ROLE OF ARYL HYDROCARBON RECEPTOR ACTIVATION BY 2,3,7,8-TETRACHLORO-DIBENZO-*P*-DIOXIN (TCDD) IN AN *IN VITRO* MODEL OF HUMAN HEMATOPOIESIS

By

D M Isha Olive Khan

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ABSTRACT

In vitro models for studying the simultaneous development of various human immune cells and hematopoietic lineages are currently lacking. Through the utilization of single-cell techniques, we have identified and characterized a stromal-cell-free *in vitro* culture system for the differentiation of human hematopoietic stem and progenitor cells (HSPCs). This system enables the concurrent development of multiple immune cell lineages.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that influences various biological processes in different cell types. Employing our *in vitro* model, we observed that the activation of AHR by the persistent environmental contaminant and high affinity ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), directs the differentiation of $CD34^+$ HSPCs derived from human umbilical cord blood toward monocytes and granulocytes. This is accompanied by a significant reduction in lymphoid and megakaryocyte lineage specification, which may lead to diminished immunocompetence.

Furthermore, using single-cell techniques, we discovered that TCDD reduces the expression of key lineage specification genes, *BCL11A* and *IRF8*, at both transcriptomic and protein levels in progenitor cells. We also identified previously unreported transcriptomic and pathway level changes in early progenitor cells and in committed hematopoietic populations at the single cell level. In summary, our *in vitro* hematopoiesis model, combined with single-cell tools, provides valuable insights into the role of AHR and TCDD in modulating hematopoietic differentiation.

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LIST OF ABBREVIATIONS

2,3,7,8-tetrachlorodibenzo-*p*-dioxin	TCDD
AHR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon nuclear translocator
bHLH	basic helix–loop–helix
cDC2	Type 2 classical dendritic cells
CFU-C	Colony-forming unit cell
CYP	Cytochrome P450
FICZ	6-Formylindolo(3,2-b)carbazole
Flt3L	FMS-like tyrosine kinase 3 ligand
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
G-CSF	Granulocyte colony stimulating factor
GMP	Granulocyte-Macrophage progenitor
GSEA	Gene set enrichment analysis
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IL-6	Interleukin-6
IL-7	Interleukin-7
Lin^-	Lineage marker negative
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
Mo-DC	Monocyte derived dendritic cells

NK cell	Natural Killer cell
PAS	PER-ARNT-SIM
RORγt	Retinoic acid receptor-related orphan receptor - γt
RPMI	Roswell Park Memorial Institute 1640 medium
Sca-1	Stem cell antigen-1
SCF	Stem cell factor
scRNA-seq	Single-cell RNA sequencing
ssGSEA	Single sample gene set enrichment analysis
T_H2	Type 2 helper T cell
T_{reg}	Regulatory T cell
T_H17	T helper 17 cell

CHAPTER 1: INTRODUCTION

1.1 Purpose of this research

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical member of the dioxin family of environmental pollutants which primarily exerts its toxic effects through the aryl hydrocarbon receptor (AHR). Its adverse effects on the functioning and development of the immune system have been studied for several decades. Although the role of TCDD on immune system function in experimental animals and humans has been explored in much detail, relatively fewer studies have assessed its effects on the development of immune cells from hematopoietic stem and progenitor cells (HSPCs) in humans. There is also limited understanding of the molecular mechanisms by which TCDD may regulate the differentiation of hematopoietic progenitor cells at the single cell level. Moreover, few assays exist to simultaneously monitor the development of human progenitor populations to specified hematopoietic lineages. My dissertation addresses these areas and aims to provide a better understanding of the role of TCDD and AHR in differentiation of human HSPCs to lineage committed cells using single-cell methods.

1.2 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is a basic helix–loop–helix (bHLH) ligand-activated transcription factor that mediates responses to exogenous and endogenous stimuli. It is expressed in multiple organs, tissues, and cell types with the highest expression in the urinary bladder, liver, lungs (Jiang *et al.*, 2010; Hanieh, 2014). It was discovered in the 1970s as the receptor that mediated the toxicity of the xenobiotic TCDD which is a side-product of industrial manufacture (Poland et al., 1976). Thereafter, it was found to be the receptor for a wide range of man-made compounds such as dioxins, furans, certain polychlorinated biphenyls, etc. whose toxic effects on the human body could be attributed to the receptor (Wall *et al.*, 2015). The importance of AHR physiologically later came into light with the use of AHR knockout studies (Fernandez-Salguero *et al.*, 1995; Esser, 2009). AHR-null mice exhibited an increase in mortality rates and different physiological abnormalities, such as reduced lymphocyte count, smaller liver size, fibrotic bile ducts, and decreased growth rate (Schmidt *et al.*, 1996). Henceforth, AHR has been found to play critical roles in other contexts. For example, AHR can modulate the microbiome and thereby intestinal metabolism as indicated from studies using $AhR^{-/-}$ mice (Korecka *et al.*, 2016). AHR is also essential for normal functioning of different immune cell types such as during differentiation of T_H17 cells, a subset of helper T cells, as well as in the development of Interleukin-22 producing $ROR\gamma t^{+}$ innate lymphoid cells and intestinal lymphoid follicles (Veldhoen *et al.*, 2008; Veldhoen *et al.*, 2009; Kiss *et al.*, 2011; Lee *et al.*, 2011; Qiu *et al.*, 2012).

Most of AHR's functions can be attributed to its canonical role as a transcription factor. Specifically, AHR is a member of the PER-ARNT-SIM (PAS) family of bHLH transcription

factors and has two PAS domains, PAS-A and PAS-B. The bHLH domain allows AHR to bind to the DNA, the PAS-A domain allows for dimerization with another bHLH transcription factor, and the PAS-B domain acts as the ligand-binding domain (Stockinger *et al.*, 2014). In the absence of any ligands, the AHR is sequestered in the cytoplasm by interacting proteins such as AHR interacting protein (AIP), heat shock protein 90 (HSP90), p23, etc. (Henry et al., 1993; Ma et al., 1997; Meyer et al., 1998; Bell et al., 2000; Petrulis et al., 2000). Upon ligand binding, AHR undergoes conformational changes and translocates to the nucleus where it dimerizes with the Aryl hydrocarbon nuclear translocator (ARNT) (**Figure 1.1**). AHR-ARNT heterodimer then binds to cognate sequences on the DNA called aryl hydrocarbon response elements called AHREs on regulatory regions of genes to regulate gene transcription (McGuire *et al.*, 1994).

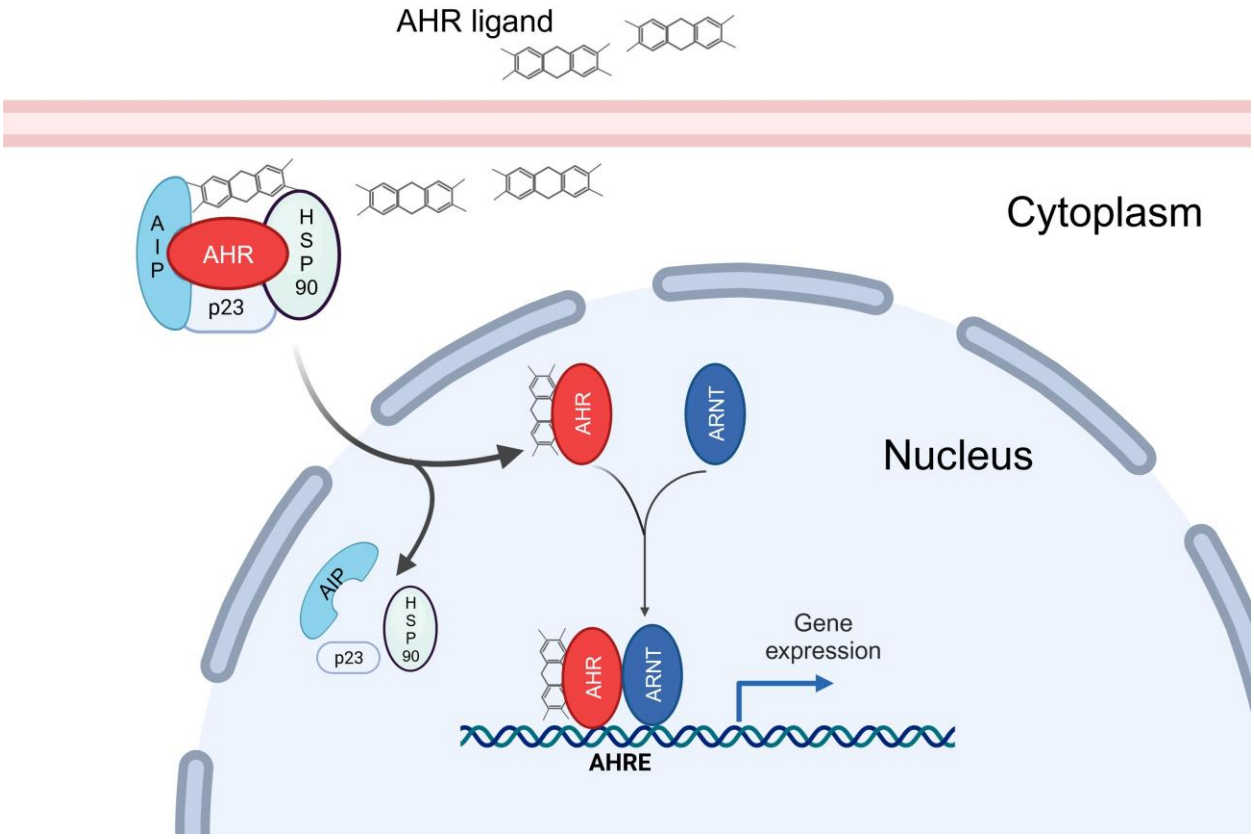

Figure 1.1 Representation of the canonical AHR signaling pathway upon binding of AHR ligand. AIP = AHR interacting protein, HSP90 = heat shock protein 90, ARNT = Aryl hydrocarbon nuclear translocator, AHRE = Aryl hydrocarbon response element.

AHR is activated by a wide variety of ligands with diverse origin and structure (Giani Tagliabue *et al.*, 2019). AHR ligands can be from either exogenous or endogenous sources. Exogenous AHR ligands of anthropogenic origin can be broadly classified into halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons. However, there are also several man-made AHR ligands that do not fall under these categories. Members of the HAHs include dioxins, furans, and dioxin-like polychlorinated biphenyls (PCBs) (Safe, 1993). AHR ligands of exogenous origin may also be derived from microbiota and cruciferous plants such as broccoli, Brussels sprouts, and cauliflower (Zelante *et al.*, 2013; Zhao *et al.*, 2019). Among others, some well-studied plant-derived

AHR ligands include certain indole-3-carbinol derivatives and flavonoids. Major categories of endogenous AHR ligands include certain tryptophan metabolites, arachidonic acid metabolites, and products of heme metabolism (Tan *et al.*, 2022). Some prominent tryptophan metabolites of endogenous origin are kynurenine, 6-Formylindolo(3,2-b)carbazole (FICZ), and their derivatives.

Anthropogenic AHR ligands such as dioxins and dioxin-like PCBs are chemically stable and have slow degradation rates in the environment (Cagnetta *et al.*, 2016). Upon intake into the body, these environmentally persistent AHR activators are poorly metabolized, have long half-lives and can induce toxic effects primarily through AHR-mediated mechanisms (Bonefeld-Jorgensen *et al.*, 2014). On the other hand, AHR ligands of endogenous sources and those derived from plants and microbes are chemically labile, readily metabolized, and, in general, have lower AHR affinity than most anthropogenic AHR ligands (Zhang, 2011; Ehrlich *et al.*, 2017). It is likely that AHR activation through these ligands are well regulated and fine-tuned to cellular requirements, and thus do not induce sustained AHR activation and subsequent toxicity as may be the case for many exogenous anthropogenic AHR ligands.

Although AHR is recognized to be physiologically involved in regulation of different biological systems through AHR knockout studies in experimental animals, the exact role of different endogenous AHR ligands is not clear. For example, in the context of the immune system, FICZ induces differentiation of T cells into inflammatory T helper 17 cells (T_H17) (Quintana *et al.*, 2008; Ehrlich *et al.*, 2018), whereas other similar endogenous and rapidly metabolized AHR ligands such as kynurenine, indole-3-pyruvic acid and 2-(1' H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) drive T cell

differentiation towards regulatory T (T_{reg}) cells that suppress helper T cells (Mezrich *et al.*, 2010; Ehrlich *et al.*, 2018; Huang *et al.*, 2023). Endogenous AHR activation by kynurenine is also associated with suppression of type I interferon responses during viral infection in mice (Yamada *et al.*, 2016). These studies highlight the fact that, in most cases, endogenous AHR activation may be associated with dampening of the adaptive immune response. It would be expected that endogenous AHR ligands would be associated with conferring protective functions. Given this premise, it is possible that endogenous AHR activation serves to prevent excessive immune activation. However, sufficient studies have not been conducted to ascertain the exact role of endogenous AHR activation in overall regulation of immune cells.

In the presence of toxicants that are AHR ligands, the AHR could be overly activated to cause aberrant perturbations of physiological functions that are regulated or influenced by the AHR, leading to adverse outcomes.

1.3 TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin)

One of the most potent AHR agonists is the dioxin, TCDD. In fact, Toxic Equivalency Factor (TEF) is calculated based on the activity of TCDD and toxicity of other dioxin-like compounds are determined relative to the activity of TCDD (Van den Berg et al., 2006; Collins et al., 2016). TCDD, a highly stable and persistent environmental pollutant, was a contaminant or by-product of industrial manufacture with the major source being the manufacture of herbicides such as the herbicide 2,4,5-trichlorophenoxyacetic acid in 1960s and 1970s (Lucier et al., 1973). At present, it is mainly produced as a contaminant or by-product of industrial manufacture of trichlorophenol (Collins et al., 2016). Small quantities of TCDD can also be produced due to incomplete combustion processes at high temperatures (Dickson *et al.*, 1987). TCDD is poorly metabolized and bioaccumulates, with a half-life of greater than seven years in humans (Pirkle *et al.*, 1989). TCDD induces enzymes, particularly certain members of the CYP family that are enriched in liver such as CYP1A1 and CYP1A2 across many species (Birnbaum, 1994).

TCDD induces a plethora of toxic effects, with most effects being similar across different species and some of the effects being species specific (Birnbaum, 1994). Some effects can vary in intensity based upon the species. For example, guinea pigs are highly sensitive to TCDD with lethality observed earlier than in other commonly employed experimental animals at high doses of TCDD. In humans, chloracne is the most consistently reported adverse effect of TCDD exposure at high doses (Neuberger *et al.*, 1999). Liver disorders, hyperlipidemia, and neuropsychological issues have been observed in workers exposed to TCDD (Neuberger *et al.*, 1999; Pelclova *et al.*, 2002). Additionally, developmental defects, reproductive toxicity, carcinogenicity in animals, and

immunotoxicity are well known toxic responses to TCDD. Suppression of the immune system functions, particularly on lymphocytes, has been observed across multiple species at relative low doses that do not cause lethality or other toxic effects.

Almost all actions by TCDD are mediated via the AHR. The fact that AHR is essential for mediating the toxicity of these compounds was illustrated using AHR knockout studies in rodents. AHR deficient mice were found to be insensitive to TCDD and exhibited little to no pathologic effects at doses of TCDD that were 10 times higher than that normally would induce toxicity in wild type mice (Fernandez-Salguero et al., 1996). Similar effects were seen with AHR knockout studies in rats where sustained TCDD administration failed to induce adverse effects in AHR knockout rats (Harrill et al., 2016).

With the immune system being a highly sensitive target of TCDD, several studies have been conducted across many decades to elucidate the effects and mechanisms of action of TCDD on immune function under different conditions. TCDD causes thymic involution and a broad suppression of immune responses to infections involving multiple immune cell types. Antibody secretion from activated B cells in mouse and humans (Sulentic *et al.*, 1998; Zhou *et al.*, 2018), and generation of high-affinity antibody forming cells in mouse is diminished due to TCDD exposure (Inouye *et al.*, 2003). Aspects of humoral immunity dependent upon T cells may also be affected as demonstrated from a decrease in the Type 2 helper T (T_H2) cell cytokines associated with antibody production in TCDD exposed mice compared to controls (Ito *et al.*, 2002). Suppressive effects on T cell mediated responses may also occur through the induction of T_{reg} cells by TCDD (Funatake *et al.*, 2005). TCDD has also been reported to suppress allergic sensitization and T cell mediated responses by modulating dendritic cells and their interactions with T

cells (Schulz *et al.*, 2013). Recently, suppressive effects of TCDD on macrophages derived from THP-1 monocyte cell line have also been described (Li *et al.*, 2021).

The deleterious effects of TCDD extend not only to mature immune cell activation and function but also to developing immune cells. Immune cells originate from hematopoietic stem cells (HSCs) which also generate erythrocytes and megakaryocytes that have traditionally been considered as non-immune cells. TCDD can modulate HSCs and their differentiation into the different hematopoietic cell lineages.

1.4 Hematopoiesis

1.4.1 Hematopoietic stem cells and the hematopoietic system

Hematopoiesis is the generation of the myriad cells that circulate in the blood. The hematopoietic system of cells can be broadly divided into the myeloid and lymphoid branches. Major myeloid cells include granulocytes, monocytes, dendritic cells, erythrocytes, and megakaryocytes. B cells, T cells, and Natural Killer (NK) cells comprise the lymphocytes. However, they have a finite life span, cannot self-renew, and need to be replenished continuously. Except for the embryonic stage where certain hematopoietic cells such as tissue macrophages, embryonic red blood cells and some innate like lymphoid cells can originate from distinct tissues within the yolk sac, the source of hematopoietic cells is the HSC (Sawai *et al.*, 2016). HSCs originate from the hemogenic endothelium in the aorta-gonad-mesenophros of the embryo and move into the fetal liver during mid-gestation through the circulatory system and finally migrate and seed into the bone marrow shortly before birth (Gao *et al.*, 2018).

Within the bone marrow, HSCs reside in specialized regions or niches. These niche regions are complex and can vary in cellular composition over time but are known to be vascularized and contain perivascular cells that comprise endothelial cells and mesenchymal stromal cells with characteristic cell markers (Morrison *et al.*, 2014). Cytokines secreted by stromal cells such as stem cell factor (SCF), CXC chemokine ligand 12 (CXCL12), etc. as well as from endothelial cells, and cell-cell interactions between these cells and HSCs regulate HSC functions such as proliferation, differentiation and lineage specification (Greenbaum *et al.*, 2013). HSCs are typically quiescent, can self-renew and have long-term repopulating capabilities where they can replenish the immune cells throughout life. As immune cells need to be replenished constantly, some long-term HSCs will leave the quiescent stage, enter the cell cycle, proliferate, and differentiate along different lineages (Hurwitz *et al.*, 2020). Various intrinsic and extrinsic factors govern the decision choice of an HSC to commit towards a particular lineage. This process is complex and although roles of specific factors have been characterized, the process is still not well understood.

1.4.2 Hematopoietic Stem Cell Differentiation and Lineage Specification

Differentiation and lineage specification of HSCs may be influenced by multiple factors, with some of these factors acting in unison. Even though the process of generation of blood cell lineages has been studied for several decades using *ex vivo* and *in vivo* methods and diverse molecular tools, there is much that remains to be understood. CD34, a transmembrane glycoprotein, is well established as a marker of both murine and human HSCs and multipotent progenitors (MPPs) (Servida *et al.*, 1996). Based on long term engraftment potential *in vivo*, human HSCs are typically characterized as Lin^- (Lineage

marker negative) $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}CD49f^{+}$ cells (Notta *et al.*, 2011; Huntsman *et al.*, 2015). HSCs with short term repopulating capabilities and immediate MPPs with limited self-renewal capacity lose their expression of CD90 and CD49f and are delineated as $Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}CD90^{-}CD49f^{-}$ cells (Huntsman *et al.*, 2015). As new markers are being identified over time, this characterization strategy remains fluid. Until recently, hematopoiesis towards different lineages was considered a discrete stepwise hierarchical process from the pluripotent HSCs that reside at the top level (**Figure 1.2**). At each intermediate stage, there is a progressive loss of potential of the progenitors to differentiate towards other lineages (Rieger *et al.*, 2012). This was gleaned from experiments where progenitor cells at different stages of development were characterized based on expression of specific cell markers and their differentiation potential for multilineage hematopoiesis. Oligopotent or bipotent progenitors were identified that had limited differentiation potential towards other lineages (Galy *et al.*, 1995; Rieger *et al.*, 2012). For example, human $Lin^{-}CD34^{+}CD38^{-}CD90^{-/lo}CD45RA^{+}CD10^{-}$ progenitors can differentiate to all hematopoietic lineages except for megakaryocyte and erythrocyte cells (Goardon *et al.*, 2011). With expression of CD10, human $Lin^{-}CD34^{+}CD45RA^{+}CD38^{+}CD10^{+}$ cells lose their ability to differentiate into myeloid cells, including megakaryocyte-erythroid cells but retain lymphoid and dendritic cell potential (Galy *et al.*, 1995). Thus, a branching tree-like concept of hematopoiesis prevailed where cells progressed along different trajectories at different stages.

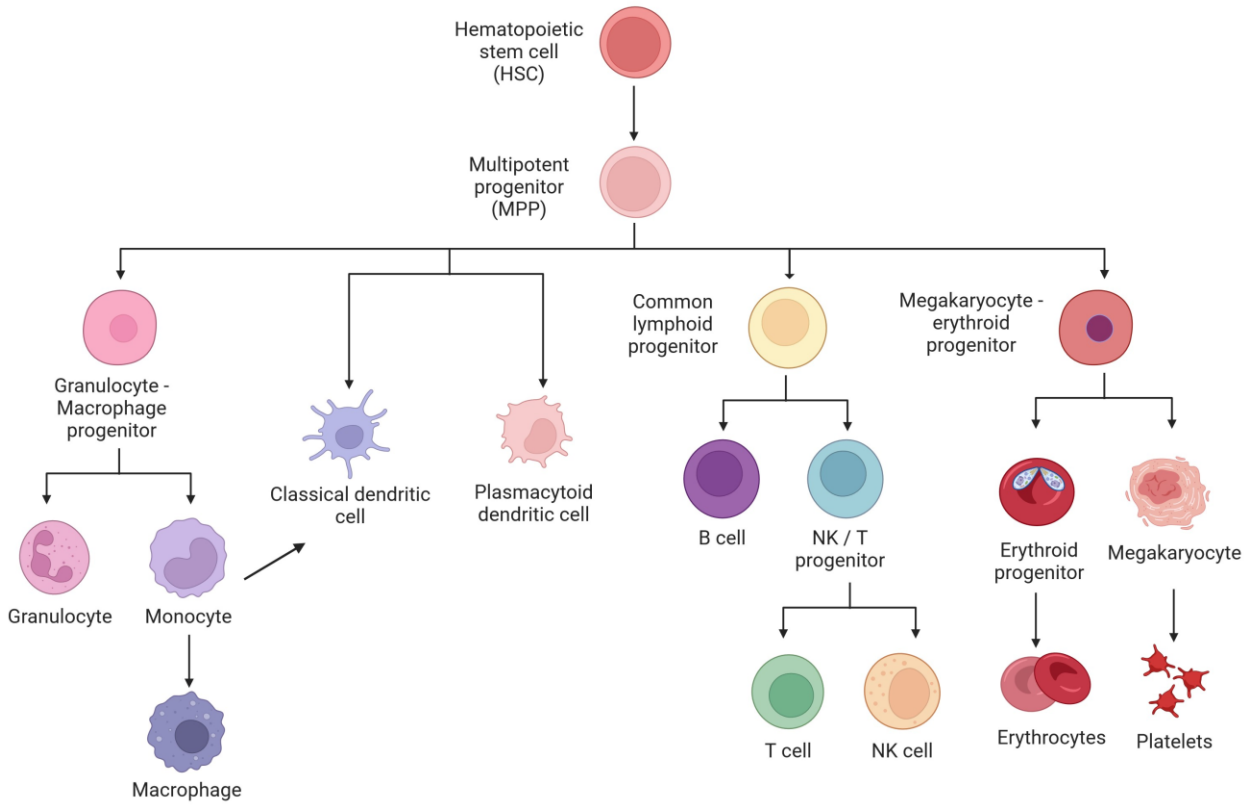

Figure 1.2 Simplified representation of a hierarchical model of human hematopoietic differentiation.

However, following the advent of technologies to study gene expression at the single cell level, the concept of discrete progenitor states has weakened. HSCs and hematopoietic progenitor cells within the same classification group have been found to be highly heterogeneous in their gene expression (Belluschi *et al.*, 2018; Karamitros *et al.*, 2018). In a study by Karamitros *et al*, different progenitor cell fractions were sorted, and single-cell RNA sequencing (scRNA-seq) was then carried out. Clustering of scRNA-seq data following dimensional reduction showed the presence of clusters that were enriched for cells of specific oligopotent fractions as well as the existence of clusters that had overlap with cells from different fractions. This suggested that the different cell fractions identified through conventional flow cytometry-based cell markers were not necessarily distinct

from each other in gene expression and the boundaries that were originally proposed between progenitor stages were not absolute. Similarly, even within human HSCs, there exists extensive heterogeneity in differentiation potential (Muller-Sieburg *et al.*, 2004; Belluschi *et al.*, 2018). Some restricted lineage potential has been identified in some HSC subsets based on level of expression of certain markers but there is no clear demarcation in potential between the different HSC populations to conclusively further divide them into a hierarchical format (Belluschi *et al.*, 2018). Thus, hematopoietic progenitors are now thought to be in a continuum of states as they differentiate from HSCs.

Studies in the mouse and humans have also pointed at alternative routes of commitment for some unipotent lineages directly from HSCs or immediate MPPs bypassing successive stages of multipotent or oligopotent states (Yamamoto *et al.*, 2013; Notta *et al.*, 2016). Based on their findings, Notta *et al* proposed a model where cells specified to be megakaryocytes and erythrocytes may branch off directly from either human HSCs, MPPs or from oligopotent progenitors and that this process was variable based upon the developmental stage of the human. However, there is greater evidence in favor of a hierarchical cloud-like continuum of states with sequential loss of multilineage potential, leading to a specific lineage commitment over that of a predisposed lineage specified HSC leading directly to a committed cell. It is of note that HSCs, even if they possess a lineage bias, retain plasticity, and can differentiate to other lineages based on the surrounding environmental conditions.

1.4.3 Mechanisms of Lineage Specification

Role of Transcription Factors

Regulation of hematopoietic differentiation is executed through intrinsic factors that may be modulated by external factors. At its core, lineage fate determination is controlled through the expression of lineage-specific transcription factors. Some of these transcription factors may serve as master regulators for lineages and through binding to regulatory regions of their target genes, promote the lineage specific programs, and repress other alternate lineage programs (Georgolopoulos *et al.*, 2021). The events that regulate the expression of specific transcription factors in an HSC or MPP are not well understood and their modulation by external influences is an active field of research.

HSCs and MPPs, in addition to expressing stem cell specific gene modules, simultaneously express lineage specific transcription factors at low levels that are antagonistic to each other, a phenomenon known as multilineage priming (Hu *et al.*, 1997; Velten *et al.*, 2017). Lineage specification may arise from titling of the balance of the transcription factor repertoire in the direction of a particular lineage in a cell. In these unstable cell states, progressive accumulation of lineage associated transcription factors, through mechanisms such as autoregulation and feed-forward loops, may finally result in generation of stable lineage specified cells (Swiers *et al.*, 2006). Stochasticity of gene expression may be at play in bringing about this effect (Raj *et al.*, 2008; Zechner *et al.*, 2020). It has been hypothesized that during cell proliferation and division, asymmetric distribution of transcription factors in the daughter cells may occur that could pave the way for a cell to progress along a particular lineage (Treichel *et al.*, 2023). However, the possibility of the existence of deterministic factors that regulate this process that are not

immediately obvious cannot be ruled out with certainty. Given that there is an unequal distribution in the output of different hematopoietic lineages in normal hematopoiesis, stochasticity perhaps cannot account for lineage specification alone. The fact that hematopoiesis may be modulated along particular trajectories in different external conditions also suggests that lineage determination, even when stochastic, is not an entirely random process.

Transcription factors, upon binding to specific motifs within gene regulatory elements, can regulate the organization of chromatin and contribute to histone and DNA modifications (Stadhouders *et al.*, 2019). These changes could allow greater accessibility and DNA binding to other transcription factors or may restrict access for some transcription factors to bind to their regulatory elements. Thus, HSCs may lose their pluripotent capacity and differentiate when specific lineage transcription factors are expressed that in turn, may prevent activity of transcription factors involved in stem cell lineage maintenance or associated with other lineages. Transcription factor levels in a cell are also important in lineage determination (Dahl *et al.*, 2003; Mak *et al.*, 2011; Kueh *et al.*, 2013). Dahl *et al*, for example, demonstrated that greater levels of the transcription factor PU.1 compared to the transcription factor CEBPα favored the development of monocytes over granulocytes and vice versa.

Role of Cytokines

External conditions such as the cytokine milieu which bathes the hematopoietic niche, stress, infections, metabolism, etc. can also modulate hematopoiesis (Pietras, 2017; Mistry *et al.*, 2023). Among these external factors that regulate HSC lineage specification, signaling through cytokines and their receptors is perhaps the most important. However,

the complete pathway from receptor activation by cytokines to downstream signaling, followed by regulation and expression of lineage-specific transcription factors is not well understood. Different cytokines preferentially facilitate the development of different lineages. For example, Granulocyte colony stimulating factor (G-CSF) and Macrophage colony stimulating factor (M-CSF) are important for differentiation, proliferation, and survival of granulocytes and monocytes/macrophages respectively (Bhattacharya *et al.*, 2015). However, many cytokines have overlapping functions and shared downstream signaling and more often than not facilitate the development of multiple hematopoietic lineages (Metcalf, 2008). Whether cytokines play a permissive or instructive role in the development of various lineages is controversial. Some studies have indicated that cytokines allow survival and proliferation of certain lineage committed hematopoietic progenitors at the expense of others, and do not necessarily enforce early HSPCs to differentiate along a particular lineage (Fairbairn *et al.*, 1993; von Muenchow *et al.*, 2016). Other *in vitro* and *in vivo* studies have demonstrated that the role played by cytokines may be instructive in nature (Kondo *et al.*, 2000; Rieger *et al.*, 2009; Tsapogas *et al.*, 2014). For example, culturing of murine bipotent granulocyte-macrophage progenitors (GMP) in G-CSF or M-CSF only allowed exclusive generation of granulocyte and macrophages respectively, and in absence of cell death, this phenomena can be attributed to the lineage instructive nature of these cytokines (Rieger *et al.*, 2009). However, it is quite difficult to ascertain the exact mechanism of action of cytokines because of technical difficulty associated with such experiments. It is also of note that some cytokines can have both instructive as well as permissive roles, and the exact role may be contingent upon the cytokine concentration or the developmental stage of the

progenitor (Brown *et al.*, 2018). The mode of action of cytokines is complicated by the fact that their action in many cases is synergistic with other cytokines and leads to several fold greater proliferation than that occurring on their own (Metcalf *et al.*, 1991).

1.4.4 Experimental models of human hematopoiesis

Study of human hematopoiesis from HSCs or HSPCs in an experimental setting requires systems that allow for the maintenance, proliferation, and differentiation of HSPCs analogous to native endogenous conditions. Xenograft models with transplantation of human HSCs or stromal cells in the mouse may be utilized to mimic human hematopoiesis (Park *et al.*, 2008; Reinisch *et al.*, 2016). However, studies that require elucidation of the effects of direct perturbations on HSCs require an *in vitro* approach. As HSPCs *in vivo* require supporting stromal cells to maintain their survival, early *in vitro* models of hematopoiesis utilized co-culture conditions of HSCs and stromal cells (McAdams *et al.*, 1996). It soon became evident that HSCs/HSPCs could be cultured without stromal cells if appropriate cytokines that are secreted by stromal cells are supplied (Verfaillie, 1992; McAdams *et al.*, 1996). Different cytokines have since been utilized for proliferation of hematopoietic progenitors and propagation of different hematopoietic cell types. Commonly used cytokines employed for *in vitro* cultures of hematopoiesis include stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3L), thrombopoietin (TPO), erythropoietin (EPO), interleukin-3, interleukin-6 (IL-6), interleukin-7 (IL-7), G-CSF, M-CSF, Granulocyte colony stimulating factor (GM-CSF), etc. Some cytokines allow for survival of proliferation of early progenitors without an apparent lineage bias whereas others have a role in specification towards particular lineages at the expense of other lineages (Kondo, 2010). Recapitulation of the exact *in vivo* cytokine milieu and their

concentrations to an *in vitro* setting is also difficult. Models of hematopoiesis that allow development of multiple lineages to an appreciable level akin to the conditions *in vivo* are therefore difficult to establish.

Models of hematopoietic differentiation can also vary depending on whether they assess lineage specification potential or whether they enable differentiation to more committed hematopoietic cells. *In vitro* colony-forming unit cell (CFU-C) assays are often used to assess the differentiation potential of HSPCs in a semi-solid medium (Sarma *et al.*, 2010). Colonies formed from a known number of input cells help inform the proliferation and differentiation potential of HSPCs. However, they are not suited to assessing the lymphoid potential of HSPCs.

1.5 Effect of AHR activation on hematopoietic stem cells and hematopoiesis

The effect of dioxin on the immune and hematopoietic systems has been noted since the 1960s. Early studies had demonstrated that TCDD caused thymic atrophy accompanied by decreased cellularity and suppression of immune responses including impaired cell-mediated immunity in experimental animals at doses lower than those associated with induction of other toxic effects (Vos *et al.*, 1973; Zinkl *et al.*, 1973). Reduced adaptive immunocompetence due to TCDD may be attributed to impaired or altered capacity of TCDD-treated/exposed mature lymphocytes to proliferate and differentiate when stimulated with appropriate antigens (Sulentic *et al.*, 2011; Ehrlich *et al.*, 2018).

However, the initial development of immune cells from hematopoietic stem cells may also be adversely affected as shown in some early studies. Mice treated with a single oral dose of TCDD at either 1 µg/kg, 10 µg/kg or 50 µg/kg had a dose-dependent acute decrease in leukocyte and lymphocyte counts after 1 week (Zinkl *et al.*, 1973). However, in the same study, a similar decrease in lymphocyte counts was not observed in rats that were given the same dose but changes in other hematopoietic cells were observed. Thrombocytopenia and greater erythrocyte counts were found in these rats. This was one of the early indications that some of the hematological effects mediated by TCDD may be species specific. Suppression of thymus-independent humoral immune responses have also been attributed to hypocellularity observed in bone marrow of TCDD treated mice (Vecchi *et al.*, 1980). Studies demonstrating reduced number of granulocyte colonies from using murine bone marrow cell cultures containing mouse lung conditioned media, and myelotoxicity inferred from decrease in granulocyte-macrophage colony-forming units

(CFU-GM) and erythroid colony-forming unit (CFU-E) also suggested that TCDD may interfere in the normal differentiation of immune cells from stem cells (Luster *et al.*, 1985; Ackermann *et al.*, 1989). However, these studies did not utilize precisely defined hematopoietic HSPCs and the maturation stage of the progenitors at which TCDD mediated its effects were obscure.

A direct effect of TCDD on murine hematopoietic stem and progenitor cells was reported by Murante *et al* whose study showed that treatment of adult C57BL6J mice with a single dose of TCDD, at 30 µg/kg, induced an increase in $Lin^{-}Sca\text{-}1^{+}$(Stem cell antigen-1)Kit^{+} (LSK) cells, over a period of approximately 30 days (Murante *et al.*, 2000). An increase in $Lin^{-}Sca\text{-}1^{+}Kit^{-}$ cells, which are downstream progenitors of LSK cells, was also observed but at a lower level than LSK cells, suggesting the effect of TCDD was more pronounced on early stem cells. TCDD treatment also leads to loss of long-term reconstitution ability of LSK cells in lethally irradiated mice (Sakai *et al.*, 2003). In human $CD34^{+}$ cells, the counterpart of murine HSPCs, induction of typical AHR regulated genes such as CYP1A1 and CYP1B1 with TCDD demonstrated that AHR is functional in these cells as well (van Grevenynghe *et al.*, 2005). However, in the previous study, at 10 nM TCDD, unlike murine HSPCs, no alterations in $CD34^{+}$ cell numbers were observed.

These studies pointed to the possibility that AHR may have a role in regulating HSCs in the absence of exogenous agonists. The physiological role of AHR in HSPCs was highlighted in studies using AHR knockout (KO) mice and AHR antagonist studies. LSK cells from $AhR^{-/-}$ mice had a 2-fold greater proliferation than cells from wildtype mice, suggesting that AHR may have role in regulating quiescence (Singh *et al.*, 2011). A similar phenomenon was described by Boitano *et al* who demonstrated that antagonism of AHR

with the compound StemRegenin-1 (SR1) led to proliferation of human cord blood derived $CD34^+$ cells of many orders of magnitude compared to control cells when they were cultured *ex vivo* for several weeks with retention of multilineage potential and reconstitution potential in irradiated mice (Boitano *et al.*, 2010).

1.5.1 Role of AHR and TCDD in development of lymphoid lineages

The effect of TCDD on development of different hematopoietic lineages with alterations in lineage specification occurring at the early progenitor level in mice was demonstrated in studies by Singh *et al*. Increase in macrophage and granulocyte progenitors were reported with a decrease in cells of B-cell lineage in bone marrow of mice exposed to TCDD (Singh *et al.*, 2009). No increase in cell apoptosis was reported in TCDD-treated mice. This was associated with a decline in colony-forming unit-preB progenitors and a temporary rise in myeloid progenitors in CFU assays, indicating a shift in the trajectory of lineage development from lymphoid to myeloid populations. This effect of TCDD on B cells was consistent with previous reports indicating B cells to be a sensitive target of AHR activation (Thurmond *et al.*, 2000a; Thurmond *et al.*, 2000b). However, studies by Thurmond *et al* had also suggested that the effect of TCDD on B cell progenitors may be at the more committed Pro-B/Pre-B cell stage of development rather than on earlier B lymphocyte progenitors (Thurmond *et al.*, 2000a). Reduced B and T lymphocyte differentiation from murine fetal liver-derived early hematopoietic progenitors in presence of TCDD in a co-culture assay demonstrated the detrimental effect of TCDD on early lymphoid differentiation (Ahrenhoerster *et al.*, 2014). The differences observed in some of these studies regarding the stage of lymphoid lineage specification affected by TCDD may partly be the result of differences associated with *in vitro* and *in vivo* studies.

Interactions between progenitors and cells in the stromal microenvironment could play a factor, with most *in vitro* assays suggesting that the effect of TCDD on B cell specification occurs at earlier stages of B cell development.

In vitro studies by Li *et al* have shown that TCDD also impairs B cell development from cord blood derived HSPCs in humans with progenitor cells being arrested at the common lymphoid progenitor stage. This was associated with a marked reduction in expression of the critical B-cell lineage associated genes, Early B cell factor 1 (EBF1) and Paired Box 5 (PAX5) in cells from the TCDD treated group (Li *et al.*, 2017a). However, the effect of TCDD on regulators upstream of EBF1 and PAX5 was not fully explored. As the culture system contains HSPCs and downstream progenitor cells, gene measurements obtained from the bulk population using qPCR in this study might not have identified other TCDD mediated small but critical changes in gene expression during early differentiation.

1.5.2 Role of AHR in the development of myeloid lineages

The role of AHR activation or TCDD treatment in development of other hematopoietic lineages from progenitor cells, especially in the context of humans, is not well established. To my knowledge, only a single study has directly investigated the effect of AHR activation on the development of monocytes and granulocytes from human HSPCs. A study by Platzer *et al* had reported that AHR ligands VAF347 and TCDD impaired differentiation of monocytes from human $CD34^+$ cells *in vitro* in media supplemented with the cytokines SCF, Flt3L, IL-6 and M-CSF through inhibition of the transcription factor PU.1 (Platzer *et al.*, 2009). No effect of VAF347 or TCDD was observed on development of granulocytes from $CD34^+$ cells in SCF and G-CSF enriched culture media. In the same study, decreased differentiation of $CD1a^+$ Langerhans cells has also been described.

Relatively more studies have been conducted on the role of AHR in the development of monocyte derived dendritic cells (mo-DCs). There are conflicting reports on how the AHR may control this differentiation process. Activation of the AHR with the AHR ligand FICZ promotes human mo-DC differentiation. Similarly inhibition of AHR by both the antagonist SR1 or silencing of AHR with lentiviral vectors decreased the proportion of mo-DCs *in vitro* (Goudot *et al.*, 2017). On the other hand, benzo(a)pyrene, a polycyclic aromatic hydrocarbon and AHR ligand, impaired *in vitro* differentiation of human monocytes to dendritic cells; whereas, TCDD had no significant effect on this differentiation (Laupeze *et al.*, 2002). The differences between these studies could be attributed to the different AHR ligands and culture conditions used in the studies. The aforementioned studies had focused on the effect of AHR activation on mo-DCs that developed directly from monocytes. Multiple murine studies have indicated that AHR activation by diverse ligands also regulates the differentiation and maturation of conventional dendritic cells from bone marrow cells. AHR activation by TCDD has been associated with a reduction in certain cell markers such as CD11c and an increase in expression for markers MHC class II (MHCII) and CD86 in bone marrow derived dendritic cells (Lee *et al.*, 2007; Vogel *et al.*, 2013); whereas, no effect on these markers was observed when bone marrow cells were cultured in presence of benzo(a)pyrene (Hwang *et al.*, 2007). Increased expression of MHCII and CD86 was also observed in bone marrow derived dendritic cells that developed in Iscove's modified Dulbecco's medium (IMDM) compared to those cells obtained from cultures in Roswell Park Memorial Institute 1640 medium (RPMI) (Ilchmann *et al.*, 2012). AHR has been implicated in this process as IMDM has at least three times greater amount of aromatic amino acids and higher endogenous AHR ligands than RPMI

(Ilchmann *et al.*, 2012). In a clinical trial study with $CD34^+$ HSPCs, AHR antagonism with the AHR antagonist SR1 *ex vivo* promotes development of myeloid dendritic cells (Thordardottir *et al.*, 2014) which suggests that AHR may negatively regulate differentiation of progenitor cells towards dendritic cells. Overall, these studies suggest that the role of AHR in development of conventional myeloid dendritic cells is multifaceted and warrants further studies.

Studies focused on the development of plasmacytoid dendritic cells (pDCs) suggest that the AHR has an inhibitory role in their development. AHR ligands Indoxyl 3-sulfate and Indole-3-carbinol impaired development of pDCs from murine bone marrow progenitor cells (Hwang *et al.*, 2018). Similarly, AHR antagonism *in vitro* in both murine (Liu *et al.*, 2014) and human (Thordardottir *et al.*, 2014) hematopoietic progenitor cells promoted development of pDCs.

The megakaryocyte-erythroid branch of hematopoietic cells is also prone to modulation by AHR as has been reported in some studies. AHR antagonism with SR1 in peripheral blood derived human $CD34^+$ cells in the presence of human mesenchymal stromal cells from bone marrow *in vitro* led to amplification of CD41 expressing megakaryocyte precursors (Strassel *et al.*, 2016), suggesting that reduced AHR activity is favorable to megakaryocyte specification. AHR may have an opposite effect on erythroid specification as observed in a study with hematopoietic progenitors derived from human induced pluripotent stem cells (Smith *et al.*, 2013). Culture of hematopoietic progenitor cells over an extended period of several weeks in a feeder-free system in presence of the AHR agonist FICZ induced a downregulation of megakaryocyte markers in megakaryocyte-erythroid precursors and an upregulation of CD235a, a marker for cells committed to the

erythroid lineage. In the same study, repression of AHR activity with a lentiviral vector containing an AHR repressor component resulted in enrichment of $CD41^+$ megakaryocyte cells and fewer $CD235a^+$ erythroid cells. This limited number of studies suggest that AHR activation may favor specification of megakaryocyte-erythroid progenitors towards the erythroid lineage over the megakaryocyte lineage.

1.6 Research rationale and hypothesis

The effect of TCDD on HSPC differentiation in humans has not been fully explored. Using culture conditions that propagate development of specific types of immune cell lineages, a handful of *in vitro* studies have demonstrated reduction in lymphoid and dendritic cell development and increase in myeloid cell populations in presence of TCDD. However, assessment of the different hematopoietic lineages simultaneously in the same culture system has not been done. This may be an important factor to consider as specific cytokine combinations in different cell cultures make it difficult to ascertain the effect of TCDD on lineage specification of HSPCs. Therefore, the effect of TCDD on human HSPC differentiation in a single culture system is not known. Moreover, the molecular mechanisms associated with HSPC differentiation that may be perturbed by TCDD treatment remain relatively unexplored.

In the study by Li *et al*, the differentiation of human HSPCs towards B cells has been investigated over a 28-day period (Li *et al.*, 2017a). On average, one-fifth of the total cells develop into cells of the lymphoid lineage. Although it was presumed that the rest of the cells were hematopoietic progenitor populations at different stages of development, the identity of these cells was not known.

Based on previous research findings, I hypothesized that TCDD treatment of human HSPCs may drive them towards the myeloid lineage by disrupting genes that regulate lymphoid-myeloid balance.

For identification of discrete cell populations that may develop as HSPCs differentiate *in vitro* and to discern transcriptomic changes brought about by TCDD treatment of HSPCs,

we employed single-cell RNA sequencing (scRNA-seq) to characterize the gene expression changes across different cell types at the single cell level.

The first part of the dissertation focuses on characterizing the effects of TCDD on human hematopoietic differentiation from human cord blood derived $CD34^+$ HSPCs in a stromal-cell free *in vitro* model using single-cell tools. The second part of the dissertation is aimed at better understanding the molecular mechanisms by which AHR activation by TCDD perturbs human hematopoiesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 *In vitro* culture of human CD34$^+$ HSPCs

Human CD34$^+$ HSPCs isolated from umbilical cord blood from mixed donors were purchased in a cryopreserved state from AllCells LLC (CA, USA). 10,000 cells per well were plated and cultured over a 28-day period in a 96-well cell culture plate in media enriched with cytokines and growth factors. The media used comprised RPMI-1640 media (Life Technologies) that is supplemented with 5% human AB serum (Valley Biomedical), 100 U/ml of penicillin (Life Technologies), 100 µg/ml of streptomycin (Life Technologies), and 50 µM 2-mercaptoethanol. SCF, Flt3L and IL-6 (each at 25 ng/ml; Miltenyi Biotec) were added on day 0 to promote proliferation of HSPCs and cells were treated with either TCDD (1 nM) or control vehicle dimethyl sulfoxide (DMSO) at 0.02%. TCDD was purchased from AccuStandard (New Haven, CT, USA) and DMSO was purchased from Sigma Aldrich (St. Louis, MO, USA).

On day 7, half of the cell culture medium was replaced with fresh medium supplanted with SCF (25 ng/ml), Flt3L (25 ng/ml) and IL-7 (20 ng/ml; Miltenyi Biotec). IL-7 was added to promote development of lymphoid progenitors and B-cells. For remainder of the study, cytokine-free fresh media was used to replace half of the medium every 7 days. As TCDD is highly lipophilic and partitions poorly into aqueous compartments, accurate estimation of TCDD remaining in the system after replacement of media is difficult. To ensure that more TCDD was not being added to the system than that being taken out, no further addition of TCDD was made.

For any treatment group, cells received the treatment only on day 0. DMSO (0.02%) was used as the solvent for TCDD and AHR antagonist CH223191. AHR antagonist

CH223191 were purchased from Sigma Aldrich (St. Louis, MO, USA). For treatments involving both TCDD (1 nM) and the AHR antagonist CH223191 (10 μM), cells were treated with the antagonist 30 minutes prior to addition of TCDD.

2.2 Single-cell RNA sequencing and data processing

Cells were harvested on day 0 from untreated HSPCs and every 7 days from three pooled 96-wells from both vehicle and TCDD treated groups. Cell suspensions containing 10,000 cells from each group were then processed using the 3' mRNA chemistry-based Gel Beads-in-Emulsion (GEM) technology from 10X Chromium Single Cell Gene Expression platform v3.2 (10X Genomics, Pleasanton, CA) to generate cDNA libraries as per company protocols. cDNA libraries were subjected to quality control analysis including a double-sided size selection. DNA fragments were selected with a size range between 400-1000 base pairs and analyzed using Agilent Bioanalyzer DNA1000 chip. cDNA libraries were sequenced on an Illumina HiSeq platform at an average sequencing depth of 50,000 paired end reads per cell. Reads were processed by CellRanger software (v 2.1) from 10X Genomics.

scRNA-seq data quality control was performed using scRNA-seq analysis package Seurat (v 4.0) (Hao *et al.*, 2021). Following generation of a cell-by-gene expression matrix, cells that have unique feature counts over 500 and less than 8000 were selected. RNA count parameters were set between 250 and 80000. Cells that have >25% mitochondrial unique gene counts were filtered out. Any cell that had >15% of its genes associated with cell cycling were filtered out. The final matrix consisted of a total of 27,340 cells calculated across 5 different time points (Day 0, 7, 14, 21, 28) from naive, vehicle and TCDD treated groups.

Gene expression data for each cell across days was concatenated into a single matrix which was analyzed with the analysis package Seurat (v 4.0). Following quality control of the single-cell gene expression matrix, data were normalized using the SCTransform method. In total, 3000 variable genes were used for dimension reduction, and cells were represented in two-dimensional space using Uniform Manifold Approximation and Projection (UMAP). Unsupervised clustering (Louvain clustering with default parameters in Seurat's 'Find Clusters' function at resolution of 0.5) of cells generated cell clusters which were annotated based on enrichment of hematopoietic lineage specific genes.

2.3 Single-cell transcriptomic data analyses

2.3.1 Developmental trajectory analysis

Cell clusters associated with the lineage of interest were selected and dimensionality reduction and projection in UMAP space were redone. This was followed by trajectory analysis and pseudotime calculations for cells using the R package 'slingshot' (Street *et al.*, 2018) (v 3.14).

For calculation of top variable transcription factors across pseudotime, the top 2000 variable genes for cells associated with a particular lineage was calculated using Seurat. A random forest-based regression model was then used to fit the variable genes against pseudotime values. This allowed ranking of the variable genes based on their variability across pseudotime. A similar approach was carried out to rank the transcription factors obtained from SCENIC analysis. Top 20 variable genes or transcription factors were then plotted against binned pseudotime values.

2.3.2 Analysis of differentially expressed genes

Differentially expressed genes (DEGs) analysis between vehicle and TCDD-treated groups was carried out using 'FindMarkers' function of Seurat (testing method called upon the MAST package and assay used was 'RNA'). Log_2 fold-change (log_2FC) threshold was set at 0.25 unless otherwise stated. Adjusted *p*-value threshold of 0.05 based on Bonferroni multiple testing correction was used as a cut-off to select significant DEGs. For gene and cluster dot-plots in the figures, an equal number of upregulated and downregulated significantly DEGs were plotted based on fold change in expression among groups. Top DEGs in each category were selected and shown in figures.

2.3.3 Inference of transcription factor activity

Transcription factor activity was estimated using the R-language based package 'SCENIC' (Holland *et al.*, 2020). SCENIC comprises a curated collection of transcription factors and their direct cis-target genes. Transcription factor activities are calculated using the mRNA expression levels of its target genes. Networks of transcription factors and their target genes across the dataset initially estimated are pruned based on significance and an arbitrary score of transcription factor activity is calculated for each cell that passes a threshold level of activity for the transcription factor.

2.3.4 Gene Set Enrichment Analysis (GSEA)

Seurat's 'FindMarkers' function was used to calculate DEGs but without any threshold for log_2 fold-change ($log_2FC = 0$). The genes were ranked based on fold change. The R package 'fgsea' (v 3.14) (Korotkevich *et al.*, 2021) was used to carry out GSEA using the pre-ranked list of genes and Hallmark gene sets from human MSigDB database.

2.3.5 Single Sample Gene Set Enrichment Analysis (ssGSEA)

To better understand the enrichment score distribution across individual cells, we also used a modification of GSEA called ssGSEA to calculate pathway enrichment on a per cell basis (Barbie *et al.*, 2009). This approach enables the quantification of an enrichment score, which reflects the extent of absolute enrichment of a gene set within each sample in a specific dataset. To calculate this score, gene expression values for a given sample were normalized by ranking, and the enrichment score was determined using the Empirical cumulative distribution functions (ECDF) of the genes within the gene set and those outside it. This process resembles GSEA, except that the ranking is based on absolute expression within a single sample. The enrichment score is computed by integrating the difference between the ECDFs. For implementation of ssGSEA to our data, we used the R package 'escape' (Easy single-cell analysis platform for enrichment) (Borcherding *et al.*, 2021).

2.3.6 Calculation of gene module scores

Genes used for calculation of gene set / module scores to portray different lineages were as follows: HSPC: *CD34, PROM1, SPINK2*; Early B cell: *CD79A, CD79B, CD19, VPREB1, VPREB3;* Promyelocyte: *AZU1, ELANE, CTSG, PRTN3;* Neutrophil: *S100A8, S100A9, CAMP, MMP8, S100P;* Monocyte/Macrophage: *CD14, F13A1, FCN1, CD68, CSF1R, APOE*; Type 2 classical dendritic cell: *CD1C, CLEC10A, ITGAM*; pDC: *SPIB, IRF8, IRF4, IL3RA, GZMB;* Megakaryocyte/Erythroid: *HBB, HBD, KLF1, ITGA2B, GP9.*
Type A CD1c cell score was calculated based on the expression of the following genes: *HLA-DPB1, HLA-DQB1, HLA-DQA1, HLA-DQA2, CD1C, P2RY14, ARL4C, CLIC2,*

FAM26F, ASAP1, SLC41A2, SLAMF7, CST7, PKIB, CXCL16, RUNX3, WDFY4, IL18R1, FCGR2B, MYO1E, AXL, PEA15, SIGLEC10, CD1E, GOLGA8B, IFITM1, INSIG1, SPATS2L, GRIP1, MCOLN2, SERTAD3, PPP1R14A, UVRAG, SIGLEC6, KPNA6, LGMN, SPIB, TOP1MT.

Type B CD1c cell score was calculated based on the expression of the following genes: *BACH1, TSC2, SHOC2, HPCAL1, PVR, RIPK2, STIM1, ID1, IKBKE, KCNN4, EMP1, GPBAR1, MKNK1, KIAA0513, FOXO3, YWHAG, TAB1, OSM, GABARAPL1, ASPH, PDLIM7, QPCT, RIN2, MRPS23, PLXND1, CLEC12A, TMEM176A, PISD, PLA2G7, TMEM141, NINJ1, AGTRAP,BLVRA, HBEGF, DMXL2, IL1B, NLRP12, SORL1, NFE2, ADAM15, CCDC69, SULT1A1, TOM1, KCNE3, PYGL, SLC11A1, HK3, ACSL1, IER3, CFD, LMNA, TREM1, PILRA, ASGR1, TXNRD1, GLUL, PSTPIP1, CSF3R, STAB1, RETN, SERPINA1, SLC7A7, CTSD, NEAT1, FPR1,CD163, S100A12, CYBB, F13A1, CES1, BST1, MTMR11, CD36, MGST1, RAB3D, PLBD1, TMEM176B, CD14, FCN1, RNASE2, VCAN, S100A8, S100A9*.

M2 macrophage module score was calculated based on the expression of the following genes: *CLEC10A, IRF4, MRC1, CCL8, CLEC7A, PDCD1LG2, SOCS2, CDH1, PPARD, PPARG*.

2.3.7 Annotation of cells using reference data set

Cells were annotated at single cell level by mapping labels from reference data (human bone marrow mononuclear cells from the Human Cell Atlas) as per the instructions in Seurat multimodal reference mapping vignette.

2.4 Statistical analysis

Statistical analysis for flow cytometry data was performed using GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA). For flow cytometry data involving comparison of two groups at a particular timepoint, a paired, two-tailed student's t-test was used to determine significance of the results. For comparison of multiple groups at any time, a repeated measures one-way ANOVA was employed, followed by a Dunnett's multiple comparisons test. Regarding SCENIC analysis and M2 macrophage score comparisons, statistical analysis for differences in transcription factor activity and M2 macrophage scores between groups was done using Wilcoxon rank sum test ('wilcox.test' function in R). For statistical analysis of differences in pathway enrichment scores between groups calculated using ssGSEA, a linear model method was used. The R package 'limma' (Smyth, 2005) was used to construct a design matrix, followed by a linear model fit. Empirical Bayes statistics were then applied through the package for differential analysis of enrichment scores.

2.5 Flow cytometric analysis

On days of cell collection, cells were harvested and washed using HBSS (pH 7.4, Invitrogen). Cell surface Fc receptors were blocked by incubating cells with human AB serum. For cell surface staining, cells were incubated with antibodies in FACS buffer (1X HBSS containing 1% BSA and 0.1% sodium azide, pH 7.4–7.6) for 30 minutes, washed and fixed using Cytofix fixation buffer (BD Biosciences) for 20 minutes (see supplementary methods for details of cell surface antibodies).

Antibodies used for flow cytometry included anti-human CD34 (Fluorophore Brilliant Violet BV510, clone 581; Biolegend), anti-human CD10 (Fluorophore BV711 / BV421, clone HI10a; Biolegend), anti-human CD19 (Fluorophore BV421, HIB19; Biolegend), anti-human CD66b (Fluorophore PE-Dazzle 594 / PerCP-Cy5.5, clone QA17A51; Biolegend), anti-human CD1c (Fluorophore PE-Cy5 / BV421, clone L161; Biolegend), anti-human CD14 (Fluorophore BV605 / Alexa Fluor 488, clone 63D3; Biolegend), anti-human CD41 (Fluorophore AF647 / AF488, clone HIP8; Biolegend), anti-human CD15 (Fluorophore Brilliant Violet 785, clone W6D3; Biolegend), anti-human CD303 (Fluorophore APC, clone AC144; Miltenyi Biotec), anti-human Ctip-1 (Fluorophore PE, clone NB600-261; Novus Biologicals), anti-human CD235a (Fluorophore Super Bright 436, clone HIR2 (GA-R2); ThermoFisher Scientific), anti-human IRF8 (Fluorophore APC, clone V3GYWCH; ThermoFisher Scientific) and anti-human AHR (Fluorophore PE-Cy7 / PE, clone FF3399; ThermoFisher Scientific). Viable cells were identified using Live/Dead Fixable IR Cell Stain (Invitrogen).

For intracellular staining, fixed cells were permeabilized by incubating in eBioscience™ Foxp3/Transcription Factor Permeabilization buffer (Invitrogen) for 20 min and incubated with relevant antibodies (anti-human Ctip-1 (*Fluorophore PE, clone NB600-261; Novus Biologicals*), anti-human IRF8 (*Fluorophore APC, clone V3GYWCH; ThermoFisher Scientific*) and anti-human AHR (*Fluorophore PE-Cy7 / PE, clone FF3399; ThermoFisher Scientific*)) for 60 minutes. This was followed by washing in the presence of the buffer. Finally, cells were resuspended in FACS buffer. Flow cytometric analysis was performed using BD FACSCanto-II cell analyzer (BD Biosciences) with FACSDiva software (BD Biosciences) or Cytek Northern Lights full spectral flow cytometer (Freemont, California).

Flow cytometry data were analyzed using FlowJo (version 10.1, Treestar Software Ashland, OR, USA).

2.6 Figure generation

Figures were generated using either R based software packages, Graphpad Prism 9.5.0, or Biorender.com.

CHAPTER 3: RESULTS

3.1 Characterization of an *in vitro* model of hematopoietic differentiation and identification of the role of TCDD in the differentiation process

3.1.1 Single-cell transcriptomic analysis of human CD34$^+$ cells reveal the development of multiple hematopoietic cell types in an in vitro model of human hematopoietic differentiation

To study the role of AHR signaling during differentiation of HSPCs, we developed an *in vitro* stromal-cell free model of human hematopoietic differentiation (**Figure 3.1A;** further details in Methods section). A minimal number of pleiotropic cytokines and growth factors at low concentrations were added at early time points to HSPCs which were cultured over a 28-day period. Combined addition of stem cell factor (SCF), fms-like tyrosine kinase 3 ligand (Flt3L) and interleukin-6 (IL-6) have been shown to expand the number of hematopoietic progenitor cells (Flores-Guzman et al., 2002; Du et al., 2015), and were added at the beginning of the experiment to promote expansion of HSPCs. Interleukin-7 (IL-7) is known to greatly enhance lymphoid and B-cell development from progenitor cells (Parrish et al., 2009), especially in combination with Flt3L and SCF (Veiby et al., 1996), and was added to the culture on day 7. For the scRNA-seq study, HSPCs were initially treated with either the control vehicle (0.02% DMSO) or TCDD (1 nM). Previous studies had shown that B cell development from HSPCs is strongly attenuated at concentrations as low as 1 nM (Li *et al.*, 2017a; Li *et al.*, 2017b). Cells from both groups were harvested at multiple time points for sequencing (see Methods section for details). Upon processing

of sequenced reads (**Figure 3.1A**), exploratory gene expression analysis in cells suggested the existence of diverse hematopoietic lineages in the system. Based upon expression of multiple genes associated with distinct hematopoietic lineages, numerous hematopoietic cell types were identified (**Figure 3.1B**).

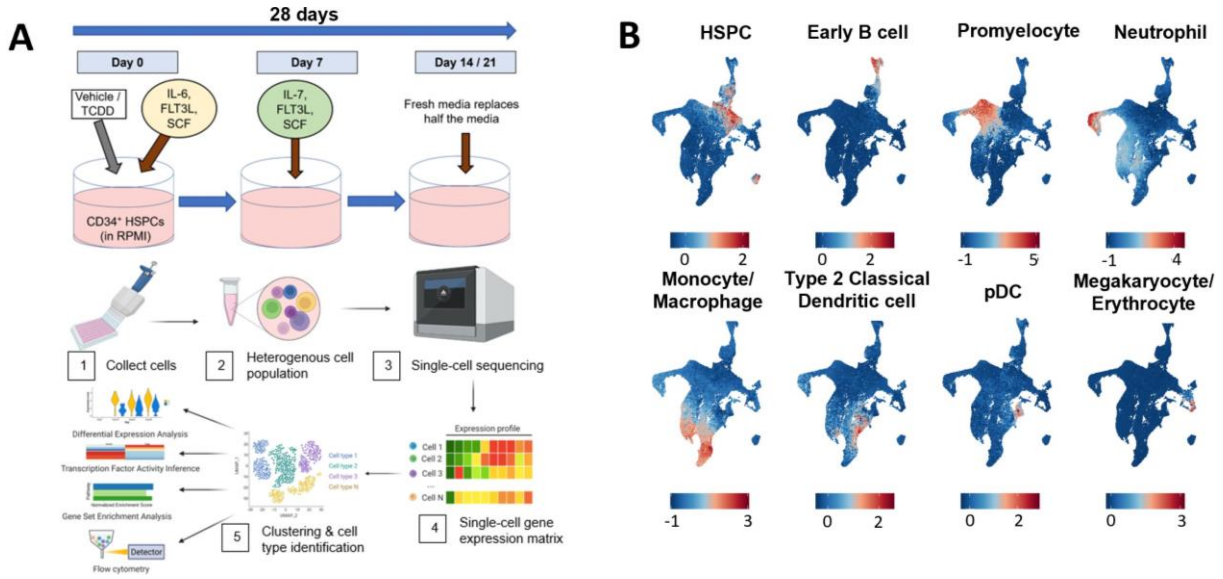

Figure 3.1 A) Experimental design: *In vitro* culture system of hematopoiesis from human cord blood HSPCs, schema of sample processing, and data analysis. (B) Different cell types were identified based on expression of a suite of lineage specific genes (see Methods 2.3.6). Legend bars represent scaled gene module score levels.

To characterize the natural development of hematopoietic progenitors in absence of TCDD, we selected only cells from Day 0 and that of the vehicle group. UMAP projection of different cell clusters reflected a natural progression of development of different hematopoietic lineages from early progenitors (**Figures 3.2A-3.2C**). Hematopoietic stem and progenitor cells (HSPCs) collected on Day 0 clustered separately from cells collected on other days and were highly enriched in hematopoietic stem cell associated genes *HLF, AVP, SPINK2* (**Figures 3.3A and 3.3B**). Clusters annotated as multipotent progenitors (MPP and MPP2) similarly expressed high levels of HSC associated genes but at a lower

level than the HSC cluster. Additionally, the MPP2 cluster had high expression of genes associated with later phases of cell cycling such as *TOP2A, SPINK2* and *MI67*. The progenitor cluster, Promyelocyte, had high expression of genes such as *AZU1, ELANE, CTSG, and PRTN3* and is adjacent to the Neutrophil cluster that expressed genes *CAMP, S100P, S100A8, and S100A9*.

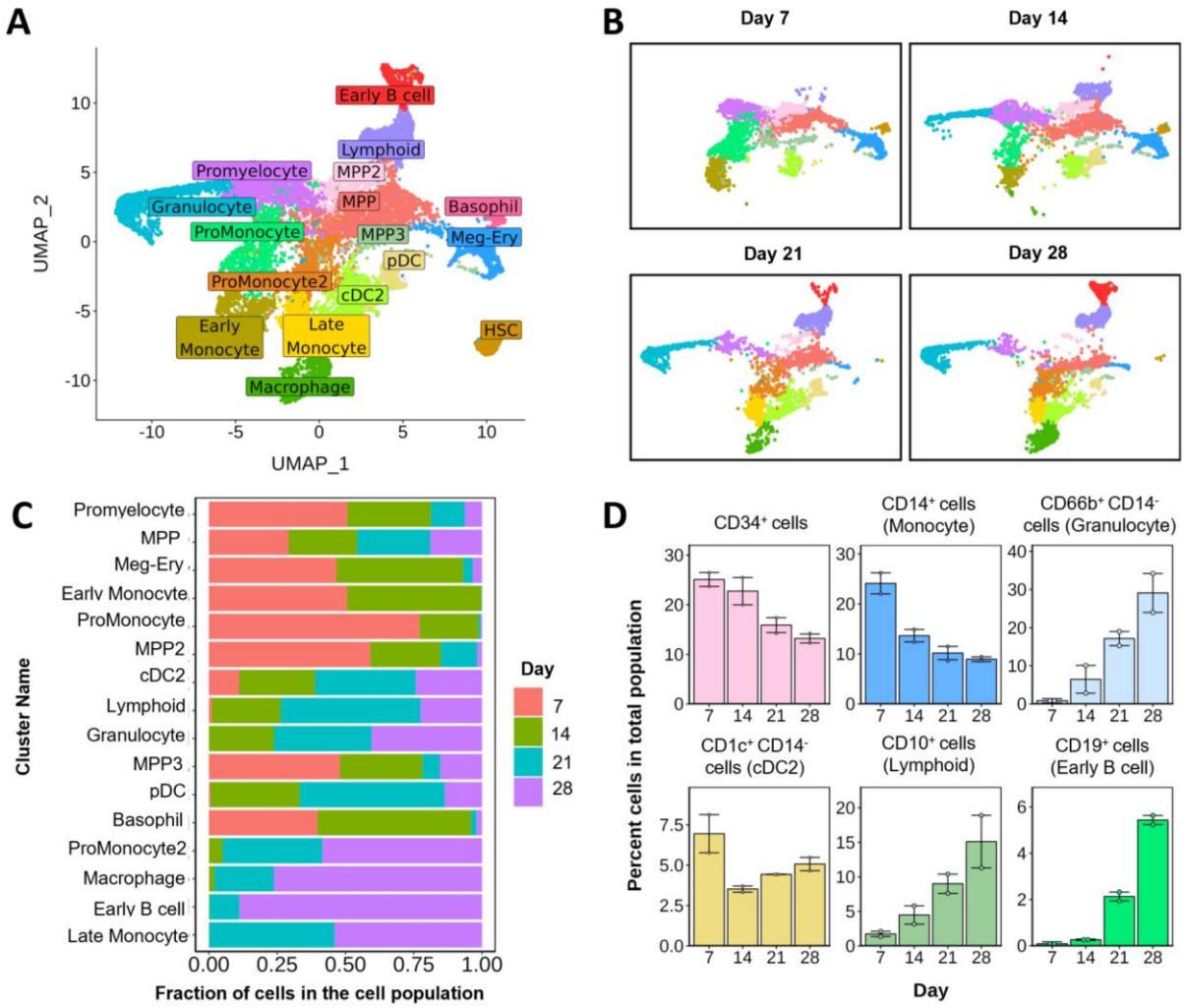

Figure 3.2 (A) Representation using UMAP of all cells from control group collected over the 28-day period; MPP = multipotent progenitors, cDC2 = Type 2 classical dendritic cells, pDC = plasmacytoid dendritic cells, HSC = hematopoietic stem cells, Meg-Ery = Megakaryocyte-Erythroid progenitors. (B) Development of cell clusters over time is shown. (C) Proportion of cells of each cluster across time. (D) Percent of cells belonging to major cell types that develop from untreated HSPCs were identified using flow cytometry. Error bars show mean ± SEM from 2 independent experiments.

The pro-monocyte clusters, Promonocyte and Promonocyte2, had high expression of genes such as *AZU1, ELANE, CTSG, and PRTN3* and is adjacent to the Neutrophil cluster that expressed genes *CAMP, S100P, S100A8, and S100A9* (**Figures 3.3A and**

3.3B). The pro-monocyte clusters, Promonocyte and Promonocyte2, developed at different times with the Promonocyte cluster developing early in the developmental period and the latter was primarily detected from Day 14 onwards (**Figures 3.2B and 3.2C**). Clusters designated as Early Monocyte (enriched for genes *CD14, F13A1, THBS1*) and Late Monocyte (*CD14, FCN1, KCTD12*) are immediately below the pro-monocyte clusters. We also detected a cluster that expressed many monocyte-associated genes but additionally had higher expression of macrophage associated genes such as *CD68, APOE, GPNMB,* which we labeled as Macrophage cluster (**Figures 3.3A and 3.3B**). The Lymphoid cluster included cells with high expression of some multipotent progenitor affiliated genes such as *CD34*, SPINK2 as well as cells associated with T (*CD7, CD3E, IRF4*) and B cell (*BCL11A*, *MEF2C, CD79A, IGHD*) development. The Early B cell cluster lies at the apex of lymphoid cells and expresses the B cell genes *EBF1, DNTT, VPREB3, and CD79B*. In addition, other cell types were identified. Clusters containing these different cells were annotated as Megakaryocyte-Erythroid (Meg-Ery), Basophil, plasmacytoid dendritic cell (pDC) and Type 2 classical dendritic cell (cDC2) clusters (**Figure 3.2C**).

Automated annotation against a dataset of human bone marrow derived cells was largely consistent with our supervised clustering (**Figure 3.4A**). To confirm the formation of *bona fide* hematopoietic lineages in our system, flow cytometry was employed using antibodies directed against markers for major hematopoietic lineages. The markers selected were informed through both literature and scRNA-seq analysis and were highly and uniquely expressed in the population of interest (**Figure 3.4B**).

Figure 3.3 (A) Dot plot of the average expression of curated hematopoietic lineage associated genes across different clusters. (B) Dot plot of the average expression of top 3 genes based on relative expression across different clusters.

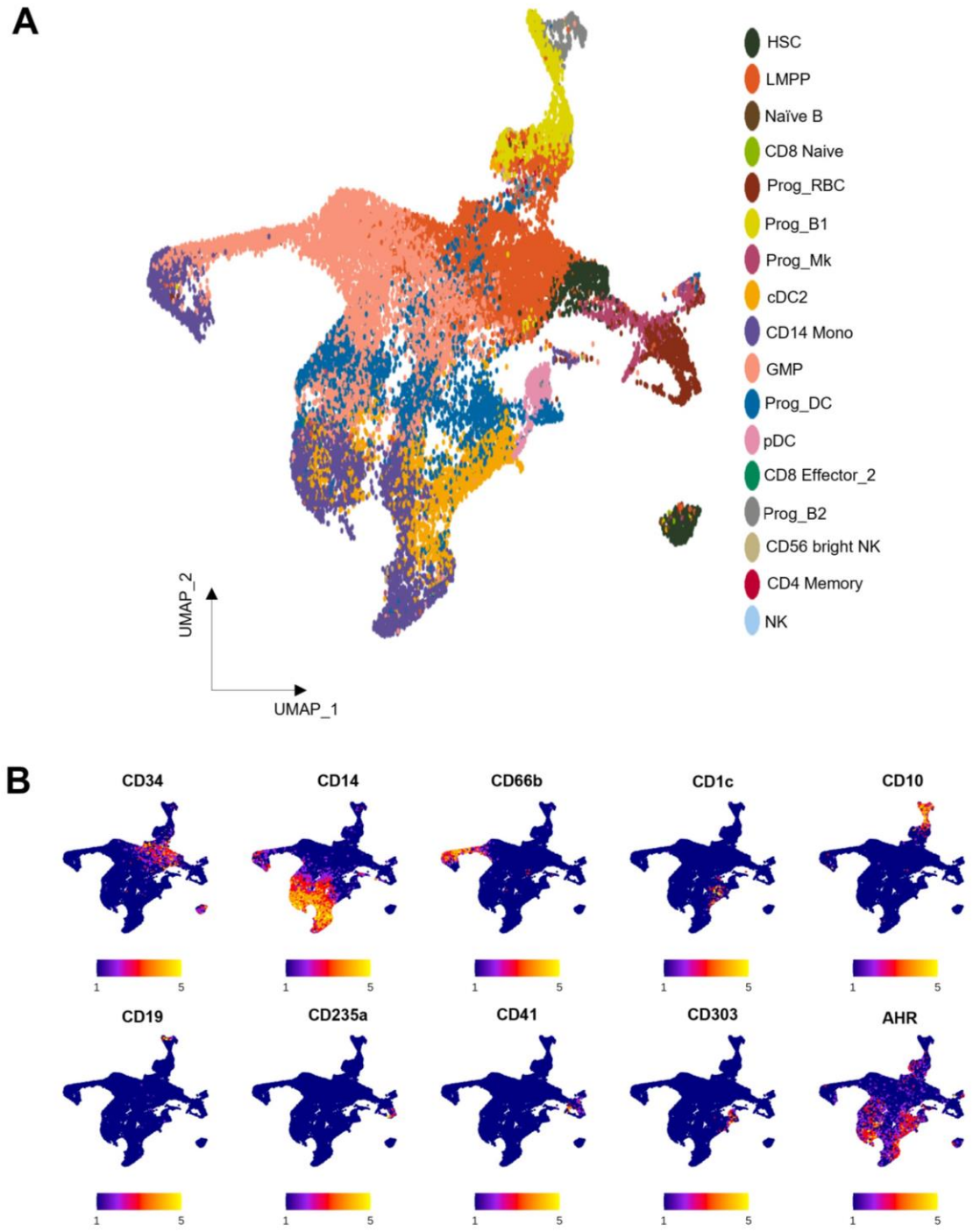

Figure 3.4 (A) Cells in single-cell RNA-seq dataset annotated using a reference dataset of human bone marrow mononuclear cells from eight individual donors produced by the Human Cell Atlas (mapped using Seurat). (B) Expression of select marker genes depicting different hematopoietic lineages in UMAP space. Legend bars represent scaled expression levels.

CD10, encoded by the gene *MME*, is a zinc metalloendopeptidase that is expressed on the surface of some hematopoietic and nonhematopoietic cells, and is also an important marker in certain malignancies (Bilalovic *et al.*, 2004). In humans, it is used to delineate common lymphoid progenitors and subsequent early progenitors of the B cell lineage (Ichii *et al.*, 2010). However, its expression decreases with further B cell maturation. Expression density of CD10 is associated with commitment propensity of lymphoid progenitors to the B cell lineage (Ichii *et al.*, 2010). CD19 is a glycoprotein and B cell marker that is involved in regulating both B cell receptor-dependent and independent cell signaling (Wang *et al.*, 2012). CD1c is a transmembrane glycoprotein and a marker for human Type 2 classical dendritic cells (Heger *et al.*, 2020). It is involved in antigen presentation by these dendritic cells to T cells. CD14 is a glycoprotein and a major marker for monocytes and macrophages (Ziegler-Heitbrock *et al.*, 1993; Zamani *et al.*, 2013). At low levels, it may also be present on dendritic cells and some neutrophils. CD14 on the cell surface of phagocytic cells is associated with Toll-like receptor 4 and acts as co-receptor for lipopolysaccharide, and thus assists in TLR4 signaling (Zamani *et al.*, 2013). CD66b is a marker of human neutrophils and eosinophils (Opasawatchai *et al.*, 2018; Bergenfelz *et al.*, 2020). It is a glycoprotein that is associated with activation, adhesion, and degranulation of granulocytes (Opasawatchai *et al.*, 2018). CD41 is an integrin that is highly expressed on human platelets (Bagamery *et al.*, 2005). It is a heterodimer that is composed of an alpha and beta chain. Along with CD61, it acts as a receptor for fibrinogen and plays an important role in thrombosis and hemostasis (Fullard, 2004). CD235a or Glycophorin A is a sialo-glycoprotein that is abundantly expressed on the surface of cells committed to the erythroid lineage, and may help prevent the aggregation

of erythrocytes (Allahyani *et al.*, 2022). CD303 or BDCA-2 (encoded by the gene *CLEC4C*) is a C-type lectin that is a highly specific marker for human pDCs and is involved in antigen presentation and regulation of interferon-α/β production (Dzionek *et al.*, 2001; Rock *et al.*, 2007).

Flow cytometric analysis of cells developing from untreated HSPCs confirmed our scRNA-seq based findings regarding the formation of several major immune cell types (**Figure 3.2D**).

3.1.2 Single-cell transcriptomic analysis identifies major genes and transcription factors associated with development of different hematopoietic lineages

To identify temporal changes in gene expression and transcription factor activity associated with development of different hematopoietic lineages, relevant cell clusters of interest were selected, and trajectory analysis was carried out (**Figure 3.5A**). MPP cluster was selected as the origin of the developmental trajectory for all lineages as this progenitor cluster was not lineage specified and a trajectory could be established to all other lineages. The top twenty variable genes and transcription factors that were altered in expression and activity respectively along the developmental trajectories were identified (**Figures 3.5B and 3.5C**). Overall, the gene expression kinetics for cells along the major lineage trajectories agreed with previous findings. Transcription factor activity derived from SCENIC (Holland *et al.*, 2020) analysis identified the main transcription factors associated with specific cell clusters (**Figure 3.6**). Transcription factor activity in each cluster was highly concordant with the cell type represented by the cluster. Compared to the Late Monocyte cluster, the Early Monocyte cluster displayed enhanced activity for transcription factors *RXRA, MAFB, JUNB, FOS* and *EGR1* that are associated with a pro-inflammatory phenotype. The Lymphoid cluster showed high *MEF2A* activity. Although *MEF2C*, a member of the MEF family of transcription factors, has been known to regulate lymphoid specification, *MEF2A* has not been previously linked to lymphoid development and is a novel finding. Interestingly, *SOX4* was identified as a regulator with high activity in B cells and low activity in myeloid cells (**Figure 3.6**).

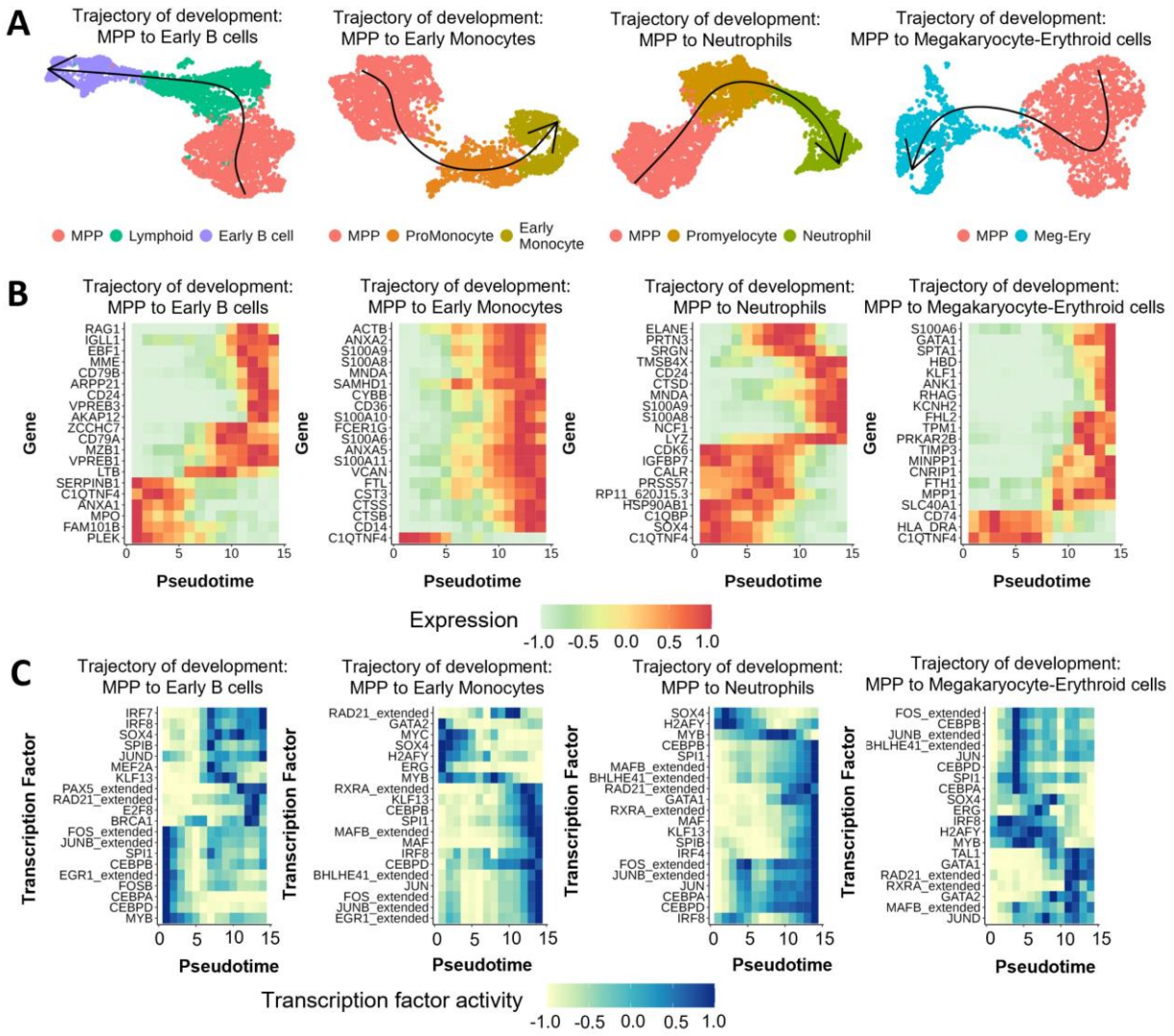

Figure 3.5 (A) Developmental trajectory of different hematopoietic lineages; MPP = multipotent progenitors, Meg-Ery = Megakaryocyte-Erythroid progenitors. (B) Top 20 variable genes as a function of developmental progression (denoted by pseudotime) along different lineages. (C) Activity of top 20 variable transcription factors as a function of developmental progression (denoted by pseudotime) along different lineages.

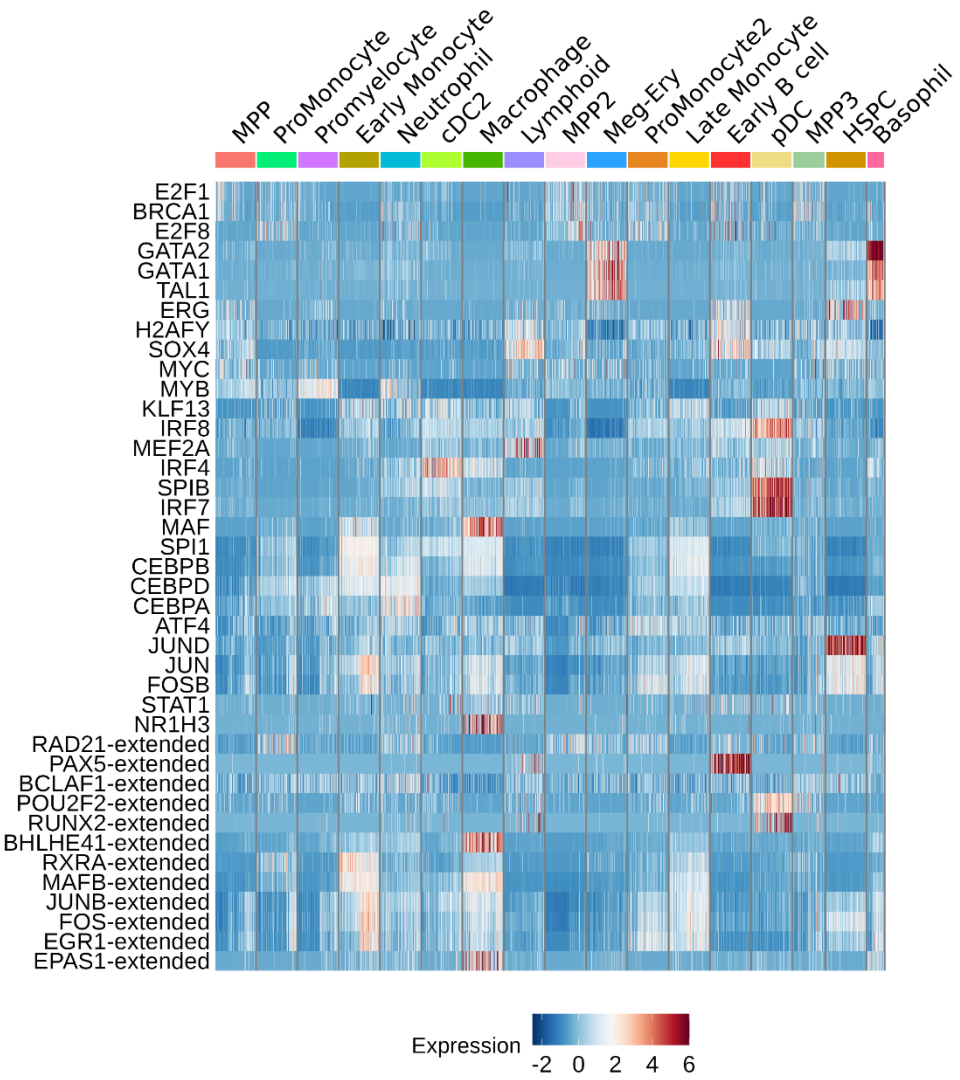

Figure 3.6 Transcription factor network active in different cell clusters (inferred using SCENIC).

Previous studies have implicated *SOX4* expression with B cell lineage specification but its role in myeloid lineage development had been inconclusive (Laurenti *et al.*, 2013). *SOX4* expression was also found to be downregulated along the MPP to neutrophil trajectory (**Figure 3.5B**), suggesting downregulation of *SOX4* is conducive to neutrophil development.

The Early Monocyte cluster had a gene expression profile associated with an activated monocyte phenotype and had higher expression of genes involved in regulation of inflammation such as *CXCL8, RNASE1* and *F13A1* compared to the Late Monocyte cluster (**Figure 3.7**). Gene set enrichment analysis (GSEA) for differentially expressed genes between the two clusters demonstrated relative downregulation of the TGF-beta signaling pathway in the Late Monocyte cluster with upregulation of oxidative phosphorylation and unfolded protein response pathways relative to the Early Monocyte cluster.

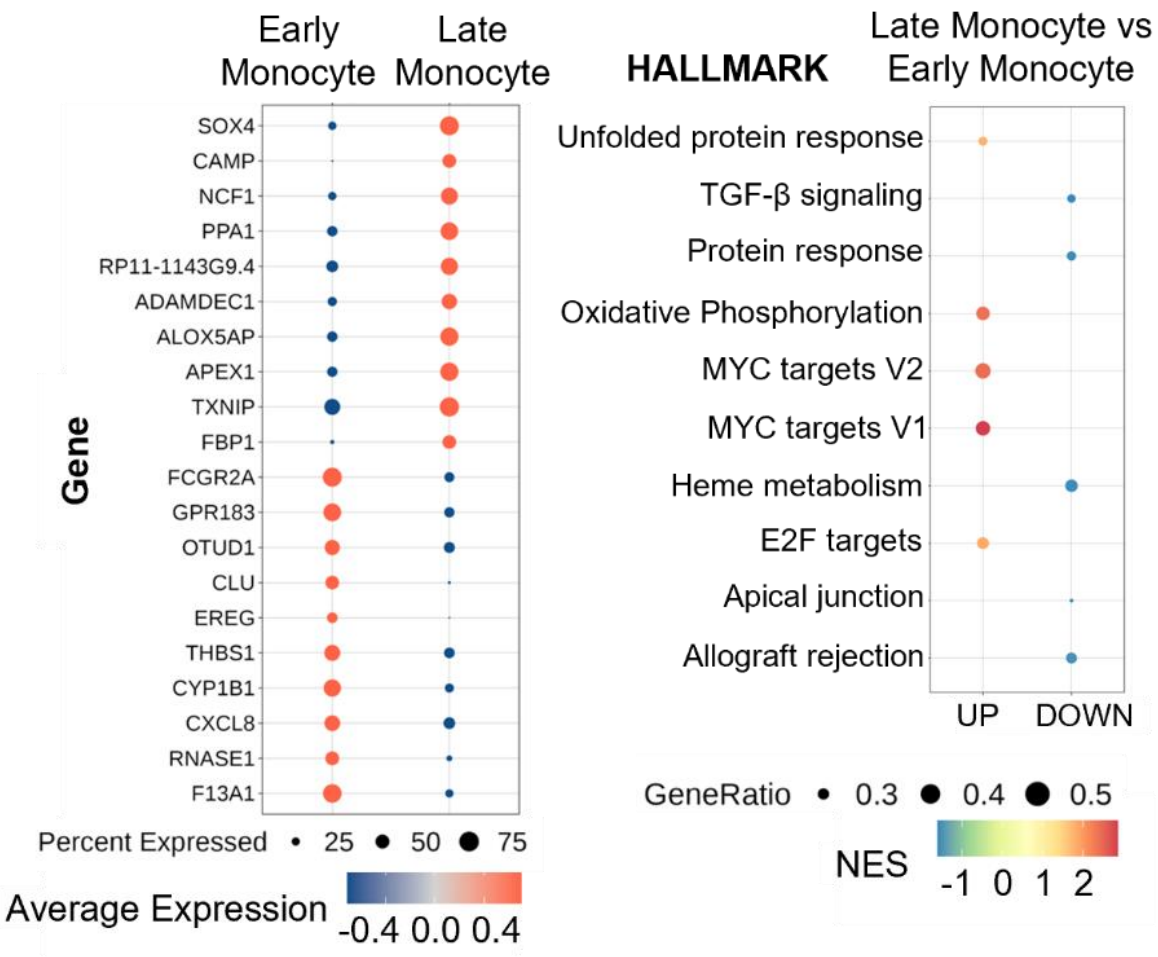

Figure 3.7 Top differentially expressed genes (DEGs) based on fold change and top enriched pathways from gene set enrichment analysis (GSEA) between Early Monocyte and Late Monocyte clusters. DEGs shown are associated with a Bonferroni adjusted *p* value < 0.05 (see Methods). NES = Normalized enrichment score.

We also carried out ssGSEA (single sample gene set enrichment analysis) to get a better idea of the distribution of pathway enrichment scores for the Hallmark database of pathways in individual cells. Pathways with distinct differences in enrichment scores over time are plotted. Over the course of the 28-day period, we observed continual reduction in pathway enrichment score for phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin(mTOR) signaling in monocytes (**Figure 3.8**). PI3K/AKT/mTOR signaling score of Late Monocyte cluster cells at day 21 was significantly lower than Early

Monocyte cluster cells at day 14. PI3K/AKT/mTOR signaling may be associated with inflammation in certain innate immune cells as suggested by a reduction in secretion of proinflammatory cytokines in LPS stimulated THP-1 monocytes and THP-1 derived macrophages with PI3K and mTOR inhibitors (Xie *et al.*, 2014). In our studies, although the culture conditions are not the same, PI3K/AKT/mTOR signaling in monocytes may potentially play a similar role and decrease in signaling intensity over time could contribute to the reduced inflammatory phenotype associated with later stage monocytes that develop in our system.

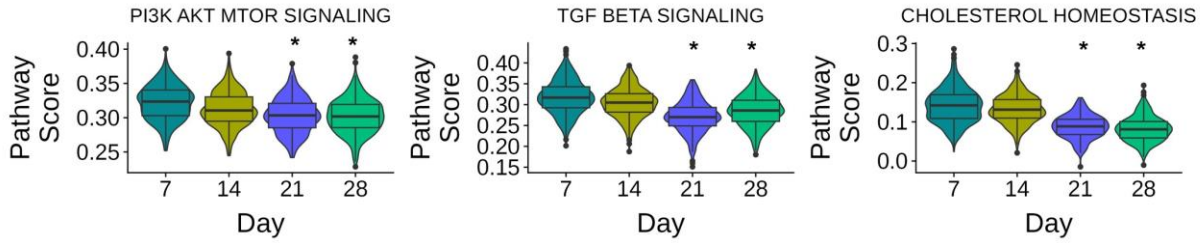

Figure 3.8 Select differentially enriched pathways in monocytes (Early Monocyte and Late Monocyte clusters) in the Vehicle group. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p*-value < 0.05) differences in pathway enrichment scores compared to days 7 and 14 are denoted by *.

Interestingly, with the later stage monocytes, there was also a significant decrease in TGF-β signaling enrichment score. TGF-β signaling has been demonstrated to play an important part in M2 macrophage polarization and is known to restrict induction of inflammation in monocytes/macrophages (Gong *et al.*, 2012; Zhang *et al.*, 2016). A lower score in late-stage monocytes would thus denote a higher inflammatory signature in these cells. However, as this is perhaps not the case, its role in the context of our *in vitro* model requires further investigation.

Figure 3.9 Select genes that are differentially expressed in monocytes (cells of Early and Late Monocyte cluster) and contribute to enrichment scores of pathway enrichment analysis.

We observed a strong reduction in enrichment score for cholesterol homeostasis with time (**Figure 3.8**). Genes associated with cholesterol import into cells such as *LDLR* (low-density lipoprotein receptor), cholesterol synthesis such as *SQLE* (squalene epoxidase) and cholesterol accumulation such as *LGALS3* (galectin-3) were lower in monocytes on days 21 and 28 (Late Monocyte cluster cells) compared to monocytes on days 7 and 14 (Early Monocyte cluster cells) (**Figure 3.9**). This suggests that late developing monocytes are lower in cholesterol content and perhaps less inflammatory as cholesterol accumulation and inflammatory phenotype in monocytes have been reported to be

inversely correlated (Cardoso *et al.*, 2021; Stiekema *et al.*, 2021). Interestingly, there was also a strong reduction in a gene important for cholesterol efflux such as CLU (clusterin). This could however be a compensatory response to the reduction in genes associated with cholesterol accumulation or vice versa. Overall, cholesterol homeostasis seems to be strongly linked to the maturation of monocytes *in vitro*.

Other clusters displayed heterogeneity in gene expression consistent with temporal development. For example, the cDC2 cluster, at a low resolution of Seurat's graph-based clustering (resolution of 0.1), split into 2 major sub-clusters with the Early DC sub-cluster representing dendritic cells on days 7 and 14, and the Late DC sub-cluster representing dendritic cells on days 21 and 28 (**Figure 3.10A**). cDC2 cluster cells on days 7 and 14 had higher expression levels of cellular proliferation associated genes such as *MKI67, S100B,* and *TOP2A*, while cDC2 cluster cells on days 21 and 28 expressed monocyte-associated genes such as *CD14, S100A8* and *ADAMDEC1* among others (**Figure 3.10B**). GSEA showed enrichment for pathways such as NF-kb signaling and complement pathway in the Late DC sub-cluster, suggesting a shift towards an inflammatory phenotype in the late-stage dendritic cells. Proliferation associated pathways such as *mTORC1* pathway were downregulated in Late DCs compared to Early DCs. scRNA-sequencing thus helped characterize the development of multiple immune cell types and their different temporal gene expression programs in our *in vitro* system.

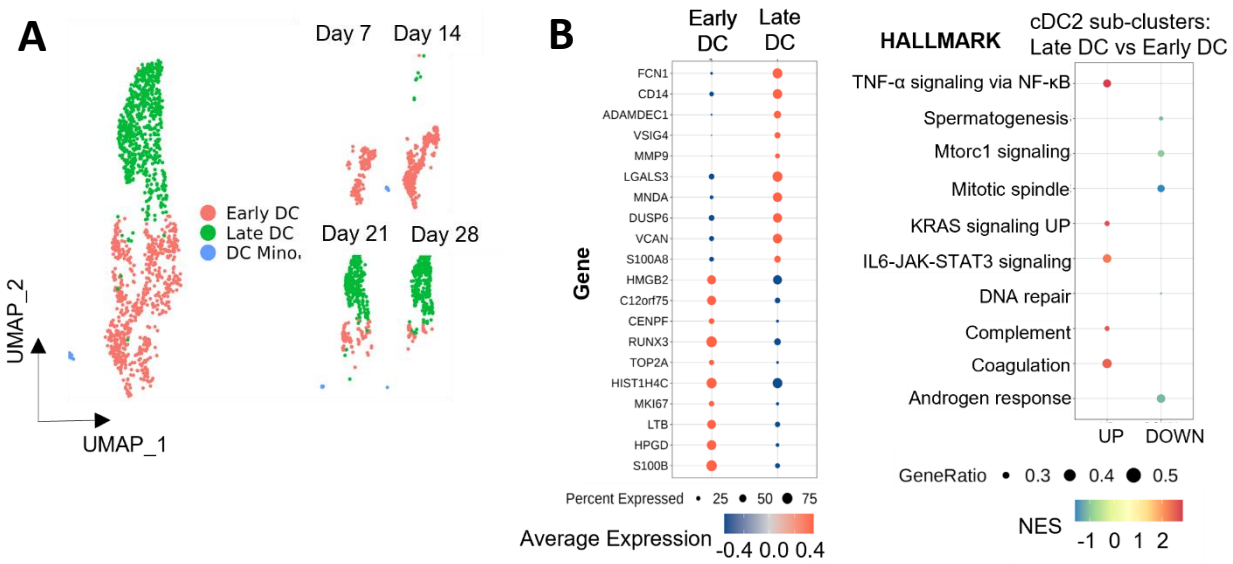

Figure 3.10 (A) Sub-clusters in cDC2 cluster re-clustered at resolution (0.1) using Seurat. (B) Top DEGs based on fold change and enriched pathways from GSEA between sub-clusters of cDC2 cluster; cDC2 = Type 2 classical dendritic cells. DEGs

In dendritic cells, the pathway enrichment score, calculated using ssGSEA, for IL6-JAK-STAT3 signaling pathway rises over time (**Figure 3.11**). This is interesting given that IL-6 levels in our culture system are lower at later time periods compared to early stages. One explanation could be that the higher enrichment score is not through increased IL-6 signaling at the top but could be via enhanced STAT3 signaling through alternate pathways that converge upon STAT3. IL6 mediated STAT3 activation has been previously linked to impaired maturation of LPS activated dendritic cells and MHC II expression on dendritic cells in the mouse (Park *et al.*, 2004; Kitamura *et al.*, 2005). A significant decrease in enrichment score for PI3K-AKT-mTOR signaling over time in cells of the cDC2 cluster was also observed. Inhibition of this pathway in mice leads to attenuation of dendritic cell maturation (Li *et al.*, 2020). The role of IL6-JAK-STAT3 and PI3K-AKT-mTOR signaling pathways in dendritic cell maturation in humans has not been well explored till now but could be potentially important pathways to investigate.

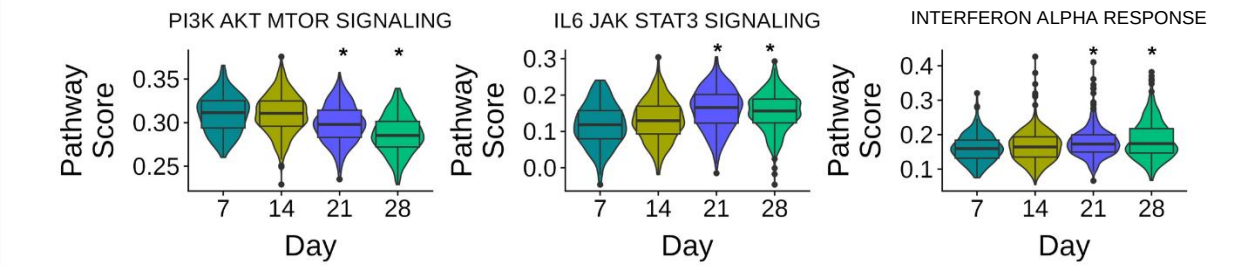

Figure 3.11 Select differentially enriched pathways in cells of the cDC2 cluster in the Vehicle group. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p*-value < 0.05) differences in pathway enrichment scores compared to days 7 and 14 are denoted by *.

3.1.3 AHR activation by TCDD alters the development of several hematopoietic lineages

In the presence of TCDD, we observed multiple changes in the hematopoietic landscape. A time and treatment-dependent split of cell clusters (scRNA-seq data) demonstrated the chronological development of different cell types and their modulation by TCDD (**Figures 3.12A and 3.12B**). With TCDD treatment, on day 7, the largest change is seen in the Megakaryocyte-Erythroid cluster with fewer cells being present in this cluster in the TCDD-treated group, relative to the vehicle group. Concomitantly, we see an increase in the proportion of cells in the ProMonocyte and Early Monocyte clusters. This trend continues to day 14. Cells belonging to the Lymphoid and plasmacytoid dendritic cell (pDC) clusters emerge on day 14 and a reduced proportion of cells belonging to these clusters is observed in the TCDD group. This was suggestive of a skewing of cells towards the monocyte lineage at the expense of other lineages due to TCDD treatment and is in agreement with previous findings reported in the mouse (Singh *et al.*, 2009). By day 21, ProMonocyte and Early Monocyte clusters give rise to ProMonocyte2, Late Monocyte and Macrophage clusters. In contrast to the vehicle group, cells belonging to the Early Monocyte cluster are still observed in the TCDD-treated group, suggesting that TCDD may delay maturation of the earlier monocytes. A substantial number of Early-B cell cluster cells appear by day 21 and progressively increase in number as seen on day 28 in the vehicle group, which is almost nonexistent in the TCDD group in agreement with our previous study (Li *et al.*, 2017a). A similar decrease is also observed for the pDC cluster with TCDD.

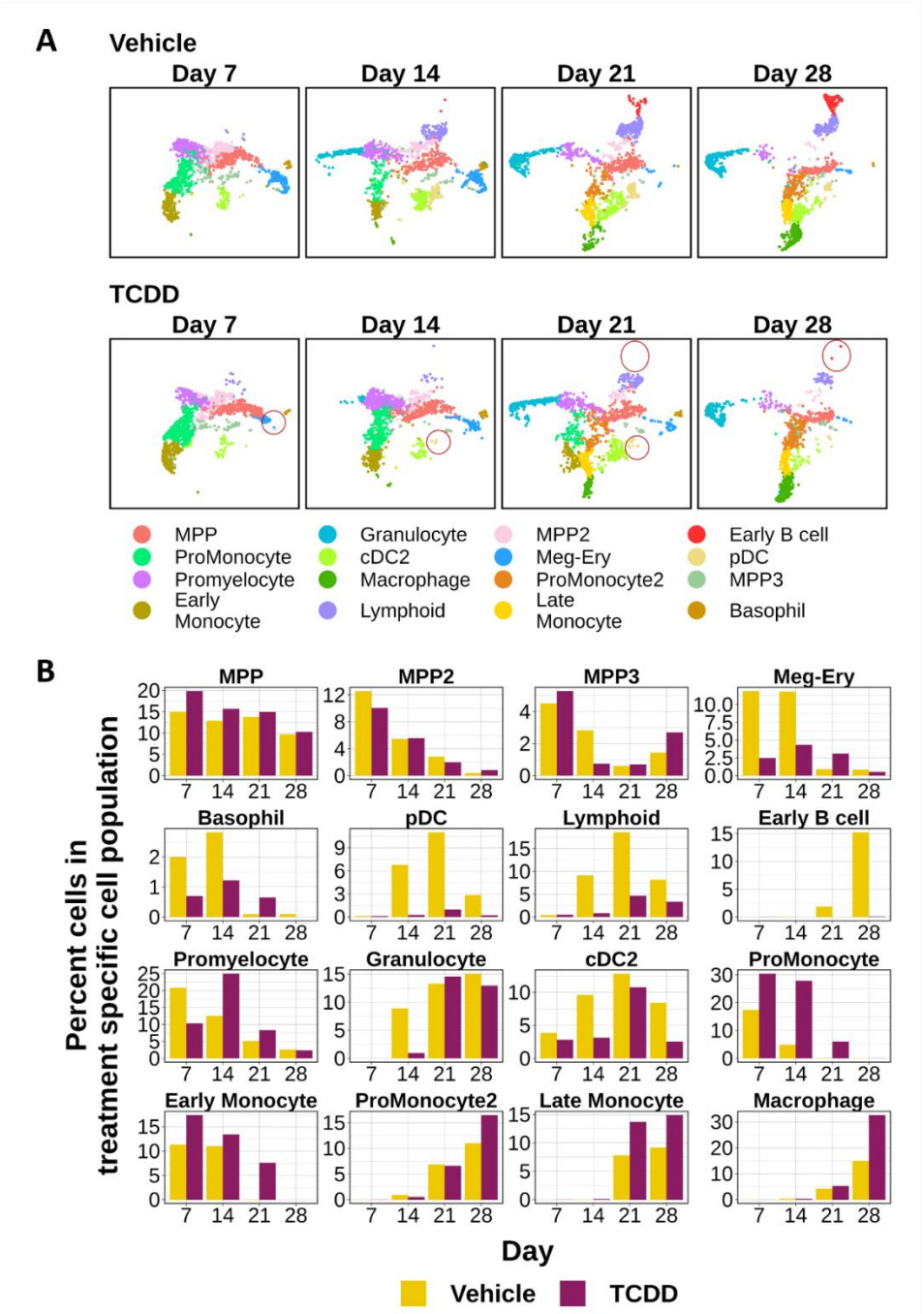

Figure 3.12 (A) Changes in cell clusters from Vehicle and TCDD groups across the 28-day developmental period. Some changes are highlighted in red circles. (B) Percent of cells belonging to each scRNA-seq data associated cell cluster for Vehicle and TCDD-treated groups across the 28-day developmental period.

Prior studies by us with TCDD had demonstrated that reduction in cell number in the presence of TCDD was not attributable to cell death by apoptosis or necroptosis (Li *et al.*, 2017b). Similarly, in our studies, we did not observe any significant difference in cell viability between cells of the vehicle and TCDD groups (**Figure 3.13)**.

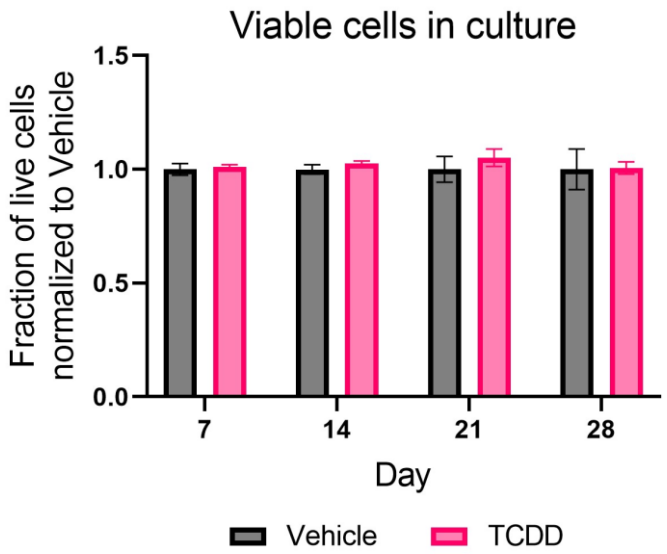

Figure 3.13 Fraction of live cells normalized to the average of percent live cells in the Vehicle groups across different samples for each day.

We next verified the changes in cell population induced by AHR activation with TCDD as observed from scRNA-seq analysis using flow cytometry. With antibodies directed against cell markers expressed on the major hematopoietic cell populations, changes in the number of different cell types with TCDD treatment were tracked over 28 days. The percent of $CD66b^{+}CD14^{-}$ cells, representing granulocytes, and $CD14^{+}$ cells (monocytes/macrophages) were significantly greater in the TCDD treatment group relative to the vehicle group on days 14, 21 and 28 of the study (**Figures 3.14A and 3.14B**). Cells populations were identified by the gating strategy shown in **Figures 3.18** and **3.19**.

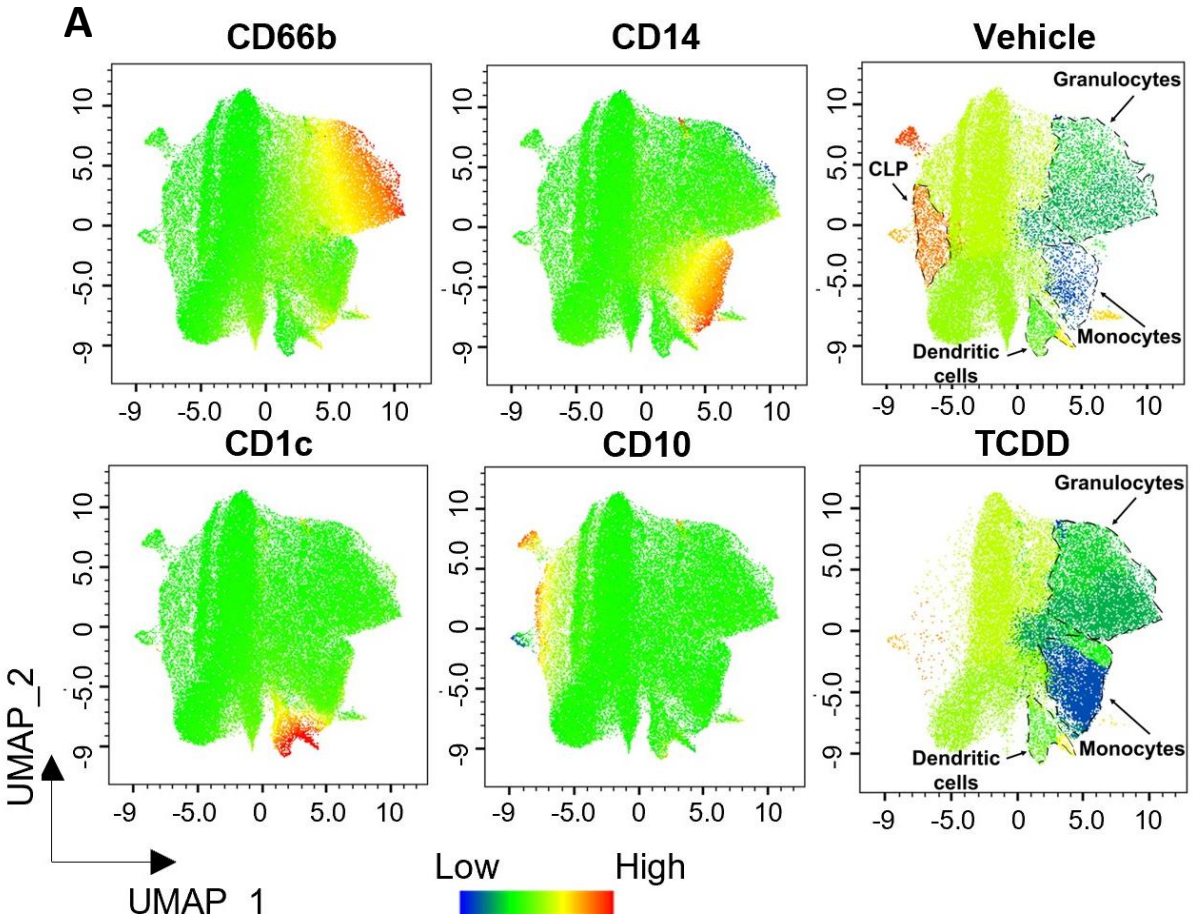

Figure 3.14 (A) Representative UMAP plots of cells captured by flow cytometry on day 21, with major cell populations identified through expression of different lineage markers. Comparison of UMAP plots of an equal number of cells (30,000) from both Vehicle and TCDD-treated groups; CLP = common lymphoid progenitor.

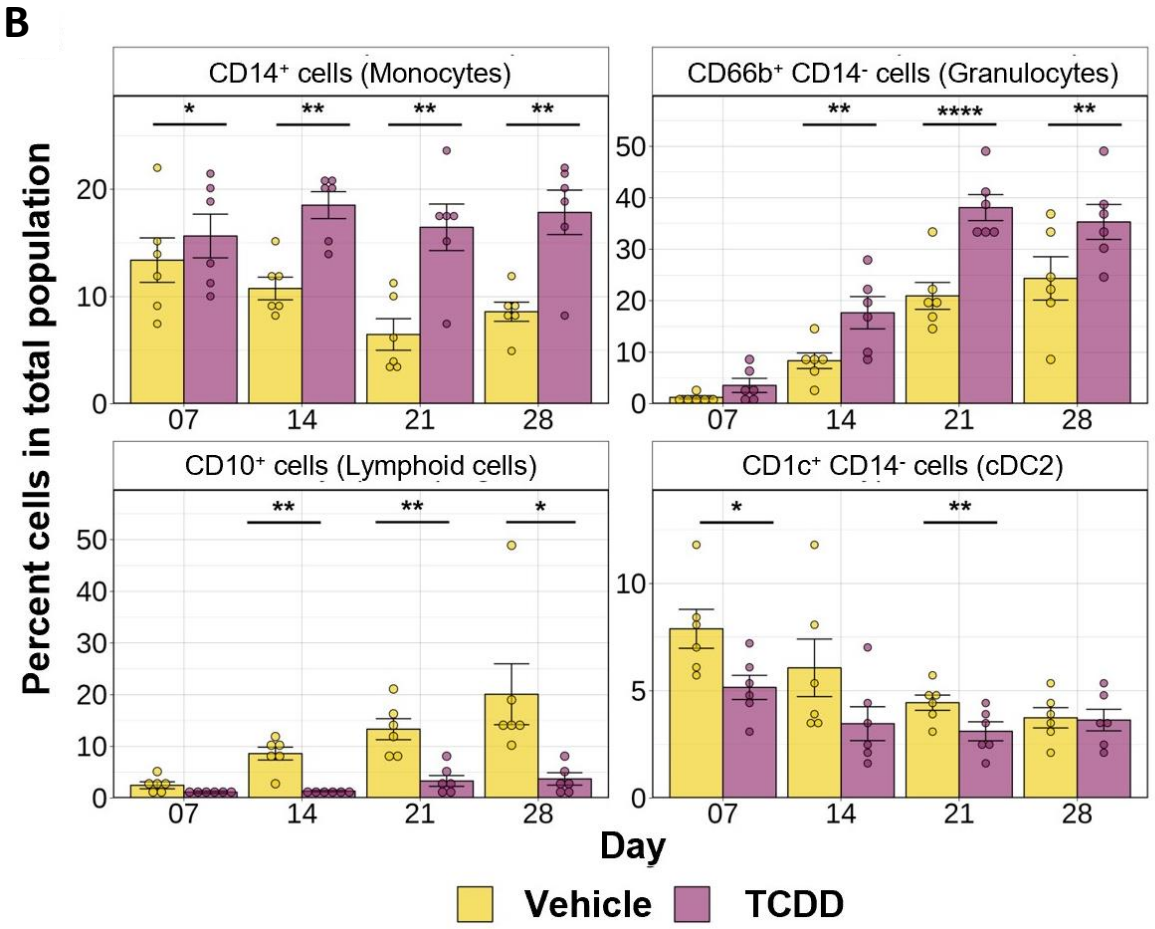

Figure 3.14 (B) Percent of cells belonging to major hematopoietic lineages captured using flow cytometry for Vehicle and TCDD-treated groups across the 28-day developmental period. cDC2 = Type 2 classical dendritic cells. Data presented is composite of 6 separate experiments. Statistical significance of differences in percentage of cells between treatments at any time point was calculated using a two-tailed paired t-test. * *p* value < 0.05, ** *p* value < 0.01, **** *p* value < 0.0001.

As eosinophils and basophils constitute a minor proportion of hematopoietic populations physiologically as well as in our system as inferred from scRNA-seq analysis, we assumed that $CD66b^{+}CD14^{-}$ cells largely represented the neutrophil population. $CD10^{+}$ cells represent the lymphoid population which can give rise to B, T and NK cell populations. The number of $CD10^{+}$ cells decreased significantly with TCDD treatment. There was a significant trend towards a decrease in $CD1c^{+}$ $CD14^{-}$ cells (Type 2 classical dendritic cells) across most days upon TCDD treatment.

TCDD is known to exert its toxic effects primarily through activation of AHR (Mimura *et al.*, 2003). To determine whether the effects on the hematopoietic profile that were observed with TCDD were mediated through the AHR, we treated HSPCs with TCDD in presence of an AHR antagonist CH223191 (**Figure 3.15**). CH223191 was initially developed to antagonize the binding of TCDD to AHR and its translocation to the nucleus, and its structure-activity relationship regarding AHR activity has been extensively investigated (Kim *et al.*, 2006; Choi *et al.*, 2012). A treatment group where cells were treated with CH223191 alone was also included. Figure 3.15 shows that the aberrant hematopoietic profile induced with TCDD treatment was abrogated in presence of the AHR antagonist, demonstrating complete antagonism of TCDD. In fact, HSPCs treated with TCDD and CH223191 had lower monocyte and granulocyte development than the vehicle group, which suggested that the antagonist not only antagonized TCDD mediated AHR activation but also putative endogenous AHR activation. HSPCs that differentiated in presence of the AHR antagonist alone demonstrated a greater skewed development along the lymphoid lineage compared to the vehicle group, suggesting again antagonism of endogenous AHR activity. These experiments demonstrated that TCDD-mediated effects on HSPCs were through AHR activation.

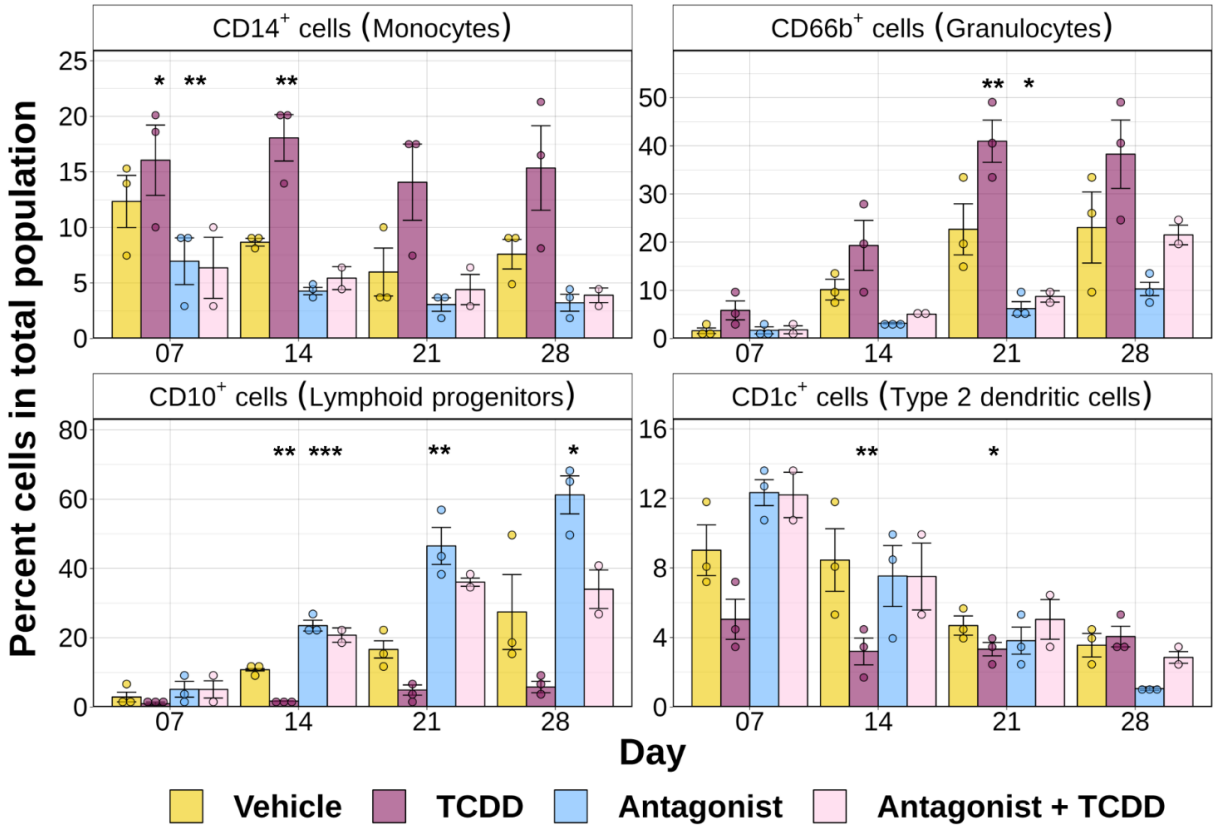

Figure 3.15 Percent of cells belonging to major hematopoietic lineages captured using flow cytometry for 4 treatment groups (Vehicle, TCDD, AHR antagonist CH223191 only, and both AHR antagonist and TCDD) groups across the 28-day developmental period. Data presented is composite of 3 separate experiments (except Antagonist + TCDD group; n = 2). Error bars show mean ± SEM. Statistical significance of differences in percentage of cells of treatments at any time point against the control Vehicle group was calculated using a repeated measures one-way ANOVA with Dunnett's multiple comparisons test. Statistical tests were not carried out for the Antagonist + TCDD group (n = 2). * *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001.

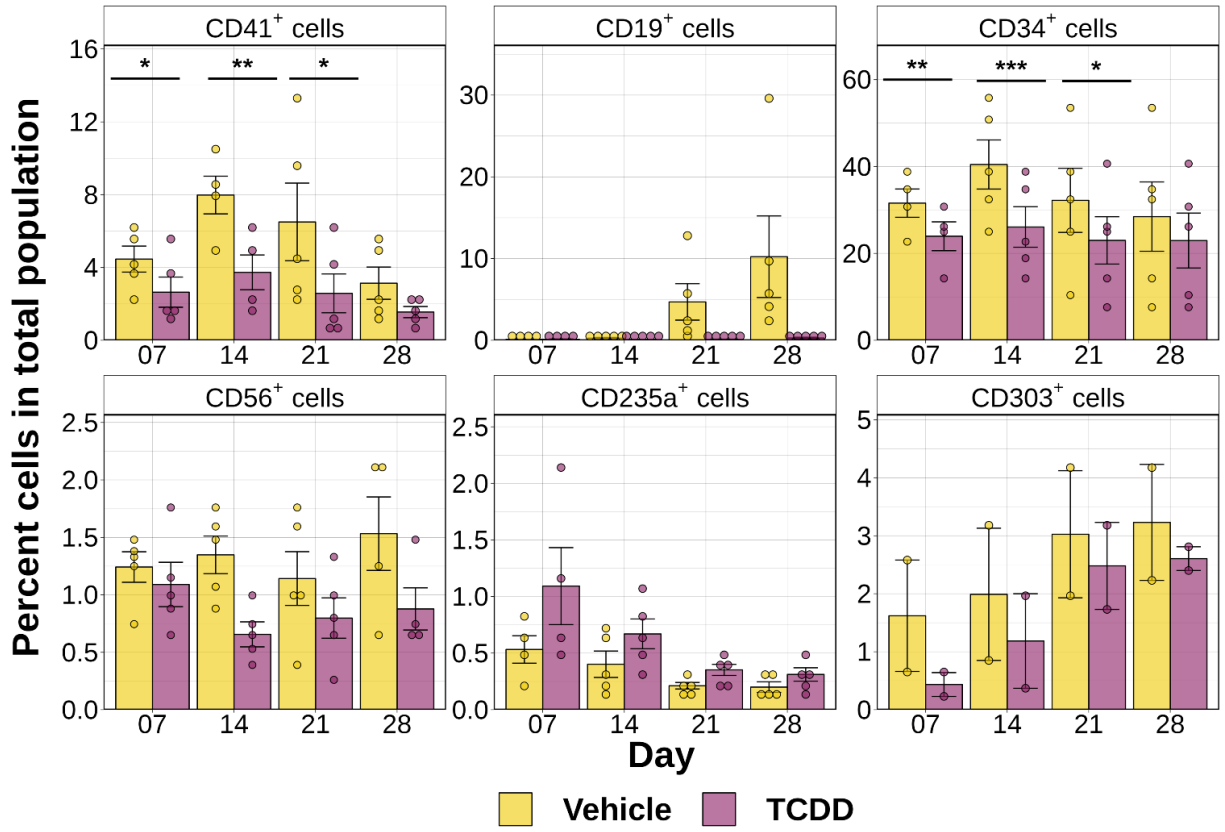

Figure 3.16 Percent of cells belonging to different hematopoietic lineages captured using flow cytometry for Vehicle and TCDD-treated groups across the 28-day developmental period. Data presented is composed of 5 separate experiments (excepting CD303; n = 2). Error bars show mean ± SEM. Statistical significance of differences in percentage of cells between treatments at any time point was calculated using a two-tailed paired t-test where appropriate. * *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001.

We used other cell markers for detecting MPPs (CD34), B cells (CD19), megakaryocytic cells (CD41), erythroid cells, (CD235a) and pDCs (CD303). Like our previous studies (Li *et al.*, 2017b), we observed strong reductions in $CD19^+$ cells and $CD34^+$ cells with TCDD treatment (**Figure 3.16**). AHR activation also resulted in a significant decrease in megakaryocyte populations (**Figure 3.16**). CD235a is a specific marker for erythroid committed cells. A trend towards a slight increase in $CD235a^+$ cells could be detected in the TCDD group. pDC population development seemed to be highly HSPC lot-specific and could not always be reliably detected. However, there was a trend towards a

decrease in CD303^{+} cells in two independent experiments (**Figure 3.16**), consistent with findings from the scRNA-seq analysis (**Figure 3.12B**).

In summary, AHR activation in HSPCs led to an increase in monocytes and granulocytes and a decrease in lymphoid, dendritic and megakaryocyte populations.

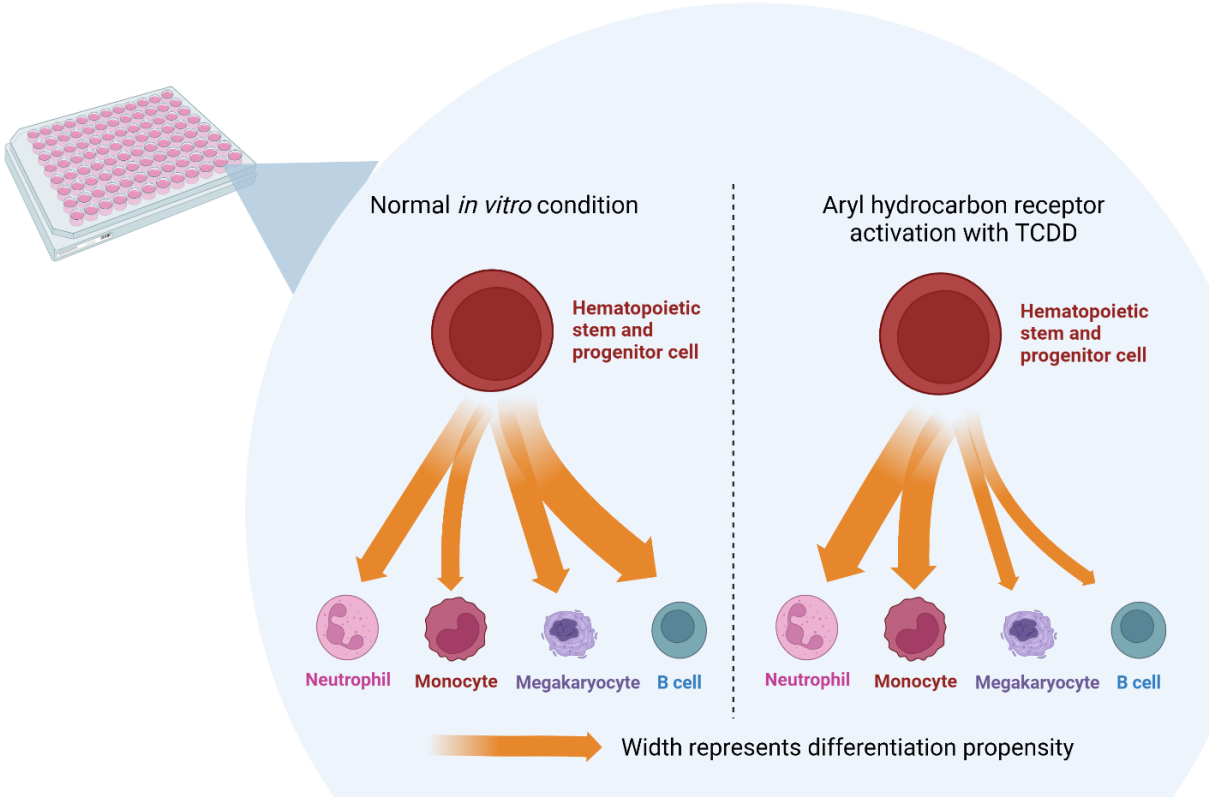

Figure 3.17 Summary of the effect of TCDD on *in vitro* differentiation of human HSPCs into different hematopoietic lineages.

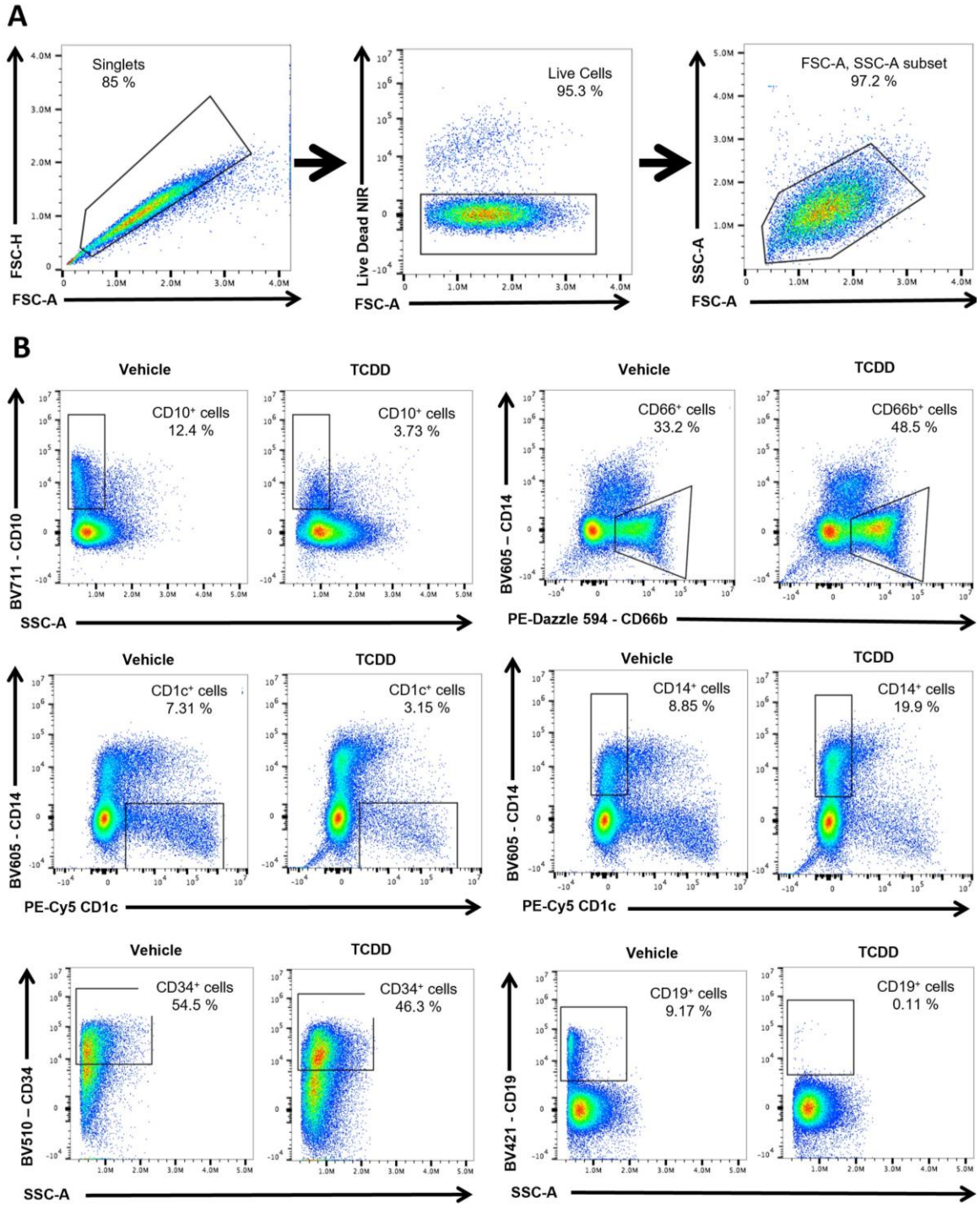

Figure 3.18 (A) Gating scheme for selecting cells: gating on singlet population, followed by selection of live cell population and gating on FSC-A, SSC-A population. (B) Representative flow cytometric plots showing the gating and percentage of $CD10^+$ cells, $CD14^+$ cells, $CD1c^+$ cells, $CD14^+$ cells, $CD34^+$ cells and $CD19^+$ cells for Vehicle and TCDD-treated groups.

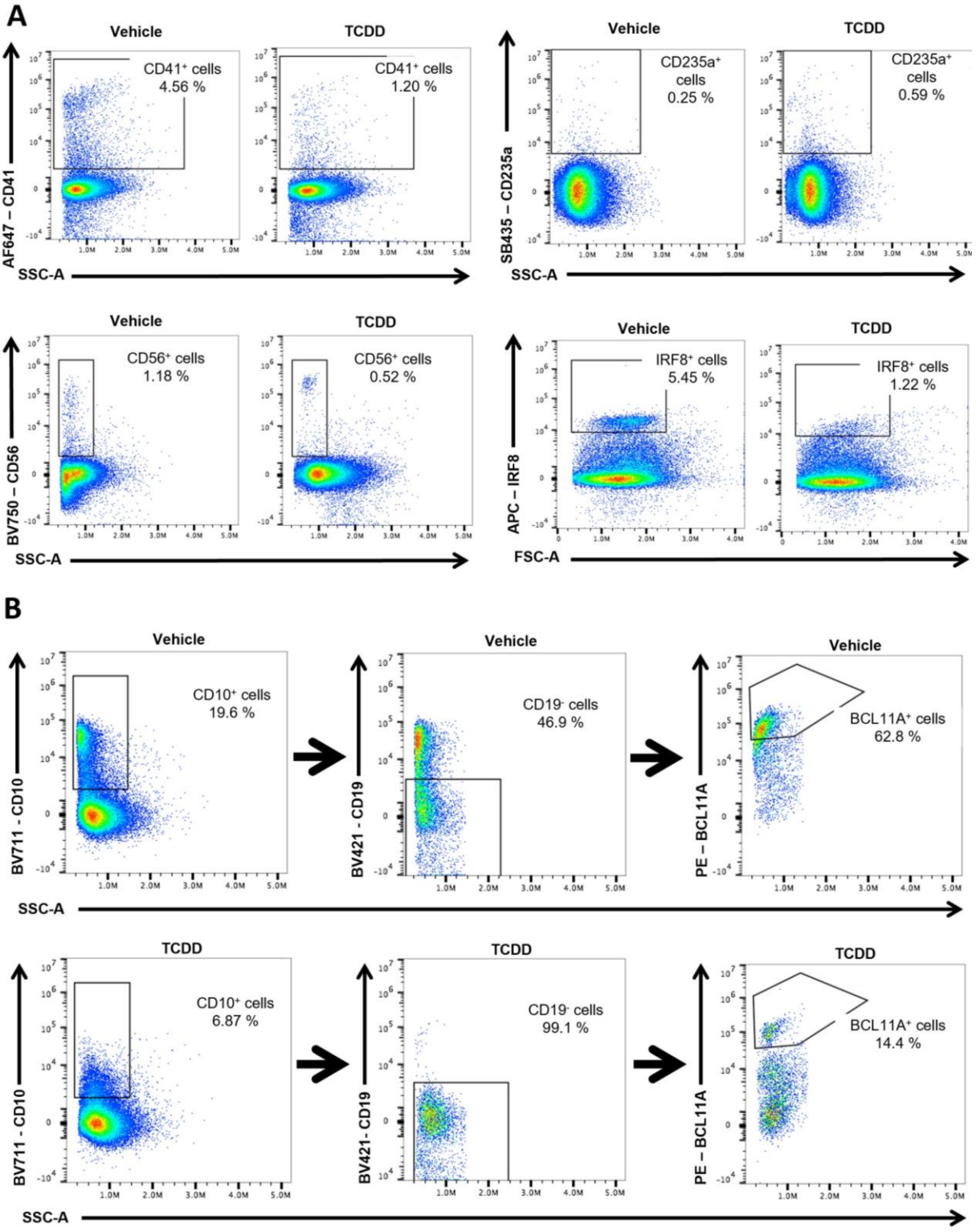

Figure 3.19 (A) Representative flow cytometric plots showing the gating and percentage of $CD41^+$ cells, $CD235a^+$ cells, $CD56^+$ cells and $IRF8^+$ cells for Vehicle and TCDD-treated groups. (B) Representative flow cytometric plots showing the gating strategy for selecting $BCL11A^+$ cells within $CD10^+CD19^-$ cell population.

3.2 Identification of molecular signatures associated with perturbed hematopoietic differentiation by TCDD

The effect of TCDD or AHR activation on the transcriptome of differentiating human HSPCs has not been explored comprehensively in previous research studies. In this chapter, I describe results of the analyses I had conducted to get an understanding of how TCDD may perturb human hematopoiesis toward certain myeloid lineages and impair lymphoid and megakaryocyte lineages. In sub-chapter *3.2.1*, I identified, in certain progenitor cell populations, putative key genes associated with the development of hematopoietic lineages, whose expression was dysregulated by TCDD. I determined whether some of these changes with TCDD were also occurring at the protein level. In sub-chapter *3.2.2,* I investigated how TCDD modulates the transcriptome of different myeloid cell populations that develop in our culture system. Cellular processes and signaling pathways that are putatively altered by TCDD have been identified and described.

3.2.1 TCDD suppresses critical genes and key transcription factors involved in lymphoid and plasmacytoid dendritic cell development

We had observed that TCDD strongly impairs lymphoid and B cell development, and favored development of certain myeloid cell types. Fate choices are more likely to occur in the early hematopoietic progenitor cells than in more downstream progenitors. We investigated changes in the transcriptomic profile of these progenitor cells in presence of TCDD, so that we could identify the early drivers of lineage regulation that may be perturbed by TCDD.

Apart from the HSC cluster, MPP cluster is an early forming cluster that is not lineage specified compared to other progenitor clusters. Early gene changes induced by AHR activation with TCDD that lead to persistent alterations in the hematopoietic profile may be gleaned from transcriptomic changes specific to this cluster. Notably, genes *IL1B* and *S100A9* were upregulated by TCDD in the MPP cluster on day 7. *IL1B* and *S100A9* have been associated with skewing of HSCs towards the myeloid lineage in mice (Oduro *et al.*, 2012; Frisch *et al.*, 2019). Higher induction of these genes could be potentially important for early myeloid skewing of human HSPCs in the presence of TCDD. We also focused upon the MPP cluster on day 14 as this was when IL-7 was present in culture to allow lymphoid cell proliferation and when lymphoid lineages were starting to develop. Among the top DEGs on day 14 in the MPP cluster were genes crucial for lymphoid and dendritic lineages such as *BCL11A, MEF2C, and TCF4*. All these genes were downregulated in the TCDD group (**Figure 3.20**). *BCL11A* is important in B cell lineage specification (Liu *et al.*, 2003). *MEF2C*, a transcription factor, is reported to regulate lymphoid vs myeloid cell fate decisions and is important in pre-B cell development in mice (Gekas *et al.*, 2009;

Stehling-Sun *et al.*, 2009). *TCF4* encodes the bHLH transcription factor E2-2, which is indispensable for pDC development (Cisse *et al.*, 2008). Suppression of these genes may explain AHR activation-mediated reduction in lymphoid cells and the pDC cluster.

Effect of TCDD on the transcriptome of lymphoid cells

Next, we focused on the lymphoid lineage to identify genes dysregulated by AHR activation that may further explain the reduced number of cells within Lymphoid and Early B cell clusters with TCDD treatment. We found expression of genes essential to lymphoid lineage and B cell lineage specification (*MME/CD10, EBF1*) to be significantly downregulated along the lymphoid trajectory as well as that of B-cell associated genes that have not previously been linked to early stages of B cell development such as *RASD1* and *FAM129C* (**Figure 3.21**). *RASD1* encodes Dexamethasone-induced Ras-related protein 1, and has very low mRNA expression in B cell deficient mice (Lindsey, 2007). *FAM129C* or B cell novel protein-1 (BCNP1) has recently been reported to be involved in B cell receptor signaling, and important in maturation of B cells in mice (Hong *et al.*, 2020). It may be particularly important in the development of B-1a cells in mice as BCNP1 knockout mice have reduced B-1a cells. Not much is known about the functions of *RASD1* and *FAM129C* in early B cell development. Our results suggest that their expression occurs during early B cell specification in humans. Based on their recently identified regulatory roles in relatively mature B cells, they could be potentially important in lymphoid to B cell transition as well.

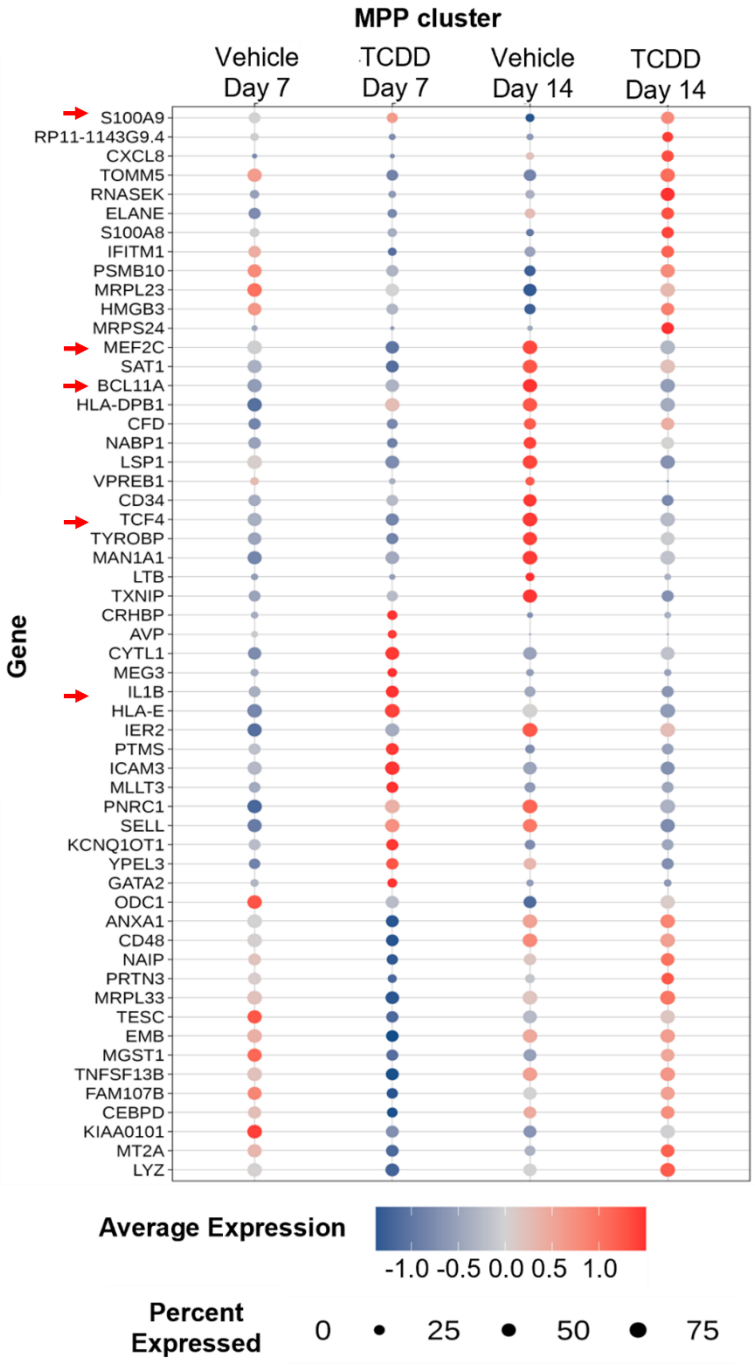

Figure 3.20 Dot plot of the average gene expression of TCDD-treatment induced differentially expressed genes (DEGs) in MPP (Multipotent progenitor) cluster on days 7 and 14. All DEGs are associated with a Bonferroni adjusted *p* value < 0.05. Genes of interest are highlighted with red arrows.

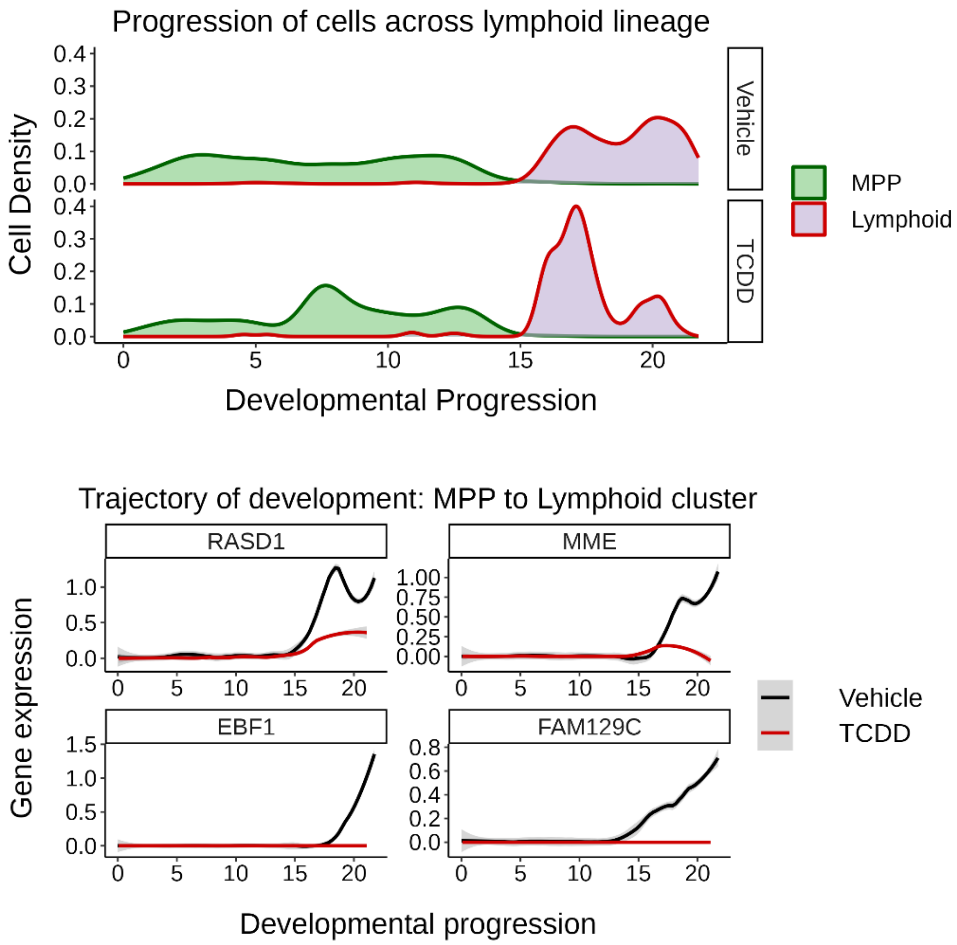

Figure 3.21 Density of cells along MPP to lymphoid cells trajectory and expression of the top four highly variable genes that are differentially expressed by TCDD treatment and are involved in the development of lymphoid cells.

As mentioned above, among the top TCDD-mediated downregulated DEGs in the MPP cell cluster on day 14 was BCL11A, a transcription factor that is known to regulate EBF1 and that is essential for B cell specification of lymphoid progenitors (Liu *et al.*, 2003). Mouse embryos with mutant *Bcl11a* do not develop B cells and fetal liver hematopoietic progenitors show absence of *Ebf1*, *Pax5* and *IL-7Rα* transcripts in *Bcl11a*-/- embryos (Liu *et al.*, 2003), highlighting the importance of *Bcl11a* in B cell development. Flow cytometric analysis confirmed a reduction in the percent of $BCL11A^{+}$ cells in the overall population with TCDD treatment across multiple days (**Figure 3.22A**).

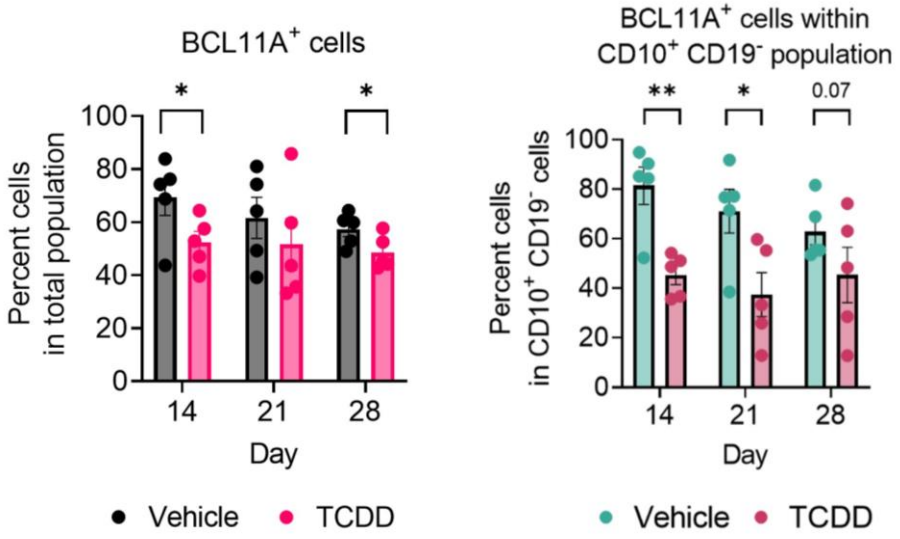

Figure 3.22 (A) Percent BCL11A protein expressing cells within the total population from 5 independent experiments. (B) Percent BCL11A protein expressing cells in $CD10^{+}CD19^{-}$ cells in the Vehicle and TCDD-treated groups across days from 5 independent experiments. (A-B) Protein expression was measured using flow cytometry. Error bars show mean ± SEM. Statistical significance of differences in percentage of cells between treatments at any time point was calculated using a two-tailed paired t-test. * *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001.

There was a significant and greater reduction in $BCL11A^{+}$ cells with AHR activation within the $CD10^{+}CD19^{-}$ cells (common lymphoid progenitors) on days 14 and 21 (**Figure 3.22B**). A decrease in BCL11A expression in lymphoid progenitors due to TCDD could thus contribute to the reduction in Early B cell formation that was seen with TCDD treatment.

Effect of TCDD on cell signaling pathways in lymphoid progenitors

We were also interested in whether any signaling pathways were modulated by TCDD in lymphoid cells, so that we have a better understanding of how TCDD may disrupt lymphoid and B cell specification. Pathways enriched in the TCDD group for cells of the Lymphoid cluster included some pathways such as xenobiotic metabolism, fatty acid metabolism, oxidative phosphorylation, and glycolysis (**Figures 3.23 and 3.24**).

Additionally, lymphoid cells of the TCDD group showed significant enrichment in G2M- and MYC- signaling. Notably, the pathway score for targets downregulated by KRAS signaling (KRAS Signaling DN) is lower in the TCDD group, which suggests that KRAS signaling may be lower in cells of this group. KRAS is a GTPase involved in cell differentiation, proliferation, and survival (Ogawa *et al.*, 2019). It may also have a role in B cell development as a study had shown that selective deletion of *KRAS* in hematopoietic cells hindered the initial stages of B cell development at the pre–B cell phase in the mouse (Chen *et al.*, 2016). Whether a lower score for KRAS signaling DN in the TCDD group is biologically relevant in humans in terms of TCDD mediated impairment of B-cell development requires further studies.

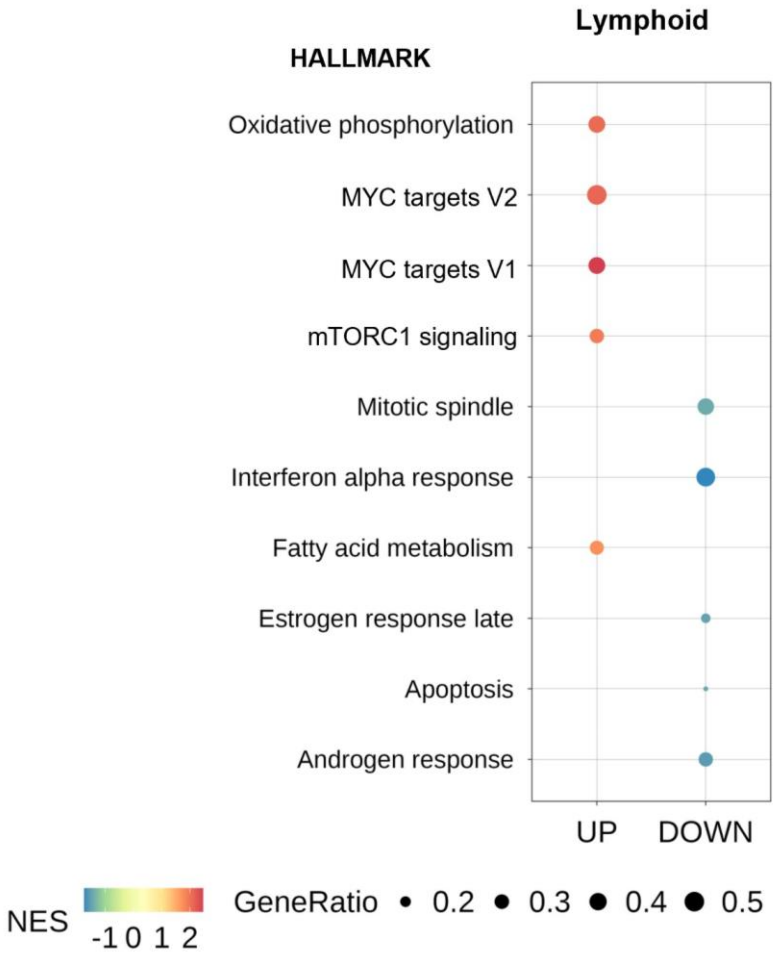

Figure 3.23 Gene set enrichment analysis using the Hallmark pathways database shows the top 10 significantly enriched pathways in cells of the Lymphoid cluster of TCDD group relative to the vehicle group on day 28. All enriched pathways have a Benjamini-Hochberg adjusted *p* value < 0.05. NES = Normalized enrichment score, UP = Upregulated pathways, DOWN = Downregulated pathways.

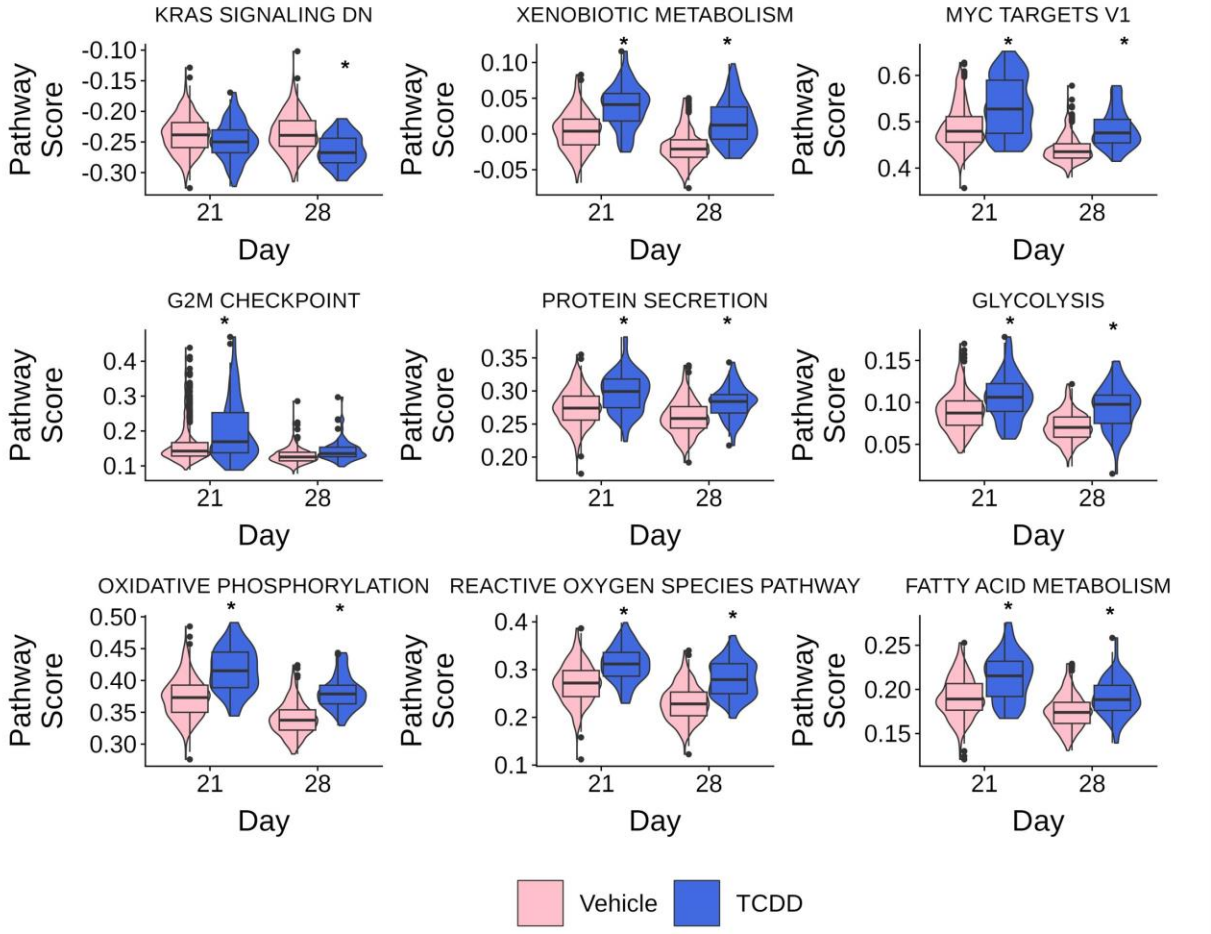

Figure 3.24 Select differentially enriched pathways in cells of the Lymphoid cluster. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p* value < 0.05) differences in pathway enrichment scores between Vehicle and TCDD groups on aligned time-points are denoted by *.

Role of TCDD in regulation of IRF8

We also observed a reduction in transcription factor activity (calculated with SCENIC) of *IRF8* in the pDC cluster for the TCDD group (**Figure 3.25A**). IRF8 plays an important role in the development of several hematopoietic lineages with increased expression being linked to development of lymphoid, pDCs, classical dendritic cells and in macrophage maturation whereas its absence is associated with an increase in granulocytes (Wang *et*

al., 2009). Using flow cytometry, we observed that the percent IRF8 protein expressing cells was also significantly reduced in the TCDD treated group on days 14 and 21 of the study (**Figure 3.25B**). The changes in IRF8 with TCDD treatment are thus consistent with the reduction in $CD10^{+}$ cells and an increase in $CD66b^{+}$ $CD14^{-}$ cells.

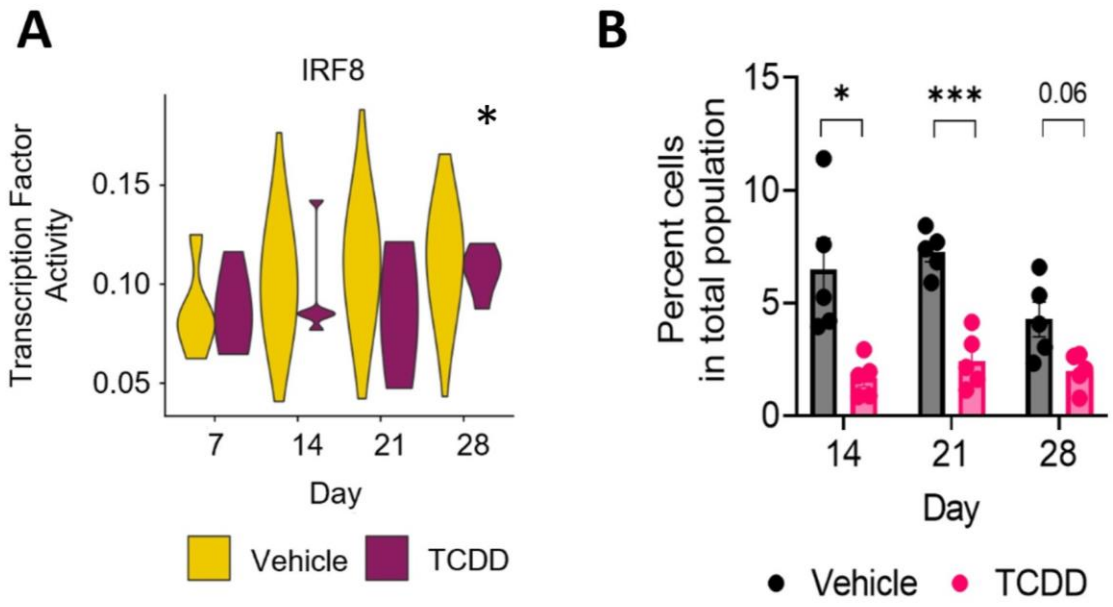

Figure 3.25 (A) Transcription factor activity of IRF8 (analyzed by SCENIC) in pDC (plasmacytoid dendritic cell) cluster. Statistical significance of differences in transcription factor activity between treatments at any time point was calculated using a Wilcoxon rank sum test. * denotes *p* value < 0.05. (B) Percent IRF8 protein expressing cells in overall population measured using flow cytometry. Data presented is composite of 5 separate experiments. Error bars show mean ± SEM. Statistical significance of differences in percentage of cells between treatments at any time point was calculated using a two-tailed paired t-test. * *p* value < 0.05, ** *p* value < 0.01, *** *p* value < 0.001.

Overall, we identified that, in early progenitor cells, AHR activation is associated with a reduction of multiple lymphoid and plasmacytoid dendritic cell associated genes at different time points and specifically demonstrated a reduction in BCL11A and IRF8 at both transcriptomic and protein levels that may contribute to the observed phenomena.

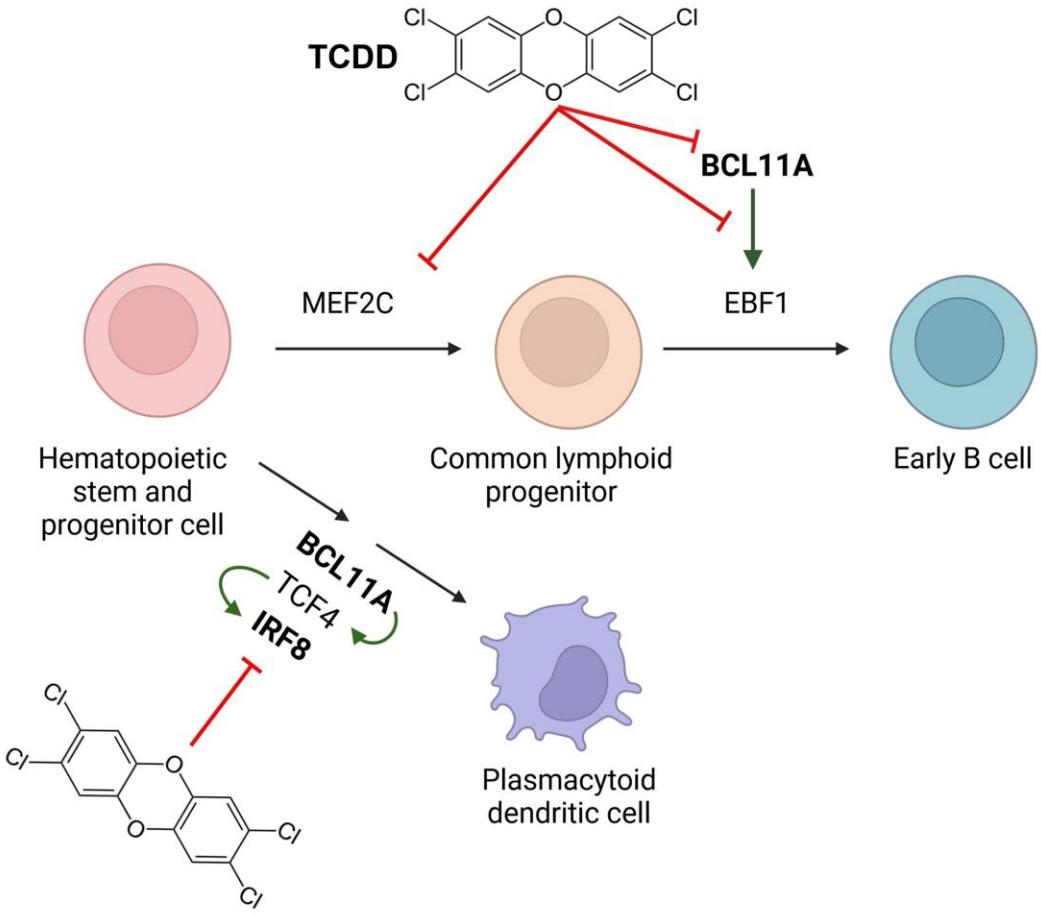

Figure 3.26 Proposed mechanism of repression of lymphoid, B cell and pDC development by TCDD.

3.2.2 Effect of TCDD on the transcriptome of myeloid cell populations

TCDD alters expression of genes associated with lipoprotein metabolism in late-stage monocytes

Along the differentiation trajectory of cells from the MPP cluster towards the Late Monocyte cluster (**Figure 3.27A**), we report an overall greater induction of lipoprotein metabolism associated genes (**Figure 3.27B**).

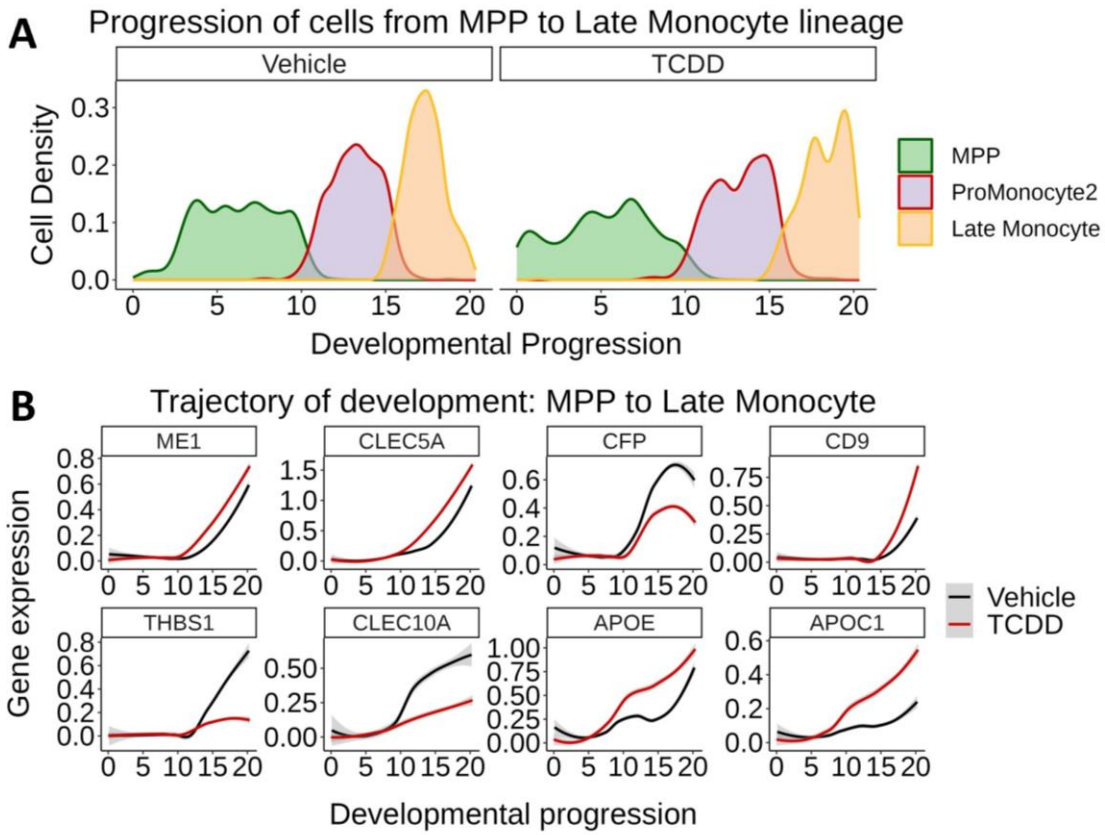

Figure 3.27 (A) Distribution of cells along pseudotime during developmental progression from MPP to Late Monocyte cluster. (B) Expression of select TCDD-treatment induced DEGs along pseudotime during developmental progression from MPP to Late Monocyte cluster. All DEGs are associated with a Bonferroni adjusted *p* value < 0.05.

Genes encoding for apolipoproteins (*APOE, APOC1*) and associated protein (*ME1*) had higher expression levels in the Late Monocyte cluster of TCDD treated cells compared to

those in the vehicle group (**Figure 3.27B**). These apolipoproteins are highly expressed in macrophage-derived foam cells (Fuior *et al.*, 2019). Foam cells are lipid rich and contribute to initiation and pathogenesis of atherosclerosis (Yu *et al.*, 2013). AHR activation has also been linked to foam cell development (Vogel *et al.*, 2004). However, the mechanism by which AHR mediates this is not known. Our findings suggest a potential link between AHR activation and apolipoprotein induction that could explain this phenomenon. In the TCDD group of these cells, cell adhesion associated genes were perturbed. *THBS1* expression was strongly reduced and CD9 expression was expression was higher in Late Monocyte cluster cells that developed in presence of TCDD (**Figure 3.27B**). *THBS1*, which encodes thrombospondin 1, is significantly low in murine macrophage-derived foam cells (Thomas *et al.*, 2015). At the same time, CD9 expression is thought to be important for foam cell formation as their development is partially impaired in $CD9^{-/-}$ macrophages in mice (Huang *et al.*, 2011). These reports are in accord with our findings. Overall, our results strongly suggest, at the transcriptomic level, that TCDD may adversely dysregulate lipoprotein metabolism in human monocytes.

Cells of the macrophage cluster developing in presence of TCDD have a reduced M2 Macrophage signature

Activated macrophages are usually classified as either M1 or M2 macrophages, where M1 macrophages are those macrophages that produce proinflammatory cytokines and M2 macrophages are those that have roles in anti-inflammatory responses (Yunna *et al.*, 2020). Even though there is an increase in proportion of cells of the Macrophage cluster with TCDD treatment (**Figure 3.12B**), cells in this cluster that developed in the presence of TCDD showed reduced expression of several markers that are usually associated with an M2 macrophage phenotype (**Figure 3.28**). These include genes that encode for complement proteins such as *C1QA, C1QC,* M2 macrophage associated marker *MRC1* (encodes CD206), and lectins such as *CLEC10A* (**Figure 3.28**) (Ilarregui *et al.*, 2019; Chen *et al.*, 2021).

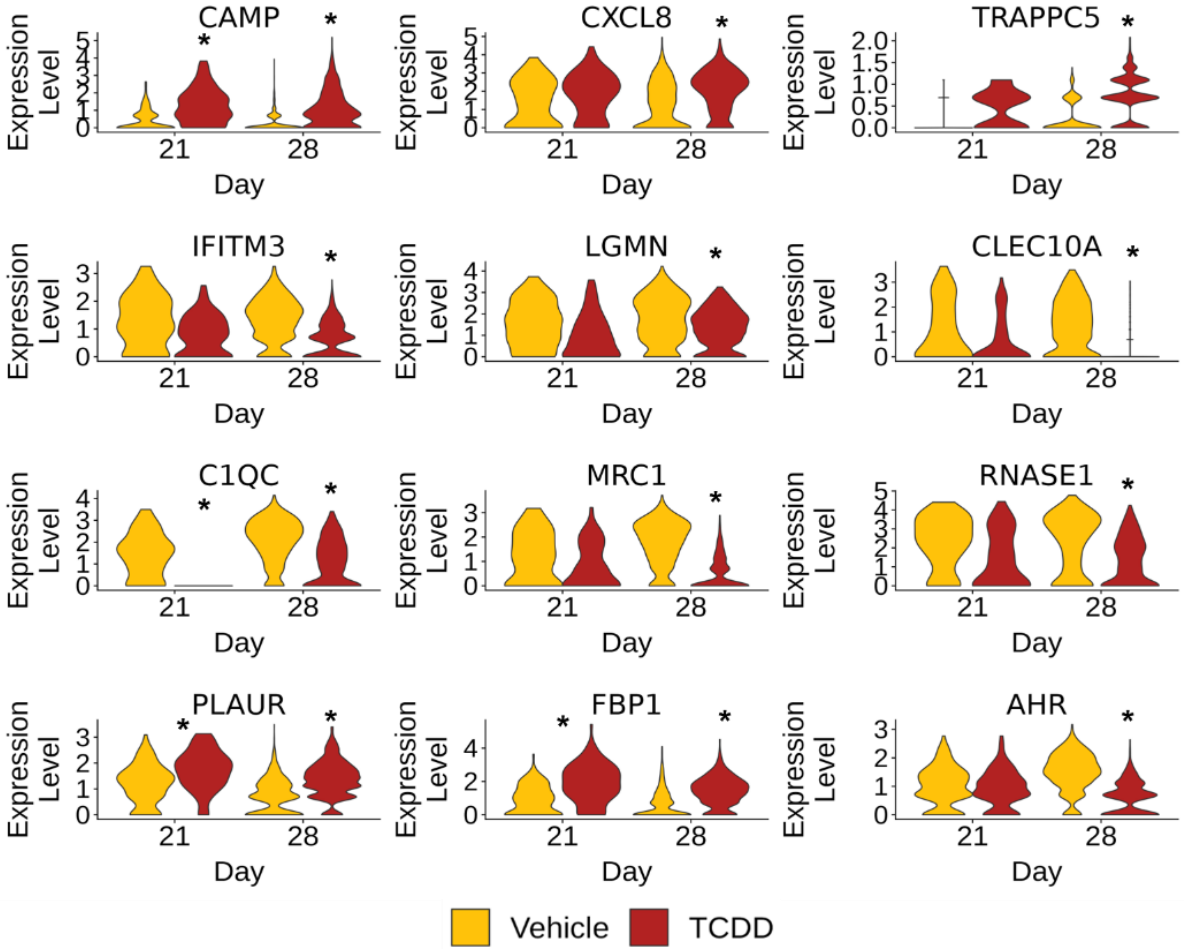

Figure 3.28 Expression of select TCDD-treatment induced differentially expressed genes in Macrophages on days 21 and 28; * denotes Bonferroni adjusted *p* value < 0.05.

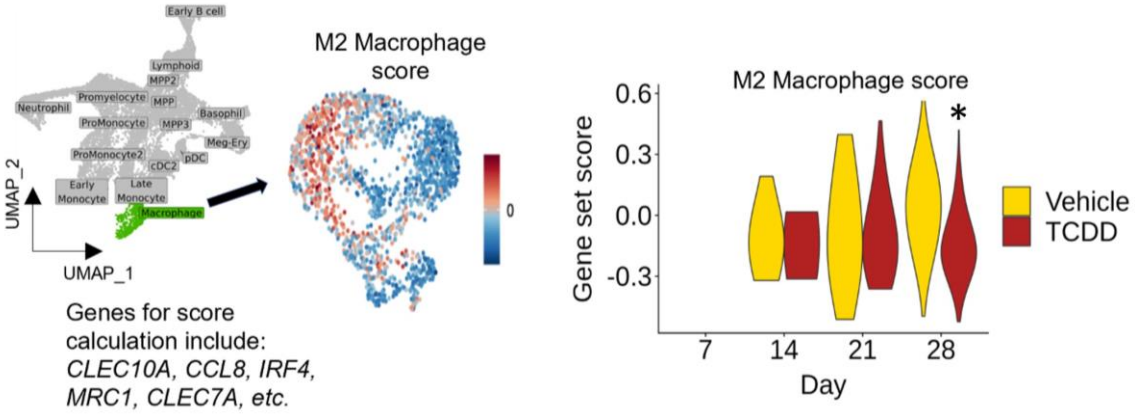

Figure 3.29 Macrophage cluster was selected, and an M2 Macrophage score was calculated based on expression of several genes (see Methods). M2 macrophage score within the Macrophage cluster, and across time for Vehicle and TCDD-treated groups is shown. Statistical significance of differences in M2 macrophage score between treatments at any time point was calculated using a Wilcoxon rank sum test. * denotes *p* value < 0.05.

A decreased M2 macrophage gene set score, calculated based on curated M2 macrophage markers, in macrophages of the TCDD-treated group (**Figure 3.29**) was also consistent with these observations. Upon calculation of transcription factor activity with SCENIC, we observed a reduction in transcription factor activity of *IRF4, MAF*, and *IRF8* among others in cells of the Macrophage cluster (**Figure 3.30**). IRF4 and MAF are known to promote M2 polarization of macrophages, whereas studies in mice have suggested that IRF8 can also play important regulatory roles in macrophage polarization (Kang *et al.*, 2017; Liu *et al.*, 2020). Overall, this suggests that AHR activation might preclude maturation of monocytes into M2 macrophages. Interestingly, a previous study using a macrophage cell line co-cultured with endometrial cells had reported that TCDD in combination with 17β-estradiol drives macrophages towards an M2 phenotype (Wang *et al.*, 2015). Albeit, the effect was not observed with TCDD alone, suggesting this

occurrence may have been a result of possible crosstalk in signaling between AHR and estrogen receptors.

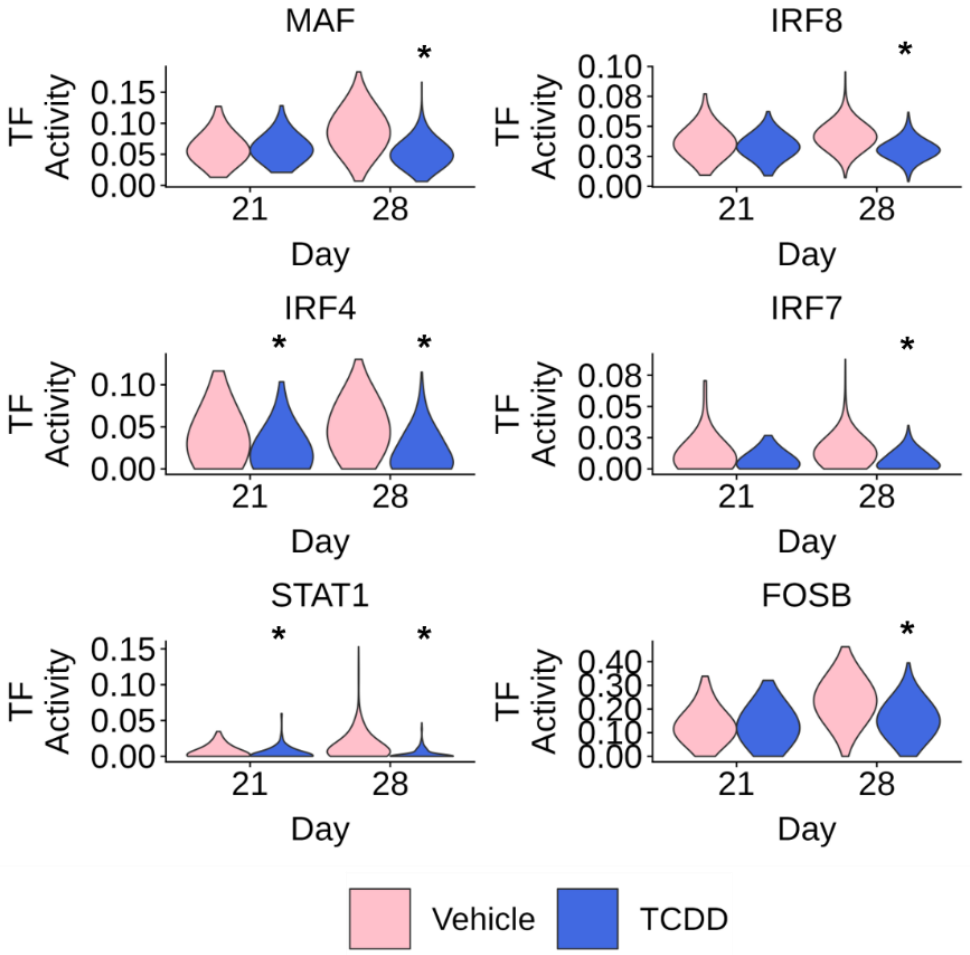

Figure 3.30 Transcription factor activity (calculated with SCENIC) in the Macrophage cluster for selected transcription factors across time is shown. Statistical significance of differences in transcription factor activity between treatments at any time point was calculated using a Wilcoxon rank sum test. * denotes *p* value < 0.05.

Effect of TCDD on cellular processes and signaling pathways in late-stage monocytes and macrophages

Using ssGSEA, we identified that cells of the Late monocyte and Macrophage cluster that developed in presence of TCDD exhibited reduced interferon-α (IFN-α) and interferon-γ (IFN-γ) signatures, mainly on day 28 (**Figures 3.31 and 3.32**). IFN-γ can prime monocytes towards macrophages and polarize them to an inflammatory phenotype (Delneste *et al.*, 2003; Ivashkiv, 2018). Interestingly, previous studies exploring the effect of TCDD on functional responses of mature B cells in mice and humans in response to antigenic stimuli have suggested that TCDD and IFN-γ may have opposing downstream actions and that IFN-γ can reverse TCDD mediated immunosuppression (North *et al.*, 2009; Blevins *et al.*, 2020). Our enrichment analysis is also suggestive of antagonism between IFN-γ signaling and AHR signaling, and shows that TCDD may interfere with downstream signaling mediators of interferons in other human cell types as well.

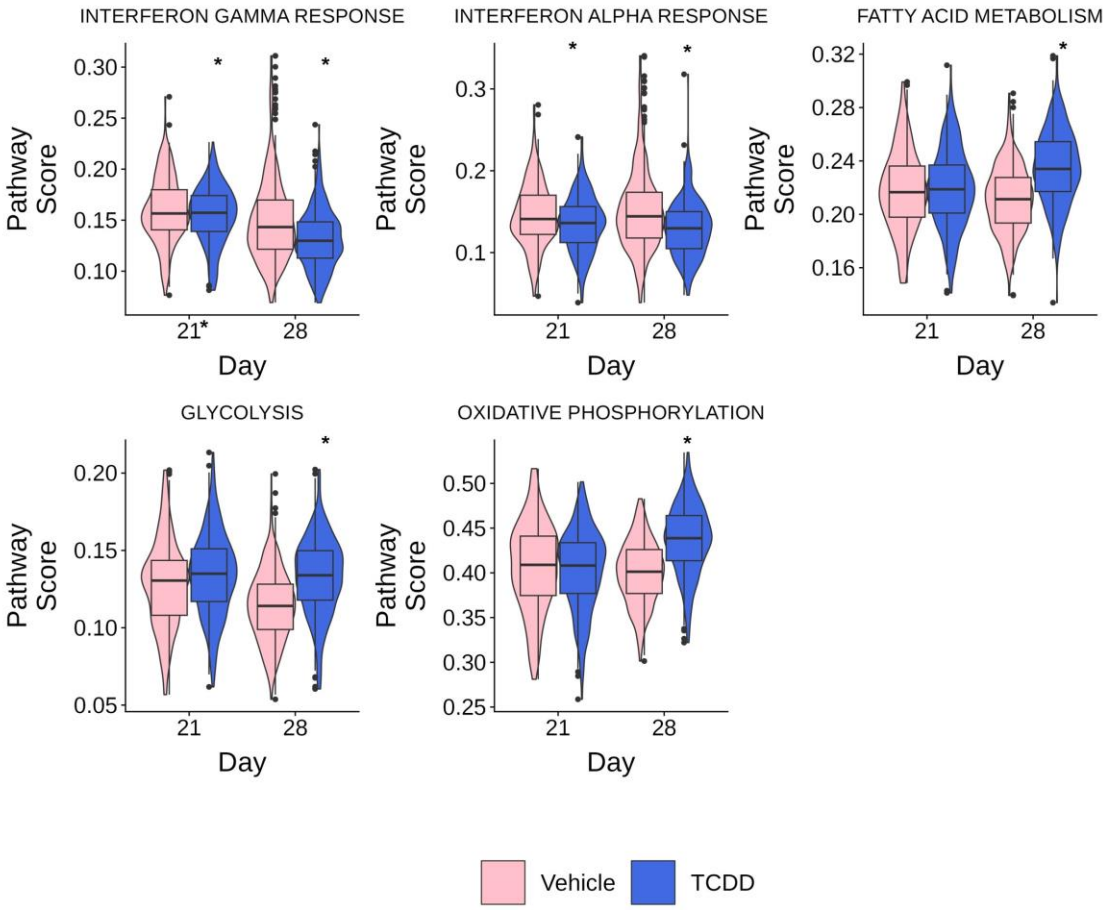

Figure 3.31 Select differentially enriched pathways in cells of the Late Monocyte cluster. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p* value < 0.05) differences in pathway enrichment scores between Vehicle and TCDD groups on aligned time-points are denoted by *.

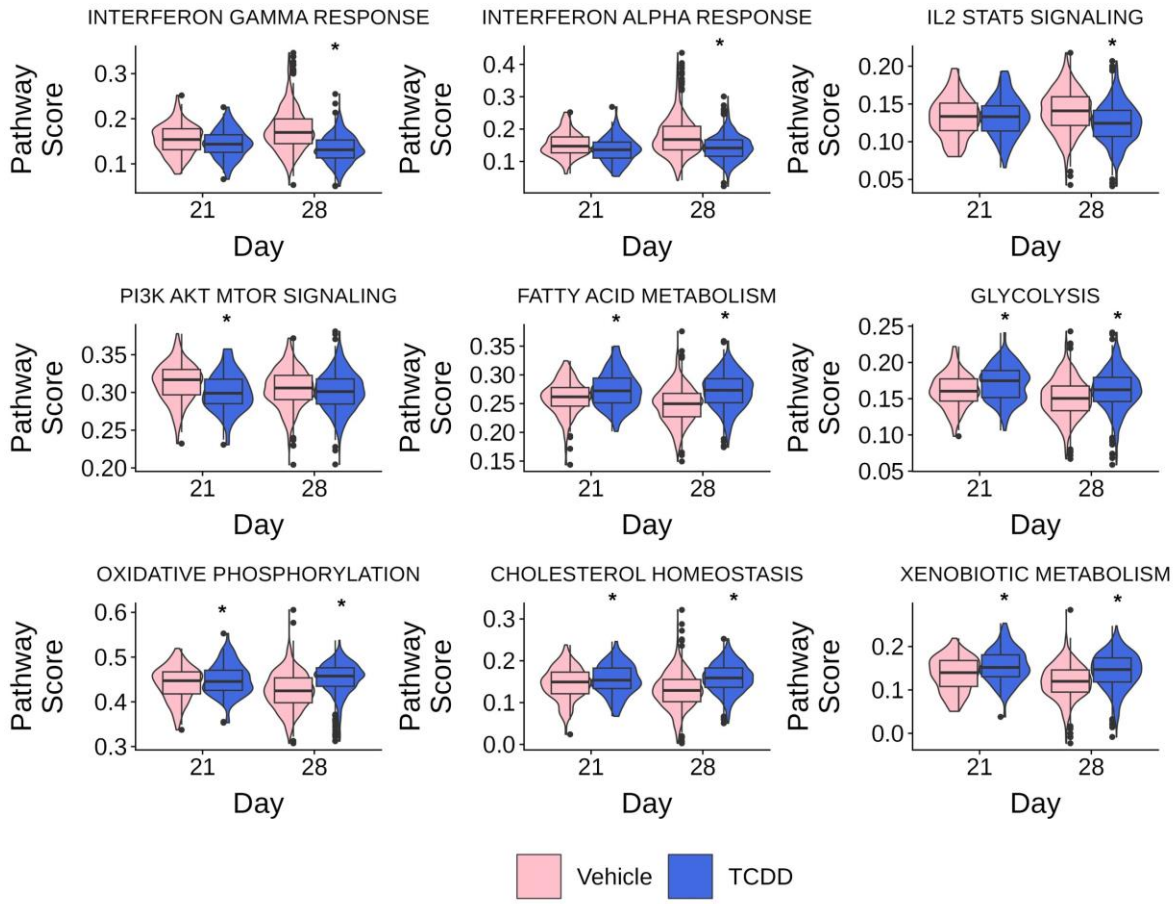

Figure 3.32 Select differentially enriched pathways in cells of the Macrophage cluster. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p* value < 0.05) differences in pathway enrichment scores between Vehicle and TCDD groups on aligned timepoints are denoted by *.

In the presence of TCDD, cells of the Late Monocyte and Macrophage cluster also showed a significant enrichment in pathways associated with fatty acid metabolism, oxidative phosphorylation, and glycolysis on day 28 (**Figures 3.31 and 3.32**). Two genes were significant contributors to the increased enrichment score for fatty acid metabolism. *MIF (Macrophage migration inhibitory factor*) and *G0S2 (G0/G1 switch protein 2*) are involved in fatty acid metabolism and genes encoding these proteins were significantly upregulated in the TCDD group (**Figure 3.33**). Upregulation of *MIF* by TCDD was similar across both clusters (2.5-fold). Increase in *G0S2* expression with TCDD was greater in cells of the Late Monocyte cluster (5.4-fold) than in cells of the Macrophage cluster (3.7-fold). *G0S2* inhibits lipolysis and *G0S2* homozygous knockout mice have reduced fat accumulation in the liver (El-Assaad *et al.*, 2015). *MIF*, a pleiotropic cytokine, is involved in metabolic dysfunction through enhanced adipogenesis in the adipose tissue in humans (Cui *et al.*, 2018). Other notable genes associated with lipid metabolism that were upregulated included *ME1* (*Malic Enzyme*) in the Late Monocyte cluster and *LGALS1* (*Galectin*) in the Macrophage cluster. Both of these genes are involved in lipid retention and obesity in mice (Yang *et al.*, 2011; Al-Dwairi *et al.*, 2012). All in all, this suggests that TCDD dysregulates lipid metabolism in monocytes/macrophages and may contribute to an increase in lipid accumulation in these cells. Thioredoxin, encoded by *TXN*, regulates glycolysis, and was significantly upregulated by TCDD in cells of the Macrophage cluster. TCDD has been associated with dysregulation of glucose homeostasis and altered lipid metabolism in mice (Ishida *et al.*, 2005; Fader *et al.*, 2015). Effects of TCDD on fatty acid metabolism and glycolysis in human primary cell populations are lacking. Our findings

suggest that TCDD deregulates these metabolic pathways in human immune cells as well.

Among the top genes contributing to the higher enrichment score in oxidative phosphorylation score with TCDD across the monocyte/macrophage clusters were genes encoding two isoforms of voltage dependent anion channels, *VDAC1* and *VDAC2*. These genes were significantly upregulated by TCDD on day 28 (**Figure 3.33**). These channels are found on the outer mitochondrial membrane and regulate transport of ATP/ADP, thus playing an important part in oxidative phosphorylation (Shoshan-Barmatz *et al.*, 2017). *VDAC1* is generally more abundantly expressed than *VDAC2*. The effect of TCDD on VDAC2 has previously been reported in 5L rat hepatoma cells with TCDD treatment inducing an upregulation of VDAC2 protein levels (Sarioglu *et al.*, 2008). To our knowledge, this is the first instance that TCDD's effect on *VDAC*s in human immune cells has been reported. TCDD has been reported to increase expression of several mitochondrial genes that are involved in the electron transport chain (ETC) and oxidative phosphorylation in mice (Forgacs *et al.*, 2010). In our study, we identified that TCDD caused significant upregulation across both Late Monocyte and Macrophage clusters of *NDUFB4* that forms complex I of the electron transport chain (ETC) as well as significant increases in expression of other components of the ETC such as *COX7C* in Late Monocyte and *COX6A1*, *COX17, COX7A2L* in the Macrophage cluster (**Figure 3.33**).

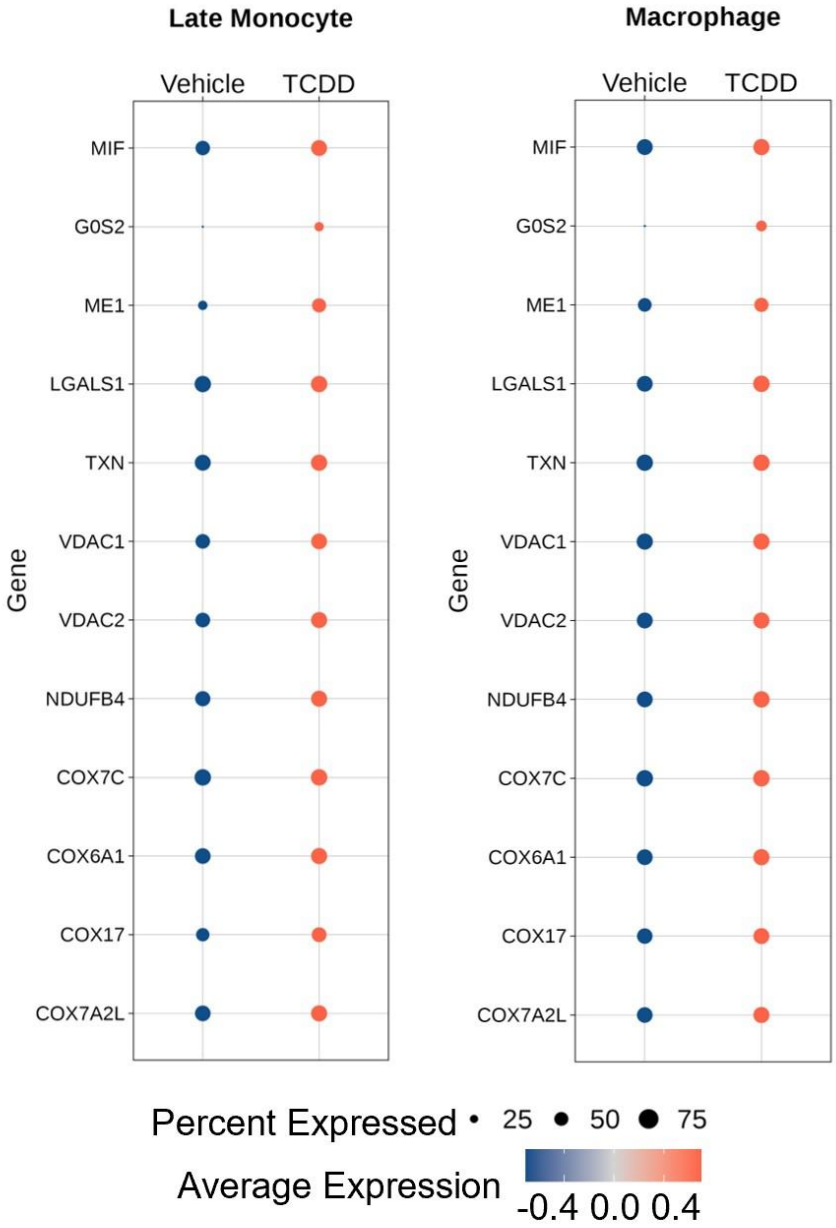

Figure 3.33 Select differentially expressed genes in cells of Late Monocyte and Macrophage clusters on day 28. All genes are associated with a Bonferroni adjusted *p* value < 0.05 unless otherwise mentioned in text.

Effect of TCDD on developing granulocytes

Granulocytes that develop in presence of TCDD show significant enrichment for multiple signaling pathways on day 28 that included IL6-JAK-STAT3 signaling, IL2-STAT5 signaling, TGF-β signaling and TNF-α signaling via NF-κβ (**Figures 3.34 and 3.35**). Enrichment of TNF-α/NF-κβ signaling is often associated with inflammation. Except for TGF-β signaling pathway, the gene CD44 is common in the gene sets for the aforementioned pathways and was significantly upregulated (4.9-fold) in the TCDD group on day 28 (**Figure 3.36**). CD44 is a cell adhesion molecule that is expressed on many cell types including neutrophils and facilitates binding of neutrophils to endothelial cells during inflammation (Pure *et al.*, 2001; McDonald *et al.*, 2015). Congruently, we also identified that granulocytes in the TCDD group possess a higher inflammatory response signature. Overall, it thus appears that granulocytes developing in presence of TCDD may be more inflammatory than those in the control group. Similar to macrophages (**Figure 3.32**), granulocytes of the TCDD group were enriched for oxidative phosphorylation, glycolysis, fatty acid metabolism, and xenobiotic metabolism pathways.

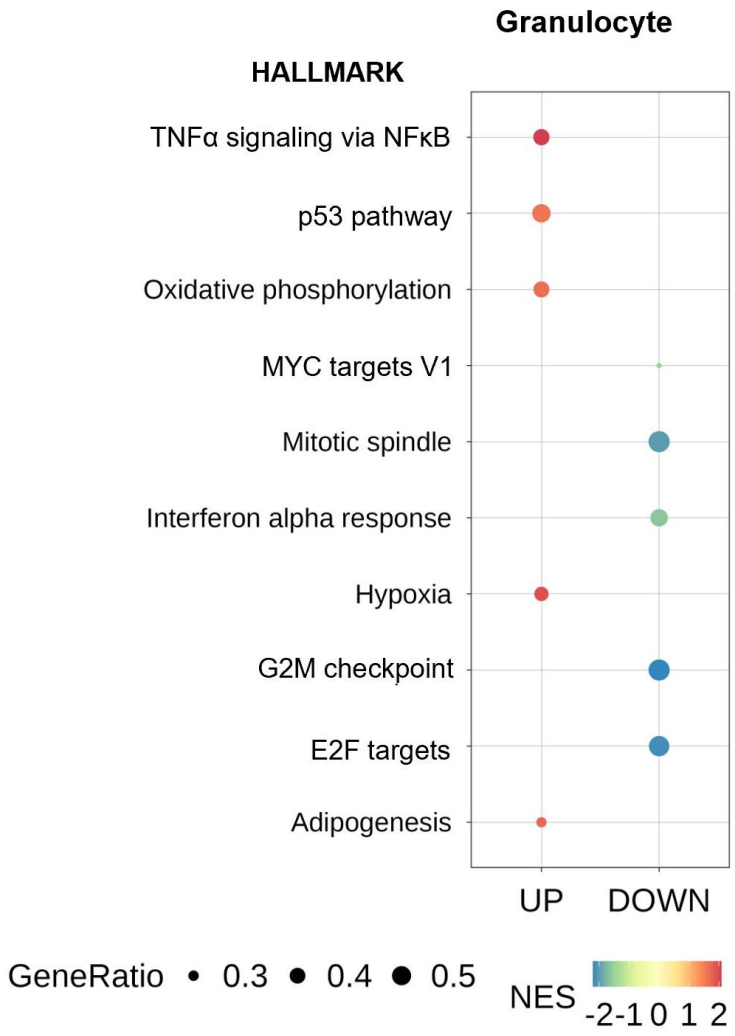

Figure 3.34 Gene set enrichment analysis using the Hallmark pathways database shows top 10 significantly enriched pathways in cells of the Granulocyte cluster of TCDD group relative to the vehicle group at day 28. All enriched pathways have a Benjamini-Hochberg adjusted *p* value < 0.05. NES = Normalized enrichment score; UP = Upregulated pathways, DOWN = Downregulated pathways.

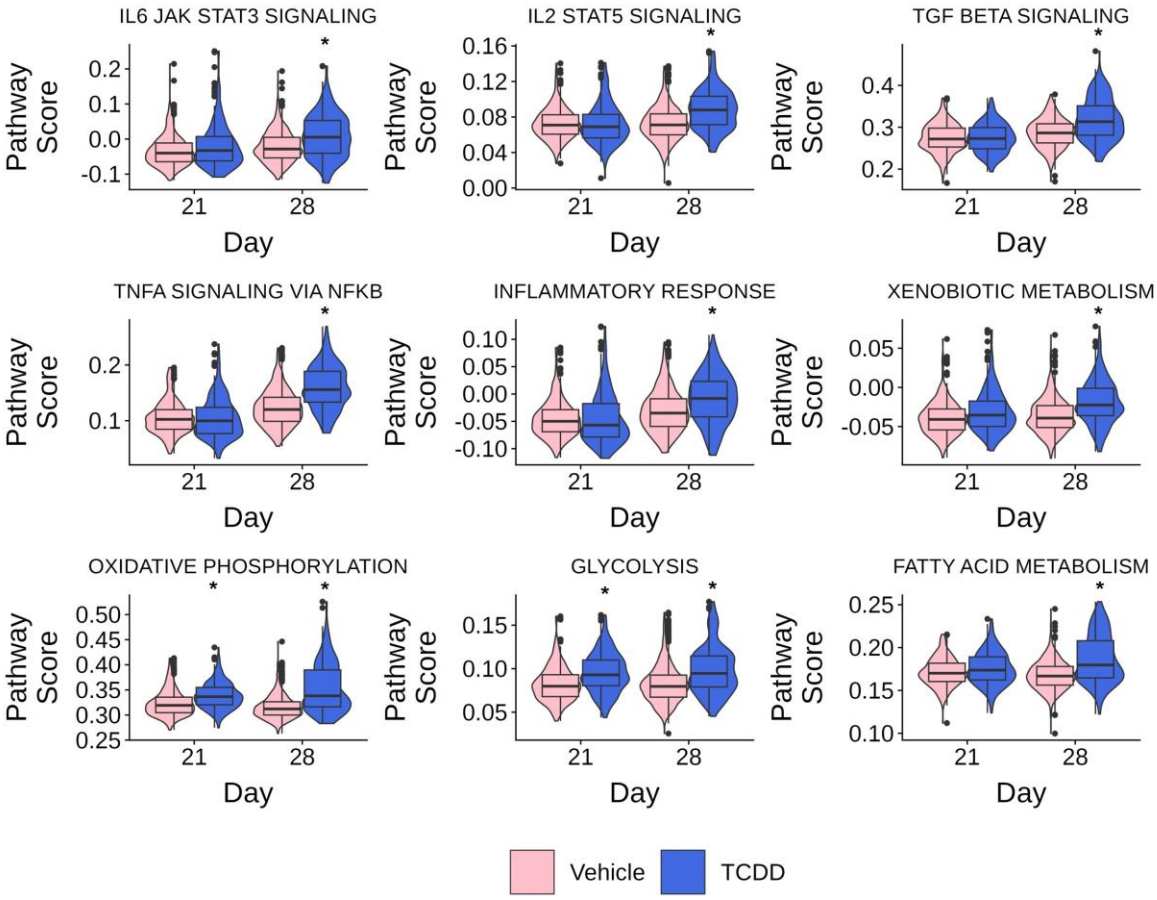

Figure 3.35 Select differentially enriched pathways in cells of the Granulocyte cluster. Pathway analysis was done using ssGSEA. Statistically significant (adjusted *p* value < 0.05) differences in pathway enrichment scores between Vehicle and TCDD groups on aligned timepoints are denoted by *.

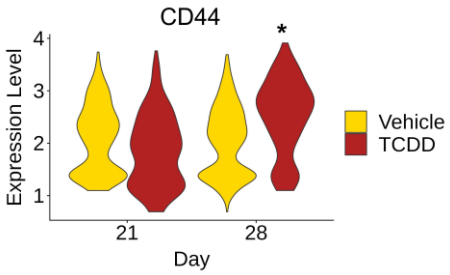

Figure 3.36 Expression of CD44 in granulocyte cluster on days 21 and 28. * denotes Bonferroni adjusted *p* value < 0.05.

TCDD dysregulates the development of classical dendritic cells

The transcriptomic signature of dendritic cells was strongly influenced by AHR activation. TCDD treatment led to a suppression of genes associated with innate immune response (*CCR6, CD37*, etc.) and anti-inflammatory (*METRNL*) responses in cells of the cDC2 cluster (**Figures 3.37 and 3.38**). The cDC2 cluster's cells in the TCDD group also had reduced expression of dendritic cell markers such as *CD1C* and *CD1E* relative to the vehicle group.

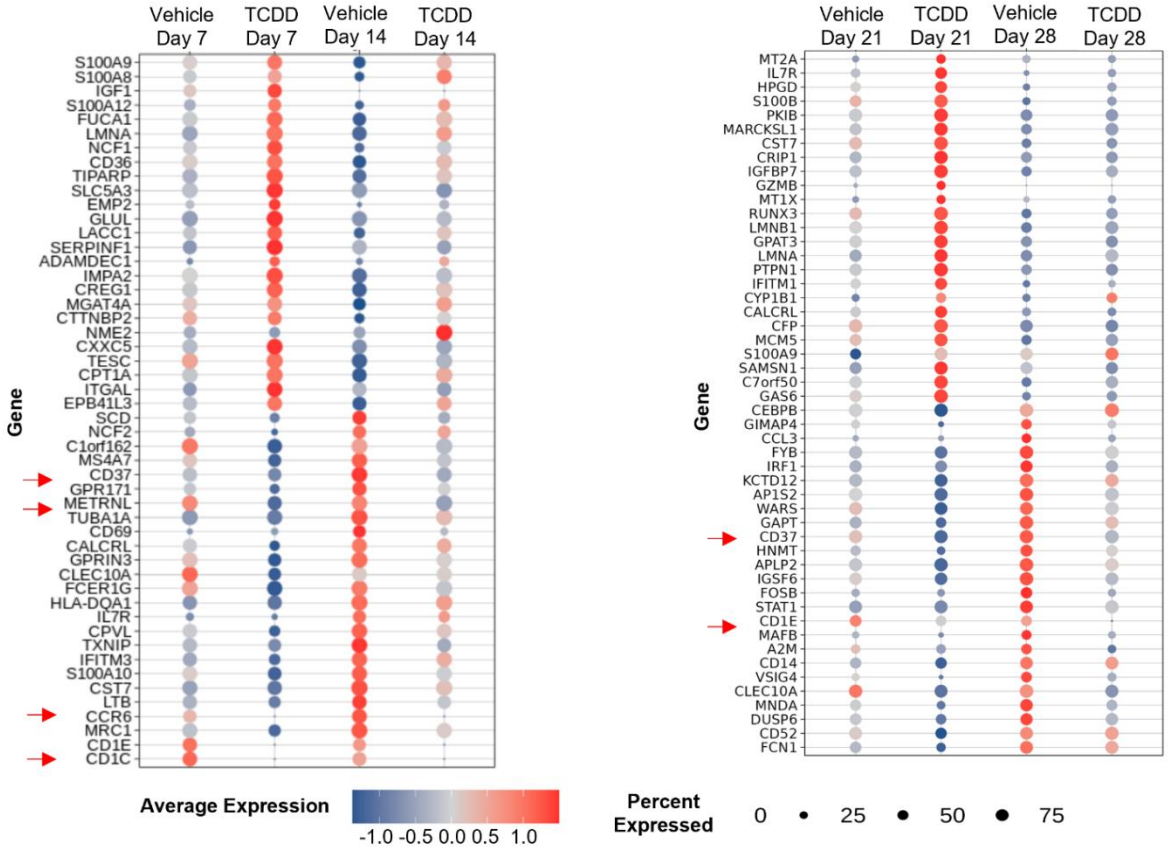

Figure 3.37 Expression of top TCDD-treatment induced DEGs based on fold change in cDC2 (Type 2 dendritic cell) cluster. All DEGs are associated with a Bonferroni adjusted p value < 0.05. Select genes of interest are highlighted with red arrows.

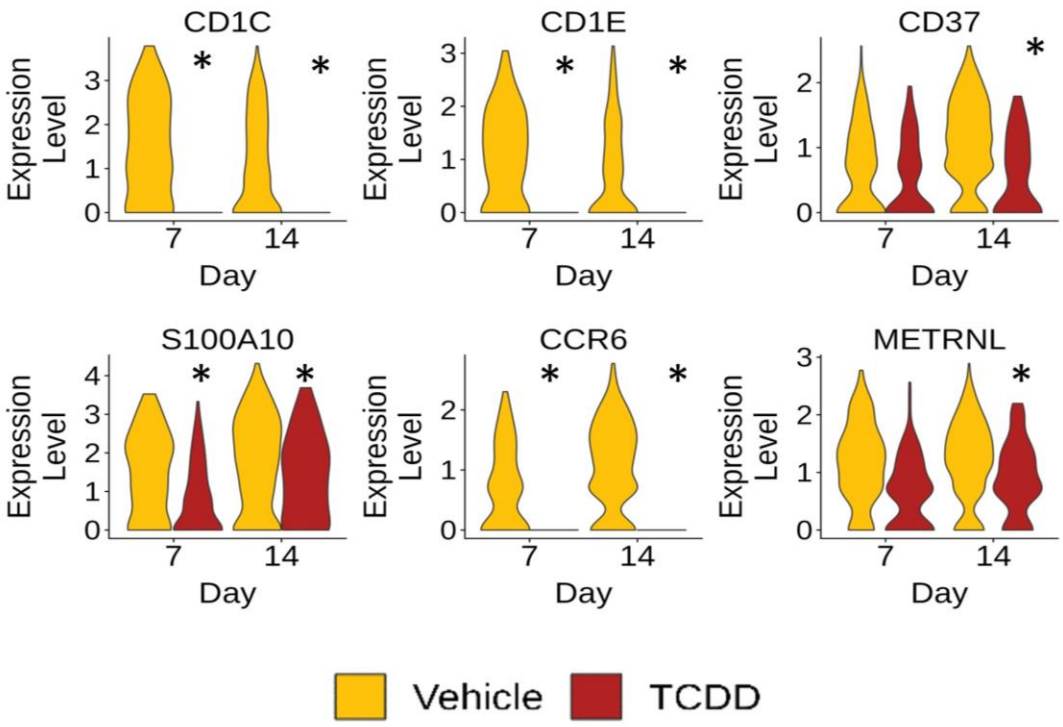

Figure 3.38 Expression of select TCDD-treatment induced DEGs in cDC2 cluster on Days 7 and 14. All DEGs are associated with a Bonferroni adjusted p value < 0.05.

At the transcriptomic level, based upon the report of Villani *et al.* of the existence of two subpopulations of CD1c$^+$ dendritic cells (Villani *et al.*, 2017), we calculated gene module scores for dendritic cells in our dataset by considering the markers associated with these two subpopulations. In absence of any exogenous AHR activation, Type A CD1c$^+$ cells, marked by higher expression of MHC class II molecules and dendritic cell markers (*CD1C* and *CD1E*) were the dominant population until day 21. Type A CD1c$^+$ cells declined in number by day 28 to give way to Type B CD1c$^+$ population (**Figure 3.39A**), which expressed higher inflammatory genes (*FCN1, S100A8, S100A9*, etc.) (also see Methods). Cells that develop in presence of TCDD had a lower gene module score for Type A CD1c$^+$ cells compared to the control for the duration of the study (**Figure 3.39B**). On the other hand, the Type B CD1c module score was consistently higher in TCDD-treated cells.

Dendritic cells are highly heterogeneous and diverse subpopulations may develop in different contexts with distinct functional roles (Said *et al.*, 2015; Villani *et al.*, 2017). Type A CD1c$^+$ cells may be deemed more important in the context of the adaptive immune system, whereas Type B CD1c$^+$ cells are perhaps better suited for mounting an innate inflammatory response.

Our findings suggest that AHR activation of human progenitor cells may lead to development of Type B CD1c$^+$ cells at the expense of Type A CD1c$^+$ cells that can have important functional implications in the context of human dendritic cell associated immunity.

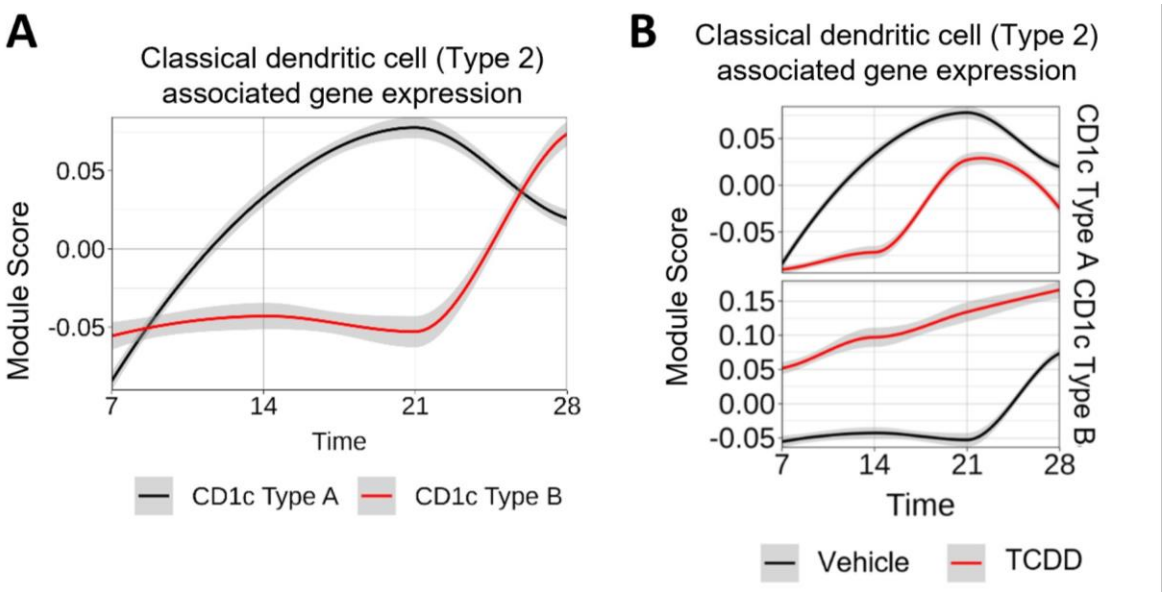

Figure 3.39 (A) Module scores of CD1c$^+$ dendritic cell sub-types across time. Module scores were calculated based on a module of curated genes from the literature. (B) CD1c subtype score in dendritic cells over time.

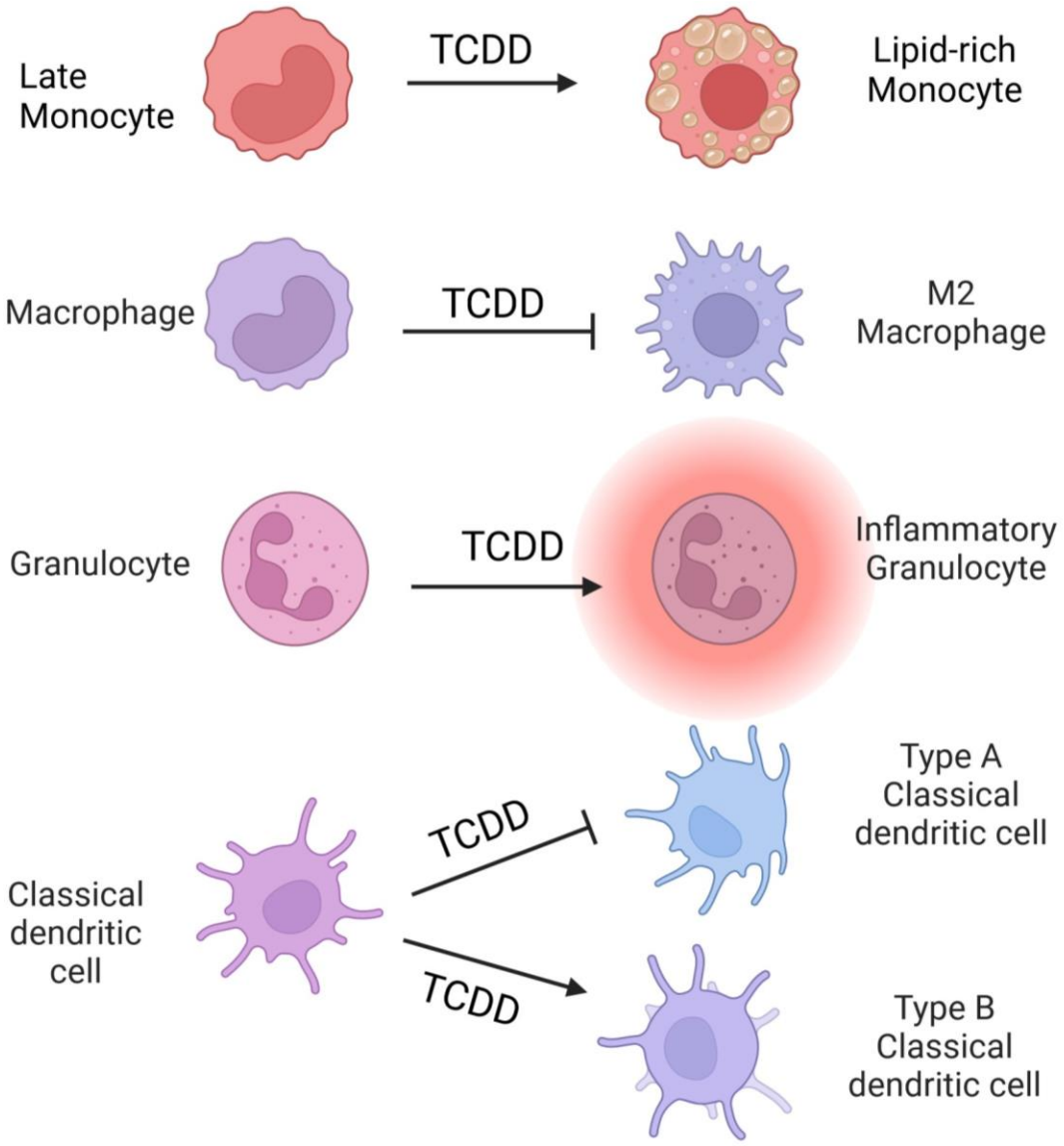

Figure 3.40 Putative effects of TCDD on different myeloid lineage cells that develop in presence of TCDD.

TCDD does not directly alter ERK1/2 signaling in HSPCs

The AHR signaling pathway participates in crosstalk with several signaling pathways such as the NF-κβ pathway and TGF-β signaling pathway. (Puga *et al.*, 2009). We investigated if any potential signaling pathway(s) was being modulated in MPPs by TCDD that may explain the perturbed hematopoietic differentiation that we observed. Among the top pathways that were modulated in cells of the MPP cluster at days 7 and 14 in GSEA, we were particularly interested in the MAPK signaling module that was suppressed by TCDD (**Figure 3.41**).

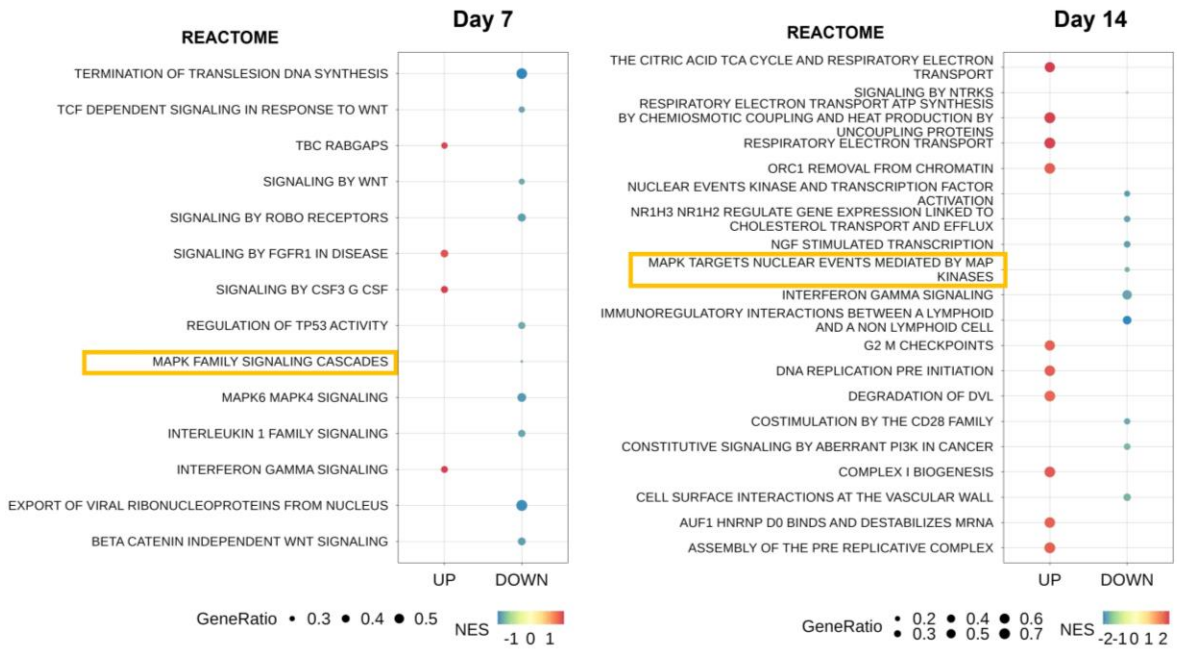

Figure 3.41 Top differentially enriched pathways in GSEA of differentially expressed genes in the MPP cluster based on the Reactome database on days 7 and 14. MAPK signaling pathway is highlighted with yellow boxes.

The MAPK signaling family are serine/threonine kinases and include the subfamilies of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinases (p38s). Within the MAPK family, ERK1/2 is modulated

more by cytokines and mitogens than the other sub-families. TCDD has been known to interact with ERK1/2 signaling in certain cell types such as cervical cells in the macaque monkey and murine fibroblasts (Enan *et al.*, 1998; Tan *et al.*, 2002). Reports of association of ERK1/2 with alterations in hematopoietic lineages are more widely reported than the other sub-families of MAPKs. Inhibiting Erk1/2 signaling in mouse lineage marker negative (Lin^-) bone marrow cells favored the development of neutrophils over monocytes when exposed to M-CSF. Consequently, sustained Erk1/2 activation led to monocyte development in response to G-CSF induction, while inhibiting Erk1/2 signaling promoted the development of neutrophils at the cost of monocyte formation in response to M-CSF (Hu *et al.*, 2015). On a similar note, inhibition of the MEK/ERK pathway with the MEK inhibitor U0126 in a murine myeloblastic cell line had a significant suppressive effect on G-CSF-triggered granulocytic differentiation and IL-6-induced monocytic differentiation (Miranda *et al.*, 2005). C/EBPα, a crucial transcription factor in the process of granulocyte formation, was discovered to undergo negative regulation through phosphorylation of serine residue 21 (S21) mediated by ERK1/2 (Jack *et al.*, 2009). This phosphorylation inhibited granulocyte development and promoted monocyte formation in the mouse. Overall, ERK1/2 activity seems to be negatively correlated with granulocyte development. The role of TCDD in relation to ERK1/2 signaling in the context of hematopoiesis has not been explored. Based on our GSEA results for the MPP cluster and our observation of enhanced granulopoiesis in presence of TCDD, we therefore investigated the role of TCDD on ERK1/2 signaling in differentiation of HSPCs. We hypothesized that TCDD inhibits ERK1/2 signaling in HSPCs which may partly explain the increased granulocyte development we observed in our study. We considered the phosphorylation of ERK1/2 at

Threonine 202 and Tyrosine 204 amino acid positions as a measure of ERK1/2 activation. As a positive control, an ERK1/2 inhibitor Ravoxertinib (GDC-0994) was used at 10 nM and added at the beginning of the study. Ravoxertinib is potent with an IC50 of 6.1 nM and 3.1 nM for ERK1 and ERK2, respectively (Yang *et al.*, 2023). ERK1/2 phosphorylation rapidly diminishes within 20 minutes and so we measured phosphorylation status of ERK1/2 at 5 and 15 minutes after cytokine stimulation of HSPCs.

Following the addition of cytokines to the culture, at 5 minutes or 15 minutes, we did not observe any significant difference in percent phospho-ERK1/2 positive cells or changes in the mean protein expression of phospho-ERK1/2 with TCDD treatment on day 0 and day 7 (**Figure 3.42**). There was also no significant difference in proportion of hematopoietic cells of different lineages that developed in presence of the ERK1/2 inhibitor compared to the vehicle group (**Figure 3.43**).

The results suggest that TCDD does not significantly alter ERK1/2 signaling in human HSPCs and perturbations in lineage specification with TCDD such as enhanced granulopoiesis are not mediated directly through this pathway.

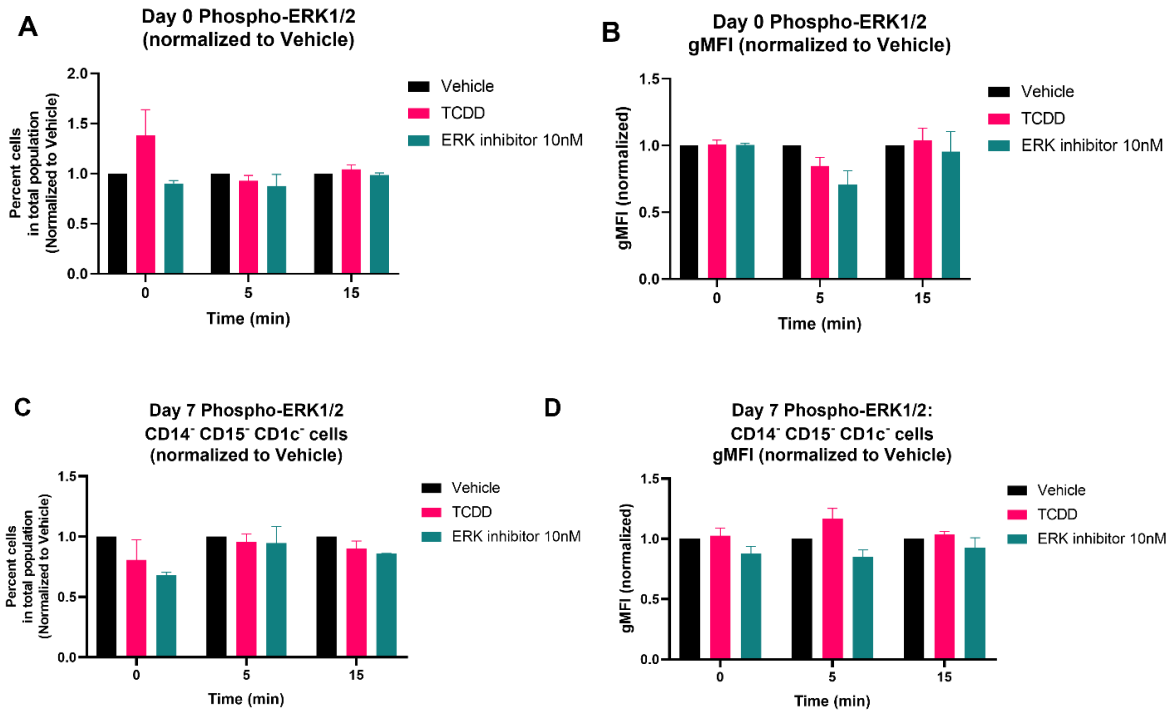

Figure 3.42 (A) Percent phosphorylated ERK1/2 positive cells in total cell population (normalized to cells of the vehicle group at corresponding timepoint) on day 0. (B) gMFI (geometric mean fluorescence intensity) of phospho-ERK1/2 protein (normalized to cells of the vehicle group at corresponding timepoint) in total cells at day 0. (C) Percent phosphorylated ERK1/2 positive cells in non-myeloid ($CD14^-$ $CD15^-$ $CD1c^-$) cell population (normalized to cells of the vehicle group at corresponding timepoint) on day 7. (D) gMFI of phospho-ERK1/2 protein (normalized to cells of the vehicle group at corresponding timepoint) in non-myeloid ($CD14^-$ $CD15^-$ $CD1c^-$) cells on day 7. Data presented is composite of 3 independent experiments.

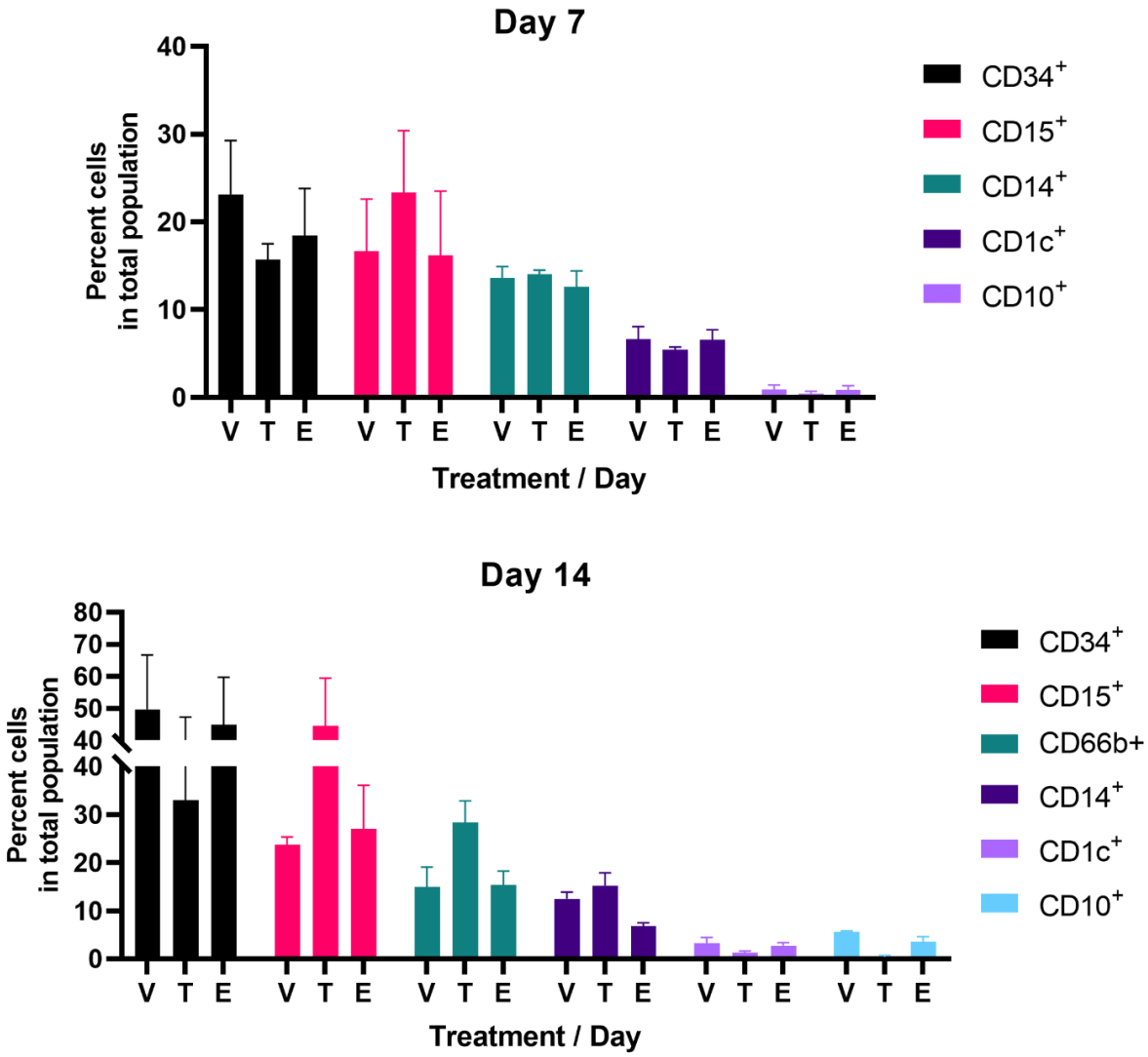

Figure 3.43 Percent cells of different hematopoietic lineages on days 7 and 14. V = Vehicle, T = TCDD, E = ERK1/2 inhibitor (10 nM). $CD15^+$ = promyelocytes, $CD66b^+$ = granulocytes, $CD14^+$ = monocytes, $CD1c^+$ = dendritic cells, $CD10^+$ = lymphoid cells. Data presented is composite of 2 independent experiments.

CHAPTER 4: DISCUSSION

We report for the first time on the use of single-cell transcriptomics to investigate the role of AHR activation by TCDD in human hematopoietic differentiation in an *in vitro* stromal cell-free model system. Earlier studies have reported the use of growth factors SCF and FLT3L to facilitate *in vitro* HSPC proliferation (Flores-Guzman *et al.*, 2002; Clanchy *et al.*, 2013). IL-6 is a highly pleiotropic cytokine that is well established to promote hematopoietic stem and progenitor cell (HSPC) proliferation (Peters *et al.*, 1998; Hunter *et al.*, 2015). We therefore added this cytokine to our culture system in conjunction with SCF and Flt3L to facilitate initial proliferation of HSPCs on day 0. IL-7, which we add to our culture system on Day 7, has been known to facilitate expansion and differentiation of B cells (Veiby *et al.*, 1996; Parrish *et al.*, 2009). The cytokine concentrations we have used are also relatively low as high cytokine concentrations can preclude differentiation of HSPCs (Zandstra *et al.*, 2000). While Kraus *et al.* have employed similar culture conditions to ours, their primary focus has been on the investigation of B-cell development (Kraus *et al.*, 2014). We have demonstrated that early megakaryocyte and erythroid progenitors also developed in the culture. As this model was established to identify effects of perturbations on early hematopoietic differentiation and not for full differentiation of all progenitors, we refrained from addition of thrombopoietin and erythropoietin to avoid further differentiation bias towards the megakaryocyte/erythroid branch at the expense of other major immune cell lineages. The current model as demonstrated through its characterization here thus allows differentiation of proliferating $CD34^+$ HSPCs and development of almost a complete spectrum of lymphoid and myeloid lineage specified cells. Our single-cell transcriptomic analysis of differentiating HSPCs collected at different

time points enabled identification of distinct cell clusters associated with multiple hematopoietic lineages and allowed us to follow their chronological development. Flow cytometric analysis confirmed existence of major cell types as identified using scRNA-seq analysis, with the exception of T cells, which are known to require Notch ligand engagement of lymphoid progenitors (Zuniga-Pflucker, 2004). In our model, the lymphoid population also emerged in sufficient numbers after day 7, following the emergence of the myeloid populations. This also recapitulates the normal physiological paradigm of hematopoiesis where megakaryocyte-erythroid cells branch off from MPP at first, followed by emergence of myeloid and later on lymphoid cell types (Rieger *et al.*, 2012). Collectively, our results suggest that this *in vitro* model may be especially useful in studies of early human hematopoiesis and in identification of agents that can alter the human developing immune system.

Results from flow cytometry-based analysis and scRNA-seq analysis were highly concordant for phenotype analysis with occasional differences. One discrepancy was in the case of erythroid progenitors. scRNA-seq analysis suggested a decrease in erythroid cells in the TCDD-treated group relative to that of the control. However, flow cytometry derived results did not show a decrease in erythroid populations in the TCDD-treated group. This disparity might reflect the formation of an overall very small number of erythroid populations *in vitro* and failure to capture these cells during processing of TCDD-treated cells for scRNA-sequencing. Differences in vehicle and TCDD mediated granulocyte populations were not always concordant between scRNA-seq data and flow-cytometry data. This may be the result of quality control analysis following harvesting of cells for scRNA-seq processing as granulocyte populations have a short lifespan and may

degrade quickly. We also observed an increase in cells of the MPP cluster in the TCDD treated group in the scRNA-seq data. This may seem contradictory to the fact that there is a decrease in $CD34^+$ cells with TCDD treatment. However, cells in the MPP cluster in scRNA-seq data are not all $CD34^+$ but have a transcriptomic profile close to the $CD34^+$ progenitors for them to be grouped in the same cluster. These are putative progenitors that have lost CD34 but have not expressed any lineage specific markers. Therefore, the presence of $CD34^+$ and $CD34^-$ early progenitors in the MPP cluster accounts for the slight increase in MPP cluster cells of the TCDD group.

Our findings that AHR activation by TCDD elicits a decrease in early B cells and megakaryocyte populations is in agreement with previous experimental and epidemiological studies (Webb *et al.*, 1987; Li *et al.*, 2017a). In contrast to previous studies, we also demonstrated that AHR activation in HSPCs increased development of both monocyte and granulocyte populations from HSPCs. A previous study using human cord blood-derived $CD34^+$ cells had reported impaired development of both monocytes and dendritic cells with AHR agonists including TCDD as well as no change in granulocytes (Platzer *et al.*, 2009). This discrepancy between our findings and the study could be due to differences in culture conditions as the study utilized diverse myeloid lineage promoting cytokines such as G-CSF, GM-CSF, and IL-4. AHR signaling is highly context specific (van Grevenynghe *et al.*, 2003) and the influence of different cytokines could modify AHR signaling, giving rise to the observed differences. As for the case of no change in granulocyte development with TCDD in presence of SCF and G-CSF as observed in the aforementioned study, it may be that the activation threshold of downstream signaling provided through G-CSF or combination of SCF and G-CSF may

be sufficiently high such that any fine tuning or modulation through AHR becomes nullified.

scRNA-seq analysis of progenitor clusters allowed identification of key genes that putatively govern the increase in monocyte and granulocyte populations at the expense of other lineages with AHR activation. We observed TCDD-mediated reduction in BCL11A and IRF8, two key transcription factors that regulate lymphoid-myeloid balance with increased expression of these transcription factors being linked to increased output of lymphoid cells and pDCs.

BCL11A (B cell lymphoma/leukemia 11A) is a transcription factor that plays critical roles in the development of different hematopoietic lineages. It is important for lymphoid lineage priming and development of common lymphoid progenitors and dendritic cells (Liu *et al.*, 2003; Ippolito *et al.*, 2014). Through its regulation of TCF4, a transcription factor and master regulator of pDC development, BCL11A is critical for development of plasmacytoid dendritic cells (Ippolito *et al.*, 2014). It is also essential for repressing the gene encoding γ-globin to enable switching of fetal hemoglobin to adult hemoglobin in erythroid progenitor cells at birth (Sankaran *et al.*, 2008). BCL11A is reported to be an upstream regulator of EBF1, and they can positively coregulate the expression of each other. EBF1 expression is observed in the lymphoid cluster and not in the MPP cluster. Given that the suppression of BCL11A was observed with TCDD in the MPP clustered cells that precede the lymphoid clustered cells, it is likely that TCDD suppression of BCL11A may play an initial role in reducing lymphoid and B cell output observed with the TCDD-treated HSPCs. Based upon screening of the data associated with a computational study on consensus binding regions for dioxins called dioxin response elements (DREs) in the human

genome, we found that BCL11A contains multiple putative DREs in the promoter region and within 1000 base pairs upstream of the gene (Dere *et al.*, 2011). It is therefore possible that TCDD via AHR directly acts on BCL11A and suppresses its expression. Further studies are required to confirm this. It cannot however be ruled out that TCDD acts on other genes that have not been linked with BCL11A expression to date or through alternate mechanisms that result in the formation of a lower number of $BCL11A^+$ cells.

IRF8 is also associated with the development of multiple hematopoietic cell types. IRF8 is essential for the development of dendritic cells, particularly pDCs and Type I classical dendritic cells. CEBPα is a transcription factor that is critical for specification towards neutrophil lineage and IRF8 negatively regulates differentiation of GMPs and monocyte-dendritic progenitors towards neutrophils through direct physical interaction with CEBPα, resulting in an increase in neutrophil populations in IRF8-/- mice (Tamura *et al.*, 2000; Kurotaki *et al.*, 2014). Other studies in mice also suggest that IRF8 skews differentiation of GMPs towards monocytes or macrophage cells (Yanez *et al.*, 2015). IRF8 also regulates lineage commitment of B cells (Wang *et al.*, 2008), differentiation of T_H17 cells (Ouyang *et al.*, 2011), and maturation of $CD8^+$ T cells into effector cells (Miyagawa *et al.*, 2012). To my knowledge, the relationship between TCDD-treatment and activation of the AHR and IRF8 expression has not been explored. Based on the role of IRF8 in modulating lymphoid and myeloid lineages, and reduced IRF8 activity as inferred from SCENIC analysis, we measured IRF8 protein expression in differentiating cells in our model using flow cytometry. Flow cytometry data show that there is a decrease in IRF8 expression in the overall population with TCDD treatment. Compared to other hematopoietic progenitors, pDCs have relatively very high IRF8 expression. $CD303^+$ cells, representing

the pDC population, are a minor population accounting for approximately 3% of the total cell population in our model and were not detected in all experiments. The percentage of $IRF8^+$ cells in the total population is greater. Therefore, a lower number of IRF8 expressing cells with TCDD treatment cannot be accounted for solely by the absence of pDCs in the TCDD-treated group. Reports from previous literature and our scRNA-seq data of *IRF8* expression show low *IRF8* expression in HSCs and cells of the MPP cluster but greater in cells of downstream progenitor clusters. It is possible that IRF8 expression is reduced at the protein level by TCDD in these downstream progenitor populations which may account for the lack of pDCs and enhanced specification of progenitor cells towards granulocytes.

It is noteworthy that regulation of hematopoietic cell fate results from the combined influence of multiple transcription factors to orchestrate lineage specific gene transcription over time (Lemon *et al.*, 2000). Modulation of a few transcription factors by AHR signaling could alter expression and activity of other transcription factors. Further experimental evidence is warranted to ascertain the exact mechanisms. Additionally, we identified decreased levels of key maturation markers in monocyte/macrophage clusters (*MRC1, CLEC10A,* etc.). This is in agreement with previous reports of attenuation of monocyte maturation to macrophages with AHR activation by xenobiotics (van Grevenynghe *et al.*, 2003). Our transcriptomic analysis identified decreased expression with TCDD treatment of several critical transcription factors involved in macrophage maturation, such as *MAF*, that have not been previously known to be modulated by AHR signaling in macrophages. Previous studies regarding the effect of TCDD-treatment on the development of dendritic cells from HSPCs have been inconclusive with a decrease in dendritic cell yield and

dendritic cell markers such as CD11c (Lee *et al.*, 2007), as well as enhanced differentiation and increase in dendritic cell markers of murine bone marrow derived dendritic cells (Vogel *et al.*, 2013) in presence of TCDD being reported. We observed a decrease in CD1c at both transcriptomic and protein levels in cells that develop in our human HSPC differentiation model. Some of these discrepancies observed in the murine studies may be attributed to differences in the mouse species used in these studies. However, our results are more concordant with a clinical study that reported an increase in dendritic cell populations with the AHR antagonist SR1 (Thordardottir *et al.*, 2014) which suggests that AHR activation may have a repressive role in human dendritic cell development.

Collectively, using scRNA-seq analysis and flow cytometry, we characterized a model of human hematopoiesis and identified gene expression programs perturbed by AHR activation during hematopoietic multi-lineage specification, thus providing a better understanding of the molecular mechanisms by which AHR signaling may impair human hematopoietic differentiation. Importantly, the model system offers an opportunity to study concurrent multi-lineage human hematopoiesis and can also be used to evaluate the effects of other small molecules or genetic perturbations on processes governing HSPC to lineage commitment. In conclusion, our study has provided important insights into the role of AHR in human hematopoiesis.

CHAPTER 5: CONCLUSION

The most toxic congener in the family of dibenzo-*p*-dioxins, TCDD, has been studied for over several decades. Almost all its toxicity is mediated through the AHR, and it is therefore a powerful probe to study the effects of continuous AHR activation. Suppression by TCDD of immune cell functions is well documented, and the immune system is recognized as a highly sensitive target of TCDD. Less is known about the effect of TCDD on the development of immune cells from early hematopoietic progenitor cells. With the recognition that AHR is functional in HSCs, more studies on elucidating the role of AHR in HSCs have been undertaken in the past few years. However, most of these studies have been conducted in mice. Although several TCDD and AHR-mediated effects are conserved across species, there are many examples when studies conducted in experimental animals do not translate well to humans. Therefore, studies of AHR in relation to human HSC-differentiation are warranted.

This is especially true in the context of identifying the role of TCDD and AHR activation in regulation of lineage specification of human HSPCs. There have been few *in vitro* studies to date that have investigated the effect of TCDD on development of specific-cell lineages from human HSPCs. In these studies, lineage specific cytokines were added to culture that have the potential to skew differentiation of HSPCs towards particular lineages. Some studies also employed supporting stromal cells in conjunction with HSPCs in culture. These studies therefore cannot effectively and directly address the role of AHR and TCDD in orchestrating cell fate decisions affecting lineage choice of human HSPCs. Knowledge about the underlying molecular processes governing hematopoietic lineage specification that are potentially perturbed by TCDD is also limited.

This study addresses these questions by employing an *in vitro* feeder cell-free model of human HSPCs differentiation with cytokines that favor growth and proliferation of progenitor cells. This is the first time that single-cell transcriptomics has been utilized to understand the role of TCDD in the regulation of the differentiation process of HSPCs at the transcriptomic level in a comprehensive, time-dependent manner.

Previous findings have demonstrated that B-cell development is impaired by TCDD in this model but whether this is due to a shift towards other lineages remained unexplored. I hypothesized that TCDD modulates differentiation of human HSPCs towards the myeloid lineage at the expense of the lymphoid lineage. Based upon this hypothesis, I initially characterized the existing *in vitro* model using single-cell transcriptomics and flow cytometry and demonstrated that it facilitates the development of multiple immune cell lineages. The results with TCDD treatment of HSPCs show that TCDD favors the differentiation of human HSPCs towards granulocyte and monocyte lineages while attenuating differentiation of dendritic, megakaryocytic, and lymphoid lineages. I also identified at transcriptomic and proteomic level that TCDD brings about reduced expression of key transcription factors *BCL11A* and *IRF8* in the hematopoietic progenitor cells that are important modulators of lymphoid and dendritic cell development and thus may explain the TCDD-mediated perturbed differentiation process. Cellular signaling pathways that are modulated in specific early hematopoietic progenitors and lineage committed cells by TCDD have also been identified. Many of these pathways were not previously known to be affected in these cells by TCDD. Thus, this research gives a better understanding of the role of TCDD in human hematopoietic differentiation and its effect on the associated molecular and cellular processes.

This knowledge of the role of AHR activation in directing lineage specification of human HSPCs may be particularly useful in areas such as regenerative medicine where development and expansion of hematopoietic lineages of interest from human HSPCs can be brought about by using AHR agonists or antagonists. AHR antagonist SR1 has been previously utilized for expansion of HSPCs in serum-free cytokine enriched media. Analogously, by modifying AHR activity in HSPCs in a differentiation promoting culture system like the one utilized here, specific hematopoietic lineages may be enriched for use in the clinical arena.

On the other hand, this study also underscores that use of AHR modulators in HSPC expansion may need to be cautiously evaluated as they may introduce bias of HSPCs towards lymphoid and dendritic cells at the expense of other lineages which can be detrimental for well-balanced hematopoietic reconstitution from HSPCs. Although the effects of the AHR antagonist would be expected to be nullified after removal of the antagonist, epigenetic alterations that are not immediately obvious cannot be ruled out. Epigenetic and chromatic modifications in HSPCs due to TCDD or AHR antagonists have not been explored in this current study and this may be an avenue to explore in future.

A balanced hematopoietic system is required to maintain an effective immune response when needed. AHR can bind a wide range of ligands with varied conformations. This includes, for example, pharmaceuticals of diverse categories such as omeprazole, nimodipine, and leflunomide, which have relatively strong AHR induction activity (Jin *et al.*, 2012). As demonstrated by our studies, AHR activation is associated with alterations in lineage bias of HSPCs and so, for drugs possessing AHR activity, the effect on hematopoiesis should perhaps be especially considered at the preclinical stage. As an

example, use of leflunomide, an anti-viral drug, has been associated with thrombotic microangiopathy with reduced platelet numbers and a temporal reduction in B cells and an increase in $CD16^+$ cells in patients (Leca *et al.*, 2008; Dona *et al.*, 2023). A link to alterations in AHR activity with leflunomide had not been investigated in these studies but some of the hematological changes observed draw close parallel with our results. Potential AHR modulation could be a causative factor for the observed changes and warrants further studies. The *in vitro* hematopoietic differentiation system we have characterized could be useful for preclinical screening to characterize the effect of similar AHR agonists or drugs on the hematopoietic system. In fact, because our *in vitro* model supports development of a broad range of hematopoietic cell types in a 96-well plate format without any use of semisolid medium as used in conventional CFU assays, this assay system is well suited for quick assessment of any agent of interest and may be employed for hematopoietic and immunotoxicity studies. This assay may also be used to identify the role of genes of interest in human hematopoietic differentiation using, for example, genome-edited HSPCs created through tools such as CRISPR/Cas9.

In our studies, we have utilized lineage specific markers to identify broad populations of hematopoietic and immune cell types using flow cytometry. In the future, by incorporating more markers, it would be possible to identify and delineate changes in sub-populations with TCDD or other AHR modulators. For our studies, we have used human $CD34^+$ HSPCs from umbilical cord blood. Although similar trends may be hypothesized, it would be interesting to test and compare how TCDD might modulate temporal differentiation of $CD34^+$ HSPCs from other sources such as those derived from fetal or adult bone marrow or from bone marrow-mobilized cells in peripheral blood.

The scRNA-seq study has been invaluable in my study of the temporal changes in gene expression in different cell types in presence of TCDD. I have utilized different bioinformatics tools to identify TCDD-mediated perturbations. The single-cell transcriptomic dataset remains a valuable tool for us and others to do further analysis with relevant bioinformatics programs as they are developed in the future. Also, as other appropriate datasets involving AHR agonists and the hematopoietic system become available, integration of those datasets with our data would allow us to make comparisons and draw interesting insights.

In conclusion, my research studies have demonstrated, for the first time, the effects of TCDD treatment of human HSPCs on hematopoietic differentiation within a system that allows multilineage hematopoietic development and identified key events that are perturbed by TCDD during this process.

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