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# Indoximod Modulates AhR-driven Transcription of Genes That Control Immune Function

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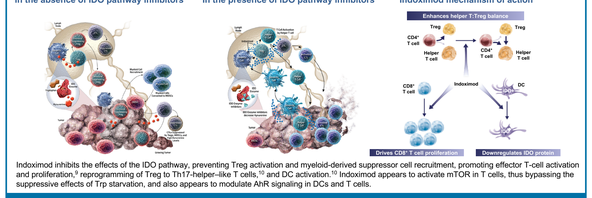
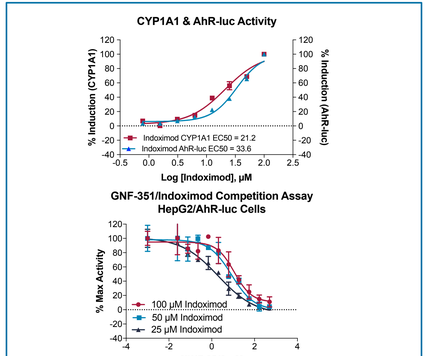
## SUMMARY


- The indoleamine 2,3-dioxygenase (IDO) pathway mediates immunosuppressive effects through the metabolization of tryptophan (Trp) to kynurenine (Kyn),[1] triggering downstream signaling through Trp sensors general control nonderepressible 2 (GCN2)[2] and mammalian target of rapamycin (mTOR),[3] and Kyn sensor aryl hydrocarbon receptor (AhR).[4] The activation of these signaling pathways has pleiotropic effects on immune cells, including influencing the differentiation of dendritic cells (DCs), helper T cells, and T regulatory cells (Treg), as well as enhancing proliferation of effector T cells and Treg[5-8]
- Indoximod is an orally administered, small-molecule IDO pathway inhibitor that reverses the immunosuppressive effects of low Trp and high Kyn that result from IDO or tryptophan 2,3-dioxygenase (TDO) activity. Indoximod has immunostimulatory effects involving three main cell types: CD8$^+$ T cells, Tregs, and DCs. Indoximod increases proliferation of effector T cells, reprograms Treg into helper T cells, and downregulates IDO expression in DCs. These effects are observed in both the presence and absence of IDO activity
- Indoximod has been demonstrated to relieve IDO-mediated immunosuppression in vitro and in vivo, by creating an artificial Trp-sufficiency signal that bypasses activation of GCN2 and inhibition of mTOR in conditions of Trp deprivation[2]
- Indoximod activates AhR-dependent transcriptional activity as evidenced by increased AhR-driven luciferase in HepG2 cells and endogenous cytochrome P450 1A1 (CYP1A1) activity in CD4$^+$ T cells
- In a Kyn-driven Treg differentiation assay, indoximod altered the transcription of genes associated with helper T and Treg phenotypes. Indoximod induced upregulation of rorc expression, a Th17-associated transcription factor, while concurrently downregulating transcription of Foxp3, which shifted the cellular phenotype from Foxp3$^+$ Treg toward Th17-producing CD4$^+$ helper T cells. Interestingly, these effects were also observed in the absence of exogenous Kyn- or Trp-deficiency, indicating that indoximod has IDO-independent activity
- Transcription of *Ido1* is controlled by interferon γ (IFNγ) and AhR response elements in its promoter.[7,8] Consistent with the role of indoximod in blocking the IDO pathway, indoximod downregulated the expression and function of IDO in in vitro–derived CD11c$^+$CD123$^+$CD83$^+$ DCs (moDCs), and in plasmacytoid DCs (pDCs) from tumor-draining lymph nodes (TDLN) that express IDO, thus affecting Kyn production and T cell proliferation
- Indoximod's effects in both T cells (Foxp3 and rorc/RORγt modulation) and DCs (IDO expression) were reverted by AhR inhibition
- Together, these data suggest that indoximod modulates AhR signaling to exert multiple immunomodulatory effects, including a shift from suppressive Foxp3$^+$ Treg toward Th17 helper T cells as well as the downregulation of IDO expression in DC, contributing to enhanced antitumor immunity

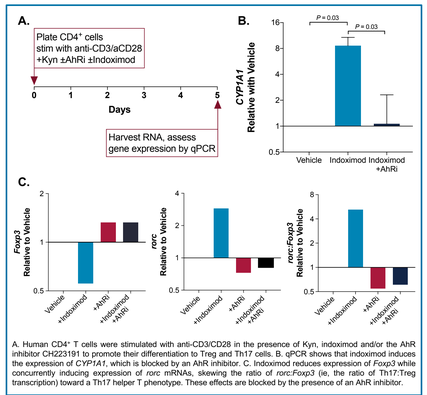

Indoximod inhibits the effects of the IDO pathway, preventing Treg activation and myeloid-derived suppressor cell recruitment, promoting effector T-cell activation and proliferation,[9] reprogramming of Treg to Th17-helper–like T cells,[10] and DC activation.[10] Indoximod appears to activate mTOR in T cells, thus bypassing the suppressive effects of Trp starvation, and also appears to modulate AhR signaling in DCs and T cells.

**Figure 1. Indoximod mechanism of action.**



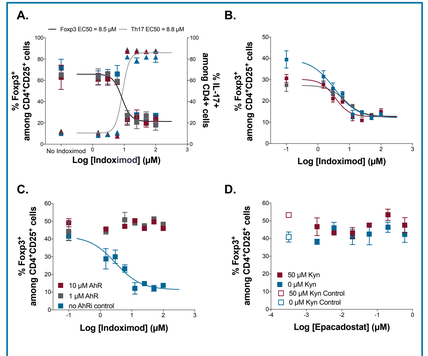

Indoximod induces expression and activity of endogenous CYP1A1 and that of luciferase controlled by an AhR-inducible promoter in HepG2 cells. These effects are suppressed by an AhR inhibitor, indicating that indoximod can induce regulation of AhR controlled genes.

**Figure 2. AhR activation by indoximod.**



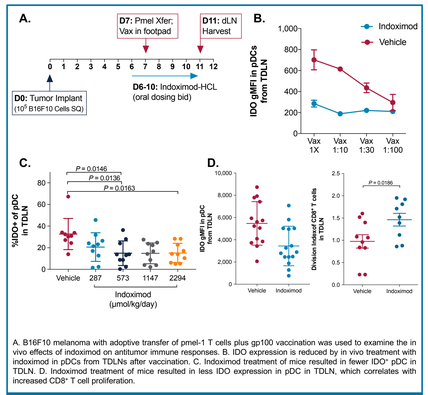

A. Human CD4$^+$ T cells were stimulated with anti-CD3/CD28 in the presence of Kyn, indoximod and/or the AhR inhibitor CH223191 to promote their differentiation to Treg and Th17 cells. B. qPCR shows that indoximod induces the expression of *CYP1A1*, which is blocked by an AhR inhibitor. C. Indoximod reduces expression of *Foxp3* while concurrently inducing expression of *rorc* mRNAs, skewing the ratio of *rorc:Foxp3* (ie, the ratio of Th17:Treg transcription) toward a Th17 helper T phenotype. These effects are blocked by the presence of an AhR inhibitor.

**Figure 3. Indoximod modulates CD4$^+$ T cell gene expression in an AhR-driven fashion.**



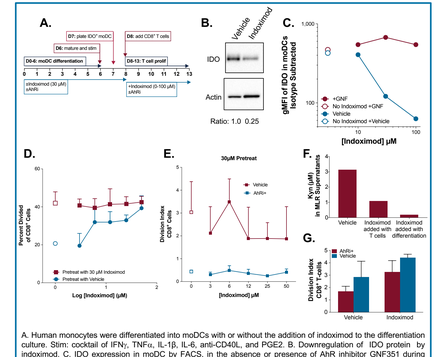

Human CD4$^+$ T cells were stimulated for 5 days with anti-CD3/CD28 in the presence or absence of Kyn to enhance their differentiation into Treg cells. Indoximod effect (A-C) or epacadostat effect (D) on the differentiation of CD4$^+$ T cells into Treg (squares) or Th17$^+$ T cells (triangles). The AhR inhibitor CH223191 reverses the effect of indoximod (C).

**Figure 4. Indoximod drives CD4$^+$ T cell differentiation toward IL-17$^+$ helper T cells via AhR independently of Kyn.**



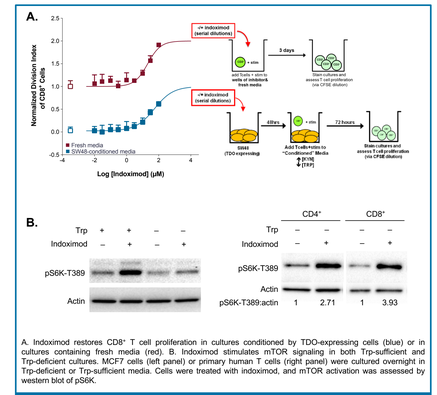

A. B16F10 melanoma with adoptive transfer of pmel-1 T cells plus gp100 vaccination was used to examine the in vivo effects of indoximod on antitumor immune responses. B. IDO expression is reduced by in vivo treatment with indoximod in pDCs from TDLNs after vaccination. C. Indoximod treatment of mice resulted in fewer IDO$^+$ pDC in TDLN. D. Indoximod treatment of mice resulted in less IDO expression in pDC in TDLN, which correlates with increased CD8$^+$ T cell proliferation.

**Figure 5. In vivo modulation of IDO expression and activity by indoximod.**



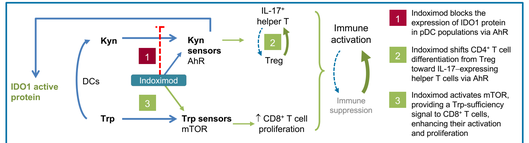

A. Human monocytes were differentiated into moDCs with or without the addition of indoximod to the differentiation culture. Stim: cocktail of IFNγ, TNFα, IL-1β, IL-6, anti-CD40L, and PGE2. B. Downregulation of IDO protein by indoximod. C. IDO expression in moDC by FACS, in the absence or presence of AhR inhibitor GNF351 during differentiation. D. moDC differentiated with or without indoximod were used in an MLR assay with allogeneic T cells, at different concentrations of indoximod. E. Same MLR as in D, in the presence or absence of GNF351 during differentiation. F. Kyn production by moDC in the MLR cultures on Day 13. G. Controls to examine toxicity of GNF351 on T cell proliferation.

**Figure 6. Modulation of IDO expression and activity by indoximod.**




A. Indoximod restores CD8$^+$ T cell proliferation in cultures conditioned by TDO-expressing cells (blue) or in cultures containing fresh media (red). B. Indoximod stimulates mTOR signaling in both Trp-sufficient and Trp-deficient cultures. MCF7 cells (left panel) or primary human T cells (right panel) were cultured overnight in Trp-deficient or Trp-sufficient media. Cells were treated with indoximod, and mTOR activation was assessed by western blot of pS6K.

**Figure 7. Indoximod activates mTOR and augments T cell proliferation in the presence or absence of IDO/TDO activity.**




**Summary of Indoximod Pharmacodynamic Effects**

| Effect | mTOR activation | AhR-dependent | IDO/TDO independent | [Trp] independent | Kyn independent |
|---|---|---|---|---|---|
| Activates transcription of genes controlled by AhR promoter elements | | ✓ | ✓ | Not tested | Yes |
| Induces transcription of CYP1A1 in CD4$^+$ T cells | | ✓ | ✓ | Not tested | Yes |
| Decreases Foxp3 mRNA and blocks CD4$^+$ T cell differentiation into Foxp3$^+$ Treg | | ✓ | ✓ | Not tested | Yes |
| Increases rorc mRNA and enhances CD4$^+$ T cell differentiation into IL-17$^+$ helper T cells | | ✓ | ✓ | Not tested | Yes |
| Downregulates IDO expression in human moDCs | | ✓ | | Not tested | Not tested |
| Increases CD8$^+$ T cell proliferation | ✓ | | ✓ | Yes | Yes |
| Increases mTOR activation in CD4$^+$ T cells | ✓ | | | Yes | Yes |
| Increases mTOR activation in CD8$^+$ T cells | ✓ | | | Not tested | Yes |

**Figure 8. Indoximod blocks IDO expression and modulates the AhR and Trp-sensing pathways.**

## CONCLUSIONS

- In the presence of indoximod, the expression of IDO protein by pDCs is inhibited in an AhR-dependent fashion, disrupting the conversion of Trp into Kyn, which breaks a feedback loop by which Kyn/AhR signaling reinforces expression of IDO protein. Concurrently, indoximod enhances helper T cell differentiation into IL-17-producing helper cells while inhibiting the formation of Treg cells. These effects also occur in an AhR-dependent and IDO/TDO-independent fashion
- Additionally, indoximod activates mTOR in Trp-deficient or Trp-sufficient conditions, enhancing proliferation of CD8$^+$ T cells
- Together, these effects shift toward immune activation in the tumor microenvironment. Of note, indoximod-driven effects on CD4$^+$ T cell differentiation and on CD8$^+$ T cell function can occur in the absence of Kyn or in conditions of Trp-sufficiency
- Thus, in addition to indoximod opposing immunosuppression mediated by the IDO pathway, indoximod drives antitumor immune responses independently from IDO
- Together, these data suggest that modulation of AhR signaling by indoximod treatment leads to the combined effects of IDO protein modulation and shifting CD4$^+$ T cells from a regulatory toward a helper phenotype, which correlates with increases in mTOR activation, T cell proliferation, and enhanced antitumor responses

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AhR, aryl hydrocarbon receptor; AhRi, AhR inhibitor; AhR-luc, AhR-luciferase; CFSE, carboxyfluorescein succinimidyl ester; CYP1A1, cytochrome P450 1A1; DC, dendritic cell; EC50, half maximal effective concentration; FACS, fluorescence activated cell sorting; gMFI, geometric mean fluorescence intensity; IDO, indoleamine 2,3-dioxygenase; IFNγ, interferon γ; IL, interleukin; Kyn, kynurenine; MLR, mixed lymphocyte reaction; moDC, CD11c$^+$CD123$^+$CD83$^+$ dendritic cell; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; PGE2, prostaglandin E2; pS6K, phosphorylated S6 kinase; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; SQ, subcutaneous; Treg, T regulatory cell; TDLN, tumor-draining lymph node; TDO, tryptophan 2,3-dioxygenase; TNFα, tumor necrosis factor α; Trp, tryptophan.