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An Interaction between Kynurenine and the Aryl Hydrocarbon Receptor Can Generate Regulatory T Cells

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The aryl hydrocarbon receptor (AHR) has been known to cause immunosuppression after binding dioxin. It has recently been discovered that the receptor may be central to T cell differentiation into FoxP3+ regulatory T cells (Tregs) versus Th17 cells. In this paper, we demonstrate that kynurenine, the first breakdown product in the IDO-dependent tryptophan degradation pathway, activates the AHR. We furthermore show that this activation leads to AHR-dependent Treg generation. We additionally investigate the dependence of TGF-β on the AHR for optimal Treg generation, which may be secondary to the upregulation of this receptor that is seen in T cells postexposure to TGF-β. These results shed light on the relationship of IDO to the generation of Tregs, in addition to highlighting the central importance of the AHR in T cell differentiation. All tissues and cells were derived from mice. The Journal of Immunology, 2010, 185: 3190–3198.
teractions (28, 29). The exact mechanistic pathway by which IDO leads to Tregs has been debated, and both tryptophan starvation and direct effects of tryptophan metabolites (including kynurenine) have been proposed (30–32). In addition to a connection via indole metabolism, the IDO–AHR interaction was particularly interesting in light of the observation that IDO may be upregulated by the AHR (33, 34) and that kynurenine and related metabolites may be AHR agonists (35–37).

In this report, we demonstrate an important role for kynurenine, the first tryptophan metabolite of the IDO pathway, in Treg generation. We provide evidence that kynurenine activates the AHR at a dose clinically relevant in humans and leads to Tregs in vitro. The role for the AHR in this process is supported by two observations. First, kynurenine does not influence Treg generation in AHR-null T cells. Second, kynurenine can be shown to activate the AHR using classical response genes, such as Cyp1al1 and Cyp1b1. In our model, the AHR in T cells is required for the generation of Tregs by kynurenine. We further define the importance of the AHR for optimal generation of Tregs by TGF-β and characterize potential mechanisms for this.

**Materials and Methods**

**Mice**

C57BL/6J wild-type (WT) and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). AHR-null (AHR-deficient B6) mice on a C57BL/6J background (13) were bred and maintained under specific pathogen-free conditions. All animal experiments were carried out according to institutional guidelines approved by the University of Wisconsin School of Medicine and Public Health Animal Care and Use Committee (Madison, WI).

**Isolation and differentiation of bone marrow-derived DCs**

The method of murine bone marrow-derived DCs (BMDCs) was performed as previously described (38). Briefly, bone marrow was obtained from mice femurs. After RBC lysis, the cells were plated in six-well plates with a density of 1 × 10^6/ml in complete RPMI 1640 media supplemented with 30 ng/ml GM-CSF. On day 3, nonadherent cells and 75% of culture media were exchanged for fresh media. On day 6, the cells were either harvested as immature DCs or cultured an additional day to maturity by again exchanging 75% of the media, with the addition of 50 ng/ml LPS. A total of 80% of the cell population stained positive for CD11c by flow cytometry. For analysis of mRNA expression, cells were treated with or without TCDD (10 nM). As mentioned above, 50 ng/ml LPS was used for maturation of BMDCs. There is a previous publication that LPS alone can lead to IDO (39). It should be clarified that this only occurred when higher doses of LPS (5 μg/ml) were used. This response is dose dependent, as seen by other investigators (33, 40, 41). As an additional control, an LPS concentration that consistently showed >90% purity for CD4^+CD62L^−DC25^− cells. An example of the separation is included in the Supplemental Material (Supplemental Fig. 2A). Viability at the beginning of culture was typically >98% as seen by trypan blue staining. For quantitative PCR (qPCR) analysis, 2.5 × 10^5 cells were cultured in each well of a 96-well round-bottom plate coated with 0.5 μg/ml anti-CD3 and anti-CD28 overnight and then washed with PBS twice before seeding the cells. The naive T cells were maintained in F10 media supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 μM penicillin, 50 μM 2-ME, 25 mM HEPES, and 2 mM t-glutamine and were treated with 10 nM TCDD, 100 nM FICZ, 2–10 ng/ml TGF-β (as specified), 50 μM kynurenine, or 25 μM each kynurenine (hydroxymethylene tryptophan, hydroxyanthranilic acid, anthranilic acid, nicotinamide, and quinolinic acid). After 5 d, the cultured cells were harvested for RNA assay. Prior to this, cells were checked for viability using live-dead staining with trypan blue. For flow cytometric analysis, purified naive T cells were stimulated with the CD3/CD28 T cell Activation/Expander Kit (Miltenyi Biotech) for 5 d. As indicated, cultures were supplemented with recombinant cytokines and reagents: human TGF-β1 (R&D Systems, Minneapolis, MN), mouse IL-6 (20 ng/ml; R&D Systems), kynurenine, FICZ (100 nM), and AHR antagonist CH-223191 (Calbiochem, San Diego, CA).

**Intracellular FoxP3 and IL-17 cytokine staining**

To stain for Foxp3, T cells were first surface stained with anti-CD4 and anti-CD25 and then fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience, San Diego, CA) for 30 min at 4°C. Following this, cells were stained with Pacific Blue-conjugated anti-Foxp3. For intracellular IL-17 staining, T cells were first stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 800 ng/ml ionomycin (Sigma-Aldrich) for 4 h in the presence of GolgiStop (BD Pharmingen, San Diego, CA); or High-capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA). The relative quantitation PCR for IDO1 (Mm00492586-m1), GAPDH (4352339-E08018), and IFN-γ (Mm9999907-m1) were performed in the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), and TaqMan Universal PCR Master Mix (Applied Biosystems) was used as a reaction reagent. The relative quantitation PCR for Foxp3, Cyp1al1, Cyp1b1, and GAPDH were performed by the Bio-Rad iCycler (Bio-Rad) and iQ SYBR Green Supermix (Applied Biosystems) used as the reaction reagent.

**Isolation of naive CD4 T cells and T cell differentiation**

Naïve CD4 T cells were isolated from spleens of C57BL/6J and AHR-null mice using the CD4 CD62L Isolation Kit (Miltenyi Biotech, Auburn, CA) and an autoMACS. This kit includes a depletion mixture, including the addition of apheresis blood and an anti-CD62L Dynabeads. CD62L is expressed on naïve T cells and downregulated upon activation. A small subset of central memory T cells also express CD62L, and could be included in this separation. These represent a very small proportion of the final separation, and we will refer to separated cells as naïve T cells. Cells were tested for purity post-sorting and consistently showed >90% purity for CD4^+CD62L^−CD25^−. An example of the separation is included in the Supplemental Material (Supplemental Fig. 2A). Viability of the beginning of culture was typically >98% as seen by trypan blue staining. For quantitative PCR (qPCR) analysis, 2.5 × 10^5 cells were cultured in each well of a 96-well round-bottom plate coated with 0.5 μg/ml anti-CD3 and anti-CD28 overnight and then washed with PBS twice before seeding the cells. The naïve T cells were maintained in F10 media supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 μM penicillin, 50 μM 2-ME, 25 mM HEPES, and 2 mM t-glutamine and were treated with 10 nM TCDD, 100 nM FICZ, 2–10 ng/ml TGF-β (as specified), 50 μM kynurenine, or 25 μM each kynurenine (hydroxymethylene tryptophan, hydroxyanthranilic acid, anthranilic acid, nicotinamide, and quinolinic acid). After 5 d, the cultured cells were harvested for RNA assay. Prior to this, cells were checked for viability using live-dead staining with trypan blue. For flow cytometric analysis, purified naïve T cells were stimulated with the CD3/CD28 T cell Activation/Expander Kit (Miltenyi Biotech) for 5 d. As indicated, cultures were supplemented with recombinant cytokines and reagents: human TGF-β1 (R&D Systems, Minneapolis, MN), mouse IL-6 (20 ng/ml; R&D Systems), kynurenine, FICZ (100 nM), and AHR antagonist CH-223191 (Calbiochem, San Diego, CA).

**Luciferase assay**

A mouse hepatoma cell line H1L6.1c3, stably carrying a dioxin-responsive element (DRE)-driven firefly luciferase reporter gene [a gift from Dr. Denison, University of California, Davis, CA (42)] was maintained with 0.3 mg/ml G418 in completed DMEM media. Briefly, 0.6 × 10^5 cells were seeded in each well of a six-well plate overnight and then were treated with different concentrations of TCDD, t-tryptophan, t-kynurenine, hydroxykynurenine, hydroxynoanthranilic acid, anthranilic acid, nicotinamide, and quinolinic acid for the time specified. All of the kynurenines (including kynurenine) were purchased from Sigma-Aldrich (St. Louis, MO), and purity was listed at >99%. The DRE DNA was purchased from HPLC. They were placed in solution in 0.5 M HCL, as recommended by the manufacturer for maximum solubility. Cells were lysed by lysis buffer (Promega, Madison, WI), and the luciferase assay was performed by using a BD monolight 3010 luminometer (BD Biosciences, San Jose, CA). The relative light unit is the indicator of luciferase expression level. All experiments were repeated three times, and each sample was tested in triplicate each time.

**Real-time quantitative PCR**

Total RNA was extracted using the reagents: RNeasy Mini Kit and RNA-Free DNase Set (Qiagen, Valencia, CA). A total of 500 ng total RNA in each group was used for RT reaction (Script cDNA Synthesis Kit, Bio-Rad, Hercules, CA; or High-capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA). The relative quantitation PCR for IDO1 (Mm00492586-m1), GAPDH (4352339-E080618), and IFN-γ (Mm9999907-m1) were performed in the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), and TaqMan Universal PCR Master Mix (Applied Biosystems) was used as a reaction reagent. The relative quantitation PCR for Foxp3, Cyp1al1, Cyp1b1, and GAPDH were performed by the Bio-Rad iCycler (Bio-Rad) and iQ SYBR Green Supermix (Applied Biosystems) used as the reaction reagent.

**Results**

**AHR activation in DCs may play a role in directing T cell differentiation.**

We first examined the role that the AHR in DCs might play in directing T cell differentiation. Based on previously published data...
The observation that IDO can be upregulated in DCs in an AHR-Ca peak at a later time point (10 h), seen in Fig. 2DRE in comparison with kynurenine. It is a potent ligand, with FICZ was also tested to compare its efficacy as an activator of the kynurenine in vitro, a substantial increase in Cyp1a1 and Cyp1b1 this cell line using real-time PCR and found that postexposure to products were examined. As seen in Fig. 2A inflammation postactivation of IDO (44) (Fig. 2A) that confirms the AHR activation in mouse BMDCs leads to IDO. BMDCs with TCDD. Interestingly, kynurenine itself showed the strongest kynurenine pathway, and compared their ability to activate the DRE (43). We tested each of the commercially available substrates in the IDO pathway for their ability to activate the AHR. We employed tryptophan breakdown products downstream to kynurenine do not above to control for any indirect affect that may have led to IDO activation of the AHR in these cells. Exposure to TCDD also in-vitro. RNA was then harvested and tested for the presence of Cyp1a1 (29, 33, 34), we considered the possibility that IDO may be a mediator of the cross talk between these cells. We initially set out to confirm that IDO could be induced by activation of the AHR. Fig. 1 shows that cultured BMDCs, when exposed to the AHR agonist TCDD, led to Cyp1a1 induction, confirming activation of the AHR in these cells. Exposure to TCDD also increased IDO mRNA levels. DCs produced Cyp1a1 and IDO in both immature and mature states, indicating they did not have to be activated to have this response. AHR-null DCs did not exhibit IDO production postexposure to TCDD. In Supplemental Fig. 3, mRNA for IDO was analyzed at earlier time points than 48 h used above to control for any indirect affect that may have led to IDO expression. As early as 7 h postculture, IDO mRNA was generated.

Kynurenine activates the AHR, whereas other tryptophan breakdown products downstream to kynurenine do not

Given that IDO can be generated by AHR activation in DCs and that tryptophan breakdown products have been known to generate AHR ligands, we examined all of the tryptophan breakdown products of the IDO pathway for their ability to activate the AHR. We employed a mouse cell line of hepatoma cells, termed Hepa1. These cells have been transfected with a luciferase reporter gene fused to the DRE (43). We tested each of the commercially available substrates in the kynurenine pathway, and compared their ability to activate the DRE with TCDD. Interestingly, kynurenine itself showed the strongest activity (Fig. 2A). Peak activity was at 5 h, with a dose (50 μm) that is comparable to levels encountered clinically in humans in areas of inflammation postactivation of IDO (44) (Fig. 2A). All other breakdown products showed less DRE activity, with decreasing peaks of activity the further down the kynurenine pathway that products were examined. As seen in Fig. 2B, we further tested this cell line using real-time PCR and found that postexposure to kynurenine in vitro, a substantial increase in Cyp1a1 and Cyp1b1 mRNA was seen, confirming activation of the AHR by this ligand. FICZ was also tested to compare its efficacy as an activator of the DRE in comparison with kynurenine. It is a potent ligand, with a peak at a later time point (10 h), seen in Fig. 2C.

Presence of the AHR is necessary in T cells for optimal generation of FoxP3+ Tregs

The observation that IDO can be upregulated in DCs in an AHR-dependent manner and that kynurenine activates the AHR led us to consider that kynurenine could generate Tregs through the AHR. Using the well-documented technique for Treg generation with TGF-β and Ab stimulation (45), we initially exposed naive CD4+- T cells from WT and AHR-null animals to 2 ng/ml TGF-β and analyzed collected mRNA for FoxP3 expression. As seen in Fig. 3A, WT cells generated FoxP3, ~40 times the response in AHR nulls. To further support this finding, we performed a similar experiment exposing WT or AHR-null cells to Ab stimulation and a higher dose of TGF-β and measured Treg generation by flow cytometry. This is represented in Fig. 3B, in which optimal Treg populations were generated in WT cells (29.1% in this representative assay), with a muted response from null cells (11.6% in this same assay). By titrating doses of TGF-β, we were able to yield increasing numbers of CD25+FoxP3+ cells seen by flow cytometry, represented graphically in Fig. 3C. When naive CD4+ T cells were separated from AHR-null animals, increasing doses of TGF-β had little effect on the generation of Tregs (Fig. 3B, 3C). To further test the importance of AHR-ligand binding in Treg generation, we repeated Ab stimulation of naive WT T cells with titrating doses of TGF-β, and this time included an AHR antagonist (CH-223191) known to competitively bind to the receptor. As seen in Fig. 3D, the addition of antagonist blocked the increase of Tregs seen by flow cytometry postexposure to TGF-β in vitro.

The above experiments all demonstrate the importance of the AHR in Ab-stimulated Treg generation via TGF-β. Given our suspicion that cell–cell contact is important in AHR-dependent Treg generation, we employed an in vitro system separating pDCs and exposing them to allogeneic naive CD4+ T cells (pDCs were derived from BALB/c mice, and naive T cells from C57BL/6J mice). This system was previously shown to be dependent on IDO for successful generation of Tregs (29). We were able to repeat the findings that pDCs exposed to CPG led to significant generation of FoxP3+ Tregs in WT allogeneic naive T cells (Fig. 3E). When naive T cells were isolated from AHR-null mice, a low percentage of Tregs were identified prior to manipulation. Addition of CpG did increase Treg generation, but the expression was dramatically less robust than in the WT cells (Fig. 3E).

Kynurenine induces generation of FoxP3+ Tregs in an AHR-dependent manner

As it is well known that IDO leads to the generation of Tregs, and it appears that kynurenine activates the AHR, the next step was to assess whether kynurenine can directly lead to FoxP3 expression. We first cultured mouse naive CD4+ T cells with Ab stimulation for 5 d in the presence of 10 ng/ml of TGF-β, kynurenine, TCDD, or FICZ. RNA was then harvested and tested for the presence of FoxP3. As seen in Fig. 4A, top panel, only TGF-β and kynurenine led to significant induction of FoxP3 RNA. We analyzed FoxP3 induction in triplicate in 11 separate biological experiments and achieved a fold change of 3.2, which was significantly increased from control with a p value of 0.017. When AHR-null cells were used (Fig. 4A, bottom panel), only TGF-β (10 ng/ml) yielded significant induction of FoxP3 RNA. To further show that kynurenine is leading to FoxP3 mRNA via an interaction with the AHR and not in some indirect way dependent on TGF-β, we cultured mouse naive CD4+ T cells as above with Ab stimulation either with or without kynurenine. RNA was then harvested and tested for Cyp1a1, Cyp1b1, and TGF-β. As seen in Fig. 4B, kynurenine exposure led to significant amounts of Cyp1a1 compared with control, but an increase of TGF-β mRNA over control was not seen, making it unlikely that kynurenine is acting indirectly by generating this cytokine. We then performed a similar experiment, exposing naive T cells to Ab stimulation with and without kynurenine and after 5 d of culture used flow cytometry.

![FIGURE 1.](image-url) AH3 activation in mouse BMDCs leads to IDO. BMDCs were generated from the bone marrow of C57BL/6j WT and AHR-null mice as described in Materials and Methods. Cells were harvested on day 6 as immature BMDCs or on day 7 as mature BMDCs following addition on day 6 of LPS at a dose of 50 ng/ml, a concentration that itself does not cause IDO expression, confirmed in Supplemental Fig. 1. BMDCs were cultured in the presence or absence of TCDD (10 nM) added on day 0 of culture. mRNA was isolated from immature or mature BMDCs and assayed for the expression of Cyp1a1 (left panel), a marker of AHR activation, and IDO1 (right panel). Data were normalized to WT control. Post ANOVA testing comparisons are to cultures without TCDD. Cyp1a1 mRNA was undetectable in all AHR-null PCR reactions. Each graph is representative of three independent experiments. ***p < 0.001.)
to assess for FoxP3. As seen in Fig. 4C, cells that were not exposed to kynurenine showed minimal FoxP3 expression, but those exposed to kynurenine had a significant shift, with 24.4% more FoxP3+ cells than untreated control. When AHR-null mice were used as the source for naïve T cells, very few Tregs were generated when exposed to pDCs. The addition of kynurenine did not cause any enhancement of Treg formation, presumably due to the lack of the AHR on the T cells. As further confirmation, we repeated the DC/T cell coculture with a 1:20 ratio of allogeneic BALB/c DCs to naive C57BL/6J T cells multiple times and present the summarized data graphically. As shown in Fig. 4E, the 1:20 ratio yielded generation of Tregs by flow, and kynurenine led to significant Treg generation as compared with untreated control. When repeated with AHR-null T cells, CpG was able to yield Tregs, but kynurenine did not support the dependence of the function of kynurenine on the expression of AHR by the T cell.

To assess the importance of the AHR on pDCs in this model, we performed this experiment using AHR-null cells as the source for pDCs (on a C57BL/6J background), and naïve T cells were taken from WT mice (BALB/c). As seen in Supplemental Fig. 4, under these circumstances, similar generation of Tregs was seen using null pDCs as WT pDCs, indicating, at least in this model, that the presence of the AHR is necessary on the T cell, and not the pDC, for optimal Treg generation.

The data in this section highlight that the AHR on T cells is activated by kynurenine and leads to Treg induction.

**TGF-β upregulates AHR expression, potentiating activation of the DRE by kynurenine**

To better understand the role of the AHR in TGF-β-dependent Treg generation, we next extracted total RNA from naïve T cells, either fresh or after 20 h or 3 d of culture, and conducted real-time PCR for AHR expression. Culture conditions included Ab stimulation with FICZ, kynurenine, or TGF-β. There is AHR expression at baseline (Fig. 5A), which increases >4-fold at 20 h with exposure to TGF-β and remains >3-fold elevated at 3 d. We additionally looked at Cyp1a1 expression at 20 h and 3 d and, as seen in Fig. 5A, FICZ and kynurenine led to 20 and 50 times mRNA production over baseline at 20 h, respectively, with Cyp1b1 levels remaining 20 times elevated at 3 d after kynurenine exposure. Culturing in the presence of TGF-β did lead to a small increase in Cyp1b1 (~4 times over baseline at 20 h), but much less than seen with FICZ or kynurenine. To assess whether AHR upregulation secondary to TGF-β would potentiate binding of ligands to the AHR, we compared the expression of Cyp1a1 and Cyp1b1 after kynurenine exposure with and without TGF-β, which is represented in Fig. 5B. The response is strongly enhanced after TGF-β exposure, shifting the curve up significantly, implying that TGF-β does potentiate the binding of kynurenine to the AHR when this ligand is present in the culture.

**Kynurenine does not lead to Th17 cell generation, whereas FICZ does**

Given that the AHR has also been implicated in the generation of Th17 cells when bound to certain ligands (FICZ), we wondered whether IDO pathway products could also favor Th17 morphology when present in a milieu favoring Th17 generation. We used the Th17-generating conditions described previously, based on exposure of naïve CD4+ T cells to IL-6 and TGF-β (21). We first repeated the finding that FICZ leads to enhancement of IL-17+ cells (23) (Fig. 6). As mentioned previously, FICZ is thought to act primarily through the AHR, confirming that the AHR can promote...
T cell differentiation to both Treg and Th17 differentiation depending on the milieu. We then tested kynurenine and found no effect on the generation of Th17 cells (Fig. 6). This indicates that activation of the AHR with different ligands can lead to entirely different outcomes depending on the surrounding milieu.

Discussion

Collectively, the data represent a novel way that IDO, via the kynurenine pathway, leads to Treg generation. It is generally believed that T cell differentiation depends on interactions between DCs and T cells (46–48), with IDO playing a role in this. The above findings establish a direct relationship between the AHR and the fate of T cells in vitro. IDO is generated by pDCs. This leads to tryptophan metabolism and kynurenine formation. Kynurenine binds to the AHR in T cells, leading to differentiation to CD25+ FoxP3+ T cells. Absence of the AHR in T cells prevents this effect. Kynurenine was previously identified in a review of AHR ligands (35), which corresponds to the knowledge that tryptophan metabolites can lead to ligands of the AHR; the finding that this ligand–receptor interaction leads to Treg generation is novel. Whereas Fig. 1 does indicate that IDO induction by DCs can be stimulated via the AHR, the physiologic significance of this needs to be further defined. IFN-γ does stimulate IDO in AHR-null DCs (data not shown), and Supplemental Fig. 4 would suggest that Tregs can be induced by AHR-null pDCs in coculture assays, but the AHR needs to be present on T cells for optimal Treg generation.

One issue that needs to be addressed is the reliance of TGF-β on the AHR. Fig. 3 indicates that optimal generation of Tregs by TGF-β is dependent on the presence of the AHR, similar to a report published previously (21). This decrease in Treg production is demonstrated both in T cells obtained from null mice and also with the use of the AHR antagonist (Fig. 3D). It is unlikely that this cytokine binds directly to the AHR, given its structure (we have tested this in a DRE luciferase assay with no response to TGF-β alone). More likely, it is secondary to the effect demonstrated in Fig. 5, which shows that TGF-β with Ab stimulation leads to an upregulation of the AHR in culture. This effect is seen in the first 24 h in culture and seems to persist at least 3 d, according to our data and the literature (21, 49). It is important to note that CD4+CD25+ T cells do express the AHR prior to its upregulation, which is demonstrated by both Western blot (21, 49) and DNA microarray (21) and in our own data (Fig.
This is further demonstrated by the fact that exposure of naive T cells to kynurenine or FICZ leads to mRNA transcription of Cyp1a1 and Cyp1b1, which would only occur in the presence of the AHR. It is possible that AHR upregulation alone leads to Treg generation after TGF-β exposure, which would correlate with a previously published experiment in which cotransfection of a construct coding for mouse AHR into a bacterial artificial chromosome with FoxP3 tagged with a Renilla luciferase reporter led to upregulation of Renilla activity (18). Another possibility is that the upregulation of the AHR allows endogenous ligands present in the system (either in media or secreted from cells during inflammation) to bind to the increased receptor with enhanced effect. This is supported by the data in Fig. 4C, which indicate that the AHR antagonist reduces the amount of FoxP3+ cells seen at baseline, as well as the fact that we do see some Cyp1a1 and Cyp1b1 induction in T cells postexposure to TGF-β (Fig. 5A, 5B). Perhaps there is ongoing binding between the AHR and endogenous ligands (which may include kynurenine in an in vivo system), and blocking the receptor blocks this Treg-generating effect. Although endogenous ligands may play a role in this differentiation, the effects of kynurenine in our assays far outweigh ligands that may already be present in the media. We tested the role of the AHR with TGF-β in Fig. 5B, in which kynurenine was titrated in culture with CD4+CD25+ T cells either in the presence of or without TGF-β. When this cytokine was present, the response of Cyp1a1 and Cyp1b1 was dramatically elevated when exposed to increasing doses of kynurenine, much higher than was seen with TGF-β alone. This would further support that kynurenine is a ligand of the AHR and that TGF-β
potentiates the effect of kynurenine binding by increasing the amount of receptor, far beyond what was seen with any ligand already present in the media.

A second important question is whether kynurenine undergoes catabolism, and is it actually a metabolite or breakdown product that is binding the AHR. It is possible that kynurenine in solution, like other small molecules, undergoes breakdown through various mechanisms. We used a 0.5 M solution of HCL to get kynurenine into solution, as per the recommendations of the manufacturer regarding maximum solubility. In an effort to test for breakdown of kynurenine, we have conducted HPLC with diode array detection to analyze kynurenine in this solution, as well as in buffered solution at a physiologic pH (50–53). In addition, the manufacturer has tested kynurenine in HCL by HPLC and found it to be >98% pure, which is similar to our findings (Supplemental Fig. 5). As can be seen in the figure, kynurenine dissolved in HCL at 1 d shows minimal decomposition, whereas kynurenine dissolved in buffer shows decreased purity. It was more difficult to dissolve kynurenine in buffered solution, which took up to 8 h at 37°C, as opposed to kynurenine in HCL, which went rapidly into solution and stayed in solution throughout the assays. We did find that kynurenine in bicarbonate buffer did not strongly activate the DRE in the luciferase assay when made fresh, but after a few days in culture displayed strong activity, which may correlate with solubility issues. When we examined kynurenine in HCL at 3 wk and 7 mo by HPLC, it continued to show minimal breakdown, indicating there is stability when placed in this solution. It is well known in the literature that the isoforms of kynurenine can be modified depending on the surrounding milieu in vivo (50–53), and it is difficult to rule out that this may occur to some degree in our assays. Nevertheless, if it is a metabolite of kynurenine binding to the AHR, it is still formed early in the kynurenine pathway, generated by effects of IDO on tryptophan. This does not diminish the importance of these findings, still linking IDO and the kynurenine pathway to the AHR.

It is fascinating that some ligands (like FICZ) activate the AHR, as seen by the luciferase assays and mRNA analysis, but do not lead to FoxP3 expression. Other ligands (kynurenine) activate this same receptor and do lead to FoxP3 on T cells. We have considered how different ligands might activate the same receptor and lead to disparate outcomes in protein generation. One hypothesis that we are investigating is that kynurenine itself may be enzymatically modified by the cytochrome P450 enzymes that are induced by the AHR, whereas FICZ may not. The product of this modification may directly lead to FoxP3 induction as opposed to a direct effect of kynurenine itself. This would explain this differential effect of these AHR ligands. This hypothesis is further supported by the fact that TGF-β can generate FoxP3 in our assays as early as 3 d, whereas the kynurenine-induced generation is typically not seen prior to 5 d (data not shown), despite the fact that the AHR is activated by kynurenine within a few hours. We will test this theory further by using known inhibitors of Cyp1a1 and Cyp1b1 enzymes [trans-stilbenes (54)] and experimenting with our recently generated DRE cluster null mice (55), which have dysfunctional Cyp1a1 and Cyp1a2 enzymes.

Regarding the concentration of kynurenine used in these experiments, the dose chosen was physiologic (44, 56), comparable to levels encountered in humans in areas of inflammation. It is also consistent with observations on the amounts of kynurenine and other tryptophan breakdown products generated in vitro by DCs.
In areas without an ongoing immune response, concentrations of the kynurenines are significantly lower, whereas when tryptophan is present to start with, additional tryptophan solution with kynurenine as the primary AHR ligand. It is still plausible that tryptophan depletion continues to play a role in the IDO-dependent generation of Tregs, and this theory, and experiments to elucidate the exact molecular responses elicited after binding of kynurenine or FICZ to the AHR are underway. The data in this manuscript further our understanding of the immune response. It is possible that both mechanisms play a role and in fact may explain why DCs are so potent at generating Tregs in coculture assays (as they generate IDO and deplete the tryptophan present in the assays). Of course, other mechanisms, including cell–cell contact and cytokine release, may also be involved. The data in this manuscript further our understanding of the emerging role of the AHR as a key player in the differentiation of T cells to Tregs, as well as the ultimate balance of regulatory and effector responses in immunity. As this pathway is further characterized, improved understanding will enhance our knowledge of T cell differentiation and yield new strategies of modulating the balance of regulation and effector response using ligands of the AHR.

Disclosures
C.A.B. has served as a scientific consultant to Dow Chemical on issues related to dioxin toxicity.

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