Dataset Paper

The Equine CD4⁺ Lymphocyte Proteome

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CD4⁺ T cells are key players in immunology and disease pathology, including relapsing autoimmune uveitis. Equine recurrent uveitis is the only spontaneous animal model for this disease in man. Knowledge about the CD4⁺ cell proteome is crucial for studies on possible changes in proteome expression of CD4⁺ effector cells in disease. For this purpose, we generated a reference dataset of the equine CD4⁺ cell proteome by sorting equine CD4⁺ lymphocytes followed by analysis of whole cell lysate as well as membrane protein fraction using mass spectrometry.

1. Introduction

CD4⁺ lymphocytes play a major role in several immunological processes and diseases, including autoimmune uveitis [1]. Several experimental animal models exist for this disease; however, due to striking immunopathological and clinical similarities, equine recurrent uveitis is the only spontaneous model for relapsing autoimmune uveitis in man [2].

Equine recurrent uveitis is an autoimmune mediated disease affecting horses worldwide [3]. It presents with painful, remitting-relapsing inflammatory attacks of inner eye structures alternating with stages of quiescence [4]. Directly prior to a uveitic attack, immune cells are activated in periphery, migrate into the eye, and attack the retina [5–7]. These cells infiltrating the eye are mainly CD4⁺ T cells with a Th1 phenotype [7, 8].

Knowledge on the protein repertoire of CD4⁺ cells is crucial for the investigation of potential changes in protein expression occurring in these cells in course of immune reactions. To create a solid fundament for further studies, we generated a table of all proteins expressed in CD4⁺ cells (Dataset

Item 1 (Table)) as well as a separate table comprising only membrane associated proteins (Dataset Item 2 (Table)).

For this purpose, CD4⁺ lymphocytes were isolated from total equine lymphocytes by fluorescence activated cell sorting. Subsequently, we extracted membrane proteins from these cells and analyzed this protein fraction using mass spectrometry. In parallel, we performed mass spectrometry analysis on whole CD4⁺ cell lysates (Figure 1).

The two dataset items presented in this study give a detailed description of the physiological CD4⁺ immune cell proteome repertoire and set a reference for further comparative proteomic studies on activated cells or those altered in course of disease.

2. Methodology

Samples were prepared as follows: lymphocytes from 6 horses were examined in this study. Equine venous blood was collected in lithium-heparin coated tubes (Kabe, Nümbrecht-Elsenroth, Germany). After rough sedimentation of erythrocytes, lymphocytes were isolated from plasma by density

FIGURE 1: Complete workflow for our study. Left panel shows isolation and staining of equine lymphocytes, middle panel shows cell sorting, and right panel shows further processing of sorted cells for mass spectrometry analysis.

gradient centrifugation (RT, 290 rcf, 25 min, brake off) using Biocoll separating solution (Biochrom, Berlin, Germany). Lymphocytes were extracted from intermediate phase, washed twice in PBS (4°C, 453 rcf, 10 min) and used immediately.

The number of cells for each sample was set at 1×10^7 per mL. Mouse IgG1 anti-equine CD4 antibody (Serotec, Puchheim, Germany) was diluted 1:2 in staining buffer (1% BSA in PBS + 0.001% NaN_3) and incubated with cells for 30 minutes at 4°C. After washing with staining buffer, goat anti-mouse IgG:Alexa488 secondary antibody (Invitrogen, Karlsruhe, Germany) was diluted 1:500 in staining buffer and added to cells for 30 min at 4°C in the dark. From each sample used in the experiment, 1×10^6 cells were stained in 96-well round-bottom plates with goat anti-mouse IgG:Alexa488 secondary antibody as a negative control (30 min, 4°C, in the dark).

Sorting of cells was performed on FACS Aria III with FACS Diva 6.1.2 software (both BD Biosciences, Heidelberg, Germany). Lymphocytes were gated according to cell size (forward scatter, FSC) and intracellular granularity (sideward scatter, SSC). Doublets were excluded in SSC-W versus SSC-H and FSC-W versus FSC-H plots. CD4^+ and CD4^- cell

subsets were collected separately from each sample, counted, and split into portions of 2×10^6 cells. Cells were used immediately for further analysis.

Cell lysis of whole CD4^+ lymphocyte protein and preparation for mass spectrometry were as follows: per sample, 2×10^6 cells were dissolved in lysis buffer (9 M urea; 2 M thiourea; 1% dithioerythritol; 4% CHAPS; 2.5 μM EDTA, complete protease inhibitor) and 200 μg of total protein per sample was loaded on gels for 1D SDS PAGE. After electrophoresis was completed, each lane was divided in upper, middle, and lower protein fraction. Each gel fraction was shrunk in 100% acetonitrile (ACN), rehydrated in 50 mM NH_4HCO_3 (shrinking and rehydration were performed twice), reduced, and alkylated with 25 mM iodoacetamide and then digested with 0.01 mg/mL trypsin (Sigma-Aldrich, Deisenhofen, Germany) in 50 mM NH_4HCO_3 overnight at 37°C. The supernatant was collected and combined with eluates of subsequent elution steps with 80% ACN and 0.1% trifluoroacetic acid (TFA) and with 100% ACN/0.1% TFA. The combined eluates were dried in a SpeedVac centrifuge and dissolved in 2% ACN and 0.1% TFA. Subsequently, each fraction was separately analyzed by mass spectrometry (LS-MSMS).

Preparation and lysis of CD4⁺ lymphocyte membrane protein fraction were as follows: membrane protein fraction of equine lymphocytes was extracted as described [9], with some alterations. Briefly, 2×10^6 cells were lysed in 800 μ L buffer 1 (2 M NaCl; 10 mM Hepes/NaOH pH 7.4; 1 mM EDTA, complete protease inhibitor) and subsequently centrifuged for 30 min at 17500 rcf and 4°C. Supernatant was collected, and pellet was dissolved in 800 μ L buffer 2 (0.1 M Na₂CO₃ pH 11.3; 1 mM EDTA) and incubated for 30 min on ice followed by centrifugation for 30 min at 17500 rcf and 4°C. Supernatant was collected. This step was performed twice. Finally, pellet was dissolved in 800 μ L buffer 3 (4 M Urea; 100 mM NaCl; 10 mM Hepes/NaOH pH 7.4; 1 mM EDTA) and centrifuged for 30 min at 17500 rcf and 4°C. Supernatant was collected and pooled with supernatants from previous steps. Membrane protein pellets were carefully washed with aqua bidest and solubilized separately in 36 μ L NH₄HCO₃ and 4 μ L RapiGest surfactant (Waters, Eschborn, Germany). Proteins were then denatured by the addition of 2 μ L 100 mM DTT, heated (60°C, 10 min), cooled, and alkylated by the addition of 2 μ L 300 mM iodoacetamide for 30 minutes at RT in the dark. Trypsine was added (5 μ L, 0.5 mg/mL) and samples were incubated at 37°C for 16 h, followed by the addition of 2 μ L trypsin and incubation for additional 20 h. Samples were acidified by addition of 3 μ L concentrated HCl and centrifuged (16000 rcf, 30 min, 4°C). Soluble phase was retrieved and used immediately for mass spectrometry analysis.

LC-MSMS mass spectrometry was performed as previously described [10]. Briefly, the digested peptides were loaded automatically to an HPLC system (Thermo Fisher Scientific) equipped with a nanotrap column in 95% buffer A (2% ACN, 0.1% formic acid (FA) in HPLC-grade water) and 5% buffer B (98% ACN, 0.1% FA in HPLC-grade water). After 5 min, the peptides were eluted and separated on the analytical column (75 μ m inner diameter $\times$ 15 cm, Acclaim PepMap100 C18, 3 μ m, 100 Å, Dionex) by a gradient from 5% to 40% of buffer B at 300 nL/min flow over 170 min followed by a 5 min gradient from 40% to 95% B in 5 min. The eluting peptides were analyzed online in an LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific) coupled to the HPLC system with a nanospray ion source. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1500) were acquired in the Orbitrap with high-resolution (60000 full-width half maximum).

The method used allowed sequential isolation of the most intense ions (up to five), depending on signal intensity, for fragmentation on the linear ion trap using collisional induced dissociation at a target value of 100000 ions. High-resolution MS scans in the Orbitrap and MS/MS scans in the linear ion trap were performed in parallel. Target peptides already selected for MS MS/MS were dynamically excluded for 30 s.

LC-MSMS-derived MS/MS spectra were analyzed using Mascot (version 2.2, Matrix Science, London, UK; <http://www.matrixscience.com/>), set up to search the Ensemble Horse protein database (version 2.66, 12722794 residues, 22644 sequences, <http://www.ensembl.org/>) setting trypsin

as digestion enzyme and allowing fragment ion mass tolerance of 0.6 Da and a parent ion tolerance of 10 ppm. One missed cleavage was allowed and iodoacetamide derivatives of cysteines as stable modifications as well as oxidation of methionine and deamidation of asparagine and glutamine as variable modifications were specified for Mascot searches.

Protein identifications were accepted if the probability based MOWSE protein score was above the $P < 0.01$ significance threshold for the database and contained at least two identified peptides with at least 80.0% probability as specified by the Peptide Prophet algorithm [11]. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

3. Dataset Description

The dataset associated with this Dataset Paper consists of 2 items which are described as follows.

Dataset Item 1 (Table). Proteins identified from whole CD4⁺ cell lysate. Using mass spectrometry analysis (LC-MSMS), a total of 1250 different proteins could be identified from the whole CD4⁺ cell lysate. The column Accession Number shows the accession number of each identified protein and the column Protein Name shows the matching protein name as listed in Ensembl Horse protein database (<http://www.ensembl.org/>). If proteins were listed as “uncharacterized” in Ensembl Horse protein database, protein names were retrieved using protein Basic Local Alignment Search Tool (BLAST) from NCBI database (<http://www.ncbi.nlm.nih.gov/>). The column Peptide Count shows the amount of peptides used for the identification of each protein. The column Total Spectral Counts contains the spectral counts and Mean Normalized Abundance contains the mean normalized abundance of each identified protein from all six specimens used in the experiment. Confidence score >30 was required for a protein to be considered as identified in Ensembl Horse protein database and is shown in the column Confidence Score.

Column 1: Accession Number

Column 2: Protein Name

Column 3: Peptide Count

Column 4: Total Spectral Counts

Column 5: Mean Normalized Abundance

Column 6: Confidence Score

Dataset Item 2 (Table). Proteins identified from membrane fraction of CD4⁺ cells. Using mass spectrometry analysis (LC-MSMS), 376 different proteins were identified from the CD4⁺ membrane fraction. The column Accession Number shows the accession number of each identified protein and the column Protein Name shows the matching protein name as listed in Ensembl Horse protein database (<http://www.ensembl.org/>). If proteins were listed as “uncharacterized” in Ensembl Horse protein database, protein names were retrieved using protein Basic Local Alignment Search Tool

(BLAST) from NCBI database (<http://www.ncbi.nlm.nih.gov/>). The column Peptide Count shows the amount of peptides used for the identification of each protein. The column Total Spectral Counts contains the spectral counts and Mean Normalized Abundance contains the mean normalized abundance of each identified protein from all six specimens used in the experiment. Confidence score >30 was required for a protein to be considered as identified in Ensembl Horse protein database and is shown in the column Confidence Score.

Column 1: Accession Number

Column 2: Protein Name

Column 3: Peptide Count

Column 4: Total Spectral Counts

Column 5: Mean Normalized Abundance

Column 6: Confidence Score

4. Concluding Remarks

The two proteomic dataset items generated in this study present a solid fundament for further investigations on altered protein expression in CD4⁺ cells, which may occur in course of immune reactions due to cell activation triggered by various conditions such as vaccination, transplant rejection, or disease.

Dataset Availability

The dataset associated with this Dataset Paper is dedicated to the public domain using the CC0 waiver and is available at <http://dx.doi.org/10.1155/2014/105312/dataset>.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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