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Analysing gene expression of IFN-α stimulated genes in patients with SLE using direct and indirect methods.

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Abstract

Systemic Lupus Erythematosus (SLE) is an autoimmune disease where the immune system is attacking the body and its tissues. This leads to a chronic inflammation that can affect different parts of the body and therefore present itself in a vast variety of symptoms. Depending on the activity of the disease it can go up in flares where the patients experience symptoms and then go down into recession. Both the adaptive and innate immune system is involved as well as both genetic and environmental factors. Among other things, it is the formation of autoantibodies, immune complexes and a decreased ability to clear cell debris from apoptotic cells that starts the cascade of events that lead to the chronic inflammation. One important factor in all this is the cytokine interferon alpha (IFN-α) which is mainly produced by plasmacytoid dendritic cells (pDCs) as a response to the cell debris and immune complexes. IFN-α then lower the threshold of B cell receptor (BCR) activation and promote B cell differentiation into autoreactive B cells, as well as promote T-cell activation. This is known as the type I IFN signature that has been observed in patients with SLE. By measuring this signature it would be possible to prevent flares of the disease and to treat the patients with drugs aimed to inhibit the type I IFN system. There is therefore a clinical need for a measuring method that can be used routinely.

This study evaluates and compares two different methods to measure the type I IFN signature, one already established a direct method where peripheral blood mononuclear cells (PBMC) are isolated from patient blood samples and the expression of seven different IFN induced genes are analysed. The other method is an indirect method where patient sera is incubated on human epithelial tumor cells (WISH cells) and the same genes are analysed and compared to the PBMC samples. The results were also compared to clinical data and the effect on freezing and thawing RNA and sera was also evaluated.

Although there were some coherent samples between the methods the overall result suggests that there is a need for improvement on the indirect method. In regard to freezing and thawing samples, RNA is stable enough for freezing and thawing once but sera is not.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that is causing inflammation in several different organ systems, including the skin, heart, joint and kidneys. This leads to a vast variety of symptoms which make the disease hard to diagnose [1]. The disease is active for periods of time, meaning that it goes up in flares where the patient is considered active in the disease (and are experiencing symptoms), and then it goes down into recession. The cause is not known, but a complex combination of environmental and genetic factors seems to be involved and the disease is much more common in women than in men [2,3]. There is no cure for the disease; the purpose of treatment of SLE is to relieve symptoms and protect from organ damage by decreasing inflammation where drugs like corticosteroids and immunosuppressants are commonly used [4].

To assess SLE disease activity a specific index, the SLE disease activity index 2000 (SLEDAI-2K) is used [5]. It includes both clinical manifestation as well as immunological abnormalities. For scientific purposes, classification criteria from the American College of Rheumatology (ACR) is used to classify the disease [6], and to measure accumulated damage from the time point of SLE diagnosis (regardless if the damage is due to SLE or not) and forward, the SLICC damage index score is used [7].

Both the innate and the adaptive immune system contribute to the pathology of SLE and high levels of antibodies directed against components of the cell nucleus including double-stranded DNA (dsDNA), histones, nucleosomes and small nuclear ribonucleoproteins are seen [8,9]. A fast and efficient removal of dying cells is important for protection against the exposure of nuclear components to the immune system and maintaining the threshold for self-tolerance. In SLE an impaired clearance of apoptotic cells is seen as well as the inability to degrade neutrophil extracellular traps (NETs). This exposure of modified nuclear components could promote an inflammatory environment where the immune tolerance is disturbed [10]. These nuclear components and their remnants are thought to be a major source of autoantigens in the disease [11]. The autoantibodies form immune-complexes (ICs) that are deposited in tissues and also cause a number of effects on immune-cells, such as activation of the type I interferon (IFN) system [12]. Elevated levels of IFN-α, the most important mediator of the IFN type I system, have been associated with disease activity and severity in SLE [10, 13].

The pDCs is one of the main producers of type I interferons being able to produce a vast amount of IFN-α at the presence of a viral infection [12].
Type I IFNs are cytokines that are important for antiviral (and some bacterial) responses and activation of the immune system. The pDCs constitutively express the endosomal Toll Like Receptors (TLRs) -7 and TLR-9 and ICs containing dsDNA and single-stranded RNA can act as TLR ligands when endocytosed, leading to an upregulation of IFN-α production (fig. 1) [9,13]. The pDCs also activate autoreactive T cells as well as stimulate CD40-activated B cells to differentiate into autoreactive plasma cells. They are able to do so through secretion of IFN-α and interleukin-6 (IL-6), where IFN-α generates non-secreting plasma blasts and IL-6 induces differentiation of the plasma blasts into Ig-secreting cells [13]. This constitutes that IFN-α can contribute to the forming of ICs and secretion of autoantibodies as a direct response to the activation of B cells [13]. The type I IFNs also activate B- and T cells through binding of ICs to the TLRs and the FcγR, promoting IFN production and creating a cascade of events increasing the immune response and impairing the tolerance (fig. 1) [13].

This type I IFN signature has been reported in patients with SLE that are active in their disease, i.e. when flares occur. The signature is defined by the increased expression of type I IFN-regulated genes (fig. 1) [14, 15]. The activation of signalling pathways that lead to the production of IFN-ɑ is therefore thought to be one of the main contributors to the pathogenesis of SLE, which is why it is of interest to be able to measure the IFN-ɑ levels. That is to be able to foresee flares of the disease in order to prevent them, as well as potentially hinder other consequences due to the IFN signature passing by unnoticed and untreated [14]. Ongoing clinical trials of potential drugs to block the type I IFN system have also published promising results [14], for instance blockage of the IFN-ɑ receptor have been proved to be effective [16]. To have a good way of measuring IFN-ɑ levels is thus also important in order to provide directed therapy with these compounds only to the patients that need it.

There are pros and cons to the methods that are used today for measuring the IFN-α levels in patients with SLE. For instance, there is a reporter cell assay where a reporter cell such as WISH cells are used and incubated with sera. If the sera contain IFN-α it will bind to the IFN-receptor, this will trigger the signal pathway leading to a gene expression of the IFN-α induced genes [17]. This indirect measuring method is reliable due to the fact that WISH cells

do not express TLR3-7-8 or -9 to any large extent which means that the cells are not generating IFN-α as a response to ICs containing RNA or DNA. This ensures that it is only the IFN-α already existing in the sample that is driving the gene expression [17].

Measurement of the IFN signature in peripheral blood mononuclear cells (PBMC) could also be used as a marker for IFN type I activity and thus, SLE disease activity. This type of cell specific method for analysing transcriptomes provides an accurate picture of the gene expression of the type I IFN induced genes at the given time point [18]. This is a reliable method, however it requires freshly taken samples.

Both reporter cell assay and PBMC methods require a real time quantitative PCR (RT-qPCR) reaction. This is a method used for determining the expression of the chosen target genes. The complementary DNA (cDNA) that has been obtained from collected mRNA (either from WISH cell or PBMC) is amplified through the RT-qPCR and the amplification is tracked to see at which time-point the amplification occurs [18]. The earlier the increase is detected, the more mRNA was present in the sample. The gene expression of IFN induced genes are normalized using a housekeeping gene, which is a gene that is normally expressed continuously in the cell, this is done to get a reference for the general gene expression in the individual [17].

Enzyme linked immunosorbent assay (ELISA) used for IFN-α detection has shown low correlation with assays based on gene expression. With this method there is a risk for detecting similar epitopes on proteins that are not IFN-α, which makes it non reliable even if it is a quick method to use [17]. Actually, most ELISAs have been unreliable or too insensitive except for single-molecule array digital ELISA (simoa) [19].

Single-molecule array digital ELISA technology (Simoa assay) is an assay that is more sensitive than ELISA and other immunoassays because it is able to count single protein molecules. This means that it allows detection of biomarkers at concentrations that are normally difficult to measure [20]. This method is based on counting immunocomplexes of proteins that have been individually enzyme-labeled and captured on paramagnetic beads. This method has also been correlated with the activity of IFN induced gene expression measured in serum which proves its reliability in this type of study. It is also easy to use but it requires specific equipment and is expensive [21].

Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) is another method that is used, and it is a time-resolved fluorescence intensity technology. DELFIA detects biomarkers using lanthanide chelate labelled reagents and is compatible with most types of samples and a variety of plate readers. This makes the method flexible to adapt to a specific study [22]. This is a highly sensitive immunoassay but could potentially be hard to use clinically [19].

Taking all of this into account, there is an unmet clinical need for an accurate way for measuring the type I IFN levels in regard to diagnosis, monitoring disease activity and future medical treatment with upcoming type I IFN inhibitors [19].

In order to improve the clinical impact of interferons as a biological marker when looking for the type I interferon signature, the methods has developed into measuring the IFN-induced transcripts (IFITs) [23], meaning that it is the expression of the genes that is analysed rather than the amount of IFN-α present. The set of the IFITs used for measurement has not been set yet and is up for debate, the same goes for the technique and materials. Different methods have had, as stated above, inconsistent results in regard to the correlation between IFN-α levels and disease activity [23].

The aim of this project is to analyse the expression of IFN-α induced genes as an indication of the presence of IFN-α in the blood of patients with SLE. Two different methods will be evaluated and compared throughout this project. The first one is a direct measuring method where PBMCs will be isolated from patient blood and the level of the gene expression of IFN-α induced genes will be measured. The other method is an indirect way of measuring the gene expression where the patient serum will be incubated with human WISH epithelial cell line cells (ATCC no. CCL-25). The genes chosen for this study have been based on literature- and clinical studies to pick the most suitable genes for this type of measurement.

2. Material and methods

2.1 Patients and healthy donors

A total of samples from 17 individuals have been collected throughout this study. Out of those, 4 were healthy donors that were pooled into a negative control (table 1) and 13 samples were from SLE patients used to compare the two methods. Clinical data were available for 8 of the SLE patients (table 2), 7 samples of SLE patients were tested for stability of RNA, and 4 samples were used for testing the stability of freezing and thawing sera.

2.2 Isolation of PBMC and collection of serum

Whole blood samples, one heparin tube and one serum tube, were obtained from individuals upon informed consent. The serum was centrifuged after 1h and stored in -80°C until use. The blood sample obtained in heparin tubes was mixed with equal volume of 0.9 % NaCl before the PBMCs was separated by density gradient on lymphoprep^{TM} (Axis-Shield PoC, Dundee, Scotland) by centrifugation for 20 minutes at 620g. Isolated cells were washed with PBS pH 7.2 by centrifugation for 5 minutes at 300g. This step was repeated before the pellet was resuspended in 1ml PBS and counted in sysmex (Sysmex Corporation).

2.3 Isolation of RNA with RNeasy Mini Kit Qiagen

Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. This is a method that produces high quality mRNA without using hazardous chemicals. It is a combination of the selective binding to a silica-based membrane together with micro-spin technology making this method easy and quick to perform. In brief, the cells were centrifuged and the pellet was resuspended in RLT which lyses the cells. Ethanol was added to create conditions that promote selective binding to the silica membrane and the mixture was transferred to a spin column before being centrifuged. RW1 and RPE was then added in a series of steps washing the sample before eluting the RNA with RNase-free water. The concentration of RNA was then measured with a DS-11 spectrophotometer (DeNovix Inc.).

2.4 Conversion of RNA into cDNA with Bio-Rad iScript™ cDNA Synthesis Kit

Total RNA was reverse transcribed using iScript™ cDNA synthesis kit (Bio Rad, Hercules) according to manufacturer's instruction to obtain cDNA for RT-qPCR. In brief, 5x reverse-transcription reaction mix containing buffer, dNTPs, primers, ribonuclease inhibitor are used together with iScript reverse transcriptase (RNaseH+ MMLV) and nuclease free water (adapted in regard to the RNA concentration) to create a cDNA mixture together with 200 ng of RNA template in a total volume of 20 ul. Lastly a PCR was performed according to the following program: 5 minutes at 25°C, 20 minutes at 46°C, 1 minute at 95°C and then down to 4°C. (PCR 2720 Thermal Cycler, Applied Biosystems).

2.5 Real Time qPCR (RT-qPCR) measuring IFN-α induced gene expression in PBMC

TaqMan™ Gene Expression assay (FAM, Applied Biosystems) was used with the following primers from ThermoFisher scientific; IFI27, RSAD2, IFI44, IFI44L, IFIT1, EIF2AK2, Mx1 and GAPDH (Table 3). Each sample was analysed in duplicate using a 96 well qPCR plate (Applied biosystems - life technologies) and a pool of cDNA from 4 healthy individuals was used as a control sample. Each primer was mixed with TaqMan Gene Expression Master Mix (containing AmpliTaq Gold DNA polymerase, Ultra-Pure, dNTPs with dUTP, Uracil-DNA glycosylase, ROX dye and optimized buffer components) and sterile water. Then, cDNA for each patient sample was mixed with the primer mix. The RT-qPCR was performed in a StepOnePlus (Applied Biosystems) by preheating for 2 minutes at 50°C, followed by 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 20 sec. Fold-change of gene expression was determined by the relative quantification method ($2^{\Delta\Delta Ct}$) after normalization to the housekeeping gene (*GAPDH*). A subsequent log-transformation was performed to achieve comparability between genes.

Normal gene expression is at 1 for the $2^{\Delta\Delta Ct}$ value (expression fold change). The definition for where a sample is considered positive for an upregulation of gene expression is set to a mean value (for all the genes tested for each individual) over 2 for $2^{\Delta\Delta Ct}$.

2.6 WISH cells incubated with patient serum

Human WISH epithelial cell line cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Cytvia) containing glucose and 5% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO_2. When cells were grown to 85% confluence the adherent cells were detached by adding Versen (0.025% trypsin diluted to 0.005% in Versen with 0.01% EDTA (R-001-100 Gibco™)). The cells were washed twice with RPMI-1640 (Cytvia) by centrifugation at 300g for 5 minutes. The cell concentration was adjusted to $1*10^6$ cells/ml before 50µl/well was added on a 96 well flat-bottomed cell culture plate (TPP). After addition of cells to the plate, 50 µl patient serum was added and for negative control, calf serum was added. All samples were analysed in duplicates and after incubation, the supernatant was removed before resuspending the cells in RLT. As previously described the RNA was isolated with RNeasy Mini Kit, converted into cDNA with Bio-Rad iScript™ cDNA Synthesis Kit, and lastly the expression of the interferon-induced genes was analysed with RT-qPCR.

2.7 Statistic analyses

Data were analysed with Wilcoxon matched-pair signed rank test and p-values below 0.05 were considered significant.

3. Ethical reflections

The individuals examined in this study, both healthy donors and SLE patients, had their blood samples taken. The samples were only taken from patients when they had other reasons for having blood samples taken, meaning that no patient samples were taken only for this study. This does not mean that potential tomophobia (fear of medical procedures including needles) or stress and discomfort in general should not be taken into account. Therefore, it is of utmost importance to not waste any blood samples provided and to treat the samples with respect.

Then there is the aspect of maintaining patient confidentiality, to protect the identity of the participants of this study a number has been assigned to each individual. Each individual has given their consent to the blood samples and its use in research studies and an ethical approval was given for this research from the Etikprövningsmyndigheten.

The knowledge gained from this study could be used to meet an unmet clinical need as it could assist in predicting future flares of the disease. It could thus prevent patients from getting seriously ill by getting the correct treatment in time. The potential gain of this study is therefore considered larger than the possible stress or hurt caused by it.

4. Results

4.1 Collection of a negative control for the direct assay

To create a negative control for the direct assay, cDNA made from RNA isolated from PBMC from healthy donors were used. The samples were individually analysed and pooled to generate an average reference value for normal expression of the chosen genes (table 1). The Ct values were normalized to the housekeeping gene (*GAPDH*) and the standard deviation was calculated for each gene between the four individuals (table 1).

4.2. Time study for the incubation time in the indirect method with WISH cells

To set the incubation time of sera with WISH cells a kinetic time study was performed with the time points 1h, 2h, 4h, and 6h in 37°C in an atmosphere containing 5% CO_2 (fig. 2). Two previously known samples were used where one of them was known to induce a higher expression of the IFN-α induced genes (positive sample) and the other was known to induce lower expression (control). The incubation time chosen was 6h, as the largest difference in expression between the control sample and the positive sample was observed at that time-point.

4.3 IFN-α induced gene expression in PBMC and WISH cells (paired samples)

RT-qPCR was done on cDNA collected from PBMCs and WISH cells in paired samples in order to validate the two methods (Table 4). The mean value was calculated for all genes for each individual before comparison. As shown in table 4, 4 patients were negative in both tests, patient number 3, 6, 8 and 12 (with mean values lower than 2 for $2^{\Delta\Delta Ct}$) and one patient, patient number 1, was positive in both tests. Other than these 5 individuals, the results from the two methods did not correlate.

4.4 Comparison of expression levels in fresh and thawed frozen samples

In order to determine the effect of freezing and thawing RNA purified from PBMC, 7 different samples were analysed for the following genes: *IFI27, RSAD2, IFI44* and *IFI44L* before and after freezing. No significant differences were seen between fresh and frozen RNA regarding the genes according to $p=0.094$, $p=0.219$, $p=0.688$ and $p=0.297$ respectively

(data not shown). This indicates that freezing and thawing RNA once do not affect the stability of the test, and when comparing the expression fold change ($2^{\Delta\Delta Ct}$) of the IFN-α genes (fig. 3) only one patient had a distinct change in the expression fold change (gene *IFI27*) after being frozen and thawed.

In regard to thawing and freezing sera before analysing the IFN-α gene expression in WISH cells, 4 samples were analysed and compared. A trend of lower values was seen after freezing and thawing the samples on the following genes *IFI27, RSAD2, IFI44* and *IFI44L* (fig. 4). Therefore, this indicates that freezing and thawing patient sera could have an impact on the test results regarding the stability.

4.5 Clinical data and disease activity

8 patients had clinical data available for comparison (table 2). The SLEDAI-2K score gives an indication of how the disease activity was at the time of sampling. The different manifestations give different scores depending on their severity. Out of these 8, 5 patients had high values of gene expression of IFN-α induced genes i.e., with a mean value of the fold change $2^{\Delta\Delta Ct}$ over 2 (patient number 2, 7, 9, 10 and 11 seen in table 4), and 3 out of these 5 had SLEDAI-2K score of 2 respectively, which were derived from low complement and presence anti-dsDNA antibodies. The other two showed no disease activity. As for the other 3 with low expression of IFN-α induced genes (patient number 6, 8 and 12), 2 of them had no disease activity while one of them had the highest with a score of 3, which was derived for thrombocytopenia and low complement.

5. Discussion

There is an unmet clinical need for routine testing of the IFN-α levels in the blood of SLE patients. This is needed in order to be able to anticipate flares of the disease and thus hinder any possible organ damage and discomfort of the patient. Therefore, two different methods were tested and compared throughout this project, one direct method with PBMC and one indirect with sera on WISH cells. The number of patient samples collected ended up being 13 (and 4 healthy donor samples). The number of samples were supposed to be higher, but due to COVID-19, very few patients attended the rheumatology department for blood sampling.

In selection of what genes to analyse for this study, there are many to choose from. Different genes are used in different studies and no clear consensus for suitable genes for this type of study has been established. The genes were chosen based on literature studies. The literature studies were based on ongoing clinical trials for drugs that intend to block the type I IFN system. That is because if the drug is later approved, it is good to have tested the same genes for analysing the expression, that is so that we can compare our data to their data. If a certain drug is used for treatment in a clinical trial to block the type I IFN system and we use the same genes for analysing the expression, we will know if the patient will have use for that certain drug or not. Examples of these type of drugs are TLR inhibitors (since TLR7 and 9 normally stimulate IFN-α production) or the use of vaccines that induce anti-IFN-α autoantibodies [15]. With that said, genes such as *IFI44, IFIT1* and *Mx1* have proved to be induced by the IFN-α present in SLE plasma samples during reporter cell assays. The expression of these genes also correlated between WISH cells and PBMCs which should make them suitable for this study [18]. Another study has used *EIF2AK2, Mx1* and *IFIT1* with WISH cells incubated with patient sera [14] and all the genes analysed in this study has been analysed by previous interferon-stimulated-gene based methods [16].

The reason for pooling the negative control in the direct assay was to save space on the RT-qPCR plate which only allows for 12 rows of wells, i.e., 6 samples per plate to be tested. It would be a better indicator for the "normal" value if several controls were used for every plate due to individual differences of gene expression ($2^{\Delta\Delta Ct}$). But this would both be more time consuming and expensive. Although, the samples for each healthy donor were individually tested before being pooled to make sure that none of them had any irregularities in regard to the gene expression of the IFN-α induced genes.

As for the time assay, 6h was chosen based on the fact that this time point had the biggest difference between the control sample and the positive sample. Also, other studies done with this method have also used the 6h incubation time [17, 18].

The results from comparing the expression of IFN-α induced genes in PBMC and WISH cells indicates that measuring IFN-α induced gene expression in PBMC is a reliable method, but that there is a need for further optimizing of the indirect method with serum stimulating WISH cells. One suggestion would be to maybe grow the WISH cells in a different medium, a medium that stresses the cells so that they are more prone to get activated. Or maybe try the method with newly bought WISH cells that has not been passaged before. Passaging cells, a process also called subculturing, means that you are removing the medium that cells are currently placed in, splitting the cells into subcultures and transferring the cells to a new flask with fresh medium. This is done so that the cells don't overgrow and die. Which allows the cells to continue growing. In addition, the quality of the sample can also affect the results. Furthermore, FBS was used as a negative control, maybe it would have given better results to use human serum. In order to do so the negative control would have to be tested individually before to make sure it was in fact negative and to make sure that each negative had the same level of expression of the IFN-ɑ induced genes. The benefit of using FBS is that it can be bought in big batches and it always provides the same level as a negative control.

The choice to compare mean values of the expression fold change (2^ΔΔCt) of all genes for each individual can also be discussed. This was done to get a quick and easy to understand overview of the individuals. At the same time, the individual gene expression of each gene could help in choosing which genes to continue to analyse. That is because some genes got higher expression fold change than others. For instance, *IFI27* more often got high 2^ΔΔCt values in PBMC than the other genes (data not shown) and the genes that got most stimulation in the WISH cells were *IFI44L* and *Mx1* (data not shown). This cannot be identified through the mean value since the poor stimulation of the other genes in the WISH cells drag the number of the expression fold change down.

As for the comparison of expression levels in fresh and thawed frozen samples, the results in this study suggests that it has no effect on the RNA (isolated from PBMC) where there was no significant difference between the two different runs (Wilcoxon test). At least not if it is

only frozen and thawed once. One needs to consider the fact that there could be an effect if the samples are frozen and thawed several times. Also, the time that the samples spend frozen could also possibly have an effect on the stability. The fact that one gene (*IFI27*) for one patient (fig. 3) was lower in the second run for the PBMC could be due to technical problems. For the sera the number of samples are too low to do any statistical analysis but when looking at the expression fold change of the genes there is a slight decrease of expression after the samples have been frozen. This indicates that the sera are more sensitive to freezing than the RNA isolated from PBMC.

Concerning the gene expression of the IFN-α induced gene and its possible connection to disease activity it is hard to see a correlation with so few patients. With that said, 3 out of the 5 with high values of gene expression did also have a SLEDAI-2K score of two. This means that at the time of sampling these patients had low complement (patient number 2 and 7) and anti-dsDNA antibodies (patient number 11), which both manifestations are an indication of activeness in the disease. At the same time, anti-dsDNA antibodies are a symptom that you can have without it having any long-term effects. This indicates that a patient can have low SLEDAI-score or not any SLEDAI-scores at all (and thus clinically regarded as not active in their disease) but actually be active with an interferon-signature. Which could, if it is allowed to go on undetected, have consequences.

6. Conclusions

To conclude, the direct method using PBMC is a reliable method and the RNA isolated from PBMC is even stable enough to freeze once. The indirect method with WISH cells needs to be optimized by perhaps using unpassaged WISH cells or grow the cells in a different media. The sera seem to be sensitive to freezing and thus, this method requires fresh samples. It is probably the IFNs in the sera that are sensitive to freezing. However, in order to get more concrete answers and to be able to draw any conclusions from this study, it needs to be performed on more patient samples.

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1515-7

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9. Tables and figures

Table 1: Average Ct values for the specific amplified genes, subtracted with the housekeeping gene, done for both the individual healthy donor and the pool used as a negative.

Donor	IFI27	RSAD2	IFI44	IFI44L	IFIT1	EIF2AK2	Mx1
1	13.269	10.349	7.513	8.583	13.037	6.817	7.444
2	12.346	9.916	7.191	8.355	13.018	7.095	6.598
3	13.826	11.015	8.111	9.337	13.494	7.761	7.765
4	14.180	10.651	8.526	9.884	13.179	7.113	7.005
Pool	12.158	9.175	7.180	7.502	11.863	5.637	5.632
Std. Deviation	0.889	0.710	0.595	0.918	0.620	0.779	0.830

Table 2:

Patient clinical data showcasing characteristics in the form of SLICC, SLEDAI-2K score and ACR criteria for 8 SLE patients.

Clinical characteristics (n=8)	
Age in yrs; median (range)	54,5 (26-69)
Gender (%)	Female (100)
SLICC; organ damage; median (range)	0 (0-3)
SLEDAI-2K score; median (range)	**2 (0-3)**
Manifestations at the time of sampling	
Thrombocytopenia, n	1
Low complement, n	3
Anti-dsDNA antibodies, n	1
ACR criteria; median (range)	**5 (3-8)**
Malar rash, n	6
Discoid rash, n	1
Photosensitivity, n	4
Oral Ulcers, n	2
Arthritis, n	6
Serositis, n	5
Renal disease, n	1
Hematological manifestations	

Leukopenia, n	4
Lymphopenia, n	2
Thrombocytopenia, n	4
Immunological manifestations	
Anti-dsDNA antibodies, n	2
False positive serologic test for syphilis, n	1
ANAs (antinuclear antibodies), n	7

Table 3: ID number for each primer (gene) used in this study.

Gene	IFI27	RSAD2	IFI44	IFI44L	GAPDH	IFIT1	EIF2AK2	Mx1
Assay ID	Hs00271467_m1	Hs00369813_m1	Hs00197427_m1	Hs00199115_m1	Hs03929097_g1	Hs01675197_m1	Hs00169345_m1	Hs0089560_m1

Table 4:

Comparison between the direct and indirect method with either PBMC or WISH cells. The table shows calculated mean values of $2^{\Delta\Delta Ct}$ for all genes for each patient.

Patient	PBMC	WISH
1	27.439	3.471
2	38.349	1.224
3	1.308	0.827
4	4.144	0.989
5	55.685	1.418
6	0.927	1.097
7	15.957	0.848
8	0.389	0.889
9	5.638	0.591
10	10.800	0.468
11	8.862	0.997
12	0.756	0.828
13	17.760	0.929

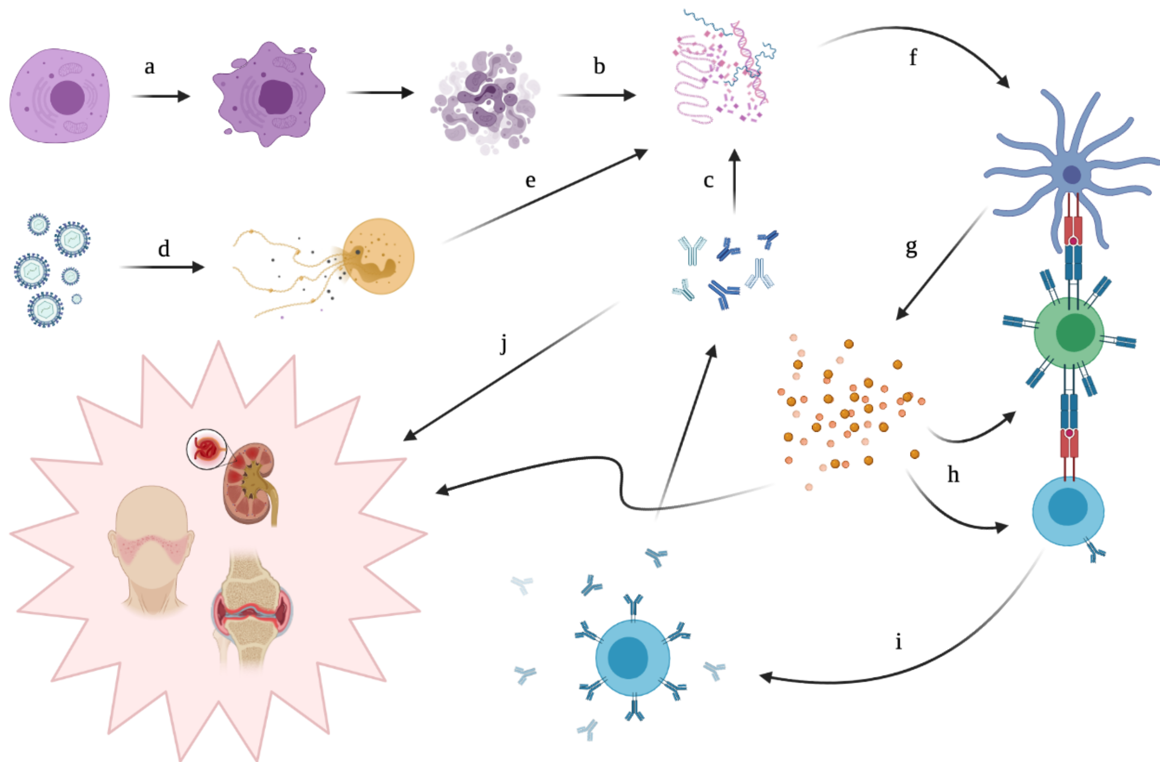

Figure 1: (a-j) schematic overview of the course of events leading to chronic inflammation in SLE. (a) Apoptosis of a cell. (b) The apoptosis leads to cell debris laying around due to decreased function of DNase I. (c) Nucleic acids, dsDNA and other components of the nucleus become targets for autoantibodies. (d) NETosis triggered by viral or bacterial infection. Neutrophil extracellular traps (NETs) are released as a defense mechanism, they are web-structures that consist of decondensed chromatin and granular molecules. (This is why an infection can trigger the cascade events of the immune system in SLE). (e) The inability to degrade NETs (which also makes it a target for autoantibodies) in patients with SLE leads to the formation of immune complexes together with the components of the nucleus. (f) The immune complexes (formed from NETs and intracellular components) are sensed and endocytosed by plasmacytoid dendritic cells. The pDC can then present the autoantigen to T cells which activate them, and the T cells can then assist in the activation of B cells. (g) The ICs also work as a ligand for the endosomal TLR7/9 in the pDC which triggers an upregulation of production of type I interferons (including IFN-ɑ). (h) The interferons also help stimulate the T and B cells and the B cells differentiate into autoreactive plasma cells (i.e., produce autoantibodies). (i) Once the B cell has been activated by a self-antigen, they begin the process of producing autoantibodies. (j) All of these steps together,

the formation of ICs, the upregulation of IFN-α, and production of autoantibodies therefore stimulate the immune system which leads to chronic inflammation in different parts of the body, such as in the joints, kidneys (nephritis) and skin (rash).

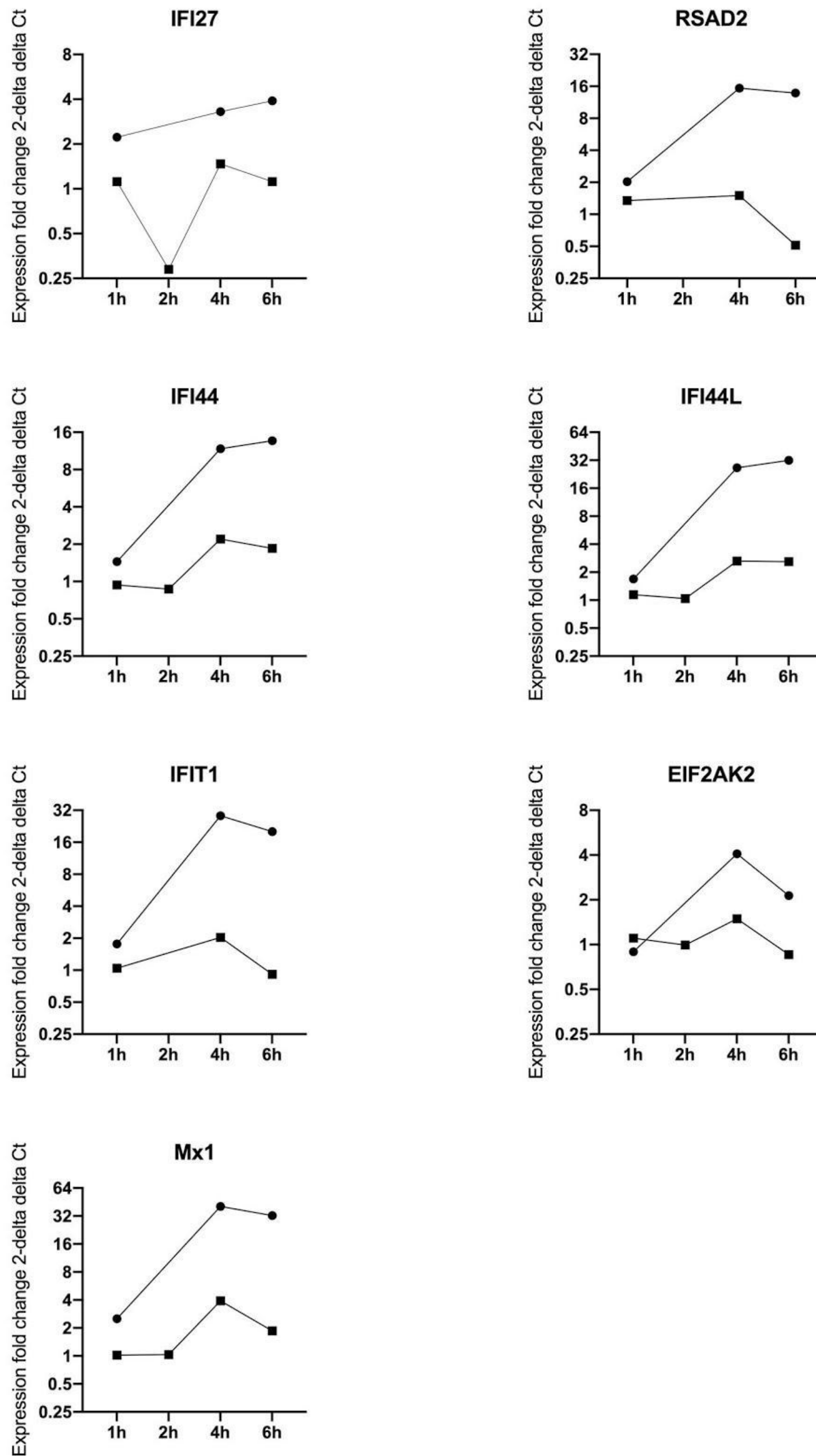

Figure 2: Measurement of gene expression in WISH cells in a time study of the incubation time with sera. Two sera were used that were previously known to provide high and low levels of expression in the IFN-α induced genes respectively. Incubation times tested were

1h, 2h, 4h and 6h, circles mark positive samples and squares mark control samples. The graphs show expression fold change 2^ΔΔCt values for each IFN-α induced gene over time and a log_2-transformation was applied to the graphs.

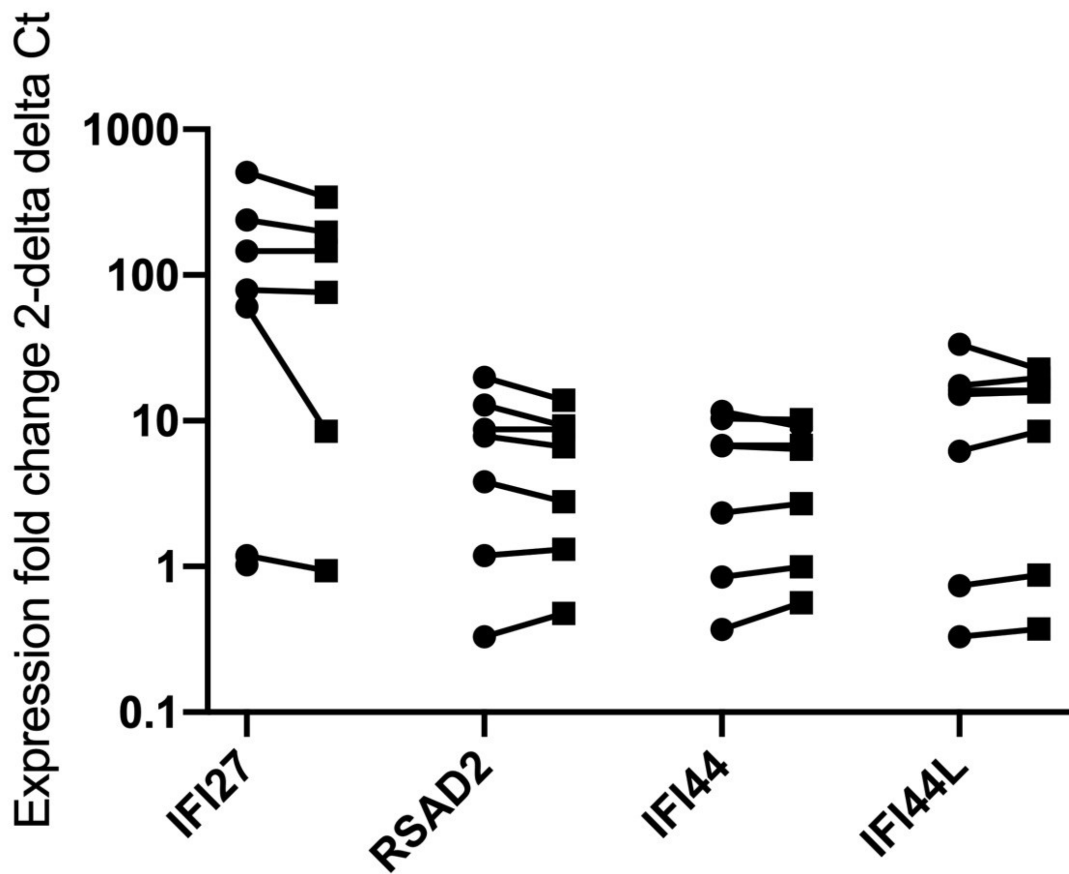

Figure 3: Measurement of gene expression in PBMCs from fresh and frozen samples. Fresh samples are marked as circles and frozen samples are marked as squares. The graph shows the expression fold change 2^ΔΔCt for paired PBMC samples. Log_{10}-transformation was applied to the graph.

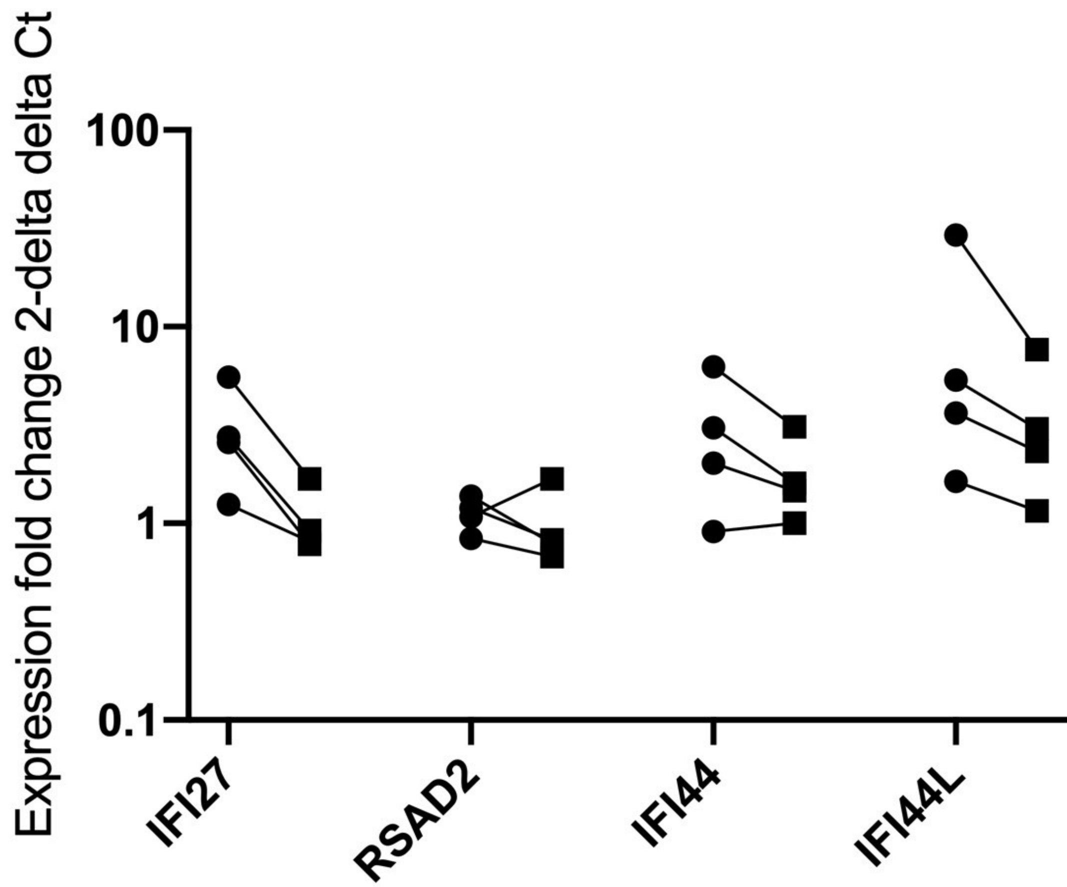

Figure 4: Measurement of gene expression in WISH cells incubated with fresh and frozen samples. Fresh samples are marked as circles and frozen samples are marked as squares. The graph shows the expression fold change 2^ΔΔCt for paired WISH/sera samples. Log_{10}-transformation was applied to the graph.