SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

INVESTIGATION OF IMMUNOREGULATORY DISORDERS IN SYSTEMIC AUTOIMMUNE DISEASES

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1. INTRODUCTION

The ability to distinguish between self and non-self molecules is essential for the maintenance of immune homeostasis. The protection of self-antigen structures encompasses several types of controlling mechanisms of the immune system. Central tolerance plays a key role in the early phase of cell maturation in the bone marrow and thymus, when lymphocytes expressing receptors for self-antigens are deactivated (clonal deletion and clonal anergy). When potentially self-reactive lymphocytes enter the periphery, the phenomenon of peripheral tolerance inhibits their activation. The complex system of peripheral immune-competent cells with regulatory characteristics plays a key role in avoiding clonal expansion of autoreactive cells, therefore maintains the inhibition of tissue and organ damage.

The first part of my work deals with the role of pathological immunoreactivity and altered regulatory function in the pathogenesis of primary Sjögren's syndrome (pSS) and systemic sclerosis (SSc), with a special emphasis on the associations with clinical symptoms. The second part of my investigation focuses on the clinical and immunomodulatory effects of extracorporeal photopheresis (ECP) in systemic sclerosis.

1.1 Sjögren's syndrome

Sjögren's syndrome is the second most common systemic autoimmune disease, after rheumatoid arthritis. It develops mostly in females during the fourth and fifth decades of life. The disease affects primarily the exocrine glands, leading to decreased lachrymal and salivary secretion. Besides the characteristic glandular symptoms, other systemic symptoms (e.g. polyarthritis, Raynaud's phenomenon, vasculitis), denoted as extraglandular manifestations (EGMs), can also be found in a subset of patients. Sjögren's syndrome is classified as either primary or secondary disease. Primary form occurs by itself, while secondary form is associated with another autoimmune disease.

The pathogenesis of the disease is still not fully understood, but there is no doubt that it is a multifactorial process, in which autoimmune cascades damage the target tissues. In exocrine glands, the infiltrating lymphocytes are predominantly CD4+ T-cells and B-cells, which contribute to the dysfunction and destruction of the affected glands by initiating an inflammatory response. Changes in the presence and suppressor function of regulatory T-cells may play an important role in the pathogenesis of Sjögren's syndrome. In salivary gland biopsies and peripheral blood of pSS patients, the number of CD4+ CD25+ regulatory T

(Treg) cells was found to be reduced compared to those in healthy individuals. However, another study investigating the same cells did not verify this reduction.

1.2. Systemic sclerosis

SSc is a systemic autoimmune disease with excessive extracellular matrix deposition and damage of small blood vessels. Inflammatory processes are prominent in skin and visceral organs, including heart, lungs, or kidneys. It develops mostly in females during the fourth decade of life. Patients with SSc are commonly classified into two clinical subsets: diffuse cutaneous SSc is dominated by rapidly progressive fibrosis of skin and visceral organs, while in limited cutaneous SSc the skin fibrosis is limited and the prevalence of internal organ involvement is low. Since therapeutic options are limited mainly to the management of the complications, the diffuse cutaneous form has the highest mortality among connective tissue diseases. The visceral organ manifestations are mainly responsible for the mortality of the disease, and pulmonary complications such as interstitial alveolitis, pulmonary fibrosis or pulmonary arterial hypertension (PAH) are the leading causes of death.

The pathogenesis of SSc is complex and still not fully understood. The three main phase of disease development are the follows: endothelial damage, pathological immune activation and fibrosis of the affected tissues. The immune dysfunction in SSc is in the focus of intense research. Previously, studies suggested that an altered balance of T-helper (Th)1 and Th2 cytokine profile may be responsible for fibrosis. In Th2 predominance, plasma level of interleukin (IL)-4 increases, which induces the transforming growth factor (TGF)-beta production leading to fibroblast proliferation and accelerated collagen synthesis. The IL-17-producing Th17 cells are major contributors to autoimmune processes, and recent studies have demonstrated that SSc patients have increased peripheral Th17 cell percentages and higher IL-17 expression. Th17 cells also induce TGF-beta synthesis and fibroblast proliferation, which raises the potential involvement of Th17 cells in the pathogenesis of SSc. Concerning regulatory T-cells, the literature is controversial about CD4+ CD25+ Tregs, both increased and decreased percentages were reported.

1.3. Extracorporeal photopheresis

ECP is a special immunomodulatory therapy, which is based on apheresis technology. The separation of leukocyte rich plasma from the red blood cell fraction is followed by its ex

vivo exposition to 8-methoxypsoralen (8-MOP) and UV-A light, and finally, the re-infusion of the treated cells. 8-MOP covalently binds and crosslinks DNA upon UV-A light exposure, which induces apoptosis in the treated lymphocytes. The apoptotic lymphoid population is phagocytosed by macrophages and dendritic cells. The presentation of antigens by antigen-presenting cells, in the absence of „danger" signals, may have implications for the establishment of self-tolerance. The complex effect of ECP leads to a shift in the cytokine profile of Th cells from Th1 to Th2, an expansion of Treg cells and elevation of IL-10 levels, thereby may attenuate autoimmune mechanisms.

ECP seems to have beneficial effects in a number of haematological and immunological diseases, and also after transplantation. Additionally, photopheresis is one of the promising therapeutic strategies in the diffuse cutan form of SSc. In a randomized, double-blind, placebo-controlled trial, ECP was shown to induce significant improvement of skin and joint involvement in SSc patients. Another study demonstrated that after ECP, the dermal echo intensity increased, while the dermal thickness reduced, which suggests that ECP is more likely to improve dermal oedema, then fibrosis. Since the complex immunobiological effects of ECP have been studied only in animal models, human graft-versus-host disease and cutaneous T-cell lymphoma, investigations focusing on the mechanism of its action in autoimmune diseases may open new avenues to enrich the modern therapeutic arsenal in autoimmune disorders.

2. OBJECTIVES

2.1. The investigation of Sjögren's syndrome

The aim of our study was to evaluate peripheral cell types with regulatory properties, reflecting overall immune-regulatory disturbances, characteristic to patients with pSS. We determined the percentages of peripheral natural killer (NK), NKT, T regulative type 1 (Tr1) and CD4+ CD25+ Treg cells, and measured the serum levels of IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-alfa and interferon (IFN)-gamma. We carried out an in vitro functional assay in order to evaluate the functional suppressor capability of CD4+ CD25+ Treg cells. We also assessed EGMs within patients, and assessed the relationship between the evaluated immune parameters and the clinical symptoms of the disease.

2.2. The investigation of systemic sclerosis

The primary aim of our investigations was to evaluate a wide spectrum of peripheral immune-competent cell types with regulatory and effector properties (B, NK, NKT, activated T, naive and memory T, Th1, Th2, Th17, Tr1 and CD4+ CD25+ Treg cells), reflecting overall immune disturbances, characteristic of patients with diffuse cutaneous SSc. Moreover, we measured the circulating IL-4, IL-10, IFN-gamma and complement levels in patients' sera, and we carried out an in vitro functional test to determine the suppressor capability of CD4+ CD25+ Treg cells. We also assessed visceral organ involvements, and evaluated the relationship between the observed changes in laboratory parameters and clinical symptoms.

2.3. The investigation of ectracorporeal photopheresis

In order to assess these possible beneficial effects of ECP therapy, we performed a wide-spectrum of analyses, ranging from clinical evaluation to serological markers and immune-competent cell analyses prior to the first ECP treatment and six weeks after each ECP cycles. We assessed the skin, joint and organ involvements, and determined a wide spectrum of circulating cytokines [TNF-alpha, IFN-gamma, IL-1-alpha, IL-1-beta, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, chemokine C-C motif ligand (CCL2), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and TGF-beta] and peripheral immune-competent

cell types with regulatory and effector properties (B, NK, NKT, activated T, naive and memory T, Th1, Th2, Th17, Tr1 and CD4+ CD25+ Treg cells), reflecting overall disturbances in immune homeostasis, characteristic to patients with diffuse cutaneous SSc. In order to determine the changes in suppressor capability of CD4+ CD25+ Treg cells, we carried out an in vitro functional test after each procedure. We also estimated how ECP may affect the parameters of serum levels of autoantibodies, and evaluated the relationship between the observed changes in clinical symptoms and laboratory parameters.

3. PATIENTS AND METHODS

3.1. Patients

Patients were recruited from the Autoimmune Outpatient Clinic of the Division of Clinical Immunology, 3rd Department of Medicine, Medical and Health Science Center, University of Debrecen, where they received regular follow-up and treatment. Informed written consent was obtained from the subjects, and the study has been approved by the Ethics Committee of University of Debrecen. All experiments carried out were in compliance with the Helsinki Declaration.

Patients with Sjögren's syndrome

Thirty-two pSS patients (1 male, 31 female; mean age 49.5 ± 7.2 years) were enrolled in the study; the diagnosis of pSS was established according to the European-American consensus criteria. Among patients with pSS, 23 had EGMs, while nine patients had only sicca symptoms. The distribution of EGMs was as follows: thyreoiditis n=1; pulmonary involvement n=2; myositis/myalgia n=3; polyneuropathy n=5; Raynaud's phenomenon n=14; vasculitis n=15 and polyarthritis n=18. A cohort of age- and sex-matched healthy individuals served as controls (n=20). No patients, or controls enrolled in this study were administered immunosuppressive or immune-modulating medications and no one had ongoing infections, either viral, or bacterial.

Patients with systemic sclerosis

In the study we enrolled 21 patients (1 male, 20 female; mean age 50.8 ± 15.2 years) with diffuse cutaneous SSc diagnosed according to the corresponding criteria. Three patients were administered methylprednisolone (4 mg/day); two patients received 15 mg methotrexate weekly; the other 16 patients received no immunosuppressive agents but were treated with 2 x 400 mg pentoxyphyllin and 2.5 mg amlodipine daily. Drug therapy was suspended in all patients 48 hours before taking blood samples. Fifteen sex- and age-matched healthy individuals served as controls. No patients, or controls enrolled in this study had infections in the last six month.

Patients underwent photopheresis therapy

Sixteen patients suffering from diffuse cutaneous SSc (14 female and 2 male; mean age 46.5 ± 13.2 years) were enrolled in the study. The diagnosis of SSc was established according to the corresponding diagnostic criteria. The mean disease duration was 3.9 years (range 0.5-7 years). For one year before the beginning of photopheresis therapy, patients received only 2 x 400 mg pentoxyphyllin and 2.5 mg amlodipine treatment. Sixteen sex- and age-matched healthy individuals served as controls for the laboratory results. No patients, or controls enrolled in this study had ongoing or previous infections during the study.

3.2. Method of extracorporeal photochemotherapy

We performed ECP procedures by using THERAKOS UVAR XTS Photopheresis System (Therakos Inc., Raritan, NJ, USA), according to the manufacturer's instructions. The patients were treated using the standard protocol in which ECP cycles were carried out once in every six weeks. Each ECP cycle consisted of two procedures on consecutive days. Patients received 6 cycles in total during the whole therapy period.

3.3. Clinical evaluation

In pSS patients, we evaluated the tear and saliva secretion by Schirmer's tear test and sialometry.

Skin involvement was evaluated by calculating modified Rodnan skin score (MRSS). In order to determine visceral organ involvement, patients underwent diagnostic procedures, including chest X-ray, high-resolution computed tomography, spirometry/diffusion capacity test, Doppler echocardiography, abdominal ultrasonography, oesophagus passage radiography and routine laboratory tests.

During ECP therapy, skin involvement was assessment by echographic measurements as well. We also examined the mobility of the upper limbs (shoulders, elbows, wrists) and lower limbs (hips, knees, ankles) on both side, registered and measured the changes in degrees.

3.4. Laboratory methods

Determination of lymphocyte subgroups

To identify lymphocyte subpopulations, we used monoclonal antibodies against CD3, CD4, CD8, CD19 and CD56 (BD Biosciences, San Diego, CA, USA and Immunotech, Marseille, France). The expression of T-lymphocyte activation markers such as CD69 and HLA-DR were also determined on CD3+ cells (BD Biosciences). The following monoclonal antibody combinations were used for phenotypic characterization of naive, and memory cells: anti-CD45RA-fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (PE) (Immunotech), anti-CD45RA-FITC/CD8-PE (AbD Serotec, Oxford, UK) and CD62L- phycoerythrin-Cy5 (PC5) (Immunotech). We used anti-CD95 (Immunotech) antibody to determine CD95 expression on lymphocytes. Measurements were performed in pSS study on a Coulter EPICS XL-4 (Beckman Coulter Inc., Miami, FL, USA), while during the investigation of SSc patients a Coulter FC500 flow cytometer (Beckman Coulter Inc.) was used. The following peripheral immune-competent cell types were investigated: T-cells (CD3+), Th cells (CD4+), cytotoxic T-cells (CD8+), B-cells (CD19+), early-activated T-lymphocytes (CD3+ CD69+), late-activated T-lymphocytes (CD3+ HLA-DR+), NK cells (CD56+) and NKT cells (CD3+ CD56+). The B, T, T-helper, activated T, NK and NKT cells were quantified as their percentages in the entire lymphocyte population. Naive and memory T-cell subsets were determined as their percentages in CD4+ or CD8+ cells, as the followings: naive helper T (CD4+ CD45RA+ CD62L+), central memory helper T (CD4+ CD45RA– CD62L+), effector memory helper T (CD4+ CD45RA– CD62L–), naive cytotoxic T (CD8+ CD45RA+ CD62L+), central memory cytotoxic T (CD8+ CD45RA– CD62L+), effector memory cytotoxic T (CD8+ CD45RA– CD62L–) and terminally differentiated effector memory cytotoxic T-cells (CD8+ CD45RA+ CD62L–).

Identification of CD4+ T-cell subsets

The following monoclonal antibodies were used for intracellular staining of CD4+ T-cell subsets: FITC-labelled anti-IFN-gamma, PE-labelled anti-IL-4, PE-conjugated anti-IL-10 (BD Biosciences), and PE-labelled anti-IL-17 (R&D Systems, Minneapolis, MN, USA). Based on intracytoplasmic staining, the phenotypes within CD4+ cells were determined as follows: Th1 cells: CD4+ IFN-gamma+ IL-4-; Th2 cells: CD4+ IFN-gamma- IL-4+; Tr1 cells: CD4+ IL10+; Th17 cells: CD4+ IL17+. Cells were quantified as their percentage in the CD4+ lymphocyte population. To identify CD4+ CD25+ Treg cells, cell surface (CD4,

CD25) staining and intracellular forkhead box P3 (FoxP3) staining was carried out on freshly isolated peripheral blood mononuclear cells (PBMCs) from heparinized blood. The following reagents were used: Ficoll (Sigma Aldrich, St Louis, MO, USA), CD4-FITC monoclonal antibody (Sigma Aldrich), CD25-phycoerythrin-Cy5 (PC5) (Immunotech), FoxP3-PE, clone: PCH101 (eBioscience, San Diego, CA, USA). Measurements were performed in pSS study on a Coulter EPICS XL-4 (Beckman Coulter Inc.), while during the investigation of SSc patients a Coulter FC500 flow cytometer (Beckman Coulter Inc.) was used.

In vitro functional assay of CD4+ CD25+ Treg cells

CD4+CD25- and CD4+ CD25+ T-cells were isolated from PBMC by using a Miltenyi Regulatory T Cell Isolation Kit with LD and MS Columns, according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany), and were cultured separately and in co-cultures in 1:1 ratio for 72 hours. For polyclonal stimulation, cells were stimulated with anti-CD3/CD28 T-cell expander microbeads (Dynal, Oslo, Norway). Cell proliferation was investigated by using the EZ4U Proliferation Kit (BioMedica Inc, San Diego, CA, USA), and optical density (OD) values were detected at 450 nm by Multiskan MS device (MTX Lab Systems Inc., Vienna, VA, USA). OD values of mixed lymphocyte cultures were corrected by OD values of CD4+ CD25+ T-cells cultured alone. Suppression activity was determined as the ratio of OD values of the CD4+CD25- T-cell cultures and mixed lymphocyte reactions.

Evaluation of circulating cytokines

Levels of IL-4, IL-6, IL-10, TNF-alfa, IFN-gamma and TGF-béta were measured by BD OptEIA enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences) according to the manufacturer's instructions. We determined a wide spectrum of serum cytokines and chemokines, including TNF-alpha, IFN-gamma, IL-1-alpha, IL-1-beta, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, CCL2, FGF, VEGF, EGF and HGF with a multiplex cytokine assay at the Tissue Engineering Laboratory of Institute of Human Physiology and Clinical Experimental Research, Semmelweis University. Measurements were carried out by using Fluorokine Multianalyte Profiling (MAP) Kits (R&D Systems), according to the manufacturer's instruction. The samples were read by a multy-analyte bioassay detection system (Luminex 200 System, Luminex, Austin, TX, USA); acquisition and preliminary analysis were carried out using Applied Cytometry System STarStation 3.0 software (Applied Cytometry, Dinnington, UK).

Determination of complement and autoantibody levels

Levels of complement C3 and C4 were measured on BN II Nephelometeren (Siemens AG, München, Germany) by using anti-C3 and anti-C4 antibodies (Dialab GmbH, Neudorf, Austria) and calibrators (Siemens AG). Anti-extractable nuclear antigen (ENA) autoantibodies were determined by ELISA technique with AUTOSTAT II kits (Hycor Biomedical, Indianapolis, IN, USA) according to the manufacturer's instructions.

3.5. Statistical analyses

The SPSS ver. 12.0 (SPSS Inc., Chicago, IL, UDA) was used for statistical analysis. To assess the distribution of data Kolgomorov-Smirnov test was used. In cases of normal distribution, we determined mean ± standard deviation (SD) values and used two-sample t test for statistical comparison of the experimental data. In cases of distributions different from that of normal, median, minimum and maximum values were calculated, and Mann-Whitney test was used. The general linear model - repeated measures ANOVA analysis was used to evaluate the significance of changes in parameters over time. When the strength of the linear relationship between two variables was evaluated, Pearson's correlation coefficient was used, while in cases of non-normal distribution, Spearman's correlation coefficient was applied. Differences were considered statistically significant at $p < 0.05$.

4. RESULTS

4.1. Results of patients with Sjögren's syndrome

Peripheral NK and NKT cells

Peripheral NK cell percentages were increased significantly in pSS patients compared to controls (15.31 ± 9.81 % vs. 10.87 ± 4.74 % respectively, $p = 0.034$). We found similar NK cell percentages in the subset of pSS with compared to pSS patients without EGMs.

In the overall pSS patient population, we found significantly elevated NKT cell percentages compared to controls (5.18 ± 4.60 % vs. 3.07 ± 2.28 % respectively, $p = 0.033$). An increased number of NKT cells in the subset of pSS with EGMs, compared to pSS patients without EGMs (6.26 ± 4.85 % vs. 2.41 ± 2.30 % respectively, $p = 0.003$) was determined, as well as pSS with EGMs compared to healthy individuals (6.26 ± 4.85 % vs. 3.07 ± 2.28 % respectively, $p = 0.005$). NKT cell percentages in patients without EGMs and controls were similar.

Peripheral regulatory T-cells

Peripheral Tr1 cell percentages were increased strongly in pSS patients compared to healthy individuals (18.51 ± 7.83 % vs. 2.73 ± 4.06 % respectively, $p < 0.001$). We found Tr1 cell percentages in patients with EGMs significantly higher than in patients without EGMs (20.02 ± 8.57 % vs. 14.81 ± 3.96 % respectively, $p = 0.028$). Both subgroups of patients have significantly elevated Tr1 cell percentages compared to controls (pSS without EGMs vs. control: 14.81 ± 3.96 % vs. 2.73 ± 4.06 % respectively, $p < 0.001$; pSS with EGMs vs. control: 20.02 ± 8.57 % vs. 2.73 ± 4.06 % respectively, $p < 0.001$).

Peripheral CD4+ CD25+ Treg cells were decreased in pSS patients when compared to healthy individuals (3.07 ± 1.41 % vs. 4.59 ± 2.34 % respectively, $p = 0.005$). Percentages of these cells were significantly increased in the pSS with EGMs group compared to patients without EGMs (3.39 ± 1.26 % vs. 2.25 ± 1.51 %, respectively $p = 0.038$). In both subgroups of patients, the percentages of CD4+ CD25+ Treg cells were significantly lower than in controls (pSS without EGMs vs. control: 2.25 ± 1.51 % vs. 4.59 ± 2.34 % respectively, $p < 0.003$; pSS with EGMs vs. control: 3.39 ± 1.26 % vs. 4.59 ± 2.34 % respectively, $p < 0.038$).

In vitro suppression assay of CD4+ CD25+ Treg cells

Seven pSS patients with EGMs, 7 patients without EGMs and 7 controls were

randomly selected for the functional in vitro suppression assay. We found that the suppression capability of CD4+ CD25+ Treg cells was lower in the overall pSS patient population compared to healthy individuals (1.526 ± 0.66 vs. 2.395 ± 0.87, p = 0.019). There was no significant difference in the suppression capability, when pSS patients with or without EGMs were compared.

Soluble cytokines

Of serum soluble cytokines, IL-6 and TNF-alpha were significantly elevated in patients compared to controls [IL-6: median: 0.8 (0.8-32.5) pg/ml vs. median: 0.8 (0.8-2.2) pg/ml respectively, p < 0.001; TNF-alpha: median: 0.8 (0.1-16.5) pg/ml vs. median: 0.4 (0.1-1.2) pg/ml respectively, p = 0.001]. We found a significant decrease of serum IL-10 levels in patients compared to healthy individuals (median: 0.79 (0-72.86) pg/ml vs. median: 5.14 (0.1-31.91) pg/ml respectively, p = 0.017). There was no significant difference in the serum levels of IL-4 and IFN-gamma between pSS patients and healthy individuals.

Associations between laboratory parameters and sicca signs

We found a negative correlation between serum IL-10 levels and the ratios of Tr1 cells (R = -0.369, p = 0.019).

A significant negative correlation was observed between the percentages of peripheral CD4+ CD25+ Treg cells and sialometry values (R = -0.538, p = 0.003).

Among pSS patients, we found 19 SS-A positive and 9 SS-A – SS-B double positive individuals. No association between the presence of autoantibodies and any investigated immune parameters was detected.

4.1. Results of patients with systemic sclerosis

Peripheral NK and NKT cells

Peripheral NK cell percentages were increased (13.33 ± 6.25 % vs. 8.74 ± 3.74 %, respectively, p = 0.014), while NKT cell percentages were decreased in patients compared to controls (1.25 ± 0.91 % vs. 2.67 ± 2.34 %, respectively, p = 0.021).

Activated, naive and memory T-cell subtypes

Among activated peripheral T-cells, unlike early-activated T-cells, late-activated T-cells were represented in increased percentages in patients when compared to controls (3.58 ±

1.83 % vs. 2.01 ± 1.57 %, respectively, p = 0.012). While CD4+ naive T-cell percentages were decreased (34.74 ± 10.39 % vs. 42.76 ± 8.09 %, respectively, p = 0.038), percentages of CD4+ central memory T-lymphocytes were increased in patients compared to healthy subjects (39.59 ± 7.77 % vs. 34.16 ± 5.43 %, respectively, p = 0.039). Similarly to the differences in CD4+ T-cell subsets, CD8+ naive T-cell percentages were lower (28.03 ± 15.13 % vs. 44.37 ± 18.93 %, respectively, p = 0.027), and CD8+ central memory T-cell percentages were higher in patients (17.75 ± 13.51 % vs. 10.05 ± 4.22 %, respectively, p = 0.035).

Peripheral T-helper cells

Th1 cell percentages were decreased significantly in patients compared to controls (12.78 ± 5.21 % vs. 18.19 ± 4.87 %, respectively, p = 0.004). Fractions of Th2 cells were similar in both patients and control groups. Regarding Th17 cells, we found significantly elevated values in patients compared to controls (1.58 ± 0.71 % vs. 1.05 ± 0.49 %, respectively, p = 0.028).

Peripheral regulatory T-cells

Tr1 cell percentages were decreased strongly in patients, compared to those found in healthy individuals (0.54 ± 0.19 % vs. 0.87 ± 0.31 %, respectively, p = 0.002). Patients had significantly lower percentages of peripheral CD4+ CD25+ Treg cells, compared to controls (5.03 ± 2.29 % vs. 6.21 ± 0.91 %, respectively, p = 0.031). Consequently, when we calculated the Th17/CD4+ CD25+ Treg ratios, elevated values were found in patients (0.39 ± 0.28 vs. 0.23 ± 0.14, respectively, p = 0.042).

In vitro suppression assay of CD4+ CD25+ Treg cells

The suppression capability of CD4+ CD25+ Treg cells was significantly reduced in SSc patients, compared to healthy individuals (1.48 ± 0.31 vs. 2.23 ± 0.79, respectively, p = 0.015).

Circulating cytokines

IL-10 levels showed a significant decrease in patients compared to control values (2.71 ± 2.13 pg/ml vs. 7.19 ± 4.16 pg/ml, respectively, p < 0.001). Both IFN-gamma and IL-4 concentrations were similar in patients and controls.

Associations between visceral organ involvement, skin symptoms and peripheral immune parameters

Twelve patients had visceral organ involvement, the distribution was as follows: PAH n = 4; pulmonary fibrosis n = 8; esophageal dysmotility n = 7; serositis n = 1. We found significantly higher NK cell percentages in patients with visceral organ involvement, compared to patients having skin symptoms only (15.41 ± 6.27 % vs. 9.21 ± 4.21 %, respectively, p = 0.014), or healthy individuals (15.41 ± 6.27 % vs. 8.74 ± 3.74 %, respectively, p = 0.005).

A negative correlation was found between MRSS values and peripheral Tr1 cell percentages (R = –0.485, p = 0.026).

Associations between complement levels, autoantibody positivity and peripheral immune parameters

Negative correlation was found between percentages of CD4+ CD25+ Treg cells and levels of both complement C3 (R = –0.531, p = 0.013) and C4 (R = –0.515, p = 0.017).

Among SSc patients, we found 14 anti-Scl-70 antibody-positive individuals. No associations between the presence and levels of autoantibodies and other peripheral immune parameters were detected.

4.3. Results of patients underwent photopheresis

Skin, joint and visceral involvements

The modified Rodnan skin score decreased as a result of the treatment. Significant reduction was observed already after the first ECP cycle compared to the baseline (29.81 ± 3.56 vs. 32.69 ± 4.36, respectively, p = 0.049), and values decreased continuously after each treatment (MRSS after the last cycle: 20.17 ± 3.76). Based on echographic measurements, the dermal thickness reduced progressively during the whole therapy period. We found significant difference between baseline values and those measured after the last cycle at each investigated places (Upper arm: 0.98 ± 0.28 mm vs. 0.84 ± 0.22 mm, p = 0.016; Forearm: 1.01 ± 0.21 mm vs. 0.90 ± 0.19 mm, p = 0.017; Hand: 1.12 ± 0.23 mm vs. 0.99 ± 0.24 mm, p = 0.013; Finger: 1.25 ± 0.24 mm vs. 1.06 ± 0.25 mm, p < 0.001). Moreover, we measured a significant increase in oral apertures after the last treatment compared to baseline values (3.84 ± 0.32 cm vs. 2.96 ± 0.53 cm, respectively, p < 0.001).

Analysis of changes in joint mobility revealed a clear improvement. ECP therapy increased the ranges of motions of both upper and lower limbs (Shoulder anteflexion: 143.75 ± 21.79° vs. 164.69 ± 12.31°, p = 0.003; Shoulder retroflexion: 47.66 ± 6.68° vs. 55.62 ± 9.46°, p = 0.010; Shoulder elevation: 70.47 ± 4.49° vs. 75.31 ± 8.06°, p = 0.044; Elbow flexion: 56.87 ± 11.27° vs. 69.37 ± 9.32°, p = 0.002; Knee flexion: 76.66 ± 14.35° vs. 87.17 ± 13.85°, p = 0.049).

The distribution of organ involvement was as follows: pulmonary fibrosis n = 9; esophageal dysmotility n = 4; PAH n = 3; serositis n = 1. Although organ involvements did not show improvement through the treatments, at least they were stabile and did not deteriorate during the ECP therapy.

Peripheral B, T, NK and NKT cells

We found no significant differences in peripheral blood CD3+, CD4+ and CD8+ T-cell and B-cell numbers and percentages between patients and controls. Interestingly, patients have significantly increased percentages of late-activated T-cell compared to control values (3.72 ± 1.59 % vs. 2.24 ± 1.43 %, respectively, p = 0.018). ECP treatment did not change significantly the number of these cell types.

Regarding CD95+ T-cells, both numbers and percentages were significantly elevated in patients, compared to healthy individuals (cell numbers: 0.485 ± 0.164 G/l vs. 0.342 ± 0.139 G/l, respectively, p = 0.007; cell percentages: 42.95 ± 7.65 % vs. 32.53 ± 5.91 %, respectively, p < 0.001), and interestingly, already after the first ECP cycle, these values significantly decreased (cell numbers: 0.373 ± 0.131 G/l vs. 0.485 ± 0.164 G/l, respectively, p = 0.035; cell percentages: 35.22 ± 6.94 % vs. 42.95 ± 7.65 %, respectively, p = 0.003) and became similar to control values. Values of CD95+ B-cells were similar in patients and controls, and did not change during the investigated period.

Both numbers and percentages of peripheral NK cells were significantly higher in patients compared to healthy individuals (cell numbers: 0.199 ± 0.102 G/l vs. 0.131 ± 0.038 G/l, respectively, p = 0.043; cell percentages: 13.16 ± 6.65 % vs. 9.01 ± 3.77 %, respectively, p = 0.038). On the other hand, values of NKT cells were significantly lower compared to controls (cell numbers: 0.0194 ± 0.0177 G/l vs. 0.0399 ± 0.0284 G/l, respectively, p = 0.041; cell percentages: 1.18 ± 1.04% vs. 2.16 ± 1.59%, respectively, p = 0.047). Based on our results, ECP treatments had no effect on NK and NKT cell counts.

Peripheral Th1, Th2 and Th17 cells

Th1 cell numbers and percentages were significantly lower in patients before treatments, compared to control values (cell numbers: 0.119 ± 0.067 G/l vs. 0.167 ± 0.045 G/l, respectively, $p = 0.034$; cell percentages: 13.72 ± 5.23 % vs. 18.25 ± 4.71 %, respectively, $p = 0.017$), and these values did not changes during the ECP therapy. Values of Th2 cells were similar in patients and controls, and did not change due to treatments.

Regarding Th17 cells, both numbers and percentages were significantly elevated in patients, compared to healthy individuals (cell numbers: 0.0129 ± 0.0062 G/l vs. 0.0073 ± 0.0034 G/l, respectively, $p = 0.007$; cell percentages: 1.61 ± 0.74 % vs. 1.09 ± 0.48 %, respectively, $p = 0.025$), and interestingly, already after the second ECP cycle, these values significantly decreased (cell numbers: 0.0129 ± 0.0062 G/l vs. 0.0085 ± 0.0032 G/l, respectively, $p = 0.021$; cell percentages: 1.61 ± 0.74 % vs. 1.14 ± 0.37 %, respectively, $p = 0.032$) and became similar to control values.

Peripheral regulatory T-cell subsets

Absolute numbers and percentages of both Tr1 and CD4+ CD25+ Treg cells were significantly lower in patients prior to receiving the first ECP treatment, compared to control values (Tr1 numbers: 0.00347 ± 0.00126 G/l vs. 0.00691 ± 0.00254 G/l, respectively, $p < 0.001$; Tr1 percentages: 0.45 ± 0.17 % vs. 0.84 ± 0.33 %, respectively, $p < 0.001$; CD4+ CD25+ Treg numbers: 0.0359 ± 0.0094 G/l vs. 0.0479 ± 0.0075 G/l, respectively, $p = 0.001$; CD4+ CD25+ Treg percentages: 4.88 ± 1.17 % vs. 6.14 ± 0.93 %, respectively, $p = 0.002$). Values of both of these regulatory cell types significantly elevated already after the second ECP cycle (Tr1 numbers: 0.00347 ± 0.00126 G/l vs. 0.00442 ± 0.00108 G/l, respectively, $p = 0.041$; Tr1 percentages: 0.45 ± 0.17 % vs. 0.59 ± 0.19 %, respectively, $p = 0.043$; CD4+ CD25+ Treg numbers: 0.0359 ± 0.0094 G/l vs. 0.0454 ± 0.0138 G/l, respectively, $p = 0.048$; CD4+ CD25+ Treg percentages: 4.88 ± 1.17 % vs. 6.02 ± 1.48 %, respectively, $p = 0.021$).

Suppressor activity of CD4+ CD25+ Treg cells

The in vitro suppressor capability of CD4+ CD25+ Treg cells was reduced in patients compared to that found in healthy individuals (1.66 ± 0.30 vs. 2.21 ± 0.75, respectively, $p = 0.014$), however, already after the first cycle, it improved significantly (2.29 ± 0.46 vs. 1.66 ± 0.30, respectively, $p < 0.001$) and became similar to control values.

Levels of circulating cytokines

According to our results, concentration of circulating IL-10 significantly elevated already after the second cycle of ECP treatment, compared to baseline (5.18 ± 4.38 pg/ml vs. 2.15 ± 2.01 pg/ml, respectively, p = 0.022). Additionally, levels of IL-1Ra showed a statistically significant increasing trend over time (F = 2.919, p = 0,028). The pro-fibrotic cytokine TGF-beta decreased significantly already after the first treatment compared to baseline values (1.28 ± 0.46 pg/ml vs. 1.77 ± 0.84 pg/ml, respectively, p = 0.049), moreover, levels of HGF showed a statistically significant upward trend over time (F = 2.687, p = 0.041). Interestingly, CCL2 chemokine levels significantly decreased already after the second treatment, compared to baseline values (377.99 ± 83.62 pg/ml vs. 463.11 ± 115.01 pg/ml, respectively, p = 0.037).

Levels of autoantibodies

Among patients, we found 15 anti-Scl-70 antibody-positive individuals. No changes in levels of autoantibodies were detected during the investigated period.

Associations between the changes of laboratory parameters

When we assessed associations between laboratory parameters, we observed significant negative correlations between the changes of the absolute numbers and percentages of peripheral CD95+ T and CD4+ CD25+ Treg cells (cell numbers: R = -0.507, p = 0.048; cell percentages: R = -0.514, p = 0.042).

Regarding early- and late-activated T lymphocytes, after the last ECP cycle, clear negative correlations developed between percentages and the functional ability of CD4+ CD25+ Treg cells (early-activated T-cells: R = -0.520, p = 0.039; late-activated T-cells: R = -0.526, p = 0.036).

Association between clinical amelioration and changes in peripheral immune parameters

Significant correlations was observed between the reduction of absolute numbers and percentages of peripheral Th17 cells and the reduction of the skin thickness measured by ultrasound scanner at the base of 3rd finger (Th17 numbers: R = 0.784, p = 0.001; Th17 percentages: R = 0.649, p = 0.009) and forearm (Th17 numbers: R = 0.532, p = 0.043; Th17 percentages: R = 0.518, p = 0.048).

5. DISCUSSION

5.1. Investigation of Sjögren's syndrome

Our findings indicated that several immune-competent cell types with regulatory capability were represented with disproportional levels in pSS compared to healthy individuals. According to our hypothesis, the elevation of NK and NKT cell levels in pSS could be part of an increased counter-regulatory reaction, presumably compensating the derailed, disproportional immune responses. We observed that NKT levels in patients with EGMs were increased significantly compared to pSS without EGMs. We found significantly elevated Tr1 cell percentages in pSS patients compared to controls, moreover, in pSS exhibiting a systemic, more pronounced course of disease (pSS with EGMs), an additional increase of Tr1 cell levels was shown. Taken together, these observations are in line with the idea that the ongoing proinflammatory autoimmune machinery in pSS could lead to an enhanced counter-regulatory reaction.

In our study, decreased percentages of CD4+ CD25+ Treg cells in patients with pSS were found, assuming that the reduced numbers of this key regulatory cell type is insufficient to dampen the autoimmune processes in pSS. Interestingly, significantly elevated levels of CD4+ CD25+ Treg cells in patients with EGMs were determined when compared to patients without EGMs. Nevertheless, these values were still significantly below control values, and our results suggest that in pSS quantitative changes of CD4+ CD25+ Treg cells has an important role in the failure of immunoregulation, which leads finally to the perpetuation of the disease.

The function of CD4+ CD25+ Treg cells is highly dependent upon the local milieu. It has been shown that inflammatory cytokines, such as TNF-alpha and IL-6 can temporarily impair the peripheral generation and function of Tregs, and render auto-aggressive T-cells resistant to Treg-mediated regulation. Interestingly, we found significantly elevated levels of both TNF-alpha and IL-6 in serum of pSS compared to healthy blood donors, raising the possibility that the increased levels of these cytokines impair the counter-regulatory function of Tregs. As the next step, we carried out an in vitro assay of CD4+ CD25+ Treg cells in order to reveal a possible functional defect in the suppression capability of these cells in pSS. We established a decreased suppression effect of Tregs in pSS, which indicates that not only are the decreased peripheral CD4+ CD25+ Treg levels responsible for their insufficient operation, but their altered function as well.

A significant decrease in serum IL-10 levels was measured in pSS patients when compared to healthy individuals. The cytokine IL-10 plays a crucial role in controlling immune responses. Interestingly, our results indicate that although as a counter-regulatory process the number of IL-10-producing Tr1 cells is increasing in patients with pSS, serum IL-10 levels are not elevated compared to healthy subjects. The significant negative correlation between the levels of Tr1 cells and of its product assume an inappropriate anti-inflammatory cytokine secreting function of Tr1 cells.

Our study revealed an association between changed peripheral immune parameters and the impairment of an important feature of exocrine secretory capability in pSS (sialometry). Decreased secretory capacity is associated with disease severity, and the deterioration of exocrine secretory function is a cornerstone of pSS pathogenesis. We found a significant negative correlation between the level of peripheral CD4+ CD25+ Treg cells and sialometry values. Along with the loss of secretory function, the intensification of a counterbalance-mechanism appears; thus levels of CD4+ CD25+ Treg cells increase as a feedback process attempting to compensate the progression of disproportional immune responses. We assume that the aforementioned qualitative and quantitative changes could be caused at least partly by elevated IL-6 and TNF-alpha levels in patients with pSS.

5.2. Investigation of systemic sclerosis

Both CD4+ and CD8+ naive T-cell percentages were decreased, while both CD4+ and CD8+ central memory T-cell percentages were increased in SSc, and along with the increased chronically activated T-cell populations, these findings represent an immunologically active state.

We found elevated peripheral blood NK cell percentages in SSc, in accordance with previous findings. Of note, when patients were divided into groups based on the presence of visceral involvement, we found further increased NK cell percentages in the more severe clinical state; however, patients with only skin symptoms had values similar to controls. The NK cell expansion may be related to the more pronounced inflammation processes and IL-2 and IL-15 cytokine production, which can induce NK cell proliferation in SSc. Concerning NK T cells, percentages were decreased in SSc patients, and taken these findings together, the alteration of both NK and NKT cells may contribute to the skewed immune responses.

When analysing the fine functional balance between Th1 and Th2 cells, we found significantly decreased Th1 cell percentages. Although Th2 cell percentages and levels of

Th1- and Th2-linked cytokines were similar in both groups and the changes in Th1/Th2 ratios did not reach significance, our findings at least partially support the hypothesis of a skewed Th1/Th2 balance.

The recognition of Th17 cells opened new avenues to explain different autoimmune features, and the essential role of these cells was underlined in several autoimmune diseases. In addition to the pronounced Th17 profile, we found decreased CD4+ CD25+ Treg cell percentages in SSc. Thus, the Th17/CD4+ CD25+ Treg ratio changed, turning the fine balance towards enhanced immune reactivity. We also carried out an in vitro assay of CD4+ CD25+ Treg cells, and observed an abnormal suppressor effect, which indicates that not only is the peripheral presence of CD4+ CD25+ Treg cells decreased, but also their altered function may be responsible for their failure in immunoregulation.

We measured lower IL-10-producing Tr1 cell percentages and decreased circulating IL-10 levels in SSc. It is known, that IL-10 is produced not only by T-cells, but also by B-cells and other PBMCs. A recent study demonstrated that IL-10 secretion by PBMCs of SSc patients and healthy individuals is similar. However, stimulated PBMCs seem to produce more IL-10 in SSc compared to controls, which may be part of an increased counter-regulatory reaction, presumably compensating for the reduced amount of IL-10 produced by Tr1 in the disease.

Moreover, there was a negative correlation between Tr1 cell percentages and MRSS, and we assume that along with the decrease in the Tr1 profile, the altered regulatory processes cannot control the enhanced autoimmune mechanisms; thus more serious clinical manifestations appear, as a result of the progression of disproportionate immune responses.

Although the complement pathway is not commonly thought to be part of the pathogenesis of SSc, the disease is often associated with hypocomplementaemia. The negative correlations between the percentages of CD4+ CD25+ Treg cells and complement levels indicate that disturbances in regulative T-cell proportions may be associated with the complement pathway in SSc.

5.3. The clinical and immunological effects of photopheresis

Our study confirmed the positive effect of ECP on clinical symptoms of SSC. During ECP therapy, MRSS scores decreased, joint mobility improved, oral aperture progressively increased and mimical function became better. Before our present investigations, the immunobiological mechanisms of ECP did not studied in scleroderma in details, thus our

laboratory results can only be compared to immunological consequences of ECP observed in other diseases.

Clinical and pathological evidence support the concept that SSc is primarily a vascular disease that is mediated by autoimmune processes and results in tissue fibrosis. Recent studies highlighted the key roles of the innate and adaptive immune system in autoimmune processes characteristic to SSc. Besides the abnormalities of NK and NKT cells, alterations in Th17, Tr1 and CD4+ CD25+ Treg cell subsets were also demonstrated in SSc. In accordance with the literature, we found increased NK and Th17 cell numbers and percentages, while values of NKT, Th1, Tr1 and CD4+ CD25+ Treg cells were decreased in SSc patients, compared to those found in controls. These results reflect that the altered Th17 and regulatory T-cells ratio may play a pathogenic role by tipping the fine balance toward enhanced immune reactivity. ECP treatment seems to have a strong effect on most of the T-cell subtypes, but not on NK and NKT cell counts. Based on studies focusing on immunological effects of ECP in GVDH, photopheresis, besides the direct elimination of autoreactive cells, may increase the proportions of peripheral CD4+ CD25+ Treg cells, contributing to the restoration of disproportional autoimmune responses. We also observed that effect on CD4+ CD25+ Treg subsets, moreover, along with the elevation of CD4+ CD25+ Treg cell counts, CD 95+ T-cell counts decreased. The CD95, also known as Fas receptor (FasR) is a death receptor on the surface of cells, its activation leads to programmed cell death. Enhanced FasR expression is characteristic on activated lymphocytes, which suggest that signalling through this type of death receptors governs homeostasis of the lymphoid system by eliminating the over-activated auto-reactive lymphocytes. According to our results, the reduction of FasR positive T-cells seems to be much more pronounced in those patients who had greater elevation in CD4+ CD25+ Treg values during the therapy. We also carried out an in vitro assay of CD4+ CD25+ Treg cells, and observed significant improvement in suppressor function during ECP therapy, moreover, after the last cycle, a negative correlation developed between the Treg suppressor capability and the levels of early- and late-activated T-cells. These findings underline the central role of CD4+ CD25+ T-cells in the attenuating effects of ECP on the ongoing autoimmune processes.

However, changes in proportions of T-cell subsets seem to be more complex. While ECP increases the numbers and percentages of IL-10-producing Tr1 cells, values of Th17 cells decrease following the therapy. According to our results, the changes in the parameters of Th17 cells seem to be much more pronounced in the patients with greater improvement in skin thickness during the therapy.

We found no significant changes in pro-inflammatory cytokine levels during the therapy. Regarding anti-inflammatory ones, we observed elevation in the concentration of IL-10, which may be the consequence of increased Tr1 cell counts. Along with IL-10 levels, concentration of IL-1RA also showed significant elevation during the therapy. The cytokine IL-1RA, by inhibiting competitively the binding of IL-1 to cell surface receptors, prevents the pro-inflammatory effects of IL-1, thus functions as a major naturally occurring anti-inflammatory protein. Since the elevated IL-1-beta levels impair the suppression ability of CD4+ CD25+ Treg cells and contribute to the generation of Th17 cells, the increase in levels of IL-1Ra, along with the increased IL-10 levels, may also contribute to the deceleration of the enhanced autoimmune responses.

Regarding fibrogenesis, growing evidence indicates the critical involvement of infiltrating activated macrophages and T-cells in the production of a variety of pro-fibrotic cytokines such as TGF-beta, CCL2, IL-2, IL-4, IL-6 and IL-17, all of which induce or promote fibrosis and fibroproliferation. We found that ECP reduces significantly TGF-beta and CCL2 levels in SSc, leading to attenuated pro-fibrotic activity, which at least partly explain the clinical amelioration observed due to the therapy. Interestingly, a recent study reported decreased levels of HGF, which is a pro-angiogenic but anti-fibrotic factor, in the disease. The deficiency or reduction of HGF may prevent vascular repair and increase tissue fibrosis in the disease. According to our results, ECP treatments increase the levels of HGF, which, together with the decrease in TGF-beta and CCL2 levels, may be an effective molecular therapeutic response resulting in attenuated fibrosis.

Although photopheresis may not improve the previously developed fibrosis in SSc, the therapy contributes to the deceleration of disease progression. Additionally, the fact that our patients did not show any adverse reaction to ECP, underlines the minimal toxicity of ECP, which is a clear advantage compared with other immunomodulatory therapies.

6. SUMMARY

The results of our study in Sjögren's syndrome (SSc) indicate that cells with certain regulatory activity are involved in the pathological immune mechanisms both at the level of innate and adaptive immune system. The increased percentages of peripheral NK, NKT and Tr1 cells may be part of an increased counter-regulatory reaction, presumably compensating the derailed, disproportional immune responses. Contrary to the other cell types, peripheral CD4+ CD25+ Treg cells showed decreased percentages in SSc. We were the first to report the decreased suppressor activity of CD4+ CD25+ Treg cells in primary Sjögren's syndrome. We demonstrated elevated IL-6 and TNF-alpha levels in the sera of the patients, which may be at least partially responsible for the functional defect in the suppression capability of these cells. In summary, our observations indicate that not only the decreased peripheral CD4+ CD25+ Treg cell percentages, but also their altered function may be responsible for their insufficient regulatory operation in pSS.

In systemic sclerosis (SSc), we observed increased Th17 cell percentages, while the ratios of the regulatory cells, such as IL-10-producing Tr1 and CD4+ CD25+ Treg cells were decreased. The levels of IL-10 and the suppressor activity of CD4+ CD25+ Treg cells were also lower in patients. Moreover, we revealed a negative correlation between the modified Rodnan skin score and Tr1 cell percentages, which indicates that beside the altered Th17/CD4+ CD25+ Treg ratio, the role of Tr1 cells may be also important in the progression of disproportionate immune responses in SSc.

During the investigation of the clinical efficacy of extracorporeal photopheresis (ECP) in SSc, in accordance with the earlier studies, we observed significant amelioration of symptoms. Since the immunobiological effects of ECP have not been investigated in SSc before our study, all of the laboratory results are novel observations in the international literature.

According to our results, ECP treatments reduce the number of Th17 cells, and increase the number of Tr1 and CD4+ CD25+ Treg cells in the peripheral blood. Moreover, the therapy improves the suppressor capacity of Treg cells in SSc patients. Levels of CCL2 and TGF-beta decreased, while levels of IL-1Ra, IL-10 and HGF increased. Thus ECP contributes to the restoration of the balance between regulatory and effector immune mechanisms, which leads to the deceleration of disease progression.

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List of publications related to the dissertation

1. **Papp, G.**, Horváth, I.F., Baráth, S., Gyimesi, E., Végh, J., Szodoray, P., Zeher, M.: Immunomodulatory effects of extracorporeal photochemotherapy in systemic sclerosis. *Clin. Immunol. 142* (2), 150-159, 2012.
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