UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

The role of glucocorticoid hormone in the differentiation of regulatory T cells

PhD thesis

Ramóna Pap

Supervisor:

Dr. Tímea Berki

Professor

PÉCS, 2022

1. INTRODUCTION

Since their discovery more than 20 years ago, regulatory T cells (Treg) have attracted great interest in both basic and clinical immunology (Shevach, 2018). Treg cells represent a subpopulation of T cells that play a key role in maintaining tolerance to self-antigens and suppressing cells in the event of antigen stimulation that triggers an excessive immune response (Sakaguchi et al., 2010). Treg cells help to maintain immunological homeostasis and reduce the risk of developing autoimmune diseases and allergies (Calzada et al., 2018). The involvement of Treg cells in the prevention of organ rejection in post-transplant patients, in maternal-fetal tolerance and in the therapeutic application of autoimmune diseases is a clinically important issue (Chandran et al., 2017; Sharabi et al., 2018). The immunosuppressive and regulatory function of Treg cells is controlled by direct cell-cell interaction or immunosuppressive cytokines, e.g.: it is enforced through the production of transforming growth factor beta (TGFβ), Interleukin (IL-) 10 or IL-35. Several cell types are targets of Treg cells, including $CD4^+$ and $CD8^+$ T cells, dendritic cells (DC), B cells, macrophages, natural killer cells (NK cells) (Shevach, 2018). The best characterized subsets of Treg cells are thymus-derived natural Treg cells and peripheral Treg cells, which are derived from $CD4^+$ T cells in the periphery (Shevach and Thornton, 2014). Tregs express CD4 and CD25 cell surface markers and are positive for the transcription factor Foxp3. Foxp3 is a lineage-specific transcription factor of Treg cells and plays a key role in regulating the immunosuppressive phenotype and function (Lu et al., 2017).

Treg cells can be induced *in vitro* from naïve T cells in the presence of TGFβ and IL-2, such cells are called induced Treg (iTreg) cells. Research is ongoing to establish a stable induced Treg population for clinical applications. Several groups have analysed the optimal conditions for *in vitro* induction of $Foxp3^+$ T cells, as this is important for future Treg therapy (Hadaschik and Enk, 2015). High yield and purity of iTreg produced *in vitro* are important, as the transfer of contaminated conventional T cells may increase the risk of unwanted autoimmunity and inflammation. The differentiation direction of a T cell upon T cell receptor complex (TCR) stimulation depends on the complex microenvironment, including the cytokine milieu, metabolites, and hormones, which can determine the fate of tolerance-mediating iTreg cells. Various methods have been proposed to enhance and stabilize Foxp3 expression, such as progesterone, retinoic acid, vitamin D3, and rapamycin (Hoeppli et al., 2015; Schmidt et al., 2016). In addition, drugs that can directly promote the conversion of naïve or effector T cells into iTreg cells *in vivo* may be useful. In our previous study, we demonstrated that thymic Treg cells are resistant to repeated high-dose *in vivo* glucocorticoid (GC) hormone treatment. We also demonstrated that both thymic and splenic Treg cells produce increased levels of immunosuppressive cytokines, IL-10 and TGFβ, following *in vivo* Dexamethasone (DX) treatment (Ugor et al., 2018). These results support the idea that GCs affect the regulatory branch of the immune system and may be relevant in clinical conditions where enhancement of Treg cell activity is expected to be beneficial. Given the widespread use of GC hormones, including DX, and the effects of such drugs on immune regulation, we aimed to investigate the effect of DX treatment on the *in vitro* expansion and cytokine profile of Treg cells for future induction of a stable, higher yield Treg population.

2. AIMS OF THE STUDY

1. The aim of our work is to investigate the role of the GC hormone in the differentiation and functioning of Treg cells.
2. We wanted to investigate the GC sensitivity of Treg cells by monitoring the Treg ratio and absolute T-cell count in the thymus and spleen of untreated and treated BALB/c mice.
3. We wanted to determine the changes in the function of Treg cells as a result of the GC treatment by measuring the cells' suppressor cytokine production and the relative mRNA expression of cytokines and the Foxp3 transcription factor.
4. Another goal was to investigate the possible colocalization of GR and Foxp3 transcription factor in Treg cells without DX and after DX treatment *in vitro*.
5. Our aim was to investigate the effect of GC on the *in vitro* differentiation of Treg cells, as well as on $CD4^+$ T cells of thymus and spleen origin under different conditions. Apply new methods to produce stable and functionally suppressive Tregs for future use in adoptive transfer experiments.
6. We wanted to determine the functional characteristics of the *in vitro* differentiated Treg cells by measuring the cytokine production of the cells and the relative mRNA expression of the cytokines and the Foxp3 transcription factor.

Obtaining a sufficient number of functionally efficient and stable Treg cells is of primary importance during the *in vitro* manipulation of Treg cells in adaptive cell therapy in diseases where immunosuppression is the desired outcome (Milward et al., 2017).

3. MATERIALS AND METHODS

Experimental animals

3–4-week-old BALB/c female mice were used for the experiments. The animals were kept under conventional conditions and received commercial mouse chow and water ad libitum. The experiment was carried out in accordance with the regulations of the Workplace Animal Welfare Committee of the University of Pécs (#BA 02/2000-16/2015). Mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA).

In vivo DX treatment

Our experimental animals were vaccinated intraperitoneally for 2-4 days with a high dose (20 mg/kg of body weight) of Dexamethasone diluted with PBS. After 24 hours from the last inoculation, the spleen and thymus of the mice were removed and placed in RPMI-1640 (RPMI) medium.

Isolation of thymocytes and spleen cells

After the mice were sacrificed, their thymus and spleen were removed. The organs were mechanically homogenized in RPMI medium, and then the suspension was filtered through cotton to remove the tissue debris. The cells were washed in 1X PBS, centrifuged for 5 minutes

at a speed of 5000 rpm, and then taken up in 1 mL of RPMI medium. The trypan blue dye exclusion technique was used to determine cell viability and cell counting. $1x10^6$ thymocytes and spleen cells were treated with a high dose of 10^{-6}M DX (10^{-2}M stock solution dissolved in dimethyl sulfoxide (DMSO) in serum-free RPMI medium and incubated at 37°C for 30 minutes for confocal microscopy and co-immunoprecipitation and overnight for flow cytometric measurements The control samples were kept under the same conditions and incubated for the same time in the presence of the solvent. The GC analogue treatment was stopped by adding ice-cold PBS-NaN_3 (1X Phosphate buffered saline, containing 0.1% Na-azide).

Isolation and purification of $CD4^+$ cells

$CD4^+$ T cells were obtained from freshly isolated spleen and thymus cells by negative selection using the EasySep Mouse $CD4^+$ T Cell Enrichment Kits selection kit. The selection was carried out based on the description recommended by the manufacturer, using the factory purple magnet EasySep Magnet. The selection was carried out in a laminar chamber. The initial cell count is 1-1.5 x 10^8 cells/mL thymocytes or spleen cells added to the selection buffer (PBS, 2% FBS, 1 mM EDTA) in a 5 mL sterile polystyrene tube. The selection was made according to the manufacturer's protocol. After magnetic selection, the supernatant contains the negatively selected T cells. To check the purity of the $CD4^+$ T-cell population obtained at the end of the selection, cell surface anti-CD4-Pacific Blue, CD8-PE labeling was performed for flow cytometric measurement and RNA was isolated. The $CD4^+$ purity of the selected T cells was >96%. After washing the cells in RPMI, the live cell number was determined in a Bürker chamber using the trypan blue dye exclusion test.

***In vitro* Treg cell expansion**

$CD4^+$ T cells were cultured *in vitro* in medium containing RPMI + 10% FCS + 100 U/mL penicillin/streptomycin + 2 mM L-glucose for 2-14 days Dynabeads CD3/CD28 T cell activating microbeads (2:1, bead:cell ratio) using 30 U/mL recombinant IL-2 ± 40 pg/mL recombinant TGF β 1 ± 10^{-6} mol/L DX in the presence of DX in a 24-well plate (1000 µL/well) at 37 °C 5% CO_2 with content. After the *in vitro* cultivation, the cells were collected, and the microbeads were removed using the EasySep Magnet. The cells were washed in 1X PBS and live splenocyte and thymocyte numbers were determined in a Bürker chamber using a trypan blue dye exclusion test. RNA was isolated from the cells using the NucleoSpin RNA XS kit, and flow cytometric measurements were performed after fluorescent labeling.

Antibodies and fluorochromes

For flow cytometric measurements, anti-CD4-FITC (YTS 191), anti-CD8-PE (53-6.7) and anti-CD25-PE-Cy5 (RM4-5) or anti-CD25-PE-Cy7 (PC61) cell surface antibodies and anti-Foxp3-PE (3G3), anti-IL-10-APC (JES5-16E3) and anti-TGFβ-PerCP (TW7-16B4) intracellular antibodies were used. For further studies of *in vitro* differentiated T cells and Treg cells, anti-IL-17A-PerCP-Cyanine5.5 (17B7), anti-IFNγ-APC (XMG1.2) and anti-IL-4-FITC (BVD6-24G2) monoclonal antibodies were used. Anti-CD4-PE (YTS 191.1.2) and anti-CD25-PE-Cy5 (PC61.5) antibodies were used for FACS separation of Treg cells. For labeling confocal microscopic sections, anti-CD4-Pacific Blue (RM4–5), anti-CD8-PE (53-6.7), anti-CD25-PE-

Cy5 (PC61), anti-Foxp3-Alexa Fluor 647 150D), anti -GR-FITC (5E4-B1) antibodies were used.

Antibody labeling and flow cytometric measurement

For cell surface labeling, an antibody cocktail was prepared with concentrations corresponding to the given antibody in 100 μL PBS/0.1% BSA/0.1% NaN_3 labeling buffer. Next, 10^6 live cells per sample were labeled with a fluorescent monoclonal antibody. The cells were incubated for 30 minutes in the dark, after which the samples were washed twice in 2 mL of PBS/NaN_3 washing buffer. The eBioscience Foxp3/Transcription Factor Staining Buffer Set kit was used for intracellular labeling. The cells were fixed for 30 minutes and permeabilized in the dark on ice with 1 mL of fixation/permeabilization buffer. After fixation, the cells were washed with 2 x 2 mL previously diluted permeabilization buffer. For intracellular labeling, the previously listed fluorescent monoclonal antibodies were used, and at the end of the 30-minute incubation, the cells were washed twice in permeabilizing buffer. At the end of the labeling, the cells were taken up in 500 μL of fixing buffer (15 μL of 35% formaldehyde + 485 μL of PBS) and stored there until the measurement. The marking was carried out based on the description specified in the kit. The samples were measured on a FACSCanto II flow cytometer, and the results were analysed using the FCS Express 4 Flow Research program. We measured 100,000 events from the lymphocyte gate per sample. Lymphocytes were identified on the basis of size and granularity (FSC/SSC) parameters, and only $CD4^+$ cells within them were examined further. Within the $CD4^+$ T-cell population, we considered the $CD25^{high+}Foxp3^+$ subpopulation as Treg cells. The ratio of cytokines was also determined within the $CD4^+$ T cell and Treg populations.

Examination of fluorescently labeled samples with a confocal microscope

After labeling the cell preparations with cell surface and intracellular antibodies for fluorescence confocal microscopic examinations, the cells measured on the sections were spun for 5 minutes at 1000 rpm using a laboratory cytocentrifuge (Cytospin III, Shandon). The Shandon Cytospin 3 centrifuge creates a monolayer of cells in a clearly defined area, the insertion is done in a tilted position to prevent cell loss. During operation, the plates are placed in a vertical position, while the cells adhere to the slide. The Cytospin 3 centrifuge helps preserve cell integrity, which is important for further testing.

The completed cytopreparations were dried at room temperature for 10 minutes. Sections were coverslipped with PromoFluor Antifade, which minimizes photobleaching of fluorochromes by stabilizing fluorochromes in fixed cells, tissues, and cell-free preparations. The completed sections were stored at 4 °C in the dark. Fluorescence images of the sections were taken using an Olympus FluoView FV-1000 confocal laser scanning microscope and a fluorescence correlation spectroscope, and an Olympus Fluoview FV-1000S-IX81 image acquisition software system. The 3 lasers of the microscope provide excitation wavelengths of 456, 470, 488, 514, 543 and 633 nm. 3 fluorescence signals can be detected simultaneously from the sample, of which 2 are spectrally resolved and one is transmitted light (DIC contrast). By scanning, images with an optical slice thickness of 0.5 μm can be produced. Colocalization was analysed using ImageJ software.

RNA preparation and Real-time PCR

The collected thymocytes and spleen cells were labeled with cell surface anti-CD4-PE and anti-CD25-PE-Cy5 antibodies according to the previously described protocol. The labeled cells were analysed using the BD FACSAriaII Cell Sorting System and BD FACSDiva Software. Within the lymphocyte gate, cells showing $CD4^+$ and high $CD25^+$ positivity were selected. RNA samples were isolated using the NucleoSpin RNA XS kit from 10^5 $CD4^+CD25^{high+}$ cells, complementary DNA was synthesized using random oligo(dT) primers. Gene expression was quantified using the SYBR Green method in the Applied Biosystems 7500 RT-PCR system. To determine the relative gene expression levels, we normalized to β-actin, and the results were represented as a multiple of the mRNA levels of untreated Treg cells (RQ value). The following primer sequences were used in the experiments: 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'; GR (Forward) 5'- TGG TGT GCT CCG ATG A-3'; GR (Reverse) 5'-AGG GTA GGG GTA AGC -3'.

PMA/ionomycin stimulation

For the activation of $CD4^+$ T cells, *in vitro* Phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation was performed on $CD4^+$ T cells isolated from the untreated spleen and thymus and selected from DX-treated animals *in vivo*. Stimulation was performed in the presence of 25 ng/mL PMA and 1 μg/mL Ionomycin in RPMI medium for 24 hours at 37 °C and 5% CO_2 concentration. At the end of the incubation period, the cells were collected and, after labeling with a fluorescent antibody, flow cytometry measurements were performed to determine the Treg cell ratios.

Statistical analysis

The statistical evaluation was performed using the SPSS V.22.0 statistics package (IBM, USA). During our work, the average of the measured data and the standard error of the averages (± SEM) were plotted on the diagrams. After a normality test (Kolmogorov-Smirnov), we used a one-way or multi-way ANOVA test with an LSD post hoc test to compare the results of the different groups, and a difference of $p < 0.05$ was accepted as statistically significant. Markings: * able to control, # difference between treatments. In the figures, the element number *n* indicates the number of experimental animals used in the different treatment groups per group. Experiments represent at least 3 independent experiments with at least 3 experimental animals per treatment group.

4. RESULTS AND DISCUSSION

In our research, we wanted to optimize the appropriate conditions for the *in vitro* differentiation and expansion of Treg cells from thymus and spleen $CD4^+$ T cells. Treg cells are essential mediators of immune regulation and maintenance of immunological tolerance and, as such, are key therapeutic targets for many diseases (Li et al., 2018; Sakaguchi et al., 2008). Adoptive transfer therapy for Treg cells is an emerging area aimed at curbing unwanted, excessive, or pathological immune responses. For adoptive Treg cell therapies to be successful, well-characterized Treg cells with high efficiency, purity, and yield are required (Vaikunthanathan et al., 2018). An important consideration in our experiments was the fact that Treg cells are at the forefront of immunological research and that glucocorticoid (GC) hormone analogs are among the most important drugs in the treatment of inflammatory and autoimmune diseases, allergies, and organ transplants and malignancies (Cain and Cidlowski, 2017). The immunoregulatory properties of glucocorticoids are used in the clinic to treat inflammatory and autoimmune diseases, as well as certain hematological malignancies, but long-term use is hampered by adverse side effects. A full understanding of the molecular events that determine the physiological effects of glucocorticoid therapy provides insight into optimal glucocorticoid therapies, a reliable assessment of patients' glucocorticoid susceptibility, and may promote the development of new GR agonists that exert immunosuppressive effects while avoiding adverse side effects. Although there is interest in both directions, information on the effect of GC analogs on Treg cells *in vivo* and *ex vivo* (*in vitro*) is relatively rare. Only a handful of studies have been published that deal comprehensively with both topics, including some seemingly contradictory results; for example (Jørgensen et al., 2019; Bruscoli et al., 2021).

The primary goal of our work was to determine the conditions under which Treg cells can be robustly proliferated. We investigated the effect of anti-CD3/CD28 stimulation, stimulation with recombinant IL-2 and rTGFβ, and the presence or absence of added DX on $CD4^+$ T cell differentiation, Treg line binding (Foxp3 expression), and immunosuppressive cytokines. important factors that are important for the functional determination of Treg cells.

Freshly isolated spleen and thymus $CD4^+$ T cells were compared and differentiated using different conditions. The best cell expansion was observed in spleen-derived T cells in the presence of anti-CD3/CD28 and rIL-2, with a relative increase in cell number of approximately 70%. When the relative proportions and absolute cell numbers of Treg cells in the CD4 + T cell population as a function of the stimulation order were examined, there was a significant increase in the percentage of Treg cells and the absolute number of cells in both thymic and spleen-derived Treg cells, *in vitro* anti-CD3 / CD28 upon stimulation in the presence of rIL-2 + rTGFβ and rIL-2 + rTGFβ + DX (Pap et al., 2019). The incorporation of DX into the model may be relevant for further understanding of the complex role of GC hormones in the immune system (Bereshchenko et al., 2018; Quatrini et al., 2021).

Foxp3 relative mRNA levels increased with rIL-2 + rTGFβ treatment and showed a further increase when DX was added to cell cultures. These data may suggest that differentiation of thymic and splenic $CD4^+$ T cells toward the Treg phenotype may be facilitated by *in vitro*

treatment with IL-2 + TGFβ + DX (Pap et al., 2019). The effect of IL-2 + TGFβ on the polarization of naive $CD4^+$ T cells toward the Treg phenotype is known (Apert et al., 2018). The cytokine IL-2 is used in clinical settings to treat a variety of autoimmune diseases and transplant patients where it has been shown to lead to *in vivo* expansion of Treg cells (Ye et al., 2018). Despite significant knowledge of the effect of TGFβ on the physiological and pathological conditions of the immune system, the direct clinical use of TGFβ has been less studied; some ongoing clinical trials have been reported for rheumatoid arthritis and osteoarthritis (van der Kraan, 2018). What mechanism can mediate the DX-induced generation of Treg cells? The upregulation between GC hormone and TGFβ signaling may be relevant. Bereshchenko and colleagues reported that GC-induced leucine zipper (GILZ), a GC-induced protein, promoted the production of Treg cells (Bereshchenko et al., 2014). Our results on the combined effect of TGFβ and DX on increased Treg cell production and Foxp3 expression are consistent with this model. In our study, the overexpression of Foxp3 as a result of TGFβ and DX treatment is remarkable, as Foxp3 gene expression is a precedent for stable transcriptional commitment to the Treg cell phenotype. Foxp3 becomes a widely recognized Treg cell epigenoma modifier by associating with molecules that mediate epigenetic modifications that affect the activation or silencing of multiple Foxp3 target genes (Bereshchenko et al., 2014; Lu et al., 2017).

We examined the production of key immunosuppressive cytokines (IL-10, TGFβ) in Treg cells. Both thymus and spleen-derived Treg cells showed an increased proportion of IL-10 and TGFβ cytokine-producing cells when treated with a combination of IL-2, TGFβ, and DX (except for thymus-derived Tregs when IL-10 alone was used). The relative level of IL-10 mRNA showed an increase compared to control cell cultures (anti-CD3/CD28 treatment), except for spleen-derived cells when cultured with IL-2 + TGFβ. The relative level of TGFβ mRNA in thymic Tregs was only significantly increased when treated with anti-CD3/CD28 microbeads + rIL-2 + rTGFβ + DX (Pap et al., 2019).

We described that *in vivo* DX treatment in mice resulted in increased IL-10 and TGFβ-producing Treg cell ratios in both the thymus and spleen (Ugor et al., 2018). This is consistent with our *in vitro* results; however, it should be noted that in our *in vitro* model, DX was used in combination with exogenously added anti-CD3/CD28 + rIL-2 + rTGFβ (Pap et al, 2019). During *in vivo* DX treatment, GC hormone is of exogenous origin (except for endogenous GC hormones produced by the endocrine system), but IL-2 and TGFβ are present as part of an endogenous pool of cytokines. It is hypothesized that increased production of IL-10 and TGFβ by Treg cells, either *in vivo* or *in vitro* treated with DX (in the latter case after transplantation into the recipient), may help such Treg cells to develop an anti-inflammatory environment for immunosuppressive cytokines and by modulating DCs. These events may promote the formation of additional Treg cells from the precursor cells and may strengthen the Treg network through directed feeding (Kim et al., 2020).

Our experiments with pre-treatment of mice with high-dose DX showed that the proportion of Treg cells in $CD4^+$ T cells increased in the thymus but decreased in the spleen. This suggests that thymic Treg cells show greater resistance to DX-induced cell death than spleen-derived Treg cells, consistent with our previous data (Ugor et al., 2018). GC hormones are known to be

formed locally in the thymus (Talaber et al., 2015), so that thymic T cells are exposed to glucocorticoids for a longer period of time *in vivo*; therefore, it can be hypothesized that the relative resistance of thymic Treg cells to DX is due in part to higher GC levels that may induce selection of thymic Treg cells for the GC-resistant phenotype. Increased differentiation of thymic T cells into Treg cells was also observed *in vitro* under all *in vitro* stimulation conditions from mice pre-treated with DX for 4 days compared to untreated (control) mice, whereas the opposite effect was observed in spleen-derived cells observed. This suggests that the population of thymic T cells may point to the Treg phenotype, as reflected by their Foxp3 expression (possibly due to local production of GCs in the thymus), which may be more strongly bound to Treg lineage than *in vivo* DX pre-treatment.

Regarding to the expression of Foxp3, IL-10 and TGFβ mRNA, a more complex picture emerged in thymic Treg cells; an increase and decrease in Foxp3 and IL-10 mRNA levels were also observed as a result of *in vivo* DX treatment of mice. In case of TGFβ mRNA, *in vivo* DX treatment of mice did not cause a significant change under subsequent *in vitro* treatment conditions, except in the *in vitro* experiments with anti-CD3/CD28 + rIL-2 + rTGFβ + DX treatment, where reduced TGFβ mRNA levels were observed. In the thymic Treg cells of mice treated with *in vivo* DX compared to untreated mice. These results may reflect the heterogeneity and plasticity of the Treg line (Sawant and Vignali, 2014).

We examined the effect of *in vitro* DX on GR expression in Treg cells, increased GR fluorescence intensity in GR expression of spleen and thymus upon treatment, and increased Foxp3 transcription factor and GR colocalization in thymus and spleen $CD4^+$ T cells after *in vitro* DX treatment. GR-Foxp3 transcription factor colocalization may explain the mechanism of the effect of GC on Treg cells.

5. SUMMARY AND NOVEL FINDINGS

Summarizing our results, our data provide further insight into the effect of GC hormones on the regulation of the immune system. The GC analogue DX promotes the expansion of thymic and splenic Treg cells, promotes $Foxp3^+$ expression, and the production of IL-10 and TGFβ immunosuppressive cytokines *in vitro*. Pre-treatment of mice with DX has a different effect on subsequent *in vitro* proliferation of Treg cells (increased thymic Treg cell expansion and decreased splenic Treg cells). This suggests that special attention should be paid to the *in vitro* expansion of Treg cells in patients receiving GC therapy. Finally, GCs may become an important drug in the optimization of therapeutic Treg cells.

1. It was possible to expand Treg cells *in vitro* from $CD4^+$ T cells isolated from the thymus and spleen using CD3/CD28 microbeads, rIL-2 and rTGFβ.
2. The absolute number of Treg cells expanded *in vitro* from the thymus showed the greatest increase in the presence of CD3/CD28 microbeads, rIL-2 and rTGFβ. The absolute cell count of Treg cells expanded *in vitro* from the spleen was significantly increased in the presence of CD3/CD28 microbeads, rIL-2 and rTGFβ and further increased upon addition of DX.

3. The relative mRNA expression of the transcription factor Foxp3 increased in the thymus-, and spleen-derived induced Treg cells in the presence of CD3/CD28 microbeads, rIL-2 and rTGFβ, which showed a further significant increase after the addition of DX in the case of Treg cells differentiated from the spleen.
4. Treg cells *in vitro* differentiated from thymus and spleen produce IL-10 and TGFβ cytokines. Treg cells showed increased IL-10 and TGFβ production under the conditions of CD3/CD28, rIL-2, rTGFβ, and the level of TGFβ increased further when DX was added. Cells differentiated *in vitro* from the spleen showed increased secretion of IL-10 and TGFβ already under the effect of CD3/CD28 microbeads, rIL-2, and TGFβ increased further in the presence of DX.
5. *In vitro* expansion of Treg cells does not cause Th1, Th2, Th17 cytokine production.
6. *In vivo* DX pre-treatment improves the *in vitro* expansion of Treg cells induced from the thymus, but not from the spleen.
7. As a result of the *in vitro* DX treatment, the GR expression of Treg cells induced from the thymus and spleen increases, and the colocalization of the Foxp3 transcription factor and GR increases.

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7. PUBLICATIONS

Publications related to thesis topic:

Pap, R., Ugor, E., Litvai, T., Prenek, L., Najbauer, J., Németh, P., & Berki, T. (2019). Glucocorticoid hormone differentially modulates the in vitro expansion and cytokine profile of thymic and splenic Treg cells. Immunobiology, 224(2), 285–295. https://doi.org/10.1016/j.imbio.2018.12.002 Q2 **IF 2.788**

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Oral or poster presentation related to thesis topic:

T, Litvai; **R, Pap**; T, Berki. Optimization of in vitro condition for regulatory T cell differentation and expansion (2019) 2019. 02. 14-15. Pécs, XVI. János Szentágothai Multidisciplinary Conference, Közlemény:31008004

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Scientometrics:

Cumulative impact factor: 69.505 (thesis: 5.586)

Citations: 170 (independent: 144)

h-index: 7

8. ACKNOWLEDGEMENTS

I would like to express my gratitude to all those without whose help my PhD work would not have been possible.

I am especially grateful to my supervisor, Prof. Dr. Tímea Berki, who made it possible for me to join the research work at the Institute of Immunology and Biotechnology, I appreciate her many supports, guidance, and practical advice during my years as a student researcher during MSc and then as a PhD student.

I would like to thank all the employees of the Institute of Immunology and Biotechnology who helped me during my work and in learning laboratory techniques.

I would like to say thank to Dr. Gergely Berta for his help in the preparation of confocal microscopic images at the Institute of Medical Biology.

I would like to express my gratitude to Dr. Katalin Sipos, and all my colleagues at the Department of Pharmaceutical Biology, especially Dr. Edina Pandur and Gergely Jánosa, for supporting me in everyday life, listening, inspiring me, and giving me constructive criticism.

I am eternally grateful to my husband, my parents, my brother, my sister, and friends who patiently and constantly supported and encouraged me during my work, stood by my side throughout my studies, and without whom I would not have been able to achieve my dreams.

My PhD work was supported by the Hungarian Scientific Research Fund (NKFI), grant number K105962, and grants GINOP-232-15-2016-00050 and EFOP361-16-2016-00004.