

Influence of Short-Term Glucocorticoid Therapy on Regulatory T Cells *In Vivo*

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Abstract

Background: Pre- and early clinical studies on patients with autoimmune diseases suggested that induction of regulatory T(T_{reg}) cells may contribute to the immunosuppressive effects of glucocorticoids(GCs).

Objective: We readdressed the influence of GC therapy on T_{reg} cells in immunocompetent human subjects and naïve mice.

Methods: Mice were treated with increasing doses of intravenous dexamethasone followed by oral taper, and T_{reg} cells in spleen and blood were analyzed by FACS. Sixteen patients with sudden hearing loss but without an inflammatory disease received high-dose intravenous prednisolone followed by stepwise dose reduction to low oral prednisolone. Peripheral blood T_{reg} cells were analyzed prior and after a 14 day GC therapy based on different markers.

Results: Repeated GC administration to mice for three days dose-dependently decreased the absolute numbers of T_{reg} cells in blood (100 mg dexamethasone/kg body weight: $2.8 \pm 1.8 \times 10^4$ cells/ml vs. $33 \pm 11 \times 10^4$ in control mice) and spleen (dexamethasone: $2.8 \pm 1.9 \times 10^5$ /spleen vs. $95 \pm 22 \times 10^5$ /spleen in control mice), which slowly recovered after 14 days taper in spleen but not in blood. The relative frequency of FOXP3⁺ T_{reg} cells amongst the CD4⁺ T cells also decreased in a dose dependent manner with the effect being more pronounced in blood than in spleen. The suppressive capacity of T_{reg} cells was unaltered by GC treatment *in vitro*. In immunocompetent humans, GCs induced mild T cell lymphocytosis. However, it did not change the relative frequency of circulating T_{reg} cells in a relevant manner, although there was some variation depending on the definition of the T_{reg} cells (FOXP3⁺: $4.0 \pm 1.5\%$ vs $3.4 \pm 1.5\%^*$; AITR⁺: 0.6 ± 0.4 vs $0.5 \pm 0.3\%$, CD127^{low}: 4.0 ± 1.3 vs $5.0 \pm 3.0\%^*$ and CTLA4⁺: 13.8 ± 11.5 vs $15.6 \pm 12.5\%$; * $p < 0.05$).

Conclusion: Short-term GC therapy does not induce the hitherto supposed increase in circulating T_{reg} cell frequency, neither in immunocompetent humans nor in mice. Thus, it is questionable that the clinical efficacy of GCs is achieved by modulating T_{reg} cell numbers.

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Introduction

Glucocorticoids (GCs) are a class of steroid hormones that bind to the glucocorticoid receptor, which is present in almost every vertebrate animal cell [1]. This explains the large variety of physiological roles played by GCs [2,3]. They are crucial modifier of the metabolic [4] and immune system [5,6], but are also important for development [7] as well as for arousal and cognition [8]. During an immune response, GCs contribute to the termination of inflammation by both suppressing and enhancing activities of the immune system [9]. At the level of the innate immune system GCs induce neutrophilia by increasing polymorphonuclear cell release

from the bone marrow while inhibiting their transmigration to inflammatory sites [10,11]. At the same time they induce apoptosis of basophils and eosinophils [12,13]. In a similar way GCs control the movement of circulating monocytes while enhancing the phagocytotic ability and antigen uptake by the tissue macrophages and therefore speed up the clearance of foreign antigens and microorganisms [14]. At the level of leukocyte gene expression, a huge number of proinflammatory cytokines (IL1 β , TNF α , IL-6, IL-8, IL-12, IL-18 etc) and chemokines (both CC and CXC) is strongly suppressed by GCs, while the anti-inflammatory cytokines IL-10 and TGF β are upregulated [15]. On the other hand, GCs favor antibody production by promoting the generation of immunoglobulin

secreting plasma cells [16]. Accordingly, GCs promote the polarization of naïve T-cells to a Th2 phenotype while at the same time promoting an immature dendritic cell phenotype [17]. Both effects may be in part responsible for a presumed upregulation of regulatory T (T_{reg}) cells by GCs [18]. In the last decades, it became clear that T_{reg} cells play an essential role in self-tolerance and for maintaining immune system homeostasis [19].

Failure of self-tolerance often leads to autoimmune diseases (AD), with an incidence of 5–10 % of the population [20]. Emergence of autoimmune diseases is not completely understood, it is however documented that T cells often play an essential role in their development (e.g. diabetes mellitus type I or type A gastritis [21]). Some of these autoimmune diseases are probably triggered by infections (e.g. Guillian Barré syndrome [22,23]), whereas in others it has been shown that tolerance defects are due to a decrease in number or defective expression pattern in the T_{reg} cell population [24,25]. Since more than 60 years GCs are often the first line therapy in inflammatory and autoimmune responses [26] and around 30 years ago a connection between GCs and a “suppressor” T cell phenotype was initially shown *in vitro* [27,28]. In the meantime, a number of different T_{reg} cell populations have been described [25,29], of which the most studied one expresses CD4 and high levels of the IL-2 receptor alpha-chain (CD25) [19,30]. Several additional surface markers have been reported to discriminate naturally occurring T_{reg} cells from other activated CD4⁺ Th2 cells such as cytotoxic T-lymphocyte antigen 4 (CTLA4) [31,32], the nuclear transcription factor forkhead box P3 (FOXP3), the activation-inducible tumor necrosis factor receptor AITR [33] and low expression of the IL-7 receptor (CD127) [34,35]. Although expression of FOXP3 is considered as the best tool to define T_{reg} cells by many authors [36,37,38], even this marker (as all other markers) is not specific to human T_{reg} cells and can be transiently induced on all effector T cell populations upon activation.

In 2004, Karagiannidis *et al.* showed for the first time that GC treatment *in vivo* (both systemic and inhaled) induces an increase in circulating T_{reg} cells (as defined by the FOXP3 and IL-10 mRNA expression of CD4⁺ T cells) in patients with asthma bronchiale [39]. However, such a positive correlation between GC treatment and the number of T_{reg} cells in the peripheral blood is still disputed. In the mouse, Chen *et al.* could demonstrate that the synthetic GC dexamethasone increased the proportion of T_{reg} cells both in peripheral blood and secondary lymphoid organs [40,41]. By contrast, Stock *et al.* showed the opposite in a mouse model of asthma [42] as did Wüst *et al.* in a mouse model of multiple sclerosis [43]. In humans, several small *in vivo* studies pointed towards a positive correlation between administration of GCs and the frequency of T_{reg} cells in patients with different autoimmune diseases [44,45,46,47,48,49,50,51,52,53,54]. However, two recently performed larger studies, both including more than 50 patients with asthma bronchiale or autoimmune connective tissue diseases, respectively, arrived at exactly the opposite conclusion [55,56]. The fact that these studies do not present a unified picture of the influence of GCs on T_{reg} cells may be explained by two aspects. Firstly, there is a huge heterogeneity in the molecular characterization of T_{reg} cells. Several studies just defined T_{reg} cells as being CD4⁺CD25^{high}. However, it is evident by now that many of these cells are not T_{reg} cells but rather activated T cells [57]. Secondly, all but one study analyzed patients with an autoimmune background and it is likely that different autoimmune diseases come along with different levels of impairment of T_{reg} cell frequency and/or function [47,48,51,53]. The only study on healthy donors so far was performed *ex vivo* using mixed PBMC cultures in the presence of dexamethasone, epinephrine and IL-2

[58]. Nonetheless, it has been already shown in mice that IL-2 topple the balance in favor of T_{reg} cells regardless of GC treatment [40,41].

Hence, the goal of this study was to determine the influence of short-term GC therapy, as frequently used in different clinical scenarios, on circulating T_{reg} cells in immunologically uncompromised mice and humans *in vivo*.

Methods

Animal experiments

Mice. C57Bl/6 mice were bred in the animal facility at the University of Göttingen Medical School, kept in individually ventilated cages under specific pathogen free conditions and used at an age of 8–12 weeks. Mice of both sexes were included in the study. Blood samples were taken by bleedings from the tail, or the mice were sacrificed using CO₂ to obtain the spleen or blood from the heart. All animal experiments were conducted according to ethical standards of humane animal care and approved by the authorities of Lower Saxonia (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES; approval ID: 33.14.42502-04-107.08).

GC treatment was performed by intraperitoneal (IP) injection of 0.8 mg/kg, 4 mg/kg, 20 mg/kg or 100 mg dexamethasone dihydrogen phosphate (Dexa-ratio-pharm®, Ratiopharm, Ulm, Germany) / kg body weight on three consecutive days [43]. Mice treated IP with 100 mg/kg were treated for additional 11 days with oral glucocorticoids with decreasing dosages of water-soluble dexamethasone (Sigma, Taufkirchen, Germany) added to the drinking water (days 4–7: 10 mg/l, days 8–11: 5 mg/l, days 12–14: 1 mg/l). The supplemented drinking water was changed every second day.

Lymphocyte analysis. Lymphocytes were isolated from the spleen of the mice on days 0, 3 and 14 by passing the freshly isolated organs through a 40 µm Nylon mesh, washed in FACS-Buffer (PBS with 0.5% BSA and 0.05% NaN₃), erythrolyzed and counted. Absolute cell numbers in blood were determined by flow cytometric analysis of a defined blood volume. Analysis of splenocytes and blood lymphocytes by six-color flow-cytometry was performed using a FACS Canto II device (BD Biosciences, Heidelberg, Germany) in combination with FlowJo software. Monoclonal antibodies directed against mouse leukocyte specific molecules were obtained from BD Biosciences unless otherwise indicated (clone name in brackets): CD3 ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD25 (PC61), TCRαβ (H57-597), GR-1 (RB6-8C5), B220 (RA3-6B2), FoxP3 (FJK-16S; eBiosciences, Frankfurt, Germany). The antibodies were directly labeled with FITC, PE, PerCP, PE-Cy7, Cy5, APC or APC-Cy7, respectively.

Analysis of the suppressive activity of T_{reg} cells *in vitro*. CD4⁺CD25⁺ regulatory T (T_{reg}) cells and conventional CD4⁺CD25⁻ helper T (T_h) cells serving as indicator cells were purified from spleens of C57BL/6 mice using the *CD4⁺CD25⁺ Regulatory T Cell Isolation Kit* (Miltenyi Biotech, Bergisch Gladbach, Germany). T_h cells (5×10^4 cells / well) and different amounts of T_{reg} cells were cocultured with γ -irradiated antigen-presenting cells (30 Gy, 10^5 cells / well) for 48 hrs in RPMI 1640 medium supplemented with 10 % FCS and 1% Pen/Strep in 96-well U-bottom plates. Polyclonal stimulation was achieved by adding Con A (2.5 µg/ml) into the cultures. Water-soluble dexamethasone (Sigma, Taufkirchen, Germany) was added to a final concentration of 5 nM where indicated. Unstimulated and stimulated T_h cells and T_{reg} cells alone served as controls. Proliferation was assessed by measuring ³H-TdR (Hartmann Analytics, Braunschweig, Germany) incorporation (37 kBq / well)

during an additional culture period of 16 hours. The labeled DNA was harvested onto Filtermat A glassfibre filters and the radioactivity was quantified using a MicroBeta² β -scintillation counter (Perkin Elmer, Rodgau, Germany). In addition, supernatants were collected from cultures after 48 hrs and Interleukin-2 levels were measured using a commercially available ELISA kit (BioLegend, San Diego/CA, USA) according to the manufacturers' instructions.

Human experiments

Patients. 16 patients (10 males and 6 females) with acute hearing loss were routinely treated in the Department of Ophthalmology and Otolaryngology of the University Hospital of Würzburg using a modified "Stennert scheme" (only GCs without pentoxifyllin as clinically indicated). All individuals were otherwise healthy and fulfilled the following inclusion criteria: age >18 years (mean patient age was 35.8 ± 13 years), no GCs or other immunosuppressive therapy in the last 8 weeks, no suspected endogenous GC excess and no evidence for any malignant or autoimmune diseases or any acute or chronic infections. All patients gave written informed consent and the study was approved by the ethic committee of the University of Würzburg (Ethik-Kommission der Medizinischen Fakultät der Universität Würzburg, Amendment 1 zu Studien-Nr. 83/05). Prednisolone was administered according to the following regimen: day 1–3: 250 mg intra venous (IV), day 4: 150 mg IV, day 5–9: 100 mg per os (P.O.), day 10–11: 75 mg P.O., day 12: 50 mg P.O., day 13: 20 mg P.O. and day 14: 10 mg P.O.

Lymphocyte analysis. Before the first injection of prednisolone (day 0) and at the end of the 14 day treatment period 5 ml EDTA and 20 ml heparinized blood were taken from the patients. The EDTA blood was analyzed in the central laboratory of the University Hospital of Würzburg for a complete blood count with white blood cell differential. The heparinized blood was diluted with 20 ml phosphate buffer saline (PBS). Subsequently, the diluted blood was applied to a Ficoll hypaque[®] gradient and separated via centrifugation at 400 G for 30 minutes [59]. The peripheral blood mononuclear cells (PBMC) were pipetted from the interface and washed twice with PBS. For a detailed characterization of cellular changes induced by GCs, the PBMCs were stained according to manufacturers' protocols using six different sets of monoclonal antibodies directed against various surface molecules (clone numbers in brackets): Set 1: CD3 (UCHT1), CD14 (M5E2), CD19 (HIB19); Set 2: CD8 (SK-1), CD25 (M-A251), CTLA-4 (BNI3); Set 3: CD4 (RPA-T4), CD25 (M-A251), CD127 (hIL-7R-M21); Set 4: CD4 (RPA-T4), CD25 (M-A251), AITR (eBioAITR); Set 5: CD4/CD 25 (RPA-T4/BC96) and staining control Set 6: no antibodies. Subsequently, the cells which had been stained with the surface markers of sets 5 and 6 were fixed and permeabilized according to the manufacturer's instructions followed by intracellular staining with rat-anti-FOXP3 (PCH101) and the appropriate isotype control antibody (eBR2a). The antibodies used in the sets 1 to 4 were obtained from BD Biosciences (Heidelberg, Germany) and those in the sets 5 and 6 from eBiosciences (Frankfurt, Germany). All antibodies were directly labeled with FITC, PE and APC. Three-color flow cytometry measurements were performed using a FACS Calibur and data analysis was done by using CellQuest pro software (both BD Biosciences, Heidelberg, Germany).

Statistics

Results are depicted as mean \pm standard deviation (SD). For statistical analysis a repeated measures Anova followed by Bonferroni comparison test was performed using Prism v.4.b.

software for Macintosh (GraphPad Software Inc., La Jolla, CA, USA). A P value <0.05 was considered statistically significant.

Results

Systemic high-dose GC treatment of mice reduces T_{reg} cells numbers in peripheral blood and spleen

We have chosen a protocol for GC treatment of mice mimicking steroid regimens widely applied to human patients. Whilst therapy of acute relapses in multiple sclerosis patients for example consists of several repeated injection of high-dose GC followed by a taper [60,61], lower concentrations of GC are used to treat rheumatoid arthritis or asthma patients. Our protocol has been further adapted to consider differences between mice and humans in terms of pharmacokinetics and handling by employing IP injections of dexamethasone [43,62]. The number of CD4⁺ T cells in the blood of the mice decreased in a dose-dependent manner dropping to levels below 10% of control animals after 3 days of IP treatment with 100 mg/kg dexamethasone (Figure 1A). CD4⁺ T cell numbers remained low even after oral steroid taper (Table 1 and Figure 1A). The number of T_{reg} cells defined as CD4⁺CD25^{high}FOXP3⁺ T cells also decreased in a dose-dependent manner after the 3 day of GC therapy and did not recover after an additional 11 days of oral treatment (Figure 1C). Importantly, also the percentage of FOXP3⁺ T_{reg} cells decreased amongst the CD4⁺ T cell population with the GC doses applied, and did not recover at day 14 (Figure 1E).

In the spleen of the same mice the situation was slightly different. Although the absolute number of CD4⁺ T cells also strongly and dose-dependently decreased after the 3 days IP treatment (Table 1 and Figure 1B), the cell numbers partially recovered during the phase of oral GC taper (Figure 1B). The same situation applies to the FOXP3⁺ T_{reg} cells, the number of which decreased after 3 days and slightly recovered after the 14 days of GC treatment (Figure 1D). Consequently, the relative proportion of T_{reg} cells amongst the CD4⁺ T cell population significantly decreased (Figure 1F) after the high-dose systemic GC treatment. Interestingly, the percentage of T_{reg} cells recovered after the additional 11 days of oral taper to values comparable to those before treatment (Figure 1F).

GC treatment does not impact the suppressive capacity of T_{reg} cells in vitro

Besides altering the absolute or relative numbers of T_{reg} cells in blood and peripheral lymphoid organs, it is also conceivable that GC impact the suppressive capacity of T_{reg} cells. This functional characteristic is best studied *in vitro*. Furthermore, a concentration of 5 nM dexamethasone was chosen since pilot experiments had revealed that this already leads to a significant regulation of T cell function while at the same time proliferation is only slightly affected (data not shown). To assess whether GC alter the functional properties of T_{reg} cells *in vitro*, we performed a standard suppression assay in the absence or presence of dexamethasone by adding decreasing amounts of T_{reg} cells to ConA-stimulated T_h cells serving as indicator cells. The activity of the T_h cells was assessed by analyzing proliferation on the basis of ³H-TdR incorporation (Figure 2A) as well as by determining IL-2 production (Figure 2B). Importantly, addition of dexamethasone into the culture did not significantly alter the suppressive capacity of T_{reg} cells, neither in terms of proliferation (42.7 ± 15.5 % at 1:1 T_{reg}:T_h ratio in control cells vs 32.0 ± 13.7 % in cells treated with dexamethasone; n.s.) nor in terms of IL-2 secretion (30.7 ± 4.5 % at 1:1 T_{reg}:T_h ratio in control cells vs 30.0 ± 1.0 % in cells treated with dexamethasone; n.s.) (Figure 2). This suggests that - at least *in vitro* - GCs do neither positively nor negatively influence the functional properties of T_{reg} cells.

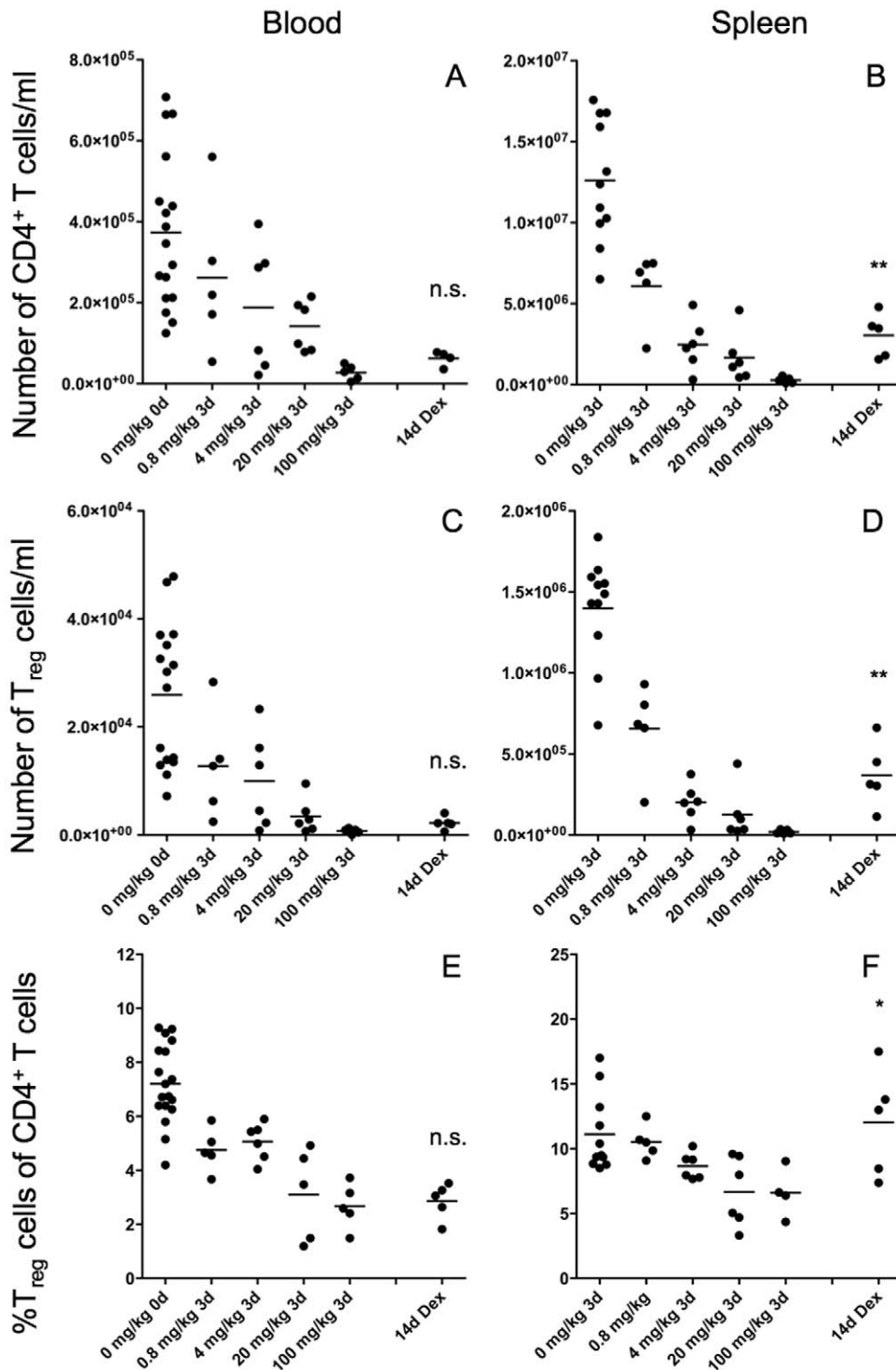


Figure 1. Modulation of CD4⁺ T cells and T_{reg} cells by GCs in mice. Peripheral blood (A, C, E) and spleen (B, D, F) cells from C57BL/6 mice were analyzed by flow cytometry before, 3 days after IP treatment with different dosages of dexamethasone and 14 days after IP treatment with 100 mg/kg dexamethasone followed by oral taper. The absolute numbers of CD4⁺ T cells (A, B) and CD4⁺CD25^{high}FOXP3⁺ T_{reg} cells (C, D) were assessed and the relative frequency of T_{reg} cells amongst all CD4⁺ T cells was calculated (E, F); *p<0.05, **p<0.01. doi:10.1371/journal.pone.0024345.g001

GCs have little impact on the relative frequency of circulating T_{reg} cells in immunologically uncompromised human subjects

The 14 days of prednisolone administration to human subjects, none of which suffered from immunological diseases, induced a

doubling of circulating leukocytes (Table 2). As expected, this was mainly attributed to a significant increase in circulating neutrophils. In addition, the number of monocytes also doubled and the number of lymphocytes increased by 30%, while the numbers of basophils and eosinophils remained unaltered. Of note, the

Table 1. Lymphocyte counts and percentages in the blood and spleen of mice before and after GC therapy *in vivo*.

cell numbers (cells / ml ± SD)						
	before therapy	3d after IP therapy with dexamethason (mg/kg)			14d after therapy	
Blood		0.8	4	20	100	
CD4 ⁺ cells	37.3×10 ⁴ ±18.8×10 ⁴	26.2×10 ⁴ ±18.9×10 ⁴	18.8×10 ⁴ ±15.7×10 ⁴	14.2×10 ⁴ ±6.2×10 ⁴	2.8×10 ⁴ ±1.8×10 ⁴	6.3×10 ⁴ ±1.8×10 ⁴
FOXP3 ⁺ cells*	2.6×10 ⁴ ±1.3×10 ⁴	1.3×10 ⁴ ±1.0×10 ⁴	1.0×10 ⁴ ±0.9×10 ⁴	0.3×10 ⁴ ±0.3×10 ⁴	0.07×10 ⁴ ±0.05×10 ⁴	0.22×10 ⁴ ±0.12×10 ⁴
%FOXP3 in CD4 ⁺ cells	7.2±1.4	4.8±0.8	5±0.7	3.1±1.7	2.7±0.8	2.9±0.7
spleen	cell numbers (cells / spleen ± SD)					
CD4 ⁺ cells	12.6×10 ⁶ ±3.8×10 ⁶	6.0×10 ⁶ ±2.2×10 ⁶	2.5×10 ⁶ ±1.6×10 ⁶	1.6×10 ⁶ ±1.5×10 ⁶	0.3×10 ⁶ ±0.2×10 ⁶	3.0×10 ⁶ ±1.4×10 ⁶
FOXP3 ⁺ cells*	1.4×10 ⁶ ±0.3×10 ⁶	0.7×10 ⁶ ±0.3×10 ⁶	0.2×10 ⁶ ±0.1×10 ⁶	0.1×10 ⁶ ±0.1×10 ⁶	0.02×10 ⁶ ±0.01×10 ⁶	0.4×10 ⁶ ±0.2×10 ⁶
%FOXP3 in CD4 ⁺ cells	11.1±2.9	10.53±1.3	8.7±1.0	6.7±2.7	6.6±1.9	12.0±1

* = as subpopulation of CD4⁺CD25^{high} cells.

‡ = % out of CD4⁺ cells ± SD.

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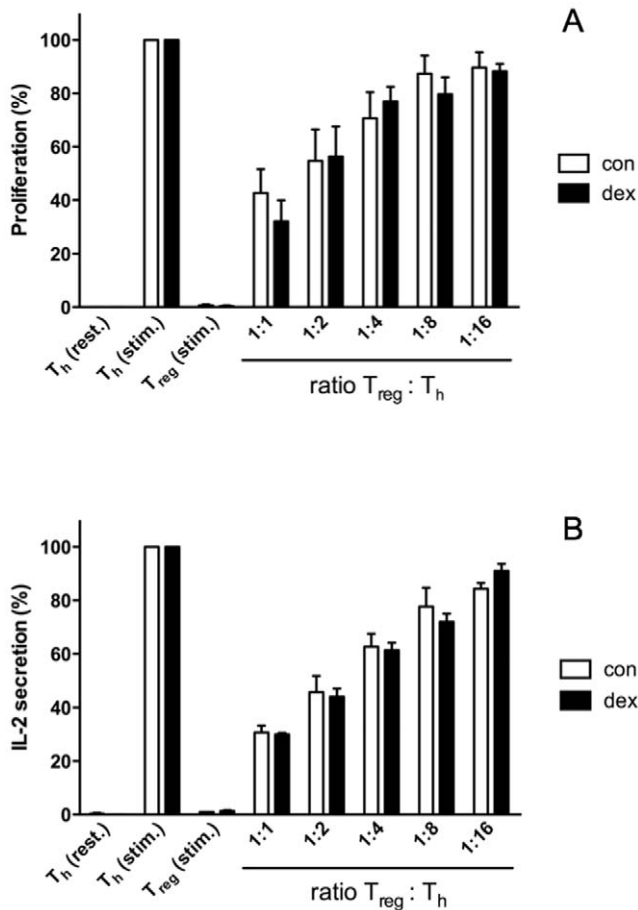


Figure 2. Effect of GC on the function of T_{reg} cells *in vitro*. T_h cells (5 × 10⁴ cells / well) were incubated with T_{reg} cells at different ratios in the presence of irradiated APC (30 Gy, 10⁵ cells / well) and Con A (2.5 μg / ml) for 48 hrs, either with dexamethasone (dex, 5 nM) or without it (con). Resting T_h cells as well as T_h and T_{reg} cells stimulated with Con A served as controls. Proliferation was determined by ³H-TdR incorporation during an additional 16 hrs culture period (A), IL-2 levels were directly measured in the supernatants by ELISA (B). All values were normalized to stimulated T_h cells treated with or without Dex, respectively. In both panels the combined data of three independent experiments are depicted. doi:10.1371/journal.pone.0024345.g002

frequency of each cell type within the leukocyte population remained largely unchanged with the exception of the eosinophils that decreased slightly (Table 2).

With regard to the T cell population, the 14 days of GC administration induced a 1.5-fold increase in the number of CD3⁺ T cells from 15 ± 5.8 × 10⁵ to 23 ± 10 × 10⁵ cells/ml (p < 0.001; Table 2) due to an increase in CD8⁺ T cell numbers (4 ± 2.5 × 10⁵ to 5.8 ± 3.1 × 10⁵ cells/ml; p < 0.001) as well as CD4⁺ cell numbers (11 ± 4.0 × 10⁵ to 18 ± 7.7 × 10⁵ cells/ml; p < 0.001; Figure 3A). In contrast, the relative frequency of all T cell subpopulations remained unaffected (58 ± 11 to 58 ± 9.0% T-cells in PBMC; 24.5 ± 9.0% to 24.3 ± 7.5% CD8⁺ T cells and 75.5 ± 9.0% to 75.7 ± 7.5% CD4⁺ T cells within the T cell population; Figure 3B). The T_{reg} cells, being a subpopulation of the CD4⁺ T cells, followed the positive trend in absolute cell numbers, however, depending on their molecular characterization, the amplitude differed (Figure 4). The number of T_{reg} cells increased only slightly when defined as FOXP3⁺ (62 ± 35 × 10³ to 82 ± 47 × 10³ cells/ml; p < 0.05; Figure 3C and 4A, B) or AITR⁺ (8.9 ± 6.8 × 10³ to 10 ± 9.0 × 10³ cells/ml; n.s.; Figure 3E and 4C, D), whereas the CD127^{low} and the CTLA4⁺ T_{reg} cells doubled from 56 ± 24 × 10³ to 100 ± 60 × 10³ cells/ml (p < 0.001; Figure 3G and 4E, F) and from 160 ± 70 × 10³ to 300 ± 200 × 10³ cells/ml (p < 0.05; Figure 3I and 4G, H), respectively. The frequency of the T_{reg} cells amongst the CD4⁺ T cells also varied depending on the molecular characterization. Whereas the frequency of FOXP3⁺ and AITR⁺ T_{reg} cells within the CD4⁺ T cell population slightly decreased after GC treatment from 4.0 ± 1.5 to 3.4 ± 1.5% (p < 0.05; Figure 3D) and 0.6 ± 0.4% to 0.45 ± 0.3% (n.s.; Figure 3F), respectively, their percentages did not increase in a relevant manner from 4.0 ± 1.3% to 5 ± 3.0% (p < 0.05; Figure 3H) and from 13.8 ± 11.5% to 15.6 ± 12.5% (n.s.; Figure 3J) when the T_{reg} cells were defined as CD127^{low} and CTLA4⁺, respectively.

Discussion

T_{reg} cells exert potent suppressive effects on the immune system through a plethora of different mechanisms. It has been previously reported that the frequency of T_{reg} cells is essential for the prevention of exaggerated inflammation and autoimmunity [24,25], and some studies suggested that T_{reg} cells are induced by GCs both in mice and humans [41,50]. It was hypothesized that this is due to the differential responsiveness of this specialized T cell subpopulation to GCs [40]. However, the respective

Table 2. Blood lymphocyte counts in immunocompetent human subjects before and after GC therapy *in vivo*.

cell type	cell numbers (cells/ml \pm SD)			frequency (% \pm SD)		
	before therapy	after therapy	p value	before therapy	after therapy	p value
leukocytes	7.3 \pm 3.0 \times 10 ⁶	13.0 \pm 4.2 \times 10 ⁶	p<0.001	-	-	-
neutrophils	4.6 \pm 2.6 \times 10 ⁶	9.3 \pm 3.7 \times 10 ⁶	p<0.001	60.4 \pm 11.6 [#]	68.0 \pm 10.3 [#]	n.s.
basophils	1.7 \pm 4.0 \times 10 ⁴	7.0 \pm 3.0 \times 10 ³	n.s.	0.2 \pm 0.5 [#]	0.1 \pm 0.3 [#]	n.s.
eosinophils	1.4 \pm 1.4 \times 10 ⁵	1.3 \pm 1.0 \times 10 ⁵	n.s.	2.5 \pm 2.6 [#]	0.9 \pm 0.7 [#]	p<0.05
monocytes	5.0 \pm 1.5 \times 10 ⁵	8.5 \pm 2.6 \times 10 ⁵	p<0.001	7.5 \pm 2.3 [#]	6.5 \pm 1.7 [#]	n.s.
lymphocytes	2.0 \pm 0.7 \times 10 ⁶	3.0 \pm 1.3 \times 10 ⁶	p<0.01	28.7 \pm 8.5 [#]	24.0 \pm 9.6 [#]	n.s.
CD3 ⁺ cells	1.5 \pm 0.6 \times 10 ⁶	2.3 \pm 1.0 \times 10 ⁶	p<0.001	58.0 \pm 11.0 [§]	58.0 \pm 9.0 [§]	n.s.
CD8 ⁺ cells	4.0 \pm 2.5 \times 10 ⁵	5.8 \pm 3.1 \times 10 ⁵	p<0.001	24.5 \pm 9.0 [§]	24.3 \pm 7.5 [§]	n.s.
CD4 ⁺ cells	11.0 \pm 4.0 \times 10 ⁵	18.0 \pm 7.7 \times 10 ⁵	p<0.001	75.5 \pm 9.0 [§]	75.7 \pm 7.5 [§]	n.s.
FOXP3 ⁺ cells*	6.2 \pm 3.5 \times 10 ⁴	8.2 \pm 4.7 \times 10 ⁴	p<0.05	4.0 \pm 1.5 [‡]	3.4 \pm 1.5 [‡]	p<0.05
AITR ⁺ cells*	8.9 \pm 6.8 \times 10 ³	10.0 \pm 9.0 \times 10 ³	n.s.	0.6 \pm 0.4 [‡]	0.5 \pm 0.3 [‡]	n.s.
CD127 ^{low} cells*	5.6 \pm 2.4 \times 10 ⁴	10.0 \pm 6.0 \times 10 ⁴	p<0.001	4.0 \pm 1.3 [‡]	5.0 \pm 3.0 [‡]	p<0.05
CTLA4 ⁺ cells*	1.6 \pm 0.7 \times 10 ⁵	3.0 \pm 2.0 \times 10 ⁵	p<0.05	13.8 \pm 11.5 [‡]	15.6 \pm 12.5 [‡]	n.s.

* = as subpopulation of CD4⁺CD25^{high} cells.

= % out of leukocyte cells \pm SD.

§ = % out of PBMC \pm SD.

‡ = % out of CD3⁺ cells \pm SD.

‡ = % out of CD4⁺ cells \pm SD.

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experiments were mostly performed not as a monotherapy but rather in combination with potent T-cell inducers like IL-2 or vitamin D3, or they were performed on vaccinated or immunodeficient mice [41,63]. To reassess the impact of GC therapy on T_{reg} cells in mice in the context of steroid monotherapy, we applied a protocol with increasing dosages of 0.8 to 100 mg/kg dexamethasone IP for 3 days. This therapy led to a dose dependant decrease of CD4⁺ T cells in peripheral blood and spleen. Surprisingly, the decrease in the number of T_{reg} cells was even more pronounced than the number of total CD4⁺ T cells. This is in agreement with the data of Stock *et al.* [42] who show in a mouse asthma model that GCs induce T_{reg} cell deficiency, which aggravates the long-term course of the allergic disease. It is also in line with the data of Wüst *et al.* [43] who reported a relative decrease of T_{reg} cell numbers in a mouse model of multiple sclerosis following high-dose GC therapy. During the additional 11 days of oral taper (from 10 mg/l to 1 mg/l) the low T cell numbers and frequencies in the blood persisted whereas the number of CD4⁺ T cells in the spleen slightly recovered and the proportion of T_{reg} cells even reached similar levels compared to before the therapy. The observed differences between peripheral blood and spleen might suggest that while under GC therapy no new T_{reg} cells are being produced by the thymus, they are constantly generated *de novo* in the secondary lymphatic organs. This corroborates data showing that GCs induce an immature dendritic cell (DC) phenotype in the periphery capable of transforming naive T cells into T_{reg} cells [64]. We therefore postulate that high-dose GC therapy in the mouse induces massive apoptosis of CD4⁺ T cells, that T_{reg} cells show an increased sensitivity to GCs, and that a prolonged lower dose of GCs favors their tolerogenic DC-induced regeneration in the periphery.

Our finding that the absolute and relative numbers of T_{reg} cells in blood and spleen are decreased by GC treatment *in vivo* does not necessarily exclude that the functional properties of the T_{reg} cells are altered. However, we found that the suppressive capacity of T_{reg} cells was indistinguishable in the presence or absence of GC

at least *in vitro*. Neither inhibition of T_h cell proliferation nor repression of their IL-2 production by T_{reg} cells was different when dexamethasone was added to the cultures. Although we cannot fully exclude that T_{reg} cell function *in vivo* is affected by GC treatment, our *in vitro* results nevertheless strongly argue that GC do not impact the functional properties of T_{reg} cells directly.

In the human subjects, 14 days of prednisolone administration in a dosage used for the treatment of many common diseases led to strong blood leukocytosis, mainly due to the already described increase in circulating neutrophils and monocytes but to a minor extent also in lymphocytes. Of note, T cells as well as their CD4⁺ and CD8⁺ subpopulations increased at the same rate. On average, the numbers of CD4⁺CD25^{high} T_{reg} cells increased independently of additional markers used for their identification. However, this change was only significant when using FOXP3⁺, CD127^{low} and CTLA4⁺ as definition of T_{reg} cells but not AITR⁺. Similarly, the frequency of FOXP3⁺ T_{reg} cells within the CD4⁺ T cell population decreased slightly, whereas the relative number of CD127^{low} T_{reg} cells increased and the one of AITR⁺ and CTLA4⁺ T_{reg} cells showed no relevant changes concerning the T_{reg}/CD4⁺ T cell ratio. We therefore conclude that GCs have no relevant impact on the frequency of circulating T_{reg} cells. However, our study also indicates that the outcome of the analysis of T_{reg} cells in peripheral blood of human subjects depends - at least in part - on the markers used for their molecular characterization. This could explain - amongst others - the partially contradictory results published before. Considering FOXP3 expression, the most accepted marker of T_{reg} cells, in our human cohort, the GC taper treatment seems to induce an increase in T_{reg} cell numbers but at the same time an even stronger increase in CD4⁺ T cells. This even results in a significant reduction of the relative frequency of T_{reg} cells as compared to the rest of the circulating CD4⁺ T cells and thereby confirms our results obtained in mice. Thus, our findings call the hypothesis that GCs exert some of their immunosuppressive capacity by inducing T_{reg} cells into question. Therefore, it is tempting to speculate that GCs rather modify cell-cell interactions

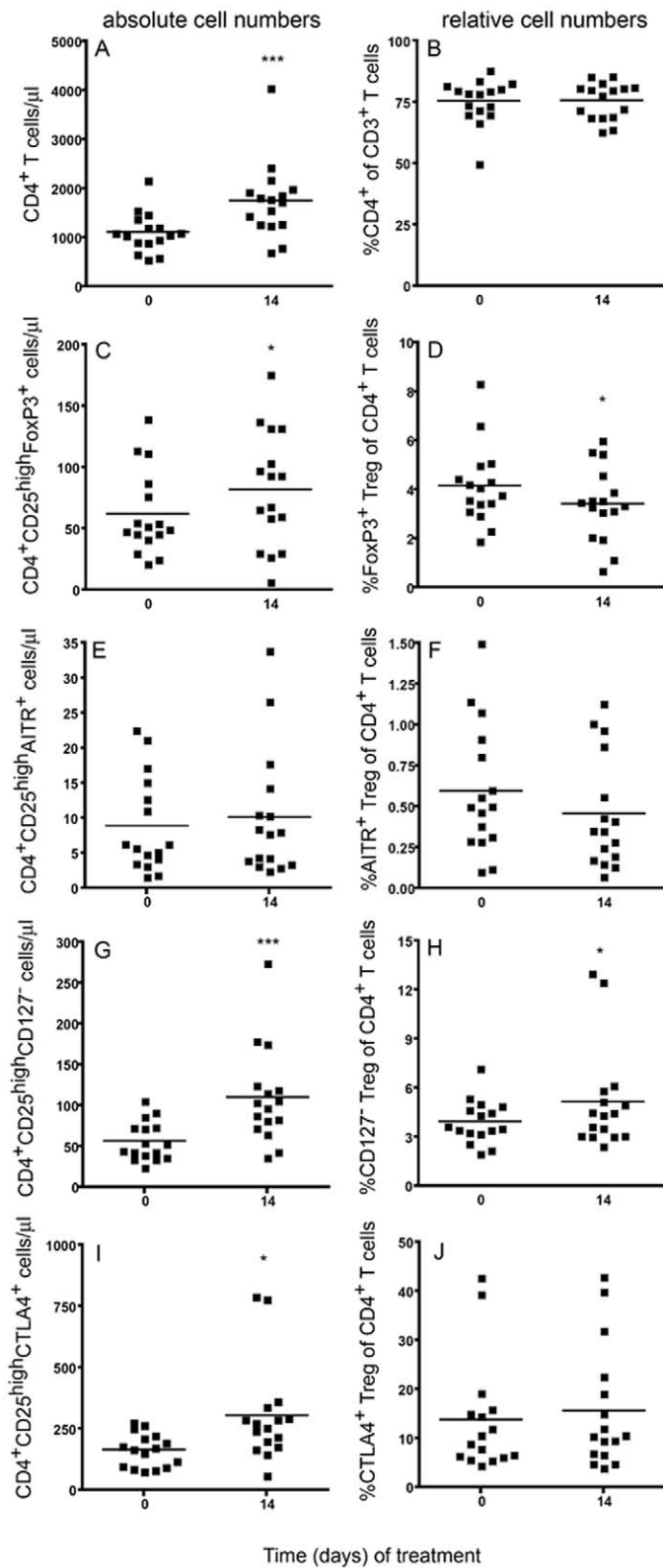


Figure 3. Modulation of CD4⁺ T cells and T_{reg} cells by GCs in humans. Peripheral blood cells from acute hearing loss patients before and 14 days after prednisolone treatment were analyzed by flow cytometry and the absolute numbers (A, C, E, G, I) and the frequency (B, D, F, H, J) of CD4⁺ T cells (A, B) and T_{reg} cells (CD4⁺CD25^{high} and FOXP3⁺ (C, D), AITR⁺ (E, F), CD127^{low} (G, H) or CTLA4⁺ (I, J)) were assessed; *p<0.05, ***p<0.001. doi:10.1371/journal.pone.0024345.g003

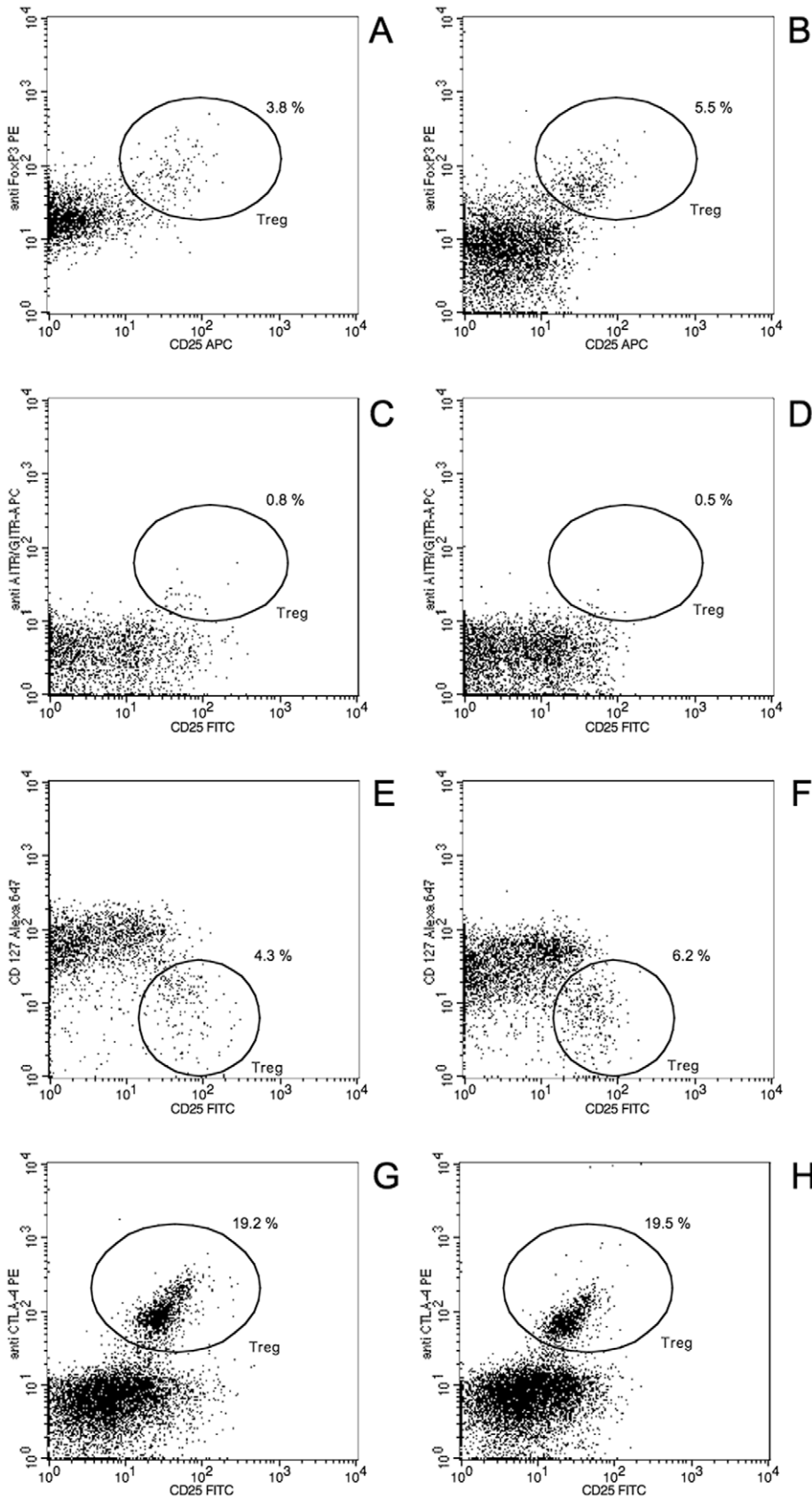


Figure 4. Flow cytometric analysis of T_{reg} cells according to different markers. Peripheral blood from one representative hearing-loss patient treated for 3 days (A, C, E, G) and 14 days (B, D, F, H) with glucocorticoid regimen was analyzed for the presence of regulatory T cells according to the following markers: $CD4^+ CD25^{high}$ and $FOXP3^+$ (A, B), $AITR^+$ (C, D) $CD127_{low}$ (E, F) and $CTLA4^+$ (G, H). Only $CD4^+$ cells are depicted and the percentages indicate the relative frequency of T_{reg} cells within this subpopulation. doi:10.1371/journal.pone.0024345.g004

of T_{reg} cells with other immune cells than increasing their frequency.

Our results are seemingly in contrast to several previously published *in vivo* studies that showed an increased number of T_{reg} cells and, even more importantly, an elevated $T_{reg}^{reg}/CD4^{+}$ T cell ratio in humans treated with GCs [44,45,50]. However, as mentioned before, most of these studies were performed in patients either suffering from hyperimmune or autoimmune diseases [51,52,53,54]. Interestingly, the same studies show a deficiency of T_{reg} cells in the same patients, probably due to thymic dysfunction, and thus the GC treatment seems to only restore their numbers to levels of healthy subjects. Based on our data we therefore hypothesize that GCs induce a tolerogenic environment in these patients leading to the generation of T_{reg} cells in the secondary lymphatic organs which compensates for the thymic dysfunction by creating an apparent advantage of the T_{reg} cells as compared to the rest of the T cells. However, the reconstitution of the T_{reg} cells will last only until the therapy ends and the *de novo* generated T_{reg} cells are recycled, which might explain the short-lived benefit and the high relapse rate described after GC therapy [65].

Our study has strengths and limitations. Firstly, the individuals of our study cohort consisting of patients with acute hear loss are most likely immunocompetent although the exact cause of the acute hear loss is unknown [66,67]. Secondly, the dosage of GCs in our prospective study was chosen rather arbitrarily and might have impact on the results. Of note, 5 of 7 patients tested on day 14 showed impaired adrenocortical function as measured by ACTH stimulation test (data not shown). In the case of our mouse

study, we administered a regimen consisting of increasing doses of dexamethasone [43], whereas in the human study we used the standard dosage for hear loss, which is also utilized for several autoimmune diseases such as lupus erythematosus [68] or idiopathic thrombocytopenic purpura [69]. Thirdly, we analyzed only selected time points and it thus remains open whether the effects that we see are only transient and would be different after shorter or longer time periods of GC treatment. Finally, we used several markers for T_{reg} cell characterization, acknowledging the fact that there is still no consensus on the best definition of T_{reg} cells in humans. However, despite the results being slightly different between each T_{reg} cell characterization, the effect on the relative frequency of circulating T_{reg} cells in general is weak.

In conclusion, short-term GC therapy did not induce the expected increase in the frequency of circulating T_{reg} cells, neither in immunocompetent human subjects nor in mice. In the same time GC treatment *in vitro* did not have any direct effect on the functional ability of the T_{reg} cells. Thus, it is doubtful whether GCs exerts their immunosuppressive effects via influencing the functionality and the (relative) number of T_{reg} cells in blood and spleen.

Author Contributions

Conceived and designed the experiments: S. Sbiera RM BA HMR MF. Performed the experiments: TD SDR KDM JvdB S. Schmall LK MB. Analyzed the data: S. Sbiera BA HMR MF. Contributed reagents/materials/analysis tools: RM SDR SW HMR. Wrote the paper: S. Sbiera TD HMR MF.

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