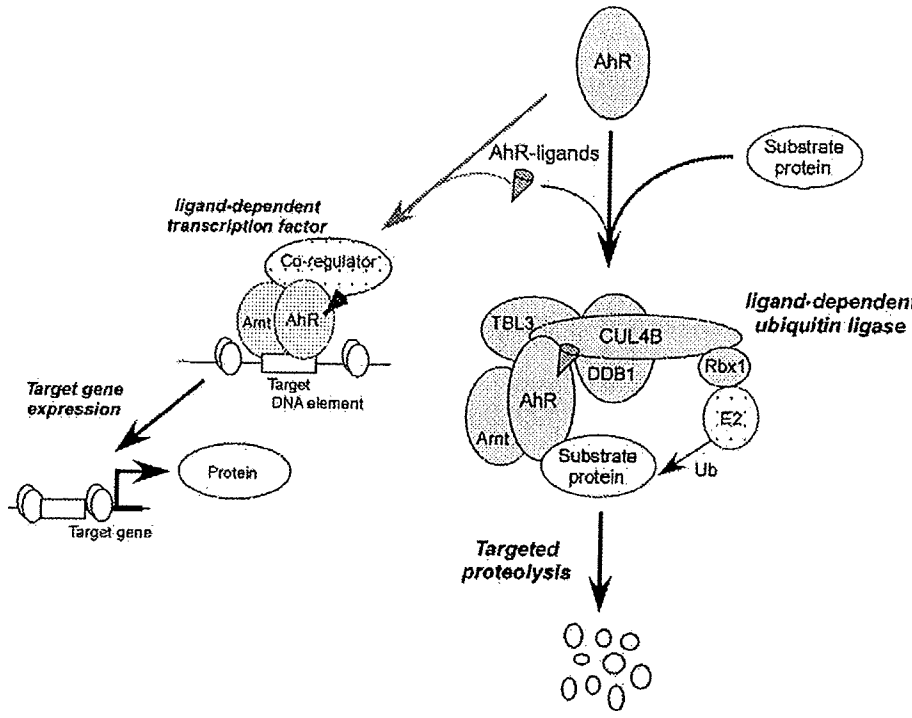

Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, $CUL4B^{AhR}$, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text for details). DDB1, damaged-DNA-binding protein 1; TBL3, transducin-beta-like 3.

tion and degradation has been demonstrated in knock-down experiments. Degradation of ERα or AR in the uterus and prostate is inducible by treatment with AhR ligands. Such degradation of ERα or AR is not seen in AhR-deficient mice [10,33]. This confirms that the AhR has E3 ubiquitin ligase activity *in vivo*. The anti-estrogenic effects of AhR ligands on estrogen-dependent uterine cell proliferation [14] appear to be mediated by the E3 ubiquitin ligase activity of AhR.

3.3. *Perspectives on the E3 ubiquitin ligase activity of AhR in cross-talk pathways*

Although it is well established that AhR is a key factor in mediating the adverse effects of dioxin-type compounds [8–10], the underlying mechanisms for this remain elusive. The putative functions of the previously identified target genes for AhR appear unlikely to fully explain the diverse range of biological actions of AhR ligands [11] (Fig. 1). The discovery of $CUL4B^{AhR}$ suggests that the adverse effects of AhR ligands in sex hormone signaling are, at least in part, attributable to the enhanced degradation of sex steroid receptors through E3 ubiquitin ligase activity of AhR [18,19] (Fig. 5). Target selectivity of the transcriptional regulatory system and the ubiquitin–proteasome system depends on specificity conferred by sequence-specific transcription factors and E3 ubiquitin ligases. To date, however, no single factor has been shown to function as a specificity factor in both target selection systems. Therefore, AhR is the first sequence-specific transcription factor identified that acts as an E3 ubiquitin ligase that also targets substrates for accelerated protein degradation. It is possible that other transcription factors, such as nuclear receptors, also function as E3 ubiquitin ligase components in some cellular contexts. Fat-soluble ligands for nuclear receptors are reported to have 'non-genomic' actions independent of transcriptional regulation-mediated effects. Considered together, ubiquitin ligase-based signaling mechanisms may possibly be involved in these non-genomic actions of various fat-soluble ligands.

From a mechanical point of view, AhR appears to be a unique and atypical type of substrate-specific component in cullin-based E3 complexes. AhR does not bear the reported signature motifs such as F-box [39], but directly associates with CUL4B. Substrate recognition by the other substrate-specific components in ubiquitin ligase complexes is usually evoked by substrate modifications such as phosphorylation [38–41] and hydroxylation [43,44,58]. However, recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type ligands, and does not occur following normal modifications of sex steroid receptors. Thus, it is plausible that activation of atypical E3 complexes may be a strategy of sensors for environmental stresses to respond to these stresses (Fig. 6). Supporting this, Hsp70 acts as an atypical substrate-specific adapter within the CHIP E3 complex in response to heat shock stress [63]. Hsp70 interacts with misfolded proteins and promotes their degradation. It later undergoes auto-catalytic degradation through CHIP [63]. In response to DNA damage, an atypical E3 complex alters the stability of TIP60, which in turn regulates ataxia-telangiectasia

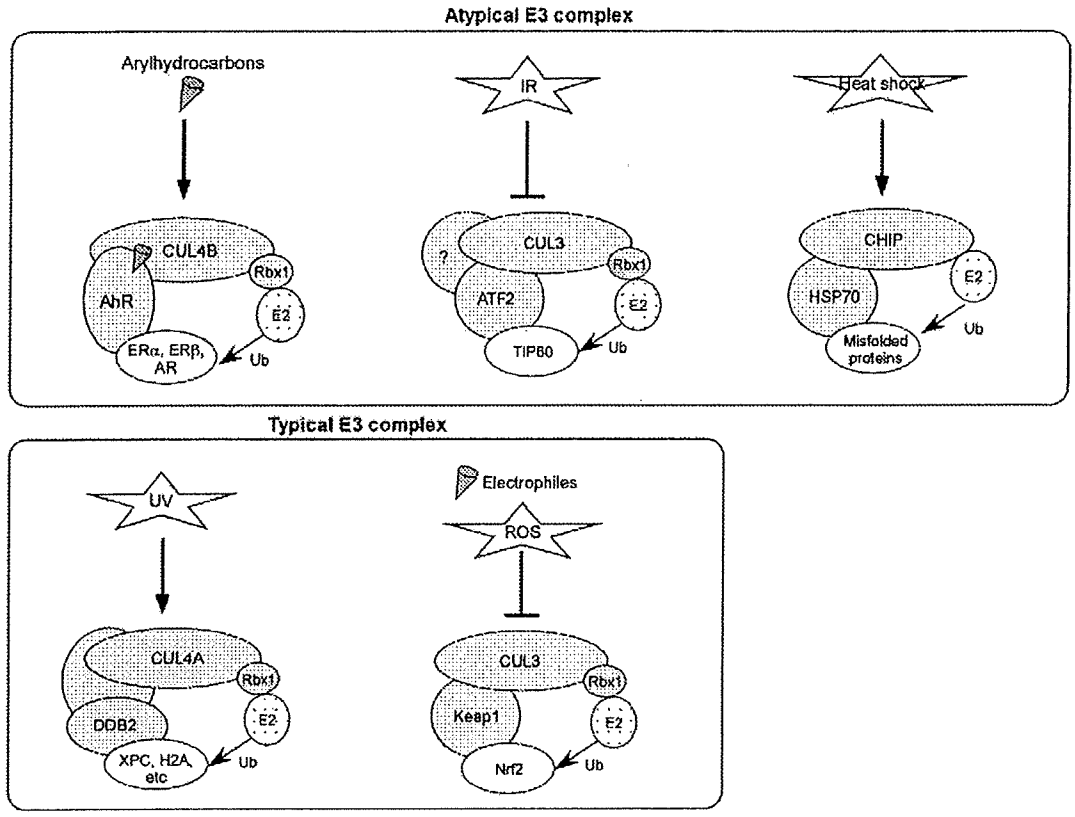

Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components with conserved signature motif act as signal-responsive factors. ATF2, activating transcription factor-2; TIP60, tat interactive protein 60; CHIP, C-terminus of Hsp70 interacting protein; Hsp70, heat shock protein 70; XPC, xeroderma pigmentosum group C; H2A, histone H2A; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; IR, ionizing radiation; ROS, reactive oxygen species.

mutated (ATM) activation in DNA repair [64]. Activating transcription factor-2 (ATF2) promotes the degradation of TIP60 by assembling a CUL3-based complex under non-stressed conditions. ATF2 dissociates from TIP60 in response to ionizing radiation (IR), resulting in enhanced TIP60 stability and activity [64]. Functional regulation of E3 components is also seen with the CUL3-based component Keap1 in the oxidative stress response [65], and CUL4A-based components DDB2 and CSA in the DNA damage response [61]. Considered together, E3 components that respond to environmental stress may be more diverse than initially believed (Fig. 6). It is possible that $CUL4B^{AhR}$ may cross-talk with these stress-responsive E3 ligases to modulate their functions. As WDXR/DWD motif containing components, including DDB2 and CSA, also bind to CUL4B [46], it is possible that AhR may associate or interfere with these CRL subunits.

The E3 ubiquitin ligase activity of AhR and the transcriptional activity of AhR appear to be responsible for a distinct set of biological events induced by AhR ligands (Fig. 5). As substrate-specific adapters of ubiquitin ligase complexes are capable of recognizing a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins may reveal new molecular links between AhR-mediated signaling and other signaling pathways and cellular events. In this regard, it is of interest that AhR interacts with various transcription factors [11], such as Rb/E2F1 [66], SF1/Ad4BP [33], and NF-κB [67], to modulate their functions. AhR has recently been shown to regulate the differentiation of Th17 and T_{reg} cells [68–70]. This may be mediated by a functional interaction with STAT1 [70]. In addition, although the underlying mechanisms remain unknown, AhR also modulates the function of transcription factors [71] such as GR and RAR [72,73]. Considering the evolutionary conservation of AhR, it is likely that the intrinsic function of AhR is to mediate the signal transduction of endogenous ligands in cross-talk pathways. A current area of interest is the identification of candidate degradation substrates for AhR which are abnormally stabilized in AhR-deficient mice. In summary, several lines of recent evidence define a novel role for AhR as a ligand-dependent E3 ubiquitin ligase to regulate target-specific protein destruction. The ubiquitin ligase activity of AhR, together with the cross-talk of AhR with nuclear receptors through direct association, provides an additional layer of complexity for AhR biology. Characterization of these new molecular aspects of AhR function may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR ligands.

Conflict of interest

The authors declare no competing financial interests.

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Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells

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IL-17-producing T helper cells (Th17) have been recently identified as a previously undescribed subset of helper T cells. Here, we demonstrate that aryl hydrocarbon receptor (Ahr) has an important regulatory function in the commitment of Th17 cells. Ahr was robustly induced under Th17-polarizing conditions. Ahr-deficient naïve T cells showed a considerable loss in the ability to differentiate into Th17 cells when induced by TGF-β plus IL-6. We were able to demonstrate that Ahr interacts with Stat1 and Stat5, which negatively regulate Th17 development. Whereas Stat1 activation returned to its basal level in Ahr wild type naïve T cells 24 h after stimulation with TGF-β plus IL-6, Stat1 remained activated in Ahr-deficient naïve T cells after stimulation. These results indicate that Ahr participates in Th17 cell differentiation through regulating Stat1 activation, a finding that constitutes additional mechanisms in the modulation of Th17 cell development.

dioxin receptor | IL-17 | IL-6 | ROR | regulatory T cells

Interleukin 17 (IL-17)-producing T helper cells (Th17) are a new subset of T helper cells. It has been demonstrated that these Th17 cells are associated with autoimmune conditions, such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) (1–3). Th17 differentiation is regulated by various cytokines. Th17 differentiation was induced by TGF-β and IL-6 in mice, and IL-1β but not TGF-β, has been shown to participate in the development of Th17 cells together with IL-6 in humans (2, 4). The development of Th17 cells is regulated negatively by IFN-γ, IL-27, and IL-2, the signals of which are dependent on Stat1 (IFN-γ and IL-27) and Stat5 (IL-2), respectively (5–7). The orphan nuclear receptors, retinoid-related orphan receptor γ (RORγ) and RORα, have been identified as the key transcription factors that determine the differentiation of Th17 lineage (8, 9). More recently, two groups have reported that the aryl hydrocarbon receptor (Ahr) activated by its ligand regulates Treg and Th17 cell development (10, 11). However, it is not clear how Ahr participates in the development of Th17 cells. In this paper, we demonstrate that Ahr is involved in the differentiation of Th17 cells by regulating Stat1 activation, which suppresses Th17 cell differentiation, under Th17-polarizing conditions.

Ahr, also known as dioxin receptor, is a ligand-activated transcription factor that belongs to the basic-helix–loop–helix-PER-ARNT-SIM family (12, 13). Ahr is present in the cytoplasm, where it forms a complex with heat shock protein (HSP) 90, Ahr-interacting protein (AIP), and p23 (14–16). Upon binding with a ligand, Ahr undergoes a conformation change, translocates to the nucleus, and dimerizes with Ahr nuclear translocator (Arnt). Within the nucleus, the Ahr/Arnt heterodimer binds to a specific sequence, designated as the xenobiotic responsive element (XRE), which causes a variety of toxicological effects (17–20). Interestingly, it has been recently reported that Ahr is a ligand-dependent E3 ubiquitin ligase (21), implying that Ahr has dual functions in controlling intracellular protein levels, serving both as a transcriptional factor to promote the induction of target proteins and as a ligand-dependent E3 ubiquitin ligase to regulate selective protein degradation. It has been reported that Ahr activated by ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) regulates the generation of regulatory T cells (Treg) and modulates the Th1/Th2 balance (22, 23). However, little is known about the molecular mechanism of how Ahr is involved in immune regulation. In this study, we demonstrated that Ahr induced by IL-6 and TGF-β, as well as ligand-activated Ahr, participates in Th17 cell differentiation and acts as a regulator of Stat1 activation under Th17-inducing conditions.

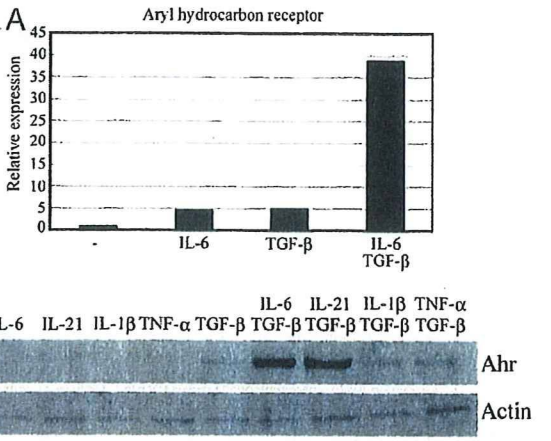

Fig. 1. Ahr is specifically expressed in Th17 cells. Isolated naïve T cells were cultured with anti-CD3/CD28 beads and the indicated cytokines for 2 days. (*A*) Gene expression profiles in nonstimulated and stimulated naïve T cells were compared by DNA microarray. (*B*) The indicated cells were lysed and subjected to Western blot analysis for the expression of Ahr and actin. Data are from one representative of three experiments.

Results

Ahr Is Induced Under Th17-Polarizing Conditions. To identify as yet unknown factors that participate in the differentiation of Th17 cells, we first used a DNA microarray for naïve T cells stimulated with IL-6 and TGF-β added either alone or in combination. This gene expression profiling analysis demonstrated that Ahr was highly expressed in naïve T cells stimulated by TGF-β plus IL-6, but not by either of these alone (Fig. 1*A*). Next, we used Western

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The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0804231105/DCSupplemental.

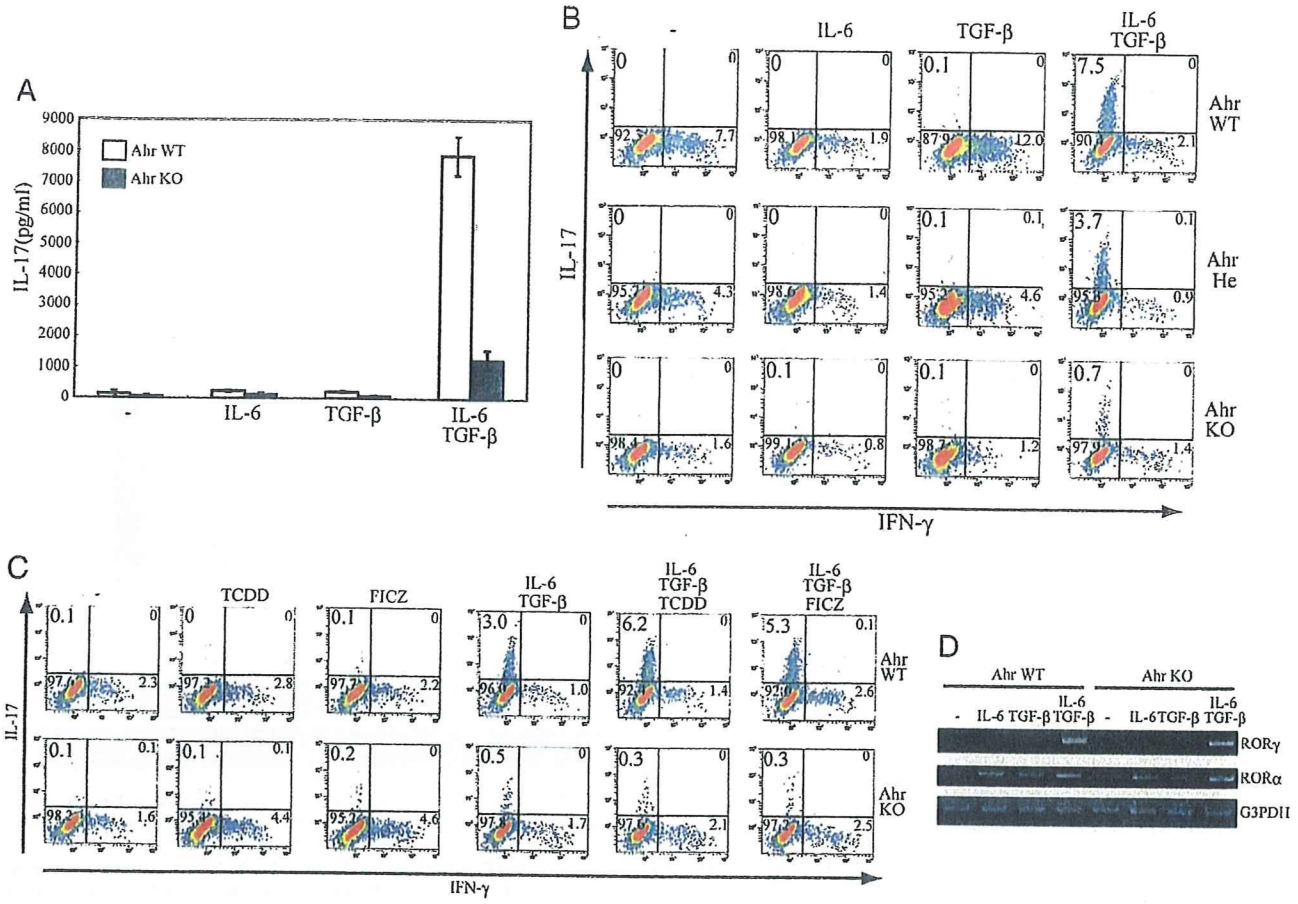

Fig. 2. Ahr deficiency reduces IL-17 production in naïve T cells. (*A*) Purified naïve T cells were stimulated with anti-CD3/CD28 beads in the presence of IL-6 or TGF-β, either alone or combined. Supernatants were collected 4 days after stimulation, and IL-17 production was measured by means of ELISA. Data show means ± SE of three independent experiments. (*B* and *C*) Dot plots show intracellular staining for IFN-γ and IL-17. (*B*) Isolated naïve T cells from Ahr WT, He, and KO splenocytes were cultured with anti-CD3/CD28 beads and the indicated cytokines for 4 days. (*C*) Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 in the presence or absence of TCDD or FICZ for 3 days. (*D*) Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and the indicated cytokines for 2 days. Total RNA and cDNA were prepared as described in Methods. RORγ and RORα induction was examined by using RT-PCR. (*B–D*) These results are representative of three independent experiments.

blot analysis to examine the expression of Ahr in naïve T cells under Th17-polarizing conditions. As shown in Fig. 1*B*, we confirmed the robust expression of Ahr under Th17-differentiating conditions. It has been reported that IL-21, like IL-6, also can initiate Th17 differentiation combined with TGF-β (24), and we also detected expression of Ahr induced by TGF-β plus IL-21 (Fig. 1*B*). Thus, Ahr is selectively induced under experimental conditions promoting Th17 cell development. However, other proinflammatory cytokines such as TNF-α and IL-1β did not induce Ahr expression even in combination with TGF-β.

Ahr Is Involved in the Differentiation of Th17 Cells. We next used Ahr deficient (KO) mice to examine whether Ahr indeed participates in Th17 development. Naïve T cells were isolated from Ahr WT and KO mice and stimulated by IL-6 or TGF-β alone or in combination. After stimulation, IL-17 production was measured with ELISA, and, as shown in Fig. 2*A*, the secretion of IL-17 was found to be drastically reduced in Ahr-deficient naïve T cells in comparison with WT naïve T cells under optimal conditions for Th17 cell development. Flow cytometry (FACS) analysis also revealed that Th17 cell differentiation was partially impaired in Ahr heterozygous (He) naïve T cells and significantly suppressed in Ahr KO naïve T cells in comparison with WT cells (Fig. 2*B*). Because TCDD (dioxin) and 6-formylindolo[3,2-b]carbazole (FICZ), which are exogenous and endogenous ligands, respectively, can bind and activate Ahr (10), we next investigated how these ligands influence Th17 cell development in Ahr WT and KO naïve T cells. TCDD or FICZ alone could not induce Th17 cell development, whereas their addition increased the percentage of IL-17-secreting cells induced by TGF-β plus IL-6 in WT cells (Fig. 2*C*). On the other hand, Ahr KO naïve T cells did not exhibit any increase in the generation of Th17 cells even in the presence of these ligands (Fig. 2*C*). Taken together, these data strongly indicate that Ahr is involved in Th17 development.

It has been reported that RORα and RORγ are required for the induction of Th17 cells (8, 9). We analyzed whether Ahr regulates their expression under Th17-polarizing conditions. Naïve T cells from Ahr WT and KO mice were stimulated with IL-6 and TGF-β, either alone or combined, followed by examination of RORα and RORγ induction by means of reverse transcriptase-PCR (RT-PCR). There was no difference in the induction of RORα and RORγ by TGF-β plus IL-6 between Ahr WT and KO naïve T cells (Fig. 2*D*). This suggests that the suppression of Th17 cell differentiation by Ahr deficiency is not because of its negative effect on the expression of RORα and RORγ.

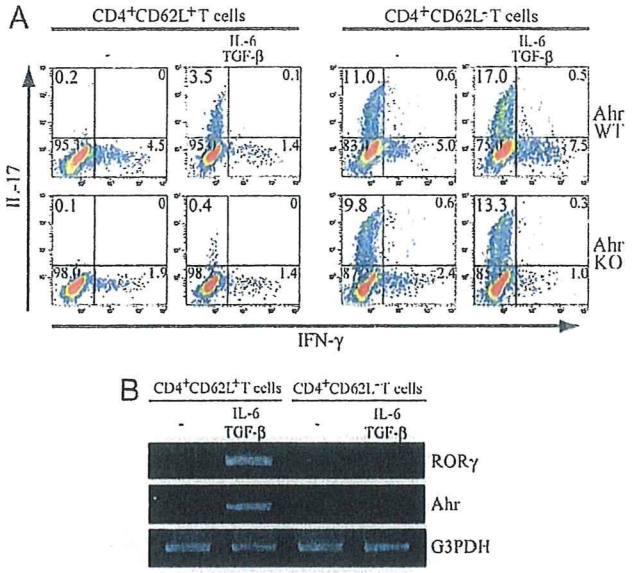

Fig. 3. Different pattern of IL-17 production between $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells. $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells isolated from WT mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6. (*A*) Three days after stimulation, cells were re-stimulated with PMA and ionomycin for 5 h and with GolgiStop (final 2 h), and then subjected to intracellular cytokine staining. Dot plots show intracellular staining for IFN-γ and IL-17. (*B*) Two days after stimulation, total RNA and cDNA were prepared as described in Methods. RORγ and Ahr induction was examined by using RT-PCR. These results are representative of three independent experiments.

IL-17 Is Produced in $CD4^+CD62L^-$ Cells Without TGF-β Plus IL-6 Treatment. In contrast to our results, a recently reported study found that $CD44^{lo}CD25^-CD4^+$ T cells from Ahr KO mice can differentiate into Th17 cells, but lack the expression of IL-22 (11). In our study, we separated $CD4^+$ T cells into $CD4^+CD62L^-$ (4–6% in the spleen cell population) and $CD4^+CD62L^+$ (15–20% in the spleen cell population) T cells and used $CD4^+CD62L^+$ T cells as naïve T cells. In contrast, Stockinger *et al.* used $CD4^+$ T cells including $CD62L^-$ fractions. We found that $CD4^+CD62L^-$ cells spontaneously produced IL-17 without TGF-β plus IL-6, and their addition promoted IL-17 production (Fig. 3*A*). Ahr and RORγ were not expressed in $CD4^+CD62L^-$ cells in the presence or absence of TGF-β plus IL-6 (Fig. 3*B*), suggesting that $CD4^+CD62L^-$ cells that produce IL-17 are distinct from a definitive Th17 cell subset. Additionally, even $CD4^+CD62L^-$ cells from Ahr KO mice could produce IL-17 with or without Th17-polarizing stimuli (Fig. 3*A*). These data collectively indicate that $CD4^+$ T cells, including $CD4^+CD62L^-$ cells, neither require Th17-polarizing stimuli nor the expression of Ahr and RORγ for IL-17 production.

Ahr Deficiency Partially Impairs Treg Development. Because Ahr was slightly induced by TGF-β alone (Fig. 1*B*), we investigated whether Ahr regulates the differentiation of Treg cells by TGF-β. We used FACS to measure Foxp3 expression in Ahr WT and KO naïve T cells stimulated by TGF-β. Compared with Ahr WT naïve T cells, Foxp3 induction was partially but significantly inhibited in Ahr KO naïve T cells (Fig. 4). Although TCDD or FICZ alone could not induce Foxp3 expression, its induction was enhanced when they were combined with TGF-β in WT cells, but not in Ahr KO cells (Fig. 4). Thus, Ahr participates in the generation of Treg cells.

Ahr Participates in Th17 Cell Development by Regulating Stat1. It was previously reported that the Stat family is essential for Th17 development, and that RORα and RORγ are induced in a Stat3-dependent manner by treatment with IL-6 and TGF-β (6, 25). On the other hand, Stat1 activation induced by IFN-γ or IL-27 inhibits Th17 polarization (5–7). Moreover, it has been demonstrated that IL-2 signaling interferes with Th17 differentiation through the activation of Stat5. Consistent with these findings, we previously reported that the combination of IL-6 and TGF-β could maintain activation of Stat3, but not of Stat1, 24 h after stimulation and that the suppressive effect of IL-27 and IFN-γ on the induction of Th17 cells is exerted through the maintenance and prolongation of Stat1 activation after IL-6 and TGF-β stimulation (26). In the current study, we investigated the relationship between Ahr induction and Stat regulation to gain a better understanding of the role of Ahr in Th17 cell differentiation. We first examined whether Ahr would bind with members of the Stat family under Th17-polarlizing conditions. Naïve T cells were stimulated with IL-6, TGF-β, or TGF-β plus IL-6, and the interaction between Ahr and the Stat family members was measured with the aid of immunoprecipitation and Western blotting. The results demonstrated that Ahr interacted with Stat1 and Stat5, but not with either Stat3 or Stat6 (Fig. 5*A*). We speculated that Ahr might participate in Th17 cell development by regulating Stat1 and Stat5. To validate this hypothesis, we next compared the inhibitory effect of IFN-γ on Th17 induction in Ahr WT and He naïve T cells, because it is known that IFN-γ serves to limit the generation of Th17 cells in a Stat1 activation-dependent manner. Because Th17 cell differentiation is significantly impaired in Ahr-deficient naïve T cells, it is not possible to examine the inhibitory effect of IFN-γ on Th17 development in Ahr-deficient naïve T cells. We, therefore, used Ahr-He naïve T cells to compare the inhibitory effect of IFN-γ with that in WT naïve T cells. As shown in Fig. 5*B*, IFN-γ suppressed Th17 cell

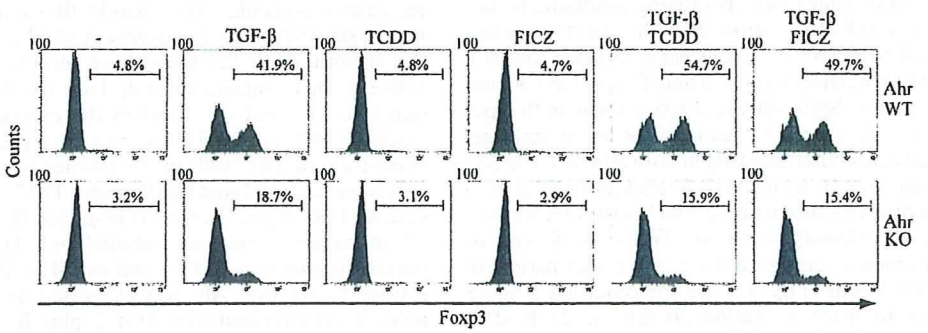

Fig. 4. Ahr partially participates in the generation of Treg cells by TGF-β. Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β with or without Ahr ligands for 2 days. Foxp3 expression was determined by staining with anti-mouse Foxp3 antibody. These data are representative of three independent experiments.

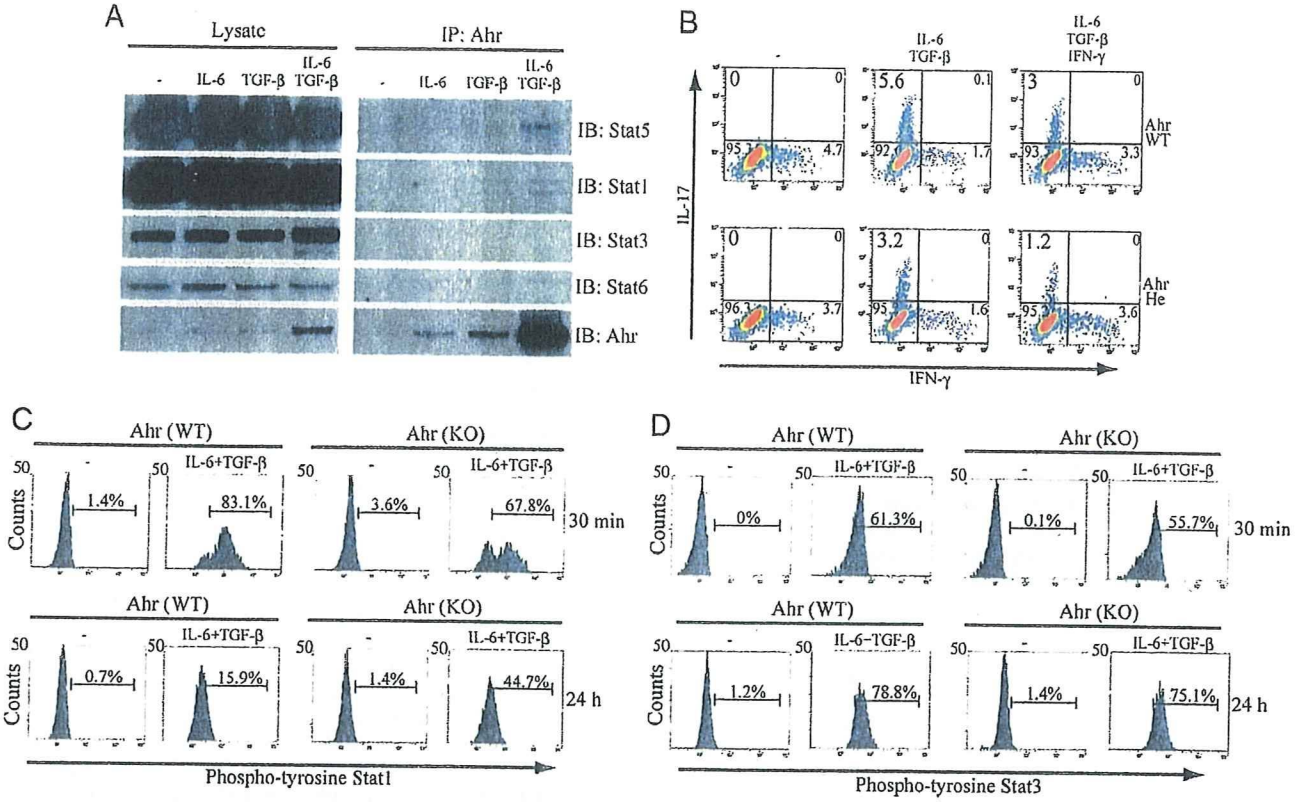

Fig. 5. Ahr regulates the activation of Stat1 in Th17 cell development. (*A*) MACS-sorted naïve T cells were cultured with anti-CD3/CD28 beads and stimulated with IL-6 or TGF-β, either alone or combined, for 2 days. Whole cell lysates were immunoprecipitated with anti-Ahr antibody, after which Stat1, Stat3, Stat5, Stat6, and Ahr were detected with Western blotting. IP, immunoprecipitation; IB, immunoblot. (*B*) Naïve T cells isolated from Ahr WT and He mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 in the presence or absence of IFN-γ for 3 days, followed by re-stimulation with PMA and ionomycin for 5 h and with GolgiStop (final 2 h), and then staining for intracellular cytokines. Dot plots show intracellular staining for IFN-γ and IL-17. (*C* and *D*) Naïve T cells isolated from Ahr WT and KO splenocytes were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6 for 30 min or 24 h, fixed and permeabilized in 90% methanol, and finally stained with Alexa Fluor 488-conjugated phospho-Stat1 and PE-conjugated phospho-Stat3. Intracellular levels of phospho-Stat1 (*C*) and Stat3 (*D*) were measured by means of flow cytometry. These results are representative of three independent experiments.

development to a higher degree in Ahr-He naïve T cells (inhibitory effect: 62.5%) than in WT cells (inhibitory effect: 46.4%). Given that IFN-γ inhibits the generation of Th17 cells via activation of Stat1, it is possible that the higher degree of inhibition of Th17 cell development by IFN-γ in Ahr-He naïve T cells is because of enhanced Stat1 activation compared to that in WT naïve T cells.

We previously reported that Stat3 remained activated under Th17-culturing conditions, whereas Stat1 activation was relatively transient and returned to the basal level during 24 h of the experimental period (26). In the current study, we compared the activation of these Stats under Th17-polarizing conditions in Ahr WT and KO naïve T cells to confirm that Ahr affects the state of the activation of Stats. Naïve T cells isolated from Ahr WT and KO mice were stimulated with IL-6 and TGF-β, and 30 min or 24 h after stimulation, Stat1 and Stat3 activation in both types of naïve T cells was measured by using intracellular staining. Stat1 was activated at a similar intensity in both Ahr WT and KO naïve T cells 30 min after IL-6 and TGF-β stimulation (Fig. 5*C*). Consistent with a previous finding (26), Stat1 activation was not maintained 24 h after stimulation in Ahr WT naïve T cells. In contrast, Stat1 remained activated 24 h after stimulation in Ahr-deficient naïve T cells (Fig. 5*C*). On the other hand, there was no difference in Stat3 activation 30 min or 24 h after stimulation between Ahr WT and KO naïve T cells (Fig. 5*D*). These results indicate that Ahr selectively regulates the activation of Stat1, but not of Stat3, under Th17-polarizing conditions.

Discussion

Th17 cells, known as a previoulsy undescribed lineage of Th cells, are associated with autoimmunity. Although it has been recently demonstrated that RORα and RORγ are key transcription factors in Th17 cells (8, 9), the mechanism of Th17 cell differentiation is not yet well understood. We previously demonstrated that IL-27 and IFN-γ suppressed the generation of Th17 cells without significant effects on the expression of RORγ (26). In this study, we confirmed that RORα, like RORγ, was expressed under Th17-polarizing conditions even in the presence of IL-27 or IFN-γ (data not shown). These results strongly suggest that regulatory molecules other than RORα and RORγ may play an important role in the development of Th17 cells. In support of this hypothesis, it has been more recently reported that Ahr, activated by its ligand, controls Treg and Th17 cell differentiation (10, 11), and we found in the current study that Ahr is markedly induced by TGF-β plus IL-6 and participates in the generation of Th17 cells in the absence of its exogenous ligand.

Ahr was induced specifically under Th17-polarizing conditions such as TGF-β plus IL-6 or TGF-β plus IL-21, but not by other inflammatory cytokines combined with TGF-β or under Th1-polarizing conditions (IL-12 and anti-IL-4) (data not shown). We further found that Ahr was expressed also in Stat1-deficient naïve T cells treated with TGF-β plus IL-6 [supporting information (SI) Fig. S1], indicating that Ahr induction is independent of Stat1. Although the exact molecular mechanism of Ahr expression in Th17 development is not clear at this point, Ahr

induction may be regulated downstream of Stat3 by IL-6 and TGF-β, similar to the induction of RORα and RORγ as reported elsewhere (8, 9). We demonstrated that Ahr deficiency significantly impaired Th17 development induced by IL-6 and TGF-β even though RORs are expressed, similar to the case of treatment with IL-27 and IFN-γ, which also indicated that Th17 development requires other regulatory mechanisms in addition to regulation by RORs. Recent studies have demonstrated that ligand-activated Ahr regulates Th17 cell development (10, 11). Stockinger *et al.* showed that $CD44^{lo}CD25^{-}CD4^{+}$ T cells from Ahr WT and KO mice can develop Th17 cells with TGF-β plus IL-6, whereas FICZ, one of the Ahr ligands, promotes the generation of Th17 cells induced by the combined usage of the two cytokines in Ahr WT $CD44^{lo}CD25^{-}CD4^{+}$ T cells, but not in Ahr KO $CD44^{lo}CD25^{-}CD4^{+}$ T cells (11). In our study, however, we could demonstrate that Th17 cell development is impaired in Ahr-deficient naïve T cells under Th17-polarizing conditions in either the presence or absence of Ahr ligands. We speculated that the reason for this discrepancy might be related to the difference in the sorted naïve T cell fractions used in the two studies. We have found that $CD4^{+}CD62L^{-}$ cells from Ahr WT and KO mice spontaneously produce IL-17 regardless of the presence or absence of TGF-β plus IL-6, despite the fact that neither Ahr nor RORγ was expressed in those cells. This may explain the discrepancies in our results and those of Stockinger *et al.*, because they used $CD4^{+}$ T cells including $CD4^{+}CD62L^{-}$ cells. Because effector memory $CD4^{+}$ T cells are characterized by $CD45RB^{low}CD44^{high}CD62L^{-}$, our isolated $CD4^{+}CD62L^{-}$ cells may belong to the effector memory $CD4^{+}$ T cell family. However, it is currently unknown whether effector memory $CD4^{+}$ T cells can produce IL-17 by anti-CD3 plus anti-CD28. Further analysis is required to develop the characteristics of this population in Th17 cell differentiation.

Th17 differentiation is positively regulated by IL-6 or IL-21 in combination with TGF-β and negatively regulated by IFN-γ or IL-27, which are controlled by Stat3 and Stat1, respectively (2, 5, 6, 7, 25). Given that Stat1 can bind with the IL-17 promoter and serve as a repressor (7), the maintenance of its activation may inhibit the interaction between ROR proteins and the IL-17 promoter by masking their binding sites. In our study, we found that Ahr binds to Stat1 and Stat5, but not to other tested members of the Stat family, raising the possibility that Ahr may regulate the generation of Th17 cells by modifying the activation of Stat1 and Stat5, which negatively regulate Th17 generation. Indeed, we found that Ahr deficiency prolonged Stat1 activation 24 h after stimulation with TGF-β plus IL-6, whereas its activation was relatively transient and returned to the basal level in WT naïve T cells during that period. On the other hand, Stat3 activation was maintained equally in both Ahr WT and KO naïve T cells. Consistent with the finding of a previous report (7), we confirmed that Th17 cell development is enhanced under Th17-polarizing conditions in the presence of neutralizing antibodies for IL-2 (data not shown), indicating that Th17 differentiation is inhibited by endogenous IL-2 secreted from naïve T cells cultured under Th17-polarizing conditions. Interaction of Ahr with Stat5 also leads us to speculate that Ahr downregulates the activation of Stat5 by IL-2 produced in naïve T cells through binding with Stat5, like Stat1, resulting in the induction of Th17 cells. At present, it is not yet understood how Ahr interacts with Stat1 and Stat5 and negatively regulates their activation in Th17 cell differentiation. It has been reported that nuclear receptors such as peroxisome proliferator-activated receptor γ (PPARγ) and estrogen receptor (ER) negatively modulate Stat3 activated by IL-6 (27). When PPARγ is activated by its ligand, the resultant PPARγ-ligand complex directly interacts with IL-6-activated Stat3 and suppresses its transcriptional activity. Although in our study, Ahr interacted with Stat1 independently of its ligand, there may be an as yet unidentified endogenous Ahr ligand that determines the interaction between Ahr and Stat1 (Stat5) in Th17 cell development by forming a complex with Ahr.

Ahr is known to have dual functions in controlling intracellular protein levels, serving both as a transcriptional factor and as a ligand-dependent E3 ubiquitin ligase (21). It also is possible that Ahr regulates the activation of Stat1 through the degradation of activated Stat1 by functioning as a ligand-dependent E3 ubiquitin ligase in the generation of Th17 cells.

At this point, we cannot exclude the possibility that Ahr may have mechanisms other than regulating the activation of Stat1 in Th17 cell differentiation. Therefore, it is important to determine the molecular basis of the interaction of Ahr with members of the Stat family and the regulation of their activation.

We were able to show that Treg induction by TGF-β was inhibited partially but significantly in Ahr-deficient naïve T cells. It has been reported that Treg differentiation is negatively regulated by IFN-γ in a Stat1-dependent manner (28). We confirmed that IFN-γ partially inhibits Treg cell development by TGF-β and that IFN-γ blocking by its neutralizing antibodies enhances Treg differentiation (Fig. S2), which suggests that the induction of Treg as well as of Th17 was disrupted under Stat1-activating conditions. Because Ahr can be slightly induced by TGF-β alone, it is expected that TGF-β–induced Ahr may regulate Treg development through the suppression of Stat1 activation by endogenous IFN-γ secreted from naïve T cells cultured under Treg-inducing conditions. We found that Treg induction by TGF-β was enhanced when Ahr was activated by TCDD or FICZ. However, Weiner *et al.* reported that FICZ inhibited Treg cell development by TGF-β, whereas Treg was induced by TCDD alone even in the absence of TGF-β (10), thus contradicting our data. At the present time, we cannot explain the reason for this major discrepancy between their results and ours.

In summary, our findings demonstrate that Ahr is essential for Th17 development through the interference of Stat1 activation under Th17-polarizing conditions. Because Ahr controls the activation of Stat1 by forming a previously undescribed complex, Ahr/Stat1, Ahr may be involved in various immune systems, including innate immunity, via Stat-dependent pathways.

Materials and Methods

Mice. C57BL/6 wild-type mice were obtained from CLEA Japan Inc., and Ahr KO mice on the C57BL/6 background were provided by Dr. Yoshiaki Fujii-Kuriyama (University of Tsukuba, Tsukuba, Japan). All mice were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience, Osaka University.

Isolation of Naïve T Cells and T Cell Differentiation. Naïve T cells were purified from spleens of C57BL/6, Ahr WT, He, or KO female mice by using the $CD4^{+}$ T cell Isolation Kit and CD62L MicroBeads (Miltenyi). Purified naïve T cells were stimulated with the Dynabeads Mouse CD3/CD28 T cell Expander (Invitrogen) for 3 days. As indicated, cultures were supplemented with recombinant cytokines: Mouse IL-6 (20 ng/ml; R&D Systems), mouse IL-21 (100 ng/ml; R&D Systems), mouse IL-1β (20 ng/ml; R&D Systems), mouse TNF-α (100 ng/ml; R&D Systems), or human TGF-β1 (2 ng/ml; R&D Systems), alone or combined. Additionally, recombinant mouse IFN-γ (20 ng/ml; R&D Systems), FICZ (100 nM; kindly donated by Dr. Yoshiaki Fujii-Kuriyama, University of Tsukuba), or TCDD (160 nM; Cerilliant) was added to some samples.

DNA Microarray. Naïve T cells were cultured with anti-CD3/CD28 beads and indicated cytokines for 2 days. cRNA was synthesized and hybridized to Murine Genome 430 2.0 microarray chips (Affymetrix). Microarray data were analyzed by Gene Spring (Agilent).

IL-17 ELISA. Naïve T cells purified from Ahr WT and KO splenocyte populations were stimulated with anti-CD3/CD28 beads and indicated cytokines. After 4 days, mouse IL-17 from the supernatants was measured by means of ELISA according to the manufacturer's instructions (R&D Systems).

Intracellular Cytokines and Foxp3 Staining. T cells were stimulated with 50 ng/ml PMA (Calbiochem), 800 ng/ml ionomycin (Calbiochem) for 5 h and GolgiStop (BD PharMingen) for the final 2 h, followed by fixation and permeabilization with Cytofix/Cytoperb (BD PharMingen). Cells were stained intracellularly with Phycoerythrin (PE)-conjugated anti-IL-17 (BD PharMingen) and FITC-labeled anti-IFN-γ (eBioscience). For Foxp3 staining, T cells were fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) for 30 min at 4°C before intracellular staining with FITC-conjugated anti-Foxp3 (eBioscience). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter).

Immunoprecipitation and Western Blotting. Purified naïve T cells were cultured with indicated cytokines for 2 days, and cells were lysed with a lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (ph 7.5), 150 mM NaCl, 10 mM Na_2VO_4, 0.5 mM DTT, and 1/100 protease inhibitor]. Ahr was immunoprecipitated with anti-Ahr (BIOMOL) and then subjected to SDS/PAGE. Whole cell lysates and the immunocomplex were analyzed with Western blotting by using anti-Stat1 (BD Transduction Laboratories), anti-Stat3 (BD Transduction Laboratories), anti-Stat5 (C-17; Santa Cruz Biotechnology), anti-Stat6 (BD Transduction Laboratories), or anti-Ahr (BIOMOL).

Reverse Transcriptase-PCR (RT-PCR). Total RNA was prepared by using RNeasy (Qiagen), and cDNA was prepared as described elsewhere (26). Reaction conditions consisted of a 45-s denaturation step at 94°C, a 30-s annealing step at 58°C, and a 30-s extension step at 72°C for 25 cycles (G3PDH), 35 cycles (RORγ), or 37 cycles (RORα). The specific primers were as follows: RORγ, sense 5′-GCGGAGCAGACACACTTACA-3′ and antisense 5′-TTGGCAAACTCCACCACATA-3′; RORα, sense 5′-AGTTTGGTCGGATGTCCAAG-3′ and antisense 5′-AGCTGCCACATCACCTCTCT-3′; G3PDH, sense 5′-TCCACCACCCTGTTGCTGTA-3′ and antisense 5′-ACCACAGTCCATGCCATCAC-3′.

Flow Cytometric Analysis of Phospho-Stat1 (Y701) and Phospho-Stat3 (Y705). Naïve T cells were cultured with TGF-β plus IL-6 for 30 min or 24 h. Cells were fixed with Fixation Buffer (BD PharMingen) for 10 min at 37°C and then permeabilized in 90% methanol for 30 min on ice. Cells were washed twice in Stain Buffer (BD PharMingen), and stained with Alexa Fluor 488-conjugated phospho-Stat1 (Y701) antibody or PE-conjugated phospho-Stat3 (Y705) antibody for 1 h at room temperature (BD PharMingen). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter).

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Benzene-induced hematopoietic toxicity transmitted by AhR in wild-type mouse and nullified by repopulation with AhR-deficient bone marrow cells: Time after benzene treatment and recovery

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ABSTRACT

Previously, we found an aryl hydrocarbon receptor (AhR)-transmitted benzene-induced hematotoxicity; that is, AhR-knockout (KO) mice did not show any hematotoxicity after benzene exposure [Yoon, B.I., Hirabayashi, Y., Kawasaki, Y., Kodama, Y., Kaneko, T., Kanno, J., Kim, D.Y., Fujii-Kuriyama, Y., Inoue, T., 2002. Aryl hydrocarbon receptor mediates benzene-induced hematotoxicity. Toxicol. Sci. 70, 150–156]. Furthermore, our preliminary study showed a significant attenuation of benzene-induced hematopoietic toxicity by AhR expression, when the bone marrow (BM) of mice was repopulated with AhR-KO BM cells [Hirabayashi, Y., Yoon, B.I., Li, G., Fujii-Kuriyama, Y., Kaneko, T., Kanno, J., Inoue, T., 2005a. Benzene-induced hematopoietic toxicity transmitted by AhR in the wild-type mouse was negated by repopulation of AhR deficient bone marrow cells. Organohalogen Comp. 67, 2280–2283]. In this study, benzene-induced hematotoxicity and its nullification by AhR-KO BM cells were further precisely reevaluated including the duration of the effect after benzene treatment and recovery after the cessation of exposure. Exposure routes, namely, intraperitoneal (*i.p.*) injection used in our previous study and intragastric (*i.g.*) administration used in this study, were also compared in terms of their toxicologic outcomes. From the results of this study, mice that had been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not show any benzene-induced hematotoxicity. The AhR-KO BM cells nullified benzene-induced toxicities in notably different hematopoietic endpoints between the *i.p.* treatment and the *i.g.* treatment; however, the number of granulo-macrophage colony-forming unit *in vitro* (CFU-GM) was a common target parameter, the benzene-induced toxicity of which was nullified by the AhR-KO BM cells.

1. Introduction

Recent studies have shown that the aryl hydrocarbon receptor (AhR) in primitive cells transmits negative signals for the proliferation of such cells (Hirabayashi et al., 2003; Garrett and Gasiewicz, 2005). This observation may require further detailed studies, because previous *in vitro* studies showed that AhR promotes cellular proliferation on one hand (Ma and Whitlock, 1996; Shimba et al., 2002), but rather suppress on the other hand (Fong et al., 2005). As we previously reported, AhR-knockout (KO) mice showed an increase in number of primitive hematopoietic progenitor cells; on the other hand, a decrease in number of relatively mature progenitor cells in a homeostatic manner (Hirabayashi et al., 2003). Therefore, there are two possibilities: one is the hierarchic positional effect of cellular differentiation and the other is a particular cell-proliferative gene alteration in *in vitro* cell lines.

We have reported that benzene-induced hematopoietic toxicity is transmitted by AhR (Yoon et al., 2002). We also found that cytochrome P450 2E1 (CYP2E1) that is, related to benzene metabolism is also up-regulated following benzene exposure in the bone marrow (BM) (Yoon et al., 2003). Therefore, it is of interest to hypothesize the important role of BM cells in hematopoietic toxicity with respect to AhR function. Accordingly, on the basis of the latest studies presented at the 25th International Dioxin Symposium, benzene-induced hematopoietic toxicity was evaluated in wild-type (Wt) mice after whole-body irradiation at a lethal dose followed by repopulation with BM cells that lack AhR or, *vice versa*, in AhR-KO mice after repopulation with Wt BM cells. As for the results, a one-day examination on day 12 after benzene exposure

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showed that the xenobiotic response of CYP2E1 up-regulation mediated by AhR for benzene hematotoxicity was metabolized specifically in the BM (Hirabayashi et al., 2005a). In this article, the detailed changes in blood parameters during the benzene exposure duration and their recovery three days after the cessation of exposure are shown.

2. Materials and methods

2.1. Animals

The establishment of homozygous AhR-KO ($AhR^{-/-}$) mice originating from the 129/SvJ strain has been described elsewhere (Mimura et al., 1997; Yoon et al., 2002). The crossing of heterozygous AhR-KO ($AhR^{+/-}$) males with $AhR^{+/-}$ females generated wild-type ($AhR^{+/+}$), $AhR^{+/-}$, and $AhR^{-/-}$mice. The neonates were genotyped by PCR screening of DNA from the tail. Male AhR-KO ($AhR^{-/-}$) mice and their Wt littermates (12 weeks old) were used in the study as donors. Eight-week-old C57BL/6 male mice from Japan SLC (Shizuoka, Japan) were used as recipients for the repopulation assay and the assay of colony-forming unit in the spleen (CFU-S). All the mice were housed under specific pathogen-free conditions at 24 ± 1 °C and 55 ± 10% relative humidity, under a 12-h light dark cycle. Autoclaved tap water and food pellets were provided *ad libitum*.

2.2. Benzene and benzene exposure

Benzene, CAS. No. 71-43-2, MW 78.11, was purchased from Wako Fine Chemical Company (Osaka, Japan). Experimental mice were intragastrically (*i.g.*) administered with freshly prepared corn oil solutions of benzene (150 mg/kg body weight) once daily for 5 days/week for 2 weeks). The dose used in this study was 150 mg/kg body weight which corresponds to the daily dose for leukemic induction, that is, 300 ppm for 6 h/day (Cronkite et al., 1984; Li et al., 2006). Both doses administered for 5 days/week for 26 weeks induce hematopoietic malignancies at the highest frequency. The aim of this study using this dose is to examine the corresponding toxicity of benzene for inducing hematopoietic malignancies. Note, this dose is over 100-fold higher than the occupational tolerable exposure dose.

2.3. Blood and BM parameters

Peripheral blood was collected from the orbital sinus. Peripheral blood leukocyte (WBC), red blood cell (RBC) and platelet (PLT) counts were determined using a blood cell counter (Sysmex K-4500, Sysmex Co., Kobe, Japan). BM cellularity was evaluated by harvesting BM cells from the femurs of each mouse (Yoon et al., 2001). The animals were sacrificed on days 5, 12, and 15 to evaluate recovery. Then, a 27-gauge needle was inserted into the femoral bone cavity through the proximal and distal edges of the bone shafts, and BM cells were flushed out under pressure by injecting 2 ml of α-MEM. A single-cell suspension was obtained by gently triturating the BM cells through the 27-gauge needle, and cells were counted using the Sysmex K-4500.

2.4. Irradiation

Recipient mice were exposed to a lethal radiation of 800.1 cGy, at a dose rate of 124 cGy/min, using a ^{137}Cs-gamma irradiator (Gammacell 40 Exactor, MDS Nordin Inc., Canada) with a 0.5 mm aluminum–copper filter.

2.5. CFU-S assay

The Till and McCulloch method (Till and McCulloch, 1961) was used for determining the number of colony-forming units in the spleen (CFU-S). Aliquots of BM cell suspensions were used for evaluating the number of CFU-S. The number of BM cells was adjusted to that appropriate for producing non-confluent spleen colonies, and the cells were then transplanted into lethally irradiated mice by injection through the tail vein. Spleens were harvested nine and 13 days after the injection, and fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inverted microscope at a magnification of 5.6×.

2.6. Assays for CFU-GM and CFU-E

Colony formation *in vitro* was assayed in a semisolid methylcellulose culture (Yoon et al., 2001; Hirabayashi et al., 2002a). Briefly, 8×10^4 BM cells suspended in 100 μl of a medium were added to 3.9 ml of a culture medium containing 0.8% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, with 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) for CFU-GM assay or 1 ng/ml murine interleukin-3 and 2 U/ml erythropoietin for erythroid CFU (CFU-E) assay. One-milliliter aliquots containing 2×10^4 BM cells were plated in triplicate in a 35-mm tissue culture plate, and incubated for six days in a completely humidified incubator at 37 °C with 5% CO_2 in air. Under an inverted microscope, CFU-GM after a six-day culture was counted at a magnification of 40× and CFU-E after a three-day culture at 100×.

2.7. BM repopulation assay

BM repopulation assay (Hirabayashi et al., 1992) was performed similarly to the assay of CFU-S, except that 1×10^6 BM cells were injected into lethally irradiated mice. One month after the transfusion of BM cells, the repopulated mice exposed to benzene.

3. Results and discussion

As previously reported, the AhR-KO mice showed a significantly higher WBC counts than the Wt mice (Fig. 1a). This was also consistent with the high number of myeloid progenitor cells, that is, CFU-S-9 and CFU-S-13, observed in the AhR-KO mice (Fig. 1b). Thus, steady-state hemopoiesis in the Wt mice, on the other hand, is presumed to be suppressed by AhR signaling because of the possible presence of a physiological ligand, which is not readily observed in the AhR-KO mice. In contrast, the numbers of mature progenitor cells in the BM, that is, CFU-E and CFU-GM, decreased (Fig. 1c). This is assumed to be due to a homeostatic negative regulation for the above-mentioned increase in the number of immature myeloid progenitor cells, although direct evidence for a possible feedback was not obtained. These lines of experimental evidences from the AhR-KO mice suggest that the numbers of mature progenitor cells, CFU-E, and CFU-GM in the Wt mice increase.

The assumption that immature progenitor cells in the Wt mice are suppressed by AhR signaling, however, is inconsistent with some reports that certain cell lines showed an enhanced proliferation in the presence of AhR signaling (Ma and Whitlock, 1996; Shimba et al., 2002). In the case of the A549 cell line, cells proliferated by AhR overexpression with the presence of ligands (Shimba et al., 2002). In the case of Hepa 1c1c7, the cell line, cell proliferation was suppressed with deceleration of cell cycling, when antisense AhR was introduced to the cell line (Ma and Whitlock, 1996). Our interpretation of these inconsistent reports is based

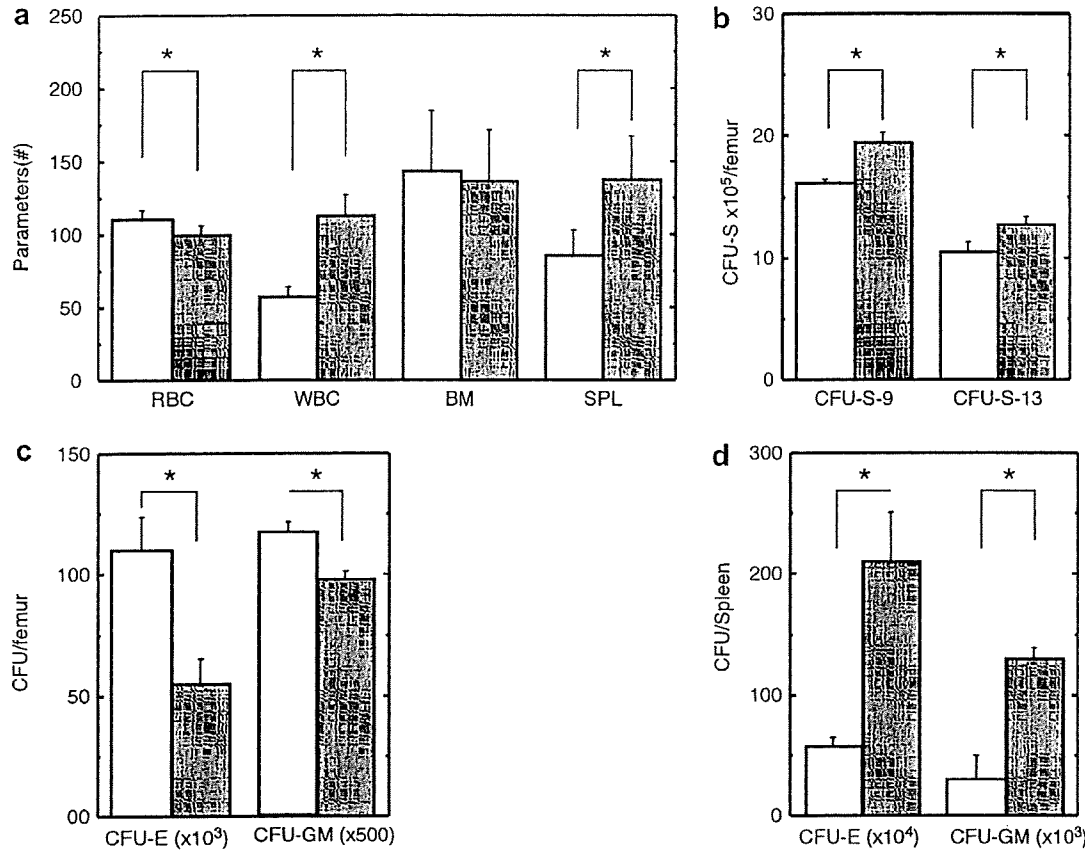

Fig. 1. Comparison of various blood parameters between Wt mice (open columns) and AhR-KO mice (shaded columns). (a) Peripheral blood, bone marrow (BM) and spleen weight. #: vertical axis "Parameters" indicate the counts of peripheral red blood cells (RBCs, $\times 10^8$/ml) and white blood cells (WBCs, $\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of the spleen (SPL, mg). (b) Number of colony-forming units in spleen (CFU-S, $\times 10^5$/femur) observed on days 9 (CFU-S-9) and 13 (CFU-S-13). (c) Numbers of *in vitro* granulocyte- macrophage CFUs (CFU-GM, ×500/femur) and erythroid CFU (CFU-E, $\times 10^3$/ femur) in femoral BM. (d) Numbers of CFU-GM ($\times 10^3$/spleen) and CFU-E ($\times 10^4$/spleen) in the spleen. *: Significant difference between Wt and AhR-KO mice determined by *t*-test at $p < 0.05$.

on *in vitro* characteristics, that is, the A549 cell line was reported to show an altered p27 expression, and Hepa 1c1c7 cells were supposed to maintain their its survival by AhR signaling. Moreover, a contradictory report was also found in which artificial AhR signaling suppression induced cellular proliferation (Fong et al., 2005). The above-mentioned evidence suggests that the receptor function in the presence or absence of a ligand may differ; therefore, it is of interest to determine whether signaling from AhR in Wt mice that suppressed cell proliferation might be altered by ligand signals.

Taking together the above lines of evidence, the function of AhR signaling is presumed to be expressed solely in immature progenitor cells, which suppresses their proliferation, maintains their quiescence, and thereby, conserves the characteristic features of stem cells, that is, stemness.

Interestingly, in response to such an AhR-null effect, the AhR-KO mice contrarily showed extensive hemopoiesis in the spleen (Fig. 1d), which resulted in a significant increase in spleen weight (Fig. 1a; most right) (Hirabayashi et al., 2003, 2005a).

Because of the above-mentioned difference in BM function between the AhR-KO mice and the Wt mice, in this study, benzene-induced hematotoxicity was evaluated in the Wt mice after subjecting them to a lethal dose of whole-body irradiation followed by repopulation with BM cells that lack AhR. Six weeks after the repopulation, the steady-state hematopoietic parameters for repopulated mice were obtained and are shown in Fig. 2a and b. The results were essentially the same between the mice repopulated with Wt BM cells (open columns) and those repopulated with AhR-KO BM cells (shaded columns) except that there is no anemic tendency, leukocytotic change, nor evidence of splenomegaly in Wt mice as compared with the AhR-KO mice as shown in Fig. 1.

Fig. 3a and b shows the percentages of RBCs (a) and WBCs (b) with respect to the control in the peripheral blood after the repopulation with BM cells. In the Wt mice repopulated with Wt BM cells and those with AhR-KO BM cells (open and closed symbols, respectively), benzene exposure induced a slight but statistically significant decrease in RBC count compared with the sham-exposure except on day 5 in the Wt groups (100% with standard deviation of the mean indicated by horizontal lines: Fig. 3a). The dose used in the present study was sufficiently high, and the decrease in RBC count was readily observed within 2 weeks of exposure.

The decreases in WBC count shown in Fig. 3b are more significant than those in RBC count throughout the exposure period except on day 5 in the AhR-KO group (the data were significantly different between Wt mice ($50.8 \pm 11.2\%$) and AhR-KO mice ($70.6 \pm 17.6\%$; $p = 0.024$)). In contrast to the previous observation (data not shown), mice repopulated with AhR-KO BM cells showed no significant difference in decrease of RBCs or WBCs. This difference in the observation of AhR-KO BM cells between the previous experiment and this study may be due to the difference in the route of benzene exposure, that is, intraperitoneal (*i.p.*) and intragastric (*i.g.*), respectively. Despite BM repopulation with AhR-KO BM cells, benzene exposure by the *i.g.* route in this study may have induced portal hepatic drug metabolism at a much greater extent than that by the *i.p.* exposure route. There was no significant differ-

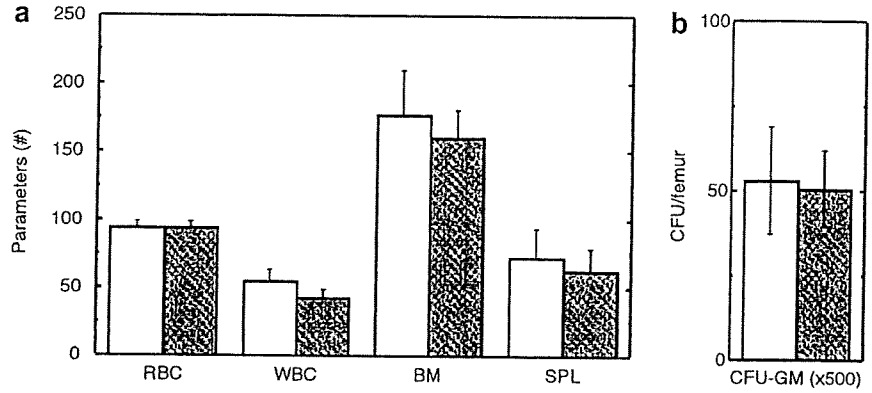

Fig. 2. Comparison of various blood parameters between mice repopulated with Wt BM (open columns) and AhR-KO BM (shaded columns) cells. (a) Peripheral blood, BM and spleen weight. #: vertical axis "Parameters" indicate the counts of peripheral RBCs ($\times 10^8$/ml) and WBCs ($\times 10^6$/ml), BM cellularity ($\times 10^5$/femur), and weight of the spleen (SPL, mg). (b) Numbers of CFU-GM (5×10^2/femur) per femur.

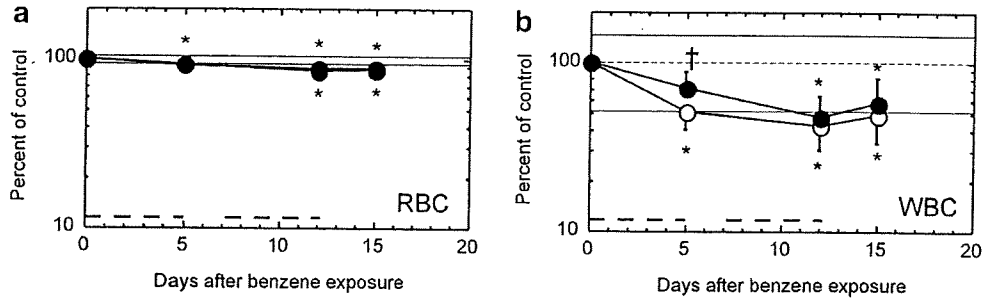

Fig. 3. Changes in percentage numbers of RBC (a) and WBC (b) of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham-exposure control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

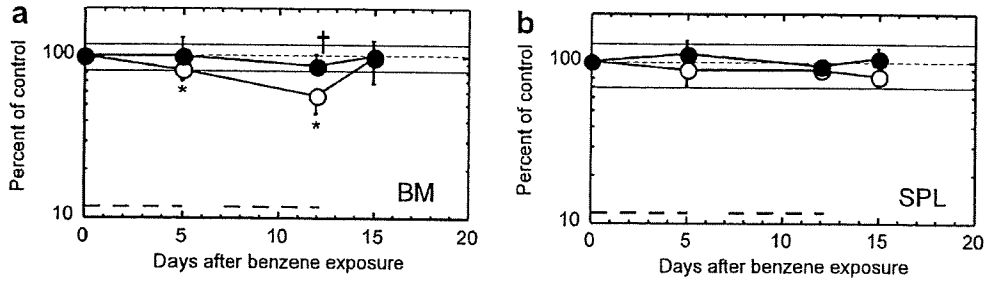

Fig. 4. Changes in percentage number of BM cells (a) and weight of spleen (b) of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

ence in data between the Wt mice and AhR-KO mice except on day 5 in the number of WBCs.

As shown in Fig. 4a, the decrease in the number of BM cells after benzene exposure is significant in the mice repopulated with Wt BM cells specifically on days 5 and 12 (82.2 ± 12.0%, $p = 0.035$ and 65.4 ± 20.3%, $p = 0.007$, respectively; number of cells obtained on day 12 was also significantly different between the Wt mice and the AhR-KO mice (86.7 ± 14.9%; $p = 0.013$)), which returned to the normal range by day 15, that is, three days after cessation of benzene treatment. In contrast to the peripheral blood parameters (Fig. 3a and b), the number of BM cells in the mice repopulated with AhR-KO BM cells did not show any decrease, but the mice showed a clear nullification of benzene-induced decrease in the number of BM cells (86.7 ± 14.9%; $p = 0.057$). Concerning the weight of the spleen, there are no significant differences among the groups regardless of the duration of benzene treatment and AhR expression (Fig. 4b).

In Fig. 5, the number of CFU-GM in the BM of mice repopulated with Wt BM cells much more significantly decreased on day 12 (open symbols, 37.8 ± 14.2%, $p = 0.019$; the number was also significantly different between Wt mice and AhR-KO mice (82.0 ± 7.0%; $p = 0.0008$)), which quickly returned to the normal range by day

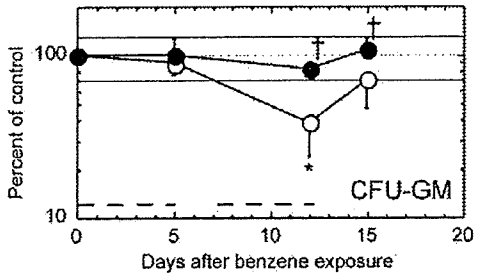

Fig. 5. Changes in percentage number of CFU-GM of mice repopulated with Wt BM (open symbols) and AhR-KO BM (closed symbols) cells during and after benzene exposure, with respect to each sham-exposure group. Vertical bars indicate the standard deviation of the mean. Horizontal dashed line indicates the mean (100%) and the solid lines indicate the standard deviation of the mean (100%) from the sham-exposure control Wt group. The dashed line at the bottom indicates benzene exposure duration. *: Significant difference between sham-exposure group and benzene-exposed group determined by *t*-test at $p < 0.05$. †: Significant difference between Wt mice and AhR-KO mice.

15, three days after cessation of benzene treatment. In this figure, interestingly, the benzene-induced decrease in the number of CFU-GM in the BM of mice repopulated with the AhR-KO BM cells (closed symbols) is clearly nullified for the Wt BM cells (open symbols), and the number stays within the range found for the sham-exposure. The reason for this much prominent decrease observed in the number of CFU-GM in the case of benzene exposure may be due, in part, to the expression of AhR, whose level is significantly high in primitive hematopoietic progenitor cells (Hirabayashi et al., 2002b, 2005b), the KO of which nullified the decrease in the number of CFU-GM much more significantly than the decrease in peripheral blood parameters.

4. Conclusions

Mice that had been lethally irradiated and repopulated with BM cells from AhR-KO mice essentially did not show any benzene-induced hematotoxicity. The present study elucidated the following: first, benzene-induced decrease in BM cellularity was clearly nullified by BM cells in mice that had been repopulated with AhR-KO BM cells. Second, we observed some differences in toxicologic phenotypes depending on the exposure route, that is, intraperitoneal, used in the previous study, or intragastric, in this study; that is, the former route induced attenuation of significant decreases in RBC number, spleen weight, and CFU-GM number, whereas the latter route induced attenuation of decrease in BM cellularity and CFU-GM number as compared with the Wt mice, respectively. Third, the marked decrease in the number of CFU-GM following benzene treatment and its nullification by repopulation with AhR-KO BM cells are the essential key discoveries of this study that may be related to the expression of AhR in primitive hematopoietic progenitor cells. Together with our previous observation and the report on the expression of CYP2E1 (Yoon et al., 2003; Ivanova et al., 2002), findings in this study may be related to the expression of CYP2E1 in BM cells.

Acknowledgments

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Disruption of Aryl Hydrocarbon Receptor (AhR) Induces Regression of the Seminal Vesicle in Aged Male Mice

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Key Words
AhR(–/–)males · Aryl hydrocarbon receptor (AhR) · 3βHsd · Mice · Seminal vesicle regression · Testes · Testosterone

Abstract
The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates diverse dioxin toxicities. Despite mediating the adverse effects, the *AhR* gene is conserved among animal species, suggesting important physiological functions for AhR. In fact, a recent study revealed that AhR has an intrinsic function in female reproduction, though its role in male reproduction is largely unknown. In this study, we show age-dependent regression of the seminal vesicles, probably together with the coagulating gland, in *AhR*(–/–) male mice. Knockout mice had abnormal vaginal plugs, low sperm counts in the epididymis, and low fertility. Moreover, serum testosterone concentrations and expression of steroidogenic 3βhydroxysteroiddehydrogenase (3βHsd) and steroidogenic acute regulatory protein (StAR) in testicular Leydig cells were decreased in *AhR*(–/–) males. Taken together, our results suggest that impaired testosterone synthesis in aged mice induces regression of seminal vesicles and the coagulating glands. Such tissue disappearance likely resulted in abnormal vaginal plug formation, and eventually in low fertility. Together with previous findings demonstrating AhR function in female reproduction, AhR has essential functions in animal reproduction in both sexes.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)-PAS (Per-AhR/Arnt-Sim) super-gene family [Burbach et al., 1992; Ema et al., 1992]. Since AhR can bind with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) [Poland et al., 1976; Ema et al., 1992], the molecular properties of AhR as a transcription factor have been extensively studied, especially focusing on the transactivation of a series of drug-metabolizing enzyme genes including *Cyp1a1* [Fujisawa-Sehara et al., 1987; Hankinson, 1995; Mimura and Fujii-Kuriyama, 2003]. In addition to these in vitro studies, in vivo gene disruption studies have revealed that AhR mediates a variety of toxicological effects of dioxin including teratogenesis, immunosuppression, tumor promotion, and estrogenic function [Poland and Knutson, 1982; Gibbons, 1993; Mimura et al., 1997; Brown et al., 1998; Shimizu et al., 2000]. Despite promoting these multiple adverse effects, the *AhR* gene is conserved across a variety of animal species from invertebrates to vertebrates [Hahn, 2002], suggesting that in addition to mediating the response to xenobiotics, there are intrinsic functions for AhR in physiological processes.

Recently, the intrinsic functions of AhR have been investigated with regards to animal reproduction and liver vasculogenesis. Indeed, recent studies in *AhR*(–/–) mice demonstrated that AhR is involved in female reproduction by regulating estradiol synthesizing *Cyp19* (P450

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aromatase) gene expression [Baba et al., 2005] and vessel remodeling in the liver [Lahvis et al., 2005]. Based on the essential functions of estradiol in the female reproductive process such as folliculogenesis, ovulation, and implantation [Fisher et al., 1998; Dupont et al., 2000; Curtis Hewitt et al., 2002], it was concluded that AhR plays an indispensable function in female reproduction. Moreover, in the case of male reproduction, dioxins were reported to reduce epididymal and ejaculated sperm number [Gray et al., 1995; Sommer et al., 1996], implicating that AhR is involved in the male reproductive process. However, there is no direct evidence for the involvement of AhR in this process.

The accessory internal reproductive systems, derived from the Wolffian duct for males and from the Mullerian duct for females, are clearly different between the two sexes. The male internal reproductive system consists of multiple tissues such as the epididymis, the deferens duct, the seminal vesicle, the coagulating gland, and the ejaculatory duct. Developmentally, all these tissues are known to be regulated by androgen signaling [Cunha, 1972; Cooke et al., 1991]. The mature seminal vesicle consists of numerous outpouchings of alveolar glands that empty into the ejaculatory duct. Although semen mostly contains materials secreted from the seminal vesicle, a definite functional relationship linking the seminal vesicle to male fertility has yet to be elucidated. The coagulating gland secretes a substance that, when mixed with the secretions from the seminal vesicle, forms a vaginal plug, and it has been thought that the vaginal plug is required for efficient pregnancy after insemination.

In this study, we define a novel phenotype of *AhR*(–/–) male mice. Interestingly, the seminal vesicle and probably the coagulating gland regressed in an age-dependent manner in the *AhR*(–/–) mouse, and such regression is possibly due to a low level of serum testosterone. These abnormalities possibly produce an abnormal vaginal plug and decrease the fertility of the male mice. This finding together with the previous finding in female reproductive processes [Baba et al., 2005] strongly suggests that AhR greatly influences animal reproduction regardless of the sex.

Materials and Methods

Mice

Targeted disruption of the *AhR* gene was performed as described previously [Mimura et al., 1997]. AhR knockout mice used in this study were backcrossed to C57BL/6J for more than eight generations in order to avoid experimental variation due to genetic background.

Antibodies

A full-length cDNA for mouse 3βHsd1 was kindly provided by Dr. A. Payne (Stanford University). A prokaryotic expression vector for 3βHsd was constructed by insertion of the 3βHsd cDNA into pET-28a (Novagen, San Diego, CA). Preparation of recombinant 3βHsd protein and immunization of rabbits were described previously [Morohashi et al., 1993]. Rabbit antibodies for AhR and Cyp19 were generously provided by Dr. R. Pollenz (University of South Florida) and Dr. N. Harada (Fujita Health University), respectively. Rabbit antibody for androgen receptor (AR) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fertility Assessment

Thirty *AhR*(+/+) wild-type males, three *AhR*(–/–) males harboring the seminal vesicle, and nine *AhR*(–/–) males lacking the seminal vesicle were mated with *AhR*(+/+) females for 5 days. Twelve days after mating, the female mice were sacrificed to determine whether they became pregnant or not. The presence of the seminal vesicle in each *AhR*(–/–) male was determined both one week prior to the mating and just after the mating was completed. Statistical analysis was performed by Fisher's exact test. All protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee of the National Institute for Basic Biology.

Immunohistochemistry and Western Blot

To detect AhR and AR, cryosections (10 μm) were prepared from the seminal vesicle treated overnight with 4% paraformaldehyde at 4°C. After washing with phosphate-buffered saline (PBS), the sections were boiled for 10 min in 10 mM sodium citrate (pH 7.0) to unmask antigen epitopes, followed by treatment with 0.3% H_2O_2 in methanol for 20 min at –20°C. The sections were incubated overnight at 4°C with anti-AhR or anti-AR antibody, washed with PBS, and then incubated with biotinylated donkey anti-rabbit IgG for 3 h at room temperature. After washing, the sections were incubated with horseradish peroxidase-conjugated streptavidin, and then visualized with diaminobenzidine at room temperature. To detect AhR and 3βHsd in the testes, 5 μm paraffin sections were prepared from 4% paraformaldehyde-fixed testes. After deparaffinization, antigen epitopes were unmasked by treatment with 20 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature for AhR or unmasked by boiling for 10 min in 10 mM sodium citrate (pH 7.0) for 3βHsd, followed by treatment with 0.3% H_2O_2 in methanol for 20 min at –20°C. The sections were incubated overnight at 4°C with the anti-AhR or anti-3βHsd antibody, washed with PBS, and then incubated with biotinylated donkey anti-rabbit IgG or Cy-3 conjugated goat anti-rabbit IgG for 3 h at room temperature. After washing, sections immunoreacted with biotinylated antibodies were incubated with horseradish peroxidase-conjugated streptavidin, and then visualized with diaminobenzidine. The sections immunoreacted with Cy-3-conjugated antibody were counterstained with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine), and then 3βHsd-positive cells were counted under fluorescence microscope.

To prepare whole tissue lysate for Western blot analysis, tissues were lysed with a cell-lysis buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA (pH 8.0), and 1% SDS. Next, 10 μg of whole tissue lysates were subjected to SDS-PAGE followed by Western blot analyses using the antibodies for AhR, AR, Cyp19, Ad4BP/SF-1 and 3βHsd, as described previously [Morohashi et al., 1994].

Determination of Serum Testosterone Concentrations

Four *AhR*(+/+) and three *AhR*(−/−) 24-week-old and eight *AhR*(+/+) and ten *AhR*(−/−) 52-week-old male mice were anesthetized with diethyl ether for collection of blood samples. After isolating the serum fraction, serum testosterone concentration was determined by enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) according to the protocol provided by the manufacturer.

Sperm Count

Count of epididymal sperm number was performed as reported previously [Bell et al., 2007]. Briefly, the cauda epididymis was dissected and pierced three times with a scalpel blade. Then, the tissue was incubated in 5 ml of PBS containing 0.57% (w/v) BSA at 37°C for 90 min. After incubation, the number of sperms was counted under a microscope.

Leydig Cell Count

Serial sections of the testes prepared from eight *AhR*(+/+) and ten *AhR*(−/−) 52-week-old mice were stained with anti-3βHsd antibody and DAPI. The number of 3βHsd-positive Leydig cells was counted under a fluorescence microscope in 32 sections (4 sections for each animal) of *AhR*(+/+) and 40 sections of *AhR*(−/−).

Quantitative RT-PCR

Quantitative RT-PCR was performed with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR master mix (Applied Biosystems). The thermal-cycling condition was 50 cycles of 15 s at 95°C and 1 min at 60°C. Primer pairs used for quantitative RT-PCR were as follows: 3βHsd (fwd), 5′-CAG ACC ATC CTA GAT GTC-3′; 3βHsd (rev), 5′-ACT GCC TTC TC GCC ATC-3′; StAR (fwd), 5′-CCG GAG CAG AGT GGT GTC A-3′; StAR (rev), 5′-GCC AGT GGA TGA AGC ACC AT-3′; Insl3 (fwd), 5′-CCT GGC TAT GTC ATT GCA ACA-3′; Insl3 (rev), 5′-TGG TCC TTG CTT ACT GCG ATC T-3′ [Cederroth et al., 2007]; and P450scc (fwd), 5′-CAG AAC TAA GAC CTG GAA GGA CCA-3′; P450scc (rev), 5′-TGG GTG TAC TCA TCA GCT TTA TTG AA-3′.

Results

Regression of Seminal Vesicles in Aged AhR(−/−) *Males*

We have previously reported that *AhR*(−/−) female mice had defective reproductive activity. In the present study, we examined the effect of *AhR* on the reproductive activity in *AhR*(−/−) males. Although the defect was milder than that observed in females, we found suppression of reproductive activity in aged *AhR*(−/−) males. Therefore, we examined whether the reproductive tissues are also affected in *AhR*(−/−) males. The seminal vesicle was completely regressed in certain population of *AhR*(−/−) males (fig. 1A). At the same time, we noticed that this tissue regression was rare in the young adult. Therefore, the regression was examined in terms of animal age. Regression of the seminal vesicle was identified in 53.8% of the 24-week-old, 66.7% of the 32-week-old, and 50.0% of the 52-week-old *AhR*(−/−) males, whereas no such regression was observed in the 8-week-old knockout males (fig. 1B). No such tissue regression was observed in age-matched *AhR*(+/+) mice, strongly suggesting that AhR is essential for the maintenance of seminal vesicle in aged mice.

Next, we quantified the regression process by measuring tissue weight. The weight of the seminal vesicles was similar in *AhR*(+/+) and *AhR*(−/−) at 8 weeks after birth (fig. 1C). However, the weight of the *AhR*(−/−) seminal vesicles did not increase after 24 weeks while that of *AhR*(+/+) increased in an age-dependent manner. We expected to observe apparent tissue regression in some of the knockout animals, but no such tissue was observed, suggesting that the regression occurs and is completed rapidly.

Since AhR is implicated in maintenance of the seminal vesicle, the expression of AhR in the seminal vesicle was analyzed by immunohistochemistry (fig. 1D) and Western blotting (fig. 2B). As shown in figure 1D, AhR was expressed in the epithelial cells of the seminal vesicle and accumulated in the cytoplasm rather than in the nuclei of these cells. This cytoplasmic localization was similar to that observed in the liver [Poland et al., 1976].

Testosterone Synthesis in AhR(−/−) *Males*

Since proliferation of the seminal vesicle epithelial cells is controlled by an androgen-mediated signal [Neubauer et al., 1981], we subsequently investigated the expression of androgen receptor (AR) immunohistochemically (fig. 2A). Similar to AhR, AR was expressed in the epithelial cells of the seminal vesicle. To assess whether regression of the seminal vesicle is due to low expression of AR in *AhR*(−/−) animal, whole tissue extracts were prepared from the seminal vesicles of *AhR*(+/+) and *AhR*(−/−) males at 8 and 32 weeks of age, and then subjected to Western blot analyses. Unexpectedly, however, no decrease in the expression of AR was observed in the absence of AhR at both 8 and 32 weeks (fig. 2B).

In addition to AR, testosterone is required for AR signaling. Therefore, we were interested in determining whether testosterone production is affected in *AhR*(−/−)

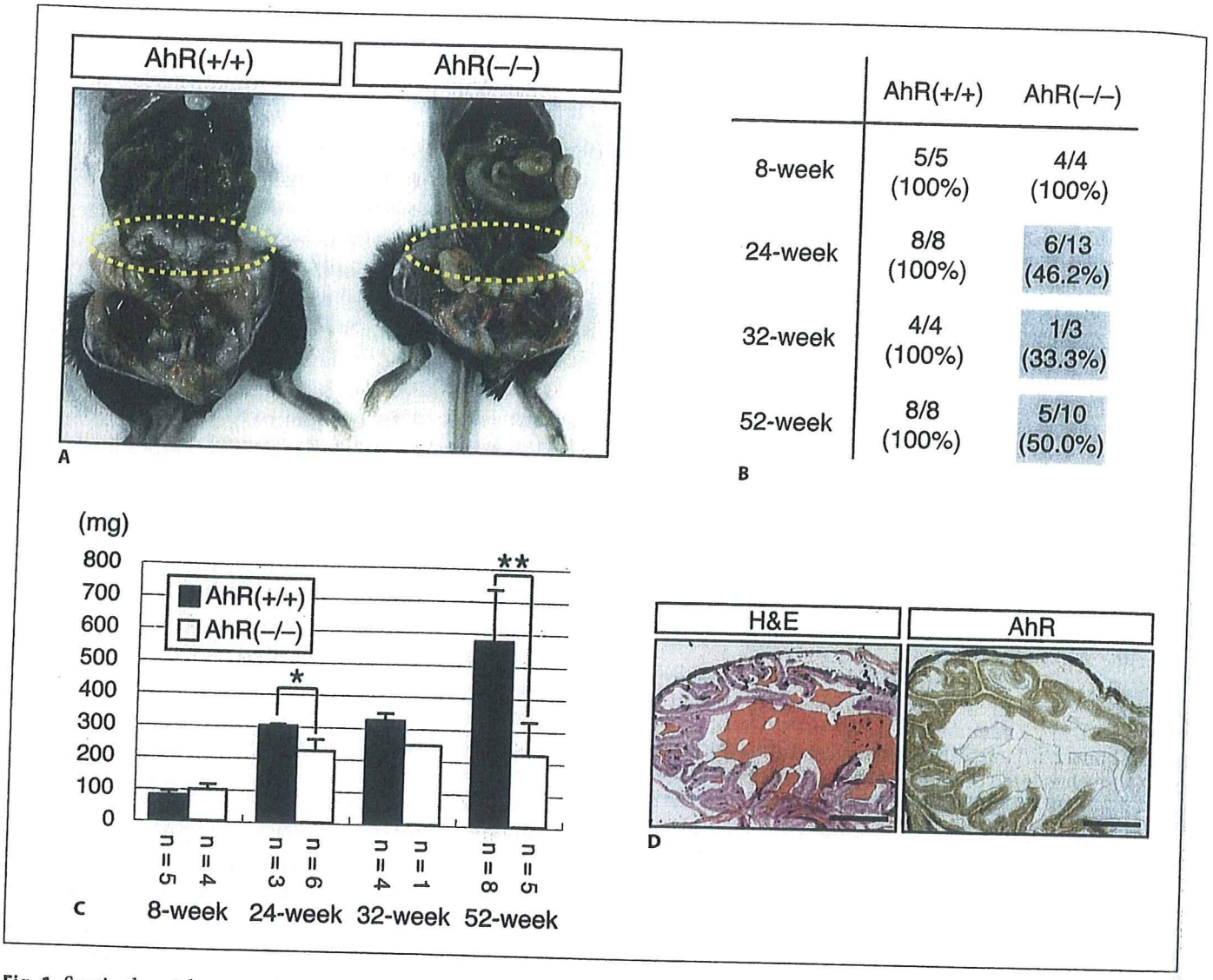

Fig. 1. Seminal vesicle regression in aged *AhR*(–/–) mice. **A** The reproductive tracts of 20-week-old *AhR*(+/+) and *AhR*(–/–) males. The seminal vesicle in the *AhR*(+/+) male is indicated by yellow dotted circles while this tissue is absent in the *AhR*(–/–) male. **B** Seminal vesicle regression in *AhR*(–/–) males is age-dependent. *AhR*(+/+) and *AhR*(–/–) males at 8, 24, 32, and 52 weeks of age were analyzed for the presence of seminal vesicles. Data are numbers of mice with intact seminal vesicles per total number of mice. **C** Comparison of seminal vesicle wet weight between *AhR*(+/+) and *AhR*(–/–) males. The seminal vesicles isolated from 8-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(–/–) males were weighed. Numbers of the mice examined are indicated. Values are mean ± SD, * $p < 0.025$, ** $p < 0.005$. Statistical analysis was not performed with the 32-week-old mice because the number of *AhR*(–/–) males harboring the seminal vesicle was small. **D** Expression of AhR in the seminal vesicle. Ten-micrometer cryosections were prepared from a 10-week-old *AhR*(+/+) seminal vesicle. The sections were stained with hematoxylin and eosin (H&E) or immunohistochemically with anti-AhR antibody (AhR). Scale bars = 200 μm.

males. The testicular weight of *AhR*(–/–) males was compared with that of age-matched *AhR*(+/+) males at 8, 24, 32, and 52 weeks after birth. As shown in fig. 3A, the weights were mostly similar in *AhR*(+/+) and *AhR*(–/–) males at the above ages, although a slight difference was observed in 52-week-old mice. We then measured serum testosterone concentration in the 24- and 52-week-old mice, and found that it had clearly decreased in *AhR*(–/–) mice to approximately one third, and half of 24- and 52-week-old *AhR*(+/+) mice (fig. 3B). This result suggested that low testosterone concentrations cause, at least in part, the defect of seminal vesicles of aged *AhR*(–/–) males.