In order to systematically analyze proteins fulfilling effector functionalities during inflammation, here we present a comprehensive proteome study of inflammatory activated primary human endothelial cells and fibroblasts. Cells were stimulated with interleukin 1-β and fractionated in order to obtain secreted, cytoplasmic and nuclear protein fractions. Proteins were submitted to a data-dependent bottom up analytical platform using a QExactive orbitrap and the MaxQuant software for protein identification and label-free quantification. Results were further combined with similarly generated data previously obtained from the analysis of inflammatory activated peripheral blood mononuclear cells. Applying a false discovery rate of less than 0.01 at both, peptide and protein level, a total of 8370 protein groups assembled from 117,599 peptides was identified; mass spectrometry data have been made fully accessible via ProteomeXchange with identifier PXD003406 to PXD003417.

Comparative proteome analysis allowed us to determine common and cell type-specific inflammation signatures comprising novel candidate marker molecules and related expression patterns of transcription factors. Cardinal features of inflammation such as interleukin 1-β processing and the interferon response differed substantially between the investigated cells. Furthermore, cells also exerted similar inflammation-related tasks; however, by making use of different sets of proteins. Hallmarks of inflammation thus emerged, including angiogenesis, extracellular matrix reorganization, adaptive and innate immune responses, oxidative stress response, cell proliferation and differentiation, cell adhesion and migration in addition to monosaccharide metabolic processes, representing both, common and cell type-specific responsibilities of cells during inflammation. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.058099, 1982–1997, 2016.

Inflammation is a complex process, which plays, especially in its chronic form, an important role in many diseases of modern civilization such as cardiovascular and neurological disorders and diverse cancers (1–3). Although it is possible to cure acute inflammation, chronic inflammation still represents a great challenge and often responds in an unsatisfying fashion to sustained treatment. In acute inflammation, the relations between cause and effects may be rather straight, so that it may be sufficient to block a single activity, for example that of COX-2, in order to achieve relieve of symptoms and subsequent healing. In chronic inflammation, these relations seem to be more complex and a simple treatment may not be successful. Actually, several different cell types are involved in inflammation, contributing to the complex signaling network necessary for the appropriate exertion and completion of this process. Chronic inflammation may occur when specific regulation mechanisms that are necessary to resolve the inflammatory process fail, resulting in an uncontrolled escalation of the ongoing processes (4). Accumulation of pro-inflammatory signaling molecules and effector cells at the site of inflammation (5), the production of new blood vessels enabling the incessant recruitment of inflammatory cells (6), or the excess deposition of extracellular matrix components resulting from an uncontrolled inflammation-related wound healing process (7) can be some of the consequences.

Different cell types may fulfill different functionalities during inflammation. Obviously, each cell type has its repertoire of specific regulatory factors and may contribute to the regulation of inflammation in a specific manner. In this way, all cell types may be cooperating to achieve the fine tuning of the complex process of inflammation. Main players of inflammation, and main targets for anti-inflammatory treatments, are leukocytes, including neutrophils and monocytes as part of the innate immune response, as well as B- and T lymphocytes, activated in the course of an inflammation-related adaptive immune response. Under normal conditions, when they have fulfilled their tasks, these cells are rapidly neutralized by induction of apoptosis (8). Stromal cells such as fibroblasts and endothelial cells are involved in the process of
inflammation as well, and these cells are capable of surviving for a longer time and may stay in their functionally activated state when the inflammatory process should be completed, thus possibly contributing to the development of chronic inflammation (9). Although the most important players of inflammation have been well described, a systematic analysis of the proteins fulfilling the effector functionalities during inflammation has not yet been undertaken. This would, however, contribute to a better understanding of the ongoing complex processes and may thus support the development of new therapeutic strategies to combat chronic inflammation and related diseases (10).

Here we present a systematic proteome study of inflammatory activated primary human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVEC). These cells have been analyzed by us previously (11, 12) demonstrating that they display all relevant cell type characteristics of stromal fibroblasts and endothelial cells, and thus represent suitable model systems. A standardized approach has been applied to semi-quantitatively determine and compare the relevant regulatory factors that were up- and downregulated by fibroblasts and endothelial cells upon inflammatory activation. To this end, NHDF and HUVEC were stimulated with the canonical inflammation mediator interleukin-1β (IL-1β) (13). Secreted, cytoplasmic and nuclear proteins were extracted from the cells and analyzed separately by shotgun proteomics using a QExactive orbitrap mass spectrometer. Results were further combined with data obtained from previous investigations on inflammatory activated peripheral blood mononuclear cells (PBMCs) (14). In this way, cell type-specific inflammation-related functionalities were determined, as well as inflammatory signatures and marker molecules that may be indicative for the inflammatory processes occurring in vivo. This motivated us to define hallmarks of inflammation - in the style of the hallmarks of cancer (15) - representing the biological processes essential for the successful resolution of inflammation and to specify responsibilities of fibroblasts, endothelial cells and leukocytes therein.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary HUVEC were purchased from (Lonza Walkersville Inc., Walkersville, MD). HUVEC were cultured in endothelial basal medium supplemented with the EGM-2 SingleQuot Kit (both Lonza), 10% FCS and 100 U/ml penicillin/streptomycin (both ATCC/LGC Standards, London, UK), according to the instructions of the manufacturer. Normal human dermal fibroblasts (NHDF) were kindly provided by Verena Paulitschke from the General Hospital of Vienna. NHDF were cultured in RPMI 1640 (Thermo Fisher Scientific, Life Technologies, Loughborough, UK) supplemented with 10% FCS and 100U/ml penicillin/streptomycin (both ATCC) at 37 °C and 5% CO2. Cells were used up to passage 7 and 22 for HUVEC and NHDF, respectively. Experiments were performed in 75 cm² culture flasks, using ~5 × 10⁵ cells per flask. Cell numbers, as well as cell viability that was consistently better than 98%, were determined using a Moxi Z cell counter (ORIFLO Technologies, Carlsbad, CA). Inflammatory stimulation with 10 ng/ml of IL-1β (Sigma-Aldrich, Vienna, Austria) was carried out for 24 h, as applied in previous studies (11, 16, 17). Control cells were incubated in parallel without IL-1β. After that, cells were washed with PBS and further cultured for 6 h in 6 ml of serum-free medium. Biological replicates were prepared for each cell type to allow statistical analyses of the resulting data.

Cell Fractionation—Supernatants of control and inflammatory activated cells were filtered through 0.2 μm filters (GE Healthcare, Freiburg, Germany) and proteins therein were precipitated overnight with ethanol at −20 °C. To obtain the cytoplasmic protein fractions as well as the nuclear and cytoskeleton rich fractions, we proceeded as previously described (14). In short, cells were lysed in isotonic lysis buffer supplemented with protease inhibitors by applying mechanical shear stress. Cytoplasmic proteins were separated from nuclei by centrifugation and precipitated overnight with ethanol at −20 °C. Nuclear proteins were extracted by incubating the nuclei in 500 mM NaCl and solubilizing the proteins in Nonidet P-40 buffer supplemented with protease inhibitors. The extracted proteins were separated from resting cell materials by centrifugation and precipitation of the resulting supernatant with ethanol at −20 °C overnight. After precipitation, all samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 0.05% SDS, 100 mM dithiothreitol (DTT)) and the protein concentrations were determined by means of a Bradford assay (Bio-Rad Laboratories, Munich, Germany).

Sample Preparation—For proteomics analyses, we prepared in-solution digests from all three subcellular protein fractions of NHDF and HUVEC, as previously described (14). In short, 20 μg of each protein sample was concentrated onto a 3kD MWCO filter (Pall Ausubl GmbH, Vienna, Austria) pre-washed with LC-MS grade water (Merck, Darmstadt, Germany). Supernatant with ethanol at −20 °C was further concentrated by centrifugation and protein pellets were dried in a speedvac and stored at −80 °C. Cells were then dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% SDS, 100 mM dithiothreitol (DTT)) and the protein concentrations were determined by means of a Bradford assay (Bio-Rad Laboratories, Munich, Germany). Proteins were extracted by incubating the nuclei in 500 mM NaCl and solubilizing the proteins in Nonidet P-40 buffer supplemented with protease inhibitors. The extracted proteins were separated from resting cell materials by centrifugation and precipitation of the resulting supernatant with ethanol at −20 °C overnight. After precipitation, all samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 0.05% SDS, 100 mM dithiothreitol (DTT)) and the protein concentrations were determined by means of a Bradford assay (Bio-Rad Laboratories, Munich, Germany).
with IAA, the proteins were digested for 18 h at 37 °C using trypsin
(Roche Diagnostics, Austria GmbH, Germany). The digested peptides
were eluted, once with 50 mM ammonium bicarbonate, and twice with
5% FA/50% ACN. The eluted peptide samples were dried and then
stored at −20 °C until further MS analyses.

**LC-MS/MS Analysis**—Dried samples were solubilized in 5 μl 30%
formic acid (FA) containing 10 fmol each of four synthetic standard
peptides (allowing us to verify the quality of the chromatographic
separation) and diluted with 40 μl mobile phase A (98% H2O, 2% ACN,
0.1% FA). Of this solution, 10 μl were injected into the nanoUPLC-system UtiMate 3000 RSLCnano (Thermo Fisher Scientific).
Peptides were first concentrated on a 2 cm x 75 μm C18 Pepmap100 pre-column (Thermo Fisher Scientific) at a flow rate of 10 μl/min using mobile phase A. Separation of the peptides was achieved by eluting them from the pre-column to a 50 cm x 75 μm Pepmap100 analytical column (Thermo Fisher Scientific) applying a flow rate of 300 nl/min and using a gradient of 8% to 40% mobile
phase B (80% ACN, 20% H2O, 0.1% FA), over 235 min for the
analysis of cytoplasmic samples and nuclear fractions, and over 95
min in case of secretome analysis. The mass spectrometric analysis
was performed on a QExactive orbitrap mass spectrometer,
equipped with a nanospray ion source (Thermo Fisher Scientific),
coupled to the nano HPLC system. For detection, MS scans were
performed in the range from m/z 400—1400 at a resolution of 70,000
(at m/z = 200). MS/MS scans were performed choosing a top 12
method for cytoplasmic samples and nuclear fractions and a top 8
method for secretome analysis; HCD fragmentation was applied at
30% normalized collision energy and analysis in the orbitrap at a
resolution of 17,500 (at m/z = 200).

**Experimental Design and Statistical Rational**—For the investigation
of functional signatures, pairs of treated and untreated cells were
compared. Technical replicates provided a measure for the coeffi-
cient of variation introduced by the applied methodology. In addition,
independent cell experiments, here designated as biological replic-
ates, were performed. Furthermore, to assess potential effects of
different donors, in case of HUVEC, three individual donors were
investigated. Two independent cell experiments each of donor 1 and
2, and three independent cell experiments of donor 3 were performed,
thus resulting in seven biological replicates. Although quantitative
differences concerning the extent of regulation of individual proteins
between the donors were evident, the actually regulated proteins
were the same in all donors. Consequently, in case of NHDF, the
investigation of three biological replicates derived from one donor
was considered as adequate to assess regulatory effects in these
cells. Combining biological and technical replicates finally resulted in
a total of fourteen individual LC-MS/MS measurements in case of
HUVEC and six in case of NHDF. All replicates were used for statis-
tical analyses. The positive identification of a large number of known
inflammation players strongly supports the present strategy. Before
statistical evaluation, identified proteins were filtered for reversed
sequences, common contaminants and a minimum of three inde-
pendent experimental identifications in at least one cell type in a given
functional state.

**Peptide and Protein Identification**—Identification of proteins as well
as label-free quantification (LFQ) and statistical analyses were per-
formed using the MaxQuant 1.5.2.8 software including the Androm-
eda search engine and the Perseus statistical analysis package (19,
20), a commonly used workflow for processing and statistical assess-
ment of shotgun proteomics data. For statistical analysis, data ob-
tained from both biological and technical replicates were used. Fur-
thermore, the obtained data from the current study were combined with
data obtained from previous investigations on inflammatory ac-
tivated PBMCs. Proteins were identified using the UniProt database
for human proteins (version 102014 with 20,195 entries, restricted to
reviewed entries only), a peptide mass tolerance of 25 ppm, an
MS/MS match tolerance of 20 ppm and a maximum of two missed
cleavages with trypsin as protease. Search criteria further included
carbamidomethylation of cysteines as fixed modification, methionine
oxidation as well as N-terminal protein acetylation as variable modi-
fications, and a minimum of two peptide identifications per protein, at
least one of them unique. Furthermore, match between runs was
performed using a 5 min match time window and a 15 min alignment
time window. For both, peptides and proteins, a false discovery rate
(FDR) of less than 0.01 was applied; the FDR was determined by the
target-decoy approach. No additional filtering concerning the Ando-
meda score for accepting MS/MS identifications was recom-
manded by the MaxQuant software when applying a strict FDR. The
mass spectrometry-based proteomics data (including raw files, result
files and peak list files, peptide sequences, precursor charges, mass
to charge ratios, amino acid modifications, peptide identification
scores, protein accession numbers, number of distinct peptides as-
signed for each identified protein, percent coverage of each identified
protein in each individual experiment and annotated MS2 spectra
for each peptide spectrum match) have been deposited to the ProteomeXchange Consortium (21) via the PRIDE partner repository
with the data set identifier PXD003406 to PXD003417 (supplemen-
tal Table S9), accessible via www.proteomexchange.org. As Max-
Quant-derived data are not yet supported for complete submissions,
here we used Proteome Discoverer 1.4 running Mascot 2.5 and
Uniprot for human proteins (version 112015 with 20,193 entries, re-
stricted to reviewed entries only) as search engine. Actually all pro-
teins found to be regulated via MaxQuant were positively identified by
Proteome Discoverer as well. Finally, for selected proteins, heat maps
representing corresponding LFQ values determined in the respective
cell type and cell state, were generated. In case of NHDF, average
LFQ-values of the technical replicates were used. In case of HUVEC,
the average LFQ-values per donor were used, in this case averaging
both technical and biological replicates. Heat maps were generated
using an R script (22) based on the raw data obtained from MaxQuant
without any further data manipulation.

**Quantification**—Label-free quantification as described in the for-
mer paragraph resulted in LFQ values for each individual protein and
was used for quantitative assessment of protein regulation. LFQ
values were obtained for all proteins from all experiments (supple-
mental Tables S6, S7, and S8) and subjected to a comparative
analysis; the same initial protein amount of 20 μg used in all experi-
ments served for normalization. Isoforms of individual proteins were
summarized into protein groups by the Andromeda software and
were not further considered here. Mutual comparisons between un-
treated and inflammatory activated cells were performed to determine
protein groups that were significantly up- or downregulated upon
inflammatory activation in each cell type. To this end, using the Per-
seus statistical analysis package, differences of LFQ values were
 calculated. Changes in protein abundance values between untreated
and stimulated cells were determined by a two-sided t test with p <
0.05 and a minimum of a twofold abundance difference. All proteins
meeting these criteria were considered in the present study as po-
tentially contributing to the regulatory effects taking place during
inflammation. In addition, to emphasize the most robust regulatory
effects observed within one kind of cell, we determined significantly
regulated proteins with a global FDR<0.05 (indicated in Tables I–IV
and supplemental Tables S1–S5) as determined by a permutation-
based method, referring to Cox et al. and Tusher et al. (23, 24).

**RESULTS**

**Proteome Profiling of Inflammatory Activated NHDF and
HUVEC**—In this study, a systematic investigation of proteins
fulfilling important effector functionalities during inflammation
has been undertaken. To this end, in-depth proteome profiling data of inflammatory activated primary human fibroblasts (NHDF) and endothelial cells (HUVEC) were generated. Secreted, cytoplasmic and nuclear proteins were extracted from cells and analyzed by shotgun proteomics using a QExactive orbitrap mass spectrometer. Comprehensive proteome profiles were generated by combining all data related to one cell type and cell state. Identification of proteins as well as determination of LFQ-values as abundance measure and statistical analyses of proteins were performed using the MaxQuant and Perseus software (19, 25). Results obtained from previous investigations about inflammatory activated PBMCs (14) were included. As a result, a total of 8370 protein groups assembled from 117,599 distinct peptides was identified and semiquantitatively assessed. Comparative proteome profiling was performed to determine proteins that were regulated in the different cell types upon inflammatory activation. Accordingly, 667 proteins were up- or downregulated (p < 0.05) with a minimum of twofold change of LFQ values between control and inflammatory activated HUVEC (supplemental Table S1). Fig. 1A shows volcano plots representing the regulation of proteins in the cytoplasm, the nucleus and the secretome of inflammation activated PBMCs (14) were included. As a result, a total of 8370 protein groups assembled from 117,599 distinct peptides was identified and semiquantitatively assessed. Comparative proteome profiling was performed to determine proteins that were regulated in the different cell types upon inflammatory activation. Accordingly, 667 proteins were up- or downregulated (p < 0.05) with a minimum of twofold change of LFQ values between control and inflammatory activated HUVEC (supplemental Table S1). Fig. 1A shows volcano plots representing the regulation of proteins in the cytoplasm, the nucleus and the secretome of

Fig. 1. Regulation of proteins in HUVEC (A) and NHDF (B) upon inflammatory activation. Differences in LFQ values (logarithmic scale to the base of two) of proteins determined in activated versus control cells, including corresponding p values (logarithmic scale), are represented as volcano plots for each subcellular fraction. Proteins related to angiogenesis and/or ECM organization, as well as proteins related to the IFN response are highlighted as indicated. Proteins that were found to be regulated in the same subcellular fraction of the other cell type, respectively, are designated as well.
Hallmarks of Inflammation: Cell Type-specific Manifestation

Inflammation—

We first investigated these results with regard to common regulatory processes. Actually, we determined 26 proteins that were found to be regulated in all three kinds of cells upon inflammatory stimulation (Fig. 2). Among these, 19 proteins, listed in Table I, were consistently up-regulated. Those included well-known pro-inflammatory mediators such as prostaglandin G/H synthase 2 (COX-2), interleukins such as IL-6 and IL-8, and C-X-C motif chemokines such as CXCL1, CXCL2, and CXCL5. Furthermore, proteins involved in the innate immune response, such as complement C3, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and receptor-interacting serine/threonine-protein kinase 2 (26), as well as carboxypeptidase D, which is involved in antigen presentation, (27) were found to be up-regulated in HUVEC, 894 in NHDF and 646 in PBMCs.

In activated NHDF, 894 proteins were found to be at least twofold up- or downregulated (p < 0.05) when compared with controls (supplemental Table S2). Corresponding volcano plots are represented in Fig. 1B. In activated PBMCs, 646 proteins had been found to be regulated (supplemental Table S3).

Common and Cell Type-specific Protein Regulation During Inflammation—We first investigated these results with regard to common regulatory processes. Actually, we determined 26 proteins that were found to be regulated in all three kinds of cells upon inflammatory stimulation (Fig. 2). Among these, 19 proteins, listed in Table I, were consistently up-regulated. Those included well-known pro-inflammatory mediators such as prostaglandin G/H synthase 2 (COX-2), interleukins such as IL-6 and IL-8, and C-X-C motif chemokines such as CXCL1, CXCL2, and CXCL5. Furthermore, proteins involved in the innate immune response, such as complement C3, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and receptor-interacting serine/threonine-protein kinase 2 (26), as well as carboxypeptidase D, which is involved in antigen presentation, (27) were found to be up-regulated in all kinds of cells. CCAAT/enhancer-binding protein delta, an important pro-inflammatory transcriptional activator, as well as jun-B, a transcription factor that contributes to the expression of pro-inflammatory molecules such as IL-1β (28), were up-regulated likewise. Besides these molecules involved in pro-inflammatory signaling, an anti-inflammatory signaling molecule was also found to be up-regulated, namely normal mucosa of esophagus-specific gene 1 protein. This protein is an important regulator of inflammation, initiating a negative-feedback loop in which toll-like receptor stimulation induces microRNA-147 to prevent uncontrolled escalation of inflammation (29).

In a second step, we focused on cell type-specific regulatory processes occurring during inflammation. In general, stimulation of the canonical IL-1β pathway induces the expression of multiple pro- or anti-inflammatory genes capable of regulating the inflammatory response. This includes activation of positive or negative feedback mechanisms able to intensify or reduce the IL-1β response. One of the positive feedback mechanisms implicates the up-regulation of IL-1β, whereas up-regulation of e.g. interleukin-1 receptor antagonist protein (IL-1RA) is part of a negative feedback mechanism (30). Up-regulation of IL-1β and IL-1RA was, however, only observed by us in inflammatory activated NHDF and PBMCs, but not in activated HUVEC. Furthermore, the regulation of IL-1β and IL-1RA was different in NHDF as compared with PBMCs. Both, IL-1β and especially IL-1RA were up-regulated mainly in the cytoplasm of NHDF, whereas they were most abundantly up-regulated in the supernatant of PBMCs (Fig. 3A); these proteins seem to have different functionalities in fibroblasts and leukocytes, as discussed below. Actually, many other proteins were found to be regulated in a cell type-specific way as well. Indeed, 471 and 699 proteins were found to be selectively regulated in activated HUVEC and NHDF, respectively, whereas only 93 proteins were found to be regulated in a similar way in both, HUVEC and NHDF (Fig. 2; supplemental Tables S4 and S5). Proteins of each of these groups are listed in Table 1B–1D.

Importantly, the determination of proteins regulated in only one kind of cell allowed us to designate marker molecules that may be helpful to assess inflammatory processes occurring in vivo in a detailed fashion. In Table I, proteins are listed that may generally indicate inflammation, whereas the marker proteins listed in Table II may indicate more specifically the involvement of distinct cell types such as fibroblasts or endothelial in biological samples. We also classified these marker molecules into secreted proteins, membrane-bound proteins from the cell surface, as well as intracellular proteins to support the most appropriate detection strategy.

Because cell type-specific protein regulation may be the result of cell type-specific transcription factor activities, we subjected the proteins specifically regulated in HUVEC, NHDF or PBMCs to the oPOSSUM software (version 3.0); this supports detecting over-represented conserved transcription factor binding sites in the corresponding sets of genes (31). In this way, NfκB and AP1 were identified as most important transcription factors acting in all kinds of investigated cells. Nonetheless, they were obviously inducing different proteins in different cells. Fig. 4 illustrates the cell type-specific expression of some proteins targets of NfκB and AP1. Furthermore, some transcription factors were apparently only present or up-regulated in one kind of cell. So, T-cell acute
TABLE I
Proteins regulated in a common as well as a cell-type-specific way in HUVEC, NHDF and PBMCs. Proteins are listed which were at least twofold up- or down-regulated (p < 0.05) in all three kinds of cells upon inflammatory activation (A); proteins which were at least twofold up- or down-regulated (p < 0.05) in inflammatory activated stromal cells, but not in activated PBMCs (B); proteins which were at least twofold up- or down-regulated (p < 0.05) in inflammatory activated NHDF only (C); and proteins which were at least twofold up- or down-regulated (p < 0.05) in inflammatory activated PBMC only (D). Acc.Nr., UniProt accession number. Significant regulation with FDR < 0.05 in one (*), two (**) or three kinds of cells (***)

| Acc.Nr. | Name | Description |
|---------|------|-------------|
| P03872** | C-X-C motif chemokine 5; CXCL5 | Proinflammatory chemokine |
| P03912** | C-X-C motif chemokine 6; CXCL6 | Proinflammatory chemokine |
| P04088** | C-X-C motif chemokine 7; CXCL7 | Proinflammatory chemokine |
| P04238** | C-X-C motif chemokine 9; CXCL9 | Proinflammatory chemokine |
| P04239** | C-X-C motif chemokine 10; CXCL10 | Proinflammatory chemokine |
| P05121** | Interleukin-6; IL6 | Proinflammatory cytokine |
| P05455** | Interleukin-8; IL8 | Proinflammatory cytokine |
| P34890** | Nicotinamide phosphoribosyltransferase | NAD metabolism enzyme |
| P00043 | Normal mucosa of esophagus-specific gene 1 protein | Mucosal cell marker |
| P05100** | Plasminogen activator inhibitor 2; PAI-2 | Tissue plasminogen activator inhibitor |
| P13954** | Prostaglandin G/H synthase 2; COX-2 | Proinflammatory enzyme |
| P01875** | C-X-C motif chemokine 2; CXCL2 | Proinflammatory chemokine |
| P041687 | Gelatinase with angiogenic factor | Vascular endothelial growth factor |
| Q14355 | RPS6abkinase 4 | Translation elongation factor |
| P30102 | Complement C3 | Inflammation mediator |
| P03976 | Carboxypeptidase D | Carboxypeptidase activity |
| P14716 | CCAAT/enhancer-binding protein delta | Transcription factor |
| P15500 | C-C motif chemokine 2; CCCL2 | Chemokine |
| P00914 | Interferon-activated protein with tetracopeptide repeat 1; RTP1 | Interferon response |
| P10528 | Ras GTPase-activating protein 2 | Ras pathway regulator |
| P12725 | Transcription factor jumB | Transcription factor |

B. Proteins regulated in stromal cells, not in PBMCs

| Acc.Nr. | Name | Description |
|---------|------|-------------|
| P04121** | Collagen alpha-1(I) chain | Extracellular matrix protein |
| P01876** | C-X-C motif chemokine 3; CXCL3 | Proinflammatory chemokine |
| P03922** | C-X-C motif chemokine 7; CXCL7 | Proinflammatory chemokine |
| P04908** | Glutamine-fructose-6-phosphate aminotransferase [isomerase] 2 | Amino acid metabolism enzyme |
| P06053** | Nuclear factor-M kappa B; NFKB | Nuclear factor activity |
| P01876** | C-X-C motif chemokine 3; CXCL3 | Proinflammatory chemokine |
| P05111** | Plasminogen activator inhibitor 1; PAI-1 | Tissue plasminogen activator inhibitor |
| P04400** | Prolyl-endopeptidase; 3-sulfoglucuronate 5-dioxygenase 2 | Peptide metabolism enzyme |
| P02184** | Stromelysin-1; MMP3 | Matrix metalloproteinase |
| P40670** | Tissue factor pathway inhibitor | Coagulation factor |
| P01201** | Complement C1r/C1s | Complement component |
| P02495** | Transmembrane protein 132A | Transmembrane protein |
| Q33490** | Tumor necrosis factor alpha-induced protein 2 | Tumor necrosis factor |
| P28374** | Cathepsin K | Peptidase activity |
| Q11241 | CMP-N-acetylneuraminic-beta-galactosamide-alpha-2,6-sialyltransferase | Sialic acid metabolism enzyme |
| P02127** | Familiar heavy chain | Immunoglobulin chain |
| Q13695 | Fibronectin type III domain-containing protein 3B | Extracellular matrix protein |
| P24965** | Fibrillin-5 | Extracellular matrix protein |
| Q03950 | Growth differentiation factor 15 | Growth factor |
| Q13751 | Laminin subunit beta-3 | Collagen family protein |
| Q06290 | Lim and canpolin homology domains-containing protein | Lim domain protein |
| P04682* | Neurillin-2 | Neural adhesion molecule |
| P05047** | PDZ and LIM domain protein 4 | PDZ domain protein |

C. Proteins regulated in HUVEC only

| Acc.Nr. | Name | Description |
|---------|------|-------------|
| Q06128** | DO kDa heat shock protein, mitochondrial | Heat shock protein |
| D09468** | Agrp1 | Neuropeptide |
| D03103** | Alkaline phosphatase | Phosphatase enzyme |
| Q18221** | Angiotensin-converting enzyme | Angiotensin metabolism enzyme |
| O95236* | Apolipoprotein L3 | Lipid metabolism enzyme |
| Q09461** | ATF3 sythase subunit B-like protein | ATP synthase |
| P05055** | Basal cell adhesion molecule | Apoptosis regulator |
| P15907** | Beta-galactoside alpha-2,6-sialyltransferase 1 | Glycosylation enzymes |
| P31830** | Biglycan | Extracellular matrix protein |
| Q09358** | Carboxyl reductase [NADP] 3 | Oxidoreductase enzyme |
| P05957** | Collagen alpha-2(IV) chain | Extracellular matrix protein |
| P03767* | Complement C1r/C1s subcomponent | Complement component |
| P05071** | Complement C1r/C1s subcomponent | Complement component |
| Q09964** | Complement component C4a receptor | Complement receptor |
| P09725** | Complement factor B | Complement component |
| Q08178** | C-type lecin domain family 14 member A | C-type lectin domain |
| Q16531** | DNA damage-binding protein 1 | DNA damage binding protein |
| Q09465** | Endothelial lipase | Lipase enzyme |
| P01641** | E-selectin | Integrin-like molecule |
| Q14607** | Esp1 | Exocyst complex protein |
| Q09464** | Farnesyl-protein transferase | Protein prenylation enzyme |
| P15800** | Fatty acid-binding protein, adipocyte | Lipid metabolism enzyme |
| Q00089** | Heterogeneous nuclear ribonucleoprotein U | RNA binding protein |
| Q07514** | ICOS ligand | B-Cell activation molecule |
| Q01385** | Interleukin-1 receptor-like 1; IL1R1 | Toll-like receptor |
| Q13478** | Interleukin-18 receptor; IL1B1R | Interleukin receptor |
| Q16362** | Laminin subunit alpha-4 | Laminin family protein |
| Q17212** | Laminin subunit beta-1 | Laminin family protein |
| P10447** | Laminin subunit gamma-1 | Laminin family protein |
| Q11757** | Laminin subunit gamma-2 | Laminin family protein |
| Q0950** | Microtubule-associated proteins 1A/1B light chain 3 | Tubulin binding protein |
| Q0950** | Microtubule-associated proteins 1A/1B light chain 3 | Tubulin binding protein |
| Q13599** | Zinc transporter ZIP14 | Zinc homeostasis enzyme |
lymphocytic leukemia protein 1 (TAL1) was specifically expressed in HUVEC (Fig. 4). Several proteins found to be regulated exclusively in activated HUVEC were effectively targets of TAL1, including for example laminin subunit gamma-2 (LAMC2) and neuronal cell adhesion molecule (NRCAM) (Fig. 4). Several of the proteins found to be regulated selectively in activated NHDF turned out to be targets of paired mesoderm homeobox protein 2 (PRRX2), such as for example leukemia inhibition factor (LIF) and thrombospondin-2 (TSP2) (Fig. 4). We were able to detect PRRX2 in NHDF, but also in HUVEC. Interestingly, PRRX1 was up-regulated in inflammatory activated NHDF, but not in activated HUVEC or PBMCs (Fig. 4); it is possible that this transcription factor may target similar proteins as PRRX2. One of the known targets of PRRX1 and PRRX2, tenasin (TNC), was determined at high levels in NHDF, even though this protein was up-regulated in inflammatory activated HUVEC likewise (Fig. 4). In conclusion, we observed both cell type-specific expression patterns of transcription factors as well as cell type-specific target gene expression.

**Biological Processes Activated During Inflammation**—The fact that each kind of inflammatory activated cell type was regulating a very specific set of proteins further raised the question whether the cells were involved in different biological processes during inflammation. To this end, we submitted all proteins found to be regulated in activated NHDF, HUVEC or PBMCs, to the DAVID Functional Annotation Tool for biological processes (32, 33). This determined the most significantly represented biological processes regulated in the cells upon inflammatory activation. Apparently, several common processes were induced in all three kinds of cells during inflammation, namely (1) innate immune response, (2) cell adhesion and migration, (3) cell proliferation and differentiation, and (4) response to oxidative stress. However, execution of these processes was apparently achieved in a cell type-specific way. Indeed, each cell type was up- or downregulating differ-
ent proteins related to these processes (supplemental Tables S4 and S5), as exemplified for several proteins in Fig. 5. Interestingly, in activated NHDF, even though several proteins related to the innate immune response were up-regulated, hardly any interferon (IFN) response was observed upon inflammatory activation. Only IFIT1 was slightly up-regulated in these cells, whereas in inflammatory activated PBMCs and HUVEC, several IFN-responsive gene products, such as interferon-induced GTP-binding protein Mx1 (Mx1), interferon-induced proteins with tetratricopeptide repeats (IFITs) and guanylate-binding proteins (GBPs) were strongly up-regulated (Fig. 3B; Table III).

Actually, two specific inflammation-related processes were evident only in fibroblasts and endothelial cells, designated here as stromal cells, namely angiogenesis and reorganization of the extracellular matrix (ECM). Table IV and Table B list regulatory and effector molecules involved in angiogenesis. Most of those were classified by applying the DAVID Functional Annotation Tool. This accounts for example for vascular endothelial growth factor C (VEGF-C), and transforming growth factor β2 (TGFβ2). Other proteins were described in literature to be involved in angiogenesis, such as for example cathepsin S. Ward et al. have shown that antibody-based blocking of cathepsin S leads to inhibition of angiogenesis (34). Plasminogen activator inhibitor 1 is an important regulatory factor of angiogenesis, which is able to both promote and inhibit angiogenesis (35, 36). Neuronal cell adhesion molecule and transforming growth factor β3-induced protein ig-h3 are involved in angiogenesis as well, as demonstrated by Aitkenhead et al. (37). Concerning proteins involved in ECM reorganization, the situation was similar. Several regulator and effector molecules related to this biological process were found significantly up- or downregulated in HUVEC and/or NHDF upon inflammatory activation (Table 4B and 4C). Proteins were again selected using both, the DAVID Functional Annotation Tool (32, 33) as well as relevant information from literature. Finally, we also determined processes that were only accomplished by inflammatory activated fibroblasts, processes related to monosaccharide metabolism and energy generation. Proteins involved in these processes are indicated in supplemental Table S6.

**Hallmarks of Inflammation—** Through the comparative analysis of inflammatory activated endothelial cells, fibroblasts and leukocytes we were thus able to determine in a comprehensive way proteins fulfilling effector functionalities during inflammation as well as corresponding inflammation-related processes. This motivated us to define hallmarks of inflammation representing the most apparent biological processes occurring during inflammation, and to specify responsibilities of fibroblasts, endothelial cells and leukocytes therein (Fig. 6).
We included (1) common processes realized by stromal cells and leukocytes, namely the innate immune response, cell adhesion and migration, cell proliferation and differentiation, as well as response to oxidative stress; (2) processes specifically executed by stromal cells, namely angiogenesis and reorganization of the ECM; (3) processes fulfilled only by fibroblasts related to monosaccharide metabolism; and (4) a process well-known to be only accomplished by leukocytes, the acquired immune response.

DISCUSSION

This study focused on inflammation-related proteins in primary human stromal cells, taking NHDF and HUVEC to represent fibroblasts and endothelial cells, respectively. Previously published data on inflammatory stimulated primary human peripheral blood mononuclear cells (PBMCs) were also considered for data interpretation. Comparative proteome profiling revealed 19 proteins that were up-regulated in a similar fashion upon inflammatory activation in all three kinds of cells (Table 1A). Obviously, pro-inflammatory agonists such as IL-1β in case of fibroblasts and endothelial cells or LPS/PHA in case of PBMCs can thus trigger similar responses in different cell types. Generally, a pro-inflammatory signal sensed on the cell surface initiates an intracellular signaling cascade, the consequent activation of transcription factors such as AP1 and NFκB and finally the expression of specific genes. Many of the common up-regulated proteins were actually NFκB and AP1 target gene products, such as for example IL-6 and COX-2. Besides these common effects, cells were also regulating proteins in a cell type-specific way (Fig. 5). The present data demonstrate that each cell type is actually contributing to inflammation in a cell type-specific way by activating its specific repertoire of proteins.

Our observations concerning the expression of the canonical inflammation inducer IL-1β itself in the different kinds of cells indeed point to complex cell type-specific regulatory mechanisms. First, up-regulation of IL-1β was only observed in activated NHDF and PBMCs, but not in HUVEC (Fig. 3A). Secondly, mechanisms controlling the functionality of IL-1β were apparently different in NHDF and PBMCs. Activated PBMCs abundantly secreted IL-1β, obviously with the aim to activate surrounding cells and reinforce the inflammatory process. However, an up-regulation and secretion of interleukin 1 receptor antagonist (IL-1RA) was observed in parallel. Although IL-1β binds to interleukin receptor 1 to induce pro-inflammatory signaling in the cell, IL-1RA competes with IL-1β, acting as an anti-inflammatory effector that counter-regulates the pro-inflammatory cascade. The ratio of IL-1β to IL-1RA determines whether the final signal becomes pro- or anti-inflammatory (38). In activated NHDF, most of the up-regulated IL-1β was found retained inside the cells and, interestingly, IL-1RA was up-regulated only in the cytoplasm.

![Figure 4. Cell type-specific regulation of proteins during inflammation.](image-url)
Inflammation-related processes realized by all cells, however, by upregulating different proteins. Heat maps of LFQ values for proteins involved in the innate immune response, cell adhesion and migration, cell proliferation and differentiation as well as response to oxidative stress are represented.
but not secreted (Fig. 3A), producing only the intracellular form of IL-1RA (39). It thus might be that IL-1β, together with IL-1RA, serves as an emergency reserve inside inflammatory activated fibroblasts. In case cells undergo cell death, dying fibroblasts would thus be able to release large amounts of IL-1β, sending rapidly an alert signal and leading to the inflammatory activation of surrounding cells. The simultaneous release of IL-1RA may serve to fine-tune the potent pro-inflammatory signaling. In activated HUVEC, themselves inducing no IL-1β at all, the IL-1β antagonist transforming growth factor β-2 (TGFβ2) (40), already abundant in untreated cells, was found up-regulated (Fig. 3A). Physiologically, TGFβ2 together with a continuous and prolonged provision of endothelial cells with IL-1β—rather derived from other cells—is capable of inducing endothelial to mesenchymal transition (EndMT) at later stages of inflammation, during resolution of inflammation and transition to the remodeling phase (40). Downregulation of endothelial markers, such as endothelial nitric oxide synthase and von Willebrand factor, accompanies ongoing EndMT and was actually observed in our experiments (supplemental Table S1). Most importantly, EndMT is capable of promoting ath...
erosclerosis and strongly correlates with the extent of atherosclerosis (41).

Furthermore, significant differences were also observed concerning the IFN response of cells (Table III; Fig. 3B; Fig. 1). Actually, in our experiments, activated HUVEC and PBMCs but not NHDF were readily upregulating several IFN response-related proteins. This observation is supported by data published from Indraccolo et al. with regard to several genes selectively induced by IFNs in endothelial cells but not in fibroblasts (42). Some of the corresponding gene products, such as CXCL10, apolipoprotein L3 and IFN-induced protein 44 were found by us to be induced in activated HUVEC, but...
not in NHDF. One possible explanation for this apparent cell type-specific difference could rely on specific Ras/MEK signaling states. Battcock et al. have actually demonstrated that type I IFN cannot establish antiviral states in cells with activated Ras/MEK (43). In HUVEC, the Ras antagonist Ras suppressor protein 1 was found to be 3.5-fold up-regulated upon inflammatory activation, whereas in NHDF this protein was found more than sevenfold downregulated (supplemental Tables S1 and S2). Furthermore, we found dual specificity mitogen-activated protein kinase kinase 1 and 2 (MEK1 and MEK2) to be up-regulated in activated fibroblasts, whereas MEK3 was downregulated in activated HUVEC (supplemental Tables S1 and S2).

These data clearly demonstrated cell type-specific regulatory mechanisms of inflammatory processes. Intriguingly several of the specifically induced proteins are targets of the common transcription factors NFκB or AP1 as exemplified in Fig. 4. This points to cell type-specific accessibility of chromatin for these transcription factors as known to account for other cell type-specific gene expression patterns (44). Besides, a cell type-specific inflammatory response might also be regulated in dependence of other, rather cell-type specific transcription factors. Actually, we were able to detect specific transcription factors such as TAL1 in HUVEC and PRRX1 in NHDF (Fig. 4). TAL1 may be responsible for the specific induction of LAMC2 and NRCAM in activated HUVEC. PRRX1 may cause the induction of its target TNC as well as the PRRX2 target genes LIF and TSP2 in activated NHDF, as PRRX1 and PRRX2 have been assigned similar functionalities in mesenchymal cells during vasculogenesis (45).

The determination of cell type- and cell state-specific proteins also allowed us to define marker molecules. The identification of pan-markers listed in Table I in complex samples such as blood or tissues could serve for the general indication of inflammation without providing information regarding the cell type of origin. In contrast, the identification of cell type-specific inflammation markers as listed in Table II would indicate inflammatory activation of fibroblasts or endothelial cells, respectively. This information may elucidate more details regarding the patho-mechanism relevant for the investigated samples. Importantly, such information may become accessible with different kinds of analysis methods including antibody-based technologies or targeted proteomics. In addition, different categories of markers were defined here that can be used for different applications (Table II): (1) blood-borne markers, i.e. proteins that are secreted by cells in the extracellular space but which do not bind to the extracellular matrix; those may be indicative for a specific inflammatory activated cell type in blood samples, using for example ELISA; (2) membrane-bound proteins from the cell surface, which can be used for FACS analyses; (3) intracellular proteins that can be used for immunohistochemistry or immunofluorescence.
Quantitative monitoring of such markers in clinical samples or appropriate model systems (46) may also help to reveal changes in the functional state of cells in response to specific treatment and, consequently, support the evaluation of specific drug effects.

Another important aim was to determine functionalities related to inflammation, which may be specific for stromal cells. Our data demonstrated that fibroblasts and endothelial cells are both involved in the regulation of angiogenesis and ECM reorganization. The relatively large number of involved proteins suggests complex regulation and fine-tuning of these important processes (Table IV; Fig. 1). Activation of specific factors such as IL-8 and IL-1β may lead to a proangiogenic signaling and the initiation of angiogenesis (47, 48). After this initial phase, other molecules such as matrix metalloproteinases (MMPs) may support the ongoing process by degrading ECM and releasing ECM-bound proangiogenic growth factors (49). Degradation of basement membranes is required to release endothelial cells into the surrounding matrix, growth factors such as VEGF and TGFβ2 supports the formation of new vessels via proliferation (50). Finally, regulatory mechanisms control undesired escalation of angiogenesis. Activation of antiangiogenic factors such as thrombospondin-1 (TSP1) and TSP2, or downregulation of proangiogenic factors such as angiostatin-converting enzyme or c-type lectin domain family 14 member A are involved here (51–54). ECM reorganization is strongly interlinked with angiogenesis and related proteins were found regulated in both cell types as well. Biglycan is a structural component of the ECM but also contributes to blood vessel remodeling, apparently being able to upregulate the expression of VEGF and thus promoting angiogenesis (55). Indeed, this protein, when proteolytically released from the ECM, acts as a danger signal stimulating pro-inflammatory signaling and activating the inflammasome (56). Other components of the ECM, for example laminins, are also important regulators of inflammation and angiogenesis. These proteins are necessary for the recruitment of immune cells to inflammatory loci (57, 58). Interestingly, laminin 8 (laminin α-4β-1γ-1), actually observed to be regulated in HUVEC, is involved in the development of inflammatory lesions of the blood brain barrier (59).

Hyaluronan, a glycosaminoglycan of the ECM plays also an important role for the recruitment of immune cells. This molecule is regulated by other components of the ECM such as TSP1 and tumor necrosis factor-inducible gene 6 protein (TSG6) (60). These two proteins were presently observed to be up-regulated in stimulated NHDF, indicating that fibroblasts may be involved in regulating the recruitment of leukocytes by endothelial cells during inflammation, which was also observed by McGGettrick et al. (61). Such interrelations between angiogenesis and ECM reorganization as well as between fibroblasts and endothelial cells are further demonstrated by the interrelation of two important proteins mentioned before, PRRX1 and TNC. The transcription factor PRRX1 regulates vascular development and angiogenesis (62, 63). The PRRX1 target gene product TNC, an ECM glycoprotein, was induced both in fibroblasts and endothelial cells upon inflammatory activation (Fig. 4). Tenascin apparently is required for PRRX1-dependent vascularization (64) and regulates angiogenesis during tumor development (65). On the other hand, TNC is also involved in promoting migration of fibroblasts to induce tissue rebuilding in response to injury (66).

Fibroblasts and endothelial cells are thus involved in similar processes during inflammation, each of them fulfilling specific functionalities therein. However, processes related to monosaccharide metabolism and energy generation were found to be specifically regulated during inflammation in fibroblasts. Several proteins related to glycolysis, such as hexokinase 2, triosephosphate isomerase and pyruvate kinase, were found to be up-regulated only in these cells, and, similarly, proteins related to the pentose phosphate pathway, such as 6-phosphogluconolactonase and transaldolase (supplemental Table S5). L-lactate dehydrogenase was found to be up-regulated as well, delivering lactate as end product of glycolysis in these cells. Up-regulation of glycolysis and production of lactate, here in the context of inflammation, has also been described to occur in cancer-supporting fibroblasts. These cells have been described to supply cancer cells via autophagy and glycolysis, producing high amounts of lactate as energy-rich fuel delivered in a paracrine fashion to cancer cells (67). Also regulators of autophagy, such as lysosome-associated membrane glycoprotein 2 (68) and ras-related protein Rab-7a (69), were found by us to be up-regulated in inflammatory activated fibroblasts (supplemental Table S5). Considering these aspects, it is not difficult to imagine how inflammation-related processes—when out of control—may contribute to cancer development or other diseases related to chronic inflammation. Especially stromal cells may be critically involved in cancer development as these cells are responsible for processes such as angiogenesis, ECM reorganization and energy supply.

We have chosen suitable model systems representative for fibroblasts and endothelial cells displaying relevant cell functions such as ECM remodeling in fibroblasts, and angiogenesis in endothelial cells. Although fibroblasts and endothelial cells derived from other tissues may display slightly different expression patterns (11), the main cell type specific characteristics will be independent from the tissue type of origin. Furthermore, despite a large number of proteins regulated upon inflammatory activation was identified they were clearly related to a rather small number of biological functions. As these biological functions represent the most important activities known to occur during inflammation, hallmarks of inflammation emerged (Fig. 6). The consideration of these hallmarks may support our understanding of complex processes occurring during inflammation and related diseases.
Conclusion and Outlook—In this study, the most important players executing complex biological processes involved in the exertion of inflammation were identified. We determined proteins regulated in inflammatory activated endothelial cells, fibroblasts and leukocytes, and elucidated mechanisms that may contribute to the observed cell type-specific regulatory effects. The present results shall contribute to a better understanding of the processes occurring during acute and chronic inflammation and may thus support the development of new therapeutic strategies to combat chronic inflammation and related diseases. The presented marker molecules may serve to accomplish such tasks by the specific detection of inflammatory activated cells in clinical samples. Furthermore, monitoring the levels of such marker proteins may also support the evaluation of drug effects.

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**Hallmarks of Inflammation: Cell Type-specific Manifestation**

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