PhD thesis

Human immunosuppressive protein, galectin-1 emerges as a novel regulatory factor in autoimmune disorders

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Abbreviations

Gal-1 – Galectin-1

Th1/Th2/Th17 – Helper T-cell subsets

Treg – Regulatory T-cell

CRD – Carbohydrate Recognition Domain

Lck – lymphocyte-specific protein tyrosine kinase

ZAP70 – Zeta-chain-associated protein kinase 70

RAS –Transforming protein p21

rGal-1 – Recombinant galectin-1

SLE – Systemic lupus erythematosus

RA – Rheumatoid Arthritis

SS – Sjögren's Syndrome

MSC – Mesenchymal Stromal Cell

ER – Endoplasmic Reticulum

CXCL9 – Chemokine C-X-C motif ligand 9

CXCL10 – Chemokine C-X-C motif ligand 10

TNFα – Tumor necrosis factor alpha

TGFβ – Transforming growth factor beta

Bcl2 – B-cell Lymphoma 2

Bax – Bcl-2-associated X protein

Bcl-xL – B-cell lymphoma-extra large

c-myc - myelocytomatosis viral oncogene homolog

ConA – Concanavalin A

PHA-L – Phytohemagglutinin-leucoagglutinin

LCA – Lens Culinaris Agglutinin

WGA – Wheat Germ Agglutinin

SNA – Sambucus Nigra Agglutinin

RCA – Ricinus Communis Agglutinin I

MGAT1-5 – N-Acetylglucosaminyltransferase I to V

MAN1A/MAN2A – Mannosidase Alpha Class 1A/2A

B4GALT1-3 – Beta-1,4-galactosyltransferase 1-3

ST3GAL3,4,6 – ST3-beta-galactoside-alpha-2,3-sialyltransferase 3,4,6

ST6GAL1 – ST6-beta-galactosamine-alpha-2,6-sialyltransferase 1

NEU1 – Neuraminidase 1

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1. Introduction

1.1. Galectin-1, a lectin in immunoregulation

Lectins are carbohydrate-binding proteins found in most eukaryotes that bind certain sugar moieties with high affinity. Lectins play a role in molecular recognition involving cells, carbohydrates, and proteins. Many are multivalent, giving rise to an agglutinating effect on cells. Due to their recognition of specific moieties, lectins have been used to map cellular glycosylation patterns [1] as well as probes to identify glycan markers of pathologies [2,3]. Lectins and their binding specificities are described in Table 1.

Galectin-1 (Gal-1), a mammalian lectin, binds to glycoconjugates, which are abundantly present on almost all cell types, and both this lectin and its ligands participates in cell adhesion [4], migration [5], and immunoregulation [6].

Structure of galectin-1: Galectin-1 is a member of a family of β-galactoside binding lectins with specific affinity to terminal N-acetyl-lactosamine motifs on multi-antennary cell surface N-glycans [7]. Its binding is partially or fully inhibited by terminal α-2-3 or α-2,6-sialylation of glycans, respectively [8–10]. In its native form, Gal-1 is a β-sandwich consisting of two anti-parallel β-sheets in which the N- and C- termini are situated at the same end of the structure, whereas the carbohydrate-binding site is at the opposite end. In solution, over 7μM concentration, Gal-1 is a homodimer, with the N- and C- termini positioned at the interface coupling the monomers with hydrophobic bounding. At lower concentrations Gal-1 dissociates into monomers, but still retains affinity for the carbohydrate ligands [10].

Intracellular functions: Gal-1 is synthetized in the cytoplasm on free ribosomes and it has intracellular roles. Two functions inside the cells has been described so far. It is known to facilitate mRNA splicing by associating with Gemin4, thereby influencing assembly of the spliceosome [11], and it forms a part of the H-RAS signalling nanocluster [12].

Extracellular functions: Gal-1 may be secreted by an unconventional fashion [13,14]. Its extracellular functions are many-fold and have been extensively studied [15,16]. Extracellular Gal-1 causes apoptosis of activated T-cells, a major immunoregulatory function of the lectin. Although a number of T-cell Gal-1-binding structure has been identified, including CD45 [17], CD7 [18], GM1 ganglioside [14,19], the exact receptor mediating Gal-1's triggered apoptosis remains unknown. Gal-1 driven apoptotic

signalling is not dependent on the Fas/FasL pathway according to studies on a Fas-resistant T-cell line and *lpr* mice [20,21]. Recently the mechanism of Gal-1-triggered cell death has extensively been identified [15,16,22–25]. Stimulation of apoptosis requires the presence of Lck and ZAP70 kinases [24,25], release of ceramide [22], decrease of mitochondrial membrane potential and caspase activation [23] when triggered with lower concentration of soluble (1,8.μM) [22–24,26,27] or cell-bound Gal-1 [15,28]. Soluble Gal-1 in higher concentration (18-20 μM) also induces apoptosis of T-cell lines or activated T-cells *in vitro* [23,27], however, this cell-death can be distinguished from that of low concentration or cell-bound Gal-1 by the lack of caspase activation and the requirement of endonuclease G for DNA degradation [23,27] (Fig 1). Regulation of T-cell viabilitywas initially examined using soluble, recombinant Gal-1 (rGal-1) in a wide range of concentrations in *in vitro* and later *in vivo* experiments. Soluble Gal-1 was efficacious in inhibiting disease development and progression in animal models of several autoimmune or inflammatory diseases. Santucci *et al.* found that Gal-1 pre-treatment could dose dependently prevent liver injury and T-cell infiltration in Concanavalin A (ConA) induced hepatitis in mice [21]. The lectin was demonstrated to suppress experimental colitis in another model, improving clinical aspects of the disease and reducing the ability of lamina propria T-cells to produce IFN-γ [29]. Soluble Gal-1 has also been studied in a mouse model of systemic lupus erythematosus (SLE), where its administration could protect SLE-prone mice by diminishing activated T-cells, raising FoxP3 expression, the definitive transcription factor of regulatory T-cells and extending survival times of the animals [30]. Also, Gal-1 exerted therapeutical effect in the animal model of rheumatoid arthritis [26]. However, Gal-1 does not occur in soluble form *in vivo* since, after secretion, it binds to the secreting or neighbouring cells or extracellular matrix proteins due to the Gal-1 binding structures on these tissue components [31,32]. Consequently, its physiological serum concentration is very low (4-10 ng/ml) [33,34] while it's functional *in vitro* concentration as well as that of the soluble Gal-1 used in the above experiments is quite high, ranging from 25-580 μg/ml [7,21,29,30].

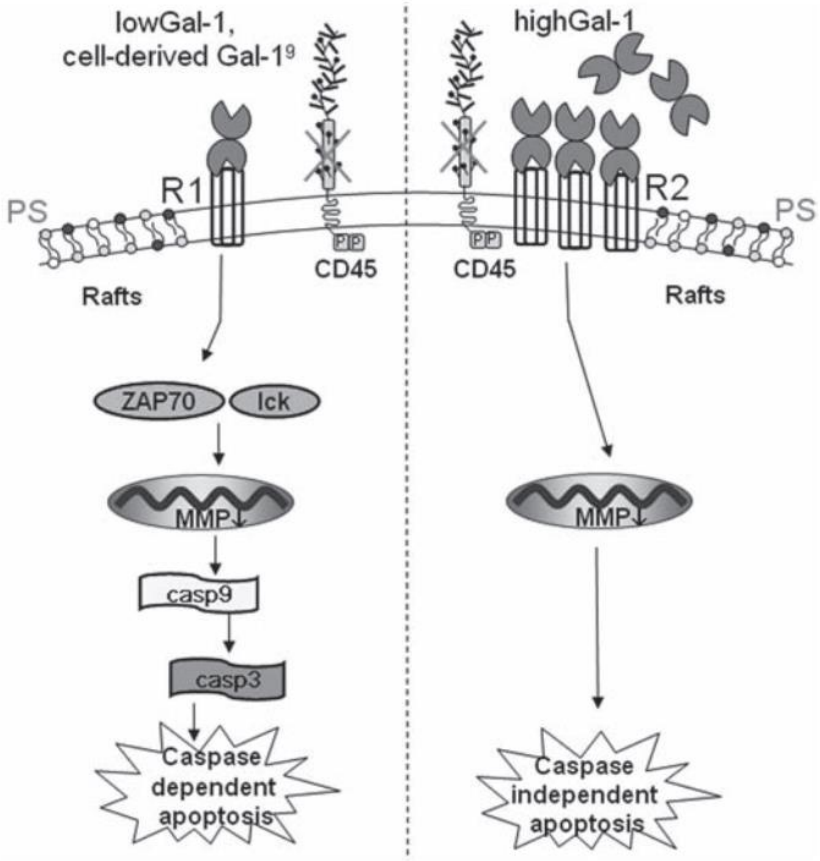


Fig1: Mechanisms of gal-1 induced T cell apoptosis. left panel: cell-death triggered by low concentration of soluble (1,8μM) and cell-derived gal-1. right panel: apoptosis induced by high concentration of gal-1 (18 μM). abbreviations: r1 and r2: receptor 1 and 2; ps: phosphatidylserine; mmp: mitochondrial membrane potential; casp9 and 3: caspase 9 and 3; endo g: endonuclease g (Figure from [23])

Effect of Gal-1 depends on the cell surface glycosylation of helper T-cells: The specific triggers for commitment to a certain lineage of $CD4^+$ T-cells, Th1, Th2, Th17 and Treg, are manifold. Factors such as strength of TCR activation, dose of antigen, type of antigen presenting cell, and cytokine milieu all influence T-cell fate [35,36]. Hence T-cell subtypes are influenced differently by Gal-1. Helper T-cells that differentiate towards Th1 and Th17 inflammatory subsets produce cell surface glycans essential for galectin-1 signalling and this sensitizes them to Gal-1 driven apoptosis. Cells that differentiate into the Th2 subset however, are protected from Gal-1 cytotoxic effect through a denser pattern of inhibitory sialylation of cell surface glycoproteins [37]. Toscano *et al.* found that Gal-1-deficient mice have stronger Th1/Th17 responses after antigen presentation in vivo, and are more susceptible to experimental autoimmune encephalomyelitis than

controls [37]. Treg cells express high amounts of Gal-1 themselves and its expression increases further after activation [38]. Furthermore, blocking Gal-1 binding with a neutralising antibody significantly reduces the inhibitory effects of both human and mouse Treg cells [38].

Gal-1 in the maintenance of immune privilege: Immune privileged sites are places in the body where foreign antigens are tolerated without triggering inflammatory immune responses. This, however is not immunological ignorance, but is maintained by a complex interplay of the local cytokine milieu [39], physical barriers against lymphatic drainage [40] and differential expression of cell surface molecules that inhibit complement activation [41] or induce immune cell apoptosis [42]. As such, immune privilege is an active process governed by factors that finely regulate the immune response. There are several immune privileged sites including the testis [43], anterior chamber of the eye [44], brain and central nervous system [40], maternal foetal interface of the placenta [45] and solid tumors [46]. Immune privileged tissues produce high levels of Gal-1. A study has found that placentas from spontaneous abortions show significantly reduced levels of Gal-1 compared to control placentas from induced abortion [47] and the lectin was tied to the maintenance of immune privilege in the trophoblast [48]. Meanwhile, Gal-1 secretion by the Sertoli cells of murine testicular tissues was found to promote development of tolerogenic dendritic cells [49]. Tumor immune escape is another aspect of immune privilege. Malignant transformation often involves the production of immunoregulatory factors such as Gal-1, allowing the tumor to mask itself from immune reactions [46]. Increased galectin-1 expression or the efficacy of Gal-1 blocking as a therapeutic possibility has been reported in colon [50], breast [51], lung [52], and several other types of cancer [53–57].

Cells expressing Gal-1: Gal-1 is expressed by stromal cells, activated endothelial cells in lymphoid organs, at sites of inflammation [58], and in immune cells, including B-cells [59], macrophages [60] and activated T-cells [20,28] as well as mesenchymal stromal cells (MSC). MSCs are a type of multipotent stem cells with immunosuppressive effects [61,62]. Because of this and their ability to differentiate towards mesodermal cell lineages such as bone, cartilage and adipose tissue, MSCs have recently been implicated in various therapeutic approaches [63]. Various immunological pathologies can be efficiently treated by MSC transplantation [64–66]. Allogeneic and autoimmune/inflammatory responses are dampened [67] by soluble and cell bound factors [68,69]. Recently, the

production and secretion of Gal-1 has also been identified in MSCs [70–73]. Although Gal-1 has been suggested to contribute to MSCs' immunosuppressive potential [73–75], its true function in their anti-inflammatory activity has been poorly examined. Nevertheless, the immunoregulation by recombinant, soluble Gal-1 has been confirmed in multiple *in vitro* and *in vivo* experimental systems [7]. Despite *in vitro* and *in vivo* data confirming the immunoregulatory functions of Gal-1, the exact role of Gal-1 in MSCs' immunosuppressive functions have not been elucidated.

Glycosylation pattern of T-cells and enzymes creating the pattern: The complex nature of glycan structures requires an expansive set of tools for its assembly, as the number of residues involved in the sugar chains grows towards higher order lifeforms [76]. Glycosylation occurs mostly in the lumen of the endoplasmic reticulum (ER) and in the Golgi apparatus. Most T-cell surface proteins in mammals carry an impressive and unique population of oligosaccharide complexes on them that are synthetized through a series of posttranslational enzymatic steps (Fig 2). The cellular repertoire of glycans that are produced is defined by the expression of glycosyltransferase and glycosidase enzymes, that have more than 200 members coded in the mammalian genome. The formation and breakdown of glycans is regulated on several levels, such as through transcriptional regulation of the enzyme coding genes, competition for substrates and interactions that influence enzyme localization in cellular organelles [76,77].

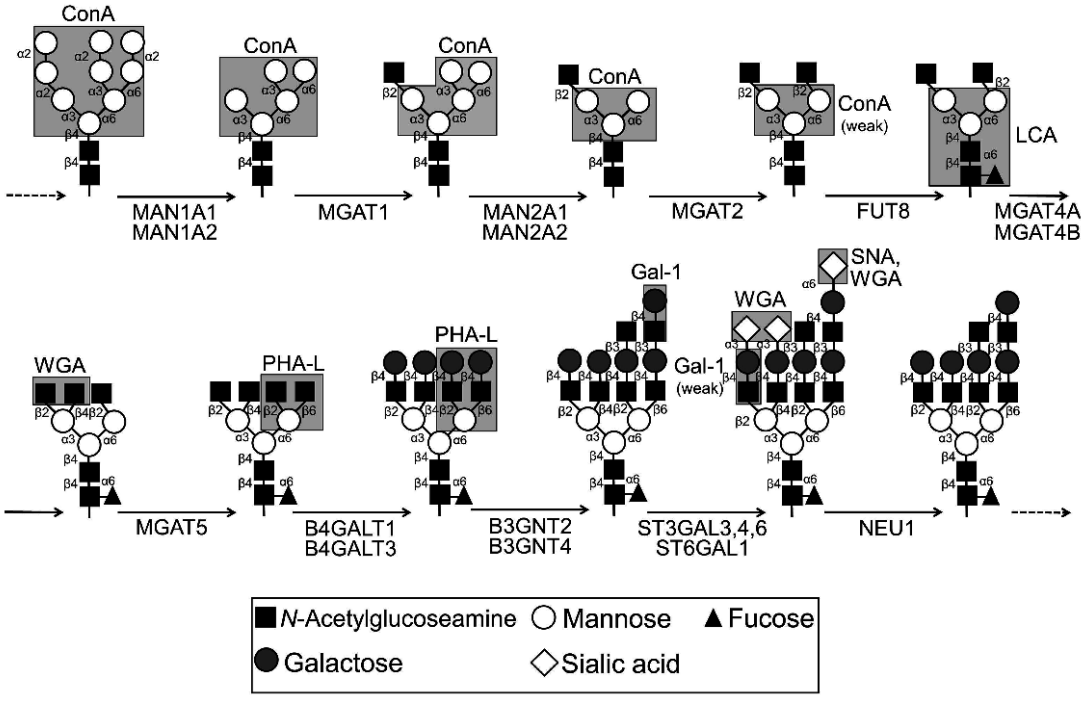


Fig 2. **Protein *N*-linked glycosylation pathway in mammals: partial enzymatic process and lectin binding sites**. Human genes encoding glycosylation enzymes relevant in each step are shown below the respective arrows. Examples for glycan determinants required for lectin binding are indicated in the grey boxed areas adopted from Cummings et al. Gene and lectin names are listed in Materials and Methods section.

Alpha mannosidases such as MAN1A1-2 and MAN2A1-2 are responsible for the removal of mannose residues from N-glycans in the Golgi apparatus. These enzymes act on early, high mannose type glycan structures [78]. Mannosidases are required to deconstruct the early, mannose rich glycan structures and without these early steps, complex, varied glycans with high affinity Gal-1 binding sites cannot arise [79].

N-Acetylglucosaminyltransferase I to V (MGAT1-5) act on the common core structure of Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn and initiate the multiple branching of N-glycans [80]. These enzymes synthetize specific branches of high complexity N-glycans based on linkage with the core mannose residues. The branches contain LacNAc units, the high affinity targets of Gal-1. The more branches present in the glycan, the higher binding affinity it shows towards Gal-1 [81]. MGAT1 transfers GlcNAc from UDP-GlcNAc to the terminal α1,3-linked mannose in Man5GlcNAc2Asn to initiate N-glycan branching [82]. MGAT4A and B enzymes catalyze the transfer of GlcNAc from UDP-GlcNAc in β-1,4 linkage to α-1,3-D-mannoside on the core structure and MGAT5 synthetizes the β(1,6) GlcNAc branch found on the α(1,6) Man.

Poly-N-acetyllactosamine chains on N-glycans can be capped through sialylation by the attachment of α-2,6 sialic acid by ST6GAL1 and α-2,3 sialic acid by ST3GAL3,4 and ST3GAL6 [83]. Terminal sialylation of branches inhibits Gal-1 binding either partially in the case of α-2,3 sialic acids, or entirely via α-2,6 sialic acids [84].

Neuraminidases act antagonistically to sialyltransferases, removing the terminal sialic acid residues from glycoproteins. Among the four neuraminidases described so far in mammals, NEU1 and NEU3 reside in the plasma membrane and are catalytically active on either α-2,3 or α-2,6 sialic acid substrates [85].

1.2. T-cell signalling and apoptosis is altered in autoimmune diseases and is affected by modified cell surface glycosylation

The wealth of glycoproteins on the cell surface are of course not without function. These glycan complexes have been shown to play a role in many fields of cellular physiology such as adhesion, migration and signal transduction into the cell [86,87]. Numerous congenital and acquired diseases show altered cell surface glycosylation, including several types of cancer and autoimmune syndromes [88–91]. Altered oligosaccharide structures have been identified on tumours and proved to be diagnostic markers of malignant phenotypes [90,92]. Protein glycosylation has become an integral part of research in autoimmunity as defective glycan structures have been described on serum immunoglobulins [93] and different glycans at certain residues on IgG subclasses have been shown to affect the effector function of autoantibodies [94,95]. Surface glycosylation of T-cells in autoimmune pathologies has not been studied in detail thus far, but it is known that defects in mucin-type O-glycan biosynthesis during embryonic development may cause Tn syndrome, a rare congenital autoimmune pathology [96]. Furthermore, mice with defective N-glycosylation of the T-cell receptor exhibit severe glomerulonephritis reminiscent of autoimmune syndromes due to a loss of self-recognition [97], and glycosylation patterns of antigens have been shown impact T-cell activity and adaptive immune reactions [98,99].

Systemic lupus erythematosus: SLE is one of the most common and devastating systemic autoimmune diseases. Clinical manifestations are very diverse, including the most frequent symptoms of polyarthritis and various skin manifestations [100]. Furthermore, glomerulonephritis, central and peripheral nervous system pathology, inflammation in the serous membranes, lung, muscles, eye and cardiovascular system as well as severe blood cytopenias also develop frequently [101–104]. Very complex immune dysregulation has been explored, including T- and B-cell functional alterations that eventually lead to the loss of peripheral tolerance [105,106]. Molecular pathology in SLE is characterized by immune-mediated inflammation in multiple organs and by the production of various autoantibodies [107]. Over the last decades, focus has shifted from B-cell dysfunction towards T-cells, which are the main mediators of B-cell activation and autoantibody production, producers of proinflammatory cytokines, and important components of the inflammatory cell infiltrate in the target organs [108]. T-cell dysfunction in SLE is known

to include widespread signal transduction alterations [105,109], decreased threshold of activation [105,109], defective proliferation [105,109] and impaired cell viability [105,109], in parallel with an increased survival and activity of autoreactive, proinflammatory T-cell clones, and defective regulatory T-cell (Treg) function [110,111]. Malfunction of apoptosis is a major factor in SLE pathophysiology [105], with T-cell death occurring by necrotic and otherwise non-physiological means, while inefficient clearance of apoptotic and necrotic bodies fuel the autoimmune state [112]. Although Gal-1 participates in the maintenance of immunohomeostasis by regulating T-cell functions and survival [27,33], its role in the lupus T-cell pathology has not yet been revealed.

Rheumatoid Arthritis: RA is a common and debilitating inflammatory syndrome. Chronic erosive inflammation of the synovial lining of the joints is the defining clinical aspect of the disease [113], but pathology often also involves skin, blood vessels, heart, lungs and muscles [114]. Studies in RA have found a key role for dysfunctional pathways of T-cell activation in the onset and maintenance of pathology [115,116]. The T-cell profile in RA is characterised by an inflated Th17 population and impaired Treg function [117]. IL-17, the defining cytokine of the Th17 subset, has been established as one of the drivers of synovitis with a strong correlation to disease index DAS28 [118]. Both $CD4^+$ and $CD8^+$ T-cells show a decreased rate of apoptosis in patients with RA, and expression of pro-apoptotic factor Bax is diminished, while anti-apoptotic Bcl-2 expression is elevated compared to healthy individuals [119].

Sjogren's syndrome: SS is marked by diffuse lymphocytic infiltration of exocrine glands and other tissues. Primary symptoms are usually the diminished function of lacrimal and salivary glands, but neurological, pulmonary and renal involvement is also possible [120]. The bulk of cells in exocrine glandular infiltrates are T lymphocytes (>75%) and $CD4^+$ T-cells constitute the majority of these cells [121]. T-cells from lesions have been found to express predominantly Th1 cytokines [122,123] and chemokines such as CXCL9 and CXCL10 [124,125]. Saliva from SS patients has increased levels of IL-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon-γ (IFN-γ) in comparison with controls, also suggesting a Th1 mediated pathology [126]. In *in vitro* experiments, apoptosis of T-cells from SS patients was significantly increased compared to controls, however mRNA levels of pro-apoptotic proteins such as Bax, Bcl-xL or c-myc don't show any alterations [127].

2. Objectives

Galectin-1, a mammalian lectin known to cause apoptosis on activated T-cells, has shown promise as a therapeutic factor in animal models of autoimmune disease including SLE [21,29,30,128,129]. Our aim was **to study *in vitro* effect and *in vivo*, systemic immunosuppressive properties of Gal-1 in an immunosuppressive cell type (MSC)**. Model of the *in vitro* analysis included co-culturing of activated T-cells as target and MSCs, as Gal-1 producing effector cells. To understand the *in vivo* effect of Gal-1 in MSC's immunosuppressive property streptozotocin induced diabetes mouse model was used. Since Gal-1 is produced by activated T-cells, we wished to **clarify the localization of the lectin (e.g. whether it is secreted or not) and whether extracellular Gal-1-induced apoptosis is affected by the *de novo* expressed Gal-1 in activated T-cells.** Localization of *de novo* Gal-1 in T-cells was ascertained using flow cytometry and fluorescence microscopy and apoptosis studies were carried out on mouse activated T-cells from wild type and Gal-1 knock-out mice.

The role of Gal-1 as an apoptotic factor of effector T-cells makes it a prime candidate for study in autoimmune pathology such as SLE, SS and RA. Our objective was to **investigate expression and function of Gal-1 in the T-lymphocytes of SLE patients** by PCR measurements of Gal-1 mRNA levels and apoptosis assays. As the presence of accessible ligands on the cell surface are required for Gal-1 activity, we set out to **study the composition of surface glycans and availability of Gal-1 binding structures on T-cells from SLE, RA and SS patients** using lectin binding assays and analysis of the expression relevant glycosylation enzymes by QPCR.

3. Materials and methods

3.1. Cells

Murine wild type and Gal-1 knockout bone marrow MSCs were isolated and obtained from Dr. Ferenc Uher of National blood Service, Budapest [130]. Wild-type and Gal-1 deficient MSCs were designated as wtMSCs and MSCgal1$^{-/-}$, respectively. MSCs were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 medium (Invitrogen), complemented with 10% fetal bovine serum (FCS, Invitrogen), 10% horse serum (Invitrogen), 50 U/mL penicillin, 50 μg/mL streptomycin (Sigma-Aldrich), and 2 mmol/L L-glutamine (Invitrogen) in a humidified CO_2 thermostat at 37°C.

Murine T-cells were isolated from lymph nodes of wild type (C57BL/6) and Gal-1 knock out mice (B6.Cg-Lgals1tm1Rob/J, 006337, Jackson Laboratory). The lymph nodes were crushed in RPMI 1640 medium until a homogenous cell suspension was achieved, then centrifuged at 300 × g for 10 min and washed twice in phosphate buffered saline (PBS). Afterwards, cells were centrifuged at 300 × g and resuspended in RPMI 1640 medium supplemented with 10% FCS and 50 μM β-mercaptoethanol (Sigma), followed by activation with 7.5 μg/ml Concanavalin A (ConA, Sigma) for 72 h.

HeLa human cervix adenocarcinoma cells were transfected with Gal-1 cDNA as described previously [15]. Mock transfected (HeLa^{mock}) or Gal-1 transgenic (HeLa^{Gal}) human cervix adenocarcinoma cells were cultured in MEM (Gibco) supplemented with 100 IU penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 10% FCS.

Human peripheral blood mononuclear cells (PBMC) were collected and separated from blood of healthy donors and patients with SLE, RA or SS using Ficoll (GE Healthcare, Chalfont St. Giles, UK) gradient centrifugation. PBMCs were stimulated with 5 μg/ml phytohaemagglutinin-M or 1 μg/ml -L (PHA-M and PHA-L) as indicated (Sigma-Aldrich) 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.

3.2. Mouse diabetes model

All mouse studies were done in strict accordance with national and international laws and regulations of animal experiments and were reviewed and approved by the Institutional Animal Care and Use Committee of the Biological Research Center of the

Hungarian Academy of Sciences, and by the Animal Care and Use Committee of the National Medical Center, Budapest, Hungary.

Streptozotocin-induced type I diabetes model: the induction of diabetes with streptozotocin (STZ) and the therapy with MSCs and bone marrow hematopoietic progenitor cells were carried out as described previously [29]. Briefly, 8- to 10-week-old female C57BL/6 mice (National Institute of Oncology, Hungary) were injected intraperitoneally with 50 mg/kg of body weight STZ in every 24 h on 5 consecutive days. Blood glucose was measured with an AccuChek Active blood glucose meter (F. Hoffmann- a Roche). Those animals were considered diabetic whose blood glucose levels were higher than 10 mmol/L on 2 consecutive days, day 14 and 15 of the experiment. Subsequently, the diabetic mice were sub lethally irradiated (250 cGy) and transplanted by tail vein injection with syngeneic bone marrow cells (BMC, 10^6 cells/animal) together with wtMSCs or MSCgal1$^{-/-}$ (10^5 cells/animal). Glucose tolerance test was carried out after overnight fasting, when mice were injected intraperitoneally with 2 g/kg of body weight glucose. Glucose disposal was analysed by measuring blood glucose at different time points.

3.3. Proliferation assay and transwell experiment

Mouse T-cells were isolated with magnetic bead separation using BD IMagnet Cell Separation Magnet (BD Bioscience) according to the manufacturer's instructions. Purity was tested with flow cytometry using FITC-labelled anti-CD3 antibody (BioLegend). Purified T-cells were stimulated with Dynabeads Mouse T-activator CD3/CD28 (Invitrogen) then labelled with CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Life Technologies) according to the manufacturer's instructions. wtMSCs and MSC$^{-/-}$ were plated into 24-well cell culture plates (Costar, Corning) and allowed to adhere to the plastic surface. After 3 hours, aforementioned T-cells were added to the wells (10^5/well) or to Transwell inserts placed above the well bottom. Proliferation was evaluated upon CFSE fluorescence intensity with cytofluorimetry after 72 hours of culture.

3.4. Gal-1 detection with indirect immunofluorescence or flow cytometry

Activated T-cells were suspended in cold PBS supplemented with 1% FCS and 0.1% NaN_3 (immunofluorescence buffer, IFB) or IFB supplemented with 0.1% Triton-X 100 for non-permeabilized or permeabilized samples, respectively and incubated with mouse

anti-human Gal-1 monoclonal antibody (clone 2c1/6, produced in our laboratory [15]) for 45 min on ice. For confocal microscopy analysis the cells were washed two times with IFB and treated with donkey anti-mouse IgG conjugated with NorthernLights557 (R&D Systems). Finally, the cells were mounted on microscopic slides with Fluoromount-G (Southern Biotech) and analysed with laser scanning confocal microscope (Olympus FV 1000). For flow cytometry analysis the anti-Gal-1 treated cells were incubated with goat anti-mouse IgG-Atto 488 (Sigma). The samples were then analysed by FACSCalibur flow cytometer using the CellQuestTM software (Becton Dickinson).

3.5. Apoptosis assays

Apoptosis of mouse T-cells: MSCs (5×10^3/sample) were plated on round coverslips (12 mm diameter, Menzel Gläser,Thermo Scientific) in 24-well plates. To remove cell surface Gal-1, wtMSCs were treated with 100 mmol/L thiodigalactoside (TDG, Carbosynth) for 30 min at 4°C, and then washed extensively with cell culture medium. Splenocytes (2×10^5 cells/sample) were activated with Con A for 72 h and then labelled with Hoechst 33342 (100 ng/mL for 30 min at 37°C) before co-culturing with MSCs. The cells were co-cultured for 16 h, fixed with 4% paraformaldehyde in PBS for 4 min at room temperature, and then the protein binding sites of the cover slips were saturated in FACS buffer. For the detection of *phosphatidylserine exposure* on the outer cell membrane, samples were washed with Annexin binding buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 140 mmol/L NaCl and 2.5 mmol/L CaCl2) and labelled with Annexin V-Alexa Fluor 488 (Molecular Probes, Life Technologies). For detection of intracellular *ceramide release*, cells were fixed and permeabilized with permeabilization buffer (PB, FACS-buffer containing 0.1% Triton X-100), and then sequentially incubated with anti-ceramide mAb (Alexis Biochemicals), biotinylated anti-mouse IgM (DAKO) and streptavidin FITC (DAKO). To analyse the *depolarization of the mitochondrial membrane*, non-fixed samples were loaded with JC-1 dye (Fluka) for 10 min at 37°C and then washed with PBS. *Caspase activation*: The active (cleaved) form of caspase-3 was detected after 24 h of T-cell/MSC co-cultures. The cells were fixed in 4% paraformaldehyde, permeabilized with PB and treated with rabbit anti-caspase-3 (recognizing the cleaved form, Cell SignalingTechnology) and then anti-rabbit IgG-TRITC (DAKO). *DNA fragmentation* was detected after 24 h co-culture using DeadEnd Colorimetric TUNEL System (Promega) according the manufacturer's instructions.

Finally, all samples on the cover slips were mounted with a drop of Fluoromount-G (Southern Biotech). To determine the apoptosis rate, samples were analysed with Axioskop 2Mot (Carl Zeiss) fluorescence microscope using AxioCam camera, AxioVision 3.1 software and 20× objective magnification. Image contrast was adjusted using Adobe Photoshop CS4 extended 11.0. In all cases, at least 100 cells/sample were analysed, and the rate of apoptosis was determined as follows: % of apoptotic cells = positive cells/total cell number × 100. Positive cells regarded the stained cells by the method used, e.g Annexin V, anti-ceramid, JC-1, propidium iodide.

Apoptosis of human activated T-cells*:* The apoptosis induced by cell-derived Gal-1 was detected as previously described [15]. Briefly, $HeLa^{mock}$ (control) or $HeLa^{Gal}$ cells (effector cells, 5×10^3 cells/sample) were plated on cover slips. Target T-cells (PHA activated T-cells, 2×10^5 cells/sample) were labelled with Hoechst 33342 (100 ng/ml for 30 min at 37 °C) and co-cultured with HeLa cells for 16 hours. The T-cells were subsequently labelled with Annexin V-Alexa Fluor 488 (Invitrogen) for 30 min and mounted with Fluoromount-G. Finally, the samples were analysed 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: relative apoptotic ratio (RAR) = % of Annexin V positive cells on $HeLa^{Gal\ -1}$- % of Annexin V positive cells on $HeLa^{mock}$.

Lectins	Abbreviation	Specificity	Ref.
Concanavalin A	ConA	Mannose, Glucose (low affinity)	[131]
Lens culinaris Agglutinin	LCA	core-fucosylated bi-antennary N-glycan	[132]
Wheat Germ Agglutinin	WGA	GlcNAc, sialic acid*	[133]
Phaseolus vulgaris Leucoagglutinin	PHA-L	β-1,6-branched tri- and tetra-antennary N-glycans	[134]
Sambucus nigra Agglutinin	SNA	α-2,6-linked sialic acid	[135]
Galectin-1	Gal-1	LAcNAc	[7]
Abbreviations: GlcNAc: N-acetylglucosamine; LAcNAc: N-acetyllactoseamine			

Enzyme Genes	Gene Symbol	Full Gene Name
Mannosidase	*MAN1A1*	Mannosidase Alpha Class 1A Member 1
	MAN1A2	Mannosidase Alpha Class 1A Member 2
	MAN2A1	Mannosidase Alpha Class 2A Member 1
	MAN2A2	Mannosidase Alpha Class 2A Member 2
Acetylglucosaminyltransferase	*MGAT1*	Mannosyl (Alpha-1,3-)-Glycoprotein Beta-1,2-N-Acetylglucosaminyltransferase
	MGAT4A	Mannosyl (Alpha-1,3-)-Glycoprotein Beta-1,4-N-Acetylglucosaminyltransferase, Isozyme A
	MGAT4B	Mannosyl (Alpha-1,3-)-Glycoprotein Beta-1,4-N-Acetylglucosaminyltransferase Isozyme B
	MGAT5	Mannosyl (Alpha-1,6-)-Glycoprotein Beta-1,6-N-Acetyl-Glucosaminyltransferase
Sialyltransferase	*ST3GAL3*	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 3
	ST3GAL4	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 4
	ST3GAL6	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 6
	ST6GAL1	ST6 Beta-Galactosamide Alpha-2,6-Sialyltranferase 1
Neuraminidase	*NEU1*	Neuraminidase 1

Table 1. Names, abbreviations and binding specificities of lectins (upper panel) as well as names and abbreviations of glycosylation enzymes (lower panel).

3.6. Quantitative Real-Time PCR

Peripheral blood mononuclear cells (PBMC) (1-3x10^6 cells) were activated with 1μg/ml PHA-L for 72 hours then washed twice with PBS. Total RNA was extracted applying Nucleospin RNA II isolation kit (MACHEREY-NAGEL GmbH, Düren,

Germany) per manufacturer's instructions. cDNA was synthesized using 2 μg of total RNA, in the presence of 50 pmol of oligo(dT_{18}) and of random hexamer primers, 0.5 mM dNTP, 20 U RiboLock RNase Inhibitor and 200 U RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Boston, MA, USA) for 60 min at 42 °C and then heated for 10 min at 70 °C. qPCR was performed using AccuPower Greenstar qPCR Master Mix (Bioneer, Daejeon, Korea) in RotoGene3000 instrument (Corbett Life Science, Quiagen, Hilden, Germany). Relative quantification of gene expression was determined by comparison of threshold cycles (Ct) related to endogenous *RPL27*. Relative mRNA amounts (R) were calculated with the equation $R = 2^{Ct(RPL27)-Ct(GENE)}$. The following primer sequences were used:

RPL27:

forward 5'-CGCAAAGCTGTCATCGTG-3'

reverse 5'-GTCACTTTGCGGGGGTAG-3'

Galectin-1 (LGALS1):

forward 5'-CGCCAGCAACCTGAATCT-3'

reverse 5'-CAGGTTCAGCACGAAGCTCT-3'.

Mannosidase Alpha Class 1A Member 1 (MAN1A1):

forward: 5'-TTGGGCATTGCTGAATATGA-3'

reverse: 5'-CAGAATACTGCTGCCTCCAGA-3'

Mannosidase Alpha Class 1A Member 2 (MAN1A2):

forward: 5'-GGAGGCCTACTTGCAGCATA-3'

reverse: 5'-GAGTTTCTCAGCCAATTGCAC-3'

Mannosidase Alpha Class 2A Member 1 (MAN2A1):

forward: 5'-CCTGGAAATGTCCAAAGCA-3'

reverse: 5'-GCGGAAATCATCTCCTAGTGG-3'

Mannosidase Alpha Class 2A Member 2 (MAN2A2):

forward: 5'-TCCACCTGCTCAACCTACG-3'

reverse: 5'-TGTAAGATGAGTGCGGTCTCC-3'

Mannosyl (α-1,3-)-Glycoprotein β-1,2-N-Acetylglucosaminyltransferase (MGAT1):

forward: 5’-CGGAGCAGGCCAAGTTC-3’

reverse: 5’-CCTTGCCCGCAGTCCTA-3’

Mannosyl (α-1,3-)-Glycoprotein β-1,4-N-Acetylglucosaminyltransferase A (MGAT4A):

forward: 5’-CATAGCGGCAACCAAGAAC-3’

reverse: 5’-TGCTTATTTCCAAACCTTCACTC-3’

Mannosyl (α-1,3-)-Glycoprotein β-1,4-N-Acetylglucosaminyltransferase B (MGAT4B):

forward: 5’-CACTCTGCACTCGCTCATCT-3’

reverse: 5’-CACTGCCGAAGTGTACTGTGA-3’

Mannosyl (α-1,6-)-Glycoprotein β-1,6-N-Acetyl-Glucosaminyltransferase (MGAT5):

forward: 5’ GCTCATCTGCGAGCCTTCT-3’

reverse: 5’-TTGGCAGGTCACCTTGTACTT-3’

Beta-1,4-Galactosyltransferase 1 (B4GALT1):

forward: 5’-CTAGCAACTTGACCTCGGT-3’

reverse: 5’-CATTTGGGTTCTGCTTTGCC-3’

Beta-1,4-Galactosyltransferase 3 (B4GALT3):

forward: 5’-GAGGATGACGACATTGCTACC-3’

reverse: 5’-TCGGTGCTTCACCATCTTATAG-3’

ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 3 (ST3GAL3):

forward: 5’-TATGCTTCAGCCTTGATG -3’

reverse: 5’-TTGGTGACTGACAAGATGG -3’

ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 4 (ST3GAL4):

forward: 5’-ATGTTGGCTCTGGTCCTG-3’

reverse: 5’-AGGAAGATGGGCTGATCC-3’

ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 6 (ST3GAL6):

forward: 5’-TCTATTGGGTGGCACCTGTGGAAA-3’

reverse: 5’- TGATGAAACCTCAGCAGAGAGGCA -3’

ST6 Beta-Galactosamide Alpha-2,6-Sialyltranferase 1 (ST6GAL1):

forward: 5’-TGGGACCCATCTGTATACCACT-3’

reverse: 5’-ATTGGGGTGCAGCTTACGAT-3’

Neuraminidase 1(NEU1):

forward: 5’- CCTGGATATTGGCACTGAA -3’

reverse: 5’- CATCGCTGAGGAGACAGAAG -3’

4. Results

4.1. Immunosuppressive effect of Gal-1 in systemic *in vivo* or *ex-vivo* modells

The Gal-1 effect is mediated by surface presentation of the lectin on producing cells or extracellular matrix [7]. Mesenchymal stromal cells are an important immunosuppressive cell type implicated in various therapeutic approaches for immunological pathologies [63–65,136] and are known to secrete Gal-1 among other anti-inflammatory factors. To understand the contribution of a powerful immunosuppressive factor, Gal-1 to the immunoregulatory function of MSCs, wild type (WT) and Gal-1 knock-out ($MSC^{gal-/-}$) MSCs, isolated from mouse bone marrow were studied in *in vivo* and *in vitro* experiments.

As one of our previous result showed, injection of MSC along with bone marrow nucleated cells was an effective therapeutical treatment in STZ-induced mouse diabetes type I model. In this set up MSCs functioned as an immunosuppressive cell type hence preventing autoimmune reaction against pancreatic β-islet cells [137]. To see the role of Gal-1 in MSCs, the effect of wtMSC was compared to $MSC^{gal-/-}$. As shown on Fig3, Gal-1 knockout MSC were as effective as wtMSCs in reducing blood glucose level (Fig 3A) and elevating the survival time (Fig 3B). These results confirmed that Gal-1 did not participate in the MSCs' immunosuppressive effect when MSCs act systemically. This data was also supported in an ovalbimune-induced DTH mouse model as wt and Gal-1 KO MSCs similarly diminished the OVA-induced paw oedema in hind limbs (data not shown) [32].

The failure detecting Gal-1 immunoregulatory effect in *in vivo* was unexpected since our previous *in vitro* results clearly showed this function regarding induction of human T-cell apoptosis [15]. Indeed, mouse wt MSCs triggered apoptosis of activated T-cell in a direct co-culture (Fig 4) by the same pathway as human Gal-1 producing tumor cells did [15]. The steps of MSC-induced T-cell death (Fig 4) included exposure of extracellular phosphatidylserine (Fig 4A), ceramide release (Fig 4B) followed by decreased mitochondrial potential (Fig 4C), caspase activation (Fig 4D) and DNA fragmentation (Fig 4E). In contrast, Gal-1 deficient MSCs (Fig 4A-E) or wtMSCs whose cell surface Gal-1 was removed with Gal-1 ligand (Fig 4A), TDG prior to apoptosis assay,

did not cause apoptosis. These results confirmed that Gal-1 was an essential factor for stimulation of T-cell death in MSCs and as we previously showed apoptosis occurred when target and effector cells were in interaction [15]. However, the *in vivo* findings regarding the similar effectiveness of wild type and Gal-1 deficient MSCs were not explained (Fig 3). A further experiment might serve an explanation: MSCs inhibited T-cell proliferation [138] and this effect required direct cell contact between MSCs and T-cells in co-culture (Fig. 5). However wild type and Gal-1 deficient MCSs caused identical inhibition of T-cell proliferation (Fig. 5), indicating that MSCs' proliferation inhibition did not depend on the expression of Gal-1. Moreover Gal-1 could be unfunctional in MSCs' apoptotic effect *in vivo,* since in systemic application the probability of direct contact of MSCs and activated T-cells was extremely low.

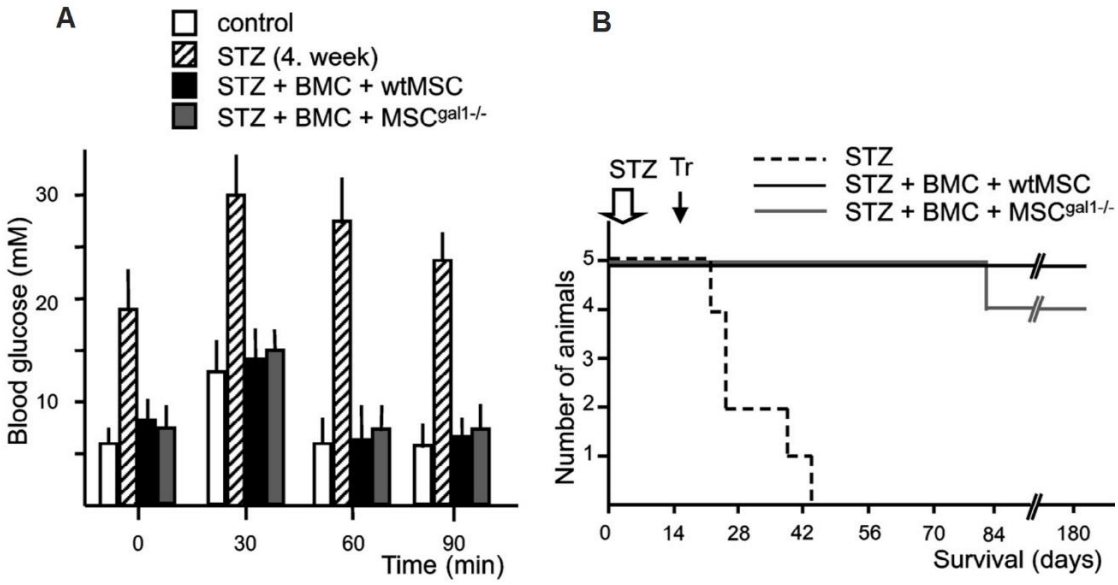


Fig 3. Galectin-1 expression does not play a role in the therapeutic effects of MSCs on STZ-induced diabetes. (A) Glucose tolerance test was carried out with mice fasting overnight and then injected with 2 g/kg of body weight glucose intraperitoneally. Blood sugar levels of control mice (white bars), STZ-induced diabetic mice (striped bars), diabetic mice treated with BMCs and wtMSCs (black bars) or MSCgal1−/− (gray bars) were measured at the indicated time points after glucose loading. (B) Survival of diabetic mice treated with BMCs and wtMSCs (black line) or $MSC^{gal1-/-}$ (gray line), or untreated (dashed line) was followed up to 180 days.

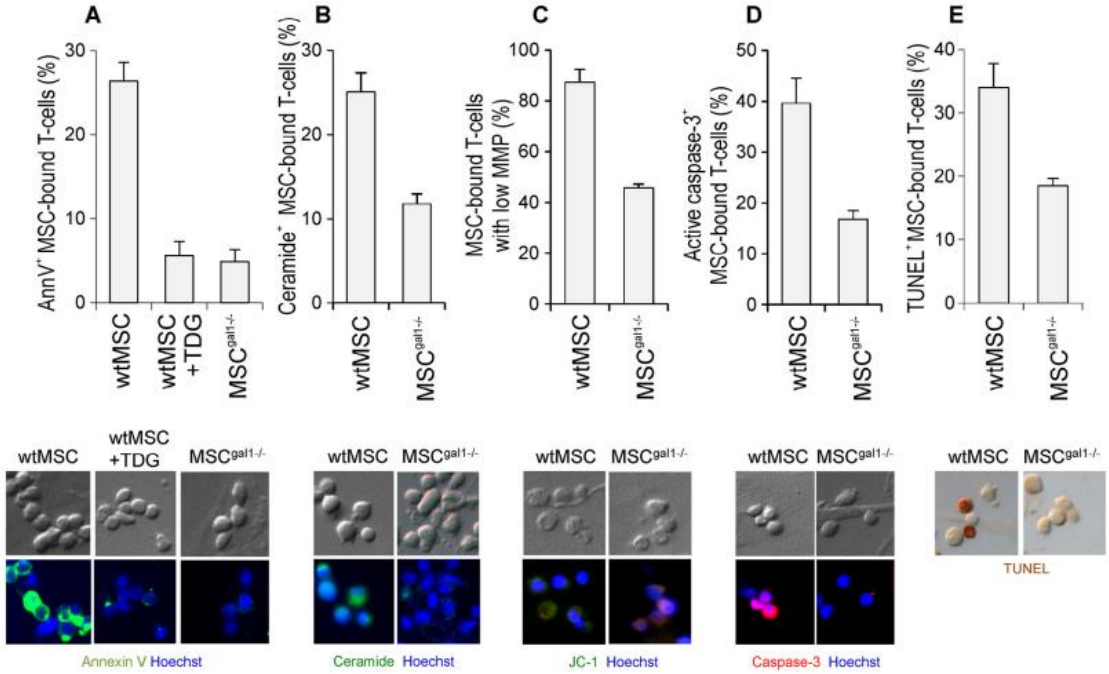


Fig 4. MSC-derived Gal-1 induces apoptosis of activated T-cells.

T-cells were activated with 7.5 μg/mL Con A for 72 h, and then the nuclei were labelled with Hoechst 33342 (blue). Activated T-cells were then co-cultured with wtMSCs or $MSC^{gal-/-}$ for 16 h (A–C) or 24 h (D, E). (A) The cells were fixed, stained with Annexin V-Alexa Fluor 488 (green) and analysed with fluorescence microscope. For removal of cell-surface Gal-1, wtMSCs were pre-incubated with TDG before co-cultures. (B) Detection of ceramide levels was carried out by incubating the samples with anti-ceramide mAb and then with biotinylated anti-mouse IgM and streptavidin-FITC (green). (C). Changes in the mitochondrial membrane potential (MMP) (red: high MMP, green: low MMP) was detected with JC-1 fluorescence dye labelling. (D) Caspase-3 activation was visualized with anti-cleaved caspase-3 antibody and anti-rabbit IgG-TRITC (red). (E) DNA fragmentation was observed with terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay (brown). Samples were analysed with fluorescence and light microscopy. Scale bar represents 10μm. The bars show mean ± SD of the percentage of positive cells, counted from at least 100 cells in 5 non-overlapping fields of view and two independent experiments.

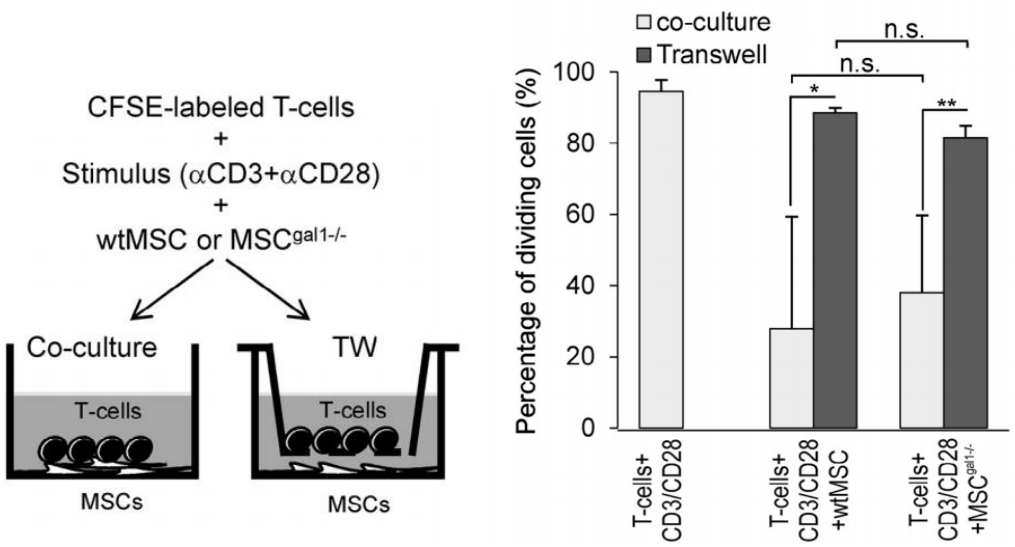


Fig 5. Inhibition of T-cell proliferation by MSC requires direct cell contact and is independent of Gal-1.

CFSE-labelled T-cells were activated with anti-CD3/CD28 beads and were then co-cultured with wtMSCs or MSCgal1−/− in direct co-cultures (grey bars) or in indirect cocultures physically separated by a Transwell insert (TW, dark bars). See experimental scheme (left panel). CFSE fluorescence intensity of the samples was analysed with flow cytometry after 72 h. The percentage of proliferating T-cells in the various culture conditions is presented as mean ± SD of data pooled from three independent experiments. Statistics: ANOVA ($P < 0.05$), followed by Fisher's least significant difference method, *$P < 0.05$, **$P < 0.01$, n.s., not significant. Samples were analysed with fluorescence and light microscopy. Scale bar represents 10μm. The bars show mean ± SD of the percentage of positive cells, counted from at least 100 cells in 5 non-overlapping fields of view and two independent experiments.

From the above data, it became apparent that the Gal-1 producing effector cells and the target activated T-cell had to be in physical closeness. It has been known that T-cells produce Gal-1 upon activation suggesting that it could function as an autocrine apoptotic factor [20]. When we analysed the cellular localization of Gal-1 in healthy activated T-cells, we showed that Gal-1 was expressed but not secreted in healthy T-cells (Fig 6) hence excluding the autocrine effect. Similar results were obtained in mouse activated T-cells and Gal-1 transgenic Jurkat cells (data not shown, [28]).

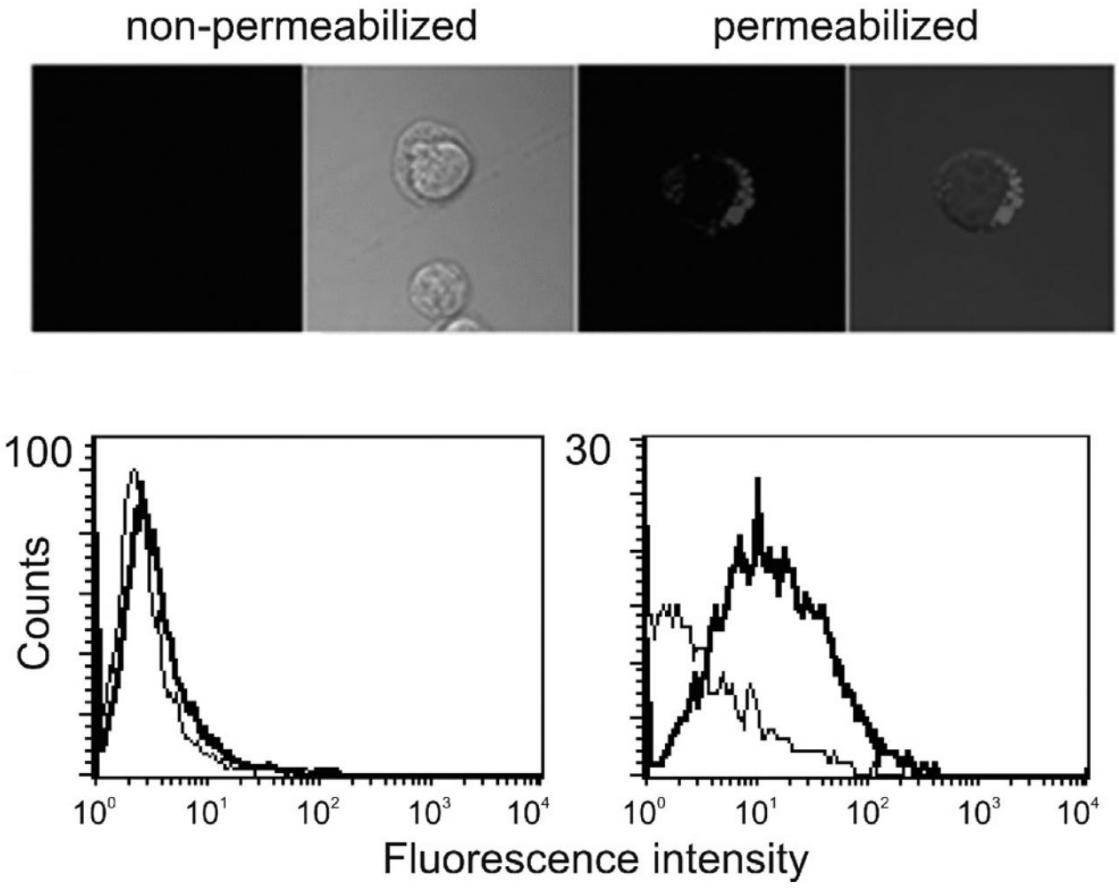


Fig 6. Activated T-cells express but do not secrete Gal-1

T-Cells were suspended in cold IFB or IFB supplemented with 0.1% Triton-X 100 for non-permeabilized or permeabilized samples, respectively. Cellular Gal-1 was detected using mouse anti-human Gal-1 monoclonal antibody followed by donkey anti-mouse IgG conjugated with NorthernLights557. Finally, the cells were mounted on microscopic slides with Fluoromount-G and analysed with laser scanning confocal microscope (upper). Alternatively, the cells were incubated with mouse mAb to Gal-1 (2C1/6) followed by goat anti-mouse IgG-Atto 488 (Sigma). The samples were analysed by FACSCalibur flow cytometer using the CellQuestTM software or subjected to flow cytometry (lower).

To clarify the role of *de novo* expressed Gal-1, Gal-1 producing and Gal-1 deficient T-cells were compared regarding their response to Gal-1 induced apoptosis. Activated mouse wild type or Gal-1 knock-out T-cells were cultured together with mouse MSCs as Gal-1 source. Gal-1 deficient T-cells displayed significantly reduced response to extracellular Gal-1 compared to wild type, Gal-1 expressing T-cells (Fig 7). Similarly, Gal-1 transgenic Jurkat cells responded more vigorously to treatment with extracellular Gal-1 than their Gal-1 non-expressing counterparts (data not shown). These results indicated that intracellular Gal-1 regulated the apoptotic response for extracellular Gal-1.

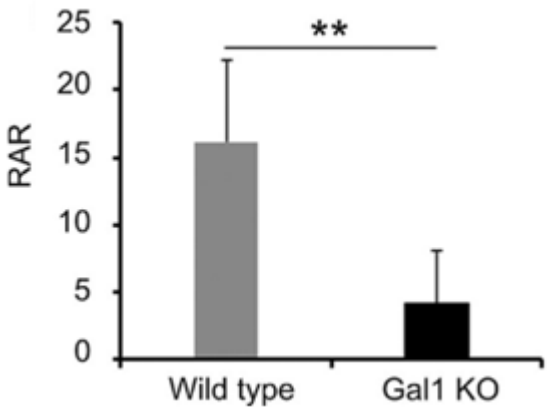


Fig 7. Absence of intracellular Gal-1 protects T-cells from the apoptotic effect of extracellular Gal-1. The Annexin V positive cells were counted in every sample (at least 100 cells/sample) and the percentage of apoptotic cells was calculated. Apoptosis calculated as relative apoptotic ratio, RAR as follows: RAR = % of Annexin V positive cells on WTMSC − % of Annexin V positive cells on KOMSC. n = 3, ** $p < 0.01$.

4.2. Role of Gal-1 in the pathomechanism of human autoimmune diseases

Previous studies have shown that expression of Gal-1 is decreased at sites of chronic inflammation [139]. However, expression of Gal-1 in T-cells has not been studied even though dysfunctional T-cell apoptosis has been documented as a crucial defect in SLE pathogenesis. Hence, Gal-1 expression was evaluated in activated T-cells of healthy, active and treated SLE patients using qPCR. Significant difference was found between the samples since active SLE T-cells expressed the lowest Gal-1 mRNA compared to healthy or inactive SLE T-cells (Fig 8). One remarkable result was that Gal-1 expression returned to healthy levels after remission.

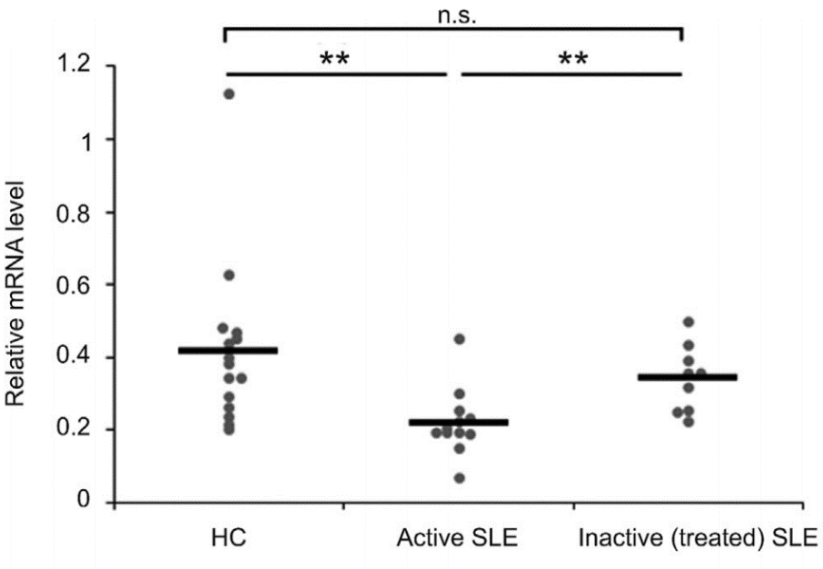


Fig 8. Relative Gal-1 mRNA level in patients' T-cells.

Gal-1 mRNA level was measured in activated T-cells by qPCR. HC: healthy control, n=16, Active SLE, n=14, Inactive (treated) SLE, patients in remission after treatment, n=9, **p<0.01. Relative mRNA level was calculated as described in Materials and methods section.

Whether the low expression of Gal-1 in pathological T-cells from SLE patients affected their sensitivity to extracellular Gal-1's apoptotic effect was investigated in a co-culture system. T-cells from 14 active SLE patients, 16 healthy controls, and 9 inactive SLE patients in remission after successful therapy were activated and co-cultured with Gal-1 producing HeLa cells. As shown in Fig 9, activated T-cells form healthy controls and form SLE patients in remission responded with apoptosis to Gal-1 while T-cells from active SLE patients were unresponsive. However, unresponsiveness of SLE T-cells to Gal-1 apoptotic effect could derive from various reasons such as modulation of the intracellular signals mediated by intracellular Gal-1 or presentation of cell surface Gal-1-binding structures in pathological situations.

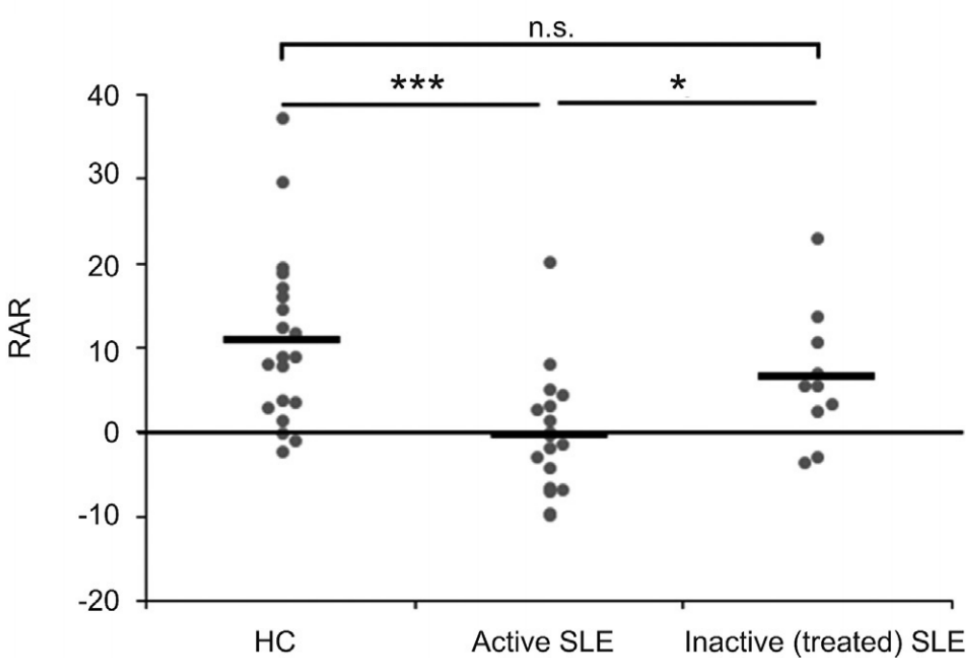


Fig 9. Presence of extracellular Gal-1 does not influence viability of T-cells of active SLE patients. Apoptosis assay was carried out in co-culture as described in Materials and Methods. RAR = (percentage of apoptotic T-cells when cultured together with $HeLa^{Gal\text{-}1}$) – (when cultured together with $HeLa^{mock}$). Zero line denotes no difference in T-cell apoptosis while cultured with $HeLa^{Gal\text{-}1}$ or $HeLa^{mock}$. HC n = 20, active SLE n = 20, inactive SLE n = 10. ***$p < 0.001$, *$p < 0.05$.

To obtain a better insight into the reason of diminished Gal-1 apoptotic effect, the cell surface glycosylation of activated autoimmune T-cells from rheumatoid arthritis (RA), Sjögren's syndrome (SS) and SLE patients was analysed and compared to that of healthy control T-cells using lectin-binding assay. For this purpose, different plant lectins with known specificities. L-PHA, ConA, LCA, Gal-1, SNA, RCA and WGA as well as a human lectin Gal-1 binding to activated T-cells was investigated. Lectin binding assay is applicable to study cell surface glycosylation pattern since the applied lectins specifically bind to distinct surface glycan structures allowing the characterisation of N-linked cellular sugar code repertoires (Fig 2, Table 1).

Generally, stimulation of the T-cells with PHA-L resulted in elevation of binding of almost all lectins (data not shown) indicating an increased density of glycans on the surface. We focused on the differences between control and patient T-cells in lectin binding after activation (Fig 10).

According to our results, healthy T-cells, RA and SS patient T-cells could bind similar amounts of Gal-1 before activation, but SLE patient T-cells bound slightly higher

amounts compared to controls (Fig 10A). The amount of bound Gal-1 was significantly elevated in healthy individuals and in RA patients after activation whereas SLE and SS individuals showed much less pronounced change (Fig 10C). All patient groups could bind significantly less of the lectin than controls after activation (Fig 10B). Reduced galectin-1 binding can be a result of at least three things: 1) reduced complexity of N-glycan antennary structures which can bind Gal-1, 2) downregulation of Gal-1 binding proteins on T-cell surface and 3) increased terminal sialylation of glycoproteins which can bind Gal-1.

Plant lectin binding was similar in resting T-cells of control, RA and SS individuals. Nevertheless, resting T-cells from SLE patients showed remarkable differences, specifically more ConA, WGA and SNA was bound than to control T-cells (Fig 10A). Upon activation of T-cells, alteration in glycosylation was remarkably different in the case of SLE and SS cells. Changes in ConA, LCA, WGA and PHA-L binding to SLE, and LCA and PHA binding to SS T-cells were significantly lower than in controls (Fig 10C).

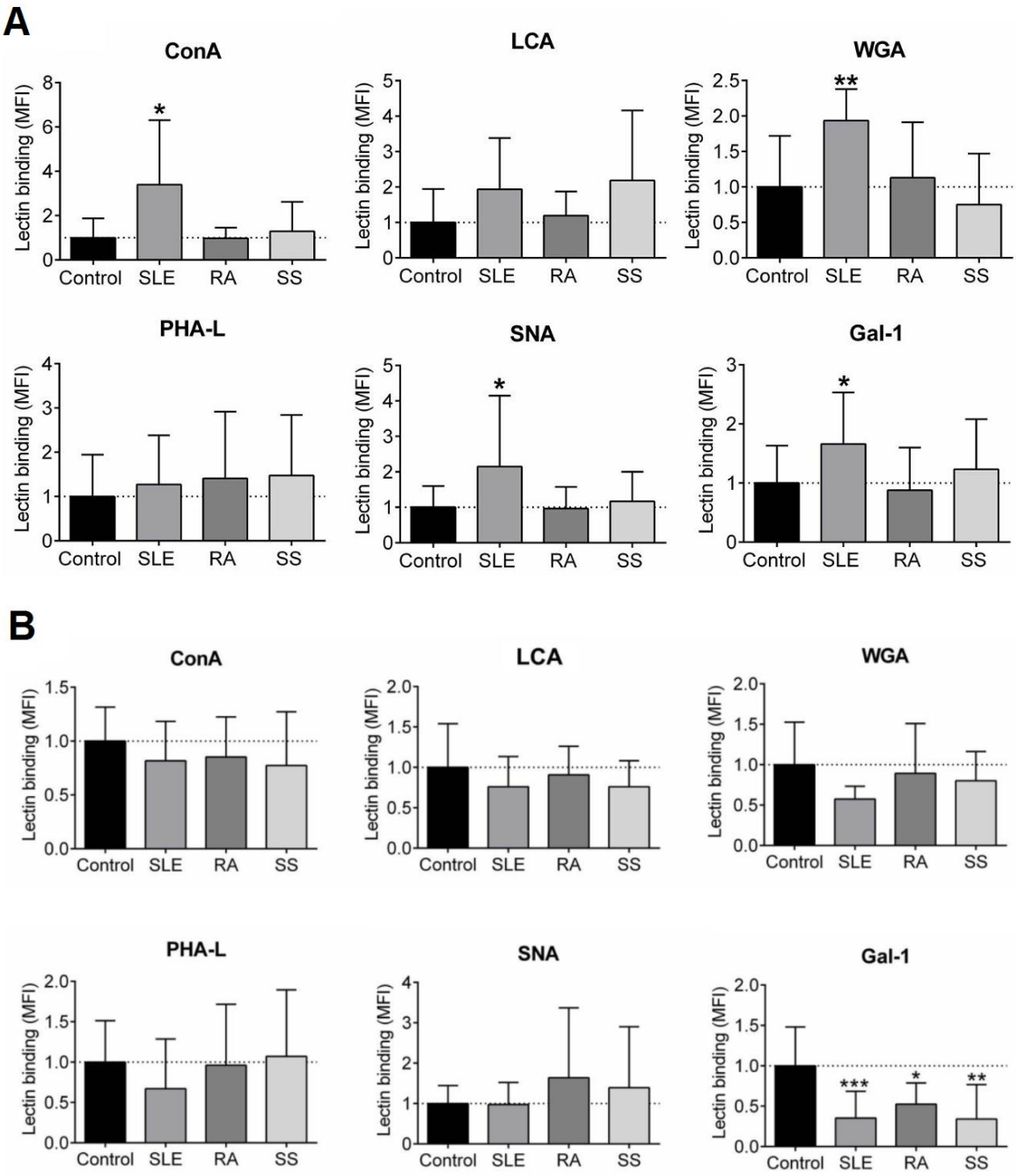
To support lectin binding data and possibly discover further changes in glycosylation, the expression levels of enzyme coding genes involved in glycosylation (Fig 11) were examined by qPCR. Gene expression levels of healthy and diseased donor T-cells were analysed after 72 h activation.

Alpha-mannosidase encoding genes (MAN1A1, MAN1A2, MAN2A1 and MAN2A2) expressed at significantly higher level in SS T-cells than in control T-cells. Higher expression of these four genes was also observed in RA T-cells, but only the difference in MAN2A2 mRNA expression between control and RA was significant. In SLE T-cells MAN1A1 gene expression was elevated compared to control, although not significantly so (Fig 11).

We found that MGAT1 gene was significantly higher expressed in SS T-cells and slightly higher expressed in SLE and RA T-cells compared to control T-cells (Fig 11). MGAT4A and MGAT4B genes were significantly higher expressed in SLE T-cells compared to controls (Fig 11). Interestingly, MGAT5 gene expression was upregulated in both SLE, RA and SS T-cells.

B4GALT1 gene expressed at similar level in all groups, while B4GALT3 expression was slightly higher in all patient groups compared to control group T-cells (data not shown).

ST6GAL1 expression was significantly elevated in RA and SS T-cells compared to control T-cells and ST3GAL6 expression was significantly higher in SLE and RA T-cells, however ST3GAL3 and ST3GAL4 expression was similar between the examined groups (Fig 12).



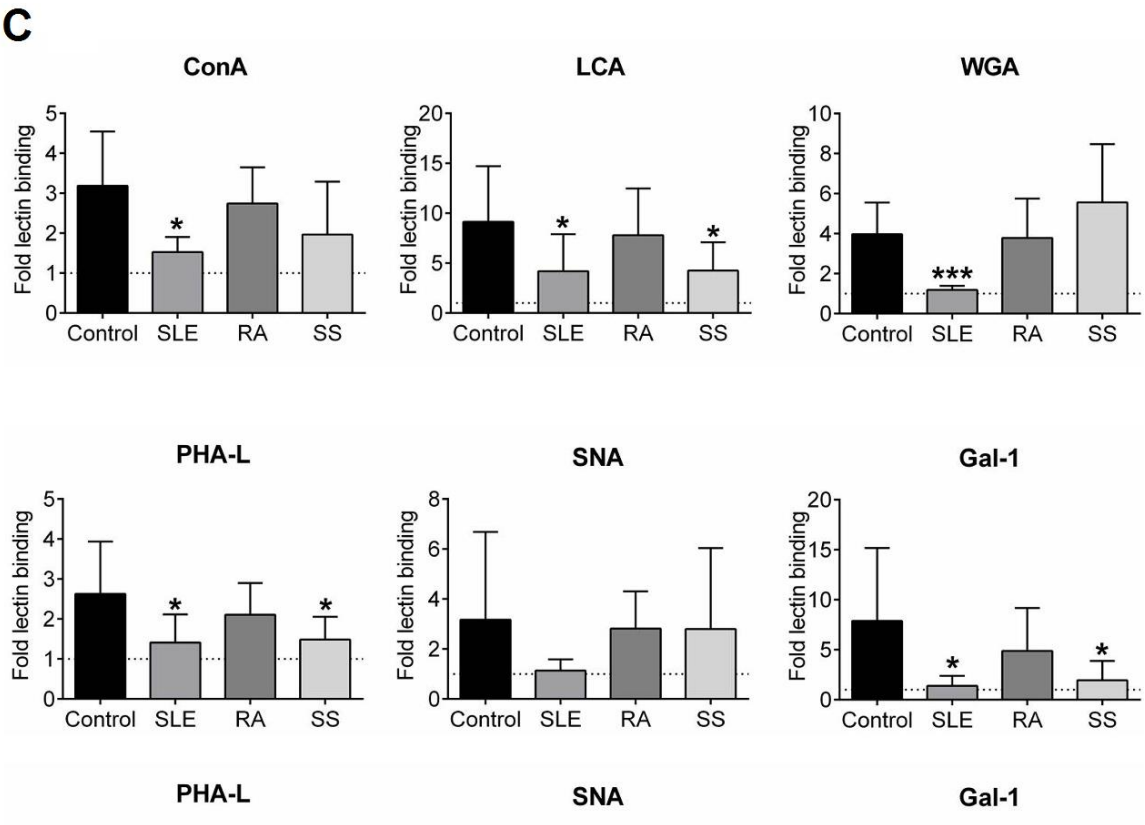


Fig 10. Lectin binding properties of peripheral blood T-cells from heathy donors and from SLE, RA and Sjögren syndrome patients. A. Resting peripheral blood derived T-cells of healthy controls (n = 15), SLE (n = 9), RA (n = 13) and SS (n = 11) patients. **B**. Activated T-cells of healthy controls (n = 15), SLE (n = 9), RA (n = 13) and SS (n = 11) individuals. **C.** Fold changes in lectin binding of T-cells after 72-hour activation period. Median fluorescence intensity (MFI) data was normalized to the 0-hour binding properties of the groups. *P < 0.05; **P < 0.01; ***P < 0.001.

During T-cell activation, the expression of NEU1 on the cell surface is upregulated strikingly although NEU3 expression remains relatively constant [140]. In our experiments, NEU1 expressed at significantly lower level in SLE, RA and SS T-cells compared to control T-cells (Fig 12).

Since sialyltransferases and neuraminidases catalyse opposite reactions, the gene expression ratios of sialyltransferases and NEU1 were calculated to highlight the sialylation status at expression level in T-cells. Significantly higher ST6GAL1/NEU1 were observed in RA and SS T-cells, and ST3GAL6/NEU1 ratio was elevated in SLE, RA and SS T-cells compared to healthy donor T-cells (Fig 12A), whereas ST3GAL3/NEU1 and ST3GAL4/NEU1 ratios were similar in all examined groups (data

not shown). Another interesting result was the correlation between SLEDAI scores and ST6GAL1/NEU1 ratio (Fig 12B).

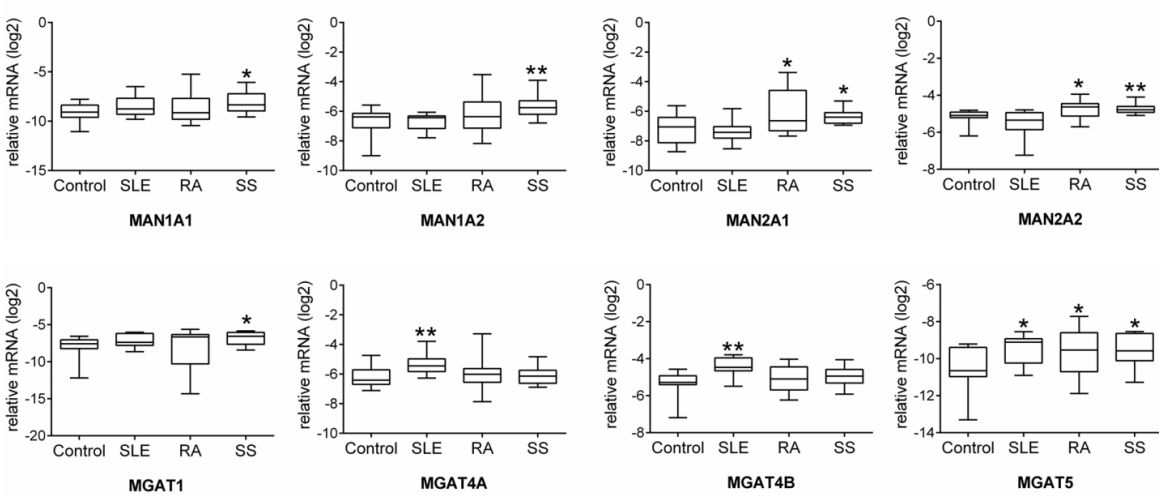
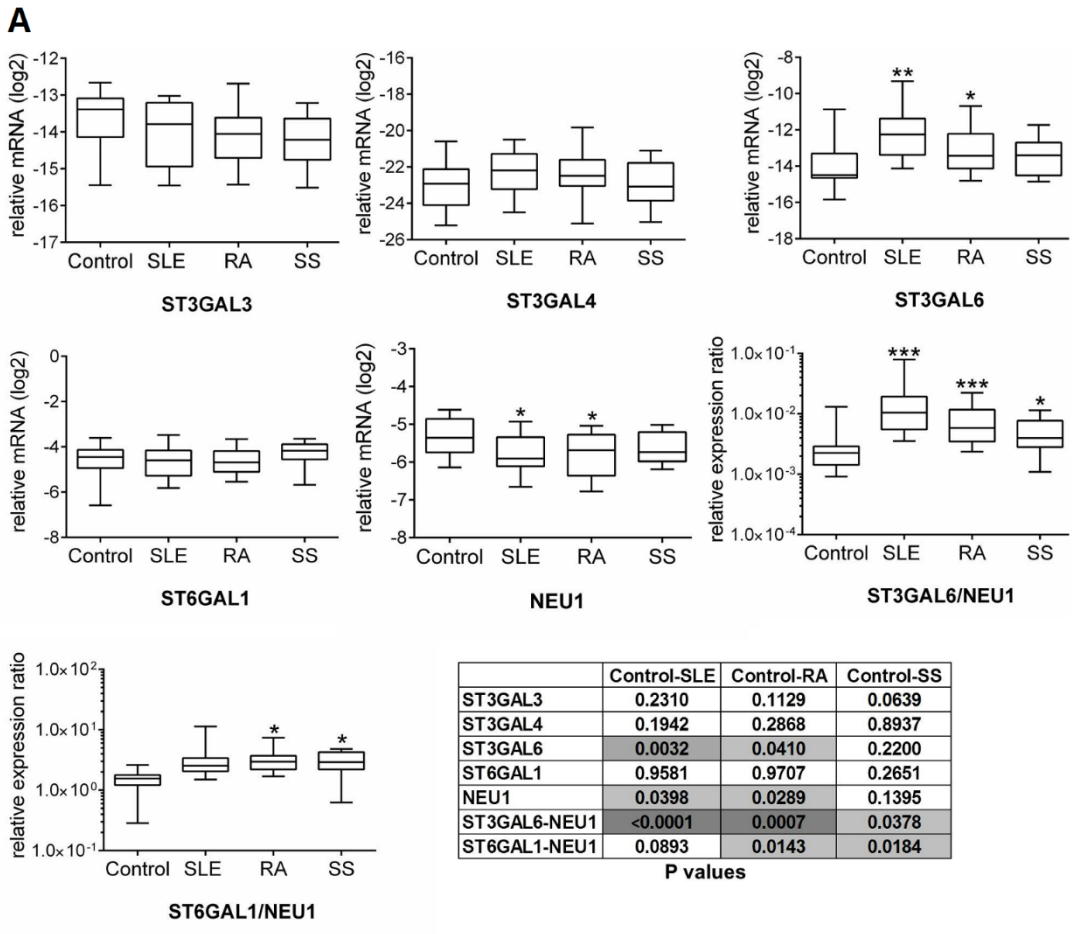


Fig 11. Expression levels of genes encoding enzymes involved in *N*-glycosylation in activated T-cells from healthy individuals, SLE, RA and SS patients. Total RNA was extracted from T-cells activated for 72 h in the presence of 1 μg/mL phytohemagglutinin L (PHA-L) and mRNA expression levels were analysed by qPCR. *RPL27* served as a housekeeping gene. The SLE (n = 9), RA (n = 13) and SS (n = 10) relative expression results ($2^{\Delta Ct}$ data) are normalized to the expression levels measured in control individuals (n = 15) + SD. *P < 0.05; **P < 0.01; ***P < 0.001. Gene names, primer sequences and calculations of relative mRNA levels are described in Materials and Methods section.



	Control-SLE	Control-RA	Control-SS
ST3GAL3	0.2310	0.1129	0.0639
ST3GAL4	0.1942	0.2868	0.8937
ST3GAL6	0.0032	0.0410	0.2200
ST6GAL1	0.9581	0.9707	0.2651
NEU1	0.0398	0.0289	0.1395
ST3GAL6-NEU1	<0.0001	0.0007	0.0378
ST6GAL1-NEU1	0.0893	0.0143	0.0184

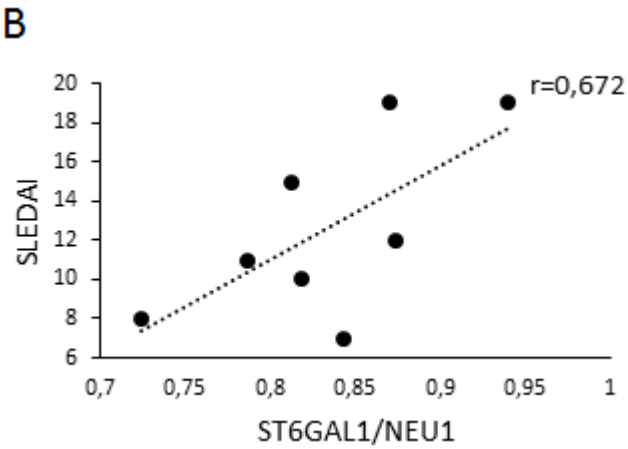


Fig 12. Expression levels of genes encoding enzymes involved in sialylation and desialylation of N-glycoproteins in activated T-cells from healthy individuals, SLE, RA and SS patients, and their correlation with SLEDAI scores. A) Total RNA was extracted from T-cells activated for 72 h in the presence of 5 μg/mL phytohemagglutinin L (PHA L) and mRNA expression levels were analysed by qPCR. The relative expression results from healthy control individuals (n = 15), SLE (n = 9), RA (n = 13) and SS (n = 10) patients are normalized to the expression levels of RPL27 housekeeping gene (log2 transformation, ΔCt). The mRNA expression ratios of each patient were calculated as follows: 2ΔCt ST3GAL6 / 2ΔCt NEU1 or 2ΔCt ST6GAL1 / 2ΔCt NEU1. Gene names, primer sequences and calculations of relative mRNA

levels are described in Table 1 and Materials and Methods section. B) Correlation between SLEDAI scores and ST6GAL/Neu1 ratio. The parametric Pearson correlation test was used to to investigate the relationships between mRNA ratios and SLEDAI scores (n = 8).

5. Discussion

The role of endogenous Gal-1 in disease development and progression has recently been shown in the animal models of autoimmune or inflammatory diseases such as arthritis, colitis, hepatitis, nephritis, encephalomyelitis and SLE [21,26,29,30,128,141]. To better understand the role that Gal-1 plays in systemic immunosuppression, we studied it in MSCs, an important immunosuppressive cell type implicated in various therapeutic approaches for immunological diseases [62–64]. Though MSCs express a plethora of immunosuppressive factors, one of their most potent anti-inflammatory molecule is Gal-1 [70,72]. Secretion of Gal-1 by MSCs has been implicated in inhibiting T-cell proliferation [73–75], and the role of MSC derived Gal-1 as an immunoregulatory factor has been confirmed [75]. Gal-1 dependence of the *in vivo* immunosuppressive potential of MSCs has been analysed in two mouse models, OVA-induced DTH [142] and STZ-induced type I diabetes [137], in which the therapeutic effect of wildtype MSCs has been previously reported.

Previous *in vitro* studies have used various approaches in studying the lectin, both through inhibition of Gal-1 with thiodigalactoside, which blocks Gal-1 binding to carbohydrate ligands [73], as well as by blocking Gal-1 with specific antibodies [74] and generation of stable Gal-1 knockdown in MSC clones [75]. However, thiodigalactoside reacts with other lectins with similar specificities to that of Gal-1, and blocking antibodies may only be partially effective [73,74]. Furthermore, the generation of stable galectin-1 knockdown MSC clones [75] bears the possibility for off-target effect of the knockdown construct and also raises the problem of generating less potent MSC clones from the substantially heterogenic MSC population, irrespective of their Gal-1 expression [143]. To avoid these disadvantages, we used wild-type and Gal-1-deficient MSCs isolated from wild-type and Gal-1 knockout mice, respectively. Both wtMSCs and $MSC^{gal1-/-}$ display the same characteristics including cell surface markers, differentiation capacity [130] and expression of the main immunosuppressive genes, *Nos2*, *Cox2* and *Tgfβ* (data not shown) [144]. According to our results, Gal-1 acts via intimate cell-to-cell contact [15,25] on

activated T-cells, and apoptosis induction of activated T-cells depends on MSC-produced Gal-1. Cell-bound Gal-1 triggers apoptosis only on those T-cells that are activated and directly couple to the Gal-1 expressing effector cells (MSCs). The molecular mechanism of the apoptosis is identical to that previously described by our laboratory for recombinant or tumour cell–derived Gal-1 [15,23]. The experiments presented here indicate that Gal-1 does not participate in the systemic immunosuppressive function of MSCs; however, it may fulfil its immunoregulatory role locally when direct cell-to-cell interaction between the secreting cells and T-cells occurs. For instance, Perone et al. clearly showed that dendritic cells (DCs) localize in the lymph nodes and spleen, the sites of immune responses [129]. In this situation, the antigen-presenting DCs and the antigen specific T-cells establish a close interaction, and cellular delivery of Gal-1 by Gal-1 transgenic DC was found to exert a therapeutic effect on diabetes in NOD mice [129]. In contrast, MSCs used as therapeutic means in diabetes model do not localize in the immune organs or in the damaged pancreas in the diabetic animals [137], indicating that their role in tissue regeneration is accounted for by an indirect rather than a local effect.

PCR measurements on T-cells of SLE patients showed decreased Gal-1 mRNA levels and treatment of the T-cells with cell-bound Gal-1 in co-culture showed that the lectin did not exert its apoptotic effect on these dysfunctional lymphocytes. Intracellular Gal-1 has not been implicated before in the process of apoptosis, but our former results showed that activated T-cells do not secrete *de-novo* expressed Gal-1 [20]. In line with the failure of production of intracellular Gal-1, the extracellular Gal-1 induced apoptosis of activated SLE T-cells also diminished. Furthermore, our results on activated murine T-cells showed that Gal-1 knock out cells similarly exhibited a decreased response to extracellular Gal-1 compared to wild type controls. Thereby endogenous Gal-1 expression upon activation seems to control susceptibility of the T-cells to extracellular Gal-1 induced apoptosis. Although there was no clear correlation between disease activity index scores or particular symptoms and Gal-1 levels (data not shown, [28]), we hypothesized that low expression and diminished responsiveness of activated SLE T-cells to Gal-1 might contribute to immune regulatory dysfunction and enhanced T-cell activity in SLE pathology [105,110,145,146]. The finding that successful immunosuppressive therapy resulted in restoration of the level of Gal-1 as well as of the apoptotic sensitivity of SLE T-cells supported our theory. Even though the signalling pathways responsible for extracellular Gal-1 driven T-cell apoptosis have largely been mapped [22–24,147,148],

many questions remain to be elucidated about the exact mechanism by which the cells were sensitized by endogenous Gal-1 to extracellular Gal-1 mediated apoptosis. This modulating effect between intracellular and extracellular forms of Gal-1 has not been observed before. While the anti-apoptotic effect of intracellular Gal-3 can be explained by the Bcl-2 homolog motif of the protein [149], Gal-1 does not have a homologous segment with any known apoptotic protein, and thus its function in apoptosis must stem from a yet unknown mechanism.

Although we found a clear correlation between the expression of intracellular Gal-1 and the target cell sensitivity to extracellular Gal-1's apoptotic effect, another regulatory mechanism can be the alteration of the cell-surface glycosylation of the pathogenic T-cells hence affecting the binding of extracellular Gal-1. Therefore, a brief study has been carried out to analyse the glycosylation pattern of autoimmune T-cells compared to that of healthy T-cells.

Our lectin binding assay resulted in the observation of distinct surface glycosylation patterns on activated T-cells from not only SLE but from patients of two other autoimmune diseases, RA and SS. SLE T-cells showed several differences from those of control both in their resting and activated state. The higher binding of WGA and Gal-1 coupling to similar binding targets, N-acetylglucosamine and N-acetyllactosamine side chains, respectively, indicated an abundance of these structures on resting SLE T-cells. Moreover, the known overexpression of GM-1 ganglioside [150] – a major ligand for Gal-1 – could also cause the elevated Gal-1 binding to resting SLE T-cells. Though SLE cells could also bind significantly more SNA than controls, indicating the presence of terminally sialylated glycan chains, this did not seem to inhibit Gal-1 binding. A general increase in lectin binding was detected in activated T-cells compared to resting T-cells in healthy controls. This was in agreement with Antonopulous *et al.* describing that activation of lymphocytes was accompanied by the upregulation of complex N-glycans [151]. However, while control T-cells presented with several folds higher binding after activation for all lectins measured, SLE T-cells lagged significantly in ConA, LCA, WGA, PHA-L and Gal-1 binding indicating a modified glycan pattern. Though such measurements were not conducted on T-cells before, a study by Hashii *et al.* found that mouse kidney cells in an SLE model presented with a less complex surface glycan structure than controls [152]. The higher binding of Gal-1 to resting SLE T-cells and the slim to no change with activation may have a connection with the activated-like

phenotype of SLE T-cells [109]. In this term, SLE T-cells show higher CD40L [153] and CD44 expression [154,155], constant membrane raft polarization and higher GM1 content [150], as well as constitutively high intracellular Ca^{2+} level [156]. Moreover, their glycosylation pattern may be related to the pathological phenotype.

Upon activation SS T-cells are similarly incapable of upregulating LCA, PHA-L and Gal-1 binding. There is currently no information about peripheral T-cell glycosylation in SS patients however our results suggest that these cells are similarly dysfunctional in N-glycan regulation as SLE T-cells are.

Resting RA T-cells did not differ significantly from controls in the sense of lectin binding. Nevertheless, in contrast to control T-cells, activation induced elevated Gal-1 binding does not occur.

Summarizing the changes of lectin bindings upon T-cell activation, SLE and SS activated-cells are defective in the creation of complex cell surface glycan structures and lack of mature sugar structures, tri- and tetra-antennary N-glycans results in diminished Gal-1 binding. On the other hand, while RA T-cells do not show similar deficiencies, they share significantly lower Gal-1 binding with SLE and SS. The common feature of diminished Gal-1 binding among SLE, SS and RA points to glycan complexity not being the only regulatory factor. The low RCA binding ability of RA T-cells both in their resting and activated state (data not shown) might serve an explanation for their decreased Gal-1 binding. A lack of terminal galactose residues recognized by RCA may modify Gal-1 binding as well although more experimental evidence is required to support this theory.

Another reason for attenuated Gal-1 binding by autoimmune T-cells can be the concealment of Gal-1 binding sites by sialylation. Indeed, analysis of the expression of glycosylation enzymes point to an overly sialylated glycan profile in activated T-cells of autoimmune patients. Difference in sialyltransferase levels and their ratios to neuraminidases shows a shift towards a denser sialylation profile. Though hyper sialylation is not visible in SNA binding measurements, that plant lectin is specific for α-2-6 sialylated chains and doesn't allow for measuring α-2,3 sialylation, which has been shown to inhibit Gal-1 binding at least partially [9].

A unique and useful finding, that ST6GAL1/NEU1 ratio in SLE activated T-cells correlates with SLEDAI scores is supported by Liou *et al.*, who found a similar correlation while studying SLE B-cells [157] . Although the correlation is unambiguous,

it must be further investigated to obtain more data as the sample size was low (n=8) in this study.

Considering that Gal-1 plays a role in apoptosis signalling in activated Th1 and Th17-cells [15,20,27,28,158] hence regulating the autoimmune and inflammatory pathologies, the low expression of intracellular Gal-1 in SLE T-cells and the reduced binding of extracellular Gal-1 to cell surface glycoproteins in activated RA, SS and SLE cells can be a novel factor in pathogenesis of autoimmune disorders and an additional tool in diagnosis.

6. Summary of novel results

- Our findings indicate that Gal-1, although a potent immunosuppressive factor, does not have a role in MSCs **systemic** anti-inflammatory effect ***in vivo***.

- Gal-1 expressing MSCs induce T-cell apoptosis in ***in vitro*** co-culture system on a Gal-1 dependent fashion with identical mechanism as soluble Gal-1 does.

- Endogenous Gal-1 is not secreted from T-cells however it essentially contributes to extracellular Gal-1 induced apoptosis by an unknown mechanism.

- Activated SLE T-cells express lower amounts of Gal-1 mRNA pointing that intracellular Gal-1 can be a relevant factor in escaping extracellular Gal-1 mediated apoptosis.

- Cell surface glycosylation is altered in SLE T-cells including lower complexity of surface glycan chains and elevated sialylation, both changes inhibiting Gal-1 binding to T-cells. Hence defective apoptotic response of SLE may derive from pathological Gal-1 expression and glycosylation. T-cells from SS patients showed similar alterations while RA T-cells showed a shift to sialylation. Sensitivity of Gal-1 induced apoptosis of SS and RA cells has not been investigated.

These data indicate, that intracellular Gal-1 expression and binding of extracellular Gal-1 may be relevant information to evaluate pathomechanism of autoimmune diseases especially that of SLE, and also these findings could be used as novel diagnostic tools after carrying out further studies.

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I.

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