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Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell–like and Foxp3$^+$ regulatory T cells

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Abstract

The aryl hydrocarbon receptor (AhR) participates in the differentiation of mouse regulatory T cells (T_{reg} cells) and interleukin 17 (IL-17)-producing helper T cells (T_H17 cells), but its role in human T cell differentiation is unknown. We investigated the role of AhR in the differentiation of human induced T_{reg} cells (iT_{reg} cells). We found that AhR activation promoted the differentiation of $CD4^+Foxp3^-$ T cells, which produce IL-10 and control responder T cells through granzyme B. However, activation of AhR in the presence of transforming growth factor-β1 induced $Foxp3^+$ iT_{reg} cells, which suppress responder T cells through the ectonucleoside triphosphate diphosphohydrolase CD39. The induction of functional $Foxp3^+$ iT_{reg} cells required coordinated action of the transcriptional regulators Smad1 and Aiolos. Thus, AhR is a potential target through which functional iT_{reg} cells could be induced in human autoimmune disorders.

In healthy people, the immune response is controlled by several subsets of regulatory T cells (T_{reg} cells) that are generated in the thymus (natural T_{reg}) and also in the periphery in response to various tolerogenic stimuli (induced T_{reg} cells (iT_{reg} cells)[1]. One of these subsets is a population of $CD4^+$ T cells characterized by expression of the transcription factor Foxp3 (A002750)[1]. In mice, Foxp3 is a specific marker for T_{reg} cells, and forced expression of Foxp3 (refs. [2,3]) or its induction with transforming growth factor-β1 (TGF-β1)[4] promotes the differentiation of functional $Foxp3^+$ T_{reg} cells. In humans, however, Foxp3 expression is not always linked to regulatory function: activated T cells transiently express Foxp3 (refs. [5,6]), and neither forced overexpression of Foxp3 (ref. [7]) nor its induction with TGF-β1 (ref. [8]) results in the differentiation of suppressive $Foxp3^+$ T_{reg} cells. Thus, additional signals beyond those controlled by Foxp3 are required for the generation of human functional $Foxp3^+$ T_{reg} cells.

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AUTHOR CONTRIBUTIONS

R.G., D.K., E.J.B., M.N. and A.L. did experiments; D.K. sorted cells by flow cytometry; B.D. provided advice; R.G., D.K., E.J.B., H.L.W. and F.J.Q. analyzed data; R.G. and F.J.Q. wrote the manuscript; and H.L.W. and F.J.Q. supervised the study and edited the manuscript.

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An additional subset of $CD4^+$ T_{reg} cells are the $Foxp3^-IL\text{-}10^+$ T cells, called type 1 regulatory T cells (Tr1 cells), initially generated after repeated cycles of *in vitro* stimulation in the presence of interleukin 10 (IL-10)[9]. Additional regimens, such as stimulation in the presence of IL-27, the addition of antibody to CD46 (anti-CD46), treatment with dexamethasone and vitamin D, or the administration of rapamycin and IL-10 can also promote the differentiation of Tr1 cells[10,11]. Tr1 cells are important in immune homeostasis and the control of graft-versus-host disease[10], but the signaling pathways that control their differentiation, especially in humans, are largely unknown.

Mice that lack functional $Foxp3^+$ T_{reg} cells develop severe auto-immunity[2], and T_{reg} cell deficits have been described in several human autoimmune diseases[12]. Because T_{reg} cells have positive effects in experimental models of autoimmunity, their induction is viewed as a promising approach for the treatment of human autoimmune disorders. Several methods have been reported to differentiate and expand human $Foxp3^+$ iT_{reg} cell populations *in vitro*, but their ability to produce substantial numbers of functional cells in a consistent manner is limited[13]. It is therefore important to characterize the pathways that control the generation of functional human $Foxp3^+$ iT_{reg} cells.

The ligand-activated transcription factor aryl hydrocarbon receptor (AhR (A000229)) controls the differentiation of mouse T_{reg} cells and IL-17-producing helper T cells (T_H17 cells) *in vitro* and *in vivo*[14–20]. Activation of AhR by its high-affinity ligand TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) *in vivo* results in the induction of $CD4^+CD25^+Foxp3^+$ T_{reg} cells[14]. These cells are functional and suppress the development of experimental autoimmune encephalo-myelitis[14], experimental autoimmune uveoretinitis[18] and spontaneous autoimmune diabetes[21]. However, it is not known whether AhR signaling can be exploited to promote the differentiation of functional human iT_{reg} cells.

To address that question, we investigated the effect of AhR ligands on the differentiation of human Tr1 and $Foxp3^+$ iT_{reg} cells. We found that AhR activation induced human Tr1-like cells that suppressed responder T cells by a granzyme B–dependent mechanism. In addition, AhR activation in the presence of TGF-β1 induced the differentiation of functional human $Foxp3^+$ iT_{reg} cells that suppressed responder T cells through the ectonucleoside triphosphate diphosphohydrolase CD39. The induction of functional $Foxp3^+$ iT_{reg} cells by the concurrent activation of TGF-β1 and AhR signaling was mediated, at least partially, by the transcription factors Smad1 and Aiolos. Thus, our data suggest that AhR might be an important target for the generation of various types of T_{reg} cells in humans and that nontoxic AhR ligands could provide new drug candidates for the induction of T_{reg} cells *in vivo* and for the management of autoimmune diseases.

RESULTS

AhR activation induces T cells that produce IL-10

AhR participates in the differentiation of mouse $Foxp3^+$ T_{reg} cells[14–18]. To investigate whether AhR contributes to the differentiation of human T_{reg} cells, we isolated naive $CD4^+$ T cells from peripheral blood mononuclear cells obtained from healthy donors and activated them with anti-CD3, anti-CD28 and IL-2 with or without the AhR ligand TCDD (Supplementary Fig. 1). Naive T cells differentiated in the presence of TCDD showed a much lower proliferative response after restimulation (Fig. 1a). Moreover, T cell activation in the presence of TCDD upregulated expression of the AhR target gene *CYP1A1* (which encodes a cytochrome p450 protein)[22] but did not modify the expression of *AHR*, as measured by quantitative real-time PCR (Fig. 1b).

To characterize the phenotype of the human T cells treated with TCDD, we analyzed expression of the lineage-specific transcription factor genes *FOXP3* (T_{reg} cells), *GATA3* (T helper type 2 (T_H2) cells), *TBX21* (T_H1 cells) and *RORC* (T_H17 cells). Activation of naive T cells in the presence of TCDD led to significant upregulation of *FOXP3* expression (Fig. 1c). However, we did not detect upregulation of Foxp3 by flow cytometry (Supplementary Fig. 2). We detected no change in the expression of *GATA3* or *RORC*, but there was significantly lower *TBX21* expression after treatment with TCDD (Fig. 1c). In addition, TCDD-treated T cells expressed significantly more *IL10* concomitant with significantly lower expression of *IFNA1* (encoding interferon-γ), *IL2* and *IL17A* than that of untreated T cells (Fig. 1d). We found no difference in the expression of *TNF* or *TGFB1* (Fig. 1d). We obtained similar results when we activated human naive $CD4^+$ T cells in the presence of the AhR ligand FICZ (6-formylindolo[3,2-b]carbazole; data not shown).

AhR transactivates the *IL10* promoter

The transcription factor c-Maf (A003947) controls the synthesis of IL-10 by mouse T cells[23–25]. To further characterize the IL-10-producing T cells induced by AhR activation, we studied the expression of *MAF* (which encodes c-Maf) by quantitative real-time PCR. We found that *MAF* was expressed by TCDD-treated and IL-27 induced Tr1 cells, but its expression was not significantly higher in those cells than in control cells (Supplementary Fig. 3).

To investigate whether the effect of TCDD on *IL10* expression was mediated by AhR, we knocked down AhR expression with small interfering RNA (siRNA). After 6 d, siRNA decreased the expression of *AHR* mRNA by 75% (Supplementary Fig. 4a). Correspondingly, the knockdown of *AHR* suppressed the induction of *IL10* by TCDD (Supplementary Fig. 4a). Thus, AhR controls *IL10* expression by TCDD-treated T cells.

Given the importance of AhR for the expression of *IL10* by TCDD-treated T cells, we hypothesized that AhR transactivates the human *IL10* promoter. Moreover, given the expression of *MAF* by TCDD-treated cells (Supplementary Fig. 3) and its reported interaction with the mouse *Il10* promoter[25], we investigated the role of c-Maf in regulating human *IL10* expression. We used bioinformatics analysis to identify a potential AhR-binding site (xenobiotic response element (XRE)) that partially overlaps with a c-Maf-recognition element (MARE) in the *IL10* promoter (Supplementary Fig. 4b).

To determine whether AhR can bind the XRE in the *IL10* promoter, we used electrophoretic mobility-shift assay to study the interaction between an oligonucleotide containing the XRE and *in vitro*–translated AhR protein in complex with the AhR nuclear translocator. *In vitro*–translated AhR–AhR nuclear translocator complexes bound the XRE in the *IL10* promoter, and this interaction was significantly lower after the addition of an excess of a competitor oligonucleotide containing an XRE found in *CYP1A1* (Supplementary Fig. 4c). To investigate whether AhR interacts with the XRE in the *IL10* promoter in TCDD-treated cells, we used chromatin immunoprecipitation (ChIP) assays. AhR bound substantially to the *IL10* promoter region that contained the XRE in TCDD-treated T cells but not in control T cells (Supplementary Fig. 4d). We also detected a substantial interaction between c-Maf and the MARE in the *IL10* promoter in TCDD-treated cells (Supplementary Fig. 4d). Thus, AhR and c-Maf interact with the XRE and MARE, respectively, in the *IL10* promoter in TCDD-treated cells.

To analyze the functional relevance of the binding of AhR and c-Maf to the *IL10* promoter, we did reporter assays using a construct containing the firefly luciferase gene under the control of the human *IL10* promoter[26]. AhR and c-Maf separately transactivated the *IL10* promoter; this transactivation was greater after activation of transfected Jurkat cells with anti-CD3 and anti-CD28 (Supplementary Fig. 4e). Notably, cotransfection with plasmids encoding both

AHR and *MAF* resulted in an additive transactivation of *IL10* (Supplementary Fig. 4e). Similarly, forced overexpression of *AHR* in naive human $CD4^+$ T cells followed by treatment with TCDD triggered *IL10* expression, which was further upregulated by forced coexpression of *MAF* (Supplementary Fig. 4f). In summary, these data suggest that AhR and c-Maf act together to control the transcription of *IL10*.

It has been shown that c-Maf interacts physically with other transcription factors whose responsive elements are located close to MARE motifs in target genes[27]. Therefore, we investigated the interaction between AhR and c-Maf in coimmunoprecipitation experiments with constructs encoding AhR tagged with hemagglutinin and c-Maf tagged with the red fluorescent protein mCherry. We found that AhR and c-Maf precipitated together when we used anti-hemagglutinin or anti-mCherry but not when we used an isotype-matched control antibody (Supplementary Fig. 4g). These data suggest that AhR interacts with c-Maf to control the transcriptional activity of the *IL10* promoter.

AhR activation induces human Tr1-like cells

The production of IL-10 and the lower proliferative response of TCDD-treated T cells resembled the phenotype of Tr1 cells, which have *in vitro* suppressive activity[9]. We therefore analyzed the suppressive activity of TCDD-treated T cells in coculture assays. Human TCDD-treated T cells showed suppressive activity *in vitro* (Fig. 2a). This suppressive activity was controlled by AhR, as it was abrogated when we knocked down *AHR* expression with a specific siRNA (Fig. 2b). To further characterize the suppressive activity of the IL-10-producing T cells induced by AhR activation, we resorted TCDD-treated T cells, washed them extensively and cultured them together with responder T cells stained with annexin V and 7-amino-actinomycin D (7-AAD) after 3 d of coincubation. Coincubation of responder T cells with TCDD-treated T cells resulted in a greater frequency of annexin V–positive, $7\text{-}AAD^-$ (early apoptotic) cells and annexin V–positive, $7\text{-}AAD^+$ (late apoptotic and/or dead) cells (Fig. 2c,d), which suggested that suppressive TCDD-treated T cells trigger apoptosis in responder T cells.

T_{reg} cells use several mechanisms to control the activity of effector T cells[28]. Using a Transwell system, we found that the suppressive activity of TCDD-treated cells required cell contact (Fig. 3a). Granzyme-triggered apoptosis can mediate the suppressive activity of Tr1 cells in a cell contact–dependent manner[29], so we used quantitative real-time PCR to analyze the expression of *GZMA* (encoding granzyme A) and *GZMB* (encoding granzyme B) in TCDD-treated T cells. We found significantly higher *GZMB* expression in TCDD-treated T cells (Fig. 3b) but no significant change in the expression of *GZMA* (data not shown). We also found significantly higher granzyme B expression when we used flow cytometry to analyze TCDD-treated T cells (Fig. 3c). To investigate whether the induction of *GZMB* by TCDD was mediated by AhR, we knocked down *AHR* expression with siRNA (Fig. 3d). Knockdown of *AHR* significantly decreased the induction of *GZMB* expression by TCDD (Fig. 3d). Thus, AhR activation directly or indirectly upregulates the expression of *GZMB*.

To assess the relevance of granzyme B for the suppressive activity of TCDD-treated T cells, we used the granzyme B inhibitor AAD-CMK (benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone). AAD-CMK abrogated the suppressive activity of TCDD-treated T cells (Fig. 3e). However, consistent with published reports[30], inhibition of caspase-3 activity had no significant effect on the suppressive activity (Fig. 3e). Moreover, knockdown of *GZMB* expression with specific siRNA abrogated the suppressive activity of TCDD-treated T cells *in vitro* (Fig. 3f). Neutralizing antibodies to other known suppressive molecules, such as IL-10, TGF-β, Fas or FasL, had no significant effect on the suppressive activity of TCDD-treated T cells (data not shown). Together these data show that activation of AhR induces Tr1-like cells that control responder T cells in a granzyme B–dependent manner.

AhR activation plus TGF-β1 induce FOXP3$^+$ T cells

TGF-β1 has an important role in the differentiation, maintenance and function of T_{reg} cells[31]. TGF-β1 promotes the differentiation of functional Foxp3$^+$ T_{reg} cells in the mouse[4]; however, although naive human T cells activated in the presence of TGF-β1 express Foxp3, they are not endowed with suppressive activity[8]. Given the reported effects of AhR[14–16,18,21] and TGF-β1 (ref. [4]) on mouse Foxp3$^+$ T_{reg} cells, we investigated the combined effect of TGF-β1 and TCDD-mediated activation of AhR on naive human T cells.

Naive T cells activated in the presence of TGF-β1 and TCDD showed a significantly smaller proliferative response after being restimulated with beads coated with anti-CD3 and anti-CD28 than did control or TGF-β1-treated T cells (Fig. 4a). Furthermore, activation of T cells in the presence of TGF-β1 or of TGF-β1 plus TCDD resulted in significant upregulation of *AHR* expression (Fig. 4b, left). The upregulation of *AHR* expression by TGF-β1 led to only a little induction of the AhR-controlled gene *CYP1A1*, but we achieved higher *CYP1A1* expression after treating cells with both TGF-β1 and TCDD (Fig. 4b, right).

We then analyzed the expression of lineage-specific transcription factors in T cells treated with TCDD and TGF-β1. The upregulation of *FOXP3* was similar in naive human T cells treated with TGF-β1 and in those treated with TGF-β1 plus TCDD (Fig. 4c). This upregulation of *FOXP3* expression was 50 times greater than the upregulation that followed treatment with TCDD alone (Figs. 1c and 4c) and we also detected it by flow cytometry (Supplementary Figs. 2 and 5). Treatment with TGF-β1 plus TCDD, but not with TGF-β1 alone, resulted in significant downregulation of the expression of *GATA3* and *TBX21* (Fig. 4c). Moreover, treatment with TGF-β1 plus TCDD suppressed the induction of *RORC* expression triggered by TGF-β1 alone (Fig. 4c). In T cells treated with TGF-β1 plus TCDD, the expression of *TGFB1* was upregulated (Fig. 4d) and the expression of *IFNA1*, *IL2* and *IL17* was downregulated, but that of *IL10* and *TNF* did not change (Fig. 4d).

TCDD plus TGF-β1 induce functional Foxp3$^+$ T_{reg} cells

Naive human T cells activated in the presence of TGF-β1 express Foxp3 but do not have suppressive activity[8]. However, T cells treated with TGF-β1 plus TCDD were suppressive *in vitro* (Fig. 5a), and their suppressive activity was greater than that of T cells treated with TCDD alone (Figs. 2a and 5a). The suppressive activity of T cells treated with TGF-β1 and TCDD was mediated by a cell contact–dependent mechanism (Fig. 5b) independently of granzyme B (Supplementary Fig. 6).

We used gene microarray to analyze the transcriptional profile of T cells treated with TGFβ-1 and TCDD to identify the mechanism of suppression used by human T_{reg} cells induced by AhR stimulation in presence of TGF-β1. We found significantly higher expression of *ENTPD1* (encoding CD39) in T cells treated with TGF-β plus TCDD (Fig. 5c). We obtained similar results when we analyzed *ENTPD1* expression in an independent set of samples by quantitative real-time PCR (Fig. 5d) and by flow cytometry (Fig. 5e). To investigate whether the induction of *ENTPD1* was mediated by AhR, we knocked down *AHR* expression with short hairpin RNA (shRNA). After 6 d, *AHR* expression was 45% lower (Fig. 5f). The knockdown of *AHR* resulted in significantly lower *ENTPD1* expression in T cells differentiated with TGF-β1 plus TCDD (Fig. 5f). Thus, AhR activation in the presence of TGF-β1 controls the expression of *ENTPD1*.

CD39 (ENTPD1) hydrolyzes ATP and mediates the suppressive activity of murine and human Foxp3$^+$ T_{reg} cells[32]. Neutralizing antibodies to CD39 abrogated the suppressive activity of T cells treated with TGF-β plus TCDD (Fig. 5g). Together these results show that activation of

AhR in the presence of TGF-β induces functional human Foxp3$^+$ T_{reg} cells that suppress effector T cells by a CD39-dependent mechanism.

AhR activation plus TGF-β1 induces Smad1 and Aiolos

Naive human T cells activated in the presence of TGF-β1 express Foxp3 but do not have suppressive activity[8]. On the basis of the finding that suppressive human Foxp3$^+$ T_{reg} cells can be induced by TGF-β1 plus TCDD, we hypothesized that AhR activation might induce additional transcription factors needed for the induction of functional Foxp3$^+$ T_{reg} cells. Gene microarray showed that expression of the transcription factors Smad1 and Aiolos (encoded by *IKZF3*) was significantly upregulated in T cells treated with TGF-β1 plus TCDD (Fig. 6a,b). We confirmed that upregulation by quantitative real-time PCR in an independent set of samples (Fig. 6c,d). To investigate whether the induction of Smad1 and Aiolos was mediated by AhR, we knocked down *AHR* expression with shRNA. Knockdown of *AHR* resulted in significantly lower expression of *SMAD1* and *IKZF3* in T cells differentiated with TGF-β1 plus TCDD (Fig. 6e,f). Thus, AhR activation in the presence of TGF-β1 upregulates the expression of Smad1 and Aiolos.

Smad1 regulates Foxp3 enhancer activity

Smad1 belongs to a family of transcription factors involved in TGF-β signaling. Smad3 binds to an enhancer in the region of positions +2079 to +2198 of human *FOXP3* and thus controls its expression[33]. Because Smad3 and Smad1 recognize similar DNA-binding motifs[34], we investigated the binding of Smad1 to the enhancer in this region (+2079 to +2198) in *FOXP3* in human T cells treated with TGF-β1 and TCDD. Using ChIP, we found that the interaction between Smad1 and the *FOXP3* enhancer located in this region was upregulated in T cells treated with TGF-β1 and TCDD compared with that of control T cells (Fig. 7a).

To investigate the functional relevance of the binding of Smad1 to the enhancer we transfected the EL4 mouse lymphoma cell line with a reporter system in which six copies of the Smad-binding motif in the *Foxp3* enhancer in the region of positions +2079 to +2198 control the expression of firefly luciferase[33] (Supplementary Fig. 7a). As reported before[33] luciferase activity was induced after cotransfection with a vector encoding Smad3 (Fig. 7b). Moreover, luciferase activity was also induced after cotransfection with a construct encoding Smad1 (Fig. 7b). We obtained similar results when we investigated the ability of constructs encoding Smad1 or Smad3 to activate a luciferase reporter controlled by the *Foxp3* enhancer in this region[33] (Fig. 7c and Supplementary Fig. 7b). Thus, stimulation with TGF-β1 concurrent with activation of AhR results in the induction of Smad1, which binds and activates the *Foxp3* enhancer in the region of positions +2079 to +2198.

To investigate the effect of the binding and activation of the *FOXP3* enhancer in that region by Smad1 on *FOXP3* expression, we knocked down *SMAD1* expression with lentivirus-delivered shRNA in naive human T cells activated in the presence of anti-CD3, anti-CD28 and IL-2 with TGF-β1 and TCDD. The expression of *SMAD1* mRNA was 80% lower after treatment with the Smad1-specific shRNA (Fig. 7d). Correspondingly, the knockdown of *SMAD1* led to significantly lower expression of *FOXP3* and *ENTPD1* by Foxp3$^+$ T_{reg} cells induced with TGF-β1 and TCDD. Conversely, *IL2* expression was significantly upregulated after knockdown of *SMAD1* (Fig. 7d). Moreover, the knockdown of *SMAD1* led to significantly lower suppressive activity of Foxp3$^+$ T_{reg} cells induced with TGF-β1 plus TCDD (Fig. 7e). Together these data show that Smad1 controls the expression of *FOXP3* and the suppressive activity in Foxp3$^+$ T_{reg} cells that have been differentiated with TCDD and TGF-β1.

Aiolos-Foxp3 interaction silences *IL2* expression

Aiolos is a transcription factor of the Ikaros family; members of this family are important in the development of hematopoietic cells[35]. Eos, another member of the Ikaros family, forms a complex with Foxp3 and mediates the silencing of target genes such as *Il2* in mouse $Foxp3^+$ T_{reg} cells[36]. DNA target sequences bound by members of the Ikaros family are highly conserved[37], and Eos interacts with the *Il2* promoter[36], so we used ChIP assays to investigate whether Aiolos binds the *IL2* promoter in human T cells activated with TGF-β1 and TCDD. The binding of Aiolos to the Eos-binding site in the *IL2* promoter was greater in naive T cells activated in the presence of TGF-β1 and TCDD (Fig. 8a).

Eos and Foxp3 form a protein complex that represses the expression of target genes[36]. To investigate whether Aiolos and Foxp3 can interact physically, we did coimmunoprecipitation studies with constructs encoding Foxp3 and Flag-tagged Aiolos. Anti-Foxp3 and anti-Aiolos precipitated a protein complex containing Foxp3 and Aiolos, but control IgG did not (Fig. 8b). Thus, Foxp3 and Aiolos physically associate with each other.

Aiolos can form homodimers and can also form complexes with other proteins through interactions mediated by zinc fingers in its C-terminal domain[37]. There are also four more zinc fingers in the N-terminal portion of the protein. To determine whether the interaction of Aiolos with Foxp3 was mediated by its C-terminal domain, we did coimmunoprecipitation studies with a vector encoding the Aio-1–5a isoform of Aiolos, which lacks the two C-terminal zinc fingers involved in homo- and heterodimerization[38] (Fig. 8c). As a control, we used the Aio-Δ3,4,5 isoform, which lacks all the zinc fingers at the N terminus but retains the C-terminal dimerization domain (Fig. 8c). Like full-length Aiolos, Aio-Δ3,4,5 interacted with Foxp3 (Fig. 8d). However, the deletion of the C-terminal domain in Aio-1–5a disrupted its interaction with Foxp3 (Fig. 8d). Therefore, like Eos, Aiolos forms a complex with Foxp3 through interactions mediated by its C-terminal zinc fingers.

Eos participates in the repression of *Il2* expression by Foxp3 (ref. [36]). Naive T cells activated in the presence of TGF-β1 plus TCDD had lower *IL2* expression (Fig. 4d) and concomitant induction of Aiolos (Fig. 6b,d). To investigate the role of Aiolos in the repression of *IL2* expression by Foxp3, we did knockdown experiments with naive human T cells activated in the presence of anti-CD3, anti-CD28 and IL-2 with TGF-β1 and TCDD using lentivirus-delivered shRNA specific for *IKZF3*. Treatment with shRNA resulted in significantly lower expression of *IKZF3* (Fig. 8e) and higher *IL2* expression in T cells treated with TGF-β1 plus TCDD (Fig. 8e). *Foxp3* expression is independent of Eos[36]. Accordingly, the knockdown of *IKZF3* did not result in significant changes in *FOXP3* expression (Fig. 8e). However, the knockdown of *IKZF3* led to significantly lower suppressive activity of $Foxp3^+$ T_{reg} cells induced with TGF-β1 plus TCDD (Fig. 8f). Together these data suggest that Foxp3 forms a complex with Aiolos to repress target genes and control the suppressive activity of $Foxp3^+$ T_{reg} cells that have been differentiated with TCDD and TGF-β1.

DISCUSSION

Here we have shown that activation of AhR promoted the differentiation of functional human Tr1 or $Foxp3^+$ iT_{reg} cells *in vitro*, depending on the cytokine context. Activation of $CD4^+$ T cells in the presence of AhR ligands promoted the differentiation of $Foxp3^-$ IL-10-producing T cells that controlled responder T cells through granzyme B. However, activation of AhR in the presence of TGF-β1 induced $Foxp3^+$ T_{reg} cells that suppressed responder T cells through CD39. The induction of functional $Foxp3^+$ T_{reg} cells by the concurrent activation of TGF-β1 and AhR signaling was mediated at least in part by the transcription factors Smad1 and Aiolos. Smad1 regulated the *FOXP3* enhancer located in positions +2079 to +2198, and Aiolos formed a complex with Foxp3 to silence *IL2* expression. Thus, in different cytokine milieus, AhR

activation can promote the differentiation of human suppressive Tr1 or Foxp3$^+$ T_{reg} cells or proinflammatory T_H17 cells[20].

Activation of naive human CD4$^+$ T cells in the presence of AhR ligands resulted in the differentiation of suppressive Tr1-like cells. Tr1 cells are Foxp3$^-$ T_{reg} cells that produce IL-10 (ref. [9]), and in different *in vitro* and *in vivo* scenarios they also produce TGF-β1, IL-5 and interferon-γ and have transient low expression of Foxp3 (refs. [10,11]). The phenotypic diversity of Tr1 cells and the disparate settings that promote their differentiation[10,11] suggest that there are several lineages of CD4$^+$IL-10$^+$ T_{reg} cells. Accordingly, transcription factors such as Sp1, Sp3, C/EBP-β, IRF1, STAT3 and c-MAF can activate the *Il10* promoter[24]. In particular, c-MAF has been proposed to be a universal transcription factor that regulates the production of IL-10 by T cells[24]. Our data, together with those of Apetoh *et al.* published in this issue of *Nature Immunology*[39], show that AhR interacts with c-Maf to control the transcription of *IL10*.

The production of IL-10 does not necessarily result in a suppressive phenotype; proinflammatory IL-9-producing helper T cells, for example, have high expression of IL-10 (ref. [40]). We found that the Tr1-like cells induced by AhR activation suppressed effector T cells by a granzyme B–dependent mechanism independently of IL-10. TCDD-induced mouse Tr1-like cells also express granzyme B[41]. However, we do not believe that AhR alone or in combination with c-Maf acts as a lineage-specification transcription factor for Tr1 cells. AhR is expressed by many lineages in addition to Tr1 cells, such as Foxp3$^+$ T_{reg} cells[14,16] and T_H17 cells14,16,20. Similarly, c-Maf is expressed not only by Tr1 cells but also by T_H1 cells24 and T_H17 cells[25]. Instead, our findings, along with those of Apoteh *et al.*[39], suggest that AhR acts in synergy with c-Maf and other transcription factors to control part of the transcriptional program linked to Tr1 cell differentiation.

In humans, neither forced Foxp3 overexpression[7] nor Foxp3 expression triggered by TGF-β1 alone[8] results in the differentiation of suppressive Foxp3$^+$ T_{reg} cells, which indicates that additional signals beyond those controlled by Foxp3 are required for the generation of functional Foxp3$^+$ T_{reg} cells. We found that the differentiation of suppressive Foxp3$^+$ T_{reg} cells by the concurrent activation of AhR and TGF-β1 signaling required the combined activities of Smad1 and Aiolos. A conserved noncoding sequence (CNS-1) in *Foxp3* controls the differentiation of Foxp3$^+$ iT_{reg} cells in gut-associated lymphoid tissue[42]. The CNS-1 region in *FOXP3* contains a functional Smad-binding site that interacts with Smad3 and drives *FOXP3* expression upon activation with TGF-β1 (ref. [33]). We found that T cell activation in the presence of TGF-β1 plus TCDD induced the expression of Smad1, which interacted with the Smad-binding motif in CNS-1 to promote *FOXP3* expression. It has been proposed that stable Foxp3 expression is achieved when the transcription factors NFAT, Smad, CREB and c-Rel form an enhanceosome that transactivates *FOXP3* (ref. [43]). In this model, various members of the Smad or NFAT family can be incorporated into the enhanceosome that drives *FOXP3* expression[43]. Our data suggest that in the Foxp3$^+$ iT_{reg} cells induced *in vitro* by the concomitant activation of TGF-β1 and AhR signaling, Smad1 alone or in combination with Smad3 and/or Smad4 interacts with CNS-1 to activate *FOXP3* expression.

Proteins of the Ikaros family share a DNA-binding domain that recognizes sequences containing the GGGA core motif[37]. Accordingly, we found that Aiolos and Eos bound the same DNA motif in *IL2* to inhibit its expression. Members of the Ikaros family control gene expression by anchoring protein complexes that regulate chromatin remodeling and histone deacetylation in target genes[44]. In addition, proteins in the Ikaros family can also repress gene expression by mechanisms independent of chromatin remodeling and histone deacetylation[45]. Together our data suggest that Aiolos interacts with Foxp3 to silence the

transcriptional program of effector T cells and further promote the differentiation of functional Foxp3$^+$ T_{reg} cells.

It has been suggested that Tr1 cells and Foxp3$^+$ iT_{reg} cells constitute alternative fates of T cell differentiation whose immunoregulatory function might be partially redundant in gut-associated lymphoid tissue[42]. Here we have reported that depending on the cytokine milieu, AhR activation promoted the differentiation of human Tr1 or Foxp3$^+$ T_{reg} cells. Thus, it is conceivable that AhR ligands provided by the diet[46] or the intestinal flora[47] influence the differentiation of Tr1, Foxp3$^+$ iT_{reg} and T_H17 cells *in vivo*. Moreover, our data suggest that AhR ligands can be used to promote iT_{reg} cell differentiation *in vitro*, which could be exploited to generate functional iT_{reg} cells *in vitro* for adoptive-transfer regimes aimed at reestablishing immune tolerance. Alternatively, nontoxic AhR ligands could constitute potential new drugs for the therapeutic induction of T_{reg} cells *in vivo* and the management of autoimmune disorders.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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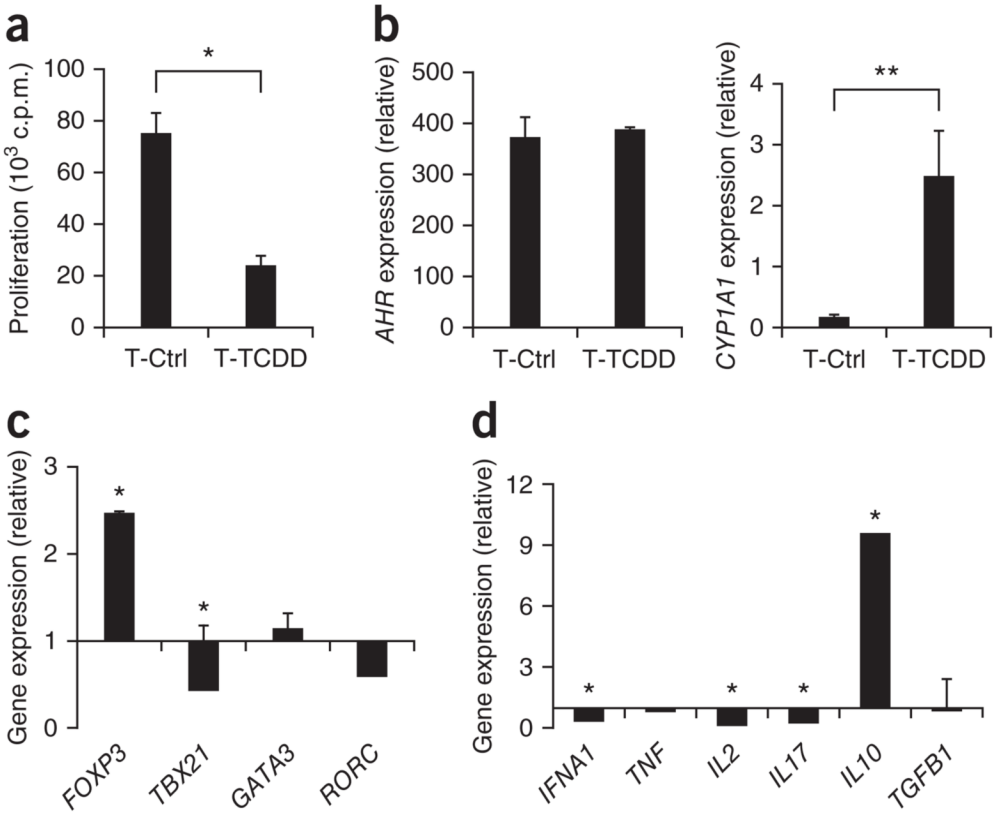

Figure 1.

AhR activation induces T cells that produce IL-10. (**a**) Proliferative response of human naive $CD4^+$ T cells activated for 6 d with plate-bound anti-CD3 and anti-CD28, with (T-TCDD) or without (T-Ctrl) TCDD, then restimulated with bead-conjugated anti-CD3 and anti-CD28. (**b**–**d**) Real-time PCR analysis of the expression of *AHR* and the AhR target *CYP1A1* (**b**), *FOXP3*, *TBX21*, *GATA3* and *RORC* (**c**) and cytokines (**d**) on differentiated T cells (only TCDD-treated cells in **c**,**d**); results are presented relative to the expression of *GAPDH* (encoding glyceraldehyde phosphate dehydrogenase; **b**–**d**) and as the ratio of expression in T-TCDD cells to that in T-Ctrl cells (**c**,**d**). *$P < 0.05$ and **$P < 0.01$, compared with T-Ctrl (Student's *t*-test). Data are representative of five experiments (**a**; mean + s.d. of triplicate wells) or represent one of three to five independent experiments (**b**–**d**; mean + s.d. of duplicates).

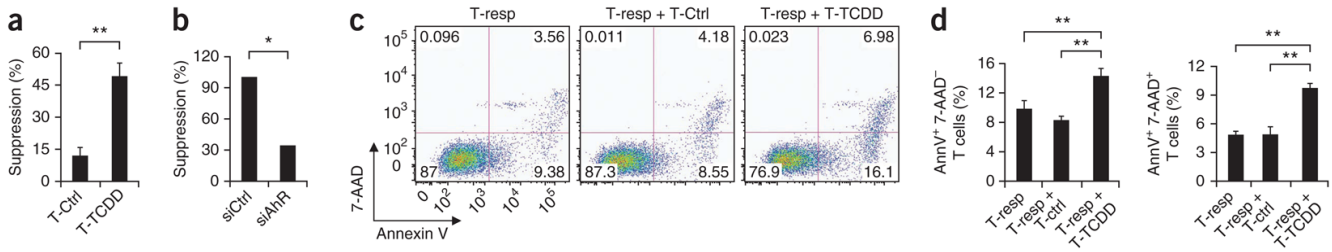

Figure 2.

AhR activation induces human Tr1-like cells. (**a**) Suppressive activity of human naive $CD4^+$ T cells activated for 6 d with plate-bound anti-CD3 and soluble anti-CD28, with or without TCDD. (**b**) Effect of *AHR* knockdown on the suppressive activity of TCDD-treated T cells transduced with non–target-specific control siRNA (siCtrl) or *AHR*-specific siRNA (siAhR). (**c**) Flow cytometry of annexin V and 7-AAD in responder human $CD4^+$ T cells (T-resp) coincubated with T cells with or without TCDD treatment. Numbers in quadrants indicate percent cells in each. (**d**) Frequency of annexin V–positive ($AnnV^+$), $7\text{-}AAD^-$ T cells (left) and annexin V–positive, $7\text{-}AAD^+$ T cells (right) in the cocultures in **c**. $^*P < 0.05$ and $^{**}P < 0.01$, compared with T-Ctrl (Student's *t*-test). Data are representative of five experiments (**a**; mean + s.d. of triplicate wells), two experiments (**b**), three to eight independent experiments (**c**) or three independent experiments (**d**; mean + s.d.).

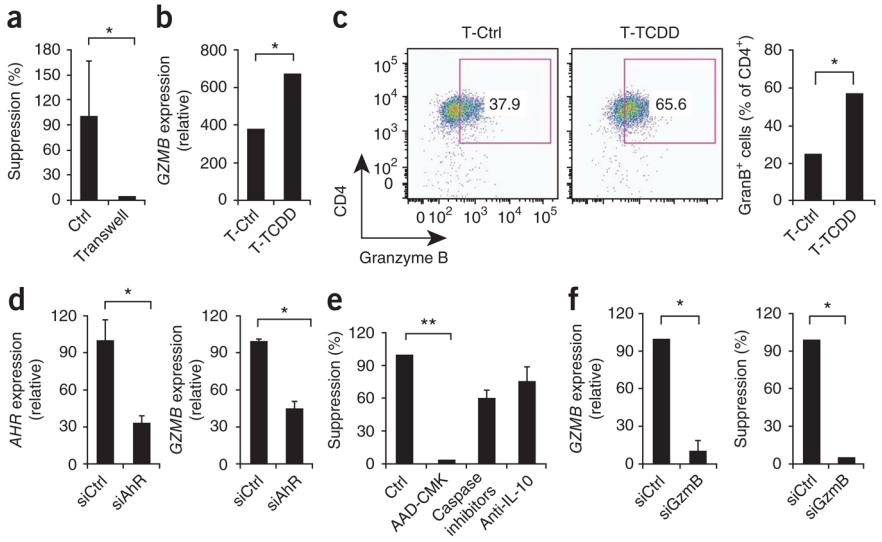

Figure 3.

The suppressive activity of Tr1-like cells induced by AhR activation is mediated by granzyme B. (**a**) Suppressive activity of human naive CD4$^+$ T cells activated with plate-bound anti-CD3 and soluble anti-CD28, with TCDD, and incubated in contact with responder T cells (Ctrl) or with a Transwell (Transwell). (**b**) Quantitative real-time PCR analysis of *GZMB* expression on T cells with or without TCDD, presented relative to *GAPDH* expression. (**c**) Flow cytometry of granzyme B expression on T cells with or without TCDD. Numbers in outlined areas (left) indicate percent CD4+ granzyme B–positive (GranB$^+$) cells. (**d**) Expression of *AHR* (left) and *GZMB* (right) in TCDD-treated cells transduced with nonspecific control or *AHR*-specific siRNA, presented relative to *GAPDH* expression. (**e**) Suppressive activity of TCDD-treated T cells left unstimulated or treated with the granzyme B inhibitor AAD-CMK, caspase inhibitors or neutralizing anti-IL-10. (**f**) *GZMB* expression (left) and the suppressive activity (right) of TCDD-treated T cells transduced with control siRNA or *GNZB*-specific siRNA (siGnzB). *$P < 0.05$ and **$P < 0.01$, compared with T-Ctrl (**b**,**c**), siCtrl (**d**,**f**) or no treatment (**e**; Student's *t*-test). Data are representative of two experiments (**a**,**d**,**f**; mean + s.d. of triplicate wells in **a** and mean + s.d. in **d**,**f**), four experiments (**b**,**c**; mean + s.d. of duplicates (**b**) or mean (right, **c**) or three experiments (**e**; mean + s.d.).

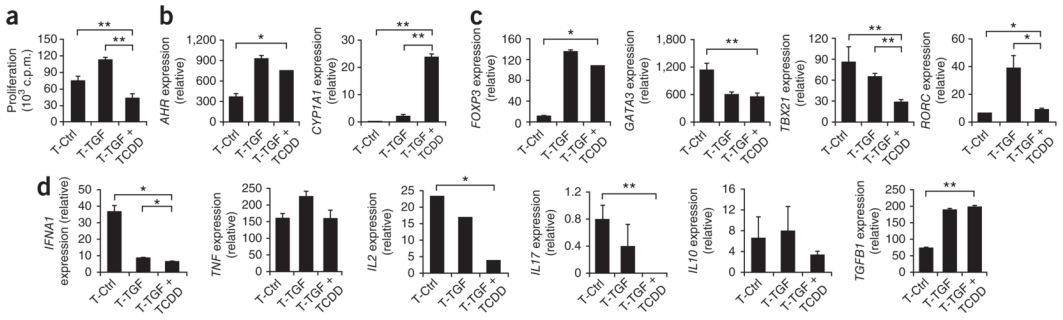

Figure 4.

AhR activation plus TGF-β1 induces Foxp3$^+$ T cells. (**a**) Proliferative response of human naive CD4$^+$ T cells activated for 6 d with plate-bound anti-CD3 and soluble anti-CD28 alone (T-Ctrl) or together with TGF-β1 alone (T-TGF) or TGF-β1 plus TCDD (T-TGF + TCDD) and restimulated with bead-conjugated anti-CD3 and anti-CD28. (**b**–**d**) Expression of *AHR* and *CYP1A1* (**b**), *FOXP3*, *GATA3*, *TBX21* and *RORC* (**c**) and cytokines (**d**) on cells treated as described in **a**; results are presented relative to *GAPDH* expression. **P* < 0.05 and ***P* < 0.01, compared with T-Ctrl or T-TGF (Student's *t*-test). Data are representative of five experiments (**a**; mean + s.d. of triplicate wells) or three to five experiments (**b**–**d**; mean + s.d. of duplicates).

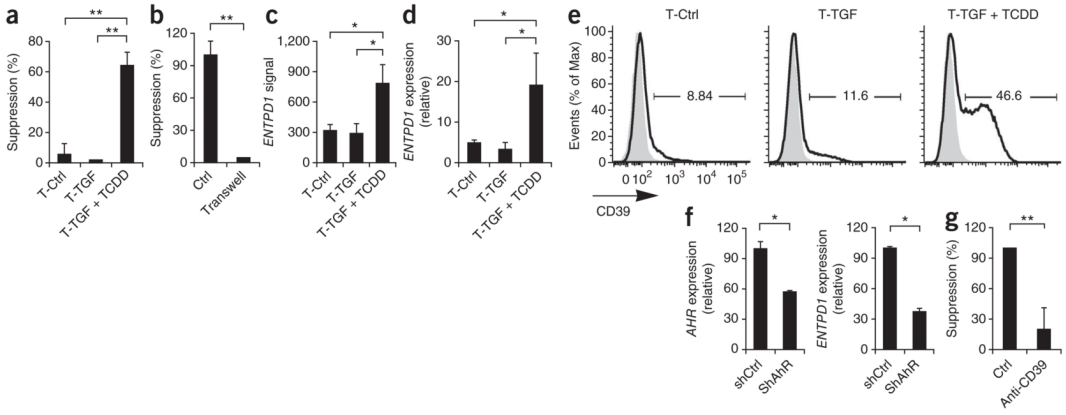

Figure 5.

AhR activation plus TGF-β1 induces functional human Foxp3$^+$ T_{reg} cells. (**a**) Suppressive activity of human naive CD4$^+$ T cells activated with plate-bound anti-CD3 and anti-CD28 alone or together with TGF-β1 alone or TGF-β1 plus TCDD (as in Fig. 4a). (**b**) Suppressive activity of T cells activated with plate-bound anti-CD3 and anti-CD28 alone together with TGF-β1 plus TCDD, incubated in contact with responder T cells (control (Ctrl)) or in a Transwell system (Transwell). (**c**,**d**) Gene microarray analysis (**c**) and quantitative PCR analysis (**d**) of *ENTPD1* expression on cells treated as described in **a**; results in **d** are presented relative to *GAPDH* expression. (**e**) Flow cytometry of CD39 (ENTPD1) expression (dark lines) on cells treated as described in **a**; gray filled histograms, isotype-matched control antibody. Numbers above bracketed lines indicate percent CD39$^+$ cells. (**f**) Expression of *AHR* (left) and *ENTPD1* (right) in T cells as described in **a** transduced with nonspecific control or *AHR*-specific siRNA, presented relative to *GAPDH* expression. (**g**) Suppressive activity of cells activated as described in **b** and treated with control antibody or anti-CD39. **P* < 0.05 and ***P* < 0.01, compared with T-Ctrl or T-TGF (**a**,**c**,**d**), shCtrl (**f**,**g**) or no Transwell (**b**; Student's *t*-test). Data are representative of five (**a**,**d**), two (**b**,**f**) or three (**e**) experiments (mean and s.d. in **a**–**d**) or three (**c**) or five (**g**) independent experiments (mean and s.d. of all).

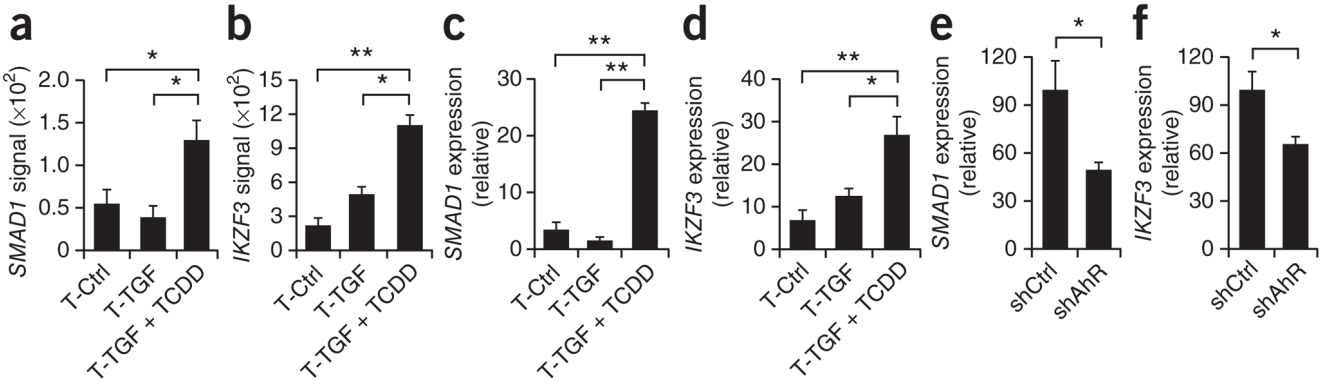

Figure 6.

AhR activation plus TGF-β1 induce the expression of Smad1 and Aiolos. (**a**,**b**) Gene microarray analysis of the expression of *SMAD1* (**a**) and *IKZF3* (Aiolos; **b**) by human naive $CD4^+$ T cells activated for 6 d with plate-bound anti-CD3 and soluble anti-CD28 alone or together with TGF-β1 alone or TGF-β1 plus TCDD (as in Fig. 4a). (**c**,**d**) Quantitative real-time PCR analysis of the expression of *SMAD1* (**c**) and *IKZF3* (**d**) on cells treated as described in **a**,**b**, presented relative to *GAPDH* expression. (**e**,**f**) Expression of *SMAD1* (**e**) and *IKZF3* (**f**) by human naive $CD4^+$ T cells activated for 6 d with plate-bound anti-CD3 and soluble anti-CD28 together with TGF-β1 plus TCDD and transduced with nonspecific control or *AHR*-specific shRNA, presented relative to *GAPDH* expression. $*P < 0.05$ and $**P < 0.01$, compared with T-Ctrl or T-TGF (**a**–**d**) or shCtrl (**e**,**f**; Student's *t*-test). Data are representative of three independent experiments (**a**,**b**; mean and s.d. of all) or five (**c**,**d**) or two (**e**,**f**) experiments (mean and s.d.).

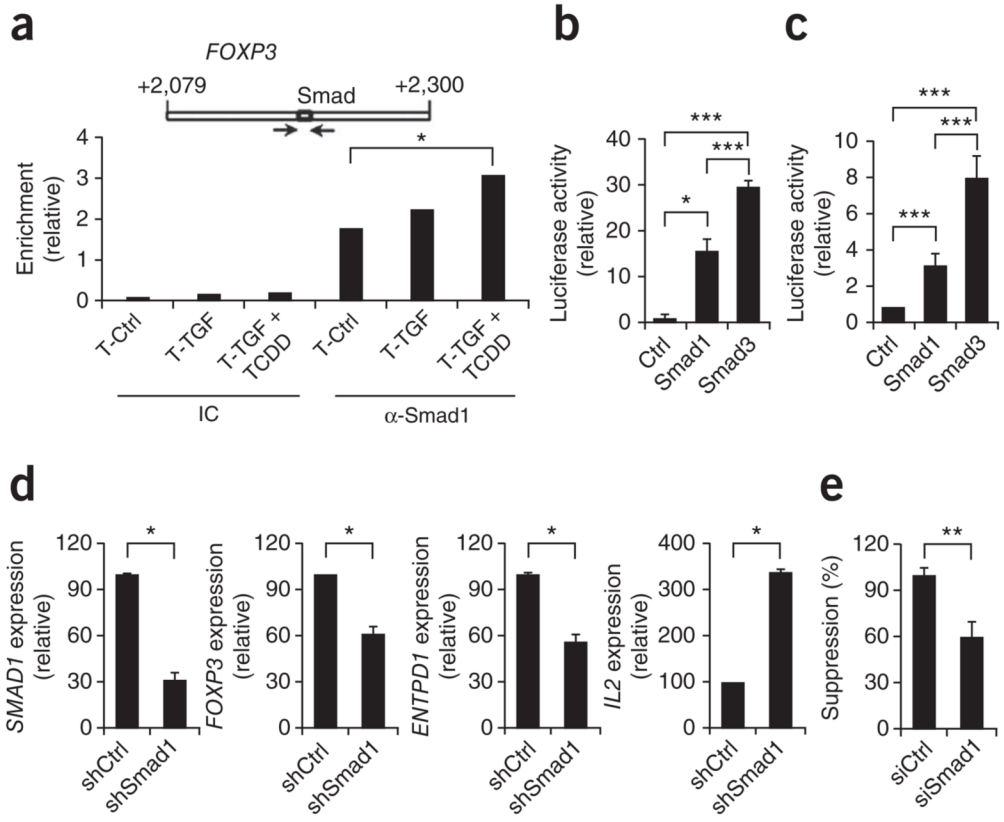

Figure 7.

Smad1 regulates *FOXP3* enhancer activity. (**a**) Smad-binding site in the *FOXP3* enhancer (top) and ChIP analysis (below) of the interaction of Smad1 with that binding site in human naive $CD4^+$ T cells activated with plate-bound anti-CD3 and soluble anti-CD28 alone or together with TGF-β1 alone or TGF-β1 plus TCDD (as in Fig. 4a), precipitated with isotype-matched control antibody (IC) or anti-Smad1 (α-Smad1); results are presented as enrichment relative to input chromatin. (**b**) Luciferase activity in EL4 cells transfected with a reporter containing six copies of the Smad-binding motif in the *FOXP3* enhancer together with empty control vector (Ctrl) or vector encoding Smad1 or Smad3, and activated in the presence of TGF-β1 (ref. [33]); results are presented relative to renilla luciferase. (**c**) Luciferase activity in EL4 cells transfected with a reporter for the *FOXP3* enhancer shown in **a**[33], together with empty control vector or vector encoding Smad1 or Smad3, and activated in the presence of TGF-β1; results are presented relative to renilla luciferase. (**d**) Expression of *SMAD1*, *FOXP3*, *ENTPD1* and *IL2* by human naive $CD4^+$ T cells activated with plate-bound anti-CD3 and soluble anti-CD28 together with TGF-β1 plus TCDD and transduced with nonspecific control shRNA (shCtrl) or *SMAD1*-specific shRNA (shSmad1), presented relative to *GAPDH* expression. (**e**) Suppressive activity of cells activated and transduced as described in **d**. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, compared with T-Ctrl (**a**), Ctrl or Smad1 (**b**,**c**), shCtrl (**d**) or siCtrl (**e**; Student's *t*-test). Data are representative of three (**a**–**c**) or two (**d**,**e**) experiments (mean and s.d.).

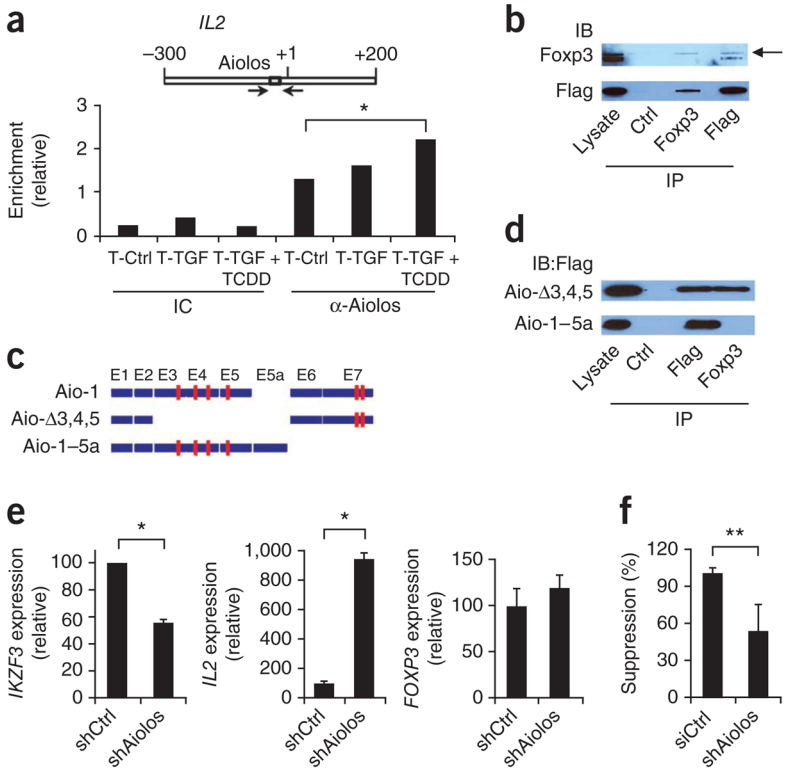

Figure 8.

Aiolos interacts with Foxp3 to silence *IL2* expression. (**a**) Aiolos-binding site in the *IL2* promoter (top) and ChIP analysis (below) of the interaction between Aiolos and that binding site in the *IL2* promoter in human naive $CD4^+$ T cells activated for 6 d with plate-bound anti-CD3 and soluble anti-CD28 alone or together with TGF-β1 alone or TGF-β1 plus TCDD (as in Fig. 4a), precipitated with isotype-matched control antibody (IC) or anti-Aiolos (α-Aiolos). (**b**) Physical interaction between Aiolos and Foxp3 in 293 human embryonic kidney cells transfected with constructs encoding Foxp3 and Flag-tagged Aiolos and lysed 24 h later, followed by immunoprecipitation (IP) with isotype-matched control antibody (Ctrl), anti-Foxp3 or anti-Flag, and analysis by immunoblot (IB) with anti-Foxp3 or anti-Flag. Arrow indicates Foxp3. Lysate, immunoblot analysis before immunoprecipitation. (**c**) Aiolos isoforms. Red boxes indicate zinc-finger motifs; E1–E7 indicate exons 1–7. (**d**) Immunoassay of 293 cells transfected with constructs encoding Foxp3 or Flag-tagged isoforms of Aiolos and lysed 48 h later, followed by immunoprecipitation with isotype-matched control antibody, anti-Foxp3 or anti-Flag, and analysis by immunoblot with anti-Flag. (**e**) Expression of *IKZF3*, *IL2* and *FOXP3* by human naive $CD4^+$ T cells activated with plate-bound anti-CD3 and soluble anti-CD28 together with TGF-β1 plus TCDD and transduced with nonspecific control shRNA (shCtrl) or *IKZF3*-specific shRNA (shAiolos), presented relative to *GAPDH* expression. (**f**) Suppressive activity of cells activated and transduced as described in **e**. $*P < 0.05$ and $**P < 0.01$, compared with T-Ctrl (**a**), shCtrl (**e**) or siCtrl (**f**; Student's *t*-test). Data are representative of three (**a**,**b**,**d**) or two (**e**,**f**) experiments (mean and s.d. in **a**,**e**,**f**).