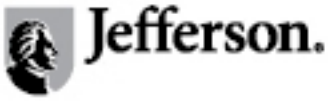

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IDO1 is an Integral Mediator of Inflammatory Neovascularization.

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Research Paper

IDO1 is an Integral Mediator of Inflammatory Neovascularization

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ABSTRACT

The immune tolerogenic effects of IDO1 (indoleamine 2,3-dioxygenase 1) have been well documented and genetic studies in mice have clearly established the significance of IDO1 in tumor promotion. Dichotomously, the primary inducer of IDO1, the inflammatory cytokine IFNγ (interferon-γ), is a key mediator of immune-based tumor suppression. One means by which IFNγ can exert an anti-cancer effect is by decreasing tumor neovascularization. We speculated that IDO1 might contribute to cancer promotion by countering this anti-neovascular effect of IFNγ, possibly through IDO1-potentiated elevation of the pro-tumorigenic inflammatory cytokine IL6 (interleukin-6). In this study, we investigated how genetic loss of IDO1 affects neovascularization in mouse models of oxygen-induced retinopathy and lung metastasis. Neovascularization in both models was significantly reduced in mice lacking IDO1, was similarly reduced with loss of IL6, and was restored in both cases by concomitant loss of IFNγ. Likewise, the lack of IDO1 or IL6 resulted in reduced metastatic tumor burden and increased survival, which the concomitant loss of IFNγ abrogated. This insight into IDO1's involvement in pro-tumorigenic inflammatory neovascularization may have important ramifications for IDO1 inhibitor development, not only in cancer where clinical trials are currently ongoing, but in other disease indications associated with neovascularization as well.

1. Introduction

Chronic inflammation provides a microenvironmental context which can foster tumor promotion through a complex and dynamic interplay between stroma and tumor that remains an area of active investigation (Peek et al., 2005). In an experimental model of two stage chemical carcinogenesis, wherein mutagenic tumor initiation and inflammatory tumor promotion are distinctly separable, IDO1 (indoleamine 2,3-dioxygenase 1) has been identified as a vital component of the inflammatory tumor promoting milieu (Muller et al., 2008). Enzymatically, IDO1 catabolizes the essential amino acid tryptophan, but it is not the enzyme responsible for maintaining normal tryptophan homeostasis, which instead is the role of the evolutionarily convergent liver enzyme TDO2 (tryptophan dioxygenase 2). Rather, IDO1 can be expressed in a variety of tissues, particularly along mucosal surfaces, and is strongly induced by the inflammatory cytokine IFNγ (interferon-γ) (Taylor and Feng, 1991). The conceptualization of IDO1 as a regulator of immune function emerged from observations that tryptophan depletion by IDO1 could suppress cytotoxic T cell activation (Munn et al., 1999). The demonstration that an IDO1 pathway inhibitor 1MT (1-methyl-tryptophan) could elicit T cell-dependent rejection of allogeneic mouse concepti (Munn et al., 1998) dramatically cemented the concept of IDO1 as a tolerogenic actor. Subsequent findings linking loss of the tumor suppressor gene *Bin1* to IDO1 dysregulation and tumoral immune escape (Muller et al., 2005) provided experimental substantiation for the corollary proposition that tumors might appropriate this mechanism for protecting the 'foreign' fetus to overcome tumor immunosurveillance. Additionally, while IDO1 can contribute to tumor development when expressed directly within tumor cells, (where immunoediting may be at play), its expression within non-malignant stroma has been shown to be contributory to tumor development as well (Munn et al., 2004).

A relatively high basal level of IDO1 is expressed in the lungs, and we previously determined that IDO1 loss was associated with markedly reduced pulmonary tumor burden and increased survival both in a transgenic mouse model of autochthonous, mutant KRAS-driven lung carcinoma and in an orthotopic graft model of metastatic breast cancer (Smith et al., 2012). Unexpectedly, a significant reduction in normal lung vascularization was also associated with the loss of IDO1,

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suggesting a possible role for IDO1 in supporting blood vessel formation (Smith et al., 2012). Neovascularization is critical for tumor outgrowth (Cao et al., 2011). However, unlike the tightly regulated process of normal tissue vascularization, tumor neovascularization is characterized by excessive and disorganized growth of blood vessels much like that induced by ischemia in tissues such as the retina and lungs (Carmeliet, 2003). One important check on tumor neovascularization is the inflammatory cytokine IFNγ. Indeed, the loss of tumor vasculature elicited by IFNγ, rather than direct tumor cell killing, has been implicated as being the primary mechanism for both $CD4^+$ and $CD8^+$ T cell-mediated tumor rejection (Qin and Blankenstein, 2000, Qin et al., 2003). However, IFNγ also drives IDO1, leading us to predict that IDO1 may act in a negative feedback capacity to dampen the tumor suppressive, anti-neovascular effects of IFNγ. A possible mechanistic explanation is suggested by our recent determination that IDO1 can potentiate the induction of another inflammatory cytokine IL6 (interleukin 6) (Smith et al., 2012), which has been implicated as a pro-neovascular factor for tumors (Middleton et al., 2014). In this report, we present evidence that IDO1 does indeed have a critical role in supporting neovascularization that corresponds with its integration at the interface between these two competing inflammatory cytokines, IFNγ and IL6.

2. Materials and Methods

2.1. Transgenic Mouse Strains

Congenic *Ido1*$^{-/-}$ mice on both BALB/c and C57BL/6 strain backgrounds were provided by A. Mellor (GHSU). The development of congenic *Ido2*$^{-/-}$ mice on the BALB/c strain background has been described previously (Metz et al., 2014). Congenic *Ifng*$^{-/-}$ and *Il6*$^{-/-}$ mouse strains on the BALB/c strain background and WT BALB/c and C57BL/6 strains were acquired from the Jackson Laboratory. All studies involving mice were approved by the Lankenau Institute for Medical Research IACUC and conform with AAALAC guidelines.

2.2. 4T1 Tumor Cell Metastasis

Pulmonary metastasis studies with the 4T1 mouse mammary carcinoma-derived cell line (Aslakson and Miller, 1992) were carried out by injecting 1×10^4 cells in 50 μl serum free medium into the number 4 mammary fatpad to establish an orthotopic tumor which then spontaneously metastasized to the lungs. For visualization of the pulmonary metastasis burden, lungs were inflated with 15% India ink dye, washed, and bleached in Fekete's solution. For immunofluorescence detection of pulmonary metastasis vasculature, lungs were inflated with 50% OCT and frozen in OCT blocks followed by 4 μm sectioning using the CryoJane tape transfer system. Blood vessels within the metastatic nodules were visualized by fluorescence staining with rabbit anti-Caveolin1 polyclonal antibody (Cell Signaling, Cat. #3238S). For quantitatively evaluating vessel density within the metastatic nodules, multiple fields were acquired per mouse lung on a Zeiss inverted microscope with 40× objective. The pixel density corresponding to the positive Cav1 signal per field was determined in Adobe Photoshop, and these values were then averaged to determine the overall mean value per mouse. For IDO1 inhibitor treatment studies, mice bearing established metastases at 3.5 weeks following 4T1 engraftment were administered 50 mg/kg epacadostat (ChemieTek) in 100 μl vehicle (3% *N*,*N*-dimethylacetamide, 10% 2-hydroxylpropyl-β-cyclodextrin) by oral gavage b.i.d. over 72 h at which point the animals were euthanized for analysis. Positive control animals were administered a single i.p. injection of 50 mg/kg cyclophosphamide (Baxter) in 100 μl sterile saline and euthanized 72 h later for analysis.

2.3. Oxygen-induced Retinopathy

Neovascularization studies in the OIR model were carried out as described (Connor et al., 2009). Neonatal mice were housed in an OxyCycler chamber set to 75% oxygen from day 7 to day 12 postpartum (P7-P12). At P12, the neonates were removed from the chamber and subsequently maintained under normoxic conditions until P17. To evaluate the retinal vasculature, the eyes were fixed in 4% paraformaldehyde and methanol. Retinas were then isolated under a dissecting microscope, stained with Isolectin B4-Alexa488, and flatmounted for fluorescence microscopy. Overlapping images were obtained on a Zeiss inverted microscope with 2.5× objective. Composite images were assembled and quantitative analysis of neovascularization was performed using Adobe Photoshop as described (Connor et al., 2009). For IDO1 inhibitor treatment studies, neonates were administered 50 mg/kg epacadostat in 50 μl (3% *N*,*N*-dimethylacetamide, 10% 2-hydroxylpropyl-β-cyclodextrin) by oral gavage b.i.d. from P12-P17 at which point the animals were euthanized for analysis. Positive control animals were administered i.p. injections of 50 mg/kg ethyl pyruvate (Aldrich) in modified Ringer's solution (130 mM sodium, 4 mM potassium, 2.7 mM calcium, 139 mM chloride) in a total volume of 50 μl b.i.d. from P12–P17.

2.4. Intravitreal Injections

For siRNA treatments (siIdo1, siVegfa Acell smart pools; GE Healthcare, Dharmacon), a single injection of 1 μg siRNA in 1 μl PBS was administered at P14, with the targeted siRNA injected into one eye and the non-targeted control siRNA injected into the contralateral eye. Prior to injection, the topical anesthetic Alcaine (0.5% proparacaine hydrochloride ophthalmic solution) was applied to the eyelids. Eyes were injected under a dissecting microscope through the cornea into the vitreous humor using a 33 gauge needle attached to a 2.5 μl glass syringe. All studies were concluded at P17 for evaluation of the impact on retinal neovascularization as described above.

2.5. Statistical Analysis

Graphing and statistical analysis was performed using Prism 6 (GraphPad Software, Inc.). Bar graphs were plotted as means ± SEM with statistical significance determined, as appropriate, by unpaired, 2-tailed Student's *t*-test (except for siRNA studies that were evaluated by paired *t*-test) or by one-way ANOVA with Tukey's multiple comparison test or Dunnett's multiple comparison test. Significance for Kaplan-Meier survival curves was determined by 2-group log-rank test. *P* value ranges are indicated as follows: ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

3. Results

3.1. IDO1 Loss Results in Reduced Neovascularization

Ido1$^{-/-}$ (*Ido1* nullizygous) mice challenged with orthotopically engrafted 4T1 mammary adenocarcinoma tumors exhibited a marked delay in pulmonary metastasis development relative to their WT (wild type) counterparts (Fig. S1a), consistent with previously published results (Smith et al., 2012). When metastases did form in *Ido1*$^{-/-}$ hosts, they were observed to have a lower vascular density than those formed in WT animals (Fig. 1a), the significance of which was confirmed by quantitative analysis (Fig. 1b; $\Delta = 2.6$-fold, $P < 0.01$). This finding suggests that neovascularization of the pulmonary 4T1 metastases is impaired in mice lacking IDO1, however, direct comparative analysis is complicated by the pronounced differential in metastatic tumor outgrowth between *Ido1*$^{-/-}$ and WT animals.

To assess the impact of IDO1 loss on neovascularization independent of other possible effects on tumor growth, studies were performed in a mouse model of OIR (oxygen-induced retinopathy). Initially developed for studying retinopathy of prematurity, OIR has been demonstrated to be a reliable and quantifiable system for modeling pathological angiogenesis (Connor et al., 2009), and this experimental model has since been employed more broadly as a surrogate system in studies of

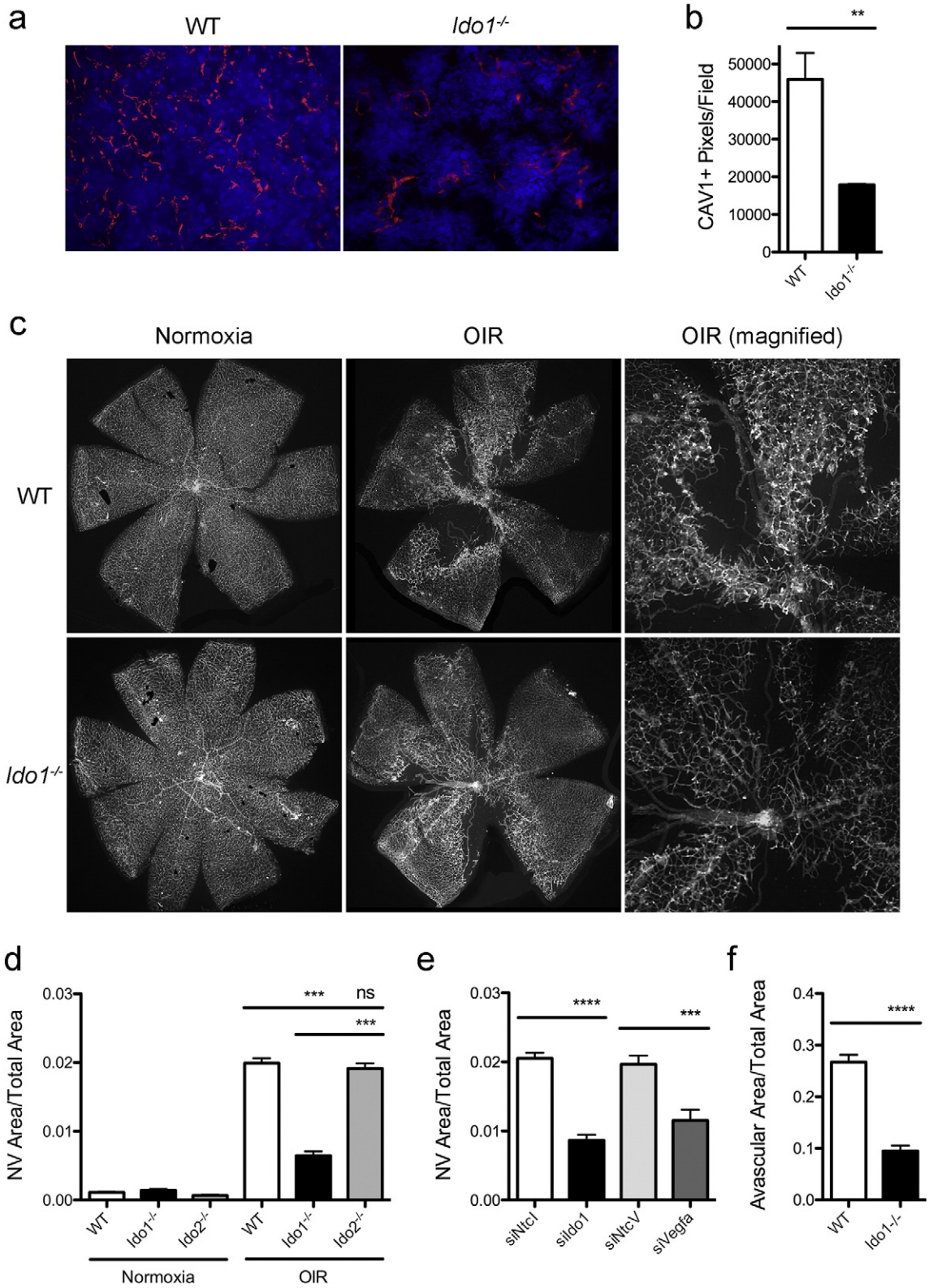

Fig. 1. IDO1 loss is associated with reduced neovascularization in pulmonary metastasis and oxygen-induced retinopathy models. (a) Representative images of immunofluorescent staining of blood vessels with anti-Cav1 (red) and of nuclei with DAPI (blue) within 4T1 lung metastases in WT and *Ido1*$_{-/-}$ mice. (b) Quantitative assessment of neovascular density as marked by anti-Cav1 positive staining within 4T1 lung metastases in WT and *Ido1*$_{-/-}$ mice ($N \geq 3$ mice). Graphed as means ± SEM with significance determined by 2-tailed Student's *t*-test (c) Representative images of B4-Alexa488-isolectin staining of blood vessels in retinal flatmounts at P17 from WT and *Ido1*$_{-/-}$ neonates maintained either under constant normoxia or exposed to hyperoxia from P7-P12 to induce OIR. At far right are higher magnifications to highlight the difference in neovascular tufts between WT and *Ido1*$_{-/-}$ mice. (d) Quantitative assessment at P17 of neovascular area over total retinal area for WT, *Ido1*$_{-/-}$, and *Ido2*$_{-/-}$ OIR cohorts ($N \geq 17$ eyes). Cohorts maintained under constant normoxia are also included for baseline comparison ($N \geq 10$ eyes). Graphed as means ± SEM with significance determined by one-way ANOVA with Tukey's multiple comparison test. (e) Quantitative comparison at P17 of neovascular area over total retinal area between WT OIR-elicited cohorts that received a single intraocular injection at P14 of the indicated siRNAs targeting *Ido1*, *Vegfa*, or the corresponding Ntc (Non-target control) ($N \geq 8$ eyes). Graphed as means ± SEM with significance determined by paired, 2-tailed Student's *t*-test. (f) Quantitative comparison at P17 of avascular area over total retinal area for WT and *Ido1*$_{-/-}$ OIR cohorts ($N \geq 27$ eyes). Graphed as means ± SEM with significance determined by 2-tailed Student's *t*-test.

tumor neovascularization (Palmer et al., 2012, Stahl et al., 2012). To test the hypothesis that IDO1 supports neovascularization, we examined the impact of *Ido1* gene disruption in the OIR model. Representative microscopy images of B4-Alexa488-isolectin stained retinal flat mounts from WT and *Ido1*$^{-/-}$ mice are shown, including higher magnification images specifically focused on the characteristic neovascular tufts (Fig. 1c). From these images it is apparent that the extent of neovascularization is markedly reduced in the *Ido1*$^{-/-}$ mouse retina. Quantitative comparison between the WT and *Ido1*$^{-/-}$ groups, (Fig. 1d), confirmed that a significant decrease in OIR-associated neovascularization was associated with the lack of IDO1 expression ($\Delta = 3.1$-fold, $P < 0.001$). A similar outcome was observed in a different inbred mouse strain background (Fig. S1b). This finding clearly establishes an important role for IDO1 in this tumor-independent model of neovascularization.

To assess the specificity of the requirement for IDO1 in OIR-associated neovascularization, mice lacking the related paralog IDO2 were evaluated. In contrast to mice lacking IDO1, *Ido2*$^{-/-}$ mice exhibited no significant impact on OIR-associated neovascularization (Fig. 1d). To determine whether the diminished retinal neovascularization observed in *Ido1*$^{-/-}$ mice is attributable to a direct effect of IDO1 loss within the context of the eye rather than a systemic developmental deficiency, siRNA targeting the *Ido1* message was delivered by intravitreal injection into one eye paired with non-target control siRNA injected into the contralateral eye. Acute, local targeting *Ido1* expression in this manner produced a significant decrease in neovascularization ($\Delta = 2.4$-fold, $P < 0.0001$) that was comparable to the effect observed with a positive control siRNA directed against *Vegfa* (Fig. 1e).

Additional control experiments were carried out to assess the broader impact of IDO1 loss on normal retinal vascularization. The regrowth of normal retinal vessels that also occurs in OIR can be quantitatively evaluated in whole mount retinas by assessment of the avascular area (Connor et al., 2009). The avascular region was significantly reduced in *Ido1*$^{-/-}$ animals relative to their wild type counterparts ($\Delta = 2.8$-fold, $P < 0.0001$) (Fig. 1f), indicative of an actual improvement in normal vessel regrowth in mice lacking IDO1. In normoxic controls, quantitative assessment of normal vascular density at P17 failed to demonstrate any effect of IDO1 loss (Fig. S1c). Likewise, at P12, when the oxygen concentration is shifted from hyperoxia to normoxia to induce neovascularization, there was no apparent difference between the retinas of WT and *Ido1*$^{-/-}$ mice maintained either under constant normoxia or exposed to hyperoxia (Fig. S2). Taken together, these results are consistent with the loss of IDO1 negatively affecting only the pathologic blood vessel formation responsible for neovascularization.

The genetic data suggest that pharmacologic inhibitors of IDO1 enzymatic activity may have therapeutic potential for reducing pathological neovascularization. To directly test this possibility, a small molecule inhibitor of IDO1, epacadostat, was administered by oral gavage to neonates following OIR elicitation. Relative to vehicle alone, epacadostat treatment resulted in a significant reduction in retinal neovascularization ($\Delta = 1.8$-fold, $P < 0.001$), which was comparable to that achieved with a positive control compound, ethyl pyruvate (Lee et al., 2013) (Fig. 2a,b). Likewise, when epacadostat was administered by oral gavage over a 72 h period to mice with established 4T1 pulmonary metastases, epacadostat treatment resulted in a significant reduction in metastatic tumor neovascularization relative to vehicle alone ($\Delta = 3.2$-fold, $P < 0.01$) that was comparable to the effect of the positive control compound cyclophosphamide (Ibe et al., 2001) (Fig. 2c,d). These results confirm that blocking the enzymatic activity of IDO1 through pharmacologic inhibition is sufficient to interfere with the ability of IDO1 to support neovascularization.

3.2. IFNγ is Required for Reduced Neovascularization and Increased Metastasis Resistance Resulting from IDO1 Loss

The inflammatory cytokine IFNγ is a major inducer of IDO1. There is substantial evidence that IFNγ is critical for effective anti-tumor immunity (Beatty and Paterson, 2001), and IFNγ-elicited reduction of the tumor neovasculature has been proposed as a mechanism of action (Qin and Blankenstein, 2000, Qin et al., 2003). To test the hypothesis that IDO1 acts as a negative feedback check on the anti-neovascular effect of IFNγ, we bred double knockout mice nullizygous for both the *Ifng* and *Ido1* alleles to evaluate in the OIR model. The concomitant loss of IFNγ together with IDO1 mitigated the reduction in OIR-associated neovascularization observed with the loss of IDO1 alone (Fig. 3a,b), such that no significant difference could be detected between the *Ifng*$^{-/-}$ *Ido1*$^{-/-}$ mice and the WT controls (Fig. 3b). The importance of IFNγ to the reduction in OIR-associated neovascularization observed with IDO1 loss was further tested by intravitreal injection of anti-IFNγ neutralizing antibody. *Ido1*$^{-/-}$ mice demonstrated a significant recovery of neovascularization in anti-IFNγ antibody treated eyes compared to the contralateral eyes treated with isotype matched negative control antibody (Fig. S3). Thus both experimental approaches yielded data consistent with the hypothesis that IDO1 supports neovascularization primarily by counteracting the negative impact of IFNγ on this process.

To examine the relevance of these findings from the OIR model in the context of a tumor setting, we examined the impact that loss of both IFNγ and IDO1 in the host animal have on neovascularization in 4T1 pulmonary metastases. Metastases that formed in *Ido1*$^{-/-}$ hosts exhibited a significantly lower vascular density relative to those that formed in the wild type controls, while the concomitant loss of both IFNγ and IDO1 in double knockout mice completely negated the effect on 4T1 metastasis neovascularization of IDO1 loss alone (Fig. 3c,d). We have previously reported that loss of IDO1 provides a significant survival benefit to mice challenged orthotopically with 4T1 tumors due to reduced pulmonary metastasis burden (Smith et al., 2012). In this context, the concomitant loss of IFNγ together with IDO1 in double knockout mice negated both the reduction in pulmonary metastasis burden and the survival benefit observed with the loss of IDO1 alone (Fig. 3e,f). These data demonstrate a striking correspondence between the countervailing effects of IDO1 and IFNγ on neovascularization and metastasis survival, suggesting that the antagonistic effect that IDO1 has on the anti-neovascular activity of IFNγ may be directly linked to its pro-tumorigenic role in this metastasis model.

3.3. IL6 Loss Results in IFNγ-dependent Reduction in Neovascularization and Increase in Metastasis Resistance

Previously we reported that loss of IDO1 was associated with attenuated IL6 induction in the 4T1 metastasis model and that metastasis susceptibility could be restored by provision of exogenous IL6 (Smith et al., 2012). To examine whether IL6 has a corresponding involvement in neovascularization, we examined the impact that genetic loss of IL6 has in OIR. Similar to the loss of IDO1, *Il6*$^{-/-}$ animals exhibited a clear reduction in neovascularization, which was reversed by the concomitant loss of IFNγ to a level that was indistinguishable from that observed with IFNγ loss alone (Fig. 4a,b). IL6 loss similarly affected neovascularization in 4T1 lung metastases, with the metastatic tumors obtained from *Il6*$^{-/-}$ animals exhibiting a significantly reduced vascular density relative to the wild type controls (Fig. 4c,d). Again, the concomitant loss of IFNγ abrogated the reduction in vascular density observed with the loss of IL6 alone (Fig. 4c,d). Loss of IL6 was likewise associated with a clear reduction in pulmonary metastasis burden and increased survival benefit for mice challenged with orthotopic 4T1 tumors, a benefit that was lost with the concomitant loss of IFNγ (Fig. 4e,f). The corresponding similarity of the effects of IL6 loss and IDO1 loss on neovascularization and metastasis susceptibility is consistent with IL6 acting as an important downstream mediator of IDO's effects on these processes.

4. Discussion

This report establishes a clear biological role for IDO1 in supporting pathologic neovascularization and provides evidentiary support for

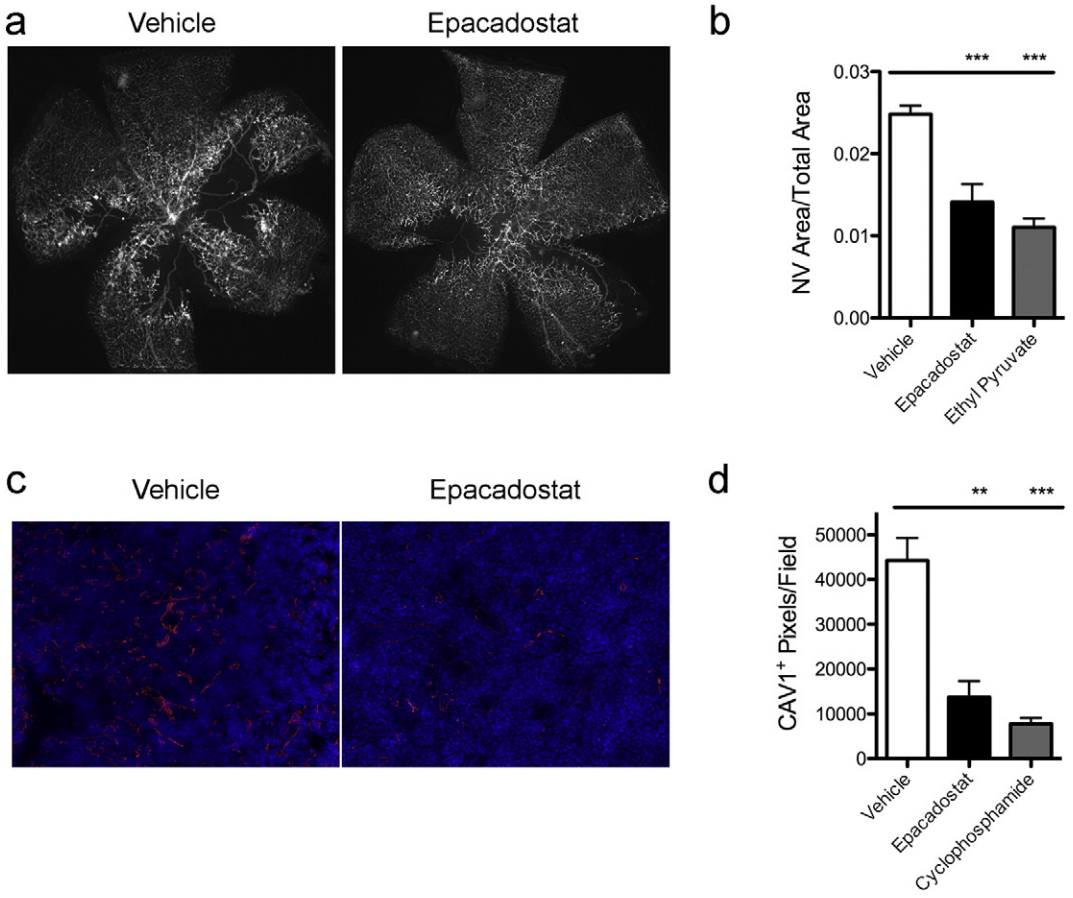

Fig. 2. IDO1 inhibitor treatment negatively affects neovascularization. (a) Representative images of B4-Alexa488-isolectin staining of blood vessels in retinal flatmounts at P17 from WT neonates exposed to hyperoxia from P7-P12 to induce OIR and then dosed p.o. with vehicle or 50 mg/kg epacadostat, b.i.d., from P12-P17. (b) Quantitative assessment at P17 of neovascular area over total retinal area for WT OIR cohorts administered either vehicle or epacadostat together with a third positive control cohort dosed i.p. with 50 mg/kg ethyl pyruvate b.i.d. ($N \geq 8$ eyes). Graphed as means ± SEM with significance determined by one-way ANOVA with Dunnett's multiple comparison test. (c) Representative images of immunofluorescent staining of blood vessels with anti-Cav1 (red) and of nuclei with DAPI (blue) within metastatic regions of lung specimens prepared from WT mice dosed p.o. with vehicle or 50 mg/kg epacadostat, b.i.d., over 3 days beginning 3.5 weeks after orthotopic 4T1 mammary tumor cell engraftment. (d) Quantitative assessment of neovascular density marked by anti-Cav1 positive staining within lung metastases of WT mice administered either vehicle or epacadostat over 3 days together with a third positive control cohort that received a single i.p. injection of 50 mg/kg cyclophosphamide at 3.5 weeks after orthotopic 4T1 mammary tumor cell engraftment for evaluation 3 days later ($N \geq 3$ mice). Graphed as means ± SEM with significance determined by one-way ANOVA with Dunnett's multiple comparison test.

the hypothesis that IDO1 promotes this outcome through its integration at the regulatory interface between two competing inflammatory cytokines, IFNγ and IL6. Neovascularization was substantially reduced with the loss of IDO1, and this effect was completely reversed by the concomitant loss of IFNγ, a primary inducer of IDO1. The loss of IL6, known to exhibit IDO1 dependent expression, likewise resulted in reduced neovascularization that was also determined to be IFNγ dependent. These effects were initially delineated in the context of OIR, a well-established model system for the quantifiable assessment of neovascularization. Direct tumor relevance was corroborated in an orthotopic 4T1 pulmonary metastasis model in which corresponding effects on neovascularization, metastatic tumor burden, and survival were observed.

Although the tolerogenic properties of IDO1 have been extensively explored, the possibility that IDO1 might also impact neovascularization has not, to this point, been generally recognized nor clearly established as biologically relevant. While two previous reports did note evidence of increased vascular density in human xenograft tumors engineered to overexpress exogenous IDO1 (Nonaka et al., 2011, Li et al., 2006) and we observed that normal pulmonary vascularization was unexpectedly diminished in *Ido1* nullizygous mice (Smith et al., 2012), the current study demonstrates, in different disease settings, the importance of endogenous IDO1 for supporting neovascularization, presents a clear correlation between IDO1-dependent neovascularization and the outgrowth of lung metastases, and provides mechanistic evidence to explain the basis for this effect by linking IDO1 to regulatory interactions with the inflammatory cytokines IFNγ and IL6.

While further details of IDO1's involvement in neovascularization remain speculative, the established literature on the cellular and molecular basis of IDO1's role in immune tolerance will almost certainly inform further investigation into the underlying basis for IDO1's role in supporting neovascularization. In addition to being expressed in immune cells, such as dendritic cells and macrophages, IDO1 expression in endothelial cells has also been identified (Blaschitz et al., 2011), and it will be important to selectively delineate where the expression of IDO1 is most relevant to its role in neovascularization. Biochemically, by catabolizing tryptophan, IDO1 can signal through two distinct metabolic pathways, one responding to downstream tryptophan catabolites and the other to the depletion of tryptophan itself. Both mechanisms have been linked to the positive regulation of IL6 (DiNatale et al., 2010, Liu et al., 2014) *via* kynurenine signaling through AHR (aryl hydrocarbon receptor) or amino acid depletion signaling through GCN2 (general nonderepressible 2), but direct relevance to IDO1's role in neovascularization remains to be determined. It is also interesting to speculate on the biological ramifications of these new findings. In the context of pregnancy where conceptual insight into IDO1's physiological relevance was first established (Munn et al., 1998), the endometrial

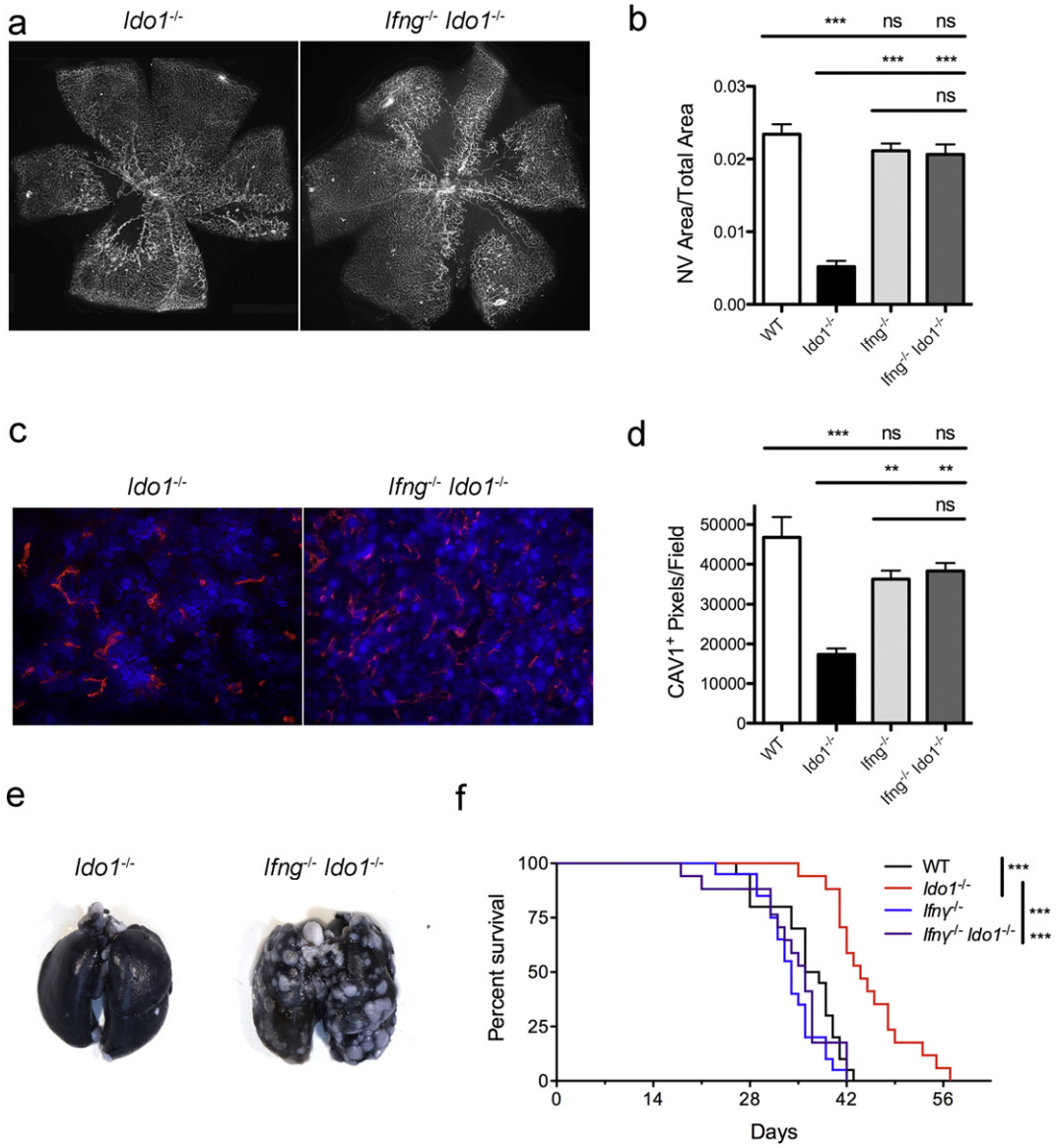

Fig. 3. IFNγ is required for the impaired neovascularization and pulmonary metastasis development that result from IDO1 loss. (a) Representative images of B4-Alexa488-isolectin staining of blood vessels in retinal flatmounts at P17 from $Ifng^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ neonates exposed to hyperoxia from P7-P12 to induce OIR. (b) Quantitative assessment of neovascular area over total retinal area at P17 for WT, $Ido1^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ OIR cohorts ($N \geq 14$ eyes). Graphed as means ± SEM with significance determined by one-way ANOVA with Tukey's multiple comparison test. (c) Representative images of immunofluorescent staining of blood vessels with anti-Cav1 (red) and of nuclei with DAPI (blue) within metastatic regions of lung specimens prepared from $Ido1^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ mice following orthotopic 4T1 mammary tumor cell engraftment. (d) Quantitative assessment of neovascular density marked by anti-Cav1 positive staining within lung metastases of WT, $Ido1^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ mice following orthotopic 4T1 mammary tumor cell engraftment ($N \geq 3$ mice). Graphed as means ± SEM with significance determined by one-way ANOVA with Tukey's multiple comparison test. (e) Staining of lungs with India ink to visualize the metastatic burden in $Ido1^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ mice at 5 weeks following orthotopic 4T1 mammary tumor cell engraftment. (f) Kaplan-Meier survival curves for cohorts of WT, $Ido1^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Ido1^{-/-}$ mice following orthotopic engraftment of 1×10^4 4T1 cells ($N \geq 17$ mice) with significance assessed by 2-group log-rank test.

inflammation that develops in response to blastocyst implantation promotes blood vessel formation as well as immune tolerance, both of which are important for sustaining placentation (Holtan and Creedon, 2011) and both of which, with these new findings, are now linked to the involvement of IDO1. Thus, the integrative role proposed for IDO1 in establishing a chronic inflammatory environment uniquely suited to sustain a developing fetus during pregnancy appears, in a different context, to play a pathogenic role in sustaining the development of tumors.

In addition to establishing IDO1's role in supporting neovascularization, we have also demonstrated that the use of a pharmacologic inhibitor has the potential to be an effective intervention strategy against neovascularization. In the OIR model, administration of epacodostat (INCB024360), a specific, small molecule inhibitor of IDO1 (Liu et al., 2010), resulted in significantly reduced neovascularization relative to vehicle alone. Likewise, in the 4T1 metastasis model, administration of epacadostat to mice harboring established, vascularized metastases resulted in a reduced level of neovascularization in response to treatment. Thus, rather than simply reiterating IDO1's role in supporting neovascular development, this result indicates that treatment with an IDO1 inhibitor has the potential to effectively degrade established neovascular networks. Positive controls for these experiments were selected based on reported anti-neovascular activity in the respective models, however, possible connections to the IDO1-based mechanism of action are intriguing. In the OIR model, the anti-neovascular effect of ethyl pyruvate was proposed to occur through suppression of HMGB1 (high-mobility group box-1) expression (Lee et al., 2013), however, the demonstration that ethyl pyruvate is an effective *in vivo* inhibitor of IDO1 expression (Muller et al., 2010), suggests that IDO1 may

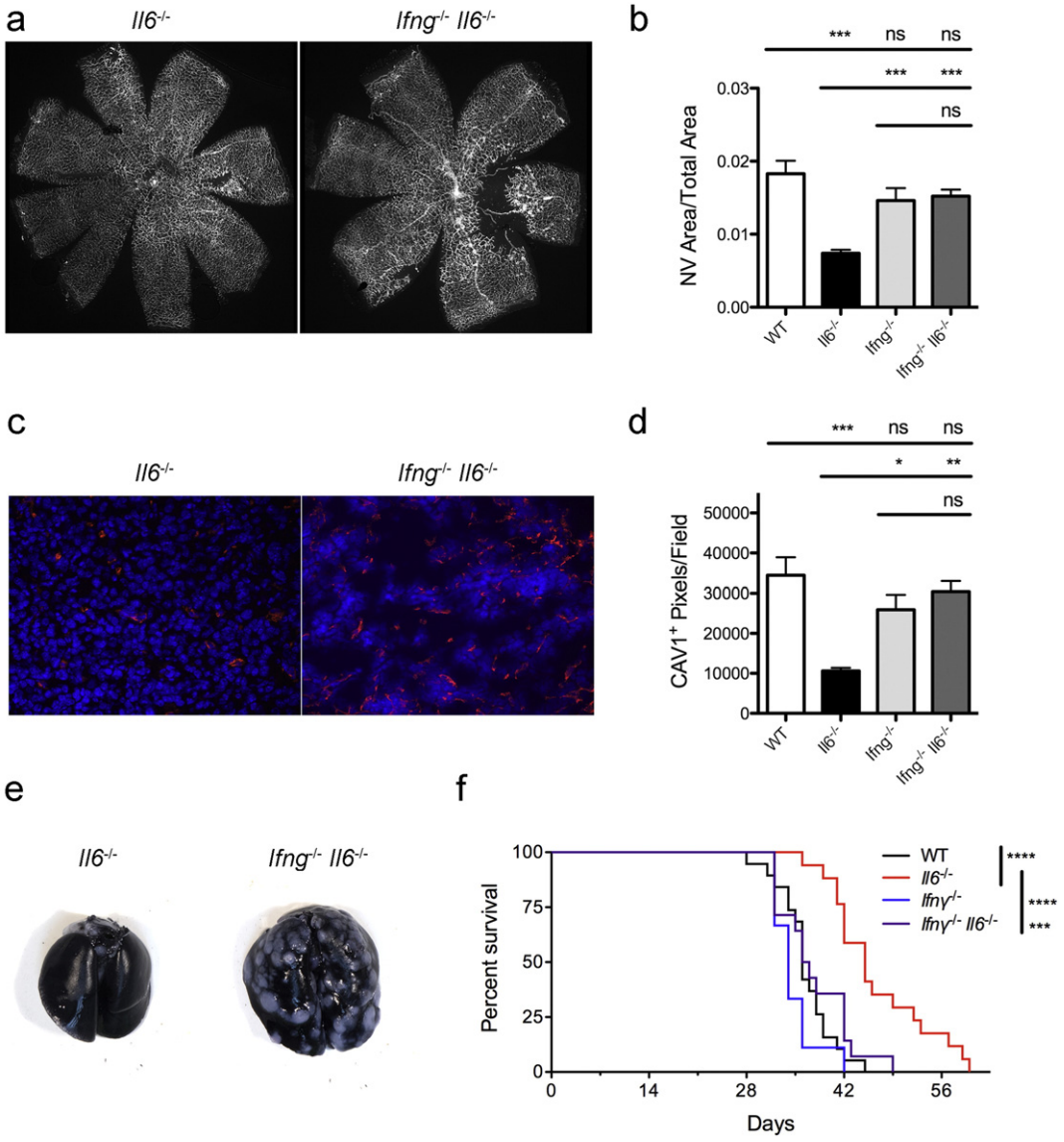

Fig. 4. IL6 loss results in IFNγ-dependent impairment of neovascularization and pulmonary metastasis development. (a) Representative images of B4-Alexa488-isolectin staining of blood vessels in retinal flatmounts at P17 from $Ifng^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ neonates exposed to hyperoxia from P7-P12 to induce OIR. (b) Quantitative assessment of neovascular area over total retinal area at P17 for WT, $Il6^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ OIR cohorts ($N \geq 15$ eyes). Graphed as means ± SEM with significance determined by one-way ANOVA with Tukey's multiple comparison test. (c) Representative images of immunofluorescent staining of blood vessels with anti-Cav1 (red) and of nuclei with DAPI (blue) within metastatic regions of lung specimens prepared from WT, $Il6^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ mice following orthotopic 4T1 mammary tumor cell engraftment. (d) Quantitative assessment of neovascular density marked by anti-Cav1 positive staining within lung metastases of WT, $Il6^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ mice following orthotopic 4T1 mammary tumor cell engraftment ($N \geq 3$ mice). Graphed as means ± SEM with significance determined by one-way ANOVA with Tukey's multiple comparison test. (e) Staining of lungs with India ink to visualize the metastatic burden in $Il6^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ mice at 5 weeks following orthotopic 4T1 mammary tumor cell engraftment. (f) Kaplan-Meier survival curves for cohorts of WT, $Il6^{-/-}$, $Ifng^{-/-}$ and $Ifng^{-/-}$ $Il6^{-/-}$ mice following orthotopic engraftment of 1×10^4 4T1 cells ($N \geq 9$ mice) with significance assessed by 2-group log-rank test.

instead be the relevant target. In established tumor models, the anti-neovascular effect of cyclophosphamide was shown to be IFNγ-dependent (Ibe et al., 2001), which, if not directly linked, also suggests a mechanistic convergence with IDO1.

IDO1 small molecule inhibitors are currently being evaluated in clinical trials for a variety of cancers based on the supposition that they will help to enable an effective immune response directed against the tumor. This study indicates, however, that a more complex conceptualization of how the tumor is affected may be required to fully understand any beneficial responses to IDO1 inhibitor treatment. The clear interconnection established between the effects of IDO1, IFNγ and IL6 loss on neovascularization and metastasis survival is consistent with other indications of the importance of the anti-neovascular effect that IFNγ exerts against tumors (Qin and Blankenstein, 2000, Qin et al., 2003), and suggests that the impact on the tumor vasculature should additionally be considered when assessing clinical results obtained with IDO1 inhibitors, particularly in the context of pulmonary metastases. Even with the data currently at hand, the concept of an IDO1 inhibitor-elicited, anti-neovascular effect may have explanative value. An emergent concept in the field of angiogenesis is that normalizing pathologic tumor vasculature will enhance the efficacy of chemo- and radiotherapies, providing another possible explanation, in addition to enhanced anti-tumor immunity, for the synergistic responses obtained with combinations of IDO1 inhibitors and chemotherapeutic agents in different mouse tumor models (Hou et al., 2007, Muller et al., 2005). Additionally, it was noted in a clinical study that the IDO1 pathway inhibitor, indoximod, appeared to sensitize tumors to salvage chemotherapy (Soliman et al., 2013) which would likewise be consistent with the

possibility of vascular normalization improving chemotherapy responses. Alternatively, IDO1 inhibition may enhance the IFNγ-dependent, anti-angiogenic effect elicited by certain chemotherapeutic drugs such as cyclophosphamide, which has been reported to produce immune-dependent rejection of large, vascularized tumors mediated by the destruction of tumor blood vessels in a manner that requires IFNγ receptor expression on normal host cells (Ibe et al., 2001). By eliminating the negative feedback constraint imposed by IDO1 on the anti-angiogenic effect of IFNγ, IDO1 inhibitors may be able to enhance this aspect of the therapeutic response to cyclophosphamide and perhaps other chemotherapeutic drugs that have also been identified as having anti-angiogenic activity, such as taxanes (Bocci et al., 2002).

Beyond cancer treatment, the results of this study suggest potential utility for IDO1 inhibitors in treating the pathologic neovascularization associated with retinopathies. Retinopathy of prematurity is the most obvious indication in which to evaluate the potential benefit of IDO1 inhibition given that this is the disease that the OIR protocol most closely models. Additionally, however, a number of other retinopathies might also benefit from IDO1 inhibitor treatment as indicated by the effectiveness of the anti-VEGF-A antibody Fab, ranibizumab, for treating diseases such as wet macular degeneration and diabetic retinopathy (Triantafylla et al., 2014). Despite the effectiveness of anti-VEGF-A antibody therapy for treating various ocular diseases, there are also notable drawbacks to its use. One significant downside of blocking VEGF-A is the collateral interference with normal angiogenesis that occurs in conjunction with the beneficial reduction in neovascularization. Evidence from the OIR model suggests that, while targeting IDO1 is comparably effective to targeting VEGF-A in interfering with neovascularization, IDO1 inhibition does not appear to interfere with normal angiogenesis based on the lack of effect of IDO1 loss on the development of retinal vasculature in normoxic mice as well as the degree of recovery that occurs in the avascular region of *Ido1*$^{-/-}$ mice in the context of OIR. Additionally, it may be possible to develop a therapeutic formulation of a small molecule IDO1 inhibitor that does not involve direct injection into the eye as is required with antibody-based therapy. Clearly, the recognition that IDO1 plays a key role in supporting inflammatory neovascularization opens up compelling avenues of basic biological investigation and potential therapeutic intervention centered around this key nodal regulator of the inflammatory environment.

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Conflicts of Interest

A.J. Muller, J.B. DuHadaway, and G.C. Prendergast declare a potential conflict of interest with regard to IDO1 due to intellectual property and financial interests with New Link Genetics Corporation, which is engaged in the clinical development of IDO1 inhibitors for the purpose of treating cancer and other diseases. No potential conflicts of interest are declared by the other authors.

Author Contributions

Conceptualization, A.J.M., A.B.N., C.S. and A.M.; Methodology, A.J.M., A.B.N., A.M. and C.S.; Investigation, A.M., J.B.D. and E.S.W.; Formal Analysis, A.M. and A.J.M; Writing –Original Draft, A.J.M. and A.M.; Writing –Review & Editing, A.B.N., C.S., G.C.P. and A.J.M.; Funding Acquisition, A.J.M. and G.C.P.; Supervision, A.J.M. and G.C.P.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.11.013.

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