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.

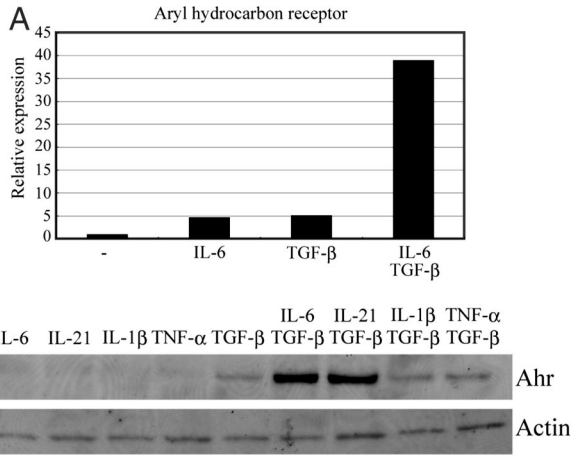

Fig. 1. Ahr is specifically expressed in Th17 cells. Isolated naïve T cells were cultured with anti-CD3/CD28 beads and the indicated cytokines for 2 days. (*A*) Gene expression profiles in nonstimulated and stimulated naïve T cells were compared by DNA microarray. (*B*) The indicated cells were lysed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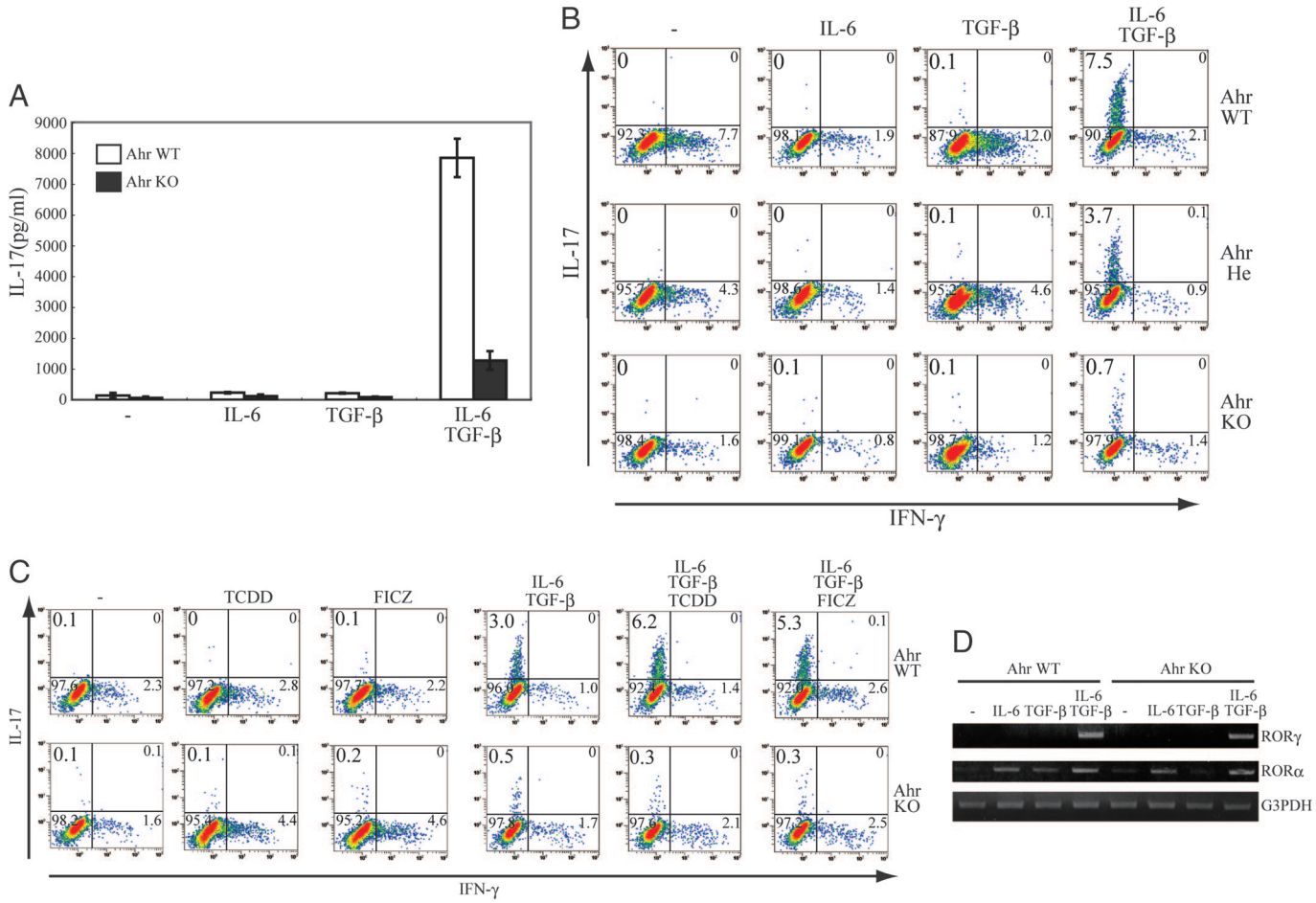

Fig. 2. Ahr deficiency reduces IL-17 production in naïve T cells. (*A*) Purified naïve T cells were stimulated with anti-CD3/CD28 beads in the presence of IL-6 or TGF-β, either alone or combined. Supernatants were collected 4 days after stimulation, and IL-17 production was measured by means of ELISA.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

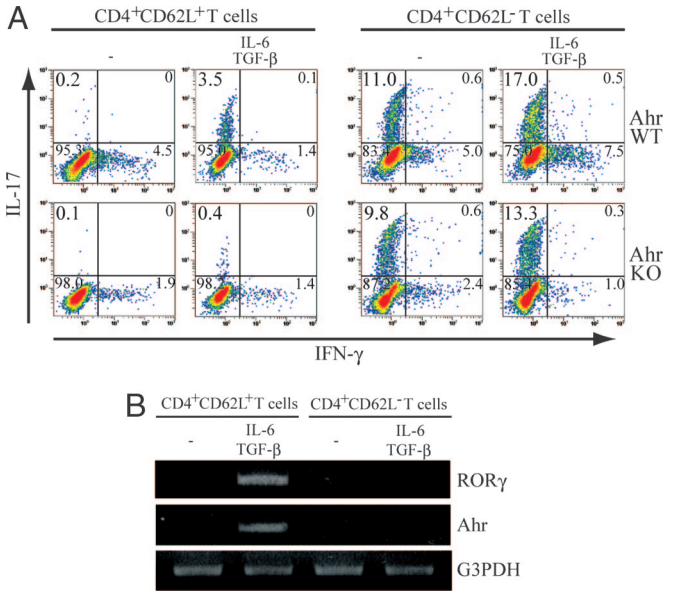

Fig. 3. Different pattern of IL-17 production between $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells. $CD4^+CD62L^-$ and $CD4^+CD62L^+$ cells isolated from WT mice were stimulated with anti-CD3/CD28 beads and TGF-β plus IL-6. (*A*) Three days after stimulation, cells were re-stimulated with PMA and ionomycin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

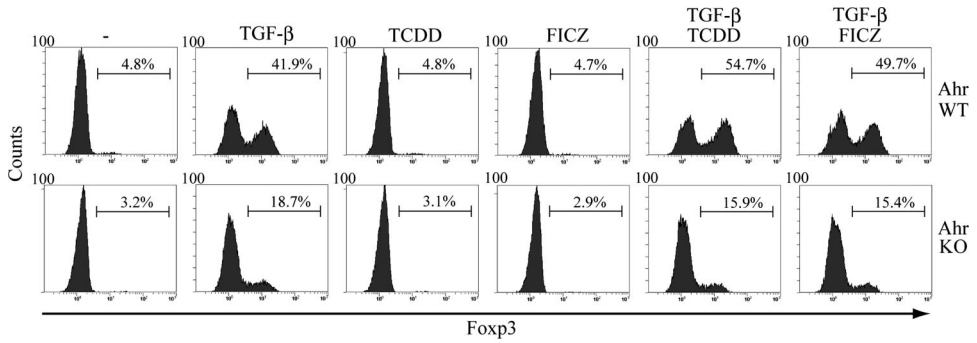

Fig. 4. Ahr partially participates in the generation of Treg cells by TGF-β. Naïve T cells isolated from Ahr WT and KO mice were stimulated with anti-CD3/CD28 beads and TGF-β with or without Ahr ligands for 2 days. Foxp3 expression was determined by staining with anti-mouse Foxp3 antibody. These data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

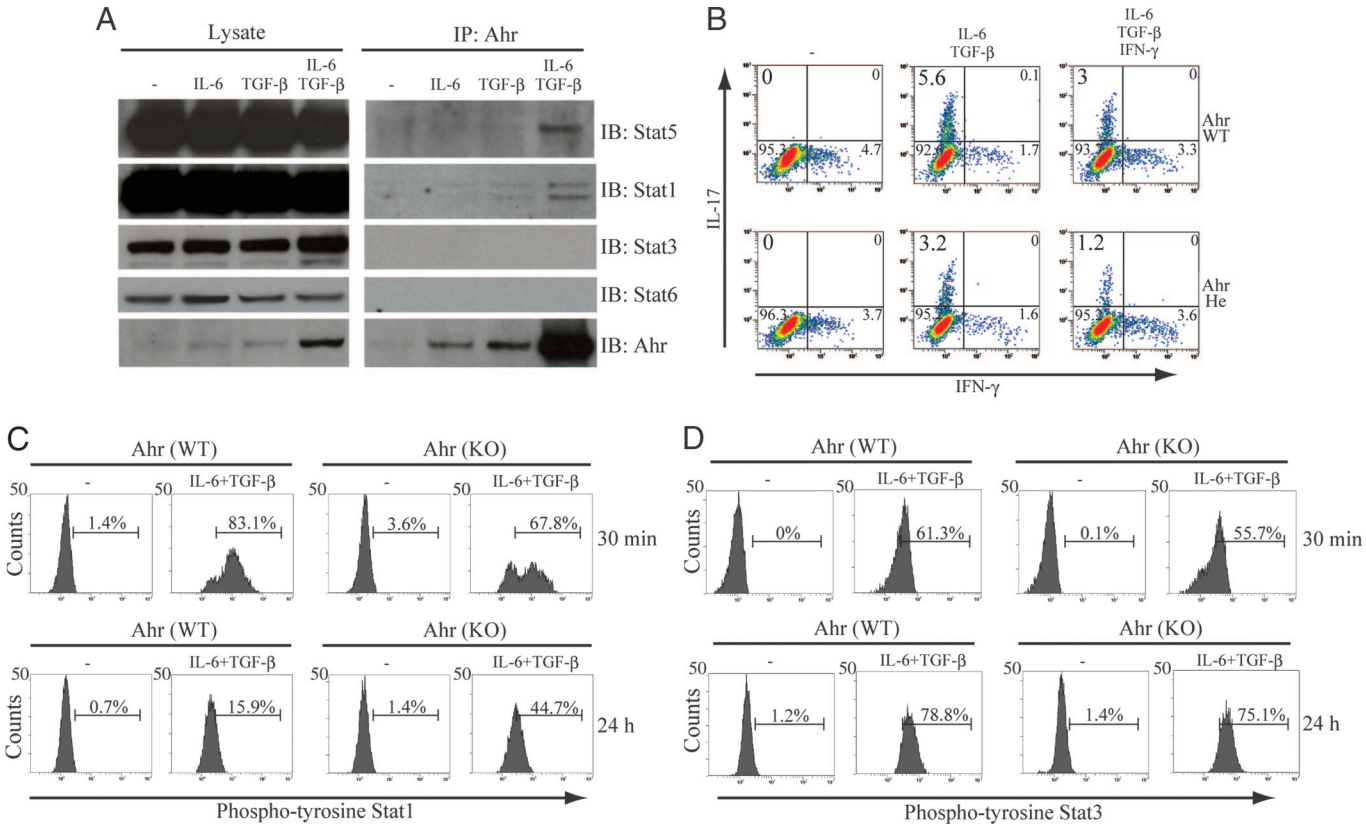

Fig. 5. Ahr regulates the activation of Stat1 in Th17 cell development. (*A*) MACS-sorted naïve T cells were cultured with anti-CD3/CD28 beads and stimulated with IL-6 or TGF-β, either alone or combined, for 2 days. Whole cell lysates were immunoprecipitated with anti-Ahr antibody, after which Stat1,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