001.

Oligonucleotide Chip Analysis of RNA Samples

Ten micrograms of total RNA from each sample was used to prepare cDNA, which was transcribed with T7 RNA polymerase to prepare a fluorescent-labeled probe, using standard methods [Lipshutz et al., 1995; Chee et al., 1996]. Each sample was hybridized to human array chip (Human GeneChip HG_U95Av2 chips), interrogating approximately 10,000 full-length genes and EST clusters from the UniGene database. A database of genes expression patterns during neutrophil priming was constructed based on differential display and Affymetrix analysis. The gene expression patterns of neutrophil priming program induced by LPS were analyzed with DNA-Chip Analyzer (dChip) (http://www.dchip.org/, Harvard University)

RESULTS AND DISCUSSION

Global Analysis of LPS-Induced Neutrophil Response Programs

In this study, we exposed human neutrophils, which were isolated from freshly drawn venous blood of healthy volunteers, to several schedules of LPS treatment. The experimental design is shown in Figure 1. LPS doses were 0 μg/ml (control dose), 10 μg/ml (lower dose), and 100 μg/ml (higher dose) over periods of 0, 30, 60, 90, and 120 min. Two groups of experiments were performed with blood from different donors. There were four conditions tested (groups 1, 2, 3, and 4) in group 1, and five (groups 5, 6, 9, 7, and 8) in group 2. Groups 1, 2, and 5 are negative control experiments in which the neutrophil cells were incubated in medium without LPS and harvested at different time points. Groups 3, 4, 6, 7, 8, and 9 examined neutrophil priming and activation. To avoid differences in responses between blood donors, we compared the samples only within each group.

In this study, we first compared the gene expression patterns between two different "control" neutrophil groups: one in which RNA was extracted immediately after cell preparation, and the other in which the neutrophils were cultured for 2 h under the same experimental conditions as the other groups, but without LPS exposure. In an oligonucleotide array analysis, 430 genes showed a change in expression levels of twofold or more. This large difference suggested that the simultaneously incubated group should be used as the negative control for the LPS incubations in this study.

To examine the effect of LPS dose, we treated neutrophils with 10 or 100 μg/ml LPS for 30 min, then washed out the LPS, and continuously incubated the cells in LPS-free medium for additional 90 min. Comparison of the expression patterns of neutrophils treated with lower dose or higher dose LPS (Fig. 1, groups 3 and 4), revealed only 33 genes differentially expressed (Table I), suggesting that there is little effect of LPS dose on the priming responses of neutrophils. On the other hand, the gene expression patterns of both the higher and lower dose LPS treatment group are significantly different from that of the control (groups 3 and 4, compared with group 2.). Using a twofold change as a cutoff, 307 genes were activated or repressed after lower dose LPS treatment and 385 genes after higher dose LPS, compared with untreated neutrophils. The two sets included many members of the interleukin, small inducible cytokine, and surface antigen families.

LPS-Induced Neutrophil Activation Program

To study responses to intermittent exposures to LPS, we compared gene expression patterns of neutrophils cultured with higher dose LPS in several temporal patterns. First, to test the effects of time in culture, we compared RNA extracted from neutrophils immediately after incubation with LPS for 30 min (group 6 in Fig. 1), with that of cells incubated in LPS-free

TABLE I. Human Neutrophil Genes Activated by LPS (100 ng/ml) for 30 min

Category	Gene	Accession number	Fold change
Chaperon			
	Heat shock 90 kDa protein 1, beta	W28616	2.23
Transporter			
	Suppressor of K^+ transport defect 1	AF038960	2.34
Transcription regulators			
	Oligodendrocyte lineage transcription factor 2	U48250	4.89
	B-cell CLL/lymphoma 10	AJ006288	3.22
	Signal transducer and activator of transcription 2, 113 kDa	U18671	3.02
	GA binding protein transcription factor, beta subunit 1 (53 kDa)	U13045	2.92
	MAX dimerization protein	L06895	2.65
	Cold shock domain protein A	M24069	2.42
	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	U65093	2.38
	Ell-related RNA polymerase II, elongation factor	U88629	2.36
Signaling			
	GTP binding protein overexpressed in skeletal muscle	U10550	2.97
	Guanine nucleotide binding protein (G protein), q polypeptide	U40038	2.39
	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	U71364	2.16
	TGFB inducible early growth response	S81439	2.06
Receptor			
	Prostaglandin E receptor 2 (subtype EP2), 53 kDa	U19487	2.21
	Prostaglandin E receptor 4 (subtype EP4)	L25124	2.19
	Eukaryotic translation initiation factor 2, subunit 3 (gamma, 52 kDa)	L19161	2.76
Metabolism			
	PAI-1 mRNA-binding protein	AL080119	2.79
	DEAD/H (Asp−Glu−Ala−Asp/His) box polypeptide 3	U50553	2.43
	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	D49817	2.27
Cytokine			
	Vascular endothelial growth factor	M63978	2.37
	Oncostatin M	M27288	2.18
Apoptosis			
	Caspase 8, apoptosis-related cysteine protease	X98175	3.37
	Testis enhanced gene transcript	W28869	2.51
	XIAP associated factor-1	X99699	2.47
Other			
	KIAA1451 protein	AL049923	3.29
	Growth arrest and DNA-damage-inducible, beta	N95168	2.94
	KIAA1096 protein	AI307607	2.37
	DKFZP564O123 protein	AL080122	2.14
	Interferon-induced protein with tetratricopeptide repeats 2	M14660	2.08
	Mitogen-activated protein kinase-activated protein kinase 2	X75346	2.25
	Glucosamine (*N*-acetyl)-6-sulfatase	Z12173	2.42
	A disintegrin and metalloproteinase domain 17	U69611	2.82

Gene accession numbers and gene names refer to National Center for Biotechnology Information database.

medium for 90 min prior to exposure to LPS for 30 min (experiment 7). There were 49 genes whose expression differed in the two groups. Comparison of each group with control neutrophils (experiment 5) revealed fewer differences between neutrophils in experiments 7 vs. 5 (28 genes) (Fig. 2) than between experiments 6 vs. 5 (111 genes) (Fig. 3). This relative difference suggests that changes in gene expression continue to develop after withdrawal of LPS. Of the genes which showed altered expression within 30 min by LPS exposure, seven are involved in apoptosis and seven encode signal transduction molecules. However, after treatment with LPS for 120 min, many more genes (246) were activated or repressed. This increase over time probably represents both an accumulation of early and later response genes together with the activation of downstream targets of very early response genes.

Neutrophils play an essential role in the body's innate immune response to infection. Therefore, a thorough understanding of the neutrophil activation program provides a foundation to understand the mechanisms of host defense against invading microorganisms. In this study, we have identified 246 genes that are differentially expressed on exposure to LPS (detailed data are presented in the supplementary materials at (http://jws-edci.interscience.wiley.com:8998/jpages/0730-2312/suppmat/89/v89.page.html). Analysis of the gene expression patterns in this "neutrophil activation program" showed that 74 genes were increased and 172 were repressed after 120 min of LPS simulation. Functional assignments include

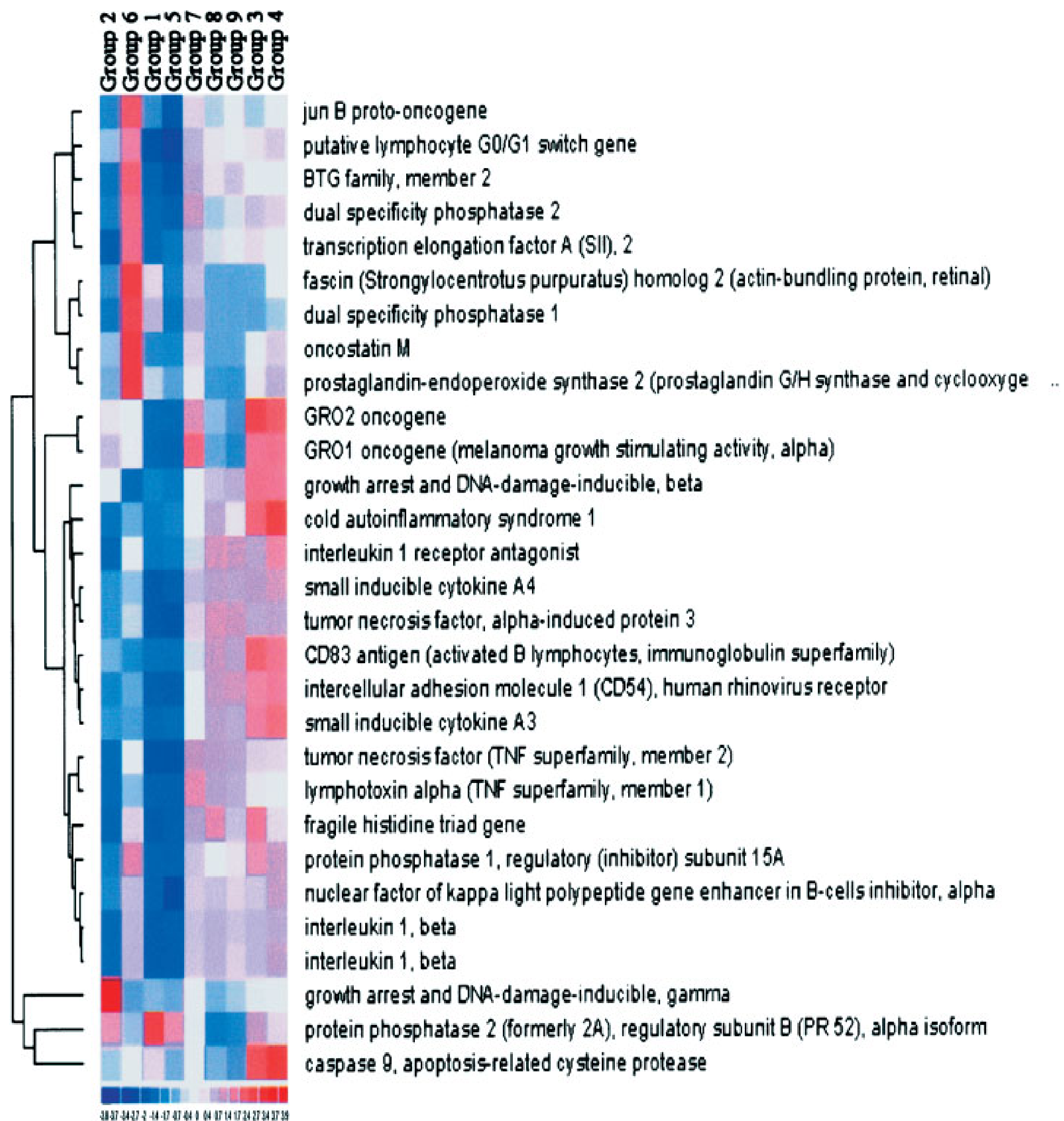

Fig. 2. Gene clustering of the differentially expressed genes in neutrophils after delayed treatment with LPS. Human neutrophils were incubated in LPS-free medium for 90 min, then with LPS (100 ng/ml) for additional 30 min (group 7 in Fig. 1). The gene expression profile was compared with that of control cells without LPS treatment (group 5) by dChip analysis of Affymetrix oligonucleotide chip data. The genes are identified for which the minimal AD values were $\geq$ 200 U and the AD difference between groups was $\geq$ two fold. Twenty-eight filtered genes are clustered based on their expression profiles across groups 5 and 7 as noted at the **top margin**; their expression patterns across the other seven groups are also presented. Dark blue represents a low expression level and dark red, a high expression level, as indicated on the scale at the **lower margin**.

87 genes involved in the cell communication, 61 signal transduction genes, and 35 immune response genes. During neutrophil activation, 24 transcription factors were identified as differentially expressed, indicating that transcriptional regulation plays an active role in the neutrophil response to LPS. The majority of these transcription factors were down-regulated, including retinoic acid receptor alpha and hematopoietically expressed homeobox, which play important roles in the myelopoiesis program as well. Among the nine up-regulated transcription factors, the expression patterns of several genes—such as Stat5a, BTG family

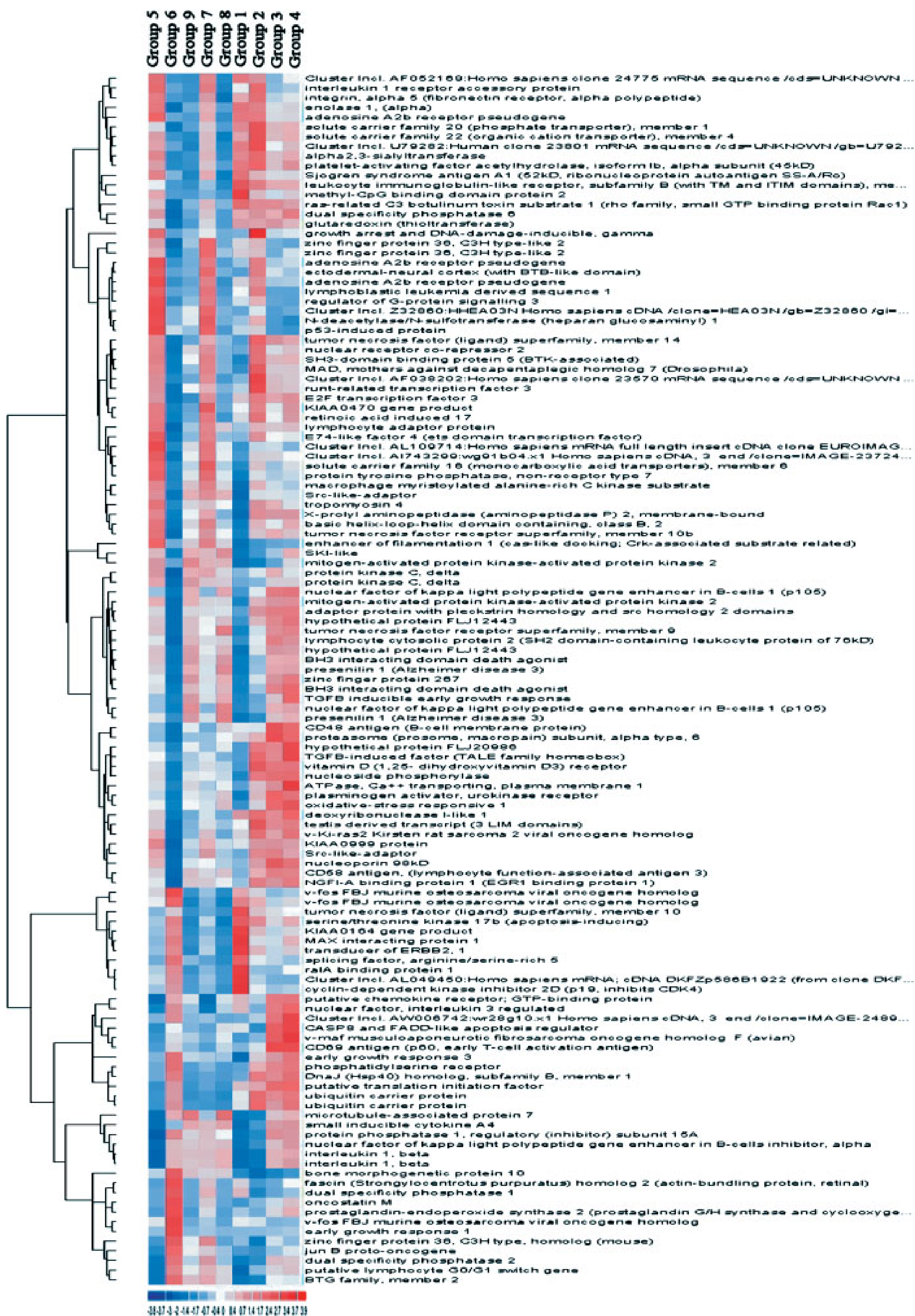

Fig. 3.

member 2, and nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) 1—were consistent with previous reports [Saban et al., 2001].

The neutrophil activation program also includes multiple genes that modulate apoptosis [Kobayashi et al., 2002]. We identified 17 differentially regulated genes involved in apoptosis, including caspase 8, apoptosis-related cysteine protease, and FADD-like apoptosis regulator. Another molecular marker, the apoptosis-related gene phosphatidylserine decarboxylase was significantly down-regulated.

In addition to full activation by LPS, neutrophils show a priming response, in which an initial exposure to LPS results in an enhancement of subsequent activation by LPS or another agonist. Analysis of neutrophil gene expression data for experiment 9, compared to experiment 6, showed that the expression of 97 genes significantly changed on re-exposure to lower dose LPS. Unsupervised hierarchical clustering of these genes revealed several prominent groups (Fig. 4). Of the 47 up-regulated genes and 50 down-regulated genes, 40 have functions related to cell communication and 23 belong to immune or defense response gene categories such as 1IL-8r, NF-κB1**,** toll-like receptor (TLR) 1, and members of the small GTPase family. Several structural protein and chaperon genes, as well as signal transduction genes, were repressed. Interestingly, comparison of response patterns in experiments 9 (rechallenge at lower dose) and (continuous exposure to higher dose) showed that there was no difference at the end points of the two modes of stimulation.

The differential display method was used in this study not only to confirm the Affymetrix data, but also to develop more information which is excluded by the latter technique. With the differential display assay, we repeated the experiments of groups 4, 5, 6, 8, as well as an additional group 10 (90 min incubation in medium, then 30 min exposure to LPS (10 ng/ml), using neutrophils from the same donor as the GeneChip experiments. Both groups 4 and 8 were compared with the control (group 5) to extract two groups of differentially expressed genes. Comparing these two groups (Fig. 5), we found that they shared 34 genes that showed similar expression patterns (shown in the central, overlapping segment of the Venn diagram).

Forty-two genes were identified only by differential display after 30 min LPS treatment (Fig. 5, left segment of Venn diagram) and included 29 up-regulated and 13 down-regulated genes. Interestingly, CCAAT/enhancer binding protein (C/EBP) beta showed up-regulation in this setting. The longer incubation with LPS led to the differential expression of 45 other genes (Fig. 5, right segment of Venn diagram). Only 33 genes showed common patterns of regulation (Fig. 5, central segment). This result suggests that the early and late neutrophil responses to LPS share some common mechanisms, but many changes in gene expression are transient or late to develop.

Toll-Like Receptor Gene Family

Members of the TLR gene family have been identified in *Drosophila*, where they are important components of antibacterial and antifungal immunity. The family of TLR genes identified in human cells encodes ten different TLR proteins [Armant and Fenton, 2002; Kaisho and Akira, 2002; Morr et al., 2002]. Five of these genes—TLRs 1, 2, 3, 5, and 6—are represented in the Human GeneChip HG_U95Av2 probe sets. The expression level of TLR3 was too low for detection, but the other four TLRs were found in human neutrophils and differentially expressed in the current LPS stimulation experiments (Fig. 6). TLR4, which also plays a role in the response to LPS [Arbour et al., 2000], was not represented on the GeneChip, but neither the protein nor mRNA were detected by flow cytometry or Northern blot assays in similarly purified neutrophils [Kurt-Jones et al., 2002].

TLR2 is the receptor for a variety of microbial ligands, including peptidoglycan from Gram-positive bacteria, yeast zymosan, and myco-

Fig. 3. Gene clustering of the differentially expressed genes in neutrophils after immediate treatment with LPS. Human neutrophils were incubated in LPS (100 ng/ml) for 30 min (group 6 in Fig. 1). The gene expression profile was compared with that of control cells without LPS (treatment group 5) by dChip analysis of Affymetrix oligonucleotide chip data. Filtering for expression level and change as in Figure 2 identified 111 genes that are clustered here, based on their expression profiles across these two groups. Expression patterns across the other seven groups are also presented. Labels and scaling are as described in the legend for Figure 2.

Fig. 4.

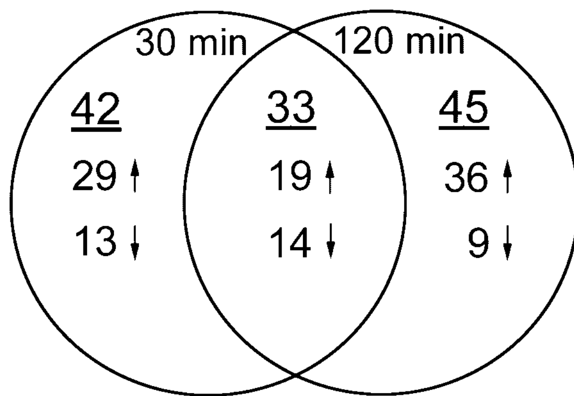

Fig. 5. Distribution of genes identified by differential display. The experiments diagrammed in Figure 1 were repeated with a differential display assay, using cells from the same donor. Groups 4 (30 min LPS incubation) and 8 (120 min LPS incubation) were compared with the control (group 5) to identify differentially expressed genes in the two groups. The Venn diagram presents the numbers of genes identified uniquely by the shorter incubation (**left segment**), the longer incubation (**right segment**), or both conditions (**overlapping segment**), as well as the breakdown of the numbers up-regulated (red) and down-regulated (green).

bacterial ara-lipoarabinomannan (ara-LAM) [Means et al., 2000; Zhang and Ghosh, 2001]. In the present study, we found TLR2 to be the family member with the highest expression level in neutrophils. The identical expression levels of TLR2 in the negative controls of the two different donors makes it is meaningful to compare its patterns between groups. LPS stimulation moderately increased the expression of TLR2, as previously reported [Muzio et al., 2000; Kurt-Jones et al., 2002]. We found that the expression level of TLR2 is LPS dose-dependent (Fig. 3, groups 3 and 4) but not strongly related to stimulation time (groups 9, 7, and 8).

Interestingly, TLR2 is expressed at a higher level in neutrophils in which LPS was removed for 90 min after an initial 30 min incubation, compared to neutrophils which were continuously stimulated or re-stimulated with LPS for the same duration. This finding suggests that TLR2 regulation may also be subject to a down-regulatory feedback in the face of continued LPS exposure.

Several studies suggest that TLR2 functions cooperatively with TLR1 or TLR6 [Hajjar et al., 2001; Kurt-Jones et al., 2002]. The cooperation of TLR family members may add greater specificity or a broader range of ligand recognition capacity to the TLR proteins, and may also enhance their signal transduction capacity. Our study showed an expression pattern for TLR2 divergent from those for TLR1 or TLR6, suggesting possible antagonism of their regulatory mechanisms.

Rel/NF-κB Gene Family

TLR family receptor activation leads to inflammatory responses through the action of transcription factors, particularly of the Rel/NF-κB family. The activation of NF-κB is strongly linked to the inhibition of apoptosis [Caamano and Hunter, 2002], at least in part due to its ability to regulate expression of anti-apoptotic genes such as *TRAF1*, *TRAF2*, *c-IAP1*, *cIAP2*, *IEX-1L*, *Bcl-*x_L, and *Bfl-1/A1* [Wang et al., 1998; Werneburg et al., 2001; Li et al., 2002]. In this study, expression of three NF-κB family members—*NFKB1*, *NFKB2*, and *RELA*— were affected by LPS. The expression patterns of NFKB1 and RELA were parallel to those of TLR2 (Fig. 6), and NFKB2 was slightly different. On the other hand, expression of nuclear factor related to kappa B binding protein (NFRKB), a factor related to inflammatory response and specific RNA polymerase II transcription, showed very low levels of expression and was not affected by LPS.

In unstimulated cells, NF-κB dimers are retained in the cytoplasm in an inactive form as a consequence of their association with proteins in the inhibitor of κB (IκB) family. We detected expression of five IκB genes in this study (Fig. 7, panel A). IKBKB and IKBKG are expressed at very low levels and did not change with LPS stimulation. IKBKAP and NFKBIE transcript levels were differentially regulated, with the former showing a slight decrease under

Fig. 4. Gene clustering of differentially expressed genes in neutrophils after restimulation with LPS. Human neutrophils were incubated with LPS (100 mg/ml) for 30 min (group 6 in Fig. 1), or stimulated with 100 ng/ml LPS for 30 min, washed, and incubated in LPS-free medium for 60 min, then restimulated with 10 ng/ml LPS for 30 min (group 8). The expression patterns were compared by dChip analysis of Affymetrix oligonucleotide chip data. Filtering for expression level and change (as in Fig. 2) identified 97 genes, shown here with clustering based on their expression profiles across these two groups. Expression patterns across the other seven groups are also presented. Labels and scaling are as described in the legend for Figure 2.

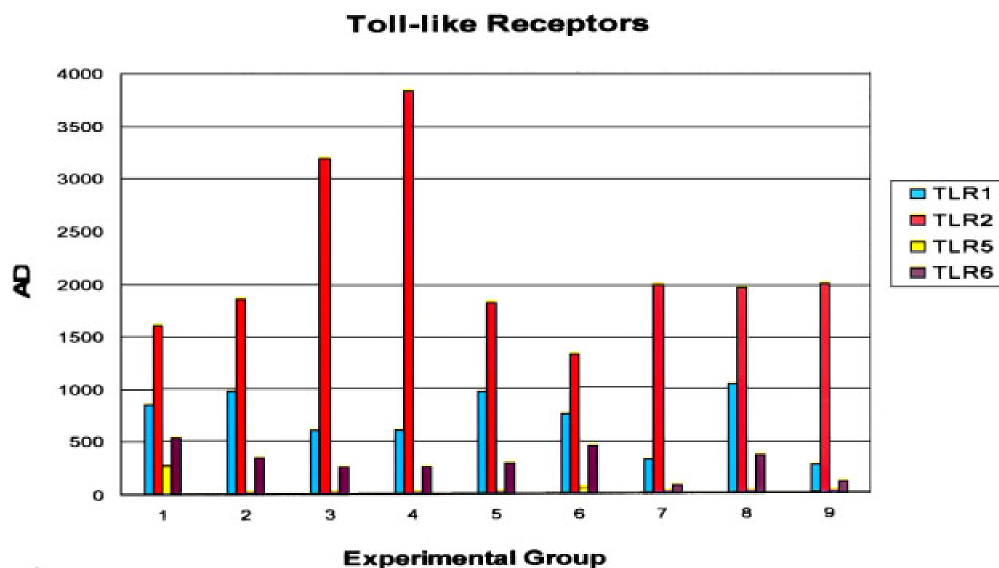

Fig. 6. Expression patterns of genes in the toll-like receptor families in human neutrophils after treatment with LPS. LPS stimulation of human neutrophils were carried out in nine different experimental groups as Figure 1. Gene expression profiles were investigated by DChip analysis of Affymetrix oligonucleotide chip data. AD: indicates average difference. Gene symbols are expanded in the supplementary materials at (http://jws-edci.interscience.wiley.com:8998/jpages/0730-2312/suppmat/89/v89.page.html). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

conditions that increased NF-κB expression and the latter showing a pattern of expression parallel to NF-κB factors, suggesting that it might act as a feedback inhibitor. However, these findings need to be interpreted in the context of rapid, largely post-translational, regulation of the NF-κB system, for which the observed changes in gene expression may provide longer-term "fine tuning" of the response.

Immune Mediator Genes

The activation and nuclear translocation of NF-κB lead to increased transcription of a number of different genes, including those coding for chemokines (e.g., IL-8), adhesion molecules (e.g., endothelial leukocyte adhesion molecules, vascular cell adhesion molecules, and intercellular adhesion molecules), and cytokines (e.g., IL-1, IL-2, TNF-α, and IL-12). These immune mediators are important components of the innate immune response to invading microorganisms and are required for the ability of inflammatory cells to migrate into areas of inflammation. In this study, IL-8 was slightly induced by LPS, but not significantly changed among the different groups (Fig. 7, panel B). IL-1B and IL-6 were induced early after treatment with both lower and higher dose LPS, whereas the expression level of IL-6 was hard to detect without stimulation. Of the six chemokine family members detected by the Affymetrix array (Fig. 7, panel D), only HM74 showed an expression pattern like those of the NF-kB family.

Adhesion molecules form another category of NF-κB target genes; Figure 7, panel C, shows

Fig. 7. Expression patterns of genes in the NF-κB, interleukin, chemokine, and adhesion molecule families in human neutrophils after treatment with LPS. LPS stimulation of human neutrophils were carried out in nine different experimental groups as in Figure 1. Gene expression profiles were investigated by dChip analysis of Affymetrix oligonucleotide chip data. **Panel A**: *NF-κB* gene family. **Panel B**: interleukin gene family members. **Panel C**: chemokine gene family members. **Panel D**: genes encoding adhesion molecules. Gene symbols are expanded in the supplementary materials at http://jws-edci.interscience.wiley.com:8998/jpages/0730-2312/suppmat/89/v89.page.html.

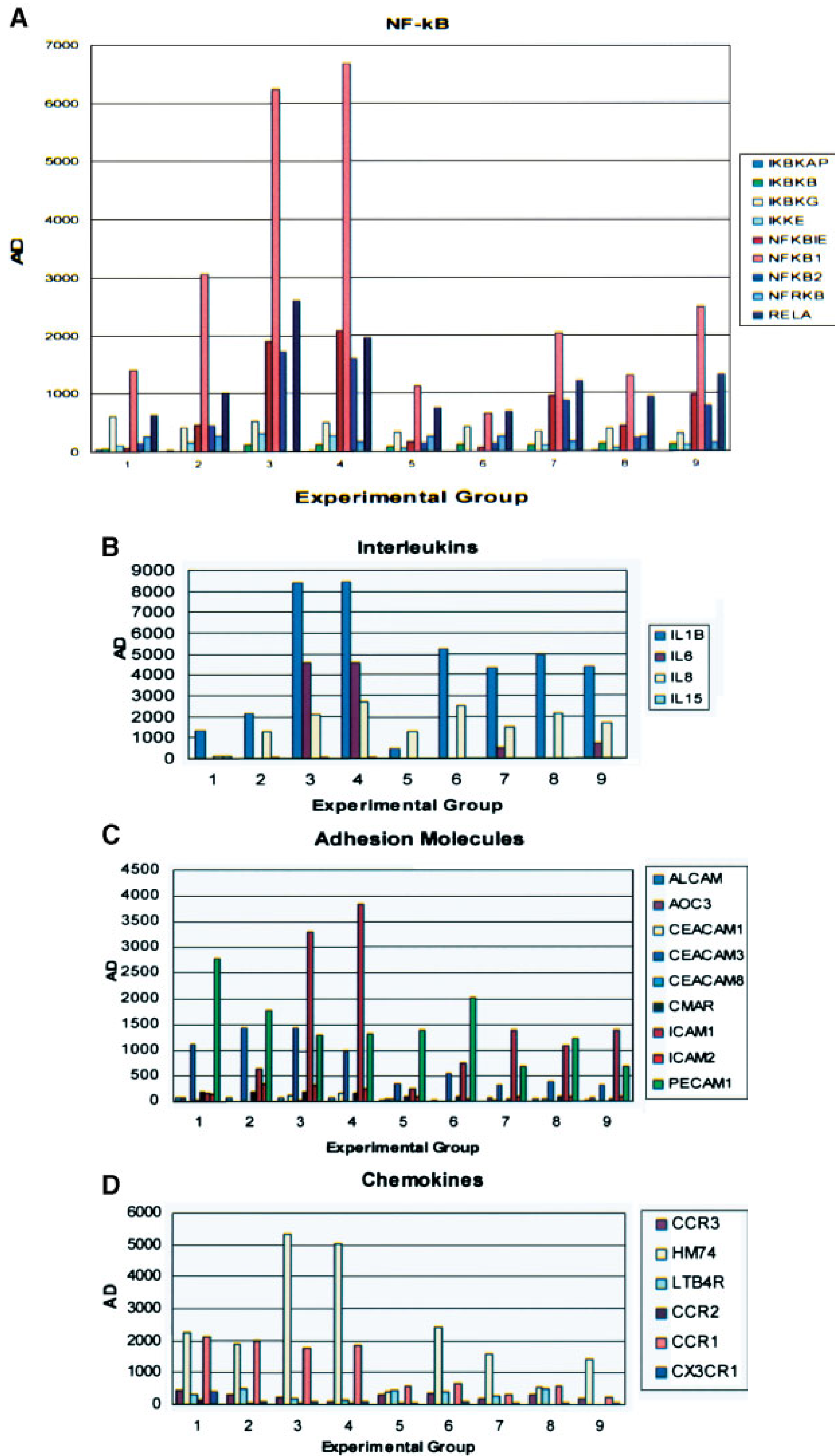

Fig. 7.

the expression patterns of several of these molecules in the current experiments. Intercellular adhesion molecule 1 (ICAM1) showed an expression pattern very similar to that of NF-κB, but platelet/endothelial cell adhesion molecule (PICAM1) responded very differently.

Fifteen TNF family members were detected as present in human neutrophils: TNFAIP1, TNFRSF8, TNFRSF9, TNFRSF10D, TNFRSF12, TNFSF8, and TNFSF13 are all expressed at very low levels but TNFAIP2 and TNFRSF1B showed expression patterns similar to NF-kB (see supplementary Fig. 1 in the supplementary materials online at http://jws-edci.interscience.wiley.com:8998/jpages/0730-2312/suppmat/89/v89.page.html).

Comparison of Differential Display and Oligonucleotide Chip Data

The differential display method was used to confirm the neutrophil RNA expression data derived from Affymetrix assay. Two restriction enzymes were used in a 3′-end differential display approach. Totally, 371 fragments corresponding to 180 transcripts were found to change substantially in expression levels. These represented approximately 150 known genes, 30 ESTs, each with a perfect or fair polyadenylation signal at an appropriate distance from the oligo-dT priming site. Among the 150 known genes, 71 were identified present, and 79 of them were absent by Affymetrix assay. Previous results in our laboratory [Lian et al., 2001] and others [Dong et al., 2001] indicate that the oligonucleotide chip assay may be a less sensitive way to detect changes in levels of gene expression than differential display or northern blot assays. Our current data offer additional evidence that the oligonucleotide chip assay may be less sensitive than the differential display method; even though, the oligonucleotide chip assay method is still a powerful technique for fast and effective studies of mRNA gene expression patterns.

Among the 71 present genes detected by both the oligonucleotide chip assay and differential display methods, 24 showed average difference (AD) values between mismatch and perfect match oligonucleotide sets of less than 100, and 15 showed at least one AD value between 100 and 200. The AD values of these 39 genes were too low to accurately compare their expression pattern changes [Lian et al., 2001]. We compared the expression of 32 genes which have at least one AD value more than 200. Twelve of these genes showed no significant changes (less than twofold) by oligonucleotide chip assay, but at least 3.5 fold changes by the differential display method. Fifteen out of 20 (75%) differentially expressed genes showed the same upward/downward trends by the two methods, only 5 (25%) of these genes showed opposing trends. These results suggest that a majority of the data acquired by oligonucleotide chip or differential display assay agree, but the differential display method maybe more sensitive [Dong et al., 2001; Lian et al., 2001]. Also, recent studies (Gingeras Science, Weissman and Snyder submitted) show there is much more transcriptional activity in animal cells than predicted from recognizable protein coding genes. These extra transcripts would be detected by differential display but not by oligonucleotide chips.

To detect the reproducibility and the variety of the expression of human neutrophil genes among various individuals and various batches of experiments, we repeated the four different sets of oligonucleotide array data from four different donors. Although the general correlation coefficient factors ranges between groups were from 0.6 to 0.8, we found that the correlation for cytoskeletal genes alone rose to 0.85–0.96, highlighting the importance of analyzing mRNA expression among different individuals or experimental batches using well-defined features and functions.

In the present studies, we have carried out an extensive investigation of gene expression in human neutrophils stimulated with several different schedules of LPS treatment. LPS signaling mimics many of effects of exposure of neutrophils to infectious agents. However, the mechanisms of LPS-induced neutrophil activation and priming have not yet been fully elucidated. The neutrophil response to LPS involves many gene families from various functional categories such as transcription factors, receptors, cytokines, and chemokines. These findings indicate that the neutrophil is a transcriptionally active cell responsive to environmental stimuli and capable of a complex series of both early and late changes in gene expression. Understanding the processes that regulate these responses should lead to a better understanding of the molecular pathology of inflammatory diseases and could also identify new therapeutic targets.

REFERENCES

Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 25:187–191.

Armant MA, Fenton MJ. 2002. Toll-like receptors: A family of pattern-recognition receptors in mammals. Genome Biol 3:REVIEWS3011.

Barroso-Aranda J, Chavez-Chavez RH, Mathison JC, Suematsu M, Schmid-Schonbein GW. 1994. Circulating neutrophil kinetics during tolerance in hemorrhagic shock using bacterial lipopolysaccharide. Am J Physiol 266:H415–H421.

Bone RC, Grodzin CJ, Balk RA. 1997. Sepsis: A new hypothesis for pathogenesis of the disease process. Chest 112: 235–243.

Caamano J, Hunter CA. 2002. NF-kappaB family of transcription factors: Central regulators of innate and adaptive immune functions. Clin Microbiol Rev 15:414–429.

Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler J, Lockhart DJ, Morris MS, Fodor SP. 1996. Accessing genetic information with high-density DNA arrays. Science 274:610–614.

Condliffe AM, Kitchen E, Chilvers ER. 1998. Neutrophil priming: Pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) 94:461–471.

Dianzani C, Lombardi G, Collino M, Ferrara C, Cassone MC, Fantozzi R. 2001. Priming effects of substance P on calcium changes evoked by interleukin-8 in human neutrophils. J Leukoc Biol 69:1013–1018.

Dong G, Loukinova E, Chen Z, Gangi L, Chanturita TI, Liu ET, Van Waes C. 2001. Molecular profiling of transformed and metastatic murine squamous carcinoma cells by differential display and cDNA microarray reveals altered expression of multiple genes related to growth, apoptosis, angiogenesis, and the NF-kappaB signal pathway. Cancer Res 61:4797–4808.

Hajjar AM, O'Mahony DS, Ozinsky A, Underhill DM, Aderem A, Klebanoff SJ, Wilson CB. 2001. Cutting edge: Functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol 166:15–19.

Kaisho T, Akira S. 2002. Toll-like receptors as adjuvant receptors. Biochim Biophys Acta 1589:1–13.

Kobayashi SD, Voyich JM, Buhl CL, Stahl RM, DeLeo FR. 2002. Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: Cell fate is regulated at the level of gene expression. Proc Natl Acad Sci USA 99:6901–6906.

Kurt-Jones EA, Mandell L, Whitney C, Padgett A, Gosselin K, Newburger PE, Finberg RW. 2002. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood 100:1860–1868.

Li X, Yang Y, Ashwell JD. 2002. TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature 416:345–347.

Lian Z, Wang L, Yamaga S, Bonds W, Beazer-Barclay Y, Kluger Y, Gerstein M, Newburger PE, Berliner N, Weissman SM. 2001. Genomic and proteomic analysis of the myeloid differentiation program. Blood 98:513–524.

Lipshutz RJ, Morris D, Chee M, Hubbell E, Kozal MJ, Shah N, Shen N, Yang R, Fodor SP. 1995. Using oligonucleotide probe arrays to access genetic diversity. Biotechniques 19:442–447.

Marie C, Muret J, Fitting C, Losser MR, Payen D, Cavaillon JM. 1998. Reduced ex vivo interleukin-8 production by neutrophils in septic and nonseptic systemic inflammatory response syndrome. Blood 91:3439–3446.

McCall CE, Grosso-Wilmoth LM, LaRue K, Guzman RN, Cousart SL. 1993. Tolerance to endotoxin-induced expression of the interleukin-1 beta gene in blood neutrophils of humans with the sepsis syndrome. J Clin Invest 91:853–861.

Means TK, Golenbock DT, Fenton MJ. 2000. The biology of toll-like receptors. Cytokine Growth Factor Rev 11:219–232.

Mollapour E, Linch DC, Roberts PJ. 2001. Activation and priming of neutrophil nicotinamide adenine dinucleotide phosphate oxidase and phospholipase A(2) are dissociated by inhibitors of the kinases p42(ERK2) and p38(SAPK) and by methyl arachidonyl fluorophosphonate, the dual inhibitor of cytosolic and calcium-independent phospholipase A(2). Blood 97:2469–2477.

Morr M, Takeuchi O, Akira S, Simon MM, Muhlradt PF. 2002. Differential recognition of structural details of bacterial lipopeptides by toll-like receptors. Eur J Immunol 32:3337–3347.

Muzio M, Bosisio D, Polentarutti N, D'Amico G, Stoppacciaro A, Mancinelli R, van't Veer C, Penton-Rol G, Ruco LP, Allavena P, Mantovani A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: Selective expression of TLR3 in dendritic cells. J Immunol 164:5998–6004.

Neufert C, Pai RK, Noss EH, Berger M, Boom WH, Harding CV. 2001. Mycobacterium tuberculosis 19-kDa lipoprotein promotes neutrophil activation. J Immunol 167: 1542–1549.

Saban MR, Hellmich H, Nguyen NB, Winston J, Hammond TG, Saban R. 2001. Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation. Physiol Genomics 5:147–160.

Subrahmanyam YV, Baskaran N, Newburger PE, Weissman SM. 1999. A modified method for the display of 3′-end restriction fragments of cDNAs: Molecular profiling of gene expression in neutrophils. Methods Enzymol 303:272–297.

Subrahmanyam YV, Yamaga S, Prashar Y, Lee HH, Hoe NP, Kluger Y, Gerstein M, Goguen JD, Newburger PE, Weissman SM. 2001. RNA expression patterns change dramatically in human neutrophils exposed to bacteria. Blood 97:2457–2468.

Sweeney JF, Nguyen PK, Omann GM, Hinshaw DB. 1998. Lipopolysaccharide protects polymorphonuclear leukocytes from apoptosis via tyrosine phosphorylation-dependent signal transduction pathways. J Surg Res 74: 64–70.

Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr. 1998. NF-kappaB antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 281:1680–1683.

Werneburg BG, Zoog SJ, Dang TT, Kehry MR, Crute JJ. 2001. Molecular characterization of CD40 signaling intermediates. J Biol Chem 276:43334–43342.

Zhang G, Ghosh S. 2001. Toll-like receptor-mediated NF-kappaB activation: A phylogenetically conserved paradigm in innate immunity. J Clin Invest 107:13–19.