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Research paper

A quantitative proteomic approach for detecting protein profiles of activated human myeloid dendritic cells

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ABSTRACT

Dendritic cells (DC) direct the magnitude, polarity and effector function of the adaptive immune response. DC express toll-like receptors (TLR), antigen capturing and processing machinery, and costimulatory molecules, which facilitate innate sensing and T cell activation. Once activated, DC can efficiently migrate to lymphoid tissue and prime T cell responses. Therefore, DC play an integral role as mediators of the immune response to multiple pathogens. Elucidating the molecular mechanisms involved in DC activation is therefore central in gaining an understanding of host response to infection. Unfortunately, technical constraints have limited system-wide 'omic' analysis of human DC subsets collected *ex vivo*. Here we have applied novel proteomic approaches to human myeloid dendritic cells (mDCs) purified from 100 mL of peripheral blood to characterize specific molecular networks of cell activation at the individual patient level, and have successfully quantified over 700 proteins from individual samples containing as little as 200,000 mDCs. The proteomic and network readouts after *ex vivo* stimulation of mDCs with TLR3 agonists are measured and verified using flow cytometry.

1. Introduction

Dendritic cells (DC) are essential antigen presenting cells (APC) that effect the innate and adaptive immune response (Banchereau and Steinman, 1998). DC derived from peripheral blood have been characterized by cell surface phenotype and divided into 2 main subsets: myeloid dendritic cells and plasmacytoid dendritic cells (mDC and pDC). They both possess toll-like receptors (TLR), antigen capturing and processing machinery, and costimulatory molecules, which allow them to act as professional APC (Banchereau et al., 2000; Jarrossay et al., 2001; Ito et al., 2002). mDCs express TLR1-6, 8, and 10 providing selectivity in response to specific pathogen associated molecular pattern signals (Liu, 2005). Therefore, TLR3 agonists such as polyinosinic:polycytidylic acid (polyI:C) can selectively activate mDC. Once activated, DC can migrate to lymphoid tissue and prime T cell responses (Banchereau et al., 2000). Elucidating the molecular mechanisms responsible for DC activation is essential towards developing a better understanding of their role and utility in combating disease (Ueno et al., 2010.).

In addition to overall mechanistic questions related to mDC function overall, important questions as to attenuations of function of mDCs against backgrounds of chronic infections and immune perturbations such as seen in HIV or HCV are very important for understanding disease progression and guiding clinical practice. A systems-wide proteomic comparison at the individual patient level to monitor alterations of DC function in disease and identify pathways that can be targeted for therapy would permit patient stratification far beyond

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current medical practice. Unfortunately, technical limitations have hindered omic-scale studies of DCs collected *ex vivo* due to insufficient sample yield from standard peripheral blood preparations. To overcome these limitations, most studies have utilized monocyte derived DC (Mo-DC) cultured *in vitro* (Wang et al., 2008; Buschow et al., 2010; Luber et al., 2010). While these studies have provided important insights with respect to the molecular mechanisms at play during DC activation, Mo-DC are thought to differ in important biological respects from mDC (Horlock et al., 2007). Recently, advanced quantitative proteomic techniques have been applied to relatively small amounts of mouse splenic DC subsets, resulting in over 5000 proteins identified from several million cells (Luber et al., 2010). This study utilized a label free proteomic technique which qualifies a peptide sequence (and associates it with its parent protein) by tandem mass spectrometry (MS/MS) identification. Peptide species are quantified by ion intensity, while individual peptides are grouped across samples based on precise mass and retention time measurements (Chelius and Bondarenko, 2002; Wang et al., 2003; Bantscheff et al., 2007). The thousands of peptides thus grouped are analyzed by well-established techniques that have been developed for analyzing high-dimensional data and permitted the variations in protein expression for different DC subsets to be established for the first time.

We have capitalized on these techniques to develop a sensitive and reproducible *ex vivo* proteomic analysis of human mDCs freshly purified from peripheral blood of individual donors. In this analysis, we performed two independent studies of label free expression of mDC which were stimulated with the TLR3 ligand poly(I:C) to test reproducibility of proteomic analysis of stimulation and response in mDCs. Importantly, we have developed a reproducible method which can analyze the proteome of DC subsets collected *ex vivo* for as few as 200,000 DC cells and reliably detect proteomic changes due to immune activation and/or chronic infection.

2. Materials and methods

2.1. Samples

Venous blood samples were collected from healthy control subjects (age 25–53 (median 41), 57% females, no known chronic viral infection or other medical illness). The label free study contained $n=6$ biological replicates which were used in a paired design that had two treatment groups. The flow cytometry validation analysis contained $n=7$ biological samples. All study subjects provided written informed consent for phlebotomy in accordance with protocols approved by the institutional review boards for human studies at University Hospitals of Cleveland and Cleveland VA Medical Center.

2.2. mDC isolation and poly(I:C) stimulation

Peripheral blood mononuclear cells (PBMC) were prepared from 100 mL of fresh peripheral blood using Ficoll (Fisher Scientific, Hudson, NH). mDC were purified from PBMCs using CD1c (BDCA-1)+ dendritic cell isolation kit for human cells (Miltenyi Biotech, Auburn, CA), where CD19+ B cells are removed by negative selection, then mDCs are isolated by CD1c (BDCA-1) positive bead selection. Purified mDCs from 6 patients were split in two and incubated separately overnight in 96 well round bottom plate containing 5% human AB serum (Gemini Bio-Products, Woodland, CA). Samples were incubated in the presence or absence of 50 μg/mL poly(I:C) (Amersham Biosciences, Piscataway, NJ). mDC were removed from 96 well plates and were washed twice in sterile PBS before snap freezing cells for proteomic analysis.

2.3. mDC purity and activation by flow cytometric analysis

mDC were stained with anti-CCR7 PE-Cy7, anti-CD11c APC, (BD Biosciences, San Jose, CA). Flow cytometric analysis was performed on an LSRII flow cytometer (BD Biosciences) with FACSDiva Software (BD Biosciences) to establish baseline purity and analysis of CCR7 upregulation in response to poly(I:C) stimulation.

2.4. Label free protein expression studies

As outlined above, the six patient samples were split into two batches and incubated either with media or poly(I:C) for 12 samples total. Each sample was lysed with a buffer of 1% Triton, 150 mM NaCl, 20 mM Tris pH 8.0 and 0.1% protease inhibitor cocktail (Sigma-Aldrich, P2714, St. Louis, MO). The sample was incubated on ice for 1.5 h and pulse-sonicated with a probe sonicator every 30 min. Following lysis, the samples were removed of detergent using the 2-D Clean-Up kit per manufacturer's instructions (GE Healthcare Bio-Sciences, Piscataway, NJ) with the exception of the final solubilization step which was performed in 10 μL 8 M urea. The samples were bath-sonicated on ice for 1 h to resolubilize the protein pellet and 10 μL of Tris pH 8.0 was added to yield a final concentration of 4 M urea. Subsequent to solubilization, total protein quantification of each sample was performed using BioRad protein assay per manufacturer's instructions (BioRad Laboraories, Herucles, CA). Each sample was adjusted to 880 ng in 13 μL with 50 mM Tris pH 8.0. Dithiotheritol was added to a final concentration of 5 mM and the samples were reduced at 37 °C for 30 min and cooled to room temperature prior to alkylation with iodacetamide at a final concentration of 10 mM for 30 min. A dual proteolytic digestion was performed with endopeptidase Lys C (Wako Chemicals, Richmond, VA) and trypsin with a final enzyme to protein ratio of 1/1 (w/w) for each protein. First, Lys C was added and incubated for 2 h at 37 °C and then subsequently adjusted to 2 M urea with 50 mM Tris pH 8.0 to accommodate the trypsin digestion which incubated overnight at 37 °C.

One hundred nanograms of each sample was analyzed by LC/MS/MS. The samples were run in two separate batches with the order of sample injections randomized for the 6 samples in a batch. Separation of peptides via capillary liquid chromatography was performed using a Dionex Ultimate 3000 capillary LC system (Dionex Sunnyvale, CA). Mobile phase A (aqueous) contains 0.1% formic acid in 5% acetonitrile and mobile phase B (organic) contained 0.1% formic acid in 85% acetonitrile. Samples were trapped and desalted on-line in mobile phase A at 10 μL/min for 10 min using a Dionex PepMap 100, (300 μm × 5 mm). The sample was subsequently loaded onto a Dionex C18 PepMap (75 μm × 15 cm) reversed phase column with 5% mobile phase B. Separation was obtained by employing a gradient of 6% to 28% mobile