DOI 10.1002/pmic.200400988 *Proteomics* 2005, *5,* 1186–1198

REGULAR ARTICLE

Changes in the proteomic profile during differentiation and maturation of human monocyte-derived dendritic cells stimulated with granulocyte macrophage colony stimulating factor/interleukin-4 and lipopolysaccharide

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Received: June 13, 2004
Revised: July 30, 2004
Accepted: August 5, 2004

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play an essential role in the immune response. We used the proteomic approach based on two-dimensional gel electrophoresis and mass spectrometry to identify the protein changes that occur during differentiation of DCs from monocytes (Mo) stimulated with granulocyte macrophage colony stimulating factor/interleukin-4 (GM-CSF/IL-4) and during the maturation of immature DCs stimulated with lipopolysaccharide. Sixty-three differentially expressed proteins (± two-fold) were unambiguously identified with sequence coverage greater than 20%. They corresponded to only 36 different proteins, because 11 were present as 38 electrophoretic forms. Some proteins such as tropomyosin 4 and heat shock protein 71 presented differentially expressed electrophoretic forms, suggesting that many of the changes in protein expression that accompany differentiation and maturation of DCs occur in post-translationally modified proteins. The largest differences in expression were observed for actin (21-fold in Mo), Rho GDP-dissociation inhibitor 2 (20-fold in Mo), vimentin (eight-fold in immature DCs), lymphocyte-specific protein 1 (12-fold in mature DCs) and thioredoxin (14-fold in mature DCs). Several proteins are directly related to functional and morphological characteristics of DCs, such as cytoskeletal proteins (cytoskeleton rearrangement) and chaperones (antigen processing and presentation), but other proteins have not been assigned specific functions in DCs. Only a few proteins identified here were the same as those reported in proteomic studies of DCs, which used different stimuli to produce the cells (GM-CSF/IL-4 and tumor necrosis factor-α). These data suggest that the DC protein profile depends on the stimuli used for differentiation and especially for maturation.

Keywords:
Dendritic cells / Lipopolysaccharide / Monocytes / Two-dimensional gel electrophoresis

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Abbreviations: DC, dendritic cell; **FBS**, fetal bovine serum; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **IL**, interleukin; **LPS**, lipopolysaccharide; **Mo**, monocytes; **PE**, phycoerythrin; **TNF**, tumor necrosis factor

1 Introduction

Dendritic cells (DCs) are specialized antigen-presenting cells essential for the induction of the immune response to antigens and for the activation of naive T cells. Immature DCs are present in nonlymphatic tissue, such as epithelium and the respiratory and gastrointestinal systems, where they capture and process antigens. From these pathogen entry sites and upon danger signals such as the presence of inflammatory cytokines, (IL-1, TNF-α) or bacterial wall components (LPS), immature DCs migrate to lymphatic organs, where they mature and initiate the immune response by efficiently presenting antigens to T lymphocytes. In addition to functional differences, immature and mature DCs have well-defined specific morphological characteristics [1, 2].

DCs are present in several tissues, but their number is limited, making it difficult to obtain enough cells for *ex vivo* studies. Several protocols have been developed to obtain DCs by *in vitro* differentiation of peripheral blood CD14+ monocytes [3] and hematopoietic precursor cells (CD34+) isolated from bone marrow [4] or umbilical cord blood [5]. These precursor cells are stimulated to differentiate into immature DCs with a mixture of cytokines (GM-CSF, IL-4) and into mature DCs by stimulation with inflammatory cytokines (TNF-α) or bacterial wall components (LPS). The development of *in vitro* protocols to produce DCs has stimulated studies of their clinical applications in immunopathogenesis [6] and as cancer vaccines [7] and also extended our understanding of antigen processing/presenting and T-cell stimulation mechanisms [8].

Comparative studies using microarrays and proteomic approaches have identified mRNAs and proteins differentially expressed during the differentiation and maturation of DCs from human monocytes (Mo). Angenieux *et al.* [9], who used GM-CSF/IL-4 for differentiation, identified 10 proteins and Le Naour *et al.* [10], using GM-CSF/IL-4 for differentiation and TNF-α for maturation, identified 18 proteins, whose expression levels change more than two-fold between Mo, immature DCs and mature DCs. Interestingly, only one (vimentin) of the differentially expressed proteins was identified in both of these studies. In the study reported here we used GM-CSF/IL-4 to stimulate the differentiation from Mo to immature DCs and LPS to mature DCs in order to evaluate the overall changes in protein expression induced by this potent maturating agent which simulates bacterial infection, thus providing a model for pathophysiological conditions. We identified 36 proteins, only three being the same as reported by Angenieux *et al.* [9] and five as reported by Le Naour [10]. These differences suggest that the pattern of protein expression during the differentiation and maturation of DCs depends on the stimuli employed.

2 Materials and methods

2.1 Differentiation and maturation of DCs from Mo

DCs were obtained from peripheral blood Mo of healthy donors at the Ribeirão Preto Regional Center for Hemotherapy. Monocytes were isolated using a Ficoll-Hypaque gradient (Hystopaque1077, Sigma Diagnostics, St. Louis, MO, USA) followed by a Percoll gradient (Amersham Biosciences, Uppsala, Sweden) as described by Almeida *et al.* [11]. Mo (2.5×10^7 cells) were distributed into two 25 mL culture flasks, one used to obtain immature DCs and the other to obtain mature DCs. Approximately 2.0×10^7 Mo were used for characterization and protein extraction. Both flasks were cultured for five days in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY, USA; lot# 1185804), 200 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ, USA) and 50 ng/mL IL-4 (PeproTech), to provide immature DCs. The cells in one of the flasks were cultured for two additional days in the same medium containing GM-CSF/IL-4 with the addition of 100 ng/mL LPS (Sigma) to obtain mature DCs. The Mo used for each preparation were obtained from different persons. After culture, cells were counted in a Neubauer chamber and aliquots of each preparation were characterized as described below in Section 2.2. The remaining cells were washed three times with PBS and pelleted by centrifugation (10 min $1000 \times g$ at 4°C) for protein extraction.

2.2 Cell characterization

Immature and mature DCs were characterized by morphology, cell surface markers and phagocytic activity. Approximately 8×10^5 immature and mature DCs were immobilized on a slide by centrifugation (Cytospin 3, Shandon, Pittsburgh, PA, USA) at $75 \times g$ for 10 min, stained with 0.2% w/v May-Grunwald for 25 min and with 1% w/v Giemsa. Slides were dried and examined with a light microscope (Axio Shop 2-plus; Carl Zeiss, Göttingen, Germany).

Cell surface antigens were identified by flow cytometry in a FACSort instrument (Becton Dickinson, San Jose, CA, USA). For each analysis, 1×10^6 cells were stained with the following monoclonal antibodies: HLA-DR-FITC and CD14-PE, CD80-PE, CD86-PE or CD1a-PE (Becton Dickinson). Mouse Ig-γ 1 and Ig-γ 2 were used as controls. Data were collected for 10 000 cells/sample.

The phagocytic activity of immature and mature DCs was determined by the procedure of Chen *et al.* [12], which measures the incorporation of FITC-dextran particles into the cells by flow cytometry. Approximately 2×10^5 cells in 500 μL of RPMI were incubated at 4°C or 37°C with 500 μg/mL FITC-dextran particles (Sigma). Two particle sizes were used: 43.2 kDa or 464.0 kDa. After 30 min, 1 h or 2 h of incubation, cells were washed four times with PBS containing 2% FBS to remove nonphagocytized particles. Finally, cells were fixed in cold PBS containing 5% w/v paraformaldehyde and submitted to analysis by flow cytometry.

2.3 2-DE

To minimize individual differences and to obtain a sufficient quantity of protein for 2-DE, after cell characterization, the protein extracts of four preparations of Mo, immature DCs and mature DCs prepared from four healthy individuals were combined to form a pool of protein extracts of each cell type.

Protein extraction was carried out with 8 M urea, 2% w/v CHAPS, 10 mM Tris-HCl and 1 mM PMSF (200 μL/5×10^7 Mo and 200 μL/1×10^7 immature and mature DCs). After three sonication cycles at 45 W for 5 min each (samples were kept on ice between sonication cycles) in a sonicator bath of 800 mL (Unique, São Paulo, Brazil) at room temperature, the samples were centrifuged at 20 000 × *g* for 30 min at 20°C. The protein content of the supernatant solution of each cell extract was quantified using the detergent compatible kit Bio-Rad DC (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The protein content of the extracts was 0.11 mg/10^7 cells for Mo, 0.87 mg/10^7 cells for immature DCs and 0.75 mg/10^7 cells for mature DCs. The difference in cell size probably contributed to the eight-fold difference in protein content *per* cell.

An aliquot containing 1 mg of each cell extract in 380 μL 8 M urea, 2% w/v CHAPS, 1 mM PMSF, 65 mM DTT, 10 mM Tris-HCl, 0.5% v/v IPG buffer and traces of bromophenol blue was applied to 18 cm pH 4 – 7 IPG strips during rehydration at 20°C for 12 h. IEF was carried out at 20°C at 50 mA/strip, 150 V for 2 h, 500 V for 1 h and 8000 V until 70 000 Vh accumulated (approximately 15 h), in a IPGPhor system (Amersham Biosciences) according to the manufacturer's instructions.

After IEF, strips were equilibrated with shaking for 15 min in 3 mL of 50 mM Tris-HCl, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 0.1% w/v bromophenol blue containing 65 mM DTT, followed by an additional 15 min in the same solution with 100 mM iodoacetamide instead of DTT. SDS-PAGE was carried out on a vertical Daltsix system (Amersham Biosciences) with 12.5%T, 3%C polyacrylamide gels (25 × 20 cm × 1.5 mm) prepared in the laboratory. Six gels were run simultaneously at 15 mA/gel at 10°C. All reagents used were PlusOne grade purchased from Amersham Biosciences. Gel staining was performed using colloidal CBB G-250 (Serva, Crescent Chemical, Hauppauge, NY, USA) as described by Neuhoff *et al.* [13]. Stained gels were digitized with an ImageScanner and analyzed with the ImageMaster V4.0 software (Amersham Biosciences).

The reproducibility of 2-DE was evaluated by preparing three replicate gels with 1 mg of protein from each cell type. Ninety-four percent of the spots were coincident in the three replicates, when the densitometric volume (related to quantity of protein) of the spots was >200, in the range of 20 to 7000. The p*I* and relative mobility were reproducible, with variations of 0.7% (± 0.021 p*I* units) and 3.0%, respectively. However, there was a variation of ± 38% when individual spot volumes stained by colloidal CBB G-250 were measured with ImageMaster software. Differential over- or under-expression was identified when the volume ratio of matched spots on two gels was >2.0 or <0.5 (variation of ± 100%).

2.4 Protein identification by MS

Spots which increased or decreased in volume (protein content) by two-fold or more when extracts of Mo, immature DCs and mature DCs were compared, were excised from the gels and washed four times with 50 mM NH_4HCO_3 containing 50% v/v ACN to remove SDS and dye. They were then washed with ACN and completely dried in a SpeedVac (Savant Instrument, Farmingdale, NY, USA). Each spot was rehydrated with 10 μL 50 mM NH_4HCO_3 containing 0.3 μg of sequence grade modified trypsin (Promega, Madison, WI, USA). After 30 min of rehydration with the trypsin solution, spots were covered with 50 mM NH_4HCO_3. The hydrolysis reaction was carried out at 37°C for 24 h and stopped by the addition of 1 μL of TFA.

Peptides were extracted twice from the gel with 40 μL 2.5% v/v TFA containing 50% v/v ACN for 3 h. Extracts were concentrated in a SpeedVac until the volume was approximately 5 μL and resuspended in 5 μL 2.5% v/v TFA containing 50% v/v ACN. One microliter of each sample was mixed with 1 μL of 10 mg/mL matrix solution (CHCA, Sigma) in 0.1% v/v TFA containing 50% v/v ACN. Samples were applied to a MALDI-TOF MS plate in duplicate, dried and submitted to PMF analysis with an Ettan MALDI-TOF Pro instrument (Amersham Biosciences). Trypsin autolysis peptides 842.51 Da and 2211.10 Da were used for internal mass calibration and [Ile7]-angiotensin III (897.53 Da) and ACTH peptide 18–39 (2465.20 Da) were used for external calibration of the instrument. The data were analyzed automatically and proteins were identified using the NCBI database. Mass lists of each spectrum were also submitted manually to the MS-Fit tool (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and the Swiss-Prot database, confirming all the identifications made automatically. Search parameters were 0.2 Da peptide mass tolerance and 1 maximum missed cleavage. An electrospray triple-quadrupole spectrometer Quattro II (Micromass, Manchester, UK) was used for sequencing some tryptic peptides from spot 6 (actin).

The functional classification of the proteins identified was based on the Gene Ontology database [14] using the FatiGO tool (http://fatigo.bioinfo.cnio.es) [15], permitting the standardization of the terms used to describe classes and functions of proteins.

3 Results and discussion

3.1 Characterization of DCs

The differentiation of peripheral blood Mo to immature DCs was stimulated using GM-CSF/IL-4 for five days and the maturation of immature DCs to mature DCs was performed

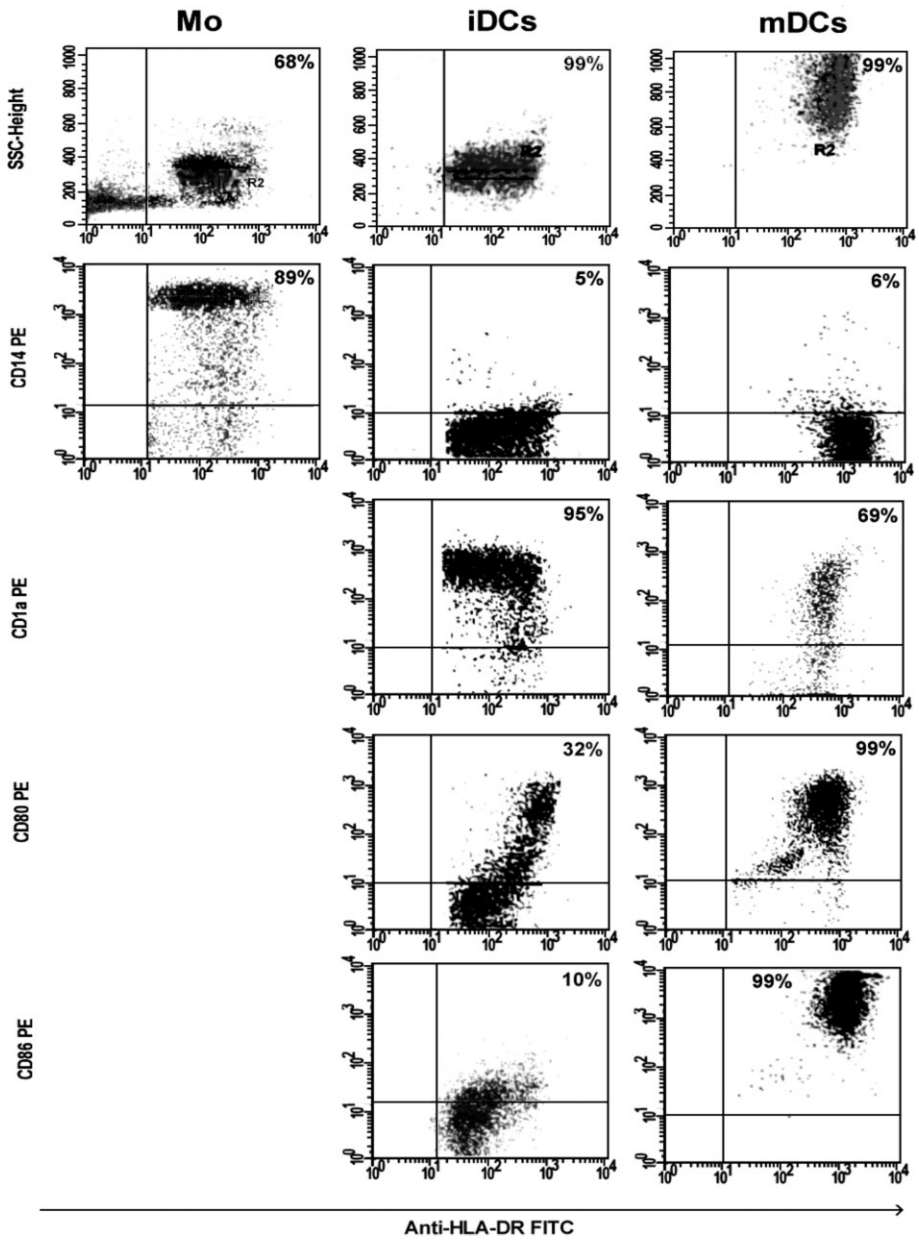

Figure 1. The figure illustrates results obtained by flow cytometry with one preparation and is representative of the four preparations used in this study. Monocyte-derived DCs were obtained after 5 days of culture in the presence of GM-CSF/IL-4 (immature DCs) and after 2 additional days in the presence of LPS (mature DCs). The specific PE-labeled antibodies used were: CD14 (Mo), CD1a (immature DCs), CD86 or CD80 (mature DCs). The data are represented as dot plot graphs indicating the percentage of cells expressing each marker. The abscissa indicates the number of cells that reacted with anti-HLA-DR-FITC (MHC class II) and the ordinate indicates the cells that also reacted with the specific PE-labeled antibodies. The top horizontal row of panels indicates the granularity (SSC-Height) of the cells and the remaining panels show the specific markers listed on the left side of the figure. Mouse Ig-γ 1 and Ig-γ 2 were used as negative controls and presented values below 0.5%.

in the presence of GM-CSF/IL-4 plus LPS for an additional two days. For each of four preparations, starting with 2.5×10^7 Mo, we obtained on average 2.10×10^7 (SD $\pm$ 0.11) immature DCs and 1.70×10^7 (SD $\pm$ 0.08) mature DCs.

The specific cell markers used for characterization by flow cytometry were CD14 for Mo, CD1a for immature DCs, and CD80 and CD86 for mature DCs. The Mo, immature DCs and mature DCs prepared from four different individuals had similar characteristics and were combined after protein extraction to minimize individual differences and to obtain a sufficient quantity of protein for 2-DE. Figure 1 illustrates the flow cytometry analysis for one of these preparations and shows the reduction of the CD14 Mo marker from 89% in Mo to 5% in immature DCs and to 6% in mature DCs as a result of differentiation and maturation. The percentage of cells expressing CD1a, considered to be a marker for immature DCs, decreased from 95% to 69% during the maturation while the mean fluorescence intensity decreased four-fold (1000 units in immature DCs to 250 units in mature DCs). Regardless of the unexpected level of cells expressing CD1a (69%) in the mature DC preparation, these cells had no phagocytic activity, as expected, and presented morphological characteristics of mature DCs, as shown below. Figure 1 also shows that after the stimulation of immature DCs with LPS, the mature DC markers CD80 and CD86 increased from 32% to 99% and from 10% to 99%, respectively.

The morphology of the DCs was similar to that described by Bykovskaia *et al.* [16]. Figure 2 shows that immature DCs have a spherical shape while mature DCs are slightly elongated and present characteristic membranous projections or dendrites. Mature DCs have a decentralized nucleus, characteristic of secretory cells and they increased in size during

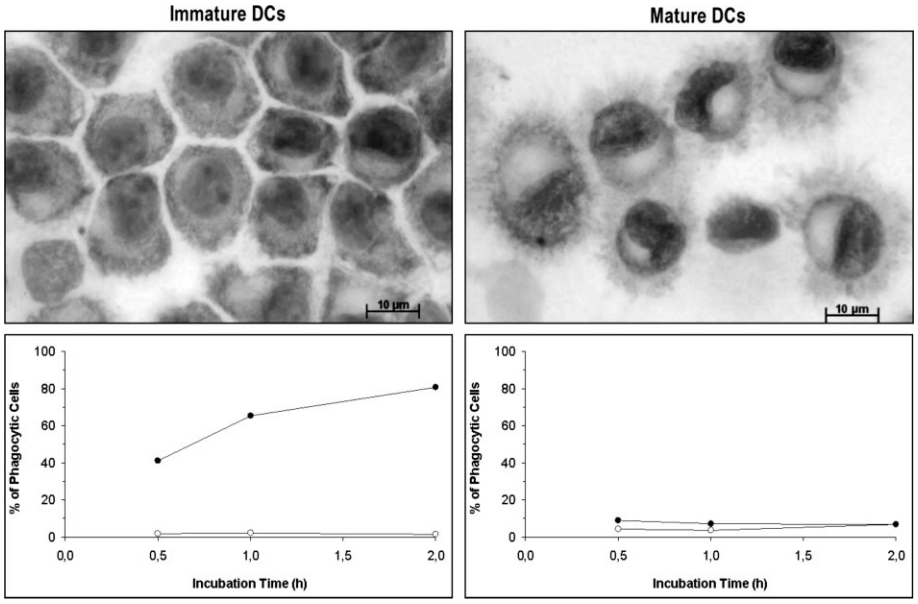

Figure 2. Morphology and phagocytic activity of immature and mature DCs obtained from monocytes. Top Panels. Morphology: DCs obtained after 5 days of culture in the presence of GM-CSF/IL-4 were spherical in shape, a morphological characteristic of immature DCs. After 2 additional days of culture in the presence of LPS, the characteristic membranous projections of mature DCs become pronounced. Cells were stained with May-Grunwald-Giemsa and observed with a light microscope (1000× magnification). Lower Panels. Phagocytic activity: cells (2.0×10^5) were incubated at 4°C (-○-) or 37°C (-●-) with 25 µg/mL FITC-dextran particles (43.2 kDa). The number of cells that phagocytosed the particles was determined by flow cytometry.

maturation. Granularity (SSC-Height) also increased by going from Mo to immature DCs to mature DCs, as demonstrated by flow cytometry data (Fig. 1, top row).

DCs were also characterized by their phagocytic activity on 43.2 kDa dextran particles labeled with FITC by flow cytometry. Figure 2 shows the percent of immature and mature DCs that incorporated the particles as a function of time. Immature DCs incorporated the particles in a time-dependent manner and after 2 h, 81% of the cells had phagocytized at least one particle. In contrast, only 7% of mature DCs presented phagocytic activity. When the same experiment was carried out at 4°C as a control, only 1% of immature DCs and 6% of mature DCs incorporated labeled particles. Similar results were obtained using larger particles of 464.0 kDa (data not shown). This assay illustrates the functional difference between immature DCs and mature DCs in terms of phagocytic activity [17].

3.2 Proteomic profile of monocytes, immature and mature DCs

2-DE was used to separate proteins and determine changes in the amount of specific proteins during the differentiation and maturation of monocyte-derived DCs. Figure 3 shows the 2-DE CBB stained gels (pH 4–7) representative of three consistent replicates of total protein extracts of Mo (top panel), of immature DCs (middle panel) and of mature DCs (bottom panel), which were used for comparative and semiquantitative spot analysis. The number of spots detected *per* gel was 336 for Mo, 385 for immature DCs and 280 for mature DCs. However, some of these spots were not visible to the naked eye or in the photograph and were detected only in high-contrast digitized gels.

Only spots having a volume greater than 200 (range 20 to 7000) were considered for detection of differential expression. The number of spots with a volume variation between cell types greater than two-fold during differentiation from Mo to immature DCs was 33 (10 increased and 23 decreased). During maturation of immature DCs to mature DCs there were 38 differentially expressed spots (22 increased and 16 decreased). However, the total number of spots that increased or decreased was 40 during differentiation and maturation, because frequently the same spot was differentially expressed during both processes. Indeed, 63 spots were detected in only one of the three stages, 26 were only present in the Mo, 24 were only present in the immature DCs and 13 were only present in the mature DCs. These 103 spots of interest (40 differentially expressed and 63 specific for each cell stage) were cut out of the 2-DE gels and submitted to MS analysis.

3.3 Identification of differentially expressed proteins

Proteins were identified by PMF with a MALDI-TOF mass spectrometer after *in situ* digestion with trypsin. Of the 103 differentially expressed spots, 63 (numbered in Fig. 3) were identified without ambiguity with sequence coverage of 20% to 79% (Table 1, column 7). These 63 spots correspond to only 36 different proteins, because 11 proteins were detected as 38 multiple spots (multiple electrophoretic forms). Actin and vimentin, for example, were identified in 6 and 10 electrophoretic forms, respectively, demonstrating the occurrence of post-translational modifications and/or differences at the genetic level.

Twenty of 63 proteins had a greater than 20% difference between the apparent molecular mass from the 2-DE gel and the mass obtained from the Swiss-Prot database, probably due to post-translational modifications (compare columns 4 and 5, Table 1). Fourteen spots presented an apparent mass greater than that reported by the database, which might

Table 1. Differentially expressed proteins during differentiation and maturation of DCs

Spot number[a)]	Protein name[b)]	Relative volume[c)] (Mo:iDCs:mDCs)			M_r / p*I* 2-DE[d)]	M_r / p*I* DataBank[a)]	Masses matched[f)] # / (%)	Protein covered (%) MS-FIT / automatic	Accession number Swiss-Prot	Biological process[g)]
1	[1] Actin, cytoplasmic 1	3	1	1	53.2/5.5	41.7/5.3	15 (57)	43/44	P02570	f
2	[1] Actin, cytoplasmic 1	7	1	2	45.1/5.7	41.7/5.3	9 (50)	30/36	P02570	f
3	[1] Actin, cytoplasmic 1	2	1	3	52.8/5.4	41.7/5.3	10 (62)	35/38	P02570	f
4	[1] Actin, cytoplasmic 1	21	1	6	53.2/5.5	41.7/5.3	13 (56)	46/44	P02570	f
5	[1] Actin, cytoplasmic 1	2	1	1	36.2/5.7	41.7/5.3	7 (70)	17/29	P02570	f
6	[1] Actin, cytoplasmic 1*	3	2	1	16.4/5.0	42.0/5.2	7 (33)	18/16	P02570	f
7	[2] Alpha enolase	2	2	1	45.8/6.1	49.5/5.8	5 (26)	14/34	Q05524	b
8	[3] ARP2/3 16 kDa	1	–	–	20.0/5.8	16.3/5.5	7 (63)	45/45	O15511	a,f
9	[4] ATP synthase beta chain	1	–	1	60.0/5.2	56.6/5.3	20 (50)	39/48	P06576	a,b
10	[5] ATP synthase D chain	–	1	2	24.7/5.4	18.5/5.2	11 (78)	73/73	O75947	a
11	[6] Calreticulin	2	4	1	56.2/4.1	48.1/4.3	9 (75)	32/31	P27797	b,c
12	[7] Chloride intracellular channel (RNCC)	3	1	1	37.2/5.3	26.9/5.1	12 (70)	64/49	O00299	a
13	[8] F-actin capping protein beta subunit	3	1	1	37.2/5.9	31.3/5.4	16 (64)	49/42	P47756	a
14	[9] Fibrinogen gamma chain [Precursor]	5	1	–	58.6/5.9	51.5/5.4	10 (40)	21/31	P02679	a,h,l,m,n
15	[10] Galectin-1	1	1	2	14.0/5.2	14.7/5.3	9 (34)	72/59	P09382	c,k,l
16	[11] Glucose-regulated protein 58 kDa (Grp58)	3	1	3	62.5/6.1	56.7/6.0	23 (67)	42/40	P30101	a,b,c
17	[11] Glucose-regulated protein 58 kDa (Grp58)	2	1	4	63.1/6.0	56.8/6.0	19 (73)	36/34	P30101	a,b,c
18	[12] Glucose-regulated protein 75 kDa (Grp75)	1	–	1	70.9/5.8	73.7/5.9	15 (71)	28/28	P38645	–
19	[13] Glucose-regulated protein 78 kDa (Grp78)	2	1	3	73.2/5.1	72.3/5.1	25 (71)	40/39	P11021	–
20	[13] Glucose-regulated protein 78 kDa (Grp78)	5	1	3	73.2/5.1	72.3/5.1	20 (80)	35/36	P11021	–
21	[13] Glucose-regulated protein 78 kDa (Grp78)	2	1	1	73.3/5.2	72.3/5.1	25 (71)	40/40	P11021	–
22	[14] Glutathione S-transferase P	4	1	1	29.3/5.8	23.3/5.4	7 (24)	55/45	P09211	b,d
23	[15] Heat shock cognate 71 kDa protein	–	3	1	49.5/5.0	70.9/5.4	9 (42)	19/22	P11142	b
24	[15] Heat shock cognate 71 kDa protein	2	–	1	71.4/5.6	70.9/5.4	24 (75)	30/47	P11142	b
25	[15] Heat shock cognate 71 kDa protein	3	1	5	69.2/5.5	70.9/5.4	10 (50)	17/24	P11142	b
26	[16] Heat shock protein 60 kDa	2	1	3	65.2/5.5	61.0/5.7	24 (92)	47/48	P10809	a,b
27	[17] Lamin B1	1	–	–	71.0/5.4	66.4/5.1	24 (82)	39/37	P20700	–
28	[18] Lymphocyte-specific protein 1	1	3	12	60.0/4.4	37.1/4.7	5 (41)	22/28	P33241	c,e,f,g
29	[18] Lymphocyte-specific protein 1	1	1	6	60.0/4.5	37.1/4.7	7 (46)	27/33	P33241	c,e,f,g
30	[18] Lymphocyte-specific protein 1	1	2	4	60.0/4.5	37.1/5.7	7 (43)	27/33	P33241	c,e,f,g
31	[19] Lymphocyte-capping protein	2	1	3	46.3/6.3	38.5/5.9	8 (50)	25/24	P40121	b,e,g
32	[19] Lymphocyte-capping protein	9	1	–	48.2/6.3	38.6/5.9	9 (37)	25/26	P40121	b,e,g
33	[20] MHC I	1	5	1	12.1/4.8	–	–	–/35	P30480	–
34	[21] Migration inhibitory factor-related (MRP14)	1	–	–	15.6/5.7	13.2/5.7	9 (64)	56/57	P06702	c,e,g
35	[21] Migration inhibitory factor-related (MRP14)	6	1	2	13.7/5.8	13.2/5.7	11 (40)	82/75	P06702	c,e,g
36	[22] Myosin light chain	2	3	1	13.3/4.4	16.9/4.6	4 (36)	31/31	P16475	d
37	[23] Myotrophin	2	1	1	12.1/5.2	12.9/5.3	4 (44)	39/48	P58546	a,b,d,j
38	[24] Proteasome subunit alpha type 5	1	–	1	32.4/4.7	26.5/4.7	6 (37)	28/30	P28066	b
39	[25] Protein disulfide isomerase precursor	4	–	1	64.2/4.8	57.1/4.8	11 (44)	25/25	P07237	b
40	[26] Ras-related protein Rap-1b	1	–	–	25.5/6.1	20.8/5.6	12 (66)	71/71	P09526	c
41	[27] Rho GDP-dissociation inhibitor 1	9	–	1	32.1/5.1	23.2/5.0	8 (61)	34/35	P52565	c
42	[28] Rho GDP-dissociation inhibitor 2	20	–	3	31.5/5.3	23.0/5.1	9 (47)	69/70	P52566	a,c,e
43	[28] Rho GDP-dissociation inhibitor 2	1	1	–	16.4/6.3	23.0/5.1	4 (30)	26/–	P52566	a,c,e
44	[29] S100 A6	1	2	–	10.1/5.2	–	–	–/46	P06703	–
45	[30] S100 A4	2	1	1	11.4/5.8	11.7/5.9	4 (57)	36/28	P26447	–

Table 1. Continued

Spot number[a]	Protein name[b]	Relative volume[c] (Mo:iDCs:mDCs)			M_r / p*I* 2-DE[d]	M_r / p*I* DataBank[a]	Masses matched[f] # / (%)	Protein covered (%) MS-FIT / automatic	Accession number Swiss-Prot	Biological process[g]
46	[31] SH3 domain-binding protein	2	2	1	10.6/4.7	10.4/4.8	5 (41)	39/40	Q9H299	–
47	[32] Strathmin	1	–	–	20.3/6.0	17.3/5.8	6 (66)	44/36	P16949	a,c
48	[33] Thioredoxin	1	3	14	13.3/5.0	11.7/4.8	9 (31)	79/52	P10599	a,b,c,f
49	[34] Tropomyosin alpha 4 chain	1	5	4	37.1/4.8	28.5/4.7	18 (48)	55/48	P07226	d
50	[34] Tropomyosin alpha 4 chain	6	1	3	37.1/4.9	28.5/4.7	15 (51)	42/35	P07226	d
51	[35] Tropomyosin alpha 4 chain	5	1	–	60.60/5.1	49.7/4.8	19 (70)	43/47	P05218	a,k,l
52	[35] Tropomyosin alpha 4 chain	3	1	–	40.5/5.2	49.7/4.8	12 (75)	31/38	P05218	a,k,l
53	[35] Tropomyosin alpha 4 chain	4	1	10	42.1/5.8	49.7/4.8	8 (38)	25/26	P05218	a,k,l
54	[36] Vimentin	1	–	–	63.1/5.3	53.7/5.1	22 (73)	52/46	P08670	–
55	[36] Vimentin	–	2	1	51.9/4.8	53.7/5.1	28 (75)	55/33	P08670	–
56	[36] Vimentin	–	3	1	32.1/4.9	53.7/5.1	18 (75)	31/25	P08670	–
57	[36] Vimentin	1	6	1	56.8/5.1	53.7/5.1	28 (90)	56/49	P08670	–
58	[36] Vimentin	1	8	2	55.0/5.0	53.7/5.1	30 (85)	57/47	P08670	–
59	[36] Vimentin	1	7	2	52.9/4.8	53.7/5.1	21 (77)	50/35	P08670	–
60	[36] Vimentin	1	6	–	60.5/5.3	53.7/5.1	15 (93)	35/32	P08670	–
61	[36] Vimentin	1	2	–	52.8/4.7	53.7/5.1	23 (82)	50/43	P08670	–
62	[36] Vimentin	–	1	4	60.4/5.5	53.7/5.1	14 (73)	34/27	P08670	–
63	[36] Vimentin	–	3	1	34.8/5.9	53.7/5.1	16 (84)	33/27	P08670	–

a) The proteins correspond to spots whose volume changed ± two-fold during differentiation of Mo to immature DCs stimulated with GM-CSF/IL-4 or maturation of immature DCs to mature DCs stimulated by LPS. Spots, numbered in Fig. 3, were cut from 2-DE gels, hydrolyzed with trypsin and submitted to MALDI-TOF MS analysis.

b) Eleven proteins were detected as 38 spots (multiple electrophoretic forms) and those having different databank accession numbers were treated as different proteins.

c) Relative volumes of matched spots determined by Image Master 4.0 (Amersham Biosciences).

d) Apparent mass and p*I* obtained from 2-DE gels.

e) Mass and p*I* obtained from Swiss-Prot database.

f) The PMFs were submitted to the Swiss-Prot database using the MS-FIT tool and to the NCBI database automatically by instrument software. # indicates the number of peptides matched and (%) reports the percentage of matched peptide masses in relation to total number of peptide masses submitted.

g) The biological process in which these proteins are involved was determined by Gene Ontology: a = cell growth/maintenance, b = metabolism, c = cell communication, d = morphogenesis, e = response to an external stimulus, f = cell motility, g = response to stress, h = circulation, i = regulation of cellular processes, j = cell differentiation, k = death, l = cell death, m = coagulation, and n = hemostasis. * Spot 6 identified as alpha cardiac actin is actually the *N*-terminal fragment of the cytoplasmic actin 1.

indicate addition of prosthetic groups (glycosylation, for example), and six spots presented mass values lower than the database mass, suggesting proteolytic processing. Spot 6 (Table 1) with 16 kDa was identified in the present study as actin. The peptides detected in the mass fingerprint of spot 6 correspond to 72% of the *N*-terminal region (residues 1 to 97) of the primary structure of cytoplasmic actin, which has a mass of 42.0 kDa in the Swiss-Prot database. We have confirmed that spot 6 corresponds to an *N*-terminal fragment of actin by completely sequencing peptides corresponding to residues 21–30 (AGFAGDDAPR), 42–52 (HQGVMVGMGQK), 87–97 (IWHHTFYNELR) using ESI-MS/MS.

Post-translational modifications such as phosphorylation or acetylation can be visualized in 2-DE gels as a "train of spots" differing only by their p*I*. These protein modifications were not characterized in the present study, but appear to be present in tropomyosin 4 (spots 49 and 50) that was identified in two molecular forms with a p*I* difference of 0.05 units (see Fig. 4) and in the lymphocyte-specific protein 1 (spots 28 to 30), identified in three molecular forms with a p*I* difference of 0.1 units. Phosphorylation is a post-translational modification already described for lymphocyte-specific protein 1, and is regulated by MAPKAP kinase 2 [18]. Note also in Fig. 4 that the molecular forms a and b of tropomyosin 4 are differentially expressed during the differentiation and maturation.

Some proteins presented differences in both mass and p*I* compared to the Swiss-Prot database. Vimentin, for example (spots 54 to 63) (Fig. 4), is present in 10 molecular forms ranging in mass from 32 – 63 kDa. The peptides detected by PMF for the two smallest forms of vimentin (spots 56 and 63) indicate that both proteins are truncated at the *C*-terminal portion, since all peptides characterized were only observed

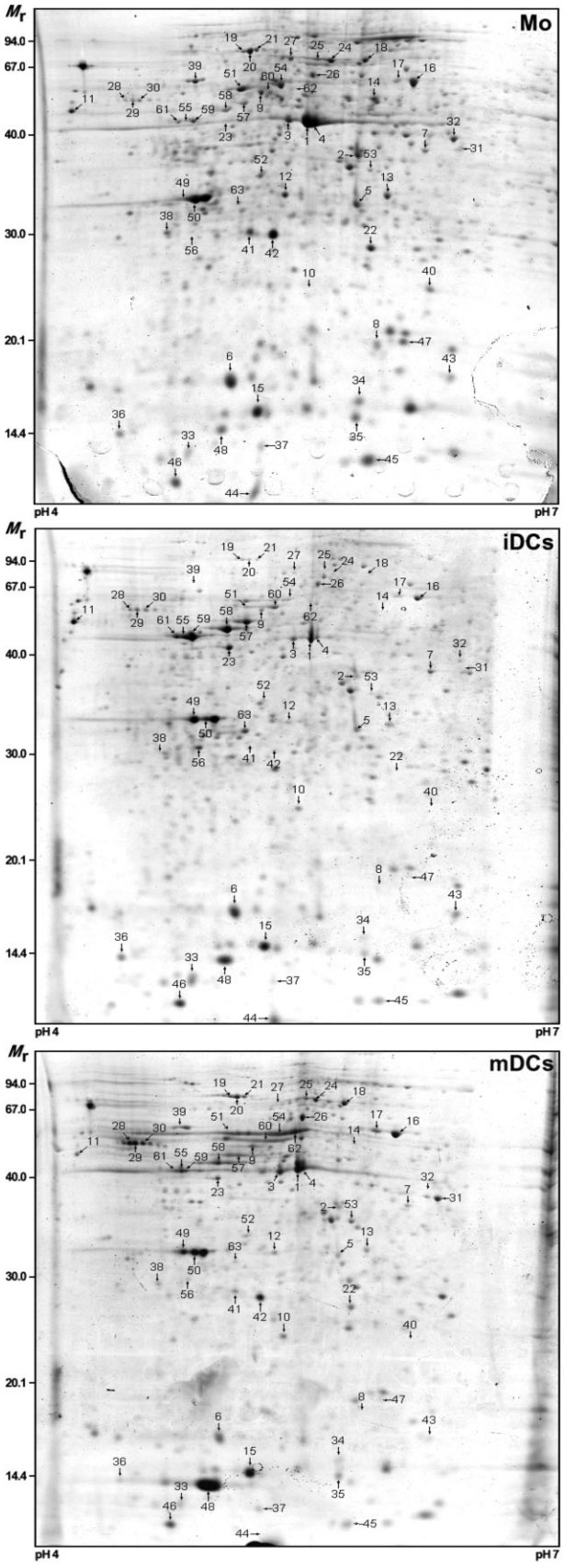

Figure 3. 2-DE gels of total extracts of monocytes (Mo), immature DCs (iDCs) and mature DCs (mDCs) are presented from top to bottom. One milligram of total cell extract protein was applied to pH 4 – 7 IPG strips and IEF was carried out for 70 000 Vh. After reduction and alkylation, IPG strips were mounted on the top of a 12.5%T, 3%C polyacrylamide gels (20×25 cm $\times$ 1.5 mm) and SDS-PAGE was carried at 15 mA/gel. Colloidal CBB was used to stain the gels. Images of each gel were analyzed with the ImageMaster V.4 software. The number of spots detected was 336 spots in the monocyte profile, 385 in the immature DC profile and 280 in the mature DC profile. The 103 differentially expressed proteins were submitted to *in situ* trypsin digestion. The 63 spots that were identified by PMF using in a MALDI-TOF MS are numbered in the gel and listed in Table 1.

in the *N*-terminal portion (data not shown). Cleavage by caspases of vimentin at Asp 85 and Asp 259 has been reported [19]. However, this probably did not occur in the present study, since the analysis of the PMF of all electrophoretic forms of vimentin showed the presence of peptides both before and after these sites of caspase cleavage. Indeed, Eriksson *et al.* [20] identified several phosphorylation sites in vimentin, which regulate the cytoskeleton intermediate filament assembly, indicating that phosphorylation can contribute to the larger number of molecular forms of vimentin described here.

Table 1 (column 3) also indicates the differential expression of the proteins in Mo, immature DCs and mature DCs. The largest differences of expression were observed for actin (spot 4, 21-fold higher in Mo), lymphocyte-specific protein 1 (spot 28, 12-fold higher in mature DCs), Rho GDP-dissociation inhibitor 2 (spot 42, 20-fold higher in Mo) and thioredoxin (spot 48, 14-fold higher in mature DCs). The functional correlation of these proteins in the cells where they were up-regulated will be discussed later in Section 3.4.

Table 2 lists all proteins identified in multiple electrophoretic forms and their regulation during differentiation/maturation of DCs. In some cases, most of the forms of one protein were up-regulated in a specific cell type. For example, 5 of the 6 electrophoretic forms of actin (spots 1 to 6) were up-regulated in Mo and 8 of the 10 forms of vimentin (spots 54 to 63) were up-regulated in immature DCs. However, other proteins were identified in the 2-DE gels as multiple spots differentially expressed in each cell stage. The heat shock cognate 71 kDa protein (spots 23 to 25) presented one electrophoretic form up-regulated in Mo, another form up-regulated in immature DCs and a third form up-regulated in mature DCs. These results suggest that the different electrophoretic forms of a protein probably have different roles during differentiation and maturation of DCs.

Thirty percent of the proteins identified (11/36) were present in multiple molecular forms of proteins described in the NCBI database, indicating that many of the proteins

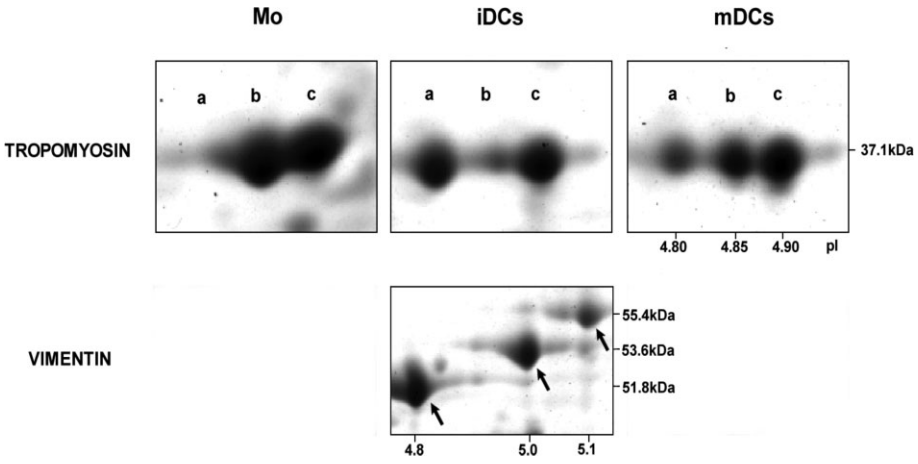

Figure 4. The two forms of tropomyosin 4 (forms a and b) was identified as two electrophoretic forms with similar migration (mass) but different p*I* values and were differentially expressed in Mo, iDCs and mDCs (see relative volume of spots 49 and 50 in Table 1, column 3). Spot c was identified as tropomyosin 3, and was expressed to the same extent by the three cell types. The protein vimentin was identified in 10 different molecular forms. In this figure we present three forms of vimentin (spots 57, 58 and 59) which showed variations in both p*I* and mass.

Table 2. Multiple electrophoretic forms of proteins detected during differentiation and maturation of DCs.

Protein[a)]	Number of multiple electrophoretic forms up-regulated		
	Mo	iDCs	mDCs
Actin, cytoplasmic 1	5	0	1
Glucose-regulated protein 58 kDa	1	0	2
Glucose-regulated protein 78 kDa	2	0	1
Heat shock cognate 71 kDa protein	1	1	1
Lymphocyte-specific protein 1	0	0	3
Macrophage capping protein	1	0	1
Migration inhibitory factor-related protein 14	2	0	0
Rho GDP-dissociation inhibitor 2	2	1	0
Tropomyosin alpha 4 chain	1	1	0
Tubulin beta-5 chain	2	0	1
Vimentin	1	8	1

a) Eleven of the 36 proteins differentially expressed during maturation and differentiation of DCs were detected as multiple spots. These proteins presented variations in p*I*, apparent molecular mass or both (see Fig. 4). Some of the multiple molecular forms of one protein were differentially expressed when going from one cell stage to another.

actually present in the cells at any one time are not exactly the same as those coded by the DNA. In addition, we have demonstrated the differential expression of some of these multiple molecular forms during differentiation and maturation. Thus our results support the view that many of the changes in protein expression that accompany differentiation/maturation of DCs occur at the post-translational level as suggested by Le Naour *et al.* [10] and Richards *et al.* [21].

3.4 Functions of the differentially expressed proteins

The 36 proteins identified in the present study were classified on the basis of presumed function by the Gene Ontology database, see column 9 in Table 1. Proteins up-regulated in Mo are mainly related to cell growth and/or maintenance (36%), metabolism (32%) and cell communication (21%). In immature DCs, up-regulated proteins are related to metabolism (30%), morphogenesis (20%) and cell communication (20%); and in mature DCs they are related to metabolism (50%), cell growth and/or maintenance (36%) and cell communication (29%). We also classified proteins on the basis of their location in the cell by Gene Ontology and observed that the proteins identified in the present study are associated with the cytoskeleton (31%), endoplasmic reticulum (14%), mitochondria (11%), and cytosol (8%), and 36% were not classified.

Most interesting is the fact that some of the 36 differentially expressed proteins identified in the present study have been associated with specific functions in DCs. In general, these proteins have been related to functional and morphological characteristics of DCs, which are associated with their stage of maturation. These proteins were reported individually in the DC literature and the integration and correlation of their regulation level, as the result of proteomic studies, should provide further understanding of the process of differentiation/maturation of DCs. Thioredoxin (Trx) was up-regulated in mature DCs (14-fold). In all organisms Trx is involved in protein disulfide reduction, in defense against oxidative stress, regulation of growth and apoptosis, but it is also secreted and has co-cytokine and chemokine activities. The extracellular human Trx has pro-inflammatory activity by potentiating cytokine release, which causes migration of T cells. The absence of cytosolic mammalian Trx is embryonically lethal and when Trx levels are elevated in cells, there is increased cell growth and resistance to normal mechanism of programmed cell death [22]. Angelini *et al.* [23] demonstrated that monocyte-derived DCs, after stimulation with LPS or TNF-α, accumulate Trx and cystine and only the con-

tact with T cells triggers the secretion of these proteins by DCs. Presumably, this provides the reducing extracellular microenvironment required for T lymphocyte activation, facilitating the immune response.

Chaperones such as grps, disulfide isomerase and calreticulin play a role in antigen processing and presentation and in immune response stimulation [24]. The expression of grp78, identified in three electrophoretic forms (all down-regulated during differentiation and two forms up-regulated during maturation), is very likely to contribute to the folding of many secreted and membrane proteins of the DCs, including the antigen presenting proteins. One phosphorylated form of grp78 showed increased expression during differentiation of a mouse myeloid leukemia cell line into macrophage-like cells by treatment with IL-6 [25]. Calreticulin, identified as up-regulated in immature DCs, contributes to the folding pathways for nascent glycoproteins and also participates specifically in the assembly of major histocompatibility complex (MHC) class I-peptide complex, a process required for antigen peptide presentation on the cell surface [26]. Heat shock proteins when released into the extracellular milieu can act as a source of antigen due to their ability to chaperone peptides and also as a maturation signal for DCs. Hsp 71 was identified in three multiple molecular forms, each one up-regulated in a specific cell stage. Hsp 60 (up-regulated in mature DCs) induces human DCs maturation and activates human DCs to secrete pro-inflammatory cytokines [27].

The expression of MHC I, up-regulated during differentiation to immature DCs and down-regulated after maturation, is consistent with the known cytoplasmic accumulation of the MHC I molecule during the antigen assembly process in immature DCs, which is then translocated to the cell surface in mature DCs.

The regulated expression of many cytoskeletal or related proteins (actin, ARP2/3, F-actin capping protein, laminin, macrophage capping, myosin, stathmin, tropomyosin, tubulin and vimentin) during differentiation and maturation of DCs is consistent with the fact that DCs undergo rapid structural changes in size (during differentiation, within 5 days) and morphology (during maturation, within 2 days). In immature DCs, for example, 8 of the 10 forms of vimentin were up-regulated, while mature DCs showed up-regulation of tubulin and lymphocyte-specific protein 1 (LSP1). These proteins up-regulated in immature DCs or mature DCs could be associated with at least two cytoskeleton-dependent phenomena: phagocytosis/vesicle traffic in immature DCs and migration/motility in mature DCs. LSP1 (12-fold increased in mature DCs) is an F-actin binding protein found in all hematopoietic cells. There is evidence suggesting that LSP1 is an important signaling molecule that regulates microfilamentous cytoskeleton structure and motile function in all leukocytes, as well as receptor-induced apoptosis [28]. LSP1 overexpression has been reported to be responsible for the morphologic and motile abnormalities of neutrophils of patients with neutrophil actin dysfunction disorder (NAD 47/89) [29]. Although several of these structural proteins are probably constitutive in many cells, they also have important functions in differentiation and maturation.

Rho GDP inhibitors, down-regulated during differentiation and maturation, act as inhibitors of Rho GTPases which have been reported to be related to regulation of morphology and function of DCs. Kobayashi *et al.* [30] reported that the inhibition of Rho GDPases results in the loss of dendrites and significantly suppresses the antigen presentation capacity of DCs. Therefore, the decreased (up to nine-fold) expression of these inhibitors is consistent with the antigen presentation functions of DCs.

Proteins not previously reported to be present in DCs have been identified in the present study. Some of them, such as alpha enolase (an essential glycolytic enzyme), ATP synthase (phosphorylation of ADP to ATP in mitochondria during oxidative phosphorylation) and RNCC (nuclear chloride channel) are related to cell metabolism. Galectin-1, up-regulated during maturation, is a galactose binding lectin reported in Langerhans cells, suggesting its importance for cell-cell contact and/or adhesion in the epidermis and for cell-extracellular matrix interaction in the dermis. Also, galectin-1 has been reported to trigger a homeostatic signal to shut off T-cell effector functions [31].

3.5 Comparison of the results of the present study with those reported in the literature

The great interest in understanding the mechanisms of differentiation, antigen presentation, maturation and migration of DCs and their clinical/immunotherapeutic applications have caused DCs to be studied recently by comparative methodologies, such as proteomics and RNA microarrays. Integrated studies at the protein and RNA level were reported in two recent papers similar to the present study. Angenieux *et al.* [9] analyzed only the differentiation of the Mo to immature DCs in RPMI medium with 10% FCS in the presence of GM-CSF (50 ng/mL)/IL-4 (40 ng/mL) for 7 days, and reported 10 differentially expressed proteins and 13 RNA transcripts (± 2-fold). Le Naour *et al.* [10] studied the differentiation of DCs from Mo in serum-free medium with GM-CSF (100 ng/mL)/IL-4 (50 ng/mL) and maturation stimulated by TNF-α (10 ng/mL). They reported that 18 proteins (spots) and 255 transcripts (RNA) were differentially expressed (± 2.5-fold) during differentiation (from Mo to immature DCs to mature DCs) and that few differences were found between immature and mature DCs. In the present study of the differentiation of Mo to immature DCs and to mature DCs, we used RPMI medium with 10% FBS, GM-CSF (200 ng/mL)/IL-4 (50 ng/mL) and LPS, a potent stimulus of DC maturation [32]. We identified 56 differentially expressed spots (± two-fold) during differentiation (from Mo to immature DCs) and 50 during maturation (from immature DCs to mature DCs), which correspond to 36 different proteins.

The large difference in the number of differentially expressed proteins identified in the present study and the fact that only three proteins were the same as reported by [9] and five as reported in [10], and that only vimentin was differentially expressed in all three studies (see Table 3), suggest significant differences between the cells studied. The two other studies [9, 10] analyzed 2-DE gels with a p*I* range of 4–8, and in the present study we used gels with a p*I* range of 4–7. However, the differences in the proteins identified between the present study and the other two studies is not due to the loss of proteins in the p*I* 7–8 range, because none of the proteins identified in the two previous studies had a predicted p$I > 7$.

The differentially expressed proteins detected during maturation stimulated by LPS cultured in presence of FBS and the larger number of proteins compared to those reported in a previous study [10] which used TNF-α in a serum-free medium, suggest that the DCs produced depend on the maturation stimuli and conditions of culture used, even when the same precursor cells (Mo) are used. There is evidence for the fact that the functional properties of DCs are different when different stimuli are used [33]. Comparative analysis of genes expressed in DCs after stimulation with TNF-α and LPS indicates a difference in modulation of gene expression. Only LPS-treated cells showed a pattern of gene expression compatible with a definitive growth arrest and with a suitable activation and control of the immune response. [34]. It has also been reported that TNF-α induces a semimaturation of DCs, detected by the high expression of MHC-II molecules but in the absence of expression of pro-inflammatory cytokines, while LPS induces a fully mature DC, which produces high levels of cytokines [35]. We can also speculate that the expression of thioredoxin, one of the most abundant proteins in mature DCs in the present study (not detected in the study by Le Naour *et al.* [10]), and which has pro-inflammatory activity by potentiating cytokine release [22] can be correlated to the fact that LPS-stimulated mature DCs have a high production of pro-inflammatory cytokines [35]. Shortman and Liu [36] have emphasized the functional plasticity of DCs pointing out that different signals for maturation induce different subtypes of DCs and consequently different immunological responses. These literature data, taken together with our results and those reported [9–10] provide additional evidence that the nature of the DCs produced depends on the stimuli employed.

The proteins identified here and in the two previous studies could be classified into two major classes: proteins related to cytoskeleton and chaperones (see Table 3). Comparison of the differentially expressed proteins identified by Angenieux *et al.* [9] with our results shows two proteins from the cytoskeleton (vimentin and ARP2/3) and 1 chaperone (grp78). However, the 16 kDa form of ARP2/3 was identified in our study (detected only in Mo) and the 34 kDa form was identified by Angenieux *et al.* [9] (up-regulated during differentiation). From the grp family, they identified grp78 and grp94-96 (both up-regulated) and we identified grp78 in three molecular forms (three down-regulated during differentiation and two up-regulated during maturation) and also grp58 and grp75. Five of the 14 differentially expressed proteins identified by Le Naour *et al.* [10] were also identified in the present study. Vimentin and macrophage capping proteins, both of the cytoskeleton, were reported in both studies. The macrophage capping protein, up-regulated during differentiation as reported by Le Naour *et al.* [10], was identified in our study in two molecular forms: two down-regulated during differentiation and one up- and one down-regulated during maturation. Calreticulin and MRP14, both belonging to the chaperone class, have been reported both here and by Le Naour *et al.* [10]. The 49 kDa protein calreticulin, identified as down-regulated during differentiation and maturation by Le Naour *et al.*, [10], was identified as up-regulated during differentiation and down-regulated during maturation in our study. However, the 32 kDa fragment of calreticulin (Crt32) reported by Le Naour *et al.* [10] as a novel form of this protein, with increased expression in immature DCs, was not detected in our study. The MRP14 protein (S100 A9) was identified by Le Naour *et al.* [10] as down-regulated during differentiation and maturation (leading to a 9- to 12-fold decrease, respectively). We identified two molecular forms of MRP14; one was only detected in Mo and the other was down-regulated during differentiation and up-regulated during maturation. In addition to MRP14, the cited investigators [10] also identified the protein MRP8 (S100 A8), reported to form a heterodimer with MRP14. We curiously did not identify MRP8 in our study. RNCC was identified as down-regulated during differentiation and maturation by Le Naour *et al.* [10] and in the present study, this protein was down-regulated only during differentiation.

Different S100 and heat shock family proteins were identified by Le Naour *et al.* [10] and in our study. The S100C protein was up-regulated during differentiation and maturation in agreement with Le Naour *et al.* [10] while in our study we identified S100 A6, up-regulated during differentiation and down-regulated during maturation, and S100 A4 down-regulated during differentiation and not changed during maturation. Le Naour *et al.* [10] reported that hsp27 and hsp73 were up-regulated during differentiation, whereas we identified hsp71 in three molecular forms (1 up- and 2 down-regulated during differentiation and 1 down- and 2 up-regulated during maturation) and hsp60 (down-regulated during differentiation and up-regulated during maturation).

The comparison of the proteins differentially expressed identified in our study with mRNA data reported in [9] and [10] was not difficult because there are very few proteins identified by both transcriptomics and 2-DE-based proteomic approaches. Three proteins (MHC I, S100 A4 and MRP14) of the 36 identified in the present study were differentially expressed among all the 255 transcripts described by Le Naour *et al.* [10] and these investigators also identified some proteins by the proteomic approach but not by RNA microarray.

Table 3. Differentially expressed proteins identified in the present study and in the studies of Angenieux *et al.* [9] and Le Naour *et al.* [10][a)].

Present study Mo → iDC → mDC (IL-4/GM-CSF and LPS plus FBS)		Angenieux *et al.* (9) Mo → IDC (IL-4/GM-CSF plus FCS)	Le Naour *et al.* (10) Mo → (iDC) → mDC IL-4/GM-CSF and TNF-α without serum)
Cytoskeleton:			
Lamin B1 ↓	**Vimentin** ↑(8)↓(1), ↑(1)↓(8)	Vimentin ↑	Vimentin ↑
Myosin light chain ↑, ↓	**ARP2/3 16 kDa** ↓	ARP2/3 34 kDa ↑	
Stathmin ↓	**Macrophage capping protein** ↓(6),↑(3)↓(1)→(2)	Annexin II ↑	Macrophage capping protein ↑
Tropomyosin alpha 4 chain ↑(1)↓(1),↑(1)↓(1)	Actin, cytoplasmic 1↓(6),↑(3)↓(1)→(2)		
Tubulin beta-5 chain ↓(3),↑(1)↓(2)	F-actin capping protein ↓,→		
Chaperones:			
Heat shock cognate 71 kDa ↑(1)↓(2),↑(2)↓1(1)	**Calreticulin** ↑,↓		**Calreticulin** ↓
Heat shock protein 60 kDa ↓,↑	***MRP14** ↓(2),↑(1)		**MRP14** ↓
S100 A6↑,↓	**Grp78** ↓(3),↑(2)→(1)	**Grp78** ↑	MRP8 ↓
*S100 A4 ↓,→	Grp58 ↑(2),↑(2)	Grp94-grp96 ↑	S100 C ↑
	Grp75 ↑,↓		Hsp73 ↑
			Hsp27 ↑
Others:			
Protein disulfide isomerase ↓,↑	**RNCC** ↓,→	Mn SOD ↑	**RNCC** ↓
Thioredoxin ↑,↑	Alpha enolase →,↓	Prx III ↑	FABP4 ↑
Myotrophin ↓,→	ATP synthase beta chain ↓,↑	Glutamate dehydrogenase ↑	Ferritin light chain ↑
Proteasome subunit alpha type 5 ↓,↑	ATP synthase D cahin ↑,↑	Glutamata dehydrogenase ↑	Ferritin light chain ↑
Ras-related protein Rap-1b ↓	Fibrinogen gamma ↓,↓	GBI-2 ↑	Guanylate kinase ↑
Rho GDP-dissociation inhibitor 1 ↓,↑	Galectin-1 →,↑		ACBP (Acyl-CoA-binding) ↑
Gho-GDP-dissociation inhibitor 2 →(1)↓(1),↑(1)↓(1)	Glutathione *S*-transferase P ↓,→		
SH3 domain-binding protein →,↓	Lymphocyte-specific protein 1 ↑(2)→(1),↑(3)		
*MHC I ↑,↓			

a) The cell stages (Mo, iDCs and mDCs) and the stimulus for differentiation and maturation are given at the head of each column. The ↑ arrow indicates proteins that were up-regulated (2-fold or more) during differentiation, the ↓ arrow indicates proteins that were down-regulated (two-fold or more) and → indicates proteins that did not change in terms of expression level. Some of the proteins in the present study are present as multiples, molecular forms, with the number given in parenthesis. When more than one arrow is given it indicates different molecular forms. When arrows are separated by a comma, they refer to two transformations: differentiation (Mo to iDCs) and maturation (iDCs to mDCs). The proteins detected in 2 or in all 3 studies compared here are printed in bold. The only differentially expressed protein identified in all 3 studies was vimentin. Table 1 lists all the proteins identified in the present study, including those with multiple molecular forms. * Proteins MHC I, S100 A4 and MRP14 identified in the present study were also reported to be differentially expressed at the RNA level by Le Naour *et al.* [10].

None of the 36 proteins identified in the present study were found to be differentially expressed among the 13 RNA transcripts reported by Angenieux *et al.* [9] and even in their own study they did not identify coincident proteins in both proteomic and SAGE approaches. Indeed, the only protein (vimentin) identified in all three proteomic studies was not detected as differentially expressed at RNA level in the two studies [9, 10]. These discrepancies in the results of mRNA and protein studies apparently are common [37] and they are the result of post-transcriptional control of protein translation and protein modifications, which are a frequent phenomenon in the human organism [38, 39].

4 Concluding remarks

Our results suggest that the protein expression profile of DCs depends on the stimulus used for differentiation/maturation on the basis of comparison with two similar proteomic studies of DCs reported [9, 10] and on the basis of the extensive recent literature about DCs [33–36]. Differences in molecular profiling between the present report and the other two indicate that there may be a multiplicity of DCs. It is tempting to speculate that such plasticity may indicate subtle adaptations of the DCs to the processing and presentation of different antigens within distinct contexts of "danger signals" postulated by Matzinger [40].

Although the proteomic approach based on 2-DE gel has several limitations in terms of the effective separation of many proteins, the detection of low abundance proteins and the separation of basic and hydrophobic proteins, the approach using 2-DE plus MS can provide information that cannot be obtained at the DNA or RNA level. As demonstrated in the present study and suggested in others, many of the changes in protein expression that accompany differentiation and maturation of DCs occur with post-translationally modified proteins. Thus, the information obtained here, such as multiple molecular forms detected for some proteins and proteins without known functions in DCs, can be used for the development of hypothesis-driven experiments that lead to further understanding of the functions of DCs in the immune system and in antigen processing and presentation.

We thank Professor Jorge Elias Kalil Filho and Professor Edecio Cunha Neto for providing access to the mass spectrometer at the Laboratório de Imunologia – Incor. We also thank Maristela Delgado Orellana and Patrícia Viana Bonini Palma for expert technical assistance, and Elettra Greene for a careful review of the manuscript. This research was supported by FAPESP (CEPID), CNPq (PRONEX) and by FAPESP fellowships to SRP and VMF.

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