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Activation of the aryl hydrocarbon receptor by 10-Cl-BBQ prevents insulitis and effector T cell development independently of Foxp3$^+$ regulatory T cells in NOD mice

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Abstract

AhR activation by high affinity ligands mediates immunosuppression in association with increased regulatory T cells, making this transcription factor an attractive therapeutic target for autoimmune diseases. We recently discovered 10-chloro-7H-benzimidazo[2,1-a]benzo[de]iso-quinolin-7-one (10-Cl-BBQ), a nanomolar affinity AhR ligand with immunosuppressive activity and favorable pharmacologic properties. Here, we tested the consequences of AhR activation in the NOD model. Oral 10-Cl-BBQ treatment prevented islet infiltration without clinical toxicity, while AhR-deficient NOD mice were not protected. Suppression of insulitis was associated with an increased frequency, but not total number, of Foxp3$^+$ Tregs in the pancreas and pancreatic lymph nodes. The requirement for Foxp3$^+$ cells in AhR-induced suppression of insulitis was tested using NOD.Foxp3^{DTR} mice which show extensive islet infiltration upon treatment with diphtheria toxin. AhR activation prevented the development of insulitis caused by the depletion of Foxp3$^+$ cells, demonstrating that Foxp3$^+$ cells are not required for AhR-mediated suppression and furthermore the AhR pathway is able to compensate for the absence of Foxp3$^+$ Tregs, countering current dogma. Concurrently, the development of disease-associated CD4$^+$Nrp1$^+$Foxp3$^-$RORγt$^+$ cells was inhibited by AhR activation. Taken together, 10-Cl-BBQ is an effective, non-toxic AhR ligand for the intervention of immune-mediated diseases that functions independently of Foxp3$^+$ Tregs to suppress pathogenic T cell development.

Introduction

Targeting T cells is a promising therapeutic strategy for the prevention or treatment of autoimmune diseases as a way to improve efficacy and minimize the toxicity that is associated with most currently used therapeutics. In this regard, the aryl hydrocarbon

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[3]Abbreviations used in this article: 10-Cl-BBQ, 10-chloro-7H-benzimidazo[2,1-a]benzo[de]Iso-quinolin-7-one; AhR, aryl hydrocarbon receptor; DC, dendritic cell; DT, diphtheria toxin; NOD, non-obese diabetic; Nrp1, neuropilin 1; PLN, pancreatic lymph node; T1D, type 1 diabetes; TCDD, 2,3,7,8-tetrachlordibenzo-*p*-dioxin; Teff, effector T cell; Treg, regulatory T cell

receptor (AhR) represents a potential new drug target as a ligand-activated transcription factor that directly alters T cell differentiation without cytotoxicity (1–3). Studies with 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), the most potent AhR ligand, have shown that AhR signaling during the early stages of $CD4^+$ T cell activation results in premature cessation of effector T cell (Teff) differentiation in association with the induction of Tr1-like regulatory T cells (Tregs) (3–5). More recently, both direct and indirect (via tolerogenic dendritic cells (DCs)) induction of $Foxp3^+$ Tregs has been associated with AhR-mediated immune suppression (2, 6, 7). Treatment of mice with TCDD has been shown to prevent or ameliorate several different types of autoimmune and allergic diseases supporting the AhR as a therapeutic target (6, 8–11).

While studies with TCDD highlight the therapeutic potential of the AhR pathway, TCDD itself has unfavorable pharmacological properties specifically related to its high lipid solubility and resistance to metabolism which leads to a half -life of approximately seven years in humans (12). Additionally, the notoriety of TCDD as an environmental toxicant would likely limit its acceptance for pharmacologic uses. To identify other high-affinity AhR ligands that exhibit more favorable pharmacokinetic properties than TCDD, we used small-molecule screening to discover a novel immunosuppressive AhR ligand, 10-Cl-BBQ (13). This nontoxic compound was shown to have an in vivo half-life of approximately two hours but could be administered to mice in a repeated dosing scheme to maintain AhR activation over time, similar to the prolonged AhR activation induced by TCDD. Using an acute-graft-vs-host model, we found that 10-Cl-BBQ, like TCDD, directly targeted the AhR in $CD4^+$ T cells to induce an AhR-dependent Treg phenotype, suppress the allogeneic CTL response and ameliorate the symptoms of GVHD (13).

In the present studies we investigated the potential for 10-Cl-BBQ to suppress the development of type 1 diabetes (T1D), a T cell-mediated autoimmune disease in which islet-specific T cells destroy the insulin-producing β-cells in the pancreas resulting in hyperglycemia (14). Multiple mechanisms have been ascribed to influencing the development of T1D, including defective control of aberrant immune responses by Tregs (15, 16). In the non-obese diabetic (NOD) mouse model for T1D, the spontaneous and progressive destruction of β-cells is suppressed by treatment with TCDD, and hyperglycemia is completely prevented (9). Suppression of T1D by TCDD was also associated with an increase in frequency of $Foxp3^+$ Tregs, in concordance with numerous other studies that have shown a correlation between autoimmune disease suppression by AhR ligands and increased frequency of $Foxp3^+$ Tregs (2, 7, 8, 10, 17–20). However, a direct role for $Foxp3^+$ Tregs as a primary mechanism of AhR-mediated immune suppression has not been clearly established.

Here we demonstrate the therapeutic efficacy of 10-Cl-BBQ in T1D as evidenced by the almost complete prevention of the development of insulitis in NOD mice with chronic treatment. Furthermore, using NOD.$Foxp3^{DTR}$ mice, we show that 10-Cl-BBQ suppresses insulitis in the absence of $Foxp3^+$ Tregs and that inhibition of Teff development does not rely on $Foxp3^+$ Tregs. These results directly challenge the widely supported proposition that induction of $Foxp3^+$ Tregs is the primary mechanism by which AhR ligands mediate suppression of immune-mediated diseases.

Materials and Methods

Animals

NOD/LtJ mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). NOD.AhR$^{-/-}$ mice were generated by backcrossing B6.129-AHR^{tm1Bra}/J onto the NOD/LtJ background for 13 generations. The Ahr locus is present on chromosome 12 (15–16.5 cM) and the nearby genes have not been linked to the diabetic process based on annotated protein homology, proposed functions, or analyses of previously published mutations. NOD.Foxp3^{DTR} mice (21) were obtained from the Juvenile Diabetes Research Foundation Center for Immune Tolerance in Diabetes at Harvard. Mice were bred and maintained under specific pathogen-free conditions at Oregon State University facilities. All experimental procedures using animals were approved by the Institutional Animal Care and Use Committee at Oregon State University.

AhR ligand treatment

10-Cl-BBQ was dissolved in DMSO-Cremaphor-Peceol (30%:20%:50%) and delivered by oral gavage. For the time-course experiments mice were treated with 10-Cl-BBQ starting at 7 weeks of age and insulitis was evaluated at 12, 15 and 20 weeks of age. The dosing strategy was continually optimized from the first 12-week experiment (alternating 60 mg/kg and 30 mg/kg, daily), to the 15-week experiment (60 mg/kg every other day), to the 20-week experiment (60 mg/kg, 3-times/week) to minimize the number of doses of 10-Cl-BBQ that needed to be administered yet would maintain activation of AhR as evidenced by *Cyp1a1* expression. We established 60mg/kg treatment with 10-Cl-BBQ by oral gavage 3x/week as the optimal dosing schedule. 12- and 15-week experiments were repeated with this optimized dosing schedule for subsequent studies. As a positive control, mice were treated with TCDD (50 μg/kg loading dose, followed by 15 μg/kg every other week) by oral gavage.

Cyp1a1 measurement

RNA was isolated from liver using RNeasy columns (Qiagen) and cDNA was synthesized using SuperArray Reaction Ready First Strand cDNA Synthesis Kit. PCR reactions were performed on an ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) using SYBR Green/ROX Master Mix (SA Biosciences). *Cyp1a1* levels were normalized to *Actb* using primers from SA Biosciences.

Assessment of disease progression

Blood glucose was measured weekly using a Glucocard Vital glucose meter (Arkray USA, Minneapolis, MN). A blood glucose level greater than 200 mg/dL for two consecutive days was considered diabetic. Insulitis was scored on sequential hematoxylin and eosin (H&E) stained pancreas sections separated by 200μm. At least 50 islets per pancreas were scored as no infiltration, peri-infiltration, less than 50% infiltration or greater than 50% infiltration.

Flow Cytometry and ELISA

At the indicated time points, mice were sacrificed, PLN and pancreata were excised, and single cell suspensions were prepared. Fc receptors were blocked with rat IgG (Jackson

ImmunoResearch, West Grove, PA) and the cells were stained with the following antibodies from eBioscience (San Diego, CA): CD4 (RM4), CD8 (53–6.7), CD19 (1D3e), Foxp3 (FJK-16s), Nrp1 (3DS304M), CD25 (PC61.5), IL-17 (eBio17B7), Tbet (eBio4B10) and RORγt (AFKJS-9). CD45 (30-F11) and IFNγ (XMG1.2) were from BD Biosciences. For intracellular staining, cells were fixed and permeabilized using the Foxp3 Fixation/ Permeabilization buffer (eBioscience). For cytokine staining, cells were stimulated with PMA, ionomycin, brefeldin A and monensin (eBioscience) for 4 hours in culture. Data were acquired using an FC-500 flow cytometer (Beckman Coulter, Brea, CA) and compensated and analyzed using FlowJo software (FlowJo, Ashland, OR). Fluorescence minus one (FMO) controls were used for setting gates for analysis.

For ELISAs, splenocytes were stimulated with PMA/ionomycin (eBioscience) for 4 hours. IFNγ and IL-17 were measured in culture supernatants according to the manufacturer's protocol eBioscience).

In vivo depletion of Tregs

Foxp3^{+} cells were depleted from NOD.Foxp3^{DTR} mice as previously described (21) with some modifications. Briefly, four to six week old NOD.Foxp3^{DTR} mice were injected i.p. with 500 ng DT (Sigma, St. Louis, MO) on days 0, 1, 3, 5 and 7 and sacrificed on day 9.

Statistical analyses

Data are presented as mean of biological replicates. For statistical comparisons of degree of islet infiltration between groups, chi-squared analyses were performed. Student's *t*-test was used for analysis of all other data. P values ≤ 0.05 were considered significant.

Results

10-Cl-BBQ treatment prevented insulitis in NOD mice

Chronic treatment of NOD mice with the prototypic AhR ligand, TCDD, was previously shown to prevent insulitis and resulting hyperglycemia as long as activation of the AhR was maintained (9). We hypothesized that the efficacy of TCDD could be recapitulated with the novel, rapidly metabolized compound, 10-Cl-BBQ (chemical structure, Figure 1A) if the dosing schedule ensured continued activation of AhR. The dose of 10-Cl-BBQ was calculated empirically by determining a concentration that would induce equivalent *Cyp1a1* expression as a body burden of 25 μg/kg TCDD, the therapeutic dose in NOD mice. Because NOD mice express the low responder AhR^{d} allele, they require a higher dose of 10-Cl-BBQ to achieve comparable activation of AhR when compared to AhR^{b} expressing C57BL/6 mice (13). Oral treatment with 60 mg/kg 10-Cl-BBQ induced commensurate expression of *Cyp1a1* as the therapeutic dose of TCDD in NOD mice (Figure 1B).

A time course was conducted to evaluate the capacity of 10-Cl-BBQ to prevent islet infiltration at both early and later stages of disease progression. Treatment was initiated at 7 weeks of age with 60mg/kg 10-Cl-BBQ administered by oral gavage on Monday, Wednesday, and Friday established as an optimal dosing schedule (Figure 1C). This regimen allowed for the maintenance of AhR activation while minimizing complications from

frequent oral administration of the vehicle carrier, and resulted in significant suppression of diabetes incidence through 20 weeks of age (Figure 1D). At 12 weeks of age, 65 ± 3.4% of islets in the pancreas of vehicle-treated mice were infiltrated with inflammatory cells with 27.9 ± 1.2 and 37.5 ± 3.1 showing less than 50% and greater than 50% of the islets infiltrated, respectively. In contrast, treatment with 10-Cl-BBQ successfully prevented infiltration, with 92 ± 2.0% of islets free from infiltration and the remaining 8% showing only minimal infiltration (Figure 1E and 1F). Similarly, at 20 weeks of age when control mice had more severe infiltration with 28.8 ± 4.5 and 56.8 ± 7.8 less than 50% and greater than 50% of the islet infiltrated, respectively, 10-Cl-BBQ significantly suppressed infiltration in 73.1 ± 6.2% of islets (Figure 1G). To determine the role of AhR in mediating the effects of 10-Cl-BBQ in suppressing type 1 diabetes, we generated AhR-deficient NOD mice. AhR expression was required for 10-Cl-BBQ to induce its therapeutic effects as the same treatment in AhR-deficient NOD mice had no effect on insulitis (Figure 1H). At the doses used, treatment with 10-Cl-BBQ was more effective at preventing islet infiltration than TCDD (Figures 1F and 1G). Since the extent of islet infiltration is directly linked to development of elevated blood glucose and overt diabetes, islet infiltration is useful as a reliable surrogate biomarker for more prolonged prevention studies. The strong suppression of islet infiltration in 10-Cl-BBQ treated mice clearly demonstrates its therapeutic potential. The absence of clinical signs of toxicity associated with prolonged treatment with 10-Cl-BBQ through 20 weeks of age also support the therapeutic potential of this novel AhR ligand (Supplemental Table I).

10-Cl-BBQ increases the proportion of Foxp3$^+$ Tregs in the pancreas and draining lymph node

Tregs are capable of suppressing islet infiltration in NOD mice (21, 22) and suppression of diabetes in TCDD-treated NOD mice was associated with an increase in Foxp3$^+$ Tregs at 30 weeks of age in the pancreatic lymph node (PLN) (9). To determine if suppression of insulitis by 10-Cl-BBQ was associated with increased Foxp3$^+$ Tregs, the leukocyte composition of the pancreas and PLN was assessed at different time points during disease progression. In the pancreas at 12 weeks of age, the total number of CD45$^+$ cells was significantly decreased in 10-Cl-BBQ treated mice (Figure 2A), correlating with the reduction of islet infiltration induced by 10-Cl-BBQ treatment. 10-Cl-BBQ treatment did not alter the proportion of CD19$^+$ B cells nor CD8$^+$ T cells in the pancreas (Figures 2B and C), but significantly reduced the percentage of infiltrating CD4$^+$ T cells (Figure 2D). In addition, the percentage of pancreatic CD4$^+$ T cells that expressed Foxp3 was increased by treatment (Figure 2E). However, as a consequence of the decrease in total CD45$^+$ cells and in the percentage of CD4$^+$ cells, the total number of Foxp3$^+$ cells in the pancreas was unaltered (Figure 2F).

As the percentage of Foxp3$^+$ cells was increased in the pancreas of 10-Cl-BBQ-treated mice at 12 weeks of age, this population was further studied over time in the PLN at 12, 15 and 20 weeks of age. At 15 and 20 weeks of age, there was a small but significant increase in the percentage of CD4$^+$ cells that were Foxp3$^+$ cells (Figure 3A and 3B); this trend was not found for total number of Foxp3$^+$ cells in the PLN (Figure 3C). Instead, at 12 weeks of age, the total number of CD4$^+$Foxp3$^+$ cells was lower in 10-Cl-BBQ mice, owing to the

reduction in the proportion of $CD4^+$ cells at this time point (Figures 3C and 3D). Similar findings were found in the PLN of TCDD treated mice, and in the spleen of 10-Cl-BBQ or TCDD mice, with small increases in the frequency of $CD4^+$ cells that were $Foxp3^+$ (Supplemental Figure 1).

To further characterize the $Foxp3^+$ Tregs that are increased by AhR activation, coexpression with the high affinity IL-2 receptor, CD25, and neuropilin 1 (Nrp1) was assessed in the PLN at 20 weeks of age. The increased percentage of $Foxp3^+$ cells in the PLN were $CD25^+$ (Figure 4A) and $Nrp1^-$ (Figure 4B) in 10-Cl-BBQ-treated mice. These results suggest that AhR activation is not expanding the population of thymically-derived Tregs but instead inducing or predominantly maintaining peripherally-derived Tregs (23).

10-Cl-BBQ suppresses Th17 cells but not Th1 cells in NOD mice

Treatment of NOD mice with 10-Cl-BBQ reduced the number of $CD4^+$ T cells in the pancreas, lymph node and spleen at 20 weeks of age, suggesting a reduction in Th1 and/or Th17 effector cells, which have been implicated in T1D pathogenesis (24). AhR activation by both 10-Cl-BBQ and TCDD suppressed IL-17 production by splenic $CD4^+$ cells as measured by intracellular staining (Figure 5A) and ELISA (Figure 5C). In contrast, IFNγ production was not altered by AhR activation (Figures 5B and 5D). These results suggest that AhR activation preferentially inhibits the development of Th17 cells in T1D.

AhR activation suppresses insulitis independently of $Foxp3^+$ Tregs

10-Cl-BBQ treatment resulted in an increased percentage of $Foxp3^+$ cells in the pancreas and PLN of NOD mice, consistent with the effect of activation of AhR in other autoimmune disease models. However, the functional significance of the AhR-induced $Foxp3^+$ Tregs in suppressing Teff cells and disease has not been clearly demonstrated. To directly determine if $Foxp3^+$ Tregs are functionally required for suppression of insulitis by AhR ligands, NOD.$Foxp3^{DTR}$ mice were employed to specifically ablate $Foxp3^+$ cells upon administration of diphtheria toxin (DT) (21). Starting at four weeks of age NOD.$Foxp3^{DTR}$ mice were injected with DT on days 0, 1, 3, 5 and 7 and sacrificed on day 9. NOD.$Foxp3^{WT}$ mice treated with DT and NOD.$Foxp3^{DTR}$ mice treated with vehicle were used as controls. Concurrently, AhR ligand-treated mice were orally dosed with 60 mg/kg 10-Cl-BBQ (days −1, 1, 3, 5 and 7) or TCDD (day -1) relative to DT treatment on day 0 (Figure 6A). Two days following the initiation of DT treatment, approximately 80% of $Foxp3^+$ cells were depleted (Figure 6B). By day 9, 50% of $Foxp3^+$ cells were depleted, consistent with the reported rebound in $Foxp3^+$ cells following DT administration (21). 10-Cl-BBQ did not alter the depletion efficacy of DT (Figure 6C). Ablation of $Foxp3^+$ cells led to rapid and extensive infiltration in the pancreas with 83.5 ±16.5% of islets in vehicle-treated mice showing infiltration (30.5 ± 23.5 and 52.4 ± 38.8 with less than 50% and greater than 50% of the islet infiltrated, respectively). Strikingly, 10-Cl-BBQ treatment prevented infiltration in 73.2 ± 25.2% of islets (Figure 6D), an effect recapitulated by TCDD treatment (Figure 6E). Thus, AhR activation was not only able to suppress infiltration in the absence of $Foxp3^+$ cells, but also compensated for the lack of $Foxp3^+$ cells to suppress infiltration in their absence.

10-Cl-BBQ does not induce a compensatory CD25$^+$ Tr1-like Treg during Foxp3 depletion

Since Foxp3$^+$ cells were not required for 10-Cl-BBQ-mediated suppression of insulitis, an alternative mechanism by which 10-Cl-BBQ might mediate suppression is through induction of a Foxp3$^-$CD25$^+$ Treg. In an acute GVHD model (3, 25), and in *in vitro* studies (26), AhR activation has been shown to induce a Tr1-like Treg, characterized by CD25 expression in conjunction with CTLA4, GITR and IL-10. Additionally, AhR activation has been recently shown to transdifferentiate Th17 cells into Tr1 cells *in vitro* (27). Although the percentage of CD25$^+$ cells increased in the PLN following depletion of Foxp3$^+$ cells, 10-Cl-BBQ treatment did not alter the percentage of CD25$^+$Foxp3$^-$CD4$^+$ cells in comparison to vehicle-treated mice (Figure 7A). Additionally, CD4$^+$ cells expressing the Tr1 markers CD49b$^+$ and Lag3$^+$ (28) were not increased by 10-Cl-BBQ treatment (Figure 7B). These results do not support an AhR-mediated induction of Tr1 cells in the NOD model.

AhR activation reduced the percentage of disease-associated CD4$^+$Nrp1$^+$Foxp3$^-$RORγt$^+$ cells

Nrp1 expression on Foxp3$^-$ cells has been associated with an activated Teff phenotype (29). Nrp1 expression was assessed on CD4$^+$Foxp3$^-$ cells to determine if Nrp1 was altered by AhR activation in the NOD Foxp3.DTR model. DT treatment, and subsequent islet infiltration, resulted in an increase in CD4$^+$Nrp1$^+$Foxp3$^-$ cells from 4.3 ± 0.6% in non-depleted mice to 12.6 ± 2.7% in DT-treated mice. This increase in Nrp1$^+$ cells was not observed in mice treated with 10-Cl-BBQ or TCDD (Figures 8A and 8B) suggesting that these cells are associated with the development of a pathogenic T cell population. To test this hypothesis, the percentage of Nrp1$^+$Foxp3$^-$ cells was plotted versus the percentage of highly infiltrated islets for each vehicle-treated mouse injected with DT; the percentage of CD4$^+$Nrp1$^+$Foxp3$^-$ cells positively and significantly correlated with disease severity (Figure 8C). CD4$^+$Nrp1$^+$Foxp3$^-$ cells were also reduced in the PLN of NOD wild-type mice treated with 10-Cl-BBQ. Likewise, CD4$^+$Nrp1$^+$Foxp3$^-$ cells were decreased, albeit non-significantly (p=0.09) in TCDD-treated mice (Figure 8D), reflecting a lower efficacy of TCDD to suppress islet infiltration in comparison to treatment with 10-Cl-BBQ (Figures 8D and E). Similar findings were observed in the spleen (Supplemental Figure 2). In contrast to CD4$^+$Nrp1$^+$Foxp3$^-$ cells, the percentage of CD4$^+$Foxp3$^+$ cells did not correlate with the degree of islet infiltration (Figure 8E).

The detrimental role of Th17 cells in exacerbating T1D has been widely established (24, 30–33). To further address whether AhR activation was inhibiting the induction of a pathogenic Teff population, coexpression of RORγt, the transcription factor for Th17 cells, with CD4$^+$Nrp1$^+$Foxp3$^-$ cells was assessed. In the CD4$^+$Foxp3$^-$ population, all RORγt$^+$ cells co-expressed Nrp1 (Figure 8F). Additionally, and consistent with the AhR-mediated reduction in CD4$^+$Nrp1$^+$Foxp3$^-$ cells, AhR activation by TCDD reduced the percentage of CD4$^+$ cells that were RORγt$^+$ (Figure 8G). TCDD treatment also reduced the percentage of CD4$^+$ cells that were Tbet$^+$ in mice that were depleted of Foxp3$^+$ cells (Supplemental Figure 3), although CD4$^+$IFNγ$^+$ cells were unaltered by AhR activation in Foxp3 replete mice. Taken together, these data demonstrate that AhR activation mediates the suppression of pathogenic Th17 cells in NOD mice and this occurs independently of Foxp3$^+$ Tregs.

Discussion

The AhR represents an unexploited target for treatment of immune-mediated diseases. Activated by exogenous compounds, AhR-driven suppression of Th1, Th2 and Th17-mediated immune responses has been extensively described (4, 6, 34). Most of our knowledge on the immune suppressive effects of AhR activation have been derived from studies using TCDD, a potent ligand that is not suitable for human therapy due to its propensity to bioaccumulate in the body. We previously reported results from a small molecule screen to identify high affinity AhR ligands with a more favorable pharmacokinetic profile than the metabolism-resistant TCDD. 10-Cl-BBQ was identified as a non-cytotoxic compound with the ability to activate the AhR at nanomolar concentrations, and with repeated dosing could maintain AhR activation despite a serum half-life of approximately two hours. Further, treatment with 10-Cl-BBQ led to similar *in vivo* immunological outcomes as the therapeutic dose of TCDD, inducing AhR-dependent $CD4^+CD25^+$ Tregs (3) as effectively as TCDD and dose-dependent increases in the expression of known AhR-associated genes in an acute parent-into-F1 GVH model (13). In the current study we examined the utility of 10-Cl-BBQ to maintain AhR activation in a chronic disease model. We found that repeated dosing with 10-Cl-BBQ effectively suppressed islet infiltration in NOD mice and treatment from seven to twenty weeks of age was not associated with clinical measures of toxicity, emphasizing 10-Cl-BBQ as a therapeutic candidate.

The mechanism by which AhR activation leads to its profound immunosuppressive effects is not fully understood. In our group, experiments in an acute GVHD model using TCDD highlight the importance of AhR signaling during early stages of T cell activation. For suppression of the CTL response, AhR activation must be initiated within the first three days following transfer of donor cells into the F1 host. This model further highlighted $CD4^+$ T cells as a critical target for AhR activation; AhR expression was required in donor CD4+ T cells in order for TCDD to induce its suppressive effects (5). In other models, DCs have been shown to play a role in AhR-driven immune suppression. For example, using an experimental autoimmune encephalitis model with Rag1- or CD11c-conditional AhR knockout mice showed that the absence of AhR in either population leads to a decrease in AhR-mediated immune suppression (35). Additional studies have described the role of both $CD4^+$ T cells and DCs as targets for AhR (2, 7, 36).

Many studies have demonstrated that AhR activation in either T cells or tolerogenic DCs leads to an increase in $Foxp3^+$ Tregs (2, 7, 9, 10, 17–20, 37). However, there is recent debate over whether or not $Foxp3^+$ Tregs are required for the AhR-mediated suppression of Teff responses (35, 37). There is also mixed evidence for AhR activation increasing the total number of $Foxp3^+$ cells or just increasing their frequency alone (8, 18, 20, 37), making it unclear if improving the Treg:Teff balance is critical for immunosuppression. Alternatively the reduction in the Teff population alone may be sufficient to minimize pathology. Several reports tried to tease apart the role of Tregs in the AhR suppressive response using anti-CD25 neutralizing antibodies to indirectly target $Foxp3^+$ cells. For example, in the peanut allergy mouse model, where TCDD treatment led to an increase in the percentage but not total numbers of splenic $CD25^+Foxp3^+$ cells, anti-CD25 treatment reduced $CD25^+Foxp3^+$

cells but did not affect $CD25^{-}Foxp3^{+}$ cells. This treatment partially abolished some, but not all, of TCDD's suppressive effects (8). Similarly, studies in an autoimmune uveitis model showed that anti-CD25 treatment partially reversed TCDD-mediated reduction of disease score (10). These results suggest that $CD25^{+}$ cells play a role in AhR-mediated immune suppression, however, because anti-CD25 may have off target effects, it is difficult to definitively implicate $Foxp3^{+}$ cells in the observed effects. In the current study we found an increase in the percentage but not total numbers of $Foxp3^{+}$ cells following AhR activation, as a result of a decrease in the number of $CD4^{+}$ cells in both the pancreas and PLN. Critically, specific depletion of $Foxp3^{+}$ Tregs in NOD.$Foxp3^{DTR}$ mice demonstrated that $Foxp3^{+}$ Tregs are not required for AhR-mediated suppression of insulitis. This conclusion was supported by a lack of correlation between the percentage of $Foxp3^{+}$ Tregs and the severity of islet infiltration in vehicle-treated NOD mice. Collectively, these results indicate that a reduction in Teff number, is the driving force of AhR-mediated immune suppression, rather than improved Treg:Teff balance.

Support for the hypothesis that the reduction in Teff number is the primary event in AhR-driven suppression of insulitis comes from the analysis of $CD4^{+}Foxp3^{-}$ cells. Nrp1 expression on $CD4^{+}Foxp3^{-}$ cells strongly correlated with the degree of islet infiltration in NOD mice. Furthermore, the coexpression of Nrp1with RORγt, the transcription factor for disease-inducing Th17 cells (30, 38) on $CD4^{+}Foxp3^{-}$ cells, strengthens the idea that these cells represent a pathogenic effector population. $Nrp1^{+}Foxp3^{-}$ cells have previously been described as activated/memory T cells that were greatly increased in TGFβRII deficient mice with autoinflammatory disease (29). Nrp1 is a receptor for TGFβ (39), which in the context of IL-6, leads to Th17 differentiation (40). As a result of the pro-inflammatory microenvironment in NOD mice (41), Th17 differentiation is consistent with the coexpression of RORγt with Nrp1 on $CD4^{+}Foxp3^{-}$ cells. Whether or not Nrp1 expression on $CD4^{+}Foxp3^{-}$ cells in the PLN is functionally involved in the development of pathogenic T cells, $CD4^{+}Nrp1^{+}Foxp3^{-}$ cells may have utility as a biomarker for the extent of islet infiltration in the pancreas. In fact, both 10-Cl-BBQ and TCDD treatments reduced the frequency of $Nrp1^{+}$ $CD4^{+}Foxp3^{-}$ cells which directly correlated with reduced islet infiltration. Functionally, Nrp1 is expressed in the immunological synapse and has been implicated in productive signaling between T cells and DCs (42). A decrease in Nrp1 expression following treatment with AhR ligands could result in altered crosstalk between T cells and DCs, impairing T cell differentiation and precluding Teff accumulation.

T1D is a chronic disease and it has recently been shown that islet inflammatory lesions are dynamic with constant influx of new T cells entering the islet (43). Since early T cell activation events are targeted by AhR signaling (4), there is continuous opportunity for AhR ligands to interfere with the activation/differentiation of new autoantigen-specific T cells. The possibility that 10-Cl-BBQ treatment will be able to halt β-cell destruction during the 'honeymoon' period in humans following early diagnosis of T1D is of particular interest (44).

Collectively, this study is the first to demonstrate that specifically depleting $Foxp3^{+}$ cells does not abrogate immune suppression by AhR ligands. These findings directly challenge the dogma that $Foxp3^{+}$ Tregs are responsible for the therapeutic effects of AhR ligands in

immune-mediated diseases. Instead, the reduction in Teff appears to be sufficient for the prevention of disease. AhR activation acts to compensate for the depletion of $Foxp3^+$ Tregs, and thus AhR ligands might be useful as an alternate treatment strategy in diseases characterized by defective Treg numbers or functionality. Our results highlight 10-Cl-BBQ as an effective and non-toxic AhR ligand for the therapeutic intervention of immune-mediated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Knutson JC, Poland A. 2,3,7,8-Tetrachlorodibenzo-p-dioxin: failure to demonstrate toxicity in twenty-three cultured cell types. Toxicol Appl Pharmacol. 1980; 54:377–383. [PubMed: 7394793]
2. Quintana FJ, Murugaiyan G, Farez MF, Mitsdoerffer M, Tukpah AM, Burns EJ, Weiner HL. An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2010; 107:20768–20773. [PubMed: 21068375]
3. Funatake CJ, Marshall NB, Steppan LB, Mourich DV, Kerkvliet NI. Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. J Immunol. 2005; 175:4184–4188. [PubMed: 16177056]
4. Kerkvliet NI, Baecher-Steppan L, Shepherd DM, Oughton JA, Vorderstrasse BA, DeKrey GK. Inhibition of TC-1 cytokine production, effector cytotoxic T lymphocyte development and alloantibody production by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Immunol. 1996; 157:2310–2319. [PubMed: 8805628]
5. Kerkvliet NI, Shepherd DM, Baecher-Steppan L. T lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): AhR expression in both CD4+ and CD8+ T cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. Toxicol Appl Pharmacol. 2002; 185:146–152. [PubMed: 12490139]
6. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, Caccamo M, Oukka M, Weiner HL. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature. 2008; 453:65–71. [PubMed: 18362915]
7. Hauben E, Gregori S, Draghici E, Migliavacca B, Olivieri S, Woisetschlager M, Roncarolo MG. Activation of the aryl hydrocarbon receptor promotes allograft-specific tolerance through direct and dendritic cell-mediated effects on regulatory T cells. Blood. 2008; 112:1214–1222. [PubMed: 18550851]
8. Schulz VJ, Smit JJ, Willemsen KJ, Fiechter D, Hassing I, Bleumink R, Boon L, van den Berg M, van Duursen MB, Pieters RH. Activation of the aryl hydrocarbon receptor suppresses sensitization in a mouse peanut allergy model. Toxicol Sci. 2011; 123:491–500. [PubMed: 21804081]
9. Kerkvliet NI, Steppan LB, Vorachek W, Oda S, Farrer D, Wong CP, Pham D, Mourich DV. Activation of aryl hydrocarbon receptor by TCDD prevents diabetes in NOD mice and increases Foxp3+ T cells in pancreatic lymph nodes. Immunotherapy. 2009; 1:539–547. [PubMed: 20174617]
10. Zhang L, Ma J, Takeuchi M, Usui Y, Hattori T, Okunuki Y, Yamakawa N, Kezuka T, Kuroda M, Goto H. Suppression of experimental autoimmune uveoretinitis by inducing differentiation of regulatory T cells via activation of aryl hydrocarbon receptor. Invest Ophthalmol Vis Sci. 2010; 51:2109–2117. [PubMed: 20007828]

11. Benson JM, Shepherd DM. Aryl hydrocarbon receptor activation by TCDD reduces inflammation associated with Crohn's disease. Toxicol Sci. 2011; 120:68–78. [PubMed: 21131560]
12. Marshall NB, Kerkvliet NI. Dioxin and immune regulation: emerging role of aryl hydrocarbon receptor in the generation of regulatory T cells. Ann N Y Acad Sci. 2010; 1183:25–37. [PubMed: 20146706]
13. Punj S, Kopparapu P, Jang HS, Phillips JL, Pennington J, Rohlman D, O'Donnell E, Iversen PL, Kolluri SK, Kerkvliet NI. Benzimidazoisoquinolines: a new class of rapidly metabolized aryl hydrocarbon receptor (AhR) ligands that induce AhR-dependent Tregs and prevent murine graft-versus-host disease. PLoS One. 2014; 9:e88726. [PubMed: 24586378]
14. Faideau B, Larger E, Lepault F, Carel JC, Boitard C. Role of beta-cells in type 1 diabetes pathogenesis. Diabetes. 2005; 54(Suppl 2):S87–96. [PubMed: 16306346]
15. Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol. 2010; 10:849–859. [PubMed: 21107346]
16. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature. 2010; 464:1293–1300. [PubMed: 20432533]
17. Yeste A, Nadeau M, Burns EJ, Weiner HL, Quintana FJ. Nanoparticle-mediated codelivery of myelin antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2012; 109:11270–11275. [PubMed: 22745170]
18. Rouse M, Singh NP, Nagarkatti PS, Nagarkatti M. Indoles mitigate the development of experimental autoimmune encephalomyelitis by induction of reciprocal differentiation of regulatory T cells and Th17 cells. Brit J Pharmacol. 2013; 169:1305–1321. [PubMed: 23586923]
19. Nugent LF, Shi G, Vistica BP, Ogbeifun O, Hinshaw SJ, Gery I. ITE, a novel endogenous nontoxic aryl hydrocarbon receptor ligand, efficiently suppresses EAU and T-cell-mediated immunity. Invest Ophthalmol Vis Sci. 2013; 54:7463–7469. [PubMed: 24150760]
20. Veiga-Parga T, Suryawanshi A, Rouse BT. Controlling viral immuno-inflammatory lesions by modulating aryl hydrocarbon receptor signaling. PLoS Pathog. 2011; 7:e1002427. [PubMed: 22174686]
21. Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. Immunity. 2009; 31:654–664. [PubMed: 19818653]
22. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med. 2004; 199:1455–1465. [PubMed: 15184499]
23. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, Anthony BA, Sverdrup FM, Head R, Kuster DJ, Ruminski P, Weiss D, Von Schack D, Bluestone JA. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J Exp Med. 2012; 209:1713–1722. S1711–1719. [PubMed: 22966003]
24. Kuriya G, Uchida T, Akazawa S, Kobayashi M, Nakamura K, Satoh T, Horie I, Kawasaki E, Yamasaki H, Yu L, Iwakura Y, Sasaki H, Nagayama Y, Kawakami A, Abiru N. Double deficiency in IL-17 and IFN-gamma signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia. 2013; 56:1773–1780. [PubMed: 23699989]
25. Marshall NB, Vorachek WR, Steppan LB, Mourich DV, Kerkvliet NI. Functional characterization and gene expression analysis of CD4+ CD25+ regulatory T cells generated in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Immunol. 2008; 181:2382–2391. [PubMed: 18684927]
26. Apetoh L, Quintana FJ, Pot C, Joller N, Xiao S, Kumar D, Burns EJ, Sherr DH, Weiner HL, Kuchroo VK. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat Immunol. 2010; 11:854–861. [PubMed: 20676095]
27. Gagliani N, Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, de Zoete MR, Licona-Limon P, Paiva RS, Ching T, Weaver C, Zi X, Pan X, Fan R, Garmire LX, Cotton MJ, Drier Y, Bernstein B, Geginat J, Stockinger B, Esplugues E, Huber S, Flavell RA. Th17 cells transdifferentiate into regulatoy T cells during resolution of inflammation. Nature. 2015; 523:221–225. [PubMed: 25924064]

28. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, Guo B, Herbert DR, Bulfone A, Trentini F, Di Serio C, Bacchetta R, Andreani M, Brockmann L, Gregori S, Flavell RA, Roncarolo MG. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med. 2013; 19:739–746. [PubMed: 23624599]
29. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, Xiong H, Dolpady J, Frey AB, Ruocco MG, Yang Y, Floess S, Huehn J, Oh S, Li MO, Niec RE, Rudensky AY, Dustin ML, Littman DR, Lafaille JJ. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J Exp Med. 2012; 209:1723–1742. S1721. [PubMed: 22966001]
30. Emamaullee JA, Davis J, Merani S, Toso C, Elliott JF, Thiesen A, Shapiro AM. Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. Diabetes. 2009; 58:1302–1311. [PubMed: 19289457]
31. Zhang J, Huang Z, Sun R, Tian Z, Wei H. IFN-gamma induced by IL-12 administration prevents diabetes by inhibiting pathogenic IL-17 production in NOD mice. J Autoimmun. 2012; 38:20–28. [PubMed: 22186068]
32. Solt LA, Banerjee S, Campbell S, Kamenecka TM, Burris TP. ROR inverse agonist suppresses insulitis and prevents hyperglycemia in a mouse model of type 1 diabetes. Endocrinology. 2015; 156:869–881. [PubMed: 25560829]
33. Li CR, Mueller EE, Bradley LM. Islet antigen-specific Th17 cells can induce TNF-alpha-dependent autoimmune diabetes. J Immunol. 2014; 192:1425–1432. [PubMed: 24446517]
34. Negishi T, Kato Y, Ooneda O, Mimura J, Takada T, Mochizuki H, Yamamoto M, Fujii-Kuriyama Y, Furusako S. Effects of aryl hydrocarbon receptor signaling on the modulation of TH1/TH2 balance. J Immunol. 2005; 175:7348–7356. [PubMed: 16301641]
35. Duarte JH, Di Meglio P, Hirota K, Ahlfors H, Stockinger B. Differential influences of the aryl hydrocarbon receptor on Th17 mediated responses in vitro and in vivo. PLoS One. 2013; 8:e79819. [PubMed: 24244565]
36. Jin GB, Winans B, Martin KC, Lawrence BP. New insights into the role of the aryl hydrocarbon receptor in the function of CD11c(+) cells during respiratory viral infection. Eur J Immunol. 2014; 44:1685–1698. [PubMed: 24519489]
37. Schulz VJ, Smit JJ, Bol-Schoenmakers M, van Duursen MB, van den Berg M, Pieters RH. Activation of the aryl hydrocarbon receptor reduces the number of precursor and effector T cells, but preserves thymic CD4+CD25+Foxp3+ regulatory T cells. Toxicol Lett. 2012; 215:100–109. [PubMed: 23041608]
38. Jain R, Tartar DM, Gregg RK, Divekar RD, Bell JJ, Lee HH, Yu P, Ellis JS, Hoeman CM, Franklin CL, Zaghouani H. Innocuous IFNgamma induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. J Exp Med. 2008; 205:207–218. [PubMed: 18195074]
39. Glinka Y, Prud'homme GJ. Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. J Leukoc Biol. 2008; 84:302–310. [PubMed: 18436584]
40. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006; 441:235–238. [PubMed: 16648838]
41. Bradshaw EM, Raddassi K, Elyaman W, Orban T, Gottlieb PA, Kent SC, Hafler DA. Monocytes from patients with type 1 diabetes spontaneously secrete proinflammatory cytokines inducing Th17 cells. J Immunol. 2009; 183:4432–4439. [PubMed: 19748982]
42. Tordjman R, Lepelletier Y, Lemarchandel V, Cambot M, Gaulard P, Hermine O, Romeo PH. A neuronal receptor, neuropilin-1, is essential for the initiation of the primary immune response. Nat Immunol. 2002; 3:477–482. [PubMed: 11953749]
43. Magnuson AM, Thurber GM, Kohler RH, Weissleder R, Mathis D, Benoist C. Population dynamics of islet-infiltrating cells in autoimmune diabetes. Proc Natl Acad Sci U S A. 2015; 112:1511–1516. [PubMed: 25605891]

44. Abdul-Rasoul M, Habib H, Al-Khouly M. 'The honeymoon phase' in children with type 1 diabetes mellitus: frequency, duration, and influential factors. Pediatric Diabetes. 2006; 7:101–107. [PubMed: 16629716]

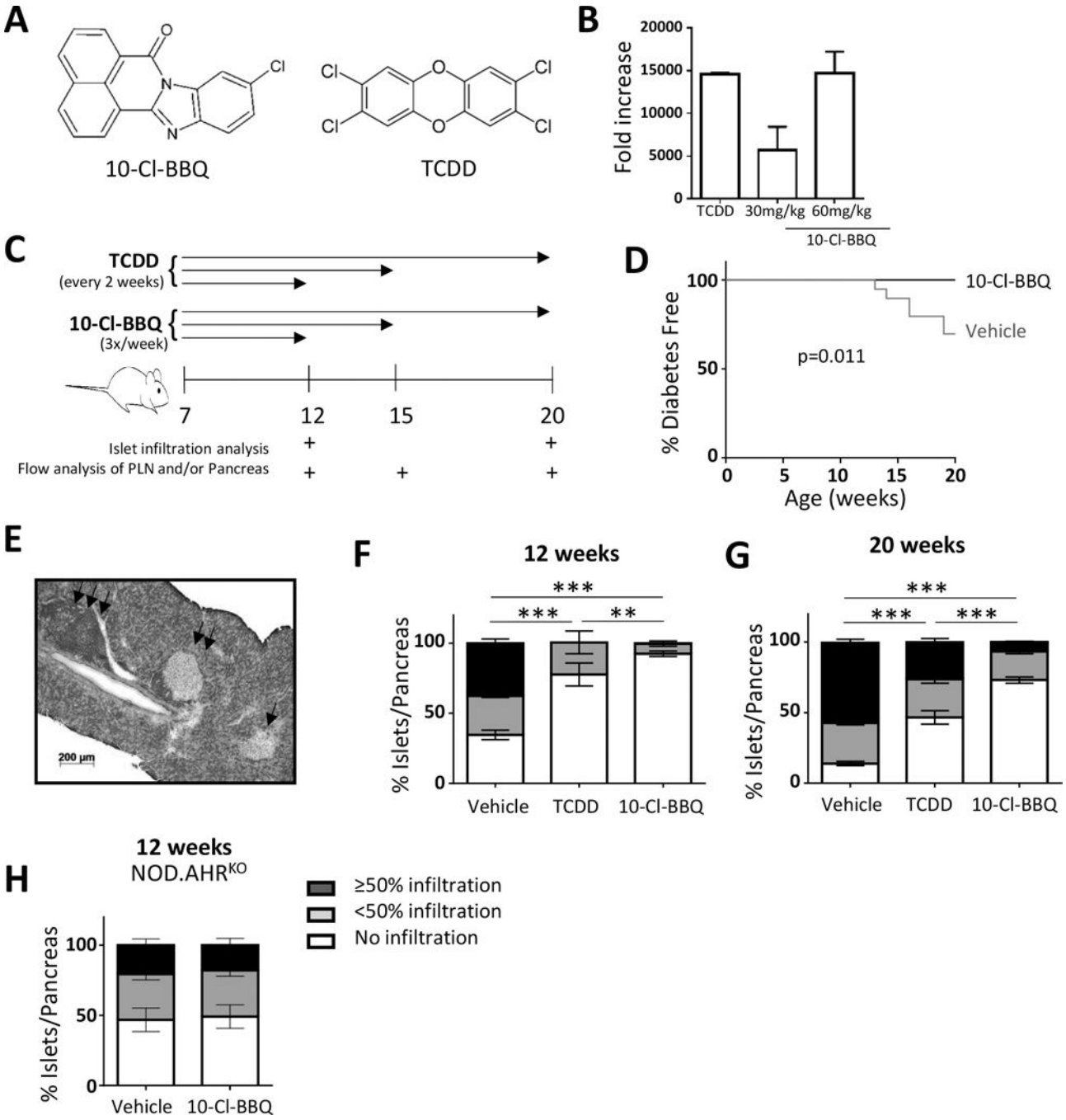

Figure 1. 10-Cl-BBQ suppresses insulitis in NOD mice

The therapeutic potential of 10-Cl-BBQ (chemical structure, **A**) to suppress islet infiltration in the NOD mouse model was assessed. **B**) *Cyp1a1* expression was measured in the liver following treatment with TCDD or 10-Cl-BBQ and compared to Vehicle treated mice. **C**) Experimental design for a time course to evaluate disease progression is shown. NOD mice were treated with 10-Cl-BBQ and TCDD starting at 7 weeks of age and blood glucose levels were measured weekly. Diabetes was categorized as two consecutive blood glucose readings ≥200 (**D**). Islet infiltration was scored as no infiltration (single arrow), less than 50%

(double arrow) and greater than 50% of islets infiltrated (triple arrow, representative scoring **E**). Data are presented as the average from each mouse per group at 12 (**F**) and 20 (**G**) weeks of age. **H**) The requirement of AhR expression was determined in NOD.AhR^{KO} mice treated with 10-Cl-BBQ starting at 7 weeks of age; islet infiltration was assessed at 12 weeks of age. n=7–9 mice/group. *, p≤0.05; ** p≤0.01; ***, p≤0.001.

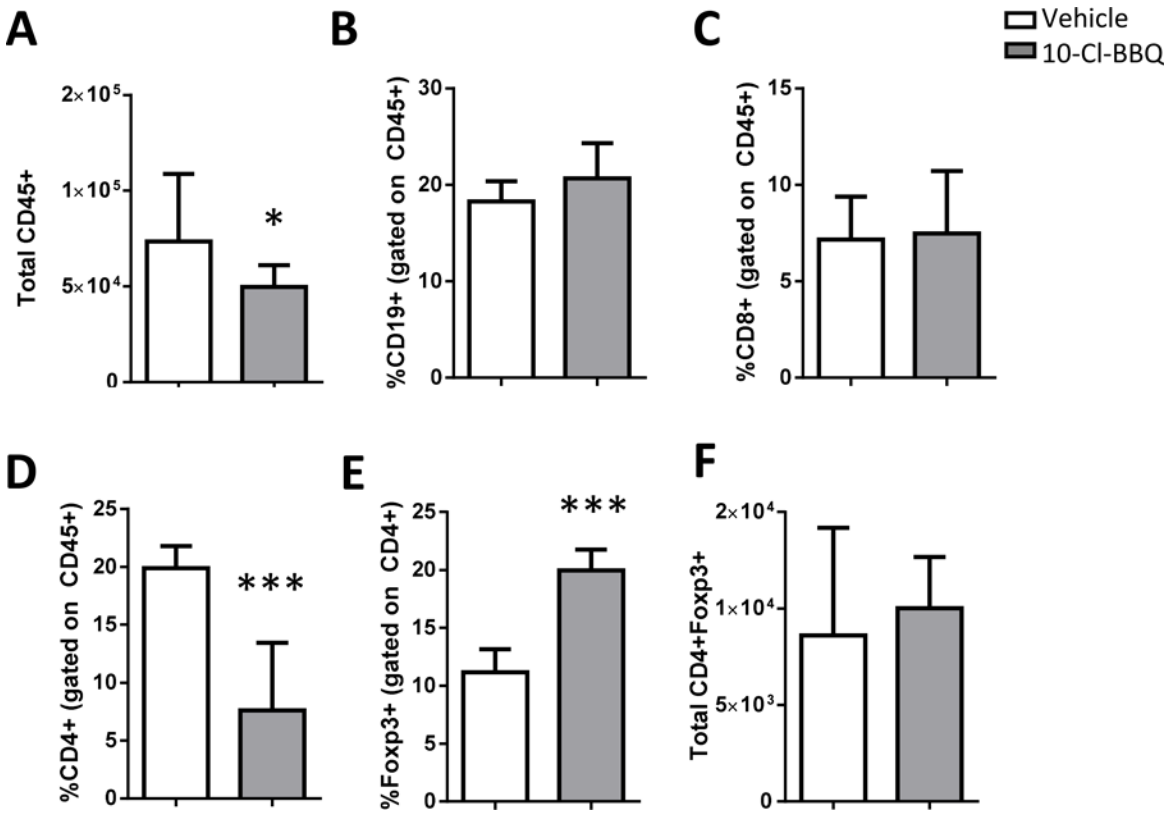

Figure 2. The percentage but not number of pancreatic CD4$^+$Foxp3$^+$ cells is increased following 10-Cl-BBQ treatment

NOD mice were treated with 10-Cl-BBQ from 7 to 12 weeks of age. At 12 weeks, pancreas were excised, digested and cell composition was assessed. Total number of CD45 (**A**) and percentages of CD19 (**B**), CD8 (**C**), CD4 (**D**) and Foxp3, gated on CD4, (**E**) are shown. Data represent one of two independent experiments. n=4–6 mice/group. *, p≤0.05; ** p≤0.01; ***, p≤0.001.

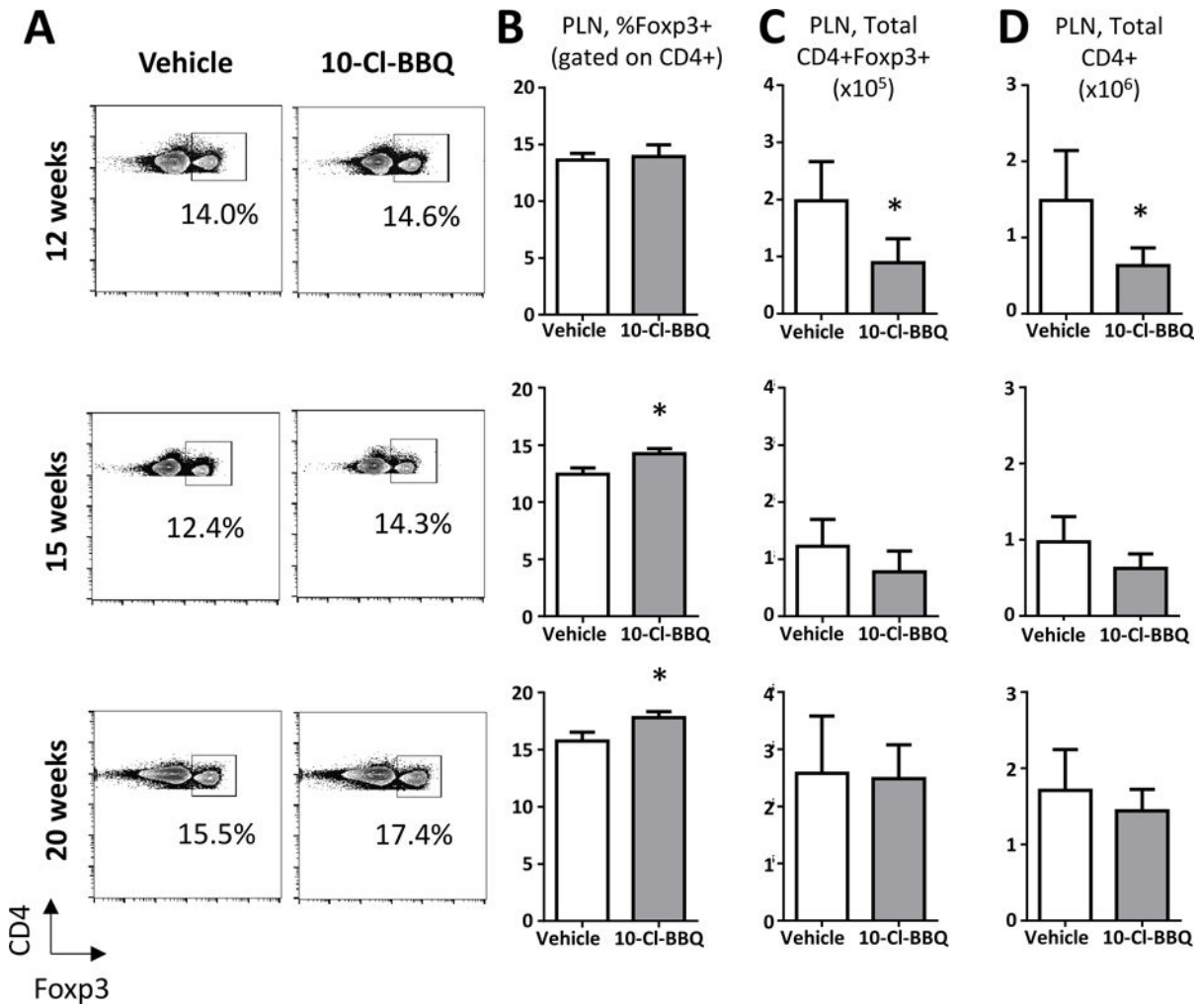

Figure 3. The percentage but not number of CD4$^+$Foxp3$^+$ cells is increased in the PLN following 10-Cl-BBQ treatment

Seven week old NOD mice were treated with 10-Cl-BBQ, 3x/week. At 12, 15 and 20 weeks, PLN were excised and stained for CD4 and Foxp3. **A**, Representative plots of Foxp3 expression gated on CD4$^+$ cells and summary data for each group (right) for PLN from mice treated with 10-Cl-BBQ is shown (**B**). Total CD4$^+$Foxp3$^+$ cells (**C**) and frequency of CD4$^+$ cells (**D**) from PLN of mice treated with 10-Cl-BBQ was determined. Data are plotted as average +/− s.d. n=5–9 mice/group. *, p≤0.05.

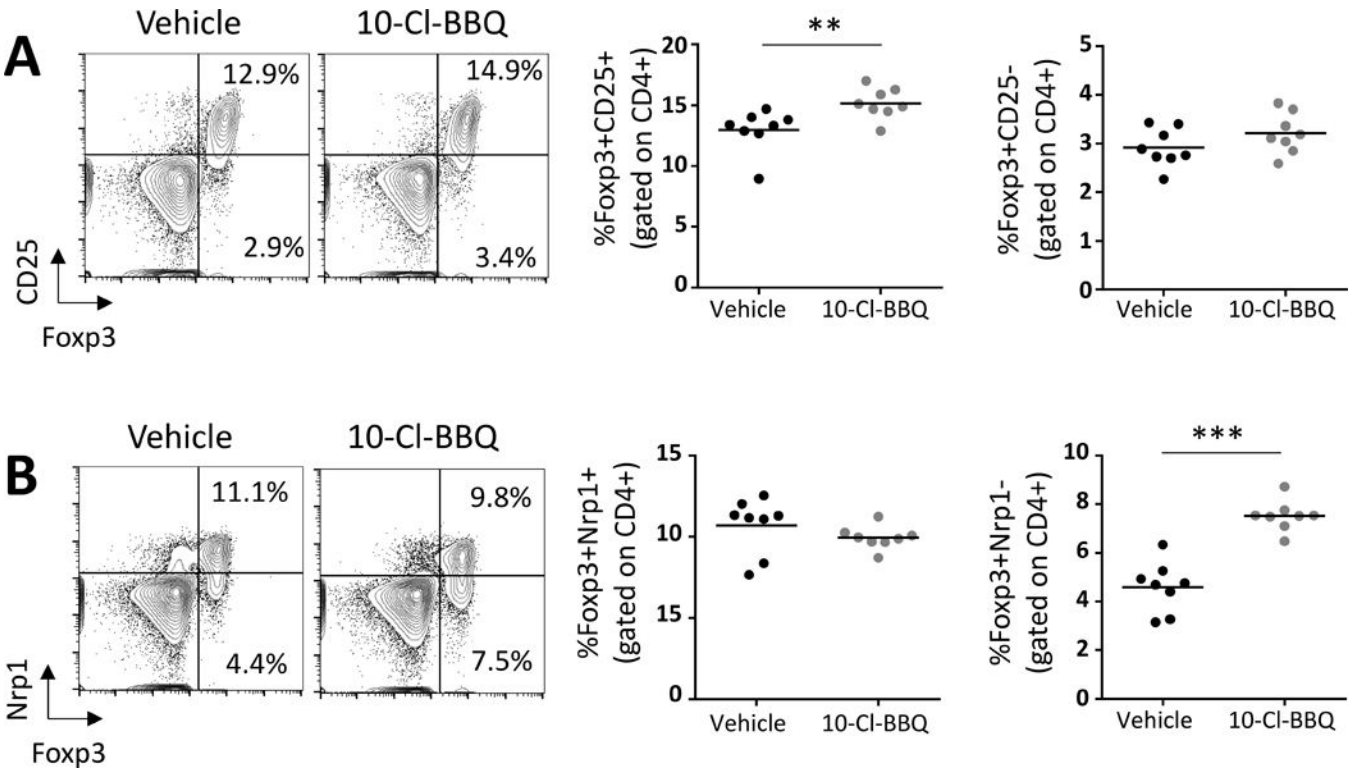

Figure 4. 10-Cl-BBQ treatment increased the percentage of $CD4^+Nrp1\text{-}Foxp3^+$ cells in the PLN at 20 weeks of age

At 20 weeks of age, PLN were excised and coexpression of $CD4^+Foxp3^+$ cells with CD25 and Nrp1 was assessed. Cells were gated on $CD4^+$ cells and the percentage of $Foxp3^+$ cells that were CD25+ and CD25- (**A**) or $Nrp1^-$ and $Nrp1^+$ (**B**) were determined. n=8–9 mice group. **, p≤0.01 ***, p≤0.001.

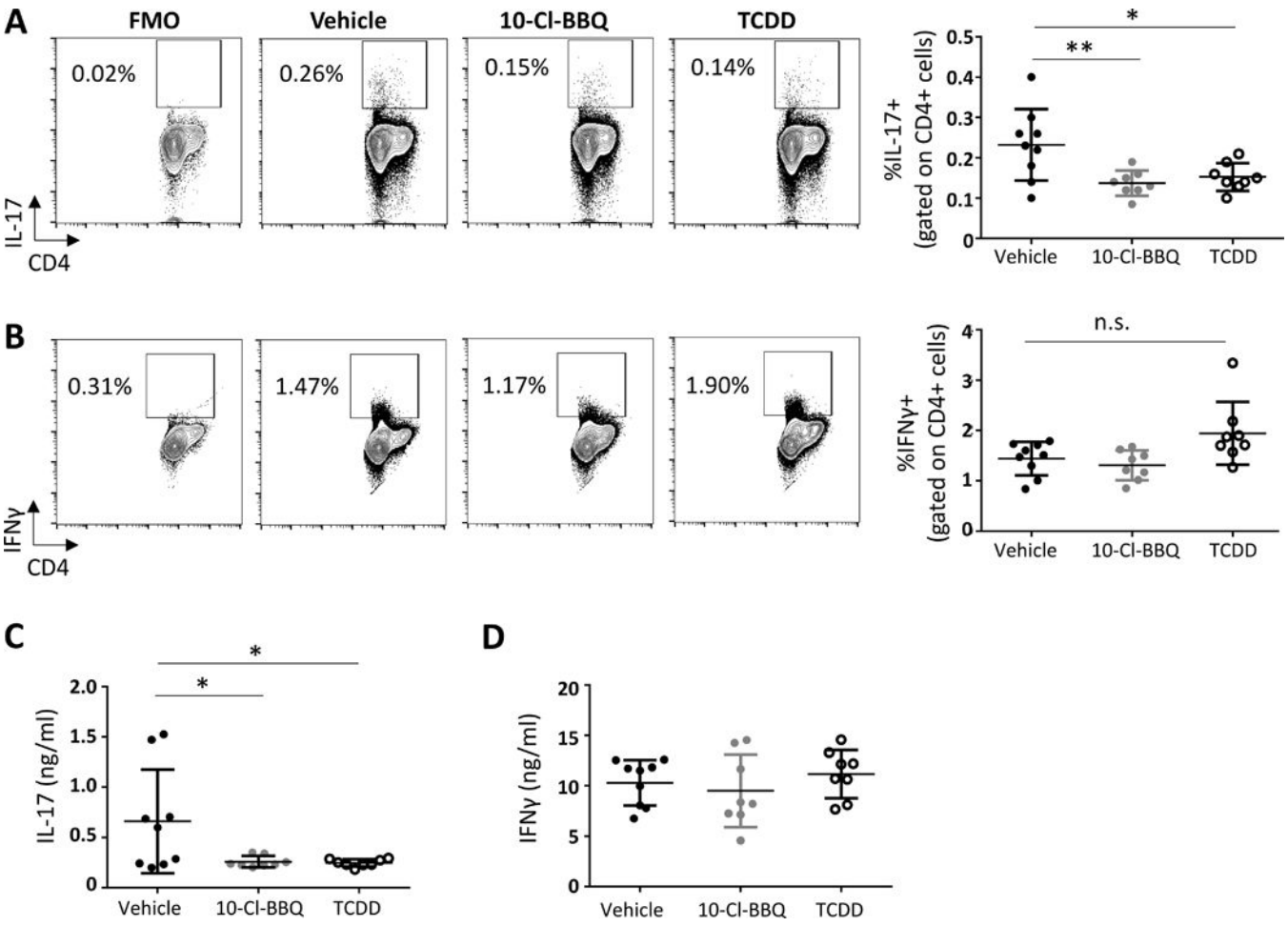

Figure 5. AhR activation reduces Th17 but not Th1 cells in splenocytes at 20 weeks of age

At 20 weeks of age, splenocytes were stimulated with PMA/Ionomycin for 4 hours. Expression of IL-17 (**A**) and IFNγ (**B**) was analyzed in CD4+ cells. Production of IL-17 (**C**) and IFNγ (**D**) in culture supernatant after 4 hours of stimulation was measured by ELISA. *, p≤0.05; ** p≤0.01

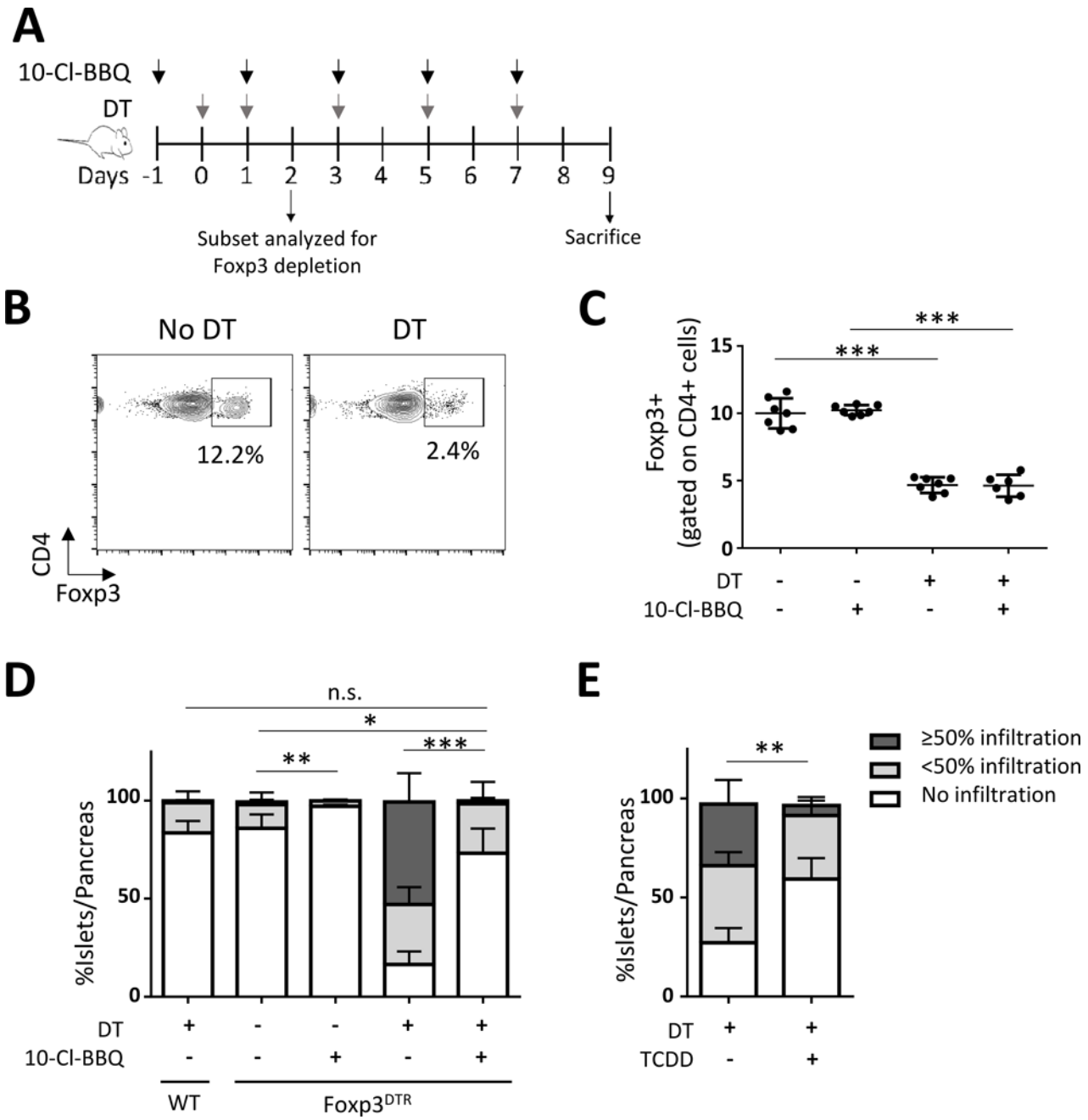

Figure 6. AhR ligands do not require Foxp3$^+$ Tregs to suppress pancreatic islet infiltration

NOD.Foxp3^{DTR} mice were utilized to specifically ablate Foxp3$^+$ cells. **A)** Four week old mice were treated with DT (500ng/mouse, i.p.) and concurrently treated with 10-Cl-BBQ or vehicle for 8 days. Depletion efficacy of Foxp3$^+$ cells was determined in a subset of mice on day 2 (n=3) (**B**) and at termination of the experiment on day 9 (**C**). Pancreatic islet infiltration was assessed by scoring H&E stained sections from wild type and NOD.Foxp3^{DTR} mice treated with DT and/or 10-Cl-BBQ (**D**) or TCDD (**E**). Data represent at two independent experiments; n=5–7 mice/group. *, p≤0.05; **, p≤0.01; ***, p≤0.001.

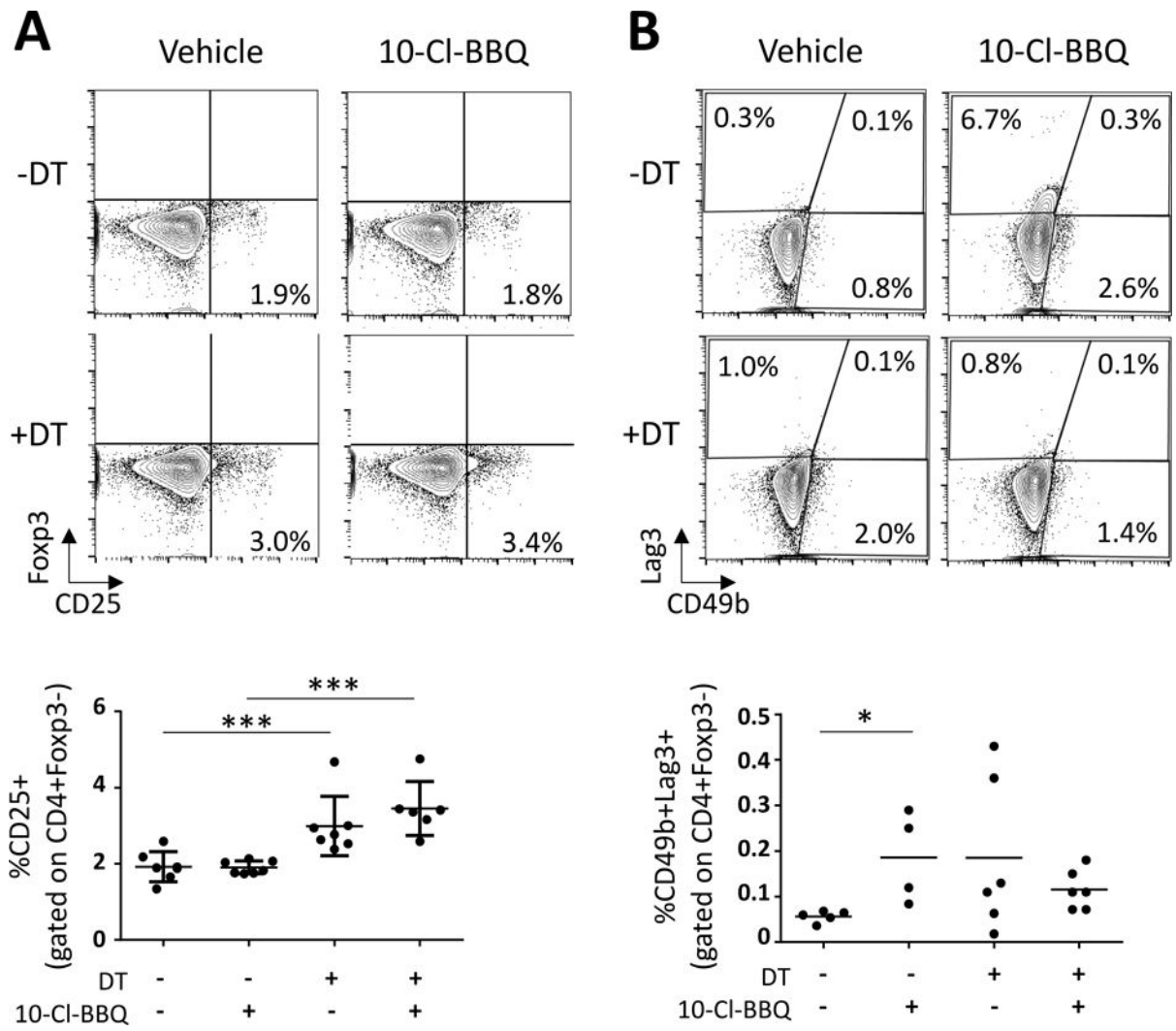

Figure 7. AhR activation does not induce CD4^{+}CD25^{+} Tr1 cells in DT treated NOD.Foxp3^{DTR} mice

Based on the ability of 10-Cl-BBQ to suppress islet infiltration in the absence of Foxp3^{+} cells, we assessed whether a compensatory CD25^{+} Foxp3^{-} Treg was induced by AhR activation in the NOD.Foxp3.DTR model. CD25 expression (A) and CD49b^{+}Lag3^{+} coexpression (B) was analyzed on CD4^{+}Foxp3^{-} cells in NOD.Foxp3^{DTR} treated with DT and/or 10-Cl-BBQ. Data represent two independent experiments; n=7 mice/group. *, p≤0.05; ***, p≤0.001.

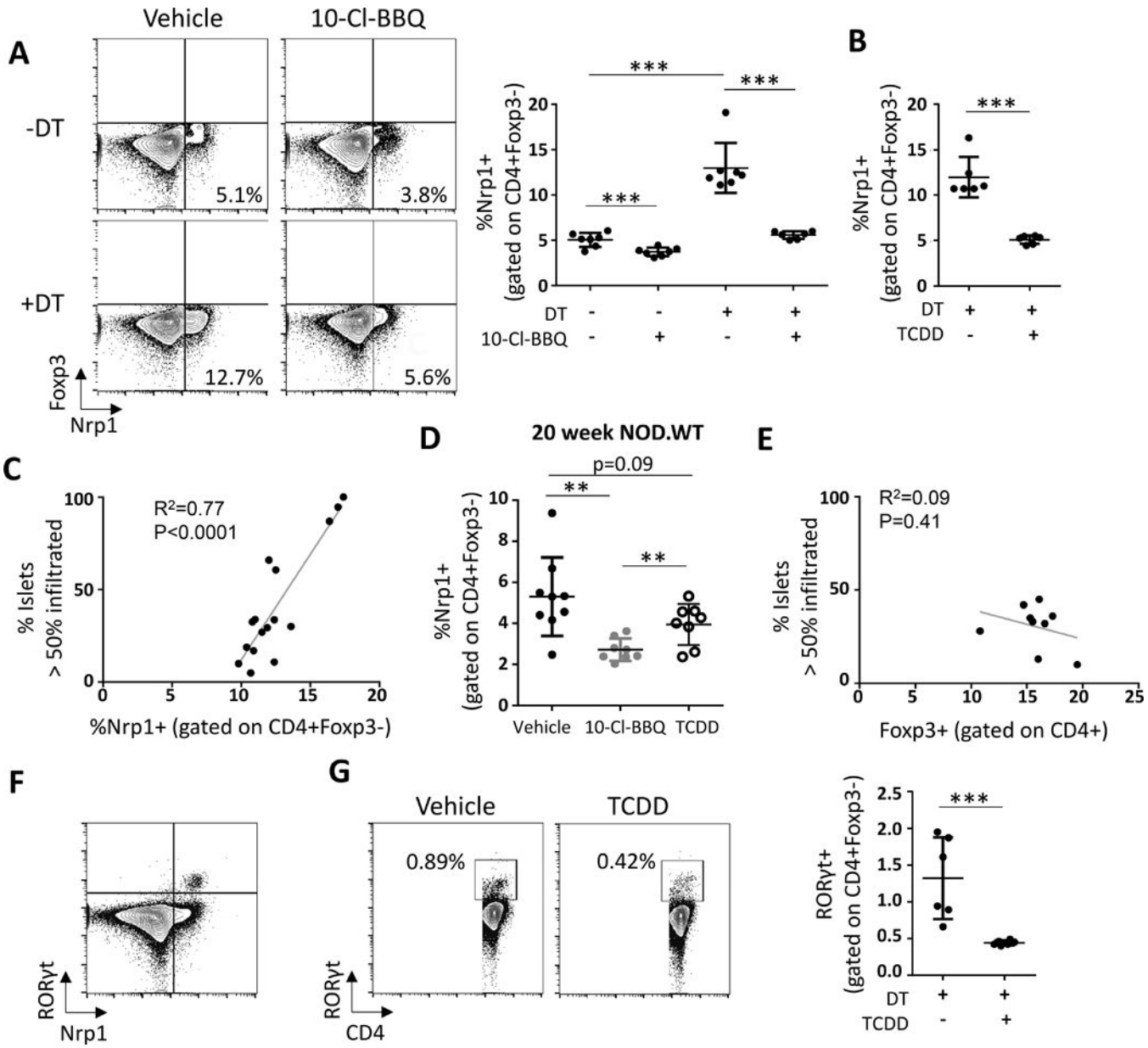

Figure 8. AhR activation reduces disease-associated CD4$^+$Nrp1$^+$Foxp3$^-$ cells

Nrp1 expression was analyzed on cells gated on CD4$^+$Foxp3$^-$ cells in NOD.Foxp3^{DTR} mice treated with 10-Cl-BBQ (**A**) or TCDD (**B**). **C)** The linear regression of the percentage of Nrp1$^+$ cells (gated on CD4$^+$Foxp3$^-$ cells) and the percentage of islets with greater than 50% infiltration in vehicle treated mice injected with DT was analyzed. **D**) Nrp1 expression was determined on cells gated on CD4$^+$Foxp3$^-$ cells in wild type NOD mice at 20 weeks of age treated with 10-Cl-BBQ or vehicle. **E**) The linear regression of the percentage of Foxp3$^+$ cells (gated on CD4$^+$ cells) and the percentage of islets with greater than 50% infiltration in vehicle treated NOD wild type mice at 20 weeks of age was calculated. **F)** Coexpression of RORγt with Nrp1 on CD4$^+$Foxp3$^-$ cells was assessed in NOD.Foxp3^{DTR} mice treated with

DT. **G)** The effects of TCDD on RORγt expression in $CD4^+$ cells was determined in NOD.$Foxp3^{DTR}$ mice on day 9. n=5–9 mice/group. p≤0.05; **, p≤0.01; ***, p≤0.001.