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

Joshua D. Mezrich,* John H. Fechner,* Xiaoji Zhang,* Brian P. Johnson,† William J. Burlingham,* and Christopher A. Bradfield‡

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: 000–000.

It has long been recognized that the immune system is in a fine balance between immunity and self-tolerance. The concept of suppressor T cells playing a role in this balance was first proposed in the 1970s (1). Efforts to identify these cells were generally unsuccessful, and their very existence was brought into question in the early 1980s by molecular biologists who failed to locate an elusive suppressor gene in the mouse MHC class II locus (2). The suppressor T cell concept was dropped and remained out of vogue until it re-emerged as the CD4+CD25+ regulatory T cell (Treg), first described in detail by Sakaguchi in 1995 (3). Since that time, numerous studies have characterized these cells and the role they play in autoimmunity, control of infection, and transplant rejection. Identification of FoxP3, a transcription factor for Treg development, has led to further characterization of the importance of regulation (4, 5). More recently, a new Th cell lineage, termed Th17, was described (6, 7). These IL-17–secreting cells are thought to play a major role as effectors in autoimmunity and transplant rejection. Interestingly, these new data have led investigators to question previously held beliefs about terminal cell differentiation and stability of Tregs, as the ability of Tregs to redifferentiate into Th17 cells in the appropriate inflammatory milieu has now been described (8, 9).

Recent publications implicate the aryl hydrocarbon receptor (AHR) as a central player in T cell differentiation. The AHR is best known as the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, or TCDD). Activation of the AHR by this environmental pollutant can lead to a range of toxic endpoints, including hepatocellular damage, epithelial changes, cancer, birth defects, thymic involution, and immunosuppression (10, 11). Although the AHR is well known for its role in toxicology, this receptor has also been shown to play a role in vascular and hematopoietic development (12, 13). Moreover, several potential endogenous ligands have been shown to bind to the AHR with variable affinity and potency (14). The fact that this receptor has been conserved in evolution (15) and that invertebrate orthologs of the AHR are well preserved and yet do not bind to TCDD (16) is consistent with the idea that a physiologically relevant endogenous ligand for this receptor exists (17).

In evaluating the mechanisms for immunosuppression seen postexposure to TCDD, it was discovered that activation of the AHR with TCDD leads to the generation of Tregs in vitro or in vivo (18), and, alternatively, activation with a different endogenous ligand, 6-formylindolo[3,2-b]carbazole (FICZ), leads to Th17 cell formation (19). Although this unusual pharmacology in which one AHR agonist diverts T cells toward regulators and another agonist generates effectors is surprising, multiple studies have confirmed the importance of the AHR in the generation of Th17 cells both in vitro and in vivo (20, 21). Regarding Treg generation, the direct relationship of regulatory cells to the AHR has been less clear (22). This has led investigators to question whether the AHR truly has a direct effect on the generation of these cells (23, 24).

Our laboratory has focused on the role of indolylic products as potential endogenous ligands of the AHR (25, 26). Therefore, we began to think about the potential for an interaction between the AHR and the IDO pathway. The IDO enzyme catalyzes the rate-limiting step of tryptophan degradation along the kynurenine pathway (27). IDO is present and activated in subsets of dendritic cells (DCs); particularly plasmacytoid DCs, or pDCs) and thought to be central to Treg generation from T cell precursors by DC–T cell in-
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 Cyp1a1 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 × 10⁶/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 the media. On day 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 concentrations of LPS (5 μg/ml) were used. This response is LPS dependent, as seen by other investigators (33, 40, 41). As an additional control, an LPS concentration of 100 ng/ml was used to check for viability 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 naive T cells. Cells were tested for purity postsorting and consistently showed >90% purity for CD4+CD125−CD25− cells. An example of the separation is shown in Supplemental Figure 2. Viability at the beginning of culture was typically >98% as seen by trypan blue staining. For quantitative PCR (qPCR) analysis, 2× 10⁵ 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 L-phenylalanine, 50 μM 2-μE, 25 mM HEPES, and 2 mM L-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 (hydroxykynurenine, 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 flow cytometry. The majority of cells were viable, but dead cells were gated out in flow cytometry analysis. For flow cytometric analysis, purified naive T cells were stimulated with the CD3/CD28 T cell Activation/Expander Kit (Miltenyi Biotec) 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 stained 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) for the final 2 h. Cells were then fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) and stained with PE-conjugated anti-IL-17. All Abs were from eBioscience. Flow cytometric analysis was performed using an LSR-II (BD Biosciences).

pDC/T cell coculture

Naive CD4+CD25− T cells were isolated from WT and AHR-null mice and cocultured with BALB/c pDCs isolated using the Miltenyi Mouse pDC Isolation Kit (Miltenyi Biotec) at a ratio of 20:1 or 10:1 (example of pDC separation in Supplemental Figure 2). Cpg, FICZ, and kynurenine were added at the start of culture. On day 5, cells were harvested and subjected to flow cytometric analysis.

Results

AHR activation in DCs leads to IDO induction

We first examined the role that the AHR in DCs might play in directing T cell differentiation. Based on previously published data...
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 postexpression 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 AHR in vitro. We employed the well-documented technique for Treg generation with Ab stimulation (45). We tested each of the commercially available substrates in the kynurenine pathway, and compared their ability to activate the DRE with standard cell lines as described in Materials and Methods. This system was previously shown to be dependent on AHR 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 CD4+ 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 that it appears that kynurenine activates the AHR, the next step was to consider that kynurenine could generate Tregs through the AHR. Using the well-documented technique for Treg generation with TGF-β and Ab activation (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 CD255FoxP3+ 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 postexpression 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).

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 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.
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 naive 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 Cyp1b1 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 naive 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. 3D).
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-\(\beta\) 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-\(\beta\) (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-\(\beta\) in Fig. 5B, in which kynurenine was titrated in culture with CD4\(^+\)CD25\(^-\) T cells either in the presence of or without TGF-\(\beta\). 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-\(\beta\) alone. This would further support that kynurenine is a ligand of the AHR and that TGF-\(\beta\)
tested kynurenine in HCL by HPLC and found it to be at a physiologic pH (50–53). In addition, the manufacturer has kynurenine, we have conducted HPLC with diode array detection to regarding maximum solubility. In an effort to test for breakdown of into solution, as per the recommendations of the manufacturer mechanisms. We used a 0.5 M solution of HCL to get kynurenine like other small molecules, undergoes breakdown through various that is binding the AHR. It is possible that kynurenine in solution, catabolism, and is it actually a metabolite or breakdown product already present in the media.

FIGURE 5. TGF-β upregulates AHR expression, potentiating activation of the DRE by kynurenine. A. Left panel, Total RNA was extracted from naive T cells (separated by magnetic beads), either fresh or after 20 h or 3 d of culture, and qPCR was performed for AHR expression. Culture conditions included Ab stimulation, FICZ 200 nM, kynurenine 50 μM, or TGF-β 3ng/ml. Right panel, Cyp1b1 mRNA expression was also examined by qPCR at 20 h and 3 d after the same culture conditions. The FICZ sample was not tested at 3 d. B, To assess whether AHR upregulation secondary to TGF-β would potentiate binding of ligands to the AHR, Cyp1a1 and Cyp1b1 expression after kynurenine exposure in culture for 3 d with and without TGF-β was measured. 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. Post ANOVA testing comparisons are against the vehicle control. *p < 0.05; **p < 0.001. mt, not tested.

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...
(57) (5–50 μM range). In areas without an ongoing immune re-
response, concentrations of the kynurenines are significantly lower,
in the nanomolar range, but increase 1000-fold in microenviron-
ments of inflammation. The fact that kynurenine concentrations
in vivo only reach the doses examined in our assays in areas of
inflammation could serve as a way to localize the IDO-AHR–
dependent Treg generation. Conceivably, this would allow an
immune response to commence, leading to IFN-γ and other inflam-
matory cytokines, which would then induce DCs to generate IDO.
Ultimately, kynurenine levels would build up sufficiently to in-
tax with the AHR and generate Tregs (and AHR expression
would be enhanced by TGF-β expression, further potentiating
the effects of kynurenine), hence muting the immune response.
In areas without inflammation, kynurenine doses would be inade-
quate to generate significant amounts of AHR-dependent Tregs.

These data shed light on the direct mechanism of IDO, which has
been controversial. Two theories on the function of IDO in Treg
generation have been proposed. The first is that IDO leads to
tryptophan depletion, and this relative starvation leads to cell cycle
arrest in some populations, favoring generation of Tregs (32). The
second theory is that the tryptophan catabolites themselves have a
more direct role in the generation of Tregs, supported by some
observational studies but without any clearly understood mecha-
nism (30, 31). The data in this paper strongly support the second
theory, and experiments to elucidate the exact molecular responses
elicited after binding of kynurenine or FICZ to the AHR are un-
derway. It is still plausible that tryptophan depletion continues to
play a role in the IDO-dependent generation of Tregs, and this
paper does not exclude this. In fact, as mentioned in our Materials
and Methods section, we did perform the assays with naive T cells
alone exposed to kynurenine in a low tryptophan media (F10),
which do not appear to be able to induce IDO. As this pathway is fur-
ther characterized and improved understanding will enhance our knowledge of
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Disclosures
C.A.B. has served as a scientific consultant to Dow Chemical on issues related to dioxin toxicity.

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Supplemental Figure 1. LPS dose-dependent upregulation of IDO1 mRNA in culture with bone marrow derived dendritic cells (BMDCs). BMDCs were generated from the bone marrow of C57BL/6J mice. On day 6 of culture, fresh media was added containing varying doses of LPS. Cells were harvested and mRNA was extracted 1 day later and then assayed for IDO1 gene expression by qPCR. Data was normalized to the vehicle control. Post ANOVA testing comparisons are against the vehicle control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Supplemental Figure 2. Following cell separation, flow cytometry was utilized to determine cell subset purity. A, Naive CD4 T cell isolation was performed by first negatively selecting for CD4+ CD25 splenocytes followed by selection of CD62L-bearing cells. Less than 5% of the naive CD4 T cells were CD44hi, while > 50% of the cells that were negatively selected for CD62L were CD44hi. B, pDCs isolated by negative immunomagnetic bead selection, were stained using PE-labeled PDCA1 (Miltenyi Biotech). Isolated cells were > 75% PDCA1 positive.
Supplemental Figure 3. AHR activation in mouse BMDCs leads to IDO. BMDCs were generated from the bone marrow of C57BL/6J WT and AHR null mice. Cells were harvested on day 6 as immature BMDCs and were treated for 7 and 20 hours with TCDD (10 nM). Following this, cells were harvested for qPCR analysis of IDO1. Post ANOVA testing comparisons are to control at each timepoint; ***, p < 0.001.
Supplemental Figure 4. The presence of the AHR on DCs is not necessary for optimal generation of Tregs in this coculture in vitro model. The experiments described in 4D were repeated using AHR null cells as the source for pDCs and naïve T cells from WT mice (pDCs in this experiment were on a C57BL/6J background, as this is the background that the AHR null is on, and WT were on a BALB/c background). Cells were treated with CpG or CpG with AHR antagonist as indicated. On day 5, cells were harvested and subjected to flow cytometric analysis. These are compared to the results using WT mice as the source for pDCs. Percentages are the fraction of gated live CD4+ cells that were FoxP3+CD25 double positive. These results are representative of 2 biologic experiments repeated in triplicate. Post ANOVA testing comparisons are to untreated vehicle control; **, p < 0.01.
Supplemental Figure 5. HPLC Testing Purity of Kynurenine in Solution. To assess the purity of kynurenine in solution, we conducted HPLC, examining the peaks representing kynurenine vs. other breakdown products. Kynurenine was either dissolved in 0.5 HCL, or in a buffered solution at physiologic pH (buffered by sodium bicarbonate, pH 7.2). These solutions were tested on HPLC at different time points after kynurenine was placed in solution, as indicated in the graph (1 day, 8 days, 3 weeks, 7 months).