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Desipramine decreases expression of human and murine indoleamine-2,3-dioxygenases

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Abstract

Abundant evidence connects depression symptomology with immune system activation, stress and subsequently elevated levels of kynurenine. Anti-depressants, such as the tricyclic norepinephrine/serotonin reuptake inhibitor desipramine (Desip), were developed under the premise that increasing extracellular neurotransmitter level was the sole mechanism by which they alleviate depressive symptomologies. However, evidence suggests that anti-depressants have additional actions that contribute to their therapeutic potential. The *Kynurenine Pathway* produces tryptophan metabolites that modulate neurotransmitter activity. This recognition identified another putative pathway for anti-depressant targeting. Considering a recognized role of the *Kynurenine Pathway* in depression, we investigated the potential for Desip to alter expression of rate-limiting enzymes of this pathway: indoleamine-2,3-dioxygenases (Ido1 and Ido2). Mice were administered lipopolysaccharide (LPS) or synthetic glucocorticoid dexamethasone (Dex) with Desip to determine if Desip alters indoleamine-dioxygenase (DO) expression *in vivo* following a modeled immune and stress response. This work was followed by treating murine and human peripheral blood mononuclear cells (PBMCs) with interferon-gamma (IFN γ) and Desip. *In vivo*: Desip blocked LPS-induced Ido1 expression in hippocampi, astrocytes, microglia and PBMCs and Ido2 expression by PBMCs. *Ex vivo*: Desip decreased IFN γ -induced Ido1 and Ido2 expression in murine PBMCs. This effect was directly translatable to the human system as Desip decreased IDO1 and IDO2 expression by human PBMCs. These data demonstrate for the first time that an anti-depressant alters expression of Ido1 and Ido2, identifying a possible new mechanism behind

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anti-depressant activity. Furthermore, we propose the assessment of PBMCs for anti-depressant responsiveness using IDO expression as a biomarker.

Keywords

desipramine; kynurenine pathway; Idol; inflammation; depression; PBMCs

1. Introduction

Currently, over 350 million people world-wide suffer from depression, with about 1 in 20 people reporting a depressive episode over the last year (Marcus et al., 2012). Even worse, depression is predicted to become the leading cause of disease burden world-wide by the year 2030 (WHO, 2011). The US population taking anti-depressants has doubled over the past 12 years further highlighting the extent of this problem (Kantor et al., 2015). Considering the poor efficacy of anti-depressants for patients suffering from Major Depressive Disorders (MDD) (Anderson, 1998; Jakubovski et al., 2016; Kirsch and Sapirstein, 1998), identifying additional targets for the creation of more-effective therapeutics is of utmost importance.

A subset of individuals with major depressive disorder present with evidence of an activated immune system (increased inflammatory biomarkers) and another subset have increased peripheral tryptophan metabolism to kynurenine (Kyn) (Murakami et al., 2016; Raison and Miller, 2011). In patients with chronic elevation of inflammatory markers, anti-inflammatory drugs have potential anti-depressant efficacy (Raison, 2017). Thus, patients with elevated Kyn production should benefit from therapeutics that modulate the *Kynurenine Pathway*. Three rate-limiting dioxygenase enzymes (DO's: indoleamine-dioxygenases Idol and Idol2, plus tryptophan-2,3-dioxygenase (Tdo2)) metabolize tryptophan into N-formyl-kynurenine that is then metabolized into Kyn. In human studies, up to 45% of patients with hepatitis C or cancer have a greater severity of depression symptoms following immunotherapy with IFN α (Capuron et al., 2002; Constant et al., 2005; Raison et al., 2009) with severity of depression symptoms associated with elevated Kyn production (Murakami et al., 2016; Raison et al., 2010), while lipopolysaccharide (LPS) administration to subjects induced inflammation and a depression phenotype (Reichenberg et al., 2001). These studies reveal that elevated inflammatory markers are linked to MDD and provide direct evidence that inflammation and the *Kynurenine Pathway* are linked to depressive symptomology (Dowlati et al., 2010; Galecki et al., 2012; Kappelmann et al., 2016).

Activation of the *Kynurenine Pathway* is associated with induction of inflammation-induced depression (Murakami et al., 2016; Raison and Miller, 2011). Expression and regulation of the DOs indicate that enhanced DO activity and elevated DO expression are correlated with depression symptomology in both human and rodent models (Brooks et al., 2016b; Capuron et al., 2002; O'Connor et al., 2009a). In preclinical studies, neuroinflammation induced by LPS administration, polyinosinic:polycytidylic acid (pI:C) treatment, peritoneal infection with *Mycobacterium bovis* or acute/chronic stress culminate in depression-like behaviors such as anhedonia and helplessness/despair (Dantzer and Kelley, 2007; Dantzer et al., 2011;

Gibney et al., 2013; Hoyo-Becerra et al., 2014; Liu et al., 2015; Moreau et al., 2008; Wang et al., 2015a). Such pre-clinical models of depression are associated with increased DO expression and/or DO activity, primarily attributed to Ido1 as diminishing DO activity by the administration of Ido1 inhibitors or using Ido1 knockout (KO) mice results in decreased inflammation- and stress-induced depression-like behaviors (Lawson et al., 2013; Liu et al., 2015; O'Connor et al., 2009b; Salazar et al., 2012). Peripheral immune challenges, largely via IFN γ , induce Ido1 and Ido2 (Brooks et al., 2016a; Brooks et al., 2016b; Browne et al., 2012; O'Connor et al., 2009a); whereas, stress hormones induce Tdo2 (Brooks et al., 2016b) in rodent models. Our recent work suggests that stress-inducible factors, glucocorticoids, synergize with IFN γ to further induce the DOs (Brooks et al., 2016a; Brooks et al., 2016b). Thus, the induction of the *Kynurenine Pathway* following inflammatory and stress-induced stimulation is intimately associated with depression (Capuron et al., 2002; Dantzer and Kelley, 2007; Dantzer et al., 2011; Gibney et al., 2013; Hoyo-Becerra et al., 2014; O'Connor et al., 2009a).

Both Ido1 and Ido2 genes are transcribed into multiple transcript variants, and it is possible to distinguish expression of DO transcripts by amplifying regions unique to each transcript. As such, the Ido1- full length (FL) transcript (comprised of exons 1,3,4,5,6,7,8,9,10,11) is poorly expressed in the naïve mouse brain, but is highly inducible by inflammation and stress. In contrast, the Ido1-variant 1 (v1) transcript (comprised of exons 2,3,4,5,6,7,8,9,10,11) is well-expressed in the brain, but far less sensitive to inflammatory stimuli (Brooks et al., 2016b). This profile suggests the use of alternate enhancer/promoters to control Ido1 expression in a transcript-dependent manner. Similarly, Ido2-FL (comprised of exons 1,2,4,5,6,7,8,9,10,11) is poorly expressed and poorly induced by inflammatory mediators or stress in the mouse brain (Brooks et al., 2016b). Ido2-v1 (comprised of exons 3,4,5,6,7,8,9,10,11) and Ido2-v3 (exon structure unknown) are well expressed within the brain and are increased following inflammatory stimuli (Brooks et al., 2016a). Similar to Ido1, differential relative expression and regulation suggest the use of distinct enhancer/promoter sequences to control Ido2 expression (Ball et al., 2009). Desipramine (Desip; a tricyclic serotonin/norepinephrine reuptake inhibitor) is a commonly prescribed anti-depressant. In preclinical studies, Desip or its precursor imipramine decreased depression-like behavior of LPS-treated (Basu Mallik et al., 2016; Elgarf et al., 2014; Jain et al., 2001; Mello et al., 2013; Park et al., 2011; Shen et al., 1999; Tomaz et al., 2014) and BCG-infected mice (Platt et al., 2013; Vijaya Kumar et al., 2014). Many such studies attributed the induction of depression-like behaviors to Ido1, but no data were provided to determine if Desip altered DO expression as a mechanism associated with diminished depression-like behaviors. As previous work has shown that Desip or its precursor imipramine can decrease LPS-induced depression-like activity (Basu Mallik et al., 2016; Elgarf et al., 2014; Jain et al., 2001; Mello et al., 2013; Park et al., 2011; Shen et al., 1999; Tomaz et al., 2014), we investigated the relationship between inflammation, Desip and DO expression.

We confirmed that LPS induces Ido1 expression *in vivo* in the brain and periphery. Interestingly, Ido1 induction was blocked by Desip in the circulation (murine PBMCs, PBMC^{-T}s, T cells and human PBMCs) and the brain (murine hippocampi, microglia and astrocytes). Using organotypic hippocampal slice cultures (OHSCs), we found that IFN γ -

induced Ido1 and Ido2 expression was blocked by Desip, and furthermore, that our previously described synergy between IFN γ and either Dex (Brooks et al., 2016b) or galectin-9 (Brooks et al., 2016a) to accentuate specific Ido1 and Ido2 transcripts were blocked by Desip.

Here we provide the first evidence that an anti-depressant drug Desip decreases Ido expression in both human and murine samples. This finding identifies a putative new mechanism that may be exploited to enhance anti-depressant activity.

2. Methods and Materials

2.1 Mice and *in vivo* reagents

C57BL/6J mice were bred in-house. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NRC), approved by the Institutional Animal Care and Use Committee. Twelve-week-old male C57BL/6J mice were used for intraperitoneal injections. Saline or Desip (20mg/kg body weight, D3900, Sigma) were administered 30min before saline, LPS (lipopolysaccharide, 330 μ g/kg, serotype 0127:B8, Sigma) or Dex (2,500 μ g/kg, 11695-4013-1, Henry Shein, Dublin, OH). Treatments were administered prior to initiation of the dark cycle and mice were sacrificed 6h post-treatment. This Desip dosage blocked LPS-induced depression-like behaviors (Park et al., 2011).

2.2 Reagents for *ex vivo* studies

Recombinant mouse IFN γ (tested at 10ng/ml, 315-05) and human IFN γ (tested at 10ng/ml, 300-02) were from Peprotech (Minneapolis, MN). Murine Gal-9 (1 μ g/ml, 3535) was from R&D Systems (Minneapolis, MN). Dex (1 μ M, D4902) was from Sigma. Desip was tested at 100 μ M.

2.3 Microglia and Astrocytes

Cells were isolated using the gentleMACS system (Miltenyi, San Diego, CA), as previously described (Matt et al., 2016; Nikodemova and Watters, 2012). Briefly, perfused brains were digested with Neural Tissue Dissociation reagents. The resulting cell suspension was passed through a 40 μ m mesh to remove debris. After centrifugation, cells were re-suspended in 30% low-endotoxin Percoll Plus (GE Healthcare) and centrifuged to remove myelin. Cells were re-suspended in PBS plus 2mM EDTA and 0.5% BSA and incubated with microbeads labeled with either anti-CD11b (microglia enrichment) or anti-ACSA-2 (astrocytes enrichment). Target cells were separated from non-antigenic cells in a magnetic field. Cells were treated for 6h in serum-free medium (Dulbecco's MEM, 1 $\times$ penicillin/streptomycin, 15mM HEPES pH 7.4, 150 μ g/ml BSA) then stored at -80° C for later extraction. Microglia enrichment was previously confirmed by flow cytometry (Gonzalez-Pena et al., 2016) and here with IL-1 β expression which was 24.0-fold higher in microglia versus astrocytes. We have confirmed cells isolated using Miltenyi CD11b⁺ microbeads as CD11b⁺, CD45^{low}, and Ly6C⁻, considered predominantly microglia, and IL-1 β staining as a positive marker for activated microglia (Matt et al., 2016). Astrocyte enrichment was confirmed by Eaat1 expression which was 31.0-fold higher in astrocytes versus microglia. Multiple studies have described using magnetic beads (ACSA-2) to separate a relatively "pure" population of

astrocytes from the brain (Feldmann et al., 2014; Holt and Olsen, 2016; Jungblut et al., 2012), and have confirmed that this population of cells express astrocytic mRNA and do not express markers/mRNA/protein specific to other population of cells in the brain (Holt and Olsen, 2016).

2.4 Murine PBMCs

Cells were isolated from heparinized blood using Ficoll-Paque PLUS (GE Healthcare) according to the manufacturer's protocol. Briefly, blood was layered above Ficoll-Paque PLUS and centrifuged. The mononuclear cell layer (PBMCs) was centrifuged (200xg) twice to remove platelets. If PBMCs were from mice treated *in vivo*, RNA was immediately extracted. If PBMCs from naïve mice were to be treated *ex vivo*, they were separated into CD90⁺ T cells and CD90⁻ PBMC⁻Ts. **T cell enrichment:** PBMCs were incubated with anti-CD90.2 microbeads (Miltenyi). Target cells were separated in a magnetic field with positive (retained-eluted T cells) and negative (fall-through PBMC⁻Ts) fractions collected. Cd3d expression (T cell marker (Haeryfar and Hoskin, 2004)) was ~700-fold higher in T cells than PBMC⁻Ts; indicating efficient T cell enrichment and removal from the PBMC⁻T fraction. Cells were rested for 1.5h, treated for 6h in serum-free medium then stored at -80°C for later extraction.

2.5 Human PBMCs

Cells (purified, characterized PBMCs) from healthy subjects (a 21 year old male and 21 year old female) were purchased from Precision For Medicine (Fredrick, MD). Cells were thawed, centrifuged to remove DMSO, then rested for 2h in serum-free medium. Cells were then treated for 6h and stored at -80°C until RNA extraction.

2.6 OHSCs

C57BL/6J pups were used to generate OHSCs as previously described (Brooks et al., 2016a; Brooks et al., 2016b). Hippocampi were sliced and placed onto membrane inserts in 6-well plates containing medium (50% Eagle's MEM, 25% HBSS, 25% heat-inactivated horse serum, penicillin/streptomycin, HEPES, 0.5% D-glucose). On day 7, media was replaced with serum-free medium. OHSCs were treated for 6h, then stored at -80°C for later extraction.

2.7 RT-qPCR

E.Z.N.A. Total RNA Kit II (Omega Bio-Tek, Norcross, Georgia) was used to extract RNA. RNA was reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA), and qPCR was performed using TaqMan universal PCR master mix (Applied Biosystems). Gene expression was normalized to GAPDH. Relative expression was calculated by comparing 2^{-C_t} , C_t =cycle threshold (Brooks et al., 2016a; Brooks et al., 2016b). Previous publications (Brooks et al., 2016a; Brooks et al., 2016b) describe probe-based assays to measure DO transcripts. Herein, we focused on 4 transcripts to quantify Ido1 (Ido1-FL, Ido1-v1) and Ido2 (Ido2-v1, Ido2-v3).

2.8 Plasma IFN γ

Blood was collected into heparinized syringes 6h post-treatment, and plasma analyzed for IFN γ (ELISA, BD Biosciences; San Diego, CA) following manufacturer's instructions.

2.9 Statistics

Data are mean $\pm$ SEM of 6 mice per treatment combination (*in vivo* experiments) and 3–4 independent PBMC^{-T}, T cell and OHSC preparations. Analysis of variance was conducted with SigmaPlot 13.0. Post-hoc analyses (Holm-Šídák) for comparisons across treatment groups were performed only with a significant interaction. Significant effects required $p < 0.05$.

3. Results

3.1 Ido1-FL

In vivo, Ido1-FL mRNA expression in mouse brain is strikingly induced by LPS (Brooks et al., 2016b; Croitoru-Lamoury et al., 2011; Fukunaga et al., 2012) (but not Dex, data not shown). We found elevated Ido1-FL expression in hippocampus (Figure 1A), astrocytes (Figure 1B) and microglia (Figure 1C) from LPS-treated mice, compared to controls. Interestingly, Ido1-FL was also induced in PBMCs (Figure 1D). Most important, LPS-induced Ido1-FL expression was blocked by Desip in hippocampus, astrocytes and PBMCs (with a similar non-significant trend in microglia).

Ex vivo, IFN γ is accepted as a major mediator of LPS-induced Ido1 expression. To test if Desip was directly blocking IFN γ activity, samples were treated *ex vivo*. When PBMCs^{-T}, T cells (Figure 1E and 1F) and OHSCs (Figure 1G and 1H) were examined, Ido1-FL expression was increased by IFN γ . We also confirmed the ability of Dex (Figure 1G) and Gal-9 (Figure 1H) to enhance IFN γ -induced Ido1-FL (Brooks et al., 2016b). Excitingly, Desip decreased IFN γ -induced Ido1-FL in PBMCs^{-T} and T cells. Desip also diminished IFN γ , IFN γ +Dex or IFN γ +Gal-9 induced Ido1-FL expression by OHSCs.

Combined, the *in vivo* and *ex vivo* data suggest that the induction and suppression of Ido1-FL in brain and OHSCs is mediated by astrocytes and microglia. These responses are mimicked by PBMCs.

3.2 Ido1-v1

In vivo, Ido1-v1 is well expressed in naïve mouse brain but only mildly (~2-fold) increased by LPS (Brooks et al., 2016a). Ido1-v1 expression was doubled in hippocampus by LPS (Figure 2A), but unaffected by Dex (not shown). Changes in Ido1-v1 are cell-type specific as LPS and Dex increased Ido1-v1 in astrocytes (Figure 2B) but not microglia (Figure 2C) or PBMCs (Figure 2D). Desip tended to depress basal PBMC Ido1-v1 expression ($p = 0.052$, Figure 2D). Unlike Ido1-FL, Ido1-v1 expression *in vivo* was relatively insensitive to Desip.

Ex vivo, IFN γ treatment did not significantly induce Ido1-v1 in PBMC^{-T} or T cells (Figure 2E and 2F), however Desip decreased basal Ido1-v1 expression in both cell populations. In OHSCs, IFN γ -induced Ido1-v1 (Figure 2G and 2H) was accentuated by Dex (Figure 2G).

As reported previously (Brooks et al., 2016b), Gal-9 did not alter Ido1-v1 (Figure 2H). In all cases, IFN γ -dependent Ido1-v1 expression by OHSCs was decreased by Desip.

Combined, *in vivo* and *ex vivo* data suggest that the induction and suppression of Ido1-v1 in OHSCs is NOT driven by astrocytes and microglia. PBMCs are unique in that Desip inhibits basal Ido1-v1 expression.

3.3 Ido2-v1

In vivo, Ido2-FL is undetectable and not inducible in hippocampus (Brooks et al., 2016a; Brooks et al., 2016b) and OHSCs (Brooks et al., 2016b). Thus, we quantified other well-expressed transcripts: Ido2-v1 and Ido2-v3. LPS (and Dex, not shown) did not increase Ido2-v1 expression in hippocampus (Figure 3A), however Ido2-v1 induction was seen in astrocytes (Figure 3B), microglia (Figure 3C) and PBMCs (Figure 3D). Desip did not affect astrocyte Ido2-v1. Desip accentuated LPS-induced Ido2-v1 in microglia but attenuated induction in PBMCs. Thus, hippocampal Ido2-v1 expression did not mimic changes seen in either astrocytes or microglia, suggesting that the induction seen in these cell types may be offset by other cell populations.

Ex vivo, Ido2-v1 expression was numerically higher in PBMC^{-T} ($p=0.08$) and significantly higher in T cells treated with IFN γ . Independent of this, Desip significantly decreased Ido2-v1 in both cell populations (Figure 3E and 3F). IFN γ also increased Ido2-v1 expression in OHSCs, an effect unaltered by Dex (Figure 3G) but accentuated by Gal-9 (Figure 3H). The induction of Ido2-v1 by Gal-9 was blocked by Desip to levels seen with IFN γ alone.

Combined the *in vivo* and *ex vivo* data suggest that induction and suppression of Ido2-v1 is cell-type specific. For the first time, Desip induced a DO transcript (microglial Ido2-v1), but in other cells Desip was either inactive (astrocytes or IFN γ +Dex OHSCs) or inhibitory (PBMC and IFN γ +Gal-9 OHSCs).

3.4 Ido2-v3

In vivo, LPS (and Dex, not shown) did not increase Ido2-v3 expression in hippocampi (Figure 4A), however, Ido2-v3 was increased by Dex in astrocytes (Figure 4B) and by LPS in both microglia (Figure 4C) and PBMCs (Figure 4D), similar to Ido2-v1 regulation. Unexpectedly, Desip increased Ido2-v3 expression slightly in astrocytes and more so in LPS-treated microglia. The induction seen in astrocytes and microglia was not evident in hippocampus, as these cells are not the major source of this transcript. Thus, the small change in astrocytes and even the 20-fold increase in microglia represent a small portion of total brain Ido2-v3. Ido2-v3 expression by PBMCs was decreased by Desip, independent of LPS or Dex.

Ex vivo, although unchanged by IFN γ , the inhibitory effect of Desip on PBMCs *in vivo* was also seen with PBMCs^{-T} and T cells (Figure 4E and 4F). In contrast, IFN γ increased Ido2-v3 expression in OHSCs (Figure 4G and 4H). Neither Dex nor Gal-9 interacted to accentuate Ido2-v3 expression, but Desip diminished the ability of IFN γ to induce Ido2-v3.

Combined, *in vivo* and *ex vivo* data suggest that the induction and suppression of Ido2-v3 is again cell-type specific. Desip induced Ido2-v3 in astrocytes and microglia, but Desip was inhibitory in PBMCs and OHSCs.

3.5 IFN γ

In vivo, previous work suggested that Desip is anti-inflammatory; for example decreasing IFN γ production by PBMCs (Diamond et al., 2006; Rink et al., 2010). As IFN γ is a potent inducer of Ido1 (Hughes et al., 2016) and Ido2 (Fatokun et al., 2013), we quantified IFN γ expression to determine if Desip decreases IFN γ to control DO expression. IFN γ expression was very low in astrocytes (and not induced, not shown) and not detected in OHSCs (not shown). IFN γ expression was increased by LPS in hippocampi (Figure 5A), microglia (Figure 5B) and PBMCs (Figure 5C). Hippocampal and microglial expression was attenuated by Desip. These data suggest that diminished Ido1 and Ido2 expression *in vivo* involves decreased IFN γ . We confirmed this by showing that LPS-induced plasma IFN γ protein was strikingly diminished by Desip (Figure 5C inset).

Ex vivo, IFN γ expression was decreased by Desip in PBMCs^{-T} (Figure 5E) but not T cells (Figure 5F, albeit T cell expression is ~7.6-fold higher than PBMCs, $p < 0.05$). Thus, *in vivo* Desip-dependent IFN γ repression may not occur in T cells per se. As T cells represent ~75% of PBMCs, their stable expression is mimicked by stable PBMC expression *in vivo*. IFN γ expression was not detected in OHSCs. This finding has a critical implication: Desip diminishes DO expression *ex vivo* by directly diminishing activity of IFN γ .

TNF α , IL-1 β and IL-6 expression were also quantified. Expression by PBMCs, microglia and astrocytes did not change in response to Desip. In contrast, there was a slight but significant increase in TNF α and IL-6 by OHSC's in response to Desip (data not shown). Thus, Desip does not appear to alter DO expression via diminished pro-inflammatory cytokine expression.

3.6 Human PBMC's

Human PBMC's were treated with IFN γ with or without Desip to confirm that the finding extended beyond rodent models. As for the mouse, there are multiple human IDO1 transcripts, we measured two IDO1 transcripts (IDO1-001 and IDO1-005) and one IDO2 transcript (IDO2-201); all three transcripts encode IDO proteins. IFN γ increased expression of IDO1-001 (Figure 6A) and IDO1-005 (Figure 6B). Desip decreased IFN γ -induced IDO1-001 expression while IDO1-005 was numerically lower but statistically unaffected by Desip. IFN γ also increased IDO2-201 expression (Figure 6C), an effect blocked by Desip.

4. Discussion

Elevated DO activity (*Kynurenine Pathway* activation) is linked to both human depression and murine depression-like symptomology (Baranyi et al., 2015; Murakami et al., 2016; O'Connor et al., 2009b; O'Connor et al., 2009c). Although the *Kynurenine Pathway* is associated with depression, there has been no work suggesting that anti-depressant activity involves modulation of the *Kynurenine Pathway*. With the incidence of depression rising, discovering mechanisms associated with successful anti-depressant therapy is vital. The

current study determined that the anti-depressant, Desip, was able to decrease DO and IFN γ expression in the brain (hippocampus, microglia and astrocytes) and the periphery (PBMCs). Since IFN γ is the most potent stimulator of Ido1 and Ido2 expression (Fatokun et al., 2013; Hughes et al., 2016), these data suggest that Desip can act by limiting IFN γ production, and thus, diminishing IFN γ -dependent Ido1 and Ido2 expression. However, our *ex vivo* work confirmed that Desip was able to directly decrease IFN γ -induced Ido1 and Ido2 expression by murine PBMC^{-T}, T cells, OHSCs and human PBMCs. In this scenario, IFN γ levels are set by the experimental conditions, and thus, Desip decreased IFN γ activity. Overall, our data suggest for the first time that Desip acts by decreasing both IFN γ levels and IFN γ activity providing a 2-prong approach to decrease DO expression and thus *Kynurenine Pathway* activation.

We and others have previously shown that Desip or its precursor imipramine block depression-like behaviors of mice (Basu Mallik et al., 2016; Elgarf et al., 2014; Jain et al., 2001; Mello et al., 2013; O'Leary et al., 2007; Park et al., 2011; Platt et al., 2013; Shen et al., 1999; Tomaz et al., 2014), and that Ido1 is necessary for murine depression-like behavior (Lawson et al., 2013; O'Connor et al., 2009b; O'Connor et al., 2009c; Salazar et al., 2012). With the current data, we highlight a new putative mechanism for Desip's anti-depressant activity: decreasing Ido1 and Ido2 expression within the brain and periphery. Determining patient DO-responsiveness to Desip may help to explain the variable effectiveness of this drug for alleviating depression symptoms. We cannot rule out that neurotransmitters play a role in Desip-mediated decrease in DO expression *in vivo*, however our *ex vivo* work support the hypothesis (Branco-de-Almeida et al., 2011) that altered neurotransmitter reuptake is not always necessary for a response to Desip. It has been suggested that anti-depressants have immunomodulatory actions outside of their ability to inhibit neurotransmitter reuptake (Diamond et al., 2006). Thus, it is not unlikely that Desip's ability to alter Ido expression within the brain and periphery is distinct from its reuptake activity. However, confirming that the *in vivo* and *ex vivo* OHSC responses to Desip on IFN γ or Ido do not involve neurotransmitter signaling is outside the scope of the current manuscript.

4.1 Ido1

Ido1-FL is highly inducible and tightly linked to depression-like behavior in rodent models (Kim et al., 2012; O'Connor et al., 2009a; O'Connor et al., 2009c) and provided the most consistent Desip responses in the current work. LPS increased Ido1-FL expression in the hippocampus, astrocytes, microglia and PBMCs; Desip decreased these responses. Although broadly considered a potent enhancer of Ido1 expression *in vivo*, LPS increases the production of multiple factors (cytokines and stress hormones/corticosteroids) which may be involved in inflammation-induced behaviors (Dantzer et al., 2008; Dantzer et al., 1999). Corticosteroid levels are also acutely elevated following LPS administration (Stinnett et al., 2015; Sulakhiya et al., 2016). We recently reported that the synthetic glucocorticoid-receptor agonist Dex enhanced the ability of IFN γ to induce Ido1-FL (Brooks et al., 2016b), suggesting that stress and inflammation work together to elevate central Ido1. We now show that not only can Desip suppress the ability of IFN γ to induce Ido1, Desip can block the synergistic actions of Dex and IFN γ . Thus, Desip may suppress inflammation and stress components of Ido system. Similarly we recently reported that LPS increases the expression

of galectins within the mouse brain (Brooks et al., 2016a). The galectins are a group of lectins that have been implicated in several central diseases and neurological disorders (John and Mishra, 2016; Liu and Rabinovich, 2010; Lutowski et al., 1997; Stancic et al., 2011; Wang et al., 2015b). Like, corticosteroids, Gal-9 interacted with IFN γ to accentuate Ido1-FL expression (Brooks et al., 2016a). Again, the ability of Gal-9 to enhance Ido induction was suppressed by Desip.

Ido1-v1 expression was only minimally increased by LPS. In the hippocampus, the magnitude of Ido1-FL induction by LPS was ~500-fold versus ~2-fold for Ido1-v1 indicating that Ido1-FL is far more responsive to peripheral inflammation. Astrocytes respond to LPS with elevated Ido1-FL and Ido1-v1, whereas microglia respond but only with enhanced Ido1-FL expression. These data confirm previous work that murine microglial and astrocyte Ido1 expression can be similarly induced by IFN γ (Kwidzinski et al., 2005; Kwidzinski et al., 2003), but extend those findings to show that this is true when quantifying Ido1-FL. We also found that astrocytes had increased Ido1-v1 expression in response to Dex suggesting that astrocytes play a unique role in the overall increase of Ido1 associated with both inflammation- and stress-induced depression-like behaviors. These data challenge the dogma that inflammation induced depression-like behaviors are solely driven by microglial Ido1-FL induction (Corona et al., 2013; O'Connor et al., 2009c).

Astrocytes generate the downstream kynurenine metabolite (kynurenic acid, KynA), a glutamate and acetylcholine receptor antagonist (Albuquerque and Schwarcz, 2013; Guillemín et al., 2001; Hilmas et al., 2001). KynA antagonizes the action of quinolinic acid and 3-hydroxykynurenine (glutamate receptor agonists), kynurenine metabolites from microglia (Guillemín, 2012; Guillemín et al., 2001; Guillemín et al., 2005; Steiner et al., 2011). Thus, astrocyte and microglia responses to inflammation lead to elevated levels of neurotransmitter antagonists and agonists (Guillemín, 2012; Guillemín et al., 2001; Guillemín et al., 2005; Steiner et al., 2011), resulting in what we have termed the 'Confused Brain' (Lawson et al., 2016). Altered microglial and astrocyte density and pathology associated with depression reflect this imbalance (Cobb et al., 2016; Sanacora and Banasr, 2013; Steiner et al., 2011; Yirmiya et al., 2015). Both astrocyte and microglial Ido1-FL expression appear to be sensitive to antidepressant treatment (Czeh and Di Benedetto, 2013; Duseja et al., 2015), indicating that both aspects of this 'confusion' are alleviated by Desip.

Possibly the most interesting aspect of these data is the PBMC response. Numerous studies have suggested that Ido1 in dendritic (Munn et al., 2002) and NK cells (Kai et al., 2004) produce Kyn to modulate the peripheral immune system (Lippens et al., 2016; Maldonado and von Andrian, 2010; Munn et al., 2002; Murakami et al., 2013). Clinical studies suggest a link between this response and depression (Baranyi et al., 2015; Murakami et al., 2016), connecting peripheral DO activity and MDD. Since Ido1-FL expression by PBMCs was increased in response to LPS (*in vivo*) and IFN γ (*ex vivo*) and blocked by Desip, our work clearly shows that Desip diminishes both CNS and peripheral DO expression.

4.2 Ido2

The primary source of brain Ido2 is neurons (Fukunaga et al., 2012). As neither Ido2-v1 nor Ido2-v3 expression in the hippocampus (an area rich with neurons) was increased by LPS,

there was no response for Desip to inhibit. This suggests that because of low *in vivo* basal expression, the LPS-induced changes in Ido2-v1 within astrocytes and Ido2-v3 in both astrocytes and microglia were insufficient to impact total hippocampal Ido2. These data suggest that neuronal Ido2 is unaltered by LPS or Desip. LPS-increased Ido2 expression by microglia (Ido2-v1 and Ido2-v3). The effect of LPS on microglia was amplified by Desip. This synergy was not found with astrocytes, PMBCs or OHSCs. These data suggest a cell-specific response. At this point, relatively little is known about Ido2 function, and thus, we can only speculate as to the significance of this synergy. However, Ido2 has a role as a negative regulator of Ido1 activity. Ido2 competes with Ido1 for heme-binding. In doing so, Ido2 (with its lower enzymatic activity) effectively diminishes Kyn production (Lee et al., 2014). Confirming such an Ido1-inhibitory effect by Ido2-induction in microglia (which produce QuinA) but not in astrocytes (which produce KynA), would support a Desip-induced balance from QuinA (neurotoxic) to KynA (neuroprotective) within the brain. Such an action supports our theory that Desip alters Kyn impact via its ability to control DO expression. In contrast, addition of IFN γ to OHSCs is sufficient to elicit a direct and detectable Ido2-inducing response. IFN γ induction of Ido2-v1 is resistant, but Ido2-v3 induction is inhibited by Desip. However, the synergy between IFN γ and Gal-9 on Ido2-v1 expression is blocked by Desip; albeit by undefined cell types. Interpreting the physiological impact of these Ido2 responses will require additional studies, but clearly indicate a complex pattern of regulation.

In the periphery, LPS increased Ido2-v1 and Ido2-v3 expression by PMBCs, responses that were attenuated by Desip. The inhibitory effect of Desip was found with PMBC^T and T cells. Our data indicate that specific cell types need to be further studied to obtain an accurate view of Ido2 regulation in response to inflammation (Brooks et al., 2016a), stress (Brooks et al., 2016b) or anti-depressants. These data further support our conclusion that brain and peripheral Ido1 as well as peripheral Ido2 expression are modulated by the anti-depressant Desip.

4.3 IFN γ

LPS increased IFN γ expression in hippocampi, microglia and PMBCs. These effects were decreased by Desip, confirming previous reports of the anti-inflammatory potential of anti-depressants (Diamond et al., 2006; Obuchowicz et al., 2014; Rink et al., 2010; Su et al., 2015). In contrast, OHSCs did not express IFN γ clearly indicating that Desip was not inhibiting DO expression via diminished IFN γ production *ex vivo*. The *in vivo* results suggest that neuroinflammation and concurrent elevation of DOs may be tempered by Desip partly via tempering IFN γ expression (Diamond et al., 2006; Rink et al., 2010). However, the OHSC results strongly suggest that in the absence of endogenous IFN γ , Desip is able to directly block the ability of added IFN γ to drive DO expression; the mechanism by which Desip attenuates DO expression is currently unknown, but under investigation.

As OHSCs do not express IFN γ , it is clear that Desip is not acting *ex vivo* by suppressing endogenous IFN γ production. However, *in vivo*, Desip could suppress Ido1 induction by altering IFN γ access to the brain. We found that circulating IFN γ protein levels are increased by LPS and diminished by Desip. IFN γ enters the brain parenchyma from the

circulation via a saturable transport system (Pan et al., 1997). By decreasing circulating IFN γ , Desip should diminish brain IFN γ levels and thus Ido1 induction. IFN γ can also be released directly into the brain by PMBCs that have infiltrated the brain. Neutrophils and macrophages are capable of producing IFN γ and both infiltrate the brain following peripheral LPS injection (Carvalho et al., 2016; Jeong et al., 2010). By diminishing IFN γ expression by PMBCs, Desip can diminish IFN γ -production by PMBCs within the brain and thus reduce Ido induction.

4.4 PBMCs

Multiple cell types (monocytes, dendritic cells, NK cells and B cells) respond to IFN γ by increasing Ido1 expression (Braidley et al., 2016; Jones et al., 2015; Kai et al., 2004; Tattavin et al., 2010). Thus, it is likely that several cell types in the human and murine PMBC responded to IFN γ . At this point it is unclear which cell type(s) was blocked by Desip. However, due to the near complete inhibition of Ido1 expression, it's clear that most cells were responding to Desip. To the best of our knowledge, this is the first report that T cells respond to IFN γ with elevated DO expression, which was diminished by Desip, similar to PMBC-Ts. Similar to murine PMBC's, IFN γ increased IDO-001 and IDO2-201 expression in human PMBCs, effects completely blocked by Desip. These data indicate that it may be possible to use DO regulation by an easily obtainable sample (PMBCs) to characterize anti-depressant responsiveness of patients.

4.5 In conclusion

The inhibitory effect of Desip on gene expression is not a general effect on transcription activity. In fact, Desip increased Ido2 expression by microglia. More importantly, Desip did not decrease a number of genes in any of our murine models (examples: Haa0, Kat2, Maoa, Qprt, Tdo2, IL-6 and Ccl5/RANTES). Desip decreased LPS- and IFN γ -induced expression of Ido1 and Ido2 in peripheral cells (PMBCs, PMBCs-T and T cells), resident brain cells (astrocytes and microglia), hippocampi and OHSCs; albeit increasing microglial Ido2. As elevated DO activity is involved in both human and mouse depression-like behaviors (Baranyi et al., 2015; Murakami et al., 2016; O'Connor et al., 2009b; O'Connor et al., 2009c), Desip's ability to block DO expression suggests an additional activity for this anti-depressant drug (Figure 7). Our data suggest that regulation of DO expression, in particular Ido1-FL, in brain and PMBCs are similarly responsive to this anti-depressant. These pioneering data offer insight into both peripheral and brain regulation of Ido1 and Ido2 during inflammation- or stressed-induced depression. We propose that monitoring anti-depressant responsiveness by assessing DO regulation in a clinically relevant sample, e.g. PMBCs, may be suitable for determining drug responsiveness on a case-by-case basis.

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Highlights

- Desipramine decreased indoleamine-2,3-dioxygenase 1 and 2 expression in brain and periphery, identifying a novel mechanism by which anti-depressants may attenuate depression
- IFN γ -induced Ido1 and Ido2 are decreased by Desip in both human and murine peripheral blood mononuclear cells, indicating translatability of our murine models
- Ido expression and regulation may be a useful biomarker for evaluation of anti-depressant responsiveness of depressed patients

Figure 1. Desip decreased Idol1-FL expression

After i.p. treatment of mice with either LPS or Dex (*in vivo*, **A–D**) or cells/brain tissue with IFN γ (*ex vivo*, **E–H**) with and without Desip, expression of Idol1-FL was quantified in **A**) Hippocampi, **B**) Astrocytes (ACSA-2⁺ enriched cells), **C**) Microglia (CD11b⁺ enriched cells), **D**) PBMCs, **E**) PBMC^T, **F**) T cells (CD90⁺ enriched cells), **G**) OHSCs $\pm$ Dex and **H**) OHSC $\pm$ Gal-9. * $p < 0.05$ for Idol1-FL induction by LPS or IFN γ ; $\delta p < 0.05$ for the inhibitory effect of Desip within LPS or IFN γ ; $\psi p < 0.05$ for the ability of Dex or Gal-9 to synergistically enhance IFN γ -stimulated gene expression.

Figure 2. Attenuation of Ido1-v1 expression by Desip

After treatment of mice i.p. with either LPS or Dex (*in vivo*, **A–D**) or cells/tissue with IFN γ (*ex vivo*, **E–H**) with and without Desip, expression of Ido1-v1 was quantified in **A**) Hippocampi, **B**) Astrocytes (ACSA-2⁺ enriched cells), **C**) Microglia (CD11b⁺ enriched cells), **D**) PBMCs, **E**) PBMC^T, **F**) T cells (CD90⁺ enriched cells), **G**) OHSCs $\pm$ Dex and **H**) OHSC $\pm$ Gal-9. * $p < 0.05$ for Ido1-v1 induction by LPS, Dex or IFN γ ; δ $p < 0.05$ for the inhibitory effect of Desip within LPS or IFN γ ; ψ $p < 0.05$ for the ability of Dex or Gal-9 to synergistically enhance IFN γ -stimulated gene expression; ϕ $p < 0.05$ main effect of Desip.

Figure 3. Attenuation of Ido2-v1 expression by Desip

After treatment of mice i.p. with either LPS or Dex (*in vivo*, **A–D**) or cells/tissue with IFN γ (*ex vivo*, **E–H**) with and without Desip, expression of Ido2-v1 was quantified in **A**) Hippocampi, **B**) Astrocytes (ACSA-2⁺ cells), **C**) Microglia (CD11b⁺ enriched cells), **D**) PBMCs, **E**) PBMC^T, **F**) T enriched cells (CD90⁺ enriched cells), **G**) OHSCs $\pm$ Dex and **H**) OHSC $\pm$ Gal-9. * $p < 0.05$ for Ido2-v1 induction by LPS or IFN γ ; δ $p < 0.05$ for the effect of Desip within LPS or IFN γ ; ψ $p < 0.05$ for the ability of Dex or Gal-9 to synergistically enhance IFN γ -stimulated gene expression.

Figure 4. Attenuation of Ido2-v3 expression by Desip

After treatment of mice i.p. with either LPS or Dex (*in vivo*, **A–D**) or cells/tissue with IFN γ (*ex vivo*, **E–H**) with and without Desip, expression of Ido2-v3 was quantified in **A**) Hippocampi, **B**) Astrocytes (ACSA-2⁺ enriched cells), **C**) Microglia (CD11b⁺ enriched cells), **D**) PBMCs, **E**) PBMC^T, **F**) T cells (CD90⁺ enriched cells), **G**) OHSCs $\pm$ Dex and **H**) OHSC $\pm$ Gal-9. * $p < 0.05$ for Ido2-v3 induction by LPS, Dex or IFN γ ; δ $p < 0.05$ for the inhibitory effect of Desip within LPS or IFN γ ; ϕ $p < 0.05$ main effect of Desip.

Figure 5. Attenuation of IFN γ expression by Desip

After treatment of mice i.p. with either LPS or Dex (*in vivo*, **A, B and C**) or cells with IFN γ (*ex vivo*, **D and E**) with and without Desip, expression of IFN γ was quantified in **A**) Hippocampi, **B**) Microglia (CD11b⁺ enriched cells), **C**) PBMCs, **D**) PBMC^T, and **E**) T cells (CD90⁺ enriched cells). * $p < 0.05$ for IFN γ induction by LPS; δ $p < 0.05$ for the inhibitory effect of Desip within LPS; ϕ $p < 0.05$ main effect of Desip.

Figure 6. Desip decreased IDO1 and IDO2 expression by human PBMCs

After human PBMCs were treated with IFN γ with or without Desip, expression of **A)** IDO1-001, **B)** IDO1-005 and **C)** IDO2-201 was quantified. * $p < 0.05$ for induction by IFN γ ; δ $p < 0.05$ for the inhibitory effect of Desip within IFN γ .

Figure 7. Proposed model for Desip blocking Kynurenine Pathway activation in brain and periphery

Multiple studies have linked inflammation and stress with major depression in humans and depressive-like behaviors in rodent models. Inflammation or stress increase peripheral pro-inflammatory cytokine release (ex. IFN γ) into the circulation, which increases IDO expression by PBMCs and most tissues. Either peripheral IFN γ or infiltrating immune cells capable of producing IFN γ enter the brain to increase IDO expression by both microglia and astrocytes. We've extended this area of research by confirming that Desip can act *in vivo* by 1) blocking peripheral IFN γ expression as a mechanism to decrease IDO expression by PBMCs, microglia and astrocytes. 2) *Ex vivo*, IFN γ increases IDO expression by PBMCs (both human and mouse) and synergizes with multiple factors (i.e. Dex or Gal-9) to increase the DOs within the brain. As such, we now show that Desip is able to directly block IFN γ -dependent IDO induction in both peripheral cells (human and murine PBMCs) and within OHSCs. The later findings suggest that 3) Desip would also act to directly block DO expression in the periphery and brain by attenuating IFN γ activity. *Kynurenine Pathway* activation (via the DOs) results in production of additional downstream kynurenine metabolites (kynurenines: quinolinic acid: QuinA and kynurenic acid: KynA) that directly alter behavior and immune activity. Thus, Desip is able to modulate DO-dependent behavior and immune responses indirectly by regulating IFN γ expression and directly by blocking IFN γ activity, both in the brain and periphery.