2,3,7,8-Tetrachlorodibenzo-p-dioxin Induces Premature Activation of the KLF2 Regulon during Thymocyte Development

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The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) causes numerous and diverse toxic events via activation of the aryl hydrocarbon receptor, including atrophy of the thymus. Exposure to TCDD induces acute thymocyte cell loss, which occurs concomitantly with proliferation arrest and premature emigration of triple negative (TN; CD4−, CD8−, CD3−) T cell progenitors. In this report, we demonstrate that TCDD exposure results in dysregulation of KLF2 (Kruppel-like factor 2) expression in developing thymocytes. The KLF2 gene encodes an Sp1-like zinc finger transcription factor that functions as a central regulator of T lymphocyte proliferation and trafficking. During normal thymocyte development, KLF2 is expressed exclusively in CD4 and CD8 single positive T cells and promotes a nonproliferative, promitogenic phenotype. In mice exposed to TCDD, however, the KLF2 gene is prematurely expressed in TN thymocytes. Administration of a 100 μg/kg dose of TCDD results in a 15-fold induction of KLF2 as early as the TN2 (CD44−, CD25+) stage of development and immediately precedes acute cell loss in the TN3, TN4, and double positive (CD4+, CD8+) cell stages. Induction of KLF2 occurs within 12 h of TCDD exposure and is fully dependent on expression of the aryl hydrocarbon receptor. In addition, TCDD exposure alters the expression of several factors comprising the KLF2 regulon, including Edg1/S1P1, β1 integrin, CD52, Cdkn2d (cyclin-dependent kinase inhibitor 2D), s100a4, and IL10Rα. These findings indicate that the pollutant TCDD interferes with early thymopoiesis via ectopic expression of the KLF2 regulon.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congener of a family of halogenated aromatic hydrocarbons produced by common industrial processes (1). Exposure to TCDD induces thymic atrophy, immune suppression, hepatic toxicity, and carcinogenic effects, including cleft palate, chloracne, hepatomegaly, cachexia, and several other diverse toxic end points (1, 2). Extensive pharmacological and genetic research has demonstrated that TCDD toxicity is mediated through activation of the aryl hydrocarbon receptor (AHR or Ah receptor), a ligand-activated member of the PAS (Per-ARNT-Sim) family of environmental sensors (3–6). Due to widespread human and animal exposure to environmental agonists of the AHR (1), these compounds pose a serious threat to public health. In the current study, we investigate how exogenous activation of the AHR provokes atrophy of the thymus.

The thymus is the primary source of the T cell repertoire and thus a critical part of the adaptive immune system. Within this organ, T cell development occurs in a series of highly regulated differentiation events (7). At the beginning of this developmental process, the thymus is seeded with pluripotent bone marrow progenitors. Interactions with resident stromal cells then induce T lineage commitment and V(D)J rearrangement of the genes encoding the α and β chains of the T cell receptor (TCR) (7). Several surface markers are coordinately regulated during this developmental process and are commonly used to represent specific stages of thymopoiesis (see Fig. 1A). The earliest progenitors within the thymus are negative for the mature surface markers CD4, CD8, and CD3 (8). These “triple negative” (TN) cells can be further categorized into four ordered developmental stages during which the TCRβ gene is rearranged and then expressed at the cell surface: TN1 (CD44+, CD25−) → TN2 (CD44+, CD25+) → TN3 (CD44−, CD25+) → TN4 (CD44−, CD25−) (8). If TCRβ rearrangement is successful, thymocytes then initiate recombination of TCRα as well as CD4 and CD8 expression (7). Although “double positive” (DP; CD4+, CD8+) cells comprise ~80% of all thymocytes, the vast majority of these cells fail to generate an appropriate TCRαβ signaling complex (7). Only cells that pass both positive and negative TCR selection are released into the bloodstream as CD4 helper or CD8 cytotoxic single positive (SP) T cells.

The process of TN development is highly sensitive to TCDD. Exposure to this environmental pollutant results in proliferation arrest of the TN3 and TN4 stages of development (9), as well as premature emigration of TN3-like thymocytes (10). Several lines of evidence suggest that these TN defects are due to cell autonomous changes in gene transcription. Prior research has demonstrated that thymocyte, but not stromal, expression of the AHR signaling pathway is required for TCDD-induced atrophy (9, 11, 12). Moreover, mutant constructs of the Ah receptor have demonstrated that AHR/DNA interactions are
required for thymic toxicity (13). Overall, these results are consistent with the idea that TCDD dysregulates TN expression of a gene, or genes, with a critical role in thymocyte proliferation and/or emigration.

In this report, we demonstrate that TCDD triggers misexpression of the KLF2 (Kruppel-like factor 2) regulon in an AHR-dependent manner. During normal thymocyte development, KLF2 is expressed exclusively in the mature SP populations and enforces (i) egress to the peripheral lymphoid organs and (ii) peripheral T cell quiescence (14–19). Here, we show that the environmental contaminant TCDD induces ectopic expression of the KLF2 transcription factor and its associated regulon during TN thymopoiesis.

**EXPERIMENTAL PROCEDURES**

**Mice**—“Wild-type” C57BL/6 and Rag-1<sup>m1Mom</sup> (Rag-1 null) mice (20) were obtained from the Jackson Laboratory (Bar Harbor, ME). AHR null mice (21) were obtained from a colony maintained at the University of Wisconsin (Madison, WI). The AHR and Rag-1 null lines were backcrossed to the C57BL/6 genotype for at least 10 generations. Animals were housed in a selective pathogen-free environment and supplied with food and water ad libitum, according to the rules and guidelines of the University of Wisconsin.

**TCDD Treatment**—5–7-week-old males were injected once intraperitoneally with 100 µg/kg body weight TCDD. Me<sub>2</sub>SO was used as vehicle control at a dose of 1.25 ml/kg body weight. Animals from age-matched litters were used for each experiment.

**Thymocyte Preparation**—Each thymus was homogenized by gentle grinding in low glucose Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (Invitrogen) and passage through a 20-gauge needle. Red blood cells were lysed in ammonium chloride buffer (0.17 M NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 1 mM EDTA, pH 7.4) for 5 min on ice. Thymocyte samples were then pelleted and resuspended in phosphate-buffered saline supplemented with 0.5% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide (both from Sigma).

**Cell Counting**—Thymus cell count was determined using a BD Biosciences FACSCalibur flow cytometer (Franklin Lakes, NJ). Each thymocyte sample was acquired for 15 s at a 0.2 µl/s flow rate, gated based on forward and side scatter, and then enumerated.

**Antibodies**—The following monoclonal antibodies were purchased from Ebioscience (San Diego, CA) or Pharmingen (Franklin Lakes, NJ) and used at a saturating concentration: fluorescein isothiocyanate (FITC)-conjugated CD4 (clone GK1.5; Ebioscience) and CD44 (IM7; Ebioscience); PE-conjugated CD8α (53–6.7; Pharmingen) and CD25 (3C7; Pharmingen); PE-Cy5-conjugated CD3e (145–2C11; Pharmingen) and β<sub>7</sub> integrin (FIB504; Pharmingen); APC-conjugated CD3ε (145–2C11; Ebioscience), CD11β (M1/70; Ebioscience), CD8α (53–6.7; Ebioscience), CD4 (RM4-5; Ebioscience).

**Flow Cytometry**—Thymocytes were incubated in staining buffer (phosphate-buffered saline plus 0.5% bovine serum albumin and 0.1% NaN<sub>3</sub>) with the appropriate antibodies for 30 min at 4 °C. Each thymocyte sample was stained using two independent antibody mixtures. The CD4/CD8 double positive and single positive cells were analyzed using fluorescein isothiocyanate–CD4 and PE–CD8 antibodies. Triple negative cells were analyzed using fluorescein isothiocyanate–CD44, PE–CD8, PE–Cy5 or APC–CD3ε, APC–CD8α, APC–CD44, APC–CD45R, and APC–CD11β antibodies. All cells negative for CD4/8/3/11β/CD45R staining were used for triple negative gating based on CD4 and CD25 expression. After antibody incubation, each sample was washed once with staining buffer and resuspended in staining buffer plus 1% (w/v) formaldehyde. Data were acquired using a FACSCalibur cytometer after calibrating for fluorescence channel overlap using the appropriate staining controls. Quantitation analysis was completed on BD Biosciences Cellquest software. The flow cytometry figures used in this publication were created using WinMDI 2.8 (Scripps Institute, La Jolla, CA) or Flojo (Ashland, OR) software.

**RNA Isolation and qRT-PCR**—Unfixed cells were sorted at 4 °C using a BD Biosciences FACSVantage SE flow cytometer. Sorting accuracy was determined to be greater than 98% in each experiment. Total RNA was isolated from each cell sample using RNeasy kits with DNase treatment (Qiagen, Valencia, CA). Quantitect RT-PCR kits (Qiagen) were then used for combined reverse transcription and amplification of samples with gene-specific primers (IDT (Coralville, IA) and Applied Biosystems (Foster City, CA)). At least three replicate reactions were assayed for each sample using the ABI Prism 7900 system (Applied Biosystems, Foster City, CA). Equal amounts of total RNA were added to each well as measured by the 2100 Bioanalyzer (Agilent, Foster City, CA); RNA loading was further normalized to endogenous levels of hypoxanthine-guanine phosphoribosyl transferase. Fold change was calculated as 2<sup>(ΔΔCT)</sup>. RT-PCR primers are provided in the supplemental materials.

**Microarray Profiling**—Total RNA (~100 ng) was isolated from TN2/TNX (CD4<sup>int</sup>, CD25<sup>high/int</sup>) cells, which were sorted from a pool of Rag-1 null mice treated with TCDD or Me<sub>2</sub>SO for 48 h (n = 8 for each treatment). Microarray comparisons were performed by the Genomics Institute of the Novartis Research Foundation (San Diego, CA), as previously described (see Refs. 22 and 23 and references therein). Briefly, each RNA was split into duplicate samples, linearly amplified, and then hybridized to GeneAtlas GNF1M high density oligonucleotide arrays (22, 23). Fluorescence values were normalized by global median scaling. Experimental variations due to differences in RNA amplification or hybridization were assessed using a two-tailed t test. Time course data (Fig. 2) were analyzed using a two-way analysis of variance with a Bonferroni posttest for differences between treatment groups.

**RESULTS**

**Exposure to TCDD Disrupts TN2 Thymopoiesis**—The overarching goal of this study was to elucidate how TCDD interferes with mammalian thymopoiesis. Toward this end, we first set out to identify which stages of thymocyte development were subject to overt TCDD toxicity. As an initial step, all thymocytes were classified into developmental stages based upon sur-
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FIGURE 1. TCDD induces a novel TN phenotype, TNX, via a decrease of CD25 surface expression in late TN2 stage cells. A, hematopoietic precursor cells undergo a series of regulated differentiation steps in the thymus in order to produce mature peripheral lymphocytes. Cells within the thymus can be classified into specific developmental stages based upon the surface expression of several developmentally regulated proteins. B, C57BL/6 mice (n = 5) were treated with TCDD or Me2SO and analyzed as described under "Experimental Procedures." Top and bottom, representative dot and contour plots, respectively, of CD44/CD25 staining from each treatment group. A novel cell population, TNX (boxed region; CD44int, CD25int) was observed after TCDD exposure. A loss of CD44intTN2 stage cells (arrowhead) was also observed. Note the concave pattern of CD25 expression during TN2 to TN3 development in TCDD-treated mice. C, Rag-1null (nontreated) mice, which have a development block at the TN3 stage, were treated with TCDD or Me2SO (DMSO) for 24 h (n = 5). Thymocytes were pooled from each treatment group and analyzed via flow cytometry. Data are presented as zebra-style contour plots.

face expression of CD3, CD4, CD8, CD25, and CD44 (see Fig. 1A). Unexpectedly, we observed that TCDD dramatically altered the canonical pattern of CD44/CD25 expression. Treatment with TCDD consistently induced a novel TN phenotype, which was intermediate in both CD44 and CD25 expression (CD44int, CD25int) (Fig. 1B, boxed region). Due to its unknown relationship to classical thymocyte development, we named this population triple negative X (TNX). In addition to TNX formation, we observed a subtle loss of the late TN2 (CD44int, CD25high) phenotype in mice treated with TCDD (Fig. 1B, arrowhead; note the concave pattern of CD25 expression during TN2 to TN3 development in TCDD-treated mice).

We hypothesized that the TN2 and TNX phenotypes were related phenomena (i.e. TN2 stage cells (CD44int, CD25high) were diverted to the TNX phenotype (CD44int, CD25int) by TCDD exposure. To test this hypothesis, we treated Rag-1 null mice, which have a profound developmental block at the TN3 stage of development (20), for 24 h with TCDD or Me2SO. We observed that TCDD treatment markedly attenuated surface expression of CD25 in TN2 stage cells (Fig. 1C). The geometric mean of CD25 expression in control and TCDD-treated TN2 cells was 11,804 and 5028 units, respectively. These results were consistent with the idea that TNX phenotype cells are a TCDD-induced derivative of the late TN2 (CD44int, CD25high) stage of development.

We next set out to determine the relationship between TNX formation and TCDD-induced thymic cell loss. Toward this end, we first determined total thymus cellularity over a time course of TCDD exposure. We found that thymocyte cell number was significantly decreased within 72 h in TCDD-treated animals; a ~90% loss of control cell number was observed by 7 days (Fig. 2A). We then asked if cell loss occurred across all thymocyte populations or was specific to select developmental stages. In order to accurately enumerate each population, we designed our fluorescence gating to exclude TNX cells from all other TN populations (Fig. 2B). We then calculated the relative number of cells in each developmental stage by multiplying the percentage of cells in each stage by the previously determined total thymus cell count. Using this method, we observed that TCDD does not significantly affect the cellularity of the TN1 and early TN2 (CD44high) stages of development (Fig. 2C). The cell populations developmentally subsequent to the TN2/TNX stage, however, were significantly reduced in cell number. The TN3, TN4, and DP cell populations each exhibited acute cell loss within 48 h of TCDD exposure.

In order to elucidate the molecular events that lead to cell loss, we set out to determine the transcriptional profile of the late TN2 (CD44int) population in both its normal and TCDD-exposed states. To obtain enough RNA for microarray analysis, we sorted TN2/TNX cells from a pool of mice, which were treated with TCDD or Me2SO for 48 h (n = 8 for each treatment). The Rag-1 null genotype was utilized for these experiments due to fluorescence overlap between the TNX and TN4 population, which is present in wild type but not Rag-1 null mice. Total RNA was isolated from each cell population and used to determine global gene expression using high density oligonucleotide microarrays (22, 23). Treatment with TCDD was found to alter the expression of ~2000 genes greater than 1.5-fold and ~350 genes greater than 5-fold in each of two arrays. The top 20 up- and down-regulated expression changes are presented in Tables 1 and 2, respectively. The entire data set is available as supplemental material.

**TCDD Induces Ectopic Expression of KLF2**—The data set of genes that were most highly altered by TCDD exposure was mined for central regulators of T cell biology. We found that KLF2, a master regulator of T cell activation and trafficking (15–19), was induced >13-fold in TCDD-treated cells (Table 1). In order to verify induction of KLF2, we sorted an independ-
ent sample of TN2/TNX cells from Rag-1 null mice, which were treated with TCDD or Me2SO for 12 h. Quantitative RT-PCR was then used to measure mRNA expression of KLF2 as well as several genes previously shown to be TCDD-inducible in the thymus, such as Cyp1b1, Scinderin, and Lgals3 (24, 25). Using qRT-PCR, we confirmed a robust increase (>5-fold) in each of these mRNA transcripts in response to TCDD, including a 15-fold increase of KLF2 (Fig. 3A).

Next, we utilized AHR null mice to determine if the KLF2 response to TCDD was mediated by the AHR and independent of the Rag-1 null genotype. Toward this end, we isolated TN3 stage cells from a pool of C57BL/6 or AHR null mice treated with TCDD or Me2SO for 24 h. The levels of KLF2 and Cyp1b1 mRNA in each treatment group were then measured via qRT-PCR. The Ahr−/− cell population exhibited a >19-fold increase in both KLF2 and Cyp1b1 mRNA in response to TCDD (Fig. 3B). The cells isolated from AHR null mice, however, did not exhibit induction of either mRNA following TCDD treatment (Fig. 3B).

**TCDD Induces Misexpression of the KLF2 Regulon**—The KLF2 protein is a member of the Sp1 family of transcription factors and a promiscuous regulator of gene expression (26). Due to the observed level of KLF2 induction, we reasoned that genes regulated by KLF2 might also be significantly altered by TCDD exposure. In order to test this hypothesis, we surveyed the literature for genes which have been shown to be highly altered (>2.5-fold) by KLF2 overexpression (19) or deficiency (18) in T lineage cells. We then examined the expression of these genes in our microarray data set. Using this approach, we identified several members of the KLF2 regulon as putative TCDD-inducible genes (Table 3).

The capacity of TCDD to alter expression of KLF2-regulated genes was then confirmed at both the protein (Fig. 4, A and B) and mRNA level (Fig. 4C). First, we determined protein expression of β7 integrin using a fluorochrome-labeled antibody against this epitope. Exposure to TCDD was found to increase protein levels exclusively in TN thymocytes (Fig. 4A). In order to determine which TN populations were affected, we further examined β7 expression using fluorescent gating similar to that shown in Fig. 2B. We observed that β7 expression was induced by TCDD exclusively in TN2 and TN3 stage cells (Fig. 4B). Although the relative increase in β7 expression was similar in

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**FIGURE 2.** Exposure to TCDD induces acute, early cell loss in the TN3, TN4, and DP cell stages. C57BL/6 mice (n = 5) were treated with TCDD or Me2SO over a 7-day time course. A, the number of cells in each thymus was determined using a flow cytometer at constant flow rate as described under “Experimental Procedures.” B, the percentage of cells in each developmental stage was determined by fluorescent gating. The gating used to analyze the TN populations was specifically designed to exclude the novel TNX population. DMSO, Me2SO. C, the relative number of cells in each developmental stage was calculated by multiplying the total thymocyte count (shown in A) by the percentage of cells in each fluorescent gate (shown in B). All data are presented as the mean ± S.D. *, p < 0.05; ***, p < 0.001.
TCDD Induces Ectopic Expression of KLF2

**TABLE 1**

Top 20 genes up-regulated by TCDD in the TN2/TNX cell population

TN2/TNX cells were sorted from a pool of Rag-1 null mice treated with TCDD or Me2SO for 48 h (n = 8 for each treatment). Global gene expression was determined by hybridization to high density oligonucleotide microarrays in duplicate. Each data point represents the fluorescence value at the designated probe location following chip-to-chip normalization.

| Common name | Full name | NCBI gene ID | TCDD-A | TCDD-B | Me2SO-A | Me2SO-B | Fold-change |
|-------------|-----------|--------------|--------|--------|---------|---------|-------------|
| kfl2        | Kruppel-like factor 2 (lung) | 10365    | 2976.7 | 2674.1 | 217.9   | 204.1   | 13.4       |
| IL12rb1     | Interleukin 12 receptor, β1 | 12594    | 545.6  | 487.2  | 29.8    | 47.4    | 1.6        |
| Vpa25       | Vacuolar protein sorting 25 | 328222   | 666.2  | 631.2  | 48.9    | 56.3    | 1.23       |
| Sxxb2       | Synovial sarcoma, X member B, breakpoint 2 | 387132   | 18.4   | 24.4   | 1      | 2.5     | 1.22       |
| Cxtn1       | Cortxin 1 | 330695    | 157.6  | 161.7  | 13.3    | 14.2    | 1.16       |
| 13000181J18Rik | RIKEN cDNA 13000181J18 gene | 223737   | 225    | 177.3  | 13.8    | 22.8    | 1.10       |
| 9130430E04  | RIKEN cDNA 9130430E04 gene | 67575    | 129.4  | 88.2   | 10.7    | 9.4     | 1.08       |
| Ret         | Ret proto-oncogene | 19713    | 180.2  | 125.4  | 18.7    | 9.7     | 2.06       |
| Ifit3       | Interferon-induced protein with tetratricopeptide repeats 3 | 115997   | 99.1   | 94.7   | 10.2    | 9.2     | 1.00       |
| Traf5       | Traf5 receptor-associated factor 5 | 22033    | 111.8  | 154.8  | 11.6    | 16.2    | 1.42       |
| Ifg7        | Integrin β7 | 16421    | 2209.4 | 2317   | 94.7    | 391.5   | 9.3        |
| Zcc2        | Zinc finger, CCHC domain-containing 2 | 227447   | 230.1  | 275.6  | 27.2    | 29.6    | 0.91       |
| Unknown     | Unclassified transcribed locus | Mm.126037 | 76     | 50.8   | 7.8     | 7       | 8.6        |
| Olf41265    | Olfactory receptor 1265 | 258340   | 233.9  | 162.1  | 20.7    | 26.3    | 0.84       |

**TABLE 2**

Top 20 genes down-regulated by TCDD in the TN2/TNX cell population

| Common name | Full name | NCBI gene ID | TCDD-A | TCDD-B | Me2SO-A | Me2SO-B | Fold-change |
|-------------|-----------|--------------|--------|--------|---------|---------|-------------|
| Cyp1b1      | Cytochrome P450 1B1 | 1545     | 5451.0 | 7606.8 | 152.2   | 245.1   | 33.1        |
| Bcl9        | B-cell CLL/lymphoma 9 | 77578    | 18.6   | 28.9   | 1       | 1.4     | 19.8        |
| Acp1        | Acid phosphatase prostate | 56318    | 288.3  | 247.7  | 14.2    | 20.3    | 15.2        |
| Tnn1S4      | Transmembrane 9 superfamily protein member 4 | 99237    | 210    | 239.5  | 17.1    | 15.1    | 14.0        |
| Klf2, Lklf  | Kruppel-like factor 2 (lung) | 10365    | 2976.7 | 2674.1 | 217.9   | 204.1   | 13.4        |
| Il12rb1     | Interleukin 12 receptor, β1 | 3594    | 545.6  | 487.2  | 29.8    | 47.4    | 1.6        |
| Vpa25       | Vacuolar protein sorting 25 | 28084    | 666.2  | 631.2  | 48.9    | 56.3    | 1.23       |
| Sxxb2       | Synovial sarcoma, X member B, breakpoint 2 | 387132   | 18.4   | 24.4   | 1      | 2.5     | 1.22       |
| Cxtn1       | Cortxin 1 | 330695    | 157.6  | 161.7  | 13.3    | 14.2    | 1.16       |
| 13000181J18Rik | RIKEN cDNA 13000181J18 gene | 223737   | 225    | 177.3  | 13.8    | 22.8    | 1.10       |
| 9130430E04  | RIKEN cDNA 9130430E04 gene | 67575    | 129.4  | 88.2   | 10.7    | 9.4     | 1.08       |
| Ret         | Ret proto-oncogene | 19713    | 180.2  | 125.4  | 18.7    | 9.7     | 2.06       |
| Ifit3       | Interferon-induced protein with tetratricopeptide repeats 3 | 115997   | 99.1   | 94.7   | 10.2    | 9.2     | 1.00       |
| Traf5       | Traf5 receptor-associated factor 5 | 22033    | 111.8  | 154.8  | 11.6    | 16.2    | 1.42       |
| Ifg7        | Integrin β7 | 16421    | 2209.4 | 2317   | 94.7    | 391.5   | 9.3        |
| Zcc2        | Zinc finger, CCHC domain-containing 2 | 227447   | 230.1  | 275.6  | 27.2    | 29.6    | 0.91       |
| Unknown     | Unclassified transcribed locus | Mm.126037 | 76     | 50.8   | 7.8     | 7       | 8.6        |
| Olf41265    | Olfactory receptor 1265 | 258340   | 233.9  | 162.1  | 20.7    | 26.3    | 0.84       |

**FIGURE 3.** KLF2 is induced by TCDD in an AHR-dependent manner. A, TN2/TNX stage thymocytes were harvested from a pool of Rag-1 null mice treated with TCDD or Me2SO for 12 h (n = 3). Expression of the depicted genes was determined by qRT-PCR. B, TN3 stage thymocytes were sorted from a pool of Rag-1 null mice treated for 48 h with TCDD or Me2SO control (n = 5 for each treatment). Global gene expression was determined by hybridization to high density oligonucleotide microarrays in duplicate. Each data point represents the fluorescence value at the designated probe location following chip-to-chip normalization.

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Mammalian exposure to the environmental pollutant TCDD provokes a broad spectrum of toxic endpoints (1, 2). The effects of TCDD on the thymus are particularly potent (Fig. 2A). We hypothesized that exogenous activation of the AHR transcription factor could lead to ectopic expression of a gene or genes deleterious to normal thymopoiesis. We therefore set out to determine (i) which stages of thymocyte development are disturbed by TCDD and (ii) which genes are misexpressed in these affected populations.

A series of flow cytometry experiments established that TCDD impairs thymopoiesis as early as the TN2 stage of development (Figs. 1 and 2). In initial studies, we observed that TCDD exposure induces the formation of a novel phenotypic derivative of the TN2 stage of development, which we termed TNX (Fig. 1, B and C). Moreover, we found that the three developmental stages downstream of TN2/TNX exhibit acute TCDD-induced cell loss, whereas the developmental stages upstream of TN2/TNX exhibit no change in cell number (Fig. 2C). These results suggested that TCDD-induced cell loss could be precipitated via interference in the transition from the TN2 to TN3 stage of thymopoiesis.

Microarray studies of TN2 cells in both their normal and TCDD-induced states (TNX) confirmed that TCDD substantially alters TN2 thymopoiesis. We found that TCDD alters the expression of over 350 genes by at least 5-fold and ~2000 genes by at least 1.5-fold. As expected, many known AHR-regulated genes were found to be induced by TCDD exposure, such as Cyp1b1 (27), Lgals3 (24), PON1 (28), NQO1 (29), AHRR (30), and Scin (25). The preponderance of gene expression changes, however, has not been previously identified as TCDD-inducible. Since the AHR is thought to be a relatively nonpromiscuous regulator of transcription, we hypothesized that this massive change in gene expression could be preceded by misexpression of a central regulator of T lineage biology.

We observed that KLF2, a member of the Sp1 family of transcription factors, is ectopically expressed in TN2 and TN3 stage thymocytes within 12 h and 24 h of TCDD exposure, respectively (Fig. 3). Similar to other members of the Sp1/KLF family, KLF2 is a potent and promiscuous regulator of transcription that binds to CACC and GC-rich regions in gene promoters (26). Although KLF2 expression is limited to specific cell lineages (31), it has been demonstrated to be a master regulator of cellular function in these cell types, including vascular endothelial cells (32–34), adipocytes (33, 35), monocytes (36), and T lymphocytes, in which the KLF2 regulon has a dual role in controlling both T cell migration and proliferation (15–19). In nor-

### TABLE 3

| Common name | Full name | NCBI gene ID | TCDD-A | TCDD-B | Me$_{50}$O-A | Me$_{50}$O-B | -fold change | TCDD | KLF2 | Reference |
|-------------|-----------|--------------|--------|--------|-------------|-------------|--------------|------|------|-----------|
| Igkb7       | Integrin β7 | 16421        | 2209.4 | 2317   | 94.7        | 391.5       | 9.3          | >10  | 16, 17|
| S100a4      | S100 calcium-binding protein A4 | 20198        | 430.3  | 388    | 10.2        | 123.3       | 6.1          | 4.1  | 17    |
| Edg1, S1P1  | G-protein-coupled receptor 1 sphingolipid G-protein-coupled receptor 1 | 13609        | 242.5  | 361.3  | 66.9        | 58.2        | 4.8          | 5.8  | 16, 17|
| Cdkn2d      | Cyclin-dependent kinase inhibitor 2D | 12581        | 767.6  | 744.8  | 129.1       | 317.6       | 3.4          | 2.6  | 17    |
| Emp3        | Epithelial membrane protein 3 | 13732        | 1895.1 | 1893.4 | 551.5       | 627         | 3.2          | 3.9  | 17    |
| IL10ra      | Interleukin 10 receptor, α | 16154        | 102.4  | 128.5  | 48.6        | 32          | 2.9          | 3.7  | 17    |
| CD52        | CD52 antigen | 23833        | 903.9  | 1221.4 | 493         | 358.5       | 2.5          | ~10  | 17    |
| Ptpn13      | Protein-tyrosine phosphatase, nonreceptor type 13 | 19249        | 131    | 116.2  | 10.2        | 123.3       | 17           |      |       |

FIGURE 4. Exposure to TCDD alters expression of the KLF2 regulon. A and B, C57BL/6 mice (n = 5) were treated with Me$_{50}$O or TCDD for 72 h. A, expression of β7 integrin was assessed using flow cytometry. A representative thymus was selected for each histo-

DISCUSSION

We observed that KLF2, a member of the Sp1 family of transcription factors, is ectopically expressed in TN2 and TN3 stage thymocytes within 12 h and 24 h of TCDD exposure, respectively (Fig. 3). Similar to other members of the Sp1/KLF family, KLF2 is a potent and promiscuous regulator of transcription that binds to CACC and GC-rich regions in gene promoters (26). Although KLF2 expression is limited to specific cell lineages (31), it has been demonstrated to be a master regulator of cellular function in these cell types, including vascular endothelial cells (32–34), adipocytes (33, 35), monocytes (36), and T lymphocytes, in which the KLF2 regulon has a dual role in controlling both T cell migration and proliferation (15–19). In nor-
mal T cell development, KLF2 is first expressed in mature SP thymocytes (14, 15) and triggers emigration to the peripheral immune organs via transcriptional control of a gene battery, which includes Edg1/S1P, and β7 integrin (18). In the peripheral T cell population, KLF2 is constitutively expressed in the absence of TCR stimulation and is a negative regulator of T lineage activation/proliferation (15, 16). It has been demonstrated that forced expression of KLF2 is sufficient to program T cell quiescence (16), whereas deficiency results in spontaneous activation (15).

In TCDD-treated mice, induction of KLF2 occurs concomitantly with misexpression of numerous KLF2-regulated genes (Fig. 4). This phenotype includes robust activation of Edg1/S1P and β7 integrin, which have a critical role in thymocyte egress (18, 37–39), as well as Cdkn2d, which negatively regulates cell cycle entry (40) (Fig. 4). Moreover, these genes likely represent only a small portion of the total number affected by premature expression of KLF2. Previous studies in vascular endothelial cells have indicated that up to 1000 genes are transcriptionally controlled through the expression level of KLF2 (33). These results indicate that ectopic expression of KLF2 may have a widespread effect on the TN transcriptome. Although the cumulative effects of these transcriptional changes on TN biology are uncertain, it is interesting to note that TCDD has been previously demonstrated to induce both premature emigration (10) and proliferation arrest of TN cells (9). Due to the prominent role of the KLF2 regulon in enforcing these phenotypes in SP biology (15–17), it is tempting to speculate that ectopic expression of KLF2 may have a similar effect on TN stage cells. Overall, these results suggest that premature expression of KLF2 has a substantial effect on TN thymopoiesis. Although these results do not deny the possible importance of additional factors, the current study identifies KLF2 as a promising molecular link between TCDD exposure and thymic toxicity.

The full mechanism underlying the interplay between TCDD and the KLF2 signaling pathways is currently unclear. Although KLF2 induction occurs during the nascent stage of TCDD toxicity and is AHR-dependent (Fig. 3B), we have not yet been able to demonstrate direct AHR/promoter interaction. Chromatin immunoprecipitation studies are highly intractable for TN stage cells, and we have not yet identified a more suitable cell line or tissue that exhibits a KLF2 response to TCDD. In the absence of such data, it remains possible that KLF2 is induced by TCDD via an indirect AHR-mediated protein and/or lipid response. Independent of mechanism, it is plausible that non-thymocyte cell populations are also affected by AHR/KLF2 interplay. Due to the substantial role of KLF2 in determining cell phenotype, future studies should be conducted to determine if AHR/KLF2 interactions influence additional end points of TCDD toxicity and/or endogenous biology. Of particular interest is vascular physiology, wherein both the AHR and KLF2 have a central role in the transcriptional response to shear stress (33, 41, 42).

CONCLUSIONS
The environmental pollutant TCDD is a highly toxic agonist of the AHR signaling pathway. In this report, we have demonstrated that TCDD induces ectopic expression of KLF2 and its associated regulon during early thymopoiesis. Overall, these results demonstrate a novel relationship between AHR and KLF2 signaling and strongly suggest that KLF2 has an important role in aspects of TCDD toxicity.

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