Agència de Gestió d'Ajuts Universitaris i de Recerca

Memòria justificativa de recerca de les convocatòries BCC, BE, BP, CTP-AIRE, DEBEQ, FI, FI-ICIP, INEFC i PIV

La memòria justificativa consta de les dues parts que venen a continuació:
1.- Dades bàsiques i resums
2.- Memòria del treball (informe científic)

Tots els camps són obligatoris

1.- Dades bàsiques i resums

Nom de la convocatòria
BP

Llegenda per a les convocatòries:

BCC	Convocatòria de beques per a joves membres de comunitats catalanes a l'exterior
BDH	Beques i ajuts postdoctorals del Programa DGR-Henkel KGaA
BE	Beques per a estades per a la recerca fora de Catalunya
BP	Convocatòria d'ajuts postdoctorals dins del programa Beatriu de Pinós
CTP-AIRE	Ajuts per accions de cooperació en el marc de la comunitat de treball dels Pirineus. Ajuts de mobilitat de personal investigador.
DEBEQ (Modalitat A3)	Beques de Cooperació Internacional i Desenvolupament
FI	Beques predoctorals per a la formació de personal investigador
FI-ICIP	Beques i ajuts per a l'etapa de formació i de recerca de personal investigador novell en els àmbits d'interès de l'Institut Català Internacional per la Pau
INEFC	Beques predoctorals i de col·laboració, dins de l'àmbit de l'educació física i l'esport i les ciències aplicades a l'esport
PIV	Beques de recerca per a professors i investigadors visitants a Catalunya

Títol del projecte: ha de sintetitzar la temàtica científica del vostre document.
Effect of Dexamethasone on cytokine secretion in CD4+ T cells in steroid refractory uveitis

Dades de l'investigador o beneficiari

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Agència
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Paraules clau: cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
Immunologia, uveitis, glucocorticoids, T helper Cells, Citocines

Data de presentació de la justificació
10/6/2011

Nom i cognoms i signatura
del/de la investigador/a

Vist i plau del/de la responsable de la
sol·licitud

6/6/2011

Generalitat de Catalunya
Departament d'Innovació,

Resum del projecte: cal adjuntar dos resums del document, l'un en anglès i l'altre en la llengua del document, on s'esmenti la durada de l'acció

Resum en la llengua del projecte (màxim 300 paraules)

Aquest treball s'ha realitzat a la facultat de Medical Sciences de la Universitat de Bristol, al laboratori del Prof. Andrew D Dick, sota la seva supervisió. El treball s'ha realitzat amb els seus col.laboradors, el Dr Richard Lee i Lauren Schewitz.
Objectiu: Els glucocorticoids (GCs) tenen diversos efectes sobre les cèl.lules T CD4+ per modular la resposta immune, *principalment mitjançant els seus efectes anti-proliferatius. Tot i això,* la dexametasona (Dex, glucocorticoid sintètic) també indueix la secreció de la citocina immunosupressora IL-10. L'objectiu d'aquest treball ha estat comparar la capacitat dels glucocorticoids en modular la producción de citocines en cèl.lules T CD4+ en pacients uveítics sensibles (SS) i resistents (SR) a esteroids.
Metodologia: Es van aïllar cèl.lules T CD4+ de pacients uveítics SS i SR. Es va induïr la producció de cèl.lules T regulatòries (Tregs) mitjançant l'estimulació amb anti- CD3/CD28 en presència d'IL-2 i Dex. Després del cultiu es van analitzar els nivells d'expressió intra-cel.lular de les citocines IL-10, IL-4, IL-9, IL-17 i IFN-y per citometria de flux. D'altra banda, també es van separar cèl.lules T CD4+ de pacients uveítics segons l'expressió de CCR6 i es van polaritzar per obtenir els fenotips Th0 i Th17 per estudiar l'efecte de Dex i ciclosporina (CsA) en aquests subtipus cel.lulars.
Resultats: Les cèl.lules T CD4+ de pacients SR no van ser capaces de produir IL-10 en resposta al tractament amb Dex. Dex no va afectar els nivells d'expressió d' IL-17, però va reduir els nivells de IL-4 i IFN-y. Els nivells d'IL-9 (marcador d'un subtipus cel.lular recentment descrit, *Th9) van ser sempre inferiors a l'1%. En canvi, el tractament amb CsA va* reduir significativamente els nivells d'IL-17 i IFN-y en cèl.lules Th17 i Th0.
Conclusions: La Dex no és capaç d'induir cèl.lules Treg funcionalment supresores en pacients uveítics SR. Aquest fenomen és independent dels efectes en l'expressió d'altres citocines. Aquests resultats suggereixen que l'efecte de la Dex sobre la funció de cèl.lules Treg és clau en el desenvolupament del fenotip SR en la uveitis.

D'altra banda, al llarg d'aquest temps he iniciat un nou projecte que ha donat lloc a un futur projecte de col.laboració. Resumidament, degut a que els nivells elevats de proteïna C-reactiva (CRP) són un factor de risc en la degeneració macular, malaltia inflamatòria crònica principal causa de ceguera en països industrialitzats, l'objectiu d'aquest altre treball ha estat iniciar un projecte per avaluar els efectes de les diferents isoformes de la CRP sobre la resposta inflamatòria de l'epiteli pigmentari retinià.

Resum en anglès (màxim 300 paraules)

This work has been performed in the Faculty of Medical Sciences of the University of Bristol under the supervision of Prof Andrew D Dick. This work has been carried out with Prof Dick collaborators' Dr Lee and Lauren Schewitz.
Purpose: Glucocorticoids (GCs) exert a range of effects on CD4+ T cells to curb the immune response. Chief among these are their anti-proliferative effects. However, dexamethasone (Dex; synthethic GC) also induces secretion of the immunosuppressive cytokine IL-10. The purpose of this study was to compare the capacity of GCs to modulate cytokine production in CD4+ T cells in steroid sensitive (SS) and steroid refractory (SR) uveitis patients.
Methods: CD4+ T cells from SS and SR uveitis patients were isolated. IL-10 secreting T-regulatory cells (Tregs) were induced by culturing CD4+ cells with anti CD3/CD28 in the presence of IL-2 and Dex. Post culture intra-cellular interleukin (IL)-10, IL-4, IL-9, IL-17 and IFN-y expression was then quantified by flow cytometry. Additionally, isolated CD4+ T cells from uveitis patients were sorted based on CCR6 expression and further polarized under Th0 and Th17 conditions to study the effect of Dex and Cyclosporin (CsA) on these T helper cell subsets.
Results: CD4+ T cells from SR patients failed to up regulate IL-10 expression in the presence of Dex. Dex did not change IL-17 expression but IL-4 and IFN-gamma were reduced. Conversely, CsA significantly reduced IL-17 and IFN-gamma expression in Th17 and Th0 cells.

Resum en anglès (màxim 300 paraules) – continuació -.

Conclusions: Dex fails to induce functionally suppressive IL-10 secreting CD4+ T cells in SR uveitis patients. This is independent of the effects on expression of other cytokines. However, whereas Dex hardly modified IL-17 expression, CsA signigicantly reduced IL-17 expression in uveitis patients. This data suggests that differential control of Dex induced Treg function is a key driver of the SR phenotype in uveitis patients.
Additionally, during this time I have started a new project, which will be continued in Barcelona as a collaboration. Briefly, since elevated levels of C-reactive protein (CRP) are a risk factor for age-related macular degeneration, a chronic inflammatory disease and the main cause of blindness in western populations, our purpose was to start a study to evaluate the direct effects of CRP isoforms on the activation of retinal pigment epithelium.

2.- Memòria del treball (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

1. Effect of Dexamethasone on cytokine secretion in CD4+ T cells in steroid refractory uveitis

This work has been performed in the Faculty of Medical Sciences of the University of Bristol under the supervision of Prof Andrew D Dick. This work has been carried out with Prof Dick collaborators' Dr Richard Lee and Lauren Schewitz. Part of this work was recently presented in the ARVO 2011 meeting as a poster communication (Schewitz L et al. D1047).

INTRODUCTION

Uveitis is a generic term used to describe the many clinical entities that comprise intraocular inflammation. The uveal tract is highly vascularized and pigmented, and has three discrete anatomical components (iris, ciliary body and choroid) that may become inflamed, either individually or in combination (1). Anterior uveitis is restricted to the iris and ciliary body, and posterior uveitis is typically restricted to the choroid and retina. Intermediate uveitis involves an area of the posterior ciliary body and the anterior or peripheral retina leading principally to vitritis, whereas panuveitis describes inflammation involving both posterior and anterior uveitis (1,2).
Pathologically, uveitis is an inflammation of the lymphovascular layer that may extend to involve other ocular structures, including the retina, vitreous and optic nerve (3,4).
On clinical presentation, the key initial decision is whether the disease is infectious or noninfectious. Noninfectious intraocular inflammation is commonly treated with steroids and immunosuppressants, while antimicrobials are used for infectious uveitis (5,6). In the latter, the profound inflammatory response may also require treatment with immunosuppressants to prevent tissue damage. Overall response to therapy in noninfectious cases is approximately 60–70%; therefore, there remains a demand to further understand immune mechanisms to develop agents to better treat and, thereby, maintain vision in affected patients (7,8).
Uveitis is estimated to affect up to 115 people per 100,000 in Western populations, (9) just under a quarter of whom will require systemic immunosuppression for sight-threatening disease (10). Glucocorticoids (GC) remain the first-line treatment for a wide range of autoimmune, allergic, and lymphoproliferative diseases. However, up to 30% of patients with ulcerative colitis (UC), asthma, Systemic Lupus Erythematosus, rheumatoid arthritis, acute lymphoblastic leukemia, and uveitis have a disease that is refractory to GC therapy (11, 12). Such steroid resistance is independent of disease severity and often results in sustained high-dose GC exposure (13). Affected individuals are therefore not only subject to the adverse sequelae of on-going tissue damage and inflammation, but also the systemic adverse effects of GCs, including centripetal obesity, skin atrophy, osteoporosis, diabetes mellitus, hypertension, and mood disturbance.

GCs exert a range of effects on CD4+ T cells to curb the immune response, mainly by inhibiting proliferation. Inhibition of lymphocyte proliferation by GCs is due to

suppression of IL-2 production and mRNA expression. However, dexamethasone (Dex; synthethic GC) also induces secretion of the immunosuppressive cytokine IL-10 (14). IL-10 is a potent antiinflammatory cytokine and inhibits Th1 and Th2 immune responses, which has led to considerable interest in its therapeutic potential to treat a wide range of immune-mediated pathologies, (15, 16). It has been shown that human IL-10–secreting Tregs (IL-10–Tregs), which express low levels of the $CD4^{+}CD25^{+}$ Treg-associated transcription factor FoxP3, can be induced following activation, through either polyclonal stimuli or antigen presented by APCs in the presence of the glucocorticoid dexamethasone (Dex) (14, 17).

Taken together the above mentioned considerations and in order to better understand the mechanisms that continue to drive inflammation in patients in whom corticosteroid taper fails, the purpose of this study was to compare the capacity of GCs to modulate cytokine production in CD4+ T cells in steroid sensitive (SS) and steroid refractory (SR) uveitis patients. The specific objectives of this work were to:

1. Compare the capacity of Dex to induce IL-10 in CD4+ T cells in steroid sensitive (SS) and steroid refractory (SR) uveitis patients
2. Study the effect of Dex and the immunosuppressant drug cyclosporin A (CsA) on the cytokine profile in Th0 and Th17 cells from uveitis patients.

METHODS

Clinical Classification of Steroid Responsiveness

As there is no accepted definition of SR disease in uveitis, a threshold of 10 mg prednisone daily was thus adopted as the standard for distinguishing between patients with SR and those with steroid-sensitive (SS) disease. To ensure that this classification was based on a pure corticosteroid response, independent of the effects of other immunosuppressants, patients' clinical records were retrospectively reviewed to establish the dose of prednisone at which the disease reactivated during their first cycle of corticosteroid monotherapy. If this was ≥10 mg daily, the patient was classified as having SR disease, and if it was <10 mg daily, the patient was classified as having SS disease.

CD4+ T cell isolation and culture

CD4+ T cells from SS and SR uveitis patients (N=10) were isolated (Rosette Sep, Stem Cell Technology) from whole blood on a density gradient (Ficoll-Paque Plus; GE Health Care, Piscataway, NJ) and cultured in RPMI-1640 with 10 % FCS, 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 20 mM HEPES buffer (Invitrogen Life Technologies, Carlsbad, CA).

Cells were seeded in 48-well U-bottomed plates (1×10^{6} per well) and incubated at 37°C in humidified air with 5% CO2 in the presence of 1×10^{-6} M dexamethasone (Dex; Sigma-Aldrich, Poole, UK). IL-2 (50 U/ml) and CD3-CD28–coated beads (Dynabeads; Dynal Biotech, Oslo, Norway) were then added (0.63 µL/well) to stimulate T-cell proliferation. Cells were cultured for 4 days.

Isolation and culture of T cell subsets (Th17 CCR6+, Th0 CCR6-)

Previously isolated $CD4^+$ cells from uveitis patients were stained for CD4, CD3, and CCR6 (BD Pharmingen) and sorted into $CD4^+CD3^+CCR6^+$ (Th17) and $CD4^+CD3^+CCR6^-$ (Th0) cells, with purities of >97%. Sorting gates were set on the basis of fluorescence minus one controls. Following sort, $CCR6^+$ were cultured under Th17 polarization conditions and $CCR6^-$ were cultured under Th0 culture conditions. For Th0 conditions cells were resuspended in complete RPMI media (with 10 % FCS, 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 20 mM HEPES buffer), whereas for Th17 conditions cells were resuspended in complete RPMI media (with 10 % FCS, 100 IU/mL penicillin G, 100 mg/mL streptomycin, and 20 mM HEPES buffer), supplemented with IL-6 (20 ng/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml),α-IFNγ (100 ng/ml), and α-IL-4 (100ng/ml). Cells were plated into wells previously coated with αCD3/αCD28 (5μg/ml) and cultured for 3 days. Afterwards, cells were removed from coated wells, plated into uncoated wells, and cultured a total of 14 days in the presence of IL-2 (50 ng/ml). At day 7 cells were reactivated with αCD3/ αCD28 (5μg/ml) for 3 days withoud IL-2. Cells were fed by removing half media and replacing with fresh media (supplemented as per Th0 or Th17 conditions). At day 13 cells were fed with fresh Th0 or Th17 media in the presence or absence of Dex ($1x10^{-6}$) or CsA (200 ng/ml) for an additional 24 h period.

Intracellular cytokine staining

CD4+ T cells were stimulated with 20 ng/ml PMA, 1 μg/ml ionomycin, and 1 μl/ml golgistop for 4 h. Stimulated cells were washed in phosphate buffered saline (PBS), incubated with Live/Dead staining for 30 min, and washed. Cells were then fixed and permeabilized (Fix/Perm solution) and stained with IL-17, IL-10, IFNγ, IL-9, IL-4 fluorescently conjugated Abs. Cells were analyzed by flow cytometry (BD LSR II).

RESULTS

1.1 SR patients fail to up regulate IL-10 in the presence of Dex

CD4+ T cells from SS and SR patients were cultured in the presence of IL-2, αCD3/CD28 and 1x10-6M Dex for 4 days. At the end of the culture, cells were harvested, stimulated with PMA, ionomycin, and Golgistop to induce intracellular cytokine production, and labelled with IL-10 and IL-17 antibodies. As seen in Figures 1 and 2 CD4+ T cells from SR produced significantly lower amounts of IL-10 than those from SS uveitis patients in the presence of Dex.

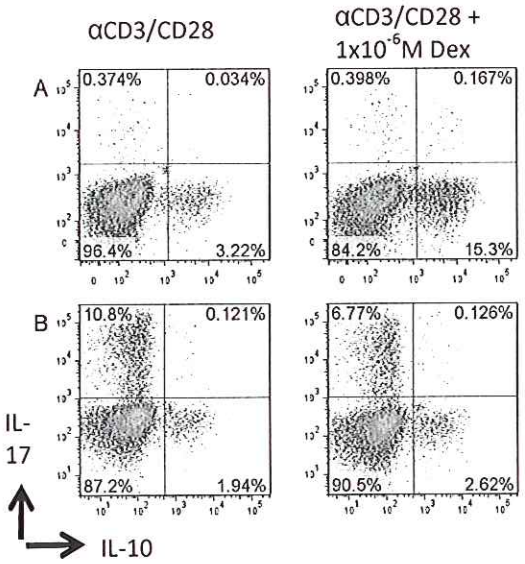

Figure 1. Representative data showing IL-10 induction from SS (A) and SR (B) uveitis patients

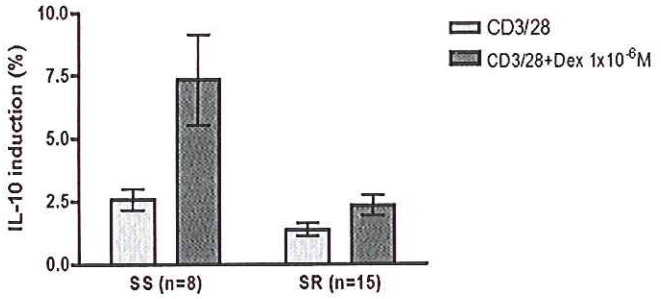

Figure 2. Compiled data of Dex IL-10 induction in CD4+ cells from SS and SR uveitis patients. CD4+ T cells were cultured as for Figure 1.

This data suggests that differential control of Dex induced Treg function is a key driver of the SR phenotype in uveitis patients.

1.2. Dex decreases IFNγ expression but not IL-17 or IL-4 from SS and SR uveitis patients

CD4+ T cells were cultured for 4 days in the presence of IL-2 and αCD3/CD28 to stimulate T cell proliferation and activation and with or without Dex (1x10-6 M). At the end of culture, cells were stimulated with PMA, ionomycin and Golgistop, and expression of IFNγ, IL17, IL4, and IL9 was measured by flow cytomtery. As seen in Figure 3, Dex decreased IFNγ expression in CD4 +T cells, regardless if those were from SS or SR patients. Conversely, Dex did not affect IL-17, nor IL-4 expression. IL-9 was detected in less than 1% of the cells (not shown).

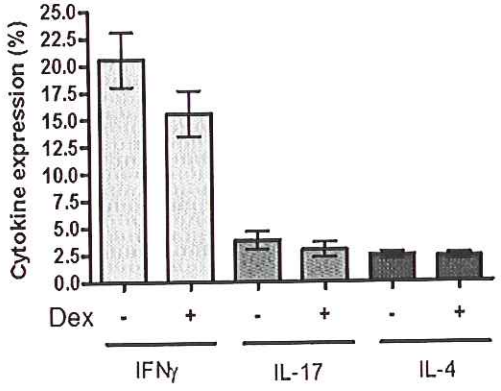

Figure 3. Combined data of cytokine expression from SS and SR CD4+ T cells after 4 days culture with (+) or without (-) Dex.

2.1 Cytokine profile of Th0 CCR6$^-$ and Th17 CCR6$^+$ cells

As described before, CD4+ T cells were sorted based on CCR6 expression. CCR6 is a cell surface protein expressed in Th17 cells. Cells positive for CCR6 were cultured under Th17 polarization conditions (in the presence of IL-2, IL-1β, IL-6, and IL-23), whereas cells that did not express CCR6 were cultured under Th0 conditions (with IL-2 only). As seen in Figure 4, 14 days after culture, Th0 CCR6- did not express IL-17 and expressed low amounts of IL-10. 48 % of Th0 CCR6- cells expressed IFNy. Conversely, Th17 CCR6+ cells expressed IL-17 (40 %) and high amounts of IFNy (75%). The high percentage of IFNy expressing cells (Th17 CCR6+) and the double positive (IFNy-IL-17) population suggest that this Th17 CCR6+ population is not a pure population of Th17 cells but also contains Th1 cells.

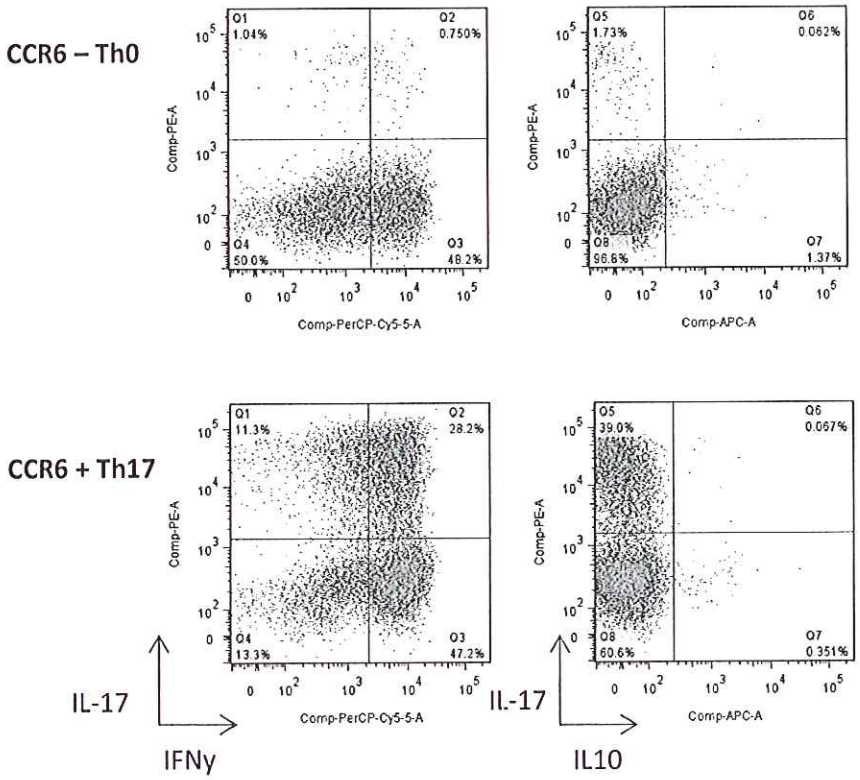

Figure 4. Representative data showing IL-17, IFNy, and IL-10 expression of CCR6- Th0 and CCR6+ Th17 cells after 14 days culture.

2.2 CsA decreases IL-17 expression in CCR6+ Th17 cells

Treatment with CsA (200 ng/ml) for 24h resulted in a dramatic decrease of IFNy in both Th0 and Th17 cells, as seen in Figure 5. Furthermore, CsA also reduced significantly the expression of IL-17 in Th17 cells (Figure 6). On the other hand, 24 h treatment with Dex only produced a small decrease in the expression of IFNy and IL-17 in Th0 and Th17 cells.

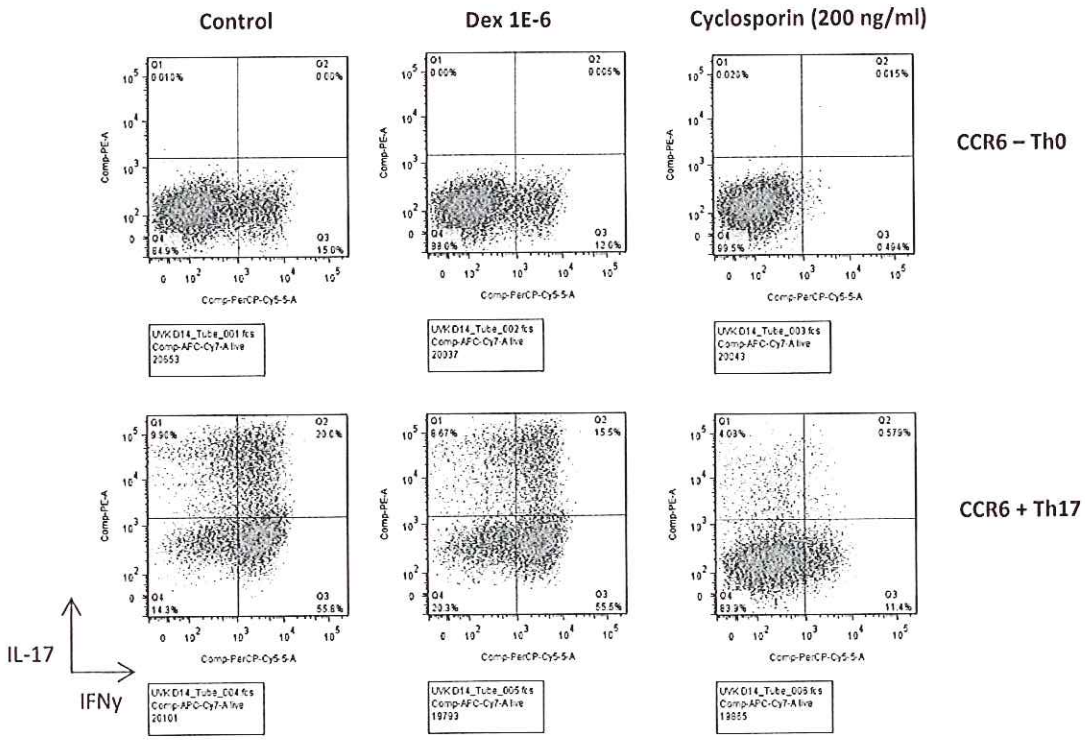

Figure 5. Representative data showing the effect of 24 h treatment with Dex or Csa on IFNy and IL-17 expression on Th0 and Th17 cells.

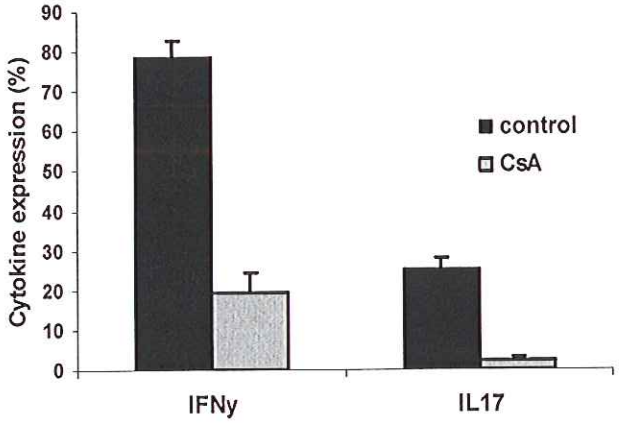

Figure 6. Compiled data (N=4) showing the effect of CsA treatment on the expression of IFNy and IL17 on Th17 CCR6+ cells.

CONCLUSIONS

This work on human T cell immunology was designed to better understand the mechanisms related to steroid responsiveness. Indeed, we found that Dex fails to induce functionally suppressive IL-10 secreting CD4+ T cells in SR uveitis patients. Furthermore, the studies aimed at studying the effect of Dex and CsA on specific T-helper cell subsets (Th0 and Th17) from uveitis patients showed that Dex did not affect IL-17 expression, whereas CsA produced a great decrease in the expression of IL-17 in Th17 cells.

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