Of many chemicals that bind to AhR, TCDD is a well-known ligand having high affinity and eliciting potent suppressive effect on both humoral and cell-mediated immune responses (Holsapple et al., 1991; Nohara et al., 2002; Kerkvliet, 2003; Inouye et al., 2003; Lawrence and Kerkvliet, 2007). Recent studies have shown that AhR activation by TCDD enhances expansion of $CD4^+CD25^+$ cells, particularly $CD62L^{low}CD4^+CD25^+$ cells, and $CD4^+Foxp3^+$ regulatory T-(Treg) cells (Funatake et al., 2005; Quintana et al., 2008). AhR activation also participates in Th17 differentiation (Quintana et al., 2008; Kimura et al., 2008; Veldhoen et al., 2008). Thus, Ahr is involved in a variety of immune responses and suppressions, while the mechanism is not fully understood.

In attempts to clarify the target cells affected by AhR activation, previous studies have shown that T-cells are direct AhR-dependent targets of TCDD-mediated immune suppression, and both $CD4^+$ and $CD8^+$ T-cells must express AhR in order for full suppression of the CTL response to alloantigen (Kerkvliet et al., 2002). Subsequent studies showed that activation of AhR in the donor T-cells was also required for TCDD-mediated induction of a subpopulation of T-cells that are $CD25^{hi}CD62L^{low}$ and exhibit suppressive activity *in vitro*, for both $CD4^+$ (Funatake et al., 2005) and $CD8^+$ T-cells (Funatake et al., 2008). However, many other cells in the immune system express AhR and are also known to be directly targeted by TCDD, including dendritic cells and B-cells (Sulentic et al., 1998; Vorderstrasse and Kerkvliet, 2001; Boverhof et al., 2004; Ruby et al., 2005). A recent study has shown that activation of AhR within hematopoietic cells, but not within $CD8^+$ T-cells, is necessary for suppression of the CTL response to influenza, suggesting that in the influenza model, the immunosuppressive effects of TCDD are mediated by the activation of AhR in immune cells other than the responding $CD8^+$ T-cells (Lawrence et al., 2006). Thus, although studies have shown that activation of AhR within the donor T-cells is required for suppression of the CTL response to alloantigen, it is not known if activation of AhR in T-cells alone is sufficient to mediate this suppression.

In order to address the exact role of the AhR in T-cells, we previously-produced transgenic (Tg) mice expressing a constitutively active AhR (CA-AhR) under the control of the T-cell lineage-specific CD2 promoter (Nohara et al., 2005). These mice express the transgene in their thymocytes, CD4 T-cells, and CD8 T-cells, and they express CYP1A1 mRNA in the thymus and spleen, showing that the CA-AhR is transcriptionally active in T-cells. These mice also display thymic atrophy (Nohara et al., 2005), which is one of the hallmarks of exposure to TCDD that occurs in an AhR-dependent manner (Laiosa et al., 2003). The immune response of these mice against Ovalbumin (OVA)-alum only partially mimicked the immune response of wild-type mice exposed to TCDD (Nohara et al., 2005, 2009). For example, splenocytes from CA-AhR Tg mice produced levels of interleukin (IL)-4 and IL-5 and antibody titers upon OVA immunization similar to those of wild-type mice (Nohara et al., 2009).

On the other hand, interferon (IFN)-γ production by the splenocytes upon OVA immunization was augmented in the Tg mice similar to that seen in wild-type mice exposed to TCDD (Nohara et al., 2009). In the present study, we used the transgenic CA-AhR mice to ask whether activation of AhR in T-cells alone was sufficient to induce the $CD25^+CD62L^{low}$ adaptive Treg-like phenotype and mediate suppression of the CTL response to alloantigen. To do so, we compared the response of CA-AhR-expressing B6 donor T-cells in B6xD2F1 mice and the response of wild-type B6 donor T-cells in B6xD2F1 mice given a single dose of TCDD in the acute graft-versus-host (GVH) model.

Materials and methods

Mice and treatment with TCDD

The generation and genotyping of the transgenic C57Bl/6-CA-AhR (CA-AhR) mice has been described (Nohara et al., 2005). The heterozygous Tg mice showed CYP1A1 mRNA expression in the thymus at the level corresponding approximately to the level induced by a single dose of 20 μg TCDD/kg (Nohara et al., 2005). The transgenic CA-AhR mice and wild-type (B6-WT) littermates used in the present studies were backcrossed to the B6 background over 10 generations. For some GVH experiments, transgenic CA-AhR mice ($CA\text{-}AhR^{+/-}$) were crossed with B6.PL-$Thy1^a$/CyJ (Thy1.1) mice, purchased from The Jackson Laboratory (Bar Harbor, ME), to generate $Thy1.1^+$ donor T-cells. $Thy1.1^+$ $CA\text{-}AhR^{-/-}$ littermates were used as B6-WT controls. B6D2F1/Crlj mice were purchased from Charles River Laboratories (Yokohama, Japan).

B6D2F1/J mice were generated by breeding C57Bl/6J female mice with DBA/2J male mice, originally purchased from CLEA Japan (Tokyo). In one study, B6 mice lacking AhR (AhrΔ2; Schmidt et al., 1996) were crossed with DBA/2 mice, (both purchased from The Jackson Laboratory). All mice were used between 2 and 5 months of age. Except where noted, B6D2F1 mice were dosed orally with 20 μg TCDD/kg body weight 1 day before the injection of donor T-cells, as previously described (Kerkvliet et al., 2003). TCDD was purchased from Cambridge Isotope Laboratories (Andover, MA) and prepared as a 4% nonane/corn oil solution. A similarly prepared solution of 4% nonane in corn oil was used as a vehicle-control. All mice were handled in a humane manner in accordance with the National Institute for Environmental Studies (NIES, Japan) guidelines for animal experiments.

Preparation of donor T-cells for the GVH model

T-Cells were purified from spleen cells of B6-WT or transgenic CA-AhR mice using the BD IMag™ and a mouse T-lymphocyte enrichment set (BD Biosciences, San Jose, CA), following the manufacturer's instructions. The percentages of $CD4^+$ and $CD8^+$ cells in the T-cell fraction were checked using flow cytometry (see below for details) and viability was assessed by trypan blue exclusion. T-Cells (i.e., $CD4^+$ and $CD8^+$ cells) were 80-93% pure and viabilities were > 90%.

The concentration of purified T-cells was adjusted so that each F1 mouse received 1.5×10^7 T-cells intravenously in 250 μl of 1X Hank's Balanced Salt Solution (HBSS). B6-WT mice were used as syngeneic recipients. In some experiments the purified donor T-cells were labeled with 0.5 μM Carboxyfluorescein Succinimydl Ester (CFSE) (Invitrogen, Carlsbad, CA) before injection into F1 or syngeneic hosts as described previously (Funatake et al., 2004).

Flow cytometry

Spleen cells were resuspended in staining buffer containing 0.1% sodium azide and 1% fetal bovine serum in phosphate-buffered saline. The cells were incubated with 200 μg/ml rat IgG (Jackson Immunoresearch, West Grove, PA) to block F_c receptors (F_cR), followed by incubation with fluorochrome- or biotin-conjugated monoclonal antibodies. Antibodies for Thy1.1, CD4, CD62L, CD19, and CD25 were purchased from eBiosciences (San Diego, CA). All other antibodies were purchased from BD Biosciences. After incubation with monoclonal antibodies, the cells were washed and incubated with streptavidin conjugated to allophycocyanin, PerCP (BD Biosciences), or PE-Cy5 (eBiosciences). After incubating the cells with streptavidin, the cells were washed and resuspended in staining buffer, and data were collected on 7000-10,000 events of interest using a BD FACSCalibur (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences).

Cytotoxicity assay

With one noted exception that used the standard ^{51}Cr-release assay, spleen cells were assayed for CTL activity using the CytoTox 96® non-radioactive cytotoxicity assay kit from Promega (Madison, WI), according to the manufacturer's instructions. Briefly, P815 cells recovered from the ascites of DBA/2 mice were added to a 96-well round bottom plate at 1×10^4/well. Spleen cells from F1 mice that had been injected with B6 donor T-cells 10 days earlier were then added to the wells in duplicate serial dilutions from 100:1 to 12.5:1. Wells containing only spleen cells were included as background controls, in addition to wells containing only P815 cells with or without lysis buffer used for maximum and spontaneous release controls, respectively. The plates were incubated for 4hr at 37°C and 5% CO_2, after which the CTL activity was measured and the percent cytotoxicity was calculated according to the manufacturer's instructions.

7-Ethoxyresorufin-O-deethylase (EROD) activity assay

Livers were homogenized and debris was removed by centrifugation at 10,000x*g* for 20min at 4°C. The microsomal fraction was isolated from the supernatants by additional centrifugation at 100,000x*g* for 90min at 4°C. The EROD activity of each microsomal fraction was determined using 2 μg of protein for the WT-F1 samples and 25 μg for the KO-F1 ($AhR^{-/-}$ B6 mice bred with DBA/2 mice to create F1 hosts that would respond poorly to AhR activation) samples. Microsomal protein was aliquoted into microtiter wells containing 200 μM 7-ethoxy-resorufin in 0.1M Tris-HCl, (pH 7.8.) NADPH was added to each well and the fluorescence was measured on a SpectraMAX Gemini plate reader using 535 nm excitation and 585 nm emission filters. Samples were assayed in triplicate and the amount of resorufin produced was calculated using a resorufin standard curve.

Statistics

All experiments were independently conducted twice with n = 3-5 mice per group per experiment. Where appropriate, data from replicate studies were combined, and all data are presented as the mean ± SEM. The differences between means were analyzed by Student's *t*-test, with $p < 0.05$ considered statistically significant.

Results

CA-AhR does not affect cell division or the number of donor T-cells in the spleen on Day 2

Previous studies have shown that exposure of F1 mice to TCDD has no adverse effect on the proliferation or number of donor T-cells in the spleen on Day 2 of the GVH response (Funatake et al., 2005, 2008). To determine if expression of CA-AhR in donor T-cells affected cell division or cell number in the spleen, we compared donor T-cells from transgenic CA-AhR mice to donor T-cells from B6-WT mice two days after injection into vehicle- or TCDD-treated F1 hosts. Donor T-cells from CA-AhR mice or wild-type littermates were labeled with CFSE before injection into F1 hosts for evaluation of cell division. As with TCDD, CA-AhR in the donor T-cells had no effect on the total number of spleen cells (Figure 1A) or on the number of donor $CD4^+$ T-cells in the spleen on Day 2 (Figure 1B). In addition, neither exposure to TCDD nor CA-AhR had any effect on the cell division of donor $CD4^+$ T-cells (Figure 1D). Exposure to TCDD led to a small but significant increase in the number of donor $CD8^+$ T-cells (Figure 1C), and there was a corresponding increase in the percentage of donor $CD8^+$ T-cells that had undergone one or more cell divisions (Figure 1E, middle panel). CA-AhR in the donor T-cells had no effect on the number of donor $CD8^+$ T-cells in the spleen (Figure 1C) or on the percentage of donor $CD8^+$ cells that were dividing (Figure 1E). As expected, in syngeneic B6 recipients, there was no difference in the total number of spleen cells or the number of donor $CD4^+$ or $CD8^+$ T-cells in the spleen two days after injection of donor T-cells from either B6-WT or CA-AhR mice (Figure 1).

CA-AhR enhances the down-regulation of CD62L but does not affect the expression of CD25

Next, we compared the effect of the CA-AhR to the effect of TCDD on the expression of CD25 and CD62L on the donor $CD4^+$ and $CD8^+$ T-cells. For donor $CD4^+$ T-cells, exposure to TCDD caused a large increase in the percentage of cells expressing CD25, whereas CA-AhR had a much more modest effect (Figure 2A and 3A). In contrast, both activation of AhR by TCDD and CA-AhR caused a substantial increase in the percentage of donor $CD4^+$ T-cells that had down-regulated CD62L (Figure 2C and 3A). For donor $CD8^+$ T-cells,

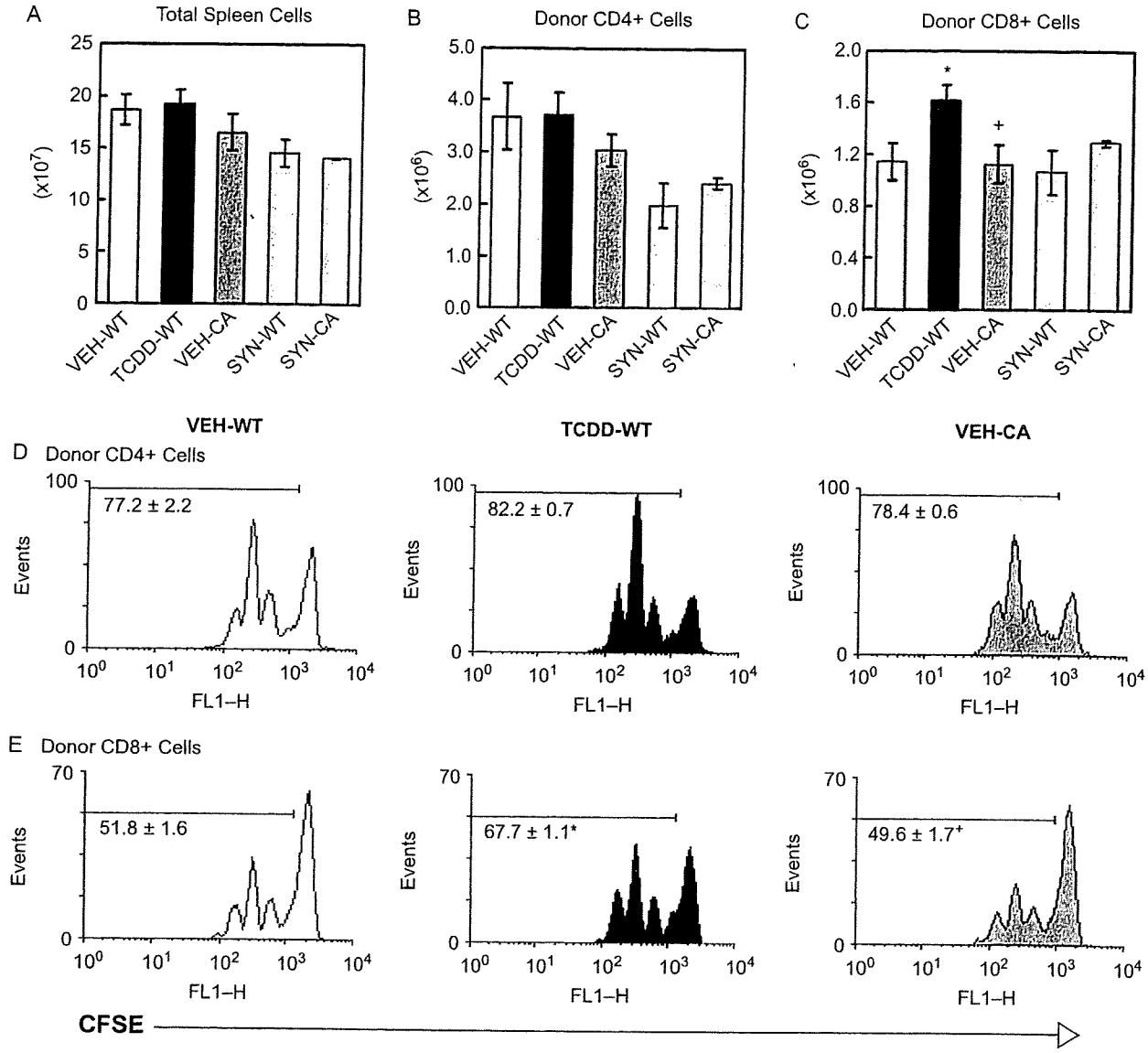

Figure 1. CA-AhR does not alter the number or proliferation of donor T-cells in the spleen on Day 2. Age- and gender-matched F1 mice were dosed with vehicle or 20 μg/kg TCDD one day before the injection of donor T-cells from B6-WT or CA-AhR mice. On Day 2 after injection of donor T-cells, the spleens from F1 mice were collected and analyzed by flow cytometry. After gating on total spleen cells (A), the donor T-cells were identified as $CFSE^+CD4^+$ (B) or $CFSE^+CD8^+$ (C). For A-C, data are combined from two independent experiments and n = 4-6 mice per group. VEH, vehicle-treated F1 recipient; TCDD, TCDD-treated F1 recipient; SYN, syngeneic B6 recipient; WT, donor T-cells from wild-type mice; CA, donor T-cells from CA-AhR Tg mice. The percentage of donor $CD4^+$ (D) or $CD8^+$ T-cells (E) that were dividing was determined on Day 2. Representative histograms are shown and the numbers on the histograms indicate the mean ± SEM of the percentage of cells in divisions 1-4 for n = 3 mice per group. *Indicates statistically significant difference compared to VEH-WT; +indicates statistically significant difference compared to TCDD-WT.

a similar phenomenon was seen. Exposure to TCDD dramatically increased the frequency of CD25-expressing cells while CA-AhR produced only a slight increase (Figure 2B and 3B). Additionally, both TCDD and CA-AhR increased the frequency of donor $CD8^+$ T-cells that had down-regulated CD62L (Figure 2D and 3B). The decrease in CD62L occurred stepwise as the T-cells divided, as indicated by the decrease in median fluorescence intensity for both TCDD-treated and CA-AhR-expressing donor T-cells as compared to vehicle-treated donor T-cells (Figures 2E and 2F). Interestingly, in syngeneic recipients, CA-AhR also caused a small increase in the frequency of donor T-cells that had down-regulated CD62L as compared to B6-WT donors for both $CD4^+$ and $CD8^+$ T-cells (Figures 2C and 2D, light grey bars).

Expression of $CD25^{hi}CD62L^{low}$ Treg phenotype

Recently, we reported that a subpopulation of donor $CD4^+$ T-cells in TCDD-treated mice is $CD25^{hi}CD62L^{low}$ and that this subpopulation has Treg-like suppressive activity *in vitro* (Funatake et al., 2005). We have also observed the same phenotype and *in vitro* suppressive activity for donor $CD8^+$ T-cells from TCDD-treated mice (Funatake et al., 2008). However, as shown above, for donor T-cells expressing CA-AhR, there were only minor changes in the expression of CD25 while at

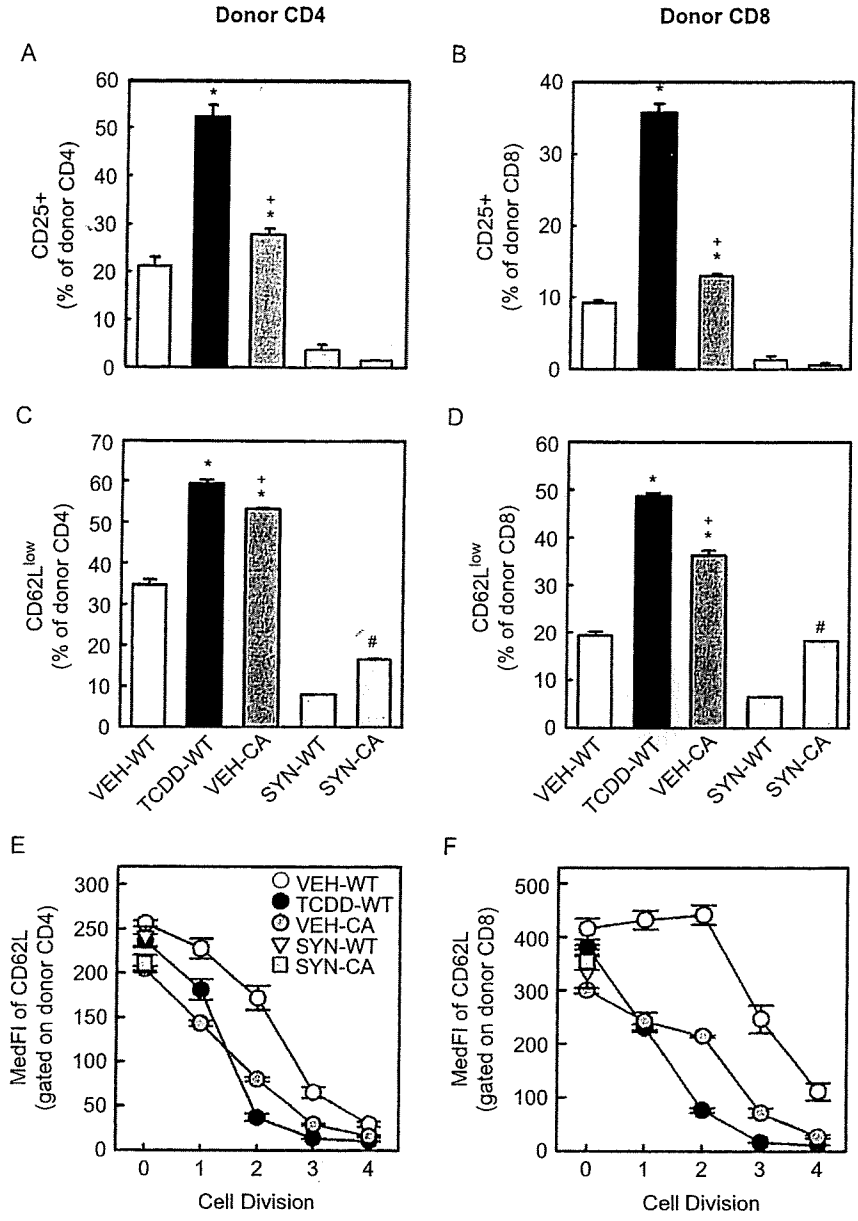

Figure 2. CA-AhR enhances the down-regulation of CD62L comparable to TCDD but has only a minimal effect on the up-regulation of CD25. F1 mice were treated with vehicle or TCDD as described for Figure 1. On Day 2, the donor CD4⁺ (A and C) and donor CD8⁺ T-cells (B and D) were analyzed for the expression of CD25 (A and B) and CD62L (C and D). For CD25, data are combined from two independent experiments with n = 4–6 mice per group. For CD62L, data are from a representative experiment with n = 3–4 mice per group. Also on Day 2, the median fluorescence intensity (MedFI) of CD62L was determined for each cell division for donor CD4⁺ (E) and CD8⁺ (F) T-cells. *Indicates statistically significant difference compared to VEH-WT; ⁺indicates statistically significant difference compared to TCDD-WT; #indicates statistically significant difference compared to SYN-WT.

the same time expression of CD62L was profoundly down-regulated. When we examined the co-expression of these two markers on the donor T-cells two days after injection into F1 hosts, exposure to TCDD greatly increased the percentage of cells that were $CD25^{+}CD62L^{low}$ for both donor $CD4^{+}$ and $CD8^{+}$ T-cells (Figures 3A and 3B, middle panel) In contrast, donor T-cells expressing CA-AhR exhibited a significant but much smaller increase in the percentage of donor $CD4^{+}$ or $CD8^{+}$ T-cells that were $CD25^{+}CD62L^{low}$ when compared to vehicle-treated mice (Figures 3A and 3B, right panel). Thus, compared to the effect from TCDD treatment, CA-AhR in the donor T-cells alone does not appear to be sufficient to induce a subpopulation of $CD25^{hi}CD62L^{low}$ donor T-cells.

Development of effector CTL and CTL activity is not impaired by CA-AhR

To determine if CA-AhR in T-cells alone was sufficient to suppress the CTL response, we injected F1 mice with donor T-cells from B6-WT or transgenic CA-AhR mice and 10 days later tested the spleen cells from F1 mice for the presence of effector CTL and CTL activity. As shown in Figure 4A, there were significantly more spleen cells in TCDD-treated

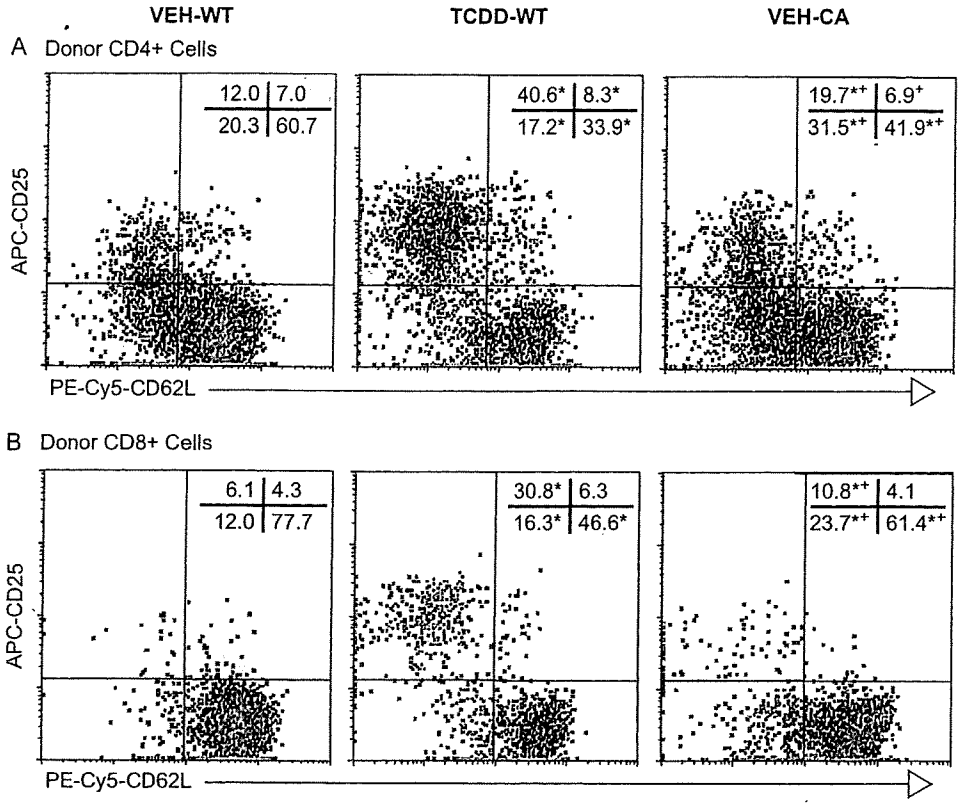

Figure 3. CA-AhR does not expand a subpopulation of $CD25^{hi}CD62L^{low}$ donor T-cells. F1 mice were treated with vehicle or TCDD as described for Figure 1. The co-expression of CD25 and CD62L was determined for the donor $CD4^+$ (A) and donor $CD8^+$ T-cells (B) on Day 2. Representative histograms are shown and the numbers on the histograms indicate the mean of the percentage of cells in each quadrant for n = 3 mice per group (SEM is excluded for clarity). *Indicates statistically significant difference compared to VEH-WT; +indicates statistically significant difference compared to TCDD-WT.

mice, while CA-AhR in the donor T-cells had no effect on the number of spleen cells on Day 10. The greater number of total spleen cells in TCDD-treated mice on Day 10 was due to greater numbers of host B-cells and host T-cells in the spleen reflecting the absence of an anti-host immune response, as shown in Figures 4D-4F. In addition, the number of donor $CD4^+$ and donor $CD8^+$ T-cells in the spleen was greatly reduced in TCDD-treated mice, while CA-AhR had no effect on the number of donor T-cells compared to vehicle-treated controls (Figures 4B and 4C).

Of the few donor $CD8^+$ T-cells present in the spleen of TCDD-treated mice on Day 10, significantly fewer expressed an effector CTL phenotype ($CD44^{hi}CD62L^{low}$) as compared to donor $CD8^+$ T-cells in vehicle-treated controls (Figure 5B), resulting in essentially no effector CTL present in the spleens of TCDD-treated mice (Figure 5C). In mice receiving CA-AhR donor T-cells, there was no difference in the percentage or number of donor-derived effector CTL as compared to vehicle-treated F1 mice that received WT donor T-cells (Figures 5B and 5C).

On Day 10, CTL activity in the spleen was measured *ex vivo* in a 4-hour non-radioactive cytotoxicity assay, as described in the Methods, and the percentage and number of effector CTL was determine by flow cytometry. As shown in Figure 5A, no CTL activity was detected with splenocytes from TCDD-treated mice. In contrast, the CTL activity of spleen cells from mice injected with CA-AhR donor T-cells was indistinguishable from CTL activity in vehicle-treated mice injected with WT donor T-cells (Figure 5A). Together, these data suggest that AhR activation only in T-cells is not sufficient to suppress CTL activity in the GVH model.

Influence of AhR responsiveness of F1 host mice on donor CTL activity

The absence of an expanded population of $CD25^{hi}CD62L^{low}$ donor T-cells and the lack of suppression of effector CTL when the donor T-cells expressed CA-AhR might suggest that activation of AhR in donor T-cells only is not sufficient to suppress the CTL response, and that activation of AhR in host cells (e.g., dendritic cells that activate the donor T-cells) contribute to the suppression of the GVH response by TCDD. To test this hypothesis, we bred $AhR^{-/-}$ B6 mice with DBA/2 mice to create F1 host mice that would respond poorly to AhR activation ("KO-F1"). If AhR activation in host cells contributes to suppression of the donor CTL response by TCDD, the CTL response should be less affected by TCDD in the KO-F1mice.

To confirm the low responsiveness of these KO-F1 mice, we measured EROD activity of CYP1A1 in the liver after treatment with TCDD. WT-F1 mice reached near-maximal EROD activity with as little as 3 μg TCDD/kg body weight, but little to no EROD activity was detected in KO-F1 mice at this dose (Figure 6A). At 15 μg TCDD/kg body weight, a dose previously shown to completely suppress the GVH CTL

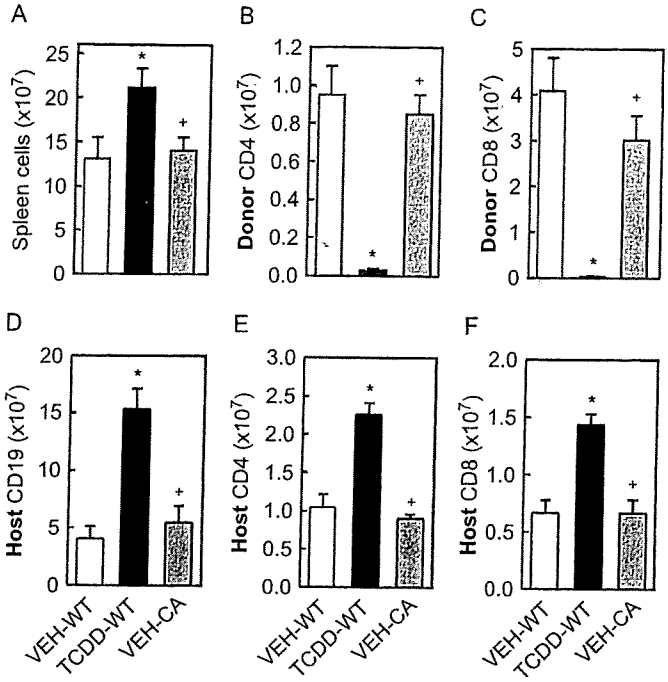

Figure 4. Analysis of the distribution of spleen cells on Day 10 after injection of donor T-cells. F1 mice were treated with vehicle or TCDD as described for Figure 1. On Day 10 after injection of donor T-cells, total spleen cells from F1 mice were counted (A) and analyzed by flow cytometry for enumeration of donor $CD4^+$ (B) and donor $CD8^+$ T-cells (C), host $CD19^+$ cells (D), host $CD4^+$ (E) and host $CD8^+$ T-cells (F). Data from a representative experiment are shown with n = 4-5 mice per group. *Indicates statistically significant difference compared to VEH-WT; +indicates statistically significant difference compared to TCDD-WT.

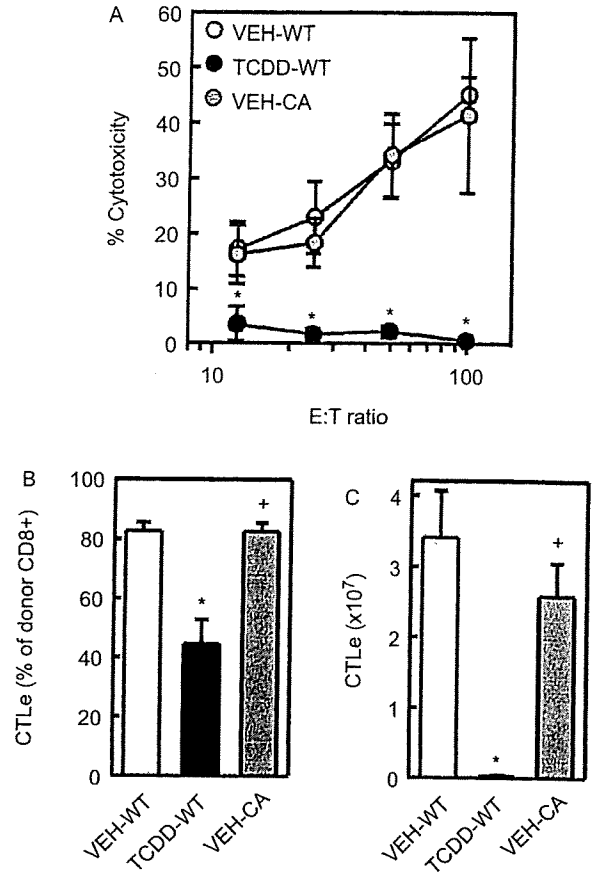

Figure 5. CA-AhR does not suppress the CTL activity or the percentage and number of CTL effectors in the spleen on Day 10. F1 mice were treated with vehicle or TCDD as described for figure 1. Ten days after in the injection of donor T-cells, the spleen cells from F1 mice were tested for CTL activity using a non-radioactive cytotoxicity assay, as described in the Methods (A). The percentage (B) of donor $CD8^+$ cells, identified as $Thy1.1^+CD8^+$ cells, expressing a CTL effector (CTLe) phenotype, defined as $CD44^{hi}CD62L^{low}$, and the number (C) of CTLe in the spleen were determined by flow cytometry. Data from a representative experiment are shown with n = 4-5 mice per group. *Indicates statistically significant difference compared to VEH-WT; +indicates statistically significant difference compared to TCDD-WT.

response (Kerkvliet et al., 2002), the EROD activity in KO-F1 mice was nearly 4 times lower than in WT-F1 mice; even at 30 μg TCDD/kg body weight, the EROD activity of KO-F1 mice was significantly less than in WT-F1 mice (Figure 6A).

Interestingly, despite the low host response to TCDD, the CTL response of AhR-WT donor T-cells in KO-F1 hosts was suppressed by TCDD to the same extent as in WT-F1 hosts (Figure 6B), suggesting that activation of AhR in host cells plays a minimal role in mediating the suppressive effects of TCDD in this model. These data suggest that CA-AhR does not behave the same as ligand (TCDD)-bound AhR in T-cells that appears to be necessary and sufficient for suppressing the CTL response. However, it remains possible that even low-level activation of AhR within key cells of the KO-F1 host treated with 15 μg/kg TCDD was sufficient to synergize with AhR-mediated signaling in T-cells to suppress the CTL response. Additional studies will be necessary to determine which of these interpretations is correct.

Discussion

In the present study, we used transgenic mice that express CA-AhR only in T-lineage cells (Nohara et al., 2005). The CA-AhR mutant has a minimal deletion in the PAS B domain which is required for binding to hsp90 and also contains part of the ligand binding domain. The minimal deletion causes the AhR to localize in the nucleus, heterodimerize with ARNT, and activate transcription by binding DRE sequences in a ligand-independent manner (McGuire et al., 2001).

In our recent study (Nohara et al., 2009), we found that CA-AhR Tg mice elicit the same level of IL-4 and IL-5 production and antibody production upon OVA immunization as wild-type mice do. We showed in the present study that CA-AhR T-cells normally proliferate and differentiate into CTL effector cells with CTL activity in B6D2F1 hosts as wild-type B6 cells. In CA-AhR Tg mice, CA-AhR is expressed in the T-cells from their fetal stage, which might affect the immune function (Nohara, et al., 2005). Nevertheless, the results obtained so far indicate that the immune system of Tg mice still maintains its normal ability to perform humoral and cellular immune responses. The findings appear to be consistent with the fact that other studies have shown that T-cells must be activated in order to see any AhR-dependent changes in phenotype or function (Shepherd et al., 2000;

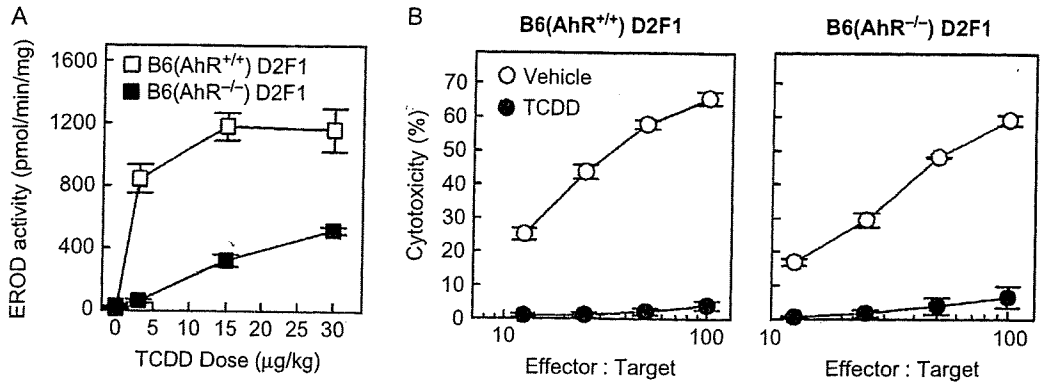

Figure 6. Host AhR does not appear to be involved in suppression of the CTL response on Day 10. (A) KO-F1 and WT-F1 mice were dosed with 0, 3, 15, or 30 μg TCDD/kg body weight and, two days later, the livers were collected and EROD activity was determined as described in the Methods. (B) KO- and WT-F1 mice received AhR-WT donor T-cells and treated with 15 μg TCDD/kg body weight. Ten days later, the CTL activity in the spleen was assessed. Data from a representative experiment are shown.

Funatake et al., 2004). On the other hand, Tg mice develop thymus atrophy (Nohara et al., 2005) and, upon OVA immunization, they showed augmented IFNγ production compared to wild-type mice (Nohara et al., 2009). These changes are also observed in wild-type mice given a single dose of TCDD (Nohara et al., 2005, 2009). The results of the present study show that CA-AhR enhanced the down-regulation of CD62L to the same extent as exposure to a single dose of TCDD (20 μg/kg body weight). Thus, CA-AhR Tg mice show an immune profile, some of which is similar to those of wild-type mice and some of which mimic those observed in wild-type mice given a single dose of TCDD.

In this study, we show that restricting the expression of CA-AhR to T-cells only partially mimics the effects of TCDD in the GVH response. Specifically, CA-AhR enhanced the down-regulation of CD62L, but had no effect on the expression of CD25 and did not suppress CTL activity on Day 10. In previous studies, the down-regulation of CD62L was found to depend strictly on expression of AhR within the affected T-cell population, whereas the up-regulation of CD25, at least for donor $CD8^+$ T-cells, was found to depend indirectly on AhR within the donor $CD4^+$ T-cells (Funatake et al., 2008). Sun et al. (2004) performed a genome-wide study of DRE located between -5000 and +2000 bp for over 11,000 mouse genes and identified eight putative DRE in the promoter region of the gene for CD62L. Three of these putative DRE had match scores equivalent to confirmed DRE for CYP1A1; interestingly, no DRE were identified in the *Il2ra* gene. Together, these findings suggest that CD62L could be a direct target of the AhR-ARNT complex, while CD25 may be an indirect target, dependent on other AhR-ARNT-regulated genes.

It is interesting that although CA-AhR had a profound effect on the down-regulation of CD62L, this did not appear to have any effect on the final outcome of the GVH response, since the CTL activity on Day 10 was unimpaired. CD62L is important for allowing entry of naïve T-cells into the lymph node through the high endothelial venules (Arbones et al., 1994; Bradley et al., 1994; Warnock et al., 1998). Once activated, T-cells down-regulate CD62L; however, this down-regulation is not necessary for the cells to exit out of a lymph node, and is more important for preventing the re-entry of activated T-cells into lymph nodes (Galkina et al., 2003). Some studies have suggested that signaling through CD62L induces conformational changes in integrins and alters expression of chemokine receptors that enhance/prolong the interaction between T-cells and antigen-presenting cells (Giblin et al., 1997; Ding et al., 2003). However, other studies have shown that the lack of CD62L has no adverse effects on the development of autoimmune diabetes (Friedline et al., 2002; Mora et al., 2004). This latter finding is consistent with our results showing that CTL activity is unimpaired on Day 10 of the GVH response despite the enhanced down-regulation of CD62L on donor T-cells expressing CA-AhR. Thus, although AhR may directly target CD62L and induce its down-regulation, this alone is not sufficient for suppression of effector function.

CA-AhR expressed in T-cells did not suppress the GVH response, suggesting that activation of AhR in other immune cells is involved in the immunosuppressive effects of TCDD. In the GVH response, host cells, in particular dendritic cells, contribute to the T-cell response by acting as antigen presenting cells. Previous studies have shown that dendritic cells, like T-cells, are sensitive, AhR-dependent targets of TCDD (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse et al., 2003; Ruby et al., 2005). In order to assess the contribution of host-expressed AhR, $AhR^{-/-}$ B6 mice were crossed with D2 mice to generate KO-F1 hosts with low responsiveness to AhR. If the host AhR contributes significantly to the suppression of the CTL response, we would expect that the CTL activity in these KO-F1 mice to be less sensitive to suppression by TCDD. However, we found that the CTL activity was suppressed to the same extent as in wild-type F1 mice. This would imply that activation of AhR in dendritic cells or other host cells does not have a major influence on the outcome of the T-cell response in this GVH model. One caveat to this interpretation is that there was still activation of AhR, albeit at a much lower level, in the KO-F1 hosts at the dose of TCDD used. This leaves open the possibility that low-level activation of AhR within the host is sufficient to synergize

with the activation of AhR within the donor T-cells, leading to the full suppression of the CTL response. To assess this possibility, generation of $AhR^{-/-}$ B6xAhR$^{-/-}$ D2 F1 would be useful.

Another possible interpretation of the results of the present study is that CA-AhR does not behave the same as ligand-bound AhR in the suppression of the GVH response. AhR-dependent changes in expression of genes, particularly those involved in the activation and differentiation of T-cells, and also non-genomic effects of AhR are reported to depend on the interaction of AhR with other proteins, such as transcription factors, co-activators, and co-repressors (reviewed in Hankinson, 2005; Harper et al., 2006). The deletion in a part of the ligand/Hsp90-binding site in the PAS B domain of AhR may affect the interaction of the CA-AhR with these other proteins and their function. In addition, it is possible that different ligands induce different conformational states, allowing for different protein interactions (Henry and Gasiewicz, 2003; Hestermann and Brown, 2003; Zhang et al., 2008). Recent studies have reported that activation of AhR by TCDD induces Treg, while activation of AhR by 6-formylindolo-[3,2-b]carbazole (FICZ) induces Th17 cells (Quintana et al., 2008; Veldhoen et al., 2008). These findings may indicate ligand-dependent regulation of AhR function. In contrast, the single-conformation available for the CA-AhR may not allow for interaction with other proteins.

In summary, our findings suggest that either activation of AhR must occur in other cells in addition to T-cells in order to cause immunosuppression or that CA-AhR does not behave exactly the same as ligand (TCDD)-bound AhR in the GvH model. Additional studies in progress of functions of CA-AhR will provide insights into the genomic and non-genomic mechanisms of AhR function.

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References

Arbones, M. L., Ord, D. C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D. J., and Tedder, T. F. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity* 1:247-260.

Boverhof, D. R., Tam, E., Harney, A. S., Crawford, R. B., Kaminski, N. E., and Zacharewski, T. R. 2004. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces suppressor of cytokine signaling 2 in murine B-cells. *Mol. Pharmacol.* 66:1662-1670.

Bradley, L. M., Watson, S. R., and Swain, S. L. 1994. Entry of naive CD4 T-cells into peripheral lymph nodes requires L-selectin. *J. Exp. Med.* 180:2401-2406.

Denison, M. S., and Nagy, S. R. 2003. Activation of the aryl hydrocarbon receptor by structurally-diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* 43:309-334.

Ding, Z., Issekutz, T. B., Downey, G. P., and Waddell, T. K. 2003. L-Selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: Implications for cellular activation during adhesion and migration. *Blood* 101:4245-4252.

Friedline, R. H., Wong, C. P., Steeber, D. A., Tedder, T. F., and Tisch, R. 2002. L-Selectin is not required for T-cell-mediated autoimmune diabetes. *J. Immunol.* 168:2659-2666.

Funatake, C. J., Dearstyne, E. A., Steppan, L. B., Shepherd, D. M., Spanjaard, E. S., Marshak-Rothstein, A., and Kerkvliet, N. I. 2004. Early consequences of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on the activation and survival of antigen-specific T-cells. *Toxicol. Sci.* 82:129-142.

Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V., and Kerkvliet, N. I. 2005. Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin generates a population of $CD4^+CD25^+$ cells with characteristics of regulatory T-cells. *J. Immunol.* 175:4184-4188.

Funatake, C. J., Marshall, N. B., and Kerkvliet, N. I. 2008. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin alters the differentiation of alloreactive $CD8^+$ T-cells toward a regulatory T-cell phenotype by a mechanism that is dependent on aryl hydrocarbon receptor in $CD4^+$ T-cells. *J. Immunotoxicol.* 5:81-91.

Galkina, E., Tanousis, K, Preece, G., Tolaini, M., Kioussis, D., Florey, O., Haskard, D. O., Tedder, T. F., and Ager, A. 2003. L-Selectin shedding does not regulate constitutive T-cell trafficking but controls the migration pathways of antigen-activated T-lymphocytes. *J. Exp. Med.* 198:1323-1335.

Giblin, P. A., Hwang, S. T., Katsumoto, T. R., and Rosen, S. D. 1997. Ligation of L-selectin on T-lymphocytes activates β1 integrins and promotes adhesion to fibronectin. *J. Immunol.* 159:3498-3507.

Hankinson, O. 2005. Role of co-activators in transcriptional activation by the aryl hydrocarbon receptor. *Arch. Biochem. Biophys.* 433:379-386.

Harper, P. A., Riddick, D. S., and Okey, A. B. 2006. Regulating the regulator: Factors that control levels and activity of the aryl hydrocarbon receptor. *Biochem. Pharmacol.* 72:267-279.

Henry, E. C., and Gasiewicz, T. A. 2003. Agonist but not antagonist ligands induce conformational change in the mouse aryl hydrocarbon receptor as detected by partial proteolysis. *Mol. Pharmacol.* 63:392-400.

Hestermann, E. V., and Brown, M. 2003. Agonist and chemopreventative ligands induce differential transcriptional cofactor recruitment by aryl hydrocarbon receptor. *Mol. Cell. Biol.* 23:7920-7925.

Holsapple, M. P., Snyder, N. K., Wood, S. C., and Morris, D. L. 1991. A review of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology* 69:219-255.

Inouye, K., Ito, T., Fujimaki, H., Takahashi, Y., Takemori, T., Pan, X., Tohyama, C., and Nohara, K. 2003. Suppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the high-affinity antibody response in C57BL/6 mice. *Toxicol. Sci.* 74:315-324.

Jeuken, A., Keser, B. J., Khan, E., Brouwer, A., Koeman, J., and Denison, M. S. 2003. Activation of the Ah receptor by extracts of dietary herbal supplements, vegetables, and fruits. *J. Agric. Food Chem.* 51:5478-5487.

Kerkvliet, N. I. 2002. Recent advances in understanding the mechanisms of TCDD immunotoxicity. *Int. Immunopharmacol.* 2:277-291.

Kerkvliet, N. I. 2003. Immunotoxicology of dioxins and related chemicals. In: *Dioxins and Health*, 2nd Edition (Schecter, A., and Gasiewicz, T. A., Eds.), New York: John Wiley & Sons, Inc., pp. 299-328.

Kerkvliet, N. I., Shepherd, D. M., and Baecher-Steppan, L. 2002. T-Lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): AhR expression in both $CD4^+$ and $CD8^+$ T-cells is necessary for full suppression of a cytotoxic T-lymphocyte response by TCDD. *Toxicol. Appl. Pharmacol.* 185:146-152.

Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y., and T., Kishimoto. 2008. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc. Natl. Acad. Sci. USA* 105:9721-9726.

Laiosa, M. D., Wyman, A., Murante, F. G., Fiore, N. C., Staples, J. E., Gasiewicz, T. A., and Silverstone, A. E. 2003. Cell proliferation arrest within intrathymic lymphocyte progenitor cells causes thymic atrophy mediated by the aryl hydrocarbon receptor. *J. Immunol.* 171:4582-4591.

Lawrence, B. P., and Kerkvliet, N. I. 2007. Immune modulation by TCDD and related polyhalogenated aromatic hydrocarbons. In: *Immunotoxicology and Immunopharmacology*, 3rd Edition (Luebke, R., House, R., and I., Kimber, I., Eds.), Boca Raton, FL: Taylor & Francis, pp. 239-258.

Lawrence, B. P., Roberts, A. D., Neumiller, J. J., Cundiff, J. A., and Woodland, D. L. 2006. Aryl hydrocarbon receptor activation impairs the priming but

not the recall of influenza virus-specific CD8⁺ T-cells in the lung. *J. Immunol.* 177:5819-5828.

Liem, A. K., Furst. P., and Rappe. C. 2000. Exposure of populations to dioxins and related compounds. *Food Addit. Contam.* 17:241-259.

McGuire, J., Okamoto, K., Whitelaw, M. L., Tanaka, H., and Poellinger, L. 2001. Definition of a dioxin receptor mutant that is a constitutive activator of transcription: Delineation of overlapping repression and ligand binding functions within the PAS domain. *J. Biol. Chem.* 276:41841-41849.

Mimura, J., and Fujii-Kuriyama, Y. 2003. Functional role of AhR in the expression of toxic effects by TCDD. *Biochem. Biophys. Acta* 1619:263-268.

Moennikes, O., Loeppen, S., Buchmann, A., Andersson, P., Ittrich, C., Poellinger, L., and Schwarz, M. 2004. A constitutively-active dioxin/aryl hydrocarbon receptor promotes hepatocarcinogenesis in mice. *Cancer Res.* 64:4707-4710.

Mora, C., Grewal, I. S., Wong, F. S., and Flavell, R. A. 2004. Role of L-selectin in the development of autoimmune diabetes in non-obese diabetic mice. *Int. Immunol.* 16:257-264.

Nguyen, L. P., and Bradfield, C. A. 2008. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem. Res. Toxicol.* 21:102-116.

Nohara, K., Fujimaki, H., Tsukumo, S., Inouye, K., Sone, H., and Tohyama, C. 2002. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on T-cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. *Toxicology* 172:49-58.

Nohara, K., Pan, X., Tsukumo, S., Hida, A., Ito, T., Nagai, H., Inouye, K., Motohashi, H., Yamamoto, M., Fujii-Kuriyama, Y., and Tohyama, C. 2005. Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. *J. Immunol.* 174:2770-2777.

Nohara, K., Suzuki, T., Ao, K., Murai, H., Miyamoto, Y., Inouye, K., Pan, X., Motohashi, H., Fujii-Kuriyama, Y., Yamamoto, M., and Tohyama, C. 2009. Constitutively active aryl hydrocarbon receptor expressed in T-cells increases immunization-induced IFNγ production in mice but does not suppress T_H2-cytokine production or antibody production *Int. Immunology*. 21:769-777.

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M., and Weiner, H. L. 2008. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65-71.

Ruby, C. E., Funatake, C. J., and Kerkvliet, N. I. 2005. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) directly enhances the maturation and apoptosis of dendritic cells *in vitro*. *J. Immunotoxicol.* 1:159-166.

Schecter, A., Cramer, P., Boggess, K., Stanley, J., Papke, O., Olson, J., Silver, A., and Schmitz, M. 2001. Intake of dioxins and related compounds from food in the U.S. population. *J. Toxicol. Environ. Health A* 63:1-18.

Schmidt, J. V., and Bradfield, C. A. 1996. Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* 12:55-89.

Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., and Bradfield, C. A. 1996. Characterization of a murine Ahr null allele: Involvement of the Ah receptor in hepatic growth and development. *Proc. Natl. Acad. Sci. USA* 93:6731-6736.

Shepherd, D. M., Dearstyne, E. A., and Kerkvliet, N. I. 2000. The effects of TCDD on the activation of ovalbumin (OVA)-specific DO11.10 transgenic CD4⁺ T-cells in adoptively transferred mice. *Toxicol. Sci.* 56:340-350.

Startin, J. R., and Rose, M. D. 2003. Dioxins and dioxin-like PCBs in food. In: *Dioxins and Health*, 2nd Edition (Schecter, A., and Gasiewicz, T. A., Eds.), New York: John Wiley & Sons, Inc., pp. 89-136.

Sulentic, C. E. W., Holsapple, M. P., and Kaminski, N. E. 1998. Aryl hydrocarbon receptor-dependent suppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin of IgM secretion in activated B-cells. *Mol. Pharmacol.* 53:623-629.

Sun, Y. V., Boverhof, D. R., Burgoon, L. D., Fielden, M. R., and Zacharewski, T. R. 2004. Comparative analysis of dioxin response elements in human, mouse and rat genomic sequences. *Nucl. Acids Res.* 32:4512-4523.

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., and Stockinger, B. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:106-109.

Vorderstrasse, B. A., and Kerkvliet, N. I. 2001. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin affects the number and function of murine splenic dendritic cells and their expression of accessory molecules. *Toxicol. Appl. Pharmacol.* 171:117-125.

Vorderstrasse, B. A., Dearstyne, E. A., and Kerkvliet, N. I. 2003. Influence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the antigen-presenting activity of dendritic cells. *Toxicol. Sci.* 72:103-112.

Warnock, R. A., Askari, S., Butcher, E. C., and von Andrian, U. H. 1998. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J. Exp. Med.* 187:205-216.

Zhang, S., Rowlands, C., and Safe, S. 2008. Ligand-dependent interactions of the Ah receptor with co-activators in a mammalian two-hybrid assay. *Toxicol. Appl. Pharmacol.* 227:196-206.

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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

Keiko Nohara[1], Takehiro Suzuki[1], Kana Ao[1], Hikari Murai[1], Yoshimi Miyamoto[1], Kaoru Inouye[1], Xiaoqing Pan[1], Hozumi Motohashi[2], Yoshiaki Fujii-Kuriyama[3], Masayuki Yamamoto[2] and Chiharu Tohyama[4]

[1]Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba 305-8506, Japan
[2]Tohoku Universtiy Graduate School of Medicine, Sendai 980-8575, Japan
[3]Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba 305-8575, Japan
[4]Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan

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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

Correspondence to: K. Nohara; Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba 305-8506, Japan; E-mail: keikon@nies.go.jp

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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′-GATGCTCTT-TCCTCCTGTGC-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).

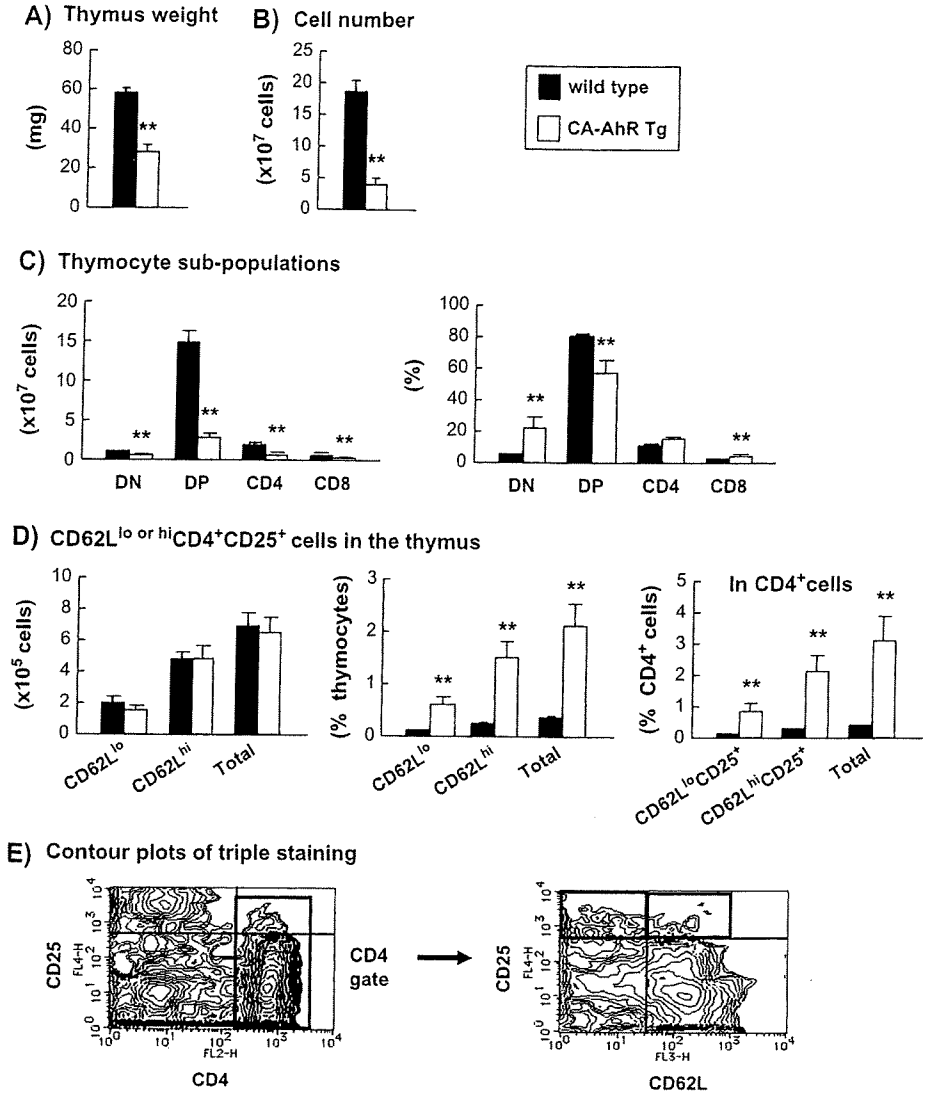

Fig. 1. The thymocytes of the CA-AhR Tg mice contained a higher percentage of $CD4^+CD25^+$ cells in the primary immune response to OVA. Female Tg (heterozygous) mice and littermate wild-type mice (n = 7 per group) were immunized with OVA/alum, and 7 days later their thymus weights (A) and thymocyte 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

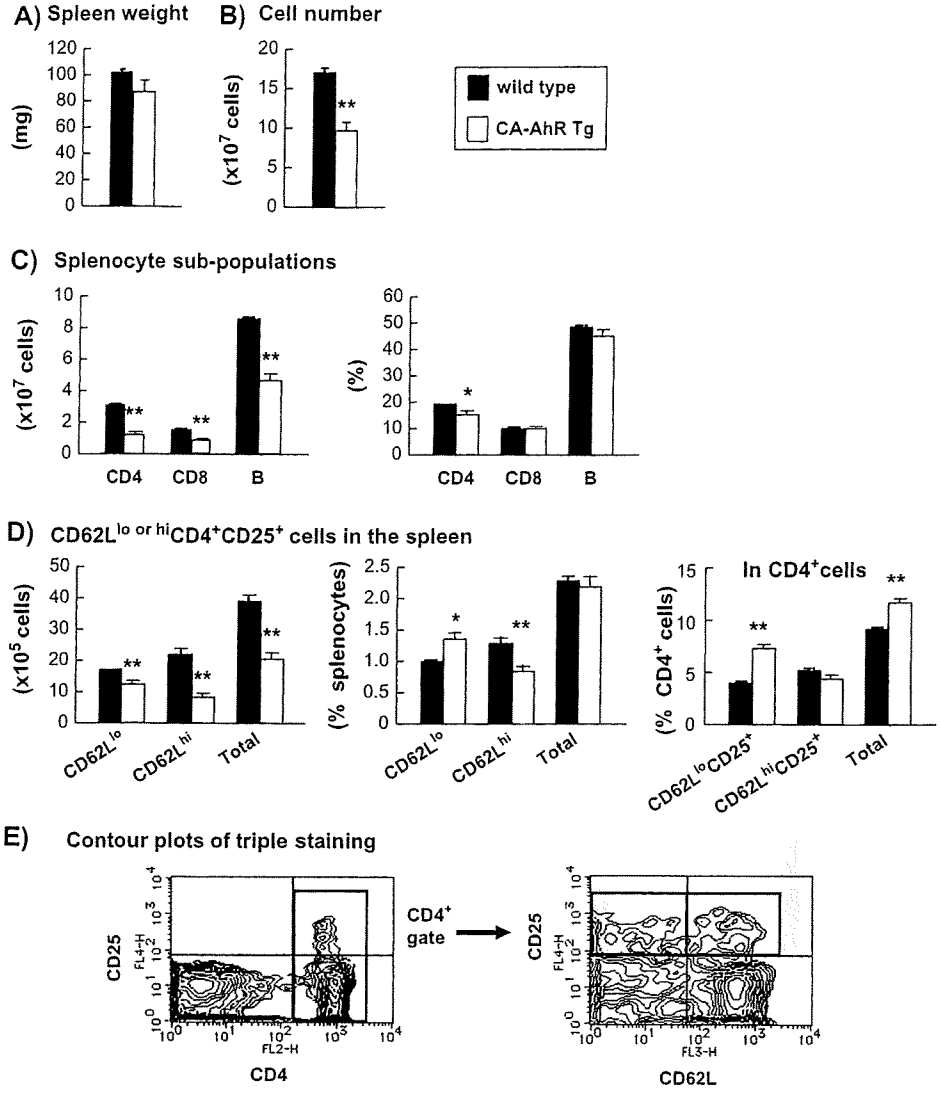

Fig. 2. The splenocytes of the CA-AhR Tg mice contained a higher percentage of $CD4^+CD25^+$ cells in the primary immune response to OVA. Spleen weight (A), splenocyte number (B), splenocyte subpopulation (C) and $CD62L^{lo\ or\ hi}$ $CD4^+CD25^+$ cells (D and E) were examined in the same mice as in 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).

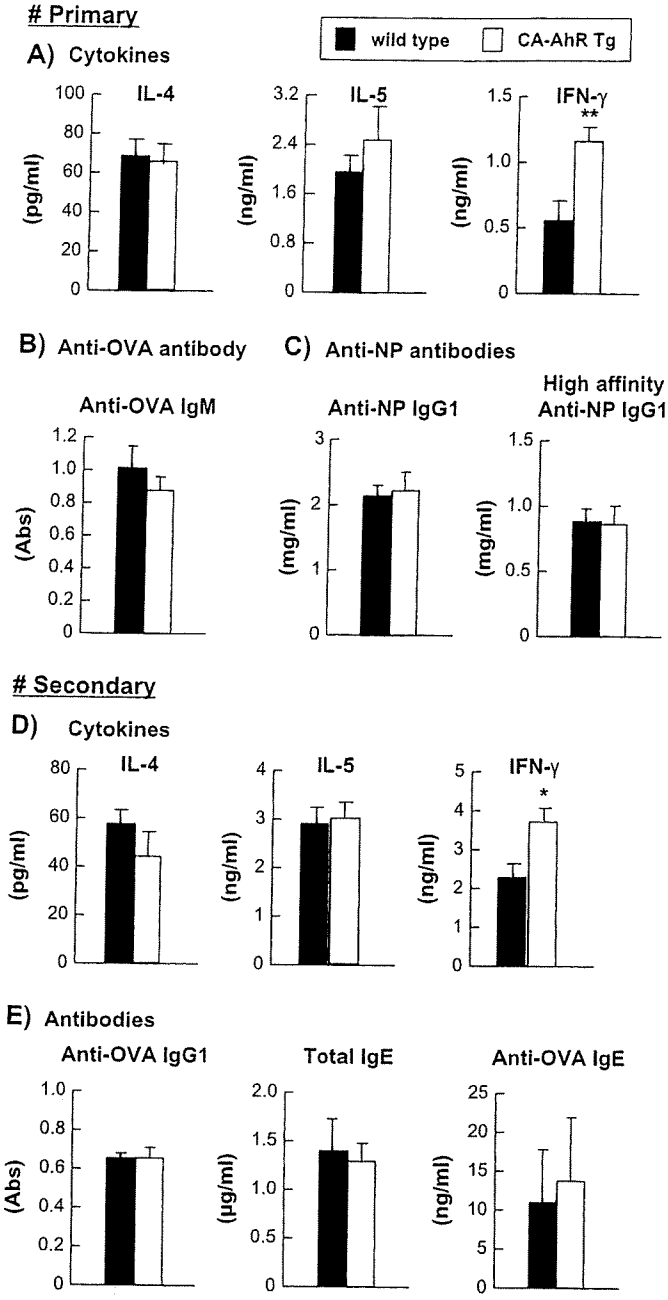

Fig. 3. CA-AhR expressed in the T cells increased IFN-γ production but did not suppress the production of T_h2-cytokines or antigen-specific antibodies. (A and B) Female Tg and wild-type mice ($n = 7$ per group) were immunized with OVA/alum. Seven days later, the mice were sacrificed and the cytokines (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

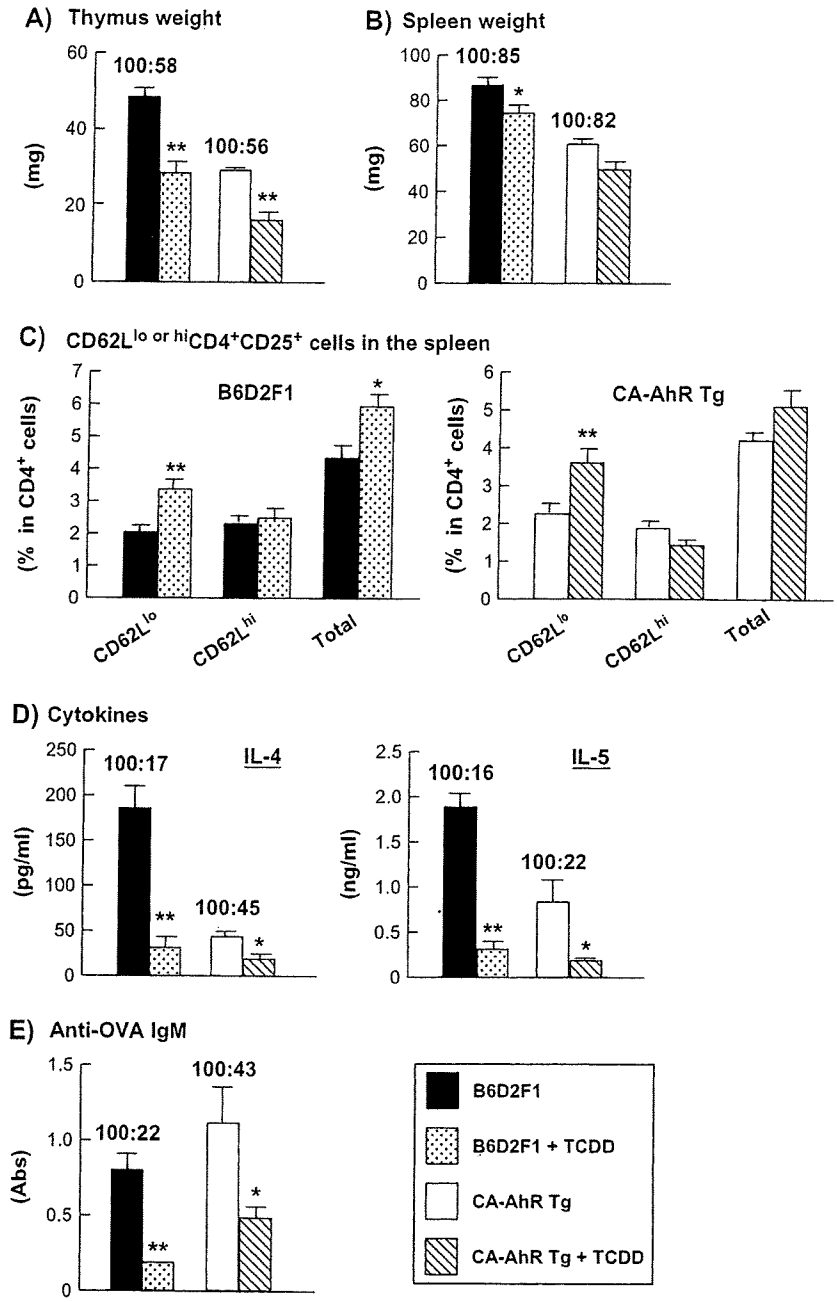

Fig. 4. TCDD exposure suppresses the immune response of CA-AhR Tg mice. Female B6D2F1 mice and CA-AhR Tg mice (n = 6/each group) were administered TCDD (40 μg kg^{-1}) or vehicle and subsequently immunized with OVA/alum. (A–D) Mice were sacrificed on Day 4 to measure thymus weight (A), spleen weight (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

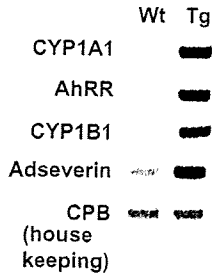

Fig. 5. Expression of AhR-target genes. Expression of AhR-target genes in the thymus of wild-type (Wt) and CA-AhR Tg (Tg) mice were measured by RT-PCR.

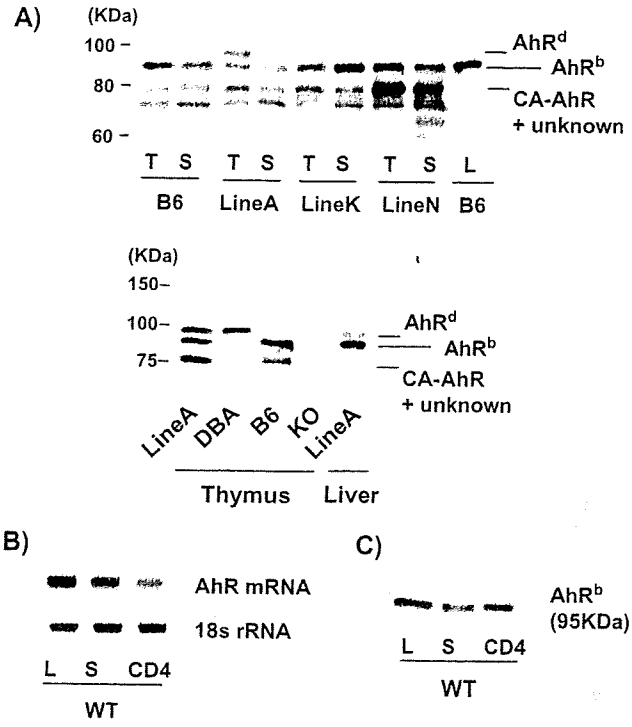

Fig. 6. Expression of AhR protein and mRNA. (A) AhR protein in the total lysate of thymus (T), spleen (S) and liver (L) was measured by western blotting. Line A, K and N heterozygous CA-AhR Tg mice were determined to have 2, 6–7 and 9–11 integrated CA-AhR genes, respectively (24). KO, AhR knockout mice.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

References

1 Schmidt, J. V. and Bradfield, C. A. 1996. Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* 12:55.
2 Mimura, J. and Fujii-Kuriyama, Y. 2003. Functional role of AhR in the expression of toxic effects by TCDD. *Biochem. Biophys. Acta.* 1619:263.
3 Okey, A. B. 2007. An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI. *Toxicol. Sci.* 98:5.
4 Schecter, A., Birnbaum, L., Ryan, J. J. and Constable, J. D. 2006. Dioxins: an overview. *Environ. Res.* 101:419.
5 Kawajiri, K. and Fujii-Kuriyama, Y. 2007. Cytochrome P450 gene regulation and physiological functions mediated by the aryl hydrocarbon receptor. *Arch. Biochem. Biophys.* 464:207.
6 McMillan, B. J. and Bradfield, C. A. 2007. The aryl hydrocarbon receptor sans xenobiotics: endogenous function in genetic model systems. *Mol. Pharmacol.* 72:487.
7 Nguyen, L. P. and Bradfield, C. A. 2008. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem. Res. Toxicol.* 21:102.
8 Laiosa, M. D., Wyman, A., Murante, F. G. *et al.* 2003. Cell proliferation arrest within intrathymic lymphocyte progenitor cells

causes thymic atrophy mediated by the aryl hydrocarbon receptor. *J. Immunol.* 171:4582.
9 Camacho, I. A., Singh, N., Hegde, V. L., Nagarkatti, M. and Nagarkatti, P. S. 2005. Treatment of mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to aryl hydrocarbon receptor-dependent nuclear translocation of NF-kappaB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J. Immunol.* 175:90.
10 Tsukumo, S., Iwata, M., Tohyama, C. and Nohara, K. 2002. Skewed differentiation of thymocytes toward CD8 T cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin requires activation of the extracellular signal-related kinase pathway. *Arch. Toxicol.* 76:335.
11 Kerkvliet, N. I., Shepherd, D. M. and Baecher-Steppan, L. 2002. T lymphocytes are direct, aryl hydrocarbon receptor (AhR)-dependent targets of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD): AhR expression in both CD4+ and CD8+ T cells is necessary for full suppression of a cytotoxic T lymphocyte response by TCDD. *Toxicol. Appl. Pharmacol.* 185:146.
12 Lawrence, B. P., Roberts, A. D., Neumiller, J. J., Cundiff, J. A. and Woodland, D. L. 2006. Aryl hydrocarbon receptor activation impairs the priming but not the recall of influenza virus-specific CD8+ T cells in the lung. *J. Immunol.* 177:5819.
13 Holsapple, M. P., Snyder, N. K., Wood, S. C. and Morris, D. L. 1991. A review of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology* 69:219.
14 Inouye, K., Ito, T., Fujimaki, H. *et al.* 2003. Suppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the high-affinity antibody response in C57BL/6 mice. *Toxicol. Sci.* 74:315.
15 Staples, J. E., Murante, F. G., Fiore, N. C., Gasiewicz, T. A. and Silverstone, A. E. 1998. Thymic alterations induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are strictly dependent on aryl hydrocarbon receptor activation in hemopoietic cells. *J. Immunol.* 160:3844.
16 Hundeiker, C., Pineau, T., Cassar, G., Betensky, R. A., Gleichmann, E. and Esser, C. 1999. Thymocyte development in Ah-receptor-deficient mice is refractory to TCDD-inducible changes. *Int. J. Immunopharmacol.* 21:841.
17 Vorderstrasse, B. A., Steppan, L. B., Silverstone, A. E. and Kerkvliet, N. I. 2001. Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. *Toxicol. Appl. Pharmacol.* 171:157.
18 Funatake, C. J., Marshall, N. B., Steppan, L. B., Mourich, D. V. and Kerkvliet, N. I. 2005. Cutting edge: activation of the aryl hydrocarbon receptor by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin generates a population of CD4+ CD25+ cells with characteristics of regulatory T cells. *J. Immunol.* 175:4184.
19 Quitana, F. J., Basso, A. S., Iglesias, A. H. *et al.* 2008. Control of T_{reg} and T_H17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453:65.
20 Kimura, A., Naka, T., Nohara, K., Fujii-Kuriyama, Y. and Kishimoto, T. 2008. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc. Natl Acad. Sci. USA* 105:9721.
21 Veldhoen, M., Hirota, K., Westendorf, A. M. *et al.* 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453:46.
22 Nohara, K., Fujimaki, H., Tsukumo, S., Inouye, K., Sone, H. and Tohyama, C. 2002. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. *Toxicology* 172:49.
23 Ito, T., Inouye, K., Fujimaki, H., Tohyama, C. and Nohara, K. 2002. Mechanism of TCDD-induced suppression of antibody production: effect on T cell-derived cytokine production in the primary immune reaction of mice. *Toxicol. Sci.* 70:46.
24 Nohara, K., Pan, X., Tsukumo, S. *et al.* 2005. Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. *J. Immunol.* 174:2770.
25 McGuire, J., Okamoto, K., Whitelaw, M. L., Tanaka, H. and Poellinger, L. 2001. Definition of a dioxin receptor mutant that is a constitutive activator of transcription. *J. Biol. Chem.* 276:41841.
26 Fujimaki, H., Nohara, K., Kobayashi, T. *et al.* 2002. Effect of a single oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on immune function in male NC/Nga mice. *Toxicol. Sci.* 66:117.
27 Inouye, K., Pan, X., Imai, N. *et al.* 2005. T cell-derived IL-5 production is a sensitive target of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Chemosphere* 60:907.
28 Nagai, H., Takei, T., Tohyama, C., Kubo, M., Abe, R. and Nohara, K. 2005. Search for the target genes involved in the suppression of antibody production by TCDD in C57BL/6 mice. *Int. Immunopharmacol.* 5:331.
29 Suzuki, T. and Nohara, K. 2007. Regulatory factors involved in species-specific modulation of arylhydrocarbon receptor (AhR)-dependent gene expression in humans and mice. *J. Biochem.* 142:443.
30 Doi, H., Baba, T., Tohyama, C. and Nohara, K. 2003. Functional activation of arylhydrocarbon receptor (AhR) in primary T cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Chemosphere* 52:655.
31 Nohara, K., Ao, K., Miyamoto, Y. *et al.* 2008. Arsenite-induced thymus atrophy is mediated by cell cycle arrest: a characteristic down-regulation of E2F-related genes revealed by a microarray approach. *Toxicol. Sci.* 101:226.
32 Okey, A. B., Vella, L. M. and Harper, P. A. 1989. Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P1-450 by 3-methylcholanthrene. *Mol. Pharmacol.* 35:823.
33 Ema, M., Ohe, N., Suzuki, M. *et al.* 1994. Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. *J. Biol. Chem.* 269:27337.
34 Ito, T., Tsukumo, S., Suzuki, N. *et al.* 2004. A constitutively active arylhydrocarbon receptor induces growth inhibition of Jurkat T cells through changes in the expression of genes related to apoptosis and cell cycle arrest. *J. Biol. Chem.* 279:25204.
35 Baba, T., Mimura, J., Gradin, K. *et al.* 2001. Structure and expression of the Ah receptor repressor gene. *J. Biol. Chem.* 276:33101.
36 Tsuchiya, Y., Nakajima, M. and Yokoi, T. 2003. Critical enhancer region to which AhR/ARNT and Sp1 bind in the human CYP1B1 gene. *J. Biochem.* 133:583.
37 Zhang, L., Zheng, W. and Jefcoate, C. R. 2003. Ah rëceptor regulation of mouse Cyp1B1 is additionally modulated by a second novel complex that forms at two AhR response elements. *Toxicol. Appl. Pharmacol.* 192:174.
38 Svensson, C., Silverstone, A. E., Lai, Z.-W. and Lundberg, K. 2002. Dioxin-induced adseverin expression in the mouse thymus is strictly regulated and dependent on the aryl hydrocarbon receptor. *Biochem. Biophys. Res. Commun.* 291:1194.
39 Schoenborn, J. R. and Wilson, C. B. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv. Immunol.* 96:41.
40 Kimura, A., Naka, T. and Kishimoto, T. 2007. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc. Natl Acad. Sci. USA* 104: 12099.
41 Allan, L. L. and Sherr, D. H. 2005. Constitutive activation and environmental chemical induction of the aryl hydrocarbon receptor/transcription factor in activated human B lymphocytes. *Mol. Pharmacol.* 67:1740.
42 Marlowe, J. L. and Puga, A. 2005. Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J. Cell. Biochem.* 96:1174.
43 Haarmann-Stemmann, T., Bothe, H. and Abel, J. 2009. Growth factors, cytokines and their receptors as downstream targets of arylhydrocarbon receptor (AhR) signaling pathways. *Biochem. Pharmacol.* 77:508.
44 Matsumura, F. 2009. The significance of the nongenomic pathway in mediating inflammatory signaling of the dioxin-activated Ah receptor to cause toxic effects. *Biochem. Pharmacol.* 77:608.
45 Ohtake, F., Takeyama, K., Matsumoto, T. *et al.* 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* 423:545.
46 Ohtake, F., Baba, A., Takada, I. *et al.* 2007. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* 446:562.
47 Richard, H., Longhi, M. P., Wright, K., Gallimore, A. and Ager, A. 2007. CD62L (L-selectin) down-regulation does not affect memory T cell distribution but failure to shed compromises anti-viral immunity. *J. Immunol.* 180:198.

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G-protein signalling negatively regulates the stability of aryl hydrocarbon receptor

Asuka Nakata[1], Daisuke Urano[1], Yoshiaki Fujii-Kuriyama[2], Norikazu Mizuno[1], Kenji Tago[1+] & Hiroshi Itoh[1]

[1]Department of Cell Biology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Takayama, Ikoma, Nara, Japan, and [2]Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

Aryl hydrocarbon receptor (AhR) is a transcription factor that works as a dioxin receptor and is also involved in various physiological phenomena, including development and cell proliferation. Here, we show that the $G\alpha_{13}$ signal destabilizes AhR by promoting the ubiquitination of AhR. $G\alpha_{13}$ interacts directly with AhR-interacting protein (AIP) and inhibits the interaction between AhR and AIP, a crucial interacting protein of AhR. Strikingly, a reporter gene assay and a quantitative reverse transcription–PCR analysis indicate that the $G\alpha_{13}$ signal shows a potent inhibitory effect on the ligand-induced transcriptional activation of AhR. $G\alpha_{13}$ results in the nuclear translocation of AhR in a ligand-independent manner. However, in the presence of active $G\alpha_{13}$, AhR fails to form the active transcriptional complex. Taken together, we propose a new negative regulation of dioxin signalling by the G protein.

Keywords: dioxin receptor; G protein; ubiquitin

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INTRODUCTION

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the bHLH-PAS (basic helix–loop–helix PER/Arnt/Sim) superfamily (Schmidt & Bradfield, 1996; Mimura & Fujii-Kuriyama, 2003). AhR is broadly expressed in various tissues and is involved both in diverse responses to dioxin and in female reproduction by regulating the expression of aromatase in ovarian cells (Baba *et al*, 2005).

In the absence of stimulation, AhR is localized in the cytoplasm and associates with two molecules of the molecular chaperone heat-shock protein 90 (HSP90), the co-chaperone p23 and the immunophilin-like protein AIP (AhR-interacting protein, also known as XAP2 or Ara9; Mimura & Fujii-Kuriyama, 2003). On ligand binding, AhR undergoes a conformational change and translocates into the nucleus. In the nucleus, AhR binds to Arnt and forms a transcriptionally active complex. AhR in the active complex binds to xenobiotic response element (XRE) in the promoter region of the target genes, which include many drug-metabolizing enzymes, such as P450/CYP1A (Fujii-Kuriyama & Mimura, 2005). At present, the endogenous ligand for AhR remains unknown; however, the transcriptional activity of AhR can be stimulated by various xenobiotic compounds, including 2,3,7,8-TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and 3-MC (3-methyl cholanthrene).

AIP facilitates the activation of AhR and contributes to the maintenance of AhR in the cytoplasm. The amino-terminal part of AIP contains regions that have homology with FK506-binding protein 12 (FKBP12) and FKBP52, but AIP does not bind to FK506 (Fig 1A). The carboxy-terminal part of AIP contains three TPR (tetratricopeptide repeat) motifs, which are involved in protein–protein interactions. AIP protects AhR from ubiquitination, resulting in stabilization (Kazlauskas *et al*, 2000; LaPres *et al*, 2000). AIP also regulates the subcellular localization of AhR, indicating that AIP is crucial in AhR signalling. It has been reported that AIP binds to other nuclear receptors, including peroxisome proliferator-activated receptor-α and thyroid hormone receptor-β1, and affects their transcriptional activity (Sumanasekera *et al*, 2003; Froidevaux *et al*, 2006). These reports indicate that AIP takes part in various nuclear receptor signalling pathways.

Heterotrimeric G proteins are composed of three subunits, α, β and γ (Gα, Gβ and Gγ), and function as molecular switches that turn on intracellular signalling cascades in response to the activation of G-protein-coupled receptors (GPCRs) by extracellular stimulation including sensory signals, hormones, neurotransmitters and chemokines in mammalian cells (Gilman, 1987; Kaziro *et al*, 1991). G proteins are typically characterized into four main classes on the basis of the primary sequence similarity of the Gα-subunits: G_s, G_i, G_q and G_{12}. Among them, the two members of the G_{12} family, G_{12} and G_{13}, have been reported to interact directly with p115RhoGEF, Na^+-H^+ exchanger, radixin, cadherin and protein phosphatase 5 (Kurose, 2003). The α-subunits of G_{12}

[1]Department of Cell Biology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan
[2]Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
+Corresponding author. Tel: +81 743 72 5444; Fax: +81 743 72 5449;
E-mail: ktago@bs.naist.jp

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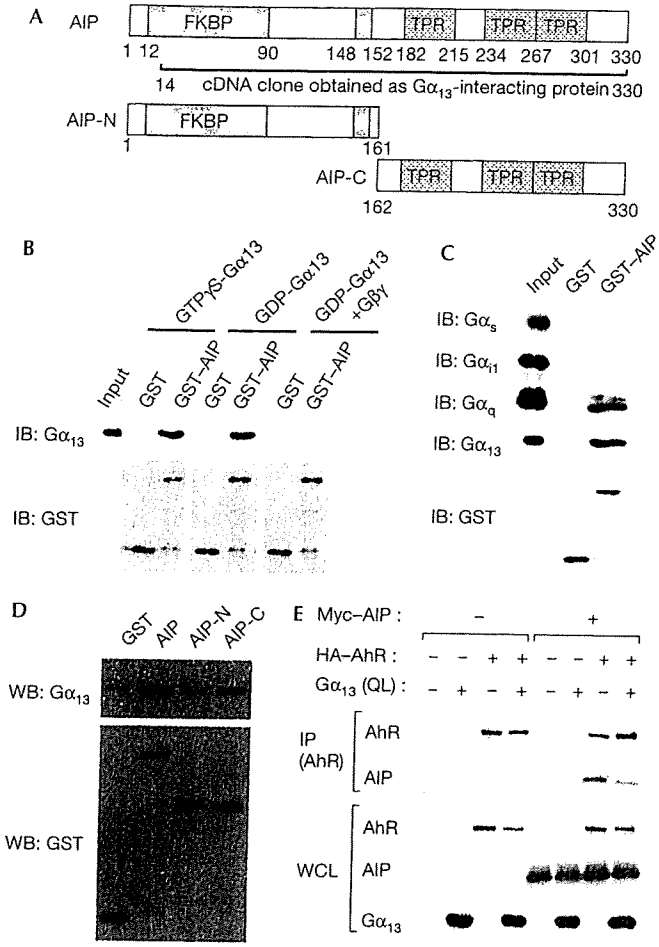

Fig 1 | AIP is identified as a $G\alpha_{13}$-interacting protein. (A) AIP contains the FKBP domain and three TPR motifs. The AIP fragment shown was isolated by a yeast two-hybrid screen. The amino- and carboxy-terminal fragments of AIP used in subsequent experiments are also shown. (B) Recombinant $G\alpha_{13}$ was treated with 10 μM GTPγS, 100 μM GDP or GDP plus Gβγ, and then mixed with GST or GST–AIP. The interaction between $G\alpha_{13}$ and AIP was detected by immunoblot (IB). (C,D) The interaction between a series of Gα subunits and GST–AIP or its mutants (illustrated in (A)) was analysed by the same procedures as in (B). (E) The whole-cell lysate (WCL) of HEK293T cells transfected as indicated was used for immunoprecipitation with the HA antibody. Experiments were performed three times, and similar results were observed. AIP, AhR-interacting protein; FKBP, FK506-binding protein; GST, glutathione *S*-transferase; HA, haemagglutinin; HEK, human embryonic kidney; TPR, tetratricopeptide repeat.

and G_{13} ($G\alpha_{12}$ and $G\alpha_{13}$) are ubiquitously expressed and coupled to the receptors, which respond to lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) and thrombin. Although the primary structure of $G\alpha_{12}$ and $G\alpha_{13}$ shows 67% similarity, the physiological roles of $G\alpha_{12}$ and $G\alpha_{13}$ seem to be different, as only $G\alpha_{13}$-deficient mice show the embryonic lethal phenotype and $G\alpha_{12}$-deficient mice do not.

In the course of our study to identify the new downstream effectors of $G\alpha_{13}$, we found that $G\alpha_{13}$ signalling represses AhR-mediated transcription by affecting the localization and stability of AhR. We propose the new concept that AhR is negatively regulated by G-protein signalling.

RESULTS AND DISCUSSION

$G\alpha_{13}$ interacts with AIP

To identify new $G\alpha_{13}$-interacting proteins, we performed a yeast two-hybrid screen by using $G\alpha_{13}$Q226L, a mutant lacking GTPase activity, as bait. From the mouse fetal brain cDNA library, we obtained two clones, both of which encoded AIP (Fig 1A). To confirm the interaction between $G\alpha_{13}$ and AIP, we prepared recombinant proteins and then performed *in vitro* pull-down analysis. As shown in Fig 1B, we observed that both the GDP and GTP forms of $G\alpha_{13}$ interacted comparably with AIP, suggesting that the interaction between $G\alpha_{13}$ and AIP is independent of GDP/GTP-binding status. Next, we tested the effect of the Gβγ-subunit on the association between $G\alpha_{13}$ and AIP. The addition of Gβγ effectively abolished the interaction between $G\alpha_{13}$ and AIP, suggesting that the dissociation of $G\alpha_{13}$ from Gβγ seems to be required for the formation of the $G\alpha_{13}$–AIP complex. Also, we tested the ability of other Gα-subunits—$G\alpha_s$, $G\alpha_{i1}$ and $G\alpha_q$—to interact with AIP. As shown in Fig 1C, $G\alpha_q$ showed less binding than did $G\alpha_{13}$; however, $G\alpha_s$ and $G\alpha_{i1}$ failed to bind to AIP. As shown in Fig 1A, the C-terminus of AIP contains three TPR motifs, which are involved in protein–protein interactions (Blatch & Lässle, 1999). Some proteins containing TPR motifs, such as protein phosphatase 5 and TPR1, interact with heterotrimeric G protein through their TPR motifs (Yamaguchi *et al*, 2002; Marty *et al*, 2003). To determine the region of AIP that binds to $G\alpha_{13}$, we prepared deletion mutants of AIP and used them for an *in vitro* binding assay. As shown in Fig 1D, $G\alpha_{13}$ could interact with both the N- and C-terminal portions of AIP. It has been reported that the TPR motifs of AIP are involved in the association of AIP and AhR (Meyer *et al*, 2000), and that $G\alpha_{13}$ also showed the ability to bind to the C terminus of AIP, suggesting that $G\alpha_{13}$ might physically disturb the interaction of AhR with AIP by competition of the TPR motifs of AIP. We tested whether $G\alpha_{13}$ counteracts the complex formation of AIP with AhR. Using human embryonic kidney 293T (HEK293T) cells expressing AIP, AhR and/or $G\alpha_{13}$Q226L, immunoprecipitation analysis was performed. As reported previously (Carver & Bradfield, 1997), AIP interacted with AhR. However, the coexpression of $G\alpha_{13}$Q226L suppressed the interaction between AIP and AhR (Fig 1E). AIP has been reported to form a complex with AhR and HSP90 in the cytoplasm, and this complex formation is necessary for the ligand-mediated activation of AhR (Meyer *et al*, 1998). Our data raise the interesting possibility that $G\alpha_{13}$ might affect the ligand-mediated activation of AhR.

Activation of $G\alpha_{13}$ inhibits AhR-mediated transcription

To examine whether $G\alpha_{13}$ affects the ligand-dependent activation of AhR, we performed an XRE-driven luciferase reporter gene assay. When AhR and AIP were exogenously expressed in COS-7 cells, 3-MC induced the AhR-dependent activation of XRE, as shown in Fig 2A. Interestingly, the expression of $G\alpha_{13}$Q226L effectively suppressed the 3-MC-induced XRE activation. Next, we examined whether a small GTP-binding protein, Rho, is involved in the inhibition of AhR by $G\alpha_{13}$, as $G\alpha_{13}$ induces the activation of Rho through p115RhoGEF. An active mutant of RhoA, RhoA(G14V), failed to affect the 3-MC-induced luciferase activity