Immunobiology 224 (2019) 285–295

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Glucocorticoid hormone differentially modulates the *in vitro* expansion and cytokine profile of thymic and splenic Treg cells

Ramóna Pap[1], Emese Ugor, Tímea Litvai, Lilla Prenek, József Najbauer, Péter Németh, Tímea Berki*

Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, H-7624, Hungary

ARTICLE INFO

Keywords:
Dexamethasone
Treg
Foxp3
Glucocorticoid hormone
IL-10
TGFβ

ABSTRACT

Objective: Functional disturbances in regulatory T cells (Treg) have been described in autoimmune diseases, and their potential therapeutic use is intensively studied. Our goal was to investigate the influence of glucocorticoid hormone on the *in vitro* differentiation of Treg cells from thymic and splenic $CD4^+$ T cells under different conditions to establish methods for generating stable and functionally suppressive iTregs for future use in adoptive transfer experiments.

Methods: Thymic and splenic $CD4^+$ T lymphocytes were isolated from 3 to 4 week-old control and *in vivo* dexamethasone (DX) pretreated BALB/c mice using magnetic bead negative selection, followed by CD25 positive selection. The cells were cultured with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGFβ and/or DX for 3–6 days. Multiparametric flow cytometry was performed using CD4, CD25, CD8, TGFβ (LAP) cell surface and Foxp3, IL-4, IL-10, IL-17 and IFNγ intracellular staining. Quantitative RT-PCR was performed to measure IL-10, TGFβ cytokine and Foxp3 mRNA levels.

Results: Differentiation of thymus-derived $CD4^+$ cells *in vitro* into iTreg cells was most effective (24–25%) when anti-CD3/CD28 beads, IL-2, and TGFβ were present. Splenic $CD4^+$ T cell expansion under same conditions resulted in a higher (44–45%) iTreg cell ratio that further increased (up to 50% Treg) in the presence of DX. Elevated immunosuppressive cytokine (IL-10 and TGFβ) production by iTregs could be measured both at protein and mRNA levels without elevation of Th1/Th2 or Th17 cytokine production. We got the highest iTreg ratio (74%) and TGFβ production when $CD4^+CD25^+$ splenic T cells were stimulated in the presence of TGFβ. *In vivo* 4 days DX pretreatment resulted in enhanced *in vitro* expansion and Foxp3 expression of thymus-derived iTregs and decreased differentiation of spleen-derived iTreg cells. In these Tregs the relative expression of IL-10 mRNA significantly decreased under all *in vitro* stimulation conditions, while TGFβ mRNA level did not change.

Conclusion: DX promotes the expansion of thymic and splenic Treg cells, and enhances $Foxp3^+$ expression and the production of immunosuppressive cytokines IL-10 and TGFβ *in vitro*. *In vivo* pretreatment of mice with DX inhibited the immunosuppressive cytokine production of *in vitro* differentiated Treg cells. We hypothesize that patients receiving GC therapy may need special attention prior to *in vitro* expansion and transplantation of Treg cells.

1. Introduction

Since their discovery more than 20 years ago, regulatory T cells (Treg) have attracted much interest in both basic and clinical immunology (Shevach, 2018). Tregs are a subpopulation of T cells that play a pivotal role in maintaining tolerance to self-antigens (Sakaguchi et al., 2010; Sharma and Rudra, 2018; Shevach and Thornton, 2014; Vignali et al., 2008) and in suppression of excessive immune responses after antigenic stimulation. Thereby Treg cells help in maintaining an immune homeostasis and in lowering the risk for developing autoimmune diseases and allergies (Calzada et al., 2018; Lu et al., 2017; Vila et al., 2009). Clinically important issues are the participation of Treg cells in the prevention of organ rejection in patients after transplantation (Chandran et al., 2017), mothers' tolerance to fetus (Kisielewicz et al., 2010), and therapeutic application in autoimmune diseases (Sharabi et al., 2018; Spence et al., 2015). The best characterized subsets of Treg cells are (1) the thymus-derived natural Treg cells (tTreg), which are generated in the thymus during negative

* Corresponding author at: Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Pécs, H-7624, Szigeti út 12, Hungary.
E-mail address: berki.timea@pte.hu (T. Berki).
[1] Current address: Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Pécs, Pécs H-7624, Hungary.

https://doi.org/10.1016/j.imbio.2018.12.002
Received 6 December 2018; Accepted 18 December 2018
Available online 27 December 2018
0171-2985/

selection process and primarily recognize self-antigens, and (2) the induced Treg (iTreg), also named peripheral Treg cells (pTreg), generated from $CD4^+$ T cells in the periphery (Kretschmer et al., 2005; Shevach and Thornton, 2014). Tregs can be expanded *in vitro* from naïve T cells in the presence of TGFβ and IL-2, such cells are called iTregs, which are generally more unstable than tTregs. There are ongoing efforts to generate stable iTregs for clinical applications (Kanamori et al., 2016). The immunosuppressive and regulatory function of Treg cells is mediated by direct cell-cell interaction or by secretion of immunosuppressive cytokines, *e.g.* TGFβ (Kretschmer et al., 2005), IL-10, or IL-35 (Saraiva and O'Garra, 2010). Numerous cell types are targets of Treg cells, including $CD4^+$, $CD8^+$ T cells, dendritic cells, B cells, macrophages, NK cells (Shevach, 2018).

Both tTreg and pTreg cells express CD4 and CD25 cell surface markers, and are positive for Foxp3 (Forkhead box protein 3) transcription factor. The Foxp3 is a lineage specific transcription factor of Treg cells, and plays a key role in regulating the acquisition of immunosuppressive phenotype and function (Kim, 2009; Lu et al., 2017; Schmetterer et al., 2012). Numerous transcription factors interact with Foxp3, and thus are involved in activation, differentiation, and functional response of $CD4^+$ T cells. There have been reports that Foxp3 may act as a transcriptional co-repressor causing inhibition of CREB (cAMP response element-binding protein), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), and RORα (retinoic acid receptor-related orphan receptor-α) (Kanamori et al., 2016; Katoh et al., 2013; Sadlon et al., 2010). Foxp3 may also interact with NFAT (nuclear factor of activated T cells) resulting in transcriptional repression of IL-2 and maintainamce of the suppressive function of Treg cells (Schmetterer et al., 2012; Selvaraj, 2013). It has been demonstrated recently that Foxp3 interacts with hnRNP F (heterogeneous nuclear ribonucleoprotein F) and modulates pre-mRNA alternative splicing (Du et al., 2018).

The cytokine TGFβ is an important regulator of T cell tolerance (Chen and Ten Dijke, 2016; Sanjabi et al., 2017). Mice with impaired or total loss of TGFβ signaling in T cells develop multifocal autoimmunity comparable to the disease observed in mice with global *Tgfb1* gene deficiency (Kulkarni et al., 1993; Shull et al., 1992). Mice with T cell-specific loss of TGFβ signaling also exhibit defects in the differentiation of thymic Treg (tTreg) cells (Liu et al., 2008), as TGFβ signaling has been shown to promote the survival of tTreg cell precursors (Ouyang et al., 2010). It was demonstrated that stimulation of T cell receptor (TCR) in the presence of TGFβ1 results in upregulation of Foxp3 expression in conventional $CD4^+CD25^-$ T cells (Chen et al., 2003). These TGFβ1-induced $Foxp3^+$ T cells displayed suppressive function and inhibited the antigen-specific expansion of $CD4^+$ T cells *in vivo*.

Several groups further analyzed the optimal conditions to induce $Foxp3^+$ T cells *in vitro* as this is important for future iTreg therapy (Hadaschik and Enk, 2015). Importantly, IL-2 is necessary for *in vitro* induction of TGFβ1-induced $Foxp3^+$ T cells and cannot be replaced by other cytokines (Davidson et al., 2007). The yield of $Foxp3^+$ T cells after induction by TGFβ1 *in vitro* crucially depends on the differentiation status of the starting cell population. High yields and the purity of *in vitro* generated iTreg are important since transfer of contaminating conventional T cells could increase the risk of unwanted autoimmunity and inflammation. The direction of differentiation of a given T cell during TCR stimulation depends on the complex microenvironment, including the cytokine milieu, metabolites, and hormones (Hoeppli et al., 2015), which may determine the generation of tolerance-mediating iTreg cells or proinflammatory Th17 cells. Various agents have been proposed to upregulate and stabilize Foxp3 expression, such as progesterone, retinoic acid, vitamin D3 and rapamycin (Hoeppli et al., 2015; Lavi Arab et al., 2015; Lee et al., 2012; Schmidt et al., 2016). Additionally, drugs that can directly promote conversion of naïve or effector T cells into iTregs cells *in vivo* will be useful.

In our previous study, we demonstrated that thymic tTreg cells are resistant to repeated high dose *in vivo* glucocorticoid (GC) hormone treatment (Ugor et al., 2018). We also showed that both thymic and splenic Treg cells produced enhanced levels of immunosuppressive cytokines IL-10 and TGFβ after *in vivo* DX treatment, which was accompanied by elevated Foxp3 mRNA expression that may reflect a stronger Treg lineage commitment. These findings supported the notion that GCs influence 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. Given the widespread use of glucocortiociod hormones, including DX, and the effect of such drugs on the regulatory arm of the immune system, we set out to investigate the effect of DX treatment on the *in vitro* expansion and cytokine profile of regulatory T cells.

2. Material and methods

2.1. Animals

BALB/c mice (3–4 weeks old) were kept in our laboratory animal facility under standard conditions and were provided food and acidified water *ad libitum.* All animal experiments complied with the regulations established by the Committee on Animal Experimentation of the University of Pécs (#BA 02/2000-16/2015).

2.2. In vivo glucocorticoid hormone treatment

Mice received intraperitoneal injection of dexamethasone (Oradexon, N. V. Organon) at 20 mg/kg bodyweight each day over 4 days. Untreated mice were used as controls. The mice were euthanized 24 h after the last injection. Thymus and spleen were removed and homogenized mechanically in RPMI (Sigma), and the homogenate was filtered using a nylon mesh. Cell viability was determined in a hemocytometer using the trypan blue dye exclusion test. The cells were used for $CD4^+$ and $CD25^+$ T cell selection and *in vitro* Treg differentiation.

2.3. Magnetic sorting of $CD4^+$ and $CD4^+CD25^+$ cells

$CD4^+$ T cells were isolated by negative selection using EasySep Mouse $CD4^+$ T Cell Enrichment Kit (Stemcell Technologies, Vancouver, Canada) as previously described (Ugor et al., 2018). In some experiments the $CD4^+$ T cells purified by negative selection were subjected to further positive selection to obtain $CD4^+CD25^+$ Treg cells using EasySep™ Mouse CD25 Treg Positive Selection Kit (Stemcell Technologies, Vancouver, Canada) following the manufacturer's instructions.

2.4. In vitro Treg cell expansion

Purified $CD4^+$ T cells were cultured in 24-well or 96-well plates (Sarstedt) in the presence of anti-CD3/CD28 T cell activator magnetic beads (Dynabeads, Life Technologies) according to the manufacturer's protocol. Cells were cultured at an initial density of 1×10^6 or 2×10^5 cells per well. RPMI medium containing 10% FBS (fetal bovine serum), 1% penicillin/streptomycin, and 1% glucose was used for Treg expansion. The culture medium contained rIL-2 (2000 U/mL), with or without rTGFβ (5 ng/mL) (both from eBioscience Inc.). To examine the effect of the GC hormone for the expanding cells, 1 μM dexamethasone (DX) was added to the culture medium. After culturing for 3 days, the cells were harvested and the magnetic beads were removed using an EasySep™ magnet (Stemcell Technologies). The collected cells were washed in PBS and stained with the appropriate antibodies. Purified $CD4^+CD25^+$ Tregs and the $CD4+CD25^-$ T cells were cultured in the presence of anti-CD3/CD28 T cell activator magnetic beads (Dynabeads, Life Technologies) with rIL-2 (2000 U/mL) and rTGFβ (2.5 ng/mL) (both from eBioscience Inc.). After 6 days of culture, the cells were harvested and the magnetic beads were removed using a magnet. The collected cells were washed in PBS and stained with the appropriate antibodies.

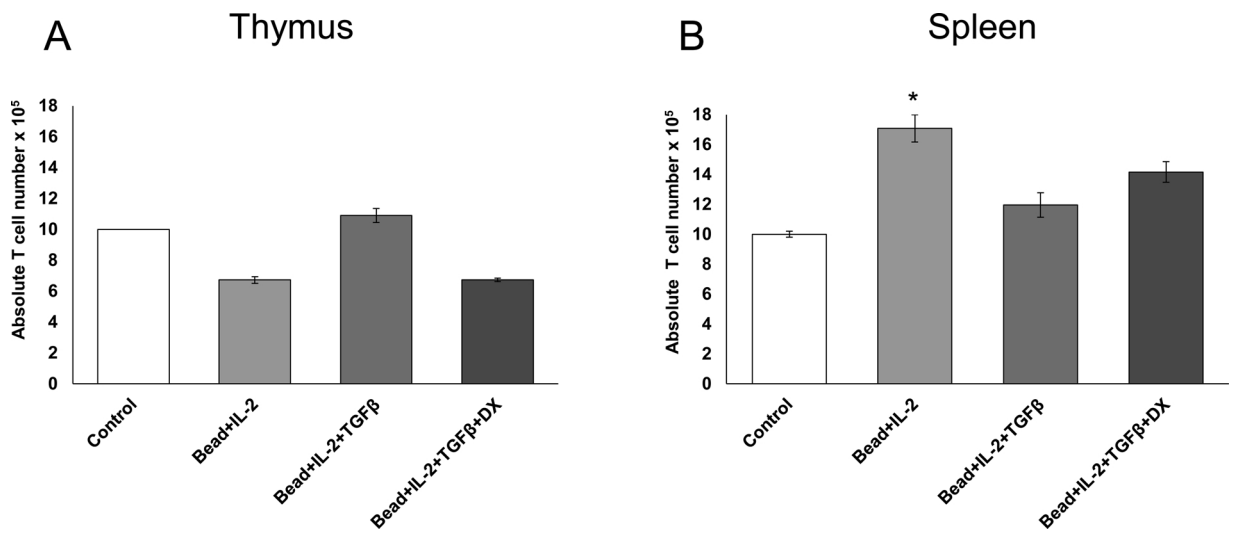

Fig. 1. Absolute numbers of thymic (A) and splenic (B) CD4 T cells after 3 days of *in vitro* stimulation. (A) The absolute thymic T cell number did not change significantly during 3 days of *in vitro* differentiation in the presence of anti-CD3/CD28 beads + rIL-2 and + rTGFβ + DX.
(B) The absolute splenic T cell number significantly increased after 3 days of *in vitro* differentiation in the presence of anti-CD3/CD28 beads + rIL-2. * indicates $p < 0.05$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX treatments. The data are shown as mean ± SEM (n = 12).

2.5. Flow cytometry and FACS analysis

Separated cells (1×10^6) were stained with cell surface specific monoclonal antibodies in binding buffer (PBS, containing 0.1% bovine serum albumin, and 0.1% NaN_3). The following antibodies were used: Pacific Blue labeled anti-CD4 (Clone RM4-5, BD Pharmingen), FITC labeled anti-CD8 (IBI clone, Department of Imunology and Biotechnology (DIB) Pécs, Hungary), PE-CY7 labeled anti-CD25 (Clone PC61, BD Pharmingen) and PerCP/Cy5.5 labeled anti-LAP (TGF-β1) (Clone TW7-16B4, BioLegend, San Diego, CA, USA). After two washing steps the cells were incubated in Foxp3 fixation/permeabilization buffer (eBioscience) and stained with the appropriate intracellular antibodies: PE labeled anti-FoxP3 (Clone 3G3, MACS, Miltenyi Biotec, Bergisch Gladbach, Germany), APC labeled anti-IL-10 (Clone JES-16E3, BioLegend, San Diego, CA, USA), FITC labeled anti-IL-4 (Clone BVD6-24G2, eBioscience Inc.), PerCP-Cyanine5.5 labeled anti-IL-17 A (Clone eBio17B7, eBioscience Inc.). After 2 washing steps, cells were fixed in 300 µl FACS-Fix (Sigma-Aldrich) and measured with a FACSCantoII flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using the FCS Express 4 Flow Research Edition software. Treg cells were determined in the CD4 gate based on their CD25/Foxp3 positivity. The cytokine ratios were measured within the $CD4^+$ T cells and within the $CD25^+Foxp3^+$ Treg subpopulation.

2.6. RNA preparation and quantitative RT-PCR

After *in vitro* expansion of $CD4^+$ T cells, RNA was isolated from 10^5 cells using NucleoSpin RNA XS kit. cDNA was synthetized using random oligo(dT) primers (Applied Biosystems). Quantification of gene expression was performed by the SYBR Green method using the Applied Biosystems 7500 RT-PCR system. The relative expression levels were normalized to the actin gene; results were presented as fold induction compared to unstimulated Treg mRNA levels (RQ). The following primer sequences were used: 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′; TGFβ1 (forward) 5′− GAC TCT CCA CCT GCA AGA CC −3′; TGFβ1 (reverse) 5′− GGA CTG GCG AGC CTT AGT TT −3′; Foxp3 (forward) 5′− TAC TTC AGA AAC CAC CCC GC −3′; Foxp3 (reverse) 5′− GTC CAC ACT GCT CCC TTC TC −3′; β-ACTIN (forward) 5′− GGG AGG GTG AGG GAC TTC C −3′; β-ACTIN (reverse) 5′− TGG GCG CTT TTG ACT CAG GA −3′

2.7. Statistical analysis

The SPSS v. 22.0 statistics package (IBM, Armonk, NY, USA) was used for statistical evaluation of data. Data were expressed as mean ± SEM. Student's *t*-test was used to compare data between the groups. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of TGFβ and GC hormone on Treg cell expansion in vitro

In our previous study we determined the Treg cell ratios in peripheral and central lymphatic organs (Ugor et al., 2018) of 3–4 weeks old BALB/c mice. We found that within the thymic $CD4^+$ T cell population there were 2.5 ± 0.2% $CD4^+CD25^{high+}Foxp3^+$ Treg cells, while in splenic $CD4^+$ T cells this ratio was 12.7 ± 2.1%. In this study, we wanted to optimize the *in vitro* conditions for Treg differentiation and expansion from thymic and splenic $CD4^+$ T cells isolated by negative selection. After 3 days *in vitro* stimulation of $CD4^+$ T cells using anti-CD3/CD28 beads and rIL-2 in the presence or absence of rTGFβ and DX, we observed little or no change in the absolute number of thymus derived $CD4^+$ T cells, whereas in the case of spleen-derived CD4 + T cells proliferation was detected by all stimulation conditions. The greatest (significant) increase of cell numbers was when anti-CD3/CD28 beads + rIL-2 were present (Fig. 1A and B).

Phenotype analysis of the *in vitro* stimulated thymic $CD4^+$ T cells by flow cytometry showed that the ratio of $CD4^+CD25^{high+}Foxp3^+$ Treg cells increased significantly (to 23.4 ± 1.9%) when anti-CD3/CD28 beads, rIL-2 and rTGFβ were present. The iTreg cell ratio did not change significantly when DX was also added to the expansion medium (24.6 ± 0.8%) (Fig. 2A). The total numbers of thymic Treg cells showed the greatest increase when stimulated with anti-CD3/CD28 beads, rIL-2 and rTGFβ without DX (Fig. 2B).

In vitro expansion of splenic $CD4^+$ cells into iTreg cells was most effective when stimulated with anti-CD3/CD28 beads, rIL-2, togehther with rTGFβ and DX. The ratio of iTregs was 44.6 ± 2.9% when TGFβ was added, which showed further increase to 50.9 ± 1.0% when *in vitro* DX was also present in the culture media. No significant *in vitro* Treg cell expansion was detected, when anti-CD3/CD28 beads and rIL-2 were only present (Fig. 3A). The absolute number of spleen-derived iTreg cells significantly increased during *in vitro* expansion when

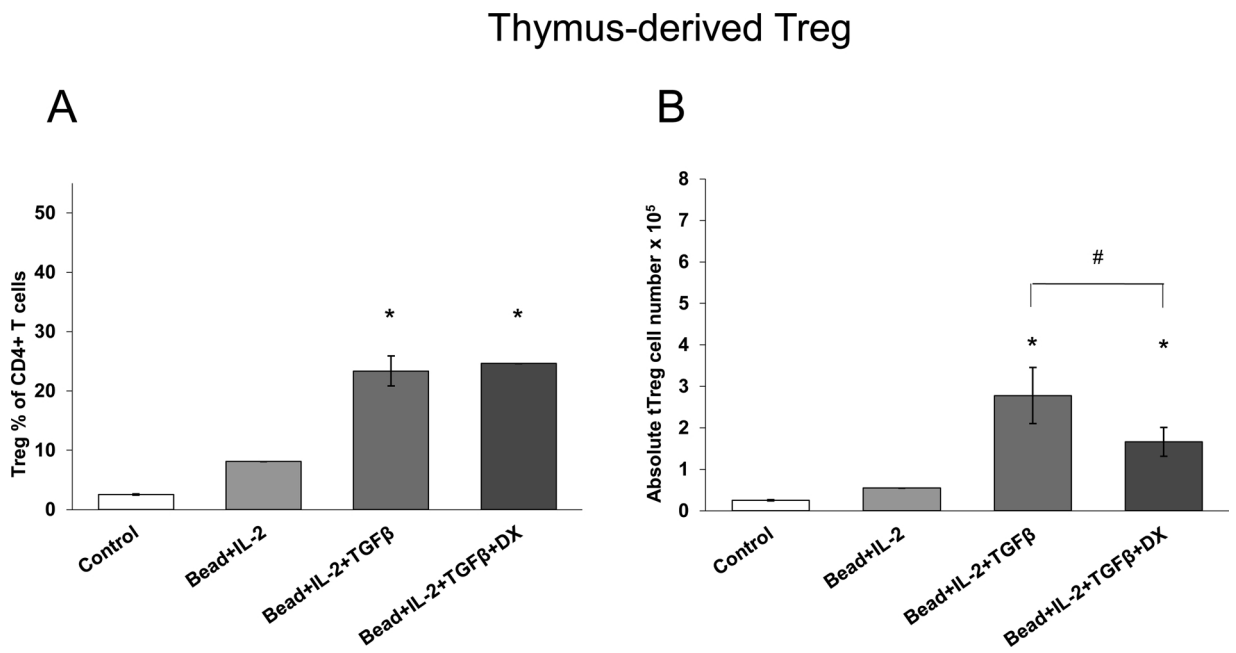

Fig. 2. Differentiation of Treg cells from thymic CD4$^+$ T cells after 3 days of *in vitro* stimulation. (A) The Treg ratios derived from thymic CD4$^+$ T cells increased after 3 days of *in vitro* expansion in the presence of anti-CD3/CD28 beads ± rIL-2 ± rTGFβ ± DX.
(B) The absolute thymic Treg cell number significantly increased after 3 days of *in vitro* differentiation in the presence of anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX. * indicates $p < 0.001$ between control (day 0) and differently treated samples, # indicates $p < 0.05$, between anti-CD3/CD28 beads + rIL-2 + rTGFβ with no DX and with DX treatment. The data are shown as mean ± SEM (n = 12).

treated with anti-CD3/CD28 beads in the presence of rIL-2, and rTGFβ. Addition of DX to the culture medium further increased the absolute spleen-derived iTreg number (Fig. 3B).

3.2. Presence of rTGFβ and dexamethasone upregulate Foxp3 expression in Treg cells in vitro

After flow cytometric measurements of Treg expansion, we wanted to assess the effect of DX on Foxp3 mRNA expression in *in vitro* differentiated Treg cells using quantitative real-time PCR analysis. In the thymic *in vitro* induced Tregs, Foxp3 mRNA expression showed significant elevation when cells were cultured together with the anti-CD3/CD28 beads, rIL-2 and rTGFβ. Addition of DX further increased the Foxp3 mRNA expression of iTreg cells (Fig. 4A). Similarly to the results observed in thymus-derived Treg cells we detected significant elevation of Foxp3 mRNA expression in the spleen-derived *in vitro* generated

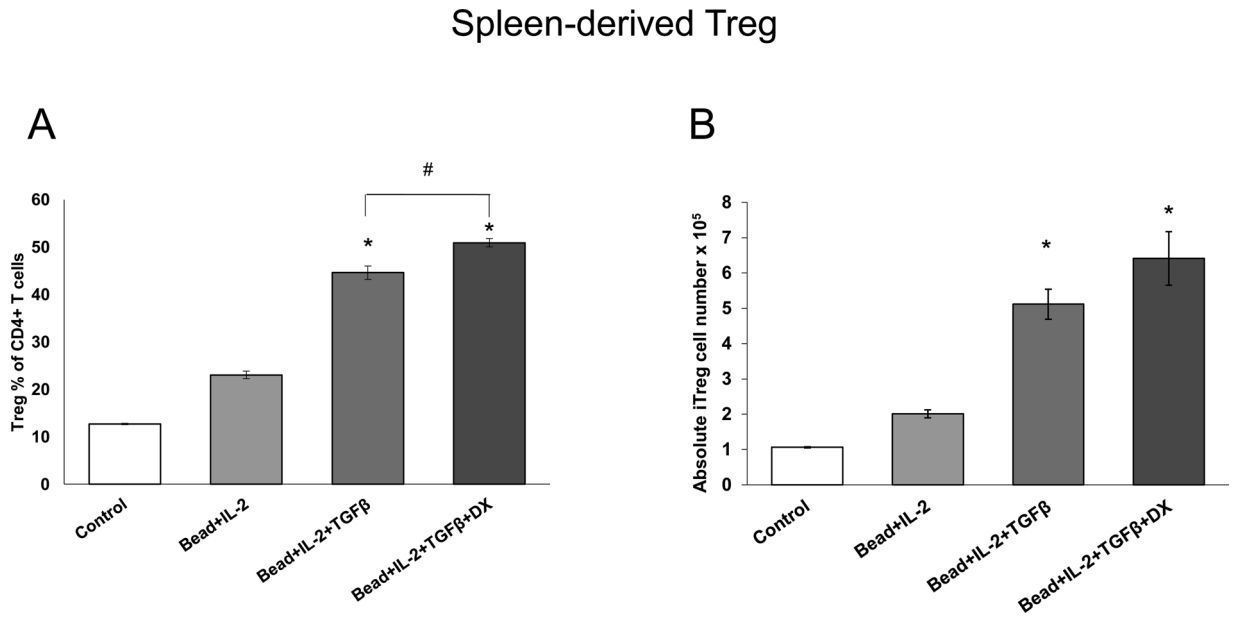

Fig. 3. Differentiation of Treg cells from splenic CD4$^+$ T cells after 3 days of *in vitro* stimulation. (A) The Treg cell ratios derived from splenic CD4$^+$ T cells increased after 3 days of *in vitro* treatments with anti-CD3/CD28 beads, rIL-2 and rTGFβ ± DX. (B) The absolute splenic iTreg cell number significantly increased after 3 days of *in vitro* differentiation in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX. * indicates $p < 0.01$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX treatments, # indicates $p < 0.05$, between stimulations in the presence or absence of DX. The data are shown as mean ± SEM (n = 12).

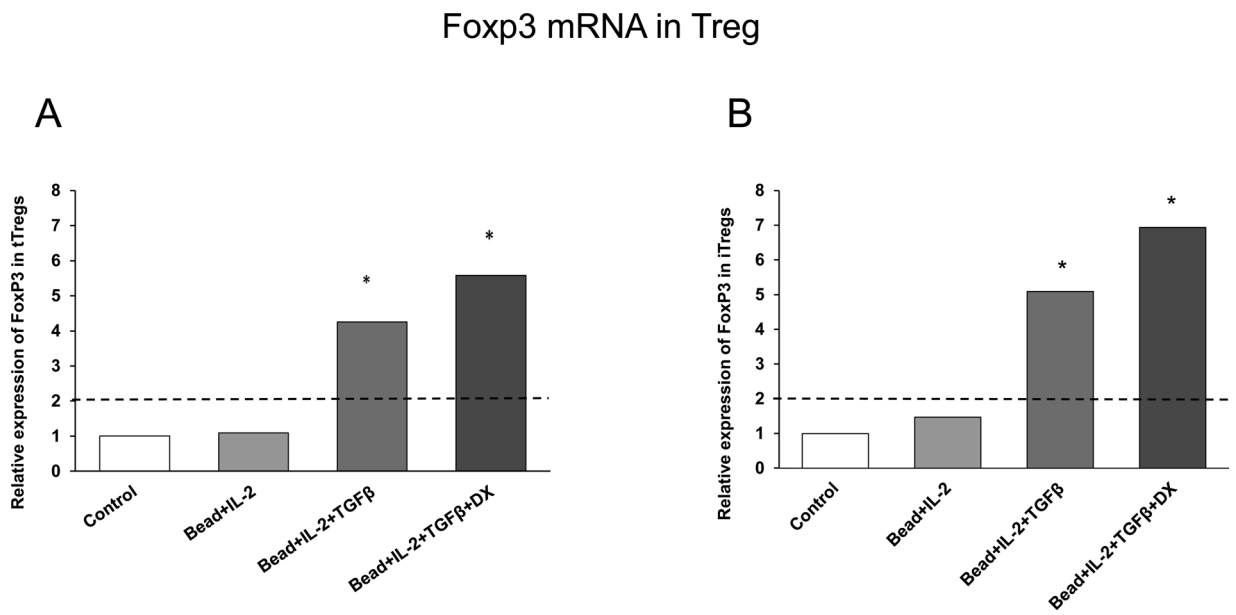

Fig. 4. Relative expression of Foxp3 mRNA in thymic and splenic Tregs after 3 days of *in vitro* differentiation. Both in thymic (A) and splenic (B) cells the relative expression of Foxp3 mRNA increased when stimulated in the presence of anti-CD3/CD28 beads + rIL-2 + rTGFβ, and futher increased in the presence of DX. * indicates $p < 0.05$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX treatments. The data are shown as mean ± SEM (n = 3).

iTreg cells, when TGFβ was present. DX further increased the Foxp3 mRNA expression *in vitro* (Fig. 4B).

3.3. Immunosuppressive cytokine production of in vitro differentiated Tregs

In order to assess the effect of various treatment conditions on the function of Treg cells, we first measured the immunosuppressive cytokines IL-10 and TGFβ produced by *in vitro* induced Tregs using intracellular detection by flow cytometry. We observed significantly higher IL-10 (9.5 ± 1.6%) and TGFβ (7.9 ± 1.2%) cytokine producing Treg ratios in anti-CD3/CD28 bead activated and differentiated thymic Tregs in the presence of rIL-2 and rTGFβ, compared to control freshly isolated tTreg cells (IL-10: 0.4 ± 0.1%; TGFβ: 1.4 ± 0.3%). We detected a further increase of TGFβ cytokine producing Tregs (12.4 ± 1.5%), when DX was present in the culture (Fig. 5A). We also investigated the relative mRNA expression of IL-10 and TGFβ cytokines in induced Tregs derived from thymic and splenic $CD4^+$ T cells after 3 days *in vitro* stimulation. We observed highly increased relative IL-10 mRNA expression in thymus-derived Tregs, when anti-CD3/CD28 beads and rIL-2 were present (Fig. 5B). Addition of rTGFβ and DX to the culture medium did not cause further increase in IL-10 mRNA level. The relative TGFβ mRNA expression increased significantly when DX was present together with anti-CD3/CD28 beads, rIL-2 and rTGFβ (Fig. 5C).

In freshly isolated, control splenic $CD4^+CD25^+Foxp3^+$ Treg cells the IL-10 producing Treg ratio was 3.6 ± 0.2%, and 2.5 ± 0.3% for TGFβ, detected by flow cytometry. After 3 days of *in vitro* expansion the immunosuppressive cytokine-producing spleen derived Treg ratio increased (IL-10: 16.9 ± 0.5% TGFβ: 19.7 ± 1.2%) when anti-CD3/CD28 beads and rIL-2 were present with a further elevation when rTGFβ was also added (IL-10: 25.1 ± 1.1% TGFβ: 30.3 ± 3.6%). A slight further increase could be detected in TGFβ production (IL-10: 25.9 ± 1.2%, TGFβ: 34.5 ± 3.1%) when DX was aslo added (Fig. 6A). Investigating the relative IL-10 mRNA expression in the spleen-derived Treg cells, we observed significant elevation by all treatment combinations, the greatest elevation was caused when anti-CD3/CD28 beads and rIL-2 were present (Fig. 6B). None of the culture treatments induced significant changes in the relative expression of TGFβ mRNA in the splenic iTregs (Fig. 6C).

3.4. In vitro Treg expansion did not cause Th1, Th2, Th17 cytokine production

In addition to the immunosuppressive cytokines (IL-10 and TGFβ), we also tested the ratios of Th1-type INFγ producing, Th2-type IL-4, and Th17-type IL-17 producing splenic $CD4^+CD25^+Foxp3^+$ Treg cells differentiated *in vitro* with anti-CD3/CD28 beads in the presence of rIL-2 ± rTGFβ ± DX. The IFNγ-producing Treg ratio was 1.02% in the control samples and was not detectable after *in vitro* Treg differentiation and expansion in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX. The IL-17 ratio was 0.72% in control and decreased after *in vitro* expansion in the presence of CD3/CD28 beads + rIL-2 ± rTGFβ ± DX. The IL-4 producing Treg ratio was 0.04% in the control sample and increased to 0.4% after *in vitro* stimulation with anti-CD3/CD28 beads + rIL-2 + rTGFβ, but was not detectable when DX was also added (Table 1).

3.5. Expansion of $CD4^+CD25^+$ splenic pTregs in vitro

To test the proliferation capacity of pTreg cells, we compared the *in vitro* expansion and differentiation of spleen-derived $CD4^+CD25^+$ and the $CD4^+CD25^-$ T cells into iTregs in the presence of anti-CD3/CD28 beads, rIL-2 and rTGFβ. We used a 6 days culture and tested the $CD4^+CD25^+Foxp3^+$ T cell ratio, absolute number and the cytokine production of the cells. The total cell number increased more then 10-fold in the case of $CD4^+CD25^-$ T cell stimulation, while this elevation was only 5.5-fold in the case of $CD4^+CD25^+$ T cell stimulation (Fig. 7A). Investigating the iTreg differentiation from the $CD4^+CD25^-$ cells, 90% of the cells started to express CD25, but 52% remained $CD25^{low+}$ and only 27% became $CD4^+CD25^{high+}Foxp3^+$ iTreg cells. Stimulation of $CD4^+CD25^+$ cells caused marked Treg expansion with Foxp3 expression in 74% of cells (Fig. 7B and 7D). The absolute iTreg number increased 20-fold in the case of $CD25^-$ T cell stimulation, and almost 40-fold when $CD25^+$ T cells were expanded (Fig. 7C). We also compared the cytokine production of the *in vitro* induced Treg subpopulations and we found higher TGFβ producing iTreg cell ratio in the $CD4^+CD25^+$ T cell-derived Treg group than in iTregs derived from $CD4^+CD25^-$ T cells (56.5% *vs* 50.3%). In contrast, IL-10 production was slightly more elevated in iTreg cells differentiated from the

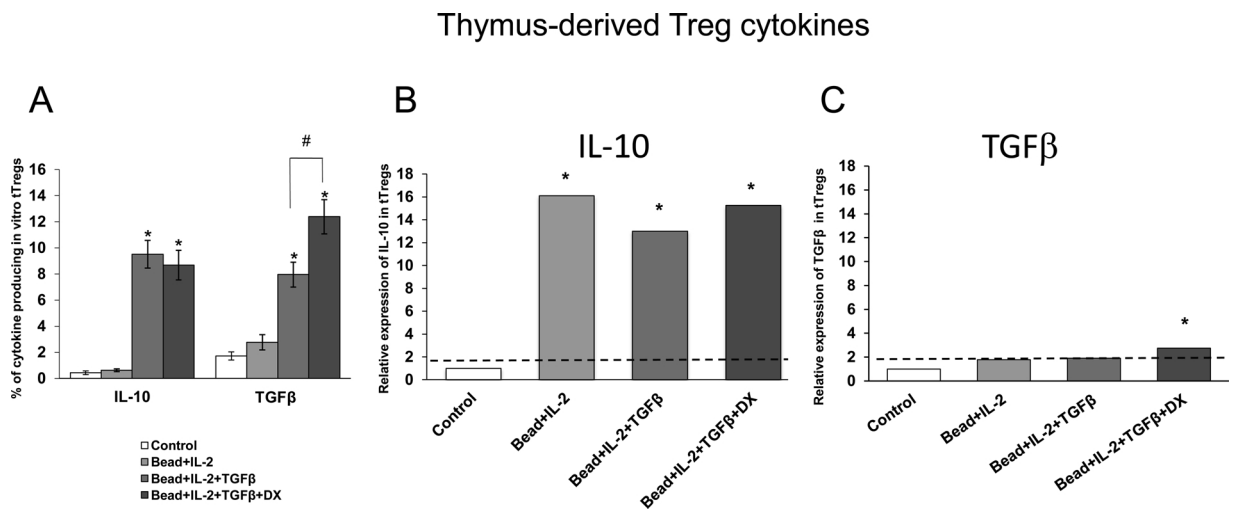

Fig. 5. Cytokine expression of thymic Treg cells after 3 days of *in vitro* stimulation. (A) Flow cytometric detection of IL-10 and TGFβ cytokine producing tTreg ratios showed significant elevation after stimulation with anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX. Treatment with DX resulted in further increase of TGFβ expression. * indicates $p < 0.001$ between control (day 0) and treatment with anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX. # indicates $p < 0.05$ between DX treated and untreated cultures. The data are shown as mean ± SEM (n = 8).
(B) Relative mRNA expression of IL-10 in tTregs increased significantly after 3 days of *in vitro* stimulation in all treatment conditions compared to the control (day 0). * indicates $p < 0.05$ between control (day 0) and treated cells. The data are shown as mean ± SEM (n = 4).
(C) Relative mRNA expression of TGFβ in tTregs increased after 3 days of *in vitro* stimulation with anti-CD3/CD28 beads + rIL-2 + TGFβ, only when DX was present. * indicates $p < 0.05$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 + rTGFβ + DX treatments. The data are shown as mean ± SEM (n = 4).

$CD4^+CD25^-$ T cells (41.5% *vs* 32.4%). Investigating the Th1/Th2/Th17 cytokine positivity of the iTreg cells, we can conclude, that none of these effector cytokines elevated after *in vitro* expansion of $CD25^-$ or $CD25^+$ T cells using anti-CD3/CD28 beads + rIL-2 + rTGFβ (Fig. 7E). Interestingly, in both iTreg groups we found an IL-10/TGFβ double-positive cell population (Fig. 7F).

3.6. Effect of in vivo DX pretreatment on Treg cell differentiation in vitro

In our previous studies we have investigated the effect of high-dose therapeutic GC hormone treatment on thymic tTreg and splenic iTreg cells that play a key role in physiological immunosuppression. We demonstrated that peripheral (*e.g.* spleen) Treg cells were sensitive to high-dose (20 mg/kg) DX treatment, in contrast, thymic Tregs were resistant. Both in the peripheral and thymic surviving Tregs, an increased IL-10 and TGFβ cytokine expression was observed (Ugor et al., 2018). In our current study, we tested how *in vivo* DX pretreatments of animals influence the *in vitro* Treg differentiation from $CD4^+$ T cells. First, we compared the $CD4^+CD25^+Foxp3^+$ Treg ratio in the thymus and spleen of DX-treated and control animals. *In vivo* 4 days of DX treatment resulted in significantly increased percentage of tTreg cells, whereas in the spleen an opposite effect was observed (Fig. 8).

To assess the funtional changes of *in vivo* DX-pretreated T cells, we determined the differentiation potential of $CD4^+$ T cells *in vitro* into induced Treg cells with the aim of generating higher numbers of Tregs. We used the same *in vitro* culture conditions as described above (anti-

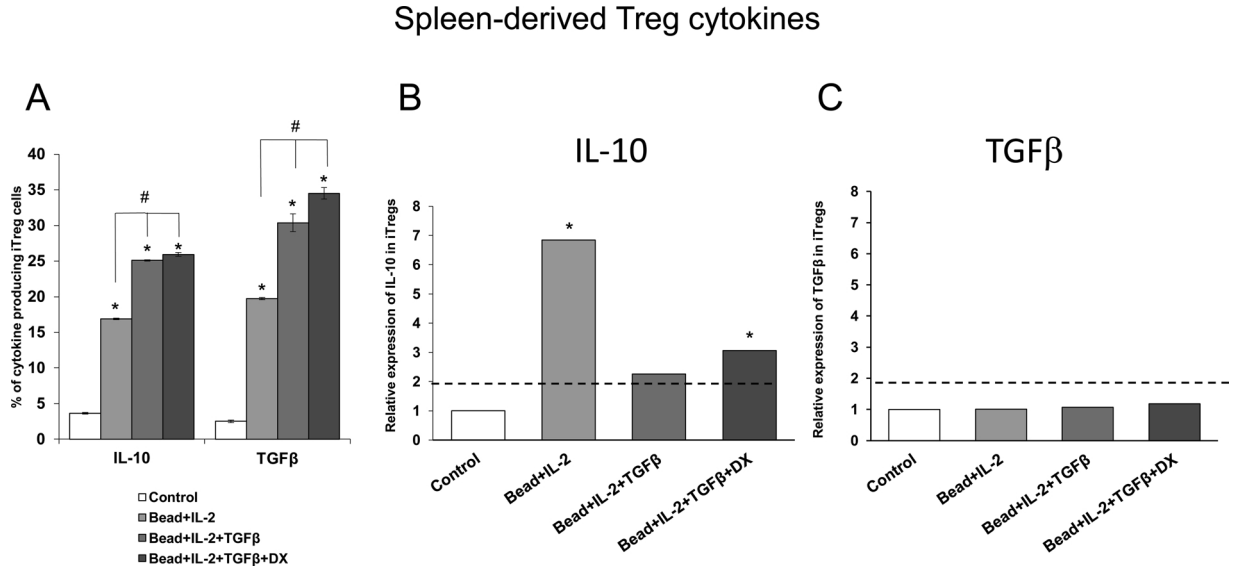

Fig. 6. Cytokine expression of splenic iTreg cells after 3 days of *in vitro* differentiation. (A) The IL-10 and TGFβ cytokine-producing splenic iTreg ratios were detected by flow cytometry in $CD4^+CD25^+Foxp3^+$ cells. The ratios of IL-10 and TGFβ producing cells increased significantly after each stimulation conditions. * indicates $p < 0.001$ between control (day 0) and anti-CD3/CD28 beads ± rIL-2 ± rTGFβ treated samples. # indicates $p < 0.05$ between anti-CD3/CD28 beads + rIL-2 in the presence or absence of rTGFβ ± DX treated cultures. The data are shown as mean ± SEM (n = 8).

Table 1
Ratios of cytokine-producing splenic iTreg cells after 3 days of *in vitro* differentiation.

Spleen	IL-10	TGFβ	IFNγ	IL-4	IL-17
Control	3.64 ± 0.1	2.52 ± 0.2	1.02 ± 0.1	0.04 ± 0.01	0.72 ± 0.02
Bead + rIL-2	16.9 ± 0.1	19.72 ± 0.1	0	0.3 ± 0.04	0.26 ± 0.04
Bead + rIL-2 + rTGFβ	25.1 ± 0.1	30.37 ± 1.9	0	0.4 ± 0.07	0.04 ± 0.01
Bead + rIL-2 + rTGFβ + DX	25.93 ± 0.4	34.52 ± 0.9	0	0	0.11 ± 0.02

The data are shown as mean ± SEM (n = 4).

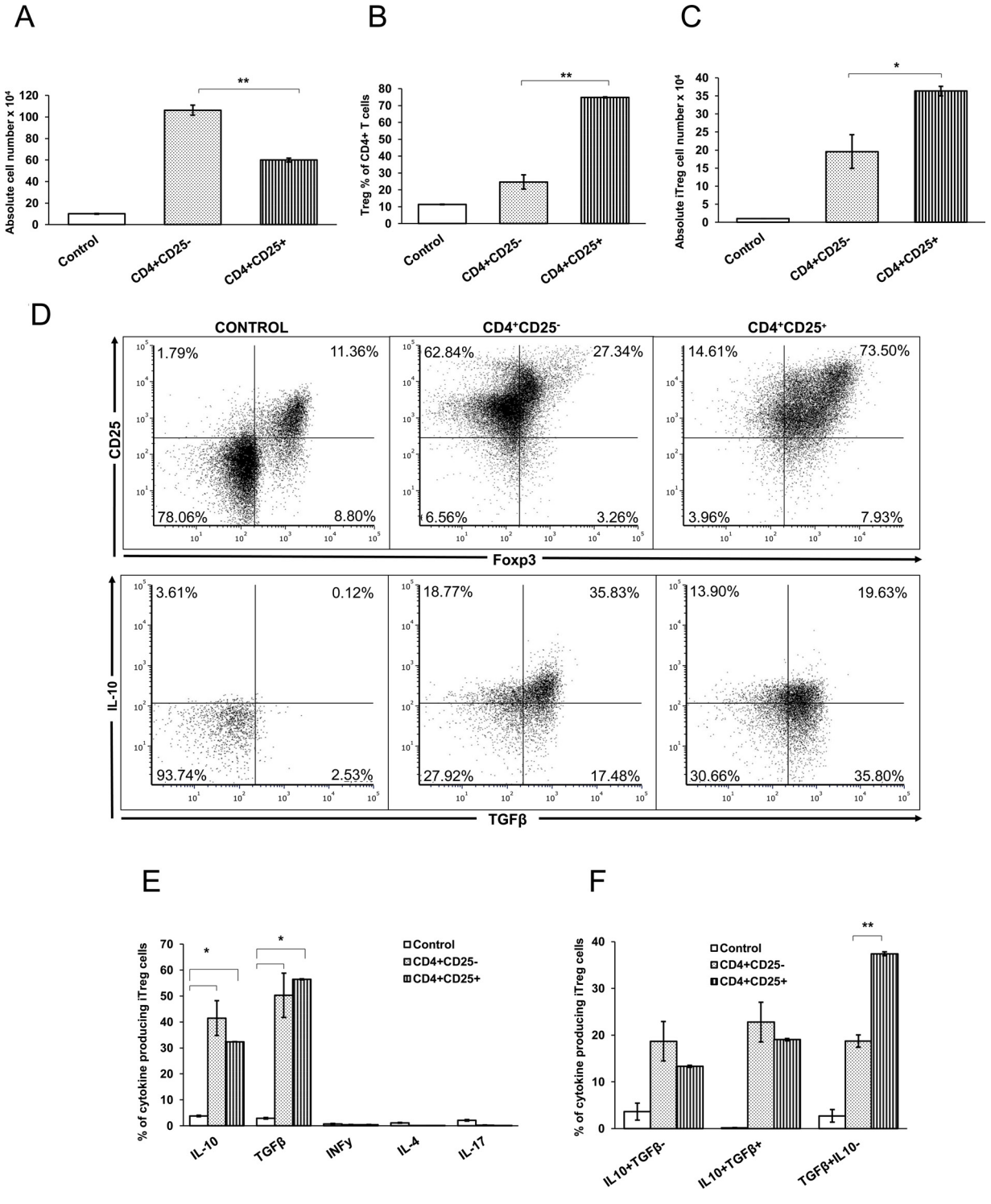

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Fig. 7. *In vitro* differentiation and cytokine production of splenic $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. (A) After 6 days of *in vitro* stimulation with anti-CD3/CD28 beads + rIL-2 and rTGFβ, the increase of the total cell numbers in samples derived from splenic $CD4^+CD25^-$ T cells was higher than in the $CD4^+CD25^+$ T cells, compared to the control (day 0). ** indicate significance at $p < 0.001$. The data are shown as mean ± SEM (n = 4).
(B) The ratios of iTreg cells derived from splenic $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells increased after 6 days of *in vitro* expansion, compared to the control (day 0). The highest ratio of Treg cells differentiated from $CD4^+CD25^+$ T cells. ** indicate significance at $p < 0.001$. The data are shown as mean ± SEM (n = 4).
(C) Absolute iTreg cell numbers derived from splenic $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells increased after 6 days of *in vitro* differentiation, compared to the control (day 0). * indicates significance at $p < 0.01$. The data are shown as mean ± SEM (n = 4).
(D) Representative flow cytometric plots of CD25 and Foxp3 labelled Treg cells in the $CD4^+$ T cell gate and their immunosuppressive cytokine (IL-10 and TGFβ) production after 6 days of *in vitro* differentiation with anti-CD3/CD28 beads + rIL-2 and rTGFβ. Left plots show the starting populations of negatively selected $CD4^+$ splenic T cells (control, day 0) that were separated into $CD25^-$ and $CD25^+$ cells and were stimulated. Upper middle ($CD4^+CD25^-$) and right ($CD4^+CD25^+$) plots show the CD25 and Foxp3 staining of differentiated cells after 6 days in culture. Lower panels show the IL-10 and TGFβ cytokine producing Treg ratios within the cell populations each from the upper right quadrants of the upper plots.
(E) Cytokine producing iTreg ratios after 6 days of *in vitro* differentiation. The IL-10 and TGFβ cytokine-producing splenic Treg ratios increased after 6 days of *in vitro* differentiation with anti-CD3/CD28 beads + rIL-2 and rTGFβ, compared to the control (day 0). The ratios of cells producing INFγ, IL-4 and IL-17 showed no increase after stimulation. * indicates significance at $p < 0.01$. The data are shown as mean ± SEM (n = 4).
(F) Immunosuppressive cytokine producing iTreg ratios after 6 days of *in vitro* differentiation. We detected increased ratios of IL-10 and TGFβ single positive and double positive cells in the Treg gate compared to the control (day 0). The ratio of TGFβ single positive Treg cells derived from $CD4^+CD25^+$ cells showed the greatest elevation after 6 days expansion. * indicates significance at $p < 0.01$. The data are shown as mean ± SEM (n = 4).

CD3/CD28 beads, rIL-2 ± rTGFβ ± DX) to stimulate the isolated $CD4^+$ T cells from the thymus and spleen of mice previously treated for 4 days with DX. In thymus-derived $CD4^+$ T cells, the *in vivo* high-dose of DX pretreatment caused increased expansion of Treg cells in the presence of anti-CD3/CD28 beads ± rIL-2 ± rTGFβ ± DX, compared to the $CD4^+$ T cells from untreated animals (Fig. 9A). In contrast to the thymic $CD4^+$ T cells, the *in vitro* differentiation of the splenic $CD4^+$ T cells into Tregs from DX pretreated animals resulted in less pronounced inscrease compared to the cells from control, untreated animals in all treatment conditions (Fig. 9B).

Since the thymus showed increased Treg cell ratios upon high-dose of DX pretreatment, we examined the Foxp3, IL-10 and TGFβ mRNA relative expression of thymus derived iTregs. The relative expression of Foxp3 mRNA increased by all *in vitro* treatment conditions in iTreg cells generated from the thymic $CD4^+$ T cells of the *in vivo* DX pretreated animals (Fig. 10A). Analysis of the functional activity of these Tregs showed that *in vivo* DX pretreatment resulted in decreased relative expression of immunosuppressive cytokines. This change was more roboust in the case of IL-10 mRNA expression (Fig. 10B), while the TGFβ mRNA relative expression showed little or no difference. Significant decrease of TGFβ mRNA relative expression was observed only in *in vitro* expanded Tregs receiving both *in vivo* and *in vitro* DX compared to control samples (Fig. 10C).

4. Discussion

Treg cells are crucial mediators of immune regulation and maintenance of tolerance, and as such, are key therapeutic targets in many diseases (Danikowski et al., 2017; Kawai et al., 2018; Li et al., 2018; Romano et al., 2017). Adoptive transfer of Treg cells is an emerging field with the aim of curbing unwanted, excessive or pathological immune reactions. For adoptive Treg cell therapies to be successful, well-characterized Treg cells of high potency, purity and yield will be required (Safinia et al., 2015; Vaikunthanathan et al., 2018). Important consideration in our experiments was the fact that Treg cells are at the forefront of research in immunology, and that glucocorticoid (GC) hormone analogues are among the most important drugs in the treatment of patients with inflammatory and autoimmune diseases, allergies, and patients with organ transplants and hematological malignancies (Cain and Cidlowski, 2017). However, despite considerable interest in both subjects, the knowledge on the effect GC analogues on Treg cells under *in vivo* and *ex vivo (in vitro)* conditions is relatively sparse. Only a handful of studies have been published data that deal with both subjects in an overarching manner (with seemingly contradictory results) (Chen et al., 2014, 2006; Hu et al., 2012; Olsen et al., 2015; Prado et al., 2011; Zhang et al., 2016).

We reported previously that thymic tTreg cells are resistant to

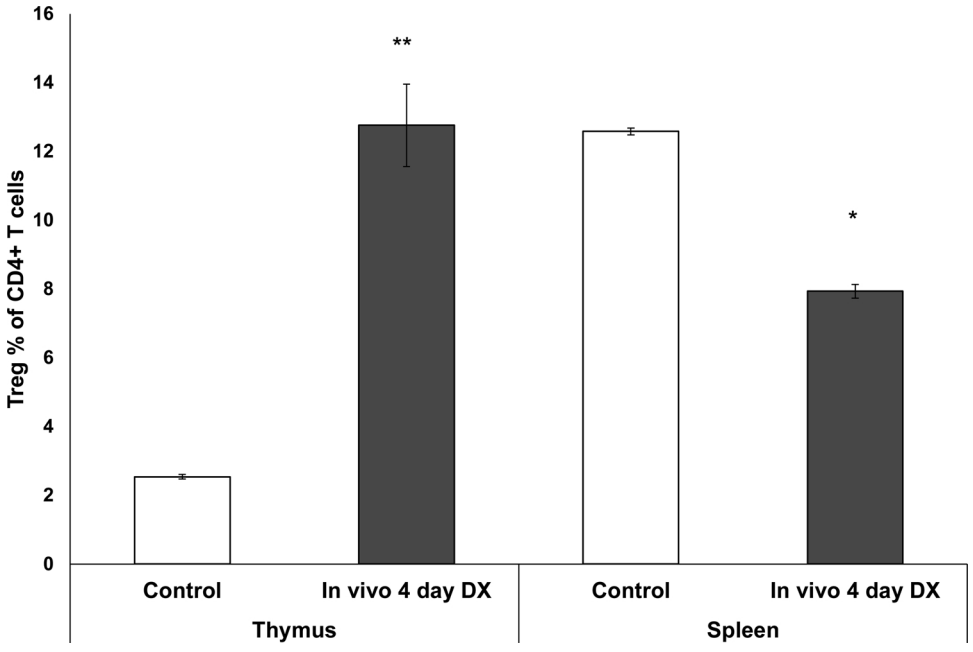

Fig. 8. Effect of *in vivo* DX treatment on the Treg ratios of thymic and splenic $CD4^+$ T cells. The Treg ratios of the thymic $CD4^+$ cells significantly increased after repeated (4 days) of high-dose (20 mg/kg) *in vivo* DX treatment, whereas the Treg ratios of splenic $CD4^+$ T cells significantly decreased. ** indicate $p < 0.001$, * indicates $p < 0.01$ between untreated control and DX-treated animals. The data are shown as mean ± SEM (n = 4).

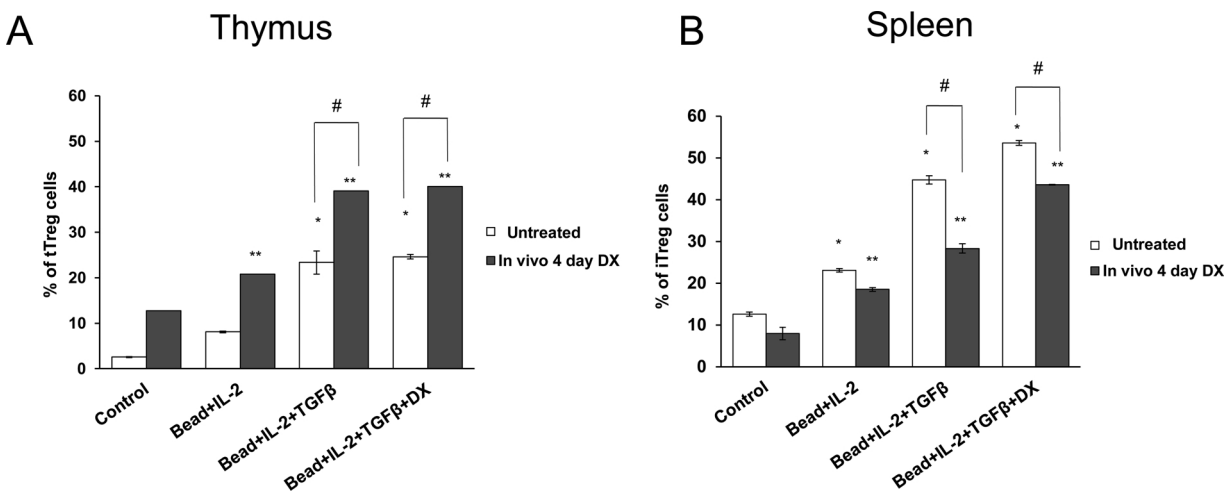

Fig. 9. Effect of *in vivo* 4 days DX pretreatment of mice on the *in vitro* expansion of thymic and splenic Treg cells.
(A) In the case of thymus-derived cells, 4 days of DX pretreatment of mice induced higher tTreg ratios when $CD4^+$ T cells were stimulated for 3 days *in vitro* in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX compared to the control (day 0).
(B) Effect of *in vivo* 4 days DX pretreatment of mice on the *in vitro* expansion of iTreg cells from splenic $CD4^+$ cells. The Treg ratios increased after 3 days of *in vitro* expansion in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX. The *in vivo* 4 days DX pretreatment resulted in lower Treg ratios derived from splenic $CD4^+$ cells in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX. * indicates $p < 0.001$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX treatments. ** indicate $p < 0.01$ between *in vivo* 4 day DX pretreated samples stimulated with anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX compared to the starting control (day 0). # indicates $p < 0.05$ between *in vivo* DX pretreated and untreated animals, when cells were expanded in the presence of anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX. The data are shown as mean ± SEM (n = 4).

repeated high dose *in vivo* DX treatment and that both thymic and splenic Treg cells produce increased levels of immunosuppressive cytokines IL-10 and TGFβ after DX treatment of mice (Ugor et al., 2018). These effects were accompanied by elevated expression of Foxp3 mRNA, which suggested a stronger Treg lineage commitment as a result of DX treatment. Therefore, the primary goal of our current study was to define the conditions under which Treg cells can be robustly expanded *in vitro*, especially the effect of TGFβ and DX when $CD4^+$ thymic and splenic T cells are stimulated under conventional conditions with anti-CD3/CD28 beads and rIL-2. We investigated the expansion, lineage commitment (Foxp3 expression) and immunosuppressive cytokine production, factors important in determining the yield and functionality of Treg cells. We found significant increases in Treg cell percentages and absolute numbers in both thymus- and spleen-derived Treg cells as a result of *in vitro* stimulation with anti-CD3/CD28 beads and IL-2 when TGFβ + DX were both present (Fig. 2 and 3). The inclusion of DX in our model might be relevant in gaining further insight into the complex role of GC hormones in the immune system (Bereshchenko et al., 2018; Cain and Cidlowski, 2017). In this context, Hippen et al. reported large *ex vivo* expansion of human natural regulatory T cells (Hippen et al., 2011). They used human nTregs purified from peripheral blood and employed repetitive cycles of restimulation using cell-based artificial antigen presenting cells (aAPCs) in the presence of rapamycin. We obtained a more modest expansion rate, but it

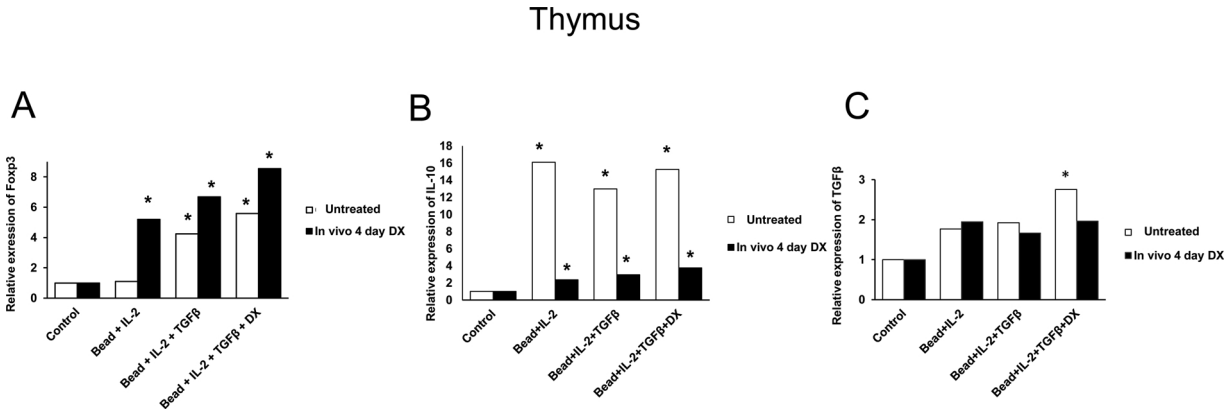

Fig. 10. Relative expression of Foxp3, IL-10, and TGFβ mRNA in control and *in vivo* 4 day DX treated tTreg cells after 3 days of *in vitro* differentiation. (A) The relative expression of Foxp3 mRNA in thymic Tregs increased after 3 days of *in vitro* expansion in the presence of anti-CD3/CD28 beads + rIL-2 ± rTGFβ ± DX in cells derived from untreated animals and futher increased in samples derived from DX pretreated mice. * indicates $p < 0.05$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX treatments in samples from both DX pretreated and untreated animals. The data are shown as mean ± SEM (n = 4).
(B) The relative expression IL-10 mRNA in both untreated and *in vivo* 4 day DX treated thymic Tregs increased after 3 days of *in vitro* expansion in the presence of anti-CD3/CD28 + rIL-2 + rTGFβ ± DX, compared to control (day 0). *In vivo* DX pretreatment resulted in substantial decrease in the IL-10 mRNA levels under all subsequent *in vitro* treatment conditions. * indicates $p < 0.05$ between control and anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX treatments and 4 day *in vivo* DX treated control and anti-CD3/CD28 beads + rIL-2 + rTGFβ ± DX treatments. The data are shown as mean ± SEM (n = 4).
(C) The relative expression TGFβ mRNA in thymic Tregs did not change after 3 days of *in vitro* expansion, except for the sample from DX untreated animals in the presence of anti-CD3/CD28 beads + rIL-2 + rTGFβ + DX. 4 days of *in vivo* DX pretreatment did not affect the TGFβ mRNA expression of *in vitro* expanded cells. * indicates $p < 0.05$ between control (day 0) and anti-CD3/CD28 beads + rIL-2 + rTGFβ + DX treatments. The data are shown as mean ± SEM (n = 4).

should be noted that the two results are hard to compare, since we used a mouse model, and cells were derived from the thymus and spleen, and the stimulation conditions were also different.

Foxp3 mRNA levels increased as a result of treatment with IL-2 + TGFβ and showed further increase when DX was added. These data suggest that differentiation of thymic and splenic $CD4^+$ T cells toward the Treg phenotype can be promoted by treatment with TGFβ + DX under *in vitro* conditions (Fig. 4). The effect of IL-2 + TGFβ on the polarization of naïve $CD4^+$ T cells towards the Treg phenotype has been well established (Apert et al., 2018; Safinia et al., 2015; Sanjabi et al., 2017). IL-2 has been used in clinical settings to treat various autoimmune diseases and transplant patients, where it has been shown to lead to *in vivo* expansion of Treg cells (Koreth et al., 2011; Saadoun et al., 2011; Ye et al., 2018). Despite significant knowledge on the effect of TGFβ on the immune system under physiological and pathological conditions, the direct clinical use of TGFβ in patients has been less explored (there have been reports of few ongoing clinical trials in rheumatoid arthritis and osteoarthritis). What mechanism may mediate the DX-induced generation of Treg cells? In this context the crosstalk between GC hormone and TGFβ signaling may be relevant. Bereshchenko et al reported that glucocorticoid-induced leucine zipper (GILZ), a protein induced by GCs, promoted Treg cell production (Bereshchenko et al., 2014). Based on their data, the authors suggested a model in which GCs induce translocation of glucocorticoid receptor to the nucleus, leading to synthesis of GILZ that cooperates with and enhances TGFβ signaling *via* Smad2 phosphorylation, nuclear translocation of the Smad complex and Foxp3 expression. Our data on the combined effect of TGFβ and DX on elevated Treg cell production and Foxp3 expression are consistent with this model. The elevated expression of Foxp3 as a result of TGFβ and DX treatment in our study is noteworthy since Foxp3 gene expression predicts stable transcriptional commitment to Treg cell phenotype. Foxp3 is becoming widely recognized as a shaper of Treg cell epigenome by its association with molecules that mediate epigenetic modifications, which affect activation or silencing of multiple Foxp3 target genes (Lu et al., 2017).

We tested the production of key immunosuppressive cytokines (IL-10, TGFβ) by the Treg cells. Both thymus- and spleen-derived Treg cells showed increased relative ratios of cells producing IL-10 and TGFβ, when treated with combinations of IL-2, TGFβ, and DX (except for thymus-derived Tregs, when treated with IL-2). The relative levels of IL-10 mRNA showed an increase, when compared to control cell cultures (treatment with anti-CD3/CD28) (except for spleen-derived cells, when cultured with IL-2 + TGFβ). The TGFβ mRNA relative level increased significantly only in thymus-derived Tregs, when treated with anti-CD3/CD28 + IL-2 + TGFβ + DX (Figs. 5 and 6). Earlier we reported that *in vivo* DX treatment of mice resulted in increased ratios of IL-10 and TGFβ-producing Treg cells both in thymus and spleen (Ugor et al., 2018). We hypothesize that GC hormone, when used *in vivo* or *in vitro*, results a stronger Treg commitment with elevated expression of not only the Foxp3 transcription factor, but also their immunsuppressive cytokines IL-10 and TGFβ. This may help such Tregs to establish an anti-inflammatory milieu by modulation of DCs and effector lymphocytes. These events may promote the formation of additional Tregs from precursor cells and amplify the Treg network in a feed-forward manner (Kendal and Waldmann, 2010; Milward et al., 2017).

We compared the proliferative potential of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, and tested the diffrentiation of these cells into $CD4^+CD25^+Foxp3^+$ iTregs *in vitro* in the presence of anti-CD3/CD28 beads, rIL-2 and rTGFβ. We found that both $CD4^+CD25^+$ pTregs and $CD4^+CD25^-$ T cells showed robust proliferation in response to stimulation for 6 days (5.5-fold and 10-fold, respectively) (Fig. 7A). Regarding iTreg differentiation from $CD4^+CD25^-$ cells, the majority (90%) of these cells expressed CD25, of which 52% remained $CD25^{low+}$, and only 27% differentiated into $CD4^+CD25^{high+}Foxp3^+$ iTreg cells. Conversely, stimulation of $CD4^+CD25^+$ cells resulted in Treg expansion, with $CD25^{high+}$ and Foxp3 expression in 74% of cells (Fig. 7B and D). Stimulation of $CD25^-$ T and $CD25^+$ T cells resulted in ~20-fold and ~40-fold increase in absolute number of iTreg cells, respectively (Fig. 7C). These data are in agreement with earlier reports on the conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^{high+}Foxp3^+$ iTreg cells *in vitro* in the presence of IL-2 and TGFβ (Chen et al., 2003; Dons et al., 2012; Zheng et al., 2002, 2007). Of note, Zheng et al reported that $CD4^+CD25^+$ Treg cells generated *ex vivo* could educate $CD4^+$CD25- T cells to become $CD4^+CD25^+Foxp3^+$ Treg cells, a process that was dependent on IL-2 and TGFβ (Zheng et al., 2004). We hypothesize that such an effect could occur in our *in vitro* model system in which the newly-generated $CD4^+CD25^+$ T cells may promote the conversion of $CD4^+CD25^-$ to $CD4^+CD25^+$ cells during stimulation with anti-CD3/CD28, rIL-2 and rTGFβ, and thus constituting a feed-forward loop of iTreg generation. We found that iTreg cells generated from both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells expressed significant levels of IL-10 and TGFβ, but they produced little or no Th1/Th2/Th17 cytokines (IFNγ, IL-4, and IL-17), which is consistent with data reported previously (Zheng et al., 2002).

Our experiments on the effect of *in vivo* pretreatment of mice with high-dose DX showed that the ratio of Treg cells among $CD4^+$ T cells increased in the thymus, but it decreased in the spleen (Fig. 8). This suggests higher resistance of thymic than splenic Treg cells to DX-induced cell death, which is consistent with our earlier data (Ugor et al., 2018). GC hormones are known to be produced locally in the thymus (Talaber et al., 2015), thus developing thymocytes are exposed to GCs *in vivo*. Therefore, it can be hypothesized that the relative resistance of thymic Tregs to DX may be due to selection of thymic Tregs towards a more GC-resistant phenotype. We also found elevated *in vitro* differentiation of thymic T cells into Treg cells (under all *in vitro* stimulation conditions) from mice that had been pretreated with DX for 4 days, when compared to non-treated (control) mice, whereas in the spleen-derived cells, an opposite effect was observed (Fig. 9). This suggests that a population of thymic T cells might be poised towards Treg phenotype, as reflected in their Foxp3 expression (perhaps by local production of GCs in the thymus, as mentioned above), which upon *in vivo* DX pretreatment may become more strongly committed to the Treg lineage. However, regarding the expression of Foxp3, IL-10, and TGFβ mRNA in thymus-derived, *in vitro* expanded Treg cells, a more complex picture emerged; we observed an increase of Foxp3, and decrease IL-10 mRNA levels (respectively), as a result of DX pretreatment of mice (Fig. 10A and 10B). Regarding TGFβ mRNA, DX pretreatment of mice did not cause significant change under any of the subsequent *in vitro* treatment conditions, except in the case of *in vitro* treatment with anti-CD3/CD28 + rIL-2 + rTGFβ + DX, where we observed decreased TGFβ mRNA level (Fig. 10C). These results may reflect the heterogeneity and plasticity of the Treg lineage (Sawant and Vignali, 2014).

Taken together, our data provide further insight into the effects of GC hormones on the regulatory arm of the immune system. The GC analogue DX promotes the expansion of thymic and splenic Treg cells, and enhances $Foxp3^+$ expression and the production of immunosuppressive cytokines IL-10 and TGFβ *in vitro*. Pretreatment of mice with DX differentially affects the subsequent *in vitro* expansion of Treg cells (increased expansion of thymic and decreased expansion of splenic Treg cells). This suggests that patients who receive GC therapy deserve special attention prior to *in vitro* expansion of Treg cells. Finally, GCs may become important drugs in optimization of therapeutic Treg cells.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

This work was supported by research grants from the Hungarian National Scientific Research Fund, OTKA K 105962 and by GINOP-232-

15-2016-00050 and EFOP361-16-2016-00004 grants.

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