Chronic TCDD exposure results in the dysregulation of gene expression in splenic B-lymphocytes and in the impairments in T-cell and B-cell differentiation in mouse model

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exposure in humans is associated with marked immune suppressions and increased incidence of lymphoblastic diseases. To elucidate mechanisms of impairments in humoral immune responses, we used a murine model. Following a 20-week administration of low doses of TCDD, we observed severely reduced antibody titers, dramatically decreased number of splenic Th1 and Th2 cells and an increase in CD19⁺ B cells. Transcriptional profiling of CD19⁺ B cells showed that markers of pre-B cells were significantly elevated, indicating delayed B cell maturation. These changes in B cells were accompanied by decreases of T helper cell numbers and reduced IgM and IgG titers. A transcriptome analysis of splenic B cells followed by Ingenuity Pathway Analysis (IPA) revealed a set of differentially expressed genes known to play roles in tumorigenesis, cell-proliferation and cell-migration. The most up-regulated transcript gene was Eph receptor A2 (EphA2), a known oncogene, and the most down-regulated transcript was ZBTB16 that codes for a negative transcriptional regulator important in epigenetic chromatin remodeling. IPA identified cAMP-responsive element modulator (CREM) and cAMP-responsive element binding protein 1 (CREB1) as top upstream regulators. Consistently, a MAPPER promoter database analysis showed that all top dysregulated genes had CREM and/or CREB1 binding sites in their promoter regions. In summary, our data showed that chronic TCDD exposure in mice caused suppressed humoral immunity accompanied with profound dysregulation of gene expression in splenic B-lymphocytes, likely through cAMP-dependent pathways. This dysregulation resulted in impairments in T-cell and B-cell differentiation and activation of the tumorigenic transcription program.

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Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a persistent environmental pollutant that targets various organs including the immune system in both acute and chronic exposure. Even at doses with no obvious signs of toxicity, TCDD alters the immune functions in virtually every species studied so far (Holapple et al., 1991; Kerkvliet, 2002). TCDD acts via the activation of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that belongs to the basic
helix-loop-helix superfamily whose members play key roles in gene expression networks underlying many essential physiological and developmental processes. Human and animal exposures to TCDD result in immune suppression that is not observed in AhR null mice (Vorderstrasse et al., 2001a). AhR is involved in both innate and acquired immune responses and has been shown to be essential for the differentiation and maintenance of the intestinal intraepithelial lymphocytes (IELs) and the innate lymphoid cells (ILCs) (Li et al., 2013; Spits and Di Santo, 2011). The role of AhR in the Th17 and T regulatory (Treg) cell development is firmly established (Ho and Steinman, 2008). B cells are also highly sensitive targets of TCDD (Sulentic and Kaminski, 2011b). Epidemiologic evidence shows that TCDD exposure depresses humoral immunity in human subjects. As such, both Seveso accident subjects with the highest TCDD exposure and Vietnam Veterans exhibited decreased plasma IgG levels and increased incidence of lymphatic tumors, especially non-Hodgkin’s lymphoma (NHU) (Sulentic and Kaminski, 2011b).

TCDD has been demonstrated to suppress the primary IgM response in purified splenic B cells (Dooley and Holsapple, 1988). This suppression, both in vivo and in vitro, involved the activation of AhR (Vorderstrasse et al., 2001b). Another TCDD effect on immune system is a shift in the Th1/Th2 balance, an important measure of the immune system fitness (Kidder, 2003). A large body of data demonstrates the effects of AhR activation on the differentiation of the T-cell subsets (Nguyen et al., 2013), including a number of genome-wide expression studies on T-cells (Li et al., 2013; Stubbington et al., 2015). While the roles of AhR in B cell differentiation are demonstrated (De Abrew et al., 2011; Zhang et al., 2013), there is a dearth of data that allows a better mechanistic understanding of the long-term consequence of the chronic TCDD exposure on humoral immunity (e.g., B cells) that underlie referenced above adverse outcomes of TCDD exposure (Kerkvliet, 2002). With the application of transcriptome analysis, we could gain a full view of the effects of TCDD on B lymphocytes at the transcriptional level. Transcriptomic studies have been performed on B cell lines (De Abrew et al., 2010); however, to our knowledge, there were no such in vivo studies reported regarding B lymphocytes isolated from mice chronically treated with TCDD. In the current study, we used a murine model that recapitulates a chronic human exposure to TCDD to evaluate the effects of TCDD on the immune phenotype and the splenic B cells transcriptome. Specifically, splenic B lymphocytes were isolated after a 20-week TCDD exposure and housed in the Animal Research Center of Tsinghua University under specific pathogen-free conditions, at a controlled temperature of 24 ± 2°C and humidity of 50% ± 10%, with a cycle of 12 hrs light and 12 hrs dark. Animals were provided with pellet foods and water ad libitum, and randomly assigned into three groups, two TCDD treated groups and one vehicle control (Mice received DMSO diluted in olive oil served as the control). TCDD (Toronto Research Chemicals Inc., Canada) was dissolved in DMSO (Sigma-Aldrich, St. Louis, USA) at stock concentrations of 0.1 μg/μL and 0.01 μg/μL, and then diluted 1000 times in olive oil as dosing solutions. The mice in two treated groups were administered with TCDD diluted in olive oil at 2 doses (low: 0.1, high: 1 μg/kg of body weight (bw)) by gavage every other week. Doses used in the current study were based on previous TCDD chronic exposure reports. In a 13–14 month-chronic exposure study, C57BL/6 mice were given 0.2 μg/kg bw TCDD for phenotype analysis of spleen, thymus and peripheral blood (Oughton et al., 1995). Body weights were measured at the time of gavage. After the 20 weeks of exposure, blood samples were collected immediately after euthanasia for serum isolation, and spleen tissues were processed for further studies. All animal experiments were conducted in accordance with animal protocols approved by the Animal Care and Use Committee of Tsinghua University.

1. Material and methods

1.1. Animals

Twenty-four male C57BL/6 mice (6 weeks old, 20 ± 1 g) were purchased from Vital River Laboratories (VRL; Beijing, China) and housed in the Animal Research Center of Tsinghua University under specific pathogen-free conditions, at a

1.3. Luminex analysis

The concentrations of selected cytokines (IL-6, IL-12p70, IL-13, and IL-22) in mouse sera were determined by Luminex assay.
with a custom kit, Mouse Th17 magnetic Bead Panel (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions. The instrument used was Luminex 200TM (Luminex, Austin, TX, USA) and read under Mag Plex. Data were analyzed using Milliplex Analyst 5.1 Software (Merck Millipore). All samples were tested in duplicate for Luminex assay.

1.4. ELISA

Levels of serum IgG, IgM and IgA were measured using ELISA kits purchased from eBioscience (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Briefly, capture antibodies (1:1000. V/V) were coated separately on microtiter plates, incubated overnight at 4°C. Capture antibodies used were purified anti-mouse IgG monoclonal antibody, purified anti-mouse IgM monoclonal antibody and purified anti-mouse IgA monoclonal antibody for IgG, IgM and IgA, respectively. After washing with PBS containing 0.05% Tween-20 (PBST), microplate wells were blocked with 5% BSA at room temperature for 2 hrs. After washing with PBST, diluted samples were added to the microplate and incubated for 2 hrs at room temperature. After washing with PBST, HRP-conjugated detection antibody was added to each well and incubated for 1 hr at room temperature. HRP-conjugated detection antibodies used were: HRP-conjugated anti-mouse IgG monoclonal antibody, HRP-conjugated anti-mouse IgH+L monoclonal antibody and HRP-conjugated anti-mouse IgA monoclonal antibody for IgG, IgM and IgA, respectively. After washing with PBST, TMB was added and incubated for 15 min at room temperature. The enzyme reaction was stopped with 1 mol/L H3PO4. The optical density was read using a microplate fluorescence reader (Tecan Infinite F200Pro Switzerland) at a wavelength of 450 nm.

1.5. Transcriptome analysis

After lysis of the red blood cells, CD19 positive cells were selected from single spleen cell suspension of control and 0.1 μg/kg bw groups using mouse CD19 positive selection kit (StemCell Technologies, Meylan, France) according to the manufacturer’s instructions. Total RNA was purified from CD19 positive cells using TRizol reagent (Invitrogen, Carlsbad, CA). Also, the RNA was sent to perform the transcriptome study.

The original image data were translated into sequence data as raw reads via base calling analysis. After a stringent filtering reads, all clean reads were mapped to the reference genome (available at ftp://ftp.ensembl.org/pub/release-75/fasta/homo_sapiens/dna/) from the Ensembl database using the Short Oligonucleotide Analysis Package alignment tool SOAP aligner/SOAP2. To identify the differentially expressed genes between control and TCDD treated group, data for each gene were normalized and calculated as RPKM (Reads Per Kilobase per Million of mapped reads) as described by Mortazavi et al. (2008). The differentially expressed genes were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using DAVID (The Database for Annotation, Visualization and Integrated Discovery) with the total mouse genome information as the background (Ashburner et al., 2000; Dennis Jr et al., 2003; Huang et al., 2009; Kanehisa and Goto, 2000). The data were further analyzed with Ingenuity Pathway Analysis (IPA). In IPA analysis, an upstream regulator analysis is a remarkable step forward in transcription regulator prediction (Krämer et al., 2014). Given the differential regulation of up- and down-regulated genes, the activation states of upstream regulators are determined by the regulation direction associated with relationship of the gene’s regulator. In IPA analysis, activation of z-score is used as an independent test to identify the significant upstream regulators.

1.6. Statistical analysis

Experimental data were analyzed by one-way ANOVA or Dunnett test using GraphPad Prism 5 (GraphPad Software, USA). Results are presented as mean ± SEM. Differences were classified as significant * for \( p < 0.05 \) and ** for \( p < 0.01 \) from control.

2. Results

2.1. Body weight showed no difference after TCDD exposure

Body weights were recorded at the time of gavage. In the 0.1 μg/kg bw treatment group, bw showed a tendency of increase, but this trend was not found in the 1 μg/kg bw treatment group. Through the 20-week exposure, no statistically significant change of bw was found (Fig. 1).

2.2. Chronic TCDD exposure dramatically reduced the numbers of Th1 and Th2 lymphocytes, shifted the Th1/Th2 ratio, and decreased the levels of pro-inflammatory cytokines IL-6 and IL-22

As shown in Table 1 and Fig. 2, chronic TCDD exposure dramatically reduced the percentages of both Th1 and Th2 lymphocytes; however, the Th1/Th2 ratio was increased following TCDD treatment at 1 μg/kg bw treatment group, suggesting the Th1/Th2 balance was shifted toward the Th1 cells. TCDD treatment only weakly affected the number of Tregs, consistent with the literature (Kerkvliet, 2009b; Negishi et al., 2005). We also found that TCDD exposure decreased the levels of pro-inflammatory cytokines IL-6 and IL-22 and increased the levels of the anti-inflammatory cytokines IL-13 and IL-12p70 (Table 2), consistent with TCDD-induced suppression of immune hypersensitivity phenotype in the mouse model of peanut allergy (Schulz et al., 2011).

2.3. Chronic TCDD exposure was associated with reduced immunoglobulin titers

Next, we tested the percentage of splenic CD19+ B lymphocytes and levels of three IgS, to evaluate effects of TCDD on the humoral immunity and B cell function. As shown in Table 3 and Fig. 3, the percentages of CD19+ B lymphocytes were increased significantly by 8.2% and 7.9% in 0.1 and 1 μg/kg bw treatment group, respectively. Serum levels of IgG were decreased significantly in both treatment groups and IgM level was decreased in the 1 μg/kg bw treatment group. IgA levels showed no obvious changes after chronic TCDD treatment.
2.4. Chronic exposure to TCDD was associated with marked changes in the splenic B cell transcriptome

To gain the insight into the mechanistic processes that underlie the observed changes in the immune functions, we performed the RNA-seq to identify a gene set that was differentially expressed in mice chronically exposed to TCDD when compared to control. TCDD exposure exerted marked changes on the splenic B cells transcriptome. Using the threshold of the absolute value of the fold change being ≥2.0, fdr corrected p-value being ≤0.001, we identified 70 differentially expressed transcripts. Ten most over-expressed and ten most under-expressed genes are shown in the Table 4. To identify which molecular pathways involved in the observed differential expression, we performed the IPA analysis. Table 5 shows the results of the Downstream Effect Analysis that revealed the most dysregulated pathways by TCDD. Consistent with the increased percentage of B cells, the most up-regulated pathway was that of lymphocyte proliferation (Z-score 2.5), with the specific B cell proliferation pathway also up-regulated (Z-score 1.1). Another set of up-regulated pathways included the pathways for invasion, migration, and movement of cells, consistent with the growing body of evidence that supports the roles of AhR in cell growth, movement and proliferation, as well as in the regulation of TGF-β activity (Carvajal-Gonzalez et al., 2009; Fernandez-Salgueso, 2010; Gomez-Duran et al., 2009; Kerkvliet, 2009a; Santiago-Josefat et al., 2004). The pathway regulating the apoptosis of cancer cells was the most down-regulated, consistent with the established lymphomagenic potential of TCDD.

2.5. The top upstream regulatory networks and the top upstream regulators identified by the Ingenuity Pathway Analysis

The analysis of the top upstream regulatory networks has identified the top downstream “diseases and functions” as vasculogenesis (consistency score 7.43) and binding of DNA (consistency score −6.0). Significant positive consistency score for vasculogenesis suggested that differentially expressed genes were involved in positive regulation of vasculogenesis that was consistent with known effects of AhR in cardiovascular physiology and disease (Lund et al., 2003). Significant negative consistency score for the “binding of DNA” module indicated that many of the differentially expressed genes were involved in gene transcription (Table 5). IPA Molecular and Cellular analysis module indicated that 31 (44% of total) differentially expressed molecules were involved in gene expression, while 39 molecules (55% of total) were involved in cellular development, growth and proliferation (Appendix A Table S1).

Top upstream transcriptional regulators identified by the IPA Core Analysis were CREM and CREB1. We used a Mapper database (Marinescu et al., 2005a), online link http://genome.ufl.edu/mapper/, to analyze 10 kb regions upstream of the ATG codon in the top 10 up-regulated and top 10 down-regulated molecules in the database (Table 6). Our analysis showed that CREM and/or CREB1 binding sites were present in all promoters but one of the analyzed genes. Interestingly, only the top up-regulated genes contained dioxin-response elements (DREs) in their promoters, whereas all top down-regulated genes lacked DREs. Other members of the cAMP-dependent signaling pathway overexpressed in our dataset included the AP1 transcription factors FosL2 (Log2 = 2.26) and JunB (Log2 = 1.03). Cyclic AMP-dependent transcription factor, ATF3, was the third most up-regulated transcript in our dataset (Log2, 2.88). Using Mapper analysis, we found one DRE, two CREM, two CREB1, and two ATF3 binding sites in the 10 kb promoter region preceding the ATG of mouse ATF3 gene, suggesting that TCDD exposure can induce a positive feedback regulation of the ATF3 transcript, potentially leading to the up-regulation of the ATF3 target genes. Taken together, these results demonstrated that chronic TCDD exposure resulted in marked and durable changes in splenic B-cell transcription program.

2.6. Chronic TCDD exposure affected mitogen-activated protein kinase signaling pathway, circadian rhythm genes and non-Hodgkin’s lymphoma-associated genes

In addition to the IPA analysis, we performed a KEGG pathway analysis and identified that mitogen-activated protein kinase (MAPK) signaling pathway and circadian rhythm pathway were dysregulated following chronic TCDD exposure. Table 7

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**Table 1 - CD4+ cell differentiation and Th1/Th2 ratio.**

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Control (μg/kg bw)</th>
<th>TCDD (μg/kg bw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Total 0</td>
<td>% Total 0.1</td>
</tr>
<tr>
<td>Th1</td>
<td>2.33 ± 0.2</td>
<td>1.73 ± 0.08**</td>
</tr>
<tr>
<td>% Ctrl</td>
<td>100</td>
<td>74</td>
</tr>
<tr>
<td>Th2</td>
<td>4.29 ± 0.68</td>
<td>3.70 ± 0.68*</td>
</tr>
<tr>
<td>% Ctrl</td>
<td>100</td>
<td>86</td>
</tr>
<tr>
<td>Treg</td>
<td>2.58 ± 0.25</td>
<td>2.09 ± 0.08</td>
</tr>
<tr>
<td>% Ctrl</td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td>Th1/Th2</td>
<td>0.54</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*p < 0.05 and **p < 0.01, data represents mean ± SEM, n = 5–8.
shows the differentially expressed genes involved in these two pathways.

Since the incidence of the non-Hodgkin's lymphoma (NHL) is significantly increased in the cases of human TCDD exposure as mentioned before, we compared our current data set of differentially expressed genes with the reported NHL-associated genes data sets, as well as with the IPA NHL-associated genes (Lan et al., 2015a). Fig. 4 shows that

**Table 2 – Cytokine changing pattern after chronic TCDD exposure.**

<table>
<thead>
<tr>
<th>Cytokine (pg/mL)</th>
<th>0</th>
<th>0.1 µg/kg bw</th>
<th>1 µg/kg bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-inflammation</td>
<td>IL-6</td>
<td>7.13 ± 2.02</td>
<td>5.03 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>IL-22</td>
<td>14.53 ± 3.87</td>
<td>16.48 ± 4.75</td>
</tr>
<tr>
<td>Anti-inflammation</td>
<td>IL-13</td>
<td>261.98 ± 36.24</td>
<td>209.60 ± 35.26</td>
</tr>
<tr>
<td></td>
<td>IL-12p70</td>
<td>15.75 ± 3.63</td>
<td>14.56 ± 2.9</td>
</tr>
</tbody>
</table>

Data represents mean ± SEM, n = 5–8.
four of the NHL-associated genes, IRF4, Tgif1, Fam46c and Dusp4, were present in our data sets.

3. Discussion

Many effects of TCDD have been studied following an acute exposure in experimental animals. In contrast, humans are exposed to the low daily doses of these chemicals inadvertently present in the environment (DeVito et al., 1995). In the current study, we used a murine model to mimic the chronic exposure of TCDD. We found dramatic changes of both humoral and cellular components of the immune system in exposed mice. After the 20-week exposure, percentages of both Th1 and Th2 subsets were decreased and cytokine patterns were changed as pro-inflammatory cytokines of IL-6 and IL-22 were decreased while anti-inflammatory cytokines of IL-13 and IL-12p70 were increased. These results, together with the significant decreases in IgG and IgM titers, indicated that the immune responses were suppressed. These results were consistent with previous reports following human accident exposures (Sulentic and Kaminski, 2011b), indicating that the selected murine model accurately recapitulated a paradigm of the chronic human exposure of TCDD and could provide information for further studies.

One of the reasons leading to dramatically reduced IgG and IgM production could be the decrease in number of the antibody-producing plasma cells, due to the arrest in the B cell maturation. B cell differentiation into the plasma cell is predominantly triggered by the contact with the T helper cells. The Th2 cells stimulate the immature B cells to proliferate into the antibody secreting plasma cells while Th1 cells promote the production of IgG (Licona-Limón et al., 2013; MacLeod et al., 2011; Smith et al., 2000). The TCDD exposure has led to a dramatic reduction in both Th1 and Th2 cells; that alone is expected to cause a partial arrest in B cell maturation into plasma cells. While we observed moderate increase in CD19+ cells, these cells exhibited a genetic signature of the immature B cells characterized by significantly increased expression of the pre-B markers, transcriptional factors Gfi1 and IRF4 (Ellmeier and Taniuchi, 2014). Thus, the splenic B-cell phenotype was characterized by the accumulation of the pre-B cells, ultimately leading to the reduction of antibody secretion following chronic TCDD exposure.

The results of the international cohort study that examined workers exposed to dioxin showed a strong positive

| Table 3 – CD19+ B cell proportion and serum antibody levels. |
|-----------------|-----------------|-----------------|
|                 | 0               | 0.1 µg/kg bw    | 1 µg/kg bw    |
| CD19+ B cell (%)| 38.84 ± 2.93    | 47.04 ± 1.72*   | 46.71 ± 1.76* |
| IgM (pg/mL)     | 3.90 ± 0.15     | 2.75 ± 0.26*    | 2.65 ± 0.31** |
| (×10^n)         |                 |                 |               |
| IgG (pg/mL)     | 74.41 ± 21.81   | 55.32 ± 20.33   | 12.74 ± 1.28* |
| (×10^n)         |                 |                 |               |
| IgA (pg/mL)     | 7.73 ± 2.82     | 3.84 ± 1        | 6.60 ± 1.83   |
| (×10^n)         |                 |                 |               |

*p < 0.05 and **p < 0.01, data represents mean ± SEM, n = 5–8.

| Table 4 – Top ten up-regulated and down-regulated genes. |
|-----------------|-----------------|-----------------|
| Gene symbol     | Gene ID         | Log2 ratio      | p-Value       |
| Epha2           | 010139          | 10.40637526     | 6.61E–06      |
| MAAF            | 010755          | 2.92290596      | 6.90E–10      |
| ATF3            | 007498          | 2.88290008      | 1.03E–13      |
| Eps8l1          | 026146          | 2.8275738007    | 9.13E–08      |
| Irs2            | 001081212       | 2.808305848     | 1.25E–12      |
| Ogtin1          | 027950          | 2.749424145     | 6.12E–07      |
| Nr4a1           | 010444          | 2.31201751      | 5.20E–12      |
| Fox2l           | 008037          | 2.256456043     | 8.08E–13      |
| Zfbp30          | 77660           | 2.23845681      | 1.34E–13      |
| Bsgnt7          | 145222          | 2.152388812     | 3.44E–13      |
| Zbkb16          | 001033324       | -9.680099889    | 1.85E–06      |
| Hspa1l          | 013558          | -5.02256871     | 1.13E–07      |
| Erdr1           | 133362          | -2.94218787     | 6.67E–51      |
| Recq1           | 001204906       | -1.712681608    | 9.05E–06      |
| Serpine2        | 009255          | -1.608845797    | 7.66E–09      |
| Zfbp318         | 013915          | -1.469410457    | 1.90E–05      |
| Zfp455          | 001048204       | -1.458645986    | 8.65E–06      |
| Tmpo            | 001080134       | -1.41569987     | 7.01E–07      |
| Zfp759          | 172392          | -1.390753513    | 1.19E–05      |
| Zfp948          | 001002008       | -1.382533006    | 1.11E–06      |

Fig. 3 – Effects of TCDD on B cell development (percentage of CD19+ B cell) in spleen. n = 5–8.
correlation between the dioxin exposure and NHL (Kogevinas et al., 1995). The increasing worldwide incidence of NHL is strongly linked to the increasing environmental pollution (Fritschi et al., 2005). As such, NHL ranked the fifth highest diagnosed cancer in Great Britain and the incidence is increasing in many regions (Shankland et al., 2012). It is widely recognized that immunosuppression, a well-established result of TCDD exposure, is a strong risk factor for NHL, leading to 50-100 fold excess risk (Evans and Hancock, 2003). However, the molecular mechanisms linking the environmental contamination to NHL are not fully elucidated. One such link can be provided by the Eph/ephrin family of the receptor tyrosine kinases known to play critical roles in lymphocyte differentiation (Cejalvo et al., 2013; Himanen et al., 2007). Upregulation of the Ephrin signaling pathway is one commonly found marker strongly associated with the oncogenic transformation (Pasquale, 2010; Xi et al., 2012). Most cases of NLH are lymphomas originated from B cell at various stages of differentiation and dysregulation of normal Ephrin signaling. According to the IPA Core Analysis, the top canonical pathway induced by the chronic TCDD exposure was Ephrin A signaling pathway. Indeed, Eph receptor tyrosine kinase Ephrin A receptor proto-oncogene Eph2A was the top induced transcript in our dataset (Log2 = 10.41). Eph2A is overexpressed in most cancer types and specifically involved in B-cell leukemia (Charmsaz et al., 2015; Trinidad et al., 2009). Hypermethylation of Eph2A was detected in leukemia cell lines (Kuang et al., 2010). Because Eph2A is found to have two DREs in its promoter region, exposure to the TCDD can potentially lead to its overexpression. Moreover, because of the presence of two CRE, two CREB1 and two ATF3 binding sites in the Eph2A promoter, it is conceivable that Eph2A overexpression would be driven by a positive feedback loop realized through the cAMP signaling pathway, as mentioned above.

In addition to the extreme overexpression of the Eph2A, other proto-oncogenes including c-Maf (Log2, 2.92), FosI2 (Log2, 2.26) and JunB (Log2, 1.03) were also highly induced. It is important to note that the c-Maf proto-oncogene was shown by CHIP analysis to be occupied by AhR (Lo and Matthews, 2012). c-Maf physically interacts with AhR to control the transcriptional activity of the IL10 promoter in Tregs (Gandhi et al., 2010); JunB was detected in leukemia cell lines (Kuang et al., 2010). Because Eph2A is found to have two DREs in its promoter region, exposure to the TCDD can potentially lead to its overexpression. Moreover, because of the presence of two CRE, two CREB1 and two ATF3 binding sites in the Eph2A promoter, it is conceivable that Eph2A overexpression would be driven by a positive feedback loop realized through the cAMP signaling pathway, as mentioned above.

composition of tissue microarray slides. To confirm results, cDNA microarray was performed to study the impact of TCDD exposure in reporter cells (Evans and Hancock, 2003). Table 5 shows the top ten up-regulated and down-regulated genes.

### Table 5 – IPA downstream effect analysis.

<table>
<thead>
<tr>
<th>Downstream effects</th>
<th>z-score</th>
<th>Genes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation of lymphocytes</td>
<td>2.533</td>
<td>BHLHE41 ADRB2 EPHA2 PIM3 AHNAK IRS2 GR1 CCR7 IRF4 BHLHE40 ZBTB16</td>
</tr>
<tr>
<td>Invasion of cells</td>
<td>1.908</td>
<td>EPHA2 AT3 IRS2 NR4A1 BHLHE40 CCR7 CD274 LITAF TP53INP2 KDM6B PLAUR AHNAK JUNB</td>
</tr>
<tr>
<td>Cell movement</td>
<td>1.537</td>
<td>EPHA2 AT5 IRS2 OSGIN1 NR4A1 FOSL2 BHLHE40 CCR7 CD274 LITAF TP53INP2 KDM6B PLAUR</td>
</tr>
<tr>
<td>Migration of cells</td>
<td>1.530</td>
<td>EPHA2 AT5 IRS2 OSGIN1 NR4A1 BHLHE40 CCR7 CD274 LITAF TP53INP2 KDM6B PLAUR</td>
</tr>
<tr>
<td>Invasion of tumor cell lines</td>
<td>1.387</td>
<td>EPHA2 AT5 IRS2 NR4A1 CCR7 TP53INP2 KDM6B PLAUR JUNB ADRB2 SERPINE2</td>
</tr>
<tr>
<td>Proliferation of B lymphocytes</td>
<td>1.117</td>
<td>IRS2 IRF4 JUNB PIM3 ZBTB16</td>
</tr>
<tr>
<td>Differentiation of myeloid cell</td>
<td>1.029</td>
<td>DUSP5 GF11 IRF4 TRB1 ZBTB16</td>
</tr>
<tr>
<td>Growth of malignant tumor cells</td>
<td>1.026</td>
<td>EPHA2 NR4A1 CD274 LITAF TP53INP2 KDM6B PLAUR ADRB2</td>
</tr>
<tr>
<td>Proliferation of cancer cells</td>
<td>1.026</td>
<td>EPHA2 NR4A1 LITAF TP53INP2 KDM6B PLAUR</td>
</tr>
<tr>
<td>Apoptosis of tumor cells</td>
<td>−0.728</td>
<td>NR4A1 DIT3 IRF4 PLAUR KLFG10 TRB1</td>
</tr>
<tr>
<td>Activation of leukocytes</td>
<td>−0.758</td>
<td>AT3 GF11 BHLHE40 CCR7 CD274 DIT3 IRF4</td>
</tr>
<tr>
<td>Apoptosis of cancer cells</td>
<td>−1.000</td>
<td>NR4A1 DIT3 IRF4 PLAUR</td>
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</table>

Table 6 – Binding sites analysis in the promoter regions of top ten up-regulated and down-regulated genes.

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<tr>
<th>Gene Symbol</th>
<th>Gene ID</th>
<th>AhR</th>
<th>CREM</th>
<th>CREB1</th>
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<tr>
<td>Epha2</td>
<td>010139</td>
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<td>2</td>
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<tr>
<td>MAFF</td>
<td>010755</td>
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<td>ATF3</td>
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<td>2</td>
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<td>Eps81</td>
<td>026146</td>
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<td>0</td>
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<td>Irs2</td>
<td>001081212</td>
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<td>1</td>
<td>1</td>
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<tr>
<td>Osgin1</td>
<td>027950</td>
<td>0</td>
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<td>0</td>
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<td>Nr4a1</td>
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<td>FosI2</td>
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<td>Tmpo</td>
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### Table 7 – KEGG pathway analysis.

<table>
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<th>Category Term</th>
<th>Term</th>
<th>p-Value</th>
<th>Genes</th>
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</thead>
<tbody>
<tr>
<td>KEGG_PATHWAY</td>
<td>mmu04010:MAPK</td>
<td>0.014</td>
<td>DDIT3, DUSP4, HSPA1B, NR4A1</td>
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<td></td>
<td>mmu04710:Grcadian rhythm</td>
<td>0.047</td>
<td>BHLHE40, BHLHE41</td>
</tr>
</tbody>
</table>
NHL related genes from previous report and IPA analysis

Current data set

57
4
66

Fig. 4 – Venn diagram of NHL related genes from the previous reports and IPA analysis with the current data set showed 4 overlapping genes, IRF4, Tgif1, Fam46c and Dusp4. NHL: non-Hodgkin's lymphoma; IPA: Ingenuity Pathway Analysis.

4. Conclusions

This study was designed to mimic human exposure to the persistent organic pollutant — TCDD. We used a transcriptome analysis to elucidate possible mechanisms of decreased humoral immunity and increased incidence of lymphomas characteristic of chronic human exposures to TCDD. Our murine model exhibited an overall immunosuppressive outcome from a 20-week TCDD exposure that was consistent with human exposure phenotypes. In addition, we identified a molecular signature of chronic TCDD exposure in splenic B cells that was consistent with the arrested maturation and lymphogenic transdifferentiation of these cells.

Using the IPA analysis, we identified CREM and CREB1 as the top upstream regulators of the transdifferentiation, and the ephrin signaling pathway as an effector of tumorigenic, proliferative and cell-migratory downstream effects. Eph2A was the most up-regulated gene and thus can serve as a possible marker and/or target of the lymphogenic B cell transdifferentiation. Our study has identified a putative link between cAMP and AhR signaling pathways in B cell differentiation and suggested that the cAMP signaling pathway could be an important regulator in AhR-mediated immune dysfunction. Further studies should be conducted to confirm the specific roles of the dysregulated genes in the observed pathological phenotype, including the specific molecular mechanisms involved in the arrested B cell maturation.
Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.11.011.

REFERENCES


