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Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy

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Immune escape is a crucial feature of cancer progression about which little is known. Elevation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) in tumor cells can facilitate immune escape. Not known is how IDO becomes elevated or whether IDO inhibitors will be useful for cancer treatment. Here we show that IDO is under genetic control of *Bin1*, which is attenuated in many human malignancies. Mouse knockout studies indicate that *Bin1* loss elevates the STAT1- and NF-κB-dependent expression of IDO, driving escape of oncogenically transformed cells from T cell–dependent antitumor immunity. In MMTV-*Neu* mice, an established breast cancer model, we show that small-molecule inhibitors of IDO cooperate with cytotoxic agents to elicit regression of established tumors refractory to single-agent therapy. Our findings suggest that *Bin1* loss promotes immune escape in cancer by deregulating IDO and that IDO inhibitors may improve responses to cancer chemotherapy.

Immune cells create a complex cytokine environment that promotes cancer cell survival, angiogenesis, invasion and metastasis[1]. To survive in this environment, however, cancer cells expressing recognizable tumor antigens must evolve strategies to thwart immune detection and destruction[2]. Immune escape is thus a hallmark of cancer progression, but its underlying molecular genetic basis remains poorly understood. The interplay between immune escape and other hallmarks of malignant conversion, such as invasion and metastasis, is similarly obscure. Aggressive and disseminated cancers can be eradicated by an appropriately activated immune system, arguing that overcoming immune escape might have broad therapeutic impact, but this expectation has yet to be realized. Small-molecule drugs are of particular interest because of their relative advantages compared to biological agents in terms of production, delivery and cost. Yet few small molecules for stimulating antitumor immunity have been described.

Studies of the BAR adapter–encoding gene *Bin1* (also known as *Amphiphysin2*) indicate that it functions in cancer suppression[3–9]. Certain Bin1 adapter isoforms associate with endocytotic complexes[10], but evidence from gene knockouts in several species suggest that *Bin1* is not essential for endocytosis[11–13]. Instead, Bin1 adapters may be important for vesicle trafficking[14], consistent with evidence that BAR domains can act as sensors of membrane curvature[15]. BAR adapter proteins may integrate signaling and trafficking processes, in some cases perhaps involving sites of action in the nucleus[4,16]. Nuclear localization of some Bin1 isoforms is important for cancer suppression; however, there is little information about the relevant effector pathways or about the precise pathophysiological consequences of attenuating the expression of nuclear isoforms, as occurs often in human malignancies[3,5–8].

We report that Bin1 is involved in controlling expression of the *Indo* gene, which encodes the IDO enzyme. IDO is emerging as an important immunoregulatory enzyme. It catalyzes the initial rate-limiting step in tryptophan catabolism, which leads to the biosynthesis of nicotinamide adenine dinucleotide. By depleting tryptophan from local microenvironments, IDO can block activation of T lymphocytes, which are particularly sensitive to loss of this essential amino acid[17,18]. Notably, IDO is needed to prevent T cell–mediated rejection of allogenic concepti[19]. IDO is overexpressed in many cancers, where it has been implicated in immune escape[20,21]. But its importance to cancer progression and therapy has yet to be gauged fully. Here we identify a mechanism for IDO elevation in cancer, and we show how pharmacological inhibitors of IDO can be used in combination with cytotoxic chemotherapeutic agents to elicit regression of established tumors.

RESULTS

Bin1 loss promotes immune escape by tumor cells

Based on evidence of interaction between Bin1 and c-Myc (encoded by *Myc*)[3,4,9,22], we investigated the effects of targeted deletion of the *Bin1* gene[12] on the malignant phenotype of primary mouse skin epithelial cells (keratinocytes) cotransformed by *Myc* plus an activated allele of *Hras1* (these cells are referred to below as MRKECs). We confirmed *Bin1* genotype and transgene expression by PCR analysis and western blot analysis respectively (**Fig. 1a**). *Bin1* deletion did not alter the phenotype

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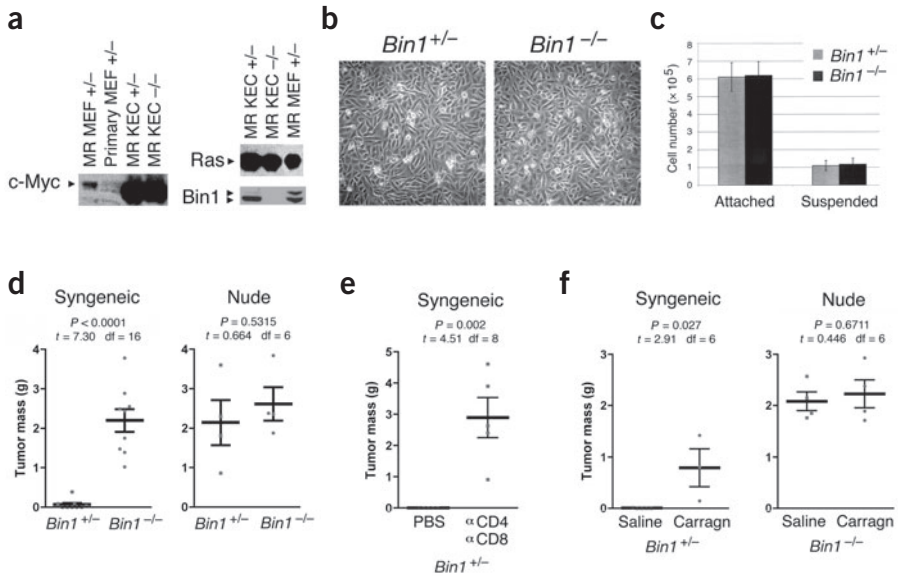


Figure 1 *Bin1* loss promotes tumor formation by facilitating immune escape. (**a**) Western blot analysis of MRKECs. (**b**) Cell morphology. (**c**) Cell proliferation. The experiment was performed twice. (**d**) Tumor formation in syngeneic versus nude mice. Tumor weight was determined 4 weeks after subcutaneous injection of MRKECs into syngeneic or nude mice. Each point on the graph represents a single tumor measurement with mean and standard error shown for each group ($t = t$ ratio (the difference between sample means divided by the standard error of the difference between the means); df = degrees of freedom ($n - 2$)). (**e**) Immune cell depletion phenocopies *Bin1* loss. *Bin1*$^{+/-}$ MRKECs were injected subcutaneously into mice depleted of both CD4$^+$ and CD8$^+$ cells and tumor formation was scored 3 weeks later. PBS, phosphate-buffered saline. (**f**) Carrageenan (Carragn) treatment partly phenocopies *Bin1* loss. *Bin1*$^{+/-}$ MRKECs were injected subcutaneously into control (saline-treated) and carrageenan-treated mice and tumor formation was scored 4 weeks later. *n* for all animal experiments can be calculated by using the formula $n = \text{df} + 2$.

or *in vitro* proliferation of MRKECs under either anchorage-dependent or anchorage-independent conditions (**Fig. 1b**,**c**). Nevertheless, *Bin1* loss substantially enhanced the outgrowth of tumors formed by MRKECs in syngeneic animals (**Fig. 1c**). The significant difference in tumorigenicity between *Bin1*$^{-/-}$ and *Bin1*$^{+/-}$ cells ($P < 0.0001$) could not be explained by increased intrinsic cell proliferation, because *Bin1*$^{+/-}$ cells were no less aggressive at forming tumors than *Bin1*$^{-/-}$ cells in T cell–deficient, athymic nude mice (**Fig. 1d**) or in syngeneic mice depleted of both CD4$^+$ and CD8$^+$ T cells (**Fig. 1e**). Because tumor cells present antigens in part by cross-priming of antigen-presenting cells (APCs), we investigated the effect of *Bin1* loss on tumors formed in syngeneic mice treated with carrageenan, a substance that depletes or inactivates phagocytic cells including macrophages, dendritic cells and neutrophils[23]. Carrageenan treatment enhanced tumor formation by *Bin1*$^{+/-}$ cells but not by *Bin1*$^{-/-}$ cells (**Fig. 1f**), consistent with the idea that cross-priming is involved in the antitumor immune response. In summary, we conclude that *Bin1* suppresses tumor formation through a cell-extrinsic, immune-based mechanism that appears to be dependent on both T cells and APCs.

IDO is under genetic control of *Bin1*

The expression of tumor antigens by most cancers means that they must evolve mechanisms to escape or subvert antitumor immunity in order to progress successfully. Two recent studies have suggested that *Bin1* functions may modulate subcellular trafficking of the STAT and NF-

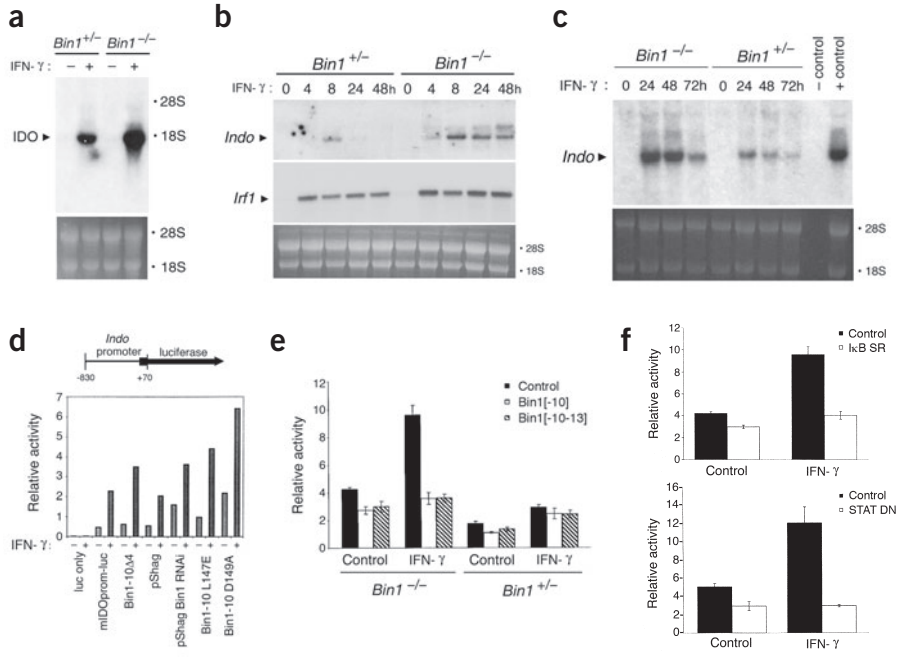


Figure 2 *Bin1* loss potentiates the NF-κB- and STAT-dependent expression of *Indo*. (**a**) *Bin1* loss elevates *Indo* expression in MRKECs treated with IFN-γ for 24 h. Steady-state RNA was analyzed by northern blotting with a mouse *Indo* cDNA probe. Ethidium bromide–stained gel is shown below as an RNA loading control. (**b**) *Bin1* loss leads to persistent induction of *Indo*. MRKECs were treated with IFN-γ for times indicated and processed for northern blot analysis as above. (**c**) *Bin1* loss accentuates induction of *Indo* in *Myc*-immortalized macrophages. Cells were treated with IFN-γ for times indicated and processed for northern blot analysis as before. (**d**) *Bin1* attenuation potentiates *Indo* transcription in human cells. HeLa cells were transfected with a mouse *IDO* promoter-luciferase reporter plus the indicated expression vectors. The day after transfection, cells were either left untreated or treated 16 h with IFN-γ and cell extracts were processed for normalized luciferase activity. (**e**) *Bin1* deletion potentiates *Indo* transcription in MRKECs. Cells were transfected with the *Indo* promoter reporter used above plus the indicated expression vectors, then treated and processed for normalized luciferase activity as before. (**f**) NF-κB and STAT1 are required for superinduction of *Indo* in *Bin1*-null cells. *Bin1*$^{-/-}$ MRKECs were cotransfected with *Indo* promoter-reporter used above plus the indicated expression vectors, then treated and processed for luciferase activity as before.

κB transcription factors, which have important roles in modulating immunity[9,24]. In considering common genetic targets of STAT and NF-κB, we identified IDO (encoded by *Indo*) as a candidate that might explain the effects of *Bin1* loss on immune escape. IDO is an extrahepatic oxidoreductase. In APCs, the expression of IDO is strongly elevated by interferon-γ (IFN-γ)[17,25]. Deletion of *Bin1* in MRKECs markedly increased IFN-γ-induced expression of IDO both quantitatively and temporally, such that the *Indo* message level was both higher and persisted longer than in *Bin1*-expressing cells (**Fig. 2a,b**). IDO was also superinduced by IFN-γ in *Bin1*$^{-/-}$ macrophages[12], showing that the *Bin1* deletion has a similar effect on IDO expression in a cell lineage in which IDO activity is known to be physiologically relevant (**Fig. 2c**). Transcription assays in human HeLa cells showed that attenuation of *Bin1* by either siRNA or dominant inhibitory strategies increased basal and IFN-γ-induced activity of the *Indo* promoter (**Fig. 2d**). Similarly, *Bin1* deletion elevated basal and IFN-γ-induced activity of the *Indo* promoter in MRKECs, and these effects were reversed by ectopic expression of cDNAs encoding the two ubiquitously expressed Bin1 splice isoforms *Bin1[-10]* or *Bin1[-10-13]* cDNAs encoding the two Bin1 proteins that are ubiquitously expressed (**Fig. 2e**). Activation of the *Indo* promoter requires the activity of STAT1 and NF-κB transcription factors[26], the regulation of which may be influenced in part by Bin1 functions[9,24]. We observed that the benefits of *Bin1* loss to IDO transcription were abolished by introduction of a 'super-repressor' mutant of IκB, which prevents NF-κB activation, as well as by a dominant negative STAT1 mutant (STAT1 Y701F), which prevents STAT1 activation (**Fig. 2f**). Taken together, these results indicate that *Indo* expression is under the genetic control of *Bin1* at the level of NF-κB- and STAT1-dependent transcription.

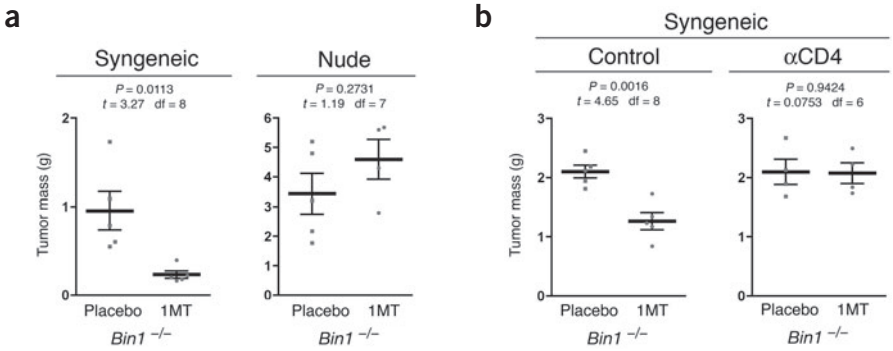


Figure 3 IDO inhibition counteracts the benefit of *Bin1* loss to tumor formation. (**a**) 1MT inhibits tumor formation by *Bin1*$^{-/-}$ cells. Mice implanted with either 1MT or placebo time-release pellets were injected subcutaneously with *Bin1*$^{-/-}$ MRKECs and tumor formation was scored 2 weeks later. (**b**) 1MT activity is abolished by T-cell depletion. Tumor formation was initiated and scored as before after subcutaneous injection of *Bin1*$^{-/-}$ MRKECs into mice treated with phosphate-buffered saline or CD4-specific monoclonal antibodies. n = df + 2.

IDO mediates immune escape by tumor cells that lack *Bin1*

To determine the importance of IDO activity to immune escape caused by *Bin1* loss, we asked whether the IDO inhibitor 1-methyl-DL-tryptophan (1MT) could specifically counteract the benefit of *Bin1* loss to MRKEC tumor growth in syngeneic mice. We confirmed that the delivery method used (subcutaneous time-release pellets) could elicit maternal immune rejection of allogeneic but not syngeneic concepti (**Supplementary Fig. 1** online), indicating sufficient systemic exposure of 1MT to achieve biological activity. In tumor-bearing animals, 1MT inhibited the growth of *Bin1*$^{-/-}$ tumors in syngeneic hosts but not in athymic nude mice

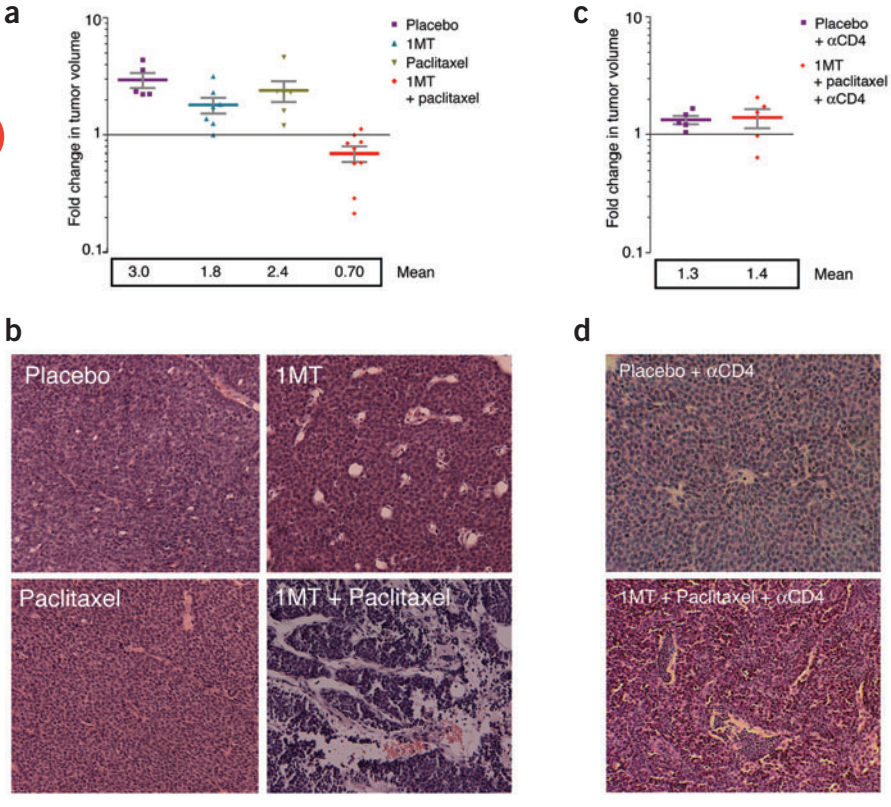


Figure 4 IDO inhibition cooperates with paclitaxel to cause regression of autochthonous MMTV-*Neu* breast tumors. (**a**) Therapeutic response. Tumor-bearing MMTV-*Neu* mice were implanted with time-release pellets containing 1MT (20 mg/d) or placebo pellets. The next day, either paclitaxel (13.3 mg/kg [MTD]) or vehicle was delivered three times per week as an intravenous bolus dose. Tumor volumes were calculated 2 weeks after therapy was initiated. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group (n = 5,7,6,9 in the order graphed). (**b**) Tumor histology at endpoint. Representative hematoxylin and eosin–stained sections were photographed using a 10x objective on an Olympus BH2 microscope. (**c**) Immune depletion abolishes the efficacy of the combination therapy. Tumor-bearing mice depleted of CD4$^+$ cells were treated with either combination therapy or vehicles and tumor volumes were calculated 2 weeks later (n = 5,5 in the order graphed). (**d**) Tumor histology at endpoint in immune-depleted mice. Photographs were taken as in **b**.

Table 1 IDO inhibition enhances the efficacy of certain commonly used cancer chemotherapeutic agents

Compound	Class	Mean ± s.e. (+1MT)	Mean ± s.e. (–1MT)	*P*	*n*	Dose (mg/kg)	Route	Schedule
Cisplatin	Alkylating agent	0.77 ± 0.18	1.7 ± 0.33	0.0419	7,8	1.0	i.v.	3×/week
Cyclophosphamide	Alkylating agent	0.81 ± 0.12	1.4 ± 0.18	0.0269	5,5	100	i.v.	3×/week
Doxorubicin	Antineoplastic antibiotic	0.79 ± 0.07	1.5 ± 0.25	0.0150	6,4	0.66	i.v.	3×/week
5-Fluorouracil	Antimetabolite	1.2 ± 0.20	1.1 ± 0.25	0.8926	8,7	50	i.v.	3×/week
Methotrexate	Antimetabolite	1.7 ± 0.28	1.7 ± 0.38	0.9047	3,3	1.0	i.v.	3×/week
Paclitaxel	Mitotic inhibitor (taxane)	0.68 ± 0.11	2.4 ± 0.43	0.0010	8,7	13.3	i.v.	3×/week
Vinblastine	Mitotic inhibitor (vinca alkaloid)	1.3 ± 0.19	1.2 ± 0.18	0.7368	10,8	1.0	i.v.	3×/week
FTI	Signal transduction inhibitor	0.67 ± 0.11	1.0 ± 0.16	0.0979	8,8	40	i.p.	qdx11
Rapamycin	Signal transduction inhibitor	0.97 ± 0.07	0.99 ± 0.25	0.9417	4,4	1.5	i.v.	qdx11
Tetrathiomolybdate	Antiangiogenic (iron chelator)	1.9 ± 0.52	2.0 ± 0.42	0.7996	3,4	40	p.o.	qdx11
Vehicle		1.7 ± 0.17	3.0 ± 0.44	0.0061	12,5			

Tumor-bearing MMTV-*Neu* mice were treated with either 1MT (+1MT) or placebo (–1MT) in combination with the cytotoxic drugs and molecular therapeutic agents indicated (at the doses indicated). We scored tumor volumes just before and 2 weeks after initiation of therapy. Fold changes in tumor volumes were determined and the means are presented for each group. i.v., intravenous; i.p., intraperitoneal; p.o., per os (orally); qdx11, once a day for 11 d. *P* values compare +1MT and –1MT groups.

(**Fig. 3a**). Moreover, immune depletion of $CD4^+$ T cells from syngeneic animals abolished the ability of 1MT to suppress *Bin1*$^{-/-}$ tumor growth (**Fig. 3b**). 1MT does not seem to be directly cytotoxic or growth inhibitory, as it did not affect tumor growth in nude or immune-depleted syngeneic mice, nor were cytotoxicity or growth inhibition observed when MRKECs were treated with 1MT *in vitro* (data not shown). We conclude that IDO elevation is a critical mediator of immune escape caused by *Bin1* loss.

IDO inhibitors potentiate cancer chemotherapy

We next investigated whether IDO is critical for tumor survival in MMTV-*Neu* mice, a well-accepted transgenic mouse model of breast cancer, which, like human malignant breast cancers, shows attenuation of Bin1 expression (**Supplementary Fig. 2** online). MMTV-*Neu* mice bearing autochthonous tumors were randomly enrolled into control and treatment groups when tumors reached a diameter of 5–10 mm. 1MT was administered as in the MRKEC tumor graft setting described above. In some trials, we combined 1MT with paclitaxel, a chemotherapeutic agent used for breast cancer treatment, based on reports that taxanes promote T-cell infiltration of tumors[27]. By itself, 1MT retarded but did not arrest outgrowth of autochthonous tumors (**Fig. 4a**). Combinations with either IFN-γ or interleukin (IL)-12 did not accentuate the effect of 1MT (data not shown), arguing that IDO inhibition could not compromise the survival of established tumor cells, even when combined with immune stimulatory cytokines.

In marked contrast, combining 1MT with various cytotoxic agents led to tumor regressions under conditions where single agents were ineffectual. Combining 1MT with paclitaxel resulted, on average, in a 30% decrease in tumor volume within 2 weeks of initiating therapy, whereas paclitaxel delivered near the maximum-tolerated dose (MTD) only slightly retarded tumor growth (**Fig. 4a**). Titrating each agent individually indicated that efficacy was retained at a sub-MTD dose of paclitaxel (4.3 mg/kg administered three times per week) whereas a partial response was observed at a one-quarter dose of 1MT (one 10-mg, 14-d release pellet) (data not shown). Histopathological analysis of tumor sections from 1MT + paclitaxel–treated mice offered evidence of increased tumor-cell death (**Fig. 4b**). Immune depletion of $CD4^+$ T cells limited tumor growth relative to control tumors during the experiment but, as expected, it abolished the ability of 1MT + paclitaxel treatment to elicit tumor regression (**Fig. 4c**). This effect correlated with a reduction in tumor-cell death as evidenced by histological analysis of tumor sections (**Fig. 4d**).

It is unlikely that a drug-drug interaction simply caused an increase in the effective dose of paclitaxel, which was administered near the MTD[28], because we did not observe characteristic neuropathies that would be expected to occur if 1MT had increased the effective dose (data not shown). Furthermore, this interpretation does not explain the effectiveness of combining 1MT with other drugs cleared by different mechanisms. We ruled out the trivial possibility that high doses of a tryptophan-like compound were sufficient by showing that DL-tryptophan was ineffective when substituted for 1MT in the regimen (data not shown). Lastly, we further confirmed the requirement for T cell–dependent immunity by showing in a tumor graft model, using an MMTV-*Neu*–derived tumor cell line, that 1MT showed effects only when the tumors were established in immunocompetent syngenic mice and was ineffectual in T cell–deficient athymic nude mice (**Supplementary Fig. 3** online).

We next evaluated the effects of combining 1MT with other cytotoxic drugs with diverse mechanisms of action that are used to treat breast cancer (**Table 1**). Among the agents tested were the DNA alkylating drugs cisplatin and cyclophosphamide, the topoisomerase inhibitor doxorubicin (adriamycin), the antimetabolites 5-fluorouracil and methotrexate, and the antimitotic agent vinblastine. We also tested several molecular targeted agents, including a farnesyl transferase inhibitor (FTI; L-744,832)[29], the mTOR pathway inhibitor rapamycin, and the angiogenesis inhibitor tetrathiomolybdate. 1MT cooperated

with cisplatin, cyclophosphamide and doxorubicin to elicit mean tumor regressions that differed significantly ($P < 0.05$) from the impact of single-agent therapy (**Table 1**). Mean tumor regression was also produced in combination with FTI; however, as a result of the growth suppression produced by FTI alone, the differential with the combination treatment did not meet the threshold for assigning significance ($P > 0.05$; **Table 1**). We conclude that IDO inhibition cooperates with diverse chemotherapeutic agents to effectively promote regression of established breast tumors that are refractory to chemotherapy.

A new inhibitor of IDO with antitumor activity

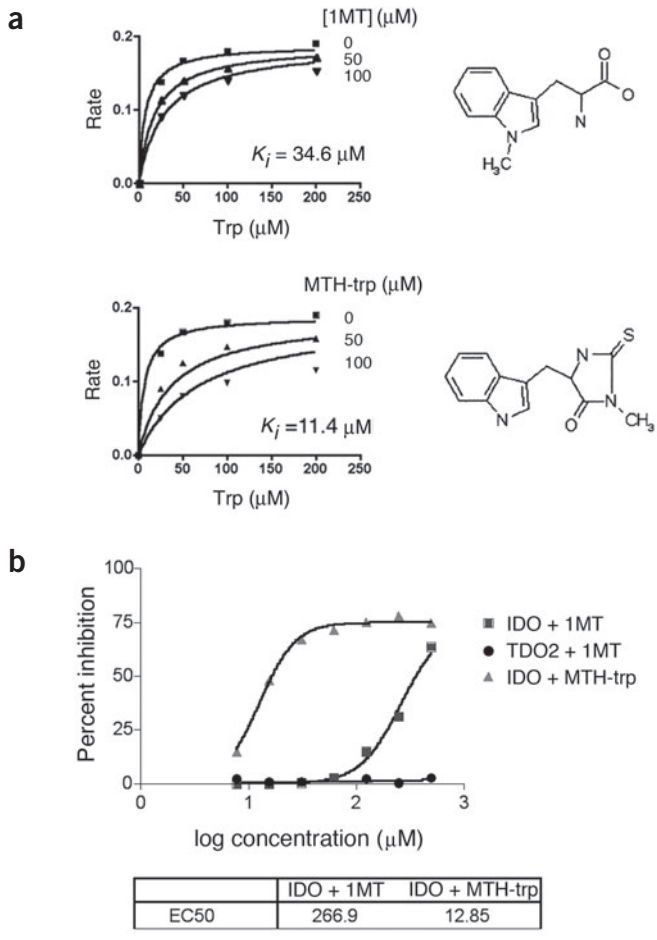
The observations described above are consistent with the presumptive specificity of 1MT for IDO; however, we wished to address off-target concerns by examining the ability of a structurally distinct inhibitor of IDO to elicit tumor regression in combination with paclitaxel. Toward this end, we screened for bioactive inhibitors among commercially available indoleamine-containing compounds, using a purified recombinant human IDO enzyme for *in vitro* assays and a human *INDO* cDNA for expression in cell-based assays. Both assays used a colorimetric method to quantify the production of kyneurinine, the product of reaction catalyzed by IDO[30]. Several compounds were identified that showed inhibition constants against recombinant human IDO that were within about two- to threefold of 1MT ($K_i = 34.2$ μM). One active compound was methyl-thiohydantoin-tryptophan (MTH-trp), which biochemical analyses showed to be a competitive inhibitor with $K_i = 11.6$ μM (**Fig. 5a**). Cell-based screens were performed after transient expression of human *INDO* cDNA in COS-1 monkey cells. As a counterscreen for selectivity, the inhibitory activity of the compounds against the structurally distinct liver enzyme TDO2 was also determined. MTH-trp was ~20-fold more potent than 1MT in the cell-based assay (**Fig. 5b**). Two other thiohydantoin derivatives of tryptophan with lower potency than MTH-trp were also identified (data not shown), confirming the IDO-inhibitory nature of this structural class and suggesting that the thiohydantoin sidechain is probably a mimetic of the amino acid backbone in tryptophan (**Fig. 5a**). Pilot pharmacology experiments have indicated that MTH-trp is more soluble in aqueous solution than 1MT but is also more rapidly cleared from serum; both compounds were found to be orally bioavailable (**Supplementary Fig. 4** online).

Using the same formulation, route of delivery and dose used previously to administer 1MT, we observed that combining MTH-trp with paclitaxel produced regression of tumors as well or better than 1MT (**Fig. 6a**). At the same 2-week endpoint, autochthonous tumors subjected to the combination therapy regressed, on average, 45% relative to the starting tumor volume. One mouse in the trial showed complete tumor regression. Histological examination of tumor sections confirmed evidence of tumor-cell death elicited by the combination therapy, as expected (**Fig. 6b**). Like 1MT, MTH-trp administered by itself retarded tumor outgrowth but did not promote regression. In addition, MTH-trp produced no evidence of gross toxicity in the mice during treatment or at necropsy. Compound titration showed that combinatorial efficacy was fully retained when the MTH-trp dose was reduced by half and partly diminished in response to a one-quarter dose (data not shown). The ability of MTH-trp to effectively combine with paclitaxel in a similar manner as 1MT strengthens the interpretation that the basis for this cooperativity is through IDO inhibition.

DISCUSSION

Immune escape is a central hallmark of cancer, but compared to other recognized hallmarks of cancer—immortalization, suppressor loss, sustained growth, apoptotic resistance, angiogenesis, invasion and metastasis[31]—much less is known about the genetics of immune escape. Here we address this gap in knowledge by defining a genetic mechanism that restricts the ability of cancer cells to escape T cell–dependent antitumor immunity. Using a mouse knockout model, we have shown that *Bin1* can restrain immune escape of oncogenically transformed cells by restricting expression of IDO, an immunoregulatory enzyme that is widely elevated in human cancer[21]. *Bin1* is likewise widely attenuated or mis-spliced in cancers of the breast, prostate, colon, brain and other organs[3,5–8,32,33] (K. Xie, L. Wang, J.B.D. and G.C.P., unpublished data). The finding that IDO is under negative genetic control by *Bin1* offers mechanistic insight into how immune escape may be enabled during cancer progression.

Translational studies prompted by our findings have led us to identify a new strategy for cancer treatment that combines IDO inhibitor–based immunomodulation with cytotoxic chemotherapy. Although gene ablation studies are needed to fully validate IDO as a therapeutic target, the chemical genetics strategy used here offers an initial line of support. Combination drug treatment for cancer is the standard of care, but few studies have explored combinations of immunomodulating agents with chemotherapy. Our findings argue that immunotherapy and chemotherapy can be combined to more effectively destroy cancer cells,



	IDO + 1MT	IDO + MTH-trp
EC50	266.9	12.85

Figure 5 MTH-trp is a potent bioactive inhibitor of IDO. (**a**) *In vitro* enzyme assay. Global nonlinear regression analysis of enzyme kinetic data obtained for human IDO in response to 1MT and MTH-trp. Computed K_i values are shown for each compound. (**b**) Cell-based assay. Results of dose-escalation studies over two logs are shown for 1MT against both IDO and TDO2 and for MTH against IDO. EC_{50} values determined by nonlinear regression are shown.

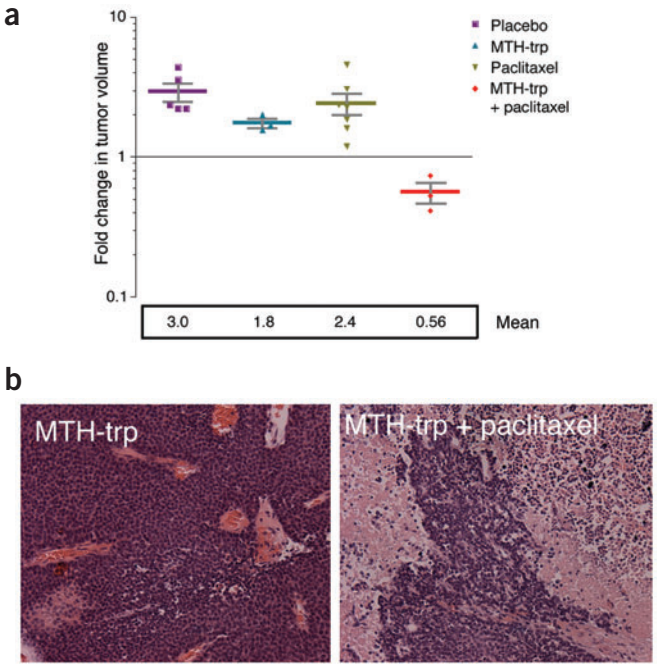


Figure 6 MTH-trp enhances paclitaxel efficacy. (**a**) Therapeutic response. Tumor-bearing mice were implanted with time-release pellets containing MTH-trp (20 mg/d) pellets. Paclitaxel was administered and tumor responses were scored as described in **Fig. 4a**. Results from placebo control and paclitaxel-only treatment groups from **Figure 4** are provided for comparison. Each point represents the fold change in volume for an individual tumor with the mean ± s.e. indicated for each group (n = 5,3,7,4 in the order graphed). The MTH-trp + paclitaxel group includes one complete tumor regression that cannot be plotted on the log scale. (**b**) Tumor histology at endpoint. Representative hematoxylin and eosin–stained sections photographed using a 10x objective on an Olympus BH2 microscope.

consistent with two other preclinical studies that focused on different immunotherapeutic principles[34,35].

Bin1 restrains IDO at the level of IFN-γ-regulated transcription by limiting the induction of IDO message by STAT1 and NF-κB, two key regulators of immunity and cancer. Precisely how *Bin1* influences the STAT1- and NF-κB-dependent transcription of *Indo* remains to be determined. Because nuclear localization is important for the suppressor activity of Bin1 isoforms[4], nuclear actions may be relevant. Because of a lack of mechanistic understanding, most studies of Bin1 adapter proteins have ignored evidence of nuclear localization of the ubiquitous isoforms[3,33,36,37], despite an established precedent for nuclear localization of other 'endocytotic-like' proteins (*e.g.*, epsin and CtBP, also known as BARS[38–40]). Nuclear localization is also intriguing given intrinsic transcriptional repression activity associated with the Bin1 BAR domain[4] (M. Huang, P.S.D. and G.C.P., unpublished data). Indeed, a recent study of the BAR adapter protein APPL reinforces this concept by showing not only its nuclear trafficking function but also its association with the chromatin remodeling complex NuRD/Mi-2 that represses transcription[16]. In summary, effects on trafficking and/or transcriptional repression by *Bin1* may be relevant to its regulation of IDO.

This study extends the evidence that *Bin1* limits cancer pathophysiology and *Myc* oncogenicity[3–9,22,41,42]. Earlier work on cell-intrinsic suppressor roles of *Bin1* are expanded here by the identification of a cell-extrinsic suppressor role that involves restraining a protoleragenic mechanism that tumor cells can exploit to escape antitumor immunity. These cell-intrinsic and extrinsic suppressor roles might be intertwined given the complex involvement of the immune stromal environment in cancer pathophysiology. *Myc* overexpression is associated with major histocompatibility complex (MHC) class I downregulation in neuroblastomas and melanomas[43,44], two cancers in which *Bin1* is often attenuated by mis-splicing[5,8]. Thus, MHC class I downregulation may cooperate with Bin1-IDO dysregulation to facilitate immune escape by cells that overexpress *Myc*. The presence of IFN-γ in the tumor microenvironment may provide a selective pressure to explain the attenuation of *Bin1* and the upregulation of IDO during cancer progression.

This is the first study to link IDO to a cancer suppression pathway. Elevated tryptophan catabolism in cancer patients has been recognized for decades[45,46], a phenomenon that can be explained by IDO overexpression in tumors[21]. IDO catalyzes the initial step in tryptophan catabolism that leads to biosynthesis of nicotinamide adenine dinucleotide. But mammals salvage rather than synthesize nicotinamide adenine dinucleotide, and a different liver-specific enzyme, tryptophan dioygenase, metabolizes dietary tryptophan. Thus, the biological role of IDO was obscure until it was shown that localized tryptophan catabolism forms the basis for a unique mechanism of establishing peripheral tolerance[19]. Because IDO inhibition is not inherently cytotoxic, identification of IDO inhibitors would elude traditional cytotoxic drug screens that are based on tumor-cell survival in tissue culture or in xenograft mouse model systems. In immunocompetent mice, we found that IDO inhibitors potentiated the efficacy of certain cytotoxic drugs without increasing their side effects. The mechanistic basis for the cooperation is not yet clear. Cooperating cytotoxic agents may induce certain types of cell death (*e.g.*, apoptotic versus nonapoptotic death) that elevate presentation of tumor antigens. Alternately, cooperating cytotoxic agents may preferentially compromise the survival of regulatory T cells relative to effector cells, contributing to a weakening of immune tolerance and stimulation of antitumor immunity[27,34,35]. In future work, it will be important to distinguish these possibilities, for example, by determining whether combinatorial efficacy is retained against drug-resistant tumor cells and whether the cytotoxic drugs that are effective in combination with IDO inhibition have similar effects on the immune system, despite their diverse cytotoxic mechanisms.

Our findings are consistent with the interpretation that IDO activity in tumor cells is the relevant target for inhibition; however, they are not incompatible with the possible involvement of stromal APCs, in which IDO can also be highly expressed (*e.g.*, as in the draining lymph nodes of breast tumors[47]). Thus, it is possible that IDO inhibitors may act in part by blocking an immune-tolerizing activity of APCs that are located at distal sites. From a therapeutic standpoint, the possibility that IDO may be a stromal target increases its appeal because of the reduced likelihood of selecting for drug resistance (relative to genetically plastic tumor cells), and because of the increased range of tumors that might be treated even in the absence of direct IDO overexpression. In closing, this study proposes IDO as an attractive and tractable target for the development of small-molecule immunomodulatory drugs to safely leverage the efficacy of standard chemotherapeutic agents.

METHODS

Drugs and chemical compounds. We purchased drugs as clinical formulations or formulated them as described in **Supplementary Methods** online. For *in vitro* studies, 1MT (Sigma) and MTH-trp (Sigma) were formulated in dimethyl sulfoxide (DMSO), including 0.1 N HCl for the less soluble compound 1MT. For *in vivo* studies, 1MT and MTH-trp were formulated in 2-week time-release pellets (Innovative Research).

Tissue culture. We obtained and cultured primary skin keratinocytes from E18.5 days post-coitus mouse embryos on a mixed 129sv/BL6 background[12], essentially as described[47]. Transformed cell populations, referred to as MRKECs, were generated by infection with ecotropic helper-free *Myc* and *Hras1* recombinant retroviruses and analyzed *in vitro* as detailed in **Supplementary Methods** online.

Northern and western blot analyses. We used standard methods as described in **Supplementary Methods** online. We generated a mouse monoclonal antibody recognizing the mouse and human IDO proteins (clone 10.1; UBI) essentially as described[49], using a bacterially expressed peptide encoding amino acids 78–184 of the human IDO protein.

Transcription assays. Cells seeded overnight in 12-well dishes were transfected with 200 ng mIDOprom900-luc, a luciferase reporter plasmid containing 900 base pairs of the mouse *Indo* promoter and 70 nucleotides of noncoding sequences in exon 1, 100 ng CMV-β-galactosidase (to normalize transfection efficiencies) and 700 ng of CMV-Bin1 plasmids as noted. Total DNA in each transfection was made up to 1,000 ng with the analogous CMV empty vector (pcDNA3-neo; Invitrogen). Detailed protocols for transfection and normalized reporter analysis, as well as siRNA sequences, are provided in **Supplementary Methods** online.

Tumor formation and drug response assays. For tumor formation by MRKECs, we injected 1×10^6 cells subcutaneously into syngeneic F1 offspring from 129S1/SvImJ and C57BL/6J breeders (Jackson Laboratories) and into immunocompromised CD-1 Nude (Crl:CD-1-nuBR) mice (Charles River Laboratories). Four weeks after cell injection, mice were killed and tumor mass was determined. We generated autochthonous mammary gland tumors in multiparous female MMTV-*Neu* mice harboring the normal rat *HER2/Neu/ErbB2* gene (Jackson Laboratories). The incidence of detectable tumors in this model is ~80% at 7 months of age and increases to nearly 95% at 8 months. To monitor drug responses, we randomly enrolled tumor-bearing mice into control and experimental treatment groups when tumors reached 0.5–1.0 cm in diameter. Based on the release rate computed by the vendor, the total dose delivered by subcutaneous time-release pellets was at least 20 mg/d, confirmed in pilot tests for a period of up to 5 d by pharmacokinetic analysis of blood serum. Control mice received placebo pellets only. Two days after pellet implantation, we delivered all chemotherapeutic agents except FTI and tetrathiomolybdate by bolus intravenous injection into the tail vein on a schedule of three times per week for a period of 2 weeks. FTI and tetrathiomolybdate were delivered daily by intraperitoneal injection on the same schedule. The doses used for each cytotoxic agent were at or near the MTD reported in the literature. At the 2-week endpoint, we determined tumor volume and wet weight. Tumors were both frozen and fixed and subsequently processed, sectioned and analyzed by standard methods as described in **Supplementary Methods** online. All methods involving mouse use were approved by the Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research.

Immune cell depletions. Immunocompetent mice used in this study were subjected to cell depletion using standard methods[23,50]. Briefly, for T-cell depletion, we isolated tissue culture supernatants from the rat hybridomas GK1.5 and 2.43 and used them as a source of CD4-specific and CD8-specific monoclonal antibodies. Mice were injected intraperitoneally with 0.5 mg antibody for 3 d consecutively. We monitored splenic T-cell numbers 3 d later and at the experimental endpoint by flow cytometry using FITC-conjugated antibodies. Cell depletion was maintained during both tumor formation and tumor therapy experiments by intraperitoneal injection of 0.5 mg antibodies every 3 d. This strategy depleted >95% of the targeted T-cell subset in all treated mice compared to control mice that were injected with phosphate-buffered saline. For depletion of macrophages and APCs, we injected mice intraperitoneally with 2 mg carrageenan at 6, 3 and 1 d before subcutaneous injection of MRKECs, after which mice were injected one time per week up to the experimental endpoint.

IDO enzyme assays and inhibitor screens. We purified recombinant human his_6-IDO from *E. coli* strain BL21DE3pLys and used it in enzymatic reactions essentially as described[30]. The biochemical and cell-based inhibitor screening assays that were used to identify new IDO inhibitors were performed in a 96-well plate format, as described in detail in **Supplementary Methods** online.

Accession numbers. The GenBank accession numbers for the human IDO protein and the *Mus musculus* strain C57BL/6J chromosome 8 genomic contig, sequences 3011229–3010328 are NP 002155 and NT_039456, respectively.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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