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The Aryl hydrocarbon Receptor (AhR) interacts with c-Maf to promote the differentiation of IL-27-induced regulatory type 1 (T_R1) cells

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Abstract

IL-10 producing regulatory type 1 (T_R1) T cells are instrumental in the prevention of tissue inflammation, autoimmunity and graft-versus-host disease. The transcription factor c-Maf is essential for T_R1 induction of IL-10, but the molecular mechanisms leading to the development of these cells remain incompletely understood. We demonstrate that the ligand–activated transcription factor aryl hydrocarbon receptor (AhR) induced by IL-27, synergizes with c-Maf to promote the development of T_R1 cells. Upon T cell activation under T_R1-skewing conditions, the AhR binds to c-Maf and promotes the transactivation of both *Il10* and *Il21* promoters, resulting in the generation of T_R1 cells and amelioration of experimental autoimmune encephalomyelitis. Manipulation of AhR signaling could therefore be beneficial in the resolution of excessive inflammatory responses.

Keywords

AhR; c-Maf; T_R1 cell differentiation; experimental autoimmune encephalomyelitis

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Authors' contribution

L.A, F.J.Q. and C.P. performed *in vitro* and *in vivo* experiments and wrote the manuscript, N.J. performed *in vivo* experiments, S.X., D.K. and E.B. performed *in vitro* experiments, D.S. provided essential reagents and hints to perform the study, H.L.W. and V.K.K. supervised the study and edited the manuscript.

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Regulatory type 1 (T_R1) cells have emerged as an important subset of $CD4^+$ T cells that is instrumental in the control of excessive inflammatory responses1. The anti-inflammatory effects of T_R1 cells rely on their secretion of interleukin-10 (IL-10), which has been shown to dampen function of antigen presenting cells and antigen-specific effector T cells to suppress tissue inflammation and autoimmunity. However, progress in molecular analysis and biological functions of T_R1 cells has been hampered due to the lack of appropriate methods to generate IL-10-producing T cells in large numbers *in vitro*.

IL-27, a heterodimeric cytokine of the IL-12 family, was initially suggested to induce the expansion of proinflammatory T helper 1 (T_H1) cells by activating the transcription factors STAT-1 and T-bet in a manner similar to IL-122. However, it was later found that IL-27 receptor-deficient (*Il27ra*$^{-/-}$) mice developed exaggerated proinflammatory T cell responses3 and autoimmunity4, suggesting that IL-27 might be directly involved in inhibiting tissue inflammation. Indeed, we5 and others6, 7 reported that IL-27 is a growth and differentiation factor for T_R1 cells. The activation of naïve $CD4^+$ cells in the presence of IL-27 or transforming growth factor (TGF)-β plus IL-27 results in the differentiation of IL-10-producing T_R1 cells with potent suppressive activity. In addition to its effects on the differentiation of T_R1 cells, IL-27 directly inhibits the differentiation of T_H17 cells4, 8 and TGF-β-induced $Foxp3^+$ T regulatory (T_{reg}) cells5.

IL-27 drives the expansion of T_R1 cells by inducing the expression of IL-21, a member of the IL-2 family of cytokines, which acts as an autocrine growth factor for T_R1 cells9–11. Like IL-10, IL-21 expression was initially reported to be T_H2 specific12, but subsequent studies demonstrated that IL-21 was also produced by T_R19, T_H1713 and T follicular helper (T_{FH})14 cells. All these cell types produce IL-10, albeit at different levels, suggesting a possible link between IL-21 and IL-10 production. Even though IL-21 promotes the expansion of T_H17 cells15, *Il21r*$^{-/-}$ mice, like *Il27ra*$^{-/-}$ mice, exhibit an increased susceptibility to the autoimmune disease, experimental autoimmune encephalomyelitis (EAE), thus suggesting a major regulatory role of IL-21 *in vivo*16.

In an effort to unravel the molecular mechanisms by which IL-27 induces T_R1 cells, we found that IL-27 directly induces the transcription factor c-Maf, which is crucial for T_R1 cell differentiation9. In the absence of c-Maf, T_R1 cell generation and expansion is compromised. Indeed, c-Maf directly transactivates the *Il10* and *Il21* promoters9, 17. Although these findings highlight the importance of c-Maf and IL-21 for the biology of T_R1 cells, the addition of recombinant IL-21 to naïve $CD4^+$ T cells alone failed to generate T_R1 cells, suggesting that additional IL-27-driven molecular signals contribute to T_R1 cell differentiation.

To explore the molecular mechanisms accounting for the effects of IL-27 in T_R1 cell differentiation, we have performed gene expression analysis of IL-27 induced T_R1 cells and found that the ligand-activated transcription factor Aryl hydrocarbon receptor (AhR) is induced by IL-27 in T_R1 cells. Furthermore, we show that during T_R1 cell differentiation, AhR physically associates with c-Maf and transactivates the *Il10* and *Il21* promoters. Mice with impaired AhR signaling showed decreased production of IL-10 and resistance to IL-27-

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mediated inhibition of EAE. Taken together, our study demonstrates that AhR and c-Maf synergize to induce IL-27-mediated T_R1 cell differentiation.

Results

High expression of *Ahr* in IL-27-induced-T_R1 cells

We first studied *Ahr* expression by real-time PCR (RT-PCR) in different CD4$^+$ T cell subsets. While *Ahr* expression levels were modest in T_H1 or T_H2 cells differentiated from naïve CD4$^+$CD25$^-$CD62L$^+$CD44^{lo} T cells, *Ahr* was expressed at very high levels in T_R1 cells induced with TGF-β plus IL-27 (Fig. 1a). Interestingly, *Ahr* expression in T_R1 cells was similar to that found in T_H17 cells, where AhR controls the production of IL-2218, 19.

We investigated the kinetics of *Ahr* expression during the differentiation of T_R1 cells with TGF-β and IL-27 and found that *Ahr* expression was significantly up-regulated 12 hours after the initiation of the culture and was sustained at high levels throughout T_R1 cell differentiation (Fig. 1b). Given that we have previously shown that T_R1 cells can also be differentiated by IL-27 without TGF-β9, we analyzed the kinetics of *Ahr* expression during the differentiation of T_R1 cells with IL-27 alone. *Ahr* expression was also induced by treatment with IL-27 alone, albeit at lower expression levels (Fig. 1b). T cells activated without any polarizing cytokines (T_H0 condition) only expressed marginal levels of *Ahr*.

The expression of the xenobiotic metabolizing enzyme cytochrome P450 encoded by *Cyp1a1* is directly controlled by the AhR which transactivates the *Cyp1a1* promoter20. To test whether the AhR is activated during T_R1 cell differentiation, we analyzed the expression of *Cyp1a1* in naïve CD4$^+$ T cells treated with IL-27, with or without TGF-β. We found that *Cyp1a1* was expressed in CD4$^+$ cells as early as 20 hours after activation (Fig. 1c). T_R1 cells differentiated with IL-27 alone showed a transient expression of *Cyp1a1*, whereas *Cyp1a1* expression was sustained at high levels in T_R1 cells differentiated with TGF-β plus IL-27 (Fig. 1c).

We have recently shown that the transcription factor c-Maf plays a major role in T_R1 cell differentiation9. Thus, we analyzed *Ahr* and *Maf* expression during the differentiation of T_R1 cells with TGF-β and IL-27. *Maf* expression was detectable as early as 6 hours after the initiation of differentiation, while *Ahr* expression was first detected 8 hours after differentiation and was expressed at lower levels than *Maf. Ahr* and *Maf* expression were sustained at high levels after 24 hours (Fig. 1d). Overall, these studies demonstrate that *Ahr*, like *Maf*, is highly expressed and active during IL-27 triggered differentiation of T_R1 cells.

AhR controls the development of T_R1 cells

Different AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypic environmental AhR agonist, or the putative endogenous ligand 6-formylindolo [3,2-b] carbazole (FICZ) regulate T_{reg} cell and T_H17 differentiation18, 19. Based on the high expression levels of *Ahr* during T_R1 cell differentiation, we hypothesized that AhR ligands might affect T_R1 cell differentiation as well. To investigate the effect of AhR signaling on T_R1 cell development, we differentiated naïve CD4$^+$ cells from IL-10.eGFP reporter mice (Vert-X)21 with TGF-β and IL-27 in the presence of either of the AhR ligands TCDD and

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FICZ. Both TCDD and FICZ doubled the frequency of IL-10- producing T cells (Fig. 2a) and increased the secretion of IL-10 by more than three-fold over controls (Fig. 2b), suggesting that AhR activation promotes T_R1 cell differentiation. Similar results were obtained with IL-27 alone (Supplementary Fig. 1). Although TCDD has been proposed to support Foxp3$^+$ T_{reg} cell development19, 22, the addition of AhR ligands together with IL-27 during T_R1 cell differentiation did not induce Foxp3 expression (Supplementary Fig. 2 and data not shown), thus ruling out any possible involvement of Foxp3$^+$ regulatory T cells in the enhanced IL-10 expression. These results are reminiscent of a previously published study demonstrating that FoxP3 is not induced in regulatory T cells generated by AhR ligands during graft versus host (GVH) responses23. To further characterize the contribution of AhR to T_R1 cell differentiation, we knocked-down *Ahr* expression using siRNA. T_R1 cells were differentiated with TGF-β and IL-27 in the presence of an siRNA specific for AhR (siAhR) or control siRNA and IL-10 expression was analyzed by RT-PCR and by flow cytometry. We found that naïve T cells, in which *Ahr* expression had been downregulated by siRNA, had a decreased ability to produce IL-10 (Fig. 2c). Similar results were obtained when *Maf* was knocked down as a positive control. In agreement with our previous results, the addition of the AhR antagonist CH-223191 during the differentiation of T_R1 cells with TGF-β plus IL-27 decreased IL-10 production (Supplementary Fig. 3a). Similarly, the ability of AhR deficient T cells to differentiate into T_R1 cells in the presence of TGF-β plus IL-27 was impaired, confirming that AhR is essential in the differentiation of T_R1 cells (Supplementary Fig. 3b). Therefore the modulation of AhR signaling either with the agonists FICZ or TCDD, the antagonist CH-223191, with siRNA mediated downregulation or genetic deletion of the AhR gene profoundly affects the development of IL-10-producing T_R1 cells.

To further demonstrate a role for *Ahr* in *Il10* expression, we retrovirally transduced primary CD4$^+$ T cells with an *Ahr*-GFP (green fluorescent protein) or a control-GFP overexpression vector and monitored *Il10* expression on GFP$^+$ cells. We observed that *Il10* (but not *Ifnγ*) expression was significantly induced when primary CD4$^+$ T cells overexpressed AhR (Supplementary Fig. 4). These results suggest that the IL-27-driven *Ahr* expression is responsible for the enhanced IL-10 secretion during T_R1 cell differentiation. Given that c-Maf was shown to be essential for T_R1 cell generation9, we decided to evaluate the relative contribution of c-Maf and AhR to the development of T_R1 cells. For this, we differentiated naïve T cells from mice overexpressing the *Maf* transgene (c-Maf-TG) under the control of the CD4 promoter without any cytokine or with TGF-β plus IL-27. In addition, to activate AhR signaling, we added AhR agonist FICZ to c-Maf transgenic T cells. In line with previous reports17, the overexpression of c- Maf was sufficient to drive IL-10 secretion from T_H0 cells (Fig. 2d). Since TGF-β plus IL-27 is required for AhR expression, FICZ alone did not alter the IL-10 secretion from c-Maf-TG cells in the absence of TGF-β and IL-27. However, we found that c-Maf overexpressing T cells dramatically increased IL-10 secretion upon differentiation with TGF-β plus IL-27 and FICZ (Fig. 2d), suggesting that the two transcription factors AhR and c-Maf cooperate to enhance IL-10 secretion from T_R1 cells.

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AhR regulates IL-21 expression in T_R1 cells

We have previously shown that IL-27 acts on T_R1 cells to trigger the production of IL-21, which acts as an autocrine growth factor for T_R1 cells9. Therefore we examined the effect of AhR signaling on IL-21 production by T_R1 cells. We first differentiated naïve $CD4^+$ T cells with TGF-β plus IL-27 in the presence of the AhR ligand FICZ and found that treatment with FICZ led to a four-fold increase in IL-21 production by T_R1 cells (Fig. 3a). Similar results were obtained when we investigated the effect of another AhR ligand TCDD (Supplementary Fig. 5).

We have previously shown that c-Maf regulates IL-21 production9, 14, thus we examined *Maf* expression in T_R1 cells differentiated in the presence of AhR ligands. We found that in addition to enhancing IL-21 expression, AhR activation by FICZ during T_R1 cell differentiation led to a significant up-regulation of *Maf* (Fig. 3b). Importantly, treatment with FICZ or TCDD in the absence of differentiating cytokines had no effect on *Maf* expression, indicating that AhR activation is not sufficient for up-regulation of *Maf* (Fig.3b and Supplementary Fig. 5). Thus, AhR activation potentiates the expression of c-Maf and IL-21 during the differentiation of T_R1 cells triggered by IL-27.

Naïve $CD4^+$ T cells from IL-21R-deficient mice have an impaired capacity to differentiate into T_R1 cells9. To test whether the effects of the AhR activation on T_R1 cell differentiation were mediated by IL-21, we differentiated naïve $CD4^+$ T cells from *Il21r*$^{-/-}$ deficient mice with TGF-β plus IL-27, with or without FICZ. IL-21 secretion was enhanced in FICZ-treated T_R1 cells from either wildtype or *Il21r*$^{-/-}$ mice (Fig. 3c). However, IL-10 production was drastically impaired in T_R1 cells derived from *Il21r*$^{-/-}$ mice (Fig. 3d). These results suggest that the effects of AhR activation on T_R1 cell differentiation are at least partly mediated by IL-21.

IL-21 is an autocrine T_R1 cell growth factor that enhances IL-10 and c-Maf expression in T_R1 cells9. Thus, we examined the effect of IL-21 on the mRNA expression of *Maf* and *Ahr* during T_R1 cell differentiation. In agreement with our previous findings, *Maf* expression in T_R1 cells was controlled by IL-21 signaling (Fig. 3e). Strikingly, there was a dramatic decrease in *Ahr* expression in T_R1 cells derived from *Il21r*$^{-/-}$ mice, while the mRNA expression level of the transcription factor T-bet, known to be induced by IL-27 during T_R1 cell differentiation24, was unaffected (Fig. 3e). This suggests that, in addition to controlling c-Maf, IL-21 further modulates T_R1 cell development by inducing and/or maintaining AhR expression. Collectively, these results demonstrate that AhR signaling controls T_R1 cell differentiation partly by regulating IL-21 production, which in turn contributes to T_R1 cell development as a positive feedback mechanism, likely enhancing AhR mRNA expression, IL-21 and IL-10 production.

AhR and c-Maf transactivate *Il10* and *Il21*

c-Maf transactivates the *Il10*9, 17 and *Il21*9 promoters. Based on our findings pointing to a key role for the AhR in the regulation of IL-10 and IL-21 during T_R1 cell differentiation, we hypothesized that the AhR might transactivate the *Il10* and *Il21* promoters in T_R1 cells as well. We first searched the *Il10* and *Il21* promoters for AhR and c-Maf binding sites. We

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found one putative AhR binding site (xenobiotic response element, XRE-1) and four putative c-Maf binding sites (Maf recognition element, MARE-1, MARE-2, MARE-3 and MARE-4) in the *Il10* promoter (Fig. 4a and Supplementary Fig. 6). Similarly, we identified two putative AhR binding sites (XRE-2 and XRE-3) and three putative c-Maf binding sites (MARE-5, MARE-6, and MARE-7) in the *Il21* promoter (Fig. 4a and Supplementary Fig. 6).

To study whether AhR binds to IL-10 and IL-21 XRE promoter sequences, we monitored whether *in vitro*-translated AhR protein would interact with an oligonucleotide containing the putative AhR binding site located in the *Il10* or the *Il21* promoter. Since binding of AhR with AhR nuclear translocator (Arnt) transforms AhR into its high affinity DNA binding form25, we studied the binding of AhR complexed with Arnt to XRE 1, 2 and 3. The AhR-Arnt complex was incubated with a radiolabeled oligomer containing the putative AhR binding site located in the *Il10* or *Il21* promoter. The AhR-Arnt-DNA protein complex was visualized by electrophoretic mobility shift assay (EMSA) (Supplementary Figure 7, lanes 1, 3 and 5). Importantly, binding of AhR to IL-10 and IL-21 XRE promoter sequences was inhibited by inclusion of a competitor oligo containing *Cyp1a1* XRE3 AhR DNA binding site26 (Supplementary Figure 7, lanes 2, 4 and 6). To confirm that AhR can also interact with its target sequences in the *Il10* and *Il21* promoters under physiological conditions, we undertook chromatin immunoprecipitation (ChIP) assays with differentiated T_R1 cells *in vitro* with IL-27 and TGF-β. AhR interacted with XRE-1 in the *Il10* promoter both in T_R1 and T_H0 cells, and with XRE-2 and XRE-3 in the *Il21* promoter in T_R1 cells (Fig. 4b, c and Supplementary Figure 6). Similarly, ChIP assays revealed a clear interaction of c-Maf with MARE 1–4 and MARE 5–7 on the *Il10* and *Il21* promoters, respectively, but only in T_R1 cells (Fig. 4d, e and Supplementary Figure 6). No interaction with the XRE or MARE sequences in either the *Il10* or *Il21* promoter was detected when we used isotype control antibodies (IgG), and no significant AhR or c-Maf binding was detected in the control sequence Untr6 (Fig. 4b-e). These data suggest that c-Maf controls the cell specificity of the *Il10* and *Il21* gene transcription.

To determine the relevance of AhR and c-Maf binding their target sequences in *Il10* and *Il21*, we studied the ability of the AhR and c-Maf to transactivate the *Il10* and *Il21* promoters in reporter assays. We used reporter constructs containing the firefly luciferase gene under the control of the *Il10* promoter (*Il10-luc*) or the *Il21* promoter (*Il21-luc*). Cotransfection of *Il10-luc* or *Il21-luc* with a construct coding for mouse c-Maf resulted in a slight up-regulation of the transcription of the *Il10* and the *Il21* gene (Fig. 4f, g). A similar upregulation was observed when *Il10-luc* or *Il21-luc* were cotransfected with a construct coding for mouse AhR (Fig. 4f, g). Notably, cotransfection of both c-Maf and AhR resulted in an additive transactivation of *Il10* as well as *Il21* expression (Fig. 4f, g), suggesting that AhR and c-Maf cooperate to control the transcriptional activity of both promoters.

The concomitant upregulation of c-Maf and AhR during T_R1 cell differentiation (Fig. 1d), their ability to bind to *Il10* promoter elements to induce IL-10 secretion (Fig. 4) and the proximity of the putative binding sites for both c-Maf and AhR on the *Il10* or *Il21* promoters led us to test whether c-Maf and AhR physically interact with each other. Indeed, AhR has been shown to interact with diverse transcription factors including NF-κB27 and the

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estrogen receptor28. To address this issue, we differentiated naïve $CD4^+$ T cells into either T_H0 or T_R1 cells and performed immunoprecipitation followed by immunoblotting. We first found that AhR and c-Maf proteins were upregulated in T_R1 cells (Fig. 4h, left panel). Moreover, we could co-precipitate c-Maf and AhR in a T_R1 cell nuclear extract by using an antibody against AhR to immunoprecipitate the complex, followed by immunoblotting with an anti-c-Maf antibody (Fig. 4h, right panel), suggesting that AhR physically interacts with c-Maf. Taken together, our results demonstrate that AhR and c-Maf interact in T_R1 cells to transactivate the *Il10* and *Il21* promoters.

AhR controls T_R1 cell generation *in vivo*

It has previously been shown that repeated *in vivo* treatment with anti-CD3 induces IL-10^+ regulatory T cells29. Given that we have shown that *AhR and Maf* are induced upon *in vitro* differentiation of T_R1 cells (Fig. 1), we assumed that they might be similarly induced *in vivo* in IL-10^+ T cells elicited by anti-CD3 treatment. To test this, we repeatedly administered anti-CD3 or an isotype control antibody (IC) to IL-10.eGFP reporter mice (Vert-X) and assessed *AhR and Maf* expression within GFP^+ IL-10-producing T cells in the spleen and mesenteric lymph nodes 4 hours after the last injection. In line with our *in vitro* findings, both *Ahr* and *Maf* were significantly induced in IL-10^+ T cells (Supplementary Fig. 8). Thus, we used this model to analyze the role of the AhR in the generation of T_R1 cells *in vivo*. We administered anti-CD3 or an isotype control antibody to wild type, mutant AhR (AhR^d) and IL-27 receptor deficient mice (*Il27ra*$^{-/-}$ mice), and studied the frequency of IL-10^+ T cells in the spleen and mesenteric lymph nodes (MLN). AhR^d mice have point mutations in the AhR ligand-binding pocket and therefore show defective AhR mediated responses *in vivo*30. Since IL-10 is produced by T_H17 cells17, $Foxp3^+$ T_{reg} cells29 and T_R1 cells, we analyzed the production of IL-10 by $Foxp3^-$IL-$17^-$$CD4^+$$CD3^+$$TCR\alpha\beta^+$ T cells (Supplementary Fig. 9). We found that the administration of anti-CD3 to wild type mice resulted in a significant induction of IL-10^+ T cells in the spleen and the mesenteric lymph nodes (MLN; Fig. 5a-c). The induction of IL-10-producing cells in this setting was mediated by IL-27, since it was not observed in anti-CD3 treated *Il27ra*$^{-/-}$ mice (Fig. 5a-c). In addition, AhR^d mice also showed a significant impairment in their ability to produce IL-10^+ T cells upon treatment with anti-CD3, both in the spleen and the MLN (Fig. 5a-c). Thus, AhR controls the generation of IL-10^+ T cells *in vivo*.

AhR controls the IL-27-mediated inhibition of EAE

To address the *in vivo* relevance of AhR in inducing IL-27-driven T_R1 cells and their effect on regulating autoimmunity and tissue inflammation, we exploited an adoptive transfer model of EAE in which IL-10, induced by IL-27, regulates EAE induced by adoptive T cell transfer7. We therefore used this model system to investigate the role of the AhR in the *in vivo* suppressive activity of IL-27-induced IL-10 production. This experimental model allowed us to exclude the effects of AhR on non-T_R1 T cells, since IL-27, if given *in vivo*, can also inhibit T_H17 and T_{reg} cell differentiation5, 31. We first immunized wild type or AhR^d mice with the myelin oligodendrocyte peptide 35–55 (MOG(35–55)) emulsified in Complete Freund's Adjuvant (CFA) and tested the secretion of IL-10 by $CD4^+$ T cells reactivated with MOG(35–55) and IL-27. We observed a significant decrease in the production of IL-10 by $CD4^+$ T cells from AhR^d mice treated with IL-27 (Fig. 6a).

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In line with previous findings7, wild type CD4$^+$ T cells reactivated solely with MOG(35–55) were poor inducers of EAE (not shown). However, reactivation of T cells in the presence of IL-12 before adoptive transfer generated MOG(35–55)-specific donor cells that induce EAE with high incidence7. Based on these observations, we compared the secretion of IL-10 by donor cells from MOG(35–55)-immunized wild type or AhR^d mice, and restimulated *in vitro* with IL-12 with or without IL-27. We found that IL-27 enhanced IL-10 production in wild type but not in AhR^d T cells (Fig. 6b). There was no effect of IL-27 on the production of interferon-γ (IFN-γ) triggered by IL-12 (Fig. 6b), and we failed to detect significant levels of IL-17 upon stimulation of donor cells with IL-12 and IL-27 (not shown). We then adoptively transferred these cells to induce EAE and found that wild type effector cells reactivated in the presence of IL-12 induced disease in the majority of the recipient mice. The incidence of EAE was significantly reduced when the donor cells were reactivated with IL-12 and IL-27 *in vitro* (Fig. 6c, d and Table 1). AhR^d donor T cells activated in the presence of IL-12 also induced EAE upon adoptive transfer. However, the activation of AhR^d T cells in the presence of IL-27 resulted in a significantly increased incidence of disease as compared with wild type T cells similarly treated (Fig. 6c, d and Table 1) suggesting that the AhR is essential for the IL-27-mediated inhibition of EAE *in vivo*. These data show that IL-27 controls the adoptive induction of EAE with wild type, but not AhR^d T cells, and that this disease inhibition by IL-27 correlates with the AhR-dependent induction of IL-10 production by IL-27.

Discussion

IL-27 has recently been shown to promote the differentiation of T_R1 cells that are instrumental in controlling autoimmunity and tissue inflammation5. In this paper, we report that AhR, like the proto-oncogene c-Maf, is strongly induced during T_R1 cell differentiation and that its expression in T_R1 cells is as high as that observed in T_H17 cells. Besides IL-10, activation of AhR by a putative endogenous ligand FICZ also increases IL-21 production in T_R1 cells, which supports their development. Furthermore, the two transcription factors (AhR and c-Maf) associate with each other to transactivate the *Il10* and *Il21* promoters. The relevance of these findings is underscored by the ability of AhR signaling to control IL-27-driven IL-10 producing T cells *in vivo*.

T_R1 cells are an important regulatory T cell type, which predominantly produce IL-10 and do not express Foxp3 but suppress tissue inflammation, GVH and autoimmunity in an IL-10-dependent manner. Although IL-10 was initially described to be a differentiation factor for T_R1 cells, IL-27 additionally generates IL-10-producing T_R1 cells5, 8. IL-27 induces both IL-10 and IFN-γ in T cells. These IL-10-IFN-γ double producing T cells have previously been reported to be generated *in vivo* following treatment with altered peptides ligands32 and regulate autoimmune tissue inflammation. Whether IL-10 and IFN-γ both contribute to the immunosuppressive function is not clear at this stage, but initial data suggest that IFN-γ produced by T_R1 cells is a potent inhibitor of T_H17 cells. This supports the view that both IFN-γ and IL-10 might be contributing to the immunoregulatory properties of T_R1 cells.

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While the ability of c-Maf to transactivate the *Il10* promoter has already been demonstrated17, previous findings showed that the ability of c-Maf to transactivate *Il10* in hepatocytes stimulated by fatty acids needed additional cofactors that were critical for inducing *Il10* gene expression33. In addition, c-Maf and AhR have been suggested to cooperate to induce the transcription of β7-integrins34. Here we show that IL-27 induces AhR, which associates with c-Maf for the generation of IL-10-producing T_R1 cells. Our results reveal that AhR and c-Maf have the ability to bind to proximal regions in both the *Il10* (XRE-1 and MARE-3) and *Il21* (XRE-2 and MARE-5; XRE-3 and MARE-6) promoters. This, together with our observation that AhR and c-Maf bind to each other to transactivate the *Il10* and *Il21* promoters, support a critical role for these two transcription factors in the development of T_R1 cells. In addition, our ChIP results show that, while AhR binds to the *Il10* and *Il21* promoters in both T_R1 and T_H0 cells, c-Maf associates with *Il10* and *Il21* promoters only in T_R1 cells, suggesting that c-Maf is controlling the tissue specificity of *Il10* and *Il21* gene transcription.

IL-21 acts as a growth factor for both T_R1 and T_H17 cells. Interestingly, IL-21 was reported to support IL-17 secretion from T_H17 cells through a self-amplifying loop35. Our results similarly suggest that during T_R1 cell differentiation, AhR and c-Maf participate in the self-amplifying feed-forward loop driven by IL-21 signaling which is essential for amplification and maintenance of the phenotype of differentiated T_R1 cells. However, the actual mechanism by which IL-21 induces and/or maintains AhR and c- Maf expression remains to be determined. IL-21 could mediate this effect by increasing the frequency of IL-10-producing T_R1 cells and also strengthen the expression of both c-Maf and AhR in a cell intrinsic manner. Overall, we propose that during the differentiation of T_R1 cells with IL-27, AhR is essential for supporting c-Maf in its ability to transactivate the *Il10* and *Il21* promoters and thus enhances the differentiation of T_R1 cells.

In addition to IL-10, AhR is essential for IL-22 production18. IL-22, a T_H17 specific cytokine, promotes acanthosis in psoriasis but also protects mice from dextran sulfate-mediated colitis and concanavalin A induced liver damage36. Interestingly, IL-22 is a member of the IL-10-related cytokine family36 and might be similarly regulated. Therefore, our results raise the possibility that the c-Maf and AhR interaction might not only control IL-10 but also IL-22 production. Indeed, motif analysis shows that the *Il22* promoter, like the *Il10* promoter, contains c-Maf and AhR binding sites in close proximity, suggesting that c-Maf and AhR might also cooperate to induce *Il22* gene transcription.

Environmentally ubiquitous polycyclic aromatic and planar halogenated hydrocarbons (PAH and HAH respectively), for which AhR is a cellular receptor, represent two major classes of environmental pollutants to which humans are regularly exposed. Endogenous ligands, although not yet completely classified37, represent an additional category of AhR activators. Notably, exposure to these chemicals can result in contrasting AhR-dependent effects on the immune response. For example, TCDD-driven AhR activation enhances inflammation in rheumatoid arthritis38 and endogenous ligand-driven AhR activation induces production of inflammatory cytokines by T_H17 cells18, 19. On the other hand, prototypic PAH and HAH can impair B and T cell proliferative responses, alter antibody isotype switching, block plasma cell differentiation, compromise antibody production, induce apoptosis in developing

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lymphocytes, inhibit NK activity, modulate cytokine production, decrease cytotoxic T lymphocyte activity, and promote tumor growth39–46. In this vein, our report, together with the study of Gandhi *et al.* (cosubmitted manuscript), provides evidence suggesting that the interaction of AhR with c-Maf is essential for the generation of mouse and human regulatory IL-10-secreting T_R1 cells that suppress inflammatory responses. These contrasting outcomes suggest that the *in vivo* immunologic effects of AhR activation are tissue- and/or ligand-specific. In the context of autoimmunity, outcomes likely depend on the type of T cell differentiation pathway activated by a given AhR ligand. Since AhR and c-Maf are expressed in T_H1717, T_{reg}, and T_R19 cells, it is unlikely that AhR alone or in combination with c-Maf acts as a specific “lineage specification” transcription factor for T_R1 cells. We would rather postulate that AhR, in combination with c-Maf, controls parts of the T_R1 cell transcriptional and differentiation program. Thus, in response to different environmental ligands, AhR can differentially induce opposing T cell subsets resulting in either tissue inflammation or immunosuppression. Therefore, while the molecular basis for the difference in differentiation pathways favored by an AhR ligand(s) is not clear at this stage, one can nevertheless predict that AhR ligands direct the nature of downstream signaling and thus provide specificity and dictate the T cell subset dominance (T_R1 vs. T_H17) during an immune response.

In summary, we have demonstrated that the AhR, together with c-Maf, regulates the generation of T_R1 cells induced by IL-27. Besides unraveling the molecular mechanisms accounting for the generation of T_R1 cells, these findings, together with other studies18, 19, suggest that the AhR is not only a receptor for environmental pollutants but an important target for regulating T cell differentiation and the quality of immune responses *in vivo*.

Online methods

Animals and induction of EAE

IL-10–eGFP reporter mice (Vert-X)21, c-Maf transgenic47 and AhR deficient48 mice have been described. C57BL/6 wild type and AhR^d mice were purchased from the Jackson Laboratories.

Adoptive transfer of EAE was performed as described previously7. AhR^d or wild type mice were immunized with 100 μg o f MOG (35–55) peptide (MEVGWYRSPFSRVVHLYRNGK) and 500 μg of M. tuberculosis extract H37 Ra (Difco). Draining lymph nodes and spleens were collected 11 days after immunization and cultured for 3 days with MOG(35–55) peptide (20 μg/ml) and carrier-free recombinant IL-12 (10 ng/ml; R&D Systems) with or without carrier-free recombinant mouse IL-27 (25 ng/ml; R&D Systems). 15×10^6 cells were subsequently transferred i.v. into naïve wild-type mice, which were injected i.p. with 200 ng of pertussis toxin on days 0 and 2. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

In vitro T cell differentiation

Naïve $CD4^+$ T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) from C57BL/6 wild type, c-Maf transgenic, *Ahr*$^{-/-}$ or *Il21*r$^{-/-}$ mice were activated with plate-bound antibodies against CD3

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(145-2C11, 2μg/ml) and CD28 (PV-1, 2 μg/ml). Mouse IL-27 (25ng/ml) and TGF-β (2 ng/ml), were all purchased from R&D Systems. TCDD (Sigma Aldrich) and FICZ (Enzo Life Sciences) were added at the start of the cultures at a final concentration of 100nM. Similarly, the AhR antagonist CH-223191 (Calbiochem) was used at a final concentration of 3μM.

siRNA

AhR- or c-Maf-specific Accell siRNAs were used according to the manufacturer's instructions (Dharmacon Inc., Lafayette, CO, USA). Naïve $CD4^+$ T cells were differentiated into T_R1 cells with anti-CD3, anti-CD28, TGF-β (3 ng/ml) and IL-27 (30 ng/ml) using T cell differentiation medium containing 3 % FBS in the presence of 1 μM siRNA.

Measurement of cytokines

Secreted cytokines were measured after 48 hours by cytometric bead array (BD Biosciences) or ELISA. For intracellular cytokine staining, cells were stimulated for 4 hours at 37 C with PMA (50 ng/ml; Sigma), ionomycin (1 μg/ml; Sigma) and monensin (GolgiStop; 1 μg/ml; BD Biosciences). After staining for surface markers, cells were fixed and permeabilized according to the manufacturer's instructions (BD Biosciences). All antibodies to cytokines were purchased from Biolegend.

Quantitative RT-PCR

RNA was extracted with RNAeasy minikits (Qiagen) and was analyzed by real-time PCR (RT-PCR) using the GeneAmp 7500 Sequence Detection System (Applied Biosystems). Expression was normalized to the expression of β-actin. Primers-probe mixtures were purchased from Applied Biosystems: IL-10 (Mm00439615-g1); c-Maf (Mm02581355-S1); IL-21 (Mm00517640-m1); AhR (Mm00478930-ml); T-bet (Mm00450960-m1); cyp1a1 (Mm00487217-m1); β-actin (Mm00446968-m1).

Chromatin immunoprecipitation (ChIP)

Cells were differentiated for 5 days into T_R1 cells with TGF-β and IL-27, fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated and sheared to an average length of 300–500 bp by sonication. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heated for de-crosslinking, followed by ethanol precipitation. AhR-bound DNA sequences were immuno-precipitated with an AhR-specific antibody (Biomol SA-210), c-Maf bound sequences were immunoprecipitated using a c-Maf specific antibody (Santa Cruz sc-7866). Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR (Q-PCR) reactions were carried out in triplicate and experimental C_t values were converted to copy numbers detected by comparison with a DNA standard curve run on the same PCR plates. Copy number values were normalized for primer efficiency using the values obtained with input DNA and the same primer pairs. Error bars represent standard deviations calculated from triplicate determinations.

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Electrophoretic mobility shift assay (EMSA)

AhR was *in vitro*-translated using a TNT-coupled reticulocyte lysate kit (Promega Corporation). To make the probes, the complementary oligonucleotides, containing the AhR binding site of either mouse *il10* or *il21* promoter were annealed and radiolabeled using [γ-32p]dATP. The oligonucleotides used for making probes are as follows: XRE-1 (5′-ATGACCTGGGAGTGCGTGAATGGAATCCACA-3′ and 5′-TGTGGATTCCATTCACGCACTCCCAGGTCAT-3′), XRE-2 (5′-TCTTCACGGAGAGCACGCTGTCTACTTAGT-3′ and 5′-ACTAAGTAGACAGCGTGCTCTCCGTGAAGA-3′), XRE-3 (5′-ATCCCTGCCCCCACACGCACACGTACACCT-3′ and 5′-AGGTGTACGTGTGCGTGTGGGGGCAGGGAT-3′). *In vitro*-translated AhR and purified Arnt proteins (OriGene Technologies Inc.) were mixed together and incubated for 60 min at 25°C in transformation buffer (25 mM HEPES (pH 7.5), 1mM EDTA, 10mM Sodium molybdate, 10% glycerol). The transformed proteins were then incubated with radiolabeled DNA probe for 15 min at 25°C in binding buffer (25mM HEPES, 200 mM KCl, 10mM DTT, 5 mM EDTA, 20% glycerol, 75 μg/mL CHAPS, and 25 ng/μL polydI:dC). DNA–protein complexes were fractionated in a 6% nondenaturing polyacrylamide gel. For identification of binding specificity, proteins were preincubated with unlabeled annealed competitor oligo (XRE; 5′-GATCTGGCTCTTCTCACGCAACTCCGGATC-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCAGATC-3′).

Immunoprecipitation and immunoblotting

Purified naïve T cells were differentiated into T_H0 cells or T_R1 cells for 6 days and lyzed with a lysis buffer [1% Nonidet NP40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM Na_2VO_4, 0.5 mM DTT and protease inhibitor]. AhR was immunoprecipitated with anti-AhR antibody (Biomol SA-210) and then subjected to SDS-PAGE. The immunocomplexes were analyzed with Western Blotting by using an anti c-Maf antibody (Santa Cruz sc-7866).

Reporter assays

10^7 EL-4 cells were electroporated as described49, activated in the presence of TGFβ1 (3 ng/ml) and analyzed after 24 h with the dual luciferase assay kit (New England Biolabs).

Retroviral infection

Naïve $CD62L^{hi}CD25^-CD44^{lo}CD4^+$ T cells were transduced with retroviruses as described50. MSCV GFP-RV retroviral DNA plasmids were transfected into the Phoenix packaging cell line and 72 h later the retrovirus-containing supernatants were collected. MACS-purified $CD4^+$ T cells were activated 24 h with plate-bound antibodies to CD3 and CD28, and infected by centrifugation (45 min at 2000 rpm) with retrovirus-containing supernatant supplemented with 8 μg/ml Polybrene (Sigma-Aldrich) and recombinant human IL-2 (25 units/ml).

In vivo treatment with anti-CD3

AhR^d, $Il27ra^{-/-}$ and control littermates were treated with 20 μg of antibodies to CD3 (clone 2C11) or isotype control, administered i.p. every 3 days for a total of 3 times. Mice were

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sacrificed 4 hours after the last treatment, single cell suspensions were prepared from mesenteric lymph nodes and spleens and IL-10 expression was analyzed by intracellular staining.

Statistical analysis

Statistical analysis was performed using Prism software (Graph Pad software, La Jolla, CA, USA). P values<0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AhR	Aryl Hydrocarbon Receptor
c-Maf	avian musculoaponeurotic fibrosarcoma v-maf
T_R1 cell	regulatory type 1 cell
EAE	experimental autoimmune encephalomyelitis

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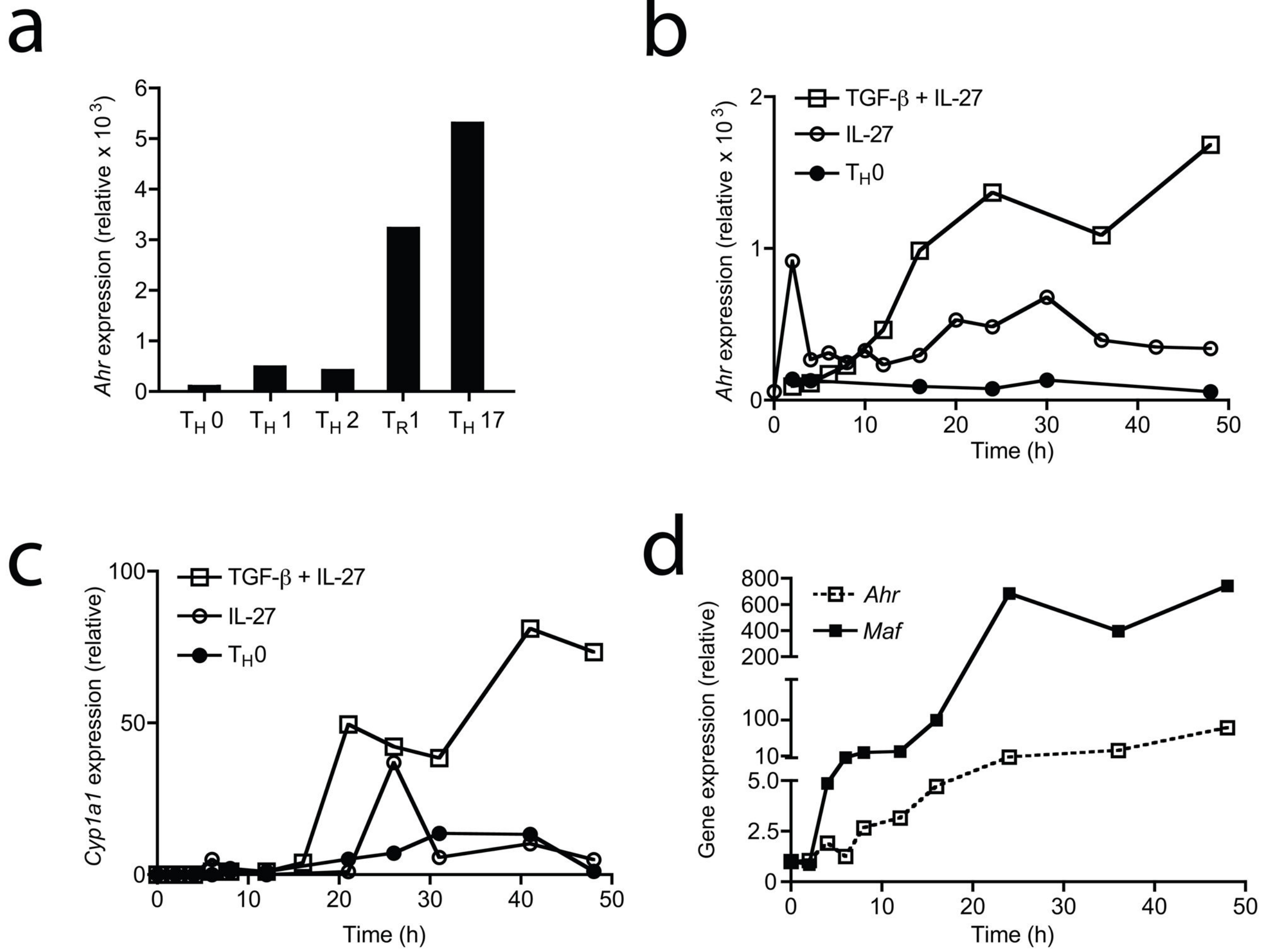

Figure 1. IL-27 upregulates AhR in T_R1 cells

RNA isolated from naïve $CD4^+CD44^{lo}CD62L^{hi}CD25^-$ cells differentiated into indicated populations in the presence of anti-CD3 and anti-CD28 antibodies was subjected to quantitative real-time PCR (RT-PCR) relative to the expression of mRNA encoding β-actin ($2^{-\ CT}$ x100000) to examine expression of *Ahr* at different time points following activation. **a**) RT-PCR analysis of *Ahr* expression at 48 hours in T_H0, T_H1, T_H2, T_H17 and T_R1 cells differentiated with either no cytokines, IL-12, IL-4, TGF-β plus IL-6 or TGF-β plus IL-27 respectively. RT-PCR kinetic analysis of **b)** *Ahr* and **c)** xenobiotic metabolizing cytochrome P450 enzyme *Cyp1a1* expression in T_H0 or T_R1 cells differentiated with IL-27 or TGF-β and IL-27. **d)** RT-PCR kinetic analysis of *Ahr* and *Maf* expression in T_R1 cells. Gene expression relative to T_H0 cells is depicted. Representative data from one of three experiments are shown.

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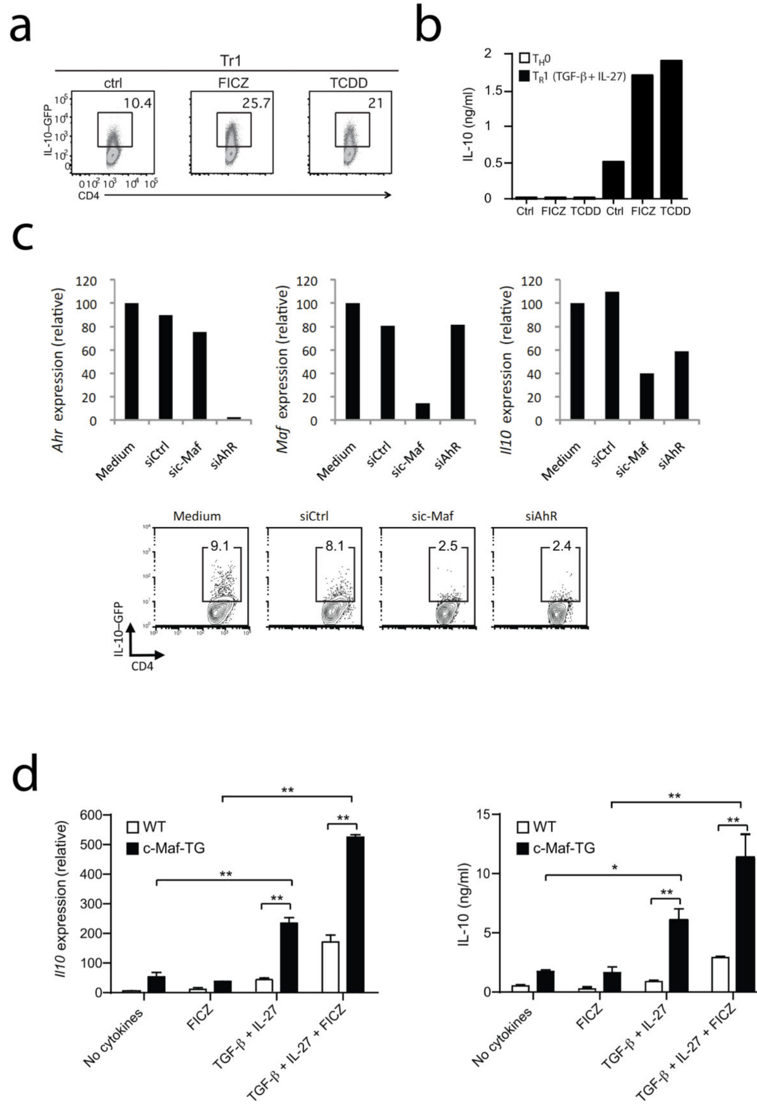

Figure 2. AhR regulates IL-10-production in T_R1 cells induced by TGF-β and IL-27

Naïve CD4+ T-cells from IL-10 reporter mice (Vert-X mice) were cultured with IL-27 and TGF-β in the absence (Control: Ctrl) or presence of the AhR agonists FICZ (100nM) or TCDD (100nM) **a)** IL-10.GFP expression was analyzed by flow cytometry after 72 hours of culture**. b)** IL-10 protein was measured by cytokine bead array analysis at 48 hours. **c)** Naïve cells from IL-10 reporter mice were cultivated with IL-27 plus TGF-β and transfected with either an irrelevant control siRNA or an siRNA against AhR or c- Maf. *Ahr*, *Maf* and IL-10 mRNA expression in T_R1 cells were assessed after 24 hours of culture by quantitative PCR (top) and IL-10.GFP expression was analyzed by flow cytometry after 48 hours (bottom). **d)** Naïve T cells isolated from wildtype (WT) or c- Maf transgenic (c-Maf-TG) mice were differentiated into T_H0 or T_R1 cells with TGF-β and IL-27 in the absence or presence of FICZ (100nM). After 48 hours of culture, *Il10* mRNA expression was assessed by quantitative PCR (left panel) and IL-10 secretion was analyzed by ELISA at 72 hours (right panel). (*p<0.05; **p<0.01)

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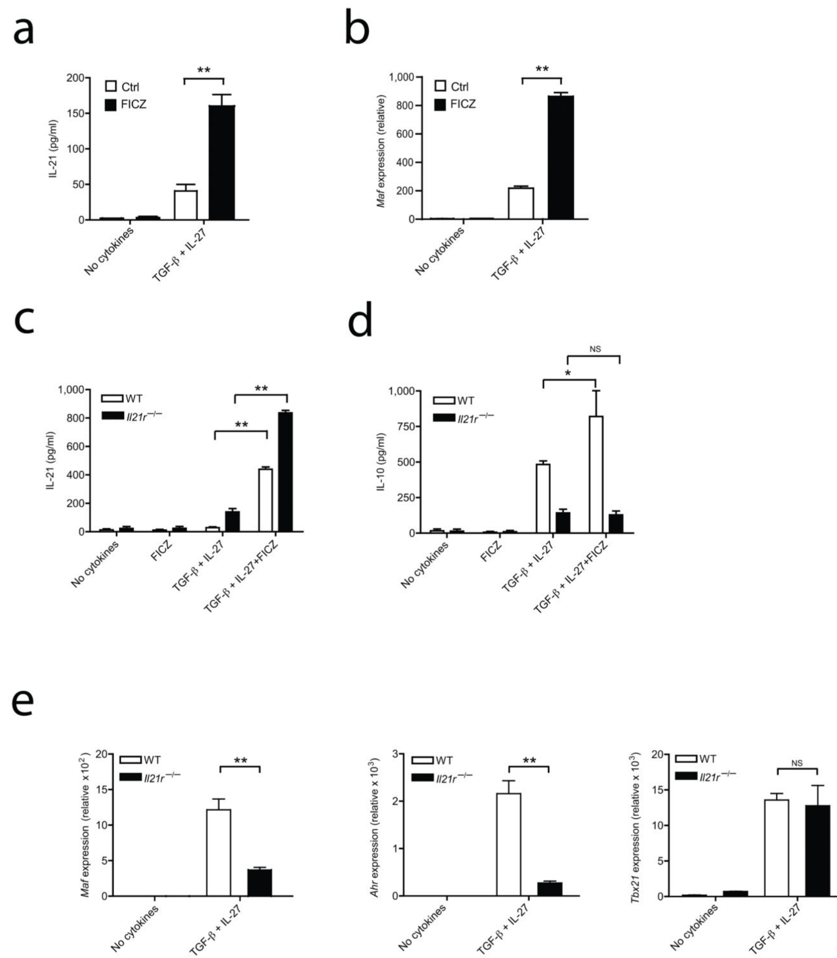

Figure 3. AhR signaling dictates IL-21 secretion in T_R1 cells

Naïve T cells were differentiated into T_R1 cells without (Ctrl) or with FICZ (100nM) and **a)** IL-21 cytokine production was assessed by cytokine bead array analysis after 72 hours of culture; **b)** The transcription factor *Maf* was quantified by RT-PCR at 48 hours **c)** and **d)** Naïve T cells from wild type and *Il21r*$^{-/-}$ mice were differentiated into T_R1 cells and IL-21 and IL-10 production were analyzed by cytokine bead array analysis after 48 hours of culture **e)** mRNA for *Maf*, *Ahr* and *Tbx21* in the cells described in **c)** was quantified by RT-PCR relative to the expression of mRNA encoding *β-actin*. Data are from one of three experiments with similar results. (*p<0.05; **p<0.01)

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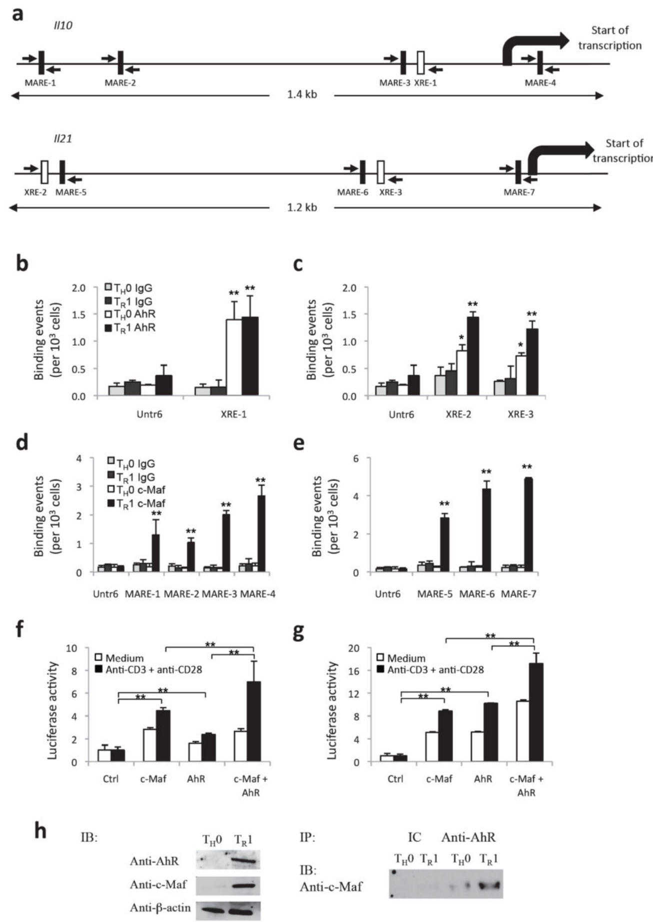

Figure 4. AhR and c-Maf transactivate the *Il10* and *Il21* promoters in T_R1 cells

a) AhR and c-Maf binding sites in the *Il10* and the *Il21* promoters. Schematic representation of the *Il10* and the *Il21* promoters, AhR binding sites (XRE) are depicted as open boxes and c-Maf binding sites (MARE) are depicted as filled boxes. **b)** ChIP analysis of the interaction of AhR or isotype control antibody (IgG) to the XRE in the *Il10* and **c)** the *Il21* promoter in *in vitro* differentiated T_R1 or control T_H0 cells. (*p<0.01; **p<0.001 between AhR vs IgG) **d)** ChIP analysis of the interaction of c-Maf or isotype control antibody (IgG) to the MARE in the *Il10* and **e)** the *Il21* promoter in *in vitro* differentiated T_R1 or control T_H0 cells. (**p<0.001 between c-Maf vs IgG) **f)** and **g)** Transactivation of the *Il10* or *Il21* promoters by c-Maf or AhR. Reporter constructs for the *Il10* **f)** or *Il21* **g)** promoters (*Il10-Luc* and *Il21-Luc*, respectively) were co-transfected in EL-4 T cells with vectors coding for AhR and/or c-Maf, and firefly luciferase activity was determined 24 hours later and normalized to the renilla luciferase activity of a co-transfected control. (**p<0.001) **h)** *In vitro* differentiated T_H0 or T_R1 cell expression of AhR and c-Maf (immunoblot; IB, left panel). AhR was immunoprecipitated from nuclear extracts with a specific antibody (IP, right panel), c-Maf and AhR complexes immunoblotted (right panel) using an anti-c-Maf antibody.

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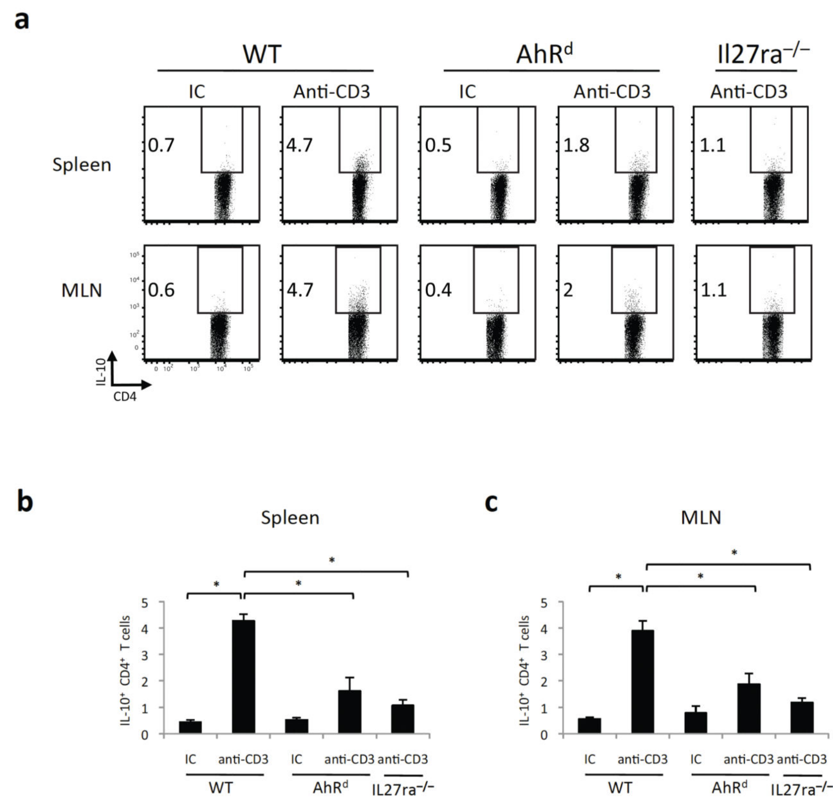

Figure 5. AhR controls the generation of T_R1 cells *in vivo*

AhR^d, wild type (WT) or *Il27ra*$^{-/-}$ mice were injected i.p. with 20 μg of antibodies to CD3 or an isotype control (IC) once every 3 days, for a total of 3 times. 4 hours after the last injection, mice were sacrificed and the expression of IL-10 in the spleen and the mesenteric lymph nodes (MLN) was analyzed by flow cytometry. **a)** Representative plots of IL-10 expression in CD4$^+$ cells in the spleen and the MLN. **b)** and **c)** Frequency of IL-10$^+$ CD4$^+$ T cells in anti-CD3 or isotype control (IC) treated mice (mean + s.d. of 3–5 mice) in spleen (**b**) or MLN (**c**). (*p<0.01)

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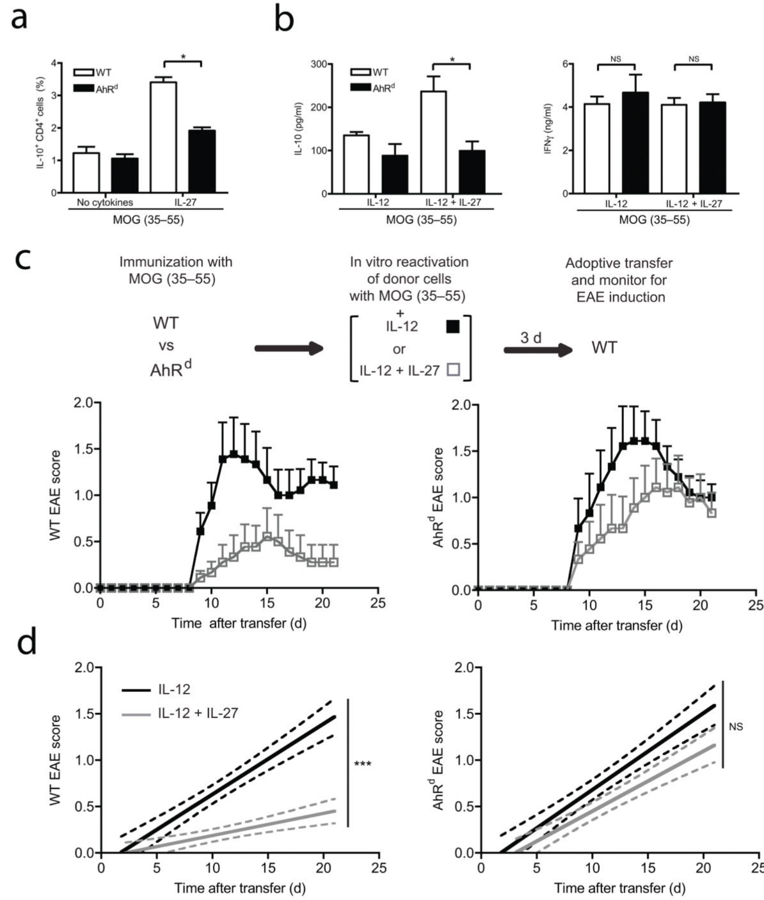

Figure 6. AhR controls the IL-27-mediated inhibition of EAE

MOG-specific cells from spleen and lymph nodes from wild type (WT) or AhR^{d} mutant mice were obtained 11 days after immunization and **a)** the percentage of IL-10-producing CD4^{+} T cells was assessed by flow cytometry after five days of culture with MOG in the presence or absence of IL-27. **b)** Cells were restimulated *in vitro* with MOG in the presence of IL-12 either with or without IL-27 and IL-10 and IFN-γ secretion was analyzed 3 days later by cytokine bead array analysis. (*$p<0.05$) **c)** and **d)** WT or AhR^{d} MOG specific cells prepared as in **b)** were adoptively transferred into WT mice and recipient animals were monitored for the development of EAE. **c)** The mean clinical disease score in each group is shown for WT or AhR^{d} donor cells. **d)** Linear regression curves of the disease for each group are shown for the experiments depicted in **c**). The disease course differs significantly between the two treatments (IL-12 versus IL-12 plus IL-27) of WT donor cells but not of AhR^{d} donor cells. The 95% confidence intervals for each curve are represented with dashed lines. (***$p<0.0001$).

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Table 1

EAE incidence after cell transfer

Group	Disease Incidence	Maximum score	Mean day of onset
WT IL-12	8/9 (89%)	2.1 ± 0.2	11.5 ± 1.5
WT IL-12 + IL-27	3/9 (33%)*	1.8 ± 0.5	10.3 ± 1.3
AhR^d IL-12	8/9 (89%)	2.4 ± 0.2	11.0 ± 0.9
AhR^d IL-12 + IL-27	8/9 (89%)	2.0 ± 0.2	12.8 ± 1.4

*P<0.05 (Fisher’s exact test)