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STREPTOZOTOCIN-INDUCED DIABETES DEPENDS ON THE BALANCE OF TH17/TREG RESPONSES AND IS MODULATED BY LIGANDS OF THE ARYL-HYDROCARBON RECEPTOR

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Abstract- IL-17-producing Th cell subsets (Th17) are thought to be involved in autoimmune disease pathogenesis. The aryl-hydrocarbon receptor (AhR) is highly expressed in Th17 cells and AhR-ligands can skew T cell polarization towards either a Th17 or Treg phenotype. Previous studies have reported effects of AhR-ligands on experimental autoimmune encephalomyelitis. However, the ability of AhR-ligands to influence other autoimmune diseases has not been investigated. Here we show that mice lacking the cytokine receptor subunit gp130 on T cells, which have amplified Treg and reduced Th17 cell development, are protected from multiple low doses of streptozotocin (MLD-STZ)-induced diabetes. Furthermore, we find that the AhR-ligands including; curcumin, quercetin, and tryptophan, significantly increase the incidence of diabetes in *C57Bl/6J* mice after sub-diabetogenic doses of STZ. Enhanced autoimmune activation correlated with increased Th17 and Th1 populations, whereas no significant effect was noticed on Foxp3+ Treg cells following the AhR-ligand administration. In contrast, the AhR-ligands FICZ and I3C decreased the incidence of diabetes, which correlated with a significant increase in Treg cells. Our findings demonstrate a T cell gp130-dependent pathway required for cell-damage-induced diabetes, and reveal the ability of different AhR-ligands to modulate autoimmunity based on their ability to alter the balance of Th17/Treg subsets.

Keywords- T helper cells, autoimmunity, gp130, curcumin, quercetin

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Introduction

The aryl-hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates a variety of different events in many tissues [1]. Previous studies have reported that different AhR-ligands can alter CD4 T cell polarization, with consequent effects on autoimmune responses [2,3].

An emerging picture of immune regulation by T cell subsets is the ability of Th cells to convert to or from Treg or other phenotypes, and this plasticity may be influenced by the cytokine environment and other extrinsic factors [4]. Whereas Treg cells develop in response to TGF-β, pro-inflammatory environments that contribute IL-6, IL-1β and IL-23, promote skewing to an IL-17-producing Th17 phenotype [5-7].

6-formylindolo[3,2-*b*]carbazole (FICZ), a tryptophan derivative and high affinity AhR-ligand, was shown to increase Th17 cell development and enhance autoimmune disease in the EAE model [2]. IL-17+ Th cells were reduced in AhR-deficient mice, and these mice exhibited delayed onset and severity of EAE. However, dioxin, (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), another highly potent AhR-ligand, increased development of Foxp3+ Treg cells and decreased the severity of EAE [3].

The ability of AhR-ligands to alter Th subsets has been further investigated in several recent studies. TCDD and FICZ promoted iTreg induction, *in vitro* [8]. In contrast, tryptophan in culture media, which may generate endogenous AhR-ligands, was shown to influence the development of Treg versus Th17 subsets [9]. Such observations raise the possibility that other naturally occurring AhR-ligands, including tryptophan metabolites, the spice curcumin and the bioflavonoid quercetin, may be able to alter effector cell populations involved in regulating autoimmunity.

AhR was shown to be highly expressed in the Th17 subset [2]. As well, AhR-ligands, such as TCDD and FICZ, can have direct effects on dendritic cells (DC), altering their maturation, cytokine production and functional properties including expression of molecules associated with tolerogenicity, such as IDO and TGF-β [10,11]. These findings suggest that T cell subset development or function may be directly altered by AhR-ligands, or that changes in DC-derived pro-inflammatory or regulatory mediators may also contribute to skew-

ing of T cell responses. The importance of Treg and Th17 cell activity in various autoimmune conditions is widely recognized. Development of EAE is significantly suppressed in IL-17-knockout mice [12]. Cytokines known to promote Th17 development have also been shown to regulate autoimmunity. Neutralization of IL-12p40, a shared subunit in IL-12 and IL-23, decreases the development of EAE, and IL-12p40 knockout mice are resistant to EAE [13]. Likewise IL-1R knockout mice showed a significantly reduced onset and severity of EAE, which correlated with a failure to induce Th17 cells [6]. Th17 responses are known to drive or occur in other autoimmune diseases, such as type-1-diabetes, rheumatoid arthritis, inflammatory bowel disease, sjogren's syndrome and psoriasis [14].

Involvement of Th17 responses in autoimmune diabetes is controversial. In the NOD mouse model of type-1-diabetes (T1D), stimulation of IFN-γ production inhibited IL-17 production and prevented diabetes [15]. Also, neutralizing IL-17 with monoclonal antibody administration starting before 10 weeks of age significantly reduced the incidence of diabetes [16]. Furthermore, IL-21 knockout mice bred on to the NOD background did not develop diabetes and had reduced numbers of Th17 cells [17]. However, recent studies showed that diabetogenic *BDC2.5* T cells transferred into *NOD.scid* mice develop into an IFN-γ-producing population during onset of diabetes, whereas IL-17 producing BDC2.5 cells only led to pancreatic insulitis without causing diabetes [18,19]. Thus Th17 cells may be involved in NOD mouse onset of diabetes, or might only cause diabetes after conversion to a Th1 phenotype.

A T cell mediated autoimmune diabetes is induced by multiple low doses (MLD) of the pancreatic islet β-cells toxin, streptozotocin (STZ), and this model has been shown to be modulated by pro-inflammatory cytokines and regulatory T cells. Cytokines related to Th17 development have been shown to play a role in onset of diabetes following STZ-induced cell damage. Blocking IL-1R signaling prevents MLD-STZ, while administration of IL-1 increases incidence of diabetes in normally resistant strains of mice [20,21]. IL-23 administration stimulated diabetes in response to sub-diabetogenic doses of STZ [22]. Recently, the ability of polarized T cell responses to influence MLD-STZ was reported [23]. Reduction of Treg populations enhanced diabetes in response to sub-diabetogenic MLD-STZ doses, and ST2 knockout mice, deficient in Th2 development, exhibited increased IL-17 production and diabetes induction in a normally resistant strain. These studies suggest that T cell polarization toward Th17 may be involved in diabetes induced by limited β-cell damage.

Based on previous studies, it is thought that AhR-ligands can potently alter T cell responses and therefore affect the onset and severity of autoimmune disease. However, it is not yet known whether different AhR-ligands can induce or inhibit diabetes in the MLD-STZ mouse model. We have studied the ability of different AhR-ligands to alter CD4 T cell polarization, *in vivo* and *in vitro*, and examined the effects on induction of diabetes in the MLD-STZ diabetes model. Using a T cell-specific knockout of the cytokine receptor signaling subunit, gp130 (*gp130$^{fl/fl}$ CD4-Cre*), which shows increased Treg responses and reduced Th17 cells development [24-26], we find that mice deficient in gp130 signaling in T cells are protected from MLD-STZ diabetes. Furthermore, administration of tryptophan-derived AhR-ligands (FICZ, I3C) increased Th1, Th17 and Treg populations, *in vivo*, whereas natural dietary ligands, including curcumin, quercetin, and the ligand precursor tryptophan, increased Th1 and Th17 subsets while no significant effect was noticed on Treg populations. The ability to stimulate Treg population in addition to Th1 and Th17 subsets correlated with reduced incidence of MLD-STZ diabetes, while AhR-ligands that increased Th1 and Th17 responses and having no significant effect on Treg production enhanced diabetes in response to sub-diabetogenic MLD-STZ. Our findings demonstrate the novel property of certain naturally occurring AhR-ligands to boost Treg cells and reduce the onset of drug-induced diabetes, or to promote Th1 and Th17 responses and increase autoimmunity in response to target cell damage.

Materials and Methods

Animals

C57Bl/6J mice were purchased from Harlan, UK. Conditional knockout mice lacking gp130 on T cells (*gp130$^{fl/fl}$ CD4-Cre*+), were kindly provided by Prof. Werner Muller, University of Manchester. All experiments using mice were performed according to the Animals (Scientific Procedures) Act (1986) under an approved UK Home Office License.

Chemicals and Antibodies

6-formyl-indolo[3,2-*b*]carbazole (FICZ) was purchased from Enzo Life Science (UK). Indole-3-carbinol (I3C), curcumin, quercetin, tryptophan, neutral buffered formalin solution (NBF), ethoxy-resorufin, salicylamide, tween-20, 2-ME, STZ, LPS (*E.coli* serotype 026:B6), PMA, ionomycin, brefeldin A, and monensin were purchased from Sigma (Poole UK). All antibodies were purchased from eBioscience (UK). Anti-CD11c-microbeads were purchased from Miltenyi (Germary). Tetra-methyl-benzidine (TMB) was purchased from Alpha Labs. WST-1 viability reagent was purchased from (Roche). Blood glucose level was measured by using Accu-Chek Advantage glucose meter with Accu-Check Advantage II test strips (Roche). Saponin was purchased from Fluka (Switzerland). RPMI, HBSS, L-glutamine, penicillin, streptomycin, fetal bovine serum were purchased from (Invitrogen, UK).

Induction of Type-1-Diabetes in C57Bl/6J Mice by MLD-STZ Injection

Male mice between the ages of 9 and 12 weeks were used. Normal blood glucose levels were confirmed by pre-bleeding all the mice. STZ at a dose of 40mg/kg in citrate buffer, pH 4.2 was freshly prepared, and (200 µl) was injected i.p for four (sub-diabetogenic dose) or five consecutive days, as previously described [50]. Mice received STZ alone and a second i.p injection 20 minutes later with (200 µl) of HBSS as a control or different AhR-ligands as described in the figure legends. Blood glucose was measured by sampling one drop of blood from the lateral tail vein, starting on day 7 following the first injection, and continued every other day until the diabetic endpoint of the experiments. Mice with blood glucose levels higher than 15mM were scored as diabetic. Diabetic mice were sacrificed after 3 readings greater than 15mM were confirmed.

EROD Assay

Cytochrome P450 induction, measured by CYP1A1-assoicated ethoxy-resorufin-*O*-de-ethylase (EROD) assay, was performed according to a previously established method [27]. Briefly, total spleen ($2x10^5$ cells per well) were prepared from control and treated mice. Spleen cells were transferred to 96-well plate, and incubated with 500 µM ethoxy-resorufin and 150mM salicylamide at 37°C, 5% CO_2, 95% humidity for 2 hrs., and the EROD activity was measured spectro-fluorimetrically using excitation 530 nm and emission 590 nm, at various time points.

Cytotoxicity Assay

Spleen cells were transferred to 96-well plate ($2x10^5$ cells per well), and incubated with doses of different AhR-ligands overnight at 37° C, 5% CO_2, 95% humidity, and the following day, cells viability was assessed by adding WST-1 reagent to all wells. Absorbance at 450 nm was measured for WST-1 using DYNEX Plate Reader and Revelation Software.

Stimulation of Spleen Cells in the Presence of Specific AhR-ligands

Murine bone marrow-derived dendritic cells (BMDC) were prepared as described previously [51]. On day 6 of development, BMDC ($3x10^4$ cells per well) were stimulated overnight with 200 ng/ml LPS, and the conditioned media (CM) containing DC-derived pro-inflammatory cytokines was collected following centrifugation at 400x*g* for 10min and stored frozen at -80°C until use. Spleen cells ($2x10^5$ cells per well) were transferred to 96- well plate, cultured in 50% BMDC+LPS-CM and stimulated with 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 in the presence or absence of 100 nM FICZ, 10 nM I3C, 100 µM tryptophan, or 10 µM curcumin. Cells were incubated for three days at 37°C, 5% CO_2, 95% humidity, and supernatants were collected for IFN-γ and IL-17A ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA plates (NUNC Immuno Maxisorp) were coated with purified capture antibody (anti-IL-17A or anti-IFN-γ, used at 1:1000 dilutions) in the recommended coating buffer (100 ng/well) and blocked with ELISA blocking buffer (PBS+2%BSA). Serial dilutions of recombinant mouse cytokines were added to the plates, and then the standard curve was prepared, and then the samples were transferred to the rest of the wells, and incubated for 2 hrs. at room temperature. After incubation and wash, biotin-conjugated anti-cytokine detection antibody (1:1000) and Avidin-HRP (1:2000) were added. After a final wash, TMB substrate solution was added to all wells and absorbance at 630 nm was measured.

CD11c-Depleted Spleen Cell Stimulation with AhR-Ligand-Treated BMDC

BMDC were stimulated overnight with 200 ng/ml LPS with or without 100 nM FICZ, 10 nM I3C, 1 mM tryptophan, 1 µM curcumin or 1 µM quercetin. Total spleen cells were depleted of CD11c+ cells using anti-CD11c-microbeads, and then followed by Auto-MACS separation. Cell separation was carried on according to the manufacturer's instructions. Pre-treated-BMDCs ($3x10^4$ cells per well) were transferred to 96-well plate, mixed with CD11c-depleted spleen cells ($2x10^5$ cells per well) and stimulated with 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 for 4 days at 37°C, 5% CO_2, 95% humidity. On day 4, the media was removed, fresh media was added, and the cultures rested for 2 days. Primed T cells were then re-stimulated for 4 hrs with 25 ng/ml PMA and 25 ng/ml ionomycin in the presence of 1 µg/ml brefeldin A and 5 µM monensin.

Flow Cytometry

Re-stimulated T cells were stained with anti-CD4-PE-Cy7 antibody in FACS buffer (PBS, 2% FBS-heat inactivated, 0.1% NaN_3) for 30 min at 4°C. Cells were washed with FACS buffer, fixed and permeabilized with Foxp3 fixation/permeabilization buffer (eBioscience) for 15 min at room temperature. After incubation time, cells were washed with PERM/Wash buffer (FACS buffer, 0.1% saponin), centrifuged, and supernatants were discarded. Cells were stained with anti- IL-17-PE or anti-IFN-γ-AlexaFluor-647 or anti-IL-10-PE, or anti-Foxp3-AlexaFluor-647 in PERM/Wash buffer and incubated at room temperature for 20min (all antibodies were used at 1:1000 dilutions of 1 mg/ml). Cells were washed and re-suspended in 200 µl NBF, and fluorescence data acquired using a FACSArray Flow Cytometer (BD Bioscience) and obtained data were analysed using FlowJo software (Tree Star, USA).

Statistics

Significant differences between experimental groups with normal distribution were determined using ANOVA by using GraphPad Prism 4 software and all error bars show SEM. Differences between Kaplan-Meier incidence curves were determined by the log-rank test.

Results

Altered Th cell Development Avoid MLD-STZ Diabetes

To assess the role of the Th subsets in the onset of diabetes induced by MLD-STZ, T cell-specific conditional knockout mice lacking the IL-6R gp130 subunit (*gp130$^{fl/fl}$ CD4-Cre*+) or littermate controls (*gp130$^{fl/fl}$ CD4-Cre*-) were injected with MLD-STZ. A high incidence of diabetes (83%) was induced in control CD4-Cre- mice [Fig -1A]. However, diabetes in *gp130fl/fl CD4-Cre*+ mice was completely prevented (0% incidence, $p<0.001$), demonstrating protection of the mice from STZ-induced diabetes [Fig-1A]. Since lack of gp130 signaling in T cells is known to prevent development of Th17 cells and to amplify Treg responses [24-26], this finding strongly suggests that altering the Th17/Treg cell balance critically affects diabetes in response to MLD-STZ.

Different AhR-ligands Change the Incidence and Severity of Diabetes

To investigate whether the ability of AhR-ligands to modulate T cell subsets would alter autoimmune diabetes induced by MLD-STZ, *C57Bl/6J* mice were given five STZ injections with or without co-administration of different AhR-ligands. STZ injection alone resulted in 100% incidence of diabetes by day 9 post injection, while co-administration of FICZ reduced diabetes incidence to 50% of mice on day 9 [Fig-1B]. Also, co-administration of I3C with STZ reduced the incidence of diabetes to 67% of treated mice [Fig-1A]. In contrast, tryptophan and curcumin co-administration resulted in 100% incidence of diabetes with a more rapid (by day 7) onset [Fig-1C]. Since the high incidence of diabetes in the control group (100% STZ-alone) may have masked the potent enhancing activity of some of the AhR-ligands, we assessed the stimulation of diabetes in response to sub-diabetogenic doses (injection of 40mg/kg of STZ for 4 consecutive days). Injecting the mice with 40mg/kg STZ alone for 4 days showed only 17% incidence of diabetes [Fig-1D]. However, co-administration of tryptophan, curcumin or quercetin significantly ($p<0.05$) increased the incidence of diabetes to 67-83% [Fig-1D]. These data suggest that FICZ and I3C are able to suppress STZ-induced diabetes, while tryptophan, curcumin and quercetin enhanced the activation of diabetes after STZ injection.

AhR-ligands Induce Cytochrome P450 Expression, *in vivo*

To verify that the aromatic compounds we chose to investigate were *bona fide* ligands and activators of the AhR transcription factor, we examined induction of AhR-regulated gene expression, following injection of mice with different putative ligands. The cyp1a1 gene product, cytochome P450 (CYP) 1A1, is controlled by the AhR -dependent dioxin-response element and can be fluorometrically measured using the EROD assay [27]. Mice injection with FICZ,

I3C, tryptophan, or curcumin, on day 0 lead to a significant increased in CYP1A1-dependent EROD activity on day 2 in isolated spleen cells [Fig-2], (p<0.0001). Therefore, these compounds can activate the AhR and result in downstream gene expression.

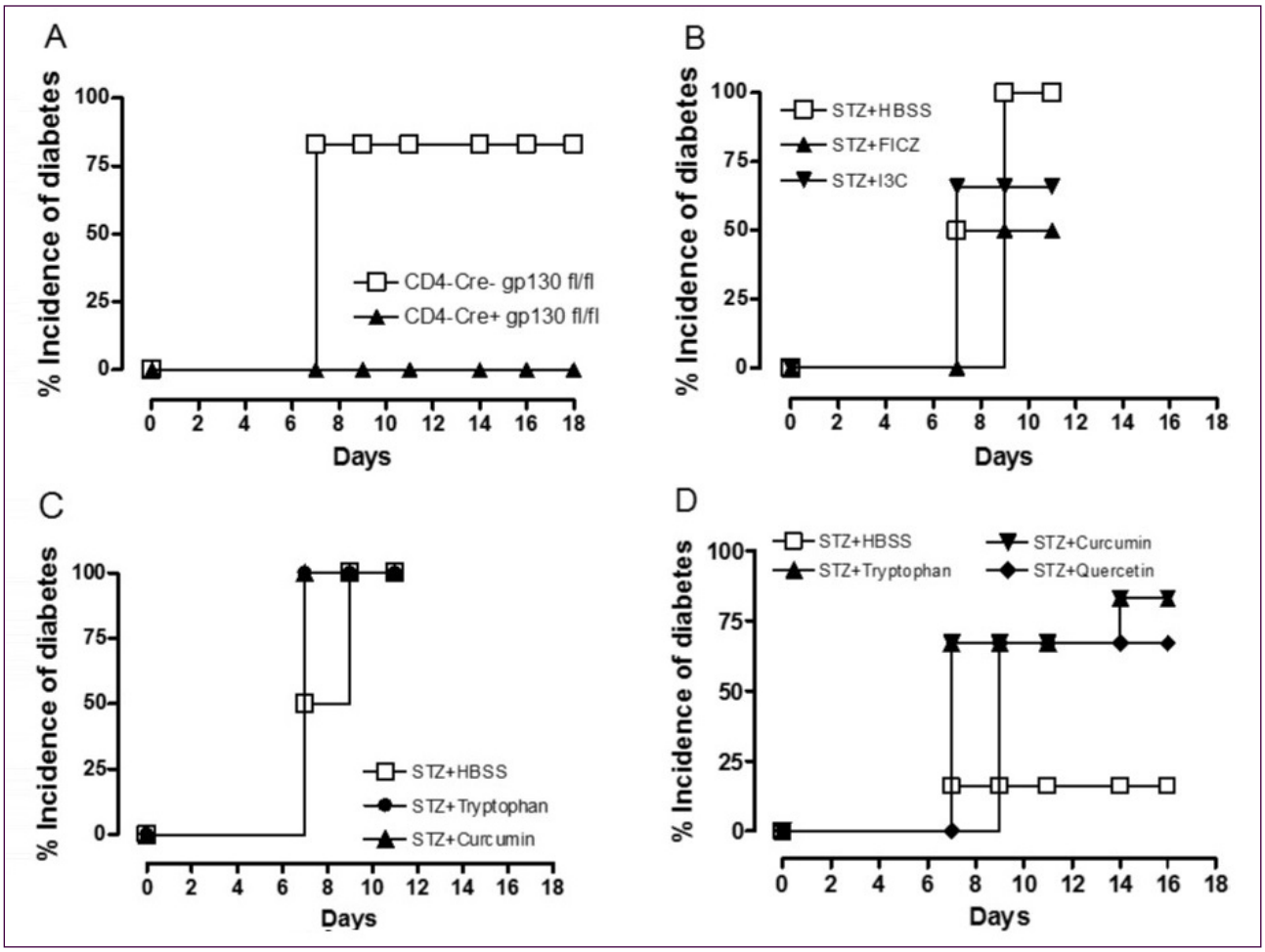

Fig. 1- Th17 Cell-specific gp130-Deficient Mice are Protected from MLD-STZ Diabetes and Co-administration of Different AhR-ligands to C57Bl/6J Mice Alters the Induction of Diabetes.

(1A) T cell-specific gp130-deficient (*gp130fl/fl CD4-Cre*+) mice, or littermate controls were treated 5 times with 40mg/kg STZ. Mice with blood glucose greater than 15mM for 3 readings were scored as diabetic. A significant difference (p<0.001) in incidence of diabetes was observed between groups. **(1B)** *C57Bl/6J* mice were injected 5 times with 40mg/kg STZ with a second i.p. injection of vehicle alone, or 20 nmol/kg FICZ or 20nmol/kg I3C. **(1C)** C57Bl/6J mice were injected 5 times with 40mg/kg STZ with a second i.p. injection of vehicle alone, or 20 µmol/kg tryptophan or 2 µmol/kg curcumin. **(1D)** *C57Bl/6J* mice were injected 4 times (sub-diabetogenic doses) with 40mg/kg STZ with a second i.p. injection of vehicle alone, or 20 µmol/kg tryptophan, or 2 µmol/kg curcumin or 2 µmol/kg quercetin. Significant differences (p<0.05) in incidence of diabetes were found for each AhR-ligand compared to STZ-alone. Data from 2 experiments with n=6 mice are shown in each group. Differences between Kaplan-Meier incidence curves were determined by log-rank test.

Administration of Different AhR-ligands Changes T cell Subsets, *in vivo*

To test the effects of AhR-ligands on Th17, Th1 and Treg subsets, *in vivo*, *C57Bl/6J* mice were treated with different AhR-ligands, and two days later, total spleen cells were re-stimulated and stained for IL-17+CD4+, IFN-γ+CD4+ and Foxp3+CD4+ T cell populations. Mice injected with FICZ, I3C, tryptophan or curcumin showed a significantly increased population of IL-17+ Th17 cells (p<0.01) compared to control mice that received buffer alone [Fig-3A], [Fig-3D], similarly, mice injected with all AhR-ligands significantly increased the IFN-γ+ Th1 cell population (p<0.01) [Fig-3B], [Fig-3E]. However, only mice injected with FICZ or I3C showed a significant increase in the population of Foxp3+ Treg cells (p<0.01), while injection of tryptophan or curcumin have no significant effect on Treg cells (p>0.05) compared to control mice that received buffer only [Fig-3C], [Fig-3F]. These results demonstrate that some AhR-ligands including FICZ and I3C alter the populations of effector and regulatory T cell subsets, while other AhR-ligands, including tryptophan and curcumin, increase the effector T cells (Th17 and Th1) while having no significant effect on the Treg population.

AhR-ligands Stimulate IL-17 and IFN-γ Secretion from Activated Spleen Cells, *in vitro*

The ability of the AhR-ligands to stimulate T cell cytokine production, *in vitro*, was then examined. To promote differentiation of T cells and production of IL-17 or IFN-γ, spleen cells were stimulated in the presence of conditioned media from LPS-stimulated BMDC (BMDC+LPS-CM), as described [5]. Spleen cell differentiation was performed in the presence of different AhR-ligands together with anti-CD3/anti-CD28 stimulation, for 3 days. Secretion of IL-17 was

significantly increased by inclusion of FICZ, I3C, tryptophan, or curcumin during spleen cell stimulation [Fig-4A]. Similarly, secretion of IFN-γ was significantly enhanced by inclusion of all four ligands [Fig-4B]. None of the ligands were cytotoxic to spleen cells at the doses used. These *in vitro* data agree with the effects of the AhR-ligands on polarization of T cell responses induced, *in vivo* [Fig-3A] & [Fig-3B], and demonstrate that AhR-ligands can promote Th17 and Th1-associated cytokine responses.

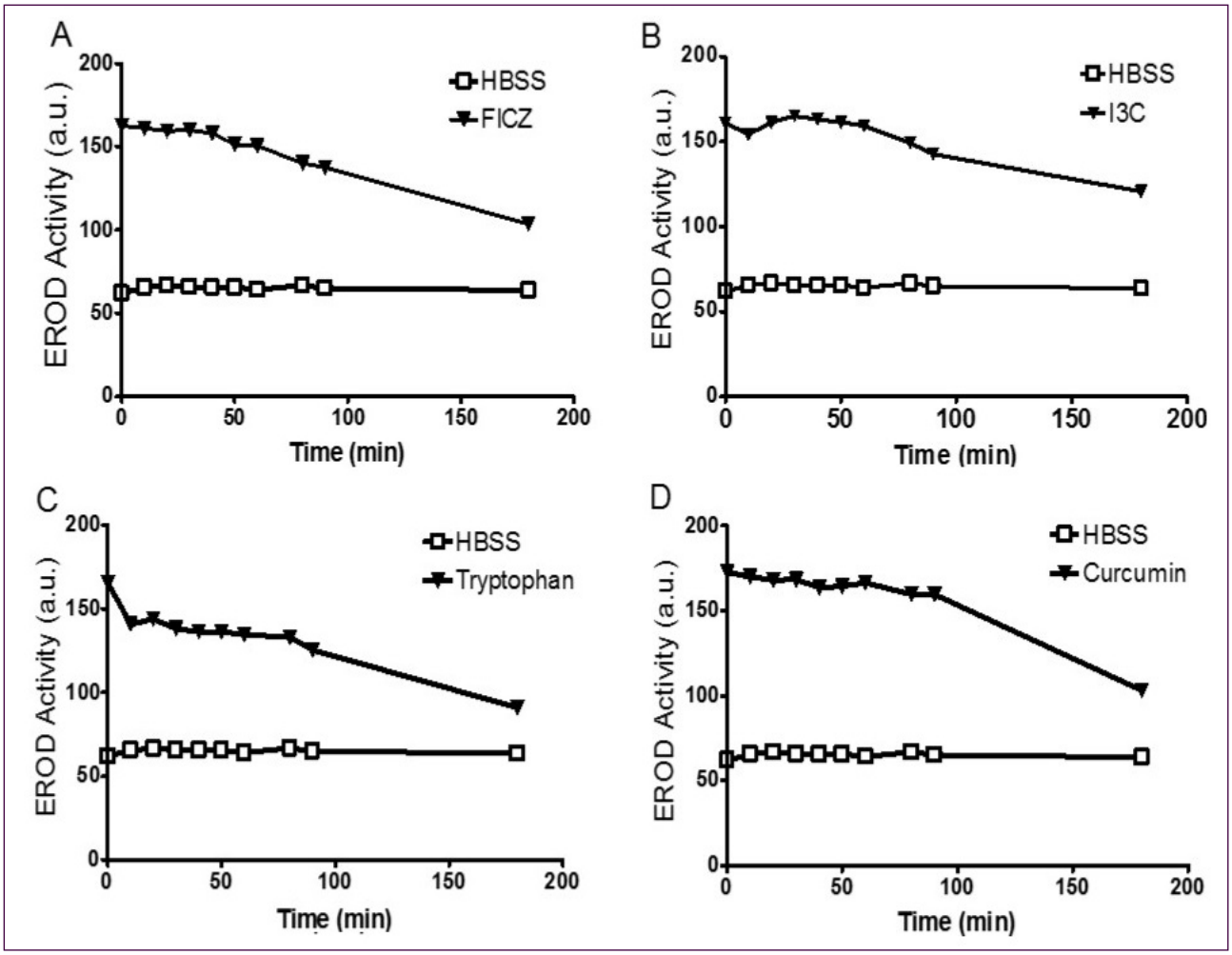

Fig. 2- Injection of AhR-ligands Induces the Expression of Cytochrome P450 in Spleen Cells.
C57Bl/6J mice were injected intraperitoneally with different AhR-ligands and two days later total spleen cells were assayed for EROD activity over time. Control mice received HBSS alone. **(2A)** FICZ (20 nmol/kg) injection significantly increased EROD activity (p<0.0001). **(2B)** I3C (20nmol/kg) injection significantly increased EROD activity (p<0.0001). **(2C)** Tryptophan (20 µmol/kg) injection significantly increased EROD activity (p<0.0001). **(2D)** Curcumin (2 µmol/kg) injection significantly increased EROD activity (p<0.0001). Representative data from 2 separate experiments using a total of 4 mice in each group. Mean values were plotted and significant differences determined by ANOVA.

AhR-ligands Promote the Development of Th1 and Th17 cells, *in vitro*

In order to stimulate potent T cell proliferation and differentiation into cytokine-producing and regulatory subsets, and to assess the effects of AhR-ligands on skewing their development, we modified our stimulation protocol to optimize detection of different subsets. First, we compared MACS-sorted naïve CD4 cells with total spleen cells, but did not find an improvement of IFN-γ and IL-17 production, even after addition of pure TGF-β and IL-6 with IL-1β or IL-23 (data not shown). Stronger increases in IL-17 and IFN-γ-expressing cells were seen following depletion of CD11c+ cells from total spleen, as suggested from previous publications (to enhance the development of Th17 population) [28,29], and stimulation with anti-CD3 plus LPS or IL-1β. We then compared the production of Th1, Th17, Treg and IL-10+CD4+ subset after stimulation in the presence of BMDC with LPS and AhR-ligand exposure [Fig-5].Treating CD11c-depleted spleen cells with either FICZ or I3C significantly increased the induction of a IL-17+ CD4+ Th17 population, compared to the control, ** p<0.01 [Fig-5A]. Also, tryptophan, curcumin, and quercetin each increased IL-17+ CD4+ cells, ** p<0.01 [Fig-5A]. The effect of different AhR-ligands on Th1 cell phenotype was also examined and FICZ, I3C, tryptophan, curcumin and quercetin all significantly (** p<0.01) increased the production of IFN-γ+ CD4+ Th1 cells [Fig-5B]. This confirms the effects of all ligands on enhancing Th17 and Th1 responses seen, *in vivo* and in the previous, *in vitro* experiment using total spleen cells [Fig-3] & [Fig-4].

AhR-ligands have Differing Abilities to Promote Treg and IL-10+CD4+ cells, *in vitro*

Using the above assay employing CD11c-depleted spleen cells with BMDC, production of Foxp3+ Treg and IL-10+CD4+ cells was detected, allowing assessment of the effects of the AhR-ligands on

regulatory T cell responses. Stimulation in the presence of either FICZ or I3C increased the induction of Foxp3+CD4+ cells, compared to the control, * $p<0.05$, ** $p<0.01$, respectively [Fig-5C]. However, tryptophan, curcumin, and quercetin were not able to increase Foxp3 expression [Fig-5C]. Similarly, stimulation in the presence of either FICZ or I3C significantly (** $p<0.01$) increased the induction of IL-10+CD4+ cells, compared to the control, while tryptophan, curcumin, and quercetin had no significant effect [Fig-5D].

These findings agree with the differential effects of some of the AhR -ligands on inducing Treg populations found, *in vivo* [Fig-3], and suggest that different AhR-ligands may promote or suppress diabetes based on their ability to reduce or enhance regulatory T cell subsets.

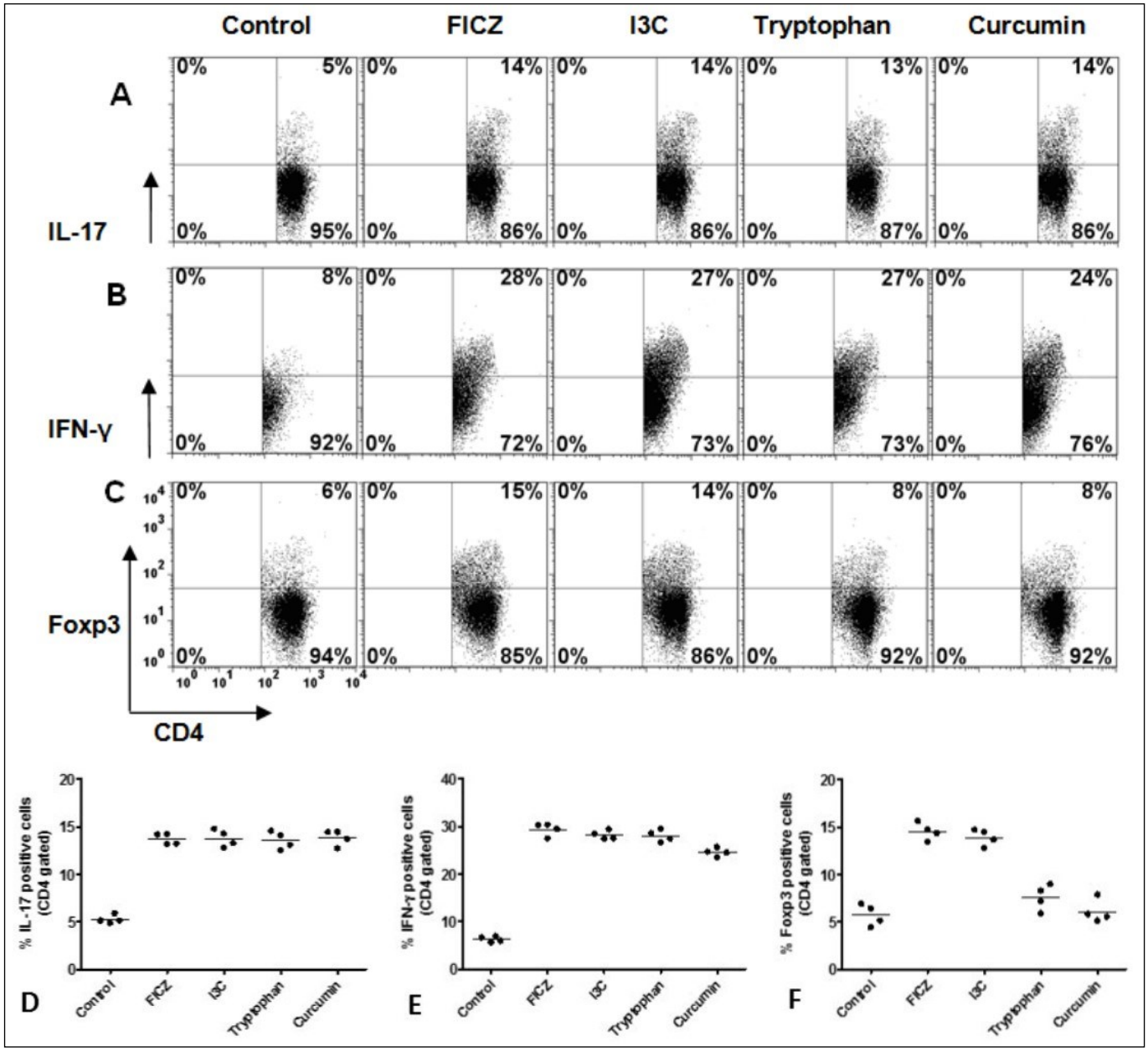

Fig. 3- The Effects of AhR-Ligands on the T cell Subsets, *in vivo*.

C57Bl/6J mice were injected with different AhR-ligands and two days later, total spleen cells were stained immediately for CD4+ and intracellular Foxp3+ Tregs, or re-stimulated with PMA/ionomycin and monensin for 4 hrs. and then stained for CD4+ and intracellular IL-17 or IFN-γ-producing cells. **(3A)** Flow cytometry dot plots of cultures double stained for IL-17 and CD4. **(3B)** Dot plots of cultures double stained for IFN-γ and CD4. **(3C)** Dot plots of cultures double stained for TGF-β and CD4. **(3D)** Mean production of IL-17+ Th17 cells, gated on CD4+ cells. All AhR-ligands significantly increased the population ($p<0.01$) compared to HBSS injection alone control. **(3E)** Mean production of IFN-γ+ Th1 cells, gated on CD4+ cells. All AhR-ligands significantly increased the population ($p<0.01$) compared to HBSS injection alone control. **(3F)** Mean production of Foxp3+ Treg cells, gated on CD4+ cells. FICZ and I3C injection significantly increased the population ($p<0.01$) as compared to the control group. Tryptophan and curcumin have no significant effect on Foxp3+ Treg population ($p>0.05$) as compared to the control group. Data shown are from 2 experiments, n=4 mice per group. Data for individual mice in each group and significant differences were determined by ANOVA.

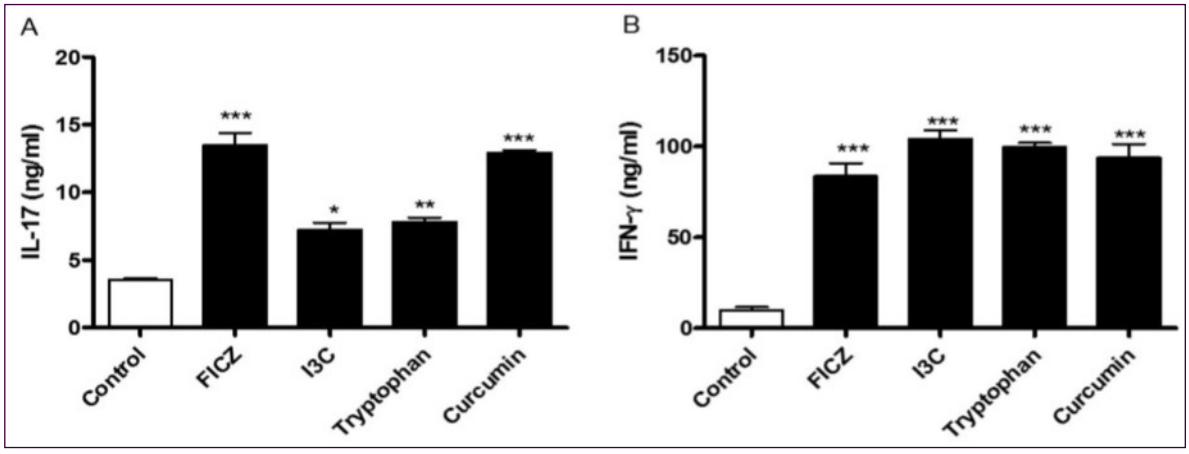

Fig. 4- AhR-ligands Increase IL-17 and IFN-γ-production from Spleen Cells, *in vitro*.

Total spleen cells from *C57Bl/6J* mice were stimulated for 3 days with anti-CD3 and anti-CD28 in the presence of LPS-treated BMDC-CM and different AhR-ligands. After the incubation, supernatants were collected and analyzed for cytokines by ELISA. **(4A)** IL-17 production in supernatants from spleen cells treated with FICZ (100 nM), I3C (10 nM), tryptophan (100 μM) or curcumin (10 μM). All ligands significantly increased IL-17 secretion compared to control. **(4B)** IFN-γ production in supernatants from spleen cells treated with FICZ (100 nM), I3C (10 nM), tryptophan (100 μM), or curcumin (10 μM). All AhR-ligands significantly increased IFN-γ secretion compared to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Representative data from 2 separate experiments using a total of 4 mice in each group. Mean values were plotted and significant differences determined by ANOVA.

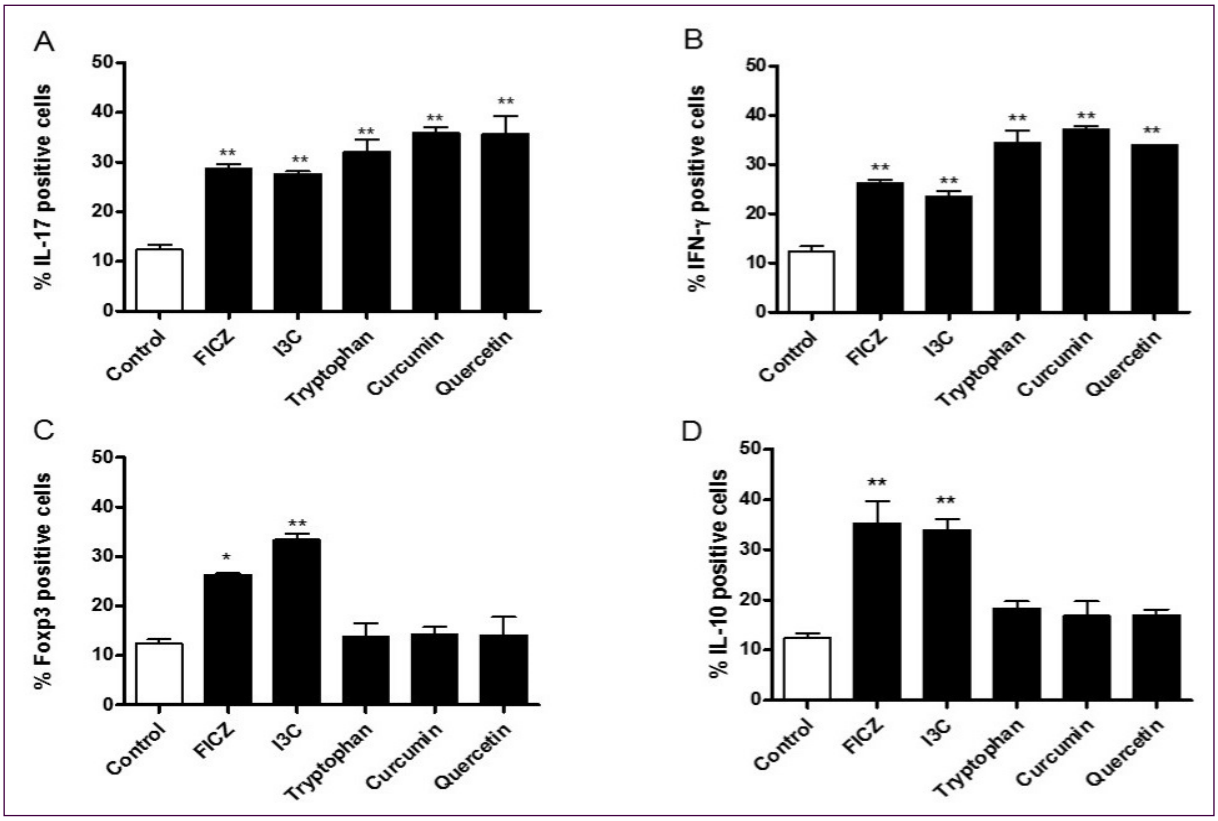

Fig. 5- AhR-ligands Enhance Development of Th1 and Th17 Populations, but have Differing Abilities to Promote Treg and IL-10-producing Subsets, *in vitro*.

Spleen cells from *C57Bl/6J* mice were depleted of CD11c+ cells, and stimulated with anti-CD3 and anti-CD28 in the presence of BMDC, which had been pre-treated with 200 ng/ml LPS and the AhR-ligands FICZ (100 nM), I3C (10 nM), tryptophan (1 mM), curcumin (1 μM), or quercetin (1 μM) for 24 hrs. **(5A)** IL-17+ Th17 population. All ligands significantly increased the Th17 population (** $p<0.01$) compared to untreated-BMDC control. **(5B)** IFN-γ+ Th1 population. All ligands significantly increased the Th1 population (** $p<0.05$) compared to untreated-BMDC control. **(5C)** Foxp3+ Treg population. Treg development stimulated by FICZ and I3C were significantly increased compared to untreated-BMDC control (* $p<0.05$, ** $p<0.01$). **(5D)** IL-10+CD4+ secreting cells. IL-10+CD4+ cells development stimulated by FICZ and I3C was significantly increased (** $p<0.01$) compared to untreated-BMDC control. Representative data from 3 separate experiments using a total of 6 mice in each group. Mean values were plotted and significant differences determined by ANOVA.

Discussion

In this study, we have investigated the effects of AhR-ligands on diabetes in the MLD-STZ mouse model and demonstrate for the first time that different AhR-ligands can either reduce the incidence of diabetes or increase the occurrence of diabetes in response to sub-diabetogenic doses of STZ. We have shown that gp130-dependent control of Th cell development determines the ability of STZ treatment to induce diabetes, and that all tested AhR-ligands increased IL-17 and IFN-γ+CD4+ T cells responses. However, groups of AhR-ligands, including FICZ and I3C, that suppressed diabetes also increased Foxp3+CD4+ populations, *in vivo* and *in vitro*, and IL-10+CD4+ cells, *in vitro*. Other groups of AhR-ligands or ligand precursor, including curcumin, quercetin, and tryptophan, that stimulated diabetes were not able to increase Treg *in vitro* and *in vivo* and IL-10+CD4+ cells development, *in vitro*. Therefore, a critical feature determining the effect of AhR-ligands on autoimmune responses is whether they can stimulate regulatory T cell responses to counter-act the elevated Th17/Th1 effector cell responses or not. Further characterization of Th17 and Treg cell responses in *gp130fl/fl CD4-Cre+* mice in response to STZ and AhR-ligand administration is currently in progress.

Previously, FICZ was reported to increase Th17 cells and promote EAE, while dioxin and ITE (a tryptophan metabolite and endogenous ligand of AhR) promoted Treg development and suppressed EAE [2,3]. However, others have reported that FICZ and dioxin can both increase Th17 cells or Treg cells, depending on the cytokine environment [30], or that both can induce Treg and IL-10+ Tr1 response [8]. Moreover, tryptophan derivatives in culture media, including FICZ, were reported to promote Th17 development, *in vitro*, [9] whereas the tryptophan metabolite kynurenine is known to enhance Treg development [31-34]. Our results agree with the reported ability of FICZ to increase both Th17 and Treg cell types and show that I3C has similar effects, *in vivo and in vitro*, and that these changes result in lower incidence of MLD-STZ diabetes. In contrast, we have found that other AhR-ligands including curcumin, quercetin, and the ligand precursor tryptophan itself, more exclusively promote Th17 and Th1 cytokine responses, while having no significant effect on Treg and IL-10+CD4+ populations, thus exacerbating autoimmune diabetes induction.

Curcumin is an abundant dietary AhR-ligand with many reported anti-inflammatory properties. Curcumin can ameliorate multiple sclerosis, type-1-diabetes, rheumatoid arthritis, and inflammatory bowel disease in human or animal models [35]. Regulation of inflammatory cytokines, in addition to associated JAK-STAT, AP-1 and NF-κB signaling pathways in immune cells is thought to be one mechanism to explain the immuno-suppressive effects of curcumin. Pre-treatment of animals with high doses of curcumin was reported to protect from MLD-STZ-induced diabetes, lowering pancreatic islet necrosis and inflammation, and preserving insulin+ cells [36]. It may be that direct effects of curcumin on β-cell resistance to cytokine-induced apoptosis or STZ-induced DNA damage resulted in protection against diabetogenic β-cell death in the previous study. In contrast, our experiments involved administration of curcumin immediately following STZ-insult, eliminating the possibility of direct protection, and allowing for subsequent effects on autoimmune responses to occur. Therefore, the timing of exposure to curcumin, whether prior to chemical damage or during damage-induced stimulation of inflammation and T cell immunity, appears to dramatically affect the outcome of diabetes induction or suppression in the MLD-STZ model.

Quercetin is found in many plants and has many reported anti-oxidative, anti-cancer, anti-inflammatory, and anti-diabetic activities [37]. Quercetin was shown to cause a time and concentration-dependent increase in the amount of CYP1A1 mRNA and CYP1A1 enzyme activity in tumours [38]. Pre-treatment of rodents with quercetin prevented STZ-induced diabetes in rats, and protected β-cells from oxidative stress-induced necrosis [39]. In a previous study, quercetin was reported to inhibit LPS-induced DC activation by reducing MHC class II and co-stimulatory molecules. In addition, quercetin was also able to suppresses the production of pro-inflammatory cytokines and chemokines, and decreased their ability to induce antigen-specific T cell activation [40]. In our experiments, quercetin and curcumin, did not suppress pro-inflammatory cytokines from BMDC (published data), but induced Th17 and Th1 cytokines while having no significant effect on Treg and IL-10+CD4+ cells [Fig-5] and enhanced incidence of STZ-induced diabetes [Fig-1]. Therefore, alteration of Th subset polarization by quercetin or curcumin is able to enhance autoimmune responses. Interestingly, a recent study has reported that quercetin can skew Th2 responses towards Th1 in a model of allergy [41].

How might different AhR-ligands, all with the ability to stimulate AhR -dependant gene transcription and promote Th17 cell development, promote either concomitant increases in Treg cells and lessen the occurrence of diabetes, or suppress Treg cell development and increase autoimmune activation? The presumed main function of AhR-induced transcriptional responses is to induce cytochrome P450 (e.g. CYP1A1) for detoxification of the detected aryl-hydrocarbon. Indeed, FICZ is rapidly metabolized in a CYP-mediated reaction, within 1-3 hrs. [42] with a corresponding drop in AhR activation [43]. Thus a transient AhR activation, even though promoting Th17 development and expansion, may ultimately terminate and allow Treg populations to emerge and dominate. In contrast, sustained AhR signaling might promote Foxp3 suppression and conversion of Treg to Th17 and Th1 cells.

Dietary AhR-ligands have also been suggested to act in an antagonistic manner to TCDD-induced AhR-activation [44,45]. Additionally, although curcumin is able to act as a substrate for CYP1A1-mediated catabolism, it was reported to be turned over after 48-72 hrs. [44] and to antagonise CYP1A1 activity [46]. Therefore, interference with full AhR function, or metabolism of the inducing AhR-ligand or other endogenous ligands, may be important in determining whether AhR-ligands result in regulatory and/or effector Th cell development.

Alternatively, certain AhR-ligands may induce distinct gene expression profiles [38,47], some promoting Th17 at the expense of Treg, others allowing Treg to also emerge. Activation of AhR in DCs by some ligands may increase tolerogenic mediators, such as IDO, which promote subsequent Treg development. In support of this mechanism, IDO expression was found to be increased in DCs by TCDD or FICZ [10].

Conversion of Treg to Th17 and Th17 to Th1 profiles have been reported and re-programming of subsets might be possible by additional cytokine provision, such as IL-23, IL-6, or removal of reinforcement factors, such as IL-23 or AhR-ligands [48]. The reported ability of IDO products (i.e. tryptophan metabolites) to suppress ROR-γt and induce Foxp3+Treg cells [49] may indicate that Th17 to Treg conversion, or shift to an IL-10-producing subset might result during exposure to some AhR-ligands.

Since some AhR-ligand treatments lead to Th17 and Th1 responses in the absence of Treg responses, allowing enhanced autoimmunity, this suggests that these ligands may be useful to promote anti-tumour immunity. It also raises the possibility that the anti-cancer effects of curcumin and quercetin may be due to their ability to promote potent effector T cell subsets in addition to suppressing some chronic inflammatory states. Another potentially beneficial use of AhR-ligands that have the ability to increase Treg populations is for the prevention or treatment of autoimmune diseases. Our results indicate that the tryptophan derivatives FICZ and I3C are most beneficial for resisting β-cell damage-induced diabetes, and may therefore be of benefit to those at risk of developing T1D. However, further understanding of the precise conditions that determine which T cell responses will result downstream of different AhR -ligands administration, and the effects of route and timing, is necessary to predict whether these compounds will be clinically beneficial or may worsen autoimmune conditions.

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