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Review

AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates the adverse effects of dioxins, including modulation of sex steroid hormone signaling. The role of AhR as a transcription factor is well described. AhR regulates the expression of target genes such as CYP1A1; however, the mechanisms of AhR function through other target-selective systems remain elusive. Accumulating evidence suggests that AhR modulates the functions of other transcription factors. The ligand-activated AhR directly associates with estrogen or androgen receptors (ERα or AR) and modulates their function both positively and negatively. This may, in part explain the sex steroid hormone-related adverse effects of dioxins. AhR has recently been shown to promote the proteolysis of ERα/AR through assembling a ubiquitin ligase complex, CUL4-B^{AhR}. In the $CUL4B^{AhR}$ complex, AhR acts as a substrate-recognition subunit to recruit ERα/AR. This action defines a novel role for AhR as a ligand-dependent E3 ubiquitin ligase. We propose that target-specific regulation of protein destruction, as well as gene expression, is modulated by environmental toxins through the E3 ubiquitin ligase activity of AhR.

Contents

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Abbreviations: AhR, arylhydrocarbon receptor; ERα, estrogen receptor; AR, androgen receptor; XRE, xenobiotic-responsive element; ERE, estrogen-responsive element; bHLH/PAS, basic helix-loop-helix/Per–Arnt–Sim; AF-1, autonomous activation function; E_2, 17β-estradiol; 3MC, 3-methylcholanthrene; βNF, β-naphthoflavone; CRL, cullin–RING ubiquitin ligase; SCF, Skp1–CUL1–F-box; CUL4B, cullin 4B; DDB1, damaged-DNA-binding protein 1.

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1. Introduction

Dioxin-type environmental contaminants, such as tetrachloro-dibenzo-*p*-dioxin (TCDD), exert toxic effects [1]. Some of these toxicities are estrogen- and androgen-related actions [2–7]. The arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor belonging to the basic helix-loop-helix/Per–Arnt–Sim (bHLH/PAS) family. AhR possesses a variety of biological and toxicological functions [8–11] (Figs. 1 and 2). AhR mediates the toxicological effects of dioxins. In addition, AhR plays a physiological role in various tissues such as the reproductive and immune systems. The transcriptional activity of AhR is regulated by direct binding of its ligands [12,13] (Figs. 1 and 2A). The unliganded AhR is sequestered in the cytosol by interacting with the Hsp90/XAP2 (also called as ARA9 or AIP) chaperon complex [8–11]. Ligand binding to the PAS-B region of AhR is thought to induce conformational changes and subsequent translocation of the AhR complex to the nucleus [8–10]. AhR then dimerizes with the AhR nuclear translocator (Arnt) in the nucleus after dissociating from the chaperon complex, recognizes the xenobiotic-responsive element (XRE), and recruits co-activators such as the histone acetyltransferase p300/CBP, chromatin remodeling factor Brg1, and the mediator (DRIP/TRAP) complex to activate transcription [8–10] (Fig. 1). The AhR/Arnt heterodimer induces the expression of target genes, such as CYP1A1, CYP1A2, and glutathione-S-transferase [1].

The actions of the direct target genes of AhR alone do not fully explain its toxicological and physiological effects. Accumulating evidence suggests that the AhR exhibits its regulatory functions by modulating the function of other transcription factors [2,11], including estrogen receptor (ERα and ERβ) [14–19] and androgen receptor (AR) [18,19] (Fig. 1). These cross-talk pathways are important mediators of the functions of endogenous and exogenous AhR ligands. The liganded AhR recently has been shown to promote the ubiquitination and proteasomal degradation of ERs and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [18,19]. Thus, complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms. In this review, we will summarize a novel role for AhR as a component of an E3 ubiquitin ligase complex, which mediates cross-talk of AhR with sex steroid receptors through promotion of proteolysis.

2. Cross-talk of AhR with ERs or AR

2.1. *Transcriptional regulatory mechanism involving nuclear receptors*

ERs and AR belong to the nuclear receptor superfamily of transcription factors [20–22] (Fig. 2). Nuclear receptors, by acting as ligand-dependent transcription factors serve as

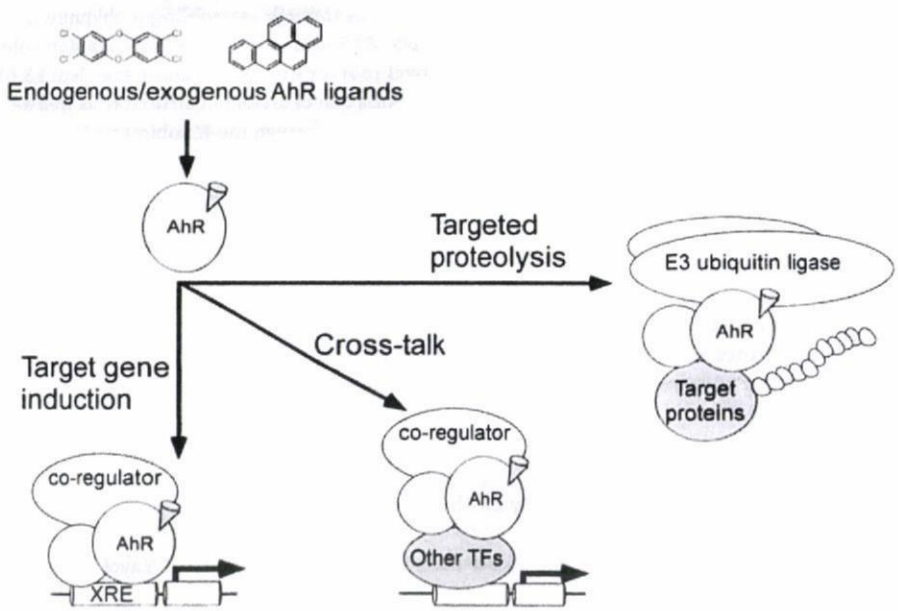

Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. In addition, cross-talk of AhR with other transcription factors, as well as the function of AhR as an E3 ubiquitin ligase, is considered important for AhR biology. XRE, xenobiotic-response element; TF, transcription factor.

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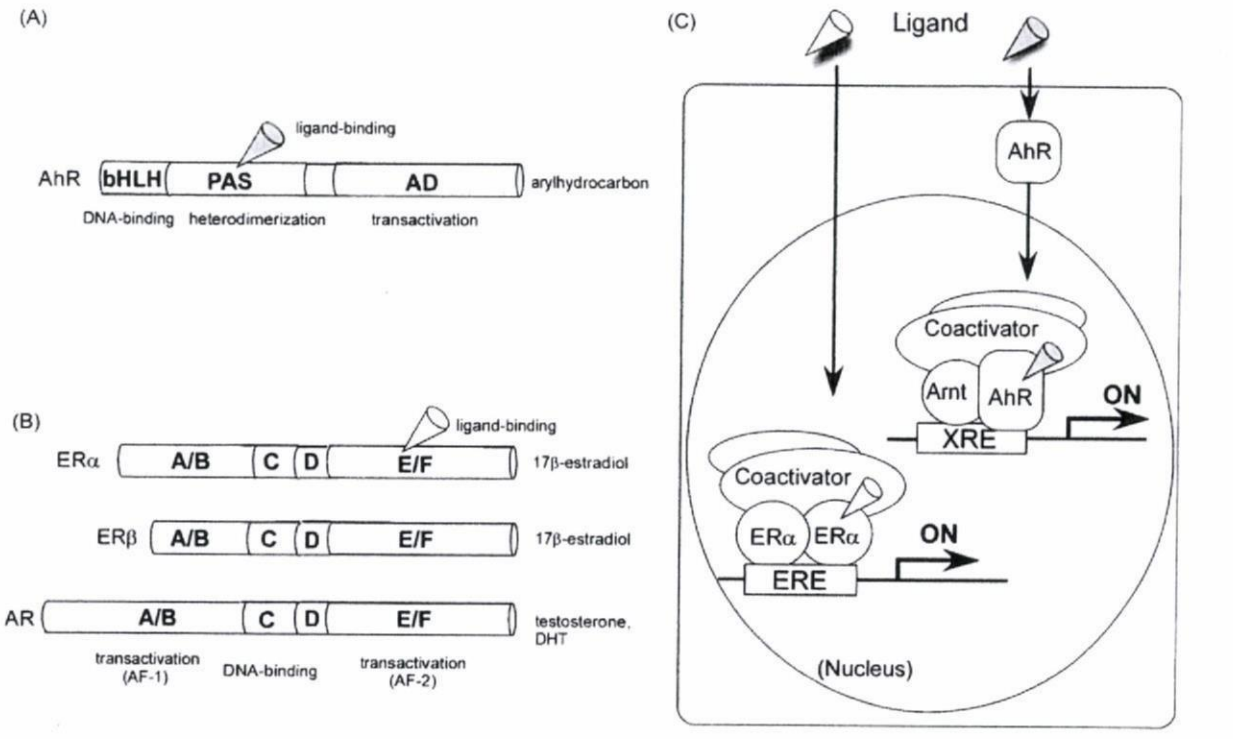

Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per–Arnt–Sim domain; AD, activation domain; AF, activation function; DHT, dihydrotestosterone. (C) Mechanisms of gene regulation mediated by AhR and nuclear receptors. ERE, estrogen-response element.

sensors for low molecular weight, fat-soluble ligands such as steroids/thyroid hormones, and vitamins A and D [20,21]. Members of the nuclear receptor gene superfamily share a common domain structure with distinct functional domains, designated A–E [21] (Fig. 2B). The ligand-binding domain is located in the C-terminal E domain. The most conserved C domain, located in the middle of the receptor, serves as the zinc finger-type DNA-binding domain. This domain specifically recognizes its cognate response elements in the target gene promoters. The N-terminal A/B domain and the C-terminal E domain are required for ligand-induced nuclear receptor transactivation functions. The autonomous activation function (AF-1) in the A/B domain is constitutively active but is presumably masked in the absence of ligand. The autonomous activation function (AF-2) in the ligand-binding E domain is, in contrast, dependent on ligand binding through the ligand-dependent conformational change of helix 12 and subsequent formation of a hydrophobic surface for the interaction with co-regulators [20] (Fig. 2).

Ligand-bound nuclear receptors recruit a number of transcriptional co-regulators and co-regulator complexes to the target gene promoters to mediate ligand-dependent transcriptional control [21,22] (Fig. 2). These complexes can be classified into three categories according to their functions. The first class of co-regulator complexes modifies histone tails covalently [23]. The amino-terminal tails of histones are subjected to various covalent modifications such as acetylation, methylation, phosphorylation, and ubiquitination by specific histone-modifying enzymes. These post-translational histone modifications are thought to serve as a 'histone code' that fine-tunes the transcriptional state through chromatin structure rearrangement [23]. The second class of complex mediates ATP-dependent dynamic remodeling of chromatin structure [22]. Chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. These chromatin remodeling complexes support the accessibility of co-regulator complexes and transcription factors to specific promoter regions. The last co-regulator complex class, the mediator complex, directly regulates transcriptional control by physically interacting with general transcription factors and RNA polymerase II. Recent evidence suggests that numerous co-regulators and nuclear receptors are recruited onto the promoters in an ordered manner, associating and dissociating transiently [24,25]. Nuclear receptors, as well as other transcription factors, serve as specific adaptors that connect co-regulator complexes and specific promoter regions.

The ligand-dependent nuclear receptor function is also regulated by other classes of signal transduction pathways. Such cross-talk pathways include at least two mechanisms: functional modulation through post-translational modification, and the association with other classes of transcription factors. MAPK, activated by EGF, phosphorylates ERα at serine 118 [26]. This in turn potentiates the ligand-dependent transactivation function of ERα [26] as well as its rapid turn-over. Phosphorylation-mediated functional modulation has been reported for a number of nuclear receptors to date.

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Complex formation-based cross-talk mechanisms are also seen in several nuclear receptors including the glucocorticoid receptor (GR) [27]. GR ligands have an anti-inflammatory action, which is mediated through ligand-dependent repression of AP-1 activity through direct association. More recently, the exchange of different classes of co-regulator complexes has been reported to underlie the signal cross-talk pathway. Ligand-activated PPARγ typically assembles co-activator complexes on its cognate promoters. In the repression of NF-κB activity, PPARγ forms a complex with NF-κB, and this complex stably associates with an NCoR co-repressor complex by inhibiting the degradation of NcoR [28]. A current view of signal cross-talk at the transcription levels is that signal/ligand-dependent transcription factors associate with each other to assemble diverse types of co-regulator complexes. These exchange dynamically and regulate transcription in a manner specific for each cross-talk pathway [22].

2.2. *Molecular mechanisms of cross-talk of AhR with estrogen or androgen receptors*

Signal cross-talk pathways are important mediators of the functions of AhR ligands in various tissues. Dioxin-type environmental contaminants exert both estrogen- and androgen-related effects [1–3,5–7,29–32] (Fig. 3). Dioxins have well-described anti-estrogenic effects, such as the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth, and target gene induction [3,7]. However, there is also evidence to the contrary as dioxins have also been shown to have estrogenic effects including the stimulation of uterine enlargement [29], induction of estrogen-responsive genes such as *VEGF*, *c-fos*, and *TERT*, and a similar pattern to estrogen of transcriptional regulation in a genome-wide study [6]. In addition, AhR-deficient mice exhibit impaired ovarian follicle maturation [33]. Using AhR-deficient cells, the importance of AhR in the proliferation of mammary cells has been confirmed [34]. These findings suggest that AhR, activated by its endogenous ligand, may modulate the estrogen signaling pathway. Similarly, dioxins exert both androgenic and anti-androgenic effects on prostate development in an age-specific manner [5]. As is true for other cross-talk pathways [22], the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context.

The molecular mechanisms of AhR modulation of ERα have been extensively studied, and both direct and indirect regulatory mechanisms have been proposed. First, TCDD/AhR either increases or decreases estrogen levels through an indirect mechanism [2,35]. TCDD promotes the clearance of estrogen, thereby repressing ER transcriptional activity [35]. AhR-deficient mice have decreased estrogen production due to impaired induction of aromatase (CYP19) gene expression [33]. Another indirect mechanism involves competitive DNA binding of AhR and ER on the responsive promoters [2]. AhR and ER, each bound to its own target promoter recruits transcriptional co-regulators

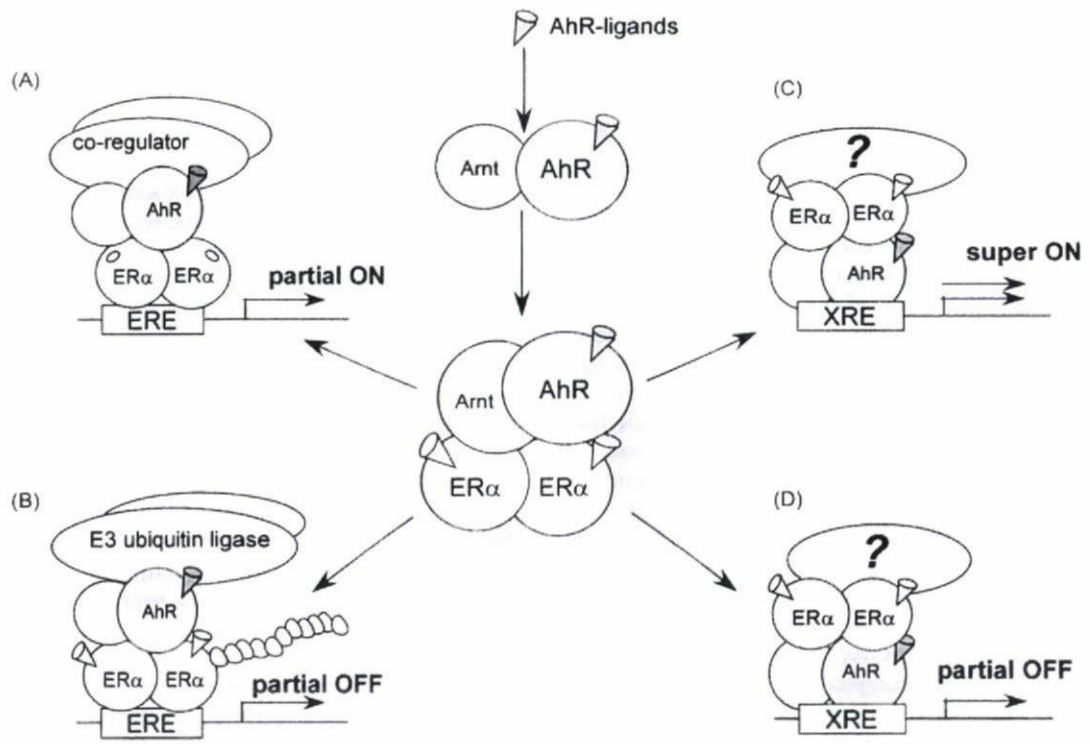

Fig. 3 – Cross-talk of AhR with ERa through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERa, ERb, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound AhR associates with unliganded ERs upon ERE and recruits transcriptional co-activators. (B) Ligand-bound AhR forms E3 ubiquitin ligase complex and recognizes ERs for proteolysis. (C) Ligand-bound ERa associates with AhR and activates transcription through XRE. (D) Association of ERa with AhR results in repression through XRE.

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in a competitive manner. This mechanism may be limited to specific genes and conditions since not all of the estrogen-responsive promoters contain XRE.

More recently, direct association of AhR with ERs has been independently reported. Ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within ERs [14–18] (Fig. 3). By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα (Fig. 3A), while it represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [14] (Fig. 3B). The interaction of AhR/ER is induced by different AhR ligands, such as TCDD, 3-methylcholanthrene (3MC), and β-naphthoflavone (βNF). The activation of AhR is thought to be sufficient for the interaction with ERα, as a constitutively active form of AhR [12] modulates ERα function in the absence of AhR ligand [19]. These results suggest that the cross-talk of AhR with ER is initiated primarily through stimulation of AhR. Supporting this, ERα is predominantly located in the nucleus, whereas AhR translocates to the nucleus upon ligand stimulation. The association of AhR/ERα has been shown by several independent approaches, including *in vitro* [36], *in vivo*, and biochemical methods [18]. Moreover, AhR/ERα cross-talk in the transcriptional regulation of ERα-responsive genes is abolished in AhR-deficient mice [10,33], confirming the specificity of the molecular pathway *in vivo* [14]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [15] (Fig. 3C) or repress [16] (Fig. 3D) AhR-mediated transcription. Considered together, the AhR/ERα complex may be able to bind to either XRE or ERE through the attachment functions of AhR or ERα, respectively. Alternatively, different complex subtypes that contain AhR/ERα may control promoter selectivity (Fig. 3). Reflecting this functional cross-talk, Arnt also acts as a co-regulator for both ERα and ERβ [37].

The proposed mechanism of AhR/ER association is a reasonable explanation for dioxin/estrogen cross-talk. First, this mechanism explains the functional AhR/ER cross-talk irrespective of differences in target gene promoters. Second, ligand-dependent AhR/ER association may result in a rapid cellular response to dioxins in terms of ER activity. The responses of ER transcriptional activity to AhR ligands are observed within a few hours in cultured cells as well as in mice, which supports the existence of direct cross-talk mechanisms. Third, variations in the AhR/ER containing co-regulator complexes may result in the complex, bi-phasic consequences of AhR/ER cross-talk. Given that complexes containing different classes of transcription factors can recruit co-regulator complexes distinct from their cognate associating complexes [22], it is possible that the AhR/ER complex, acting as a functional unit, may recruit different types of complexes depending on the cellular context. A current area of interest is the identification of the molecular determinants by which the activity of the AhR/ER complex is controlled.

3. Ubiquitin ligase activity of AhR

3.1. *The ubiquitin–proteasome system*

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels in response to various cellular contexts in metazoans (Fig. 4A). Whereas the transcriptional regulatory system is targeted by environmental fat-soluble ligands, the involvement of the ubiquitin–proteasome system in the adverse effects of these environmental toxins remains largely unknown. The target selectivity of these systems depends on the recognition of specific DNA elements by sequence-specific transcription factors [20–22] and recognition of degradation substrates by E3 ubiquitin ligases [38–41] (Fig. 4B). These transcription factors and E3 ubiquitin ligases primarily serve as specific adapters to subsequently recruit enzymes such as transcriptional co-

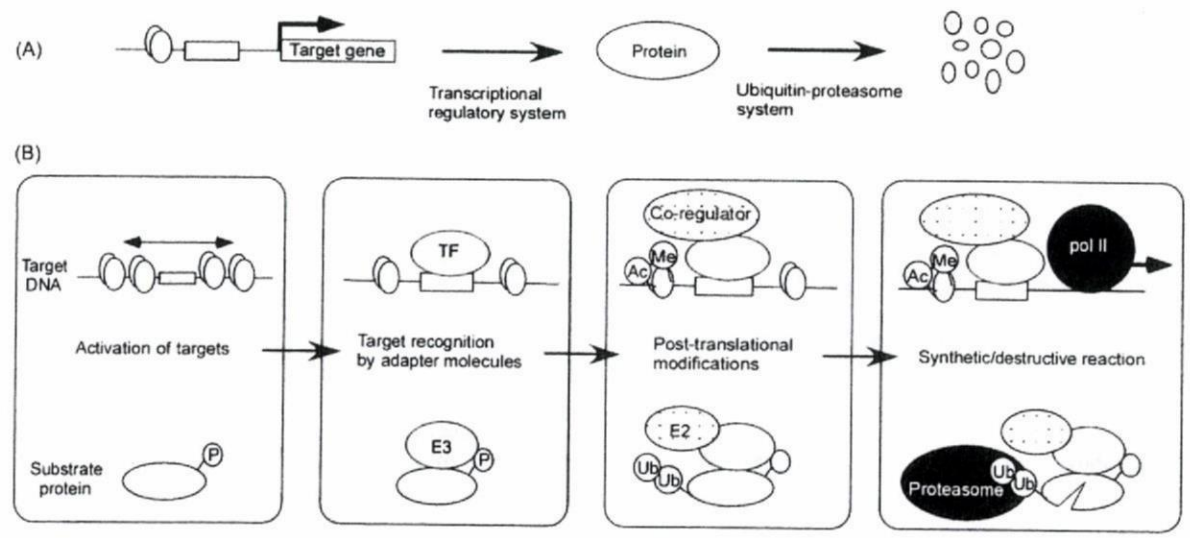

Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters in these systems. Ub, ubiquitin; P, phosphorylated serine/threonine; Ac, acetylated lysine; Me, methylated lysine; Pol-II, RNA polymerase II.

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regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. Considering the functional analogy of E3 ubiquitin ligase and transcription factors, it is possible that E3 ubiquitin ligase also serves as a target of environmental toxins.

The ubiquitin–proteasome system, which regulates cellular protein degradation, plays a pivotal role in cellular homeostasis [38–41]. Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes. Ubiquitin is covalently attached to lysine (Lys) residues of substrate proteins. Ubiquitination of proteins is catalyzed by sequential reactions involving ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Ubiquitin is conjugated either as one molecule (mono-ubiquitination) or as a tandem polymer (poly-ubiquitination). Poly-ubiquitination can occur at any of seven lysine residues in the ubiquitin molecule. The Lys48-linked poly-ubiquitin chain is then recognized by the 26S proteasome for subsequent proteolysis (Fig. 4B).

Among E1, E2, and E3 enzymes, the E3 ubiquitin ligases are most diverse and therefore possess substrate specificity. E3 acts as a bridge between E2 and the substrate, maintaining the appropriate distance. E2 then conjugates ubiquitin to the substrate [38–41]. Of the RING-type E3s, the largest class is comprised of the cullin–RING ubiquitin ligases (CRLs) [40–44]. CRLs are multisubunit complexes that include a cullin (CUL1, 2, 3, 4A, 4B, or 5) subunit, a RING finger protein Rbx1/Roc1 or Rbx2/Roc2, and a substrate-recognition subunit. Cullin serves as a scaffold protein, binding to the substrate-recognition subunit or adapter protein at its N-terminus while binding to Rbx1 at its C-terminus [41]. Rbx1 binds to E2 enzymes through RING finger to support efficient conjugation of ubiquitin to the substrates. Their diverse substrate-recognition subunits enable CRLs to target numerous substrates. The best characterized CRLs are the SCF (Skp1–CUL1–F-box) complexes. In SCF complexes, F-box proteins function as a substrate-recognition subunit by binding to Skp1, which is bound to the N-terminal region of CUL1. F-box proteins and other types of substrate-recognition subunits serve as adapters for target-specific substrates. Therefore, any protein binding to E3 core components can potentially act in a manner similar to substrate-recognition subunits. More interestingly, F-box proteins and other types of substrate-recognition subunits are rapidly degraded through an auto-catalytic mechanism once they are integrated into the CRL core complexes [42]. In this way, CRLs can efficiently ubiquitinate different substrates by associating with different substrate-recognition subunits. This raises the possibility that F-box and F-box 'equivalent' proteins act either as substrates or as adapter components, as in the case of DDB2 in the CUL4-based CRL complex [45–50].

3.2. *AhR is an E3 ubiquitin ligase*

As discussed above, dioxins, through activating the AhR, have well-described effects on the transcriptional regulatory system. TCDD is also reported to decrease the uterine ERα protein level in the rat [51], suggesting that AhR may also be involved in the control of protein stability. Somewhat unexpectedly, our own study has shown that in a ChIP analysis, the ligand-bound AhR does not block co-activator recruitment of liganded ERα. In addition, repression of ERα transcriptional activity by AhR is not observed when ERα is over-expressed in transient reporter assays (Ohtake et al., unpublished data). These observations imply that the ligand-activated AhR has an additional molecular role beyond transcriptional regulation, at least in the modulation of sex hormone signaling.

Exploring the functions of AhR in sex hormone signaling, we found that upon activation of AhR by binding of AhR ligands such as 3MC and βNF, as well as by expression of constitutively active AhR, protein levels of endogenous ERα, ERβ, and AR, were drastically decreased without alteration in mRNA levels [19] (Fig. 5). Since ligand-bound AhR and ERα proteins are ubiquitinated for proteasome-mediated degradation [52–57], we tested whether the functional modulation of ERs and AR by activated AhR is related to this degradation system. 3MC-enhanced degradation of sex steroid receptors is attenuated in the presence of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERα is consistently observed irrespective of E2 binding. MG132 treatment abrogates the transcriptional modulation of liganded sex steroid receptor function by activated AhR. This indicates that the ubiquitin–proteasome system mediates the repressive AhR–ER cross-talk pathway.

These experiments provide evidence that AhR acts as an E3 ubiquitin ligase component. First, FLAG–AhR immunoprecipitated complexes exert a self-ubiquitination activity in an E1/E2 enzyme-dependent manner *in vitro*. Second, 3MC-dependent recognition of ER and AR by AhR [14] appears to induce ubiquitination of ER/AR. Third, degradation of AhR itself is accelerated upon activation of degradation of sex steroid receptors, which is a typical sign of self-ubiquitination of the E3 component [42]. Taken together, these properties of AhR resemble that of classical adapter components of the E3 ubiquitin ligase complex such as F-box proteins in the SCF complex [39,42], DDB2/CSA in the CUL4A complex [45–49], and VHL in the CUL2 complex [58]. Therefore, we reasoned that activated AhR might serve as an E3 ubiquitin ligase component.

Supporting this idea, an AhR associating ubiquitin ligase complex has been biochemically purified [59] from HeLa cells. This complex includes cullin 4B (CUL4B) [39,60], damaged-DNA-binding protein 1 (DDB1) [61,62], and Rbx1 [39] together with subunits of the 19S regulatory particle (19S RP) of 26S proteasome as well as Arnt and transducin-beta-like 3 (TBL3) (Fig. 5). The core complex appears to constitute a CRL-type E3 ligase, and therefore is referred to as $CUL4B^{AhR}$. Although the typical CUL4B-type CRL complex contains substrate-recognition components having a WDXR/DWD motif [45–49], no such component has been identified in this complex. AhR directly interacts with the N-terminal region of CUL4B in GST pull-down assays. Together with the direct interaction of AhR with ER, it appears that AhR may act as a substrate-recognition component in the $CUL4B^{AhR}$ complex. Using an *in vitro* reconstituted ubiquitination assay, the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ERα is dependent only on 3MC, and not on E_2. This suggests that $CUL4B^{AhR}$ has the unique property of being able to respond to ligand signals by complex assembly and ubiquitin ligase activity (Fig. 5). The importance of the $CUL4B^{AhR}$ components for the promotion of ERα ubiquitina-

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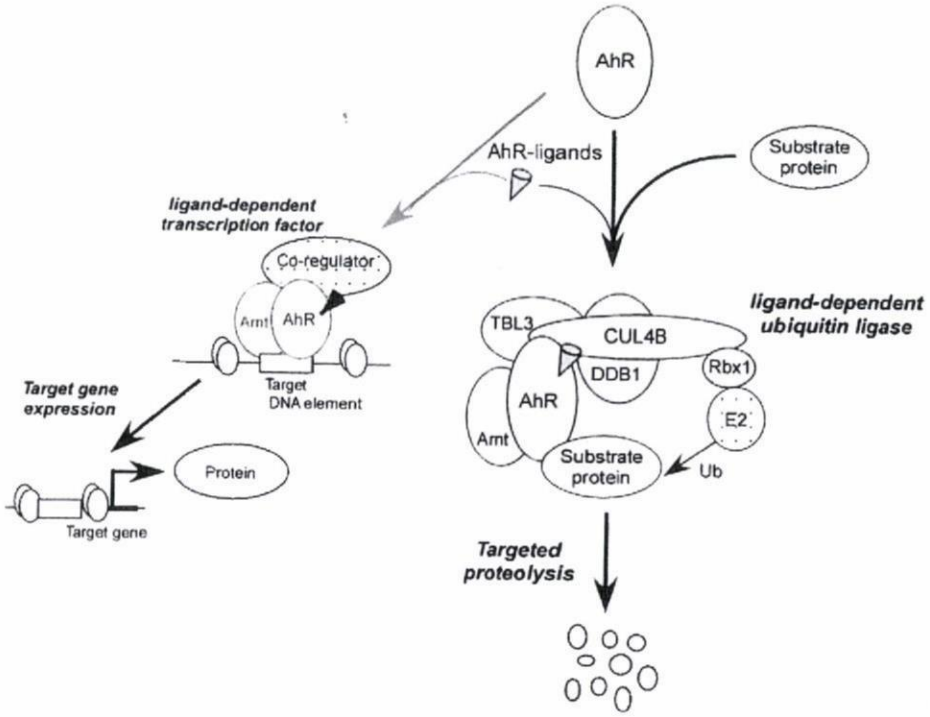

Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, CUL4B^{AhR}, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text 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

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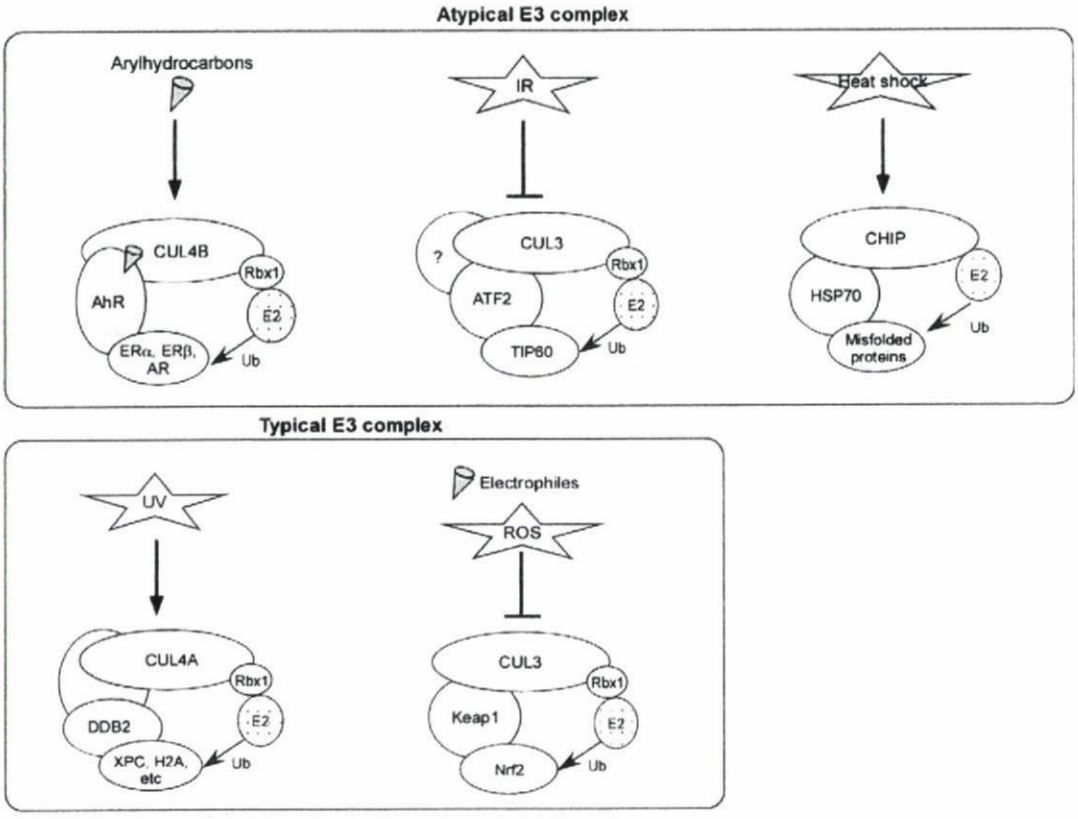

Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components 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.

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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.

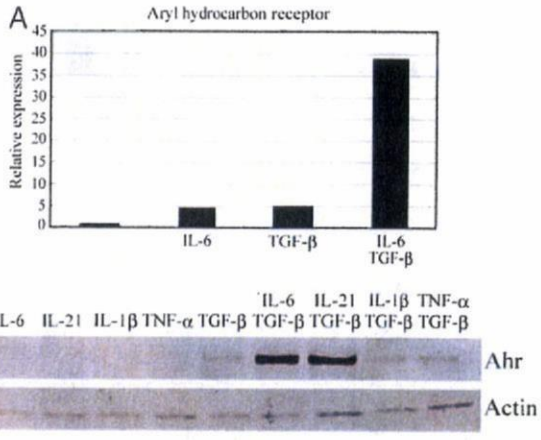

Fig. 1. Ahr is specifically expressed in Th17 cells. Isolated naïve T cells were cultured with anti-CD3/CD28 beads and the indicated cytokines for 2 days. (*A*) Gene expression profiles in nonstimulated and stimulated naïve T cells were compared by DNA microarray. (*B*) The indicated cells were lysed 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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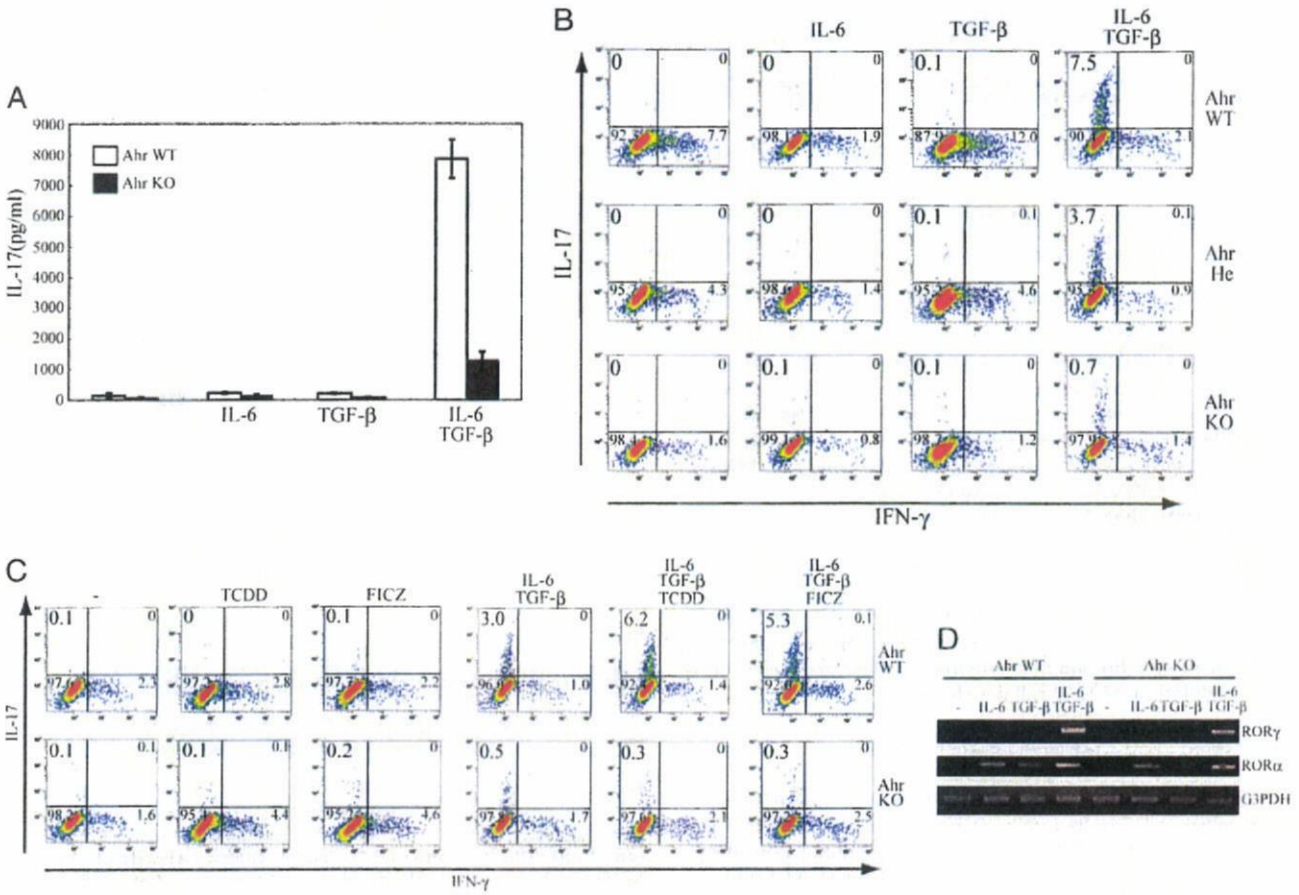

Fig. 2. Ahr deficiency reduces IL-17 production in naïve T cells. (*A*) Purified naïve T cells were stimulated with anti-CD3/CD28 beads in the presence of IL-6 or TGF-β, either alone or combined. Supernatants were collected 4 days after stimulation, and IL-17 production was measured by means of ELISA. 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.

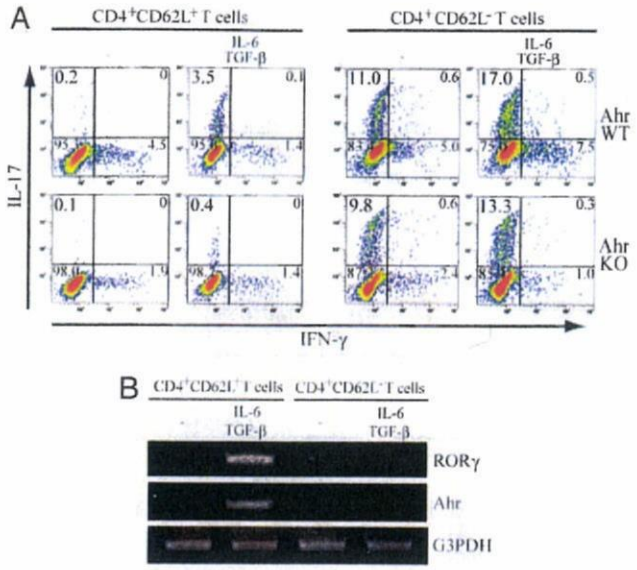

Fig. 3. Different pattern of IL-17 production between $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells. $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells isolated from WT mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6. (*A*) Three days after stimulation, cells were re-stimulated with PMA and ionomycin 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

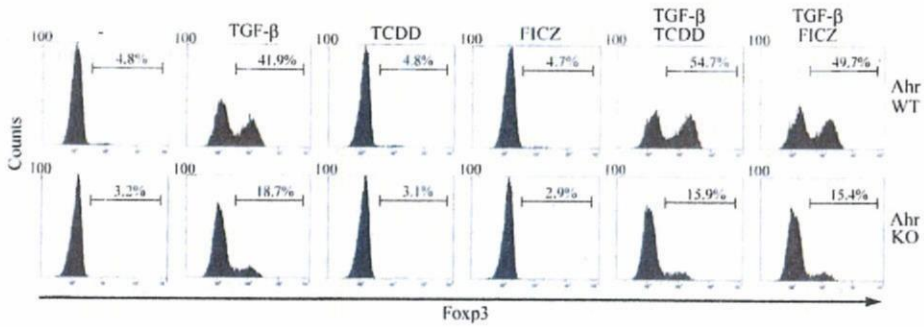

Fig. 4. Ahr partially participates in the generation of Treg cells by TGF-β. Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β with or without Ahr ligands for 2 days. Foxp3 expression was determined by staining with anti-mouse Foxp3 antibody. These data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

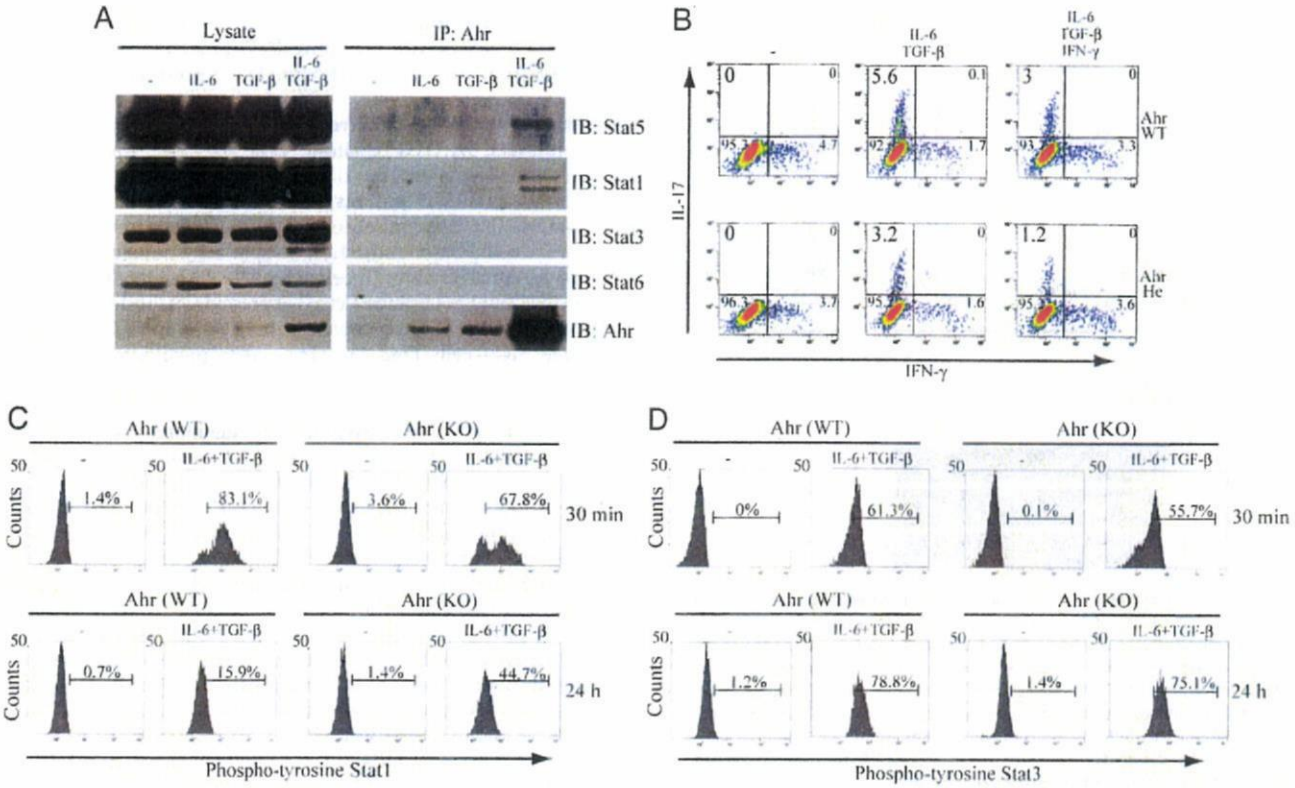

Fig. 5. Ahr regulates the activation of Stat1 in Th17 cell development. (*A*) MACS-sorted naïve T cells were cultured with anti-CD3/CD28 beads and stimulated with IL-6 or TGF-β, either alone or combined, for 2 days. Whole cell lysates were immunoprecipitated with anti-Ahr antibody, after which Stat1, 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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