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Thymic Alterations Induced by 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Are Strictly Dependent on Aryl Hydrocarbon Receptor Activation in Hemopoietic Cells

J. Erin Staples,* Francis G. Murante,† Nancy C. Fiore,* Thomas A. Gasiewicz,† and Allen E. Silverstone2*

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related congeners affect the immune system, causing immunosuppression and thymic atrophy in a variety of animal species. TCDD is believed to exert its effects primarily through the ligand-activated transcription factor, the aryl hydrocarbon receptor (AhR). Although the AhR is found at high levels in both thymocytes and thymic stroma, it is uncertain in which cells TCDD is activating the AhR to cause alterations in the thymus. Some investigators have suggested that stromal elements, primarily epithelial cells, within the thymus are the primary targets for TCDD. Others have suggested that atrophy is due to a direct effect on thymocytes, either by apoptosis or by altering the development of progenitor cells. By producing chimeric mice with TCDD-responsive (AhR+/+) stromal components and TCDD-unresponsive (AhR−/−) hemopoietic components, or the reverse, we have clarified the role of stromal vs hemopoietic elements in TCDD-induced thymic alterations. Our results show that the targets for TCDD-induced thymic atrophy and phenotypic alterations are strictly in the hemopoietic components, and that TCDD activation of epithelial cells in the stroma is not required for thymic alterations. Furthermore, changes observed in the putative stem cell populations of these chimeric mice are also dependent on TCDD activation of the AhR in hemopoietic elements. The Journal of Immunology, 1998, 160: 3844–3854.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix (bHLH) superfamily of DNA binding proteins. The bHLH proteins are associated with tissue growth and differentiation processes (1, 2). Although the AhR is known to readily bind to exogenous aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3), the natural endogenous ligand and function of the receptor are not known. The recent generation of AhR knockout mice has provided evidence of a role for this protein in the normal development of liver and possibly in the immune system (4–7). Studies using TCDD and its related congeners to activate the AhR also suggest that the receptor plays a role in the immune system. TCDD, even at doses well below the lethal level, causes thymic atrophy and immunosuppression in all animal species examined (8, 9). It is also known that of all tissues, the thymus has one of the highest expression levels of the AhR or its mRNA (10–13).

Unbound AhR is found in the cellular cytoplasm complexed with multiple proteins, including hsp90 (14) and a recently identified protein, the AhR-interacting protein (15). AhR-interacting protein has sequence homology to proteins that are necessary for cell cycle and mRNA synthesis. Once a ligand, such as TCDD, enters the cell and binds to the AhR, the ligand-receptor complex is translocated to the nucleus where it forms a heterodimer with another bHLH protein, the AhR nuclear translocator. This complex then binds to a specific sequence in various genes, called the dioxin response element (16–18). Dioxin response elements are found in the regulatory region of many genes, including cytokines and growth factors (19, 20).

TCDD, via the AhR, has been shown to have a variety of effects on T cell development and function, including decreasing the number of thymocytes and altering the effector functions of mature Th and T killer cells (2, 21, 22). Although a variety of studies have been performed to determine how TCDD is influencing T cells and the thymus, the mechanism and targets of its actions are still unclear. It has been suggested that in the thymus both the stromal cells (23–26) and the hemopoietic cells, including the thymocytes (27–30) and their precursors (31), are sensitive to TCDD. Since TCDD exposure has multiple consequences on the thymus, such as decreasing the number of thymocytes and shifting their phenotypic profile (22, 24, 32, 33), it is possible that TCDD might be targeting both thymocytes and stromal elements in different ways to account for its effects. Additionally, since thymocyte precursors in the bone marrow are known to exhibit sensitivity to TCDD (31), it is also possible that marrow resident hemopoietic cells and/or stromal cells represent targets for TCDD.

The thymus itself is a complex organ that is responsible for the maturation and education of most peripheral T cells. It consists of two main components: the thymocytes and the supporting stromal...
elements. Stromal elements are a heterogeneous group of cells, including radioresistant epithelial cells and radioresistant, macrophage, and dendritic cells (DC) (34, 35). The epithelial cells are found throughout the thymus, and the current consensus is that their main function is to mediate positive selection of double-positive thymocytes (36–38). DC and macrophages are derived primarily from bone marrow hemopoietic precursor cells (35, 39, 40), although DC can arise from intrathymic thymocyte precursors (41, 42). While the exact roles of DC and macrophages in the thymus are not fully understood, it has been shown, using various models, that DC are essential for negative selection of thymocytes (36, 43, 44), while macrophages have multiple roles, including phagocytosis and negative selection (34, 40).

We wanted to further determine the role of hemopoietic cells and stromal cells in TCDD-induced thymic atrophy. To accomplish this we constructed radiation chimeras using mice that lack the dioxin-binding receptor (AhR−/−) and are, therefore, unresponsive to the effects of TCDD even at high toxic doses (45). By taking bone marrow cells, which contain hemopoietic stem cells, from these AhR−/− mice and using them to reconstitute lethally irradiated B6Ly5.1 (AhR+/+) congenic mice, we effectively created mice that have stromal cells that are sensitive to the actions of TCDD, while the hemopoietic cells are insensitive. We then treated these chimeric mice with TCDD and compared the results from these mice to radiation chimeras that contained both sensitive stromal and hemopoietic elements. These studies were additionally validated by lethally irradiating AhR+/− mice, reconstituting them with AhR+/+ marrow, and treating them with TCDD. Our results show that TCDD targets hemopoietic elements directly to cause alterations in the thymus, including thymic atrophy and phenotypic shifts, and that the radioresistant stromal elements do not play a significant role in these processes.

Materials and Methods

Chemicals

TCDD was obtained from Cambridge Isotopes (Cambridge, MA). A stock solution, in p-dioxane, was diluted to an appropriate concentration in olive oil (F. Berio, Hackensack, NJ) to yield a treatment solution containing 6 μg of TCDD/ml.

Experimental animals

Strains used. C57BL/6 Ly5.1 congenic mice were originally obtained from Dr. E. A. Boyse (Memorial Sloan-Kettering Cancer Center, New York, NY) and maintained at our own facility. The 129/SV × C57BL/6N AhR+/+ and AhR−/− mice were obtained from P. Fernandez-Salguero and F. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD) (4). These mice have the B6 Ly5.2 phenotype and either lack the AhR gene (AhR−/−) or have the AhR+,sensitive allele (AhR+/+) from the B6 founder. These mice will be called B6Ly5.2 AhR+/+ or AhR−/− for brevity. All mice were housed and cared for according to The Guide for the Care and Use of Laboratory Animals (46). Specifically, the mice were housed in isolation cages at constant temperature and humidity on a 12-h light/dark cycle, with food and water provided ad libitum. Due to increased susceptibility to infection in the colony of B6Ly5.2 AhR mice (5), they were maintained prophylactically on acid water (pH 3.0) and oxytetracycline HCI (Terramycin, Pfizer, New York, NY).

Chimeric mice. Radiation bone marrow chimeras are designated bone marrow donor—irradiated host. Four-week-old B6Ly5.1 or B6Ly5.2 AhR−/− mice were irradiated by a calibrated x-ray source (Philips MGC 30 x-ray machine, Philips, Hamburg, Germany) in two doses of 550 rad spaced at a 4-h interval. By splitting the dose we can reduce the toxicity to the gut (47) while delivering a cumulative dose of 1100 rad, which is effectively lethal for hemopoietic cells in this strain of mice (48). One half-hour after their second irradiation the mice were given 1 × 10⁸ bone marrow cells from B6Ly5.1 AhR−/−, B6Ly5.2 AhR−/−, or B6Ly5.2 AhR−/− by tail vein injection. B6Ly5.2 AhR+/+ or AhR−/− bone marrow was used to reconstitute the B6Ly5.2 AhR−/− hosts to avoid a graft-vs-host reaction that could have resulted from B6Ly5.1 bone marrow cells recognizing the 129/SV component of the B6Ly5.2 AhR−/− mice. The reconstructed mice were allowed to recover for 4 wk before they were treated to be sure the thymus was fully reconstituted (49) and releasing mature cells of donor origin (50). The mice were weighed twice a week after being irradiated, and mice that had significant or continuing weight loss, a sign of incomplete reconstitution, were excluded from the analysis. All chimeric mice were given acidic water with terramycin approximately 2 to 3 days before irradiation and were maintained on it until they were killed. To ensure that the irradiation of the host mice was successful, one irradiated mouse in each experiment was not reconstituted. All nonreconstituted irradiated mice died or were killed because of morbidity within 9 to 12 days after irradiation.

Treatment protocol

Eight-week-old chimeric or B6Ly5.1 male mice were injected i.p. with either 30 μg/kg of TCDD in olive oil or olive oil only (0.1 ml/20 g). All mice were killed 10 days after receiving an injection. Each experiment was performed with randomized, age-matched litters (±3 days). A minimum of four mice were used per treatment group, although the chimera studies were performed with at least five mice per group. Mice were analyzed individually. Each experiment was performed at least twice.

Cell isolation

For cell counting and flow cytometry. Mice were euthanized by CO₂ asphyxiation, and the thymus were removed and dissected free of lymph nodes and blood vessels. Spleens, along with femurs and tibias from both legs, were also removed. Individual thymi and spleens were weighed and then mashed with the flat end of a 1- or 5-ml syringe plunger, to release the cells, in cold MEM with Hank's salts (Life Technologies, Grand Island, NY; catalog no. 41200-072) containing 5% FBS (Life Technologies) and penicillin-streptomycin (PS; 100 U/ml penicillin and 0.1 mg/ml streptomycin; Life Technologies). The tissues were triturated, and debris was eliminated by passing the cell suspension through a Pasteur pipette stuffed with 80- gauge nylon mesh (Textco, Kansas City, MO). Bone marrow was processed similarly, with the bones first being cleaned of muscle and connective tissue, then <1 mm of the end of each bone was removed. The marrow cavity was flushed with MEM with 5% FBS and P/S using a 25-gauge needle. The marrow was then suspended by passing the cells successively through a 22-gauge needle and a 25-gauge needle. The suspension was finally passed through 80-gauge nylon mesh. All leukocyte preparations were then pelleted by centrifugation. After the initial pelleting, the splenocytes and bone marrow cells were resuspended in 1 ml of ACK buffer (0.17 M NH₄Cl, 10 mM KHCO₃, and 1 mM EDTA) and incubated for 4 min at room temperature to lyse the RBC. These cells were washed once and resuspended. Cells were resuspended in MEM, 5% FBS, and P/S for cell counting. The cell yield was enumerated by diluting the cell counts and counting at least two samples for each cell preparation with a Neubauer hemocytometer (Reichert, Buffalo, NY). Cell viability was determined to be >90% from all animals by trypan blue dye (0.08%) exclusion.

For reconstitution. Bone marrow cells were obtained as described above with the following exception. After the cells were counted, they were re- pelleted and resuspended in MEM and P/S without FBS at a concentration of 5 × 10⁶ cells/ml; 0.2 ml (1 × 10⁶ cells) was then injected via tail vein into the irradiated recipients.

Abs

The following mAbs were used at predetermined saturating levels: FITC- and biotin-conjugated anti-CD8α (clone 53-6.7, rat IgG2a); FITC- and PE-conjugated anti-CD4 (clone RM4-5, rat IgG2a); FITC- and PE-conjugated anti-CD3e (clone 145-2C11 and clone 500A2, hamster IgG); PE- and biotin-conjugated anti-IFN-γ (clone R4-6A2, rat IgG2a); PE-conjugated anti-CD44 (clone IM7, rat IgG2b); biotin-conjugated anti-CD25 (IL-2Rα, clone 7D4, rat IgM); FITC-conjugated anti-CD45.1 (Ly5.1, clone A20, rat IgG2a); biotin-conjugated anti-TER-119 (clone TER-119, rat IgG2b); biotin-conjugated anti-Gr-1 (Ly-6G, clone RB6-8C5, rat IgG2b); biotin-conjugated anti-Mac-1α (CD11b, clone M1/70, rat IgG2b); FITC-conjugated anti-ε-Kit (CD117, clone 2B8, rat IgG2b); and PE-conjugated anti-Sca-1 (Ly-6A/E, clone E13-161.7, rat IgG2a; Pharmingen, San Diego, CA). Biotin-conjugated anti-CD45.2 was made at our facility using Ly5.2 hybridoma clone. A-20 1.7 (mouse IgG2a) obtained from Dr. Shoji Kimura (Memorial Sloan-Kettering Cancer Center).

Flow cytometric staining and analysis

Freshly isolated thymocytes and splenocytes were pelleted by centrifugation and washed in PBS containing 0.5% BSA and 0.1% sodium azide. One
hundred thousand cells were then incubated with FITC and biotin-conjugated mAb in 50 μl for 30 min at 4°C. Cells were washed twice with 1 ml of PBS-0.5% BSA and 0.1% sodium azide and were then incubated with PE-conjugated mAb and streptavidin-conjugated Red 670 (Life Technologies) for 30 min at 4°C. After two more washes, the cells were fixed in 1% paraformaldehyde in PBS. Ten thousand or more fixed cells were also analyzed on a Becton Dickinson (Mountain View, CA) FACStar Plus flow cytometer using the Becton Dickinson LYSYS II program. Single and dual stainings of fresh thymocytes from untreated young adults were used in assorted combinations to set compensation. Negative staining was performed with either isotype or total Ig controls stained appropriately.

Freshly isolated bone marrow cells were pelleted by centrifugation and washed in HBSS with Ca2+ and Mg2+ (Life Technologies, catalog no. 14025-092) containing 0.2% BSA (HBSS-0.2% BSA). Aliquots of 8 × 10^6 cells were then incubated for 30 min in a mixture of biotin-conjugated mAbs (anti-Mac-1, anti-Gr-1, anti-TER-119, anti-CD3ε, anti-CD8α, and anti-B220) diluted with HBSS-0.2% BSA and 0.1% sodium azide and were then incubated with FITC-conjugated anti-c-Kit mAb. The cells were washed once with HBSS-0.2% BSA and were then incubated for 30 min in Dulbecco’s PBS containing 1% BSA and streptavidin-conjugated Red 670. After one rinse in HBSS-0.2% BSA, the cells were fixed in 1% paraformaldehyde in PBS. Data on the fixed bone marrow cells were acquired on a Becton Dickinson FACScan flow cytometer using the Becton Dickinson LYSYS II program and were analyzed with Becton Dickinson CellQuest software. A mean linear fluorescence-3 value was then used to define a low fluorescence-3 gate. A second data file of 50 × 10^6 fixed bone marrow cells, and a fluorescence-3 intensity equal to 1/100 of the mean fluorescence-3 value was then used to define a low fluorescence-3 gate. A second data file of 50 × 10^6 fixed bone marrow cells that satisfied the predetermined low fluorescence-3 gate for each sample was acquired. Single stainings of vehicle-treated bone marrow cells with FITC-anti-c-Kit, PE-anti-Sca-1, or biotinylated Ab mixture and streptavidin-Red 670 was used to set compensation. Spleen and bone marrow cells were preblocked with anti-FcγII/IIIIR (clone 2.4G2, rat IgG2b; Pharmingen) to reduce nonspecific binding.

RNA analysis for CYP1A1 induction

Total RNA was isolated from liver sections using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Twenty micrograms of total RNA was separated by electrophoresis through a 1% agarose formaldehyde gel and transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, IL) by capillary action. RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Duplicate blots were prehybridized for 1 to 4 h and hybridized overnight at 42°C in hybridization solution (50% formamide, 5× Denhardt’s solution, 5× SSPE, 0.5% SDS, 20 mg/ml salmon sperm DNA, and 3H-labeled cDNA probe for CYP1A1 expression). Blots were also probed with 32P-labeled L32 DNA (51) for normalization of lanes. Blots were washed twice for 10 min each time with 2× SSPE/0.1% SDS at room temperature and twice for 15 min each time with 1× SSPE/0.1% SDS at 65°C. Specific bands were visualized by autoradiography. Results shown represent duplicate analyses.

Statistics

Two-tailed Student’s t test for paired and unpaired variables was used to evaluate differences between treatment groups and their respective vehicle-treated controls. Results were considered statistically significant at p < 0.05.

Results

Irradiation and reconstitution do not alter the response to TCDD

To create receptor chimeric mice, we irradiated B6Ly5.1 mice and reconstituted them with bone marrow cells from various donors. Since radiation is known to have an adverse effect on many cells and cellular systems, it was possible that the cells remaining after irradiation in the host mouse had an altered sensitivity to TCDD. To rule out this possibility, we took 4-wk-old B6Ly5.1 male littermates and divided them into two groups (Table I, groups 1 and 2). One group of mice was irradiated and reconstituted with bone marrow cells from another B6Ly5.1 mouse (designated 5.1→5.1), while the second group of mice (designated 5.1→5.2) was not irradiated or reconstituted. At 8 wk of age, half of each group was treated with 30 μg/kg of TCDD, while the other half received vehicle alone.

There was no difference between irradiated and nonirradiated mice in their gross response to TCDD (Fig. 1). In 5.1→5.1 mice treated with TCDD, thymic weight and thymic cell number were 37 and 18% of control values, respectively, while in 5.1→5.2 mice the values were 35 and 21%, respectively. Similar alterations were seen in their thymocyte phenotypes with respect to CD4, CD8, and

FIGURE 1. Similar responses of 5.1→5.1 and 5.1→5.2→5.1 mice to TCDD. Eight-week-old 5.1→5.1 mice or radiation chimeric (IR), 5.1→5.1 mice were treated with 30 μg/kg of TCDD or with oil alone (Control). Ten days after the treatment the mice were killed. Body weights, thymic weights, and thymic cell counts were determined for each individual mouse. Results are expressed as the mean ± SD (n = 4–8). *** indicates p < 0.001.
CD3 expression in response to TCDD, as was shown previously (31). These results confirm that the process of irradiating and reconstituting mice does not alter their sensitivity or response to TCDD.

Construction and validation of AhR chimeric mice

Once we found that irradiation and reconstitution did not alter the sensitivity of the chimeric mice to TCDD, we developed hemopoietic chimeras to test whether stromal elements, hemopoietic elements, or both were involved in TCDD-induced changes in the thymus (Table I, groups 3 and 4). To do this we took bone marrow cells from a B6Ly5.2 AhR−/− mouse and used them to reconstitute irradiated B6Ly5.1 (AhR+/+) mice. In doing this we effectively created mice that have stromal elements that are sensitive to TCDD, while the hemopoietic elements are insensitive, B6Ly5.2 AhR−/−→B6Ly5.1 (designated 5.2−→5.1−). Simultaneously, we reconstituted another set of irradiated B6Ly5.1 mice with bone marrow from a B6Ly5.2 AhR+/+ mouse derived from the same heterozygous cross that led to the B6Ly5.2 AhR−/− mice. The B6Ly5.2 AhR+/+→B6Ly5.1 mice (designated 5.2+→5.1−), in contrast to the AhR−/− reconstituted mice, have both stromal and hemopoietic elements that are sensitive to TCDD.

To validate that there was successful radiation-induced ablation of the host immune cells and reconstitution with donor cells in the chimeric mice, we performed two-color flow cytometry using Ly5.1 (host) vs Ly5.2 (donor) markers. On the average, <1% of the cells in the thymus (Fig. 2A), bone marrow (Fig. 2B), and B cell compartment of the spleen (not shown) contained Ly5.1 on their surface. Further analysis of the 5.1− thymocytes showed that >98% were labeled with the donor phenotype, Ly5.2 (Fig. 2C).

To verify that the chimeric mice were effectively exposed to TCDD and that AhR+/+ tissues were responsive to this treatment, RNA was isolated from the livers of control and treated mice and analyzed for CYP1A1 induction. CYP1A1 is a gene responsible for the production of cytochrome P450A1 and is readily induced by TCDD (1). Figure 3 shows that while there was no detectable CYP1A1 mRNA in the oil-treated controls, there was induction in the liver of CYP1A1 in both 5.2−→5.1− and 5.2−→5.1+ chimeric mice. Furthermore, the induction obtained in 5.2−→5.1− chimeric mice was similar to that in 5.2−→5.1+ chimeric mice.

5.2−→5.1+ mice are unresponsive to TCDD’s ability to induce thymic atrophy

As shown in Figure 4, A and B, there was significant thymic atrophy in the 5.2−→5.1− chimeric mice treated with TCDD, with respect to both thymic weight (36% control) and thymic cell number (18% control). These results are consistent with those in 5.1− mice and 5.1+ mice treated with TCDD. A small, but significant (p < 0.03), decrease in the weight of the spleen of treated mice was also seen, but a similar decline in splenocyte numbers was not seen when the leukocytes were counted.

The most striking finding was that when the 5.2−→5.1+ chimeras were treated with TCDD, no alterations were seen in thymic weight or thymic cell number (Fig. 4, C and D). Other parameters, such as body weight and spleen weight, did not show any significant difference from the controls.

Phenotypic profile of various immune compartments is unaltered in 5.2−→5.1+ mice

Given that the roles of stromal cells in hemopoietic tissues are to support the maturation, differentiation, and, in the thymus, selection of thymocytes, it is possible that TCDD could be acting on the stromal elements to cause shifts in the phenotypic profile of the hemopoietic cells independently of thymic atrophy. Three-color flow cytometry was performed to assess phenotypic shifts among the thymocytes, splenocytes, and bone marrow cells from chimeric mice treated with TCDD.

Figure 5A shows the phenotypic alterations seen in the thymus when chimeric mice containing both sensitive stroma and hemopoietic elements (5.2−→5.1−) were treated with TCDD. The phenotypic results of all mice from all experiments are averaged in Table II. Slight, but statistically significant, shifts in the percentage of CD4+CD8−, CD4+CD8+, and CD4+CD8− cells were seen. The cells were also stained with CD3, and there was a modest increase in cells with a high level of CD3 on their surface (14.5 to 19.8%), as we would have expected from the increases seen with the single-positive CD4 and CD8 cells.

Since significant changes in the percentage and number of CD4+CD8−, double-negative (DN), cells in TCDD-treated mice were observed, we further analyzed this population using T cell maturation markers, CD44 and CD25. Pro-T cells are seeded from...
the bone marrow expressing CD44; once in the thymus, these cells up-regulate their CD25 to become CD44$^+$CD25$^+$. CD44 is then down-regulated, resulting in the CD44$^-$CD25$^+$ pre-T cell. Finally, CD25 is down-regulated before the cells move onto the next stage of thymic development, mainly CD4$^+$CD8$^+$. When CD44 and CD25 were used to stain CD3$^+$, CD4$^+$, and CD8$^+$ cells in TCDD-treated mice, there were significant decreases in the percentage and the number of CD25$^+$ cells in the triple-negative population. There was also a significant increase in the percentage of CD44$^+$ cells, although the absolute number of cells was reduced (Table III). These results suggest that TCDD is affecting the DN population at a specific stage of maturation.

When the chimeric mice containing sensitive stroma but insensitive hematopoietic cells (5.2$^-$→5.1$^+$) were treated with TCDD, no major alterations were seen in the CD4 and CD8 phenotypes of their thymocytes compared with the controls (Fig. 5B). There was, however, a slight decline in the percentage of CD4$^+$ cells (Table II), but this was not a consistent finding among all the experiments. Expression levels of CD44 and CD25 in the triple-negative population were not altered significantly when these mice were treated with TCDD (Table III). These phenotypic results indicate that the radioresistant stromal cells were not responsible for alterations

FIGURE 3. CYP1A1 RNA induction in TCDD-treated AhR chimeric mice is normal. Twenty micrograms of total RNA isolated from the livers of TCDD and oil-treated AhR chimeric mice was run on a 1% agarose formaldehyde gel. The RNA was transferred to a nylon membrane and hybridized with a $^{32}$P-labeled CYP1A1 cDNA. $^{32}$P-labeled L32 was used for normalization of lanes. A, Two TCDD-treated and two oil-treated 5.2$^-$→5.1$^+$ mice. B, Two TCDD-treated and two oil-treated 5.2$^-$→5.1$^+$ mice. Results shown are representative of 20 mice (five of each type and treatment).

FIGURE 4. 5.2$^-$→5.1$^+$ mice do not respond to TCDD. Eight-week-old 5.2$^-$→5.1$^+$ (A and B) or 5.2$^-$→5.1$^+$ (C and D) were treated with 30 μg/kg of TCDD or with oil alone (Control). Ten days later the mice were sacrificed, and the following parameters were measured: body weight, thymic weight, and splenic weight (A and C) and thymic and splenic cell numbers (B and D). Results are expressed as the mean ± SD (n = 5). * indicates p < 0.05; *** indicates p < 0.001.

FIGURE 5. 5.2$^-$→5.1$^+$ thymocyte subpopulations are unaffected by TCDD treatment. Eight-week-old 5.2$^-$→5.1$^+$ mice (A) or 5.2$^-$→5.1$^+$ mice (B) were treated with 30 μg/kg of TCDD or with oil (Control). After 10 days the mice were sacrificed, and cell suspensions of their thymocytes were stained with anti-CD4 and anti-CD8. Shown are representative dot plots in logarithmic scales from cells falling within a viable lymphocyte size gate, as determined by forward and side scatter. Numbers refer to the frequency of cells within each quadrant.
seen in the double-positive (CD4+CD8+), single-positive CD8 cells, or DN populations of 5.2→5.1+ TCDD-treated mice.

Analysis of the splenocytes failed to show any significant alterations in CD4, CD8, CD3, or B220 expression for either chimera, 5.2→5.1+ or 5.2→5.1−, treated with TCDD (data not shown). Therefore, even when both the stromal and hemopoietic elements were sensitive to TCDD, there were no significant alterations in spleen lymphocyte phenotypes.

Since TCDD is known to impair the thymic reconstitution capacity of bone marrow prothymocytes (31) and reduce the expression of lymphocyte-specific markers in the bone marrow (31, 53), it is possible that changes in bone marrow contribute to TCDD-induced thymic alterations in the AhR chimeric mice. To determine this, we analyzed the bone marrow of AhR chimera mice for phenotypic shifts in the putative hemopoietic stem cell populations. Although there is continuing controversy over the exact correlation of phenotypic markers in the bone marrow with T-lineage commitment (54–59), there is some general agreement about which bone marrow populations contain cells that have thymic reconstituting ability (60–62). Efforts to isolate and characterize stem cells by surface phenotype have found that the murine lineage-negative (lin−) low Thy-population is enriched for cells with long term multilineage reconstitution activity and day 12 CFU spleen and thymic repopulating ability (60). Cells in this compartment that stain for Sca-1 have more stem cell potential than those lacking this marker (60, 61). Other studies showed that c-kit+Sca-1 lin− cells contained more stem cell activity than the c-kit− set (61, 62). Based on these data, we examined the consequence of TCDD exposure on the c-kit and Sca-1 bone marrow populations in the chimeric mice. Figure 6A shows phenotypic alterations to the lin− fraction of bone marrow cells with respect to Sca-1 and c-kit expression when chimeric mice containing both sensitive stroma and hemopoietic elements (5.2→5.1+) were treated with TCDD. By contrast, Figure 6B shows a lack of effect on these populations when stroma is sensitive but hemopoietic elements are not. Phenotypic results for lin− populations from chimera with positive stroma are summarized in Table IV. Statistically significant increases were seen in the lin−c-kit+Sca-1− and the lin−c-kit−Sca-1− populations, while a significant decrease was seen in the lin−c-kit+Sca-1+ set. Chimeras lacking the AhR in their hemopoietic compartment did not show statistically significant alterations. These results suggest that TCDD is altering the hemopoietic stem cell population through activation of the AhR in hemopoietic cells and not through radioreistant stromal cells.

### Tables

**Table II. Phenotypic alterations in the thymocyte profile of 5.2→5.1+ and 5.2→5.1− mice treated with TCDD**

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<th>CD4+CD8−</th>
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</tbody>
</table>

* Eight-week-old 5.2→5.1+ and 5.2→5.1− mice were injected with either 30 µg/kg, i.p., of TCDD or 0.1 ml/20 g, i.p., of oil and sacrificed 10 days later. Thymocytes were harvested as described and stained with anti-CD4 and anti-CD8, and analyzed by flow cytometry. Values represent an average of four to five mice (±SD). 5.2→5.1+ or mice m (5.2→5.1−). *p < 0.05; **p < 0.01; ***p < 0.001.

* Percent of viable lymphocyte size cells (determined by forward and side scatter).

* Absolute number of thymocytes within each CD4/8 subset was calculated according to the frequency of the subset and the total number of cells per thymus.

### Table III. Phenotypic alterations in the triple negative thymocyte compartment of 5.2→5.1+ and 5.2→5.1− mice treated with TCDD

<table>
<thead>
<tr>
<th></th>
<th>CD44+CD25+</th>
<th>CD44+CD25−</th>
<th>CD44−CD25+</th>
<th>CD44−CD25−</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2→5.1+ Percentage</td>
<td>Control</td>
<td>5.14 ± 0.93</td>
<td>2.63 ± 1.38</td>
<td>33.07 ± 3.71</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>8.57 ± 1.34**</td>
<td>3.61 ± 0.75</td>
<td>26.22 ± 2.95*</td>
</tr>
<tr>
<td>5.2→5.1− Percentage</td>
<td>Control</td>
<td>0.33 ± 0.05</td>
<td>0.16 ± 0.08</td>
<td>2.10 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>0.12 ± 0.02***</td>
<td>0.05 ± 0.007*</td>
<td>0.39 ± 0.10***</td>
</tr>
</tbody>
</table>

* Eight-week-old 5.2→5.1+ and 5.2→5.1− mice were injected with either 30 µg/kg, i.p., of TCDD or 0.1 ml/20 g, i.p., of oil and sacrificed 10 days later. Thymocytes were harvested as described and stained with anti-CD4/8a/e FITC in combination with anti-CD4/4PE and anti-CD25Bio. Using flow cytometry, cells that were CD4/8− and fell within a lymphocyte size gate were collected and analyzed for their expression levels of CD44 and CD25. Values are an average of four to five mice (±SD) from a representative experiment. There were variations noted in the basal level of staining for controls between experiments but the shifts in the percentages that occurred after treatment were consistent. *p < 0.05; **p < 0.01; ***p < 0.001.

* Absolute number of thymocytes within each subset was calculated according to the frequency of the subset and the total number of cells in the triple negative compartment.
group 6) to further substantiate that AhR−/− chimeras are completely unresponsive to the effects of TCDD in the thymus and that the process of irradiation/reconstitution did not alter their unresponsiveness.

Figure 7, A and B, shows that there was significant thymic atrophy in the 5.2−→5.2+ chimeric mice treated with TCDD with respect to both thymic weight and thymic cell number. By cell number there was an average 69% reduction in all TCDD-treated mice compared with that in the 5.2−→5.1+ mice (Table IV). The bone marrow data from these mice also paralleled those from the 5.2−→5.1+ treated mice with an increase in the c-kit−Sca-1+ phenotype from 5.5 ± 0.5 to 11.9 ± 3.9 (p < 0.01), an increase in the c-kit−Sca-1+ phenotype from 4.2 ± 1.3 to 6.0 ± 1.6 (p < 0.01), and a decrease in the c-kit−Sca-1+ phenotype from 58.6 ± 7.5 to 38.6 ± 9.9. In comparison, phenotypic results for both the thymus (data not shown) and the bone marrow cells of TCDD-treated 5.2−→5.2+ mice showed no change from control values (10.4 ± 3.9 to 8.9 ± 2.1 for c-kit−Sca-1+, 4.6 ± 0.9 to 4.3 ± 1.0 for c-kit−Sca-1−, and 42.5 ± 7.9 to 44.5 ± 4.5 for c-kit−Sca-1−). Splenocytes from treated 5.2−→5.2+ and 5.2−→5.2− mice showed no difference from controls in CD3, CD4, CD8, and B220 populations (data not shown).

Discussion

The AhR is known to mediate most, if not all, of the effects of TCDD and its related congeners (1). In the immune system TCDD has been shown to suppress the immune response and to cause thymic atrophy (8, 9). Some investigators have suggested that stromal elements in the thymus, specifically epithelial cells, are the primary targets for TCDD induction of thymic atrophy (24–26). Others have suggested that atrophy is due to a direct effect on thymocytes, either by directly inducing apoptosis (27–30) or by arresting the development of progenitor cells (31, 63). By constructing radiation chimeric mice using hemopoietic stem cells from AhR knockout mice and then challenging these chimeras with TCDD, we were able to determine whether epithelial elements play a role in mediating thymic atrophy.

Our results indicate that hemopoietic elements of the immune system play the major, if not the exclusive, role in mediating the effects of TCDD on the thymus. When chimeric mice containing unresponsive (AhR−/−) hemopoietic elements and responsive (AhR+/+) stromal elements were treated with TCDD, no signs of thymic atrophy were seen (Fig. 4, C and D). In addition, there were no significant alterations in the phenotype of the thymus (Fig. 5B and Table II). This lack of atrophy and phenotypic alterations were also seen in chimeras containing both insensitive stromal and sensitive hemopoietic elements (Fig. 7, C and D). In contrast, chimeric mice containing sensitive hemopoietic and stromal elements and mice that had only sensitive hemopoietic elements had similar and significant declines in thymic weight and cell numbers after TCDD treatment (p < 0.001; Fig. 4, A and B, and Fig. 7, A and B). Both these chimeras had similar phenotypic alterations, including a reduction in the percentage of CD4+CD8− and an increase in the percentage of CD4−CD8− and CD4−CD8+ cells compared with the control percentages (Fig. 5A and Tables II and V). These observations agree with those reported by us and other investigators in other mouse strains after TCDD treatment (22, 24, 32, 33, 53).

### Table IV. Phenotypic alterations in the lineage-negative bone marrow fraction of 5.2−→5.1+ and 5.2−→5.1+ mice treated with TCDD

<table>
<thead>
<tr>
<th></th>
<th>c-kit−Sca-1+</th>
<th>c-kit−Sca-1−</th>
<th>c-kit+Sca-1+</th>
<th>c-kit+Sca-1−</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2−→5.1+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>Control</td>
<td>TCDD</td>
<td>Control</td>
<td>TCDD</td>
</tr>
<tr>
<td>8.29 ± 1.69</td>
<td>10.61 ± 1.09*</td>
<td>6.20 ± 1.61</td>
<td>19.36 ± 3.47***</td>
<td>46.80 ± 9.29</td>
</tr>
<tr>
<td>5.2−→5.1+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>Control</td>
<td>TCDD</td>
<td>Control</td>
<td>TCDD</td>
</tr>
<tr>
<td>3.37 ± 0.49</td>
<td>3.31 ± 0.25</td>
<td>6.70 ± 1.45</td>
<td>8.32 ± 0.82</td>
<td>43.71 ± 2.19</td>
</tr>
</tbody>
</table>

* Eight-week-old 5.2−→5.1+ and 5.2−→5.1+ mice were injected with either 30 μg/kg, i.p., of TCDD or 0.1 ml/20g, i.p., of oil and sacrificed 10 days later. Bone marrow was harvested as described, stained with a panel of lineage antibodies together with anti-Sca-1 and anti-c-kit, and analyzed by flow cytometry. Total cell yield within the lineage negative compartment was not altered with TCDD treatment. Values represent an average of multiple experiments ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.

* c-kit− incorporates both c-kit+ and c-kit−.
Since there were alterations in the percentage and number of DN cells in TCDD-treated chimeras containing sensitive hemopoietic cells, we analyzed this population using CD44 and CD25 markers (52). After TCDD treatment, there was a decrease in the percentage of immature CD44^1 cells compared with that in the CD25^1 cells obtained from TCDD-treated chimeras (Table III), suggesting that TCDD must be directly targeting hemopoietic cells to cause this early thymocyte developmental block.

Besides examining the thymus, we also analyzed the effects of TCDD on spleen and bone marrow in these chimeras. Both 5.2^+→5.1^+ and 5.2^−→5.1^− chimeras manifested loss in spleen weight, although the decline only achieved significance in the 5.2^+→5.1^+ mice (Fig. 4, A and C). This decline in weight, however, did not translate into a decline in leukocyte cell number (Fig. 4, B and D). A feasible explanation for this discrepancy could be a loss of RBC, which has been reported previously (64). Analysis of spleen cell phenotypes did not reveal differences in the major T cell classes or in total B220^+ B cells in any of the AhR chimeras studied. This, too, has been reported for nonchimeric mice of other strains (32).

Bone marrow data from chimeric mice with sensitive (AhR^+/+) hemopoietic elements showed an increase in lin^-c-kit^-Sca-1^- and lin^-c-kit^-Sca-1^- populations regardless of whether the radioresistant stromal elements were AhR^+/+ or AhR^-/- (Fig. 6, Table IV, and text). Since the total number of bone marrow cells isolated and the percentage of lin cells obtained from TCDD-treated chimeras were not significantly different from the control values, these results suggest that shifts are occurring within the putative hemopoietic stem cell populations, including those cells that could give rise to thymocytes.

### Table V. Phenotypic alterations in the thymocyte profile of 5.2^+→5.2^− mice treated with TCDD^a^

<table>
<thead>
<tr>
<th></th>
<th>CD4^+ CD8^-</th>
<th>CD4^+ CD8^-</th>
<th>CD4^+ CD8^-</th>
<th>CD4^+ CD8^-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percentage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>82.27 ± 3.82</td>
<td>6.86 ± 1.89</td>
<td>3.24 ± 0.55</td>
<td>5.82 ± 1.78</td>
</tr>
<tr>
<td>TCDD</td>
<td>68.48 ± 14.86**</td>
<td>12.94 ± 5.94*</td>
<td>8.59 ± 3.86***</td>
<td>9.58 ± 6.07*</td>
</tr>
<tr>
<td><strong>Cell count</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.53 ± 4.11</td>
<td>0.97 ± 0.43</td>
<td>0.37 ± 0.17</td>
<td>0.62 ± 0.23</td>
</tr>
<tr>
<td>TCDD</td>
<td>2.77 ± 2.51***</td>
<td>0.34 ± 0.21***</td>
<td>0.22 ± 0.13*</td>
<td>0.23 ± 0.12***</td>
</tr>
</tbody>
</table>

^a^ Eight-week-old 5.2^+→5.2^− mice were injected with either 30 μg/kg, i.p., of TCDD or 0.1 ml/20 g, i.p., of oil and sacrificed 10 days later. Thymocytes were harvested as described, stained with anti-CD4 and anti-CD8, and analyzed by flow cytometry. Values represent an average of 14 mice (±SD) from three separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

^b^ Percent of viable lymphocyte size cells (determined by forward and side scatter).

^c^ Absolute number of thymocytes within each CD4/8 subset was calculated according to the frequency of the subset and the total number of cells per thymus.
These results supplement our previous studies that demonstrated that bone marrow cells from mice exposed to TCDD had reduced thymic reconstituting potential (31) and a decided reduction in the expression of lymphocyte-specific markers such as terminal deoxynucleotidyl transferase protein (31) and terminal deoxynucleotidyl transferase and recombinase-activating gene-1 mRNA (53). Since there is still a fair amount of controversy about which cell marks define the potential T cell progenitors or pluripotent hematopoietic stem cells in the bone marrow (57–62, 65, 66), it is difficult to correlate our current findings of lin−c-kit+Sca-1 alterations to our previous findings of a reduction in lymphoid stem cell activity and markers. Nevertheless, the results from the chimeric mice suggest that TCDD is directly affecting putative hematopoietic stem cell populations in the bone marrow independent of the bone marrow stromal elements. In addition, these data are consistent with the possibility that TCDD-induced thymic alterations may be mediated at least in part by effects of TCDD directly on the hematopoietic compartment in the bone marrow.

Although we did not see significant alterations in the thymus or bone marrow of TCDD-treated 5.2−→5.1+ chimeras, we did see induction of CYP1A1 RNA in liver from these animals. The degree of induction in the 5.2−→5.1+ chimeras was similar to that of 5.2−→5.1+ chimeras (Fig. 3), confirming that AhR−/− hematopoietic cell-containing mice treated with TCDD produce a normal hepatocyte response. In preliminary observations we also noted that there were fewer significant lesions in the livers of TCDD-treated 5.2−→5.1+ chimeras than in the livers of TCDD-treated 5.2−→5.1− chimeras, which had focal necrosis with infiltration of immune cells in the perivascular regions. T.S. Thurmond, manuscript in preparation). This observation suggests that some liver lesions induced by TCDD may be a result of immune cell activation. TCDD treatment has been shown by others to potentiate the response of lymphoid cells to anti-CD3 T cell activation (67). Therefore, these chimeras could be used to determine whether the effects of TCDD and congeners are due to a direct effect on a tissue or an indirect effect mediated by activation of immune cells.

Our results show that TCDD-induced thymic atrophy is mediated by hematopoietic cells, but these studies do not allow us to determine the specific cell type(s) that is the direct target(s) for mediating this effect. Both DC and macrophages of the thymic stroma are believed to be sensitive to irradiation (39) and should therefore be ablated in recipients during the construction of the chimeras. Since these elements are bone marrow derived (40, 68), they are reconstituted with either AhR−/− or AhR+/+ cells depending on the donor. These hematopoietic elements of stroma might be the cellular targets for TCDD activation of the AhR, rather than developing lymphoid cells. However, both DC (6, 43, 44) and macrophages (34, 40) have been shown to be actively involved in negative selection, and since TCDD does not seem to alter negative selection (33, 69), it is less likely that nonlymphoid hematopoietic cells are the decisive cellular targets. This evidence again suggests that the hematopoietic elements are the main target of TCDD action. Previous work by others has also provided some evidence that hematopoietic elements mediate TCDD suppression of the T cell-dependent primary Ab response (70). Additionally, we have provided evidence that some of the lymphoid targets for the effects of TCDD could include T cell progenitors in the bone marrow (31, 53).

Although our results support a direct action on developing thymocytes or lymphoid stem cells by TCDD, they contradict the results of others (24–26) who have argued that epithelial cells mediate the action of TCDD in the thymus. These previous investigations, however, were performed using higher doses of TCDD or in vitro culture systems. For example, De Waal et al. (26) saw changes in the cortical-epithelial ultrastructure of the rat thymus following the administration of 150 μg/kg of TCDD. However, at 50 μg/kg such epithelial changes were not detectable, although there was significant atrophy. We observed atrophy induction at an even lower dose, so the epithelial effects observed by De Waal et al. might be due to additional toxic effects of TCDD at higher doses.

Greenlee (25) showed that thymocytes cultured on thymic epithelial layers incubated with TCDD had reduced responses to T cell mitogens. This result does not explain thymic atrophy induction, since the main responders in the mitogen assay should be the mature population in the thymus, and the mature cells, according to our data, do not appear to be the target population. Kremer et al. (24) used fetal thymic organ cultures to show that epithelial elements exposed to TCDD failed to support proliferation of early thymocytes. This difference from our in vivo results has precedent (21), possibly because factors present in serum of the cultures or interactions of TCDD with serum factors (71) could be creating in vitro-specific effects. The in vitro systems are also unable to evaluate the contributions of bone marrow precursors, thymic ultrastructure, and neuroendocrine signals (72). One might argue that fetal thymic organ cultures address some of these deficiencies, but it is hard to make a direct connection between the in vitro effects of TCDD on an immature (gestational day 15) thymic structure and a block in development to TCDD effects on the adult organ that clearly involve a loss of cells. For example, DC are not prominent in the thymus until gestational day 17 (73), and in the developing organ there could be multiple targets (74).

Our overall conclusions are the following. 1) TCDD induces thymic atrophy by directly targeting the T cell progenitors (31, 53). This leads to a reduction of cells entering the thymus and a decline in the absolute number of DC cells (Tables II and V). 2) TCDD exposure inhibits the development of cells in the DN compartment through a direct effect on hematopoietic cells. This causes a decline in the number of maturing DN CD25+ cells (Tables III and VI), which, in turn, leads to a decline in the number of cells leaving the

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**Table VI. Phenotypic alterations in the triple negative thymocyte compartment of 5.2−→5.2− mice treated with TCDD**

<table>
<thead>
<tr>
<th>Percentage Control</th>
<th>CD4+CD25−</th>
<th>CD4−CD25−</th>
<th>CD4−CD25+</th>
<th>CD4+CD25+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.27 ± 0.85</td>
<td>0.25 ± 0.26</td>
<td>20.40 ± 5.95</td>
<td>77.09 ± 6.04</td>
</tr>
<tr>
<td>TCDD</td>
<td>4.39 ± 1.42***</td>
<td>0.34 ± 0.49</td>
<td>16.50 ± 8.57</td>
<td>78.76 ± 9.61</td>
</tr>
</tbody>
</table>

**a** Eight-week-old 5.2−→5.2− mice were injected with either 30 μg/kg, i.p., of TCDD or 0.1 ml/20 g, i.p., of oil and sacrificed 10 days later. Thymocytes were harvested as described and stained with anti-CD4/8a/α/β FITC in combination with anti-CD4/α/β negative and fell within a lymphocyte size gate were collected and analyzed for their expression levels of CD44 and CD25. Values are an average of 14 mice (±SD) from three separate experiments. There were variations noted in the basal level of staining for controls between experiments, but the shifts in the percentages that occurred after treatment were consistent. ***p <0.001.

**b** Absolute number of thymocytes within each subset was calculated according to the frequency of the subset and the total number of cells in the triple negative compartment.

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DN compartment and contributing to the double-positive compartment. 3) The shifts seen in double-positive, CD8 single-positive, and DN cells in the thymus after TCDD treatment are also caused by a direct effect on hematopoietic cells as chimeras, with AhR<sup>-/-</sup>-hemopoietic elements do not show these shifts (Table II).

Finally, our results further support the idea that the AhR plays a role in the immune system, as suggested by the development of AhR knockout mice. It was reported that AhR<sup>-/-</sup> mice, in which exon 1 of the AhR gene is knocked out, developed perturbations in observed in a second AhR<sup>2/-</sup> mouse. Further studies

**References**


