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Cell Proliferation Arrest within Intrathymic Lymphocyte Progenitor Cells Causes Thymic Atrophy Mediated by the Aryl Hydrocarbon Receptor

Michael D. Laiosa,* Amber Wyman,† Francis G. Murante,† Nancy C. Fiore,* J. Erin Staples,* Thomas A. Gasiewicz,† and Allen E. Silverstone‡

Activation of the aryl hydrocarbon receptor (AHR), a basic helix-loop-helix transcription factor, in lymphocytes by the immunosuppressive environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been shown to cause thymic atrophy in every species studied. We set out to identify the specific hemopoietic cellular populations in which the AHR was activated to lead to thymic atrophy and to determine the effect of AHR activation in those cellular populations. Initially, we examined whether AHR activation in intrathymic dendritic cells could mediate TCDD-induced thymic atrophy. It was found that thymic atrophy occurred only when the AHR could be activated in the thymocytes but not hemopoietic-derived dendritic cells or other APCs. We next analyzed the effect of TCDD on the proliferation of thymocytes in vivo. There was a significant increase in the percentage of thymocytes in the G1 phase of the cell cycle and a significant decrease in the percentage of S plus G2/M thymocytes, especially in the CD4<sup>+</sup> CD8<sup>−</sup> triple-negative intrathymic progenitor cell population 24 h after exposure to 30 µg/kg TCDD. Furthermore, by 12 h after exposure to TCDD, we observed ~60% reduction of 5-bromo-2-deoxyuridine incorporation in specific intrathymic progenitor cell populations. This reduction persisted for at least 6 days. These data indicate that intrathymic progenitor cells are direct targets of TCDD in the thymus and suggest that TCDD causes thymic atrophy by reducing entrance into cell cycle in these populations. The Journal of Immunology, 2003, 171: 4582–4591.
this extensive and persistent cell loss, we cannot detect an increase in thymocyte apoptosis (11, 13).
Thymic atrophy induced by TCDD is mediated by binding to and activation of the aryl hydrocarbon receptor (AHR) as shown in AHR-deficient mice (14). The AHR is a basic helix-loop-helix (bHLH) transcription factor possessing a Period/AHR nuclear translocator/Singleminded (PAS) domain. The PAS domain is named for its shared homology with the Drosophila proteins Period and Singleminded, and the human AHR and AHR nuclear translocator (15, 16). The normal physiological function of the AHR is currently unknown, although proteins with PAS domains may be involved in circadian rhythms (17). Additionally, observations in the AHR−/− mice suggest a possible role in normal development of the liver, immune system, aging processes, ovarian follicle numbers, mammary gland development, vascular remodeling, and cardiac physiology (18–23). However, AHR activation by TCDD leads to the modulation of a broad array of genes, including detoxification enzymes, factors involved in cellular growth, proliferation, and cell death (24–26). The precise relationships between elicited gene modifications and AHR function in physiology and/or development of any tissue has not been established.

Previous studies, using chimeric mice with the AHR in either hemopoietic or stromal tissues, proved that thymic atrophy was secondary to AHR activation in hemopoietic cells, not stromal tissues (27). There are two potential cellular targets within the hemopoietic compartment, including the hemopoietic progenitor cells and hemopoietically derived dendritic cells (DCs). In this study, we wanted to further clarify the specific intrathymic cellular targets and the effects that lead to thymic atrophy following AHR activation.

The first potential intrathymic cellular targets we examined were hemopoietic-derived DCs and/or APCs, which could mediate alterations of thymocyte selection after AHR activation. To do this, we created a model where the APCs (DCs and macrophages) were responsive to TCDD, but the thymocytes were not. Using this model, we found that thymic atrophy required that AHR activation occur in developing thymocytes directly.

Having determined that the AHR must be activated in developing lymphoid cells, and given the 80% reduction of thymocyte cellularity within 10 days after exposure (8, 11, 12), we hypothesized that AHR activation was causing a reduction in cell proliferation, and this reduction occurred in the intrathymic progenitor cell compartment. We determined that TCDD inhibits cell proliferation within 9–12 h after exposure. This inhibition of cell cycling was limited to specific subsets in the intrathymic lymphocyte progenitor cell compartment. We believe this to be the primary cause of AHR-mediated thymic atrophy.

Materials and Methods

Experimental animals

Mixed hemopoietic chimeras were generated by reconstituting C57BL/6J Ly 5.1 congenic mice, originally obtained from Dr. E. A. Boyse (Memorial Sloan-Kettering Cancer Center, New York, NY) with bone marrow cells from Ly 5.1 (CD45.1) Ahr−/− and Ly 5.2 (CD45.2) recombinase-activating gene (Rag1)−/− donors. AHR−/− mice were originally obtained from Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD) and were bred and backcrossed 10 generations onto the Ly 5.1 C57BL/6J congenic background at State University of New York (SUNY) Upstate Medical University. Rag1−/− mice on the C57BL/6J background (Ahr−/−; TCDD-sensitive allele) were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed in isolation at SUNY Upstate Medical University for 1 wk before euthanasia and harvesting of bone marrow stem cells.

For the time course studies, 4- to 5-wk-old male C57BL/6J mice were purchased from The Jackson Laboratory and were allowed to acclimate for 1 full week after arrival at the University of Rochester. Mice were main-
tained on a 12-h dark, 12-h light cycle and were provided food (5001 rodent diet; Purina Mills, St. Louis, MO) and water ad libitum. All mice were housed and cared for according to protocols approved by the animal welfare committees at both SUNY Upstate Medical University and University of Rochester.

Chemicals and treatment protocol

TCDD was obtained from Cambridge Isotopes (Andover, MA). A stock solution in the solvent p-dioxane (Sigma-Aldrich, Milwaukee, WI) was diluted to an appropriate concentration in olive oil (F. Berio, Lucca, Italy) to yield a treatment solution containing 6 µg/ml TCDD. For the mixed chimera studies, mice were injected i.p. with either 30 µg/kg TCDD in olive oil per kilogram of body weight, or olive oil alone in a volume of 0.1 ml per 20 g of body weight 10 days before euthanasia.

For the time course studies, mice were injected i.p. with either 30 µg/kg TCDD or olive oil alone at 3, 6, 9, 12, 24, 48, 96, or 144 h before sacrifice. 5-Bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) was diluted in a stock concentration of 10 mg/ml in PBS. All mice were injected with 1.0 mg of BrdU in 0.1 ml of PBS 2 h before sacrifice.

For the apoptosis study, mice were injected with oil, TCDD, or 5 mg/kg dexamethasone (Dex) in PBS 12 h before euthanasia. All mice were injected with 1.0 mg of BrdU in 0.1 ml of PBS 2 h before sacrifice.

Bone marrow lymphocyte isolation and production of hemopoietic chimeras

Bone marrow cells were isolated and prepared as previously described (12, 27). Briefly, the marrow cavity was flushed with MEM plus 100 U/ml penicillin and 0.1 mg/ml streptomycin (P/S; Life Technologies, Rockville, MD) and 5% FBS. Cells were degaggretated, and debris was eliminated by passing the cell suspension through a Pasteur pipette containing 80-gauge nylon mesh (Tetko, Kansas City, MO). Cells were pelleted and depleted of erythrocytes by treatment with ACK buffer (0.17 M NH4Cl, 10 mM KHCO3, and 1 mM EDTA). Cell yield and viability were enumerated with a Neubauer hemocytometer (Reichert, Buffalo, NY). Cell viability was determined to be >95% from all animals by trypan blue dye exclusion. Cells were suspended in MEM without FBS in an appropriate concentration for reconstitution.

Generation of irradiation-reconstitution chimeras was performed as previously described (27), with the following modifications: C57BL/6J Ly 5.1 were reconstituted with either 1 × 106 bone marrow cells in a 1:1 ratio of Rag1−/−; Ahr−/− cells or 5 × 106 bone marrow cells in a 9:1 ratio of Rag1−/−; Ahr−/− cells. Mice were allowed to reconstitute for a minimum of 5 wk to allow for reconstitution of bone marrow-derived lymphocyte, myeloid, and DC populations.

Four weeks after reconstitution, chimeric mice were transferred to University of Rochester and allowed to acclimate for 1 full week before dosing with TCDD. Mice were injected with either 30 µg/kg TCDD or olive oil alone. All mice were euthanized 10 days after receiving the injection. A minimum of five mice was used per treatment group, and cells from each mouse were analyzed separately.

Thymocyte isolation

Mice were euthanized by CO2 asphyxiation at the appropriate time point after TCDD treatment. The thymus were removed, and individual cell suspensions were made in cold MEM, containing 5% PBS and P/S. Debris was eliminated as described above. Cell viability was determined to be >95% for all thymocyte preparations.

Thymus and splenic DC enrichment

DCs were isolated as previously described (28, 29). Briefly, either intact spleens or thymus were injected with a 25-gauge needle filled with 100 Mandl U/ml of collagenase D (Boehringer Mannheim, Indianapolis, IN), diluted in HBSS (Life Technologies). After injecting −1 ml of collagenase, the thymus or spleen was torn open, and a cell suspension was made. The cells in suspension were collected and held on ice for later manipulation. The remaining spleen and thymus fragments were incubated with 400 U/ml collagenase D at 37°C and 5% CO2 for 90 min. Next, the digested thymus and spleen fragments were filtered through a sterile sieve, rinsed with Ca2+/Mg2+-free HBSS, and added to the cells obtained from the initial suspension step. The collagenase-digested splenocyte or thymocyte suspensions were then pelleted and subsequently resuspended in a 35% BSA (Cohn fraction V; Intergen, Purchase, NY) solution, which was layered over 1.5 ml of RPMI 1640 in a 15-ml conical tube. Low-density collagenase-digested mononuclear cells were collected after 30 min of centrifugation at 900 × g by removing the cells at the interface of the RPMI 1640 and BSA layers using a Pasteur pipette. Low-density lymphocytes were...
washed once with 50 ml of RPMI 1640 and pelleted at 350 × g for 10 min. Low-density lymphocytes were resuspended in 0.5 ml of RPMI 1640 containing 5% FBS, P/S, 10 mM HEPES, and 50 μM 2-ME (supplemented RPMI 1640) per thymus or 1 ml of supplemented RPMI 1640 per spleen. The DCs were then enriched overnight using plastic adherence in 35-mm tissue culture plates. After incubation for 10 h, adherent DCs were removed with a Pasteur pipette, pelleted, counted, and prepared for flow cytometry staining. Each spleen yielded between 0.5 and 1 × 10^6 DCs, and each thymus yielded between 0.5 and 1 × 10^5 DCs (data not shown) as reported by others (28, 29).

**Enrichment of intrathymic progenitor cells**

For the time course studies, 5 × 10^6 thymocytes from each mouse were washed twice with 5 ml of label buffer (PBS containing 2 mM EDTA). Next, thymocytes were resuspended in 0.5 ml of label buffer containing 3.75 μg each of biotin-conjugated anti-CD8α (clone 53-6.7, rat IgG2a) and anti-CD4 (clone RM4-5, rat IgG2a) Abs. Cells were incubated on ice for 25 min with mixing approximately every 10 min and washed twice with 5 ml of label buffer. After the second wash, thymocytes were resuspended in 0.45 ml of label buffer, and 0.05 ml of MACs streptavidin coupled to magnetic microbeads (Miltenyi) Bioturm, Aubern, CA, and incubated for 25 min. Bead-conjugated thymocytes were washed twice with label buffer and resuspended in 0.5 ml of PBS plus 2 mM EDTA plus 0.5% BSA. Enrichment of CD4^+ CD8^− DN thymocytes was accomplished by running the labeled cell suspensions through the MACs streptavidin magnet using a BS column (Miltenyi Bioturm). Each individual mouse thymus yielded between 0.8 and 1.5 × 10^6 cells with >96% purity (data not shown). Mature DN TCR-expressing cells were eliminated during analysis using HSA (CD24) (30, 31).

**Abs and lymphocyte staining for flow cytometry**

The following mAbs were used at predetermined saturating levels for staining freshly isolated cells: anti-CD8α (clone 53-6.7, rat IgG2a) conjugated to either FITC, biotin, or allophycocyanin; anti-CD4 (clone RM4-5, rat IgG2a) conjugated to PE; anti-CD3e (145-2C11, hamster IgG) conjugated to FITC or biotin; anti-CD11c (clone HL3, hamster IgG) conjugated to PE; anti-HSA (CD24; clone D24U, clone M1/69, rat IgG2b) conjugated to PE; anti-CD8 (clone 53-6.7, rat IgG2b) conjugated to biotin; anti-CD11c (clone 7D4, rat IgM) conjugated to biotin; anti-CD44 (clone IM7, rat IgG2b) conjugated to allophycocyanin or PE; anti-LY 5.1 (CD45.1; clone A20; mouse IgG2a) conjugated to FITC or biotin; anti-LY 5.2 (CD45.2; clone A20, mouse IgG2a) conjugated to FITC or biotin. All Abs were purchased from BD PharMingen (San Diego, CA).

Cells were first stained with FITC-, allophycocyanin-, and biotin-conjugated mAbs, washed with PBS containing 0.1% Na2, and 0.5% BSA, and then stained with PE-conjugated mAbs and either streptavidin-Red 670 (Life Technologies) for acquisition on the FACStarProl flow cytometer (BD Biosciences, Mountain View, CA) or streptavidin-PerCP (BD PharMingen) for acquisition on the FACScalibur flow cytometer (BD Biosciences).

For the detection of BrdU incorporation in thymocytes, intracellular staining was performed following the staining of cell surface Ags as previously described (4). Briefly, cells were fixed and permeabilized using the BD PharMingen Cytofix/Cytoperm buffer for 15 min. Cells were washed twice with the BD PharMingen Perm/Wash buffer and then stained for 20 min with a solution of anti-BrdU (clone B44) conjugated to FITC, containing DNase I (BD Biosciences). After a final wash, cells were fixed in 1% paraformaldehyde and stored in the cold and dark overnight before acquisition on the flow cytometer.

For cell cycle determination, cells were first stained with a mixture of anti-CD3, -CD4, and -CD8 FITC, followed by fixation with 1% paraformaldehyde for 15 min. Cells were subsequently permeabilized with 70% ethanol (−20°C) for at least 48 h. After permeabilization, thymocytes were treated with 1 mg/ml RNase, and then DNA was stained with 50 μg/ml propidium iodide (PI). Cell cycle analysis was performed using FlowJo software (Tree Star, San Carlos, CA).

Detection of apoptotic thymocytes by TUNEL was performed as previously described (11), with the exceptions that DN cells were enriched first using magnetic separation, and fixation and permeabilization was performed with the BD PharMingen Cytofix/Cytoperm kit. Briefly, TdT was used with digoxigenin-conjugated dUTP to label the ends of nicked DNA. The TdT is followed by fluorescein-conjugated antidigoxigenin Ab to visualize the cells with DNA breaks. A control tube omitting the TdT step was used to establish the background level for the anti-digoxigenin Ab.

At least 20,000–50,000 viable cells were collected from each sample using either a three-color FACStarProl flow cytometer or a four-color FACScalibur flow cytometer (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

**Statistics**

For each experiment, we individually analyzed three to five mice per treatment group. Each time point was performed on at least two separate occasions. Results are presented as the mean ± SEM with the number of mice per group appropriately noted. A one-way ANOVA was performed on each set of data using Statview SAS (Cary, NC) software for Macintosh. Comparisons between the means were performed using Tukey's t test. Values of p ≤ 0.05 were considered statistically significant.

**Results**

**Expression of the AHR in thymocytes is required for mediating TCDD-induced thymic atrophy**

TCDD-induced thymic atrophy is due to the activation of the AHR, and this activation must occur strictly in the hematopoietic compartment (27). However, it is possible that bone marrow-derived DCs or myeloid APCs could mediate the inhibitory effects of TCDD on T cell development through an indirect mechanism, such as increased negative selection of thymocytes or failure to support positive selection. To address this possibility, we generated mixed hematopoietic chimeras by reconstituting lethally irradiated C57BL/6J Ly 5.1 mice with a mixture of bone marrow from Rag1−/−/Ly 5.2 mice on the C57BL/6J background and Ahr−/−/Ly 5.1 mice. Although T cell development is blocked in the Rag−/−/Ly 5.1 mice, previous studies have shown that development of the intrathymic dendritic and myeloid lineage is unaffected (32–33). Therefore, the Rag1−/− bone marrow was used as a source of Ahr−/− DCs and APCs that could potentially mediate TCDD-induced thymic atrophy indirectly, by affecting the TCDD-insensitive Ahr−/− thymocytes (14, 27). We generated a 1:1 and 9:1 mixture of Rag1−/−; Ahr−/− bone marrow radiation-reconstitution chimeras and treated each set with either 30 μg/kg or 0.5 μg/kg TCDD.

To verify that donor cells reconstituted the lethally irradiated thymus in the proportions we expected, we analyzed LY 5.1 expression in both total thymocytes and bone marrow-derived DC/myeloid-enriched cells. As shown for the 9:1 mixed chimeras in Fig. 1A, high and low peaks of LY 5.1 fluorescence can be observed. The LY 5.1high cells represent thymocytes derived from the Ahr−/− donor (and perhaps a small proportion of residual radioresistant host cells: ≤5%), which make up ~90% of the total thymocyte population. The LY 5.1low cells represent the Rag−/−-derived donor thymocytes. The LY 5.1low cells were independently confirmed to express LY 5.2 (data not shown). As expected, the LY 5.1low cells are blocked in the DN stage of thymocyte development (Fig. 1A, middle dot plot). In comparison to the Rag−/−-derived thymocytes, the LY 5.1high (Ahr−/−-derived cells) exhibit normal proportions of thymocytes in each major subset (Fig. 1A, right dot plot), indicating that competition with an excess of Rag−/−-derived bone marrow progenitors does not inhibit development of the Ahr−/− thymocytes. In contrast to the thymocyte compartment, where there are ~10 times the number of Ahr−/−-derived thymocytes to Rag−/−-derived thymocytes, the low-density plastic adherent cells (29) enriched for DC and myeloid lineages are skewed toward the LY 5.1high, or Rag−/−-derived donors (Fig. 1B). Using HSA expression for the thymic DCs, or CD11c for splenic DCs (34, 35), reveals the predicted 9:1 ratio of LY 5.1low to LY 5.1high cells corresponding to the input of bone marrow progenitors from the Rag−/− and Ahr−/− donors (Fig. 1B, lower two panels).

If Ahr+/− DCs contribute to TCDD-induced thymic atrophy in an indirect manner, then in the mixed chimeras, there would be a reduction in the number of Ahr−/− thymocytes after TCDD exposure. As shown in Table I, the total number of Ahr−/−-derived cells was the same in both the vehicle- and TCDD-treated mixed chimeras. This was true whether the 1:1 or 9:1 Rag1−/−:Ahr−/− ratio was used. In comparison, the number of Rag1−/−-derived
thymocytes was reduced in both sets of chimeras by $\geq 60\%$, and this reduction achieved statistical significance in the 9:1 group ($p \leq 0.001$). When mixed chimeras are generated with $\text{RAG}^{1-/-}$: $\text{Ahr}^{+/+}$ bone marrow, TCDD induced a $> 70\%$ reduction in the number of the AHR $^{+/+}$-derived thymocytes (Table I). Moreover, when the absolute number of DN, DP, and CD4 and CD8 single-positive thymocytes are determined for each mixed chimera, the when the absolute number of DN, DP, and CD4 and CD8 single-positive thymocytes is shown above the gates in the representative histogram. The phenotypic distribution of $\text{Ahr}^{+/+}$ or $\text{RAG}^{+/+}$ thymocytes was obtained by analyzing the CD4 vs CD8 distribution within LY 5.1 $^{\text{high}}$ (middle dot plot) or LY 5.1 $^{\text{low}}$ (right dot plot) thymocytes. The proportion of viable lymphocyte-sized cells as determined by forward light scatter and side light scatter discrimination for each thymocyte subpopulation is shown in the upper left quadrant of each CD4 vs CD8 dot plot. B. DC/myeloid cells from pooled thymus or individual spleens were enriched as described in Material and Methods. DCs in the thymus and spleen were stained with Abs against HSA or CD11c, and LY 5.1. DC enrichment was confirmed by first gating on viable cells (by forward light scatter vs side light scatter) and then either HSA (thymic DCs; left histogram) or CD11c (splenic DCs; right histogram). Reconstitution by each donor was determined by analyzing the ratio of LY 5.1 $^{\text{high}}$ (AHR $^{+/+}$) derived to LY 5.1 $^{\text{low}}$ (RAG $^{1-/-}$) expression within the respective gates.

**TCDD causes an accumulation of thymocytes in the G$_1$ stage of the cell cycle**

Given that thymocytes are sensitive targets of TCDD, we set out to identify what effect AHR activation has on these cells and to identify which stages of thymocyte differentiation are immediately affected. Previous studies had demonstrated an inhibitory effect of TCDD on cell proliferation (13, 36–38). However, the exact mechanism in vivo and the specific thymocyte cell populations where alterations of cell cycle progression are occurring have yet to be elucidated. To determine whether TCDD inhibits entry into the cell cycle in vivo, we injected 4- to 5-wk-old C57BL/6J mice with 30 $\mu$g/kg TCDD. Twenty-four hours after injection, we analyzed the distribution of either total or CD8/CD4/CD3 triple-negative (TN) thymocytes within the cell cycle. We analyzed TN thymocytes by electronic gating and determined PI fluorescence by applying the Dean and Jett algorithm to determine the percentage of thymocytes in G$_1$, S, and G$_{2}$/M phase of the cell cycle (39).

In total thymocytes from TCDD-treated mice, there was a slight but significant increase (3% increase; $p \leq 0.05$) in the percentage of total thymocytes in the G$_1$ phase of the cell cycle and $\approx 80\%$ decrease in both the percentage and number of cells in the S plus G$_{2}$/M phase ($p \leq 0.01$). In TN thymocytes, which have a higher proportion of cycling cells, there is also a significant decrease in the proportion and number of cells within the S plus G$_{2}$/M phase of the cell cycle. As shown in Fig. 2, C and D, after exposure to TCDD for 24 h, the proportion of cells in S plus G$_{2}$/M declines from $\approx 15$ to 7%, and there is a $\approx 64\%$ reduction in the absolute number of cells possessing a DNA content of $\geq 2N$ ($p \leq 0.01$).

**Identification of the developmental stages where cell proliferation is inhibited**

After determining that TCDD-induced atrophy is due to a direct effect on thymocytes, and that the primary effect is a reduction of TN cells in the S plus G$_{2}$/M phase of the cell cycle, we set out to determine whether inhibition of cell proliferation is more pronounced in a specific DN stage of thymocyte development. To identify which cell population(s) in the thymus were most susceptible to the effects of TCDD on cell proliferation, we analyzed BrdU incorporation in each DN progenitor cell population.

As shown in Fig. 3, viable-sized lymphocytes were first electronically gated using forward and side light scattered parameters. The lymphocyte-sized cells expressing HSA (CD24; Fig. 3A, middle histogram) were gated and then analyzed for CD44 and CD25 expression (A, top right dot plot). HSA was used as a marker for all populations, except the DN1 (CD44$^{+/+}$CD25$^{+/+}$) population. The earliest DN1 cells have previously been reported to have low surface expression of HSA and are cells that undergo a low rate of cell cycling. In contrast, DN1 HSA$^{++}$ cells are in the process of expressing CD25 on their surface as they mature into the DN2 population (2, 4). HSA expression was used for staging the DN2 through DN4 populations, because it confirms that these cells are primarily immature thymocytes and not mature CD4$^{-}$CD8$^{-}$ $\gamma$$\delta$ or $\alpha$$\beta$ T cells that would not be eliminated using our depletion protocol (30, 31). For these studies, we further separated the DN4 cells into a DN4A (HSA$^{+}$CD44$^{+}$CD25$^{+/+}$) and DN4B (HSA$^{+}$CD44$^{+}$CD25$^{low}$) subset based on the difference in BrdU incorporation observed in cells expressing intermediate levels of CD25 when compared with the CD25$^{low}$ cells in the control-treated mice. As shown in Fig. 3A, an electronic gate was set surrounding the DN2 through DN4B populations for analysis of BrdU incorporation. A separate analysis was performed for determination of cell proliferation in the DN1 population by removing the electronic HSA gate (R2).

As shown in Fig. 3B, BrdU incorporation in the DN3 thymocyte population was reduced by $> 60\%$ 24 h after TCDD exposure compared with DN3 thymocytes from vehicle-treated mice ($p < 0.001$). There were also statistically significant decreases in BrdU incorporation in the DN4A and DN4B populations from TCDD-treated mice (Fig. 3, C and D; $p < 0.001$). The reduction of BrdU
incorporation in the DN3, DN4A, and DN4B thymocyte progenitor cells indicates that these cells are sensitive targets for AHR activation.

Kinetics of the effects on DN cell proliferation

For AHR activation to cause significant reductions in total cell number as soon as 3 days after exposure and for the reductions to gradually increase in severity over time (8, 11, 12), AHR activation must result in the inhibition of proliferation in the progenitor cell populations within 1–2 days, and the inhibition must be persistent. As shown in Fig. 4A, BrdU incorporation in DN1 cells was unaffected until 144 h after TCDD treatment, at which point a slight increase was observed. However, the observed increase in BrdU incorporation in DN1 cells was not statistically significant. There were also no statistical differences between TCDD- and vehicle-treated mice in the DN2 population (Fig. 4B). However, 12 h after TCDD exposure, there were statistically significant reductions in BrdU incorporation in the DN3 and DN4A populations (Fig. 4, C and D; p < 0.01). Even earlier reductions in BrdU incorporation were observed at 9 h (p < 0.01) and at 12 h after TCDD exposure in the DN4B population (Fig. 4E; p < 0.05). Moreover, this decrease in BrdU incorporation at these early time points (≤12 h) precedes the time where any cell loss is observed. BrdU incorporation remained inhibited for at least 144 h in the DN3, DN4A, and DN4B stages of thymocyte development.

Effects on the total number of proliferating cells

Following analysis of BrdU incorporation in each DN progenitor cell population, the kinetics of cell loss was determined. Absolute cell number for each DN population was calculated by multiplying the total thymic cellularity at each time point after TCDD exposure by the percentage of viable thymocytes from each phenotype by the total number of thymocytes per mouse and represents the total cellularity × 10^7.

<table>
<thead>
<tr>
<th>Mixed Chimera</th>
<th>Treatment</th>
<th>LY 5.1\textsuperscript{hi}\textsuperscript{h}</th>
<th>LY 5.1\textsuperscript{lo}\textsuperscript{w}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Rag\textsuperscript{−/−}: Ahr\textsuperscript{−/−}</td>
<td>Percentage\textsuperscript{b}</td>
<td>Vehicle 96.83 ± 0.51</td>
<td>1.55 ± 0.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 98.31 ± 0.24</td>
<td>0.22 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 10.31 ± 1.91</td>
<td>0.36 ± 0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 10.88 ± 1.49</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>9 Rag\textsuperscript{−/−}: Ahr\textsuperscript{−/−}</td>
<td>Percentage</td>
<td>Vehicle 91.03 ± 1.70</td>
<td>6.36 ± 1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 96.27 ± 0.76\textsuperscript{d}</td>
<td>1.98 ± 0.33\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 4.85 ± 1.35\textsuperscript{e}</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 5.08 ± 0.73</td>
<td>1.01 ± 0.03\textsuperscript{d}</td>
</tr>
<tr>
<td>9 Rag\textsuperscript{−/−}: Ahr\textsuperscript{+/+}</td>
<td>Percentage</td>
<td>Vehicle 89.25 ± 1.83</td>
<td>7.15 ± 1.56</td>
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<tr>
<td></td>
<td></td>
<td>TCDD 84.37 ± 2.06</td>
<td>10.26 ± 1.31</td>
</tr>
</tbody>
</table>

\textsuperscript{a} C57BL/6J LY 5.1 mice were lethally irradiated and reconstituted with a mixture of cells from RAG1\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h}) and AHR\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h}) bone marrow in either a 1:1 (5 × 10\textsuperscript{5} each) or 9:1 ratio (4.5 × 10\textsuperscript{5}:5 × 10\textsuperscript{5}). Five weeks after reconstitution, mice were injected with either oil or 30 μg/kg TCDD, and mice were euthanized and thymus were removed 10 days after injection. Data are from a representative experiment, with each group of chimeras repeated at least once.

\textsuperscript{b} Thymi were removed, cell suspensions were made, and thymocytes were counted and stained with fluorochrome-labeled Abs directed against LY 5.1, CD8, and CD4. Data were analyzed as shown in Fig. 1 by flow cytometry.

\textsuperscript{c} Cell number was obtained by multiplying the percentage of viable LY 5.1 thymocytes by the percentage of cells from each phenotype (data not shown) by the total number of thymocytes per mouse. Numbers in the table are ×10\textsuperscript{7}.

\textsuperscript{d} Statistically different compared with vehicle-treated mice in the same mixed chimera group, p ≤ 0.01.

\textsuperscript{e} The reduced BrdU incorporation in the DN3, DN4A, and DN4B thymocyte progenitor cells indicates that these cells are sensitive targets for AHR activation.

\textsuperscript{f} The cell number was obtained by multiplying the percentage of viable LY 5.1 thymocytes by the percentage of cells from each phenotype by the total number of thymocytes per mouse and represents the total cellularity × 10\textsuperscript{7}.

\textsuperscript{g} Statistically different compared with vehicle-treated mice in the same mixed chimera group, p ≤ 0.01.

\textsuperscript{h} The reduced Ahr\textsuperscript{−/−} thymocyte-derived cell numbers observed in the 9:1 mixed chimeras could be attributed to competition with Rag\textsuperscript{−/−} cells for the stem cell environment.

Table II. \textit{TCDD causes a reduction in AHR\textsuperscript{−/−} thymocytes directly}\textsuperscript{a}

<table>
<thead>
<tr>
<th>Donor Cells</th>
<th>Treatment</th>
<th>CD4\textsuperscript{−}CD8\textsuperscript{−}</th>
<th>CD4\textsuperscript{+}CD8\textsuperscript{−}</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h})</td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 0.10 ± 0.04</td>
<td>4.02 ± 1.18</td>
<td>0.56 ± 0.11</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 0.12 ± 0.03</td>
<td>4.54 ± 0.71</td>
<td>0.30 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>RAG1\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h})</td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 24.46 ± 4.31</td>
<td>ND\textsuperscript{d}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCDD 7.43 ± 2.45\textsuperscript{e}</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AHR\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h})</td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 0.04 ± 0.01</td>
<td>2.24 ± 0.51</td>
<td>0.26 ± 0.06</td>
<td>0.06 ± 0.01 \textsuperscript{e}</td>
</tr>
<tr>
<td>RAG1\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h})</td>
<td>Cell number (×10\textsuperscript{7})</td>
<td>Vehicle 0.01 ± 0.001\textsuperscript{e}</td>
<td>0.40 ± 0.12\textsuperscript{e}</td>
<td>0.06 ± 0.02\textsuperscript{e}</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a} C57BL/6J LY 5.1 mice were lethally irradiated and reconstituted with a mixture of cells from RAG1\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h}) and AHR\textsuperscript{−/−} (LY 5.1\textsuperscript{hi}\textsuperscript{h}) bone marrow in a 9:1 ratio. Five weeks after reconstitution, mice were injected with either oil or 30 μg/kg TCDD, and mice were euthanized and thymus were removed 10 days after injection. Thymus were removed, cell suspensions were made, and thymocytes were counted and stained with fluorochrome-labeled Abs directed against LY 5.1, CD8, and CD4. Data were analyzed as shown in Fig. 1 by flow cytometry.

\textsuperscript{b} The cell number was obtained by multiplying the percentage of viable LY 5.1 thymocytes by the percentage of cells from each phenotype (data not shown) by the total number of thymocytes per mouse. Numbers in the table are ×10\textsuperscript{7}.

\textsuperscript{c} Cell number was obtained as described above. The numbers are ×10\textsuperscript{7}.

\textsuperscript{d} ND, None detected.

\textsuperscript{e} Statistically different compared with vehicle-treated mice in the same mixed chimera group, p ≤ 0.01.
cell compartment (or HSA− for DN1 only) (data not shown). The number of BrdU+ cells was calculated by multiplying the total DN cell number by the percentage of cells demonstrating BrdU incorporation in that population at each time point. There were no statistical differences in cell number at any time point after injection with the vehicle, and therefore, the average from all control-treated mice was used for comparisons with TCDD-treated mice.

Twenty-four hours after injection, total thymic cellularity (data not shown) and the absolute cell number for each DN thymocyte population in TCDD-treated mice do not differ significantly from those in vehicle-treated mice (Fig. 5A). However, 48 h after TCDD treatment (still at a time point before any significant total cell loss), there is ~3-fold reduction in the number of DN3 thymocytes compared with the same population in vehicle-treated mice (p ≤ 0.01). This reduction in the DN3 population remains essentially constant through the first 144 h after TCDD exposure. In the DN4A and DN4B populations, the cell numbers are reduced starting at 48 h postexposure, and this reduction reaches maximal levels by 96 h (p < 0.01). In comparison, the absolute cell numbers for the DN1 and DN2 populations remain unchanged at all time points evaluated.

As shown in Fig. 5B, there was a 3.6-fold reduction in the absolute number of BrdU+ cells in the DN3 population 24 h after
These trends of decreasing numbers of BrdU/H11001 in each successive time point (p < 0.01) are a decrease in intrathymic progenitor cell proliferation with no detectable increase in apoptosis. Subsequent studies attempting to support this finding in vivo have reported an increase in thymocyte apoptosis, but only at a very high dose of TCDD and only after overnight culture of the thymocytes in vitro (41). However, in a recent review by Robertson and Orrenius (42) of toxicological agents known to induce apoptosis, the authors conclude that the recent data do not support apoptosis as a primary mechanism for TCDD-induced thymic atrophy. To address the question of whether a loss of BrdU incorporation was a result of increased thymocyte apoptosis, particularly in the DN compartment, we compared DNA fragmentation in oil-, TCDD-, and Dex-treated mice using the TUNEL assay 12 h after exposure. As shown in Fig. 6A, BrdU incorporation is unaffected in DP thymocytes 12 h after exposure to either oil or TCDD. In comparison, BrdU incorporation in DP thymocytes from mice treated with 5 mg/kg Dex is reduced by ~5-fold. Fig. 6B shows that, when assaying for apoptotic DP thymocytes, only 1–2% of the cells in both oil- and TCDD-treated mice are apoptotic, whereas ~15% of the DP thymocytes from the mice treated with Dex are TUNEL positive. Importantly, there are no TUNEL + DN3 or DN4 thymocytes in either the oil- or TCDD-treated mice, whereas there is a small population of TUNEL + cells in the DN3 and DN4 cells from the Dex-treated mice (Fig. 6C). These data support a model where the earliest effects of TCDD in the thymus are a decrease in intrathymic progenitor cell proliferation with no detectable increase in apoptosis.

Discussion

It is well established that TCDD has potent suppressive effects on T cell development (6–8, 38, 43). However identification of the
mice. Additionally, a significant reduction in BrdU incorporation was observed. Inhibition of BrdU incorporation was cell stage specific, occurring in the DN3 and DN4 stages of T cell development. It should be noted that there was a slight decrease in BrdU incorporation even in the vehicle-treated mice in the first three time points examined. Although this decrease was not statistically significant, it might be due to elevated stress/glucocorticoid levels in the mice after receiving two i.p. injections (oil and BrdU in PBS). Because the oil-alone effect was not statistically significant, it would be inappropriate to go beyond this without undue speculation. Nevertheless, the decrease in BrdU incorporation in TCDD-treated mice was ultimately more severe and achieved statistical significance as early as 9 h after exposure in the DN4B population and 12 h after exposure in the DN3 and DN4A subsets. This reduction in BrdU incorporation at these early time points occurs before measurable cell loss is observed. Moreover, because BrdU was administered 2 h before sacrifice, our results suggest AHR activation causes alterations in the DNA replication machinery by 7 h after exposure in vivo. The 60% reduction in BrdU incorporation can be directly correlated to the net reduction in the total number of cells synthesizing DNA in the DN3, DN4A, and DN4B thymocyte populations 24 h after TCDD exposure, and this reduction is persistent for at least 6 days after exposure. These data suggest that the mechanism behind TCDD-induced thymic atrophy is mainly due to the inhibition of cell proliferation in the DN progenitor cell compartment and is not due to apoptosis as suggested by other investigators (40, 41). Moreover, we cannot detect an increase in thymocyte apoptosis in either DN or DP thymocytes by the TUNEL assay in vivo. It is possible that apoptotic cells are rapidly cleared by resident macrophages (44); however, we can detect TUNEL+ cells 12 h after exposure to Dex, but not TCDD (Fig. 6). It is also possible that TCDD causes a delayed apoptosis where first cell cycle is inhibited, and this is rapidly followed by activation of death pathways. However, we have shown both in vitro and vivo that lck proximal promoter-driven Bcl-2 transgenic mice are sensitive to TCDD-induced thymic atrophy but are protected from Dex-induced atrophy (11). In comparison to the Bcl-2 transgenic mice, it has been shown in fetal thymic organ cultures that mice deficient in the G1→S cell cycle regulator, p27Kip, are partially protected from TCDD-induced thymic atrophy, adding further support that TCDD activation of the AHR results in modulation of the cell cycle to cause atrophy (36).

From the decrease in BrdU incorporation detected in the DN3, DN4A, and DN4B populations 7–10 h after TCDD exposure, we conclude that these populations are the primary cellular targets in the thymus. If AHR activation was targeting an earlier progenitor cell population, it is possible that this earlier population might continue to differentiate but fails to proliferate once it reaches the DN3 or DN4 stage of maturation. However, it has been estimated that the average murine thymocyte doubling time is between 10 and 12 h, and that it can require two cellular divisions for cells in the DN2 population to differentiate into the DN3 population (4, 45). Therefore, because we observed a decrease in BrdU incorporation in the DN3 and DN4 populations 10 h after TCDD exposure, it is highly likely that the vast majority of cells in the DN3, DN4A, and DN4B populations have decreased DNA synthesis directly. This reduction is most likely dependent on activation of the AHR, because in preliminary studies using Ahr−/− mice, there was no difference in BrdU incorporation within any DN cell population in either control- or TCDD-treated mice (data not shown).

These data demonstrating that TCDD-induced thymic atrophy is caused by inhibition of cell cycle within the intrathymic progenitor population complement earlier studies by Frey et al. (1) where they demonstrated that, in the absence of bone marrow input, the thymus begins to undergo profound thymic atrophy within 1 wk after the intrathymic progenitor population is exhausted. We have found that TCDD-induced thymic atrophy can be due to inhibition of bone marrow reconstitution of the thymus (43, 46) and inhibition of cell proliferation within the intrathymic progenitor population.

**FIGURE 6.** TCDD does not cause an increase in thymocyte apoptosis. Mice were injected with oil, 30 μg/kg TCDD, or 5 mg/kg Dex, and all mice were injected with 1 mg of BrdU 2 h before euthanasia. Twelve hours after oil, TCDD, or Dex injection, mice were euthanized, thymi were removed, and cell suspensions were made as described above for the time course studies. A representative histogram from four mice per group is shown. A, DP thymocytes were gated and analyzed for BrdU incorporation. The percentage of BrdU+ cells is shown above the analysis gate for each treatment, and the treatment is listed above each column of histograms. B, DP thymocytes were gated and the percentage of TUNEL+ cells is shown above the analysis gate for each treatment. A control tube lacking TdT was used to determine the background and is shown as the shaded overlay. C, TUNEL staining in enriched DN thymocytes gated on the DN3 and DN4 cell populations from oil-, TCDD-, or Dex-treated mice.
LYMPHOCYTE PROGENITORS ARE TARGETS OF AHR ACTIVATION BY TCDD

In fact, based on our data, one might expect an accumulation of DN3 cells in the thymus if they are not dying and if there is continual input from the bone marrow. A consideration of the present and previous data (43, 46) would indicate that a lack of accumulation is due to the finding that TCDD also inhibits bone marrow progenitor reconstitution of the thymus. The result of AHR activation in both the bone marrow and intrathymic progenitors is a statistically significant reduction in overall thymocyte cellularity beginning 3 days after exposure, reaching maximal levels (80% reduction) by day 10, and persisting for at least 24 days after exposure to TCDD at this dose (11, 12, 27).

The reduction of cell proliferation in specific stages of thymocyte development by AHR activation raises some intriguing possibilities for the biological role of the AHR at these particular developmental checkpoints. Specifically, the stages of development where inhibition seems to be most profound (DN3 and DN4) are normally characterized by RAG expression, TCR gene recombination, and pre-TCR signaling. Additionally, genetic studies have demonstrated critical roles for several bHLH transcription factors, including HEB and Id3 (47–49). It is tempting to speculate that the AHR may play some unknown role at this stage of development in coordination with one or more of these other factors. This role may be disrupted in the presence of TCDD, leading to the observed atrophy following AHR activation. However, studies in the Ahr−/− mouse have not revealed any obvious deficiencies in this particular stage of thymopoiesis (18, 27), indicating a possible redundancy with other bHLH proteins during normal development.

An alternative mechanism for the observed inhibition of cell proliferation in immature thymocytes comes from studies demonstrating that TCDD-induced AHR activation results in the up-regulation of the gelsolin-like actin binding protein aseverin (50, 51). Aseverin induction occurred exclusively in the DN3 and DN4 stages of development, but not in earlier or later stages of thymocyte development (52). Perhaps aseverin induction by the AHR interferes with actin polymerization, which could mediate a block or reduction in cell cycle progression in the DN3 and DN4 thymocytes.

In conclusion, the primary cellular targets in which the AHR is activated in the thymus are DN3, DN4A, and DN4B intrathymic thymocytes. The effect of TCDD in these cell populations is an arrest of cell proliferation, which can account for the observed thymic atrophy.

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References

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