Clinical Immunology (2013) 149, 400–410

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

# Regulatory and effector T-cells are differentially modulated by Dexamethasone ☆

Julieta Pandolfi [a,b], Plácida Baz [a], Pablo Fernández [a], Ailén Discianni Lupi [a,b], Florencia Payaslián [a], Luis Ariel Billordo [a], Leonardo Fainboim [a,b], Lourdes Arruvito [a,b,*]

[a] Institute of Immunology, Genetics and Metabolism (INIGEM), Clinical Hospital, University of Buenos Aires, National Council for Scientific and Technological Research, Buenos Aires, Argentina
[b] Department of Microbiology, School of Medicine, University of Buenos Aires, Argentina

Received 16 July 2013; accepted with revision 30 September 2013

**KEYWORDS**
Regulatory T cells;
Effector T cells;
Apoptosis;
Glucocorticoids;
Cytokines

**Abstract** It is assumed that the ratio between effector T cells (Teff) and regulatory T cells (Tregs) controls the immune reactivity within the T-cell compartment. The purpose of this study was to investigate if Dexamethasone (Dex) affects Teff and Tregs subsets. Dex induced on Tregs a dose and time-dependent apoptosis which resulted in a relative increase of Teff. After TCR activation, Dex induced a strong proliferative inhibition of Teff, but a weaker proliferative inhibition on Tregs. These effects were modulated by IL-2, which not only restored the proliferative response, but also prevented Dex-induced apoptosis. The highest dose of IL-2 prevented apoptosis on all FOXP3 + CD4+ T cells. Meanwhile, the lowest dose only rescued activated Tregs (aTregs), probably related to their CD25 higher expression. Because Dex did not affect the suppressor capacity of aTregs either, our results support the notion that under Dex treatment, the regulatory T-cell compartment maintains its homeostasis.


## 1. Introduction

Regulatory T cells (Tregs) have been characterized as CD4+ T cells expressing CD25, FOXP3 and very low amounts of CD127. This low expression of CD127 allows differentiation of Tregs from naive and memory conventional T cells.

As recently reported, FOXP3 + CD4+ T cells include three phenotypic and functionally distinct cellular subpopulations. Two of them having *in vitro* suppressive activity, were

*Abbreviations:* Tregs, regulatory T cells; rTregs, resting Tregs; aTregs, activated Tregs; Teff, effector T cells; GC, glucocorticoids; Dex, Dexamethasone; GR, glucocorticoid receptor; APCs, antigen presenting cells; geoMFI, geometric mean fluorescence intensity.

☆ This work was supported by grants from ANPCyT (PICT Bicentenario 2010 n° 0392 and PICT 2011 n° 1367) and a grant from UBA (UBACyT n° 0361).

* Corresponding author at: Institute of Immunology, Genetics and Metabolism (INIGEM), Clinical Hospital, University of Buenos Aires, National Council for Scientific and Technological Research. Av. Córdoba 2351 (1120), Buenos Aires, Argentina. Fax: +54 11 5950 8758.
*E-mail address:* lourarruvito@yahoo.com.ar (L. Arruvito).

1521-6616/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.clim.2013.09.008

characterized as FOXP3$^{low}$CD45RA + resting Treg cells (rTregs) and FOXP3$^{high}$CD45RA- activated Tregs (aTregs). A third subset of FOXP3$^{low}$CD45RA- cells was found to be a cytokine-secreting cell population without suppressor activity, and was identified as FOXP3+ non-Tregs [1,2].

Under physiological conditions, the magnitude of the immune reactivity within the T-cell compartment is largely proportional to the ratio between effector T cells (Teff) and Tregs. In this context, an impaired function and/or homeostasis of Tregs have implications in the development of several common autoimmune or inflammatory diseases.

However, Tregs not always operate under physiological conditions, and the immune state might be altered by drugs frequently used in the medical practice, some of them having cytotoxic activity.

Glucocorticoids (GC) are potent anti-inflammatory and immunosuppressive agents used in the treatment of numerous autoimmune and inflammatory diseases. GC mediate their biological effects through the binding to an intracellular receptor that translocates to the nucleus and targets specific DNA sequences resulting in the blockage of several inflammatory pathways [3,4] and the induction of apoptosis [5]. These events lead to the inhibition of T cells [6,7] and/or altering the function of dendritic cells [8,9].

The sensitivity of Tregs to steroid drugs such as Prednisone and Dexamethasone (Dex) has been the subject of conflicting data. In the murine system, some studies reported that Dex increased the proportion of Tregs, both in peripheral blood and secondary lymphoid organs [10,11]. However, additional studies in mice models of asthma [12] or multiple sclerosis [13] indicated that GC induced a decrease in the number of Tregs. In humans, a first report [14] claimed that the treatment of patients with bronchial asthma with GC (either systemic or inhaled) induced the increase of circulating Tregs. Later on, several small *in vivo* studies also pointed out towards there being a positive correlation between the administration of GC and the frequency of Tregs in patients with different autoimmune diseases [15–17]. By contrast, two recently larger studies performed in patients with bronchial asthma or autoimmune connective tissue diseases, arrived at the opposite conclusion [18,19]. These contradictory results may have at least two possible explanations. Firstly, there is a large heterogeneity in the characterization of Tregs. Several studies only defined Tregs as being CD4+ CD25$^{high}$, but it is well known that many of these cells represent activated T cells instead of Tregs [20–22]. Secondly, most of these studies were performed in patients with an autoimmune background and it is likely that these individuals already had an impairment of Tregs frequency and/or function.

Tregs differ in many aspects from cytotoxic Teff. The sensitivity of Tregs to apoptosis is of outmost importance to sustain the equilibrium between effector and suppressor forces. Tregs have a variable sensitivity to apoptosis which is influenced by factors such as the cytokines secreted within the inflammatory environment, the type of antigenic stimulation and the proliferation rates [23].

Our study provides evidence that during the course of an immune response, GC exert differential effects on both, effector and regulatory T cells by inducing a strong inhibition of the proliferation of Teff and a differential apoptosis of Tregs. Finally GC effects can be modulated by IL-2, which even in very low amounts could differentially impact on Tregs survival and function.

## 2. Material and methods

### 2.1. Subjects

Buffy coat was obtained from 1 unit of blood collected from 15 male and 15 non-pregnant healthy female donors (average 34 years, range 27–42 years), and processed immediately after volunteer's donations. Infectious and endocrine disorders were cleared in all patients, who were not undergoing any kind of treatment. This study has been approved by the Investigation and Ethics Committee at the Hospital de Clínicas "José de San Martín" and informed consent was obtained from all donors.

### 2.2. Peripheral blood mononuclear cell (PBMCs) isolation

PBMCs were obtained from buffy coats through a Ficoll–Hypaque (GE Biosciences) density gradient centrifugation.

### 2.3. Cell sorting

CD4+ T cells were purified by negative selection by using CD4+ T cell MACS beads (Miltenyi Biotec), following manufacturer's instructions. Different subsets of FOXP3 + CD4+ T cells and Teff were isolated by staining purified CD4+ T cells with anti-CD4 PerCP, anti-CD25 PE and anti-CD45RA FITC antibodies (all from BD Biosciences) and sorting with a FACSAria II Flow cytometer (Becton Dickinson), yielding four populations: CD25$^{low}$CD45RA + (rTregs), CD25$^{high}$CD45RA− (aTregs), CD25$^{low}$CD45RA− (FOXP3+ non-Tregs), CD25− (Teff). Cells were collected into RPMI 1640 medium (Hyclone) plus 50% heat-inactivated fetal calf serum and washed once for further studies. The expression of FOXP3 in sorted cells determined by Flow cytometry was detected in >90% of aTregs, >80% of rTregs and FOXP3+ non-Tregs, and in less than 0.5% of Teff.

### 2.4. Cell lines

HeLa human cervix adenocarcinoma cells were maintained in complete culture medium following the recommendations from the American Type Culture Collection.

### 2.5. Flow cytometry

Freshly isolated or *in vitro*-cultured cells were stained with anti-CD4 (PerCP or APC), anti-CD25 (PE or APC-Cy7), anti-CD45RA (PE-Cy7, APC or FITC), and anti-CD127 (PE), all from BD Biosciences. Intracellular detection of FOXP3 with anti-FOXP3 (PE or Alexa Fluor 488), Ki-67 antigen with anti-Ki-67 (FITC) and BCL-2 with anti-BCL-2 (FITC) antibodies was performed on fixed and permeabilized cells following the manufacturer's instructions. Negative control samples were incubated with an isotype-matched mAb. Data was acquired using a FACSAria II (Becton Dickinson) and was analyzed with FlowJo software. Statistical analyses are based on at least 100,000 events gated on the population of interest.

### 2.6. Intracellular staining for detection of Glucocorticoid receptor (GR)

1–2 × $10^6$ PBMCs were fixed and permeabilized by using the BD Pharmingen™ Human FOXP3 Buffer set (BD Biosciences). Cells were incubated with a purified mouse anti-human GR α isoform mAb (clone 41/GR, BD Biosciences) or its isotype control during 1 h at RT. Then, cells were washed twice with PBS and GR mAb binding was detected using PE-goat anti-mouse-IgG (Dako) and washed once as described above. Intracellular detection of FOXP3 and surface markers were performed according to the manufacturer's instructions. Data was analyzed using a FACSAria II cytometer.

### 2.7. Apoptosis assay

Preliminary experiments were performed to identify the optimal concentration of Dex and the incubation time that achieved the best compromise between minimizing spontaneous cell death and maximizing Dex-induced apoptosis. The rate of spontaneous apoptosis varied between different donors.

An incubation time of 24 h was considered optimal as this time point was short enough for the untreated control cells to remain sufficiently viable, yet long enough to observe significant and discriminatory Dex-induced cell death.

The lowest concentration of Dex that induced close-to-maximal killing at all time points was 1 × $10^{-7}$ M. This concentration was therefore adopted as the standard for most experiments, unless otherwise indicated.

Briefly, 1 × $10^6$ PBMCs were cultured in complete culture medium at different time points (6, 12, 24, and 48 h) in a 48 multiwell plate (GBO), in the absence or presence of different concentrations of Dex (1 × $10^{-5}$ M, 1 × $10^{-7}$ M, and 1 × $10^{-9}$ M; Sigma-Aldrich). Because expression of CD127 correlates inversely with FOXP3 expression on Tregs [21,22], we used this molecule as a surface marker to discriminate between FOXP3 + CD4+ T cells and activated FOXP3-CD4+ T cells in those experiments in which we needed to analyze cell viability/death. In order to discriminate between live and apoptotic cells, PBMCs were labeled with anti-CD4, anti-CD127, anti-CD25 and anti-CD45RA antibodies. CD4 + CD127+ cells were gated as CD25− (Teff). CD4 + $CD127^{-/low}$ cells were gated as $CD25^{low}CD45RA$ + (rTregs), $CD25^{high}CD45RA$− (aTregs), and $CD25^{low}CD45RA$− (FOXP3+ non-Tregs). Percentage of apoptosis was determined by using FITC-Annexin V and 7-AAD, respectively (BD Biosciences) and analyzed with a FACSAria II cytometer using FlowJo software. To investigate whether the apoptotic effect of Dex could be antagonized by GR antagonist, cells with Dex (1 × $10^{-7}$ M) were incubated in the presence or absence of RU 486 (RU; Sigma-Aldrich) at 1 × $10^{-6}$ M for 24 h. Results were analyzed as described above.

### 2.8. Cultures of PBMCs in the presence of IL-2

In some experiments cells were activated with anti-CD3 (1.2 ug/ml, Beckman Coulter) and CD28 (1 ug/ml; BD Pharmingen), and Dex (1 × $10^{-7}$ M) was added at the beginning of the culture or after 24 h, when levels of IL-2 secreted by PBMCs could be detected. After 72 h, the proliferation of different subsets was analyzed by using the Ki-67 staining.

In other experiments, PBMCs or sorted cells were incubated with different concentrations of IL-2 (100, 10 or 0.1 ng/ml, PeproTech) in presence or absence of Dex (1 × $10^{-7}$ M) for 12 or 24 h. Then cells were collected and analyzed by Flow cytometry or RT-PCR.

### 2.9. Quantitative RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen) and subjected to reverse transcription using Improm-II Reverse Transcriptase (Promega). PCR analysis was performed with a real-time PCR detection system (Mx3000P, Stratagene) using SYBR Green as fluorescent DNA binding dye. The primer sets used for amplification were: BCL-2-F: 5′-ttgagttcggtggggtcatg-3′; BCL-2-R: 5′-acagttccacaaaggcatcc-3′; BAX-F: 5′-gctctgagcagatcatgaag-3′; BAX-R: 5′-tgagacactcgctcagcttc-3′; GCCR-F: 5′-ttcctctgagttacacaggc-3′; GCCR-R: 5′-gtcagttgataaaaccgctgc-3′; GAPDH-F: 5′-cgaccactttgtcaagctca-3′; GAPDH-R: 5′-ttactccttggaggccatgt-3′. All primer sets yielded a single product of the correct size. Relative expression levels were normalized against GAPDH.

### 2.10. Suppression assay

The suppressive capacity of cell sorting-isolated aTregs was assayed as described [24]. In brief, fix numbers of purified Teff (5 × $10^4$) were cultured with autologous Antigen Presenting Cells (APCs, 3 × $10^4$ cells, obtained from CD3-depleted PBMCs) and decreasing numbers of sorted autologous aTregs (2.5 × $10^4$, 1.25 × $10^4$ and 0.625 × $10^4$). Cells were stimulated with anti-CD3/CD28, and cultured in a 96-well U-bottom plate for 5 days. Cell proliferation was measured by [$^3$H] thymydine uptake (Perkin ElmerLife).

To evaluate the effect of Dex on suppressor capacity, aTregs were pre-incubated during 12 h or 24 h with Dex (1 × $10^{-7}$ M) or medium; afterwards cells were washed twice and used in the suppression assay.

Effector T cells co-cultured with APCs and stimulated with anti-CD3/CD28 were used as a control to measure baseline proliferation before suppression by aTregs (maximum response). Teff co-cultured with APCs only were included as an autologous control.

### 2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. Two groups were compared using the Wilcoxon signed-rank test. Three or more groups were compared using the Kruskall–Wallis test or Friedman test followed by Dunn multiple comparison tests, for unpaired samples or paired samples respectively. A p value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Dex modulates FOXP3 + CD4+ T cell frequency in a dose- and time-dependent manner

To investigate the effect of Dex on Teff and Tregs, PBMCs from 10 healthy donors were cultured in the presence or

absence of Dex at $1 \times 10^{-7}$ M. At different time points (24, 48 or 72 h), cells were collected and the different subsets of FOXP3 + CD4+ T cells (rTregs, aTregs, and FOXP3+ non-Tregs) and FOXP3-CD4+ T cells (Teff) were analyzed by Flow cytometry after staining with a combination of anti-CD4, anti-CD45RA and anti-FOXP3 antibodies as described in [2].

Treatment with Dex during 72 h induced a significant decreased frequency of all FOXP3 + CD4+ T cells ($p < 0.01$) accompanied by a significant relative increase of Teff frequency ($p < 0.01$; Fig. 1A and B.) The analysis on each FOXP3 + CD4+ T cell subset revealed that a significant reduced frequency was detected in all three subsets: rTregs ($p < 0.05$), aTregs and FOXP3+ non-Tregs ($p < 0.01$, Fig. 1C).

Knowing that FOXP3 + CD4+ T cells and Teff differ in functional and phenotypic features [25–27], we first compared the proliferative status of each subpopulation by measuring the expression of Ki-67. As previously reported by us and others [1,2] at the steady state, Ki-67 is detected in almost half of aTregs, but hardly detected in rTregs, FOXP3+ non-Tregs or Teff. The proliferative capacity of Teff or FOXP3 + CD4+ T cells was not affected by the addition of Dex during 24 h to unstimulated PBMCs (data not shown).

Trying to explain the decreased frequency of FOXP3 + CD4+ T cells, we next investigated how the dose and time of exposure to Dex affect the viability of the different subsets of CD4+ T cells. PBMCs were cultured in the presence of decreasing doses of Dex ($1 \times 10^{-5}$ M, $1 \times 10^{-7}$ M, and $1 \times 10^{-9}$ M) or medium alone at several time points (6, 12, 24 and 48 h), and then stained with 7-AAD/FITC-Annexin V. Because expression of CD127 correlates inversely with FOXP3 expression on Tregs [21,22], we used this surface marker to discriminate between unfixed FOXP3 + CD4+ T cells and Teff and to perform the apoptosis assay.

Apoptosis was almost exclusively observed on FOXP3+ CD4+ T cells, with negligible effect on Teff ($p < 0.01$, $n = 10$; Fig. 2A and B). Moreover, the analysis on each subset of FOXP3 + CD4+ T cell showed that aTregs were the subpopulation more susceptible to apoptosis induced by Dex ($p < 0.0001$, Fig. 2C).

Six independent experiments showed a significant dose-dependent increase of the cell death of all FOXP3 + CD4+ T cell subsets exposed during 24 h to Dex (Fig. 2D). This effect was also observed in Teff exposed to increasing doses of Dex.

Additionally, Dex effect was time dependent. Apoptosis was already detected as early as 12 h, reaching significant levels of apoptosis on aTregs and FOXP3+ non-Tregs after 24 h of exposure to Dex ($p < 0.05$). After 48 h, all FOXP3 + CD4+ T cell subsets showed a high level of apoptosis ($p < 0.01$). At 48 h, a significant level of apoptosis was also detected in Teff, but as expected, it was lower than the one observed in FOXP3 + CD4+ T cells (Fig. 2E).

Hence, apoptotic mechanisms contribute to Dex-induced decreased frequency of FOXP3 + CD4+ T cells. The higher sensitivity to Dex-induced apoptosis detected on aTregs may be related to their faster basal turnover rates.

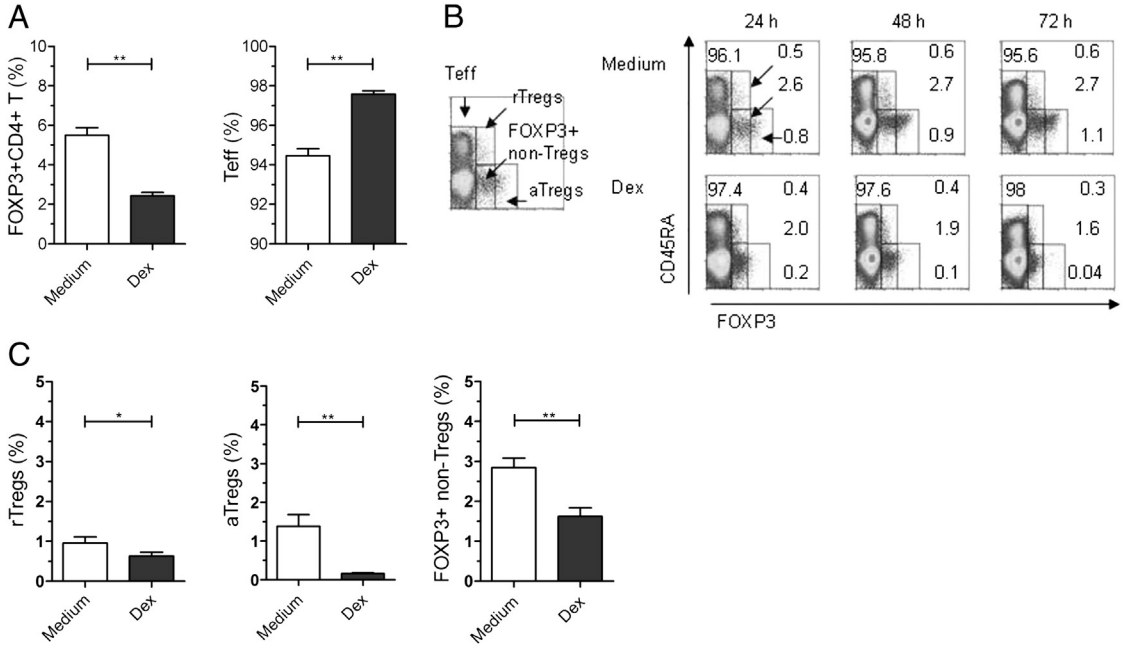


**Figure 1** Differential effect of Dex on the frequency of FOXP3 + CD4+ T cells and Teff. PBMCs (n = 10) were cultured for 24, 48 or 72 h in the absence or presence of Dex ($1 \times 10^{-7}$M). Based on the expression of CD45RA and FOXP3, four subsets of CD4+ T cells were defined by Flow cytometry: FOXP3$^{low}$CD45RA + (rTregs), FOXP3$^{high}$CD45RA− (aTregs), FOXP3$^{low}$CD45RA− (FOXP3+ non-Tregs) and FOXP3− (Teff). (1A) Shows the relative frequency of FOXP3 + CD4+ T cells and Teff after 72 h of culture. (1B) Representative dot plots show the different subsets of FOXP3 + CD4+ T cells and Teff during the culture. (1C) Shows the relative frequency of rTregs, aTregs and FOXP3+ non-Tregs after 72 h of Dex treatment. Data is presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

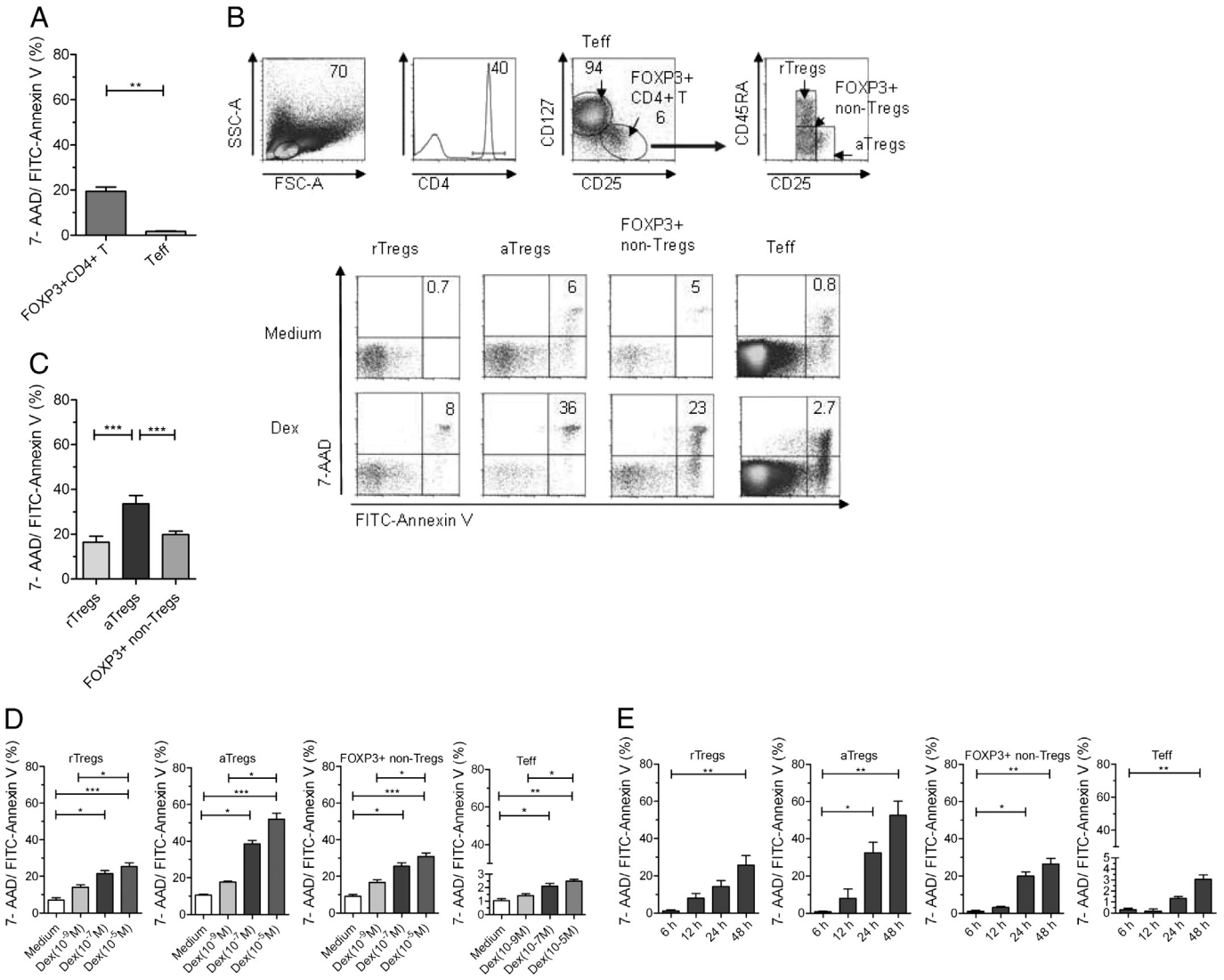


**Figure 2** FOXP3 + CD4+ T cells are more susceptible to Dex induced apoptosis. PBMCs (n = 10) were cultured in the absence or presence of Dex (1 x$10^{-7}$M) during 24 h. The assay discriminates between live and apoptotic cells labeled with anti-CD4, anti-CD127, anti-CD25 and anti-CD45RA antibodies. CD4 + $CD127^{-/low}$ cells were gated as $CD25^{low}CD45RA$ + (rTregs), $CD25^{high}CD45RA-$ (aTregs), and $CD25^{low}CD45RA-$ (FOXP3+ non-Tregs); CD4 + CD127+ cells were gated as CD25− (Teff). (2A) Shows the % of apoptosis in all FOXP3 + CD4+ T cells and Teff in the presence of Dex. (2B) Representative dot plots show the strategy of gating and the % of cell death detected on rTregs, aTregs, FOXP3+ non-Tregs and Teff after 24 h of incubation with Dex. (2C) Shows the % of cell death in the different subsets of FOXP3 + CD4+ T cells in the presence of Dex. (2D) Shows the apoptotic dose dependent effect of Dex in each subset of FOXP3 + CD4+ T cells and Teff (n = 6). (2E) Shows the apoptotic time dependent effect of Dex in each subset of FOXP3 + CD4+ T cells and Teff (n = 6). Data is presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

### 3.2. IL-2 induces FOXP3 + CD4+ T cell resistance to steroids and prevents Dex-induced apoptosis

IL-2 has been shown to be able to rescue T cells from apoptosis induced by a variety of stimuli, and has the capacity to create a state of steroid resistance [28–30]. It is known that Dex antagonizes TCR-induced IL-2 production [31–33]. Hence, in the next set of experiments the impact of IL-2 on Dex-mediated effects on CD4+ T cells was investigated.

The proliferative response of PBMCs stimulated during 72 h with anti-CD3/CD28 was evaluated in the presence of Dex ($1 \times 10^{-7}$ M) added at t = 0 or at t = 24 h post activation, having assumed that IL-2 had already been secreted [34]. The response was measured as Ki-67 expression and showed that Dex added at t = 0 h induced more than 40% of inhibition of Teff proliferative capacity, which differed from the 23% observed on rTregs, 11% on FOXP3+ non-Tregs, and non inhibition induced on aTregs (n = 6, Fig. 3A and B). In contrast, the addition of Dex at t = 24 h did not affect the proliferative capacity of FOXP3 + CD4+ T cells, and reduced but did not abrogate the inhibitory effect induced on Teff proliferation.

IL-2 secreted during the next 24 h that follow T-cell activation might represent the contribution exerted by this cytokine to maintain the homeostasis altered by the use of Dex during the course of an immune response.

In addition, this cytokine also prevented apoptosis induced by Dex ($1 \times 10^{-7}$ M) in all FOXP3 + CD4+ T cells. This effect was variable depending on the dose of IL-2 used. Fig. 3C, shows that the highest dose of IL-2 (100 ng/ml) prevented apoptosis induced by Dex on all FOXP3 + CD4+ T cells ($p < 0.01$, n = 5). Moreover, a smaller dose (10 ng/ml) was able to rescue aTregs and FOXP3+ non-Tregs ($p < 0.05$) and the lowest one (0.1 ng/ml) was sufficient to rescue aTregs (probably related to their higher expression of CD25; $p < 0.05$; Fig. 3D). By contrast, any dose of IL-2 was able to rescue the marginal apoptosis induced by Dex on Teff.

### 3.3. Altered balance between BAX and BCL-2 influences the intrinsic susceptibility to apoptosis of aTregs

Because the cellular response to signals of death or proliferation is modulated by members of the BCL-2 protein family

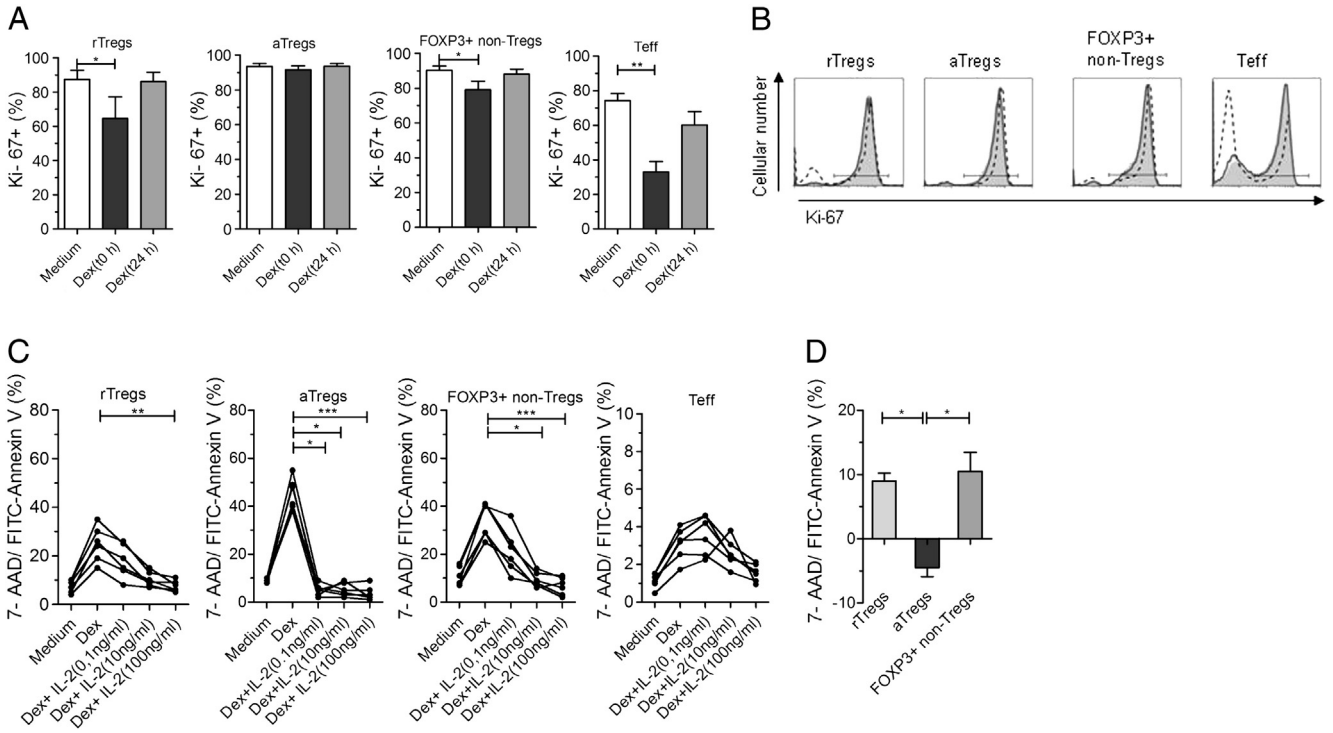


**Figure 3** Dex effects on FOXP3 + CD4+ T cells and Teff can be differentially modulated by IL-2. (3A) PBMCs from six independent donors were activated with anti-CD3/CD28 during 72 h and Dex ($1 \times 10^{-7}$M) was added at the beginning of the culture (t = 0) or after 24 h of cell activation (t = 24). The expression of the proliferation marker Ki-67 for each cell subset was analyzed by flow cytometry. Figure shows the % of Ki-67 expressed in each FOXP3 + CD4+ T cell subset and Teff. (3B) Representative histograms of experiments performed as described in 3A. Figure shows Ki-67 expression in each CD4+ T subset in the absence of Dex (filled histogram) or in the presence of Dex (continuous line represents Dex added at t = 24 and dotted line represents Dex added at t = 0). (3C) Resting PBMCs were incubated with 100, 10 or 0.1 ng/ml of IL-2 and cultured in the presence of Dex ($1 \times 10^{-7}$M) during 24 h. Percentage of apoptosis was analyzed as described in Fig. 2 and shows the % of cell death in all subsets of FOXP3 + CD4 + T cells and Teff. (3D) Shows the % of cell death in all FOXP3 + CD4 + T cells in the presence of Dex and 0.1 ng/ml of IL-2 after subtraction of the corresponding controls (cells cultured in medium only). Data is presented as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

[35–37], a comparison has been drawn among the constitutive expression of the anti-apoptotic protein BCL-2 in the different subsets of CD4+ T cells. BCL-2 was expressed in both FOXP3 + CD4+ T cells and Teff. However, the geometric mean fluorescence intensity (geo MFI) was significantly lower in those cells expressing FOXP3 ($p < 0.05$; Fig. 4A), with the lowest geo MFI detected on aTregs ($p < 0.01$; Fig. 4B). These results suggested that BCL-2 might be one of the factors controlling the survival of aTregs.

After T cell activation, the expression of BCL-2 is known to be maintained by the secretion of IL-2 [38,39]. Additionally, previous studies have demonstrated that IL-2 can induce steroid resistance in T cells [30,40]. In the present study, the addition of 10 ng/ml of IL-2 to PBMCs exposured to Dex ($1 \times 10^{-7}$ M) restored BCL-2 geo MFI, both in aTregs ($p < 0.01$) and FOXP3+ non-Tregs ($p < 0.05$, Fig. 4C). These findings suggested that the increased expression of BCL-2 induced by IL-2 could be implicated in the inhibition of Dex-induced apoptosis in these subsets.

We next compared the relative expression of mRNA of two BCL-2 family members (BAX and BCL-2). Sorted cells were cultured during 12 h in the presence of medium, Dex, Dex plus IL-2 or IL-2 and the relative expression of BAX and BCL-2 mRNA was quantified by RT-PCR. Samples of seven different individuals were analyzed and BAX mRNA was detectable in six of them showing marked variability among subjects. As depicted in Fig. 4D, in comparison with BCL-2, the basal expression of BAX in aTregs was higher.

Because an appropriate balance between pro- (BAX) and anti-(BCL-2) apoptotic proteins are required to sustain cell survival, the BAX/BCL-2 ratio was analyzed in the different culture conditions as mentioned above. As depicted in Fig. 4E, the ratio of BAX/BCL-2 in cells maintained only in medium showed a higher ratio in aTregs. Unexpectedly, Dex treatment did not increase the ratio BAX/BCL-2 in all samples analyzed. Moreover, the addition of Dex plus IL-2 or IL-2 alone induced a decreased ratio of BAX/BCL-2 on aTregs and FOXP3 non-Tregs, as a consequence of the increase in BCL-2 (not shown). This decreased ratio was not detected on rTregs or Teff.

Collectively, these data suggest that the constitutive higher BAX/BCL-2 ratio of aTregs may be responsible for their cell death rate in the presence of a pro-apoptotic stimulus such as Dex. Moreover, in the presence of low doses of IL-2 aTregs are capable of maintaining an appropriate balance between pro- (BAX) and anti-(BCL-2) apoptotic proteins, and sustaining survival by a mechanism that is independent of Dex.

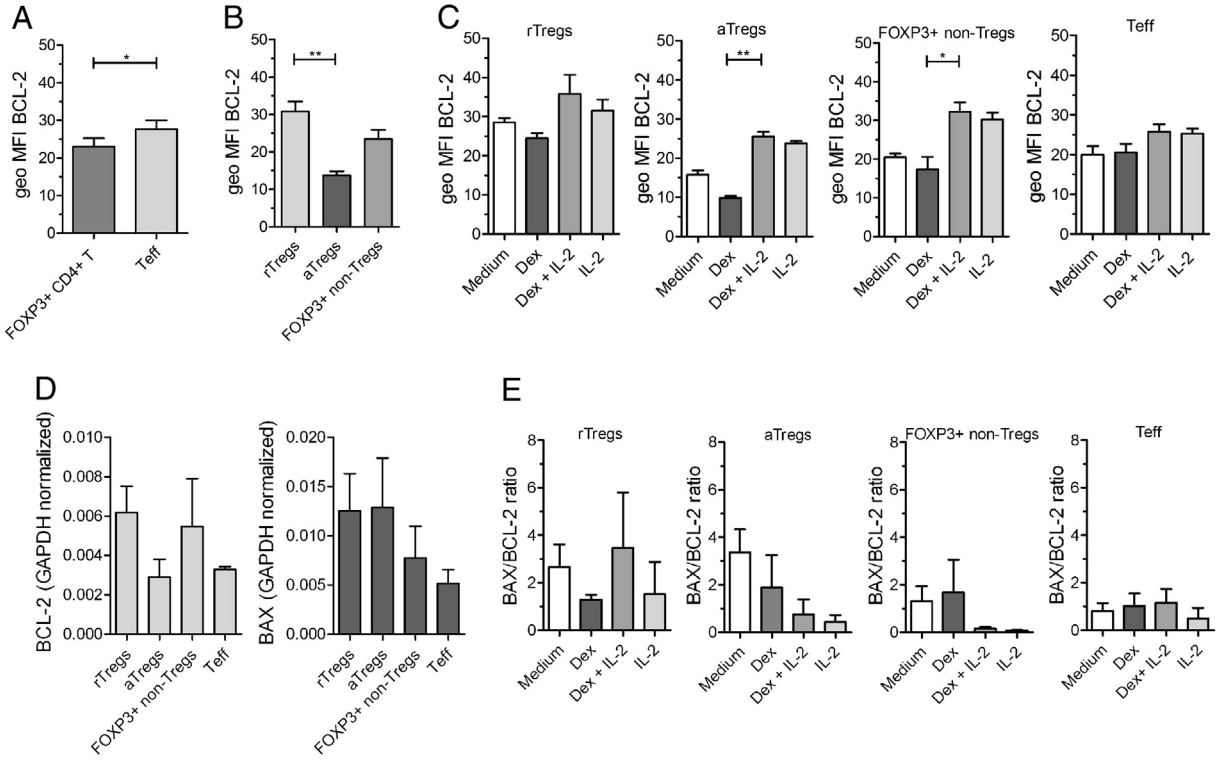


**Figure 4** The balance between BAX and BCL-2 influences the intrinsic susceptibility to apoptosis of aTregs. (4A) PBMCs from 6 different donors were analyzed for the constitutive geo MFI expression of BCL-2 in all FOXP3 + CD4+ T cells and Teff. (4B) Shows the constitutive geo MFI expression of Bcl-2 in rTregs, aTregs and FOXP3+ non-Tregs. (4C) PBMCs were cultured in the presence of Dex ($1 \times 10^{-7}$M), IL-2 (10 ng/ml), Dex plus IL-2 or medium during 24 h (n = 4). Geo MFI of BCL-2 was analyzed in the different subsets of CD4+ T cells. (4D) Shows the constitutive expression of BAX and BCL-2 mRNA in sorted cells from 6 different donors. (4E) Shows the BAX/BCL-2 ratio of sorted cells cultured during 12 h in the presence of Dex, IL-2, Dex plus IL-2 or medium. The relative expression levels of BAX and BCL-2 were normalized against GAPDH. Data is presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

### 3.4. Mifepristone, an antagonist of GR overcomes Dex-induced apoptosis

The effects of GC hormones are mediated by an intracellular GR located in the cytoplasm, which upon ligand-induced activation, translocates to the nucleus where it can initiate transcription of specific target genes. To evaluate if Dex induced-apoptosis was mediated by GR, the ability of a GR antagonist (Mifepristone or RU 486; RU) to block the hormone effect was studied. PBMCs were cultured for 24 h in the presence of Dex ($1 \times 10^{-7}$ M), RU ($1 \times 10^{-6}$ M) or Dex plus RU. As depicted in Fig. 5A, RU was able to inhibit apoptosis induced by Dex on all FOXP3 + CD4+ T cells (Dex vs. RU plus Dex, $p < 0.001$, n = 9). On the contrary, the addition of RU did not affect cell viability in the absence of Dex, as no difference between the apoptosis level was found between RU and medium alone. This demonstrates that Dex has a pro-apoptotic effect directly on cells mediated by binding to GR.

The presence of GR was assessed by FACS and RT-PCR. As a positive control the cervix cancer cell line HeLa was used, as it expresses high GR levels. By Flow cytometry GR was detected on most of the cervix cancer cell line (94.85% ± 0.85) and was also highly expressed (range 82 to 96 %) on FOXP3 + CD4+ T cells and Teff from 6 different individuals (Fig. 5B). We next performed RT-PCR on each purified cell subset and confirmed that the α GR isoform was expressed at similar levels in all CD4+ T cell subsets, (Fig. 5C) indicating that differences in GR expression do not account for a higher susceptibility to Dex-induced apoptosis.

### 3.5. Dex does not affect the suppressor function of aTreg cells

Human Tregs must be activated through their TCR to be functionally suppressive [41–44]. Activated Tregs maintain their suppressor activity after being fixed with paraformaldehyde [45], indicating that once activated, they do not need to be viable to mediate suppression. This is in line with the observation that aTregs remain highly suppressive after becoming apoptotic following *in vitro* TCR stimulation [1].

As described in [2], Dex pro-apoptotic effect was confirmed by Tripan Blue staining on purified aTregs by cell sorting and exposed to Dex for 24hs (Fig. 6A, n = 5).

To study how this short time exposure to Dex could impact on its suppressor ability, a classic three cell suppression assay was performed as previously described in [2]. The assay included isolated aTregs exposed to $1 \times 10^{-7}$ M Dex (Dex-treated aTregs) for 12 h or 24 h. After being extensively washed to remove any presence of Dex, cells were co-cultured with purified Teff supplemented with autologous APCs. Fixed numbers of Teff were co-cultured with decreasing numbers of Dex-untreated aTregs or Dex-treated aTregs to reach decreasing ratios of Teff: aTregs (2:1, 4:1 and 8:1). Because Dex impacts on Teff proliferative capacity, only isolated aTregs were exposed to Dex treatment. After 12 or 24 h of exposition to Dex, and at all ratios evaluated, no significant differences between the suppressor ability of Dex-treated or untreated aTregs were found. (Fig. 6B, n = 5).

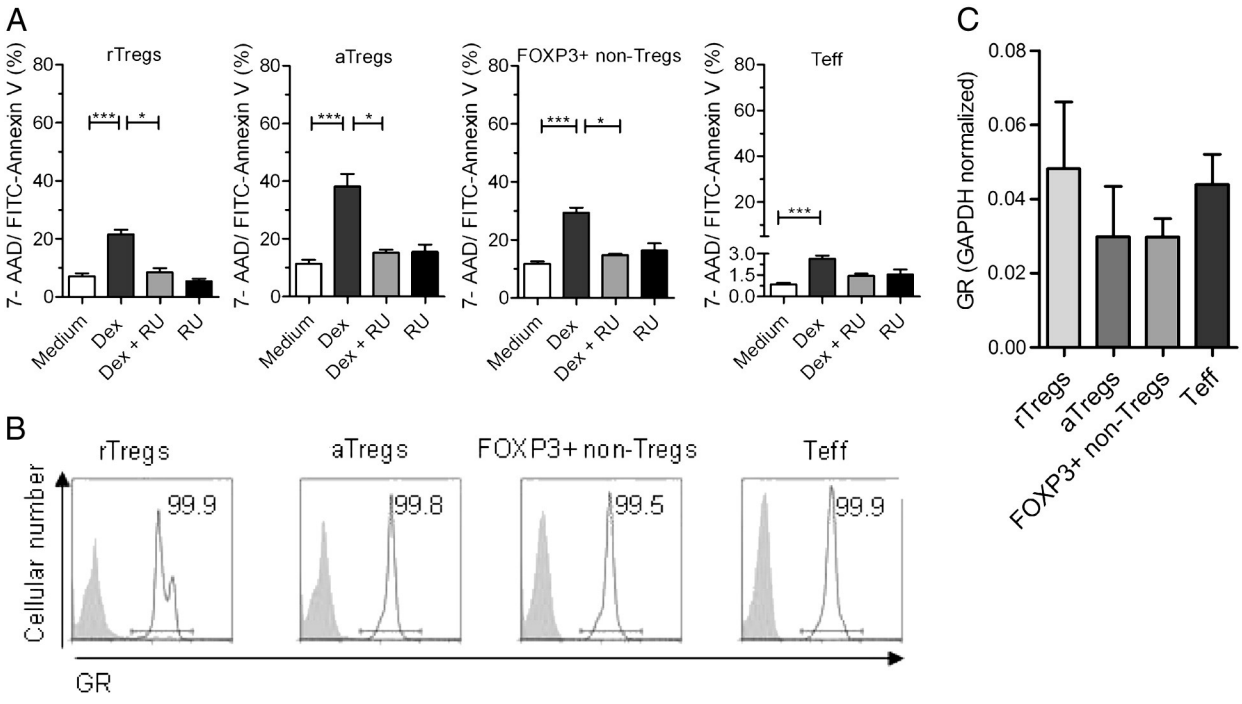


**Figure 5** FOXP3 + CD4+ T cells and Teff express similar levels of GR. (5A) PBMCs were cultured in medium, Dex ($1 \times 10^{-7}$M), RU ($1 \times 10^{-6}$M) or Dex plus RU during 24 h. Percentage of cell death in rTregs, aTregs, FOXP3+ non-Tregs and Teff was analyzed as described in Fig. 2 (n = 9). (5B) Histograms showing the GR expression (open histogram) or an isotype control (filled histogram) in each CD4+ T cell subset. (5C) Relative amounts of mRNA of GR, from the different subsets of CD4+ T cells quantified by RT-PCR (n = 3). The relative expression levels of GR were normalized against GAPDH. Data is presented as the mean ± SEM. * $p < 0.05$, *** $p < 0.0001$.

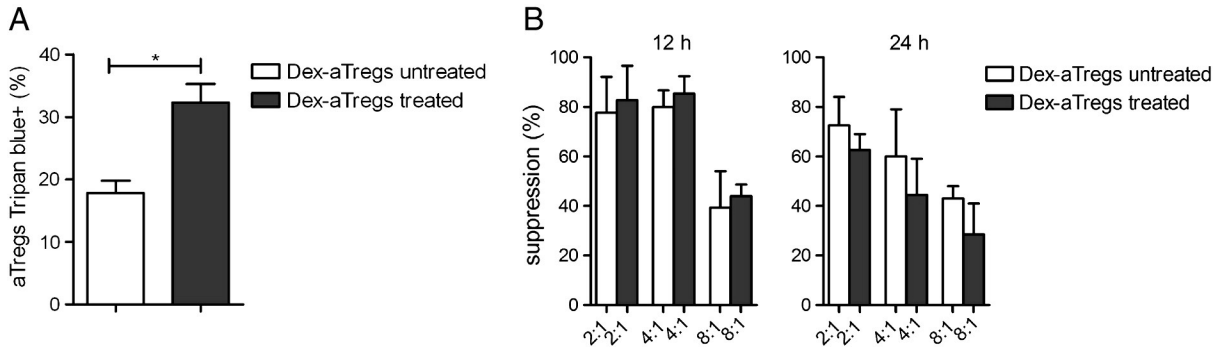


**Figure 6** Dex does not affect the suppressor activity of aTregs. CD4 + $CD25^{high}CD45RA-$ (aTregs) and CD4 + CD25− (Teff) cells were isolated by cell sorting. Teff were co-cultured with autologous APCs. Purified aTregs were exposed to Dex ($1 \times 10^{-7}$ M-Dex-treated aTregs) or medium (Dex-untreated aTregs) for 12 or 24 h, washed and added to the culture. (6A) Cell viability was evaluated by Tripan blue staining after 24 h of culture. (6B) Five independent experiments compared the suppressor capacity between Dex-treated and Dex-untreated aTregs on Teff proliferation. Data is presented as mean ± SEM. * $p < 0.05$.

Our results clearly suggest that in the presence of an inflammatory environment, the induction of apoptosis induced by Dex does not alter aTregs suppressive capacity.

## 4. Discussion

The present study was designed to validate the immunoregulatory role of Dex during the course of a T-cell response.

Natural Tregs are a subset of thymus-derived CD4+ T cells with imprinted genetic configuration [46,47], that show many differences with Teff. To exert their functions, Teff require TCR activation and clonal expansion. In contrast, the suppressor function of Tregs is independent of their proliferative status.

Several studies in mice and humans have suggested that Tregs might be expanded by GC [9,11,14–17,48,49]. However, we should take into account that in most of those previous studies, Dex was used in vaccinated individuals, or in combination with potent T-cell inducers, but not as monotherapy.

The present study shows that GC, *in vitro*, modulate the course of the T-cell mediated response through at least two mechanisms: 1 — inducing a greater inhibition of the proliferation of Teff; 2 — inducing a greater rate of aTregs apoptosis in a dose- and time-dependent manner.

Under steady state (unstimulated) conditions, FOXP3 + CD4+ T cells are very sensitive to Dex-induced apoptosis with mortality rates significantly higher than those detected on Teff. This sensitivity to apoptosis varies between subsets of FOXP3 + CD4+ T cells; with relative resistance on slow-cycling cells (rTregs) and more sensibility on fast-cycling cells (aTregs). Furthermore aTregs were found to express the highest constitutive BAX/BCL-2 ratio among all the CD4+ T cells.

In relationship with the use of Dex in autoimmune and/or inflammatory diseases, it is a relevant finding that the apoptotic effect on aTregs can be overcome by TCR-stimulation or by IL-2 supplementation. These results suggest that aTregs are intrinsically more capable of maintaining an appropriate balance between pro- (BAX) and anti-(BCL-2) apoptotic proteins for ensuring survival, even in the presence of very low doses of IL-2. Furthermore, the constitutive expression of CD25 on Tregs might represent an adaptive advantage over Teff, by which minimal levels of IL-2 could inhibit the effect of Dex on Tregs but not on Teff. In this context, it has recently been described that FOXP3 is a pro-apoptotic and lethal transcription factor to Tregs unless that a counterbalance by common γc-dependent cytokine signals exists [50].

As previously reported [51], this study demonstrates that GR is similarly expressed on all CD4+ T cells subsets, and as expected Dex-induced apoptosis could be abrogated by the addition of the GR antagonist RU. This demonstrates that Dex has a pro-apoptotic effect directly on cells mediated by binding to GR. Moreover, the differential Dex-induced cell death on Tregs and Teff cannot be attributed to the expression of GR as its expression was found to be similar in all CD4+ T cell subsets.

The present study demonstrates that GC modulates Teff function in many ways. Dex induced very low levels of apoptosis on Teff which contrasted with a strong inhibition of Teff proliferation. This issue is relevant because the biological function of Teff is dependent on their clonal expansion. In addition, many studies have demonstrated an inhibitory effect of GC on IFN-γ and IL-17 production by T cells [52,53]. These effects were observed after a short time exposure to Dex demonstrating that GC could modulate the immune system rapidly. By contrast, our finding that Dex treatment diminishes the relative numbers of FOXP3 + CD4+ T cells does not necessarily imply that functional properties of Tregs are altered. In agreement with Sbiera et al. [54], we found that a brief Dex-treatment of aTregs had no effect on their suppressor capacity. Although, we cannot fully exclude that Tregs function *in vivo* is affected by GC treatment, our *in vitro* results strongly argue that under Dex environment and in the presence of low doses of IL-2, the regulatory T-cell compartment could maintain its homeostasis.

In summary, our results support the notion that GC effects on the immune response mediated by effector and regulatory T cells are dynamically modulated by several factors in the microenvironment. The functional cross-talk between GC, cytokines and cell-activation could determine the biological response to GC explaining individual variations in the development of side effects at low doses. Finally, a study of the use of

GC in patients with an autoimmune disease, will allow us to confirm whether our *in vitro* results are also observed in an *in vivo* model involving self-antigens activation.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

We thank all blood donors who kindly accepted to participate in this trial. We also thank Luciano Cortiñas for assistance in digital art.

## References

[1] M. Miyara, Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, S. Sakaguchi, Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor, Immunity 30 (2009) 899–911.

[2] L. Arruvito, J. Sabatte, J. Pandolfi, P. Baz, L.A. Billordo, M.B. Lasala, H. Salomon, J. Geffner, L. Fainboim, Analysis of suppressor and non-suppressor FOXP3+ T cells in HIV-1-infected patients, PLoS One 7 (2012) e52580.

[3] D. Refojo, A.C. Liberman, F. Holsboer, E. Arzt, Transcription factor-mediated molecular mechanisms involved in the functional cross-talk between cytokines and glucocorticoids, Immunol. Cell Biol. 79 (2001) 385–394.

[4] T. Rhen, J.A. Cidlowski, Antiinflammatory action of glucocorticoids — new mechanisms for old drugs, N. Engl. J. Med. 353 (2005) 1711–1723.

[5] J.D. Ashwell, F.W. Lu, M.S. Vacchio, Glucocorticoids in T cell development and function*, Annu. Rev. Immunol. 18 (2000) 309–345.

[6] F. Paliogianni, S.S. Ahuja, J.P. Balow, J.E. Balow, D.T. Boumpas, Novel mechanism for inhibition of human T cells by glucocorticoids. Glucocorticoids inhibit signal transduction through IL-2 receptor, J. Immunol. 151 (1993) 4081–4089.

[7] W.Y. Almawi, H.N. Beyhum, A.A. Rahme, M.J. Rieder, Regulation of cytokine and cytokine receptor expression by glucocorticoids, J. Leukoc. Biol. 60 (1996) 563–572.

[8] N. Vanderheyde, V. Verhasselt, M. Goldman, F. Willems, Inhibition of human dendritic cell functions by methylprednisolone, Transplantation 67 (1999) 1342–1347.

[9] G. Stary, I. Klein, W. Bauer, F. Koszik, B. Reininger, S. Kohlhofer, K. Gruber, H. Skvara, T. Jung, G. Stingl, Glucocorticosteroids modify Langerhans cells to produce TGF-beta and expand regulatory T cells, J. Immunol. 186 (2011) 103–112.

[10] X. Chen, T. Murakami, J.J. Oppenheim, O.M. Howard, Differential response of murine CD4 + CD25+ and CD4 + CD25− T cells to Dexamethasone-induced cell death, Eur. J. Immunol. 34 (2004) 859–869.

[11] X. Chen, J.J. Oppenheim, R.T. Winkler-Pickett, J.R. Ortaldo, O.M. Howard, Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+)CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE, Eur. J. Immunol. 36 (2006) 2139–2149.

[12] P. Stock, O. Akbari, R.H. DeKruyff, D.T. Umetsu, Respiratory tolerance is inhibited by the administration of corticosteroids, J. Immunol. 175 (2005) 7380–7387.

[13] S. Wust, J. van den Brandt, D. Tischner, A. Kleiman, J.P. Tuckermann, R. Gold, F. Luhder, H.M. Reichardt, Peripheral T cells are the therapeutic targets of glucocorticoids in experimental autoimmune encephalomyelitis, J. Immunol. 180 (2008) 8434–8443.

[14] C. Karagiannidis, M. Akdis, P. Holopainen, N.J. Woolley, G. Hense, B. Ruckert, P.Y. Mantel, G. Menz, C.A. Akdis, K. Blaser, C.B. Schmidt-Weber, Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma, J. Allergy Clin. Immunol. 114 (2004) 1425–1433.

[15] N.A. Azab, I.H. Bassyouni, Y. Emad, G.A. Abd El-Wahab, G. Hamdy, M.A. Mashahit, CD4 + CD25+ regulatory T cells (TREG) in systemic lupus erythematosus (SLE) patients: the possible influence of treatment with corticosteroids, Clin. Immunol. 127 (2008) 151–157.

[16] E. Xystrakis, S. Kusumakar, S. Boswell, E. Peek, Z. Urry, D.F. Richards, T. Adikibi, C. Pridgeon, M. Dallman, T.K. Loke, D.S. Robinson, F.J. Barrat, A. O'Garra, P. Lavender, T.H. Lee, C. Corrigan, C.M. Hawrylowicz, Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients, J. Clin. Invest. 116 (2006) 146–155.

[17] M. Braitch, S. Harikrishnan, R.A. Robins, C. Nichols, A.J. Fahey, L. Showe, C.S. Constantinescu, Glucocorticoids increase CD4CD25 cell percentage and Foxp3 expression in patients with multiple sclerosis, Acta Neurol. Scand. 119 (2009) 239–245.

[18] L. Banica, A. Besliu, G. Pistol, C. Stavaru, R. Ionescu, A.M. Forsea, C. Tanaseanu, S. Dumitrache, D. Otelea, I. Tamsulea, S. Tanaseanu, C. Chitonu, S. Paraschiv, M. Balteanu, M. Stefanescu, C. Matache, Quantification and molecular characterization of regulatory T cells in connective tissue diseases, Autoimmunity 42 (2009) 41–49.

[19] P. Majak, B. Rychlik, I. Stelmach, The effect of oral steroids with and without vitamin D3 on early efficacy of immunotherapy in asthmatic children, Clin. Exp. Allergy 39 (2009) 1830–1841.

[20] B. Zhang, X. Zhang, F. Tang, L. Zhu, Y. Liu, Reduction of forkhead box P3 levels in CD4 + CD25high T cells in patients with new-onset systemic lupus erythematosus, Clin. Exp. Immunol. 153 (2008) 182–187.

[21] W. Liu, A.L. Putnam, Z. Xu-Yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D.M. Soper, S.F. Ziegler, J.A. Bluestone, CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells, J. Exp. Med. 203 (2006) 1701–1711.

[22] N. Seddiki, B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, A. Kelleher, B. Fazekas de St Groth, Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells, J. Exp. Med. 203 (2006) 1693–1700.

[23] E.S. Yolcu, S. Ash, A. Kaminitz, Y. Sagiv, N. Askenasy, S. Yarkoni, Apoptosis as a mechanism of T-regulatory cell homeostasis and suppression, Immunol. Cell Biol. 86 (2008) 650–658.

[24] L. Arruvito, M. Sanz, A.H. Banham, L. Fainboim, Expansion of CD4 + CD25 + and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction, J. Immunol. 178 (2007) 2572–2578.

[25] S. Jaleco, L. Swainson, V. Dardalhon, M. Burjanadze, S. Kinet, N. Taylor, Homeostasis of naive and memory CD4+ T cells: IL-2 and IL-7 differentially regulate the balance between proliferation and Fas-mediated apoptosis, J. Immunol. 171 (2003) 61–68.

[26] S. Yarkoni, A. Kaminitz, Y. Sagiv, I. Yaniv, N. Askenasy, Involvement of IL-2 in homeostasis of regulatory T cells: the IL-2 cycle, Bioessays 30 (2008) 875–888.

[27] B. Fritzsching, N. Oberle, N. Eberhardt, S. Quick, J. Haas, B. Wildemann, P.H. Krammer, E. Suri-Payer, In contrast to effector T cells, CD4 + CD25 + FoxP3+ regulatory T cells are highly susceptible to CD95 ligand — but not to TCR-mediated cell death, J. Immunol. 175 (2005) 32–36.

[28] B.H. Nelson, IL-2, regulatory T cells, and tolerance, J. Immunol. 172 (2004) 3983–3988.
[29] T.R. Malek, A.L. Bayer, Tolerance, not immunity, crucially depends on IL-2, Nat. Rev. Immunol. 4 (2004) 665–674.
[30] E. Goleva, K.O. Kisich, D.Y. Leung, A role for STAT5 in the pathogenesis of IL-2-induced glucocorticoid resistance, J. Immunol. 169 (2002) 5934–5940.
[31] T.J. Creed, R.W. Lee, P.V. Newcomb, A.J. di Mambro, M. Raju, C.M. Dayan, The effects of cytokines on suppression of lymphocyte proliferation by Dexamethasone, J. Immunol. 183 (2009) 164–171.
[32] E. Arzt, D. Kovalovsky, L.M. Igaz, M. Costas, P. Plazas, D. Refojo, M. Paez-Pereda, J.M. Reul, G. Stalla, F. Holsboer, Functional cross-talk among cytokines, T-cell receptor, and glucocorticoid receptor transcriptional activity and action, Ann. N. Y. Acad. Sci. 917 (2000) 672–677.
[33] Q. Tang, J.Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C.A. Piccirillo, B.L. Salomon, J.A. Bluestone, Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction, Immunity 28 (2008) 687–697.
[34] S. Gillis, K.A. Smith, Long term culture of tumour-specific cytotoxic T cells, Nature 268 (1977) 154–156.
[35] J.M. Adams, S. Cory, The Bcl-2 protein family: arbiters of cell survival, Science 281 (1998) 1322–1326.
[36] A. Strasser, The role of BH3-only proteins in the immune system, Nat. Rev. Immunol. 5 (2005) 189–200.
[37] S. Wojciechowski, P. Tripathi, T. Bourdeau, L. Acero, H.L. Grimes, J.D. Katz, F.D. Finkelman, D.A. Hildeman, Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis, J. Exp. Med. 204 (2007) 1665–1675.
[38] G. Deng, E.R. Podack, Suppression of apoptosis in a cytotoxic T-cell line by interleukin 2-mediated gene transcription and deregulated expression of the protooncogene bcl-2, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 2189–2193.
[39] X. Wang, A.L. Szymczak-Workman, D.M. Gravano, C.J. Workman, D.R. Green, D.A. Vignali, Preferential control of induced regulatory T cell homeostasis via a Bim/Bcl-2 axis, Cell Death Dis. 3 (2012) e270.
[40] E. Goleva, L.B. Li, D.Y. Leung, IFN-gamma reverses IL-2- and IL-4-mediated T-cell steroid resistance, Am. J. Respir. Cell Mol. Biol. 40 (2009) 223–230.
[41] C. Baecher-Allan, J.A. Brown, G.J. Freeman, D.A. Hafler, CD4 + CD25high regulatory cells in human peripheral blood, J. Immunol. 167 (2001) 1245–1253.
[42] M.K. Levings, R. Sangregorio, M.G. Roncarolo, Human cd25(+) cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function, J. Exp. Med. 193 (2001) 1295–1302.
[43] L.S. Taams, M. Vukmanovic-Stejic, J. Smith, P.J. Dunne, J.M. Fletcher, F.J. Plunkett, S.B. Ebeling, G. Lombardi, M.H. Rustin, J.W. Bijlsma, F.P. Lafeber, M. Salmon, A.N. Akbar, Antigen-specific T cell suppression by human CD4 + CD25+ regulatory T cells, Eur. J. Immunol. 32 (2002) 1621–1630.
[44] H. Jonuleit, E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, A.H. Enk, Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood, J. Exp. Med. 193 (2001) 1285–1294.
[45] C. Baecher-Allan, V. Viglietta, D.A. Hafler, Inhibition of human CD4(+)CD25(+high) regulatory T cell function, J. Immunol. 169 (2002) 6210–6217.
[46] J.D. Fontenot, M.A. Gavin, A.Y. Rudensky, Foxp3 programs the development and function of CD4 + CD25+ regulatory T cells, Nat. Immunol. 4 (2003) 330–336.
[47] R.S. McHugh, M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, M.C. Byrne, CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor, Immunity 16 (2002) 311–323.
[48] N. Seissler, E. Schmitt, F. Hug, C. Sommerer, M. Zeier, M. Schaier, A. Steinborn, Methylprednisolone treatment increases the proportion of the highly suppressive HLA-DR(+)-Treg-cells in transplanted patients, Transpl. Immunol. 27 (2012) 157–161.
[49] W.W. Unger, S. Laban, F.S. Kleijwegt, A.R. van der Slik, B.O. Roep, Induction of Treg by monocyte-derived DC modulated by vitamin D3 or Dexamethasone: differential role for PD-L1, Eur. J. Immunol. 39 (2009) 3147–3159.
[50] X. Tai, B. Erman, A. Alag, J. Mu, M. Kimura, G. Katz, T. Guinter, T. McCaughtry, R. Etzensperger, L. Feigenbaum, D.S. Singer, A. Singer, Foxp3 Transcription Factor Is Proapoptotic and Lethal to Developing Regulatory T Cells unless Counterbalanced by Cytokine Survival Signals, Immunity 38 (2013) 1116–1128.
[51] D. Marchetti, N.T. Van, B. Gametchu, E.B. Thompson, Y. Kobayashi, F. Watanabe, B. Barlogie, Flow cytometric analysis of glucocorticoid receptor using monoclonal antibody and fluoresceinated ligand probes, Cancer Res. 49 (1989) 863–869.
[52] X. Liu, P. Yang, X. Lin, X. Ren, H. Zhou, X. Huang, W. Chi, A. Kijlstra, L. Chen, Inhibitory effect of Cyclosporin A and corticosteroids on the production of IFN-gamma and IL-17 by T cells in Vogt–Koyanagi–Harada syndrome, Clin. Immunol. 131 (2009) 333–342.
[53] A. Sharabi, A. Haviv, H. Zinger, M. Dayan, E. Mozes, Amelioration of murine lupus by a peptide, based on the complementarity determining region-1 of an autoantibody as compared to Dexamethasone: different effects on cytokines and apoptosis, Clin. Immunol. 119 (2006) 146–155.
[54] S. Sbiera, T. Dexneit, S.D. Reichardt, K.D. Michel, J. van den Brandt, S. Schmull, L. Kraus, M. Beyer, R. Mlynski, S. Wortmann, B. Allolio, H.M. Reichardt, M. Fassnacht, Influence of short-term glucocorticoid therapy on regulatory T cells in vivo, PLoS One 6 (2011) e24345.