The Australian National University

Understanding the Aryl Hydrocarbon Receptor (AhR)-Mediated Generation of the Intraepithelial Lymphocytes.

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

Mature conventional T cells with α and β T-cell receptor (TCR) chains are largely composed of two distinct major subsets: CD4 helper T (Th) cells and CD8 cytotoxic T (Tc) cells. Th cells essentially regulate immune responses by mediating the activation and controlling the function of other immune cell types, whereas Tc cells predominantly confer immune protection by performing cytotoxic functions on infected or cancerous cells. Although the fate to become a Th or Tc lineage is pre-determined during T-cell development in the thymus, recent studies have revealed that, in special cases, mature Th cells, when losing Th lineage-determining transcription factor ThPOK, could upregulate Tc lineage-determining transcription factor Runx3, express CD8 and gain cytotoxic function in the peripheral. The cytotoxic reprogramming of Th cells is particularly observed in a significant population of intraepithelial lymphocytes (IELs). These cells are also shown to suppress intestinal inflammation.

With the emerging evidence highlighting a fundamental role of gut immune homoeostasis for human health, our lab aims to investigate molecular signals that regulate the generation and function of CD8αα^{+}CD4^{+} IELs, particularly those signals with the potentials to be targeted for immunomodulation and immunotherapies. My PhD project focused on aryl hydrocarbon receptor (AhR) as a physiological regulator for CD8αα^{+}CD4^{+} IELs and also TCRαβ^{+}CD8αα^{+} IELs. AhR is an intracellular sensor of aryl hydrocarbons, which are enriched in the mammalian mucosal environment. Our results show that certain AhR ligands induce CD8 expression on both mouse and human CD4 T cells *ex vivo*. Correspondingly, AhR-deficient mice demonstrated a defective generation of CD8αα^{+}CD4^{+} IELs and TCRαβ^{+}CD8αα^{+} IELs.

We further characterised the role of T-cell-specific transcription factor 1 (TCF1) in mediating the regulation of AhR for the generation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs. TCF1 is a key regulator that maintains ThPOK expression while we discovered that AhR through direct binding to the *Tcf7* promoter, inhibits its transcript, leading to the suppression of TCF1 expression in IELs.

My study provides the first detailed mechanism underlying AhR-mediated regulation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs. Such mechanism provides a key piece knowledge to understand how AhR regulates T cell homeostasis in the mucosal environment. Therefore, this new knowledge will pave the way for rationale-based design of immune interventions to improve gut health and treat diseases, such as inflammatory bowel disease.

Declaration

I declare that this thesis entitled, 'Understanding the Aryl Hydrocarbon Receptor (AhR)-Mediated Generation of the Intraepithelial Lymphocytes', contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: ……

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Date: 03/02/2020

Publications

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Contents

CHAPTER I

Introduction

1.1. What is the aryl hydrocarbon receptor (AhR)?

1.1.1. AhR is a bHLH-PAS transcription factor

The aryl hydrocarbon receptor (AhR) is a transcription factor that contains two common domains: bHLH (basic helix-loop-helix) and PAS (period [per]-aryl hydrocarbon receptor nuclear translocator [ARNT]-single minded [SIM]). Through well-conserved bHLH and PAS domains, bHLH-PAS transcription factors heterodimerise to form a functional DNA binding complex to regulate transcription (McIntosh, Hogenesch, & Bradfield, 2010). In the AhR protein, the bHLH domain locates near the amino terminal and performs DNA binding function. The PAS domains, composed of PAS-A and PAS-B, mediate vital interaction with ARNT to co-translocate into the nucleus. The ligand binding docks mainly sit in the PAS-B domains. The chaperone heat shock protein 90 (HSP90) binds to AhR through the region largely overlapping with the ligand binding docks. Such interaction between HSP90 and AhR silences its downstream signals but can be activated upon ligand stimulation. Transcriptional activation binding domain exists close to the carboxyl terminal, which contains a proline-rich (Q-rich) region (Murray, Patterson, & Perdew, 2014; Stockinger, Di Meglio, Gialitakis, & Duarte, 2014). The schematic structure of AhR is depicted in **Figure 1.1A**.

1.1.2. Ligand-induced activation of the AhR pathway

Activation of AhR signalling is primarily driven by the stimulation of its ligands. In steady state, inactivated AhR exists within a cytosolic complex also comprising HSP90, AIP, p23 and actin filament. Upon ligand binding, the AhR complex dissociates so that AhR translocates into the nucleus where another bHLH-PAS transcription factor family member ARNT dimerises with the dissociated AhR to form a functional DNA binding complex. The AhR/ARNT dimer recognises genomic sequences containing AhR-responsive elements

(AhRE, also called Dioxin- or xenobiotic- responsive element, DRE or XRE, core sequence 5'-GCGTG-3' **Figure 1.1B**) to regulate gene transcription (Shen & Whitlock, 1992). The putative AhR-binding sites are mapped across the whole genome (Yao & Denison, 1992). Many studies have demonstrated that AhR can regulate a large number of gene expressions with diversified functions (De Abrew, Kaminski, & Thomas, 2010; Lo & Matthews, 2013). Notably, a well-characterised downstream response of ligand-induced AhR signalling is to trigger the negative feedback loop to limit AhR signal (Bersten, Sullivan, Peet, & Whitelaw, 2013). First, the activation of AhR induces the expression of key metabolizing enzymes, such as CYP1A1 (Cytochrome P450, family 1, subfamily A, polypeptide 1), to bio-transform or eliminate the AhR ligands. Second, the AhR-induced AhR repressor (AhRR) is also upregulated to compete with AhR for the dimerization of ARNT, thus reducing the amount of activated AhR/ARNT dimer. Third, ligand activated AhR is exported from the nucleus and degraded by the ubiquitin/proteasome pathway. A simplified model of ligand-induced AhR activation is shown in **Figure 1.1C**.

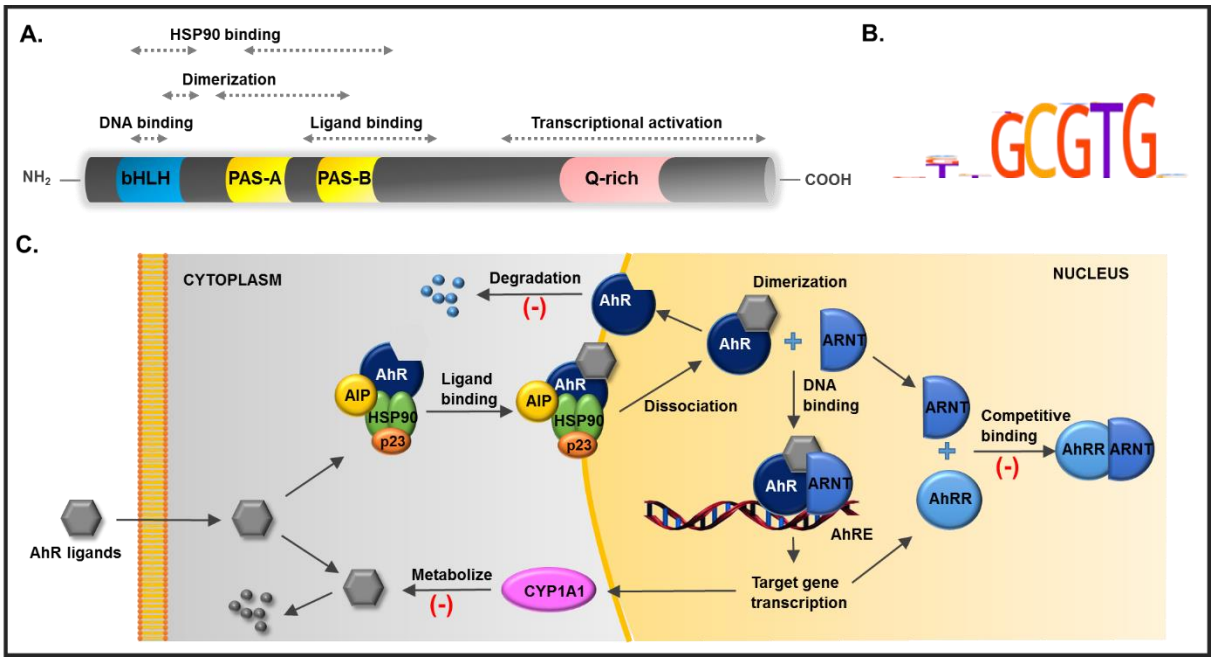


Figure 1.1, the regulation of the AhR activation. ***A)*** *Functional domains of the AhR protein. AhR includes three major functional domains: bHLH (basic helix-loop-helix), PAS (PER-ARNT-SER) and Q-rich (proline rich). The bHLH domain performs DNA binding and dimerization, while the two PAS domains participate in dimerization and ligand binding. Both the bHLH and PAS are required for HSP90 interaction. Q-rich domain contributes to transcriptional activation.* ***B)*** *The core motif of AhR response elements (AhRE).* ***C)*** *The model of the activation of AhR. Ligands penetrates cell membrane and bind to the cytoplasmic AhR complex which remains inactive by interacting with HSP90, p23 and AIP (AhR interacting protein). The activated AhR then translocate into the nucleus to dimerize with ARNT. The dimer recognises AhRE to transactivate target genes such as Cyp1a1 and AhRR. CYP1A1 catalyses the metabolism of ligands. AhRR competes with AhR to dimerize with ARNT. Ligand-activated AhR is also imported to the plasma for degradation, mainly by proteasome. "Red minus" symbols indicate the negative feedback pathways to reduce the activation of AhR. Figure adapted from (H. Wang, Wei, & Yu, 2015).*

The ligands of AhR constitute an expanding family with new members being continuously added. Generally, ligands can be categorised into four major classes characterised by the source of ligands. These four classes are known as endogenous metabolites, dietary metabolites, microbial derivatives and xenobiotics (**Figure 1.2**) (Denison & Nagy, 2003; Murray et al., 2014; Stejskalova, Dvorak, & Pavek, 2011). Such categorisation is general rather than strict. Many AhR ligands are derived from tryptophan as a result of various biological and physiochemical processes. They are related to each other and may fall into more than one class. AhR ligands vary greatly in their chemical structures and binding affinities, with equilibrium dissociation constant (K_D) ranging from the pM range (strong ligands) to the μM range (weak ligands) (Denison & Nagy, 2003). The potency of ligands is also determined by the bioavailability and pharmacokinetics *in vivo*. For example, 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) is considered as one of the most toxic AhR ligands due to both high binding affinity (the pM range) and long half-life (~seven years in humans) (Pirkle et al., 1989). 6-formylindolo[3,2-b]carbazole (FICZ) is another comparably high affinity ligands with a K_D of 0.07nM (Adachi, Mori, Matsui, & Matsuda, 2004; Busbee, Nagarkatti, & Nagarkatti, 2014; Ema et al., 1994; Wincent et al., 2009). Both K_D and EC50 of these high affinity ligands are listed in the **Table 1.1**. To be noted, AhR ligands can be often classified as agonists or antagonists. One of the well-studied antagonists is CH223191 (Zhao, Degroot, Hayashi, He, & Denison, 2010). In many occasions, antagonists compete with agonists by comparable binding affinities but elicit only weak to no AhR signal (Stejskalova et al., 2011). The AhR ligands have been comprehensively reviewed by (Denison & Nagy, 2003; Stejskalova et al., 2011).

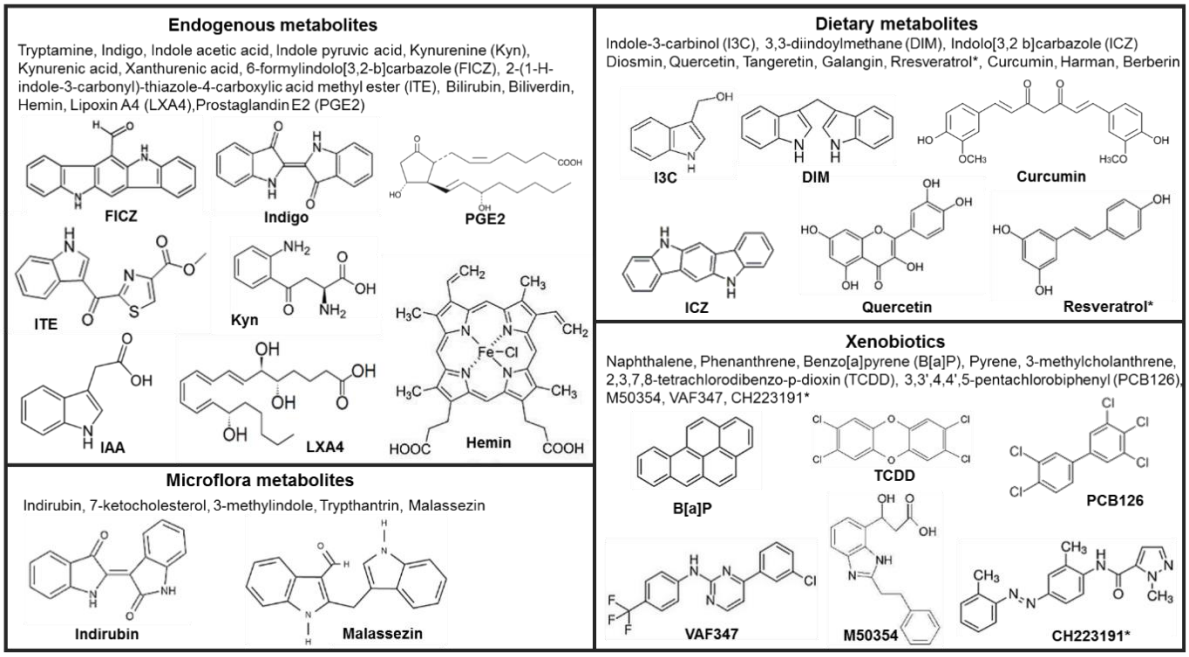


Figure 1.2, General classification of AhR ligands. AhR ligands can be categorized into four major sources: endogenous metabolites, dietary metabolites, microbial derivatives and xenobiotics. Examples and chemical structures for each category are shown. Most ligands are classified as the AhR agonists and the antagonistic ligands are labelled with asterisks. Figure adapted from (H. Wang et al., 2015).

AhR Ligands	**K_D**	**EC50**
TCDD	0.27nM-16nM	1pM-10nM
FICZ	0.07nM	30pM-830pM

Table 1.1, Reported K_D and EC50 of high affinity AhR ligands (Adachi et al., 2004; Busbee et al., 2014; Ema et al., 1994; Wincent et al., 2009).

1.1.3. AhR regulates lymphocytes in the periphery

In the canonical model, immune responses are initiated by the innate immune system that recognises unique molecular patterns expressed by pathogens, referred to as pathogen-associated molecular patterns (PAMPs). The adaptive immune system (T and B lymphocytes) is then primed by stimulated innate immune cells through antigen presentation, costimulatory signals and cytokine milieu. This cascade serves as a main mechanism for the immune system to discriminate self v.s. non-self (Chaplin, 2010). The wide expression of AhR in lymphocytes provides a complementary tunnel to directly communicate with the environment through AhR ligands-induced signals. As to be discussed later and summarized in **Figure 1.3**, AhR functions have been mostly reported to regulate effector lymphocytes in the periphery. Therefore, AhR mediates a mechanism to respond rapidly to environmental change and tune the magnitude of immune responses. And this pathway has great potential in controlling lymphocyte function.

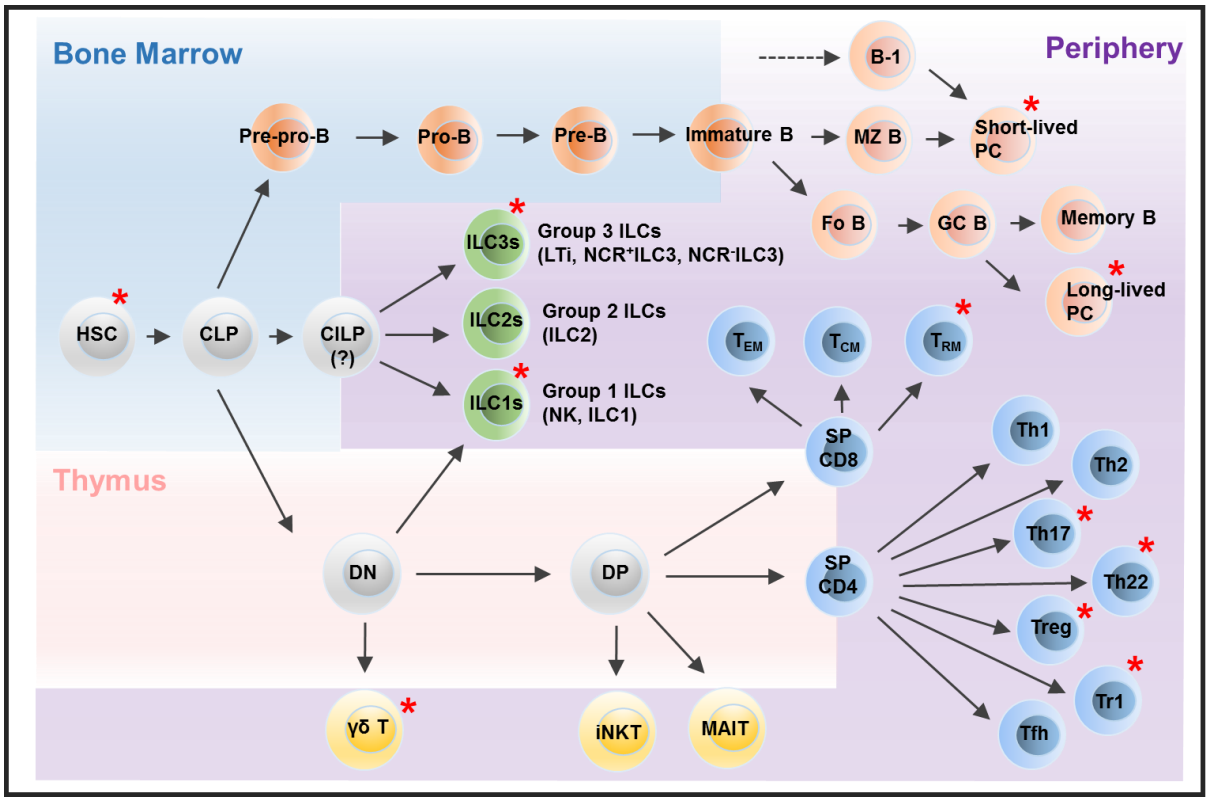


Figure 1.3, AhR mostly regulates the effector lymphocytes in the periphery. A schematic diagram of the lymphocyte development. The lymphocyte populations reported to be regulated by AhR are labelled with red asterisks. Abbreviations: HSC (hematopoietic stem cell), CLP (common lymphoid progenitor), CILP (common innate lymphoid progenitor - its identity still under debate), DN (double negative T cell), DP (double positive T cell), iNKT (invariant natural killer T cell), MAIT (innate mucosal-associated invariant T cell), SP CD8 (single positive CD8 T cell), SP CD4 (single positive CD4 T cell), LTi (Lymphoid tissue inducer cell), MZ B (marginal zone B cell), Fo B (Follicular B cell), GC B (germinal center B cell), PC (plasma cell). Figure adapted from (H. Wang et al., 2015).

1.1.4. Difference between human and mouse AhR

Human and mouse AhR are relatively conserved. Based on the amino acid sequence, the bHLH domain is 100% identical and the PAS domain retains more than 85% similarity, while the Q-Rich domain is less conserved between mouse and human, with a sequence similarity of 59.5% (Ema et al., 1994). Even though amino acid sequences in the main functional domain of AhR in both mouse and human are highly conserved, they differ for binding affinities to ligands (Ema et al., 1994; Flaveny & Perdew, 2009). There are at least two reasons: i) the key amino acid in the ligand binding domain between C57BL6 mouse AhR A375 and the human AhR V386 are different, and ii) the canonical human AhR protein contains 848 aa, while the mouse AhR is 43 aa shorter (Ema et al., 1994; Flaveny & Perdew, 2009). To be noted, different mouse strains also show diverse AhR proteins. For instance, the C57BL6 mouse expresses the canonical 804aa AhR, while DBA mouse expresses the 848aa one (Ema et al., 1994; Flaveny & Perdew, 2009). All these differences potentially lead to variable strength of AhR pathway when stimulated by same ligands. Human AhR preferentially binds and responds to Indole while mouse AhR does not (Hubbard et al., 2015). Although the difference between mouse and human AhR does exist, both can also highly respond to same ligands such as TCDD and FICZ, measured by the expression of the well-studied downstream target CYP1A1 (Ehrlich, Pennington, Bisson, Kolluri, & Kerkvliet, 2018; Kiyomatsu-Oda, Uchi, Morino-Koga, & Furue, 2018; Kovalova, Nault, Crawford, Zacharewski, & Kaminski, 2017; Nebert et al., 2000).

1.2. Important role of AhR in the mononuclear phagocyte system

1.2.1. Dendritic cells

Dendritic cells (DC) are critical for priming the immune system while the AhR signalling pathway negatively regulates this process. AhR can induce immunosuppression which was reported to be mediated by the modulation of DC function (Navid et al., 2013). DC enhances immune tolerance by inducing naïve T cell to differentiate towards Treg through the production of indoeamine-2,3-dioxygenase (IDO) and its metabolites (Van Voorhis, Fechner, Zhang, & Mezrich, 2013). Notably, IDO is the rate limiting enzyme catalysing the metabolism of indole compounds and tryptophan, leading to the production of AhR ligands. Therefore, IDO boosts the production of AhR agonists and activates AhR pathway, which in turn upregulates IDO expression and strengthens Treg differentiation (Van Voorhis et al., 2013). The upregulation of IDO in DCs by the AhR signalling pathway was considered extremely consistent, even in the presence of stimuli such as LPS or CpG (Nguyen et al., 2010). The immunosuppressive role of AhR in DCs is also supported by the evidence that the deficiency of AhR signalling significantly reduces the production of IL-10 by DCs (Nguyen et al., 2010). Together, these studies suggested the negative role of AhR in regulating immunity via IDO pathway in DC.

The immunosuppressive function of AhR in DCs was also supported by the regulation and degradation of the transcription factors NF-κB and AP-1 (Quintana, 2013b). Published results showed that dietary AhR ligand indole-3-carbinol (I3C) and indirubin-3-oxime (IO) can upregulate *ALDH1A, IDO* and *TGFB,* and down-regulate NF-κB p65 (Bankoti, Rase, Simones, & Shepherd, 2010; Benson & Shepherd, 2011; Simones & Shepherd, 2011; Vogel, Goth, Dong, Pessah, & Matsumura, 2008). Both studies suggested that the reduction of NF-κB is mostly due to an interaction between its signalling compounds and AhR components (Benson &

Shepherd, 2011; Quintana, 2013a). Besides the key role in modulating immunosuppression by regulating the differentiation of Tregs, AhR can also reduce secretion of pro-inflammatory cytokines, such as IL-6 in DCs (Quintana, 2013a).

Although AhR regulates the immunosuppressive function in DCs, it can promote DC maturation (J. A. Lee et al., 2007; Nguyen, Hanieh, Nakahama, & Kishimoto, 2013; Vogel et al., 2008). By blocking the AhR signalling pathway using 3'-methoxy-4'-nitroflavone (MNF) as an antagonist, DC maturation was largely limited (Vogel et al., 2008). Consistently, adding TCDD, FICZ or BaP dramatically promoted DC development and/or maturation (Hwang, Lee, Cheong, Youn, & Park, 2007; J. A. Lee et al., 2007; Vogel et al., 2008). However, the function of AhR on DC differentiation was shown site-specific. For example, TCDD treatment doubled the absolute number of $CD11c^{+}CD103^{+}$DC in the spleen specifically via the AhR pathway while reducing the absolute number of $CD11c^{+}CD103^{+}$DC in the mesenteric lymph node (Schulz et al., 2013). The increase of splenic DC numbers was suggested to result from the increased availability of IL-10, which may also suppress the adaptive immune response (Schulz et al., 2013).

1.2.2. Macrophages

AhR signalling restricts the inflammatory response of macrophages mainly by suppressing the secretion of inflammatory cytokines and also by the inhibition of the differentiation of macrophages (Kimura et al., 2009; Quintana, 2013a; van Grevenynghe et al., 2003). The suppression on the cytokine secretion was reported to be limited to a specific subset of macrophages. This was shown that the stimulation with LPS upregulates AhR expression only in M1 macrophages (encourage inflammation), while the expression of AhR in the M2

macrophages (decrease inflammation and promote tissue repair) was largely unchanged (Mills, 2012; Nguyen et al., 2013). In AhR deficient macrophages, IL-6 and TNF-α production was increased upon LPS stimulation, compared to WT macrophages (Kimura et al., 2009; Nguyen et al., 2013) while the production of anti-inflammatory cytokines such as IL-10 was inhibited (Hao & Whitelaw, 2013). However, not all pro-inflammatory cytokines are suppressed by AhR. The production of IFN-γ in macrophages in lung during influenza infection was promoted by TCDD treatment (Neff-LaFord, Teske, Bushnell, & Lawrence, 2007). The change was suggested to be dependent on AhR and also the increased expression of iNOS as a result of the interaction of AhR and NF-κB p65 subunit (Neff-LaFord et al., 2007). Taken together, AhR mainly limits pro-inflammatory cytokine secretion in M1 macrophage and dampening immune response.

1.3. Important role of AhR in the innate lymphoid cells

B lymphocytes and T lymphocytes including non-conventional T cells express BCRs and TCRs, which are required for them to recongnise very diversified antigens. In contrast, innate lymphoid cells (ILCs) are defined by a similar lymhpocyte-like morphology but the lack of BCR or TCR. Importantly, ILCs and B and T lymphocytes share common lymphoid progenitors for their development (Lanier, 2013). The unifying nomenclature that entails the classification of ILCs into three groups has been proposed (Spits et al., 2013). Group 1 ILCs are defined by the production of the signature cytokine IFN-γ but not Th2 cell- and Th17 cell-associated cytokines. The prototypical member of this group is the natural killer (NK) cell. ILC1s are the subset of group 1 ILCs distinct from NK cells in terms of the cytotoxicity. Group 2 ILCs produce Th2 cell-associated cytokines such as IL-5, IL-13, IL-9 and IL-4. Group 3 ILCs are defined by their capacity to produce the cytokines IL-17 and/or IL-22 and characterised by

the expression of RORγt. The prototypical group 3 ILCs are lymphoid tissue-inducer (LTi) cells, which are crucial for the formation of secondary lymphoid organs during embryogenesis. ILC3s represents a subset group 3 ILCs that are distinct from LTi cells based on the ontogeny (Mills, 2012). NCR^+ (natural cytotoxicity triggering receptor) ILC3s produce IL-22 but not IL-17, sometimes called ILC22. NCR^- ILC3s produce IL-22, IL-17 and IFN-γ.

1.3.1. Group 1 ILCs/NK cells

NK cells develop normally in AhR-deficient mice (J. S. Lee et al., 2012; Shin et al., 2013). The activation of NK cells by IL-2, IL-15 and IL-12 is associated with the increased expression of AhR (Shin et al., 2013; Wagage et al., 2014). *Ahr*$^{-/-}$ NK cells showed a reduced cytotoxicity compared to wildtype NK cells whereas various dietary AhR agonistic ligands, such as kynurenine, DIM and 13C enhanced the production IFN-γ in NK cells (Shin et al., 2013). The defective production of IL-10 from NK cells was also observed when treated with the AhR antagonist CH-223191 *in vitro* or in *Ahr*$^{-/-}$ mice infected with *Toxoplasma gondii* (Wagage et al., 2014). These data indicate that AhR signalling promotes the effector function of NK cells. AhR expression is low in non-NK ILC1s (Fuchs et al., 2013), and the functional study for AhR in this cell type has not been reported.

1.3.2. Group 2 ILCs

Compared with other innate cells types, the effect of AhR in type 2 innate lymphoid cells (ILC2s) lacks extensive investigations. One recent publication showed that AhR intrinsically inhibited the generation and function of ILC2s, leading to a severer helminth infection (S. Li et al., 2018). Other studies suggested that AhR can reduce ILC2s differentiation when

coordinating with Notch 1 or GATA3 (Mjosberg, Bernink, Peters, & Spits, 2012). Therefore, AhR may negatively regulate ILC2s but this requires further validation.

1.3.3. Group 3 ILCs/RORγt$^+$ ILCs

AhR is highly expressed by the RORγt$^+$ ILCs. Collectively, AhR is reported to positively regulate the maintenance of RORγt$^+$ ILCs via three key mechanisms. First, AhR can promote the maturation of RORγt$^+$ ILCs by enhancing the Notch signalling pathway (Kiss & Vonarbourg, 2012). TCDD treatment was shown to upregulate both Notch1 and Notch2 in RORγt$^+$ ILCs (Mjosberg et al., 2012). Second, AhR can sustain the survival of RORγt$^+$ ILCs via IL-7 pathway. Results showed that lacking AhR significantly reduced IL-7 and IL-7R expression, and impaired the expression of anti-apoptotic proteins such as Bcl2 (Kiss & Vonarbourg, 2012; Qiu & Zhou, 2013). Last, AhR can directly bind to the tyrosine kinase (Kit) receptor promoter to enhance its expression ,which promoted the maintenance of RORγt$^+$ ILCs (Kiss & Diefenbach, 2012).

On the other hand, AhR controls the function of RORγt$^+$ ILCs by regulating the production of IL-22 (Kumar, Rajasekaran, Palmer, Thakar, & Malarkannan, 2013; Qiu et al., 2013; Qiu & Zhou, 2013). Such effect is mediated by the interaction of AhR and RORγt, resulting the binding of AhR approximately to the promoter in the *Il22* locus (Qiu et al., 2012; Qiu & Zhou, 2013). In line with this, the lack of AhR increased the apoptosis of RORγt$^+$ ILCs and decreased IL-22 production (Kiss et al., 2011; Qiu et al., 2012). To be noted, not all RORγt$^+$ ILCs require AhR. At the fetal stage, the RORγt$^+$ ILCs such as fetal LTi cells appear not to be dependent on AhR (Qiu et al., 2012; Qiu & Zhou, 2013). These results together highlight the significant role of AhR in the maintenance and function RORγt$^+$ ILCs.

1.4. Important role of AhR in the adaptive immunity

Peripheral conventional αβ T cells are composed of CD4 or CD8 single positive lymphocytes. During T-cell development in the thymus, immature T cells progress through a rearrangement of TCR α and β chain, of which the affinity to major histocompatibility complex (MHC) is critical for positive selection by cortical epithelial cells (Parel & Chizzolini, 2004). These immature T cells can express both CD4 and CD8 (so-called immature double positive T cells, DP), which locate at thymus cortex. After positive selection, these DP T cells differentiate into either CD4 or CD8 single positive lineage, and then go through a negative selection by dendritic cells based on the affinity to MHC (Parel & Chizzolini, 2004). These cells, known as mature naïve T cells, migrate into periphery playing either regulatory or cytotoxic roles, which are commonly identified as $CD4^+$ T cells (helper or regulatory T cells) and $CD8^+$ T cells (cytotoxic T cells) respectively.

Peripheral B cells are critical for humoral responses to clear pathogenic invaders. Once B cells encounter antigen, they can either differentiate into short-lived plasma cells to secret relatively low-affinity immunoglobulin (Ig) or initiate germinal centres (GCs). After the selection in GCs, long-lived plasma cells will be produced with the capability to secrete high-affinity antibodies. Another product of GCs are memory B cells, which, together with memory T cells, mount rapid and more robust immune responses to o protect the host from repeated pathogen challenges. The role of AhR in B cell development, differentiation and function has not been well studied.

1.4.1. CD4$^+$ T cells

CD4$^+$ T cells, also known as helper T cells, play a key role in the regulation of adaptive immune response. Different CD4$^+$ T-cell subsets can provide either supportive or suppressive function by producing remarkable cytokines. These subsets are generally categorised by their functions, including Type 1 helper T (Th1) cells, Type 2 helper T (Th2) cells, IL-17-producing T helper (Th17) and Foxp3$^+$ regulatory CD4$^+$ T (Treg). AhR is highly expressed in Th17 cells, lower in Treg cells and almost undetectable in Th1 and Th2 cells (Duarte, Di Meglio, Hirota, Ahlfors, & Stockinger, 2013; Quintana et al., 2008; Veldhoen et al., 2008), suggesting an important role of AhR in Th17 and Treg cells.

Th17 cells are characterised by the production of IL-17, IL-17F, IL-21 and IL-22. Th17 cells recruit neutrophils to induce tissue inflammation that is not only important for the host defence against bacterial and fungal infections but also involved in inflammatory and autoimmune diseases (Korn, Bettelli, Oukka, & Kuchroo, 2009). As a closely related subset, Th22 cells are characterized by the high production of IL-22, but not IL-17. Th22 cells regulate epithelial cells and keratinocytes to counteract the destructive effects of the immune response and limit tissue damages (Akdis, Palomares, van de Veen, van Splunter, & Akdis, 2012).

High expresssion level of AhR in Th17 cells suggests its important role in the homeostatsis of Th17 cells (Duarte, Di Meglio, Hirota, Ahlfors, & Stockinger, 2013; Quintana et al., 2008; Veldhoen et al., 2008). The presence of natural AhR agonists in culture medium is necessary for the optimal induction of Th17 differentiation *in vitro* (Veldhoen, Hirota, Christensen, O'Garra, & Stockinger, 2009). The addition of FICZ or other AhR agonists in culture could further enhance Th17 cell differentiation in an AhR-dependent manner (Duarte et al., 2013;

Veldhoen et al., 2009; Veldhoen et al., 2008). Notably, AhR-deficient mice demonstrated a very mild reduction of normal Th17 cell differentiation in the steady state (Kimura, Naka, Nohara, Fujii-Kuriyama, & Kishimoto, 2008) or immunization-induced experimental autoimmune encephalomyelitis (EAE) model (Duarte et al., 2013; Veldhoen et al., 2008), suggesting either other Th17-intrisinc signals redundant to AhR or some extrinsic factor regulated by AhR that influences Th17 cells *in vivo*. It was probably more surprising that systemic administration of AhR agonists TCDD and FICZ via intraperitoneal injection inhibited EAE in mice (Duarte et al., 2013; Quintana et al., 2008). Given that AhR is differentially expressed by the immune system and also on epithelial and stromal cells, the full explanation of *in vivo* effects of the activation of AhR in the EAE model remains to be elucidated. In our view, the influence of ligand-induced AhR activation on Th17 cells might be offset by the effects of ligand-induced AhR activation on Treg cells, which will be discussed below. In the model of collagen-induced arthritis, mice with conditional deletion of the *Ahr* gene in T cells ($Ahr^{flox/flox}$ x Lck-Cre) developed a milder disease with reduced Th17 cells in the draining inguinal lymph nodes, compared to those in wildtype mice (Nakahama et al., 2011). This provides a convincing evidence for AhR in the control of Th17 cells *in vivo*.

The mechanisms by which the AhR pathway promotes the Th17 differentiation are still under debate. Early studies revealed that AhR interacted with STAT1 and STAT5 (but not STAT3 or STAT6), two negative regulators of Th17 differentiation, but without affecting the production of IL-2 (Kimura et al., 2008; Veldhoen et al., 2009). A later study, however, suggested AhR cooperated with STAT3 to induce the expression of the epigenetic modifier Aiolos, which silenced IL-2 expression (Quintana et al., 2012). Recently, the AhR signalling was shown as a potent inducer for the expression of IL-1 receptor (Duarte et al., 2013). As the

IL-1/IL-1r signal induces Th17 differentiation and is critical for the effector function of Th17 cells (Y. Chung et al., 2009), this could be another putative mechanism.

During the investigation of AhR in Th17 cells, a striking observation was that the production of IL-22 was profoundly affected by AhR signalling than IL-17 (Veldhoen et al., 2009; Veldhoen et al., 2008). The lack of AhR almost completely abolished IL-22 production in Th17 cells (Veldhoen et al., 2008). Therefore, AhR is crucial for the differentiation of Th22 cells. The specific induction of IL-22, but not IL-17, by the AhR agonists TCDD and FICZ was also observed in vitro in human $CD4^+$ T cell cultures (Ramirez et al., 2010; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). *In vivo*, the treatment of FICZ in mice induced the production of IL-22 and protected mice from trinitrobenzene sulfonic acid (TNBS)-, dextran sulfate sodium (DSS)-, and T-cell transfer-induced colitis in an AhR-dependent manner (Monteleone et al., 2011). The source of IL-22 in this study could be Th22 cells or group 3 ILCs. Mechanistically, AhR cooperates with the transcription factor RORγt to induce IL-22 production in Th22 cells (Trifari et al., 2009).

Treg cells are essential in preventing excessive inflammation and autoimmunity in mice and humans (Sakaguchi, Miyara, Costantino, & Hafler, 2010). AhR-binding sites were identified in the *Foxp3* gene locus by bioinformatic analysis. Chromatin immunoprecipitation (ChIP) and luciferase reporter assay confirmed the direct binding of AhR to the genomic elements and its transcriptional activity, demonstrating that AhR regulates Foxp3 expression in Treg cells (Quintana et al., 2008). TCDD had a mild effect on Foxp3 expression in activated $CD4^+$ T cells *in vitro* (Quintana et al., 2008). Once TGF-β, a major inducer for Treg differentiation, was added into the culture, TCDD could not further enhance TGF-β-induced Treg cell

differentiation (Duarte et al., 2013; Gandhi et al., 2010). Without expanding Treg cell numbers, TCDD enhanced the suppressive function of TGFβ-induced Treg cells by upregulating the expression of IL-10 and ectonucleotide triphosphate diphosphohydrolase CD39. The concurrent activation of TGF-β and AhR signalling is mediated, at least partially, by the transcription factors Smad1 and Aiolos (Gandhi et al., 2010). As aforementioned inhibition of the EAE model by treating mice with TCDD, there was no significant changes in the frequency of Treg cells either in the draining paraaortic lymph node or in the spinal cord (Duarte et al., 2013; Quintana et al., 2008). This observation is in line with the *in vitro* data, again indicating that AhR activation controls the functionality rather than the quantity of Treg cells.

Type 1 regulatory T (Tr1) cells secret IL-10 to suppress tissue inflammation and autoimmunity (Pot, Apetoh, & Kuchroo, 2011). The expression of AhR is strongly upregulated in Tr1 cells that are induced by TGF-β and IL-27 (Apetoh et al., 2010). Both TCDD and FICZ significantly increased IL-10 production in Tr1 cells *in vitro* (Apetoh et al., 2010; Gandhi et al., 2010). In mice carrying the *d* allele of the Ahr gene (Ahr^d) which encodes a mutant AhR protein with much reduced affinity (10 to100-fold) for ligands (Okey, Vella, & Harper, 1989), reduced differentiation of Tr1 cells was reported. In the Tr1-skewing condition, ligand-activated AhR interacted with c-Maf to bind the promoter of *Il10* gene and transactivate its expression (Apetoh et al., 2010; Gandhi et al., 2010).

Several studies reported a very low AhR expression in Th1 and Th2 cells (Apetoh et al., 2010; Duarte et al., 2013; Quintana et al., 2008; Veldhoen et al., 2008). However, *in vitro* $CD4^+$ T cells stimulated with AhR agonists produced more IFNγ and less IL-4, indicating the AhR

signalling can still modulate Th1/Th2 balance (Negishi et al., 2005), although the physiological significance of this observation remains to be determined.

1.4.2. CD8$^+$ T cells

Compared to a wealthy collection of studies that have revealed many roles of AhR in CD4$^+$ T cell subsets, such evidence for CD8$^+$ T cells is scarce. The CD8$^+$ T cells in AhR-deficient mice or mice treated with TCDD were examined in viral infection models and found to have reduced function. Both studies suggested AhR likely regulated APCs rather than directly having an effect in CD8$^+$ T cells (Jin, Moore, Head, Neumiller, & Lawrence, 2010; Lawrence, Roberts, Neumiller, Cundiff, & Woodland, 2006). The very low expression of AhR in most CD8$^+$ T cell populations might underpin the lack of direct regulation of CD8$^+$ T cells by AhR signalling. A recent study revealed a dramatic increase of AhR expression in tissue-resident memory T (T_{RM}) cells (Zaid et al., 2014). In the skin, these memory CD8$^+$ T cells reside in the epidermis after being recruited to this site by infection or inflammation and protect against subsequent infection. Compared to wildtype cells, *AhR*$^{-/-}$ T_{RM} disappeared from the skin over time, showing that AhR facilitates T_{RM} persistence in the epidermis (Zaid et al., 2014).

1.4.3. B cells

In defined mouse B cell populations, the expression of AhR is negligible in bone marrow pro- and pre-B cells, is increased in transitional B cells and remains low in follicular, marginal zone, germinal centre B cells and plasma blasts in spleen. The highest expression of AhR was identified in mature plasma cells in bone marrow (Green et al., 2011; Sherr & Monti, 2013). Little expression of AhR in pro-/pre- B cells seems at odds with the fact that AhR ligands

induces apoptosis of pro-/pre-B cells and suppresses B cell development. This can be explained by the fact that the suppression is mainly mediated by AhR-expressing bone marrow stromal cells whose function is impaired by the AhR activation. More details are discussed in (Sherr & Monti, 2013).

In mature B cells, the expression of AhR can be induced by various stimulations including lipopolysaccharide (LPS), CpG, anti-CD40 and IL-4 (Allan & Sherr, 2005; Marcus, Holsapple, & Kaminski, 1998; Tanaka et al., 2005). It is of particular interest to study AhR in plasma cells due to their highest expression level. Using an in vitro culture system to stimulate CD40 on purified human B cells for plasma cell differentiation, the AhR ligand benzo[a]pyrene (B[a]P) was identified to specifically suppress plasma cell differentiation (Allan & Sherr, 2010). TCDD also suppressed B cell differentiation and antibody production in a mouse CH12.LX B-cell line (Sulentic, Holsapple, & Kaminski, 1998). B lymphocyte-induced maturation protein-1 (Blimp1, encoded by the Prdm1 gene) is a transcription factor that critically regulates plasma cell differentiation and antibody production (Shapiro-Shelef & Calame, 2005). Two putative mechanisms have been proposed to link the AhR activation and the downregulation of Blimp1. First, the AhR activation downregulates the activator protein 1 (AP-1) transcription factor (c-Jun) which transactivates the Prdm1 gene (D. Schneider, Manzan, Yoo, Crawford, & Kaminski, 2009). Second, the AhR activation upregulates Bach2, a Prdm1 repressor (De Abrew, Phadnis, Crawford, Kaminski, & Thomas, 2011). A study by the combination of whole-genome, microarray-based ChIP (ChIP-on-chip) and time course gene expression microarray analysis in TCDD-treated CH12.LX cells revealed AhR-mediated impairment of B-cell differentiation occurs at multiple nodes of the B-cell differentiation network and potentially through multiple mechanisms (De Abrew et al., 2010). All these works demonstrate AhR regulates the expression of Blimp1 to control B-cell differentiation into

plasma cells. The physiological significance of AhR in B-cell differentiation in vivo needs to be evaluated. This can be achieved by conditional deletion of the *Ahr* gene in mature B cells.

AhR is emerging as a special mediator to enable immune cells to sense environmental cues directly through a large array of AhR ligands. Such important regulatory function of AhR is summarised in **Figure 1.4**. Interestingly, endogenous and dietary AhR ligands are usually enriched in tissues and organs in contact with the environment, such as skin, gastrointestinal tract and lung. Presumably by evolutionary selection, AhR signalling may acquire a prominent role in controlling the effector function and homeostasis of immune cells that reside at the tissue-environment interface.

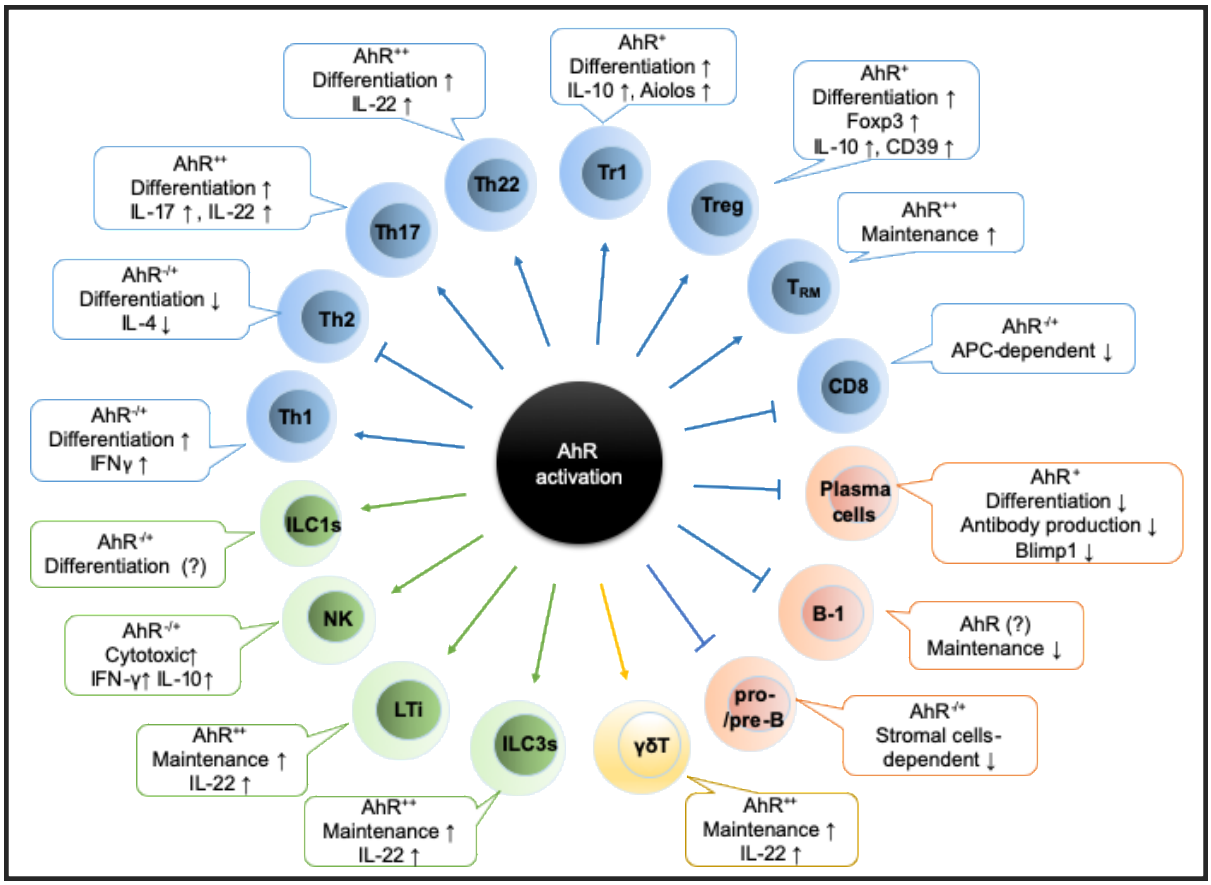


Figure 1.4, A summary of the known expression and functions of AhR in immune cells. Figure adapted from (H. Wang et al., 2015).

1.5. AhR and mouse and human gut immune homeostasis

AhR has been previously identified to be involved in carcinogenic pathway, and is critical for the regulation of WNT signalling in tumour models (Dietrich & Kaina, 2010; Falahatpisheh & Ramos, 2003; Liu et al., 2018; Mathew, Sengupta, Ladu, Andreasen, & Tanguay, 2008; Mathew, Simonich, & Tanguay, 2009; A. J. Schneider, Branam, & Peterson, 2014; C. Wu, Yu, Tan, Guo, & Liu, 2018). More recently, the important role of AhR in regulating the immune homeostasis in the gut is emerging (Hooper, 2011), with a strong implication in bowel diseases. Indeed, studies reported that the AhR pathway, activated by diet derived ligands, is important to limit inflammatory responses in gut (Bock, 2019; Koch et al., 2017; Sun, Ma, He, Johnston, & Ma, 2019). Due to the similar function executed by mouse and human AhR, there is a great potential to use mouse models to develop novel strategies targeting AhR pathway to treat bowel disease. In this section, the important role of AhR in bowel diseases including both inflammatory and infectious diseases will be discussed.

1.5.1. Inflammatory bowel disease (IBD)

Inflammatory bowel disease is a common disease including a broad range of complications. IBD can also lead to cancers in digestive tracts including colorectal cancer, small bowel adenocarcinoma, and intestinal lymphoma etc (Askling et al., 2001; Axelrad, Lichtiger, & Yajnik, 2016; Greenstein, Sugita, & Yamazaki, 1989; Gyde, 1989; Itzkowitz, 1997; S. K. Jain & Peppercorn, 1997; Jawad, Direkze, & Leedham, 2011; Lai, Kuo, & Liao, 2019; Pohl, Hombach, & Kruis, 2000; Schechter, 1995; Tsianos, 2000; Xie & Itzkowitz, 2008). IBD are generally classified into two types: Ulcerative Colitis (UC) and Crohn's Disease (CD). In both diseases, the differential expression of AhR was detected, which suggested a potential role in

the pathology of IBD (Neavin, Liu, Ray, & Weinshilboum, 2018). Recent studies provided promising evidence to target AhR, via its anti-inflammatory property in the treatment of IBD.

AhR has been linked to IBD pathology. Specifically, either the expression of AhR or the abundance of its ligands in patients were shown significantly reduced (Qiu & Zhou, 2013; Rothhammer et al., 2016). On the other hand, high affinity AhR ligands TCDD and FICZ have been shown effective in reducing the pathology in murine models for colitis (Lv, Wang, Qiao, Yang, et al., 2018; Lv, Wang, Qiao, Dai, & Wei, 2018). This effect of AhR signalling might be attributed to enhancing the differentiation of iTreg cells in the gut, which is otherwise impaired by inflammation (Lv, Wang, Qiao, Yang, et al., 2018; Singh et al., 2011). Meanwhile, the activation of AhR by FICZ was also reported to sustain the tight junction (TJ) integrity by maintaining the expression of TJ protein and inhibiting the increases in the phosphorylation of MLC, consequently protected mice from the DSS-induced colitis (M. Yu et al., 2018). Dietary AhR ligands such as tryptophan derivatives including I3C and DIM, despite of their relatively low-affinity to AhR, were reported to play a beneficial role in suppressing the inflammatory response in the gut (Busbee, Rouse, Nagarkatti, & Nagarkatti, 2013). Collectively, both low and high-affinity AhR ligands, and the activation of the AhR pathway have been shown the anti-inflammatory activities in improving IBD.

1.5.2. Gut infections

Besides IBD, infections in the gut are also a major threat to human health. Gut infections are usually caused by parasites or bacteria but also by viruses.

Parasites in gastrointestinal track can cause severe sickness in host. For example, *Toxoplasmosis gondii* can introduce heavy burdens in the immune system of the infected host causing an out of control inflammation. While IL-10 was known as a key suppressor for anti-inflammatory response, the sources of IL-10 at the early stage of *T.gondii* infection was limited to NK cell (Roers et al., 2004). Interestingly, AhR is required for optimal IL-10 expression in the NK cells in this scenario (Wagage et al., 2014). This again emphasises the important immunosuppressive function of AhR.

Listeria monocytogenes is one of the infectious bacteria that can causing severe listeriosis affecting gut health. AhR was required for an effective clearing of the infection via reducing the death of macrophage by the induction of the apoptosis inhibitor of macrophages, and increasing the production of reactive oxygen species (ROS) in infected macrophages (Kimura et al., 2014). On the other hand, AhR is capable to inhibit the caspase-1 activation as well as NLRP3 expression in the macrophages, further reducing the strength of inflammasome activation, supporting and maintaining the homeostasis of the immune cells in the gut (Huai et al., 2014). In AhR-deficient mice, the secretion of IL-1, IL-6 and TNF was enhanced in the model mimicking bacterial infections by LPS treatment (Kimura et al., 2009; Sekine et al., 2009; Stockinger, Hirota, Duarte, & Veldhoen, 2011). Soon after the LPS treatment, the expression of hepatic enzyme that regulates the metabolism of tryptophan was induced and led to the production of AhR ligand IDO1, which subsequently activated the AhR signalling to turn down the early inflammatory gene expression (Bessede et al., 2014). Moreover, when the host was exposed to a secondary LPS challenge, AhR was again required for the induction of the tolerance and the fitness of the gut in the host (Bessede et al., 2014). In line with this, the deletion of AhR in mice increased the susceptibility to endotoxemia (Sekine et al., 2009). Besides pathogens, the AhR pathway can also mediate the communication between the host

and commensal gut microbiome. Such communication can further affect neurodevelopment and neurodegeneration in the central nervous system including (H. U. Lee, McPherson, Tan, Korecka, & Pettersson, 2017).

In terms of virus infections, little is known about the effect of AhR on the differentiation of specific subsets. However, AhR is required to control the ocular herpes simplex virus induced inflammatory lesion through the promoted apoptosis of non-Treg $CD4^+$ T cells (Veiga-Parga, Suryawanshi, & Rouse, 2011). This result suggested the potential of AhR in controlling virus infections in the gut.

1.6. Specific regulatory role of AhR in the cytotoxic T cells in gut

While many studies have revealed the anti-inflammatory role of AhR in protecting gut health through the fine-tuning of the function of DCs, macrophages and $Foxp3^+$ Treg cells, much less attention has been given to cytotoxic T cells. These T cells primarily consist of mesenteric lymph nodes, Intraepithelial and lamina propria lymphocytes (Cesta, 2006; Liebler-Tenorio & Pabst, 2006). T cells within the mucosal epithelium, termed as intraepithelial lymphocytes (IELs), are essential to orchestrate the balance between host and microbiotas in the gastrointestinal track. More importantly, over 90% of IELs are T cells (Olivares-Villagomez & Van Kaer, 2018) and majority of the T cells in the IELs, are $CD8\alpha^+$ T cells, also known as cytotoxic T cells (Yap & Marino, 2018). These T cells are able to provide effective and specific killing against pathogens in the gut mucosal environment, thus maintaining the gut fitness. Despite AhR was initially discovered to sense environmental pollutants, a common consensus has been reached that AhR signalling is crucial for the development and maintenance of gastrointestinal homeostasis, (Hooper, 2011; Lawrence & Sherr, 2012). However, it remained

largely unknown whether the AhR signalling regulates cytotoxic T cells in the gut at the time when I started this project for my PhD.

1.6.1. Intraepithelial lymphocytes (IELs) safeguard the gut homeostasis

In the intestinal track, many of the beneficial commensal bacteria exist, meanwhile, potential chance for the exposure to varies pathogens is also high. IELs provide a solid defence for the host in the first line to fight against potential invaders (Sheridan & Lefrancois, 2010). Cytotoxic T cells in IELs are categorised into two major classes based on the expression of different TCR chains as TCRαβ or TCRγδ. CD8α expression is detected in both TCRαβ and TCRγδ classes. $CD8\alpha^+$ TCRαβ cells consist of three subsets: CD8αα, CD8αβ which is equivalent to the conventional CD8αβ TCRαβ cells dominantly in peripheral lymphoid tissues such as spleen and lymph node, and also $CD8\alpha^+CD4^+$ cells. All these T cells have been reported to effectively produce cytotoxic molecules such as granzymes and interferons to perform or assist killing that facilitates the elimination of invasive pathogens (Sheridan & Lefrancois, 2010). Interestingly, IELs can directly communicate with intestinal epithelial cells (IECs) (Konjar, Ferreira, Blankenhaus, & Veldhoen, 2017). On one hand, IELs express a high level of CD103, which can interact with IECs in the epithelium to mediate the homing of these cytotoxic T cells (Konjar et al., 2017). On the other hand, the IECs can secret cytokines especially IL-15 which has been shown to regulates the proliferation, survival and function of cytotoxic T cells in the epithelium (Konjar et al., 2017; Sheridan & Lefrancois, 2010). Such regulatory feedback can enhance the homeostasis of the epithelium tissues, which has been shown to protect the host from infections such as S. *tuphimurium*, *T. gonddi*, and *Nippostrogylus brasiliensis* (Dalton et al., 2006; Inagaki-Ohara, Sakamoto, Dohi, & Smith, 2011; Ismail et al., 2011; Lepage, Buzoni-

Gatel, Bout, & Kasper, 1998; Sumida, 2019; Yap & Marino, 2018). In addition, a population of CD8α^+ IELs do not express TCR (Ettersperger et al., 2016), which was reported to expand in patients with celiac disease and execute NK-like cytotoxicity (Olivares-Villagomez & Van Kaer, 2018). However, the development and function of this population is still largely unknown.

1.6.2. Enrichment of AhR ligands in the mucosal environment

Ligands binding is essential for the activation of the AhR signalling pathway and initiate all downstream activities. Therefore, the bioavailability of AhR ligands should be considered for AhR-mediated regulation. While pollutants constitute a large group of AhR ligands which may do harm to human beings after the exposure, AhR ligands are produced and enriched at mucosal environmental inside human body. Some AhR ligands are component of diet so human being access them through food intakes. More AhR ligands are generated by the mentalism of human being or commensal microbiota. It is important to understand the type and amount of AhR ligands available in the gut of mammalian animals including humans.

Indeed, scientists found out that the AhR ligands are enriched in the gastrointestinal tracks in mammals, due to the metabolism of microbiota largely. More than 26 strains of commensal bacteria, includes *Lactobacillus reuteri* and *E. coli* in gastrointestinal tracks have been reported to be an effective producer of indole derivatives (Lamas, Natividad, & Sokol, 2018). Notably, high fat diet was shown to induce metabolic syndrome and dramatically impair the production of AhR agonists from microbiota (Natividad et al., 2018). On the other hand, the depletion of AhR ligands in the gut significantly affected the composition of microbiota, indicating the important regulatory role of the AhR ligand for the symbiosis of gut microorganisms (Brawner et al., 2019).

Even though indole derivatives are not high in food, tryptophan, the major source to generate indoles is abundant in *Brassica* genus (including broccoli cauliflower, brussel sprouts, and cabbages) (Gutierrez-Vazquez & Quintana, 2018; Lamas et al., 2018). Tryptophan can be metabolised into diverse AhR ligands primarily through the kynurenine pathway by the gut microbiota and the host (Gutierrez-Vazquez & Quintana, 2018; Lamas et al., 2018; Sun et al., 2019). With the light stimulation, tryptophan can be catalysed into FICZ, which is an important AhR ligand with a high affinity to AhR comparable to the exogenic carcinogenic TCDD (Gutierrez-Vazquez & Quintana, 2018; Lamas et al., 2018). Another food enriched material, Glucobrassins are another food-derived source to be metabolised and generate AhR ligands via the digestive reactions (Lamas et al., 2018).

1.6.3. AhR modulates the generation of γδ T cells

The AhR expression is detected in all γδ T cells which help to maintain the epithelial integrity and mucosal homeostasis (Y. Li et al., 2011; Stange & Veldhoen, 2013). AhR appears to be not essential for the overall generation of γδ T cells in lymph nodes, spleen, and thymus (Y. Li et al., 2011). However, the AhR expression is higher in the immature γδ T cells with TCRγ1 and TCRγ5 cells than those with other TCR (Stange & Veldhoen, 2013). The AhR deficiency specifically reduced certain γδ T cells subsets including Vγ5 γδ T cells and Vγ3 γδ T cells, particularly in IELs (Y. Li et al., 2011; Stange & Veldhoen, 2013). These results suggest the importance of AhR in γδ T cells.

1.6.4. The AhR signalling pathway is required for the differentiation of TCRαβ$^+$CD8αα$^+$ T cells

TCRαβ$^+$CD8αα$^+$ T cells in IELs were reported to differentiate from two precursors in the thymus: nascent TCRβ$^+$CD4$^-$CD8$^-$PD-1$^+$ thymocytes and TCRβ$^+$CD4$^-$CD8$^-$T-bet$^+$ thymocytes (Lambolez, Kronenberg, & Cheroutre, 2007; Ruscher, Kummer, Lee, Jameson, & Hogquist, 2017). One study suggested that the TCRαβ$^+$CD8αα$^+$ IELs were matured locally in the cryptopatches (Lambolez & Rocha, 2001). The deficiency of AhR in the mice resulted in a significant loss of the TCRαβ$^+$CD8αα$^+$ population in the IELs (Y. Li et al., 2011). Since the expression of AhR in the thymocytes is moderate, it remains unclear whether AhR is involved in the migration of the precursors from thymus to the local compartment play or the maturation or expansion of TCRαβ$^+$CD8αα$^+$ T cells in the gut. AhR appears not to be required for the homeostasis or function of conventional TCRαβ$^+$CD8αβ$^+$ T cells in the IELs. Consistently, the expression level of AhR in the TCRαβ$^+$CD8αα$^+$ T cells is similar to the γδ T cells and higher than TCRαβ$^+$CD8αβ$^+$ T cells (Y. Li et al., 2011). To be noted, when ARNT is specifically deleted in T cells, the differentiation of TCRαβ$^+$CD8αα$^+$ T cells in the IELs were also dramatically impaired, consistent with the intrinsic requirement of the AhR signalling pathway for the formation of this population (Nakajima et al., 2013). The mechanism underlying AhR in the generation of TCRαβ$^+$ CD8αα$^+$ T cells in IELs is not entirely clear but some studies suggested AhR cooperates with other molecular pathways to do so (Reis, Hoytema van Konijnenburg, Grivennikov, & Mucida, 2014). Transcription factor T-bet may be downstream of AhR signalling and work cooperatively to control the formation of TCRαβ$^+$ CD8αα$^+$ T cells (Reis et al., 2014). Interestingly, different from mouse IELs, TCRαβ$^+$ CD8αα$^+$ T cell population is a minor compartment of IELs of human gut, (Mayassi & Jabri, 2018). The reasons for the diverse frequencies of this population between human and murine is unclear.

1.6.5. A unique CD8αα$^+$CD4$^+$ T cell population in the intraepithelium

In contrast to T cells in peripheral lymphoid organs such as spleens or lymph nodes with single CD4$^+$ or CD8$^+$ phenotypes, T cells with a unique CD8αα$^+$ CD4$^+$ phenotype were identified in IELs. Compared to immature CD4$^+$ CD8$^+$ DP T cells in thymus that express both CD8α and CD8β, the DP T cells in IELs are mature and express only CD8α but no CD8β (Das et al., 2003; Mucida et al., 2013; Parel & Chizzolini, 2004). Importantly, these cells are still restricted to recognise MHC class II-peptide complex (Mucida et al., 2013), indicating that these mature DP T cells are most likely derived from CD4$^+$ T cell lineage. This was supported by experimental evidence. After being transferred into Rag$^{-/-}$ mice, mature CD4$^+$ T cells that migrated and resided in the intestinal epithelium could acquire CD8α expression (Das et al., 2003).

Similar to cytotoxic CD8$^+$ T cells, CD8αα$^+$ CD4$^+$ IELs also express cytotoxic molecule granzyme B and the marker of degranulation CD107a, which are lacking in their progenitor CD4$^+$ T cells. *In vitro* experiments demonstrated CD8αα$^+$ CD4$^+$ T cells can effectively kill target cells, showing similar function as cytotoxic CD8$^+$ T cells (Mucida et al., 2013; Reis, Rogoz, Costa-Pinto, Taniuchi, & Mucida, 2013).

1.6.6. AhR is required to the generation of IEL CD8αα$^+$CD4$^+$ T cells

When I studied my PhD project, one key hypothesis to be tested was that AhR also mediated the generation of IEL CD8αα$^+$CD4$^+$ T cells in the gut. In 2017, soon after I started and had generated some preliminary but exciting results to support our hypothesis, a paper came out in *Science*, showing *Lactobacillus reuteri* regulates the generation of IEL CD8αα$^+$CD4$^+$ T cells

via an AhR dependent manner (Cervantes-Barragan et al., 2017). However, the molecular mechanism underlying the AhR-mediated differentiation of IEL CD8αα$^+$CD4$^+$ T cells was not clearly studied.

1.7. Transcription factors intrinsically control the cytotoxic differentiation of T cells

1.7.1. Runx3 and ThPOK control lineage determination of CD8$^+$ and CD4$^+$ T cells in thymus

T cells undergo immature development in the thymus and exit as mature CD4$^+$ or CD8$^+$ mature T cells. Before maturing into CD4$^+$ or CD8$^+$ single positive (SP) mature T cells, CD4$^+$ and CD8$^+$ DP thymocytes are selected based on their capability to interact with MHC class I or class II – peptide complex to allow fate determination. This is a critical step for T cell development, with two key transcription factors that function antagonistically and orchestrate the DP to SP differentiation. They are ThPOK and Runx3. During the T cell development in thymus, the transcription factor ThPOK induces the fate determination of CD4$^+$ T cells and prevents the differentiation into CD8$^+$ T cells whereas the other transcription factor Runx3, oppositely, terminates CD4 expression and promote the differentiation of CD8$^+$ T cells (Egawa, 2014; Egawa & Littman, 2008; He et al., 2005; Kappes, 2010; Luckey et al., 2014; Muroi et al., 2008; Vacchio et al., 2014; L. Wang & Bosselut, 2009). A schematic graph of ThPOK and Runx3 function in T-cell lineage determination is drafted in **Figure 1.5**.

1.7.2.TCF1 and LEF1 regulate the CD4$^+$ T cell lineage by maintaining ThPOK expression in the thymus

Emerging evidence have revealed that transcription factors T-cell-specific transcription factor 1 (TCF1) and lymphoid enhancer binding factor 1 (LEF1) also participate in the regulation of the fate decision of T cells during the thymic development. TCF1, encoded by Tcf7, is the downstream regulator that interacting with β-Catenin mediates signalling in the canonical Wnt pathway (Roose et al., 1998; Tiemessen et al., 2012; Y. Wang et al., 2019). It also plays a crucial role in T cell differentiation in the peripheral, such as promoting Th2 and Tfh differentiation (Maier et al., 2011; Xu et al., 2015; Q. Yu, Sharma, & Sen, 2010). LEF1, encoded by *Lef1*, is not only essential in the Wnt signalling, but also critical for the stem cell maintenance, tumorigenesis and organ development (Aoki, Hecht, Kruse, Kemler, & Vogt, 1999; Behrens et al., 1996; Schepers & Clevers, 2012; Singhi et al., 2014). It can suppress Th2 differentiation (Hebenstreit et al., 2008). When coordinated together, TCF1 and LEF1 can negatively regulate the activation of human naive CD8$^+$ T cell (Willinger et al., 2006).

In thymus, TCF1 and LEF1 are reported as the upstream regulator for ThPOK expression and function (**Figure 1.5**) (Steinke et al., 2014). Such effect was shown critically through the intrinsic HDAC activity of both TCF1 and LEF1, since genetically silencing the HDAC function in TCF1 and LEF1 induced the expression of CD4$^+$ lineage genes in CD8$^+$ T cells (S. Xing et al., 2016).

TCF1 and LEF1 are closely related and functionally redundant members of the high-mobility group (HMG) transcription factor family. Using chromatin immunoprecipitation and deep

sequencing (ChIP-seq), the genome-wide regulation between TCF1 and LEF1 were compared. TCF1 and LEF1 shared 3536 common binding loci but there were still 12841 unique binding loci by TCF1 and 949 unique binding loci by LEF1 that should be unignored (Emmanuel et al., 2018). TCF1 showed around 10 times more unique binding sites compared with LEF1, suggesting a dominant regulatory function of TCF1 in T cell (Emmanuel et al., 2018). Notably, TCF1 can directly bind to the *Lef1* locus to suppress its expression. Therefore, the deficiency of TCF1 results in a significant upregulation of LEF1, thus compensating the loss of TCF1 function (S. Yu et al., 2012). Collectively, the above evidence supports the notion that TCF1 plays a major role in regulating T cells while LEF1 partially compensates in particular in the absence of TCF1.

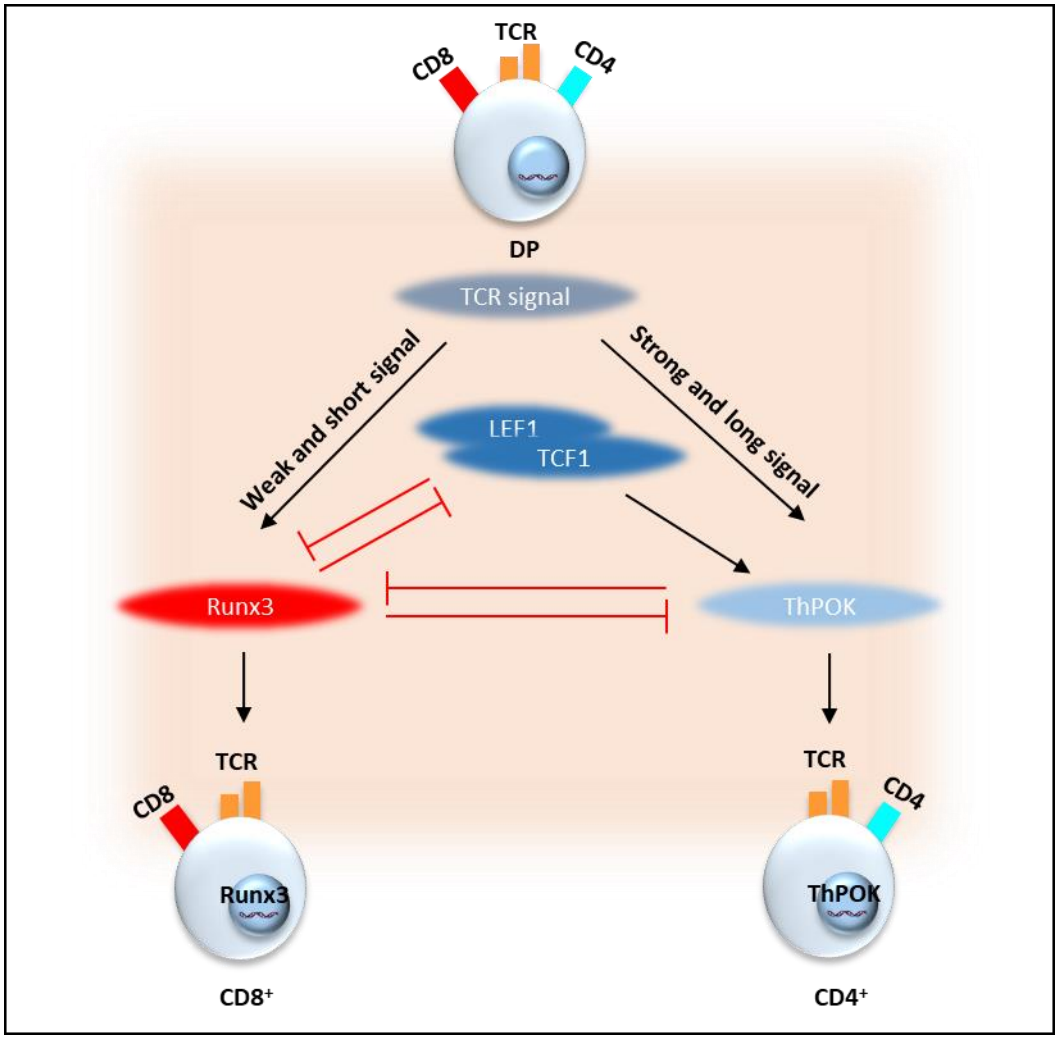


Figure 1.5, A schematic graph of the gene regulation network for the T-cell fate decision in the thymus. ThPOK and Runx3 regulate $CD4^+$ v.s. $CD8^+$ lineage decision. TCF1 and LEF1 promote and maintain $CD4^+$ lineage. Figure adapted from (Mookerjee-Basu & Kappes, 2014; Shan et al., 2017).

1.7.3. Loss of ThPOK promotes the differentiation of CD8αα$^+$CD4$^+$ T cells in IELs

Although cytotoxic CD8αα$^+$CD4$^+$ T cells have been identified for over 20 years (Takimoto et al., 1992), the mechanism that regulates their differentiation had remained largely unknown until recently. In 2013, several studies revealed that the mutually antagonising transcription factor pair Runx3-ThPOK also plays a central role in controlling the differentiation of CD8αα$^+$ CD4$^+$ T cells in IELs (Mucida et al., 2013; Reis et al., 2013; Vacchio et al., 2014). These studies provided the evidence that mature CD4$^+$ T cells in periphery could lose the expression of fate-determining transcription factor ThPOK and further differentiate into cytotoxic CD8αα$^+$ CD4$^+$ T cells in IELs (Mucida et al., 2013; Reis et al., 2013). By tracking the expression of Runx3 and ThPOK, the study found that the Runx3 upregulation succeeded the downregulation of ThPOK (Reis et al., 2013). More importantly, the differentiation of CD8αα$^+$ CD4$^+$ T cells was abolished in the absence of Runx3 (Reis et al., 2013). Collectively, these studies propose a model that the loss of ThPOK initiates the cytotoxic differentiation of CD4$^+$ T cells, while the upregulation of Runx3 realise the differentiation by further suppressing the expression of ThPOK. Since CD8αα$^+$ CD4$^+$ T cells are predominantly present in IELs but hardly in spleen or lymph nodes, environmental clues are required to promote their generation. However, it was unclear what is the environmental signal that acts upstream of the loss of ThPOK to drive IEL CD4$^+$ T cells to differentiate into CD8αα$^+$ CD4$^+$ T cells.

1.7.4. AhR: bridging the environment and the intracellular regulation

Transcriptional and post-transcriptional regulations that govern the T cell development and differentiation have been heavily studied. In contrast, much less is known for the role of the environmental regulators except for cytokines.

As being introduced above, a great deal of evidence supports the bridging role of AhR to mediate intracellular responses to extracellular stimuli and consequently fine-tune proliferation, metabolism, adhesion, migration and so on (Kung, Murphy, & White, 2009; Larigot, Juricek, Dairou, & Coumoul, 2018). For the immune system, it has been reviewed by others and us that AhR ligands, as important extracellular signals, are critical for modulating the immune response particularly at the sites of body-environment interfaces, such as the gut (Gutierrez-Vazquez & Quintana, 2018; H. Wang et al., 2015). Therefore, AhR critically bridges the intracellular regulatory network and environmental stimuli.

1.8. Project outline – How does the AhR signalling exactly regulate intracellular regulatory network in T cells? An unanswered question.

1.8.1. Overall hypothesis: AhR suppresses the expression of TCF1 to promote the differentiation of IEL CD8αα$^+$CD4$^+$ T cells

My PhD project was initially designed to dissect the role of the AhR signalling in regulating the generation of IEL CD8αα$^+$ CD4$^+$ T cells. The publication in *Science* scoped my discovery that the AhR signalling is required for the generation of the AhR signalling. Therefore, my PhD project has slightly changed to focus on the mechanism underlying the regulation executed by the AhR signalling.

This is an important question. Despite the important role of AhR in the regulation of gut immune cells, especially cytotoxic IELs including CD8αα$^+$ CD4$^+$ T cells, very little is known for the mechanism underlying the observations. Using bioinformatic tools, we predict multiple AhREs within the promoter region of the *Tcf7* gene. Published data also support a suppression of the expression of TCF1 in AhR-regulated IEL T cells We thus hypothesize that the AhR ligands in the gut stimulate the AhR pathway to suppress TCF1 expression, which induces the loss of ThPOK to initiate the differentiation of CD8αα$^+$ CD4$^+$ T cells. The specific objectives for this PhD project are outlines as follows.

1.8.2. Objectives

Aim 1: To characterise the role of AhR in regulating the generation and function of cytotoxic IEL cells.

Hypothesis: AhR is required for the generation of both TCRαβ$^+$CD8αα$^+$ IELs and CD8αα$^+$CD4$^+$ IELs through a mechanism by downregulating ThPOK.

Aim 2: To test whether TCF1 is required for the development of cytotoxic IEL cells.

Hypothesis: TCF1 is required for the generation of both TCRαβ$^+$CD8αα$^+$ IELs and CD8αα$^+$CD4$^+$ IELs

Aim 3: To investigate the mechanism underlying AhR-mediated downregulation of TCF1.

Hypothesis: AhR directly binds to the promoter of *Tcf7/Lef1* loci to supress their transcription.

Results of each aim will constitute one result chapter (Chapter 3-5). Part of Chapter 1 was published as a review article: **Wang, H.**, Wei, Y., & Yu, D. (2015). Control of lymphocyte homeostasis and effector function by the aryl hydrocarbon receptor. Int Immunopharmacol. 28: 818-824.

1.8.3. Significance

Unconventional T cell populations in gut, TCR$\gamma\delta^+$, TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ or CD8$\alpha\alpha^+$CD4$^+$ T cells, are regulated by AhR for their generation but the mechanism underlying this regulation is largely unknown. The proposed project will investigate the role and mechanism of AhR in the differentiation of cytotoxic T cells (TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ or CD8$\alpha\alpha^+$CD4$^+$ T cells) in intraepithelium. Such knowledge will also help us to understand how AhR participates in the transcriptional regulatory network in T cells and shed lights on how T cells respond to environmental signals. The results from this study will help to develop new strategies to regulate gut immune homeostasis and function by targeting the AhR pathway, such as through AhR agonists or antagonists.

CHAPTER II

Material and Methods

2.1. Material

2.1.1. Reagents

AhR ligands were dissolved in DMSO and stored at -20°C or -80°C followed by instruction. AhR ligands used in experiments are listed in the **Appendix table 1**.

Fluorescent antibodies that were used in the flow cytometry experiments are listed in the **Appendix table 2**.

Cytokines and antibodies used in cell culture are listed in the **Appendix table 3**.

2.1.2. Buffers

Buffers used are listed as following. Final concentrations are shown in brackets.

- Complete RPMI: 500ml of RPMI; 50ml of heat-inactivated FCS (10%); 5ml Penicillin Streptomycin Glutamine (1%); 5ml of sodium pyruvate (1%); 500µl of β-mercaptoethanol (1%); 10ml of 1M HEPES (2%)
- Complete DMEM: 500ml of DMEM; 50ml of heat-inactivated FCS (10%); 5ml of Penicillin Streptomycin Glutamine (1%); 5ml of sodium pyruvate (1%)
- 2%RPMI: 500ml of RPMI; 10ml of heat-inactivated FCS (2%)
- Sorting Buffer: 500ml of PBS; 10ml of heat-inactivated FCS (2%); 500µl of 2mM EDTA (1mM)
- FACS Buffer: 500ml of PBS; 10ml of heat-inactivated FCS (2%); 500µl of 2mM EDTA (1mM); 0.1% sodium azide
- TBE: 20L of distil water; 216g Tris (10.8g/L); 110g boric acid (5.5g/L); 80ml of 0.5M EDTA (0.2%); pH8
- 90% Percoll: 45ml of 100% percoll; 5ml of 10X PBS (1X)
- IEL digestion buffer: 500ml of PBS; 10ml of heat-inactivated FCS (2%); 1ml of 0.5M EDTA (1mM); 500µl of 1M DTT (1mM)

2.1.3. Experiment apparatus

All the experiment instruments are listed in the **Appendix table 4**.

2.2. Mice

C57BL6 mice were purchased from MARP (Monash Animal Research Platform) or APF (The Australian Phenomics Facility). All mice were bred under high barrier in the animal facility by MARP or APF unless specified. Mice used in experiments were 8-16 weeks old unless specified. All the mice breeding and experiment protocols were approved by ethic committees.

For the AhR flox mice (The Jackson Laboratory), the loxP sites were inserted on either side of exon 2 of *Ahr*. For CD4 cre mice (The Jackson Laboratory), CD4 enhancer, promoter and silencer sequences drive the expression of a Cre recombinase gene. $CD4^{cre}Ahr^{flox/flox}$ mice were generated by breeding above two strains and genotyped by following primers: $CD4^{cre}$ geno For: ATCTGGCATTTCTGGGGATTG; $CD4^{cre}$ geno Rev: GGCAACACCATTTTTTCTGACC; $Ahr^{flox/flox}$ For: CAGTGGGAATAAGGCAAGAGTGA; $Ahr^{flox/flox}$ Rev: GGTACAAGTGCACATGCCTGC

The Tcf7-GFP mice were imported from Xue group as a gift and described in (Yang et al., 2015). For Tcf7-GFP mouse strain, the full-length TCF1 isoforms (p45 and p42) are transcribed from an upstream promoter using exon 1. To generate a Tcf7GFP reporter allele, an enhanced green fluorescent protein (EGFP) expression cassette was inserted and a neomycin-resistant gene (neo^r) cassette into the least conserved region in the first intron of Tcf7. The EGFP expression cassette contains a strong En2 gene-splicing acceptor to facilitate

splicing of Tcf7 exon 1 to the reporter, an internal ribosome entry site to facilitate independent translation of EGFP, and the EGFP cDNA followed by a polyadenylation sequence. Two Frt sites were inserted to flank the EGFP and neo^{r} cassettes. One loxP site was inserted immediately downstream of the second Frt site in intron 1, and another loxP site was inserted into the least conserved region in intron 2. These features, although not used in this study, were designed to remove the EGFP and neor cassettes with Flippase, converting the Tcf7-GFP reporter allele to a Tcf7 exon 2–floxed allele, to conditionally target the long TCF1 isoforms. Mice were genotyped by following primers: p45 typing p1: TCCTCGTGGGTCCCATCTC; En2P2: CAGACCTTGGGACCACCTCA; p45 typing p4: TCCAGACCTCCACTTCGCG.

The TCF1 flox mice were imported from Xue group as a gift and described in (Steinke et al., 2014). The Tcf7 gene was conditionally targeted by the International Knockout Mouse Consortium (IKMC, project 37596). The exon 4 of Tcf7 was flanked by two LoxP sites, and deletion of this exon results in a nonsense frame-shift mutation. Mice were genotyped by following primers: Tcf7-Type-F: AGCTGAGCCCCTGTTGTAGA; Tcf7-Type-R1: TTCTTTGACCCCTGACTTGG; Tcf7-Type-R2: CAACGAGCTGGGTAGAGGAG.

The lef1 flox mice were imported from Xue group as a gift and described in (Steinke et al., 2014). For the lef1 flox, a bacterial artificial chromosome (BAC) clone containing the entire Lef1 locus, RP23-185B8, was obtained from Life Technologies. A 13.8 kb segment encompassing introns 4-9 of the Lef1 gene was first retrieved into the pL253 plasmid. LoxP sites and the neomycin-resistant gene (NEO) cassette were inserted to flank exons 7 and 8 using standard recombineering approach. All PCR amplified targeting arms were verified by DNA sequencing, and all sequences surrounding the insertion sites were verified to ensure no

unwanted mutations. The construct was then electroporated into embryonic stem (ES) cells (F1 hybrid of C57BL/6 and 129/Sv), and ES clones with expected homologous recombination were screened by Southern blotting following previously published protocols (Xue et al., 2008). Blastocyst injection of the ES cells was performed at the Transgenic Animal Model Core facility, University of Michigan (headed by Dr. Thom Saunders). Germline-transmitted animals were then crossed with the Flippase transgenic mice to remove Frt site-flanked NEO cassette, which was verified by Southern blotting. The resulting allele was designated floxed Lef1 allele ($Lef1^{fl/+}$). Mice were genotyped by following primers: Lef1a: ACGTTGCTCCTGTATAGACG; Lef1b: GCAGATATAGACACTAGCACC; Lef1c: TCCACACAACTAACGGCTAC.

2.3. *In vitro* cell culture

2.3.1. Cell sorting

Donor mice were sacrificed, and lymph nodes, MLN and spleen were dissected and stored in Eppendorf tube with 1ml of 2% RPMI (2% FCS). LN and spleens were homogenized through strainer on a 6-well plate, cells were flushed with 2%RPMI to wash out all cell chunks stuck on strainer. The homogenized cells were transferred into a 15ml Falcon tube and centrifuged at 800x g for 5min. Supernatants were discarded, and tubes were dried on tissue. The cell pellets were resuspended with sorting buffer and centrifuged at 800x g for 5min. Supernatants were discarded, and tubes were dried on tissue again. LN pellets were resuspended in 800µl of fluorescent antibody staining master mix and placed on a rotator in cold room (10°C) for 30min in dark. Splenocyte pellets were resuspended in 2ml of RBC and proceeded red blood lysis for 8min. RBC lysine was neutralized by adding 15ml of sorting buffer and tubes were centrifuged at 800x g for 5min to remove the remained RBC buffer. Repeating washing once, and then the

splenocyte pellets were resuspended in 1ml of fluorescent antibody staining master mix. Tubes were placed on a rotator in cold room (10°C) for 30min in dark. After staining, cells were washed twice with sorting buffer and isolated by centrifuging at 800x g for 5min. And the washed cells were resuspended in 1ml of sorting buffer and transferred to sorting tube. For cell collection, 15ml falcon tubes were prepared by adding 1ml of 2% FCS. The collection tubes were fully rotated to coat FCS on wall in order to increase the survival of sorted cells. Whole procedures should be performed on ice and sterile environment if not mentioned specifically.

The fluorescent antibody staining mix for sorting was prepared in sorting buffer **Table 2.1**:

Sorting staining panel		
Channel	**Antibody**	**Dilution**
7AAD		1:800
BV605	CD8α	1:400
V500	CD44	1:200
E450	CD62L	1:400
APC-Cy7	CD4	1:400
PE-Cy7	CD25	1:200

Table 2.1, sorting staining panel

Naïve $CD4^+$ T cells were sorted as $7AAD^-$ $CD4^+$ $CD8\alpha^-$ $CD44^-$ $CD25^-$ $CD62L^+$ on the influx FACS sorting machine.

2.3.2. In vitro cell differentiation

Sorted cells were washed with CR and centrifuged at 800x g for 5min. The cytokine mix was prepared in CR for different polarization conditions. Th1 polarization condition: IL-12 50ng/ml, anti-IL-4 10µg/ml, IL-2 20ng/ml; TH17 polarization condition: IL-6 50ng/ml, TGF-b 1ng/ml, anti-IL-4 10µg/ml, anti-IFN-g 10µg/ml, IL-2 20ng/ml. AhR ligand mix was prepared in CR for different ligand dosages. 0.1% DMSO was prepared in CR, which is used as vehicle control since AhR ligands are dissolved in DMSO. Sorted cells were counted and resuspended in the prepared cytokine mix. 50k of sorted cells were seeded on the normal binding 96-well plate. The plate was pre-coated with anti-CD3 and anti-CD28 5µg/ml overnight at 4°C. AhR ligand mix was added right after seeding cells. After seeding, the plate was shortly span (10sec), and placed in a 37°C CO_2 incubator for 3 - 5 days according to conditions of specific experiments. Medium was changed if the colour started turning yellow (usually on day 3). Cytokines and AhR ligands were re-added when replacing fresh medium.

For pre-differentiation experiment, cytokines were added at the start of cell culture for 3 days as mentioned above, but AhR ligands were not. On day 3, old medium was carefully removed and fresh medium with IL2 20ng/ml and AhR ligands was added. Cells were cultured for 24hr more.

2.3.3. In vitro re-stimulation

After differentiation, medium was carefully removed by pipetting and then 20ng/ml PMA, 1µg/ml inomycin, 3µg/ml brefeldin A and 2µM monensin in fresh medium was added. The 96-well plate was placed in CO_2 incubator for 4hr at 37°C.

2.3.4. In vitro IEL culture

Isolated IELs were washed with completed RPMI twice and centrifuged at 800x g 4°C for 5min. 300k cells were counted and seeded in a U-bottom Plate. Medium was pre-mixed with DMSO, FICZ 100nM and FICZ 1000nM, respectively. Cells were collected at 12hr, 24hr, and 48hr.

2.4. Tissue isolation

2.4.1. IEL isolation

Mice were sacrificed to collect small intestine which was then placed on paper towel and pre-wetted with 2%RPMI. Fat was carefully removed from the one side of intestine. And feces were pushed out, while dissecting out Peyers' patches. The remained intestine was opened longitudinally and washed in cold PBS for 3 times to completely remove feces. The washed small intestine was chopped into 5mm pieces tissue and transferred into 50ml Falcon tubes with 50ml of IEL digestion buffer. The digestion buffer was prepared freshly. The digestion is proceeded in a shaker at 20x g and 37°C for 20min. Completely shaking and suspending the tissue was required in order to guarantee complete digestion. The digested solution was passed through a stainless-steel sieve to remove the remained intestinal tissue. The pass-through was kept and centrifuged at 1300x g 4°C for 12min. Supernatants were discarded, and the cell pellets were resuspended with 5ml of 2%RPMI. The suspension was filtered by the BD strainer once to completely remove undigested tissue.

90% percoll was prepared during IEL digestion. For 70% percoll phase, 2ml of 2%RPMI was mixed with 7ml of 90%percoll for each sample. For 40% percoll phase, 4ml of 90% percoll

was added into 5ml of resuspended sample. The 9ml of the 40% percoll phase was slowly overlaid onto 3ml of the 70% percoll phase, without destroy the interface. The tubes were centrifuged at 1300x g room temperature for 20min at 2 accelerate and 0 brake. IECs were removed from top of the upper 40% percoll section, and IELs were collected from the 40%-70% percoll interface and transferred into a 15ml tube. The IELs were washed with sorting buffer or FACS buffer for further usage.

2.4.2. Tissue Collection

Spleen, thymus, lymph nodes and intestine were dissected from mice and temporarily kept in RPMI media for isolation or homogenisation before antibody staining. Livers were dissected from donor mice and collected in Eppendorf tubes, and immediately placed in dry ice to reduce RNA degradation. Small intestine and colon tissue were cut from donor mice for about 1cm long and collected in Eppendorf tube and immediately frozen in dry ice. All samples should be quickly collected and stored in -80°C freezer for RNA extraction.

2.5. Cloning

The original constructs of vector for TCF1WT and TCF1mut5aa were provided by Prof Xue and described (S. Xing et al., 2016). Both plasmids were reconstructed into pR-GFP vector (**Appendix Figure 1**). Cloning was conducted using NEBuilder HiFi DNA Assembly Cloning Kit (NEB#E5520) followed by manufacturer's protocol.

2.6. Retrovirus packaging

2x 10^6 GPE86/Phoenix cells were seeded in T75 flask with complete DMEM (10% FBS, 1% PSG, 1% Sodium pyruvate). When the confluency reached 50-60%, cells were treated with 25μm chloroquine, 60μg plasmid, 75μl of 2.5M CaCl2 and 750μl of 2x HeBs. Then the cells were left at 37°C, for 4hrs. GFP was checked under fluorescence microscope and cell sorting was applied to purify the GFP high populations.

2.7. Transduction

To prepare primary $CD4^+$ T cells for transduction, naive $CD4^+$ T cells were purified from mice and stimulated with plate bound 5μg/ml anti-CD3 and 5μg/ml anti-CD28 for 48 hours. For the transduction, cells were spinoculated on an 8μm/cm^2 Retronectin pre-coated plate, with the supernatant that was collected from viral vectors stably transfected GPE86 cell culture, at 800x g for 1 hour at 33°C. Cells were rested in fresh complete RPMI media containing 20 ng/mL of rmIL2. After 48 hours, GFP^+ $CD4^+$ T cells were sorted and 300k sorted of each type of cells were mixed well and transferred into $Rag^{-/-}$ mouse.

2.8. Flow cytometry

2.8.1. Surface staining

Samples were transferred to a 96-well V-bottom plate and centrifuged at 800x g for 5min. Supernatant was removed by flipping the plate and wiping upon paper towel. Multiple flips should be avoided to keep cells in the plate. FACS buffer was added to resuspend and wash the cells pellet. The plate was span down to remove the supernatant. Cells were resuspended in fluorescent antibody staining mix and place on ice or cold room for 30min in dark. Wash

twice with FACS buffer and resuspend in 200µl or 100µl of FACS buffer for cytometry analysis.

The fluorescent antibody staining mix was prepared in FACS buffer as **Table 2.2**:

Surface staining		
Channel	**Antibody**	**Dilution**
7AAD		1:800
BV605	CD8α	1:400
APC-Cy7	CD4	1:400

Table 2.2, surface staining panel

2.8.2. Intracellular staining

Samples were transferred to a 96-well V-bottom plate and centrifuged at 800x g for 5min. Supernatant was removed, and the cells were washed once with FACS buffer. Surface staining was performed as mentioned above before intracellular staining. Different surface staining panels were applied based on particular cell markers. After surface staining, cells were washed twice with FACS buffer and proceeded to permeabilization. 100µl of BD IC permeabilization or eBioscience Foxp3 staining permeabilization was added to suspend cell pellet and the plate was placed on ice or cold room for 30min in dark. Cells were washed twice with Perm Wash buffer to remove the permeabilization buffer. The fluorescent antibody mix for IC staining was prepared in Perm Wash buffer. Cell pellets were resuspended in 100µl of IC fluorescent antibody staining master mix and placed on ice or cold room for 40min in dark. Cells were washed twice with Perm Wash buffer and finally resuspend in 200µl of FACS buffer for flow cytometry analysis.

The surface fluorescent antibody staining master mix for IEL was prepared in FACS buffer; IC fluorescent antibody staining master mix was prepared in Perm Wash buffer as in **Appendix Table 5**.

The surface fluorescent antibody staining master mix for *in vitro* cultured Th17 cells was prepared in FACS buffer; The IC fluorescent antibody staining master mix for *in vitro* cultured Th17 cells was prepared in Perm Wash buffer as in **Appendix Table 6**.

2.9. PCR

2.9.1. TRIzol total RNA extraction

1ml of TRIzol reagent was added to tubes, and tissue was homogenized using the power homogenizer until fully dissolved. 200µl of chloroform was added per 1ml of TRIzol reagent used and vigorously mixed. The samples were centrifuged at 12,000x g for 16min at 4°C. The aqueous phase was isolated by pipetting carefully and transferred to an autoclaved Eppendorf tube. 500µl of 100% isopropanol was added to the sample per 1ml TRIzol used. The tubes were sitting at room temperature for 10min and then centrifuged at 12000x g 10min at 4°C. Supernatant was remove by pipetting and the RNA pellets were washed by 1ml of 75% ethanol. Tubes were centrifuged at 7500x g 5min at 4°C. The RNA pellets were air-dried and resuspended in 20-50µl of RNase-free water. Resuspended RNA was incubated in heating block for 10min at 60°C in order to increase solubility.

2.9.2. RNeasy Mini Kit RNA extraction

Cell pellets were resuspended in 350µl of Buffer RLT and mixed with 350µl of 70% ethanol. All samples were transferred to the RNeasy Mini spin columns and centrifuged for 15s at 8000x g. 700µl of Buffer RW1 was added to the columns and then the columns were span at 8000x g for 15s. 500µl of Buffer RPE was added to the columns and then the columns were span at 8000x g for 15s. 500µl of Buffer RPE was added again and the columns were centrifuged at 8000x g for 2min. The columns were then placed in the sterile collection tubes, 50µl of RNase-free water was added to release RNA by centrifuging for 1min at 8000x g. (Quick-StartProtocol QIAGEN)

2.9.3. RT-PCR and quantitative PCR

The RT template was dissolved completely by pipetting 18µl of DD water in the tube. 2µl of extracted RNA was added into the tube and quick spin mixed. The reverse transcription process was proceeded as following, 22°C 5min, 45°C 30min and 85°C 5min.

The formed cDNA was added to the qPCR plate together with DD water, dyes and qPCR primers. The qPCR machine was run following manuscripts. qPCR primers are designed as in **Appendix Table 7**.

2.10. Chromatin Immunoprecipitation

EL4 cells were fixed using 1% formaldehyde and stored in -80 between lysis. Cell pellets were resuspended in 400 µl fresh ChIP-lysis buffer and lysis on ice. Genomic DNA was fragmented by sonication with 20 cycles 30sec on and 20sec off in cold alublock. Chromatin fragments were collected by centrifuge the samples at 4000x g for 10min at 4°C. Washed samples were

incubated with antibodies for 2 hours at 4°C with rotation and followed by an overnight incubation with the protein G DNA binding slurry at 4°C with rotation. Immunoprecipitated DNA was then washed and elute with elution buffer overnight at 65°C. Phenol-Chloroform was later applied to purified enriched DNA samples. The samples were ready for further analysis on qPCR or library preparation.

Primers used to detect the enriched DNA fragments were listed in **Appendix Table 8**

2.11. RNA-seq

IELs were collected and purified from C57BL/6 mice. RNeasy Micro Kit (Qiagen) was used to extract RNA from IELs. RNA integrity was analysed using Agilent RNA 6000 Nano Kit (Agilent). PolyA-enrichment was used for library preparation and 50 bp one-end sequencing was performed by NovaGene

To analyse RNA sequencing data, RPKM (Read per kilobase per million) were obtained from raw dataset and log2 transformed after replacing zero by the minimum value in the dataset. The log2 RPKM data were quantile normalized. The R statistical software (version 3.3.4) was used to calculate differentially expressed genes between CD8αα, CD8αβ, CD8ααCD4 and CD4 T cells. Genes satisfying the following criteria were chosen for analysis: first, the average count is more than 100 in at least one sample group and second, the global false discovery rate (FDR) is controlled at 0.05 with a minimum fold-change of 2.

2.12. Chromatin immunoprecipitation and sequencing (ChIP-seq)

ChIP-exonuclease (ChIP-exo) was performed on EL4 cells treated with ligands at 30min and 90min. The precipitated genomic DNA was amplified with the KAPA Real Time Amplification kit (KAPA Biosystems). Cluster generation and sequencing was carried out using the Illumina HiSeq 2000 system with a read length of 50 nucleotides according to the manufacturer's guidelines.

To analyse ChIP-seq data, Bowtie2.2.8 has been used for alignment with mouse mm9 as reference genome, MACS2.1.0 for peak calling with p value 10e–10 as cut-off. The peak table with the peak starting and ending location was mapped to genes with the following strategies: Peaks were assigned to genes in a stepwise manner by prioritizing genes containing peaks in their promoter and/or gene body. For this, peaks with -2.5 kb to TSS and $^{+}$2.5 kb to TES were first assigned to the corresponding gene. Other peaks within 50kb to gene body were assigned to the nearest gene for long distance regulation. R functions Venn in g-plots package and barcode plots in limma package were used to generate the figures.

2.13. Statistical analysis

Preliminary experiments were performed using 2-5 mice to determine the expected means and sample distributions of the control and experimental groups. The means and sample distributions were then used to calculate the sample size required to test the hypothesis in subsequent experiments using MedCalc (exact numbers of samples were indicated in figure). Statistic of data were analysed by GraphPad Prism. Paired t test or student t test were applied to analyse the significance of data as specified in figure legend. ***** = $P < 0.05$; ****** = $P < 0.01$; ******* = $P < 0.001$, ns = $P \geq 0.05$.

CHAPTER III

AhR mediates the generation of cytotoxic IELs

3.1. Preamble

Cytotoxic IELs including TCRαβ$^+$CD8αα$^+$ or CD8αα$^+$CD4$^+$ IELs were discovered more than two decades ago (Takimoto et al., 1992). However, the mechanism of how these populations are generated and the physiological function of these cells have remained largely unclear. These cells distribute abundantly in the intraepithelial site of small intestine, which suggests such unconventional cytotoxic T cells might participate in the regulation of gut homeostasis.

In 2013, two studies significantly advanced our understanding of the generation of CD8αα$^+$CD4$^+$ IELs, which revealed that ThPOK and Runx3 cooperatively orchestrate the development of CD8αα$^+$CD4$^+$ IELs (Mucida et al., 2013; Reis et al., 2013). It was suggested that the intrinsic loss of ThPOK is the key driver to promote the cytotoxic reprogramming of CD4$^+$ T cells into CD8αα$^+$CD4$^+$ T cells. In the same year, a breakthrough was also made to the generation of TCRαβ$^+$CD8αα$^+$ IELs. A study demonstrated that the deletion of ARNT, known as the key mediator in the AhR pathway by forming an active heterodimer complex with AhR to bind DNA and regulate transcription, largely diminished the generation of TCRαβ$^+$CD8αα$^+$ IELs in the gut (Nakajima et al., 2013). Although these studies nicely uncovered the key transcriptional factors sustaining the generation of TCRαβ$^+$CD8αα$^+$ and CD8αα$^+$CD4$^+$ IELs, the upstream regulators that activate these pathways and initiate the generation of special IEL subsets were not well understood. When I started my PhD in 2016, I set the goal to understand the generation of TCRαβ$^+$CD8αα$^+$ or CD8αα$^+$CD4$^+$ IELs. Based on the preliminary data from my group, my hypothesis was that AhR played a key role. It was a major surprise for me that, in the late of 2017, one study published in *Science* revealed the pivotal role of AhR in controlling the generation of CD8αα$^+$CD4$^+$ IELs and suggested the AhR ligand in the gut could derived from gut microbial *L. reuteri* (Cervantes-Barragan et al., 2017). At that time, I had

already obtained the results from examining AhR deficient mice and found the requirement of AhR in the development of TCRαβ$^+$CD8αα$^+$ or CD8αα$^+$CD4$^+$ IELs.

In this chapter, I characterised the requirement of the AhR pathway in the generation of TCRαβ$^+$CD8αα$^+$ and CD8αα$^+$CD4$^+$ IELs. Although some of results were scooped by the paper published in *Science* (Cervantes-Barragan et al., 2017), I hereby not only performed the mouse study in more details and also extended the investigation in human T cells.

3.1.1. Characterisation of IEL subsets by flow cytometry

IEL populations can be distinguished by measuring the expression of surface markers including TCRγδ, TCRαβ, CD4, CD8α and CD8β. IELs express the hematopoietic marker CD45. Following the gating on CD45.2$^+$/CD45.1$^+$ hematopoietic population, more than 80% of cells express either TCRγδ or TCRαβ, suggesting T cells predominate IELs. The ratio of these two TCR$^+$ populations is around 1:1 (Kuo, El Guindy, Panwala, Hagan, & Camerini, 2001.). In TCRγδ$^+$ T cells, all cells express CD8α, while only a very small population co-expresses CD8β. In TCRαβ$^+$ T cells, a subpopulation expresses CD4. In the CD4$^+$ population, a variable percentage of cells co-express CD8α but not CD8β. The percentage of CD8αα$^+$CD4$^+$ in total CD4$^+$ T cells varies in mice with different age or housing environment, which will be illustrated in next session. In the CD4$^-$ T cell population, all cells express CD8α with around 50% of them co-expressing CD8β, typically in 12-week old mice. A sample of FACS gating strategy is showed in **Figure 3.1**.

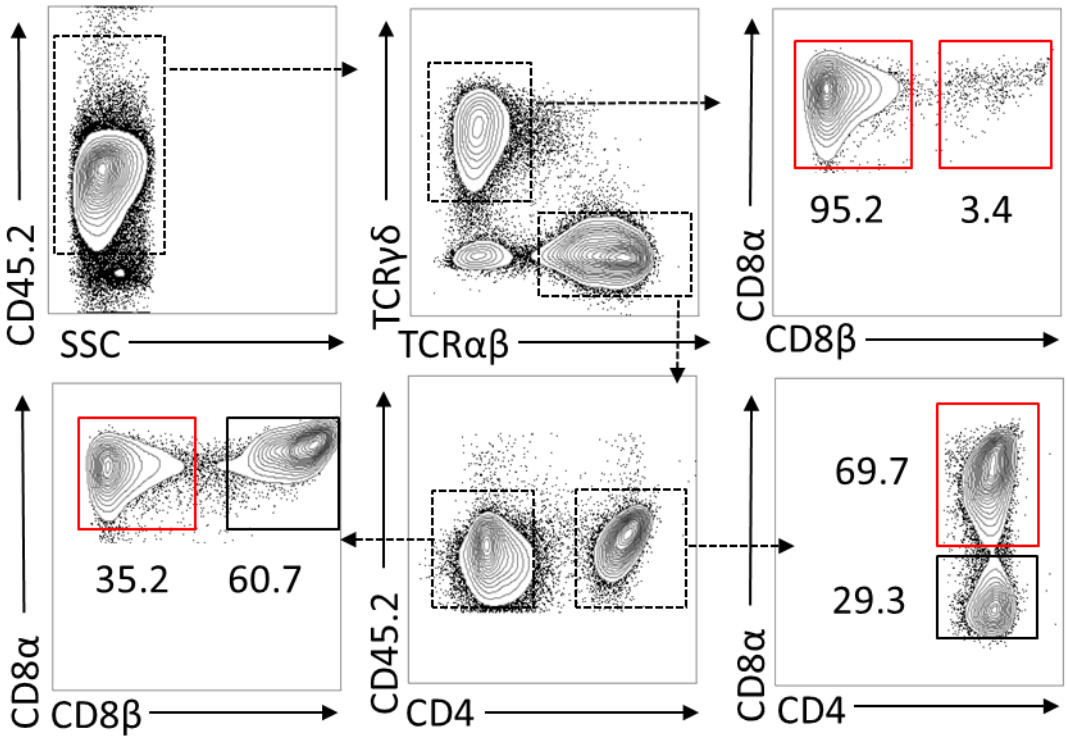


Figure 3.1, Flow cytometric identification of cytotoxic IELs. IELs were collected from a 12-week old C57BL/6 mouse and stained with indicated markers. CD45.2$^+$ lymphocytes were divided into three groups: TCRγδ$^+$, TCRαβ$^+$ and TCRγδ$^-$TCRαβ$^-$. The TCRγδ$^+$ T cells were further grouped as CD8αα$^+$ and CD8αβ$^+$ (a minor population) T cells. The TCRαβ$^+$ T cells can be further grouped as CD8αα$^+$CD4$^+$, CD4$^+$, CD8αβ$^+$ and CD8αα$^+$ T cells. Red gates label the unconventional cytotoxic IELs.

3.1.2. Accumulation of cytotoxic IELs along with age despite low affinity AhR allele

The intestinal environment and microbiota in mice have been shown to be substantially affected by the housing environment (H. Chung et al., 2012). The effect of the stimulation from diet including AhR ligands and symbiotic or pathogenic microbials likely accumulate and gradually change the gut homeostasis. In order to understand how housing environment and age effect the differentiation of cytotoxic IELs, IELs from mice in different animal facilities and at different ages were collected and compared, in which the result indicated that the proportion of $CD8\alpha\alpha^{+}CD4^{+}$ IELs largely expanded in elder mice from East Wing (**Figure 3.2)**. We also found that the accumulation of $CD8\alpha\alpha^{+}CD4^{+}$ IEL was positively correlated with increased age in mice housed in the East Wing animal facility. Such accumulation showed a linear correlation rather than exponential. However, murine from the APF (The Australian Phenomics Facility) exhibited rather higher ratio of $CD8\alpha\alpha^{+}CD4^{+}$ IELs at early ages (**Figure 3.2**), although these facilities hold a same level of air filter and pathogen barriers. It is wondered that the gut microbiota of mice from different facilities may be different, and some could preferentially promote the generation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs. Thus, even at the early age, these mice already developed a high proportion of $CD8\alpha\alpha^{+}CD4^{+}$ IELs.

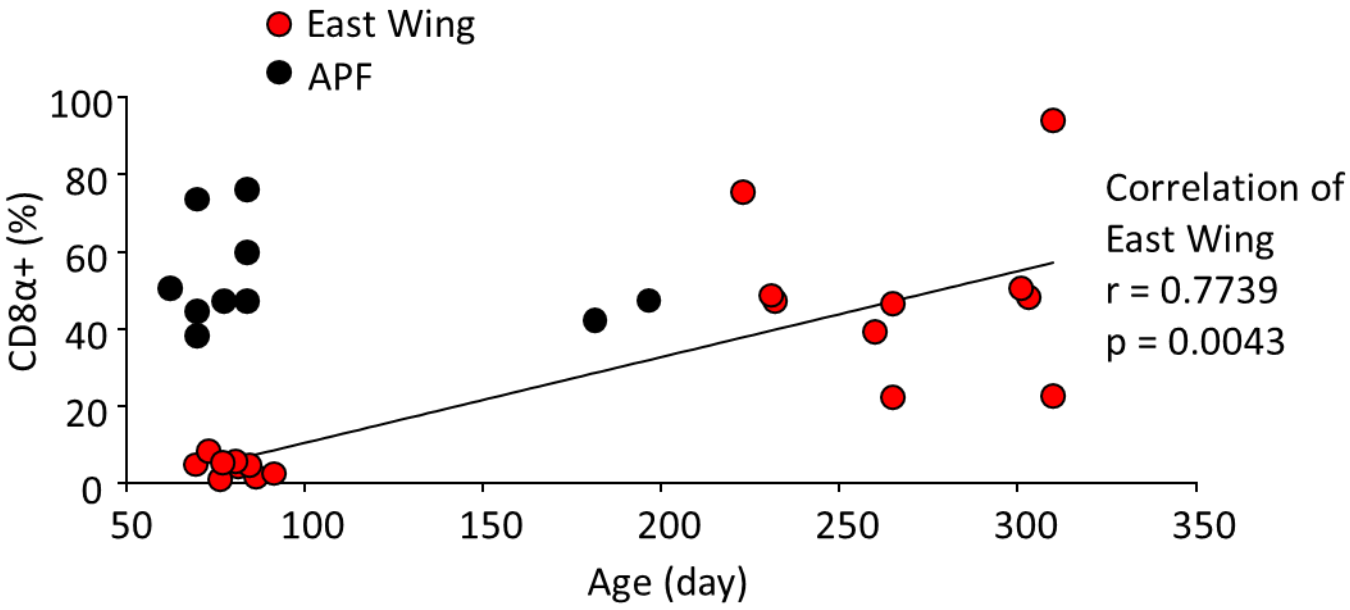


Figure 3.2, The proportion of CD8αα^{+}CD4^{+} IELs accumulate with age in mice and differed between facilities. IELs were collected from C57BL/6 mice with age ranged from 8 weeks to 46 weeks followed by FACS staining for flow cytometric analysis. Mice were housed in different animal facilities including East Wing at Monash University and Australian Phenomics Facility (APF) at Australian National University. Correlation was calculated based on the data from mice housed in East Wing.

3.1.3. Low-affinity allele of Ahr gene significantly impaired the differentiation of CD8αα^{+}CD4^{+} IELs

It has been shown that different mouse strains carry polymorphic AhR alleles with distinct binding affinities to AhR ligands (Ema et al., 1994; Flaveny & Perdew, 2009). I sequenced the PASb domain of the *Ahr* gene that mediates the binding of ligands and found that C57BL/6 mice at Monash University and Australian National University carried polymorphism (**Figure 3.3A**). A single nuclear acid change from C to T leads to the change of Alanine into Valine at amino acid position 375 counted from the N-terminal of AhR protein. Mice carrying V^{375} polymorphism showed much lower percentages of CD8αα^{+}CD4^{+} IELs than mice carrying the V^{375} polymorphism (**Figure 3.3B**). According to the structure of PASb domain (Pandini et al., 2009; Y. Xing et al., 2012), the AA 375 site is proximal to the ligand binding pocket (**Figure 3.3C**). The A375V change might reduce ligand binding affinity, which suggested the AhR signalling regulates the generation of CD8αα^{+}CD4^{+} IELs.

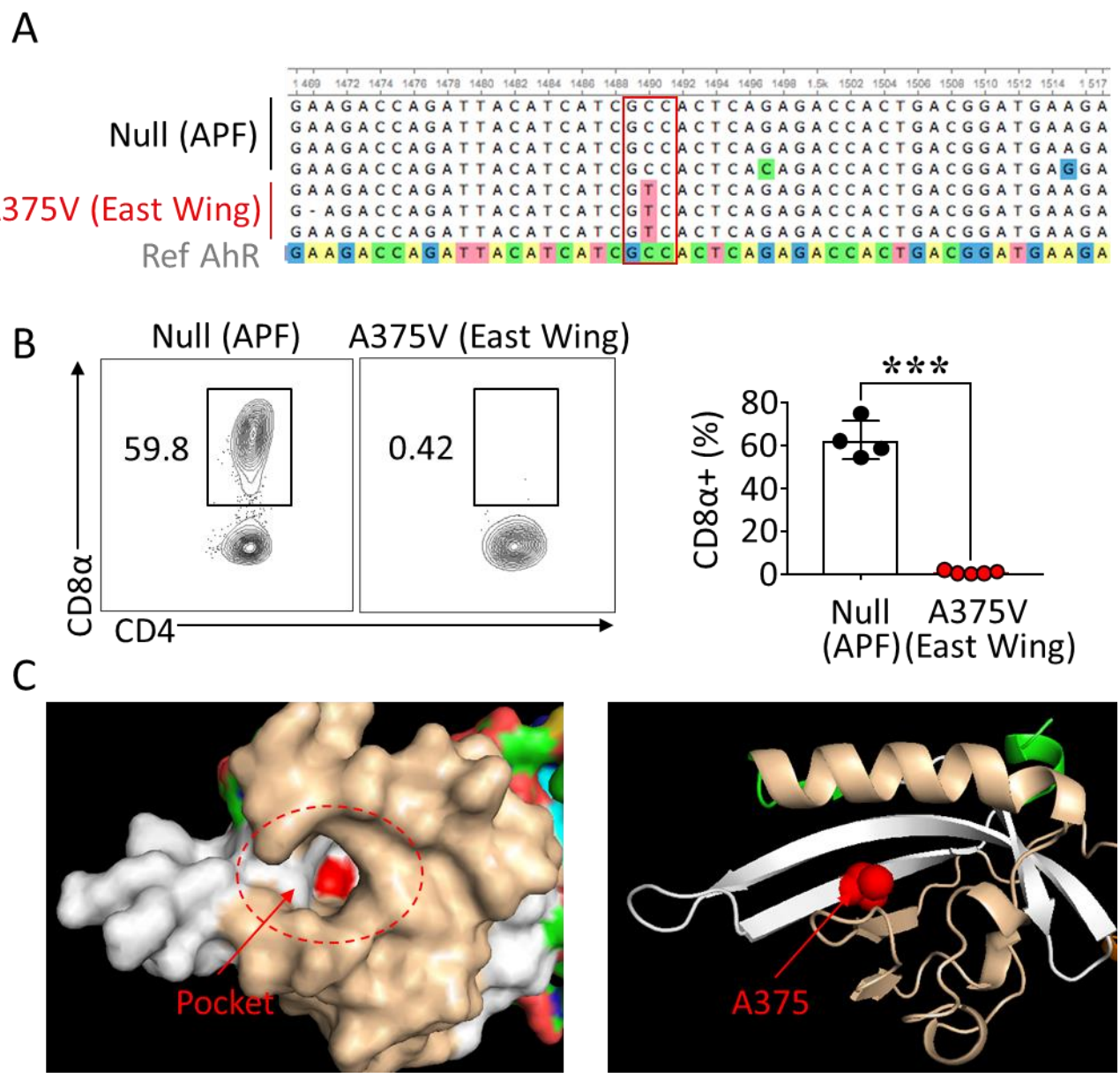


*Figure 3.3, The polymorphism of the Ahr allele affect the generation of CD8αα^{+}CD4^{+} IELs. A) sanger sequencing of partial Ahr gene in 10-week-old C57BL6 mice from East Wing and APF. Red blank labels the gene encoding amino acid at position 375. Top 4 lanes are the Ahr sequence of mice in APF, following 3 lanes are the Ahr sequence of mice in East Wing, while the bottom lane is the reference Ahr sequence of B6 mouse. Each lane represents one mouse. B) flow cytometry plot and statistics of mice with null mutation and A375V. C) simulation of PASb ligand binding pocket domain by PyMOL. Dashed circle indicates the ligand binding pocket of PASb domain. A375 amino acid is coloured in red. Statistical analysis by student t test. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.*

3.1.4. Chapter outline

These preliminary results suggested AhR played a role in the differentiation of cytotoxic IELs. To address this question, we bred $CD4^{cre}$ mice with $AhR^{fl/fl}$ mice. Resulting offspring will generate the specific deletion of AhR in both $CD4^+$ and $CD8^+$ T cells. Meanwhile, we will examine how the treatment of AhR ligand affects the phenotype of human $CD4^+$ T cells *in vitro*.

3.2. AhR is required for the generation of CD8αα$^+$CD4$^+$ IELs

The first key question is whether the generation of CD8αα$^+$CD4$^+$ IELs requires AhR signalling pathway. Hence, mice with a Cre recombinase cassette inserted in *Cd4* gene were bred with mice carrying loxP flanked *Ahr* gene. The breeding generated mice that have the AhR expression silenced in both $CD4^+$ and $CD8^+$ T cells. Compared to the control mice with heterozygous of floxed AhR, $CD4^{Cre}$:$AhR^{flox/flox}$ mice demonstrated drastically reduced proportion of CD8αα$^+$CD4$^+$ IELs, supporting the conclusion that the generation of CD8αα$^+$CD4$^+$ IELs requires AhR signalling pathway (**Figure 3.4**).

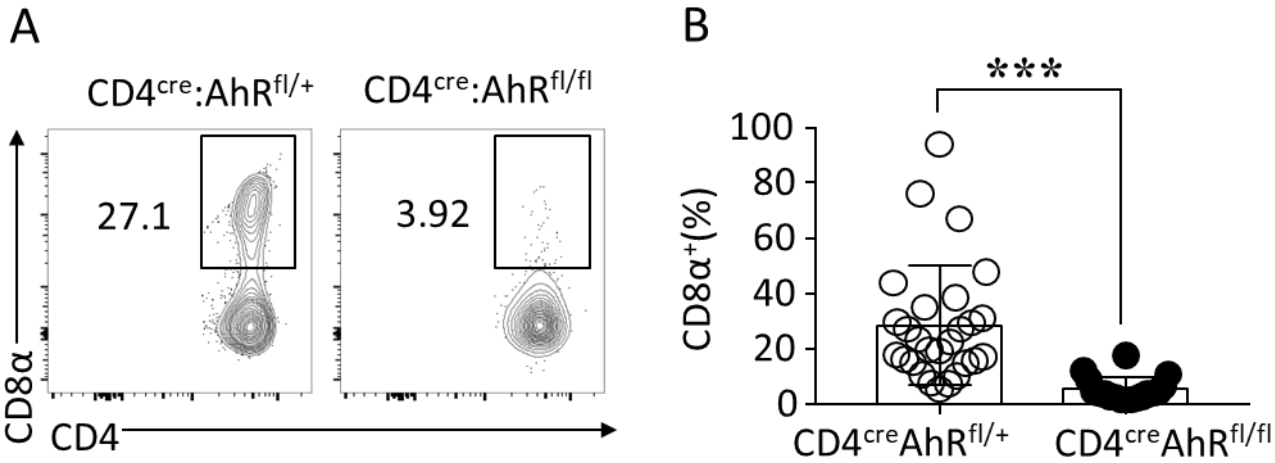


Figure 3.4, AhR is required for the generation of CD8αα^{+}CD4^{+} IELs. A) contour plot of the proportion of CD8αα^{+}CD4^{+} IELs. IELs were collected from CD4^{cre}:AhR$^{fl/+}$ or CD4^{cre}:AhR$^{fl/fl}$ mice between 8 to 46 weeks. Population shown are pre-gated on CD45.2^{+}TCRαβ^{+}CD4^{+}. B) statistics of the proportion of CD8αα^{+}CD4^{+} IELs. Results are compiled of three independence experiments. Data shown are mean value ± SD. Statistical analysis by student t test. ***** = P < 0.05; ****** = P < 0.01; ******* = P < 0.001, ns = P≥0.05.*

3.3. AhR is required for the generation of TCRαβ$^+$CD8αα$^+$ T cells in IELs

I also examined whether AhR also modulates the formation of another unconventional cytotoxic T cell subset - TCRαβ$^+$CD8αα$^+$ IELs and found that the deletion of AhR in T cells led to about 3-fold reduction of TCRαβ$^+$CD8αα$^+$ IELs population (**Figure 3.5**), which is consistent with the published results that AhR pathway is critical for the differentiation of cytotoxic IELs including TCRαβ$^+$CD8αα$^+$ and CD8αα$^+$CD4$^+$ T cells (Cervantes-Barragan et al., 2017; Nakajima et al., 2013).

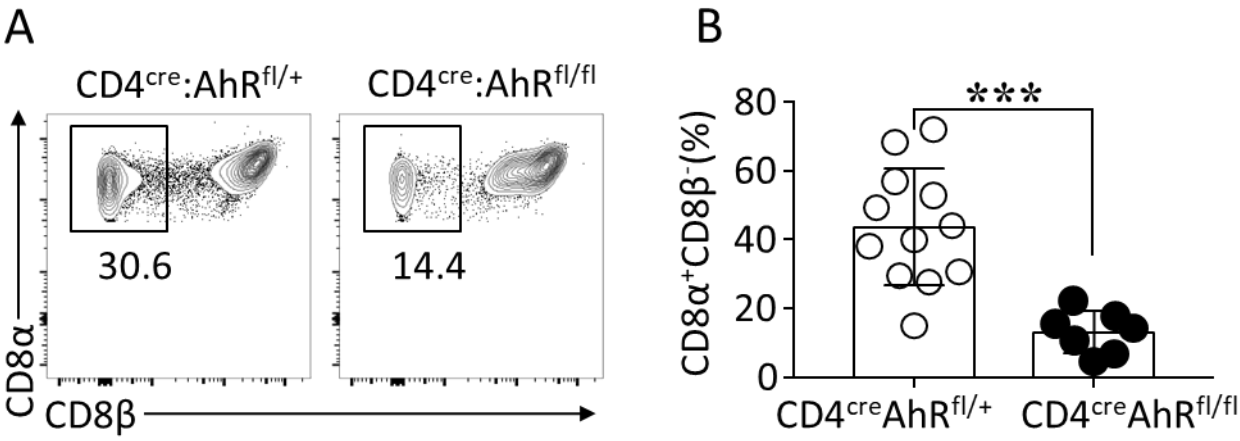


Figure 3.5, AhR is required for the generation of TCRαβ$^+$CD8αα$^+$ IELs. A) contour plot of the proportion of TCRαβ$^+$CD8αα$^+$ IELs. IELs were collected from CD4^{cre}:AhR$^{fl/+}$ or CD4^{cre}:AhR$^{fl/fl}$ mice between 8 to 16 weeks. Population shown are pre-gated on CD45.2$^+$TCRαβ$^+$CD4$^-$. B) statistics of proportion of TCRαβ$^+$CD8αα$^+$ IELs. Results are compiled of three independence experiments. Data shown are mean value ±SD. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

3.4. AhR intrinsically regulates the differentiation of CD8αα^{+}CD4^{+} T cells

While previous data proved that AhR is important to modulate the differentiation of two subsets of cytotoxic IELs, whether the regulation is extrinsic or intrinsic remained unclear. In order to address this question, an adoptive transferred model was adopted. In this mouse model, mature CD4^{+} T cells were isolated from the spleen of both CD4^{cre}:AhR$^{fl/+}$ and CD4^{cre}:AhR$^{fl/fl}$ mice by flow cytometry. Purified CD4^{+} T cells were adoptively transferred into Rag$^{-/-}$ recipient mice, which are lack of CD4^{+} or CD8^{+} T cells. Mice were rested for approximate one month to allow transferred cells to migrate and reconstitute in the gut. The adoptive transfer of CD4^{+} T cells into Rag$^{-/-}$ recipients might induce colitis but the phenotype of transferred cells was analysed after 4 weeks before the development of significant colitis (Ostanin et al., 2009). In this transfer model, the lack of AhR in transferred CD4^{+} T cells significantly impaired the cytotoxic differentiation of CD4^{+} T cells into CD8αα^{+}CD4^{+} IELs (**Figure 3.6**), suggesting that AhR intrinsically regulates the gain of CD8α expression and associated cytotoxic differentiation of CD4^{+} IELs.

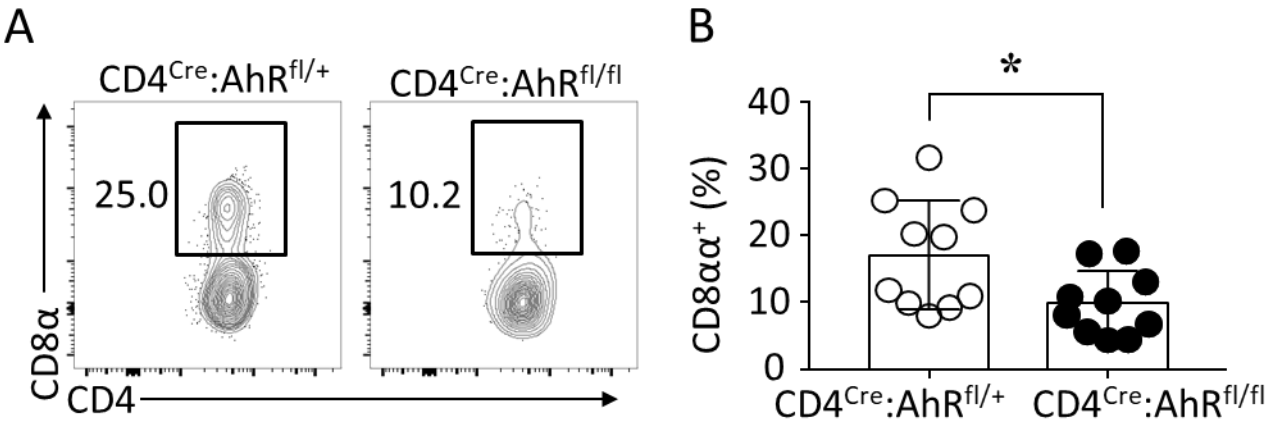


Figure 3.6, AhR intrinsically regulate the differentiation of CD8αα $^{+}$CD4^{+} IELs. IELs were collected from Rag$^{-/-}$ mice that adoptively transferred with CD4^{+} T cells isolated from CD4^{cre}:AhR$^{fl/+}$ or CD4^{cre}:AhR$^{fl/fl}$ mice and rested for 4 weeks before tissue collection. Population shown are pre-gated on CD45.2^{+}TCRαβ^{+}CD4^{+}. Results are compiled of two independence experiments. Data shown are mean value ± SD. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

3.5. AhR ligands induce CD8 upregulation on $CD4^+$ T cells

After showing that AhR is required for the *in vivo* formation of cytotoxic IELs, I next tested whether AhR agonists could directly induce CD8α expression on $CD4^+$ T cells. In this experiment, I chose AhR agonists from different categories. $CD4^+$ T cells were polarised *in vitro* under Th17 conditioning environment (anti-CD28, anti-IFNγ, anti-IL-4, IL-6, TGF-β, IL-2), due to reported relative high expression level of AhR in Th17 cells (Stockinger et al., 2014), in order to maximise the sensitivity of cells to AhR agonists. Importantly, ligands induce variable expression level of CD8α from minimum to high (**Figure 3.7**). Ligands in the category of exogenous agonists or environmental pollutants induced stronger expression of CD8α including BaP, DacA and BPE (listed in **Figure 1.2** and described in section 1.1.2), although they also induced cell apoptosis. While endogenous derivatives such as I3C and Ind (listed in **Figure 1.2** and described in section 1.1.2) were weaker in promoting the expression of CD8α. To be noted, FICZ, which is classified as an endogenous metabolite, effectively induced CD8α expression. However, the exogenous AhR ligand TCDD with a high binding affinity barely stimulated CD8α expression. Such variation indicates that the capability of AhR ligands in inducing the cytotoxic differentiation of $CD8\alpha\alpha^+CD4^+$ IELs is not solely determined by the binding affinity.

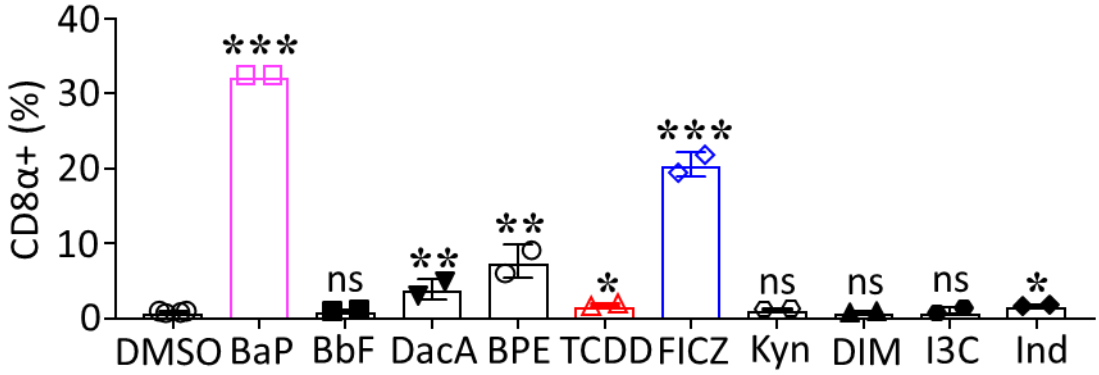


Figure 3.7, Differential upregulation of CD8α in cultured mouse CD4$^+$ T cells stimulated by various AhR agonists. Naïve CD4$^+$ T cells were purified from C57BL/6 mice and cultured in vitro in the Th17 differentiation condition (anti-CD3, anti-CD28, anti-IFNγ, anti-IL-4, IL-6, TGF-β, IL-2) for 3 days. AhR ligands were added to the medium at the concentration of 1μM from the start of culture. Results are representative of two independence experiments. Data shown are mean value ± SD. Statistical analysis by student t test. ***** = *P* < 0.05; ****** = *P* < 0.01; ******* = *P* < 0.001, *ns* = *P*≥0.05.

3.6. AhR ligands fail to upregulate CD8α on AhR-deficient $CD4^+$ T cells

To further test whether the upregulation of CD8α expression by AhR ligands was via the AhR signalling pathway, the effect of AhR ligands on wildtype $CD4^+$ T cells was compared to that of AhR-deficient $CD4^+$ T cells. Consistently to the previous experiment, FICZ strongly induced the expression of CD8α on $CD4^+$ T cells but failed to do so on AhR-deficient $CD4^+$ T cells (**Figure 3.8**). TCDD upregulated CD8α expression in a much smaller extent compared to FICZ and such upregulation was also dependent on AhR signalling pathway, shown by no upregulation of CD8α in AhR-deficient $CD4^+$ T cells (**Figure 3.8**).

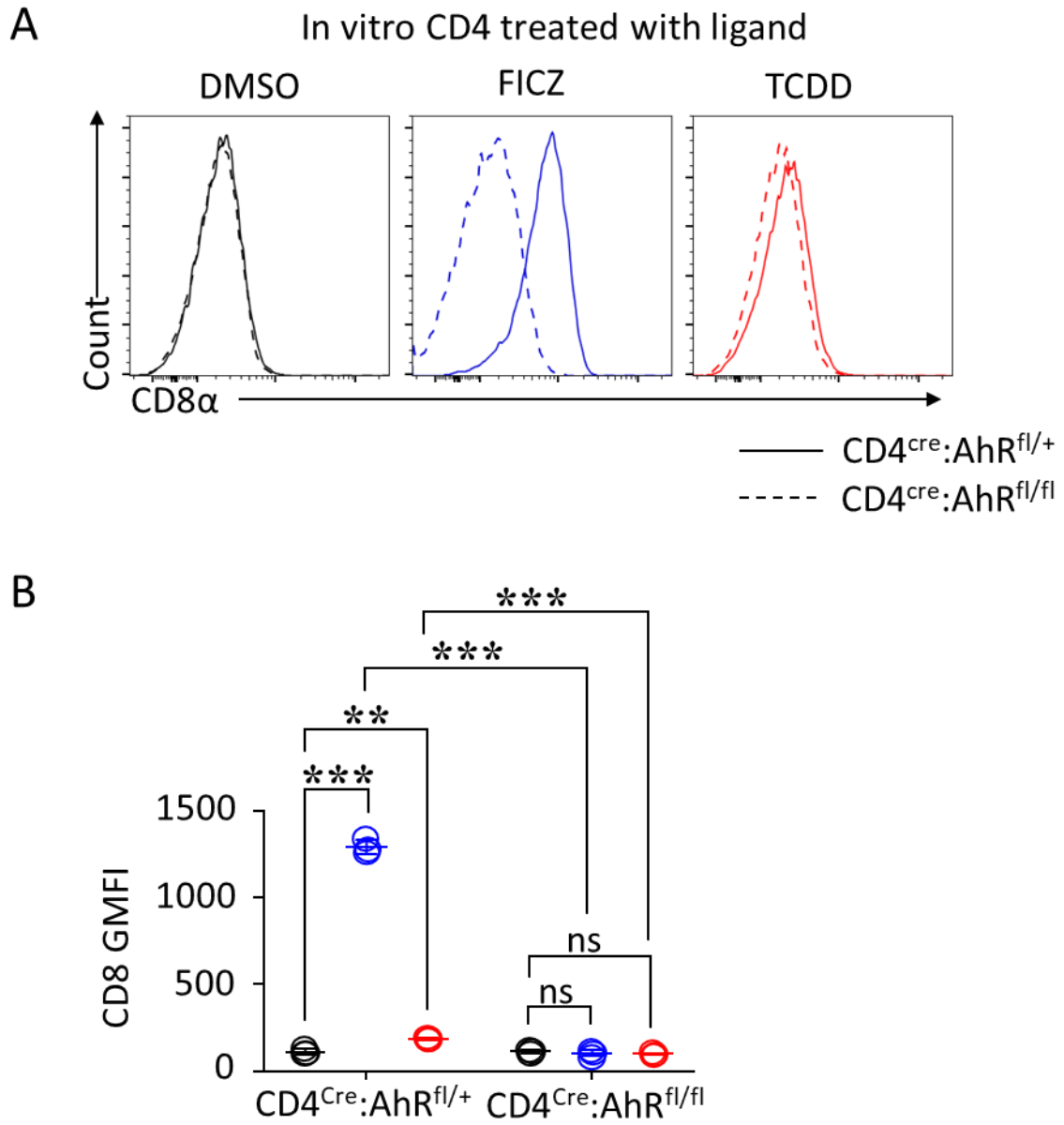


*Figure 3.8, High affinity ligand FICZ and TCDD induced the expression of CD8α on $CD4^+$ T cells in an AhR-dependent manner. A) histogram plot of the expression of CD8α on the $CD4^+$ T cells. B) statistic of CD8α GMFI on the $CD4^+$ T cells. Naïve $CD4^+$ T cells were purified from $CD4^{cre}$:$AhR^{fl/+}$ (solid line) or $CD4^{cre}$:$AhR^{fl/fl}$ (dash line) mice and cultured in vitro with stimulation of anti-CD3, anti-CD28, anti-IFNγ, anti-IL-4, IL-6, TGF-β, IL-2 for 3 days. Ligands were added to the medium at the concentration of 1μM from the start of culture. Results shown are the representative of three independent experiments and mean value ± SD. Statistical analysis by student t test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, ns = $P \geq 0.05$.*

3.7. AhR ligand upregulates CD8α on $CD4^+$ IELs

While previous experiments tested $CD4^+$ T cells from the spleen, it remained to be tested whether IELs actually respond to AhR agonists. Therefore, I isolated IELs from mouse small intestine and cultured *in vitro* with AhR ligands. Due to the prolonged and harsh isolation procedure and their intrinsic nature, IELs poorly survived *in vitro*. Thus, IELs were cultured up to 48 hours when 95% of cultured IELs were $7AAD^+$, a sign of cell death. Consistent with its function on cultured splenic $CD4^+$ T cells, FICZ also upregulated CD8α expression on $CD4^+$ IELs after 24 hours (**Figure 3.9**). TCDD was weaker than FICZ in the upregulation of CD8α expression on splenic $CD4^+$ T cells. It could not do so on IEL $CD4^+$ T cells, either due to the shorter culture time or the difference of cells from two resources (**Figure 3.9**).

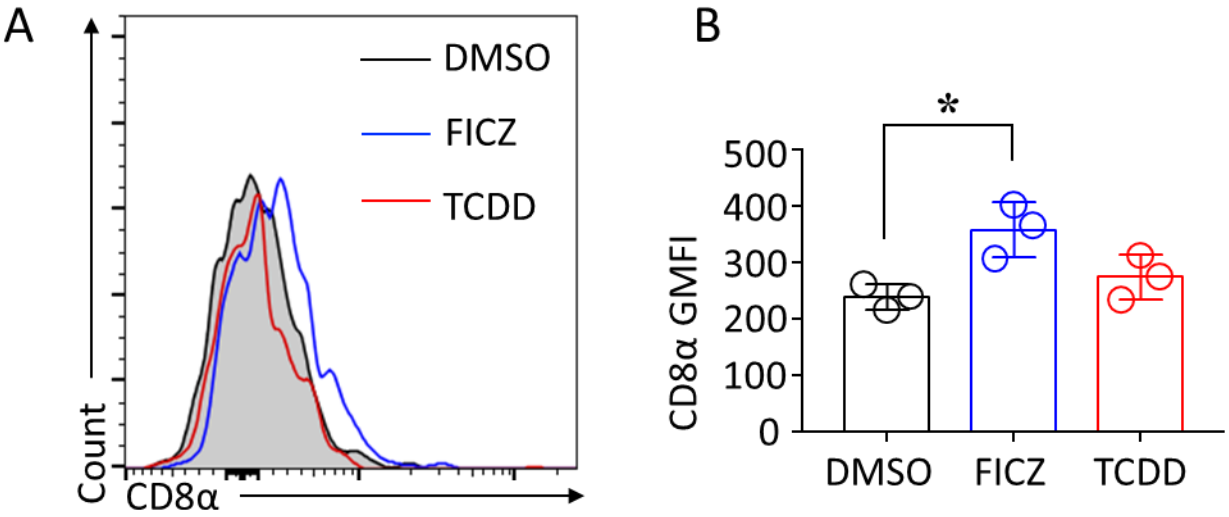


*Figure 3.9, FICZ promotes the expression of CD8α on CD4⁺ IELs in vitro. A) histogram plot of the expression of CD8α on the CD4⁺ IELs. B) statistic of CD8α GMFI on the CD4⁺ IELs. IELs were purified from C57BL/6 mice and cultured in vitro with stimulation of IL-2 for 24 hours. Ligands were added to the medium at the concentration of 1μM from the start of culture. Results shown were the representative of three independent experiments and mean value ± SD. Statistical analysis by student t test. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.*

3.8. AhR ligand induces CD8α upregulation on human $CD4^+$ T cells

AhR shows a conserved function between human and mouse species. I further tested whether AhR ligand could upregulate the expression of CD8α on human $CD4^+$ T cells. Several AhR ligands were tested in a series of concentrations to examine their effect on cultured human T cells. The expression levels of CD8α was increased in a dose-dependent manner **(Figure 3.10A)**. Interestingly, the dosage of FICZ exhibited an exponential correlation with the expression of CD8α on $CD4^+$ T cells, while BaP showed a linear correlation. To test whether the upregulation of CD8α was dependent on AhR, I applied an AhR antagonist CH223191 to block the AhR signalling, which had been previously showed able to effectively compete with other AhR ligands without activating AhR signal simultaneously (Zhao et al., 2010). Consistent with the results from mouse experiments, the blocking of AhR signalling blunted the effects of AhR ligands to induce CD8α expression, suggesting a dependence of AhR (**Figure 3.10B**).

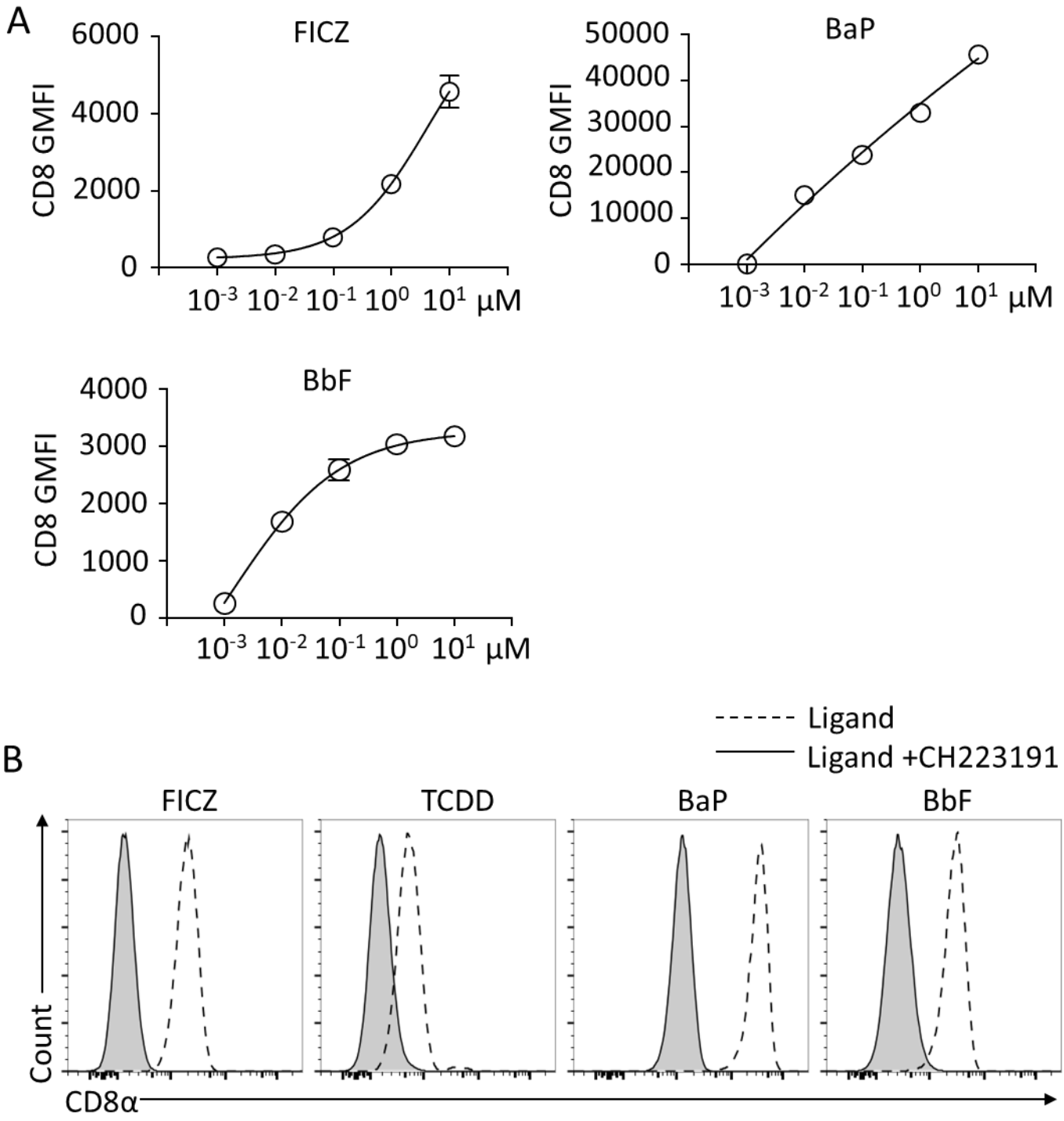


Figure 3.10, AhR signalling upregulates the CD8α expression on human CD4^{+} T cells in vitro. A) correlation of CD8 GMFI with dosage of ligand. B) histogram plot of CD8 expression in cultured T cells. Human PBMC were purified and cultured in vitro with stimulation of ligands at specified concentration (A) or 1μM (B) for 3 days. Results shown are the representative of two independent experiments.

3.9. Discussion

The phenomenon that IEL $CD4^+$ T cells are able to differentiate into $CD8\alpha\alpha^+CD4^+$ T cells improved our understanding of distinct $CD8^+$ and $CD4^+$ T cell lineages, which were used to be considered as terminally differentiated and exclusive for each other. In the gut, $CD4^+$ T cells are not terminally differentiated and show a plasticity for further differentiation, reflecting the dynamics of T-cell homeostasis in the mucosal environment. Our results demonstrated that AhR mediates the differentiation of $CD4^+$ IELs into $CD8\alpha\alpha^+CD4^+$ IELs. AhR can also promote the generation of $TCR\alpha\beta^+CD8\alpha\alpha^+$ T cells. Collectively, these observations indicated a strong pro-cytotoxic feature of the AhR signalling at the interface between gut epithelium/IEL and the gut environment (Funatake, Marshall, & Kerkvliet, 2008; Kerkvliet, 2002; Nakajima et al., 2013).

One key feature is that $CD8\alpha\alpha^+CD4^+$ IELs accumulated along with age (Ghia et al., 2007), probably due to the chronic exposure to increased concentrations of AhR ligands. To be noted, $CD8\alpha\alpha^+CD4^+$ IELs were rarely enriched in young and adult mice (<150days) in the East Wing facility at Monash University, but high percentage of $CD8\alpha\alpha^+CD4^+$ IELs were consistently observed in young mice that housed in APF at Australian National University. No evidence indicates that air filter quality and pathogen barrier are different between these two animal facilities. The difference was at least partially caused by different *Ahr* alleles that encode AhR proteins with distinct binding affinities to ligands. Another possibility is that the environment of two animal facilities may differentially regulate the gut microbiota in housed mice. The bacterial strain, *L. reuteri*, was reported critical to help to produce AhR ligands and promote the formation of $CD8\alpha\alpha^+CD4^+$ IELs (Cervantes-Barragan et al., 2017). However, due to the time limitation, the microbiota of mice housed by two facilities has not been tested. To answer

this question, the analysis on the microbiota in feces collected from different mice in different facilities by measuring and comparing the 16S rRNA sequence is recommended in the future.

In mice, CD8αα$^+$CD4$^+$ IELs are particularly enriched in intestinal intraepithelial site (Mucida et al., 2013). AhR ligands are also enriched in intestine through multiple metabolic pathways (Lamas et al., 2018), which may initialise the reprograming of IEL CD4$^+$ T cells to CD8αα$^+$CD4$^+$ T cells (Cervantes-Barragan et al., 2017). Our data demonstrate that the reprograming is mediated by AhR signalling pathway *in vivo* and AhR ligands directly initialise the process. These data together suggested a direct regulatory function of AhR in controlling the cytotoxicity of IELs.

However, in *ex vivo* models, CD8α upregulation was not consistent among different T-cell polarisation conditions despite the same dose of ligand added. Polarisation towards Th17 cells made CD4$^+$ T cells highly sensitive to AhR ligands, while unpolarised or Th1 polarisation did not make CD4$^+$ T cells effectively response to AhR ligands, in regards of CD8α expression. This may due to the highest expression level of AhR in Th17, moderate in Foxp3$^+$ Tregs and almost undetectable in Th1 and Th2 cells (Duarte, Di Meglio, Hirota, Ahlfors, & Stockinger, 2013; Quintana et al., 2008; Veldhoen et al., 2008), which suggests an more important role of AhR in controlling Th17 or Tregs in the mucosa barrier, as Th17 and Tregs are major CD4$^+$ T cells in IELs.

Besides CD8αα$^+$CD4$^+$ IELs, TCRαβ$^+$CD8αα$^+$ IELs are another cytotoxic IELs. Although a previous study indicated that ARNT is required for the generation of TCRαβ$^+$CD8αα$^+$ IELs, the direct evidence for the requirement of AhR is not provided. It should be noted that ARNT

can interact with other partner proteins, such as HIF-1α (Hu et al., 2006; Mandl & Depping, 2017; Mandl, Lieberum, & Depping, 2016; Weir, Robertson, Leigh, Vass, & Panteleyev, 2011). By examining $CD4^{cre}$:$AhR^{fl/fl}$ mice with the specific deletion of AhR in T cells, I provided the direct evidence that AhR is also required for the generation of $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs.

Unlike $CD8\alpha\alpha^{+}CD4^{+}$ IELs, which can be differentiated directly from $CD4^{+}$ IELs locally, $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs are proposed to derive from T-bet^{+} or $TAK1^{+}$ DN precursor cells in the thymus (Ruscher et al., 2017). However, how these precursor cells migrate into IELs or whether AhR regulates the relocation of these precursor cells are unclear. Indeed, AhR ligands were reported to be less abundantly present in the thymus but the expression of AhR in thymocytes is high. Further study is required to understand whether AhR regulates the generation of $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs primarily in the thymus or in the gut.

My results provide the evidence showing the ligands-AhR axis mediated the regulation of T cells. Generally, AhR ligands can be classified into four categories. Ligands from both xenobiotics and endogenous metabolites exhibited relatively stronger abilities to upregulate CD8α expression on $CD4^{+}$ T cells, while those from dietary and microbial metabolites (some paper classified Ind as endogenous) were weaker to do so. It is possible that xenobiotics and endogenous metabolites are the major cues for the development of $CD8\alpha\alpha^{+}CD4^{+}$ IELs. Notably, many ligands are derived from tryptophan, which suggests the importance of microbiota and the food intakes in regulating the gut immune homeostasis.

The binding affinity of AhR ligand is also critical in trigger different levels of signal. Ligands with high binding affinity usually induce stronger changes of downstream AhR target genes than those with low binding affinity. However, the ligand binding affinity was not in line with the ability of inducing the expression of CD8α. As showed in our result, FICZ and BaP are two effective ligands promoting this cytotoxic reprogramming progress in $CD4^+$ T cells. It has been reported that the half-maximal effective concentration of FICZ can reach 30pM (Busbee et al., 2013). Thus, even low dosage of FICZ was able to induce significantly CD8α upregulation on $CD4^+$ T cells. BaP is a photo-degradable ligand when dissolved in DMSO at the temperature higher than 24°C (D. Dabrowska, 2008). Additionally, BaP did not exhibit high binding affinity to AhR (Moriguchi et al., 2003). Unexpectedly, BaP strongly induced CD8α upregulation on $CD4^+$ T cells in our results. TCDD has been considered as one of the strongest agonists for activating AhR pathway (Pirkle et al., 1989). However, in our results, TCDD merely induced $CD8\alpha\alpha^+$ $CD4^+$ T cells even at high dosage. Despite of the chemical and physical properties, the high affinity AhR ligands FICZ and TCDD were significantly different in term of cytotoxically reprogramming $CD4^+$ T cells, which requires further investigation. The difference of FICZ and TCDD in modulating T-cell homeostasis was previously reported. FICZ and TCDD play contrary roles in the differentiation of Th17 and Treg bi-axis (Duarte et al., 2013; X. M. Li, Peng, Gu, & Guo, 2016; Singh et al., 2011). One recent study also revealed the DNA binding function of AhR-ARNT heterodimer can be affected by conformational changes of the binding domain and their dimerization (Seok et al., 2017b). Therefore, it is possible that different ligands induce functionally conformational change of AhR, resulting in binding different target DNA loci. Due to the physiological importance of AhR, the investigation on the molecular mechanism of ligand-induced structural changes of AhR receptor is highly sought after in the future.

To summarise this chapter, AhR plays an important role in orchestrating the homeostasis of both $CD8\alpha\alpha^+$ $CD4^+$ IELs and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs. In heathy mucosal environment, epithelial cells build up a barrier protecting intestinal milieu against threats. When the barrier loses the integrity such as during an invasion by pathogens, AhR ligands may penetrate into the epithelium. Therefore, AhR-induced $CD8\alpha\alpha^+$ $CD4^+$ IELs and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs could act as an emergency-responding mechanism to kill potential infections and maintain healthy gut.

CHAPTER IV

TCF1/LEF1 inhibit the generation of cytotoxic IELs

4.1. Preamble

Mature TCRαβ$^+$ conventional T cells are classified into two distinct subsets: CD4$^+$ T cells and CD8$^+$ T cells. CD4$^+$ T cells essentially regulate immune responses by mediating the activation and controlling the function of other immune cell types, whereas CD8$^+$ T cells predominantly perform cytotoxic function to eliminate infected or cancerous cells. Although the fate to become a CD4$^+$ or CD8$^+$ T cell lineage is primarily determined during T-cell development in the thymus, both recent studies and our results have revealed that mature CD4$^+$ T cells, when losing helper lineage-determining transcription factor ThPOK, could upregulate cytotoxic lineage-determining transcription factor Runx3, express CD8α and gain cytotoxic function. The cytotoxic reprogramming of CD4$^+$ T cells is particularly observed in IEL. These cells were also reported to suppress intestinal inflammation by secreting IL-10 (Sujino et al., 2016).

Our data demonstrated that cytotoxic reprograming of peripheral CD4$^+$ T cell is intrinsically mediated by AhR signalling pathway. In the *in vitro* culture, AhR ligand upregulated the expression of CD8α on CD4$^+$ T cells but the level of upregulated CD8α expression on cultured mouse CD4$^+$ T cells was lower than that on CD8αα$^+$ CD4$^+$ IELs. In mice, CD8αα$^+$ CD4$^+$ T cells are rare in the spleen or lymph nodes and are mainly located in IELs. These results suggest the gut environment provides a special condition favourable to the generation of CD8αα$^+$ CD4$^+$ T cells although the reason is unclear (Mucida et al., 2013). One possibility is that the generation requires a prolonged exposure to AhR ligands that exist in the gut but not in another lymphoid tissues or in the culture. It is also possible that other stimulus synergises with the AhR pathway to achieve effective reprogramming. Nevertheless, both *ex vivo* and *in vivo* experiments suggested an important regulatory role of AhR in the development of CD8αα$^+$ CD4$^+$ IELs, but how AhR regulates this cytotoxic reprogramming is not understood.

To understand the molecular signatures involved in the formation of cytotoxic IELs, Yu group used RNA-seq to profile gene expression in specific mouse IEL subsets. The RNA-seq data identified several differentially expressed transcription factors including ThPOK, Runx3, TCF1 and LEF1 that are well known for their regulatory function in determining $CD4^+$ and $CD8^+$ lineage differentiation during T-cell development. Previous studies showed that during T-cell development in the thymus, the transcription factor ThPOK guides the fate determination of $CD4^+$ T cells and prevents the development of $CD8^+$ T cells, whereas the transcription factor Runx3 terminates CD4 expression and promotes the generation of $CD8^+$ T cells (Egawa & Littman, 2008; He et al., 2008; Luckey et al., 2014; Reis et al., 2013; Sakaguchi et al., 2015; Twu & Teh, 2014; L. Wang et al., 2008). More recently, TCF1 and LEF1 have been shown to guard the expression of ThPOK during T-cell development in the thymus (Steinke et al., 2014; Yu et al., 2012). Studies on IELs revealed that the ThPOK-Runx3 axis also plays a central role in the differentiation of $CD8\alpha\alpha^+$ $CD4^+$ T cells (Mucida et al., 2013; Reis et al., 2013; Vacchio et al., 2014). These studies proposed a model of epigenetic regulation whereby gut $CD4^+$ T cells, including lamina propria Treg cells, lose the expression of ThPOK and gain CD8α expression (Mucida et al., 2013; Reis et al., 2013; Sujino et al., 2016). Collectively, the downregulation of ThPOK is the key event for the formation of $CD8\alpha\alpha^+$ $CD4^+$ IELs. It remained unknown whether TCF1/LEF1 execute a similar function as they do in the thymus to support the expression of ThPOK in IELs and regulate the generation of $CD8\alpha\alpha^+$ $CD4^+$ IELs.

4.1.1. RNA-seq results identified potential transcription factors underlying the development of cytotoxic IELs

To study the molecular pathways involved in the differentiation of CD8αα$^+$ CD4$^+$ IELs, we used the RNA-seq technology to quantify the transcripts in all TCRαβ$^+$ populations in IELs including TCRαβ$^+$CD8αα$^+$, TCRαβ$^+$CD8αβ$^+$, CD8αα$^+$ CD4$^+$ and CD4$^+$ IELs . Unsupervised hierarchical clustering on overall gene expression demonstrated that CD8αα$^+$CD4$^+$ IELs exhibited a closer relationship to TCRαβ$^+$CD8αα$^+$ IELs or TCRαβ$^+$CD8αβ$^+$ IELs (**Figure 4.1A**). By comparing CD8αα$^+$ CD4$^+$ and CD4$^+$ IELs, there appeared more downregulated genes in CD8αα$^+$ CD4$^+$ IELs. I selected a few makers and functional regulators involved in the regulation of CD4$^+$ and CD8$^+$ T cell lineages, which include *Cd4*, *Cd8a*, *Cd8b*, *Zbtb7b* (encoding ThPOK), *Runx3*, *Tcf7* (encoding TCF1) and *Lef1*. I also included the key topic of this project, *Ahr*. The transcripts for *Zbtb7b*, *Tcf7* and *Lef1* showed more than 4-fold reduction in CD8αα$^+$CD4$^+$ IELs compared with CD4$^+$ IELs, while *Cd8α* had a more than 6-fold upregulation (**Figure 4.1B**). Similar trend of the gene expression pattern of these selected candidates except *Zbtb7b* was found when comparing TCRαβ$^+$CD8αα$^+$ IELs with TCRαβ$^+$CD8αβ$^+$ IELs (**Figure 4.1**). The differential expression of TCF1 and LEF1 suggested that they may play roles in regulating CD8αα$^+$ CD4$^+$ IELs differentiation. Notably, LEF1 is generally considered compensatory in the TCF1-LEF1 regulatory network (Haynes et al., 1996; Okamura et al., 1998). Thus, this chapter focused more on the function of TCF1 in regulating the differentiation of cytotoxic IELs.

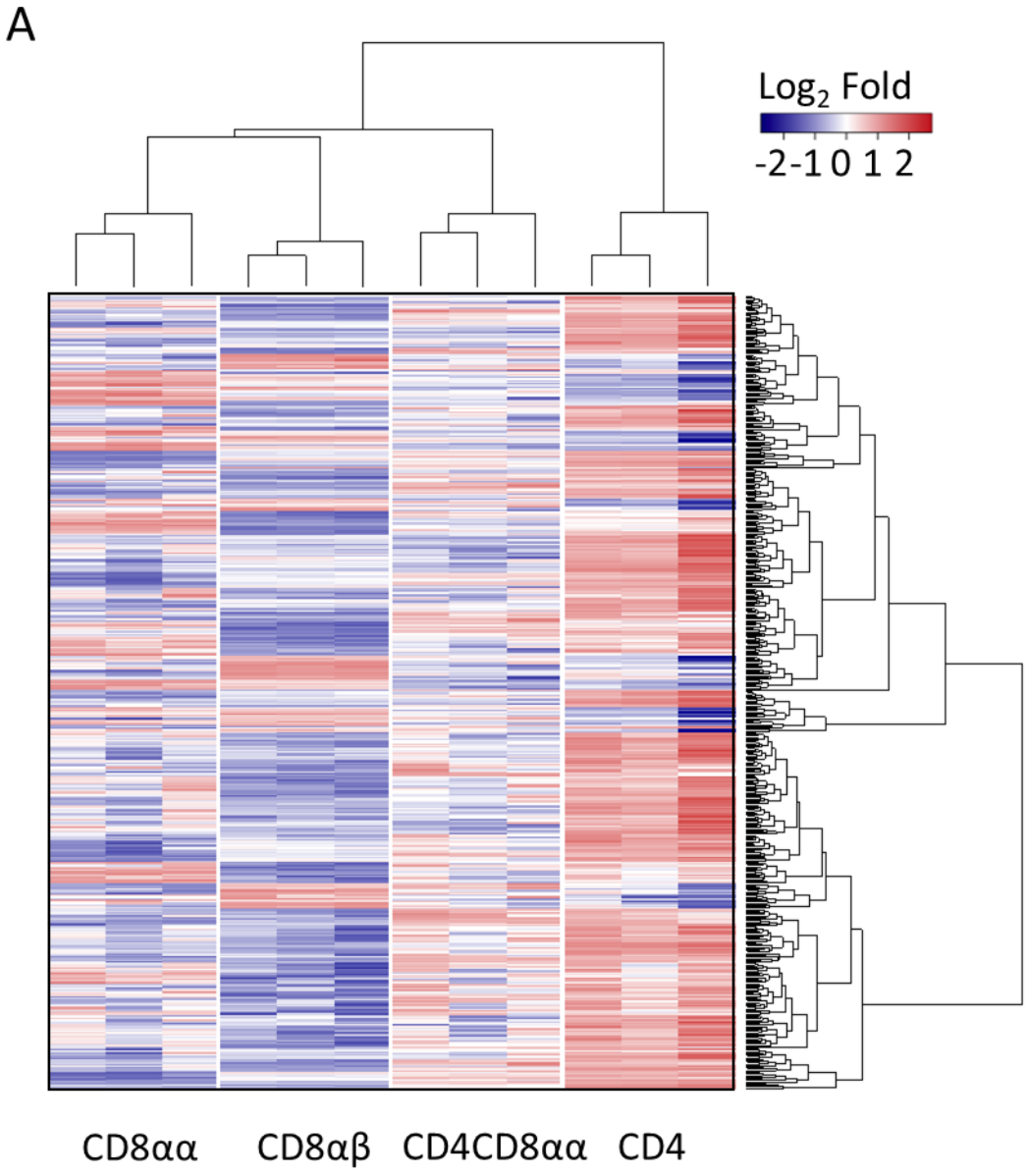

A
Log2 Fold
-2-1 0 1 2
CD8αα
CD8αβ
CD4CD8αα
CD4
B
Ahr
Runx3
Cd8b1
Tcf7
Zbtb7b
Lef1
Cd4
Cd8a
CD8αα
CD8αβ
CD4CD8αα
CD4

Figure 4.1, Transcriptomic analysis by RNA-seq for the sub-populations of TCRαβ$^+$ IELs. A) heatmap of top 500 genes in the four TCRαβ$^+$ IELs subpopulations: CD8αα$^+$CD4$^+$, CD4$^+$, CD8αβ$^+$ and CD8αα$^+$. Each group contains three replicates. B) heatmap of selected genes for CD4$^+$ and CD8$^+$ T cell lineage determination. IELs were purified from C57BL/6 mice and RNA was extracted by Qiagen RNeasy kit. Sequencing was performed by illumine HiSeq 2000. Raw counts were generated by pair-end alignment and analysis was conducted using R-studio.

4.1.2. The quantification of the expression of TCF1 protein in IEL subsets.

To validate the differential expression of *Tcf7* gene revealed by RNA-seq, a p45-GFP reporter mouse strain was deployed. In this transgenic mouse strain, the GFP expression cassette was inserted in the *Tcf7* promoter region, resulting in the expression of GFP in tandem with *Tcf7* without affecting its expression or function (Yang et al., 2015). Therefore, the expression of TCF1 can be directly and conveniently traced by the expression of GFP. Respectively compared to $CD4^+$ IELs and $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ IELs with a heterogenous positive and negative expression of *Tcf7*/GFP, the expression of *Tcf7*/GFP was mainly negative $CD8\alpha\alpha^+$ $CD4^+$ and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs (**Figure 4.2A&B**). Additionally, the *Tcf7* transcription was also quantified in the adoptive transfer model where transferred $CD4^+$ T cells differentiating into $CD8\alpha\alpha^+$ $CD4^+$ IELs in $Rag^{-/-}$ recipient mice. Again, $CD8\alpha\alpha^+$ $CD4^+$ IELs significantly reduced TCF1 positive fractions compared to $CD4^+$ IELs (**Figure 4.2C&D**).

IELs consist of different types of T cells expressing TCRαβ or TCRγδ. $TCR\alpha\beta^+$ IELs are classified into four subsets: $CD4^+$, $CD8\alpha\alpha^+$ $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ (**Figure 3.1**). To be noted, AhR signalling is not only important for the generation of $CD8\alpha\alpha^+$ $CD4^+$ IELs and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs, but also enhanced the formation of $TCR\gamma\delta^+$ T cells (Ji et al., 2015; Y. Li et al., 2011; Nakajima et al., 2013). All these AhR regulated populations are highlighted in red in **Figure 4.2**.

In addition to quantify the transcripts of *Tcf7*, flow cytometric analysis of TCF1 protein expression was performed. The protein expression of TCF1 was binary, similar as the pattern

seen by the Tcf7/GFP reporter. Several IEL populations showed a main expression of low TCF1 (**Figure 4.2E-G**). Importantly, these populations including TCRγδ$^+$, CD8αα$^+$ CD4$^+$ and CD8αα$^+$ IELs are those subsets that require the AhR pathway for their generation (**Figure 4.2E-G**), suggesting a potential interaction between the AhR pathway and the expression of TCF1.

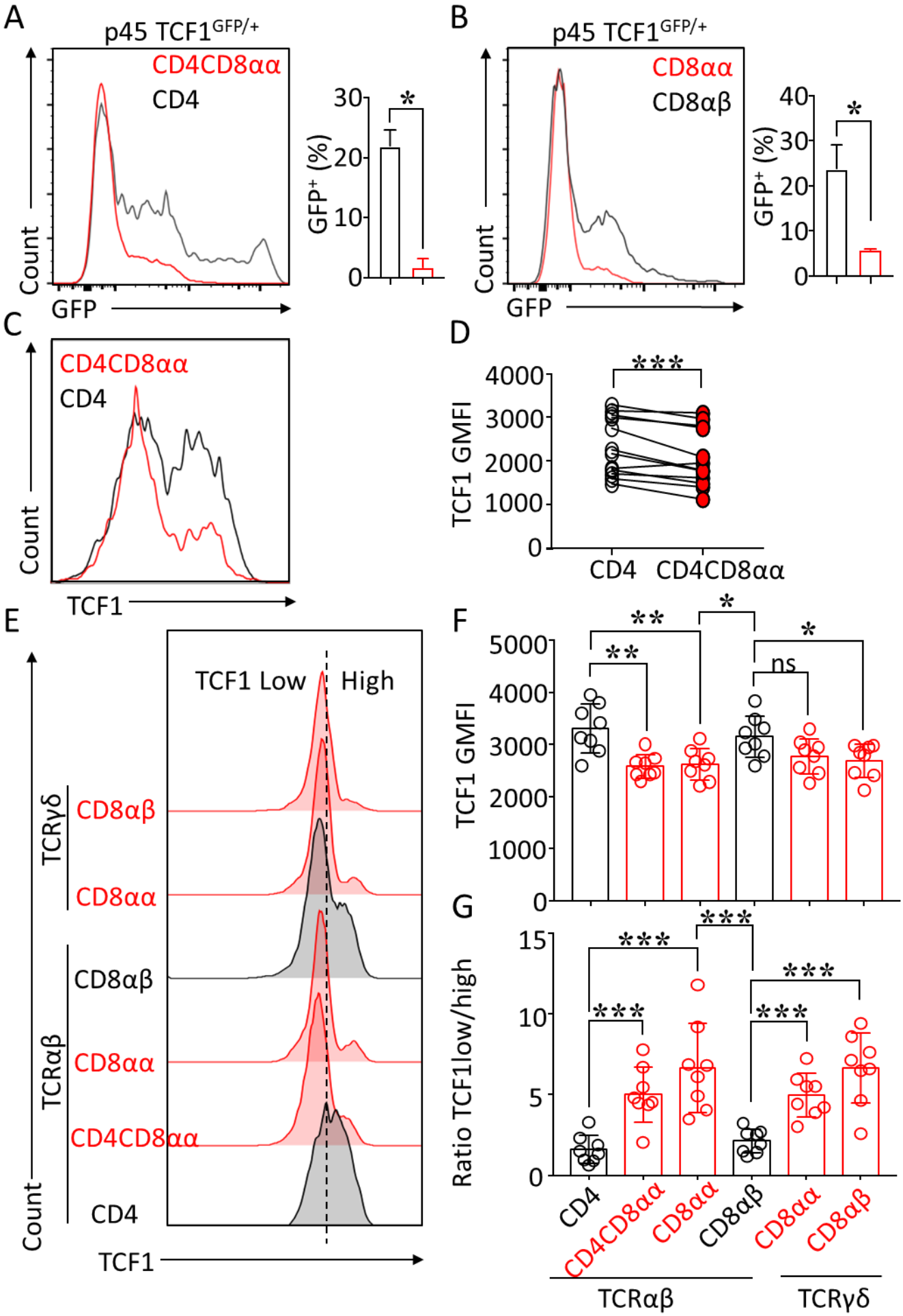

A
p45 TCF1GFP/+
CD4CD8αα
CD4
Count
GFP
GFP+ (%)
30
20
10
0
*
B
p45 TCF1GFP/+
CD8αα
CD8αβ
Count
GFP
GFP+ (%)
40
30
20
10
0
*
C
CD4CD8αα
CD4
Count
TCF1
D
TCF1 GMFI
4000
3000
2000
1000
0
***
CD4
CD4CD8αα
E
TCF1 Low
High
TCRγδ
CD8αβ
CD8αα
TCRαβ
CD8αβ
CD8αα
CD4CD8αα
CD4
Count
TCF1
F
TCF1 GMFI
5000
4000
3000
2000
1000
0
**
**
*
ns
*
G
Ratio TCF1low/high
15
10
5
0
***
***
***
***
***
CD4
CD4CD8αα
CD8αα
CD8αβ
CD8αα
CD8αβ
TCRαβ
TCRγδ

Figure 4.2, Low TCF1 expression in IEL populations that require the AhR pathway for the generation. IELs were collected from C57BL/6 mice. A&B) IELs were collected from p45-Tcf7$^{GFP/+}$ mice. Histograms and statistics of GFP in IELs of p45-TCF1$^{GFP/+}$ mice. C) Mice were adoptively transferred with purified CD4^{+} T cells and reconstituted for 4 weeks. Histograms and statistic of Tcf7 expression of reconstituted IELs in Rag$^{-/-}$ mice. D) Statistics of Tcf7 GMFI in the adoptive transferred T cells. E) Histograms of TCF1 expression in IELs. F&G) Statistic of TCF1 GMFI (F) and low to high ratio (G) in IEL subsets. Red colour highlights IEL populations that required AhR for the development or maintenance. Results shown are compiled of two independent experiments and mean value ± SD. Statistical analysis by student t test for A,B,F&G; paired t test for D. ***** = P < 0.05; ****** = P < 0.01; ******* = P < 0.001, ns = P≥0.05.*

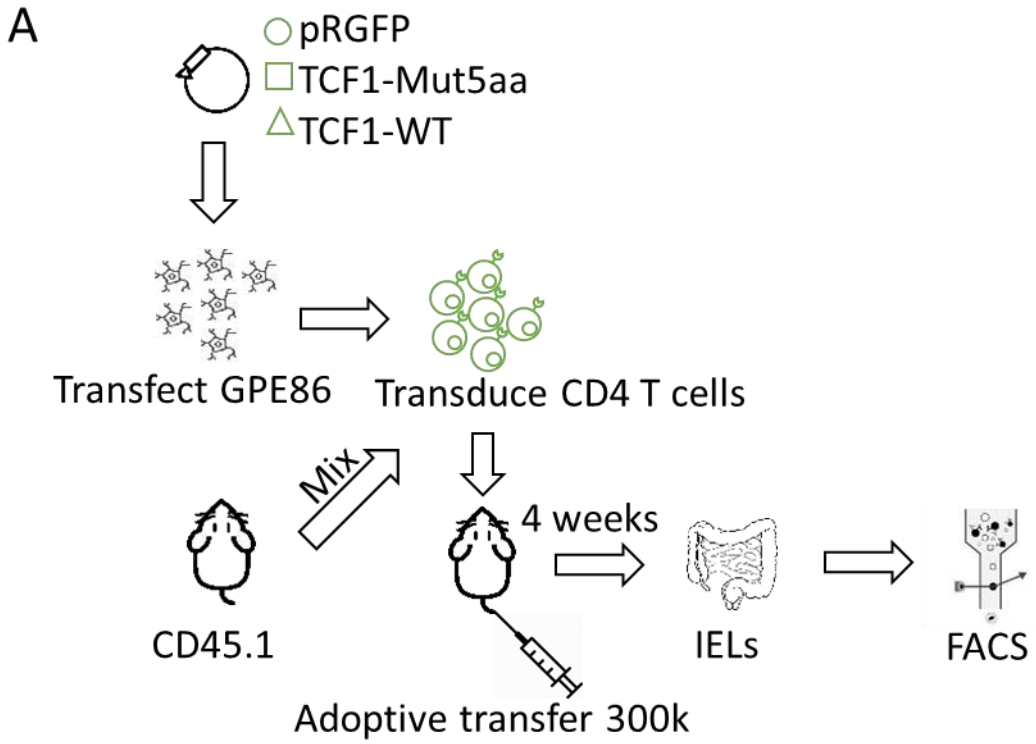
4.1.3. Chapter outline

The above results suggested that AhR might downregulate TCF1/LEF1 in promoting the generation of CD8αα^{+}CD4^{+} and TCRαβ^{+}CD8αα^{+} IELs. In this chapter, I will test whether TCF1 regulates the development of cytotoxic IELs. In order to answer the question, I bred CD4^{cre} mice with TCF1$^{fl/fl}$ and TCF1$^{fl/fl}$ x AhR$^{fl/fl}$ to generate the specific deletion of TCF1 and TCF1/AhR in T cells. The overexpression of TCF1 by viral vector will be constructed to test whether the overexpression of TCF1 suppresses the differentiation of CD8αα^{+} CD4^{+} IELs.

4.2. Downregulation of TCF1 is required for the differentiation of CD8αα^{+} CD4^{+} IELs

To test the function of TCF1 in the differentiation of CD8αα^{+} CD4^{+} IELs, I constructed a GFP reporter retroviral vector to overexpress TCF1 in T cells. Original TCF1 plasmids were generously provided by Prof. Haihui Xue and were described in the paper (S. Xing et al., 2016). The protein coding region for TCF1-WT and TCF1-Mut5aa were sub-cloned into the bicistronic GFP-expression retroviral vector (**Appendix Figure 1**), which allow the overexpression of target gene and GFP as a marker in transduced cells. TCF1-Mut5aa contains five amino acids mutations in the HDAC domain of TCF1, resulting in the loss of intrinsic HDAC function, which is required for the TCF1 to sustain ThPOK expression during T-cell development in the thymus (S. Xing et al., 2016). GPE86 cell line was used to package the retrovirus. The supernatant containing viruses was collected for the transduction of CD4^{+} T cells. The expression level of TCF1 was valid by flow cytometry. To examine the function of TCF1 in modulating CD8αα^{+} CD4^{+} IELs, transduced CD45.2^{+} CD4^{+} T cells purified by flow cytometry were adoptively transferred into Rag$^{-/-}$ mice together with CD45.1^{+}CD4^{+} T cells (internal control) in a 50:50 mixture for four weeks. The experiment designed is outline in

Figure 4.3A. Transduced cells maintained a high GFP expression 4-week after adoptively transferred into $Rag^{-/-}$ mice, indicating a consistent overexpression of TCF1/GFP in transduced T cells (**Figure 4.3B**).


A
pRGFP
TCF1-Mut5aa
TCF1-WT
Transfect GPE86
Transduce CD4 T cells
Mix
4 weeks
CD45.1
IELs
FACS
Adoptive transfer 300k

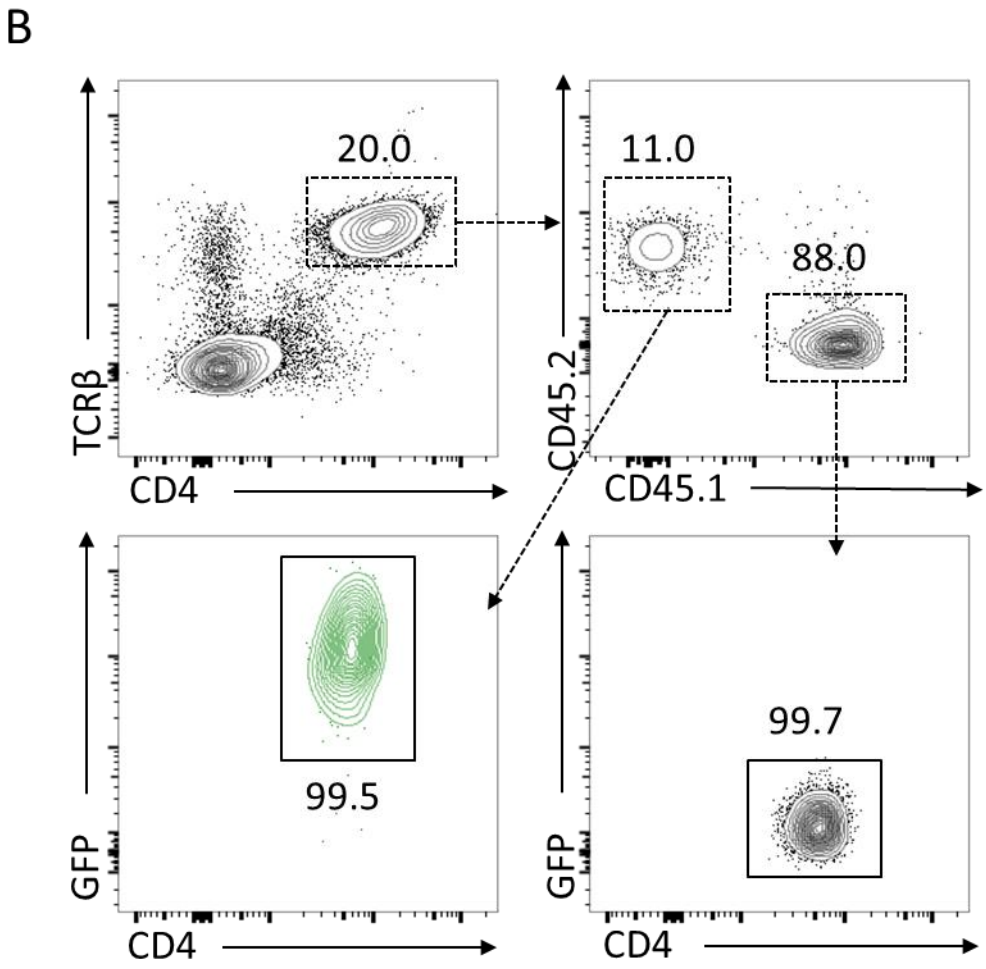

B
20.0
11.0
88.0
TCRβ
CD4
CD45.2
CD45.1
99.5
99.7
GFP
CD4
GFP
CD4

Figure 4.3, Experiment set up for testing the TCF1 function in the generation of cytotoxic IELs. A) schematic outline of retrovirus packaging, transduction of TCF1 in the $CD4^+$ T cells and adoptive transfer of $CD4^+$ T cells into $Rag^{-/-}$ mice. $CD45.2^+CD4^+$ T cells were purified from C57BL/6 mice and transduced with constructed vectors including pRGFP (empty vector), TCF1-Mut5aa and TCF1-WT perspective. Transduced $CD45.2^+CD4^+$ T cells were mixed equally with $CD45.1^+CD4^+$ T cells and adoptively transferred together into $Rag^{-/-}$ mice. $CD45.1^+CD4^+$ T cells were the internal control. B) contour plot of transduced $CD4^+$ T cells remaining high expression of GFP in the $Rag^{-/-}$ mice. IELs were collected from the $Rag^{-/-}$ mice reconstituted with transduced $CD4^+$ T cells for 4 weeks. Green colour highlights GFP expression.

Since TCF1 enhanced the expression ThPOK during $CD4^+$ T cell development in the thymus, we hypothesised that TCF1 overexpression may also support the expression of ThPOK in $CD4^+$ IELs and thus inhibit the generation of $CD8\alpha\alpha^+$ $CD4^+$ IELs. In the co-transfer of $CD45.1^+$ cells and pR-GFP empty vector-transduced $CD45.2^+$ cells, we observed comparable frequencies of $CD8\alpha\alpha^+$ $CD4^+$ IELs. In contrast, the overexpression of TCF1-WT in $CD4^+$ T cells significantly inhibited the differentiation of $CD8\alpha\alpha^+CD4^+$ IELs from the reconstituted $CD4^+$ T cells (**Figure 4.4**). Furthermore, the overexpression of TCF1-Mut5aa did not affect the generation of $CD8\alpha\alpha^+$ $CD4^+$ IELs from $CD4^+$ T cells in $Rag^{-/-}$ mice, indicating the HDAC function of TCF1 was required for the inhibitory function of TCF1 for the generation of $CD8\alpha\alpha^+$ $CD4^+$ IELs .

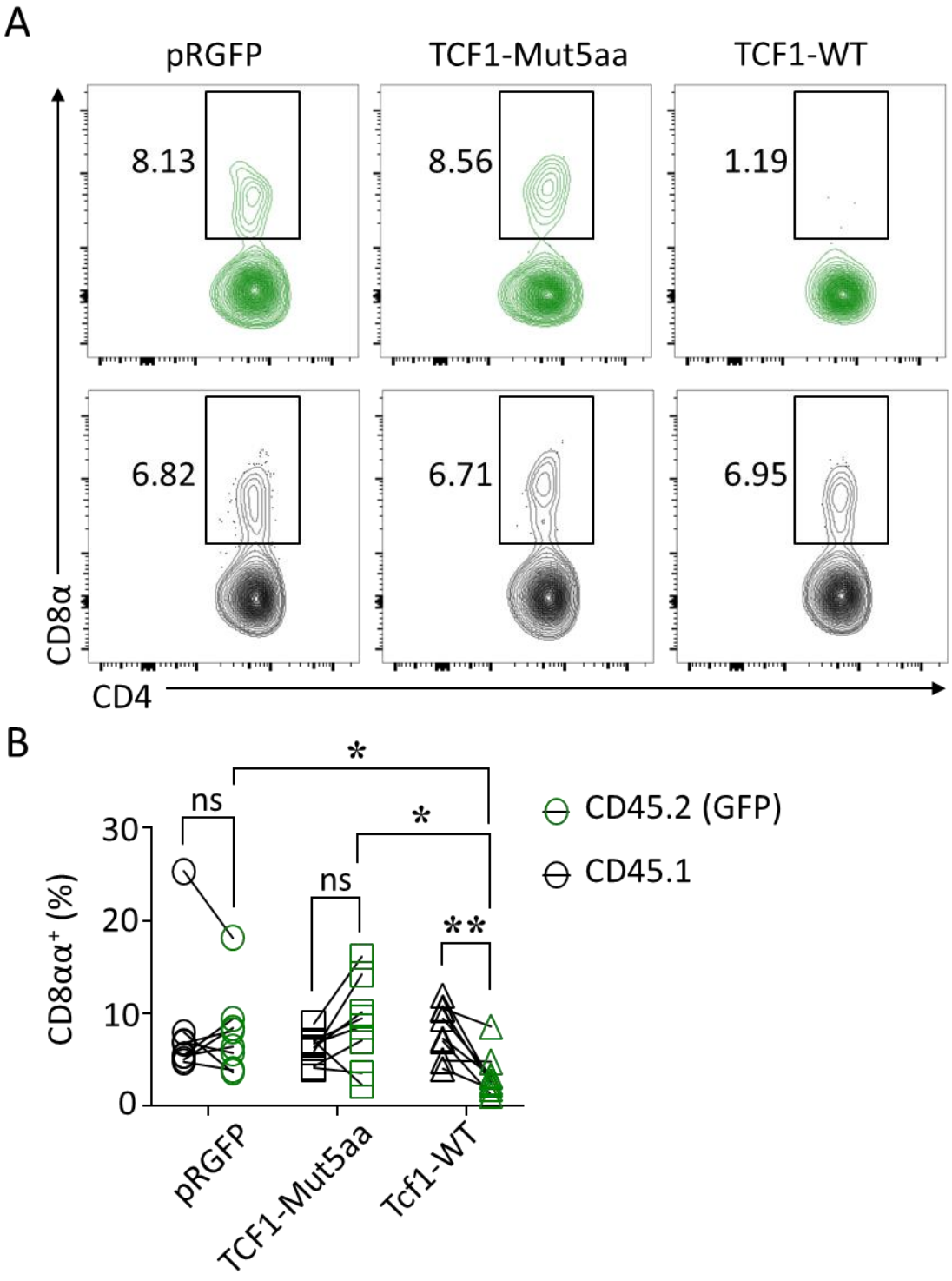

A
pRGFP
TCF1-Mut5aa
TCF1-WT
8.13
8.56
1.19
6.82
6.71
6.95
CD8α
CD4
B
*
ns
*
ns
**
CD45.2 (GFP)
CD45.1
CD8αα⁺ (%)
30
20
10
0
pRGFP
TCF1-Mut5aa
Tcf1-WT

Figure 4.4, Overexpression of TCF1 prohibited the differentiation of CD8αα$^+$CD4$^+$ IELs. A) contour plot of CD8α$^+$ proportion in the transferred CD4$^+$ T cells in Rag$^{-/-}$ mice. Populations were pre-gated on CD45.2$^+$ (Top) or CD45.1$^+$ (Bottom). B) statistics of CD8α$^+$ percentage in CD4$^+$ IELs. Green colour highlights GFP expression. Results shown are compiled of two independent experiments. Statistical analysis by paired t test within groups; student t test between groups. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

4.3. TCF1 suppresses the expression of CD8α via its intrinsic HDAC activity

The intrinsic HDAC activity was reported critical for the regulatory function of TCF1 in determining CD8$^+$ T cell lineage in the thymus (S. Xing et al., 2016). My results showed that the Mut5aa mutation in the HDAC functional domain of TCF1 led to the loss of the capability of TCF1 in suppressing CD8α expression. I next tested whether the intrinsic HDAC activity of TCF1 regulated the expression of CD8α in CD4$^+$ T cells using a complementary approach of tubacin treatment, which was reported specifically inhibit the intrinsic HDAC activity of TCF1 without affecting other HDACs (S. Xing et al., 2016). CD4$^+$ T cells from both wildtype and AhR deficient mice were purified and cultured *in vitro* with or without tubacin treatment. I observed that the treatment of tubacin, presumably by inhibiting the HDAC activity of TCF1, increased a small but consistent expression of CD8α on CD4$^+$ T cells even without the stimulation by AhR agonists (**Figure 4.5**). Moreover, such gain of CD8α expression was independent of AhR signalling, as AhR deficient CD4$^+$ T cells exhibited comparable CD8α upregulation to that of WT T cells.

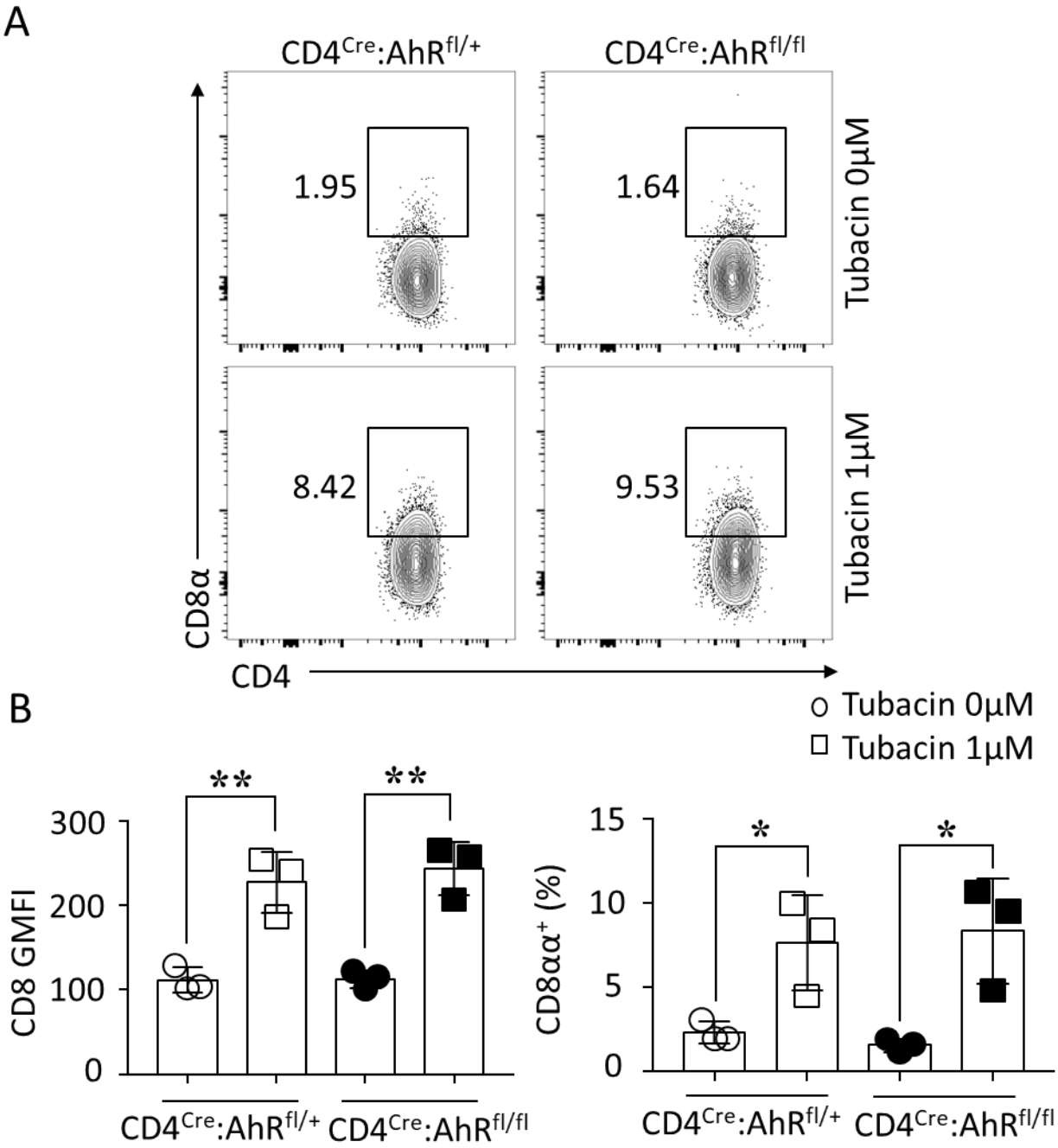


Figure 4.5, Inhibition of the HDAC activity of TCF1 promotes the expression of CD8α on T cells. Naïve CD4$^+$ T cells were purified from mice and cultured in vitro with stimulation of anti-CD3, anti-CD28, anti-IFNγ, anti-IL-4, IL-6, TGF-β, IL-2 for 3 days. Tubacin was treated at the start of culture. A) Contour plots of CD8a expression on CD4$^+$ T cells. B) Statistics of CD8α GMFI and proportion on CD4$^+$ T cells after treatment. Results shown are representative of two independent experiments and mean value ± SD. Statistical analysis by student t test.

**** = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.***

4.4. Loss of TCF1 rescues TCRαβ^{+}CD8αα^{+} IELs in AhR deficient mice

I hypothesised that TCF1 may be an important downstream target of AhR to mediate the formation of cytotoxic IELs. If so, the loss of TCF1 would rescue the defects of cytotoxic IELs in AhR deficient mice. In order to test this, I bred CD4^{cre}:AhR$^{fl/fl}$ and Tcf7$^{fl/fl}$ mouse strains to specifically delete both TCF1 and AhR in T cells. The deletion of TCF1 restored the generation of TCRαβ^{+}CD8αα^{+} IELs in AhR-deficient mice (**Figure 4.6**). However, I did not observe such rescue effect on CD8αα^{+}CD4^{+} IELs (**Figure 4.7**), which will be discussed below.

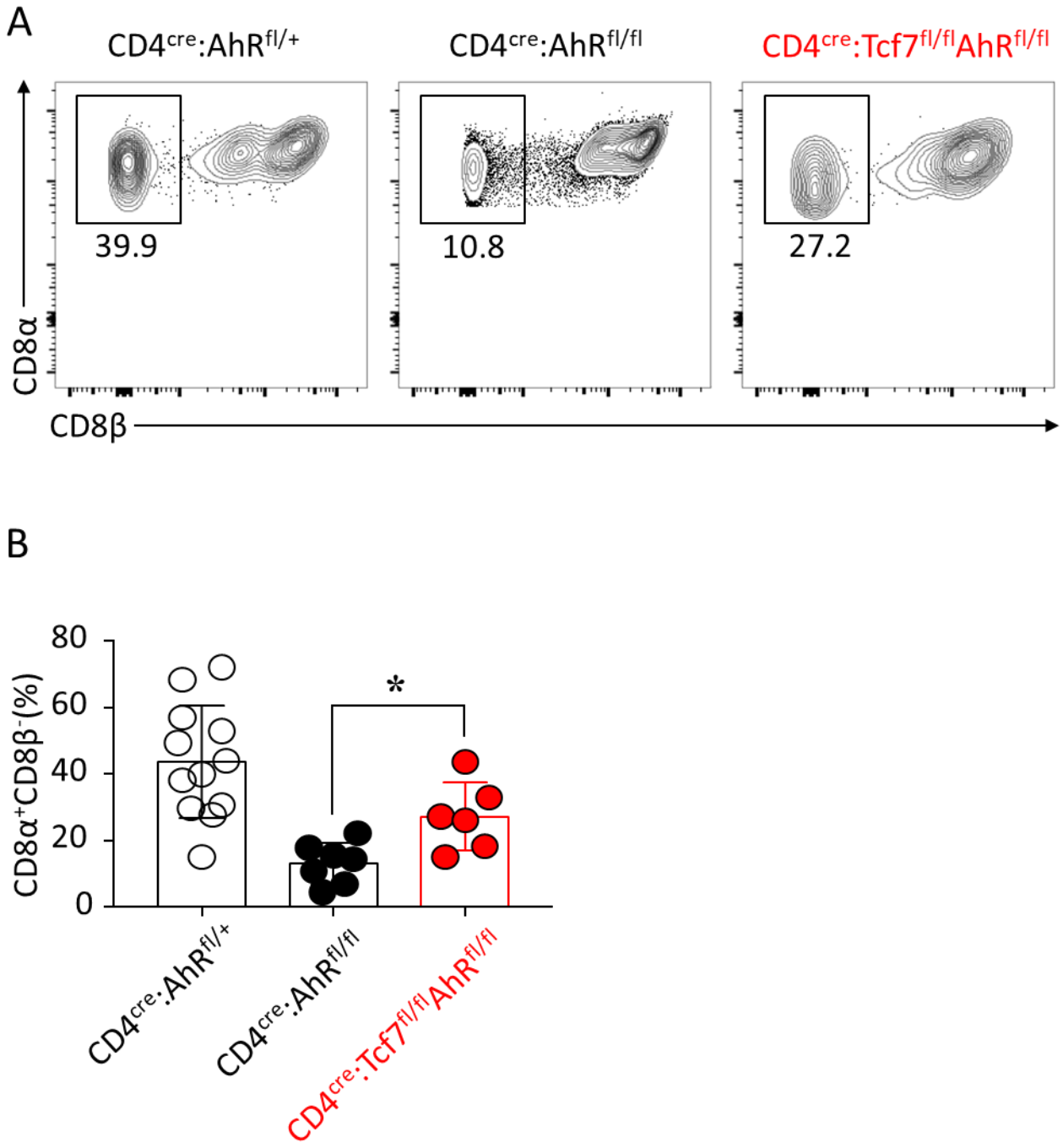


*Figure 4.6, Deletion of TCF1 restores the generation of TCRαβ$^+$CD8αα$^+$ IELs in AhR deficient mice. IELs were collected from CD4^{cre}:AhR$^{fl/+}$, CD4^{cre}:AhR$^{fl/fl}$ or CD4^{cre}:Tcf7$^{fl/fl}$AhR$^{fl/fl}$ mice between 8 to 16 weeks. Cells were pre-gated on CD45.2$^+$TCRαβ$^+$CD4$^-$. A) Contour plots showing the proportion of TCRαβ$^+$CD8αα$^+$ IELs. B) statistics of the CD8αα$^+$ proportion in IELs. Results shown are compiled of two independent experiments and mean value ± SD. Statistical analysis by student t test. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.*

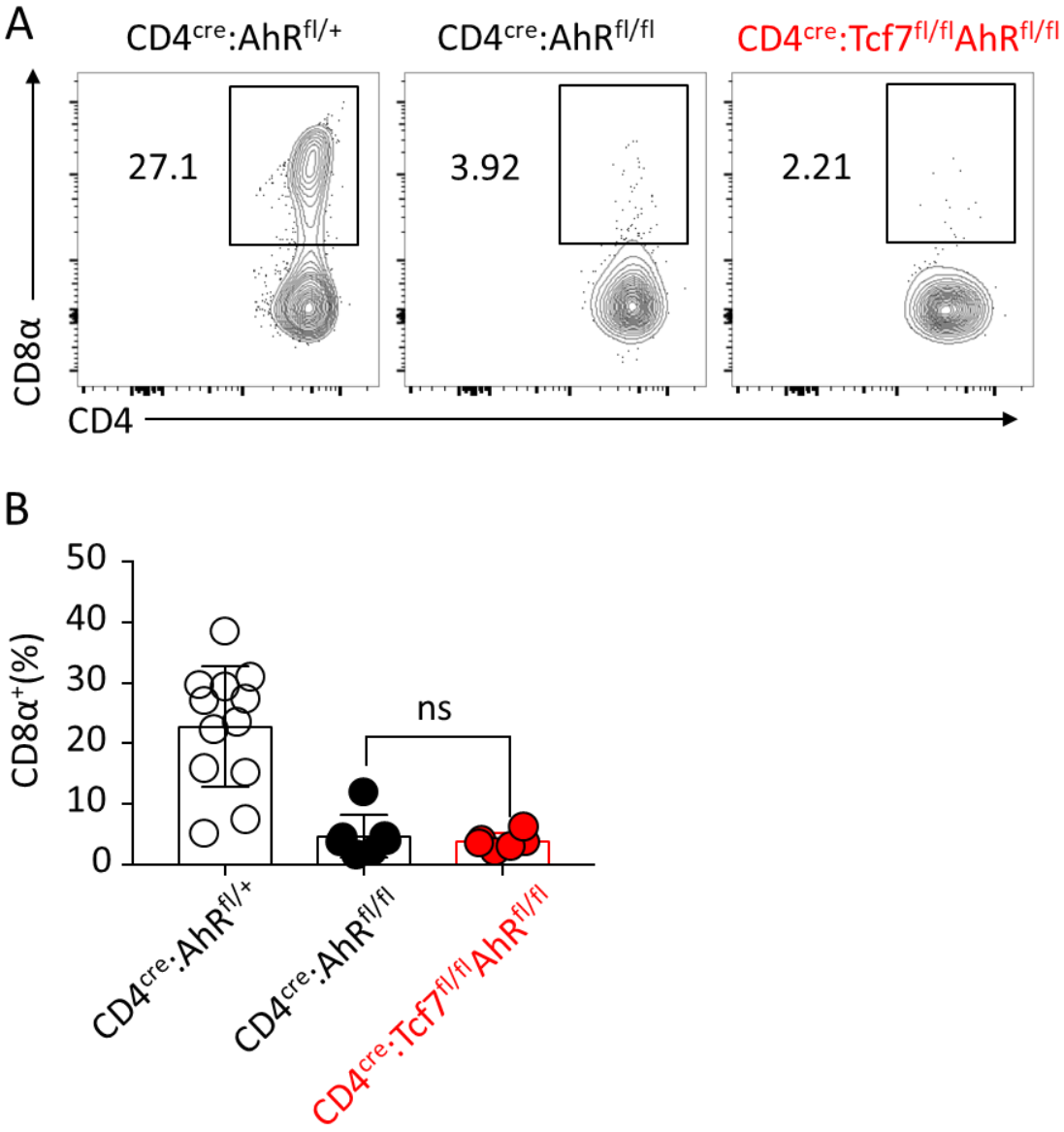


*Figure 4.7, Deletion of TCF1 does not rescue the generation of CD8αα$^+$CD4$^+$ IELs in AhR deficient mice. IELs were collected from CD4^{cre}:AhR$^{fl/+}$,CD4^{cre}:AhR$^{fl/fl}$ or CD4^{cre}:Tcf7$^{fl/fl}$AhR$^{fl/fl}$ mice between 8 to 16 weeks. Cells were pre-gated on CD45.2$^+$TCRαβ$^+$CD4$^+$. A) Contour plots showing the proportion of CD8αα$^+$CD4$^+$ IELs. B) statistics of the CD8αα$^+$CD4$^+$ proportion in IELs. Results shown are compiled of two independent experiments and mean value ± SD. Statistical analysis by student t test. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.*

4.5. Genetic deletion of TCF1 leads to the upregulation of LEF1

I observed that the generation of CD8αα^{+}CD4^{+} IELs was not rescued by deleting TCF1 in AhR deficient mice. Since LEF1 can partially compensate the loss of TCF1 function by sharing common binding loci (Emmanuel et al., 2018), I measured the LEF1 expression. I observed that the deletion of TCF1 significantly enhanced the expression of LEF1 (**Figure 4.8**). A drastic increase of LEF1 was particularly recorded in the thymus but also in CD4^{+} IELs. Therefore, I proposed that the upregulation of LEF1 in CD4^{+} IELs might inhibit the generation of CD8αα^{+}CD4^{+} IELs in CD4^{cre}:Tcf7$^{fl/fl}$AhR$^{fl/fl}$ mice.

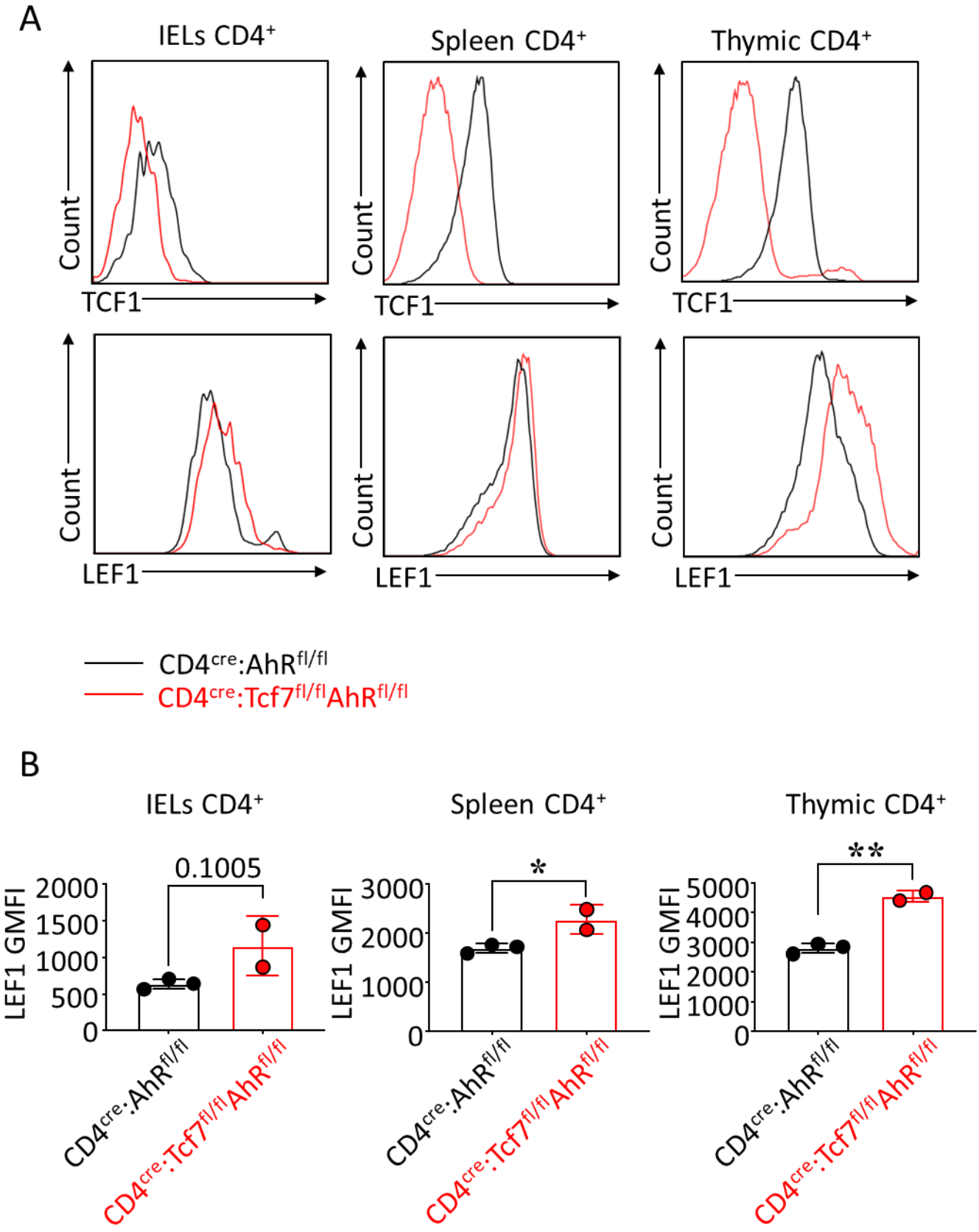

A
IELs CD4+
Spleen CD4+
Thymic CD4+
Count
TCF1
LEF1
CD4cre:AhRfl/fl
CD4cre:Tcf7fl/flAhRfl/fl
B
IELs CD4+
Spleen CD4+
Thymic CD4+
LEF1 GMFI
0.1005
*
**
CD4cre:AhRfl/fl
CD4cre:Tcf7fl/flAhRfl/fl

Figure 4.8, The upregulation of LEF1 in TCF1 deficient mice. IELs, spleens and Thymus were collected from $CD4^{cre}$:$AhR^{fl/+}$, $CD4^{cre}$:$AhR^{fl/fl}$ or $CD4^{cre}$:$Tcf7^{fl/fl}AhR^{fl/fl}$ mice. A) Histograms showing TCF1 and LEF1 expression. Red colour highlights TCF1 knock out. Results shown are the representative plot. B) statistics of the LEF1 GMFI. Results shown are representative of two independent experiments and mean value ± SD. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

4.6. Discussion

My results demonstrate that AhR is required for the formation of both CD8αα$^+$CD4$^+$ IELs and TCRαβ$^+$CD8αα$^+$ IELs, but how AhR controls such unconventional reprogramming process has not been well-studied. Previous studies indicated that AhR may have a potential role in suppressing ThPOK, leading to the differentiation of CD8αα$^+$CD4$^+$ IELs from CD4$^+$ IELs (Mucida et al., 2013; Reis et al., 2013; Sujino et al., 2016). However, no further work has been conducted to explain how AhR suppressed ThPOK either directly or indirectly (Cervantes-Barragan et al., 2017; Sujino et al., 2016). In this chapter, the intracellular mechanism underlying the AhR-mediated gain of CD8α expression on CD4$^+$ T cells as well as the generation of cytotoxic IELs were investigated. I focused on TCF1/LEF1 as candidate intermediators between the regulation of AhR and ThPOK during the differentiation of CD8αα$^+$CD4$^+$ IELs. TCF1 is an important regulator to promote CD4$^+$ T cell formation from the DP cells in the thymus by controlling the ThPOK-Runx3 axis (Steinke et al., 2014). Therefore, a model whereby AhR suppresses TCF1/LEF1 to reduce ThPOK expression is a plausible mechanism for AhR-mediated gain of CD8α expression on T cells.

The hypothesis was inspired by the RNA-seq result which indicated a potential of AhR/TCF1 axis to control the differentiation of cytotoxic IELs. I also examined the expression of TCF1 in published RNA-seq data (GSE84615)(Bilate et al., 2016), in which transcriptomes were compared between CD8αα$^+$CD4$^+$ IELs and CD4$^+$ IELs generated from a model of adoptively transferring CD4$^+$ T cells in Rag$^{-/-}$ mice. Consistent to my data, the transcript for *Tcf7* was downregulated in CD8αα$^+$CD4$^+$ IELs compared to CD4$^+$ IELs (data not shown). I also used the Tcf7-GFP reporter mice to confirm the downregulation of Tcf7 in CD8αα$^+$CD4$^+$ IELs.

Collectively, these data suggested that the suppression of TCF1 accompanied with the generation of cytotoxic IELs.

I confirmed that the protein level of TCF1 was significantly lower in CD8αα$^+$CD4$^+$ IELs and TCRαβ$^+$CD8αα$^+$ IELs compared respective control populations by flow cytometry. Even in TCRγδ$^+$ cells, the TCF1 expression was largely diminished. The consistent association between low TCF1 expression in certain IEL subsets and the requirement of AhR for the generation of these subsets suggests the suppression of TCF1 by AhR which will be examined in the next chapter. To answer whether TCF1 downregulation is required to generate CD8αα$^+$CD4$^+$ IELs, I overexpressed TCF1-WT with both empty vector pR-GFP and dysfunctional TCF1-Mut5aa as controls to evaluate the function of TCF1 in the modulation of CD8αα$^+$CD4$^+$ IELs. TCF1-Mut5aa can help me to test whether the intrinsic HDAC function of TCF1 is required to regulate the cytotoxic differentiation of CD4$^+$ IELs. By the overexpression of TCF1-WT, the expression of ThPOK was maintained (S. Xing et al., 2016), resulting in an inhibition of the development of CD8αα$^+$CD4$^+$ IELs from CD4$^+$ IELs. The overexpression of TCF1-Mut5aa failed to inhibit the differentiation of CD8αα$^+$CD4$^+$ IELs, suggesting the maintenance of ThPOK by TCF1 required its intrinsic HDAC activity. Hence, TCF1 via the intrinsic HDAC function prevents the differentiation of cytotoxic IELs.

In addition to the gain of function experiment, I also conducted the loss of function experiment. I found that the deletion of TCF1 is capable to rescue the development of TCRαβ$^+$CD8αα$^+$ IELs in AhR deficient mice. However, the differentiation of CD8αα$^+$CD4$^+$ IELs was not rescued. One explanation is that LEF1, the close family member of TCF1 was significantly

upregulated and thus compensated the loss of HDAC function of TCF1 since the HDAC activity of LEF1 was reported partially redundant to TCF1 (Xing et al., 2016).

According to the RNA-seq result, both TCF1 and LEF1 expression were lower in $CD8\alpha\alpha^{+}CD4^{+}$ IELs compared to $CD4^{+}$ IELs, suggesting a synergistic reduction of both TCF1 and LEF1 might be required for the differentiation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs (Haynes et al., 1996; Okamura et al., 1998). To further investigate the redundancy of LEF1, a vector for LEF1 overexpression was also constructed. This experiment will be conducted for preparing the publication manuscript in the near future. Meanwhile, we are currently breeding $CD4^{cre}Rosa26^{GFP/GFP}$ mice crossing with $AhR^{fl/fl}TCF1^{fl/fl}LEF1^{fl/fl}$ in order to generate a mouse model with dual deletion of TCF1 and LEF1 in T cells in AhR deficient mice, which can help to examine whether the redundancy of LEF1 inhibits the AhR-TCF1 mediated development of $CD8\alpha\alpha^{+}CD4^{+}$ IELs.

Taken together, TCF1 plays an inhibitory role in the formation of cytotoxic IELs. Our results clearly showed that the reduction of TCF1 was required for the regeneration $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs, suggesting the possibility that AhR acts upstream of TCF1/LEF1 to modulate the cytotoxic differentiation of IELs. The molecular mechanism on how AhR suppresses TCF1 will be investigated in next chapter.

CHAPTER V

The molecular mechanism underlying the suppression of TCF1/LEF1 by AhR

5.1. Preamble

In the previous two chapters, I have demonstrated that both AhR and TCF1 critically regulate the generation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs. The association between low TCF1 expression in IEL subsets including $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs and the dependence of these subsets on AhR for their generation suggested a potential interaction between these two mechanisms. TCF1/LEF1 are the major end point mediators of WNT signalling (Cadigan & Waterman, 2012). Previous studies suggested that AhR suppresses the activation of WNT pathway (Mathew et al., 2009; A. J. Schneider et al., 2014; Shiizaki, Kido, & Mizuta, 2019). However, it remains unknown whether AhR directly regulates TCF1/LEF1 expression.

Previously, we attempted ChIP-qPCR to test whether AhR can directly bind to *zbtb7b* and *Runx3*. Rare enrichment of AhR near the *Zbtb7b* and *Runx3* regulatory elements were found. Consistently, the mRNA of Runx3 was not significantly increased in both our and published RNA-seq data. Therefore, our focus in this chapter is on studying the direct regulation of AhR on TCF1/LEF1.

5.1.1. Prediction of AhR bindings on the Tcf7 promoter

AhR recognises the binding motif GCGTG. By binding to the promoter or other regulatory regions for genes, AhR regulates the expression of targeted genes. By searching the GCGTG binding motif, I found 21 potential binding sites in the mouse *Tcf7* gene with more than half of them are proximal to the promoter **(Figure 5.1**). The high enrichment of the AhR binding motif suggests that AhR might directly bind to the *Tcf7* and regulate its transcription.

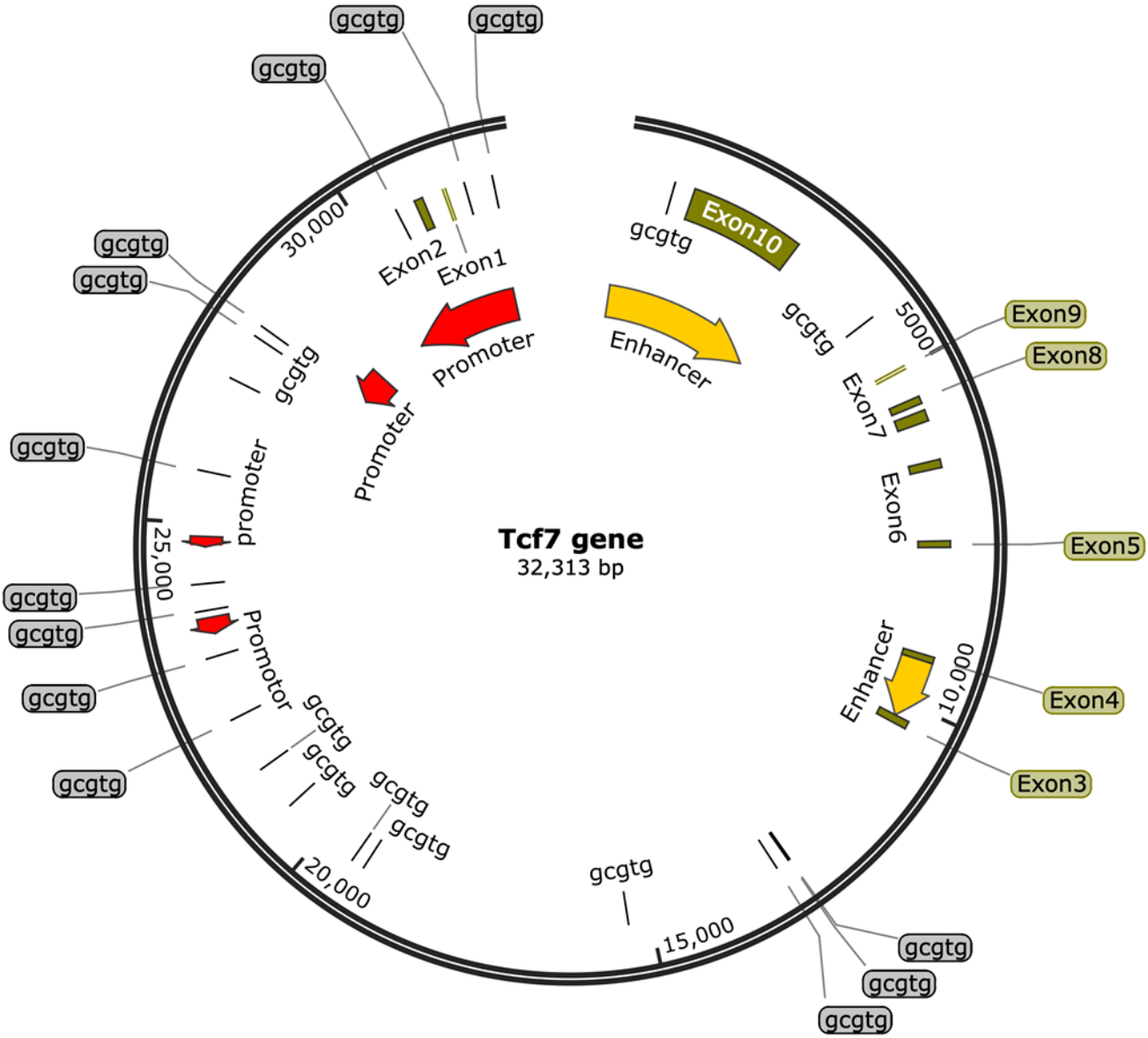


Figure 5.1, Enriched GCGTG motif in Tcf7 gene. Tcf7 sequence was obtained from UCSC database. Map was generated using SnapGene Viewer. Red block represents promoter, yellow block represents enhancer, grey block represents GCGTG motif, green block represents exons.

5.1.2. Chapter outline

In this chapter, I will investigate how AhR regulates the expression of TCF1/LEF1. Different $CD4^+$ T cell populations from thymus, spleen and IELs as well as EL4 cell line will be stimulated with AhR ligands including FICZ and TCDD, and I will use quantitative real-time PCR to measure the change in Tcf7 transcription after the stimulation. After examining the suppression of TCF1 expression by AhR, I will use ChIP-qPCR and ChIP-seq to identify the direct binding of AhR to the predicted regulatory elements in *Tcf7/Lef1 genes*.

5.2. AhR signalling pathway negatively regulates TCF1 expression

First, I examined whether AhR is required to induce the downregulation of TCF1 *in vivo*. $CD4^+$ T cells isolated from either $CD4^{cre}AhR^{fl/+}$ or $CD4^{cre}AhR^{fl/fl}$ mice were adoptively transferred into $Rag^{-/-}$ mice. After 4 weeks, IELs derived from the transferred cells were collected to measure the expression of TCF1 by flow cytometry. The expression of TCF1 was significantly higher in AhR deficient $CD4^+$ IELs than that of WT counterparts (**Figure 5.2A**), indicating that the AhR pathway is required to downregulate TCF1 expression. Furthermore, the enhanced expression of TCF1 in AhR deficient $CD4^+$ IELs was accompanied by the enhanced expression of ThPOK (**Figure 5.2B**). This was expected since the persistence of TCF1 expression sustained the expression of ThPOK. In contrast, Runx3 expression was comparable between two groups, suggesting Runx3 played a minor if any role in controlling the cytotoxic differentiation of $CD4^+$ IELs.

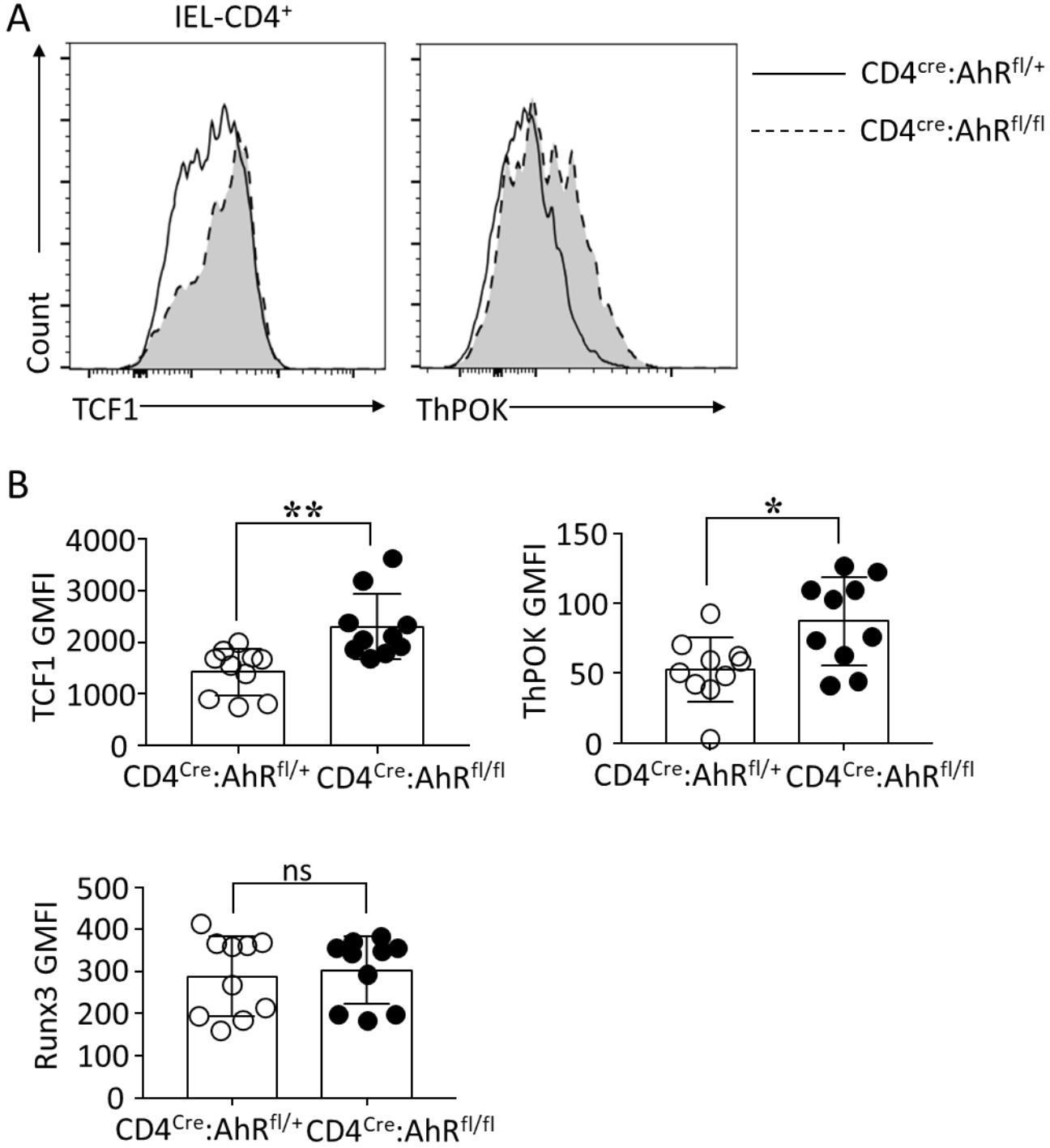


Figure 5.2, Deletion of AhR results in increased expression of TCF1 and ThPOK in IELs. IELs were collected from Rag$^{-/-}$ mice that were adoptively transferred with CD4 T cells isolated from CD4^{cre}:AhR$^{fl/+}$ or CD4^{cre}:AhR$^{fl/fl}$ mice. A) Histograms of TCF1 and ThPOK expression in IELs. B) Statistics of TCF1, ThPOK and Runx3 GMFI in IELs. Results are compiled of two independence experiments and mean value ± SD. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

In my previous experiments, FICZ has been shown to efficiently induce CD8α expression on $CD4^+$ T cells isolated from the spleen or lymph nodes, while TCDD moderately upregulated CD8α expression. To test whether the treatment of AhR agonists can directly suppress the expression of TCF1 in T cells, the expression level of TCF1 was measured in cultured $CD4^+$ T cells treated with AhR ligands FICZ and TCDD. In line with the differential upregulation of CD8α expression on the $CD4^+$ T cells, FICZ significantly downregulated TCF1 expression in cultured $CD4^+$ T cells while TCDD did so to less extent (**Figure 5.3A&B**). Notably, the downregulation of TCF1 is dependent on the AhR signalling pathway since FICZ or TCDD was unable to downregulate TCF1 in AhR deficient cells. Consistently, ligand treatments also suppressed TCF1 level in thymic $CD4^+$ T cells (**Figure 5.3C**).

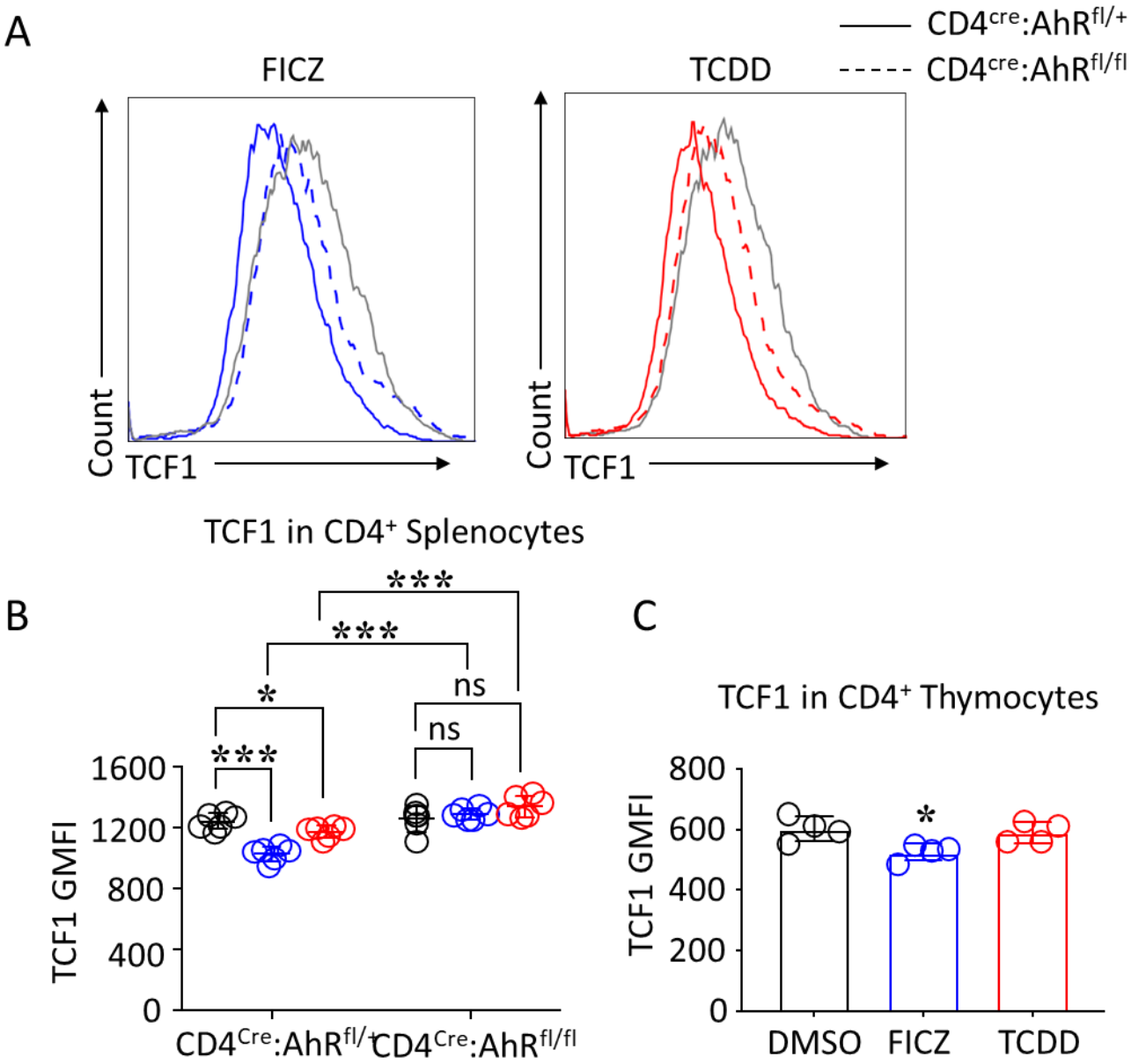


*Figure 5.3, AhR ligands reduced TCF1 in the CD4⁺ T cells in vitro. Naïve CD4⁺ T cells were purified from CD4^{cre}:AhR$^{fl/+}$ or CD4^{cre}:AhR$^{fl/fl}$ mice and cultured in vitro with stimulation of anti-CD3, anti-CD28, anti-IFNγ, anti-IL-4, IL-6, TGF-β, IL-2 for 3 days. A) Histogram plot of TCF1 expression in CD4⁺ T cells in vitro. Grey line represents DMSO treated group. B) Statistics of TCF1 GMFI in splenic CD4⁺ T cells. C) Statistics of TCF1 GMFI in thymic CD4⁺ T cells. Thymic CD4⁺ T cells were purified from B6 mice and cultured for two days with ligand treatments. Results shown are representative of two independent experiments and mean value ± SD. Statistical analysis by student t test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, ns = $P \geq 0.05$.*

Finally, the effect of AhR ligands on isolated $CD4^+$ IELs was tested. As expected, FICZ reduced TCF1 expression and simultaneously upregulated CD8α expression in $TCF1^{low}$ $CD4^+$ IELs (**Figure 5.4**). TCDD did not significantly suppress TCF1 in cultured $CD4^+$ IELs, probably due to a short treatment time, which is in line with its modest effect in the upregulation of CD8α on $CD4^+$ IELs. Overall, FICZ suppressed TCF1 in $CD4^+$ T cells via AhR signalling pathway.

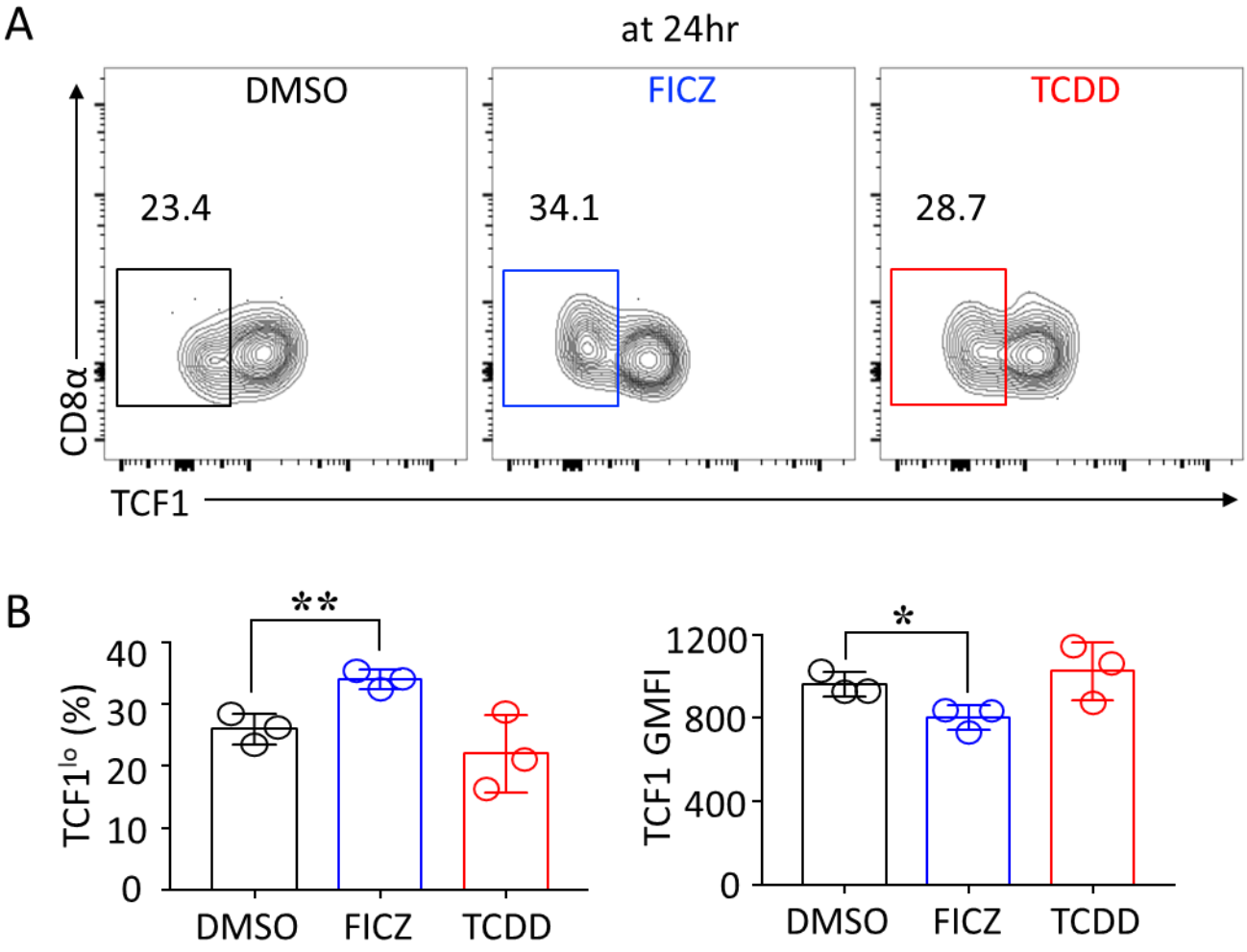


Figure 5.4, FICZ inhibits the expression of TCF1 in IELs in vitro. IELs were purified from C57BL/6 mice and cultured in vitro with stimulation of IL-2 for 24 hours. Ligands were treated at 1μM from the start of culture. A) contour plot of CD8α and TCF1 expressions on the CD4$^+$ IELs. Cells were pre-gated on CD45.2$^+$TCRαβ$^+$CD4$^+$. Results shown are representative of two independent experiments and mean value ± SD. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

5.3. Prolonged stimulation of AhR ligands strongly inhibits TCF1 expression

AhR has several feedback mechanisms to negatively regulate its activity, which includes i) the activation of AhR induces the expression of key metabolizing enzymes, such as CYP1A1, ii) the upregulation of AhRR and iii) the ubiquitin/proteasome pathway that can degrade the ligands or suppress its activation strength, hence, maintaining its physiological signal strength (Bersten et al., 2013). Therefore, prolonged stimulation of AhR ligands are required for stronger effects. My previous experiments demonstrated that the effects of AhR agonists on T cells were determined by the duration of stimulation, with shorter stimulation time associated with weaker the upregulation of CD8α or less effective downregulation of TCF1. I next investigated whether continuous supplement of AhR ligands can further enhance the reduction of TCF1. Due to the reduced survival of primary T cells in extended culture time, I switched to EL4 cell line, which was derived from thymic T cells and express high level of TCF1 and AhR. EL4 cells were cultured with AhR ligands for 48 hours or 144 hours with culture media replaced every 48 hours. The viability of EL4 cells at 48h and 144h were above 95%. Both FICZ and TCDD modestly reduced TCF1 expression in EL4 cells after 48 hours but the effect became much more prominent after 144 hours (**Figure 5.5A and B**).

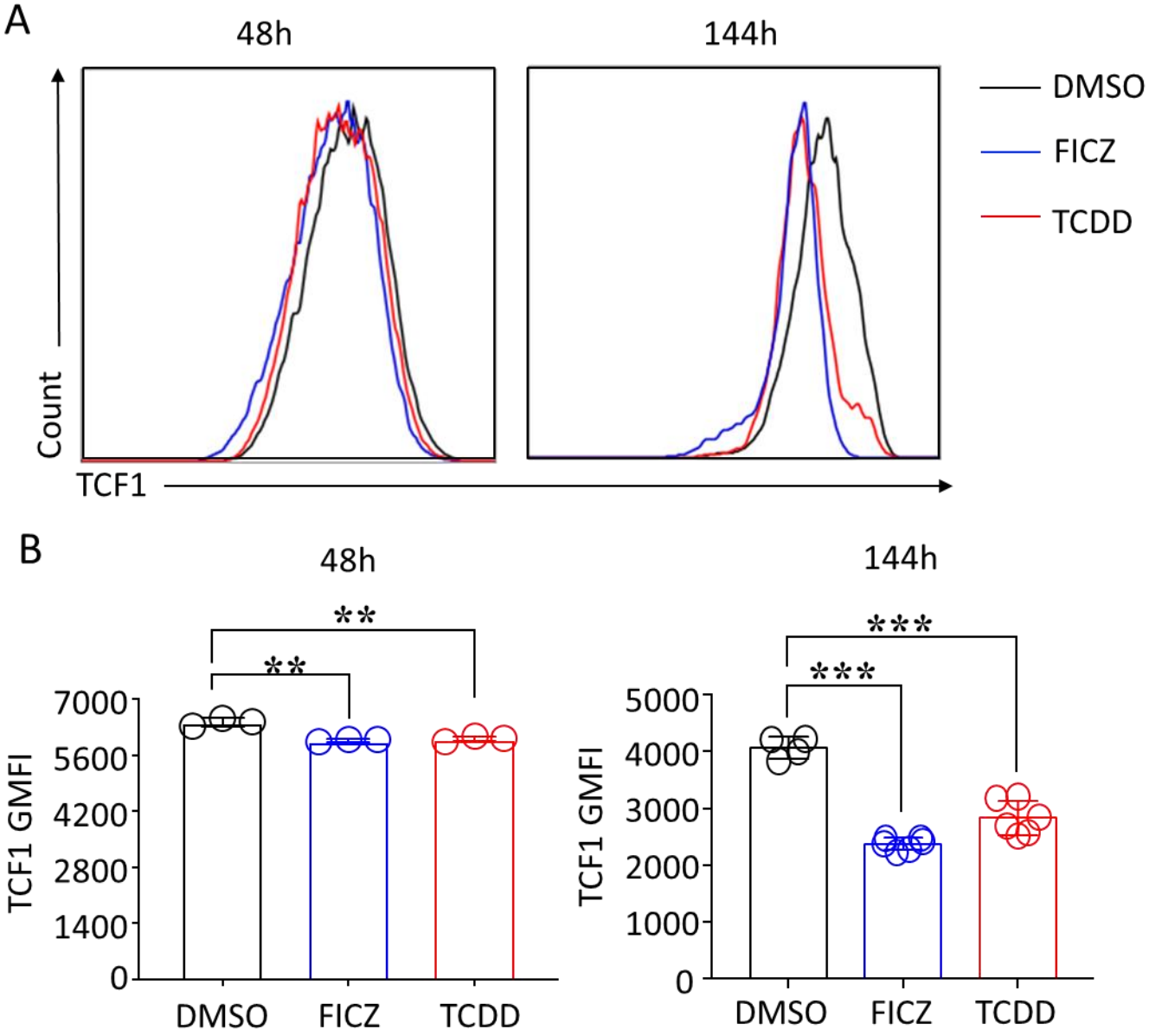


Figure 5.5, AhR ligands suppress the expression of TCF1 in EL4 cells in vitro. A) Histograms of TCF1 expression in EL4 cells at 48 hours or 144 hours of ligand treatments. B) Statistics of TCF1 GMFI of EL4 cells at 48 or 144 hours of ligand treatments. Ligands were refreshed every two days. Results shown are the representative of two independent experiments and mean value ± SD. Statistical analysis by student t test. ***** = P < 0.05; ** = P < 0.01; *** = P < 0.001, ns = P≥0.05.*

5.4. AhR promptly suppresses the *Tcf7* transcription

To understand the kinetics of AhR signal to modulates the transcription of *Tcf7*, I measured *Tcf7* mRNA by qPCR at different timepoints ranging from 1.5 hours to 48 hours. The results demonstrated that the stimulation of AhR agonists induced the downregulation of *Tcf7* mRNA for about 3-fold at 5 hours after the stimulation. Such effect began to decline, showing about 2-fold downregulation at 10 hours after the simulation (**Figure 5.6A**). In contrast, the signature downstream target of AhR pathway, *Cyp1a1*, was not upregulated at early timepoint by the ligand stimulation but responded sharply at 10 hours after the ligand stimulation (**Figure 5.6B**). Therefore, the suppression of *Tcf7* by AhR happened promptly but the lasting effect might need a prolonged and/or multiple stimulation.

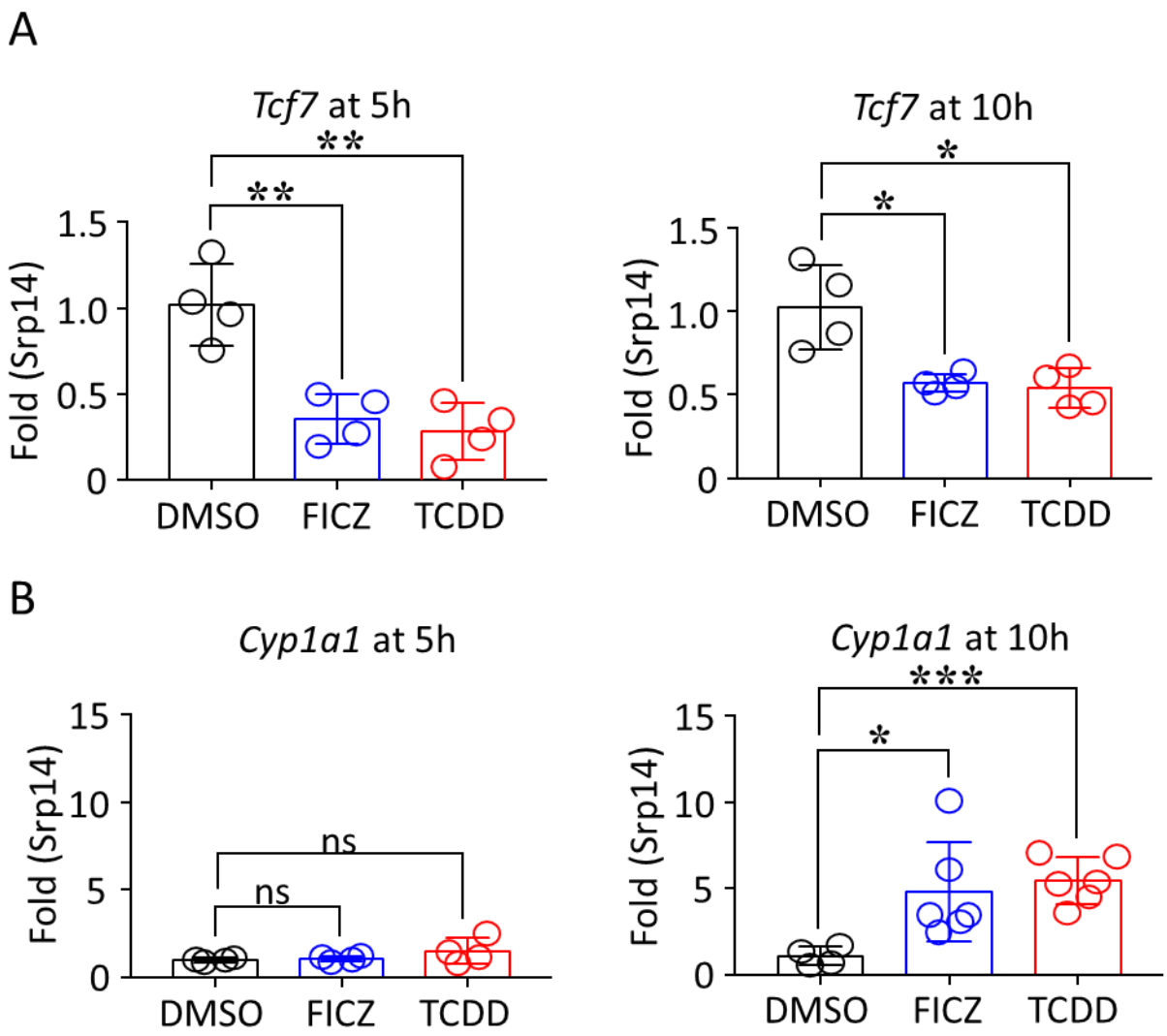


Figure 5.6, Ligand treatments suppresses the transcription of Tcf7 in EL4 cells. A) fold change of TCF1 mRNA in EL4 cells at 5 hours or 10 hours after ligand treatment. B) fold change of CYP1A1 mRNA in EL4 cells at 5 hours or 10 hours after ligand treatment. Results shown are the representative of two independent experiments and mean value ± SD. Statistical analysis by student t test. ***** = P < 0.05;* ****** = P < 0.01;* ******* = P < 0.001, ns = P≥0.05.*

5.5. The binding of AhR on the regulatory elements of *Tcf7* and *Lef1* genes

The result from my previous experiment showed the rapid transcriptional suppression of *Tcf7* by the stimulation of AhR agonists, suggesting a direct binding and transcriptional regulation of *Tcf7* gene by AhR. To test this, ChIP-qPCR experiment was conducted. Primers to detect the binding of AhR on the *Tcf7* gene were designed based on the predicted binding loci containing the AhR binding motif (**Figure 5.7A**). I used antibodies specifically for AhR and RNA polymerase II (Pol2) for immunoprecipitation to pull down DNA fragments so that both the sites bound by AhR and the levels of active transcription can be determined. The RNA polymerase is a family of enzymes for gene transcription, in which Pol2 typically transcribes DNA into precursor mRNA (Kornberg, 1999; Sims, Mandal, & Reinberg, 2004). When the transcription of gene is activated, Pol2 will be recruited to DNA and walk through the whole transcriptional region to generate mRNA. When transcription being suppressed, the enrichment of Pol2 bindings along the transcriptional region will be reduced. By combining AhR-ChIP-qPCR and Pol2-ChIP-qPCR, AhR interaction with Tcf7 can be addressed. According to the results, binding peaks were detected on P1 and P2 region on the *Tcf7* gene suggested that ligands induced binding of AhR to *Tcf7* promoter. A weaker binding on P5 and P6 region was also observed (**Figure 5.7B**). Furthermore, there was drastic loss of Pol2 bindings across the *Tcf7* region started at P2 region and the suppressed binding occurred over the *Tcf7* promoter and enhancer (**Figure 5.7B**). Such loss of Pol2 binding suggested that there was a suppressed transcription of *Tcf7*, which resulted in the loss of TCF1 expression .

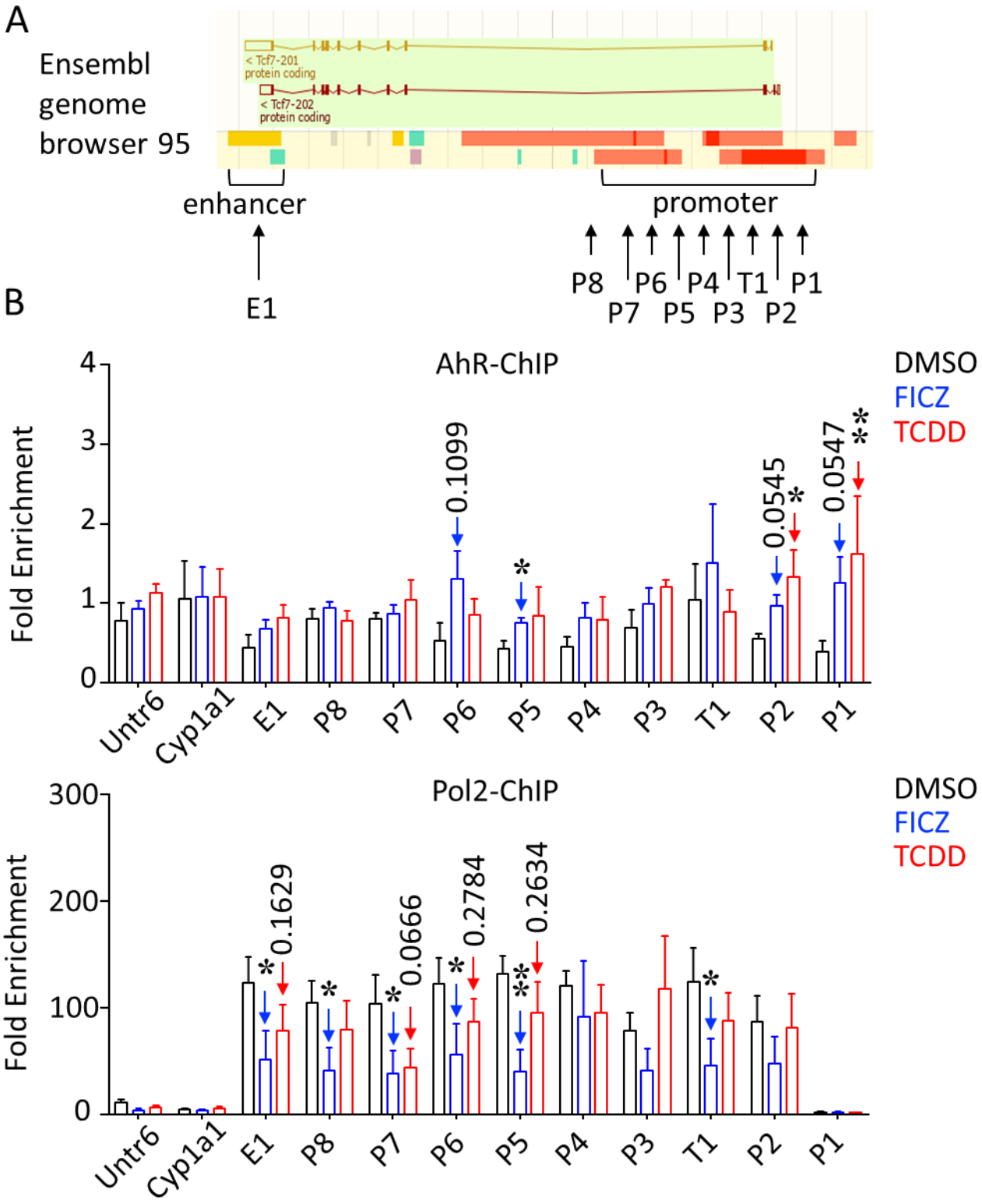

A
Ensembl
genome
browser 95
< Tcf7-201
protein coding
< Tcf7-202
protein coding
enhancer
promoter
E1
P8
P7
P6
P5
P4
P3
T1
P2
P1
B
AhR-ChIP
DMSO
FICZ
TCDD
Fold Enrichment
0.1099
0.0545
0.0547
Untr6
Cyp1a1
E1
P8
P7
P6
P5
P4
P3
T1
P2
P1
Pol2-ChIP
0.1629
0.0666
0.2784
0.2634

Figure 5.7, AhR directly binds to the promoter of Tcf7 and inhibits the transcription. EL4 cells were treated with AhR ligands for 90min and fixed by 1% formaldehyde, followed by chromatin immunoprecipitation and DNA purification. A) design of probes detecting the binding of AhR on TCF1 gene. B) ChIP-qPCR of AhR and Pol2. Fold enrichment was tested by q-PCR with designed probes. Results shown are compile of five independent experiments and mean value ± SEM. Statistical analysis by student t test. ***** *= P < 0.05;* ****** *= P < 0.01;* ******* *= P < 0.001, ns = P≥0.05.*

5.6. Discussion

Activation of AhR and loss of TCF1 are required for the development of both CD8αα$^+$CD4$^+$ IELs and TCRαβ$^+$CD8αα$^+$ IELs, but the interaction between these two pathways has not been studied previously. Our transcriptomic analysis of IEL subsets using RNA-seq provided a clue that AhR activation might be associated with low TCF1 expression in IEL subpopulations. I then performed a set of experiments to validate this regulation. From the *in vitro* culture of CD4$^+$ T cells from thymus, spleen or IELs, I confirmed that the stimulation of AhR ligands downregulated TCF1 expression. I found FICZ strongly suppressed TCF1 expression in primary CD4$^+$ T cells from different resources and thymocyte-derived EL4 cell line, while of the deficiency of AhR pathway abrogated this suppression. In comparison, TCDD only modestly induced CD8α expression or reduced TCF1 in primary CD4$^+$ T cells but showed more significant downregulation of TCF1 in EL4 cells. Although it is unclear why TCDD induced significant downregulation of TCF1 in EL4 cell line while not or weak in primary T cells, these results collectively demonstrate the ability of strong AhR agonists in reducing protein expression of TCF1.

At the transcription level, the transcripts of *Tcf7* at different timepoint were measured. I found that the suppression of *Tcf7* transcription occurred promptly after the stimulation of AhR agonists. Notably, this response was induced faster than AhR-induced transcription of *Cyp1a1*, the signature downstream target of AhR pathway. Interestingly, without continuously supplying the agonists, the suppression of *Tcf7* transcription was relaxed gradually. My experiment revealed that the replenishing of the ligands in culture induced a much stronger suppression of TCF1 expression, confirming that a persistent AhR signal is required to effectively inhibit the expression of TCF1.

The downregulation of Tcf7 transcripts was prior to the suppression of TCF1 protein suggested a direct transcriptional inhibition. To further investigate the molecular interaction between AhR and the *Tcf7* gene, I performed ChIP-qPCR and ChIP-seq to test the specific binding of AhR on *Tcf7* gene. In the ChIP-qPCR experiment, primers were designed to cover potential sites proximal to *Tcf7* gene that contain AhR binding motif. Among them, several binding events were detected. Even though each binding strength was not high, the synergistic effect of multiple binding sites is likely pronounced, similar example of AhR multiple binding for a single gene reported for other AhR regulated genes, such as *Cyp1a1* (Denison & Nagy, 2003; Hestermann & Brown, 2003; Kobayashi, Sogawa, & Fujii-Kuriyama, 1996; Patel, Kim, Peters, & Perdew, 2006; Tian, Ke, Chen, & Sheng, 2003). Since accumulative stimulation of AhR ligands is required to effectively inhibit TCF1 expression, the weak AhR-*Tcf7* binding after a single dose of ligand treatment might be expected. To further confirm that the binding of AhR to *Tcf7* can modulate the transcription, I applied the Pol2 ChIP to measure the change of active transcription. Using the Pol2-ChIP-qPCR, I found that the Pol2 bindings across the *Tcf7* gene was reduced after the stimulation of AhR ligands, which suggested that AhR not only bound to the *Tcf7* gene but also suppressed its transcription. While additional repeats will be conducted to validate the above results, these results support a model whereby the activation of AhR induces its binding to the *Tcf7* gene and impose a suppression on its transcription, which leads to the suppression of TCF1 expression. Probably due to the strong feedback inhibitory mechanism of AhR signalling pathway, a persistent stimulation of AhR ligands is required for effective suppression of TCF1 expression.

To understand genome-wide binding events of AhR beyond the binding to *Tcf7*, I conducted ChIP-seq. In addition, I chose two different timepoints to understand the kinetics of binding events. Although the data quality was not great, I can still see that the binding peaks were

enriched mainly in the promoter flank of both *Tcf7* and *Lef1* , which also showed enrichment of AhR binding motif GCGTG. On the contrary, no binding peaks were observed in controls with vehicle treatment or technic controls of ChIP without antibody. Interestingly, the binding events induced by FICZ seemed to happen earlier (30 minutes) than events induced by TCDD. Both FICZ and TCDD induced AhR binding to *Tcf7* and *Lef1* in sites enriched with AhR binding motif GCGTG. Published results indicated that the loss of ThPOK is the major reason for the increased CD8α expression in the IELs (Sujino et al., 2016). I did not find significant binding peaks proximal to *Zbtb7b* gene, suggesting that AhR induced downregulation of ThPOK is most likely an indirect event of AhR signal. The results support the notion that AhR-induced suppression of TCF1/LEF1 represents a major driving force for the downregulation of ThPOK in IELs.

CHAPTER VI

Discussion

6.1. AhR: a direct sensor of adaptive immune cells to environmental stimuli

The adaptive immune system in mammals requires a restrict orchestration to benefit the host health. Such comprehensive system builds up the final defencing line against pathogens from the environment. Effective activation of the adaptive immune response usually relies on a sufficient communication between the immune system and the environment, such as the antigen presentation by DCs (Guermonprez, Valladeau, Zitvogel, Thery, & Amigorena, 2002). By this way, the critical information from pathogens, as a form of antigens can be effectively delivered to T cells, and consequently induce the activation and differentiation of $CD8^+$ T cells to specifically eliminate the invaders with the help of primed $CD4^+$ T cells. Although immunocompromised individuals are vulnerable to pathogens, excessive reaction of immune responses can lead to harmful autoimmunity. Therefore, fine-tuning of adaptive immune responses is essential. Even though extensive studies have reported that cytokines produced by the immune system and other tissues are critical in modulating T-cell immunity (Arango Duque & Descoteaux, 2014; Banyer, Hamilton, Ramshaw, & Ramsay, 2000; Belardelli & Ferrantini, 2002; Hansson, Libby, Schonbeck, & Yan, 2002; A. Jain & Pasare, 2017), an concept is emerging that environmental stimuli especially aromatic chemicals can also directly modulate the function of T cells. Aromatic chemicals are highly enriched in our living environment with diet and gut microbial metabolites as the major resource. Therefore, it is very important to study how T cells sense these compounds.

Aromatic chemicals can be categorised into two major classes: non-polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). Although PAHs used to be considered primarily from environmental carcinogens and harmful for human health (H. Yu,

2002), recent studies revealed that many PAHs are enriched in diet especially vegetables and beneficial to human health by regulating the immune system through the AhR signalling pathway (Gutierrez-Vazquez & Quintana, 2018; Lamas et al., 2018). AhR is broadly expressed in both innate and adaptive immune cells (Ambrosio et al., 2019) with a high level of expression in some populations such as Th17 cells (de Lima et al., 2018). This receptor was initially discovered as a de-toxic pathway in the hepatocytes that generate enzymes CYP1A1 or CYP1B1 to catalyse the degradation of chemicals commonly contains in environmental pollutants (Ehrlich et al., 2018; Kiyomatsu-Oda et al., 2018; Kovalova et al., 2017; Nebert et al., 2000). It is also considered playing an important role in carcinogenesis (Xue, Fu, & Zhou, 2018). In the past decade, a new function of AhR in the regulation of immune homeostasis was emerging (Gutierrez-Vazquez & Quintana, 2018; Quintana & Sherr, 2013; Stockinger et al., 2014; Stockinger et al., 2011; H. Wang et al., 2015).

AhR can regulate T cells in many aspects such as survival, proliferation and function (Gutierrez-Vazquez & Quintana, 2018; Quintana & Sherr, 2013; Stockinger et al., 2014; Stockinger et al., 2011; H. Wang et al., 2015). The detailed information of AhR in the regulation of the immune system has been summarised in the introduction chapter. One interesting feature should be noted is that of the AhR signalling pathway can execute different or event opposite functions in the adaptive immunity, depending on the type of ligands. For example, FICZ promotes the Th17 differentiation and function while TCDD mainly favours the Treg differentiation (Quintana et al., 2008). In our study, FICZ strongly induced $CD8\alpha\alpha^{+}CD4^{+}$ IELs while TCDD was much weaker to do so, even though both can induce high levels of CYP1A1 expression in T cells. Yet, it is not clear why the ligand-induced AhR signal activation can result in drastically different effects on T cell differentiation and function. One potential explanation is the distinct conformational changes of AhR protein upon different

ligand stimulation. Structures of truncate AhR proteins have been solved (Sakurai, Shimizu, & Ohto, 2017; Schulte, Green, Wilz, Platten, & Daumke, 2017; Seok et al., 2017a), but the structural analysis of a full length AhR together with different ligands has not been achieved due to technical limitations on crystallisation. Such information together with the profiling of genome-wide transcriptional regulation will provide new insight for ligand-specific regulation of the AhR signalling pathway.

Studies highlighted that AhR influences the adaptive immunity particularly at barrier sites such as skin, lung and gut (Stockinger et al., 2014). Indeed, the expression of AhR varies in the same type of T cells residing in different tissues. High expression of AhR was recorded in T cells of intestinal epithelium. In line with the low expression of AhR in secondary lymphoid tissue such as spleen or lymph nodes, the deletion of AhR did not alter the frequency of T cell subset significantly (Fernandez-Salguero et al., 1995). This strongly indicates the regulatory function of AhR is tissue-specific. Notably, the gut microenvironment is one of the largest barrier sites that can easily access to a variety of AhR ligands (Brawner et al., 2019; Lamas et al., 2018; Natividad et al., 2018). Therefore, AhR is critical for the homeostasis of immune cells in the gut, including not only γδ T cells, Th17 and Treg but also ILC1s and ILC3s (Kiss & Vonarbourg, 2012; Y. Li et al., 2011; Quintana et al., 2008; Shin et al., 2013; Stange & Veldhoen, 2013). Our results suggest a direct and intrinsic regulatory role for AhR in the development of both $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs in responding to aromatic compounds in the gut environment.

6.2. Why is AhR important for the gut immune homeostasis?

The primary function of gastrointestinal track is for digestion and absorption of nutrients, which require to maintain the symbiosis with commensal microbiota and the homeostasis of gut associated lymphoid tissues to prevent food-associated allergy and pathogenic infections. Therefore, a normal gut is critical for body's health. Notably, AhR ligands are abundantly produced and accumulated in the gut, through activities including but not limited to intaking food that contains high concentrations of indoles, metabolism of commensal microorganisms, and potential absorption of environmental pollutants (Brawner et al., 2019; Lamas et al., 2018; Natividad et al., 2018). The intestinal epithelium, including IELs, is therefore exposed to a large quantity and variety of AhR ligands and has evolved to respond to the changes of AhR ligands to maintain a dynamic homeostasis.

In response to the mucosal environment, IELs not only dynamically interact with epithelial cells but also migrate from and to lamina propria (Edelblum et al., 2012; Hoytema van Konijnenburg et al., 2017; Sumida, 2019). Such activities provides efficient immune surveillance to protect from infections and maintain dynamic immune homeostasis for environmental stimuli (Sumida, 2019). Particularly, cytotoxic T cells that are highly enriched in IELs, providing a non-TCR dependent fast response to pathogens such as stimulations by tissue damage, cell stress factors or metabolic alterations, which is unlike conventional T cells (Konjar et al., 2017). Such defence mediated by cytotoxic T cells of IELs secures the balance between the symbiosis with commensal microbiota and the elimination of pathogens (Edelblum et al., 2012; Hoytema van Konijnenburg et al., 2017; Sumida, 2019).

AhR is essential for the development of cytotoxic T cells among IELs. In this project, I have shown that AhR induces the plasticity of IELs that is otherwise rare for $CD4^+$ T cells in spleen or lymph nodes. In these secondary lymphoid tissues, $CD4^+$ T cells are terminally differentiated and maintain their helper but not cytotoxic functions to support immune response. However, in the mucosal environment, $CD4^+$ T cells are able to acquire the expression of CD8α and the ability to secrete cytotoxic molecules such as granzyme B and gain cytotoxicity to kill pathogen invaded cells (Konjar et al., 2017; Olivares-Villagomez & Van Kaer, 2018; H. C. Wang, Zhou, Dragoo, & Klein, 2002). $TCR\alpha\beta^+CD8\alpha\alpha^+$ and $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs are cytotoxic. It is therefore intriguing why it is necessary to arm $CD4^+$ T cells with acquired cytotoxicity. Notably, it was reported that Treg cells could loss the expression of ThPOK and transform into $CD8\alpha\alpha^+CD4^+$ T cells in IELs (Sujino et al., 2016). Even though the physiological consequence of this cytotoxic reprogramming was not illustrated, it is possible that transformed Treg can assist in cytotoxic killing while reducing the inflammation at the same time. This is supported by the evidence that $CD8\alpha\alpha^+CD4^+$ IELs demonstrate substantial anti-inflammatory function (Sujino et al., 2016). This notion requires further investigation.

Unlike the dependence on AhR for $CD8\alpha\alpha^+CD4^+$ and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs, conventional $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs are not dependent on AhR signalling pathway for their development and function. This was demonstrated by negligible changes of $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs in AhR deficient mice. The distinctive developmental mechanisms suggest conventional ($TCR\alpha\beta^+CD8\alpha\beta^+$) and unconventional ($CD8\alpha\alpha^+CD4^+$ and $TCR\alpha\beta^+CD8\alpha\alpha^+$) T cells of IELs might be induced and/or expanded by different type of infections or stimuli. Further study also needs to examine whether these two types of cytotoxic T cells kill target cells in different manners.

6.3. How does AhR regulate the generation of cytotoxic IELs?

An important role of AhR in controlling the homeostasis of IELs including TCR$\gamma\delta^+$, TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ and CD8$\alpha\alpha^+$CD4$^+$ have been reported. This conclusion was largely based on the analysis of the phonotype of AhR-deficient mice (Cervantes-Barragan et al., 2017; Ji et al., 2015; Martin, Hirota, Cua, Stockinger, & Veldhoen, 2009; Nakajima et al., 2013). However, none of these studies has illustrated the mechanism underlying the AhR-mediated regulation. Understanding the mechanism is highly sought-after since such knowledge may improve the knowledge for regulatory network for the AhR pathway and support the development of new therapeutic strategies to enhance gut health.

Unconventional cytotoxic IELs: TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs and CD8$\alpha\alpha^+$CD4$^+$ IELs share several key phenotypes such as the expression of CD8α but not CD8β, and the secretion of cytotoxic molecules such as granzyme B. However, these subsets are considered to be generate along different routes even though both populations are dependent on AhR signalling pathway. TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs were reported to be derived from DN precursors in the thymus and terminally differentiated in IELs (Ruscher et al., 2017). However, how AhR participates in the development of this population remained unclear. My results showed that TCF1 is a key transcription factor that modulates the generation of these TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs. By the deletion of TCF1 in AhR deficient mice, the generation of TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs were significantly restored, indicating that the downregulation of TCF1 by AhR is a conceivable mechanism that promotes the formation of TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs.

CD8$\alpha\alpha^+$CD4$^+$ IELs, unlike TCR$\alpha\beta^+$CD8$\alpha\alpha^+$ IELs, are generally regarded directly derived from local CD4$^+$ IELs in an AhR dependent manner (Cervantes-Barragan et al., 2017). It was

demonstrated that the loss of ThPOK was the major step that initiates the differentiation of $CD4^+$ IELs into $CD8\alpha\alpha^+CD4^+$ IELs (Cervantes-Barragan et al., 2017; Mucida et al., 2013; Reis et al., 2013; Sujino et al., 2016). The question to be answered is how AhR signalling pathway interacts with the loss of ThPOK in $CD4^+$ IELs. In my project, TCF1 was shown to play an important role to sustain the expression of ThPOK in $CD4^+$ IELs, while the expression of TCF1 in $CD8\alpha\alpha^+CD4^+$ IELs was reduced compared to $CD4^+$ IELs, suggesting AhR might also downregulate TCF1 in promoting the generation of $CD8\alpha\alpha^+CD4^+$ IELs. However, the deletion of TCF1 was insufficient to restore the generation of $CD8\alpha\alpha^+CD4^+$ IELs in AhR deficient mice. I found the loss of TCF1 led to the upregulation of its family member LEF1, as reported (Haynes et al., 1996; Okamura et al., 1998; S. Yu et al., 2012). Since LEF1 showed a partially functional redundancy of TCF1 in the transcriptional regulation of targeted genes, which was demonstrated by significant overlapping of genomic loci bound by these two family members (Emmanuel et al., 2018), it is plausible that the upregulation of LEF1 caused by TCF1 deficiency compensated the loss of function of TCF1. Due to the time limit of my PhD, I have not been able to finish the analysis of the phenotype of AhR, TCF1 and LEF1 triple deficient mice. Such results will critically examine the above hypothesis.

Taken together, I conclude that AhR suppresses the expression of TCF1 to promote the generation of cytotoxicity of IELs in the mucosa. A model of such cytotoxic reprograming process of IEL subsets is shown in **Figure 6.1**. The next key question is how AhR suppresses the TCF1 expression in IELs.

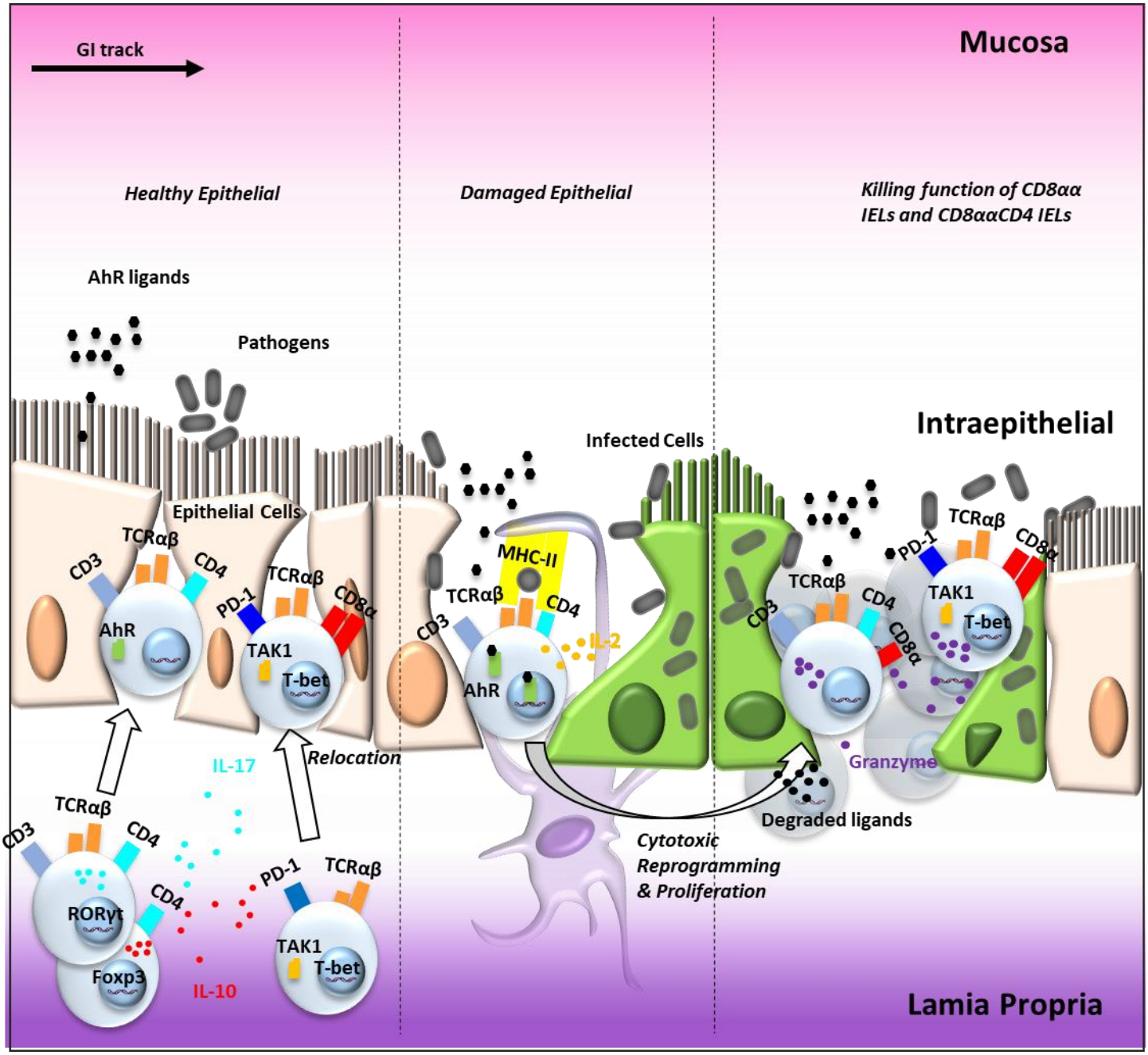


Figure 6.1, A model for the formation and function of cytotoxic IELs. Epithelial cells form a barrier to protect the gut from pathogens. In normal gut, Th17 (RORγt$^+$) cells, Treg (Foxp3$^+$) cells and CD8αα$^+$ precursor cells (TAK1$^+$/T-bet$^+$ and PD1$^+$) can migrate into intraepithelial site. When the epithelial integrity is compromised, the concentration of AhR ligands will increase and stimulate CD4$^+$ IELs to differentiate into CD8αα$^+$CD4$^+$ IELs which could not only perform cytotoxic killing but also suppress inflammation. TCRαβ$^+$CD8αα$^+$ IELs are also expanded (or matured) by the stimulation of AhR ligands to enhance cytotoxic function.

6.4. The AhR-TCF1/LEF1 axis in the regulation of cytotoxic IELs and potentially beyond

AhR plays a key role in the generation of cytotoxic IEL subsets and sustain the gut immune homeostasis (Cervantes-Barragan et al., 2017; Nakajima et al., 2013). My data suggest that AhR executes the role in enhancing the differentiation of TCRαβ$^+$CD8αα$^+$ and CD8αα$^+$CD4$^+$ IELs at least partially by downregulating key transcriptional axis of TCF1 (also possible LEF1). How does AhR suppress TCF1/LEF1? Using ChIP-qPCR or ChIP-seq, I revealed a clear binding pattern of AhR proximal to the promoter region of *Tcf7* gene after the AhR ligand treatment, indicating that the ligand-induced AhR/ARNT dimer may bind and directly suppress the *Tcf7* transcription. I also observed the recruitment of AhR/ARNT dimer to the *Lef1* promoter. In the transcriptomic analysis to compare CD8αα$^+$CD4$^+$ and CD4$^+$ IELs by RNA-seq, the expression of LEF1 was lower in CD8αα$^+$CD4$^+$ IELs compared to CD4$^+$ IELs. The evidence collectively suggests that AhR might also suppress the expression of LEF1 in additional to TCF1. This also provides an explanation why the single knockout of TCF1 was insufficient to rescue the generation of CD8αα$^+$CD4$^+$ IELs in AhR deficient mice. Additional deletion of LEF1 is likely to be required to achieve this. In contrast, the single deletion of TCF1 is sufficient to rescue the formation of TCRαβ$^+$CD8αα$^+$ IELs in AhR knockout mice. The reason for the differential redundancy between TCF1 and LEF1 in these two cell types remains to be further investigated.

The downregulation of ThPOK, the key transcription factor to sustain the CD4 lineage and suppress CD8 lineage, was reported to initiate the differentiation of CD4$^+$ IELs into CD8αα$^+$CD4$^+$ IELs (Mucida et al., 2013; Reis et al., 2013; Sujino et al., 2016). I was unable to detect the direct binding of AhR to *Zbtb7b* (the gene encoding ThPOK) or the suppression of

ThPOK expression by a short period of the AhR ligand treatment. Therefore, AhR does not directly regulate ThPOK expression and additional signals might synergise with AhR to efficiently induce the loss of ThPOK.

AhR has been shown to also enhance the generation of γδT cells (Kadow et al., 2011; Martin et al., 2009; Witherden, Ramirez, & Havran, 2014; D. Wu, Wu, Qiu, Wei, & Huang, 2017). I also noticed that TCF1 expression was low in γδT cells of IELs, suggesting a possible role of regulation by the AhR-TCF1/LEF1 axis in γδT cells. Interestingly, both AhR and TCF1 were reported to show dynamic expression and regulate the generation of certain subsets of ILCs (Harly et al., 2019; S. Li, Bostick, & Zhou, 2017; Mielke et al., 2013; Seillet et al., 2016; Yang et al., 2015). It is of interest to examine the role of AhR-TCF1/LEF1 axis in ILCs, which also critically participate the gut immune homeostasis. In summary, I, for the first time, revealed an important role of the AhR-TCF1/LEF1 regulatory axis in cytotoxic IEL subsets, which may be also applicable in other lymphocyte differentiation and function (**Figure 6.2**).

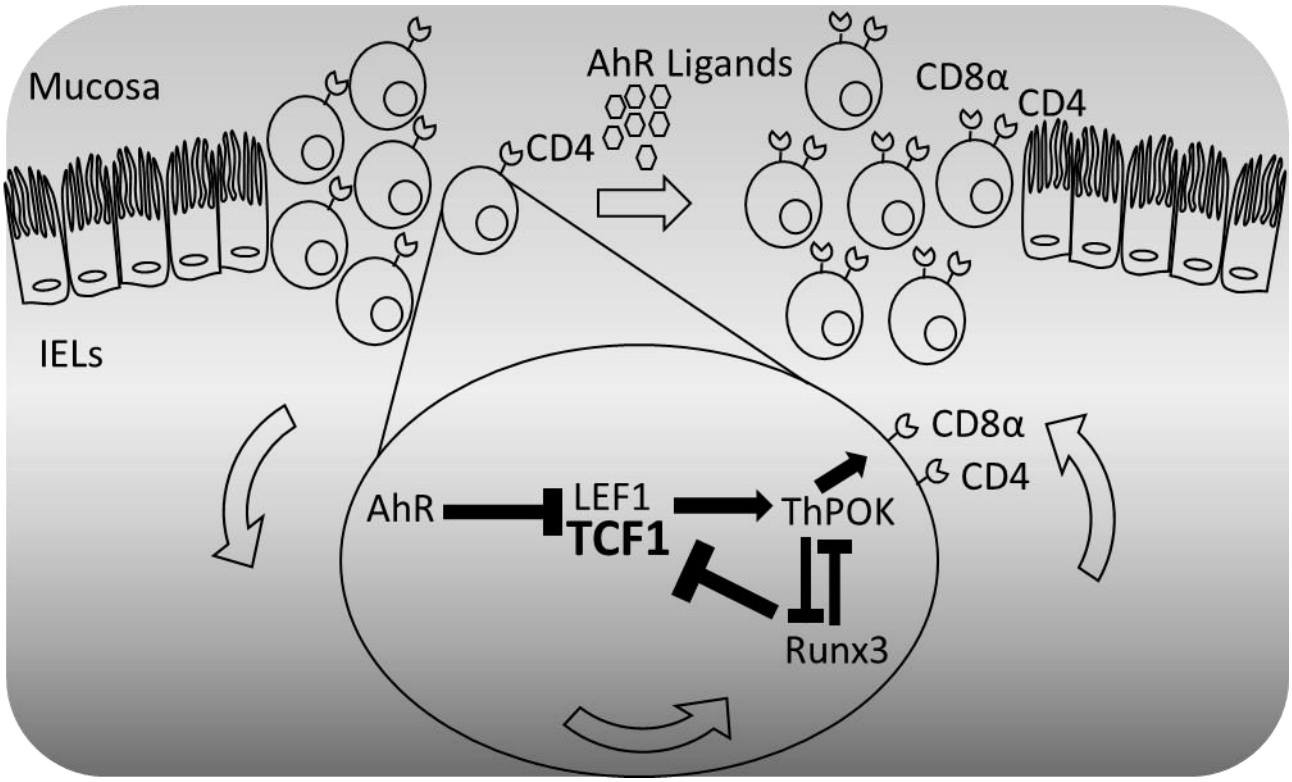


Figure 6.2, A model of the mechanism underlying AhR-mediated molecular circuit for the generation of cytotoxic IELs. AhR directly suppresses TCF1/LEF1 to induce the loss of ThPOK and the expression of CD8α. Such regulatory axis is initiated and strengthened by prolonged exposure to AhR ligands which are enriched in the gut mucosal environment.

6.5. Concluding remark and future direction

In conclusion, my PhD project has achieved three major findings: i) AhR is required for the generation of unconventional cytotoxic IELs including $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs; ii) The downregulation of TCF1 is required to generate $CD8\alpha\alpha^{+}CD4^{+}$ IELs and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs and iii) AhR directly binds to the *Tcf7* and possibly *Lef1* promoters to suppress their transcription, underlying the downregulation of TCF1/LEF1 to promote the generation of $CD8\alpha\alpha^{+}CD4^{+}$ and $TCR\alpha\beta^{+}CD8\alpha\alpha^{+}$ IELs.

Two additional experiments are undertaken to confirm the results and further prove the hypotheses. First, we will perform an optimised ChIP-exo-Seq experiment by introducing both AhR and Pol2 ChIP to confirm the genome-wide binding of AhR and the transcription of AhR-targeted gene. Second, to further address whether the redundancy of LEF1 block the differentiation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs in the TCF1 and AhR dual deficient mice, a TCF1-LEF1-AhR triple knock out mice model was introduced by cross-breeding $hCD2^{cre}$ x $Rosa26^{GFP/GFP}$ mice with $TCF1^{fl/fl}$ x $LEF1^{fl/fl}$ x $AhR^{fl/fl}$ mice. Due to the time limitation and difficulties of generating homozygous triple knockout mice, the results are not yet available in this thesis, but will be tested in the near future. An anticipated result will be knockout of TCF1 and LEF1 in AhR deficient mice rescues the generation of $CD8\alpha\alpha^{+}CD4^{+}$ IELs.

Future experiments are also required to test whether such mechanism is conserved in human IELs. It is especially important to test the specificity of human IELs to AhR ligands since there are considerable difference of binding affinity and downstream activities of between human and mouse AhR for specific ligands. Finally, efforts should be made to translate the discovery in the development of potential therapeutic or supplementary strategies to target the AhR

pathway for the treatment of human diseases, especially gut infections and inflammatory bowel disease.

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Appendices

Appendix Table 1, AhR ligands

Category	Chemical name	Abbr.	CAS#	Company
Xenobiotics	Benzo(α)pyrene	B[a]P	50-32-8	Sigma-Aldrich
	Benzo(β)fluoranthene	B[b]F	205-99-2	Sigma-Aldrich
	Dibenzo(a,c)anthracene	D[a,c]A	215-58-7	Sigma-Aldrich
	Benzo(g,h,i)perylene	BPE	191-24-2	Sigma-Aldrich
	2,3,7,8-tetrachlorodibenzo-ρ-dioxin	TCDD	1746-01-6	AccuStandard
Endogenous	6-formylindolo(3,2-β)carbazole	FICZ	172922-91-7	Santa Cruz
	L-kynurenine	Kyn	2922-83-0	Sigma-Aldrich
Dietary	3,3-diindoylmethane	DIM	1968-05-4	Sigma-Aldrich
	Indole-3-carbinol	I3C	700-06-1	Santa Cruz
Microbial	Indirubin	Ind	479-41-4	Sigma-Aldrich

Appendix Table 2, Fluorescent antibodies

Channel	Antibody	CAT#	Company
PECy7	B220	103222	Biolegend
PECy7	CD25	102016	Biolegend
PECy7	CD45.2	560696	BD
PECy7	IFN-γ	557649	BD
AF647	B220	103226	Biolegend
AF647	CD8β	126612	Biolegend
AF647	IL17A	560184	BD
AF700	B220	557957	BD
AF700	CD8α	100730	Biolegend
APCCy7	B220	552094	BD
APCCy7	CD4	100526	BD
PE	AHR	12-5925-80	Ebioscience
PE	CD4	553049	BD
PE	TCRγδ	12-5711-82	Ebioscience
AF488	CD8α	100723	Biolegend
FITC	CD8α	553031	BD
BB515	CD8α	564422	BD

PB	B220	103227	Biolegend
BV421	TCRαβ	562839	BD
E450	CD62L	48-0621-82	Ebioscience
V500	CD44	560780	BD
V500	CD45.2	562129	BD
V500	Zombie Aqua	423101	Biolegend
BV605	CD4	563151	BD

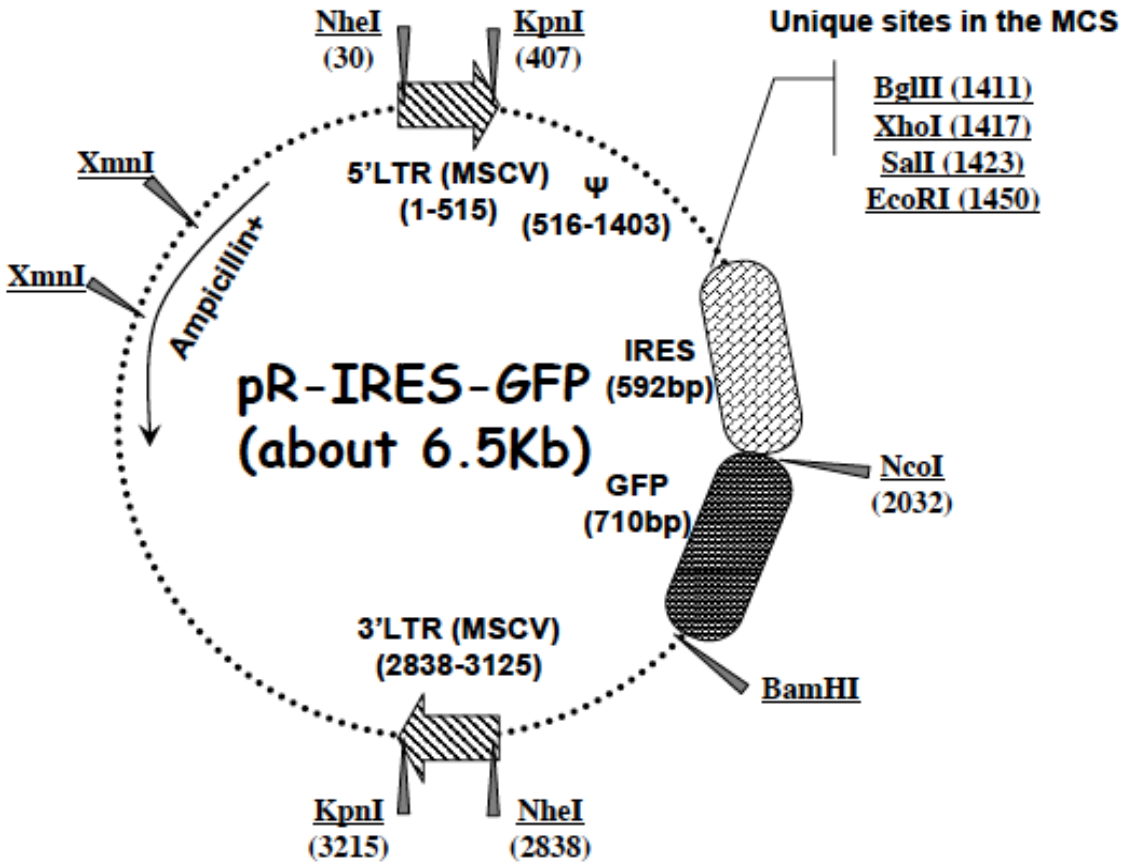
Appendix Table 3, cytokines and antibodies for cell culture

Cytokines and antibodies	CAT#	Supplier
Recombinant human IL-2	200-02	PeproTech
Recombinant murine IL-2	212-12	PeproTech
Recombinant murine IL-6	216-16	PeproTech
Recombinant murine IL-12	210-12	PeproTech
Recombinant human TGF-β	100-21	PeproTech
Anti-Murine IL-4	500-P54	PeproTech
Anti-IFN-γ	500-P119	PeproTech
Anti-CD3		Walter and Eliza Hall
Anti-CD28		Walter and Eliza Hall

Appendix Table 4, experiment apparatus

Instruments	Company
Centrifuge	Thermos scientific
PCR machine	Kyratech
Pipet set	Thermos scientific
Eppendorf Tube	Axygen
2ml Tube	Axygen
15ml Tube	Falcon
50ml Tube	Falcon
1ml syringe	Terumo
5ml cell strainer	Falcon

Appendix Figure 1, Plasmid map of pR-GFP retrovirus construct for overexpression of genes in mouse cells



Appendix Table 5, IEL FACS staining panel (Antibodies in same channel were stained separately)

surface and IC staining		
Channel	**Antibody**	**Dilution**
7AAD		1:400
BV605	CD8α	1:400
APC-Cy7	CD4	1:400
PE-Cy7	CD45.2	1:400
BV421	TCRαβ	1:200
AF647	CD8β	1:400
PE	TCRγδ	1:200
V500	ZA	1:200
BV650	CD45.1	1:200
AF488	GFP	1:200
PE	AhR (IC)	1:100
PE	TCF1 (IC)	1:200
PE	Runx3 (IC)	1:100
APC	ThPOK (IC)	1:100
AF488	LEF1 (IC)	1:100

Appendix Table 6, Th17 FACS staining panel

Surface and AhR staining		
Channel	**Antibody**	**Dilution**
7AAD		1:800
BV605	CD4	1:400
AF700	CD8α	1:400
PE	AhR (IC)	1:100
PE-Cy7	IFN-γ (IC)	1:500
APC	IL17A (IC)	1:100

Appendix Table 7, qPCR primers

Oligo Name	Company	Sequence
mRunx3 long-F	Sigma Aldrich	AGGCCGAGTCCTTGCCACT
mRunx3 long-R	Sigma Aldrich	CTGGTGCTCGGGTCTCGTAT
mRunx3 both-F	Sigma Aldrich	GGGCGATTGTCAGTGGGATT
mRunx3 both-R	Sigma Aldrich	GGGTGGCTGTTTCAGGTGC
mRunx3 short-F	Sigma Aldrich	TTCTTTGGCACAGTCTAACCG
mRunx3 short-R	Sigma Aldrich	GCCCACGAATCGAAGGTC
FM1_Cd4	Sigma Aldrich	TAGCAACTCTAAGGTCTCTAAC
RM1_Cd4	Sigma Aldrich	GATAGCTGTGCTCTGAAAAC
FM1_CD8α	Sigma Aldrich	ATAAGTACGTTCTCACCCTG
RM1_CD8α	Sigma Aldrich	GAGTTCACTTTCTGAAGGAC
FM1_CD8β1	Sigma Aldrich	ACTACCCTGAAGATGAAGAAG
RM1_CD8β1	Sigma Aldrich	GCACACAGTAAAAGTAGACG
FM1_Cyp1b1	Sigma Aldrich	ACTATTACGGACATCTTCGG
RM1_Cyp1b1	Sigma Aldrich	ATCTGGTAAAGAGGATGAGC
FM1_Zbtb7b	Sigma Aldrich	CGGAAAGCTTTTCTTCAAAC
RM1_Zbtb7b	Sigma Aldrich	CAACCATCTCTTCTTCTTCG
AhR FW	Sigma Aldrich	GGCTTTCAGCAGTCTGATGTC

AhR RV	Sigma Aldrich	CATGAAAGAAGCGTTCTCTGG
Arnt1	Sigma Aldrich	TCTCCCTCCCAGATGATGAC
Arnt2	Sigma Aldrich	CAATGTTGTGTCGGGAGATG
Cyp1a1 FW	Sigma Aldrich	GACCCTTACAAGTATTTGGTCGT
Cyp1a1 RV	Sigma Aldrich	GGTATCCAGAGCCAGTAACCT
Tcf7-171121-F	Sigma Aldrich	CAATCTGCTCATGCCCTACC
Tcf7-171121-R	Sigma Aldrich	CTTGCTTCTGGCTGATGTCC
Lef1-171121-F	Sigma Aldrich	TGAGTGCACGCTAAAGGAGA
Lef1-171121-R	Sigma Aldrich	CTGACCAGCCTGGATAAAGC
Srp14 F	Sigma Aldrich	CAGCGTGTTCATCACCCTCAA
Srp14 R	Sigma Aldrich	GGCTCTCAACAGACACTTGTTTT
GAPDH F	Sigma Aldrich	TGAAGCAGGCATCTGAGGG
GAPDH R	Sigma Aldrich	CGAAGGTGGAAGAGTGGGAG

Appendix Table 8, ChIP-qPCR primers

Oligo name	Company	Sequence
TCF1 P1F	Sigma Aldrich	AGCTGGACTCGGGCGG
TCF1 P1R	Sigma Aldrich	GCGCTGTCGCGATTCTTATC
TCF1 P2F	Sigma Aldrich	TGGTGAATGAGTCCGAAGGC
TCF1 P2R	Sigma Aldrich	TACCAACTCGGGACGAGGTC
TCF1 P3F	Sigma Aldrich	ATGCCATTCCCTAGCGTGAT
TCF1 P3R	Sigma Aldrich	CCAGTCTTCTCACACCCGAG
TCF1 P4F	Sigma Aldrich	CTAGGCCAATCGCCATGGAT
TCF1 P4R	Sigma Aldrich	CAGGGACCTGATGCTAAGCC
TCF1 P5F	Sigma Aldrich	GCCCGAACTATTAGGCTCCC
TCF1 P5R	Sigma Aldrich	GGAGGAAGGCTGACACTCAC
TCF1 P6F	Sigma Aldrich	AGCTCATCAGACCAAGGCAG
TCF1 P6R	Sigma Aldrich	GAAGGGTGCTTGCTAGTCCA
TCF1 P7F	Sigma Aldrich	GACTGAACTCTGGTGGGCAG
TCF1 P7R	Sigma Aldrich	GCTGTGGACCCTTGTACCTC
TCF1 P8F	Sigma Aldrich	CACCTTGACTGGCTCACCAA
TCF1 P8R	Sigma Aldrich	AGTGGCATGGTGATTGGAGG
TCF1 T1F	Sigma Aldrich	TAGGGAGTCAAGTCCCGGTC

TCF1 T1R	Sigma Aldrich	GGGTCCCCTTCAAGTTCGTG
TCF1 E1F	Sigma Aldrich	TGGCCTGCGTGATTGTACTG
TCF1 E1R	Sigma Aldrich	TGCCAATCTGCAAAAGCCAAA
CYP1A1 F	Sigma Aldrich	AGGCTCTTCTCACGCAACTC
CYP1A1 R	Sigma Aldrich	CTGGGGCTACAAAGGGTGAT
UNTR6 F	Sigma Aldrich	TCAGGCATGAACCACCATAC
UNTR6 R	Sigma Aldrich	AACATCCACACGTCCAGTGA