Aus dem Deutschen Rheumaforschungszentrum Berlin
und der Medizinischen Klinik mit Schwerpunkt Rheumatologie und Klinische Immunologie
der Medizinischen Fakultät Charité - Universitätsmedizin Berlin

DISSERTATION

Polychromatic flow cytometry and immune cell-specific gene expression profiling: An integrated approach to identify and validate new biomarkers and signatures of immune responses in chronic inflammatory rheumatic diseases.

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
Charité Universitätsmedizin Berlin

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Datum der Promotion: 01.02.2013

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Abbreviations

APC	allophycocyanine
AS	ankylosing spondylitis
CCR	C-C chemokine receptor
CXCR	C-X-C chemokine receptor
DC	dendritic cells
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide, bacterial endotoxin
NK cells	natural killer cells
PAM	prediction analysis for microarray
PE	phycoerythrin
RA	rheumatoid arthritis
Siglec-1	Sialic acid–binding immunoglobulin-like lectin-1
SLE	systemic lupus erythematosus
TNF	tumor necrosis factor

1.1 Abstract

Autoimmune diseases like ankylosing spondylitis (AS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are characterised by chronic inflammatory immune responses towards the body's own tissues.

Uncertain diagnosis especially in early states and not fully understood etiopathology hamper a curative breakthrough in these diseases.

This work introduces a novel multicolour flow cytometry approach for the development of diagnostic procedures based on a complex characterization of the inflammatory condition by simultaneous assessment of numerous peripheral blood leukocyte subsets from the expression of 50 surface CD antigens. A newly developed bioinformatic strategy for the analysis of 800 immunophenotypic parameters revealed a distinct pattern of differentially expressed surface molecules from AS patients compared to healthy individuals. In phenotypes, that determined the pattern of AS from the peripheral blood, the expression of surface molecules CD14, CD1c, CD1a, CXCR4, CD62L and CD69 was most pronounced. An even more enhanced expression of the above-mentioned molecules has also been demonstrated on cells from the synovial fluid of patients with AS, which showed impressively that this pattern typical for a local inflammation could be evaluated already from the analysis of immune cells in the peripheral blood. A comparable analysis of the peripheral blood from AS, RA and SLE patients indicated that not a single marker but a characteristic pattern of differently regulated surface molecules, in terms of an immunophenotypic signature, made it possible to distinguish between various rheumatic diseases.

In addition, complex flow cytometric immunophenotyping provides an attractive opportunity to validate candidate genes at the protein level, which were identified by gene expression studies.

Cell-specific gene expression analysis of peripheral monocytes from SLE patients showed a dominant type-I interferon (IFN) signature. As a reliable surrogate marker for type I IFN-induced responses, the sialic acid-binding immunoglobulin-like lectin 1 (Siglec-1) could be validated by flow cytometry. The frequency of Siglec-1 expressing peripheral monocytes correlating with disease activity proved to be useful as a potential biomarker for the therapeutic follow-up.

The gene expression signature identified in monocytes after *in vitro* stimulation with tumor necrosis factor α (TNFα) compared to the signature in peripheral monocytes from RA patients showed an overlapping profile of predominantly down-regulated genes. This finding emphasizes the contribution of TNFα to the disease immunopathology at the transcriptional level. A therapeutic blockade of TNFα reverses this transcriptional signature, suggesting that one could predict treatment response or failure from the peripheral blood prior to initiation of therapy.

In summary, comprehensive immunophenotyping of peripheral leukocytes by polychromatic flow cytometry provides a promising systems biology approach to characterizing aberrant immune responses. The presented disease-specific cytometric immunophenotypic signatures along with validated biomarkers and immune cell-specific gene expression signatures could contribute to the development of new diagnostic procedures for classification of diseases, assessment of disease activity and stratification of the therapy.

1.2 Zusammenfassung

Autoimmunerkrankungen wie Spondylitis ankylosans (AS), rheumatoide Arthritis (RA) und systemischer Lupus erythematodes (SLE) sind durch eine chronisch-entzündliche Immunantwort gegen eigene Gewebe gekennzeichnet. Unsicherheit in der Diagnose vor allem in frühen Stadien und eine bisher nicht vollständig verstandene Ätiopathologie erschweren eine kurative Therapie dieser Erkrankungen.

Diese Arbeit stellt ein neues Konzept zur Entwicklung diagnostischer Verfahren auf der Basis polychromatischer Durchflusszytometrie vor, bei dem der Entzündungszustand durch die gleichzeitige Beurteilung von 50 CD-Oberflächenantigenen und damit zahlreichen Leukozyten-Subpopulationen im peripheren Blut umfassend beschrieben wird. Eine neu entwickelte Bioinformatik-Strategie für die Analyse von 800 immunphänotypischen Parametern ergab bei AS-Patienten ein eindeutig typisches Muster von Oberflächenantigenen im Vergleich zu gesunden Personen. In den Phänotypen, die das Muster für die AS aus dem peripheren Blut bestimmten, war die Expression der Oberflächenmoleküle CD14, CD1c, CD1a, CXCR4, CD62L und CD69 am markantesten. Eine noch deutlicher erhöhte Expression der oben genannten Moleküle auf Zellen aus der Synovialflüssigkeit von AS-Patienten zeigte eindrucksvoll, dass dieses für eine lokale Entzündung typische Muster schon durch die Analyse von Immunzellen aus dem peripheren Blut bewertet werden kann. Die vergleichende Analyse des peripheren Blutes von AS-, RA- und SLE-Patienten zeigte an, dass nicht einzelne Marker sondern erst ein charakteristisches Muster verschiedenartig regulierter Oberflächenmoleküle, im Sinne einer immunphänotypischen Signatur, eine Unterscheidung der verschiedenen rheumatischen Erkrankungen erlaubt. Darüber hinaus bietet die komplexe durchflusszytometrische Immunphänotypisierung eine attraktive Möglichkeit, die Relevanz von Kandidatengenen, die durch Genexpressionsanalysen identifiziert wurden, auf Proteinebene zu prüfen. So zeigten zellspezifische Genexpressionsanalysen peripherer Monozyten von SLE-Patienten eine dominante Typ-I-Interferon-(IFN)-Signatur. Für diese Typ-I-IFN-induzierten Antworten konnte als zuverlässiger Surrogatmarker das Sialinsäure-bindende Immunglobulin-ähnliche Lektin 1 (Siglec-1) mit Hilfe der Durchflusszytometrie validiert werden. Die Frequenz der Siglec-1-exprimierenden peripheren Monozyten korrelierte mit der Krankheitsaktivität und könnte sich damit als potentieller Biomarker in der Verlaufskontrolle als nützlich erweisen. Die Genexpressionssignatur von Monozyten nach in-vitro-Stimulation mit Tumornekrosefaktor α (TNFα) zeigte im Vergleich zur Signatur peripherer Monozyten von RA-Patienten ein überlappendes Profil überwiegend herunterregulierter Gene, was den Beitrag von TNFα zur Krankheitsimmunpathologie auf der Transkriptionsebene unterstreicht. Eine therapeutische Blockade von TNFα hebt diese transkriptionelle Signatur auf, was darauf hinweist, dass Therapieansprechen oder -Versagen bereits vor Beginn der Therapie aus dem peripheren Blut vorhergesagt werden könnte. Zusammenfassend bietet umfassende Immunphänotypisierung der peripheren Leukozyten mittels polychromatischer Durchflusszytometrie ein vielversprechendes neues systembiologisches Herangehen, um die aberranten Immunantworten zu charakterisieren. Die vorgestellten krankheitsspezifischen zytometrischen Signaturen unter Einbeziehung der validierten Biomarker könnten in Verbindung mit den immunzellspezifischen Genexpressionsignaturen zur Entwicklung neuer diagnostischer Verfahren hinsichtlich der Klassifizierung von Krankheiten, Beurteilung der Krankheitsaktivität und Therapiestratifizierung beitragen.

2 Introduction

2.1 Immunological background for pathogenesis of chronic inflammatory rheumatic diseases

Chronic rheumatic diseases such as ankylosing spondylitis (AS), systemic lupus erythematodes (SLE) or rheumatoid arthritis (RA) are classified as autoimmune diseases. Different concepts relating to immunological findings attempt to elucidate their pathology. Allotypes of human leukocyte antigen (HLA) molecules associated with autoimmune diseases have been established to define genetic predisposition: HLA-B27 with AS and other diseases of the spondyloarthritis group [1-4] or HLA-DR4 and HLA-DR1 [5, 6] with RA. Other important risk factors for development of RA include non-major histocompatibility complex genes like protein tyrosine phosphatase (PTPN22) and smoking [7]. Additional environmental factors are crucial in arthritis disease induction [8, 9]. In this context, it is interesting to know that HLA-B27 has been shown to prolong intracellular survival of enterobacterial pathogens in monocytic cells [10, 11]. A specific HLA class-II haplotype (DRB1*1501/DQB1*0602) has been shown to contribute to the outcome of invasive streptococcal infection in patients by the negative regulation of cytokine responses to streptococcal superantigens [12]. Specific HLA allotypes might determine susceptibility for development of arthritis via reduced intensity and efficiency of immune response to particular infectious factors.

Most hypotheses focus on the contribution of adaptive immune responses such as loss of tolerance due to escape from clonal selection of self-reactive lymphoid cells described by MacFarlane Burnet as early as 1968 [13], shared epitopes [14], molecular mimicry based on similarities of pathogen and host antigens [15-17], idiotype cross-reactivity to the host cell receptor [18, 19], or cytokine dysregulation [20, 21]. More recent concepts suggest insufficient function of suppressor cell populations like regulatory T cells [22, 23], aberrant B cell receptor-mediated feedback [24] or impaired clearance of apoptotic material by macrophages [25, 26]. All of them emphasize the pathological role of individual immune cell-related effects, but none might alone explain the complex disease immuno-pathology with respect to genetic risk factors, specific organ involvement, clinical course of the disease and the relation to contributing environmental factors.

2.2 Diagnosis and therapeutic decisions in chronic inflammatory rheumatic diseases

Early diagnosis and start of therapy are crucial for disease progress and consequently for the quality of life. Typically, 5 to 10 years pass after the appearance of the first symptoms and final AS diagnosis [27]. The main reason lies in the difficulty to differentiate this inflammatory spine disease from the large group of chronic back pain ailment of different origins. Multiple clinical symptoms such as inflammatory back pain, enthesitis, uveitis or peripheral arthritis in combination with laboratory parameters such as HLA-B27 positivity or increased C-reactive protein, and X-ray or magnetic resonance imaging of sacroiliitis are necessary for diagnosis [28-30]. Thus, long-term assessment of the disease activity in AS and in RA, including the disease progress in local inflammatory sites, requires extensive and frequent diagnostic procedures for respective therapeutic decisions. Due to the availability of effective therapy options, such as immunological targeting tumor necrosis factor (TNF), achievement of remission or only very low disease activity [31] has become in recent years a new standard. More and more research efforts focus on biomarkers that enable early diagnosis and stratification of diseases with respect to therapy resistance [32-34]. Other systemic rheumatic diseases like SLE, scleroderma,

polymyositis, dermatomyositis and Sjögren syndrome often present overlapping clinical and immunological findings complicating their classification. Generally, single parameters are of limited value for diagnosis and prognosis. Another issue of growing importance is the diagnosis of dangerous infectious complications in immunocompromised rheumatologic patients. Biologic agents functionally blocking cytokines like TNF are responsible for atypical presentation and location of inflammatory symptoms of infectious processes [35-37]. Even approved diagnostic laboratory tests might be of limited value since for example C-reactive protein induced by microbial phosphocholine is suppressed by a drug response mechanism blocking interleukin (IL)-6 (Tocilizumab) [38, 39]. Thus, new methods to detect infectious processes under immuno-suppressive treatment regimens are needed. A combination of established clinical parameters supported by additional immune cell-dependent signatures of inflammatory conditions could improve diagnosis and facilitate therapeutic decisions.

3 Aim of the study: The application of global strategies like cytomics and transcriptomics to identify new biomarkers and immune cell-dependent signatures in the field of rheumatic diseases

Although the activities of autoreactive T cells and autoantibody-producing plasma cells are the hallmarks of autoimmune processes, most autoimmune diseases are characterized by an enhanced activity and involvement of immune cell types of both the innate and the adaptive immune systems. This includes enhanced antigen presentation, changed phagocytosis and migration or increased secretion of inflammatory mediators. Defining "fingerprints" of immune cell-dependent activities from small biological samples, that simultaneously assess as many immune cell subsets as possible, should provide information on cell types contributing to the pathology and also on involved cellular mechanisms.

Global molecular and cellular approaches, such as multicolour flow cytometry and whole genome expression analyses, provide promising technical requirements for a multiplex assessment of cellular and molecular parameters in a hypothesis-driven and in a hypothesis-generating manner. Combining results from these two state-of-the-art technologies most notably allow identification of new biomarkers and the validation of disease-related signatures. This provides a basis to expand the available spectrum of clinical and immunological diagnostic parameters as well as to newly define disorders and stratify for therapeutic procedures.

Exemplifying the immuno-pathologies of AS, SLE and RA, this study focused on the following points:

1) The development and validation of a reproducible multi-color flow cytometry data collection and data analysis system for identification of disease-relevant immuno-phenotypic signatures with the potential for it's automation and introduction into the diagnostic application (cytometric profiling).
2) Proof-of-principle study using cytometric profiling of AS in comparison to healthy donors in samples of peripheral blood and synovial effusion from inflamed joints.
3) Validation of sialic acid-binding immunoglobulin-like lectin (Siglec)-1 as a potential surrogate marker for type-I IFN-induced immune responses and of its diagnostic value as a disease activity marker in SLE at the single cell level by flow cytometry.
4) Comparison of transcriptional signatures in monocytes generated *in-vitro* by TNFα with imprints in monocytes of RA patients before and after successful treatment with a TNFα-blocking antibody in order to test the potential of the cytokine-specific transcriptional signature to monitor therapeutic effects.

4 Methods

4.1 Patients and samples

Patients enrolled in the study were selected according to criteria of the American College of Rheumatology for the classification of SLE and RA and modified New York diagnostic criteria for AS [40-42]. The Ethics Committee of the Charité - Universitätsmedizin Berlin approved the studies using human immune cells originating from peripheral blood and joint fluid. Freshly collected heparinized blood or joint fluid samples were treated with erythrocyte lysing buffer according to the manufacturer's instructions (Qiagen). For estimation of absolute leukocyte numbers, a CASY® cell counter (Schärfe System) was used.

4.2 Multiparametric flow-cytometric assessment of leukocyte subpopulations in ankylosing spondylitis (AS), systemic lupus erythematodes (SLE) and rheumatoid arthritis (RA)

Total blood leukocytes were simultaneously analysed for the expression of 50 different cell-surface markers. Ten 7-colour staining mixtures [see Table 1 in ref. 40] combined appropriate fluorescence-conjugated antibodies. The following dyes including the cyanine-dye family (Cy) were used: fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5, PE-Cy7, allophycocyanine (APC), APC-Cy7 and Alexa Fluor 405 excited by three lasers (blue: 488 nm, red: 633 nm, violet: 405 nm). A wide range of leukocyte populations, such as memory and naive B- and T-cell subsets, inflammatory and resident monocyte populations, natural killer (NK) cells, plasma cells, plasmacytoid and myeloid dendritic cells (DC), neutrophils and eosinophils were assessed. Lineage markers were combined with antigens involved in complement and antibody binding, migration and cell activation. Some staining cocktails contained different antibodies conjugated to the same fluorescent dye. This strategy was applied for markers exclusively expressed by defined cell populations, which allowed a higher complexity in data acquisition, which could be unravelled by sequential cell-specific gating [see Fig. 1 in ref. 40]. Cells were pre-incubated with Fc receptor-blocking reagent (Beriglobin; CSL Behring). After staining, cells were fixed with paraformaldehyde. Unstained samples were used as controls. Experiments were measured on an LSR II (BD Biosciences) with standard laser and filter configurations [see Table 2 in ref. 40]. Standardized Rainbow Calibration Particles (G. Kiesker) were acquired in parallel in order to calculate normalized, relative fluorescence signals. At least $6x10^5$ events were collected for each stained sample. Primary data were analysed with the BD FACS Diva software (v 4.1; BD Biosciences). Data were compensated with single stained antibody capture beads (CompBeads; BD Biosciences) and automated spectral overlap correction. In total, nearly 800 immuno-phenotypic parameters were obtained for each sample described by relative cell numbers, mean and median fluorescence intensities. Original data volume of 300-400 MB per sample of files generated by the BD FACS Diva software was reduced by about 5,000 fold to 64 kB when only calculated statistic values were exported as comma separated value (csv)-file format and integrated into a custom-made Access database. This database allowed normalising fluorescent signals of populations to intensities of Rainbow Calibration Particles, relating absolute cell numbers to 1 µl whole blood and finally performing statistical group analyses. A two-tailed, unpaired t-test with Welch's correction was used to identify significant parameters. These were further analysed by hierarchical clustering using the Genes@Work software (IBM Research) and

prediction analysis for microarray (PAM). The primarily analysed AS group in comparison to healthy donors was expanded to 12 AS and 12 healthy individuals and subsequent analysis of 5 RA and 6 SLE blood samples. Validation of parameters identified by the global cytometric approach was performed by the screening of independent groups of AS and healthy donors with the reduced set of antibodies. Two staining cocktails were used as follows: 5-color; CD3 Pacific Blue, CD19 APC- Cy7, CD1c PE, CD1a PE-Cy5, CD14 FITC, and 7-color; CD3 Pacific Blue, CD4 APC-Cy7, CD8 APC, CCR7 PE-Cy7, CD45RA FITC, CD62L PE-Cy5, CXCR4 PE. In addition to peripheral blood, synovial fluid samples were analyzed in parallel to compare systemic and local inflammatory events. Blood and joint fluid, processed as described above, was assessed using a FACS Canto II analyser (BD Biosciences) with standard filter and laser configuration. To ensure data reproducibility, the newly accessible quality control tool Cytometer Setup and Tracking system (BD Biosciences) was employed and instrument settings were adjusted to baseline setup directly before cells were acquired. Positive events were determined by cells stained as so-called Fluorescence Minus One (FMO) controls leaving one reagent at a time out of the mixture. The non-parametric Mann-Whitney test was used to identify significant parameters.

4.3 Identification of SLE-specific monocyte transcription profile and validation of the prominent candidate gene sialic acid–binding immunoglobulin-like lectin (Siglec)-1 as potential type-I interferon (IFN) surrogate marker by flow cytometry

Peripheral blood monocytes were prepared and subjected to gene transcription analysis. The surface expression of Siglec-1 on these monocytes was validated by flow cytometry using an unconjugated Siglec-1 (CD169) antibody and a secondary FITC-conjugated goat anti-mouse immunoglobulin G. After the blockade of remaining F(ab') binding sides by mouse immunoglobulin, cells were counterstained with CD14 PE and CD16 APC-Cy7. Samples as measured on an LSR II were analysed for inflammatory $CD169^{+}$ $CD14^{high}$ $CD16^{-}$ and resident $CD14^{+}$ $CD16^{+}$ monocytes. Frequencies of inflammatory and resident monocyte subsets of 52 SLE and 38 control samples were analysed by an unpaired t-test and correlated with clinical parameters determining disease activity [41].

4.4 Defining tumor necrosis factor (TNF)α- and lipopolysaccharide-induced gene signatures in monocytes after whole-blood *in-vitro* stimulation and monitoring of anti-TNFα-induced effects in monocytes of Adalimumab-treated RA patients

To assess stimulus-dependent monocyte transcriptome signatures, whole blood samples were acquired from four healthy individuals. One part of the samples was immediately processed to isolate monocytes, and the other part was incubated without stimulus, with TNFα or lipopolysaccharide (LPS) before monocytes were isolated and subjected to Affymetrix Gene Chip technology. Selected differentially expressed transcripts were validated by their expression (IL-1β, IL-6, IL-8, TNFα and nuclear factor-κB phosphorylation) by flow cytometry in parallel to gene expression assessment. To test the potential of TNFα-induced signatures to identify the contribution of this cytokine to RA-specific gene expression in monocytes, disease profiles from freshly isolated monocytes of RA patients were compared to monocyte profiles obtained by *in-vitro* stimulation with TNFα [42].

5 Results

5.1 Immuno-phenotypic classification of AS by cytometric profiling

Steinbrich-Zöllner M, Grün JR, Kaiser T, Biesen R, Raba K, Wu P, Thiel A, Rudwaleit M, Sieper J, Burmester GR, Radbruch A, Grützkau A. From transcriptome to cytome: integrating cytometric profiling, multivariate cluster, and prediction analyses for a phenotypical classification of inflammatory diseases. Cytometry A 2008;73:333-40.

This is the first study using an unbiased multiparametric flow cytometry approach to define a cytometric signature of immuno-phenotypic parameters that classified AS patients and healthy donors. Eighty immune cell-related parameters were differentially regulated in peripheral leukocyte populations (n=7; $p < 0.022$ by Welch t-test) specifying systemic immune presentation of AS. Hierarchical cluster analysis using these 80 parameters clearly distinguished the two sample groups. The predictive power was verified by PAM and validated with four independent samples originally not included for parameter selection [see Fig. 3 and Fig. 4 in ref. 40]. Identified parameters included expression of: CD1c on B cells, CD69 on $CD4^+$ T cells, CXCR4 on $CCR7^+$ $CD45RA^-$ $CD8^+$ memory and $CCR7^-$ $CD45RA^{-/+}$ $CD8^+$ effector T cells and on a $CD62L^+$ $CD45RA^+$ NK cell subpopulation. Moreover, relocations were ascertained within the $CD8^+$ T-cell compartment. The frequency of memory T cells was decreased and the pool of $CCR7^-$ $CD45RA^+$ terminally differentiated effector T cells was significantly increased in AS samples. A final analysis including 12 AS patients' and 14 healthy donors' samples (two healthy donors were measured as replicates) confirmed 77 out of identified 80 parameters. These parameters also allowed a correct classification by hierarchical clustering and PAM classification (Fig. 1).

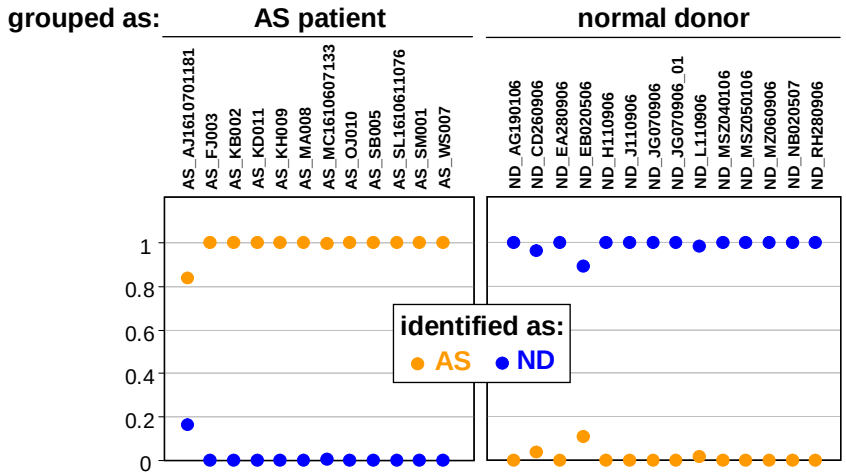

Fig. 1. **Cross validation of AS patients or normal donors using PAM classification.** X-axis presents samples from peripheral blood of 12 AS patients and 14 normal donors (including two replicates). These were tested with 77 significant phenotypic parameters with Welch t-test ($p<0.05$ at PAM threshold = 0). Cross-validation and probability estimation, shown on Y-axis, demonstrates correct classification of all samples into respective groups with a probability of more than 80%.

Blood samples from patients with other active inflammatory rheumatic diseases were recently analysed by mean fluorescence intensities in comparison to normal donors (n=12). CD1c over-expressed on B cells of AS patients (n=12; p=0.0005) was not significantly altered in SLE (n=6; p=0.8), but marginally in RA (n=5; p=0.05).

CXCR4, important for T-cell chemotaxis, was significantly up-regulated on $CD8^+$ T cells not only in AS (n=12; naive: p=0.007, effector: p=0.0005) but also in RA (n=5; naïve: p=0.001, effector: p=0.002) and SLE (n=6; naive: p=0.02, effector: p=0.003). The early activation marker CD69 was up-regulated on $CD4^+$ lymphocytes in all tested samples from patients (data unpublished). Up-regulation of CD14 on monocytes was significant in AS (n=12; p=0.03) and in RA (n=5; p=0.002) but not in SLE (n=6; p=0.8).

Ongoing studies focus on the expression of molecules of the CD14 and CD1 family that are important to detect pathogens and their products. For further validation of candidate parameters, appropriate antibodies have been arranged in 5-color and 7-color cocktails and cells were analysed in independent peripheral blood samples of 12 AS patients and 12 healthy controls. CXCR4, CD62L, CD14, CD1a and CD1c proved to be differentially expressed in AS compared to normal donors. CD1c was up-regulated on B cells and myeloid antigen-presenting cells. CXCR4 showed over-expression on naïve, memory and effector $CD8^+$ T cells. In AS patients, expression of CXCR4 was significantly up-regulated also on naïve $CD4^+$ T cells and CD62L was down-regulated on monocytes. These candidate surface molecules were monitored by application of the above staining cocktails not only in peripheral blood of AS patients and healthy controls, but also in synovial fluid of inflamed joints of four AS patients. Interestingly, the phenotypes detected in peripheral blood were also ascertainable in synovial fluid to a much higher extent. Figure 2 shows expression levels of CD14 on myeloid antigen-presenting cells as an example.

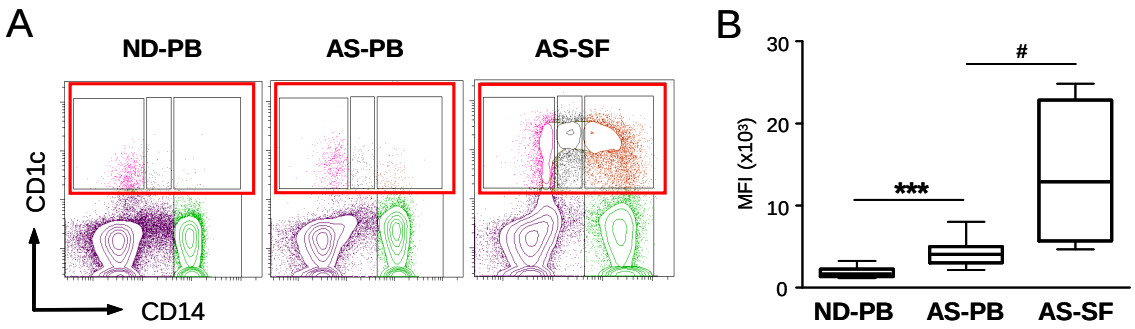

Fig. 2. **Comparison of CD14 expression levels on $CD3^-$ $CD19^-$ $CD1c^+$ leukocytes in peripheral blood and synovial fluid immune cells.** Peripheral blood (PB) of 12 normal donors (ND) and 12 AS patients as well as synovial fluid samples (SF) of four AS patients were stained for multicolour flow cytometry and gated for the $CD3^-$ $CD19^-$ leukocyte subset. (**A**) Representative dot-plot analysis for the expression of CD14 and CD1c. $CD1c^+$ events evaluated for each sample are encircled in red. (**B**) Mean fluorescence intensity (MFI) of CD14 on the $CD1c^+$ cell subset (***p=0.0005 by Mann Whitney t-test; #p=0.04 by t-test for paired samples of PB and SF of four AS patients).

In general, CD14 was up-regulated on peripheral blood monocytes of AS patients compared to normal donors, but the most striking difference was found in the particular subset of $CD1c^+$ $CD14^{+/low}$ antigen presenting cells. This subset simultaneously expressed significantly elevated levels of CD1c and CD1a. The synovial fluid was characterized by the appearance of an abundant subset of $CD14^{+/low}$ cells positive for both CD1c and CD1a important for glycolipid antigen presentation. Similarly, synovial monocytes were mainly $CD62L^{-/low}$ (L $selectin^{-/low}$) and this significant down-regulation of CD62L was already detectable on peripheral monocytes.

Taken together, the results suggest that most of the molecules expressed under inflammatory conditions on leukocyte subsets alone do not point to specific disease pathology. However, extensive immuno-phenotyping of inflammatory diseases by multicolour flow cytometry from easily accessible blood samples will allow not only to identify potential single biomarkers, but also to unravel disease specific patterns. As an example, AS-specific up-regulation of CD1c on B cells and monocyte subsets not only provides new combinatory diagnostic markers but also opens up the discussion for new mechanistic insights into the immuno-pathology of the disease. A manuscript that will include cited unpublished data is in preparation.

5.2 Evaluation of type-I IFN-inducible Siglec-1 gene on the protein and cell surface level as the disease activity biomarker

Biesen R, Demir C, Barkhudarova F, Grün JR, **Steinbrich-Zöllner M,** Backhaus M, Häupl T, Rudwaleit M, Riemekasten G, Radbruch A, Hiepe F, Burmester GR, Grützkau A. Sialic acid-binding Ig-like lectin 1 expression in inflammatory and resident monocytes is a potential biomarker for monitoring disease activity and success of therapy in systemic lupus erythematosus. Arthritis Rheum 2008;58:1136-45.

Gene transcription analysis of purified blood monocytes of 9 SLE patients and 7 normal donors identified 1,032 differentially regulated transcripts which allowed a clear discrimination of samples by hierarchical cluster analysis and revealed a prominent cluster of 132 transcripts known to be modulated by IFNα. Among other tightly co-expressed transcripts, which have not been previously described to be affected by IFNα treatment, one of the most prominent was the sialoadhesin Siglec-1 (CD169). Its expression was known to be specific for tissue macrophages, but was never shown for peripheral monocytes. Comparison of monocyte transcriptomes from samples of patients with other rheumatic diseases revealed up-regulation of Siglec-1 in a subgroup of systemic sclerosis patients.

Up-regulation of Siglec-1 in SLE at the transcriptional level was validated by significantly increased frequencies of Siglec-1$^+$ monocytes of inflammatory and resident subsets as determined by flow cytometry in independent blood samples from SLE patients and normal healthy controls ($p<0.0001$ by Welch t-test). Frequencies of Siglec-1$^+$ monocyte subsets positively correlated with disease activity according to the SLE Disease Activity Index (SLEDAI) as well as with levels of anti-dsDNA antibodies and negatively with complement C3 levels. Seven days of high-dose glucocorticoid therapy of SLE patients with active disease significantly reduced the frequencies of Siglec-1$^+$ monocytes in inflammatory and resident subsets. In contrast, 9 days of therapy with INFα2a demonstrated significant increase in Siglec-1 expression in both monocyte subsets in a patient with rare Erdheim-Chester disease.

Taken together, Siglec-1, detected at the transcriptional level and validated by flow cytometry at the protein level from defined cell population as monocytes, represents a promising biomarker in SLE and related type-I IFN-driven diseases.

5.3 Effect of exogenous or endogenous biological stimuli on transcriptome signatures of *in vitro*-stimulated monocytes and estimation of cytokine-specific immune responses in peripheral monocytes of RA patients

Smiljanovic B, Grün JR, **Steinbrich-Zöllner M**, Stuhlmüller B, Häupl T, Burmester GR, Radbruch A, Grützkau A, Baumgrass R. Defining TNF-alpha- and LPS-induced gene signatures in monocytes to unravel the complexity of peripheral blood transcriptomes in health and disease. J Mol Med 2010;88:1065-79.

Gene transcription analysis of monocytes isolated from human whole blood that was stimulated *in vitro* with TNFα or LPS revealed differentially expressed genes compared to unstimulated controls. Both TNFα and LPS induced considerably overlapping expression but each stimulus alone also induced specific changes. Functional classification with the Database for Annotation, Visualisation and Integrated Discovery (v 6.7; http://david.abcc.ncifcrf.gov/ 2009) revealed up-regulated genes involved in signal transduction, apoptosis and immune/inflammatory responses. Down-regulated genes were found to be primarily involved in metabolic processes.

Genes regulated in treated monocytes were investigated in more detail with regard to the cytokine/chemokine compartments and factors involved in apoptosis and cell survival. Of these differentially expressed genes, 37 genes were interleukins and their receptors. TNFα and LPS up-regulated the enzyme that converts IL-1 family members into their active forms (caspase 1) as well as members of the IL-1 family themselves, such as IL-1α, IL-1β and IL-18. Up-regulation of IL-1F9, a recently identified new member, was specific for LPS. The production of IL-1β, IL-6 and IL-8 upon *in vitro* stimulation of macrophages with TNFα or LPS was confirmed at protein level by flow cytometry. Overall, cytokine production was stronger in response to LPS than to TNFα and correlated with their up-regulation at the transcriptional level.

Interestingly, 29 differentially regulated genes were members of the CC/CXC chemokine families (CCL/CXCL) and their receptors. Here, CCL and CXCL were mostly up-regulated by TNFα or LPS and the expression of chemokine receptors was concordant. In the chemokine/chemokine receptor groups only a few genes were exclusively regulated by one or the other stimulus. In monocytes, TNFα as well as LPS induced up-regulation of TNF/TNF receptor family members like TNFα or ADAM17, the enzyme responsible for cleavage of membrane-bound TNFα to release its soluble form. The apoptosis/cell survival profile of *in vitro*-stimulated macrophages comprised 240 genes with a special preference for caspase and bcl2 family members.

The signature of differentially expressed genes after *in-vitro* stimulation with TNFα reflected an imprint in monocyte gene expression profile of peripheral blood-derived monocytes of RA patients before and after successful treatment with the TNFα-blocking antibody Adalimumab [43]. Within 471 treatment-regulated probe sets (457 up- and 14 down-regulated), 179 probe sets were comparable for both gene expression profiles that used different array types and allowed clear classification of TNFα-stimulated and un-stimulated samples by hierarchical cluster analysis. The majority (174/179) of the TNFα-stimulated probe sets were down-regulated and clustered as up-regulated in RA samples after treatment.

Thus, the pattern of macrophage responses to defined stimuli *in vitro* helped to elucidate the contribution of single stimuli to the intricate pathology in chronic inflammatory diseases.

6 Discussion

Immuno-phenotyping by flow cytometry is routinely applied in the diagnosis of haematologic malignancies and immune deficiencies, as well as for monitoring of lymphocytes in human immuno-deficiency virus-infected patients and for enumeration of $CD34^+$ progenitor cells in haematopoietic transplants. Other applications are rare and include diagnosis of paroxysmal nocturnal hemoglobinuria, systemic mastocytosis, primary thrombocytopathies or the immuno-phenotyping of bronchoalveolar lavage. Despite encouraging research in the fields of infectious and autoimmune diseases, flow cytometry has very limited use in clinical diagnostics. Comprehensive assessment of a current immune status by a cell-based analysis of multiple phenotypes from a single blood sample presents an attractive approach to monitoring inflammatory diseases, especially chronic rheumatic autoimmune diseases. New diagnostic immune cell-based tools might be helpful in the early diagnosis and classification of disease states, assessment of disease activity and therapy follow-up especially using biological agents.

The experimental strategy, presented here, analysing simultaneously around 800 phenotypic parameters of all major cell types from a single peripheral blood sample, by far exceeds existing diagnostic methods using flow cytometry with regard to the amount of accessible information. The staining protocol included fluorescence-conjugated antibodies for 50 CD antigens combined to ten 7-colours staining cocktails and is adaptable to any cytometer equipped with a blue, red and violet laser. Careful handling of samples and reagents and, particularly important, rigorous monitoring of instrument with use of standardized beads and Cytometer Setup and Tracking system minimized technical sources of variation. This strategy markedly improved reproducibility of data acquired on different days over a period of one year and allowed for the comparison of fluorescence intensity-based parameters.

The primary labour-intensive manual pre-analysis was done with BD FACS Diva software. Cytometry data were analysed with reference to established standards [44, 45]. Further analysis of the extensive data pools generated by multicolour cytometry protocol required novel analytic solutions. Feasible data storage, including these phenotypic parameters and additional clinical data such as age, gender, diagnosis, applied medications as well as flexible statistical analysis of experimental groups like AS patients and healthy controls were performed using the specially adapted Access database [40]. Identified significant parameters could be further analysed and graphically presented by the use of hierarchical cluster and prediction analyses. The applied bioinformatics strategy presents an attractive analytical system that has a potential for automation.

The presented cytometric array covered most major leukocyte populations of the blood and demonstrated effects at single-cell level in contrast to the generalizing level of gene expression arrays from whole blood samples. Moreover, not single marker molecules but an entire set of biomarker candidates was directly validated at protein level. No comparable study has been described so far. Although multicolour protocols using up to 17 differently labelled antibodies have been already published, these studies were designed to identify rare antigen-specific T-cell subsets and can be categorised as a typical hypothesis-driven research [46]. The unique software solution (Access database) introduced with the presented study allowed assessment of the expression of cell surface molecules by systematic comparison of all relative fluorescence intensities on all

accessible cell populations. Such analysis can be at least in part classified as non-hypothesis driven in contrast to multicolour cytometry studies based exclusively on manually managed analysis [47].

CXCR4, CD62L and CD69 molecules modulated under inflammatory conditions, as identified in this study, were changed significantly in peripheral blood not only in AS, but in RA and SLE compared to normal donors. Similarly, CD14 over-expression on monocytes was significant in AS and RA blood samples. Up-regulation of CXCR4 and CD69 on T-cells has been already shown in RA [48, 49]. Shinohara et al showed elevated expression of CD14 on peripheral blood monocytes in RA patients compared to healthy controls [50]. $CD14^+$ monocytes accumulating in the synovial-pannus junction, differentiate into macrophages/osteoclasts and contribute to cartilage and bone destruction by enhanced proinflammatory cytokine production [51, 52]. The up-regulation of CD14 expression on monocytes is known to be induced by LPS. Although LPS is considered its main ligand, CD14 also recognizes other pathogen-associated molecular patterns. Down-regulation of CD62L was so far found on peripheral blood-derived myeloid DC in psoriatic arthritis and RA [53]. CD62L is known as an adhesion receptor supporting the rolling process of leukocytes on endothelium that directly precedes the extravasation step of leukocyte entry to inflamed tissue [54, 55]. Its down-regulation assessed from peripheral blood might represent enrichment of intravascular cells that already lost the expression due to involvement in the rolling process. An unexpected and intriguing finding was the up-regulation of CD1c, a molecule for glycolipid presentation on $CD19^+$ B cells in the blood of AS patients [40] that has not been shown so far.

The AS phenotypic pattern assessed from peripheral blood in the presented study that includes elevated expression of CD14, CD1c and CD1a molecules on antigen presenting cells was also ascertained in the local joint compartment. Since human monocytes differentiate into DC *in vitro* under cytokine conditions that resemble an inflamed environment [56, 57], numerous $CD14^{+/low}$ $CD1c^+$ $CD1a^+$ cells in the synovial fluid may represent local maturation of monocytes into myeloid DC at the inflammatory site *in vivo*. Local inflammatory sites are rather ambiguous research subjects due to difficulties in conclusive differentiation of immune cells related to or responsible for the inflammation from other cells constituting the affected organs and from non-relevant steady-state immune cells. Joint fluid containing pure effusions of immune cells during inflammation is especially attractive for studies on local immune reactions and their systemic impact. Subsets with prominent inflammatory leukocyte phenotypes revealed in the synovial fluid were demonstrated to be significantly altered in the peripheral blood of AS patients compared to healthy controls and defined the cytometric AS signature. Thus, this local immuno-pathological pattern reflected by the blood-derived leukocytes could be detected without joint puncture. Interestingly, the pattern of synovial fluid leukocytes revealed up-regulation of CD14, a part of the LPS receptor, which was accompanied by up-regulation of CD1a and CD1c molecules involved in glycolipid presentation to T cells. These findings at cellular level support a mechanistic concept of the activity of adaptive immunity towards its own proteins that is induced by non-protein adjuvants [58] and of the involvement of CD1 as non-canonical major histocompatibility complex molecules for glycolipid presentation in T cell priming [59, 60]. An AS-specific signature of prominent $CD14^{+/low}$ $CD1c^+$ leukocyte populations also opens the discussion on a potential role of non-protein bacterial products in terms of haptens modifying own proteins in

the complex autoreactivity phenomena of arthritis pathogenesis [61]. Thus, besides their diagnostic and potential prognostic value in the clinical routine, these findings might broaden our understanding of arthritis immuno-pathology that to date for the most part is attributed to protein-dependent involvement of T and B cells during inflammation.

Monocytes are believed to contribute to SLE pathology due to impaired phagocytic activity responsible for the clearance of apoptotic cell material [25, 26] and to increased apoptosis and release of nucleosomes that represent a major target for autoantibody-dependent immune responses [62]. Gene expression profiles from peripheral monocytes of active SLE established within this work revealed a prominent imprint of transcripts clustering with type-I IFN-response genes [41]. Thus, monocytes were imprinted in a way that allowed conclusions to be drawn on the pro-inflammatory milieu, which may lead to the identification of essential single markers or patterns of mediators for this particular disease. One of the most differentially expressed genes that co-clustered with numerous type-I IFN response genes was Siglec-1 encoding a lectin-like adhesion molecule that is known to be expressed by a subpopulation of macrophages and involved in mediation of cell-cell interactions. Siglec-1 could be validated at protein level by cytometry presenting itself as a potential valuable biomarker to monitor disease activity and treatment efficiency. Siglec-1 could be easily determined in peripheral blood and was exclusively expressed on monocytes. Its expression correlated with disease activity and it was down-regulated under intensive immuno-suppressive treatment. Moreover, Siglec-1 expression reflected type-I IFN serum activity as demonstrated *in vivo* by up-regulation under IFNα2a (Roferon-A) treatment [41]. Over-expression of Siglec-1 on blood monocytes has been shown in other inflammatory diseases that involve activation of type-I IFN-dependent pathways like systemic scleroderma or in patients with high viral loads of the human immuno-deficiency virus-1 [63, 64]. Patterns that comprise multiple markers at protein and/or transcriptome level to identify subtle shifts in biomolecular characteristics of immune cell populations may help to define and to understand differences, and even more importantly, similarities between autoimmune pathology and the immuno-pathology of infection. Comparison of these patterns at cellular and general levels e.g. from SLE to viral infection might renew the views of the chronic inflammatory diseases with frequent flares as predominantly autoimmune origin.

An *in vitro*-stimulation system for whole blood samples and established stimulus/cell-specific gene expression profiles may facilitate exploration of major inflammatory mediators specific or required in particular diseases. TNFα as a prototypic endogenous pro-inflammatory cytokine and LPS, a bacteria-derived exogenous factor, showed considerable overlap of genes induced in peripheral blood cells [42]. Besides this common profile, resulting from LPS-binding to the CD14 receptor complex, subsequent interaction with Toll-like receptor-4 that induces production of IL-1β, IL-6, IL-8 and TNFα via activation of the nuclear factor κB pathway [65, 66], both stimuli also induced a specific gene expression. This expression specifically induced by LPS or TNFα might help to find out whether and to what extent exo- or endogenous stimuli are the basis for the immune etiopathology. Monocyte TNFα-induced gene expression signature, determined after *in-vitro* stimulation, could

be identified within monocyte gene expression profile of RA patients with active disease and was reversed upon therapy blocking TNFα. Mainly down-regulated/silenced genes composing a TNFα profile in RA patients that were up-regulated upon successful treatment demonstrated the therapeutic success at transcriptional level and might also prove useful for therapy response assessment. Effects of exo- or endogenous biological stimuli like LPS and TNFα, as identified via their stimulus/cell-specific signatures generated *in vitro,* may help uncover the most important mediators within the disease-related signatures and might help to monitor therapeutic responses or even support the discovery of relevant detrimental factors.

Complex immune status assessment at cell type and/or cellular level is appealing for not only autoimmune diseases studied here, but also for other inflammatory conditions e.g. infection, allergy and intoxication. Libraries of immune cell-related signatures that delineate different pathologic conditions might establish disease-specific phenotypic patterns as a feasible diagnostic tool to support therapeutic decisions. Moreover, these disease-specific patterns may help uncover similarities between disorders of known and unknown etiopathology, which may help to direct research efforts. Taken together, multiplex immune signatures for defined inflammatory diseases with local and systemic involvement of immune cells as obtained by the combination of polychromatic flow cytometry and immune cell-specific gene expression as state-of-the art techniques within this work may extend the range of diagnostic possibilities for the rationalization of therapeutic decisions. With special respect to rheumatic diseases, characterized by local inflammatory responses in intrinsically sterile compartments, it offers new avenues to compare and to functionally link them to chronic inflammations and infections that for example involve the mucosal barrier. It provides valuable information to answer the most challenging question on endo- or exogenous starting points of inflammatory concerts and the mechanisms underlying the vicious circle that has to be broken to cure the diseases.

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ANHANG

Danksagung

Meinem Doktorvater Herrn Prof. Dr. rer. nat. Andreas Radbruch möchte ich sehr herzlich für die Überlassung des Themas danken. Herrn Dr. rer. nat. Andreas Grützkau gebührt mein herzlicher Dank für seine Betreuung und Supervision meiner experimentellen Arbeiten sowie Begleitung und Unterstützung aller Schritte. Dr. rer. nat. Joachim Grün verdanke ich die Hilfe bei der erfolgreichen Erzeugung der Datenergebnisse und Präsentationen.
Bei Herrn Prof. Dr. med. Joachim Sieper möchte ich mich sehr herzlich bedanken für sein Interesse an meinem weiteren Beitrag zur Entwicklung der Analyse der multiparametrischen zytometrischen Daten. Einen ganz besonderen Dank möchte ich Dr. rer. nat. Ulrike Erben aussprechen für die Mitbetreuung meiner wissenschaftlichen Arbeit in ihrer finalen Phase.
PD Dr. med. Thomas Häupl, PD Dr. med. Heiner Appel, Prof. Dr. rer. nat. Andreas Thiel, Dr. med. Robert Biesen und Dr. rer. nat. Biljana Smiljanovic möchte ich herzlich danken für lehrreiche Diskussionen und fortwährende Unterstützung bei dieser Promotionsarbeit. Dipl. Ing. Thoralf Keiser, Dipl. Ing. Katharina Raba, Peihua Wu, Rebecca Scheer und Silvia Pade bin ich sehr dankbar für Hilfeleistung bei Spender Akquisition, Durchführung der Experimente und sonstige Unterstützung.
Für die Geduld, das Interesse an meiner Arbeit und die vielen kleinen und großen Hilfestellungen danke ich meinem Ehemann und meiner Mutter.

Diese Arbeit wurde im Rahmen des Netzwerkes The Marie Curie Research Training Network EURO-RA, durch das Bundesministerium für Bildung und Forschung (BMBF) im Rahmen des Netzwerkes für Infektion und Entzündung: Signatures, Pathways, Genes (SIPAGE) des Nationalen Genomforschungsnetzes (NGFN) und durch den Sonderforschungsbereich 633 der Charité – Universitätsmedizin Berlin gefördert.

Selbständigkeitserklärung

Ich, Marta Steinbrich-Zöllner, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Polychromatic flow cytometry and immune cell-specific gene expression profiling: An integrated approach to identify and validate new biomarkers and signatures of immune responses in chronic inflammatory rheumatic diseases.“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Marta Steinbrich-Zöllner
(Promovendin)

Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Originalarbeiten, die in die Publikationspromotion einfließen

1. Smiljanovic B, Grün JR, **Steinbrich-Zöllner M**, Stuhlmüller B, Häupl T, Burmester GR, Radbruch A, Grützkau A, Baumgrass R.
   Defining TNF-α- and LPS-induced gene signatures in monocytes to unravel the complexity of peripheral blood transcriptomes in health and disease.
   J Mol Med 2010;88:1065-79.
   Impact Factor J Mol Med (2009): 5.004

2. **Steinbrich-Zöllner M**, Grün JR, Kaiser T, Biesen R, Raba K, Wu P, Thiel A, Rudwaleit M, Sieper J, Burmester GR, Radbruch A, Grützkau A.
   From transcriptome to cytome: Integrating cytometric profiling, multivariate cluster and prediction analyses for a phenotypical classification of inflammatory diseases.
   Cytometry A 2008; 73:333-40.
   Impact Factor Cytometry A (2007): 2.978
   Preisträger beim XXIV International Congress Cytometry in the Age of Systems Biology der International Society of Analytical Cytometry; Budapest, Hungary, 17.–21.05.2008

3. Biesen R, Demir C, Barkhudarova F, Grün JR, **Steinbrich-Zöllner M,** Backhaus M, Häupl T, Rudwaleit M, Riemekasten G, Radbruch A, Hiepe F, Burmester GR, Grützkau A.
   Sialic Acid- Binding Ig-Like Lectin 1 in Inflammatory and Resident Monocytes Is a Potential Biomarker for Monitoring Disease Activity and Success of Therapy in Systemic Lupus Erythematosus.
   Arthritis Rheum 2008;58:1136-45.
   Impact Factor Arthr. Rheum (2007): 7.677

Erklärung über den Anteil an den Publikationen

Die Studien dieser Promotion wurden im Rahmen eines Kooperationsprojektes zwischen Deutsches Rheumaforschungszentrum Berlin und der Medizinischen Klinik m.S. Rheumatologie und Klinische Immunologie, Charité - Universitätsmedizin Berlin durchgeführt.

Die Promovendin Marta Steinbrich-Zöllner hatte folgenden Anteil an den vorgelegten Publikationen:

Publikation 1: Steinbrich-Zöllner M, Grün JR, Kaiser T, Biesen R, Raba K, Wu P, Thiel A, Rudwaleit M, Sieper J, Burmester GR, Radbruch A, Grützkau A. From transcriptome to cytome: integrating cytometric profiling, multivariate cluster, and prediction analyses for a phenotypical classification of inflammatory diseases. Cytometry A. 2008

65 Prozent: Etablierung des zytometrischen Färbprotokolls und Auswertungsystems, Optimierung der Reproduzierbarkeit und Qualitätskontrolle, Studien- und Versuchsplanung, Durchführung sämtlicher Experimente, Datenauswertungen und statistischer Analysen, Schreiben des Manuskripts, Bearbeitung der Reviewer-Fragen

Publikation 2: Biesen R, Demir C, Barkhudarova F, Grün JR, **Steinbrich-Zöllner M,** Backhaus M, Häupl T, Rudwaleit M, Riemekasten G, Radbruch A, Hiepe F, Burmester GR, Grützkau A. Sialic acid-binding Ig-like lectin 1 expression in inflammatory and resident monocytes is a potential biomarker for monitoring disease activity and success of therapy in systemic lupus erythematosus. Arthritis Rheum 2008

15 Prozent: Beteiligung an Durchführung der Experimente, Datenauswertung und Interpretation, Beitrag zum Manuskriptentwurf.

Publikation 3: Smiljanovic B, Grün JR, **Steinbrich-Zöllner M,** Stuhlmüller B, Häupl T, Burmester GR, Radbruch A, Grützkau A, Baumgrass R. Defining TNF-alpha- and LPS-induced gene signatures in monocytes to unravel the complexity of peripheral blood transcriptomes in health and disease. J Mol Med 2010

15 Prozent: Beteiligung an dem Entwurf des Studienprotokolls, Literaturrecherche, Beitrag zu Datenauswertung und Interpretation.

Prof. Dr. rer. nat. Andreas Radbruch	Marta Steinbrich-Zöllner
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