Inhibition of experimental auto-immune uveitis by the A3 adenosine receptor agonist CF101

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Abstract. Uveitis is an inflammation of the middle layer of the eye with a high risk of blindness. The Gi protein associated A_3 adenosine receptor (A_3AR) is highly expressed in inflammatory cells whereas low expression is found in normal cells. CF101 is a highly specific agonist at the A_3AR known to induce a robust anti-inflammatory effect in different experimental animal models. The CF101 mechanism of action entails down-regulation of the NF-κB-TNF-α signaling pathway, resulting in inhibition of pro-inflammatory cytokine production and apoptosis of inflammatory cells. In this study the effect of CF101 on the development of retinal antigen interphotoreceptor retinoid-binding protein (IRBP)-induced experimental autoimmune uveitis (EAU) was assessed. Oral treatment with CF101 (10 μg/kg, twice daily), initiated upon disease onset, improved uveitis clinical score measured by fundoscopy and ameliorated the pathological manifestations of the disease. Shortly after treatment with CF101 A_3AR expression levels were down-regulated in the lymph node and spleen cells pointing towards receptor activation. Downstream events included a decrease in PI3K and STAT-1 and proliferation inhibition of IRPB auto-reactive T cells *ex vivo*. Inhibition of interleukin-2, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production and up-regulation of interleukin-10 was found in cultured splenocytes derived from CF101-treated animals. Overall, the present study data point towards a marked anti-inflammatory effect of CF101 in EAU and support further exploration of this small molecule drug for the treatment of uveitis.

Introduction

Uveoretinitis is an inflammation of the uvea and retina, the middle layers of the eye that are critical to vision. The disease can be caused by autoimmune disorders or by infection. Symptoms of uveitis consist of redness of the eye, blurred vision, sensitivity to light, dark floating spots in the vision and eye pain. Possible complications of chronic uveitis include glaucoma, cataracts, accumulation of fluids within the retina, retinal detachment and vision loss (1-3). The current treatment approach entails mostly steroids, which may be given as single treatment or may be combined with anti-metabolites (methotrexate), T cell inhibitors (cyclosporine A) or alkylating agents (cyclophosphamide). Recently, biological drugs such as interferon-α (IFN-α) and anti-tumor necrosis factor-α (anti-TNF-α) (4-6) were introduced for the treatment of uveitis.

The Gi protein associated A3 adenosine receptor (A_3AR) was recently defined as a new specific anti-inflammatory target. A_3AR is highly expressed in inflammatory cells whereas low or almost no expression is found in normal cells (7,8). The highly selective A_3AR agonist CF101 acts as a potent oral anti-inflammatory agent in experimental animal models of rheumatoid arthritis, osteoarthritis and inflammatory bowel disease (7-10). The mechanism of action mediating the anti-inflammatory effect of CF101 includes down-regulation of the NF-κB-TNF-α signaling pathway, resulting in inhibition of pro-inflammatory cytokine production and apoptosis of inflammatory cells (7-10).

Overexpression of A_3AR in different eye cells under pathological conditions has been reported. Schlötzer-Schrehardt *et al* (11), observed that the receptor is highly expressed in non-pigmented ciliary epithelium derived from pseudoexfoliation syndrome eyes, with and without glaucoma, in comparison to normal and glaucomatous control eyes (12). A_3AR overexpression has also been reported in retinal ganglion cells which upon agonist treatment showed reduced Ca^{2+} levels and cell rescue from apoptosis following activation of P2X7 or NMDA receptors (13-15). This protective effect of A_3AR agonists was also demonstrated *in vivo* by Hu *et al* (16).

The anti-inflammatory and the protective effects mediated via A_3AR prompted us to examine the effect of CF101 on a model of experimental autoimmune uveitis (EAU) which represents human uveitis with an autoimmune etiology. CF101 inhibited the clinical and pathological manifestations of interphotoreceptor retinoid-binding protein (IRBP) induced uveitis, suggesting further exploration of its effect in this autoimmune disease.

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Materials and methods

Reagents. Human IRBP peptide 1-20 was synthesized by Anaspec, Inc. (San Jose, CA). Purified *Bordetella pertussis* toxin was purchased from List Biological Laboratories (Campbell, CA). Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich (St. Louis, MO). *Mycobacterium tuberculosis* strain H37RA extract was purchased from Difco (Detroit, MI).

The A_3AR agonist 1-deoxy-1-[6-[[(iodophenyl)methyl] amino]-9H-purine-9-yl]-N-methyl-(-D-ribofuranuronamide) (IB-MECA), was synthesized by Can-Fite BioPharma and designated as CF101. A stock solution was prepared in DMSO and further diluted in PBS.

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonal antibodies against mouse A_3AR (developed against amino acids 151-230 with the internal region of the A_3AR); the signaling protein PI3K (p110β) rabbit polyclonal antibody against amino acids 189-390 and β-actin. The STAT-1 rabbit polyclonal antibody against STAT-1 phosphorylated at Tyr701, was purchased from Cell Signaling Technology (Beverly, MA).

IRBP-induced EAU and CF101 treatment. All studies were performed under an approved institutional animal protocol in the animal resource center of the National Institutes of Health. Animal management complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EAU was induced by immunizing C57BL/6 mice subcutaneously in the thighs and base of the tail with an emulsion of the retinal antigen IRBP, residues 1-20 sequence (GPTHLFQ PSLVLDMAKVLLD, 300 μg/mouse) in complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37RA to 2.5 mg/ml.

In addition, *Pertussis* toxin (300 ng/mouse) was injected intraperitoneally. Treatment with CF101 (10 μg/kg p.o., twice daily) was initiated 7 days after immunization and continued until Day 20 when the experimental cells were harvested.

Clinical assessment of disease was conducted in a masked fashion by fundoscopy, upon anesthetization and pupil dilatation utilizing a binocular microscope at Day 20 after immunization. Scores were assigned semi quantitatively. The presence and number of white focal lesions and white linear lesions affecting blood vessels, retinal hemorrhaging and retinal detachment were analyzed. The fundoscopy scores were assigned according to the following: 0, no change; 0.5, trace; few (1-2), very small. Peripheral focal lesions, minimal vasculitis/viritis; 1, mild vasculitis, <5 small focal lesions, ≤1 linear lesion; 2, multiple (>5) chorioretinal lesions and/or infiltrations; severe vasculitis (large size, thick wall, infiltrations); few linear lesions (<5).

For histopathological analysis, eyes were collected and prefixed for 1 h in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processing. Tissue sections (3-6 μm) were stained with hematoxylin and eosin (H&E). Incidence and severity of disease were examined in a masked fashion by an ophthalmic pathologist.

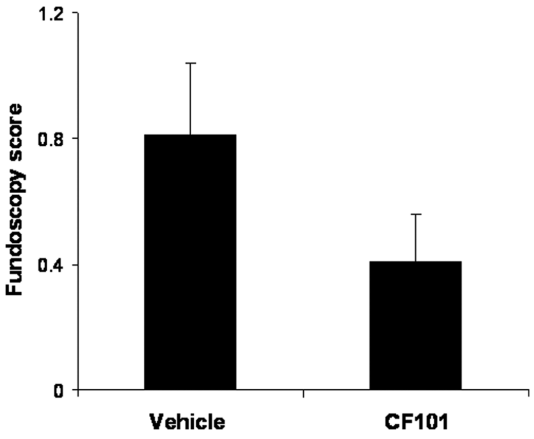

Figure 1. Inhibition of EAU by CF101: fundoscopy. Mice were immunized with a uveitogenic regimen of IRBP and treated with CF101 from Day 7 after immunization. Eyes were examined for disease after pupil dilation under a binocular microscope. Shown are results on Day 20 following immunization.

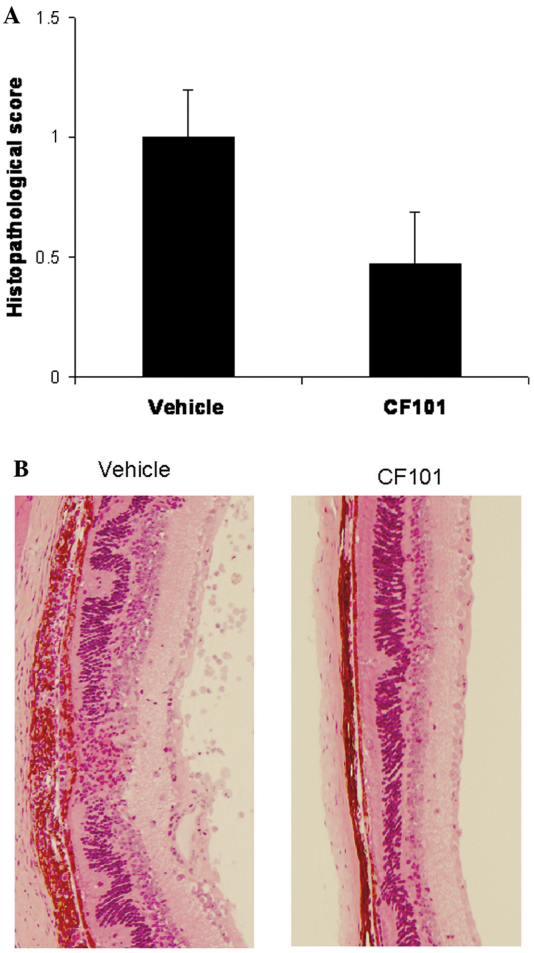

Figure 2. Inhibition of EAU by CF101: histopathology. Mice were immunized with a uveitogenic regimen of IRBP and treated with CF101 from Day 7 after immunization. On Day 20 eyes were collected, fixed and tissue sections were stained with hematoxylin and eosin. (A) Histopathology scores. (B) Pictures of representative slides.

Protein extract preparation. At the end of the study mononuclear cells from spleen and draining lymph node tissues were pooled within the groups, rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (RIPA buffer: 50 mM Tris-buffer pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, and 0.1% SDS). Cell debris was removed by centrifugation for

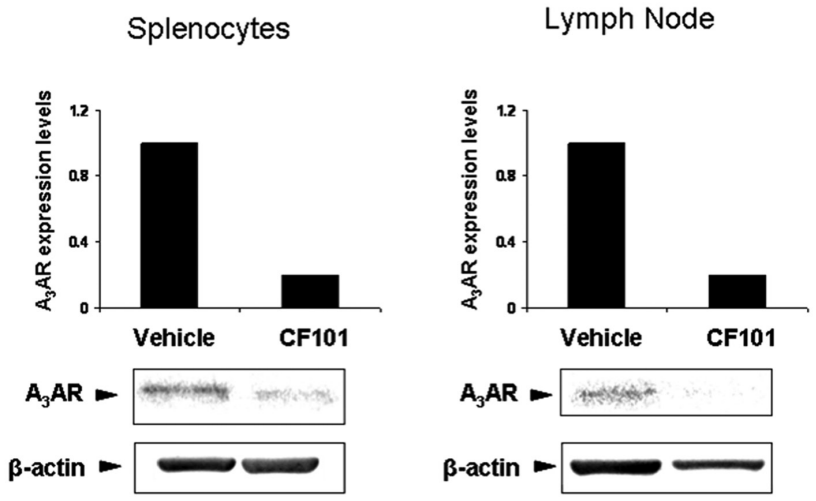

Figure 3. A_3AR down-regulation upon CF101 treatment. Mononuclear cells from draining lymph nodes and splenocytes were collected from EAU mice upon treatment with vehicle or with CF101. Cell protein extracts were subjected to Western blot analysis.

10 min, at 7500 x g. Protein concentrations were determined using the Bio-Rad protein assay dye reagent.

Western blot analysis. Western blot analysis was carried out according to the following protocol. Equal amounts of protein (50 μg) were separated by SDS-PAGE electrophoresis, using 10% polyacrylamide gels (Invitrogen). The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were blocked with 3% non-fat milk and incubated with the desired primary antibody (dilution 1:1,000) for 24 h at 4˚C. Blots were then washed and incubated with a secondary antibody (0.1% non-fat milk) for 1 h at room temperature. Bands were recorded using the BCIP/NBT color development kit (Promega, Madison, WI). Western blot analyses were normalized against the housekeeping protein, β-actin.

Measurement of immunological responses. To test the immunological effects of CF101, the antigen-specific responses of T cells were evaluated using an *in vitro* antigen-driven proliferation assay and pro-inflammatory cytokine production.

At the end of the experiment draining lymph nodes (inguinal and iliac) were collected from both vehicle and CF101-treated groups. For the proliferation assay the cells ($5x10^5$/well) were cultured for 48 h in RPMI-1640 containing 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin $1x10^5$ 2M β-mercaptoethanol and 10% fetal bovine serum, in the presence of graded doses of IRBP (0.2-20 μg/ml). The proliferation rate was evaluated by an ^{3}[H]-thymidine incorporation assay.

For cytokine assays, cultures were set up as for proliferation. Supernatants collected after 72 h were quantitated using the Pierce Multiplex SearchLight Arrays technology (Thermo Fisher Scientific).

Results

Effect of CF101 on the development of IRBP-induced EAU. The IRPB-induced EAU model was utilized to evaluate the effects of CF101 treatment on the development of the clinical and pathological manifestations of uveitis. Mice were immunized with a uveitogenic protocol of IRBP. Fundus examination was performed on Day 20 demonstrating that the severity of EAU was ameliorated in CF101-treated mice compared with vehicle-treated mice (Fig. 1). Histological examination performed on eyes collected 20 days after immunization showed that CF101 inhibited the development of EAU (Fig. 2A).

In the representative pictures of H&E stained eye slides derived from vehicle-treated mice, infiltration of immune cells, retinal folds and focal retinal detachments were observed. In contrast, eyes from CF101-treated mice exhibited a better preserved retina with less extensive lesions. In addition, minor infiltration of immune cells into the eye is noted (Fig. 2B). These results indicate that systemic administration of CF101 is effective in suppressing ocular inflammation and preserving retinal architecture.

Effect of CF101 on the expression levels of A_3AR and downstream signaling proteins. It has been established that upon agonist binding to a G protein coupled receptor, the receptor is internalized and degraded in the lysosomes (17). Draining lymph node cells and splenocytes were collected on Day 20 after immunization from CF101-treated and vehicle-treated animals. Protein extracts were prepared and subjected to Western blot analysis. A_3AR expression levels were downregulated in the CF101-treated lymph nodes and splenocytes, suggesting that receptor activation took place (Fig. 3).

Further mechanistic studies revealed a significant reduction in the expression levels of both PI3K and STAT-1 (Fig. 4).

Immunological effects of CF101. The effect of CF101 on antigen-specific responses of T cells was evaluated using an *in vitro* antigen-driven proliferation assay. Draining lymph nodes (inguinal and iliac) were collected from the IRBP-immunized mice treated with vehicle or CF101 20 days after immunization. The cells were cultured for 48 h in the presence of graded doses of IRBP (0.2-20 μg/ml) and proliferation was evaluated by an ^{3}[H]-thymidine incorporation assay. Inhibition

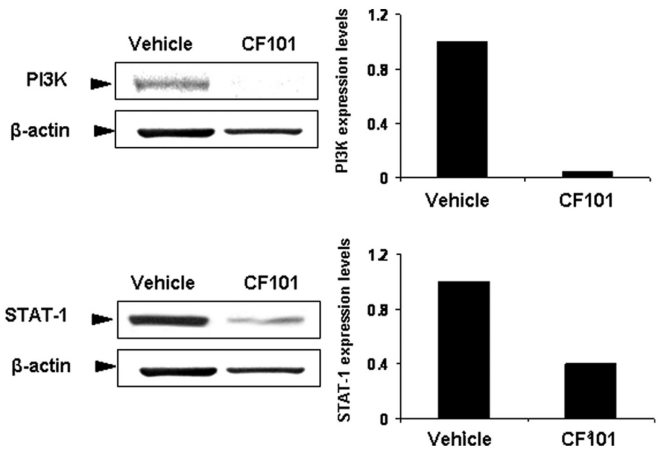

Figure 4. Modulation of signal transduction proteins upon CF101 treatment. Mononuclear cells from draining lymph nodes were collected from EAU mice upon treatment with vehicle or CF101. Protein cell extracts were subjected to Western blot analysis. PI3K and STAT-1 were markedly down-regulated upon CF101 treatment.

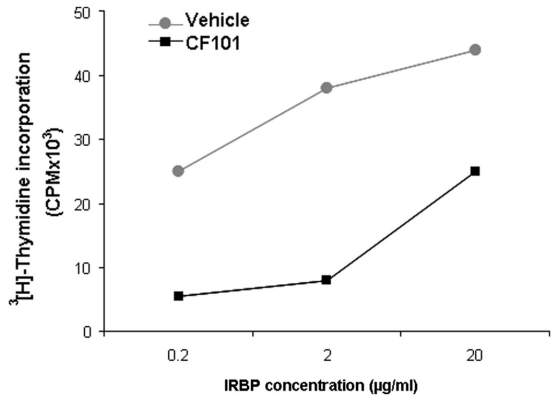

Figure 5. CF101 treatment inhibits the *ex vivo* proliferation of antigen specific lymphocytes. Draining lymph nodes were collected from vehicle and from the CF101-treated IRBP immunized mice, The cells were cultured for 48 h in the presence of different IRBP concentrations (0.2-20 μg/ml) and proliferation was evaluated by an ^{3}[H]-thymidine incorporation assay.

of cell proliferation was noted in cells derived from CF101-treated animals in comparison to vehicle-treated animals in all doses of IRBP examined. These data demonstrate the ability of CF101 to suppress the antigen-specific proliferation of autoreactive T cells (Fig. 5).

To test the effect of CF101 treatment on *ex vivo* cytokine production, splenocytes from either vehicle or CF101-treated mice were harvested on Day 20 after immunization and assayed for cytokine secretion upon IRBP stimulation. In cells derived from the CF101-treated mice, a decrease in IL-2, TNF-α and IFN-γ was noted whereas an increase of IL-10 production was observed (data not shown).

Discussion

The data presented in this study demonstrate the anti-inflammatory effect of CF101 in an experimental animal model of uveitis. CF101 administered orally improved the clinical score of uveitis as measured by fundoscopy, and ameliorated the pathological manifestations of the disease. The drug also acted as an immunomodulatory agent and was able to suppress antigen-specific proliferation and cytokine production of autoreactive T cells.

The anti-inflammatory effect of CF101 had been described earlier in experimental animal models of rheumatoid arthritis, osteoarthritis and inflammatory bowel disease (7-9). Upon CF101 binding to the A_3AR, receptor down-regulation takes place, giving rise to the modulation of downstream signal transduction pathways (7). More specifically, CF101 induces de-regulation of the PI3K-NF-κB pathway resulting in inhibition of TNF-α and induction of apoptosis of inflammatory cells (7-9). In this study, we could show a similar pattern of responses, manifested by A_3AR down-regulation in splenocytes and lymph node cells derived from the CF101-treated animals compared to vehicle-treated animals. Receptor down-regulation in response to the drug supports the conclusion that the inhibition of EAU development upon CF101 treatment is specifically mediated via A_3AR activation.

An additional interesting finding of the present study is the reduction of PI3K expression levels in the lymph nodes upon CF101 treatment (18,19). PI3K controls multiple cellular activation functions, among them IFN-γ production by Th1 cells and production of downstream pro-inflammatory cytokines, such as TNF-α (18,19). Modulation of cellular function by A_3AR engagement could also have other effects relevant to ocular pathology, including effects on eye-resident cells. Earlier studies showed that in retinal pigmented epithelial cells, inhibition of PI3K by LY294002 markedly decreased IL-6, TNF-α and CCL2 production (20). Furthermore, CF101 also decreased the expression levels of STAT-1, known to act as a transcription factor of inflammatory cytokines involved with the etiology and pathogenesis of uveitis (20-22).

EAU is a T cell mediated disease, manifested by an increase in IL-2, IFN-γ and TNF-α producing cells (23,24). Interestingly, CF101 inhibited antigen-induced production of these inflammatory cytokines, whereas the production of interleukin-10 (IL-10) was up-regulated. Earlier data have indicated that IL-10 has a role in controlling EAU and may be involved in natural resolution of the disease (25). Furthermore, basal expression of the IL-10 gene may confer a greater resistance to experimental autoimmune uveitis in some rat strains (26). The inhibition of antigen specific production of IL-2, TNF-α and INF-γ from primed lymphoid cells of CF101-treated mice is compatible with its effects on PI3K and STAT-1.

Taken together, the present study data suggest that CF101 may be useful for the treatment of uveitis, similarly to novel biologics such as IFN-α and anti-TNF-α currently used in the treatment of uveitis (5,6). Remarkably, CF101 is currently in phase 3 clinical development stage for the treatment of dry eye syndrome and phase 2 study data demonstrated that the drug is efficacious and has an excellent safety profile (27). Therefore, CF101 may be a good drug candidate for uveitis based on its anti-inflammatory characteristics and good safety profile proven in ongoing phase 2 and 3 clinical studies.

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