Investigation of immunological alterations influencing the clinical picture of systemic lupus erythematosus

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PhD Thesis

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Szeged, 2015

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Abbreviations

aCL - anti-cardiolipin

ACR - American College of Rheumatology

AECC - American-European Consensus Criteria

AGSE - peptide aa184-227 from the second extracellular loop of the m3AChR

ANA - antinuclear antibody

anti-MCV - anti-mutant citrullinated vimentin

anti-RNP - anti-ribonucleoprotein

anti-Sm - anti-Smith

anti-SSA - anti-Sjögren’s syndrome A

anti-SSB - anti-Sjögren’s syndrome B

APA – antiphospholipid antibody

APC - antigen presenting cell

APS - antiphospholipid syndrome

aß2GPI - anti-beta2glycoprotein I

BAGSE - BSA-AGSE multiple-conjugated peptide

BSA - bovine serum albumin

C3 - complement 3

C4 – complement 4

CMV - cytomegalovirus

DNA – desoxiribonucleic acid (ds – double stranded)

EBV - Epstein-Barr virus

ELISA – enzyme-linked immunosorbent assay

ESR - erythrocyte sedimentation rate

FACIT - Functional Assessment of Chronic Illness Therapy

FcRγ – Fc receptor γ

FCS - foetal calf serum

GAGSE - GST-AGSE fusion peptide

Gal-1 – Galectin-1 (in – intracellular, ex – extracellular)

GST - glutathion-S-transferase

GYNIP - GST-YNIP fusion peptide

HeLaGal - Gal-1 transgenic human cervix adenocarcinoma cells

HeLamock - Mock transfected human cervix adenocarcinoma cells

HLA – human leucocyte antigen

i.v. – intravenous

Ig – immunoglobuline

IL – interleukin

LA - lupus anticoagulant

Ly – lymphocyte count

m3AChR - muscarinic acetylcholine receptor subtype-3

MEM - Minimum Essential Medium

mRNA - messenger ribonucleic acid

NMDA - N-methyl-D-aspartate

OD - optical density

p56lck - lymphocyte-specific protein tyrosine kinase 56

PBMC - peripheral blood mononuclear cells

PCR – polymerase chain reaction

PHA – phytohaemagglutinin

RA - rheumatoid arthritis

RAR - relative apoptotic ratio

RF - rheumatoid factor

RPMI - Roswell Park Memorial Institute medium

SD – standard deviation

SF-36 – short form 36 health survey

SLE – systemic lupus erythematosus

SLEDAI-2K – systemic lupus erythematosus disease activity index 2000

SLICC - Systemic Lupus International Collaborating Clinics

SPSS - Statistical Package for the Social Sciences Software

SS - Sjögren's syndrome (p – primary, s – secondary)

Syk - Spleen tyrosine kinase

TCR – T cell receptor

Th – helper T cell

TLR - Toll-like receptor

TNFα – tumour necrosis factor α

Treg – regulatory T cell

TRIC - peptide aa360-377 from the third intracellular loop of the m3AChR

WBC – white blood cell count

YNIP - peptide aa506-521 from the third extracellular loop of the m3AChR

ZAP70 - Zeta-chain-associated protein kinase 70

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by disorders of both the cellular and humoral immune responses, production of autoantibodies and formation of immune complexes leading to a diverse array of clinical manifestations. It is a multisystem disease involving the immune-mediated inflammation in multiple organs affecting predominantly the joints, kidneys, mucocutaneous and nervous system and causing haematological abnormalities. The course of the disease is tipified by exacerbations and remissions. The severity of the clinical picture is greatly affected by the number and nature of the various organ manifestations, while the mortality of patients with SLE is still remarkable. Fatal outcome is not only the result of lupus activity when vital organs are involved, but also the complications of immunosuppressive treatment, in particular infections, or the long-term complications such as cardiovascular disease.(1, 2).

SLE afflicts mainly women, with the ratio of 9:1 compared with men. The disease can develop at any age with varying characteristics (3). Typically, the manifestation of the disease tends to be more acute in those of younger age. However, patients with late onset lupus have a much higher mortality rate, nearly 50% of them die due to complications of infection. This is most likely the impact of older age, since the manifestation of their disease is usually less severe than in younger patients. Women of childbearing age are at particular risk of the development of SLE.

The diagnosis of SLE is established on expert opinion and is aided by the latest classification criteria of the Systemic Lupus Collaborating Clinics (SLICC), who revised and validated the American College of Rheumatology (ACR) SLE classification criteria in 2012 (4, 5). From the seventeen identified SLICC criteria, the diagnosis of SLE requires either the fulfillment of at least four criteria, with at least one clinical and one immunologic criterion, or lupus nephritis as the sole clinical criterion in the presence of antinuclear antibody (ANA) or anti-dsDNA antibodies. Although SLE usually occurs alone, many patients have symptoms characteristic of one or more of other connective tissue diseases, such as Sjögren's syndrome (SS), antiphospholipid syndrome (APS), rheumatoid arthritis (RA), scleroderma, polymyositis-dermatomyositis, and various forms of vasculitis. An overlap diagnosis can be made when a patient meets the official criteria for two or possibly more autoimmune diseases.

The complex etiopathogenesis of SLE still remains elusive (6,7). Various genetic, environmental and immunomodulatory factors contribute to the expression of SLE. The already identified heritable factors associated with SLE include the combined effect of genes that are involved in nucleic acid sensing, interferon production, antigen-presentation (HLA-associations), and in T or B cell signaling pathways. Epigenetic changes such as DNA hypomethylation in CD4 T cells also play an important role in the regulation of gene expression. Exposure to ultraviolet light, various environmental toxins (e.g. smoking), viral infections including parvovirus B19, cytomegalovirus (CMV), and Epstein-Barr virus (EBV) are known risk factors. Pathogen-associated nucleic acids can exacerbate SLE pathology through stimulation of Toll-like receptors (TLRs), moreover, the discrimination by TLRs between pathogenic and self nucleic acids is not perfect, thus endogenous nucleic acids of apoptotic cells and necrotic debris may also activate TLRs.

The immune system is broadly compromised in patients with SLE, and the dysregulation of single elements induces further, multiple alterations at virtually every component of the system. A central role for T cells is suggested in the pathogenesis of the disease, and intrinsic aberrations of SLE T cells are considered to be the major factor in the pathologic process (7-9). An important step is the enhanced T cell activation due to abnormal antigen presenting cell (APC) function. Multiple activation and signaling defects have been reported in SLE T cells. The key players in the enhanced early signaling are the altered T cell receptor/CD3 complexes (CD3ζ is replaced by the FcRγ chain, that associates with Syk kinase), and the aggregation of lipid rafts on the cell surface (membrane zones rich in signaling molecules, thus facilitating T cell activation). The abnormal gene transcription profile observed in lupus T cells is also complex, in some aspects it shows the phenotype of activated T cells, and in others shares characteristics with anergic cells. Furthermore, the abnormally increased expression of the adhesion molecule CD44 on SLE-T cells grants them an increased capacity to migrate into inflamed organs.

Activated SLE T cells provide excessive help to B cells, infiltrate target organs causing damage, while failing to produce sufficient interferon-γ and interleukin-2 (IL-2). This low IL-2 level may account for the known decreased cytotoxic activity, defective Treg function, and decreased activation-induced cell death in SLE patients (7). Moreover, sera of patients with lupus contain abnormally high levels of IL-17, that amplifies the inflammatory response by recruiting effector cells to target organs, and contributes to the survival and

proliferation of B cells and their transformation into antibody-secreting cells. B cell overactivity accounts for the abnormal production of a wide spectrum of autoantibodies against soluble and cellular constituents, but most commonly against intranuclear antigens (ANAs) (9). They form immune complexes that deposit in tissues propagating a chronic inflammatory process that destroys organ parenchyma and results in end-stage organ failure. It is only a handful of these autoantibodies that have been shown to contribute convincingly to disease-related tissue injury, for exaple anti-blood-cell antibodies that activate complement and cause cytopenias, and anti-dsDNA antibodies that are thought to contribute to the development of nephritis, and crossreact with N-methyl-D-aspartate (NMDA) receptors in the brain thereby causing neuropsychological manifestations (7).

1.1. Investigation of the relationship of exocrine gland dysfunction with autoantibodies to muscarinic acetylcholine receptor-3 and mental health status parameters in SLE overlapping with Sjögren's syndrome

In SLE, one of the most frequent overlapping autoimmune disease is SS. In this syndrome the fundamental symptoms are decreased tear and saliva production, which lead to xerostomia and keratoconjuctivitis sicca. The pathogenesis of SS is characterized by the chronic inflammation of the exocrine glands, particularly the lachrymal and salivary glands, and a wide variety of further immune-mediated organ involvements may develop (10). SS often overlaps with other systemic autoimmune diseases including RA or systemic sclerosis, but it can also present as a separate entity (primary SS). An associated SS alters the immunological phenotype of SLE including the organ manifestations or the immunoserological profile (11).

Similarly to SLE, polyclonal B cell activation dominates the immunological alterations of SS, and in the affected secretory tissues focal lymphocytic infiltration can be detected. It has previously been observed that the extent of salivary gland tissue damage caused by lympocytic infiltration is not proportional to the salivary flow impairment (12). An immune-mediated mechanism has been proposeded to impair the glandular function by means of autoantibodies which block the functionally dominant muscarinic acetylcholine receptor subtype-3 (m3AChR) on acinar cells (13-15). These postsynaptic muscarinic receptors modulate the saliva production upon the signals of the autonomic nervous system. The

presence of circulating autoantibodies directed against the m3AChR has previously been demonstrated in patients with pSS (16-22), and their binding to muscarinic receptors on human salivary gland acinar cells has also been confirmed (23). These anti-m3AChR autoantibodies have been hypothesised to block the parasympathetic neurotransmission (15, 24), thereby serving as an example of how a pathological immunological process can lead to organ dysfunction by inhibiting the normal function of the autonomic nervous system.

Although the role of anti-m3AChR antibodies seems to be established in pSS by experimental settings, involving rodent tissues and cultured human salivary glands (24-26), formal proof of their functional role in humans is still lacking because a validated immunodiagnostic test is still not available. The exact epitope-specificity of anti-m3AChR antibodies has also remained yet unidentified, nevertheless several epitopes on the m3AChR were proposed as targets of the immune response in SS, specifically on the second and the third extracellular loop (20-22, 24, 27). The prevalence and pathogenic role of the various anti-m3AChR antibodies have not been systematically examined to date in other systemic autoimmune diseases including SLE overlapping with SS.

Sicca symptoms are common not only in definite SS. A sicca syndrome clinically indistinguishable from SS also develops in a considerable proportion of patients with organ-specific autoimmune diseases, such as primary biliary cirrhosis, multiple sclerosis or gluten-sensitive enteropathy (28), and in other chronic non-immune-mediated illnesses, such as fibromyalgia or chronic fatigue syndrome (29, 30). In the background of the observed exocrine insufficiency, the effect of chronic stress and an altered neuro-endocrine homeostasis have been presumed, which are modulated by an autonomic nervous system dysfunction. Several data raised the concept that chronic stress itself has direct impact on saliva production, and it may lead to chronic xerostomia (31-34). It is suggested that the impairment of mental health based on chronic stress, depression and the long-standing distress associated with systemic autoimmune diseases such as SLE (35, 36) may also negatively influence the salivary gland function. No data are available whether mental health alterations may correlate with the development of a sicca syndrome in SLE, in addition to the immune-mediated mechanisms.

1.2. Investigation of the risk of the development of non-thromboembolic organ manifestations in systemic lupus erythematosus associated with antiphospholipid syndrome

In SLE, the production of a boundless spectrum of autoantibodies is observed. Some of these SLE-related antibodies correlate with the disease activity (e.g. anti-dsDNA), while others are markers of specific disease subsets (e.g. anti-Ro/SSA-positivity increases the likelihood of subacute cutaneous lupus and congenital lupus including atrioventricular conduction block). The formation of antiphospholipid antibodies (APAs) is definitely pathogenic (37,38) and their presence defines a specific disease subset. APA positivity itself is considered a prothrombotic condition, since these autoantibodies exert widespread effects on the coagulation cascade and on the cells involved in haemostasis including endothelial cells and thrombocytes. Moreover, APAs facilitate atheroma formation, leading to accelerated atherosclerosis (39-41). Knowledge on the associations of autoantibody positivity and organ manifestations facilitates a more accurate and timely recognition and a better prevention or early treatment of the respective clinical manifestations of SLE.

The APAs form a heterogeneous group of autoantibodies, that are directed against anionic membrane phospholipids and associated proteins. The most common and intensely studied members are lupus anticoagulant (LA), anti-cardiolipin (aCL) and anti-beta2-glycoprotein I (aβ2GPI). The latter two antibodies can be present in IgG, IgM and IgA isotypes, and the IgG class antibodies in particular are of clinical significance (40, 42, 43). The reported prevalence of APAs in SLE varies between 15 and 35% (44-47).

When the presence of APAs is accompanied by arterial or venous thrombotic events or an adverse pregnancy outcome, this clinical condition is called antiphospholipid syndrome (APS) (48-50). APS is predominantly associated with SLE of the systemic autoimmune diseases, but it can also present as a secondary feature in malignant processes and infections, or it may develop as a primary, independent entity. The leading APS-related clinical features are the results of hypercoagulability and the specific vaso-occlusive, ischaemic lesions. Patients with persistently positive APAs, particularly those who exhibit LA or triple autoantibody positivity, are at higher risk for thrombosis, and the risk is also higher in patients with arterial thrombosis or those with recurrent thrombosis despite antithrombotic therapy. In addition, immune-mediated mechanisms also play role in the development of several

manifestations of APS including thrombocytopenia, pregnancy complications and neurological symptoms.

APS complicates SLE by adding a vaso-occlusive factor to the inflammatory component that adversely affects the prognosis. APS modifies the clinical picture of SLE also in other aspects, for example, cutaneous laesions (e.g. Raynaud's phenomenon, livedo reticularis), Coombs-positive haemolytic anaemia and non-bacterial endocarditis have been found to be more common in overlapping disease subsets than in SLE without APS (48, 51). APS is also a major predictor of irreversible organ damage and death in patients with SLE (51), in part because the development of cardiovascular and cerebrovascular diseases cause a substantial morbidity. In summary, the combination of SLE and APS appears to be of greater concern than either entity alone. However, little is known about the distinct influence of APS on the non-thrombotic disease manifestations of SLE.

The variable clinical phenotype and heterogenity in the autoantibodies that are associated with SLE and APS result in challenges in defining the optimal treatment. The high incidence of recurrent thromboembolism that characterizes this condition carries the necessity of antithrombotic therapy. While APA positivity itself requires the use of single antiplatelet medication, the recommendations for the treatment of APS include long-term anticoagulation. In addition, hydroxychloroquine has been shown to reduce the risk of an initial thrombotic event in SLE patients with or without APAs, and is frequently used in combination with antithrombotic therapy in these patients. When the inflammatory responses predominate the clinical picture, immunosuppressive treatments are administered according to the severity of the disease. The adverse events in correlation with the treatment eg. bleeding, toxicity or infections represent additive risk in the management of SLE + APS patients.

1.3. Investigation of the role of Galectin-1 in the apoptosis of T cells in systemic lupus erythematosus

T cell homeostasis and survival plays a crucial role in the loss of peripheral tolearance and thus in the development of autoimmune diseases. T cell dysfunction, including the malfunction of apoptosis is a major factor in SLE pathophysiology, and dysregulated T cell apoptosis in SLE has been reported previously (8). Galectins, a family of beta-galactoside

binding lectin-type proteins, are prominent contributors in innate and adaptive immune responses. Galectin-1 (Gal-1) has well-defined functions in maintaining immunohomeostasis (52, 53), partly *via* the induction of apoptosis of activated T cells, and growing evidence suggests its impact in autoimmune processes. Regarding the T-helper (Th) cell-centered autoimmunity and the apototic disturbances of SLE T cells, the possible role of Gal-1 can be suggested in the pathogenesis of SLE.

Gal-1 is present in peripheral lymphoid organs and inflammatory sites, and exerts various biological effects as an anti-inflammatory agent and as a suppressor of various T cell functions. It has been shown that Gal-1 inhibits cytokine production and induces apoptosis in activated Th1 and Th17 cells. This function has been attributed to its secreted form (54). The mechanism of Gal-1-triggered cell death has been extensively studied *in vitro* (55, 56). The stimulation of apoptosis requires direct interaction between T cells and the surrounding environment (cells or extracellular matrix) (55). The further signaling intracellular pathways involve the p56lck and ZAP70 kinases, release of ceramide, decrease of mitochondrial membrane potential, and activation of caspases (55, 57, 58). Immunoregulatory function of Gal-1 has also been confirmed by *in vivo* experiments in rodents (56). The differential impact of Gal-1 on the T cell subpopulations shifts the immune response from the inflammatory Th1 to the Th2 direction (59). While inhibiting the cytokine production and triggering apoptosis of activated Th1 and Th17 cells, Gal-1 induces cytokine production of Th2 and Treg cells (60). T cells are targets of extracellular Gal-1 (exGal-1), nevertheless they produce Gal-1 themselves upon activation (61). The function of the *de novo* expressed Gal-1 (intracellular Gal-1 - inGal-1) has remained largely elusive. It has been suggested by Blaser *et al*. that Gal-1 might act as an apoptotic factor via autocrine or paracrine mechanisms (61). However, it has not been sufficiently proven whether Gal-1 is secreted from the cells or remains intracellularly, and possibly contributes to cell death as an intracellular protein. In contrast, recent studies of our group have proven that Gal-1 remains intracellularly during T cell activation, and it implies that Gal-1 can not perform its physiological activities in the hypothesized autocrine or paracrine way. Consequently, we have also proposed that soluble Gal-1 is not an appropriate experimental method to study the apoptotic effects of Gal-1, but rather, Gal-1 requires direct cell-cell contact to exert its apoptotic effects on T cells. Previous experiments from our laboratory have confirmed that exGal-1 deriving from Gal-1 transgenic HeLa tumour cells, expressed on the surface of these cells, is a suitable experimental setting for the study of Gal-1-induced T cell apoptosis (55).

The role of Gal-1 in the development and progression of various immune-mediated diseases and malignancies has been addressed in some studies. It has been revealed that Gal-1 expression is diminished at the sites of severe chronic inflammation such as psoriatic skin (62). In contrast, the overexpression of Gal-1 is typical in malignant diseases, and this promotes tumour immune-escape from anti-tumoural immune surveillance, tumour cell dissemination, thereby leading to poor prognosis of cancer patients (52, 53, 104). Furthermore, the impact of Gal-1 treatment has recently been shown by *in vivo* experiments including the animal models of autoimmune or inflammatory diseases such as arthritis, colitis, hepatitis, nephritis, encephalomyelitis and SLE (63-69). All parameters of experimentally induced arthritis, such as incidence, clinical score and paw edema have been found to be significantly higher in Gal-1 knockout mice than in wild type animals and Gal-1 therapy has been revealed to be efficacious in amelioration of the disease (69). Recombinant Gal-1 injections suppress the clinical and histological signs of autoimmune encephalomyelitis, a T cell mediated disease directed against myelin basic protein, whereby it blocks the sensitization of encephalitogenic T cells (68). Regarding the results in experimental SLE, the administration of recombinant Gal-1 to SLE-prone mice reduced lymphocyte activation, inhibited serum anti-dsDNA IgG antibody production, decreased the incidence of proteinuria, and increased survival rate. In addition, aged Gal-1-deficient mice had higher serum levels of antibodies against dsDNA. *Ex vivo* T cells from lupus-mice treated with recombinant Gal-1 were less efficient in the elicitaion of lipid raft clustering and exhibited less proliferation in response to TCR stimulation (65). These therapeutic results suggest the role of underexpression of Gal-1 in the pathogenesis of autoimmune disorders including SLE.

Gal-1 expression in pathological T cells, i.e. activated T lymphocytes from patients with autoimmune diseases, has not been specifically investigated so far. There are no published investigations available about the expression and immunomodulatory activity of Gal-1 in human SLE. Considering the Th1 and Th17 driven autoimmunity and the apototic dysregulation of T cells, SLE is suitable to study the function of Gal-1 in pathological conditions.

2. Objectives

The exact epitope-specificity, the prevalence and the pathogenetic role of the various anti-m3AChR antibodies have not yet been studied in SLE overlapping with SS. In this work we wished **to determine the antigenic epitope of m3AChR which interacts with autoantibodies from SLE, RA and pSS patients**. Rheumatoid arthritis was included in the study as the most common inflammatory rheumatic disease, that may also overlap with SS in a significant proportion of the patients. **We attempted to develop an appropriate immunodiagnostic method for the detection of these anti-m3AChR antibodies**, and to compare their prevalence in the studied disease groups. Furthermore, we also aimed to assess the various disease-specific clinical correlates of the anti-m3AChR autoantibodies in SLE patients. With regard to the complex pathogenesis of impaired exocrine gland function, our further objective was **to address the relative contributions of** immunological factors, in particular anti-m3AChR antibodies, and **mental health status** (as assessed with the SF-36 and the Functional Assessment of Chronic Illness Therapy - FACIT scales) **to the elicitation of the sicca complex arising in SLE.**

The presence of antiphospholipid antibodies in SLE patients bears the risk of the development of certain micro- and macrovascular organ involvements in which acute or chronic thrombotic mechanisms are key pathogenetic factors. However, there are no available data whether patients with concomitant APS are predisposed to further non-thrombotic SLE-related morbidities. In this work we set out to define the impacts of APA production alone, and an associated APS on the clinical presentation of SLE. Our objective was **to compare the frequencies of the various non-thrombotic SLE manifestations between the subgroups of APA-positive and APA-negative patients, and between the patients with and without definitive APS**. We aimed to address differences in disease severity and progression between the studied patient subsets. Furthermore, we were also searching for correlations of the therapeutic requirements in these patient subgroups as this can further modify the clinical outcome in addition to the SLE-specific disease manifestations.

Gal-1 has emerged as an important immunomodulatory factor in autoimmune and inflammatory diseases. Although Gal-1 participates in the maintenance of immunohomeostasis by regulating T cell functions and survival, its role in the lupus T cell

pathology has not yet been explored. In this work, the possible pathogenetic role of Gal-1 was studied in SLE. Based on the reported data and the previous results of our study group, we hypothesised that the expression of Gal-1 is diminished in SLE T cells compared to healthy control cells and, as a consequence, these pathological T cells are less sensitive to the exGal-1-induced cell death. The aim of our work was **to analyse the sensitivity of SLE T cells to the exGal-1-mediated apoptotic signal.** Furthermore we were searching for a correlation between disease activity and the Gal-1 related apoptotic disturbances of SLE T cells. We aimed to identify whether different disease activity markers predispose SLE patients to decreased T cell apoptosis, thereby contributing to the development of the disease.

3. Patients and methods

3.1. Investigation of the relationship of exocrine gland dysfunction with autoantibodies to muscarinic acetylcholine receptor-3 and mental health status parameters in systemic lupus overlapping with Sjögren's syndrome

3.1.1. Patients

In this cross-sectional, case-control study, specific parameters of an overlapping SS were studied in 103 SLE patients, and compared with those on 65 patients with RA, 76 patients with pSS and 50 healthy controls. The mean age of the SLE patients (9 male and 94 female) was 48 years (25-74 years), that of the RA patients (11 male and 54 female) was 58 years (22-82 years), and that of the pSS patients (2 male and 74 female) was 56 years (30-76 years). The disease groups were classified according to the appropriate international criteria: the modified 1982 SLE criteria of the American College of Rheumatology (5), the 2010 RA criteria of the American College of Rheumatology/European League against Rheumatism (70), and the 2002 American-European Consensus Criteria (AECC) for SS (71).

The presence of sicca complex was evalualed in the SLE and RA patients. Corresponding to the AECC for SS, objective exocrine deficiency was evaluated by means of the Schirmer test and the measurement of unstimulated whole saliva production, while subjective sicca complaints were regarded as present if at least one of the three questions included in these criteria was answered positively. Sicca complex was defined when at least one positive objective test on at least two separate occasions was associated with subjective sicca symptoms of both the eye and the mouth. Labial salivary gland biopsy was not performed in several patients for ethical reasons, this limited the determination of the presence of secondary SS (sSS) as classified by the AECC.

Numerous disease-specific clinical and immunoserological data were collected on the SLE and RA patients to assess the correlates of sicca complex. Selected clinical parameters, including the immunoserological profile of SLE and RA, are presented in *Table 1*.

SLE (n=103)	%	RA (n=65)	%
polyarthritis	89.2	rheumatoid nodules	12.5
photosensitivity	70.6	pulmonary fibrosis	4.7
nephritis	30.4	RF	84.4
serositis	28.4	anti-MCV	77.9
nervous system	21.6	anti-SSA	9.5
lymphopenia	51,5	anti-SSB	7.1
anti-dsDNA	74.2		
anti-SSA	50.5		
anti-SSB	38.6		
low C3, C4	67.6		

Table 1. Characteristic clinical and laboratory variables of SLE (n=103) and RA (n=65). anti-dsDNA: antibody to double-stranded DNA; C3, C4: complement-3 or -4; RF: Rheumatoid factor; anti-MCV: anti-mutant citrullinated vimentin

The patients were on usual immunosuppressive therapies, the most frequently administered therapies for SLE being low dose corticosteroid, chloroquine, azathioprine, cyclopsosphamide or mycophenolate mofetil, and for RA low dose corticosteroid, methotrexate, leflunomide, TNFα inhibitor or anti-CD20 biologic agents. Most of the pSS patients received only symptomatic treatment, but a significant minority was also treated with immunosuppressive therapy, most commonly corticosteroid, chloroquine, or azathioprine. The impact of the immunosuppressive drugs as well as those with anticholinergic effects, specifically antihistaminic, antidepressant, antipsychotic, antispasmodic, antiemetic and antiepileptic drugs, on the exocrine function was assessed.

3.1.2. Organ involvements

The diagnosis of the lupus-related organ involvements were established on standard clinical methods, radiological and histological findings. The manifestations involving the heart, the lungs, the kidneys and the central nervous system were considered as major organ manifestations, as these have a profound impact on the outcome of the disease. Only the manifestations directly attributable to the disease were considered lupus-related, and other causes of organ damage or any other pathological condition e.g. drug or infection-related symptoms were excluded. The definitions of selected organ manifestations were as follows:

Pulmonary involvement: specific signs of parenchymal lung disease, including interstitial pneumonitis, chronic fibrosing alveolitis (pulmonary fibrosis) or acute alveolitis demonstrated by radiological examination.

Nephritis: biopsy-proven lupus glomerulonephritis exhibiting a characteristic histological picture (class I-V. lupus nephritis) or, if a biopsy was not performed, the presence of proteinuria >/= 0.5 g/die and/or microscopic hematuria and/or cylindruria not explained by any condition other than active SLE.

Skin vasculitis: clinical presence of cutaneous vasculitis including periungual vasculitis, digital vasculitis, nodular vasculitis, livedo vasculitis, urticaria vasculitis, purpura or crural ulcers, in selected cases verified by histological examination.

Neuropathy: cranial and peripheral inflammatory neuropathies.

Organic brain syndrome: diffuse brain tissue damage with acute or chronic psycho-organic syndrome (acute confusional state or accelerated cognitive dysfunction).

Overlapping Sjögren's syndrome: presence of objective and subjective sicca symptoms affecting the eyes and/or the mouth, with decreased tear and/or saliva production, meeting the AECC for Sjögren’s syndrome (71).

3.1.3. Laboratory tests

The tests were performed at the Department of Laboratory Medicine of our University by means of routine diagnostic methods (ELISA, nephelometry, LA-sensitive coagulation tests, etc.). The evaluated laboratory features of SLE included leucopenia (WBC <4.0 G/l), lymphopenia (Ly count <1.5 G/l), haemolytic and non-haemolytic anaemia (haematocrit

<35%), and thrombopenia (platelet count <100 G/l). We also studied the immunoserological profile of the patients: anti-dsDNA, ANA, anti-SSA, anti-SSB, anti-RNP, anti-Sm, LA, aCL, aβ2GPI and hypocomplementaemia (C3 and C4) were assessed.

3.1.4. Antigen preparation

The computer software "Peptide Companion" Version 1.231 (Coshisoft/PeptiSearch) was used to identify immunodominant epitopes of the human m3AChR, and peptide aa184-227 (AGSE) from the second extracellular loop of the receptor; peptide aa506-521 (YNIP) from the third extracellular loop; and peptide aa360-377 (TRIC) from the third intracellular loop have been selected as antigens. AGSE peptide is localised in the ligand binding region of the m3AChR.

Then we aimed to test whether the modification of m3AChR pepides could amplify the antigenicity and enhance the detection of the autoantibodies specific to the respective peptides with ELISA. Various forms of these antigens were prepared: First, short linear peptides were synthesised with solid-phase peptide synthesis as described in an earlier publication of our group (16). Afterwards, we constructed a recombinant fusion protein containing the peptides AGSE and YNIP fused with glutathion-S-transferase (GST). With this modification of the protein microenvironment, we expected to facilitate the assumption of an appropriate physiological conformation of the studied peptides (72). Briefly, peptide-coding DNA sequences were assembled from synthetic oligonucleotides, and cloned into expression vectors in fusion with GST; the fusion product was expressed in Escherichia coli and purified by affinity chromatography (the details are to be seen in reference 16). AGSE peptide was predicted to possess the strongest antigenicity, so finally this epitope was also prepared in multiple conjugation to bovine serum albumin (BSA), aiming to boost the sensitivity of the assay. The results of the ELISA studies using the described three different forms of the antigens were compared.

3.1.5. ELISA techniques

The specific antigens were coated on microtitre plates as follows: 1 μg/ml of the AGSE, 2 μg/ml of the YNIP and TRIC synthetic peptides, and 10 μg/ml of the GST-fusion products and the BSA-conjugated antigen. The prepared plates were incubated overnight at 4 °C, then patient sera were added at a dilution of 1:200 in PBS-TWEEN followed by

peroxidase-labelled anti-human IgG (1:2500 in PBS-TWEEN; Sigma-Aldrich Hungary, Budapest). After adding ortho-phenylene diamine to the samples, optical density (OD) was read at 492 and 620 nm. For the GST- or BSA-conjugated proteins, the specific corrected OD characterizing the m3AChR-specific epitope within an individual sample was calculated by subtracting the OD for the GST or BSA protein from that for the GST-m3AChR peptide fusion protein or BSA-AGSE conjugate. Appropriate numbers of negative controls were used for every microtitre plate, and measurements were made in duplicate. As the absolute OD values for the negative controls varied from plate to plate, the OD values of the patients' samples were normalized to the mean of the controls for every plate by dividing the OD of the test sample by the cut-off (mean + 2SD of the negative control OD values). The resulting relative OD values were used for the calculations.

3.1.6. Mental health status assessments

By means of specific questionnaires we have assessed the physical and mental health of the patients. The FACIT fatigue scale and the SF-36 survey were applied (73, 74). The FACIT fatigue scale is a short, 13-item query that shows the patient's level of fatigue during the usual daily activities over the past week (73). The SF-36 questionnaire is set up of 36 questions which represent multiple indicators of physical and mental health, including behavioural function and dysfunction, distress and well-being, objective reports and subjective ratings, and both favourable and unfavourable self-evaluations of the general health status (74).

3.1.7. Statistical methods

The statistical analyses were performed with SPSS 15.0 software. Levels of $p < 0.05$ were taken as statistically significant. The demographic, clinical and immunoserological parameters, the levels of anti-m3AChR antibodies specific to the various antigens and the SF-36 and FACIT test results were compared between the SLE and RA patients with or without sicca complex by means of the Student t-test or Fisher's exact test as appropriate, depending on whether continuous or categorical variables were tested. To test whether the administration of certain immunosuppressive drugs or those with anticholinergic effects had an effect on the exocrine function, the chi^2 test was applied. The differences of mean autoantibody concentrations between the studied groups were assessed with analysis of variance with Bonferroni's correction as a *post hoc* test. The occurrences of positivities to the various

antigenic epitopes were compared between the different patient and control groups with the chi^2 test.

3.2. Investigation of the risk of the development of non-thromboembolic organ manifestations in systemic lupus erythematosus associated with antiphospholipid syndrome

3.2.1. Patients

In this retrospective cohort study on an unselected group of consecutive SLE patients, we have assessed the risk of the development of non-thromboembolic disease manifestations of SLE with relation to the presence of APAs or APS. Data of 224 adult patients (age >18 years) were evaluated. All of the enrolled patients fulfilled the updated ACR criteria for the classification of SLE (5). The mean age of the patients at the time of inclusion was 49 (20-92) years, while the average length of time since the diagnosis was established was 13 (0-49) years. The proportion of female patients was 91% (n= 204)

The presence of APS was established following the Sydney criteria (75). The diagnosis of APS required APA positivity coexisting with documented obstetric and/or thrombotic complications. APA-s were considered positive when at least two laboratory tests 12 weeks apart were positive for LA or aCL or aβ2GPI IgG and/or IgM.

Selected clinical and laboratory parameters of SLE and APS were compared between patients with or without APA positivity. In addition, data of the patients in whom SLE was associated with APS were also evaluated in comparison with those of the SLE patients without APS. We studied the following three categories: organ involvement, laboratory features and immunosuppressive therapy. Thirty-one different organ involvements of SLE were included in the analysis. The definition of selected manifestations is detailed in Section 3.1.2. Laboratory measurements were performed with the methods described in 3.1.3.

3.2.2. Immunosuppressive therapy

The patients were treated according to the current international guidelines. Milder (e.g. chloroquine) or more potent immunomodulatory agents (e.g. cyclophosphamide) were administered depending on the severity of the disease. Medication with oral or i.v.

corticosteroid, chloroquine, azathioprine, methotrexate, cyclosporine or i.v. cyclophosphamide was recorded. The number of patients taking mycophenolate mofetil or rituximab was small, and therefore statistical analyses were not feasible.

3.2.3. *Statistical methods*

In the studied patient subgroups (SLE +/- APA, SLE +/-APS) the differences between the occurrence of the various organ manifestations, the immunoserological parameters and the specific treatment modes were calculated with the chi^2 test. Levels of $p < 0.05$ were taken as statistically significant.

3.3. Investigation of the role of Galectin-1 in the apoptosis of T cells in systemic lupus erythematosus

3.3.1. Patients

We performed a prospective, controlled study, involving 18 adult SLE patients (age >18 years). All of the enrolled subjects fulfilled the updated ACR criteria for the classification of SLE (5). All patients were female, their mean age at the time of inclusion was 42 (19-71) years, while the average length of time since the diagnosis was established was 4 (0,2-16) years. The control group consisted of 20 age- and sex- matched healthy volunteers.

Disease activity was evaluated with the SLE Disease Activity Index 2000 (SLEDAI-2K) (76), anti-dsDNA antibody levels, and erythrocyte sedimentation rate (ESR). The inclusion criteria for the patients were set at SLEDAI-2K score of 7 or above („active SLE" group). Newly diagnosed patients without SLE specific medication were preferred for enrollment (11/18). Moreover, patients with relapsing disease on low dose immunomodulatory drugs - methylprednisolone </= 12 mg/day (7/18), azathioprine <150 mg/day (2/18) and methotrexate 10 mg/week (2/18) - were also included.

The first blood samples were taken at the time of active SLE before starting or intensifying an immunosuppressive therapy. The second blood samples were taken when the enrolled patients' disease has become quiescent after treatment ("inactive SLE" group - SLEDAI-2K < 7 or decrease in SLEDAI-2K >/= 7). Ten SLE patients were eligible for the

second measurements. Treatments stably maintained for at least two months at the second blood sampling included low-dose (< 20 mg prednisolone) corticosteroid, chloroquine, azathioprine, methotrexate, cyclosporine or epratuzumab. Selected characteriscics of the patients were also recorded, including the immunological and haematological laboratory findings at the time of enrollment and the subsequently applied therapies. The upper limit of cytopenias were set as follows: anaemia - haematocrit < 35%, leucopenia - WBC < 4.0 G/l, lymphopenia - Ly count < 1.5 G/l, thrombopenia - platelet count < 100 G/l (*Table 2*).

Autoantibodies	**%**	**Cytopenia**	**%**	**Therapies**	**%**
ANA	63,6	Anaemia	63,6	Oral steroid	95,5
anti-dsDNA	90,9	Coombs positivity	18,2	I.v. steroid	68,2
anti-SSA	36,4	Leucopenia	50	Azathioprine	63,6
anti-SSB	22,7	Lymphopenia	50	Chloroquine	59,1
anti-Sm	27,3	Thrombopenia	13,6	Cyclophosphamide	36,4
anti-RNP	27,3			Methotrexate	31,8
anti-CL	13,6			Mycophenolate	9,1
anti-ß2GPI	9,1			Cyclosporine A	4,5
LA	9,1			Epratuzumab	9,1
low C3	63,6				
low C4	40,9				

Table 2. Prevalence of autoantibodies, cytopenias and therapies applied ever during the disease course in the cohort of SLE patients of section 3.3.1 (n=18). ANA: anti-nuclear antibodies, anti-dsDNA: anti-double stranded DNA, anti-SSA: anti-Sjögren's syndrome A, anti-SSB: anti-Sjögren's syndrome B, anti-Sm: anti-Smith, anti-RNP: anti-ribonucleoprotein, aCL: anti-cardiolipin, anti- ß2GPI: anti-beta2glycoprotein I, LA: lupus anticoagulant, C3: complement 3, C4: complement4, i.v.: intravenous.

3.3.2. Cells

Blood samples from SLE patients and healthy donors were separated using Ficoll (GE Healthcare) gradient centrifugation. Activated T cells were prepared as follows: peripheral blood mononuclear cells (PBMC) were stimulated with 5 μg/ml Phytohaemagglutinin (PHA-M, Sigma-Aldrich) and were cultured for 72 hours at 37 °C in RPMI-1640 medium (Gibco) supplemented with 10% FCS, 2 mM L-glutamine, 100 IU penicillin and 100 μg/ml streptomycin.

The studied HeLa human cervix adenocarcinoma cells were transfected as described previously (55). Mock transfected ($HeLa^{mock}$) or Gal-1 transgenic ($HeLa^{Gal}$) human cervix adenocarcinoma cells were cultured in MEM (Gibco, Invitrogen) supplemented with 100 IU penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 10% FCS.

3.3.3. Gal-1 induced T cell apoptosis in co-culture

Apoptosis induced by cell-derived Gal-1 was assessed as previously described by our group (55) To detect the Gal-1 induced T cell death, we applied co-cultures of HeLa cells and the SLE patients' T cells. Normally, T cells die when co-cultured with Gal-1 expressing ($HeLa^{Gal}$) but survive with Gal-1 non-expressing ($HeLa^{mock}$) tumour cells.

$HeLa^{mock}$ (control) or $HeLa^{Gal}$ cells (effector cells, 5×10^3 cells/sample) were plated on cover slips. The nuclear DNA of activated target T cells (2×10^5 cells/sample) was labelled with Hoechst 33342 fluorescent dye (100 ng/ml for 30 min at 37 °C) and co-cultured with both HeLa cell lines for 16 hours. Then T cells were labelled for phosphatidyl-serine exposure on the outer cell membrane, which is an early apoptotic signal of the cells, using fluorescent staining by Annexin V-Alexa Fluor 488 (Invitrogen) for 30 min and mounted with Fluoromount-G. Finally, the samples were analyzed with Carl Zeiss (Axioskop 2Mot) fluorescence microscope using AxioCam camera, AxioVision 3.1 software and 20 × objective magnifications. The contrasts of the images were adjusted using Adobe Photoshop CS4 Extended.

The degree of apoptosis was determined by counting at least 100 cells/ sample and was calculated as follows: % of apoptotic cells = (Annexin V positive cells/ total cell number) × 100. The exGal-1 induced T cell apoptosis was quantified as relative apoptotic ratio (RAR),

that represents the difference of the apoptotic cell ratios between the co-cultures applying $HeLa^{Gal}$ and $HeLa^{mock}$ cells.

3.3.4. Statistical methods

Data had normal distribution, therefore, the Student t-test was used for the comparison of RAR values between both SLE patient groups and the controls and also between the two SLE groups. Correlations between Gal-1 mRNA expression levels and RAR values with each other, and with disease activity parameters were assessed with Pearson's correlation test, or Spearman's signed rank test for the ESR. Data are presented as mean ± SD, and $p < 0.05$ values were regarded as statistically significant.

4. Results

4.1. Investigation of the relationship of exocrine gland dysfunction with autoantibodies to muscarinic acetylcholine receptor-3 and mental health status parameters in SLE overlapping Sjögren's syndrome

4.1.1. Anti-m3AChR detection

Figure 1 shows the mean levels of anti-m3AChR antibodies depending on the method of antigen preparation and epitope-specificity in the SLE, RA and pSS patients and the healthy controls. Significantly higher mean autoantibody levels were detected in both the SLE and the pSS patients than in the controls using the single peptide corresponding to the second (AGSE) and the third (YNIP) extracellular loops, while the RA patients exhibited only a borderline significant difference versus controls ($p=0.067$). This method applying short peptide sequences failed to make further distinctions between the SLE, pSS, and RA patients. The studies with the AGSE epitope in fusion with GST displayed significantly lower mean antibody levels in RA as compared with the other two patient groups. When the BSA conjugate was presented in ELISA, the mean autoantibody levels to this antigen were higher in all three diseases than in the controls, thus this modification failed to identify antibodies with sufficient group-discriminative power. The TRIC peptide, which is located in the intracellular portion of the m3AChR cannot be regarded antigenic in these diseases, since it reacted with only a small proportion of the sera.

The prevalence of the studied antibodies is demonstrated in *Figure 2*. Autoantibodies to the short synthetic peptides of AGSE and YNIP were detected at significantly higher frequencies in all three disease groups than in the controls. In addition, the presentation of GST-fusion forms also discriminated the patient groups: the prevalences of GST-AGSE and GST-YNIP were elevated in both SLE and pSS as compared with RA, furthermore antibodies to GST-YNIP also revealed differences between SLE and pSS patients, as these antibodies occurred at significantly higher frequency in pSS than in SLE, though in SLE it was still significantly more prevalent than in RA or in the controls.

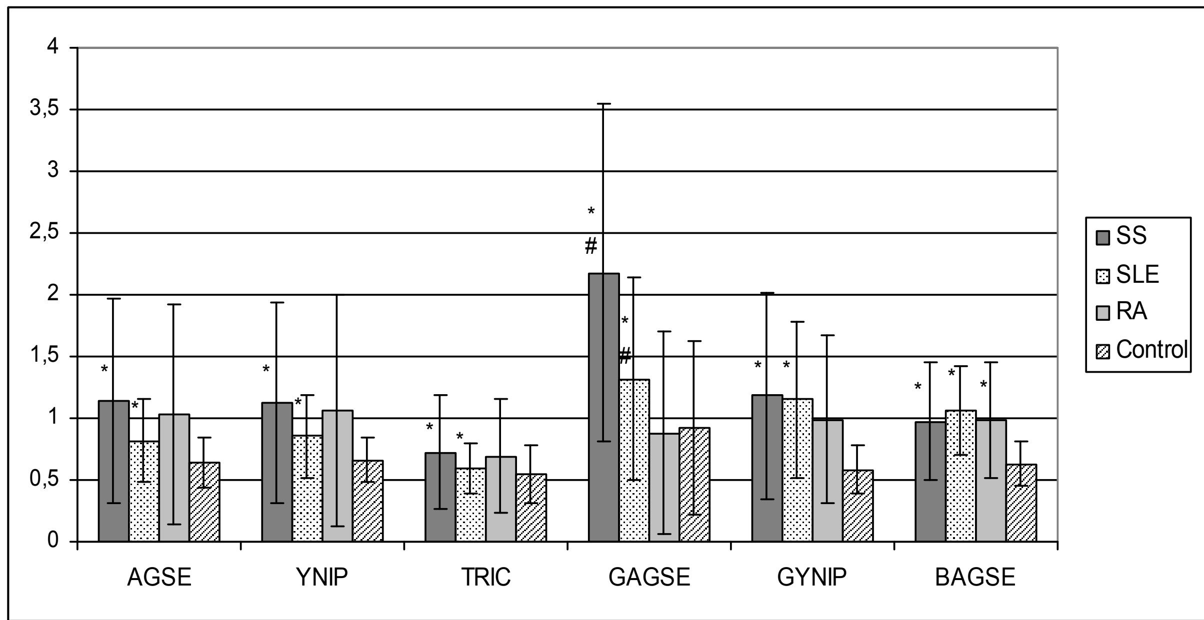

Figure. 1. Mean relative OD values of anti-m3AChR antibodies measured with ELISA. GAGSE: GST-AGSE fusion peptide, GYNIP: GST-YNIP fusion peptide, BAGSE: BSA-AGSE multiple-conjugated peptide; SS: primary Sjögren's syndrome; SLE: Systemic lupus erythematosus; RA: Rheumatoid arthritis,* p<0,05 vs control; # p<0,05 vs RA

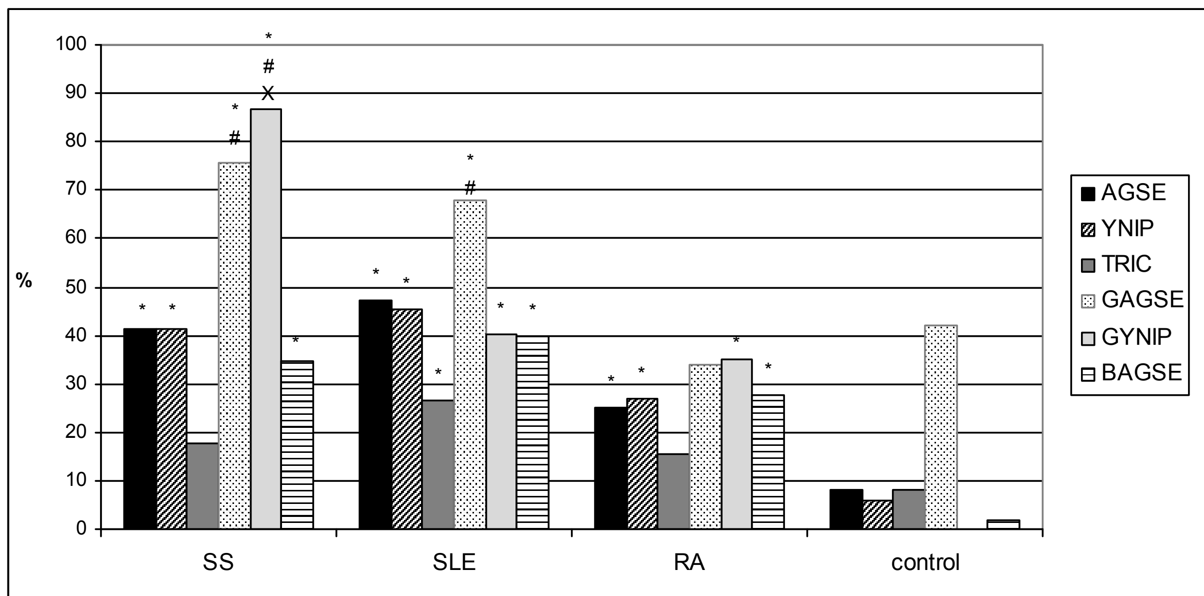

Figure 2. The prevalences of anti-m3AChR antibody-positivities in the three diseases and the controls. For the abbreviations, see the legends of *Figure 1*. * p<0,05 vs control; # p<0,05 vs RA; x p<0,05 vs SLE

After these assessments it became evident that short peptide sequences detect anti-m3AChR with relatively high specificity but low sensitivity, whereas fusion with GST is able to preserve the specificity and enhance the sensitivity of detection of anti-GST-YNIP. Multiple epitope conjugation to BSA does not improve the sensitivity of the ELISA technique (*Table 3*).

	Sensitivity	**Specificity**
AGSE	41,2%	92,0%
YNIP	41,2%	94,0%
TRIC	17,6%	92,0%
GAGSE	75,6%	58,1%
GYNIP	86,5%	100,0%
BAGSE	34,7%	98,2%

Table 3. Sensitivity and specificity of the tests for the detection of anti-m3AChR antibodies. For the abbreviations, see the legends of *Figure 1*.

4.1.2. Correlations of the sicca complex with clinical, immunoserological and mental health parameters

The clinical presentation of SS overlapping with SLE was analysed, and compared with that in RA. Sicca symptoms were common in both patient groups: sicca complex was established in 27 (26.2%) SLE and 14 (21.5%) RA patients. We aimed to identify clinical, immunological and psychosomatic correlates of the sicca complex. We studied the sicca and non-sicca patients regarding age, disease duration, disease-specific organ involvements, medication, immunoserology, mental health status parameters, as well as the anti-muscarinic receptor antibody positivity and epitope specificity.

As described before, anti-m3AChR antibodies specific to the second or the third extracellular loops and to the third intracellular loop occurred in both SLE and RA patients, however the prevalence (*Table 4*) and the mean levels of these autoantibodies were not statistically different between the patient subgroups of SLE or RA with or without sicca complex (mean level data not shown).

	SLE + sicca	SLE - sicca	RA + sicca	RA - sicca
AGSE	43,7%	48,6%	22,2%	25,6%
YNIP	43,7%	45,9%	33,3%	25,6%
TRIC	37,5%	21,6%	11,1%	16,3%
GAGSE	81,2%	64,4%	18,2%	37,8%
GYNIP	17,6%	48,0%	55,5%	29,0%
BAGSE	25,9%	44,7%	28,6%	27,4%

Table 4. Prevalence of the anti-m3AChR autoantibodies in the subgroups of SLE and RA with or without sicca complex. For the abbreviations, see the legends of *Figure 1*.

Decreased saliva production correlated with increasing age in both SLE and RA patients as expected. Furthermore, this study revealed that those SLE patients who had sicca complex developed organic brain syndrome, stroke and anti-SSA antibody positivity more frequently, whereas they presented with nephritis and lymphopenia more rarely than the non-sicca SLE patients, all with borderline significance (*Table 5*). Regarding the mental status, the FACIT fatigue scale did not reveal differences between the subgroups of sicca and non-sicca SLE patients. However, in the SF-36 survey, sicca SLE patients proved to have significantly impaired mental health parameters, specifically, in the “role-physical”, the vitality, the social function, the mental health domain, and the mental composite score as compared with the SLE patients without sicca syndrome (*Table 5*). In contrast, the RA patients showed no clinical or immunological correlates other than increasing age that could discriminate between those with or without sicca complex. Interestingly, none of the parameters that characterize the fatigue (FACIT) or the quality of life (SF-36) were found to correlate with the presence of sicca syndrome in RA. Finally, the impact of the immunosuppressive drugs as well as those with anticholinergic effects was assessed. Five SLE patients were on anticholinergic therapy.

The treatment with none of the studied medications showed a correlation with the presence of sicca complex (data not shown).

selected SLE-related parameters	**non-sicca (%)**	**sicca (%)**	**p**
organic brain syndrome	1,3	11,1	0,056
stroke	0	7,4	0,068
anti-SSA	45,9	62,9	0,098
nephritis	34,6	18,5	0,091
lymphopenia	57,3	37	0,056
SF-36	**mean values**		**p**
role-physical	39,33	35,05	0,081
vitality	46,71	41,70	**0,005**
social functioning	42,68	38,04	**0,041**
mental health	42,12	36,82	**0,048**
mental composite score	44,01	38,52	**0,035**

Table 5. Differences in the occurrence of selected clinical or laboratory paremeters and SF-36 items of the sicca and non-sicca SLE patients. Significant differences are written in bold. Higher SF-36 mean values represent better health status.

4.2. Investigation of the risk of the development of non-thromboembolic organ manifestations in systemic lupus erythematosus associated with antiphospholipid syndrome

4.2.1. Characteristics of the overall cohort

The most common disease manifestations, immunoserological variables and immunosuppressive mediacations are presented in *Table 6*.

	%		%
Arthritis	89.3	ANA	87.1
Photosensitivity	65.2	anti-dsDNA	76.3
Non-haemolytic anaemia	56.3	anti-SSA	46.0
Raynaud's phenomenon	54.9	anti-SSB	33.9
Leucopenia	54.0	anti-Sm	21.4
Lymphopenia	46.0	anti-RNP	16.1
Other skin manifestations	32.1	anti-CL	35.3
Lymphadenomegaly	31.3	anti-ß2GPI	23.2
Pleuritis	30.4	LA	24.6
Pericarditis	24.1	low C3	62.9
Butterfly erythema	20.5	low C4	46.4
Venous thromboembolism	19.2	oral corticosteroid	90.2
Skin vasculitis	18.8	i.v. corticosteroid	37.5
Thrombopenia	17.9	chloroquin	62.9
Overlap Sjögren's syndrome	17.9	azathioprin	34.4
Haemolytic anaemia	14.7	i.v. cyclophosphamide	25.9
Oral ulceration	10.3	methotrexate	21.0
Repeated spontaneous abortion	5.2*	cyclosporin A	9.8
Nephritis	**35.3**		
Pulmonary involvement	**8.9**		
Organic brain syndrome	**7.1**		
Stroke	**4.5**		
Myocarditis	**3.6**		
Convulsion	**3.6**		
Psychosis	**2.7**		
Endocarditis	**1.3**		

Table 6. Prevalences of the most common and the major clinical manifestations, the immunoserological abnormalities and the immunosuppressive therapies in the studied SLE patients. SLE: systemic lupus erythematosus, aCL: anti-cardiolipin, anti-ß2GPI: anti-beta2-glycoprotein I, LA: lupus anticoagulant, ANA: anti-nuclear antibodies, anti-dsDNA: anti-double stranded DNA, anti-SSA: anti-Sjögren's syndrome A, anti-SSB: anti-Sjögren's syndrome B, i.v.: intravenous, Numbers indicate percentages. * in female patients. The major manifastations are written in bold.

The APS-related clinical manifestations were venous thromboembolism (39 patients), stroke (8 patients), and repeated spontaneous abortion or intrauterine death (9 patients). The

frequency of the distinct APAs ranged between 20 and 33%. One hundred and five (47%) patients were found to produce at least one type of APA according to the Sidney criteria, and 52 of these APA-positive patients (23% of the total) fulfilled the criteria for APS *(Figure 3)*. The frequency of APS among our patients is in accord with the literature findings that 15-30% of SLE cases are associated with APS (48, 49, 77).

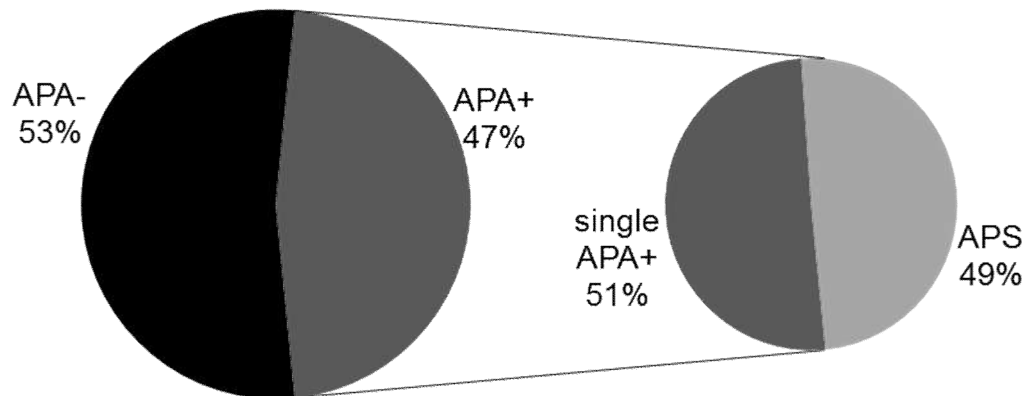

Figure 3. Proportions of APA-positive, APA-negative and APS patient subgroups. APA: antiphospholipid antibody, APS: antiphospholipid syndrome.

4.2.2. The impact of antiphospholipid antibody positivity

Several clinical distinctions were identified between the APA-positive (n=105) and negative patients (n=119) (*Table 7).*

	Mean number of total organ involvements	Endocarditis (%)	Venous thrombo-embolism (%)	Haemolytic anaemia (%)	Thrombopenia (%)
APA+	7.6	2.8	29.5	21.9	25.7
APA-	6.8	0	10.1	8.4	10.9
p	<0.05	0.101	0.000	0.007	0.005

Table 7. Clinical differences between the APA+ and the APA- SLE patients.
APA: antiphospholipid antibody, SLE: systemic lupus erythematosus.

The APA-positive patients exhibited significantly higher morbidity than the APA-negative patients, as indicated by the significantly higher total number of organ involvements detected during the course of the disease. In addition to the expected complication of venous thromboembolism, haemolytic anaemia, thrombopenia and endocarditis were also observed nearly three times more often in the APA-positive patients than in those without APAs. Endocarditis occurred exclusively in APA-positive patients, supporting the role of APA positivity as a risk factor in the development of non-bacterial endocarditis.

4.2.3. The clinical presentation of SLE with secondary antiphospholipid syndrome

Significant clinical differences were detected in the disease course of the patients in whom SLE was accompanied by secondary APS in comparison with the APS-negative SLE subgroup *(Table 8).*

	SLE + APS	**SLE withoutAPS**
Stroke	15.4%	1.2%
Venous thromboembolism	75.0%	2.3%
Repeated spontaneous abortion	17.3%	1.25%
Pleuritis	40.4%	24.3 %
Nephritis	53.9%	29.4%
Myocarditis	7.7%	2.3%
Pulmonary involvement	17.3%	6.4%
Organic brain syndrome	13.5%	5.2%
Thrombopenia	26.9%	17.8%

Total number of organ involvements per patient	8.1	6.9
Total number of major organ involvements per patient	1.2	0.5
i.v. corticosteroid	56%	32%
azathioprine	52%	29%
cyclophosphamide	39%	22%

Table 8. Differences in the frequency of selected organ involvements and in the therapeutical requirements between the SLE + APS and SLE without APS patients ($p<0.05$).
APS: antiphospholipid syndrome, SLE: systemic lupus erythematosus, i.v: intravenous.

Corresponding to the definition of APS, venous thromboembolism, stroke and spontaneous abortion were more common in this patient subgroup than in the absence of APS. Moreover, our results revealed that a significantly higher proportion of the APS cases developed nephritis, interstitial pulmonary involvement, pleuritis, myocarditis, organic brain syndrome or thrombopenia than in the non-APS group (*Table* 8). Renal involvement is known to be crucial in determining the outcome of the disease, thus it is noteworthy that lupus glomerulonephritis was present in more than half of the SLE + APS cases.

APS patients also proved to develop a higher total number of organ involvements compared with the APA-negative patients (8.1 vs 6.9; $p <0.05$), similarly to that seen in the cases with APA-positivity alone. In addition, major SLE manifestations were diagnosed significantly more frequently in patients with APS than in patients without APS (average numbers per patient: 1.2 vs 0.5; $p < 0.05$). Stroke, as a thrombotic event typically present in APS, was excluded from the major organ involvements in this comparison. However, APA positivity alone did not lead to an increased incidence of major SLE manifestations.

The assessments of the therapeutic medications also confirmed the more severe disease course in APS, since it implies the need for more aggressive therapy. When SLE was complicated by APS, the patients required i.v. corticosteroid, cyclophosphamide, or azathioprine significantly more often than in the absence of APS *(Table 8)*

4.3. Investigation of the role of Galectin-1 in the apoptosis of T cells in systemic lupus erythematosus

4.3.1. SLE disease activity

Disease activity was evaluated with the SLEDAI-2K index, anti-DNA antibody levels, and ESR at the time of enrollment and after therapy. The characteristics of disease activity in the patient subgroups of active and inactive SLE are shown in *Table 9.*

Patient No.	SLEDAI 1.	SLEDAI 2.	anti-dsDNA 1.	anti-dsDNA 2.	ESR 1.	ESR 2.
SLE1	14	0	200	17	63	12
SLE2	17	2	220	30	41	16
SLE3	23	6	151	39	76	31
SLE4	18	8	220	84	54	39
SLE5	18	2	220	75	60	7
SLE6	17	10	65	57	70	6
SLE7	7	2	130	140	63	NA
SLE8	15	4	220	51	95	11
SLE9	26	2	220	27	39	11
SLE10	12	0	12	5	93	70
SLE11	11		220		54	
SLE12	9		74		120	
SLE13	12		220		12	
SLE14	7		13		37	
SLE15	28		220		58	
SLE16	12		81		26	
SLE17	12		220		53	
SLE18	7		67		73	

Table 9. Parameters of disease activity in SLE patients before (1. – active, n=18) and after (2. – inactive, n=10) therapy.

SLE: systemic lupus erythematosus, SLEDAI: SLE disease activity index 2000, anti-dsDNA: anti-double stranded DNA, ESR: erythrocyte sedimentation rate, NA: not available.

4.3.2. *Apoptotic response of activated SLE T cells to extracellular Gal-1*

Recent studies of our group confirmed that the presence of endogenous, inGal-1 in the target T cells has an impact on the apoptotic response induced by exGal-1. T cells expressing Gal-1 upon activation display higher sensitivity to the exGal-1 induced apoptosis.

In the present work the apoptotic response of activated SLE T cells to exGal-1 was measured in co-culture experiments, in which exGal-1 was delivered by direct cell-cell contact between activated T cells and Gal-1 producing HeLa tumour cells. In active phase of the disease, SLE T cells displayed nearly zero apoptotic response to the effects of exGal-1, while, as expected, the healthy controls exhibited an apoptotic reaction in many T cells, similarly to the previous observations of our group. The difference was highly significant (mean RAR of -0,19 ± 7,26 vs 11,06 ± 10,19; p=0,0004). The susceptibility to apoptosis increased after the disease has become quiescent, as reflected by the significant mean increase of RAR from -0,19 ± 7,26 to 6,59 ± 7,85 (p=0,029). Results were compared between healthy controls and inactive SLE patients as well, and no significant difference could be detected between the two groups (mean RAR of 11,06 ± 10,19 vs. 6,59 ± 7,85; p=0,26) (*Figure 4*). Representative images of T cell apoptosis in co-culture with Gal-1 producing HeLa cells from a patient with active SLE and from a healthy control is presented in *Figure 5.*

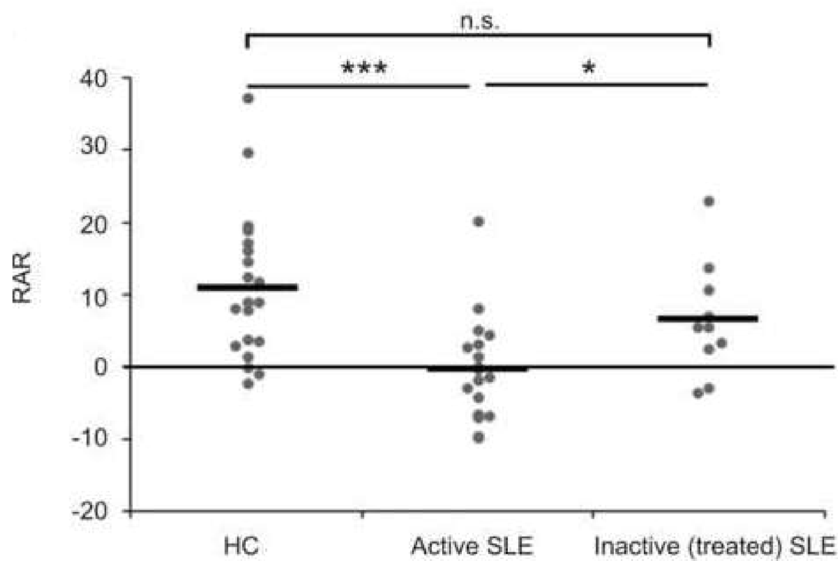

Figure 4. Apoptotic response of activated SLE T cells to extracellular Gal-1in the active and inactive phase of the disease, compared to healthy controls

HC: healthy control, n= 20; Active SLE, n= 18; Inactive (treated) SLE, n=10; RAR: relative apoptotic ratio; *** $p < 0.001$; * $p < 0.05$; n.s.: not significant

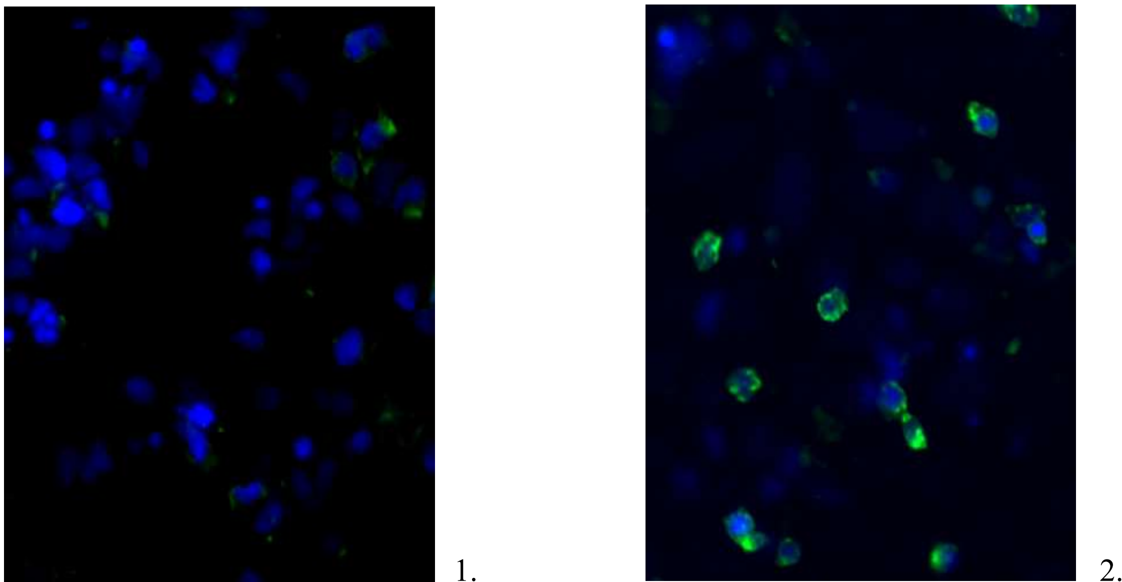

Figure 5. T cell apoptosis after 16 hours of co-culture with Gal-1 producing HeLa cells in a patient with active SLE (1.) and in a control (2). Image under fluorescecnce microscope, edited by Adobe Photoshop. T cell nuclear DNA was stained with blue fluorescent Hoechst 33342 dye, and phosphatidyl-serine exposure on the outer cell membrane of apoptotic T cells was revealed by means of staining with green fluorescent Annexin V-Alexa Fluor 488 dye. T cells with Annexin V-positive cell membrane display apoptosis.

In parallel with the co-culture experiments, our group has also measured the expression of inGal-1 by means of quantitative real time PCR in SLE and control activated T cells. Gal-1 expression represented by Gal-1 mRNA level was diminished significantly in active SLE patients compared to the controls. After treatment, SLE T cells produced significantly more Gal-1 mRNA, reaching a similar level to that of healthy controls (*data not shown*).

We have analyzed the correlation between the inGal-1 expression and the apoptotic sensitivity of the same samples of activated T cells of the individual patients and controls. We could not determine direct correlation between the studied parameters neither in SLE nor in healthy T cells ($p > 0,05$). We were also searching for correlations between both Gal-1 mRNA expression levels and RAR values with disease activity measures in lupus patients. The individual values of the Gal-1 mRNA levels and also the RAR did not correlate with the parameters of disease activity such as SLEDAI-2K, anti-dsDNA antibodies or ESR ($p > 0,05$).

5. Discussion

5.1. Investigation of the relationship of exocrine gland dysfunction with autoantibodies to muscarinic acetylcholine receptor-3 and mental health status parameters in systemic lupus overlapping with Sjögren's syndrome

This work has revealed two distinct neuroimmune alterations that may contribute to the complex pathophysiology of exocrine gland dysfunction in SLE overlapping with SS: autoantibody production that directly targets m3AChR, the specific autonomic neurotransmitter receptor, and an altered mental health status that may be regarded as a central inhibitory mechanism of the parasympathetic innervation of the salivary glands.

Our group has previously demonstrated the production of anti-m3AChR antibodies in the majority of pSS patients (16, 17) by means of an ELISA method involving the second extracellular loop of the human m3AChR as antigen. The presence of these autoantibodies has been confirmed also by other investigators, moreover further epitopes were proposed on the m3AChR as the targets of immune response in pSS, specifically on the third extracellular loop (20-22, 24, 78); however others have failed to confirm our results (27, 79, 80). The production of anti-m3AChR antibodies has not yet been examined in SLE and RA, however an overlapping SS occurs in a large proportion of these common systemic autoimmune diseases. Our present results appear to confirm that the second extracellular loop is antigenic not only in pSS, but also in some SLE and RA patients. In addition, our findings support the recent results that the third extracellular loop also contains antigenic sequences in a considerable proportion of all these patients groups.

The diagnostic efficiency of the different antigen preparation modes for ELISA was also assessed. We demonstrated that the GST-fusion construct is an appropriate mode of antigen presentation. Actually, anti-GST-YNIP directed against the third extracellular loop of the m3AChR has emerged as the diagnostically most useful antibody, as it discriminates the pSS, SLE and RA patient groups from each other and from the controls with the best balance of specificity and sensitivity. These autoantibodies occurred most frequently in pSS, and a decreasing prevalence was observed in the following order: SLE > RA > controls. It should be

noted that the putative functional significance of the anti- m3AChR autoantibodies is rather expected with regard to those potentially inhibitory antibodies that target and bind to the second extracellular loop, where the ligand-binding region is located. Moreover, our data provide no indication whether the presence of anti-m3AChR antibodies may be directly related to the pathogenesis of SS overlapping with SLE or RA. Therefore, further validation of these results on larger patient populations and potentially a more precise epitope mapping are needed to elucidate the exact epitope specificity and functional importance of these autoantibodies.

The development of SS overlapping with different systemic autoimmune diseases, including SLE or RA is rather variable. The probability of sicca symptoms in RA has been suggested to be correlated with the cumulative disease activity and the degree of functional impairment, i.e. the burden of the chronic disease (81), while others did not find such associations (82). In the present work we have demonstrated new data about the correlates of sicca syndrome in SLE. Certain clinical and immunoserological characteristics – organic brain syndrome, stroke, nephritis, lymphopenia, and anti-SSA-positivity – displayed differences with borderline significance between the SLE patients with and without sicca syndrome. Manoussakis et al. presented similar results in a larger cohort of patients (11), and the concordant findings of a recent meta-analysis (83) also confirm the assessment of sicca syndrome in our SLE cohort. It has to be emphasized that although some of the investigated SLE and RA patients with sicca symptoms did not strictly meet the classification criteria of secondary SS, they can definitely be regarded as patients with SS in clinical practice. The main reason of the incomplete diagnostic confirmation is that very few patients underwent labial salivary gland biopsy once the diagnosis of SLE and RA had been verified, mostly because of the lack of specific therapeutic consequences, or anticoagulant therapy. However, all the sicca syndrome patients had definitive objective and subjective glandular dysfunctions, since they showed markedly and repeatedly low salivary and lachrymal flow rates and they consequently gave positive answers to the sicca questionnaire on at least two separate occasions.

Regarding the psychosocial well-being, our study revealed marked differences in the SLE + sicca syndrome patients compared to those patients in whom sicca symptoms were not present. The sicca patients versus non-sicca patients presented a significantly higher occurrence of psychological dysfunction including lower vitality, a worse perception of their

mental health, difficulties in physical functions reflecting the disturbances of usual daily activities such as work, studies or household duties, and worse scores in the SF-36 composite index, which indicates a lower overall level of mental well-being. The correlation of salivary dysfunction and the worse health-related quality of life has similarly been addressed previously (84). Our results suggest an association between the insufficient psychological parameters and the evolution of sicca symptoms, however this study is not sufficient to draw conclusions of causal relationships. Nevertheless, the chronic stress has been shown to have direct impact on saliva production, leading to chronic xerostomia (33, 85), and the salivary levels of cortisol and chromogranin A have also been found elevated in patients with low salivary flow rates (31, 32, 34). The mental alterations based on chronic stress, depression and the distress associated with SLE (35) may further impair the salivary gland function in addition to the immune-mediated mechanisms, and are factors that should be addressed during the care of these patients.

In summary, the presented results have revealed a probably polyclonal autoimmune reaction to multiple epitopes on the m3AChR in SLE, but also in RA similarly to that in pSS. Our data indicate that the fusion of these antigenic peptides to GST may be useful in the development of a laboratory test for the diagnosis of SS both in primary and in overlapping cases. Furthermore, a dominant stress response and a deteriorated psychosocial well-being are suggested as contributors to the pathogenesis of SS in SLE.

5.2. Investigation of the risk of the development of non-thromboembolic organ manifestations in systemic lupus erythematosus associated with antiphospholipid syndrome

The disease course of SLE is highly variable with a wide spectrum of organ involvements ranging from mild, intermittent symptoms to life-endangering flares or a frequently relapsing clinical picture. The burden of the disease is greater when major manifestations occur, e.g. central nervous system, cardiac, pulmonary and renal symptoms. In view of the heterogeneity of SLE, it is important to identify prognostic clinical, demographic and laboratory parameters that would facilitate the prediction of the disease outcome in a condition in which a precise early assessment and risk-stratification are crucial to prevent life-threatening complications. Any parameter of predictive value with regard to the severity of

the disease is highly informative for both the patient and the treating physician to decide on the appropriate treatment, including immunosuppressive agents.

The assessment of specific autoantibody positivities (86-89) and some demographic parameters (e.g. age at onset, gender) (90-95) are useful in defining the prognosis of SLE. The development of various organ manifestations is related to the production of certain autoantibodies. Prominent examples are the anti-C1q antibodies, that indicate the appearance of nephritis (87), and anti-ribosomal-P antibodies that are associated with neuropsychiatric SLE (88, 89). Screening for these autoantibodies facilitates the early recognition of the related serious disease manifestations, and this may help prevent irreversible organ damage. The detection of APAs is of utmost importance in the determination of thromboembolic risk, hence the treatment guidelines of patients with such autoantibody positivity propose preventive platelet aggregation inhibitor therapy even without previous thrombotic events (77, 96). The presence of APAs not only predisposes to the development thrombosis and accelerated atherosclerosis (97), but has also been verified to be associated with higher lupus-related organ damage (51) and a poorer survival (2, 51).

Our knowledge of the APS-specific symptoms is well established in SLE, however the impact of an associated APS on the other lupus-related morbidities has not been sufficiently studied to date. No detailed analysis is available regarding the contribution of APS to the risk of non-thromboembolic disease manifestations in SLE. In our investigations, we have obtained new information on the disease course of SLE, as we have detected that several non-thromboembolic SLE-specific morbidities present more frequently in relation to APS. Similarly to the previous literature data, our results also showed that a significantly higher proportion of the APA-positive than of the APA-negative SLE patients developed Coombs-positive haemolytic anaemia, thrombopenia or endocarditis. Nevertheless, our studies revealed remarkable differences in the clinical presentation of SLE when it was complicated by APS. In these patients various non-thromboembolic symptoms, including pleuritis, interstitial lung disease, myocarditis, nephritis and organic brain syndrome occurred significantly more often than in the absence of APS.

The presented assessements verify that SLE patients with APS are at a higher overall mobidity risk and they are predisposed to more severe SLE disease course. The total number of major organ manifestations and the total number of the organ manifestations that have ever

occurred in an individual patient were both higher in the SLE + APS patients than in those without APS. In contrast, the presence of APA autoantibodies alone was accompanied only by an increased incidence of the above-mentioned organ involvements.

The treatment of active SLE requires widespread experience and expert knowledge, but when APS is complicating the clinical picture it implies further challenges. The presented results indicate that SLE patients with APS frequently need specific and intense immunosuppressive therapies in addition to the long-term anticoagulant treatment. The management of these patients involved significantly more often the administration of immunosuppressive drugs including i.v. corticosteroid, i.v. cyclophosphamide and azathioprine than that of the non-APS patients.

Summarizing our findings, when SLE is associated with APA production or with APS, the risk of the development of non-thromboembolic disease manifestations is higher, further influencing the clinical picture of SLE in addition to the APA-related symptoms. The burden of the disease is even greater regarding the fact that APS usually appears in young or middle-aged SLE patients (98), and this determines a predisposition to more extensive organ damage during a longer disease course with enhanced disease severity. These characteristics and our results indicate that screening for APA autoantibodies is necessary at the time of the diagnosis of SLE. The higher morbidity and mortality rate of these patients warrants close attention to the potential presentation of nephritis, interstitial lung disease or neuropsychiatric manifestations, thereby providing opportunity to prevent life-threatening organ involvements and complications with the appropriate therapy.

5.3. Investigation of the role of Galectin-1 in the apoptosis of T cells in systemic lupus erythematosus

Gal-1 has been proposed as a possible candidate for immunosuppressive therapy in many earlier studies (63-69, 99), and the impaired Gal-1 expression has emerged as an important pathogenetic factor in the development of various immune-mediated diseases. The previous rodent experiments suggested a correlation between Gal-1 deficiency and lupus pathology, however the confirmation of this hypothesis in human disease is still lacking, and

the exact role of Gal-1 in the facilitation of the development of autoimmunity remained to be elucidated. Therefore we focused on the investigation of the impact of Gal-1 on the T cell pathophysiology in SLE.

It has been shown that in physiological conditions T cells express Gal-1 upon activation, and the apoptotic effect of exGal-1 on activated T cells has also been described (54, 55, 57, 61, 100). Even though the signaling pathways responsible for this effect have largely been mapped (57, 58, 101-103), the exact mechanism by which the T cells are sensitized to exGal-1 driven apoptosis remained to be clarified. Blaser *et al*. have proposed that the apoptotic effect of exGal-1 on T cells might stem from an autocrine loop that is created by secretion of the protein (61), however Gal-1 is normally present in the serum only in very low quantities (several ng/ml) which is far below the apoptosis inducing concentration of the soluble protein (55, 100, 104). The previous results of our group have shown that Gal-1 produced in activated T cells is not secreted to the extracellular space in any detectable amount, thus excluding the possibility of rebinding of this protein to cell surface glycoconjugates.

The intracellular form of Gal-1 has not yet been implicated in the process of exGal-1-related apoptosis, therefore our group intended to examine whether the intracellular, *de novo* expressed Gal-1 plays a role in the eventual fate of T cells. The studies were performed on a Gal-1 transgenic Jurkat model system and on wild type versus Gal-1 knockout murine activated T cells. The co-culture based experiments were designed specifically to create a physiologically more accurate environment to study the cytotoxic effect of cell-bound exGal-1 in its native form (55). The analysis of Gal-1 producing and non-producing Jurkat clones and murine activated T cells co-cultured with cell-bound exGal-1 showed that Gal-1 deficient cells exhibited significantly lower susceptibility to apoptosis than did the Gal-1 expressing counterparts. The modulating effect of inGal-1 content on the apoptotic reaction of cells to exGal-1 was a novel finding, however the underlying exact mechanism remained to be elucidated.

Based on these previous results the aim of this work was to investigate the role of Gal-1 in the apoptosis of T cells under pathologic autoimmune conditions in SLE. We analyzed the sensitivity of SLE T cells to the exGal-1-mediated apoptotic signal. Since lupus T cells show mixed characteristics of activated and anergic cells and the inGal-1 content is crucial in

this mechanism, it is important to assess the ability of these cells to express Gal-1 upon activation. The severity of the disease might also influence this process. Our group has recently demonstrated that in active phase of the disease, activated SLE T cells in general contained significantly lower levels of Gal-1 mRNA than healthy controls, and after successful treatment, the T cells of the same SLE patients produced significantly more Gal-1 mRNA, thus regaining corresponding levels to that of healthy controls.

Our findings on the Gal-1 triggered apoptotic reactivity of activated SLE T cells revealed the expected outcome analogous to that in the preceding experiments. T cells of active lupus patients showed nearly no sensitivity to the cytotoxic effect of exGal-1, while the immunosuppressant therapy restored the T cells' ability to undergo apoptosis induced by exGal-1. These cured lupus T cells behaved similarly to the T cells from healthy controls in the apoptosis assays. While we could not find a significant direct correlation between the exact level of inGal-1 mRNA in SLE T cells and their susceptibility to apoptosis, it is noteworthy that the observed tendencies and the apparent favorable effect of immunosuppressive therapy still indicate an association between diminished Gal-1 expression and the consequent downregulation of apoptosis in lupus T cells.

Neither the apoptotic disturbances of SLE T cells, nor the individual values of the Gal-1 mRNA levels correlated with particular parameters of the disease activity such as SLEDAI-2K scores, anti-dsDNA antibody titers or ESR. This finding is possibly influenced by the relatively low number of studied patients, and by the fact that SLE has widespread immunological and clinical manifestations, which can hardly be reflected by disitinct single characteristics of the disease. Moreover, our findings do not provide information on how the Gal-1 deficiency modulates the clinical picture of SLE.

In conclusion, we demonstrated that inGal-1 regulates the responsiveness of activated T cells to the apoptosis-inducing effect of exGal-1 in SLE similarly to the physiological process. The analysis of activated T cells from SLE patients shows a clear diminution in Gal-1 expression and concomitant resistance to exGal-1 triggered apoptosis. These findings serve as potential novel markers to SLE pathogenesis. Gal-1 related apoptotic disturbances might contribute to immunoregulatory dysfunction and enhanced T cell activity in SLE pathology. This mechanism is supported by the finding that successful immunosuppressive therapy results in restoration of the level of Gal-1 as well as the apoptotic sensitivity of SLE T cells. In addition to the defective Gal-1 expression, impaired binding of exGal-1 on T cells or the

presence of anti-Gal-1 autoantibodies might also contribute to this novel pathogenetic mechanism, therefore further studies are needed to assess their roles.

Acknowledgement

The author is grateful to László Kovács for his outstanding scientific support and supervision of this work, and Éva Monostori for her expert contribution, furthermore to Ákos Hornung, Roberta Fajka-Boja and Julianna Novák for their profound help in completing the experiments and the statistics of Galectin-1, and to Szilvia Pördi and Andrea Gercsó for their precise laboratory assistance. The work was supported by EU FP.7 framework grants TÁMOP-4.2.2/B-10/1-2010-0012, and TÁMOP-4.2.2.A-11/1/KONV-2012-0035 and an investigator-initiated research grant from UCB.

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