Investigating the role of glucocorticoid (GC) signaling in the differentiation and function of regulatory T cells

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We followed the work plan and investigated the in vivo and in vitro effect of **glucocorticoid hormone (GC) on regulatory T cell differentiation and function, in a BALB/c** mouse model.

1. Our first aim was to determine the natural (tTreg) and induced Treg (iTreg or pTreg) cell ratio and absolute Treg cell numbers in thymus, lymph nodes, spleen and Peyer's patches of control and 2 or 20 mg/kg (high dose) Dexamethasone (DX) treated Balb/c mice based on CD4/CD25 and intracellular Foxp3/HELIOS staining. We found a major difference in the Treg cell ratios of the central and the peripheral lymphatic organs: 3,5% in the thymus, $15.09 \pm 0.43\%$ in the spleen, $10,0 \pm 0,61$ lymph nodes (LN), $6,78 \pm 0,51$ in the Peyer's patches (PP). Based on HELIOS staining the tTreg : iTreg ratio was 90 : 10 in the thymus, 65 : 35 in spleen, 53 : 47 in LN and 48 : 52 in PP.

   After 2-4 days DX treatment we found elevated Treg ratio (8-11%) in the thymus, with unchanged total Treg numbers (3×10^5/thymus) and decrease in both the Treg ratio and absolute cell number in the spleen: from 3×10^6 it changed to 5×10^5 cells/spleen. This reflects on a different GC sensitivity of the natural/thymic and peripheral, induced Foxp3+ Treg cells. We spent a lot of time with HELIOS transcription factor expression based differentiation of tTreg and iTreg cells, but we could not find any cell surface marker for the differentiation of these two cell populations. Therefore in the further experiments we worked with thymic/natural and splenic/peripheral/induced Treg cells and compared their functional and activation behavior.

Publications: *Ugor E. és mtsai Immunológiai szemle VI.(3): 8-12. 2014. Domestic and International Congress abstracts (see below). Diploma Thesis of 2 students.*

2. Then we studied the effects of *in vivo GC treatment* on Treg functions and measured their IL-10, IL-35 and TGFβ production by flow cytometry and rtPCR. For flow cytometric intracellular cytokine detection cells were stimulated with PMA/ionomycin/brefeldin followed by intracellular cytokine staining of CD4/CD25/Foxp3+ cells. For rtPCR analysis of Treg cytokines we spent a lot of time with the establishment of a reliable high capacity Treg cell sorting technique for further experiments. We compared the magnetic two step sorting (EasySep CD4 negative selection followed by CD25 positive selection) and different cell surface based labelling methods. Finally we used the conventional cell surface Treg staining and the CD4+CD25high+ cells were separated (resulted >90% purity) on FACSAriaII cell sorter and the mRNA was isolated.

   In the thymus, the ratio of IL-10 and TGFβ positive tTreg cells was similar ($11.0 \pm 2.3\%$ and $13.5 \pm 3.1\%$), but DX treatment resulted in significant increase in both cytokine secreting tTreg ratios ($17.6 \pm 1.4\%$ and $21.0 \pm 4.9\%$). In the splenic pTreg cells of control animals, we detected significantly higher TGFβ positivity ($13.7 \pm 2.0\%$), compared to IL-10 positivity ($3.6 \pm 0.5\%$). As a result of DX treatment the percentage of both IL-10 and TGFβ positive Tregs increased significantly, but the fold-increase for IL-10 was higher than for TGFβ.

   We also compared the DX treatment-induced relative IL-10 and TGFβ mRNA expression in purified (sorted) $CD4^{+}CD25^{high+}$ Tregs. In the thymic tTreg cells the cytokine mRNA expression showed an increasing tendency, especially the relative elevation of IL-10 mRNA level as a result of repeated (2x) high-dose GC treatment. In the splenic pTreg cells DX treatment induced an increased relative expression of TGFβ mRNA, whereas

the relative IL-10 mRNA expression remained unchanged. We also measured the effect of DX treatment on another Treg suppressor cytokine, IL-35, which was unchanged in thymus and showed an elevation in splenic pTreg at mRNA level.

Based on these results we can conclude, that repeated high dose in vivo GC treatment cause elevated secretion of immunosuppressive cytokines. In thymic Treg cells IL-10 while in the splenic Treg cells TGFβ is the dominant immunosuppressive cytokine detected both at mRNA and protein level.

Next we studied the relative quantitative changes of Foxp3 transcription factor expression, which plays a role in determining the functions of Treg cells [1]. Foxp3 mRNA levels were very similar in purified $CD4^{+}CD25^{high+}$ thymic and splenic Treg cells (data not shown), but showed an increasing tendency after DX treatment in thymic Tregs and a significant elevation in splenic Tregs. The DX-induced increased Foxp3 mRNA expression is consistent with a higher Treg cell commitment and production of immunosuppressive cytokines.

Publication: *Domestic and International Congress abstracts (see below). Diploma Thesis of 2 students. Ugor et al. Immunobiology 2018; 223(4-5):422-431.*

Since Treg cells have major role in the development of **autoimmune diseases** we also investigated the role and function of Treg cells in systemic sclerosis (SSc)

3. Treg subpopulations and their cytokines, IL-10 and TGF-β in the peripheral blood of early stage, untreated dcSSc patients were investigated with epigenetically regulated methylation state of the FOXP3 promoter and enhancer regions. CD4+CD25+Foxp3+CD127- Treg cells were significantly elevated in patients with diffuse cutaneous SSc and in patients with anti-Scl-70/RNA-Pol-III autoantibody positivity and with lung fibrosis. Increased CD62L+Treg cells were present in all SSc subgroups. The production of immunosuppressive cytokines by both CD127- and CD62L+ Tregs was diminished. We observed reduced methylation of Treg specific FOXP3 enhancer regions, and elevated FOXP3 gene expression in active SSc cases with negative correlation in the frequency of CD62L+IL-10+ Tregs. Our data indicate an inappropriate distribution and cytokine production of Treg cells in early form SSc.

Publication: *Domestic and International Congress abstracts (see below). Diploma Thesis of 2 students. Ugor et al. Clin Immunol. 2017;184:54-62.*

4. The **GC induced apoptosis** sensitivity of different cell types is influenced both by the **expression level of the glucocorticoid receptor (GR)** and the ligand-induced GR translocation pattern (ie. nuclear or mitochondrial translocation). We compared the GR expression of different T lymphocyte subpopulations and their GC sensitivity and found that both in thymus the SP CD8+ T cells and the peripheral CD8+ cytotoxic T cells express the highest GR level and they are the less sensitive to GC induced apoptosis, followed by CD4+ T helper cells and the pTreg cells which express similar GR level. Since we observed a relative GC resistance of tTreg cells, we looked for a possible relationship between the observed GC resistance and their GR expression. We measured intracellular GR protein levels by flow cytometry and GR mRNA levels by qRT-PCR in purified thymic and splenic $CD4^{+}CD25^{high+}$ Treg cells. In thymic tTreg cells of untreated animals we detected significantly lower GR protein levels than in splenic pTreg cells. After repeated high-dose DX treatment we measured a significantly 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 was characteristic of SP thymocytes and mature T cells (Berki et al., 2002b; Boldizsár et al., 2006). We did not observe detectable changes in GR mRNA expression in thymic and splenic Tregs.

Since the most GC sensitive double positive (DP) thymocytes express the lowest GR level and that short-term high dose in vitro GC treatment induced the **GR mitochondrial translocation** in 30 min, we investigated which Bcl-2 family proteins could participate in GC-induced thymocyte apoptosis. In subcellular fractions of DP cells we investigated the GR interaction with Bcl-2 family proteins and the subcellular localization of them using immunoprecipitation and confocal microscopy. After short-term DX treatment the mitochondrial GR showed association with pro-and anti-apoptotic members of Bcl-2 family like Bak, Bim and Bcl-x_L and Bax accumulated in mitochondria, followed by Cytochrome C release, and elevation of active caspase-3,-8, and-9 levels. These results support that in early phase of GC-induced thymocyte apoptosis, the mitochondrial pathway plays a crucial role, with accumulation of GR and pro-apoptotic Bcl-2 proteins into the mitochondria. The activation of caspase-8 was presumably due to the cross talk between the intrinsic- and extrinsic apoptotic signaling pathways.

Interestingly investigating the in vitro DX treatment induced Treg apoptosis pathways we found elevated Annexin-V positivity and loss of mitochondrial function (diminished CMX-Ros positivity) and weak activation of both caspase-8 and 9 in tTreg cells. Weak caspase 9 activation was found in both tTreg and pTreg cells. These results do not support our in vivo findings, that tTreg cells are resistant to DX induced apoptosis. Another possibility is that in vivo DX induced apoptosis of tTreg cells is inhibited by other signals in the thymus. To clarify this, we need further experiments.

Publication: *Domestic and International Congress abstracts (see below). Diploma Thesis of 2 students. Prenek L. et al.: Immunológiai szemle VI.(3): 4-8. 2014, Prenek L. et al. Apoptosis 2017. 22: (2) pp. 239-253., Ugor et al. Immunobiology 2018; 223(4-5):422-431,*

5. Based on confocal microscopic investigation GR showed ligand induced nuclear translocation in Treg cells. We also investigated the GR and Foxp3 localization in thymic and splenic Treg cells with and without previous in vivo DX treatment using confocal microscopy. We observed a characteristic nuclear localization of both GR and Foxp3 in CD4+CD25+Foxp3+ tTreg and pTreg cells. In untreated samples Foxp3 highly colocalized with GR both in thymic and splenic Treg cells. Upon in vitro high dose DX treatment (30 min) colocalization further increased in the splenic pTreg cells, while in thymic tTregs this association showed no change. We also started FRET measurements in collaboration with György Vereb (DE) for the detection of the physical association of the two molecules. We found co-localization between GR and Foxp3 in pTreg cells which further elevated due to in vitro short term DX treatment.

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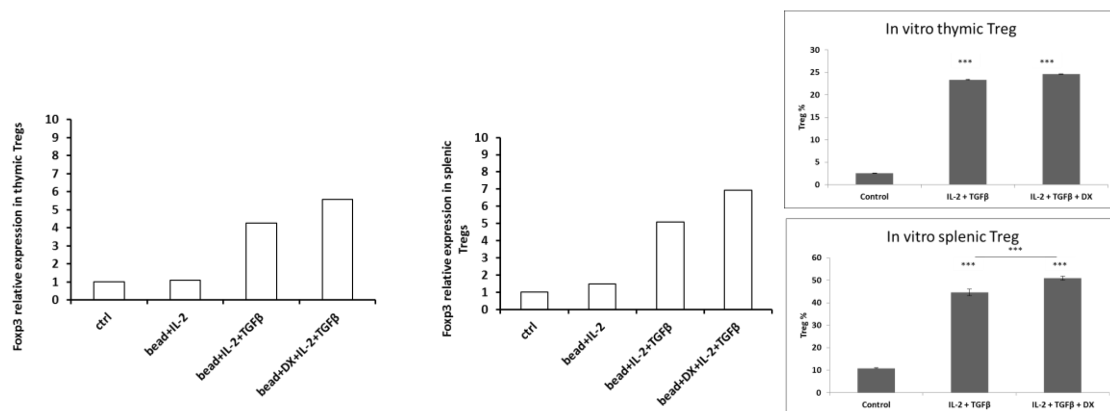
6. To enrich Treg cells for further in vitro and in vivo experiments, we used different strategies. Since we found major differences in the OX40, GITR or CD45RB staining of Treg cells, we did not use these markers to separate them. The tTreg absolute cell number is very low in the thymus, even the elevated Treg ratio in DX treated animals allowed us to isolate low Treg numbers and the in vitro expansion of tTreg cells resulted low cell number and low cytokine producing tTreg ratios. Therefore we isolated splenic CD4+ T cells with negative selection followed by separation of CD25+ and CD25- cell populations with magnetic cell sorting. These cells were cultured in vitro with anti-CD3/CD28 magnetic beads in the presence of IL-2, ±TGFbeta and ±Dexamethasone to

optimize the Treg expansion conditions. We measured Foxp3 protein and mRNA level in the samples and cytokine expression. Splenic CD4+ T cell expansion resulted in a higher (44-45%) pTreg cell ratio, which further increased (to 50%) in the presence of GC, with elevated immunosuppressive cytokine (IL-10 and TGFbeta) producing cell ratios.

Table 1.: Cytokine production of in vitro expanded splenic Treg cells

	IL-10	TGFβ	IFNγ	IL-4	IL-17
Control	4,265	4,145	0,51	1,275	1,34
Bead + IL-2	11,115	13,96	0,055	1,145	0,13
Bead + IL-2 + TGFβ	15,915	22,775	0	0,2	0,02
Bead + IL-2 + TGFβ + DX	18,605	35,77	0	0,945	0,055



Ratio of Foxp3+ Treg cells and their Foxp3 expression elevated in the presence of DX in in vitro expanded Treg cells

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7. Since T cells are important mediators of autoimmune diseases, to test the function of Tregs we used the recombinant human aggrecan G1-domain (rhG1)-induced arthritis model (GIA), which resembles the human rheumatoid arthritis. To evaluate the effect of repeated (4-times) DX pre-treatment (2 and 20 mg/kg, before antigen induction) on the development and severity of the disease BALB/c mice were injected daily with DX before the arthritis induction by immunization with rhG1 antigen once every three weeks for three times. The clinical symptoms were assessed using a scoring system and the splenocytes were *in vitro* stimulated to investigate antigen-specific T cell proliferation and cytokine production (IL-1β, IL-4, IL-6, IL-10, IL-17, IFNγ, TNFα, TGFβ). The pTreg count and their cytokine production did not show significant changes, but the DX pre-treated mice developed less severe arthritis compared to controls and the T cell cytokine production were altered. Then we transferred *in vivo* differentiated Treg cells ($CD4^{+}/CD25^{high+}$ 10^{6} i.v/animal) from DX pre-treated arthritic and non-arthritic mice into healthy Balb/c mice prior the arthritis induction. Mice, receiving Tregs from DX pre-treated donors with less severe arthritis, developed less pronounced arthritis than the control group. In conclusion, the high dose DX pre-treatment reduced the severity of arthritis in GIA model, and GC pre-treated, probably antigen-specific, Tregs might play a role in decreasing the severity of arthritis.

Publication: *Domestic and International Congress abstracts. Publication is under preparation.*

Presentation of the results:

Original papers:

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Congress abstracts:

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Berki T.: Functionally impaired regulatory T cell subsets are elevated in systemic sclerosis 4th International Congress on Controversies in Rheumatology and Autoimmunity (CORA)" Bologna 2017.

Prenek L.: Functional role of Dexamethasone pre-trerated regulatory T cell tarnsfer in the development of murine autoimmune arthritis. 11th International Congress on Autoimmunity 2018. (előadás)