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Cytoplasmic Proteome and Secretome Profiles of Differently Stimulated Human Dendritic Cells

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Dendritic cells (DCs), the most potent and specialized antigen-presenting cells, play a key role in the regulation of the adaptive immunity. Immature DCs were generated by *in vitro* culturing of peripheral blood monocytes and functionally activated with the classical pathogen-associated molecular pattern lipopolysaccharide (LPS). Alternative activation resulting in Th-2 polarization was induced with lipid oxidation products derived from 1-palmitoyl-2-arachidoyl-*sn*-glycerol-3-phosphorylcholin (OxPAPC). Tolerogenic cells were obtained by treating DCs with human rhinovirus (HRV). The aim of this study was the identification of proteome profiles related to the functionally different dendritic cell phenotypes. Cytoplasmic proteins were analyzed by shotgun proteomics resulting in the identification of 1690 proteins. While mature and alternatively activated DCs displayed highly distinct protein expression profiles, HRV-treated DCs showed minor proteome alterations. As DCs exert many specific functions via secretion, we investigated the secretomes by a combination of 2D-PAGE and shotgun proteomics. We successfully identified a broad variety of cytokines (e.g., GM-CSF, TNF-alpha, interleukin-1beta, 6, 12 beta, 28B and 29), chemokines (e.g., CCL3, 5, 8, 17, 18, 19, 24, CXCL1, 2, 9 and 10) and growth factors (growth/differentiation factor 8, C-type lectin domain family 11 member A). The relative composition of secretome profiles, although comprising much less proteins, was found to be much more affected by functional alteration of cells than the cytoplasmic protein composition. In conclusion, we demonstrate that functional distinct subsets of DCs display distinct proteome profiles which comprise biomarker candidates. These proteins may prove useful for the interpretation of complex clinical proteomics data.

Keywords: 2D-PAGE • shotgun • LC−MS/MS • dendritic cell • secretome, proteome

Introduction

Mass spectrometry-based protein identification has enabled in-depth analysis of biological samples for biomedical research.[1] The identification of biomarkers and thereby improvement of our understanding of patho-physiological mechanisms belong to the most obvious aims of this research.[2] While a large number of clinical proteome studies rely on the comparison of samples related to healthy and disease states, we here envisage another research strategy. Many kinds of disease-related protein alterations may be attributed to altered cell states, which may be causally related to the corresponding disease. The identification of marker proteins indicative for characteristic cell states by the use of appropriate *in vitro* model systems shall aid the interpretation of complex proteome profiling data derived from clinical samples.

Many diseases are caused or accompanied by acute and/or chronic inflammation. Dendritic cells play a key role in the adaptive immune system and are main regulators of inflammation. Immature DCs are the most specialized antigen-presenting cells (APCs), recognizing and responding to invading pathogens with the help of their pattern recognition receptors (PRRs) which interact with so-called pathogen-associated molecular patterns (PAMPs) that are present on microbiological surfaces.[3,4] During their migration to T cell areas, immature DCs lose their ability to identify and target other invaders and as mature DCs they are able to initiate T cell- and consequently immune response.[5−7] Actually, mononuclear phagocytes exhibit a tremendous functional diversity.[8] They may promote the orientation of adaptive responses in a type I or type II direction and may display specialized and polarized effector functions.[9,10] While mature (type I or M1) DCs induce Th-1

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responses causing type I inflammation initiating, for example, killing of intracellular pathogens, alternatively activated (type II or M2) DCs induce Th-2 responses causing type II inflammation rather related to allergy, immunoregulation, killing of parasites and tumor promotion.[10] Furthermore, DCs treated with human rhinovirus may induce a deep anergic state in T cells.[11]

It was the aim of this study to assess proteome profiles of human DCs at different functional states simulated by appropriate *in vitro* models. [12] Studies demonstrated that *in vitro* culturing of peripheral blood monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) provides an optimal source to generate immature DCs.[3,13−15] Starting from those, we generated mature DCs by the treatment with lipopolysaccharide (LPS). Alternatively activated DCs were generated by the treatment of immature DCs with oxidized phospholipids 1-palmitoyl-2-arachidoyl-*sn*-glycerol-3-phosphorylcholin (OxPAPCs).[4] Generally, OxPAPCs result from the defense of the innate immune system against invading microbes[16,17] and were detected in different types of cells and tissues, for example in apoptotic cells,[18] inflamed tissues, and oxidized low density lipoproteins.[17,19] In addition, other studies showed that OxPAPCs regulate DC activation by preventing LPS-activation of DCs via TLR4 and TLR3, so they limit pathogen induced activation and maturation of DCs[4] similar to immature DCs.

DCs inducing the formation of tolerogenic T cells were generated by the treatment of immature DCs with human rhinovirus-14, a member of the major group HRV family. HRV is the major elicitor of upper respiratory tract infections and of the common cold.[20] Studies proved the fact that HRVs play a key role in predisposing or causing otitis media, sinusitis, exacerbations of asthma and other lower respiratory tract disorders.[21] Infection with human rhinovirus appears to be limited to ciliated epithelial cells of the upper respiratory tract.[22] Despite the recruitment of immature dendritic cells, the adequate immune response seems to be inhibited or deregulated in the respiratory tract upon HRV infection.[23] HRV-14 inhibits antigen-specific T cell responses by inducing IL-10, which is involved in terminating inflammatory reactions.[23] In addition, it has been demonstrated that immature dendritic cells stimulated with HRV-14 induce the expression of accessory molecules on the surface of DCs, namely the expression of the inhibitory receptor B7−H1 and sialoadhesin, rendering cocultured T cells tolerogenic.[11,23]

Until now, dendritic cells have been analyzed by proteome profiling, primarily by employing 2D-PAGE, whereas the main focus was the detection of changes in the proteome profile during the maturation process from monocytes to dendritic cells.[14,24−26] The high abundance of antioxidant proteins in DCs was described by Rivollier et al.[26] By using proteome profiling methods, a "limited maturation" phenotype of Th2-inducing DCs generated with helminth antigens was previously described.[27] Comparison of proteome profiles of monocyte-derived dendritic cells with KG-1 cells, a myeloic cell line, was performed by Horlock et al.[28]

For the generation of proteome profiles, we analyzed subcellular fractions rather than total cell lysates to improve reproducibility and reliability.[29] Here, we focused on cytoplasmic and secreted proteins. To get access to low abundant proteins, we mainly employed shotgun analysis. Secreted proteins of activated dendritic cells were investigated by Chevallet et al.[30] Extending the existing literature with respect to protein identifications, here we realized a comprehensive comparative study of four different dendritic cell states including cytoplasm and secretome. Interpretation of data was supported by the database of the Clinical Proteomics Laboratories at the Medical University of Vienna (CPL/MUW-database).[31] The reliability and specificity of our data was supported by the independent reconfirmation of established cytokine and chemokine profiles of human DCs.[10] The characteristic protein expression profiles presented in this study suggest the existence of several marker proteins potentially indicative for the presently investigated functional states.

Experimental Section

Antibodies and Reagents. The following murine mononuclear antibodies were kindly provided by O. Majdic, Institute of Immunology, Vienna: VIM13 (CD14), mouse antihuman MxA (clone 383/7D4).

Rhinovirus Preparation and Purification. HRV-14 was obtained from ATCC and routinely grown in suspension cultures of HeLa cells (strain Ohio; Flow Laboratories). Preparation and purification of rhinoviruses were performed as described.[23] Working dilutions of used HRV-14 stock preparation contained 10 pg LPS/mL.

Monocyte-Derived DC Preparation and Stimulation. PBMCs were isolated from heparinized whole blood of four different healthy donors by standard density gradient centrifugation with Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Subsequently, T cells and monocytes were separated by magnetic sorting using the MACS technique (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.[32] Monocytes were enriched by using the biotinylated CD14 mAb VIM13 (purity 95%) as previously described.[32] DC were generated by culturing purified blood monocytes for 7 days with a combination of GM-CSF (50 ng/mL) and IL-4 (100 U/mL). Maturation of DCs was induced by adding 100 ng/mL LPS from *Escherichia coli* (serotype 0127-B8, Sigma Chemie) for 24 h. For lipid treatment, DCs were treated with 50 μg/mL OxPAPC for 24 h as previously described[4] and for HRV treatment, HRV-14 was added for 48 h at a titer of 1 TCID50 (50% tissue culture infectious dose) per cell.[11] For the isolation of protein fractions scheduled for subsequent shotgun analysis, the cells were washed after treatment and further incubated in serum-free AIM-V Medium supplemented with L-glutamine for 6 h at 37 °C. For metabolic labeling, cells were washed after treatment and incubated in serum-free, methionine- and cysteine-free RPMI-1640 medium (MP Biomedicals, Solon, Ohio) supplemented with L-glutamine in the presence of ^{35}S-labeled methionine and cysteine (Trans35label, Biomedica, MP Biomedicals) for 4 h at 37 °C.

Assessment of Cytokine Production. Supernatants of DCs were analyzed for production of TNF alpha by sandwich ELISA using matched-pair Abs (R&D Systems, Minneapolis, MN). The lower detection limit was 20 pg/mL.

FACS Analysis. For MxA cytoplasmic staining DC were harvested, fixed with fixation solution (An der Grub, Kaumberg, Austria) for 20 min, washed twice with PBS, and permeabilized for 20 min with permeabilization solution (An der Grub) in the presence of anti-MxA antibody. After washing twice Oregon Green-conjugated goat antimouse-Ig (Molecular Probes Inc., Eugene, OR) was used as second step reagent.

Unlabled Sample Preparation. The DCs were routinely cultivated in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Cells in the density of 1×10^6 cells/mL were

incubated in RPMI-1640 medium supplemented with L-glutamine for 6 h at 37 °C.

^{35}S-Metabolic Labeling. The DCs were routinely cultivated in RPMI-1640 supplemented with 10% fetal calf serum (FCS). Cells in the density of 1×10^6 cells/mL were incubated in serum-free, methionine- and cysteine-free RPMI-1640 medium (MP Biomedicals, LLC) supplemented with L-glutamine in the presence of ^{35}S-labeled methionine and cysteine (Trans35label, Biomedica, MP Biomedicals) for 6 h at 37 °C.

Subcellular Fractionation. For isolation of the secreted protein fraction, the cell supernatant was collected, sterile filtrated (0.2 μm, FP POINT 2-S, Schleicher & Schuell, Whatman, London, Great Britain) to remove cellular debris and precipitated by the addition of ethanol as previously described.[29] For the isolation of cytoplasmic proteins, all buffers were supplemented with protease inhibitors: PMSF (1 mM), aprotinin, leupeptin and pepstatin A (each at 1 μg/mL). Cells were lysed in hypotonic lysis buffer (10 mM HEPES/NaOH, pH 7.4, 0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 0,5% Triton X-100) and pressed through a 26 g syringe to open the cells by rupture. The cytoplasmic fraction was separated from the nuclei by centrifugation and precipitated by the addition of ethanol. All protein samples were dissolved in sample buffer (7.5 M urea, 1.5 M thiourea, 4% CHAPS, 0.05% SDS, 100 mM DDT).

2D Polyacrylamid Gel Electrophoresis (2D-PAGE). Proteins were loaded by passive rehydration of IPG strips pH 5−8, 17 cm (BioRad, Hercules, CA) at room temperature. IEF was performed in a stepwise fashion (1 h 0− 500 V linear; 5 h 500 V; 5 h 500−3500 V linear; 12 h 3500 V). After IEF, the strips were equilibrated with 100 mM DTT and 2.5% iodacetamide according to the instructions of the manufacturer (BioRad). For SDS-PAGE using the Protean II xi electrophoresis system (BioRad), the IPG strips were placed on top of 1.5 mm 12% polyacrylamide slab gels and overlaid with 0.5% low-melting agarose. The gels were stained with a 400 nM solution of Ruthenium II tris (bathophenanthroline disulfonate) (RuBPS) as described.[33] Fluorography scanning was performed with the FluorImager 595 (GE Healthcare, Fairfield, CT) at a resolution of 100 μm. After scanning, the radioactively labeled gels were dried using the slab gel dryer SE110 (Hoefer, San Francisco, CA). After exposure to phosphor screens (Molecular Dynamics), the screens were scanned using the Phosphorimager SI (Molecular Dynamics) at a resolution of 100 μm as previously described.[34] All 2D gel data were independently reproduced for at least four times.

1D-PAGE for Subsequent Shotgun Analysis. Cytoplasmic protein fractions were loaded on 12% polyacrylamid gels, electrophoresis was performed until complete separation of a prestained molecular marker (Dual Color, Biorad, Hercules, CA) was visible. Gels were fixed with 50% methanol/10% acetic acid and subsequently silver stained as described below. Gel lanes were cut out of the gel and digested with trypsin as described below.

MS-Compatible Silver Staining Procedure. Gels were fixed with 50% methanol, washed and sensitized with 0.02% $Na_2S_2O_3$. The gels were stained with 0.1% $AgNO_3$ ice cold for 20 min, rinsed with bidistillated water and subsequently developed with 3% Na_2CO_3/0.05% formaldehyde as previously described.[35]

Tryptic Digest. Protein lanes were devided into slices and were cut out of the gel. The gel-pieces were destained with 15 mM $K_3Fe(CN)_6$/50 mM $Na_2S_2O_3$ and intensively washed with 50% methanol/10% acetic acid. The pH was adjusted with 50 mM NH_4HCO_3, and proteins were reduced with 10 mM DTT/50 mM NH_4HCO_3 for 30 min at 56 °C and alkylated with 50 mM iodacetamide/50 mM NH_4HCO_3 20 min in the dark. Afterward the gel-pieces were treated with acetonitrile (ACN) and dried in a vacuum centrifuge. Between each step, the tubes were shaken 5−10 min (Eppendorf thermomixer Comfort). Dry gel-spots were treated with trypsin 0.1 mg/mL (Trypsin sequencing grade, Roche Diagnostics, Germany)/50 mM NH_4HCO_3, for 20 min on ice, afterward covered with 50 mM NH_4HCO_3 and were subsequently stored overnight at 37 °C. The digested peptides were eluted by adding 50 mM NH_4HCO_3, the supernatant was transferred into silicon coated tubes, and this procedure was repeated two times with 5% formic acid/50% acetonitrile. Between each elution step the gel-spots were ultrasonicated for 10 min. Finally the peptide solution was concentrated in a vacuum centrifuge to an appropriate volume.

Mass Spectrometry Analysis. Peptides were separated by nanoflow LC (1100 Series LC system, Agilent, Palo Alto, CA) using the HPLC-Chip technology (Agilent) equipped with a 40 nl Zorbax 300SB-C18 trapping column and a 75 μm × 150 mm Zorbax 300SB-C18 separation column at a flow rate of 400 nL/min, using a gradient from 0.2% formic acid and 3% ACN to 0.2% formic acid and 50% ACN over 60 min. Peptide identification was accomplished by MS/MS fragmentation analysis with an iontrap mass spectrometer (XCT-Ultra, Agilent) equipped with an orthogonal nanospray ion source. The MS/MS data, including peaklist-generation and search engine, were interpreted by the Spectrum Mill MS Proteomics Workbench software (Version A.03.03, Agilent) allowing for two missed cleavages and searched against the SwissProt Database for human proteins (Version 20080409 containing 19038 entries or, alternatively, version 14.3 containing 20328 entries) allowing for precursor mass deviation of 1.5 Da, a product mass tolerance of 0.7 Da and a minimum matched peak intensity (%SPI) of 70%. Due to previous chemical modification, carbamidomethylation of cysteines was set as fixed modification. No other modifications were considered here. The listed peptides were identified with the indicated scores. The scores were essentially calculated from sequence tag lengths, but also consider mass deviations. To assess the reliability of the peptide scores, we performed searches against the corresponding reverse database; 5.9% positive hits were found with peptides scoring >9.0, whereas 0.21% positive hits were found with peptides scoring >13.0. Consequently, we set the threshold for protein identification to at least one peptide scoring higher than 13.0. To eliminate redundancy in the case of peptides matching to multiple members of a protein family, a selection procedure was applied. In all cases, the most plausible protein was selected out of a list of potential candidates considering gene expression profiles and other established data of the candidate proteins. If several proteins were of similar plausibility, the first entry of the isoforms in question was chosen (one before two, A before B etc.) as described by Zhang et al.[36] Proteins not fulfilling these criteria were negatively selected.

Data Assembling and Interpretation. Mass data were organized by a homemade SQL-database. Reproducibility of peptide identifications was higher than 90% in case of independent samples from the same donor and around 80% in case of similar samples from different donors. In order to avoid misinterpretation due to variations derived from individual differences and some random priorisation of peptides in the mass spectrometer, we summarized the data of four independent experiments with protein samples from four different donors. Proteins displaying amino acid sequences only, which

also occur in bovine, were deleted. From all experiments per condition investigated, the best peptide scores were chosen. These summaries were considered as reference proteome maps (Table S1, Supporting Information). Amino acid sequences of candidate secreted proteins were searched for signal sequences using the signalP 3.0 algorithm (www.cbs.dtu.dk/services/SignalP/).[37]

All proteome analysis data presented in the following, including MS^2 peptide fragmentation spectra of all identified peptides, are accessible via PRIDE database (http://www.ebi.ac.uk/pride/),[38,39] accessions 3381−3384 and 8858 − 8883 (Table 1).

Results

Generation of Dendritic Cells with Different Functional Capabilities. Immature DCs of four different donors were generated by *in vitro* cultivation and treatment of purified human peripheral blood monocytes with GM-CSF and IL-4. Maturation and modulation of human dendritic cells as presented in this work have been performed as previously described.[4,11] Upon treatment with LPS, immature DCs differentiated into mature DCs with strong T cell stimulatory capacity.[40] Alternatively, immature DCs were treated with OxPAPC. Consequently, type II or alternatively activated macrophages[4] were generated, which have lost the capability to activate T cells. To obtain another different functional DC phenotype, immature DCs were treated with human rhinovirus. These DCs favored the formation of tolerogenic T cells that negatively regulate immune response[11,23] (Figure 1). To confirm cell functionality, aliquots of supernatants were analyzed in parallel by ELISA for the expression of TNF-alpha. 625 ± 138 pg TNF-alpha per ml were detected in the supernatant of mature DCs. No TNF-alpha was detectable in immature DCs, OxPAPC-treated or HRV activated DCs (three independent experiments, data not shown).

Generation of Proteome Reference Maps for Cytoplasmic Proteins of Human DCs. To improve the reliability of data, we preferred to analyze cytoplasmic fractions instead of total cell lysates as previously described.[29] Shotgun analysis was performed after a 1D-PAGE prefractionation step. Lanes were cut and digested with trypsin, peptides were separated by nano-LC−MS/MS and identified by fragmentation analysis in the mass spectrometer. Reference maps of specific cell states were generated by summarizing the mass analysis data of at least three independent experiments (biological replica). A total of 1690 proteins was identified (Table S1, Supporting Information), compiled from the analysis of the DCs at the four different conditions. The overlap between the different conditions was consistently above 80% (Figure 2). The average sequence coverage achieved was 19.9%, 1280 proteins were identified with at least two peptides, 999 proteins with three peptides or more. 924 proteins (54.7%) were found at each of the four conditions, whereas 1435 proteins (84.9%) were identified in at least two different conditions.

Beside a large number of proteins with ubiquitous or broad expression specificity,[41] several proteins were identified which are known to be characteristic and biologically important for macrophages and dendritic cells. These included cluster of differentiation antigens CD1a, and b, CD9, CD11d, CD43, CD44, CD45, CD54, CD59, CD68, CD81, CD85j, CD97, CD143, CD147, CD172a, CD206 (macrophage mannose receptor), CD209 (dendritic cell-specific ICAM-3-grabbing nonintegrin 1), CD276, several HLA class II histocompatibility antigens, integrins alpha-5 (CD49e), alpha-D (CD11d), alpha-M (CD11b), alpha-X (CD11c), beta-1 (CD29), beta-2 (CD18) and beta-7, and macrophage derived chemokines (CCL3, CCL17, CCL18, CCL22, CCL23).

Table 1. List of all PRIDE Accession Numbers and the Corresponding Experiments

PRIDE accession	
3381	immature human DCs, cytoplasmic protein fraction
3382	immature human DCs, cytoplasmic protein fraction
3383	immature human DCs, cytoplasmic protein fraction
3384	immature human DCs, cytoplasmic protein fraction
8858	immature human DCs, secreted protein fraction
8859	immature human DCs, secreted protein fraction
8860	immature human DCs, secreted protein fraction
8861	immature human DCs, secreted protein fraction
8862	human DCs, treated with human rhinovirus, cytoplasmic protein fraction
8863	human DCs, treated with human rhinovirus, cytoplasmic protein fraction
8864	human DCs, treated with human rhinovirus, cytoplasmic protein fraction
8865	human DCs, treated with human rhinovirus, secreted protein fraction
8866	human DCs, treated with human rhinovirus, secreted protein fraction
8867	human DCs, treated with human rhinovirus, secreted protein fraction
8868	human DCs, treated with human rhinovirus, secreted protein fraction
8869	mature human DCs, cytoplasmic protein fraction
8870	mature human DCs, cytoplasmic protein fraction
8871	mature human DCs, cytoplasmic protein fraction
8872	mature human DCs, cytoplasmic protein fraction
8873	mature human DCs, secreted protein fraction
8874	mature human DCs, secreted protein fraction
8875	mature human DCs, secreted protein fraction
8876	mature human DCs, secreted protein fraction
8877	human DCs, treated with oxidized phospholipids, cytoplasmic protein fraction
8878	human DCs, treated with oxidized phospholipids, cytoplasmic protein fraction
8879	human DCs, treated with oxidized phospholipids, cytoplasmic protein fraction
8880	human DCs, treated with oxidized phospholipids, secreted protein fraction
8881	human DCs, treated with oxidized phospholipids, secreted protein fraction
8882	human DCs, treated with oxidized phospholipids, secreted protein fraction
8883	human DCs, treated with oxidized phospholipids, secreted protein fraction

Accessibility of Protein and Peptide Identification Data. All present proteome analysis data, including peptide sequences, identification scores and MS^2 spectra of each identified peptide, have been made accessible in full detail via the PRIDE database at (http://www.ebi.ac.uk/pride/).[38,39] The identification details can be also accessed when searching a single protein in the uniprot database at www.uniprot.org[42] following the PRIDE-

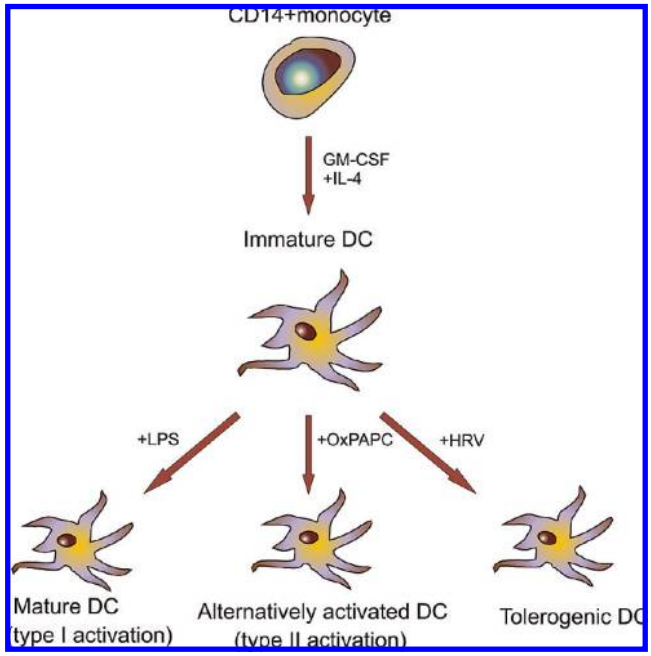

Figure 1. Treatment of immature dendritic cells. CD14+monocytes were treated with GM-CSF in combination with IL-4 for 7 days to generate immature DCs which served as control. These immature DCs were further stimulated with either LPS (mature DCs, type I activation), OxPAPC (alternatively activated DCs, type II activation) or with HRV (tolerogenic DCs).

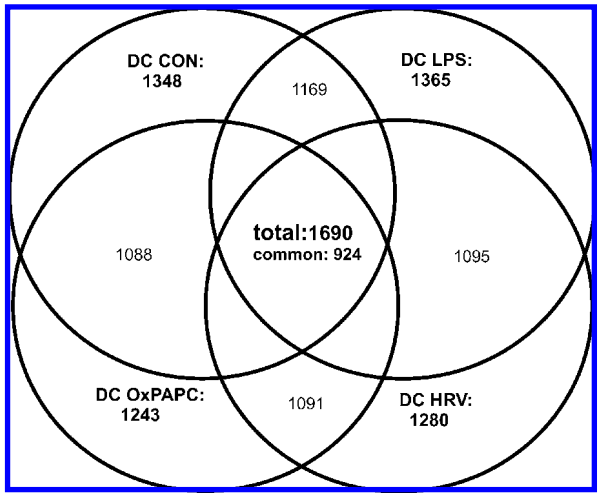

Figure 2. Number of proteins identified in different functional DC cell states. Numbers in the intersections indicate overlapping protein identifications between the differently treated DCs. DC CON, immature DCs; DC LPS, mature DCs; DC OxPAPC, alternatively activated DCs; DC HRV, tolerogenic DCs.

link there. In addition, proteome reference maps of the dentritic cells can be assessed and downloaded via the CPL/MUW database at http://www.meduniwien.ac.at/proteomics/database. Following the "List all cells" link, the dendritic cells at the desired cell state can be selected. The protein list which returns upon selection corresponds to the sum of proteins and peptides identified in all shotgun experiments of the selected cell type. These protein lists can be sorted, further selected or downloaded. The specificity of protein expression can be assessed in the following way. Searching for a protein name or an accession number and confirming the selection returns two pieces of information. The "Identification Details"-window lists the number of different kinds of cells expressing the selected protein. Following the "View cell" link returns the identity of these cells. A protein with restricted expression pattern such as CD43 or CD209 will return only a single cell type, while others such as CD44 return several cell types. Furthermore, all identified peptides related to the selected proteins are listed with further identification details accessible. The list of peptides identified for a single protein as obtained in each of the different cells can be obtained upon selection of a cell type in the "View cell" window. For interpretation of data as outlined below, we made use of these tools.

Identification of Cytoplasmic Proteins Related to Characteristic Functional Cell States. A direct comparison between the proteins identified at the four different dendritic cell states suggested 255 proteins (15.1%) to be differently expressed. Two-hundred twenty-two of those, however, were identified with one peptide only, indicating detection at the sensitivity limit. Some, but not all, of the proteins which were identified only in DCs at a single functional state may be truly specific for the corresponding cell state. Evidently, protein identifications based on a single peptide are hard to reproduce in independent experiments and may therefore simulate a specific expression pattern. To filter the most plausible specifically expressed proteins, we made use of the CPL/MUW database, which allows to compare proteome profiles of DCs with those of other cells, including fibroblasts, keratinocytes, endothelial cells and others.[31,41] For example, the small inducible cytokines A3 (CCL3), CCL22 and lymphotoxin-beta (tumor necrosis factor C), all identified with only one peptide, were presently identified in mature DCs but not in any other cell type. Indeed, these proteins have been described previously to be specific for mature macrophages and we therefore consider them as truly LPS-induced (Table 2).[10] 3-Mercaptopyruvate sulfurtransferase, to give a different example, presently as well identified in mature DCs only, was elsewhere identified in lymphocytes, neutrophils, endothelial cells, hepatocytes and others (see www.meduniwien.ac.at/proteomics/database, simple search). Such a finding suggests that the identification of this protein in mature DCs was rather accidental in this case and does not imply that the protein was induced by LPS. Following this strategy, we considered only those proteins as specifically related to functional activation, which in addition to function-associated expression as well showed a restricted pattern regarding cell-specific expression. In this way, we identified a total of 34 candidates for function-related marker proteins (Table 2). LPS-induced maturation displayed the most pronounced effect, resulting in the up-regulation of 22 proteins (Table 2). These included several interferon-induced proteins in addition to signal transduction proteins and secreted proteins, which were identified in the cytoplasmic fraction. Six proteins were found induced by OxPAPC (Table 2). Three of them were exclusively identified in DCs, two only in OxPAPC-treated DCs. Due to the high expression level, the protein heme oxygenase may be well suitable as marker protein for type II activated DCs. Another six proteins were found induced by treatment with HRV (Table 2), three of them were exclusively identified in HRV-treated DCs. It needs to be further established whether one of those might be a suitable marker protein.

Several proteins were quantitatively regulated rather than specifically expressed. The higher the relative concentration of a protein, the higher is the chance to identify a larger number of peptides. We described a correlation between protein abundances as determined from 2D gel spot intensities and

Table 2. Cytoplasmic Proteins Specifically Related to Functional Cell States of Differently Stimulated Human Dendritic Cells[a]

AccNr	protein	CON	LPS	OX	HRV
	LPS-induced				
P07384	Calpain-1 catalytic subunit	1	6	1	1
P21926	CD9 antigen (p24)		2	1	
O60271	**C-jun-amino-terminal kinase-interacting protein 4**		2	1	
P05362	Intercellular adhesion molecule 1 precursor (CD54 antigen)	1	5	1	1
P05161	Interferon-induced 17 kDa protein precursor	1	7		
P20591	Interferon-induced GTP-binding protein Mx1 (MxA)		12		
P20592	Interferon-induced GTP-binding protein Mx2		3		
P32455	Interferon-induced guanylate-binding protein 1	1	5		1
P09914	Interferon-induced protein with tetratricopeptide repeats 1		2		
P09913	**Interferon-induced protein with tetratricopeptide repeats 2**		4		
O14879	**Interferon-induced protein with tetratricopeptide repeats 3**		8		
Q96AZ6	Interferon-stimulated gene 20 kDa protein (ISG20)		4		
P01584	Interleukin-1 beta precursor (IL-1 beta)		1		
Q06643	**Lymphotoxin-beta**		1		
Q9Y639	Neuroplastin precursor (Stromal cell-derived receptor 1)		2		1
Q05655	Protein kinase C delta type		2		1
P07332	**Proto-oncogene tyrosine-protein kinase Fes/Fps**		3		
Q92974	**Rho/Rac guanine nucleotide exchange factor 2 (GEF-H1)**	1	4		
O00626	**Small inducible cytokine A22 precursor (CCL22)**		1		
P10147	**Small inducible cytokine A3 precursor (CCL3)**		1		
P02778	Small inducible cytokine B10 precursor (CXCL10)		2		
P24557	Thromboxane-A synthase		2		
	OxPAPC-induced				
Q14011	Cold-inducible RNA-binding protein			3	
Q6UWP2	**Dehydrogenase/reductase SDR family member 11 precursor**			1	
P25685	DnaJ homologue subfamily B member 1			2	
O60739	**Eukaryotic translation initiation factor 1b**	1		5	
P09601	Heme oxygenase 1			13	
O15027	**Protein KIAA0310**			4	
	HRV-induced				
P56378	**6,8 kDa mitochondrial proteolipid**				2
P27986	Phosphatidylinositol 3-kinase regulatory subunit alpha				2
Q9HAB8	**Phosphopantothenate--cysteine ligase**				2
Q9NQP4	Prefoldin subunit 4 (Protein C-1)				2
Q15276	**Rab GTPase-binding effector protein 1**				2
P43405	Tyrosine-protein kinase SYK				3

[a] Proteins were identified by shotgun proteomics. Numbers indicate distinct peptides identified per protein in the respective cell states. Proteins which were identified by us as indicated, but not in any other cell type included in the CPL/MUW database are marked in bold. CON, immature dendritic cells; LPS, mature dendritic cells; OX, DCs treated with OxPAPC; HRV, DCs treated with human rhinovirus.

the relative number of peptides identified per protein of the same samples analyzed by shotgun proteomics.[31] Consequently, the number of distinct peptides identified per protein was used to semiquantitatively assess relative protein amounts (Table 2). To provide full access to the present results, Table S1 in the Supporting Information lists all 1690 proteins including the number of distinct peptides identified at the corresponding cell states.

Secretome Analysis. The supernatant of cultured cells is usually composed of a mixture of secreted proteins in addition to cytoplasmic proteins, which are derived from dying cells and of residual fetal calf serum proteins or other contaminants originating from cell culture medium.

Secretomes were analyzed by using a combination of fluorescence detection and metabolic labeling. Figure 3A shows a

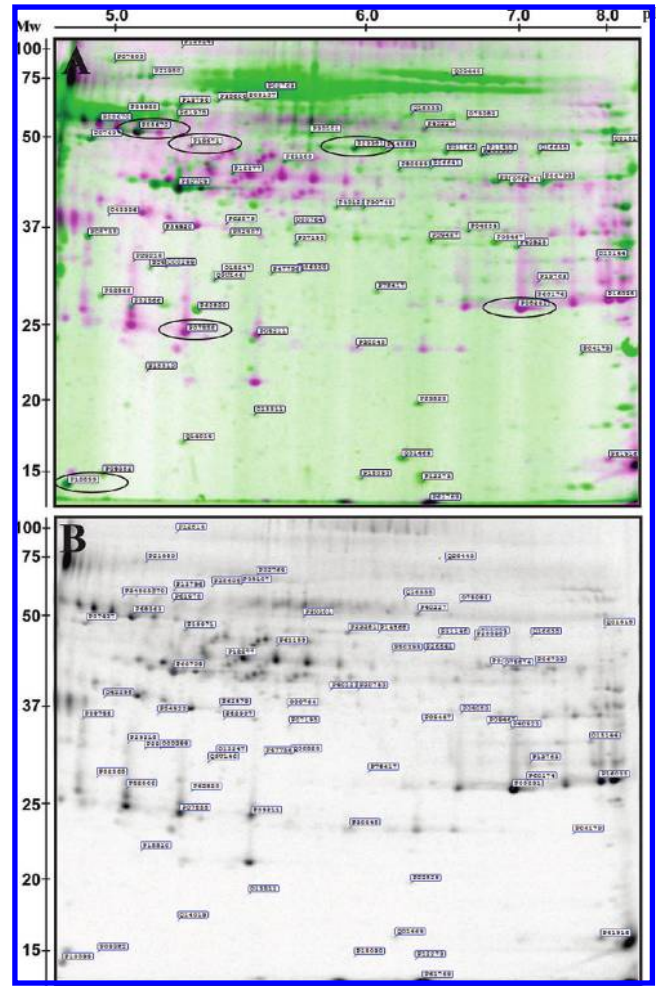

Figure 3. Differential detection of secreted and most abundant proteins in a 2D gel of proteins isolated from mature dendritic cell supernatant. (A) Overlay of fluorescence detection (green) and autoradiographic detection (magenta). Many abundant proteins (intense green) do not display autoradiographic signals and are thus clearly identified as contaminants. In contrast, many strongly secreted proteins (intense magenta) such as interleukin-6 (P05231, encircled) do not display a fluorescence stain and are thus present only in minute amounts. Several proteins (also encircled) were found secreted but were also detected in significant amounts in the cytoplasm. (B) Autoradiograph of the same 2D gel. The clear and distinct spot pattern suggests selective detection of secreted proteins.

fluorescence scan (green) of cell supernatant proteins isolated from mature DCs and separated by 2D-PAGE overlaid to the corresponding autoradiograph (magenta). Tryptic digestion and mass analysis of isolated spots identified the main constituents as serum albumin and serotransferrin derived from residual fetal calf serum, in addition to other cytoplasmic proteins (Figure 3). Obviously, genuinely secreted proteins were hardly detectable by fluorescence detection. On the other hand, almost all abundant spots detectable by fluorescence (green) in Figure 3A, were not detectable by autoradiography (magenta). This finding clearly identified these proteins as contaminants from cytoplasm released from dead cells or fetal calf serum. Autoradiography of the same gel (magenta in Figure 3A, and Figure 3B), however, selectively detected proteins which were genuinely secreted by living cells as previously described.[34] Many secreted proteins are of very low abundance as clearly exempli-

fied in Figure 3A for interleukin-6, which is a predominant spot in the autoradiograph (pink), but still undetectable by fluorescence detection (green). Other proteins such as vimentin, tryptophanyl-tRNA synthetase, thymidine phosphorylase (platelet-derived endothelial cell growth factor), cathepsin B and thioredoxin were clearly detectable by both techniques, indicative for active secretion in addition to abundant cytoplasmic occurrence.

The same set of samples was analyzed by shotgun proteomics, following an analysis strategy similar to that applied to the cytoplasmic fractions. Four independent experiments were summarized per condition, the number of distinct peptides identified per protein again served as a semiquantitative measure for protein abundance. Of a total set of 995 proteins identified, 227 comprised a signal sequence that potentially targets proteins to be secreted.[43] After the exclusion of proteins that are most probably contaminants derived from residual fetal calf serum, the number was reduced to 691 distinct proteins (Table S2, Supporting Information) containing 132 proteins with a signal sequence or secreted by nonclassical pathways according to published literature, which we consider as genuinely secreted (Table 3). If all identified peptides of a protein were compatible with the bovine amino acid sequence and did not comprise human-specific sequences, we considered it as fetal calf serum contaminant. Indeed, we identified some serum proteins, such as alpha-1-acid glycoprotein, in the supernatant of dendritic cells by human-specific sequences (Table 3). Furthermore, the secretome comprised cytokines (e.g., GM-CSF, TNF-α, interleukins 1β, 6, 8, 12β, 28B and 29), chemokines (CCL3, 5, 8, 17, 18, 19, 24, CXCL1, 2, 9 and 10), growth factors (growth/differentiation factor 8, stem cell growth factor), growth factor binding proteins (IGFBP-2); proteases (MMP-9, ADAM DEC1, ADAMTS-17, ADAMTSL-4, cathepsins B, D, S, and Z) and peptidases (aminopeptidase B and N), protease inhibitors (TIMP-1, TIMP-2), acute phase proteins (alpha-1-acid glycoprotein 1 and 2), apolipoproteins CIII, D and E, complement factors (C1q, C3, C4-A), extracellular matrix proteins (fibronectin, extracellular matrix protein 1, laminin alpha-2) and many others (Table 3).

Identification of Secreted Proteins Related to Characteristic Functional Cell States. Figure 4 displays 2D-PAGE autoradiographs of the secreted protein fraction of the differently treated DCs. As determined from the sum of spot intensities and compared to immature DCs, LPS-treatment induced protein secretion 2.4-fold (± 0.4 from three independent experiments), treatment with OxPAPC reduced the secretion by 18% (± 11%), and stimulation with HRV again induced protein secretion 2.1-fold (± 0.45). The spot patterns at all investigated conditions showed striking similarities (Figure 4). Proteins were identified from silver-stained gels prepared from unlabeled protein samples which were always generated in parallel (not shown). Several spots such as beta-2-microglobulin were detected at all conditions with similar intensity. Proteins were considered as up-regulated if the average integrated spot intensity exceeded the corresponding control value at least 2-fold. LPS induced the secretion of several proteins which are hardly or not detectable in immature DCs such as vimentin, hsp70, tryptophanyl-tRNA synthetase, cathepsin B and IL-6 (Figure 4B). The most pronounced OxPAPC-induced effect detected in 2D gels was the induction of adipocyte fatty acid-binding protein (AFABP) (Figure 4). Tolerogenic HRV-treated DCs secreted considerably less vimentin, cathepsin B and IL-6 in comparison to mature DCs, but displayed induced secretion of endothelial cell growth factor (Figure 4D).

While many proteins were found in the secretome of DCs at more than one cell state, several proteins appeared to be specifically expressed. As outlined above, we again made use of the CPL/MUW database and referred to published literature to support appropriate protein selection. Besides pro-inflammatory cytokines characteristic for mature DCs such as IL-1β, IL-6, IL-8, IL-12 and TNF-α, we successfully identified proteins previously established as markers for mature dendritic cells such as decysin (Table 3).[44] The identification of chemokines CCL3/5/8 and 19 as well as CXCL1, 9, and 10 also confirmed known expression profiles.[10] We also reconfirmed the expression pattern of IL-29.[45] Furthermore, here we found for the first time that mature DCs are capable of secreting stem cell growth factor and vasorin (Table 3). Also unexpected was the finding that DC maturation is accompanied by the secretion of the plasma proteins complement factor B, leucine-rich alpha-2-glycoprotein and zinc-alpha-2-glycoprotein (Table 3). Nine proteins, including ADAMTSL-4 and growth/differentiation factor 8, appeared to be specifically expressed in OxPAPC-treated cells. Eight proteins, including ADAMTS-17, appeared to be specifically expressed in HRV-treated cells (Table 3). Eighty-nine proteins, including several cytokines and chemokines such as CXCL2, CCL17, 18, and 24 identified in the secretome of dendritic cells, showed no clear correlation to one of the investigated functional cell states (Table 3).

Discussion

Dendritic Cells As Important Model System for Clinical Proteomics. Dendritic cells are main regulators of inflammation and thus critical players for a large variety of diseases. Recent data suggest a critical role of inflammation and thus an active regulatory role of dendritic cells also for the development of cancer.[46] Here we performed a proteome profiling study of human dendritic cells at four different functional states corresponding to immature cells, mature cells mediating acute or type I inflammation, alternatively activated cells mediating type II inflammation and tolerogenic cells inhibiting T-cell activation (Figure 1).

Clinical proteomics aims at the identification of marker proteins with diagnostic and/or prognostic value. Proteome profiling of clinical materials is a great challenge, from an analytical perspective as well as with respect to data interpretation. We, therefore, consider the analysis of representative *in vitro* models for patho-physiologically relevant cell states as useful strategy to support the interpretation of clinical proteomics data. There is still a lack of valid references with respect to protein expression profiles of human cells. In many cases it is hard to verify whether an experimentally obtained proteome profile is truly representative for the investigated sample. If the profiling data are representative, many proteins known to be expressed in a cell type and function-specific fashion should be present accordingly, while others known to be specific for other cells should be absent. Working with dendritic cells offers plenty of opportunities to test the validity of shotgun-derived proteome profiles, because a lot of protein expression data obtained from immunological studies are available. We were thus able to assess the reliability and comprehensiveness of our data by comparing the apparent expression specificities suggested by our proteome profiles to published data derived from gene expression analysis, ELISA or other protein identification strategies.

Table 3. Secretome Profiles of Differently Stimulated Human Dendritic Cells[a]

AccNr	protein	CON	LPS	OX	HRV
	LPS-induced				
O15204	**ADAM DEC1 precursor**		1		
P00751	Complement factor B precursor		2		
Q9Y240	C-type lectin domain family 11 member A precursor (stem cell growth factor)		1		
P04141	**Granulocyte-macrophage colony-stimulating factor precursor (GM-CSF)**		1		
P09341	Growth-regulated protein alpha precursor (CXCL1)		3		
P01584	Interleukin-1 beta precursor (IL-1 beta)		1		
P29460	**Interleukin-12 subunit beta precursor (IL-12)**		1		
Q8IZI9	**Interleukin-28B precursor (IL-28B)**		1		
Q8IU54	**Interleukin-29 precursor (IL-29)**		2		
P05231	Interleukin-6 precursor (IL-6)		8		
P10145	Interleukin-8 precursor (IL-8)		2		
P02750	Leucine-rich alpha-2-glycoprotein precursor (LRG)		4		
P26022	Pentraxin-related protein PTX3 precursor		7		
P83105	**Probable serine protease HTRA4 precursor**		1		
P10147	**Small inducible cytokine A3 precursor (CCL3)**		1		
P80075	**Small inducible cytokine A8 precursor (CCL8)**		2		
P02778	Small inducible cytokine B10 precursor (CXCL10)		5		
Q99731	**Small-inducible cytokine A19 precursor (CCL19)**		2		
P13501	Small-inducible cytokine A5 precursor (CCL5)		1		
Q07325	**Small-inducible cytokine B9 precursor (CXCL9)**		1		
P01375	Tumor necrosis factor precursor (TNF-alpha)		5		
P98066	Tumor necrosis factor-inducible protein TSG-6 precursor		2		
Q6EMK4	Vasorin precursor (Protein Slit-like 2)		1		
P25311	Zinc-alpha-2-glycoprotein precursor		14	7	
	OxPAPC-induced				
Q6UY14	**ADAMTS-like protein 4 precursor (ADAMTSL-4)**			1	
Q16610	**Extracellular matrix protein 1 precursor (Secretory component p85)**			1	
O14793	**Growth/differentiation factor 8 precursor (GDF-8)**			1	
P05107	Integrin beta-2 precursor			2	
Q04912	**Macrophage-stimulating protein receptor**			1	
Q12765	Secernin-1			1	
P62328	Thymosin beta-4			1	
Q15582	Transforming growth factor-beta-induced protein ig-h3 precursor	3	1	8	3
Q9NQW7	Xaa-Pro aminopeptidase 1			1	
	HRV-induced				
Q8TE56	**ADAMTS-17 precursor**				1
Q9H4A4	Aminopeptidase B				1
P15144	Aminopeptidase N				3
P08107	Heat shock 70 kDa protein 1			1	4
P05120	Plasminogen activator inhibitor 2 precursor (PAI-2)				1
O76061	Stanniocalcin-2 precursor				1
P06126	T-cell surface glycoprotein CD1a precursor (CD1a antigen)				1
P29017	T-cell surface glycoprotein CD1c precursor (CD1c antigen)				1
	Not regulated				
P35318	ADM	1			
P55008	Allograft inflammatory factor 1	2	2	1	2
P02763	Alpha-1-acid glycoprotein 1 precursor (AGP 1)	1	5	4	
P19652	Alpha-1-acid glycoprotein 2 precursor (AGP 2)		5	4	
P02771	Alpha-fetoprotein precursor	5	6	5	6
P02760	AMBP protein precursor	1	6	6	1
P12821	Angiotensin-converting enzyme, somatic isoform precursor	4		2	3
P01008	Antithrombin-III precursor	3	1	4	2
Q8NCW5	Apolipoprotein A-I-binding protein precursor	1	2		3
P02656	Apolipoprotein C−III precursor	1		1	1
P05090	Apolipoprotein D precursor		3		
P02649	Apolipoprotein E precursor	8	1	3	4
P02749	Beta-2-glycoprotein 1 precursor	1	2	7	1
P61769	Beta-2-microglobulin precursor	3	6	4	4
Q13867	Bleomycin hydrolase	1			
P06703	Calcyclin	2	3		1
P07858	Cathepsin B precursor	13		11	14
P07339	Cathepsin D precursor	2		1	
P25774	Cathepsin S precursor	5		6	4
Q9UBR2	Cathepsin Z precursor	2		1	3
P16070	CD44 antigen precursor (Phagocytic glycoprotein I)	1		1	
O00299	Chloride intracellular channel protein 1	5	3	6	7
Q9Y696	Chloride intracellular channel protein 4	4	1	3	4
P00488	Coagulation factor XIII A chain precursor	10		7	4
P02745	Complement C1q subcomponent subunit A precursor	1		1	1
P02746	Complement C1q subcomponent subunit B precursor	1	3	4	5
P02747	Complement C1q subcomponent subunit C precursor	3	3	4	4
P01024	Complement C3 precursor	7	3	6	3
P0C0L4	Complement C4-A precursor (Acidic complement C4)		2	2	
P00746	Complement factor D precursor	2			3
P01034	Cystatin C precursor	8	7	10	6
O76096	Cystatin F precursor (Leukocystatin)	1	1	1	1
Q86TI2	Dipeptidyl peptidase 9	1			
P61916	Epididymal secretory protein E1 precursor	2	2	3	5
P30042	ES1 protein homologue, mitochondrial precursor				1
P15090	Fatty acid-binding protein, adipocyte (AFABP)	3	1	3	2
P02751	Fibronectin precursor	7	10	5	7

Table 3. Continued

AccNr	protein	CON	LPS	OX	HRV
P17931	Galectin-3	1	2	4	3
Q08380	Galectin-3-binding protein precursor	1			
P47929	Galectin-7		1		
O00182	Galectin-9	1			1
P13284	Gamma-interferon-inducible lysosomal thiol reductase precursor	1			1
P06396	Gelsolin precursor	16	4	12	12
P01903	HLA class II histocompatibility antigen, DR alpha chain precursor	3		3	4
P13760	HLA class II histocompatibility antigen, DRB1−4 beta chain precursor	4		5	5
P18065	Insulin-like growth factor-binding protein 2 precursor	2	2	1	
P11215	Integrin alpha-M precursor	6	2	5	7
P20702	Integrin alpha-X precursor		1	2	2
P05362	Intercellular adhesion molecule 1 precursor (ICAM-1)	1			1
P18510	Interleukin-1 receptor antagonist protein precursor (IL-1ra)	3	4	1	5
P02788	Lactotransferrin precursor	2	2	1	2
P24043	Laminin alpha-2 chain precursor	1	1	1	1
O00754	Lysosomal alpha-mannosidase precursor	1			
P61626	Lysozyme C precursor	3	2	5	6
P07333	Macrophage colony-stimulating factor 1 receptor precursor	1		1	1
P19875	**Macrophage inflammatory protein 2-alpha precursor (CXCL2)**	1	1		
P22897	Macrophage mannose receptor 1 precursor	14	7	8	10
P14174	Macrophage migration inhibitory factor	1	1	1	1
P14780	Matrix metalloproteinase-9 precursor	11	6	7	3
P01033	Metalloproteinase inhibitor 1 precursor (TIMP-1)	2	2	2	2
P16035	Metalloproteinase inhibitor 2 precursor (TIMP-2)	5	3	3	4
Q12904	Multisynthetase complex auxiliary component p43	1			1
Q96PD5	N-acetylmuramoyl-L-alanine amidase precursor	3	7	3	3
O14786	Neuropilin-1 precursor	1		1	
O95497	Pantetheinase precursor			1	1
P23284	Peptidyl-prolyl cis−trans isomerase B precursor	5	5	3	6
Q15063	Periostin precursor	1		1	
P36955	Pigment epithelium-derived factor precursor (PEDF)	5	1	4	2
P68402	Platelet-activating factor acetylhydrolase IB beta subunit			1	2
Q15102	Platelet-activating factor acetylhydrolase IB gamma subunit		1		1
P07602	Proactivator polypeptide precursor	5	1	2	4
Q969H8	Protein C19orf10 precursor	2	2	2	
Q8IWL2	Pulmonary surfactant-associated protein A1 precursor	1		1	
Q53FA7	Putative quinone oxidoreductase		1		
Q93091	Ribonuclease K6 precursor	2	2	1	
P26447	S100 calcium-binding protein A4 (Metastasin)	4	2	2	3
Q13103	Secreted phosphoprotein 24 precursor	1			
P10124	Secretory granule proteoglycan core protein precursor	5	3	3	3
Q92583	**Small inducible cytokine A17 precursor (CCL17)**	4	2	1	2
P55774	**Small inducible cytokine A18 precursor (CCL18)**	4	3	1	1
O00175	**Small inducible cytokine A24 precursor (CCL24)**	2		2	
O00391	Sulfhydryl oxidase 1 precursor	7		5	1
P05452	Tetranectin precursor	2	1	2	
P10599	Thioredoxin	5	5	4	4
P19971	Thymidine phosphorylase precursor	6	1	4	6
P05543	Thyroxine-binding globulin precursor	1	1	2	
O14773	Tripeptidyl-peptidase I precursor	1		1	1
Q53FT3	Uncharacterized protein C11orf73	1			
P12955	Xaa-Pro dipeptidase	1		1	

[a] Proteins were identified in the corresponding cell supernatant fractions. Only proteins with a signal sequence or described to be secreted by non-classical pathways are listed. Annotations as in Table 2.

Cytoplasmatic Proteins Characteristic for DCs. A large number of proteins identified in the cytoplasmic protein fraction indeed matched known protein expression data. This is the most obvious in the case of the successful identified CD antigens, whose expression patterns are very well established. When comparing the present data to protein expression profiles obtained in the case of endothelial cells, fibroblasts and keratinocytes,[41] we were able to identify proteins of relative high abundance which appear to be quite specifically expressed by DCs or connatural cells. Proteins such as rho-GTPase-activating protein 4 and formin-like 1 protein were found abundant in DCs and not detectable in other nonleukocyte cell types. Such systematic analysis may unravel somewhat unexpected expression patterns, as observed in case of chloride intracellular channel protein 2, which was found abundant in DCs but not detectable by us neither in nonleukocyte cell types nor in monocytes. This is remarkable as the corresponding gene expression data do not suggest such a specific expression pattern.

Cytoplasmatic Proteins Characteristic for Different Functional States. Alterations of the protein expression accompanying the mature cell state comprised several interferon-inducible proteins (Table 2). This observation is compatible with established literature as outlined in the following. MxA was described to mediate antiviral host defense,[47,48] which poses a main duty for mature dendritic cells. Interferon-stimulated gene 20 kDa protein (ISG20) is an interferon-induced RNase specific for single-stranded RNA supporting antiviral strategies of the innate immune system.[49] Interferon-induced protein with tetratricopeptide repeats 3 is considered to be involved in immune response and the corresponding gene was found induced e.g. in inflammatory murine microglia,[50] which are closely related to mature dendritic cells. It has been described to promote differentiation of monocytes into DC-like cells.[51] Among other genes, interferon-induced protein with tetratricopeptide repeats 1 was described in a transcriptomics study as specific for LPS-induced microglia.[50] Other proteins point to the regulation of migration and homing of mature DCs. Lymphotoxin-beta (tumor necrosis factor C), that was found to be specifically expressed by mature DCs, provides the membrane anchor for the attachment of DCs to stromal cells in lymphoid tissue.[52] The proto-oncogene tyrosine-protein kinase Fes/Fps was described to modulate the immune re-

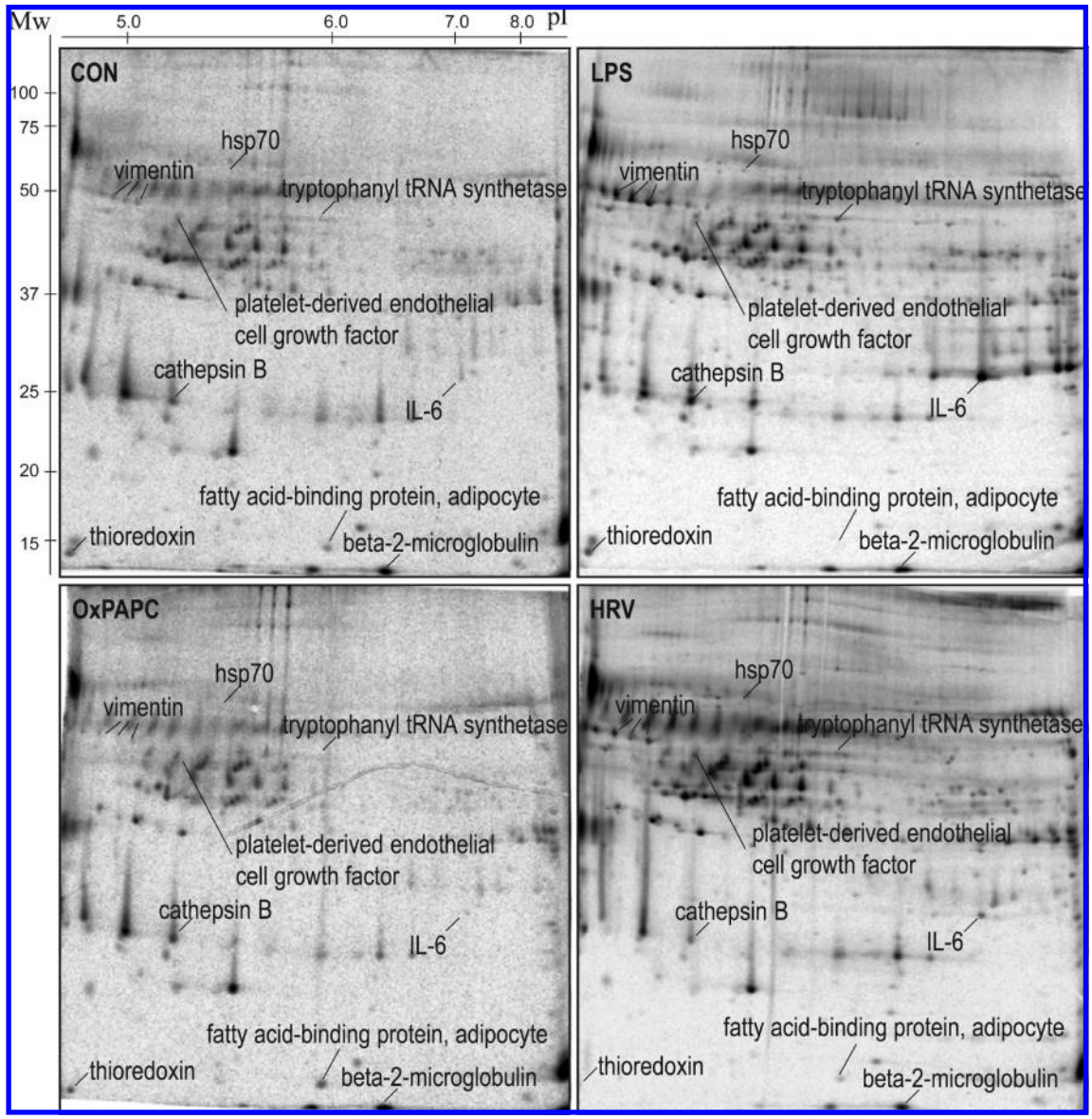

Figure 4. Comparative secretome analysis of differently stimulated DCs by 2D autoradiographs. Immature DCs were considered as control, mature DCs were generated by treatment with LPS, OxPAPC-treatment resulted in loss of T-cell activation capability, tolerogenic DCs were generated by human rhinovirus (HRV) stimulation. Secretion of beta-2-microglobulin was almost constant at all tested conditions. Mature DCs displayed induced secretion of vimentin, hsp70, tryptophayl tRNA synthetase, cathepsin B and IL-6. the synthesis of IL-6 (P05231) is clearly up-regulated when treated with LPS. Adipocyte fatty acid binding protein (P15090) was up-regulated in OxPAPC treated DCs. HRV-stimulated DCs showed induced secretion of platelet-derived endothelial cell growth factor.

sponse of macrophages to LPS[53] and regulated leukocyte recruitment during inflammation.[54] Rho/Rac guanine nucleotide exchange factor 2 (GEF-H1) is involved in cell motility and polarization,[55] dendritic spine morphology, antigen presentation and leukemic cell differentiation.[56]

Less specific effects accompanying DC maturation may as well contribute to characteristic protein expression (see Table S1, Supporting Information). Nicotinamide phosphoribosyltransferase was described to be up-regulated in inflammatory activated neutrophils and mediates protection against apoptosis.[57] Alpha-centractin has been described to be involved in cell motility and vesicle transport,[58] which plays an important role during DC maturation.[59] Myoferlin, which was not described in DCs until now, plays a critical role to maintain membrane integrity,[60] which may plausibly be challenged during inflammatory activation. Other proteins apparently up-regulated by LPS (Table S1, Supporting Information) may relate to a general increased metabolic activity such as increased demand for iron or elevated requirement to stabilize the redox-system.

OxPAPC-treated DC displayed fewer proteins with a specific expression profile. Heme oxygenase 1, however, was strongly and exclusively expressed by these cells (Table 2). Mainly involved in erythrocytes degradation processes, heme oxygenase 1 was found to play an important role in the attenuation of inflammatory processes to support healing.[61,62] The induction of the secreted protein transforming growth factor-beta-induced protein ig-h3 by OxPAPC, otherwise observed upon ingestion of apoptotic cells[63] may as well be related to healing processes.

HRV also induced several proteins (Table 2). A direct relation to the tolerogenic cell phenotype was not obvious for us.

Secretome Analysis. The cytoplasmic fraction contains a large number of so-called house-keeping proteins that are needed for basic cellular functions.[41] Such proteins are expressed in different cell types. In contrast to this, secreted proteins are more specifically related to the current biological task of the cells. This is why secreted proteins generally are of high interest for proteome profiling focusing on specific cell functions.

Mainly due to their capability to secrete different chemokines and cytokines, DCs are able to initiate, prolong and regulate immune response. Thus, the secretome of these cells is a source of potential biomarkers for regulatory processes of inflammation.

Secretome analysis represents a true technical challenge, due to the usually very low abundance of secreted proteins in addition to two further major obstacles: contamination of the cell supernatant by proteins released from dead or dying cells and residual serum proteins derived from the culture medium.[30,34] Cell vitality and functionality, which is usually supported by the addition of fetal calf serum to the medium, is required for full secretory activity. Under standardized conditions with 10% FCS, however, a secreted protein fraction would contain up to 50 000 times more serum proteins than secreted proteins, rendering their identification very difficult. For this reason, cells have to be washed before secreted proteins can be cumulated in serum-free medium. Here resides the second problem: lack of serum proteins may cause cell stress and the induction of cell death, eventually resulting in the cessation of protein secretion and release of cytoplasmic proteins into the supernatant. Consequently, some of the proteins currently described to be secreted by immature dendritic cells may have been induced by cell stress. The experimental conditions were similar for the different functional states and many of the currently identified proteins reproduce previous findings as outlined above and thus make sense from a biological point of view. This gives us confidence that our results are meaningful.

Protein Secretion Dependent on the Functional State. The present data confirm dendritic cells as a rich source of secreted proteins including cytokines, chemokines, proteases, protease inhibitors, growth factors and various modulators of protein and cell functions. Immature dendritic cells secreted proteases such as lysozyme C, *N*-acetylmuramoyl-L-alanine amidase and matrix metalloproteinase-9; protease inhibitors such as cystatins C and F; chemokines such as CXCL2, CCL17, CCL18 and CCL24, and galectin-9; redox regulators such as glutathione peroxidase 3, an angiogenesis inhibitor pigment epithelium-derived factor, the vasoconstrictor angiotensin-converting enzyme (CD143)[64] and many others (Table 3). Upon stimulation with LPS, the secretion of several cytokines and chemokines in addition to stem cell growth factor and other proteins was found strongly induced (Table 3). In addition, several membrane proteins were found in the supernatant, including intercellular adhesion molecule 1 (ICAM-1), HLA class II histocompatibility antigens and CD81, which might result from membrane shedding. On the other hand, several proteins identified in immature DCs were no more detectable in mature DCs including bleomycin hydrolase and galectin-9.

In the case of OxPAPC treatment, most of the LPS-induced cytokines were not detectable any more as expected from the biological phenotype. The observed decrease in secretion activity (Figure 4), which was also observed in other studies,[4] did not result from a general decrease in protein synthesis, because the synthesis of cytoplasmic proteins was rather elevated (data not shown). ADAMTS-like protein 4 (TSRC1), potentially a positive regulator of apoptosis,[65] was specifically detected in OxPAPC-treated DCs.

Rhinovirus stimulated DCs again displayed characteristic features. Human rhinovirus leads to inhibition of adequate antigen-specific T cell response, that is involved in termination of inflammatory response.[23] As in the case of OxPAPC-treated DCs, most cytokines affecting T cells, which were found induced by LPS, were not detectable. This was accompanied by shedding of the metalloprotease aminopeptidase N (CD13), which has been described as a receptor for human viruses and may as well contribute to tumor invasion.[66]

Conclusion

Shotgun proteomics enabled the identification of a large number of proteins in differently stimulated human dendritic cells and the assignment of functional proteome signatures to these cells. Here we present several candidate proteins which may serve as biomarkers for the recognition of dendritic cell activities in clinical proteomics samples. Consideration of known functions of the identified proteins corroborated the significance of our findings, while several other proteins were identified, which were not yet assigned to dendritic cells or the corresponding cell states. Secretome profiling of these cells suggested that the observed functional phenotypes may not be assigned to the effect of single proteins but rather result from the combined effects of several secreted proteins including agonists, antagonists and modulators.

Abbreviations: APC, antigen-presenting cell; CD, cluster of differentiation; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; HRV, human rhinovirus; IL, interleukin; LPS, lipopolysaccharid; OxPAPC, 1-palmitoyl-2-arachidoyl-*sn*-glycerol-3-phosphorylcholin; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; TLR, toll-like receptor.

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Supporting Information Available: Table S1: List of all 1690 proteins identified in the cytoplasmic fraction of human DCs. Proteins are sorted according to protein names and identified by their SwissProt Accession numbers. The numbers listed in rows indicate the number of distinct peptides identified by mass spectrometry corresponding to each functional cell state. Coverage, amino acid coverage of the identified peptides; CON, immature dendritic cells; LPS, mature dendtitic cells; OX, DCs treated with OxPAPC; HRV, DCs treated with human rhinovirus. Table S2: List of all identified proteins in the cell supernatant of human DCs. In addition to genuinely secreted proteins (Table 2), this Table contains all cytoplasmic proteins identified in the same fraction, which are derived from dead cells. Annotations as in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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