Doctoral (PhD) thesis

Glucocorticoid hormon sensitivity of regulatory T cells in mouse modell and their aberrations in systemic sclerosis

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1. Introduction

Regulatory T cells (Treg) are a specialized subpopulation of T cells that play a key role in maintaining tolerance to self-antigens and in suppression of excessive immune response after antigenic stimulation, thereby helping to keep immune homeostasis and lower the risk for developing autoimmune diseases and allergies. Some of the clinically important issues are their participation in prevention of organ rejection after transplantation and tolerance to a fetus by the mother. The thus far best described subsets of Treg cells are the naturally arising thymus-derived (tTreg) Treg cells and the induced Treg (iTreg) cells that arise in the periphery recently also called peripheral Treg cells (pTreg). Both tTregs and pTregs express the characteristic transcription factor Foxp3 (Forkhead box protein 3), CD25, and CD4.

Foxp3 is a member of the forkhead transcription factor family, which is defined by a common DNA-binding domain, termed the forkhead box or winged helix domain. The expression of Foxp3 is sufficient to generate T cells with a suppressive phenotype. Lineage specification factors play an important role in cellular differentiation by modulating expression of a broad set of genes whose expression patterns define functional and phenotypic properties of a given cell type. Foxp3 represents a rare example of a lineage specification factor with a specialized role in supporting differentiation and function of a single cell type, namely the Treg cells. Therefore, Foxp3 is considered a “master regulator” of Tregs. There are, however, emerging data suggesting that Foxp3 expression per se may not be sufficient for stable maintenance of Treg suppressive function or reliably defining functional Tregs. For example, activated effector T cells (Teff) can also transiently express Foxp3 without acquiring Treg suppressive activity and even produce proinflammatory cytokines upon activation. As a transcription factor, Foxp3 interacts with multiple transcription factors known to be involved in activation, differentiation, and response of $CD4^+$ T cells to T cell antigen receptor (TCR) stimulation. On the other hand, Foxp3 may also act as a transcriptional co-repressor because it inhibits the activity of NFκB, CREB, and RORα. Thus, Foxp3 functions as a transcription activator and repressor by interacting with other transcription factors, and programs T cells in a direction to promote immune tolerance. For example, Foxp3 transcriptionally represses IL-2 and maintains suppressor functions of Tregs by interacting with a transcription factor, NFAT. Reciprocally, some transcription factor partners of Foxp3 facilitate the gene expression of Foxp3.

TGFβ is a regulatory cytokine from the transforming growth factor beta superfamily produced by Treg cells, macrophages and many other cell types, acting through receptor serine/threonine kinase signaling pathways. It is a pleiotropic cytokine that promotes the differentiation of naïve T cells to Th17 cells, together with IL-6, as well as in the absence of IL-6 to induced Treg cells (pTreg cells). Treg cells also produce IL-10, which is also produced by other cell types (e.g. Th1, Th2, Th17, B cells) under certain circumstances, and these cytokines act as suppressors of immune responses and inflammation.

It is currently unknown how glucocorticoid (GC) treatment affects the activity or frequency of Tregs exactly, but there is evidence that utilizing GCs may help induce Tregs. Therefore, GCs are used therapeutically if there is a loss of peripheral tolerance against self-antigens that cause diseases such as autoimmune disorders, allergies and also for preventing transplant rejections. Since both Treg cells and GC hormones exert their immunosuppressive effects by influencing cytokine production and cell activation, it was an interesting question to

investigate their synergistic effects. Studies about the sensitivity of Treg cells to GCs in mice reported both increased and decreased proportions of Treg cells after dexamethasone (DX) treatment. In BALB/c mice an increased proportion of $CD4^{+}CD25^{+}$ Treg cells in thymus and spleen and higher levels of Bcl-2 and GR in $CD4^{+}CD25^{+}$ than in $CD4^{+}CD25^{-}$ cells were observed. Administration of IL-2 and DX in another study also resulted in higher proportion of $Foxp3^{+}CD4^{+}CD25^{+}$ Treg cells in secondary lymphoid organs. On the other hand, studies investigating Treg cells in disease models such as asthma in a mouse model suggested that treatment with corticosteroids limits the development of Treg cells, but did not investigate Treg cells with any specific surface or intracellular marker.

SSc is an autoimmune disease affecting multiple organs and is characterized by fibrosis of the skin and visceral organs, vascular damage and immune dysfunction. Activation of T lymphocytes is considered to be a key stimulus in promoting the vascular abnormalities and fibrosis observed in SSc. As a result, activated T lymphocytes, especially $CD4^{+}$ T cells, are readily detected in the circulation and in the affected organs of SSc patients. In addition, many studies also implicate other elements of the immune system in the pathology of SSc, including the presence of autoantibodies and elevated cytokine levels. Due to its involvement in the development of fibrosis in affected organs and also in Treg development and function, transforming growth factor-beta (TGF-ß) probably plays a role in SSc pathogenesis. Several reports have shown that the ratio of Tregs is elevated in the PBMC compartment in SSc, while some studies have reported normal or decreased Treg levels. Nevertheless, it is generally thought that abnormal immune suppression by Treg cells in SSc is not only due to a change in the frequency of Tregs, but also to their dysfunction.

Beside the conventional definition of Tregs as CD4+CD25high+Foxp3+ T cells, numerous studies have defined further Treg subsets based on additional cell surface staining. Low levels, or no expression, of CD127 (interleukin-7 receptor alpha chain) has been proposed as a Treg marker amongst CD4+CD25high+ T cells, allowing the identification and purification of live Treg cells. The most rigorous and precise approach to identify Tregs remains expression of CD4, CD25 and transcription factor Forkhead-box-protein-3 (Foxp3) in conjunction with CD127 negativity. CD62L (L-selectin), a homing receptor of lymphocytes to lymph nodes, has been proposed to further distinguish the CD62L+ Treg subpopulations with an active recirculation into lymph nodes. These cells predominantly suppress naive T cell activation within the lymph nodes, whereas CD62L- Treg cells primarily migrate into inflammatory sites and suppress inflammation in peripheral tissues.

Recent evidence suggests that Foxp3 expression in Treg cells is under epigenetic control. A distinct DNA methylation pattern combined with the formation of characteristic histone modifications establishes an open chromatin structure, thereby imprinting *FOXP3* expression in Treg cells. In addition to three conserved non-coding sequences (CNS) demethylated or hypomethylated CpG regions in promoter, upstream enhancer and intronic enhancer provide stable long-term expression of the *FOXP3* gene. These regions are the primary targets of epigenetic regulation and are necessary to modulate its expression depending on the environmental cues T cells receive. In thymically derived natural nTreg cells these regions are generally unmethylated resulting in stable Foxp3 expression, whereas in induced iTreg cells they are hypomethylated while in effector T cells are fully methylated. Given the importance of stable Foxp3 expression, we hypothesize that the methylation status of the

FOXP3 promoter and upstream enhancer regions are altered in SSc patients resulting in an imbalance between iTregs defined by CD62L- and nTregs defined by CD62L+.

2. Aims

1. Our aim was to examine the effect of high dose *in vivo* GC hormone treatments on Treg cells in the thymus and in peripheral lymphoid organs since the results of recently published studies were inconsistent.

2. We aimed to determine the effect of GC hormone therapy on the function of Treg cells by mesauring the production and relative mRNA expression of suppressor cytokines accompanied by the quantification of relative mRNA of the transcription factor Foxp3.

3. We planned to examine the expression of GR, which greatly determines GC sensitivity, both at protein and mRNA manner in Treg cells.

4. We aimed to investigate the morphological changes of GC hormone induced GR translocation in Treg cells with confocal microscopy. The possible co-localization of the ligand bound GR and transcription factor Foxp3 was also planned to be assessed by confocal micoscopy.

5. It was also our aim to identify Treg cell subsets in the peripheral blood of early SSc patients and to analyze possible differences in their ratio and cytokine (IL-10 and TGFβ) production and Foxp3 expression when compared to healthy controls.

6. We planned to analyze the possible differences in defined subgroups of early SSc patients.

7. In search of the potential epigenetic background of the alterations of Treg cells in SSc we aimed to investigate the methylation state of the promoter and upstream enhancer regions of Foxp3 since this transcription factor is a major element of the Treg phenotype.

3. Materials and methods

3.1 Treg's in mouse modell

3.1.1 Animals

Four to six-weeks-old BALB/c mice were kept under conventional conditions and provided with pelleted rodent chow and acidified water ad libitum. All animal experiments were carried out in accordance with the regulations set out by the University's committee on animal experimentations (#BA 02/2000-16/2015).

3.1.2 In vivo glucocorticoid treatment

Mice were treated each day with intraperitoneal injection of 20 mg/kg bodyweight of dexamethasone (Oradexon, N. V. Organon) for 48-96 h (2-4 days), untreated mice served as controls. The mice were euthanized 24 hours after the last injection. Thymus, spleen, peripheral lymph nodes and Peyer's patches were removed and homogenized mechanically in PBS containing 0.1% BSA and 0.1% NaN_3, followed by filtration through a nylon mesh. Cell viability was determined using a hemocytometer and trypan blue dye exclusion test.

3.1.3 Antibodies and fluorochromes

The following antibodies were used for flow cytometry: anti-CD4-FITC (IBI clone YTS 191; Department of Immunology and Biotechnology (DIB) Pécs, Hungary), or anti-CD4-PE-Cyanine5 (PE-Cy5) (clone RM4-5) and anti-CD25-PECy7 (clone PC61) and anti-CD8-PE (clone: 53-6.7) (all from BD Pharmingen, San Jose, CA, USA) for cell surface antigens. Intracellular anti-Foxp3-PE (clone 3G3, Exbio, Prague, Czech Republic), anti-IL-10-APC (clone JES5-16E3, BioLegend, San Diego, CA, USA), and anti-TGFβ-PerCP (BioLegend, clone TW7-16B4) antibodies according to the staining procedure of the eBioscience Foxp3 staining kit (eBioscience, San Diego, CA, USA).

For confocal microscopy the following antibodies were used: anti-CD4-Pacific Blue (BD Pharmingen, clone RM4-5), anti-mouse/rat/human Foxp3-Alexa Fluor 647 (BioLegend, clone 150D), anti-GR-FITC (IBI Clone 5E4-B1, DIB, Pécs, Hungary). PromoFluor Antifade Reagent (PromoKine, Heidelberg, Germany) was used to prevent bleaching.

For FACS separation of Treg cells anti-CD4-PE (clone YTS 191.1.2, ImmunoTools, Friesoythe; Germany), and anti-CD25-PE-Cy5 (clone PC61.5, eBioscience) antibodies were used.

3.1.4 Stimulation and staining of cells for cytokine detection

$1x10^6$ cells were stained with anti-CD4-FITC, anti-CD25-PerCP antibodies followed by two washing steps. Then the cells were incubated in e-Bioscience fixation/permeabilization buffer and stained with anti-Foxp3-PE and anti-GR-FITC intracellular antibodies. After 2 washing steps, cells were fixed in 300 µl FACS-Fix [9 µl 35% formaldehyde (Sigma-Aldrich) + 291 µl phosphate-buffered saline] and measured in a FACSCantoII flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using the FCS Express 4 Flow Research Edition software. Thymocyte subpopulations were gated based on their cell surface CD4/CD8 staining. Treg cells were determined in the CD4 gate based on their CD25/Foxp3 positivity. GR expression of T cell subpopulations and Treg cells was determined on histogram overlays by analyzing the FL-1 mean fluorescence intensities (MFI) separately.

For cytokine detection thymocytes or splenocytes of DX treated and control animals were stimulated with 25 ng/ml PMA (Sigma-Aldrich), 1 µg/ml Ionomycin (Sigma-Aldrich) and 10 µg/ml Brefeldin A (Sigma-Aldrich) in RPMI-1640 medium (Sigma-Aldrich) /10% FCS (Gibco) at 37°C in a CO_2 incubator for 24 hours. Cytokine detection was performed on 10^6 cells/samples in binding buffer [PBS containing 0.1% bovine serum albumin, BSA and 0.1% NaN_3 (both from Sigma-Aldrich)] after anti-CD4-FITC, anti-CD25-PECy7, and anti-LAP (TGFβ)-PerCP cell surface staining and intracellular anti-Foxp3-PE, anti-IL-10-APC antibody labeling according to the staining procedure of the eBioscience Foxp3 staining kit. Samples were measured in a FACS Canto II cytometer and analyzed using the FCS Express 4 Flow

Research Edition program. In the lymphocyte gate $CD4^+/CD25^+$ T cells were gated and the $Foxp3^+TGF\beta^+$ and $Foxp3^+IL\text{-}10^+$ cell ratios were determined.

3.1.5 RT-PCR

Isolation of Treg cells for RT-PCR

Thymocytes and splenocytes were collected and stained with anti-CD4-PE and anti-CD25-PE-Cy5. Stained cells were then analyzed using BD FACSAriaII Cell Sorting System with BD FACSDiva Software (BD Biosciences). Cells in the lymphocyte gate were sorted based on CD4 expression and high expression levels of CD25.

RNA preparation and quantitative RT-PCR

RNA was isolated from 10^5 $CD4^+CD25^{high+}$ cells using NucleoSpin RNA XS kit, and cDNA was prepared using random oligo(dT) primers (Applied Biosystems). Gene expression was quantified by the SYBR Green method using the Applied Biosystems 7500 RT-PCR system. The relative expression levels were determined by normalization to actin 'housekeeping gene', and results were presented as fold induction compared to unstimulated Treg mRNA levels (RQ). Primer sequences were as follows: β-ACTIN (Forward) 5'- GGG AGG GTG AGG GAC TTC C -3'; β-ACTIN (Reverse) 5'- TGG GCG CTT TTG ACT CAG GA -3'; IL-10 (Forward) 5'- GTG AAG ACT TTC TTT CAA ACA AAG -3'; IL-10 (Reverse) 5'- CTG CTC CAC TGC CTT GCT CTT ATT -3'; Foxp3 (Forward) 5'- TAC TTC AGA AAC CAC CCC GC -3'; Foxp3 (Reverse) 5'- GTC CAC ACT GCT CCC TTC TC -3'; TGFβ1 (Forward) 5'- GAC TCT CCA CCT GCA AGA CC 3'; TGFβ1 (Reverse) 5'- GGA CTG GCG AGC CTT AGT TT-3'; mouse GR (Forward) 5'- TGG TGT GCT CCG ATG A-3'; mouse GR (Reverse) 5'-AGG GTA GGG GTA AGC -3'.

3.1.6 Statistical analysis

Statistical evaluation was performed using SPSS v. 22.0 statistics package (IBM, Armonk, NY, USA). Variables were expressed as mean ± SEM. Student's t-test was used to compare data between the investigated groups. P values < 0.05 were considered significant.

3.2 Treg's in systemic sclerosis

3.2.1 Patients

We recruited 26 SSc patients with early onset of the disease [20 females and 6 males; mean age (SD): 54.7 (12.6) years with the mean duration of disease (SD): 2.5 (1.7) years] at the University of Pécs, Department of Rheumatology and Immunology, classified as dcSSc (19) or lcSSc (7) based on the criteria proposed by LeRoy et al. All patients fulfilled the 2013 ACR/EULAR SSc classification criteria. The onset of SSc was defined as the date of the first SSc-specific symptom other than Raynaud's symptom. Ten age- and gender-matched healthy volunteers were investigated. All participants gave their informed consent to the study. The study was approved by the Hungarian National Ethics Committee (84-256/2008-1018EKU) and was performed according to the principles of the Declaration of Helsinki 2011. Blood samples were collected using standard procedure in vacuum tubes containing heparin. Pulmonary fibrosis was characterized by detection of fibrosis with high resolution CT and/or decreased forced vital capacity (FVC<80%). Disease activity was also recorded according to the European

Scleroderma Study Group (EScSG) disease activity index and the disease was considered active with index values > 3. Thirty one percent of all SSc patients (42% of dcSSc) received immunosuppressive therapy (low dose corticosteroid (6-8 mg methylprednisolone) (3 patients) or cyclophosphamide + corticosteroid (5 patients) at the time of blood collection.

3.2.2 Autonatibody measurements

Disease specific autoantibodies were measured using conventional ELISA tests or by immunoblotting. After positive anti-nuclear-antibody (ANA) screening test (ANA-Ease ELISA Kit, Genesis, GD74), anti-CenpB (Orgentec, ORG 633) and anti-Scl-70 (Orgentec, ORG 212-24) antibodies were detected using antigen-specific ELISA tests. Anti-RNA polymerase III (RNA-Pol-III) antibody was detected by immunoblot method (Euroimmune, DL 1532-1601 G), which was also used to confirm the anti-CenpB and anti-Scl-70 autoantibody positivity.

3.2.3 Flow cytometric detection of regulatory T cell subpopulations

Multiparametric flow cytometry was performed on PBMCs isolated by Ficoll density gradient centrifugation. Regulatory T cells and their subgroups were determined using FITC conjugated anti-CD4 (Becton Dickinson, RPA-T4), APC conjugated anti-CD25 (Becton Dickinson, M-A251), PE-conjugated anti-Foxp3 (Becton Dickinson, 259D/C7), PacificBlue conjugated anti-CD127 (BioLegend, AO19D5) and APC/Cy7 conjugated anti-CD62L (BioLegend, DREG-5C) antibodies. Intracellular staining was performed by using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer’s instructions. Fluorescence of labelled cells was recorded using a FACS Canto II flow cytometer (Becton Dickinson, USA) and analyzed using FCS Express 4 software (De Novo Software, USA). Lymphocytes were gated based on forward and sideward scatter (FSC and SSC). CD25+Foxp3+ conventional Treg cells were determined as proportion of CD4+ cells. CD4+ cells were analyzed for CD25 and CD127 expression, and then the CD25+CD127- cells for Foxp3 positivity. CD62L+ Tregs (an activated subgroup) were detected in the gated CD4+CD25+CD127-Foxp3+ cells.

3.2.4 Cytokine detection

For functional analysis, PBMCs were stimulated with 25ng/ml PMA (Sigma)/ 1µg/ml Ionomycin (Sigma)/10µg/ml Brefeldin (Sigma) in RPMI for 4 hours at 37°C and their IL-10 and TGF-β cytokine production was investigated by flow cytometry using PerCP/Cy5.5 conjugated anti-TGF-β1 (BioLegend, TW4-2F8), PE/Cy7 conjugated anti-IL-10 (BioLegend, JES3-9D7) antibodies. Following cell surface CD4/CD25/CD127/CD62L labelling, cells were washed and intracellular staining was performed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer’s instructions. Fluorescence of labelled cells was measured using a FACS Canto II flow cytometer (Becton Dickinson, USA) and analyzed using FCS Express 4 software (De Novo Software, USA). Determination of IL-10 and TGF-β cytokine production within the CD4+CD25+CD127-Foxp3+ cells and in the CD62L+ Treg cells was performed.

3.2.5 Methylation analysis by pyrosequencing

Genomic DNA from B cell depleted lymphocytes was obtained using the QIAamp DNA Micro Kit (Qiagen). Assays for quantification of methylation levels of CpGs in the target regions of *FOXP3* promoter and upstream enhancer were designed by using the PyroMark Assay Design software (Qiagen, Hilden, Germany). Primers and sequences are listed in

Supplementary Table 1 and 2. Bisulfite conversion of DNA obtained from PBMCs of all (n_{HC}=10; n_{SSc}=24) was performed using the EpiTectFast 96 Bisulfite Kit (Qiagen) according to the manufacturer's protocol. PCR amplification steps were performed on a Vapo-Protect^{TM} (Eppendorf, Wesseling-Berzdorf, Germany) with an initial denaturation step at 95°C for 5 min, 37 cycles of 95°C for 30 s, primer-specific annealing temperature of 60°C for 30 s, 72 °C for 45 s, and a final extension step at 72°C for 7 min. The reaction mixture consisted of 5 µl 10x PCR buffer with $MgCl_2$, 1 µl 10 mM dNTP mix, 2.5 µl of each forward and reverse primer (final concentration 0.5 µM), 0.4 µl (final concentration 1 U) Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 36.6 µl PCR-grade water, and 2 µl template DNA (70-200 ng). PCR products were visualized by electrophoresis in 1.0% agarose gel.

Bisulfite pyrosequencing was performed on a PyroMarkTMQ96 MD Pyrosequencing System with the PyroMark Gold Q96 CDT Reagent Kit (Qiagen). Pyro Q-CpG software (Qiagen) was used for data analysis.

3.2.6 Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from B cell depleted lymphocytes using RNeasy PlusMikro kit (Qiagen). Complementary DNA (cDNA) was generated from 25-400 µg RNA using oligo(dT)$_{18}$ primers (Thermo Scientific, Waltham, MA, USA) for reverse transcription with Maxima reverse transcriptase (Thermo Scientific). Real-time PCR was performed using the applied Biosystems® Real Time PCR 7500 (Applied Biosystems, Darmstadt, Germany) utilizing iTaquniver SYBR green according to the manufacturer's instructions (Bio-Rad, Ismaning, Germany). Amplification was conducted for 40 cycles. Relative expression of *FOXP3* was determined by normalizing expression of each gene to β2-microglobulin. Primers: Foxp3 (forward) 5'- TCA TCT GTG GCA TCA TCC GA -3'; Foxp3 (reverse) 5'- GGA ACT CTG GGA ATG TGC TG -3'; β2M (forward) 5'- CCA GCA GAG AAT GGA AAG TC -3'; β2M (reverse) 5'- GAT GCT GCT TAC ATG TCT CG -3'.

3.2.7 Statistikal analysis

Statistical evaluation was performed using SPSS v. 22.0 statistics package (IBM, USA). Shapiro-Wilk test was used to test for normal distribution, before applying Student's t-test for normally-distributed and non-parametric Mann-Whitney-U for not normally-distributed independent variables. Normally-distributed variables were expressed as mean ± standard error of the mean (SEM). Not normally-distributed variables were expressed as medians and interquartile ranges (IQR). Correlations between variables were identified by Spearman's rank correlation coefficient. Given the explorative nature of the study, no correction for multiple testing was performed. P values < 0.05 were considered significant.

4. Results

4.1 Treg's in mouse modell

4.1.1 Effect of DX treatment on Treg cells

Since GCs are used for immunosuppression, first we wanted to investigate the effect of high-dose DX treatment on the ratio and numbers of thymocytes and peripheral T cell subgroups in different lymphoid organs. After repeated (48 h) DX treatment, in the thymus the immature double-positive (DP) thymocytes largely disappeared, their proportion dropped from 77.9% to 7% (Fig. 1A). The proportion of most immature double-negative (DN) and single-

positive (SP) thymocytes increased (DN: 3.7-fold, CD4 SP: 4-fold, CD8 SP: 4.9-fold). The CD8 SP thymocytes were the most resistant to GC-induced apoptotic effects.

In the spleen, the CD4/CD8 ratio did not change significantly, but we detected a decrease in the proportion of non-T cells (B and NK) and a relative increase of Th cell frequencies from 22.7% to 27.5%, and Tc cell ratios from 12.2% to 14.1%.

Next we investigated the normal distribution of $CD4^+CD25^+Foxp3^+$ Treg cells in the primary (thymus) and secondary (spleen, lymph nodes and Peyer's patches) lymphoid organs of control mice. In the thymus the proportion of Tregs in the $CD4^+$ T cell gate was approximately 3.5%, whereas in the peripheral lymphoid organs this ratio was higher between 7-15% (spleen ~15%, lymph nodes ~10%, and Peyer's patches ~7%). When we followed the proportion of Treg cells in the thymus after repeated (2x) DX treatment, we detected significantly increased Treg proportions in the $CD4^+$ T cell gate. Among the peripheral lymphoid organs, in the spleen we detected a small but significant decrease in the Treg ratio, while no change was detected in the lymph nodes and Peyer's patches.

We also measured the total numbers of Treg cells in the thymus and peripheral lymphoid organs of mice after repeated (4x) DX treatment and compared to untreated controls. Of note, the number of thymic Treg cells did not change, whereas the total number of Treg cells in the peripheral lymphoid organs decreased significantly. This differential change in thymic versus peripheral Treg cell numbers was due to the relative resistance of thymic Tregs to DX, which resulted in a robust (16-fold) increase in thymic Treg cell ratios, while the total thymocyte number dropped to one sixth of original value (from $8x10^7$ to $1.3x10^7$cells/thymus). In the peripheral lymphoid organs, DX treatment resulted in significant drop in total numbers of lymphocytes, together with numbers of Treg cells (Table 1), which was accompanied by dramatic decrease in the size of these organs (data not shown), as we reported previously. These data suggest that mature T cells and Tregs of peripheral lymphoid organs are sensitive to GC.

We also investigated the time-kinetics of a single high-dose GC treatment on the Treg cells in the peripheral blood. As shown in Fig. 2B, 4 h and 8 h after DX treatment, we detected a significant increase in the Treg cell ratios, which by 24 h returned to the starting control levels, and remained at this level at 48 h post-treatment.

4.1.2 Effect of DX treatment on cytokine production and Foxp3 expression of Treg cells

Next we investigated whether GC treatment, in addition to the effects on Treg cell ratios, has an effect on Treg cell function, therefore we tested the IL-10 and TGFβ production and Foxp3 expression by tTreg and pTreg cells.In the thymus, the ratio of IL-10 and TGFβ-positive tTreg cells were similar (11.0±2.3% and 13.5±3.1%), but DX treatment resulted in significant increase in both cytokine secreting Treg ratios (17.6±1.4% and 21.0±4.9%). In the splenic Treg cells of control animals, however we detected significantly higher TGFβ positivity (13.7±2.0%), compared to IL-10 positivity (3.6±0.5%). As a result of 48 h DX treatment the positivity of Tregs for both cytokines increased significantly, but the fold-increase for IL-10 was higher than for TGFβ.

We also compared the GC treatment-induced relative mRNA expression of IL-10 and TGFβ in purified $CD4^+CD25^{high+}$ Tregs. In the thymic Treg cells the cytokine expression did not show a clear change as a result of repeated (2x) high-dose GC treatment. Similarly, the IL-

10 mRNA expression in splenic Treg cells did not change significantly after in vivo GC treatment, but it induced a 3.4-fold higher relative expression of TGFβ mRNA.

Next we studied the relative quantitative changes of Foxp3 transcription factor expression, which plays a role in determining the functions of Treg cells. The Foxp3 mRNA levels were very similar in purified $CD4^{+}CD25^{high+}$ thymic and splenic Treg cells, but increased after DX treatment in splenic Tregs, but not in the thymic Tregs. This is consistent with the functional changes observed in splenic Treg cells and may explain the TGFβ mRNA upregulation in splenic Tregs described above.

4.1.3 Effect of DX treatment on GR expression

The GC sensitivity of a given cell type is largely determined by its levels of GR expression. Therefore, we looked for possible relationship between the observed GC resistance of tTregs and their GR expression. For this purpose we measured the levels of intracellular GR by flow cytometry, and GR mRNA levels by qRT-PCR in purified thymic and splenic $CD4^{+}CD25^{high+}$ Treg cells.

After repeated high-dose DX treatment we detected an increased GR protein level in tTregs surviving in the thymus (cells resistant to GC), when compared to controls. Opposite to this, the control splenic pTregs that expressed higher levels of GR showed a decrease in their GR expression after DX treatment. This GC-induced upregulation of GR expression was characteristic in DP thymocytes while downregulation of GR expression is characteristic of SP thymocytes and mature T cells, an effect that we had observed in earlier studies both in mouse and human cells.

24 hours after the last DX treatment (2x), we did not observe detectable change in GR mRNA expression in thymic and splenic Treg cells.

In one of our previous studies we have shown that in GC-sensitive DP thymocytes DX treatment caused mitochondrial translocation of the GR instead of nuclear translocation, which indicated, that, apart from the actual level of GR expression, the subcellular localization is equally critical in determining the apoptosis sensitivity. Therefore, we now investigated the GR and Foxp3 localization in thymic and splenic Treg cells with and without previous in vivo GC treatment (2x) using confocal microscopy. The colocalization of GR and Foxp3 was analyzed on negatively selected $CD4^{+}$ T cells. In untreated samples Foxp3 highly colocalized to GR both in thymic and splenic Treg cells. Upon DX treatment the Foxp3-GR spatial association increased further in the splenic Treg cells, while in thymus this association showed no change.

4.2 Treg's in systemic sclerosis

4.2.1 Identification of Treg cells

In order to identify Treg cells in SSc patients with early form of the disease and in HC subjects we used three combinations of markers based on data of previous studies on Treg cells. We compared 'conventional' CD4+CD25+Foxp3+Treg cell staining with an 'extended' cell surface labeling including CD4+CD25+CD127-, and a 'combined' staining of CD4+CD25+Foxp3+CD127-, respectively. First we tested the correlation of Foxp3+ and CD127- cells within the CD4+CD25+ T cell population of all study subjects and found a strong correlation between the proportions of CD127- and Foxp3+ cells ($p < 0.001$, $r = 0.896$), which agrees with previous reports. Then we compared Treg ratios in SSc and HCs using different

marker combinations for identification of Tregs. Using the conventional Treg markers and the cell surface staining alone, not significant but similarly elevated Treg cell proportions were detected in SSc patients.

With the CD4+CD25+Foxp3+CD127- marker combination we detected significantly increased proportions of Treg cells in SSc patients, when compared to the HC group ($p < 0.05$). Based on these data, in our subsequent experiments we used the CD4+CD25+Foxp3+CD127- markers for identification of Treg cells (CD127-Treg), which is considered to be the most accurate and rigorous marker combination.

4.2.2 Elevated CD127-Treg cell frequencies with decreased IL-10 cytokine production in dcSSc

Since we detected a significantly increased CD127-Treg cell frequency within the CD4+ T cells of SSc patients using the 'combined' labeling strategy, then we separated the SSc patients into subgroups such as limited or diffuse cutaneous SSc (lcSSc and dcSSc), the presence of autoantibodies and the presence or absence of pulmonary fibrosis. We found that patients with dcSSc, anti-Scl-70 and anti-RNA-Pol-III autoantibody seropositivity and patients with pulmonary fibrosis had significantly increased frequencies of CD127- Treg cells when compared to the HC group ($p < 0.05$).

In addition, we also characterized Treg cells based on their cytokine production, including IL-10 and TGF-β. In SSc patients, we found a significantly lower proportion of IL-10 producing Tregs ($p < 0.05$) compared to HC, which was mainly attributed to the lcSSc group. When stratifying the SSc group according to the presence of autoantibodies, the ACA+ (anti-centromere antibody) group showed a significantly decreased frequency of IL-10 positive Treg cells ($p < 0.05$), when compared to HC. The proportion of TGF-β producing Treg cells did not differ in any subgroups of the disease.

4.2.3 Elevated proportions of CD62L+Tregs with decreased TGF-β production in SSc

In order to analyze Treg cells with different homing nature, we also measured the frequency of CD62L+ (L-selectin) Tregs within the CD4+CD25+Foxp3+CD127- Treg cell subgroup and their cytokines. A significantly higher proportion of CD62L+Tregs were detected in SSc patients, when compared to HCs ($p < 0.05$). We then stratified SSc patients into subgroups according to disease type (lcSSc, dcSSc), the presence of autoantibodies, the presence or absence of pulmonary fibrosis and disease activity. Significantly increased proportions of CD62L+Tregs were detected in all subgroups, except in the aAb-, and ACA+ cases.

Then we analyzed the frequency of cytokine producing CD62L+ Treg cells in HCs and found a much higher proportion of IL-10 and TGF-β producing cells (IL-10: $4.3 \pm 0.6\%$; TGF-β: $49.1 \pm 9.6\%$) than in the total CD127- Treg population (IL-10: $1.3 \pm 0.2\%$; TGF-β: $1.4 \pm 0.2\%$). In both lcSSc and dcSSc patients we found decreased frequency of TGF-β producing CD62L+ Tregs compared to HC ($p < 0.05$). The proportion of IL-10 producing CD62L+Treg cells did not differ. When stratifying the SSc group according to the presence of autoantibodies, the aAb-, ACA+, and Scl-70+/RNA-Pol-III+ subgroups showed significantly decreased proportions of TGF-β positive CD62L+Treg cells ($p < 0.05$), when compared to HC.

4.2.4 Abundance of CD4+CD25-Foxp3+ T cells

The evidence that human CD4+CD25- cells containing Foxp3+ T cells convert to a CD25+ Treg phenotype upon homeostatic expansion, prompted us to investigate whether this CD4+CD25-Foxp3+ reservoir population is affected in SSc patients. We detected a non-significant tendency to lower percentages of CD4+CD25-Foxp3+ T cells in SSc, when compared to HC. In SSc patients without pulmonary fibrosis the percentage of CD4+CD25-Foxp3+ T cells was significantly lower compared to the SSc group with pulmonary fibrosis ($p < 0.05$). Consequently we hypothesize that CD4+CD25-Foxp3+ T cells turn into Treg cells at such high rate that results in the relative shortage/lack of CD4+CD25-Foxp3+ T cells.

4.2.5 FOXP3 gene expression is elevated in active SSc patients

We also assessed *FOXP3* gene expression with qRT-PCR in B cell depleted lymphocytes of SSc patients. Relative gene expression values (RQ) were calculated by comparing the individual gene expression level in SSc patients to the average of HCs samples adjusted to RQ=1. We found a significantly higher number of patients (7 of 14) with upregulated *FOXP3* expression (RQ>2) in the active disease group compared to the inactive disease group (1 of 11). When we analyzed the correlation between *FOXP3* gene expression and the previously defined Treg cell subgroups we found an inverse correlation between *FOXP3* RQ values and the proportion of IL-10+CD62L+ Treg cells (p=0,009; $r_{\text{Spearman Rank}}$ = -0,580). This correlation supports the functional impairment of the CD62L- effector memory Treg cells in SSc.

4.2.6 CpGs within the FOXP3 upstream enhancer are hypomethylated in SSc patients

As *FOXP3* gene expression was up-regulated in several SSc patients, we determined the methylation status of the proximal promoter and upstream enhancer CpG regions of the *FOXP3* gene locus. No significant differences in the promoter methylation status were observed in any groups of SSc patients compared to HCs. Methylation analysis of the *FOXP3* upstream enhancer 1 and 2 regions revealed lower percentages of methylation at specific CpGs in SSc patients compared to HCs. Significant hypomethylation was found in CpG 3 and 7 sequences of the *FOXP3* upstream enhancer 2 region. This suggests the increase of a cell population with stable expression of Foxp3 in patients with SSc.

5. Discussion

5.1 Glucocorticoid hormone sensitivity of regulatory T cells in mouse modell

By inhibiting the immune response against allo- and autoantigens Treg cells are important members and therapeutic targets of the immune regulation and the maintenance of tolerance. GC analogues are one of the most important drugs in the treatment of inflammatory and autoimmune diseases and patients with organ transplants, however, the effect of GC on Treg cells remains to be elucidated.

In our work we investigated the effect of GC treatment on tTreg and pTreg cells in central and peripheral lymphoid organs. The ratio of tTregs increased significantly after DX treatment, and it continued to increase in parallel with the duration of the treatment, whereas the overall number of thymocytes decreased dramatically, which can be explained by apoptosis

of GC-sensitive, DP thymocytes. The absolute number of Treg cells did not change, thus the observed increase in their ratio was the result of their survival. This suggests that the tTregs are resistant to GC-induced apoptosis.

The ratio of pTreg cells in peripheral lymphoid organs was much higher than in the thymus. Upon repeated DX treatment, the ratio of pTregs slightly decreased in the spleen, and the total number of pTreg cells also decreased. Based on these results we hypothesized that tTregs and pTregs differ in their sensitivity to GC.

Of note, the relative numbers of Treg cells in the peripheral blood showed a particular time-dependent kinetics after DX treatment: with a transitory increase in percentage of Treg cells, peaking at ~4-8 hours after DX treatment, and returning to approximately pre-treatment levels. This suggests that DX treatment may cause a transitory increase in immunosuppression in the blood. Since Tregs may play a role during various microbial infections, an increase of Treg cell relative numbers may affect the susceptibility of the host to infectious diseases, too.

The GC sensitivity of a given cell is dependent on its level of GC expression and its cross-talk with different signaling pathways. Previous research by our group showed that the immature DP thymocytes express the lowest level of GR in the thymus, and despite of this, they are the most sensitive cell population to GC-induced apoptosis. The mature, peripheral lymphocytes are more resistant to treatment with GC analogues and a significant downregulation of the GR can be observed in such cells. Our current data shows that the GR expression of tTregs in the thymus is lower than in the spleen of control animals, which may be a consequence of local microenvironmental influences (e.g. local GC production). Upon GC treatment the GR expression increased in thymic Treg cells, while it was downregulated in the spleen, which may reflect the different GC sensitivity of Tregs in the thymus and peripheral organs. However, the signaling pathways activated by GC in Treg cells need further investigation.

Importantly, we measured increased ratios of IL-10 and TGFβ-producing Treg cells after DX treatment. Both of these cytokines possess immunosuppressive activity, and TGFβ has been called a 'purveyor of immune privilege'. IL-10 and TGFβ are major mediators of inhibitory activity exerted by Treg cells on effector T cells and other cell types of the immune system. Our data indicate that DX treatment can induce a relative increase in IL-10 and TGFβ-expressing Treg cell numbers in the thymus and spleen. The relative expression of these cytokines at the mRNA level also showed an increase, except in the case of IL-10 mRNA in the spleen. We can only speculate regarding the lack of correlation between IL-10 protein and mRNA in the spleen, however this result may not be surprising in the light similar data observed for a wide range of proteins and corresponding mRNAs in various cell types and organisms.

Nevertheless, these data suggest that the immunological milieu dictated in part by Treg cells in the thymus and the spleen may shift towards an immunosuppressive direction. Thus, in addition to numerous mechanisms of action by glucocorticoids, DX (and possibly other glucocorticoids) may affect major immunoregulatory pathways and mechanisms by promoting selective survival of Treg cells and increased production of immunosuppressive cytokines by such cells.

Finally, we detected physical colocalization of GR and Foxp3 in the thymic and splenic Treg cells. The degree of this colocalization increased in splenic Treg cells upon DX treatment of mice. These data suggest that GR and the Foxp3 transcription factor may be recruited to certain regions of chromatin, where these molecules may regulate gene transcription, either

individually or in collaboration. Earlier studies have shown that GR interacts with nuclease accessible sites in open chromatin regions, and Foxp3 exploits the pre-existent enhancer landscape during Treg cell specification. In the light of these data, it is logical to ask: Is there evidence that of crosstalk between GR and Foxp3 transcriptional networks? Relevant to this, genome-wide ChIPseq and microarray analysis was used to identify GR-binding sites in DX-treated mouse cells. Among many genes, the FOXP3 gene was identified that contained GR-binding region. This suggests that GR may affect the activity of the FOXP3 gene in mammalian cells, and Foxp3 may in turn regulate the activity of multiple target genes. Our data showing colocalization of GR and Foxp3 proteins in Treg cells may support the hypothesis that glucocorticoids may directly modify FOXP3 gene activity in Tregs, and thus may influence a major regulatory arm of the immune system.

Another interesting question, based on the present data, whether there could be additive or synergistic effect between the increased expression of Foxp3, IL-10 and TGFβ in Treg cells. We hypothesize that such effects may exist based on the known crosstalk between these factors at the genome (epigenome) level and protein signaling pathways. First, expression of Foxp3 at low levels induces a limited Treg cell phenotype with little or no suppressive function, and the full suppressive effect is only gained when Foxp3 is highly expressed. Second, Foxp3 gene induction in Treg cells is promoted by TGFβ signaling (via Smad2/3 binding to the CNS1 region of the Foxp3 gene) and TGFβ is part of a positive feedback loop in the generation of $Foxp3^{+}$ Treg cells. Third, IL-10 acts in a paracrine manner on Treg cells to maintain their Foxp3 expression.

Data obtained in human subjects suggests that glucocorticoid therapy increases Treg cell ratio and/or function in various disease conditions. For example, glucocorticoids increase $CD4^{+}CD25^{high}$ cell percentage and Foxp3 expression in patients with multiple sclerosis, and it has been suggested that this may be a mechanism whereby steroids expedite recovery from relapses in such patients. In patients with Graves’ disease, dexamethasone therapy improved the function of Treg cells, whereas in patients with nickel allergy, oral glucocorticosteroid therapy resulted in enhanced Treg response in the skin.

Taken together, our data suggest that treatment with the glucocorticoid analogue dexamethasone results in increased relative numbers of Treg cells, and that such Treg cells produce enhanced levels of immunosuppressive cytokines IL-10 and TGFβ. These findings add to the knowledge on the biological effects of glucocorticoids influencing the regulatory arm of the immune system, and may have relevance to clinical conditions where enhancement of Treg cell activity is expected to be beneficial.

5.2 Analysis of the composition and function of Treg cells in systemic sclerosis

SSc is a chronic progressive disease with extensive dermal and visceral inflammation, vascular damage and autoantibody production, indicating the involvement of immunological processes. Treg cells have been identified as crucial cells in limiting the extent of cellular and humoral immune responses. The number and activity of Treg cells may significantly affect the course of the disease. Our first objective in this study was the identification of Treg cells within the CD4+ T cell population using various cell surface and intracellular marker combinations, including the detection of the intracellular Foxp3 transcription factor involved in CD4+CD25+ Treg differentiation. For live Treg cell isolation cell surface labeling is conventionally used and the CD4+CD25high+CD127- phenotype based Treg analysis showed close correlation between

the Foxp3+ and CD127- Tregs also in our study. However, using both Foxp3 positivity and CD127 negativity of the CD4+CD25+ T cells for Treg cell identification revealed significant differences between the Treg cell frequencies of SSc patients and HCs.

Earlier investigations suggested increased Treg proportions, while others reported decreased levels in SSc patients. One of our consistent findings is that the proportion of CD127- Treg cells is elevated in the early disease stage of SSc patients compared to HC subjects. The statistically significant higher Treg proportions were more pronounced in severe forms of the diseases, such as dcSSc cases with Scl-70 and RNA-Pol-III autoantibody positivity and pulmonary fibrosis. Although no consensus has been reached regarding changes in Treg subpopulations in SSc, our data support the finding of increased Treg levels in the peripheral blood of SSc patients due to the elevated frequency of CD62L+Tregs representing the natural or thymic Treg cell population and a group of central memory Tregs both with a recirculation pattern into the lymphatic organs suppressing the activation of naive CD4+ T cells. As an important subset within the Treg cells in murine studies, CD62L+ Treg cells have been shown to possess significantly higher inhibitory capacity than their CD62L- Treg counterpart. In another study in coeliac disease patients CD62L+ Treg cells were described as lymphoid tissue homing proportion of Tregs, while CD62L- Tregs as mucosal homing cells responsible for the tissue immune homeostasis.

In SSc, there is not only an imbalance between the CD62L positive and negative Treg cells, but their functional capacity is also disturbed in terms of reduced IL-10 secretion by the total Treg group and in terms of TGF-β production by the CD62L+ Treg subset . Since the Treg-derived anti-inflammatory cytokines play a central role in suppression of immune response and in preventing inflammatory and autoimmune pathologies, such a decrease in IL-10 positive Treg cells in the face of increased proportions of Treg cells could be due to a functional exhaustion of the Treg cells, similar to other T cell populations. Of note, in our study, no decreased production of IL-10 was observed in the CD62L+Treg cell population, which may suggest that the CD62L-Treg cell subpopulation(s), e.g. Tr1 or CD62L- effector memory Treg cells are defective in IL-10 production. This cell group has been shown to be responsible for the balance of tissue homeostasis, and inhibition of inflammatory responses in the skin and mucosa. Our finding, that elevated *FOXP3* gene expression is characteristic in SSc patients with active forms of the disease and inversely correlate with the proportion of CD62L+IL-10+ Tregs also support the functional impairment of the effector memory Treg cells in SSc. Another important cytokine in the functioning of Treg cells is TGF-β, levels of which were decreased in the CD62L+Treg cell population, but not in the total Treg cells. This finding supports the functional impairment of CD62L+ nTregs and central memory Tregs, while the peripherally induced Th3 subpopulation of Treg cells, which is known to produce TGF-β is not affected. The mechanism underlying the functional changes in IL-10 and TGF-β expression by FoxP3+ Treg cells is not well defined, however there is emerging evidence that epigenetic mechanisms, such as DNA methylation status, histone modifications and non-coding RNAs might be involved in SSc pathogenesis. Methylation status of the proximal promoter and upstream enhancer CpG regions of the *FOXP3* gene locus may play a role in stabilization of the FoxP3-positive Treg cell phenotype. Unmethylated FOXP3 gene locus promoter and enhancer regions provide stable long-term expression of the *FOXP3* gene which is characteristic for the thymically derived nTreg cells, hypomethylated *FOXP3* enhancer region is characteristic for induced iTregs and it is fully methylated in conventional T cells. Our methylation analysis of

the *FOXP3* genes revealed hypomethylation at all CpG sites of the *FOXP3* enhancer regions in SSc T cells compared to that of HCs with significantly diminished methylation in 2 specific CpG sites. This observation support an elevated stable FoxP3-expressing cell population in SSc patients which is consistent with our flow cytometric finding regarding the increased CD62L+ FoxP3+ Treg proportions in SSc patients as found by others.

We observed a tendency of decreased CD4+CD25-Foxp3+ T cell frequency in all SSc patients but significant alteration was only in patients without lung fibrosis. Of note, the CD4+CD25-Foxp3+ T cells are considered to comprise a reservoir pool for Treg cells that can be recruited to the CD25+ pool upon homeostatic expansion and/or activation. We hypothesize that depletion of the CD4+CD25-Foxp3+ T cell pool could be due to a robust increase in Tregs, as suggested by our data. Mechanistically this could reflect a high conversion rate of the CD4+CD25-Foxp3+ T cells into the CD4+CD25+Foxp3+ Treg pool that outpaces the generation of CD4+CD25-Foxp3+ T cell reservoir. Additionally, it can be envisioned that exhaustion of the CD4+CD25-Foxp3+ T cell population, or changes in T cell populations that are involved in the generation of the CD4+CD25-Foxp3+ pool (similarly to that described in conditions of chronic antigen persistence, e.g. chronic infections or cancer), could underlie the reduction in CD4+CD25-Foxp3+ T cells in SSc. Since these cells might be the source of induced iTregs, it would not be surprising if an imbalance between nTreg and iTreg cells also existed in SSc patients.

In summary, our data demonstrate an imbalance (increased relative proportion) of regulatory T cell subsets and abnormalities in TGF-β and IL-10 cytokine production by Treg cell subsets in SSc patients. The proportional changes were attributable to disease stage and auto-antibody status. We also detected hypomethylation of CpG sites in the enhancer region of *FOXP3* in SSc patients. These data suggest that epigenetic mechanisms may underlie, at least in part, the imbalance of Treg cell function in SSc. Further studies should reveal the impact of such immunological changes on the pathogenesis and progression of SSc.

6. List of publications

Publications the thesis is based on

1. Ugor Emese, Simon Diána, Pap Ramóna, Nikola Kraljik, Németh Péter, Boldizsár Ferenc, Berki Tímea
   Regulatórikus T-sejtek glukokortikoidhormon-érzékenységének vizsgálata
   IMMUNOLÓGIAI SZEMLE 6:(1-2) pp. 17-24. (2014)

2. Emese Ugor, Diána Simon, Giovanni Almanza, Ramóna Pap, József Najbauer, Péter Németh, Péter Balogh, Martina Prelog, László Czirják, Tímea Berki
   Increased proportions of functionally impaired regulatory T cell subsets in systemic sclerosis
   CLINICAL IMMUNOLOGY (2017); 184:54-62. ***IF: 3,990***

3. Emese Ugor, Lilla Prenek, Ramóna Pap, Gergely Berta, Dávid Ernszt, József Najbauer, Péter Németh, Ferenc Boldizsár, Tímea Berki

Glucocorticoid hormone treatment enhances the cytokine production of regulatory T cells by upregulation of Foxp3 expression
IMMUNOBOLOGY (2017) DOI: 10.1016/j.imbio.2017.10.010 ***IF: 2,720 (from this 1,904 is used for this dissertation)***

Other publications

4. Prenek L, Boldizsar F, Kugyelka R, Ugor E, Berta G, Nemeth P, Berki T
The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells.
APOPTOSIS 22:(2) pp. 239-253. (2017) ***IF: 3,833***

5. Prenek Lilla, Ugor Emese, Papp Ramóna, Boldizsár Ferenc, Berki Tímea
A glukokortikoid hormon nem genomikus hatásai a T-sejtek jelátvitelére és apoptózisára
IMMUNOLÓGIAI SZEMLE 6:(3-4) pp. 54-58. (2014)

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