The investigation of lymphocyte activation processes and an inflammatory biomarker in autoimmune connective tissue diseases

Ph.D Thesis

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Contents

Publications related to the subject of the Thesis

I. Legány N, Toldi G, Distler J.H.W, Beyer C, Szalay B, Kovács L, Vásárhelyi B, Balog A. Increased plasma soluble urokinase plasminogen activator receptor levels in systemic sclerosis: possible association with microvascular abnormalities and extent of fibrosis. Clinical Chemistry and Laboratory Medicine**.** 53(11):1799-1805. (2015)

II. Legány N, Toldi G, Orbán Cs, Megyes N, Bajnok A, Balog A. Calcium influx kinetics, and the features of potassium channels of peripheral lymphocytes in primary Sjögren's syndrome. Immunobiology. 221(11):1266-1272. (2016)

III. Legány N, Berta L, Kovács L, Balog A, Toldi G. The role of B7 family costimulatory molecules and indoleamine 2,3-dioxygenase in primary Sjögren's syndrome and systemic sclerosis. Immunologic Research. 65(3):622-629. (2017)

Abbreviations

ACR	American College of Rheumatology
AECG	American European Consensus Group
Ag	antigen
APC	antigen presenting cell
AUC	area under the curve
BAFF	B-cell activating factor
$[Ca^{2+}]_{cyt}$	cytoplasmic calcium concentration
CaM	calmodulin
ChTX	charybdotoxin
CRAC	calcium release-activated calcium channel
CRP	C-reactive protein
CTLA-4	cytotoxic T-lymphocyte antigen 4
DAG	diacylglycerol
DC	dendritic cell
dcSSc	diffuse cutaneous systemic sclerosis
DLCO	diffusing capacity for carbon monoxide
DMSO	dimethyl-sulfoxide
EAE	experimental autoimmune enchephalomyelitis
ER	endoplasmic reticulum
ESR	erythrocyte sedimentation value
ESSDAI	EULAR Sjögren' Syndrome disease activity index
ESSPRI	EULAR Sjögren' Syndromme patient reported index
EULAR	European League Against Rheumatism
FDA	Food and Drug Administration
FVC	forced vital capacity
GPI	glycosy-phosphatidylinozitol
HLA	human leukocyte antigen
ICOS	inducible co-stimulator of T-cell
ICOSL	inducible co-stimulator of T-cell ligand
IDO	indoleamine 2,3-dioxygenase
IL	interleukin
IP3	inositol 1,4,5-triphosphate
IRF5	interferon regulatory factor
I IFN	I interferon
KYN	kynurenine
lcSSc	limited cutaneous systemic sclerosis
Max	maximum
MFI	mean fluorescence intensity
MGTX	margatoxin
MHC	major histocompatibility complex
NC	nailfold capillaroscopy

NFkB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
P2X	purinergic
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PD-1	programmed cell death-1
PD-L1	programmed cell death ligand 1
PF	pulmonary fibrosis
PHA	phytohaemaglutinin
PHT	pulmonary arterial hypertension
PIP2	phosphatidylinositol 4,5-bisphosphate
PMCA	plasma membrane calcium ATPase
pSS	primary Sjögren's syndrome
SERCA	sarco/endoplasmic reiculum calcium ATPase
SGEC	salivary gland epithelial cell
ShK	stichodactylia helianthus
SLE	systemic lupus erythematosus
SOCE	store-operated calcium entry
SSc	systemic sclerosis
STAT4	signal transducer and activation of transcription 4
STIM1	stromal interaction molecule 1
suPAR	soluble urokinase-type plasminogen activator receptor
Tcm	central memory T cell
TCR	T cell receptor
Tfh	follicular T helper cell
TGF-B	transformin growth factor B
Tmax	time to reach maximum value
TNF-α	tumor necrosis factor-α
TRAM	triarylmethane
Treg	regulatory T cell
Trp	tryptophyan
TRP	transient receptor potential
uPAR	urokinase-type plasminogen activator receptor

1. Introduction

In recent years, major progress has been made in our understanding of the pathomechanism of autoimmune connective tissue diseases (e. g. primary Sjögren's syndrome, systemic sclerosis). Thanks to these new results, the therapeutic possibilities have greatly improved and expanded following the usage of targeted/biologic therapies. In spite of this progress, a multitude of open questions regarding these autoimmune disorders and their treatments remain. Therefore, more accurate knowledge of these immunological processes and their precise mechanisms will help to better understand the pathomechanism of these diseases and lead to more effective treatment solutions. In my PhD thesis, we investigated the alteration of T cell function and activation, including the secondary costimulatory and coinhibitory signals (e. g. CD28, CTLA-4, ICOS, and PD-1 molecules and their receptors), the activity of indoleamine 2,3-dioxygenase and the regulation of the intracellular calcium signal in primary Sjögren's syndrome and systemic sclerosis compared to healthy controls. In addition to these investigations, our studies focused on the role of an inflammatory biomarker, the soluble urokinase plasminogen activator receptor (suPAR) in systemic sclerosis, as the importance of this molecule in inflammatory processes and autoimmune rheumatic diseases has recently emerged.

1.1. Primary Sjögren's Syndrome

Primary Sjögren's Syndrome (pSS) is among the most frequent systemic autoimmune diseases, as the estimated prevalence is between 0.01 and 0.1% of the caucasian population, with a female to male ratio of 9:1 (1,2). The main clinical features are ocular and oral dryness, fatigue and a significant proportion of patients develop extraglandular manifestations, such as arthritis, vasculitis, neuropathy, interstitial lung disease, or nephropathy. Sometimes these can cause severe systemic complications, which may require intensive immunosuppressive therapy. Moreover, in case of patients with long-standing primary Sjögren's syndrome, the potential risk of lymphoma is significant. In practice, two disease activity indices exist for the assessment and monitoring of pSS: the EULAR Sjögren's syndrome disease activity index (ESSDAI) and the EULAR Sjögren's syndrome patient reported index (ESSPRI). Besides these indices, hypocomplementemia, thrombocytopenia, high anti-SSA/SSB level, as well as

the presence of vasculitis all are indicators of a worse prognosis. The etiopathogenesis of pSS is not completely clear, but this systemic autoimmune disease represents a multi-genetic and multi-factorial process that leads to target organ dysfunction and lesion. There are some unique features in pSS that distinguish it from other systemic autoimmune diseases, such as glandular and mucosal germinal center formation or lymphomagenesis. The possible causes of pSS may be epithelial and lymphocytic disturbances. Genetic predisposition is mostly caused by the alleles within the major histocompatibility complex (MHC) class II gene region, in particular human leukocyte antigen-DR (HLA-DR) and HLA-DQ alleles. These gene associations may vary between different populations. Besides the HLA genes, other studies confirmed the association of pSS with key genes in the homeostasis of the immune system, such as interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4), and various cytokines (3,4). Besides genetic predisposition, environmental factors such as infectious agents can also initiate the cascade of events leading to autoimmune manifestation. It is known that viruses (hepatitis C virus, Epstein-Barr virus, human T cell leukemia virus-1) can promote autoantibody production through molecular mimicry mechanisms resulting in tissue destruction (5). At first, an external factor may trigger type I interferon (I IFN) production by plasmocytoid dendritic cells due to a glandular insult and lead to the undesirable epithelial cell surface exposure of autoantigens (e. g. SS-A/Ro, SS-B/La, α-fodrin, β-fodrin, and muscarinic M3 receptor antibodies) (6). This glandular destruction induces autoimmune activation and migration of dendritic cells and lymphocytes (T cells and B cells) to the gland. The innate and adaptive immune responses both play an important role in this pathological process. Growing evidence suggests that CD4+ Th cells are predominant, and the Th1/Th2 balance shifts in favor of Th1 in exocrine glands. Other studies show that in the early lesion, Th2 cytokines dominate, while in the later stages of pSS, Th1 cytokines are increasingly secreted by lymphocytes (7). Another T cell subset, the Th17 cells may participate in the maintenance of inflammation as well. CD8+ T cells accumulate in these glands as well, leading to the destruction of epithelial cells by apoptosis and perforin-granzyme secretion (8). The high levels of IFNs stimulate the production of B-cell activating factor (BAFF) by epithelial cells, dendritic cells, T cells, and B cells. BAFF promotes the maturation and differentiation of B cells into autoantibody-secreting plasma cells (9). Elevated plasma BAFF levels are associated with autoantibody level increase in pSS, including anti-SSA/SS-B (10). In addition to the proinflammatory process, failure of

suppressive mechanisms may also result in autoimmune inflammation in glandular tissues. Recent studies confirm the active involvement of epithelial cells in the induction and maintenance of inflammation (11***).*** In previous years, if a new pSS patient was diagnosed, only symptomatic interventions (e. g. tear/saliva substitution, non-steroidal anti-inflammatory drugs) or if necessary, systemic immunosuppressive drugs (e. g. methyl-prednisolone, chloroquine, methotrexate, azathioprine) could be used for treatment. However, in recent years, based on the expanding knowledge regarding the pathomechanism of pSS, there has been considerable progress made in the treatment options of pSS. For instance, monoclonal antibodies targeting specific molecules that are now known to play a crucial role in the pathogenetics process, such as rituximab and belimumab have been introduced, albeit only off-label so far. Both of these molecules are B cell inhibitors. Rituximab is a chimeric monoclonal antibody to CD20, and leads to B cell depletion. Rituximab can improve the systemic manifestations of pSS, and could also prevent and treat Sjögren-associated lymphoma. But the efficacy of rituximab and clinical outcomes vary between studies (12). Belimumab is a human monoclonal antibody that inhibits BAFF, and in this way suppresses B cell proliferation. Belimumab is currently in phase II trials and the early results are promising (13).

1.2. Systemic sclerosis

Systemic sclerosis (SSc) is an infrequent autoimmune connective tissue disease. The prevalence and incidence rate lies in a broad range. Depending on the geographical location, the prevalence ranges from 30-440/million, while the incidence from 4-22/million/year. SSc is more frequent in the USA and Australia than in Europe and Japan. There is female predominance, with a female to male ratio of 3-9:1 (14). Systemic sclerosis (SSc) is characterized by autoimmune disorder, microvascular injury, and excessive collagen deposition in the skin and internal organs, all of which lead to hypoperfusion and multiple organ dysfunction. There are some risk factors such as female gender, African-American race, age, family history, and genetic factors. A large genome-wide association study found that susceptibility depends on genetic factors and the involved genes are both in the HLA/MHC region and in the non-MHC region (15). For instance, HLA-DRB1*0407 and HLA-

DRB1*1304 alleles increase the risk of renal crisis in SSc (16). SSc was further classified as limited cutaneous or diffuse cutaneous SSc according to LeRoy *et al*. (17). In the limited form, the most prominent features are vascular manifestations and the thickening of the skin distal to the elbows and knees, while the internal organ involvement is usually less severe and the progression is slower than in the diffuse form. The anti-centromere antibody is the most frequent disease specific autoantibody in this form. In diffuse cutaneous SSc, the fibrosis of the skin, lungs, and other internal organs progress more rapidly, and therefore visceral organ complications develop at an early stage. The most frequently affected organs are the skin, lung, heart, gastrointestinal tract, and the kidney, while the typical disease-specific autoantibody is the anti-topoisomerase I antibody. Activation of the immune system is the likely first step in the pathological process. An early histological feature is the infiltration of the dermis by inflammatory cells, followed by endothelial cell damage (18) and excessive extracellular matrix deposition. Therefore, the activation of autoreactive T lymphocytes plays a crucial role in the initiation and maintenance of inflammation. The affected tissues are infiltrated predominantly by T cells, fibroblasts, and monocytes. T cells predominantly exhibit a Th2 pattern of cytokine production. In fact, the Th2 cytokine response, with the production of interleukin-4 (IL-4), IL-10, IL-13, and transforming growth factor-β (TGF-β) leads to tissue fibrosis, whereas Th1 and Th17 cytokines promote inflammation in SSc (19, 20). In addition, Th1 cytokines induce the production of anti-fibrotic and anti-angiogenic chemokines by fibroblasts (21). CD8+ lymphocytes take part in not only the cell-mediated cytolytic process, but in T helper function, as these cells produce Th2 cytokines, such as IL-4 or IL-13 in this autoimmune disorder (22-24). This multiplex role in the pathological inflammatory process highlights the relevance of CD8+ lymphocytes in SSc. T lymphocytes seem to be major players in the relationships between the microvascular endothelium, fibrosis, and autoimmunity. Currently, depending on the specific organ involvement of a given patient with SSc, there are many symptomatic drugs available (e.g. calcium-channel blockers, pentoxyphyllin, proton pump inhibitors, ACE inhibitors), and systemic immunosuppressive treatment (e.g. methyl-prednisolone, methotrexate, cyclophosphamide, mycophenolate mofetil) is also an option. Additionally, recent years have seen the development of several novel symptomatic drugs (e.g. prostacyclin analogs, phosphodiesterase inhibitors, endothelin receptor antagonists), which have led to an improvement in the clinical outcomes, however, no disease-specific therapies, which could reverse or slow the progression of the disease, are

thus far available. Although recent studies with the monoclonal antibody drug rituximab have been promising in the specific treatment of SSc (25), more options and further in-depth studies are required.

1.3. Activation of T-lymphocytes and intracellular processes

1.3.1. T-lymphocyte co-stimulation and co-stimulatory molecules

T lymphocyte activation is a complex mechanism, requiring multiple signaling pathways. The first step in this process is the recognition of the antigen (Ag) by the specific T cell receptor (TCR). This recognition requires that the antigen is presented on antigen presenting cell (APC) surfaces by the MHC. However, Ag recognition alone is insufficient for T cell activation. It is necessary to have an additional second signal to achieve an effective immune response. In the absence of co-stimulation, TCR-mediated antigen recognition induces a state of hypo-responsiveness, and T cells transition into an anergic state (26). The second impulse can result in either a stimulatory or inhibitory effect, largely depending on the cell surface co-stimulatory molecule expression.

1.3.1.1. CD28 Ig superfamily and B7 molecules

The members of the CD28 superfamily, such as CD28, cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death-1 (PD-1), and inducible co-stimulator of T cell (ICOS), play a crucial role in providing the second signal after Ag recognition. While the CD28 and ICOS molecules have a co-stimulatory effect, the CTLA-4 and PD-1 molecules have a co-inhibitory effect on lymphocyte activation. These molecules are expressed on T cell surfaces and are able to bind to the B7 receptor family members, such as CD80, CD86, programmed cell death ligand-1/2 (PD-L1/2), inducible co-stimulator of T cell ligand (ICOSL) on the surface of APCs. (Fig.1.)

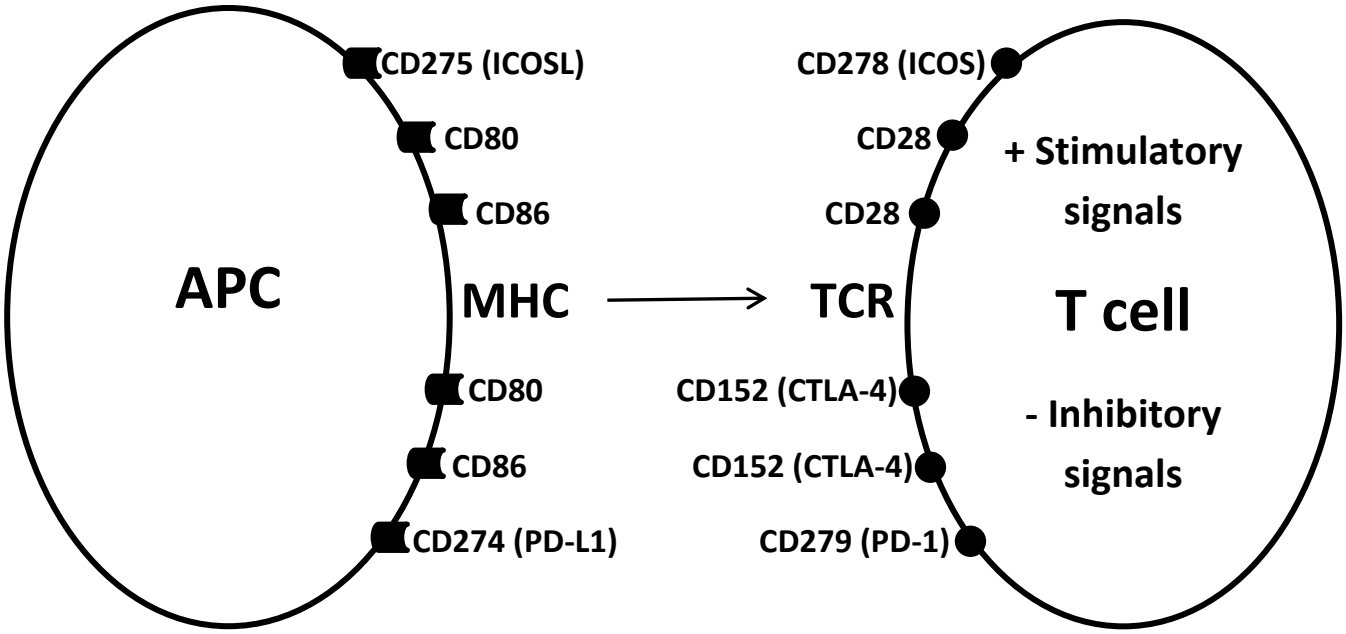


Figure 1: CD28 Ig superfamily members expressed on T cells, and their receptors (B7 family members) on the antigen presenting cell (APC). MHC - major histocompatibility complex; TCR - T-cell receptor; ICOS - inducible costimulator; ICOSL - inducible costimulator ligand, CTLA-4 - cytotoxic T-lymphocyte antigen-4; PD-1 - programmed death-1 receptor; PD-L1 - programmed death-1 receptor ligand.

1.3.1.1.1. CD28 - CD80/86 molecules

The CD28 molecule was the first detected co-stimulatory molecule, discovered in the 1980s. The stimulatory effect of CD28 on T cell proliferation was first observed at the end of that decade (27). Both TCRs and CD28 are constitutively expressed by most naive T cells, enabling them to respond to the antigen being presented (28). There are two ligands of the CD28 receptor, the inducible CD80 molecule, expressed on activated APCs (dendritic cells, activated B cells, monocytes/macrophages), and the CD86 molecule, which is constitutively expressed on resting APCs. The co-stimulatory signal from CD80 or CD86 via CD28 induces the production of IL-2 in T cells, and this interleukin promotes T cell activation, cytokine production, proliferation, and protects these cells from apoptosis and anergy (29). Without co-stimulation via CD28, the antigen recognition by TCRs induces the tolerance of T cells to their cognate antigen instead of being activated (30). (Fig. 1.)

1.3.1.1.2. CTLA-4 - CD80/86 molecules

Members of the CD28 Ig superfamily mediate not only stimulatory, but also inhibitory effects on T cell activation. The CTLA-4 is inducibly-expressed on T cells, by way of the engagement of TCRs with the presented Ag. CTLA-4 receptors are the same as those of-, CD28 (i.e. CD80, CD86) but they bind to CTLA-4 with much higher affinity than to CD28 (31). Therefore, CTLA-4 competes with CD28 for receptor binding, and because of its higher affinity, this molecule can inhibit T cell co-stimulation, IL-2 production, and the resulting activation (32). (Fig.1.) In contrast to conventional T cells, regulatory T (Treg) cells express CTLA-4 constitutively, furthermore, during the activation of Treg-cells, this basic expression is further upregulated (33). These cells are essential for maintaining immunological self-tolerance and immune homeostasis**.** Additionally, the biological agent abatacept, which is a fully human fusion molecule of IgG-Fc and CTLA-4, modulates CD28-mediated T cell costimulation. This soluble CTLA-4-Ig molecule binds to CD80/86 receptors on APCs with high affinity and outcompetes CD28 for CD80/86 ligation. In this way, abatacept administration results in an effective blockade of stimulatory signalling by CD28 (34). Abatacept was approved by the United States Food and Drug Administration (FDA) in 2005 for use in patients with rheumatoid arthritis. Besides inhibition, enhancement of this costimulatory process via CD28 is also a possibility. Ipilimumab is an anti-CTLA-4 monoclonal antibody-, that acts to activate the immune system through the CD28 pathway. Ipilimumab was approved by the United States FDA in 2011 for the treatment of metastatic melanoma (35).

1.3.1.1.3. PD-1 - PD-L1/2 molecules

The PD-1 molecule plays a crucial role in the maintenance of self-tolerance, and controlling activation of the immune system during infection, thus preventing excessive tissue damage. On naive T cells, the PD-1 is absent or present only at very low levels, its expression is induced upon TCR stimulation (36). The PD-1 receptors of T/B lymphocytes bind to PD-L1 or PD-L2 on APCs. PD-L1 is expressed in different somatic cells (lung, heart, thymus, spleen, kidney, placenta, liver, epithelial cells, leukocytes, cancer cells), and this heterogeneous appearance is important in the regulation of peripherial immunotolerance. Meanwhile, PD-L2 is expressed in relatively few cells, as it is largely restricted to APCs (37, 38). (Fig.1.) The function of the PD-1 receptor is to set an immune-checkpoint. Antigen encounter without

inflammation causes a rapid increase in PD-1 expression, thus inducing apoptosis or anergy of self-reactive T cells, decreasing T cell proliferation, as well as decreasing production of several inflammatory cytokines (e.g. tumor necrosis factor- α (TNF-α), IFN-ɤ, IL-2), to prevent autoimmunity. In this way, this co-inhibitory signal may determine the extent of T cell activation and consequently the balance between tolerance and autoimmunity (39). The PD-1 pathway is a new tumor-immunology target, because of the capability of tumor cells to enhance the expression of PD-L1/L2 on their surface leading to the inhibition of anti-tumor immune reactions. Nivolumab is an anti-PD-1 monoclonal antibody which blocks the interaction of PD-L1/L2 with T cells and increases the proliferation and function of anti-tumor T cells (40). The United States FDA approved nivolumab in 2014 for the treatment of melanoma, non-small cell lung cancer, renal cell carcinoma, Hodgkin's lymphoma, urothelial carcinoma, colorectal cancer, and hepatocellular carcinoma.

1.3.1.1.4. ICOS - ICOSL molecules

The ICOS receptor has a complex role in the regulation of the adaptive immune system. This co-stimulatory molecule is not constitutively expressed on resting T cells, only on activated CD4+ and CD8+ T cells (41). Its ligand is ICOSL, the expression of which is largely restricted to APCs (dendritic cells, monocytes/macrophages, B-cells), although it is also expressed in epithelial and endothelial cells to a lesser degree. (Fig.1.) Contrary to CD28, after TCR engagement, ICOS cross-linking is followed by IL-2 independent T cell proliferation and differentiation. Instead of IL-2, these T cells produce IL-4, IL-10, and INF-ɣ (42). The ICOS receptor is the regulator of the Th1/Th2 ratio, which is determined by the antigen environment. ICOS receptors expressed in follicular T helper (Tfh) cells can promote their IL-21 production, and the germinal center reaction of B cells, which is an important pathological process in pSS (43).

1.3.2. Indoleamine 2,3-dioxygenase

The main role of the tryptophan (TRP) catabolic enzyme indolamine 2,3-dioxygenase (IDO) is the inhibition of activated T cell proliferation, and therefore induction of immune tolerance. IDO is an intracellular enzyme, which is expressed by APCs and catalyzes the degradation of TRP into kynurenine (KYN) (44). Proinflammatory signals, especially IFN-ɣ, induce IDO expression (45). Moreover, soluble CTLA-4, or CTLA-4 on the surface of Treg cells

enhances IDO production of activated dendritic cells (DCs), as a negative feed-back mechanism (46). As a result of the effect of IDO, TRP is converted to KYN, which is then further metabolized by different enzymes. The local depletion of TRP and the production of proapoptotic TRP metabolites of the KYN pathway, such as 3-hydroxyanthranilic acid and quinolinic acid, are among the mechanisms potentially responsible for the immunosuppressive effects of IDO (47). KYN and its metabolites inhibit effector T cell function and proliferation, and furthermore increase T cell apoptosis and facilitate their differentiation to regulatory T cells (48). The immunosuppressive activity of IDO represents a counter-regulatory effect against excessive immune activation. In this way, this mechanism can play an important role in the suppression of autoimmune processes**.** In an animal model, inhibition of IDO activity or knockout of the gene encoding IDO, leads to an increase in the severity of collagen-induced arthritis with elevated numbers of Th1 and Th17 lymphocytes in the joints and lymph nodes. Conversely, if IDO activity was induced with an adenoviral vector encoding IDO, the clinical and the histopatological severity were reduced. Besides enhanced IDO activity, the therapeutic administration of L-kynurenine also slowed the progression of experimental arthritis (49).

1.3.3. Intracellular calcium signaling during T-lymphocyte activation

The elevation of the cytoplasmic calcium concentration $[Ca^{2+}]_{cyt}$ has a key role in the regulation of lymphocyte activation, proliferation, differentiation, and apoptosis. This is a very complex process which is induced by TCR engagement. The process is initiated when TCR recognizes a specific antigen presented bound to a MHC molecule, and concurrently, a second co-stimulatory signal is also delivered, triggering several signal transduction processes. First, multiple tyrosine kinases are activated, which then activate phospholipase Cɣ1, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane. In the following step, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) are generated from PIP_2. DAG then activates protein kinase C, a molecule that promotes the nuclear factor ƙB (NFƙB) pathway, ultimately activating gene transcription. IP_3 binds to IP_3 receptors on the endoplasmic reticulum (ER), and induces calcium outflow from the ER into the cytoplasm (50-52). When the calcium level decreases in the ER, the stromal interaction molecule 1 (STIM1) senses this alteration in the ER membrane and, following oligomerization, STIM1 directly interacts with the pore-forming plasma membrane protein

ORAI1 in the plasma membrane, which is a subunit of the calcium release-activated calcium channel (CRAC). Therefore, the oligomerization of STIM induces Ca^{2+} influx from the extracellular space through CRAC channels. This process is termed store-operated calcium entry (SOCE) (53). Notably, CRAC channels are selective for Ca^{2+} (54). After the opening of CRAC channels, the Ca^{2+} influx is determined by the electrochemical gradient of Ca^{2+}, thus there is an increase in Ca^{2+} influx if the membrane potential is more negative. In recent years, several families of calcium channels and transporters have been described besides CRAC channels, all taking part in the regulation of cytoplasmic calcium levels in lymphocytes, such as transient receptor potential (TRP) channels, N-methyl D-aspartate (NMDA) receptors, and purinergic (P2X) receptors; however, these molecules are not selective for Ca^{2+} and the overall relevance of these transport molecules may be less (55-57). In lymphocytes, the most important participant in the control of elevated cytoplasmic Ca^{2+} levels are the sarco/endoplasmic reticulum Ca^{2+}ATPase (SERCA) and the plasma membrane Ca^{2+}ATPase (PMCA) pumps, ion transporters which transfer back the Ca^{2+} to the ER and the extracellular space, thus decreasing $[Ca^{2+}]_{cyt}$ after lymphocyte activation (58). To a lesser extent, mitochondria may also take up and store Ca^{2+} from the cytoplasm (59). Following TCR engagement, the cytoplasmic calcium signal depends on a lot of various mechanisms, and as a result of these processes, the pattern of $[Ca^{2+}]_{cyt}$ may vary from T cell to T cell. This variation in cytoplasmic Ca^{2+} kinetics leads to differences in activation, cytokine secretion, proliferation, and differentiation in lymphocytes.

1.3.4. The role of potassium channels in lymphocyte activation

The elevation of $[Ca2+]_{cyt}$ leads to a decrease in the electrochemical potential gradient. Membrane depolarization attenuates the calcium signal. The maintenance of the electrochemical potential gradient and calcium influx is ensured by potassium ion outflow from the cytoplasm to the extracellular space through potassium channels. In lymphocytes, two potassium channels are expressed, the voltage-gated Kv1.3 and the calcium-activated IKCa1 potassium channels, together providing a counterbalancing cation efflux (60). (Fig. 2.)

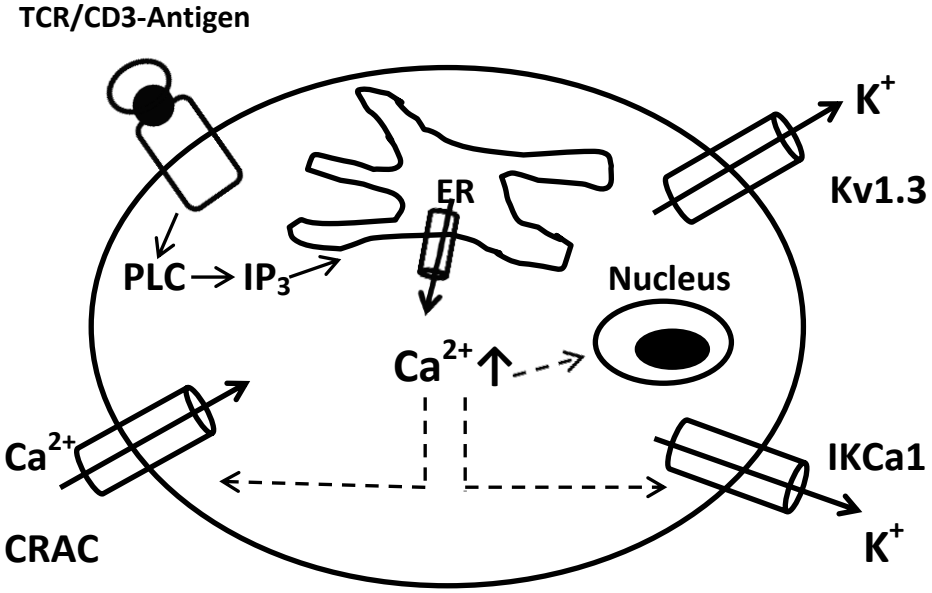


Figure 2: The mechanism of the cytoplasmic free calcium level elevation during lymphocyte activation. TCR – T-cell receptor; PLC - phospholipase-C; IP_3 – inositol trisphosphate; ER – endoplasmic reticulum; CRAC – calcium release activated calcium channel; Kv1.3 – voltage gated potassium channel; IKCa1 – calcium-activated potassium channel.

1.3.4.1. Kv1.3 potassium channel

The Kv1.3 channel was discovered in human T cells in 1984 (61). The Ca^{2+} influx through CRAC channels reduces the depolarized potential of cells. This depolarization signal activates the voltage-gated Kv1.3 channels and leads to a conformational change. The functional channel consists of four subunits, the K^+ ions flow through the center of this tetramer. One Kv1.3 subunit contains 6 transmembrane segments, a pore region, and intracellular N- and C-termini (60). The activating range is between -50 mV and -60 mV. As this value is close to the resting membrane potential in lymphocytes, the Kv1.3 channels open rapidly when the electrochemical potential gradient is altered (62). This is a mostly potassium-specific channel, although other ions can pass through it, like Rb^+, NH_4^+, Cs^+, and Na^+, but in very low amounts. If the membrane potential remains depolarized, the Kv1.3 channel is inactivated by a slow-type inactivation process caused by a conformational change. Therefore, repeated depolarization signals result in use-dependent inactivation (63). Approximately 200-400 Kv1.3 channels are expressed in resting human T cells. After T cell activation, there is a small increase in the number of these channels compared to channels on the surface of naive T cells.

In terminally differentiated effector memory T cells (T_{EM}) however, Kv1.3 channels are upregulated to 1500 per cell. Thus, a selective blocker of Kv1.3 potassium channels may be more effective on effector memory cells, compared to naive T cells (60, 64). The importance of Kv1.3 in T cell activation shows that specific Kv1.3 channel blockers can suppress T cell proliferation and cytokine production.

1.3.4.2. IKCa1 potassium channel

The Ca^{2+}-activated K^+ channel (IKCa1) was identified in T cells in 1992 (65). Similar to Kv1.3 channels, IKCa channels consist of four subunits, which contain six transmembrane segments as well. This channel is activated by an increase in $[Ca^{2+}]$ in the cytoplasm following lymphocyte activation. In a resting T cell with a low basal $[Ca^{2+}]_{cyt}$, IKCa1 channels are closed, but when the $[Ca^{2+}]_{cyt}$ rises above 300 nM, IKCa1 channels open rapidly (66). On the C-terminus of IKCa1, a Ca^{2+} sensing molecule, calmodulin (CaM) is bound, and plays an important role in the channel activation (67). In a human T cell, 5-35 IKCa1 channels are expressed per cell. Following T cell activation, this number increases to 500 per cell in naive T cells and central memory T cells (T_{CM}). Therefore, selective inhibitors of IKCa1 channels may have an effect in naive and T_{CM} cells.

1.3.4.3. Selective Kv1.3 and IKCa1 potassium channels inhibitors

Selective inhibition of potassium channels in lymphocytes leads to membrane depolarization, inhibition of Ca^{2+} influx, and consequently to lymphocyte activation. Thus, it may be a possible therapeutic approach for the treatment of autoimmune diseases. Structurally diverse molecules, like peptides, small molecules, and metal ions can block these potassium channels. As both the Kv1.3 and IKCa1 channel are especially selective for K^+, the competitive effect on other metal ions, like Rb^+, Cs^+, Na^+, are negligible. Two important drug targets for immunomodulation include small molecules and peptides with K^+ channel inhibiting potential.

1.3.4.3.1. Peptide type potassium channel blockers

Peptide blockers were first detected and isolated from scorpion venom and sea anemones. These peptides bind at the external entrance to channel pores, and this mechanism leads to a physical blockage of the pore (68). The first peptide blocker identified was charybdotoxin

(ChTX), which inhibits both Kv1.3 and IKCa1 channels to a similar extent (69). Most of the subsequently identified peptide blockers preferentially inhibit the Kv1.3 channel, rather than the IKCa1. Maurotoxin is an exception, as it most potently inhibits IKCa1 channels. Furthermore, stichodactyla helianthus toxin (ShK) and margatoxin (MGTX) are potent blockers of Kv1.3 channels at low concentrations, however they are not totally selective for Kv1.3. For instance, ShK not only blocks Kv1.3, but Kv1.1 and Kv1.6 potassium channels in neurons, therefore this effect leads to neurotoxicity *in vivo* (70, 71). In *in vitro* experiments, MGTX is used frequently in the laboratory, as in the case of selected cells it is a potent inhibitor of Kv1.3. Designed peptide inhibitors can be the solution for this problem, for instance Dalazatide (ShK-186), a fluoresceinated analogue of ShK, is a more selective blocker of Kv1.3 channels, moreover it is also detectable by flow cytometry. Besides toxicity, the other difficulty is that low molecular mass peptide blockers are eliminated rapidly through renal excretion. For this reason, there have been attempts to increase the molecular mass of these blockers by PEGylation or conjugation with an antibody Fc domain or human serum albumin. In animal models, the peptide blockers of Kv1.3 have been shown to be effective in rheumatoid arthritis, experimental autoimmune enchephalomyelitis (EAE), multiple sclerosis, pristane-induced arthritis, and asthma (72). In human phase 1a studies, dalazatide (ShK-186) was shown to be well tolerated in healthy individuals. In phase 1b studies, dalazatide caused improvements in patients with plaque psoriasis (73). Phase 2a trials are currently planned with dalazatide in systemic lupus erythematosus with nephritis (74)**.**

1.3.4.3.2. Small-molecule potassium channel blockers

The affinity and selectivity of small molecule inhibitors to potassium channels are lower compared to peptide inhibitors, as these molecules only bind to a small surface of potassium channels. Consequently, the effective dose is higher as well. These blockers are able to pass through the plasma membrane, and then block the channels under the so-called selectivity filter at the intracellular part of the channel. The first detected small molecule inhibitor was tetraethylammonium-chloride. In contrast to other small-molecule inhibitors, this molecule binds to the extracellular part of potassium channels, but with a very low affinity and specificity. At low affinities, some small molecule calcium channel inhibitors such as verapamil, diltiazem, and nifedipin can also block IKCa1 and Kv1.3 channels, but their effects are negligible. The most potent small molecule inhibitor of the Kv1.3 channel is the

Psora4 molecule, but its selectivity for Kv1.3 is limited, as it can also block the cardiac Kv1.5 channel, so it could present a risk to use this molecule in clinical practice (75). The antimycotic clotrimazole inhibits the IKCa1 channel, but unfortunately blocks cytochrome P450 enzymes as well. The designed inhibitor pyrazole-substituted triarylmethane (TRAM 34) is currently the most promising IKCa1 channel blocker, as it has over 100-fold selectivity for IKCa1 channels over other K^+ channels (76). It has been used successfully in models of acute organ graft rejection, EAE, and rheumatoid arthritis in animal trials without showing severe side effects.

1.4. Soluble urokinase plasminogen activator receptor

The urokinase-type plasminogen activator receptor (uPAR) is expressed on the surface of various immune cells (such as macrophages, dendritic cells, lymphocytes, fibroblasts), endothelial cells, smooth muscle cells, and cancer cells. uPAR is a glycosylated, 50–60 kDa protein, which consists of three extracellular domains, and is anchored in the plasma membrane by a COOH-terminal glycosyl-phosphatidylinositol (GPI) molecule. uPAR has no intracellular or transmembrane segment, thereby concentrating its serine-protease activity in the pericellular region (77, 78). In the case of healthy individuals, the presence of uPAR is detectable, but at low levels. Immune activation up-regulates the expression of this receptor. There are multiple functions of uPAR, as it plays an important role not only in the regulation of degradation of the extracellular matrix, but in the migration, adhesion, and proliferation of immune cells. The interaction of uPAR with the extracellular matrix is necessary for the reorganization of cytoskeletal architecture. In this way, uPAR is a central mediator of growth factor-induced endothelial cell adhesion, migration, proliferation, and capillary tube formation. In uPAR -/- cells, angiogenic activity is decreased and there are alterations in endothelial cell morphology in murine cells (79). In systemic sclerosis, uPAR is truncated by matrix metalloproteinase-12 on microvascular endothelial cell surfaces. This abnormal uPAR cleavage negatively regulates the activities of uPAR, subsequently the β2 integrin–mediated connection of uPAR with the actin cytoskeleton is decreased in these endothelial cells. These alterations lead to reduced angiogenic properties of microvascular endothelial cells in SSc (80). When the effects of uPAR in dermal fibrosis were investigated, it was observed that the

absence of uPAR, in uPAR$^{-/-}$ mice, attenuates the activity of matrix metalloproteinase-2, and -9 in the skin. In addition, collagen synthesis and the number of myofibroblasts was higher in uPAR gene-deficient mice, consequently the absence of uPAR induces dermal fibrosis (81).

Soluble urokinase plasminogen activator receptor (suPAR) is the soluble form of uPAR, which is derived from the cleavage and release of the membrane-bound protein. The suPAR is also detectable in low amount in the blood sample of healthy individuals and it is present in plasma, urine, and cerebrospinal fluid too. The serum level of suPAR is increased as a result of immune system activation, such as infectious, autoimmune, neoplastic, and pregnancy-related diseases. In this way, suPAR is a biomarker, as the elevated presence of this molecule is a suitable indicator of systemic inflammation. In most cases, elevated suPAR level signals worse prognosis in these diseases. SuPAR seems to be a stable biomarker, and is simply measurable from a plasma sample. The suPAR level is stationary in serum throughout the day in healthy individuals. Previous research by our research team showed that in rheumatoid arthritis, the suPAR level is a more sensitive indicator of low-level inflammatory activity in remission compared to CRP or ESR (82). In systemic lupus erythematosus (SLE), suPAR and ESR values were higher in SLE patients than in control patients. Moreover, suPAR levels in patients with high disease activity were higher than in patients with moderate disease activity or patients in remission, while ESR level was comparable in all subgroups (83). Therefore, we hypothesized that this protein could not only be an inflammatory biomarker in SSc, but also act as a pathological factor in fibrosis and endothelial dysfunction.

2. Aims

The aims of this study were to investigate the process of altered lymphocyte activation from the co-stimulatory/co-inhibitory signal to the cytoplasmic calcium signal in primary Sjögren syndrome and systemic sclerosis compared to healthy controls. Moreover, a new inflammatory biomarker, suPAR level was also analyzed in systemic sclerosis. The studies presented in this thesis were designed to address the following aims:

Aim 1. To determine the expression of CD28 Ig superfamily members (CD28, CTLA-4, PD-1, ICOS) on the surface of CD3+ T cells and CD4+ T helper cells, and to analyse the expression of their receptors, the B7 family members (CD80, CD86, PD-L1/2, ICOSL) on CD11b+ antigen presenting cell surfaces in pSS and SSc patients and compare it with healthy controls.

Aim 2. To determine the expression of indoleamine 2,3-dioxygenase in CD3+ T cells and CD4+ T helper lymphocytes and CD11b+ monocytes in pSS and SSc patients, and compare it with healthy controls.

Aim 3. To determine the elevation of cytoplasmic calcium concentration after lymphocyte activation in CD4+ Th1, Th2, and CD8+ lymphocytes in pSS and SSc patients, and compare it with healthy controls.

Aim 4. To determine the cytoplasmic calcium concentration alteration following the employment of potassium channel inhibitors in activated lymphocytes in the previous lymphocyte subgroups (CD4+ Th1, Th2 and CD8+) in pSS and SSc patients relative to healthy controls.

Aim 5. To determine the cell surface expression of Kv1.3 potassium channel on the CD4+ Th1, Th2, and CD8+ T cells in pSS and SSc patients in comparison with healthy control subjects.

Aim 6. To determine the level of suPAR in serum from patients with SSc with different internal organ involvements, and compare it to healthy individuals.

3. Patients and methods

3.1. Patients

3.1.1. Patient groups in the study of costimulatory/inhibitory signals and IDO expression

15 patients with pSS, 9 patients with SSc, and 20 age-and gender-matched healthy individuals were enrolled in this study. The average age was 52 (36-77) in pSS, 53 (39-65) in SSc, and 53 (42-61) in the healthy group. The female to male ratio was 14:1 in pSS, 7:2 in SSc, and 6:2 in the healthy group. The patients were selected from the Department of Rheumatology and Immunology, Albert Szent-Györgyi Health Center, University of Szeged. pSS patients fulfilling the American European Consensus Group (AECG) 2002 (84) or American College of Rheumatology (ACR) 2012 classification criteria (85) were included. The ESSDAI scores were calculated at the time of sampling. The median ESSDAI score was 2 which is consistent with low disease activity by longstanding immunosuppressive therapy (chloroquine, low doses of methyl-prednisolone, methotrexate, and azathioprine). All pSS patients had anti-SSA and/or anti-SSB autoantibody positivity and 80% of the pSS patients had a positive labial salivary biopsy. The following clinical symptoms occurred in pSS patients' history: sicca syndrome (100%), blood cytopenia (anemia or leukopenia) (87%), arthritis (67%), vasculitis (53%), renal involvement (13%), myositis (7%), and peripheral neuropathy (20%).

SSc patients fulfilled the 2013 ACR/EULAR classification criteria for scleroderma (86). Patients were further classified as those with limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to LeRoy et al. (17). In this study, there were 4 lcSSc and 5 dcSSc patients. The disease showed a low activity without active internal organ inflammation at the moment of sampling. Only 55% of the SSc patients were taking immunosuppressive drugs at the time of sampling (low doses of methyl-prednisolone, azathioprine, or methotrexate). The following clinical symptoms occurred in SSc patients' history: Raynaud syndrome (88%), digital ulcers (33%), interstitial lung disease (66%), gastrointestinal involvement (66%), pulmonary hypertension (33%), and pericarditis (22%). In this investigation we had 20 age- [53 years (42-61)] and gender- (the male/female ratio - 5/15) matched healthy control individuals.

3.1.2. Patient groups in the study of intracellular calcium influx kinetics and potassium channel inhibitors

In this study, we investigated the same pSS group, as in the co-stimulation study.

The SSc group was different from the previous study group, as this measurement was performed later in another laboratory with a different flow cytometer. Because of the new flow cytometer, calibration was necessary with a new control group. In the new groups, there were 16 SSc patients with an average age of 55 (39-65), and 12 healthy individuals, who were age- [52 years (28-72)] and gender- (male/female ratio - 3/9) matched. Similar to the previous study, all SSc patients fulfilled the currently accepted classification criteria for the disease and all patients were treated at the Department of Rheumatology and Immunology, Albert Szent-Györgyi Health Center, University of Szeged. In this study group, there were 9 lcSSc and 7 dcSSc patients. Besides the longstanding disease duration [10 years (1-33)], these patients also showed a low disease activity. The following symptoms occurred in these patients' disease history: Raynaud's syndrome (100%), digital ulcers (37%), gastrointestinal involvement (87%), interstitial lung disease (62%), and pulmonary hypertension (31%). 9 patients were undergoing immunosuppressive therapy at the time of the blood sampling.

3.1.3. Patient groups in the study of soluble urokinase plasminogen activator receptor

This study is a result of an international collaboration with the Department of Internal Medicine III and Institute for Clinical Immunology in the University of Erlangen-Nürnberg. Therefore, it was possible to analyze a large number of SSc samples. 83 patients with SSc fulfilling the criteria proposed by the 2013 American College of Rheumatology and the European League Against Rheumatism (ACR/EULAR) classification criteria for scleroderma (67 women and 16 men; median age 51.5 years, range 44 – 60, and median disease duration 5 years, range 2 – 10.5) were enrolled. Patients were further classified as those with limited cutaneous SSc (n=56) or diffuse cutaneous SSc (n=27). In these patients' medical history, Raynaud syndrome (94%), digital ulcers (21%), interstitial lung disease (45%), gastrointestinal involvement (60%), and pulmonary arterial hypertension (33%) occurred. In this investigation, there were 29 age- and gender-matched healthy individuals (Table 1.).

3.1.4. Healthy controls

In all measurements, healthy individuals had a negative history of rheumatic symptoms and negative status upon detailed physical and laboratory examination. No co-morbidities were detected in patients and controls that could have influenced our investigation, nor did they take any medication that could have interfered with the measurements.

3.1.5. Ethical committee

Written informed consent was obtained from all subjects, and our studies were reviewed and approved by an independent ethical committee of the University of Szeged. Laboratory studies and interpretations were performed on coded samples lacking personal and diagnostic identifiers. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

3.2. Investigation of the expression of CD28 and B7 family members, and indoleamine 2,3-dioxygenase

3.2.1. PBMC isolation

For the isolation of peripheral blood mononuclear cells (PBMCs), we used freshly drawn peripheral blood, which was collected in lithium heparin-treated tubes. PBMCs were separated by a standard density gradient centrifugation (FicollPaque, Amersham Biosciences AB, Uppsala, Sweden), by means of centrifugation for 25 min. The PBMCs were washed with phosphate buffer saline (PBS) twice, and after washing, cells were kept at −80 °C in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO) until analysis.

3.2.2. Cell staining

After thawing, PBMCs were suspended in 1.5 ml PBS, and aliquoted to 3 Eppendorf tubes equally. In the first tube, we stained the CD28 Ig superfamily members on the surface of CD3+ T cells- and CD4+ T helper cells. PBMCs were incubated for 30 min at room temperature in the dark with the following conjugated antibodies: 2 ul PerCP-conjugated CD3, 2 ul PE Cy7-conjugated CD4, 5 ul PE-conjugated CD28, 5 ul APC-conjugated CD152 (CTLA-4), 4 ul FITC-conjugated CD278 (ICOS) and 5 ul APC Cy7-conjugated CD279 (PD-

1)mAbs. In the second tube, we stained the CD80 and ICOSL from the B7 family on the cell surface of monocytes, thus the PBMCs were incubated with 2 ul PerCPconjugated CD3, 3 ul PE Cy7-conjugated CD11b, 5 ul APCconjugated CD80, and 5 ul PE-conjugated CD275 (ICOSL) mAbs, according to the previous method. In the third tube, we stained CD86 and PD-L1 from the B7 family on monocytes with 2 ul PerCP-conjugated CD3, 3 ul PE Cy7-conjugated CD11b, 5 ul APC-conjugated CD86, and 5 ul PE-conjugated CD274 (PD-L1) mAbs in separate tubes (BioLegend, San Diego, CA, USA). After washing, cells were fixed with fixation/permeabilization solution and treated with permeabilization buffer according to the manufacturer's instructions (eBioscience, San Diego, CA, USA), in order to mark IDO molecules not only on the cell surface, but intracellularly as well. Samples were then stained with a mouse anti-human IDO monoclonal antibody (Millipore, USA) for 30 min at 4 °C in the dark. After washing with PBS, cells were stained with FITC-labeled goat anti-mouse IgG antibody for 15 min at 4 °C in the dark.

3.2.3. Flow cytometry

After washing, cells were analyzed using a BD FACSAria flow cytometer (BD Biosciences) equipped with 488- and 633-nm excitation lasers. The members of the CD28 Ig superfamily were monitored on the cell surface of all CD3+ T cells, and on the CD4+ T helper subset of CD3+ cells, whereas the members of the B7 molecule family were investigated on the surface of CD11b+ monocytes. The presence of IDO molecules were detected both on the surface of CD3+ and CD11b+ cells and also in the intracellular space. Data were processed using the FACSDiVa software. One hundred thousand cells were recorded. The populations of lymphocytes and monocytes were gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics.

3.3. Investigation of the intracellular calcium influx kinetics and the effect of potassium channel inhibitors

3.3.1. PBMC isolation and staining

PBMCs were isolated from peripheral venous blood by density gradient centrifugation as already described, and we kept in a modified RPMI medium (calcium concentration 2 mM). We aliquoted the PBMCs to three equal samples and an extra vial, then incubated these

samples with conjugated anti-human monoclonal antibodies in order to differentiate T lymphocyte subsets. The following anti-human monoclonal antibodies were applied: 3 ul anti-CD4 PE-Cy7, 3 ul anti-CD8 APC-Cy7, 8 ul anti-CXCR3 APC, and 8 ul anti-CCR4 PE (all from PharmMingen, San Diego, CA, USA). CD4+CXCR3+ cells were regarded as Th1 lymphocytes, and the CD4+CCR4+ cells were regarded as Th2 subsets. The extra vial was treated with 1 ul anti-CD4 PE-Cy7, 1 ul anti-CD8 APC-Cy7, 3 ul anti-CXCR3 APC, 3 ul anti-CCR4 PE (all from PharmMingen, San Diego, CA, USA) and 10 ul anti-Kv1.3 channel FITC (Sigma-Aldrich, St. Louis, MO, USA), to mark the Kv1.3 potassium channel on the T cells surface. Unfortunately, antibody against IKCa1 potassium channel was not available at the time of the study. Afterwards, the three equal PBMC samples were loaded with calcium sensitive fluorescent stains, Fluo-3 and Fura-Red (Invitrogen, Carlsbad, CA, USA) for monitoring $[Ca^{2+}]_{cyt}$. We used all dyes according to the manufacturer's recommendations. The first one, from the three equal samples, was the control, which was not treated with potassium channel inhibitor. In the second sample, Kv1.3 channels were blocked with margatoxin (MGTX, 60 nM), and in the third sample, IKCa1 channels were blocked with triarylmethane compound (TRAM, 60 nM). PBMCs were activated by the addition of 20 μg phytohemagglutinin (PHA), following 2 minutes' monitoring of basic cell fluorescence.

3.3.2. Investigation of the cell surface expression of Kv1.3 potassium channel on T cells

We measured the extra vial, which was treated with anti-Kv1.3 antibodies, using a standard flow cytometry method. One hundred thousand cells were recorded and the T cells were gated from PBMCs according to forward scatter characteristics and side scatter characteristics. The cell surface expression of Kv1.3 potassium channel on T cells was monitored in this manner.

3.3.3. Kinetic method of flow cytometry measurements

We monitored the change of fluorescence absorbance in lymphocytes with a kinetic method. This approach gives an insight into kinetic alterations of the intracellular processes related to short-term lymphocyte activation, based on the change of intracellular calcium level. The advantage of this flow cytometry method is that we can investigate a large number of different cell types simultaneously within sequentially measured cells. The fluorescence data of pSS samples were measured using a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA) equipped with 488- and 633-nm excitation lasers, and data were processed using the

FACSDiVa software. The SSc samples with their healthy controls were measured at the Department of Biochemistry, University of Szeged, using a different flow cytometer (Partech), so these results are not comparable with the pSS samples, although all settings and methodology were consistent with the previous measurements. The cell fluorescence data were measured and recorded for 10-15 minutes using a kinetic method. Cells were gated according to forward scatter and side scatter characteristics, and then the gated events were plotted with the measured fluorescent channel against time. (Fig.3.)

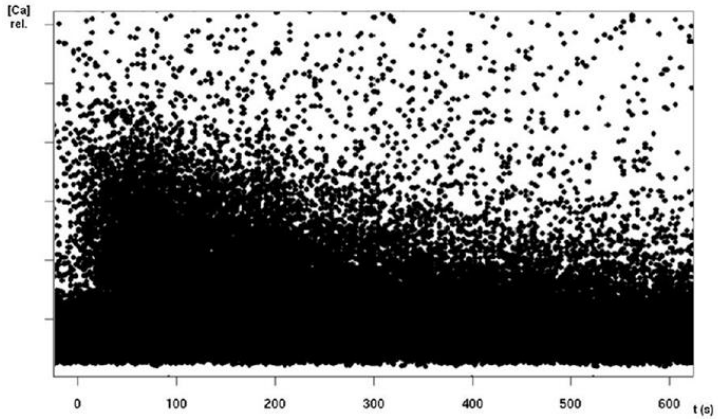


Figure 3: The median fluorescence of calcium binding dyes in lymphocytes against time. Every point is equivalent to a lymphocyte.

Our collaborators at the First Department of Pediatrics at Semmelweis University, introduced a novel algorithm to characterize cytoplasmic Ca^{2+} signal after short-term activation of lymphocytes that allows a description of kinetic changes in an objective manner (87). This specific software (FacsKin) was used for the evaluation of data from kinetic flow cytometry measurements. Analysis with FacsKin is based on the calculation of a double-logistic function for each Ca^{2+} influx recording. This function is used to characterize measurements that have an increasing and a decreasing intensity as time passes. The software calculates parameter values describing each function, such as the area under the curve (AUC), maximum (Max), time to reach maximum (Tmax), and Slope value. AUC values correspond to the sum of $[Ca^{2+}]_{cyt}$ increase, which further corresponds to the level of lymphocyte activation. Max values represent the peak value of the calcium influx curve upon lymphocyte activation. Tmax values describe how soon the peak value of the calcium influx curve is reached. The slope value reflects how rapidly the peak of calcium influx is reached (87, 88). With this new

flow cytometry method, we could follow the intracellular calcium level changing simultaneously in a large amount of T cells as time passes. (Fig. 4.)

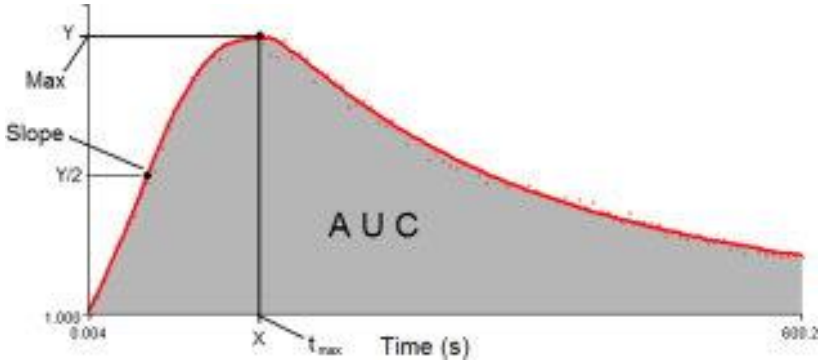


Figure 4: Parameter values are calculated by FacsKin. The vertical axis represents the relative fluorescent intensity. AUC - area under the curve; Max - maximum value; Tmax - time to reach maximum.

3.4. Investigation of the suPAR level in SSc patients' serum

We collected EDTA anticoagulated blood samples from SSc patients and healthy individuals. Plasma was isolated from peripheral venous blood and stored at – 80 °C until measurement. suPAR concentrations were measured with the suPARnostic Flex ELISA assay (ViroGates A/S, Birker ø d, Denmark). Then, we compared the suPAR level with the c-reactive protein (CRP) and erythrocyte sedimentation values (ESR) in the limited cutaneous and diffuse cutaneous SSc groups, depending on the clinical features. We obtained CRP and ESR results from central laboratory data.

3.5. Statistical analysis

Data are expressed as median and interquartile ranges. In the investigation of co-stimulatory molecules, comparisons between sample populations were made with the Kruskal-Wallis test. Correlation analyses were performed using Spearman's signed ranked tests. In the investigation of potassium channels, comparisons between two sample groups were made

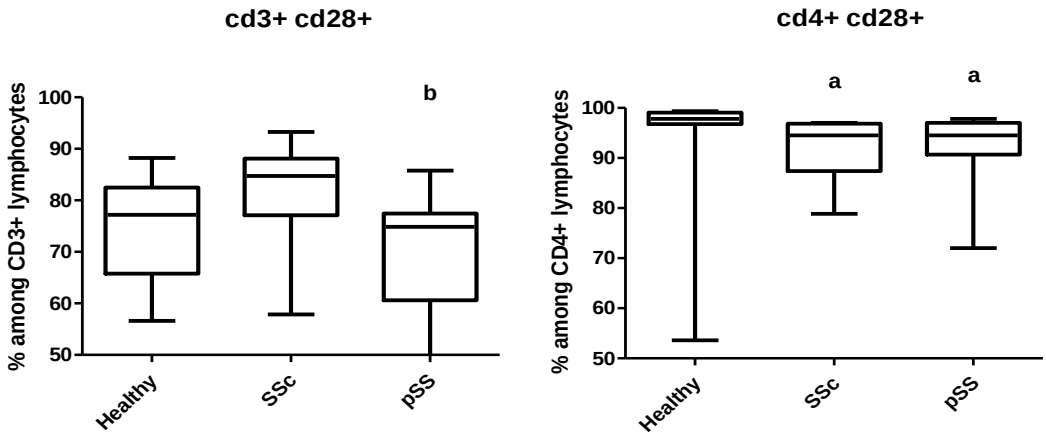
with the Mann–Whitney test. For comparisons between paired values in the same group, Wilcoxon tests were applied. In the investigation of serum suPAR level, controls and SSc patient subgroups and their clinical characteristics were compared using Mann-Whitney U and Kruskal-Wallis tests, respectively. P values less than 0.05 were considered significant. Statistics were calculated using the GraphPad Prism software (version: 5.00, GraphPad Software Inc., La Jolla, CA, USA).

4. Results

4.1. Cell surface expression of the CD28/B7 Ig superfamily in primary Sjögren syndrome and systemic sclerosis

4.1.1. Expression of CD28 Ig superfamily on T lymphocytes

We investigated the expression of these molecules on the surface of CD3+ T cells as well as specifically focusing on the subset of CD4+ T helper lymphocytes within the CD3+ cells. In the case of the CD28 molecule, no difference was observed in the prevalence of CD3+ CD28+ cells in SSc or pSS samples compared with healthy samples, although there was a significant increase in SSc as compared with pSS. In the T-helper subset, the prevalence of CD4+ CD28+ T-helper cells was significantly lower in both SSc and pSS than in healthy group. There was no difference in the prevalence of CD3 + CTLA-4+ cells between the study groups, but within T-helper cells, the amount of CD4+ CTLA-4+ T-helper cells was higher in pSS compared with healthy samples. (Fig. 5.)



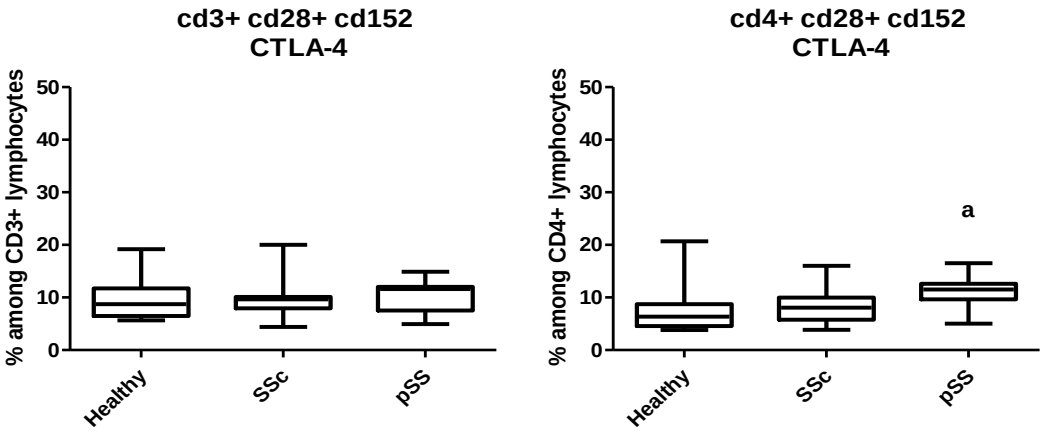
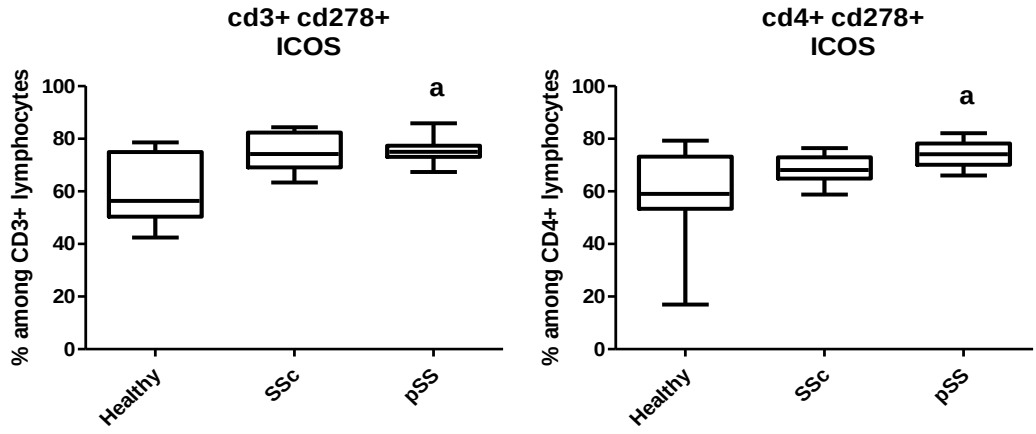


Figure 5: Frequency of T lymphocytes which expressed CD28/CTLA-4 molecules. a: lymphocytes from SSc or pSS patients were compared to lymphocytes from healthy control, $p<0.05$. b: lymphocytes from pSS samples were compared with lymphocytes from SSc samples, $p<0.05$.

When we investigated the expression of the ICOS molecule, we observed that the prevalence of CD3+ICOS+ and CD4+ICOS+ lymphocytes was higher in pSS than in healthy controls. Moreover, the prevalence of ICOS+ T cells was higher in the SSc group compared to the healthy controls, but not significantly. The frequency of CD3+PD1+ and also CD4+PD1+ lymphocytes was lower in both SSc and pSS samples than in the healthy samples. (Fig. 6.)



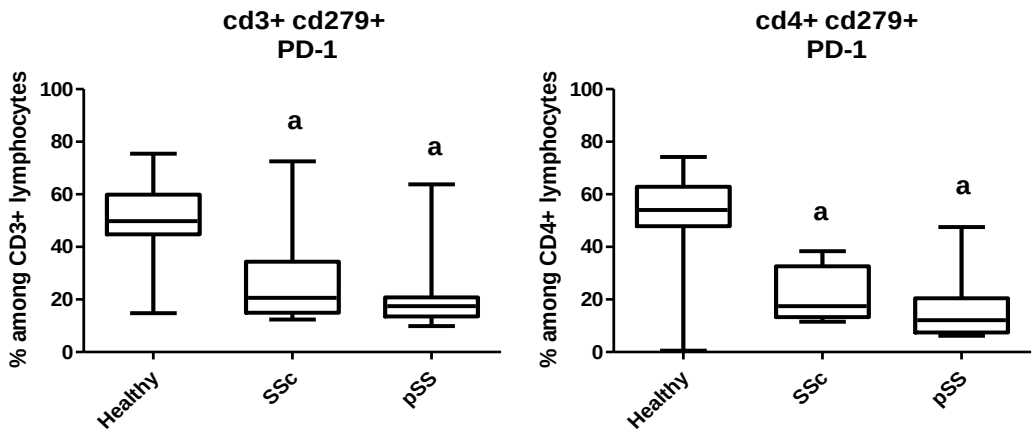
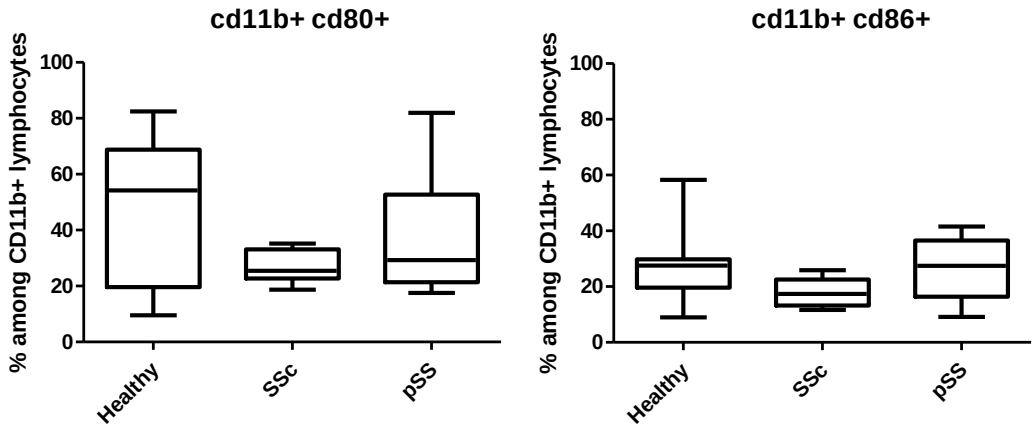


Figure 6: Frequency of T lymphocytes which expressed ICOS/PD-1 molecules. a: cells from SSc or pSS patients were compared to cells from healthy control, $p<0.05$.

4.1.2. Expression of B7 molecules on monocytes

When we analyzed the members of the B7 molecule family on the surface of CD11b+ monocytes, no significant difference was observed in the prevalence of CD80+, CD86+, and PD-L1+ expressing monocytes between SSc, pSS, and healthy control groups. However, the frequency of ICOSL+ monocytes was lower in SSc than in the healthy group, and higher in pSS than in SSc. (Fig. 7.)



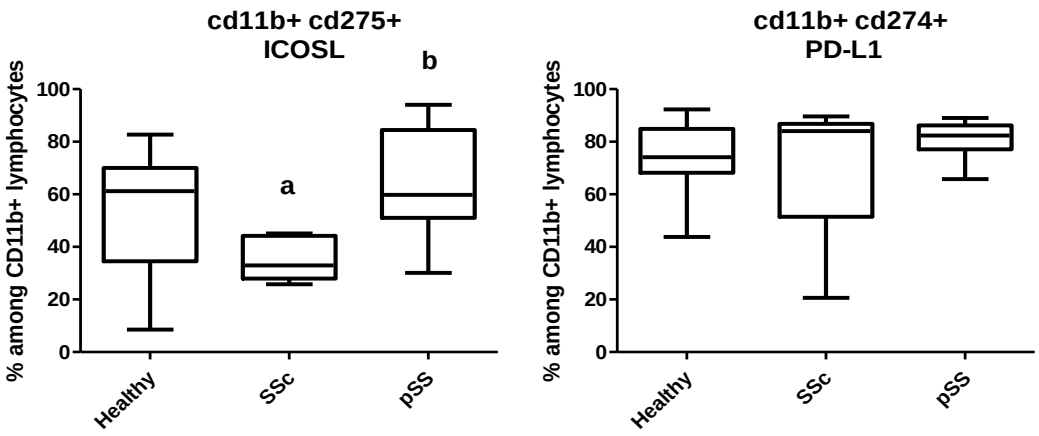


Figure 7: The frequency of APCs which expressed CD80/CD86/ICOSL/ PD-L1 molecules. a: cells from SSc or pSS patients were compared to cells from healthy control, $p<0.05$. b: cells from pSS samples compared with cells from SSc samples, $p<0.05$.

4.2. Presence of IDO in primary Sjögren syndrome and systemic sclerosis

The expression of IDO molecules was measured both on the surface of CD3+ T lymphocytes' and CD11b+ monocytes and also in their intracellular space in the pSS, SSc, and healthy groups. In the case of CD3+ T cells, no difference was observed in the prevalence of CD3+IDO+ lymphocytes; however, IDO MFI values in CD3+ cells, which characterize the intracellular IDO content, were higher in pSS samples compared with SSc or healthy control samples. The prevalence of IDO+ monocytes was higher only in pSS samples compared with the other groups, but there was no difference in the MFI of IDO in monocytes between the study groups. In order to explore the presence of reverse signaling via CD80 and CD86, correlation analyses were performed. No correlation was revealed between IDO-expressing T cells and monocytes and the prevalence of CD80+ or CD86+ monocytes. (Fig. 8.)

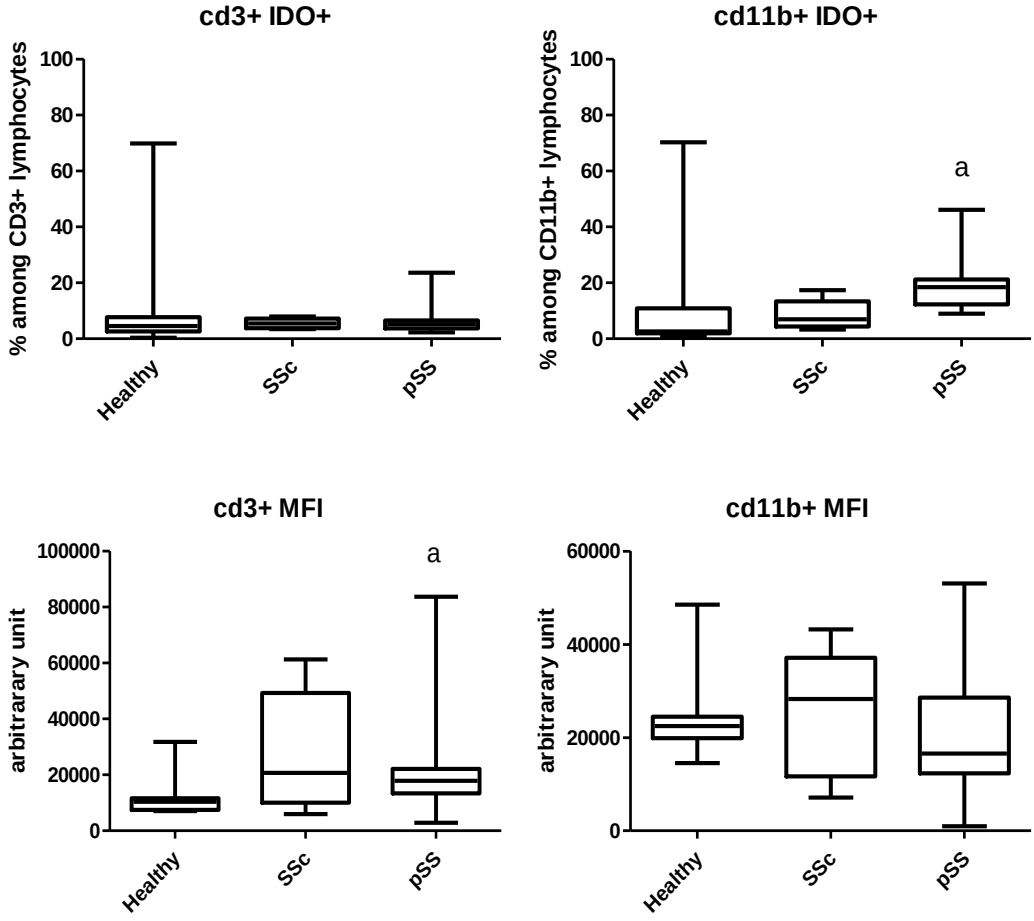


Figure 8: IDO expression on CD3+/CD11b+ cell surface and in their cytoplasm. MFI - mean fluorescence intensity. a: cells from pSS patients were compared with healthy controls, $p < 0.05$.

4.3. The cytoplasmic calcium influx kinetics in T lymphocytes in primary Sjögren syndrome and systemic sclerosis

4.3.1. Basal cytoplasmic calcium level in the investigated lymphocyte subsets

Before lymphocyte activation, we measured the basal intracellular calcium levels of these cells, characterizing the resting state. Specifically, we evaluated the ratio of the basal median fluorescence of calcium binding dyes in lymphocytes of the study groups. In pSS, the basal median fluorescence of calcium binding dyes was lower in both Th1 and Th2 lymphocyte subsets compared to the healthy control. However, there was no difference between the study

groups in CD8+ lymphocyte fluorescence. In SSc, there was no difference in the basal cytoplasmic calcium levels compared to healthy samples. The basal cytoplasmic calcium level was higher in the CD8+ subsets than in the CD4+ and Th1 subsets in the healthy control, but in the pSS samples, there was no difference among the lymphocyte subsets. (Fig.9.) In the SSc samples, the basal cytoplasmic calcium level was significantly higher in the Th2 cells compared to the Th1 cells. (Fig. 10.)

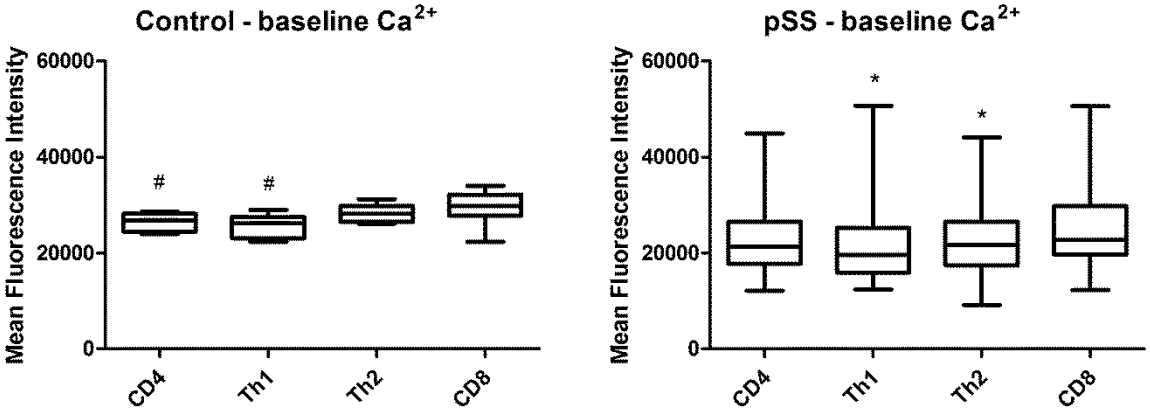


Figure 9: The basal mean fluorescence intensity of calcium binding dyes in the investigated lymphocyte subsets in pSS and the healthy samples. # $p<0.05$ vs. CD8+ healthy control lymphocytes. * $p<0.05$ vs. healthy control.

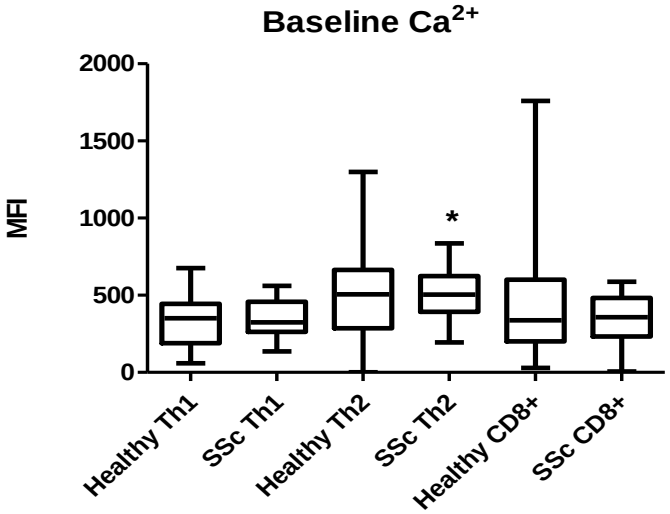


Figure 10: The basal cytoplasmic Ca^{2+} concentration in the investigated lymphocyte subsets in the two study groups. MFI – mean fluorescence intesity; SSc – systemic sclerosis. * $p < 0.05$ vs. Th1.

4.3.2. Calcium influx kinetics after lymphocyte activation

After lymphocyte activation with PHA, intracellular calcium influx kinetics were measured using calculated parameter values (AUC, Max, Tmax, and slope) in healthy subjects, and in pSS, and SSc patients. In the pSS group, we observed that AUC and Max values were lower in the CD4+ population compared to the healthy control. Furthermore, the AUC value was also lower in the Th1 population in the pSS group than in the healthy control. This alteration showed a lower level of lymphocyte activation in the pSS group in these T cell subsets. (Fig. 11.) In the case of Th2 and CD8+ lymphocytes, the calculated parameter values showed no significant difference from healthy controls.

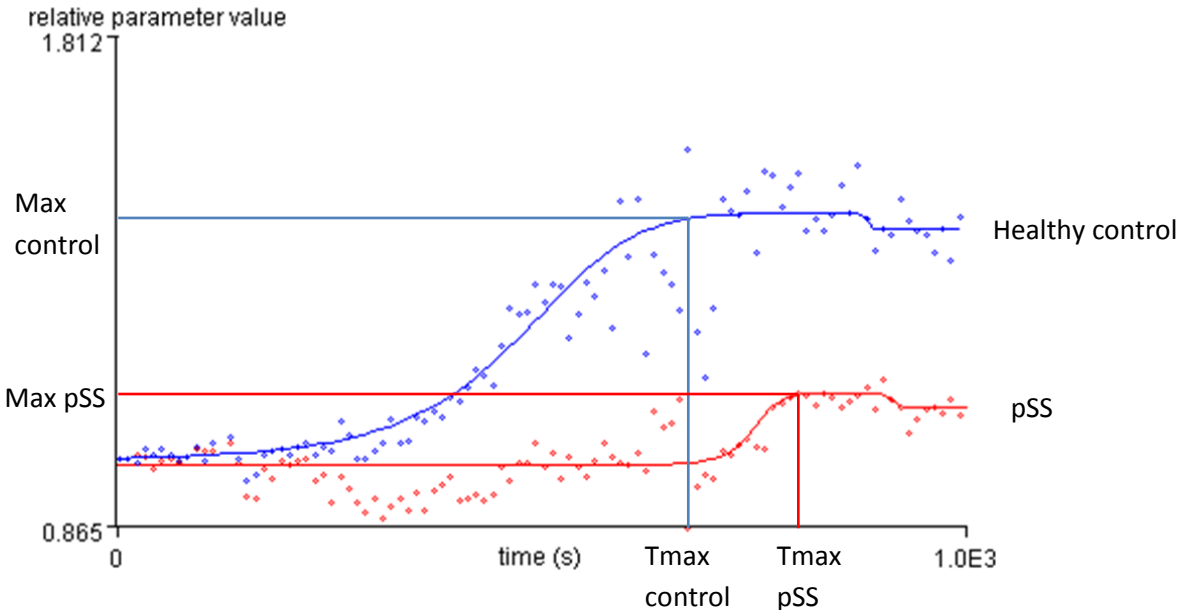


Figure 11: Calcium influx kinetics in Th1 lymphocytes of healthy individuals and pSS patients upon activation of samples. Data of a representative sample from each group. Max - maximum value, Tmax - time to reach maximum.

When studying the SSc patients, the AUC of CD8+ lymphocytes was lower in SSc compared to healthy CD8+ lymphocytes (SSc: 671.6[643.5-685.9], healthy: 707.3[672.4-784.1] in units). The same trend was observed in the case of Th1 and Th2 lymphocyte subsets in SSc, but the differences were not significant. Moreover, in the Th1 population, Tmax values were higher in SSc than in the healthy group (SSc: 748.8[602.6-888.5], healthy: 239[178.1-736.3] in seconds). This data indicates a slower Th1 activation in the SSc group compared to healthy Th1 lymphocytes. Within the SSc group, the Max value of CD8+ lymphocytes was higher compared to Th1 lymphocytes (CD8+: 1.258 [1.169-1.1.357], Th1: 1.145[1.099-1.197] in relative parameter value), therefore the CD8+ lymphocyte subset is activated mostly in the SSc group. In the healthy population, the calcium influx kinetics showed no significant difference among the investigated lymphocyte subsets (CD4+, Th1, Th2, and CD8+).

4.4. The effect of potassium channel inhibitors on lymphocyte calcium influx kinetics

We monitored the effect of potassium channel inhibitors (MGTX, TRAM) in the pSS, SSc, and healthy study groups. We observed different sensitivity to potassium channel inhibition in the different lymphocyte subsets in the healthy group. Treatment with TRAM, the specific inhibitor of the IKCa1 channel, decreased calcium influx in Th2 cells to a lower extent than in Th1 cells. In contrast to IKCa1, the inhibition of Kv1.3 channels with MGTX resulted in a larger decrease of calcium entry in Th2 than in Th1 cells. Thus, TRAM preferentially inhibited Th1 lymphocytes, while MGTX decreased Th2 lymphocyte activation to a higher extent. In healthy individuals, CD4 cells were more sensitive to the inhibition of Kv1.3 channels with MGTX, when compared to the CD8 subset. Interestingly, in the pSS group, neither of the inhibitors induced alteration in the calcium influx of lymphocytes. (Fig.12.) In the SSc samples, treatment with TRAM decreased the AUC and Max values in the Th1 cells, as was the case in the healthy population. MGTX altered the dynamics of calcium influx kinetics, decreasing the Tmax values in the Th1 and Th2 lymphocyte subsets in SSc. (Fig.13.)

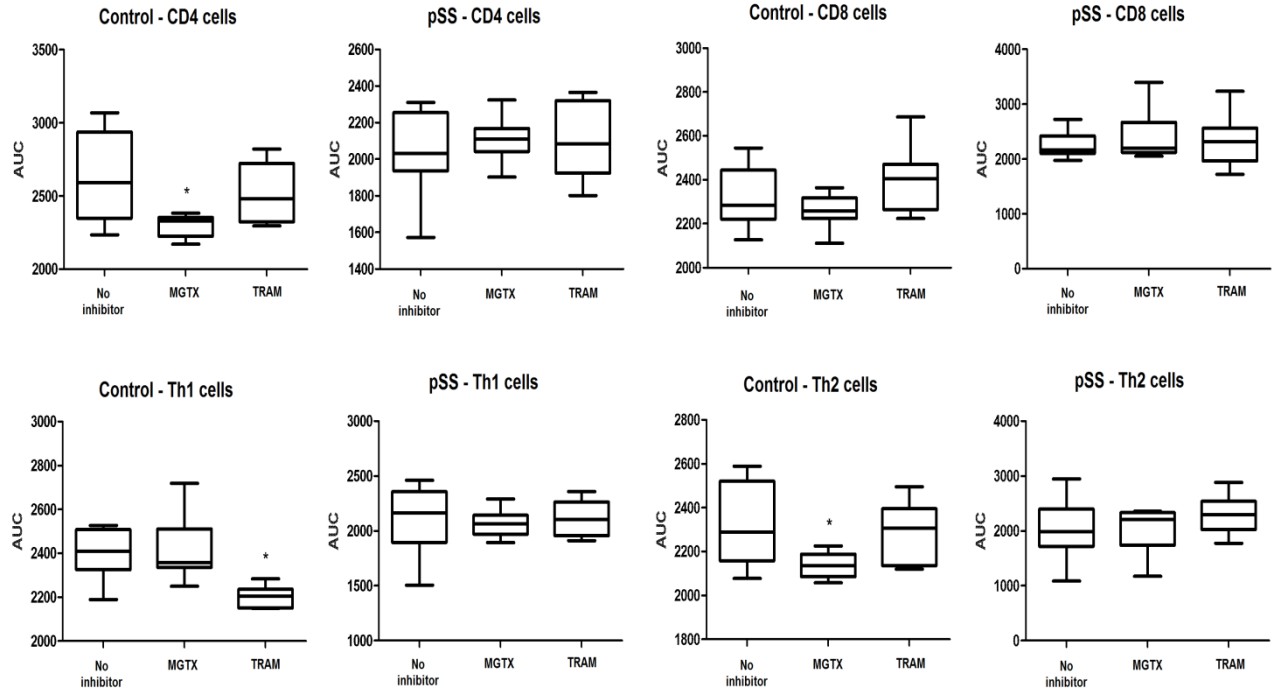


Figure 12: The effect of specific inhibitor of the Kv1.3 channel, margatoxin (MGTX) and the specific inhibitor of the IKCa1 channel, triarylmethane (TRAM) on the AUC of the intracellular calcium content elevation in different lymphocyte subgroups from healthy controls and pSS patients. AUC - area under the curve in units (U). * $p<0.05$, vs. healthy control.

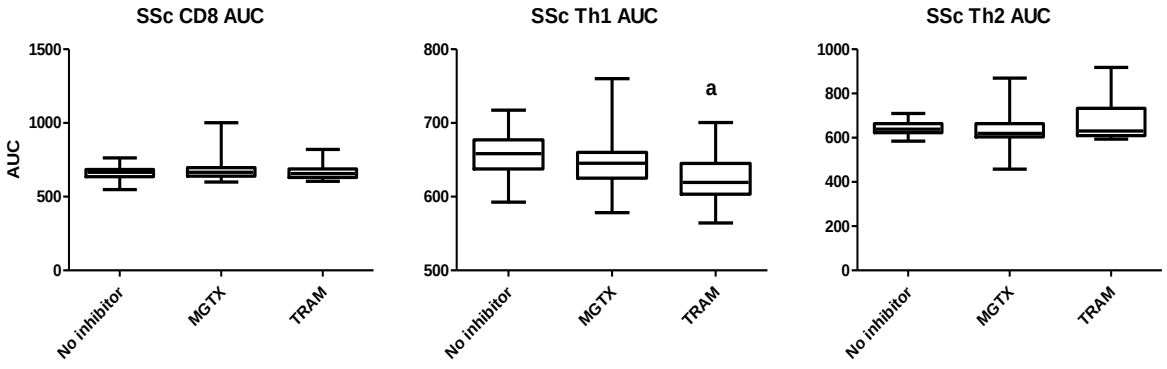


Figure 13: The effect of specific inhibitor of the Kv1.3 channel, margatoxin (MGTX) and the specific inhibitor of the IKCa1 channel, triarylmethane (TRAM) on the AUC of the

intracellular calcium content elevation in different lymphocyte subgroups from SSc patients. AUC - area under the curve in units (U). * $p<0.05$, vs. healthy control.

4.5. Kv1.3 channel cell surface expression on the investigated lymphocyte subsets

We evaluated the median fluorescence of the antibody against Kv1.3 channels on the surface of lymphocytes. Unfortunately, because of technical limitations (the lack of IKCa1 channel antibody), we could only investigate the expression of Kv1.3 channels on the surfaces of lymphocytes. We observed no significant difference in Kv1.3 channel expression among the several healthy lymphocyte subsets. In the pSS group however, Kv1.3 channel expression was significantly higher in CD4+, Th2, and CD8+ lymphocyte subsets compared with the control healthy lymphocytes and also with the Th1 cells in the pSS group. (Fig. 14.)
In the SSc group, we measured higher Kv1.3 expression on Th2 cells than on the other lymphocyte subsets. (Fig. 15.)

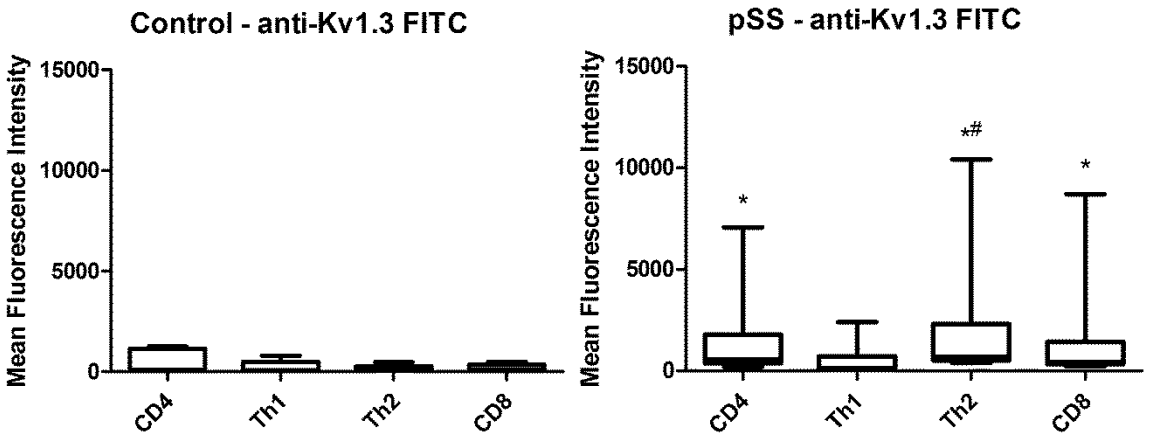


Figure 14: Kv1.3 channel expression on lymphocytes from pSS patients compared with healthy controls. *: pSS samples vs. healthy samples, $p<0.05$. #: Th2 cells from pSS samples vs. Th1 lymphocytes from pSS samples, $p<0.05$.

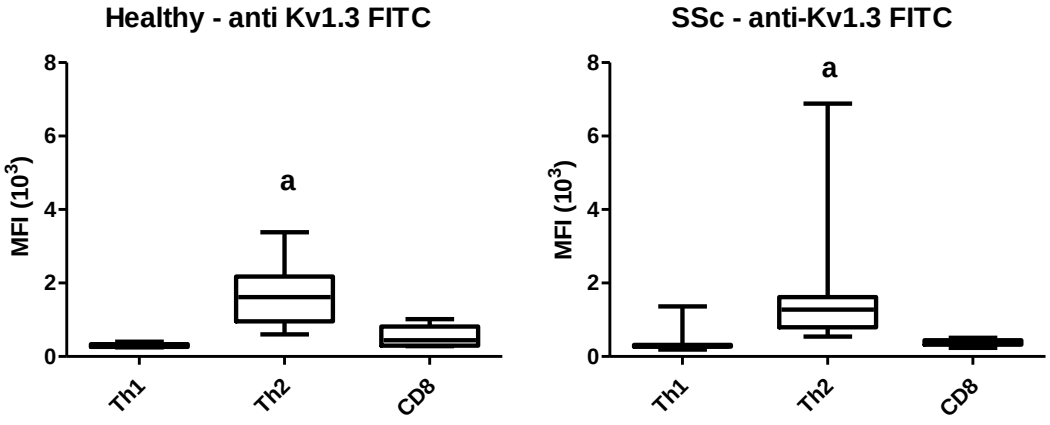


Figure 15: Kv1.3 channel expression on lymphocytes from SSc patients compared with healthy controls. MFI – mean fluorescence intesity. a: the Kv1.3 channel expression on Th2 lymphocyte subsets compared to other lymphocyte subgroups, $p<0.05$.

4.6. Plasma soluble urokinase plasminogen activator receptor level in systemic sclerosis

The investigated parameters (suPAR, CRP, ESR) showed that suPAR and ESR levels were higher in the SSc group compared to healthy controls, while the CRP level was comparable in these study groups. (Table 1.)

	Healthy controls	**SSc**
Age	55 [46-69]	51.5 [44-60]
Gender (male/female)	10/19	16/67
suPAR (ng/l)	2.8 [2.06-3.42]	4.02[a] [3.19-5.53]
CRP (mg/l)	2.7 [BLD-4.15]	3.5 [1.8-8.4]
ESR (mm/h)	10 [7-14]	18[a] [8-28]

Table 1: The demography characteristics and inflammatory markers are presented as median [interquartile range]. BLD - below the level of detection. [a] : results of SSc vs. healthy controls, $p<0.05$.

Moreover, suPAR level was higher in dcSSc than in lcSSc, and correlated with anti-Scl-70 autoantibody-positivity. When we analyzed the organ involvement of the disease, we observed that high suPAR level correlated with severe interstitial lung disease, with pulmonary fibrosis (PF), and pulmonary arterial hypertension (PHT). Furthermore, while CRP only differentiated patients with moderate forced vital capacity (FVC) from mild FVC, and ESR differentiated patients with moderate diffusing capacity for carbon monoxide (DLCO) and with PHT from controls, suPAR level also correlated with the degree of severity of interstitial pulmonary involvement. Specifically, we observed a negative correlation between suPAR levels compared to DLCO values. Besides interstitial lung disease and pulmonary arterial hypertension, arthritis and microvascular changes, like digital ulcers, Raynaud phenomenon, and nailfold capillaroscopy (NC) pattern also correlated with high suPAR levels. The NC patterns were divided into normal and abnormal pattern. Further correlation between suPAR and capillaroscopic patterns (early, active, late) was not performed due to the limited number of NC data at the time of plasma sampling. We observed no significant difference in the suPAR level depend on the skin and gastrointestinal involvement in the SSc group, but we measured higher suPAR level in these SSc groups compared to healthy controls as well. Other clinically relevant differences in ESR and CRP values in patient subgroups divided according to microvascular and pulmonary characteristics were not observed. (Table 2.)

Clinical characteristics of SSc patients	**suPAR (ng/mL)**	**CRP (mg/L)**	**ESR (mm/h)**
lcSSc (67.5 %)	3.54[a] [2.93-5.03]	3.57 [2.00-7.2]	16 [5-30]
dcSSc (32.5%)	5.38[a,b] [3.79-6.24]	2.85 [2.00-4.15]	22[a] [7-35]
Anti-Scl-70 (27.7 %)			
(+)	4.95[a,b] [3.75-5.78]	3.85 [2.0-6.2]	21[a] [6-47]
(-)	3.27 [2.83-4.59]	3.45 [1.68-5.7]	16 [7-28]
Anti-centromere (44.6 %)			
(+)	3.57[a] [3.01-5.31]	3.55 [2.00-5.7]	14 [6-23]
(-)	4.55[a] [3.03-5.48]	3.28 [1.62-5.2]	18 [4-32]
Modified Rodnan skin score			
<15 (41.3%)	3.85[a] [3.35-5.78]	2.56 [1.45-4.57]	13 [4-28]
>15 (58.7%)	3.89[a] [3.09-5.48]	3.89 [2.05-7.18]	20 [12-32]
Interstitial lung disease, pulmonary fibrosis (45.8%)			

(+)	5.43[a,b] [4.52-6.37]	2.00 [1.88-5.30]	21[a] [13-30]
(-)	3.26 [2.90-3.93]	2.00 [0.90-5.15]	16 [6-27]
FVC (data available from 39/83 of patients)			
Normal (64.1 %)	3.57[a] [2.91-5.4]	2.88 [2.00-6.1]	10 [4-23]
Mild decrease (15.4 %)	3.20 [2.40-4.18]	1.7 [0.62-2.00]	17 [9-34]
Moderate decrease (20.5 %)	5.18[a] [3.91-6.42]	5.30[b] [1.25-7.38]	13 [6-33]
DLCO (data available from 58/83 of patients)			
Normal (51.7 %)	3.10 [2.68-3.66]	2.75 [2.00-5.7]	11 [5-21]
Moderate decrease (34.5 %)	4.98[a,c] [3.6-6.39]	3.65 [2.00-6.5]	21[a] [13-30]
Severe decrease (13.8%)	6.14[a,c] [4.68-6.45]	4.15 [1.24-7.38]	9 [6-14]
Pulmonary arterial hypertension (33.7%)			
(+)	5.09[a,b] [4.42-6.24]	3.5 [1.93-6.27]	22[a] [14-27]
(-)	3.39 [2.91-5.31]	2.00 [1.4-4.40]	17 [7-23]
Digital ulcers (21.7 %)			
(+)	4.55[a] [3.21-6.01]	3.40 [1.98-6.75]	11 [5-26]
(-)	3.42 [2.96-5.03]	2.00 [1.10-4.40]	18[b] [12-30]
Raynaud phenomenon (94.0 %)			
(+)	4.22[a] [3.19-5.58]	2.00 [1.78-5.15]	17[a] [8-27]
(-)	3.79 [3.10-6.87]	1.90 [0.60-5.4]	23[a] [11-47]
Nailfold capillaroscopy pattern (data available from 21/83 of patients)			
Scleroderma pattern (76.2%)	7.86[a,b] [3.12-10.3]	2.90 [2.00-7.13]	16 [5-29]
Normal (23.8%)	3.6[a] [2.96-5.93]	2.00 [1.20-5.05]	10 [2-24]
Gastrointestinal involvement (60.2 %)			
(+)	3.85[a] [3.12-5.45]	3.85 [1.90-5.10]	18 [8-32]
(-)	4.20[a] [3.28-6.29]	3.20 [1.60-4.85]	16 [4-22]
Arthritis (9.6%)			
(+)	4.42[a] [3.21-5.89]	4.80 [2.00-8.20]	22 [8-37]
(-)	3.57 [2.96-5.10]	3.58 [2.00-5.50]	17 [6-30]

Table 2: Clinical characteristics of SSc patients are presented as median [interquartile range]. Numbers in brackets in the first column indicate the overall prevalence of the parameter in SSc patients; (+) indicates positivity, (–) means indicates negativity based on serology and the presence of clinical signs and symptoms. [a]: SSc patients were compared to healthy individuals, $p<0.05$. [b]: SSc subgroup were compared to the corresponding other SSc subgroup, $p<0.05$. [c]: other subgroup were compared to normal subgroup according to DLCO, $p<0.05$. FVC - forced vital capacity, FVC with mild restrictive dysfunction (70%<FVC<90%), FVC with moderate restrictive dysfunction (50%<FVC<69%); DLCO - diffusing capacity for carbon monoxide: mild (normal reference lab test values>DLCO>60%), moderate (40%<DLCO<60%), severe (DLCO<40%).

5. Discussion

5.1. The role of the CD28 Ig superfamily and B7 molecules in the pathomechanism of primary Sjögren's syndrome and systemic sclerosis

T cell activation is a complex process, as single antigen recognition by a TCR is not enough without a second costimulatory signal or in conjunction with an inhibitory signal for activation. Thus, during the development of an autoimmune disease, there are several checkpoints which can fail, resulting in a pathological autoimmune alteration. The presentation of autoantigens will only initiate an autoimmune response if costimulatory signals overcome the checkpoints of peripheral tolerance. The members of the CD28 Ig superfamily on T cells, in addition to B7 receptors on APCs are the most important players in the second signal mechanisms and are essential to the maintenance of the delicate balance between immune potency and suppression of the healthy immune system. The importance of these receptor families in regulating immune responses is clear from their demonstrated roles in the development of immunodeficiency, autoimmune disease, and cancer (89-94). Furthermore, as a result of research from the last several decades, we can manipulate these signal pathways using monoclonal antibodies against these molecules, therefore these CD28 and B7 molecule families show a great potential in the treatment of cancer or autoimmune diseases. The aim of our study was to investigate the role of the CD28 Ig superfamily on T lymphocytes, as well as B7 family molecules on APCs in primary Sjögren's syndrome and systemic sclerosis.

5.1.1. CD28, CTLA-4 and CD80/86 in pSS and SSc

In our study, the expression of CD28 in the T helper subset was lower in both pSS and SSc samples than in the control group. This finding indicates a decreased costimulation via CD28 in T-helper cells in these diseases. At the same time, the frequency of CTLA-4+ T helper cells was higher in pSS compared to the control healthy group. In addition, the expression of CD80 and CD86 costimulatory molecules on APCs was comparable in all three study groups. The lower level of CD28 receptors on T-helper cells in pSS and SSc and the higher level of CTLA-4+ T-helper cells in pSS suggest a protective status of costimulation between APCs

and T cells that reduces the T cell-dependent autoimmune response. One reason for this alteration could be that the enrolled patients had relatively long disease durations (pSS: 5 [2-14], SSc: 11 [1-33] years) which could lead to exhausted T cells and a hyporesponsive state. Additionally, these patients not only had long-standing autoimmune disease, but they took different immunosuppressive drugs, which could also have caused this immunosuppressed state of T helper cells in pSS and SSc. Interestingly, abatacept, a soluble CTLA-4-Ig fusion protein was tested in several other autoimmune diseases, like systemic lupus erythematosus, multiple sclerosis, and psoriasis vulgaris. In an open-label pilot study of abatacept in active pSS, patients with a recent onset of pSS and high disease activity were monitored. During the intravenous abatacept treatment, ESSDAI, ESSPRI, rheumatoid factor, and IgG levels decreased significantly, and fatigue and health-related quality of life improved significantly (95). Abatacept also induces clinical improvement in patients with severe and treatment-resistant diffuse SSc (96). Selective modulation of costimulation represents a reasonable therapeutic option, therefore a double – blinded, randomized, placebo-controlled study in these investigated autoimmune disorders would be a worthwhile undertaking.

5.1.2. ICOS and ICOSL in pSS and SSc

We have demonstrated that on T lymphocytes, the expression of ICOS, a stimulator of T cell activation, is elevated in pSS. Moreover, the tendency is similar in SSc, but the result was not significant. But its corresponding costimulatory molecule, ICOSL, was strongly decreased in SSc compared to controls. Therefore, this pathway appears to play a more dominant role in pSS. Previous data of others fall in line with our results, supporting the notion that ICOS/ICOSL receptors have an important role in the pathomechanism of pSS. ICOSL was found to be expressed not only in APCs, but also in salivary gland epithelial cells (SGECs) in pSS. These SGECs play a crucial role in the differentiation of naive CD4+ T cells into follicular helper T cells by the secretion of IL-6. Interactions between ICOSL on SGECs and ICOS on follicular helper T cells results in enhanced IL-21 secretion by follicular helper T lymphocytes, which cytokine’s augmented secretion is a hallmark of several autoimmune diseases. IL-21 might be a relevant biomarker and/or therapeutic target in primary Sjögren's syndrome (97). In another study, ICOSL expression by CD11c+ myeloid cells was associated with enhanced T cell survival in the kidneys of lupus-prone mice. Local activation of ICOS by CD11c+ myeloid cells drives organ inflammation in lupus. These results suggest an

autoantibody-independent role for ICOS in self-reactive T cell survival in target organs (98). Similarly to our findings, a greater frequency of circulating T follicular helper (Tfh) cells express ICOS in systemic lupus erythematosus (SLE) compared to healthy controls (99). Accumulating evidence suggests that ICOS is instrumental in T cell-driven multi-organ inflammation in autoimmune diseases (100). According to these findings, our results point to a potential role of ICOS in the pathomechanism of pSS, potentially by means of the maintenance of a sustained stimulation of T cells within the target organs, including the salivary gland.

5.1.3. PD-1/PD-L1 in pSS and SSc

In our results, the frequency of PD-1 expressing T lymphocytes was found to be decreased both in pSS and SSc compared with controls, while there was no difference in the PD-L1 expression of APCs between the investigated study groups. PD-1 regulates self-tolerance against many organs and prevents autoimmunity by setting at least two checkpoints that control the induction and maintenance phases of the anergic state. It is known that PD-1 or PD-L1 knockout mice develop spontaneous autoimmunity. In a mouse with a germline null-mutation of PD-1, a late-onset lupus-like syndrome developed (101). Additionally, myocarditis, pneumonitis, and type I diabetes were also observed in PD-1 or PD-L1 deficient animal models (102, 103). Therefore, the decreased frequencies of PD-1 expressing T cells we observed may reflect deficiencies in this regulatory pathway and it can promote the autoimmune processes in these two connective tissue diseases.

5.2. Indoleamine 2,3-dioxygenase

In our investigations, we found that the frequency of IDO-expressing APCs, as well as intracellular IDO content in T cells were higher in pSS than in controls. IDO is the first and also rate-limiting step in the KYN pathway, and is thought to play a key role in immune homeostasis through depletion of tryptophan and accumulation of KYN metabolites. Normally, the activation of the immune system increases the expression of IDO, which inhibits inflammation and induces immunological tolerance, to prevent an excessive inflammatory process. In support of our results, in other studies, a higher percentage of IDO expressing APCs was observed in pSS than in healthy controls (104). Furthermore, increased

KYN concentration and KYN/TRP ratio, reflecting the activity of IDO, were also measured in the peripheral blood of pSS patients (105). In contrast to the previous studies, the inhibition of IDO activity, or knockout of the gene encoding IDO, was shown to cause an increase in the severity of collagen-induced arthritis, providing an animal model of rheumatoid arthritis (106). There is abundant evidence of immune cell hyper-activation in pSS and SSc, so it is possible that the increased IDO activity tries to control the chronic autoantigen stimulation in pSS and plays a role in the counter-regulatory response.

5.3. Basal cytoplasmic calcium level and its alteration during lymphocyte activation in primary Sjögren's syndrome and systemic sclerosis

Previously, when we and our collaborator partner examined different autoimmune disorders, rheumatoid arthritis (RA), multiple sclerosis, and type I diabetes, we found similar intracellular calcium kinetics (107-109). In RA, the basal cytoplasmic calcium level was elevated in the CD4+, as well as the CD8+ lymphocyte subsets. Moreover, the CD4+ cells responded more quickly to stimulation than the healthy controls. So in this example, autoimmunity can be triggered by exuberant T cell responses. In contrast to our previous studies, we found that the basal $[Ca^{2+}]$cyt was lower in pSS compared to the control group. Furthermore, lymphocytes isolated from pSS patients responded slower to stimulation than those of healthy individuals. This is represented by higher Tmax values, i.e. calcium influx reaches its peak later upon activation, and a lower AUC compared to healthy controls. Several animal models have already demonstrated that the reduced TCR signal causes increased susceptibility to autoimmune diseases. SKG mice have a genetic defect in ZAP-70, a key signal transduction molecule in T-cells leading to T-cell mediated autoimmune arthritis, and other autoimmune disorders. This defect attenuates the TCR signal, therefore negative and positive selection of T cells is impaired in the thymus promoting self-reactive hyporesponsiveness of T-cell selection (110). Therefore, the hyporesponsive state does not exclude the development of autoimmunity. Recently, however, growing evidence has suggested that the pro-inflammatory cytokine interleukin (IL)-17 plays a pivotal role in the pathogenesis of pSS (111). Additionally, presence of different T cell subsets was found in pSS, in peripheral blood and in salivary glands. At least six Th subsets exist: Th0, Th1, Th2,

Th17, regulatory T (Treg), and follicular helper T (Tfh) cells, which are suggested to be involved in the pathogenesis of pSS. Furthermore, besides conventional CD4+ Th17 cells, another IL-17-producing T-cell subpopulation, lacking both CD4 and CD8 surface molecules (double negative), has a key role in the pathogenesis of pSS (112). Consequently, our results of the investigation of Th1 and Th2 lymphocyte subsets do not show the complete immune status in these autoimmune disorders. Additionally, presence of different T cell subsets was shown in pSS in peripheral blood and in the salivary gland. The other reason of the hyporesponsive state could be that all enrolled patients had long-standing disease with chronic immunosuppressive therapy. Moreover, at the time of blood sampling, these pSS patients had low disease activity (low ESSDAI) without any severe systemic manifestations. So these non-specific immunosuppressive drugs (chloroquine, methylprednisolone, methotrexate, azathioprine) could have caused this attenuated immune status and subsequently low disease activity. In the previous study with RA patients, we enrolled early RA patients, who had not received anti-rheumatic (anti-inflammatory, immunosuppressive) treatment, but in the case of pSS patients, this was not possible, as this is a more rare autoimmune disease and we did not have enough newly diagnosed, previously untreated pSS patients at our disposal. Notably in our results, not only the time when the peak of calcium influx was reached increased significantly, which alteration indicates that these cells are in a state of sustained reactivity due to the ongoing autoimmune reaction, but the peak of calcium influx decreased significantly as well, indicating that these cells are in an insensitive state suggesting the distinct pathomechanism of pSS.

In the SSc group, in contrast to our previous results in a recent study, we found no difference in the basal $[Ca^{2+}]$cyt between the lymphocytes from patients with systemic sclerosis and from healthy individuals. It must be noted that there was a significantly higher basal $[Ca^{2+}]$cyt in Th2 cells compared to other lymphocyte subsets in SSc, indicating an elevated activated state in these cells. Previous studies have shown that fibrosis is associated with Th2 polarized responses, characterized by the production of IL-4, IL-5, and IL-13 cytokines (113,20). Besides the importance of Th2 cells, we observed the elevated activation of CD8+ cells compared to Th1 lymphocytes in SSc seen by increased Max value. The CD8+ lymphocytes take part in not only the cell-mediated cytotoxic process, but in T helper function, as these cells produce Th2 cytokines, like IL-4 or IL-13 in this autoimmune disorder (22,23,24). In addition, depletion of CD8+ lymphocytes in peripheral blood could be an immunological

mark of SSc. Target tissue migration of CD8+ cells can cause this decreased level of CD8+ cell circulation (114). This multiplex role in the pathological inflammatory process highlights the relevance of CD8+ lymphocytes in SSc. According to our results, in SSc, the Th1 cells not only reach a lower Max value after activation, but the Tmax value is also higher compared to healthy Th1 lymphocytes, meaning slower T cell activation. The inhibitory effect of the Th2 cytokines may well be the reason for the hyporesponsivness of Th1 cells in SSc. In general, it is accepted that there is Th2 predominance during early phases of the disease, while in later phases, there is an increase in IL-12 serum levels, which promote Th1 cell differentiation, when fibrosis tends to regress (115,116).

5.4. The effects of potassium channel inhibitors on lymphocyte calcium influx in primary Sjögren's syndrome and systemic sclerosis

Taken together, our results in rheumatoid arthritis, multiple sclerosis and type I diabetes suggest that selective modulation of lymphocyte activation through specific inhibition of potassium channels may be a possible therapeutic approach for the treatment of autoimmune disease. In case of healthy individuals, treatment with TRAM decreased calcium influx in the Th1 subset in a higher extent, while treatment with MGTX resulted in a larger decrease in calcium entry in Th2 subsets. Additionally, the CD4+ lymphocytes were more sensitive to the effect of MGTX. In contrast to these results, the selective potassium channel inhibitors did not influence the calcium influx kinetics in pSS lymphocytes, as they were ineffective in all T cell subsets in pSS. The reason of this alteration could be in the distinct calcium kinetics. The selective potassium channel inhibitors could not decrease the calcium level in these hyporesponsive T cells any further. As discussed earlier, hyporesponsiveness may be due to several factors, most likely the low disease activity and the long immunosuppressive therapy could be the cause of this insensitive from the clinical aspect. On the other hand, the originally lower basic calcium level of the investigated T cells and the slower activation could partly explain this insensitive T cells status.

In the SSc group, TRAM treatment affected lymphocytes similarly as in the healthy group, decreasing the activation of Th1 cells. However, the effect of MGTX surprisingly differed in SSc, as instead of the inhibition of the lymphocyte activation, we observed quicker response

to stimulation in the Th1 and Th2 cells. Since the expression of the Kv1.3 potassium channels was the same in SSc and in healthy controls, the significantly different inhibitor effect of MGTX may raise the possibility of a difference in structure and cell surface pattern or functionality of Kv1.3 channels.

5.5. Kv1.3 channel expression on the investigated lymphocyte subsets in primary Sjögren's syndrome and systemic sclerosis

The insensitivity of peripheral T-lymphocytes in pSS to potassium channel inhibitors may be due to altered functionality or changes in the expression of Kv1.3 and IKCa1 channels. Unfortunately, due to the lack of commercially available antibodies against the IKCa1 channel, we could not perform a similar measurement in the case of IKCa1. Therefore, we analysed cell surface expression of the Kv1.3 channel. In this analysis, we observed significantly higher Kv1.3 cell surface expression on CD4+, Th2, and CD8+ lymphocytes in pSS, than on healthy controls. Similarly to this alteration, we also observed higher Kv1.3 expression on T cells in RA compared to healthy samples in a study with RA patients (105). These findings support the data of others, that in autoimmune diseases, the naive T cells often differentiate into effector memory T (T_{EM}) cells by repeated stimulation of their cognate autoantigen, and these T_{EM} cells upregulate Kv1.3 expression on their cell surfaces while the expression of IKCa1 does not change significantly (60,72). These T_{EM} cells may be Th1 and/or Th17 depending on their cytokine profile. In these autoimmune diseases, Kv1.3 channel inhibition could be more effective than the blockage of IKCa1 potassium channels. Thus, the Kv1.3 channel could be a perfect therapeutic target, without compromising the low Kv1.3 expressing naive T cells and central memory T cells, lymphocytes which have important roles in the protective immune response (117). In the case of our study, in spite of the increased Kv1.3 expressing T cells, the selective Kv1.3 blockade with MGTX was ineffective, supposedly because of the existing hyporesponsive immune state. Functional alterations of the Kv1.3 channel may also play a role in differential sensitivity upon inhibition detected between pSS patients and healthy controls.

In the study with SSc patients, we observed increased Kv1.3 channel expression in the case of Th2 lymphocytes in both study groups, SSc and healthy samples. The Kv1.3 channel

upregulation on Th2 cells lead to more K^+ ion efflux to the extracellular space and consequently larger Ca^{2+} ion influx in the cytoplasm. Accordingly, we measured elevated basal Ca^{2+} levels in the Th2 lymphocyte subset in SSc, an alteration which contributes to the augmented activation state of Th2 cell subset. It is known that fibrosis is a Th2 polarized process in SSc. Th2 cytokines, like IL-4 and IL-13, induce pro-fibrotic and pro-angiogenic IL-8 production by fibroblasts (21). Thus, the Th2 lymphocyte subset has an important role in the pathogenesis of SSc.

5.6. Elevated plasma soluble urokinase plasminogen activator receptor level in systemic sclerosis

Activation of the immune system and the development of an inflammatory response lead to elevated plasma suPAR concentration. suPAR has previously been described as a valuable indicator of the activation of the immune system in immune-related rheumatic diseases including rheumatoid arthritis, systemic lupus erythematosus, and further autoimmune diseases such as multiple sclerosis (82,83,118). We also found higher suPAR levels in SSc compared to healthy controls as the consequence of chronic inflammation and autoimmune mechanisms. While CRP was higher only in SSc patients with moderately restricted FVC, suPAR levels were significantly elevated in numerous other patient subgroups. Further analysis of patients with different stages and types of SSc also revealed that high suPAR levels within the SSc population are associated with a more severe disease manifestation that is characterized by microvascular, lung, and articular involvement. Interestingly, all microvascular changes including NC abnormalities, Raynaud phenomenon, and the presence of digital ulcers also correlated with higher suPAR level. Microvascular damage and dysfunction represent the earliest morphological and functional markers of SSc, and these complications are clinically reflected by Raynaud phenomenon and capillary morphological analysis. Raynaud phenomenon and NC alterations are available to predict the early microvascular abnormalities in SSc, but laboratory markers of this process are still not available. It was previously shown that experimental deficiency of uPAR lead to decreased angiogenic function and altered endothelial cell morphology (79,80). Besides the microvascular damage, the clinical characteristics of interstitial lung disease, including the

reduction of DLCO and FVC parameters and the presence of PF or PHT are correlated with significantly higher suPAR values than those without the respective characteristics. This may suggest that suPAR is a potential marker correlating with the severity of interstitial pulmonary involvement. Recently, uPAR –/– mice, novel animal models were described that closely mimic SSc histopathological features. Experimental deficiency of uPAR lead to decreased angiogenic function, altered endothelial cell morphology and resulted in skin and lung fibrosis (119). These results support the notion that the uPA/uPAR system plays an active role in the pathogenesis of fibrosis in SSc. On the other hand, a dysfunctioning uPA/uPAR system with extensive uPAR cleavage simultaneously lead to high peripheral suPAR levels indicating that suPAR may be a marker of this process (80,120). Moreover, the arthritis subgroup also showed higher suPAR levels than the non-arthritis subgroup or healthy controls, in accordance with our previous results supporting suPAR as a sensitive inflammatory marker as well (82). In this context, suPAR is not only a marker of the activated immune system in SSc, but it potentially also could have a functional role in the pathomechanism of the disease. Therefore, the higher suPAR level as a "decoy" receptor of uPA might decrease the function of the uPA/uPAR system in endothelial cells which could lead to microvascular abnormalities and impaired fibrosis. In practice, suPAR has further benefits compared to traditional markers such as CRP. In contrast to CRP, suPAR levels are biologically and chemically stable; they are not affected by circadian rhythm or fasting state, and are largely insensitive for preanalytical procedures such as freeze-thaw procedures (121).

6. Summary: conclusions and potential significance

- The lower level of CD28 receptors on T-helper cells in pSS and SSc and the higher level of CTLA-4 receptors on T-helper cells in pSS suggest that this costimulatory pathway is enervated.
- The increased ICOS expression on T lymphocytes in pSS may be connected to the previously observed salivary gland epithelial cell ICOSL expression and subsequently with an enhanced tendency to the differentiation of the follicular Th lymphocytes.
- Decreased PD-1 expression may promote the enhancement of autoimmune processes in both investigated connective tissue diseases (pSS, SSc).
- In accordance with the data of others, we found that the frequency of IDO-expressing APCs, as well as intracellular IDO content in T cells were higher in pSS than in healthy controls. This alteration may be an attempt to control the chronic autoantigen stimulation in pSS and could play a role in the counter-regulatory response.
- We observed not only lower basal intracellular calcium levels in Th1 and Th2 lymphocytes in pSS compared to healthy samples before activation, but the calcium influx was also lower in CD4+ cells than in healthy samples following activation as well.
- In the SSc group, the basal intracellular calcium level was comparable with healthy controls, but in the Th2 lymphocyte subset, we measured increased basal $[Ca^{2+}]_{cyt}$ compared to other lymphocyte subsets in SSc.
- We also observed decreased calcium influx in SSc compared to healthy controls. Within the SSc group, the Max value of CD8+ lymphocytes was the highest following lymphocyte activation.
- The potassium channel inhibitors (MGTX, TRAM) had no effect on the hyporesponsive pSS lymphocytes.
- In the SSc group, TRAM decreased the calcium influx in the Th1 subset, similarly to the healthy group. But as an effect of MGTX, the Tmax value decreased in Th1 and Th2 subsets, thus the lymphocyte activation became faster.
- We measured higher Kv1.3 expression on CD4+, Th2, and CD8+ lymphocytes in pSS compared to healthy controls.

- In the SSc group, there was higher Kv1.3 channel expression on Th2 cells compared to other lymphocytes subgroups in SSc, but there was no different from healthy controls.
- We measured elevated suPAR level in the SSc group compared to healthy controls, moreover suPAR level was higher in dcSSc than in lcSSc and correlated with anti-Scl-70 autoantibody.
- SuPAR level correlated with the presence and severity of interstitial lung disesase, furthermore with the presence of microvascular changes (e. g. Raynaud phenomenon, digital ulcer, abnormal nailfold capillaroscopy pattern), pulmonary arterial hypertension, and arthritis.

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I.

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Increased plasma soluble urokinase plasminogen activator receptor levels in systemic sclerosis: possible association with microvascular abnormalities and extent of fibrosis

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Abstract

Background: Urokinase plasminogen activator receptor (uPAR) is a key component of the fibrinolytic system involved in extracellular matrix remodeling and angiogenesis. Novel animal models supported the key role of uPAR not only in fibrosis but also in systemic sclerosis (SSc)-related microvascular abnormalities. The aim of this study was to investigate plasma soluble uPAR (suPAR) levels in SSc, and their association with organ-specific involvement.

Methods: suPAR concentrations were measured by ELISA in SSc patient (n=83) and in healthy controls (n=29). Simultaneously, CRP and ESR were assessed. Detailed clinical data including skin, lung, heart and microvascular characteristics were evaluated at sampling.

Results: suPAR values were higher in SSc patients than in controls. Subgroup analysis showed higher suPAR values in diffuse cutaneous- than in limited cutaneous SSc and correlated with anti-Scl-70+. suPAR levels also associated with pulmonary function test parameters of fibrosis, presence of microvascular lesions (e.g., Raynaud phenomenon, naifold capillaroscopic abnormalities and digital ulcers) and arthritis.

Conclusions: Our data indicate that suPAR might be a valuable early diagnostic marker of SSc which also correlates with disease severity.

Keywords: fibrosis; microvascular abnormalities; pulmonary fibrosis; soluble urokinase plasminogen activator receptor; systemic sclerosis.

Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disorder characterized by microvascular injury, fibrosis and autoimmunity that affects the skin and internal organs [1]. To date, there is still neither an SSc-specific treatment nor systemic markers that support the follow-up of organ-specific disease activity. Currently, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels are used to assess SSc disease activity, severity, and predict survival [2]. Biomarkers of endothelial dysfunction and impaired angiogenesis have been also suggested in SSc. These include ADAMTS-13 enzyme, endothelin-1, endothelial growth factor receptor-1 and -7, and matrix metalloproteinase-12 [3, 4].

The urokinase plasminogen activator receptor (uPAR) plays an important role in the regulation of degradation of the extracellular matrix (ECM) [5]. Indeed, uPAR is a central mediator of growth factor-induced endothelial cell migration, and experimental deficiency of uPAR leads to decreased angiogenic function and altered endothelial cell morphology [6]. Abnormal uPAR cleavage and loss of function of the uPA/uPAR system in endothelial cells

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are implicated in SSc-related microvascular abnormalities and impaired angiogenesis [7].

Evidence also suggests that uPAR gene deficiency may be involved in the pathogenesis and progression of dermal fibrosis in mice [8]. Additionally, the cleavage/inactivation of uPAR was shown to be a crucial step in the transdifferentiation of fibroblasts into myofibroblasts, which are mainly responsible for the excessive ECM production and deposition in SSc [9]. Soluble uPAR (suPAR) has also been described as a valuable

Table 1: Clinical and demographic characteristics of the 83 patients with SSc used for collection of plasma samples and for healthy controls.

Characteristics	SSc patients n=83	Healthy controls n=29
Age, years	51.5 [44–60]	55 [46–69]
Genders male/female	16/67	6/23
suPAR, ng/mL	4.02[a] [3.19–5.53]	2.80 [2.06–3.42]
CRP, mg/L	3.50 [1.80–8.40]	2.70 [BLD–4.15]
ESR, mm/h (data available from 47/83 of patients)	18[a] [8–28]	10 [7–14]
Disease duration, years,	5 [2–10.5]	–
Disease subset,		–
lcSSc	56 (67.5)	
dcSSc	27 (32.5)	
Autoantibody positivity,		
ANA	70 (84.3)	–
Anti-Scl-70	23 (27.7)	–
ACA	37 (44.6)	–
Presence of digital ulcers	18 (21.7)	–
Raynaud phenomenon	78 (94.0)	–
Nailfold capillaroscopy pattern, (data available from 21/83 of patients)		–
Scleroderma pattern	16 (76.2)	
Normal	5 (23.8)	
Skin score[b]	10 [5–14.5]	–
Interstitial lung disease		–
Pulmonary fibrosis[c]	38 (45.8)	
FVC[d] (data available from 39/83 of patients)		
Normal	25 (64.1)	
Mild	6 (15.4)	
Moderate	8 (20.5)	
DLCO[e] (data available from 58/83 of patients)		
Normal	30 (51.7)	
Moderate	20 (34.5)	
Severe	8 (13.8)	
Pulmonary arterial hypertension[f]	28 (33.7)	–
Gastrointestinal involvement	50 (60.2)	–
Arthritis	8 (9.6)	–

Data are expressed as median [interquartile range]. [a]$p<0.05$ vs. healthy controls. Dichotomical and continuous variable are presented as n, (%) of subjects and median [interquartile range], respectively. ACA, anticentromere antibodies; ANA, antinuclear antibodies; Anti-Scl-70, anti-Scl-70 antibodies; BLD, below the limit of detection; CRP, C-reactive protein; dcSSc, diffuse cutaneous SSc; ESR, erythrocyte sedimentation rate; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis; suPAR, soluble urokinase plasminogen activator receptor; [b]modified Rodnan skin thickness score; [c]determined by high-resolution computer tomography; [d]FVC, forced vital capacity, FVC with mild restrictive dysfunction (70%<FVC<90%), FVC with moderate restrictive dysfunction (50%<FVC<69%); [e]DLCO, diffusing capacity for carbon monoxide: mild (normal reference lab test values>DLCO>60%), moderate (40%<DLCO<60%), severe (DLCO<40%); [f]determined by right heart catheterization.

indicator of the activation state of the immune system and inflammation [10].

On this basis, we hypothesized that plasma suPAR level might be a potential biomarker to assess organ-specific involvement of SSc and to characterize SSc subtypes.

Materials and methods

Eighty-three patients with SSc fulfilled the criteria proposed by 2013 ACR/EULAR classification criteria for scleroderma [11] (67 women and 16 men; median age 51.5 years, range 44–60, and median disease duration 5 years, range 2–10.5) were enrolled. Patients were further classified as those with limited cutaneous SSc (lcSSc; n=56) or diffuse cutaneous SSc (dcSSc; n=27) according to LeRoy et al. [12]. Clinical and laboratory data (including routinely assessed CRP levels in all, ESR values in 47, and nailfold capillaroscopy (NC) in 21 SSc patients) were recorded at the time of sampling of 2 mL EDTA anticoagulated blood (for details see Table 1). As controls, 29 age-, and gender-matched healthy blood donors served who had no current or prior signs and symptoms suggesting rheumatic disease. This study was approved by an independent Ethical Committee of the institution. Written informed consent was obtained from all subjects. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Laboratory procedures

Plasma was isolated and stored at −80 °C until measurement. suPAR concentrations were measured with the suPARnostic Flex ELISA assay (ViroGates A/S, Birkerød, Denmark).

Statistical analysis

Data are expressed as median [interquartile range] due to non-normal distribution of data. CRP values below the level of detection (1 mg/L) were regarded as 1 mg/L. Controls and SSc patients, controls and SSc patient subgroups were compared with Mann-Whitney U and Kruskal-Wallis tests, respectively.

Results

While CRP levels were comparable, suPAR and ESR values were higher in SSc patients than in controls (Table 1). Tables 2 and 3 and Figure 1 show the association of suPAR levels with the clinical characteristics in SSc patients. suPAR values were higher in dcSSc than in lcSSc and correlated with anti-Scl70+ (Figure 1A). Interstitial lung disease assessed by diffusing capacity for carbon monoxide (DLCO) and forced vital capacity (FVC) was more severe in patients with high suPAR (Figure 1B). SSc patients with pulmonary fibrosis (PF) and pulmonary arterial hypertension (PHT) also exhibited higher suPAR levels than those without PF or PHT (Figure 1B). Microvascular changes including the presence of digital ulcers (21.7%), Raynaud phenomenon (94.0%) and NC scleroderma pattern (76.2%) and arthritis (9.6%) were also more prevalent with high suPAR values (Figure 1C and Table 2). Figure 1D and E indicate CRP and ESR values in SSc subgroups. CRP and ESR differentiated just patients with moderate from those with mild FVC (Figure 1D) and patients with moderate DLCO and with PHT from controls (Figure 1E), respectively. Further clinically relevant differences in ESR and CRP values in patient subgroups according to microvascular and pulmonary characteristics were not observed (Table 3).

Table 2: Correlation of suPAR levels with clinical characteristics of patients with SSc.

Clinical characteristics of SSc patients	suPAR, ng/mL
dcSSc (32.5)	5.38[a,b] [3.79–6.24]
lcSSc (67.5)	3.54[a] [2.93–5.03]
Anti-Scl-70 (27.7)	
(+)	4.95[a] [3.75–5.78]
(–)	3.27[b] [2.83–4.59]
ACA (44.6)	
(+)	3.57[a] [3.01–5.31]
(–)	4.55[a] [3.03–5.48]
Skin score[c] 10 [5–14.5]	
≤35 (41.3)	3.85[a] [3.35–5.78]
>35 (58.7)	3.89[a] [3.09–5.48]
Gastrointestinal involvement (60.2)	
(+)	3.85[a] [3.12–5.46]
(–)	4.20[a] [3.28–6.29]
Arthritis (9.6)	
(+)	4.42[a] [3.21–5.89]
(–)	3.57 [2.96–5.10]
SSc patients according to CRP values	
CRP ≤5 (59.0)	3.67[a] [2.90–5.61
CRP >5 (41.0)	4.62[a] [3.44–5.65]
SSc patients according to ESR values (data available from 47/83 of patients)	
≤20 (55.32)	3.02 [2.65–3.55]
>20 (44.68)	4.60[a,b] [3.60–6.12]

Dichotomical and continuous variable are presented as [n, (%)] of subjects and median [interquartile range], respectively; (+) indicates positivity; (–) means indicates negativity based on serology and the presence of clinical signs and symptoms. [a]$p<0.05$ vs. healthy individuals; [b]$p<0.05$ vs. corresponding other subgroup; c modified Rodnan skin thickness score. ACA, anticentromere antibodies; ANA, antinuclear antibodies; Anti-Scl-70, anti-Scl-70 antibodies; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis.

Table 3: Correlation of suPAR levels with microvascular and pulmonary characteristics of patients with SSc.

Clinical characteristics of SSc patients	suPAR, ng/mL	CRP, mg/L	ESR, mm/h
Digital ulcers (21.7)			
(+)	4.55[a] [3.21–6.01]	3.40 [1.98–6.75]	11 [5–26]
(–)	3.42 [2.96–5.03]	2.00 [1.10–4.40]	18[a] [12–30]
Raynaud phenomenon (94.0)			
(+)	4.22[a] [3.19–5.58]	2.00 [1.78–5.15]	17[a] [8–27]
(–)	3.79 [3.10–6.87]	1.90 [0.60–5.40]	23 [11–47]
Nailfold capillaroscopy pattern (data available from 21/83 of patients)			
Scleroderma pattern (76.2)	7.86[a,b] [3.12–10.3]	2.90 [2.00–7.13]	16 [5–29]
Normal (23.8)	3.60[a] [2.96–5.93]	2.00 [1.20–5.05]	10 [2–24]
Interstitial lung disease pulmonary fibrosis[d] (45.8)			
(+)	5.43[a] [4.52–6.37]	2.00 [1.88–5.30]	21[a] [13–30]
(–)	3.26[b] [2.90–3.93]	2.00 [0.90–5.15]	16 [6–27]
FVC[e] (data available from 39/83 of patients)			
Normal (64.1)	3.57[a] [2.91–5.40]	2.00 [2.00–6.10]	10 [4–23]
Mild (15.4)	3.20 [2.40–4.18]	1.70 [0.62–2.00]	17 [9–34]
Moderate (20.5)	5.18[a] [3.91–6.42]	5.30[b] [1.25–7.38]	13 [6–33]
DLCO[f] (data available from 58/83 of patients)			
Normal (51.7)	3.10 [2.68–3.66]	2.00 [2.00–5.70]	11 [5–21]
Moderate (34.5)	4.98[a,c] [3.60–6.39]	3.65 [2.00–6.50]	21[a] [13–30]
Severe (13.8)	6.14[a,c] [4.68–6.45]	4.15 [1.24–7.38]	9 [6–14]
Pulmonary arterial hypertension[g] (33.7)			
(+)	5.09[a] [4.42–6.24]	3.50 [1.93–6.28]	22[a] [14–27]
(–)	3.39[a,b] [2.91–5.31]	2.00 [1.40–4.40]	17 [7–23]

Dichotomical and continuous variable are presented as [n, (%)] of subjects and median [interquartile range], respectively; (+) indicates positivity; (–) means indicates negativity based on serology and the presence of clinical signs and symptoms; [a]$p<0.05$ vs. healthy individuals, [b]$p<0.05$ vs. corresponding other subgroup, [c]$p<0.05$ vs. Normal subgroup according to DLCO; [d]determined by high-resolution computer tomography; [e]FVC, forced vital capacity, FVC with mild restrictive dysfunction (70%<FVC<90%), FVC with moderate restrictive dysfunction (50%<FVC<69%); [f]DLCO, diffusing capacity for carbon monoxide: mild (normal reference lab test values>DLCO>60%), moderate (40%<DLCO<60%), severe (DLCO<40%), [g]determined by right heart catheterization.

Discussion

Vascular complications are considered as the earliest clinical manifestations of SSc. Vascular remodeling and fibrosis were suggested as crucial events in the pathogenesis of SSc. Microvascular damage and dysfunction represent the earliest morphological and functional markers of SSc, and these complications are clinically reflected by Raynaud phenomenon and capillary morphological analysis. However, laboratory markers of this process are still not available.

Recently, the uPAR$^{-/-}$ mice, a novel animal model was described that closely mimicked SSc histopathological features. Experimental deficiency of uPAR lead to decreased angiogenic function, altered endothelial cell morphology and resulted in skin and lung fibrosis [13]. Dysfunctioning uPA/uPAR system with extensive uPAR cleavage lead to high peripheral suPAR levels at the same time raising the notion that suPAR may be a marker of this process. Additionally, a recent study also found possible associations of uPAR gene variation with vascular complications of SSc [14].

Activation of the immune system and the development of an inflammatory response lead to elevated plasma suPAR concentrations. suPAR was already described as a valuable indicator of the activation of the immune system in immune-related rheumatic diseases including rheumatoid arthritis, systemic lupus erythematosus, and further autoimmune diseases such as multiple sclerosis [10, 15]. We also found higher suPAR levels in SSc as the consequence of the chronic inflammation and autoimmune mechanisms. However, the higher suPAR level as a "decoy" receptor of uPA might decrease the function of the uPA/uPAR system in endothelial cells which could lead to microvascular abnormalities and impaired fibrosis. In this content suPAR is not only the marker of the activated immune system in SSc but also it has some potential functional role in the patomechanism of the disease.

suPAR has further benefits compared to traditional markers such as CRP. In contrast to CRP, suPAR levels are biologically and chemically stable; they are not affected by circadian rhythm, fasting state and are largely insensitive for preanalytical procedures such as freeze-thaw procedures [16]. We observed higher than normal plasma

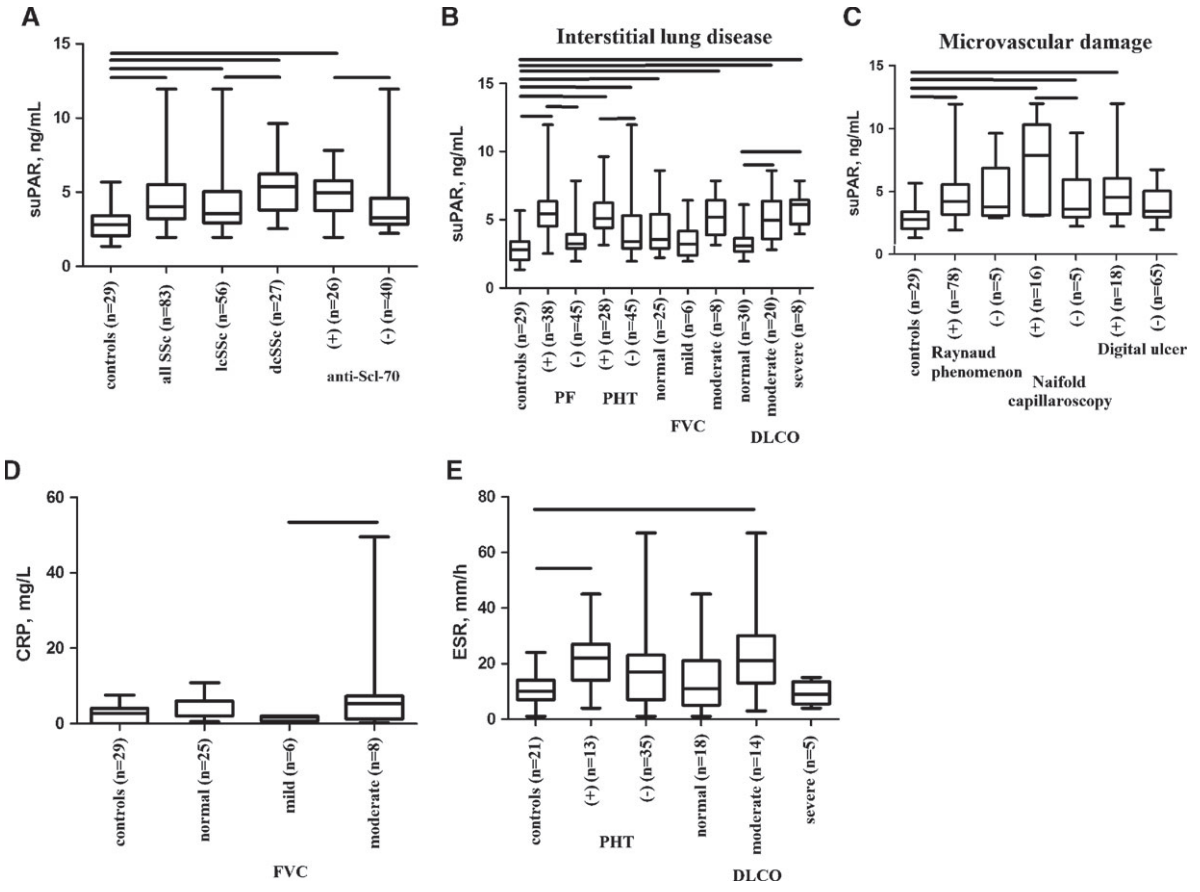

Figure 1: Correlation of suPAR, CRP and ESR levels with clinical characteristics of patients with SSc.
(+) Indicates positivity; (–) indicates negativity based on serology and the presence of clinical signs and symptoms; Number of patients is presented as (n=). Data are shown as box plots. Each box represents the 25th–75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. Lines above the boxes indicate the significant difference between the two groups, $p<0.05$. ANA, antinuclear antibodies; Anti-Scl-70, anti-Scl-70 antibodies; CRP, C-reactive protein; dcSSc, diffuse cutaneous SSc; ESR, erythrocyte sedimentation rate; lcSSc, limited cutaneous SSc; PF, pulmonary fibrosis determined by high-resolution computer tomography; PHT, pulmonary hypertension determined by right heart catheterization; SSc, systemic sclerosis. FVC, forced vital capacity, FVC with mild restrictive dysfunction (70%<FVC<90%), FVC with moderate restrictive dysfunction (50%<FVC<69%); DLCO, diffusing capacity for carbon monoxide: mild (normal reference lab test values>DLCO>60%), moderate (40%<DLCO<60%), severe (DLCO<40%).

suPAR levels in SSc which corresponds to previous results [17]. While CRP was higher only in SSc patients with moderately restricted FVC, suPAR levels were significantly elevated in numerous other patient subgroups. Further analysis of patients with different stage and type of SSc also revealed that high suPAR levels within SSc population are associated with a more severe disease that is characterized by microvascular, skin and lung involvement.

We demonstrated that all the investigated parameters concerning to microvascular changes including NC abnormalities, Raynaud phenomenon and the presence of digital ulcers also correlated with higher suPAR levels than those without the respective characteristics. The NC capillaroscopic patterns were divided into normal and abnormal pattern. Further correlation between suPAR and capillaroscopic patterns (early, active, late) was not performed due to the limited number of NC data at the time of plasma sampling.

The clinical characteristics of interstitial lung disease including the reduction of DLCO and FVC parameters and the presence of PF or PHT are correlated with significantly higher suPAR values than those without the respective characteristics. It may suggest that suPAR is a potential marker correlating with the severity of interstitial pulmonary involvement.

The arthritis subgroup of SSc also showed higher suPAR levels than the non-arthritis subgroup or healthy controls in accordance with our previous results which supports that suPAR is a sensitive inflammatory marker as well [10].

Our results are the first data that evaluation of plasma suPAR concentrations could provide information about

SSc severity including vascular involvement, skin and lung fibrosis. This finding is of particular importance as so far just subjective complaints and some clinical signs and symptoms such as Raynaud phenomenon and NC alterations are available to predict the early microvascular abnormalities in SSc. Reduced capillary density on NC correlates with a high risk of developing digital skin ulcers and the presence of pulmonary arterial hypertension, and can therefore be used as a marker of SSc severity [18]. Correlation between suPAR and these clinical characteristics may suggest that suPAR may be the first laboratory marker that indicates microvascular abnormalities and ongoing fibrosis.

Albeit these results are quite promising, several issues should be clarified before the routine use of suPAR in patient monitoring could be recommended. First, the number of patients in our cohort is still low, therefore, increasing the number of observations would provide exact values to discriminate between different SSc subtypes. Second, suPAR was demonstrated to be sensitive in a number of immune mediated disorders and it is less clear whether it was suitable to distinguish between different diagnoses. Third, follow-up studies with repeated measurements of suPAR with repeated assessment of patients' condition would add invaluable information regarding the usefulness of this potential novel biomarker in SSc.

In conclusion, our preliminary data indicate that suPAR may be a candidate biomarker in SSc that would reflect early diagnosis, disease severity.

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Calcium influx kinetics, and the features of potassium channels of peripheral lymphocytes in primary Sjögren's syndrome

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ABSTRACT

Objective: The transient increase of the cytoplasmic free calcium level plays a key role in the process of lymphocyte activation. Kv1.3 and IKCa1 potassium channels are important regulators of the maintenance of calcium influx and present a possible target for selective immunomodulation.
Design: Case–control study.
Subjects and methods: We took peripheral blood samples from 8 healthy individuals and 15 primary Sjögren's syndrome (pSS) patients. We evaluated calcium influx kinetics following activation in peripheral T lymphocytes. We also assessed the sensitivity of T lymphocytes to specific inhibition of the Kv1.3 and IKCa1 potassium channels, and the Kv1.3 channel expression.
Results: The basal cytoplasmatic calcium levels were lower in both Th1 and Th2 lymphocytes in pSS compared to controls. The peak of calcium influx in lymphocytes isolated from pSS patients is reached later, indicating that they respond more slowly to stimulation compared to controls. In healthy individuals, the inhibition of the IKCa1 channel decreased calcium influx in Th2 and CD4 cells to a lower extent than in Th1 and CD8 cells. On the contrary, the inhibition of Kv1.3 channels resulted in a larger decrease of calcium entry in Th2 and CD4 than in Th1 and CD8 cells. In the pSS group, neither of the inhibitors induced alteration in calcium influx. Expression of Kv1.3 channels on CD4, Th2 and CD8 lymphocytes in pSS was significantly higher compared to controls.
Conclusion: The altered expression and specific inhibition of potassium channels seem to be related to altered calcium influx kinetics in pSS which distinguish pSS either from healthy controls or other systemic autoimmune diseases.

1. Introduction

Pathways of calcium homeostasis participate in a number of cellular processes that determine short and long-term function of T lymphocytes. The increase of the cytoplasmic calcium concentration from intra- and extracellular sources (i.e., the endoplasmic reticulum and store-operated calcium entry through the plasma membrane) is the cornerstone of T lymphocyte activation and functionality. Over the recent year, an increasing number of calcium channels and transporters have been described that play a key role in balancing cytoplasmic calcium levels in T cells. (Toldi, 2013; Feske, 2013).

In the course of lymphocyte activation, potassium channels maintain the driving force for sustained calcium influx from the extracellular milieu as they grant the efflux of potassium from the cytoplasm, thus conserving an electrochemical potential gradient between the intra- and extracellular spaces. There are two major types of potassium channels in T cells: the voltage gated Kv1.3 and the calcium-activated IKCa1 channels (Orbán et al., 2013). The relation between the calcium currents through calcium release activated calcium (CRAC) channels and the efflux of potassium makes the proliferation and activation of lymphocytes sensitive to pharmacological modulation of Kv1.3 and IKCa1 channels, and

Abbreviations: AUC, area under the curve; $[Ca^{2+}]_{cyt}$, cytoplasmic free calcium level; CRAC, calcium release activated calcium; Max, maximum value; MGTX, margatoxin; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; TCM, central memory T cell; TEM, effector memory T cell; Th, T helper; t_{max}, time to reach maximum value; TRAM, triarylmethane.

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provides an opportunity for targeted intervention. Specific inhibition of these channels results in a diminished calcium influx in lymphocytes and a lower level of lymphocyte activation. Previous data suggest that selective modulation of lymphocyte activation through specific inhibition of potassium channels may be a possible therapeutic approach for the treatment of autoimmune disease (Beeton et al., 2006; Rangaraju et al., 2009).

Furthermore, a different characteristic potassium channel phenotype of effector memory T cells was described in multiple sclerosis (MS): terminally differentiated effector memory T (TEM) cells exhibit Kv1.3high IKCa1low channel phenotype, contrasting naïve, and central memory T cells, which exhibit a Kv1.3low IKCa1high channel phenotype (Wulff et al., 2003).

Although results from animal models are promising, limited data is available on the effects of potassium channel inhibition on T cell function in humans. Furthermore, besides naïve and memory T cells, alterations in the activation pattern of effector (CD4+ helper and CD8+ cytotoxic) T lymphocytes have not been described upon Kv1.3 and IKCa1 inhibition. Although these cells might have a less-specific role in the maintenance of autoreactivity compared to TEM cells, their inhibition have important consequences on the overall immune response.

Therefore, over the recent years, we have investigated calcium influx characteristics in effector T cell subsets in a number of autoimmune diseases (Toldi et al., 2010, 2011, 2013, 2015).

In this study we focused on primary Sjögren's syndrome (pSS). pSS is a chronic autoimmune inflammatory disorder that primarily affects exocrine glands leading to their functional impairment. Although pSS etiology is not fully elucidated, it is well established that the interplay between genetic, environmental and hormonal factors represents the trigger of aberrant autoimmune response with B and T lymphocyte hyperactivity, autoantibody production and progressive destruction of target organs. The pathological hallmark of pSS is a chronic mononuclear cell infiltrate affecting exocrine glands mainly driven by T helper (Th) 1-type cytokines. The innate and adaptive immune responses both play an important role in this pathological process. Growing evidence suggests that CD4+ Th cells are predominant, and the Th1/Th2 balance shifts in favor of Th1 in gland, but Th2 cytokine repertoire prevails in sera (Tzioufas et al., 2012).

Therefore, in this study we aimed to characterize the effects of lymphocyte potassium channel inhibition on short-term peripheral blood T lymphocyte activation in major lymphocyte subsets of patients diagnosed with pSS. We employed a kinetic flow cytometry method to describe calcium influx characteristics of the CD4, Th1, Th2 and CD8 subsets and its sensitivity to the inhibition of Kv1.3 and IKCa1 lymphocyte potassium channels.

2. Materials and methods

2.1. Patients

We enrolled 8 healthy individuals and 15 patients with primary Sjögren's Syndrome (pSS). pSS patients fulfilling American European Consensus group (AECG) 2002 or American College of Rheumatology (ACR) 2012 classification criteria were included (Vitali et al., 2002; Shiboski et al., 2012). Clinical parameters of study participants are summarized in Table 1. The following clinical symptoms occurred in pSS patients history: sicca parameters (100%), cytopenia and anemia of autoimmune origin (87%), arthritis (67%), vasculitis (53%), anti-SSA/B antibody positivity (100%), low C3/C4 complement level (47%), renal involvement (13%), fever and night sweats (20%), lymphadenopathy (20%), lymphoma (0%), weight loss (33%), myositis (7%), peripheral neuropathy (20%). The EULAR Sjögren's syndrome disease activity (ESSDAI) scores were calculated at the time of sampling (Seror et al., 2015). The ESSDAI score was 2 corresponding to low disease activity. All pSS patients received a variety of disease modifying antirheumatic drugs (DMARDs), containing at least one of the following components: chloroquine, methylprednisolone, methotrexate and azathioprine.

Table 1
Clinical characteristics of study participants.

Characteristics	Healthy individuals n = 8	pSS patients n = 15
Age (years)	53 (42–61)	52 (36–77)
Gender (male/female)	2/6	1/14
pSS duration (years)	–	5 (2–14)
Anti-SSA/B positivity	–	15
LSG biopsy positivity	–	12
ESSDAI		2 (0–4)
ESR (mm/h)	8 (5–12)	27* (7–55)

Data are expressed as median (interquartile range) for continous variables and as number for categorical variables. pSS, primary Sjögren's Syndrome; LSG biopsy, focal lymphocytic sialadenitis (FLS) with focus score ≥ 1 in labial salivary gland (LSG) biopsy; Anti-SSA/B anti-SSA/B autoantibodies; ESSDAI, EULAR Sjögren's syndrome disease activity; ESR, erythrocyte sedimentation rate.
* $p < 0.05$ vs. healthy individuals.

Healthy controls had a negative history of rheumatic symptoms and negative status upon detailed physical and laboratory examination. No co-morbidities were detected in patients and controls that could have influenced our investigation, nor did they take any medication that could have interfered with the measurements. Written informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the university. Laboratory studies and interpretations were performed on coded samples lacking personal and diagnostic identifiers. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

2.2. Fluorescent staining

Our measurements were carried out as described earlier (Toldi et al., 2011). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by a standard density gradient centrifugation from 9 mL of freshly drawn peripheral venous blood and afterwards kept in a modified RPMI medium (calcium concentration: 2 mM) throughout the following steps of the procedure. PBMCs were then incubated with the following conjugated anti-human monoclonal antibodies in order to differentiate T lymphocyte subsets: anti-CD4 PE-Cy7, anti-CD8 APC-Cy7, anti-CXCR3 APC (for the determination of Th1 cells) and anti-CCR4 PE (for the deter-mination of Th2 cells) (all from PharMingen, San Diego, CA, USA), as well as anti-Kv1.3 channel FITC (Sigma–Aldrich, St. Louis, MO, USA), according to the manufacturers' instructions. For monitoring [Ca2+]cyt, PBMCs were loaded with calcium sensitive Fluo-3 and Fura-Red dyes according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA).

2.3. Flow cytometry

PBMCs were equally distributed into three vials. The first vial was used as control. The second vial was treated with margatoxin (MGTX, 60 nM), a selective blocker of the Kv1.3 channel. The third vial was treated with a triarylmethane compound (TRAM, 60 nM), a specific inhibitor of the IKCa1 channel. PBMCs were activated by the addition of 20 μg phytohemagglutinin (PHA) and the measurements were initiated directly afterwards on a BD FACSAria flow cytometer. Cell fluorescence data were measured and recorded for 15 min in a kinetic manner.

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Table 2
Prevalence of lymphocyte subsets in the overall lymphocyte population gated according to Forward Scatter Characteristics (FSC) and Side Scatter Characteristics (SSC) in 8 healthy individuals and 15 pSS patients.

Subset	Healthy	pSS
CD4+/ly	37.7(32.4–44.6)	43.4(30.6–52.1)
CXCR3+/CD4	29.8(17.6–34.7)	20.4(15.7–22.9)
CCR4+/CD4	18.9(12.5–26.3)	13.1 (8.8–23.6)
CD4+ CXCR3+/CD4+ CCR4+ ratio	1.89(1.27–2.88)	1.25(0.84–2.56)
CD8+/ly	15.4(9.02–25.6)	17.7(14.6–23.3)

Data are expressed as median [interquartile range]. CD4+ CXCR3+—Th1 subset, CD4+ CCR4+—Th2 subset, ly—overall lymphocyte population, pSS—primary Sjögren's syndrome.

2.4. Data evaluation

Recordings were evaluated with our specific software (FacsKin), based on the calculation of a double-logistic function for each recording (Toldi et al., 2011). This function is used to characterize measurements that have an increasing and a decreasing intensity as time passes. The software also calculated parameter values describing each function, such as the Area Under the Curve (AUC), Maximum (Max), time to reach maximum (tmax), and Slope values. AUC values correspond to the sum of [Ca2+]cyt increase, which further corresponds to the level of lymphocyte activation. Max values represent the peak value of the calcium influx curve upon lymphocyte activation. Tmax values describe how soon the peak value of the calcium influx curve is reached. The Slope value reflects how rapidly the peak of calcium influx is reached. A detailed description of the evaluation process can be found at www.facskin.com.

Statistics Data are expressed as median and interquartile range. Comparisons between two sample groups were made with the Mann–Whitney test. For comparisons between paired values in the same group Wilcoxon tests were applied. P values less than 0.05 were considered significant. Statistics were calculated using the R software.

3. Results

3.1. Clinical data

As seen in Table 1, the age and gender distribution of participants were similar in both study groups. Inflammatory parameters (erythrocyte sedimentation rate and C reactive protein values) were higher in pSS patients than in healthy controls.

The number of patients in different subgroups according to therapeutic intervention was limited, and no significant differences in the investigated parameters were observed between the subgroups.

3.2. The frequency of the investigated lymphocyte subsets

We determined the frequency of CD4, Th1, Th2 and CD8, cells in samples of both study groups. CD4+ CXCR3+ cells were regarded as Th1 lymphocytes, while CD4+ CCR4+ cells were regarded as the Th2 subset. It was no detected significant difference regarding individual cell subsets among the study groups (Table 2).

3.3. Basal cytoplasmatic calcium levels in the investigated lymphocyte subsets

We evaluated the ratio of the basal median fluorescence of calcium binding dyes in lymphocytes of both study groups. Before the lymphocyte activation, the basal median fluorescence of calcium binding dyes was lower in both Th1 and Th2 lymphocyte subsets in the pSS group (CD4+ CXCR3: 24,494 [12,365–50,658], CD4+ CCR4+: 22768 [9110–44,026]) than in the healthy group CD4+ CXCR3: 26,151 [22,324–28,949], CD4 + CCR4: 28,187 [26,149–31,163] arbitrary units, $p < 0.05$ (median [interquartile range]) respectively (Fig. 1).

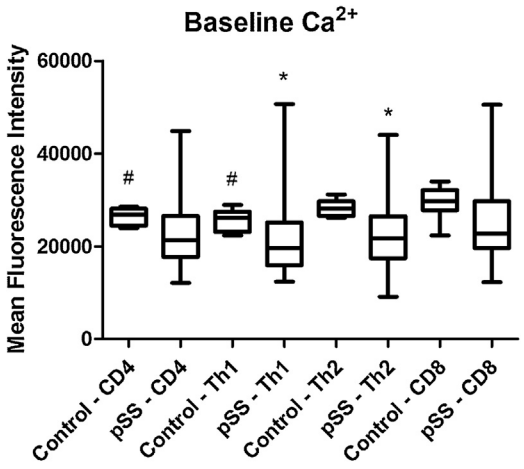

Fig. 1. The basal mean fluorescence intensity of calcium binding dyes in the investigated lymphocyte subsets in healthy individuals and primary Sjögren's syndrome patients. * $p < 0.05$ vs. control # $p < 0.05$ vs CD8.

There was no difference between the study groups in CD8+ pSS: 26,483 [12,251–50,510], healthy: 29,797 [22,307–33,994] lymphocyte fluorescence. The basal cytoplasmatic calcium level was higher in the CD8+ subsets than in the CD4+ and Th1 subsets in the control group (CD8+: 29,797 [22,307–33,994], CD4+: 26,843 [23,925–28,502], CD4 + CXCR3: 26,151 [22,324–28,949] $p < 0.05$ (median [interquartile range])), but in the pSS samples, there was no difference among the lymphocyte subsets (CD4+: 25,167 [12,126–44,988], CD4+ CXCR3: 24,494 [12,365–50,658], CD4+ CCR4: 22,768 [9110–44,026], CD8+: 26,483 [12,251–50,510] (median [interquartile range])) (Fig. 1).

3.4. Calcium influx kinetics

After lymphocyte activation with PHA, intracellular calcium influx kinetics were measured with calculated parameter values (AUC, Max, tmax, and slope) in healthy subjects and in pSS patients (Table 3). In the CD4+ population, AUC and Max values were lower in the pSS group compared to the healthy group. For Th1 lymphocytes, the AUC value was lower in pSS samples than in the control group. In the case of Th2 and CD8+ lymphocytes, the calculated parameter values showed no significant difference among the study groups (Table 3, Fig. 2).

3.5. The effects of potassium channel inhibitors on lymphocyte calcium influx

We measured the effect of potassium channel inhibitors (MGTX, TRAM) in both pSS and healthy groups. Our results revealed that the sensitivity of Th1 and Th2 cells to IKCa1 channel inhibition was different in lymphocytes isolated from healthy individuals (Table 3). Treatment with TRAM, the specific inhibitor of the IKCa1 channel decreased calcium influx in Th2 cells to a lower extent than in Th1 cells. On the contrary to IKCa1, the inhibition of Kv1.3 channels resulted in a larger decrease of calcium entry in Th2 than in Th1 cells. In healthy individuals, CD4 cells were more sensitive to the inhibition of Kv1.3 channels than the CD8 subset, responding with a higher level of decrease of the AUC value upon the application of MGTX. However, upon treatment with TRAM, CD8 cells showed a larger decrease in AUC and Max values than CD4 cells (Table 3).

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Table 3
The effects of the specific inhibitor of the Kv1.3 channel, margatoxin (MGTX) and the specific inhibitor of the IKCa1 channel, triarylmethane (TRAM) application on parameter values (AUC – Area under the curve in units (U), Max – maximum value in relative parameter value (rpv), t_{max} – time to reach maximum value in seconds (s)) of calcium influx kinetics in peripheral lymphocytes obtained from 8 healthy individuals and 15 pSS patients.

		No inhibitor		MGTX (60 nM)		TRAM (60 nM)	
Subset		Healthy	pSS	Healthy	pSS	Healthy	pSS
CD4+	AUC (U)	2591 (2349–2939)	2033[a] (1936–2255)	2328[b] (2226–2353)	2110 (2043–2168)	2483 (2325–2723)	2084 (1923–2319)
	Max (rpv)	1.46 (1.249–1.608)	1.115[a] (1.036–1.215)	1.207[b] (1.141–1.22)	1.085 (1.049–1.121)	1.303 (1.198–1.483)	1.143 (1.033–1.227)
	t_{max} (s)	631.9 (471.7–930.1)	504.3 (355.4–576.3)	913.5 (666.2–1351)	598.4 (427.2–799.3)	755.2 (564.4–972.7)	464.2 (131.6–578.4)
CD4+ CXCR3+	AUC (U)	2411 (2325–2508)	2162[a] (1896–2358)	2358 (2336–2511)	2067 (1972–2142)	2204[b] (2151–2235)	2104 (1958–2262)
	Max (rpv)	1.284 (1.203–1.335)	1.122 (1.022–1.298)	1.134[b] (1.096–1.155)	1.081 (1.053–1.166)	1.231 (1.199–1.34)	1.066 (1.024–1.198)
	t_{max} (s)	674.8 (472.7–1003)	552.8 (189.1–646.6)	901.6 (756–990)	604.1 (432.5–782.3)	872.9 (645.3–1096)	652.4 (511.2–1100)
CD4+ CCR4+	AUC (U)	2289 (2158–2521)	1984 (1718–2396)	2136[b] (2086–2189)	2204 (1737–2331)	2306 (2136–2396)	2297 (2027–2537)
	Max (rpv)	1.239 (1.099–1.361)	1.097 (1.006–1.222)	1.088 (1.057–1.12)	1.278 (1.144–1.359)	1.192 (1.09–1.249)	1.164 (1.104–1.407)
	t_{max} (s)	791.1 (626.6–1046)	615.3 (345.3–906.1)	1065 (889.2–1215)	677.5 (535.6–711.1)	1034 (885.5–1149)	900.3 (382.8–1117)
CD8+	AUC (U)	2285 (2220–2444)	2164 (2105–2423)	2258 (2224–2318)	2199 (2121–2667)	2404 (2265–2471)	2322 (1964–2566)
	Max (rpv)	1.181 (1.141–1.317)	1.213 (1.09–1.365)	1.156 (1.141–1.198)	1.174 (1.137–1.355)	1.213 (1.067–1.521)	1.257 (1.159–1.284)
	t_{max} (s)	648.1 (519.4–1014)	531.7 (436.3–682.5)	988.1 (671.6–1076)	659.5 (455.8–856.5)	606.6 (541.9–927.9)	590.2 (388.9–1001)

CD4+, CD4+ CXCR3+, CD4+ CCR4+ and CD8+ cells were gated from the overall lymphocyte population. Data are expressed as median [interquartile range]. CD4+ CXCR3+ – Th1 subset, CD4+ CCR4+ – Th2 subset, pSS – primary Sjögren's syndrome. [c] MGTX and TRAM treated samples were compared with samples with no inhibitor application within lymphocytes isolated from pSS patients, $p < 0.05$.

[a] Lymphocytes isolated from pSS patients were compared with lymphocytes isolated from healthy individuals within samples with no inhibitor application, $p < 0.05$.

[b] MGTX and TRAM treated samples were compared with samples with no inhibitor application within lymphocytes isolated from healthy individuals, $p < 0.05$.

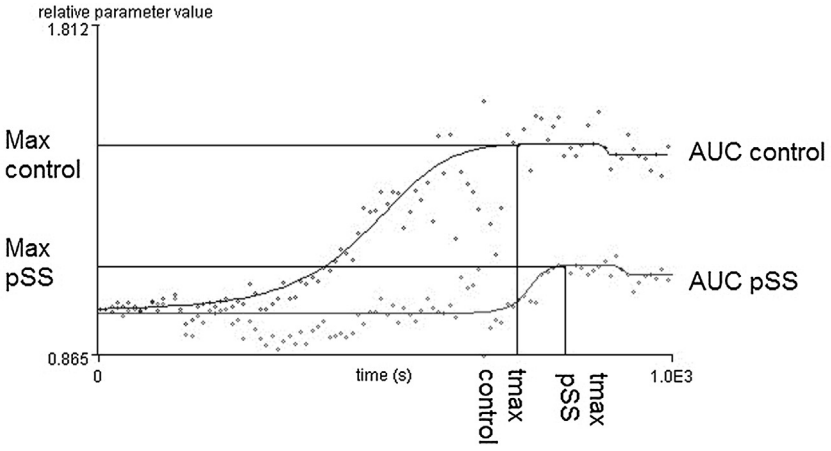

Fig. 2. Calcium influx kinetics in peripheral Th1 lymphocytes of healthy individuals and pSS patients upon activation of samples with phytohemagglutinin (data of a representative sample from each group). For Th1 lymphocytes, the AUC value was lower in pSS samples than in the control group. The basal [Ca2+]cyt was lower in pSS compared to healthy individuals. This is represented by higher tmax values, i.e. calcium influx reaches its peak later upon activation, and a lower Max compared to healthy controls.
Abbreviations: pSS—primary Sjögren syndrome, AUC—area under the curve, Max—maximum value, tmax—time to reach maximum

In the pSS group, neither of the inhibitors induced alteration in calcium influx of lymphocytes (Table 3, Fig. 4).

3.6. Kv1.3 channel expression in pSS in the investigated lymphocyte subsets

We observed a significantly higher Kv1.3 channel expression in CD4+, Th2, and CD8+ lymphocyte subsets in pSS samples than in control lymphocytes. The median fluorescence of the study groups was similar in the case of the Th1 lymphocyte subsets. There was no difference among the several control lymphocyte subsets (Fig. 3).

4. Discussion

4.1. Calcium influx kinetics during lymphocyte activation

We applied a novel flow cytometry-based approach for the detection of calcium influx. Until the recent past, single-cell techniques were used for the investigation of calcium influx during lymphocyte activation. There has been no high-throughput method

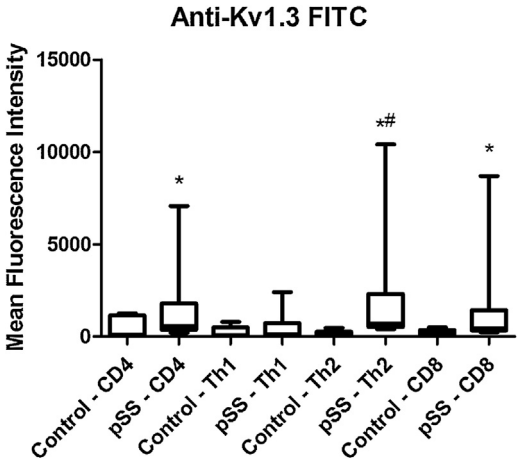

Fig. 3. Mean fluorescence intensity of the anti-Kv1.3 channel antibody in the investigated lymphocyte subsets in healthy individuals and primary Sjögren's syndrome patients. * $p < 0.05$ vs. control # $p < 0.05$ vs Th1.

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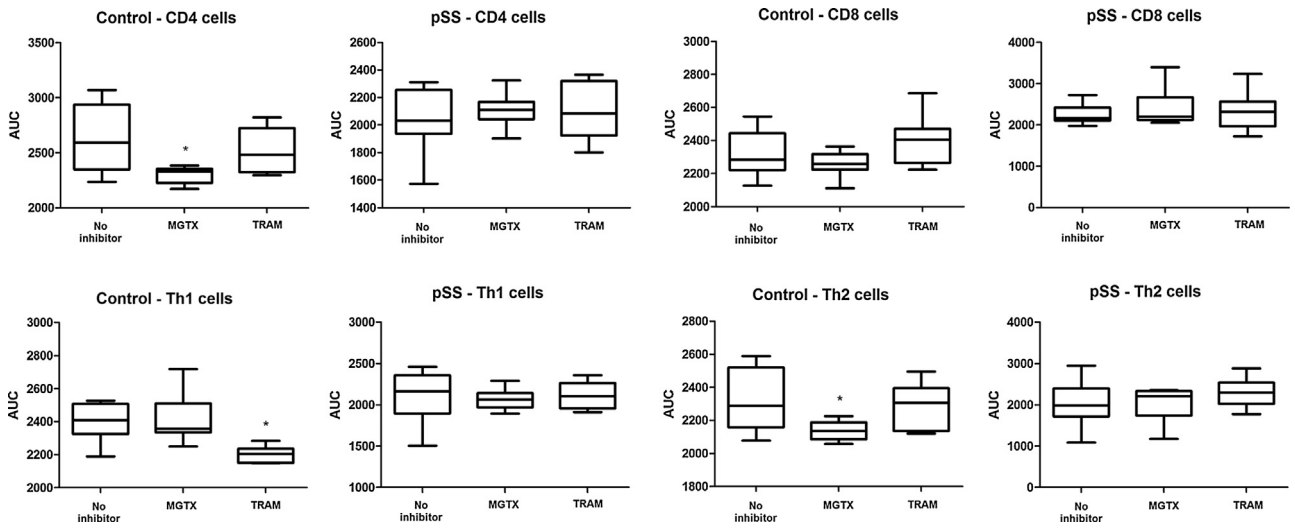

Fig. 4. The effects of the specific inhibitor of the Kv1.3 channel, margatoxin (MGTX) and the specific inhibitor of the IKCa1 channel, triarylmethane (TRAM) application on parameter values AUC—area under the curve in units (U) in peripheral lymphocytes obtained from 8 healthy individuals and 15 and primary Sjögren's syndrome patients. *$p < 0.05$ vs. control.

available to study the kinetics of lymphocyte activation in more subsets at the same time. Single-cell techniques are restricted by not being capable of characterizing this process in complex cellular systems, thus ignoring the interaction between the different lymphocyte subsets that may modulate the course of their activation. Therefore, over the recent years we have developed a novel algorithm that allows simultaneous monitoring of calcium influx in several lymphocyte subsets. Our software (FacsKin) fits functions to median values of the data of interest and calculates relevant parameters describing each function. By selecting the best fitting function, this approach provides an opportunity for the mathematical analysis and statistical comparison of kinetic flow cytometry measurements of distinct samples (Toldi et al., 2011).

4.2. Basal cytoplasmatic calcium levels and calcium influx kinetics in the investigated lymphocyte subsets

Several animal models have already demonstrated that the reduced TCR signal causes increased susceptibility to autoimmune diseases. SKG mice have a genetic defect in ZAP-70, a key signal transduction molecule in T-cells leading to T-cells mediated autoimmune arthritis, and other autoimmune disorders. This defect attenuates the TCR signal therefore negative and positive selection of T cells is impaired in the thymus promoting self-reactive hyporesponsiveness of T-cell selection. On the other hand alloantigens induce sufficient response in ZAP-70 deficient autoreactive T-cells. Our previous observation in type one diabetes and rheumatoid arthritis are in line with animal models (e.g. collagen induced arthritis) where autoimmunity can be triggered by exuberant T cell responses. However, our results in primary Sjögren syndrome are rather comparable to SKG animal models with sub-optimal T cell activation (Sakaguchi et al., 2012).

In contrast with our previous studies in autoimmune disorders, multiple sclerosis, rheumatoid arthritis, and type one diabetes, we found that lymphocytes isolated from pSS patients respond slowler to stimulation than those of healthy individuals (Toldi et al., 2013). Furthermore, the findings of our study indicate that basal [Ca2+]cyt was lower in pSS compared to healthy individuals. This is represented by higher tmax values, i.e. calcium influx reaches its peak later upon activation, and a lower AUC compared to healthy controls (Table 3, Fig. 2). This finding is especially relevant in the Th1 subset, also indicating that Th1 responses peripherally might be decreased in pSS to control the ongoing inflammation (Cornec et al., 2014).

The pathological hallmark of pSS is a chronic mononuclear cell infiltrate affecting exocrine glands mainly driven by T helper (Th) 1-type cytokines (Beeton et al., 2006). Recently, however, growing evidence has been suggesting that the pro-inflammatory cytokine interleukin (IL)-17 plays a pivotal role in the pathogenesis of pSS (Gong et al., 2014). Additionally, presence of different T cell subsets was evidenced in pSS, in peripheral blood and in salivary glands. At least six Th subsets exist: Th0, Th1, Th2, Th17, regulatory T (Treg), and follicular helper T (Tfh) cells, which are suggested to be involved in the pathogenesis of pSS (Toldi et al., 2011). The salivary glands are predominantly infiltrated by CD4 + T helper (Th) cells at an early stage of pSS, and in advanced stage, B cells predominate and these infiltration extends to occupy the acinar epithelium (SjögrenIgG4) (Nocturne and Mariette, 2013). Additionally, besides conventional CD4+ Th17 cells, another IL-17-producing T-cell subpopulation, lacking both CD4 and CD8 surface molecules (double negative, DN) has a key role in the pathogenesis of pSS (Alunno et al., 2014).

Furthermore, an epigenome-wide DNA methylation study identified several genes which were hypo or hypermethylated in peripheral naïve CD4 + T cells from pSS patients compared to healthy controls (Altorok et al., 2014). Hypomethylated genes were mainly involved in lymphocyte activation and immune response, whereas hypermethylated genes were involved in antigen processing and presentation.

In this study, all pSS patients were enrolled after longer onset of disease (years after the presentation of the diagnosis) without any severe systemic manifestations (low ESSDAI) at time of blood sampling. Theoretically, all of these observations in pSS including the role of different Th subsets, the presence of different T cell subsets in peripheral blood and in salivary glands might partially explain the decreased reactivity of peripheral Th1 and Th2 lymphocytes in pSS and the absence of significant difference regarding individual cell subsets among the study groups.

Muscarinic acetylcholine receptor (M3R) is expressed in exocrine glands and plays crucial roles in exocrine secretion. Acetylcholine binds to and activates M3R on salivary gland cells, causing a rise in intracellular Ca2+ concentration via inositol 1, 4, 5-triphosphate (IP3) and IP3 receptors. Similarly to T cell activation, the increase of the cytoplasmic calcium concentration is the

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cornerstone of salivary gland cells and functionality. Several anti-M3R antibodies (Abs) were described in pSS. These anti-M3R Abs cause salivary dysfunction in pSS via reduction in Ca2+ influx and down-regulation of M3R molecules on epithelial cells of salivary glands. Next to the destruction of affected tissues (mainly salivary and lacrimal glands) another feature of pSS is B cell hyperactivity, as attested by abundant production of autoantibodies (Sumida et al., 2014). Autoimmune responses are mainly polyclonal, targeting multiple epitopes within the same or interacting autoantigens. Despite extensive studies, the mechanisms operating for the exposure of certain intracellular autoantigens to the immune system remain rather elusive. It is tempting to speculate potential Abs cause Th1 and Th2 peripheral lymphocytes dysfunction via altered calcium influx kinetics in pSS.

Based on our results, the time when the peak of calcium influx was reached decreased in autoimmune patients compared to healthy individuals, indicating that these cells are in a state of sustained reactivity due to the ongoing autoimmune reaction. Contrary, not only the time when the peak of calcium influx was reached increased significantly, but the peak of calcium influx decreased significantly too, indicating that these cells are in an insensitive state which suggests the distinct pathomechanism of pSS (Table 3, Fig. 2).

4.3. The effects of potassium channel inhibitors on lymphocyte calcium influx

Earlier studies were limited to the investigation of potassium channels in naive and memory lymphocytes. We have extended these findings to significant effector T lymphocyte subsets, and found a different pattern of sensitivity to the inhibition of lymphocyte potassium channels in Th1 cells of autoimmune patients (RA, MS, T1DM) compared to healthy individuals (Toldi et al., 2010, 2011, 2013). In the investigated autoimmune patients a greater decrease of calcium influx upon the inhibition of the Kv1.3 channel than that of the IKCa1 channel was observed in Th1 cells. This finding is of special interest, since Th1 cells are regarded as key players in the mediation of pro-inflammatory responses. However, the selectivity of the investigated inhibitors was limited in our experiments, as they did not only affect a single subset, as previously suggested. Although in earlier observations the inhibition of Kv1.3 channels specifically blocked the function of TEM cells, our investigations extending to significant effector T lymphocyte subsets demonstrated that the inhibitory effect is present not only in disease-associated CD8 and Th1 cells, but also in the anti-inflammatory Th2 subset.

However, previous data harmonizing with our results suggest that selective modulation of lymphocyte activation through specific inhibition of potassium channels may be a possible therapeutic approach for the treatment of autoimmune disease, peripheral T-lymphocytes in pSS were insensitive to the potassium channel inhibitors.

4.4. Kv1.3 channel expression in pSS in the investigated lymphocyte subsets

The insensitivity of peripheral T-lymphocytes in pSS to the potassium channel inhibitors results may be due to altered functionality or changes in the expression of Kv1.3 channels. We therefore analyzed cell surface expression of Kv1.3 channel by measuring median fluorescence of specific antibodies. Changes to the sensitivity of Kv1.3 channel inhibition seem to be related to its altered expression, and therefore, might explain our findings. Hence, functional alterations of the Kv1.3 channel must also play a role in differential sensitivity upon inhibition detected between pSS patients and healthy controls. Of note, due to the lack of commercially available antibodies against the other investigated potassium channel, we could not perform a similar measurement in case of IKCa1.

5. Conclusion

In conclusion, the altered expression of Kv1.3 channels and specific inhibition of potassium channels seem to be related to altered calcium influx kinetics in pSS which distinguish pSS either from healthy controls or other systemic autoimmune diseases. These observations support the differential pathomechanism of pSS. Therefore, further studies, including the analysis of other lymphocytes subsets and functional consequences of specific inhibition of potassium channels such as cytokine production would add valuable information.

Conflict of interest

All authors declare no conflict of interest related to this manuscript.

Contributorship

Study conception and design: NL, GT, ABalog; Data collection and analysis: NL, NM, CO, ABajnok; review and approve the final manuscript: GT, ABalog Analysis and interpretation of data: NL, GT, ABalog; NL, GT, ABalog wrote the manuscript.

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III.

The role of B7 family costimulatory molecules and indoleamine 2,3-dioxygenase in primary Sjögren's syndrome and systemic sclerosis

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ORIGINAL ARTICLE

The role of B7 family costimulatory molecules and indoleamine 2,3-dioxygenase in primary Sjögren's syndrome and systemic sclerosis

Nóra Legány[1] · László Berta[2] · László Kovács[1] · Attila Balog[1] · Gergely Toldi[2]

Abstract B7 costimulatory molecules are present on antigen-presenting cells (APCs) and influence intracellular expression of indoleamine 2,3-dioxygenase (IDO), a molecule with important immunoregulatory functions. We determined the frequency of activated (CD11b+) monocytes expressing B7-1, B7-2, B7-H1, and B7-H2 molecules, and that of CD3+ and CD4+ T cells expressing the corresponding CD28, CTLA-4, PD-1, and ICOS receptors in peripheral blood samples of 20 healthy adults and 9 SSc and 15 pSS patients using flow cytometry. We also examined the intracellular expression of IDO. The expression of CD28 was lower in both SSc and pSS patients. The frequency of CTLA-4 was increased in pSS. The expression of ICOS, a stimulator of T cell activation, was elevated in pSS, but not in SSc, while that of its corresponding costimulatory molecule, B7-H2, was strongly decreased in SSc compared to controls. The frequency of PD-1 expressing T lymphocytes was decreased in both pSS and SSc. The frequency of IDO-expressing APCs, as well as intracellular IDO content in T cells was higher in pSS than in controls. Our investigation identified a number of differences in B7 costimulation between SSc and pSS patients which may play a role in the distinct pathogenesis and clinical features of these autoimmune disorders.

Keywords Antigen presentation · B7 · ICOS · Indoleamine 2,3-dioxygenase

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Introduction

Antigen presentation and costimulation are the initial steps in the activation of T cells, and the nature and intensity of costimulatory signals are key determinants of the coordination of pro- and anti-inflammatory events.

B7 costimulatory molecules are expressed on antigen-presenting cells (APCs), including dendritic cells and monocytes, and are important regulators of T cell activation, as well as of cytokine production in T helper cells. Upon the engagement of the T cell receptor (TCR), the costimulatory signal from B7-1 (CD80) or B7-2 (CD86) via CD28 induces the production of IL-2 in T cells, thus protecting them from apoptosis and anergy. Both the TCR and CD28 are constitutively expressed by most naive T cells, enabling them to respond to the antigen being presented [1]. Without costimulation, the signal from the TCR induces the tolerance of T cells to their cognate antigen instead of being activated [2].

Nevertheless, B7 family members mediate not only stimulatory, but also inhibitory effects on T cells [1]. Engagement of the TCR induces the phosphorylation of cytotoxic T lymphocyte antigen 4 (CTLA-4), resulting in its stabilization on the cell surface. Thereby, CTLA-4 will compete with CD28 for B7 binding, and because its affinity is higher than that of CD28, it will block the costimulatory signal, and prevent further IL-2 production [3].

Another B7 family member, B7-H1 or PD-L1, has predominantly, but not exclusively, inhibitory effects on T cells. Its inhibitory function is carried out by signaling through the programmed death-1 receptor (PD-1), inducing apoptosis or anergy of self-reactive T cells [4]. Genetic deletion of PD-1 results in severe autoimmunity due to the loss of peripheral tolerance of self-reactive T cells [5].

B7-H2 or ICOSL serves as the ligand for inducible costimulator of T cells (ICOS), and promotes T cell activation,

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differentiation, and effector responses [6]. Costimulation through ICOS can augment induction of both Th1 and Th2 cytokines, including IL-4, IL-10, and INF-γ [7].

Besides the initiation of signal transduction in T lymphocytes, ligation of CD80 and CD86 may back-signal into the APC and modulate their function [8]. For instance, reverse signaling through CD80 and CD86 after ligation by a soluble form of CTLA-4 was shown to upregulate the tryptophan (TRP) catabolic enzyme, indoleamine 2,3-dioxygenase (IDO) [9]. In the first steps of the kynurenine (KYN) pathway, TRP is transformed into KYN by IDO. KYN is then further metabolized by different enzymes. The local depletion of TRP and the production of proapoptotic TRP metabolites of the KYN pathway, such as 3-hydroxyanthranilic acid and quinolinic acid, are among the mechanisms potentially responsible for the immunosuppressive effects of IDO [10]. KYN and its metabolites suppress effector T cell function and facilitate their differentiation to regulatory T cells [11]. Since the TRP metabolic pathway is activated by proinflammatory stimuli, the anti-inflammatory effect of KYN metabolites provides a feedback mechanism in the downregulation of the immune response (Fig. 1).

Primary Sjögren's syndrome (pSS) is characterized by lymphocytic infiltration of the salivary and lacrimal glands, leading to the destruction of exocrine glands, but the autoimmune inflammatory process often involves other organs too. Systemic sclerosis (SSc), also termed scleroderma, is characterized by autoimmune activation, microvascular injury, and fibrosis in multiple organs, including the skin, lung, heart, and gastrointestinal tract. Being chronic systemic autoimmune diseases, permanent activation of the adaptive immune system is obvious in both pSS and SSc [12, 13]. The recognition of the outstanding importance of the costimulatory regulation in many autoimmune diseases and in tumor-immunology has lead to highly effective targeted therapies in both fields [14–16].

However, data on the alterations in the expression of costimulatory molecules and their receptors in pSS and SSc are very limited. We hypothesize that the degree and the balance of the expression of the components of the B7 family member molecules and the consequent alterations in the IDO pathway may significantly determine the differences observed in the immunopathogenesis of autoimmune rheumatic disorders, included in pSS and SSc. In this study, we therefore aimed to investigate the prevalence of a major APC subset, CD11b + activated monocytes expressing CD80, CD86, PD-L1, and ICOSL costimulatory molecules, and that of CD3+ T cells and CD4+ T helper cells expressing CD28, CTLA-4, PD-1, and ICOS, as well as the expression of IDO in these cell types in pSS and SSc.

Materials and methods

Patients

We enrolled 15 patients with pSS, 9 patients with SSc, and 20 age- and gender-matched healthy individuals. Demographic and laboratory data are summarized in Table 1.

pSS patients fulfilling American European Consensus Group (AECG) 2002 [17] or American College of Rheumatology (ACR) 2012 classification criteria [18] were included. The following clinical symptoms occurred in pSS patients' history: sicca syndrome (100%), labial salivary gland biopsy positivity for focal lymphocytic sialadenitis with focus score ≥ 1(80%), blood cytopenia (anemia or leukopenia) (87%), arthritis (67%), vasculitis (53%), anti-SSA/SSB antibody positivity (100%), low C3/C4 complement level (47%), renal involvement (13%), fever and night sweats (20%), lymphadenopathy (20%), weight loss (33%), myositis (7%), and peripheral neuropathy (20%). The EULAR Sjögren's syndrome disease activity (ESSDAI) scores were calculated at the

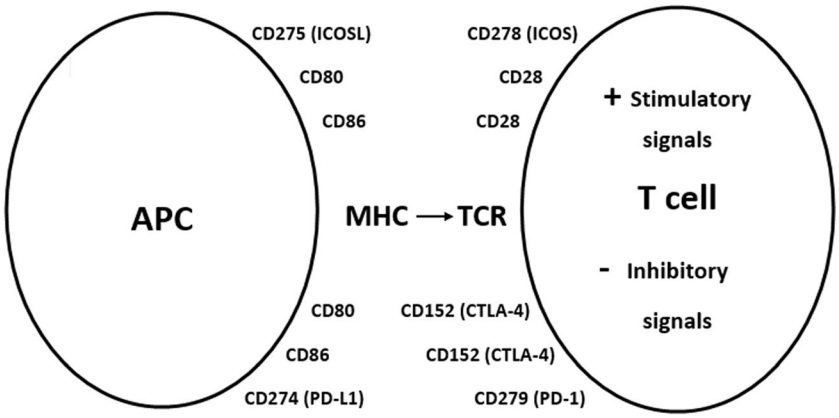

Fig. 1 B7 co-stimulatory molecules expressed on antigen presenting cells (APCs), and their stimulatory or inhibitory ligands on T cells. *MHC* major histocompatibility complex, *TCR* T cell receptor, *ICOS* inducible costimulator, *ICOSL* inducible costimulator ligand, *CTLA-4* cytotoxic T lymphocyte antigen-4, *PD-1* programmed death-1 receptor, *PD-L1* programmed death-1 receptor ligand

Table 1 Clinical characteristics of patients with SSc and pSS as compared with healthy controls

Characteristics	Healthy $n = 20$	SSc patients $n = 9$	pSS patients $n = 15$
Age (years)	53 (42–61)	53 (39–65)	52 (36–77)
Gender (male/female)	2/6	2/ 7	1/ 14
Disease duration (years)		11 (1–33)	5 (2–14)
ESR (mm/h)	8 (5–12)	24[a] (1–53)	27[a] (7–55)
CRP	<2	5.47[a] (2.0–16.0)	4.8[a] (2.0–13.8)
Autoantibody positivity:			
Anti-SSA and/or anti-SSB		0	15
Anti-Scl-70		5	0
Anti-centromere		2	0
Antirheumatic drugs			
Methyl-prednisolone		3	7
Chloroquin		0	10
Methotrexate		1	3
Azathioprin		2	1

Data are expressed as median (interquartile range) for continuous variables and as number for categorical variables

SSc systemic sclerosis, *pSS* primary Sjögren's syndrome, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein

[a] $p < 0.05$ vs. healthy individuals

time of sampling [19]. The median ESSDAI score was 2 which is consistent with low disease activity. SSc patients fulfilled the 2013 ACR/EULAR classification criteria for scleroderma [20]. Patients were further classified as those with limited cutaneous SSc or diffuse cutaneous SSc according to LeRoy et al. [21]. The following clinical symptoms occurred in SSc patients' history: Raynaud syndrome (88%), digital ulcers (33%), interstitial lung disease (66%), pulmonal hypertension (33%), and pericarditis (22%).

Healthy controls had a negative history of rheumatic symptoms. No co-morbidities were detected in patients and controls that could have influenced the investigated parameters, nor did they take any medication that could have interfered with the measurements. Written informed consent was obtained from all subjects, and our study was reviewed and approved by the ethics committee of the institution.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (FicollPaque, Amersham Biosciences AB, Uppsala, Sweden), by means of centrifugation for 25 min, 400*g*, 22 °C from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). Cells were kept at −80 °C in fetal bovine serum containing 10% DMSO until analysis.

Flow cytometry

After thawing, PBMCs were incubated for 30 min at room temperature in the dark with the following conjugated antibodies: PerCP-conjugated CD3, PE Cy7-conjugated CD4, PE-conjugated CD28, APC-conjugated CD152 (CTLA-4), FITC-conjugated CD278 (ICOS) and APC-Cy7-conjugated CD279 (PD-1)mAbs, or PerCP-conjugated CD3, PE Cy7-conjugated CD11b, APC-conjugated CD80 and PE-conjugated CD275 (ICOSL) mAbs, or PerCP-conjugated CD3, PE Cy7-conjugated CD11b, APC-conjugated CD86, and PE-conjugated CD274 (PD-L1) mAbs in separate tubes (BioLegend, San Diego, CA, USA). After washing, cells were fixed with fixation/permeabilization solution and treated with permeabilization buffer according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). They were then stained with a mouse anti-human IDO monoclonal antibody (Millipore, USA) for 30 min at 4 °C in the dark. After washing, cells were stained with FITC-labeled goat anti-mouse IgG antibody for 15 min at 4 °C in the dark. After washing, cells were analyzed on a BD FACSAria flow cytometer (BD Biosciences) equipped with 488- and 633-nm excitation lasers. Data were processed using the FACSDiVa software. One hundred thousand cells were recorded. The populations of lymphocytes and monocytes were gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics.

Statistics

Data are expressed as median and interquartile range. Comparisons between sample populations were made with the Kruskal-Wallis test. Correlation analyses were performed using Spearman's signed ranked tests. p values less than 0.05 were considered significant. Statistics were calculated using the GraphPad Prism software (version: 5.00, GraphPad Software Inc., La Jolla, CA, USA).

Results

Cell frequency results are summarized in Table 2.

In case of T lymphocytes, no difference was observed in the prevalence of CD3+ CD28+ cells in SSc or pSS samples compared with healthy controls, although there was a significant increase in SSc as compared with pSS. In the T-helper subset, the prevalence of CD4 + CD28+ T-helper cells was significantly lower in both SSc and pSS than in controls. There was no difference in the prevalence of CD3 + CTLA-4+ cells between the study groups, but within T-helper cells, the amount of CD4 + CTLA-4+ T-helper cells was higher in pSS compared with controls. The prevalence of CD3+ ICOS+ and CD4 + ICOS+ lymphocytes was higher in pSS than in controls.

The frequency of CD3 + PD1+ and also CD4 + PD1+ lymphocytes was lower in both SSc and pSS samples than in the controls (Fig. 2). Regarding the APCs (CD11b+ monocytes), there was no difference observed in the prevalence of CD80+, CD86+, and PD-L1 + expressing monocytes between SSc, pSS and healthy control samples. However, the frequency of ICOSL+ monocytes was lower in SSc than in controls, and higher in pSS than in SSc.

No difference was observed in the prevalence of CD3+ IDO+ lymphocytes; however, IDO MFI values in CD3+ cells were higher in pSS samples compared with controls (Fig. 3).

The prevalence of IDO+ monocytes was higher only in pSS samples compared with controls, but there was no difference in the MFI of IDO in monocytes between the study groups.

In order to explore the presence of reverse signaling via CD80 and CD86, correlation analyses were performed. No correlation was revealed between IDO-expressing T cells and monocytes and the prevalence of CD80+ or CD86+ monocytes.

Table 2 Frequency of the investigated cell surface and intracellular markers

	Healthy control (n = 20)	SSc (n = 9)	pSS (n = 15)
CD3+ CD28+ cells/CD3+ lymphocytes	77.18 (65.78–82.50)%	84.73 (77.09–88.12)%	74.87[b] (60.60–77.43)%
CD3+ CD28+ CTLA-4+ cells/CD3+ CD28+ lymphocytes	8735 (6.463–11.73)%	9650 (7.920–10.09)%	11.60 (7.500–12.00)%
CD3+ ICOS+ cells/CD3+ lymphocytes	56.35 (50.36–74.92)%	74.24 (69.09–82.36)%	75.08[a] (73.09–77.34)%
CD3+ PD-1+ cells/CD3+ lymphocytes	49.83 (44.79–59.85)%	20.66[a] (15.00–34.42)%	17.43[a] (13.55–20.80)%
CD4+ CD28+ cells/CD4+ lymphocytes	97.82 (96.76–99.08)%	94.54[a] (87.38–96.85)%	94.54[a] (90.70–97.02)%
CD4+ CD28+ CTLA-4+ cells/CD4+ CD28+ lymphocytes	6340 (4565–8743)%	8060 (5785–9975)%	11.50[a] (9.660–12.60)%
CD4+ ICOS+ cells/CD4+ lymphocytes	59.05 (53.39–73.23)%	68.12 (64.87–72.95)%	74.11[a] (70.14–78.22)%
CD4+ PD-1+ cells/CD4+ lymphocytes	54.03 (47.89–62.85)%	17.45[a] (13.32–32.59)%	12.11[a] (7440–20.46)%
CD11b + CD80+ cells/CD11b + monocytes	54.22 (19.63–68.76)%	25.48 (22.75–33.12)%	29.32 (21.35–52.69)%
CD11b + CD86+ cells/CD11b + monocytes	27.57 (19.62–29.78)%	17.35 (13.21–22.52)%	27.47 (16.34–36.51)%
CD11b + PD-L1+ cells/CD11b + monocytes	74.09 (68.22–84.90)%	84.00 (51.43–86.79)%	82.40 (77.16–86.21)%
CD11b + ICOSL+ cells/CD11b + monocytes	61.23 (34.52–70.04)%	32.97[a] (27.93–44.23)%	59.81[b] (51.08–84.46)%
CD3+ IDO + cells/CD3+ lymphocytes	4500 (2615–7708)%	5430 (3785–7205)%	5170 (3730–6510)%
IDO MFI in CD3+ IDO + cells (arbitrary unit)	10,400 (7490–11,625)	20,737 (10,012–49,270)	17,857[a](13,396–22,117)
CD11b + IDO + cells/CD11b + monocytes	2585 (1.970–10.84)%	6930 (4.365–13.40)%	18.46[a] (12.30–21.21)%
IDO MFI in CD11b + IDO + cells (arbitrary unit)	22,450 (19,875–24,475)	28,278 (11,670–37,101)	16,588 (12,338–28,590)

Data are presented as median (interquartile range)

CTLA-4 cytotoxic T lymphocyte antigen-4, *ICOS* inducible costimulator, *PD-1* programmed death-1 receptor, *PD-L1* programmed death-1 receptor ligand, *ICOSL* inducible costimulator ligand, *IDO* indoleamine 2,3-dioxygenase, *MFI* mean fluorescence intensity

[a] Frequencies of cells isolated from SSc and pSS patients were compared with those of cells isolated from healthy individuals, $p < 0.05$

[b] Frequencies of cells isolated from pSS patients were compared with those isolated from SSc patients, $p < 0.05$

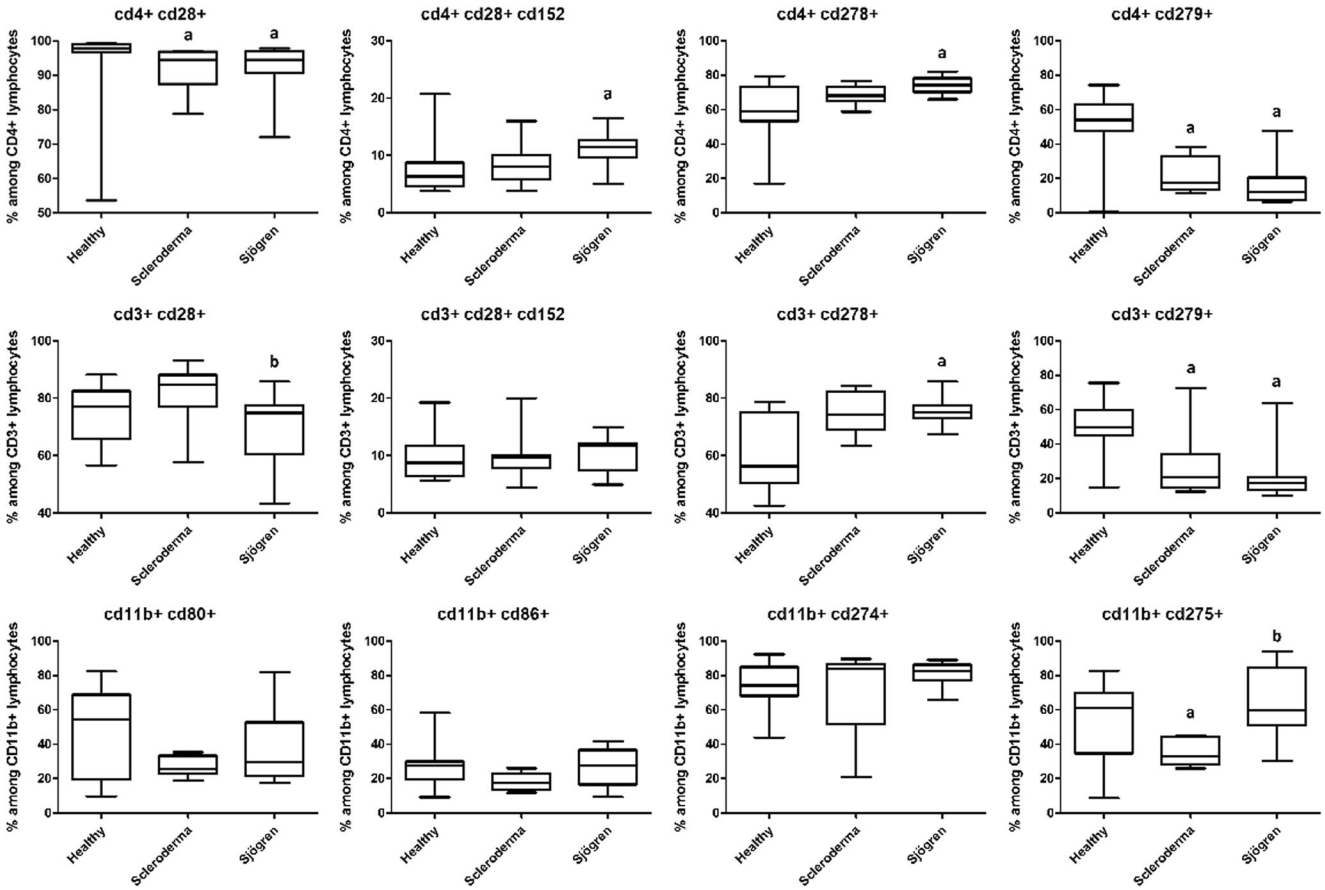

Fig. 2 Frequency of the investigated cell surface and intracellular marker-positive cells. The expression of CD28 on T helper lymphocytes and CD279 (PD-1) on T lymphocytes were lower in both SSc and pSS. The frequency of CD152 (CTLA-4) in T helper lymphocytes and CD278 (ICOS) in T lymphocytes were increased in pSS but not in SSc. The expression of CD275 (ICOSL) was strongly decreased in SSc on antigen presenting cells. *SSc* systemic sclerosis, *pSS* primary Sjögren's syndrome, *PD-1* programmed death-1 receptor, *CTLA-4* cytotoxic T lymphocyte antigen-4, *ICOS* inducible costimulator, *ICOSL* inducible costimulator ligand. *a* Frequencies of cells isolated from SSc or pSS patients were compared with those of cells isolated from healthy individuals, $p < 0.05$. *b* Frequencies of cells isolated from pSS patients were compared with those isolated from SSc patients, $p < 0.05$.

Discussion

The key event in the development of an autoimmune disease is the activation of effector CD4+ T cells that recognize a self peptide. There are several checkpoints that must be overcome to result in this, including the failure of central tolerance and peripheral regulation. The presentation of autoantigens will only initiate an autoimmune response if costimulatory signals overcome the checkpoints of peripheral tolerance induction. The aim of our study was to investigate the role of B7 family molecules and IDO pathway molecules on monocytes and their corresponding receptors on T lymphocytes in two systemic autoimmune diseases. To the best of our knowledge, our study is the first to investigate several members of costimulatory molecules at the same time in pSS and SSc.

The B7 family is one of the most important second signal mechanisms and is essential to the maintenance of the delicate balance between immune potency and suppression of autoimmunity. The importance of the B7 family in regulating immune responses is clear from their demonstrated role in the development of immunodeficiency, cancer, and autoimmune diseases [22]. Manipulation of the signals delivered by B7 ligands shows a great potential in the treatment of cancer or autoimmune disorders including pSS and SSc.

CD80/CD86, CD28, and CTLA-4

The upregulation of CD80/CD86 costimulatory molecules and their corresponding receptor CD28 lead to an increase in APC activity and the augmentation of T cell responses. In our study, the expression of CD80 and CD86 costimulatory molecules was comparable in all three study groups. At the same time, their corresponding receptor's expression of CD28 was lower in the T-helper subset both in pSS and SSc patients than in controls. This finding indicates a decreased costimulation via CD28 in T-helper cells in these diseases.

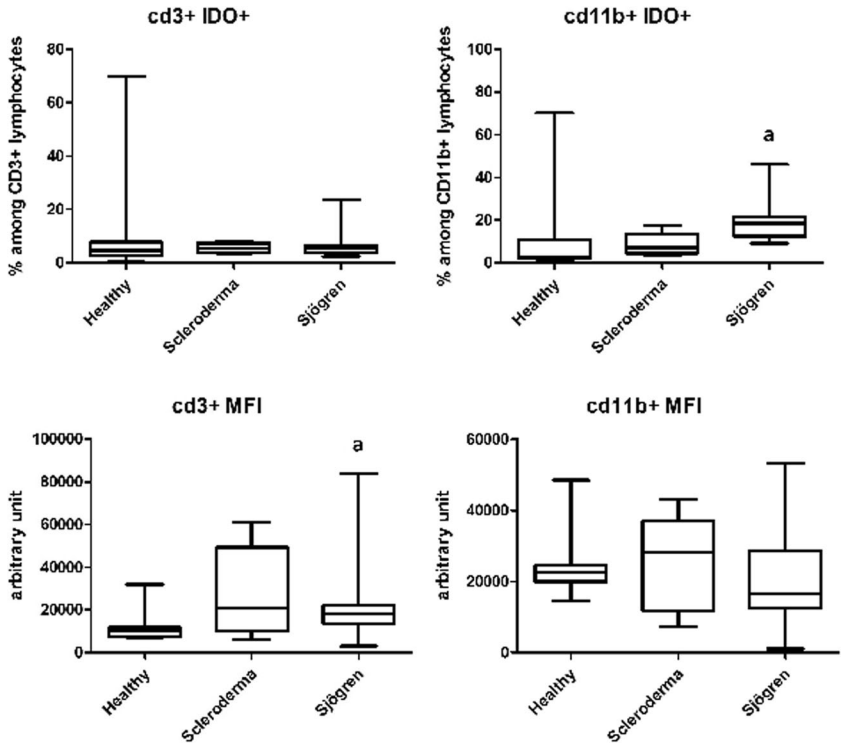

Fig. 3 IDO expression is elevated in pSS samples. While no difference was observed in IDO expression between SSc and pSS patients and controls in CD3+ lymphocytes, the frequency of IDO-expressing APCs was higher in pSS samples than in healthy controls. The intracellular IDO content (mean fluorescence intensity) was higher in CD3+ lymphocytes in pSS than in controls. There was no difference in intracellular IDO content in APCs in SSc and pSS samples compared with healthy controls. *IDO* indoleamine 2,3-dioxygenase, *SSc* systemic sclerosis, *pSS* primary Sjögren's syndrome, *APCs* antigen-presenting cells, *MFI* mean fluorescence intensity. *a* Cells from pSS patients were compared with healthy controls, $p < 0.05$.

Furthermore, in our study, the frequency of CTLA-4+ T-helper cells was higher in pSS than in controls. CTLA-4 is located on the same chromosomal region as CD28, and binds B7 more strongly. The B7 signal blockade using a CTLA-4 Ig construct leads to suppression of humoral and cell-mediated immune responses. Multiple mechanisms for CTLA-4-mediated immunosuppression include competition for CD28 binding and induction of suppressive intracellular signaling pathways such as cytokine-dependent tryptophan catabolism and upregulation of IDO in B7-expressing APCs [9, 23]. Furthermore, a rapidly developing fatal lymphoproliferation and multisystemic autoimmunity was demonstrated in Ctla-$4^{-/-}$ mice [24].

No biological agent has yet been approved either for pSS or SSc. Abatacept is a fully human fusion molecule of IgG-Fc and CTLA-4 that modulates CD28-mediated T cell costimulation. Given the mechanism of action of abatacept and the recognized role of APCs and T and B cells in pSS and SSc, selective modulation of costimulation represents a reasonable therapeutic option. In an open-label pilot study of abatacept in active pSS, patients with recent onset of pSS and high disease activity were monitored. During the intravenous abatacept treatment, ESSDAI, ESSPRI, rheumatoid factor, and IgG levels decreased significantly, and fatigue and health-related quality of life improved significantly [16]. Abatacept also induces clinical improvement in patients with severe and treatment-resistant diffuse SSc [15]. In our study, the lower level of CD28 receptors on T-helper cells in pSS and SSc and the higher level of CTLA-4+ T-helper cells in pSS suggest a "protective" status of costimulation between APCs and T cells that reduces the T cell-dependent autoimmune response. The enrolled patients with pSS and SSc in our study were selected randomly from our outpatient clinic. All investigated patients with pSS or with SSc had a low disease activity according to ESSDAI, ESSPRI, mRSS, and EScSG activity indices, respectively. All patients had relatively long disease duration, and took immunosuppressive therapy. It may be hypothesized that either the long disease duration (chronic autoimmune process) or the long-term effect of immunosuppressive agents might have lead to the desensitization or lower expression of CD28 receptors and higher level of CTLA-4 on T-helper cells in pSS and SSc.

ICOS

We have demonstrated that in T lymphocytes, the expression of ICOS, a stimulator of T cell activation, is elevated in pSS, but not in SSc, while that of its corresponding costimulatory

molecule, ICOSL, was strongly decreased in SSc compared to controls. Therefore, this pathway appears to play a more dominant role in pSS.

Accumulating evidence suggests that ICOS is instrumental in T cell-driven multi-organ inflammation in autoimmune diseases [25]. ICOSL was found to be expressed not only in APCs, but also in salivary gland epithelial cells (SGECs) in pSS. These SGECs play an important role in the differentiation of naive CD4+ T cells into follicular helper T cells by the secretion of IL-6. Interactions between ICOSL on SGECs and ICOS on follicular helper T cells results in enhanced IL-21 secretion by follicular helper T lymphocytes [26]. In another study, ICOSL expression by CD11c+ myeloid cells was associated with enhanced T cell survival in the kidneys of lupus-prone mice. Local activation of ICOS by CD11c+ myeloid cells drives organ inflammation in lupus. These results suggest an autoantibody-independent role for ICOS in self-reactive T cell survival in target organs [27]. According to these findings, our results point to a potential role of ICOS in the pathomechanism of pSS, potentially by means of the maintenance of a sustained stimulation of T cells within the target organs, including the salivary gland.

Similarly to our findings, a greater frequency of circulating T follicular helper (Tfh) cells express ICOS in systemic lupus erythematosus (SLE) compared to healthy controls. Furthermore, in vitro ICOS costimulation of peripheral T cells from patients with active SLE resulted in greatly enhanced IFN-γ production relative to normal controls, and ICOS ligation preferentially induced production of isotype-switched α-double-stranded DNA (α-dsDNA) antibodies during co-culture with autologous B cells. In animal models, splenic ICOS+ CD4+ T cell production of IL-21 was linked to renal disease and early mortality. In a different study, DC ICOSL expression was correlated with kidney nephritis and proteinuria. As a whole, by way of enhancing autoantibody formation and accelerating organ inflammation, these data strongly suggest that ICOS plays a direct role in promoting SLE disease progression [28]. Therapeutic interventions designed to disrupt ICOS/ICOSL signaling may therefore be similarly promising in pSS as in SLE, taking our current findings into consideration.

PD-1

The frequency of PD-1 expressing T lymphocytes was found to be decreased both in pSS and SSc compared with controls, while there was no difference in the PD-L1 expression of APCs between the investigated study groups.

PD-1 regulates self-tolerance against many organs and prevents autoimmunity by setting at least two checkpoints that control the induction and maintenance phases of the anergic state. In addition, the innate immune system may inhibit autoreactive lymphocyte proliferation by PD-1-induced pathways [29]. Decreased frequencies of PD-1 expressing T cells may reflect deficiencies in this regulatory pathway in these two connective tissue diseases.

IDO

There is abundant evidence of immune cell hyper-activation in pSS and SSc. This raises the question of whether there is corresponding activation of the IDO pathway, in an attempt to regulate uncontrolled T cell responses. IDO is the first and rate-limiting step in the KYN pathway, and is thought to play a key role in immune homeostasis through depletion of tryptophan and accumulation of KYN metabolites. Inhibition of IDO activity, or knockout of the gene encoding IDO, was shown to cause an increase in the severity of collagen-induced arthritis, an animal model of rheumatoid arthritis [30]. On the other hand, a higher percentage of IDO-expressing APCs was observed supported in pSS than in healthy controls [31]. Increased KYN concentration and KYN/TRP ratio, which reflects the activity of IDO, were also measured in the peripheral blood of pSS patients [32]. In our study, we found that the frequency of IDO-expressing APCs, as well as intracellular IDO content in T cells, was higher in pSS than in controls. Therefore, it is possible that the increased IDO activity tries to control the chronic autoantigen stimulation in pSS and plays a role in the counter-regulatory response.

Our results also supported that the induction of IDO activity resulting in the activation of the KYN pathway is an attractive therapeutic approach. However, further investigation of the downstream members of the KYN pathway is needed to confirm or refute this hypothesis.

A limitation of our study is the lack of data on changes in cytokine and chemokine expression related to alterations observed in the costimulatory pathways. These linked mechanisms also play a key role in the pathomechanism of pSS and SSc and need further investigation based on the current results. Another limitation may be the heterogeneity of our patient populations in terms of treatment with different immunosuppressive agents. In future studies, patients with more active disease state should also be included.

Conclusion

Our understanding of the B7 family has expanded enormously since the description of costimulatory pathways. Contemporary models of immune regulatory networks reflect this complexity. Defining the signals by which specific types of APCs and costimulatory molecules drive T cell autoimmunity, and elucidating whether these signals play a disease

specific role, will advance our knowledge towards the development of new therapeutics that rely on disrupting T cell-APC interactions. The promise of modification of autoimmune mechanisms through pharmacologic manipulation of this pathway shows substantial potential. Furthermore, our results also suggest that the expression of costimulatory molecules differs between distinct autoimmune disorders, although a limitation of the current study is the low number of patients. Therefore, sub-classification of the investigated patients' group according to clinical characteristics (disease activity, disease duration, medication, etc.) would provide further details about the role of costimulatory pathways.

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