DIOXIN CAUSES THYMIC ATROPHY BY INHIBITING ENTRY INTO CELL CYCLE IN THE EARLIEST THYMIC LYMPHOCYTE DEVELOPMENTAL STAGE.

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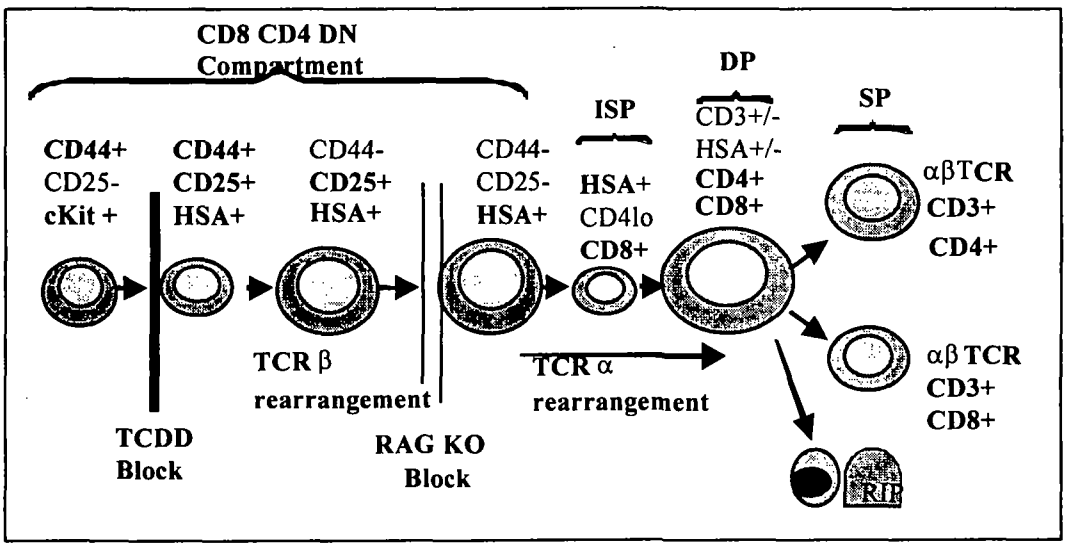
Introduction:
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and its related congeners affect the immune system, causing immunosuppression, and, in all mammalian and avian species examined, thymic atrophy[1]. The thymus is the organ in which the T-cell population is created and educated. Utilizing bone marrow radiation chimeras with AhR-KO mice, we have demonstrated that thymic atrophy (and other immune system effects) are mediated by the direct activation of the AhR in hemopoietic cells[2]. Furthermore, using fetal thymic organ cultures (FTOCs) as well as normal adult animals, we have shown that TCDD treatment causes an inhibition of thymic development in the earliest stage of development[2,3].

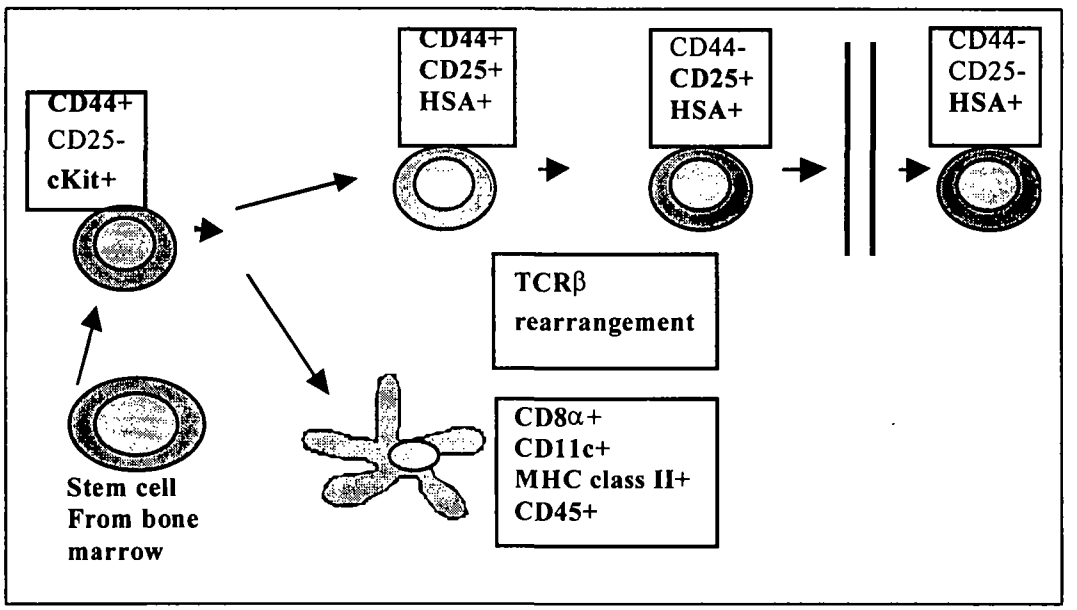
Figure 1.



Although our previous work showed that the AhR must be activated in the bone marrow derived hemopoietic cells to cause thymic atrophy[2], it is possible that the effect is mediated indirectly through bone marrow derived macrophage and dendritic cells which are also derived from bone marrow precursors rather than by a direct effect on the developing lymphocytes. That is, CD11c

positive cells can develop in the thymus (and bone marrow) prior to commitment to lymphopoeisis with induction of the gene rearranging recombinase genes (RAG) as shown in Figure 2. These non lymphoid cells could provide signals for development, and since they also will lack a functional AhR in the chimeras we previously constructed, they would be unresponsive to the effects of TCDD

Figure 2



Using a strategy that we have confirmed will permit us to reconstitute the non lymphoid hemopoietic compartment with AhR positive cells, while the developing lymphoid population lacks the AhR, we here demonstrate that the AhR effect is mediated directly on the developing thymic lymphocyte population and furthermore, that the primary effect is a reduction of thymic stem cells enetering cell cycle. This supports our work[3] and that of others[4] that suggest TCDD's primary effect is cell cycle inhibition and not induction of apoptosis.

Materials and Methods.

Radiation Chimeras: Recombinase activating gene (RAG) knock-outs and Aryl hydrocarbon receptor (AhR) knockout mouse mixed chimeras were produced by hematopoietic reconstitution of lethally irradiated mice as described previously[2]. 4-5 week old C57BL6/Ly5.1 congenic mice exposed to 110 Gy of x-irradiation were innoculated with 5-10 x 10^5 nucleated bone marrow cells from AhR-KO mice (and conversely) with or without 5 or 50 x 10^5 nucleated bone marrow cells from BL6 Ly5.2 RAG-KO mice. After 4-5 weeks these mice were treated with TCDD (30μg/kg) in a single IP injection in olive oil. Controls were injected with olive oil alone. After 10 days, thymus was removed, weighed, cell suspensions made and enumerated as described[3]. Cells were stained with monoclonal antibodies for Ly5.1 and Ly5.2.

Fetal Thymic Organ Cultures (FTOC) were prepared as previously described. from the mating of C3H female mice with male p56lck^{Pr}-*bcl-2* heterozygous C3H-trangenic mice. On gd 15, mice were euthanized, fetal livers removed for genotype analysis using DNA slot blotting with a ^{32}P-dCTP labeled human *bcl-2*/GH probe and thymuses placed on filters for cultivation and treatment[6] Following treatment with TCDD, diethylstilbestrol (DES) (20 μM), or Dexamethasone (DEX) (1μM), cells were harvested stained for surface markers, then fixed and permeabilized for cell cycle analysis by propidium iodide staining and for apoptosis using a FACS based TUNEL assay as described previously[5,6].

Results and Discussion

When lethally irradiated BL6 mice are reconstituted with mixtures of bone marrow from AhRKO and Recombinase negative (RAG-KO) mice, their thymuses and bone marrow contain myeloid and dendritic cells and thymic DN lacking and containing the AhR in proportion to their input (data not shown). On the other hand, all maturing thymocytes (after the DN stage) and all maturing B cells will lack the AhR as RAG-KO cells cannot develop because they cannot rearrange their T-cell receptor or Immunoglobulin genes. As shown in Table 1, AhR Positive Dendritic Cells (from RAG-KO mice) are not able to induce atrophy in response to TCDD in chimeras lacking the AhR in their thymocytes populations.

Table 1.

AhR recipient Genotype	AhR Donor genotype	RAG-KO Stem cells	Response to TCDD
+/+	+/+	None	Atrophy
KO	KO	None	No atrophy
+/+	KO	None	No atrophy
KO	+/+	None	Atrophy
+/+	KO	= to AhRKO donor	No Atrophy
+/+	KO	10X AhRKO donor	No atrophy

Even in the presence of a vast excess of positive epithelial, myeloid, and dendritic cells, TCDD cannot induce atrophy if the maturing thymic lymphocyte population lacks the AhR.

There continues to be some debate about whether atrophy results from the induction of apoptosis (in cells maturing beyond the CD44+25- stage)[7] or whether it is due to developmental arrest and if so, is this arrest due to or merely associated with inhibition of proliferation[5,6]. Utilizing fetal thymic organ cultures from mice expressing elevated levels of the anti-apoptotic oncogene, bcl-2 under control of a thymocyte specific promoter we have demonstrated that proliferation arrest is the cause of thymic atrophy and not apoptosis. The results of these studies are shown below.

Table 2.

Treatment	% Thymocytes in cycle	% DN Thymocytes in cycle	% Thymocytes apoptotic	% DN Thymoctyes in cycle
Control	16 (7)	30 (19)	2(1)	4 (1)
TCDD	11 (5)	11 (5)	1 (1)	2 (1)
DES	32 (24)	34 (37)	9 (2)	23 (2)
DEX	ND	ND	14 (1)	ND

(Results in parenthesis are from bcl-2-tg+ mice).

TCDD strikingly reduces the percent of cells in cycle, especially in the stem cell compartment, while DES increases the percentage in cycle. Apoptosis is seen with DES and Dexamethasone (the latter at 12 hours for maximum, DES at 24 hours, maximum), and bcl-2 protects against this. No apoptosis is seen with the TUNEL assay or a highly sensitive Pulse Field Gel Assay in TCDD treated cultures.

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