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Constitutively active aryl hydrocarbon receptor expressed in T cells increases immunization-induced IFN-γ production in mice but does not suppress T_h2-cytokine production or antibody production

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Abstract

The ligand-dependent transcription factor aryl hydrocarbon receptor (AhR) has been implicated in various immune functions. Our previous studies have shown that AhR activation by exposure of ovalbumin (OVA)-immunized mice to the potent ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases immunization-induced IFN-γ production in the spleen and suppresses the production of T_h2 cytokines and OVA-specific antibodies. In the present study, we used transgenic (Tg) mice that express a constitutively active mutant of aryl hydrocarbon receptor (CA-AhR) specifically in T-lineage cells to clarify the role of AhR activation in T cells in these reactions. The results of this study clearly demonstrated that AhR activation only in the T cells augments IFN-γ production upon OVA immunization. By contrast, production of T_h2 cytokines and antibodies were not significantly suppressed by CA-AhR in the T cells. These results suggest that suppression of T_h2 cytokines and antibodies production require AhR activation not only in T cells but also in other cell types as caused by TCDD exposure. Alternatively, these results may indicate that IFN-γ augmentation and T_h2 cytokines and antibodies suppression depend on different ways of functions of AhR in the T cells and that CA-AhR does not replicate the suppressive effect of TCDD-activated AhR on T_h2 cytokines and antibodies. Expression of CA-AhR in the T cells was also shown to increase the percentage of $CD25^+$ cells among $CD4^+$ cells in the thymus and spleen. Thus, studies using T-cell-specific CA-AhR Tg mice provide a way to dissect the role of AhR in individual cell types and how the AhR functions.

Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor and a member of the basic helix–loop–helix (bHLH) -PAS superfamily (1–3). While its physiological ligand has yet to be identified, a variety of xenobiotic chemicals, such as dioxins and polycyclic aromatic hydrocarbons, have been found to act as ligands. Among the dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is well known to be the most stable ligand with the highest affinity for AhR. In the absence of such ligands, the AhR exists in the cytoplasm in the form of a complex with Hsp90, XAP2 and p23 proteins (2). Upon binding to ligands, the AhR becomes activated and translocates into the nucleus, where the receptor dissociates from the Hsp90s and dimerizes with another transcription factor, aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer specifically binds DNA sequences called xenobiotic-responsive elements (XREs) and induces or modulates expression of various genes, which leads to the development of numerous

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biological and toxic reactions (1–4). Evidence of the intrinsic physiological roles of the AhR has been accumulating in recent years (5, 6), and numerous endogenous compounds, including indigoids and tryptophan metabolites, have been proposed as endogenous and physiological ligands (7).

In the immune system, the AhR has been shown to be associated with a variety of immune reactions. Exposure of mice to TCDD induces thymus atrophy (8–10), suppresses CTL activity (11, 12) and reduces antibody production (13, 14), all of which have been attributed to AhR activation, since none of these effects has been observed in AhR-deficient mice (15–17). Recent studies have shown that AhR activation by TCDD enhances expansion of $CD4^+CD25^+$ cells, particularly $CD62L^{lo}CD4^+CD25^+$ cells, and $CD4^+Foxp3^+$ regulatory T (Treg) cells (18, 19), and the AhR is also involved in T_h17 differentiation (19–21). In our previous studies, we found that exposing mice immunized with ovalbumin (OVA) to TCDD suppressed antigen-induced production of T_h2-type cytokines (IL-4, IL-5 and IL-6) and increased production of IFN-γ by splenocytes prior to suppression of antigen-specific antibody production (22, 23). We also found that TCDD suppressed immunization-induced increases in splenic T cells and B cells (14, 23). These findings suggest that AhR activation by TCDD targets the T cells and suppresses antibody production by inhibiting T_h2 cytokine production and expansion of T cells and B cells. However, the function of AhR activation only in the T cells in these reactions has not been clarified, since TCDD exposure to mice activates all the AhR in various cell types in the body.

In order to address the exact role of the AhR in the T cells, we previously generated transgenic (Tg) mice that specifically express a constitutively active mutant of aryl hydrocarbon receptor (CA-AhR) in T-lineage cells by expressing a CA-AhR under the regulation of a CD2 promoter (24). The mutant form of the AhR, which has a minimal deletion in the C-terminal portion of the ligand/Hsp90-binding site in the PAS-B domain, constitutively localizes to the nucleus, heterodimerizes with ARNT and activates transcription by binding XRE sequences in a ligand-independent manner (25). The mice generated express the transgene in their thymocytes, CD4 T cells and CD8 T cells, but not in their B cells, and they express the representative AhR-target gene *CYP1A1* in their thymus and spleen (24). The mice develop thymus atrophy and, upon OVA immunization, exhibit suppressed splenocyte expansion, showing that AhR activation in the T cells alone induces these reactions (24).

In the present study, we further investigated the primary and secondary immune responses of the Tg mice to OVA in order to clarify the contribution of AhR activation only in T cells to the individual immune responses.

Methods

Tg mice and immunization

CA-AhR Tg mice (line A) expressing a CA-AhR mutant and GFP in the T-lineage cells under the regulation of a CD2 promoter were generated as described previously (24) and maintained by backcrossing with C57BL/6J mice. The level of CYP1A1 mRNA expression in the thymus of the heterozygous Tg mice corresponded approximately to the level induced by a single dose of 20 μg TCDD kg^{-1} (24). The heterozygous Tg mice used for most of the experiments were backcrossed to C57BL/6J mice for 11 generations. AhR knockout mice were backcrossed to C57BL/6J.

To investigate the primary immune reactions, alum-precipitated OVA (OVA/alum) was prepared as described previously (14, 24), and the heterozygous Tg mice and their wild-type littermates (7–8 weeks old) were intra-peritoneally immunized with the OVA/alum (100 μg OVA per mouse) on Day 0. The animals were sacrificed for examination of cytokine production by splenocytes on Day 4 or 7, based on the results previously reported (23). The antibodies in plasma were examined on Day 7 or 10, based on the results previously reported (14). In some experiments (4-hydroxy-3-nitrophenyl)acetyl (NP) linked to chicken γ-globulin was used for immunization instead of OVA (14). To examine the secondary immune response (22, 26), heterozygous Tg mice and wild-type littermates (6–7 weeks old) were intra-peritoneally immunized with 10 μg OVA/2 mg $Al(OH)_3$ on Day 0 and were boosted with OVA 3 weeks later (Day 21). One week after the boost (Day 28), they were sacrificed for examination of their plasma and splenocytes.

TCDD exposure

Female B6D2F1 mice were purchased from Charles River Laboratories, Japan. TCDD (purity, 98%) was obtained from Cambridge Isotope Laboratory (Andover, MA, USA). A single dose of TCDD (40 μg kg^{-1}) or vehicle was administered to B6D2F1 mice or CA-AhR Tg mice by gavage, and the animals were subsequently immunized intra-peritoneally with OVA/alum as described previously (14). They were sacrificed on Day 4 for examination of the thymus, spleen and cytokine production or on Day 10 for antibody measurement.

Antibody measurement by ELISA

Plasma was prepared from immunized mice on the days stated above. The amounts of OVA-specific IgM and IgG1 in the plasma were measured by ELISA as described previously (14). The amounts of total IgE and OVA-specific IgE in the plasma were measured by ELISA as described previously (22, 26). Total or high-affinity NP-specific IgG1 was measured by using high-NP-haptenated or low-NP-haptenated BSA as described previously (14).

Cytokine measurement by ELISA

Splenocytes were prepared from immunized mice on the days stated above and cultured for 3 days at 1×10^6 cells in 200 μl in complete medium with or without OVA (100 μg ml^{-1}) (22, 27). The culture medium was then collected, and the IL-5 and IFN-γ in the supernatant were measured by ELISA using the BIOSOURCE Immunoassay Kit (Invitrogen Co.) and the High Sensitivity ELISA Ready-SET-Go! (eBioscience), respectively, according to the manufacturer's instructions.

FACS analyses of cellular composition

Single-cell suspensions of thymus and spleen were prepared and analyzed for cell surface markers with a FACSCalibur (BD Biosciences) as described previously (22, 24). The following monoclonal antibodies were purchased from eBioscience: anti-CD4-PE (clone GK-1.5), anti-CD8-biotin (53-6.7), anti-CD3ε-PE (145-2C11), anti-CD25-APC (PC61.5)

and anti-CD62L-PE-Cy5 (MEL-14). Anti-CD19-biotin (ID3) and streptavidin–APC were obtained from BD Biosciences.

Amounts of AhR protein and mRNA in CD4T cells

CD4 T cells were isolated from the spleens of C57BL/6 mice by positive selection with antibody-labeled magnetic particles (28). Briefly, the splenocytes were labeled with IMag Mouse CD4 Particles-DM (BD Biosciences), and the particle-labeled cells were isolated by passing them over a magnet according to the manufacturer's instructions. Western blotting was carried out as described previously (29). Antibody against AhR (SA-210) was purchased from BIOMOL. AhR mRNA was analyzed by reverse transcription (RT)–PCR as described previously (30). Mouse AhR sense primer 5′-acataacggacgaaatcctgacc-3′ and anti-sense primer 5′-tcaactctgcaccttgcttagga-3′ and HPRT sense primer 5′-gctggtgaaaaggacctct-3′ and anti-sense primer 5′-cacaggactagaaaatat-3′ were used.

Affymetrix GeneChip analysis and RT-PCR

Affymetrix GeneChip analysis was performed as previously described (28, 31). A single total RNA sample was prepared from three thymuses of the Tg mice or their wild-type littermates, and the fold change in gene expression in the Tg mice in comparison to the value in the wild-type mice was calculated. Two independent experiments were carried out, and the average of the results in the two experiments was given. RT-PCR of CYP1A1, adseverin (24) and AhRR (28) were performed as described previously. The sequences of primers used were 5′-GGACAAGGACGGCTTCATTA-3′ and 5′-ACTGATGAGCGAGGATGGAG-3′ for CYP1B1 and 5′-AGACTGTTCCAAAAACAGTGGA-3′ and 5′-GATGCTCTTTCCTCCTGTGC-3′ for cyclophilin B.

Results

CA-AhR expression increases the percentage of CD25$^+$ cells among CD4$^+$ cells

Consistent with the results of our previous study (24), the CA-AhR Tg mice immunized with OVA had lower thymus weights and lower cell numbers in their thymuses and spleens than their wild-type littermates did (Figs 1A and B and 2A and B). The cell numbers of all sub-populations examined in the thymuses and spleens were reduced (Figs 1C and 2C). A recent study in a mouse graft-versus-host model reported that TCDD exposure increases the percentage of donor CD62L^{lo}CD4$^+$CD25$^+$ cells that display the functional characteristics of Treg cells (18). In the present study, the thymocytes of the immunized CA-AhR Tg mice showed much higher percentages of CD62L^{lo}CD25$^+$ as well as CD62L^{hi}CD25$^+$ cells among their CD4$^+$ (CD4$^+$CD8$^+$ double positive and CD4$^+$ single positive) cells than their wild-type littermates did (Fig. 1D, right panel). The splenocytes also showed higher percentage of CD62L^{lo}CD4$^+$CD25$^+$ cells and total CD4$^+$CD25$^+$ cells among their CD4$^+$ cells compared with the wild-type littermates (Fig. 1D, right panel). The same results were observed in the thymus and spleen of unimmunized Tg mice (data not shown). These results show that AhR activation in T cells alone induces the phenotypic changes in the T cells in the thymus and spleen. However, the numbers of CD62L^{lo}CD25$^+$ and CD62L^{hi}CD25$^+$ cells were similar in the thymus of the Tg mice and the wild-type littermates (Fig. 1D, left panel), since the total numbers of CD4$^+$ cells in the Tg mice were lower than in the wild-type littermates (Fig. 1C). In the spleen, the numbers of CD62L^{lo}CD4$^+$CD25$^+$ cells and total CD4$^+$CD25$^+$ cells were rather lower in the Tg mice compared with the wild-type littermates (Fig. 2D, left panel) due to the reduced number of CD4$^+$ cells in the Tg mice (Fig. 2C).

CA-AhR expression increases IFN-γ production but does not suppress production of T_h2 cytokines or anti-OVA antibodies

Consistent with the results of a previous study on TCDD exposure of OVA-immunized mice (22), IFN-γ production by the splenocytes was also increased in the Tg mice in the primary (Fig. 3A) and the secondary (Fig. 3D) immune response. These results demonstrate that AhR activation in the T cells alone plays a part in augmenting IFN-γ production in the spleen.

We previously found that IL-5 production by the splenocytes of OVA-immunized mice is a very sensitive end point for detecting a suppressive effect of TCDD on the immune system (27). The expression of CA-AhR in the T cells in the Tg mice, however, did not suppress IL-5 production in either the primary (Fig. 3A) or secondary (Fig. 3D) immune response. IL-4 production was also not suppressed in the splenocytes of the Tg mice (Fig. 3A and D). Furthermore, no suppression of anti-OVA IgM, IgG1 or IgE production was observed in the Tg mice in either the primary (Fig. 3B) or secondary (Fig. 3E) immune response. Thus, these results were not consistent with the results of exposure of wild-type mice to TCDD, which suppressed production of T_h2-type cytokines and antigen-specific antibodies in the primary and secondary immune responses (14, 22, 23). Production of high-affinity antibodies against antigen (Fig. 3C) was also not suppressed in the Tg mice, in contrast to the TCDD-exposed wild-type mice (14).

TCDD exposure of CA-AhR Tg mice suppresses production of T_h2 cytokines and antibodies

Since T_h2-cytokine and antibody production was not suppressed in the CA-AhR Tg mice, we investigated whether these reactions in Tg mice were affected by TCDD exposure which activates all the intrinsic AhR in the body by comparing the effects of TCDD on the immune reactions in the Tg mice and wild-type mice. The CA-AhR Tg mice were derived from C57BL/6 × DBA/2 eggs (24). Line A Tg mice express not only AhR^b coded by the *b* allele from C57BL/6 mice but also AhR^d coded by the *d* allele of the *AhR* gene from DBA/2 mice even after backcrossing 17 times with C57BL/6 mice and selecting for CA-AhR (Fig. 6A). Thus, the CA-AhR transgenes are assumed to have inserted very close to the *AhR^d* locus of the gene in the C57BL/6 × DBA/2 egg and remain accompanied by AhR^d on the same chromosome. We therefore used B6D2F1 wild-type mice for a comparative reference purpose. Since AhR^d has much weaker affinity for its ligands than AhR^b does (32, 33), we dosed the mice with 40 μg kg^{-1} of TCDD (Fig. 4).

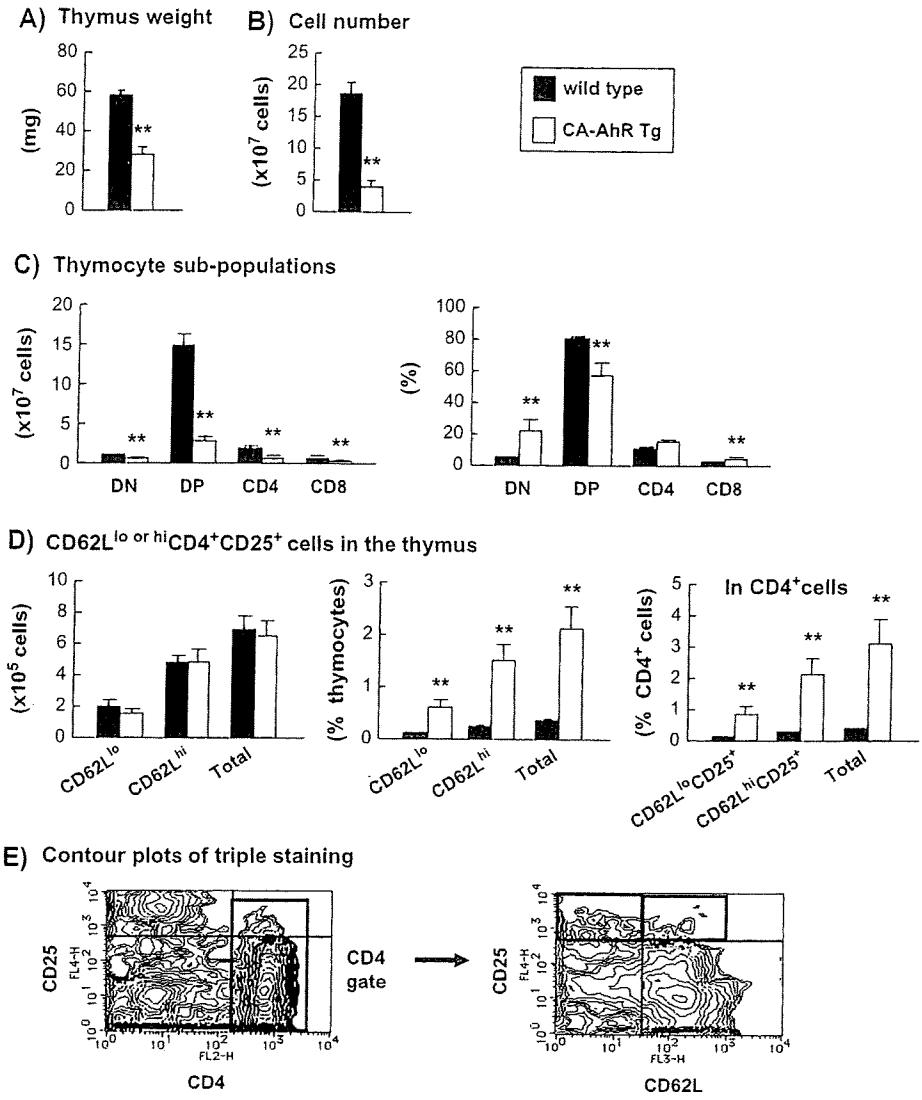

Fig. 1. The thymocytes of the CA-AhR Tg mice contained a higher percentage of $CD4^+CD25^+$ cells in the primary immune response to OVA. Female Tg (heterozygous) mice and littermate wild-type mice (n = 7 per group) were immunized with OVA/alum, and 7 days later their thymus weights (A) and thymocyte numbers (B) were examined. (C) Thymocytes were stained with a combination of anti-CD4-PE and anti-CD8-biotin/streptavidin–APC and measured by flow cytometry. The numbers (left panel) and percentages (right panel) are shown. (D and E) Thymocytes were stained with a combination of anti-CD4-PE, anti-CD25-APC and anti-CD62L-PE-Cy5 and measured by flow cytometry. CD4-positive cells were gated and analyzed for CD25 and CD62L staining. The differences between the Tg mice and wild-type mice were analyzed by Student's t-test. The data are expressed as means ± SDs. $^*P < 0.05$, $^{**}P < 0.01$

TCDD exposure further reduced the thymus weight in the OVA-immunized Tg mice, and the extent of the reduction by TCDD was very similar to that observed in the immunized B6D2F1 mice (Fig. 4A). Spleen weight was also lowered by TCDD to a similar extent in both the Tg mice and B6D2F1 mice (Fig. 4B). TCDD exposure of B6D2F1 mice increased the percentages of $CD62L^{lo}CD25^+$ cells and total $CD25^+$ cells in the spleen $CD4^+$ cells (Fig. 4C, left). The effect of a single dose of TCDD on the $CD4^+$ cells was very similar to that of CA-AhR expression in the T cells in the Tg mice (Fig. 2D, right). The percentages of $CD62L^{lo}CD25^+$ cells and total $CD25^+$ cells in the $CD4^+$ cells were also increased in the Tg mice by TCDD to a similar extent observed in the B6D2F1 mice (Fig. 4C). Although IL-4, IL-5 and anti-OVA IgM productions were not reduced in the Tg mice in comparison with their wild-type littermates (Fig. 3), TCDD exposure suppressed the production of these cytokines as well as IgM in the Tg mice (Fig. 4D and E). These results demonstrate that the immune system of the Tg mice retains sensitivity to TCDD. They also indicate that expression of CA-AhR in the T cells alone does not suppress the production of T_h2 cytokines or antigen-specific antibodies.

Anti-OVA IgG1 was unaffected by exposure of the Tg mice to the 40 μg kg^{-1} dose of TCDD, the same as the B6D2F1 mice (data not shown), suggesting that IgG1 production is less sensitive to AhR activation than IgM production and IFN-γ production was also unaffected in the Tg mice and B6D2F1 mice by TCDD exposure. IL-4 and IL-5 production was much higher in the B6D2F1 mice than in the C57BL/6-background Tg mice (Fig. 4D), suggesting

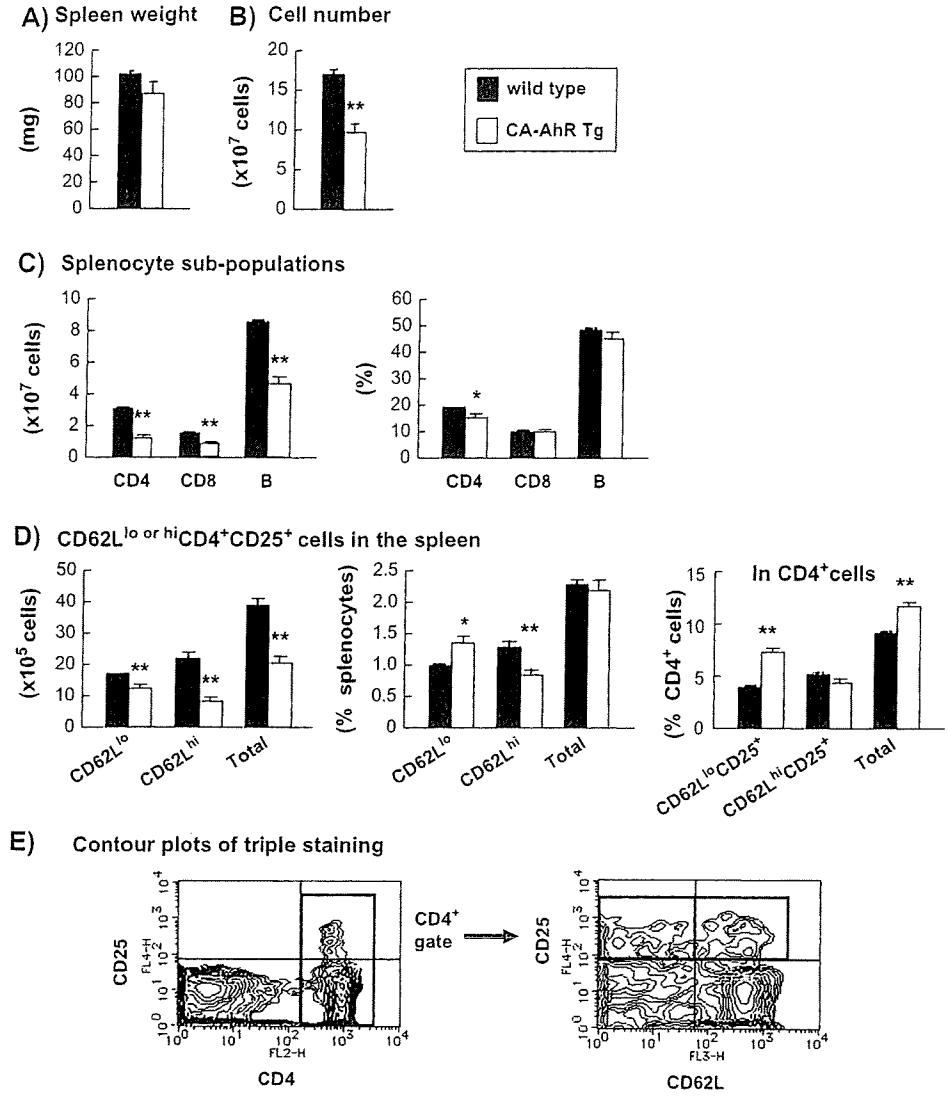

Fig. 2. The splenocytes of the CA-AhR Tg mice contained a higher percentage of $CD4^+CD25^+$ cells in the primary immune response to OVA. Spleen weight (A), splenocyte number (B), splenocyte subpopulation (C) and $CD62L^{lo\ or\ hi}$ $CD4^+CD25^+$ cells (D and E) were examined in the same mice as in Fig. 1. The differences between the Tg mice and wild-type mice were analyzed by Student's *t*-test. The data are expressed as means ± SDs. $^*P < 0.05$, $^{**}P < 0.01$.

that the DBA/2 background augments the production of these cytokines.

AhR-dependent gene expression in the Tg mice

The CA-AhR we used has been demonstrated to induce expression of the XRE-dependent AhR-target gene *CYP1A1* (24, 34). We further investigated the expression of AhR-dependent genes in the thymus of Tg mice. Analyses of gene expression with Affymetrix GeneChips showed that expression of XRE-dependent AhR-target genes *AhRR* (35) and *CYP1B1* (36, 37), in addition to *CYP1A1*, in the Tg mice was up-regulated 367-, 30- and 382-fold, respectively, compared with their expression in the wild-type littermates. The expression of another AhR-target gene *adseverin* was also up-regulated in the Tg mice 13-fold compared with the wild-type littermates, while XRE dependency of its induction is unknown (38). The expression changes of those genes were confirmed by RT-PCR (Fig. 5). These results support the notion that the CA-AhR functions in a manner similar to that of TCDD-activated wild-type AhR at least such as an XRE-dependent transcription factor.

Decreased AhR mRNA expression in cultured CD4 T cells has been reported (20, 21). A recent study showed that AhR expression was restricted to T_h17 cells among the several subsets derived from mouse CD4 T cells *in vitro* and that expression in other subsets, including T_h0, was negligible compared with expression in the liver (21). In the present study, we confirmed the expression of AhR mRNA and protein in CD4 T cells freshly isolated from the spleen of wild-type mice. The freshly prepared CD4 T cells contained a significant level of AhR mRNA, although the level was lower than in the liver and spleen (Fig. 6B). Furthermore, the level of AhR protein in the CD4 T cells was found to be similar to its level in the liver and spleen (Fig. 6C).

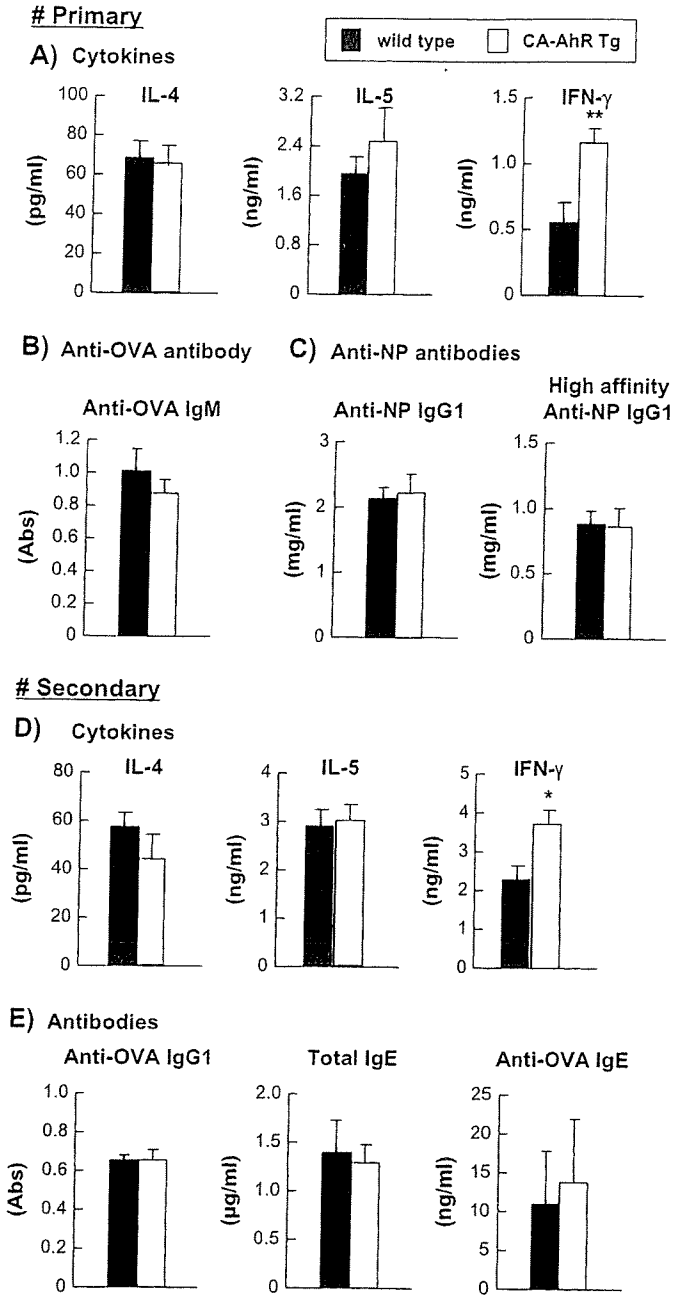

Fig. 3. CA-AhR expressed in the T cells increased IFN-γ production but did not suppress the production of T_h2-cytokines or antigen-specific antibodies. (A and B) Female Tg and wild-type mice (n = 7 per group) were immunized with OVA/alum. Seven days later, the mice were sacrificed and the cytokines (A) and anti-OVA antibody (B) were examined as described in the Methods section. (C) Female Tg and wild-type mice (n = 3 or 4) were immunized with NP-CG, and 10 days later total and high-affinity NP-specific antibodies were measured as described in the Methods section. (D and E) Female Tg and wild-type mice (n = 6 per group) were immunized with OVA/alum on Day 0, boosted on Day 21 and sacrificed for examination of the secondary immune reaction on Day 28. The differences between the Tg mice and wild-type mice were analyzed by Student's t-test. The data are expressed as means ± SDs. $^*P < 0.05$, $^{**}P < 0.01$

Discussion

The results of the present study have demonstrated that CA-AhR expression in T cells only augmented IFN-γ production. While IFN-γ is crucial for anti-viral and anti-tumor immunity, aberrant or excessive IFN-γ production is associated with autoimmune diseases and pathology, such as severe inflammation and tissue damage (39). IFN-γ is also involved in the suppression of T_h17 cell induction (40). Thus, AhR activation in the T cells might be involved in not only host defense but also in immune pathogenesis. The previous findings that TCDD exposure of OVA-immunized mice suppresses T_h2 cytokine production and augments IFN-γ production by splenocytes (22, 23) suggested that AhR activation in T cells causes skewing of the T_h1/T_h2 balance toward T_h1. However, the results of the present study have demonstrated that AhR activation in the T cells alone enhances IFN-γ production without suppressing T_h2 cytokine production in the spleen after OVA immunization. These results indicate that the T_h2 cytokines and IFN-γ were affected by AhR activation in an independent manner. The primary sources of IFN-γ in the adaptive immune response are T_h1 cells and CD8 effector cells (39). Precise studies on the time course of IFN-γ production by individual cell types will be required to clarify the molecular mechanism of the association between AhR and IFN-γ production in the course of immune reactions.

On the other hand, the CA-AhR only in the T cells did not suppress the production of IL-4 or IL-5 upon OVA immunization. The present study showed that the CA-AhR not only induced expression of *CYP1A1* but also of the other AhR-target genes, *AhRR* (35), *CYP1B1* (36, 37) and *adseverin* (38). The induction of *AhRR* (35) and *CYP1B1* (36, 37) has been shown to be dependent on AhR/ARNT/XRE, as has been widely known for CYP1A1 induction. These findings suggest that the CA-AhR replicates the function of ligand-bound activated AhR, at least as an XRE-dependent transcription factor. If the CA-AhR functions in exactly the same manner as the liganded wild-type AhR, the results of the present study indicate that the suppression of T_h2 cytokine production by TCDD is induced by the activation of AhR in the T cells as well as additional cell types, such as antigen-presenting cells, or indirectly induced by activation of cells other than T cells. The TCDD-induced suppression of antibody production might be a consequence of the lowered T_h2 cytokine level or due to direct effects of AhR activation in B cells as previously reported (13, 41).

Alternatively, another possible explanation for the unchanged level of T_h2 cytokines in the Tg mice is that CA-AhR does not fully replicate ligand-bound activated AhR. In addition to acting as a transcription factor, the AhR has been shown to be involved in non-genomic signaling pathways through interaction with a variety of proteins (20, 42–46). The AhR can modulate estrogen receptor (ER) function in a positive way as a coactivator-like factor (45) and in a negative way as a ligand-dependent E3 ubiquitin ligase (46). Assessment of the interaction between the AhR and the ER in the AhR ΔPAS(Δ117-424) mutant showed a greatly decreased interaction, and the AhR Δ327-516 mutant showed a slightly weaker interaction with the ERα-binding domain compared with full-length AhR (45). Since the CA-AhR we used has a minimal deletion Δ277-418 in the C-terminal

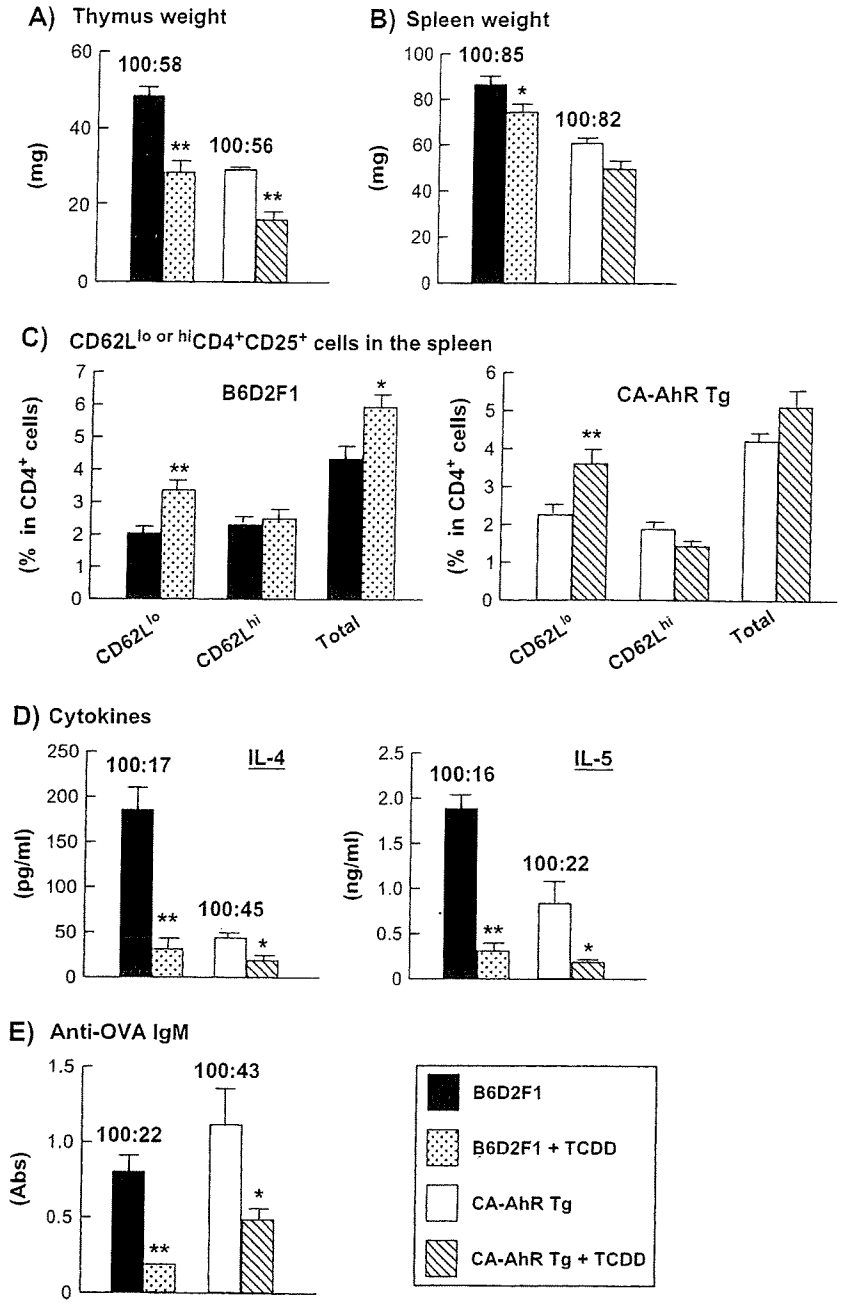

Fig. 4. TCDD exposure suppresses the immune response of CA-AhR Tg mice. Female B6D2F1 mice and CA-AhR Tg mice (n = 6/each group) were administered TCDD (40 μg kg^{-1}) or vehicle and subsequently immunized with OVA/alum. (A–D) Mice were sacrificed on Day 4 to measure thymus weight (A), spleen weight (B), $CD62L^{lo\ or\ hi}$ $CD4^+CD25^+$ cells (C) and IL-4 and IL-5 productions (D). (E) Antigen-specific antibody was measured on Day 10. The absorbance of plasma samples from non-immunized mice was 0.13–0.28. The differences between the Tg mice and wild-type mice were analyzed by Student's t-test. The data are expressed as means ± SDs. $^*P < 0.05$, $^{**}P < 0.01$

portion of the ligand/Hsp90-binding site in the PAS-B domain (24), it may interact more weakly with the ER. If the AhR acts through such an interaction, the CA-AhR may function differently from liganded wild-type AhR. A recent study reported that AhR activation by 6-formylindolo[3,2-b]carbazole (FICZ), another potent AhR ligand, inhibited Treg cell development by transforming growth factor (TGF)-β, while AhR activation by TCDD induced Treg cells even in the absence of TGF-β (19). These findings imply the existence of an as yet unknown mechanism of ligand-dependent AhR function. These results also suggest possible differences between functions of liganded AhR and CA-AhR. Kimura *et al.* (20), however, showed that AhR activation by both TCDD and FICZ enhanced Treg induction by TGF-β, and the reason for this discrepancy is not known.

Another different feature between CA-AhR in the Tg mice and wild-type AhR activated by TCDD exposure is the persistent activation of the CA-AhR. When CA-AhR expression was examined by flow cytometry analysis of co-injected GFP expression in the thymocytes, the transgene expression

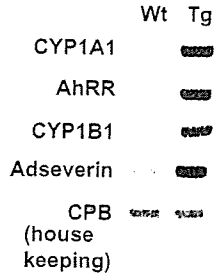

Fig. 5. Expression of AhR-target genes. Expression of AhR-target genes in the thymus of wild-type (Wt) and CA-AhR Tg (Tg) mice were measured by RT-PCR.

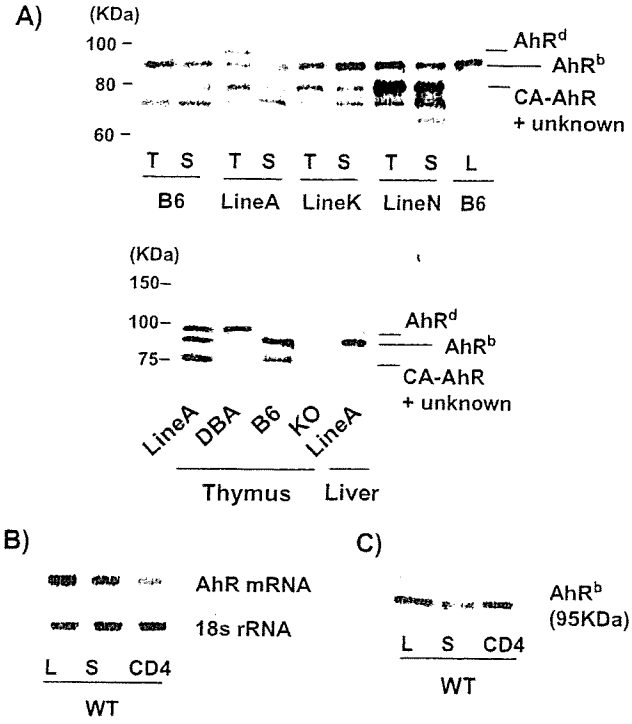

Fig. 6. Expression of AhR protein and mRNA. (A) AhR protein in the total lysate of thymus (T), spleen (S) and liver (L) was measured by western blotting. Line A, K and N heterozygous CA-AhR Tg mice were determined to have 2, 6–7 and 9–11 integrated CA-AhR genes, respectively (24). KO, AhR knockout mice. (B) Expression of AhR mRNA in the liver (L), spleen (S) and CD4 T cells of a wild-type mouse. (C) The amount of AhR protein in the liver, spleen and CD4 T cells was measured by western blotting.

was detected as early as on gestational day 16.5 (data not shown). The persistent expression of CA-AhR in the T cells has been shown not to dull the sensitivity of the immune system to TCDD exposure, as TCDD exposure of Tg mice suppressed T_h2 cytokines and antibodies in the present study. Furthermore, the results of the present study have shown that the persistent CA-AhR expression in the T cells induces IFN-γ augmentation in a similar manner to the effect of a single exposure to TCDD.

We have also shown that CA-AhR Tg mice have higher percentages of CD62L^{lo} and CD62L^{hi}CD25$^+$ cells among the thymus CD4$^+$ cells and higher percentage of CD62L^{lo}CD25$^+$ cells among the spleen CD4$^+$ cells compared with wild-type mice. The same characteristics were observed in both Tg mice and TCDD-exposed wild-type mice after OVA immunization. Since not only induction of CD25 but also down-regulation of CD62L accompanies T-cell activation (47), the apparent increase in the percentage of CD25$^+$ cells, particularly CD62L^{lo}CD25$^+$ cells, by AhR activation observed in the present study seems to imply that the AhR is involved in the signaling pathway downstream of the T-cell receptor. The mechanism by which AhR activation increases the percentage of this population in the CD4$^+$ cells and whether the increase relates to functional Treg generation have yet to be clarified.

In summary, we have demonstrated that expression of CA-AhR activation in T cells alone increases IFN-γ production by splenocytes upon OVA immunization, while it does not induce the suppression of T_h2 cytokine production or antibody production that were observed by TCDD-induced AhR activation in OVA-immunized mice. The studies using CA-AhR Tg mice give clues how to dissect the role of the AhR in different cell types and the way the AhR functions in immune reactions.

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Abbreviations

AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
CA-AhR	constitutively active mutant of aryl hydrocarbon receptor
ER	estrogen receptor
FICZ	6-formylindolo[3,2-b]carbazole
NP	(4-hydroxy-3-nitrophenyl)acetyl
OVA	ovalbumin
RT	reverse transcription
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Tg	transgenic
TGF	transforming growth factor
Treg	regulatory T
XRE	xenobiotic-responsive element.

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Early embryonic losses in mice induced by diethylstilbestrol.

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Abstract

Estrogens cause embryonic lethality and the disturbance of early placental development in mice. Diethylstilbestrol (DES) at 1, 10, or 100 microg/kg was orally administered to Institute of Cancer Research mice on gestational days (GD) 4 through 8, and the uterus and placenta were examined histopathologically on GD 9. Decidua of DES-treated mice showed insufficient development, and the uterine lumen at the implantation site did not effectively minimize. The trophoblast giant cell layer was not separated from the uterine lumen by the decidua capsularis, and hemorrhage from the denuded trophoblast giant cell layer into the uterine lumen was noted at the peripheral part of the decidua basalis. The results of the present study suggest that decidual hypoplasia and subsequent placental hemorrhage causes fetal death due to the administration of DES during the early stage of pregnancy.

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—Original—

Effects of Transmaternal Exposure to Genistein in Hatano High- and Low-Avoidance Rats

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Abstract: Hatano high- and low-avoidance (HAA and LAA) rats are separated by breeding from Sprague-Dawley rats by high versus low rates of avoidance responses in a shuttle-box task. In addition, compared to HAA rats, LAA rats show lower running-wheel activity, later sexual maturation, 5-day estrous cycling, lower sperm motility, more pronounced immunological reactions, and are generally less reactive to stress. The present study was designed to compare the effects of transmaternal exposure to genistein on these characteristics between HAA and LAA rats. To this aim, litters from both strains were fostered onto Sprague-Dawley rats receiving genistein by gavage with 5 mg/animal/day from day 17 of pregnancy through day 21 of lactation. Inhibited growth after weaning and reduced uterine weight at weaning were observed in the LAA offspring reared by genistein-treated dams. IgM antibody production in response to sheep red blood cells was significantly decreased in the HAA offspring reared by genistein-treated dams. During restraint stress, the plasma concentration of corticosterone was significantly lower in the LAA offspring reared by genistein-treated dams. Strain-related differences were detected in shuttle-box avoidance performance, running-wheel activity, estrous cycling, and sperm motility. The results demonstrate that transmaternal exposure to genistein potentially affects the immunological and stress responses as well as the post-weaning growth of the offspring. It suggests that a comparative study using Hatano rats would be useful for studying the influence of endocrine active chemicals on the whole body systems.

Key words: endocrine disruptor, genistein, inbred strain, offspring

Introduction

From the viewpoint of behavioral genetics, consideration of genetic control is necessary for animal studies of neurobehavioral teratology. Therefore, we have separated two inbred strains from Sprague-Dawley (SD) rats, Hatano high- and low-avoidance (HAA and LAA) rats, which show uniform behavior within the strain, but different baseline behaviors between strains in a shuttle-box avoidance task [27]. The selection criterion is based on the number of avoidance responses obtained during four daily sessions of 60 trials, with HAA rats being

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identified by a high rate of avoidance responses and LAA rats by a low rate. Using these two strains, we have developed new methods for the risk assessment of the toxicological effects of substances on the behavior of the next-generation [28, 30]. Although the Hatano rats are separated by their avoidance performance as mentioned above, characteristic differences between the strains are not only observed in their behaviors but also in their reproductive function [2, 35, 36, 38], immunological reactions [26], and stress responses [1, 3, 31]. For example, compared to HAA rats, LAA rats show lower running-wheel activity, later sexual maturation, 5-day estrous cycling, lower sperm motility, more pronounced immunological reactions, and are generally less reactive to stress. In addition, the data of these two strains falls within the normal range of variation for SD rats, from which the Hatano rats are derived.

In this study, we studied the effects of an endocrine active compound that has the potential to stimulate the normal endocrine system and focused on its effects on the nervous, endocrine, and immune systems. The results of animal studies on endocrine active compounds have often varied study by study, especially in their effects on the next generation. These variations in results may originate in genetic variations in the animals used [39], and we hoped to clarify the situation by using the HAA and LAA inbred strains, which show little inter-individual variation and whose nervous, endocrine, and immune interactions are well characterized.

Genistein (GN) is a typical phytoestrogen. Phytoestrogens are naturally occurring constituents of plants such as soy, and are known to exhibit estrogenic activity in rodent uterotrophic assays [13]. Soy-containing infant formulas and the breast milk of mothers that consume soy-based foods are rich in isoflavones [8, 37]. In animal studies, GN is reported to have had effects on behavior [7, 18], the reproductive system [4, 19, 23, 34], and the immune system [9, 15, 41].

In the present study, we used non-selected SD rats (background strain of Hatano rats) as the foster dams to rear pups of Hatano rats and administered the test compound, genistein, to the foster dams in order to avoid the influence of strain differences in maternal behavior [32].

Materials and Methods

Newborns of ten litters from HAA and LAA strains, maintained at the Hatano Research Institute were used for this experiment. In addition to the newborns, 20 pregnant SD rats purchased from Charles River Laboratories Japan, Inc. were prepared as foster dams for the newborns. The animals were kept in an animal room maintained under a 12-h light-dark cycle (lights on from 07:00 to 19:00), with a room temperature of 22 to 24°C and a relative humidity of 50 to 65%. GN (Purity: minimum 98%) was purchased from Sigma Chemical Co. (St. Louis, MO), and was suspended in corn oil (Nacalai Tesque, Co.) and mixed in a mortar to prepare the dosing sample (5 mg/ml). A stomach tube attached to a syringe was used to orally administer 5 mg/animal/day of GN to ten pregnant SD rats from day 17 of pregnancy through day 21 of lactation. Based on the average body weight of 0.3 kg for a foster dam rat, the dose of 5 mg/animal/day was estimated as being approximately equal to a dose of 16 mg/kg/day. This dose was within the range of human exposure levels. Another ten pregnant SD rats, used as a control group, were administered with 1 ml/animal/day of corn oil in the same manner. All of the pregnant females were housed individually with wood-chip bedding, and free access to food (CE-2, Clea Japan Inc.) and water. On the day after parturition, designated as postnatal day 1 (PD 1), eight newborns (4 males and 4 females where possible) from HAA and LAA dams were fostered onto SD dams receiving GN administration. The ages of the litters reared by the foster dams were within ± 24 h of their own litters. The HAA and LAA offspring were subjected to the tests noted below. The animal experiments in this study were conducted in accordance with the "Guidance for Animal Experiments in Hatano Research Institute, Food and Drug Safety Center".

Body weight and physical development

All offspring were weighed on PDs 1, 4, 7, 14, and 21, and weanlings were further weighed at 4, 5, 6, 7, 8, 9, and 10 weeks of age. Test offspring were examined daily for the following developmental landmarks as previously described: eyelid opening [29] from PD 12, vaginal opening [38] from PD 28, and preputial separa-

tion [36] from PD 35.

Behavioral tests and estrous cycle

Litters weaned at PD 21, were caged in pairs of the same sex and kept in the same animal room as before. Four offspring (2 males and 2 females where possible) from each litter were examined using the following tests as previously described [30]: shuttle-box avoidance and running wheel activity tests at 7 and 8 weeks of age, respectively. From 9 weeks of age, vaginal smears were taken daily from three females of each litter for 3 weeks to determine the stage of the estrous cycle.

Organ weight, sperm motility, and hormone levels

At PD 21, two offspring (1 male and 1 female where possible) from each litter were sacrificed by exsanguination under sodium pentobarbital anesthesia. The adrenal glands, thymus, testes, epididymides, ovaries, and uterus were weighed.

At 12 weeks of age, four offspring (1 male and 3 female where possible) from each litter were anesthetized with sodium pentobarbital, and blood was collected in heparinized tubes from the posterior vena cava. Females were sacrificed at various times during the estrous cycle (12 and 18 h of pro-estrus, 12 h of estrus). The adrenal glands, spleen, thymus, testes, epididymides, seminal vesicles, ventral prostate, ovaries, and uterus were weighed. The characteristics of motile sperm in the caudal epididymis were determined using a Hamilton-Thorne IVOS analyzer as previously described [35]. Blood collected at necropsy was centrifuged and the plasma was separated and stored at –20°C until determination of progesterone, LH, and FSH [2].

Immune response to sheep red blood cells (SRBC)

At 19 weeks of age, one male from each litter was given a single intravenous injection of 0.7 ml of 1% SRBC four days prior to necropsy. The animals were anesthetized with sodium pentobarbital, and blood was collected from the posterior vena cava, prior to sacrifice. The spleen was weighed, and the spleen cells were subjected to a plaque-formation cell (PFC) assay as previously described [26]. The blood's lymphocyte count was analyzed by an automated hematology analyzer (Cell-Dyn3500, Abbott Laboratories), and then the serum was separated and stored at –80°C until determination of anti-SRBC-IgM [26].

Hormonal response to stress

At 6 months of age, one male from each litter was sacrificed by decapitation following 30 min of immobilization in a plastic bag as previously described [3]. On the day before immobilization, a blood sample was collected from the tail vein as previously described [31] to assess the basal level of hormones. The blood sample was collected in heparinized tubes containing aprotinin and centrifuged. The plasma was separated and stored at –20°C until it was assayed for ACTH, corticosterone and prolactin [3]. The testes, epididymides, and ventral prostate were weighed at necropsy.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) with transmaternal exposure (GN, oil) and strain (HAA, LAA) as between-subject factors, and day as a within-subject (repeated measure) factor. Data were analyzed separately for males and females. Significant interactions were further analyzed using simple-effect ANOVA at each level of interaction to localize the major effects. The offspring data used the litter average as the unit of statistical analysis. Statistical significance was assumed at P values of 0.05 or less.

Results

No effects of GN exposure on the body weight of the offspring during the pre-weaning period were observed in either the HAA or LAA offspring. The body weights after weaning are shown in Figs. 1A to 1D. In the LAA offspring, the post-weaning weights of the GN group were significantly lower than those of the control group in both males [$F(1,48)=6.56$, $P<0.05$] and females [$F(1,54)=5.53$, $P<0.05$]. In the HAA offspring, no significant effects of GN exposure were observed in the post-weaning weights of either sex.

The mean ages of eyelid opening, vaginal opening and preputial separation are shown in Table 1. No influence of GN exposure on eyelid opening was observed in either the HAA or LAA offspring. In the LAA offspring, in which sexual maturation is observed later than HAA

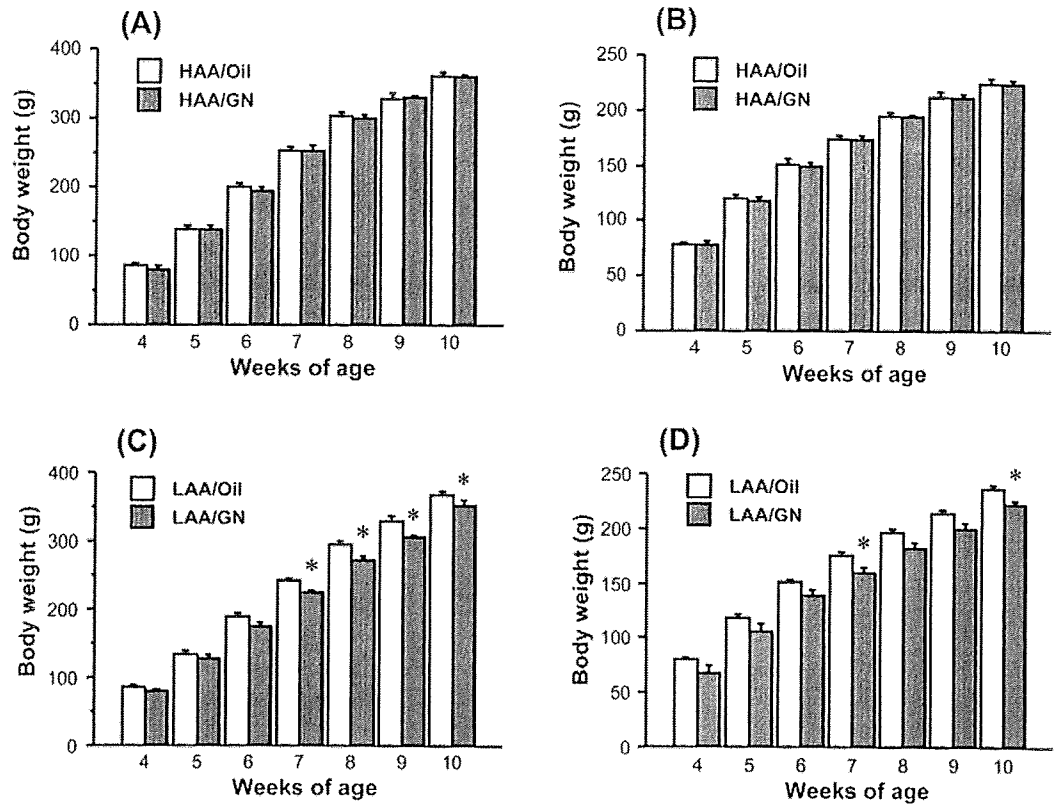

Fig. 1. Body weights of male HAA (A), female HAA (B), male LAA (C), and female LAA (D) offspring from 4 to 10 weeks of age following transmaternal genistein exposure. Data are expressed as the litter mean ± SE. *$P<0.05$ compared to the respective controls.

Table 1. Physical development of HAA and LAA offspring following lactational genistein exposure

Strain	HAA		LAA	
Treatment	Control	Genistein	Control	Genistein
Eyelid opening	14.2 ± 0.2 (32)	14.0 ± 0.1 (39)	14.7 ± 0.2 (48)	14.6 ± 0.2 (39)
Vaginal opening	32.5 ± 0.6 (11)	32.2 ± 0.3 (16)	34.7 ± 0.4 (17)	36.5 ± 0.6* (15)
Preputial separation	40.4 ± 0.9 (13)	41.2 ± 0.5 (12)	46.9 ± 0.4 (19)	48.3 ± 0.7* (13)

Data are expressed as the mean ± SE in postnatal days until the criterion was met. Parentheses show the number of animals examined. *$P<0.05$ compared to the respective controls.

offspring, both vaginal opening [$F(1,30)=6.40$, $P<0.05$] and preputial separation [$F(1,30)=5.93$, $P<0.05$] were further delayed in the GN exposure groups. The differences were marginally significant when individual data were used for analysis, but did not reach significant levels when litter means were used. In the HAA offspring, no influence of GN exposure was observed in either sex.

No significant differences between the control and GN groups were observed in the rate of avoidance responses during 2 days (60 trials per day) of shuttle-box avoidance tests, in spite of clear strain differences between HAA and LAA rats.

The number of revolutions in the running wheel activity test was significantly higher in HAA than LAA offspring over 3 consecutive days. There were, however, no significant effects of GN exposure on the number of revolutions in either HAA or LAA offspring.

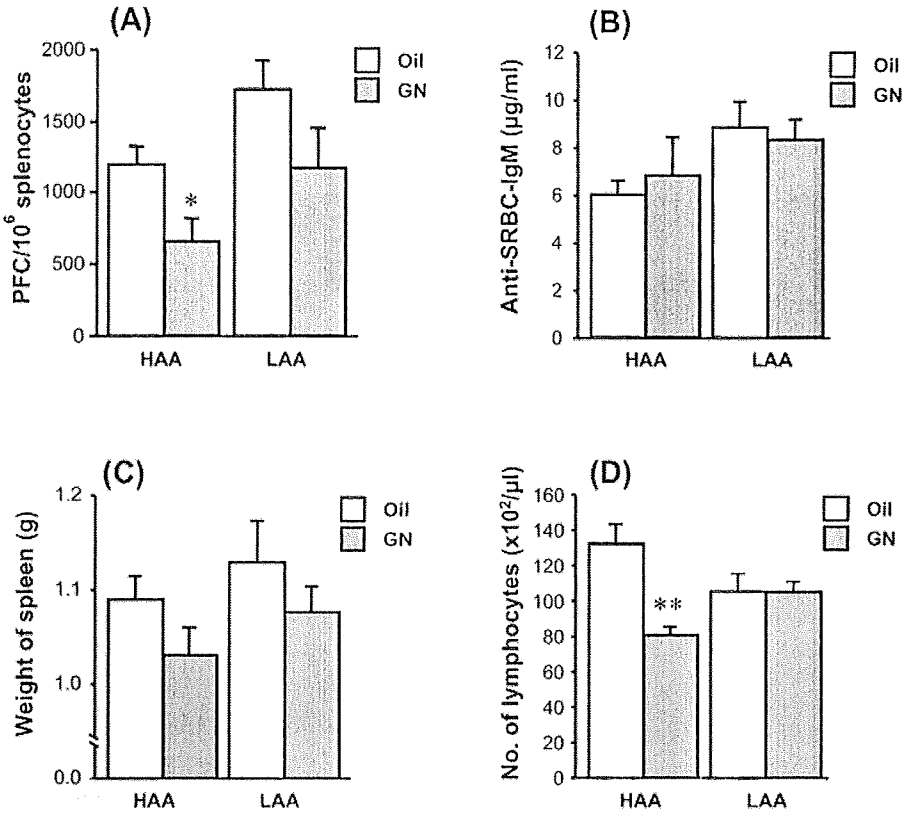

Fig. 2. PFC assay (A), anti-SRBC-IgM (B), spleen weight (C), and the number of lymphocytes (D) in HAA and LAA offspring at 19 weeks of age following transmaternal genistein exposure. Data are expressed as the mean ± SE. $*P<0.05$, $**P<0.01$ compared to the respective controls.

In the monitoring of estrous cycle from 9 to 12 weeks of age, all of the HAA offspring showed a regular 4-day estrous cycle in both the control and GN groups. In the LAA offspring, 24 and 41% of the control rats exhibited regular 4- and 5-day cycles, respectively, as did 7 and 46% of the GN group. The rest of the control (35%) and GN (47%) groups showed repeated 4- and 5-day cycles, and the mean cyclic lengths of the control (4.6 days) and GN (4.7 days) groups were not significantly different.

At weaning of LAA offspring, the mean uterus weight of the GN group (27.0 mg) was significantly lighter [$F(1,10)=6.53$, $P<0.05$] than that of the control group (30.3 mg). There were no significant differences between the control and GN groups in the weights of the adrenal glands, thymus, testes, epididymides, or ovaries in either strain.

In male offspring at 12 weeks of age, the weights of the seminal vehicles, adrenal glands, and thymus were significantly lighter, and the weight of the testes was significantly heavier in LAA than in HAA rats. There were no significant effects of GN exposure on any organ of either strain. The percent of motile sperm was significantly higher in HAA than in LAA rats. The values of VAP, VSL, and VCL, which represent the swimming speed, and ALH, which reflects the oscillation width of a sperm head, were significantly higher in HAA than in LAA rats. There were, however, no significant effects of GN exposure on any of the parameters of sperm motion. A strain difference between HAA and LAA rats was observed in the plasma levels of FSH, but no significant effects of GN on progesterone, LH, or FSH of male offspring were noted.

In female offspring at 12 weeks of age, the weight of the thymus was significantly lighter, and that of the ovaries was significantly heavier in LAA than in HAA rats. No influence of GN exposure was observed in any organ

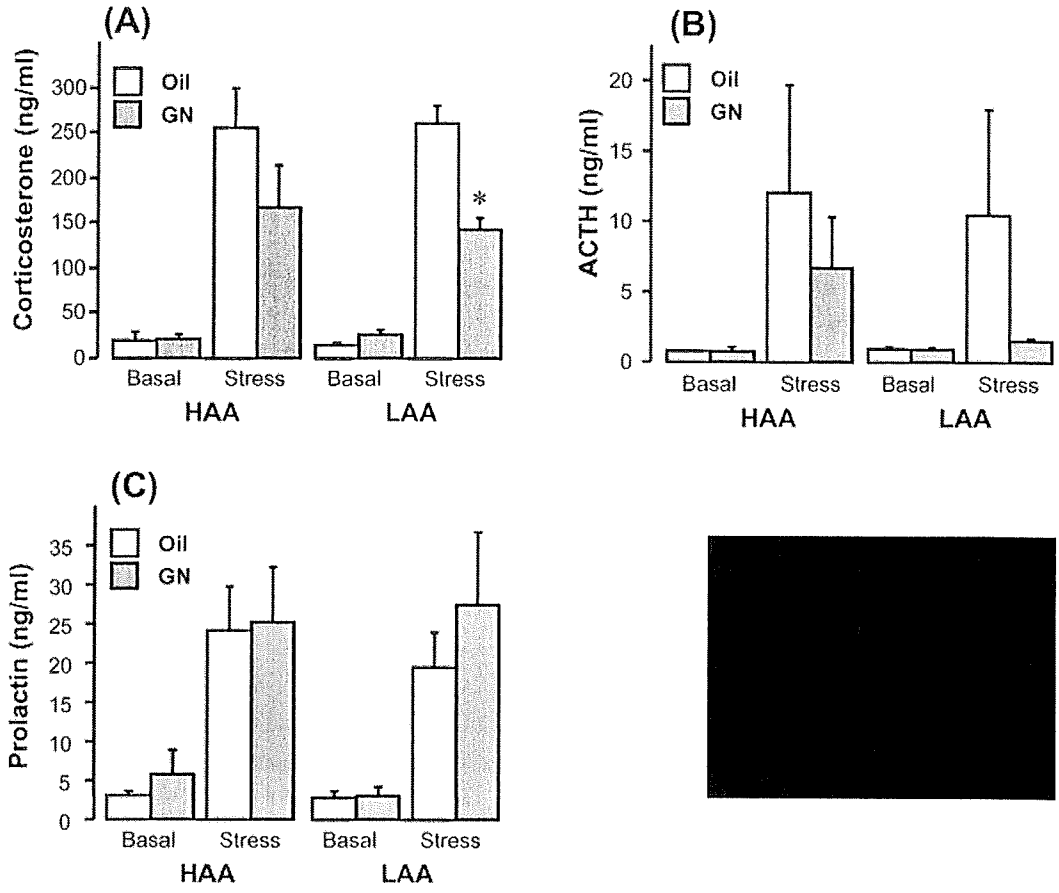

Fig. 3. Plasma levels of corticosterone (A), ACTH (B), and prolactin (C) in HAA and LAA offspring during restraint stress at 6 months of age following transmaternal genistein exposure. Data are expressed as the mean ± SE. *$P<0.05$ compared to the respective controls.

of either strain. Strain differences were observed in the plasma levels of progesterone, LH, and FSH, but no significant effects of GN on these hormones were noted.

The results of the PFC assay, the anti-SRBC IgM, spleen weights, and lymphocyte counts are shown in Figs. 2A to 2D. The value of the PFC assay in the HAA offspring was significantly decreased by GN exposure [$F(1,6)=6.42, P<0.05$]. The same tendency was observed in the LAA offspring, but the difference was not significant. The anti-SRBC IgM level was higher in LAA than in HAA offspring. There was, however, no significant effect of GN exposure on anti-SRBC IgM levels. No effect of GN exposure was observed on the spleen weights of either strain. The number of lymphocytes was significantly decreased in HAA offspring reared by GN-treated dams [$F(1,6)=18.92, P<0.01$]. No significant effect of GN exposure was observed in the LAA offspring.

The results of the restraint-stress challenge test are shown in Figs. 3A, 3B, and 3C. During restraint stress, the plasma concentrations of corticosterone, ACTH, and prolactin increased in both strains. A significant effect of GN was found on the corticosterone concentrations of LAA offspring [$F(1,9)=16.53, P<0.01$], which were significantly lower in the GN group than in the control group. Similarly, the corticosterone concentrations of the HAA offspring and the ACTH concentrations of both strains tended to decrease in the GN group during stress, but these changes were not significant. There was no significant effect of GN on plasma prolactin concentrations. The weights of the testes and the ventral prostate at 6 months of age were significantly heavier in LAA

than in HAA offspring. However, there were no significant effects of GN on the weights of the testes, epididymides, or ventral prostate at 6 months of age.

Discussion

The influence of transmaternal GN exposure on body weight was observed in both sexes of LAA offspring after weaning. The newborns of SD rats directly given 12.5 mg/kg/day or more of GN by gavage from PD 1 to PD 5 exhibited a decrease in the body weight of both sexes after weaning [23]. It has been reported that in juvenile female mice given GN (20 and 80 mg/kg/day s.c.), fat pad weight decreases dose dependently and that lipoprotein lipase mRNA also decreases [22]. Oral GN treatment (150 and 1,500 mg/kg diet) in ovariectomized mice is also reported to result in reduced body weight and apotosis of adipose tissue [14]. In addition, GN (5 mg/kg) caused a slight increase in blood glucose concentration with a concomitant drop in insulin level in male rats [40]. Although the mechanisms of the body weight reduction induced by transmaternal GN exposure in the present study are not clear, the strain difference in the effect of GN on body weight is interesting, and this difference is probably dependent on the genetic backgrounds of the animals.

Sexual maturation was delayed in both sexes of LAA offspring. This was possibly caused by the body weight effect, because a high dose of GN as well as other estrogenic compounds is expected to accelerate vaginal opening.

No obvious effects of GN were observed on any parameters of shuttle-box avoidance or running-wheel activity for either the HAA or LAA strain. Therefore, transmaternal exposure to GN does not affect avoidance learning or locomotion activity independently of baseline behavior.

When the organ weights were measured at weaning of the LAA offspring, the mean uterine weight in the GN group was significantly lighter than that in the control group. Although postnatal exposure to a high dose of GN is expected to increase uterine weight, decreased uterine weight following lactational exposure was reported at low doses of GN [4, 6]. Low doses of diethylstilbestrol or ethynylestradiol also induced uterine weight reduction by neonatal treatment [5]. Uterine weight reduction may be caused by down regulation of uterine estrogen receptor during neonatal treatment with estrogens.

There were no effects of GN exposure on the estrous cycles of the HAA and LAA offspring. It was shown that irregular estrous cycles occur following neonatal exposure of rats to GN for prolonged periods during estrus [24]. Jefferson *et al.* [12] reported alterations in the estrous cycle of CD-1 mice following neonatal exposure to GN at doses of 0.5 to 50 mg/kg and these were exacerbated more at 6 months than at 2 months of age. The dose of GN used in the present study probably did not affect the estrous cycle of offspring, because no significant effects of GN were observed on reproductive organ weight or hormone levels in adulthood. However, estrous cycle observation in the present study was made only at a young age. Further study at an older age is needed to evaluate the estrous cycle of offspring.

Although no effect of GN exposure was observed on anti-SRBC IgM levels, the value of the PFC assay was decreased by GN exposure in the HAA offspring. Furthermore, the number of lymphocytes was decreased in the HAA offspring reared by GN-treated dams. These results suggest that GN induces immunosuppression *in vivo*. GN at a dose of 80 mg/kg/day produced impairments in humoral immunity reducing keyhole limpet haemocyanin-specific antibody titers in mice [41]. In ovalbumin-immunized mice, GN at a dose of 20 mg/kg/day suppressed ovalbumin-specific IgG levels [16]. However, an increased splenic T-cell number was observed in SD rats exposed to GN during gestation and lactation [10]. Sakai and Kogiso [34] suggest that the effect of GN on immunity is immune cell-dependent.

Long-Evans rats that were given a high phytoestrogen diet showed decreased anxiety, as expressed in elevated plus maze results [18]. During restraint stress in the present study, the plasma concentrations of corticosterone and ACTH were lower in the GN group than in the control group in both strains. These results seem to agree with the decreased anxiety reported for GN offspring as described above. The opposite result was reported in hooded Lister rats that were fed 150 μg of GN plus daidzein for 14 days [11]. Furthermore, male Long-Evans rats on a lifelong high phytoestrogen diet (600 μg/g of

diet) showed higher plasma ACTH but similar corticosterone levels after stress [17]. In addition, serum corticosterone levels tended to decrease in male Wistar rats that were administered subcutaneously with GN (40 mg/kg/day) for 3 weeks after weaning [25]. These reports suggest that GN alters the negative feedback of stress hormones and/or steriodogenesis in the adrenal gland of rats. However, the perinatal effect of GN on the hypothalamic-pituitary-adrenal (HPA) axis is not clear. Further study is needed to evaluate the effects of GN on the relationship between anxiety stress and the HPA axis.

The dosage of 5 mg/animal/day (approximately 16 mg/kg/day) of GN, that was used in the present study, was chosen to be comparable to the normal range of human exposure levels during lactation [8, 37]. Lewis *et al.* [20] reported that SD rats administered with GN in a single oral dose of 16 mg/kg during lactation had a milk GN level of 0.17 μg/ml, while the plasma level of dams was 1.8 μg/ml. Thus, the amount of GN expressed into milk is low. However, some alterations were detected in offspring reared by GN-treated dams in the present study suggesting that this dose of GN caused some effects in the next generation.

This comparative study using HAA and LAA rats, which have different characteristics between their strains and uniform characteristics within strains, may provide useful information on individual differences in sensitivity to compounds with estrogen activity such as GN. Transmaternal exposure to GN inhibited growth and reduced uterine weight in the LAA offspring. Antibody production was inhibited in the HAA offspring and a reduced stress response was observed in the LAA offspring. These results suggest that the HAA and LAA strains are useful animal models for studying the influence of endocrine active chemicals found in the environment and for estimating their influences on the whole body systems.

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RESEARCH ARTICLE

Expression of constitutively-active aryl hydrocarbon receptor in T-cells enhances the down-regulation of CD62L, but does not alter expression of CD25 or suppress the allogeneic CTL response

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Abstract

Activation of aryl hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in T-cells is required for TCDD-induced suppression of the allogeneic CTL response and for induction of $CD25^{hi}CD62L^{low}$ adaptive regulatory T-cells. Here, the ability of a constitutively-active AhR (CA-AhR) expressed in T-cells alone to replicate the effects of TCDD was examined. The response of CA-AhR-expressing B6 donor T-cells in B6xD2F1 mice was compared to the response of wild-type B6 donor T-cells in B6xD2F1 mice given a single dose of TCDD. Expression of CA-AhR in donor T-cells enhanced the down-regulation of CD62L on Day 2 after injection, similar to a single oral dose of TCDD, but did not induce up-regulation of CD25 on Day 2 or affect CTL activity on Day 10. This suggests that activation of AhR in T-cells alone may not be sufficient to alter T-cell responses in this acute graft-versus-host (GvH) model. Since host APC are responsible for activating the donor T-cells, we examined the influence of the F1 host's AhR on donor T-cell responses by creating an $AhR^{-/-}$ B6xD2F1 host that had a greatly diminished AhR response to TCDD compared to wild-type F1 mice. As in $AhR^{+/+}$ B6xD2F1 mice, the CTL response in $AhR^{-/-}$ B6xD2F1 mice was completely suppressed by TCDD. This suggests that either CA-AhR dose not fully replicate the function of TCDD-activated AhR in suppression of the CTL response, or that minimal activation of AhR in host cells is required to combine with activation of AhR in T-cells to elicit the immunosuppressive effects of TCDD.

Keywords: *AhR; TCDD; regulatory T-cells; CTL response; transgenic mouse*

Introduction

The AhR is an intracellular receptor and transcription factor well known for mediating the immunosuppressive effects of a large group of environmental contaminants, including certain polycyclic aromatic hydrocarbons and polychlorinated dibenzo-furans and -dioxins (Kerkvliet, 2002, 2003). Human exposure to dioxins and related chemicals occurs primarily through the consumption of food, particularly meat and dairy products, because these chemicals are highly fat-soluble and bioaccumulate (Liem et al., 2000; Schecter et al., 2001; Startin and Rose, 2003). In addition to these man-made ligands, many natural chemicals found in plants as well as products of normal cellular metabolism have been shown to bind and activate AhR (Denison and Nagy, 2003; Jeuken et al., 2003; Nguyen and Bradfield, 2008). Ligand binding leads to nuclear translocation of the AhR and dimerization with its transcriptional partner, ARNT, which in turn binds to the consensus DNA sequence, termed the dioxin response element (DRE). DRE has been found in the promoters of many genes, including one of the most sensitive gene targets, *CYP1A1* (Schmidt and Bradfield, 1996; Mimura and Fujii-Kuriyama, 2003).

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