Title

Cushing syndrome and glucocorticoids: T-cell lymphopenia, apoptosis, and rescue by IL-21.

Permalink

Journal

Authors

Hwang, SuJin
Tatsi, Christina
Kuehn, Hye
et al.

Publication Date

2022

DOI

Peer reviewed

Cushing syndrome and glucocorticoids: T-cell lymphopenia, apoptosis and rescue by IL-21

SuJin Hwang, Ph.D.[#1], Christina Tatsi, M.D., Ph.D.[#2], Hye Sun Kuehn, Ph.D.[#1], Julie E. Niemela, MS, MLS.[1], Jennifer Stoddard, BS[1], Yan Su, BS[1], Maya Lodish, M.D., MHSc.[2], Gulbu Uzel, M.D.[3], Rosanne Spolski, Ph.D.[4], Warren J Leonard, M.D.[4], Steven M Holland, M.D.[3], Thomas A Fleisher, M.D.[1], Constantine A. Stratakis, M.D., D(Med)Sc.[2], Sergio D. Rosenzweig, M.D., Ph.D.[1,*]

[1.]Immunology Service, Department of Laboratory Medicine, NIH Clinical Center, NIH, Bethesda, MD, USA, 20892

[2.]Section on Endocrinology and Genetics, Eunice Kennedy Shriver NICHD, NIH, Bethesda, MD, USA, 20892

[3.]Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda, MD, USA, 20892

[4.]Laboratory of Molecular Immunology, Immunology Center, NHLBI, NIH, Bethesda, United States

[#] These authors contributed equally to this work.

Abstract

Background: Pediatric endogenous Cushing syndrome is mainly caused by pituitary corticotropin-producing adenomas and most glucocorticoid-dependent effects progressively regress upon tumor removal. Endogenous Cushing syndrome reproduces long-term high-dose glucocorticoid therapy, representing a clean, natural and unbiased model in which to study glucocorticoid *bona-fide* effects on immunity.

Objectives: To perform extensive immunologic studies in otherwise healthy pediatric patients with endogenous Cushing syndrome before and 6–13 months after tumor resection, as well as in *in-vitro* glucocorticoid-treated control cells.

Methods: Flow cytometry, immunoblotting, ELISA, qRT-PCR and RNA-seq techniques were used to characterize patients' and *in-vitro* glucocorticoid treated cells.

Results: Reduced thymic output, decreased naïve T cells, diminished proliferation, and increased T-cell apoptosis were detected before surgery, and all these defects eventually normalized after tumor removal in patients. *In-vitro* studies also showed increased T-cell apoptosis, with

*Corresponding author: Sergio D. Rosenzweig, M.D., Ph.D., Immunology Service, Dept. of Laboratory Medicine, NIH Clinical Center, NIH; 10 Center Dr., 2C410F, Bethesda, MD, 20892; (301) 451-8971; srosenzweig@cc.nih.gov.

correspondingly diminished NF-κB signaling and IL-21 levels. In this setting, IL-21 addition upregulated anti-apoptotic BCL2 expression and rescued T-cell apoptosis in a PI3K pathway-dependent manner. Similar and reproducible findings were confirmed in endogenous Cushing syndrome patient cells as well.

Conclusions: We identified decreased thymic output and lymphocyte proliferation, together with increased apoptosis, as the underlying causes to T-cell lymphopenia in endogenous Cushing syndrome patients. IL-21 was decreased in both natural and *in-vitro* long-term high-dose glucocorticoid environments, and *in-vitro* addition of IL-21 counteracted the pro-apoptotic effects of glucocorticoid therapy. Thus, our results suggest that administration of IL-21 in patients receiving long-term high-dose glucocorticoid therapy may contribute to ameliorate lymphopenia and the complications associated to it.

Graphical Abstract
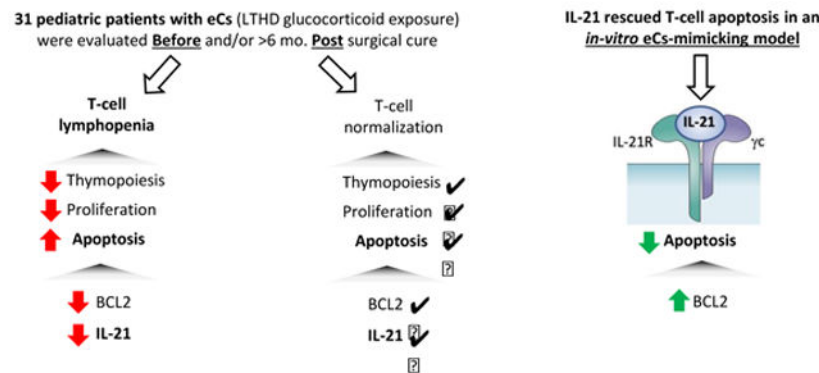
eCs: endogenous Cushing syndrome; LTHD: long-term high-dose; Check marks indicate within normal ranges; Red arrows indicate abnormal results; Green arrows indicate improved results

Capsule summary:

Pediatric patients with endogenous Cushing syndrome, and an *in-vitro* model, were evaluated to determine the *bona-fide* glucocorticoid effects on immunity. IL-21, regulated by the NF-κB and acting through the PI3K pathways, controlled glucocorticoid-mediated T-cell apoptosis.

Keywords

NF-κB; PI3K; BCL2; cytokines; interleukins; infections

Introduction

Glucocorticoids have been part of the medical armamentarium for almost 80 years, first as replacement therapy for adrenal insufficiency starting in the 1930s and then for the treatment of rheumatologic diseases beginning in the 1940s[1]. Since then, long-term high-dose (LTHD) glucocorticoid treatment has been the therapeutic cornerstone of an extensive list of diseases including but not restricted to rheumatologic diseases (e.g., systemic lupus erythematous, polyarteritis nodosa, Churg-Strauss disease, giant cell arteritis, and rheumatoid arthritis among others), renal diseases (e.g., nephrotic syndrome and

membranous glomerulonephritis), allergic diseases (e.g., atopic dermatitis), pulmonary diseases (e.g. asthma, allergic bronchopulmonary aspergillosis), dermatological disease, gastrointestinal diseases, liver diseases, as well as non-malignant and malignant hematologic diseases (e.g., autoimmune thrombocytopenia, leukemias and lymphomas)[2–4]. Taken in aggregate, these various conditions affect ~1% of adults living in developed counties and receiving LTHD glucocorticoid treatment, and this percentage is increasing[5]. In a study conducted between 1999 and 2005 based on the National Health and Nutrition Examination Survey (NHANES), the weighted prevalence of LTHD glucocorticoid use in the United States among individuals   20 was 1.2% (95% CI 1.1–1.4)[6], and a study run over 20 years in the United Kingdom in individuals   18 years old showed, LTHD glucocorticoid treatment in 0.75%, with a 34% increase in use over the course of the study[7].

As the use of LTHD glucocorticoid has increased over the years, its effects on immune system cells have become more evident[8, 9]. However, recognition of the specific *bona-fide* effects of LTHD glucocorticoid therapy on immune function have been limited by comorbidities and the underlying confounding immunologic conditions in patients receiving such therapy[10–13]. Here, we focused our studies on pediatric endogenous Cushing syndrome (eCs) patients with corticotropin (ACTH)-producing pituitary adenomas as a means of studying long-term effects of steroids *in-vivo*. All patients were previously healthy and lacked comorbidities. Importantly, their eCs phenotype resembled LTHD glucocorticoid use, and clinical and immunological parameters tended to progressively normalize over 6–13 months after successful tumor removal (Post). Our immunological studies were performed pre- and post-surgery, representing LTHD glucocorticoid therapy and withdrawal. Moreover, beyond characterizing the LTHD glucocorticoid *bona-fide* impact on human immunity, we also explored the role of cytokines in particular IL-21, rescuing such effects. As previously shown by our and other groups, IL-21 seemed to have a protective and restorative effect on murine models regarding thymopoiesis/lymphopoiesis under particular conditions, including aging, hematopoietic stem cell transplantation and glucocorticoid treatment[14, 51–54].

Methods

Patients

All study participants and their parents provided assent and informed consent, respectively, to this study, which was approved by the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) institutional review board (IRB). Samples from 31 pediatric patients with eCs due to ACTH-producing adenoma were collected and evaluated as follows: paired samples from 15 eCs patients (age range 7–16 years old, 8 females) were collected before and 6–13 months post-surgical treatment (Post); non-paired samples from 8 eCs (age range 11–16 years old, 6 females) and 8 Post (age range 9–19 years old, 4 females) different patients were also available for study.

Studies

Immunological studies included: a) white blood cell counts and extensive lymphocyte subset enumeration, including extracellular and intracellular proteins, T cell receptor (TcR) Vβ repertoire, as well as functional responses to cytokine stimulation; b) lymphocyte

proliferation to mitogens and TcR stimulation; c) lymphocyte apoptosis in response to glucocorticoids and other apoptosis inducers in the presence or absence of IL-2, IFN-α or IL-21; d) IL-21 plasma levels, mRNA expression, and intracellular protein expression; e) IκBα degradation; and f) RNAseq on CD4+ naïve T cells. Technical details are described in the Supplementary Appendix, Materials and Methods.

Results

eCs patient's diagnosis and characterization

Diagnosis of eCs in the 31 patients studied was established according to the Endocrine Society guidelines by increased midnight serum cortisol and urinary free cortisol levels as previously reported[15–19] (Figure 1A). Diagnosis of ACTH-producing adenoma was confirmed by histopathology analysis in each case (not shown). Patient demographic data is summarized in Table S1.

T cell lymphocytes in eCs patients

We first analyzed cell counts in eCs and Post pediatric patients. Total white blood cell (WBC) and polymorphonuclear leukocytes (PMN) cell counts were both markedly increased in eCs patients; monocytes and dendritic cells showed no statistical differences between the two groups; and lymphocytes were significantly lower in eCs than in Post patients (Figure 1B, Figure S1A Table S2). These results are consistent with findings previously reported in patients with eCs and patients undergoing glucocorticoid treatment[15–19]. We next further characterized the lymphocyte subsets in eCs and Post clinical settings. No significant differences were detected in the absolute number of B cells and NK cells (Figure S1B–D, Table S2), but marked differences were observed in T cells, with paired patient samples showing significantly reduced T cells in the eCs condition as compared to samples at 6–13 months post-surgery (Post) (Figure 1C). This effect was greatest on CD4+ T cells, whereas no significant difference in absolute CD8+ T cell numbers was observed between eCs and Post (Figure 1D). Significantly lower total CD4+ T cells were detected in the eCs condition, as was the case when CD4+ specific T cell subsets, including recent thymic emigrants (RTE) (Figure 1E), naïve CD4+ T cells (Figure 1F), CD4+ T regulatory (Treg) cells (Figure S1E) and T follicular helper (Tfh) cells (Figure S1F), were individually evaluated. Evaluation of Th0, Th1, Th2 and Th17 populations based on their intracellular cytokine production pattern (e.g., IL-2, IFNγ, IL-4, and IL-17), demonstrated no change in relative percentage were detected between eCs and Post conditions (Figure S2A). Moreover, no differences were detected either in T cell receptor (TcR) variable beta ($V_β$) family distribution in CD4+ or CD8+ T cells (Figure S3) or absolute CD57+ and PD-1+ senescent CD8+ cells in eCs versus post patients (Figure S1G and H). While the low lymphocyte thymic output, as evidenced by significantly reduced RTE cells, likely contributed to the T cell lymphopenia in the eCs patients under LTHD glucocorticoid exposure, other possible mechanisms were also evaluated. Lymphocyte proliferation by both CD4+ and CD8+ T cells was markedly decreased in response to TcR-mediated stimulation (anti-CD3 + anti-CD28 antibodies) or mitogen (phytohemagglutinin) in the eCs condition (Figure 2A). As glucocorticoids are known to induce lymphocyte apoptosis[20, 21], we also assessed apoptosis in eCs and Post patient cells using Annexin V staining of total CD4+ and CD8+ T cells, as well as of

naïve CD4$^+$ and naïve CD8$^+$ T cells. Annexin V interacts strongly and specifically with phosphatidylserine, a phospholipid that is externalized relatively early during apoptosis. While other methods are available, Annexin V corresponding signal provides a sensitive, specific and validated method for detecting cellular apoptosis[22–26]. Apoptosis was strongly increased in CD4$^+$ and CD8$^+$ T cells in general, particularly in naïve cells in eCs patients (Figure 2B, C). Taken together, these findings suggest that lymphopenia in eCs patients is multifactorial and may be explained by the sum of reduced thymic output of T cells, reduced proliferation capacity (to both TcR-dependent and - independent triggers), and increased levels of apoptosis-mediated T-cell death.

Cytokine modulation of apoptosis and IL-21-mediated effects

To evaluate apoptosis and its cytokine-mediated modulation using an eCs-mimicking condition, peripheral blood mononuclear cells (PBMC) from healthy controls (HC) were incubated *in-vitro* with dexamethasone 1.0 μg/mL as reported elsewhere[27–29] to reproduce the apoptosis levels detected in eCs patients. Primary CD4$^+$ T cells from eCs patients' PBMC showed apoptosis levels 10.2–34.1% (Figure 2C). When different concentrations of dexamethasone were evaluated (i.e., 0/.001/0.01/0.1/0.2/0.5/1.0/2.0/5.0/10.0 μg/mL) on HC PBMC, apoptosis ranges similar to those shown on eCs patients' CD4$^+$ T cells were achieved on the 0.1–1.0 μg/mL dose range (Figure S4). Dexamethasone was chosen as it is a long-acting glucocorticoid that does not require re-stimulation during overnight culture periods (consistent with LTHD glucocorticoid effect).

Prednisolone 1 and 5 μg/mL were also evaluated in the absence or presence of IL-2, IFNα and IL-21 to evaluate their modulatory effects of apoptosis associated with another type of glucocorticoid exposure (Figure S5). Apoptosis was determined in total CD4$^+$ and CD8$^+$ T cells as well as in naïve CD4$^+$ T cells. (Figure 3A, Figure S6A). While incubation of PBMC from HC with glucocorticoids increased apoptosis of total and naïve CD4$^+$ T cells (arbitrarily defined as 100%), the addition of IL-2 or IFNα reduced it by ~50%, whereas addition of IL-21 to control PBMC under the same conditions, reduced glucocorticoid-induced apoptosis by more than 80%, representing a level of apoptosis similar to that seen at baseline/non-glucocorticoid stimulated condition (Figure 3A and 3B). To determine the specificity of the IL-2, IFNα and IL-21-mediated rescue of apoptosis in glucocorticoid-treated cells, we tested their effects on staurosporine- and FAS-mediated apoptosis. Neither IL-2, IFNα, or IL-21, modulated apoptosis upon stimulation with either of these pro-apoptotic agents (Figure S7). Next, we evaluated the expression of BCL6, an IL-21-regulated molecule; and BCL2, an anti-apoptotic molecule. As anticipated, BCL6 expression was only upregulated in the glucocorticoid+IL-21 stimulated condition, but not in non-glucocorticoid stimulated cells, glucocorticoid stimulated cells or following IL-2 or IFNα addition in naïve CD4$^+$ T cells (Figure 3C) or CD8$^+$ T cells Figure S6B). On the other hand, while no cytokine alone modulated BCL2 expression, its level was upregulated in response to glucocorticoid+IL-21 in CD4$^+$ (Figure 3D and E) and CD8$^+$ (Figure S6C) T cells. When we assessed apoptosis in PBMC from eCs patients who were exposed to endogenous LTHD glucocorticoids (Figure S8A), IL-21 was again more anti-apoptotic than IL-2, and this correlated with a specific upregulation of BCL2 levels; paralleled increases in BCL6 were also detected, linking IL-21 signaling to these effects (Figure S8B and C).

To further assess the role of BCL2 as the anti-apoptotic driver following IL-21stimulation in glucocorticoid-stimulated cells and to discriminate it from the potential role of BCL6, we tested the effects of the inhibitor of BCL2 (iBCL2) S55746 and the inhibitor of BCL6 (iBCL6) 79–6 on BCL2 and BCL6 expression and the level of apoptosis in unstimulated, as well as glucocorticoid, and glucocorticoid+IL-21 stimulated cells. First, we measured the effect of iBCL2 and iBCL6 on BCL2 and BCL6 expression (Figure S9). While glucocorticoids decreased and IL-21 rescued BCL2 expression, iBCL2 and iBCL6 had no direct effect on BCL2 expression in any of the testing conditions. Regarding BCL6, glucocorticoids had minimal or no effect on BCL6 baseline expression, but addition of IL-21 markedly upregulated it in the absence or presence of iBCL2 and iBLC6. Moreover, addition of iBCL6 did not affect BCL6 expression either by itself, with or without glucocorticoids or IL-21. When the antiapoptotic effects of iBCL2 and iBCL6 were evaluated (Figure 3F), iBCL2 but not iBCL6 increased the apoptosis rates in unstimulated cells (from ~23% to ~47%). When cells were incubated with glucocorticoids or glucocorticoids+iBCL6, apoptosis was increased similarly (both ~50%) but was further increased following dexamethasone+iBCL2 treatment (~80%). Finally, IL-21 was able to rescue apoptosis in cells incubated with glucocorticoid or glucocorticoid+iBCL6 (~25%), but not after glucocorticoid+iBCL2 incubation (~75%). It is noteworthy that the levels of apoptosis found in cells treated with glucocorticoid+iBCL2 or glucocorticoid+IL-21+iBCL2 were very similar (~80% vs. ~75%) (Figure 3G). Other BCL2-family members, including MCL1 (anti-apoptotic) and BIM (pro-apoptotic), had no effect on apoptosis after cytokine stimulation (i.e., IL-2, IFNα or IL-21), in the presence or absence of glucocorticoids (Figure S10A and B). These data confirm that the IL-21 mediated antiapoptotic effect seems almost exclusively dependent on BCL2 with very little evidence for the involvement of other BCL2-family members, including no antiapoptotic role for BCL6 detected in this model system either.

On further testing, the rescue of apoptosis and modulation of BCL2 and BCL6 expression by IL-21 were not observed in cells from an *IL21R*-deficient patient carrying a known, disease-causing homozygous mutation (*IL21R,* c.240_245delCTGCCA; p.Cys81_His82del), providing extra support that the anti-apoptotic effects observed were dependent on IL-21 signaling (Figure S11A–C). Taken together these results show that glucocorticoids, whether exogenous or endogenous, are inducers of T-cell apoptosis. Furthermore, although glucocorticoid-induced apoptosis was decreased by the addition of IL-2, IFNα or IL-21, IL-21 had the most dramatic anti-apoptotic effect and this effect correlated with increased expression of the anti-apoptotic molecule BCL2.

IL-21 biology in eCs and Post patients

To evaluate IL-21 biology in patients under LTHD glucocorticoid exposure, we measured IL-21 plasma concentrations, IL-21 receptor (R) expression and IL-21 signaling in eCs and Post patients. While IL-21R expression (Figure S12) and IL-21 (as well as IL-2 and IFNα signaling (Figure S13) were not different among eCs, Post, and HC, IL-21 levels in plasma were significantly lower in the eCs patients when compared to HC or Post patients (Figure 4A). Furthermore, IL-21 mRNA levels correlated with IL-21 plasma levels in all clinical settings (Figure 4B), strongly suggesting that a glucocorticoid-mediated

reduction in IL-21 transcription underlies the lower plasma levels of the cytokine in eCs patients. To confirm these results, we evaluated IL-21 intracellular expression in our eCs-mimicking *in-vitro* model (PBMC from HC incubated with glucocorticoids), demonstrating a marked reduction in this cytokine too (Figure 4C). We then explored the mechanism by which IL-21 is regulated in a high glucocorticoid exposure condition. We first analyzed the IL-21 gene structure *in silico* and found three canonical glucocorticoid responsive elements (GRE) in the cytokine promoter region, suggesting a direct regulation of IL-21 by glucocorticoids (Figure S14). Moreover, IL-21 gene transcription and expression is known to be up-regulated by the NF-κB family transcription factor c-Rel[30], and glucocorticoids are potent inhibitors of NF-κB activation through induction of IκBα synthesis[31, 32]. Interestingly, the IκBα promoter itself also contains GRE that presumably mediate its gene expression by binding to GR[33]. In this regard, we assessed the kinetics of IκBα levels following phorbol-myristate-acetate (PMA)/Ionomycin (Iono) stimulation in eCs and Post patients, as well as in HC. Stimulated PBMC from HC, eCs and Post patients showed a similar time course of IκBα levels with a nadir at 30 min and a rebound peak expression at 60 min. However, the baseline levels of IκBα, as well as the difference between nadir and peak and the IκBα peak level at 60 min. were significantly increased in eCs when compared to HC and Post patients (Figure 4D and E). To explore the relation between NF-κB signaling and IL-21 production, we tested IL-21 mRNA and protein levels in PBMC from HC and a patient with a known, disease-causing hemizygous *IKBKG/NEMO* mutation (*IKBKG,* c.−16+1G>C) leading to reduced NF-κB signaling. While HC cells elicited a strong IL-21 mRNA and protein production response following PMA/Iono stimulation, this effect was virtually abrogated after glucocorticoid incubation and mimicked the null response observed in PBMC carrying an *IKBKB/NEMO* mutation impairing NF-κB signaling (Figure 4F and G; of note, *IKBKB/NEMO* cells showed normal proliferative responses to TcR stimulation with anti-CD3+anti-CD28, data not shown).

As IL-21 has been reported to sustain inflammatory signals[34], we tested it effects on canonical NF-κB signaling of eCs and Post patients' cells by evaluating p65 phosphorylation in response to IL-21 and TNF-α. As seen in Figure 4H, there was no evidence of canonical NF-κB signaling by IL-21 alone or any potentiation of the effect of TNF-α in any of the conditions tested. Taken altogether, these data suggest that LTHD glucocorticoid exposure reduces the transcription and translation of IL-21 possibly by directly regulating its promoter, but also in a NF-κB canonical pathway signaling-dependent manner by increasing IκBα baseline and rebound levels. Importantly, despite the low IL-21 transcripts and protein levels, IL-21R expression is sustained and responsiveness to this cytokine remained intact upon extrinsic IL-21 stimulation in eCs patient cells. Moreover, addition of IL-21 to eCs or Post patients' cells does not seem to increase NF-κB-dependent inflammation or sustain NF-κB activation through other agonists (i.e., TNF-α), arguing against a potential deleterious consequence of IL-21 use in a glucocorticoid-environment.

Transcriptomic effect of LTHD glucocorticoid exposure in lymphocytes

To further evaluate the global transcriptional impact of glucocorticoid on CD4+ naïve T cells (the lymphocyte subset most affected by LTHD glucocorticoid exposure), we performed RNAseq analysis of these cells (>90% enriched from PBMC) at their baseline state in eCs

and Post patients, as well as HC. Correlation analysis with hierarchical clustering revealed significantly differentially expressed transcripts between naïve CD4+ cells from eCs patients when compared with either HC or Post patient cells. No differences were detected when naïve CD4+ T cells from HC and Post patients were compared (Figure 5A). When the analysis was stratified on immune-related genes, FOS and FOSL2 were among the top differentially transcribed (reduced) in eCs vs. Post patients (Figure 5B, Table S3). We also performed Ingenuity Pathway Analysis (IPA) of the differentially expressed (DE) genes (eCs vs. Post and HC) and built a custom pathway by combining information from the Ingenuity Pathways Knowledge Base (IPKB) and our in-house experimental data (Figure S15). This analysis revealed glucocorticoid-induced downregulation of genes encoding Ap1 complex proteins (*FOS, FOSL2, FOSB, ATF3*), as well as downstream pro-inflammatory cytokines (*IFNG, IL1B*) and cytokine receptor (*IFNGR1*), with concomitant downregulation of PI3K (*IRS1, IRS2, GAB2, FOS*) and NF-κB (*REL, BCL3, BCL6*) signaling. Although a single transcript of *BCL2* was targeted and covered by the RNAseq experiment, it was not determined to be differentially expressed. Nonetheless, the lower transcription of *BCL6* and *BCL3*, whose antiapoptotic gene products affect BCL2 expression, was consistent with the lower BCL2 protein expression and increased apoptosis that we observed in separate experiments. The under-expression of *IRS1* and *IRS2*, whose gene products bind BCL2, also correlated with the lower BCL2 protein expression observed separately. The PELI1 under-expression was consistent with the *BCL6* under-expression and is another indicator of downregulated NF-kB activation. As noted above, *FOS* and *FOSL2* were among the top DE genes (decreased) in eCs vs. Post patients (Figure 5B, Table S3). The FOS gene family consists of 4 members: *FOS*, *FOSB*, *FOSL1*, and *FOSL2*, encoding leucine zipper proteins that dimerize with proteins of the JUN family, forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation[35]. While PI3 Kinase (PI3K) induces c-FOS transcription[36], IL-21 promotes T cell survival through the PI3K pathway[37].

We next evaluated phosphorylated S6 (pS6, downstream the PI3K/AKT/mTOR signaling pathway) in TcR stimulated CD4+ and CD8+ T cells followed by re-stimulation with cytokines (i.e., IL-2, IFNα and IL-21) in our eCs-mimicking *in-vitro* model (Figure 6A and S16). While TcR-stimulation strongly upregulated pS6 in the presence or absence of cytokines, glucocorticoids markedly decreased this. However, when cytokines were added to the system, IL-21 showed the strongest upregulation of pS6 with a ~50% higher induction of phosphorylation when compared to all the other cytokines tested. To evaluate the role of the PI3K pathway in the IL-21-dependent apoptosis modulating effect under glucocorticoid exposure, we added LY294002, a PI3K-specific inhibitor, in our eCs-mimicking condition (Figures 6B and C, S17, and S18). 10uM LY294002 effectively and almost completely inhibited S6 phosphorylation in T cell blasts upon TcR stimulation (Figure S17). As previously shown, IL-21 was able to rescue T cell apoptosis under glucocorticoid stimulation, but this effect was eliminated with PI3K inhibition (and apoptosis was further enhanced); the more limited apoptosis rescuing effects of IL-2 or IFNα were not affected by PI3K inhibition Figures 6B and S18). Interestingly, LY294002 by itself was able to induce apoptosis to similar levels as glucocorticoid, but neither additive or synergistic effects were evidenced when LY294002+glucocorticoid were used together

in the presence of cytokines other than IL-21 (Figures 6B and S18A). As previously determined, IL-21 was also able to upregulate BCL2 expression to a greater extent than the other cytokines, but this effect was also abrogated by PI3K inhibition (Figures 6C and S18B). Together, these experiments demonstrate that LTHD glucocorticoids in eCs patients not only affect naïve CD4 T cells quantitively (as concluded from previous experiments) but also functionally at their transcriptomic profile (as determined by the RNAseq results), but these effects were no longer observed 6–13 months post successful surgical therapy. The PI3K pathway was particularly affected by the lower transcription of FOS and FOSL2 in LTHD glucocorticoid-exposed eCs patients. Although the contribution of other parallel pathways cannot be formally excluded, signaling through the PI3K pathway was both central and specific to the IL-21-dependent apoptosis rescue, as blocking this pathway decreased the expression of the antiapoptotic molecule BCL2, markedly increasing the level of apoptosis.

Discussion

Endogenous Cushing syndrome in children is a rare disorder most frequently caused by pituitary tumors[38]. This rare condition is also a natural model in which to evaluate the *bona fide* effects of glucocorticoids on immunity, among other systems. While the effects of gonadal or non-glucocorticoid adrenocortical steroids on the immune system cannot be completely ruled out, they are likely minor or neglectable in the conditions studied. In the course of pediatric eCs, patients have the hormonal changes associated with puberty arrested and for about a year following successful surgery. Moreover, mineralocorticoids are typically suppressed because the elevated circulating cortisol binds to the mineralocorticoid receptor as well[39–41]. On the other hand, the study of healthy controls following single doses of glucocorticoids, although interesting, does not reflect the situation of common medical practice involving protracted glucocorticoid administration[42]. In our study we not only found T cell lymphopenia associated with eCs and LTHD glucocorticoid treatment/exposure consistent with previously reports, but here we have markedly expanded what is known in this setting by establishing reduced thymic output, decreased lymphocyte proliferation, and increased apoptosis as critical regulators of glucocorticoid-induced lymphopenia. We also identified and mechanistically defined the roles of several cytokines that affect glucocorticoid-mediated apoptosis. IL-21 had a distinctive effect on glucocorticoid-exposed T cells by preventing apoptosis and restoring more normal T-cell homeostasis. This effect was specific, as IL-21 did not modulate the apoptotic effects of anti-Fas or staurosporine on T cells. Through our work we showed that IL-21 acts by augmenting expression of BCL2 and thereby promoting cellular survival and diminishing apoptosis in a PI3K-dependent manner[43, 44]. In fact, blocking PI3K signaling using a specific inhibitor, or testing cells from a patient with IL-21 receptor deficiency, prevented BCL2 upregulateion following glucocorticoid+IL-21 stimulation, that was associated with persistence of increased T-cell apoptosis (glucocorticoid effects on T cells summarized in Figure S19).

When IL-21 mRNA and protein levels were analyzed, both were significantly reduced in eCs patients but restored 6–13 months postoperatively, while other cytokines examined were not affected.

Glucocorticoids negatively regulate the NF-κB canonical pathway by means of increased IκBα levels, which seems to play a central role in IL-21 regulation. Normally following stimulation, IκBα is phosphorylated, ubiquitinated, and degraded in the cytosol, allowing the NF-κB transcription factors c-Rel/p65 and p50 to be translocated to the nucleus to initiate transcription of NF-κB-regulated genes. The markedly increased IκBα levels detected in LTHD glucocorticoid-exposed eCs patient cells both at baseline and 60 minutes following stimulation prevented this response, thereby diminishing IL-21 transcription and translation. This effect was replicated using cells from a patient with an *IKBKG/NEMO* defect, which prevented c-Rel/p65 and p50 nuclear translocation and was associated with virtually absent IL-21 mRNA and protein levels. These results were also confirmed using HC PBMC that were incubated *in-vitro* with glucocorticoids.

Glucocorticoids can regulate the expression of particular genes by direct GRE binding, or indirectly via tethering to other proteins such as the AP-1 family of transcription factors[45]. Both the presence of GRE (Fig. S14) and preferential utilization of AP-1/JUN[46] have been documented for the IL-21, strongly supporting the glucocorticoid-mediated IL-21 regulation hypothesis we propose.

For our eCs mimicking *in-vitro* model we used HC PBMC incubated overnight with dexamethasone 1μg/mL. While this model was carefully selected, it does not exactly technically mimic the long-term aspect of LTHD glucocorticoid exposure in eCs patients who are glucocorticoid exposed for weeks/months prior to their surgical treatment. On the other hand, this *in-vitro* model does reproduce the glucocorticoid-dependent effects on T cells from eCs patients, particularly when evaluating apoptosis and IL-21 modulation. Besides, we also demonstrated that these effects are glucocorticoid-dependent but not dexamethasone-specific as similar results were obtained when prednisolone, a more commonly used glucocorticoid clinically, was tested.

These data provide novel insights and possible therapeutic options to explore in LTHD glucocorticoid-treated lymphopenic individuals affected with severe opportunistic infections. IL-2 and IL-7 have been used in cancer immunotherapy, as well as some immune deficient states[47, 48]. Recombinant human (rh) IL-21 has been both well tolerated and exhibits antitumor effects in metastatic melanoma and renal cancers[49]. Primarily produced by Tfh and Th17 T cells, and to a lesser extent by NKT cells, IL-21 is well known to elicit pleiotropic responses that affect the differentiation and function of lymphoid and myeloid cells[50]. Of note, the effects of IL-21 observed in therapeutic models to date seem to differ from those induced by IL-21 when in a glucocorticoid-rich environment, as described both in mouse models and herein[51–54]. In preclinical studies conducted on non-human primates (Cynomolgus Macaques), rhIL-21 generated a dose-dependent thymic involution but did not result in changes in the total number of circulating T-lymphocytes, T-helper lymphocytes, or T-cytotoxic/suppressor lymphocytes[55]. In cancer patients, peripheral blood lymphocyte counts were reduced to less than half of baseline during rhIL-21 treatment, followed by a strong rebound after completion of cytokine treatment, likely caused by T cell activation/migration/redistribution[49]. These data suggest that glucocorticoid sufficiently alters thymocytes and T cells in such a way that addition of IL-21 causes distinctive functional effects under these different circumstances.

In summary, our dissection of the immunophenotype and mechanisms for T cell lymphopenia in pediatric patients with eCs before and 6–13 months after surgical cure of their disease has identified the *bona fide* effects of LTHD glucocorticoid on the immune system and its function, that is directed primarily on T cells. Our testing model of an *in-vitro* system to assay therapeutic alternatives (e.g., rhIL-21) allows us to develop and test agents to diminish the lymphopenia and lymphoid dysfunction that threaten so many.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations:

ACTH	corticotropin
BCL	B-cell lymphoma
eCs	endogenous Cushing syndrome
GRE	glucocorticoid responsive elements
HC	healthy controls
iBCL	inhibitor of BCL
IFN	interferon
IL	interleukin
IL21R or IL-21R	IL-21 receptor
Iono	Ionomycin
IPKB	Ingenuity Pathways Knowledge Base
LTHD	long-term high-dose
NF-κB	nuclear factor-kappa B
PBMC	peripheral blood mononuclear cells

PI3K	phosphatidylinositol 3- kinase
PMA	phorbol-myristate-acetate
PMN	polymorphonuclear leukocytes
pS6	phosphorylated S6
RTE	recent thymic emigrants
TcR	T cell receptor
Tfh	T follicular helper
TNF	tumor necrosis factor
WBC	white blood cells

References

1. Hench P Effects of cortisone in the rheumatic diseases. Lancet 1950; 2:483–4. [PubMed: 14785191]

2. Flammer JR, Rogatsky I. Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms. Mol Endocrinol 2011; 25:1075–86. [PubMed: 21511881]

3. Griffin GD, Charron D, Al-Daccak R. Post-traumatic stress disorder: revisiting adrenergics, glucocorticoids, immune system effects and homeostasis. Clin Transl Immunology 2014; 3:e27.

4. Oppong E, Cato AC. Effects of Glucocorticoids in the Immune System. Adv Exp Med Biol 2015; 872:217–33. [PubMed: 26215996]

5. Fardet L, Petersen I, Nazareth I. Common Infections in Patients Prescribed Systemic Glucocorticoids in Primary Care: A Population-Based Cohort Study. PLoS Med 2016; 13:e1002024. [PubMed: 27218256]

6. Overman RA, Yeh JY, Deal CL. Prevalence of oral glucocorticoid usage in the United States: a general population perspective. Arthritis Care Res (Hoboken) 2013; 65:294–8. [PubMed: 22807233]

7. Fardet L, Petersen I, Nazareth I. Prevalence of long-term oral glucocorticoid prescriptions in the UK over the past 20 years. Rheumatology (Oxford) 2011; 50:1982–90. [PubMed: 21393338]

8. Migliorati G, Nicoletti I, Pagliacci MC, Riccardi C. Glucocorticoid-induced thymocyte apoptosis: inhibition by interleukin-2 and interleukin-4. Pharmacol Res 1992; 25 Suppl 1:15–6. [PubMed: 1508795]

9. Nieto MA, Gonzalez A, Gambon F, Diaz-Espada F, Lopez-Rivas A. Apoptosis in human thymocytes after treatment with glucocorticoids. Clin Exp Immunol 1992; 88:341–4. [PubMed: 1572099]

10. Azab NA, Bassyouni IH, Emad Y, Abd El-Wahab GA, Hamdy G, Mashahit MA. CD4+CD25+ regulatory T cells (TREG) in systemic lupus erythematosus (SLE) patients: the possible influence of treatment with corticosteroids. Clin Immunol 2008; 127:151–7. [PubMed: 18299252]

11. Braitch M, Harikrishnan S, Robins RA, Nichols C, Fahey AJ, Showe L, et al. Glucocorticoids increase CD4CD25 cell percentage and Foxp3 expression in patients with multiple sclerosis. Acta Neurol Scand 2009; 119:239–45. [PubMed: 18771523]

12. Baris HE, Baris S, Karakoc-Aydiner E, Gokce I, Yildiz N, Cicekkoku D, et al. The effect of systemic corticosteroids on the innate and adaptive immune system in children with steroid responsive nephrotic syndrome. Eur J Pediatr 2016; 175:685–93. [PubMed: 26833050]

13. Wirsum C, Glaser C, Gutenberger S, Keller B, Unger S, Voll RE, et al. Secondary Antibody Deficiency in Glucocorticoid Therapy Clearly Differs from Primary Antibody Deficiency. J Clin Immunol 2016; 36:406–12. [PubMed: 26980224]

14. Moretto MM, Hwang S, Chen K, Khan IA. Complex and Multilayered Role of IL-21 Signaling during Thymic Development. J Immunol 2019; 203:1242–51. [PubMed: 31341076]

15. Tatsi C, Boden R, Sinaii N, Keil M, Lyssikatos C, Belyavskaya E, et al. Decreased lymphocytes and increased risk for infection are common in endogenous pediatric Cushing syndrome. Pediatr Res 2018; 83:431–7. [PubMed: 29211058]

16. Masri-Iraqi H, Robenshtok E, Tzvetov G, Manistersky Y, Shimon I. Elevated white blood cell counts in Cushing's disease: association with hypercortisolism. Pituitary 2014; 17:436–40. [PubMed: 24078318]

17. Saffar AS, Ashdown H, Gounni AS. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. Curr Drug Targets 2011; 12:556–62. [PubMed: 21504070]

18. Cox G Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. J Immunol 1995; 154:4719–25. [PubMed: 7722324]

19. Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. Cell Mol Life Sci 2006; 63:60–72. [PubMed: 16314919]

20. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980; 284:555–6. [PubMed: 6245367]

21. Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function*. Annu Rev Immunol 2000; 18:309–45. [PubMed: 10837061]

22. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994; 84:1415–20. [PubMed: 8068938]

23. van Genderen H, Kenis H, Lux P, Ungeth L, Maassen C, Deckers N, et al. In vitro measurement of cell death with the annexin A5 affinity assay. Nat Protoc 2006; 1:363–7. [PubMed: 17406257]

24. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998; 31:1–9. [PubMed: 9450519]

25. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995; 182:1545–56. [PubMed: 7595224]

26. Cummings BS, Schnellmann RG. Measurement of cell death in mammalian cells. Curr Protoc Pharmacol 2004; Chapter 12:Unit 12 8.

27. Potter A, Kim C, Gollahon KA, Rabinovitch PS. Apoptotic human lymphocytes have diminished CD4 and CD8 receptor expression. Cell Immunol 1999; 193:36–47. [PubMed: 10202111]

28. Tonomura N, McLaughlin K, Grimm L, Goldsby RA, Osborne BA. Glucocorticoid-induced apoptosis of thymocytes: requirement of proteasome-dependent mitochondrial activity. J Immunol 2003; 170:2469–78. [PubMed: 12594272]

29. Torres-Roca JF, Tung JW, Greenwald DR, Brown JM, Herzenberg LA, Herzenberg LA, et al. An early oxygen-dependent step is required for dexamethasone-induced apoptosis of immature mouse thymocytes. J Immunol 2000; 165:4822–30. [PubMed: 11046005]

30. Chen G, Hardy K, Bunting K, Daley S, Ma L, Shannon MF. Regulation of the IL-21 gene by the NF-kappaB transcription factor c-Rel. J Immunol 2010; 185:2350–9. [PubMed: 20639489]

31. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS Jr. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 1995; 270:283–6. [PubMed: 7569975]

32. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 1995; 270:286–90. [PubMed: 7569976]

33. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997; 336:1066–71. [PubMed: 9091804]

34. De Simone V, Ronchetti G, Franze E, Colantoni A, Ortenzi A, Fantini MC, et al. Interleukin-21 sustains inflammatory signals that contribute to sporadic colon tumorigenesis. Oncotarget 2015; 6:9908–23. [PubMed: 25839161]

35. Bamberger AM, Milde-Langosch K, Rossing E, Goemann C, Loning T. Expression pattern of the AP-1 family in endometrial cancer: correlations with cell cycle regulators. J Cancer Res Clin Oncol 2001; 127:545–50. [PubMed: 11570575]

36. Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. Science 1995; 268:100–2. [PubMed: 7701328]

37. Ostiguy V, Allard EL, Marquis M, Leignadier J, Labrecque N. IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade. J Leukoc Biol 2007; 82:645–56. [PubMed: 17554014]

38. Lodish MB, Keil MF, Stratakis CA. Cushing's Syndrome in Pediatrics: An Update. Endocrinol Metab Clin North Am 2018; 47:451–62. [PubMed: 29754644]

39. Leong GM, Mercado-Asis LB, Reynolds JC, Hill SC, Oldfield EH, Chrousos GP. The effect of Cushing's disease on bone mineral density, body composition, growth, and puberty: a report of an identical adolescent twin pair. J Clin Endocrinol Metab 1996; 81:1905–11. [PubMed: 8626856]

40. Storr HL, Isidori AM, Monson JP, Besser GM, Grossman AB, Savage MO. Prepubertal Cushing's disease is more common in males, but there is no increase in severity at diagnosis. J Clin Endocrinol Metab 2004; 89:3818–20. [PubMed: 15292311]

41. Tatsi C, Stratakis CA. Pediatric Cushing Syndrome; an Overview. Pediatr Endocrinol Rev 2019; 17:100–9. [PubMed: 31763802]

42. Franco LM, Gadkari M, Howe KN, Sun J, Kardava L, Kumar P, et al. Immune regulation by glucocorticoids can be linked to cell type-dependent transcriptional responses. J Exp Med 2019; 216:384–406. [PubMed: 30674564]

43. Wan G, Mahajan A, Lidke D, Rajput A. Bcl-2 together with PI3K p110alpha regulates cell morphology and cell migration. Cell Death Dis 2015; 6:e2006. [PubMed: 26633712]

44. Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, et al. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J Biol Chem 2000; 275:10761–6. [PubMed: 10753867]

45. Vockley CM, D'Ippolito AM, McDowell IC, Majoros WH, Safi A, Song L, et al. Direct GR Binding Sites Potentiate Clusters of TF Binding across the Human Genome. Cell 2016; 166:1269–81 e19. [PubMed: 27565349]

46. Li P, Leonard WJ. Chromatin Accessibility and Interactions in the Transcriptional Regulation of T Cells. Front Immunol 2018; 9:2738. [PubMed: 30524449]

47. Levy Y, Lacabaratz C, Weiss L, Viard JP, Goujard C, Lelievre JD, et al. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. J Clin Invest 2009; 119:997–1007. [PubMed: 19287090]

48. Sereti I, Anthony KB, Martinez-Wilson H, Lempicki R, Adelsberger J, Metcalf JA, et al. IL-2-induced CD4+ T-cell expansion in HIV-infected patients is associated with long-term decreases in T-cell proliferation. Blood 2004; 104:775–80. [PubMed: 15090457]

49. Thompson JA, Curti BD, Redman BG, Bhatia S, Weber JS, Agarwala SS, et al. Phase I study of recombinant interleukin-21 in patients with metastatic melanoma and renal cell carcinoma. J Clin Oncol 2008; 26:2034–9. [PubMed: 18347008]

50. Leonard WJ, Spolski R. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. Nat Rev Immunol 2005; 5:688–98. [PubMed: 16138102]

51. Al-Chami E, Tormo A, Khodayarian F, Rafei M. Therapeutic utility of the newly discovered properties of interleukin-21. Cytokine 2016; 82:33–7. [PubMed: 26748727]

52. Al-Chami E, Tormo A, Pasquin S, Kanjarawi R, Ziouani S, Rafei M. Interleukin-21 administration to aged mice rejuvenates their peripheral T-cell pool by triggering de novo thymopoiesis. Aging Cell 2016; 15:349–60. [PubMed: 26762709]

53. Rafei M, Dumont-Lagace M, Rouette A, Perreault C. Interleukin-21 accelerates thymic recovery from glucocorticoid-induced atrophy. PLoS One 2013; 8:e72801. [PubMed: 24023776]

54. Tormo A, Khodayarian F, Cui Y, Al-Chami E, Kanjarawi R, Noe B, et al. Interleukin-21 promotes thymopoiesis recovery following hematopoietic stem cell transplantation. J Hematol Oncol 2017; 10:120. [PubMed: 28615039]

55. Waggie KS, Holdren MS, Byrnes-Blake K, Pedersen S, Ponce R, Hughes S, et al. Preclinical safety, pharmacokinetics, and pharmacodynamics of recombinant human interleukin-21 in cynomolgus macaques (Macaca fascicularis). Int J Toxicol 2012; 31:303–16. [PubMed: 22723515]

Clinical Implications:

IL-21 was identified as a key mediator in glucocorticoid-induced T-cell apoptosis via BCL2 expression, suggesting IL-21 use in clinical settings may provide benefit controlling lymphopenia and its associated consequences.
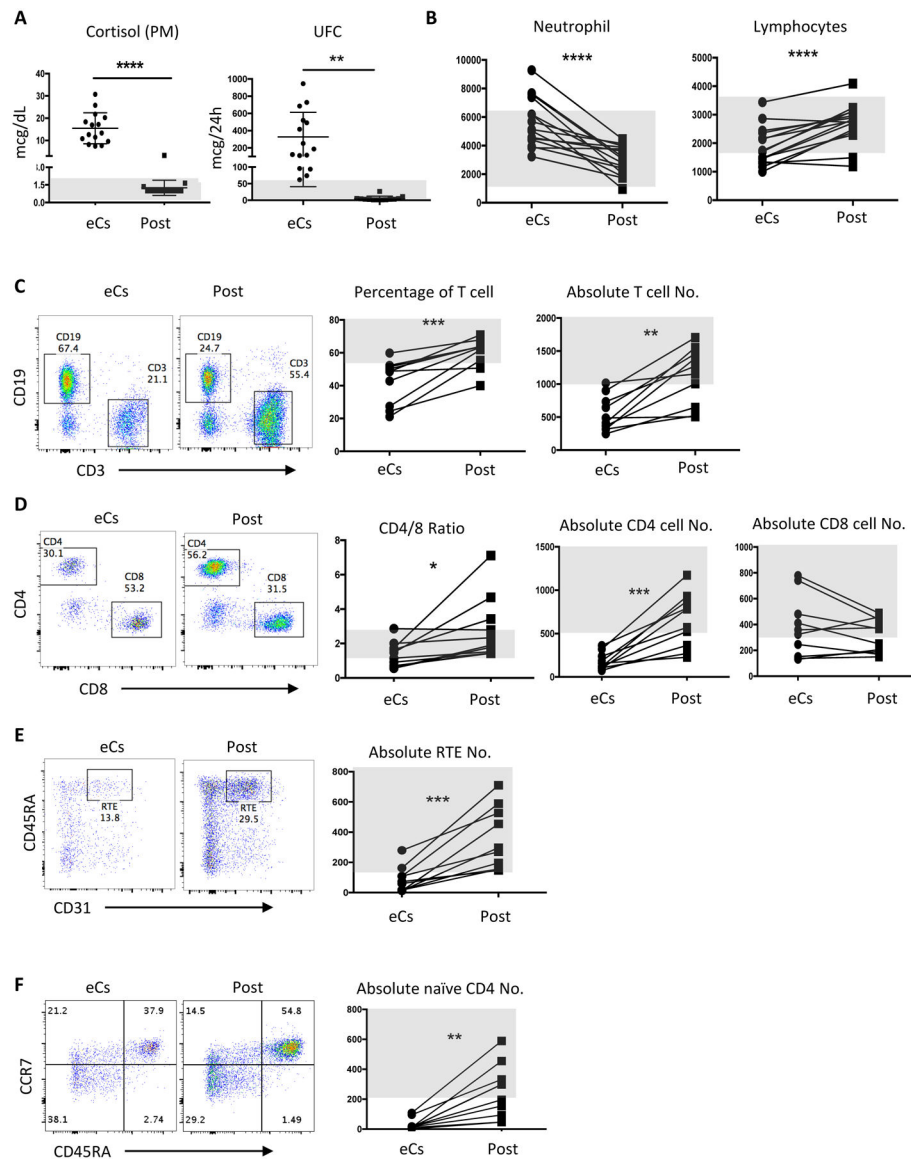
Figure 1.

Cortisol (PM and free urinary), white blood cells, and basic lymphocyte phenotype in eCs and Post patients

A. Midnight serum cortisol values (left), UFC (Urinary Free Cortisol, right) in eCs and Post patients. B. Lined dots indicate the absolute neutrophil count (left), and absolute lymphocytes (right) in each individual patient (n= 15). C. Representative flow cytometry plots of CD3+ (T cells) versus CD19+ (B cells) on the left; on the right, percentage and absolute numbers of CD3+ T cells. D. CD4/8 ratio in eCs and Post patients (n= 10). The CD4/8 ratio (left), absolute CD4+ (middle) and CD8+ T cells (right) is represented by lined dot graphs (n= 10). E. Recent Thymic Emigrants (RTE) were detected by staining CD3+CD4+CD45RA+CD31+ in eCs and Post patients (n= 10). F. Naïve CD4+ T cells; CD3+ gated T cells were analyzed by CD4+CD45RA+CCR7+. The gray shade indicates the normal
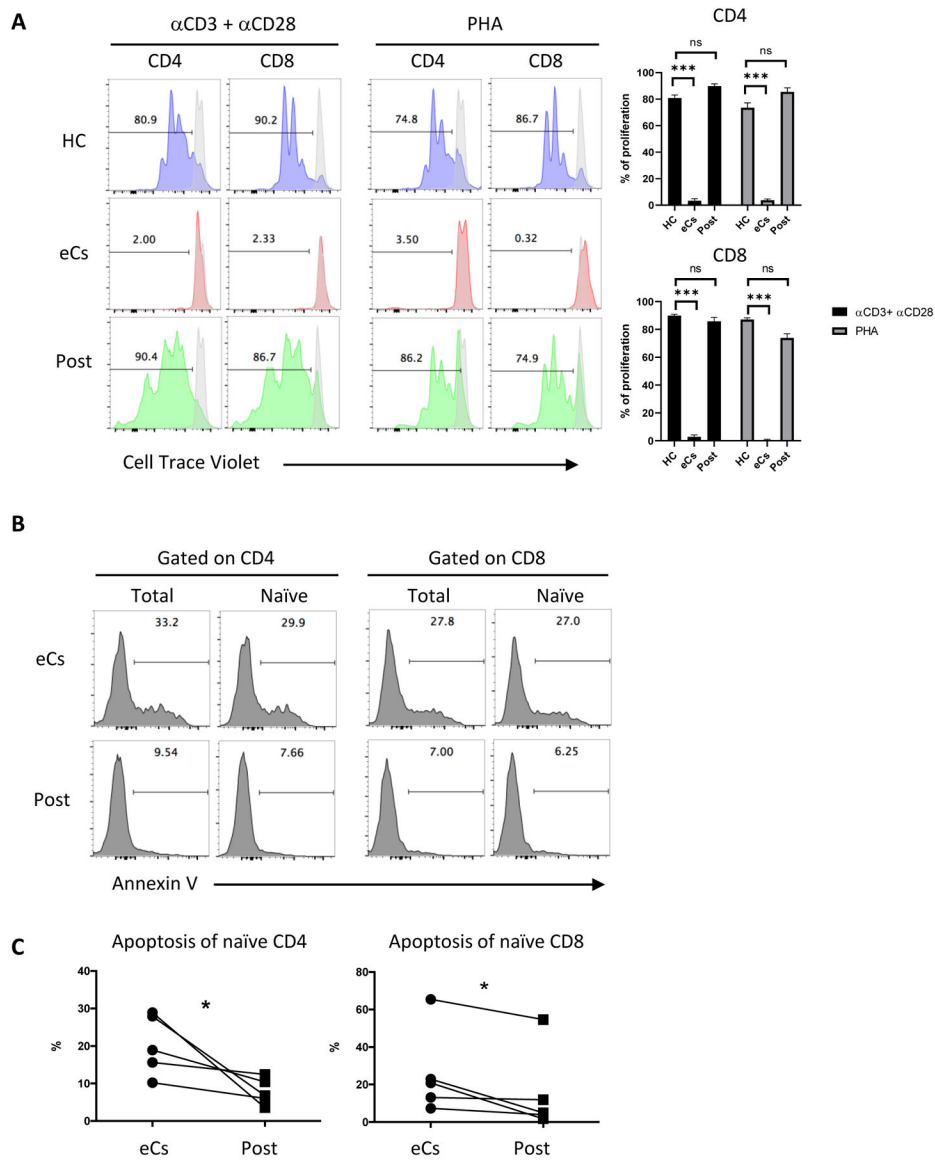
pediatric range of each test. PBMCs from 10 paired patients were analyzed by paired *t*-test in each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$.

Figure 2.

Cell proliferation and cell death via apoptosis in eCs and Post patients

A. Gated on $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell proliferation via TcR signaling (anti-CD3 + anti-CD28) or mitogenic stimulation (PHA) in HC, eCs and Post patients. The paired patients PBMC were analyzed by unpaired *t*-test in each group (n=4). Representative (left, histograms) and aggregate data of n=4 individuals per group tested (right, bar graphs) are shown. B. Apoptosis was measured by Annexin V staining on CD4, naïve CD4 T cells ($CD3^+CD4^+CD45RA^+CCR7^+$), CD8 and naïve CD8 T cells ($CD3^+CD8^+CD45RA^+CCR7^+$) in eCs (top) and Post patients (bottom). C. The lined dot graph indicates the percentage of apoptotic cells in each cell subsets between eCs and Post patients (n=5). PBMCs from 5 paired patients were analyzed by paired *t*-test in each group. * *P* < 0.05.

Figure 3.

Regulation of T cell apoptosis by IL-21 treatment

A. Healthy donors' PBMC were incubated without (left) or with (right) dexamethasone (1μg/mL) in the different combination with IL-2, IFNα and IL-21 for overnight. Apoptosis was measure by Annexin V staining. B. The bar graph shown the percentage of apoptosis (Annexin V) on naïve CD4+ T cells following treatment with dexamethasone (n=5). The bar graph represented the percentage of apoptosis by normalization with DEX solely treated sample as 100%. C-D. BCL6 (C) and BCL2 (D) expression was detected in the absence or presence of dexamethasone (1μg/mL) with additional incubation with IL-2, IFNα or IL-21. E. The bar graph represents the BCL2 (MFI) after dexamethasone and indicated cytokine incubation (n=3). F-G. Apoptosis was determined by Annexin V staining after dexamethasone +/− IL-21 overnight incubation of PBMCs with BCL2 inhibitor (iBCL2) or BCL6 inhibitor (iBCL6) (vehicle (DMSO) was included in the negative control). Statistical

analysis for B and E: one-way ANOVA correcting for multiple comparison using the Tukey method; for G: and two-way ANOVA correcting for multiple comparison using the Sidak method. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
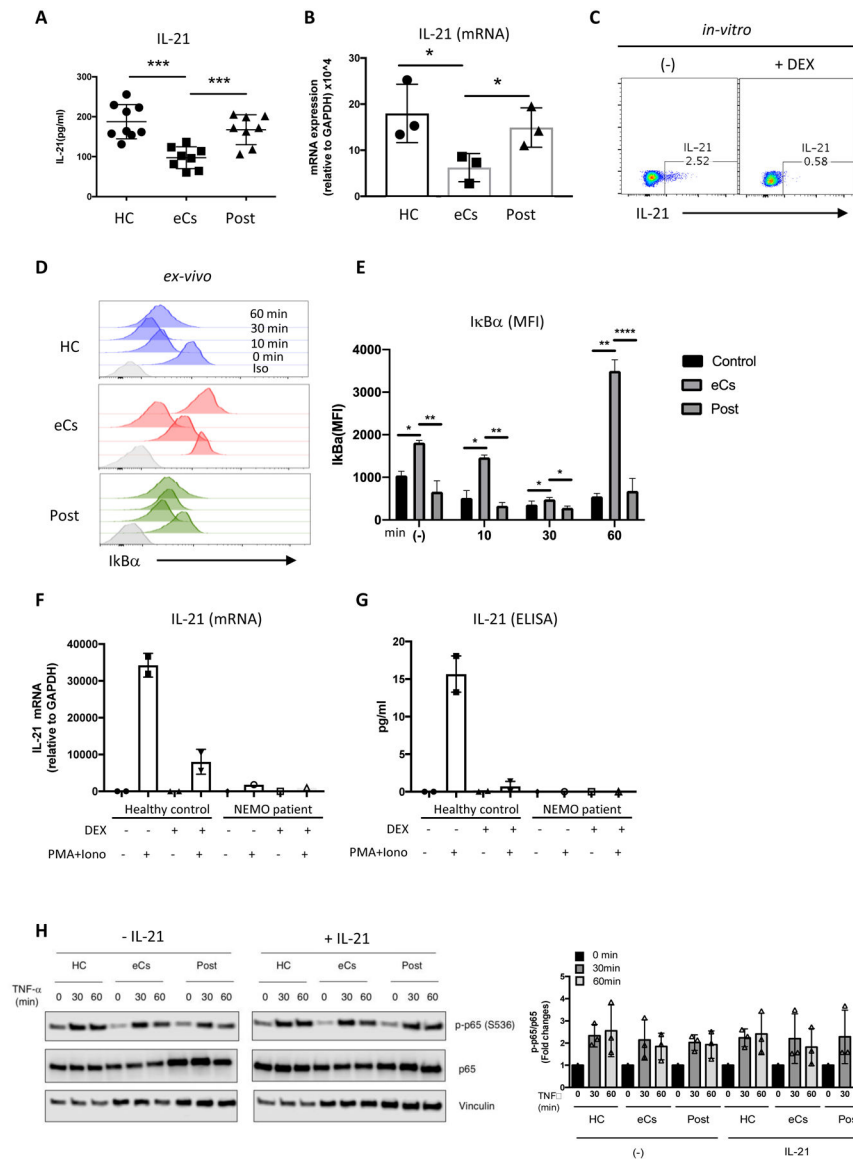
Figure 4.
IL-21 regulation in eCs and Post patients, and NF-κB pathway evaluation

A-B. IL-21 in plasma (n=8) (A) and IL-21 mRNA (n=3) (B) was detected by qRT-PCR and ELISA in eCs and Post patients. C. Healthy donors' PBMC was incubated without (left) or with (right) dexamethasone (1μg/mL) for 3 hours after PMA/ionomycin/brefeldin A stimulation for 5 hours, and measured IL-21 intracellular staining (n=4). D. IκBα degradation was performed at 0, 10, 30 and 60 min after PMA/ionomycin stimulation. E. The bar graph shows the MFI of IκBα on CD3$^+$ T cells in eCs, Post and HC (n=3). F-G. IL-21 mRNA (F) and IL-21 in the supernatant (G) was detected by qRT-PCR and ELISA in PBMC from 2 HC and 1 NEMO patients stimulated with PMA/ionomycin in the absence or presence of dexamethasone (1μg/mL). (H) IL-21 effect on NF-κB pathway was evaluated by p65 phosphorylation on PBMCs from eCs and Post patients. Representative (left) and aggregated data (right) are shown. IL-21 did not increase p65 phosphorylation in

the absence or presence of TNF-α on eCs or Post patients' cells. Quantified values for p65 phosphorylation (p-p65) were normalized to total p65 expression and shown as fold changes compared to each untreated (0 minute) samples. Data are means ± SD (n = 3 independent experiments). Vinculin was used as a loading control. Statistical analysis for A and B: one-way ANOVA correcting for multiple comparison using the Tukey method; for E, F and G: two-way ANOVA correcting for multiple comparisons using the Sidak method, mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.
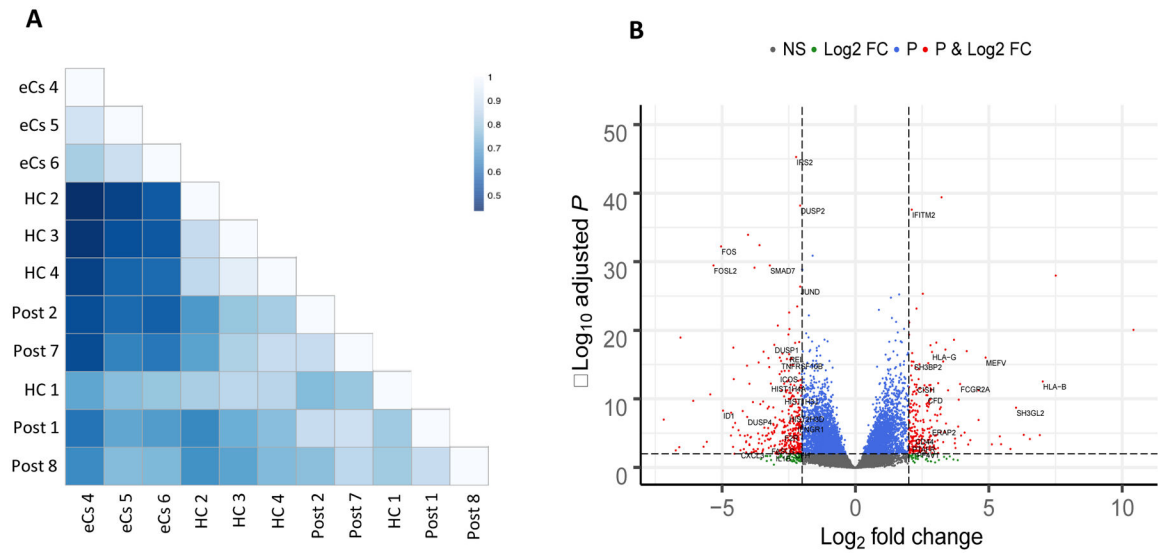
Figure 5.

RNAseq from naïve $CD4^+$ T cells from eCs and Post patients

A. Pearson correlation analysis of the variance stabilized RNASeq counts matrix for all differentially expressed genes (eCs vs. Post); Individuals in the eCs group (n=4) clustered together. Individuals in the Post patient group (n=3) clustered with the healthy controls (n=4) showing that there is a "normalizing" treatment effect on the targeted transcriptome.

B. Volcano plot of differentially expressed immune-related genes (eCs vs. Post); NS=not significant; FC=fold change (maximum likelihood estimation); P=Wald test adjusted p-value.

Figure 6.

PI3K pathway evaluation in eCs-mimicking conditions

A. pS6 (pS240) was assessed in blasted healthy control CD4^{+} T cells upon anti-CD3+ProteinA stimulation with or without dexamethasone incubation (n=5). The histogram (representative data, left) and bar graph (aggregated data of n=5, right) show phosphorylated S6 (pS6) under dexamethasone incubation with IL-2, IFNα or IL-21. B. PI3K inhibitor (LY294002: 10μM) was added 1 hour prior to dexamethasone incubation with cytokines (IL-2, IFNα or IL-21) and apoptosis was measured by Annexin V staining (n=5). C. Intracellular BCL2 expression was detected with LY294002 + dexamethasone in cytokines treatment (n=5; vehicle (DMSO) was added to the negative control (−) condition); the bar graph shows the statistical analysis: two-way ANOVA correcting for multiple comparisons using the Sidak method, mean ± SD. ** $P < 0.01$