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Indoleamine 2,3-dioxygenase 1 inhibition targets anti-PD1-resistant lung tumors by blocking myeloid-derived suppressor cells

Ailin Li¹, Hampartsoum B. Barsoumian², Jonathan E. Schoenhals², Taylor R. Cushman², Mauricio S. Caetano², Xiaohong Wang³, David R. Valdecanas³, Sharareh Niknam², Ahmed I. Younes², Guang Li¹, Wendy A. Woodward², Maria Angelica Cortez³, and James W. Welsh^{2,*}

¹Department of Radiation Oncology, The First Hospital of China Medical University, China

²Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, USA

³Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, USA

Abstract

Indoleamine 2,3-dioxygenase 1 (IDO1), involved in the catabolism of tryptophan (Trp) to kynurenine (Kyn) is an important regulator of tumor-mediated immunosuppression implicated in resistance to anti-PD1 immunotherapy. We investigated the role of IDO1 in an anti-PD1-resistant lung cancer model (344SQ_R) compared to the parental 344SQ tumors (344SQ_P). IDO1 was overexpressed in tumor-infiltrating leukocytes, and plasma Kyn levels were increased, in 344SQ_R vs. 344SQ_P. The IDO1 inhibitor INCB023843 retarded tumor growth and reduced lung metastases in 344SQ_R. IDO1 was expressed at higher levels in F4/80⁺Gr1^{int}CD11b⁺ myeloid-derived suppressor cells (MDSCs) that were prominent in 344SQ_R. The INCB023843 reduced IDO1 expression and percentages of these MDSCs while increasing CD8⁺ T cells infiltration, hence reactivating antitumor T-cell responses in 344SQ_R. Therefore, IDO1 inhibition holds promise for treating lung cancer that does not respond to anti-PD1 therapy.

Keywords

IDO1; anti-PD1 resistance; lung cancer; immunotherapy; myeloid-derived suppressor cells

Introduction

Anti-programmed cell death 1 (anti-PD1) monoclonal antibodies are among the most effective cancer immunotherapeutic agents available; however, a substantial proportion of patients present with or develop resistance to them [1]. Notably, partial or complete response with PD1 inhibitors are achieved in only 15% to 20% of patients with non-small cell lung

*Corresponding author: James W. Welsh, MD, Department of Radiation Oncology, Unit 97, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030 USA. Tel 713-563-2447; jwelsh@mdanderson.org.

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cancer (NSCLC), and the 1-year progression-free survival rate for such patients is only 19% to 21% [2-4]. Until recently, little was known about the mechanisms underlying resistance to anti-PD1, leading to numerous efforts to clarify and overcome such mechanisms [5].

One potential explanation for tumor resistance to immunotherapies (including anti-PD1) involves immunosuppressive events that impede the accumulation of tumor-infiltrating T effector cells [6, 7]. Indoleamine-2,3-dioxygenase 1 (IDO1) is an important regulator of tumor-mediated immunosuppression [6, 8]; high IDO1 activity has been associated with solid tumor progression and metastasis, and overexpression of IDO1 in the peritumoral stroma has been linked with poor prognosis in patients with NSCLC [9-12]. IDO1 is expressed by tumor-associated macrophages, myeloid-derived suppressor cells (MDSCs), tolerogenic dendritic cells (DCs) [13-15], and tumor cells [16]. IDO1 is an intracellular enzyme that initiates the rate-limiting step in the catabolism of the essential amino acid tryptophan (Trp) to kynurenine (Kyn) [17]. IDO1 restricts the availability of Trp and promotes the accumulation of Kyn, which decreases CD8⁺ T-cell proliferation, promotes apoptosis and anergy of T cells in the tumor microenvironment (TME) [17]. Tumor-expressed IDO1 has also been shown to correlate with recruitment and activation of MDSCs with negative effects on T cells [14, 18]. IDO1 inhibitors have been shown to produce durable suppression of IDO1 activity in plasma, decrease MDSCs, and abolish their suppressive function on effector T cells; IDO1 inhibitors have also shown antitumor activity in clinical trials [18-20].

Knockout of IDO1 in mice with melanoma treated with anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) was linked with the accumulation of effector T cells and enhanced antitumor effects [7]. Combining anti-PD1 and anti-programmed cell death ligand 1 (PDL1) treatment in IDO1-deficient mice also resulted in reduced melanoma tumor growth and improved survival [7]. Little is known as to whether IDO1 mediates anti-PD1 resistance and whether IDO1 inhibitors would reactivate antitumor immune response in anti-PD1-resistant NSCLC. Here we report that high IDO1 expression in tumor-infiltrating leukocytes (TILs) is involved in anti-PD1 resistance mechanisms in a preclinical anti-PD1-resistant lung adenocarcinoma model (344SQ_R, previously generated from the 344SQ parental model 344SQ_P) [21]. In addition, our findings suggest that the IDO1 inhibitor INCB023843 can reactivate antitumor immune responses specifically by blocking IDO1-expressing F4/80⁺Gr1^{int}CD11b⁺ MDSCs in anti-PD1 resistant tumors.

Materials and Methods

Cell lines and IDO1 inhibitor

The 344SQ murine lung cancer cell line (344SQ_P) was a generous gift from Dr. Jonathan Kurie (MD Anderson Cancer Center). Cells were maintained in RPMI-1640 supplemented with 10% FBS [22]. An anti-PD1-resistant cell line (344SQ_R) originated from implanted 344SQ_P cells treated with anti-PD1 and passaged in 129Sv/Ev mice as described previously [21]. Both cell lines were validated by DDC Medical (<http://ddcmedical.com>; Fairfield, OH) by short-tandem-repeat (STR) DNA fingerprinting. The IDO inhibitor INCB023843 was synthesized by Incyte [20, 23]. 1-MT (Xcessbio M60291-2s) and NLG919 (Seleckchem S7111) were obtained commercially.

Animal experiments

All animal procedures were approved by UT MD Anderson Cancer Center Animal Care and Use Committee. For the one-tumor model, 8 to 12 week old 129Sv/Ev mice received a subcutaneous injection of 0.5×10^6 344SQ_P or 344SQ_R cells in the right leg only. For the two-tumor model, primary tumor (0.5×10^6 344SQ_P or 344SQ_R cells) was injected in the right leg while secondary tumor (0.2×10^6 cells) was injected in the left leg on the same day. When tumors became visible, INCB023843 was reconstituted in 0.5% methylcellulose (Sigma-Aldrich, Cat#M0430) dissolved in deionized water; mice were weighed and given a 300 mg/kg dose by oral gavage once per day. Tumors were measured every other day using calipers, and tumor volume was calculated as $0.5(\text{length} \times \text{width}^2)$. The INCB023843 treatment started on day 3 after inoculation in the one-tumor model and on day 8 in the two-tumor model. For assessment of lung metastasis, lungs were collected 2 h after the final dose of INCB023843, fixed in Bouin's solution (Sigma-Aldrich, Cat#HT10132), and metastatic nodules were counted. Tumors and tumor-draining lymph nodes (TDLNs) were collected on day 14 after inoculation, and blood samples were collected on day 20, 2 h after INCB023843 treatment. Tumors were harvested after 7 doses of INCB023843 for immune profiling and IDO1 expression.

Trp and Kyn quantification

Trp and Kyn levels in mice plasma were quantified using an analytical method that combined protein precipitation and extraction with trichloroacetic acid and liquid chromatography coupled with tandem mass spectrometry analysis (LC/MS/MS). The linear range of this assay was 20 to 10,000 nmol/L for Kyn and 200 to 100,000 nmol/L for Trp when analyzing 0.1 mL samples. Aqueous standards were prepared to avoid the need to adjust for endogenous Trp and Kyn present in biological samples [20].

Isolation of F4/80-positive TILs and treatment with IDO1 inhibitor

TILs were collected from ten 344SQ_R tumors. F4/80-positive TILs were isolated using anti-F4/80 microbeads ultrapure kit (Miltenyi Biotec, Cat#130-110-443) and column (Miltenyi Biotec, Cat#130-042-401). Then, F4/80-positive TILs were counted and cultured in 12-well plates. IDO1 inhibitor was added at 0, 0.3, 1, or 3 μM and cells were collected 48h later.

RNA extraction and qPCR

Total RNA was isolated from TILs, TDLNs, and F4/80-positive TILs by using the RNeasy Mini Kit (Qiagen, Cat#74104). RNA was extracted from tumors with the chloroform/phenol method after frozen tissues were homogenized in Trizol (Thermo Fisher Scientific, Cat#15596018). RNA quality and quantity were assessed with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). Next, cDNA was synthesized using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Cat#1708841). Quantitative polymerase chain reaction was done with a Fast SYBR Green Master Mix (Thermo Fisher Scientific, Cat#4385612) with Bio-Rad's PrimePCR SYBR Green Assay IDO1 mouse primers (Unique Assay ID qMmuCID0021139).

Cell staining and flow cytometry

TILs isolation was done as previously described [21]. TDLNs were processed, suspended in RPMI-1640, then washed and filtered. Tumor cells or TILs were blocked with anti-mouse CD16/32 (FcR blocker; BioLegend, Cat#101302) for 10 min at room temperature, and then stained with Pacific blue-CD45 (BioLegend, Cat#103126), APC/Cy7-CD4 (BioLegend, Cat#100414), PerCP/Cy5.5-CD8a (BioLegend, Cat#100734), PE-CD11c (BioLegend, Cat#117308), FITC-F4/80 (BioLegend, Cat#123108), APC/Cy7-CD11b (BioLegend, Cat#101226), and PE/Cy7-Gr-1 (BioLegend, Cat#108416) at room temperature for 30 min. For intracellular staining cells were fixed and permeabilized according to the manufacturer's instructions (eBioscience, Cat#00-5523-00) and stained with PE-FOXP3 (eBioscience, Cat#12-5773-82) and Alexa Fluor 647-IDO1 (BioLegend, Cat#654004). All samples were analyzed with LSRII flow cytometer and FlowJo software (version 10.0.7).

CCL22 enzyme-linked immunosorbent assay

Levels of CCL22 in plasma were measured with a mouse CCL22 ELISA kit (R&D Systems, Cat#MCC220) as described by the manufacturer. Absorbance values were read at 450 nm with a PerkinElmer Multimode plate reader (PerkinElmer, Inc. Waltham, MA). Protein concentrations in experimental samples were calculated by using GraphPad Prism 6.

Statistical analyses

Data were analyzed with GraphPad Prism 6 software. Statistical significance was determined by Student's *t*-tests or multiple *t*-tests between two groups. A two-tailed *P*-value 0.05 was considered statistically significant. Wherever indicated, two-way ANOVA was conducted to compare treatment groups. All experiments were repeated two to four times.

Results

Kyn and Kyn:Trp in plasma is higher in anti-PD1 resistant tumors compared to control

Kyn levels and Kyn:Trp of plasma were analyzed as a measure of IDO1 activity in vivo [8]. Mice with 344SQ_R implants had Kyn levels that were 4.03 times higher than in mice with 344SQ_P implants (mean $\pm$ standard error of the mean [SEM] 3347 ± 987.8 nM in 344SQ_R vs. 830.2 ± 116.9 nM in 344SQ_P, Fig. 1A) and a 2.78-fold increase in Kyn:Trp (38.29 ± 12.10 in 344SQ_R vs. 13.77 ± 0.6762 in 344SQ_P, Fig. 1B). Because Kyn levels also correlate with tumor burden [24], we next analyzed Kyn levels normalized by tumor volume (mm^3) and found that the Kyn level/tumor volume was 1.89 times higher in 344SQ_R than in 344SQ_P (2.339 ± 0.7923 nM/ mm^3 in 344SQ_R vs. 1.235 ± 0.03888 nM/ mm^3 in 344SQ_P, Fig. 1C). Thus IDO1 activity was higher in the anti-PD1-resistant 344SQ_R model than in the anti-PD1-sensitive 344SQ_P model.

IDO1 inhibition suppresses tumor growth and lung metastases in anti-PD1 resistant tumors

Since IDO1 activity was different in the 344SQ_P and 344SQ_R one-tumor model, we tested the effect of INCB023843 on tumor growth and metastasis. We confirmed that plasma Kyn levels and Kyn:Trp were higher in 344SQ_R than in 344SQ_P, and they were inhibited

by INCB023843 in both models (Fig. 2A). However, tumor growth was impeded only in the 344SQ_R (Fig. 2B). In the two-tumor model, plasma Kyn levels and Kyn:Trp were found to increase gradually with tumor growth in the untreated control groups (Fig. 2C), suggesting that IDO1 expression (or activity) correlates with more extensive disease. Moreover, Kyn levels and Kyn:Trp were higher in the 344SQ_R than in the 344SQ_P (Fig. 2C). Treatment with INCB023843 decreased both Kyn and Kyn:Trp to similarly low levels in 344SQ_P and 344SQ_R at all time points (Fig. 2C). However, INCB023843 inhibited tumor growth only in the 344SQ_R (Fig. 2D). IDO1 inhibitors have been tested in clinical trials of patients with metastatic disease [19], therefore we analyzed lung metastasis in 344SQ_P and 344SQ_R two-tumor models. The numbers of lung surface metastatic nodules were higher in the 344SQ_R than in the 344SQ_P and were also reduced only in 344SQ_R. (Fig. 2E and 2F). No tumor growth differences were observed in the secondary tumors between control and INCB023843 treatment groups (Fig. S1A). As a measure of gross toxicity, changes in mouse body weights were not detected with treatment (Fig. S1B).

IDO1 is overexpressed in TILs in 344SQ_R vs. 344SQ_P tumors and is decreased by IDO1 inhibitor only in 344SQ_R tumors

In an attempt to determine the source of the high IDO1 in the 344SQ_R model, we measured IDO1 expression in TILs, tumors, and TDLNs. IDO1 mRNA expression in TILs was 11.84 times higher in the 344SQ_R than in TILs from 344SQ_P (11.84 ± 6.605 , Fig. 3A). However, no difference was found in IDO1 mRNA expression in tumors from the 344SQ_P vs. 344SQ_R (Fig. 3B). IDO1 mRNA expression was drastically lower in the TDLNs than in TILs in both 344SQ_P and 344SQ_R (0.1292 ± 0.01498 for 344SQ_P and 0.01928 ± 0.002082 for 344SQ_R, Fig. S2A), but no difference in IDO1 expression was noted between them (Fig. S2A). Flow cytometry analysis also showed the presence of an IDO1⁺ population among CD45⁺ TILs but nearly no IDO1⁺ cells among CD45⁺ cells in TDLNs (Fig. S2B). We further found that CD11b⁺ cells were rare in the TDLNs (Fig. S2C), which may explain the relative lack of IDO1⁺ myeloid cells in TDLNs. Since IDO1 activity was higher with two transplanted tumors (Fig. 2C) and IDO1 inhibition impeded primary tumor growth in the two-tumor model (Fig. 2D), we carried out the next experiments with mice bearing two tumors. IDO1 mRNA expression was decreased after IDO1 inhibition only in 344SQ_R (Fig. 3C). More importantly, more CD45⁺ TILs expressed IDO1 in 344SQ_R tumors than in 344SQ_P, and that percentage significantly decreased after INCB023843 treatment in the 344SQ_R tumors only (Figs. 3D and 3E). Moreover, the majority of IDO1⁺ cells were CD11b⁺ of myeloid origin in both 344SQ_P and 344SQ_R models (Figs. 3F and 3G). The same cell subtype expressed IDO1 and IFN γ in 344SQ_P and 344SQ_R tumors (Fig. 3H). The IFN γ mRNA expression was 2.374 ± 0.5861 times higher in TILs from 344SQ_R versus 344SQ_P tumors and dramatically decreased after IDO1 inhibition only in 344SQ_R tumors (Fig. 3I).

IDO1 inhibition reactivates CD8⁺ T cells in 344SQ_R tumor by targeting IDO1-expressing F4/80⁺Gr1^{int}CD11b⁺ MDSCs

Evaluation of MDSCs (Gr1⁺CD11b⁺) in our two-tumor model revealed two subpopulations: one expressed high Gr1 (Gr1^{hi}CD11b⁺) and the other low or intermediate Gr1 (Gr1^{int}CD11b⁺) (Fig. 4A). Interestingly, very few Gr1^{hi}CD11b⁺ MDSCs were IDO1⁺ (Fig. 4B), but an

IDO1⁺ population was clearly present among the Gr1^{int}CD11b⁺ MDSCs and nearly all were F4/80⁺ (Fig. 4C). Moreover, the percentage of Gr1^{int}CD11b⁺ MDSCs (among CD45⁺ TILs) was higher in 344SQ_R than in 344SQ_P ($P=0.0011$, Fig. 4D) and that percentage was reduced by INCB023843 only in the 344SQ_R ($P=0.01$, Fig. 4D). The percentage of F4/80⁺Gr1^{int}CD11b⁺ MDSCs (among CD45⁺ TILs) was higher in 344SQ_R than in the 344SQ_P ($P=0.0023$, Fig. 4E) and was reduced by INCB023843 in 344SQ_R ($P=0.007$, Fig. 4E), which contributed to the difference evident in Figure 4D. The same pattern was apparent for the percentage of IDO1⁺F4/80⁺Gr1^{int}CD11b⁺ MDSCs (among CD45⁺ TILs; higher in 344SQ_R than in 344SQ_P, $P=0.0016$; and decreased by INCB023843 in 344SQ_R, $P=0.002$; Fig. 4F). Finally, the percentage of IDO1⁺ cells among F4/80⁺Gr1^{int}CD11b⁺ MDSCs was significantly higher in untreated 344SQ_R than in untreated 344SQ_P ($P=0.009$, Fig. 4G) and that percentage was reduced by INCB023843 only in 344SQ_R ($P=0.011$, Fig. 4G). Some may argue that IDO1 inhibitor antagonizes only IDO1 activity but not IDO1 expression, which may reflect inconsistency with our flow cytometry findings (Fig. 4G). For that purpose, we separated F4/80⁺ cells from TILs of 344SQ_R primary tumors in the two-tumor model and incubated with different concentrations of IDO1 inhibitor (0, 0.3, 1, or 3 μ M) for 48h. The qPCR findings showed that IDO1 expression in F4/80⁺ TILs was downregulated in a dose-dependent manner (expression totally abrogated with 3 μ M INCB023843, Fig. 4H). This was not the case for the IDO inhibitors 1-MT and NLG-919 (GDC-0919) where downregulation in IDO1 mRNA expression was not detected (Fig. S3). Looking more into cellular mechanisms, the percentage of CD8⁺ T cells was lower in the untreated 344SQ_R than in the untreated 344SQ_P ($P=0.0003$, Figs. 4I and 4J), and treatment with INCB023843 increased this percentage but only in 344SQ_R ($P=0.0332$, Figs. 4I and 4J). We further noted an increase in plasma CCL22 level in 344SQ_R relative to 344SQ_P that was significantly reduced by INCB023843, but only in the 344SQ_R ($P=0.008$, Fig. S4A). However, we found no difference in T regulatory cells (Tregs) between the 344SQ_R versus the 344SQ_P, and INCB023843 did not affect the percentage of Tregs (Fig. S4B and S4C).

IDO1 inhibition reduces the percentage of IDO1⁺CD11b⁺ DCs in 344SQ_R tumors

We next found two subpopulations of DCs (CD11c⁺CD11b⁺ and CD11c⁺CD11b⁻, Figs. 5A and 5B). CD11c⁺CD11b⁺ DCs accounted for about 80% of DCs in TILs and majority of them were CD8⁻ (Fig. 5C). IDO1 was expressed only in the CD11c⁺CD11b⁺ DCs (Figs. 5B), and the percentage of IDO1⁺CD11b⁺ DCs (among CD11c⁺ cells) in TILs was reduced by INCB023843 only in the 344SQ_R ($P=0.0302$, Fig. 5D). CD11b⁺ DCs and IDO1⁺ DCs were not found in the TDLNs (Figs. 5E and 5F).

Discussion

Anti-PD1 antibodies are effective immunotherapeutic agents for NSCLC; however, anti-PD1 resistance is common, underscoring the need to clarify the mechanisms of action and find other effective immunotherapeutic approaches. Anti-PD1 antibodies have been reported to remain functional when resistance develops and maintain the ability to prevent inhibitory signals through PD1, which suggests that mechanisms other than PD1- PDL1 interaction contribute to anti-PD1 resistance [25]. One such mechanism may be due to the

immunosuppressive TME via suppression of CD8⁺ effector cells [6, 26]. IDO1 catabolizes Trp to Kyn, which is cytotoxic and inhibits the clonal expansion of effector T cells [27] and generates and activates MDSCs [17, 18]. In one example, IDO1-mediated immunosuppression has been linked to immunotherapy resistance, where overexpression of IDO1 in melanoma cells was found to confer resistance to CTLA4 blockade compared with wild-type melanoma cells [7]. This evidence led us to explore the differences in IDO1 expression (or activity) in our anti-PD1-resistant mouse model (344SQ_R) as compared to anti-PD1-sensitive 344SQ parental mouse model (344SQ_P).

In our experiments, 344SQ_R showed higher plasma Kyn levels and Kyn:Trp than the 344SQ_P, which links high endogenous IDO1 expression (or activity) to anti-PD1 resistance. Although most studies have reported that IDO1 expression by tumor cells drives local immunosuppression [16, 20], we found no difference in IDO1 expression between 344SQ_P and 344SQ_R tumors. We found higher IDO1 expression in TILs from 344SQ_R tumors, which is supported by evidence from others showing that IDO1 expression by host immune cells is important to immunotherapy resistance [7, 28-30]. In addition, two other dioxygenases that generate Kyn, indoleamine 2,3-dioxygenase 2 (IDO2) and Tryptophan 2,3-dioxygenase 2 (TDO2), could not be detected in TILs in these two models (data not shown), which excluded the possibility of inference by IDO2 and TDO2.

Increased IDO1 activity levels have not only been reported in patients with liquid tumors such as chronic lymphocytic leukemia (CLL) [31], but also in patients with colorectal cancer [28], gynecological cancer [29], and melanoma [30]. Our findings corroborate that tumor-infiltrating myeloid cells were the main source of IDO1 production, leading us to explore the various subsets that expressed IDO1. In mice, most MDSCs express both CD11b and Gr-1 [13, 32]. We found that IDO1 was expressed mainly in Gr-1^{int}CD11b⁺ MDSCs and that the percentages of Gr-1^{int}CD11b⁺ MDSCs were higher in CD45⁺ TILs from 344SQ_R vs. 344SQ_P. The CD11b⁺Gr-1^{int} MDSC subset, consisting mainly of monocytic M-MDSCs, strongly suppress CD8⁺ T-cell activation in an antigen-nonspecific manner relative to CD11b⁺Gr-1^{hi} MDSCs, with the extent of suppression proportional to the abundance of these cells [18, 32-34]. Holmgaard et al. reported that tumor-expressed IDO1 induced the expansion and migration of CD11b⁺Gr-1^{int} MDSCs in the TME in a B16 breast cancer model and that their activation to functional MDSCs in tumors was IDO1-dependent [18]. However, that group showed that the source of IDO1 and the immunosuppressive effects was the tumor, and the contribution of host-derived IDO1 was absent or minor [18]. In contrast, Jitschin et al. reported that CLL cells induced the conversion of monocytes into high-IDO1-expressing M-MDSCs, which suppressed T-cell proliferation; and the presence of these MDSCs correlated negatively with T-cell counts in patients. The interpretation was that accumulation of these high IDO1-expressing M-MDSCs aggravated immunosuppressive effects and accelerated the growth of malignant cells [31], underscoring the importance of IDO1 derived from immune cells. We also found IDO1 was expressed only in F4/80⁺ subpopulation of CD11b⁺Gr-1^{int} MDSCs and the percentages of F4/80⁺Gr-1^{int}CD11b⁺ MDSCs were higher in CD45⁺ TILs in 344SQ_R compared to 344SQ_P. Liu et al. reported that naïve F4/80⁺Gr-1^{int}CD11b⁺ MDSCs could not inhibit T cell activation and had the ability to induce apoptosis of activated T cells only after they entered the TME [35]. Taken together, we hereby showed that F4/80⁺Gr-1^{int}CD11b⁺ MDSCs are the main and important

source for IDO1 expression in tumor-infiltrating immune cells, hence generating an immunosuppressive environment capable of driving resistance to anti-PD1 therapy.

Previous encouraging results have demonstrated the feasibility of MDSC-based therapy [36]. In line with our results, Holmgaard and colleagues demonstrated in a murine melanoma model, IDO1 inhibition resulted in decreased MDSCs within the TME [18]. However, melanoma is considered highly immunogenic and responds well to immunotherapies relative to less immunogenic cancers. Yu et al. reported that inhibition of IDO enzymatic activity by 1-MT significantly attenuated MDSC-mediated suppression on T cell proliferation and promotion of T cell apoptosis. However, to date, there was no data regarding the role of subtype-specific IDO inhibitors in attenuating MDSC-mediated immunosuppression [14]. Our data fill this knowledge gap by demonstrating that IDO1-specific inhibitor INCB023843 decreased the percentages of F4/80⁺Gr1^{int}CD11b⁺ MDSC subtype, consequently reactivating CD8⁺ T cells and thus increasing anti-tumor immune responses.

We also found that INCB023843 decreased IDO1 expression level in these F4/80⁺ cells in TILs in a dose-dependent fashion in vitro. Previous studies reported that another IDO1-specific inhibitor (INCB024360) inhibits IDO1 enzymatic activity and not its expression in HeLa cells and DCs in vitro [8]. The same group also reported INCB023843 treatment did not impact the IDO1 expression of CT26 tumors but decreased Kyn levels in vivo [20]. To our knowledge, our study is the first to report IDO1 inhibition decreased IDO1 expression of F4/80⁺Gr1^{int}CD11b⁺ MDSCs within the TME. In our model, INCB023843 reduced IFN γ levels that can impact IDO1 expression. However, it is also shown that the expression could rely on the IFN γ independent Kynurenine-Aryl hydrocarbon receptor (Kyn-AhR) axis. The IDO activity sustains its own expression via an autocrine AhR-IL-6-STAT3 signaling loop [37]. With that rationale, the IDO inhibitor (INCB023843) binds to IDO1 leading to the downregulation of Kyn levels. This in turn leads to decreased AhR activation and reduction in IDO1 mRNA expression [38, 39]. In contrast, Moyer BJ et al. [40] show that IDO1 inhibitors may have dual function and rather activate AhR which stimulates a pro-inflammatory response, but may also lead to IDO production down the loop which requires further investigation.

In conclusion, our study provides preclinical evidence that anti-PD1 resistance in lung cancer is associated with IDO1 overexpression in F4/80⁺Gr1^{int}CD11b⁺ MDSCs. Treatment with an IDO1 inhibitor reduced IDO1 expression and blocked the accumulation of F4/80⁺Gr1^{int}CD11b⁺ MDSCs, leading to the reactivation of antitumor immune responses (Fig. 6). These findings suggest that high IDO1 levels in immune cells, as well as high Trp levels and Kyn:Trp in plasma, may be useful biomarkers of anti-PD1 treatment resistance, a finding that would be valuable for screening and clinical trials. Therefore, IDO1 inhibition represents an alternative immunotherapeutic strategy to overcome the immunosuppressive TME in anti-PD1-resistant tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PD1	programmed cell death protein 1
NSCLC	non-small cell lung cancer
IDO1	Indoleamine 2,3-dioxygenase 1
MDSCs	myeloid-derived suppressor cells
DCs	dendritic cells
Trp	Tryptophan
Kyn	Kynurenine
TME	tumor microenvironment
CTLA4	cytotoxic T-lymphocyte-associated antigen 4
PDL1	programmed cell death ligand 1
TILs	tumor-infiltrating leukocytes
TDLNs	tumor-draining lymph nodes

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Figure 1.

Generation of an anti-PD-1-resistant lung tumor mouse model. A, Schematic illustration. 344SQ mouse lung cancer cells were subcutaneously injected into the flank the syngeneic 129Sv/ev mice on day 0. Anti-mouse PD-1 or isotype control (ctrl) IgG antibodies (10 mg/kg) were administered intraperitoneally on days 3, 7, 10, and 14. Tumor growth was monitored for up to 4 weeks. A nonresponsive tumor was isolated and digested into a single-cell suspension. Tumor cells were cultured *in vitro* for 2 to 3 weeks and then reinoculated into 129Sv/ev mice, followed by anti-PD-1 treatment. This procedure was repeated for four cycles. B, Representative tumor growth curve of parental 344SQ cells and the anti-PD-1-resistant 344SQ cells upon control IgG or anti-PD-1 treatment. Data represented as mean $\pm$ SD from an n of 5. ***, $P < 0.001$, multiple t tests; experiments were also repeated at least three times. C, Representative picture of hematoxylin and eosin staining of parental and anti-PD-1-resistant tumors (magnification, x200). Red enclosed area, tumor necrosis; black arrows, mitotic tumor cells.

Figure 2.

Downregulation of MHC molecule expression on anti-PD-1-resistant tumors. A, Flow cytometry studies of MHC class I (H-2D^b and H-2K^b) and MHC class II (I-A/I-E) expression on 344SQ_P and 344SQ_R tumor cells (gated on CD45⁺) after cocultured with syngeneic splenocytes (gated on CD45^b). MFI, mean fluorescence intensity. B, H-2K^b and I-A/I-E expression on the parental and anti-PD-1-resistant tumors isolated from tumor-bearing mice with anti-PD-1 or control (ctrl) IgG treatment. C, Western blotting of b2M in parental and resistant tumor tissues. Vinculin was used as a loading control. *, $P < 0.05$; ***, $P < 0.001$ in Student t tests; data represent means $\pm$ SD for an n of 5, with experiments repeated at least three times.

Figure 3.

Reduced infiltration and function of TILs in anti-PD-1-resistant tumors. A, Representative flow cytometry staining of CD4^b and CD8^b T cells in immune cells isolated from parental and anti-PD-1-resistant tumors. Ctrl, control. B, Percentages of CD45^bCD4^b and CD45^bCD8^b T cells in the gated lymphocytes isolated from tumors. C and D, Total numbers of tumor-infiltrating immune cells (CD45^b; C) and CD45^bCD4^b and CD45^bCD8^b T cells (D) isolated from parental and anti-PD-1-resistant tumors. E, Total numbers of IFNγ-producing CD4^b and CD8^b TILs in tumors. Data represent means ± SD for an *n* of 5, with experiments

repeated at least three times. *, $P < 0.05$; **, $P < 0.01$ in Student t tests; N.S., not statistically significant.

Figure 4.

Radiation increases MHC class I expression and overcomes anti-PD-1 resistance. Mice bearing anti-PD-1-resistant 344SQ tumors were either untreated or irradiated with three 12-Gy fractions. At 5 days after radiation, tumors were isolated, digested into single cells, and stained with live/dead dye, fluorochrome-conjugated CD45, and H-2D^b and H-2K^b antibodies. Tumor cells were gated on a CD45⁺ population. A, Radiation significantly increased MHC class I (H-2D^b) expression on the anti-PD-1-resistant tumors. MFI, mean fluorescence intensity. B, Radiation resensitized tumors, which allowed the tumors to

respond to anti-PD-1 treatment. 344SQ_R cells were inoculated into syngeneic 129Sv/ev mice. At 8 days after inoculation, tumors had reached about 100 mm³ and were irradiated with three 12-Gy fractions over 3 days. The first dose of anti-PD-1 (10 mg/kg) was given on the same day as the first radiation dose and continued for three more doses (twice per week). $P < 0.001$ for XRT versus control groups at days 19, 21, 23, and 26; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for XRT þ anti-PD-1 versus XRT groups at days 21, 23, 26, and 28. Data represent means $\pm$ SD for an n of 7 to 8 per group, with experiments repeated three times.

Figure 5.

Radiation plus anti-PD-1 induces tumor regression and increases proportions of CD8⁺ T cells in both irradiated and nonirradiated tumors. A, Radiation plus anti-PD-1 synergistically enhanced the antitumor response by controlling both irradiated tumors and other, nonirradiated, tumors and by reducing the number of spontaneous lung metastases in the 344SQ parental tumor model. 2°, secondary. Primary tumors were established in the right leg by subcutaneous injection of 1×10^6 344SQ_P cells into 129Sv/ev mice. Other nonirradiated tumors were established on the left leg by subcutaneous inoculation of the

same amount of 344SQ_P cells in the same mouse on day 10 after primary tumor inoculation. On day 14, when the primary tumors had reached about 100 mm³, primary tumors were irradiated with three 12-Gy fractions over 3 days. Anti-PD-1 treatment was given as described for Fig. 4B; $n \frac{1}{4}$ 7 to 8 per group. In the primary irradiated tumors, $P < 0.01$ for XRT þ anti-PD-1 versus anti-PD-1 groups from day 21 to 42 (the endpoint), and $P < 0.05$ or <0.01 for XRT þ anti-PD-1 versus XRT groups from day 33 to 42. In the nonirradiated tumors, P values were <0.05 or <0.01 for control (ctrl) versus anti-PD-1 groups from day 13 to 31 (the endpoint), and P values were <0.01 or <0.001 for XRT versus XRT þ anti-PD-1 groups from day 13 until the end of the experiment. B, Radiation plus anti-PD-1 treatment increased the proportion of CD8^b T cells in both irradiated and nonirradiated tumors. For these experiments, 344SQ_P cancer cells (1×10^6) were injected in the right leg and 2×10^5 cells in the left leg of mice on the same day. Radiation and anti-PD-1 treatments were the same as described for A. Tumors and spleen were collected on day 7 after radiation treatment. $n \frac{1}{4}$ 5 per group. Data represent means $\pm$ SD, with experiments repeated three times. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 6.

Radiation induced IFN β , but not IFN γ , production. Mice bearing 344SQ_R tumors were either untreated or irradiated to a total dose of 36 Gy, given in three daily 12-Gy fractions. Five days later, plasma was collected for cytokine detection (via Multiplex). (B) Both histograms and (C) bar graphs of mean fluorescence intensity from flow cytometry show dose-dependent induction of MHC-I (H-2Kb and H-2Db) and MHC class II (I-A/I-E) by IFN β . For these studies, 344SQ_P and 344SQ_R cells were cultured in vitro and treated with IFN β (100–10,000 U/mL) for 24 h. Cells were then stained for cell-surface expression of H-2Kb, H-2Db, and I-A/I-E and subjected to flow cytometry. Experiments were repeated twice. (D) Radiation-induced sensitization to anti-PD-1 is blunted by antibody blockade of the IFN α/β receptor I (IFNAR1). Mice bearing anti-PD-1-resistant 344SQ tumors were treated with radiation (36 Gy in three daily 12-Gy fractions) and anti-PD-1 (10 mg/kg, intraperitoneal injection), with or without anti-IFNAR1 (1 mg/kg, intratumoral injection). Tumor growth was monitored. P values were <0.05 or <0.01 for XRT+anti-PD1 vs XRT +anti-PD1+anti-IFNAR1 groups from day 17 to 24 (the end point). Data represent means $\pm$ SD for an n of 7, with experiments repeated twice. (E) Model of anti-PD-1 resistance. Tumors that respond to treatment with anti-PD-1 express higher levels of MHC class I, which can stimulate tumor-infiltrating lymphocyte proliferation and activation, promoting strong antitumor immune responses that cause tumor regression. In contrast, anti-PD-1-resistant tumors express relatively lower levels of MHC class I, and thus have less T cell proliferation and activation. As a result, defective antitumor immunity cannot control tumors, and tumors continue progressing. Radiation induces type I IFN, which enhances MHC I expression, resulting in resensitization to anti-PD-1 treatment.

Table 1

Morphologic features of parental and resistant tumors

Morphologic features	Tumor cell type and treatment condition			
	Parental p Control IgG	Parental p Anti-PD-1	Resistant p Control IgG	Resistant p Anti-PD-1 Tubular formation ^d
1 Tubular formation ^a	Yes	Yes	None or little	None or little
Necrosis rate mean (%±SD) ^a	7.3 ± 2.9	22.8 ± 18 ^b	9 ± 4.8	10.9 ± 13.7
Cell size ^c	>4 RBC	>4 RBC	>4 RBC	>4 RBC
Nuclear/cytoplasm ratio ^c	50–80	50–80	>80	>80
Nuclear pleomorphism ^a	Mild	Mild	Marked	Marked
Mitotic count median per field ^c (25%–75%)	8 (5–10)	9 (5–10)	31 (30–50)	37 (30–50)
Immune cell infiltration ^a	Moderate	High	Discrete	Discrete

Abbreviation: RBC, red blood cell.

^aFeatures are evaluated in ×200 magnifications.

^b*P* < 0.05 versus parental plus control IgG group.

^cFeatures are evaluated in ×400 magnifications.