Animal Proteomics

Dataset Brief

Proteome dynamics in biobanked horse peripheral blood derived lymphocytes (PBL) with induced autoimmune uveitis

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Abstract

Equine recurrent uveitis (ERU) is the only spontaneous model for recurrent autoimmune uveitis in humans, where T cells target retinal proteins. Differences between normal and autoaggressive lymphocytes were identified in this study by analyzing peripheral blood derived lymphocytes (PBL) proteomes from the same case with IRBP-induced uveitis sampled before (day 0), during (day 15) and after uveitic attack (day 23). Relative protein abundances of PBL were investigated in a quantitative, label-free differential proteome analysis in cells that were kept frozen for 14 years since the initial experiment. Quantitative data could be acquired for 2632 proteins at all three time-points. Profound changes (≥ 2 fold change) in PBL protein abundance were observed when comparing day 0 to 15, representing acute inflammation (1070 regulated proteins) and day 0 to 23 (cessation; 1571 regulated). Significant differences applied to proteins with functions in integrin signaling during active uveitis, involving "Erk and pi-3 kinase are necessary for collagen binding in corneal epithelia", "Integrins in angiogenesis" and "Integrin-linked kinase signaling" pathways. In contrast, at cessation of uveitic attack, significantly changed proteins belonged to pathways of "nongenotropic androgen signaling", "classical complement pathway" and "Amb2 integrin signaling". Several members of respective pathways were earlier shown to be changed in naturally occurring uveitis, underscoring the significance of these findings here and proofing the value of the induced model in mimicking spontaneous autoimmune uveitis. All MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [1, 2] (dataset identifier PXD005580).

Abbreviations

ACN Acetonitrile

CFA Complete freund's adjuvant

Da Dalton

DMSO Dimethylsulfoxide

EAU Experimental autoimmune uveitis

EPHB Ephrin B receptor

ERU Equine recurrent uveitis

FA formic acid

FCS Fetal calf serum

FOS Proto-Oncogene, AP-1 Transcription Factor Subunit

ILK Integrin-linked kinase

IRBP Interphotoreceptor retinoid binding protein

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B-cells

PBL Peripheral blood derived leukocytes

TNF-α Tumor necrosis factor alpha

THY1 Thy-1 Cell Surface Antigen (CD90)

Equine recurrent uveitis (ERU) is a naturally and frequently occurring disease in horses. It is an important disease in veterinary medicine, but has additionally important translational qualities as disease model for autoimmune uveitis and as organ-specific autoimmune diseases in general [3]. In this spontaneous disease, T cells target and subsequently damage retinal proteins [4, 5]. Identification of a shared autoantigen between horses and patients with recurrent autoimmune uveitis evidenced the translational quality of ERU [4]. For proof-of-principle experiments, it is possible to test hypotheses of pathophysiology in experimental horses, which allows direct comparison of the naturally occurring disease with the induced one. From several models of induced experimental uveitis in horses, one mimicked the spontaneous disease best. This model based upon subcutaneous injection of Interphotoreceptor retinoid binding protein (IRBP) with complete Freund`s adjuvant (CFA)

that induced an autoaggressive T cell response in respective horses [6]. IRBP-specific lymphocytes then left peripheral blood, migrated into the eyes and destroyed the retinal architecture with similar pathological findings as in ERU [6]. This outcome was very important since it proofed that an autoimmune response of PBL to a retinal protein is sufficient to induce severe intraocular inflammation by these lymphocytes with a clinical picture that is indistinguishable from the naturally occurring disease [6, 7]. But how these autoaggressive lymphocytes differ from physiologically responding cells and which molecular mechanisms enable them to transmigrate blood-retinal barrier, detect the target protein behind this barrier and carry out their damaging inflammatory response is not understood so far.

Earlier, we identified differences in the immune cell proteome of spontaneous ERU cases with differential proteome analyses using 2D gel approaches and detected 7 differentially expressed proteins in lymphocytes and 17 in leukocytes, amongst them proteins with functions for cell migration and immune response [5, 8]. This experiment reflected the situation of the naturally occurring disease in biological replicates of ERU cases.

Additionally, we had stored very interesting specimens of the IRBP-induced uveitis cases in our biobank. The samples were taken in close intervals during these experiments [6], therefore we could investigate PBL proteomes before any manipulation of the immune system and after immunization with IRBP and CFA during uveitic attack and in abatement of uveitis in one and the same animal. This allowed us to identify autoimmune-related differences in PBL in this study. As a side-effect, we tested identification rate of proteins in primary PBL stored for 14 years at -80°C. We undertook these analyses now, because analytical-depth of identifications has largely increased in proteomics using gel-free approaches and novel mass spectrometers [9]. We analyzed PBL before IRBP immunization (day 0), during the acute uveitic attack (day 15) and at regression of the respective attack (day 23) [6]. PBL were separated from heparinized blood samples through density gradient centrifugation as described [5] and 2 x 10^7 lymphocytes each were frozen in vials containing 90% FCS/10% DMSO. After storage for 15 years at -80°C, cells were thawn in a 37 °C waterbath and immediately transferred into 10 ml prewarmed RPMI medium. Cells were

washed in PBS and filtered through a 70 µm cell strainer. After two additional washing steps, cells were pelleted and shock frozen at -80 °C. Cells were lysed and processed for LC-MS/MS with the in-StageTip method as described [10, 11] with slight adaptations. Briefly, cells were lysed in lysis buffer (6M GdmCl, 100 mM Tris pH 8.5), boiled for 8 min at 95 °C and subsequently sonicated for 5 x 1 min using a water bath sonicator (Elma Transsonic T310-H). Protein content was determined using BCA assay and 15 µg protein lysate were reduced and alkylated before proteolysis with Trypsin and Lys-C. Resulting peptides were fractionated after loading to activated SDB-RPS (Empore, 3M Bioanalytical Technologies) by successive elution in buffers 1-3 (Buffer 1, 100 mM NH4HCO2 40 % ACN 0.5 % FA; Buffer 2, 150 mM NH4HCO2 60 % ACN 0.5 % FA 50 mM NaOH; Buffer 3, 5 % NH4OH 80 % ACN). Eluates were collected, dried, and resolved (2 % ACN, 0.5 % TFA) and used for LC-MS/MS analysis performed as described [10] on an LTQ OrbitrapXL. Briefly, peptides were automatically loaded to a nano trap column before separation by reversed phase chromatography with a gradient of increasing ACN concentrations. The 10 most abundant peptide ions were selected for fragmentation in the linear ion trap, with a dynamic exclusion of 60 seconds. During fragment analysis, a high-resolution (60,000 full width at half-maximum) MS spectrum was acquired with a mass range from 200 to 1500 Da. Label-free quantitative analysis was performed as described [10, 12] using Progenesis QI software (Nonlinear Dynamics, Waters, Newcastle upon Tyne, U.K.) [10] with raw files imported, followed by automatic peak picking and retention time alignment and normalization of total peak intensities across all samples to minimize loading differences. All MS/MS spectra were exported from the Progenesis QI software as Mascot generic files (mgf) and used for peptide identification with Mascot (version 2.5.1) using the Ensembl Horse protein database (version 2.75, 22654 sequences, http://www.ensembl.org). Search parameters used were 10 ppm peptide mass tolerance, 0.6 Da fragment mass tolerance, one missed cleavage allowed, carbamidomethylation was set as fixed modification, and methionine oxidation and deamidation of asparagine and glutamine as variable modifications. Mascot integrated decoy database search was set to a false discovery rate (FDR) of 1% when searching was performed on the concatenated mgf files with a percolator ion score cut-off of 15 and an appropriate significance threshold p. Identifications were re-imported into Progenesis QI and

redundancies grouped following the rules of parsimony to generate a minimal set of proteins explaining the data. For quantification, all unique peptides of an identified protein were included and the total cumulative normalized abundance was calculated by summing up the abundances of all unique peptides allocated to the respective protein. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD005580 and 10.6019/PXD005580.

We identified a total of 2632 proteins in PBL in all states analyzed (see supporting information tables 1 and 2 including statistical evaluation of protein ratios), which is a significant increase compared to our previous study applying 2D gel-based proteomics in PBL from ERU cases [13] and well in line with other studies investigating PBL by label-free LC-MS/MS (e.g. 1738 proteins identified in PBL from HIV infected patients [14]. Profound expression changes (≥ 2 fold change) were observed with 570 up- and 500 downregulated proteins when comparing samples before and during the uveitic attack (day 0 to 15), 735 up- and 398 downregulated proteins comparing samples during and after uveitic attack (day 15 to 23), and most 1005 up- and 566 downregulated when comparing samples before and after uveitic attack (day 0 to 23). Amongst the identified proteins were high numbers of plasma membrane proteins (e.g. CDs and integrins), as well as important signaling proteins altering their abundance during the inflammatory response. For visualization of dynamic protein expression over time, proteins with a minimal fold change of 2 in at least one group comparison were clustered with GProX software (Version 1.1.16) [15] using normalized abundances per protein expressed as proportion of 1 for each time point. Regulated proteins were distributed across clusters, with highest numbers in cluster 4 (Figure 1A; supporting information table 3). In addition, ≥ 2-fold changed proteins were hierarchically clustered based on euclidian distance (Fig. 1B). The resulting heat map underlines the profound proteomic changes accompanying acute uveitic attack and disease regression. In total, 75% (1973) of all identified proteins (2632) were subjected to distinct (≥ 2 fold change) expression changes throughout the course of the induced disease. The heat map further reveals that only a small proportion of proteins had returned to pre-immunization expression levels after regression of uveitic attack.

We were especially interested in enrichment analyses of canonical signal transduction pathways of clusters 6 and cluster 1, because they represent ≥ 2 fold changed proteins during active uveitic attack at day 15 (cluster 6) and at abatement of respective attack at day 23 (cluster 1). We analyzed the data with gene ranker (Genomatix version 3.7) for enrichment of canonical pathways. This revealed proteins comprised in pathways "Erk and pi-3 kinase are necessary for collagen binding in corneal epithelia", "Integrins in angiogenesis" and "Integrin-linked kinase signaling" top candidates in active inflammation (Table 1).

Talin 1 is a member of both top regulated pathways ("Erk and pi-3 kinase are necessary for collagen binding in corneal epithelia" and "Integrins in angiogenesis"). This confirms earlier proteomics studies by us with PBL of spontaneously diseased horses, where Talin 1 was found to be an important protein with changed expression [8, 16]. It further proofs, that the IRBP induced uveitis model [6] closely mimics the changes occurring in the spontaneous disease [8, 16]. Talin 1 is closely involved in integrin function, which fits well to the finding of two pathways dealing with integrin function as next top regulated pathways, namely "Integrins in angiogenesis" and "Integrin-linked kinase (ILK-) signaling" (Table 1). ILK is stimulated by integrins, growth factors and chemokines and has many different downstream targets [17]. One of them is Akt that was also detected as changed protein during active inflammation in this study (Table 1). ILK regulates immune-cell survival via the Akt pathway through inhibition of apoptosis [17]. Further, ILK renders T cells more responsive to chemokine stimulation [18]. ILK is a serine-threonine kinase that regulates cellular responses to many further stimuli like cell-matrix interactions, cytoskeletal organization, and cell signaling [19]. For example, it was shown that ILK is a critical regulator for the NF-κB-mediated pro-inflammatory signaling pathway by transcriptional induction of TNF-α [19].

Interestingly, completely different canonical signal transduction pathways are enriched at regression of disease (day 23, Table 2). Signaling events mediated by nongenotropic Androgen signaling, the classical complement pathway and Amb2 Integrin signaling came to the fore, indicating a profound change in PBL in quiescent stage of uveitis (Table 2).

The involvement of proteins from "nongenotropic Androgen signaling" during cessation of

the acute uveitic attack (Table 2) was especially interesting since FOS was among the changed candidates. FOS plays important roles in the regulation of immune responses. For example, the endogenous FOS expression level ensures IL-2 expression within a memory CD4+ T cell population [20]. This is probably important to understand the recurrence of the autoimmune response after a variable stage of quiescence in this and other relapsing autoimmune diseases. Proteins from "classical complement pathway" and "Amb2 Integrin signaling" were already detected by us in the naturally occurring disease model, indicating again that IRBP-induced uveitis closely models several changes occurring in PBL during autoimmune uveitis. We described participation of classical complement factors as changed on PBL very early, at that time a surprising finding enabled by proteome analyses [21]. THY1, a member of the "Amb2 Integrin signaling" family was found as Talin 1 interactor (through interaction proteomics) in ERU leukocytes and significantly regulated candidate in ERU by us [16].

Participation of these pathways was important information from this experiment, since it linked previously unknown candidates to the dysregulated immune response in active attack, as well as in downturn of this naturally occurring disease. Their significance for ERU will be further investigated in the future. Enriched signaling pathways from all clusters are listed in the supporting information table 4. Taken together, proteomic profiling of PBL allowed greater insight in the multifaceted changes occurring on protein level during a spontaneous autoimmune reaction that could be monitored from initiation to full activity to cessation in PBL and could additionally be directly compared to the naturally disease that it models. Additionally, we gained the information that primary cells consistently stored at minus 80°C still allow complex identification of their proteomes. Here, we demonstrated identification of 2632 proteins during different states of an inflammatory attack and the detection of known and novel pathways involved in the dysregulation of lymphocytes leading to severe attack of self-antigens.

A recent proteomic profiling analysis in plasma and aqueous humor of EAU rats revealed that the differentially expressed proteins were associated with complement and coagulation cascades, metabolic pathways, NF-kappa B, PI3K-Akt, Toll-like receptors and autophagy

[22]. The finding that the differentially expressed proteins in EAU rat plasma were mainly involved in the complement cascades fits to our finding here, that the classical complement pathway changed on T cells in the late phase of uveitis (Table 2). Upregulation of complement and coagulation cascade pathways in plasma and aqueous humor of the experimental rats with IRBP induced uveitis [22] confirm our findings of upregulation of respective pathways in vitreous of spontaneously diseased horses with uveitis [23]. The critical importance of complement activation for EAU was shown by transfer of *in vitro* primed antigen-specific CD4+ T cells, demonstrating a role in the induction of antigen specific T-cell responses in autoimmune uveitis [24, 25]. Additionally, presence of complement in respective experimental rats was indispensable for the production of inflammatory cytokines and integrins in the course of EAU [24, 25], linking integrin and complement activation to inflammatory T cells in uveitis. Activation of the complement pathway was also shown in patients with autoimmune uveitis [25]. As a recent study of common C3 gene variants in uveitis patients could not link a C3 gene variant to uveitis susceptibility [26], further investigations in uveitis are needed to clarify the role of the complement system in autoimmune uveitis. The study conducted here confirms the importance of the complement pathway, albeit other components than C3. Further it added evidence about further pathways that are involved in active inflammation of autoimmune uveitis during active and abating uveitis in biobanked samples. Nevertheless, there are clearly limitations in the current study. We used PBL instead of cells from intraocular locations like aqueous humor or vitreous, those probably do not reflect the entire characteristics of the local inflammatory cells. The reason was that we wanted to analyze the changes throughout a well-documented course of induced uveitis [6] in sequential samples. Repetitive sampling of vitreal cells in such short intervals would not be possible because it requires general anesthesia to obtain these cells. Further, cell numbers significantly vary during uveitis; further complicating quantitative analyses [27]. However, PBL is an important specimen in uveitis, because adoptive transfer experiments in rodents clearly proved that PBL invade the inner eye immediately before uveitic attack and therefore reflect an important part of uveitis pathogenesis [28].

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Conflict of interest statement

The authors declare no conflict of interest.

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Table 1: Canonical signal transduction pathway enrichment during uveitis (day 15).

Signaling pathway	p-value	# Proteins (observed)	List of observed proteins [gene symbols]
Erk and pi-3 kinase are necessary for collagen binding in corneal epithelia	2.33E-06	6	ZYX, PFN1, ROCK1, TLN1, VCL, DIAPH1, MAPK3
Integrins in angiogenesis	3.74E-06	8	ROCK1, FN1, TLN1, ILK, VCL, AKT1, PTPN11, MAPK3
Integrin-linked kinase signaling	2.95E-05	7	ZYX, PPP1R12A, ILK, PPP1R14A, LIMS1, AKT1, DIAPH1

Table 2: Enriched canonical signal transduction pathways in PBL at abatement of uveitis.

Signaling pathway	P-value	# Proteins (observed)	List of observed proteins [gene symbols]
Nongenotropic Androgen signaling	1.27E-04	5	GNB1, GNAI2, AR, GNG2, FOS
Classical complement pathway	2.39E-04	4	C7, C5, C1R, C1QA
Amb2 Integrin signaling	3.16E-04	6	LRP1, FGR, ITGB2, LYN, PLAUR, THY1

Figure 1. Visualization of PBL protein dynamics during induced equine uveitis. (A) Clustering of ≥ 2 fold expression changed proteins in PBL during uveitic attack, measured before (day 0), during (day 15) and after (day 23) intraocular visible inflammation. (B) Hierarchical Cluster Analysis of PBL protein expression before (day 0), during (day 15) and at regression of acute uveitic attack (day 23). All proteins which were at least 2-fold changed between any of the three experimental conditions were used as input. Proteins (left dendrogram) and samples (top dendrogram) were clustered based on the respective protein abundances applying hierarchical clustering based on Euclidian distances. Highly abundant proteins are presented in green and low abundant proteins in red.