Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteome and phosphoproteome analysis of commensally induced dendritic cell maturation states

Ali Giray Korkmaz[a,*], Todor Popov[a], Loulou Peisl[a], Marius Cosmin Codrea[b], Sven Nahnsen[b], Alexander Steimle[a], Ana Velic[c], Boris Macek[c], Martin von Bergen[d], Joerg Bernhardt[e], Julia-Stefanie Frick[a]

[a] Institute of Medical Microbiology and Hygiene, University of Tübingen, Germany
[b] Quantitative Biology Center, University of Tübingen, Germany
[c] Proteome Center, University of Tübingen, Germany
[d] Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany
[e] Ernst-Moritz-Arndt Universität Greifswald, Institute for Microbiology, Germany

ARTICLE INFO

Keywords:
Commensals
B. vulgatus
Dendritic cells
Inflammation
Shotgun proteomics
Expression analysis

ABSTRACT

Dendritic cells (DCs) can shape the immune system towards an inflammatory or tolerant state depending on the bacterial antigens and the environment they encounter. In this study we provide a proteomic catalogue of differentially expressed proteins between distinct DC maturation states, brought about by bacteria that differ in their endotoxicity. To achieve this, we have performed proteomics and phosphoproteomics on murine DC cultures. Symbiont and pathobiont bacteria were used to direct dendritic cells into a semi-mature and fully-mature state, respectively. The comparison of semi-mature and fully-mature DCs revealed differential expression in 103 proteins and differential phosphorylation in 118 phosphosites, including major regulatory factors of central immune processes. Our analyses predict that these differences are mediated by upstream elements such as SOCS1, IRF3, ABCA1, TLR4, and PTGER4. Our analyses indicate that the symbiont bacterial strain affects DC proteome in a distinct way, by downregulating inflammatory proteins and activating anti-inflammatory upstream regulators.

Biological significance

In this study we have investigated the responses of immune cells to distinct bacterial stimuli. We have used the symbiont bacterial strain *B. vulgatus* and the pathobiont *E. coli* strain to stimulate cultured primary dendritic cells and performed a shotgun proteome analysis to investigate the protein expression and phosphorylation level differences on a genome level. We have observed expression and phosphorylation level differences in key immune regulators, transcription factors and signal transducers. Moreover, our subsequent bioinformatics analysis indicated regulation at several signaling pathways such as PPAR signaling, LXR/RXR activation and glucocorticoid signaling pathways, which are not studied in detail in an inflammation and DC maturation context. Our phosphoproteome analysis showed differential phosphorylation in 118 phosphosites including those belonging to epigenetic regulators, transcription factors and major cell cycle regulators. We anticipate that our study will facilitate further investigation of immune cell proteomes under different inflammatory and non-inflammatory conditions.

1. Introduction

The human gut microbiota is composed of approximately 100 trillion bacteria that belong to over 1000 species. Humans and their gut microbial ecosystem have coevolved to exist in homeostasis, and the bacteria residing in the gut provide numerous benefits to the human host such as aiding digestion, supporting gut development, and preventing pathogenic bacteria to colonize the gut. Two important and prevalent species of gut microbiota are the gram negative bacteria *E. coli* and *B. vulgatus*, which have notable differences in terms of their interaction with the host and their effects on gut homeostasis. Waidmann et al. have shown that monocolonization of germ free IL2$^{-/-}$ mice with *E. coli* leads to colitis whereas monocolonization with *B. vulgatus* does not. Moreover, co-colonization with *B. vulgatus* protects

* Corresponding author.
E-mail address: giray.korkmaz@med.uni-tuebingen.de (A.G. Korkmaz).

https://doi.org/10.1016/j.jprot.2017.11.008
Received 17 February 2017; Received in revised form 18 September 2017; Accepted 14 November 2017

mice from inflammatory bowel disease (IBD) inducing effects of *E. coli* [1]. This suggests that the two bacteria species have significant differences in regulating the immune responses of the host; however, their effects on the immune cells and regulation of immunological pathways are unknown.

Dendritic cells are potent regulators of immune responses and exist in different maturation states. DC maturation states have been previously shown to be influential in eliciting tolerance and protecting from disease [2,3]. Our group has previously shown that DCs isolated from colitic mice show increased expression of cell surface activation markers and proinflammatory cytokines, whereas DCs isolated from protected mice show a semi-mature phenotype. This maturation state difference of DCs have also been shown *in vitro*, where *E. coli* stimulation of immature DCs leads to high levels of proinflammatory cytokine secretion. On the other hand, stimulation of BMDCs with *B. vulgatus* leads to low levels of proinflammatory cytokine secretion and renders them unresponsive to further stimulus [4,5]. These findings prompted us to investigate the differential immune responses mounted against these bacteria, and we performed a label free quantitative proteome analysis to catalogue the differences in protein expression patterns between two different maturation states.

Our proteomics experiments revealed relative abundance differences in 301 proteins between different dendritic cell maturation states (any protein that exhibit a relative abundance difference among the 3 pairwise comparisons of *E. coli vs.* PBS, *B. vulgatus vs.* PBS, *B. vulgatus vs. E. coli* stimulations is included). These 301 differentially expressed proteins were then used as the experimental data set for further bioinformatics downstream analysis. Functional grouping and pathway analysis revealed differences in major immune regulatory pathways and processes such as IRF signaling, TLR signaling and leukocyte activation pathways. Besides these pathways which are directly tied to central immune functions, pathways such as PPAR signaling, LXR/RXR activation and JAK/Stat signaling were observed to be taking part in creating the distinct maturation phenotypes. Our further analyses were targeted to major upstream regulators and revealed 37 proteins that were differentially activated between *B. vulgatus* and *E. coli* stimulated DCs. Among these are factors such as PTGER4, ABCA1, DUSP1 and SOCS1 that regulate inflammatory processes. Taken together, we created a proteome catalogue of different dendritic cell maturation states, and investigated the cellular processes mediating their phenotypical differences. We anticipate that our results will contribute to dissecting the dendritic cell maturation process in further detail, and thereby improve our understanding of immune regulation in inflammatory diseases.

2. Materials and methods

2.1. Bacterial strains

Bacteria used for stimulating mouse dendritic cells were *Escherichia coli* and *Bacteroides vulgatus* [1]. The *E. coli* strain was cultured in Luria-Bertani (LB) medium at 37 °C under aerobic conditions, the *B. vulgatus* strain was cultured in Brain-Heart-Infusion (BHI) medium at 37 °C under anaerobic conditions. Bacterial concentration was determined by photometry at OD_{600}.

2.2. Murine bone marrow dendritic cell culture

The mice used are 6–10 weeks old female C57BL/6NCrl, purchased from Charles River Laboratories and kept under specific pathogen-free conditions for two weeks before bone marrow isolation. Necessary animal experimentation permission was granted by the local authority on animal experimentation (Regierungspräsidium Tübingen, Anzeige 01.12.11). All proteome and phosphoproteome analyses were done in two biological replicates, each biological replicate consisting of cells pooled from 8 mice. The animals were sacrificed by euthanasia followed by cervical dislocation. Bone marrows of femur and tibia were aspirated, and the resulting cells were cultured in VLE-RPMI-medium (Biochrom, FG1415) supplemented with 10% heat inactivated fetal calf serum (Sigma, F7524), 1% non-essential amino acids (Biochrom, K0293), 100 Units/mL Penicillin, 100 µg/mL Streptomycin, 50 µmol/l 2-Mercaptoethanol and 0.5 ng/mL GM-CSF produced in house using the murine myeloma cell line P3X63. A detailed protocol for obtaining and culturing BMDCs was described before [6], and used with the modification of shortening the culture time to 7 days instead of 10. After 7 days of culture, the resulting population was analyzed by flow cytometry for the presence of DCs, harvested and stimulated with the bacterial strains *E. coli* and *B. vulgatus* at an MOI1 for 16 h under cell culture conditions. At the end of the stimulation, dendritic cell activation/maturation was analyzed by flow cytometry (cellular fraction) and ELISA (culture supernatant).

2.3. Flow cytometry analysis

After stimulation with bacterial strains for 16 h, cells were harvested and stained with fluorophore conjugated primary antibodies CD11c-APC (BD, 550261), MHC class II-FITC(BD, 553623), CD40-FITC(553723). Stained cells were measured by LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Version 7.6, Tree Star Inc.).

2.4. ELISA

Enzyme linked immunosorbent assays have been performed on cell culture supernatants of DCs stimulated as indicated above. Cytokine concentrations in supernatants have been determined using ELISA kits for IL-6 (BD, 555240), IL-1β (BD, 559603), TNF-α (BD, 555268), and IL12p40 (BD, 555165). ELISAs were performed as described in manufacturer's protocols.

2.5. Protein digestion

For the analysis of phosphoproteome two milligrams of the protein extracts derived from the DC culture upon bacterial stimulation were digested in solution with trypsin as described previously [7,8]. For proteome analysis immunoprecipitated proteins of 20 µg of sample were loaded on a 10% SDS-PAGE gel. After short gel run and brief Coomassie staining each gel piece was cut into small pieces. Destaining was performed by washing three times with 10 mM ammonium bicarbonate (ABC) and acetonitrile (ACN) (1:1, v/v). This was followed by protein reduction with 10 mM dithiothreitol (DTT) in 20 mM ABC for 45 min at 56 °C, and alkylation with 55 mM iodoacetamide (IAA) in 20 mM ABC for 30 min at room temperature in the dark. The gel pieces were then washed twice for 20 min in destaining solution followed by dehydration with can in a vacuum centrifuge. The residual liquid was removed and gel pieces were rehydrated at room temperature by adding 13 ng/µl sequencing grade trypsin in 20 mM ABC. Digestion of proteins was performed at 37 °C overnight. The resulting peptides were extracted in three subsequent incubation steps: 1) with 30% ACN/3% TFA; 2) with 80% ACN/0.5% acetic acid; and 3) with 100% ACN. Supernatants were combined, ACN was evaporated in a vacuum centrifuge and peptides were desalted using C18 StageTips.

2.6. Phosphopeptide enrichment

Phosphopeptide enrichment and phosphoproteome analysis was done as described previously [9], with minor modifications: about 95% of the peptides were separated by strong cation exchange (SCX) chromatography with a gradient of 0 to 35% SCX solvent B resulting in eleven fractions that were subjected to phosphopeptide enrichment by TiO2 beads. Fractions containing a high amount of peptides were subjected to TiO2 enrichment multiple times. Elution from the beads