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Plasma membrane proteomes of differentially matured dendritic cells identified by LC–MS/MS combined with iTRAQ labelling

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ABSTRACT

Dendritic cells (DCs) play a pivotal role in polarising Th lymphocyte subsets but it is unclear what molecular events occur when DCs generate Th2-type responses. Here, we analysed plasma membrane-enriched fractions from immature, pro-Th1 and pro-Th2 DCs and used a combination of iTRAQ labelling and LC–MS/MS to quantify changes in the proteomes. Analysis was performed on triplicate biological samples and changes verified by flow cytometry. MHC class II molecules and CD29 were up-regulated in pro-Th1 DCs whilst CD18 and CD44 were up-regulated in pro-Th2 DCs. One of the most down-regulated molecules in pro-Th1 DCs was YM-1 whilst the greatest decrease in pro-Th2 DCs was NAP-22. Other molecules up-regulated in pro-Th2 DC compared to pro-Th1 DCs included some potentially involved in protein folding during antigen processing (clathrin and Rab-7), whilst other non-membrane proteins such as enzymes/transporters related to cell metabolism (malate dehydrogenase, pyruvate kinase, and ATPase Na^+/K^+) were also recorded. This suggests that pro-Th2 DCs are more metabolically active while pro-Th1 DCs have a mature 'end state'. Overall, although several molecules were preferentially expressed on pro-Th2 DCs, our proteomics data support the view of a 'limited maturation' of pro-Th2 DCs compared to pro-Th1 DCs.

1. Introduction

Dendritic cells (DCs) are critical in development of immunity against pathogens [1] and are able to interpret different pathogen-inherent signals to play a pivotal role in polarising Th lymphocyte subsets [2]. In general, pathogen-associated molecular patterns (PAMPs) that drive DCs to promote Th1-type responses, such as bacterial lipopolysaccharide (LPS), bind to

Abbreviations: 0–3hRP, zero-to-three hours released proteins; Arp2/3, actin-related protein 2/3 complex; BM, bone marrow; CD, cluster of differentiation; DC, dendritic cell; E/S, excretory/secretory; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM-CSF, granulocyte–macrophage colony-stimulating factor; GNBP, guanine nucleotide-binding protein; LPS, lipopolysaccharide; MFI, median fluorescence intensity; NAP-22, 22 kDa neuronal tissue-enriched acidic protein; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; SEA, schistosome egg antigen; Th, T helper.

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pattern recognition receptors (PRRs) on the DC plasma membrane leading to subsequent activation and maturation of the DCs. Conversely, molecules from helminths, which are potent inducers of Th2-type responses, stimulate DCs with a 'modified' phenotype [3–7] but it is unclear how/why these DCs promote Th2-type immunity. Pro-Th2 DCs may have a distinct phenotype, defined by a unique profile of signature molecules [4,5], or may resemble immature DCs, stimulating Th2 polarisation *via* a 'default' pathway in the absence of Th1-inducing stimuli [8]. An 'inhibition model' in which Th2-stimuli inhibit Th1 polarisation by DCs through competitive signalling pathways has also been proposed [9].

Changes in DC gene expression correlate poorly with changes in the level of protein expression [10,11]. Therefore, as protein expression is a better indicator of cell phenotype and function, a number of studies have examined the proteomes of differentially matured DCs [12–15]. For example, 2-DE and MS/MS have revealed changes in the proteome of pro-Th1 DCs matured with LPS *versus* pro-Th2 DCs stimulated with excretory/secretory (E/S) material from a parasitic helminth *Schistosoma mansoni* [14]. This E/S material, released by the parasite during infection and known as '0–3hRP', is an important stimulant of innate immune cells in the skin [16,17] enabling DCs to promote Th2 responses *in vitro* and *in vivo* [7]. Proteomic analysis of pro-Th1 DCs revealed up-regulated expression of cytoskeletal proteins and chaperone molecules whereas pro-Th2 DCs, stimulated with 0–3hRP, exhibited a proteome intermediate between that of immature DCs and pro-Th1 DCs; thus termed a 'limited maturation' phenotype [14]. As soluble cytosolic molecules dominated the cell extracts used in the study, immune-associated proteins from the plasma membrane (*e.g.* PRRs, adhesion molecules, MHC complexes and costimulatory molecules) were not readily detected. Although such molecules are likely to be highly relevant with respect to differential maturation of DCs, their low abundance and hydrophobic nature makes them difficult to isolate for proteomic characterisation.

In order to 'home in' on the detection of specific plasma membrane proteins which are likely to be important in differential DC maturation, we compared proteins enriched from plasma membranes of immature DCs, pro-Th1 DCs stimulated with LPS and pro-Th2 DCs stimulated with schistosome egg antigen (SEA) which is a well characterised pro-Th2 helminth product [3]. First, DC plasma membrane-enriched fractions were analysed by shotgun LC–MS/MS to establish a list of proteins associated with DCs. Second, a gel-free technique using iTRAQ [18,19] was used to quantify changes in protein expression following differential DC maturation. By performing three biological replicates of each type of DCs and confirming proteomic data by flow cytometry, we identified a number of proteins that were differentially expressed by pro-Th1 *versus* pro-Th2 DCs.

2. Materials and methods

2.1. Generation and maturation of DCs from bone marrow

Bone marrow-derived dendritic cells (BM-DCs) from female C57BL/6 strain mice were cultured in RPMI medium containing 10% low endotoxin FCS plus 20 ng/mL GM-CSF (Peprotech, London, UK) as previously described [7,14]. All experimental procedures were undertaken with the guidelines of the United Kingdom Animal's Scientific Procedures Act 1986 and approved by the University of York Ethics committee. On day 6, immature BM-DCs were seeded at 1×10^6/mL and cultured for 18 h alone (MED-DCs), or in the presence of 40 μg/mL SEA [3] (SEA-DCs), or 10 ng/mL LPS (from *Escherichia coli* strain 0111:B4, Sigma-Aldrich, Poole, UK; LPS-DCs) [14]. After overnight culture, cells were harvested and prepared for proteomic analysis.

2.2. Preparation of DC plasma membrane-enriched fractions

Plasma membrane proteins from MED-DCs, SEA-DCs and LPS-DCs were extracted and purified using a plasma membrane protein extraction kit (BioVision, Mountain View, USA). All steps were performed at 4 °C. Briefly, BM-DCs were mechanically homogenised in an ice-cold glass cell grinder and then spun at 700 *g*. The resulting supernatants were spun at 10,000 *g* for 30 min to yield total membrane protein (*i.e.* plasma and cellular organelle membranes) enriched pellets which were re-suspended in 'Upper Phase solution' and mixed with an equal volume of 'Lower Phase solution' before centrifugation at 1000×*g* for 5 min. The upper phase was spun at 25,000 *g* for 10 min, and the resulting plasma membrane-enriched pellet solubilised in 0.5% Triton X-100. Total protein content was assessed by densitometry of SYPRO Ruby stained 1-D electrophoresis gels (NuPAGE 4–12%) against known quantities of cytosolic fractions as standards separated on the same gel.

2.3. Digestion and iTRAQ labelling

Plasma membrane-enriched fractions (35–50 μg) were reduced with 2 mM tris-(2-carboxyethyl)phosphine in 0.5 M triethylammonium bicarbonate (Sigma-Aldrich) pH8.5, at 60 °C for 1 h, alkylated with 10 mM methyl methanethiosulfonate (Sigma-Aldrich) at room temperature for 10 min and digested overnight with sequencing-grade porcine trypsin (Promega, Madison, USA) at 37 °C. The iTRAQ labelling reagents (114, 115, and 116; Applied Biosystems, Framingham, USA) were reconstituted in isopropanol and added to the digests. After 2 h, labelled peptides were combined and purified using cation-exchange and C18 cartridges. Although iTRAQ allows for the multiplexing of several samples in a single run, comparison is performed in a pair-wise manner. In this respect, a common reference sample between iTRAQ analyses is essential. However, a pooled standard is not universally required if the samples are suitably similar, as in our study, where MED-DC is taken as a common standard across all runs [20].

2.4. LC–MS/MS

Peptides were separated using a Dionex polystyrene-divinylbenzene column and fractions collected directly onto a target plate with addition of CHCA matrix. Positive-ion MALDI mass spectra were acquired using an Applied Biosystems 4700 Proteomics Analyzer in reflectron mode over a mass range of *m/z* 800–4000. Monoisotopic masses were obtained from centroids of raw, unsmoothed data. The 20 most intense peaks with a S/N≥50 from each fraction were selected for CID-MS/MS