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Article

Activation of α2B/2C adrenergic receptor ameliorates ocular surface inflammation through enhancing regulatory T cell function

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A B S T R A C T

There is an unmet need for effectively treating dry eye disease (DED), a T cell-mediated chronic, inflammatory ocular surface disorder. Given the potential of nonneuronal adrenergic system in modulating T cell response, we herein investigated the therapeutic efficacy and the underlying mechanisms of a specific alpha 2 adrenergic receptor agonist (AGN-762, selective for α2B/2C receptor subtypes) in a mouse model of DED. Experimental DED was treated with the AGN-762 by oral gavage, either at disease induction or after disease establishment, and showed sustained amelioration, along with reduced expression of DED-pathogenic cytokines in ocular surface tissues, decreased corneal MHC-II^{+}CD11b^{+} cells and lymphoid Th17 cells, and higher function of regulatory T cells (Treg). In vitro culture of DED-derived effector T helper cells (Teff) with AGN-762 failed to suppress Th17 response, while culture of DED-Treg with AGN-762 led to enhanced suppressive function of Treg and their IL-10 production. Adoptive transfer of AGN-762-pretreated DED-Treg in syngeneic B6.*Rag1*$^{-/-}$ mice effectively suppressed DED Teff-mediated disease and Th17 response, and the effect was abolished by the neutralization of IL-10. In conclusion, our findings demonstrate that α2B/2C adrenergic receptor agonism effectively ameliorates persistent corneal epitheliopathy in DED by enhancing IL-10 production from Treg and thus restoring their immunoregulatory function.

Introduction

Dry eye disease (DED) is a chronic, inflammatory ocular surface disorder affecting hundreds of millions of people worldwide.[1] T cells have been shown to play a central role in the pathogenesis of DED, supported by the experimental evidence that adoptive transfer of T cells from DED mice to naïve animals induces the same disease in the recipients,[2,3] as well as the approval of two topical T cell inhibitors, Restasis® (Cyclosporine ophthalmic emulsion, 0.05 %) and Xiidra® (Lifitegrast ophthalmic solution, 5 %), for the treatment of DED.[4] It is understood that desiccating stress or other environmental stimulus elicits the release of the pro-inflammatory cytokines, such as IL-1β and IL-6, from the ocular surface epithelial cells, which subsequently activate corneal resident antigen-presenting cells (APC) by up-regulating their expressions of MHC-II, costimulatory molecules, and trafficking molecules. These activated APC then migrate to the eye-draining lymph nodes (DLN) and engage with the naïve T cells, promoting their differentiation into effector T cells (Teff), primarily Th17 cells.[4] The activated Th17 cells, through their specific expression of chemokine receptors, are recruited to the ocular surface where they cause ocular surface damage via secreting IL-17 or both IL-17 and IFN-γ (the so-called 'Th17/1′ cells).[3] IFN-γ has been implicated in promoting DED-associated ocular surface pathology, including corneal epithelial damage, conjunctival epithelial and specialized goblet cell damage, and corneal nerve damage.[5–11] On the other hand, activated Th17 cells impair the function of regulatory T cells (Treg) via IL-17, and thus are resistant to the suppression of Treg.[12] Therefore, current focus of investigation on new therapies for DED aims to restore the immune homeostasis by suppressing effector T cells while improving Treg function.

The immune system and nervous system are intimately connected.[13]

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It is well-documented that lymphoid organs, including the lymph nodes, are innervated by sympathetic nerve fibers, which predominantly secrete norepinephrine.[14] The catecholamine released from the sympathetic nerves and adrenal glands exerts their immunomodulation effects on innate and adaptive immune cells through adrenergic receptors (AR).[13,15] The AR are a class of G protein-coupled receptors, consisting of α and β family members, with 9 subtypes: α1A, α1B, α1D, α2A, α2B, α2C, β1, β2 and β3.[16,17] The functional roles of α1- and α2-AR in regulating inflammation are inconclusive – while some studies show that α1-AR and α2-AR activation promote cytokine secretion and neutrophil recruitment,[18] others show that α2-AR activation decreases pro-inflammatory cytokine production.[13,19] Specifically on T cells, they have been shown to express both α1- and α2-AR, and activation of α2-AR, but not α1-AR, in T cells leads to suppression of their proliferation and IFN-γ and IL-4 secretion.[13] It remains largely unknown whether activation of the subtypes of α2-AR alters T cell immunity and their function in modulating Th17-mediated chronic inflammatory diseases. In a recent parallel study, our colleagues have specifically demonstrated the expression of α2B-AR by $CD4^+$ T cells and other immune cells.[20]

In the present study, we show the direct immunomodulatory effect of α2B/2C activation using a specific α2B/2C-AR agonist in a well-established T cell-mediated DED model and provide the novel precise mechanisms by which α2B/2C-AR agonism modulates inflammation. We demonstrate that α2B/2C agonism leads to significant and sustained disease amelioration by restoring Treg function to effectively suppress Th17 activity and ocular inflammation, which is dependent on the enhanced IL-10 production by Treg.

Results

Treatment with the α2B/2C agonist suppresses the induction and progression of acute corneal epitheliopathy in DED

Mice challenged with desiccating stress for 14 days exhibited an acute, progressive disease course characterized by overt corneal fluorescein staining (CFS) as early as day 3 (mean score of ~ 8) with subsequently steady increase to the disease peak by day 14 (mean score of ~ 11) (untreated group in Fig. 1**A**). However, mice treated with the α2B/2C agonist (AGN-762) at the start of DED induction showed significantly decreased CFS scores at day 3 through day 14 with about 70 % reduction compared to untreated or vehicle-treated groups, accompanied by decreased expression levels of corneal IL-1β and IL-6 as well as conjunctival IL-17A (Fig. 1**A**). Next, to determine whether AGN-762 was effective in treating established disease, mice were induced for DED for 7 days when the treatment started, which lasted for 7 days (until day 14). Those treated with AGN-762 demonstrated similarly significant reduction of CFS scores at both days 10 and 14 compared to untreated or vehicle-treated groups, accompanied by decreased expression levels of corneal IL-1β as well as conjunctival IL-17A and IFN-γ (Fig. 1**B**).

Treatment with the α2B/2C agonist ameliorates chronic corneal epitheliopathy in DED

Given that patients are often in chronic stage of the disease when they seek clinical care, we next evaluated the therapeutic efficacy of the α2B/2C agonist in mice with established chronic disease. After initial 14 days in the desiccating environment, mice were transferred to a standard non-desiccated vivarium for another 7 days (day 21) when the disease became chronic at relatively lower severity (mean score of ~ 6), and mice were treated from day 21 to day 28.[21] After 7 days of treatment with the AGN-762, mice with DED showed significantly lower CFS scores than the untreated or vehicle-treated groups (Fig. 2**A**), along with decreased $MHC\text{-}II^+CD11b^+$ cells and significantly reduced cytokine levels of IL-1β and IL-6 in the cornea (Fig. 2**B**). As Th17 immunity, including their derived IFN-γ, plays a central role in DED,[3] we examined the Th17 response in the conjunctivae and local DLN. AGN-762 treatment led to reduced $CD4^+$ T cell infiltration and significantly decreased IL-17A, IL-17F and IFN-γ expressions in the conjunctivae (Fig. 2**C**), as well as significantly decreased Th17 cells in DLN (Fig. 2**D**). In addition, AGN-762 treatment restored the suppressive function of Treg which was impaired in DED[12] (Fig. 2**E**).

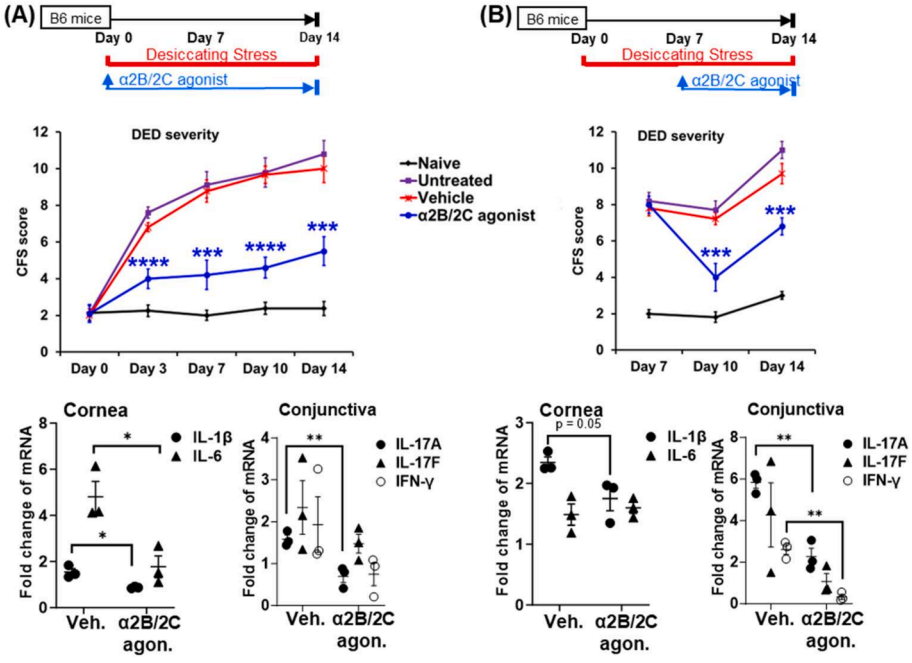

Fig. 1. α2B/2C agonism suppresses induction and progression of acute corneal epitheliopathy in dry eye disease (DED). (A) Treatment with the α2B/2C agonist (AGN-762) at the time of DED induction effectively prevents the development of severe corneal epitheliopathy, and decreases the ocular surface inflammation. **(B)** Treatment with the α2B/2C agonist after DED induction effectively suppresses the acute progression of corneal epitheliopathy, and decreases the ocular surface inflammation. Corneal fluorescein staining (CFS) score was used to assess disease severity. ***, $p < 0.001$; ****, $p < 0.0001$ vs untreated or vehicle group; n = 10 eyes (5 mice) /group; data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test. Cytokine mRNA levels were quantified relative to the naive (non-DED) group (relative level = 1) at the end of treatment; both eyes of each mouse were pooled together for analysis; *, $p < 0.05$; **, $p < 0.01$; data were analyzed by unpaired *t* test.

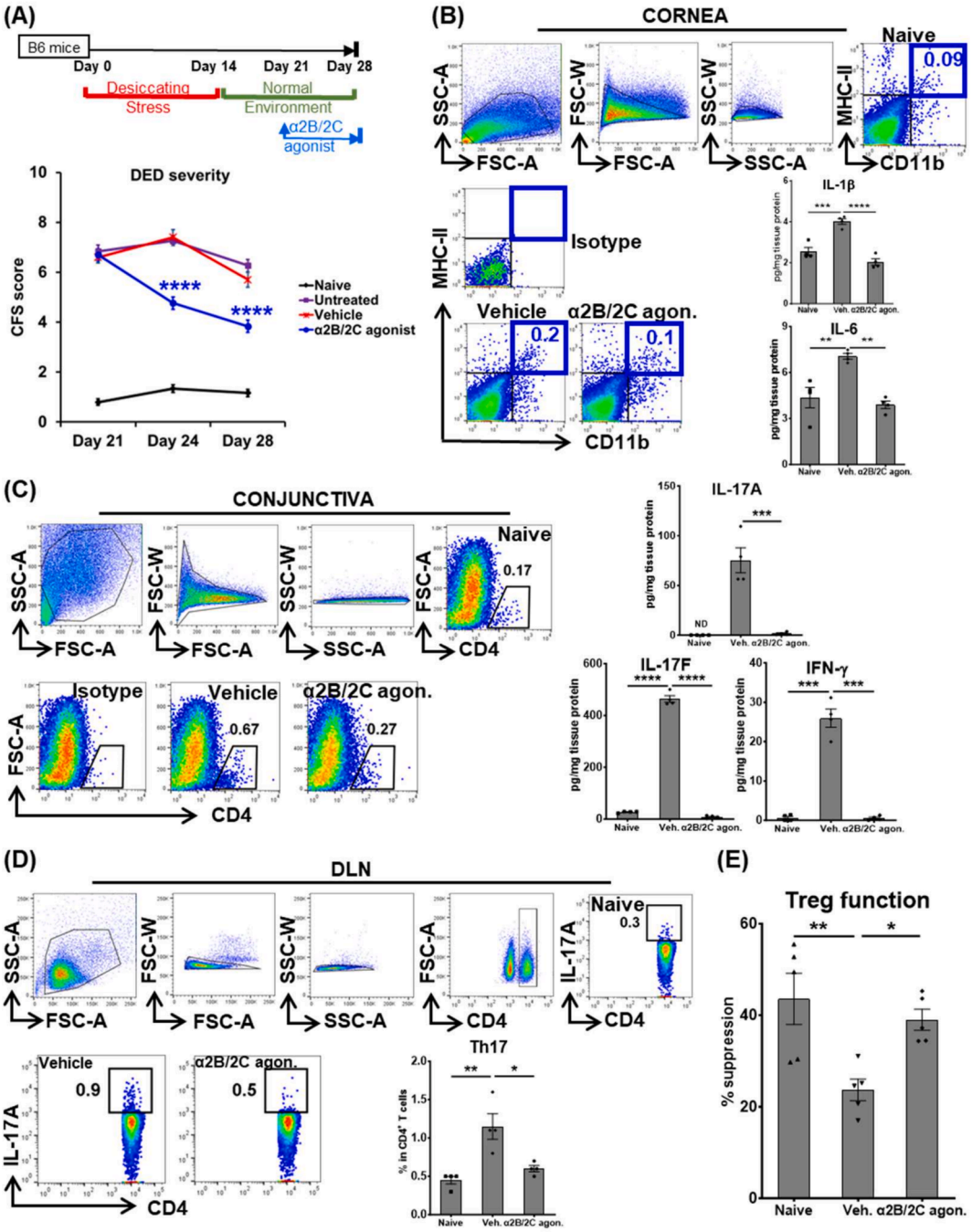

Fig. 2. α2B/2C agonism suppresses chronic corneal epitheliopathy in dry eye disease (DED) and restores immunohomeostasis. (A) Treatment with the α2B/2C agonist (AGN-762) after the development of chronic DED effectively ameliorates the disease, assessed by corneal fluorescein staining (CFS) score. ****, $p < 0.0001$ vs untreated or vehicle group; n = 30 eyes (15 mice) /group from two independent experiments. **(B)** Corneal inflammation was assessed at the end of treatment by quantifying mature MHC-II$^+$CD11b$^+$ cells using flow cytometry and protein levels of IL-1β and IL-6 using ELISA. The upper panel shows the gating strategy for flow cytometry analysis. **(C)** Conjunctival inflammation was assessed by quantifying infiltrating CD4$^+$ T cells using flow cytometry and protein levels of IL-17A, IL-17F, and IFN-γ using ELISA. The upper panel of the flow plots shows the gating strategy for flow cytometry analysis. **(D)** Draining lymph node (DLN) Th17 response was determined by flow cytometry. The upper panel shows the gating strategy for flow cytometry analysis. **(E)** The suppressive function of Treg cells on cell proliferation was quantified using the BrdU incorporation assay and compared with the proliferative responses in the absence of Treg (0 % suppression). For flow cytometry of corneal and conjunctival tissues, samples were pooled from 5 eyes in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; data shown were from one representative experiment out of two performed and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

Treatment with the α2B/2C agonist leads to sustained amelioration of chronic corneal epitheliopathy in DED and suppresses disease exacerbation

To evaluate the long-term effect of the α2B/2C agonist, after the treatment ended on day 28, mice with chronic DED were continuously assessed for disease severity until day 56. In contrast to the untreated or vehicle-treated groups showing stable, persisting corneal epitheliopathy (mean score of ~ 5) during the prolonged phase (day 28–56), those previously treated with AGN-762 exhibited sustained disease amelioration (significantly lower mean score of ~ 2.5) (Fig. 3**A**). At day 56, diseased animals were re-challenged with the desiccating stress for 1 week. The untreated or vehicle-treated groups showed significant

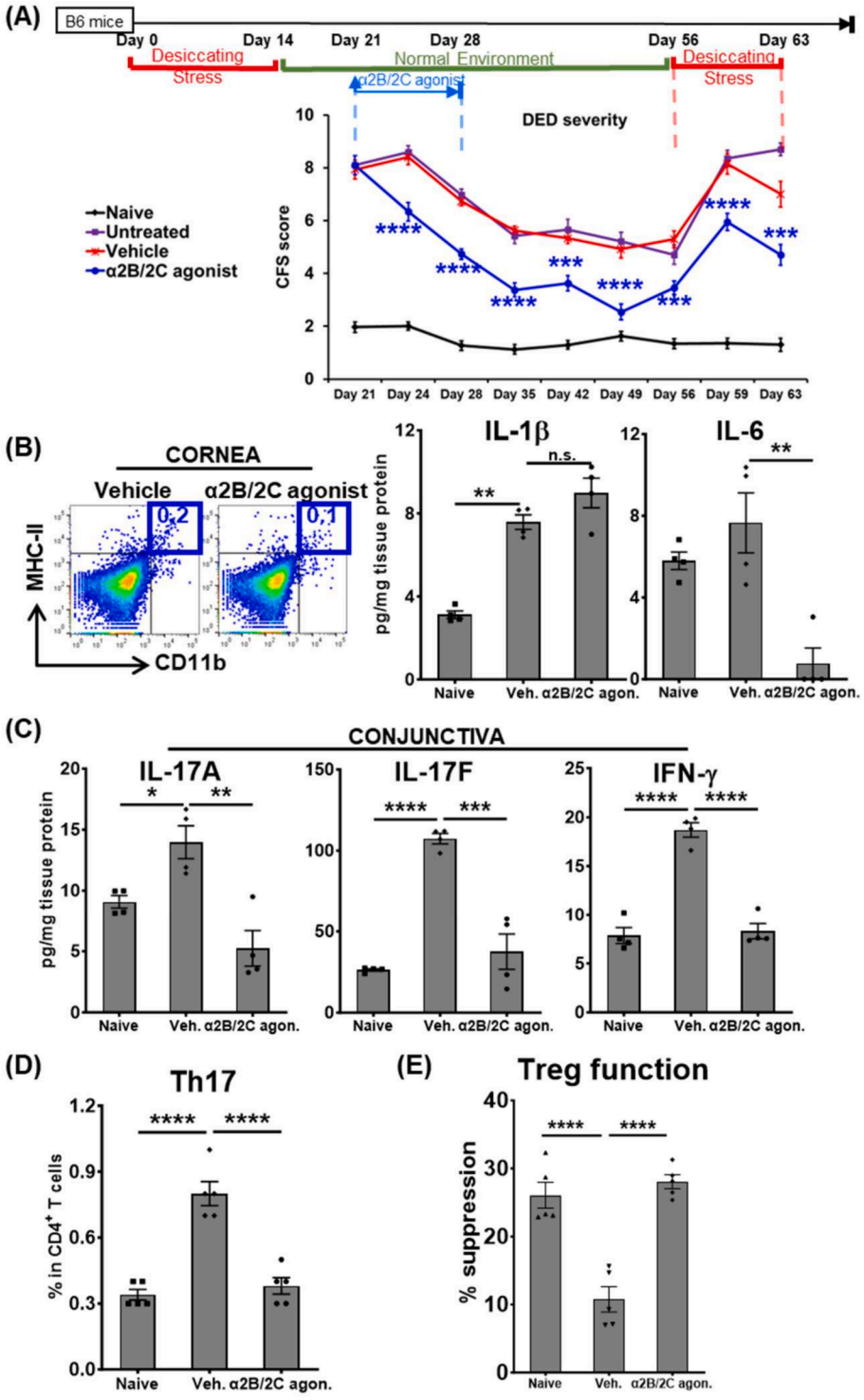

Fig. 3. α2B/2C agonism provides sustained amelioration of corneal epitheliopathy and suppresses disease exacerbation. (A) A 7-day treatment with the α2B/2C agonist (AGN-762) in chronic DED (day 21 – 28) effectively maintains therapeutic efficacy for months (day 28 – 56), and suppresses disease exacerbation in treated animals upon re-challenged with desiccating stress (day 56 – 63). Disease severity was assessed by corneal fluorescein staining (CFS) score. ***, $p < 0.001$; ****, $p < 0.0001$ vs untreated or vehicle group; $n = 20$ eyes (10 mice) /group from two independent experiments. **(B)** Corneal inflammation was assessed at day 63 by quantifying mature MHC-II^{+}CD11b^{+} cells using flow cytometry and protein levels of IL-1β and IL-6 using ELISA. The same gating strategy for flow cytometry analysis was used as shown in Fig. 2B. **(C)** Conjunctival inflammation was assessed at day 63 by quantifying protein levels of IL-17A, IL-17F, and IFN-γ using ELISA. **(D)** Draining lymph node Th17 response was determined by flow cytometry at day 63. The same gating strategy for flow cytometry analysis was used as shown in Fig. 2D. **(E)** The suppressive function of Treg cells on cell proliferation was quantified using the BrdU incorporation assay and compared with the proliferative responses in the absence of Treg (0 % suppression). For flow cytometry of corneal and conjunctival tissues, samples were pooled from 5 eyes in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.s., not significant; data shown were from one representative experiment out of two performed and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

disease exacerbation from day 56 to day 63, while those previously treated with AGN-762 showed significantly suppressed exacerbation (Fig. 3**A**). At the end of the re-challenge, immune response in the ocular surface and DLN was examined. Compared to the vehicle treatment, AGN-762 treatment decreased MHC-II^{+}CD11b^{+} cells and IL-6 expression in cornea (Fig. 3**B**). The expressions of IL-17A, IL-17F, and IFN-γ in the conjunctivae and the frequency of Th17 cells in the DLN were significantly up-regulated in the vehicle-treated DED group, while they were reduced to the baseline non-DED levels by the AGN-762 treatment (Fig. 3**C and 3D**). The suppressive function of Treg in the vehicle-treated group remained impaired after the secondary challenge, and AGN-762 treatment effectively restored their function (Fig. 3**E**).

The α2B/2C agonist acts on DED-derived Treg to restore their function in vitro

To investigate the mechanisms by which the α2B/2C agonist ameliorates DED, we first examined the expression of α2B and α2C by Treg and Teff in DED. Compared to Treg from normal non-DED mice, Treg from DED showed a significant 6-fold upregulation of α2C expression, while the α2B expression in both normal and DED Treg was relatively low and did not show any significant difference. On the other hand, Teff expressed relatively low levels of α2B and α2C and showed further downregulation of α2B from normal Tconv (Supplemental Fig. 1).

Next, we isolated Treg cells from the DLN of DED mice (Fig. 4**A**) and cultured them with 1 μM or 10 μM AGN-762. After 24 h, cultured cells were collected for analysis of Treg phenotypes and function. Compared to the vehicle-treated cell cultures, AGN-762-treated groups did not show any significant changes in Treg frequency or their Foxp3 expression levels (Fig. 4**B**). However, analysis of the cell culture supernatants showed significantly increased IL-10 protein in 1 μM, but not in 10 μM,

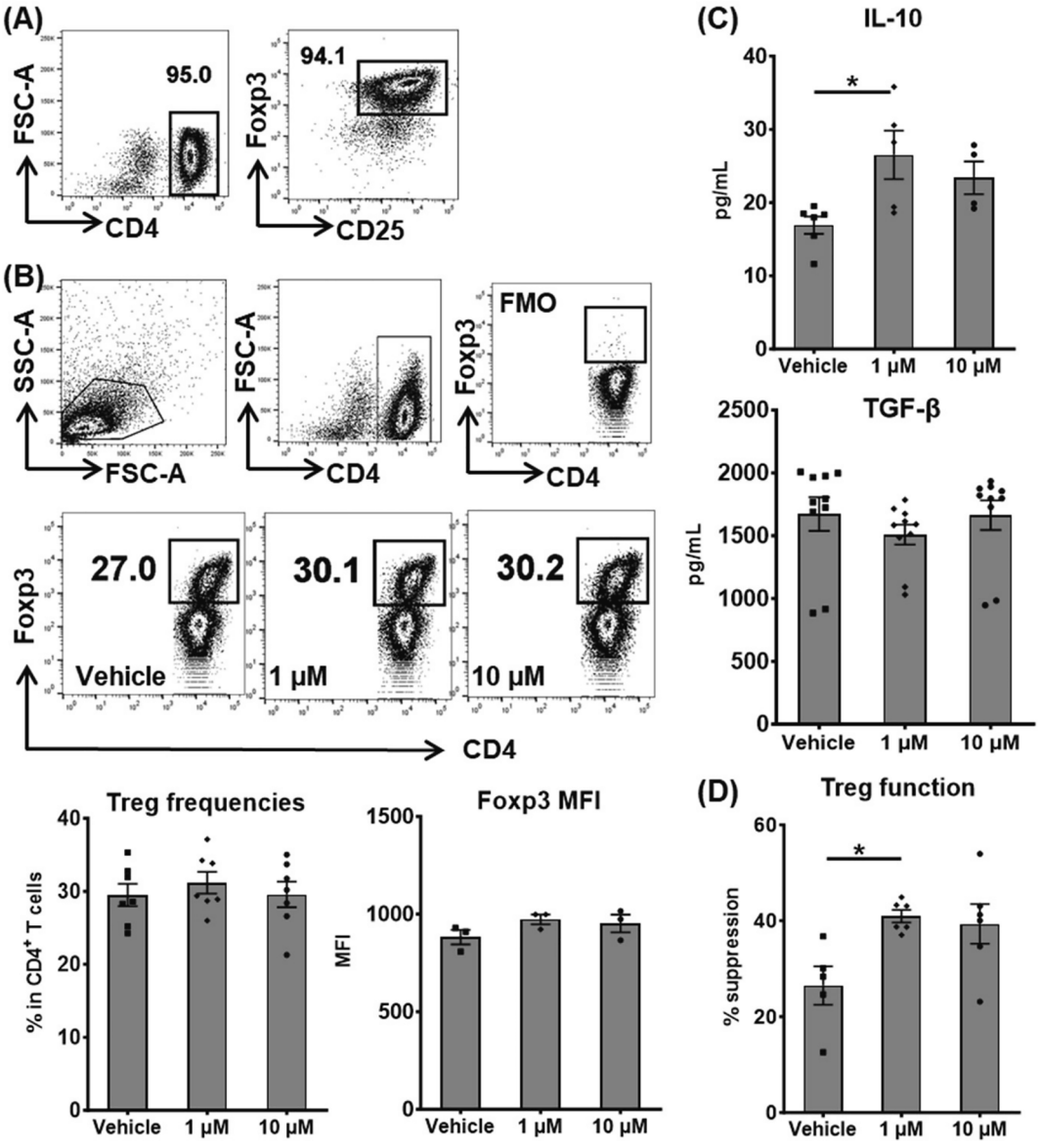

Fig. 4. α2B/2C agonism by AGN-762 promotes IL-10 production by DED-Treg and restores the function of DED-Treg in vitro. (A) Flow cytometry plots show the purity of freshly sorted Treg cells defined as CD4^{+}CD25^{+}Foxp3^{+} cells that were subjected to cell cultures in vitro. **(B)** Flow cytometric analysis of DED-Treg after 24 h culturing with AGN-762, with representative dot plots shown (the upper panel shows the gating strategy), and frequencies and mean fluorescence intensity (MFI) of Foxp3 summarized in the bar graphs. The frequencies were summarized from three independent experiments and MFI was summarized from one representative experiment. **(C)** ELISA assay on the culture supernatants for the levels of IL-10 and TGF-β. Data were summarized from two independent experiments. **(D)** The suppressive function of Treg cells on cell proliferation was quantified using the BrdU incorporation assay and compared with the proliferative responses in the absence of Treg (0 % suppression). *, $p < 0.05$; data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

AGN-762-treated groups; neither AGN-762 groups showed significant changes in TGF-β protein levels (Fig. 4**C**). Further, the AGN-762- or vehicle-treated Treg were washed and collected for suppressive function assay (without the further presence of AGN-762 or its vehicle), and the results showed that although both AGN-762-treated groups showed restored Treg function, only 1 μM AGN-762 treatment led to significantly higher levels (similar to normal Treg function shown in Supplemental Fig. 2**D** — the vehicle group) than the vehicle treatment group (Fig. 4**D**). In addition, we also examined the effects of α2B/2C agonist on normal Treg by culturing isolated Treg from normal non-DED mice with AGN-762. Similar results with DED-Treg culturing were found with no significant changes in Treg frequency or their Foxp3 expression while significantly increased secretion of IL-10 by AGN-762-treated normal Treg (Supplemental Fig. 2).

High, but not low, concentration of the α2B/2C agonist enhances DED-derived Teff function in vitro

Next, we evaluated the potential effects of the α2B/2C agonist on Teff isolated from the DLN of DED mice (Fig. 5**A**). After 24 h of culturing, flow cytometric analysis showed no significant differences in the frequencies of Th17, Th17/1, or Th1 cells among vehicle, 1 μM AGN-762, and 10 μM AGN-762-treated groups, except that 10 μM AGN-762-treated group exhibited significantly increased Th1 cells than the vehicle group (Fig. 5**B**). Consistently, the 10 μM AGN-762-treated Teff also secreted significantly higher protein levels of IFN-γ, along with a higher trend of IL-17A (Fig. 5**C**). We further examined the effects of α2B/2C agonism on conventional T cells (Tconv) isolated from normal non-DED mice (Supplemental Fig. 3**A**). We found that co-culture with 10 μM AGN-762 increased the frequencies of Th17, Th17/1, and Th1 cells (Supplemental Fig. 3**B and 3C**). Under the in vitro Th17 polarization condition, naive $CD4^+$ T cells differentiated into Th17 lineage more efficiently in the presence of the AGN-762 (Supplemental Fig. 3**D**). These findings are consistent with the lower expression levels of α2B and α2C in Tconv and Teff (Supplemental Fig. 1).

DED Treg treated with the α2B/2C agonist in vitro effectively suppresses corneal epitheliopathy severity in DED and Th17 immunity in vivo

Our previous study has demonstrated that Treg in DED is functionally impaired in suppressing Th17 immunity,[12] and thus we wondered whether in vitro treatment of DED Treg with AGN-762 can rescue their in vivo immunosuppressive function. Based on the above in vivo and in vitro results, along with the higher expression of α2B and α2C in Treg, we decided to use the concentration of 1 μM for AGN-762 in the subsequent experiments and focus the mechanisms of action by AGN-762 on Treg. To this end, we reconstituted the B6.*Rag1*$^{-/-}$ mice (lacking their own T or B cells) with syngeneic wild-type DED mice-derived Teff to induce DED in the recipients, as we have done previously.[3,22] Some of those recipients also receive in vitro treated Treg (AGN-762 or vehicle was washed out before transfer) simultaneously. As expected, Teff alone recipients showed significant clinical disease by day 6 post-transfer, and those receiving both Teff and vehicle pre-treated DED Treg showed similar severity of disease. However, the group receiving both Teff and AGN-762 pre-treated DED Treg exhibited significantly reduced disease severity at day 6, which was comparable to the group receiving both Teff and normal (non-DED) functional Treg (Fig. 6**A**). Flow cytometric analysis of the DLN of recipients showed no significant differences in $CD3^+CD4^+$ T cells among groups (Supplemental Fig. 4), but significantly lower frequencies of total IL-17-producing $CD4^+$ T cells (including both Th17 and Th17/1 cells) in the groups receiving AGN-762 pre-treated DED Treg or normal functional Treg, as compared to the group receiving Teff alone (Fig. 6**B**).

α2B/2C agonism-mediated restoration of Treg function is dependent on their IL-10 production

Given that AGN-762 restored the suppressive capacity of dysfunctional Treg along with enhanced IL-10 production by Treg (Fig. 4), we next investigated whether the recovered function of Treg was primarily attributed to IL-10. As expected, DED-derived Treg showed significantly reduced suppressive function than normal Treg, and pre-treatment of DED Treg with 1 μM AGN-762 in vitro effectively restored their function to a similar level as normal Treg; however, addition of an IL-10 neutralizing antibody to the AGN-762-treated DED Treg significantly reduced their function back to the level of DED Treg (Fig. 7**A**). Furthermore, we performed the adoptive transfer experiment using the B6.*Rag1*$^{-/-}$ mice to determine the contribution of IL-10 to the in vivo effects mediated by AGN-762-treated DED-Treg. The B6.*Rag1*$^{-/-}$ recipients were injected with the freshly sorted Teff from DED mice along with DED-Treg that were pre-incubated with AGN-762 before transfer. Recipients were then treated with the anti-IL-10 antibody or isotype IgG. The antibody-treated group showed prominent disease induction and significantly higher disease scores at days 4 and 6 post-transfer than the isotype IgG-treated group (Fig. 7**B**). Flow cytometric analysis of the DLN of recipients showed significantly higher frequencies of $CD4^+$ T cells and the IL-17-producing subsets (Th17 and Th17/1) in the antibody-treated group (Fig. 7**C**).

Discussion

DED is the most common ocular disease, resulting from the loss of balance between immune effectors and regulators that leads to the generation of pathogenic Th17 immunity.[12] In the present study, we demonstrate that activation of specific subtypes of AR – α2B/2C in T cell compartment can effectively rescue the function of the key regulator – Treg to suppress effector Th17 response, and thus restore the immune balance at the ocular surface and achieve the sustained amelioration of DED corneal epitheliopathy, assessed by corneal fluorescein staining, which is a core measurement of corneal barrier function and consistently correlated to corneal epithelial morphological assessment.[23–25] It is noteworthy that the most recent Dry Eye Work Shop (DEWS II) report defines DED with three key components, including tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities.[26] Our chronic model of DED used in the present study, which more accurately reflects the clinical reality where patients predominantly experience a chronic course of the disease, does not show tear deficiency but instead is characterized by ocular surface inflammation and damage[21] which is extensively evaluated by fluorescein staining.[27] This is consistent with clinical observations that many DED patients present with corneal epitheliopathy without a decrease in tear production. Therefore, aqueous tear production was not assessed in the present study. Additionally, our lab is currently establishing ocular pain-associated behavioral tests in our chronic DED model, and these tests have not been validated in our model when this study was performed, but can certainly be included in the future. Moreover, standardization of acquiring digital images of corneal fluorescein staining for grading can be considered to improve the assessment objectivity.

Treg play a critical role in maintaining peripheral self-tolerance, suppressing immune response to self-antigens, and thus protecting hosts from self-destructive autoimmunity.[28] The immunopathogenesis of DED is ascribed to the loss of the suppressive function (but not numerical reduction) of Treg on Th17 effectors.[12] Restoration of immunological homeostasis in DED by recruiting more Treg or improving Treg function has been shown successfully to mitigate disease severity.[28,29] Our study showed that in vivo treatment of DED mice with an α2B/2C-AR agonist substantially recovered their suppressive function to a level similar to normal Treg. The understanding of AR-mediated modulation of immune responses is evolving as studies have demonstrated that activation of α2- and β2-AR suppresses inflammation, whereas activation of α1-AR amplifies inflammation.[13,15] In contrast, the complexity of α2-AR modulation of immune responses is further exemplified by α2-AR stimulation by broad agonism across α2A, 2B and 2C subtypes can enhance innate and adaptive immune responses and indicates that non-selective α2 agonism is not immunosuppressive.[30] Indeed, the immune

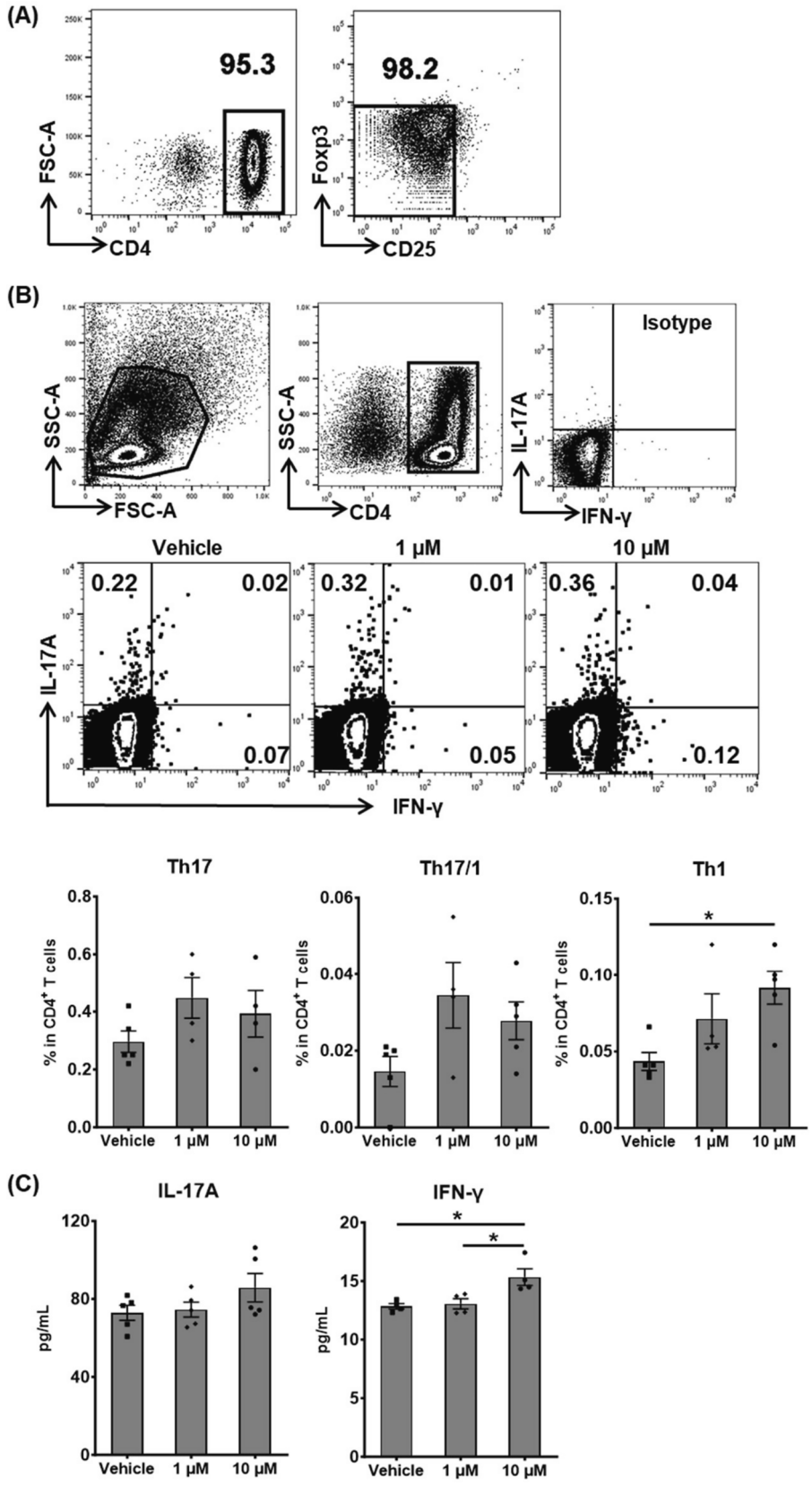

Fig. 5. α2B/2C agonism (by AGN-762 at the higher concentration) enhances DED-derived effector T cells (Teff) in vitro. (A) Flow cytometry plots show the purity of freshly sorted Teff cells defined as $CD4^+CD25^-Foxp3^-$ cells that were subjected to cell cultures in vitro. **(B)** Flow cytometric analysis of $CD4^+CD25^-$ Teff after 24 h culturing with AGN-762, with representative dot plots shown (the upper panel shows the gating strategy), and frequencies of IL-$17A^+$IFN-γ^- Th17, IL-$17A^+$IFN-γ^+ Th17/1, and IL-$17A^-$IFN-γ^+ Th1 summarized in the bar graphs. **(C)** ELISA assay on the culture supernatants for the levels of IL-17A and IFN-γ. Data shown were

from one representative experiment out of two performed. *, $p < 0.05$; data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

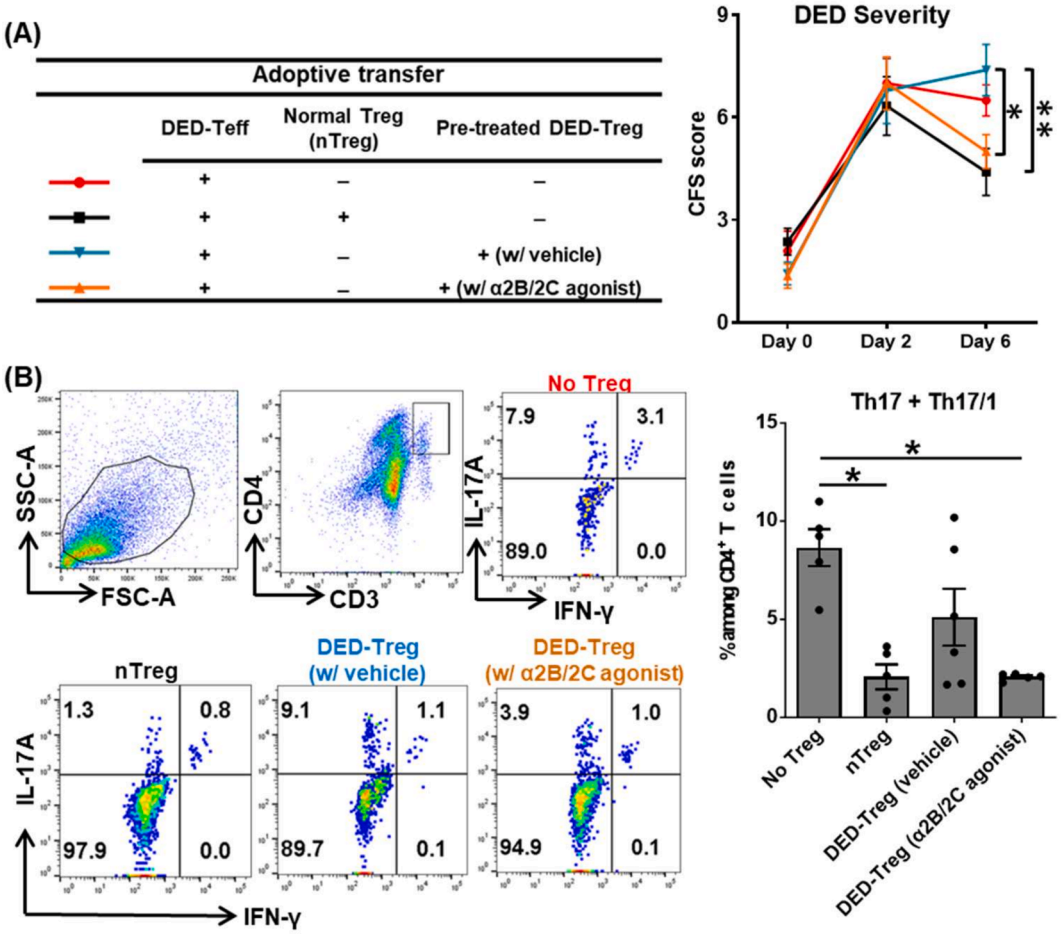

Fig. 6. Adoptive transfer of ex vivo AGN-762-treated DED-Treg effectively reduces corneal epitheliopathy severity and Th17 immunity. (A) Treg cells were isolated from DED mice and in vitro cultured with 1 μM AGN-762 or vehicle for 24 h. The freshly sorted effector $CD4^+$ T cells from DED (Teff) along with normal Treg, DED-Treg pre-treated with the vehicle, or DED-Treg pre-treated with AGN-762 were adoptively transferred to *Rag1*$^{-/-}$ mice, which were subsequently exposed to desiccating stress for 6 days. Disease severity was assessed by corneal fluorescein staining (CFS) score, and summarized from two independent experiments. n = 10–14 eyes (5–7 mice) /group. **(B)** Flow cytometric analysis of T cell response in the draining lymph nodes of *Rag1*$^{-/-}$ recipients at day 6, with representative dot plots shown on the left (the upper panel shows the gating strategy) and frequencies of IL-17-producing CD4 + T cells (including both IL-17A$^+$IFN-γ$^-$ Th17 and IL-17A$^+$IFN-γ$^+$ Th17/1) summarized from two independent experiments on the right. *, $p < 0.05$; **, $p < 0.01$; data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

response outcome mediated by α2-AR can be subtype and cell dependent: Recent cancer immunotherapy research supports enhanced anti-tumor T cell responses are dependent on α2A stimulation of macrophages.[30]

To date, only a few reports specifically investigating the activation of AR on Treg are available and the results are divergent between nonselective and selective activation of AR subtypes. One study has shown that activation of β2-AR in Treg enhances their in vitro suppressive activity in a PKA-dependent manner as well as promotes naïve T cells to differentiate into Treg.[31] In other studies, blockade of universal AR or α-AR through ablation of peripheral sympathetic nerves leads to expansion of Treg,[28,32] suggesting that nonselective activation of AR can limit Treg pool. The discrepancy in the affinity of sympathetic catecholamines binding to AR subtypes may explain the divergent effects of nonselective versus selective activation of AR on modulating Treg.[32,33] Therefore, enhancing Treg function by selectively activating α2B/2C-AR or β2-AR has considerable promise as a novel immunomodulatory therapy in autoimmunity and chronic inflammation.

Foxp3 is the master transcriptional factor to critically induce and maintain the suppressive function of Treg. Mutation of *foxp3* causes severe multi-system autoimmunity along with the absence of $CD4^+CD25^+$ Treg.[34,35] In DED, reduced expression of Foxp3 along with the impaired suppressive function of Treg has been detected.[12] In our study, α2B/2C-AR agonism did not significantly up-regulate the Foxp3 expression levels by DED Treg, suggesting that their enhanced function by α2B/2C-AR activation is mediated through other mechanisms. One of the most studied direct suppression mechanisms in Treg is their production of the anti-inflammatory cytokines IL-10 and TGF-β, which mediate Foxp3-independent Treg function in inducing immune tolerance and alleviating colitis.[36] The results of our study suggest that enhanced IL-10 production is the primary underlying mechanism by which α2B/2C-AR agonism restores Treg function. IL-10 expressed by Treg is known to suppress phagocyte function, antigen presentation, co-stimulatory molecule expression, as well as T cell proliferation and their IL-2 and IFN-γ production.[37] IL-10 has also been demonstrated as a critical functional factor produced by regulatory B cells (Breg) to inhibit follicular T helper cells in primary Sjögren's syndrome.[38] In line with our findings of the α2B/2C-AR agonist, the long-acting β2-AR agonist

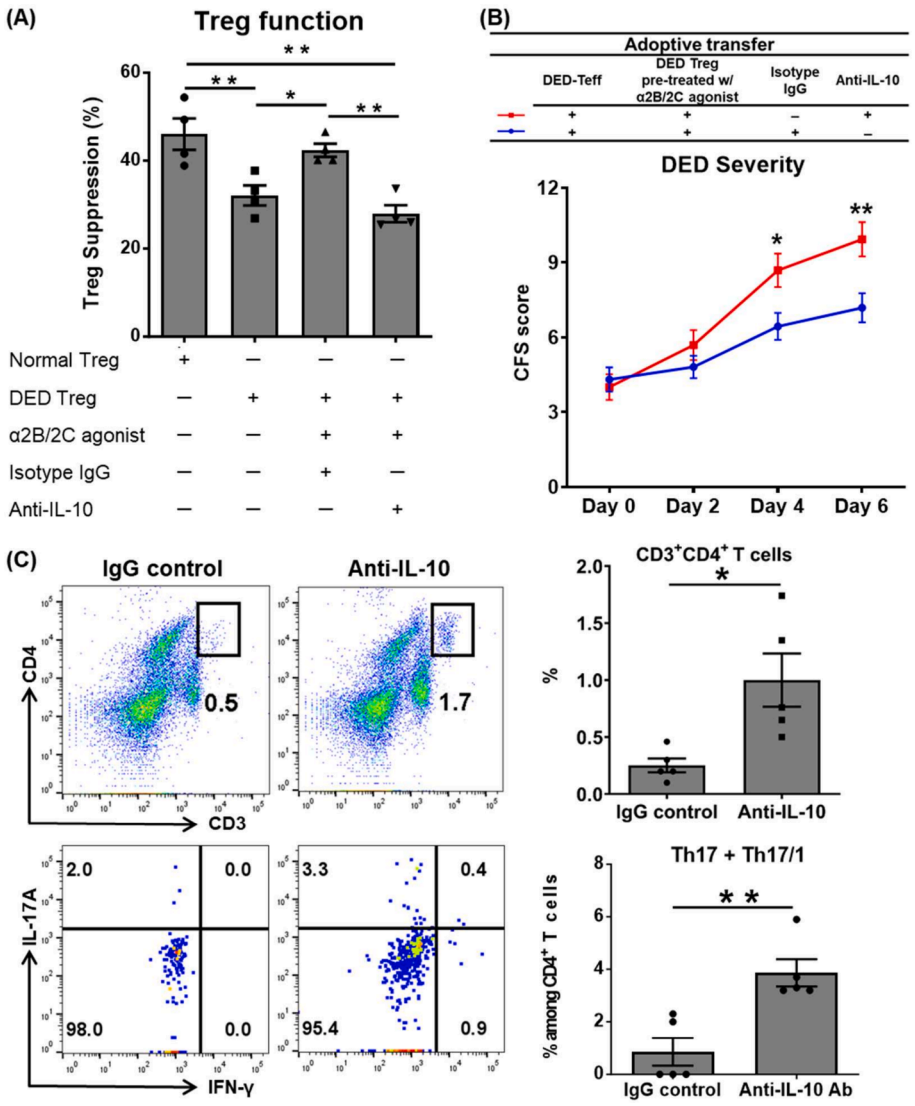

Fig. 7. Neutralization of IL-10 abrogates AGN-762-mediated restoration of Treg immunosuppressive function. (A) DED-Treg cells were pre-treated with 1 μM AGN-762 or vehicle for 24 h and then subjected to suppressive function assay in the presence of anti-IL-10 neutralizing antibody or isotype IgG control. **(B)** Treg cells were isolated from DED mice and in vitro cultured with 1 μM AGN-762 for 24 h. The freshly sorted effector $CD4^+$ T cells from DED (Teff) along with DED-Treg pre-treated with AGN-762 were adoptively transferred to *Rag1*$^{-/-}$ mice, which were subsequently exposed to desiccating stress for 6 days. The recipients received treatment with anti-IL-10 antibody or control IgG immediately after adoptive transfer (day 0) as well as at day 3. Disease severity was assessed by corneal fluorescein staining (CFS) score, and summarized from two independent experiments. n = 16 eyes (8 mice) /group **(C)** Flow cytometric analysis of T cell response in the draining lymph nodes of *Rag1*$^{-/-}$ recipients at day 6, with representative dot plots shown on the left and frequencies of total T cells and IL-17-producing CD4 + T cells (including both IL-17A$^+$IFN-γ$^-$ Th17 and IL-17A$^+$IFN-γ$^+$ Th17/1) summarized from two independent experiments on the right. *, $p < 0.05$; **, $p < 0.01$; data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test (A) or unpaired *t* test (B and C).

similarly increases IL-10 secretion by $CD4^+$ T cells,[39] suggesting that IL-10 may be a common anti-inflammatory mechanism in AR-mediated T cell modulation. Our in vitro suppression assay showed that IL-10 mediated the α2B/2C-AR agonism-enhanced DED-Treg function, and the in vivo systemic IL-10 neutralization abolished the function of α2B/2C-AR agonist-treated DED-Treg in suppressing disease induction and Th17 response. The in vivo effect could still, in part, be attributed to the blockade of non-Treg-derived IL-10. Therefore, the direct function of Treg-derived IL-10 in suppressing the specific Th17 subset will need to be further examined in future studies. While α2B/2C-AR agonism simultaneously enhanced the IL-10 production and suppressive function of dysfunctional DED-Treg, it did not further enhance the suppressive capacity of normal Treg despite their increased IL-10 production. This suggests that untreated normal Treg may already exert maximal suppressive function on Tresp in vitro.

Our study showed treatment with the α2B/2C-AR agonist in DED mice not only enhanced Treg function but effectively suppressed Th17 immunity, the key effectors causing corneal epitheliopathy in DED. However, in vitro culture results showed no direct suppression on Th17 effectors by the α2B/2C-AR agonist. Contrary to our findings, Bao et al. showed that a broad α2-AR agonist attenuated ConA-induced lymphocyte proliferation and IFN-γ and IL-4 production.[13] This discrepancy can be due to differential effects between specific α2B/2C- versus broad α2 activation as well as distinct lymphocyte populations between the two studies. We used sorted Teff by removing the Treg, but Bao et al. analyzed unsorted T cells including the Treg, and thus their readout on T cell proliferation and cytokine production was affected by the co-existing Treg in the experimental system. Of note, our in vitro assay showed that the high, but not the lower, concentration of the α2B/2C-AR agonist increased the expression of inflammatory cytokines IL-17 and IFN-γ by DED-derived Teff, which is consistent with their lower expression levels of α2B and α2C, suggesting that the in vivo suppression

of Th17 immunity by α2B/2C-AR agonism, at the dosage we used, in DED is not a direct effect on Teff. In addition, our in vivo experiments showed a suppressive potential of α2B/2C agonist treatment on corneal $CD11b^+$ cells; these cells serve as APC to activate Th17 immunity in DED.[40] A previous study found skin APC do not express α2-AR[41] and the recent tumor immunotherapy study found macrophages in tumor microenvironment express high levels of α2A and moderate levels of α2C but do not express α2B,[30] suggesting that our in vivo observation of α2B/2C agonist in suppressing corneal $CD11b^+$ may be primarily due to enhanced Treg function; however, any potential direct effect of the α2B/2C agonist on $CD11b^+$ cells needs further investigation. Taken together with our adoptive transfer data, the suppression of Th17 immunity in the α2B/2C agonist-treated DED is primarily through the restoration of the function in Treg, which either directly inhibit activated Th17 cells, or hinder APC activation and subsequent Th17 generation.[42] On the other hand, the in vivo biological effect of α2B/2C-AR agonist may alter the receptor binding of intrinsic catecholamine on different types of AR, such as β-AR, which contributes to the anti-inflammatory effect as well. Therefore, it is possible that the in vivo biological effect of α2B/2C-AR agonist observed in this study could also be mediated through enhancing other AR signaling regulated by endogenous catecholamine.

In summary, we demonstrate that α2B/2C-AR agonism effectively ameliorates DED corneal epitheliopathy and ocular surface inflammation by restoring the immune homeostasis through recovering Treg function which is at least partially mediated by their enhanced production of IL-10. Our study broadens our knowledge of the impact of AR on immune system and potentially provides novel avenues to design effective immunotherapy to treat DED and other chronic inflammatory diseases.

Methods

Animals and experimental model of DED

Female 8- to 10-wk-old wild-type (WT) C57BL/6 mice (Charles River Laboratories) and B6.*Rag1*$^{-/-}$ mice (The Jackson Laboratory) were used for this study. All animal experiments were approved by the Schepens Eye Research Institute Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were placed in a controlled-environment chamber (relative humidity < 15 %, airflow of 15 L/min, temperature of 21–23 °C) for 14 consecutive days without scopolamine injection to induce acute DED. To induce chronic DED, acute DED mice were thereafter transferred to a standard non-desiccated vivarium (relative humidity of 40–60 %, no direct airflow, temperature of 21–23 °C) and housed for additional 14 – 42 days.[22] For rechallenge experiments, established chronic DED mice were placed back into the controlled-environment chamber for additional 7 days.[22]

Treatment of DED mice and corneal epitheliopathy severity assessment

Active compound AGN-762 (1.5 mg/ml) dissolved in 5 % dimethyl sulfoxide (DMSO) or 5 % DMSO as the vehicle (both provided by AbbVie Inc.) in 100 μl volume was administered to unanesthetized mice by oral gavage three times a day from day 0 – 14 or from day 7 – 14 in acute DED, or from day 21 – 28 in chronic DED. Oral gavage was conducted by holding the head of awake mice in vertical alignment with the esophagus, inserting a disposable sterile feeding needle (20G X 1.5″, Cadence Science) into the mouth and threading down the esophagus, and injecting the substance slowly. Experimental mice were housed in individual cages, with animals from multiple groups randomly assigned to each cage. A masked observer assessed all cages on the same day with breaks between each cage to minimize bias and reduce the impact of observer fatigue. Disease severity was evaluated using corneal fluorescein (Sigma-Aldrich) staining (CFS) on both eyes of each mouse and scored by a masked investigator according to the National Eye Institute (Bethesda, MD) grading system of 0–3 for each of the five areas of the cornea—central, superior, inferior, nasal, and temporal.[43]

Conventional T cells (Tconv), effector T cells (Teff) and regulatory T cells (Treg) isolation and culture

The DLN of acute DED or normal mice were collected and pooled respectively (5–7 DED or 8 normal mice in each experiment). $CD4^+CD25^-$ Teff from DED mice, $CD4^+CD25^-$ Tconv from normal mice, and $CD4^+CD25^+$ Treg were sorted using the MACS kit (Miltenyi Biotec). The 1×10^6 sorted Teff or Tconv or 1×10^5 sorted Treg were cultured with AGN-762 in a 96-well U bottom plate at a series of concentrations or its vehicle for 24 h. Additional Treg cells were cultured with AGN-762 in the presence of the anti-IL-10 antibody (1 μg/ml) or isotype IgG. All the Treg cultures were supplemented with 20 ng/mL IL-2. There was no addition of any stimulating molecules or cells in Tconv or Teff cultures. At 24 h, supernatants were collected for subsequent ELISA analysis, and cells were harvested for flow cytometry or Treg suppression assay as detailed below.

T cell adoptive transfer

Teff and Treg were sorted from DLN using the Treg MACS kit (Miltenyi Biotec). 5×10^5 Teff freshly isolated from the DLN of DED mice, along with 2×10^5 Treg freshly isolated from the DLN of DED mice or after pre-incubation with AGN-762 or vehicle (DMSO, 1 μl in 10 ml of culture medium), were intravenously injected into B6.*Rag1*$^{-/-}$ mice which were then immediately placed in the controlled-environment chamber for 6 days. Additional B6.*Rag1*$^{-/-}$ mice receiving AGN-762-pretreated DED Treg were intraperitoneally injected with an anti-IL-10 antibody (1 mg/ml in 100 μl PBS) or isotype IgG (1 mg/ml in 100 μl PBS) on the day of cell transfer and 3 days thereafter. Then, the DLN of B6.*Rag1*$^{-/-}$ mice were harvested for flow cytometric analysis of transferred T cells.

Real-time PCR

Conjunctivae and corneas were harvested and stored in TRIzol reagent (Invitrogen, Carlsbad, CA) at −80 °C until RNA was isolated and reverse-transcribed using RNeasy micro kit (Qiagen, Valencia, CA) and SuperScript III kit (Invitrogen). Treg, Tconv, and Teff cells were sorted from the DLN of normal and DED mice and processed for RNA extraction and reverse transcription. Real-time PCR was performed using TaqMan Universal PCR Master Mix and predesigned primers for IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-17A (Mm00439618_m1), IL-17F (Mm00521423_m1), IFN-γ (Mm01168134_m1), α2B (Mm00477390_s1), α2C (Mm00431686_s1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) (Applied Biosystems, Carlsbad, CA). Samples were analyzed using a real-time PCR system (LightCycler 480 II System; Roche Applied Science, Indianapolis, IN). The GAPDH gene was used as an endogenous control for each reaction. The results of quantitative PCR were analyzed by the C_T method, in which the target change $= 2^{-\Delta\Delta CT}$. The results were normalized by the C_T value of GAPDH, and the mean C_T of relative mRNA level in the normal (non-DED) group was used as the calibrator. Alternatively, the copy numbers of target genes were calculated relative to 10^6 copies of GAPDH.

Flow cytometry analysis

Corneas, conjunctivae, and DLN of mice were collected and single cell suspensions were prepared as previously described.[22] Briefly, cornea and conjunctival tissues were first cut into small fragments and digested with 2 mg/ml DNase I and 2 mg/ml collagenase type IV (Roche) in RPMI 1640 (Invitrogen) for 1 h at 37 °C with agitation. The suspension was then triturated through a 30-gauge needle to homogenize the remaining tissue and filtered through a 70-μm cell strainer (BD Biosciences) to prepare single cell suspensions. For corneal and conjunctival tissues, samples were pooled from 5 eyes in each group. For DLN, single cell suspensions were prepared from pooled superficial cervical LN of each individual animal directly using a 70-μm cell strainer. Trypan blue exclusion assay confirmed cell viability.[22] The following antibodies were used: PerCP-Cy5.5-conjugated anti-CD11b, BV421-conjugated anti-MHC-II, PE-conjugated anti-CD3, FITC- or BV421-conjugated anti-CD4 (all form Biolegend), PE- or APC-conjugated anti-IL-17A, FITC-

conjugated anti-IFN-γ, and APC-conjugated anti-FoxP3 (all from eBioscience). For intracellular cytokine staining, cells were first stimulated with PMA plus ionomycin (Sigma-Aldrich) for 5 h at 37 °C and 5 % CO2 in the presence of GolgiStop (BD Biosciences). Stained cells were examined with an LSR II flow cytometer (BD Biosciences), and the results were analyzed using FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay (ELISA)

Cornea and conjunctivae of mice were harvested and stored in cold sterile PBS containing protease inhibitors (Sigma-Aldrich) at −80 °C until used. The samples were homogenized on ice and centrifuged. The supernatants were assayed using commercial ELISA kits for levels of the total protein (Thermo Scientific), IL-1β and IL-6 for cornea, IL-17A, IL-17F, and IFN-γ for conjunctivae (Invitrogen or Biolegend). For cell cultures, the supernatants were collected and assayed using commercial ELISA kits for levels of IL-10 (Biolegend) and TGF-β (Invitrogen) for Treg cultures, and IL-17A, IL-17F, and IFN-γ for Teff or Tconv cultures.

Treg suppression assay

To evaluate Treg function, 1×10^5 responder $CD4^+CD25^-$ T cells (Tresp) isolated from the DLN of normal mice were cocultured with 5×10^4 Treg from the DLN of different experimental groups, 1×10^5 T cell-depleted syngeneic splenocytes (by CD90.2 depletion kit, Miltenyi Biotec), and 1 μg/ml anti-CD3 Ab for 3 days as previously described.[12] Proliferation was measured using the BrdU cell proliferation kit (Millipore, Billerica, MA) and a colorimetric microplate reader. The proliferation measured in the cell co-culture without adding Treg cells was considered the control proliferation with 0 % suppression, and the percentage of suppression was calculated using this formula: %suppression = [(Tresp proliferation without Tregs – Tresp proliferation with Treg)/ Tresp proliferation without Treg] × 100.

In vitro Th17 cell polarization

Th17 cell polarization were performed using Mouse Th17 cell differentiation kit (CDK017, R&D system) according to the manufacturer's instructions. In brief, naïve $CD4^+$ T cells were sorted from normal mouse splenocytes using the MACS kit (Miltenyi Biotec). Naïve $CD4^+$ T cells were suspended in the mouse Th17 differentiation media at 1×10^6 cells/mL with AGN-762 at a concentration of 0, 1, or 10 μM and cultured in the anti-mouse CD3 antibody-coated plate. Mouse Th17 differentiation media with AGN-762 were replenished on day 3. On day 6, cells were collected for flow cytometry.

Statistical analysis

The statistical software GraphPad Prism 9.5 (GraphPad Software, La Jolla, CA, USA) was used for all analyses. For comparison of multiple groups, ANOVA followed by Bonferroni's multiple comparisons post hoc test was used to determine the statistical significance. For comparison of two groups, an unpaired, two-tailed Student's *t*-test was used for statistical analysis. Results are presented as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

Data Availability

Core data generated or analyzed during this study are included in this published article and its supplementary information files. Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi. Replicate data from some experiments are not included and are available from the authors on reasonable request.

CRediT authorship contribution statement

Nai-Wen Fan: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Man Yu:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Shudan Wang:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Tomas Blanco:** Investigation, Formal analysis. **Zala Luznik:** Investigation. **Sunil K. Chauhan:** Investigation. **Veena Viswanath:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Daniel Gil:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Katherine Held:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Yihe Chen:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Reza Dana:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was supported by a sponsored research award from AbbVie Inc. to Reza Dana and Yihe Chen. Veena Viswanath and Daniel Gil are former employees of Allergan Inc./AbbVie Inc.. Katherine Held is a current employee of AbbVie Inc.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mucimm.2024.11.002.

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