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Identification of Cinnabarinic Acid as a Novel Endogenous Aryl Hydrocarbon Receptor Ligand That Drives Th22 Differentiation

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Identification of Cinnabarinic Acid as a Novel Endogenous Aryl Hydrocarbon Receptor Ligand That Drives Th22 Differentiation

by

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CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

A revised version of Chapters II through IV is in submission as "Identification of Cinnabarinic Acid as a Novel Endogenous Aryl Hydrocarbon Receptor Ligand That Drives Th22 Differentiation" at *PLoS One*. The co-authors on this publication are Jeff E. Mold[1], Bittoo Kanwar[1,2], Yong Huang[3], Alexander Louie[3], Michael P. Pollastri[4], Cuihua Wang[4], Guatam Patel[4], Diana G. Franks[5], Jennifer Schlezinger[6], David H. Sherr[6], Allen E. Silverstone[7], and Mark E. Hahn[5]. Joseph M. McCune[1] supervised the work.

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Identification of Cinnabarinic Acid as a Novel Endogenous Aryl Hydrocarbon Receptor Ligand That Drives Th22 Differentiation

Margaret Lowe

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a cytosolic transcription factor that recognizes and induces metabolic enzymes in response to a wide variety of xenobiotics. However, more recently the AHR has been proven critical in a diverse array of biological processes, including organ development, circadian rhythm, and immune response. In particular, AHR activation has been shown to affect T cell differentiation for both inflammatory T cells that produce IL-17 (Th17) and IL-22 as well as regulatory T cells (Treg) involved in tolerance. Given that the balance between inflammatory and immunoregulatory T cells has been implicated in pathological processes in contexts such as HIV infection and autoimmunity, understanding the role AHR and its ligands play in immune cell diffentiation is critical. While environmental AHR ligands can alter T cell differentiation, endogenous ligands are likely to be more relevant in host immune responses. We investigated downstream metabolites of tryptophan as potential AHR ligands because (1) tryptophan metabolites have been implicated in regulating the balance between Th17 and Treg cells and (2) many of the AHR ligands identified thus far are derivatives of tryptophan. We characterized the ability of tryptophan metabolites to bind and activate the AHR and to alter T cell differentiation. We report that the tryptophan metabolite, cinnabarinic acid (CA), is an AHR ligand that stimulates the differentiation of human and mouse T cells producing IL-22. We compare the IL-22-stimulating activity of CA to that of other tryptophan metabolites and define stimulation conditions that lead to CA production from immune cells. Our

findings link tryptophan metabolism to AHR activation and define a novel endogenous AHR agonist with potentially broad biological functions.

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CHAPTER I: INTRODUCTION

Activation of the aryl hydrocarbon receptor (AHR) within the immune compartment can dictate numerous, diverse immune responses in a ligand-specific manner. Given that such responses can range from immunosuppressive to immunostimulatory, understanding which ligands are available during a local immune response becomes critical to predicting the outcome of such a response. Interestingly, numerous tryptophan derivatives and metabolites have been identified as AHR ligands, and tryptophan metabolism is also tightly regulated in the context of immune activation. Given that induction of tryptophan metabolism is also associated with poorer outcomes in HIV infection, identifying the immunological effects tryptophan metabolites exert through AHR activation may allow for a better understanding of the immune response to HIV infection.

Part A: The aryl hydrocarbon receptor and immune response

The transcriptional activity of the AHR

The AHR is a cytosolic transcription factor that was initially identified as a xenobiotic sensor, upregulating metabolic enzymes in response to foreign compounds such as 2,3,7,8-tetracholrodibenzo-*p*-dioxin (TCDD) (1). Upon binding to a ligand, the AHR dissociates from its cytosolic binding partner, Hsp90, and enters the nucleus. There, it associates with the AHR nuclear translocator (ARNT) and binds to characteristic nucleotide sequences, referred to as dioxin-response elements (DREs), within the promoter of responsive genes (2). Such genes include metabolic enzymes cytochrome P450 1A1 (*CYP1A1*), *CYP1A2*, *NQO1*, and *ALDH3A1* (3). However, AHR-driven gene transcription can occur independently of binding to consensus

DREs and recruitment of ARNT (4), and there is considerable cross-talk with other cell signaling pathways (3), making complete characterization of AHR functional activity difficult. In addition to the effect of AHR on metabolic genes, additional AHR gene batteries affecting diverse pathways including cell cycle regulation, cardiovascular function, calcium regulation, and immune response have been described (5,6).

Though AHR orthologs exist within most vertebrates as well as some non-vertebrate species, sequence identity and ligand responsiveness can be highly divergent even between closely related species (7). Indeed, the initial discovery of the AHR was facilitated by polymorphisms existing between strains of inbred mice: C57BL/6 mice showed increased hydroxylase activity in response to dioxin while DBA/2J mice did not (8). Interestingly, the selectivity of AHR for ligand in humans may be more similar to the allele expressed in DBA/2J mice (9). Therefore, when considering translation to human biology, care must be taken in results obtained from mouse models. Fortunately, within human populations, only polymorphisms within the AHR with relatively minor effects on drug metabolism and response have been described to date (10,11).

Ligand specific effects of AHR activation

The ligands that can bind to and activate the AHR are highly structurally diverse. Planar, hydrophobic compounds structurally similar to TCDD, as well as nonplanar, polar compounds have been shown to have AHR-dependent effects on gene transcription. Not only may diverse ligands activate the AHR, they may do so with highly differential outcomes. Characterization of the immediate transcriptional effects of multiple AHR ligands has revealed that the majority of responses are not shared between ligands (12). The mechanism of this transcriptional diversity is

still being investigated. Some groups have described changes in preference of the AHR for different promoter response elements by specific ligands (13). However, when Degroot et al. characterized the DNA sequences bound by AHR when activated by six structurally diverse ligands, little to no diversity in promoter response element recognition was found among the ligands tested (14), indicating some additional mechanism must account for the differential effects seen in this group of ligands. Differential recruitment of co-activators by different AHR ligands has additionally been observed (15). Additionally, effects of AHR activation can vary between cell types (16), making comparisons between studies within different systems challenging. Therefore, the end result of AHR activation by a particular ligand may prove difficult to predict.

Endogenous ligands of the AHR

Though xenobiotic compounds have important effects on AHR activity, studies in AHR knockout mice have revealed roles of AHR activation in development and initiated interest in endogenous ligands for the AHR (17). Though independently generated AHR knockout lines have exhibited some distinctive phenotypes, universally observed effects include a reduction in liver size, abnormal liver development, reduced body size as neonates, and lower constitutive expression of certain metabolic enzymes such as *Cyp1a2* (18). More recently, a requirement of AHR expression for certain immune development and response pathways has been described. *AHR*-/- mice are greatly diminished in their capacity to produce IL-22 (19), and AHR expression is critical for the development of certain gastrointestinal immune structures (20,21). Though some of the ligands responsible for this development are diet-derived (21), removal of such

compounds does not affect all AHR-dependent immune cell subsets (20). Therefore, the ligands responsible for AHR-driven immune development still demand investigation.

Numerous compounds have been described as endogenous activators of the AHR (22). Some, such as 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), have been detected in tissue isolates, though the biosynthetic pathway for this compound remains undescribed (23). Others, such as 6-formylindolo[3,2-b]carbazole (FICZ), are potentially formed as a result of oxidative processes; UV irradiation of tryptophan may catalyze FICZ formation within the skin, though its effects may extend throughout the body (24,25). More recently, kynurenic acid and L-kynurenine, metabolites derived directly downstream of tryptophan, have been shown to activate the AHR (26,27). Due to the large number of products that may interact with the AHR, many of which are tryptophan-derived, the overall control of AHR-related activity may stem from the sum of several ligands instead of one sole regulator.

Regulation of AHR expression and response within the immune compartment

As previously mentioned, since the initial characterization of the AHR, multiple roles for this transcription factor in immune development and response have been identified (28). AHR expression within the immune compartment is highly regulated from the first stages of immune cell differentiation. Hematopoietic stem cells express AHR, and AHR signaling regulates their homeostatic proliferation and differentiation (29,30). Dendritic cells, monocytes, and macrophages also can respond to AHR ligands; AHR activation has been shown to alter monocyte-derived macrophage and dendritic cell maturation (31,32). However, the response to AHR activation in different immune cell subsets is not identical; the AHR-dependent gene battery has little overlap between dendritic cells and T cells (6).

Within $CD4^+$ T cells, AHR expression is tightly controlled through development. In naïve cells, AHR expression is low; activation, particularly with the addition of Th17 polarizing cytokines such as IL-6 and TGFβ, upregulates AHR expression (19,33). More recently, AHR up-regulation under Th17 driving conditions has been ascribed to action of the transcription factor, Ikaros, through alterations in methylation at the AHR promoter (34). Th17 driving conditions are not solely responsible for AHR expression changes following naïve CD4 T cell activation. Treg driving conditions also lead to AHR up-regulation, as does IL-27, an immunosuppressive Tr1-driving cytokine (35,36). Given that AHR expression may occur in both inflammatory and immunoregulatory states, it is not surprising that AHR ligands have important effects on T effector cell differentiation.

Effects of AHR activation on T cell differentiation

Both xenobiotic and endogenous AHR ligands have been shown to affect the immune response, though often through highly distinct, ligand-specific mechanisms. In particular, differentiation of T cells into inflammatory Th17/Th22 cells or immunosuppressive regulatory T cells (Treg) may both be influenced by AHR activation (see Table 1).

TCDD was one of the first AHR ligands reported to induce differentiation of T cells into Tregs *in vitro* and *in vivo* (37). Initially, it was postulated that Tregs might be less sensitive to the apoptotic effects of TCDD and would accordingly expand in a preferential manner due to the indirect effects of TCDD toxicity. However, data revealing that *AHR*-/- mouse-derived T cells are less effective at differentiating into Tregs *in vitro* and evidence of epigenetic modification of the Foxp3 promoter by TCDD both lend credence to a direct effect of TCDD, and potentially other AHR ligands, on Treg differentiation (33,38). Therefore it is not surprising that other AHR

ligands, including L-kynurenine, ITE, 3,3'-diindolylmethane, and indole-3-carbinol also promote Treg differentiation through AHR activation (27,39,40).

While many AHR ligands induce immunosuppression, partially through increasing Treg differentiation, others have an alternate effect on immune function. Th17 cells are inflammatory effector T cells that secrete cytokines such as IL-17, IL-22, IL-21, and IL-23 in order to promote activation and recruitment of other immune cells, including neutrophils, to sites of inflammation (41). Notably, the tryptophan photoproduct, FICZ, has been shown to increase Th17 differentiation both *in vitro* as well as *in vivo* in mouse models of multiple sclerosis and colitis (19,42). Interestingly, the increase in Th17 cells in the multiple sclerosis model resulted in greater pathology, while in the context of colitis they were beneficial, suggesting pathological outcome of AHR activation also depends upon the greater context of the inflammatory disease in question. Other AHR agonists that increase Th17 differentiation include indoxyl 3-sulfate and β-naphthoflavone (43,44).

Though traditionally characterized as a Th17 related cytokine, IL-22 is sometimes expressed independently of IL-17 within $CD4^+$ T cells as well as within innate cell subsets such as innate lymphoid cells (ILCs) (reviewed in (45)). The mechanism of AHR-driven IL-22 production appears to be due to RORγt-facilitated recognition of DREs in the *Il22* promoter (46). AHR activation does not necessitate that IL-17 and IL-22 are co-produced. Some ligands, such as microbiome-derived indole-3-aldehyde, appear to have a stronger effect on IL-22 production than on IL-17 production (47). Other AHR ligands appear to increase IL-22 while having neutral or suppressive effects upon IL-17. The small molecule VAF347 increases IL-22 through AHR activation without IL-17 induction (48). Additional studies have also reported induction of Tregs by VAF347 (49). The functional effects of VAF347 may therefore be more

similar to TCDD than to FICZ; a case report of TCDD exposure in a human patient also demonstrated expansion of IL-22 producing T cells *in vivo* (50).

It is important to note that the effects of certain AHR ligands on T cell differentiation may be driven additionally by effects on innate immune cells. Treatment with ITE was protective for mice in a multiple sclerosis model through generation of immunosuppressive dendritic cells that expanded Tregs *in vivo* (39). VAF347 not only directly increased IL-22 production in naïve T cells, but also programmed monocyte-derived dendritic cells to polarize naïve T cells towards IL-22 production (48). Notably, the immunosuppressive effects of VAF347 in an allograft tolerance model was also mediated by AHR-dependent effects on both dendritic cells and T cells (49). It will be interesting to determine whether additional ligands with known immunological effects have similar functional activity in other compartments of the immune system.

Part B: Indoleamine 2,3 dioxygenase (IDO) and immune response

Several known AHR ligands are tryptophan derivatives (such as the photoproduct FICZ) or tryptophan metabolites (i.e. kynurenic acid and L-kynurenine) produced downstream of the interferon-induced tryptophan metabolic enzyme indoleamine 2,3-dioxygenase (IDO). IDO activation has long been known to be important in regulating inflammatory immune responses in multiple diverse contexts. Thus, understanding the biology of the tryptophan metabolic pathway is critical for dissecting the roles of these AHR metabolites in immune function.

Regulation of IDO expression and activity

In humans, three enzymes are capable of catalyzing the irreversible, rate-limiting conversion of the non-essential amino acid tryptophan into N-formyl kynurenine: indoleamine

2,3-dioxygenase 1 (IDO1), IDO2, and tryptophan 2,3-dioxygenase (TDO) (51). In general, TDO has been described as a constitutive regulator of tryptophan concentrations in peripheral blood and is expressed primarily in the liver, while IDO1 expression appears to regulate tryptophan metabolism locally within tissues in response to inflammatory stimuli (52). IDO1 expression and activity have been observed within the eye, the placenta, the kidney, within astrocytes and microglia in the CNS, as well within antigen-presenting cells including macrophages and dendritic cells (53-58). An enzyme closely related to IDO1, IDO2, is less completely characterized. IDO1 and IDO2 appear to be expressed in similar organs, such as the kidney, but within distinct cell types within tissues (59).

IDO1 expression is induced by inflammatory signals provided by stimuli including interferon-gamma (IFNγ) or lipopolysaccharide (LPS), but through independent pathways (60). IDO2 expression does not appear to be similarly affected by interferon signaling: IFNγ deficient mice maintain IDO2 expression, and IDO2 is not induced during malaria infection (59). Interestingly, AHR agonists are also capable of increasing both IDO1 and IDO2 expression within bone marrow-derived dendritic cells (27,61). However, IDO expression does not always parallel functional activity. For instance, though both IDO1 and IDO2 mRNA and protein may be induced in B cells, consumption of tryptophan under such conditions was not observed (62). Even within monocyte-derived macrophages and dendritic cells, which are both known to be capable of functional IDO1 expression, induction of mRNA and detection of protein did not necessarily lead to metabolism of tryptophan (63-65). Potentially, this may be due to either failure of the cells to transport extracellular tryptophan for catabolism or additional mechanisms modulating IDO translation and activity. Some post-transcriptional and post-translational

regulatory mechanisms for IDO1 that have been observed *in vitro* include inhibition by nitric oxide and, separately, TGFβ (66,67).

Given the relative paucity of information on IDO2 and because much work relating to tryptophan metabolism was conducted before its identification, the rest of this discussion will use the term "IDO" to describe in general the metabolism of tryptophan to kynurenine occurring within immune cells, which is assumed to be mostly mediated by IDO1.

Effects of tryptophan deficiency through IDO induction on the immune response

IDO induction within the immune compartment was initially understood to be a protective antimicrobial mechanism through its capability to affect local tryptophan concentrations (56). Monocytes, exposed to stimuli such as IFN-γ or lipopolysaccharide (LPS), are able to remove tryptophan from the microenvironment through upregulation of IDO. Bacteria, dependent upon exogenous tryptophan for proliferation, are unable to compensate for this loss. However, local depletion of tryptophan does not only suppress proliferation of bacteria, but also the proliferation of cells of the immune system, such as T cells. IDO activity was first found to be immunosuppressive within the context of maternal-fetal tolerance (68). Inhibition or genetic deletion of IDO led to immune rejection of an allogeneic fetus. This immunosuppressive effect of IDO was solely ascribed to depletion of tryptophan from the microenvironment of proliferating immune cells, which resulted in activation of a sensor of amino acid deficiency in lymphocytes (69).

Effects of metabolites downstream of IDO on the immune response

However, further research found that IDO could exert immunosuppressive effects beyond those caused by tryptophan depletion. Terness et al. showed that metabolites generated downstream of IDO are capable of affecting the immune response through cytotoxic effects that inhibit proliferation *in vitro* (70), while Jasperson et al. demonstrated that administration of a mixture of kynurenine metabolites prevents immunorejection in a graft-versus-host disease (GVHD) model (71). Platten et al. likewise reported that such metabolites were capable of treating an autoimmune model of multiple sclerosis (72).

While kynurenine metabolites were initially characterized as generally immunosuppressive and often administered in combination within disease models, individual metabolites appear to have unique effects. Thus, L-kynurenine (L-KYN) has been shown to activate the AHR and to induce differentiation of T cells into regulatory T cells (Treg) (27). 3-hydroxyathranilic acid (3-HAA), likewise, has been shown to increase Tregs, and has also been shown to suppress IL-17 production (73,74). Kynurenic acid (KYA) has also been shown to activate the AHR, yet it increases IL-6 production and has not been shown to have an effect on Tregs (26). The diverse effects of these tryptophan metabolites makes understanding the sum effect of IDO induction difficult.

Regulation of metabolism downstream of IDO within the immune compartment

The tryptophan metabolic pathway (see Figure 1) downstream of IDO branches extensively, and the degree to which other metabolic enzymes are regulated in the immune compartment remains incompletely understood. Within primary human macrophages and monocyte-derived cell lines, many enzymes (including kynurenine 3-hydroxylase, kynureninase, and kynurenine aminotransferase) are expressed and functional at baseline and are not affected

by IFN-γ stimulation (75,76). Interestingly, while cells derived from non-immune tissues (i.e., lung and brain) are capable of kynurenic acid formation, kynurenine 3-hydroxylase, kynureninase, or 3-hydroxyanthranilate 3,4 dioxygenase activity have only been observed in immune tissues, indicating that these metabolic pathways may be of particular immunological importance. Though most of these enzymes are poorly characterized within the T cell compartment, one report has shown Th17 cells to preferentially express kynurenine monoxygenase, which appears to have an inhibitory effect on IL-17 production potentially through regulating metabolites downstream of IDO (77). Establishing the activity of tryptophan metabolic enzymes in specific cell subsets during the immune response will help to predict the influence of tryptophan metabolites.

Part C: The relationship between HIV infection and IDO expression

Targets for Therapeutic Agents in HIV Infection

Infection with HIV in humans and SIV in non-human primates leads to numerous immunological changes in response to viral presence. Certain species of non-human primates, such as African green monkeys and sootey mangabeys, do not develop pathology such as loss in $CD4^+$ T cells, despite sustained viral loads. Conversely, rhesus and pigtail macaques develop a clinical disease that is similar to that which occurs in the majority of people infected with HIV, namely a long-term loss in CD4 counts coupled with a progressive rise in viral load (78).

Though most HIV-infected patients, without treatment, will progress to end-stage AIDS, the clinical progression of these patients exists on a spectrum. Some patients infected with HIV will control virus without therapy, while others will progress much more rapidly, in spite of

adequate treatment (79). Some potential genetic causes for such diversity in response, such as protective or deleterious HLA alleles, have been identified; aggressive, early treatment regimens consisting of Highly Active Anti -Retroviral Therapy (HAART) have also been demonstrated to lead to better prognosis long-term.

However, the differences between HIV-infected patients who progress and those who do not remain incompletely understood; in parallel, the differences between pathogenic and non-pathogenic monkey models of SIV infection are not fully grasped. Identifying such differences would potentially identify novel targets for treating the immunological deficits that develop following HIV infection.

HIV and tryptophan metabolism

Decreases in serum tryptophan concentration in HIV-infected patients have been noted as early as 1990 (80). Fuchs et al. later correlated this degradation of tryptophan with increased serum IFN-γ in HIV-infected patients (81). This link between IFN-γ and tryptophan degradation led to the hypothesis that interferon-driven induction of IDO was responsible for these observations. Though induction of IDO during the course of HIV infection was implied by these findings, direct evidence of this was not demonstrated until ten years later. Grant et al. infected human monocyte-derived macrophages (MDM) in vitro, finding that certain strains of HIV transiently induced both IDO protein expression and activity (82). Direct evidence of IDO induction in HIV-infected patients did not arrive until Boasso et al. demonstrated increased IDO mRNA in patients versus non-infected controls (83). Conversely to what had been previously hypothesized, Boasso et al. showed that blocking interferon *in vitro* did not abrogate IDO up-regulation, indicating HIV is able to induce IDO through some other mechanism.

The relationship between IDO expression and HIV appears to be bi-directional. The presence of virus is directly responsible for IDO induction and, as expected, adequate treatment of HIV and viral suppression restores to some degree the normal ratio of tryptophan to kynurenine in the serum (84). However, inhibition of IDO also appears to have some effect on HIV; in a pathogenic SIV monkey model, monkeys treated with anti-retroviral therapy (ART) and with an IDO inhibitor had lower viral loads than monkeys treated with ART alone (85).

Interestingly, induction of IDO in the context of HIV and SIV infection does not follow a uniform pathway. In both non-pathogenic and pathogenic acute SIV infection models, IDO is induced. However, following acute infection, non-pathogenic monkey models show reduced IDO expression, in contrast to pathogenic monkey models in which IDO expression is sustained (86,87). Given that IDO expression and activity are correlated with viral load (88), it is not surprising that humans infected with HIV who sustain higher CD4 counts have lower mRNA expression of IDO as compared to noncontrollers (89). Finally, IDO activity, as measured by the ratio of kynurenine to tryptophan (K/T ratio), is predictive of loss of CD4 cells in progressive HIV infection (73). Therefore, understanding the biology of tryptophan metabolism in the context of the immune response to HIV could provide additional targets for therapy as well as better explain why patients experience different outcomes following HIV infection.

HIV and T cell differentiation

Given that chronic viral infection results from the failure of the adaptive immune system to adequately clear virus, the immunological changes induced by HIV upon the T cell compartment are of considerable interest. Observations in both tissues and peripheral blood, in acute and chronic infection, and in human patients as well as SIV model systems have revealed

sustained immune activation to be a predictor of progression to AIDS (reviewed in (90)). Multiple studies have been conducted to understand the stimuli that continue to influence immune activation, even when virus is absent in the peripheral blood.

Early events during infection appear to set the stage for such a chronic inflammatory state. One contributing factor may be inflammatory signals from impaired gastrointestinal barrier function. Prior to widespread availability of HAART, gastrointestinal pathology, including changes in gastrointestinal permeability, was frequently noted in HIV-infected patients (91,92). These detrimental changes begin early during the course of infection (93,94). A depletion of $CD4^+$ T cells from gut-associated lymphoid tissue (GALT) has been noted during the first months of infection and precedes the loss of peripheral blood $CD4^+$ T cells (93,95,96). This loss is not always restored by administration of HAART, despite adequate recovery of peripheral blood CD4 counts. However, early administration of HAART may in some cases allow for eventual complete recovery of CD4s in the GALT (97). Like the peripheral blood, immune cells in the GALT also develop increased markers of immune activation (98,99). $PD\text{-}1^+CD38^+$ $CD4^+$ and $CD8^+$ T cells were both significantly increased in frequency in the GALT and in the peripheral blood in HIV non-controllers versus seronegative controls (98). Similar gastrointestinal dysfunction was found in SIV pathogenic models (95,100). As in human patients, mucosal $CD4^+$ T cells were restored by early ART (101), and changes in immune activation markers have also been noted (102).

Multiple groups have noted that the loss of CD4 T cells from the gut mucosa corresponds to an alteration in the subsets of immune cells that are present (87,103,104). Changes in the frequency of immunosuppressive Tregs versus IL-17 and IL-22 producing Th17 and Th22 cells could potentially have a large impact on systemic immune responses through their modulation of

the gut mucosal barrier. Observations relating to these subsets in the context of HIV and SIV and their correlated impact on gut and peripheral immunity are described in further detail below.

Treg. Despite an overall loss of $CD4^+$ T cells in the peripheral blood and tissues during the course of HIV infection, a relative expansion of Tregs has been observed in both the peripheral blood and tissues in multiple studies. Untreated HIV-infected patients and SIV infected monkeys both exhibit increases in Treg accumulation in tissue, which corresponds to higher IDO expression; this accumulation of Tregs was corrected after administration of HAART (98,105,106). However, not all studies have resulted in identical findings; Mozos et al. observed increased Tregs in the peripheral blood with unchanged FOXP3 protein expression in lymphoid tissue (107). The relatively lower and more heterogeneous viral loads within that cohort could explain such a discrepancy; indeed, patients able to control HIV viremia without therapy have also exhibited lower Treg frequencies (108,109). Finally, greater sensitivity to Treg-mediated suppression may in some cases compensate for reduction in Treg numbers (110).

The means by which Tregs may influence HIV disease progression are still being investigated. Though Tregs are capable of being infected by HIV and are therefore a potential reservoir for virus (111), they are not preferentially infected above other CD4 cell subsets (112). Putative Tregs (identified as $CD4^+CD25^+$ cells) from patients infected with HIV are capable of suppressing HIV-specific T cell responses, leading to the hypothesis that an expansion of tolerogenic Tregs allows for viral persistence (113-115). Additionally, Tregs expanded in the context of HIV may suppress IL-2 production of activated T cells, which has been previously shown to inhibit HIV-specific T cell responses (116,117).

The mechanism of this expansion of Tregs following HIV infection is incompletely described. Altered methylation patterns within the gut mucosa that correlated with increased

FOXP3 expression have been observed in cells from infected patients (118). HIV itself appears to have a direct effect on the suppressive function and survival of Tregs (119). Finally, changes in tryptophan metabolism caused by induction of IDO may influence Treg differentiation in the context of HIV (73,120). In particular, the tryptophan metabolite, 3-HAA, was found to expand Tregs *in vitro*; interestingly, it did so while concurrently suppressing IL-17 production, which also plays a critical role in the context of HIV.

IL-17. An expansion of Tregs is not the sole change in T cell subsets that has been observed during HIV infection. The depletion in $CD4^+$ T cells in the gut mucosa is reflected by a relatively greater loss of Th17 cells that serve as key regulators of mucosal immune homeostasis, a loss that correlates with markers of immune proliferation in the periphery (121,122). Similar to what has been observed in human patients, reductions in Th17 cells in the gut mucosa have also been observed in SIV models (87). This reduction may have an effect on disease progression, as viral load appears to be negatively correlated with Th17 frequency in both humans and monkey models (123,124).

This alteration of Th17 cells within the gut mucosa is thought to lead to ineffective regulation of the gut microbiota. Not only are Th17 cells important in mediating pro-inflammatory responses to defend against pathogens, they may also gain the capacity within the intestine to limit their own inflammatory effects, allowing for resolution of inflammation (125). Up-regulation of IL-17 within the lamina propria in response to Salmonella infection has been shown to be blunted in SIV infection; greater bacterial dissemination within these monkeys as well as in Salmonella infected *IL17ra*-/- mice was also observed (126). Additionally, markers of microbial product translocation and damage to the gastrointestinal epithelium have also been found in the context of SIV models and HIV infection (102,127). Lacking the capability of

protecting the gastrointestinal barrier following a loss of Th17 cells, HIV-infected patients may be exposed to a greater burden of inflammatory stimuli.

Given that Th17 cells and Tregs exert opposing effects on the immune response, several groups have begun to look at the ratio of Th17 to Tregs within HIV and SIV models (73,87,128). The Th17 to Treg ratio in both the peripheral blood and gastrointestinal mucosa is correlated with markers of immune activation in HIV; Chevalier et al. additionally found it to be predictive of CD8 T cell activation six months later (128). Likewise, in monkey models, the relative proportion of inflammatory Th17 cells to immunosuppressive Tregs within peripheral blood, lymph nodes, and colon following SIV infection also correlates to markers of proliferation (87). Therefore, it is important to consider changes in multiple T cell subsets when evaluating disease progression and the likelihood of pathological outcomes.

IL-22. Though interleukin-22 (IL-22) was initially characterized as a Th17-type cytokine, it can be produced by both T cells and innate lymphoid cells in the absence of IL-17 (129). IL-22 signals through the IL-22 receptor complex, composed of IL-22R1 and IL-22R2, to promote cell survival and proliferation. IL-22 receptors are expressed on epithelial, endothelial, and pancreatic stromal cells, amongst other cell types; however, no hematopoetic cells have been shown to be responsive to IL-22. IL-22 is particularly critical at epithelial barriers such as the gut mucosa, stimulating the secretion of antimicrobial peptides (45). In an otherwise healthy organism, loss of IL-22 does not appear to be overtly detrimental. However, in inflammatory settings, lack of IL-22 leads to increased weight loss in mouse models of inflammatory bowel disease due to inability of the epithelium to repair damage (130). Therefore, understanding the changes in IL-22 production occurring in the gastrointestinal tract in the context of HIV could prove important.

Given that the gut mucosa is weakened during chronic HIV infection, it is not surprising that IL-22 production in the lamina propria is decreased in pathogenic SIV models as well as within human patients. Klatt et al. found that in rhesus macaques (RMs) infected with SIV, $CD4^+$ T cells as well as innate cells producing IL-22 were lost from the lamina propria, a deficit that correlated with markers of loss of gut mucosal barrier integrity. IL-22, produced by both T cells and innate cells, was also diminished following HIV infection and returned following viral suppression (131). It will be important to look at the entire picture of IL-17 production, IL-22 production, and Treg suppression in both the gastrointestinal tract and the periphery in future studies of HIV.

Conclusion

Given the evidence that IDO induction occurs and is sustained following infection with HIV and that IDO induction leads to downstream metabolites that can influence T cell differentiation in ways that parallel the changes observed in the context of HIV, it is likely that IDO-derived metabolites play a role in the outcome of HIV infection. AHR activation can lead to the generation of T cell subsets that block effective immune responses against HIV (e.g., Tregs) as well as those that can serve to prevent or even reverse HIV-mediated pathology (e.g., Th17 and Th22 cells). Given the plethora of AHR ligands that are or that resemble metabolites of tryptophan, identification of a metabolite downstream of IDO that leads to IL-22 and IL-17 production without expanding regulatory T cells could allow for novel targets for HIV therapeutics.

Table 1. AHR Ligands and Immune Effects

AHR Ligand	Immune Effects	Assays conducted	Citation
2,3,7,8-Tetrachlorodibenzodioxin (TCDD)	Increase in Tregs; suppression of IL-17	• Tregs at higher frequency in cells isolated from mice treated with TCDD • Tregs increased from naïve T cells stimulated in the presence of TCDD *in vitro* • IL17 was suppressed in mice treated with TCDD during EAE model	Quintana et al. (37)
	Increase in IL-22	• Increase in IL-22 mRNA in CD4 T cells under Th0 and Th17 conditions • Increase in IL-22^{+} cells in a human patient exposed to TCDD	Rohlman et al. (35) Brembilla et al. (50)
6-formylindolo[3,2-b]carbazole (FICZ)	Increase in IL-17 and IL-22 Suppression of IFNγ and IL-17, increase in IL-22	Naïve T cells differentiated *in vitro* in the presence of FICZ • Lamina propria mononuclear cells stimulated *in vitro* in the presence of FICZ • mRNA from colons of mice treated with FICZ during TNBS and DSS colitis models	Veldhoen et al. (19) Monteleone et al. (42)
2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)	Increase in Tregs; suppression of IL-17	• Cells isolated from mice treated with ITE in a multiple sclerosis model • Treatment of DCs with ITE generated tolerogenic DCs that induced Tregs and suppressed IL17	Quintana et al. (39)
	Suppression of IL-17 and IFNγ	Cells from draining lymph nodes in uveitis model following treatment with ITE	Nugent et al. (132)
L-kynurenine (L-KYN)	Increase in Tregs; no effect on IL-17	• Tregs increased in naïve T cells cultured *in vitro* with L-KYN • No effect on IL-17 under Th17 conditions	Mezrich et al. (27)
Kynurenic Acid (KYA)	Increase in IL-6	IL-6 mRNA was detected in MCF-7 cell line incubated with IL1 β in the presence of KYA	DiNatale et al. (26)
3,3'-diindolylmethane	Suppression of IL17	• Serum of mice treated with DIM during multiple	Rouse et al. (133)

(DIM)	and TNFα; increase in Tregs	sclerosis model (EAE) • Frequency of IL-17 producing cells and Tregs measured from EAE mice • DIM reduced IL-17 in naïve cells cultured under Th17 conditions	
indole-3-carbinol (I3C)	Suppression of IL17 and TNFα	• Serum of mice treated during multiple sclerosis model treated with I3C • IL-17 producing cells also measured *ex vivo* • I3C reduced IL-17 in naïve cells cultured under Th17 conditions	Rouse et al. (133)
	Increase in Tregs	Bone marrow derived dendritic cells treated with I3C generated Tregs *in vitro*	Benson et al. (134)
indoxyl 3-sulfate (I3S)	Increase in IL-17	• Naïve $CD4^+$ T cells incubated with I3S • Cells from mice treated with 13S during multiple sclerosis model produced more IL-17	Hwang et al. (43)
indole-3-aldehyde (IAld)	Increase in IL-22	Supernatants of $NKp46^+$ cells stimulated *in vitro*	Zelante et al. (47)
VAF347	Increase in IL-22; decrease in IL-17	• Monocyte-derived dendritic cells generated in the presence of VAF347 increased IL-22 and suppressed IL-17 in co-cultured T cells • T cells stimulated directly with VAF347 increased IL-22 production	Baba et al. (48)
VAG539 (prodrug of VAF347)	Increase in Treg survival and Treg frequency	• Dendritic cells from VAG539 treated mice promoted transferrable graft tolerance through increase in Tregs • Tregs treated with VAF347 survived longer *in vitro*	Hauben et al. (49)
Beta-napthoflavone (βNF)	Increase in IL-17	Splenocytes from mice sensitized to peanut allergen and treated with βNF were restimulated *ex vivo* with peanut allergen	Schulz et al. (44)

Figure 1

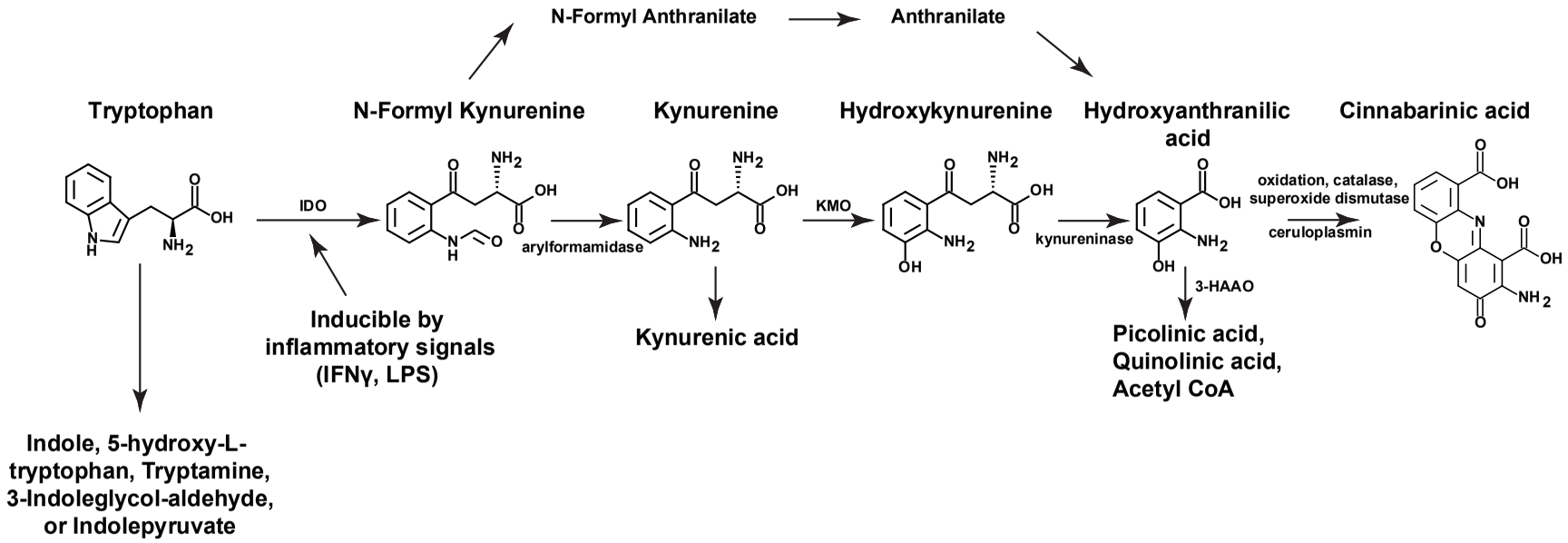

Figure 1. Tryptophan metabolic pathways downstream of IDO. A simplified depiction of tryptophan metabolism following the rate-limiting step mediated by IDO. Metabolic pathways are derived from the KEGG database (135,136).

CHAPTER II: THE EFFECTS OF THE TRYPTOPHAN METABOLITE CINNABARINIC ACID ON T CELL DIFFERENTIATION

Abstract

The effects of tryptophan metabolites 3-hydroxykynurenine (3-HKA), 3-hydroxyanthranilic acid (3-HAA), picolinic acid (PA), and quinolinic acid (QA) on IL-22 production were profiled. Although 3-HAA dramatically increased IL-22 production in $CD4^+$ T cells in an AHR-dependent manner, it was not found to bind or activate the AHR. The dimerization product, CA, was shown to increase IL-22 production more effectively in human naïve T cells. The effects of CA on IL-22, IL-17, and IFNγ production and Treg differentiation were profiled in both human and mouse naïve T cells. CA-mediated induction of IL-22 was conserved between both humans and mice, while differential effects were observed in IL-17 and IFNγ production.

Introduction

Numerous tryptophan metabolites downstream of indoleamine 2,3 dioxygenase (IDO) have been shown to have an effect on immune response. These metabolites, which belong to a family known as kynurenines, act in part through their effects on T cell differentiation. Mixtures of kynurenine metabolites have been shown to be immunosuppressive in contexts such as graft versus host disease and a mouse model of multiple sclerosis (70,72). More recently, L-kynurenine has been described as an AHR agonist that induces differentiation of naïve T cells into Tregs (27). Considering the importance of IDO induction in numerous settings of disease, including HIV, understanding the immunological effects of kynurenine metabolites is critical.

We have previously shown that 3-HAA, one metabolite in the kynurenine family, suppresses IL-17 production while inducing Treg differentiation (73), effects that mirrors changes in immune cell subsets observed during the course of SIV and HIV infection *in vivo*.

Treg and Th17 cells share similar developmental pathways and may arise from a common progenitor (137). Differentiation into a Treg or Th17 cell may be governed by the presence of inflammatory cytokines (138), retinoic acid (139), and/or activation of the AHR (19,37). The AHR is a cytosolic transcription factor that is involved in many biological processes, including development, cellular differentiation and proliferation, xenobiotic metabolism, and the immune response (37). To date, the best-studied AHR ligands are halogenated and polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (2). Only a few candidate endogenous ligands have been identified, many of which are tryptophan derivatives such as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), tryptamine, indirubin, 6-formylindolo[3,2-b]carbazole (FICZ), L-kynurenine, and kynurenic acid (2,26,27,140). The highly conserved nature of the AHR signaling pathway has prompted the search for additional natural ligands that can be directly linked to physiological functions and established as true endogenous ligands.

Although the AHR was initially proposed to affect Treg and Th17 development, a Th17-associated cytokine, IL-22, is even more specifically dependent upon AHR activation (19). *Ahr*-/- mice retain the ability to generate some Th17 cells but are compromised in terms of IL-22 production (19). Human T cell differentiation also exhibits distinct requirements for the AHR: activation of the AHR in stimulated human T cells was found to inhibit Th17 differentiation and to promote the differentiation of $CD4^+$ T cells that produce IL-22 (Th22). Finally, though certain AHR agonists preferentially stimulate IL-17 production over Treg differentiation or vice versa,

many of these ligands concurrently drive IL-22 (19,35,48). For instance, FICZ stimulates production of both IL-17 and IL-22 while having a suppressive effect on Treg differentiation (37). In contrast, TCDD induces Treg differentiation and IL-22 production while having no effect on IL-17 (35). Thus, regulation of IL-22 production by AHR agonists is not predictable by their effects on other cell subsets. We therefore investigated the effects of tryptophan metabolites 3-HKA, 3-HAA, PA, and QA on IL-22 production on IL-22 production and identified a byproduct of 3-HAA, CA, with potent effects on IL-22.

Materials and Methods

Chemicals and mice. Cinnabarinic acid was synthesized utilizing a single-step reaction sequence. A suspension of 1 g 2-amino-3-hydroxybenzoic acid (1 g, 6.53 mmol) in 250 mL of methanol was stirred at 25 °C for 15 min. Diacetoxyiodobenzene (4.31 g, 13.39 mmol) was added to the reaction mixture in portions. The color of the reaction mixture gradually changed from pale yellow-pink to red. Stirring was continued for 12 h at room temperature. The fine red precipitate was collected by filtration and washed with methanol, affording 0.58 g of cinnabarinic acid (29.6 % yield). ^{1}HNMR (400 MHz, d_6-DMSO) δ 9.71 (s, 1H), 8.78 (s, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.75 (d, J = 8.0 Hz, 1H), 7.59 (dd, overlapped, 1H), 6.59 (s, 1H). ^{13}CNMR (100.4 MHz, d_6-DMSO) δ 178.1, 169.1, 166.3, 152.5, 150.5, 147.6, 142.4, 129.0, 128.8, 127.9, 126.2, 120.2, 104.9, 92.7. LCMS found 301.2, [M+H]+. The purity of CA was 96.9% as measured by LC/MS with UV detection; FICZ was not present as determined by single ion monitoring of the MS spectrum. FICZ was synthesized as described previously (141). 4-fluoro-3-hydroxyanthranilic acid (4-F-3-HAA) was synthesized by Drs. Bill Todd and Barry

Carpenter (142) and provided by Robert Schwarcz. 3-Hydroxyanthranilic acid (3-HAA) (>99.6% purity), quinolinic acid (QA), picolinic acid (PA), 3-hydroxykynurenine (3-HKA) were purchased from Sigma. All chemicals were of the highest purity commercially available—typically ≥98%. The AHR antagonist, CH-223191 (2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide) (143), was purchased from CalBiochem. TCDD was purchased from Ultra Scientific. 1-(1-propynyl)pyrene (1-PP) was the generous gift of Cornelis Elferink (Univ. of Texas Medical Branch, Galveston, TX). C57BL/6 mice were purchased from Jackson Laboratory. Mice were housed under specific pathogen free conditions at San Francisco General Hospital and were fed standard chow. Mouse experiments were performed in compliance with the University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

***In vitro* human cell culture**. Heparinized blood from healthy volunteers was collected under protocols approved by the University of California, San Francisco Committee on Human Research. Subjects gave written informed consent in accordance with the Declaration of Helsinki. For assays involving total PBMCs, $3x10^5$ cells obtained by ficoll-hypaque density gradient centrifugation were plated with $5x10^4$ irradiated allogeneic cells in a 96 well U-bottom plate in 200 μL RPMI with 10% FBS. Cells were stimulated with plate-bound anti-CD3 (0.5 μg/mL in PBS, BD, SP34) and soluble anti-CD28 (0.5 μg/mL, BD) in the presence of tryptophan metabolite or DMSO control. Metabolites and inhibitors were replenished on day 2 or 3.

For naïve $CD4^+$ T cell sorting, human PBMCs from adult donors or from cord blood were stained with anti-CD3-Alexa700 (BD), anti-CD4-ECD (Invitrogen), anti-CD8-PeCy5.5 (Invitrogen), anti-CD45RA-FITC (BD), anti-CD95-APC (BD), anti-CD25-PE (BD), anti-CCR7-

PeCy7 (BD), and anti-CD27-APCCy7 (eBioscience). Naïve $CD4^+$ T cells were sort-purified as $CD3^+CD4^+CD8^-$ $CD45RA^+CCR7^+CD95^-CD25^-CD27^+$. $1x10^5$ cells were plated with $5\text{-}10x10^4$ irradiated allogeneic stimulators in 200 μL XVIVO-20 serum free media (Lonza) in 96 well U-bottom plates. Cells were incubated with CA or DMSO under polarizing conditions with plate-bound 0.5 μg/mL anti-CD3 (SP34) and 0.5 μg/mL soluble anti-CD28, 10 ng/mL IL-21 (eBioscience), IL-1β, IL-23, 10 μg/mL anti-IFNγ, and 5 μg/mL anti-IL-4 and anti-IL12 blocking antibodies (R&D). Cord blood samples were instead stimulated with CD3/CD28 Dynabeads (Invitrogen) and additionally incubated with TGF-β (R&D). Cytokines, blocking antibodies, and metabolite were added again on days 2 and 4.

The optimal day for restimulation was determined to be day 5 or 6; cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of brefeldin A (BFA) and stained for cytokine production Cells were stained extracellularly with anti-CD4-ECD and anti-CD8-PeCy5.5, and Aqua viability dye (Invitrogen), and fixed and permeabilized (BD Cytofix/Cytoperm). Samples were stained intracellularly with anti-CD3-Alexa700 or anti-CD3-APC-Cy7 (BD) to detect internalized CD3 on activated cells, as well as anti-IL-17-AlexaFluor647 (eBioscience), anti-IL-22-PE (R&D), and anti-IFNγ-PB (eBioscience) antibodies. All events were acquired on an LSRII (BD) and analyzed with FlowJo v7-9.3.2 (Treestar). $CD4^+$ cells were gated as live, $CD3^+$, $CD4^+CD8^-$ lymphocytes. At least 10,000 $CD4^+$ events were analyzed per sample.

Human regulatory T cell differentiation. Naïve $CD4^+$ T cells were sorted as described above. $2x10^5$ naïve T cells were incubated in a 96 well U-bottom plate in 200 μL XVIVO-20 media and stimulated with CD3/CD28 Dynabeads (Invitrogen) in the presence of CA or DMSO vehicle

control. Some cells were additionally treated with TGF-β (R&D). Cells were re-fed by removing 100 μL of media and adding 100 μL of 2x metabolite/TGF-β on days 2 and 4. Cells were stained on day 6 with Aqua viability dye, anti-CD3-APCCy7, anti-CD4-ECD, anti-CD8-PeCy5.5, and anti-CD25-PeCy7, and then were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained intranuclearly with anti-Foxp3-APC or PE (eBioscience, clone PCH101).

Human Treg suppressor assay. Tregs were generated as described above, in the presence of CA (10 μM) or DMSO. On day 6 of culture, Dynabeads were removed, and viable cells were quantified following propidium iodide staining on the Accuri C6 (BD). Autologous PBMCs were isolated and labeled with 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE), and 100,000 PBMCs were plated in a 96-well U bottom plate in XVIVO-20 with varying ratios of Tregs. Cells were stimulated with plate-bound 0.5 μg/mL anti-CD3 (HIT3a) and 0.5 μg/mL soluble anti-CD28 for four days and then stained with anti-CD3, anti-CD4, and anti-CD8.

***In vitro* mouse cell culture.** Naïve $CD4^+$ T cells were sort-purified from mouse splenocytes following depletion of non-$CD4^+$ T cells with the MACS $CD4^+$ T cell Isolation Kit II (Miltenyi). Cells were stained with anti-CD3-PB (BD), anti-CD4-PE (BD), anti-CD62L-PeCy7 (BD), anti-CD25-APCCy7 (eBioscience), and anti-CD45Rb (BD), and sorted as $CD3^+CD4^+CD25^-CD62L^+CD45Rb^{bright}$. Cells were stimulated with 4 μL anti-CD3/CD28 Dynalbeads/well (Invitrogen), 10 ng/mL IL-1β (Peprotech), 20 ng/mL IL-6 (Peprotech), 10 ng/mL TGF-β (R&D), and 10 μg/mL anti-IFNγ and anti-IL-12/23 (UCSF Hybridoma Core) in 200 μL XVIVO-20 media. CA in varying concentrations was added to individual wells; an equivalent volume of

vehicle (DMSO) was added to control wells. On day 2, cells were transferred to a 48-well plate. Cytokines, blocking antibodies, and metabolites, enzymes, or inhibitors were added at 2X concentration on days 2 and 4.

Cells were re-stimulated on day 5 (a day determined to be optimal in pilot experiments), with PMA/ionomycin in the presence of BFA for 4-6 hours and stained for cytokine production. Cells were stained extracellularly with anti-CD4-QDot 605 (Invitrogen) and anti-CD8-PeCy5.5 or anti-CD8-PB (Caltag), Aqua viability dye, and intracellularly with anti-CD3-PB or anti-CD3-PeCy5 (BD), anti-IL-22-PE (eBioscience), anti-IL-17-APC (BD), and anti-IFNγ-APCCy7 (BD).

RNA was collected following restimulation with PMA and ionomycin for 5 hours in RLT lysis buffer. RNA was purified with Qiagen RNeasy columns. RNA input was standardized per experiment by Nanodrop before cDNA transcription reactions (Omniscript). Taqman primers for *Il22* (Mm00444241_m1) and *Hprt1* (Mm00446968_m1) were used to quantify cDNA transcript in reactions with Taqman Universal PCR master mix. Reactions were run in a StepOnePlus analyzer.

Mouse regulatory T cell differentiation. Naïve $CD4^+$ T cells were sorted from mouse splenocytes as described above. $2x10^5$ cells were stimulated with 4 μL/well CD3/CD28 Dynabeads in 96 well U bottom plates in 200 μL XVIVO-20 media in the presence of CA or DMSO vehicle control. TGF-β was added to some wells. On days 2 and 4, 100 μL of media were removed, and 100 μL of 2X cytokine/metabolite were re-added. On day 5, Dynabeads were removed magnetically and cells were stained for flow analysis. Cells were stained with anti-CD4-QDot 605, anti-CD8-PeCy5.5, Aqua viability dye, anti-CD3-PB, and anti-CD25-APCCy7

(BD). Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained intranuclearly with anti-Foxp3-PE (eBioscience).

Statistical analysis. Statistical tests used to analyze data are denoted individually within figure legends (GraphPad Prism v.4.0c). P values < 0.05 (following corrections for multiple comparisons) were considered statistically significant.

Results

3-Hydroxyanthranilic acid (3-HAA) increases the frequency of IL-22-expressing CD4$^+$ T cells in vitro

Human PBMCs were stimulated with antibodies against CD3 and CD28 in vitro in the presence of different tryptophan metabolites, including 3-hydroxykynurenine (3-HKA), 3-hydroxyanthranilic acid (3-HAA), picolinic acid (PA), and quinolinic acid (QA). 3-HKA and 3-HAA, but not the downstream metabolites PA or QA, were able to promote IL-22 production in stimulated CD4$^+$ T cells (Fig. 1A). Though donors differed in the frequency of IL-22$^+$ cells detected following 3-HAA exposure, 3-HAA was able to promote a 2-fold or greater expansion of IL-22-producing cells in each (Fig. 1B). Statistically significant expansion of these cells was seen beginning at 25 μM 3-HAA (Fig. 1B, aggregate donors). Within PBMC cultures stimulated in the presence of 3-HAA, upregulation of IL-22 was only seen in CD4$^+$ T cells (Fig. 1C) and not in CD8$^+$ T cells (Fig. 1D). These IL22$^+$ CD4$^+$ T cells frequently co-expressed IFNγ but not IL-17A, consistent with the phenotype of Th22 cells identified in humans (Fig. 1C) (144). 3-HAA suppressed IL-17 production within CD4$^+$ T cells, as has been previously reported (73).

3-HKA and 3-HAA are not AHR ligands but may be precursors to an AHR ligand

To determine whether the expansion of IL-22-producing cells within this population was AHR-dependent, we stimulated human PBMCs in the presence or absence of a potent AHR antagonist (CH-223191) and observed that CH-223191 abolished the increase in IL-22 production caused by 3-HAA (Fig. 2, A and B). The above studies identified 3-HAA, and, to a lesser extent, 3-HKA, as potential ligands of the AHR. The ability of these metabolites as well as that of PA to bind the AHR was assayed using a well-established assay that measures the ability of compounds to displace [^{3}H]TCDD from full-length human AHR protein expressed *in vitro*. Modest binding of 3-HAA and PA was evident, but only at very high concentrations (1000 μM, Fig. 2C). While it is possible that 3-HAA was acting through the AHR to induce IL-22 production, we suspected that other metabolic byproducts of 3-HAA might be responsible for IL-22 production in our *in vitro* assays.

Inhibition of 3-HAA metabolism increases IL-22 production

Because neither PA nor QA, the primary metabolites downstream of 3-HAA, was found to have any impact on IL-22 production, we thought it unlikely that either was an AHR ligand. The enzyme upstream of PA and QA, 3-hydroxyanthranilate 3,4-dioxygenase (HAAO), is also expressed by monocytes and macrophages, the same cell populations that express IDO under inflammatory conditions (145). To determine whether downstream intermediates of 3-HAA generated through HAAO were acting as AHR ligands, the specific inhibitor, 4-F-3-HAA, was used to block HAAO activity (Fig. 2, D and E). Contrary to our expectations, HAAO inhibition within stimulated PBMC cultures did not block the ability of 3-HAA to up-regulate IL-22

production; rather, at higher concentrations of 3-HAA, HAAO inhibition increased IL-22 production (Fig. 2E). This suggested that either 3-HAA was a weak AHR ligand or that an alternative pathway of 3-HAA metabolism might give rise to an AHR ligand.

Identification of CA as a metabolite downstream of 3-HAA capable of inducing IL-22

3-HAA is susceptible to oxidation, resulting in the formation of CA through 3-HAA dimerization, both in solution (146) and in cell culture (141). As a tricyclic aromatic compound, the structural features of CA resemble those of some AHR ligands. Additionally, CA has potent effects on thymocyte maturation (141), similar to those observed in cultured thymocytes treated with the AHR agonist, TCDD (147). Assays described in Chapter III describe further the capacity of CA to bind and interact with the AHR.

Treatment of adult human PBMCs with CA resulted in up-regulation of IL-22, but not IL-17, within human $CD4^+$ T cells in a dose-dependent manner (Fig. 3A). To determine whether CA was acting on T cells directly, adult human and mouse naïve $CD4^+$ T cells were sort-purified and incubated with tryptophan metabolites under polarizing conditions (see Methods for details). In the presence of 25 μM CA, human naïve $CD4^+$ T cells upregulated the production of IL-22 and IFN-γ to a greater extent than those incubated with an equivalent concentration of 3-HAA (Fig. 3B). Like 3-HAA, CA had suppressive effects on IL-17 when cells were stimulated under conditions favoring both IL-22 and IL-17 production (Fig. 3C). CA treatment of mouse naïve $CD4^+$ T cells also resulted in significant expansion of $IL\text{-}22^+$ cells as well as a trend towards an expansion of IL-17 producing cells, unlike that seen in humans (Fig. 3D and 3E). Also in contrast to human cells, no expansion of IFNγ-producing cells was observed within mouse naïve cell cultures (Fig. 3F).

Given the role of the AHR in regulating Treg differentiation (37), we additionally tested whether CA was able to affect the differentiation of Tregs as measured by expression of FOXP3 and functional activity. An expansion of $FOXP3^+$ cells was seen in human naïve $CD4^+$ T cells stimulated in the presence of CA (Fig. 4A). However, T cells exposed to CA were not more suppressive than DMSO treated cells, despite the greater abundance of $FOXP3^+$ cells (Fig. 4B). Unlike in human cells, incubation of mouse naïve cells with CA suppressed generation of $Foxp3^+$ cells (Fig. 4C). Nevertheless, the lack of suppressive activity in human cells treated with CA indicates that this phenotypic difference between species may not result in a change in function.

Discussion

CA is a tryptophan metabolite capable of driving IL-22 production in both mouse and human naïve $CD4^+$ T cells. In contrast to its conserved effects between humans and mice regarding IL-22 production, it differentially affects IL-17 in mouse and human cells. CA appears to increase IL-17 production in mouse cells while having neutral to suppressive effects on IL-17 in human cells. Likewise, the effects of CA on IFNγ production was also discordant between humans and mice, with CA having no effect on mouse T cells but driving more IFNγ in humans cells. Finally, CA did not productively increase Treg differentiation *in vitro* in either mouse or human cells.

Though 3-HKA and 3-HAA also had the ability to increase IL-22 production, the effects of CA were much more potent. Furthermore, unlike 3-HKA and 3-HAA, CA is capable of interacting with the AHR (see Chapter III for further details). It is interesting to note that, like CA's effects in human cells, at least one AHR ligand has also demonstrated the capacity to increase IFNγ *in vitro* (148), and the human Th22 subset is also known to co-express IFNγ

(144). Though differential effects were seen between human and mouse T cells treated with CA, the divergence of the AHR, particularly in terms of the ligand-binding domain, between mice and humans may explain some of these outcomes (9).

Chapters III and IV will expand upon this initial identification of CA as a mediator of IL-22 production. Analysis of its interaction with the AHR, a comparison of CA to other tryptophan metabolites that interact with the AHR, and assessment of the capability of CA to be produced by immune cells will help clarify its role in T cell differentiation. Understanding how immune response may be affected by metabolites downstream of IDO, an enzyme induced by inflammatory stimuli, is critical to anticipating the outcome and developing treatment strategies for immunopathological events such as those associated with HIV infection.

Figures

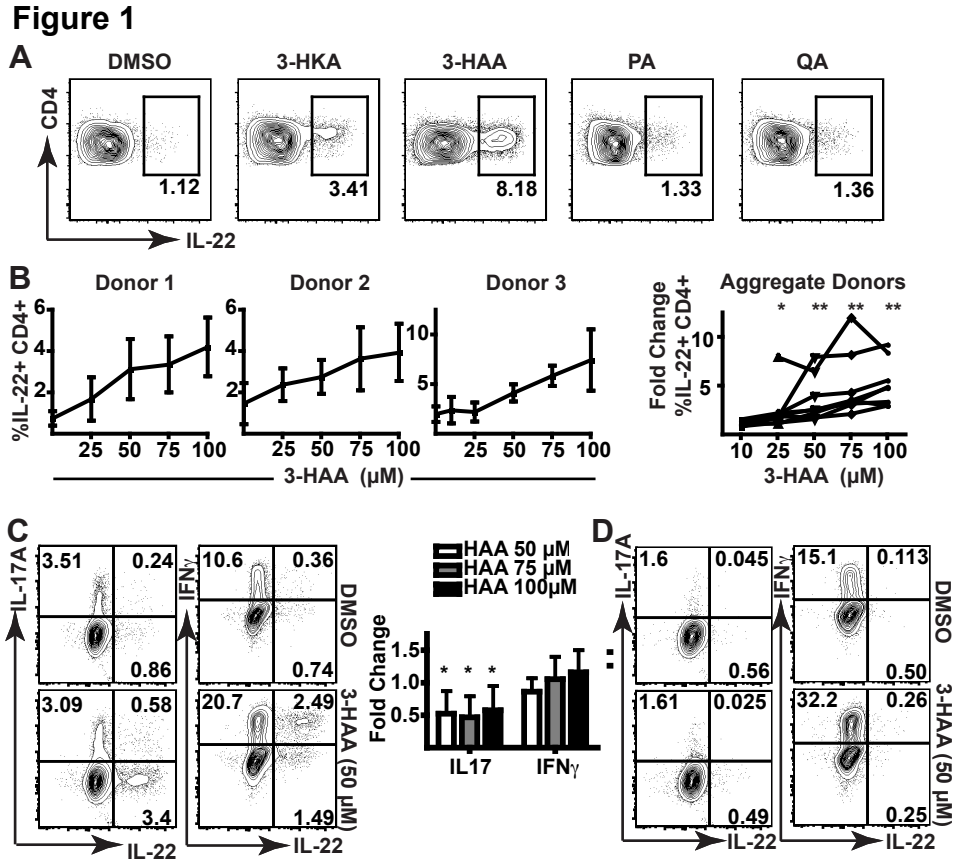

Figure 1. 3-HKA and 3-HAA promote IL-22 expression in stimulated human CD4⁺ T cells. (A) Flow cytometric analysis of $CD4^+$ T cells following stimulation of human PBMCs in the presence of 100 μM 3-HKA, 3-HAA, PA, or QA for six days. Data represent at least three independent experiments. (B) Flow cytometric analysis of $CD4^+$ T cells from individual and aggregate donors following stimulation of PBMCs in the presence of increasing concentrations of 3-HAA (μM) for six days. Individual donor data are pooled from at least three independent experiments. Error bars indicate SD. Fold change in IL-22 expression versus vehicle control is statistically different from 1 (Wilcoxon signed rank test; *, p=0.0312; **, p=0.0078; N=8 donors). (C) Cytokine production for live $CD3^+CD4^+CD8^-$ T cells from human PBMC cultures that were stimulated with anti-CD3 and anti-CD28 antibodies and allogeneic APCs for six days

with DMSO or 50 μM 3-HAA. Panel (right) depicts fold-changes in 9 donors relative to DMSO. *, p<0.05 indicates data analyzed by Wilcoxon signed rank test is statistically different than 1. (D) Cytokine production for live $CD3^+CD8^+CD4^-$ T cells in PBMC cultures, stimulated as above with DMSO or 3-HAA (50 μM). Data are representative of three experiments.

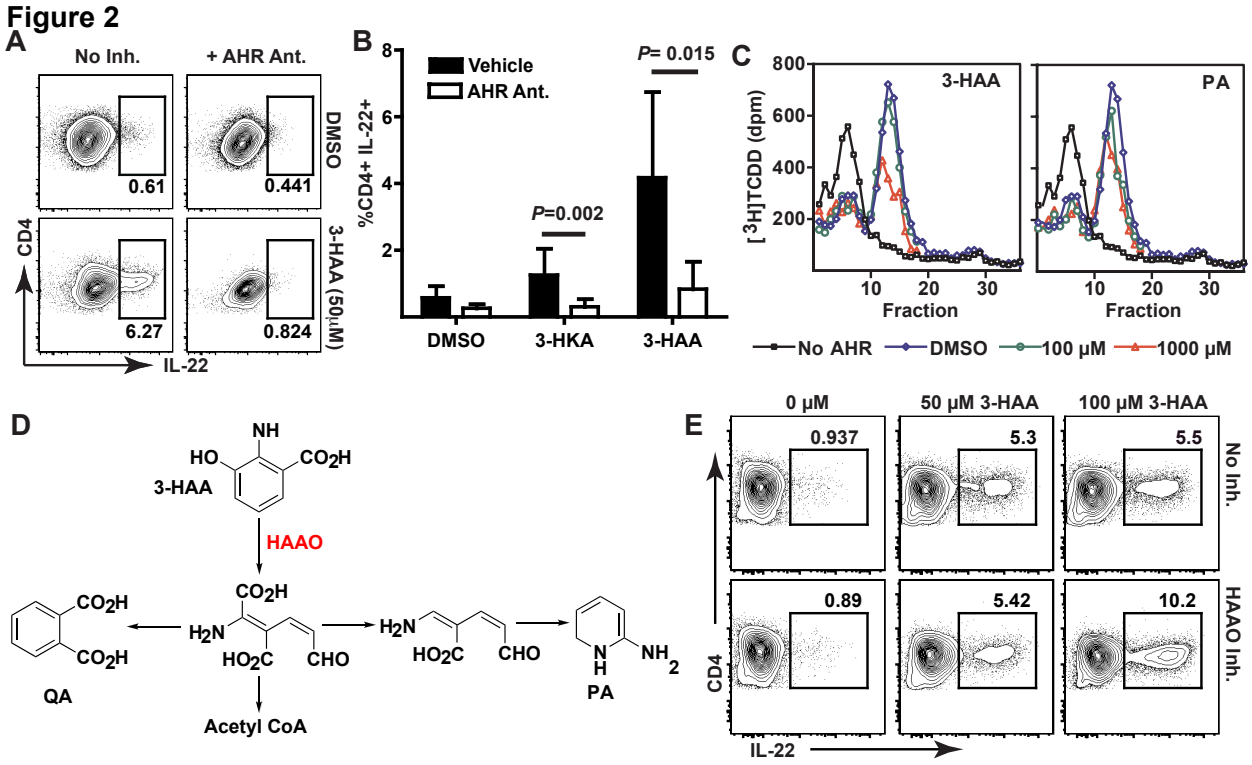

Figure 2. IL-22 production stimulated by HAA requires AHR activation, but HAA does not bind or activate the AHR. (A) Flow cytometric analysis of $CD4^+$ T cells following stimulation of human PBMCs in the presence of 3-HAA +/- the AHR antagonist, CH-223191. Data are representative of at least three independent experiments. (B) Comparison of IL-22 production in $CD4^+$ T cells following stimulation of human PBMCs in the presence of DMSO, 3-HKA (50 μM), or 3-HAA (50 μM), with or without an AHR antagonist, N=6. P values were calculated by

Mann-Whitney. Error bars indicate SD. (C) Displacement of [^{3}H]TCDD from the human AHR in the presence of 3-HAA or PA. Data for 100 μM concentrations were from 2-4 experiments; data for 1000 μM concentrations were from one or two experiments. (D) Metabolic pathways downstream of 3-HAA catalyzed by the enzyme, 3-hydroxyanthranilate 3,4-dioxygenase (HAAO), including intermediates upstream of PA and QA. (E) Flow cytometric analysis of $CD4^+$ T cells in human PBMCs stimulated in the presence of varying concentrations of 3-HAA (50 μM or 100 μM) with or without the HAAO inhibitor, 4-F-3-HAA (50 μM). Data are representative of three experiments.

Figure 3

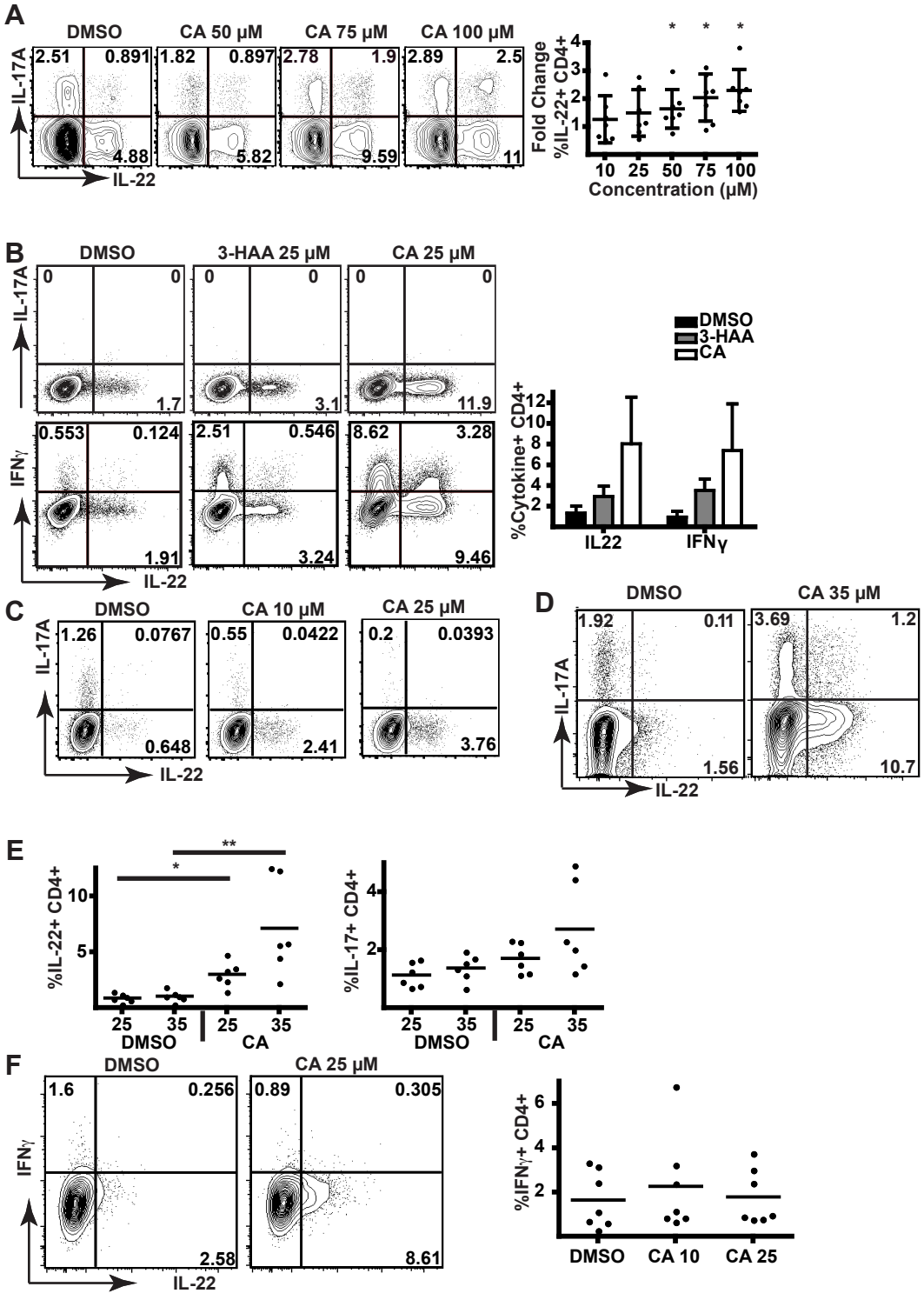

Figure 3. CA increases the differentiation of IL-22$^+$ human and mouse CD4$^+$ T cells *in vitro*.

(A) Flow cytometric analysis of CD4$^+$ T cells from human PBMCs stimulated in the presence of

DMSO or increasing doses of CA (left). Fold change in IL-22 production in $CD4^+$ T cells from human PBMCs from multiple donors stimulated in the presence of CA versus DMSO control (right panel). Data were analyzed by Wilcoxon signed rank test for significant deviation from a theoretical median of 1.000. *$p<0.05$. (B) Flow cytometric analysis of sorted naïve human $CD4^+$ T cells stimulated under polarizing conditions (with IL-21, IL-1β, IL-23, anti-IFNγ, anti-IL-4 and anti-IL12) with DMSO, 3-HAA (25 μM), or CA (25 μM). Data on IL-22 and IFNγ production from three independent experiments are shown in the panel on the right. Error bars are SD. (C) Flow cytometric analysis of sorted naïve human $CD4^+$ T cells from cord blood stimulated under polarizing conditions (with TGFβ, IL-21, IL-1β, IL-23, anti-IFNγ, and anti-IL-4) in the presence of DMSO or CA. (D) Flow cytometric analysis of IL-17 and IL-22 production in sorted naïve mouse $CD4^+$ T cells from C57BL/6 mice stimulated under polarizing conditions (with IL-1β, IL-6, TGF-β, anti-IFNγ, and anti-IL12/23) in the presence of DMSO or CA (35 μM). (E) Flow cytometric data (top panel) from six independent experiments in C57BL/6 mice were analyzed by Kruskal-Wallis ANOVA and Dunn's Multiple Comparison test. *, $p<0.05$. **, $p<0.01$. DMSO controls for the 25 and 35 μM CA experiments are shown separately. (F) Flow cytometric analysis of IFN-γ production in sorted naïve wild-type mouse $CD4^+$ T cells stimulated under polarizing conditions (as in panel D) in the presence of DMSO or CA. Data are from seven independent experiments (right) were analyzed by Friedman ANOVA; no statistically significant differences were found.

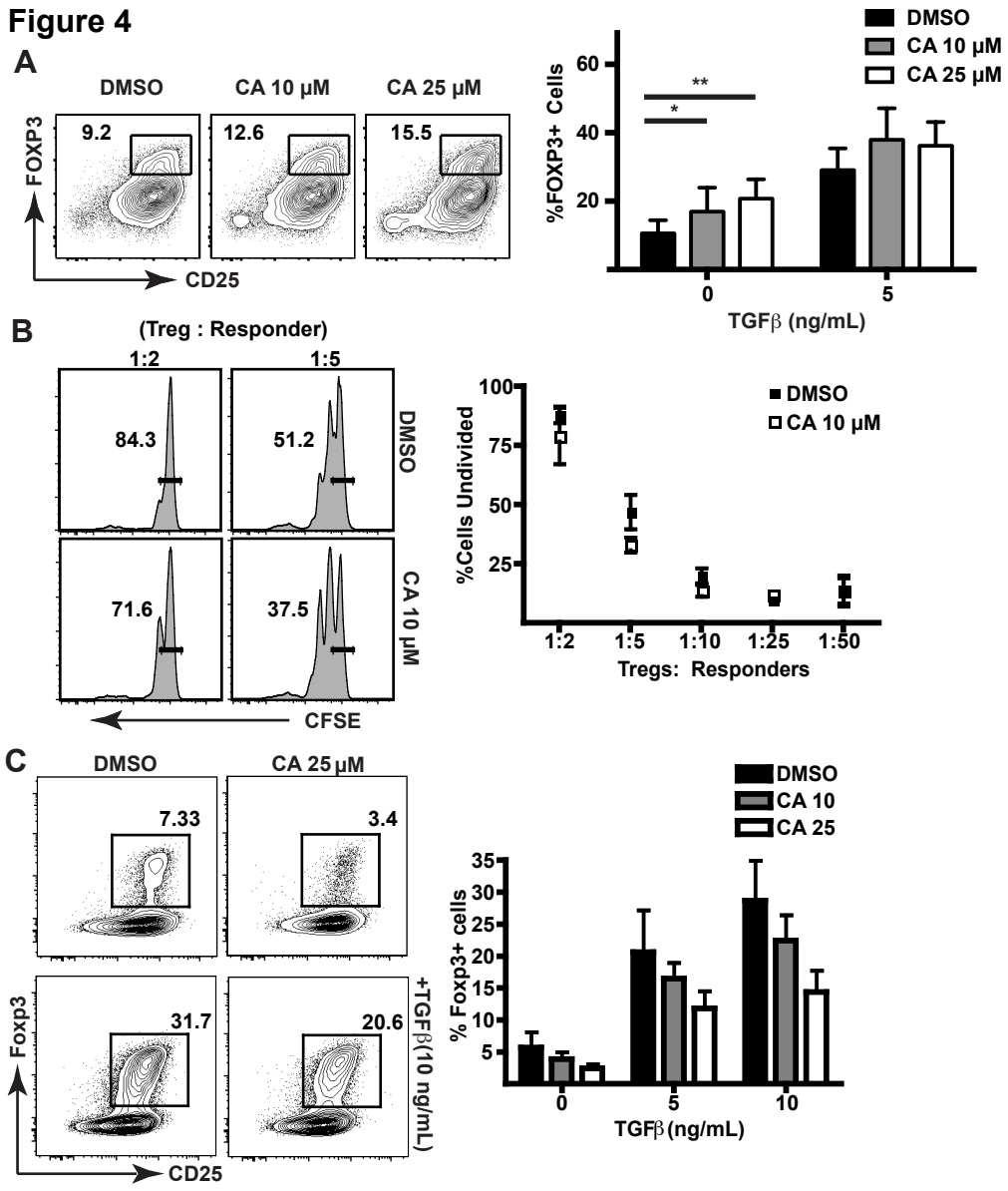

Figure 4. CA decreases FoxP3 expression in mouse cells and does not induce functional Tregs in human cells.

(A) Flow cytometric analysis of FOXP3 staining following stimulation of sorted naïve human $CD4^+$ T cells in the presence of DMSO or CA without addition of TGF-β. Quantification of $\%FOXP3^+CD25^+$ T cells of $CD4^+$ cells from at least seven donors per condition in four independent experiments (right panel) with or without 5 ng/mL TGF-β. Data were analyzed by

two-way ANOVA and Bonferroni post-tests. *, $p<0.05$. **, $p<0.01$. Error bars are SD. (B) Flow cytometric analysis of CFSE-labeled responder $CD8^+$ T cells incubated with autologous Tregs generated in the presence of CA or DMSO control. Data are representative of three independent experiments. (C) Flow cytometric analysis of Foxp3 expression in sorted naïve wild-type mouse $CD4^+$ T cells stimulated with increasing concentrations of TGFβ. Quantification of $\%Foxp3^+CD25^+$ T cells of $CD4^+$ cells from four independent experiments is shown in the panel on the right. Error bars are SD.

CHAPTER III: THE ABILITY OF THE TRYPTOPHAN METABOLITE CINNABARINIC ACID TO BIND AND ACTIVATE THE AHR

Abstract

Given that several tryptophan metabolites and derivatives are AHR agonists, further characterizing the interactions of kynurenine family metabolites with the AHR is highly relevant. The tryptophan metabolites CA, 3-3-HAA, 3-HKA, PA, and QA were interrogated for their ability to activate or inhibit activation of an AHR reporter construct. CA was found to induce the AHR-mediated transcription and was further determined to bind to the AHR and to modestly induce AHR-responsive gene *Cyp1a1*. Finally, the induction of IL-22 mediated by CA was determined to be dependent on AHR activation.

Introduction

The tryptophan metabolite, CA, is capable of increasing IL-22 production in human and mouse naïve $CD4^+$ T cells, an activity that is characteristically influenced by AHR activation

(19). While the tryptophan metabolite 3-HAA did not bind to the AHR (see Chapter II, Fig. 2C), it was necessary to directly test the ability of CA to bind and to activate the AHR. Multiple assays are useful for determining whether a compound has the ability to bind the AHR and facilitate its activation of gene transcription. Displacement of other known AHR ligands from AHR may demonstrate binding, while electromobility shift assays (EMSA) may measure ability of the ligand-AHR complex to recognize DNA (149). Effective activation of the AHR can also be demonstrated by AHR-responsive reporter cell lines or transcription of known AHR-responsive genes (150). Importantly, different AHR ligands may demonstrate activation of differential batteries of genes (151), and some AHR-mediated responses are independent of binding to DNA (152) and do not necessarily lead to induction of traditional AHR-responsive gene products (153). The mechanisms by which the latter "selective AHR modulators" (SAhrMs) work is still an area of active debate and investigation.

Due to the high variability of ligand structure and observed activities on gene transcription, it has recently been hypothesized that the induction of AHR-responsive genes by certain ligands is not due to a direct effect on the AHR but rather alteration of the metabolism of another AHR ligand, such as the tryptophan photoproduct FICZ (154). Wincent et al. observed that inhibitors of CYP1A-mediated metabolism were frequently able to induce CYP1A-mediated metabolism. While one hypothesis would be that the compounds themselves are AHR ligands, another possibility is that they affect metabolism of an AHR ligand such as FICZ. Given that the induction of CYP1A by many of these ligands is diminished in media reconstituted with crystallized tryptophan (which should thus lack FICZ), the authors concluded that inhibition of FICZ metabolism by these ligands boosted FICZ intracellularly, leading to a readout of AHR activation. Thus, assessing direct binding of CA for the AHR as well as testing the effects of CA

on CYP1A-mediated metabolism is important to characterize fully the means by which it is affecting IL-22 production.

Materials and Methods

Chemicals and mice. The origins for most chemicals and mice are as described in Chapter II. Additionally, FICZ was synthesized as described previously (155). α-Naphthoflavone (α-NP) was purchased from Sigma. 1-(1-Propynyl)pyrene (1-PP) was the generous gift of Cornelis Elferink (Univ. of Texas Medical Branch, Galveston, TX). *Ahr*$^{-/-}$ mice on a B6 background were derived from the line created by Schmidt et al (156). Mice were housed under specific pathogen free conditions at San Francisco General Hospital and were fed standard chow. Mouse experiments were performed in compliance with the University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

***In vitro* AHR competitive-binding assay.** Assays were performed as in Chapter II.

AHR reporter assay. Mouse H1G1.1c3 cells (courtesy of Dr. M. Denison, UC Davis) were prepared as described previously (150), except that $6x10^4$ cells were added to each well of a 96-well, black-sided plate in 200 μl of selective medium and incubated at 37°C for 24 hours. The medium was replaced with 100 μl of non-selective medium prior to compound application. A TCDD standard curve plate was prepared by adding vehicle (DMSO, 0.5%) or TCDD (10^{-14}-10^{-9}M), with each concentration added to 8 wells. For agonist experiments, vehicle or test compound was added at a single concentration in each column, excluding two untreated columns. For antagonism experiments, compound application was immediately followed by

dosing with either vehicle or TCDD (10^{-10}M). The plates were incubated at 33°C for 24 hours and EGFP fluorescence was read with a fluorometric plate reader (Synergy2, Biotek Instruments). Excitation and emission wavelengths were 485 nm (20 nm bandwidth) and 530 nm (25nm bandwidth). Untreated well fluorescence was subtracted from experimental well fluorescence. Data were averaged from eight replicate wells. The gain was adjusted between experiments so that wells exposed to 10^{-10}M TCDD wells produced 15,000 RFUs per well; subsequent plates in the experiment were analyzed with that gain setting.

The specificity of the fluorescence measured in the H1G1 cells treated with CA, HAA, and tryptamine was determined by concurrently treating Hepa-1 cells (the parent line of the H1G1 cell line) with CA, HAA and tryptamine at the same concentrations. Treatment, incubation, and analysis were carried out as above. Fluorescence measured in Hepa-1 cells was subtracted from the fluorescence measured in H1G1 cells treated with the same concentration of CA, HAA, or tryptamine to correct for background fluorescence.

***Cyp1a1* induction.** Zebrafish embryos [TL strain; 48 or 72 hours post fertilization (hpf)] were exposed to CA (100 μM) or DMSO for 6 hours. Following exposure, three replicate groups of 20 embryos from each treatment group were frozen in liquid nitrogen. In one experiment, 72-hpf embryos exposed for 6 hr to CA or DMSO were subsequently held in clean water and sampled at 96 hpf. Total RNA was isolated from frozen embryos using RNA STAT-60 (Tel-Test B, Inc.). cDNA was synthesized from 2 μg of total RNA using Omniscript reverse transcriptase (Qiagen). Real-time RT-PCR for *cyp1a* and *β-actin* was performed using the iQ SYBR Green Supermix (Bio-Rad) in an iCycler iQ Real-Time PCR Detection System (Bio-Rad), as described previously (157). Fold induction of *cyp1a* by CA was calculated using the $\Delta\Delta C_T$ method (158).

Human PBMCs were isolated by ficoll hypaque density gradient centrifugation and plated in 48-well plates at a density between 0.5-2 x 10^6 cells per well in 1 mL of RPMI. Cells were stimulated with 1 μg/mL PHA in the presence of tryptophan metabolite or DMSO for 8-20 hours and harvested in RLT lysis buffer. RNA was purified with Qiagen RNeasy columns. RNA input was standardized per experiment by Nanodrop before cDNA transcription reactions (Omniscript). Taqman primers for *CYP1A1* (Hs00153120_m1) and *HPRT1* (Hs99999909_m1) were used to quantify cDNA transcript in reactions with Taqman Universal PCR master mix. Reactions were run in a StepOnePlus analyzer.

Mouse lymphocytes were isolated from brachial, axillary, and inguinal lymph nodes, and were then plated in 96 well plates at a density of 1x10^6 cells/well in 200 μL of RPMI, cultured for 4 hours, and lysed in RLT lysis buffer. RNA isolation and RT-PCR was handled as with human cells, except with Taqman mouse *Cyp1a1* (Mm00487217_m1) and *Hprt1* (Mm00446968_m1) primers.

CYP1A1 inhibition. 1Human CYP1A1 + P450 Reductase Supersomes (1.8 pmol) (BD) were used per reaction in the luminescent-based P450-Glo CYP1A1 Assay (Promega). Briefly, CYP1A1 Supersomes, luciferin-CEE substrate, and test compound were equilibrated in white opaque 96-well plates (Pierce) for 10 minutes at 37ºC per kit protocol. NADPH Regenerating Solution (Promega) was added, and reactions were terminated after 15 minutes at 37ºC by addition of luciferase detection reagent. Luminescence was read with the SpectraMax M2 microplate reader using SoftMax Pro software (Molecular Devices) and averaged over 6-8 reads per well.

Results

AHR agonism and antagonism by tryptophan metabolites

We first measured AHR activation and antagonism by tryptophan metabolites 3-HKA, 3-HAA, PA, and QA within a reporter cell line expressing AHR responsive elements upstream of a GFP reporter (Fig. 1, A and B). None of the metabolites tested either activated the AHR or inhibited TCDD-mediated AHR activation within the concentrations tested. While a slight increase in AHR activity measured by fluorescence was found during more extensive dose-response testing using 3-HAA as an agonist (Fig. 1C), this failed to reach significance. In contrast, CA induced GFP expression in the AHR reporter cell line, with significant increases occurring at 50 μM (Fig. 1D) Furthermore, CA also bound to *in vitro*-expressed human AHR, as measured by displacement of [^{3}H]TCDD at much lower concentrations than those required for 3-HAA and 3-HKA (Fig. 1E).

Inhibition of CYP1A1 mediated metabolism by CA

Next, CA was tested for its ability to induce the well-known AHR responsive gene, *CYP1A1*, and its orthologs *in vivo* and *in vitro*. In zebrafish embryos, a model vertebrate *in vivo* system, *Cyp1a* was strongly induced after 6 hours of exposure to CA (Fig. 2A, upper panel); this effect was lost 18 hours after washout of CA (Fig. 2A, lower panel). The degree of induction (25- to 50- fold) was comparable to that produced by FICZ under similar experimental conditions (~30-fold) (159). CA also caused statistically significant induction of *Cyp1a1* in mouse lymphocytes *in vitro* (Fig. 2B). No *Cyp1a1* induction was detected in lymphocytes derived from *Ahr*$^{-/-}$ mice (Fig. 2B), demonstrating that CA was acting through the AHR. Human

PBMCs also exhibited a statistically significant, although modest, induction of *CYP1A1* (Fig. 2C).

Given that some AHR ligands with weaker effects on *CYP1A1* induction have been reported to induce AHR-responsive genes indirectly by inhibiting the CYP1A1-mediated metabolism of FICZ (154), CA was tested for its effects on CYP1A1 metabolic activity. CA was incubated with CYP1A1-loaded microsomes and a CYP1A1 substrate. Dose-dependent inhibition of CYP1A1 metabolism was seen with CA; however, this inhibition was much less than that caused by the known CYP1A1 inhibitors α-napthoflavone and 1-(1-propynyl)pyrene (1-PP) (Fig. 3A). 1-PP in particular has been shown to activate the AHR indirectly through inhibition of CYP1A1-dependent metabolism of an endogenous AHR ligand (160). Thus, 1-PP was tested in mouse naïve cell cultures to determine whether Cyp1a1 inhibition could induce IL-22 in this system. Importantly, concentrations of 1-PP capable of completely inhibiting Cyp1a1 were unable to induce IL-22, providing evidence that induction of IL-22 by CA is not the result of Cyp1a1 inhibition (Fig. 3B). CA in the presence of 1-PP retained its ability to induce IL-22, showing that 1-PP does not affect the ability of the cells to respond to CA. Therefore, it seems unlikely that CA is exerting its effects by altering FICZ metabolism.

Finally, the dependence of CA upon the AHR to induce IL-22 *in vitro* was tested directly. Mouse naïve T cells from *Ahr*-/- mice exposed to CA under polarizing conditions failed to induce IL-22 (Fig. 4A). Given that *Ahr*-/- mouse cells could be impacted by developmental differences that could block IL-22 production, an AHR antagonist was also used on wild-type C56BL/6 mouse cells in the presence of CA. Like the *Ahr*-/- mouse cells, cells exposed to an AHR antagonist also failed to produce IL-22 in the presence of CA (Fig. 4B). Finally, the ability

of the AHR antagonist to block CA-driven activation of the AHR-responsive reporter cell line was verified (Fig. 4C). Cells treated with the AHR antagonist were also unable to respond to CA.

Discussion

The increase in IL-22 production mediated by CA is most likely due to its role as an AHR ligand. The conclusion that CA is acting via AHR is supported by several lines of evidence, including CA displacement of [^{3}H]TCDD from the human AHR, induction of *Cyp1a* in zebrafish embryos *in vivo* and in human and mouse lymphocytes, AHR-dependent reporter gene induction in H1G1 cells, loss of effects in cells from *Ahr*$^{-/-}$ mice, and the ability of an AHR antagonist to block the stimulation of IL-22 production by CA in cells from wild-type mice. However, the effects of CA upon *CYP1A1* induction in humans and mice were rather modest, allowing for the possibility that another mechanism exists for the effect of CA on IL-22.

Other characterized AHR agonists have effects that appear to be dependent upon presence of a second AHR ligand (154). These agonists appear to work indirectly through inhibition of that ligand's metabolism. The possibility that CA could be acting through CYP1A1 inhibition was tested directly by studying the effects of known CYP1A1 inhibitor, 1-PP. Addition of this compound to cell cultures did not lead to production of IL-22, implying that CA-mediated CYP1A1 inhibition would be insufficient to cause the changes in T cell differentiation observed. Furthermore, most agonists characterized to work through metabolic inhibition do not bind well to the AHR (154). Thus, the aggregate evidence from AHR binding studies, reporter gene assays, modest induction of *CYP1A1*, and inhibition of CA-stimulated IL-22 production by an AHR antagonist all point to a role for the AHR in mediating the effects of CA on IL-22.

Figures

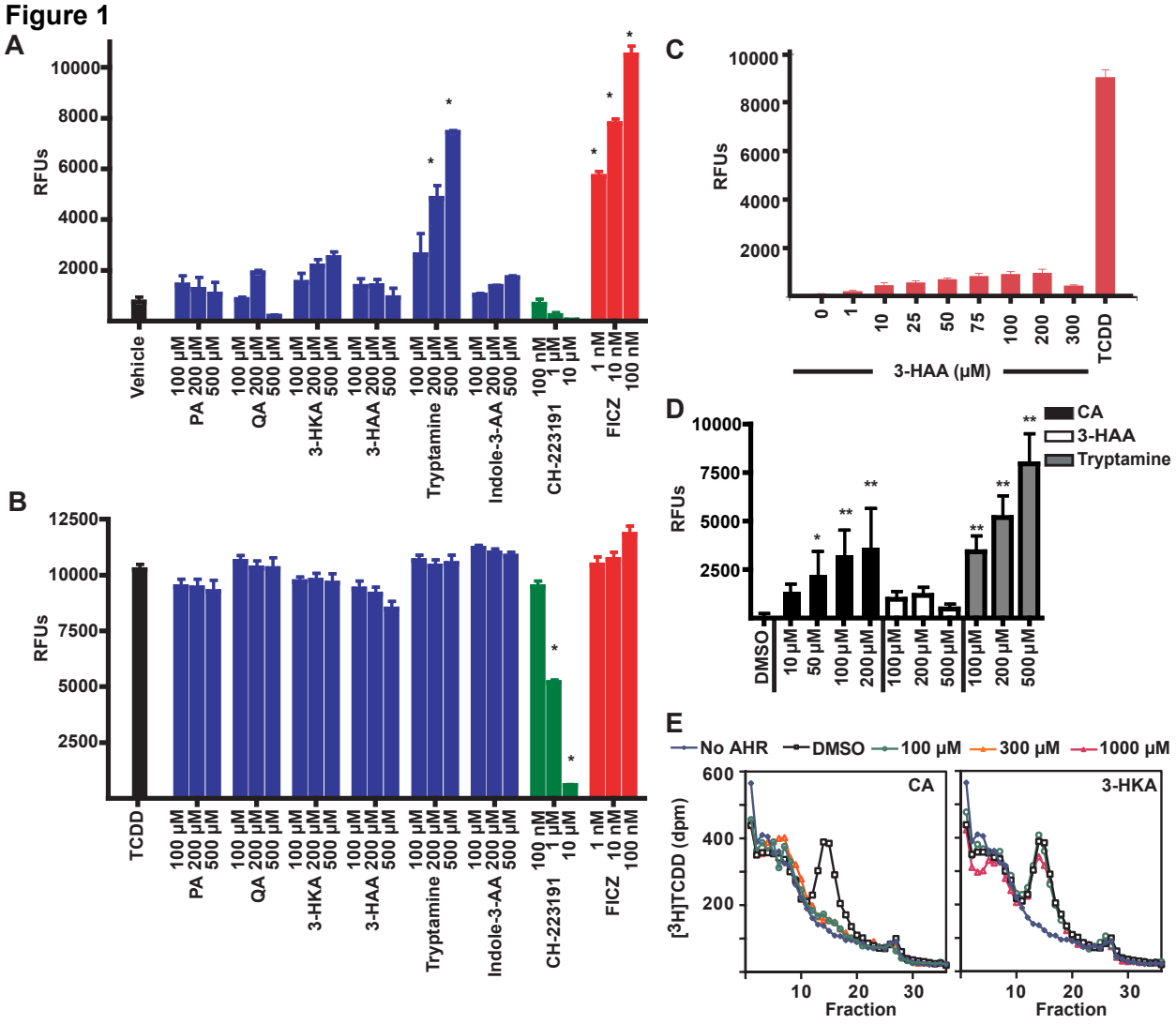

Figure 1. CA activates reporter constructs while other tryptophan metabolites do not.

(A) Fluorescence of the AHR-reporter construct following incubation of transfected cells with 3-HAA, 3-HKA, PA, or QA. Tryptamine and FICZ are positive controls. (B) Inhibition of TCDD (10^{-10}-M)-induced activation of the AHR reporter construct. CH-223191 is a positive control. *, $p<0.05$ (ANOVA, Scheffe's). Error bars are SD. (C) Fluorescence of an AHR-responsive reporter construct measured after incubation of a stably transfected murine hepatoma cell line in the presence of increasing concentrations of 3-HAA. TCDD ($5x10^{-11}$M) is a positive

control. Error bars are SD. N=6. (D) Fluorescence [measured in relative fluorescence units (RFUs)] of the AHR-responsive reporter construct in cells incubated with varying concentrations of 3-HAA or CA. Tryptamine was a positive control. *, $p<0.05$ (ANOVA, Scheffe's). Error bars represent SD. (E) [^{3}H]TCDD displacement from *in vitro* translated human AHR protein by incubation with varying concentrations of CA compared to 3-HKA. Data are representative of four (CA) or two (3-HKA) independent experiments.

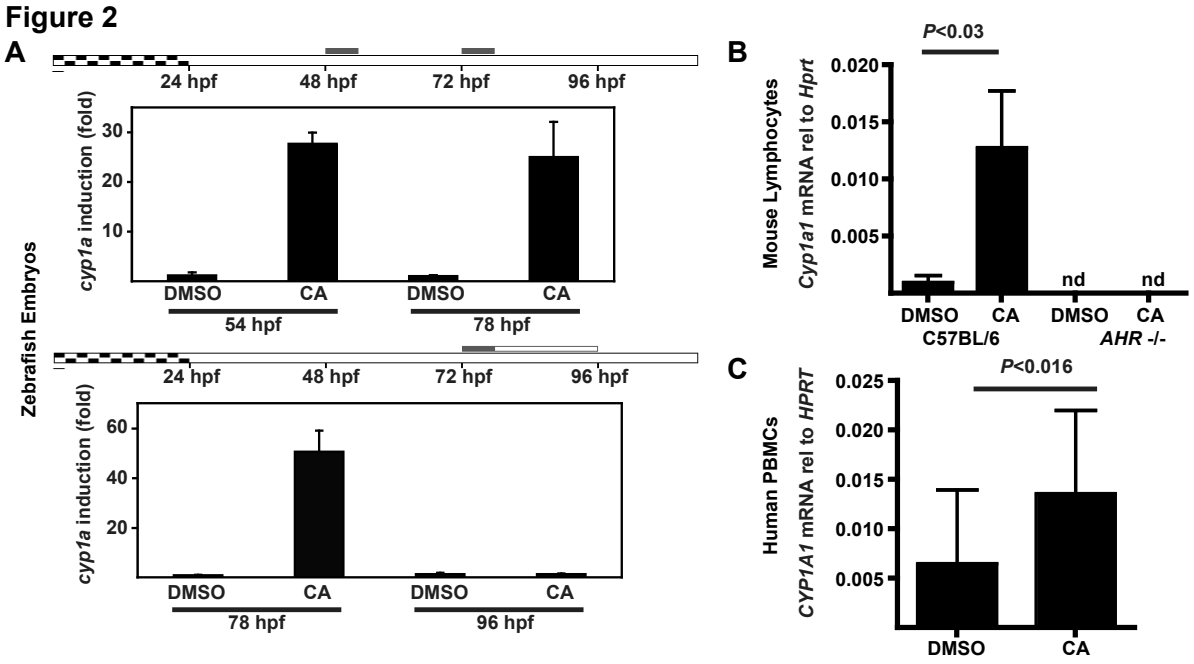

Figure 2. CA induces *Cyp1a* orthologs in zebrafish, humans, and mice.

(A) Induction of *cyp1a in vivo* as measured by qRT-PCR. Zebrafish embryos at 48 or 72 hours post fertilization (hpf) were exposed to CA (100 μM) for 6 hours, and either sampled immediately (54 or 78 hpf; top panel) or placed in clean water and sampled at 96 hpf (24 hours after beginning of exposure; bottom panel). *Cyp1a* mRNA was normalized to *β-actin* and to the average DMSO value. (DMSO values for 78 and 96-hpf embryos were similar). Values represent fold-change in CA-treated versus DMSO-treated embryos; each panel represents an experiment

sampling three replicate groups of twenty embryos per group. (B) Induction of *Cyp1a1* in wild-type or *Ahr*-/- mouse total lymphocytes incubated with CA (50 μM) in RPMI for 4 hours. *Cyp1a1* was measured by qRT-PCR and normalized to *Hprt*. Error bars represent SD. P values were calculated with the Mann-Whitney test for lymphocyte cultures from three individual mice. (C) Induction of *CYP1A1* PHA-stimulated human total PBMCs after 12-20 hours of incubation with CA (50 μM) in RPMI. *CYP1A1* was measured by qRT-PCR and normalized to *HPRT*. Data shown are pooled experiments from six individual donors. Error bars are SD. P values were calculated with the Mann-Whitney test.

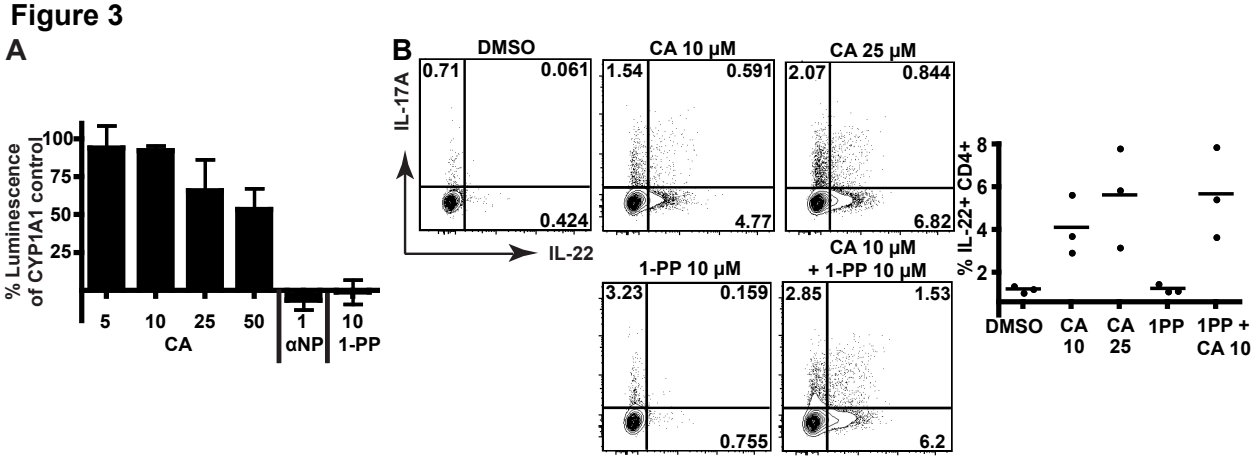

Figure 3. Inhibition of FICZ metabolism does not affect IL-22 production.

(A) Inhibition of CYP1A1 activity measured by luminescence of CYP1A1-driven metabolism of luciferin-CEE. Background readings in the absence of CYP1A1 supersomes are subtracted and luminescence is shown as a percentage of the CYP1A1 only control. Test compound concentrations are in μM. α-NP and 1-PP are positive controls. Values are averaged from three replicate wells. Data are representative of three independent experiments. (B) Flow cytometric analysis of mouse naïve $CD4^+$ T cells stimulated under polarizing conditions (as described in

Chapter II) in the presence of the Cyp1a1 inhibitor, 1-PP. Data are from three independent experiments are graphed (right).

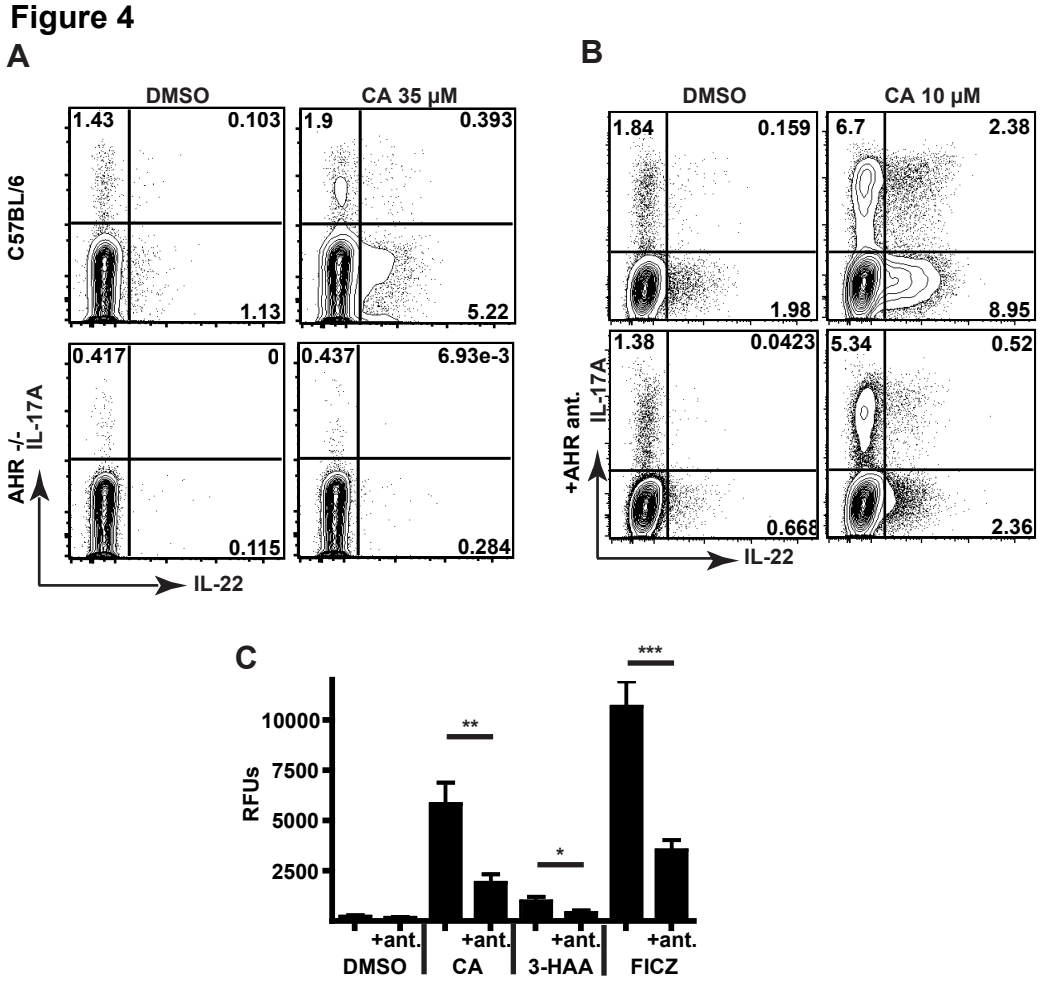

Figure 4. Experiments in *Ahr-/-* mice and addition of an AHR antagonist abrogates the effects of CA *in vitro*. (A) Flow cytometric analysis of IL-17 and IL-22 production in sorted naïve mouse $CD4^+$ T cells from *Ahr-/-* mice stimulated under polarizing conditions (with IL-1β, IL-6, TGF-β, anti-IFNγ, and anti-IL12/23) in the presence of DMSO or CA (35 μM). (B) Flow cytometric analysis of IL-17 and IL-22 production in C57BL/6 mouse naïve $CD4^+$ T cells stimulated under polarizing conditions in the presence of DMSO or CA, with or without the

AHR antagonist CH-223191 (10 μM). Data are representative of three independent experiments. (C) Fluorescence of an AHR-responsive reporter construct measured following incubation of H1G1.1c3 cells with DMSO control, CA (200 μM), 3-HAA (200 μM), or the positive control FICZ (100 nM), with or without the AHR antagonist CH-223191 (50 μM). *, $p<0.01$. **, $p<0.001$. ***, $p<0.0001$. (2-way ANOVA, Bonferroni's post-test). Error bars are SD.

CHAPTER IV: A COMPARISON OF CINNABARINIC ACID TO OTHER TRYPTOPHAN METABOLITES THAT ACTIVATE THE AHR

Abstract

Other tryptophan metabolites and photoproducts have been shown to affect cytokine production and T cell differentiation. CA was compared to kynurenic acid (KYA) and L-kynurenine (L-KYN) and was found to be more effective at increasing IL-22 production within naïve $CD4^+$ T cells. Surprisingly, CA was much less effective than these metabolites at increasing *Cyp1a1* transcription *in vitro*. A dose response curve measuring CA-induced IL-22 production was created and compared to concentrations produced by immune cells stimulated *in vitro*.

Introduction

As discussed in Chapters II and III, CA is a tryptophan metabolite capable of binding and activating the AHR and increasing IL-22 production in $CD4^+$ T cells. Other tryptophan metabolites, L-kynurenine (L-KYN) and kynurenic acid (KYA), have also been shown to

activate the AHR and to have effects on cell differentiation or cytokine production. Additionally, the tryptophan photoproduct FICZ has been shown to increase IL-22 production in $CD4^+$ T cells through AHR activation and may play a role in endogenous immunostimulatory processes through formation in the skin (19,25). Therefore, understanding (a) how CA compares to other kynurenine metabolites that are AHR ligands and (b) how CA compares to known inducers of IL-22 is critical to understand its relevance in T cell differentiation *in vivo*.

L-KYN has been shown to induce Treg differentiation in mouse $CD4^+$ T cells stimulated *in vitro* at concentrations around 50 μM (27). Endogenous plasma concentrations of L-KYN range from 0.7 to 3 μM, but in the context of HIV infection concentrations can reach 5 μM (73,120,161). Conversely, KYA synergized with IL1β to induce IL-6 in a human cell line at concentrations as low as 100 nM (26). However, KYA concentrations in the periphery of healthy donors is much lower than L-KYN, ranging from 6 to 54 nM (161). Plasma concentrations of KYA have not been documented during HIV infection, but KYA in the cerebrospinal fluid of HIV-infected patients is elevated versus controls, implicating that similar changes in concentration may occur systemically during HIV (162).

Given that many of the effects of these metabolites may be exerted locally, assessing their concentrations within tissue is also important. A thorough documentation of these metabolites in a rat model of renal insufficiency revealed that increases in plasma concentrations were mirrored by increases in tissues, though whole tissue concentrations were on the order of pmol/mg tissue, which is difficult to equate to concentrations used *in vitro* (163). Though concentrations of these metabolites in the peripheral blood are below those which induce changes in cytokine production or T cell differentiation *in vitro*, it must be emphasized that local concentrations of these metabolites may be effectively higher, much as cytokine production may

be concentrated at immunological synapses (164). Additionally, *in vitro* assays may be complicated by reductions in bioavailability to cells caused by binding to serum proteins (165). Relating effective concentrations *in vitro* to endogenous immunological effects will be a critical and challenging area of future research.

While relating effective *in vitro* concentrations of KYA and L-KYN to *in vivo* effects has proven to be difficult, so to has it been hard to analyze the biological role of the tryptophan photoproduct, FICZ. Produced readily upon ultraviolet irradiation of cell culture media, FICZ has been shown to have a critical role in cell culture assays, potently increasing IL-22 and IL-17 production *in vitro* (19,166). At concentrations of 250 nM, FICZ has been reported to induce IL-22, and its activity on other AHR-responsive genes such as *Cyp1a1* has been reported in the picomolar range (166). However, the effects of FICZ *in vitro* are limited by its self-induced metabolism (167). Furthermore, FICZ has not yet been detected endogenously, though the presence of sulfated products of FICZ metabolism in the urine implies that endogenous formation of FICZ is feasible (25).

Endogenous CA has only recently been detected in rat tissues including the lung, liver, spleen, and kidney at relatively low concentrations ranging from 7 to 60 pg/mg tissue (168). Administration of LPS increased CA in both the spleen and kidney as well as in the brain, where in normal rats it was below the limits of detection. While endogenous concentrations of CA are less understood, CA production *in vitro* is better studied. Multiple enzymes are capable of converting 3-HAA into CA, including catalase, superoxide dismutase, the fungal enzyme laccase, and its human ortholog, ceruloplasmin (146,169). Additionally, CA may be formed non-enzymatically under oxidative conditions (170), as may occur in the context of inflammatory responses (171). Interestingly, thymocytes incubated with 300 μM 3-HAA *in vitro*

formed 30 μM of CA after 6 hours of culture, indicating that cell mediated synthesis of CA is feasible (141).

To better understand the relevance of CA endogenously, the effect of CA on *Cyp1a1* induction was compared to L-KYN and KYA. Next, the ability of CA to affect IL-17 and IL-22 production was compared to tryptophan metabolites, L-KYN, KYA, and FICZ. Then, the ability of immune cells to produce CA following stimulation with cytokines and TLR agonists known to induce IDO and generate kynurenine metabolites was tested. Finally, the concentrations of CA obtained from immune cells were compared to concentrations effective at altering IL-22 production *in vitro*.

Materials and Methods

Chemicals and mice. Chemicals and mice were obtained as described in Chapters II and III. Additionally, kynurenic acid (KYA), L-kynurenine (L-KYN), and laccase (from *T. versicolor*) were purchased from Sigma. For some experiments, laccase was heat killed by incubation at 95˚C for five minutes.

In vitro human cell culture. Naïve human T cell assays were performed as in Chapter II.

In vitro mouse cell culture. Naïve mouse T cell assays were performed as in Chapter II.

***Cyp1a1* induction.** Assays were performed as described in Chapter III.

CA detection. Human PBMCs were plated at a concentration of 2.5-5x10^6 cells/well in 200 μL of RPMI. Cells were left unstimulated or incubated with 50 μg/mL LPS, 10 ng/mL PMA, 100 ng/mL IFNγ, or 1-5 μg/mL concanavalin A (conA) for 16 hours. Supernatants were frozen for detection of CA by LC/MS/MS.

Sort depletion. Human PBMCs were stained with anti-CD14-QDot605 (Invitrogen), anti-HLADR-PECy7 (BD), anti-CD3-Alexa700 (BD), and anti-CD16-PB (BD). Cells designated as all cells were sorted as singlets; cells designated as CD3 depleted were sorted as singlet $CD3^-$ events. Cells designated as CD14 depleted were sorted by combining singlet $CD3^+$ cell gate with the $CD3^-CD14^-$ cell gate into one sort stream. Supernatants were collected from cells stimulated with IFNγ, as described under "CA detection."

LC/MS/MS. CA was measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Samples (20 μl) were mixed with 100 μl of internal standard, piroxicam (100 ng/ml) in acetonitrile, vortexed for 1 min, and centrifuged at 3000 rpm for 10 min. The supernatant was transferred to an autosampler vial and 8 μl was injected onto the LC/MS/MS system. The standard curve was generated by serial diluting CA standard solution in water. The mass detector was an API 5000 triple quadrapole (Applied Biosystems), equipped with a Turbo Ion Spray source. The system was set in positive ionization mode. The ion spray voltage was 5500 V and the source temperature was 600°C. The values for CAD, CUR, GS1, and GS2 were 6, 15, 55, and 75, respectively. The multiple reaction monitor was set at 301.1 – 265.0 *m/z* for CA and 332.0 – 94.9 *m/z* for piroxicam. A Shimadzu system was used for the HPLC, consisting of a pump, solvent degasser, autosampler and column oven, which was set to 30°C. The mobile

phase, consisting of 40% acetonitrile, 0.1% trifluoroacetic acid containing 5 mM ammonium acetate, was pumped through a Synergi Polar RP (4.6 x 75 mm, 4 μm particle size) column with a flow rate of 1.0 ml/min. Data were acquired and processed by Analyst 1.5.1 software.

The limit of quantification (LOQ) and limit of detection (LOD) for CA were 7.81 ng/ml and 3 ng/ml, respectively. The tryptophan, L-KYN, and 3-HAA levels were measured in API-5000 with a similar method as reported before (73). CA values below LOQ were treated as ½ LOQ for statistical analysis (172).

Results

First, the ability of CA to induce transcription of the AHR-responsive gene, *Cyp1a1*, was compared to other tryptophan metabolites, KYA and L-KYN, which have recently been identified as AHR agonists (26,140). In both mouse (Fig. 1A) and human (Fig. 1B) lymphocytes, CA induced *Cyp1a1* but was less effective than these other tryptophan metabolites. Next, KYA and L-KYN were compared to CA in mouse naïve T cells at concentrations where they were more effective than CA at inducing *Cyp1a1* (Fig. 2, A and B). However, CA was much more effective at increasing IL-22 production in these cells; neither KYA nor L-KYN was able to increase significantly IL-22 or IL-17 production. Given that KYA is more effective at activating the AHR in human cells as compared to mouse cells (26), CA was also compared to these metabolites in naïve T cells isolated from human cord blood (Fig. 2C). Likewise, CA proved effective at increasing IL-22 production while the other metabolites did not under the conditions and concentrations tested. Finally, CA was directly compared to FICZ in mouse naïve cell cultures (Fig. 2D). Although FICZ did induce some IL-22 production, IL-22 production was not greater than that caused by CA, even when FICZ was titrated to 10 μM (Fig. 2E).

Generation of CA from immune cells

We next sought to determine whether immune cells are capable of producing CA. To date, CA has only been identified within human cell cultures in which 3-HAA has been exogenously added (141). However, the formation of CA *in vivo* in rats injected with LPS has recently been described (168). We accordingly asked whether human PBMCs are capable of generating CA when stimulated with LPS or other immunostimulatory compounds. After culture for 16 hours, CA at concentrations up to ~1 μM appeared in the cell culture supernatants of cells stimulated with LPS, IFNγ, or concanavalin A, yet remained low or below the LOQ in unstimulated (NS) control wells (Fig. 3, A and B). Production of CA was correlated with both the endogenous 3-HAA concentration in the supernatant (Fig. 3C) and with the supernatant kynurenine/tryptophan ratio (Fig. 3D), the latter of which is indicative of IDO-driven tryptophan metabolism (73). To our knowledge, this is the first characterization of CA secretion from human cells *in vitro*. Next, we asked whether certain cell populations were critical to CA production. Removal of $CD14^+$ cells from the cultures abrogated CA production, while removal of $CD3^+$ cells did not cause a significant difference (Fig. 3E). Of note, the process of sort-purifying cells stimulated CA production at baseline, which was also prevented in $CD14^+$ depleted samples.

We next asked whether concentrations of CA produced in the supernatant of these assays would be effective at increasing IL-22 production. Human naïve T cells were differentiated in the presence of decreasing doses of CA. Concentrations as low as 1 μM were found to significantly increase IL-22 production (Fig. 3F). Finally, we tested whether conditions likely to lead to CA generation could affect IL-22 production *in vitro*. The fungal enzyme, laccase, has

been described as capable of catalyzing the formation of CA from 3-HAA (169). When laccase alone was introduced into mouse naïve $CD4^+$ T cell cultures under polarizing conditions, IL-22 production was doubled, possibly from formation of CA or a related dimerization product from tryptophan metabolites in the media (Fig. 3G). Laccase that had been heat killed was unable to increase IL-22 production, demonstrating a requirement for its enzymatic activity.

Discussion

We have directly compared the ability of CA to induce IL-22 to that of other reported tryptophan-derived AHR agonists (e.g., FICZ, L-KYN and KYA) (19,140,173). Amongst the metabolites downstream of IDO (L-KYN and KYA), the ability of CA to increase IL-22 production from naïve T cells is comparable to that observed with the tryptophan photoproduct, FICZ (Fig. 2D). Neither L-KYN nor KYA increased IL-22 production as effectively as CA in mouse or human T cells under the tested concentrations and conditions, despite the ability of these two compounds to much more effectively induce *CYP1A1*, an AHR-responsive gene, in human and mouse lymphocytes. CA may be a selective AHR modulator (SAhRM) (2), more potently inducing *IL-22* than *CYP1A1*. Indeed, several AHR ligands that bind the AHR and elicit AHR-dependent effects, but are weak inducers of *CYP1A1*, have been described previously (174-177). The actions of such SAhRMs can be cell- and species-specific (2,178), and SAhRMs with selective immunomodulatory activity (although not involving IL-22) have been reported previously (153). The molecular mechanism by which AHR activation leads to enhanced IL-22 expression is not yet well understood, but it appears to involve interaction of AHR with RORγt at the *Il22* gene (46). Whether this mechanism involves direct DNA binding by the CA-activated

AHR or a DNA-binding-independent mechanism (such as tethering to DNA-bound RORγt) (152) remains to be investigated.

In addition to demonstrating that CA is an AHR agonist that promotes IL-22 production, we show that CA can be produced by stimulated human PBMCs, in the absence of exogenous 3-HAA. Potential enzymatic modulators that can regulate the generation of CA from 3-HAA would predictably affect the resolution of inflammation. Such enzymes include ceruloplasmin (169), superoxide dismutase (146), catalase (146), and the fungal virulence factor, laccase (169). It is interesting to note that ceruloplasmin recently has been shown to be protective in mouse models of inflammatory bowel disease, where IL-22 has also been shown to be protective (179). CA also might be generated through non-enzymatic reactions favored under oxidizing conditions (170), such as those found in the context of inflammatory responses. For instance, neutrophils, which produce reactive oxygen species (ROS) in an antimicrobial oxidative burst, also express high levels of IDO in the setting of fungal infections (171). In such cells, co-expression of IDO and enzymes involved in generating ROS might skew the tryptophan metabolic pathways towards the generation of CA over PA or QA. It is important to note CA is effective at driving IL-22 production only at the upper limit of secreted concentrations detected in our assays (~1 μM) (Figs. 3B, 3F). However, the ability of CA to be generated intracellularly at the site of an inflammatory immune response may allow for relatively high effective concentrations to be achieved locally. Future study will be required to understand the roles of IDO-derived metabolites in mediating immune responses within tissues.

Figures

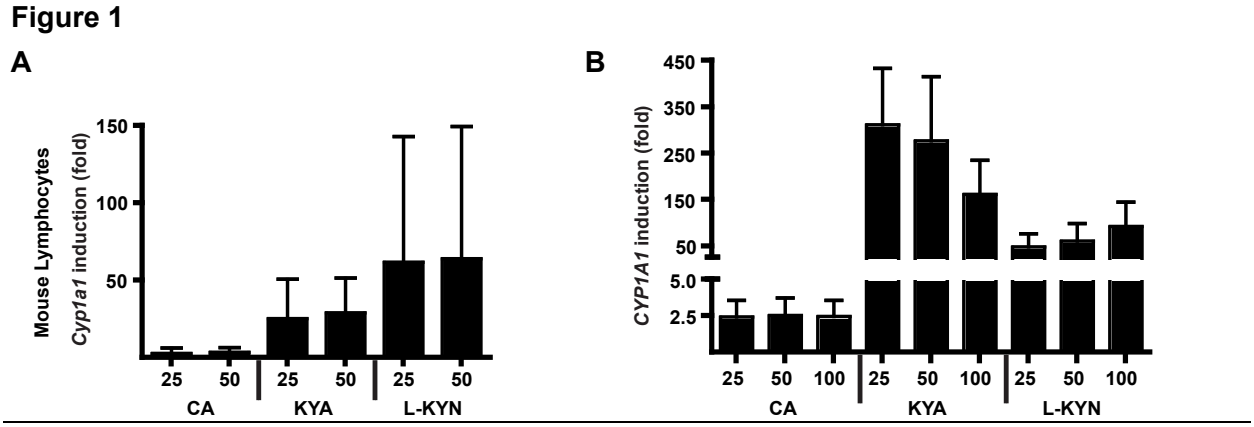

Figure 1. CA is less effective at inducing *Cyp1a1* transcript when compared to KYA and L-KYN. Induction of mouse *Cyp1a1* (A) and human *CYP1A1* (B) measured by qRT-PCR relative to *Hprt* or *HPRT*. Mouse lymphocytes (A) and PHA-stimulated human PBMCs (B) were incubated with CA, KYA, L-KYN. Values are pooled from at least three independent experiments and represent fold-change compared to DMSO control. Metabolite concentrations are in μM. Error bars represent SD.

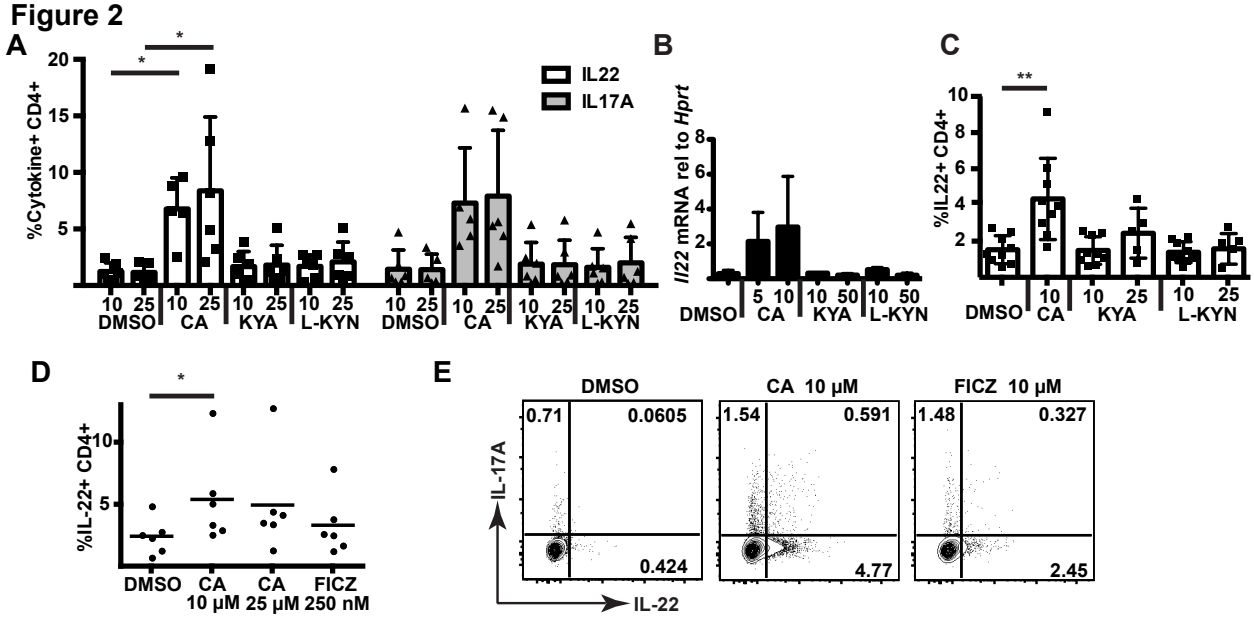

Figure 2. CA increases IL-22 and IL-17 production more efficiently than other tryptophan metabolites. (A) Flow cytometric analysis of mouse naïve $CD4^+$ T cells for IL-22 (left, white bars) and IL-17 (right, gray bars) production following stimulation under polarizing conditions (with IL-1β, IL-6, TGF-β, anti-IFNγ, and anti-IL12/23) in the presence of DMSO, CA, L-KYN, or KYA. Data are from six independent experiments. Metabolite concentrations are in μM. Data from individual experiments are depicted by separate data points and were analyzed by analyzed by Kruskal-Wallis ANOVA and Dunn's Multiple Comparison test. *, $p<0.05$. (B) qRT-PCR data were analyzed from mouse naïve $CD4^+$ T cells stimulated under polarizing conditions for the expression of *Il22* transcripts measured relative to *Hprt*. Data are pooled from at least three independent experiments per sample; metabolite concentrations are in μM. (C) Flow cytometric analysis of naïve $CD4^+$ T cells isolated from human cord blood and stimulated under polarizing conditions (with IL-1β, IL-6, IL-23, TGF-β , anti-IFNγ, and anti-IL4) in the presence of DMSO, CA, L-KYN, or KYA, with concentrations noted in μM. Data from six independent experiments with nine (10 μM) or five (25 μM) individual donors were analyzed by one-way ANOVA (Kruskal-Wallis) and Dunn's Multiple Comparisons Test. **, $P<0.01$. (D) Flow cytometric analysis of mouse naïve $CD4^+$ T cells stimulated under polarizing conditions in the presence of DMSO, CA, or FICZ. Data from six independent experiments were analyzed by the Friedman test and Dunn's Multiple Comparison test. *, $p<0.05$. Error bars represent SD. (E) Flow cytometric analysis of mouse naïve $CD4^+$ T cells stimulated under polarizing conditions in the presence of equimolar concentrations of DMSO, CA, or FICZ. Data are representative of three independent experiments.

Figure 3

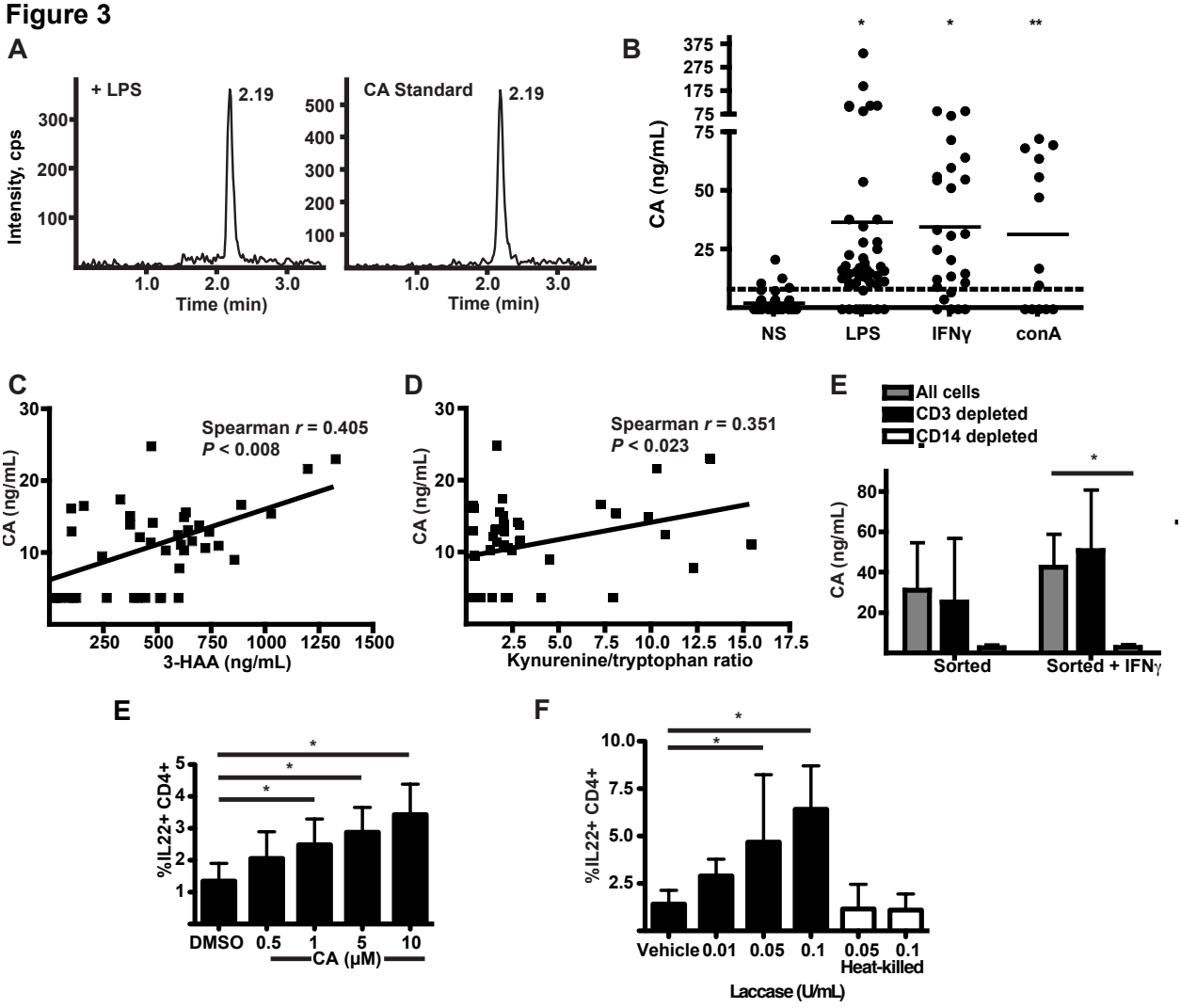

Figure 3. CA is generated by human PBMCs stimulated *in vitro*. (A) Detection of CA by LC/MS/MS in supernatants of human PBMCs stimulated with LPS (50 μg/mL). The left chromatogram is representative of LPS-treated samples, and the right represents a CA standard peak. (B) Measurement of CA in supernatants of human PBMCs cultured with LPS (50 μg/mL), IFNγ (100 ng/mL), or conA (1μg/mL) for 16 hours versus non-stimulated (NS) controls. Data points represent individual treated wells from human donors. The dashed line represents the limit of quantification (LOQ = 7.81 ng/mL) of CA. Data points below LOQ were treated as ½ LOQ, while data points below the limit of detection (LOD = 3 ng/mL) were treated as zero. Data from

different stimulation conditions were compared by one-way ANOVA (Kruskal-Wallis, Dunn's Multiple Comparison). *P<0.001, **P<0.01 versus non-stimulated wells. (C) Correlation of the CA concentration in supernatants of human PBMCs with the concentration of 3-HAA (Spearman's rank correlation). Each data point represents an individual sample treated +/- LPS, LPS/PMA, IFNγ, or conA. Samples with CA below LOQ were assigned a value of ½ LOQ. (D) Correlation of CA secretion in supernatants of human PBMCs with the ratio of kynurenine/tryptophan in the supernatant (Spearman's rank correlation). (E) Measurement of CA in supernatants of sort-depleted human PBMCs cultured with or without IFNγ (100 ng/mL) for 16 hours. Data were analyzed by Kruskal-Wallis ANOVA with Dunn's Multiple Comparison test. *, $p<0.05$. (F) Flow cytometric analysis of cord blood naïve $CD4^+$ T cells stimulated under polarizing conditions in the presence of decreasing concentrations of CA. Data from four experiments were analyzed by repeated measures ANOVA and Dunnett's Multiple Comparisons Test. *, $p<0.05$. (G) Incubation of sorted naïve mouse $CD4^+$ T cells with DMSO, the fungal enzyme laccase, or heat-killed laccase under polarizing conditions (as in panels A, B). Data pooled from four independent experiments were analyzed by one-way ANOVA (Kruskal-Wallis with Dunn's Multiple Comparison Test). * $p<0.05$. Error bars are SD.

CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

In summary, we have identified CA as an endogenous tryptophan metabolite generated downstream of IDO induction that is capable of increasing IL-22 production through AHR activation. In addition to its effects on IL-22, we have also shown it to have differential effects

on IL-17 between humans and mice, increasing IL-17 in mice only. Finally, CA exhibited no effective induction of suppressive Tregs *in vitro* in either species. We have shown that CA is capable of binding and activating the AHR, and that the effects of CA upon IL-22 production are inhibited by an AHR antagonist. We have compared CA to other IDO-derived tryptophan metabolites and find that it is a weaker inducer of certain AHR-responsive genes, such as *CYP1A1*, but even more effectively increases IL-22 production. Finally, we show that CA may be produced by human immune cells and that the concentrations produced *in vitro* are in the range of those required to induce IL-22 *in vitro*.

Given the above, it will be of great interest to further understand the regulation of CA formation and metabolism, and its role in AHR-mediated immune regulation. While both endogenous enzymes as well as more spontaneous oxidative processes have been described to lead to CA formation, which mechanism is most critical in either health or disease remains to be described. Future research identifying potential inducers or inhibitors of CA formation will allow for a greater understanding of how CA may impact immune responses *in vitro* and *in vivo*. While generating a model organism incapable of producing CA may prove difficult due to the number of potential enzymatic modulators, studying formation of IL-22 in mice deficient in enzymes such as ceruloplasmin or catalase may reveal a role for enzymatic formation of an AHR ligand such as CA.

The effects of CA on immune cell differentiation appear to be unique from other tryptophan-derived metabolites such as L-KYN and 3-HAA, which preferentially expand Tregs. We have shown previously that tryptophan catabolism can result in a loss of Th17 cells in the context of HIV disease through generation of 3-HAA (73). We hypothesize that this loss, particularly within the gut mucosa, allows for ongoing inflammation due to continued microbial

translocation. Conversion of 3-HAA into CA could reverse the effects of 3-HAA within immune cells, thereby restoring Th17 and Th22 cells in the context of increased IDO activity. This would allow for the resolution of the inflammatory signaling cascade by strengthening the mucosal barrier, thus stopping a vicious cycle that might otherwise drive disease progression (73). Although IL-22 was initially linked to IL-17 as a pro-inflammatory cytokine, recent evidence suggests that it plays an independent immunoregulatory role in the context of non-hematopoietic cells, maintaining epithelial cell homeostasis in the mucosal tissues (129,130,180). If so, the pathways that lead to the generation of CA may operate in tandem with the immunosuppressive mechanisms linked to tryptophan metabolism to generate a population of Th22 cells that plays a specific role in tissue repair following inflammation (129). These findings prompt future investigation into the potential roles that CA may play in numerous biological settings in which the AHR is involved.

The number of AHR ligands that have been identified to play a role in immunological development and response has expanded greatly in recent years. Environmentally generated ligands for the AHR have been recently shown to affect homeostasis between the immune system and commensal microflora in the gut mucosa (21), and commensal microflora themselves have been proven capable of producing AHR agonists that affect the immune response (47). AHR activation was found to be critical for maintenance of local intraepithelial lymphocyte subsets that in turn regulate the homeostasis of and prevent bacterial dissemination across the mucosal epithelium. Additional immune cell types, including innate lymphoid cells producing IL-22 (ILC22) in the gut have also been shown to be AHR-dependent (181). Identifying the source of these ligands is critical for understanding and affecting AHR-dependent immune responses. In some of these cases, AHR activation was induced by exogenous ligands;

for gastrointestinal immunity, the presumptive AHR ligands were dietary, whereas tryptophan photoproducts such as FICZ may be generated by UV exposure of the skin. By contrast, removal of dietary AHR ligands had no effect on the function of ILC22s (21), suggesting that another source of AHR agonists must exist. While the gastrointestinal microbiota is an appealing candidate (47), given the wide breadth of tissues and cell types regulated by AHR activation at multiple stages in development, it is likely that multiple, independently generated AHR agonists, including CA, work in separate compartments to regulate AHR-dependent processes globally.

Footnotes

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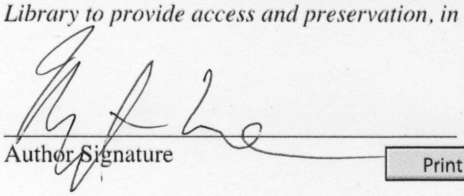

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