Microenvironment and Immunology

Immunotherapeutic Suppression of Indoleamine 2,3-Dioxygenase and Tumor Growth with Ethyl Pyruvate

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Abstract

Efforts to improve cancer care in the developing world will benefit from the identification of simple, inexpensive, and broadly applicable medical modalities based on emergent innovations in treatment, such as targeting mechanisms of tumoral immune tolerance. In this report, we offer preclinical evidence that the low-cost, anti-inflammatory agent ethyl pyruvate elicits a potent immune-based antitumor response through inhibition of indoleamine 2,3-dioxygenase (IDO), a key tolerogenic enzyme for many human tumors. Consistent with its reported ability to interfere with NF-κB function, ethyl pyruvate blocks IDO induction both *in vitro* and *in vivo*. Antitumor activity was achieved in mice with a noncytotoxic dosing regimen of ethyl pyruvate shown previously to protect against lethality from sepsis. Similar outcomes were obtained with the functional ethyl pyruvate analogue 2-acetamidoacrylate. Ethyl pyruvate was ineffective at suppressing tumor outgrowth in both athymic and *Ido1*-deficient mice, providing *in vivo* corroboration of the importance of T-cell–dependent immunity and IDO targeting for ethyl pyruvate to achieve antitumor efficacy. Although ethyl pyruvate has undergone early-phase clinical testing, this was done without consideration of its possible applicability to cancer. Our findings that IDO is effectively blocked by ethyl pyruvate treatment deepen emerging links between IDO and inflammatory processes. Further, these findings rationalize oncologic applications for this agent by providing a compelling basis to reposition ethyl pyruvate as a low-cost immunochemotherapy for clinical evaluation in cancer patients. *Cancer Res; 70(5); 1845–53. ©2010 AACR.*

Introduction

Mounting evidence argues that immune escape through the establishment of dominant, immune tolerance is a fundamental hallmark of tumor progression (1), and it is likely that successful immunotherapy will require the implementation of strategies to overcome this barrier through targeting of tolerogenic determinants protecting the tumor (2). One promising target for pharmacologic intervention in this regard is the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO; refs. 3, 4). Two closely related IDO isoforms encoded by the genes *IDO1* and *IDO2* have been identified. *IDO2* appears to be expressed in a more restricted range of tissue types than *IDO1* (5, 6). The functional and physiologic relevance of the IDO2 isoform has yet to be clearly delineated, while genetic evidence clearly supports the im-

portance of IDO1 in tumoral immune escape (7, 8). In numerous clinical studies, IDO upregulation in cancer patients has been associated with a less favorable prognosis (9), whereas, in various animal models of cancer, systemic blockade of IDO activity with small-molecule inhibitors suppresses the outgrowth of tumors and cooperates with chemotherapy, radiotherapy, or cancer vaccines to trigger regression of tumors that are otherwise recalcitrant to treatment (7, 10, 11). These encouraging outcomes have sparked interest in further discovery and development of inhibitors of IDO signaling to evaluate as cancer therapeutics (12–16). One approach to facilitating this process is to consider whether any existing agents might leverage this immunologic mechanism to permit repositioning for cancer treatment.

In earlier studies of IDO dysregulation in cancer, we showed that, in addition to the well-established JAK/STAT signaling requirement, NF-κB signaling is also essential for IDO induction in oncogenically transformed skin epithelial cells (10). In a skin carcinogenesis model, we subsequently showed that IDO is critical for inflammation-based tumor promotion (8). Given the central involvement of NF-κB signaling in both cancer and inflammation, we speculated that relieving IDO-mediated tumor tolerance may be a key mechanism whereby clinical agents that interfere with NF-κB signaling might exert an immunotherapeutic effect in cancer. Among clinically evaluated anti-inflammatory agents that have been shown to inhibit NF-κB signaling, ethyl pyruvate is particularly notable as a simple, inexpensive, nontoxic food

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additive that, via i.p. or i.v. route of administration, displays *in vivo* efficacy in mouse models of sepsis and other inflammatory disorders (17). Here, we report preclinical evidence that ethyl pyruvate can induce robust antitumor immune responses through its ability to inhibit the *in vivo* expression of IDO.

Materials and Methods

Chemical compounds. Chemicals were purchased from the following vendors: ethyl pyruvate (Aldrich), 2-acetamidoacrylate (2-AA; Fluka), 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ; Biomol), 1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[*h*]-imidaz[4,5-*f*]isoquinolin-7-one (DBI; Calbiochem), and methyl-thiohydantoin tryptophan (MTH-Trp or Necrostatin 1; Biomol). Stock solutions were prepared in DMSO for use in cell-based assays.

Cell culture. Myc + Ras-transformed keratinocytes from a Bin1-deficient mouse (Bin1$^{-/-}$ MR KECs) described previously (10) and B16-F10 mouse melanoma cells (American Type Culture Collection) were cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Mediatech) at 37°C and 5% CO_2. U937 human monocytic cells (American Type Culture Collection) were cultured in RPMI 1640 (Mediatech) supplemented with heat-inactivated 10% fetal bovine serum (Mediatech), 55 μmol/L β-mercaptoethanol, and 1% penicillin-streptomycin (Mediatech) at 37°C and 5% CO_2. Recombinant human IFN-γ (R&D Systems) was used at a final concentration of 100 ng/mL, and lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma) were used at a final concentration of 100 ng/mL.

Mice. C57BL/6 mice and athymic NCr-*nu/nu* (nude mice) were obtained from National Cancer Institute-Frederick. Congenic, homozygous *Ido1*-null mice on the C57BL/6 strain background (*Ido1*-KO) described previously (18) were a kind gift from Dr. Andrew Mellor (Medical College of Georgia). All studies involving mice were approved by the Institutional Animal Use Committee of the Lankenau Institute for Medical Research.

IDO enzyme assays. To assess IDO activity in U937 cells, kynurenine levels in the medium at 24 h post-induction were analyzed by high-performance liquid chromatography coupled to electrospray ionization-tandem mass spectroscopy analysis as described (19) using a Varian 320-MS triple-quadrupole mass spectrometry system. Quantitation of kynurenine was based on analysis of two daughter ions.

Transcription assay. The transient transfection procedure was adapted from ref. 20 for electroporation of U937 cells, specifically 2 μg huIDOpro1245-luc with the luciferase gene controlled by the human IDO1 promoter (-1,207 to +38) adapted from ref. 21, 1 μg CMV-β-galactosidase (to normalize for transfection efficiencies), and 2 μg pcDNA3.1 or CMV4-IκBα-SR expressing the (S32A/S36A) superrepressor mutant of IκBα (22). Total plasmid DNA in each transfection was made up to 25 μg with pUC19 carrier plasmid DNA in a total of 50 μL of 0.1× TE and added to 0.3 mL complete medium containing 5×10^6 U937 cells. Electroporation was carried out at 960 mF and 220 V using a Gene

Pulser (Bio-Rad) using disposable electroporation cuvettes with a 0.4 cm electrode gap (Denville Scientific). After electroporation, cells were left to recover for 48 h and then stimulated with a combination of IFN-γ + LPS for 24 h and then harvested for luciferase and β-galactosidase activity as described previously (10).

Northern and Western blot analyses. Northern blot analysis of IDO was conducted using a human full-length cDNA probe essentially as described (23). Western blot analysis was done using standard methods. Antibodies to IDO1 (clone 10.1; ref. 10) and actin (1-19; sc-1616; Santa Cruz Biotechnology) were used as described (24) or as recommended by the vendor. Immunoprecipitation of Ido1 protein from mouse lung tissue with purified rabbit polyclonal antibody (25) followed by Western blot–based detection with rat monoclonal antibody (clone mIDO-48; Biolegend) was done as described (25).

Pharmacodynamic assay. IDO was induced in 8- to 10-week-old C57BL/6 mice by intrapulmonary delivery of LPS from *E. coli* 0111:B4 (Sigma). Mice anesthetized by inhalation of isoflurane were instilled intranasally with 25 μg LPS in 25 μL sterile saline. Ethyl pyruvate was administered by i.p. injection at 40 mg/kg in 500 μL sterile modified Ringer's ethyl pyruvate solution (in which ethyl pyruvate substitutes for lactate; ref. 26) immediately before LPS challenge and with repeated dosing at 6 and 24 h post-challenge. Mice were euthanized at 24 h post-challenge by cervical dislocation, and lung tissues were weighed and frozen on dry ice. For analysis of kynurenine, frozen lung tissue samples were homogenized in PBS (1:4, w/v) and subjected to three rounds of freeze/thaw lyses. Deproteinated lysates were analyzed by high-performance liquid chromatography coupled to electrospray ionization-tandem mass spectroscopy analysis as described (19) using a Varian 320-MS triple-quadrupole mass spectrometry system. Quantitation of kynurenine was based on analysis of two daughter ions.

Tumor formation and drug response. Tumor graft experiments were carried out in 8- to 10-week-old mice injected s.c. with 1×10^6 Bin1$^{-/-}$ MR KEC cells or 1×10^5 B16-F10 melanoma cells. Treatment with either vehicle alone or ethyl pyruvate or 2-AA in modified Ringer's solution (26) injected i.p. at 40 mg/kg twice daily was initiated at day 7 following initial tumor cell engraftment. Tumor growth was monitored by performing caliper measurements of orthogonal diameters, and the estimated tumor volume was calculated based on the formula for determining a prolapsed elliptoid ($d^2 \times 1/0.52$), where d is the shorter of the two orthogonal measurements. Graphing and statistical analysis of the data was done using GraphPad Prism 4 software (GraphPad Software).

Results

***Ethyl pyruvate inhibits IDO expression* in vitro *and* in vivo.** Ethyl pyruvate is a stable aliphatic ester of pyruvate that has been reported to impair nuclear translocation and DNA binding by NF-κB through covalent modification of the

p65 RelA subunit at Cys[38] (27, 28). To evaluate the effects of ethyl pyruvate on IDO expression, we employed U937 monocytic cells as a model system in which IDO is upregulated by exposure to IFN-γ and LPS. Under assay conditions in which the cells are maintained in medium supplemented with low endogenous endotoxin serum, we found that LPS is required in conjunction with IFN-γ to substantially induce IDO gene expression and enzymatic activity that is otherwise marginally induced with either factor alone (Fig. 1A). At concentrations of ethyl pyruvate that interfere with NF-κB activity (27), we found that ethyl pyruvate could suppress the level of kynurenine (the product of tryptophan catabolism by IDO) in the medium by ~90% after IFN-γ + LPS treatment (Fig. 1A). This degree of IDO inhibition was comparable with that achieved with the unrelated NF-κB inhibitory compound QNZ (29) as well as with the pan-JAK inhibitory compound DBI (30). In all three instances, the inhibition of IDO activity by these compounds correlated with suppression of IDO1 protein expression (Fig. 1B). By way of comparison, these compounds were at least as effective at inhibiting IDO activity as the bioactive, competitive inhibitor MTH-Trp (10), which did not suppress the level of IDO1 protein expression (Fig. 1).

Titration of ethyl pyruvate in this cell-based assay yielded an EC_{50} of ~2.2 mmol/L (Supplementary Fig. S1), a value consistent with previous studies of this agent as an NF-κB inhibitor (27). When ethyl pyruvate was titrated against purified recombinant IDO1 enzyme in the same manner as in the cell-based assay, no inhibitory activity was observed (Supplementary Fig. S1). Western blot analysis of IFN-γ + LPS–stimulated U937 cells showed that ethyl pyruvate acted by blocking the induction of IDO1 protein expression within the effective dose range (Supplementary Fig. S2). We confirmed the absence of cytotoxicity at the levels of ethyl pyruvate exposure evaluated in these studies by flow cytometric analysis of propidium iodide–stained cells (Supplementary Fig. S3). A substantial increase in sub-G_1 cells was observed after IFN-γ + LPS stimulation, but this was not exacerbated by exposure to ethyl pyruvate. Northern blot analysis revealed that the primary effect of ethyl pyruvate treatment on IDO1 expression was to decrease the level of induced message (Fig. 2). Following the removal of IFN-γ + LPS from the culture medium, there was no demonstrable contribution of newly synthesized message to the *IDO1* mRNA pool as shown by treatment with actinomycin D, and the level of *IDO1* mRNA declined precipitously past 4 h irrespective of the presence or absence of ethyl pyruvate (Fig. 2A). As opposed to the lack of evidence for destabilization of IFN-γ + LPS–induced *IDO1* mRNA, evaluation of the effect of ethyl pyruvate on IFN-γ + LPS–stimulated *IDO1* promoter activity in a transcriptional reporter assay supported the conclusion that ethyl pyruvate interferes with new message synthesis (Fig. 2B). Expression of the mutated inhibitor of NF-κBα (IκBα) "superrepressor" protein (31), which interferes with so-called "canonical" NF-κB signaling, had a comparable effect to that of ethyl pyruvate in suppressing *IDO1* promoter activity in this reporter assay (Fig. 2B), consistent with the interpretation that ethyl pyruvate blocks IDO induction
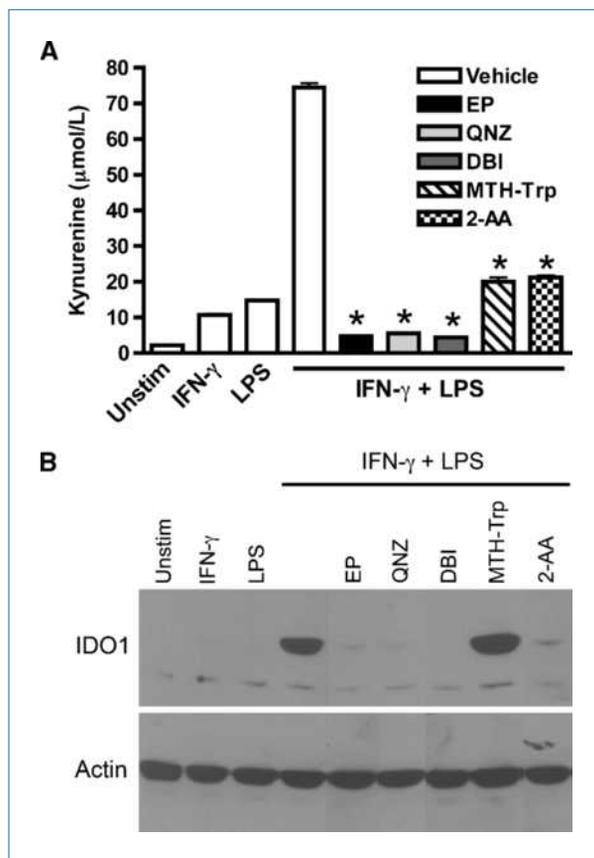

Figure 1. Ethyl pyruvate suppresses induction of IDO. U937 cells stimulated for 24 h with 100 ng/mL IFN-γ and 100 ng/mL LPS in the absence or presence of the following compounds: 10 mmol/L ethyl pyruvate (EP), 1 μg/mL QNZ (NF-κB inhibitor), 500 nmol/L DBI (pan-JAK inhibitor), 100 μmol/L MTH-Trp (IDO inhibitor), and 10 mmol/L 2-AA (functional analogue of ethyl pyruvate) were evaluated for (A) kynurenine output by liquid chromatography-tandem mass spectroscopy analysis of culture supernatant (done in triplicate) and (B) IDO1 protein expression by Western blot analysis. Unstimulated cells (Unstim) and cells treated with either IFN-γ or LPS alone were also evaluated as controls. The kynurenine data are plotted as mean ± SD. For statistical comparisons, each compound-treated IFN-γ + LPS–stimulated group was compared with the IFN-γ + LPS–stimulated group not treated with compound using a two-tailed Student's *t* test. Statistical significance between groups, denoted with an asterisk, was determined at $P < 0.05$. All five compounds tested produced responses with $P < 0.0001$.

through its ability to target p65 RelA, which is a major component of the canonical NF-κB signaling pathway (22).

Lung has long been recognized as a tissue in which high levels of IDO activity can be induced in response to exposure to bacterial LPS (32, 33). Based on these data, we have developed a pharmacodynamic assay whereby IDO is induced in the lungs of mice in response to pulmonary exposure to LPS (34–37). In isolated lung tissue, the kynurenine level, which is reflective of IDO activity, was elevated by ~4-fold at 24 h following LPS administration (Fig. 3). Mice with a homozygous disruption of the *Ido1* gene displayed a lower baseline level of kynurenine in the lungs than did wild-type
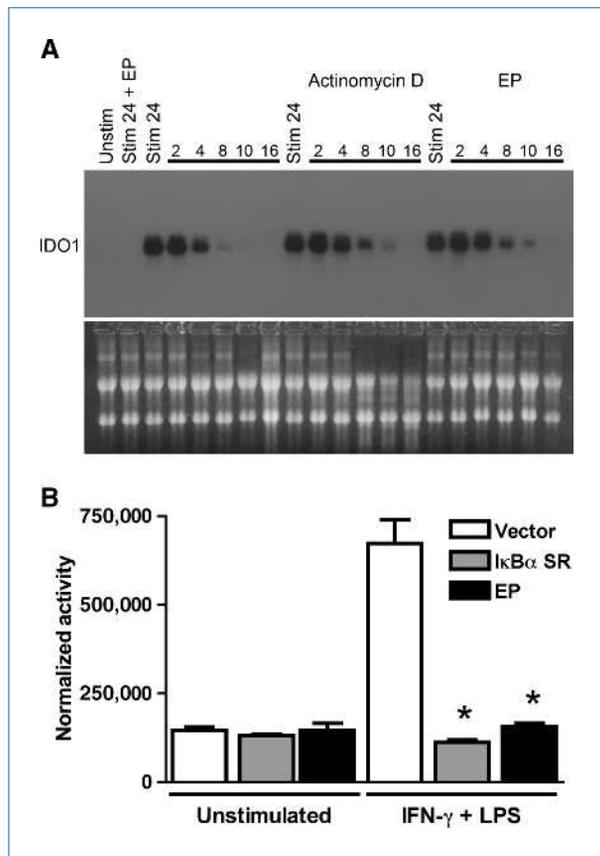
Figure 2. Ethyl pyruvate interferes with induction of IDO mRNA synthesis. A, ethyl pyruvate does not decrease IDO1 message stability. U937 cells stimulated with 100 ng/mL IFN-γ and 100 ng/mL LPS for 24 h were cultured in either fresh medium alone or in medium containing either 10 μg/mL actinomycin D or 10 mmol/L ethyl pyruvate and subsequently harvested at 0, 2, 4 8 10, and 16 h time points for Northern blot analysis. RNA prepared from unstimulated cells and from IFN-γ + LPS–stimulated cells concurrently treated with 10 mmol/L ethyl pyruvate for 24 h was also included in the analysis. Top, image of the blot hybridized with a probe for IDO1; bottom, image of the ethidium bromide–stained gel before transfer. B, ethyl pyruvate blocks IDO promoter activity. U937 cells were electroporated with a human *IDO* promoter/luciferase reporter and CMV promoter/β-galactosidase reporter either without or with an IκB superrepressor expression vector. Two days post-transfection, cells were left untreated or treated with 100 ng/mL IFN-γ and 100 ng/mL LPS either without or with 10 mg/mL ethyl pyruvate. At 24 h, cell extracts were prepared and processed to determine β-galactosidase–normalized luciferase activity. Each assay was done in triplicate and plotted as mean ± SD. Statistically significant effects, denoted with an asterisk, were determined at $P < 0.05$ using a two-tailed Student's t test (in the context of IFN-γ + LPS stimulation $P = 0.0002$ for IκB superrepressor and $P = 0.0001$ for ethyl pyruvate).

mice and also exhibited no significant elevation in kynurenine in response to LPS exposure, showing the specificity of this assay for assessing Ido1 enzyme activity (Fig. 3A). In this pharmacodynamic assay, administration of ethyl pyruvate at a dose level previously identified as sufficient to interfere with *in vivo* NF-κB activity (34–37) as well as produce a positive survival outcome in a mouse sepsis model (26) suppressed kynurenine elevation by 83% to

<1.5-fold above baseline (Fig. 3A). Western blot analysis confirmed the expectation that the reduction in kynurenine elevation by ethyl pyruvate correlated with the suppression of Ido1 protein induction in lung tissue from ethyl pyruvate–treated animals (Fig. 3B). Taken together, our results indicate that, at concentrations where it has been shown to mediate anti-inflammatory effects, ethyl pyruvate inhibits IDO expression *in vitro* and *in vivo*.

Ethyl pyruvate treatment suppresses tumor outgrowth. Compounds that directly interfere with IDO-mediated tryptophan catalysis have shown antitumor activity. The ability of ethyl pyruvate to block IDO activity by suppressing its expression suggested that it might be capable of exerting similar antitumor effects. We have tested this prediction in two tumor models, Bin1$^{-/-}$ MR KECs and B16-F10 melanoma cells, both of which have been used previously to examine the antitumor activity IDO inhibitors (7, 10, 14, 38).

Our earlier work had identified IDO as a gene that is dysregulated as a result of homozygous deletion of the *Bin1* tumor suppressor gene in MR KEC cells and had shown that an IDO-dependent immune escape mechanism renders these Bin1$^{-/-}$ MR KECs more aggressively tumorigenic in syngeneic, immunocompetent animals than their wild-type counterparts (10). The Bin1$^{-/-}$ MR KEC cell line also serves as a model in which IDO induction is NF-κB dependent (10). Ectopic expression of the IκBα superrepressor
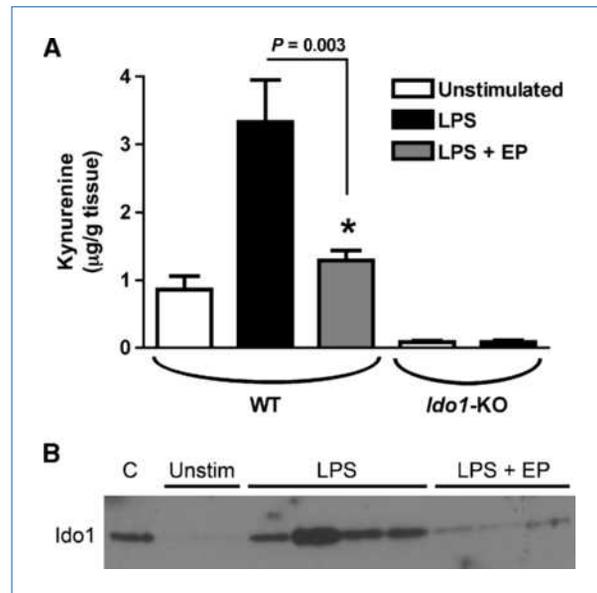

Figure 3. Ethyl pyruvate blocks IDO induction *in vivo*. Lungs were evaluated for (A) kynurenine levels by liquid chromatography-tandem mass spectroscopy analysis and (B) Ido1 protein expression by IP-Western blot analysis of tissue lysates at 24 h after exposure of mice to intrapulmonary administration of 25 μg LPS (C; epididymis lysate positive control). Ethyl pyruvate (40 mg/kg) was administered by i.p. injection three times at 0, 6, and 24 h. Results were obtained from both C57BL/6 (WT) and B6-congenic, homozygous Ido1-null (Ido1-KO) mice. The kynurenine data are plotted as mean ± SD. Statistical significance, denoted with an asterisk, was determined at $P < 0.05$ using a two-tailed Student's t test.
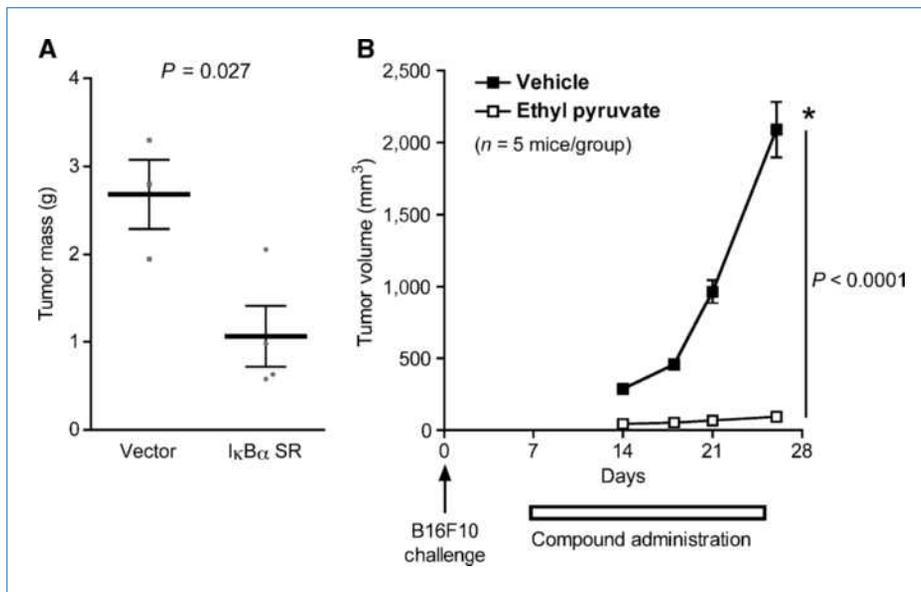
Figure 4. Ethyl pyruvate blocks NF-κB–dependent outgrowth of $Bin1^{-/-}$ MR KECs. A, two sublines of $Bin1^{-/-}$ MR KECs, one stably expressing the IκB superrepressor mutant and the other expressing a vector-transfected control, were injected s.c. at 1×10^6 cells per mouse into C57BL/6 mice. At 4 wk, weights of resected tumors were measured and plotted as the mean ± SD. Statistical significance between the two groups, denoted with an asterisk, was determined at $P < 0.05$ using a two-tailed Student's t test. B, C57BL/6 mice were injected s.c. with 1×10^6 $Bin1^{-/-}$ MR KECs, and treatment with ethyl pyruvate or vehicle was initiated 7 d later. Ethyl pyruvate was administered i.p. at 40 mg/kg twice daily 5 days/wk until termination of the experiment. Caliper measurements of tumors were recorded biweekly. Mean ± SE from these measurements is plotted for each group. At the conclusion of the study, the difference in tumor volumes between treatment and nontreatment groups was assessed using a two-tailed Student's t test to determine the indicated P value. Statistical significance, denoted with an asterisk, was determined at $P < 0.05$.

in $Bin1^{-/-}$ MR KECs effectively suppressed the ability of these cells to form tumors (Fig. 4A), confirming the requirement for NF-κB signaling to support IDO-dependent outgrowth in this tumor model. Ethyl pyruvate treatment likewise suppressed tumor growth when administered at the same dose level that blocked IDO activity and expression in the pharmacodynamic assay (Fig. 4B). Thus, as predicted, ethyl pyruvate treatment can suppress the outgrowth of tumors in this model, and this correlates with its ability to inhibit the NF-κB–dependent expression of IDO.

Because $Bin1^{-/-}$ MR KECs represent a rather specialized tumor model, we extended these studies to evaluate ethyl pyruvate treatment in the widely used B16-F10 melanoma isograft tumor model. Unlike the $Bin1^{-/-}$ MR KECs, IDO is not expressed detectably in B16-F10 tumor cells themselves but rather has been found to be expressed in antigen-presenting cells within the tumor-draining lymph nodes of the host animal (39). B16-F10 cells form highly aggressive, poorly immunogenic tumors that have been shown to be resistant to a variety of immunotherapeutic strategies; however, direct inhibitors of the IDO enzyme can elicit robust single-agent responses in this model (14, 38). Comparing final mean tumor volumes between ethyl pyruvate–treated and control animals at the ∼4-week endpoint, we found that ethyl pyruvate treatment of B16-F10 challenged mice caused significant suppression of tumor growth (Fig. 5A), which equates to a T/C ratio of 5.9% (a T/C ratio of <42% is indicative of efficacy according to standard National Cancer Institute criteria; ref. 40).

This finding corroborates our observations made in the $Bin1^{-/-}$ MR KEC model regarding the antitumor activity of ethyl pyruvate.

To independently test the proposed biological basis for ethyl pyruvate antitumor activity, we examined the effects of a functional analogue, 2-AA, which has also been reported to inhibit nuclear translocation and DNA binding by p65 RelA (28). Although appearing to be structurally dissimilar, 2-AA has been postulated to actually mimic the enol tautomer of ethyl pyruvate, which may represent the biologically active form (28). In support of ethyl pyruvate and 2-AA having a shared mechanism of action, we found that 2-AA also inhibited IDO expression in U937 cells (Fig. 1A). Furthermore, when administered to B16-F10 tumor-bearing mice, 2-AA suppressed tumor outgrowth as effectively as ethyl pyruvate (Fig. 5B). Thus, the key predictions that a distinct compound sharing the NF-κB–dependent mechanism of action of ethyl pyruvate would both inhibit the induction of IDO activity and suppress tumor growth were effectively borne out.

T-cell immunity and IDO targeting are essential for antitumor efficacy. As a therapeutic class, IDO enzyme inhibitors require intact T-cell function to suppress tumor outgrowth in mice (7, 10, 14, 16, 38). Therefore, if the biological consequences of ethyl pyruvate treatment are primarily mediated through its ability to block IDO induction, it should exhibit similar requirements as well. To evaluate the importance of T-cell–dependent immunity to the antitumor
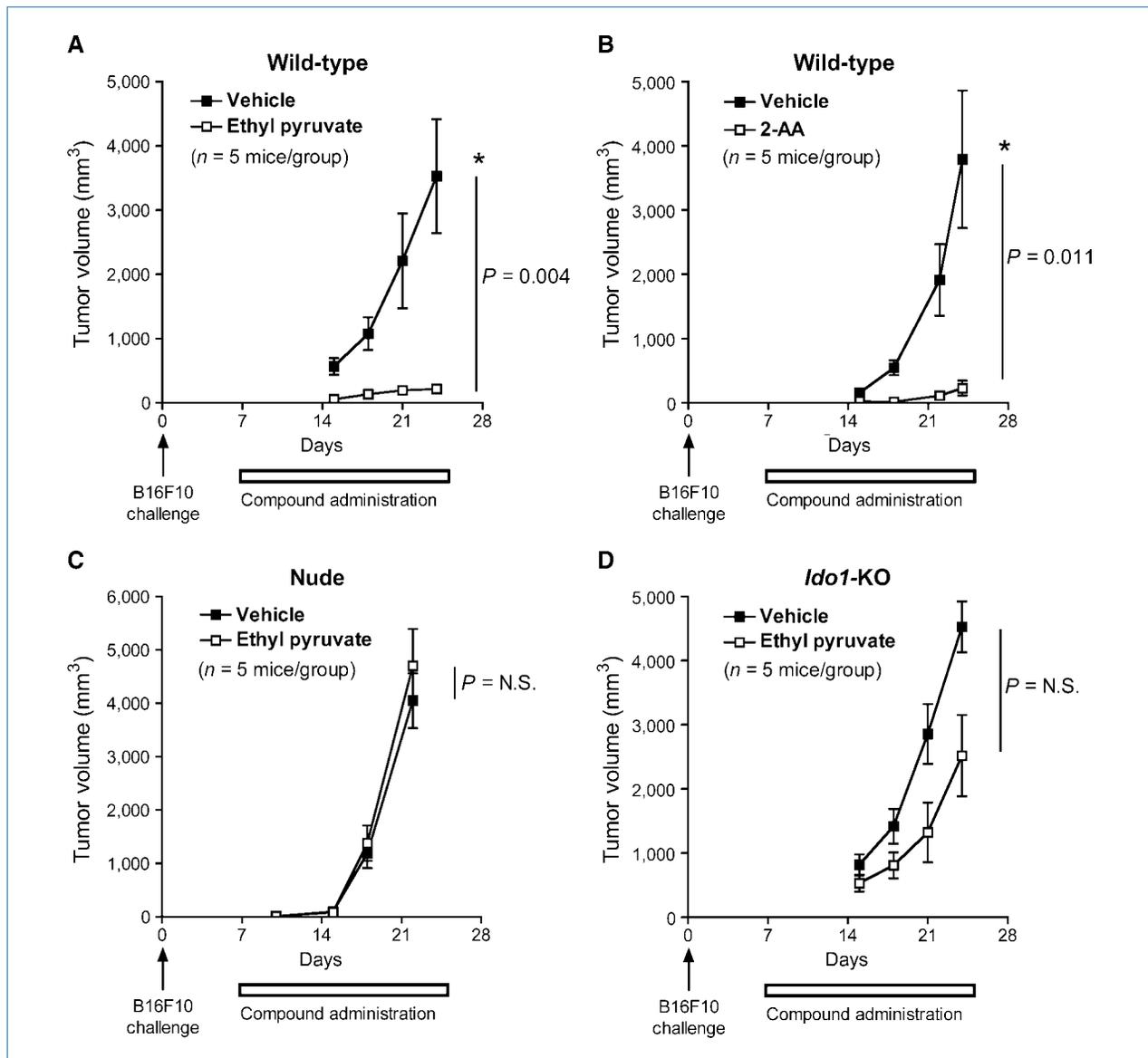
Figure 5. B16-F10 melanoma outgrowth suppression by ethyl pyruvate is dependent on T cells and IDO targeting. Treatment with either ethyl pyruvate or 2-AA was initiated 7 d following s.c. challenge of the indicated mouse strains with 1×10^5 B16-F10 s.c. injected melanoma cells. Each compound was administered i.p. at 40 mg/kg twice daily 5 days/wk until termination of the experiment. Caliper measurements of tumors were recorded biweekly. Mean ± SE from these measurements is plotted for each group. At the conclusion of each study, the difference in tumor volumes between treatment and nontreatment groups was assessed using a two-tailed Student's t test to determine the indicated P value (N.S., not significant). Statistical significance, denoted with an asterisk, was determined at $P < 0.05$.

activity of ethyl pyruvate, athymic "nude" mice that are deficient in mature T cells were challenged with B16-F10 tumors. In the context of these mice, ethyl pyruvate treatment had no discernible effect on tumor outgrowth and tumor growth rate in both ethyl pyruvate untreated and treated mice was somewhat accelerated relative to tumor growth rate in wild-type mice (Fig. 5C). Because IDO is expressed only in normal host cells in the B16-F10 model and not the tumor cells themselves, $Ido11$-KO mice were employed to genetically evaluate the direct relevance

of Ido1 blockade to the mechanism of action of ethyl pyruvate. As predicted, ethyl pyruvate treatment was ineffective at suppressing the outgrowth of B16-F10 tumors in $Ido1$-KO mice (Fig. 5D), although its antitumor efficacy appears not to have been completely abolished as was observed previously with direct IDO inhibitors (14, 38). These findings support the concept that ethyl pyruvate exerts its antitumor effects in a T-cell–dependent manner primarily mediated through its ability to block the induction of IDO.

Discussion

The finding that ethyl pyruvate elicits IDO-directed, immune-based antitumor responses reinforces emerging concepts about the important role of inflammatory processes in supporting cancer pathophysiology. Prompted by observations of Fink and colleagues in rodent models of ischemia/reperfusion injury, hemorrhagic shock and sepsis (26, 35, 41), ethyl pyruvate treatment has been investigated for several years in a variety of animal models of acute and chronic inflammatory disorders (17), while clinical testing thus far has been limited to the evaluation of ethyl pyruvate as an i.v. agent for the prevention of single- and multiple-organ dysfunction in patients undergoing cardiopulmonary bypass surgery (42). Early studies of IDO have noted its elevation in response to bacterial infections or LPS exposure (32, 43). More recently, it has been reported to be a mediator of endotoxic shock-associated lethality (44). It is intriguing to speculate that diseases as seemingly disparate as cancer and sepsis may be linked at some underlying level of pathophysiology through a shared dysregulation of IDO. In this light, the interaction of cancer cells with the host may bear some similarity to unresolved infections that result in the clinical manifestations of sepsis. Survival following the onset of severe sepsis as modeled in the mouse can be dramatically improved by the administration of ethyl pyruvate (26), and it will be important to assess whether this benefit is also linked to the ability of ethyl pyruvate to inhibit IDO.

Many studies have suggested that correcting imbalances in NF-κB signaling in cancer may have important benefits in the context of both the tumor cell and the inflammatory tumor microenvironment, but the concept of correcting immune escape via this signaling pathway has received relatively little attention. Given the centrality of NF-κB as a signal transduction node, the degree to which ethyl pyruvate antitumor activity was found to rely specifically on IDO targeting in the host might be considered somewhat surprising. However, this outcome aligns with a concept we have termed "tolerance addiction," proposed as a result of previous studies of IDO inhibitory compounds (14, 38). As noted previously, B16-F10 melanomas are illustrative of a class of tumors that appear to preferentially use IDO as an immune escape mechanism, such that, once a tumor is established, continued IDO activity must be sustained to maintain the immunoprivileged state of the tumor. In this context, acute disruption of IDO activity, as with a pharmacologic agent, causes an immunologic unmasking that promotes rejection. However, if upregulation of IDO activity is not an available option (as in the IDO-deficient animal), alternate immune escape mechanisms can apparently be accessed by the developing tumor in which case IDO-targeting compounds are ineffectual. The specific target of ethyl pyruvate, p65 RelA, is an important component of canonical NF-κB signaling but plays no apparent role in the noncanonical pathway (22). This specifically implicates canonical NF-κB signaling as the regulatory pathway controlling IDO expression, a conclusion that is further supported by the demonstration that the IκBα superrepressor, which also selectively interferes with canonical NF-κB signaling, also effectively suppresses induction of IDO promoter activity. These results appear to counter the evidence that noncanonical NF-κB signaling is important for IDO induction mediated through GITR signaling (45). Our data do not, however, necessarily contradict these findings but rather indicate that the regulation of IDO expression is likely to be complex and that the relative importance of canonical versus noncanonical NF-κB signaling in controlling this process may be contextual.

Although our findings are consistent with published evidence of ethyl pyruvate as a NF-κB inhibitor, they do not rule out alternative mechanisms that may be germane to its *in vivo* effects. Ethyl pyruvate has also been reported to exert anti-inflammatory effects through ROS scavenging (46) and through blocking HMGB1 release (26), and it is not inconceivable that elevated ROS or HMGB1 release may support dysregulated expression of IDO. One group has recently reported that ethyl pyruvate can exert antitumor activity in a liver metastasis model and has suggested that ethyl pyruvate may produce anti-inflammatory and proapoptotic effects responsible for its antitumor activity though mechanistic validation was lacking (47). From our studies it is clear that direct cytotoxicity is not sufficient to account for the antitumor efficacy of ethyl pyruvate against B16-F10 tumors *in vivo*, as no evidence of antitumor activity was observed when this compound was administered to athymic, tumor-bearing mice. Furthermore, it is clear from the loss of ethyl pyruvate efficacy in *Ido1*-deficient mice that the relevant regulatory pathway targeted by ethyl pyruvate directs an immune escape mechanism that is predominantly orchestrated through the elevation of IDO activity.

While direct inhibition of the IDO enzyme is presently being explored by many groups as an interventional approach, ethyl pyruvate may offer an alternative, low-cost, readily accessible tool to indirectly block IDO for therapeutic purposes. It is likely that IDO inhibitors will prove most effective when combined with other cancer treatment modalities (7, 10, 11), and ethyl pyruvate, as a safe and inexpensive food additive, could readily be evaluated as an adjuvant to standard-of-care treatments with minimal risk of adverse side effects. Given accumulating evidence that elevated IDO activity may have a pathophysiologic role in other diseases such as chronic infections and autoimmune disorders (25, 48), ethyl pyruvate may find other clinical applications as an IDO inhibitory strategy as well. Fink and colleagues have described a simple formulation to administer ethyl pyruvate (49) by substituting it for lactate in Ringer's lactate solution that is usually given i.v. for fluid resuscitation after blood loss or as a conduit for drug delivery. Insofar as IDO inhibitors have been shown to cooperate with different types of cancer therapy in mouse tumor models, we suggest the same Ringer's formulation as a route to administer ethyl pyruvate with standard i.v. chemotherapeutics. Repositioning ethyl pyruvate for an oncology study in this manner would be a straightforward strategy to clinically evaluate the potential of ethyl

pyruvate as a cutting-edge immunochemotherapeutic agent that could address the acute need in developing countries for simple, low-cost advances in cancer treatment.

Disclosure of Potential Conflicts of Interest

G.C. Prendergast and A.J. Muller: commercial research grant, ownership interest, and consultant/advisory board, New Link Genetics. J.B. DuHadaway: ownership interest, New Link Genetics. R. Metz: employee and ownership interest, New Link Genetics. The other authors disclosed no potential conflicts of interest. The Editor-in-Chief of *Cancer Research* is an author of this article. In keeping with the AACR's Editorial Policy, a member of the AACR's Publications Committee had the article reviewed independently of the journal's review process and made the decision concerning acceptability.

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Correction: Immunotherapeutic Suppression of Indoleamine 2,3-Dioxygenase and Tumor Growth with Ethyl Pyruvate

In this article (Cancer Res 2010;70:1845–53), which was published in the March 1, 2010 issue of *Cancer Research* (1), the x-axis was incorrectly labeled in Fig. 4B. The authors have corrected the figure and regret their error. The corrected figure, with the x-axis labeled "MR KEC challenge," appears below:
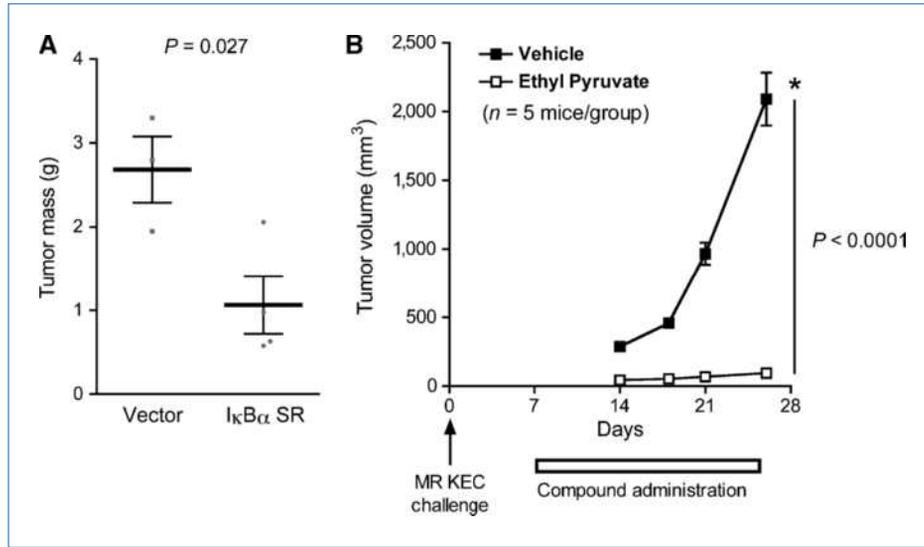

Figure 4.

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1. Muller AJ, DuHadaway JB, Jaller D, Curtis P, Metz R, Prendergast GC. Immunotherapeutic suppression of indoleamine 2,3-dioxygenase and tumor growth with ethyl pyruvate. Cancer Res 2010; 70:1845–53.

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Immunotherapeutic Suppression of Indoleamine 2,3-Dioxygenase and Tumor Growth with Ethyl Pyruvate

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