Journal of Autoimmunity 100 (2019) 75–83

Contents lists available at ScienceDirect

Journal of Autoimmunity

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

Transcriptomic and proteomic analysis of iris tissue and aqueous humor in juvenile idiopathic arthritis-associated uveitis

Lena Wildschütz[a,*], Doreen Ackermann[b], Anika Witten[c], Maren Kasper[a], Martin Busch[a], Shirin Glander[c], Harutyun Melkonyan[d], Karoline Walscheid[a], Christoph Tappeiner[e], Solon Thanos[d], Andrei Barysenka[c], Jörg Koch[a], Carsten Heinz[a,f], Björn Laffer[a,f], Dirk Bauer[a], Monika Stoll[c], Simone König[b], Arnd Heiligenhaus[a,f]

[a] Department of Ophthalmology and Ophtha-Lab at St. Franziskus-Hospital, Münster, Germany
[b] Interdisciplinary Center for Clinical Research, Core Unit Proteomics, University of Münster, Germany
[c] Core Facility Genomics, University of Münster, Germany
[d] Institute of Experimental Ophthalmology at University of Münster, Germany
[e] Department of Ophthalmology, Inselspital, University of Bern, Bern, Switzerland
[f] Department of Ophthalmology, University of Duisburg-Essen, Essen, Germany

ARTICLE INFO

Keywords:
Juvenile idiopathic arthritis
Uveitis
B cells
Cytokines
Plasma cell niche
HLA-B27

ABSTRACT

Gene and protein expression profiles of iris biopsies, aqueous humor (AqH), and sera in patients with juvenile idiopathic arthritis-associated uveitis (JIAU) in comparison to control patients with primary open-angle glaucoma (POAG) and HLA-B27-positive acute anterior uveitis (AAU) were investigated. Via RNA Sequencing (RNA-Seq) and mass spectrometry-based protein expression analyses 136 genes and 56 proteins could be identified as being significantly differentially expressed (DE) between the JIAU and POAG group. Gene expression of different immunoglobulin (Ig) components as well as of the B cell-associated factors ID3, ID1, and EBF1 was significantly upregulated in the JIAU group as compared to POAG patients. qRT-PCR analysis showed a significantly higher gene expression of the B cell-related genes CD19, CD20, CD27, CD138, and MZB1 in the JIAU group. At the protein level, a significantly higher expression of Ig components in JIAU than in POAG was confirmed. The B cell-associated protein MZB1 showed a higher expression in JIAU patients than in POAG which was confirmed by western blot analysis. Using bead-based immunoassay analysis we were able to detect a significantly higher concentration of the B cell-activating and survival factors BAFF, APRIL, and IL-6 in the AqH of JIAU and AAU patients than in POAG patients. The intraocularly upregulated B cell-specific genes and proteins in iris tissue suggest that B cells participate in the immunopathology of JIAU. The intracameral environment in JIAU may facilitate local effector and survival functions of B cells, leading to disease course typical for anterior uveitis.

Abbreviations: ANA, anti nuclear antibodies; APRIL, a proliferation-inducing ligand; AU, anterior uveitis; AAU, acute anterior uveitis; AqH, aqueous humor; BAFF, B cell-activating factor; BCMA, B cell maturation antigen; BSA, bovine serum albumin; CD, cluster of differentiation; cDNA, complementary desoxyribonucleic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DE, differentially expressed; DMARD, disease-modifying anti-rheumatic drug; DTT, Dithiothreitol; f, female; EBF1, early B cell factor 1; FDR, false discovery rate; fw, forward; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Gpr83, G Protein-Coupled Receptor 83; HLA, human leukocyte antigen; HKG, housekeeping gene; ID, inhibitor of DNA binding; Ig, immunoglobulin; IL, interleukin; ILAR, International League of Associations for Rheumatology; IOP, intraocular pressure; JIA, juvenile idiopathic arthritis; JIAU, juvenile idiopathic arthritis-associated uveitis; LC-IMS, liquid chromatography coupled ion mobility mass spectrometry; LLPC, long-lived plasma cell; m, male; mRNA, messenger RNA; Mmrn2, Multimerin 2; MTX, methotrexate; MZB1, marginal zone and B1 cell-specific protein; Nedd9, Neural Precursor Cell Expressed; PC, plasma cell; PCA, principle component analysis; POAG, primary open-angle glaucoma; qRT-PCR, quantitative real-time polymerase chain reaction; RA, rheumatoid arthritis; RF, rheumatoid factor; RIN, RNA integrity number; rlog, regularized logarithm; RNA, ribonucleic acid; RNA-Seq, RNA Sequencing; rv, reverse; SD, standard deviation; SE, standard error; SDC-1, Syndecan-1; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SLE, systemic lupus erythematosus; SS, Sjögren syndrome; SUN, standardization of uveitis nomenclature; TBS-T, Tris-buffered saline with Tween 20; TCEP, tris(2-carboxyethyl)phosphine); Th, T-helper; UPLC, Ultra Performance Liquid Chromatography

* Corresponding author. Department of Ophthalmology and Ophtha-Lab at St. Franziskus Hospital, Hohenzollernring 74, 48145, Muenster, Germany.
E-mail address: lena.wildschuetz@uveitis-zentrum.de (L. Wildschütz).

https://doi.org/10.1016/j.jaut.2019.03.004
Received 23 January 2019; Received in revised form 4 March 2019; Accepted 4 March 2019
Available online 15 March 2019
0896-8411/

1. Introduction

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic disease in childhood and associated uveitis (JIAU) represents its most common extra-articular manifestation (9–13%) [1,2]. JIA constitutes a hetergeneous group of clinically distinct arthritic disorders, with onset before the age of 16 years [3]. Approximately two thirds of patients refer to the oligoarthritis subtype [2,4]. Children of young age at JIA onset, antinuclear (ANA) antibody positivity [2,4,5], and genetic predespositions such as female gender and HLA-DRB1 alleles [6,7] are considered as patients at risk for uveitis. JIAU typically involves the iris and ciliary body [8] with insidious onset of flare and frequent development of vision-threatening complications, for example, posterior synechiae, cataract formation, glaucoma, and macular edema [9].

The pathogenesis of JIAU is still unknown. Similar to other autoimmune disorders, genetic and environmental factors may be involved [7,10]. Previous immunohistochemical studies from iris biopsies in end-stage JIAU revealed a predominance of B cells in the intraocular inflammatory infiltrate, with plasma cells and CD20 $^{+}$ B cells being the most abundant cell populations [8,11–14]. Elevated amounts of immunoglobulin (Ig) isotypes G, M, and A were observed in the vitreous fluid of a patient with JIAU [15]. The important role of B cells is further supported by the production of ANA-associated autoantibodies targeting the anterior uveal tissues, which can be detected frequently in JIAU patients [16]. Thus, these results indicate an involvement of B cells in the pathogenesis of JIAU. However, no data are available defining the characteristic intraocular environment that may facilitate B cell dominance, B cell precursors, or plasma cell (PC) survival in the so-called plasma cell niche. The soluble B cell maturation antigen (BCMA) ligands APRIL and BAFF are indispensable for the longevity of PC [17]. Evidently, long-lived plasma cells (LLPC) persist during several autoimmune disorders at the site of inflammation [18–20]. In the present study, we therefore performed transcriptomic and proteomic analysis of iris specimens of JIAU patients and compared them to control patients.

2. Materials and methods

2.1. Sample collection

Samples were collected from 2013 to 2018 at the Department of Ophthalmology at St. Franziskus Hospital Münster. JIA (study group) was diagnosed by cooperating pedriatric rheumatologists according to the International League of Associations for Rheumatology (ILAR) criteria [3] and uveitis was assessed ophthalmologically in agreement with the Standardization of Uveitis Nomenclature (SUN) Working Group recommendations [21]. As a disease control group, we have chosen HLA-B27 positive acute anterior uveitis (AAU) as another well-defined anterior uveitis entity (acute onset of flare, anterior uveitis anatomic type). The study was performed according to the principles of the Declaration of Helsinki and was approved by the local ethics committee. For futher information about the sample collection see suppoting information.

2.2. RNA library preparation, sequencing, and gene expression analysis

For detailed information about RNA processing, RNA library preparation, sequencing, and gene expression analysis see supporting information (RNA library preparation and sequencing – procedure, Gene expression analysis – procedure).

2.3. Quantitative real-time polymerase chain reaction

After isolating total RNA from JIAU and POAG iris samples, reverse transcription to cDNA of the entire RNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Foster City, CA, USA) according to the manufacturer's instructions. For further information about the primer sequences used (BioLegio, Nejmegen, Netherlands) and the procedure of qRT-PCR see the supporting information (Supplemental Table 1, Quantitative real-time polymerase chain reaction - procedure).

2.4. Protein preparation for expression analysis

Iris samples were homogenized to a fine powder with a micro pestle for 5 min in the presence of liquid nitrogen. Then, 200 µl lysis buffer (7 M urea, 2% w/v CHAPS, and 10 mM TECEP) was added, followed by sonification (15 min) and centrifugation (30,000 × *g* 30 min). Supernatants were processed as described with slight modifications [22,23]. Detailed information about the protein preparation and expression analysis are listed in the supporting information (Protein preparation for expression analysis – procedure).

2.5. Western-blot analysis

For western blot analysis, SDS-PAGE (S260 Mighty Small II Deluxe Mini verticale Electrophoresis unit, Hoefer Inc., Holliston, USA) was performed using 8–16% gradient gels (SERVAGel™ TG PRiME™ 8–16%, Serva Electrophoresis, Heidelberg, Germany) with a 20 µl loading volume. After electrophoresis, the proteins were blotted onto a PVDF membrane (Amersham Hybond P 0.45 µm pore size, GE Healthcare Life Science, Freiburg, Germany) in a semi-dry blotting system (BlueFlash Semi-Dry Blotter). For detailed information about the blotting procedure see supporting information (Western blot – procedure).

2.6. LEGENDplex™assay

To simultanously quantify the concentration of the soluble targets APRIL, BAFF, and IL-6 we analyzed AqH and sera by using the bead-based multiplex LEGENDplex™ from JIAU patients and compared the results to the POAG and AAU group. For detailed information see supporting information (LEGENDplex™assay – procedure).

3. Results

The demographic data of patients included in the study were representative for the specific groups (Table 1). Briefly, JIAU patients had female predominance, were mostly ANA positive, and presented with early onset of disease and a high rate of uveitis-related ocular complications, and most had received systemic DMARD treatment to achieve uveitis inactivity (Supplementary Table 1). AAU patients had different associated systemic diseases (spondyloarthritis n = 10, spondyloarthritis with chronic inflammatory gut disease n = 1, psoriasis arthritis n = 2). Elderly POAG patients had received surgery, while AAU patients were middle aged and predominantly of male gender. All of the uveitis patients had inactive uveitis at the time of surgery.

3.1. RNA-seq analysis

To identify global changes in gene expression in iris tissue from the patient groups with JIAU or POAG, we perfomed transcriptomic profiling by RNA-Seq. Detailed information about the RNA-Seq reads are listed in the supporting information (RNA-Seq analysis, Supplemental Table 2).

3.2. Hierarchial cluster analysis

To obtain an overview of similarity over all samples, we performed hierarchical cluster analysis by determining sample-to-sample distances (Fig. 1). The samples JIAU 3, 2, and 4 built one cluster. Equally, the samples POAG 2, 3, and 1. The outliersamples POAG 4 and JIAU 1 were removed from further analyses.

Table 1
Clinical data of patient groups included in the study.

	Study group JIAU	Control group AAU	Control group POAG	p-value
Patients (*n*)	30	18	20	
Female gender n (%)	27 (90)	10 (56)	10 (50)	[1] 0.0035
Age (mean ± SD)	11 ± 6	45 ± 13	70 ± 7	[2] 0.001
Associated systemic immune-mediated disease	30 (100)	13 (72)	0 (0)	[1] 0.0006
ANA positivity *n* (%)	25 (83)a	n. d.	n. d.	
RF positivity *n* (%)	0 (0)b	n. d.	n. d.	
Preoperative IOP (mean ± SD)*	20 ± 9	16 ± 6	19 ± 4	[3] 0.07
Glaucoma**	21 (70)	6 (33)	20 (100)	[4] 0.0551
Cataract or other uveitis-related eye complications *n* (%)	27 (90)	16 (89)	n. a.	
Inactive uveitis# *n* (%)	30 (100)	18 (100)	n. a.	
Systemic DMARDs *n* (%)***	25 (83)	15 (83)	n. d.	[4] 0.658
Topical corticosteroids# *n* (%)	28 (93)	17 (94)	12 (60)	[1] 0.0026

ANA = antinuclear antibodies; RF = rheumatoid factor; IOP = intraocular pressure; * under combined anti-glaucomatous medication, if required,; ** secondary glaucoma in case of uveitis patients, primary open-angle glaucoma in the control group; n. d. = not determined, n. a. = not applicable, ***DMARDs = disease-modifying anti-rheumatic drugs; a = not determined in 4; b = not determined in 11; # in the eye, then receiving surgery.[1] Chi²-test, [2] One-way ANOVA, [3] Kruskal-Wallis Test, [4] Fisher's exact test.

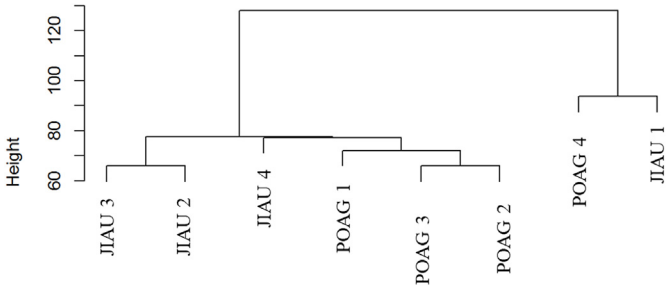

Fig. 1. Cluster dendrogram by hierarchical clustering of all samples. The samples POAG 4 and JIAU 1 were outliers. All other samples built 2 clusters.

3.3. Principle component analysis (PCA)

PCA was conducted to visualize sample relationships (Fig. 2). The PCA plot shows that the samples of the JIAU or POAG group could be separated into two clusters, according to their gene expression profile. Based on these results, differential gene expression analysis followed comparing JIAU and POAG.

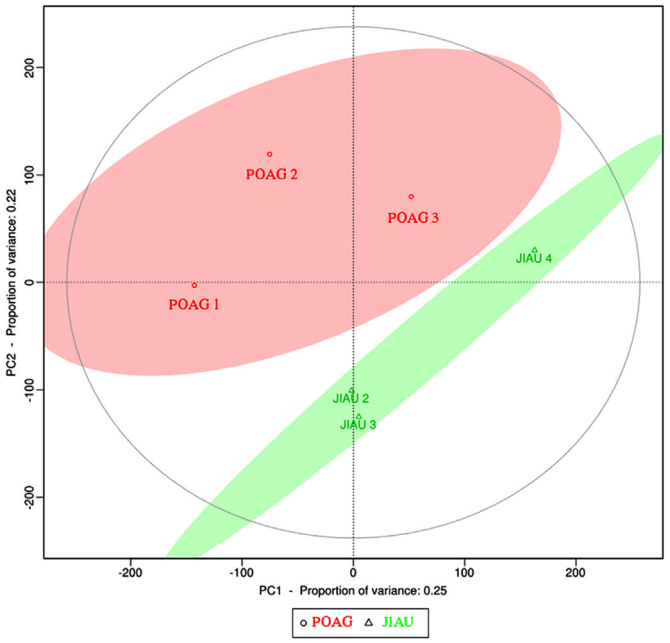

Fig. 2. PCA of gene expression patterns over all genes and samples. JIAU and POAG samples constitute two clusters. The first principle component (PC1, x-axis) accounts for 25% and the second principle component (PC2, y-axis) 22% of total variance.

3.4. Differential gene expression analysis

After filtering out weakly expressed genes, 43,567 genes with nonzero total count remained for differential expression analysis. After correction for multiple testing, a total of 136 genes showed significantly differential expression in the JIAU group compared to the POAG group, whereby the majority of these genes (110 genes) were upregulated, while 26 genes were downregulated (Fig. 3). For detailed information, see the supporting information (Supplemental Table 3).

Concerning B cells, expression of several Ig components was significantly higher in the JIAU group, of which two were the most

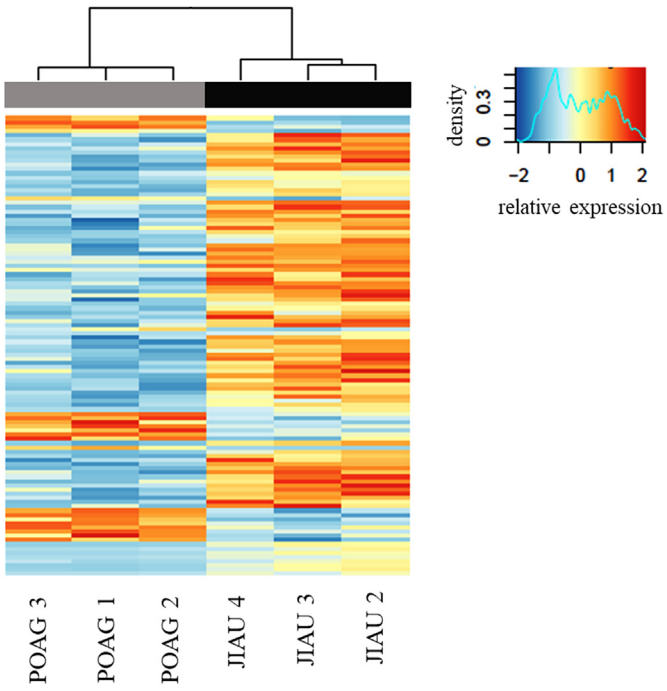

Fig. 3. Heatmap of the 136 significantly DE genes among all samples. Each row represents one gene, each column one sample. Samples were clustered due to their gene expression similarities. The color key indicates the relative gene expression intensity, where red symbolizes higher and blue indicates lower gene expression. Normalized read counts were used.

Table 2
Upregulated B cell-associated genes in the JIAU group compared to the POAG group ($p \leq 0.05$).

Ensemble Gen ID	Symbol	*BaseMean*	log2 *fold change* (JIAU vs. POAG)	adj. p-value
ENSG00000211892	IGHG4	289.67	3.44	2.44E-32
ENSG00000211598	IGKV4-1	126.95	3.29	8.56E-29
ENSG00000211666	IGLV2-14	53.91	2.44	1.46E-14
ENSG00000117318	ID3	1907.50	1.01	1.83E-06
ENSG00000211663	IGLV3-19	24.35	1.55	1.73E-05
ENSG00000125968	ID1	261.65	1.08	4.08E-04
ENSG00000211943	IGHV3-15	15.88	1.32	6.41E-04
ENSG00000164330	EBF1	138.45	1.07	3.77E-03

significant DE genes as compared to the POAG group: First, the Ig heavy chains IGHG4 (log2 fold change = 3.44, p = 2.44E-32), IGHV3-15 (log2 fold change = 1.32, p = 6.41E-04), and the light chains IGKV4-1 (log2 fold change = 3.29, p = 8.56E-29), IGLV2-14 (log2 fold change = 2.44, p = 1.46E-14), and IGLV3-19 (log2 fold change = 1.55, p = 1.73E-05), which are known for participating in antigen recognition [24] (Table 2) and being strongly associated with Ig-secreting PC [25]. Secondly, the expression of the B cell associated genes Id 3 (Inhibitor Of DNA Binding 3, log2 fold change = 1.01, p = 1.83E-06), Id1 (log2 fold change = 1.08, p = 4.08E-04), and Ebf1 (Early B Cell Factor 1, log2 fold change = 1.07, p = 3.77E-03) was significantly upregulated in the JIAU group.

3.5. qRT-PCR

We performed qRT-PCR to validate RNA-Seq data (Fig. 4). Here, we selected genes that showed a highly differential expression in the JIAU group (Id1, Nedd9 (neural precursor cell expressed, developmentally downregulated 9, and Gpr83 (G protein-coupled receptor 83)) compared to the POAG group, or that were known to be B cell associated (Id3, Ebf1, Fig. 4A). Similar to the RNA-Seq data, qRT-PCR showed a significantly differential expression between JIAU and the POAG groups for all selected genes except Ebf1. A significantly increased mRNA level of Id1 in the JIAU group could be observed by RNA-Seq analysis (fold change = 2.11 ± 0.34 [mean ± standard error], p = 0.00041) and qRT-PCR analysis (fold change = 5.13 ± 2.87, p = 0.04) and likewise for the mRNA level of Id3 in the RNA-Seq analysis (fold change = 2.01 ± 0.25, p = 1.83E-06) and qRT-PCR (fold change = 1.28 ± 0.1, p = 0.04) relative to those measured in the POAG group (fold change = 1 ± 0). In addition, Nedd9 showed a significantly increased upregulation of mRNA in the RNA-Seq analysis (fold change = 1.6 ± 0.21, p = 0.07) and qRT-PCR (fold change = 3.94 ± 2.76, p = 0.04). Inconsistent with the RNA-Seq analysis, the mRNA level for Ebf1 (fold change = 2.1 ± 0.38, p = 3.77E-03) was non-significantly higher in the qRT-PCR analysis (fold change = 1.33 ± 0.18, p = 0.49). Gpr83 showed a consistent statistically significant mRNA downregulation by RNA-Seq analysis (fold change = 0.49 ± 0.11, p = 0.05) and qRT-PCR (fold change = 0.14 ± 0.08, p = 0.04) compared to the mRNA levels measured in the POAG group.

To provide more evidence for the concept of B cell involvement in JIAU, we further analyzed mRNA levels of genes that are typically associated with different B cell stages, including CD19, MS4A1 (CD20), CD24, CD27, CD38, syndecan-1 (SDC1), and MZB1 (Marginal Zone B and B1 cell-specific protein) (Fig. 4B). Expression of all analyzed genes was significantly higher in the JIAU samples than in the POAG samples.

3.6. Proteome analysis

We then performed LC-IMS analysis from iris samples to detect the proteomic differences in the JIAU group as compared to the POAG group. Similar to the RNA-Seq analysis, we first conducted a PCA to visualize sample relationships (Fig. 5). The samples were clearly separated into two clusters (4 samples JIAU, 3 samples POAG).

A total of 1082 proteins were differentially expressed (Tables S4 and S5, fold change ≥ 1.2) between the JIAU and POAG group. Of these, 56 proteins reached the significance level (ANOVA $p \leq 0.05$), with expression of 17 proteins being higher in JIAU and 39 proteins lower in POAG.

Consistent with the RNA-Seq data, we found a significantly differential expression of Ig-specific/associated proteins. Moreover, expression of B cell-specific protein MZB1 was 3.5-fold higher in the JIAU group than in the POAG group (Table 3).

To determine the relevance of the proteomic findings, western blot

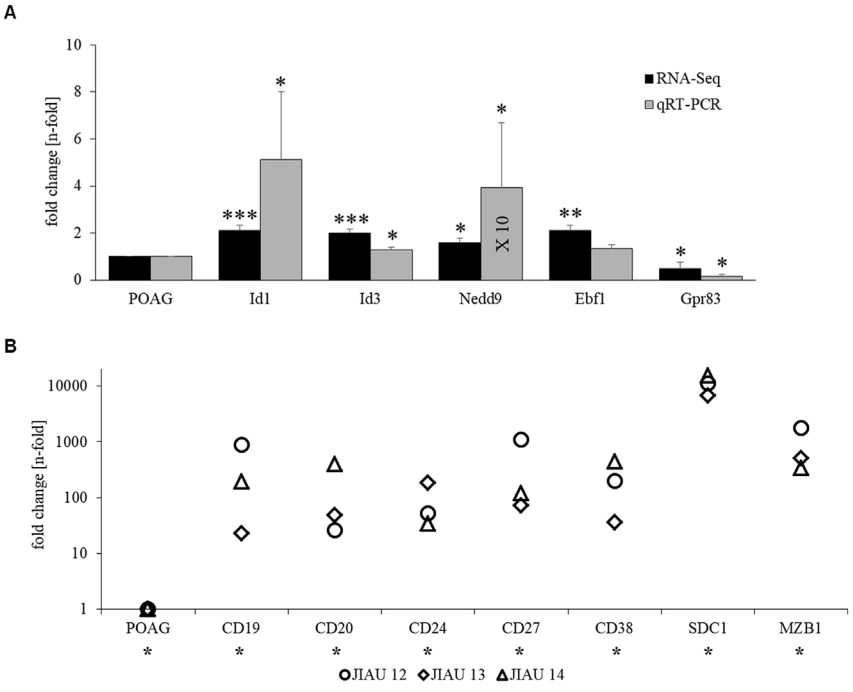

Fig. 4. qRT-PCR results of mRNA levels in JIAU samples relative to those measured in the POAG samples. (A) RNA-Seq data were verified by qRT-PCR, (B) qRT-PCR analysis of selected B cell-associated genes. RNA-Seq analysis: $*p \leq 0.1$, $**p \leq 0.01$; $***p \leq 0.001$; qRT-PCR: $*p \leq 0.05$; n = 3. A: mean ± SE; B: single values.

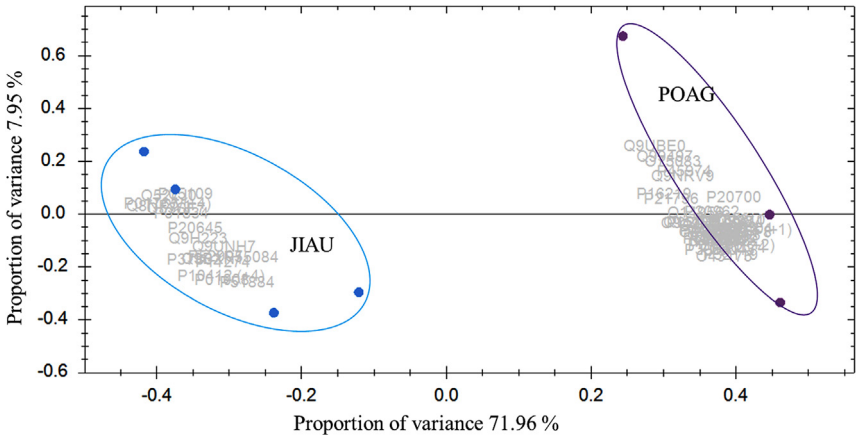

Fig. 5. PCA of protein expression patterns of all proteins with ANOVA $p \leq 0.05$. JIAU and POAG samples were clearly separated into two clusters. n = 4 (JIAU), n = 3 (POAG).

analysis from iris samples of MZB1 was performed and related to the expression intensity of the housekeeping protein vinculin (Fig. 6). An upregulation of MZB1 was detected in the iris of JIAU as compared to POAG.

As our results support published data concerning B cells residing in the iris during JIAU, we simultaneously performed multiplex quantification of BAFF and APRIL related to B cell survival and activation (Fig. 7A and B) and IL-6 (Fig. 7C) in AqH and serum samples of JIAU, POAG, and AAU patients. In the AqH of AAU and JIAU patients, a significantly increased amount of BAFF, APRIL, and IL-6 was observed compared to the POAG group. The concentration of IL-6 was also significantly increased in the serum from AAU and JIAU patients compared to the POAG group.

4. Discussion

Anterior uveitis is the most common extra-articular manifestation of JIA, whereby a considerable number of children develop vision-threatening complications [26]. The immunopathologic mechanisms are still not well understood and, currently, no adequate animal model representing the pathomechanisms of JIAU exists. Thus, genomic and proteomic analysis of the anterior uveal tract, the primary site of this inflammation, could provide important information regarding the pathogenesis of JIAU.

Gene and protein expression studies have been widely used to identify novel factors or pathways implicated in the pathogenesis of autoimmune diseases [27,28]. To our knowledge, this is the first comprehensive gene and protein expression profiling study focusing on B cell involvement performed on iridectomy specimens from JIAU patients.

The significantly upregulated gene and protein expression of B cell-specific factors, especially the upregulation of several Ig components, provided evidence for the presence of B cells at the anatomic site of inflammation in JIAU patients. These results were supported by qRT-PCR analyses, showing a significant increase in the pan B cell markers CD19 and CD20, and also of CD27 as a marker for activated B cells. Furthermore, elevated gene expression of CD138 (SDC-1) specific for PC – the final differentiation stage of B cells – was detected. These

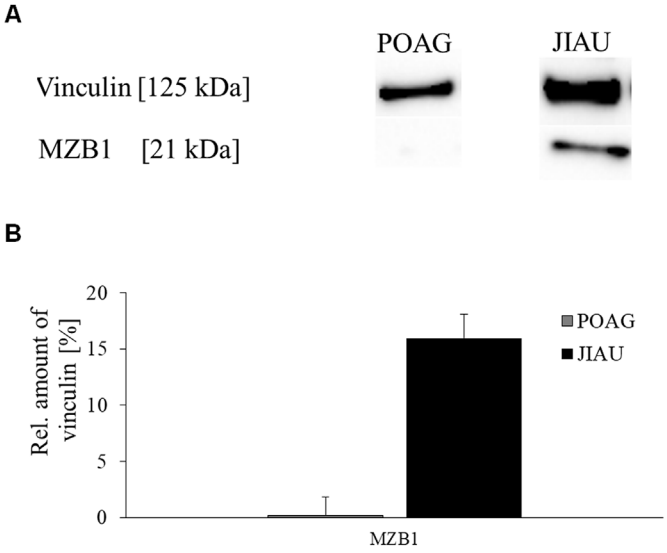

Fig. 6. Gray value (A) and densitometric analyses of MZB1 (B) by western blot. Expression was normalized to the housekeeping protein expression of vinculin (125 kDa).

findings are in line with earlier, mainly immunohistochemical studies identifying PC as the most prominent cell type infiltrating the iris in JIAU, and indicating a remarkable role for B cells in the pathogenesis of this disease [8,11–14]. Likewise, a local Ig production of B cells has been detected, although the target antigens are still unknown [8,11,12]. Recently, significantly higher amounts of intraocularly produced antibodies specific to Parvovirus B19 in JIAU patients compared to children with AU of unknown etiology was detected, while Parvovirus B19 DNA was absent. Hence, this suggests either that the virus titer was low or that a B cell-mediated autoimmune reaction was initially triggered by Parvovirus B 19 [29]. In addition to the apparent predominance of PC in the iris specimens from the JIAU patients that were analyzed, the increased expression of other B cell-related factors like CD20 and the transcription factors Ebf1 and Id3, or MZB1 found in

Table 3
Differentially expressed Ig and MZB1 proteins in iris tissue of the JIAU group.

UniProt Protein ID	Name	No. of peptides	P value (ANOVA)	*n-fold*
P01834	Ig kappa chain C region	6	0.033	11.4
P01765	Ig heavy chain V-III region TIL	2	0.031	4.1
Q8WU39	Marginal zone B- and B1-cell-specific protein	6	0.180	3.5

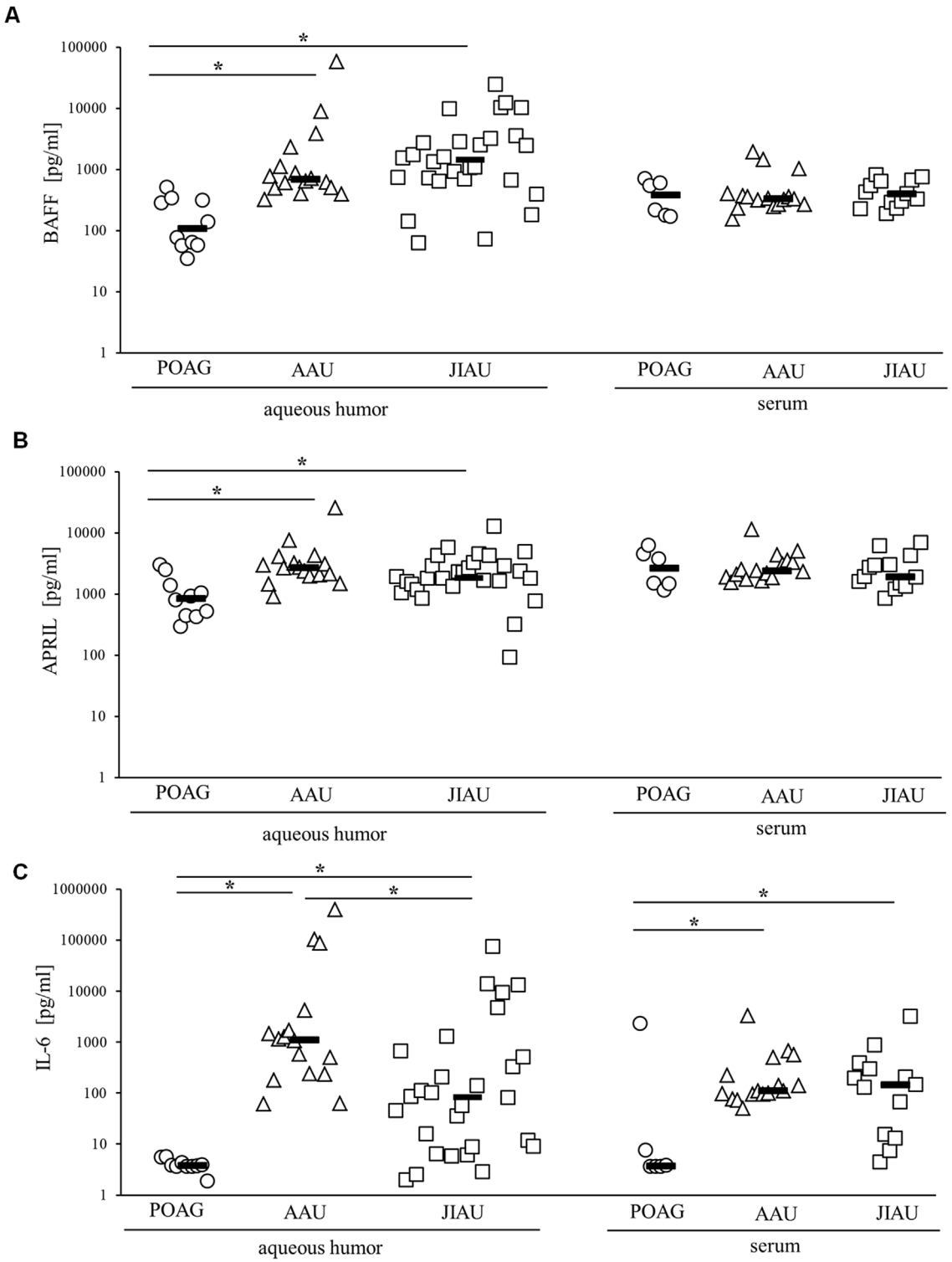

Fig. 7. Quantification of B cell-associated factors in the aqueous humor and sera of POAG, AAU, and JIAU patients. The B cell survival factors BAFF (**A**), APRIL (**B**), and the B cell stimulating factor IL-6 (**C**) were quantified (pg/ml). Black bar = median; aqueous humor: POAG n (patients) = 9, n (eyes) = 10, AAU n = 16, JIAU n = 28; serum: POAG n = 6, AAU n = 19, JIAU n = 13; $^{*}p \leq 0.05$.

the present study, are associated with different B cell stages [30–33]. This may be due to diverse duration and disease stages of JIA patients, as has been previously reported [34,35]. Recent JIA patients with early onset of disease (< 6 years) showed higher expressions of B cell-associated genes by peripheral blood mononuclear cells (PBMC) than late-onset JIA patients [34]. Thus, the pathologic mechanisms may vary with JIA- and related uveitis disease onset and duration, by displaying a variable number of B cells.

Tissue-damage is decisively provoked by immune cell infiltration in autoimmune disorders [36–39], and our results suggest that there is lymphocytic infiltration at the primary anatomic site of uveitis, in accordance with previous studies – therefore, we aimed to characterize the intraocular conditions that are possibly responsible for B cell activation and stimulation. The analysis of AqH is a valuable method to

obtain information about the intraocular micromilieu that is likely charateristic of uveitic diseases [40–43]. Here, we additionally compared our data to AAU, another well-defined uveitis entity. AAU is the most common uveitis-entitiy in western europe and the USA, accounting for about 30% of uveitis patients with onset frequently observed in children and adolescents [44]. Cytokine analysis of AqH in the present study showed significantly increased concentrations of BAFF, APRIL, and IL-6 in the AqH of JIAU and AAU, but not in POAG. Among others, these factors are essential for establishing and maintaining a microenvironment that enables PC to become long-lived [45–47]. BAFF and APRIL provide pro-survival effects by inducing the antiapoptotic molecule Mcl 1 (myeloid leukemia cell differentiation protein), which is essential for PC survival [48]. Transgenic BAFF-overexpressing mice show a SLE-like phenotype and SS-like symptoms with inflammation of the salivary glands [49] as well as abnormal B cell maturation and B cell expansion, mainly of splenic transitional B cells and marginal zone (MZ) B cells [50]. Eliminating MZ B cells reduced autoimmune symptoms [51–53]. It has been suggested that MZ-like B cells have different target receptors [54], which allow circulation and infiltration in peripheral tissues due to higher BAFF expression; however, it is not the total amount of MZ B cells that correlates with autoimmunity, but rather their localization and function [50,55,56]. Thus, the increased gene and protein expression of MZB1 in the iris of JIAU patients found herein is probably the result of significantly increased BAFF expression. Regarding IL-6 as another critical PC suvival factor [47], increased concentrations of IL-6 were detected in the AqH of JIAU patients, which is in agreement with the literature [40]. Thus, the presence of B cells in the iris of JIAU patients, together with the upregulation of IL-6, BAFF, and APRIL could now provide information about the long-term survival of B cells (in so-called PC niches), even during phases of disease inactivity, suggesting their contribution to uveitis chronicity. As the AAU patients also showed significantly increased levels of these factors, it is probably not specific for JIAU. The AAU specimens showed even higher concentrations of IL-6 and APRIL than the JIAU group, although all specimens were obtained during the inactive stage of disease. This may be due to the fact that inflammation is more intense during recurrent attacks, which is typical for AAU as compared to JIAU, characterized by high hypopyon and fibrin development and a higher amount of inflammatory cells in the anterior chamber, causing severe complications [57].

In the sera BAFF and APRIL concentrations detected in the JIAU group did not differ from those in AAU and POAG, which suggests an intraocular production. Previously, increased BAFF and APRIL concentrations could be detected in the sera of RA and SLE patients [58,59] as well as in the sera of children with JIA and SLE [60,61]. An increased concentration during the early phases of RA indicated an activation of B cells and also the development of an autoreactive B cell-mediated immune response [62]. The significant upregulation of IL-6 in the sera of both AAU and JIAU patients in comparison to POAG patients is probably related to the associated systemic diseases in AAU or JIAU.

Further evidence that B cells are involved in the pathogenesis of JIAU has been shown by studies demonstrating a therapeutic benefit, particularly the clinical response to rituximab (anti-CD20), or tocilizumab (IL-6 receptor inhibitor) treatment. Indeed, both biologics have proven to be effective even for severe JIAU that is refractory to corticosteroids and conventional synthetic DMARDs [63–67]. Another treatment which therefore might be beneficial in those cases aims to inhibit BAFF and APRIL. In a randomized phase-I study, belimumab (*anti*-BAFF) significantly reduced the B cell population in SLE patients when compared to a placebo control group [68]; moreover, in a phase-II study a reduction of naive, activated CD20 $^{+}$ B cells and also *anti*-dsDNA antibody titers was observed [69]. In a phase-II study in RA patients treated with atacicept (BAFF- and APRIL-inhibitor), a dose-dependent reduction in IgM, IgA, and IgG as well as a decreased number of mature B cells and PC were observed [70]. BAFF and APRIL both play important roles in the pathogenesis of diseases, in which autoreactive B cells are involved. Thus, treatment approaches targeting BAFF and APRIL might also be considered for JIAU.

The present study has some limitations. Our analysis was done on iris samples from patients with long-standing courses of disease, probably not reflecting immunopathogenesis in early disease stages. Due to the small size of the iris specimens, however, an absolute quantification of gene and protein expression was often not possible. The majority of the JIAU patients had received a systemic DMARD treatment that might have influenced the results. Owing to the heterogeneity of individual cases of disease, sample pooling was not advisable.

5. Conclusion

In summary, our study showed an intense intraocular gene and protein expression of B cell and PC-associated molecules in iris tissues from JIAU patients, indicating a crucial role of these cells in JIAU. The concurrently increased concentrations of the B cell survival factors BAFF, APRIL, and IL-6 in the AqH of patients might possibly be responsible for the longevity of PC in the affected tissues, even during phases of inactive disease. Future studies identifying possible target antigens of PC-produced antibodies and characterization of the conditions responsible for the stimulation and long-term suvival of PC are required.

Acknowledgments

The authors thank Gerrit Randau (Core Facility Genomics, University of Münster, Germany) for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.03.004.

Funding

This work was funded in part by German Ophthalmological Society (DOG) section uveitis.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The current affiliation for S. G. is codecentric AG, Münster, Germany.

The current affiliation for H. M. is Administration of the scientifically employed at the university hospital, Münster, Germany.

S. T. is retired since 2017.

Due to the sensitive nature of the questions asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

Author contributions

L. W. designed this study, performed experiments, analyzed the data, and wrote the paper. D. A. performed experiments. A. W., M. S., C. T., and D. B. contributed to research design. M. K. designed study, performed experiments and revised the manuscript critically. M. B. performed experiments, analyzed statistical data and revised the manuscript critically S. G. and A. B. analyzed data. H. M. and S. T. supported experimental procedure. K. W. provided clinical data and revised the manuscript critically. J. K., C. H., and B. L. supported sample collection. S. K. contributed to research design and analyzed the data. A. H. designed the study and revised the manuscript critically.

References

[1] L. Berntson, B. Andersson Gare, A. Fasth, T. Herlin, J. Kristinsson, P. Lahdenne, et al., Incidence of juvenile idiopathic arthritis in the Nordic countries. A population based study with special reference to the validity of the ILAR and EULAR criteria, J. Rheumatol. 30 (2003) 2275–2282.

[2] A. Heiligenhaus, M. Niewerth, G. Ganser, C. Heinz, K. Minden, German Uveitis in Childhood Study G. Prevalence and complications of uveitis in juvenile idiopathic arthritis in a population-based nation-wide study in Germany: suggested modification of the current screening guidelines, Rheumatology 46 (2007) 1015–1019.

[3] R.E. Petty, T.R. Southwood, P. Manners, J. Baum, D.N. Glass, J. Goldenberg, et al., International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001, J. Rheumatol. 31 (2004) 390–392.

[4] K. Kotaniemi, H. Kautiainen, A. Karma, K. Aho, Occurrence of uveitis in recently diagnosed juvenile chronic arthritis: a prospective study, Ophthalmology 108 (2001) 2071–2075.

[5] P.E. Carvounis, D.C. Herman, S. Cha, J.P. Burke, Incidence and outcomes of uveitis in juvenile rheumatoid arthritis, a synthesis of the literature, Graefes Arch. Clin. Exp. Ophthalmol. 244 (2006) 281–290.

[6] S.T. Angeles-Han, C. McCracken, S. Yeh, S.R. Jang, K. Jenkins, S. Cope, et al., HLA Associations in a cohort of children with juvenile idiopathic arthritis with and without uveitis, Investig. Ophthalmol. Vis. Sci. 56 (2015) 6043–6048.

[7] A.J.W. Haasnoot, M.W. Schilham, S. Kamphuis, P.C.E. Hissink Muller, A. Heiligenhaus, D. Foell, et al., An amino acid motif in HLA-DRbeta1 distinguishes patients with uveitis in juvenile idiopathic arthritis, Arthritis Rheumatol. 70 (2018) 1155–1165.

[8] J.G. Parikh, K.A. Tawansy, N.A. Rao, Immunohistochemical study of chronic nongranulomatous anterior uveitis in juvenile idiopathic arthritis, Ophthalmology 115 (2008) 1833–1836.

[9] C. Tappeiner, J. Klotsche, S. Schenck, M. Niewerth, K. Minden, A. Heiligenhaus, Temporal change in prevalence and complications of uveitis associated with juvenile idiopathic arthritis:data from a cross-sectional analysis of a prospective nationwide study, Clin. Exp. Rheumatol. 33 (2015) 936–944.

[10] S. Prahalad, D.N. Glass, A comprehensive review of the genetics of juvenile idiopathic arthritis, Pediatr Rheumatol Online J 6 (2008) 11.

[11] W.A. Godfrey, C.B. Lindsley, F.E. Cuppage, Localization of IgM in plasma cells in the iris of a patient with iridocyclitis and juvenile rheumatoid arthritis, Arthritis Rheum. 24 (1981) 1195–1198.

[12] J.C. Merriam, L.T. Chylack Jr., D.M. Albert, Early-onset pauciarticular juvenile rheumatoid arthritis. A histopathologic study, Arch. Ophthalmol. 101 (1983) 1085–1092.

[13] R. Sabates, T. Smith, D. Apple, Ocular histopathology in juvenile rheumatoid arthritis, Ann. Ophthalmol. 11 (1979) 733–737.

[14] V. Kalinina Ayuso, M.R. van Dijk, J.H. de Boer, Infiltration of plasma cells in the Iris of children with ANA-positive anterior uveitis, Investig. Ophthalmol. Vis. Sci. 56 (2015) 6770–6778.

[15] Q.D. Nguyen, R.L. Humphrey, J.P. Dunn, M.S. Humayun, Elevated vitreous concentration of monoclonal immunoglobulin manifesting as schlieren in juvenile rheumatoid arthritis-associated uveitis, Arch. Ophthalmol. 119 (2001) 293–296.

[16] K. Walscheid, M. Hennig, C. Heinz, S. Wasmuth, M. Busch, D. Bauer, et al., Correlation between disease severity and presence of ocular autoantibodies in juvenile idiopathic arthritis-associated uveitis, Investig. Ophthalmol. Vis. Sci. 55 (2014) 3447–3453.

[17] B.P. O'Connor, V.S. Raman, L.D. Erickson, W.J. Cook, L.K. Weaver, C. Ahonen, et al., BCMA is essential for the survival of long-lived bone Marrow plasma cells, J. Exp. Med. 199 (2004) 91–98.

[18] E.A. Szyszko, K.A. Brokstad, G. Oijordsbakken, M.V. Jonsson, R. Jonsson, K. Skarstein, Salivary glands of primary Sjogren's syndrome patients express factors vital for plasma cell survival, Arthritis Res. Ther. 13 (2011) R2.

[19] G. Cassese, S. Lindenau, B. de Boer, S. Arce, A. Hauser, G. Riemekasten, et al., Inflamed kidneys of NZB/W mice are a major site for the homeostasis of plasma cells, Eur. J. Immunol. 31 (2001) 2726–2732.

[20] H.J. Kim, V. Krenn, G. Steinhauser, C. Berek, Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis, J. Immunol. 162 (1999) 3053–3062.

[21] D.A. Jabs, R.B. Nussenblatt, J.T. Rosenbaum, Standardization of uveitis nomenclature working G. Standardization of uveitis nomenclature for reporting clinical data. Results of the first international workshop, Am. J. Ophthalmol. 140 (2005) 509–516.

[22] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359–362.

[23] S. Konig, K. Hadrian, S. Schlatt, J. Wistuba, S. Thanos, M.R.R. Bohm, Topographic protein profiling of the age-related proteome in the retinal pigment epithelium of Callithrix jacchus with respect to macular degeneration, J Proteomics (2018).

[24] M.P. Lefranc, Immunoglobulin and T Cell receptor genes: IMGT((R)) and the birth and rise of immunoinformatics, Front. Immunol. 5 (2014) 22.

[25] H.W. Schroeder Jr., L. Cavacini, Structure and function of immunoglobulins, J. Allergy Clin. Immunol. 125 (2010) S41–S52.

[26] A. Skarin, R. Elborgh, E. Edlund, E. Bengtsson-Stigmar, Long-term follow-up of patients with uveitis associated with juvenile idiopathic arthritis: a cohort study, Ocul. Immunol. Inflamm. 17 (2009) 104–108.

[27] X. Wang, S. Xia, B. Fu, RNAseq analysis of synovial fibroblasts in human rheumatoid arthritis, Mol. Med. Rep. 10 (2014) 241–247.

[28] Q.Y. Lu, Q.H. Han, X. Li, Z.C. Li, Y.T. Pan, L. Liu, et al., Analysis of differentially expressed genes between rheumatoid arthritis and osteoarthritis based on the gene co-expression network, Mol. Med. Rep. 10 (2014) 119–124.

[29] J.D. de Groot-Mijnes, J. Dekkers, L. de Visser, A. Rothova, A.M. van Loon, J.H. de Boer, Antibody production against B19 virus in ocular fluid of JIA-associated uveitis patients, Ophthalmology 122 (2015) 1270-2.e1.

[30] P. Stashenko, L.M. Nadler, R. Hardy, S.F. Schlossman, Characterization of a human B lymphocyte-specific antigen, J. Immunol. 125 (1980) 1678–1685.

[31] H. Lin, R. Grosschedl, Failure of B-cell differentiation in mice lacking the transcription factor EBF, Nature 376 (1995) 263–267.

[32] S. Chen, M. Miyazaki, V. Chandra, K.M. Fisch, A.N. Chang, C. Murre, Id3 orchestrates germinal center B cell development, Mol. Cell Biol. 36 (2016) 2543–2552.

[33] M. Rosenbaum, V. Andreani, T. Kapoor, S. Herp, H. Flach, M. Duchniewicz, et al., MZB1 is a GRP94 cochaperone that enables proper immunoglobulin heavy chain biosynthesis upon ER stress, Genes Dev. 28 (2014) 1165–1178.

[34] M.G. Barnes, A.A. Grom, S.D. Thompson, T.A. Griffin, L.K. Luyrink, R.A. Colbert, et al., Biologic similarities based on age at onset in oligoarticular and polyarticular subtypes of juvenile idiopathic arthritis, Arthritis Rheum. 62 (2010) 3249–3258.

[35] W.T. Shearer, H.M. Rosenblatt, R.S. Gelman, R. Oyomopito, S. Plaeger, E.R. Stiehm, et al., Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study, J. Allergy Clin. Immunol. 112 (2003) 973–980.

[36] S. Takemura, P.A. Klimiuk, A. Braun, J.J. Goronzy, C.M. Weyand, T cell activation in rheumatoid synovium is B cell dependent, J. Immunol. 167 (2001) 4710–4718.

[37] J.C. Crispin, M. Oukka, G. Bayliss, R.A. Cohen, C.A. Van Beek, I.E. Stillman, et al., Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys, J. Immunol. 181 (2008) 8761–8766.

[38] R. Hohlfeld, E. Meinl, K. Dornmair, B- and T-cell responses in multiple sclerosis: novel approaches offer new insights, J. Neurol. Sci. 274 (2008) 5–8.

[39] L. Adalid-Peralta, A. Mathian, T. Tran, L. Delbos, I. Durand-Gasselin, D. Berrebi, et al., Leukocytes and the kidney contribute to interstitial inflammation in lupus nephritis, Kidney Int. 73 (2008) 172–180.

[40] K.M. Sijssens, G.T. Rijkers, A. Rothova, J.S. Stilma, P.A. Schellekens, J.H. de Boer, Cytokines, chemokines and soluble adhesion molecules in aqueous humor of children with uveitis, Exp. Eye Res. 85 (2007) 443–449.

[41] K.M. Sijssens, G.T. Rijkers, A. Rothova, J.S. Stilma, J.H. de Boer, Distinct cytokine patterns in the aqueous humor of children, adolescents and adults with uveitis, Ocul. Immunol. Inflamm. 16 (2008) 211–216.

[42] A.M. Haasnoot, J.J. Kuiper, S. Hiddingh, P.A. Schellekens, W. de Jager, S.M. Imhof, et al., Ocular fluid analysis in children reveals interleukin-29/interferon-lambda 1 as a biomarker for juvenile idiopathic arthritis-associated uveitis, Arthritis Rheum. 68 (2016) 1769–1779.

[43] V. Kalinina Ayuso, J.H. de Boer, H.L. Byers, G.R. Coulton, J. Dekkers, L. de Visser, et al., Intraocular biomarker identification in uveitis associated with juvenile idiopathic arthritis, Investig. Ophthalmol. Vis. Sci. 54 (2013) 3709–3720.

[44] C.A. McCannel, G.N. Holland, C.J. Helm, P.J. Cornell, J.V. Winston, T.G. Rimmer, Causes of uveitis in the general practice of ophthalmology. UCLA community-based uveitis study group, Am. J. Ophthalmol. 121 (1996) 35–46.

[45] M.J. Benson, S.R. Dillon, E. Castigli, R.S. Geha, S. Xu, K.P. Lam, et al., Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL, J. Immunol. 180 (2008) 3655–3659.

[46] B. Huard, T. McKee, C. Bosshard, S. Durual, T. Matthes, S. Myit, et al., APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa, J. Clin. Investig. 118 (2008) 2887–2895.

[47] G. Cassese, S. Arce, A.E. Hauser, K. Lehnert, B. Moewes, M. Mostarac, et al., Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals, J. Immunol. 171 (2003) 1684–1690.

[48] V. Peperzak, I. Vikstrom, J. Walker, S.P. Glaser, M. LePage, C.M. Coquery, et al., Mcl-1 is essential for the survival of plasma cells, Nat. Immunol. 14 (2013) 290–297.

[49] F. Mackay, S.A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, et al., Mice transgenic for baff develop lymphocytic disorders along with autoimmune manifestations, J. Exp. Med. 190 (1999) 1697–1710.

[50] M. Batten, J. Groom, T.G. Cachero, F. Qian, P. Schneider, J. Tschopp, et al., BAFF mediates survival of peripheral immature B lymphocytes, J. Exp. Med. 192 (2000) 1453–1466.

[51] E. Peeva, D. Michael, J. Cleary, J. Rice, X. Chen, B. Diamond, Prolactin modulates the naive B cell repertoire, J. Clin. Investig. 111 (2003) 275–283.

[52] C.M. Grimaldi, D.J. Michael, B. Diamond, Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus, J. Immunol. 167 (2001) 1886–1890.

[53] D. Zeng, M.K. Lee, J. Tung, A. Brendolan, S. Strober, Cutting edge: a role for CD1 in the pathogenesis of lupus in NZB/NZW mice, J. Immunol. 164 (2000) 5000–5004.

[54] J. Groom, S.L. Kalled, A.H. Cutler, C. Olson, S.A. Woodcock, P. Schneider, et al., Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome, J. Clin. Investig. 109 (2002) 59–68.

[55] H. Amano, E. Amano, T. Moll, D. Marinkovic, N. Ibnou-Zekri, E. Martinez-Soria, et al., The Yaa mutation promoting murine lupus causes defective development of marginal zone B cells, J. Immunol. 170 (2003) 2293–2301.

[56] S. Atencio, H. Amano, S. Izui, B.L. Kotzin, Separation of the New Zealand Black genetic contribution to lupus from New Zealand Black determined expansions of marginal zone B and B1a cells, J. Immunol. 172 (2004) 4159–4166.

[57] A. Rothova, W.G. van Veenedaal, A. Linssen, E. Glasius, A. Kijlstra, P.T. de Jong, Clinical features of acute anterior uveitis, Am. J. Ophthalmol. 103 (1987) 137–145.

[58] A. Becker-Merok, C. Nikolaisen, H.C. Nossent, B-lymphocyte activating factor in systemic lupus erythematosus and rheumatoid arthritis in relation to autoantibody levels, disease measures and time, Lupus 15 (2006) 570–576.

[59] J.-E. Gottenberg, C. Miceli-Richard, B. Ducot, P. Goupille, B. Combe, X. Mariette, Markers of B-lymphocyte activation are elevated in patients with early rheumatoid arthritis and correlated with disease activity in the ESPOIR cohort, Arthritis Res. Ther. 11 (2009) R114–R.
[60] T.A. Gheita, I.H. Bassyouni, Y. Emad, A.M. el-Din, E. Abdel-Rasheed, H. Hussein, Elevated BAFF (BLyS) and APRIL in Juvenile idiopathic arthritis patients: relation to clinical manifestations and disease activity, Joint Bone Spine Revue du rhumatisme 79 (2012) 285–290.
[61] S.D. Hong, A. Reiff, H.T. Yang, T.S. Migone, C.D. Ward, K. Marzan, et al., B lymphocyte stimulator expression in pediatric systemic lupus erythematosus and juvenile idiopathic arthritis patients, Arthritis Rheum. 60 (2009) 3400–3409.
[62] R.A. Moura, R. Cascao, I. Perpetuo, H. Canhao, E. Vieira-Sousa, A.F. Mourao, et al., Cytokine pattern in very early rheumatoid arthritis favours B-cell activation and survival, Rheumatology 50 (2011) 278–282.
[63] A. Heiligenhaus, E. Miserocchi, C. Heinz, V. Gerloni, K. Kotaniemi, Treatment of severe uveitis associated with juvenile idiopathic arthritis with anti-CD20 monoclonal antibody (rituximab), Rheumatology 50 (2011) 1390–1394.
[64] E. Miserocchi, I. Pontikaki, G. Modorati, F. Bandello, P.L. Meroni, V. Gerloni, Rituximab for uveitis, Ophthalmology 118 (2011) 223–224.
[65] C. Tappeiner, C. Heinz, C. Specker, A. Heiligenhaus, Rituximab as a treatment option for refractory endogenous anterior uveitis, Ophthalmic Res. 39 (2007) 184–186.
[66] C. Tappeiner, M. Mesquida, A. Adan, J. Anton, A.V. Ramanan, E. Carreno, et al., Evidence for tocilizumab as a treatment option in refractory uveitis associated with juvenile idiopathic arthritis, J. Rheumatol. 43 (2016) 2183–2188.
[67] V. Calvo-Rio, M. Santos-Gomez, I. Calvo, M.I. Gonzalez-Fernandez, B. Lopez-Montesinos, M. Mesquida, et al., Anti-Interleukin-6 receptor tocilizumab for severe juvenile idiopathic arthritis-associated uveitis refractory to anti-tumor necrosis factor therapy: a Multicenter study of twenty-five patients, Arthritis Rheum. 69 (2017) 668–675.
[68] R. Furie, W. Stohl, E.M. Ginzler, M. Becker, N. Mishra, W. Chatham, et al., Biologic activity and safety of belimumab, a neutralizing anti-B-lymphocyte stimulator (BLyS) monoclonal antibody: a phase I trial in patients with systemic lupus erythematosus, Arthritis Res. Ther. 10 (2008) R109.
[69] D.J. Wallace, W. Stohl, R.A. Furie, J.R. Lisse, J.D. McKay, J.T. Merrill, et al., A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active, Syst. Lupus Erythematosus. Arthritis Rheum. 61 (2009) 1168–1178.
[70] R.F. van Vollenhoven, N. Kinnman, E. Vincent, S. Wax, J. Bathon, Atacicept in patients with rheumatoid arthritis and an inadequate response to methotrexate: results of a phase II, randomized, placebo-controlled trial, Arthritis Rheum. 63 (2011) 1782–1792.