IDO Is a Nodal Pathogenic Driver of Lung Cancer and Metastasis Development

Courtney Smith¹, Mee Young Chang¹, Katherine H. Parker³, Daniel W. Beury³, James B. DuHadaway¹, Hollie E. Flick¹,⁴, Janette Boulden¹, Erika Sutanto-Ward⁵, Alejandro Peralta Soler¹,⁷, Lisa D. Laury-Kleintop¹, Laura Mandik-Nayak¹, Richard Metz², Suzanne Ostrand-Rosenberg³, George C. Prendergast¹,⁵,⁶, and Alexander J. Muller¹,⁶
INTRODUCTION

Inflammatory tissue microenvironments contribute strongly to tumor progression, but due to the complex multifactorial nature of inflammation, there remains limited understanding of specific pathogenic components that might be targeted to effectively treat cancer (1). In this context, the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) has emerged as an intriguing target implicated in tumoral immune escape (2, 3). IDO-inhibitory compounds have entered clinical trials based on evidence of immune-based antitumor responses in a variety of preclinical models of cancer (4–10). Meanwhile, inadvertent IDO targeting may already be providing benefits to patients as illustrated by recent evidence that the clinically approved tyrosine kinase inhibitor imatinib dampens IDO induction as a key mechanism for achieving therapeutic efficacy in the treatment of gastrointestinal stromal tumors (11).

Although results with IDO pathway inhibitors are provocative, the conclusions that can be drawn are inherently limited by drug specificity concerns, especially in the absence of independent genetic validation. Addressing this issue, our studies on the impact of Ido1 gene deletion on 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA)-elicited skin papillomagenesis established that IDO has an integral tumor-promoting role in the context of phorbol ester–elicited inflammation (12, 13), but interpretation of these results is tempered by the possibility that the chemical exposures in this model may produce anomalies irrelevant to the majority of spontaneous tumors. The lungs present a particularly compelling physiologic context in which to further investigate the role of IDO in tumorigenesis as IDO is known to be highly inducible in this tissue (14, 15), and there is an urgent unmet medical need for effective therapeutic options to treat primary lung tumors and metastases. In this report, we investigated the consequences of IDO loss through genetic ablation in the context of well-established, pulmonary models of oncogenic KRAS-induced adenocarcinoma and orthotopic breast carcinoma metastasis. Our findings reveal previously unappreciated roles for IDO in vascularization and in the production of the proinflammatory cytokine interleukin (IL)-6 that in turn dictates the development of protumorigenic, myeloid-derived suppressor cells (MDSC).

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) enzyme inhibitors have entered clinical trials for cancer treatment based on preclinical studies, indicating that they can defeat immune escape and broadly enhance other therapeutic modalities. However, clear genetic evidence of the impact of IDO on tumorigenesis in physiologic models of primary or metastatic disease is lacking. Investigating the impact of Ido1 gene disruption in mouse models of oncogenic KRAS-induced lung carcinoma and breast carcinoma–derived pulmonary metastasis, we have found that IDO deficiency resulted in reduced lung tumor burden and improved survival in both models. Micro-computed tomographic (CT) imaging further revealed that the density of the underlying pulmonary blood vessels was significantly reduced in Ido1-nullizygous mice. During lung tumor and metastasis outgrowth, interleukin (IL)-6 induction was greatly attenuated in conjunction with the loss of IDO. Biologically, this resulted in a consequential impairment of protumorigenic myeloid-derived suppressor cells (MDSC), as restoration of IL-6 recovered both MDSC suppressor function and metastasis susceptibility in Ido1-nullizygous mice. Together, our findings define IDO as a prototypical integrative modifier that bridges inflammation, vascularization, and immune escape to license primary and metastatic tumor outgrowth.

SIGNIFICANCE: This study provides preclinical, genetic proof-of-concept that the immunoregulatory enzyme IDO contributes to autochthonous carcinoma progression and to the creation of a metastatic niche. IDO deficiency in vivo negatively impacted both vascularization and IL-6-dependent, MDSC-driven immune escape, establishing IDO as an overarching factor directing the establishment of a protumorigenic environment. Cancer Discov, 2(8); 722–35. © 2012 AACR.
Figure 1. IDO deficiency extends the survival of mice with KRAS-induced lung adenocarcinomas despite an elevated number of early lesions. A, Kaplan-Meier survival curves for cohorts of Lox-Kras\textsuperscript{G12D} (n = 23) and Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} (n = 14) mice infected with 2.5 × 10\textsuperscript{7} plaque-forming units (pfu) of Ad-Cre virus. B, Kaplan-Meier survival curves for cohorts of Lox-Kras\textsuperscript{G12D} (n = 8) and Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} (n = 5) mice infected with 1.25 × 10\textsuperscript{8} pfu Ad-Cre virus. Significance for both data sets was assessed by 2-group log-rank test at P < 0.05. C, total lung DNA prepared from 3 mice per time point was analyzed for the presence of the viral Cre gene by real-time PCR at 0, 1, 3, and 7 days postinfection. Relative Cre levels determined from this analysis are plotted as means ± SEM. D, representative hematoxylin and eosin (H&E)-stained sections depicting the observed difference in early lesions between the lungs of Lox-Kras\textsuperscript{G12D} and Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} mice at 6 weeks postinfection. E, quantitative histopathologic assessment of lesion frequency in H&E-stained sections of lung biopsies from Lox-Kras\textsuperscript{G12D} and Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} mice at 6 and 12-week postinfection (n ≥ 5). The number of lesions identifiable under low magnification within a defined region of each specimen are graphed on the scatter plot with the means ± SEM. Significance was determined by 2-tailed Student t test at P < 0.05. NS, not significant.

(Ad-Cre) to activate the latent oncogenic Kras\textsuperscript{G12D} allele (16). These RAS-induced adenocarcinomas elicit a robust inflammatory response (17) wherein IDO may impart a protumorigenic skew (2). To investigate this hypothesis in an autochthonous lung tumor setting, we introduced Ido1\textsuperscript{-/-} (homozygous Ido1-null) alleles (18) into the LSL-Kras\textsuperscript{G12D} mouse strain. Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} mice displayed significantly increased survival relative to Lox-Kras\textsuperscript{G12D} mice at 2 different multiplicities of Ad-Cre infection (Fig. 1A and B). Similar levels of Cre were present in the lungs of both strains at 0, 1, 3, and 7 days postinfection (Fig. 1C). Unexpectedly, histopathologic examination at 6 weeks revealed that the frequency of early precancerous lesions was actually about 3-fold higher in the Ido1\textsuperscript{-/-} Lox-Kras\textsuperscript{G12D} mice (Fig. 1D and E), substantiating
that IDO deficiency does not interfere at the stage of Ad-Cre–mediated oncogenic RAS activation required to initiate these tumors (ref. 16; Supplementary Fig. S1A). While early tumorigenesis may be negatively impacted by IDO-mediated tryptophan catabolism, as previously proposed (19), this phenomenon was transient with the differential no longer significant by 12 weeks (Fig. 1E).

IDO Deficiency Impairs Tumor Outgrowth and Vascular Development in the Lung

To assess the impact of Ido1 loss on overt lung tumors, noninvasive micro-computed tomographic (CT) scans were conducted on groups of Lox-KrasG12D and Ido1−/−Lox-KrasG12D mice at 18 and 24 weeks following Ad-Cre administration (Fig. 2A).

**Figure 2.** IDO deficiency impairs the outgrowth of overt lung adenocarcinomas and reduces normal pulmonary vascularization. A, representative axial micro-CT images and 3D reconstructions of Lox-KrasG12D and Ido1−/−Lox-KrasG12D mouse lungs acquired at 0, 18, and 24 weeks postinfection. Tumor and vasculature, which have indistinguishable X-ray densities, are designated in red in the individual CT images (left) or red/orange for exterior/interior location in the 3D reconstructions (right). B, volumetric image analysis of tumor and vasculature conducted on the 3D reconstructions of lung micro-CT images. The data are graphed as a scatter plot with the means ± SEM (Δ; fold difference). Significance was determined by 2-tailed Student t test at P < 0.05. C, immunofluorescent staining of blood vessels with antibody to caveolin 1 (red) and 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei (blue) in representative lung tissue specimens from BALB/c WT and Ido1−/− mice. D, quantitation of blood vessel areas. The vessel area totals measured within each field are graphed as a scatter plot with the means ± SEM (Δ; fold difference). Significance was determined by 2-tailed Student t test at P < 0.05. E, distribution of pulmonary vessels within specified size ranges. The total number of small (<500 μm²), medium (500–5,000 μm²), and large (>5,000 μm²) vessels identified within the defined fields evaluated in D are plotted on a bar graph (Δ; fold difference). Also see Supplementary Fig. S1C for a graph of individual vessel measurements rank ordered across the entire size range.
Semi-automated quantitative image analysis (20) was conducted on 3-dimensional (3D) reconstructions of the thoracic cavity excluding the heart to assess the combined tumor and vascular volume within this space. Although lung tumor burden did increase progressively in both cohorts, it was significantly reduced in the Ido1−/− Lox-KrasG12D mice relative to the corresponding Ido1-competent Lox-KrasG12D mice (Fig. 2B). Individual micro-CT scan images paired with 3D reconstructions of total chest space and functional lung volume visually highlighted the difference in lung tumor burden between representative Ido1−/− Lox-KrasG12D and KrasG12D animals (Fig. 2A; Supplementary Videos). These results indicate that IDO deficiency mitigates overt lung tumor outgrowth, consistent with the increased survival exhibited by these mice.

Micro-CT analysis additionally revealed that the density of normal vasculature in the lungs of uninfected animals was substantially diminished in the Ido1−/− animals (Fig. 2A and B). Intriguingly, the difference in vascular density between IDO-deficient and IDO-competent cohorts was proportionately comparable with the difference in overt lung tumor burden at the 18- and 24-week time points (Supplementary Fig. S1B), suggesting an association between the extent of the underlying basal vasculature and the capacity of the lungs to support tumor formation. Immunofluorescent staining of blood vessels in the lungs confirmed the decrease in pulmonary vascular density in Ido1−/− animals (Fig. 2C). The area within the lungs occupied by vessels was reduced by about 1.6-fold in Ido1−/− animals (Fig. 2D), in line with the differential identified by micro-CT data analysis. Further analysis revealed that the reduction in vascular density occurred predominantly at the level of small- to medium-sized vessels, which were nearly twice as abundant in the wild-type (WT) animals, whereas there was little difference in the number of large vessels (Fig. 2E; Supplementary Fig. S1C).

**IDO Promotes IL-6 Elevation during Lung Tumor Formation**

In the lungs, IDO is highly responsive to pathogen or cytokine exposure (14, 15). To determine whether lung tumorigenesis also stimulates IDO, we compared steady-state levels of the tryptophan catabolite kynurenine at various times after KrasG12D activation. Although baseline levels of kynurenine in the lungs of uninfected Lox-KrasG12D mice were significantly higher than in their IDO-deficient counterparts (Fig. 3A), these levels remained constant during lung tumorigenesis (Fig. 3A). In contrast, a multiplexed analysis of inflammatory cytokines at 19 and 26 weeks revealed IL-6 to be elevated by about 25- and 68-fold, respectively, in lungs from tumor-bearing Lox-KrasG12D mice but only by about 1- and 3-fold in Ido1−/− Lox-KrasG12D mice (Fig. 3B). This finding was notable given the known tumor-promoting role of IL-6 in this model (21). Although not of the same magnitude, induction of CCL2/MCP1 [chemokine (C-C motif) ligand 2] was likewise attenuated in tumor-bearing Lox-KrasG12D mice lacking Ido1 (Fig. 3C). In contrast, Ido1 loss did not significantly affect the relative levels of IL-10, IFN-γ, TNF-α, or IL-12p70 (data not shown).

**IDO Deficiency Impedes the Development of Pulmonary Metastases**

Given the evidence that Ido1−/− mice are resistant to the outgrowth of primary lung tumors, we asked whether Ido1−/− animals might exhibit reduced susceptibility to pulmonary metastasis development as well. This question was investigated by orthotopic engraftment of mice with highly malignant 4T1 breast carcinoma cells, which metastasize efficiently to the lungs. Survival was increased significantly in Ido1−/− hosts compared with WT hosts after challenge with either a 4T1-luciferase–expressing subclone or with parental 4T1 cells, despite an overall shift in the curves (Fig. 4A and B). No difference in primary tumor growth rate was observed (Supplementary Fig. S2A and S2B), but metastatic lung nodules at necropsy were unambiguously less pronounced in Ido1−/− mice (Fig. 4C). Noninvasive micro-CT imaging also confirmed a marked reduction in metastatic burden in Ido1−/− mice (Fig. 4C), which was quantified by an ex vivo colony-forming assay (ref. 22; Fig. 4D). The metastasis differential was not attributable to reduced intravasation because the same numbers of tumor cells were present in peripheral blood samples from both strains (Fig. 4D). In contrast to lung,
Figure 4. IDO deficiency delays the development of pulmonary metastases. Kaplan–Meier survival curves for cohorts of WT and Ido1−/− mice following orthotopic engraftment of 1 × 10⁴ (A) 4T1-luc (n = 25) or (B) 4T1 (n ≥ 9) tumor cells. Significance was assessed by 2-group log-rank test at P < 0.05. The survival benefit observed in Ido1−/− mice was independently replicated at University of Maryland Baltimore County. C, Staining of lungs with India ink and axial images from micro-CT scans depicting the difference in pulmonary metastasis burden between WT and Ido1−/− mice at 5 weeks following orthotopic 4T1 tumor cell engraftment. At 5 weeks following (D) orthotopic engraftment of 4T1 cells (n = 6) or (E) orthotopic engraftment of 4T1 cells and resection of the primary tumor at 18 days postengraftment (n ≥ 11), colony-forming assays were conducted to assess the relative tumor cell burden in the blood (neat) and lungs (1:1,000). Individual data points are graphed on a log scale scatter plot with the means ± SEM and significance assessed by 2-tailed Student t test at P < 0.05 (NS, not significant).
no difference in metastatic burden was observed in liver, although the presence of 4T1 cells was also nearly too low to detect in this tissue (Supplementary Fig. S2C). Because excision of the primary tumor can alter immune-based effects on metastasis (23), we evaluated the metastasis burden in resected mice. *Ido1−/−* mice continued to exhibit significant resistance to metastasis development (Fig. 4E), indicating that IDO-mediated support of metastatic development in lung is not dependent on the presence of the primary tumor. We also examined pulmonary VEGF levels but found that these increased comparably in both WT and *Ido1−/−* lungs during metastasis development and were actually somewhat higher at baseline in the *Ido1−/−* lungs (Supplementary Fig. S2D).

**IDO Is Activated during Metastatic Lung Colonization and Potentiates IL-6 Induction**

In WT mice, IDO1 protein and kynurenine levels both increased in the lungs during 4T1 metastasis development, particularly at 5 and 6 weeks postengraftment (Fig. 5A and B). The principal source of IDO1 expression in this context appears to be the native stroma rather than the engrafted 4T1 tumor cells because no IDO1 protein was detectable in the lungs of *Ido1−/−* mice (Fig. 5A), even at 7 weeks postengraftment when...
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Figure 6. IDO-dependent potentiation of IL-6 production. A, supernatant from U937 cells stimulated for 24 hours with IFN-γ (100 ng/mL) and/or LPS (100 ng/mL) was analyzed for kynurenine and IL-6. Results from triplicate wells are plotted as the means ± SEM. Methylthiohydantoin tryptophan (MTH-Trp, 100 μmol/L) was included during induction where indicated and significance relative to the corresponding induced level without MTH-Trp was determined by 2-tailed Student t test (*, P ≤ 0.0001). B, supernatant from HL-60 cells stimulated for 24 hours with IFN-γ (100 ng/mL) and LPS (100 ng/mL) was analyzed for kynurenine and IL-6. Results from duplicate wells are plotted as the means ± SEM. Methylthiohydantoin tryptophan (MTH-Trp, 100 μmol/L) was included during induction where indicated and significance relative to the corresponding induced level without MTH-Trp was determined by 2-tailed Student t test (**, P ≤ 0.0001). C, HL-60 cells treated in triplicate with Ido1-targeting (si-Ido1) or nontargeting (si-Gapdh) siRNAs were stimulated for 24 hours with IFN-γ (100 ng/mL) and LPS (100 ng/mL). Pooled cell lysates were analyzed by Western blot analysis for Ido1 and β-actin (left). Ido1 induction was suppressed by approximately 89.7% as assessed by densitometric analysis and normalization to actin. Individual cell supernatants were analyzed for IL-6 (right). The IL-6 data are plotted as the means ± SEM with the significance of the difference between specific Ido1-targeting versus nontargeting results determined by 2-tailed Student t test (**, P ≤ 0.0001). Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

the metastatic tumor burden was high. However, a weak but significant increase in kynurenine was observed in the lungs of Ido1−/− mice (Fig. 5B), suggesting that metastasis development may be associated with induction of an alternative mechanism of kynurenine production, such as IDO2 (24) or TDO2 (tryptophan 2,3-dioxygenase; ref. 25), either in conjunction with or in the absence of IDO1.

As in the Kras-driven primary lung tumor model, Ido1 competence in the pulmonary metastatic setting was linked to enhanced elevation of IL-6, with levels increasing up to 15-fold over baseline in WT animals (Fig. 5C). On the other hand, the IL-6 levels in Ido1−/− lungs remained about 2- to 4-fold over baseline even when evaluated at an extended time point to account for the differential in tumor burden (Fig. 5C). Thus, like the autochthonous lung tumor studies, results from this lung metastasis model led us to infer a positive regulatory link between Ido1 and IL-6 production. Direct interrogation of this hypothesis was carried out in a cell-based assay with known IDO inducers. Lipopolysaccharide (LPS) induced both IDO activity and IL-6 production in monocyctic U937 cells whereas IFN-γ on its own elicited little response but greatly elevated the level of IDO activity in combination with LPS that was mirrored by a comparable enhancement of IL-6 production (Fig. 6A). In both instances, inclusion of the competitive IDO-inhibitory compound MTH-tryptophan (8) significantly suppressed the observed increases in IDO activity as well as IL-6 production (Fig. 6A). MTH-tryptophan-mediated suppression of IL-6 induction was confirmed in a second monocytic cell line HL-60 (Fig. 6B). Likewise, siRNA-mediated interference with Ido1 gene expression also significantly suppressed IL-6 induction (Fig. 6C). Taken together, these results are consistent with our in vivo findings suggesting that IDO activity can potentiate the elevated production of IL-6.

IDO Drives MDSC Expansion and Imnosuppressive Function

Studies in Il1r−/− (IL-1 receptor-nullizygous) mice have shown a crucial role for IL-6 in 4T1 pulmonary metastasis development (26). At the cellular level, IL-1β enhances development
of tumor-promoting MDSCs with IL-6 serving as a critical downstream mediator of this process (26). Because Ido1 loss attenuated IL-6 induction and metastatic colonization in the lung, we hypothesized that MDSCs may be compromised at some level in tumor-bearing Ido1−/− mice. MDSCs isolated from WT and Ido1−/− mice did not differ phenotypically (Fig. 7A; Supplementary Fig. S3A); however, an early delay in the expansion of Gr1+CD11b+ cells in Ido1−/− mice, similar to that observed in Il1r−/− animals (26), was noted (Fig. 7B). Moreover, circulating MDSCs isolated from Ido1−/− hosts were functionally impaired in their ability to suppress T cells (Fig. 7C). We did not detect IDO1 protein in Gr1+CD11b+ cells.
obtained from tumor-bearing WT hosts (Supplementary Fig. S3B), consistent with the hypothesis that the observed functional impairment of MDSCs is a non–cell-autonomous effect of IDO deficiency in which IL-6 may act as a key intermediary.

**IL-6 Is Critical to IDO-Driven MDSC Activity and Pulmonary Metastasis**

To directly test the ability of IL-6 to functionally restore MDSC-suppressive activity in Ido1<sup>−/−</sup> mice, orthotopic tumors were established using 4T1–IL-6 cells (26), a 4T1 cell population engineered to constitutively express IL-6. MDSCs isolated from Ido1<sup>−/−</sup> mice engrafted with 4T1–IL-6 cells exhibited an elevated T-cell-suppressive activity similar to that of MDSCs isolated from WT hosts engrafted with parental 4T1 cells (Fig. 7D). Further enhancement of MDSC-suppressive activity could be achieved by engrafting 4T1–IL-6 cells into WT mice (Fig. 7D), indicating that the endogenous IL-6 levels stimulated by parental 4T1 tumor cells in WT animals were not fully saturating with regard to promoting MDSC suppressor function.

We next asked whether restoring IL-6 levels could also reverse the metastatic resistance exhibited by Ido1<sup>−/−</sup> mice. In the orthotopic setting, high levels of IL-6 produced in primary tumors formed by 4T1–IL-6 cells complicated the analysis by impairing the efficiency of pulmonary metastasis [possibly reflecting the recruitment of metastatic cancer cells back to IL-6–expressing primary tumors as documented previously (ref. 27)]. However, as our results in orthotopically engrafted mice had indicated that the Ido1 allelic status does not affect 4T1 intravasation, we reasoned that a valid assessment of the impact of IDO deficiency on pulmonary metastasis could be made by introducing the metastatic tumor cells directly into the circulation. Accordingly, we confirmed that intravenously engrafted Ido1<sup>−/−</sup> mice maintained their resistance to pulmonary metastasis formation, with the apparent mean metastatic tumor burden being 30.4- and 31.6-fold lower in Ido1<sup>−/−</sup> versus WT mice challenged with 4T1 and 4T1–IL-6 cells, respectively (Fig. 7E). The proportional increase in metastatic burden observed in the 4T1–IL-6 challenged cohorts is also in line with the proposed interpretation of the MDSC functional data that IL-6 is not being produced at saturating levels in the 4T1-challenged WT animals. Because of the significantly higher metastasis burden produced by 4T1–IL-6 cells, comparison of 4T1–IL-6-challenged Ido1<sup>−/−</sup> mice to 4T1-challenged WT mice yielded a differential in mean metastatic tumor burden of only 4.8-fold (Fig. 7E). Thus, IL-6 supplementation not only rescued WT levels of MDSC suppressor function in 4T1 tumor-challenged Ido1<sup>−/−</sup> mice but also markedly restored their susceptibility to pulmonary metastasis development.

**DISCUSSION**

The idea of immune escape as a “hallmark of cancer” (28, 29) represents a groundbreaking although still largely untested paradigm within the field of cancer biology. The presumption that tumors exploit IDO activity as a mechanism of immune escape, initially inferred from the pioneering studies on maternal immune tolerance of Munn and colleagues (30), has become increasingly accepted despite a fundamental deficit in genetic support for the role of IDO in tumor development. This study addresses this gap with direct genetic validation of the importance of IDO in well-established models of lung cancer and metastasis that offers novel insights into the impact of IDO on tumor pathogenesis. Moreover, these findings strongly encourage the prioritization of clinical investigations into the use of IDO pathway inhibitors for treating lung adenocarcinomas and pulmonary metastases where more effective modalities are urgently needed.

While IL-6 activity was not elevated in lung tissue beyond baseline levels during KRAS-driven lung tumor development, the observed reduction in pulmonary vascularization in Ido1<sup>−/−</sup> animals even before initiation of tumorigenesis implied that the loss of steady-state IDO in this context was sufficiently consequential to impact physiologic processes important to tumor outgrowth. Enhanced tumor vascularization has been reported in tumor xenograft models involving exogenous IDO overexpression (31, 32), but our study is the first to identify a role for IDO in supporting vascular development under native physiologic conditions. Our findings likewise genetically establish the importance of IDO activity in nontumor cells for supporting pulmonary metastasis. In this manner, IDO activity may influence metastatic dissemination to tissues such as the lung where its expression is particularly robust. This may, however, be less relevant when IDO (or tryptophan 2,3-dioxygenase (ref. 25)) activity is substantially elevated within the tumor cells themselves (8, 33), enabling the malignancy to preemptively shape its surroundings through intrinsic tryptophan catabolism. As such, IDO activity that originates from stromal cells of the tumor microenvironment or from the tumor cells themselves may contribute to direct tumor outgrowth.

The positive association between IDO and IL-6 in lung tumorigenesis and metastasis was not necessarily anticipated, given that it runs counter to expectations based on IDO-mediated induction of liver-enriched inhibitory protein (LIP), a negative regulatory isoform of the Il6 gene expression promoting transcription factor C/EBPβ (24, 34). The precise regulatory impact of LIP on Il6 expression is not clear, however, insofar as other findings have indicated that LIP can interact with NF-κB to induce rather than limit Il6 transcription (35). Our findings are also consistent with evidence that a downstream product of IDO-mediated catabolism, kynurenic acid, can potentiate IL-6 production in the context of inflammation by signaling through the aryl hydrocarbon receptor (36). IL-6 is a pleiotropic cytokine that is widely implicated in supporting neoplastic outgrowth in the context of chronic inflammation (37). Clinically, IL-6 has been established as a marker of early relapse of resected lung tumors (38). Analyses of DNA polymorphisms in the IL-6 promoter region have identified positive correlations between IL-6 inducibility and lung cancer susceptibility in the context of concurrent inflammatory disease (39) as well as micrometastatic disease in patients with high-risk breast cancer (40). Functionally, IL-6 induction has been identified as an essential downstream component of RAS-induced tumorigenesis (41) that is directly linked to lung tumor development in the Lox-Kras<sup>G12D</sup> transgenic mouse model (21).

Numerous other studies indicate that IL-6 can also contribute to tumor promotion by supporting angiogenesis and neovascularization of tumors (42, 43). Thus, biologically,
the epidemiologic and functional data for IL-6 are consistent with the tumor-promoting activity that we have ascribed to IDO through mouse genetics.

Tumor responses to IDO-inhibitory compounds require functional host immunity (5, 6, 8, 9), but the mechanisms through which IDO promotes immune escape have yet to be fully delineated. Connecting IL-6 to IDO provides valuable insight in this regard. IL-6 has previously been identified in the 4T1 metastasis model as critical to the induction of MDSCs, which act as potent inhibitors of antitumor immunity (44). MDSC accumulation is known to be driven by several factors that are produced by tumor cells and the tumor stroma, including the potent inflammatory mediators prostaglandin E2 and IL-1β (26). In this context, our findings identify IDO as a key determinant of IL-6–elicited MDSC accumulation and suppressor activity. Interestingly, IL-1β may dynamically potentiate the contribution of IDO to IL-6 induction given that IL-1β may promote the upregulation of IFN-γR1 (48) that enhances IDOL inducibility in response to IFN-γ. In contrast, IL-6 may exert a counter-regulatory feedback effect by inducing SOCS3 (suppressor of cytokine signaling 3), which not only attenuates IL-6 signaling (49) but also limits IDO transcription and IDO enzyme stability (50, 51).

Our results deepen the concept that IDO activity profoundly influences the pathogenic character of the tumor microenvironment by identifying the cytokine IL-6 as a crucial IDO effector for establishing “cancer-associated” inflammation. IL-6 is a far-reaching, pleiotropic signaling molecule that can elicit both intrinsic and extrinsic effects on tumor development (i.e., increased malignancy and survival as well as increased angiogenesis and immune escape). The ramifications of our results thus extend beyond the constrained effects that local IDO-mediated tryptophan catabolism might exert on the proximal microenvironment, and one would expect the potentiation of IL-6 expression by IDO to affect diverse aspects of tumor development with the relative weighting of each aspect being an important focus of future study. Indeed, further investigations of IDO as a nexus for control of tumorigenic inflammation, vascularization, and immune escape will be invaluable in formulating rational strategies to guide the best application of IDO inhibitors that have entered clinical development.

METHODS

Transgenic Mouse Strains

Congenic Ido1−/− mice on C57BL/6 and BALB/c strain backgrounds were provided by A. Mellor (Georgia Health Sciences University, Augusta, GA), and corresponding control strains were purchased from Jackson Laboratory. LSL-KrasG12D Cre-inducible transgenic mice on a mixed 129SvJ-C57BL/6 strain background (16) were obtained through the Mouse Models of Human Cancer Consortium (NCI-Frederick, Frederick, MD). Administration of Ad-Cre virus to activate the latent KrasG12D allele in lungs of LSL-KrasG12D transgenic mice (referred to as Lox-KrasG12D mice; ref. 16) was carried out as described (52). Doubly mutant Ido1−/− LSL-KrasG12D mice were generated through breeding of the 2 transgenic strains. Mating pairs of BALB/c and T-cell receptor (TcR) transgenic DO11.10 BALB/c mice (I-Ak-restricted, specific for chicken ovalbumin 323–339) were obtained from The Jackson Laboratory. Mating pairs of TcR transgenic Clone 4 BALB/c mice [H-2Kd–restricted, specific to influenza hemagglutinin (HA) peptide 318–326] and TcR transgenic TS1 BALB/c mice (I-Ek–restricted, specific to HA peptide 110–119) were provided by E. Fuchs (Johns Hopkins, Baltimore, MD). All procedures involving mice were approved by either the Lankenau Institute for Medical Research (LIRM, Wynnewood, PA) or University of Maryland Baltimore County (UMBIC) Institutional Animal Care and Use Committee (IACUC).

Micro-CT Scanning

Three-dimensional micro-CT images were acquired from anesthetized mice using an Impekk Micro-CT scanner operated at 40-kVp, 500-μA, 250-millisecond per frame, 5 frames per view, 360 views, and 1-degree increments per view. Contiguous axial DICOM-formatted images through each mouse thorax, with voxels of dimensions 91 μm × 91 μm × 91 μm were compiled into 3D format using Amira v5.1 software and normalized to Hounsfield units. Using the segmentation editor, manual selections of the chest cavity minus the heart were conducted on every other slice following interpolation of these selections. Magic wand tool selection was conducted at the threshold range defining air (determined to be between −750 and −350) to define the functional lung volume, which was automatically subtracted from the total chest space to identify the volume representing vasculature and tumors (20).

4T1 Tumor Cell Metastasis

Parental 4T1 mouse mammary carcinoma cells and 4T1-derived cell lines expressing luciferase (4T1-luc) or mouse IIF (4T1–IL-6) were maintained as described (5, 22, 26). Primary tumor growth was monitored by caliper measurements of orthogonal diameters. Tumor volume was calculated using the formula for determining a prolated ellipsoid [(dF × dL)/0.52], where d is the shorter of the 2 orthogonal measurements. To enhance visualization of metastatic nodules, lungs were insufflated with India ink dye, washed, and bleached in Fekete’s solution. The clonogenic assay to assess metastatic burden was conducted as described (22).

Real-time PCR

Lung DNA was analyzed by Real Time-PCR containing SYBR green PCR master mix (Applied Biosystem) and primers to amplify Cre (5′-GGAGCGCCGCAGAGATA-3′ and 5′-GCCACCAGTGTGACAT-3′) and endogenous mouse Cd81 (5′-TGCCGAAGGATGGAAGCA-3′ and 5′-CATTGTTGGCATCATCATCCA-3′). Assays were conducted in quadruplicate, and relative quantitation of the viral Cre gene present in lung tissue was calculated using the comparative threshold cycle (Ct) method (User Bulletin 2, Applied Biosystem) normalizing the target Ct values to the internal housekeeping gene (Cdh1).

Histology

Tissues were isolated and fixed in 10% neutral-buffered formalin or 4% paraformaldehyde, sectioned, and stained for histopathologic analysis with hematoxylin and eosin using standard methods. For immunofluorescent staining, 4-μm paraffin sections were deparaffinized in xylene and rehydrated with a graded alcohol series. Following antigen retrieval (vector), sections were washed and placed in 0.1% Triton for 10 minutes. Tissue was blocked in 40 μg/ml goat anti-mouse IgG-Fab (H+L) (Jackson ImmunoResearch) followed by

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10% normal goat serum (Jackson ImmunoResearch). Rabbit anti-mouse caveolin-1 (1:200; Cell Signaling) was incubated overnight at 4°C. Sections were washed and incubated with goat anti-rabbit Cy3 (1:200; Jackson ImmunoResearch). Tissues were mounted using Prolong Gold with DAPI (Invitrogen). To quantitate the blood vessel areas present within defined fields of caveolin-1–stained lung samples, 4 images were acquired per mouse from 5 WT and 5 Idel mice. Vessel boundaries were identified by caveolin-1 staining, and the area of every vessel within each field was determined using AxioVision Release 4.6 software.

Immunoprecipitation–Western Blot Analysis

Immunoprecipitation of IDO1 protein from mouse lung tissue with purified rabbit polyclonal antibody (7) followed by Western blotting–based detection with rat monoclonal antibody (clone mIDO-48; BioLegend) was carried out as described (9).

Flow Cytometry for Cytokine and Cell Analysis

Flow cytometric data were acquired on a FACSCanto II or Cyan ADP flow cytometer and analyzed using FACSDIVA (BD Biosciences) or Summit v4.3.02 (Beckman/Coulter) software. Multiplexed cytokine analysis was conducted using the Inflammation Bead Array (BD Biosciences). Flow cytometric analysis was conducted by the authors. Twenty-four–hour treatment of every vessel within each field was determined using AxioVision.

Kynurenine Assay

Lungs were homogenized in PBS containing dithiothreitol (DTT) and protease and phosphatase inhibitors (1:3 wt/vol). Deproteinated lysates were analyzed by high-performance liquid chromatography (HPLC) coupled to electrospray ionization liquid chromatography/ tandem mass spectroscopy (LC/MS-MS) analysis as described (9).

Cell Culture

U937 and HL-60 monocytic cell lines (American Type Culture Collection) were expanded for frozen storage after receipt and freshly thawed cells cultured in Dulbecco’s Modified Eagle’s Media + 10% FBS were used at early passage for experiments. No additional authentication was conducted by the authors. Twenty-four–hour treatment of cells with LPS (100 ng/mL; Sigma) and/or IFN-γ (100 ng/mL; R&D systems) was carried out in triplicate on 1 × 10⁶ cells per well in a 96-well dish. MTH-Trp (methylthiohydantoin-DL-tryptophan; 100 μmol/L; Sigma) was also included at the time of induction as indicated. Kynurenine and IL-6 levels in the supernatant were analyzed as described above. Idel gene “knockdown” studies were conducted with siRNAs (Dharmacon) targeting Idel (catalog no. E-010337-00) or Gapdh (catalog no. D-001930-01) using the Accell siRNA Delivery System (Dharmacon) as described by the manufacturer. HL-60 cells were plated at 1 × 10⁶ per well in a 96-well dish and cultured with 1% FBS in the Accell growth media. Twenty-four–hour treatment of cells with LPS and IFN-γ was initiated at 48 hours following incubation with siRNA. Western blotting to detect IDO1 protein in cell lysates was conducted following standard procedures using rabbit polyclonal anti-IDO1 (7) and rabbit monoclonal anti-β-actin (13E5; Cell Signaling) as a loading control. Detection was carried out with goat anti-rabbit IgG, horseradish peroxidase (HRP)- linked secondary antibody (catalog no. 7074; Cell Signaling) using the SuperSignal West Femto Chemiluminescent substrate (Thermo Scientific).

T-cell Suppression Assay

MDSC-suppressive activity was measured as previously described (53) using transgenic splenocytes and their cognate peptides in the presence of 25 Gy–irradiated, blood-derived MDSCs from 4T1 tumor–bearing mice. HA110-119, HA138-146, and Ova257-264 peptides were synthesized in the Biopolymer Core Facility at the University of Maryland, Baltimore, MD. ELISA duo set mAbs for mIL6 were from R&D Systems. Monoclonal antibody V88.1.8.2-PE was from BD PharMingen.

Disclosure of Potential Conflicts of Interest

G.C. Prendergast, A.J. Muller, and J.B. DuHadaway declare a potential conflict of interest with regard to IDO due to intellectual property, financial interests, grant support, and consultancy roles with New Link Genetics Corporation, which is engaged in the clinical development of IDO inhibitors for the purpose of treating cancer and other diseases. R. Metz is an employee of New Link Genetics Corporation as Director of Research and has financial and intellectual property interests in the company. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: C. Smith, S. Ostrand-Rosenberg, G.C. Prendergast, A.J. Muller


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Smith, M.Y. Chang, K.H. Parker, D.W. Beury, J.B. DuHadaway, J. Boulden, E. Sutanto-Ward, S. Ostrand-Rosenberg


Writing, review, and/or revision of the manuscript: C. Smith, D.W. Beury, A.P. Soler, L.D. Laury-Kleintop, L. Mandik-Nayak, R. Metz, S. Ostrand-Rosenberg, G.C. Prendergast, A.J. Muller

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Smith, J. Boulden, A.P. Soler, S. Ostrand-Rosenberg

Study supervision: C. Smith, S. Ostrand-Rosenberg, A.J. Muller, G.C. Prendergast

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