Gene expression analysis illuminates the transcriptional programs underlying the functional activity of ex vivo-expanded granulocytes

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Huang LT, Paredes CJ, Papoutsakis ET, Miller WM. Gene expression analysis illuminates the transcriptional programs underlying the functional activity of ex vivo-expanded granulocytes. Physiol Genomics 31: 114–125, 2007. First published June 5, 2007; doi:10.1152/physiolgenomics.00053.2007.—Global gene expression analysis established the temporal expression patterns and programs underlying the development of functional activity of ex vivo-expanded (EXE) human granulocytes, as well as differences compared with peripheral blood (PB) granulocytes. CD34+ progenitor cells were cultured for 3 wk to induce rapid expansion and granulocytic differentiation, with 40% CD15+ cells by day 3 and 90% by day 12. Phagocytic and respiratory burst activity increased with the fraction of CD15+CD11b+ cells (myelocytes to segmented) and peaked by day 17. However, only 25% of CD15+CD11b+ cells were phagocytic, and respiratory burst activity was one-third that of PB granulocytes. EXE granulocytes from later days and PB granulocytes showed similar expression of Fcγ receptors (-1A, -2A, -2C, -3A) and complement receptors (-1, -3, -4). Later downregulation of CD36 (expressed by macrophages) suggests lineage plasticity early in granulocytic differentiation. Expression in mature EXE and PB granulocytes was similar for most Fcγ receptor-mediated phagocytosis signaling proteins, including high-level expression of Hck, Fgr, and the actin-related protein 2/3 complex. Lower expression of Lyn, Cdc42, pleckstrin, and PKCζ by EXE granulocytes may explain decreased phagocytosis. PB and mature EXE granulocytes expressed similar levels of NADPH oxidase complex genes and receptors for fMLP-mediated respiratory burst. Lower burst activity by EXE granulocytes may result from lower expression of Raf1 and PKCζ. Elevated expression of toll-like receptor (TLR)2, TLR1, and CD14 in mature EXE and PB granulocytes supports a role for the TLR2 and CD14 pathway in zymosan-mediated respiratory burst activity. Lower activity in EXE granulocytes may be due to greater expression of IRAK3, which inhibits TLR-mediated signaling.

DNA microarray; neutrophil; phagocytosis; respiratory burst; cell culture

Neutrophils provide the first line of defense against microbial invasion. Granulocytes produced in culture have the potential to mitigate neutropenia associated with chemotherapy or severe infections. Numerous studies have demonstrated that ex vivo-expanded (EXE) myeloid cells derived from CD34+ hematopoietic stem and progenitor cells (HSPCs) can decrease the period of neutropenia following high-dose chemotherapy and HSPC transplantation (9, 37, 43, 46, 49). However, it has proven difficult to allocate specific beneficial effects to the different cell populations. Granulocyte transfusion to treat infections, which requires ~3 × 1010 cells/dose, has experienced renewed interest because the availability of granulocyte colony-stimulating factor (G-CSF) has greatly increased the neutrophil harvest (1, 6, 45). However, widespread use of granulocyte transfusions is limited by donor fatigue because of the need for daily transplants and the potential for alloimmunization. Ex vivo production of granulocytes from autologous CD34+ cells has the potential to overcome both limitations (22–24, 40), but it must be demonstrated that EXE granulocytes are functionally active.

EXE granulocytes typically exhibit lower phagocytic and respiratory burst activity than peripheral blood (PB) neutrophils (18, 22, 24, 25, 40). Differences in reported EXE granulocyte functional activity may be attributed to variability in the extent of granulocytic (G) maturation due to different culture conditions, harvest times, cell sources, and cytokines. However, the complex cellular programs underlying function acquisition make it difficult to elucidate possible mechanisms that might explain such differences. Targeted genomic analysis is well suited for investigating complex phenotypes. Here, we explored temporal gene expression analysis as a means for explaining the differences in functional activity between EXE and PB granulocytes. Moreover, coordinated temporal analysis, during ex vivo culture, of functional activity and the expression profile of genes associated with the respective activation and/or signaling pathways may provide insight into the development and regulation of functional activity during G maturation. Microarray analysis has been used to evaluate changes in gene expression with G maturation in vivo (58) and ex vivo (27). However, functional activity was not measured, the use of granulocyte-macrophage CSF (GM-CSF) resulted in only 50–75% G purity (27), and the ex vivo culture was carried out for only 11 days, whereas respiratory burst activity likely peaks at later time points (25).

In this study, we carried out a comprehensive analysis of G differentiation, respiratory burst activity, phagocytosis, and gene expression over 24 days in cultures initiated with CD34+ cells from mobilized PB using cytokines that resulted in 10,000-fold cell expansion with 95% G purity. We also evaluated PB granulocytes from normal donors to provide a direct comparison with mature EXE granulocytes. Our objectives were to correlate functional activity with the stage of G cell maturation in culture and to use temporal gene expression microarray analysis to 1) investigate the gene expression profile that underlies increased functional activity with G maturation, 2) explain differences in functional activity between EXE and PB granulocytes, and 3) understand the basis for differences in respiratory burst activity after incubation of EXE and PB granulocytes with different agonists.
Materials and methods

Cells and culture conditions. Cultures were initiated with fresh or frozen G-CSF-mobilized PB CD34+ cells from normal donors (AllCells, Emeryville, CA). Cells were cultured in medium supplemented with 12.5% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 12.5% heat-inactivated horse serum (Stem Cell Technologies, Vancouver, BC, Canada), 10 ng/ml IL-3 (R&D Systems, Minneapolis, MN), 10 ng/ml IL-6 (PeproTech, Rocky Hill, NJ), 50 ng/ml stem cell factor (SCF; Amgen, Thousand Oaks, CA) and 10 ng/ml G-CSF (Amgen) as described (23). Cultures were maintained between 0.3 and 3 x 10^6 cells/ml in a humidified 37°C incubator under 5% CO₂ and either 5 or 20% O₂. Since G-CSF degrades rapidly at 37°C (52), G-CSF (10 ng/ml) was supplemented every 2 days. PB granulocytes for the phagocytosis and respiratory burst assays were obtained from normal donors after informed consent. Briefly, blood was collected into Vacutainer tubes with sodium heparin (BD Biosciences, San Jose, CA) by medical technologists at the Northwestern University Health Service Clinical Laboratory. Samples were transported to our laboratory under ambient conditions (~20 min) and centrifuged at 250 g for 10 min to recover plasma. Cells were resuspended in PBS, layered onto 2% dextran (Sigma) in PBS, and sedimented on the bench for 30 min. The granulocyte-containing fraction was used immediately for the phagocytosis and respiratory burst assays. This study was approved by the Northwestern University Institutional Review Board.

Flow cytometry. The expression of surface markers CD34, CD15, CD11b, and CD66b was measured using flow cytometry (23). Briefly, 2 x 10^6 cells/tube were stained with phycoerythrin (PE)-labeled anti-CD34 (clone 581, IgG1), fluorescein isothiocyanate (FITC)-labeled anti-CD15 (clone MMa, IgM), PE-labeled anti-CD11b (clone ICRF44, IgG) and/or FITC-labeled anti-CD66b (clone G10F5, IgM); fluorocytically conjugated isotype controls were used for comparison (all antibodies from BD Biosciences). Propidium iodide (0.2 mg/ml; Sigma) was used to exclude dead cells from analysis. Data were acquired using a BD FACSscan flow cytometer (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or WEASEL (WEHI, Melbourne, Australia) software.

Wright-Giemsa staining. Cells were centrifuged onto microscope slides using Cytospin 3 (Thermo Electron, Louisville, CO). Slides were fixed in fresh methanol for 10 s and stained with Quik Stain II (Microsystems, Bannockburn, IL). Stained cells were imaged using a Leica DMRB Upright Light Microscope (Leica Microsystems, Bannockburn, IL).

DNA microarray experiments and analysis. EXE granulocytes were harvested at each sampling time point, washed twice with RNase-free PBS, flash-frozen in liquid nitrogen, and stored at −80°C. We did not enrich for CD15+ cells before analysis because the cultures contained 40% CD15+ cells by day 3, and this increased to 90% by day 12, when substantial functional activity was observed. Unless noted, all materials and methods used in microarray experiments and data analysis were from Agilent Technologies (Palo Alto, CA) and were performed as described previously (16). Briefly, RNA from input CD34+ cells or cells harvested from culture were purified from frozen cell pellets. Reference RNA was obtained from Stratagene (La Jolla, CA; Universal Human Reference RNA), and RNA from PB granulocytes (96–99% CD15+ cells) was obtained from AllCells. Purified RNA was amplified and reverse-transcribed into labeled cDNA and hybridized to Agilent 1A(2) microarrays. Hybridizations were performed in a reference design; each biological sample was labeled with cyanine-3 (Cy3) and co-hybridized with reference RNA labeled with Cy5. The resulting data were normalized as previously described, and data from technical replicates (performed on ~15% of all samples) were averaged (62). Data from multiple probes for the same transcript variant were averaged after verification of the probe sequences against the National Center for Biotechnology Information (NCBI) sequence database (http//www.ncbi.nlm.nih.gov/BLAST/). For PB granulocytes, RNA from three donors was analyzed. For EXE granulocytes, RNA from three CD34+ cell donors was analyzed. CD34+ cells from each donor were used to initiate two cultures, one maintained under 5% O₂ and the other under 20% O₂. Similar gene expression patterns were observed for cells maintained under 5 or 20% O₂ (Supplemental Fig. S1; supplemental data are available at the online version of this article), so data from all six cultures were combined for analysis (n = 6 from 3 donors). Raw and normalized data were deposited in the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/(GSE5922, GSE6792). Subsequent data analysis was performed using the MultiExperiment Viewer 3.0 (MeV; Institute for Genomic Research, Rockville, MD) (50). Differentially expressed genes were identified using the MeV analysis of variance (ANOVA) feature with P < 0.05.

Quantitative RT-PCR. cDNA was obtained from total RNA samples using the High-Capacity cDNA Archive kit, and quantitative RT-PCR (QRT-PCR) was performed with Assays-on-Demand kits (Applied Biosystems, Foster City, CA) (16). The amount of mRNA for each sample was normalized using the average of two housekeeping genes (GUSB and RPLPO) (15, 16, 48). The primer codes were as follows: GUSB (Hs99999908_m1), RAB20 (Hs00215134_m1), and STAT3 (Hs00234174).

Phagocytosis assay. Phagocytic activity was measured with the Phagotest kit (Orpegen Pharma, Heidelberg, Germany). Briefly, 5 x 10^5 cells were incubated with autologous plasma and 1.25 x 10^7 FITC-labeled opsonized Escherichia coli at 37°C for 10 min. Cells were washed to remove E. coli and labeled with the fluorescent DNA-staining solution provided to distinguish cells from E. coli. The percentage of phagocytic cells was measured using a FACSscan flow cytometer (BD Biosciences).

Respiratory burst assay. The respiratory burst activity stimulated by three agonists, N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP), zymosan, and phorbol 12-myristate 13-acetate (PMA) (all Sigma), alone or in the presence of TNF-α (R&D Systems) or the PKC inhibitor GF109203X HCl (Sigma), was detected by measuring the H₂O₂-catalyzed hydrolysis of nonfluorescent 2,7'-dichlorofluorescein diacetate (DCF-DA) to fluorescent DCF. The assay was performed as described (34), except that residual erythrocytes were removed with an ammonium chloride lysing solution, autologous plasma (5% vol/vol final concentration) was added at the same time as fMLP (90 pM), cells were incubated for 15 min after stimulus addition, and TNF-α (20 ng/ml) or GF109203X HCl (1 μM) was preincubated with cells for 30 or 15 min, respectively, before DCF-DA addition. DCF fluorescence intensity was measured on a FACSscan flow cytometer (BD Biosciences).

Results

CD34+ cells undergo selective G differentiation ex vivo and exhibit phagocytic and respiratory burst activity. We first examined how the functional activity of EXE granulocytes correlated with the extent of G maturation, and also compared this to the functional activity of PB granulocytes. The number of cells in cultures initiated with PB CD34+ cells expanded ~11,000-fold by day 24 (data not shown). G maturation is marked by sequential expression of CD15 and CD11b as follows: CD15+/CD11b− (myeloblasts), CD15+/*/CD11b− (promyelocytes/early myelocytes), and finally CD15+/CD11b+ (early myelocytes to band and segmented stages) (57). There was rapid commitment to the G lineage, with 40% CD15+ cells on day 3 and >80% by day 10 (Fig. 1A). The percentage of CD15+/CD11b− cells peaked at ~25% on day 3 and that of CD15+/CD11b− cells peaked at ~50% on days 8–9 (Fig. 1B). Very few cells expressed CD34 after day 9. The CD15+/*/CD11b+ cell fraction increased rapidly after day 7 and reached...
a plateau of \( \sim 80\% \) by day 17. Since CD15 and CD11b are also present on monocytes, we confirmed the presence of granulocytes using the G-specific marker CD66b, which appeared several days earlier than CD11b and was expressed by \( \sim 90\% \) of the cells by day 14 (Fig. 1A). Cells at days 5 and 7 exhibited oval nuclei characteristic of the myeloblast/promyelocyte stages (Fig. 2, A and B), whereas cells at days 9 and 11 showed concave and kidney bean-shaped nuclei characteristic of the myelocyte/metamyelocyte stages (Fig. 2, C and D). The large fraction of CD15\(^{++}\)/CD11b\(^{+}\) cells after day 12 is consistent with the presence of a large fraction of band stage cells on day 14 (Fig. 2E) and of segmented stage cells by day 17 (Fig. 2, F–H). For comparison, the PB CD15\(^{+}\) cells were at least 99% positive for CD11b and CD66b, with >95% segmented stage cells (data not shown).

The percentage of phagocytic cells increased together with that of CD15\(^{++}\)/CD11b\(^{+}\) cells (Fig. 1A). This is consistent with the finding that granulocytes isolated from bone marrow exhibit high levels of phagocytic activity beginning at the metamyelocyte stage (17). However, phagocytic cells com-

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**Fig. 1. Phenotypic characteristics and functional activity of ex vivo-expanded (EXE) granulocytes.**

A: percentage of cells expressing the surface markers CD15 (circle), CD66b (diamond), and high levels of CD15 together with CD11b (CD15\(^{++}\)/CD11b\(^{+}\)) (triangle) \((n = 13)\) and percentage of cells capable of phagocytosing FITC-labeled opsonized E. coli (square) \((n = 6)\), all assessed by flow cytometry. B: percentage of cells expressing CD34 (circle), low levels of CD15 but no CD11b (CD15\(^{++}\)/CD11b\(^{-}\)) (light grey triangle), high levels of CD15 but no CD11b (CD15\(^{++}\)/CD11b\(^{-}\)) (grey triangle), and high levels of CD15 together with CD11b (CD15\(^{+}\)/CD11b\(^{+}\)) (black triangle). C: representative flow cytometry plots of day 9 EXE granulocytes in the absence or presence of DCF, fMLP, and/or opsonized zymosan (ZAS). D: respiratory burst activity of cells stimulated with fMLP (black diamond) or opsonized zymosan (black square) alone \((n = 4)\) or following preincubation with the PKC inhibitor GF109203X HCl [fMLP (open diamond); opsonized zymosan (open square) \((n = 1)\)]. E: respiratory burst activity of cells stimulated with phorbol 12-myristate 13-acetate (PMA) (black triangle) alone \((n = 5)\) or following preincubation with the PKC inhibitor GF109203X HCl (open triangle) \((n = 2)\). In A, D, and E, the error bars represent the standard error (SE) of the mean.
prised only ~25% of the CD15+/CD11b+ population in our cultures. The phagocytic cell content did not increase above ~20% after day 14, even though the segmented stage cell content increased from 10 to 50% between days 14 and 19 (data not shown). In contrast, 91% (±6) (n = 3) of PB granulocytes were phagocytic.

Respiratory burst capacity was evaluated using three agonists, fMLP, zymosan, and PMA, that stimulate the respiratory burst via different signaling pathways. Incubation of EXE granulocytes with DCF-DA in the absence of agonists resulted in the production of DCF+ cells (Fig. 1C). About two-thirds of unstimulated cells were DCF+ on day 7, and this increased to 75% on day 9. By day 11, ~90% of unstimulated cells were DCF+. This is consistent with other reports of substantial unstimulated respiratory burst activity in EXE granulocytes (24, 40).

Stimulation with fMLP, zymosan, and PMA produced very different responses. fMLP stimulation did not increase the fraction of DCF+ cells and barely increased their mean fluorescence intensity (MFI) (Fig. 1, C and D). In contrast, stimulation with opsonized zymosan increased both the fraction of DCF+ cells and their MFI (Fig. 1C and data not shown). This resulted in a three- to fourfold greater DCF MFI in stimulated cells after day 14 (Fig. 1D). PMA stimulated even greater respiratory burst activity, with a maximum of ~14-fold higher DCF MFI in stimulated cells on days 19–21 (Fig. 1E). The temporal profile for PMA-stimulated respiratory burst activity is similar to that reported for cord blood (CB) CD34+ cell cultures (25). The trend observed for respiratory burst activity stimulation with the different agonists (PMA > zymosan > fMLP) is consistent with previous reports for PB and EXE granulocytes (13, 22, 34, 42).

The respiratory burst activity (relative to unstimulated controls) for normal PB granulocytes was similar to that for mature EXE granulocytes (days 17–24) for each of the agonists (Table 1). The values obtained for PB granulocytes were much lower than those typically reported for H2O2-based flow cytometry assays (13, 34, 42). The relatively low values obtained for PB granulocytes were likely due to partial activation of the cells during the 20-min sample transit between blood collection and assay performance. This is consistent with very high ratios for unstimulated control vs. isotype control (Table 1). Thus the ratio of the agonist-stimulated response to the isotype control is a more appropriate measure of the inherent activation potential of the cells. Using this ratio for comparison, we find that PB granulocytes exhibited a 3-fold

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**Fig. 2.** Morphological changes of EXE granulocytes. Representative Wright-Giemsa-stained images of cells at days 5 and 7 with oval nuclei characteristic of cells in the myeloblast/promyelocyte stages (A and B), days 9 and 11 showing concave and kidney bean-shaped nuclei characteristic of the myelocyte/metamyelocyte stages (C and D), day 14 showing band nuclei characteristic of the band stage (E), and days 17–21 showing segmented nuclei characteristic of the most mature, or segmented, stage of granulocyte maturation (F, G, and H). Scale bar in each image represents 13 μm.
greater response to fMLP and a 3.5-fold greater response to zymosan than did EXE granulocytes (Table 1). The similar activation potential values for PMA stimulation may be due to the nonphysiological nature of this agonist. We conclude that, although EXE granulocytes exhibited similar surface marker expression and morphology as PB granulocytes, they exhibited lower levels of phagocytosis and respiratory burst activity.

**Transcriptional analysis design provides direct comparison of temporal EXE granulocyte gene expression with quiescent PB granulocytes.** We employed two complementary analyses. 1) The gene expression level at each time point beginning on day 0 for EXE granulocytes was compared against the average gene expression level in EXE cells from days 1–4. This comparison identifies genes that are differentially expressed in cells containing a large fraction of committed granulocytes compared with the populations of days 1–4, which also express genes of other hematopoietic and tissue lineages (data not shown). 2) The average gene expression level of EXE granulocytes from days 7–21 in culture was compared against that of CD34+ cells from day 0. This comparison identifies genes that are differentially expressed in committed granulocytes compared with the CD34+ cells used to initiate the cultures. For both comparisons, the gene expression level of PB granulocytes was also compared against the average gene expression level of the respective reference cells. This allows for direct comparison between gene expression profiles in EXE vs. PB granulocytes. We note that partial activation of EXE granulocytes during culture would be reflected in their gene expression profile. In contrast, the gene expression pattern of PB granulocytes should reflect their full activation potential, because RNA levels would not be expected to change very much during cell transportation and processing by AllCells (<2 h at ambient temperature). Of the ~18,000 genes probed by the arrays, 9,142 genes were identified as being differentially expressed using ANOVA analysis of all time points from day 1 to day 21 (complete list in Supplemental Table S1). The biological replicate cultures showed highly reproducible gene expression patterns (Supplemental Fig. S1). Thus, for simplicity and ease of presentation, we averaged the gene expression data across cultures for presentation in the figures. QRT-PCR was used to validate the microarray results of three differentially expressed genes (Fig. 3B). As we previously reported (15, 16), data from the Agilent microarrays strongly correlated with the QRT-PCR results, although QRT-PCR data generally show larger fold changes than microarray analysis.

**Expression of phagocytosis-associated receptors increases with G maturation and is similar in mature EXE and PB granulocytes.** The receptors and signaling pathways that mediate phagocytosis have mostly been studied using monocytic cells and cell lines. To facilitate the analysis of genes whose expression has not previously been identified in granulocytes, we used Gene Ontology and two recent reviews (36, 55) to generate a list of phagocytosis-associated genes expressed in diverse phagocytic cells (Supplemental Table S2). The temporal transcriptional program of these genes, which have not previously been evaluated as a system, was examined in an effort to better understand the development of phagocytic capability with G maturation and explain the lower level of phagocytosis in EXE granulocytes.

PB granulocytes exhibited high expression levels for most of the phagocytosis-related receptors, including the Fcγ receptors CD64 (FCGR1A), FCGR2A, FCGR2C, and FCGR3A and the complement receptors CR1, CR3 (CD11b/CD18), and CR4 (CD11c/CD18) (Fig. 3A). The exceptions include CD36, FCER1A, CALR, CD91, and ITGAV, all of which have been primarily associated with phagocytosis in other cell types (11, 14, 20, 41, 56). Receptor expression patterns in EXE granulocytes at the later time points were very similar to (and in some cases higher than) those in PB granulocytes. Most of the receptors were upregulated only in cells cultured for >7 days, which is consistent with acquisition of functional capacity by more mature cells. Later expression of CR1 compared with CD64 and CD18 is consistent with protein expression of the Fcγ receptor and CR3 beginning at the promyelocyte stage in vivo, while that of CR1 is observed only at the band and segmented stages (17). The broader perspective and finer resolution of our temporal analysis also revealed new information on phagocytic receptor gene expression with G maturation. For example, lower downregulation of FCER1A and CD36, which are normally expressed by related basophils, mast cells (FCER1A) and macrophages (CD36) (14, 32, 35), suggests some lineage plasticity early in G differentiation. Decreasing expression of CALR during the maturation of EXE granulocytes is consistent with its role as a molecular chaperone during myeloperoxidase synthesis (39), which occurs at intermediate stages of G maturation (8), and with its low expression levels in PB granulocytes (Fig. 3A). The similar patterns in mature EXE and PB granulocytes suggest that altered receptor expression is not responsible for less extensive phagocytosis by EXE granulocytes.

**Lower expression of Lyn, Cdc42, pleckstrin, and PKCβ correlates with lower levels of phagocytosis in EXE granulocytes.** Genes for phagocytosis-associated downstream signaling and activation proteins (identified as discussed above, using Gene Ontology and Refs. 36, 55; Supplemental Table S2) that were differentially expressed either temporally (days 1–24; P < 0.05) in EXE granulocytes or between EXE (days 21) and PB granulocytes (2-fold) are shown in Fig. 4A. There was a general increase with G maturation of gene expression for proteins responsible for downstream events in Fcγ receptor-mediated phagocytosis (Fig. 4B). Expression levels in mature EXE and PB granulocytes were similar for most of these genes, including high-level expression of the Src family kinase HCK and FGR and several subunits (ARPC2 and

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**Table 1. Respiratory burst activity for EXE and PB granulocytes stimulated by different agonists**

<table>
<thead>
<tr>
<th>Agonist Stimulated/Control</th>
<th>EXE Granulocytes (days 17–24)</th>
<th>Normal PB Granulocytes</th>
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<tbody>
<tr>
<td>fMLP/unstimulated</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>fMLP/isotype</td>
<td>145 ± 44</td>
<td>456 ± 113</td>
</tr>
<tr>
<td>Zymosan/unstimulated</td>
<td>3.5 ± 0.3</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Zymosan/isotype</td>
<td>346 ± 89</td>
<td>1,200 ± 244</td>
</tr>
<tr>
<td>PMA/unstimulated</td>
<td>11.4 ± 2.9</td>
<td>8.4 ± 4.6</td>
</tr>
<tr>
<td>PMA/isotype</td>
<td>4,070 ± 760</td>
<td>5,940 ± 2,500</td>
</tr>
</tbody>
</table>

Agonist-stimulated responses are normalized against those of unstimulated controls or isotype controls, as indicated: n = 3 for peripheral blood (PB) granulocytes; for ex vivo-expanded (EXE) granulocytes, n = 4 (3 donors) for N-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP) and zymosan, and n = 5 for phorbol 12-myristate 13-acetate (PMA).
Fig. 3. Transcriptional analysis of phagocytic receptors and microarray data validation. A: expression profiles of genes encoding phagocytic receptors (additional details in Supplemental Table S2) in EXE and peripheral blood (PB) granulocytes. The first block shows expression ratios in EXE granulocytes (G) at days 5, 7, 9, 11, 14, 17, and 21 with respect to the average gene expression in EXE granulocytes from days 1–4. The second block shows the expression ratio in PB granulocytes with respect to the average gene expression in EXE granulocytes from days 1–4. The third block shows the average expression ratio in EXE granulocytes from days 7–21 with respect to the day 0 CD34+/H11001 cells used to initiate the cultures. The fourth block shows the expression ratio in PB granulocytes with respect to day 0 CD34+/H11001 cells. Genes were hierarchically clustered using data in blocks 1 and 2. The color denotes the degree of differential expression: saturated red, 4-fold upregulation; saturated green, 4-fold downregulation. For EXE granulocytes, n = 3 for days 1, 2, 4, and 21; n = 3 for day 0; n = 4 for day 17; and n = 6 for days 3, 5, 7, 9, 11, and 14. For PB granulocytes, n = 3. At right, the median expression ratio of EXE granulocytes on days 5, 7, 9, 11, 14, 17, and 21 with respect to the average expression from days 1–4, averaged among all experiments, is provided in “Median” (a negative value represents downregulation), and the maximum (for upregulated genes) or minimum (for downregulated genes) ratio averaged among all experiments is provided in “Max.” The superscripts following each gene name indicate the type of cell the receptor is involved in phagocytosing: A, apoptotic cells; GN, gram-negative bacteria; GP, gram-positive bacteria; Y, yeast. Genes highlighted in blue are differentially expressed, as identified by ANOVA analysis of time points from day 5 to day 21 (P < 0.05). B: quantitative RT-PCR (QRT-PCR) validation of microarray results across multiple granulocyte culture samples. Microarray log expression ratios for each sample are compared with values obtained using QRT-PCR for each of 3 genes: ENC1 (square), RAB20 (triangle), and STAT3 (circle). Solid gray line represents the x = y curve.
Fig. 4. Expression analysis of genes involved in phagocytic signaling. A: expression profiles of genes encoding members of the Fc-, complement-, and other receptor-mediated phagocytic signaling pathways (additional details in Supplemental Table S2) that are differentially expressed as identified by either ANOVA analysis of all time points from day 1 to day 21 (P < 0.05) or at least a 2-fold difference in expression between the values in block 2 and the day 21 column of block 1. Expression ratios, samples, and color-coded text are represented as described in the legend to Fig. 3A. B: schematic of key genes involved in the FcγR-mediated phagocytosis signaling pathway (adapted from Ref. 38). Full names and gene expression of FcγR, Lyn, Hck, Fgr, Syk, Cdc42, and Rac are shown in Figs. 3A (FcγR) and 4A. The transcriptional profiles of Wiskott-Aldrich syndrome (Was); WAS protein family, member 1 (Wasf1); and ADP-ribosylation factor-6 (Arf6) were not identified as being differentially expressed, using the criterion described in the legend to Fig. 4A.
Expression of NADPH oxidase complex genes responsible for respiratory burst activity is similar in mature EXE and PB granulocytes. Transcriptional analysis revealed similar or greater expression of all NADPH oxidase complex components (2, 29) in mature EXE vs. PB granulocytes (Fig. 5A). Genes encoding p91phox, p47phox, and p67phox were highly upregulated with G maturation. Genes encoding p22phox and p40phox were only slightly upregulated but were already expressed at high levels in CD34+ cells (data not shown). These patterns are consistent with the finding that p22phox and p40phox are expressed in both immature and mature cells, whereas the other subunits are expressed only in mature granulocytes (47). Of the two RAC GTPases implicated in the NADPH oxidase complex, RAC2 was upregulated by twofold compared with cells at days 1–4, whereas RAC1 was downregulated by twofold. Furthermore, RAC2 expression was among the highest of all genes expressed in CD34+ cells (data not shown). This is consistent with an important role for RAC2 in respiratory burst activity (7, 19). Since lower respiratory burst activity in EXE vs. PB granulocytes could not be explained by lower expression of genes associated with the NADPH oxidase complex, we examined the signaling pathways associated with the respective agonists.

Low expression of downstream signaling components is consistent with low fMLP-mediated respiratory burst activity. fMLP is a formyl peptide derived from E. coli that signals through both high-affinity (FPR1) and low-affinity (FPRL1) fMLP receptors (51). Preincubation with proinflammatory cytokines such as TNF-α upregulates fMLP receptor expression and increases fMLP-mediated neutrophil respiratory burst activity (61). In neutrophils, fMLP activates the phosphatidylinositol 3-kinase-γ/Ras/Raf/MEK/ERK cascade and also directly activates PKCζ (5, 12, 60). However, the transcriptional programs of the receptors and the signaling cascade have not been examined systematically or holistically. Here, our transcriptional analysis revealed increasing fMLP and TNF-α receptor expression with G maturation and generally similar patterns of gene expression for mature EXE vs. PB granulocytes (Fig. 5B). The receptors were upregulated by at least threefold compared with EXE days 1–4. However, most of the downstream components, and especially RAS, which was already expressed at low levels in CD34+ cells (data not shown), were expressed at lower levels. This may explain low activation by fMLP in both EXE and PB granulocytes. One exception to this pattern is that RAF1 expression was 2.5-fold higher in PB granulocytes, and this may contribute to greater fMLP-mediated respiratory burst in PB vs. EXE granulocytes. The gene expression data suggest that MEK1 is more important than MEK2 for fMLP-mediated signaling. Greater fMLP-mediated respiratory burst in PB granulocytes may also be due to greater expression of PRKCB1 (Fig. 5D, bottom). The broad PKC inhibitor GF109203X HCl (GFX) had no effect on the already low fMLP-mediated respiratory burst activity of EXE or PB granulocytes (Fig. 1D and data not shown). Others have reported that GFX substantially inhibits fMLP-mediated rat PB neutrophil respiratory burst activity (60), so the lack of inhibition that we observed for PB granulocytes may be due to their partial activation during processing. Preincubation with TNF-α did not affect the fMLP-stimulated respiratory burst of EXE or PB granulocytes (data not shown). Although PB granulocytes displayed greater expression of TNFRSF1A, the receptor primarily responsible for mediating inflammatory effects in neutrophils (61), their partial activation may have obscured any priming by TNF-α.

Zymosan-stimulated respiratory burst activity in granulocytes is likely mediated in part by toll-like receptors and PKCζ. Zymosan is a carbohydrate from yeast cell wall that activates the alternative complement pathway via binding of the Fcγ receptor (CD64) and CR3 (31). As discussed above, CD64 and CR3 (CD11b/CD18) were upregulated to a similar extent in EXE and PB granulocytes (Fig. 3A). Lower expression of LYN, CDC42, PRKCB1, and PLEK (Fig. 4), discussed above in association with the lower phagocytic activity of EXE granulocytes, may also explain lower levels of zymosan-stimulated respiratory burst activity. However, since the fraction of EXE granulocytes exhibiting zymosan-stimulated respiratory burst activity was much greater than the fraction of phagocytic cells, we also examined other zymosan-mediated signaling pathways.

Toll-like receptor (TLR) agonists are known to activate neutrophil respiratory burst activity (21, 28, 44). In particular, GM-CSF priming of zymosan-induced respiratory burst activity has been associated with a transient increase in cell surface expression of TLR2 and CD14 (28). Transcriptional analysis showed that expression of TLR2, the TLR2 partner TLR1, and CD14 increased with G maturation, and that all three genes were expressed at similar high levels in mature EXE and PB granulocytes (Fig. 5C, top). TLR4 expression was also very high but increased later than that for TLR1 and TLR2. The TLR2 partner TLR6 was expressed at lower, but similar, levels in EXE and PB granulocytes. This expression pattern supports a role for the TLR2 and CD14 pathway in zymosan-mediated respiratory burst activity in granulocytes. Expression of the downstream signal transduction genes (Fig. 5C, bottom; from Ref. 4) was much lower than that for TLR2, TLR1, and CD14 but was generally similar in PB and EXE granulocytes. One exception is IRAK3, which negatively regulates the pathway (26). Greater expression of IRAK3 in EXE granulocytes may
Fig. 5. Transcriptional analysis of NADPH oxidase complex components and proteins involved in the fMLP-, zymosan- and PMA-stimulated respiratory burst pathways. A: expression profiles of genes encoding components of the NADPH oxidase complex. B: expression profiles of genes encoding receptors and downstream components of the fMLP signaling pathway. C: expression profiles of genes encoding receptors (top) and downstream components (bottom) of the toll-like receptor (TLR) signaling pathway activated by opsonized zymosan. D: expression profiles of genes encoding all PKC isoforms present on the Agilent Human 1A(v2) microarray. PMA activates both conventional (top) and novel (middle) PKC isoforms but not atypical (bottom) isoforms. Expression ratios, samples, and color-coded text are represented as described in the legend to Fig. 3A. Additional information on all of the genes is included in Supplemental Table S2.
explain their lower level of zymosan-mediated respiratory burst activity.

Recently, opsonized zymosan stimulation in monocytes was also shown to induce PKCδ (PRKCD) phosphorylation of p67phox (64). PRKCD expression was upregulated with G maturation and was similar in EXE and PB granulocytes (Fig. 5D, middle). Previous reports suggest that PKC inhibitors decrease zymosan-mediated respiratory burst activity, and that larger doses are needed than for the fMLP-mediated response (34, 60). The PKC inhibitor GFX decreased zymosan-mediated respiratory burst activity of EXE granulocytes by 50% (Fig. 1D), which suggests that PKCδ contributes to zymosan-mediated respiratory burst activity in granulocytes. GFX did not inhibit zymosan-mediated respiratory burst activity in PB granulocytes (data not shown). As discussed above for fMLP, this may be due to partial activation of PB granulocytes before the assay.

**DISCUSSION**

This is the first study to relate temporal changes in functional activity during ex vivo G maturation with changes in the expression of genes associated with functional activity and to compare these patterns with the functional activity and gene expression profile of PB granulocytes. DNA microarray-based transcriptional analysis was carried out without G enrichment, because the cultures contained >50% CD15 + cells on day 7 and ~90% CD15 + cells beginning on day 11, when substantial levels of functional activity were first detected (Fig. 1). In their gene expression analysis of ex vivo hematopoietic cell maturation, Komor et al. (27) used a similar approach, directly extracting RNA from cells removed from cultures containing 48–75% G cells. Their study identified only three functionally related genes, fMLP receptor FPRL1 and phagocytosis receptors CR1 and FCGR2B, as being differentially expressed in granulocytes, either temporally or with respect to erythrocytes or megakaryocytes. We also identified differential expression of FPRL1 (Fig. 5B) and CR1 (Fig. 3A). FCGR2B is not present on the Agilent 1At(v2) microarrays, but we identified differential expression of Fcy receptors CD64, FCGR2A, FCGR2C, and FCGR3A (Fig. 3A).

There were many more differentially expressed genes in common with the analysis of in vivo G maturation by Theilgaard-Mönch et al. (58). For example, 35 of the phagocytosis-associated genes shown in Figs. 3 and 4 were identified as differentially expressed by Theilgaard-Mönch et al.; 32 of these showed similar expression profiles for in vivo vs. ex vivo G maturation, including high-level expression of HCK. Twenty-five of the respiratory burst-associated genes shown in Fig. 5 were identified as differentially expressed by Theilgaard-Mönch et al., and most of these showed similar in vivo vs. ex vivo expression patterns. For the five genes with different in vivo vs. ex vivo expression profiles, we saw a similar trend between EXE vs. PB granulocytes, including much lower expression of PRKCB1 in EXE granulocytes.

While there were many differentially expressed genes in common between our study and that of Theilgaard-Mönch et al. (58), there were also important differences. Their study did not include genes for a number of important receptors (CD64, FCGR3A, TLR4) and downstream signaling molecules (RAC1 and -2; ARPC5, -2, and -1B; PRKCE and -Z). For example, only MYD88 was included from the downstream components of the TLR pathway shown at bottom in Fig. 5C. Of greater importance, this is the first time that the temporal expression profile of genes related to granulocyte functional activity has been examined holistically. Also, the many samples taken during ex vivo culture allow for a more sensitive analysis of temporal changes in gene expression with G maturation. For example, we were able to determine that p67phox, p47phox, and gp91phox expression is upregulated in that order (Fig. 5A).

The trend for phagocytosis by EXE granulocytes is consistent with results for bone marrow granulocytes (17), in that phagocytic activity was only observed for CD15 +/CD11b + cells. However, only 25% of the CD15 +/CD11b + cells were phagocytic (Fig. 1A). Other investigators have reported phagocytosis by ~40% (25) and 95% (24) of EXE granulocytes. It should be noted that these higher values were obtained via uptake of fluorescent latex beads rather than FITC-labeled opsonized E. coli.

Previous reports indicate that EXE granulocyte respiratory burst activity may be much less than or nearly equal to that for PB granulocytes. Using a cytokine cocktail (IL-1, IL-3, IL-6, SCF, and G-CSF) similar to the one used in the present study, Neildez-Nguyen et al. (40) found that PMA-stimulated O2 production by day 14 bone marrow CD34 + cells derived from PB granulocytes were both ~25% of the value exhibited by PB granulocytes. Similarly, Hino et al. (24) found that PMA- and fMLP-stimulated O2 production by day 14 EXE cells (derived from PB CD34 + cells cultured with IL-3, SCF, G-CSF, and GM-CSF) was ~50% of that for PB granulocytes. These results are consistent with our findings (Table 1) of a three- to fourfold greater activity by PB granulocytes. In contrast, Haylock et al. (22) observed that fMLP-stimulated O2 production by day 14 EXE cells (derived from PB CD34 + cells cultured with IL-1, IL-3, IL-6, SCF, G-CSF, and GM-CSF) was similar to that for PB granulocytes. In the studies by Neildez-Nguyen et al. and Hino et al., EXE cells showed higher levels of unstimulated activity than PB granulocytes. This suggests that EXE granulocytes were partially activated in culture and is consistent with our results. By culturing the cells for 7 days with G-CSF alone, after 7 days in the cytokine cocktail, Hino et al. obtained lower unstimulated activity for EXE granulocytes, with similar PMA- and fMLP-stimulated O2 production as for PB granulocytes. The impact of different cytokines has also been demonstrated by Jung et al. (25), who found that peak (day 15) O2 production in cultures derived...
from CB CD34+ cells differed by as much as 20-fold, depending on the cytokine cocktail employed.

The combination of ex vivo G differentiation and gene expression microarray analysis provides a powerful tool for exploring the organization of genes responsible for G functional activity and their temporal orchestration. The ability to simultaneously examine the time-dependent expression of large numbers of related genes facilitates analysis of linkages between pathways. Our analysis is also useful for identifying the factors responsible for differences in the functional activity of different G cell populations. One important general conclusion from the present study is that, although receptor gene expression levels were similar between EXE and PB granulocytes, the expression level of key genes associated with downstream signaling events was generally lower in EXE granulocytes. This was true for phagocytosis, as well as fMLP- and zymosan-mediated respiratory burst activity, and is likely responsible for the lower functional capacity of EXE granulocytes.

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GRANTS

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DISCLOSURES

We declare no competing financial interests.

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