Alpha-tocopherylquinone-mediated activation of the Aryl Hydrocarbon Receptor regulates the production of inflammation-inducing cytokines and ameliorates intestinal inflammation

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Abstract

This study investigated the role of Alpha-tocopherylquinone (TQ) in regulating the intestinal immune system and the underlying mechanisms. In the experimental dextran sodium sulfate and T cell-mediated colitis models, TQ significantly reduced the mRNA levels of interleukin (IL)-6, IL-1β, IL-17A, IL-23, and tumor necrosis factor (TNF)-α and the abundance of proinflammatory macrophages, T helper (Th)17 cells, and ILC3s in the colons of wild-type mice. TQ also prevented lipopolysaccharide (LPS)-induced activation of NFκB and signal transducer and activator of transcription (Stat)-3 pathways in the human macrophage U937 cells. Pharmacological inhibition or CRISPR-Cas-9-mediated knockout of Aryl hydrocarbon Receptor (*AhR*) prevented the anti-inflammatory effects of TQ in the LPS-treated U937 cells. Furthermore, TQ reduced the mRNA levels of the LPS-induced pro-inflammatory cytokines in the WT but not *Ahr*$^{-/-}$ mice splenocytes. TQ also reduced IL-6R protein levels and IL-6-induced Stat-3 activation in Jurkat cells and *in vitro* differentiation of Th17 cells from wild-type but not *Ahr*$^{-/-}$ mice naive T cells. Additionally, TQ prevented the pro-inflammatory effects of LPS on macrophages and stimulation of T cells in human PBMCs and significantly reduced the abundance of tumor necrosis factor-α, IL-1β, and IL-6^{hi} inflammatory macrophages and Th17 cells in surgically resected Crohn's disease

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(CD) tissue. Our study shows that TQ is a naturally occurring, non-toxic, and effective immune modulator that activates AhR and suppresses the Stat-3-NFκB signaling.

INTRODUCTION

Homeostasis of the immune system forms a key foundation of host health. Carefully orchestrated and stringently regulated signaling pathways that activate and subsequently inactivate the innate and adaptive branches of the immune system mediate robust protection from foreign antigens while protecting against bystander self-damage. In diseases with immune dysregulation like rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease (IBD), including Crohn's disease (CD), and ulcerative colitis (UC), however, these homeostatic regulatory circuits are often compromised by the loss of the inhibitory signaling of the immune system, resulting in persistent, unresolved inflammation[1,2].

Aberrant activation and loss of regulation of the IL-6 signaling axis have been implicated in inflammatory conditions including IBD, Systemic Lupus Erythematosus, etc[3]. Interleukin (IL)-6 is a pleiotropic cytokine that is produced by multiple immune cell types and serves as a key regulator of the signal relay between the innate and humoral immune responses. IL-6 cis- or transsignaling activates the Janus kinase/signal transducer (Jak) and activator of transcription (Stat) kinases and alters the transcriptional state of the target cells to initiate inflammation[4]. Moreover, other than being a standalone regulator of proinflammatory responses, IL-6 is one of the key precursors for the differentiation of T helper (Th)17 cells, a special subset of the CD4$^+$ T cells that secrete IL-17A/F and IL-22 as their signature cytokines. IL-6, combined with tumor growth factor-β, and IL-23 promotes the differentiation of naive T cells into the Th17 lineage while inhibiting the development of the anti-inflammatory Treg cells[5]. Th17 cells serve a key role in multiple immunological responses against pathogens and tissue repair; however, like IL-6, unregulated proliferation and the hyperactivation of Th17 cells have been attributed to several diseases with immune dysregulation, such as IBD[6,7].

As an environmental sensor, the AhR binds to a variety of synthetic and natural compounds and acts as a class I, basic helix-loop-helix transcriptional regulator[8]. Upon activation, the AhR dimerizes with the AhR nuclear translocator protein, which then binds xenobiotic-responsive element (XRE; also known as dioxin response element) sites in gene regulatory regions, regulating the expression of a diverse set of genes. Genetic deletion studies have demonstrated important functions of AhR in the immune responses of innate immune cells, like macrophages, and in adaptive immunity, like its regulatory role in T cell differentiation. Furthermore, AhR activation has been shown to improve colitis outcomes in animal IBD models[9–20]. According to a current hypothesis, decreased intestinal AhR expression or defective AhR activation contributes to intestinal barrier dysfunction, immune dysregulation, and dysbiosis, thus favoring chronic inflammation and IBD[21].

Vitamin E derivatives are believed to have several clinical benefits, including regulation of oxidative stress and cell death[22], although the molecular basis of the many physiological functions of vitamin E remains unknown. Alpha-tocopherylquinone (TQ) is a quinone-structured, non-toxic, less reactive, cell membrane integrity-promoting oxidation product

of vitamin E[23,24]. In this study, we examined the immunomodulatory role of TQ. Our studies show that TQ has remarkable immunomodulatory abilities, including inhibiting pro-inflammatory IL-6 production by macrophages and IL-17A/F production by effector CD4^{+} T cells and preventing the differentiation of naive CD4^{+} T cells to Th17 cells. Consequently, TQ significantly reduced intestinal inflammation and Th17 abundance in experimental colitis. Additionally, TQ reduced IL-17A/F production by effector T cells from CD patient-derived tissues. We also show that TQ activates AhR, and the immunomodulatory effect of TQ is AhR-dependent. Thus, our study shows that TQ offers a naturally occurring, non-toxic, potential tool for use as an anti-inflammatory agent in immune-mediated disease conditions.

RESULTS

TQ treatment decreases the production of inflammatory cytokines in mouse colon in experimental colitis models

In our previous studies, we have observed that TQ enhances the intestinal epithelial tight junction barrier in an AhR-dependent mechanism and ameliorates disease activity and inflammation in various models of experimental colitis[25]. The role of TQ in the regulation of intestinal immune responses is not known. Thus, we investigated the effect of TQ on the production of inflammatory cytokines in experimental colitis. TQ administration significantly reduced the DSS-induced increase in the levels of IL-1β, IL-6, IL-17A, IL-23, and tumor necrosis factor (TNF)-α transcripts during the acute and chronic DSS colitis (Figs. 1A and 1B) and T cell-mediated colitis (Fig. 1C). Having observed this anti-inflammatory effect of TQ in the different experimental colitis models, we next directly assessed the abundance of pro-inflammatory inflammatory macrophages, Th17 cells, and ILC3s[26], cells that have been implicated in the development and pathology of IBD, in the mouse colons. We observed that TQ administration in both the acute DSS (Figs. 2A–C) and chronic DSS colitis models (Figs. 2D–F) significantly reduced the abundance of all three cell types within the colons of WT mice. Taken together, our data show that administration of TQ dampens excessive colonic immune response in experimental colitis.

We next sought to directly investigate the anti-inflammatory role of TQ by using murine splenocytes. We observed that activation of the splenocytes by lipopolysaccharide (LPS) or a cocktail of PMA and ionomycin significantly increased the mRNA levels of the inflammatory cytokines IL-1β, IL-6, IL-17A, and IL-23, which was prevented upon TQ treatment (Fig. 3A). We next assessed if this reduction in transcript levels was a consequence of increased cell death induced by TQ administration. For this, the activated splenocytes with and without TQ treatment were treated with a cell viability stain, and the proportion of live cells was analyzed by flow cytometry. We observed no changes in the proportion of live cells between any treatments (Fig. 3B); however, the proportion of inflammatory macrophages and Th17 cells within these live cell populations was reduced by TQ treatment (Figs. 3C and 3D). Thereby, our data show that the reduction of inflammatory cytokine production observed upon TQ treatment is not due to alteration in cell viability or the overall number of macrophages or CD4^{+} T cells but due to the relative reduction in the abundance of intracellular inflammatory cytokines within these cells.

TQ reduces the production of inflammatory cytokines by human macrophage U937 cells in an AhR-dependent mechanism

Having observed the anti-inflammatory role of TQ in the murine models of colitis and splenocyte activation, we next investigated the effect of TQ on the human U937 cell line to assess the underlying mechanism. Activation of the human macrophage U937 cells by LPS treatment increased the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α; however, treating cells with LPS in the presence of 25 μM TQ decreased the stimulatory effects of LPS. (Fig. 4A). TQ treatment also reduced the protein levels of IL-6 and IL-1β in cell supernatants as assessed by ELISA (Supplementary Fig. 1). Since TQ was identified as an activator of the AhR by a chemical screen, we examined if TQ activates AhR in immune cells and if its immune-modulatory effect is AhR dependent. To this end, we assessed the nuclear localization of AhR by confocal-immunofluorescence microscopy in the vehicle and TQ-treated U937 cells and observed increased localization of AhR in the nucleus upon TQ treatment (Figs. 4B and 4C). Also, the mRNA level of *CYP1A1*, the prototypic target of AhR, was elevated in a dose-dependent manner upon TQ treatment, with the maximum effect observed at the 25 μM concentration of TQ (Fig. 4D). Furthermore, in an XRE-luciferase reporter assay, we observed increased luciferase activity in U937 cells treated with TQ (Supplementary Fig. 2), confirming that TQ activates AhR.

To test if AhR was involved in the TQ-induced regulation of immune responses in the macrophages, we treated U937 cells with LPS and assessed the levels of IL-6 transcripts in the presence or absence of TQ along with the known AhR inhibitors GNF-531(20 μM), SGA-360 (20 μM), or CH-223191(10 μM)[27–29]. Of the three AhR inhibitors, only GNF-531 treatment successfully inhibited the anti-inflammatory effect of TQ on the production of IL-6 (Fig. 5A). These observations allude to a mechanism where TQ-mediated suppression of inflammation is AhR dependent; however, given that GNF-531 is a cytoplasmic competitive inhibitor of AhR, the anti-inflammatory effect of TQ may be independent of the nuclear translocation of AhR.

To account for the non-specific effects of the pharmacological inhibitors and directly assess the anti-inflammatory role of AhR, we employed CRISPR/Cas9 to generate $AHR^{-/-}$ U937 cells. Targeting the *AHR* locus by sgRNA produced a near-complete deletion of AhR in U937 cells (Fig. 5B). The $AHR^{-/-}$ cells produced high baseline levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α compared to the non-target guide RNA-transfected U937 cells (Scr), which were amplified upon LPS treatment. Additionally, unlike the Scr cells, where TQ treatment successfully quelled the effect of LPS, TQ treatment produced an exacerbated inflammatory effect in the $AHR^{-/-}$ cells, increasing the transcript levels of the cytokines (Figs. 5–E), potentially due to the combination of LPS-mediated inflammatory response and loss of anti-oxidative response to electrophilic stress caused by TQ, in the absence of AhR[30]. Furthermore, like the $AHR^{-/-}$ U937 cells, TQ did not affect the LPS-induced elevation of IL-1β, IL-6, IL-23, and TNF-α mRNA levels in the splenocytes isolated from $Ahr^{-/-}$ mice (Fig. 5F). Additionally, in acute DSS colitis, unlike the WT mice, TQ did not reduce the abundance of the IL-1β[hi], TNF-α[hi], and IL-6^{hi} pro-inflammatory macrophages and Th17 cells in $Ahr^{-/-}$ mice colons (data not shown). Taken together,

our data show that TQ-mediated dampening of inflammatory cytokine production is AhR-dependent.

TQ uniquely dampens LPS-mediated cytokine production by inhibiting the activation of the NFκB and Stat-3 pathways

Having established the role of AhR in regulating the TQ-mediated modulation of the immune responses, we further examined the mechanism underlying the anti-inflammatory role of TQ. LPS is a bacterial antigen that causes inflammatory cytokine production through the activation of the NFκB pathway[31]. To this end, we assessed the localization of p65, one of the subunits of the NFκB complex, upon LPS treatment of U937 cells in the presence and absence of TQ. Confocal-immunofluorescence imaging showed that while LPS treatment significantly increased the nuclear localization of p65, TQ significantly reduced the LPS-induced p65 nuclear localization (Figs. 6A and 6B). In a complementary approach, we also assessed the protein levels of p65 and IkBα in the U937 cells and observed increased levels of p65 and a corresponding decrease in IkBα upon LPS treatment, indicating successful activation of the NFκB pathway. The LPS-activated NFκB pathway, however, was inhibited by TQ (Fig. 6C). We next examined the effect of *AHR* knockdown on the NFκB pathway. Assessing for the NFκB promoter activity by luciferase reporter showed that TQ treatment significantly reduced the LPS-induced increase in NFκB promoter activity in Scr cells but not *AHR*$^{-/-}$ cells (Fig. 6D), confirming the AhR dependence of the ability of TQ to inhibit NFκB signaling.

As functional interactions between NFκB and Stat-3 in immune cells control the production of pro-inflammatory cytokines[32], and our previous studies also showed inhibition of Stat-3 by TQ in the intestinal epithelium[25], we also assessed the activation of the Stat-3 in U937 cells in the presence and absence of TQ. TQ significantly inhibited the LPS-induced phosphorylation and hence activation of Stat-3 (Figs. 7A and 7B) and Stat-3-dependent promoter activity in luciferase assay (Fig. 7E). Furthermore, upon testing the AhR dependence of these anti-inflammatory roles of TQ, we observed that while TQ successfully inhibited the phosphorylation of Stat-3 and the increase in Stat-3 promoter activity in the Scr cells, this effect was nullified in the *AHR*$^{-/-}$ U937 cells (Figs. 7C–E). Taken together, our data show that TQ mediates its anti-inflammatory action by inhibiting the NFκB and Stat-3 pathways in a process dependent on AhR.

TQ inhibits the IL-6-IL-6R signaling axis in T cells

Considering the specific effect of TQ in the suppression of IL-6 production, an essential cytokine necessary for Th17 cell differ entiation[33], and the reduction of IL-17A/F transcript levels in the different models of colitis and activated murine splenocytes, we assessed the effect of TQ on different components of the IL-6 signaling in the human T cell line Jurkat cells. IL-6 signaling is initiated by the binding of IL-6 to the receptor IL-6R[34]. Treatment of Jurkat cells with TQ significantly reduced the levels of IL-6R (Figs. 8A and 8B). Also, TQ significantly reduced the levels of IL-6R in primary WT mouse-derived CD4$^+$ T cells (Supplementary Figs. 3A and 3B), indicating that this is not a cell-line-specific effect. Furthermore, this effect was unique to TQ as treatment with known AhR agonists 6-Formylindolo-(3,2-b) carbazole (FICZ) (100 nM) or β-naphthoflavone (β-NF) (50 μM)

did not affect the levels of IL-6R (Figs. 8C and 8D). We next investigated the effect of TQ on the downstream signaling cascade of the IL-6R. IL-6-IL-6R-mediated activation of the Jak1-Stat-3 pathway is a characteristic hallmark of Th17 differentiation, which upon activation promotes the transcription of RORγt, the signature transcription factor of Th17 cell lineage, and locks in the fate of the naive CD4^{+} T cell[35]. Having observed the reduction of IL-6R levels by TQ, and our data showing inhibition of Stat-3 activation by TQ in the macrophages, we also investigated its effect on Stat-3 activation in Jurkat cells. IL-6 treatment of Stat-3-luciferase reporter-transfected Jurkat cells significantly increased the luciferase activity, which was inhibited when combined with TQ. Additionally, only TQ but not FICZ or β-NF inhibited the IL-6-induced increase in Stat-3-luciferase activity (Fig. 8E), further demonstrating the uniqueness of TQ in regulating Th17 development. Taken together, our data highlight the distinctive effect of TQ-mediated AhR activation in blocking the IL-6-IL-6R signaling axis in T cells.

TQ uniquely activates AhR and reduces Th17 differentiation *in vitro*

In the intestinal mucosa, CD4^{+} T lymphocytes are key regulators of immune homeostasis through the production of pro and anti-inflammatory cytokines. De-regulation of this homeostatic state plays a central role both in the induction and in the persistence of chronic inflammation in IBD. Among the different CD4^{+} T cell differentiated subsets, the Th17 cells have been of particular interest in IBD[36]. While multiple studies have pointed to the protective role of the IL-17A/F cytokines by the Th17 and γδ T cells in infection and tissue healing, uncontrolled and persistent Th17 responses are implicated in intestinal inflammation and IBD development[37,38]. Based on the uniqueness of TQ in regulating the IL-6-IL-6R signaling axis, we next investigated whether TQ can regulate the differentiation of naive CD4^{+} T cells to the Th17 lineage. For this, lymphocytes from the thymus and spleen of WT mice were isolated, and naive CD4^{+} T cells were sorted as CD4^{+}, CD25^{−}, CD44^{lo/−}, and CD62L[hi/+] cells (Supplementary Fig. 4). The isolated cells were then allowed to differentiate in Th17 differentiation media in the presence of α-CD3 and α-CD28 antibodies in the presence or absence of TQ. We observed successful differentiation of naive T cells into the effector Th17 cell type in the presence of the α-CD3 and α-CD28 antibodies, as evidenced by their expression of CD44, CD28, and increased production of IL-17A/F. However, in combination with TQ, the naive T cells did differentiate to effector CD4^{+} T cells, as evidenced by high CD44 and CD28 expression, but they produced a significantly lower amount of IL-17A. Furthermore, in the absence of α-CD3 and α-CD28 antibodies, no differentiation was observed, indicating the specificity of the differentiation. To ascertain the involvement of AhR in the process, we next assessed the role of TQ in the differentiation of naive CD4^{+} isolated from *Ahr*^{−/−} mice to Th17 cells. Incubation of naive CD4^{+} T cells in the Th17 differentiation media promoted Th17 differentiation; however, TQ treatment had no effect in inhibiting the production of IL-17A in these *Ahr*^{−/−} mouse-derived cells (Figs. 9A and 9B and Supplementary Figs. 5 and 6).

Previous studies have highlighted the role of AhR activation by known agonists like FICZ and β-NF in promoting the differentiation of naive CD4^{+} T cells into the Th17 lineage[39,40]. Since we observed an opposite phenomenon with TQ and established the requirement of AhR for the activity of TQ, we investigated the specificity of TQ in regulating the

differentiation of naive T cells to Th17 cells. For this, naive CD4^{+} T cells from WT mice were allowed to differentiate in the presence or absence of FICZ, β-NF, or TQ. We observed successful differentiation of naive T cells into the effector Th17 cell type in the presence of the α-CD3 and α-CD28 antibodies, as evidenced by their expression of CD44 and CD28 and increased production of IL-17A. As expected, IL-17A responses were heightened by FICZ and β-NF in the presence of CD3 and CD28 antibodies. In the case of TQ, however, while it did not impede the differentiation of the naive T cell to the effector cell type, it specifically impeded the production of IL-17A by these effector CD4^{+} T cells (Fig. 9C). Taken together, our data show that TQ uniquely inhibits the differentiation of Th17 cells in an AhR-dependent process.

TQ reduces inflammation in human tissues

To demonstrate the clinical relevance of our findings in the *in vitro* and mouse studies, we next assessed the effect of TQ on human peripheral blood mononuclear cells (PBMCs). For this, we initially incubated PBMCs obtained from healthy human subjects with LPS or PMA + ionomycin in the presence of vehicle or TQ. We observed that activation by both LPS and PMA+ ionomycin significantly increased the mRNA abundance of IL-6, IL-23, and TNF-α, which was prevented by TQ (Fig. 10A). Also, we observed no cytotoxic effect on human PBMCs treated with TQ (Fig. 10B). Next, we examined the effect of TQ on inflammatory macrophages and Th17 responses, specifically in the human colon. For this, surgically resected paired diseased and adjoining normal human colonic tissues (0.5 cm^2) from CD patients were incubated with vehicle or TQ for 18 hours. TQ treatment significantly reduced the abundance of inflammatory macrophages and Th17 cells compared to vehicle treatment in both normal tissue (NT) and CD tissues without reducing the overall abundance of macrophages or CD4^{+} T cells (Figs. 10C and 10D). Taken together, these data indicate the ability of TQ to dampen the inflammatory response in the immune cells in the human gut.

DISCUSSION

It is well established that exaggerated and dysregulated immune response is a key pathogenic factor in IBD, including CD and UC. Dysregulation of both innate and adaptive immune responses is known to contribute to the aberrant intestinal inflammatory response in IBD patients. The cytokines and cytokine-producing immune cells play a crucial role in IBD pathogenesis, as evidenced by the identification of several cytokine and chemokine receptor signaling genes as IBD susceptibility or risk loci[41]. In this study, we show that TQ acts as a non-cytotoxic immunomodulatory agent that ameliorates colonic inflammation in a mechanism dependent on the AhR. Several studies have established the critical role of AhR in the regulation of the intestinal immune system and immune cell functioning[42,43]. We show that TQ ameliorated the inflammation-promoting immune responses and reduced the mRNA levels of pro-inflammatory cytokines in the experimental colitis models of acute and chronic DSS administration and T cell transfer-mediated colitis. Furthermore, we also show that TQ reduced the population of inflammatory macrophages, CD4^{+} T cells, and ILC3 cells enriched in IL-17A/F in the mouse colon in the colitis models. The upregulation of the inflammatory cytokines as a key contributing factor for intestinal inflammation has

been established in the different models of colitis[44,45]. In addition, we also demonstrated the anti-inflammatory role of TQ in murine splenocytes without eliciting cytotoxicity. Therefore, our data suggest that TQ-mediated immune modulation is an important factor in the amelioration of experimental colitis.

As a mechanism for TQ-mediated immunomodulation, we demonstrated that TQ activates AhR signaling based on the nuclear translocation of AhR, XRE reporter assay, and *CYP1A1* gene transcription. Beyond the xenobiotic response, AhR has been shown to play several homeostatic roles, including intestinal differentiation, stem cell homeostasis, and maintenance of the gut microbiome[46,47]. Moreover, AhR activation by various endogenous or exogenous agents has been shown to be protective against experimental colitis via various mechanisms, including inhibition of inflammatory cytokines, induction of regulatory immune responses, microbiome homeostasis, and maintenance of gut barrier integrity[9–20]. Our data showed that while TQ activated AhR and promoted its nuclear localization, unlike the conventional AhR agonists like FICZ and β-NF, its function was independent of the nuclear translocation of AhR. We showed that TQ prevents the nuclear localization and transcriptional activity of NFκB and the phosphorylation and activation of Stat-3, two key transcription factors involved in the transcription of proinflammatory genes. We also show that the effect of TQ on these two disparately activated pro-inflammatory pathways was dependent on the AhR protein. Pharmacological or genetic inhibition of the AhR completely blocked the effectiveness of TQ in preventing the activation of the NFκB and Stat-3 proteins. This further establishes the AhR dependence of the activity of TQ, pointing to a unified model of the regulation of the NFκB and Stat-3 pathways via the cytoplasmic activity of the activated AhR protein. However, whether this activity is directly mediated by the AhR protein or through intermediaries needs further investigation.

Another key finding of this study is the effect of TQ on the pleiotropic pro-inflammatory cytokine IL-6 and the CD4$^+$ Th17 cells. The inflammatory cytokine IL-6 is an essential component in the immune arsenal to fend off foreign antigens; however, hyperactivation of the IL-6 signaling axis has been implicated in numerous chronic inflammatory diseases, including psoriasis and IBD[48]. IL-6 signaling in intestinal epithelium also contributes to increased transcription of the pore-forming protein claudin-2, which diminishes the epithelial barrier and is often associated with the leaky gut phenotype associated with IBD[49]. Furthermore, IL-6 signaling has also been implicated in prolonged T-cell responses by inhibiting T-cell apoptosis, which further contributes to intestinal inflammation in IBD[50]. In this study, we successfully show that TQ inhibits IL-6 production in mouse intestinal tissue and splenocytes and also by the human macrophage cell line U937 cells. We highlight the role of AhR in regulating this inhibitory effect of TQ and also show the inhibition of the downstream signaling of IL-6 that occurs by Stat-3 activation. We also show the effect of TQ on another key downstream event of IL-6 signaling, that is the development of Th17 cells from naive T cells. The hyperactivation of Th17 cells has been implicated in numerous immune disorders, including IBD[51,52]. Targeting the Th17 cell response has also been an active area of research for the treatment of IBD; however, the efficacy of said treatment in patient cohorts has not been efficient[53,54]. Our data show that TQ reduced the mRNA levels of IL-17A, the key cytokine produced by Th17 cells, and also Th17 abundance in the different models of colitis. TQ impeded the differentiation of Th17 cells from naive murine

CD4^{+} T cells in an AhR-dependent manner, as TQ was only able to elicit this response in naive CD4^{+} T cells isolated from WT but not $Ahr^{-/-}$ mice, which have been shown to have increased susceptibility to experimental colitis[13]. Moreover, consistent with the ligand and tissue-specific effects of AhR signaling[55], we observed that the Th17 suppressive role is unique to TQ-mediated AhR activation. Activation of AhR by other known agonists like FICZ and β-NF was unable to inhibit Th17 development. As an underlying mechanism, we show that TQ uniquely reduced the levels of the IL-6 receptor CD126/IL-6Rα on the human T cell line Jurkat cells and primary murine CD4^{+} T cells, and as in the case of U937 cells, it also reduced Stat-3 activation and transcriptional activity, two essential factors that contribute to the development of Th17 cells. In this regard, both elevated IL-6R, as well as noncanonical Stat-3 functions, have been implicated in IL-17 production[33,56]. Besides the above mechanisms, the contradicting regulatory effect of TQ, compared to known AhR agonists such as FICZ and β-NF, on IL-6R and IL-6 expression and the abundance of Th17 cells can be attributed to cell-specific AhR metabolism, coactivators, and corepressors, affinity for AhR binding, and activation of canonical *vs.* noncanonical AhR pathways. Whereas FICZ and β-NF-dependent AhR activity relies primarily on the nuclear translocation of AhR, TQ-mediated anti-inflammatory effects were dependent on AhR activation and a resultant inhibition of NFκB and Stat-3 signaling, which was accomplished by neither FICZ nor β-NF. The overall reduction of IL-17A production in the presence of TQ may occur due to the reduction of Stat-3 signaling. Stat-3 activation by the IL-6-IL-6R axis is known to promote the transcription of *RORγt*, the hallmark transcription factor for Th17 cells. The RORγt protein together with active Stat-3 associates with the transcription machinery and binds to conserved non-coding sequence 2 (CNS2) cis-regulatory elements upstream of the *IL-17A* gene locus to promote the transcription of the cytokine[57]. We recognize that further investigations are needed into these factors to clarify the differential effect of TQ compared to known AhR ligands. Overall, our data show that TQ employs a unified mode of action, operating via AhR and regulating the pro-inflammatory IL-6, NFκB, and Stat-3 pathways in different cell types, thereby exerting anti-inflammatory responses and protecting against intestinal inflammation.

In addition to the murine and cell line studies, we also showcase the efficacy of TQ on human blood-derived and human colonic tissue-derived immune cells. We show that TQ successfully reduced the mRNA abundance of IL-6, IL-23, and TNF-α by human PBMCs without any cytotoxicity. Finally, we demonstrated the efficacy of TQ in reducing the abundance of pro-inflammatory TNF-α, IL-1β, and IL-6 -enriched inflammatory macrophages and Th17 cells in colonic tissues derived from CD patients, indicating the potential to use TQ in the treatment of IBD.

In summary, we demonstrated the unique immunomodulatory ability of TQ in experimental colitis as well as in healthy and diseased human colonic mucosa. We provided pharmacological and genetic evidence for AhR signaling as an underlying mechanism through which TQ exerts its immune-modulatory effect. Very complex ligand and tissue-specific effects of AhR activation, toxicity considerations, and inadequate potency have precluded the application of AhR modulators against IBD[58]. Nevertheless, due to the ligand-dependent modulation of AhR activity, an effective, non-toxic AhR modulator is of great scientific interest[8]. Although only transient prolongation of clotting time without

any adverse events was noted in a clinical study[59], the anti-coagulative activity of TQ must be considered in its clinical use. Our results indicate that TQ may offer a naturally occurring, non-toxic intervention for the regulation of a broad spectrum of intestinal immune responses and therapeutic for inflammatory conditions.

METHODS

Chemicals and antibodies

D-alpha-tocopheryl quinone (TQ) (T2283) was obtained from TCI (Portland, OR, USA). The primary antibodies used included AhR (MA1–514) and CYP1A1 (PA5–15212) from Life Technologies (Waltham, MA, USA); p65 (AbCam, Boston, MA, USA, AB532536); IkBα (10268-AP), IL-6R (23457–1-AP), Stat-3 (10253–2-AP), and CYP1A1 (13241–1-AP) from ProteinTech (Rosemont, IL, USA); and phospho-Stat-3 (9145) and GAPDH (2118) from Cell Signaling Technologies, Danvers, MA, USA. The anti-rabbit HRP (AB97051) and anti-mouse HRP (AB205719) secondary antibodies were purchased from AbCam (Boston, MA, USA). For confocal-immunofluorescence microscopy, AF488-tagged anti-rabbit antibody (A11008) and Cy3-tagged anti-mouse antibody (APC124C) were purchased from Invitrogen and Sigma-Aldrich, respectively. AhR activators FICZ (SML-1489) and βNF (N3633), Lipopolysaccharide [LPS O127:B8 (L3129)], PMA (79346), and ionomycin (I3909) were purchased from Sigma-Aldrich (St. Louis, MO). Brefeldin A solution 1000X (420601) and all antibodies used for flow cytometry and fluorescence-assisted cell sorting (FACS) sorting were purchased from BioLegend, San Francisco, CA, USA. TaqMan-tagged primers for assaying the expression of all the target genes by qRT-PCR were purchased from Life Technologies, Carlsbad, CA, USA. The AhR inhibitors SGA360 (AOB1971), GNF-531 (182707), and CH223191 (C8124) were obtained from Aobious (Gloucester, MA, USA), EMD Millipore (Burlington, MA, USA), and Sigma-Aldrich, respectively. The untagged cytokines used for T-cell differentiation were purchased from ACROBiosystems (Newark, DE, USA).

Cell culture and *in vitro* differentiation

Human U937 cells (CRL-1593.2, ATCC) and Jurkat cells (TIB-152, ATCC) were maintained in RPMI-medium (Corning, 10–040CV) supplemented with 10% heat-inactivated FBS (R&D Systems, Minneapolis, MN, USA, S11150H) and antibiotics in a 37 °C incubator with 5% CO_2. The U937 cells were activated with 10 ng/ml LPS or a cocktail of 1 μg/ml each of PMA and ionomycin. Mouse T cells were maintained in T cell media (RPMI-1640 + 10% heat-inactivated FBS + antibiotics + 50 μM 2-mercaptoethanol). Jurkat and mouse spleen-derived CD4+ T cells were activated by incubation with 1 μg/ml anti-CD3 antibody (α-CD3) and 1 μg/ml anti-CD28 antibody (α-CD28). Mouse naive CD4+ T cells were isolated and differentiated into Th17 cells by culturing naive cells in T cell media supplemented with 20 ng/ml IL-6, 3 ng/ml tumor growth factor (TGF)-β, 5 ng/ml IL-2, 10 ng/ml IL-23, 5 μg/ml anti-IL-4 antibody (α-IL-4) and 5 μg/ml anti-IFN-γ antibody (α-IFN-γ) in the presence of 2 μg/ml α-CD3 and 1 μg/ml α-CD28.

Isolation of lymphocytes from the murine lymph nodes and spleen

WT and $Ahr^{-/-}$ C57BL.6 mice were anesthetized using 5% isoflurane, and their spleen and mesenteric lymph nodes were harvested in ice-cold PBS supplemented with 2% heat-inactivated FBS. The spleen and lymph nodes were pulverized by gentle rubbing between two frosted slides (Fisher Scientific, 22–034486). Spleen samples were then incubated in $1\times$ RBC lysis buffer (100 mM NH_4Cl + 10 mM $NaHCO_3$ + 1 mM EDTA) for 5 minutes at room temperature, followed by the addition of enriched RPMI-1640 medium to stop lysis and centrifugation to remove lysed RBCs. The lymph and spleen cells were then subjected to 40%–80% gradient percoll centrifugation, and the lymphocyte band was isolated for further experimentation. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics.

Cell treatment

TQ treatment of cells in culture was performed as described previously[60]. Briefly, cells in culture were treated with TQ using bovine serum albumin (BSA) as a carrier for TQ, dissolved first in ethanol, and subsequently added to a 7.5% BSA solution (Sigma-Aldrich, A8412) at 37 °C with mixing, producing a 20-fold concentrated stock. This solution was then added to the standard growth medium at a 5% concentration (i.e. a final con centration of 0.4% BSA) to produce a 25 μM concentration of TQ in the culture medium.

Western blot analysis

Cells were rinsed twice with ice-cold PBS (Corning, Glendale, AZ, 21–040) and lysed using RIPA buffer (Sigma-Aldrich, R0278) containing protease inhibitors (Sigma-Aldrich, 11836170001). The cell lysates were centrifuged at 12,000 rpm for 5 minutes, and the clear supernatant was used for protein quantification. Protein quantification of the extracted aliquots was performed (BCA protein assay kit, Thermo Scientific 23225), and Laemmli gel loading buffer (Invitrogen, Waltham, MA, USA, NP007) was added to the lysate and boiled at 95 °C for 5 minutes. An equal amount of protein was loaded on a 4%–15% SDS-PAGE gel (BioRad, Hercules, CA, USA, 4561086) and transferred to a nitro-cellulose membrane (BioRad, 1620112). The membrane was incubated for 1 hour in the blocking solution (5% non-fat dry milk in TBS-Tween 20 buffer), followed by overnight incubation with the appropriate primary antibody in the blocking solution. After incubation with the primary antibody, the membrane was washed in TBS-0.1% Tween 20 buffer, incubated in the appropriate secondary antibody, and developed using the SuperSignal West Pico PLUS kit (Thermo Scientific, 34580). The densitometry analysis was performed using ImageJ software (NIH, USA).

Quantitative Real-Time PCR

Cells and tissues were suspended in TRIZol (Invitrogen, 15596026), followed by RNA isolation using the Direct-zol RNA Miniprep Plus kit (Zymo Research, Irvine, CA, R2072) by following the manufacturer's instructions. Transcript levels of the different markers were normalized to GAPDH transcript levels.

Flow cytometry and FACS sorting

Flow cytometry was performed by modifying the previously described protocol[44]. Briefly, cells were washed with FACS buffer (RPMI-1640 + 1% FBS + 1X DPBS + 5 mM EDTA), followed by staining with fixable viability Dye-eFluor780 (Thermo Scientific, 65-0865-18) for 10 minutes at 4 °C and F_c blocking (Thermo Scientific, 16-0161-82 for mice and BioLegend 422302 for humans) for 10 minutes at room temperatures. To assess intracellular cytokine marker staining, cells were treated with brefeldin A (5 μg/ml) for the last 4 hours of incubation before prepping cells for flow cytometry. Cells were permeabilized using $1\times$ Permeabilization buffer (Thermo Scientific, 00-8333-56) at 37 °C for 30 minutes, followed by staining with cognate fluorescently tagged antibodies (BioLegend). Cells were then either sorted using a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ) or were analyzed using an LSR Fortessa flow cytometer (BD Biosciences) using appropriate compensations, isotype, and FMO controls. Flow data were analyzed using Flow Jo V10 software (BD Biosciences). For the subtyping of the murine intestine and spleen-derived immune cells, inflammatory macrophages were identified as the live $CD45^+$, $MHC\text{-}II^+$, $F4/80^+$, $CD64^+$, and $CXC3CR1^+$ with $TNF\text{-}\alpha^{hi}$, and IL-6 ^{hi} intracellular staining; Th17 cells were identified as the live $CD45^+$, $CD4^+$, $CD28^+$, $ROR\gamma t^+$, $CCR6^+$, and $CD25^-$ cells with IL-17A and IL-17F^{hi} intracellular staining; and ILC3 cells were identified as the live $CD45^+$, $CD4^-$, $ROR\gamma t^+$, and $CCR6^+$ cells with IL-17A and IL-17F^{hi} intracellular staining. The gating strategy for each is shown in Supplementary Figs. 7A (macrophages), 7B (Th17 cells), and 7C (ILC3).

Lentiviral-mediated CRISPR/Cas9 knockout of AhR

The plasmid Cas9 nuclease CP-LVC9NU (Genecopoeia, Rockville MD, USA) was used individually with a single guide RNA (sgRNA) targeting the region 5'-CAAGTCGGTCTCTATGCCGCT-3' of the *AHR* gene, or scrambled sgRNA for control in pCRISPR- LVSG03 (Genecopoeia), to generate respective lentiviral particles packaged in Lenti-X 293T cells using PMD2.G (12259) and pPAX2 (12260) (Addgene, Watertown MA). The lentiviral particles obtained were used to transduce U937 cells in the presence of polybrene (Sigma-Aldrich, TR-1003) and were selected in their respective antibiotic selection media to generate stable knockout U937 cells. *AHR* knockout was confirmed using Western blot analysis.

Transient transfection and reporter gene assay

U937 and Jurkat cells were cultured in 12-well tissue culture plates for 24 hours before transfection at 60%–80% confluency. For the measurement of either NFκB or Stat-3 activity in the presence and absence of TQ, the cells were transiently co-transfected with 1 mg/well pLminP_Luc2P_RE1 (NFκB reporter, Addgene 90335) or pLminP_Luc2P_RE5 plasmid (Stat-3 reporter, Addgene 90339) expressing plasmid and 0.1 mg/well Renilla luciferase (hRluc) reporting plasmid (pGL4.74) using Lipofectamine 2000 (Invitrogen, 11668019). After 24 hours of transfection, cells were treated with LPS (for U937 cells) or IL-6 (for Jurkat cells) in the presence or absence of TQ for 48 h, after which the cells were lysed and assayed for luciferase activity using Dual-Luciferase Reporter Assay system (Promega, E1960) on the GloMax 20/20 Luminometer (Promega, Madison, WI, USA). The activity

of firefly luciferase was normalized against Renilla reporter activity (control) in the same samples.

Confocal-Immunofluorescence imaging

Confocal Immunofluorescence for AhR and NFκB was performed by standard methods. Cells were washed twice with cold PBS and fixed with 2% paraformaldehyde for 20 minutes. The cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 minutes. The cells were blocked in normal serum and labeled with primary antibodies in a blocking solution overnight at 4 °C. After PBS washes, the cells were incubated in Alexa Fluor-488 or Cy-3 conjugated secondary antibodies (Invitrogen). ProLong Gold antifade reagent (Invitrogen, P36931) containing DAPI as a nuclear stain was used to mount the sections on glass slides. The slides were examined using a confocal fluorescence microscope, Leica SP8. Images were processed with LAS X software (Leica Microsystems).

Experimental animals

Experimental methodologies used in the study were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine (approval number 201800202). The mice were housed under standard housing conditions with a 12-h light-dark cycle and *ad lib* standard chow diet and water supply. The WT (Jackson Laboratories, Bar Harbor, ME, Strain #000664) and $Ahr^{-/-}$ mice with C57BL/6J background of both sexes were randomly allotted to the experimental groups. The experimental murine DSS colitis (acute and chronic DSS) models were established using standard protocols and as described previously[44,61,62]. In the acute colitis model, 8–10-week-old C57BL/6J mice received 2.5% DSS dissolved in auto-claved water for 7 days. TQ treatment (50 mg/kg/day) was initiated two days prior and continued throughout DSS treatment. In the chronic DSS colitis model, 8–10-week-old C57BL/6J mice received three cycles of 5 days of 1.5% DSS in drinking water followed by 5 days of plain water. Mice were treated with TQ in the last cycle of water and DSS. For T cell transfer colitis, FACS was performed to isolate donor T cells from WT mice. Briefly, the mice's spleen was harvested and ground in between the rough edges of two frosted slides. The resultant cell slurry was incubated for 5 minutes at room temperature in RBC lysis solution, followed by a wash with RPMI-1640 complete media (RPMI-1640 + 10% FBS). Cells obtained from the lysis step were subjected to an 80%–40% Percoll (Sigma-Aldrich) gradient centrifugation to enrich the leukocyte population and staining for flow cytometry. Effector and memory T cells were sorted as the CD45RB-FITC$^+$ and CD4-APC$^+$ cells. Cells were sorted using a BD-Aria Cell sorter (BD Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using FlowJo version 10 software (BD Biosciences). Approximately 0.5×10^6 cells were then intraperitoneally injected into recipient $Rag^{-/-}$ mice (Jackson Laboratory, Strain #002216). Isolated T cells from male donor mice were injected into male recipient mice, and cells from female donor mice were administered into female recipient mice. Mice were weighed twice per week and monitored for disease onset for 5 weeks post-injection. Upon the appearance of diarrhea-like symptoms at week 5 postinjection, mice were separated into two groups, with one being gavaged vehicle and the other being gavaged TQ for 7 days. The groups' allocation, treatments, and outcomes were measured in a blinded fashion. The

experimental colitis data represent a minimum of three independent experiments with n 3 mice per group.

Human samples and treatment

The surgically resected human colon samples were obtained freshly from the Department of Surgery, Division of Colon and Rectal Surgery, as per the protocols approved by the Institutional Review Board (STUDY00010256). Samples were deemed normal or diseased by Anatomic Pathologists. The colonic tissues were washed and incubated in supplemented RPMI-1640 for 18 hours[63] in the presence of vehicle or TQ. Tissues were then processed for flow cytometry. For human blood samples, healthy volunteer blood PBMCs were isolated by incubating the samples in 1X RBC lysis solution for 5 minutes at room temperature, followed by percoll gradient centrifugation to obtain PBMCs. Cells were then incubated overnight in supplemented RPMI-1640 culture medium with 10 ng/ml LPS or a cocktail of 1 μg/ml each PMA + ionomycin with or without 25 μM TQ. Cells were then used for RNA isolation and quantitative RT-PCR. Similar to the murine cells, for the human colonic biopsy-derived immune cells, the inflammatory macrophages were identified as the live CD45$^+$, HLA-DR$^+$, CD14$^+$, CD80 $^+$, CD 86$^+$, and CD16$^+$ cells with TNF-α ^{hi}, IL-1β^{hi}, and IL-6 ^{hi} intracellular staining, and Th17 cells were identified as the live CD45$^+$, CD4$^+$, CD28$^+$, RORγt$^+$, and CD25$^−$ cells with IL-17A^{hi} intracellular staining.

Statistical analysis

Data are reported as means ± SEM. Whenever needed, data were analyzed by using the Student's t-test or analysis of variance for repeated measures (Sigma Stat, Systat Software, San Jose, CA, USA). Tukey's test was used for post hoc analysis between treatments following analysis of variance ($p < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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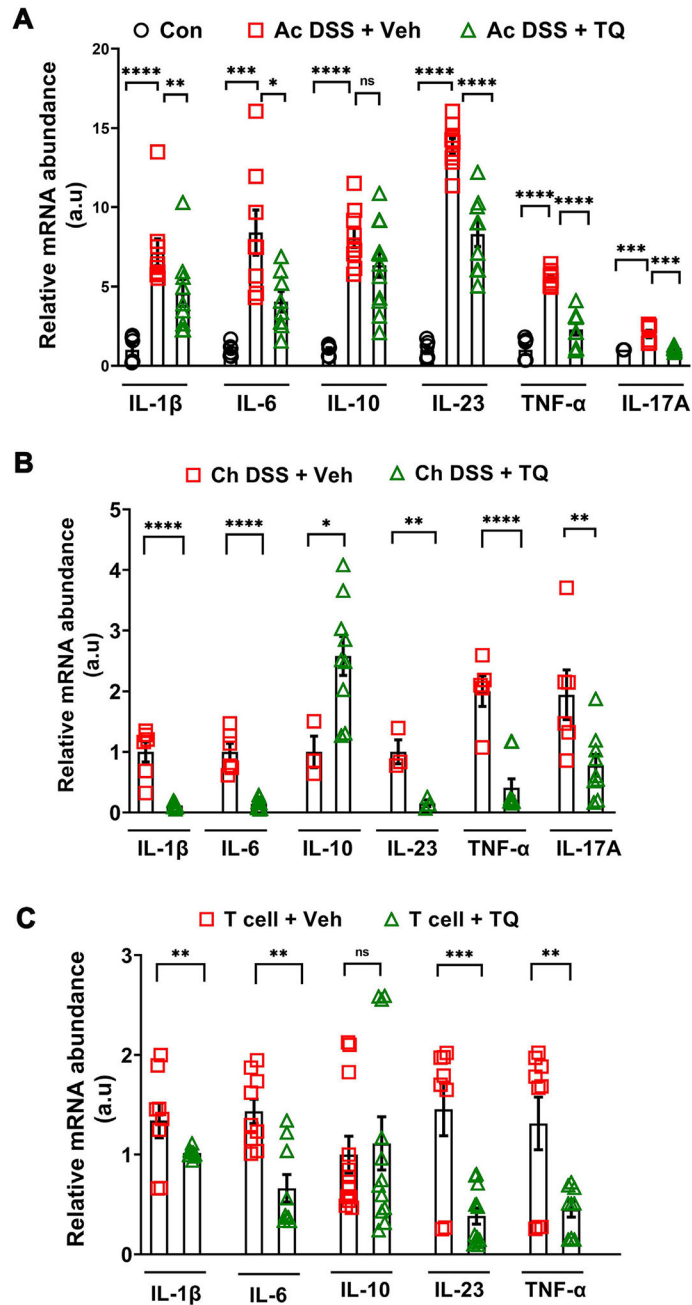
Fig 1.

TQ ameliorates colonic inflammation in WT mice. (A) In the acute DSS (Ac DSS) model of colitis, TQ reduced the mRNA levels of key inflammatory cytokines IL-1β, IL-6, IL-17A, IL-23, and TNF-α in the colons of WT B6 mice. TQ, however, had no effect on IL-10 mRNA levels in this acute DSS colitis model. (B) In the chronic DSS (Ch DSS) model of colitis, TQ reduced the mRNA levels of key inflammatory cytokines IL-1β, IL-6, IL-23, TNF-α, and IL-17A in the colons of WT B6 mice compared to vehicle (Veh) administered mice. In this model of colitis, TQ also increased the levels of IL-10 mRNA. (C) In the T cell colitis model, TQ gavaging reduced the mRNA levels of the inflammatory cytokines IL-1β,

IL-6, IL-23, and TNF-α in the colons from $Rag^{-/-}$ B6 mice. Like the Ac DSS model, TQ had no significant effect on IL-10 mRNA levels in this model of colitis. Data represented as mean ± SEM, n 3/group from two independent experiments. Student's t-test or one-way analysis of variance with Tukey's post test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns = not significant; a.u = arbitrary units.
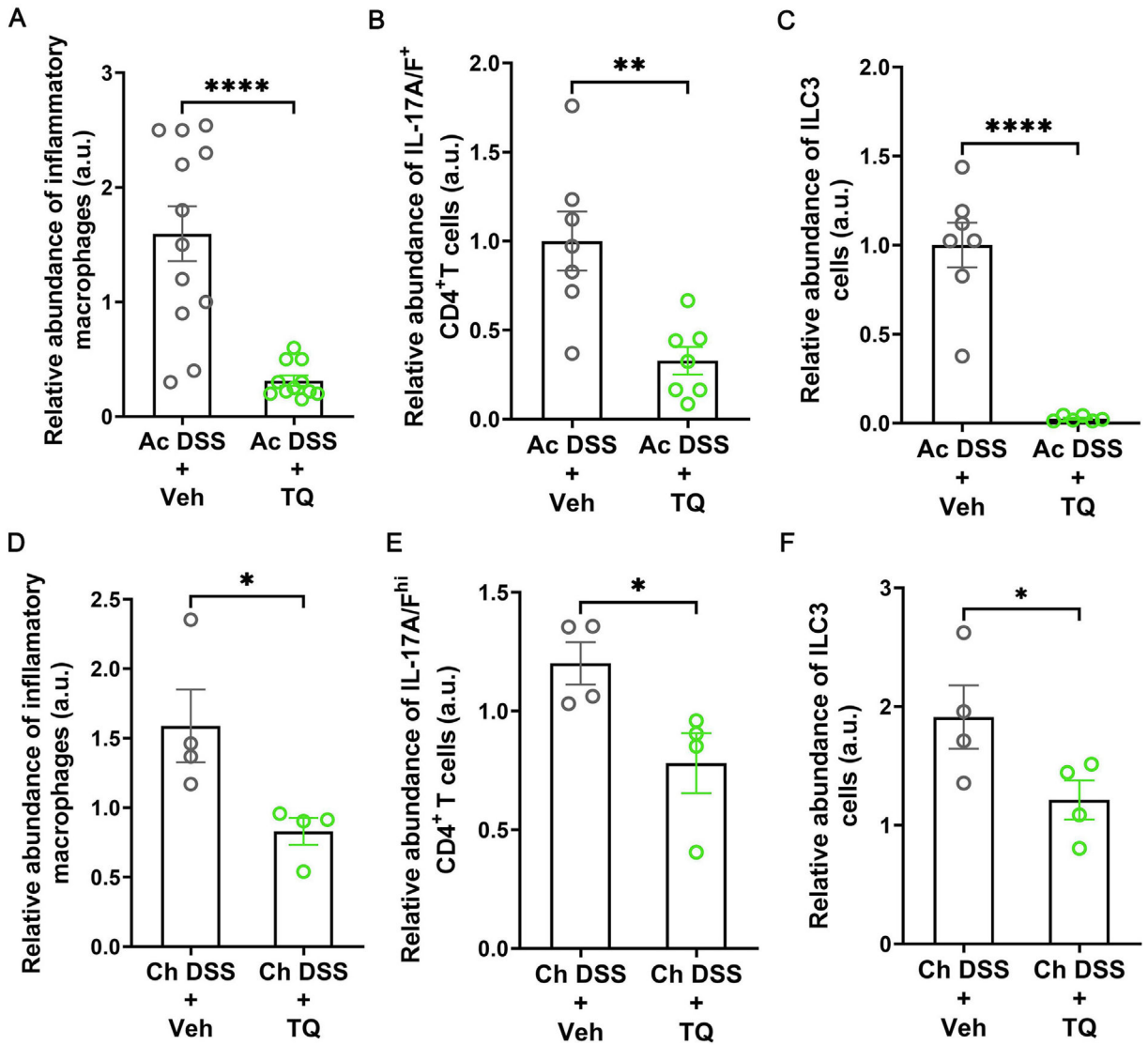
Fig. 2.
TQ ameliorates intestinal inflammation by reducing the abundance of inflammatory cell types. In acute DSS colitis (Ac DSS), administration of TQ along with DSS reduced the abundance of IL-6, TNF-α, and IL-1β -enriched inflammatory macrophages (A), IL-17A/F enriched RORγt$^+$ CD4$^+$ T cells (B), and IL-17A/F$^+$ IL-22$^+$ CD4$^-$ innate lymphoid cells type 3 (ILC3) (C) in the colons of WT mice. In chronic DSS colitis (Ch DSS), administration of TQ along with DSS reduced the abundance of IL-6, TNF-α, and IL-1β -enriched inflammatory macrophages (D), IL-17A/F enriched RORγt$^+$ CD4$^+$ T cells (E), and IL-17A/F$^+$ IL-22$^+$ CD4$^-$ innate lymphoid cells type 3 (ILC3) (F) in the colons of WT mice. The flow cytometry data are represented as the ratio of the absolute number of IL-6, and TNF-α - enriched inflammatory macrophages to the total number of macrophages (A and C) or the ratio of the absolute number of IL-17A-enriched CD4$^+$ T cells to the total number of CD4$^+$ T cells (B and D) or IL-17A and IL-17F-enriched cells to the total RORγt$^+$ CCR6$^+$ cells (C and F), normalized to the average values of similar ratio of the

vehicle-treated control (Veh) mice. Data represented as mean ± SEM. $n \geq 3$/group from two independent experiments for Ac DSS and $n = 4$/group for Ch DSS. Student's t-test. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.
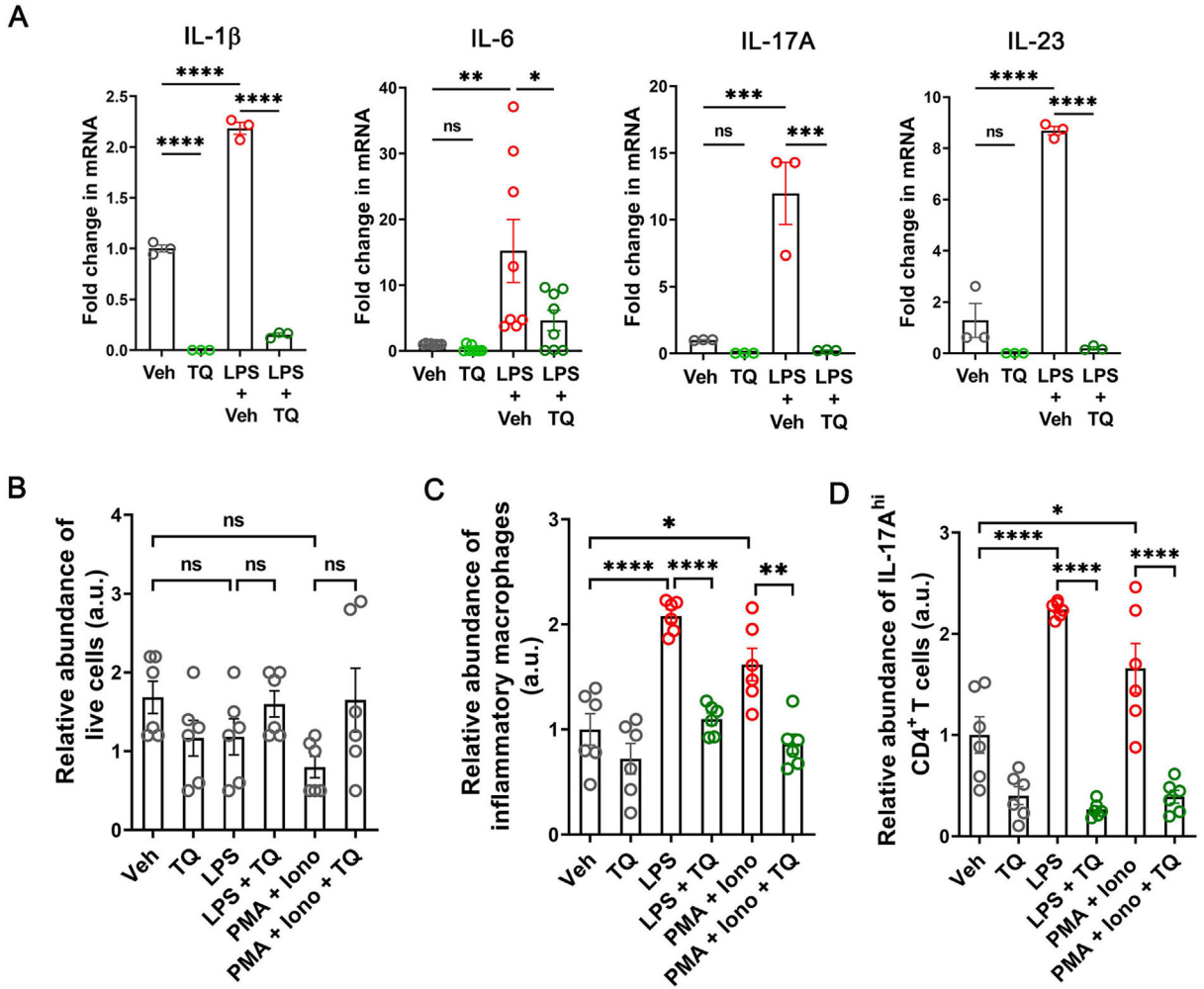
Fig. 3.

TQ efficiently reduces the effect of inflammatory stimulus in murine splenocytes. (A)
TQ (25 μM) reduced the LPS-induced upregulation in the mRNA levels of IL-1β, IL-6,
IL-17A, and IL-23 by splenocytes isolated from WT mice. (B) TQ alone or in combination
with any of the activators did not affect the survival of the splenocytes. (C) TQ reduced
the abundance of IL-6, TNF-α, and IL-1β-enriched inflammatory macrophages within the
splenocyte population when they were activated with LPS (10 ng/ml) or a cocktail of PMA
and ionomycin (1 μg/ml each). (D) TQ reduced the abundance of IL-17A-enriched RORγt+
CD4+ T cells within the splenocytes when they were activated with LPS or a cocktail of
PMA and ionomycin. The flow cytometry data are represented as the ratio of the absolute
number of IL-6, and TNF-α -enriched inflammatory macrophages to the total number of
macrophages (C) or the ratio of the absolute number of IL-17A-enriched CD4+ T cells to the
total number of CD4+ T cells (D), normalized to the average values of the similar ratio of the
vehicle-treated control (Veh) cells. Data represented as mean ± SEM, n 3/group. One-way
analysis of variance with Tukey's post test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <
0.0001$, ns = not significant.
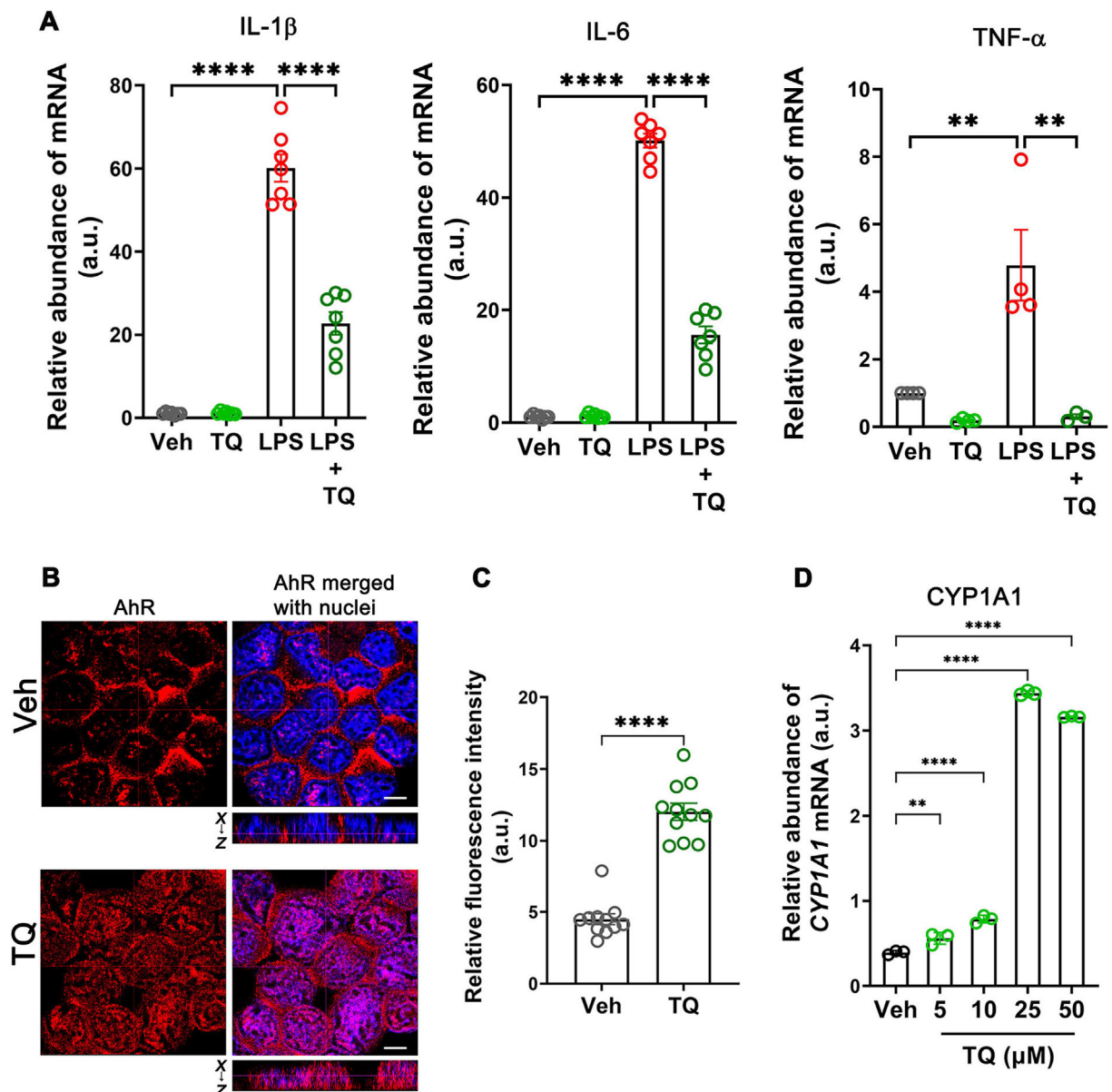
Fig. 4.

TQ exerts its anti-inflammatory effect by activating the AhR in U937 cells. (A) TQ prevented the LPS-induced (10 ng/ml) increase in the mRNA levels of IL-1β, IL-6, and TNF-α in the human U937 cell line. (B) Confocal images of AhR (red) and nuclei (blue) in U937 cells. The images showed that TQ treatment (4 h) increased AhR levels as well as its nuclear translocation, indicating that TQ activates AhR. The dotted lines represent the optical level for the x-y and x-z planes. White bar: 5 μm. These data are representative of multiple areas from independent samples, $n = 5$. (C) Quantification of AhR fluorescence in the nucleus from (B). (D) The mRNA levels of *CYP1A1*, the prototypic transcriptional target of AhR, showed a dose-dependent increase in abundance with increasing concentrations of TQ with the maximum effect observed with 25 μM concentration of TQ. Data represented as mean ± SEM, $n > 3$ from 3 independent

experiments. Student's t-test (C) and one-way analysis of variance with Tukey's post test (A and D). **$p < 0.01$, ****$p < 0.0001$.
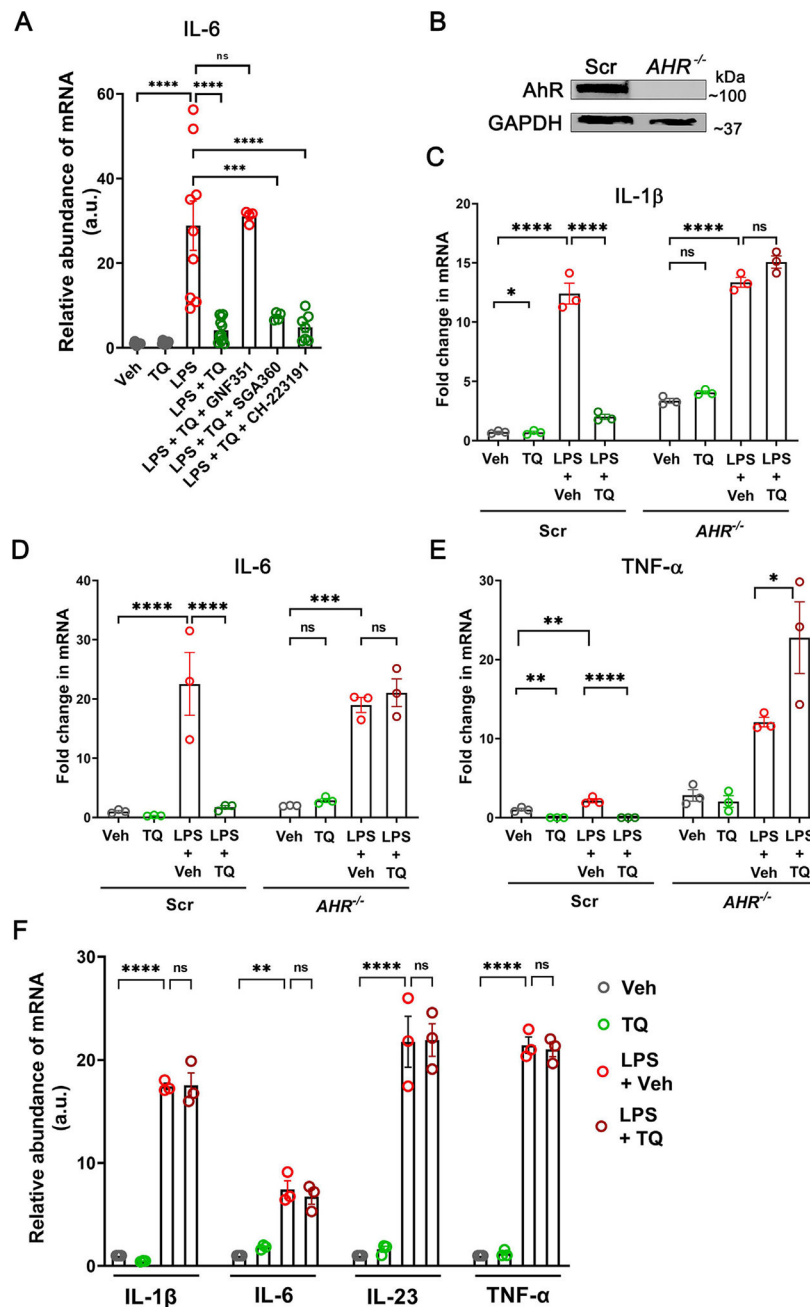
Fig. 5.
The anti-inflammatory effect of TQ is dependent on AhR. (A) The anti-inflammatory effect of TQ, as measured by the mRNA levels of IL-6 in U937 cells upon activation with LPS, was prevented by the competitive inhibitor of AhR, GNF-531 (20 μM). Other AhR inhibitors SGA-360 (20 μM) and CH-223191 (10 μM) failed to inhibit the anti-inflammatory effect of TQ. (B) CRISPR/Cas9-mediated targeting of the *AHR* gene produced a complete knockout of AhR in U937 cells. The GAPDH levels are shown as the loading control. (C–E) In the absence of AhR, TQ failed to prevent the LPS-induced upregulation in the mRNA levels of IL-1β (C), IL-6 (D), and TNF-α (E) by U937 cells. Scr- non-target scrambled control. (F)

TQ did not prevent the LPS-induced upregulation of IL-1β, IL-6, IL-23, and TNF-α mRNA levels in the *Ahr*$^{-/-}$ mouse splenocytes ($n = 3$/group). Data represented as mean ± SEM, n 3 from 3 independent experiments. One-way analysis of variance with Tukey's post test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ns = not significant.
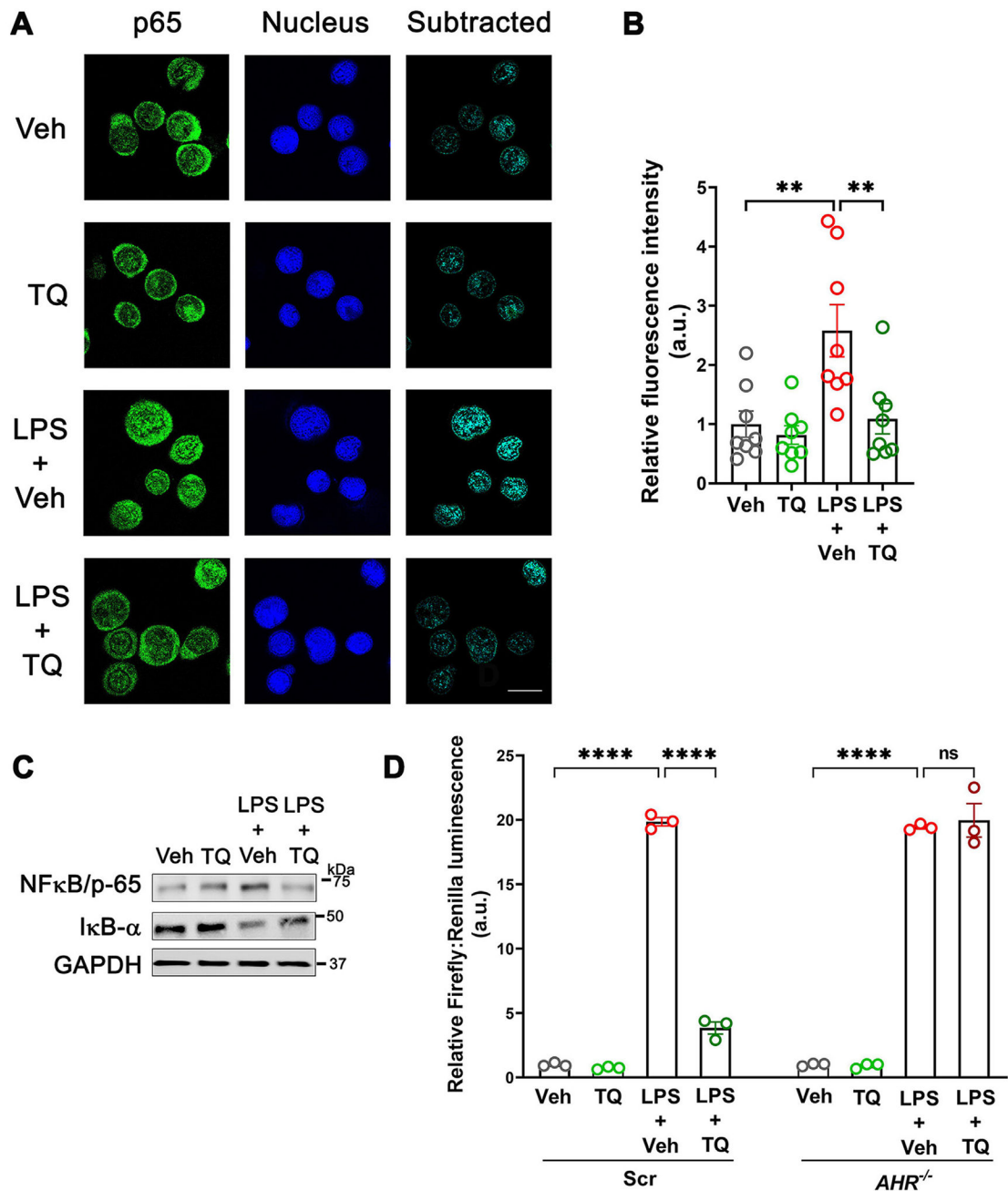
Fig. 6.
TQ reduces signaling along the NFκB axis. (A) Confocal immunofluorescence imaging showed that TQ reduced the LPS-induced nuclear localization of the NFκB protein p65 (green) in U937 cells. White bar = 10 μM. Image representative 3 independent experiments with > 30 individual fields captured per experimental round. (B) Quantification of the p65 fraction localized to the nucleus from (A). (C) TQ prevented the LPS-induced increase in p65 protein levels and the associated decrease in the levels of IκBα in U937 cells. The GAPDH levels are shown as the loading control. Representation of three independent experiments. (D) In $AHR^{-/-}$ U937 cells, TQ did not prevent the LPS-induced upregulation

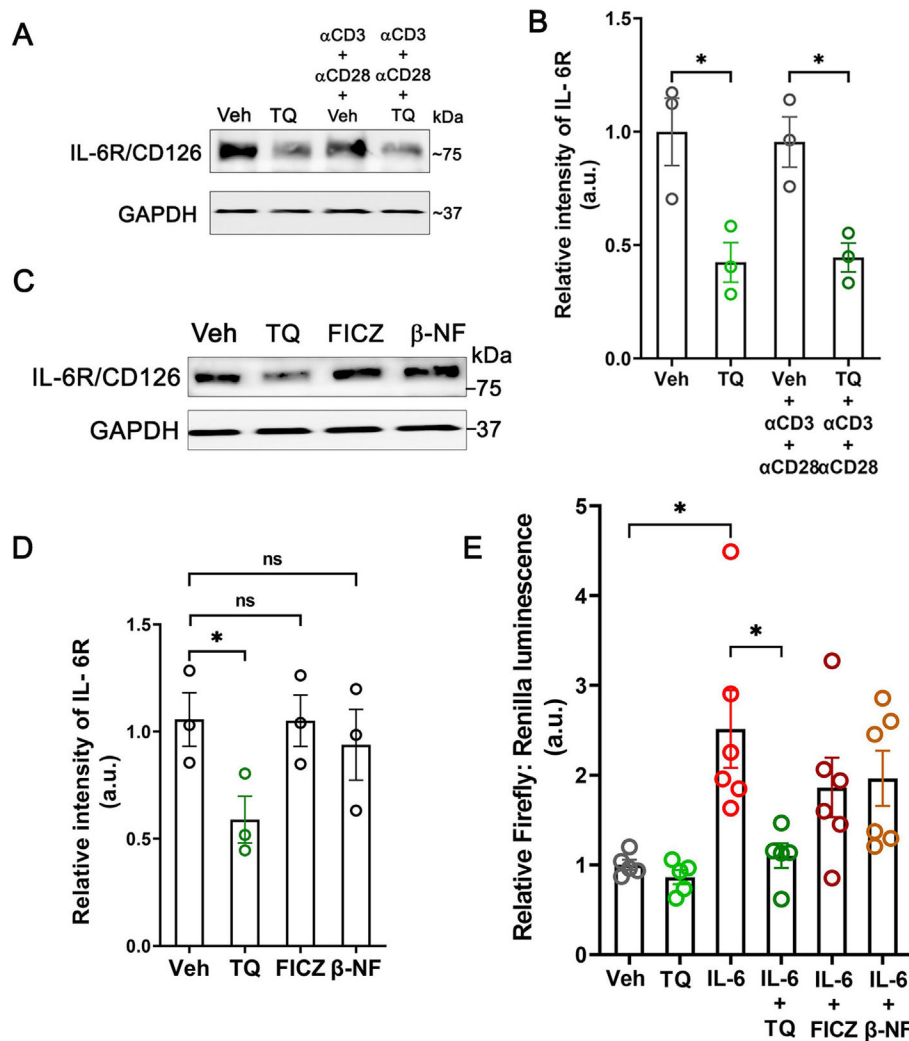of luciferase activity where the luciferase reporter was placed downstream of the NFκB binding site. Representation of three independent experiments. Data represented as mean ± SEM. One-way analysis of variance with Tukey's post test. **$p < 0.01$, ****$p < 0.0001$. ns = not significant.
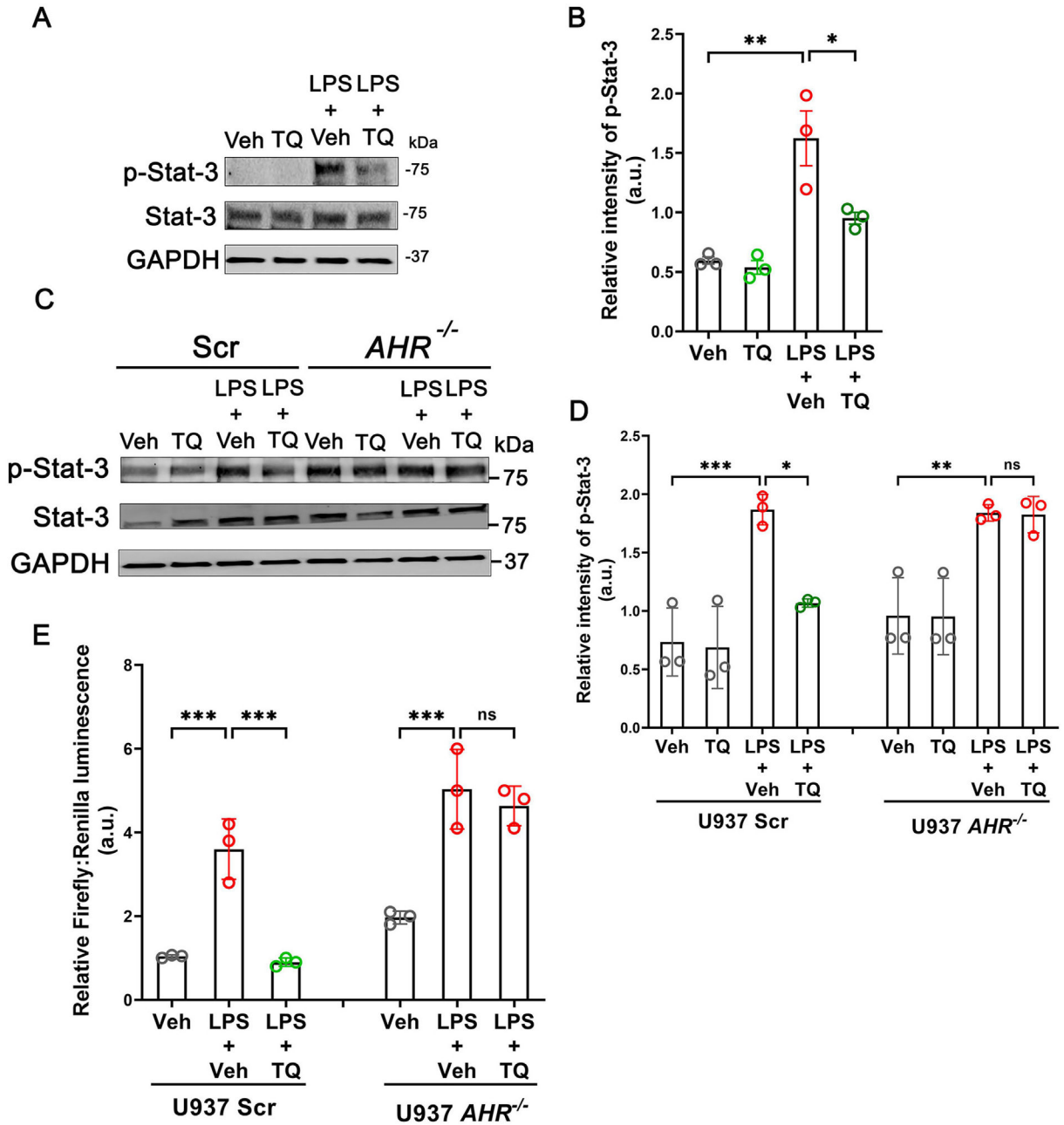
Fig. 7.
TQ reduces Stat-3 signaling. (A) TQ reduces the LPS-induced phosphorylation and
activation of Stat-3 in U937 cells. The GAPDH levels are shown as the loading control.
Representation of three independent experiments. (B) Quantification of p-Stat-3 band
intensity normalized to total Stat-3 from (A). (C) The inhibitory effect of TQ on Stat-3
activation was dependent on AhR as $AHR^{-/-}$ U937 cells failed to reduce the LPS-induced
phosphorylation of Stat-3 upon incubation with TQ. The GAPDH levels are shown as the
loading control. Representation of three blots. (D) Densitometric quantification of phospho-
Stat-3 bands normalized to total Stat-3 from (C). (E) TQ failed to prevent the LPS-induced
upregulation of luciferase activity in the $AHR^{-/-}$ U937 cells where the luciferase reporter

was placed downstream of the Stat-3 dimer binding site. Representation of 3 independent experiments. Data represented as mean ± SEM. One-way analysis of variance with Tukey's post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns = not significant.
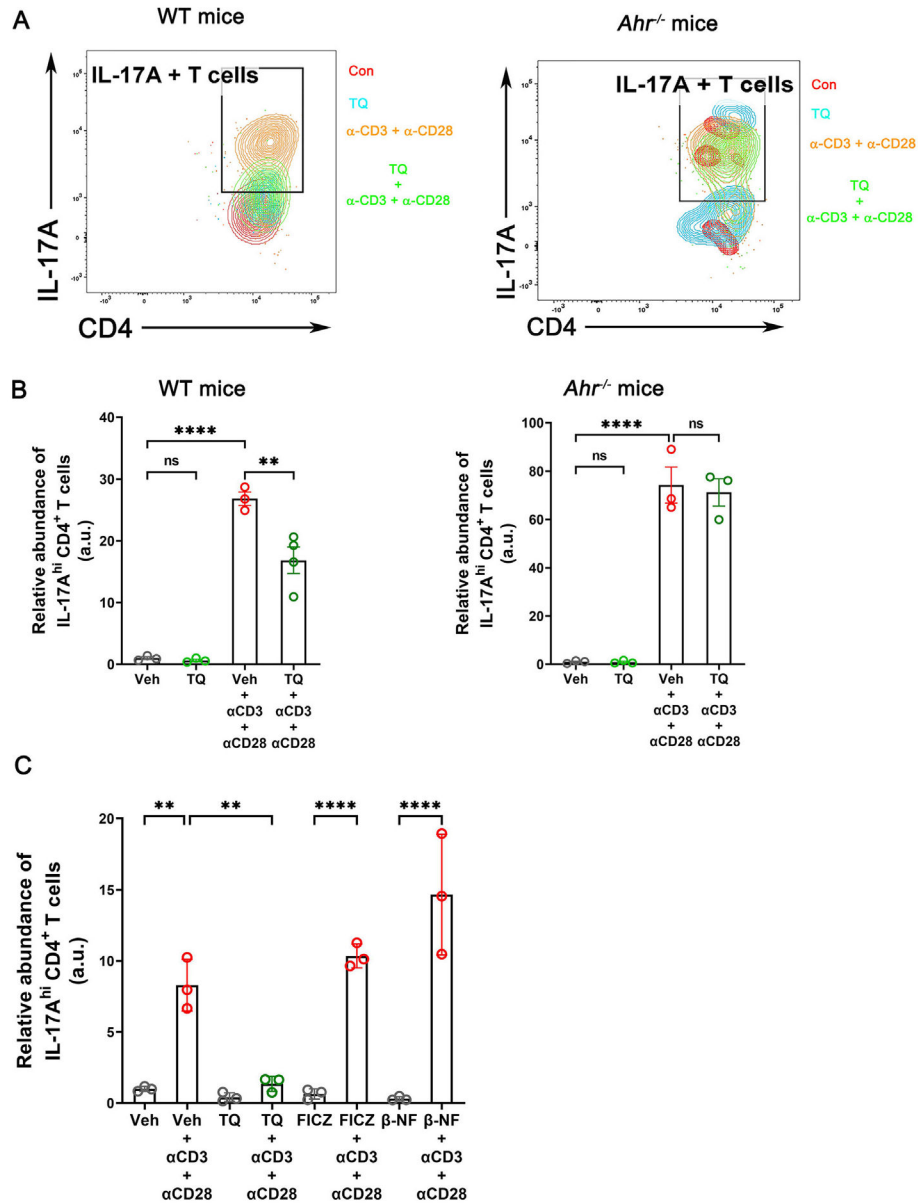
Fig. 9.
TQ occludes the differentiation of Th17 cells. (A) Flow panel showing the distribution of IL-17^{hi} CD4$^+$ Th17 populations differentiated from naive CD4$^+$ T cells isolated from WT and $Ahr^{-/-}$ mice in Th17 cell differentiating conditions *in vitro*. (B) Quantification of WT and $Ahr^{-/-}$ naive CD4$^+$ T cells differentiated Th17 cells under each test condition. (C) TQ but not the other AhR agonists FICZ and β-NF reduced the *in vitro* differentiation of WT mouse-derived naive CD4$^+$ T cells to Th17 cells. Flow cytometry quantification data are represented as the ratio of the absolute number of IL-17A-enriched CD4$^+$ T cells to the total number of CD4$^+$ T cells, normalized to the average values of vehicle-treated control (Veh) without α-CD3 and α-CD28. Data represented as mean ± SEM, $n = 3$/test group. One-way analysis of variance with Tukey's post test. **$p < 0.01$, ****$p < 0.0001$, ns = not significant.
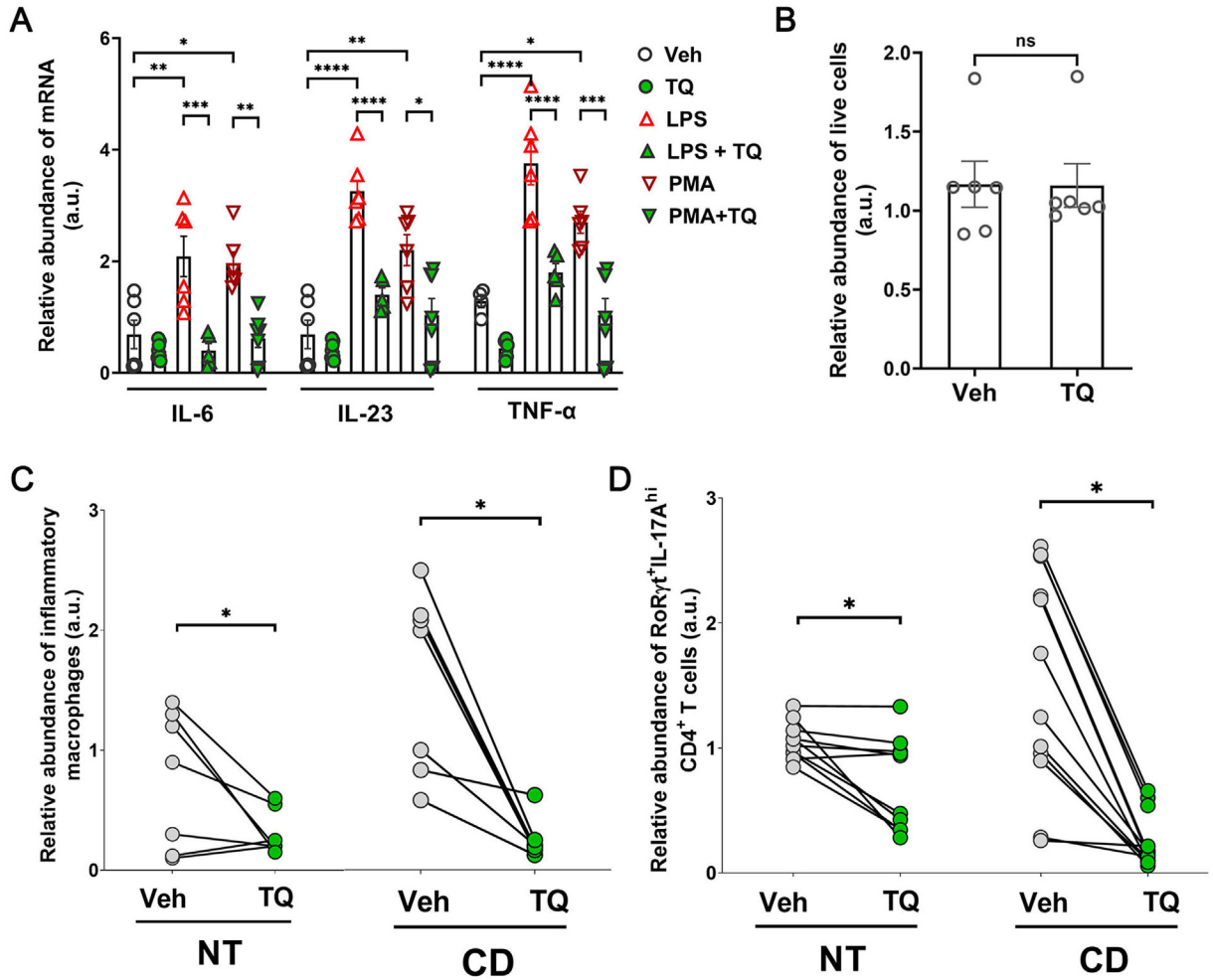
Fig. 10.

TQ effectively reduces inflammation in human tissues. (A) TQ effectively reduced the upregulation of IL-1β, IL-6, and TNF-α mRNA in human PBMCs when activated with LPS or a cocktail of PMA + ionomycin (PMA). (B) TQ did not affect the cell viability of human PBMCs. (C) In human Crohn's disease (CD) patient-derived inflamed colonic tissues, TQ treatment reduced the abundance of IL-6, TNF-α, and IL-1β -enriched inflammatory macrophages. Flow cytometry quantification data are represented as the ratio of the absolute number of IL-6, TNF-α, and IL-1β -enriched inflammatory macrophages to the total number of macrophages, pairwise normalized to the average values of three technical replicates for each patient vehicle-treated NT. (D) In human Crohn's disease (CD) patient-derived inflamed and adjoining normal colonic tissues (NT), TQ treatment reduced the abundance of IL-17A-enriched RORγt+ CD4+ T cells. The flow cytometry quantification data are represented as the ratio of the absolute number of IL-17A-enriched CD4+ T cells to the total number of CD4+ T cells, pairwise normalized to the average values of three technical replicates for each patient vehicle-treated NT. Data represented as mean ± SEM, n 5/ treatment group. Paired Student's t-test or one-way analysis of variance with Tukey's post test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. ns = not significant.