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

Ph.D. Thesis

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Publications

Publications related to the thesis:

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). IF: 3.017

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). IF: 2.72

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). IF: 2.905

Citable abstracts related to the thesis:

Balog A, Legány N, Toldi G, Distler JH, Beye C, Szalay B, Kovács L,Vásárhelyi B. Increased plasma soluble urokinase plasminogen activator receptor level in systemic sclerosis with impaired microvascular

abnormalities and fibrosis**. ANNALS OF THE RHEUMATIC DISEASES** 74:(Suppl. 2)p. 956.(2015)EULAR 2015.

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Introduction

Over the course of my Ph.D. studies, I investigated alterations in the pattern of leukocyte activation of patients with primary Sjögren's syndrome (pSS) and systemic sclerosis (SSc) compared to healthy controls. Additionally, an inflammatory biomarker, the soluble urokinase-type plasminogen activator receptor (suPAR) was studied in the plasma of SSc patients in relation to the internal organ involvement.

Primary Sjögren's Syndrome (pSS) is a systemic autoimmune disease, which leads to autoimmune inflammation in the exocrine glands and fatigue. A significant proportion of patients develop extraglandular manifestations, such as arthritis, vasculitis, neuropathy, interstitial lung disease, or nephropathy. In the case of patients with long-standing primary Sjögren's syndrome, the potential risk of lymphoma is higher as well. The possible causes of pSS may be epithelial and lymphocytic disturbances. The innate and adaptive immune responses both play an important role in this pathological process. The histological features include lymphocyte infiltration

of the target tissues and germinal center formation. CD4+ T helper (Th), CD8+ T cytotoxic (Tc) and B cells take part in the development of these autoimmune processes equally. As the effect of the activation of pathological B cells, autoantibodies are produced (e. g. anti-SSA/SSB, anti-fodrin, and anti-muscarin M3 receptors). Recent studies confirm the active involvement of epithelial cells in the induction and maintenance of inflammation.

SSc also relates to autoimmune connective tissue diseases, and is characterized by autoimmune disorder, including microvascular injury, and excessive collagen deposition in the skin and internal organs, all of which lead to hypoperfusion and multiple organ dysfunction. The most frequently affected organs are the skin, lung, heart, gastrointestinal tract, and the kidney. SSc was further classified into those with limited cutaneous, or diffuse cutaneous SSc according to LeRoy criteria. An early histological feature is the infiltration of the dermis by inflammatory cells (e. g. lymphocytes, fibroblasts, monocytes), followed by endothelial cell damage and excessive extracellular matrix deposition. 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. The CD8+ lymphocytes play an important role in the cell-mediated cytolytic process.

The process of specific Ag recognition by T cell receptors (TCRs) 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 may induce an anergic state of the T cell. This second impulse can result in either a stimulatory or inhibitory effect, depending on the cell surface co-stimulatory molecule expression. The members of the CD28 Ig superfamily, such as CD28, CTLA-4, PD-1, and 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, therefore the inflammatory processes can be modulated in various directions. These molecules are expressed on T cell surfaces and are able to

bind to the B7 receptor family members, like CD80, CD86, PD-L1/2, and ICOSL on the surface of antigen presenting cells (APCs).

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme, which is expressed by APCs and regulatory T cells and catalyzes the degradation of tryptophan (TRP) into kynurenine (KYN). Proinflammatory signals, especially INF-ɣ, induce IDO expression. As a result of IDO's effect, the proapoptotic KYN and its metabolites are produced, which inhibit effector T cell function and proliferation, and furthermore increase T cell apoptosis and facilitate their differentiation into regulatory T cells. The immunosuppressive activity of IDO represents a counter-regulatory effect against excessive immune 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. The elevation of $[Ca^{2+}]$cyt leads to a decrease in the electrochemical potential gradient and this alteration attenuates the Ca^{2+} influx. The maintenance of the electrochemical potential gradient and calcium influx

is controlled 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. Selective inhibition of potassium channels in lymphocytes leads to inhibition of Ca^{2+} influx, and subsequently to lymphocyte activation.

SuPAR is the soluble form of urokinase plasminogen activator receptor (uPAR). The serum level of suPAR is increased as a result of the activation of the immune system, in infectious, autoimmune, and neoplastic diseases, as well as pregnancy. In animal models, it has been suggested that uPAR may have a functional role in endothelial dysfunction and the induction of excessive fibrosis in SSc.

Aims:

Aim 1. To determine the expression of CD28 Ig superfamily members (CD28, CTLA-4, PD-1, ICOS) on the surface of CD3+ and CD4+ T cells, and to analyze the expression of their receptors, the B7 family members (CD80, CD86, PD-L1/2, ICOSL) on CD11b+ antigen presenting cells derived from pSS and SSc patients compared to healthy control subjects.

Aim 2. To determine the expression of IDO in CD3+ T cells and CD11b+ monocytes in pSS and SSc comparing to healthy controls.

Aim 3. To determine the elevation of cytoplasmic Ca^{2+} concentration after lymphocyte activation in CD4+ Th1, Th2, and CD8+ lymphocytes in pSS and SSc compared to healthy controls.

Aim 4. To determine the cell surface expression of Kv1.3 potassium channel on CD4+ Th1, Th2, and CD8+ T cells in pSS and SSc in comparison with healthy control patients.

Aim 5. 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 relative to healthy controls.

Aim 6. To determine the level of suPAR in serum from patients with systemic sclerosis with different internal organ involvement, and compare it to healthy samples.

Patients and methods

15 patients with pSS, 9 patients with SSc, and 20 age- and gender-matched healthy individuals were enrolled in the study of the CD28-B7 molecule family. pSS patients all fulfilled the AECG 2002 or American College of Rheumatology ACR 2012 classification criteria, while SSc patients fulfilled the 2013 ACR/EULAR classification criteria for scleroderma. Mononuclear cells were isolated from peripheral blood, and then were incubated with conjugated monoclonal antibodies, in order to stain the members of the CD28-B7 molecule family on the surface of T lymphocytes and monocytes. The different leukocytes were also separated by the cell surface antigens. Cells were fixed with fixation/permeabilization solution and treated with

permeabilization buffer, in order to mark IDO molecules not only on the cell surface, but intracellularly as well. Cells were analyzed using flow cytometeric measurements.

For the investigation of intracellular Ca^{2+} level and the function of K^{+} channels, we enrolled 15 patients with pSS, 16 patients with SSc and 20 healthy control individuals. After mononuclear cell isolation, we incubated these samples with conjugated anti-human monoclonal antibodies in order to differentiate T lymphocyte subsets. Mononuclear cells were loaded with calcium sensitive fluorescent stains for monitoring the intracellular Ca^{2+} concentration. The cells were marked with anti-Kv1.3 channel FITC for the measurement of the expression of Kv1.3 potassium channels on the surface of the T cells. In the investigated lymphocytes, the Kv1.3 channels were blocked with margatoxin (MGTX) and the IKCa1 channels were blocked with triarylmethane compound (TRAM). Lymphocytes were activated using phytohemagglutinin (PHA). The expression of Kv1.3 channels was observed using a standard flow cytometry method and the alteration of Ca^{2+} kinetics and the

function of K^+ channels were monitored with a kinetic method. The advantage of this flow cytometry method is that it is possible to investigate a large number of different cell types simultaneously within sequentially measured cells. The results were evaluated over time.

83 patients with SSc fulfilling the criteria proposed by the 2013 ACR/EULAR classification criteria for scleroderma were enrolled with 29 age- and gender-matched healthy individuals. Patients were further classified as those with limited cutaneous SSc (n=56), or diffuse cutaneous SSc (n=27). SuPAR levels were measured with the suPARnostic Flex ELISA assay. The serum suPAR level were compared to the serum c-reactive protein (CRP) and erythrocyte sedimentation values (ESR) in the SSc subgroups, depending on the clinical features.

Results

During the course of the investigation of co-stimulatory molecules, we observed lower expression of CD28 in pSS and SSc, but in the case of the ICOS molecule, the expression was higher on CD3+ and CD4+ cells in the pSS group compared to healthy controls. The co-inhibitory CTLA-4 expression was higher on CD4+ lymphocytes in pSS, whereas the expression of PD-1 molecules was higher in both patient groups compared to T cells from healthy individuals. On the surface of CD11b+ APCs, the expression of CD80, CD86 and PD-L1 molecules were comparable with the healthy samples, but in SSc, we measured lower expression of ICOSL.

In the case of IDO, we measured elevated intracellular IDO content in CD3+ lymphocytes and higher IDO expression on the surfaces of CD11b+ monocytes in pSS. In the SSc samples, there was no significant difference compared to the control group.

In pSS, the basal intracellular Ca^{2+} level was lower in both Th1 and Th2 lymphocyte subsets compared to healthy controls. A similar trend was observed after

lymphocyte activation; the calcium influx was lower in the CD4+ and Th1 cell in pSS, than in the healthy controls. In SSc patients, there was a higher resting Ca^{2+} level in the Th2 subsets compared to other lymphocyte subgroups, but there was no difference compared to healthy controls. Following activation, the Ca^{2+} influx in the SSc group also lagged behind the healthy group, but within the group, we observed the highest Ca^{2+} in the CD8+ subgroup.

The cell surface expression of Kv1.3 channels was considerably higher on CD4+, Th2, and CD8+ cells in the pSS group than in the healthy group, whereas the results of SSc samples were comparable with healthy samples. However, within the SSc group, Kv1.3 expression in Th2 lymphocytes was elevated compared to other lymphocyte subgroups.

The K^+ channel inhibitors (MGTX, TRAM) did not prove to be effective in pSS; neither of the inhibitors induced any alterations in the calcium influx of lymphocytes. In the SSc group, TRAM decreased the Ca^{2+} influx in the Th1 subgroup similarly to the healthy

group, but interestingly MGTX had an opposite effect, as it accelerated the Ca^{2+} influx in the Th1 and Th2 cells.

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. Moreover, suPAR level was higher in dcSSc than in lcSSc, and correlated with anti-Scl-70 autoantibody-positivity. We also observed that higher suPAR level correlated with interstitial lung disease and its severity, pulmonary arterial hypertension, all forms of microvascular injury (Raynaud syndrome, digital ulcers, and nailfold capillaroscopy), and arthritis. Meanwhile, 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) or pulmonary arterial hypertension from controls.

Discussion

When investigating the CD28 and CTLA-4 molecules, which bind to the same ligands (CD80/CD86), the CD28 stimulatory pathway seems to have decreased activity in both autoimmune diseases. By contrast, elevated ICOS expression is in accordance with the known ICOSL expression of salivary gland epithelial cells and consequently with the differentiation of follicular T helper cells. The decreased frequencies of PD-1 expressing T cells may promote the survival of autoreactive lymphocytes, and ultimately lead to the increased activity of autoimmune processes in these two connective tissue diseases.

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 against autoimmunity.

The decreased resting Ca^{2+} level in pSS and lower Ca^{2+} influx following activation in both diseases lead to a decreased responsiveness and an exhaustion of abnormal lymphocytes. Chronic autoimmune inflammatory

processes or the longstanding immunosuppressive therapy may potentially explain these alterations.

The overexpression of Kv1.3 channel on T cells in pSS may be an indication that in some autoimmune diseases, the number of high Kv1.3 expressing effector memory T cells can increase by repeated stimulation of their cognate autoantigen. In SSc, Kv1.3 channel upregulation on Th2 cells may play a role in the elevated basal Ca^{2+} level in this lymphocyte subgroup.

We found a relationship between the elevation of suPAR level and the presence and severity of SSc, therefore it has the potential to be an adequate activity biomarker. In light of others results supporting the close connection between suPAR level and pulmonary and microvascular involvement, the uPAR system's role in the pathomechanism of SSc is suggested.

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