International Journal of Immunology Research
ISSN: 0976-4909 & E-ISSN: 0976-4917, Volume 3, Issue 1, 2013, pp.-29-39.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000229

ARYL HYDROCARBON RECEPTOR LIGANDS STIMULATE AUTO-REACTIVE TC1/TC17 T CELL ACTIVATION AND ENHANCE SELF-ANTIGEN-INDUCED DIABETES

ABU-REZQ H.A.[1,2]* AND MILLAR D.G.[2]

[1]Kuwait Institute for Scientific Research (KISR), Environment and Life Sciences Research Center (ELSRC), Aridland and Agricultural Production Program (AAPP), P.O. Box 24885, Safat, 13109, Kuwait.
[2]Faculty of Life Sciences, The University of Manchester, United Kingdom.
*Corresponding Author: Email- haborizq@kisr.edu.kw

Received: September 09, 2013; Accepted: September 24, 2013

Abstract- Previous studies have reported that ligands of the aryl-hydrocarbon receptor (AhR) can influence the induction of different autoimmune diseases by altering the development of IL-17+ CD4+ Th17 cells or Foxp3+ CD4+ Treg cells. CD8+ T cells are also involved in mediating or regulating autoimmune destruction, and are known to polarize into different subsets. However, the effects of AhR-ligands on CD8+ T cell phenotypes and effector functions have not yet been investigated. In the present research, the effects of specific AhR-ligands, and the ligand precursor, tryptophan, on CD8+ T cell mediated autoimmunity were investigated for the first time using the *RIP-LCMV-GP* transgenic diabetes model. We find that, the co-injection of tryptophan, curcumin, or quercetin together with immunization with mature bone marrow-derived dendritic cells carrying self-antigen, increased the incidence of diabetes induced in *RIP-GP/P14 TCR* transgenic mice. In contrast, co-injection with the AhR-ligands FICZ and I3C failed to stimulate diabetes. The activation of diabetes following injection of tryptophan, curcumin, or quercetin, correlated with their ability to increase IL-17+CD8+ (Tc17) and IFN-γ+CD8+ (Tc1) cell populations, *in vivo*. Furthermore, *in vitro* experiments showed that tryptophan, curcumin, or quercetin treatments directly promoted significantly increased Tc1 and Tc17 cell development, whereas FICZ and I3C treatments directly promoted IL-10+CD8+ T cell development while having no significant effect on either Tc1 or Tc17 subsets. These novel findings indicate that some AhR-ligands can increase CD8+ T cell-mediated autoimmune responses by skewing T cell polarization towards effector subsets.

Keywords- Autoimmunity, Transgenic Mice, Curcumin, Tryptophan, Quercetin

Citation: Abu-Rezq H.A. and Millar D.G. (2013) Aryl Hydrocarbon Receptor Ligands Stimulate Auto-Reactive Tc1/Tc17 T Cell Activation and Enhance Self-antigen-Induced Diabetes. International Journal of Immunology Research, ISSN: 0976-4909 & E-ISSN: 0976-4917, Volume 3, Issue 1, pp.-29-39.

Introduction

Accumulating evidence has shown that different AhR-ligands can alter CD4+ T cell polarization, with consequent effects on autoimmune responses [1,2]. However, the effects of these ligands on polarized effector functions of CD8+ T cells have not yet been reported, and potential effects on CD8+ T cell-mediated autoimmune responses remain to be investigated.

CD8+ T cell are known to play critical roles in the development and progression of multiple autoimmune diseases [3]. Multiple sclerosis (MS), characterized by demyelination and chronic inflammation in the central nervous system, is thought to be mediated by CD4+ Th cells and macrophages, but is also characterized by CD8+ T cells reactive against self-antigens [4]. A recent study showed that a high production of IL-17 from both CD4+ and CD8+ cells is found in active MS patients [5]. Furthermore, in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, results have shown that CD8+ T cells can play an active role in disease induction [6,7]. However, other studies have also shown participation of antigen-specific CD8+ Treg cells in the recovery phase and suppression of ongoing EAE [8,9].

Type-1-diabetes (T1D) is another autoimmune condition with a clear role of auto-reactive CD8+ T cells in the induction of disease. T1D, characterized by destruction of pancreatic islet β-cells, was found to be associated with CD8+ CTL (also referred to as Tc cells) reactive against β-cell antigens [10]. CD8+ T cells reactive against insulin B chain were shown to be responsible for β-cell destruction in T1D islet transplant patients [11]. CD8+ T cells have been shown to infiltrate islets in recently diagnosed T1D patients [12] and both CD4+ and CD8+ peripheral blood lymphocytes from newly diagnosed T1D patients secrete elevated levels of IL-17 [13]. In the NOD mouse model of T1D, CD8+ and CD4+ T cells are required for diabetes, and diabetogenic islet antigen-specific CD8+ and CD4+ T cell clones have been identified [14].

CD4+ Th cells have the ability to develop into polarized subsets, including Th1, Th2, Th17 and Treg, depending on the cytokines in the surrounding environment, such as TGF-β, IL-12, IL-4, IL-6, and

IL-23 [15]. CD8+ T cells are also known to differentiate into different effector subsets [16]. Type 1 CD8+ cytotoxic T cells (Tc1) are characterized by the secretion of high amounts of both IFN-γ and IL-2. Type 2 CD8+ cytotoxic T cells (Tc2) are capable of secreting other cytokines, such as IL-4, IL-5 and IL-10. Natural and induced suppressive CD8+ cells have been described previously [17,18]. Natural CD8+ Treg were found in both human and mice and express both CD25 and Foxp3 [19]. Adaptive CD8+ Treg can express a variety of markers and effector molecules including Foxp3, CD25, CD28, CTLA-4, GITR, CD122, IL-10 and TGF-β [18].

Recently, the plasticity of CD8+ T cell subsets and their differentiation into other cytokine-producing phenotypes has begun to be investigated. CD8+ T cells producing IL-17 (Tc17), were produced in the same polarizing conditions used to produce Th17 cells, *in vivo* and *in vitro* [20]. STAT-3 and ROR-γt transcription factors were demonstrated to be important for the production of Tc17 cells [21]. It was reported that the Tc17 cells expressed reduced granzyme-B and perforin, compared to Tc1 cells, and had reduced ability to mediate cell lyses, *in vitro*. The stability of Tc17 and Tc1 subsets was also tested by transferring both sub-populations into hemagglutinin transgenic (Tg) mice. Production of IL-17 by Tc17 cells was stable; however these cells acquired additional production of IFN-γ and lead to immune pathology [20]. This suggests that IL-17 secreting Tc17 cells generated, *in vitro* may convert to IFN-γ-producing cells in certain inflammatory cytokine environments. In another study investigating an adoptive-transfer model of diabetes, Tc17 cells were demonstrated to express perforin and granzyme B and to have cytolytic activity, and after exposure to IL-23 these cells could induce diabetes upon transfer into target antigen Tg mice [22].

Few studies have examined the effects of AhR-ligands on CD8+ T cell polarization. 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), a highly potent ligand for AhR, was reported to increase the suppressive activity of CD25+CD4+Treg and CD25+CD8+Treg populations [23]. The effect on Treg activity was mediated by AhR in both CD4+ and CD8+ subsets and was characterized by expression of CD25, CTLA -4, CD28 and GITR, as well as transcription of IL-10 and TGF-β genes. These findings suggest that signaling of AhR in CD8+ T cells can influence their polarization towards a regulatory phenotype.

However, AhR-dependent effects of dioxin and other ligands have been demonstrated in non-T cells, including dendritic cells (DC) and macrophages [24]. AhR-ligands have been reported to either suppress or enhance DC maturation and function [25,26] (Abu-Rezq & Millar, manuscript in preparation), and were found to promote IDO expression and Treg development [27,28]. Thus, indirect effects of AhR-ligands on cells such as DC can alter production of inflammatory or regulatory cytokines and other regulatory enzymes that might also alter differentiation of regulatory or effector CD8+ T cell subsets [29].

We have investigated direct and indirect effects of AhR-ligands on the differentiation of CD8+ T cells to different subsets and on the induction of autoimmunity using the *RIP-GP/P14 TCR* double Tg mouse model of diabetes. These mice express lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) in the pancreatic β-cells and express a TCR specific for the immuno-dominant epitope of LCMV-GP (gp33-41) on CD8+ T cells [30]. We find that co-administration of the AhR-ligands quercetin, curcumin, or the ligand precursor tryptophan, but not FICZ or I3C, together with LPS-pretreated and self-antigen pulsed bone marrow-derived dendritic cells (BMDC) resulted in enhanced autoimmune diabetes. The ligands that enhanced diabetes also increased Tc1 and Tc17 responses, *in vitro* and *in vivo*, while ligands that failed to stimulate diabetes were less able to increase Tc1 and Tc17 populations, but did increase the IL-10+CD8+ population. Our findings demonstrate novel effects of AhR-ligands on Tc1, Tc17 and IL-10+CD8+ T cell responses that can influence induction of autoimmunity in response to DC vaccination.

Materials and Methods

Animals

RIP-GP/P14 TCR is a double transgenic mouse model of autoimmune diabetes bred from previously described RIP-GP and P14 TCR strains on the C57Bl/6J background for more than 10 generations [30,31]. Adult *RIP-GP/P14 TCR* mice were used for *in vivo* and *in vitro* experiments. *C57Bl/6J* mice were used for BMDC preparations. 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-formylindolo (3, 2-*b*) carbazole (FICZ) was purchased from Enzo Life Science (UK). Indole-3-carbinol (I3C), curcumin, quercetin, tryptophan, neutral buffered formalin solution (NBF), ethoxyresorufin, salicylamide, tween-20, 2-ME, streptozotocin (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 (Germany). Tetra-methyl-benzidine (TMB) was purchased from Alpha Labs. WST-1 viability reagent was purchased from Roche. Peptide gp33-41 (KAVYNNFATM) was synthesized by Sigma-Genosys. Blood glucose level was measured by using an Accu-Chek Advantage glucose meter with Accu-Chek Advantage II test strips (Roche). Saponin was purchased from Fluka (Switzerland). RPMI, HBSS, L-glutamine, penicillin, streptomycin, and FBS were purchased from GIBCO, Invitrogen (UK).

CD11c-depleted Spleen Cell Stimulation with AhR-ligand Pretreated BMDC

Total spleen cells from *RIP-GP/P14 TCR Tg* mice were depleted of CD11c+ cells using anti-CD11c-microbeads followed by AutoMACS separation. Cell separation was carried on according to the manufacturer's instructions (Miltenyi). Murine BMDC were prepared as described previously [32]. On day 6 of development, BMDC were stimulated overnight with the following AhR-ligands; 100nM FICZ, 10nM I3C, 100μM tryptophan, 10μM curcumin, or 10μM quercetin. Treated BMDC ($3x10^4$ cells per well) were mixed with CD11c-depleted spleen cells ($2x10^5$ cells per well) and stimulated with 30-100ng/ml LPS and $1x10^{-9}$ M gp33-41 peptide 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 4hrs with 25ng/ml PMA and 25ng/ml ionomycin in the presence of 1μg/ml brefeldin A and 5μM monensin.

Spleen Cell Stimulation in the Presence of Different AhR-ligands

CD11c-depleted spleen cells ($2x10^5$ cells per well) were co-cultured with immature BMDC ($3x10^4$ cells per well) in 96-well plate and the following AhR-ligands; 100nM FICZ, 10nM I3C, 100μM tryptophan, 1μM curcumin, or 1μM quercetin. The cultures were additionally

stimulated with 30-100ng/ml LPS and $1x10^{-9}$ M gp33-41 peptide for 4 days at 37°C, 5% CO_2, 95% humidity. On day 4, the media was removed, fresh media added, and the cultures rested for 2 days. Primed T cells were then re-stimulated for 4hrs with 25ng/ml PMA and 25ng/ml ionomycin in the presence of 1µg/ml brefeldin A and 5µM monensin.

Flow Cytometry

Re-stimulated T cells were stained with anti-CD8-APC or anti-CD8-PE antibody in FACS buffer (PBS, 2% FBS-heat inactivated, 0.1% NaN_3) for 30min at 4°C. Cells were washed with FACS buffer, then fixed and permeabilized with Foxp3 fixation/permeabilization buffer (eBioscience) for 15min 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, anti-IFN-γ-AlexaFluor-647 or anti-IL-10-PE 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 100µl NBF, and fluorescence data acquired using a FACSArray Flow Cytometer (BD Bioscience) and obtained data were analyzed using FlowJo software (Tree Star, USA).

EROD Assay

Cytochrome P450 induction, measured by CYP1A1-assoicated ethoxy-resorufin-*O*-de-ethylase (EROD) assay, was performed according to a previously established method [33]. Briefly, total spleen cells from RIP-GP/P14 TCR Tg mice were prepared from control and AhR-ligand-treated mice. Spleen cells at $2x10^5$ cells per well were transferred to a 96-well plate and incubated with 500µM ethoxy-resorufin and 150mM salicylamide at 37°C, 5% CO_2, 95% humidity for 2 hours, and the EROD activity was measured spectrofluorimetrically using excitation 530nm and emission 590nm, at various time points.

Induction of Diabetes Autoimmune Disease in RIP-GP/P14 TCR Tg Mice

Method 1: BMDC on day 5 were incubated overnight with 200ng/ml LPS and AhR-ligands. The following day, BMDC were centrifuged at 400x*g* for 5min, supernatants were discarded, and pellets were re-suspended in HBSS at $1x10^7$cell/ml, cells were pulsed with $1x10^{-7}$ M gp33-41 peptide and incubated for 90min at 37°C, 5% CO_2, 95% humidity. Mice received 300µl of AhR-ligand-treated BMDC ($3x10^6$ cells s.c.) as described in figure legends. BMDC treatments and injections were repeated every other day for a total of 3 injections.

Method 2: BMDC on day 5 were matured with 200ng/ml LPS overnight. The following day, BMDC were centrifuged at 400x*g* for 5min, supernatants were discarded, and pellets were re-suspended in HBSS at $1x10^7$cell/ml and pulsed with $1x10^{-7}$ M gp33-41 peptide. All mice received 300µl of $3x10^6$ BMDC s.c. with a second i.p injection with either 200µl HBSS as a control or different AhR-ligands as described in figure legends. BMDC treatments and injections were repeated every other day for a total of 3 injections.

In both methods, pre-glucose measurements were taken to confirm healthy, non-diabetic animals (glucose between 5-12mM). Blood glucose level 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.

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. Significant differences between Kaplan-Meier incidence curves were determined by the log-rank test.

Results

Development of Tc1, Tc17, and IL-10+CD8+ T Cells in Response to AhR-ligands-pre-treated BMDC, *in vitro*

In order to investigate the development of different CD8+ T cell subsets, *in vitro*, we used the stimulation of CD11c-depleted spleen cells from *P14 TCR Tg* mice, activated with epitope peptide gp33-41, LPS and additional BMDC, which was previously determined to give a BMDC-dependent increase in IFN-γ-producing Th1 cells. BMDC were additionally pre-treated with different AhR-ligands to assess their ability to alter the differentiation of different CD8+ subsets.

Cultures of P14 TCR T cells incubated in the presence of BMDC and gp33-41 peptide and LPS alone generated few IFN-γ+CD8+ Tc1-type cells ([Fig-1A] 'Control' i.e. BMDC+LPS). Including BMDC that had been pre-treated with the AhR-ligands FICZ or I3C, had no significant effect on the Tc1 response [Fig-1A], [Fig-1D]. However, including BMDC that had been pre-treated with tryptophan, curcumin, or quercetin significantly (*** $p<0.001$) increased the induction of IFN-γ+CD8+ T cells compared to Control [Fig-1A], [Fig-1D]. The effect of BMDC treated with different AhR-ligands on stimulation of a Tc17 phenotype was also examined. The exposure of BMDC to FICZ or I3C did not alter the development of IL-17+CD8+ T cells, compared to control [Fig-1B], [Fig-1E]. However, including BMDC that had been pre-treated with tryptophan, curcumin, or quercetin significantly (** $p<0.01$) increased the induction of IL-17+CD8+ T cells compared to control [Fig-1B], [Fig-1E]. Also, the effect of the ligands on the production of IL-10+CD8+ T cells was investigated. Cultures of control BMDC with P14 TCR T cells generated few IL-10-expressing cells ([Fig-1C] 'Control' i.e. BMDC+LPS'). FICZ or I3C-pre-treated BMDC caused a significant (*** $p<0.001$) increase in IL-10+CD8+T cells [Fig-1C], [Fig-1F]. Tryptophan, curcumin, or quercetin pre-treatment had no significant effect on IL-10 expression [Fig-1C], [Fig-1F]. Overall these results suggest that some AhR-ligands have effects on BMDC that enhance Tc1 and Tc17 development, while other AhR-ligands (FICZ and I3C) promote potentially regulatory IL-10+CD8+ T cells without affecting Tc1 and Tc17 development.

Efficacy of BMDC Immunization is not Increased by pre-treatment with AhR-ligands

Since we and others have found that AhR-ligands can promote pro-inflammatory cytokine production from BMDC [26], and with the results shown in [Fig-1], we hypothesized that BMDC exposed to some of the AhR-ligands might be more immuno-stimulatory and be able to activate auto-reactive T cells, resulting in autoimmunity. To test whether AhR-ligand-treated BMDC could promote activation of autoimmune diabetes in *RIP-GP / P14 TCR Tg* mice, we immunized mice with self-antigen peptide-loaded BMDC stimulated with LPS and different AhR-ligands. The incidence of diabetes in the control group was low, indicating that LPS-matured BMDC carrying self-

antigen is poor stimulators of autoimmunity [Fig-2A]. Pre-treatment of BMDC with FICZ or I3C did not significantly increase the incidence of diabetes [Fig-2A]. The incidence of diabetes after injection of curcumin or tryptophan pre-treated BMDC was not significantly different than control [Fig-2B]. Thus, although AhR-ligands pre-treatment of BMDC can alter their ability to cause effector CD8+ T cells polarization, *in vitro*, this effect did not significantly contribute to enhanced autoimmune effects, *in vivo*.

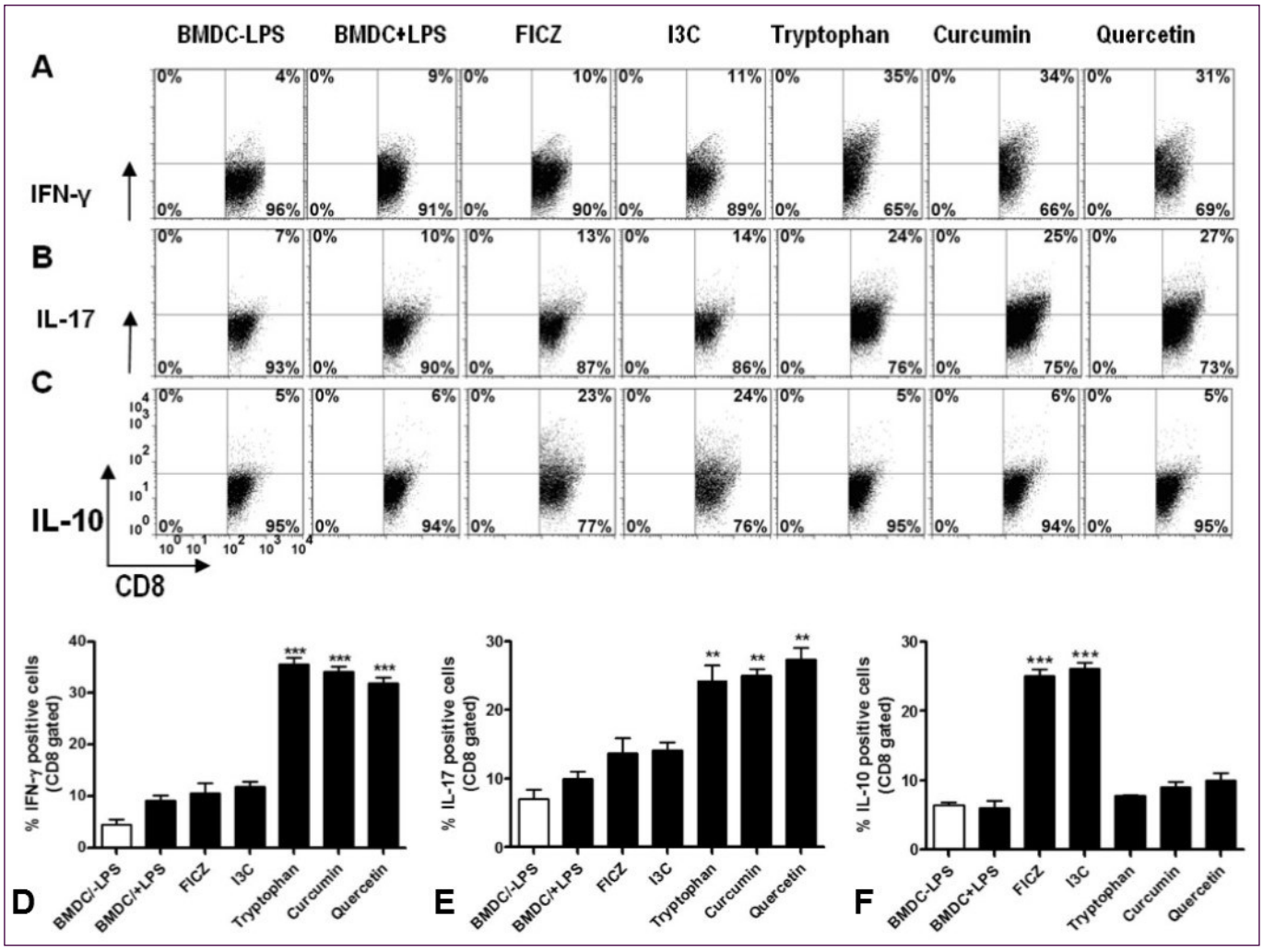

Fig. 1- Pre-treatment of BMDC with Different AhR-ligands Enhances the Development of Tc1 and Tc17, or IL-10+CD8+ Subsets, *in vitro*. Immature BMDC were pre-treated overnight with AhR-ligands (FICZ (100nM), I3C (10nM), tryptophan (100μM), curcumin (10μM), or quercetin (10μM)), then washed and mixed with CD11c-depleted spleen cells from RIP-GP/P14 TCR Tg mice. Co-cultures were stimulated with 1x10-9 M gp33-41 peptide and LPS for 4 days. Following rest in fresh media and re-stimulation, samples were stained for CD8+ and cytokine-positive populations. **(1A)** Flow cytometry dot plots of cultures double stained for IFN-γ and CD8. **(1B)** Dot plots of cultures double stained for IL-17 and CD8. **(1C)** Dot plots of cultures double stained for IL-10 and CD8. **(1D)** Mean production of Tc1 cells. Significant differences were observed after tryptophan, curcumin, or qurecetin treatment compared to control (*** p<0.001). **(1E)** Mean production of Tc17 cells. Tryptophan, curcumin, and quercetin significantly (** p<0.01) increased the Tc17 population. **(1F)** Mean production of IL-10+CD8+ T cells. FICZ and I3C both significantly (*** p<0.001) increased the population compared to control. Representative cytometry data from 2 separate experiments are shown (n=4). Mean value were plotted and significant differences were determined by ANOVA.

Direct Exposure of CD8+ Spleen Cells to Some AhR-ligands Induces Tc1, Tc17, and IL-10+CD8+ T Cells, *in vitro*

The ability of the AhR-ligands to directly stimulate T cell subset development, *in vitro*, was then examined. To promote differentiation of T cells, CD11c-depleted spleen cells from *P14 TCR Tg* mice were stimulated in the presence of control BMDC and different AhR -ligands together with LPS and gp33-41 peptide. Following rest in fresh media and re-stimulation, cytokine-positive CD8+ populations were identified [Fig-3].

As shown previously in [Fig-1], culture of P14 TCR T cells incubated in the presence of BMDC and gp33-41 peptide and LPS alone generated few IFN-γ+CD8+ Tc1-type cells ([Fig-3A] 'Control i.e. BMDC+LPS'). Including the AhR-ligands FICZ or I3C during T cell stimulation, had no significant effect of the Tc1 response [Fig-3A], [Fig-3D]. However, including tryptophan, curcumin, or quercetin in the cultures significantly (*** p<0.001) increased the induction of IFN-γ+ CD8+ T cells compared to the control [Fig-3A], [Fig-3D]. Similarly, culture of P14 TCR T cells incubated in the presence of BMDC and gp33-41 peptide and LPS alone generated few IL-17+CD8+ Tc17-type cells ([Fig-3B] 'Control i.e. BMDC+LPS'). Including the AhR-ligands FICZ or I3C did not alter the development of IL-17+CD8+ T cells, compared to control [Fig-3B], [Fig-3E]. How-

ever, including tryptophan, curcumin, or quercetin significantly (** p<0.01) increased the induction of IL-17+CD8+ T cells compared to the control [Fig-3B], [Fig-3E]. In contrast, inclusion of FICZ or I3C during T cell stimulation caused a significant (** p<0.01, * p<0.05, respectively) increase in IL-10+CD8+ T cells compared to the control [Fig-3C], [Fig-3F]. However, adding tryptophan, curcumin, or quercetin to the culture had no significant effect on IL-10 expression [Fig-3C], [Fig-3F]. Overall these results suggest that some of the AhR-ligands (tryptophan, curcumin, and quercetin) directly enhance Tc1 and Tc17 differentiation, while FICZ and I3C can skew differentiation towards an IL-10+CD8+ T cells phenotype rather than Tc1 and Tc17 subsets.

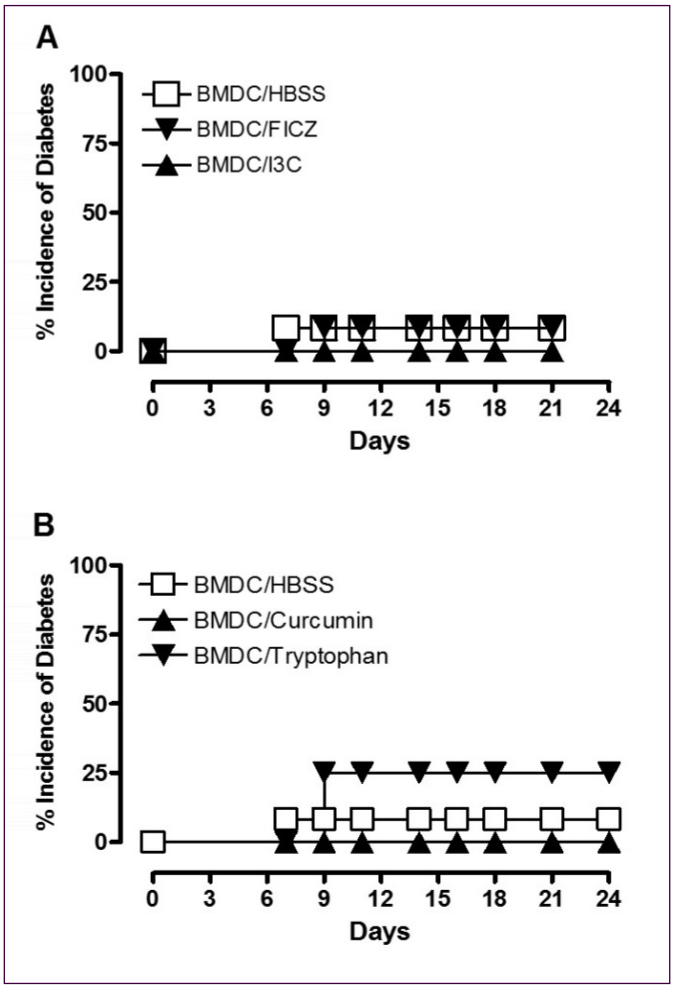

Fig. 2- RIP-GP/P14 TCR Tg mice Injected with AhR-ligands Pre-treated BMDC have no Significant Effect on the Incidence of Diabetes

Immature BMDC were pre-treated overnight with 200ng/ml LPS and AhR-ligands (FICZ (100nM), I3C (10nM), curcumin (10μM), or tryptophan (100μM)) or HBSS buffer alone, then washed and pulsed with $1x10^{-7}$ M gp33-41 peptide. RIP-GP/P14 TCR Tg mice were then injected three times, every other day, with the different BMDC preparations. **(2A)** The incidence of diabetes in the control group was 8.3%, and the incidence of diabetes using FICZ or I3C pre-treated BMDC was not significantly different. **(2B)** The incidence of diabetes using tryptophan pre-treated BMDC increased to 25% while injection of curcumin pre-treated BMDC gave 0% incidence. Data are from 3 experiments with n=12 mice. Differences between Kaplan-Meier incidence curves were determined by log-rank test.

Efficacy of BMDC Immunization is Increased by co-administration of Tryptophan, Quercetin or Curcumin

To determine whether a direct effect of the AhR-ligands on T cells would alter the ability of LPS-matured BMDC to stimulate autoimmune responses, we co-administered AhR-ligands during BMDC immunization of Tg mice. BMDC were matured with LPS alone overnight, pulsed with the peptide gp33-41 the next day, and then administered s.c. to *RIP-GP/P14 TCR Tg* mice, along with a second i.p injection of AhR-ligands. As shown in [Fig-4A], co-administration of BMDC with either FICZ or I3C did not significantly increase the incidence of diabetes compare to control buffer co-injection. In contrast, co-administration of BMDC with tryptophan resulted in a significantly (p<0.01) increased incidence of diabetes [Fig-4B]. Similarly, co-administration of curcumin also resulted in an increase in the incidence of diabetes; however this did not achieve statistical significance [Fig-4C]. Co-administration of BMDC with quercetin resulted in a significantly (p<0.01) increased incidence of diabetes, similar to tryptophan [Fig-4D]. These results suggest that FICZ and I3C are unable to increase a diabetogenic immune response induced by LPS-matured, self-antigen-pulsed BMDC, while co-injection with tryptophan, curcumin and quercetin can enhance development of diabetes. These effects are also in agreement with previous results which showed that co-injection of the AhR-ligands tryptophan, curcumin, or quercetin increased the incidence of diabetes induced by multiple low dose streptozotocins.

Induction of Cytochrome P450 Expression, By specific AhR-ligands, *in vivo*

Next, we confirmed that all the AhR-ligands used were capable of activating the AhR transcription factor in *P14 TCR Tg* mice. Cytochrome P450 1A1, controlled by AhR-dependent dioxin-response element, can be fluorometrically measured using the EROD assay [33]. Injection of *P14 TCR Tg* mice with FICZ, I3C, tryptophan, curcumin, or quercetin on day 0 lead to a strong increase in cytochrome P450-1A1-dependent EROD activity on day 2 in isolated spleen cells, compared to buffer injection alone [Fig-5A], [Fig-5D].Therefore, these compounds are effective in TCR Tg mice at activating AhR-dependent gene expression.

Administration of Different AhR-ligands Alters CD8+ T cell Subsets, *in vivo*

To assess the direct of effects of AhR-ligands on Tc1 and Tc17 populations, *in vivo*, *RIP-GP/P14 TCR Tg* mice were injected with different AhR-ligands and two days later, total spleen cells were re-stimulated and stained for IL-17+CD8+ and IFN-γ+CD8+ cells. Mice injected with FICZ, I3C, tryptophan, curcumin or quercetin showed increased populations of IFN-γ+CD8+ T cells compared to control mice that received buffer alone [Fig-6A]. However, tryptophan, curcumin and quercetin increased the Tc1 population significantly more than FICZ and I3C (p<0.001). Injection of FICZ and I3C increased significantly the Tc17 population [Fig-6B]. However, the AhR-ligands tryptophan, curcumin, and quercetin, were found to significantly (p<0.001) increase IL-17+CD8+ T cells compared to control, and either FICZ or I3C injections [Fig-6B]. These results demonstrate that tryptophan, curcumin, and quercetin, can promote enhanced effector T cell development (Tc1 and Tc17), whereas FICZ and I3C have also promote Tc1 and Tc17 cells, *in vivo*, but to a lesser degree.

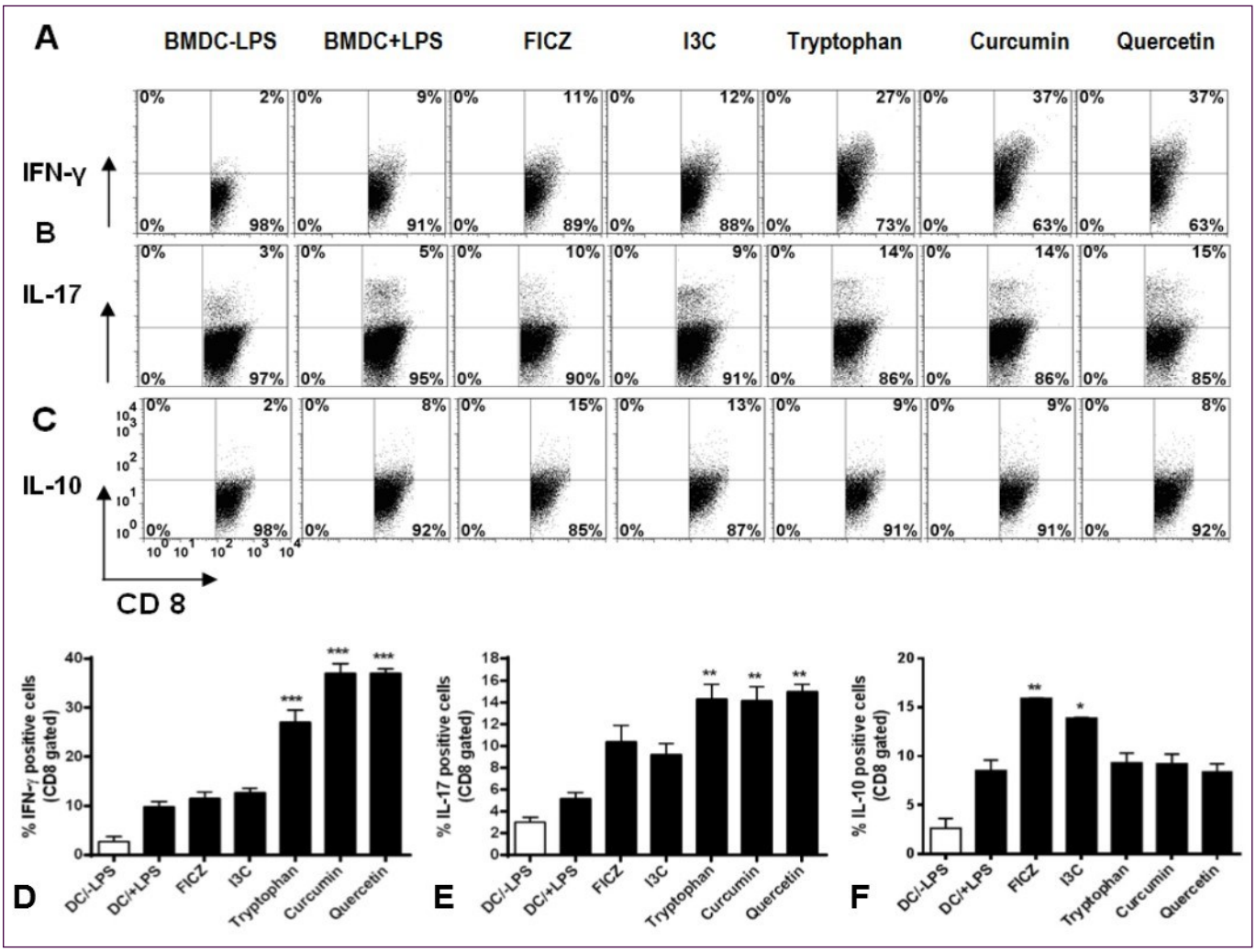

Fig. 3- Some AhR-ligands Directly Enhance Development of Tc1 and Tc17 Populations, but have Differing Abilities to promote an IL-10+CD8+ subset, *in vitro*.

Spleen cells from RIP-GP/P14 TCR Tg mice were depleted of CD11c+ cells and stimulated with untreated BMDC, $1x10^{-9}$ gp33-41 peptide, and LPS for 4 days in the presence of different AhR-ligands (FICZ (100nM), I3C (10nM), tryptophan (100μM), curcumin (1μM), or quercetin (1μM)). Following rest in fresh media and re-stimulation, samples were stained for CD8+ and cytokine-positive populations. **(3A)** Flow cytometry dot plots of cultures double stained for IFN-γ and CD8. **(3B)** Dot plots of cultures double stained for IL-17 and CD8. **(3C)** Dot plots of cultures double stained for IL-10 and CD8. **(3D)** Mean production of Tc1 cells. Significant differences were observed after tryptophan, curcumin, or qurecetin treatment compared to control (*** $p<0.001$). **(3E)** Mean production of Tc17 cells. Tryptophan, curcumin, and quercetin significantly (** $p<0.01$) increased the Tc17 population. **(3F)** Mean production of IL-10+CD8+ T cells. FICZ and I3C both significantly (** $p<0.01$, *$p<0.05$, respectively) increased the population compared to control. Representative cytometry data from 2 separate experiments are shown (n=4). Mean value were plotted and significant differences were determined by ANOVA.

Discussion

In the present study, the effects of different AhR-ligands on the induction of autoimmune diabetes in the *RIP-GP/P14 TCR Tg* mouse model were investigated for the first time. Our results show that some AhR-ligands can increase the occurrence of diabetes during immunization with BMDC carrying self-Ag. We have shown that co-administration of self-antigen-pulsed-BMDC with tryptophan, curcumin, or quercetin increased the incidence of diabetes, and the results were correlated with an increase in IL-17+CD8+ Tc17 cells and IFN-γ+CD8+ Tc1 cell populations, *in vivo*. On the other hand, other AhR-ligands including FICZ and I3C, were not able to increase the incidence of diabetes and these AhR-ligands had a reduced ability to generate Tc17 and Tc1 populations, and had the additional ability to increase the induction of IL-10+CD8+ T cells, *in vivo*. Therefore, the main difference between the two groups of ligands associated with activation of diabetes is the extent to which they skew towards Tc1/Tc17 cell development rather than towards IL-10+CD8+ T cells.

Th17 cells have been widely suggested to be involved in autoimmune diseases [34]. IL-17A is known to enhance other inflammatory cytokines and chemokines and promote recruitment of neutrophils and other immune cells. Neutralization or deficiency of IL-17 has been shown to protect against some models of autoimmunity [34]. The requirement for TGF-β in both Th17 and Treg development is thought to indicate a reciprocal relationship between these two subsets, whose balance determines the extent of immune activation or tolerance. Previously, we have reported that suppression or enhancement of streptozotocin-induced diabetes depended on

the development of Treg or Th17 responses, and these responses were modulated by co-administration of AhR-ligands. Similar findings were reported for the AhR-ligands TCDD and FICZ, which had different abilities to promote Treg or Th17 responses and consequent effects on EAE [1,2]. The main sources of IL-17 have been presumed to be Th17 cells, as well as γδ-T cells and innate immune cell types [35,36]. However, CD8+ T cells can also develop into an IL-17 producing Tc17 phenotype.

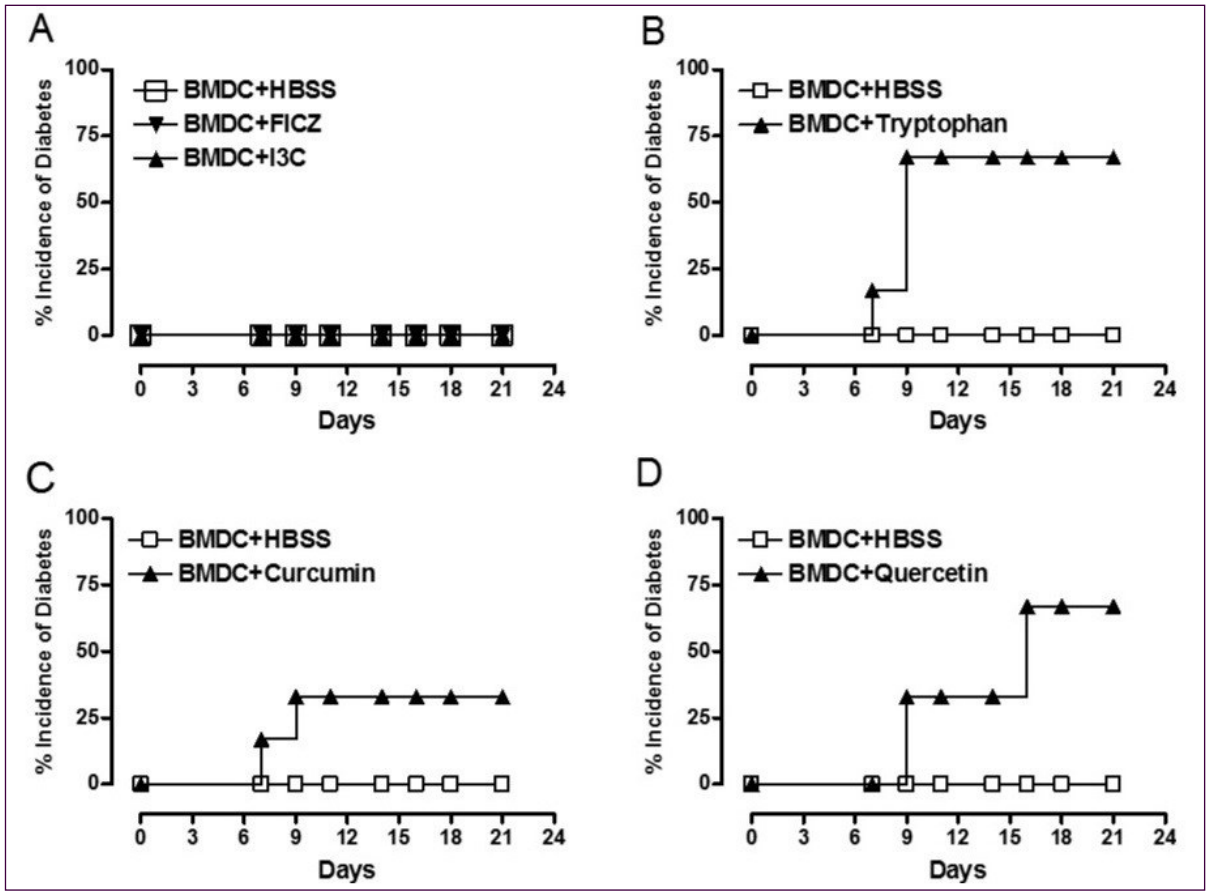

Fig. 4- Co-administration of Some AhR-Ligands and LPS-Matured BMDC Increases the Incidence of Diabetes

RIP-GP / P14 TCR Tg mice were injected s.c. three times every other day with LPS-matured BMDC pulsed with gp33-41 peptide, and a second i.p. injection with either HBSS or one of the following AhR-ligands: FICZ (2nmol/kg), I3C (2nmol/kg), tryptophan (20μmol/kg), curcumin (2μmol/kg), or quercetin (2μmol/kg). **(4A)** The incidence of diabetes in the control group was 0%, and the incidence of diabetes with co-injection of FICZ or I3C was not significantly different. **(4B)** The incidence of diabetes with tryptophan co-injection increased significantly ($p<0.05$) to 67%. **(4C)** Co-injection of curcumin increased the incidence to 33%. **(4D)** Quercetin co-injection significantly ($p<0.05$) increased the incidence of diabetes to 67%. Data from 2 experiments with n=6 mice per group are shown. Differences between Kaplan-Meier incidence curves were determined by log-rank test.

The role of IL-17 secretion from CD8+ T cells in autoimmune conditions has not yet been investigated. One would expect that IL-17 secreted from CD8+ cells would have the same effect on inflammation and cell recruitment as Th17 cells. In conditions involving MHC class I-restricted self-antigen exposure, initial stimulation of CD8+ T cells in a TGF-β and IL-6 rich environment may produce Tc17 cells that promote autoimmunity. Self-antigen and TCR Tg models of CD8+ T cell-mediated diabetes have shown that Tc1-type CTL responses, involving perforin and IFN-γ, are important for autoimmune β-cell destruction [37,38]. However, recent characterization of Tc17 CD8+ cell phenotype and function has shown that this subset can express perforin and granzyme B, have cytolytic activity, and mediate autoimmune destruction [22]. Tc17 cells, like Th17 cells, can acquire expression of IFN-γ, and lead to additional immune pathology [20,39]. The cytokine IL-23 may be decisive in allowing Th17 and Tc17 cells to acquire IFN-γ production and for executing a pathogenic autoimmune function. The combined effects of Tc17 cells with IFN-γ-secretion on recruitment of cells and maintaining inflammation, together with unmasking of β-cell self antigens, may provide the necessary conditions for complete immune-mediated β-cell destruction.

A previous study investigating the effects of TCDD on CD8+ T cell polarization showed induction of both CD25+CD4+ Treg and CD25+CD8+ Treg population with the ability to suppress allogeneic immune responses [23]. Both Treg subsets were characterized by high expression of CD25, CTLA-4, CD28 and GITR, and the ability to secrete IL-10 and TGF-β cytokines. Our study has revealed multiple effects of different AhR-ligands on CD8+ T cell populations and the consequences for autoimmune destruction. The effects of FICZ and I3C included expansion of an IL-10+CD8+ T cell population, *in vivo*, together with a small increase in Tc1 and Tc17 cells, which failed to enhance induction of diabetes [Fig-4]. However, tryptophan, curcumin, and quercetin, strongly enhanced Tc1 and Tc17 cells without increasing IL-10+CD8+ cells, and their co-administration with BMDC vaccination, increased diabetes. This

suggests that FICZ and I3C may resemble TCDD in promoting suppressive T cell functions, but highlights the different abilities of some AhR-ligands to influence effector CD8+ T cell subset differentiation.

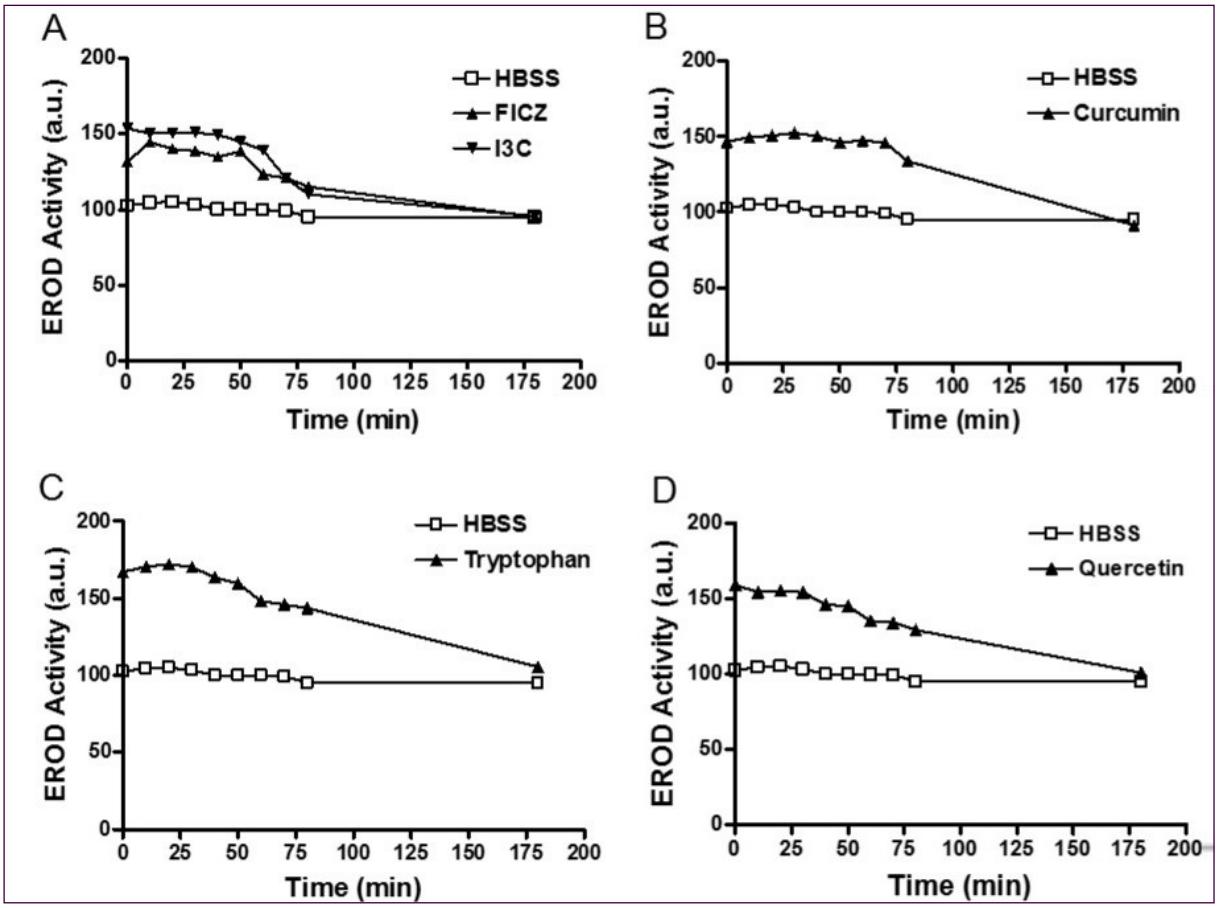

Fig. 5- Injection of AhR-ligands Induces Expression of Cytochrome P450, in Spleen Cells

RIP-GP/P14 TCR Tg mice were injected with different AhR-ligands and two days later total spleen cells were assayed for EROD activity over time. Control mice received HBSS alone. **(5A)** FICZ (2nmol/kg) increased significantly EROD activity as compared to control group (p<0.0001). I3C (2nmol/kg) increased significantly EROD activity as compared to control group (p<0.0001). **(5B)** Curcumin (2µmol/kg) increased significantly EROD activity as compared to control group (p<0.0001). **(5C)** Tryptophan (20µmol/kg) increased significantly EROD activity as compared to control group (p<0.0001). **(5D)** Quercetin (2µmol/kg) (p<0.0001). Representative data from 2 separate experiments with n=4 mice per group are shown. Mean value were plotted and significant differences were determined by ANOVA.

Several studies have reported contact-dependent suppressive functions of, *in vitro* generated adaptive CD8+ Treg cells. However, naturally-occurring Foxp3+CD8+ T cells isolated *ex vivo*, were described to have a lower suppressive capacity compared to CD4+ nTreg [40] and their role in maintaining tolerance, *in vivo*, has not yet been demonstrated. In contrast, expansion of inducible Foxp3+CD25+CD8+ Treg cells, for example by anti-CD3 treatment, has been reported to enhance tolerance in diabetes patients [41]. Antigen-specific CD8+ T cells have been described, that can suppress and reverse diabetes in NOD mice [42]. These cells expressed CD122 and suppression of diabetes required, a significant suppression in antigen presentation by APC, which could be mediated by different mechanisms including; suppression of the level of IFN-γ secreted, enhancing the expression of IDO, and killing APC via perforin. Other suppressive CD8+ T cell populations expressing CD122 have also been described. A study was made to investigate the role of CD8+CD122+ Treg in EAE. Results showed that depleting CD8+CD122+ Treg cells from *C57Bl/6J* mice by the injection of anti-CD122 mAb, *in vivo*, caused persistent EAE disease and decreased the production of IL-10 significantly, while injecting these mice with CD8+CD122+ cells increased the production of IL-10 significantly and reduced the EAE symptoms [9]. The differentiation of CD8+ T cells to a suppressive phenotype can be achieved by stimulation with IL-4 and IL-12, *in vitro* [43]. The suppressive function of this subset appears to be due to a contact-dependent mechanism, as well as their ability to secrete IL-10. During our studies, in addition to observing altered IL-10+CD8+ development during treatment with some AhR-ligands, *in vitro*, we also noticed a decrease in Foxp3+ expression in *P14 TCR* CD8+ T cells during exposure to the AhR-ligands that enhanced diabetes (data not shown). It will be of interest to investigate whether Foxp3+ P14 TCR T cells or IL-10+ P14 T cells would be able to suppress diabetes in response to immunogenic BMDC or peptide vaccination in future studies.

Enhancement of self-reactive CD8+ cytotoxic T cell activation is an important component of cancer treatment [44]. CD8+ T cells can differentiate into Tc1, which may fight tumors by IFN-γ-dependant mechanisms. CD8+ T cells skewed to produce IL-17 were able to mediate anti-tumor responses, but also converted to IFN-γ-producers, *in vivo* [45]. *In vitro* generated Tc17 cells were reported

to secrete IL-17A, IL-17F, TNF-α, IL-21 and IL-22 and it was shown that transferring OVA-specific Tc17 cells into OVA+ tumor-bearing mice could control tumor growth in early and late stage of melanoma [46]. The Tc17 cells also secreted multiple chemokines causing recruitment of Th1 and Tc1 cells which helped in tumor regression, and IL-17-dependant recruitment of neutrophils further enhanced control of tumor growth [46]. These findings suggest that properties of both Tc17 and Tc1 cells have desirable effects on anti-tumor immunity. However IL-17+CD8+ T cells in hepato-carcinoma patients were found to accumulate in tumors and were associated with disease progression [47] and IL-17 has been reported to promote angiogenesis and tumor development [48,49]. Therefore, while stimulating self-reactive Tc17 cell expansion by administration of AhR-ligands may be beneficial for inducing anti-tumor immunity, caution is needed to avoid tumor promoting effects, as well as inducing potentially damaging autoimmune side effects.

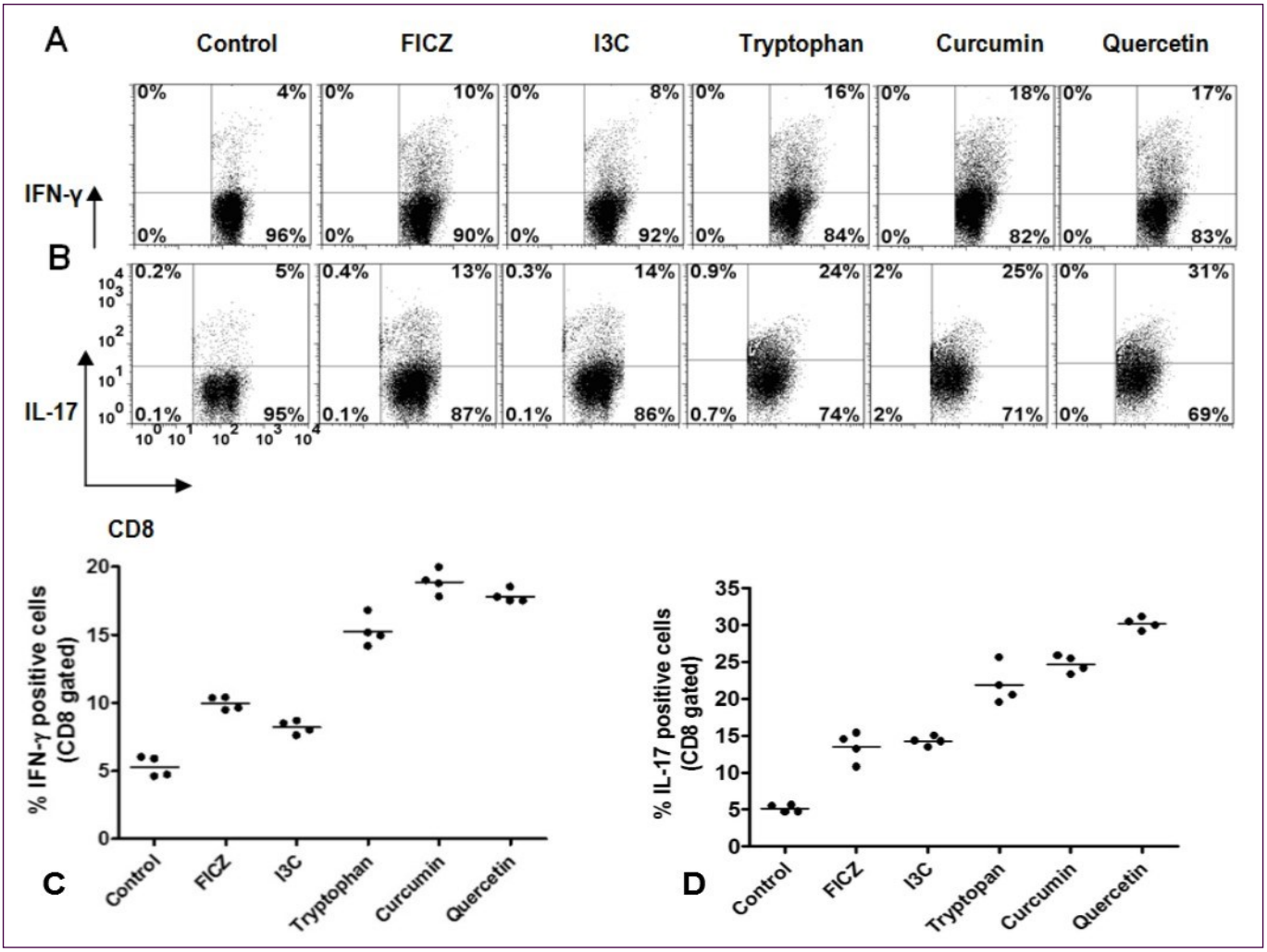

Fig. 6- The Effects of AhR-ligands on T cell Subset Populations, *in vivo*

RIP-GP/P14 TCR Tg mice were injected with different AhR-ligands and two days later, total spleen cells were re-stimulated with PMA/ionomycin and monensin for 4 hours and then stained for CD8 and intracellular IL-17 or IFN-γ. **(6A)** Flow cytometry dot plots of cultures double stained for IFN-γ and CD8. **(6B)** Flow cytometry dot plots of cultures double stained for IL-17 and CD8 **(6C)** Population of IFN-γ+CD8+ Tc1 cells. Tryptophan, curcumin, and quercetin significantly (p<0.001) increased the population of Tc1 compared to the control or FICZ or I3C injections. **(6D)** Population of IL-17+CD8+ Tc17 cells. Tryptophan, curcumin, and quercetin significantly (p<0.001) increased the Tc17 population compared to the control or FICZ or 13C injections. Data shown are representative of 2 experiments, n=4 mice per group. Data for individual mice in each group and the mean value is shown and significant differences were determined by ANOVA.

Acknowledgement

This study was supported by a New Investigator Award from Medical Research Council (UK) and a Biotechnology and Biological Sciences Research Council (BBSRC) Project Grant (to D.G.M) and PhD Studentship from the Government of Kuwait

Conflict of Interest: The authors have no conflict of interest to announce.

References

[1] Veldhoen M., Hirota K., Westendorf A.M., Buer J., Dumoutier L., Renauld J.C. and Stockinger B. (2008) *Nature,* 453, 106-109.

[2] Quintana F.J., Basso A.S., Iglesias A.H., Korn T., Farez M.F., Bettelli E., Caccamo M., Oukka M. and Weiner H.L. (2008) *Nature,* 453, 65-71.

[3] Walter U. and Santamaria P. (2005) *Current Opinion in Immunology,* 17, 624-631.

[4] Zang Y.C., Li S., Rivera V.M., Hong J., Robinson R.R., Breitbach W.T., Killian J. and Zhang J.Z. (2004) *The Journal of Immunology,* 172, 5120-5127.

[5] Tzartos J.S., Friese M.A., Craner M.J., Palace J., Newcombe J., Esiri M.M. and Fugger L. (2008) *The American Journal of Pathology,* 172, 146-155.

[6] Sun D., Whitaker J.N., Huang Z., Liu D., Coleclough C., Wekerle H. and Raine C.S. (2001) *The Journal of Immunology,* 166, 7579-7587.

[7] Huseby E.S., Liggitt D., Brabb T., Schnabel B., Ohlen C. and Goverman J. (2001) *The Journal of Experimental Medicine,* 194, 669-676.

[8] Faunce D.E., Terajewicz A. and Stein-Streilein J. (2004) *The Journal of Immunology,* 172, 1991-1995.

[9] Lee Y.H., Ishida Y., Rifa'i M., Shi Z., Isobe K. and Suzuk H.i. (2008) *The Journal of Immunology,* 180, 825-832.

[10] Panina-Bordignon P., Lang R., van Endert P.M., Benazzi E., Felix A.M., Pastore R.M., Spinas G.A. and Sinigaglia F. (1995) *The Journal of Experimental Medicine,* 181, 1923-1927.

[11] Pinkse G.G., Tysma O.H., Bergen C.A., Kester M.G., Ossendorp F., van Veelen P.A., Keymeulen B., Pipeleers D., Drijfhout J.W. and Roep B.O. (2005) *Proceedings of the National Academy of Sciences of the United States of America,* 102, 18425-18430.

[12] Richardson S.J., Willcox A., Bone A.J., Morgan N.G. and Foulis A.K. (2011) *Seminars in Immunopathology,* 33, 9-21.

[13] Marwaha A.K., Crome S.Q., Panagiotopoulos C., Berg K.B., Qin H., Ouyang Q., Xu L., Priatel J.J., Levings M.K. and Tan R. (2010) *The Journal of Immunology,* 185, 3814-3818.

[14] Lieberman S.M., Takaki T., Han B., Santamaria P., Serreze D.V. and DiLorenzo T.P. (2004) *The Journal of Immunology,* 173, 6727-6734.

[15] Murphy K.M. and Stockinger B. (2010) *Nature immunology,* 11, 674-680.

[16] Mosmann T.R., Li L. and Sad S. (1997) *Seminars in Immunology,* 9, 87-92.

[17] Shevach E.M. (2006) *Immunity,* 25, 195-201.

[18] Suzuki M., Konya C., Goronzy J.J. and Weyand C.M. (2008) *Human Immunology,* 69, 781-789.

[19] Cosmi L., Liotta F., Lazzeri E., Francalanci M., Angeli R., Mazzinghi B., Santarlasci V., Manetti R., Vanini V., Romagnani P., Maggi E., Romagnani S. and Annunziato F. (2003) *Blood,* 102, 4107-4114.

[20] Yen H.R., Harris T.J., Wada S., Grosso J.F., Getnet D., Goldberg M.V., Liang K.L., Bruno T.C., Pyle K.J., Chan S.L. anders R.A., Trimble C.L., Adler A.J., Lin T.Y., Pardoll D.M., Huang C.T. and Drake C.G. (2009) *The Journal of Immunology,* 183, 7161-7168.

[21] Huber M., Heink S., Grothe H., Guralnik A., Reinhard K., Elflein K., Hunig T., Mittrucker H.W., Brustle A., Kamradt T. and Lohoff M. (2009) *European Journal of Immunology,* 39, 1716-1725.

[22] Ciric B., El-behi M., Cabrera R., Zhang G.X. and Rostami A. (2009) *The Journal of Immunology,* 182, 5296-5305.

[23] Funatake C.J., Marshall N.B. and Kerkvliet N.I. (2008) *Journal of Immunotoxicology,* 5, 81-91.

[24] Kerkvliet N.I. (2009) *Biochemical Pharmacology,* 77, 746-760.

[25] Platzer B., Richter S., Kneidinger D., Waltenberger D., Woisetschlager M. and Strobl H. (2009) *The Journal of Immunology,* 183, 66-74.

[26] Bankoti J., Rase B., Simones T. and Shepherd D.M. (2010) *Toxicology and Applied Pharmacology,* 246, 18-28.

[27] Vogel C.F., Goth S.R., Dong B., Pessah I.N. and Matsumura F. (2008) *Biochemical and Biophysical Research Communications,* 375, 331-335.

[28] Nguyen N.T., Kimura A., Nakahama T., Chinen I., Masuda K., Nohara K., Fujii-Kuriyama Y. and Kishimoto T. (2010) *Proceedings of the National Academy of Sciences of the United States of America,* 107, 19961-19966.

[29] Hauben E., Gregori S., Draghici E., Migliavacca B., Olivieri S., Woisetschlager M. and Roncarolo M.G. (2008) *Blood,* 112, 1214-1222.

[30] Ohashi P.S., Oehen S., Buerki K., Pircher H., Ohashi C.T., Odermatt B., Malissen B., Zinkernagel R.M. and Hengartner H. (1991) *Cell,* 65, 305-317.

[31] Alam M.U., Harken J.A., Knorn A.M., Elford A.R., Wigmore K., Ohashi P.S. and Millar D.G. (2009) *The Journal of Immunology,* 183, 5728-5737.

[32] Lutz M.B., Kukutsch N., Ogilvie A.L., Rossner S., Koch F., Romani N. and Schuler G. (1999) *The Journal of Immunology Methods,* 223, 77-92.

[33] Kennedy S.W. and Jones S.P. (1994) *Analytical biochemistry,* 222, 217-223.

[34] Hemdan N.Y., Birkenmeier G., Wichmann G., . Abu El-Saad A.M, Krieger T., Conrad K. and Sack U. (2010) *Autoimmunity Reviews,* 9, 785-792.

[35] Roark C.L., Simonian P.L., Fontenot A.P., Born W.K. and O'Brien R.L. (2008) *Current Opinion in Immunology,* 20, 353-357.

[36] Cua D.J. and Tato C.M. (2010) *Nature Reviews,* 10, 479-489.

[37] Kagi D., Odermatt B., Ohashi P.S, Zinkernagel R.M. and Hengartner H. (1996) *The Journal of Experimental Medicine,* 183, 2143-2152.

[38] von Herrath M.G. and Oldstone M.B. (1997) *The Journal of Experimental Medicine,* 185, 531-539.

[39] Hirota K., Duarte J.H., Veldhoen M., Hornsby E., Li Y., Cua D.J., Ahlfors H., Wilhelm C., Tolaini M., Menzel U., Garefalaki A., Potocnik A.J. and Stockinger B. (2011) *Nature Immunology,* 12, 255-263.

[40] Mayer C.T., Floess S., Baru A.M., Lahl K., Huehn J. and Sparwasser T. (2011) *European Journal of Immunology,* 41, 716-725.

[41] Bisikirska B., Colgan J., Luban J., Bluestone J.A. and Herold K.C. (2005) *The Journal of Clinical Investigation,* 115, 2904-2913.

[42] Tsai S., Shameli A., Yamanouchi J., Clemente-Casares X., Wang J., Serra P., Yang Y., Medarova Z., Moore A. and Santamaria P. (2010) *Immunity,* 32, 568-580.

[43] Noble A., Giorgini A. and Leggat J.A. (2006) *Blood,* 107, 4475-4483.

[44] Steer H.J., Lake R.A., Nowak A.K. and Robinson B.W. (2010) *Oncogene,* 29, 6301-6313.

[45] Hinrichs C.S., Kaiser A., Paulos C.M., Cassard L., Sanchez-Perez L., Heemskerk B., Wrzesinski C., Borman Z.A., Muranski P. and Restifo N.P. (2009) *Blood,* 114, 596-599.

[46] Garcia-Hernandez Mde L., Hamada H., Reome J.B., Misra S.K. and Tighe M.P. (2010) *The Journal of Immunology,* 184, 4215-4227.

[47] Kuang D.M., Peng C., Zhao Q., Wu Y., Zhu L.Y., Wang J., Yin X.Y., Li L. and Zheng L. (2010) *The Journal of Immunology,* 185, 1544-1549.

[48] Numasaki M., Fukushi J., Ono M., Narula S.K., Zavodny P.J., Kudo T., Robbins P.D., Tahara H. and Lotze M.T. (2003) *Blood,* 101, 2620-2627.

[49] Wang L., Yi T., Zhang W., Pardoll D.M. and Yu H. (2010) *Cancer Research,* 70, 10112-10120.