

Protein-induced changes during the maturation process of human dendritic cells: a 2-D DIGE approach.

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Protein-induced changes during the maturation process of human dendritic cells: a 2-D DIGE approach.

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Abbreviations:

APC: antigen presenting cell; CCL19: chemokine (C-C motif) ligand 19; CCR7: chemokine (C-C motif) receptor 7; DC: dendritic cell; FACS: fluorescent-activated cell sorting; FITC: fluorescein isothiocyanate; GM-CSF: granulocyte/macrophage colony-stimulating factor; IFN-γ: interferon gamma; IL: interleukin; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cell.

Keywords:

2-D DIGE, dendritic cell, maturation, network, proteomics

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Summary

Dendritic cells (DCs) are unique antigen presenting cells (APCs), which upon maturation change from a specialized antigen-capturing cell towards a professional APC. In this study, a 2-D DIGE analysis of immature and mature DCs was performed, to identify proteins changing in expression upon maturation. The protein expression profile of immature and mature DCs, derived from $CD14^+$ peripheral blood monocytes was investigated using 2 pH ranges (pH 4-7 and 6-9) (n=4). Ninety one differentially expressed spots ($p<0.01$) were detected, from which we identified 74 spots (81.32%) corresponding to 41 different proteins. The proteins identified play a role in diverse processes, such as antigen processing/presentation, vesicle transport and cytoskeleton remodeling. In addition, a protein interaction network contained 29 (out of 41) proteins, suggesting that, although they functionally originate from distinct classes, these proteins are acting as a protein-interactome. In conclusion, the proteins shown here to be altered in expression upon maturation are in line with the morphological and functional changes observed during the maturation process, providing a better understanding of the processes involved. This will open new avenues for investigating treatment regimens for immune-associated disorders.

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1 Introduction

Dendritic Cells (DCs) are unique antigen presenting cells (APCs) with the ability to induce and modulate primary immune responses and to induce immunological tolerance. They are derived from bone marrow progenitors, giving rise to a highly specialized antigen-capturing cell, the immature DC. These cells are present in peripheral tissues, such as skin and mucosae, as well as in the interstitial spaces of many organs, lymphoid tissues and blood [1]. In the absence of inflammatory signals, immature DCs lack T cell costimulatory molecules (CD40, CD54 and CD86), are highly phagocytic and continue to capture/process antigens and present them inefficiently to T cells. The interaction between immature DCs and T cells promotes T cell induction of apoptosis, anergy [2,3] or differentiation into regulatory T cells [4].

In the presence of inflammatory or infectious agents, immature DCs undergo a maturation process, up-regulation of costimulatory molecules and migration to the lymphoid organs, i.e. spleen and lymph nodes [5]. There, they complete their maturation, attract T and B cells by releasing chemokines [6] and maintain the viability of recirculating T lymphocytes [7]. Mature DCs have a reduced capacity for antigen uptake, but a high efficiency to stimulate T cells [8].

Several studies, both by microarray and by proteomic analysis, have been undertaken to investigate the global gene/protein expression pattern related to the different stages of the lifecycle of DCs, to gain a better insight into the processes involved [9-18]. Although these studies have identified many important proteins involved in the

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differentiation/maturation process of DCs, the picture is far from complete. We therefore performed in the present study a proteomic analysis making use of the recently developed 2-D DIGE technique. This technique couples the well-known separation and resolution capacity of 2-DE to the labeling with fluorescent cyanine-dyes, which bind covalently to the lysine residues of proteins. In addition, the ability to run multiple samples within a single gel avoids much of the variability inherent to classical 2-DE, making it a more reproducible technique, eventually resulting in the identification of subtle differences between proteins with high statistical certitude.

Using this technique, we compared protein expression profiles between immature and mature human DCs, making use of an overlap of 2 different pH ranges (pH4-7 and pH6-9) (n=4). This allowed us to detect 91 differential protein spots ($p<0.01$), of which 71 were unambiguously identified by MALDI-TOF/TOF, corresponding to 41 different proteins. Many of these have not been described before as playing a role in DC maturation [13-19]. In addition, 29 out of the 41 differentially expressed proteins were shown to be linked to each other, suggesting that the maturation process is a complex process in which proteins of different classes/pathways are acting closely together, rather than as single entities, finally resulting in the typical phenotype of a mature DC.

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2 Materials and methods

2.1 In vitro generation of human dendritic cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of four healthy donors by density gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway) and monocytes were purified subsequently by CD14-specific positive magnetic cell sorting according to the supplier's protocol (MACS, Milteny Biotec, Bergish Gladbach, Germany). Isolated monocytes (~ 90% pure, $1x10^6$ cells/mL) were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with Glutamax-I (Gibco), 25 mM HEPES (Gibco), 5 µg/ml geneticin (Life Technologies, Rockville MD, USA) and 10% heat-inactivated fetal calf serum (FCS, Gibco), in $75cm^2$ tissue culture flasks (TPP, Transadingen, Switzerland). For inducing DC differentiation, 1000U/mL recombinant human IL-4 (Gentaur, Brussel, Belgium) and 800 U/mL recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) (Gentaur) were added. Medium and cytokines were refreshed on day 3. On day 6, immature DCs were harvested for functional/morphological analysis or for protein extraction, and maturation was induced in the remaining cultures, by addition of 100 ng/mL Escherichia coli-derived LPS (Sigma, St Louis, MO), 1000 U/mL recombinant human IFN-γ (Roche Diagnostics, Indianapolis, IN) and 800 U/mL GM-CSF. Mature DCs were harvested after an additional 48 hours for functional/morphological analysis or for protein extraction.

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Three additional maturation protocols were performed in order to investigate whether altered protein expressions were dependent on the conditions used for in vitro maturation. Therefore, immature DCs were generated from two different donors using IL-4 and GM-CSF as described above. The maturation protocols lasted for 48 hours and were as follows: A) 1000U/mL IL-4, 800 U/mL GM-CSF and 100ng/mL LPS; B) 800 U/mL GM-CSF, 100 ng/mL LPS and 1000 U/mL IFN-γ C)100ng/mL LPS and D) 1000 U/mL IL-4, 800 U/mL GM-CSF and 10ng/mL recombinant human TNFα (Strathmann Biotec, Hamburg, Germany). The different subsets of immature and mature DCs were harvested for protein extraction and Western blotting analysis.

2.2 Phenotypic analysis of human dendritic cells

Analysis of DC surface markers

2.5 x 10^5 DCs were incubated for 30 min at 4°C with the following FITC- or PE-conjugated human antibodies: CD1a, CD14, CD80, CD54, CD40 (all from eBiosciences, San Diego, CA); HLA-DR, CD83, CD86, CD206, CCR7 (all from Becton Dickinson, San Jose, CA) and corresponding control antibodies, all diluted in PBS. After washing and fixing in 1% paraformaldehyde, the cells were analyzed on a FACSsort (Becton Dickinson). Expressed data were corrected for control antibodies. FACS analysis was performed on DCs from 3 different donors.

Chemotaxis assay

Active migration towards CCL19 was evaluated by a transwell assay. Briefly, lower chambers of polycarbonate membrane Transwell plates (6.5mm-diameter, 5.0µm-pore

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size, Corning, New York, USA) were filled with 600 µl complete medium containing 100 ng/mL recombinant human MIP-3β/CCL19 (Peprotech). A control condition, without chemokine, was included. Immature and mature DCs (2.5 x 10^6 cells) were added to the upper chambers in 100 µL complete medium. Cells were allowed to migrate for 2 hours at 37°C. Migrated cells were collected from the lower chambers and counted in a Bürker chamber. The percentage of migrating cells was calculated as a ratio between the number of migrating cells and start number of cells. Specific migration was calculated by subtracting the mean number of spontaneously migrating cells (migration to medium only) from the mean number of cells that migrated in response to the chemokine. The assay was performed on DCs from 2 different donors with duplicates per assay.

Endocytosis assay

Immature and mature DCs (2 x 10^5) were pre-cooled at 4°C for 30 min and then incubated with either FITC-dextran (1 mg/mL, Sigma), for the analysis of mannose receptor-mediated endocytosis, or Lucifer Yellow (1 mg/mL, Sigma), for the evaluation of fluid-phase endocytosis, at 37°C for 90 min. As a negative control, cells were incubated with either one of the reagents at 4°C for 90 min. Cells were washed 3 times in ice-cold PBS supplemented with 1% FCS, fixed in ice-cold 1% paraformaldehyde and immediately analyzed by FACS. Specific endocytosis was calculated by subtracting the percentage of endocytotic cells at 4°C from the percentage of endocytotic cells at 37°C. The assay was performed on DCs from 2 different donors with duplicates per assay.

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Analysis of cytokine production

Immature and mature DCs (5 x 10^5) were stimulated for 24 hours with CD40-ligand (5 /mL; Peprotech) after which supernatants were collected. IL-12p70 levels were determined by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (R&D Systems, Minneapolis, MN). ELISA was performed on DCs from 2 different donors with duplicates per assay.

2.3 2-D DIGE

Four independent cell culture experiments were performed, each of them originating from a different healthy human blood donor. Immature and mature DCs, obtained as described above, were washed with ice-cold PBS (Cambrex, Verviers, Belgium) and protein extraction followed by the 2-D DIGE experiments were carried out as previously described [20]. Briefly, after extraction, protein content was determined. 50µg of protein was labeled with 200 pmol of amine reactive Cyanine dyes, Cy2, Cy3 or Cy5 (GE Healthcare). *The Cy2 sample consisted of a pool of equal amounts of all samples used in the experiment (internal standard).* Reverse labeling was performed for Cy3 and Cy5 to eliminate any preferential or non-specific labeling. The pooled samples were loaded into individual 24-cm IPG strips, pH4-7 and pH6-9 and isoeletric focusing was carried out on an Ettan IPGphor II manifold (GE Healthcare) at 20°C for about 70kV.h with a maximum current per strip of 50 µA.

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After isoelectric focusing, the IPG strips were equilibrated and run on a 1 mm thick 11,5% polyacrylamide SDS gel (25 x 20 x 0.5 cm; Ettan Dalt Six, GE Healthcare). The gels were run overnight at 20°C with a constant current of 8 mA/gel for 1 hour, followed by 16 mA/gel until the end of the run. After the second dimension, the gels were scanned on a Typhoon 9400 gel imager (GE Healthcare) with 100 µm resolution and image analysis was performed using the Batch Processor module of the DeCyder™ V 6.5 software (GE Healthcare), following the manufacturer's recommendations.

Two preparative gels in each pH range containing 350 µg of protein were run for spot picking. The gels were then post-stained with Deep Purple (GE Healthcare) according to the manufacturer's protocol and the spot matching was carried out on the BVA module of the DeCyder software. A pick list was generated and exported into the Spot Picker V1.20, the software that controls the Ettan Spot Picker (GE Healthcare). Spot manipulation was made as previously described [20]. After digestion and extraction of the tryptic peptides, the peptide samples were concentrated and desalted with ZipTip C-18 (Millipore, Bedford, MA) and analyzed by mass spectrometry on a MALDI-TOF/TOF 4800 instrument (Applied Biosystems, Foster City, USA). Data interpretation was carried out using the GPS Explorer software (V3.5) and database searching was carried out using the Mascot program (version 2.0.00). *MS/MS searches were conducted with the following settings: SwissProt 20050315 (homo sapiens taxonomy) as database, MS/MS tolerance between 0.2 Da and 1 Da depending on the sample, methionine oxidation as variable modification and carbamidomethylation of cysteine as fixed modification. Using these parameters, the probability-based MOWSE*

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scores greater than the given cut-off value for MS/MS fragmentation data are significant ($p<0.05$).

2.5 Western blotting

Total protein extracts of immature and mature DCs from three different healthy donors were prepared as described above. Equal amounts (10µg) of protein from each extract were solubilized in loading buffer (Invitrogen, Merelbeeke, Belgium) and subjected to SDSpolyacrylamide gel (4-12% gradient gels, Invitrogen) electrophoresis. Proteins were transferred to PVDF membranes, which were blocked (5% milk in PBST 0.1%) for one hour and then incubated with the following antibodies: mouse monoclonal anti-Fascin 1 (55K-2); goat polyclonal anti-PA28beta (PSME2) (L-19); goat polyclonal anti-TrpRS (WARS) (N-17); goat polyclonal anti-PRX I (N-19); rabbit polyclonal anti-Stat5a (L-20); rabbit polyclonal anti-Cathepsin S (H-50), all purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA; rabbit polyclonal anti-MnSOD (Upstate – Millipore, Brussels, Belgium) and rabbit polyclonal anti-Calreticulin (Abcam, Cambridge, UK). The membranes were then incubated with horseradish peroxidase-conjugated appropriated secondary antibody (donkey anti-goat, from Santa Cruz; anti-rabbit and anti-mouse, from Dako, Leuven, Belgium). Chemiluminescence was performed according to the manufacturer's instructions (PerkinElmer, Zaventem, Belgium). As a loading control, the membranes were reprobed with anti-GAPDH mouse monoclonal antibody (Applied Biosystems, Lennik, Belgium). Quantitative densitometry was carried out on the Typhoon 9400 gel imager (GE Healthcare). The detected bands were scanned with a 100 µm resolution and image analysis was

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performed using the 1-D gel analysis of ImageQuant software (GEHealthcare). The volume density of the chemiluminescent bands was calculated as the ratio between main antibody and loading control antibody after background correction.

2.6 Human protein interaction network

A human protein interaction network was constructed as recently reported [21,22]. Data were downloaded from MINT 48, BIND 49, IntAct 50, KEGG and Reactome. In order to increase the coverage of interactions, interolog data 53 (the transfer of protein interactions between orthologous protein pairs in different organisms) were included. Interactions were transferred from 17 eukaryotic organisms and added to the network. Orthology was assigned using the Inparanoid database 55 using stringent thresholds. The statistical significance of the networks was estimated using a randomization scheme in which networks were generated from random input sets of the same size as in the present experiment. We performed 10,000 randomizations from which we derived a probability distribution that was used to calculate the significance of the networks in this study [22].

2.7 Statistical analysis

The statistical differences between group average ratios were determined by unpaired two-tailed Student's t-test, performed automatically by the BVA module of the DeCyder V6.5. $P < 0.01$ was considered statistically significant.

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Results

3.1 Characterization of DCs

DCs were generated from human $CD14^+$ monocytes, isolated from PBMCs of healthy donors. Immature DCs were obtained after 6 days of culture in the presence of IL-4 and GM-CSF, while mature DCs were cultured for an additional 2 days in the presence of GM-CSF, LPS and IFN-γ. Morphologically, immature DCs were spherical non-adherent cells expressing short cytoplasmic projections on their surfaces, whereas mature DCs were slightly elongated and had longer cytoplasmic projections. FACS analysis of cell surface receptors showed that CD1a was expressed both in immature and mature DCs, whereas CD14 was almost absent in both populations. The mannose receptor CD206 had its expression slightly increased upon maturation. As expected, HLA-DR, CD83, CCR7 and the T cell costimulatory molecules CD80, CD54, CD86 and CD40 were all strongly induced upon maturation (Table 1). Finally, functional analyses showed that immature DCs could more efficiently capture antigens, assayed both by FITC-dextran (65.8±4.8% vs. 32.7±5.1% in mature DCs, $p<0.05$) and Lucifer Yellow (54.5±4.7% vs. 23.2±4.5% in mature DCs, $p<0.05$) uptake. Immature DCs released less CD40-ligation triggered IL-12p70 (38±26.87 pg/mL vs. 584.1±31.75 pg/mL, by mature DCs, $p<0.01$) and were less able to migrate actively towards the CCR7 ligand, CCL19 (0.5±1.7% vs. 23.1±1.5% migrated in mature DCs, $p<0.01$). Taken together, these results are consistent with previous reported typical characteristics of immature and mature DCs and confirm the phenotype of the cells for further investigation.

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3.2 Detection and identification of differentially expressed proteins.

2-D DIGE was used to investigate the differences in protein expression profile between immature and mature DCs. For this purpose, quadruplicate experiments were performed, using samples originating from four independent DC cultures from four healthy blood donors. Each sample was separated at two different pH ranges (4-7 and 6-9). A total of 1823±87 spots in the pH 4-7 and 1787±90 spots in the pH 6-9 could be detected. Out of these, 91 spots (62 in the pH 4-7 and 29 in the pH 6-9 range) were differentially expressed upon maturation, and these spots were present in all four replicates ($p<0.01$) (Fig. 1). A total of 74 (81.3%) differentially expressed protein spots were unambiguously identified by MALDI-TOF/TOF after in-gel trypsin digestion. These corresponded to 41 different proteins, indicating that many proteins were represented in multiple spots, due to the presence of post-translational modifications *(Table 2 and supplementary table 1)*. All identifications were based on the sequencing of more than one peptide.

The identified proteins were classified in different functional groups, based on gene ontology classification, as follows: protein biosynthesis/proteolysis (40,0%), metabolism (16,0%), cell growth/cytoskeleton proteins (11,0%), signal transduction (16,0%), RNA processing/regulation of transcription (4,0%), immune response (3,0%), response to oxidative stress (7,0%) and transport (3,0%). Irrespective of this classification, they are involved in many different functional pathways, such as pyruvate metabolism (pyruvate kinase isozymes M1/M2 (PKM2), pyruvate carboxylase (PCB), dihydrolipoyl dehydrogenase (DLD), alpha enolase (ENO1), malate

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dehydrogenase (MDH1)); cell-cell interaction and cell motility (CD11b, PTP-1B, RhoGDP inhibitor, cathepsin B (CTSB)); oxidative stress/defense (superoxide dismutase (SOD2), carbonyl reductase); cytoskeleton reorganisation (lamin A, actin, fascin); vesicle transport (N-ethylmaleimide sensitive fusion protein (NSF), Interferon-induced GTP-binding protein Mx1 (Mx1)) and antigen processing and presentation (Proteasome activator complex subunit 2 (PSME2), Proteasome 26S subunit 2 (PSMD2), cathepsin D (CSTD), cathepsin S (CTSS), tryptophan t-RNA ligase (WARS), cytosol aminopeptidase (LAP3)) (*Fig 2*).

Interestingly, a high number of proteins were present in more than one spot (63,7%), indicating that PTMs are crucial in the maturation process. Although the scope of the present study was not to further characterize these PTMs, it is noteworthy that the enzymes WARS and LAP3 were found in 11 and 8 multiple electrophoretic forms, respectively. Other proteins present in multiple forms were CTSD and SOD2 (6 spots), CD11b (4 spots) and Mx1, STAT5a, actin and fascin (2 spots). The appearance of these multiple electrophoretic forms could be due to different PTMs, i.e. phosphorylation, glycosylation or even proteolytic cleavage.

Of note, some proteins were up-regulated to a very high level in mature DCs, i.e. WARS, SOD2, PCB and Mx1; these were all more than 5-fold induced in at least one of their identified forms. In addition, all forms of CTSD (6 spots) were significantly down-regulated, with one spot more than 5-fold down-regulated in mature DCs (Table 2).

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To validate the data obtained from 2-D DIGE and spot identification, we confirmed the regulation profiles and the identity of six differentially expressed key proteins by Western blotting, namely Stat5a, WARS, PSME2, Fascin and CTSS (n=5, Fig 3) and SOD2 (n=2, Fig 4). This revealed a very comparable differential expression pattern for all of these six proteins when compared to the 2-D DIGE results, indicating the high level of confidence obtained using this proteomic technique.

In addition, Western blotting for calreticulin, CTSS, fascin, Prx1, PSME2, SOD2, Stat5a and WARS in DCs maturated in 4 different ways (see Materials and Methods) indicated that all protocols induced similar changes in protein expression for these selected proteins, although to a different extent (Fig. 4).

3.3 Protein- protein interactions during DC maturation: Network analysis

To investigate whether the identified proteins possibly interact with each other, a protein network analysis was performed. For this purpose, the identified proteins were placed in a protein interaction network recently developed by us [21,22]. This analysis revealed that 29 out of the 41 identified proteins indeed interact with each other, interconnecting different pathways (p=2.8e-5) (Fig. 5). This network contains proteins from different functional classes, such as antigen presentation/processing, cytoskeleton organization, metabolism, signal transduction and RNA metabolism.

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4 Discussion

The combination of classical immunological techniques and 2-D DIGE coupled to MS/MS allowed us to confirm many known events in the differentiation from immature to mature DCs and most importantly, it allowed us to identify novel proteins involved in this process and to identify networks of proteins interacting during maturation.

From the 91 differentially expressed spots between immature and mature DCs, 74 (81.32%) were identified, corresponding to 41 different proteins. Many of these could be associated with specific well-known functions in DCs, such as antigen processing and presentation, including proteins related to the MHC class I and MHC class II pathways. Not surprisingly, since one of the major differences between immature and mature DCs is their ability to capture, process and present antigens. Proteins identified, directly or indirectly linked to the MHC class I pathway were cytosol aminopeptidase (LAP3), proteasome activator complex subunit 2 (PSME2) and 26S proteasome non-ATPase regulatory subunit 2 (PSMD2) (Fig 2). Interestingly in this regard, LAP3 as well as PSME2, are also known to be induced by IFN- γ [23,24]. Also three endosomal proteases, cathepsin B, D and S (CTSB, CTSD and CTSS) were down-regulated, consistent with a role in the MHC II pathway, which is mainly occurring in immature DCs (Fig. 2). CTSD is an aspartic protease, found in 6 different spots, whereas CTSB is involved in remodeling of extracellular matrix (ECM) and in antigen presentation [25]. CTSS is the only cathepsin thought to be essential for antigen presentation by MHC II molecules and for degradation of the invariant chain, at least in its final stages [26-28].

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Another group of proteins found to be highly regulated during DC maturation, were proteins involved in vesicle transport, namely interferon-induced GTP-binding protein Mx1 (Mx1), cytoplasmic dynein 1 intermediate chain 2 (DYNC 1|2) and N-ethylmaleimide sensitive fusion protein (NSF). Mx1 is localized in the smooth ER where it is involved in membrane trafficking to or from this organelle [29]. The protein is highly regulated by IFN-γ, which can next to inducing total levels of Mx1, also induce PTMs with altered pI [30]. Also important for vesicle transport is dynein, responsible for the transport of peptide-bound MHC II molecules to the cell membrane in compartments towards the minus end of microtubules [31].C-dynein binds to dynactin with the help of the dynein-associated intermediate filaments [32]. The vesicle will dock to the plasma membrane, where it is fused in a process mediated by the SNARE family proteins [33]. NSF, found to be up-regulated in the present study, is responsible for disruption of the SNARE complex, necessary for vesicle recycling (Fig. 2).

In addition to these, we were able to identify proteins involved in cytoskeleton remodeling, metabolism, cell-cell interaction and cell motility - all processes known to be affected during DC maturation. In line with the morphological changes observed during DC maturation, a major induction in the expression of fascin was observed. In DCs, fascin is known to be highly expressed during the maturation process, playing an important role in cytoskeleton remodeling [1], and functioning as a maturation marker. This protein was described by Horlock et al. [16], where peripheral blood myeloid DCs (mDCs), as well as monocyte-derived DCs or KG-1 DC-like cells were matured by

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LPS. Fascin was found as highly up-regulated in both mDC models when compared to the original population. Next to this, fascin induction is apparently taking place upon maturation of DCs irrespective of the inflammatory stimulus used for maturation, as revealed by Western blotting in our study. This at first sight inconsistency with other proteomic studies, where differential expression of fascin was not reported, points to differences in capacities of the different proteomic techniques [13-15,17,18].

When comparing data from different proteomic approaches [13-19] on human DCs, it is striking that only very few proteins are found in common. One issue of debate is evidently the technical differences between the different proteomics studies performed, such as 1) the sensitivity and accuracy of 2-D DIGE compared to classical 2-DE, 2) differences in pH and MW range and 3) the use of full cell lysates as compared to fractionated samples. Another issue of debate is the difference obtained due to the maturation protocols. We used a combination of LPS and IFN-γ, Pereira et al. and Watarai et al. used GM-CSF, IL-4 and LPS, the groups of Horlock and Rivollier used LPS only and Le Naour et al. used a combination of IL-4, GM-CSF and TNFα to induce maturation. Especially the effect of IFN-γ was clearly noticeable in our study, with different IFN- γ inducible proteins being picked up, such as LAP3, PSME2, WARS and Mx1.

Considering that proteins are not merely acting as single entities, but rather in a complex network of protein-to-protein interactions, it was interesting to find that a large portion of the differentially expressed proteins (29 out of 41) could indeed be

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linked to each other, suggesting that they may act as a protein-interactome. Interestingly, proteins involved in biosynthesis/proteolysis seem to interact with proteins playing a role in antigen processing and presentation, such as the cathepsins B, D, S and Z, PSME2, PSMD2 and WARS. Moreover, these proteins also interact with proteins involved in cytoskeleton rearrangement and metabolism, such as PKM2, SOD2, DYNC1|2, NSF and actin itself. This suggests that upon triggering of an immature DC, many different pathways are activated which all together will result in a mature DC consistent with the changes observed in its morphological and functional characteristics. Translating this to a more clinical situation, where upon an immune or inflammatory attack a combination of different stimuli is most probably present/activated, the picture will be even more complex.

In conclusion, many of the proteins shown here to be involved in the maturation of DCs, linked to processes such as metabolism, vesicle transport, cytoskeleton and antigen processing/presentation, have not been previously described. The use of 2-D DIGE, together with the different stimuli used for the maturation of DCs, resulted in new findings that together with the construction of a protein interaction network*, adds new information leading to a more complete understanding on the maturation process of these cells.*

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Figure legends

Figure 1. Images of 2-D gels from human DCs. Protein lysates from immature and mature human DC samples were reversed labeled with Cy3 or Cy5, mixed with the pooled internal standard (labeled with Cy2) and run into a single gel, at two different pH ranges (4-7 and 6-9). Separate images of immature (upper gels) and mature DC samples are shown. Highlighted spots in yellow are differentially expressed upon analysis using the DeCyder software (n=4, $p<0.01$). One representative experiment out of 4 is shown.

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Figure 3. Protein expression levels of Fascin, PSME2, WARS, CTSS and Stat5a. Immature and mature human DCs were lysed and the protein levels were analyzed by Western blotting with appropriate specific antibodies. As loading control, the membranes were re-probed with anti-GAPDH antibody. The immunoblots shown are representative of a total of five independent experiments. The corresponding histograms on the right-hand side of the figure show the relative increase of all normalized densitometric values, with the results obtained for the immature DCs set as 1. The results are presented as the mean±S.D. of five independent experiments.

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Table1. Surface marker expression of immature (IM) and mature (M) DCs[a]

	CD1a	CD14	HLA-DR	CD83	CD80
IM	56[a]	4	745	19	1
M	32	4	2,294	75	29

	CD40	CD86	CD54	CCR7	CD206
IM	59	582	596	ND[b]	114
M	259	2,250	2,198	13	137

a) Values are expressed as mean fluorescence intensity; one representative experiment out of 3 experiments is shown. b)ND: Non detectable.

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Table 2. Identified proteins with altered expression upon DC maturation

pH range	Spot n°	Full protein name	gene name	AC number	M/IM ratio	Peptides matched	Coverage (%)	ion score
Protein biosynthesis/ Proteolysis								
4-7	8	26S proteasome non-ATPase regulatory subunit 2	PSMD2	Q13200	2,03	8	12%	255
	45	Cathepsin B precursor - **heavy chain**	CTSB	P07858	-3,22	5	30%	262
	48	Cathepsin D precursor - **heavy chain**	CTSD	P07339	-4,00	4	14%	77
	49				-3,78	6	18%	165
	51				-2,40	5	14%	53
	52				-5,79	5	14%	103
	54				-4,86	4	14%	42
	55				-2,79	5	14%	108
	42	Cathepsin Z	CTSZ	Q9UBR2	-2,03	4	20%	203
	25	Cytosol aminopeptidase (leucine aminopeptidase)	LAP3	P28838	2,17	9	20%	112
	26				1,63	5	12%	261
	28				1,97	8	17%	236
	29				1,59	9	24%	482
	31				2,32	11	31%	600
	32				-2,93	6	12%	144
	30				1,86	5	8%	154
	38	Serpin B9 – proteinase inhibitor 9	SERPINB9	P50453	3,02	8	23%	388
	14	tryptophan-tRNA ligase (Tryptophanyl-tRNA synthetase)	WARS	P23381	4,96	5	11%	127
	15				3,52	5	11%	82
	16				6,62	6	14%	312
	17				5,86	12	31%	461
	18				6,82	12	32%	588
	19				7,77	8	17%	362
	20				6,74	9	20%	378
	21				2,82	4	10%	80
	24				8,04	7	18%	353
	36				2,20	8	20%	291

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	37				2,02	6	16%	195
6-9	69	Cathepsin S [Precursor]	CTSS	P25774	-2,74	3	11%	120
	65	Cytosol aminopeptidase (leucine aminopeptidase)	LAP3	P28838	2,21	13	39%	855
Metabolism								
4-7	41	Apolipoprotein-L2	APOL2	Q9BQE5	2,63	4	14%	208
	53	Beta-hexosaminidase beta chain [Precursor] (A and B chain)	HEXB	P07686	-2,48	3	14%-A 8%-B	55
	58	Biliverdin Reductase A [precursor]	BLVRA	P53004	1,71	8	25%	219
	44	Carbonyl reductase [NADPH] 3	CBR3	O75828	3,29	8	38%	511
	57	Ganglioside GM2 activator [Precursor]	GMA2	P17900	-2,72	2	15%	63
	43	Malate dehydrogenase	MDH1	P40925	-1,41	3	13%	83
	5	Pyruvate carboxilase, Pyruvic carboxilase, PCB	PCB	P11498	5,77	8	9%	303
	33	Pyruvate kinase isozymes M1/M2	PKM2	P14618	-1,50	7	4-7	221
6-9	66	Alpha enolase	ENO1	P06733	1,91	10	32%	791
	67	Cytosolic acyl coenzyme A thioester hydrolase	ACOT7	O00154	-1,67	5	14%	73
	64	Dihydrolipoyl dehydrogenase, mitochondrial [Precursor]	DLD	P09622	2,85	3	8%	96
Cell growth/cytoskeleton protein								
4-7	40	Actin, cytoplasmic 2 ; Gamma-actin	ACTG	P63261	-3,18	7	26%	332
	56				-2,38	9	29%	465
	9	Cytoplasmic dynein 1 intermediate chain 2 (Isoform 2C)	DYNC1I2	Q13409	1,50	3	8%	165
	22	Fascin	FSCN1	Q16658	3,14	5	10%	196
	10	N-ethylmaleimide sensitive fusion protein	NSF	P46459	1,53	8	11%	298
	50	Rho GDP-dissociation inhibitor 1, Rho-GDI alpha	ARHGDIA	P52565	-1,41	4	13%	71
6-9	62	Fascin	FSCN1	Q16658	1,60	5	10%	111
	63				3,27	4	10%	105
	59	Lamin A	LMNA	P02545	-1,98	5	9%	97
Signal transduction								
4-7	2	Cell surface glycoprotein CD11b precursor	ITGAM	P11215	-1,83	8	8%	224
	1				-2,06	9	9%	252
	3				-2,11	11	11%	262
	4				-2,23	7	7%	109

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	11	Interferon-induced GTP-binding protein Mx1	MX1	P20591	2,99	11	21%	497
	12	Interferon-induced GTP-binding protein Mx1	MX1	P20591	5,13	6	9%	134
	13	Serine/threonine-protein kinase 4	STK4	Q13043	2,06	7	17%	188
	39	Serine-threonine kinase receptor-associated protein	STRAP	Q9Y3F4	-1,27	4	17%	250
	23	Thymidine phosphorylase [Precursor],	ECGF1	P19971	1,86	5	11%	168
	35	Tyrosine-protein phosphatase non-receptor type 1	PTP1B	P18031	6,26	9	24%	403
6-9	61	Proto-oncogene tyrosine-protein kinase Src	SRC	P12931	2,53	6	12%	159
RNA processing/regulation of transcription								
4-7	27	Heterogeneous nuclear ribonucleoprotein H, hn RNP H	HNRPH1	P31943	-1,96	9	30%	492
	6	Signal transducer and activator of transcription 5A (STAT5A)	STAT5A	P42229	2,04	5	7%	113
	7				3,61	5	7%	100
Immune response								
4-7	46	Proteasome activator complex subunit 2	PSME2	Q9UL46	1,89	3	18%	112
6-9	60	SAM domain and HD domain-containing protein 1	SAMHD1	Q9Y3Z3	-1,44	8	14%	129
Response to oxidative stress								
6-9	70	Superoxide dismutase [Mn], mitochondrial [Precursor]	SOD2	P04179	7,18	5	28%	302
	71				4,11	4	20%	112
	72				6,62	3	25%	106
	73				4,99	4	23%	97
	74				6,51	5	27%	112
	75				6,76	4	23%	189
Transport								
4-7	47	Chloride intracellular channel protein 2 (XAP121)	CLIC2	O15247	1,92	5	24%	89
	34	Serum albumin precursor	ALB	P02768	-2,50	2	4%	107

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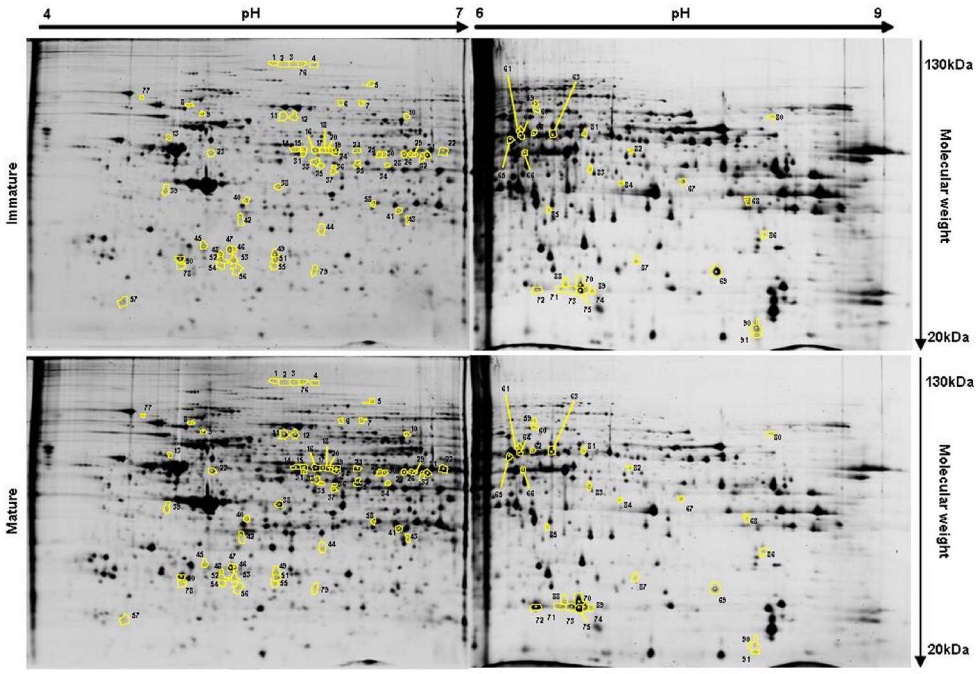


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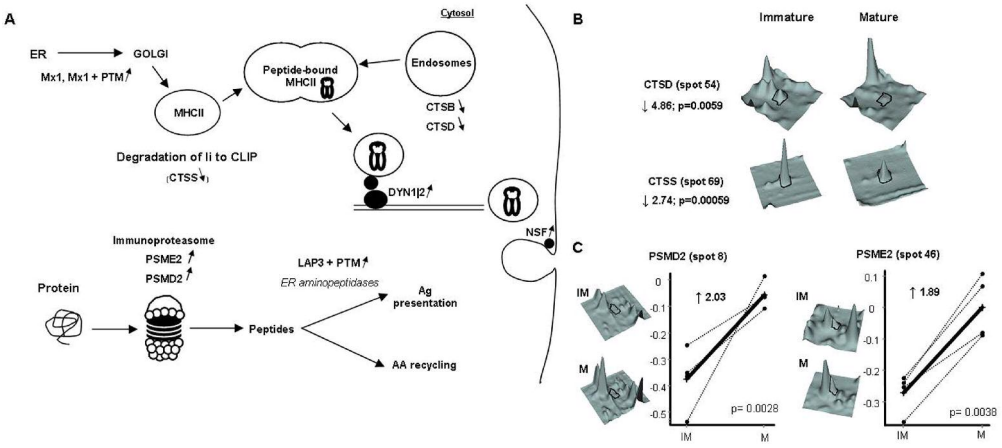


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View

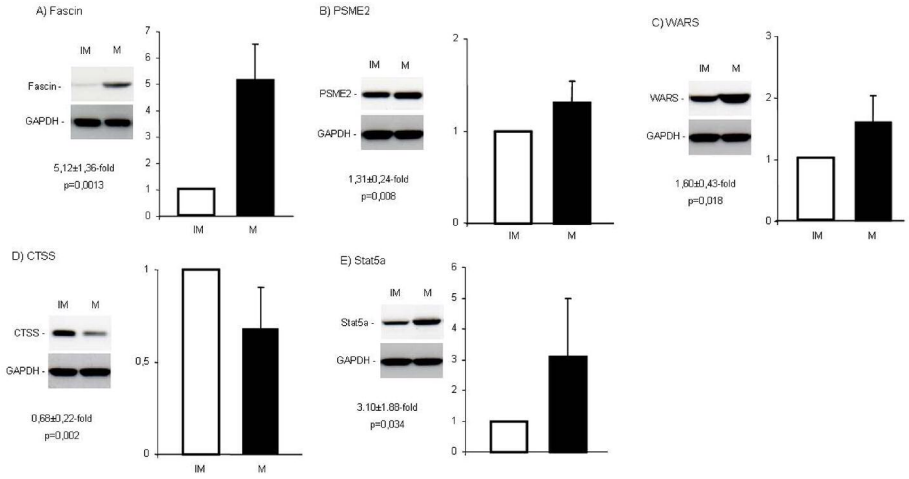
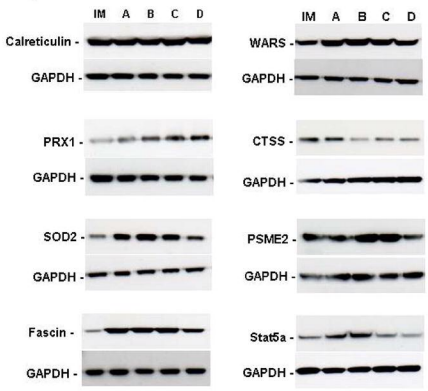


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228x128mm (400 x 400 DPI)

eview

A)



B)

Protein expression between distinct maturation protocols

Protein	IM	A	B	C	D
Calreticulin	1	0,93	0,86	0,73	0,83
CTSS	1	0,45	0,68	0,29	0,49
Fascin	1	3,47	5,12	3,99	2,82
Prx1	1	1,49	1,65	1,90	1,89
PSME2	1	1,28	1,31	3,90	4,85
Stat5a	1	3,13	3,10	2,55	1,43
SOD2	1	1,99	2,76	2,09	1,40
WARS	1	1,55	1,60	1,76	1,49

Figure 4. Protein expression levels of SOD2, Calreticulin, PRX1, Fascin, TrpRS, CTSS, PA28beta and Stat5a. Immature DCs were submitted to 4 different maturation protocols for 48 hours. The maturation cocktails contained A) GM-CSF, IL-4 and LPS; B)GM-CSF, IL-4 and IFN-γ (protocol used for the 2-D DIGE experiments); C) LPS alone; and D) GM-CSF, IL-4 and TNF-α. A) Immunoblots of the 8 different antibodies. Membranes were reprobed with anti-GAPDH antibody for loading control. B) Normalized densitometric values, with the results obtained for the immature DCs set as 1. The results are presented as the mean of two independent experiments, except for the results of protocol B for CTSS, fascin, PSME2, Stat5a and WARS ,which were performed in 5 independent experiments, p<0.05).
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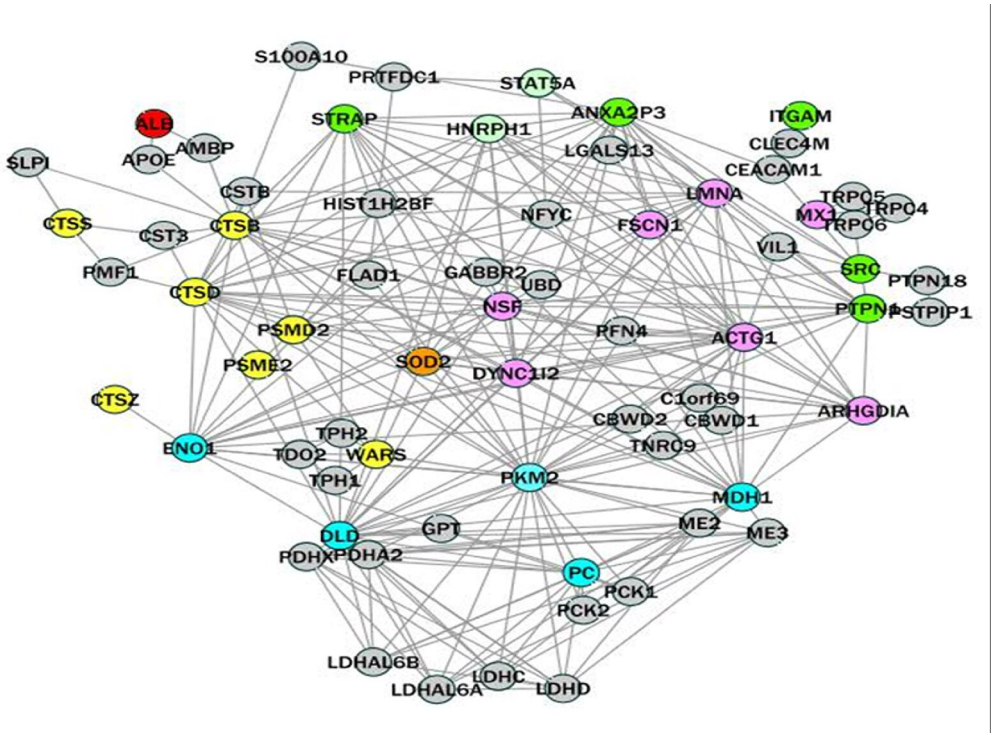


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150x109mm (400 x 400 DPI)