Neutrophils in Cystic Fibrosis Display a Distinct Gene Expression Pattern

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We compared gene expression in blood neutrophils (polymorphonuclear leukocytes, or PMNs) collected from healthy subjects with those of cystic fibrosis (CF) patients devoid of bacterial colonization. Macroarray analysis of 1050 genes revealed upregulation of 62 genes and downregulation expression of 27 genes in CF blood PMNs. Among upregulated genes were those coding for vitronectin, some chemokines (particularly CCL17 and CCL18), some interleukin (IL) receptors (IL-3, IL-8, IL-10, IL-12), all three colony-stimulating factors (G-, M-, GM-CSF), numerous genes coding for molecules involved in signal transduction, and a few genes under the control of γ-interferon. In contrast, none of the genes coding for adhesion molecules were modulated. The upregulation of six genes in CF PMNs (coding for thrombospondin-1, G-CSF, CXCL10, CCL17, IKKε, IL-10Rα) was further confirmed by qPCR. In addition, the increased presence of G-CSF, CCL17, and CXCL10 was confirmed by ELISA in supernatants of neutrophils from CF patients. When comparison was performed between blood and airway PMNs of CF patients, there was a limited difference in terms of gene expression. Only the mRNA expression of amphiregulin and tumor necrosis factor (TNF) receptor p55 were significantly higher in airway PMNs. The presence of amphiregulin was confirmed by ELISA in the sputum of CF patients, suggesting for the first time a role of amphiregulin in cystic fibrosis. Altogether, this study clearly demonstrates that blood PMNs from CF patients display a profound modification of gene expression profile associated with the disease, suggesting a state of activation of these cells.

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INTRODUCTION

The properties of circulating neutrophils from cystic fibrosis (CF) patients differ from those of healthy subjects. These observations probably reflect that mutation or deletion of the CF transmembrane conductance regulator (CFTR) may lead to disturbance of blood polymorphonuclear leukocytes (PMNs), either directly (1) or as a consequence of an ongoing inflammation and infection (2). Particularly, circulating PMNs from CF patients appear to be primed and can release increased levels of interleukin (IL)-8 (3), elastase (4), lipoxygenase products (5), myeloperoxidase (6), and chloramine (7) in response to proper activators. Furthermore, CF blood PMNs display enhanced oxidative activity in response to platelet-activating factor and untreated or opsonized zymosan (8). In contrast, circulating PMNs display a decreased chemotactic response to leukotriene B4 (9) and decreased chlorination of phagocytosed bacteria (1). Other modifications of cell-surface markers have been reported such as reduced expression of Fcγ RIII (CD16) (8) and reduced shedding of L-selectin (CD62L) upon stimulation with either IL-8 or formyl-methionyl-leucyl-phenylalanin (FMLP) (10). Apoptosis of blood PMNs appears similar between CF patients and healthy subjects, but the interaction of CF airway PMNs with CF respiratory epithelial cells reduces their apoptosis (11,12).

In this study, for the first time, we undertook an analysis of gene expression in the neutrophils of CF patients. We compared PMNs derived from blood and airway of CF patients to blood PMNs from healthy individuals. We performed a macroarray analysis to investigate the mRNA expression of 1050 different genes coding for cytokines, chemokines and their receptors, apoptosis-related molecules, cellular signaling molecules, and different cellular metabolism actors.

MATERIALS AND METHODS

Patient Characteristics

Five CF patients (two boys, three girls) with different genotypes (ΔF508/ΔF508; G542X/G542X; N1303K/347delCC; ΔF508/G542X; ΔF508/NI), mean age 10.7 ± 5.6 years, clinical score 80 ± 10, were included in this study. In all patients, the diagnosis of CF was confirmed by a sweat chloride concentration of >60 Meq/L and by CFTR gene mutations (13). Results of physical examination, chest radiographs, Schwachman-

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Kulczycki score (14), pulmonary function tests with determination of forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1), oxygen saturation (SaO_2), and sputum quantitative bacterial cultures were recorded at the time of the study. For pulmonary function tests, the mean values expressed as percentage of predicted values were FVC 65.5% ± 18% and FEV_1 55% ± 16%. None of the CF children tested positive for *Pseudomonas aeruginosa*. The control group included three healthy young adults from the medical staff, mean age 30.8 ± 3.2 years, without history of lung disease and with normal lung function. Written informed consent was obtained from all CF patients or their guardians. The study was approved by the Ethic Committee of St. Louis University Hospital (Paris, France) (reg. no. CCPPRB 2004/15). For ethical reasons, it was not possible to obtain blood samples from healthy children.

Isolation of PMNs from Blood Samples

The PMNs from venous blood of CF patients were obtained as described (3). In brief, blood PMNs from CF patients were isolated by employing glucose dextran and Ficoll (Amersham Pharmacia Biotech, France) method. To allow the elimination of contaminating monocytes, blood PMNs were further purified after incubation with pan-antihuman HLA class II–coated magnetic beads (Dynabeads M450; Dynal, Oslo, Norway) (3,15). The purity and viability of blood PMNs were assessed by blue trypan and May-Grünwald-Giemsa staining.

Isolation of PMNs from Airway Samples

Spontaneous sputa were collected in sterile cups and processed immediately. Airway PMNs in sputa were isolated in accordance with a previously adopted procedure (3). In brief, the airway was incubated with trypsin-EDTA, the mixture was shaken vigorously at 37°C, and the reaction was stopped with trypsin-inhibitor and washed with cold PBS. Airway PMNs were not prepared from healthy subjects, because we found in a previous study (3) that induced sputa from healthy subjects does not contain enough PMNs for isolation and culture. As in the procedure described above for the blood PMNs, contaminating monocytes were eliminated after incubation with pan-antihuman HLA class II–coated magnetic beads. We followed a similar cell-counting procedure and assessment of viability with trypan blue dye exclusion. The purity of the PMN suspension was >99% as assessed by May-Grünwald-Giemsa staining.

Cultures of Blood PMNs

PMNs were cultured in RPMI 1640 supplemented with L-glutamine, antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin; Life Technology), and 5% heat-inactivated normal human serum (pool of sera from healthy volunteers) in absence of any stimuli. Aliquots (0.5 mL; 5×10^5 cells) of PMN suspension were incubated in a 5% CO_2 incubator in 24-well multidish plates (Nunc; ATGC Biotechnology, Marne La Vallée, France) for 18 h at 37°C. At the end of the culture, the supernatants were harvested, centrifuged for 10 min at 300*g* and 15°C, and kept at –20°C before cytokine measurements.

Measurement of Amphiregulin, CCL17, CXCL10, and G-CSF by ELISA

Amphiregulin, CXCL10, and G-CSF were measured with a DuoSet from R&D System (Abingdon, UK) and CCL17 with a Quantikine kit (R&D Systems) as recommended by the manufacturer.

Macroarray Hybridization and Analysis

The macroarray experiments were carried out using membranes that were characterized and validated in two published studies (16,17). Briefly, PCR products of 1050 human genes were spotted in duplicate on positively charged nylon membranes as described (16). Macroarray design can be found on the ArrayExpress website (www.ebi.ac.uk/arrayexPress) with the accession number A-MEXP-141. cDNA labeling and hybridization scanning were also described (16). After recording of the signals for each gene with ArrayVision and quality control of hybridizations using the luciferase signal intensity, data corresponding to all the membranes were transformed in a log2 scale and normalized by a method derived from the variance analysis (ANOVA) to give an equal median signal to all membranes. This statistical method estimates the weight and significance of variability sources on experimental data. For each condition, 4 biological replicates were performed and hybridized onto macroarrays, where PCR products were spotted in duplicate; eight signals for one gene were used for one experimental condition. Comparative analyses between baseline (control blood) and experiment (CF blood or airway) were done with the dChip software (18), using an unpaired Welch *t* test with a *P* value threshold of 0.05. This software was also used for hierarchical clustering using Euclidian distance and average as a linkage method. Before clustering, the expression values for one gene across all samples were standardized to have a mean of zero. Increased or decreased values were then ranged compared with this mean. Macroarray hybridization and analysis were performed on blood-derived PMNs from four patients and on airway-derived PMNs from three CF patients. They were compared with blood PMNs from three healthy controls.

Quantitative Real-Time PCR

Total RNA of PMN was prepared using the RNeasy Mini Kit (Qiagen). Purified RNA was reverse-transcribed with Superscript II RNase H (Invitrogen) and an oligodT 12-18 primer (Invitrogen) according to the manufacturer's protocol. The expression levels of genes of interest and GAPDH were determined by real-time quantitative PCR, using a Brilliant SYBR Green qPCR master mix (Stratagene) and Mx3005P (Stratagene). The primer sequences are listed in Table 1. All results were normalized to the expression of GAPDH. To confirm the specificity of the PCR products, the melt-

Table 1. Oligonucleotides used for qPCR

Gene	Sense oligonucleotide	Antisense oligonucleotide
GAPDH	5′-GAGTCAACGGATTTGGTCGT-3′	5′-TTGATTTTGGAGGGATCTCG-3′
Thrombospondin-1	5′-AAAGGATAATTGCCCCAACC-3′	5′-CGGTCTCCCACATCATCTCT-3′
G-CSF	5′-GCTTGAGCCAACTCCATAGC-3′	5′-TCCCAGTTCTTCCATCTGCT-3′
CXCL10 (IP-10)	5′-AGGAACCTCCAGTCTCAGCA-3′	5′-CAAAATTGGCTTGCAGGAAT-3′
IKKε	5′-GTGCACAAGCAGACCAGTGT-3′	5′-GCCCTTGGCAGTGTTGTAAT-3′
CCL17 (TARC)	5′-CTGCAAAGCCTTGAGAGGTC-3′	5′-CATGGCTCCAGTTCAGACAA-3′
IL-10Rα	5′-GGATTCACTGAGGGGAGACA-3′	5′-GCAGCAAAGTGAGGATGTGA-3′

ing profile of each sample was determined by heating from 60°C to 95°C at a linear rate of 0.10°C/s while measuring the fluorescence emitted. Analysis of the melting curve demonstrated that each pair of primers amplified a single product. In all cases, the PCR products were checked for size by agarose gel separation and ethidium bromide staining to confirm that a single product of the predicted size was amplified. Each run consisted of an initial denaturation time of 10 min at 95°C and 40 cycles at 95°C for 30 s, 58°C for 60 s, and 72°C for 30 s.

Statistical Analysis

The significance of the differences between PMNs from healthy controls, CF blood, and CF airway was determined by ANOVA and Fischer protected least significant difference (PLSD). A value of $P < 0.05$ was the criterion for statistical significance. Statistical analysis was performed with Statview software (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Gene Expression Profile of Blood PMNs

As shown in Table 2, the expression of 62 genes in CF PMNs was significantly enhanced compared with non-CF PMNs. Genes related to many aspects of PMNs activity were upregulated. It is worth mentioning the upregulation of numerous genes coding for chemokines (CCL17, CCL18, CXCL12, XCL1, XCL2), including two chemokines induced by γ-interferon (IFNγ) [CXCL9 (MIG), and CXCL10 (IP-10)]. Interestingly, two other genes related to the action of IFNγ (including 1-8B gene from IFN-inducible gene family and interferon regulatory factor-1) are also upregulated, the former being the most upregulated gene among all those studied. In contrast, the expression of the genes of two related chemokines [CXCL5 (ENA78) and CXCL8 (IL-8)] that specifically act on neutrophils are downregulated (Table 3). All three main colony-stimulating factors (M-CSF, G-CSF, GM-CSF) had their genes upregulated, as well as some cytokine receptors (IL-3Rα, IL-10Rα, IL-12Rβ1, M-CSFR, CXCR2). The upregulation of numerous genes of molecules involved in cell signaling (including IKKε, IKKγ, MAPK, MAPKK, transcription factor DP1 and DP2, ras homolog gene family, and TRAF4), as well as genes of molecules involved in ubiquitination, strongly suggest an activation process within the cells of the CF patients.

Twenty-seven genes were downregulated in PMNs from CF patients compared with PMNs from healthy donors (Table 3). Surprisingly, whereas that of capsase-1 (the IL1β-converting enzyme) is enhanced, that of IL-1β is decreased.

Confirmation of Macroarray Results by qPCR and ELISA

To confirm some of the results obtained with the macroarrays, we performed qPCR for several genes upregulated in neutrophils from blood of CF patients. We amplified genes related to cell adhesion (thrombospondin-1), coding for chemokines (CCL17, CXCL10), involved in signal transduction (IKKε), and a cytokine receptor (IL-10Rα) and a growth factor (G-CSF). As shown in Figure 1, for all six genes, we found increased expression in CF patients versus healthy controls. We also confirmed this upregulation at the protein level, in culture supernatants of neutrophils, for two chemokines (CCL17 and CXCL10) and one growth factor (G-CSF) (Figure 2).

Comparison between Blood and Airway PMNs from CF Patients

The levels of mRNA expression of CF blood PMNs was compared with that of airway PMNs from three CF patients. Only two genes were significantly more upregulated in airway than in blood: amphiregulin (1.53×; $P = 0.0017$) and TNFR p55 (1.60×; $P = 0.026$). The similar gene expression profile for blood and airway from CF patients is also evident in the gene clustering presentation of the macroarray (Figure 3), which shows the increased (red) or decreased (blue) expression of genes, compared with the healthy controls. We investigated the presence of amphiregulin in airway PMN culture supernatants, but its level was below detection. In contrast, we detected significant amounts of amphiregulin in crude sputum from six of seven CF patients (range 54–100 pg/mL).

DISCUSSION

A comparative macroarray analysis between healthy blood PMNs and CF blood and airway PMNs was performed on 1050 different genes. For ethical reasons, we could not obtained PMN from healthy children, but there are probably few differences between PMNs from children and young adults.

We mainly focus our discussion on genes whose expression was significantly enhanced in CF PMNs compared with healthy PMNs. Vitronectin is one of

Table 2. Upregulated genes in CF PMNs compared with PMNs from healthy donors

Biological process	Acc. no.	Gene coding for	Fold change	*P* value
Apoptose	XM_054989	Similar to caspase 8	2.11	0.004
	U13699	Caspase 1	2.50	0.017
	AL049703	PAC 179D3	1.54	0.023
	AF310105	nalp1	1.65	0.034
Carbohydrate metabolism	X52486	Uracil-DNA glycosylase 2	1.87	0.002
Cell adhesion	X14787	Thrombospondin 1	1.73	0.035
	X03168	Vitronectin	1.75	0.038
Cell cycle	D13639	Cyclin D2	1.51	0.001
	D63878	Neural precursor cell expressed, develop	1.57	0.006
	U18291	CDC16 (cell division cycle 16)	1.72	0.005
Chemokine	NM_002995	XCL1 (lymphotactin)	1.75	0.002
	NM_003175	XCL2	1.52	0.004
	NM_002416	CXCL9 (MIG)	1.84	0.004
	BC010954	CXCL10 (IP10)	1.83	0.023
	U16752	CXCL12 (SDF1)	1.91	0.012
	D43767	CCL17 (TARC)	3.01	< 0.001
	AF111198	CCL18 (PARC)	2.43	0.002
Cytokine and chemokine receptors	AB032734	CXCR2	2.89	< 0.001
	M74782	IL-3 receptor α (low affinity)	5.35	0.001
	U00672	IL-10 receptor α	4.08	< 0.001
	U03187	IL-12 receptor β1	1.80	< 0.001
	X03663	CSF-1 receptor	1.87	< 0.001
Cytoskeleton	X00351	β-Actin	1.85	0.003
DNA repair	L07541	Replication factor C (activator 1) 3 (38)	2.00	0.023
	NM_005916	MCM7	1.62	< 0.001
Electron transport	M12792	Cytochrome P450, subfamily XXIA (steroid)	1.61	0.003
Growth factor	NM_000757	M-CSF	1.99	0.008
	X03438	G-CSF (1)	3.20	<0.001
	M13207	GM-CSF (1)	2.13	0.031
	U66197	Fibroblast growth factor 12	2.08	<0.001
	K03222	Transforming growth factor α	2.08	<0.001
	M34309	Epidermal growth factor	2.22	0.038
	M86528	Neurotrophin 5 (neurotrophin 4/5)	1.59	0.016
IFN-related	X57351	1-8D gene from IFN-inducible gene family	6.06	<0.001
	X14454	IFN regulatory factor 1	1.83	<0.001
Metabolism	M64082	GAPDH	1.51	<0.001
	M64082	Flavin containing monooxygenase 1	1.78	0.046
	X14672	*N*-acetyltransferase 2 (arylamine N-acety)	1.95	0.016
Protein amino acid phosphorylation	U43522	Protein tyrosine kinase 2 beta	1.59	0.002
	U24152	P21/Cdc42/Rac1-activated kinase 1 (yeast)	1.73	0.044
Protein folding	U07550	Heat shock 10-kDa protein 1 (chaperonin 10)	1.69	0.045
Receptor	M38690	CD9 antigen (p24)	1.92	0.008
Regulation of transcription	U35113	Metastasis associated 1	1.55	< 0.001
Sensory perception	L10035	Crystallin β B2	1.59	0.010
Signal transduction	AF074382	Ikk γ	2.48	0.001
	AF241789	Ikk ε	2.60	0.001
	X75208	EphB3	1.51	0.019
	U07349	Mitogen-activating protein kinase kinase kinase kinase 2	1.93	0.026
	U77129	Mitogen-activated protein kinase kinase kinase kinase 5	1.55	0.018
	L05624	Mitogen-activated protein kinase kinase 2	1.69	0.018
	U39657	Mitogen-activated protein kinase 6	2.17	0.015
	D85815	ras homolog gene family	1.71	0.022
	U81002	TRAF4	1.72	0.014
	U18422	Transcription factor Dp-2	2.43	< 0.001
	L23959	Transcription factor Dp-1	2.68	< 0.001
Transcription factor	L24804	v-myc	1.57	< 0.001
	XM_113786	cfos	1.71	0.023
Transferase activity	U94352	Manic fringe (*Drosophila*) homolog	1.59	0.007
Ubiquitination	BC005980	Ubiquitin-conjugating enzyme E2D1	1.51	0.016
	NM_003592	Cullin 1 (cdc53)	1.55	0.004
	NM_004788	Ubiquitin factor E4A	1.78	0.007
	NM_014248	Rbx-1	1.84	0.009

Table 3. Downregulated genes in CF PMNs compared with PMNs from healthy donors

Biological process	Acc. no.	Gene coding for	Fold change	*P* value
Antigen presentation	M16276	MHC Class II HLA-DR2-Dw12	–1.51	0.001
cAMP biosynthesis	D14874	Adrenomedullin	–2.14	< 0.001
Cell cycle	U49089	Discs, large (*Drosophila*) homolog 3 (neu)	–2.14	<0.001
Chemokine	X78686	CXCL5 (ENA78)	–1.52	0.002
	Y00787	CXCL8 (IL-8)	–2.19	0.003
Cytokine activity	A14844	IL-2	–1.79	0.016
	NM_000576	IL-1β	–1.67	0.001
Defense response	L08044	Intestinal trefoil factor	–1.62	0.001
DNA repair	M29971	*O*-6-methylguanine-DNA methyltransferase	–1.54	0.003
Growth factor	M27968	Fibroblast growth factor 2 (basic)	–1.69	0.001
	M60828	Fibroblast growth factor 7	–1.57	0.014
IFN related	X01992	IFNγ	–1.71	0.012
Immune response	AB021288	β2-microglobulin	–1.92	0.004
Inflammatory response	M69043	MAD-3 (IK-B like activity)	–1.60	0.012
Ion transport	AF043233	H^+/oligopeptide transporter	–1.95	0.017
Metabolism	X68060	Topoisomerase (DNA) II β (180 kDa)	–1.58	<0.001
Protein folding	U15590	Heat shock 27-kDa protein 3	–1.78	0.031
	M86752	Stress-induced-phosphoprotein 1 (Hsp70/H)	–1.71	0.018
	S67070	Heat shock 27-kDa protein 2	–1.62	0.003
Receptor	L24804	Inactive progesterone receptor, 23 kDa	–1.97	<0.001
Regulation of transcription	M83221	I-Rel	–1.55	0.034
	U12767	MINOR	–1.55	< 0.001
Signal transduction	L25081	ras homolog gene family, member C	–1.72	0.001
	X66360	PCTAIRE protein kinase 2	–1.68	< 0.001
Transcription factor	X16416	c-alb p150	–1.75	< 0.001
	M95712	v-raf murine sarcoma viral oncogene homo	–1.52	< 0.001
Ubiquitination/degradation	BC002979	Proteasome α6	–1.67	<0.001

them. Vitronectin is a multifunctional glycoprotein present in blood and anchored to the extracellular matrix (19). It promotes cell adhesion, spreading, and migration by interaction with specific integrins. Vitronectin is involved in fibrinolysis and also in the immune defense through its interaction with the terminal complex of complement and in hemostasis through its binding to heparin. If released in the airways, vitronectin can potentially regulate the proteolytic degradation of the matrix. All these properties make vitronectin an important molecule associated with the inflammatory process in the airways of CF patients.

Among other genes whose expression was significantly enhanced in CF blood PMNs, it is worth mentioning the chemokines CCL17, CCL18, XCL1, CXCL9, and CXCL10. The upregulation of two, CCL17 and CXCL10, was further confirmed by qPCR and ELISA. Interestingly, these chemokines are different from those for which gene expression was upregulated in vitro by lipopolysaccharide (LPS) (20), after receptor-mediated phagocytosis (21), or after bacterial-induced apoptosis (22). Our analysis was performed in patients negative for *P. aeruginosa*. Thus, the difference between CF and healthy PMNs is of interest because it suggests that the absence of the active CFTR molecule is sufficient to initiate a reprogramming of gene expression in circulating PMNs in the absence of microbial stimuli. This modification may be a direct consequence of the CFTR mutation itself (1), or an indirect effect of the inflammatory process occurring early after birth in CF patients (2).

Among the chemokines whose gene expression was upregulated, CXCL9 and CXCL10 are both induced in response to IFNγ. Two other IFNγ-related genes were also upregulated, 1-8D and interferon responsive factor-1 (IRF-1). Mainly induced by IFNγ, the function of 1-8D protein is unknown at present. IRF-1 is a transcription factor that acts as a regulator of cell cycle and apoptosis and negatively regulates cell growth. A role for IFNγ in the pathophysiology of cystic fibrosis is thus suggested by these observations. Indeed, IFNγ has been detected in sputa (23) and circulating γδT cells of CF patients with *P. aeruginosa* -infection (24).

Another striking finding is the upregulation of the G-CSF gene revealed by macroarray analysis and confirmed by qPCR. In addition, an increased presence of G-CSF in supernatants of CF blood neutrophils was observed. G-CSF was previously detected in the airway of CF patients (25) and in their serum (23,26). It is known that G-CSF regulates the production, maturation, function, and sur-

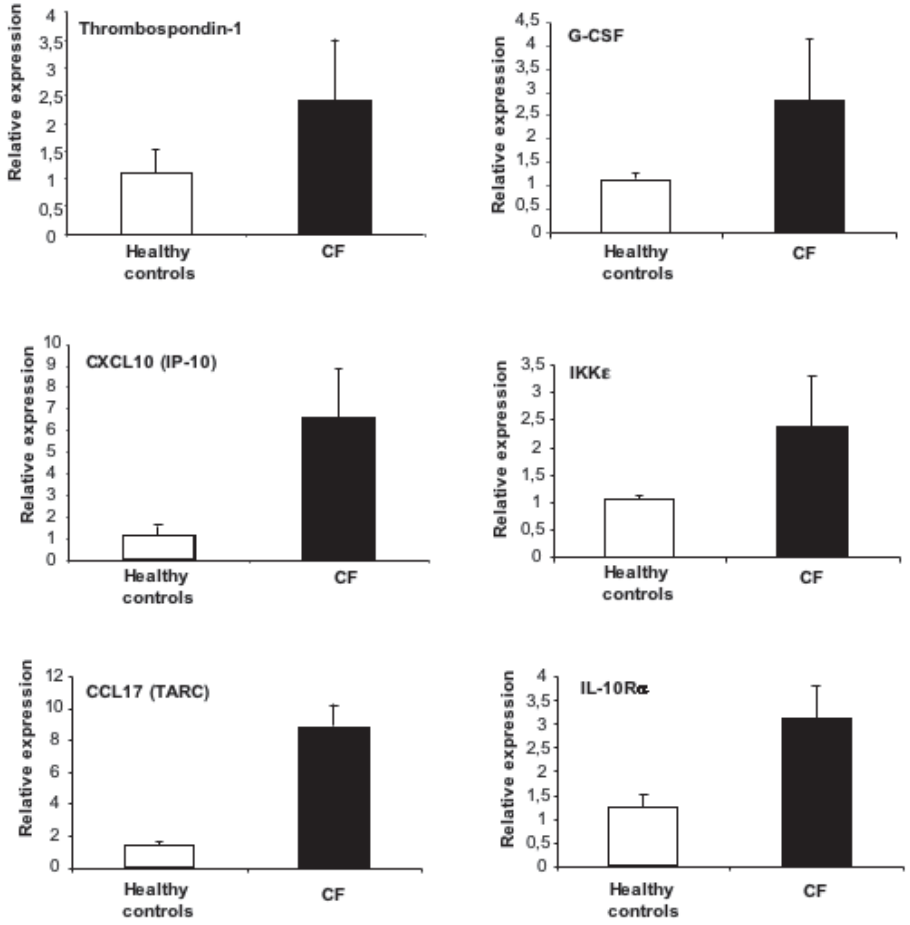

Figure 1. Gene expression in blood neutrophils analyzed by real-time PCR. mRNA was purified from blood neutrophils of CF patients (n = 5) and healthy individuals (n = 4). cDNA was prepared and subjected to qPCR. The expression of genes of interest was normalized to that of GAPDH and compared with mean values of healthy controls. Values are mean ± SEM.

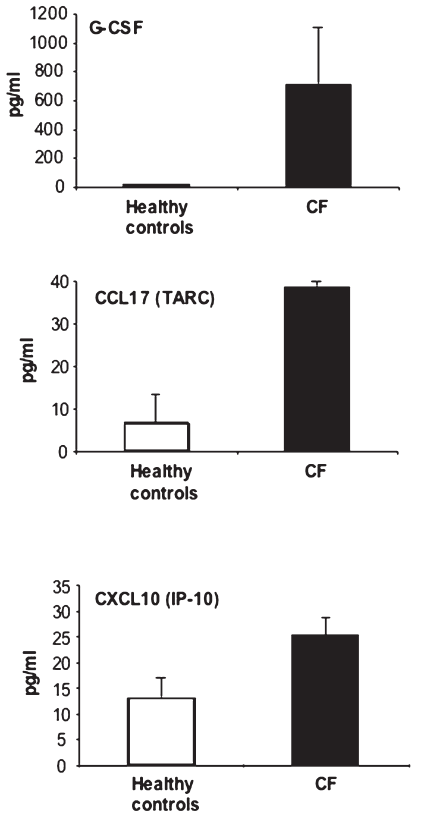

Figure 2. Dosage of G-CSF, CCL17, and CXCL10 in culture supernatants of blood neutrophils. Blood neutrophils from CF patients (n = 4) and healthy individuals (n = 6) were cultured overnight in RPMI supplemented with 5% heat-inactivated normal human serum without any stimulation. The presence of spontaneously released chemokines and growth factor was assessed in the culture supernatants by specific ELISA. Values represent mean ± SEM.

vival of neutrophils, and it can also exacerbate underlying inflammatory diseases (27). Whereas many cells can produce G-CSF, it is interesting to note that neutrophils could contribute to their own activation process in an autoregulatory loop. Accordingly, G-CSF could be a natural factor present in the serum of CF patients that contributes to the activation status observed for patients' neutrophils, even in the absence of infection.

Another interesting observation was the upregulated expression of genes coding for cytokine receptors. This was particularly the case for IL-3R. Although IL-3 is not as potent as GM-CSF to activate or prime mature PMNs (28,29), it may act synergistically with other cytokines such as IFNγ (30) or more efficiently if the number of receptors is enhanced. The upregulation of the gene expression of CXCR2 further suggests that IL-8, found in large amounts in CF sputa (31) and in supernatants of CF airway epithelial cell/PMN coculture (12), is acting on PMNs. This result also correlates nicely with the fact that CF blood PMNs showed significantly increased migration to IL-8 (32). Among cytokine receptors, IL-10Rα gene expression was found to be upregulated by both macroarray and qPCR. It is worth mentioning that blood PMNs from CF patients are responsive to the anti-inflammatory effects of IL-10, which can inhibit IL-8 production in vitro in response to bacterial LPS or peptidoglycan (33).

We also found an upregulation of the expression of several genes regulating the NF-κB transcription factor in both blood and airway PMNs of CF patients. In unstimulated cells, NF-κB dimers are maintained in the cytoplasm through interaction with inhibitory proteins, the IκBs. In response to cell stimulation, a multisubunit protein kinase, IκB kinase (IKK), is rapidly activated and phosphorylates two critical serines in the

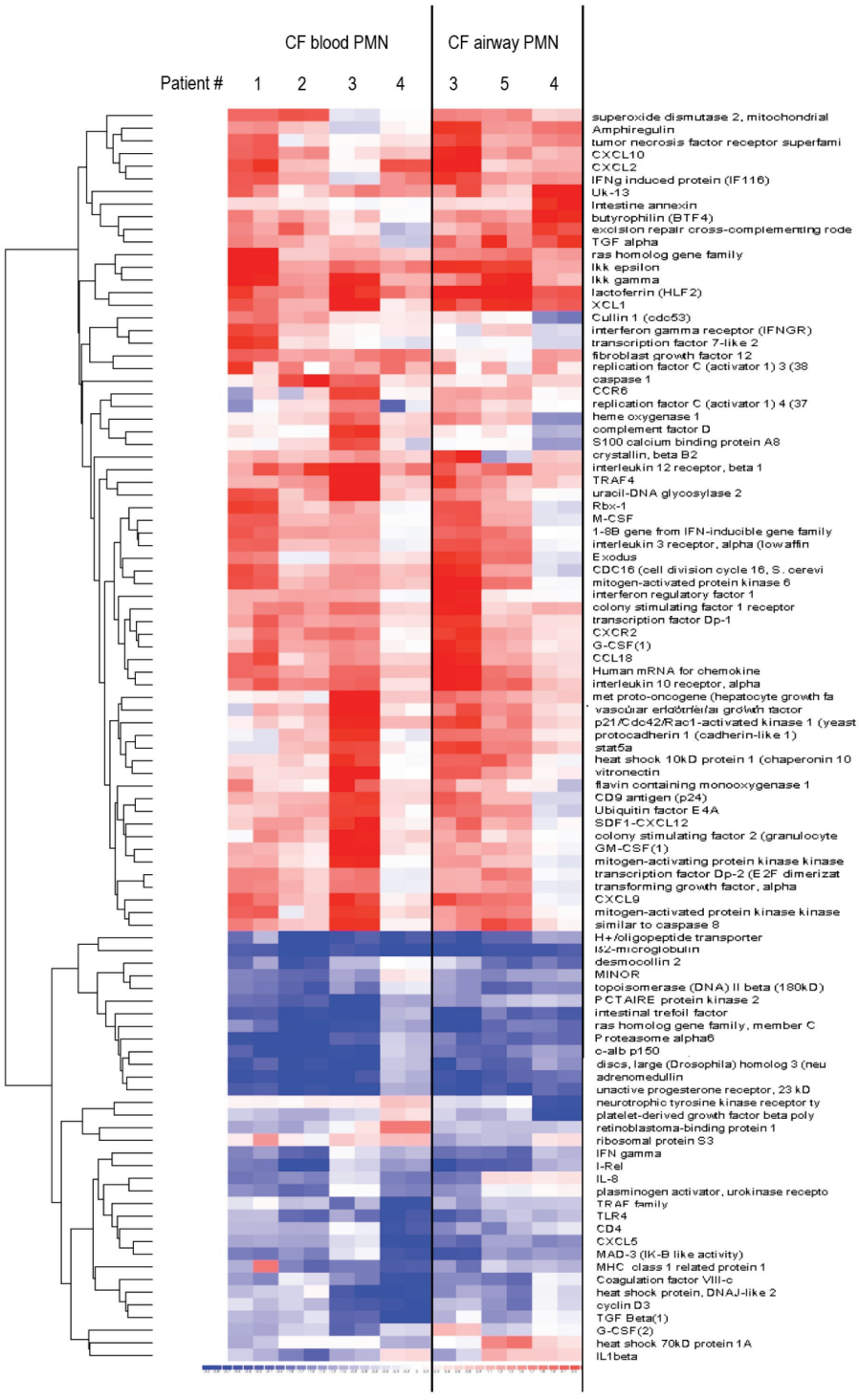

Figure 3. Gene clustering of the differentially expressed genes in blood PMNs (samples 1–4), and airway PMNs (samples 5–7) of CF patients. The gene expression profile was compared with that of blood PMNs from healthy subjects (n = 3). Blue represents a lower expression level and red a higher expression level compared with the mean expression in normal blood PMNs.

N-terminal regulatory domain of the IκBs. IKK complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (NEMO) (34), is the master regulator of NF-κB–mediated innate immune and inflammatory responses. The reported upregulation of the gene coding for IKKγ further illustrates that circulating PMNs are activated. Upregulated expression of the gene coding for IKKε was also observed. IKKε and TBK1 (TANK-binding kinase 1) synergize with TANK (TRAF family member-associated NF-κB activator) to promote their interaction with the IKKs, allowing IKKε and another kinase (TBK1) to modulate NF-κB activation (35). In addition, IKKε is also a key element in the signaling pathway downstream of the Toll/interleukin-1 receptor domain-containing adapter protein (TRIF) and TBK1. This pathway leads to IFNβ production through activation of IFN regulatory factor (IRF)-3 that is directly phosphorylated by TBK1 and IKKε (36). Our data on the upregulated genes coding for IKKγ and IKKε in CF blood PMNs are in good agreement with recent observations by Srivastava et al. (37) that show differentially overexpressed proteins of the TNF-α/NF-κB signaling pathway (IKKα, I-TRAF, and IKKε) in sera of CF patients. According to those authors, pooled sera from CF patients, characterized by a CF versus non-CF serum proteomic signature using an antibody microarray platform, are enriched in protein mediators of inflammation that may be selectively expressed in CF-affected tissues such as lung.

Among other genes that give evidence that CF blood PMNs are activated is the up-regulation of the gene coding for DP1. DP1 is a binding partner for E2F transcription factors. Target genes include those involved in DNA synthesis, cell cycle, and apoptosis (38). Of course, the macroarray approach does not allow measurement of some other aspects of cell signaling, where activation is related to protein phosphorylation or cleavage.

We did not find any modulation of the genes coding for α-integrin (-2, -3, -4, -5,

-6, -8, -9), β-integrin (-1, -2, -3, -5, -7, -8), or other adhesion molecules (ICAM-1, ICAM-2, CD11a). This is consistent with the literature, where a very limited modulation of these genes has been reported, if one ignores the downregulation of α-5 integrin by LPS (39) and the upregulation of α-3 integrin, α-7 integrin, and ICAM-1 by receptor-mediated phagocytosis (21,40). Among cell adhesion molecules, we found increased expression of thrombospondin-1 that was confirmed by qPCR. Except for caspase 1, we failed to find major modulation of apoptosis-related genes (bcl-2 and bcl-2 family members, caspase-2, -3, -4, -6, -7, -8, -9, -10). In the literature, the gene expression of caspase-1 was shown to be upregulated by phagocytosis, and the gene expression of caspase-3, -8, and -9 was shown to be downregulated by LPS or phagocytosis (21,39,40).

We also compared blood PMNs to those prepared from sputum of CF patients. There was a limited difference between CF blood- and airway-derived PMNs. In spite of different CFTR mutations, we could find important homogeneity in terms of gene expression in the neutrophils from these two compartments. Among the 1050 studied genes, only six genes were significantly more reduced and two genes more upregulated among CF airway PMNs compared with CF blood PMNs, including amphiregulin. Although we failed to detect amphiregulin in PMN culture supernatants, we found significant amounts of amphiregulin in crude sputum from six of seven patients. Amphiregulin is an epidermal growth factor receptor ligand that activates epithelial cells and contributes to TNF-induced IL-8 release by human airway epithelial cells (41). It also activates human lung fibroblasts and favors their proliferation (42). Furthermore, amphiregulin enhances transmigration of human neutrophils through epithelial cell monolayers after alteration of E-cadherin–dependent tight junctions (43). Our findings and all these properties make amphiregulin a new marker of CF-associated lung inflammation and an interesting putative target molecule. Thus, a greater understanding of molecular mechanisms by which CF airway PMNs upregulated amphiregulin production will identify novel therapeutic windows of opportunity. Altogether, this study clearly demonstrates that PMNs from cystic fibrosis patients display a profound modification of their gene expression profile associated with the disease. Most interestingly, the nature of the expressed mRNA of blood PMNs, in the absence of obvious interaction with inflammatory and/or infectious foci, was altered as compared to blood PMNs from healthy donors, and there was a very limited difference between the PMNs isolated from blood and airway in terms of gene expression.

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