Aus der Medizinischen Klinik
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DISSERTATION

Analysis of SIGLEC1 as a surrogate marker for a type I interferon signature in autoimmune congenital heart block and primary Sjögren's syndrome

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1. Abstract

1.1 English abstract

Interferon-α (IFN-α), belonging to the family of type I interferons, is a cytokine involved in the pathogenesis of many autoimmune diseases, including systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS). As there are no standardised tests for the direct measurement of IFN-α, the expression of the myeloid cell surface receptor SIGLEC1 on CD14$^+$ monocytes was examined as potential biomarker for a type I interferon signature. While several studies have analysed the SIGLEC1 expression in SLE patients, further research is required into other autoimmune diseases, including pSS and congenital heart block (CHB). It was therefore the aim of this study to analyse the expression of SIGLEC1 on CD14$^+$ monocytes, and how it is influenced by certain immunomodulatory medication, in pSS patients and in females with a CHB pregnancy complication.

It was found that patients with pSS had an increased expression of SIGLEC1 compared to healthy individuals, with levels comparable to those found in SLE patients. PSS patients with a systemic or extraglandular disease manifestation (n = 16) had a significantly higher expression of SIGLEC1 compared to patients where the disease was restricted to the glandular organs (n = 15; p = 0.0001, Mann-Whitney U test (MWU)). A total of 9 pregnant females were included in the study whose children developed CHB *in utero*, and were compared to 14 pregnant at-risk females with anti-Ro autoantibodies who bore unaffected children. We found a significantly higher expression of SIGLEC1 and significantly higher plasma concentrations of IFN-α in the affected group compared to the unaffected group (p = 0.0034 and p = 0.0135, respectively, MWU), while there was no significant difference for plasma concentrations of interferon-γ induced protein 10 (IP10; p = 0.14, MWU). The expression of SIGLEC1 was reduced in patients with SLE, pSS, and females with a CHB pregnancy complication upon introduction of glucocorticoids and hydroxychloroquine.

In summary, this study highlights a potential clinical use of the biomarker SIGLEC1 as a biomarker indicative of a more severe disease manifestation in patients with pSS, for dose titration and monitoring treatment response to hydroxychloroquine and glucocorticoids, as well as to other medication still in development, and for risk evaluation in pregnant females with anti-Ro antibodies.

Further studies are needed to fully understand the pathogenetic and clinical implications of an increased SIGLEC1 expression, reflecting an activated type I interferon system, for patients with autoimmune diseases.

1.2   German abstract

Das Zytokin Interferon-α (IFN-α) aus der Familie der Typ-I-Interferone ist am Pathomechanismus vieler Autoimmunerkrankungen beteiligt, wie z.B. dem systemischen Lupus Erythematodes (SLE) und dem primären Sjögren-Syndrom (pSS). Dennoch gibt es keine standardisierten Testverfahren zur direkten Messung von IFN-α, sodass die Expression des auf Myelozyten exprimierten Oberflächenmoleküls SIGLEC1 als potentieller Biomarker für eine Typ-I-Interferon-Signatur untersucht wurde. Während vorherige Studien die Expression von SIGLEC1 in Patienten mit SLE untersucht haben, besteht weiterhin Forschungsbedarf in anderen Autoimmunerkrankungen, wie beispielsweise dem pSS und dem kongenitalen Herzblock (CHB). Es war daher das Ziel dieser Studie, in Patienten mit pSS und Frauen mit einer CHB-Schwangerschaftskomplikation sowohl die Expression von SIGLEC1 auf $CD14^+$-Monozyten, als auch deren Beeinflussung durch immunmodulierende Medikamente zu untersuchen.

Die Studie ergab, dass Patienten mit pSS gegenüber gesunden Kontrollprobanden eine erhöhte Expression von SIGLEC1 aufwiesen, die vergleichbar war zu der Expression in Patienten mit SLE. PSS-Patienten mit einer systemischen bzw. extraglandulären Krankheitsmanifestation (n = 16) hatten eine signifikant höhere Expression als Patienten, bei denen das Krankheitsgeschehen auf das Drüsengewebe begrenzt war (n = 15; p = 0.0001, Mann-Whitney-U-Test (MWU)). Es wurden 9 schwangere Frauen in die Studie eingeschlossen, deren Kinder im Uterus einen Herzblock entwickelten und mit 14 schwangeren Frauen verglichen, die mit Anti-Ro-Antikörpern zwar ein Risikoprofil aufwiesen, jedoch gesunde Kinder gebaren. Hier fanden wir ebenfalls eine signifikant höhere SIGLEC1-Expression, wie auch signifikant höhere IFN-α-Konzentrationen bei den betroffenen Frauen (p = 0.0034 und p = 0.0135, MWU), während die Konzentrationen von Interferon-γ-induziertem Protein 10 (IP-10) sich zwischen den beiden Gruppen nicht signifikant unterschieden (p = 0.14, MWU). Die Expression von SIGLEC1 konnte bei Patienten mit SLE und pSS, sowie bei Frauen mit einer CHB-Schwangerschaftskomplikation durch Glukokortikoide und durch Hydroxychloroquin gesenkt werden.

Zusammenfassend erweitert diese Studie den potentiellen klinischen Nutzen von SIGLEC1, sowohl als Biomarker, der auf einen schwereren Krankheitsverlauf bei Patienten mit pSS hindeuten kann, zur Dosisfindung und Therapiekontrolle einer Therapie mit Glukokortikoiden und Hydroxychloroquin, bzw. auch von Medikamenten, die sich derzeit noch in der Entwicklung befinden, sowie zur Risikoevaluation bei schwangeren Frauen mit anti-Ro-Antikörpern. Weitere Studien sind erforderlich, um die vollständige pathogenetische und klinische Bedeutung einer erhöhten SIGLEC1-Expression als Indikator eines aktivierten Typ-I-Interferonsystems für Patienten mit Autoimmunerkrankungen zu verstehen.

2. Introduction

The vertebrate immune system is based on complex interactions between the various components of an immune response, which in a physiological condition allows the effective elimination of pathogens, while maintaining self-tolerance towards autoantigens. Cytokines act as signalling molecules that orchestrate and balance an immune response within a complex cytokine network. Autoimmune diseases, such as systemic lupus erythematosus (SLE), primary Sjögren's syndrome (pSS) and rheumatoid arthritis (RA), are characterised by a break in self-tolerance, resulting in chronic inflammation. In these and other autoimmune diseases, various cytokines such as interferon (IFN)-α, tumor necrosis factor (TNF)-α, Interleukin (IL)-6 and B-cell activating factor (BAFF) are considered to play a crucial role in the pathogenesis of the disease and the maintenance of inflammation.(1-3)

IFN-α belongs to the family of type I interferons (IFNs) with antiviral, antiproliferative and immunomodulatory effects on both the innate and the adaptive immune response. One mechanism of IFN-α induction is through endosomal/lysosomal binding of immune complexes containing nucleic acids through Toll-like receptors (TLRs) 7 and 9 in plasmacytoid dendritic cells (pDCs), the main producers of IFN-α in humans.(4) SLE is an example of an autoimmune disease characterised by the presence of immune complexes, which consequently leads to a high production of IFN-α, resulting in the induction of interferon-inducible genes and a distinct "interferon signature" in the peripheral blood mononuclear cells (PBMCs) of around 40-75% of SLE patients.(5-7) In most cases, an "interferon signature" is associated with a more severe disease phenotype and with higher autoantibody titres, especially those reactive with RNA binding proteins.(6, 8)

Because IFN-α itself is difficult to measure, especially in a clinical context, due to test limitations and the low stability of the molecule,(9) potential surrogate parameters have been studied with regards to their ability to monitor disease activity and predict flares. Here, the expression of the cell surface molecule SIGLEC1 (sialoadhesin, CD169) on circulating monocytes has emerged as a promising IFN-α surrogate parameter.(10-12) In a previous study, Biesen et al. showed that SIGLEC1 was one of the genes most prominently upregulated by IFN-α in patients with SLE (see also **figure 1**), and that the SIGLEC1 expression on CD14$^+$ monocytes correlated with the patient's disease activity.(10) These findings were further supported by Rose et al.,(13) who recently also provided biomarker thresholds for clinical practice.(14)

While the role of IFN-α in SLE has been well studied, and it is becoming feasible to use the biomarker SIGLEC1 in clinical practice in patients affected by SLE, there is still a need for further research into other autoimmune diseases. These include pSS, where strong evidence for a key role of IFN-α exists, but the biomarker SIGLEC1 has not been sufficiently studied.(15-17) On the other hand, immunological disorders exist where a potential role of IFN-α has not yet been investigated, for example in autoimmune congenital heart block (CHB). CHB is a rare pregnancy complication in females with anti-Ro (SS-A) autoantibodies, in which the foetal heart's conduction system is damaged by an immunological process. Since CHB development is often associated with a maternal diagnosis of pSS or SLE, we hypothesized that IFN-α may also be a central cytokine in CHB development, and, furthermore, that SIGLEC1 may also be a viable biomarker in this disease.

The overall aim of this study was therefore to further investigate SIGLEC1 as a biomarker in autoimmune diseases, using the well-studied "interferonopathy" SLE as a reference. Here, the main focus was laid on the diseases pSS and CHB. We wanted to study whether higher levels of IFN-α, with a subsequent upregulation of SIGLEC1, could be associated with a more severe disease phenotype in pSS, or with the development of heart block in CHB. Additionally, we wanted to further analyse the effects of immunosuppressive/immunomodulatory medication on the SIGLEC1 expression. This would further support the rationale of targeting the type I interferon pathway with immunomodulatory medication in certain autoimmune diseases, and would additionally provide a means of monitoring treatment response.
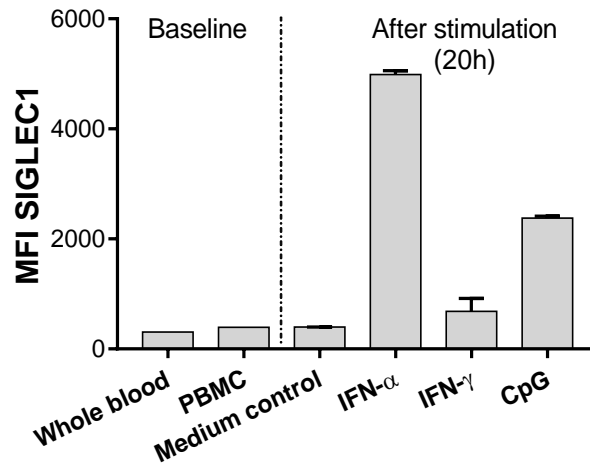
Figure 1: Induction of SIGLEC1 on peripheral blood mononuclear cells (PBMCs) by interferon-α (IFN-α), interferon-γ (IFN-γ) or the TLR9 agonist CpG. PBMCs from a healthy donor were stimulated with either medium, or with medium containing IFN-α2A (5 ng/ml), IFN-γ (5 ng/ml) or CpG (0,5 µg/ml). The median fluorescence intensity (MFI) of SIGLEC1 was determined by flow cytometric measurement from either whole blood, from PBMCs directly after isolation, or from PBMCs after 20 hours of stimulation. Shown are the mean and standard deviation of technical duplicates. MFI; median fluorescence intensity; PBMC, peripheral blood mononuclear cells; IFN, interferon; CpG, CpG oligodeoxynucleotide.

3. Materials and Methods

A detailed description of the materials and methods can be found in the corresponding publications.(18-20)

3.1 Flow cytometric analysis of SIGLEC1 expression on CD14$^+$ monocytes

The expression of SIGLEC1 on CD14$^+$ monocytes was measured in patients of the Department of Medicine/Rheumatology and Clinical Immunology at the Charité – Universitätsmedizin Berlin, as well as in healthy donors. An informed consent form was obtained from each individual and the ethics committee of the Charité – Universitätsmedizin Berlin approved the study. The detailed materials and methods can be found in corresponding publications by Lisney et al. and Rose et al.(18, 19) After blood collection and erythrolysis, the cells were washed twice with PBS, 0.5% BSA, 2 mM EDTA (PBE), and, after blocking the Fc receptor, stained for 15 minutes at room temperature with phycoerythrin Cy7 (Pe-Cy7)-labeled anti-CD19, fluorescein isothiocyanate (FITC)-labeled anti-CD27, allophycocyanin H7 (APC-H7) labeled anti-CD14, Pacific Blue (PacBlue)-labeled anti-CD3, Alexa Fluor 647 (AF647)-labeled anti-SIGLEC1 and Pacific Orange (PacO)-labeled anti-CD20. The flow cytometric measurement was performed within one hour on a FACS Canto II flow cytometer (Becton Dickinson, Heidelberg, Germany).

3.2   T, B and NK ("TBNK") cell staining

The bead-based BD Multitest™ 6-color "TBNK" system was used together with BD Trucount™ tubes (both Becton Dickinson) according to the manufacturer's instructions in order to determine the percentages and absolute numbers of T, B, and natural killer (NK) cells, as well as of the $CD4^+$ and $CD8^+$ T cell subpopulations in the peripheral blood (see **figure 2**).
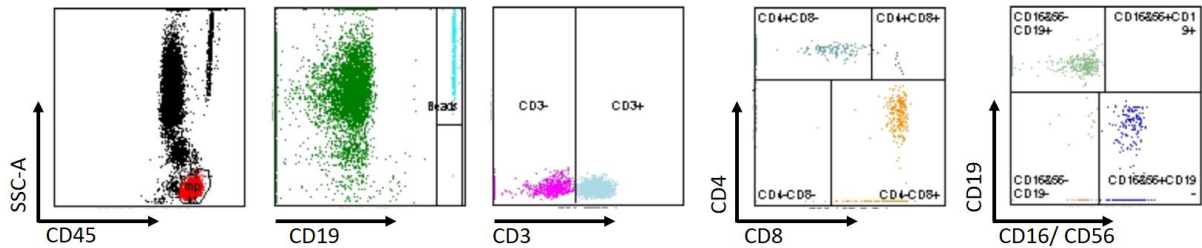

Figure 2: **Gating strategy for the bead-based „TBNK" staining.**

3.3   Measurement of IFN-α and IP-10 in serum and plasma

Plasma concentrations of IFN-α were determined by ELISA using the VeriKine-HS Human Interferon Alpha Multi Subtype Serum ELISA Kit (Pbl Assay Science, New Jersey, USA) according to the manufacturer's instructions. Plasma concentrations or serum concentrations of IP-10 (CXCL10) were determined by Bioplex® technology (Bio-Rad Laboratories, Inc., Hercules, USA) and analysis was performed using a Bio-Plex® 200 system reader system (Bio-Rad) according to the manufacturer's instructions. Double measurements (technical replicates) were performed for each sample, and the mean values considered for further analysis.

3.4   Statistical data analysis

Statistical analysis was performed using either GraphPad Prism 7.0 (GraphPad, LaJolla, California, USA) or SPPS 23.0 (IBM, Armonk, New York, USA). Statistical comparisons were performed under the assumption that samples did not follow a Gaussian distribution for most experiments. The non-parametric Mann-Whitney U test (MWU) was used for group comparisons and the Wilcoxon test for paired comparisons. The Kruskal-Wallis test (KWT) was used together with Dunn's correction for multiple comparisons. The Spearman's rank test (SRT) was used for correlation analyses. The statistical test and p-values are included in the figure legends. P-values of less than 0.05 (two-tailed) were considered to be of significance.

4. Results

4.1 SIGLEC1 expression in different autoimmune diseases

The expression of SIGLEC1 on CD14$^+$ monocytes from a total of 763 measurements of 438 individuals is shown in **figure 3**. A detailed description of the patients' clinical and epidemiological characteristics can be found in the appendix (**table 1**). In line with previously published data,(10, 13) patients with SLE had a significantly higher expression of SIGLEC1 on CD14$^+$ monocytes compared to healthy donors (HD) (mean MFI: 915.6 vs. 208.5, p < 0.0001, KWT). The same applied for patients with pSS, where IFN-α is also believed to play a central role in the pathogenesis (mean MFI: 882.7, p < 0.0001, KWT).(15) Interestingly, pregnant females bearing children affected by congenital heart block (CHB) also had a significantly higher SIGLEC1 expression compared to healthy donors (mean MFI: 1457.0, p < 0.0001, KWT). The expression of SIGLEC1 in pSS and CHB will be discussed in more detail in the following sections. Patients with RA and other diseases pathogenically driven by TNF-α, such as psoriasis arthritis (PsA) and axial spondyloarthritis (SpA),(21) did not have significantly different levels of SIGLEC1 expression compared to healthy donors.
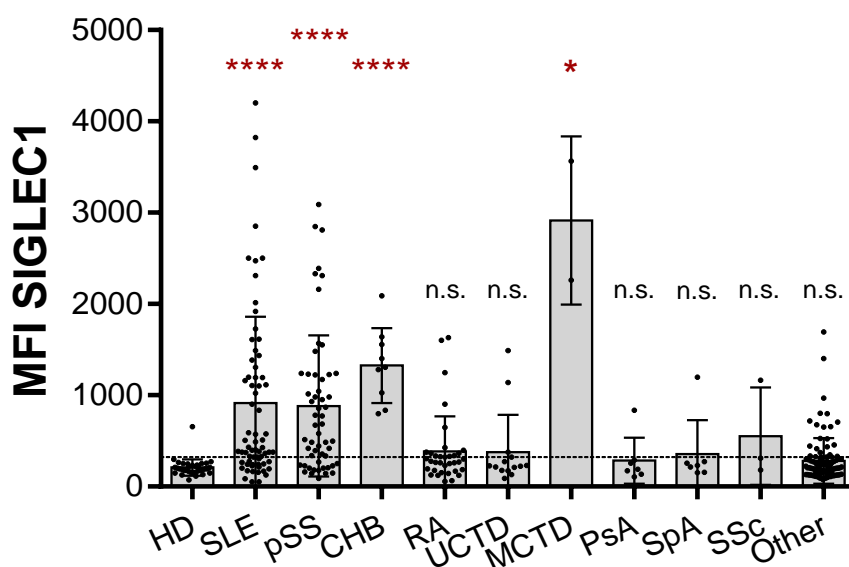
Figure 3: Expression of SIGLEC1 in rheumatological patients according to clinical diagnosis. Of individuals with more than one measurement, the mean is shown. (Error) bars represent mean ± standard deviation. Asterisks indicate the significance according to the Kruskal-Wallis test with Dunn's correction for multiple comparisons for HD vs. each disease group (****: p < 0.0001; *: p < 0.05, ns: p > 0.05). The dotted black line indicates the calculated threshold for SIGLEC1 (MFI 324.4). HD, healthy donor; SLE, systemic lupus erythematosus; pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; UCTD, undifferentiated connective tissue disease; MCTD, mixed connective tissue disease; PsA, psoriasis arthritis; SpA, axial spondyloarthritis; SSc, systemic sclerosis; CHB, congenital heart block; MFI, median fluorescence intensity; n.s., not significant.

4.2 SIGLEC1 is an indicator of extraglandular manifestation in primary Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is an autoimmune disease that is characterised by lymphocytic infiltration of the glandular organs.(22) The involvement of lacrimal and salivary glands leads to the typical "sicca symptoms" with dryness of the mouth and eyes. A distinct group of patients also suffers from extraglandular (systemic) symptoms, such as arthralgia and Raynaud's syndrome, and these patients are at an especially high risk of overall morbidity and mortality.(22, 23) The systemic disease manifestation of pSS has many overlapping features with a subset of systemic lupus erythematosus (SLE), with similar presentation, overlapping antibody profiles (anti-Ro/SS-A) and the common activation of the type I IFN system as a hallmark in the pathophysiology.(16, 22, 24)

While SIGLEC1 was shown to correlate with disease activity in SLE,(10, 13) it did not correlate with disease activity in pSS patients with inactive to mildly active disease.(17) It was therefore the aim of the study by Rose et al. (*publication 2*) to analyse whether patients with a more systemic disease presentation have an upregulation of SIGLEC1, reflecting an activated type I interferon system.(19) In the study, the expression of SIGLEC1 on CD14^{+} monocytes was examined in 31 patients with pSS which was either restricted to the glandular organs (n = 15) or which also had a systemic component (n = 16), defined as at least low activity in any organ domain of the "EULAR Sjögren's syndrome disease activity index" (ESSDAI) score.(19, 25)

It was found that the expression of SIGLEC1 significantly differed between the two groups (p = 0.0001; Mann-Whitney U test), with a positive predictive value for SIGLEC1 of 80%. The expression of SIGLEC1 also correlated with the patients' disease activity evaluated using the ESSDAI score (p = 0.005, r_s = 0.54, SRT).(25) Additionally, IP-10 levels in the patients' sera were measured by Bioplex®, but these did not significantly differ between the two groups (p = 0.65, MWU), or correlate with disease activity (p = 0.58, SRT). Both the MFI of SIGLEC1 and the serum levels of IP-10 were significantly higher in patients with pSS compared to a control group of 13 healthy donors (p < 0.0001 and p = 0.005, respectively, MWU).

4.3 Enhanced SIGLEC1 expression in pregnant females with offspring affected by congenital heart block

Autoimmune congenital heart block (CHB) is a severe heart disease that, like pSS, is associated with anti-Ro autoantibodies. It is estimated that around 2% of neonates born to females with anti-Ro autoantibodies are affected by CHB.(26) Affected children most commonly suffer from conduction defects at the AV-node, where histological analyses have shown fibrosis, macrophage infiltration and IgG accumulation.(27, 28) While it is rare, with an estimated incidence of 1 in 20,000 pregnancies, it is clinically relevant due to its high foetal morbidity and mortality.(26)

Previous studies have aimed to identify maternal and foetal risk factors for CHB development. However, the pathophysiology behind the disease is still incompletely understood. This is especially relevant, as around ⅔ of affected mothers themselves do not show signs of an autoimmune disease at the time of pregnancy,(26) and are therefore often not in rheumatological care at critical time points during pregnancy. The most common diseases in clinically symptomatic females are pSS and SLE,(26) both of which are pathogenically driven by type I IFN. As a potential role of type I IFN in CHB development had not yet been investigated, this was the aim of the study by Lisney et al. (*publication 1*).(18)

In the study, 9 pregnant females with a CHB pregnancy complication were included and compared to 14 at-risk females with anti-Ro autoantibodies who bore healthy children, as well as to 30 healthy pregnant females without the respective autoantibodies. Both the plasma levels of IFN-α (ELISA) and interferon-gamma induced protein 10 (IP-10, Bioplex®), as well as the expression of SIGLEC1 on CD14^{+} monocytes, were measured.

It was found that the affected females had significantly higher levels of IFN-α (mean: 1.38 pg/ml vs. 0.22 pg/ml, p = 0.0135, MWU) and a higher expression of SIGLEC1 compared to at-risk females who bore healthy children (mean MFI: 731, p = 0.0034, MWU). There was no significant difference between the two groups for IP-10 (mean: 918 pg/ml vs. 848 pg/ml, p = 0.14, MWU). Healthy pregnant females had the lowest levels for all three parameters (see **figure 4**).
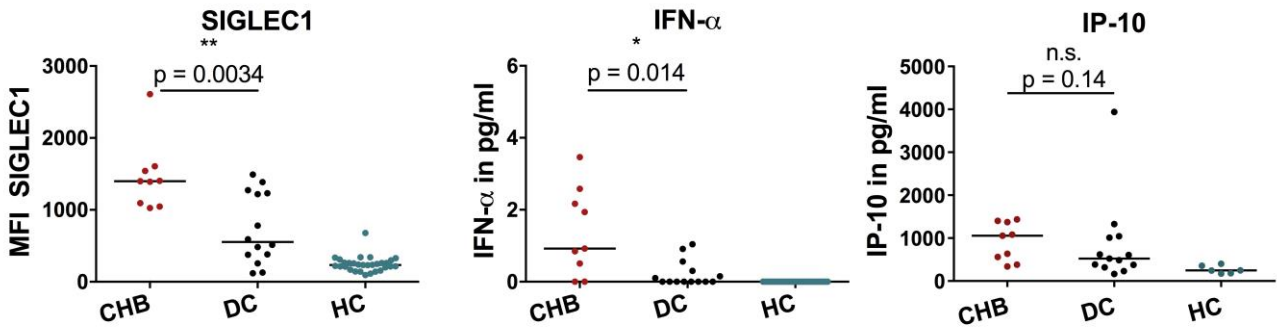
Figure 4: SIGLEC1 expression on peripheral CD14$^+$ monocytes and plasma levels of IFN-α and IP-10. SIGLEC1 expression on CD14$^+$ monocytes and IFN-α plasma levels were significantly higher in the CHB group than in at-risk females bearing healthy children ("disease controls", DC) (p = 0.0034 and p = 0.014, both MWU). IP-10 did not significantly differ between CHB and DC (p = 0.14, MWU). Bars represent the median. MFI, median fluorescence intensity; CHB, congenital heart block; DC, disease controls; HC, healthy control pregnancies; IP-10, interferon-gamma induced protein 10.

4.4 Treatment effects of immunomodulatory medication on SIGLEC1 expression

Besides the potential use of SIGLEC1 to monitor disease activity, SIGLEC1 has been proposed as a biomarker to monitor treatment response to certain immunomodulatory medications.(10) Biesen et al. showed that the expression of SIGLEC1 was reduced in four SLE patients upon introduction of high dose glucocorticoids.(10) We also found a reduction of SIGLEC1 upon introduction of oral glucocorticoids, not only in SLE, but also in patients with pSS and females with a CHB pregnancy complication. Additionally, we found that the same applied for hydroxychloroquine (see **figure 5**). No significant effect was observed for treatment with methotrexate, azathioprine, mycophenolic acid, or TNF-α inhibitors (data not shown).
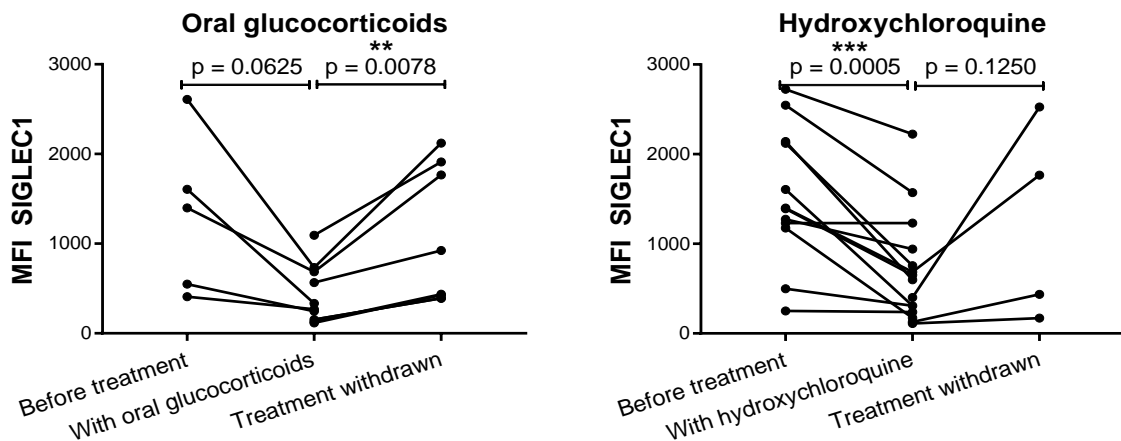
Figure 5: Treatment effects of hydroxychloroquine and oral glucocorticoids in patients with SLE, pSS and females with a CHB pregnancy complication. The MFI of SIGLEC1 was significantly reduced upon introduction of hydroxychloroquine (mean dose: 307.7 ± 125.6 mg) and upon withdrawal of oral glucocorticoids (mean dose (prednisolone equivalent): 11.4 ± 7.9 mg) (p = 0.0005 and p = 0.0078, respectively, Wilcoxon test). MFI, median fluorescence intensity; pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; CHB, congenital heart block.

4.5 Comparison of immune cell subsets

We were also interested in the immune cell composition, where disease-specific differences have been described, and which is also known to be influenced by IFN-α.(29) In line with previously published data by our group and others,(30-32) patients with SLE had a significantly higher percentage of plasmablasts (defined as $CD19^+$/ $CD20^-$/ $CD27^{++}$) within the $CD19^+$ B cell compartment in the peripheral blood compared to healthy donors (see **figure 6**). Interestingly, this also applied to mothers with a CHB pregnancy complication. The latter also had reduced numbers of memory B cells, which were comparable to levels found in patients with pSS.

Analysis of the bead-based "TBNK" staining revealed that all three patient groups had reduced numbers of natural killer (NK) cells ($CD16^+$/ $CD56^+$), B ($CD19^+$) and T ($CD3^+$) cells compared to healthy donors (see **figure 7**). Patients with SLE had the lowest cell numbers for all three cell types. There was no significant difference between the groups for $CD14^+$ monocytes (data not shown). A correlation analysis of SIGLEC1 with immune cell subsets and immunological parameters can be found in the appendix (**table 2**).

Figure 6: Immune cell subsets in healthy donors and patients with SLE, pSS and females with a CHB pregnancy complication. Shown are results from the SIGLEC1 staining. Asterisks indicate significant differences between the groups based on the Kruskal-Wallis test with Dunn's correction for multiple comparisons (****: $p < 0.0001$; ***: $p < 0.001$; **: $p < 0.01$, *: $p < 0.05$) (where not indicated, the p-value is ≥ 0.05). (Error) bars represent the mean ± standard deviation. HD, healthy donor; SLE, systemic lupus erythematosus; pSS, primary Sjögren's syndrome; CHB, congenital heart block.
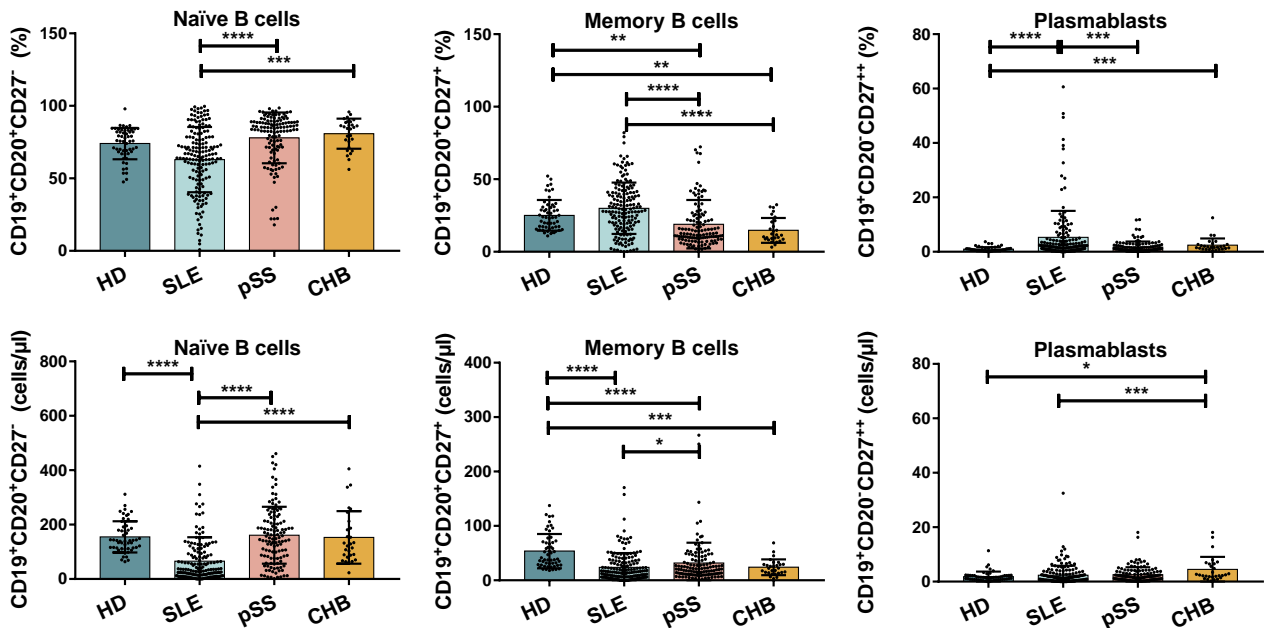
Figure 7: Immune cell subsets in healthy donors and patients with SLE, pSS and females with a CHB pregnancy complication. Shown are results from the bead based "TBNK" staining. Statistical analysis according to the Kruskal-Wallis test with Dunn's correction for multiple comparisons. (Error) bars represent the mean ± standard deviation. HD, healthy donor; SLE, systemic lupus erythematosus; pSS, primary Sjögren's syndrome; CHB, congenital heart block.
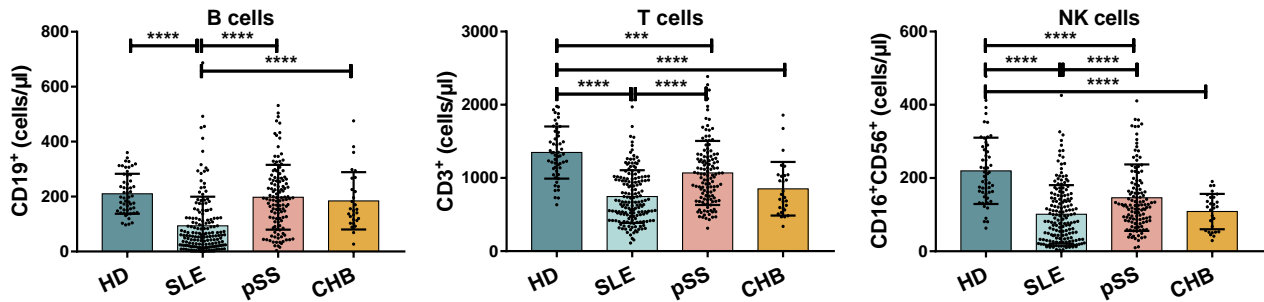
4.6   Relationship between SIGLEC1 expression and anti-RBP autoantibodies

We subsequently analysed the SIGLEC1 expression in relationship to anti-RNA binding protein (anti-RBP) autoantibodies available from the hospital's clinical database, since these have been shown to induce IFN-α production.(8) For pSS, patients with anti-Ro and/or anti-La antibodies had a significantly higher expression of SIGLEC1 compared to patients without the respective antibodies ($p < 0.0001$, MWU; see **figure 8A**). For patients with SLE, we also included anti-U1RNP, anti-RNP70 and anti-Sm antibodies into the analysis, and found that the SIGLEC1 expression differed significantly between patients who tested positive for one or more of these antibodies, and patients who tested negative for all five ($p = 0.0015$, MWU; see **figure 8B**). When analysing the antibodies individually in patients with SLE, we only found a significant difference for anti-Ro antibodies ($p = 0.0015$, MWU). The analysis of SIGLEC1 expression in relationship to anti-Ro/anti-La antibodies in females with a CHB pregnancy complication can be found in the corresponding publication by Lisney et al. (*publication 1*).(18)
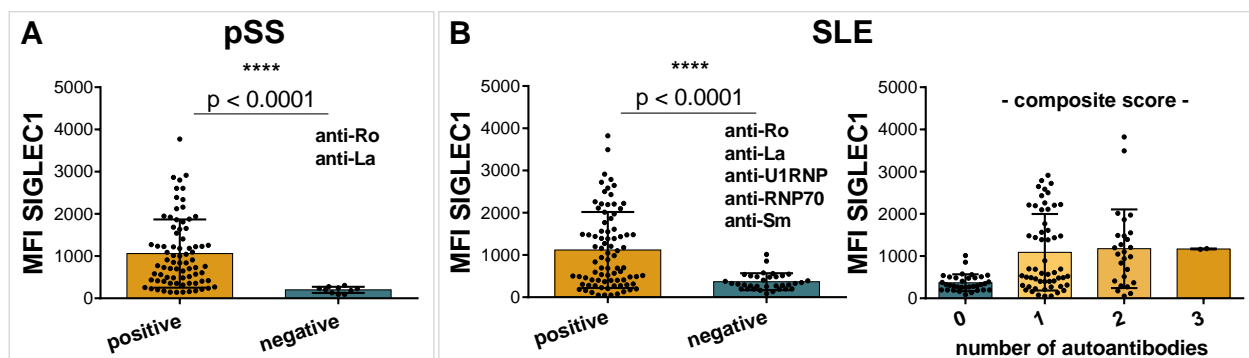
Figure 8: Relationship between SIGLEC1 expression and anti-RBP autoantibodies. Shown is the SIGLEC1 expression in patients with pSS who tested positive or negative for anti-Ro and/ or anti-La antibodies (A) and patients with SLE who tested positive or negative for anti-Ro, anti-La, anti-U1RNP, anti-RNP70 and/or anti-Sm antibodies (B). P-values according to the Mann-Whitney U test. pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; MFI, median fluorescence intensity.

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4.7 Plasmablasts with a mucosal phenotype contribute to plasmacytosis in SLE

Systemic lupus erythematosus (SLE) is a chronic-relapsing autoimmune disease affecting mostly women of childbearing age. A study showing increased levels of IFN-α in patients with SLE was published by Hooks et al. as early 1979,(24) and the central role of IFN-α in the disease's pathogenesis has since been further verified in various publications.(33, 34) Consequently, SLE was also one of the first diseases where surrogate markers to monitor the type I IFN system were studied, and here, SIGLEC1 emerged as a promising candidate that was shown to correlate with disease activity, both cross-sectionally and longitudinally.(10, 13, 14)

Among many roles in SLE, IFN-α also supports critical points in the maturation of B cells into antibody-secreting plasma cells.(35) These, and their direct precursors, the plasmablasts, are a central cell type in SLE. Plasmablasts are known to be expanded in SLE (see also **figure 6**), which is directly associated with various disease-defining phenotypes, such as hypergammaglobulinemia, and the production of autoantibodies. Plasmacytosis has been shown to correlate with disease activity,(30) and also weakly correlated with SIGLEC1 in our study ($p < 0.0001$, $r_s = 0.30$, SRT). It was the aim of the study by Mei et al. (*publication 3)* to further analyse the known plasymacytosis in SLE.(20) In the study, 58% of plasmablasts expressed IgA, and 75% of plasmablasts at least one molecule of IgA, CCR10 or β7 integrin. In conclusion, it was found that phenotypically mucosal plasmablasts significantly contribute to the expansion of plasmablasts, indicating an overly active mucosal immune system in SLE.

5. Discussion

The cell surface molecule SIGLEC1 is emerging as a promising surrogate marker for a type I interferon signature across different autoimmune diseases characterised by an upregulation of IFN-α, such as SLE. The cell-specific expression of SIGLEC1 on myeloid cells has an advantage over the analysis of an interferon signature in peripheral blood mononuclear cells (PBMCs) on a genome level, as the cell composition can be influenced by IFN-α, thus changing the relative contribution of individual cell types to the overall signature.(29) Additionally, the measurement of SIGLEC1 by flow cytometry is time and cost-efficient, and can be highly standardised, which makes it a suitable parameter for clinical practice. However, while SIGLEC1 is well-studied as a biomarker in SLE, further research is required into other autoimmune diseases. The present study therefore aimed to further investigate the biomarker SIGLEC1, mainly focusing on the diseases pSS and CHB.

Within this study, we found that patients with pSS had an increased expression of SIGLEC1 compared to healthy controls, with levels comparable to those found in patients with SLE. Additionally, we found that the SIGLEC1 expression differed significantly between patients with a systemic disease manifestation and patients where the disease was restricted to the glandular organs. These findings contrast with those of a previous study by Maria et al., who did not find a correlation between SIGLEC1 expression and disease activity evaluated by ESSDAI score in pSS patients whose disease activity was inactive to mildly active.(17) We therefore hypothesize that a higher disease activity with a systemic disease manifestation is required for the production of type I interferons, leading to a subsequent upregulation of SIGLEC1. Maria et al. proposed MxA as a type I interferon-regulated biomarker in pSS, which was not assessed in our study. Future research should therefore compare the two biomarkers SIGLEC1 and MxA in pSS patients with a broader range of disease severity, also taking into account the clinical feasibility of each biomarker. Interestingly, SIGLEC1 correlated with IgM rheumatoid factor in the study by Maria et al., which is also reflected by our data. In summary, strong evidence exists that type I interferon-regulated biomarkers can be valuable clinical parameters in patients with pSS, and that an upregulation can be indicative of a more severe disease course, possibly requiring targeted (systemic) immunomodulatory therapy.

In addition to patients with SLE and pSS, we also found that females whose children developed congenital heart block *in utero* had a higher expression of SIGLEC1 compared to at-risk females who bore healthy children. This is probably the most significant finding of the present study, since CHB is a rare disease and therefore difficult to study. Its development is associated with maternal autoantibodies directed against the ribonucleoprotein Ro, which is also present in many patients with pSS and, to a lesser extent, patients with SLE – diseases in which a pathogenic role of IFN-α is well documented.(4) While previous studies have focused on the cytokine TNF-α in CHB development,(36) our study was the first to find an association with maternal levels of IFN-α. This may be important for better understanding the pathogenesis of the disease, since interferons are known to upregulate Ro52 and to induce apoptosis,(37, 38) which may make foetal tissue more accessible to maternal antibodies. Although a potential role of IFN-α in CHB development has also been acknowledged by other groups,(39, 40) much research is still required to fully understand the implications of increased maternal IFN-α levels and SIGLEC1 expression for CHB development. This is especially relevant for risk-stratification in pregnant females with anti-Ro autoantibodies, as well as for treatment decisions during pregnancy.

In addition to the potential use of SIGLEC1 for monitoring disease activity, SIGLEC1 has also been proposed as a biomarker for monitoring treatment response.(10) While a suppression of IFN-α through glucocorticoids is known,(7) and a suppression of SIGLEC1 could be found in patients taking high-dose glucocorticoids,(10) the effects of hydroxychloroquine on IFN-α levels are less clear. It is thought that hydroxychloroquine acts through interference with Toll-like receptors (TLR) and stabilization of microsomes.(41) In the present study, we observed a reduction of SIGLEC1 following treatment with oral glucocorticoids in patients with SLE, pSS and females with a CHB pregnancy complication. Additionally, we found that the same applied for treatment with hydroxychloroquine. SIGLEC1 may therefore be a valuable biomarker for monitoring treatment response and for dose titration for these medications, as well as for medication that is still in development.(42) Also, the proposed beneficial effects of hydroxychloroquine on CHB development may be linked to a reduction of IFN-α levels.(36) Since medications directly targeting the IFN-α pathway are currently being investigated in clinical studies,(42) the importance of SIGLEC1 as a biomarker for monitoring treatment response may further increase in the future.

In summary, this study adds important new information to the body of knowledge that already exists on SIGLEC1 as a surrogate marker for type I interferons in autoimmune diseases. We were able to confirm previously published data in SLE, and, for the first time, described an association between IFN-α and SIGLEC1 expression and CHB development. Furthermore, the role of SIGLEC1 in pSS, and the effects of immunomodulatory medication on the SIGLEC1 expression were further investigated. Together, these may have direct implications for clinicians when caring for patients with autoimmune diseases. Nevertheless, further research is still required in order to fully understand the significance of an increased SIGLEC1 expression in a clinical context.

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Appendix

Table 1: Clinical and epidemiological characteristics of patients and healthy donors. Medication is baseline data.

	HD	SLE	pSS	CHB	RA	UCTD	MCTD	PsA	SpA	SSc	Other
Number of individuals	38	68	55	9	35	14	2	7	7	3	95
Total measurements	54	165	124	28	74	18	8	10	10	5	295
Age in years (mean ± SD)	31.4 ± 9.9	42.1 ± 14.5	52.1 ± 17.0	32 ± 3.8	51.2 ± 17.0	54.9 ± 20.6	54.6 ± 16.0	48.3 ± 5.4	61.4 ± 14.3	58.2 ± 10.8	48.6 ± 15.8
Female (%)	68.4	95.6	96.4	100	91.4	92.9	100	71.4	71.4	100	72.6
Medication: (n (%))											
Oral glucocorticoids	0	44 (65)	13 (24)	2 (22)	20 (57)	4 (29)	1 (50)	2 (29)	2 (29)	0	11 (12)
Hydroxychloroquine	0	29 (43)	9 (16)	3 (33)	3 (9)	1 (7)	1 (50)	0	0	0	3 (3)
Azathioprine	0	19 (28)	1 (2)	0	1 (3)	1 (7)	0	0	0	0	0
Methotrexate	0	2 (3)	0	0	8 (22)	1 (7)	0	2 (29)	1 (14)	0	2 (2)
Cyclosporine	0	2 (3)	0	0	0	0	0	0	0	0	0
Cyclophosphamide	0	2 (3)	0	0	0	0	0	0	0	0	0
Rituximab	0	0	0	0	1 (3)	0	0	0	0	0	0
TNF-α inhibitors	0	0	0	0	3 (9)	0	0	1 (14)	2 (29)	0	1 (1)
BAFF inhibitors	0	2 (3)	0	0	0	0	0	0	0	0	0
Other biologicals	0	0	0	0	4 (11)	0	0	1 (14)	0	0	0
Leflunomide	0	0	0	0	3 (9)	0	1 (50)	0	0	0	0
Sulfasalazine	0	0	0	0	1 (3)	0	0	0	0	0	1 (1)
Mycophenolate mofetil	0	2 (3)	0	0	0	1 (7)	0	0	0	0	2 (2)

HD, healthy donor; SLE, systemic lupus erythematosus; pSS, primary Sjögren's syndrome; CHB, (autoimmune) congenital heart block; RA, rheumatoid arthritis; UCTD, undifferentiated connective tissue disease; MCTD, mixed connective tissue disease; PsA, psoriasis arthritis; SpA, ankylosing spondyloarthritis; SSc, systemic sclerosis.

Table 2. SIGLEC1 correlation analysis. Shown is the correlation of SIGLEC1 with age, immunological parameters and results from the flow cytometric analysis according to the Spearman's rank test for all patients (left column), and for patients without immunomodulatory medication (right column).

		SLE		pSS		CHB		HD
		All	No med.	All	No med.	All	No med.	All
		n = 165	n = 21	n = 124	n = 62	n = 28	n = 13	n = 54
Age	Spearman's rank	-0.093	-0.69	-0.32	-0.31	0.12	-0.074	0.14
	p-value	0.23	0.0006	0.0002	0.015	0.53	0.81	0.32
CRP (mg/l)	Spearman's rank	-0.082	-0.40	-0.10	-0.36	0.66	-0.054	n.a.
	p-value	0.38	0.22	0.32	0.011	0.0047	0.88	n.a.
Neutrophils (cells/nl)	Spearman's rank	-0.090	-0.62	-0.19	-0.29	-0.030	-0.095	n.a.
	p-value	0.27	0.0044	0.036	0.029	0.89	0.77	n.a.
ANA titre	Spearman's rank	0.15	0.37	0.37	0.44	0.35	0.23	n.a.
	p-value	0.086	0.16	0.0002	0.0028	0.12	0.49	n.a.
Anti-dsDNA (ELISA) (U/ml)	Spearman's rank	0.043	-0.49	-0.31	-0.90	-0.43	n.a.	n.a.
	p-value	0.70	0.36	0.32	0.083	0.35	n.a.	n.a.
RF IgA (U/ml)	Spearman's rank	0.19	0.49	0.40	0.34	0.11	0.46	n.a.
	p-value	0.049	0.078	0.0002	0.024	0.64	0.23	n.a.
RF IgM (U/ml)	Spearman's rank	0.35	0.57	0.45	0.52	0.33	0.47	n.a.
	p-value	0.0002	0.037	0.0001	0.0003	0.15	0.14	n.a.
ACPA (U/ml)	Spearman's rank	0.13	0.35	0.38	0.16	0.24	0	n.a.
	p-value	0.24	0.27	0.0008	0.34	0.33	1.00	n.a.
Complement c3 (mg/l)	Spearman's rank	-0.12	-0.8	-0.097	0.036	n.a.	n.a.	n.a.
	p-value	0.51	0.13	0.78	0.95	n.a.	n.a.	n.a.
Complement c4 (mg/l)	Spearman's rank	0.054	-0.7	-0.25	-0.36	n.a.	n.a.	n.a.
	p-value	0.76	0.23	0.46	0.44	n.a.	n.a.	n.a.
T cells (cells/µl)	Spearman's rank	-0.19	-0.51	-0.18	0.015	-0.031	-0.23	0.046
	p-value	0.012	0.017	0.041	0.91	0.87	0.46	0.74
B cells (cells/µl)	Spearman's rank	0.070	-0.27	0.33	0.29	0.37	0.42	0.012
	p-value	0.37	0.24	0.0002	0.023	0.054	0.16	0.93
NK cells (cells/µl)	Spearman's rank	-0.15	-0.63	-0.21	-0.19	0.14	0.49	-0.012
	p-value	0.05	0.0021	0.019	0.13	0.47	0.093	0.93
Naïve B cells (cells/µl)	Spearman's rank	0.12	0.0013	0.39	0.35	0.35	0.29	0.17
	p-value	0.12	1.00	0.0001	0.0048	0.070	0.34	0.22
Memory B cells (cells/µl)	Spearman's rank	-0.12	-0.45	-0.028	-0.15	-0.042	0.016	-0.14
	p-value	0.12	0.038	0.75	0.24	0.83	0.96	0.32
Plasmablasts (cells/µl)	Spearman's rank	0.30	0.13	0.43	0.45	0.59	0.32	-0.13
	p-value	0.0001	0.58	0.0001	0.0003	0.0009	0.28	0.33

negative	p < 0.001	p < 0.005	p < 0.05		p < 0.05	p < 0.005	p < 0.001	positive

No med., no (immunomodulatory) medication; SLE, systemic lupus erythematosus; pSS, primary Sjögren's syndrome; CHB, (autoimmune) congenital heart block; HD, healthy donor; CRP, c-reactive protein; ANA, antinuclear antibodies; RF, rheumatoid factor; ACPA, anti-citrullinated peptide antibodies; n.a., not available.

Affidavit (Eidesstattliche Versicherung)

I, Anna R. Lisney, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic

"**Analysis of SIGLEC1 as a surrogate marker for a type I interferon signature in autoimmune congenital heart block and primary Sjögren's syndrome".**

I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date   Signature

Declaration of any eventual publications

Anna Lisney had the following share in the publications listed below:

Publication 1: A. R. Lisney, F. Szelinski, K. Reiter, G. R. Burmester, T. Rose and T. Dorner (2017). "High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block." Ann Rheum Dis **76**(8): 1476-1480. *Impact factor (2016):* 12.811

> Contribution in detail: Together with Prof. Dr. Thomas Dörner recruitment of patients and of healthy donors; drawing of blood; composition and analysis of clinical data; flow cytometric measurement of SIGLEC1 and of T cells, B cells and NK cells; measurement of IFN-α (ELISA) and of IP-10 (Bioplex®); measurement of autoantibody profiles (Immunoblot); statistical data analysis. Drafting and revising the manuscript.

Publication 2: Rose, T., F. Szelinski, A. Lisney, K. Reiter, S. J. Fleischer, G. R. Burmester, A. Radbruch, F. Hiepe, A. Grützkau, R. Biesen and T. Dörner (2016). "SIGLEC1 is a biomarker of disease activity and indicates extraglandular manifestation in primary Sjögren's syndrome." RMD Open **2**(2): e000292. *Impact factor (2016): N/A*

> Contribution in detail: Preparation of figure 3 in the manuscript, including the flow cytometric measurement of SIGLEC1 and of T cells, B cells and NK cells, the composition and analysis of clinical data and the statistical analysis; discussion and interpretation of data. Contribution to the drafting of the manuscript.

Publication 3: Mei, H. E., S. Hahne, A. Redlin, B. F. Hoyer, K. Wu, L. Baganz, A. R. Lisney, T. Alexander, B. Rudolph and T. Dorner (2017). "Plasmablasts with a mucosal phenotype contribute to plasmacytosis in SLE." Arthritis Rheumatol. **69**(10): 2018-2028. *Impact factor (2016):* 6.918

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Prof. Dr. Thomas Dörner   Anna R. Lisney

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Publications

Publication 1:

High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block, Ann Rheum Dis, 2017

Publication 2:

SIGLEC1 is a biomarker of disease activity and indicates extraglandular manifestation in primary
Sjögren's syndrome, RMD Open, 2016

http://dx.doi.org/10.1136/rmdopen-2016-000292

Publication 3:

Plasmablasts With a Mucosal Phenotype Contribute to Plasmacytosis in Systemic Lupus
Erythematosus, Arthritis Rheumatol, 2017

Curriculum vitae

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

Full list of publications

Scientific publications:

- Rose, T., F. Szelinski, A. Lisney, K. Reiter, S. J. Fleischer, G. R. Burmester, A. Radbruch, F. Hiepe, A. Grützkau, R. Biesen and T. Dörner (2016). "SIGLEC1 is a biomarker of disease activity and indicates extraglandular manifestation in primary Sjögren's syndrome." RMD Open **2**(2): e000292.

- Lisney, A. R., F. Szelinski, K. Reiter, G. R. Burmester, T. Rose and T. Dörner (2017). "High maternal expression of SIGLEC1 on monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block." Ann Rheum Dis **76**(8): 1476.

- Mei, H. E., S. Hahne, A. Redlin, B. F. Hoyer, K. Wu, L. Baganz, A. R. Lisney, T. Alexander, B. Rudolph and T. Dörner (2017). "Plasmablasts with a mucosal phenotype contribute to plasmacytosis in SLE." Arthritis Rheumatol. **69**(10): 2018-2028.

Conference presentations:

- Lisney, A. R., F. Szelinski, K. Reiter, G. R. Burmester, C. Scholz, T. Rose and T. Dörner (2017). "High maternal expression of SIGLEC1 on CD14+ monocytes as a surrogate marker of a type I interferon signature is a risk factor for the development of autoimmune congenital heart block." Ann Rheum Dis **76** (supplement 2): 184.

  (Oral presentation at the EULAR conference in Madrid, June 2017)

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