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# Down-Regulation of Proinflammatory Capacity During Apoptosis in Human Polymorphonuclear Leukocytes

Scott D. Kobayashi,<sup>1</sup> Jovanka M. Voyich,<sup>1</sup> Kevin R. Braughton, and Frank R. DeLeo<sup>2</sup>

Polymorphonuclear leukocytes (PMNs) are essential to innate immunity in humans and contribute significantly to inflammation. Although progress has been made, the molecular basis for termination of inflammation in humans is incompletely characterized. We used human oligonucleotide microarrays to identify genes encoding inflammatory mediators that were differentially regulated during the induction of apoptosis. One hundred thirty-three of 212 differentially expressed genes encoding proinflammatory factors, signal transduction mediators, adhesion molecules, and other proteins that facilitate the inflammatory response were down-regulated during the induction of apoptosis following PMN phagocytosis. Among these, 42 genes encoded proteins critical to the inflammatory response, including receptors for IL-8 $\beta$ , IL-10 $\alpha$ , IL-13 $\alpha$ 1, IL-15 $\alpha$ , IL-17, IL-18, C1q, low-density lipoprotein, IgG Fc (CD32), and formyl peptide, Toll-like receptor 6, platelet/endothelial cell adhesion molecule-1 (CD31), P-selectin (CD62), IL-1 $\alpha$ , IL-16, and granulocyte chemoattractant protein-2 were down-regulated. Many of these genes were similarly down-regulated during Fas-mediated or camptothecin-induced apoptosis. We used flow cytometry to confirm that IL-8R $\beta$  (CXCR2) and IL-1 $\alpha$  were significantly down-regulated during PMN apoptosis. We also discovered that 23 genes encoding phosphoinositide and calcium-mediated signal transduction components, which comprise complex pathways essential to the inflammatory response of host cells, were differentially regulated during PMN apoptosis. Importantly, our data demonstrate that PMNs down-regulate proinflammatory capacity at the level of gene expression during induction of apoptosis. These findings provide new insight into the molecular events that resolve inflammation following PMN activation in humans. *The Journal of Immunology*, 2003, 170: 3357–3368.

Human polymorphonuclear leukocytes (PMNs)<sup>3</sup> are the first line of defense against invading microorganisms and eliminate pathogens by a process known as phagocytosis. During phagocytosis, PMNs produce reactive oxygen species (ROS) and release cytotoxic granule components into phagocytic vacuoles to kill ingested microbes (1). PMNs accumulate rapidly at sites of infection; therefore, the regulation of PMN turnover following phagocytosis is important for preventing damage to healthy tissues that would otherwise occur in the event of necrotic cell lysis. Removal of PMNs containing ingested microbes by programmed cell death (apoptosis) likely prevents such damage and is presumably critical for the resolution of inflammation due to PMN activation (2, 3). In support of this hypothesis, recent evidence indicates that phagocytosis initiates a molecular cascade of events that accelerate apoptosis in human PMNs (4). However, there is little direct evidence to indicate that resolution of inflammation

following PMN activation is due to down-regulation of proinflammatory capacity as a result of apoptosis.

During the course of microbial infections, signal transduction events that facilitate host cell responses are relayed through PMN surface receptors, including those specific for complement receptors (CRs) and Ab (FcRs) (5), IL-8–10 (6–8), IL-13 (9), IL-15 (10, 11), and IL-18 (12), and multiple receptors for bacteria and microbial products, such as the Toll-like receptors (13). Expression of these receptors is essential for PMN functions such as chemotaxis, phagocytosis, ROS production, and release of cytokines, chemokines, and growth factors that potentiate innate immune and inflammatory responses. Importantly, recent studies from multiple laboratories indicate that mature PMNs synthesize de novo many cytokines, chemokines, and growth factors, including IL-1 $\beta$ , IL-6, IL-8, growth-regulated gene products (GROs)  $\alpha$  and  $\beta$ , TNF- $\alpha$ , TGF- $\beta$ , oncostatin M (OSM), macrophage inflammatory protein-1 $\alpha$ , and vascular endothelial growth factor (VEGF) (14–16). Although significant progress has been made toward understanding the role of specific receptors and inflammatory mediators during the innate immune response, very little is known about their regulation during PMN apoptosis.

Phosphoinositide metabolism and calcium signal transduction mediate key PMN functions including chemotaxis, phagocytosis, ROS production, degranulation, and cell fate (17–24). For example, phosphoinositide 3-kinase (PI3K)-Akt/PKB signal transduction represses programmed cell death by activating and inhibiting transcription factors that mediate cellular survival (25–28) and induce apoptosis (29), respectively. Moreover, the inositol triphosphate (IP3) receptor and IP3-mediated calcium spikes control mitochondrial permeability transition pore involvement in programmed cell death (30, 31). Thus, PI3K and calcium signal transduction appear critical for modulating PMN function during the course of the inflammatory response and for termination of inflammation.

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<sup>3</sup> Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; CR, complement receptor; LB, latex bead; CPT, camptothecin; ROS, reactive oxygen species; ADI, average difference intensity; ER, endoplasmic reticulum; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; VEGF, vascular endothelial growth factor; OSM, oncostatin M; PI3K, phosphoinositide 3-kinase; GRO, growth-regulated gene product; IP3, inositol triphosphate; ITPR1, IP3 receptor 1.

We recently proposed that programmed cell death in human PMNs is regulated by an apoptosis differentiation program, a final stage of transcriptional maturity that facilitates resolution of inflammation (32). Thus, multiple cellular processes in human PMNs are gene-regulated during apoptosis. In the present study, we used human oligonucleotide microarrays to demonstrate that the proinflammatory capacity of human PMNs is down-regulated at the level of gene expression during apoptosis. We propose that repression of PMN proinflammatory capacity is crucial for resolution of inflammation due to neutrophil activation in humans.

## Materials and Methods

### Materials

Sterile water and 0.9% sodium chloride (both Irrigation, U.S. Pharmacopeia) were purchased from Baxter Healthcare, Deerfield, IL. Dextran T-500 and Ficoll-Paque PLUS were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). PE-conjugated Abs specific for IL-8R $\beta$ , CD31, CD54, CD59, CD89, and IL-1 $\alpha$  and corresponding isotype control Abs were purchased from BD Biosciences (Los Angeles, CA). Rabbit Ab specific for human serum albumin and human IgM was purchased from ICN Biomedicals (Costa Mesa, CA). Anti-Fas Ab (IgM clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY). RPMI 1640 medium was from Invitrogen (Rockville, MD). Latex beads (LB; 2.0  $\mu$ M) were purchased from Polysciences (Warrington, PA). Unless specified, all other reagents were from Sigma-Aldrich (St. Louis, MO).

### Isolation of human PMNs

Human PMNs were isolated from venous blood of healthy individuals as described previously (4) in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Allergy and Infectious Diseases. Briefly, PMNs were purified using dextran sedimentation followed by Hypaque-Ficoll gradient centrifugation (33). Cell preparations contained >99% PMNs and all reagents used contained <25.0 pg/ml endotoxin. Eosinophils accounted for  $5.2 \pm 2.8\%$  of the PMNs from the three individuals used in the oligonucleotide array experiments, and comprised  $5.8 \pm 3.0\%$  of the human PMNs from a random sampling of preparations ( $n = 13$  donors). We have previously reported that contaminating lymphocytes and monocytes account for  $0.71 \pm 0.26\%$  and  $0.11 \pm 0.07\%$  ( $n = 30$  PMN preparations) of the cells in our PMN preparations (4).

### PMN phagocytosis and gene expression analysis

Preparation of complement (C3bi)- and Ab (IgG)-coated LB and phagocytosis experiments were performed as described by Kobayashi et al. (4). Briefly, PMNs ( $10^7$ ) were combined on ice with or without IgG and/or C3bi-coated LB ( $8 \times 10^7$ ) in wells of a 12-well tissue culture plate (precoated with 20% normal human serum) and centrifuged at  $350 \times g$  for 8 min at 4°C to synchronize phagocytosis. Following centrifugation, plates were incubated at 37°C in a CO<sub>2</sub> incubator for up to 24 h. Alternatively, PMNs ( $10^7$ ) were combined with 10  $\mu$ M camptothecin (CPT) or 500 ng/ml anti-Fas Ab (CH-11) and incubated at 37°C in a CO<sub>2</sub> incubator. At the indicated times, tissue culture medium was aspirated from the plate (or cells were centrifuged in microfuge tubes before aspiration of the media) and PMNs were lysed directly with RLT buffer (Qiagen, Valencia, CA). Purification of PMN RNA and subsequent preparation of labeled cRNA target (12  $\mu$ g) was performed as described (4). Labeling of samples, hybridization of cRNA with Hu95A $\nu$ 2 oligonucleotide arrays (Affymetrix, Santa Clara, CA), and scanning were performed according to standard Affymetrix protocols ([http://www.affymetrix.com/pdf/expression\\_manual.pdf](http://www.affymetrix.com/pdf/expression_manual.pdf)) as described previously (4). Experiments were performed with three separate donors at each time point using a separate oligonucleotide array for each donor.

Gene expression data were analyzed as reported previously with GeneSpring expression analysis software version 4.04 (Silicon Genetics, Redwood City, CA) (4). Briefly, genes were defined as differentially expressed if the average expression level changed at least 2-fold in one of the treatments compared with that from time-matched unstimulated cells over three experiments, and were called "Present" in at least two experiments by GeneChip Suite (Affymetrix). Receptor-specific changes were determined as described previously (4). Graphs generated with GeneSpring expression analysis software version 4.04 (Silicon Genetics) were annotated in CorelDraw, version 10 (Corel Corporation, Ottawa, Ontario, Canada).

Relative levels of differentially expressed genes were assigned based on average difference intensity (ADI) values determined by GeneChip Suite.

(A) = genes called absent; (VL) very low expression = ADI <50; (L) low expression = ADI 50–200; (M) moderate expression = ADI 200–500; (H) high expression = ADI 500–1500; (V<sub>H</sub>) very high expression = ADI >1500. ADI is an approximation of transcript abundance and not caused by oligonucleotide primer hybridization bias.

### TaqMan real-time RT-PCR analysis

Phagocytosis experiments and RNA preparation for TaqMan analysis were done with conditions identical with those used for the microarray analysis. TaqMan analysis of samples from three blood donors was performed in triplicate with an ABI 7700 thermocycler (Applied Biosystems, Foster City, CA) as described (4).

### Flow cytometric analysis of receptor surface expression, IL-1 $\alpha$ production, and cell viability

PMN apoptosis following phagocytosis was measured with flow cytometry using a modified TUNEL assay (Apo-BRDU Apoptosis Detection kit; BD Biosciences) or with annexin V-FITC (Annexin V-FITC Apoptosis Detection kit II; BD Biosciences) as described by the manufacturer. For Fas- or CPT-induced PMN apoptosis experiments, PMNs ( $2 \times 10^6$ ) were combined with 10  $\mu$ M CPT or 500 ng/ml anti-Fas Ab (CH-11) and incubated at 37°C in a CO<sub>2</sub> incubator for the desired times. PMN viability was determined by propidium iodide staining with flow cytometry, and was unaffected by incubation at 37°C for at least 9 h after phagocytosis (32). PMN viability at the start of the assay after the centrifugation step (0 min) was  $96.4 \pm 1.3\%$ , and was reduced only slightly to  $90.0 \pm 2.6\%$  in unstimulated PMNs after incubation for 24 h at 37°C (eight separate determinations). However, PMN lysis occurred more rapidly after 24 h in culture (data not shown).

To measure surface expression of IL-8R $\beta$ , CD31, CD54, and annexin V-FITC, phagocytosis assays were performed as described above, but with the following modifications. PMNs ( $10^6$ ) were mixed on ice with IgG and/or C3bi-coated LB ( $8 \times 10^6$ ) in 96-well microtiter plates precoated with 20% normal human serum and centrifuged at  $350 \times g$  for 8 min at 4°C to synchronize phagocytosis. Following centrifugation, plates were incubated at 37°C in a CO<sub>2</sub> incubator for up to 9 h as indicated. Alternatively, PMNs were treated with 500 ng/ml anti-Fas Ab (CH-11) as described above. Cells were stained with the indicated Abs and with annexin V-FITC as recommended by the manufacturer (BD Biosciences). Staining for intracellular IL-1 $\alpha$  was performed 6, 9, and 16 h following phagocytosis in the presence of GolgiPlug (brefeldin A) as suggested by the manufacturer (BD Biosciences). The percentage of unstimulated PMNs staining positive for intracellular IL-1 $\alpha$  was routinely 6–13% (individual variability) at 9 h, and this percentage increased significantly over time to 21–28% after 16 h due to accumulating cytokine in the endoplasmic reticulum (ER) after treatment with brefeldin A. Increasing the time of brefeldin A treatment increased the level of detectable IL-1 $\alpha$ . Thus, it is likely that all PMNs constitutively produce IL-1 $\alpha$ , albeit not to detectable levels. Despite individual variability in IL-1 $\alpha$  staining, there was always less intracellular IL-1 $\alpha$  in FcR/CR-stimulated PMNs compared with unstimulated PMNs. All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and a single gate was used to eliminate debris.

### Statistics

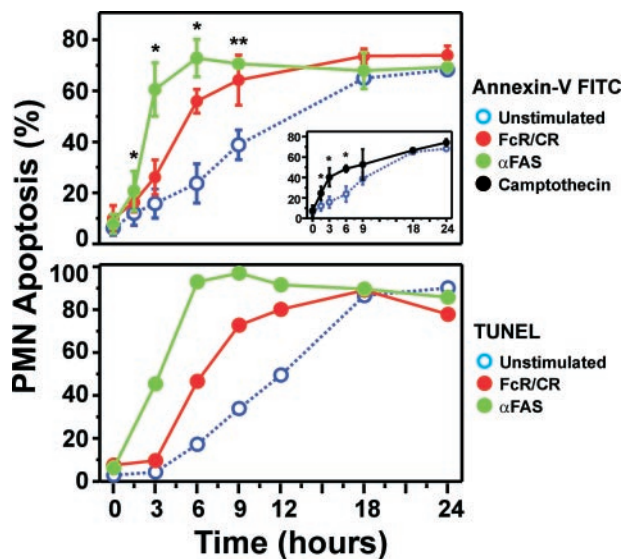
One-way ANOVA with Dunnett's correction for multiple comparisons, Kruskal-Wallis ANOVA on ranks, and Student's *t* test were performed in Sigma Stat for Windows, version 2.03 (SPSS, Chicago, IL) where indicated.

## Results

### Phagocytosis-, Fas-, and CPT-induced PMN apoptosis

Unstimulated human PMNs undergo spontaneous or constitutive apoptosis, which presumably facilitates normal cell turnover in vivo (Ref. 2; Fig. 1). PMN apoptosis is accelerated significantly following receptor-mediated phagocytosis (phagocytosis) (4, 32), Fas ligation (34), and treatment with the topoisomerase inhibitor CPT (Ref. 35; Fig. 1, upper panel). The initial stages of phagocytosis-induced apoptosis occurred 3–6 h after PMN activation (Fig. 1). Therefore, analysis of gene regulation within this time period is critical for understanding molecular processes related to cell fate in PMNs, and ultimately, resolution of inflammation due to PMN activation.





**FIGURE 1.** Phagocytosis-, Fas-, and CPT-induced PMN apoptosis. Apoptosis was measured with an annexin V-FITC assay (upper panel) or a TUNEL assay (lower panel) in unstimulated human PMNs (○), following FcR/CR phagocytosis (red circle), after incubation with anti-Fas Ab (green circle), or CPT (●, inset) as indicated. Results for annexin V-FITC are the mean  $\pm$  SD of two to eight experiments. \*,  $p < 0.001$  vs CTL for each stimulus; \*\*,  $p < 0.03$  vs CTL for each stimulus (ANOVA). Results for TUNEL are from a representative experiment performed with each of the three individuals used in the oligonucleotide array experiments.

#### Regulation of inflammatory response mediators during the initial stages of PMN apoptosis

We screened ~12,500 human genes for changes in expression 3–6 h following phagocytosis, and compared expression of the up- and down-regulated genes in cells treated with anti-Fas Ab or CPT (Figs. 2 and 3, and Table 1<sup>4</sup>). A total of 212 differentially regulated genes encoded proteins that mediate inflammatory response-related processes, signal transduction, adhesion, or cytoskeletal/structural function. A total of 133 genes (62.7%) were down-regulated and 79 genes (37.2%) were up-regulated.

Sixty genes encoding receptors and proinflammatory surface molecules were differentially regulated during the induction of PMN apoptosis following receptor-phagocytosis (Fig. 2). Forty-seven of these phagocytosis-regulated genes were induced or repressed after Fas ligation and/or treatment with CPT (Fig. 2). Genes encoding the IL-9R, CCR1, CCRL2, HM74, CD48, CD54, CD89, CD200, and other key immune function modulatory proteins were up-regulated after phagocytosis compared with unstimulated cells (Fig. 2A). The IP3 receptor 1 (ITPR1) gene (*ITPR1*) was also up-regulated and a potential role for the regulation of this gene during apoptosis is described below. The gene encoding CD59, a protein that protects host cells from the membrane attack complex of serum complement, was up-regulated (Fig. 2). This finding is consistent with a previous report describing up-regulated surface expression of CD59 just before spontaneous PMN apoptosis (36). Although PMNs are not widely considered as typical APCs, several genes encoding proteins involved in Ag presentation, HLA-F, HLA-DQA1, HLA-DQB1, and CD83 were up-regulated during the initial stages of PMN apoptosis (Fig. 2). Of note, the gene encoding HLA-DQA1 was up-regulated by all apoptosis stimuli (Fig. 2A). Expression of these proteins on the surface of PMNs has not been associated with apoptosis, but they

might facilitate PMN clearance and/or potentiate the acquired immune response. Several key proinflammatory mediators were also up-regulated during the induction of phagocytosis-induced PMN apoptosis (Fig. 2). Genes encoding 10 cytokines and growth factors including GRO $\beta$ , GRO $\gamma$ , VEGF, OSM, and IL-6 were up-regulated, as were those encoding acute-phase reactant proteins, pentraxin 3 and orosomucoid 1 (Fig. 2). Several of these genes were correspondingly up-regulated during Fas- and/or CPT-induced PMN apoptosis (Fig. 2A). We reported previously that genes encoding several of these cytokines were induced between 30 and 90 min after phagocytosis, and that the gene encoding TNF- $\alpha$  was up-regulated between 3 and 6 h after phagocytosis (4).

During the induction of phagocytosis-induced apoptosis, 32 genes encoding key surface molecules including receptors for IL-8 $\beta$ , IL-10 $\alpha$ , IL-13 $\alpha$ 1, IL-15 $\alpha$ , IL-17, IL-18, C1q, and formyl peptide, Toll-like receptor 6, P-selectin (CD62), carcinoembryonic Ag-related cell adhesion molecule-4, platelet/endothelial cell adhesion molecule-1 (CD31), syndecan 1, Fc $\gamma$ RIIB (CD32), two Ig-like receptors (LILRB2 and LILRA1), and three purinergic receptors were down-regulated (Fig. 2). Consistent with the idea that there is general down-regulation of proinflammatory capacity during programmed cell death, 24 of these genes were down-regulated during Fas- and/or CPT-induced apoptosis (Fig. 2A). Down-regulation of these key PMN effector molecules, especially the IL-8R $\beta$ , would alter host-pathogen interactions normally mediated by PMNs (37). Genes encoding IL-1 $\alpha$  and IL-16, key proinflammatory cytokines, and granulocyte chemoattractant protein 2 were significantly down-regulated (Fig. 2). Moreover, genes encoding sialic acid binding lectin 7 (SIGLEC7), peptidoglycan recognition protein (PGLYRP), cytotoxic granule-associated RNA binding protein (TIAL1), and calgranulin C, all involved directly in host defense, were down-regulated (Fig. 2). Taken together, these data suggest that key components of PMN proinflammatory and bactericidal capacities are regulated at the level of gene expression during the early stages of apoptosis.

#### TaqMan confirmation of microarray data

We selected several up- and down-regulated genes ( $n = 7$ ) representative of the microarray data set for confirmation by TaqMan real-time PCR (Fig. 2B). There was almost absolute correlation ( $r = 0.99$ ) of gene expression levels between the two methods at either 3 or 6 h following phagocytosis, including those for *CD59* and *CD54* (Fig. 2B). Microarray data for the other genes confirmed by TaqMan in Fig. 2B are presented below. These results are consistent with the strong positive correlation we reported between TaqMan and microarray data previously (4, 32). Importantly, these results confirm that regulation of key signal transduction mediators occurs at the level of gene expression during the initial stages of phagocytosis-induced apoptosis.

#### Comparison of gene expression during phagocytosis-, Fas-, and CPT-induced PMN apoptosis

To gain insight into common components of PMN apoptosis, we compared expression of selected apoptosis-related genes shown previously to be up- or down-regulated after phagocytosis (4) with gene expression in cells after Fas ligation or CPT treatment (Fig. 3A). Genes encoding glycoprotein NMB, BTG3, early growth response 2, TR3 orphan receptor, TNF- $\alpha$ , and optineurin (FIP2) were up-regulated by all of the apoptosis-inducing stimuli (Fig. 3A). Furthermore, 13 genes encoding apoptosis-related proteins such as apoptosis inhibitor 5, DRAKs 1 and 2, and DEFCAP were down-regulated in all treatments (Fig. 3A). These findings provide compelling evidence that regulation of these genes is important for PMN apoptosis per se (rather than specific to phagocytosis).

<sup>4</sup> The online version of this article contains supplemental material.

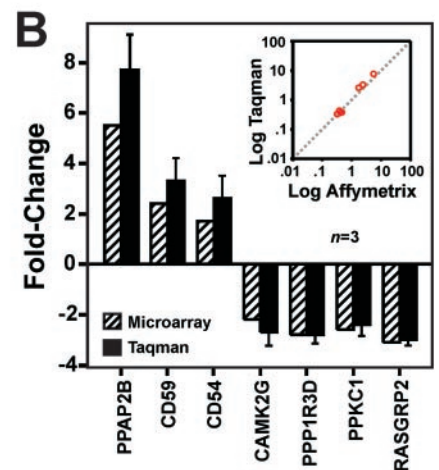
## A

Receptors & surface molecules (60)	Phago.	$\alpha$ Fas	CPT
*CD22 (SIGLEC2)	+	+	+
*zona pellucida glycoprotein 3A (ZP3A)	+	NC	NC
*fms-related tyrosine kinase (FLT1)	+	+	NC
*IL-9 receptor (IL9R)	+	NC	NC
*phosphatidylserine receptor (PTDSR)	+	NC	NC
CD48	+	NC	-
CD59	+	NC	NC
CD71, transferrin receptor, p90 (TFRC)	+++	-	NC
CD200 (MOX2)	+	+	**
purinergic receptor P2X4 (P2RX4)	+	**	-
inositol 1,4,5-trisphosphate receptor 1 (ITPR1)	+	NC	-
prostanoid E receptor 2 (PTGER2)	+	+	+
prostanoid E receptor 4 (PTGER4)	++	**	+
integrin, $\alpha$ 7 (ITGA7)	+++	+	+
chemokine receptor 1 (CCR1)	+	NC	**
**CD83	+	NC	NC
**MHC class I, F (HLA-F)	+	-	NC
**MHC class II, DQ $\alpha$ 1F (HLA-DQA1)	+	+	+
**MHC class II, DQ $\beta$ 1F (HLA-DQB1)	+	+	-
**leukocyte specific transcript 1 (LST1, B144)	+	NC	+
**CD54, (ICAM1)	+	-	NC
**CD89, IgA receptor (FCAR)	+	+	-
**leukocyte immunoglobulin-like receptor, B4 (LILRB4)	+	+	NC
**chemokine receptor (HM74)	+	+	-
**polio-virus receptor-related 1, nectin (PVRL1)	+	+	+
**chemokine receptor-like 2 (CCRL2)	+	+	+
**CMRF35 leukocyte Ab-like receptor (CMRF35)	+	+	+
**sortilin-related receptor (SORL1)	+	+	-
*IL-15 receptor $\alpha$ (IL15RA)	-	NC	NC
*lymphocyte antigen 94 homolog (LY94)	-	NC	-
*low density lipoprotein receptor (LDLR)	-	NC	-
*CD86 (CD86)	-	+	+
IL-17 receptor (IL17R)	---	NC	NC
IL-8 receptor, $\beta$ (CXCR2, IL8RB)	-	**	**
Toll-like receptor 6 (TLR6)	-	**	**
purinergic receptor P2Y (P2RY2)	-	**	-
CD31, PECAM-1 (PECAM1)	---	**	**
*IL-10 receptor, $\alpha$ (IL10RA)	-	-	-
**IL-13 receptor, $\alpha$ 1 (IL13RA1)	-	NC	NC
**IL-18 receptor 1 (IL18R1)	-	+	-
**IL-1 receptor accessory protein (IL1RAP)	-	NC	-
**formyl peptide receptor 1 (FPR1)	-	NC	-
**CD1, member D (CD1D)	---	NC	-
**CD32, Fc $\gamma$ RIIb, IgG receptor (FCGR2B)	-	NC	NC
**CD62, P-selectin (SELP)	-	+	+
**CGM7, CEA family adhesion molecule (CEACAM4)	-	-	NC
**glutamate receptor, ionotropic, AMPA3 (GRIA3)	-	-	-
**coagulation factor II (thrombin) receptor-like (F2RL1)	-	-	-
**leukocyte receptor cluster (LRC) member 4 (LENG4)	-	-	NC
**leukocyte immunoglobulin-like receptor, B2 (LILRB2)	-	-	NC
**leukocyte immunoglobulin-like receptor, A1 (LILRA1)	-	NC	-
**complement component C1q receptor (C1QR)	-	NC	-
**lymphocyte antigen 75 (LY75)	-	-	-
**advanced glycos. end product-specific receptor (AGER)	-	NC	NC
**purinergic receptor (P2Y10)	-	+	-
**purinergic receptor P2X (P2RX1)	-	NC	NC
**retinoic acid receptor (RARA)	-	-	NC
**syndecan 1 (SDC1)	-	+	+
**cell matrix adhesion regulator (CMAR)	-	NC	NC
**peptidoglycan recognition protein (PGLYRP)	-	NC	NC

Cytokines & growth factors (13)	Phago.	$\alpha$ Fas	CPT
oncostatin M (OSM)	+++	**	+
GRO- $\beta$ (CXCL2, GRO2)	+++	-	NC
GRO- $\gamma$ (CXCL3, GRO3)	++	NC	+
vascular endothelial growth factor (VEGF)	+++	-	**
IL-6 (IL6)	+++	**	**
MIP-3 $\alpha$ (CCL20, SCYA20)	+	+	+
MCP-1 (CCL2, SCYA2)	+	+	**
**MCP-3 (CCL7, SCYA7)	+	++	+
**MIP-1 $\alpha$ (CCL3, SCYA3)	+	NC	NC
**galectin 3 (LGALS3)	+	-	-
IL-1 $\alpha$ (IL1A)	-	NC	+
IL-16 (IL16)	-	NC	+
granulocyte chemotactic protein 2 (CXCL6, SCYB6)	-	-	NC

Host defense (9)	Phago.	$\alpha$ Fas	CPT
surfactant, pulmonary-associated protein C (SFTPC)	+	+	NC
orosomucoid 1 (ORM1)	+	**	+
**pentraxin-3 (PTX3)	+	NC	+
**viperin (CIG5)	+	NC	NC
*cytotoxic granule-assoc. RNA binding protein (TIAL1)	-	-	NC
glioma pathogenesis-related protein (RTVP1)	-	NC	**
sialic acid binding Ig-like lectin 7 (SIGLEC7)	---	**	**
**collagenase homologue (PM5)	---	-	-
**calgranulin C (S100A12)	-	NC	+

up-regulated	down-regulated	
+	-	1.5-2 fold
+	-	2-5 fold
++	-	5-8 fold
+++	---	>8 fold
		NC= no change



**FIGURE 2.** Differential regulation of genes encoding receptors, cytokines, chemokines, and growth factors, and proteins that mediate host defense during the induction of PMN apoptosis. **A**, Differential gene expression was measured with Affymetrix human oligonucleotide microarrays during the induction of PMN apoptosis 3–6 h after receptor-mediated phagocytosis (Phago.), or following treatment with anti-Fas Ab ( $\alpha$ Fas) or CPT as described in *Materials and Methods*. Changes in gene expression are represented as relative increase (+) or decrease (–) compared with unstimulated PMNs as follows: (+)/(–), 2- to 5-fold change; (++)/(–), 5- to 8-fold change; (+++)/(–), 8- or greater fold change; gray symbols, 1.5- to 2.0-fold change. \*, 3 h after activation; \*\*, 6 h after activation; unmarked genes, 3 and 6 h after activation. Black lettering, genes regulated similarly in all three types of phagocytosis (FcR, CR, and FcR/CR); red lettering, FcR and/or FcR/CR (FcR-specific); blue lettering, CR and/or FcR/CR (CR-specific); green lettering, FcR/CR only; NC, no change. Results for each gene are based on expression changes in PMNs from three separate individuals (and three separate Affymetrix Hu95A2 GeneChips) as described in *Materials and Methods*. A detailed and complete listing of changes in PMN gene expression is provided as Table I. **B**, TaqMan verification of microarray results. Genes ( $n = 7$ ) identified as differentially transcribed by Affymetrix microarrays (▨) were selected from the overall dataset for confirmation by TaqMan real-time PCR (■) following FcR/CR-mediated phagocytosis. *Inset*, The correlation ( $r = 0.99$ ) between real-time PCR and microarray analyses. Data represent the mean  $\pm$  SD of fold changes in gene expression from the three donors used in the microarray experiments. Microarray data are presented as the mean fold change of the three individuals.

In contrast, 24 genes encoding mediators of programmed cell death were differentially regulated during Fas- and/or CPT-induced apoptosis, but not following phagocytosis (Fig. 3B). Genes encoding UBL1 and IER3, inhibitors of Fas- and TNFR-mediated apoptosis, were down-regulated after Fas-ligation, consistent with concomitant induction of apoptosis (Fig. 3B). In contrast, the gene encoding TRAILR4 decoy receptor (apopto-

sis inhibitor) was up-regulated, and genes encoding CD95 (Fas), TRAILR2, BID, and BNIP3L (each promotes apoptosis) were down-regulated (Fig. 3B). Although these findings seem at variance with induction of apoptosis (see Fig. 1), several of these genes function in the initial stages of apoptosis (e.g., Fas and TRAILR2) rather than being required for downstream effector processes.



<b>A</b>			
<b>Shared apoptosis-related genes (43)</b>			
	Phago.	$\alpha$ Fas	CPT
**glycoprotein nmb (GNPMB)	+++	+++	+
tumor necrosis factor- $\alpha$ (TNFA)	++	**+	**+
TR3 orphan receptor (NR4A1)	+	**+	++
**BTG family, member 3 (BTG3)	+	+	+
early growth response 2, Krox-20 homologue (EGR2)	+	**+	**+
**optineurin (FIP2)	++	+	+
**tumor suppressing transferable candidate 3 (TSSC3)	+	NC	+
*modulator of apoptosis 1 (MOAP1, MAP-1)	+	NC	+
**FOSB	+	NC	+
**cathepsin L (CTSL)	+	NC	+
dual specificity phosphatase 10 (DUSP10)	++	**	-
*p21-activated kinase (PAK2)	+	**	**
**TNF-receptor-associated factor 3 (TRAF3)	+	-	-
**MAP4K4	+	-	-
**NF- $\kappa$ B inhibitor, epsilon (NFKBIE)	+	-	-
glucosylceramide synthase (UGCG)	+	-	**
**Toll-like receptor 2 (TLR2)	+	-	NC
**CD120b, p75 (TNFRSF1B)	+	-	**
**Ras homologue enriched in brain 2 (FIP1, RHEB2)	+	**	-
SKI-like oncoprotein (SKIL)	-	-	---
MAP2K4	-	**	---
serine/threonine (apoptosis-inducing) kinase 17a (DRAK1)	-	**	---
*apoptosis inhibitor 5 (API5)	-	-	---
protein kinase, DNA-activated (PRKDC)	-	-	---
B-cell CLL/lymphoma 11A (BCL11A)	-	**	---
*caspase 9 (CASP9)	-	-	---
**death effector CED-4 like apoptosis protein (DEFCAP)	-	-	---
**serine/threonine (apoptosis-inducing) kinase 17b (DRAK2)	-	**	**
xeroderma pigmentosum, complementation group C (XPC)	-	**	**
erythropoietin receptor (EPOR)	-	**	**
**TGF- $\beta$ homologue, BMP6 (TGFB1)	-	-	---
TGF- $\beta$ induced protein (TGFB1)	-	+/**	-
myocyte enhancer factor 2C (MEF2C)	-	NC	---
**TGF- $\beta$ induced anti-apoptotic factor (TIAF1)	-	NC	-
**forkhead box O1A (FOXO1A)	-	NC	-
CARD-containing antagonist of caspase 9 (TUCAN)	-	NC	-
*glucocorticoid receptor (NR3C1)	-	NC	-
**calcineurin binding protein1 (CABIN1)	-	-	NC
*GADD45G	-	-	NC
tumor protein p53-binding protein (TP53BP1)	-	**	NC
*caspase 8 (CASP8)	-	-	+
c-fos (FOS)	-	-	+
**NURR1 orphan receptor (NR4A2)	-	+	+

<b>B</b>			
<b>Fas &amp; CPT apoptosis-related genes (24)</b>			
	$\alpha$ Fas	CPT	
**TRAILR4 (TNFRSF10D)	+	+	
**TNF receptor-associated factor 5 (TRAF5)	+	+	
**etoposide-induced mRNA (PIG8)	+	+	
*tumor protein 63 kDa (TP63)	+	+	
X-ray repair complementing defective repair protein (XRCC2)	+	**+	
**B-cell CLL/lymphoma 3 (BCL3)	+	+	
**baculoviral IAP repeat-containing 3 (BIRC3)	NC	+	
Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1)	-	---	
TRAF family member-associated NF- $\kappa$ B activator (TANK)	-	-	
**TNF receptor-associated factor 1 (TRAF6)	-	-	
*receptor-interacting serine-threonine kinase 2 (RIPK2)	-	-	
CD95, Fas (TNFRSF6)	+	-	
forkhead box O3A (FOXO3A)	**	-	
ubiquitin-like 1 (sentrin) (UBL1)	-	-	
**BCL2/adenovirus E1B interacting protein 3-like (BNIP3L)	-	-	
**apoptosis antagonizing transcription factor (DED, AATF)	-	-	
BH3 interacting domain death agonist (BID)	-	-	
TRAILR2 (TNFRSF10B)	**	-	
*TRAILR3 (TNFRSF10C)	NC	-	
NF- $\kappa$ B transcription factor (NFKB1)	NC	---	
*programmed cell death 4 (PDCD4)	NC	-	
*baculoviral IAP repeat-containing 2 (BIRC2)	NC	-	
**CASP8 and FADD-like apoptosis regulator (CFLAR)	NC	-	
*immediate early response 3 (IER3)	-	NC	

**FIGURE 3.** Differential regulation of genes encoding apoptosis-related proteins during phagocytosis-mediated and Fas- and CPT-induced PMN apoptosis. **A**, Expression of selected apoptosis-related genes identified as up- or down-regulated 3–6 h following phagocytosis was examined after treatment with anti-Fas Ab ( $\alpha$ Fas) and CPT. Differential gene expression was measured as defined in the legend for Fig. 1. Some of the gene expression changes measured following PMN phagocytosis were published previously (see Refs. 4 and 32) and are used for reference. **B**, Comparison of selected Fas- and CPT-induced gene changes not regulated following phagocytosis.

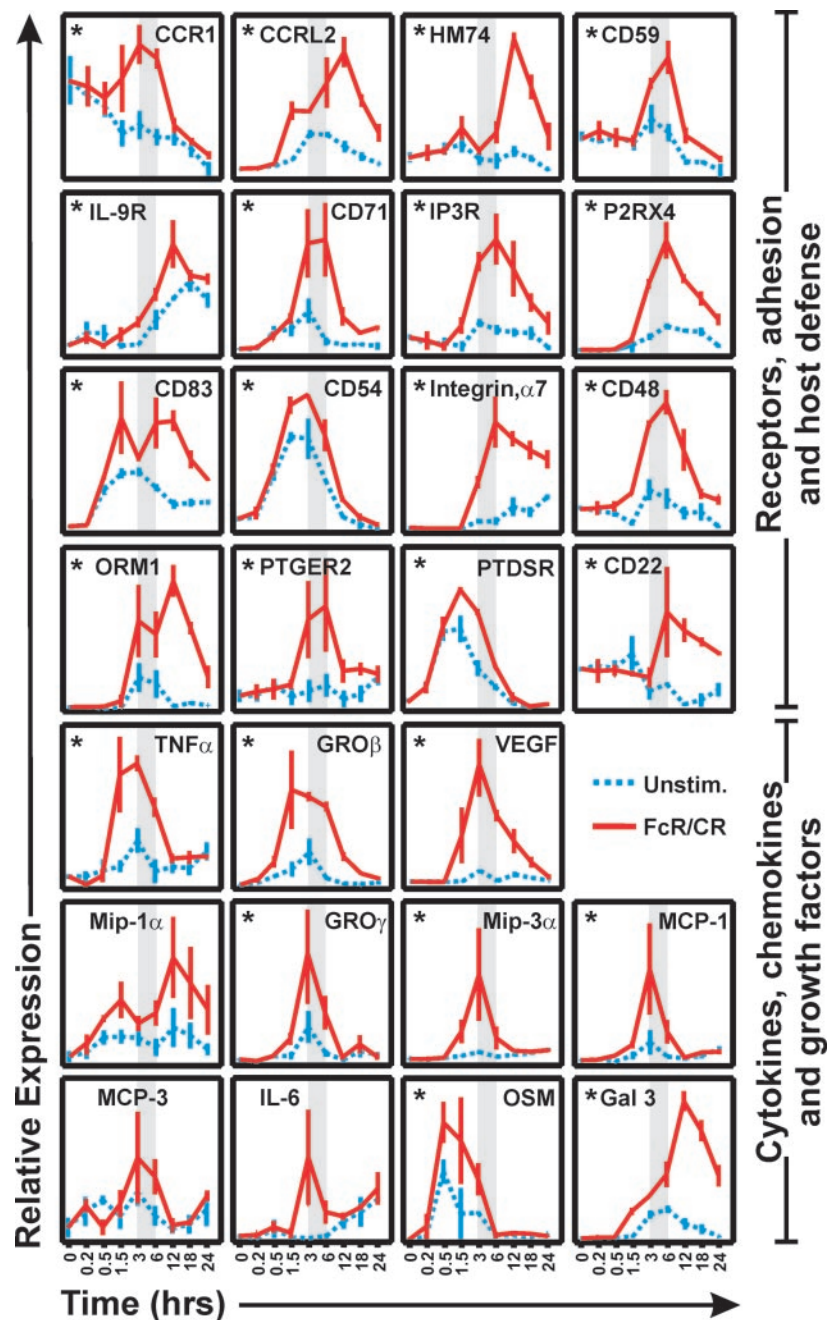
### Down-regulation of proinflammatory mediators during PMN apoptosis

Although there were nearly equivalent numbers of up- and down-regulated receptors, surface molecules, and proinflammatory mediators during the onset of PMN apoptosis (Fig. 2), we hypothesized that expression of up-regulated proinflammatory molecules would diminish as apoptosis progressed. To test that hypothesis, we extended the time-course for analysis of up-regulated genes to 24 h after PMN stimulation (Fig. 4). In 20 of 28 up-regulated genes examined, expression decreased after 3 or 6 h of phagocytosis; albeit, expression was still generally greater than that in unstimulated PMNs (Fig. 4). For example, increased expression of genes encoding CCR1 and CD48 peaked at 3 and 6 h, respectively, following phagocytosis and then decreased over the next 21 h (Fig. 4). Diminishing expression levels for up-regulated cytokines, chemokines, and growth factors was even more striking at this time period, as 9 of 11 up-regulated cytokines and growth factors decreased markedly (Fig. 4). Decreasing expression of *CCR1*, *CD48*, *CD59*, *CD71*, *TNF- $\alpha$* , and *GRO $\beta$*  was especially striking after 3 or 6 h (Fig. 4). By 24 h, expression of all but *Galectin 3*, *CD22*, and *Integrin,  $\alpha$ 7* had returned to levels of expression comparable if not identical with that in unstimulated PMNs (Fig. 4). We note that expression of genes encoding proinflammatory molecules in unstimulated PMNs routinely decreased after 6 h in culture, concomitant with induction of spontaneous apoptosis in those cells (compare Figs. 1 and 4). These results suggest that PMNs terminate production of immune response mediators during apoptosis. Importantly, the implication of this discovery is that a significant portion of PMN proinflammatory capacity is down-regulated at the level of gene expression during the initial stages of apoptosis.

### Surface expression of inflammatory mediators during PMN apoptosis

To determine whether differential expression of proinflammatory genes resulted in concomitant regulation of protein levels, we measured surface expression of IL-8R $\beta$  (CXCR2; Ref. 6), CD31, and CD54 during phagocytosis- and Fas-induced PMN apoptosis (Fig. 5). Freshly isolated human PMNs expressed all three proteins (Fig. 5, 0 h). Compared with unstimulated cells, expression of IL-8R $\beta$ , a receptor critical to the inflammatory response, was significantly reduced at 1.5, 3, and 6 h following phagocytosis, and 3 and 6 h after Fas ligation ( $p < 0.006$  vs unstimulated PMNs for each stimulus; Fig. 5A). Importantly, decreased surface expression of IL-8R $\beta$  correlated with increased staining for annexin V following phagocytosis and Fas ligation, confirming that the receptor is down-regulated during PMN apoptosis (Fig. 5B). Surface expression of CD31 was also significantly reduced 1.5 h following phagocytosis ( $p < 0.006$  vs unstimulated PMNs; Fig. 5A). However, CD31 was surface-expressed on neither stimulated nor unstimulated cells by 3 or 6 h (Fig. 5A). Although plasma membrane-associated CD54 decreased slightly over time, surface expression was increased significantly compared with unstimulated cells at 6 h ( $p < 0.004$  vs unstimulated PMNs; Fig. 5, A and B). We note that an overall decrease in expression of genes encoding CD31 and CD54 in unstimulated PMNs after 6 h correlated with the initial stages of spontaneous apoptosis (compare Figs. 1 and 5). Compared with unstimulated cells, surface expression of CD59 and CD89 was also up-regulated during the early stages of activation-induced apoptosis (+13.7% and +7.7% for CD59, and +34.0% and +27.3% for CD89 compared with unstimulated cells at 3 and 6 h, respectively,  $n = 3$ ;  $p = 0.01$  for surface expression of CD89 in stimulated vs unstimulated PMNs at 6 h), consistent with gene expression (Fig. 2A). Taken together, there was general correlation

**FIGURE 4.** Expression of genes encoding proinflammatory molecules decreases after induction of apoptosis. Measurement of the expression of genes encoding inflammatory mediators and host defense identified as up-regulated between 3 and 6 h after phagocytosis (Fig. 1) in unstimulated PMNs (blue dotted line, Unstim.) or in PMNs after FcR/CR-mediated phagocytosis (red solid line, FcR/CR) was extended to 24 h. Specific categories of genes are indicated at the right. The gray shaded area in each graph represents 3–6 h after phagocytosis, a time period reflecting induction of apoptosis and the time at which genes were identified as differentially expressed in Fig. 1. \*,  $p \leq 0.05$  vs gene expression in unstimulated PMNs at either 3 or 6 h. Results are illustrated as the mean  $\pm$  SD of relative gene expression from three separate individuals.



of gene transcription and expression of receptors at the cell surface (compare panels in Fig. 5).

#### Down-regulation of proinflammatory capacity

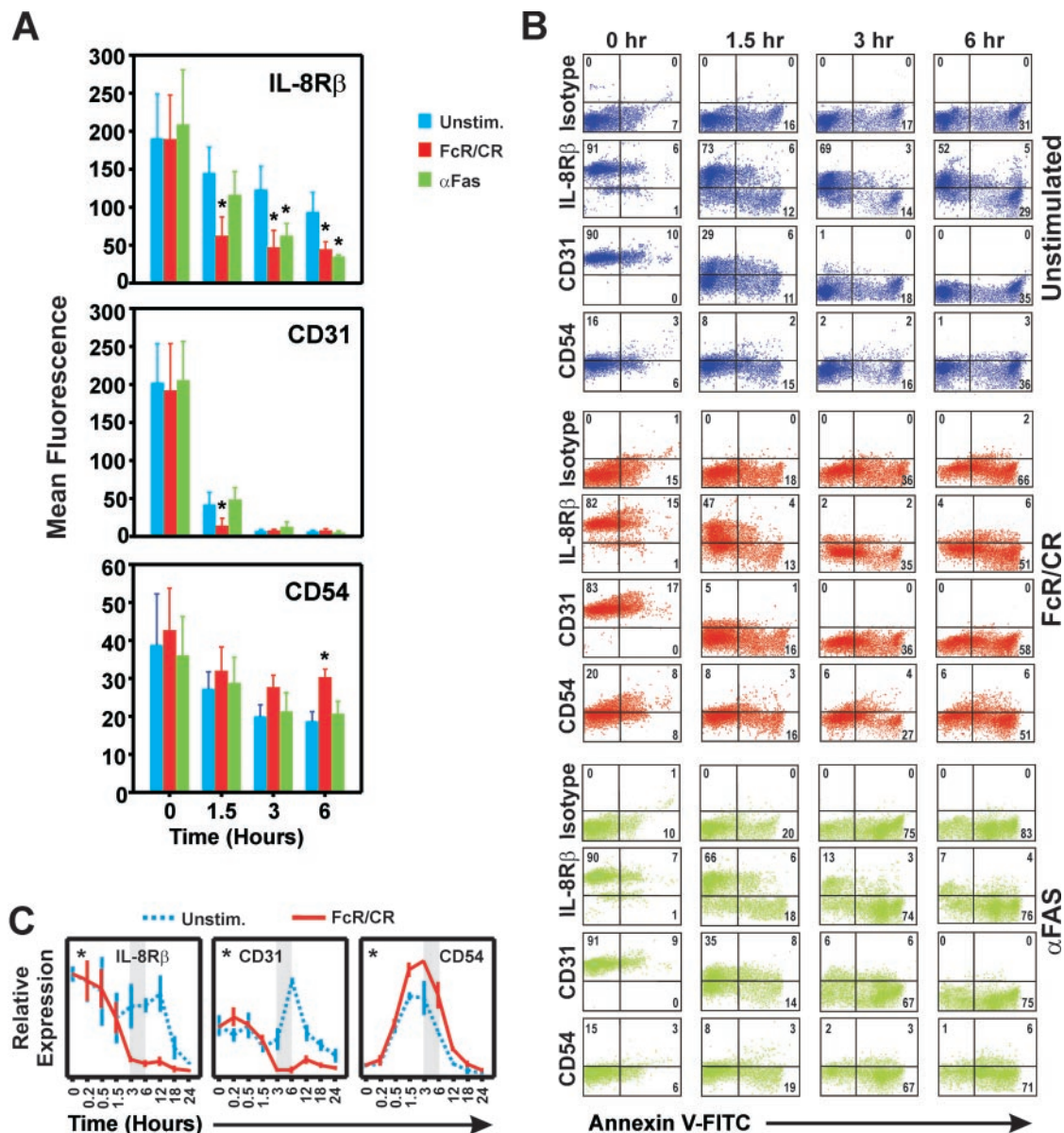
IL-1 $\alpha$  is a critical mediator of inflammatory processes, especially those mediated by PMNs, and its down-regulation during the induction of apoptosis would likely be important in the resolution of inflammation. To determine whether down-regulation of the gene encoding IL-1 $\alpha$  resulted in a concomitant decrease in protein levels, we measured intracellular IL-1 $\alpha$  at 6 and 9 h following phagocytosis in human PMNs (Fig. 6A). There was a significant decrease in PMN-associated IL-1 $\alpha$  at both 6 and 9 h after phagocytosis (during PMN apoptosis) compared with unstimulated cells (reduced by  $44.8 \pm 9.3\%$  and  $45.8 \pm 24.5\%$ , respectively; \*,  $p = 0.03$  and  $0.009$  vs unstimulated PMNs, respectively; Fig. 6B). Down-regulation of the gene encoding IL-1 $\alpha$  correlated with decreased expression of IL-1 $\alpha$  protein at those times, indicating that

IL-1 $\alpha$  is down-regulated at the level of gene expression (compare A and C in Fig. 6).

#### Expression of pro- and anti-inflammatory mediators in PMNs not differentially regulated

Although a focus of these studies was to identify differentially expressed inflammatory mediators during apoptosis, we hypothesized that many genes encoding pro- and anti-inflammatory molecules are expressed in human PMNs, but not differentially regulated. Therefore, we examined expression of 114 genes that encode a significant number of the known inflammatory mediators, but were not up- or down-regulated following phagocytosis by our criteria (Fig. 7). Of these genes, there were a limited number (38) identified as present in human PMNs, and most encoded receptors rather than cytokines (Fig. 7). Importantly, the gene encoding IL-1R antagonist (*IL1RN*) was very highly expressed, consistent





**FIGURE 5.** Down-regulation of surface-expressed PMN proinflammatory molecules. **A**, Surface expression of IL-8R $\beta$ , CD54, and CD31 was measured on unstimulated PMNs (blue square), after FcR/CR-mediated phagocytosis (red square), and during Fas-induced apoptosis (green square) as indicated. \*,  $p < 0.006$  vs unstimulated PMNs (ANOVA). **B**, Coexpression of IL-8R $\beta$ , CD54, CD31, and annexin V-FITC was determined in unstimulated PMNs (blue dot plots), after FcR/CR-mediated phagocytosis (red dot plots), and during Fas-induced apoptosis (green dot plots). Numbers indicate the percentage of cells within each quadrant. Results are from a representative experiment performed five to eight separate times. **C**, Measurement of the expression of genes encoding IL8R $\beta$ , CD54, and CD31 in unstimulated PMNs (Unstim., blue dotted line) or in PMNs after FcR/CR-mediated phagocytosis (red solid line, FcR/CR) was extended to 24 h.

with down-regulation of IL-1 $\alpha$  and proinflammatory capacity during this period of time (Fig. 7). We also note that genes encoding receptors which mediate apoptosis, such as TRAILR2 and CD120a, although not differentially regulated following phagocytosis, were highly expressed in human PMNs (Fig. 7).

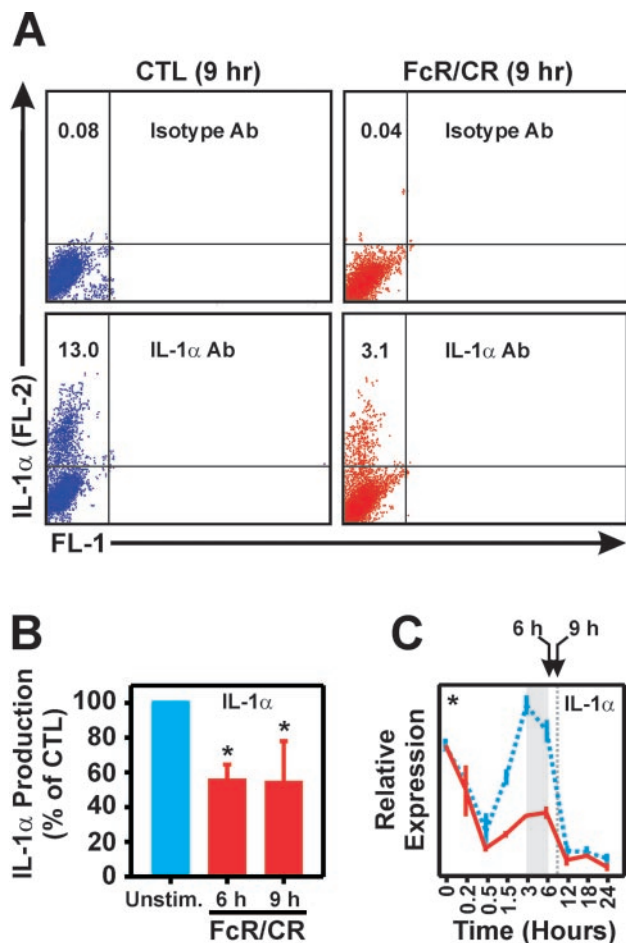
#### Regulation of signal transduction mediators and structural/cytoskeletal proteins

Inasmuch as signal transduction is coupled to regulation of PMN function following receptor ligation, we identified 80 signal transduction-related genes differentially expressed between 3 and 6 h after phagocytosis (Fig. 8). Sixty-three of these genes were down-regulated and 17 were up-regulated (Fig. 8). Thirty of 34 genes encoding kinases and associated proteins were down-regulated, in-

cluding key signal transduction regulators such as class I PI3K, p110 subunits  $\alpha$ ,  $\delta$ , and  $\gamma$ , and calcium/calmodulin-dependent kinases II $\gamma$  and  $\beta$  (Fig. 8). Moreover, genes encoding 12 phosphatases and 14 of 24 G-protein-related molecules were down-regulated during induction of phagocytosis-induced apoptosis (Fig. 8). Genes encoding phospholipases D2 and C $\epsilon$ 2, critical regulators of receptor-mediated signal transduction, were down-regulated (Fig. 8). We also identified 27 differentially regulated genes that encode structural proteins or those comprising and/or involved in modifying the actin cytoskeleton (Fig. 8). For example, genes encoding  $\alpha$ 2-actin, myosin 5A, and isoforms 1 and 4 of tropomyosin were down-regulated during the induction of PMN apoptosis (Fig. 8).

There were only a limited number of up-regulated genes encoding signal transduction mediators at this time (Fig. 8). Of note,





**FIGURE 6.** Down-regulation of IL-1 $\alpha$  during the induction of phagocytosis-induced apoptosis. **A**, IL-1 $\alpha$  was measured in unstimulated (blue dots) or FcR/CR-stimulated (red dots) human PMNs with flow cytometry as described in *Materials and Methods*. Percentage of staining from a representative experiment is indicated within the quadrant. Staining with an IgG1 isotype control Ab is shown in the top half of the panel (dotted line, Isotype). **B**, Quantitation of IL-1 $\alpha$  produced in unstimulated (blue square) and stimulated (red square) PMNs. The graph represents percentage of PMNs staining positive for intracellular IL-1 $\alpha$  after FcR/CR-mediated phagocytosis compared with unstimulated cells. Intracellular IL-1 $\alpha$  was detected in  $1.78 \pm 0.38\%$  and  $8.1 \pm 3.0\%$  of unstimulated PMNs at 6 and 9 h, respectively, compared with  $0.95 \pm 0.05\%$  and  $3.9 \pm 1.3\%$  at those same times in PMNs following FcR/CR-mediated phagocytosis. \*,  $p = 0.03$  and  $0.009$  vs unstimulated PMNs, respectively; Student's  $t$  test. Data represent the mean  $\pm$  SD from three to seven separate individuals. **C**, Measurement of the expression of the gene encoding IL-1 $\alpha$  in unstimulated PMNs (blue dotted line, Unstim.) or in PMNs after FcR/CR-mediated phagocytosis (red solid line, FcR/CR) was extended to 24 h. \*,  $p \leq 0.05$  vs gene expression in unstimulated PMNs at 3 and 6 h.

*PIK3C2B*, the gene encoding a class II PI3K, was significantly up-regulated (Fig. 8). To determine whether expression of *PIK3C2B* diminished at later times during apoptosis, we extended the time-course of phagocytosis out to 24 h and then measured gene expression (Fig. 8B). There was a sharp decrease in the expression of *PIK3C2B* between 6 and 12 h after phagocytosis, consistent with results for genes encoding up-regulated proinflammatory regulators during this same period of time (compare Figs. 4 and 8B). Taken together, these data indicate that PMNs down-regulate capacity for signal transduction at the level of gene expression during apoptosis.

#### Gene-regulated phosphoinositide and calcium-mediated signal transduction

Twenty-three of the signal transduction-related genes identified as differentially regulated during the initial stages of PMN apoptosis (21 down-regulated and 2 up-regulated) comprised complex pathways for phosphoinositide- and calcium-mediated signal transduction (Fig. 8). The observation that genes encoding all three known class I PI3Ks (*PIK3CA*, *PIK3CD*, and *PIK3CG*), Akt1 (*AKT1*) and Akt2 (*AKT2*), and anti-apoptotic transcription factors CREB1 (*CREB1*) and Forkhead box O1A (*FOXO1A*) were down-regulated is consistent with the parallel finding that PMNs are undergoing the initial stages of apoptosis (Ref. 4, Fig. 8, and Table I<sup>4</sup>). We previously described down-regulation of *AKT1* and *FOXO1A* during phagocytosis-induced PMN apoptosis (4). Although *CREB1* encodes a transcription factor, it was included in this study because of its critical importance as a downstream effector for PI3K activation. PI3Ks are also critical for the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), and repression of these genes is a potential mechanism to regulate IP3 synthesis, and thus, calcium signal transduction (Fig. 8). Consistent with those findings, genes encoding proteins directly involved in calcium-mediated signal transduction, such as protein kinase C $\epsilon$  and calcium/calmodulin-dependent protein kinases II $\beta$  and II $\gamma$ , were down-regulated during phagocytosis-, Fas-, and CPT-induced apoptosis (Fig. 8).

Of the differentially expressed genes comprising PI3K/calcium signal transduction, only genes encoding the *ITPR1* and *PI3K, C2 $\beta$*  (*PIK3C2B*) were up-regulated (Figs. 2A and 8A). This finding is intriguing because class II PI3Ks phosphorylate phosphatidylinositol and phosphatidylinositol 4-phosphate rather than PIP2, and their role in leukocyte signal transduction remains unclear (38). Up-regulation of *ITPR1* is at variance with the observation that other critical regulators of calcium signal transduction are down-regulated (Fig. 8). However, *ITPR1* has been implicated in T lymphocyte programmed cell death (39), and it is probable that it has a similar role in PMN apoptosis.

#### Discussion

Several lines of evidence indicate that proinflammatory capacity of human PMNs is regulated at the level of gene expression during apoptosis. First, genes encoding 133 key proinflammatory mediators or signal transduction molecules were down-regulated during the initial stages of phagocytosis-induced apoptosis. These genes encode a significant number of proteins known to moderate essential PMN innate immune and inflammatory responses. The observation that proinflammatory genes are repressed is not likely caused by general down-regulation of transcription 3–6 h after phagocytosis, because 98 genes encoding metabolism and vesicle trafficking are up-regulated during that time (32). Previous studies of Whyte and coworkers (40) demonstrated diminished chemotaxis, degranulation, ROS production, and phagocytosis during spontaneous apoptosis. Our observation that genes encoding proinflammatory mediators and accompanying signal transduction regulators are down-regulated during induction of phagocytosis-induced PMN apoptosis is consistent with those findings. Importantly, our results demonstrate that repression of genes encoding innate immune effectors accompanies reduced PMN function during apoptosis. Second, expression of genes encoding the majority of up-regulated proinflammatory molecules decreased rapidly after the 3- to 6-h “window” of the induction of PMN apoptosis. This finding is especially significant in the context of the expression of genes encoding 9 of 11 up-regulated proinflammatory cytokines, chemokines, and growth factors, including TNF- $\alpha$ ,

Gene (cytokines and chemokines)	Expression	Gene (receptors/anti-inflammatory)	Expression
<b>IL-1<math>\beta</math> (IL1B)</b>	<b>High</b>	<b>IL-1 receptor antagonist (IL1RN)</b>	<b>Very High</b>
IL-2 (IL2)	Absent	<b>IL-1 receptor, <math>\beta</math> (IL1R<math>\beta</math>)</b>	<b>High</b>
IL-3 (IL3)	Absent	IL-2 receptor, $\alpha$ (IL2RA)	Absent
IL-4 (IL4)	Absent	IL-2 receptor, $\beta$ (IL2RB)	Absent
IL-5 (IL5)	Absent	<b>IL-2 receptor, <math>\gamma</math> (IL2RG)</b>	<b>High</b>
IL-7 (IL7)	Absent	<b>IL-3 receptor, <math>\alpha</math> (IL3RA)</b>	<b>Moderate</b>
<b>IL-8 (IL8)</b>	<b>Moderate</b>	<b>IL-4 receptor (IL4R)</b>	<b>High</b>
IL-9 (IL9)	Absent	<b>IL-5 receptor, <math>\alpha</math> (IL5RA)</b>	<b>Low</b>
IL-10 (IL10)	Absent	<b>IL-6 receptor (IL6R)</b>	<b>High</b>
IL-11 (IL11)	Absent	<b>IL-7 receptor (IL7R)</b>	<b>Low</b>
IL-12 $\alpha$ (IL12A)	Absent	<b>IL-8 receptor, type A (IL8RA)</b>	<b>High</b>
IL-12 $\beta$ (IL12B)	Absent	<b>IL-10 receptor, <math>\beta</math> (IL10RB)</b>	<b>Low</b>
IL-13 (IL13)	Absent	IL-11 receptor, $\alpha$ (IL11RA)	Absent
IL-14 (IL14)	Absent	IL-12 receptor, $\beta$ 1 (IL12RB1)	Absent
IL-15 (IL15)	Absent	IL-12 receptor, $\beta$ 2 (IL12RB2)	Absent
IL-17 (IL17)	Absent	IL-13 receptor, $\alpha$ 2 (IL13RA2)	Absent
<b>IL-18 (IL18)</b>	<b>Very Low</b>	<b>GCSF receptor 3, CD114 (CSF3R)</b>	<b>High</b>
Interferon, $\alpha$ 1 (IFNA1)	Absent	<b>GM-CSF receptor, <math>\alpha</math> CD116 (CSF2RA)</b>	<b>Low</b>
Interferon, $\alpha$ 2 (IFNA2)	Absent	<b>GM-CSF receptor, <math>\beta</math> CDw131 (CSF2RB)</b>	<b>Very High</b>
Interferon, $\alpha$ 4 (IFNA4)	Absent	Chemokine receptor 2 (CCR2)	Absent
Interferon, $\alpha$ 5 (IFNA5)	Absent	<b>Chemokine receptor 3 (CCR3)</b>	<b>Moderate</b>
Interferon, $\alpha$ 6 (IFNA6)	Absent	Chemokine receptor 4 (CCR4)	Absent
Interferon, $\alpha$ 8 (IFNA8)	Absent	Chemokine receptor 5 (CCR5)	Absent
Interferon, $\alpha$ 10 (IFNA10)	Absent	Chemokine receptor 6 (CCR6)	Absent
Interferon, $\alpha$ 14 (IFNA14)	Absent	<b>Chemokine receptor 7 (CCR7)</b>	<b>Moderate</b>
Interferon, $\alpha$ 16 (IFNA16)	Absent	Chemokine receptor 8 (CCR8)	Absent
Interferon, $\alpha$ 21 (IFNA21)	Absent	Chemokine receptor 9 (CCR9)	Absent
Interferon, $\beta$ 1 (IFNB1)	Absent	<b>Interferon <math>\gamma</math> receptor 1 (IFNGR1)</b>	<b>Very High</b>
Interferon, $\gamma$ (IFNG)	Absent	<b>Interferon <math>\gamma</math> receptor 2 (IFNGR2)</b>	<b>High</b>
Interferon, $\omega$ 1 (IFNW1)	Absent	<b>Interferon (<math>\alpha</math>,<math>\beta</math>,<math>\omega</math>) receptor 2 (IFNAR2)</b>	<b>Low</b>
<b>Colony stimulating factor 1 (CSF1)</b>	<b>High</b>	<b>Interferon (<math>\alpha</math>,<math>\beta</math>,<math>\omega</math>) receptor (IFNAR1)</b>	<b>Low</b>
Colony stimulating factor 2 (CSF2)	Absent	<b>TNF receptor superfamily member 1A, CD120a (TNFRSF1A)</b>	<b>High</b>
Colony stimulating factor 3, granulocyte (CSF3)	Absent	TNF receptor superfamily member 4, OX40 (TNFRSF4)	Absent
Small inducible cytokine, C1, lymphotactin (SCYC1)	Absent	<b>TNF receptor superfamily member 5, CD40 (TNFRSF5)</b>	<b>Moderate</b>
<b>Small inducible cytokine, B5, ENA-78 (SCYB5)</b>	<b>Very Low</b>	<b>TNF receptor superfamily member 6, CD95 (TNFRSF6)</b>	<b>Moderate</b>
Small inducible cytokine, B9B (SCYB9B)	Absent	TNF receptor superfamily member 6B, (TNFRSF6B)	Absent
Small inducible cytokine, B10, IP-10 (SCYB10)	Absent	TNF receptor superfamily member 7, CD27 (TNFRSF7)	Absent
Small inducible cytokine, B11 (SCYB11)	Absent	<b>TNF receptor superfamily member 8, CD30 (TNFRSF8)</b>	<b>Low</b>
Small inducible cytokine, B13 (SCYB13)	Absent	<b>TNF receptor superfamily member 9, CD137 (TNFRSF9)</b>	<b>Low</b>
Small inducible cytokine, A1(SCYA1)	Absent	<b>TNF receptor superfamily member 10B, TRAILR2 (TNFRSF10B)</b>	<b>High</b>
<b>Small inducible cytokine, A5, RANTES (SCYA5)</b>	<b>Moderate</b>	<b>TNF receptor superfamily member 10C, TRAILR3 (TNFRSF10C)</b>	<b>High</b>
Small inducible cytokine, A8, MCP-2 (SCYA8)	Absent	<b>TNF receptor superfamily member 10D, TRAILR4 (TNFRSF10D)</b>	<b>Low</b>
Small inducible cytokine, A11, eotaxin (SCYA11)	Absent	TNF receptor superfamily member 11A, (TNFRSF11A)	Absent
Small inducible cytokine, A13, MCP-4 (SCYA13)	Absent	TNF receptor superfamily member 11B, (TNFRSF11B)	Absent
Small inducible cytokine, A14, MIPF-1 (SCYA14)	Absent	<b>TNF receptor superfamily member 12, DDR3 (TNFRSF12)</b>	<b>Moderate</b>
Small inducible cytokine, A16 (SCYA16)	Absent	TNF receptor superfamily member 17, (TNFRSF17)	Absent
Small inducible cytokine, A17 (SCYA17)	Absent	TNF receptor superfamily member 21, DR6 (TNFRSF21)	Absent
<b>Small inducible cytokine, A18 (SCYA18)</b>	<b>Low</b>	<b>TNF receptor superfamily member 3, CD18 (LTBR)</b>	<b>Moderate</b>
Small inducible cytokine, A19, MIP-3 $\beta$ (SCYA19)	Absent	Oncostatin M receptor (IL6ST)	Absent
Small inducible cytokine, A21 (SCYA21)	Absent		
Small inducible cytokine, A22 (SCYA22)	Absent		
Small inducible cytokine, A23 (SCYA23)	Absent		
Small inducible cytokine, A25 (SCYA25)	Absent		
Small inducible cytokine, A27 (SCYA27)	Absent		
<b>TNF superfamily member 3, Lymphotoxin <math>\beta</math> (LTB)</b>	<b>High</b>		
TNF superfamily member 4, OX40L (TNFSF4)	Absent		
TNF superfamily member 5, CD40L (TNFSF5)	Absent		
TNF superfamily member 6, CD95L (TNFSF6)	Absent		
TNF superfamily member 7, CD27L (TNFSF7)	Absent		
TNF superfamily member 8, CD30L (TNFSF8)	Absent		
TNF superfamily member 9, (TNFSF9)	Absent		
TNF superfamily member 11, (TNFSF11)	Absent		
TNF superfamily member 12, APO3 (TNFSF12)	Absent		
TNF superfamily member 13, APRIL (TNFSF13)	Absent		
<b>TNF superfamily member 14, HVEM-L (TNFSF14)</b>	<b>High</b>		

**FIGURE 7.** Expression levels for pro- and anti-inflammatory mediators not differentially regulated. Gene expression was measured with Affymetrix human oligonucleotide microarrays 3–6 h after receptor-mediated phagocytosis as described in *Materials and Methods*. Expression levels for genes called “Present” (boldface type) by GeneChip Suite (Affymetrix) were determined based upon ADI values as described in *Materials and Methods*. Genes called “Absent” by GeneChip Suite are listed as absent. In some cases, several probe sets were used to determine the expression level for a single gene.

VEGF, OSM, IL-6, and GROs  $\beta$  and  $\gamma$  (Fig. 4). These results indicate that proinflammatory signals up-regulated because of phagocytosis/PMN activation are subsequently repressed at the onset of apoptosis.

Repression of genes encoding CD31 and IL-8R $\beta$  correlated with down-regulation of the surface expression of these proteins. Down-regulation of CD31, a molecule required for transendothelial migration of PMNs (41), has been shown to accompany spontaneous PMN apoptosis, although no link was provided between gene and protein expression (42). Although it could be argued that decreased surface expression of IL-8R $\beta$  and CD31 in stimulated cells is caused by internalization mediated by endocytosis (43), we found no significant difference in the surface expression of IL-8R $\beta$

(between unstimulated and stimulated PMNs) until there was concomitant change in gene expression (Fig. 5). Of note, little evidence has been provided for the modulation of IL-8R $\beta$  during PMN apoptosis. The gradual decrease in IL-8R $\beta$  on the PMN surface in our studies (over several hours) contrasts with very rapid (within 10 min) down-regulation observed during endocytotic IL-8R $\beta$  internalization (43). Loyd and coworkers (44) previously demonstrated a link between IL-8R $\beta$  gene transcription and expression of protein on the PMN surface, which occurred on the order of hours rather than minutes, just as we found in our studies. Moreover, down-regulation of IL-8R $\beta$  surface expression occurred at a point in time in which PMN secretion of IL-8 and other CXCR2 ligands was likely diminished compared with earlier time



A

Kinases & associated proteins (34)	Phago.	$\alpha$ Fas	CPT
*A kinase anchor protein 2 (AKAP2)	+	+	-
dual-specificity TYR-phos. regulated kinase 3 (DYRK3)	+	NC	+
phosphoinositide 3-kinase, class 2 $\beta$ (PIK3C2B)	++	NC	+
*tousled-like kinase 1 (TLK1)	+	NC	-
*phosphoinositide 3-kinase, p110 $\alpha$ (PIK3CA)	-	NC	-
*serine/threonine kinase 3 (STK3)	-	NC	-
protein kinase C, $\tau$ (PRKC1)	-	+	-
CaM kinase II $\gamma$ (CAMK2G)	-	**	**
CaM kinase II $\beta$ (CAMK2B)	-	**	**
vaccinia-related kinase 2 (VRK2)	-	NC	-
phosphoinositide 3-kinase, p110 $\delta$ (PIK3CD)	-	NC	-
phosphoinositide 3-kinase, p110 $\gamma$ (PIK3CG)	-	NC	-
PTEN induced putative kinase 1 (PINK1)	-	NC	NC
protein kinase, lysine deficient 1 (PRKWINK1)	-	NC	+
protein kinase C binding protein 1 (PRKCBP1)	-	+	+/**
protein tyrosine kinase 9 like (PTK9L)	-	**	NC
**c-fes/fps (FES)	-	NC	**
*spleen tyrosine kinase (SYK)	-	-	-
*tyrosine kinase 2 (TYK2)	-	-	-
**casein kinase 1, $\gamma$ 2 (CSNK1G2)	-	-	-
**MAP/microtubule affinity reg. kinase 2 (MARK2)	-	-	-
*G protein-coupled receptor kinase 5 (GPRK5)	-	-	-
*PI 4-phosphate 5-kinase, II $\beta$ (PIP5K2B)	-	NC	NC
*protein kinase, AMP-activated, $\beta$ 1 (PRKAB1)	-	NC	NC
*protein kinase C-like 2 (PRKCL2)	-	-	-
*serine/threonine kinase 38-like (STK38L)	-	+	NC
*serine/threonine kinase (STK38)	-	NC	-
*diacylglycerol kinase, $\zeta$ (DGKZ)	-	-	-
*c-mer tyrosine kinase (MERTK)	-	+	+
*activated p21cdc42Hs kinase (ACK1)	-	NC	NC
*Bruton agammaglobulinemia tyrosine kinase (BTK)	-	+	NC
**Akt2 (AKT2)	-	-	-
*A kinase anchor protein 8 (AKAP8)	-	-	NC
*A kinase anchor protein 9 (AKAP9)	-	NC	-

G-proteins & associated molecules (23)	Phago.	$\alpha$ Fas	CPT
*guanine nucleotide exchange factor for Rap1 (GFR)	+	+	NC
*ADP-ribosylation factor-like 4 (ARL4)	+	NC	NC
G-protein-coupled receptor induced protein (GIG2)	+	**	+
rho GDP dissociation inhibitor, $\alpha$ (ARHGDI1)	+	**	+
reg D protein (RAGD)	+	**	**
*pseudautosomal GTP-binding protein-like (PGPL)	+	-	NC
*rho7 (RH07)	+	+	+
*ARF GTPase activating protein 1 (ARFGAP1)	+	+	-
*guanine nucleotide binding protein, $\alpha$ 2 (GNAI2)	+	-	NC
*IQ motif GTPase activating protein 2 (IQGAP2)	-	NC	NC
regulator of G-protein signaling 14 (RGS14)	-	-	NC
regulator of G-protein signaling 3 (RGS3)	-	**	-
G-protein-coupled receptor kinase interactor 2 (GIT2)	-	-	-
G-protein-coupled receptor 107 (GPR107)	-	+	-
HTCD37	-	NC	NC
*membrane interacting protein of RGS16 (MIR16)	-	NC	NC
*guanylate binding protein 2 (GBP2)	-	-	-
*guanine nucleotide binding protein, $\gamma$ 7 (GNG7)	-	NC	NC
*ras p21 activator 1 (RASA1)	-	NC	-
*ralA binding protein 1 (RALBP1)	-	-	-
*ras guanyl releasing protein 2 (RASGRP2)	-	-	-
*P114-RHO-GEF	-	-	-
*signal-induced proliferatio-associated gene 1 (SIPA1)	-	-	-

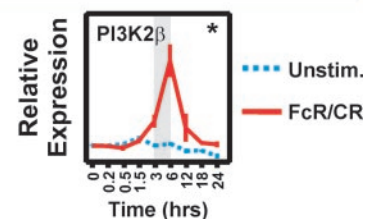
Phosphatases & related proteins (12)	Phago.	$\alpha$ Fas	CPT
*protein phosphatase 1, reg. subunit 3D (PPP1R3D)	-	NC	NC
*SHP2 interacting transmembrane adaptor (SIT)	-	NC	NC
*protein tyrosine phosphatase, non-receptor 1 (PTPN1)	-	-	-
*protein tyrosine phosphatase, type IVA, 1 (PTP4A1)	-	NC	+
*protein tyrosine phosphatase, type IVA, 2 (PTP4A2)	-	-	-
*protein tyrosine phosphatase, receptor N (PTPRN)	-	+	+
*protein tyrosine phosphatase, receptor N2 (PTPRN2)	-	NC	NC
*inositol polyphosphate 4-phosphatase, 1 (INPP4A)	-	NC	-
*protein phosphatase 1, catalytic $\gamma$ subunit (PPP1CC)	-	-	-
*inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2)	-	-	-
*P/S/T phosphatase interacting protein 1 (PSTPIP1)	-	-	NC
*myotubularin related protein 1 (MTMR1)	-	NC	-

B

Structural/cytoskeletal proteins (27)	Phago.	$\alpha$ Fas	CPT
*talin 2 (TLN2)	+	+	+
*transglutaminase 3 (TGM3)	+	**	**
* $\alpha$ 1 liprin (PPF1A1)	+	NC	NC
transgelin 2 (TAGLN2)	+	**	**
$\beta$ -spectrin, non-erythrocytic 1 (SPTBN1)	++	+	-
$\beta$ 2 liprin (PPFIBP2)	+	NC	+
*dynein (DNAH17)	+	NC	+
*osteopontin (SPP1)	++	NC	NC
*radixin (RDX)	+	+	+
*kinesin 3B (KIF3B)	-	NC	-
* $\beta$ 1 crystallin superfamily (AIM1)	-	-	-
*fascin, actin-bundling protein (SNL)	-	NC	-
*cytohesin-2 (PSCD2)	-	NC	NC
tubulin, $\alpha$ 1 (TUBA1)	-	NC	**
$\beta$ -amyloid precursor protein-binding protein (APPBP2)	-	NC	-
*myosin 5A (MYO5A)	-	+	-
*catenin, $\delta$ 1 (CTNND12)	-	+	NC
* $\alpha$ -tropomyosin 1 (TPM1)	-	NC	NC
* $\alpha$ -tropomyosin 4 (TPM4)	-	NC	-
*kinesin 3C (KIF3C)	-	+	NC
*actin, $\alpha$ 2 (ACTA2)	-	-	NC
*nuclear mitotic apparatus protein 1 (NUMA1)	-	-	-
*clathrin light chain (CLTB)	-	NC	NC
*plectin 1 (PLEC1)	-	-	NC
*fibrinogen-like 2 (FGL2)	-	-	-
*CLIP-associating protein 1 (CLASP1)	-	NC	-
*golgi reassembly stacking protein 1 (GORASP1)	-	NC	NC

Miscellaneous (34)	Phago.	$\alpha$ Fas	CPT
*GPI-PLD1 (GPLD1)	+	+	+
*parvalbumin (PVALB)	++	NC	+
*haptoglobin-related protein (HPR)	+	+	+
linker for activation of T-cells (LAT)	+	+	+
alpha integrin binding protein 63 (AIBP63)	+	+	+
adipophilin (ADFP)	+++	**	+
coagulation factor III (F3)	+++	**	+/**
semenogelin 1 (SEMG1)	+++	**	+/**
serine protease inhibitor, Kazal type 1 (SPINK1)	+++	**	**
serine (or Cys) proteinase inhibitor, B2 (SERPINB2)	+	**	**
tetraspan 3 (TSPAN3)	+	NC	**
*annexin A2 (ANXA2)	++	+	+
*serine (or Cys) proteinase inhibitor, B9 (SERPINB9)	+	+	-
*calphastin (CAST)	+	+	NC
*secretory leukocyte protease inhibitor (SLPI)	+	-	NC
*protein associated with SH3 domain of STAM (AMSH)	-	-	NC
*cdc42 effector protein (CDC42CEP2)	-	NC	-
*centaurin, $\delta$ 1 (CENTD1)	-	-	-
thrombospondin (THBD)	-	+	NC
phospholipase D2 (PLD2)	-	**	+
phospholipase C, $\epsilon$ 2 (PLCL2, PLCE2)	-	NC	-
coagulation factor V (F5)	-	+	**
butyrophilin, 3A2 (BTN3A3)	-	-	-
DiGeorge syndrome critical region gene 2 (DGCR2)	-	+	+/**
cAMP responsive element binding protein 1 (CREB1)	-	NC	+
*CDP-DAG-inositol 3-phosphatidytransferase (CDIPT)	-	+	NC
*myelin protein zero-like 1 (MPZL1)	-	+	NC
*sec7 homolog (TIC)	-	NC	-
*arrestin, $\beta$ 2 (ARRB2)	-	NC	NC
*seven in absentia homolog 2 (SIAH2)	-	-	NC
*enigma, LIM domain binding protein (ENIGMA)	-	-	-
*flotillin 2 (FLOT2)	-	-	NC
*syntrophin, $\beta$ 2 (SNTB2)	-	NC	NC
*TBC1 domain family member 1 (TBC1D1)	-	-	-



**FIGURE 8.** Differential regulation of genes encoding signal transduction mediators and structural/cytoskeletal proteins. A, Differential gene expression was measured with Affymetrix human oligonucleotide microarrays during the induction of PMN apoptosis 3–6 h after receptor-mediated phagocytosis (Phago.), or following treatment with anti-Fas Ab ( $\alpha$ Fas) or CPT. Symbols and colors are defined in the legend for Fig. 1. B, Measurement of the expression of the gene encoding the class II PI3K2 $\beta$  in unstimulated PMNs (blue dotted line, Unstim.) or in PMNs after FcR/CR-mediated phagocytosis (red solid line, FcR/CR) was extended to 24 h. \*,  $p \leq 0.05$  vs gene expression in unstimulated PMNs at either 3 or 6 h.

points (Fig. 2). Because IL-8 is a potent chemokine that modulates key leukocyte responses such as chemotaxis (45), we propose that down-regulation of IL-8R $\beta$  during apoptosis is critical for the resolution of inflammation following PMN activation. Of note, the

gene encoding  $\beta_2$  arrestin (ARRB2), a protein that modulates IL-8R internalization, was down-regulated (Fig. 7).

Phagocytosis-induced PMN apoptosis was accompanied by significant decrease in the gene encoding IL-1 $\alpha$  and importantly, the

corresponding level of IL-1 $\alpha$  protein was diminished significantly. Although a seemingly small population of PMNs expressed detectable IL-1 $\alpha$  in our assays (Fig. 6A), we found that the percentage of PMNs with detectable IL-1 $\alpha$  increased significantly over time because of accumulating cytokine in the ER after treatment with brefeldin A. As a result, when the time of brefeldin A treatment was extended, the level of detectable IL-1 $\alpha$  increased (from  $1.78 \pm 0.38\%$  IL-1 $\alpha$ -positive PMNs at 6 h, to  $8.1 \pm 3.0\%$  and  $24.2 \pm 4.9\%$  at 9 and 16 h, respectively). Most significantly, there was always less intracellular IL-1 $\alpha$  in stimulated PMNs compared with unstimulated cells (Fig. 6). Production of IL-1 $\alpha$  by PMNs has not been characterized, but the cytokine has a critical role in the innate immune and inflammatory responses in humans (46). Pro-IL-1 $\alpha$ , the fully active IL-1 $\alpha$  precursor, is released upon cell death (47), and would occur during necrotic PMN lysis. Because IL-1 $\alpha$  is an important inflammation-inducing cytokine, its down-regulation by human PMNs during apoptosis has important implications for termination of the inflammatory response. In support of these findings, the gene encoding IL-1R accessory protein, a molecule critical for IL-1 signal transduction (48), was down-regulated (Fig. 2).

Multiple PMN functions are regulated by phosphoinositide metabolism and calcium-mediated signal transduction (reviewed in Refs. 20–24). Ligation of cell surface receptors, including IL-8R $\beta$ , activates PI3Ks, which in turn moderate a balance between PIP2 and PIP3, important second messengers for downstream signal transduction events and subsequent cell activation. PIP3 concentrations increase dramatically upon PMN stimulation because of the conversion of PIP2 to PIP3 by class I PI3Ks (49). By comparison, PIP2 is present at high constitutive levels in human PMNs (49) and is hydrolyzed to IP3 and diacylglycerol by phospholipase C. It is PIP2-derived IP3 that binds to the ITPR1 and elicits efflux of calcium from the ER into cytosol, thus facilitating subsequent calcium-regulated cellular processes. Of note, PI3K signal transduction has been directly linked to ROS generation in PMNs (17–19). Therefore, regulating PI3K and calcium-mediated signal transduction is important in maintaining a balance between pro- and anti-inflammatory signals in the cell. The observation that at least 21 genes encoding PI3K and calcium signal transduction pathways were down-regulated suggests that the ability of the cell to respond to proinflammatory stimuli, such as IL-8, was limited. Thus, proinflammatory mediators and the signal transduction pathways linking them to cellular responses are down-regulated at the level of gene expression during PMN apoptosis. Importantly, these studies define precisely the regulation of specific components of complex signal transduction pathways essential for PMN function.

In conclusion, we discovered that proinflammatory capacity is 1) down-regulated during the induction of PMN apoptosis, and 2) regulated at the level of gene expression. We recently proposed that an apoptosis differentiation program regulates, in part, cell fate, energy metabolism, and responses to oxidative stress, and represents a final stage of transcriptionally regulated PMN maturation (32). Based on the findings reported herein, the apoptosis differentiation program down-regulates proinflammatory capacity as well. We propose that these processes are critical for the resolution of inflammation and bacterial infections in humans.

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