Induction of Genes Mediating Interferon-dependent Extracellular Trap Formation during Neutrophil Differentiation*

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Interferons (IFNs) are cytokines that possess potent anti-viral and immunoregulatory activities. In contrast, their potential role(s) in anti-bacterial defense and neutrophil activation mechanisms is less well explored. By comparing gene expression patterns between immature and mature human neutrophils, we obtained evidence that intracellular proteases and other anti-bacterial proteins are produced at earlier stages of maturation, whereas the genes for receptors and signaling molecules required for the release of these effector molecules are preferentially induced during terminal differentiation. For instance, mature neutrophils strongly expressed genes that increase their responses to type I and type II IFNs. Interestingly, granulocyte/macrophage colony-stimulating factor was identified as a repressor of IFN signaling components and consequently of IFN-responsive genes. Both IFN-α and IFN-γ induced strong tyrosine phosphorylation of STAT1 in mature but not in immature neutrophils. Functional in vitro studies suggested that IFNs act as priming factors on mature neutrophils, allowing the formation of extracellular traps upon subsequent stimulation with complement factor 5α (C5α). In contrast, both IFN-α and IFN-γ had only little capacity to prime immature cells in this system. Moreover, both IFNs did not have significant anti-proliferative effects on immature neutrophils. These data contribute to our understanding regarding changes of gene expression during neutrophil differentiation and IFN-mediated anti-bacterial defense mechanisms.

Neutrophils are a critical component of the innate immune system with several effector and immunoregulatory functions (1). They are generated in the bone marrow under the influence of cytokines, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF), from hematopoietic stem cells. Interestingly, G-CSF is not expressed in normal bone marrow cells under physiologic conditions (2), suggesting that it drives myeloid differentiation in a hormonal manner. Multiple cell types such as endothelial cells, epithelial cells, fibroblasts, and macrophages are able to produce G-CSF and GM-CSF (3, 4). All these cells make early contact with invading microorganisms and/or their products, resulting in increased cytokine production after infection. For instance, blood G-CSF levels have been described to rise from 25 to up to 10,000 pg/ml under pathologic conditions (5). Moreover, systemic injection of G-CSF (6) or GM-CSF (7) results in a dramatic increase of neutrophil production. Taken together, G-CSF and GM-CSF have been demonstrated to be major regulators of neutrophil production. Under conditions of stress, such as infections, neutrophil numbers in blood can increase as a consequence of cytokine-forced neutrophil differentiation.

Although immature neutrophils can be classified by morphology as well as by the expression of more or less specific surface proteins (8), it is difficult to obtain pure cell populations characterized by a certain maturation stage. Therefore, most of the studies trying to understand neutrophil differentiation at the molecular level were performed by using cell lines derived from leukemias. Previously published work resulted in the identification of genes that may play critical roles in the differentiation of neutrophils (9). Moreover, a gene expression profile of neutrophils has been established (10). Despite these previous studies, the underlying molecular events of normal neutrophil differentiation are not well understood, and many of the genes that are expressed by mature neutrophils have not been related to function.

The objective of this study was to compare the transcriptional repertoire of immature and mature human neutrophils by using oligonucleotide microarrays. In addition we investigated whether certain differences in gene expression are reversible by in vitro re-exposure of mature neutrophils with GM-CSF. Although multiple genes were more expressed in mature compared with immature cells, it was interesting to see that mature neutrophils also demonstrated higher expression of genes, which transduce signals of type I and type II interferons. Consequently, several known IFN-responsive genes had elevated expression levels in mature compared with immature cells. The subsequently obtained functional data demonstrate the importance of increased IFN sensitivity of mature neutrophils for the formation of extracellular traps, which consist of antimicrobial agents able to kill bacteria (11).

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† The abbreviations used are: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; C5α, complement factor 5α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; Jak, Janus kinase; MPO, myeloperoxidase; PBMC, peripheral blood mononuclear cells; STAT, signal transducer and activator of transcription; Ab, antibody; mAb, monoclonal Ab.
**MATERIALS AND METHODS**

**Reagents**—Human IFN-α was purchased from PBL Biomedical Laboratories (Alexis Corp., Lausen, Switzerland), and human IFN-γ was from R&D Systems (Abingdon, United Kingdom). Human GM-CSF was from Novartis Pharma GmbH (Nurnberg, Germany), and human G-CSF was from Aventis Pharma AG (Zurich, Switzerland). Anti-phycocerythrin secondary Abs microbeads from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Phytohemagglutinin was from Roche Diagnostics. Propidium iodide and Sytox Orange were both obtained from Molecular Probes (distributed by Invitrogen). Unless stated otherwise, all other reagents were from Sigma.

**Cells**—Immature neutrophils were isolated from bone marrow aspirates with normal cellular morphology and distribution as previously described (12). Briefly, after centrifugation on a two-step discontinuous Percoll density gradient, cells were negatively isolated using anti-CD7 and anti-CD36 mAbs to eliminate contaminating lymphoid and erythroid precursors. The resulting cell population contained 97% cells of the neutrophil lineage as determined by MPO staining and analysis of lineage-associated surface proteins as well as with Diff-Quik (Medion GmbH, Duedingen, Switzerland) and light microscopy. Table I demonstrates the distribution of the different maturation stages within the immature neutrophil populations used in this study. On average we

### Table I

**Relative cellular distribution of purified bone marrow neutrophils**

| No. | CD15<sup>low</sup> CD16<sup>a</sup> CD11b<sup>a</sup> (myeloblasts<sup>a</sup>) | CD15<sup>+</sup> CD16<sup>-</sup> CD11b<sup>-</sup> (promyelocytes<sup>a</sup>) | CD15<sup>+</sup> CD16<sup>-</sup> CD11b<sup>-</sup> (early myelocytes<sup>a</sup>) | CD15<sup>+</sup> CD16<sup>-</sup> CD11b<sup>-</sup> (metamyelocytes<sup>a</sup>) | CD15<sup>+</sup> CD16<sup>-</sup> CD11b<sup>-</sup> (mature neutrophils<sup>a</sup>) |
|-----|-------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| %   | %                                               | %                               | %                               | %                               | %                               |
| 1   | 28                                              | 9                               | 20                              | 5                               | 35                              |
| 2   | 11                                              | 18                              | 32                              | 6                               | 30                              |
| 3   | 9                                               | 6                               | 45                              | 14                              | 20                              |
| 4   | 1                                               | 3                               | 30                              | 35                              | 25                              |
| 5   | 24                                              | 2                               | 21                              | 11                              | 39                              |
| 6   | 7                                               | 4                               | 23                              | 13                              | 47                              |
| 7   | 29                                              | 54                              | 4                               | 1                               | 10                              |
| 8   | 3                                               | 17                              | 7                               | 25                              | 43                              |
| 9   | 13                                              | 19                              | 10                              | 33                              | 22                              |
| 10  | 24                                              | 16                              | 19                              | 7                               | 28                              |
| 11  | 24                                              | 8                               | 8                               | 14                              | 34                              |
| 12  | 7                                               | 21                              | 19                              | 30                              | 22                              |
| Mean| 15.0                                            | 14.1                            | 20.1                            | 16.2                            | 29.6                            |
| S.E.| 2.9                                             | 4.1                             | 3.6                             | 3.4                             | 3.1                             |

<sup>a</sup> Note that the majority of the cells represent the indicated cell type. However, some overlap between different maturation stages can occur. For instance, some early myelocytes are also present within the subpopulation indicated as promyelocytes, and metamyelocytes are usually seen in the subpopulation defined as myelocytes.

### Table II

**Primers and PCR conditions**

| Gene/Primers | Sequence | Amplicon size | Annealing temperature |
|--------------|----------|---------------|-----------------------|
| STAT1 (forward) | 5'-TGG GCT GAT CTC CAA CGT CAG-3' | 113 | 55 |
| STAT1 (reverse) | 5'-CAC ATG GTG GAG TCA GGA AGA-3' | | |
| STAT2 (forward) | 5'-GAG GCC TCA ACT CAG ACC AG-3' | 200 | 56 |
| STAT2 (reverse) | 5'-GCC TTC ATT CCA GAG AT-3' | | |
| Jak1 (forward) | 5'-TGC TCC TGA GTG TGT TGA GG-3' | 192 | 60 |
| Jak1 (reverse) | 5'-AGG TCA GCC AGC TCC TTA CA-3' | | |
| Jak2 (forward) | 5'-TGG GCA GAA TTA GCA AAC CT-3' | 200 | 60 |
| Jak2 (reverse) | 5'-TGT GGT GTT CAA TCC TCT-3' | | |
| IFNABR-1 (forward) | 5'-TTG CTC TCC CGT TTG TCA TTT A-3' | 395 | 60 |
| IFNABR-1 (reverse) | 5'-GAC CTC AGG CTC CCA GTG TAA C-3' | | |
| IFNABR-2 (forward) | 5'-CCC TTA AAA TGC ACC CTC CT-3' | 200 | 60 |
| IFNABR-2 (reverse) | 5'-GCA TTC CAC CCG TTA GTG AG-3' | | |
| IFNGR-1 (forward) | 5'-TCC TCG ATT GTC TTC GTG ATG C-3' | 225 | 60 |
| IFNGR-1 (reverse) | 5'-GTT CTT AGC CGT GTC TAT CTG T-3' | | |
| IFNGR-2 (forward) | 5'-TGG GGT CAC CTC AAT GTT TTC T-3' | 104 | 60 |
| IFNGR-2 (reverse) | 5'-TTC AAT GTC ACT CTA CGC CTT CG-3' | | |
| GAPDH | GAPDH quantification kit (Search LC, GmbH, Dossenheim, Germany) | | |
counted 15% myeloblasts, 14% promyelocytes, 36% myelocytes, 30% metamyelocytes and band cells, and 5% mature bone marrow neutrophils.

Mature peripheral blood neutrophils and peripheral blood mononuclear cells (PBMC) were purified from healthy normal individuals by Ficoll-Hypaque centrifugation (4, 13). The resulting cell populations contained less than 5% contaminating cells. Eosinophil contamination was always less than 2%. Written informed consent was obtained from all participants and control individuals who donated bone marrow aspirates and blood, respectively. The study was approved by the ethics committee of the Canton Bern.

Cell Cultures—Human immature and mature neutrophils were cultured at 1 x 10⁶/ml in complete culture medium (RPMI 1640 containing 10% fetal calf serum) in the presence and absence of GM-CSF (50 ng/ml) or IFN-γ (500 units/ml), IFN-α (500 units/ml), and IFN-γ (250 units/ml) for the indicated time periods.

Gene Expression Profiling—Total RNA was isolated using the TRIzol reagent (Invitrogen). Double-stranded cDNA was generated using a Superscript cDNA synthesis kit (Invitrogen) using an oligo(dT)₄₅ primer containing a T7 RNA polymerase promoter at the 3′ end (Megascript T7 kit, Ambion (Europe) Ltd., Huntingdon, UK) in the presence of biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY) and purified using RNeasy columns (Qiagen AG, Basle, Switzerland). Labeled cRNA was prepared from each gene measured. The overall fluorescence intensity was scaled to a mean of 0 and a cut-off of 4 (2 for GM-CSF treatment) in both directions; 3) the P calls had to coincide with the regulation in question, 4) the P calls in mature (GM-CSF treated cells), triplicate samples in the immature (untreated group) had to have a P call, and 5) the P calls in mature neutrophils or in GM-CSF-treated versus not treated neutrophils: 1) Student’s t test with a multiple testing correction (Benjamini-Hochberg) significance level p < 0.05; 2) fold-change cut-off of 4 (2 for GM-CSF treatment) in both directions; 3) the P calls had to coincide with the regulation in question, e.g., if a candidate gene were to be called “up-regulated” in mature (GM-CSF treated cells), triplicate samples in the “treated” group had to be called P, and vice versa, if a gene were to be called “down-regulated,” triplicate samples in the immature (untreated) group had to have a P call. The selected differentially expressed genes were normalized by the absolute GAPDH mRNA copy number/l. To compensate the variance in quantity and quality of starting mRNA, the absolute gene copy number/l was normalized by the absolute GAPDH mRNA copy number/l.

Functional category

| Functional category | Numbers of up-regulated genes |
|---------------------|-------------------------------|
| Immature neutrophils |                               |
| Protein biosynthesis | 58                            |
| Metabolism           | 44                            |
| Cell cycle           | 39                            |
| Control of transcription | 29                   |
| Immune/inflammatory response | 23                    |
| Mitosis              | 21                            |
| Signal transduction  | 13                            |
| Proliferation        | 13                            |
| DNA repair           | 11                            |
| Cell growth          | 9                             |
| Organ development    | 8                             |
| Cell adhesion        | 8                             |
| Apoptosis/cell death | 7                             |
| Anti-proliferative    | 7                             |
| Cell-cycle signaling | 5                             |
| Anti-apoptosis        | 2                             |
| Chemotaxis           | 1                             |

Mature neutrophils

| Functional category | Numbers of up-regulated genes |
|---------------------|-------------------------------|
| Signal transduction | 97                            |
| Immune/inflammatory response | 72                   |
| Control of transcription | 60                   |
| Apoptosis/cell death | 37                            |
| Cell growth          | 30                            |
| Organ development    | 27                            |
| Metabolism           | 26                            |
| Cell cycle           | 24                            |
| Cell adhesion        | 24                            |
| Proliferation        | 23                            |
| Cell-cycle signaling | 17                            |
| Chemotaxis           | 9                             |
| Anti-proliferative    | 7                             |
| Anti-apoptosis        | 7                             |
| Mitosis              | 7                             |
| Protein biosynthesis | 2                             |

Gene Array Data Analysis

The expression data for all genes were exported to GeneSpring 5.0 (Silicon Genetics, Redwood City, CA). The overall fluorescence intensity was scaled to a mean of 0 and a cut-off of 4 (2 for GM-CSF treatment) in both directions; 3) the P calls had to coincide with the regulation in question, 4) the P calls in mature (GM-CSF treated cells), triplicate samples in the immature (untreated group) had to have a P call, and 5) the P calls in mature neutrophils or in GM-CSF-treated versus not treated neutrophils: 1) Student’s t test with a multiple testing correction (Benjamini-Hochberg) significance level p < 0.05; 2) fold-change cut-off of 4 (2 for GM-CSF treatment) in both directions; 3) the P calls had to coincide with the regulation in question, e.g., if a candidate gene were to be called “up-regulated” in mature (GM-CSF treated cells), triplicate samples in the “treated” group had to be called P, and vice versa, if a gene were to be called “down-regulated,” triplicate samples in the immature (untreated) group had to have a P call. The selected differentially expressed genes were normalized by the absolute GAPDH mRNA copy number/l. To compensate the variance in quantity and quality of starting mRNA, the absolute gene copy number/l was normalized by the absolute GAPDH mRNA copy number/l.

Functional groupings of genes differentially regulated in immature versus mature neutrophils obtained by screening NetAffx™ annotation database (www.affymetrix.com/analysis/index.affx)

| Functional category | Numbers of up-regulated genes |
|---------------------|-------------------------------|
| Immature neutrophils |                               |
| Protein biosynthesis | 58                            |
| Metabolism           | 44                            |
| Cell cycle           | 39                            |
| Control of transcription | 29                   |
| Immune/inflammatory response | 23                    |
| Mitosis              | 21                            |
| Signal transduction  | 13                            |
| Proliferation        | 13                            |
| DNA repair           | 11                            |
| Cell growth          | 9                             |
| Organ development    | 8                             |
| Cell adhesion        | 8                             |
| Apoptosis/cell death | 7                             |
| Anti-proliferative    | 5                             |
| Cell-cycle signaling | 2                             |
| Anti-apoptosis        | 2                             |
| Chemotaxis           | 1                             |

Mature neutrophils

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| Cell cycle           | 24                            |
| Cell adhesion        | 24                            |
| Proliferation        | 23                            |
| Cell-cycle signaling | 17                            |
| Chemotaxis           | 9                             |
| Anti-proliferative    | 7                             |
| Anti-apoptosis        | 7                             |
| Mitosis              | 7                             |
| Protein biosynthesis | 2                             |

Noniodit P-40, 150 mM sodium chloride, 50 mM Tris, pH 7.4) supplemented with a protease inhibitor mixture (Sigma) and 10 μM phenylmethylsulfonyl fluoride (Roche Applied Science) on ice for 30 min (17, 18). For phospho-STAT1 immunoblots, radioimmune precipitation assay buffer was used, which contained 1% Triton X-100. After a 10-min centrifugation to remove insoluble particles, loading buffer was added, and equal amounts of the cell lysates were loaded on NuPage gels (Invitrogen) and separated by electrophoresis on polyvinylidene difluoride membranes (Immobilon-P, Millipore, Volketswil, Switzerland) except for phospho-STAT1 immunoblots where we used Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The filters were incubated overnight with anti-IFNABR-1 (1/5000), anti-IFNABR-2 (1/2000), anti-IFNAR-1 (1/3000), anti-IFNAR-2 (1/1000), anti-Jak1 (1/500), anti-Jak2 (1/500), anti-STAT1 (1/1000), and anti-STAT2 (1/1000) Abs at 4 °C in Tris-buffered saline, 0.1% Tween 20, 3% nonfat dry milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat dry milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat dry milk. Anti-phospho-STAT1 Abs were incubated with the appropriate horseradish peroxidase-conjugated secondary Ab in Tris-buffered saline, 0.1% Tween 20, 3% nonfat milk saline (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat dry milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat milk. Anti-phospho-STAT1 Ab was diluted in 1% bovine serum albumin (essentially globulin-free, Sigma-A-7638), Tris-buffered saline, 0.1% Tween 20, 1% nonfat milk.
volumes of immunocomplexes from immature than from mature cells on NuPage-Gels. The gels were analyzed using the imaging system Typhoon 9200 (Amersham Biosciences).

**TABLE IV**

| Accession no. | Gene name | Gene symbol | -Fold increase |
|---------------|-----------|-------------|----------------|
| Immature neutrophils | | | |
| M96326 | Azurocidin 1 (cationic antimicrobial protein 37) | AZU1 | 292 |
| M34379 | Neutrophil elastase 2 | ELA2 | 291.4 |
| J04739 | Bactericidal/permeability-increasing protein | BPI | 243.4 |
| A1762213 | Lipocalin 2 | LCN2 | 241.1 |
| M19507 | Myeloperoxidase | MPO | 231.9 |
| M16117 | Cathepsin G | CTSG | 177.2 |
| J05556 | Matrix metalloproteinase 8 (neutrophil collagenase) | MMP8 | 163.3 |
| A1250799 | Defensin, α4 (corticostatin) | DFFA4 | 162.1 |
| J04990 | Cathepsin G | CTSG | 155.4 |
| AL036554 | Neutrophil-specific defensin, α3 | DFFA3 | 84.7 |
| Z35026 | Cathelicidin antimicrobial peptide | CAMP | 24.9 |
| X04011 | CYO-245, β-polypeptide | CYO-245 | 7.42 |

**Mature neutrophils**

| Accession no. | Gene name | Gene symbol | -Fold increase |
|---------------|-----------|-------------|----------------|
| ABO11406 | Alkaline phosphatase | ALPL | 172.7 |
| LI9598 | Interleukin-8 receptor β | IL8RB | 76.2 |
| X16868 | Receptor for Fc fragment of IgG, low affinity IIIb (CD16) | FCR3B | 65.4 |
| ABO00220 | Semaphorin 3C | SEMA3C | 62.9 |
| D10923 | Putative human chemokine receptor | HM74 | 42.6 |
| X07834 | Mitochondrial superoxide dismutase 2 | SOD2 | 42.3 |
| J01462 | Receptor for Fc fragment of IgG, low affinity IIIa (CD16) | FCR3A | 42.1 |
| M58130 | Interleukin-8 | IL8 | 42.1 |
| U14550 | Sialyltransferase | STHM | 40.6 |
| U11870 | Interleukin-8 receptor α | IL8RA | 36.2 |
| X80890 | Receptor for Fc fragment of IgG, low affinity Ia (CD32) | FCR2A | 31 |
| M59820 | G-CSF 3 receptor | CSF3R | 11.2 |
| M59818 | G-CSF 3 receptor | CSF3R | 10.7 |
| M02011 | Neutrophil cytosolic factor 2 | NCF2 | 5.67 |
| M62840 | Neutrophil acyloxyacyl hydrolase | AOAH | 5.03 |

**Fig. 1.** IFN-inducible gene expression signatures in mature and immature neutrophils determined by two-way hierarchical clustering analysis. Normalized intensities are presented for each gene as the S.D. of log intensity above the mean (red) and below the mean (green) across samples. Increasing distance from the mean is represented by increasing color intensity (see the bar on the bottom right). Genes are identified by their GenBank accession number and symbol. IFN-inducible genes are highlighted in red. They have clustered close together with multiple other genes with a similar expression pattern but different functions.
CSF for 30 min with the concentrations indicated in Cell cultures. Cells were subsequently stimulated with 10^7 M C5a for 10 min and fixed with 4% paraformaldehyde solution. RNAs were digested by addition of 1 mg/ml RNase in phosphate-buffered saline for 15 min at room temperature. For DNA detection slides were treated with 2.5 g/ml propidium iodide. Specimens were washed with phosphate-buffered saline and mounted in a drop of anti-bleaching mounting medium (5% 1,4-diazabicyclo-[2.2.2]octane, 11% glycerol, 22% polyvinyl alcohol, 56 mM Tris-HCl, pH 9.0) and analyzed with a confocal laser scanning microscope (LSM 510, Carl Zeiss).

To quantify DNA release, neutrophils were seeded into 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and stimulated under the same conditions as described above. Five μl Sytox Orange, a non cell-permeable DNA binding dye, was added (11). The plates were read using the imaging system Typhoon 9200 using a filter setting of 532 nm (excitation)/580 nm (emission) and TotalLab software (Amersham Biosciences). In addition, plates were analyzed by light microscopy and photographed.

For MPO release analysis the same protocol for cell activation was used. Released MPO was measured in the supernatants by spectroscopy (20). Briefly, 10 μl of supernatant and 10 μl of 20 mg/ml o-dianisidine were added to 155 μl of 100 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. The reaction was started by adding 25 μl of 2 mM hydrogen peroxide solution. Absorbance of 450 nm of visible light was measured at 37 °C for 20–30 min. MPO concentrations were calculated using an MPO standard.

**Proliferation Assay**—Proliferation of immature neutrophils was induced by GM-CSF or G-CSF in complete culture medium. As control PBMC were stimulated with 10 μg/ml phytohemagglutinin. These proliferation assays were carried out in the presence and absence of interferons (IFN-α and IFN-γ). Total culture times were 72 h (immature neutrophils) and 48 h (PBMC), respectively, and pulsing the cells with 1 μCi/ml [methyl-^3H]thymidine (Amersham Biosciences) was always performed for 16 h. [^3H]Thymidine incorporation was measured by using a liquid scintillation counter (Wallac ADL, Hünenberg, Switzerland (21)).

**Statistical Analysis**—Besides the gene array data, results are expressed either as single data (usually triplicate experiments) or as the means ± S.E. for the indicated number of independent experiments. Student’s t test was used to identify statistical significant differences. The calculated p values are indicated in the figures.

**RESULTS**

**Differentially Expressed Genes in Immature Versus Mature Human Neutrophils**—To gain an understanding of the molecular processes that occur during differentiation and infection in neutrophils, we screened 12,599 genes for changes in gene expression using the following neutrophil populations: 1) immature neutrophils isolated from bone marrow aspirates, 2) immature neutrophils isolated from peripheral blood, and 3) mature neutrophils stimulated in vitro with GM-CSF for 7 h. Based on our filtering criteria, we identified 1049 genes that were differentially transcribed in immature and mature neutrophils (459 genes up-regulated and 590 down-regulated in immature cells). To facilitate further analysis, each gene was
assigned to a defined functional category according to available annotations (NetAffx). As shown in Table III, immature neutrophils showed preferential expression of genes involved in protein biosynthesis, metabolism, and transcriptional control as well as in cell cycle, mitosis, and proliferation. In contrast, mature neutrophils appeared to up-regulate genes that regulate signal transduction, inflammatory responses, transcription, and apoptosis.

A detailed analysis of neutrophil lineage-associated genes suggested high mRNA expression of azurocidin 1, neutrophil elastase 2, bactericidal/permeability-increasing protein, lipocalin 2, MPO, cathepsin G, and neutrophil collagenase as well as the defensins α3 and α4 in immature neutrophils. In contrast, mature neutrophils appeared to up-regulate genes that regulate signal transduction, inflammatory responses, transcription, and apoptosis.

Increased Expression of IFN Signaling and IFN-regulated Genes in Mature Compared with Immature Human Neutrophils—Two-way unsupervised hierarchical clustering analysis, a mathematical approach that essentially organizes the data by grouping genes with similar expression pattern, yielded two major array clusters (i.e. immature and mature neutrophils) with this set of 1049 differentially expressed genes. In the analysis shown in Fig. 1 all the indicated genes were highly expressed in mature compared with immature neutrophils. Many of these genes are known to be regulated by IFN (22). Other genes with an apparently similar expression pattern encode proteins involved in multiple cellular functions, including adhesion, signal transduction, and apoptosis. Fig. 2A demonstrates a quantitative analysis of the induction of the IFN-regulated genes (up to 70-fold) in mature as compared with immature neutrophils.

Some of these genes are activated in response to type I IFNs (IFIT1, IFIT2, IFIT4, 2'5' OASL, Mxβ protein), whereas others are induced by type II IFNs (GBP2, IP-30) or both (IFITM1–3). Furthermore, we observed the up-regulation of genes involved in type I (IFNABR-2, Jak1, STAT1, p48) and type II (IFNFR-1, IFNFR-2, Jak1, STAT1) IFN signaling pathways.
Additional genes were higher expressed in mature compared with immature neutrophils that have also been shown to be induced by IFNs. For instance, we observed increases of caspase-1 (9.6-fold) and Pim-1 (5.5-fold) expressions as previously reported in IFN-α-stimulated PBMC (23). Similarly, we found increases in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression (17.3-fold) in blood neutrophils, confirming recently published work obtained in IFN-β-stimulated melanoma cells (24). Together, these data strongly suggest the activation of IFN-inducible genes during terminal neutrophil maturation.

Interestingly, when we stimulated mature neutrophils with GM-CSF, a known growth factor of immature neutrophils, we observed that the expression of many of the IFN-regulated genes declined (Fig. 2B). This suggests that the induction of these genes in neutrophils may occur as a consequence of decreased GM-CSF exposure during terminal differentiation and/or following the bone marrow exit into the circulation. Because proximal and more distal elements of the IFN-signaling pathways are among these genes, it is possible that the transcript changes of the IFN-responsive genes reflect different in vivo efficacies in IFN signaling in immature and mature neutrophils. Over all, the gene expression responses of mature neutrophils toward GM-CSF revealed that 234 genes were up-regulated, and 414 were down-regulated.

We used LightCycler™ real-time PCR to verify changes in expression detected by microarray analysis (Fig. 3). We selected eight genes known to be important for type I and type II IFN signal transduction. There was a good correlation between real-time PCR data and microarray gene expression profiles. In particular, there was not a single difference in gene expression observed by using microarray analysis that could not be confirmed by the PCR technique. Because of the higher sensitivity of the LightCycler™ PCR, additional gene expression differences were detected that have not been seen by microarray analysis. For instance, Jak2 and IFNABR-1 were not found to be repressed in immature cells compared with mature cells using the microarray analysis (Fig. 2A and 3). In addition, we found decreased expression of IFNGR-2 and IFNABR-2 in GM-CSF-stimulated mature neutrophils compared with unstimulated mature neutrophils (Fig. 2B and 3). Taken together, microarray gene expression analysis seems to be highly specific but less sensitive as compared with real-time PCR measurements.

To investigate whether the observed differences in mRNA expression also result in differences in protein expression, we performed immunoblot analysis. As shown in Fig. 4, the protein expression analysis largely confirmed the microarray and LightCycler™ gene expression data. Expression of Jak1, Jak2, and STAT1 was clearly increased in mature compared with immature neutrophils. Two IFN receptors (IFNABR-1 and IFNGR-1) were also more expressed in the mature cells. The differences in IFNABR-2 expression were less pronounced. The protein expression of IFNGR-2 did not appear to differ between mature and immature neutrophils. STAT2 expression in immature neutrophil populations varied between low expression and normal levels when compared with mature neutrophils. The differences in IFNABR-2 expression were less pronounced. The protein expression of IFNGR-2 did not appear to differ between mature and immature neutrophils. STAT2 expression in immature neutrophil populations varied between low expression and normal levels when compared with mature neutrophils.

**IFN Signaling Is Accelerated in Mature Compared with Immature Human Neutrophils**—The different expression of some

**Fig. 4.** Expression of different genes involved in IFN I/II-type signaling in immature and mature neutrophils as assessed by immunoblotting. Filters were also probed with anti-GAPDH mAb to ensure equal loading of the gels. For both neutrophil populations, results from four different donors are shown. The determined approximate molecular sizes of the molecules are indicated at the right.

**Fig. 5.** IFN-induced STAT1 phosphorylation in mature but not immature neutrophils. A, both IFN-α and IFN-γ induced tyrosine 501 phosphorylation of STAT1 in mature but not immature neutrophils. Cells were stimulated for the indicated time periods and then analyzed by immunoblotting. B, incorporation of total phosphate into STAT1 after stimulation with IFNs was observed in mature but not immature neutrophils. STAT1 immunoprecipitates were analyzed by gel electrophoresis and phosphorimaging (autoradiogram). The immunoprecipitation efficiency was controlled using anti-STAT1 Ab and immunoblotting. Note: we loaded three times larger volumes of STAT1 immunoprecipitates in immature than in mature neutrophils.
IFN receptors and the Jak kinases in immature and mature neutrophils implied that the two cell populations differentially transduce type I and type II IFN signals. To estimate the efficacy of IFN signaling, we measured the phosphorylation of Tyr-701 on STAT1 upon stimulation with type I and type II IFNs in both immature and mature neutrophils (25). As shown in Fig. 5A, both IFN-α and IFN-γ increased tyrosine phosphorylation of STAT1 within 15 min of stimulation in mature neutrophils. In contrast, both IFNs induced no or only very weak responses in immature cells, and clear increases in tyrosine phosphorylation of STAT1 were also not evident at later time points. To exclude the possibility that equal increases in phosphorylation of STAT1 occurred in both neutrophil populations but were not detectable in immature neutrophils due to very low STAT1 levels, we immunoprecipitated STAT1 after 32P-labeling of the cells. This allowed loading of larger concentrations of STAT1 on the gels, most likely resulting in a higher sensitivity for the detection of phosphorylated STAT1. However, also under these conditions and in contrast to mature neutrophils, we observed no significant increases in STAT1 phosphorylation in IFN-γ-stimulated immature neutrophils (Fig. 5B). In conclusion, STAT1 occurs at lower levels and cannot efficiently be tyrosine-phosphorylated upon IFN stimulation in immature compared with mature neutrophils.

**IFN Priming for Extracellular Trap Formation in Mature but Not Immature Human Neutrophils**—To understand the biological relevance of the observed differences between immature and mature neutrophils regarding the efficacy of IFN signal transduction, we established two functional in vitro assays. First, we investigated the effect of IFN-α and IFN-γ, respectively, on the formation of extracellular traps in both immature and mature neutrophils. Second, we analyzed IFN effects on growth factor-induced proliferative responses in immature neutrophils.

Neutrophil extracellular traps are structurally composed of granule and nuclear constituents that disarm and kill bacteria (11). Because DNA is a major structural component of neutrophil extracellular traps, we used a propidium iodide staining procedure to make the extracellular structures visible. Priming with IFN-α or IFN-γ and subsequent C5a stimulation resulted in the formation of extracellular fibers in mature but not immature neutrophil populations (Fig. 6). GM-CSF was also able to function as a priming factor in this system. In both neutrophil populations, stimulation with the cytokines or C5a alone was not followed by visible extracellular trap generation (data not shown).

Neutrophil extracellular trap formation was quantified by two ways. First, we measured the amount of released DNA using a DNA dye that is excluded from cells. Second, we analyzed the amount of released MPO, which is another using a DNA dye that is excluded from cells. Second, we analyzed the amount of released DNA and MPO (Fig. 7A) but not immature (B) neutrophil populations. GM-CSF was also able as a positive control. The figure demonstrates representative data from five independent experiments. **Bar, 10 μm.**

**DISCUSSION**

During a large scale survey investigating gene expression profiles in neutrophils, we obtained evidence for the induction of genes encoding receptors for antibodies, cytokines, and chemokines as well as molecules involved in signal transduction during the terminal differentiation of these cells. Among these genes we identified many molecular components of the type I and type II IFN pathways. Moreover, the mRNA expression of several of these molecules involved in IFN signaling in mature neutrophils was reduced by GM-CSF in *vitro*. If freshly isolated immature and mature neutrophils were compared, we noticed that the differences in gene expression of the IFN signaling components correlated with the expression of IFN-regulated genes. This pointed to the possibility that the increased levels of expression of the IFN signaling components in mature neutrophils might result in increased *in vivo* IFN sensitivity of mature compared with immature neutrophils.

Indeed, we obtained evidence for more efficient type I and type II IFN signal transduction in mature compared with immature cells. For instance, STAT1 was not only expressed at lower levels in immature neutrophils; it was also not phosphorylated on Tyr-701 (25) upon both IFN-α and IFN-γ stimulation of these cells. Based on the gene expression and STAT1 phosphorylation findings, we were puzzled by two additional questions. Why do mature neutrophils, which are known to be involved in the defense against bacteria (1), require a signaling pathway(s) for IFNs that are believed to be important in antiviral immune responses (26)? On the other hand, what is the advantage of immature neutrophils to respond to IFNs with lower efficacy?

Increased IFN(α) production has been demonstrated during bacterial infections. For instance, LPS released IFN-γ, in an interleukin-12/18-dependent manner from PBMC (27), IFN-dependent genes were induced in these cells upon contact with bacteria (28), and IFN-β was generated after TLR4 activation in macrophages (29). Recently, it has been shown that activated neutrophils release granule proteins and chromatin that together form extracellular fibers able to bind both Gram-positive and -negative bacteria. These so-called neutrophil extracellular traps were able to degrade virulence factors and to kill bacteria (11).

Because IFN-γ has been shown to enhance anti-fungal activities of neutrophils (30–32), we asked whether IFN-α or IFN-γ might be able to help in the formation of neutrophil extracellular traps. Indeed, using two different techniques we demonstrated that IFN priming and subsequent C5a stimulation...
resulted in DNA release in mature but not in immature neutrophils. Similar data were obtained when we analyzed the release of MPO from azurophilic cytoplasmic granules (33) into the extracellular space, where MPO is a component of the extracellular traps (11). These data suggest that both IFN-α and IFN-γ are able to participate not only in anti-fungal but also in anti-bacterial neutrophil defense mechanisms.

Treatment of patients with IFN-α (34) and IFN-γ (35) is often associated with reduced leukocyte numbers, probably due to their anti-proliferative activities on bone marrow cells. In our system using immature neutrophils cultured in complete culture medium, we were able to mimic the known proliferative effects of G-CSF and GM-CSF on hematopoietic precursor cells (6, 7). Both IFNs partially blocked the cytokine-mediated proliferative responses, but this effect was not significant. In contrast, IFN-α significantly blocked proliferative responses in T cells, probably by interfering in early signaling events (36). These data suggested that, despite almost undetectable STAT1 tyrosine 701 phosphorylation, immature neutrophils are able to transduce IFN signals, but with reduced efficacy. We hypothesize at this point that a fully active IFN-signaling pathway(s) in immature neutrophils might be dangerous because patients suffering from common viral infections would carry a high risk for developing neutropenia and subsequently severe immunodeficiencies as well as for damaging the bone marrow due to the release of toxic neutrophil mediators.

There was only little interindividual variability regarding gene expression in immature and mature neutrophils. This suggested that the individuals from whom we obtained the bone marrow aspirates and the blood were indeed healthy. In contrast, when we mimicked the situation of an infectious disease condition by exposing mature neutrophils to GM-CSF in vitro, we observed dramatic changes in gene expression. This is in agreement with previously published work, which analyzed gene expression modification related to cancer and bacterial infection in blood leukocytes (37). Interestingly, it appears that the number of genes that are down-regulated as a consequence of GM-CSF exposure is greater than the number of induced genes.

We investigated gene expression profiles between human immature bone marrow neutrophils and human mature blood neutrophils. A similar analysis in specific immature neutrophil
subsets would be of great interest but is hampered by the non-availability of suitable purification techniques. Highly purified cell populations would be required to precisely detect the exact time periods of expression of individual genes. Although we used a mixture of immature neutrophils, gene expression profiles demonstrated only little variability among different donors. It is possible that the inter-individually variability is higher under in vivo pathologic conditions. Therefore, further work is required to compare gene expression pattern between normal and infectious diseases blood neutrophils (e.g. cystic fibrosis, sepsis).

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