Glucocorticoid-resistant Th17 cells are selectively attenuated by cyclosporine A

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Glucocorticoids remain the cornerstone of treatment for inflammatory conditions, but their utility is limited by a plethora of side effects. One of the key goals of immunotherapy across medical disciplines is to minimize patients' glucocorticoid use. Increasing evidence suggests that variations in the adaptive immune response play a critical role in defining the dose of glucocorticoids required to control an individual's disease, and Th17 cells are strong candidate drivers for nonresponsiveness [also called steroid resistance (SR)]. Here we use gene-expression profiling to further characterize the SR phenotype in T cells and show that Th17 cells generated from both SR and steroid-sensitive individuals exhibit restricted genome-wide responses to glucocorticoids in vitro, and that this is independent of glucocorticoid receptor translocation or isoform expression. In addition, we demonstrate, both in transgenic murine T cells in vitro and in an in vivo murine model of autoimmunity, that Th17 cells are reciprocally sensitive to suppression with the calcineurin inhibitor, cyclosporine A. This result was replicated in human Th17 cells in vitro, which were found to have a conversely large genome-wide shift in response to cyclosporine A. These observations suggest that the clinical efficacy of cyclosporine A in the treatment of SR diseases may be because of its selective attenuation of Th17 cells, and also that novel therapeutics, which target either Th17 cells themselves or the effector memory T-helper cell population from which they are derived, would be strong candidates for drug development in the context of SR inflammation.

Th17 | glucocorticoid | steroid resistance | calcineurin inhibition | uveitis

Glucocorticoids are the single most commonly used drug to treat all inflammatory diseases in man (1). However, their benefits are balanced against a plethora of side effects, including centripetal obesity, diabetes mellitus, hypertension, and osteoporosis, resulting in a substantial iatrogenic burden of ill health and reduced life expectancy (2). This finding has particular relevance to so-called steroid-resistant (SR) individuals, who require intolerable doses of glucocorticoids to maintain disease remission, and account for up to a third of patients with inflammatory diseases across all medical specialties (3). Better understanding of the biological mechanisms that underlie the SR phenotype therefore promises to reveal novel strategies to minimize glucocorticoid use in clinical practice.

The effects of glucocorticoids are mediated through the glucocorticoid receptor (GR), which regulates a broad spectrum of physiological processes, and genetic mutations and polymorphisms of the GR gene (*NR3C1*) give rise to well-described rare SR syndromes (4). However, in the absence of such a premorbid abnormality in steroid signaling there is no unifying explanation of the SR phenotype. Candidate mechanisms include overexpression of the inhibitory β-isoform of the GR (GR-β) (1, 5), but there is also increasing evidence to suggest that specific variations in the adaptive immune response to glucocorticoid therapy play a key role (6–8).

Th17 cells represent a distinct $CD4^+$ lineage defined by the secretion of characteristic cytokines, in particular IL-17A (hereafter referred to as IL-17), IL-17F, and IL-22 (9). In humans, Th17 cells are subsets of effector memory T-helper cells that express the chemokine receptor CCR6 (10), and they play a vital role in host defense against intracellular bacterial and fungal infections (9). These cells are also key drivers in the development of autoimmune conditions (11). Increasing evidence supports the concept of a SR Th17 phenotype, and their adoptive transfer, or the overexpression of their canonical transcription factor retinoid-related orphan receptor (ROR)-γt, has been shown to induce SR disease in a murine model of asthma (12, 13). In humans, glucocorticoid-resistant Th17 cells express the multidrug resistance type 1 protein, which is inhibited by cyclosporine A (CsA) (14), and historically CsA was one of the first drugs used to successfully rescue SR diseases (1). We therefore hypothesized that CsA would selectively inhibit human Th17 cells.

In this article, we compare the effects of glucocorticoids and CsA on human and murine Th17 cells. First, we quantified the in

Significance

Cyclosporine A was one of the first drugs used in clinical practice to successfully rescue glucocorticoid-resistant inflammatory diseases. In this article we extend the characterization of glucocorticoid-resistant human Th17 cells, and demonstrate that this effector memory T-cell subset is reciprocally attenuated by cyclosporine A. This therapeutic paradigm was confirmed in a murine model of autoimmunity, refining our understanding of cyclosporine A's effect on the adaptive immune response. These data support the rationale for Th17-targeting therapies in the treatment of glucocorticoid-resistant inflammation.

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Conflict of interest statement: R.W.J.L., R.B.N., L.W., A. D. Dick, I.G., and L.P.S.-B. are named inventors on a US patent application (no. 61/919,404), which incorporates calcineurin inhibitor-based conjugates for treating inflammatory diseases.

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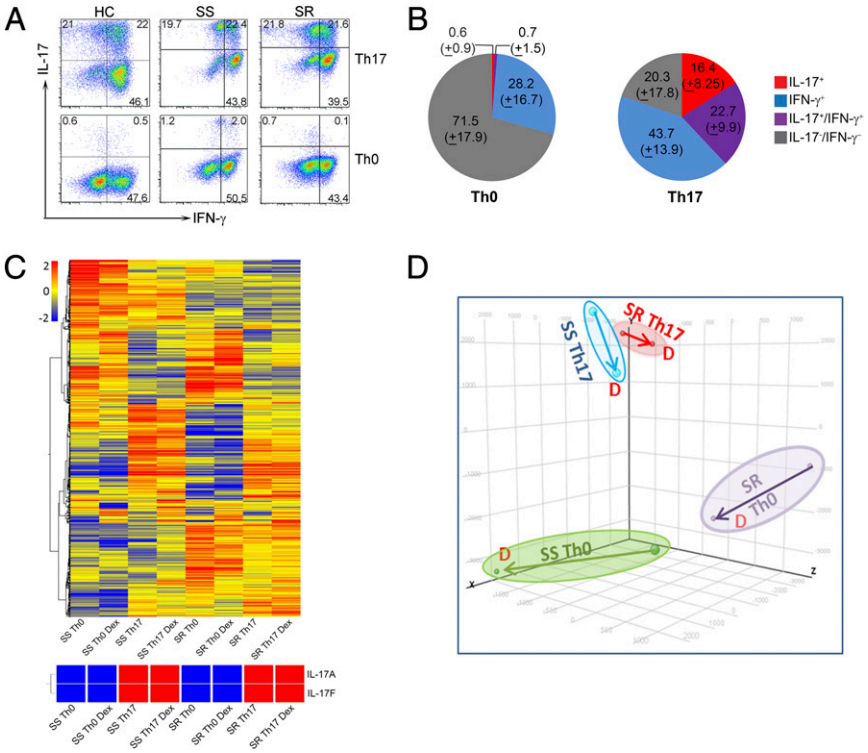

Fig. 1. Human Th17 cells exhibit a restricted genome-wide response to glucocorticoids. (*A*) Intracellular IL-17 and IFN-γ expression in $CD4^+CCR6^+$ and $CD4^+CCR6^-$ cells following 14-d culture in Th17 or control (Th0) conditions. Representative FACS plots from HC, SS patients, and SR patients are shown. (*B*) Percentage (± SEM) of intracellular IL-17 and IFN-γ single-positive cells and IL-17/IFN-γ double-positive or double-negative cells in $CD4^+CCR6^+$ and $CD4^+CCR6^-$ cells following 14-d culture in Th17 or control (Th0) conditions, calculated according to the gates shown in *A* ($n = 25$). The data for HC, SS, and SR donors has been compiled as there was no difference in the phenotype of cells across these groups (Fig. S1*A*). (*C*) Hierarchical clustering analysis of SS and SR Th17 and Th0 cells (untreated and Dex-treated). Up-regulated genes are shown in red on the heat map and down-regulated genes are in blue. The IL-17A and IL-17F expression in each culture is highlighted. (*D*) PCA of genes with at least twofold changes between any two of the four conditions. The shift in gene expression in response to Dex is proportional to the length of the arrow. These are presented as untreated and Dex treated Th17 and Th0 cells derived from SS or SR patients; D, post-Dex treatment.

vitro expression of IL-17 in effector memory $CD4^+$ T cells from the peripheral blood of patients with an autoimmune disease (uveitis) for which the SR phenotype represents a significant clinical challenge (7), and compared this with controls. Second, we characterized the effect of glucocorticoids on the gene-expression profile of human Th17 cells in vitro and examined whether this was affected by differential GR nuclear translocation or isoform expression. Third, we interrogated the SR Th17 paradigm in enriched murine transgenic Th17 cells in vitro, and also in vivo using an allied mouse model of organ-specific autoimmunity, to test the hypothesis that the calcineurin inhibitor CsA would have a reciprocal effect to glucocorticoids on IL-17 expression. Fourth, we assessed the effects of CsA on human Th17 cells to see if the results seen in mouse were replicated in man. These data show that Th17 cells are refractory to glucocorticoid suppression at a genome-wide level in a GR-independent manner and are conversely sensitive to inhibition with CsA.

Results

Human Th17 Cells Exhibit a Restricted Genome-Wide Response to Glucocorticoids. To profile the glucocorticoid response of Th17 cells (Fig. 1 *A* and *B*), memory $CD4^+CCR6^+$ cells were sorted from the peripheral blood of independent donors and cultured for 14 d in the presence of polarizing cytokines, whereas control $CD4^+CCR6^-$ T cells were simultaneously sorted and cultured in the absence of polarizing cytokines (10). As shown in Fig. S1*A*, we did not find a difference in the proportion of $CD4^+$ cells expressing IL-17 in either culture condition from steroid sensitive (SS) patients, SR patients, or healthy control (HC) donors. Total IL-17 production was 39.1% from $CCR6^+$ cells (hereafter referred to as human Th17 cells) compared with 1.3% from the non-Th17 control cells cultured in the absence of polarizing cytokines, which defaulted to expression of IFN-γ (hereafter referred to as human Th0 cells, which are similar to murine Th1 cells, in terms of IFN-γ and Tbet expression, and lack of IL-17 and RORC expression) (Fig. 1*B*).

We then analyzed the genome-wide expression profiles in Th17 cells versus Th0 cells treated with or without the synthetic glucocorticoid dexamethasone (Dex) using Affymetrix U133 2.0 GeneChips. A total of 24 samples were analyzed (triplicates for each patient group and in vitro condition), and Dex was used at 1×10^{-6} M (Fig. S1*B*). We found that the expression of classic Dex-inducible genes, such as *FKBP5* and *TSC22D3*, were elevated in both Th17 and Th0 cells. Hierarchical clustering analysis confirmed the up-regulation of IL-17A and IL-17F in human Th17 cultures (Fig. 1*C*). However, principal component analysis (PCA) of all of the differentially expressed genes (defined as a twofold change with a P value <0.05) revealed that the genome-wide expression changes induced by Dex treatment in Th17 cells from both SS and SR individuals were significantly restricted compared with Th0 cells (Fig. 1*D*). Analysis of the combined dataset confirmed that, overall, human Th17 cells are less responsive to glucocorticoids compared with Th0 cells (Fig. S1*C*). This finding was also evident in terms of T-cell proliferation (Fig. S1*D*). Furthermore, we also observed that the total number of genes responding to Dex in human Th17 cells was much less than in Th0 cells (Fig. S1*C* and Table S1). In particular, the expression of nuclear factor κ light polypeptide gene enhancer in B-cells inhibitor ζ (*NFKBIZ*), which promotes IL-17 expression (15), was suppressed in Th0 cells, whereas its expression was increased by Dex in Th17 cells.

The Failure of Human Th17 Cells to Respond to Glucocorticoids Is GR-Independent. Glucocorticoid-induced nuclear translocation of the GR is the first step in the process of transactivation of genes containing glucocorticoid-response elements (16). To determine whether a defect in GR nuclear translocation could explain the glucocorticoid refractory nature of human Th17 cells, we examined the subcellular localization of GR in Th17 cells using confocal microscopy. Immunofluorescent staining showed that GR, which was primarily cytoplasmic in resting cells, translocated to the nucleus following Dex treatment (Fig. 2*A* and Movies S1–S4). The nuclear fluorescent intensity of GR was also

assessed in control Th0 cells and indicated that GR translocation is not significantly perturbed in human Th17 cells (Fig. 2*B*). Overexpression of the functionally inactive GR-β isoform could also be responsible for glucocorticoid resistance (5), and we therefore interrogated the expression of total GR and GR-β. We did not find a significant difference in the expression of GR or GR-β between human Th17 and Th0 cells, and this was not altered by the addition of Dex (Fig. 2 *C* and *D*). These results suggest that the failure of human Th17 cells to respond to glucocorticoids was not because of differences in total GR expression, GR nuclear translocation, or overexpression of the dominant-negative GR-β isoform.

Murine Th17 Cells Are SR and Are Preferentially Suppressed with CsA. Given the success of CsA in clinically rescuing SR inflammation (1) and that Th17 cells are resistant to Dex, we hypothesized that Th17 cells are susceptible to CsA inhibition. To test this, we first used a murine in vitro system to generate more highly enriched populations of IL-17– and IFN-γ–expressing $CD4^+$ T cells to interrogate the comparative effect of Dex (Fig. S2) and CsA on these two canonical T-cell subsets. Using naïve $CD4^+$ cells from hen egg lysozyme (HEL)-specific T-cell receptor (TCR) transgenic mice on a B10.BR background (3A9), we generated T cells that were highly enriched for the expression of IL-17 ("Th17"), and control cells that were highly enriched for the expression of IFN-γ ("Th1") (Fig. 3*A*). Consistent with our human data, murine Th17 cells continued to proliferate despite the presence of Dex at a concentration that fully suppressed Th1 cells (Fig. 3*B*). Conversely, the reverse was seen in CsA-treated cultures, in which the division of Th17 cells was fully suppressed at a dose that was unable to inhibit Th1 cells (Fig. 3*B*). These observations were then replicated, first using $CD4^+$ cells from ovalbumin (OVA)-specific OT-II mice (on a C57BL/6 background), confirming that this was not a phenomenon limited to a particular TCR transgenic strain (Fig. 3 *C* and *D*), and second with an alternative calcineurin inhibitor, tacrolimus (Tac) (Fig. S3). Hence,

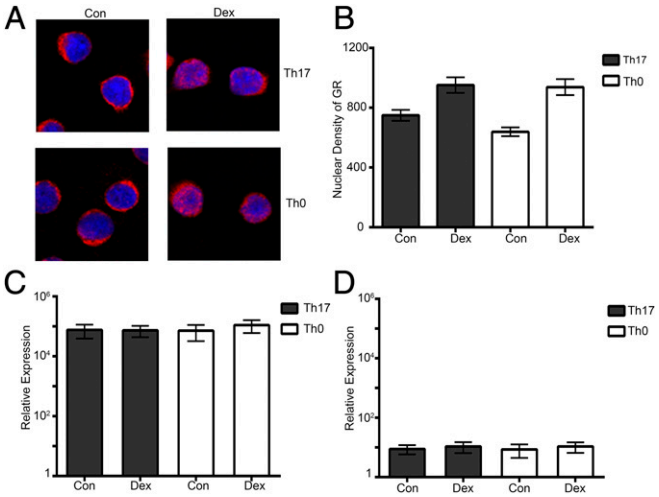

Fig. 2. GR expression in human Th17 cells. (*A*) GR expression in $CD4^+CCR6^+$ and $CD4^+CCR6^-$ cells following 14-d culture in Th17 or control (Th0) conditions. Cells were treated for 30 min with Dex. Con, Control. A representative of five independent experiments is shown. (Magnification: 1,260×.) (*B*) Quantitative image analysis of GR nuclear density in control and Dex-treated cells. Data represented as mean ± SEM (n = 5; 163 ± 26.16 cells analyzed per experiment over five fields of view). (*C*) Real-time PCR analysis for total GR in Th17 and Th0 cells cultured for 14 d and then treated with or without Dex for 30 min (n = 5). Data represented as mean ± SEM. (*D*) Relative expression of the GR-β isoform with or without Dex treatment for 30 min (n = 5). Data represented as mean ± SEM.

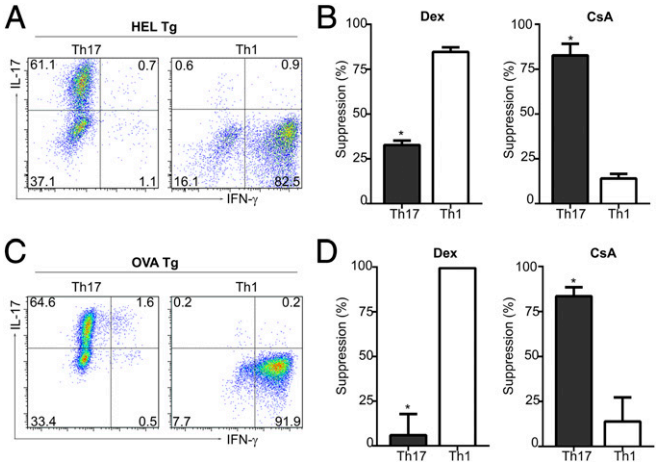

Fig. 3. In vitro cultured murine glucocorticoid-resistant Th17 cells are controlled by CsA. (*A*) $CD4^+$ T cells with a transgenic TCR specific for HEL were cultured under Th17 or Th1 polarizing conditions before activation with PMA/ionomycin for 4 h. Cells were stained intracellularly for the expression of IL-17 and IFN-γ. Plots are gated on live $CD4^+$ cells and representative of three independent experiments. (*B*) Percentage suppression of proliferation (± SEM) quantified by tritiated thymidine incorporation in Th17 and Th1 cells treated with Dex or CsA for 48 h (n = 5; $^*P < 0.05$; Mann–Whitney nonparametric test). (*C* and *D*) Experiments were carried out as for *A* and *B*, except $CD4^+$ T cells with a transgenic TCR specific for OVA (OT-II) (n = 3; $^*P < 0.05$; using a Mann–Whitney nonparametric test).

Dex and calcineurin inhibition with either CsA or Tac had reciprocal effects on Th1 and Th17 cell proliferation, with Th17 cells being less responsive to glucocorticoids, and more responsive to calcineurin inhibition, than Th1 cells.

To examine whether Dex and CsA had reciprocal effects on Th1 and Th17 cell proliferation in vivo, we used the organ-specific model of Th1/Th17-driven inflammation, experimental autoimmune uveitis (EAU) (17). For comparison of the effect of CsA and Dex on T-cell subsets, drug concentrations were titrated to establish the minimum dose at which equivalent suppression of inflammation was achieved, as measured by direct visualization of the organ-specific immune response in the eye using topical endoscopic fundal imaging (TEFI) (Fig. S4*A*) (18). We then treated EAU mice with either Dex or CsA and evaluated their effects by a combination of TEFI and histology (Fig. 4 *A*–*D*). This finding demonstrated a significant reduction of disease severity with both drugs following the first and subsequent treatments. Furthermore, there was a significant and equal reduction of the total number of organ-infiltrating $CD4^+$ T cells in both Dex and CsA treated mice, and the proportion of infiltrated $Ly6G^+$ neutrophils and $CD11b^+$ myeloid cells was also similarly reduced in both treatment groups (Fig. 4*E*). However, despite achieving equivalent suppression of $CD4^+$ T-cell numbers with each drug, the IL-17 and IFN-γ cytokine profiles of tissue infiltrating $CD4^+$ cells was strikingly different. Dex suppressed IFN-γ expression by 45%, whereas the proportion of IL-17–expressing cells was relatively increased. In contrast, CsA almost completely ablated IL-17 expression with a 91% reduction in the proportion of cells expressing this cytokine compared with control mice. IFN-γ expression was also significantly reduced by CsA (Fig. 4*F*). This drug-specific cytokine skewing was particularly evident when the ratio of IL-17/IFN-γ was calculated. Dex decreased IFN-γ more than IL-17, and CsA—which decreased both cytokines—inhibited IL-17 expression more than IFN-γ (Fig. S4*B*). Consistent with this finding, mRNA expression of the Th17 associated cytokines *Il17a*, *Il17f*, and *Il22* in ocular-infiltrating $CD4^+$ T cells from Dex-treated mice was increased compared with the infiltrating $CD4^+$ T cells from control

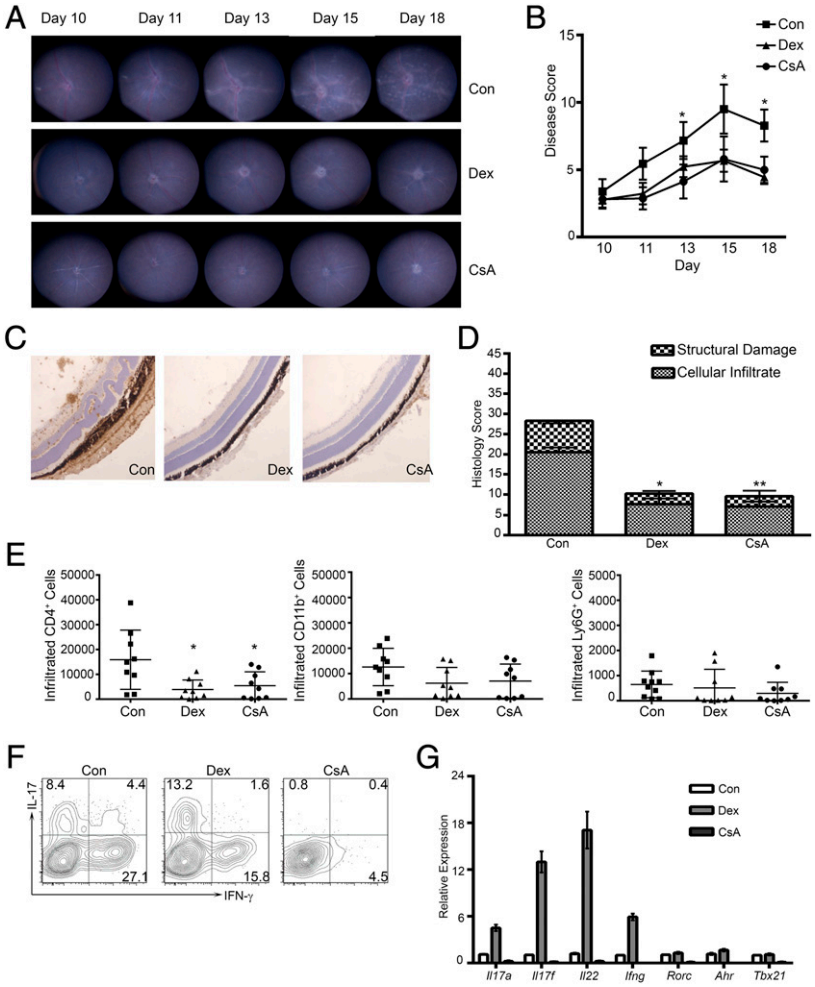

Fig. 4. In vivo murine glucocorticoid-resistant Th17 cells are controlled by CsA. (*A*) B10.RIII mice were immunized with IRBP-$3_{161–180}$ and pertussis toxin and from day 10 postimmunization TEFI was used to clinically monitor disease. On days 11, 13, 15, and 17 postimmunization, mice were treated with Dex or CsA, or left untreated (Con). Photographs are representative of a dilated, right eye from one mouse from each group. (*B*) Disease scores following immunization and treatment with or without Dex or CsA. Both right and left eyes were scored and data are representative of three experiments. Data represented as mean ± SEM ($n = 9$ for each group; $P < 0.05$; ANOVA). (*C*) Staining of 12-μm-thick frozen sections with H&E of day 18 retinal tissue from immunized mice following treatment or control. (Magnification: 20×.) (*D*) Summary of histological cellular infiltrate and structural damage scores from treated or control mice. Data represented as mean ± SEM ($n = 3$; $*P < 0.05$, $**P < 0.005$). (*E*) Total number of living $CD4^+$ T cells, $CD11b^+$ myeloid cells and $CD11b^+Ly6G^+$ neutrophils/eye from retina and ciliary body tissue extracts at day 18 ($n = 9$ in each group, $*P < 0.05$; Mann–Whitney nonparametric test). (*F*) $CD4^+$ T cells from day 18 retina and ciliary body tissue were stimulated with PMA/ionomycin for 4 h. Cells were stained intracellularly to quantify IL-17 and IFN-γ expression by flow cytometry. Plots are gated on live cells ($n = 9$ in each group). (*G*) Real-time PCR analysis of gene expression in $CD4^+$ T cells from day 18 retina and ciliary body tissue. Data represented as mean ± SEM ($n = 9$ for each group).

(untreated) animals. Moreover, only CsA treatment significantly reduced the expression of Th17- and Th1-specific transcription factors, *Rorc*, *Tbx21*, and *Ahr*, quantified by PCR (Fig. 4*G*). Therefore, these data demonstrate that Th17 cells escape Dex suppression in vivo, whereas calcineurin inhibition controls both Th17- and Th1-mediated inflammation in this organ-specific model of autoimmunity.

Human Th17 Cells Are also Exquisitely Sensitive to Calcineurin Inhibition. To determine whether the dominant anti-Th17 effects of CsA seen in mice would be replicated in man, human Th17 and Th0 cells generated using identical conditions to the glucocorticoid experiments presented in Fig. 1 were treated with CsA for 24 h. This process suppressed the expression of both IL-17 and IFN-γ in human Th17 cells (Fig. 5*A*). However, as seen in murine $CD4^+$ T cells, IL-17 expression was suppressed by CsA to a greater extent than IFN-γ (Fig. 5*B*). This suppression of Th17 cells by calcineurin inhibition was replicated using Tac (Fig. S5), and further reflected in reduced expression of the key Th17 cell transcription factor *RORC*, with concomitant ablation of *IL17A* mRNA, and an over 90% reduction in *IL17F*. This result was despite the continued expression of *AHR*. Conversely, the expression of *IFN-γ* (*IFNG*) was only reduced by 43%, and expression of the key transcription factor for Th1 differentiation *TBX21* was not changed in CsA-treated cells (Fig. 5*C*).

RNA-seq technology (Illumina) was used for gene-expression profiling of human Th17 and Th0 cells to assess genome-wide changes in response to CsA treatment. PCA of all genes with at least a twofold difference in their expression between any two conditions showed that significant changes in gene expression were induced in human Th17 cells following CsA treatment, but only a small expression pattern change was seen in Th0 cells (Fig. 5*D*). Importantly, 210 of 246 genes uniquely expressed in Th17 cells were suppressed by CsA, including *IL17A*, *IL22*, *IL26*, *IL23R*, and *CD161* (Fig. S6*A* and Table S2), whereas the expression of only 2% of all Th0 specific genes was changed by CsA

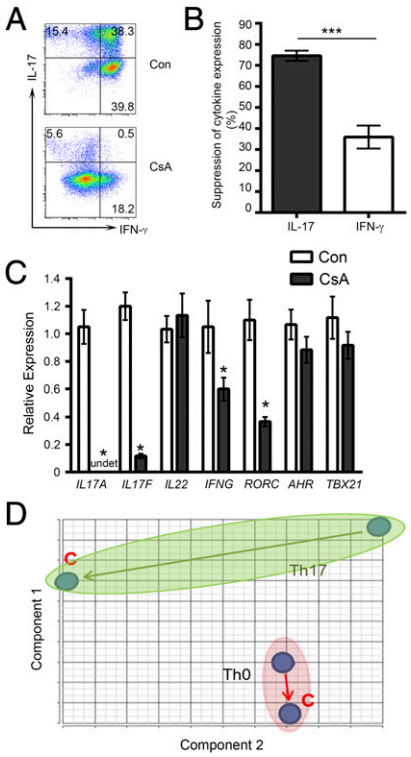

Fig. 5. CsA suppresses human Th17 cells. (*A*) $CD4^+CCR6^+$ and $CD4^+CCR6^-$ cells cultured in vitro for 14 d in Th17 conditions were treated with CsA for 24 h before stimulation with PMA/ionomycin for 4 h. Cells were stained intracellularly to quantify IFN-γ and IL-17 expression by flow cytometry. Plots are gated on live $CD4^+$ cells and are representative of 21 independent experiments. (*B*) Percentage cytokine suppression (± SEM) of $CD4^+$IL-17^+ and $CD4^+$IFN-γ^+ cells in Th17 cultures treated with or without CsA treatment for 24 h as determined by intracellular cytokine staining ($n = 21$; $^{***}P < 0.0001$; Mann–Whitney nonparametric test). (*C*) Real-time PCR analysis of gene expression in Th17 cells following 24 h treatment with or without CsA. Error bars indicate SD. Undet, undetectable. ($n = 6$; $^{*}P < 0.05$). (*D*) PCA of genes with at least twofold changes between any two of the four conditions (untreated and CsA treated Th17 and Th0 cell; C, CsA-treated). Each condition was run in duplicate (eight RNA-seq with two donors for each condition).

treatment (Fig. S6*B*). These data demonstrate that CsA results in suppression of the human Th17 transcriptome at a concentration that had a comparatively limited effect on control Th0 cells.

Discussion

In this study we have extended previous reports of attenuated Th17 cell responses to glucocorticoids, demonstrating that this is exhibited at a genome-wide level in human $CD4^+$ T cells and is independent of GR isoform expression and nuclear translocation. In addition, we have shown in both mouse and man that there is a reciprocal sensitivity of Th17 cells to calcineurin inhibition with CsA. These observations are consistent with the clinical efficacy of CsA in the treatment of SR conditions, suggesting that one of its key effects on the adaptive immune response is to selectively suppress Th17 cells that escape glucocorticoid inhibition.

The SR Th17 paradigm has already been described in terms of restricted suppression of cell proliferation in murine in vitro and in vivo systems in comparison with IL-4, IL-5, and IL-13 expressing Th2 cells (12). This result has also recently been replicated in human in vitro cultures in comparison with IFN-γ–expressing cells (14). We therefore examined whether effector memory $CD4^+CCR6^+$ T cells from patients with clinically defined SR disease were biased to express IL-17 when exposed to the Th17 cell-promoting cytokines IL-1β, IL-23, and IL-6, and found that there was no difference between SR and SS patients, or HCs in terms of IL-17 expression (Fig. 1*A* and Fig. S1*A*). This finding suggests that effector memory cells from SR patients have no greater propensity to produce IL-17 than those from SS individuals when cultured under Th17 polarizing conditions. Th17 cells, as a subtype of effector T cells, are characterized by the expression of not only IL17A, but also a set of genes encoding cytokines, chemokines, and key signaling molecules promoting inflammation and antimicrobial immunity. Therefore, at a genome-wide level, our PCA analysis of microarray data demonstrated that restriction of the response to glucocorticoid treatment was attributable to the T-cell lineage (i.e., Th17 vs. control Th0 cells) rather than whether the T-cell donor was SS or SR. This finding suggests that beyond cytokine production, Th17 cells have intrinsic factors that are hyporesponsive to glucocorticoid treatment. For example, NFKBIZ is essential for Th17 cell polarization and directly regulates IL17 expression by binding to its promoter in mouse (19). We found it was induced in Th17 cells by Dex (Table S1). Therefore, the persistence of *NFKBIZ* in Dex-treated human Th17 cells (Table S1) could be key to their maintenance of *RORC* expression (20) and may also interfere with GR function (21, 22). In addition, recent reports of genome-wide binding profiles have demonstrated the transcription factors NF-κB and Stat3, both of which are activated in Th17 cells, may antagonize GR functions by changing the DNA binding sites of GR (23). Furthermore, it is possible that altered GR binding affinity at glucocorticoid response elements plays a role (19).

CsA's effect on IL-17– and IFN-γ–expressing cells is strikingly opposite to that of glucocorticoids; it was shown to selectively suppress Th17 more than Th1 cell proliferation in vitro using different types of murine $CD4^+$ T cells (with two transgenic TCRs: HEL- and OVA-specific) (Fig. 3). Furthermore, in an in vivo model of organ-specific autoimmunity, which is driven by both Th1 and Th17 cells (24), *Il17a*, *Il17f*, and *Il22* continued to be expressed in Dex-treated animals, despite the total cell number being markedly reduced compared with control animals. Conversely, there was complete ablation of the expression of IL-17, IFN-γ, and the Th1- and Th1-associated transcription factors *Rorc*, *Ahr*, and *Tbx21* in residual tissue-infiltrating $CD4^+$ T cells following CsA treatment. This finding confirms that depletion of either Th1 or Th17 cells can lead to clinical ablation of murine intraocular inflammation (24). However, the complete ablation of $CD4^+$ T-cell IL-17 expression by chronic calcineurin inhibitor treatment could also have deleterious long-term effects at sites of inflammation, given the important role of IL-17 in tissue repair (25).

The clear bias CsA exhibited toward the suppression of IL-17 more than IFN-γ (Fig. 5*B*) was further reflected in the complete inhibition of *IL17A* mRNA and profound reduction of *IL17F* and *RORC* expression in CsA-treated human Th17 cells, whereas the expression of the Th1 transcription factor *TBX21* was maintained with less than 50% reduction in *IFNG* by CsA in human Th17 cells (Fig. 5*C*). Furthermore, our RNA-seq data demonstrated that the genome-wide response to CsA treatment of human IFN-γ–expressing Th0 cells compared with Th17 cells is greatly restricted and the previously identified human Th17 signature genes, including *IL22*, *IL26*, *IL17A*, *IL23R* (26), and *CD161* (27), were among the top six genes that were highly suppressed by CsA treatment (Fig. S6 and Table S2). This finding is again opposite to the effect of Dex on the gene-expression profile of these cells (Fig. 5*D* and Fig. S1*C*), suggesting the difference of CsA responses between Th17 and Th0 cells is also across the whole transcriptome, as well as on signature cytokines and cell-surface markers for human Th17 cells.

Although others have previously shown that when CsA is used to treat inflammation, it reduces IL-17 concentrations in the serum of patients, and also in mixed cultures of cells in vitro

(28, 29), this is, to our knowledge, the first time this T-helper cell subtype-specific effect has been demonstrated. Similar suppression of IL-17 expression and cell division in Th17 cells treated with Tac also suggests that this effect is common across calcineurin inhibitors. Furthermore, our affirmation of the human Th17 SR phenotype makes the presence of these cells, either in peripheral blood or inflamed tissue, a candidate biomarker for the SR disease. In addition, our data suggest that one of the principal actions of CsA is its selective attenuation of IL-17–secreting $CD4^+$ T cells, which is supported by its efficacy in the treatment of Th17-associated disorders, such as inflammatory bowel disease and psoriasis (30, 31), and this is likely to have effects beyond the inhibition of IL-17 alone. Hence, alternative approaches to treatment that have minimal effects on nonimmune tissues and exquisitely interfere with Th17 cell function, either by targeting Th17 cells themselves or the effector memory $CD4^+$ population from which they are derived, would be strong candidates for drug development in the context of SR inflammation.

Methods

Patient and Healthy Control Details. Peripheral blood mononuclear cells were obtained from up to 100 mL of blood from HC and patients following informed consent in accordance with Institutional Review Board-approved protocols at the NIH (08-EI-0099), and National Health Service Research Ethic Committee-approved protocols at University Hospitals Bristol (National Health Service) Foundation Trust, United Kingdom (04/Q2002/84). Further details are described in *SI Methods*, as are further details for all of the techniques listed below.

EAU Induction, Treatment, Clinical Assessment, and Organ Digestion. B10.RIII mice were immunized for EAU induction. Dex was administered subcutaneously and CsA was administered by oral gavage. TEFI was used to assess the clinical score of EAU (Table S3) (18). A single-cell suspension of tissues was isolated using a tissue dissociation method.

Cell Culture and T Cell Proliferation Assays. Murine Th1 and Th17 cultures were cultured, as previously reported (32). Human peripheral $CD4^+CCR6^-$ and $CD4^+CCR6^+$ cells were cultured in IL-2 alone (Th0) or with Th17 polarizing cytokines (Th17). T-cell proliferation was measured by pulsing with 37 kBq [^{3}H]thymidine (Perkin-Elmer) per well for the final 12–16 h of cultures.

Flow Cytometry and FACS Sorting. All flow cytometry experiments were conducted on the BD LSR II and FACS sorting was carried out using the BD Influx system.

Quantitative Real-time PCR. Total RNA was extracted and real-time PCR assays were performed using Taqman reagents (Applied Biosystems).

Immunofluorescence. GR trafficking into the nucleus was optimized and quantified using a Leica SP5 confocal imaging system.

Affymetrix Microarray Data Collection, Analysis, and RNA-seq. RNA was hybridized to GeneChip U133 plus 2.0 arrays (Affymetrix) according to the manufacturer's protocols. RNA-seq libraries were sequenced with paired-end 50-bp reads on an Illumina HiSEq. 2000.

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