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14. ABSTRACT
Evidence of loss or attenuation of the Bin1 gene in human breast cancers has implicated Bin1 as a tumor suppressor or negative modifier gene in mammary gland epithelial cells. We have discovered that Bin1 loss can promote tumorigenesis through an immune escape mechanism. This correlates with the negative regulatory impact that we have found Bin1 to exert on the important immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO). Previously we have reported how, in combination with certain chemotherapeutic agents, inhibitors of IDO can be employed in a non-obvious therapeutic regimen to successfully treat pre-established, autochthonous breast tumors in MMTV-Neu transgenic mice. During this reporting period, we have obtained evidence in mouse models that IDO expressed in plasmacytoid dendritic cells that accumulate in the tumor draining lymph node may be the relevant mechanism of immune escape in breast cancers, not direct expression of IDO in the tumor. We have further found that inhibiting IDO may not be an effective chemopreventive strategy for breast cancer but may be an effective strategy for suppressinig breast cancer metastasis.

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Table of Contents

INTRODUCTION

Loss or attenuation of expression of the *Bin1* anti-cancer gene in patient biopsies has been associated with malignant breast carcinoma (*1*) as well as other prevalent cancers. Our previous studies have indicated that *Bin1* loss can have a striking effect in promoting tumoral immune escape and that this can be more important to tumor formation than the impact of *Bin1* loss on intrinsic growth properties(*2*). We have identified the immunomodulatory gene *Indo* as a negatively-regulated downstream target of Bin1. *Indo* encodes indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme that has been demonstrated to play a physiologically essential role in protecting the allogeneic fetus during pregnancy by suppressing T cell activation. Our work is the first to connect the IDO to a known cancer suppression pathway, and dovetails nicely with the observation of increased IDO-mediated tryptophan catabolism that has been frequently reported in cancer patients. In a well-established transgenic mouse model for breast cancer, the MMTV-*Neu* mouse, we have demonstrated that IDO inhibitors can exhibit impressive therapeutic cooperativity when used in combination with specific chemotherapeutic agents (*2*). Identification of this non-obvious combination of immunotherapeutic and chemotherapeutic-based regimens presents a clear path forward for translational development. Our current studies are aimed at addressing the links between *Bin1* loss, IDO dysregulation, and host immunity in mouse breast cancer models. The overall goals are to evaluate how mammary gland-targeted *Bin1* loss and pharmacological inhibition of IDO impact tumor development, as well as examine the roles played by specific immune cell populations in anti-tumor rejection and tolerance. These studies are essential for understanding how IDO inhibitors operate in the *in vivo* context of mammary gland tumorigenesis and will establish the preclinical conceptual basis for future breast cancer drug development.

BODY

Task 1. Examine Bin1 and IDO expression in autochthonous MMTV-*Neu* tumors

In the previous year we reported evidence that Bin1 function in MMTV-*Neu* tumors may be attenuated both through decreased expression and mislocalization during the process of malignant transformation. We indicated that technical difficulties had precluded the evaluation of IDO in these tumors. We have not been able to resolve this difficulty because we have not obtained an antibody that gives specific staining of IDO in mouse tissues that is convincing. We have recently established a collaboration with Dr. David Munn at the Medical College of Georgia. His laboratory has successfully performed immunohistochemical analysis of IDO expressed in mouse tissues (*3*) and they are willing to evaluate these tissues from our mice as part of a collaboration that we have established. In a preliminary experiment, we have evaluated IDO in tumors formed by 4T1 breast carcinoma isografts (described in more detail in the next section) implanted ectopically into the mammary fatpad (**Fig. 1**). Interestingly, although there was no evidence of IDO expression in the tumor, IDO expression was detected in the tumor draining lymph node in what appear morphologically to be plasmacytoid dendritic cells. This is the same sort of staining pattern that this group has previously observed in a melanoma isograft

model, which has led to the hypothesis that, in the case of some tumors, immune escape can be mediated by IDO expression in the tumor draining lymph node. They have collected data from human breast cancer patients indicating that this may be the more common mechanism of immune escape for breast cancer and we are now collaborating to publish on these observations. Currently, we are evaluating tumors and tumor draining lymph nodes from MMTV-*Neu* mice for IDO staining and my expectation is that a similar pattern of IDO staining in the tumor draining lymph node but not the primary tumor will be the most likely outcome.

Task 2. Directly determine the impact of *Bin1* loss on tumor development

To address the goal of investigating how *Bin1* loss affects tumorigenesis driven by lactation-dependent expression of the c-*Neu* proto-oncogene in the mammary gland, we reported last year crossing the MMTV-*Neu* transgene onto the Wap-*Cre*$^{+/-}$*Bin1*$^{flox/KO}$ background, in which the *Bin1* gene undergoes tissue targeted disruption in the mammary epithelial cells of parous female mice. Experimental and control groups were evaluated under conditions that we have previously found to result in nearly 100% of MMTV-*Neu* transgenic female mice on the FVB strain background developing mammary gland tumors by 8 months. However, we were unable to obtain interpretable data in the Bin1 loss studies because tumor formation was dramatically suppressed as a consequence of the mixed non-FVB strain background in which these experiments were conducted. In order to circumvent this problem, we have performed the necessary breeding to make it possible to perform the same experiment on an FVB strain background as described in the Potential Pitfalls and Alternative Approaches section of Task 2. This required backcrossing three different transgenic lines to FVB for at least 5 generations each (to bring the genetic background to > 95% FVB) and then performing a relatively complex breeding strategy (diagrammed in **Fig. 2**) to produce mice with all of the requisite genetic elements in place. We currently have generated cohorts of FVB-strain MMTV-*Neu*$^{+/-}$Wap*Cre*$^{+/-}$ *Bin1*$^{flox/KO}$ experimental mice and MMTV-*Neu*$^{+/-}$Wap*Cre*$^{+/-}$*Bin1*$^{flox/wt}$ control mice that we are currently following for tumor formation, but the experiment is not yet far enough along to report any results. We have also pursued additional backup strategies that utilize alternative mechanisms to MMTV-*Neu* transgene expression for studying autochthonous and orthotopic mammary gland tumors in mice and these backup strategies are yielding positive data. In particular we have ongoing studies to evaluate breast tumors induced by chemical carcinogenesis and a metastatic breast carcinoma isograft model.

We reported last year that, although Bin1 is not essential for mammary gland development, it does facilitate lobular development prior to and during pregnancy but that compensatory development apparently minimizes this difference following parturition. In long term experiments, over a period of nearly 2 years, no significant impact of mammary gland targeted *Bin1* loss was observed on the frequency of spontaneously developing breast cancer in parous females (**Table 1**). To evaluate *Bin1* as a negative modifier of tumorigenesis, we have assessed the effect of *Bin1* loss on the mammary gland tumors initiated by treatment with the carcinogen 7, 12-dimethylbenz[α]anthracene (DMBA). In these experiments, the synthetic progesterone medroxyprogesterone acetate (MPA) was also been administered to increase tumor frequency, decrease tumor latency and reduce non-tumor related morbidity and mortality (*4*). After weaning their first litter, uniparous Wap*Cre*$^{+/-}$ *Bin1*$^{flox/KO}$ and Wap*Cre*$^{+/-}$*Bin1*$^{flox/wt}$ mice received

subcutaneous implants in the intrascapular area of two 20 mg MPA compressed pellets. Three weeks later, mice received the first of four 1x/week doses of 50 mg/kg DMBA, administered by oral gavage in cottonseed oil, with the three subsequent doses delivered 1, 3 and 4 weeks after the first dose. Wap*Cre*$^{+/-}$ *Bin1*$^{flox/KO}$ and Wap*Cre*$^{+/-}$*Bin1*$^{flox/wt}$ groups showed similar tumor latency and multiplicity. However, while Wap*Cre*$^{+/-}$*Bin1*$^{flox/wt}$ mice developed well-differentiated mammary tumors with robust tubule formation, minimal nuclear pleomorphism, and low mitotic indices, Wap*Cre*$^{+/-}$ *Bin1*$^{flox/KO}$ mice developed poorly differentiated mammary tumors with limited tubule formation, marked nuclear pleomorphism, and high mitotic indices (**Fig. 3**) We first reported on this observation last year and finalized the data collection during this study period (**Table 1**). A similar trend towards more aggressive tumors developing in the context of Bin1 loss was observed in the tumors that developed spontaneously in the absence of carcinogen exposure as well with the caveat that these data sets were very small (**Table 1**). In contrast, breast tumors that developed through introduction of a transgenic element in which expression of the c-Myc proto-oncogene is controlled by the mouse mammary tumor virus (MMTV) promoter resulted in poorly differentiated tumors regardless of Bin1 status. These data are consistent with the idea that Bin1 is on the same oncogenic pathway as c-Myc and that enforced over-expression of c-Myc can overcome the impediment that physiological levels of Bin1 expression present to cellular transformation (**Table 2**). These data were published recently in *Cancer Research* (*5*).

Task 3. Investigate the chemopreventative activity of IDO inhibitor treatment in relation to *Bin1* status

The rationale for this Task was predicated on the assumption that Bin1 loss in the MMTV-*Neu* model would promote tumor development. Because of difficulties encountered with lack of tumor susceptibility in mixed strain background that we originally used, we are currently still working to test the initial hypothesis as explained in detail under Task 2. One assumption in the proposal that we are now beginning to question is that actively ablating Bin1 in the mammary gland epithelium will help make the tumors better able to escape host immunity through dysregulated IDO. Since beginning work on this project, we are becoming increasingly convinced that the relative importance of tumor-expressed IDO may be contextual and that breast cancer may be a tumor type in which this is less important to primary tumor outgrowth than IDO activity in the stroma. This idea is based in large part on studies that we have performed using the highly metastatic 4T1 breast carcinoma cell line. 4T1, which was isolated from a spontaneously arising BALB/c-strain mammary gland tumor (*6*), forms progressively growing primary tumors and that spontaneously metastasize to the lungs, liver, blood, lymph nodes, brain, and bone marrow within two weeks after an initial orthotopic injection of 1 x 10^4 cells in a 50µl volume into the no. 4 mammary gland (*7*). Using a luciferase expressing 4T1 derivative, we have found that higher dose cyclophosphamide (100 mg/kg) in combination with the IDO inhibitory compound1MT produces cooperative anti-tumor activity that results in tumor regressions (**Fig. 4A**). This is similar to the type of response we have been able to achieve in the MMTV-*Neu* model using paclitaxel in combination with oral dosing of 1MT on a twice a day (bid) schedule (**Fig. 4B**). With lower dose cyclophosphamide (25 mg/kg) we have, in the absence of an effect on primary tumor growth (data not shown), been able to demonstrate that combining with 1MT produces a significant increase in survival from metastatic disease (**Fig. 5A**). This cooperativity occurred specifically with the D rather than the L isomer of 1MT. We

also found the L isomer to be more active in the MMTV-*Neu* breast cancer model (**Fig. 5B**) while our collaborators Drs. David Munn and Andrew Mellor have made a similar observation in a melanoma isograft model. In collaboration with Drs. Munn and Mellor, we have generated data that suggest that the D isomer of 1MT selectively inhibits IDO activity in tumors while the L isomer selectively inhibits IDO activity in tolerogenic dendritic cells (*8*). Consistent with these data, tumors formed by 4T1 cells and by B16-F10 cells exhibit no evidence of IDO expression by immunohistochemical staining, while clear evidence of IDO staining was found in both cases to be present in the tumor draining lymph nodes in cells morphologically characterized as plasmacytoid dendritic cells. As indicated for Task 1, we do not as of yet have immunohistochemical staining data for MMTV-Neu tumors, but because of the selective activity of the D isomer of 1MT in this model, we anticipate a similar pattern. Further confirming the importance of stromal IDO in this context, our collaborators have shown that combination therapy with cyclophosphamide and 1MT was no longer effective against B16-F10 tumors in the context of an IDO knockout mouse (**Fig. 5C**). These data were recently published in a second *Cancer Research* article (*8*).

The combination therapy data in the 4T1 model suggested to us the possibility that in the case of this breast cancer model IDO might be more relevant to the establishment of metastases than to primary tumor outgrowth and that the relevant expression of IDO is more likely to be in the stroma rather than the tumor. As indicated in the Potential Pitfalls and Alternate Approaches section to Task 3, we were clearly cognizant that this sort of question could be ideally addressed using an IDO knockout mouse, but we deemed creation of such a mouse to be beyond the scope of the project. We did not, however, anticipate at the time that we would be able to obtain the IDO knockout mice through the establishment of a collaboration with Drs. Munn and Mellor. Acquiring their IDO knockout mouse line has allowed us to perform experiments aimed at dissecting the role of IDO in tumor development more directly than would be possible with just the use of small molecule IDO inhibitors as we had originally proposed. Based on our IDO inhibitor treatment data, we anticipated that we would have to provide chemotherapy to IDO knockout mice challenged with 4T1 tumor cells in order to produce a survival benefit. Instead we found that the IDO knockout mice without any additional treatment had improved survival over wild type mice that was comparable to what was achieved with the combination of 1MT + cyclophosphamide in the wild type mice (**Fig. 6**). Furthermore, the additional treatment of IDO knockout mice with cyclophosphamide did not provide any greater survival benefit over vehicle treatment. Pharmacokinetic analysis of 1MT levels has indicated that we do not achieve sufficient exposure to achieve 100% enzyme inhibition in an *in vitro* cell based assay so it is unlikely that we are completely blocking IDO enzyme activity in animals with 1MT treatment. The knockout data suggest that better inhibitors might be able to supplant the need to combine with a chemotherapeutic agent in order to achieve anti-tumor efficacy, at least against metastatic disease.

We have used IDO knockout mice to further evaluate the idea that blocking IDO might be an effective breast cancer chemopreventive strategy, thereby addressing the major goal for this Task. Our preliminary results suggest that there will not be a simple straightforward answer, but instead indicate that the answer may depend on the tumor type and how it arises. We have performed carcinogenesis in IDO knockout mice to preferentially induce the development of breast tumors using DMBA and MPA as described in the report on Task 2. Using this

carcinogenesis regimen, we observed no significant difference in the incidence of breast tumors that developed in IDO knockout mice as compared to wild type in either the C57BL/6 or a BALB/c strain backgrounds (**Fig. 7A**). In contrast, in a parallel set of experiments for a different project, we have also employed the classical two step DMBA/TPA carcinogenesis procedure to evaluate the impact of IDO loss on skin tumor development. In this case there was a dramatic impact of IDO loss, with papilloma formation significant reduced in the IDO knockout mice as compared to the wild type in the two strain backgrounds tested (**Fig. 7B**). We had also previously found that Bin1 loss had no significant impact on the incidence of breast tumors induced by chemical carcinogenesis (**Table 1**) but, consistent with the IDO knockout data, loss of Bin1 in the skin resulted in a clear increase in papilloma formation (**Fig. 7B**). Thus it appears that the chemopreventive potential for IDO inhibition may not be of much consequence in hormone driven breast cancer but may be much more significant in tumors in which local inflammation is more involved in the establishment of the disease, such as perhaps colon or lung cancer. When the MMTV-*Neu* mice on the FVB strain background are available, we do plan to test this hypothesis in this tumor model as well.

Task 4. Profile tumor-associated immune cell populations and functionally characterize the involvement of specific T cell populations.

We have just begun to address experiments proposed in this final task. In very preliminary studies, we have stained MMTV-*Neu* tumors for CD3 positive cells. We have observed an apparent increase in the number of CD3 positive cells present in tumors from mice treated with a combination of paclitaxel + 1MT relative to the tumors from vehicle alone treated mice (**Fig. 8**). Preliminary FACS analysis of CD69 positive cells further suggests that the number of activated lymphocytes in the tumor is approximately 3-4 fold higher in mice treated with combination therapy as compared to vehicle treated controls (data not shown). These data are consistent with an active immune response being mounted against the tumor, and we will continue with the experiments outlined for this task to explore the underlying immunologic basis for this response.

KEY RESEARCH ACCOMPLISHMENTS

- Established a collaboration with Drs. David Munn and Andrew Mellor to evaluate IDO staining in MMTV-*Neu* mouse mammary gland tumors and tumor draining lymph nodes as part of a larger project to evaluate the relevance to breast cancer of IDO expression in these two compartments. Have obtained preliminary immunohistochemical staining data from an orthotopic breast carcinoma isograft model consistent with IDO expression being predominantly associated with plasmacytoid dendritic cells in the tumor draining lymph node rather than in the tumor itself.

- Backcrossed all of the necessary genetic elements onto the FVB strain background and performed all of the subsequent crosses needed to evaluate the impact of mammary gland targeted deletion of the Bin1 gene on MMTV-*Neu* driven breast cancer. The experiment to determine this is currently underway.

- Demonstrated that mammary gland targeted Bin1 loss does not significantly impact the frequency or latency of carcinogen-induced breast cancer, but does consistently result in

disease that scores as histopathologically more aggressive. These data were recently published in *Cancer Research*.

- Demonstrated that the D isomer of the IDO inhibitor, which selectively targets stromal rather than tumoral IDO, effectively delays metastatic disease progression in an orthotopic breast carcinoma isograft in combination with cyclophosphamide treatment. These data were recently published in a second article in *Cancer Research*.

- Through our collaboration with Drs. David Munn and Andrew Mellor, were able to use an IDO knockout mouse to demonstrate that the absence of stromal IDO is sufficient to effectively delay metastatic disease progression in an orthotopic breast carcinoma isograft model.

- Further used the IDO knockout mouse to address the relevance of IDO as a chemoprevention target by demonstrating that the lack of IDO does not significantly affect the incidence or latency of carcinogenesis-induced breast tumors while it results in dramatic suppression of the incidence of carcinogenesis-induced skin tumors.

- Have performed preliminary analysis of tumor-associated immune cell populations which is consistent with there being an active immune response against MMTV-*Neu* tumors following treatment with paclitaxel plus the IDO inhibitor 1MT.

REPORTABLE OUTCOMES

- **Manuscripts**

Gaspari, P., T. Banerjee, W.P. Malachowski, **A.J. Muller**, G.C. Prendergast, J. DuHadaway, S. Bennett and A.M. Donovan. Structure-activity study of brassinin derivatives as indoleamine 2,3-dioxygenase inhibitors. *J. Med. Chem.* **49**:684-692 (2006).

Muller, A.J. and P.A. Scherle. Targeting the mechanisms of tumoral immune tolerance with small molecule inhibitors. *Nat. Rev. Cancer* **6**:613-625 (2006).

Chang, M.Y., J. Boulden, E. Sutanto-Ward, J.B. DuHadaway, A.P. Soler, **A.J. Muller**, G.C. Prendergast. Bin1 ablation in mammary glands delays tissue remodeling and drives cancer progression. *Cancer Res.* **67**:100-107 (2007).

Hou, D.-Y., **A.J. Muller**, M. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, A.L. Mellor, G.C. Prendergast, D.H. Munn. Inhibition of IDO in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with anti-tumor responses. *Cancer Res.* **67**:792-801 (2007).

Muller, A.J. and G.C. Prendergast. Indoleamine 2,3-dioxygenase in immune suppression and cancer. *Curr. Cancer Drug Targets* **7**:31-40 (2007).

- **Abstracts/Presentations**

11th Meeting of International Study Group for Tryptophan Research
Tokyo, Japan July 4-7, 2006
Abstract presented: "Inhibition of IDO, an Immunoregulatory Target of the Cancer Suppression gene *Bin1*, Potentiates Cancer Therapy"
(Invited speaker)

American Association for Cancer Research 97th Annual Meeting. Washington, DC. April 1-5, 2006.
Abstract presented: "Development of brassinin derivatives as IDO inhibitors for combinatorial cancer treatment"
(Poster)

Molecular Targets 2007: Mechanism and Therapeutic Reversal of Immune Suppression in Cancer. Clearwater Beach, FL. January 25-28, 2007.
Abstract presented: "Cancer immunotherapy targeting IDO-mediated tryptophan catabolism"
(Poster)

CONCLUSION

Our ongoing studies in mouse breast cancer models have helped to establish a case for this particular tumor type being more dependent on IDO activity expressed in the stroma for mediating tumoral immune escape than on IDO activity directly expressed in the tumor cells. This is consistent with observations made by Drs. David Munn and Andrew Mellor, with whom we are now collaborating, in the B16-F10 melanoma isograft tumor model. In particular, our IDO staining data indicate that, similar to the melanoma studies, the accumulation of plasmacytoid dendritic cells with elevated IDO in the tumor draining lymph node appears to be associated with the outgrowth of orthotopic breast carcinoma isografts. It will be very interesting to see if similar results are obtained with autochthonously developing MMTV-*Neu* tumors, which should even more closely recapitulate the full developmental process of human breast cancers.

The observation that IDO is differentially targeted by the two isomers of the IDO inhibitor 1-methlyl-tryptophan (1MT), so that the L form is more effective against IDO expressed in tumors while the D form is more effective against IDO expressed in the stroma, is interesting from both a basic research as well as a clinical development perspective. From a basic research perspective, this brings up the obvious question of what is different between IDO in these two compartments – a question that we plan to actively pursue in future research projects. From a clinical development perspective, both the NCI, through the CTEP program, as well as the biotechnology company NewLink Genetics Corporation, are in the process of preparing to file an IND to bring the compound D-1MT forward into early phase clinical trials. Thus, it is imperative to acquire as much information as possible regarding the tumor settings in which this compound may most likely be efficacious as well as where it may not. The data we have generated point to

breast cancer as potentially being a clinically relevant tumor type in which to evaluate D-1MT because it appears to be more dependent on stromal rather than tumor expressed IDO. Furthermore, our data suggest that prevention of primary tumor development may not be the most effective application of IDO inhibitors in breast cancer, but that other cancers may be tractable to an IDO chemoprevention strategy due, perhaps, to their greater degree of dependence on an inflammatory microenviroment. On the other hand, IDO inhibition may be an effective approach to impair breast cancer metastasis. Our studies are the first to demonstrate that IDO inhibition can be effective against tumor metastases, and clinically this may be even more relevant than the data that have been generated regarding the activity of IDO inhibitory compounds against primary tumors.

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Bin1 Ablation in Mammary Gland Delays Tissue Remodeling and Drives Cancer Progression

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Abstract

Genes that modify oncogenesis may influence dormancy versus progression in cancer, thereby affecting clinical outcomes. The *Bin1* gene encodes a nucleocytosolic adapter protein that interacts with and suppresses the cell transforming activity of Myc. *Bin1* is often attenuated in breast cancer but its ability to negatively modify oncogenesis or progression in this context has not been gauged directly. In this study, we investigated the effects of mammary gland–specific deletion of *Bin1* on initiation and progression of breast cancer in mice. *Bin1* loss delayed the outgrowth and involution of the glandular ductal network during pregnancy but had no effect on tumor susceptibility. In contrast, in mice where tumors were initiated by the *ras*-activating carcinogen 7,12-dimethylbenz(*a*)anthracene, *Bin1* loss strongly accentuated the formation of poorly differentiated tumors characterized by increased proliferation, survival, and motility. This effect was specific as *Bin1* loss did not accentuate progression of tumors initiated by an overexpressed mouse mammary tumor virus-c-*myc* transgene, which on its own produced poorly differentiated and aggressive tumors. These findings suggest that *Bin1* loss cooperates with *ras* activation to drive progression, establishing a role for *Bin1* as a negative modifier of oncogenicity and progression in breast cancer. [Cancer Res 2007;67(1):100–7]

Introduction

Breast cancer is currently the second leading cause of cancer-related death among women. In recent years, medical advances have improved detection at earlier stages, such that women with small localized breast tumors will tend to have a good prognosis after treatment. However, up to 20% of patients with 'good prognosis' will nevertheless relapse within 5 years with advanced disease. Conversely, patients considered to have a poorer prognosis are not necessarily fated to relapse with disease. Thus, the limited prognostic information available may cause some patients to be treated too aggressively, increasing therapy-related morbidity, and other patients to be treated too conservatively, increasing disease-related mortality. One way to help improve the management of breast cancer would be to use markers that can accurately predict disease course.

Modifier genes may offer usefulness in this regard given their effects on dormancy versus progression in the context of certain oncogenic pathways that drive neoplasia (1, 2). Alterations in the structure or regulation of a candidate modifier gene that correlates with progression status can offer one line of evidence for a marker. By evaluating alterations in an animal model, one can directly determine whether they are coincidental or causal to disease. To identify disease modifier genes, classic genetics can be used to map genes by "top-down" designs or reverse genetics can be used to assess candidates via "bottom-up" designs, with the understanding that a candidate will be phenotypically silent in the absence of relevant oncogenic lesions. In the present study, we used the latter approach to test the hypothesis that *Bin1* acts as a negative modifier of breast cancer progression.

Bin1 encodes a nucleocytosolic BAR adapter protein that can interact with the c-Myc oncoprotein and inhibit its cell transforming activity (3–5). c-Myc is involved in the development of many human breast cancers where its overexpression has been associated with poor prognosis (6). At least 10 splice isoforms of Bin1 exist, with differences in the pattern of tissue distribution, subcellular localization, and protein interactions that indicate diverse functional roles (7–10). BAR adapter proteins include a signature fold termed the BAR domain that recognizes curved vesicular membranes (11). Although BAR adapter proteins have a canonical function in membrane dynamics (12), in certain family members that localize to the nucleus (e.g., including Bin1 and APPL proteins), a moonlighting function in transcriptional regulation has been suggested (4, 5, 13). Notably, only those Bin1 isoforms that are capable of localizing to the nucleus are capable of suppressing oncogenic transformation, facilitating cell suicide, and promoting immune escape of transformed cells in various model systems (3, 4, 14–20). Although attenuation of Bin1 by silencing or missplicing is a frequent event in many human cancers, including breast cancer (3, 16), the consequences of Bin1 loss on tumor progression have not been addressed directly in a preclinical model of disease. Therefore, we tested whether such losses were sufficient to drive initiation or progression of cancers in mice harboring mammary gland–specific deletions of *Bin1*.

Materials and Methods

Production of transgenic mouse strains. A *Bin1*-targeting plasmid with the structure shown in Fig. 1 was introduced by electroporation into embryonic stem (ES) cells derived from 129sv mice. Briefly, a neomycin resistance gene (*neo*) cassette flanked by wild-type (WT) loxP sites was inserted into a genomic targeting vector spanning introns 2 to 5 of the mouse

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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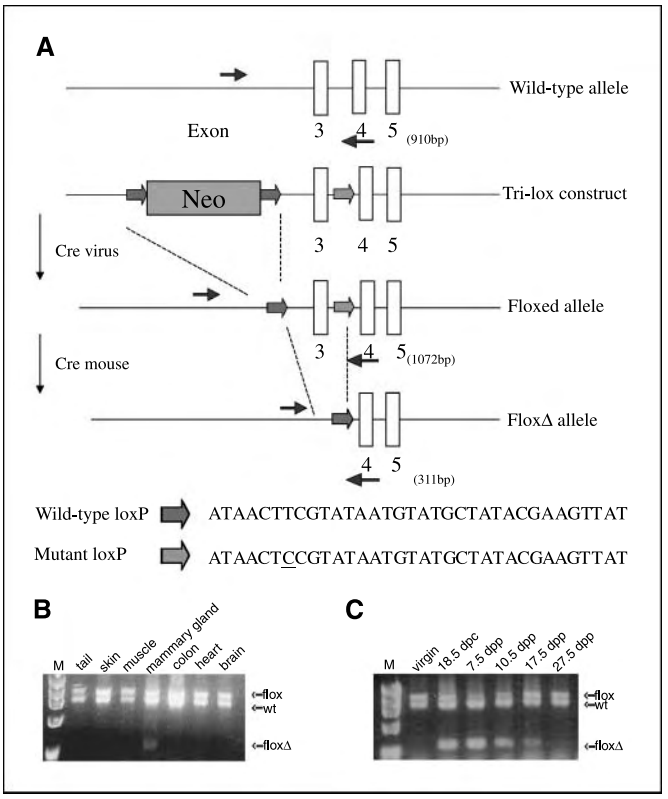

Figure 1. Tissue-specific deletion of *Bin1* in murine mammary gland. *A,* Bin1 flox targeting construct. *White boxes,* exons. *Colored arrows,* the WT (*solid*) and variant mutant (*hatched*) loxP sites; *thin arrows,* location of PCR primers with the size of the predicted amplification products given in bp. The structure of the tri-lox-targeting plasmid is noted along with the structure of the desired floxed or floxΔ alleles generated by Cre-mediated recombination in ES cells *in vitro* or *in vivo*, respectively. The position of the T→C mutation introduced into the variant loxP site. *B,* mammary gland–specific recombination in wap-cre;+/flox mice. Genomic DNA isolated from various tissues was subjected to PCR using the *Bin1* primers presented above (*blue arrows*). Conversion to the floxΔ allele occurred specifically only in the mammary glands of parous female mice. *C,* kinetics of wap-cre–mediated recombination. Generation of the floxΔ allele is apparent during and after pregnancy and weaning.

Bin1 gene (21). Three ES cell lines with the desired homologous recombination event were infected with a recombinant Cre adenovirus and subcloned to identify cell colonies that had selectively lost the neo marker, leaving intact the desired floxed exon 3 segment. These correctly targeted ES cell lines were microinjected into C57BL/6J blastocysts, and chimeric animals with germ-line transmission of the floxed *Bin1* allele were generated. *Bin1* is haplosufficient for viability (22). Therefore, to establish the most efficient system for producing Bin1-expressing or nonexpressing cells by a single Cre-mediated excision event, we crossed the 'floxed' allele (flox) onto a strain with the 'straight' knockout (KO) allele (22). Cre recombinase was introduced by interbreeding with B6129-Tg(wap-cre)11738 Mam/J(wap-cre mice) (The Jackson Laboratory). Activated c-*myc* was introduced by interbreeding with mouse mammary tumor virus (MMTV)-c-*myc*. FVB-N mice (Charles River Laboratories) under a license from DuPont Medical Products (Wilmington, DE).

Genotype analysis. PCR was used to genotype mice as follows. Mouse tissue samples were digested overnight at 60°C in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 100 mmol/L NaCl, 1% SDS, 30 μg/mL proteinase K]. DNA-containing supernatant was diluted 1:50 in 10 mmol/L Tris-Cl (pH 8.0) and 2 μL of diluted supernantant were used for PCR in a final volume of 20 μL in a PTC-2000 Peltier Thermal Cycler (MJ Research). Amplification products were separated by electroporation on 2% agarose gels prestained with ethidium bromide, using *Hae*III-digested φX174 phage DNA (Fisher) as a molecular size marker. The primers used to monitor the Bin1 flox allele were 5′-TGGAGTCTGCCACCTTCTATCC-3′ (loxP1) and 5′GCTCATACACCTCCTGAAGACAC3′ (loxP2; Integrated DNA Technologies, Inc.) with expected sizes of 0.9, 1.07, and 0.31 kb for WT, flox, and recombined flox (floxΔ) alleles, respectively. Following a 4-min denaturation at 94°C, 35 cycles of PCR were done at 94°C for 20 s, 58°C for 1 min, and 72°C for 1 min with the addition of a 10-min final elongation step at 72°C. The primers and PCR conditions used to monitor the Bin1 KO allele have been described (22). The primers used to monitor the *wap-cre* gene were 5′-GCGGTCTGGCAGTAAAAACTATC-3′ (Wap1) and 5′-GTGAAACAG-CATTGCTGTCACTT-3′(Wap2) with allele-positive mice identified by a single 100-bp agarose gel band. PCR conditions for the *wap-cre* gene were as follows: after a 4-min denaturation at 94°C, 40 cycles of PCR were done at 94°C for 30 s, 62°C for 1 min, and 72°C for 1 min with the addition of a 5-min final elongation step at 72°C. The primers used to monitor the MMTV-c-*myc* gene were 5′-CCCAAGGCTTAAGTAAGTTTTTGG-3′ (Myc1) and 5′-GGGCATAAGCACAGATAAAACACT-3′ (Myc2) with allele-positive mice identified by a single 880-bp agarose gel band. PCR conditions for the MMTV-c-myc gene were as follows: after a 3-min denaturation at 96°C, 39 cycles of PCR were done at 96°C for 30 s, 58°C for 1 min, and 72°C for 1 min with the addition of a 5-min final elongation step at 72°C.

Mammary gland carcinogenesis. After one round of pregnancy, female mice received s.c. implants in the intrascapular area of two compressed pellets of 20 mg medroxyprogesterone acetate (Hormone Pellet Press). Three weeks later, we gave the first of four weekly doses of 50 mg/kg 7,12-dimethylbenz(*a*)anthracene (DMBA; Sigma, St. Louis, MO), given p.o. in cottonseed oil, with the three subsequent doses delivered 1, 3, and 4 weeks after the first dose. On this regimen, we observed mammary tumors to appear with a frequency of ~100% with an average latency of 112 days,[4] not significantly longer than the 99 days reported for CD2F1 (BALB/CXDBA/2) mice (23).

Cell biology. Murine mammary epithelial cells (MMEC) explanted from breast tumors were cultured in DMEM containing 10% fetal bovine serum (FBS; HyClone) and antibiotics. Cells were passaged multiple times at a 1:4 passage ratio to rid explanted tissue of contaminating fibroblasts and other cells. Western blot and immunofluorescence analyses with E-cadherin and β-catenin antibodies (see below) were done to confirm the epithelial nature of MMEC cultures established in this manner. Cell proliferation assays were done by seeding 1×10^6 cells in 100-mm dishes and harvesting at various times later for counting by trypan blue exclusion (24). For serum deprivation, cells were treated the day after seeding them into culture for 24 h with DMEM containing 0.1% FBS. For anchorage-independent growth, 1×10^4 cells were seeded in soft agar, and colony formation was documented as described previously (24). For flow cytometry, cells were harvested, washed once with PBS, fixed in 70% ethanol, stained with propidium iodide, and analyzed on a FACScan device (Becton Dickinson). For motility assays, 1×10^6 cells were seeded in a 100 μL droplet in individual wells of a six-well plate and incubated for 16 h. When cells reached confluency within the droplet, its center was scratched, 2 mL DMEM plus 10% FBS was added to the well, and motility was documented at 48 h by photomicrography.

Western blot analysis. Cells were harvested by washing thrice in PBS before lysis in 1× radioimmunoprecipitation assay buffer [1× PBS containing 1% NP40, 0.5% sodium-deoxycholate, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride] with 10 μL/mL Protease Inhibitor Set II and III (Calbiochem). Protein was quantitated by Bradford assay and 50 μg protein per sample was analyzed by SDS-PAGE. Gels were processed by standard Western blotting methods using the Bin1 antibody 2F11 (ammonium sulfate supernatant, 1:200 dilution) and horseradish peroxidase (HRP)–conjugated goat anti-mouse secondary antibody (1:2,000 dilution; Cell Signaling). For actin, a primary anti-actin goat polyclonal antibody was used (1:500 dilution; Santa Cruz Biochemicals) and HRP-conjugated rabbit anti-goat secondary antibody (1:5,000 dilution; Southern Biotechnology Associates). For cell adhesion proteins, primary antibodies

[4] M.Y. Chang, unpublished observation.

used included anti-E-cadherin (1:1,000 dilution; clone 36, Transduction Laboratories), anti-β-catenin (1:1,000 dilution; clone 5H10, Zymed), anti-N-cadherin (1:1,000 dilution; clone 3B9, Zymed), anti-vimentin (1:1,000 dilution; clone Vim13.2, Sigma), and goat anti-mouse secondary antibody (1:1,000 dilution; Southern Biotechnology Associates). Detection was done routinely using a commercial kit (enhanced chemiluminescence-Western blot, Amersham).

Zymogram analysis. Gelatinase activity in protein extracts from established MMEC tumor cell lines was monitored as described.[5]

Orthotopic tumor formation assay. Cells (1×10^7) were suspended in 200 μL DMEM and injected orthotopically into the mammary fat pads of syngeneic F1 offspring from FVB-N and C57BL/6J breeders (The Jackson Laboratory) or immunocompromised CD-1 nude (Crl:CD-1-nuBR) mice (Charles River Laboratories). When tumors reached ~20 mm in diameter, mice were euthanized and tumor weight(s) and volume(s) were calculated via caliper (volume = width^2 × length × 0.52).

Results

Targeted deletion of *Bin1* delays remodeling and involution of the mammary gland. To determine whether Bin1 attenuation could directly affect initiation or progression of breast cancer, we embarked on an investigation of the consequences of deleting *Bin1* in the mammary gland of the mouse. Previous work established that homozygous deletion causes perinatal lethality (22); therefore, for this work, we generated a conditional mutant using Cre-lox technology. The design is presented in Fig. 1 along with results confirming the desired *in vivo* operation of the 'floxed' allele in the mammary gland. In the 'tri-lox' scheme used, deletion of exon 3 leads to exon 2 to 4 splicing, producing an out-of-frame stop codon in exon 4 that abolishes protein expression from all alternately splice isoforms of *Bin1* RNA. In one variation of the standard design, a mutant loxP site incorporating a T→C mutation was included, such that exon 3 was flanked on its 5′ side by a WT loxP site and on its 3′ side by the mutated loxP site (Fig. 1*A*). This variation conferred a selective advantage to Cre-mediated excision of the neo cassette *in vitro*, without compromising the subsequent ability of Cre to delete the floxed target sequence *in vivo*.[4] Chimeric mice generated from targeted ES cell transfectants were interbred with transgenic mice to produce strains that included the WT *Bin1* allele (+), floxed KO allele (flox), and 'straight' KO allele (22) along with a breast-specific *wap-cre* transgene and, in some experiments, a MMTV-c-*myc* transgene. In animals carrying the *wap-cre* gene, loxP-mediated recombination in females was induced by parity because the whey acidic protein (wap) promoter is activated in mammary epithelial cells during pregnancy. Bin1 is haplosufficient for survival (22), so the breeding scheme compared mice with +/flox or KO/flox genotypes to compare the effects of functional ablation. As expected, mice with a wap-cre;KO/flox genotype exhibited tissue-specific conversion of the floxed allele to the desired 'floxΔ' allele in genomic DNA isolated from mammary gland from late pregnancy through weaning (Fig. 1*B* and *C*). To simplify nomenclature, in the text that follows, we refer to mice with a wap-cre;+/flox or wap-cre;KO/flox genotype as Bin1+mam or Bin1Δmam mice, respectively, indicating the retention of one functional allele or the loss of both alleles in the mammary gland. In work to be reported elsewhere,[6] we confirmed that the floxΔ allele is functionally inactivated based on its ability to phenocopy a 'straight' KO allele with regard to myocardial hypertrophy and perinatal lethality (22). These experiments confirmed that the model system operated as required to investigate the effect of *Bin1* ablation on remodeling and tumorigenesis in the mammary gland.

To evaluate whether *Bin1* loss affected mammary gland remodeling induced by pregnancy, female mice were set up for timed pregnancies by monitoring for vaginal plugs. After parturition, litter sizes were normalized to five pups and nursing was continued 1 week to ensure full lactation before pups were removed to induce mammary gland involution. Mammary gland tissues were isolated for analysis from virgins (control) or at 18.5 days post coitum (dpc), 7.5 days post partum (dpp; full lactation), 10.5 dpp (early involution), 17.5 dpp (late involution), and 27.5 dpp (full regression). A delay in the kinetics of ductolobular development was apparent at 18.5 dpc, at which time Bin1Δmam mice showed significantly less glandular remodeling than Bin1+mam mice (Fig. 2). However, during lactation at 7.5 dpp, this defect had resolved, such that no deficiencies were apparent in nursing and pups showed no signs of malnutrition. During glandular involution, a delay in remodeling again became apparent, such that ductolobular regression was achieved with somewhat slower kinetics in Bin1Δmam mice. We concluded that *Bin1* was non-essential for formation of a fully functional lactating mammary gland but that it was needed to optimally support the rapid kinetics of ductolobular remodeling in the gland during pregnancy and weaning.

Bin1 attenuation drives progression of *ras*-dependent mammary carcinomas. We evaluated *Bin1* as a classic suppressor or negative modifier gene in breast cancer by investigating whether its deletion was sufficient (*a*) to increase the incidence of mammary tumor formation, in the manner of an inactivated suppressor, or (*b*) only to increase the progression of mammary tumors initiated by primary oncogenic lesions, in the manner of an inactivated negative modifier.

To evaluate *Bin1* as a suppressor gene, we compared the effect of tissue-specific ablation in three cohorts of Bin1+mam and Bin1Δmam female mice carried out as nonparous animals (virgin), uniparous animals (one round of pregnancy), or multiparous animals (seven rounds of pregnancy). After birth, litters were normalized to five pups, nursed 10 days, and then removed. By 2 years of age, both strains of mice developed mammary gland tumors with the same low frequency (Table 1). No differences were seen between uniparous and multiparous groups, which were combined as parous. Although the tumors that arose in the cohort of Bin1Δmam mice were relatively more poorly differentiated, the similarly low incidence observed argued against the notion that *Bin1* functioned as a classic breast tumor suppressor gene.

To evaluate *Bin1* as a negative modifier gene, we compared the effect of its tissue-specific ablation in tumors initiated by the carcinogen DMBA, which acts through *ras* activation (23), a MMTV-c-*myc* transgene, or both. We chose these well-established models of mammary carcinoma based on the evidence that *Bin1* can suppress neoplastic transformation of primary cells by c-*myc*+*ras* (3, 4, 19). In the initial trials, Bin1+mam and Bin1Δmam female mice were treated with DMBA and monitored for tumor formation. Both cohorts displayed similar rates of tumor latency, multiplicity, and lung metastasis (Table 1). However, whereas Bin1+mam mice developed well-differentiated tumors, characterized by high tubule formation, low mitotic indices, and limited nuclear pleomorphism, Bin1Δmam mice developed poorly

[5] http://www.chemicon.com/techsupp/protocol/gelatinzymograph.asp.
[6] M.Y. Chang and G.C. Prendergast, in preparation.

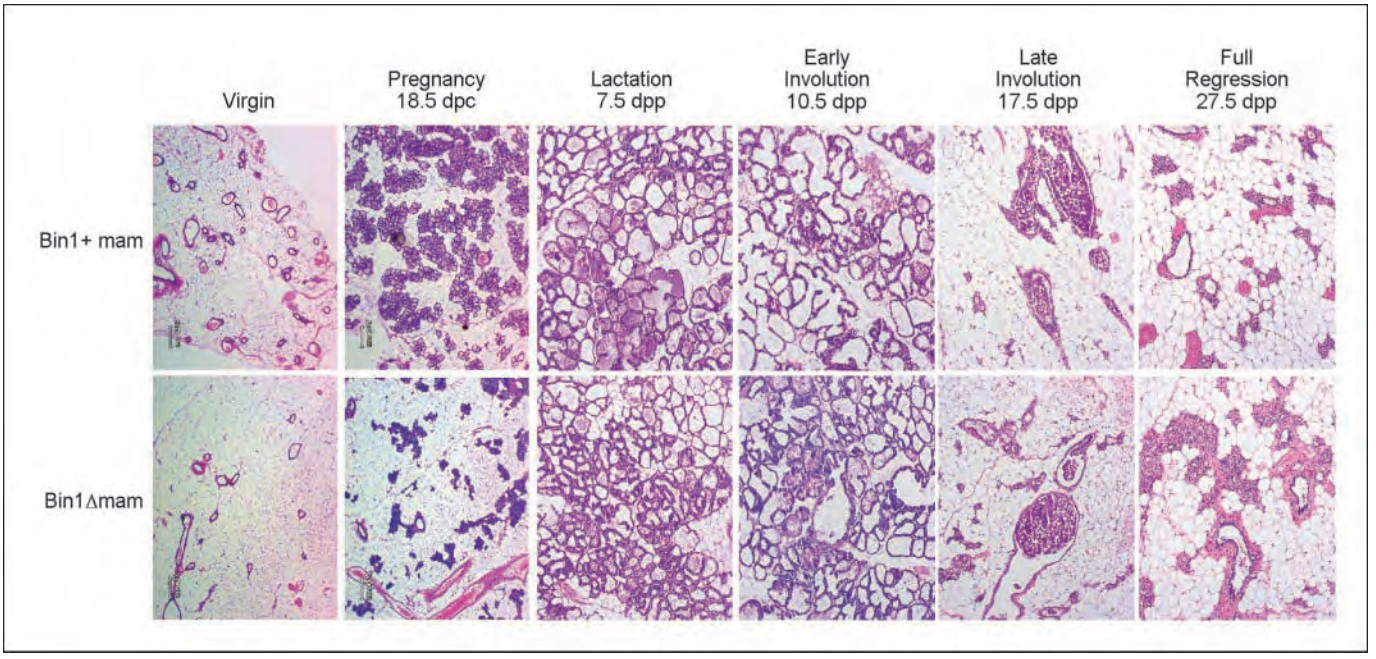

Figure 2. *Bin1* ablation delays mammary ductolobular remodeling during pregnancy. At the times indicated, mammary gland tissue sections were prepared and processed from Bin1+mam and Bin1Δmam mice for histologic analysis.

differentiated tumors, characterized by low tubule formation, high mitotic indices, and high degrees of nuclear pleomorphism (Table 1). Nuclear pleomorphism was particularly increased by *Bin1* loss (Fig. 3). Additionally, Bin1Δmam mice displayed a relative increase in lymphocyte infiltration compared with Bin1+mam mice (57% versus 20% of tumors; A.P.S., data not shown). In parallel experiments done in a mosaic model, we saw a similar pattern of development of more poorly differentiated mammary tumors in Bin1Δmam mosaic mice.[4] Together, these observations suggested that *Bin1* functioned as a negative modifier to restrict the progression of tumors initiated by activation of the *ras* pathway.

An interesting feature of the mammary tumor-bearing Bin1Δmam cohort was that it displayed a coincident elevation in uterine endometritis and ovarian granulosa cell tumors, implying either haploinsufficiency or a non–cell autonomous effect of Bin1 loss in the uterus and ovary. DMBA treatment is known to cause such lesions in addition to mammary tumors (e.g., ref. 25) but not at the relatively higher penetrance observed in mice from the Bin1Δmam cohort. The transcriptional activity of the wap promoter is restricted to brain and the mammary gland during the lactational stage of pregnancy (26), and consistent with this pattern of expression, we did not detect recombination of the Bin1 floxed allele in the ovary or uterus of Bin1Δmam mice.[4]

Table 1. Bin1 loss drives progression of DMBA-induced mouse mammary carcinomas

Genotype	Regimen	No. mice w/ tumors (%)	Tumors per mouse	Latency (d)*	Grade† T,N,M, (sum)	Differentiation status	Lung metastasis (%)
Bin1+	Nonparous	0/6 (0)	0	NA	ND	NA	0/0 (0)
Bin1+mam	Parous	1/19 (5)	1	NA	ND	WD	0/1 (0)
Bin1Δmam	Parous	2/24 (8)	1	NA	ND	PD	0/2 (0)
Bin1+mam	DMBA	8/8 (100)	2.3 ± 1.3	128 ± 47	1.6,1.5,2.3 (5.4)	WD	4/8 (50)
Bin1Δmam	DMBA	14/14 (100)	2.2 ± 1.4	115 ± 36	2.3,2.5,2.9 (7.8)	PD	7/14 (50)

NOTE: Nonparous and parous mice not treated with DMBA were monitored for their full life span for breast tumor formation. In these groups, the small number of tumors that arose was all seen in elderly animals of >1 year of age. Uniparous animals treated with DMBA exhibited similar latencies for mammary tumor formation regardless of genotype that were not significantly longer than 99 days reported in CD2F1 mice (23). All DMBA-treated animals were carefully examined at necropsy for lung metastases, other neoplasms, and other pathologic lesions in major organs (see text), with any suspected lesions confirmed by histologic analysis.

Abbreviations: NA, not applicable; ND, not determined; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

*Number of days after last DMBA treatment.

†Grade according to the Nottingham modification of the Bloom-Richardson system (three variables): T (tubule formation), 1-3; N (nuclear pleomorphism—nuclear variation in size and shape), 1-3; M (mitotic count—mitoses), 1-3.

Nevertheless, there was an increased incidence of DMBA-induced ovarian granulosa cell tumors in Bin1Δmam mice (43%) relative to Bin1+mam mice (13%), all of whom also had mammary tumors at diagnosis (Supplementary Figure S1; Supplementary Table S1). In the uterus, a similar incidence of cystic hyperplasia was observed but only the Bin1Δmam cohort displayed endometritis (Supplementary Figure S2; Supplementary Table S1). These observations corroborated the concept of *Bin1* as a negative modifier of lesions produced by DMBA treatment, due to either haploinsufficiency or a non–cell autonomous mechanism of action in the ovary and uterus.

In contrast to the above observations, we found that *Bin1* deletion had little effect when tumor formation was initiated by a c-*myc* transgene. In female MMTV-c-*myc* mice carried out under multiparous conditions to activate transgene expression, mammary adenocarcinomas develop at a frequency approaching 100% with a latency of 7 to 10 months (27). In multiparous c-Myc;Bin1+mam and c-Myc;Bin1Δmam females, we observed the development of similar moderate to poorly differentiated mammary adenocarcinomas, with similar latencies, high tumor grades, and robust metastatic propensities (Supplementary Table S2). These tumors were characterized by large round cells with histologic evidence of an abundance of infiltrating macrophages and apoptotic cells (data not shown). When these mice were treated with DMBA, we observed the development of similar poorly differentiated mammary carcinomas. However, c-Myc;-Bin1Δmam mice also developed aggressive lymphomas that appeared in some animals before mammary carcinomas had formed (Supplementary Table S2). As above, this observation suggested either haploinsufficiency or a cell nonautonomous modifier effect on DMBA-induced tumors (28) in cooperation with MMTV-c-*myc* (the expression of which is leaky in lymphoid cells). Taken together, these findings argue that the effects of *Bin1* loss were selective insofar as cooperation was only observed in the absence of c-Myc overexpression (which on its own was sufficient to drive formation of poorly differentiated high-grade mammary carcinomas). We concluded that *Bin1* loss cooperated specifically with DMBA-induced *ras* activation to drive breast tumor progression.

Aggressive characteristics of mouse mammary tumor cells lacking Bin1. To gain insight into how *Bin1* loss facilitates tumor progression, we compared the behavior of MMECs established from several DMBA-induced tumors excised from Bin1Δmam and Bin1+mam mice. Bin1Δmam cell lines grew to higher densities and displayed a spindle morphology consistent with the more aggressive features displayed by the tumors from which they were derived (Fig. 4*A*). The status of *Bin1* in cell lines was confirmed by PCR and Western blot analysis and the analysis of a representative pair is presented below (Fig. 4*B*; data not shown). Western blot analyses confirmed the expression of E-cadherin and β-catenin, but not of vimentin, which is expressed strongly in mammary myoepithelial cells and fibroblasts (Fig. 4*C*). Immunohistochemical staining of primary tumors confirmed common expression of E-cadherin and β-catenin (data not shown), consistent with the likelihood that the established tumor cell populations are indeed epithelial in character. N-cadherin was also expressed in these cell lines; however, because there was no correlation to Bin1 status, this mesenchymal marker was interpreted as a general feature of DMBA-induced breast carcinogenesis in the mouse (Fig. 4*C*). Bin1Δmam cells displayed a 3–4 times higher rate of *in vitro* proliferation under anchorage-dependent conditions (Fig. 4*D*). Under conditions of anchorage-independent growth in soft agar culture, only Bin1Δmam cells displayed detectable colony formation activity in parallel with their more aggressive growth character (data not shown). Bin1Δmam cells also exhibited severalfold greater resistance to apoptosis elicited by serum deprival (Fig. 4*E*), extending evidence of a proapoptotic role for *Bin1* in neoplastic cells (14–16, 18, 19). Lastly, Bin1Δmam cells displayed an increased motility in monolayer culture associated with increased gelatinase activity attributable to activated matrix metalloproteinase (MMP-9; Fig. 4*F* and *G*). Taken together, these results strengthened the evidence that *Bin1* acts in the guise of a negative modifier in cancer.

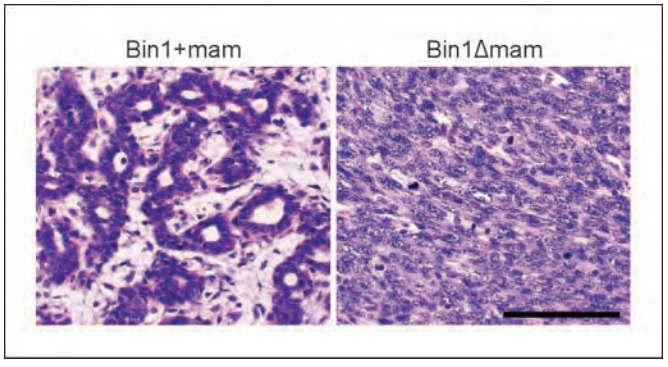

Figure 3. Increased histopathologic grade of DMBA-induced mammary tumors lacking Bin1. DMBA-induced mammary gland tumors excised at necropsy from Bin1+mam and Bin1Δmam mice were fixed in paraffin, and tissue sections were processed for H&E staining.

We compared the ability of Bin1Δmam and Bin1+mam MMECs to form orthotopic tumors in syngeneic immunocompetent mice and immunocompromised nude mice, based on an earlier demonstration that *Bin1* loss could promote immune escape of *myc+ras*-transformed keratinocytes (20). Unfortunately, none of the MMEC populations established from DMBA-induced mammary tumors could form tumors in syngeneic FVB/N × BL/6 F1 female mice, preventing us from exploring this issue further. In contrast, after orthotopic injection of 10^7 cells into the fat pads of immunocompromised female nude mice, Bin1Δmam cells formed tumors efficiently, whereas Bin1+mam cells formed mainly indolent nodules up to 5 months after seeding (Fig. 4*H*). The more profound *in vivo* growth differences observed could not be fully explained by the *in vitro* differences documented, suggesting that *Bin1* loss may provide an additional undefined benefit *in vivo*. Nude mice retain natural killer (NK) cell and some B-cell immune functions, but we observed a similar pattern of tumor growth in severe combined immunodeficient (SCID) and SCID/beige murine hosts, which completely lack T/B and T/B/NK cell functions, respectively (data not shown). In summary, we concluded that *Bin1* acts to limit the progression of DMBA mammary tumors at several intrinsic levels, including by negatively modifying the proliferation, survival, and motility of tumor cells.

Discussion

This study provides evidence that *Bin1* functions as a negative modifier or antiprogression gene during breast tumorigenesis. In the parous gland, Bin1 was dispensable for function, based on normal patterns of nursing and development in newborn pups. However, histologic analysis revealed a requirement for the rapidity

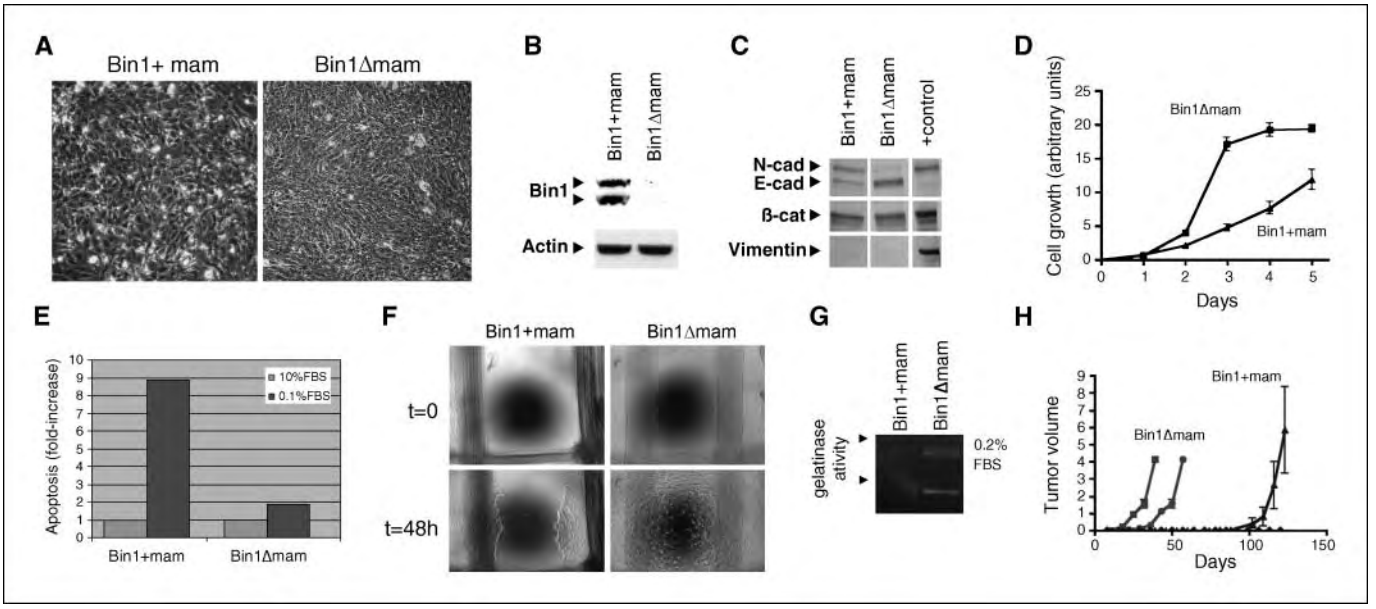

Figure 4. Advanced neoplastic character of MMECs established from DMBA-induced mammary tumors lacking Bin1. *A,* morphology. Cells were photographed using phase optics. *B,* Bin1 status in representative pair of MMEC tumor cell lines. Total cellular protein was extracted MMEC tumor cell lines and Bin1 status was monitored by Western blot analysis. *C,* epithelial character of MMEC tumor cell lines. Western blot analysis of cellular protein extracts for the epithelial markers E-cadherin and β-catenin and the mesenchymal markers N-cadherin and vimentin was done. *D,* proliferation. Cells were seeded into growth medium and viable cell count was determined at the times indicated. Relative outgrowth. *E,* resistance to apoptosis triggered by serum deprival. Cells were seeded overnight into growth medium and then incubated in medium containing 0.1% FBS before being harvested 48 h later for flow cytometric analysis. Relative levels of apoptosis were defined by the proportion of the cellular population exhibiting sub-G_1–phase DNA profile. *F,* motility. Cells were incubated as above, except that the monolayer was scratched with a pipette tip before photomicrography 24 h later. *G,* MMP-9 zymogram. Cells were incubated as above before preparation and analysis of protein extracts. *H,* graft tumor formation. Two tumor cell populations of each genotype (10^7 cells each) were injected orthotopically into the mammary fat pads of syngeneic FVB × B6 offspring of FVB-N and C57BL/6J breeders or into immunocompromised CD-1 nude (Crl:CD-1-nuBR) mice. Tumor growth was monitored by caliper measurements, and final volume and wet weight were determined at necropsy.

of the kinetics of ductolobular remodeling that occurs during pregnancy. Given an involvement of the yeast homologues of *Bin1* in stress signaling (25, 29), the significance of its role in remodeling might depend on stresses in the natural environment that could limit milk production. In DMBA-induced tumors, we observed effects of *Bin1* status on the activity of the MMP-9, which can contribute to remodeling of the mammary gland during pregnancy (30). However, because dysregulation of MMP-9 has significantly more pronounced effects on the normal mammary gland than that produced by *Bin1* loss, we do not favor the interpretation that the phenotype produced by *Bin1* loss relates to MMP-9 dysregulation.

We observed no long-term effects of *Bin1* deletion on cancer incidence in virgin or parous animals, indicating that this gene does not function as a classic tumor suppressor in the mammary gland. In contrast, when mammary tumors were initiated by DMBA in *Bin1*-deficient mice, we found that high-grade carcinomas emerged that exhibited increased proliferation, survival, and motility relative to tumors induced in control mice expressing *Bin1*. Interestingly, we noted a coincident increase of ovarian tumors or lymphomas in Bin1Δmam mice, which reflected either haploinsufficiency or a non–cell autonomous mechanism of action in these settings. Although the underlying mechanisms of these effects were undefined, they provided further corroboration of the concept of *Bin1* as a negative modifier in breast cancer.

In previous work in transformed mouse keratinocyte and fibroblast models, we observed that *Bin1* loss strongly affected the capacity for immune escape with less effect on proliferation and survival (19, 20). In particular, in the transformed keratinocyte model, we had identified a role for indoleamine 2,3-dioxygenase (IDO) in mediating immune escape (20). Unfortunately, we could not evaluate effects of *Bin1* loss on IDO-mediated immune escape in the DMBA mammary carcinogenesis model because none of the tumor MMEC populations had the ability to form grafts in immunocompetent hosts. In any case, other evidence suggests that in breast cancer, the mechanism of immune escape based on IDO elevation may be more relevant in the peripheral immune cells in tumor-draining lymph nodes than in the tumor cells themselves (31), the latter of which do not tend to overexpress IDO like other solid tumors (32). Therefore, breast models may not be especially pathophysiologically germane to evaluate how *Bin1* attenuation in tumor cells affects immune escape. In assessing cell-intrinsic qualities, differences in the effects of *Bin1* on proliferation and

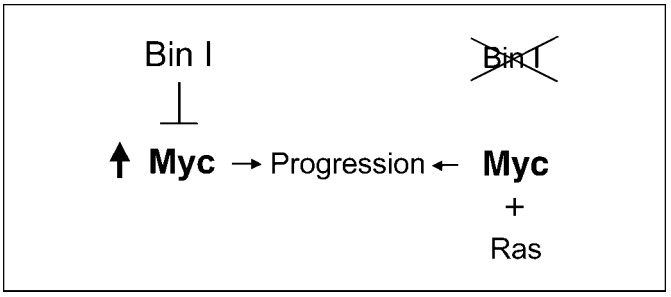

Figure 5. Model. Maintaining Myc expression is sufficient to drive cell division and tumorigenesis, yet in many human cancers, Myc is both deregulated and overexpressed, implying the existence of a selection for high Myc levels to provide a progression benefit. The model proposes that acquisition of this progression benefit can be phenocopied by Bin1 loss in cooperation with *ras* activation.

survival in the keratinocyte and fibroblast models may reflect their *in vitro* generation, where strong selections for survival and proliferation are imposed (perhaps defeating the benefits of losing a negative modifier). In contrast, the findings from the *in vivo*–generated breast model reported here corroborate the findings of a large number of reports showing the ability of *Bin1* to limit cell proliferation and survival (3, 4, 14–19, 33, 34). In this study, we also observed increased motility and elevated MMP-9 activity in MMEC tumor cell populations lacking *Bin1*, a finding that we have since corroborated in the transformed keratinocyte and fibroblast models characterized previously.[7] Further assessment of the mechanism of MMP-9 dysregulation as well as the effects of *Bin1* loss on proliferation and survival is currently being conducted in a mosaic model where direct *in vivo* evaluations in other tissues are possible.

It is apparent that the effects of *Bin1* loss in the mammary gland were selective because of the specific cooperation of *Bin1* loss in driving progression with *ras* activated by DMBA but not c-*myc* overexpressed from the MMTV promoter. These data imply that the functional effects of *Bin1* loss and *myc* overexpression must overlap to some extent because of the ability of either *Bin1* loss or *myc* activation to cooperate with *ras* activation to drive breast tumor progression (present study; ref. 35). *Bin1* loss obviously does not fully phenocopy *myc* activation. Thus, along with evidence that nuclear Bin1 proteins functionally interact with c-Myc protein (3–5), a logical inference is that *Bin1* acts to limit a subset of *myc* functions that are selectively important to progression in cooperation with *ras*. In this context, it is interesting to note that although maintaining the expression of *myc* throughout the cell cycle is sufficient to prevent cell cycle exit and to drive tumorigenesis, many human cancers not only deregulate *myc* but overexpress it (36). Following the implication that *myc* overexpression may benefit tumor progression, our data support a model where *Bin1* loss partly or fully phenocopies such benefits in cooperation with *ras* activation (Fig. 5).

In considering models that incorporate a relationship between these genes, we note that recent genetic studies of *myc* in *Drosophila* seem to relate vesicle trafficking processes to a facet of Myc function that drives cell competition in tissues (37). Using the imaginal disc as a model system, a function for *myc* in driving cell competition was defined that could be fully phenocopied by overexpression of the small GTPase Rab5 (37), a well-characterized regulator of vesicle trafficking. Specifically, it was shown that high-Myc cells and high-Rab cells could compete equally for internalization of limiting growth factors in a tissue (37, 38), surviving at the cost of low-Myc or low-Rab cells that lost the competition and perished (37). The genetic complementarity these findings suggest that the cell competition function of Myc may rely on trafficking events of the type that involve Rab5 and Bin1 (12), which biochemically interact through Rin3, a Rab5 guanine nucleotide exchange factor that binds Bin1 on early endosomes (39). In future work, the conditional mutant mouse and pathologic footing developed here will provide a solid foundation to address questions about the precise mechanism(s) by which *Bin1* loss facilitates tumor progression.

One clinical implication of our findings is that losses of nuclear Bin1 may predict poor prognosis of breast cancers when c-Myc is not overexpressed but Ras signaling is deregulated, for example, due to deregulation of an upstream growth factor receptor. Although some fraction of breast carcinomas overexpress c-Myc, signaling poor prognosis (40–44), and immunohistochemical losses of Bin1 that seem to occur more frequently (16, 45) may be useful in the larger number of cases where c-Myc is not overexpressed. The findings of this study prompt an examination of Bin1 in retrospective or prospective studies where its potential usefulness as a progression marker can be further evaluated (46).

[7] J.B. Duhadaway, unpublished observation.

Acknowledgments

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Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Antitumor Responses

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Abstract

Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme that contributes to tolerance in a number of biological settings. In cancer, IDO activity may help promote acquired tolerance to tumor antigens. The IDO inhibitor 1-methyl-tryptophan is being developed for clinical trials. However, 1-methyl-tryptophan exists in two stereoisomers with potentially different biological properties, and it has been unclear which isomer might be preferable for initial development. In this study, we provide evidence that the D and L stereoisomers exhibit important cell type–specific variations in activity. The L isomer was the more potent inhibitor of IDO activity using the purified enzyme and in HeLa cell–based assays. However, the D isomer was significantly more effective in reversing the suppression of T cells created by IDO-expressing dendritic cells, using both human monocyte–derived dendritic cells and murine dendritic cells isolated directly from tumor-draining lymph nodes. *In vivo*, the D isomer was more efficacious as an anticancer agent in chemo-immunotherapy regimens using cyclophosphamide, paclitaxel, or gemcitabine, when tested in mouse models of transplantable melanoma and transplantable and autochthonous breast cancer. The D isomer of 1-methyl-tryptophan specifically targeted the *IDO* gene because the antitumor effect of D-1-methyl-tryptophan was completely lost in mice with a disruption of the *IDO* gene (IDO-knockout mice). Taken together, our findings support the suitability of D-1-methyl-tryptophan for human trials aiming to assess the utility of IDO inhibition to block host-mediated immunosuppression and enhance antitumor immunity in the setting of combined chemo-immunotherapy regimens. [Cancer Res 2007;67(2):792–801]

Introduction

The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated as an immunosuppressive and tolerogenic mechanism contributing to maternal tolerance toward the allogeneic fetus (1), regulation of autoimmune disorders (2–5), and suppression of transplant rejection (6, 7). IDO can also be expressed by cancer cells in a variety of human malignancies (8, 9). In murine models, transfection of immunogenic tumor cell lines with recombinant IDO renders them immunosuppressive and lethally progressive *in vivo*, even in the face of otherwise protective T-cell immunity (8). In humans, expression of IDO by ovarian and colorectal cancer cells has been found to be a significant predictor of poor prognosis (9, 10).

IDO can also be expressed by host antigen-presenting cells (APC). APCs with the potential to express IDO include human monocyte–derived macrophages (11), human monocyte–derived dendritic cells cultured under specific conditions (12–19), and certain subsets of murine dendritic cells (20–25). In murine tumor models, IDO^+ dendritic cells displaying a plasmacytoid phenotype ($CD11c^+B220^+$) have been found at increased levels in tumor-draining lymph nodes (22). These have been shown to suppress T-cell responses *in vitro* and create antigen-specific T-cell anergy *in vivo* (22, 25). In humans, IDO^+ cells of host origin have been shown in draining lymph nodes of patients with melanoma, breast cancer, and other tumors (13, 22, 26, 27). In patients with malignant melanoma, the presence of these IDO-expressing cells in sentinel lymph node biopsies was correlated with significantly worse clinical outcome (22, 28). Thus, expression of IDO, either by host cells or by tumor cells, seems associated with poor outcome in a number of clinical settings.

These findings have prompted interest in development of IDO inhibitor drugs for cancer immunotherapy (29). The most widely studied of these has been 1-methyl-tryptophan (30–32). Recently, it was shown that 1-methyl-tryptophan displays marked synergy with a number of clinically relevant chemotherapeutic agents when used in combined chemo-immunotherapy regimens (33). In that study, the combination of 1-methyl-tryptophan with cyclophosphamide, cisplatin, doxorubicin, or paclitaxel was able to cause regression of established tumors in a demanding model of autochthonous HER-2/*neu*–induced murine breast cancers (33). From a clinical standpoint, combining an immunomodulatory agent, such as 1-methyl-tryptophan, with conventional chemotherapy drugs represents an attractive strategy, and a sound mechanistic rationale supporting such chemo-immunotherapy approaches is now being elucidated (34–36).

However, a key unanswered question regarding 1-methyl-tryptophan has been which of the two available stereoisomers (D and L) should be developed initially for clinical trials. The two isomers differ significantly in their effects on the recombinant IDO enzyme *in vitro* (37), and they could potentially have different biological effects, bioavailability, and off-target toxicities. Most of the studies in the literature have employed the racemic (DL) mixture of 1-methyl-tryptophan comprising both isomers, thus leaving unanswered the question of which stereoisomer would be

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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best suited for use in chemo-immunotherapy regimens. The goal of the present study was to compare the biological activity of the D and L isomers of 1-methyl-tryptophan *in vitro* and *in vivo*, to ask whether their pattern of efficacy *in vitro* correlated with their observed antitumor effect *in vivo*.

Materials and Methods

Additional methods available online. Detailed description of mice, published methods, and statistical analyses are available online at http://cancerres.aacrjournals.org/.

Reagents. 1-Methyl-D-tryptophan (45,248-3), 1-methyl-L-tryptophan (44,743-9), and 1-methyl-DL-tryptophan (86,064-6) were obtained from Sigma-Aldrich (St. Louis, MO). For *in vitro* use, these were prepared as a 20 mmol/L stock in 0.1 N NaOH, adjusted to pH 7.4, and stored at −20°C protected from light.

Autochthonous breast cancer model. Multiparous female MMTV-*Neu* mice, maintained as described (33), have a high incidence of autochthonous mammary gland carcinomas. Tumor-bearing mice were enrolled randomly into experimental groups when tumors reached 0.5 to 1.0 cm in diameter. Tumor volume was measured at the beginning and end of the 2-week treatment period.

B16F10 and 4T1 tumor models. B16F10 melanoma (American Type Culture Collection, Manassas, VA) were established in B6 mice by s.c. injection of 5×10^4 cultured cells. B78H1-GM-CSF (38), gift of H. Levitsky, (Johns Hopkins University, Baltimore, MD) was implanted by s.c. injection of 1×10^6 cells. Orthogonal diameters were measured, and the $x \cdot y$ product (tumor area) was reported. The use of the orthotopically implanted 4T1 breast cancer line (39) has been described in detail (40). Tumors were implanted by injection of 1×10^4 cells in 50 μL volume into the mammary fat pad of 6- to 10-week-old BALB/c females. In some experiments, luciferase-transfected 4T1 cells (4T1-luc) were used for bioluminescence imaging, as described in the Supplementary Material.

Administration of 1-methyl-tryptophan and chemotherapeutic agents. Detailed protocols for administration of 1-methyl-tryptophan, orally and by s.c. pellets, in conjunction with chemotherapy, are given in the Supplementary Material.

Human and mouse mixed lymphocyte reactions. Human and murine allogeneic mixed lymphocyte reactions (allo-MLR) were done as detailed in the Supplementary Material and have been previously described (14, 22).

Western blots. Western blots were done using affinity-purified polyclonal rabbit antibody against peptides from the NH_2-terminal and COOH-terminal portion of human IDO, as previously described (13) and as specified in detail in the Supplementary Material.

Results

Cooperativity effect of s.c. DL-1-methyl-tryptophan with chemotherapy or radiation in B16F10 melanoma. We first evaluated the racemic DL mixture of 1-methyl-tryptophan as a component of chemo-immunotherapy using three tumor models: a stringent established (day 7) B16F10 melanoma, orthotopically implanted 4T1 breast carcinoma, and autochthonous breast tumors arising in HER-2/*neu*–transgenic mice. Figure 1*A* shows established B16F10 tumors treated with DL-1-methyl-tryptophan (20 mg/d by 14-day s.c. copolymer pellet; ref. 1), with or without a single injection of cyclophosphamide (150 mg/kg). DL-1-methyl-tryptophan alone had no effect on tumor growth, and cyclophosphamide alone induced only a transient growth delay. However, the combination of DL-1-methyl-tryptophan + cyclophosphamide resulted in a sustained growth delay and prolonged survival. In all experiments, the end of the study period was defined as the time when all of the mice in the vehicle-only group reached their ethical surrogate end point (tumor area ≥300 mm^2). At the point when all mice in the control group had reached this end point, all mice in the

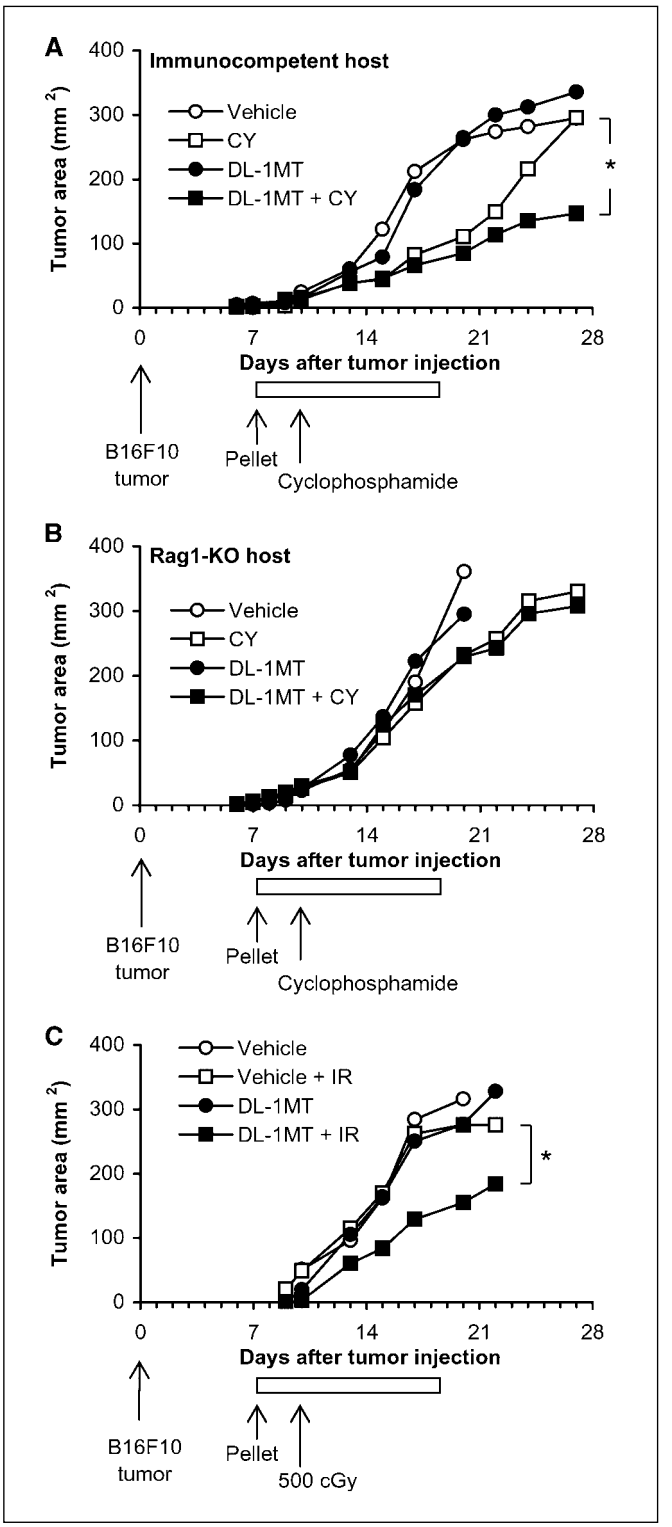
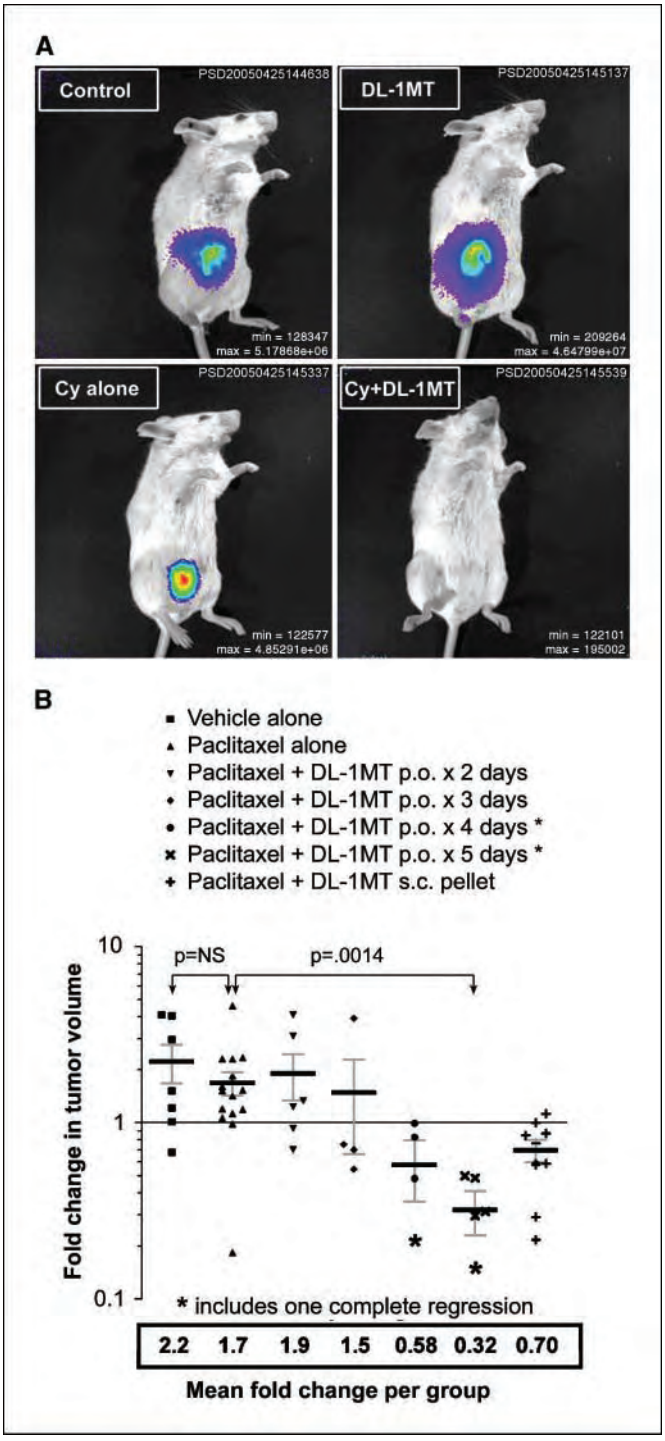


Figure 1. Effect of parenteral DL-1-methyl-tryptophan (*DL-1MT*) in B16F10 tumors. *A,* B16F10 tumors were implanted in syngeneic C57BL/6 mice. Beginning on day 7, mice were treated as shown with timed release s.c. pellets of DL-1-methyl-tryptophan (20 mg/d) plus cyclophosphamide (*CY*; 150 mg/kg i.p. × 1 dose). Three identical experiments were done (a representative example is shown), and the pooled results were analyzed in a three-experiment × 2 group ANOVA. *, $P < 0.05$. *B,* identical experimental design showing that the effect of DL-1-methyl-tryptophan was lost when hosts were immunodeficient Rag1-KO. Groups were not significantly different by ANOVA. *C,* similar experimental design, except that 500 cGy of whole-body cesium-137 irradiation replaced the cyclophosphamide. One of four similar experiments. *, $P < 0.05$, ANOVA.

DL-1-methyl-tryptophan + cyclophosphamide group were still surviving. Figure 1*B* shows that the effect of DL-1-methyl-tryptophan was lost in immunodeficient Rag1-knockout (Rag1-KO) hosts, indicating that the antitumor effect of DL-1-methyl-tryptophan was entirely immune mediated.

Whole-body irradiation has many of the same effects as chemotherapy when combined with antitumor immunotherapy (41). We tested DL-1-methyl-tryptophan in combination with 500 cGy whole-body irradiation (Fig. 1*C*). In these experiments, there was considerable variability in the effect of the radiation component alone on tumor growth, but in all experiments, the effect of DL-1-methyl-tryptophan plus radiation was superior to radiation alone.



Cooperativity between oral DL-1-methyl-tryptophan and cyclophosphamide in treating 4T1 breast carcinoma isografts. We next asked whether DL-1-methyl-tryptophan showed efficacy via the oral route. For these studies, we tested chemo-immunotherapy of the poorly immunogenic 4T1 breast tumor model, implanted orthotopically in mammary tissue of syngeneic hosts. Because orthotopic 4T1 tumors are highly invasive and their margins are difficult to measure conventionally, we followed the tumor size using luciferase-transfected 4T1 (4T1-luc) tumors imaged following luciferin challenge. Oral DL-1-methyl-tryptophan was given by gavage twice daily, five times a week, combined with a weekly single i.p. dose of cyclophosphamide, beginning at the time of tumor implantation. As shown in representative scans in Fig. 2*A*, cyclophosphamide alone produced a modest reduction in tumor size, but the combination of cyclophosphamide + DL-1-methyl-tryptophan produced a marked decrease in tumor size (survival studies in this model are presented below).

Oral administration of DL-1-methyl-tryptophan in combination with paclitaxel can elicit regression of autochthonous breast tumors. We next tested the efficacy of varying durations of oral DL-1-methyl-tryptophan in combination with paclitaxel for the treatment of autochthonous tumors arising in MMTV-*Neu* mice (33). Mice with tumors were randomly assigned to treatment with paclitaxel for 2 weeks, with or without addition of 2 to 5 days of oral DL-1-methyl-tryptophan during the first week, as indicated in Fig. 2*B*. Paclitaxel alone caused a minor reduction in the rate of tumor growth, but tumors continued to increase in size during the study period despite paclitaxel. The addition of oral DL-1-methyl-tryptophan produced a progressive reduction in the rate of tumor growth with increasing duration of 1-methyl-tryptophan, such that treatment with 4 and 5 days of DL-1-methyl-tryptophan reversed tumor growth, and caused regression of the established tumors during the treatment period. Five days of administration via the oral route was at least as effective as parenteral delivery of the drug at a comparable daily dose, using implantable s.c. pellet (the last treatment group and the route reported in our previous study; ref. 33).

***In vitro* comparison of D versus L isomers of 1-methyl-tryptophan.** We next used *in vitro* models to compare the different isomers of 1-methyl-tryptophan for their biological effects, using two readouts: (*a*) activity of the IDO enzyme measured as

Figure 2. Oral DL-1-methyl-tryptophan in orthotopic 4T1 and autochthonous MMTV-*Neu* tumors. *A,* orthotopic tumor isografts were established in the mammary fat pad. Treatment was initiated concurrent with tumor challenge, using cyclophosphamide i.p. at 100 mg/kg, once a week and DL-1-methyl-tryptophan oral gavage at 400 mg/kg per dose, twice daily, five times a week. Bioluminescence imaging of 4T1 tumor cell line transfected with luciferase, showing the effect of each treatment on tumor burden. Treatment received by each mouse is indicated. Images were produced at 4 wks following the initiation of treatment. *B,* MMTV-*Neu* mice bearing 0.5 to 1.0 cm spontaneous tumors were treated for 2 wks with either vehicle alone, paclitaxel alone (13.3 mg/kg i.v. q. M/W/F), or paclitaxel plus oral DL-1-methyl-tryptophan (400 mg/kg i.v. twice daily, given for up to 5 d during the first week, as indicated in the legend). Paclitaxel was given i.v. at over the 2-wk treatment period. The last group received s.c. pellets of 1-methyl-tryptophan, as in Fig. 1. Fold changes in individual tumor volumes over the 2-wk period are plotted for each group. *Points,* mean fold change for each group (also listed in the box below the graph); *bars,* SE. *, fully regressed tumors are included in the calculation of the mean and SE. For the statistical analyses (*arrows*), the two comparisons of interest were vehicle alone versus paclitaxel alone and paclitaxel alone versus paclitaxel + D-1-methyl-tryptophan × 5 d. Significance was determined at $P < 0.025$ using a two-group Wilcoxon exact test.

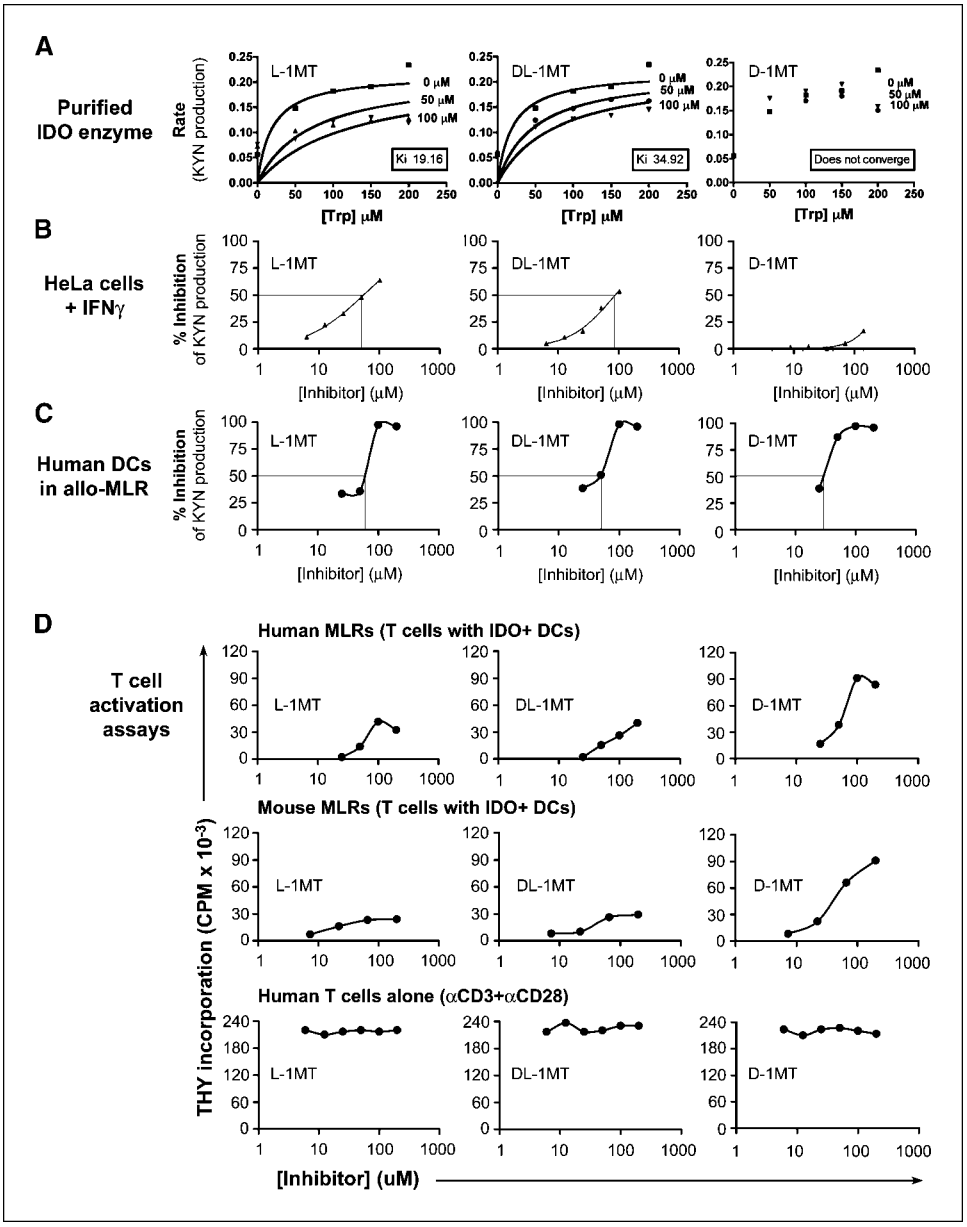


Figure 3. Effect of different isomers on *in vitro* enzyme assays and T-cell proliferation. *A,* enzyme kinetics, measured as kynurenine (*KYN*) production in cell-free assay, for purified recombinant human IDO, showing the effect of the L, DL, and D forms of 1-methyl-tryptophan in the presence of varying concentrations of L-tryptophan substrate. *B,* intracellular IDO enzyme activity (measured as kynurenine production in culture supernatants) by IFNγ-activated HeLa cells, showing inhibition by different isomers of 1-methyl-tryptophan. % Inhibition of maximal kynurenine production; lines show interpolated EC_{50} for each isomer. *C,* intracellular IDO activity (kynurenine production in MLR supernatants) by human monocyte–derived dendritic cells (*DC*) activated in allo-MLRs; lines show EC_{50}. Combined average of three experiments using three different donors. *D,* effect of 1-methyl-tryptophan isomers on T-cell proliferative responses. Proliferation was measured by thymidine incorporation in allo-MLRs using either human T cells stimulated by IDO-expressing human monocyte–derived dendritic cells (1 of 10 experiments, using a variety of different donor combinations), or mouse T cells stimulated by IDO-expressing plasmacytoid dendritic cells from tumor-draining lymph nodes, as described in Materials and Methods (one of three experiments). As controls, purified human T cells without dendritic cells were activated with immobilized anti-CD3 + anti-CD28 antibodies (one of three experiments).

production of kynurenine from tryptophan and (*b*) a biological readout measured as the ability to prevent the suppression of T-cell proliferation caused by IDO-expressing dendritic cells.

Figure 3*A* shows enzyme kinetics (kynurenine production) using recombinant human IDO enzyme in a cell-free assay system. Using the recombinant enzyme, the L isomer of 1-methyl-tryptophan functioned as a competitive inhibitor (K_i = 19 μmol/L), whereas the D isomer was much less effective (no K_i found at 1-methyl-tryptophan concentrations up to 100 μmol/L). The DL mixture was intermediate, with a K_i of 35 μmol/L. These values are consistent with the published literature for studies using cell-free enzyme assays for IDO (37).

We next tested the different isomers in a biological assay, based on the intracellular IDO enzyme expressed by living cells (in this case, HeLa cells activated with IFNγ; Fig. 3*B*). Kynurenine production by HeLa cells showed a pattern of inhibition similar to that of the cell-free recombinant enzyme, with L-1-methyl-tryptophan being more effective than D-1-methyl-tryptophan. In other studies (data not shown), similar results were obtained using the murine MC57 tumor cell line transfected with recombinant mouse IDO and also the simian COS cell line transfected with human IDO: in each of these transfected cell lines, L-1-methyl-tryptophan was superior to D-1-methyl-tryptophan at inhibiting kynurenine production.

In contrast to the behavior of cell lines, when primary human monocyte–derived dendritic cells were used as the IDO-expressing cells (Fig. 3*C*), the D isomer of 1-methyl-tryptophan was found to be at least as effective as the L isomer in its ability to inhibit IDO activity (measured as kynurenine production in culture supernatants). In these assays, dendritic cells were activated physiologically by exposure to T cells in allo-MLRs, rather than with recombinant IFNγ, because we have previously shown that IFNγ alone is not sufficient to activate functional IDO in dendritic cells prepared by this protocol (13, 14).

In addition to kynurenine production, we and others have shown that IDO suppresses proliferation of T cells responding to antigens presented by IDO$^+$ dendritic cells (13, 14, 22). Figure 3*D* shows a comparison of the different 1-methyl-tryptophan isomers on human T-cell proliferation in allo-MLRs stimulated by IDO$^+$ monocyte–derived dendritic cells (similar to the MLRs shown in Fig. 3*C*, but using T-cell proliferation as the readout). Using this readout, the D isomer was found to be reproducibly superior to either the L isomer or the DL mixture, typically eliciting a 2- to 3-fold greater maximum level of T-cell proliferation. A similar pattern was seen using murine T cells (Fig. 3*D*). For mice, allo-MLRs were done using IDO$^+$ dendritic cells isolated directly from murine tumor-draining lymph nodes, as previously described (22). These tumor-activated dendritic cells were used to present a constitutive allo-antigen to BM3 TCR-transgenic T cells (specific for the H2K^b antigen expressed by the C57BL/6 dendritic cells). In this model, just as in the human system, the D isomer of 1-methyl-tryptophan was superior in supporting activation and proliferation of T cells, compared with either the L or DL forms.

To test for nonspecific (off-target) effects of 1-methyl-tryptophan on the T cells themselves, control experiments were done using purified human T cells stimulated by immobilized anti-CD3 + anti-CD28 antibodies (i.e., without any dendritic cells present to express IDO). Under these conditions, none of the 1-methyl-tryptophan preparations had any detectable effect on T-cell proliferation (Fig. 3*D*). Additional studies (shown in Supplementary Fig. S1) were done further evaluating the D isomer, using MLRs stimulated by dendritic cells derived from mice with a targeted disruption of the *IDO* gene (IDO-KO mice). MLRs using IDO-KO dendritic cells showed that the effects of the D isomer were completely lost when the stimulating dendritic cells lacked IDO. Thus, the D isomer of 1-methyl-tryptophan exerted its effects in MLR specifically by targeting the *IDO* gene expressed by the dendritic cells, not through an off-target effect.

Western blots suggest the possible existence of more than one isoform of IDO. The cell type–specific effects of the different isomers of 1-methyl-tryptophan prompted us to ask whether there might be more than one form of IDO expressed in different cells. Published databases suggested potential alternate splicing isoforms of human IDO differing primarily in the COOH-terminal portion of the molecule.[8] Therefore, we generated polyclonal antibodies against peptide sequences in the NH_2-terminal and COOH-terminal portions of the IDO molecule for use in Western blots, as described in the Supplementary Material.

Figure 4*A* shows Western blots using the two different antibodies. Samples were prepared from human monocyte–derived macrophages, as a known source of IFNγ-inducible IDO (11). As shown in Fig. 4*A*, the NH_2-terminal antibody detected a band of ~44 kDa, which was present both before and after IFNγ stimulation, and which showed little apparent change with IFNγ. In contrast, the COOH-terminal antibody detected an antigen of ~42 kDa, which was only visible after IFNγ treatment. A similar pattern of two different constitutive and inducible bands has been described for IDO expression by in other cell types (42). We and others have also shown that IDO can be expressed constitutively at the protein level (e.g., as with the higher molecular weight band) without necessarily showing enzymatic activity until activated (13, 43). In other experiments (data not shown), HeLa cells showed the same pattern of bands and the same response to IFNγ, as did the monocyte-derived macrophages in Fig. 4*A*.

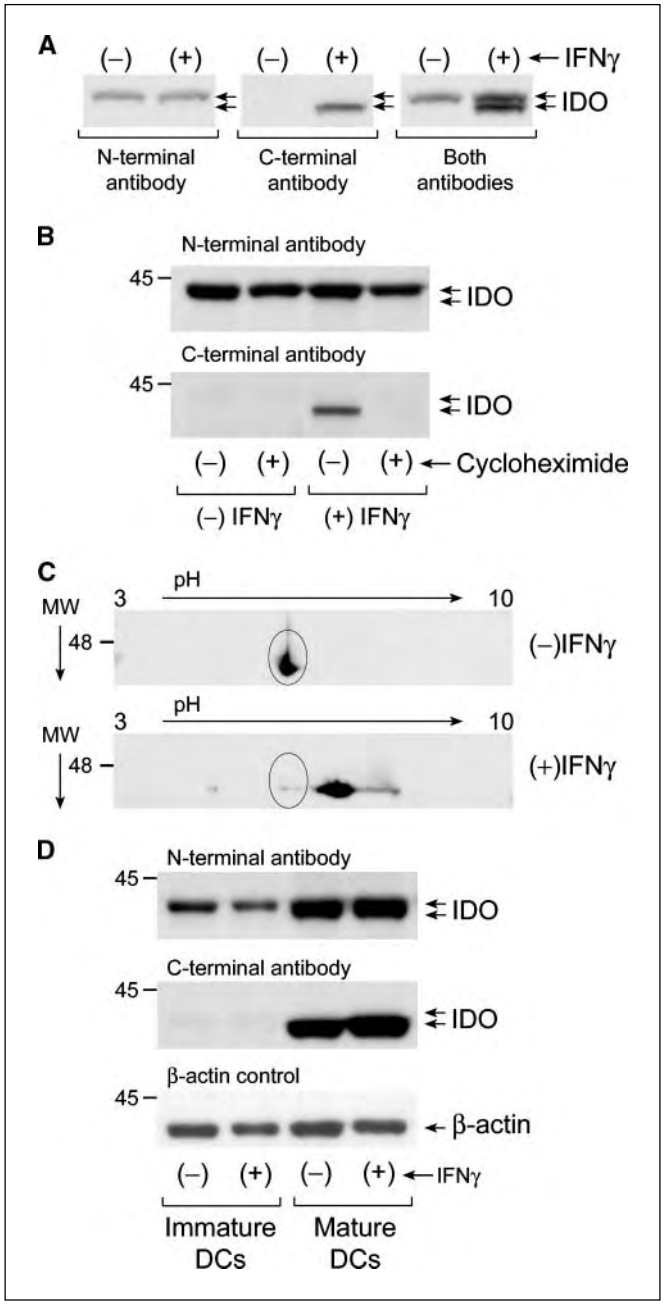


Figure 4. Evidence for two possible isoforms of human IDO. *A,* human monocyte–derived macrophages were prepared as described (11), with or without IFNγ treatment for the final 24 h. Lysates were analyzed by Western blot using antibodies against the NH_2-terminal portion of IDO, the COOH-terminal portion, or a mixture of the two antibodies. All blots were stripped and reprobed for β-actin (data not shown) to confirm even loading. *B,* macrophages, as above, were treated with or without IFNγ, in the presence or absence of cycloheximide (10 μg/mL). β-Actin blots (data not shown) confirmed even loading. *C,* lysates of macrophages with and without IFNγ pretreatment were analyzed by two-dimensional electrophoresis, followed by Western blotting with the NH_2-terminal–specific anti-IDO antibody. *D,* human monocyte–derived dendritic cells were cultured for 7 d as described in Materials and Methods, with or without addition of a maturation cocktail during the final 48 h. IFNγ was added during the last 24 h. Western blots were done as in (*B*), with the same blot stripped and reprobed for each anti-IDO antibody and the β-actin loading control.

[8] J. Thierry-Mieg et al. AceView: identification and functional annotation of cDNA-supported genes in higher organisms—*Homo sapiens* gene INDO, encoding indole-amine-pyrrole 2,3 dioxygenase. Available from http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly.

Figure 4*B* shows that expression of the IFNγ-inducible (lower molecular weight, COOH-terminal) band was blocked by cycloheximide, suggesting that it represented a newly synthesized protein, rather than a posttranslational modification of the larger isoform. Although conventional Western blot analysis did not reveal any obvious change in the larger molecular weight (NH_2-terminal) isoform in response to IFNγ, two-dimensional Western blots (Fig. 4*C*) revealed that there was a significant IFNγ-induced shift in isoelectric point (up to 2 pH units). Thus, these data revealed that both forms of IDO were in fact IFNγ responsive, with the larger form appearing to undergo some IFN-induced posttranslational modification, whereas the smaller form seemed to be synthesized *de novo*.

Regulation of IDO activity in dendritic cells is more complex than in macrophages, with multiple factors reported to influence both protein expression and enzymatic activity (17, 19). When we analyzed human monocyte–derived dendritic cells by Western blot (Fig. 4*D*), there was significant up-regulation of the larger (NH_2-terminal) isoform with dendritic cell maturation, whereas IFNγ treatment had no discernible effect on this band in dendritic cells. The smaller (COOH-terminal) isoform showed no expression in immature dendritic cells and was not inducible in dendritic cells by IFNγ. However, the COOH-terminal isoform underwent marked up-regulation with dendritic cell maturation (again independent of IFNγ). Thus, the regulation of the two IDO isoforms in dendritic cells was complex and differed from their regulation in macrophages. However, the essential point was similar for dendritic cells: that more than one species of IDO was present, and that the pattern of expression was regulated by biologically relevant cytokine signals.

Efficacy of the D isomer of 1-methyl-tryptophan in chemo-immunotherapy. Based on the superiority of the D isomer in supporting T-cell activation *in vitro*, we tested the D isomer of 1-methyl-tryptophan *in vivo* using the B16F10 model. Established (day 7) B16F10 tumors were treated with cyclophosphamide plus D-1-methyl-tryptophan in a design similar to Fig. 1*A*. However, in these studies, the dose of the D isomer was reduced 4-fold compared with the dose of the DL mixture used in Fig. 1*A*, based on its superior efficacy *in vitro*. Even at the lower dose, D-1-methyl-tryptophan + cyclophosphamide showed significant growth delay compared with cyclophosphamide alone (Fig. 5*A*). Similar results were seen with a second chemotherapeutic agent gemcitabine (Fig. 5*B*). Neither gemcitabine alone nor D-1-methyl-tryptophan alone had a significant effect on B16F10 tumor growth, but together, the combination produced a significant growth delay.

D-1-methyl-tryptophan had no effect on B16F10 tumors when used as a single agent, but B16F10 is not a highly immunogenic tumor; we therefore asked whether D-1-methyl-tryptophan alone might show an effect if a more immunogenic tumor was used. B78H1-GM-CSF is a subline of B16 that has been transfected with granulocyte macrophage colony-stimulating factor (GM-CSF) to increase recruitment of APCs to the tumor and draining lymph nodes (44). The tumor is modestly immunogenic, although if implanted without irradiation, the tumors invariably grow and kill the host (45). In this somewhat more immunogenic model, D-1-methyl-tryptophan, as a single agent, was found to have a modest but reproducible and statistically significant effect on the growth (Fig. 5*C, left*). This modest antitumor effect was lost when the hosts were immunodeficient Rag1-KO mice (Fig. 5*C, middle*), showing that the effect of D-1-methyl-tryptophan was immune mediated. Likewise, the effect of D-1-methyl-tryptophan was lost when the less immunogenic parental tumor (without GM-CSF) was used in place of B78H1-GM-CSF (Fig. 5*C, right*). Thus, D-1-methyl-tryptophan did show some modest effect as a single agent when used with an artificially immunogenic tumor. However, this was substantially less potent than the effect of 1-methyl-tryptophan in combination with chemotherapy.

Comparison of D versus L isomers in chemo-immunotherapy. We next did side-by-side comparisons of the different isomers of 1-methyl-tryptophan in chemo-immunotherapy regimens. Figure 6*A* shows a comparison of D versus L versus DL forms of 1-methyl-tryptophan in orthotopic 4T1-luc tumors. Each 1-methyl-tryptophan preparation was given in combination with low-dose cyclophosphamide (25 mg/kg/dose by oral gavage once per week). Although minor effects were observed with the other combinations, only D-1-methyl-tryptophan with cyclophosphamide showed a statistically significant prolongation of survival relative to cyclophosphamide alone (for clarity, these two groups are re-graphed together in the second plot). A second, similar experiment showed the same results, reproducing the survival advantage of D-1-methyl-tryptophan over L-1-methyl-tryptophan in combination with cyclophosphamide.

Figure 6*B* compares the D versus L isomers of 1-methyl-tryptophan in the autochthonous MMTV-*Neu* breast tumor model. Both isomers were delivered orally for 5 days, as in Fig. 2*C*, in combination with paclitaxel. In this model also, D-1-methyl-tryptophan was found to be superior to L-1-methyl-tryptophan (in these studies, the L isomer showed no effect compared with chemotherapy alone).

Specificity of the D isomer for host IDO *in vivo*. Finally, one critical outstanding question was the target specificity of the D isomer *in vivo*. We had shown in Supplementary Fig. S1 (Supplementary Material) that the D isomer of 1-methyl-tryptophan specifically targeted the *IDO* gene *in vitro*. However, it was possible that *in vivo*, D-1-methyl-tryptophan might exert an antitumor effect via some other off-target mechanism. Figure 6*C* addresses this question by comparing tumors grown in wild-type (IDO sufficient) mice versus tumors grown in IDO-KO mice, each treated with cyclophosphamide + D-1-methyl-tryptophan. The tumors that grew in the IDO-KO hosts would, by definition, have been selected for their lack of dependence on IDO (i.e., they must necessarily be escape variants that could grow in the absence of IDO). Thus, if D-1-methyl-tryptophan truly targeted IDO, then treating tumors grown in IDO-KO mice with D-1-methyl-tryptophan should have no effect on tumor growth; conversely, if D-1-methyl-tryptophan was not specific for IDO, then any off-target effects should be retained in the IDO-KO hosts. Figure 6*C* shows that tumors grown in IDO-KO mice became completely refractory to the effects of D-1-methyl-tryptophan, thus confirming that IDO was the target of D-1-methyl-tryptophan *in vivo*, as hypothesized. More specifically, these studies suggested that in this model, the relevant target for D-1-methyl-tryptophan was IDO expressed by host cells, rather than by tumor cells, because the tumor cells were the same in both cases.

Discussion

In the current study, we show significant differences in biological activity between the D and L stereoisomers of 1-methyl-tryptophan. The L isomer was superior at inhibiting activity of purified recombinant IDO enzyme in a cell-free assay and also at inhibiting IDO enzymatic activity in HeLa cells and other cell lines. In

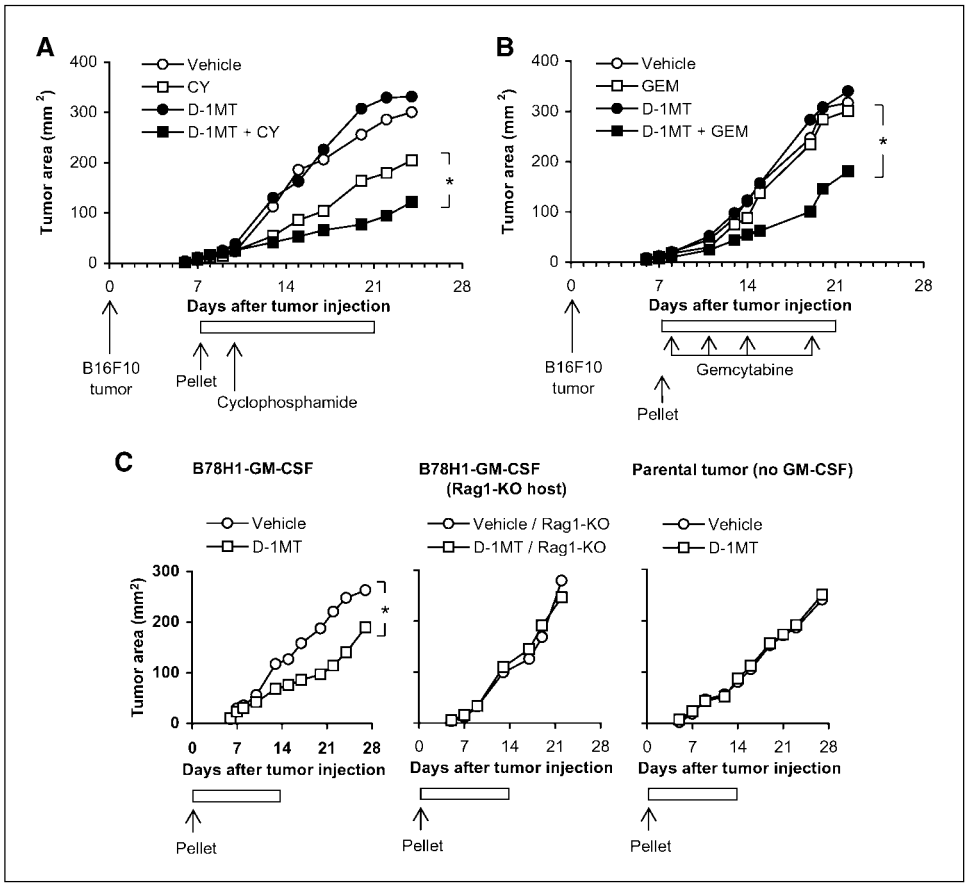


Figure 5. Effect of parenteral D-1-methyl-tryptophan in the B16F10 model. *A,* mice with B16F10 tumors were treated in a design similar to Fig. 1*A*, except using the D isomer of 1-methyl-tryptophan at a 4-fold lower dose (5 mg/d by timed release pellets). Cyclophosphamide was given at 150 mg/kg i.p. Three identical experiments were pooled and analyzed by ANOVA. *, $P \leq 0.05$. *B,* experimental design similar to (*A*), using gemcitabine 120 mg/kg i.p. on days 8, 11, 14, and 19 following B16F10 tumor implantation. Three experiments were pooled and analyzed by ANOVA. *, $P < 0.05$. *C,* B78H1-GM-CSF tumors, or parental tumors without the GM-CSF transgene, were implanted as indicated. Beginning at the time of implantation, mice received 14-day pellets of D-1-methyl-tryptophan (5 mg/d) or vehicle control. *Left,* three experiments were pooled and analyzed by ANOVA. *, $P = 0.011$. *Middle,* all hosts were Rag1-KO. *Right,* tumors lacked the GM-CSF transgene (neither of these groups showed significant differences).

contrast, the D isomer was at least as effective as the L isomer at inhibiting IDO enzymatic activity expressed by human or mouse dendritic cells. Unexpectedly, the D isomer was found to be significantly superior to both the L form and the DL mixture when tested by the biologically important readout of T-cell activation in MLRs. *In vivo*, a head-to-head comparison of the antitumor effect of the two isomers showed that the D isomer was more effective than the L isomer, using two different tumors and different chemo-immunotherapy regimens. Thus, the *in vitro* superiority of the D isomer for enhancing T-cell activation in MLRs seemed to correctly predict the superior *in vivo* antitumor efficacy in the models tested, whereas the results of the cell-free enzyme assays did not.

The superiority of the L isomer in the cell-free enzyme assay was expected from the literature (37). However, to our knowledge, no comparison of the two isomers of 1-methyl-tryptophan has been previously reported using assays based on intact cells. Such cell-based systems are important because different cell types may respond differently to the two isomers, as we have now shown. The molecular basis for these cell type–specific differences is not yet known. Possibilities include differential transport into or out of the cells, different subcellular compartmentalization of the inhibitors, or altered metabolism by cellular enzymes. It is also possible that there may be different isoforms of IDO (as could be suggested by our Western blot data), and these might have different sensitivities to the two isomers, although this is currently speculative. Finally, it may be that 1-methyl-tryptophan exerts some of its inhibitory effects on IDO not by competing directly for the catalytic site but by altering enzyme activity in another way that does not register in the cell-free enzyme assay.

Others have also reported efficacy of the D isomer of 1-methyl-tryptophan for enhancing T-cell responses *in vitro* and *in vivo* (46, 47). Importantly, our data unambiguously showed that the T cell–enhancing effect of D-1-methyl-tryptophan *in vitro* was completely lost when APCs were derived from IDO-KO mice; and, likewise, the antitumor efficacy of D-1-methyl-tryptophan *in vivo* was lost when the tumor-bearing hosts were IDO-KO. Thus, the molecular target of D-1-methyl-tryptophan was indeed IDO, and the efficacy of D-1-methyl-tryptophan was not due to some off-target effect. This would also be consistent with recent studies using RNA–knock-down techniques, which concluded that the major molecular target of the DL-mixture of 1-methyl-tryptophan was IDO, rather than an off-target effect (48).

One critical reason underlying the superior activity of the D isomer *in vivo* may be our observation that the L isomer seemed actively inhibitory for T-cell activation in MLRs. Both isomers were equally effective at blocking the enzymatic activity of IDO in MLRs (measured as kynurenine production in the supernatant); yet, the L isomer could not produce the same high levels of T-cell proliferation achieved by the D isomer. Revealingly, the DL mixture also proved less effective than the D isomer alone, suggesting that the presence of the L isomer actively inhibited T-cell proliferation. The nature of this inhibition is currently unknown. However, it did not seem to be due to a direct toxic effect of L-1-methyl-tryptophan on the T cells themselves because T cells stimulated by mitogen (i.e., in the absence of IDO-expressing dendritic cells) were no longer affected by L-1-methyl-tryptophan. This suggests that the off-target inhibitory effect of the L isomer might be due to a toxic effect of L-1-methyl-tryptophan on the IDO-expressing dendritic cell itself

(e.g., rendering it less able to present antigen to the T cells). Perhaps consistent with such an off-target effect on dendritic cells, it has recently been reported that exposure of dendritic cells *in vitro* to the DL-mixture of 1-methyl-tryptophan at 1,000 μmol/L (much higher than the maximum concentration used in the current study) caused alteration in dendritic cell function, which did not seem related to the effect of DL-1-methyl-tryptophan on IDO itself (49). Alternatively, the T cells might be sensitive to some metabolite of the L isomer generated by the dendritic cells. In either case, it seems that the D isomer of 1-methyl-tryptophan escaped this off-target inhibitory effect on T-cell activation, perhaps precisely because it was not the "natural" stereoisomer.

Although the D isomer showed superior efficacy in our chemo-immunotherapy models, the L isomer proved better at inhibiting IDO in HeLa cells and in mouse tumor cell lines transfected with IDO. Thus, it may be that in certain biological contexts the L isomer

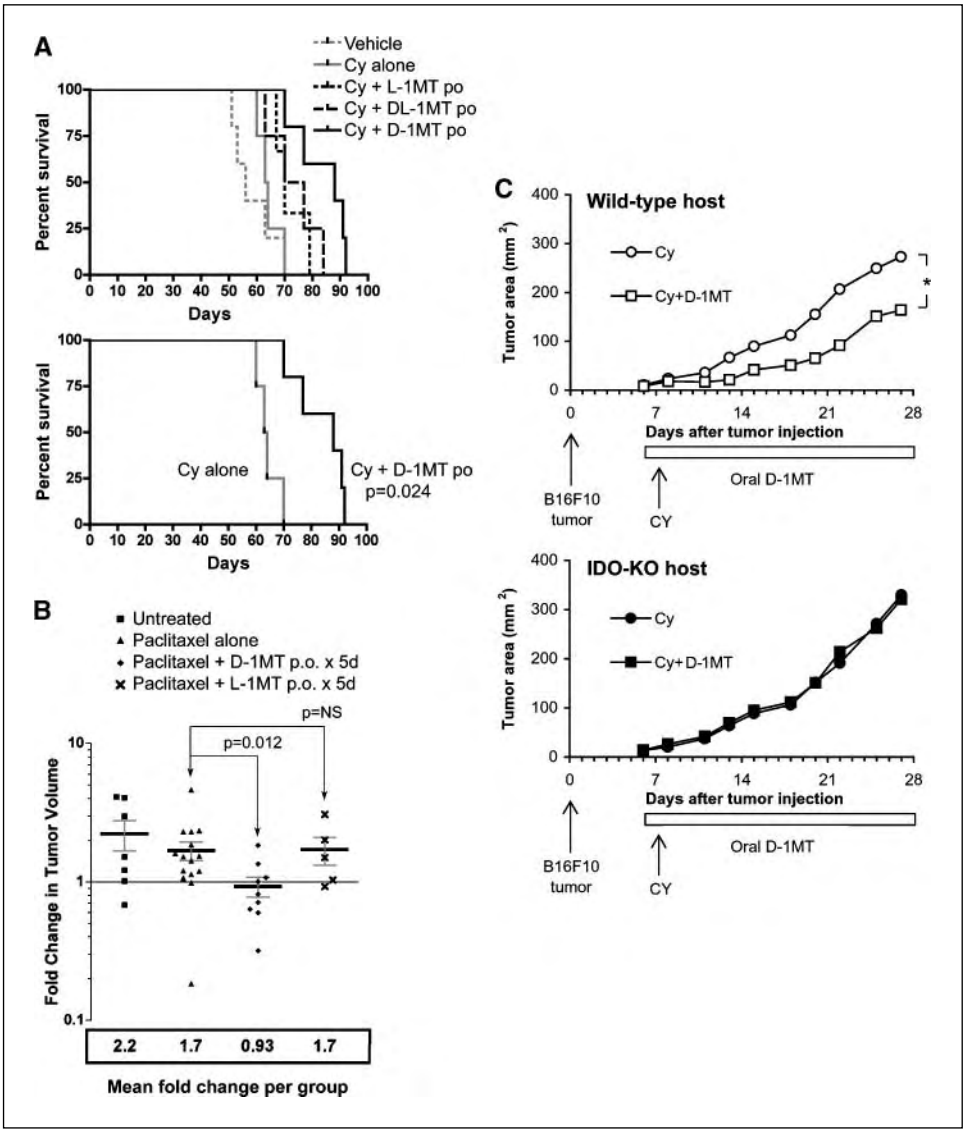


Figure 6. D-1-methyl-tryptophan provides greater survival benefit in combination therapy, in an IDO-dependent fashion. *A,* 4T1-luc orthotopic isografts were established in the mammary fat pad. Cyclophosphamide was given at 25 mg/kg orally once a week, and 1-methyl-tryptophan (D, L, or DL) given at 400 mg/kg by oral gavage twice daily, five times a week by gavage, beginning at the time of tumor implantation. *Top,* time to endpoint for all groups; *bottom,* only the cyclophosphamide versus cyclophosphamide + D-1-methyl-tryptophan groups, for clarity. The comparisons of interest were between D-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide and L-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide. Because survival data were not censored, groups were analyzed using a two-group Wilcoxon exact test; statistical significance was determined at $P < 0.025$. The combination of D-1-methyl-tryptophan + cyclophosphamide showed a significant survival benefit over cyclophosphamide alone ($P = 0.024$), whereas L-1-methyl-tryptophan + cyclophosphamide was not different from cyclophosphamide alone ($P = 0.14$). *B,* MMTV-*Neu* mice with tumors were treated for 2 wks as in Fig. 2*B*, receiving either vehicle alone, paclitaxel alone, or paclitaxel (13.3 mg/kg q. MWF) plus oral D-1-methyl-tryptophan or L-1-methyl-tryptophan for 5 d, as indicated. For statistical analysis, the comparisons of interest were D-1-methyl-tryptophan + paclitaxel versus paclitaxel alone and L-1-methyl-tryptophan + paclitaxel versus paclitaxel alone. Significance was determined at $P < 0.025$ using a two-group Wilcoxon exact test. The fold change of the D-1-methyl-tryptophan + paclitaxel group was significantly smaller than that of paclitaxel alone ($P = 0.012$), whereas paclitaxel + L-1-methyl-tryptophan was not different from paclitaxel alone ($P = 0.85$). *C,* effects of the D isomer of 1-methyl-tryptophan require an intact host *IDO* gene. B16F10 tumors were grown in either wild-type B6 hosts or IDO-KO hosts on the B6 background. All groups received cyclophosphamide, with or without oral D-1-methyl-tryptophan (2 mg/mL in drinking water). Analysis by ANOVA showed that cyclophosphamide + D-1-methyl-tryptophan was significantly different (*, $P < 0.05$) than cyclophosphamide alone for the wild-type hosts, but there was no effect of D-1-methyl-tryptophan when tumors were grown in IDO-KO hosts.

might be preferable, whereas in other contexts, the D isomer is superior. This might become relevant where the target of 1-methyl-tryptophan is IDO expressed by the tumor cells themselves, rather than by host dendritic cells. However, the data from our *in vitro* T-cell activation models and from our *in vivo* chemo-immunotherapy models suggest that in these systems, the beneficial effect of the D isomer on T-cell activation is the key advantage, rendering the D isomer superior in these settings. Furthermore, based on the fact that efficacy of D-1-methyl-tryptophan was lost when the host mice were genetically deficient in IDO (Fig. 6*C*), our data suggest that the molecular target of D-1-methyl-tryptophan in our system was the IDO activity expressed specifically by host APCs, not by the tumor cells themselves.

In the murine models used in this study, relatively high doses of 1-methyl-tryptophan were required to see an antitumor effect. However, this seems to represent a peculiarity of 1-methyl-tryptophan pharmacokinetics in mice. Preclinical pharmacology studies in both rats and canines (to be published elsewhere) show that these animals require significantly lower doses per kilogram to achieve plasma levels in the same range. These lower doses should be readily achievable clinically.

The combination of 1-methyl-tryptophan with chemotherapy (cyclophosphamide, paclitaxel or gemcitabine) was more potent against established tumors than either 1-methyl-tryptophan or chemotherapy alone. Regimens featuring chemotherapy plus immunotherapy are receiving increasing attention (34, 35). In part, this is because they are readily applicable in the clinic because patients do not have to be denied standard chemotherapeutic agents to receive immunotherapy. In addition, there is a sound mechanistic rationale underlying combined chemo-immunotherapy. Chemotherapy causes death of tumor cells, thus releasing tumor antigens into the host antigen-presentation pathway (34). In addition, certain chemotherapy drugs seem to decrease the number and activity of regulatory T cells (50, 51), which may assist the immunotherapy regimens in breaking tolerance to tumor antigens. Finally, the recovery phase from chemotherapy-induced lymphopenia seems to constitute a favorable window for reactivating previously tolerized T cells (41). However, despite these effects, chemotherapy alone does not elicit an effective antitumor immune response. We hypothesize that one reason for this failure is because the antigens released by chemotherapy are presented first in the tumor-draining lymph nodes. We and others have previously shown that tumor-draining lymph nodes are a highly tolerogenic microenvironment (52), due at least in part to the presence of IDO-expressing APCs (22, 25). Thus, IDO^+ host APCs may play an important pathogenic role in helping the tumor re-establish immunologic tolerance toward itself after it is disrupted by chemotherapy. Based on our current data, we hypothesize that the addition of an IDO inhibitor drug during this post-chemotherapy period may allow the tumor-bearing host to mount an effective immune response to tumor antigens during this post-chemotherapy window of opportunity.

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TUMOUR MICROENVIRONMENT

Targeting the mechanisms of tumoral immune tolerance with small-molecule inhibitors

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Abstract | Cancer immunotherapy has been predominantly focused on biologically based intervention strategies. However, recent advances in the understanding of tumour–host interactions at the molecular level have revealed targets that might be amenable to intervention with small-molecule inhibitors. In particular, key effectors of tumoral immune escape have been identified that contribute to a dominant toleragenic state that is suspected of limiting the successful implementation of treatment strategies that rely on boosting immune function. Within the context of the pathophysiology of cancer-associated immune tolerance, this Review delineates potential molecular targets for therapeutic intervention and the progress that has been made in developing small-molecule inhibitors.

Since 1863, when Virchow proposed that tumours could originate from sites of chronic inflammation, it has become well-established that cancer and inflammation are closely associated[1]. Chronic inflammatory diseases, such as inflammatory bowel disease, or agents that induce inflammation, such as phorbol-12-myristate-13-acetate (TPA), greatly exacerbate local tumour susceptibility. Furthermore, developing tumours actively promote a pro-inflammatory environment comprised of cytokines, chemokines and growth factors, activated stroma, and DNA-damaging agents, all of which can promote tumorigenesis[2]. However, inflammation is also a key step in activating both innate and adaptive immune responses, which should inherently increase the likelihood of immune-mediated tumour rejection. This dichotomy imparts a strong selective pressure on the tumour to overcome immune surveillance to maintain the benefits of an inflammatory milieu without the danger of immune-based elimination. Cancer cells might accomplish this by reducing the external signals they present that elicit immune recognition, a process that has been referred to as 'immune editing'[3]. In this vein, evidence of decreased expression levels of major histocompatability complex (MHC) class I and specific tumour-associated antigens has been reported. However, increasing evidence indicates that an important mechanism of tumoral immune escape is the establishment of active, pathological immune tolerance[4], which might represent an important corollary to the concept of immune editing.

Immunotherapeutic strategies have focused predominantly on promoting immune-effector functions based on the hypothesis that immune stimulation will enable the recognition of weak antigenic determinants that are expressed by the tumour. The development of small-molecule agonists of immune function tends to be conceptually problematic, and biological agents, including cell, cytokine and vaccine-based agents, currently dominate the arena of immunotherapeutics. Overall, however, these efforts have produced only a small proportion of positive clinical responses[5], and dominant mechanisms of tumoral immune tolerance might account for this low success rate[4]. In principle, it should be more straightforward to antagonize key toleragenic effector proteins with traditional small-molecule inhibitory compounds, which have a number of advantages relative to biological agents in terms of production, delivery, titratability and cost. As the underlying causes of tumoral immune escape are elucidated (BOX 1), several targets for possible intervention with small-molecule inhibitors have become apparent. These include enzymes, receptors and cytokine signalling pathways that are present in stromal cells and in the tumour cells themselves (FIG. 1), and which can, through various mechanisms, lead to the pathophysiological immunosuppression that is observed in cancer patients. This review will focus on key molecular nodal points for tumoral immune tolerance that might be amenable to pharmacological blockade with small molecules (FIG. 2).

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At a glance

- Tumours dominantly suppress anti-tumour immunity through various pro-toleragenic mechanisms. A particularly attractive approach to target these mechanisms might be through the development of small-molecule immunotherapeutic drugs.
- The indoleamine 2,3-dioxygenase (IDO) enzyme, which catabolizes the essential amino acid tryptophan, promotes tumoral immune tolerance by blocking the activation of effector T cells. Cancer cells express IDO, as do antigen-presenting dendritic cells that are present in tumour-draining lymph nodes, and both might contribute to immune escape. A small-molecule IDO inhibitor is in preclinical development.
- The arginase (ARG)1 enzyme, which is expressed by tumour cells, myeloid suppressor cells and tumour-associated macrophages, has been implicated in suppressing anti-tumour immunity through catabolism of the amino acid arginine. Inducible nitric-oxide synthase (iNOS), another enzyme that catabolizes arginine, generally shows reciprocal regulation to ARG. However, in the context of some tumours, increased expression of both enzymes might contribute to immune suppression. Nitroaspirin, which inhibits both enzymes, is currently the most promising small molecule in development.
- The cyclooxygenase 2 (COX2) enzyme has a central role in regulating the mechanisms of immune suppression and promotes the generation of regulatory T cells. Selective COX2 inhibitors have demonstrated evidence of additional clinical benefit in combination with chemotherapeutic agents, but recent concerns over safety have slowed testing. Inhibitors that target the prostaglandin receptors might represent an alternative.
- The transforming growth factor β (TGFβ) cytokine can have profound immunosuppressive effects, and cancer cells adapt by becoming refractory to TGFβ signalling. Several approaches aimed at inhibiting TGFβ signalling are currently being investigated in terms of therapeutic potential, including small-molecule inhibitors of the TGFβ receptor tyrosine kinase.
- Targeting downstream Janus kinase (JAK)–signal-tansducer-and-activator-of-transcription (STAT) signalling pathways might be an option for interfering with toleragenic cytokine activity, as might the vascular-endothelial-growth-factor (VEGF) receptor FMS-related tyrosine kinase 1 (FLT1), which can promote defective dendritic-cell maturation. Chemokines have also been implicated in the recruitment of immunosuppressive effector cell types to the local tumour microenvironment. Small-molecule inhibitors of the chemokine receptors CCR4, CXCR4 and CCR2 are currently in preclinical development.
- One potential application of anti-toleragenic, small-molecule immunotherapeutics might be in combination with standard cytotoxic chemotherapies. The possibility that these new agents might improve standard treatments should facilitate clinical testing.

Indoleamine 2,3-dioxygenase

The enzyme indoleamine 2,3-dioxygenase (IDO), which is encoded by the *INDO* gene, regulates immune responses by suppressing effector T-cell function through its capacity to catabolize the essential amino acid tryptophan[6] (BOX 2). The biological relevance of IDO-mediated tryptophan catabolism to peripheral immune tolerance was first established by Munn *et al.* when they showed that treating female mice with a small-molecule inhibitor of IDO, 1-methyl-tryptophan (1MT), could break the toleragenic state that protects allogeneic concepti from the maternal immune system[7]. These findings were consistent with *in vitro* results indicating that catabolism of tryptophan by cultured macrophages could block T-cell activation by inducing G1 arrest[8]. Increased tryptophan catabolism has been associated with various malignancies as well as autoimmune disorders and infections. In the context of cancer, increased IDO activity has been regarded as a tumoricidal consequence of interferon γ (IFNγ) exposure[9,10]. This might indeed be the case, at least initially, but tumours that adapt to the selective pressure of growing in a low-tryptophan environment might benefit from the toleragenic consequences of local tryptophan catabolism. This would be similar to the adaptive exploitation by tumours of other immunosuppressive factors that might, at least initially, be deleterious to tumour-cell growth, such as transforming growth factor β (TGFβ; discussed below).

IDO can be expressed by tumour cells and by the surrounding stromal cells (FIG. 1), but which is most relevant to tumoral immune escape has yet to be fully resolved, and the relative contribution of either compartment to pathological immune tolerance might vary for different tumours. IDO is overexpressed by various tumour-cell types[11], and increased expression of IDO in tumour cells has been shown to be an independent prognostic variable for overall survival in patients with serous ovarian cancer[12] and colorectal cancer[13]. Ectopic expression of IDO in tumour cells has been shown to inhibit T-cell responses[14] and can potentiate immune escape and growth of grafted tumours in animals that have been pre-immunized against a specific tumour antigen[11]. In this context, loss of the *Bin1* anti-cancer gene, which results in IFNγ-driven superinduction of *Indo* gene expression, might be a pathophysiological mechanism for IDO deregulation in tumours[15].

An alternative possibility is that the relevant induction of IDO occurs in the stroma. This could occur either in cells within direct proximity of the tumour or in cells at more distal sites, such as the tumour-draining lymph nodes (TDLNs), which are the primary sites for immune activation. Antigen-presenting cells (APCs) are particularly well situated to use this mechanism in their capacity as immune modulators. The first indications of a mechanistic involvement of IDO in the suppression of T-cell activation were obtained using macrophages that had been exposed to colony-stimulating factor 1 (CSF1)

Toleragenic state
A state of immune-system unresponsiveness that is actively promoted by tumour cells to avoid immune recognition.

Antigen-presenting cells
(APCs). Cells that process antigen and present antigen fragments to other cells of the immune system (cross-presentation) to initiate an immune response. Dendritic cells are the most potent APCs.

Box 1 | **Cellular effectors of tumoral immune tolerance**

Tumour cells
- Suppress immunity both directly and indirectly (through negative-regulatory cells).
- Toleragenic mechanisms include: enzymes, cell-surface markers, cytokines and chemokines.

Regulatory T cells (T_{reg} cells)
- Immune-suppressive T cells that are vital to maintaining peripheral immune tolerance.
- 'Natural' T_{reg} cells are a discreet lineage of self-reactive T cells ($CD4^+CD25^+$; $FOXP3^+$) — they elicit immune suppression through direct cell–cell contact.
- 'Induced' T_{reg} cells are derived from conventional T cells through alternative differentiation — there are two subtypes; T regulatory 1 (T_R1) cells and T helper 3 (T_H3) cells. These cells secrete pro-toleragenic T helper 2 (T_H2) cytokines; T_R1 cells secrete interleukin 10 (IL10) and T_H3 cells secrete transforming growth factor β (TGFβ).

Dendritic cells (immature/mature)
- A heterogeneous class of leukocytes that is central to orchestrating immune responses.
- 'Immature' dendritic cells are toleragenic by default until external inputs promote differentiation. They produce pro-toleragenic cytokines such as IL10 and vascular endothelial growth factor (VEGF).
- 'Mature' toleragenic dendritic-cell subgroups also exist, although it is unclear whether they arise through alternative differentiation or as distinct lineages. They can promote T_H2 polarization and expand the population of T_{reg} cells.

Myeloid suppressor cells (MSCs)
- A heterogeneous population of immature myeloid precursors that restrain cellular immunity.
- Identified operationally as a cell population that is positive for both monocyte (CD11b, also known as MAC1) and granulocyte (Gr1 also known as Ly6G) cell-surface markers.
- MSCs can interfere with the ability of $CD8^+$ T cells to produce interferon γ (IFNγ). They produce immunosuppressive cytokines (such as TGFβ) and can also inhibit T-cell activity through arginase-mediated reactive oxygen species (ROS) production.

Tumour-associated macrophages (TAMs)
- Macrophages can be polarized towards a T_H1 (M1 macrophage) or T_H2 (M2 macrophage) phenotype in a lymphocyte-independent manner.
- Classical (M1) macrophages produce pro-inflammatory cytokines and effect cell killing.
- Alternatively, polarized (M2) TAMs dampen inflammation and promote angiogenesis. Tumour-derived factors (such as IL6, IL10 and colony-stimulating factor 1 (CSF1)) promote TAM development and survival.
- TAMs secrete pro-toleragenic factors (such as IL10, TGFβ and prostaglandin E2 (PGE2)).

Natural killer T cells (NKTs)
- Subpopulation of innate T cells with NKT cell-surface markers.
- Express semi-invariant T-cell receptors to self-antigens (lipids or carbohydrates).
- T-cell receptor (TCR) ligation elicits rapid, robust cytokine production (both T_H1 and T_H2 cytokines).

M1 macrophage
A macrophage subtype that produces pro-inflammatory cytokines and acts as an effector of cell killing.

M2 macrophage
A macrophage subtype that acts to dampen inflammatory responses and scavenge debris, as well as promote angiogenesis and tissue remodelling and repair.

Mixed lymphocyte reaction
(MLR). An *in vitro* assay designed to measure the T-cell proliferation that occurs in response to cells bearing allogeneic MHC class I and class II molecules. Used to assess T-cell functional capacity.

Plasmacytoid morphology
There are two main subsets of dendritic cell in the human system; myeloid and plasmacytoid. Plasmacytoid dendritic cells look like plasma cells (antibody-producing B cells), and can produce high amounts of interferon-α.

in vitro, which promotes the induction of IDO activity in these cells in the context of the mixed lymphocyte reaction (MLR). Alternatively, in human non-small-cell lung cancer (NSCLC) biopsies, expression of IDO has been reported to occur in infiltrating granulocytes[16]. Most attention, however, is currently focused outside the immediate vicinity of the tumour on the dendritic cells (DCs) that localize to the TDLNs. In the context of tumour development, IDO-positive cells have been observed in the TDLNs of mice bearing IDO-negative tumour isografts. In this model, treatment with 1MT inhibited tumour growth, which indicates that protection from immunity was being afforded by the host rather than by any tumour-derived IDO activity[17].

Tumours formed by the B16 melanoma-derived cell line that had been engineered to constitutively express granulocyte-macrophage colony-stimulating factor (GMCSF) elicited a particularly robust accumulation of IDO-expressing cells in the TDLNs. These cells were identified as a subset of $CD11c^+$ DCs with plasmacytoid morphology that were also expressing the B-cell-lineage marker CD19 (REFS 18,19). This cell population was potently toleragenic, and the suppressor function depended on IDO activity, as it could be prevented by 1MT treatment or by using plasmacytoid DCs that were obtained from *Indo*-null mice. This latter control is particularly important because it addresses legitimate concerns regarding possible off-target effects of 1MT[20] (see below). Many mechanisms for inducing toleragenic IDO activity in splenic DC subsets *in vivo* have been identified[21–25], leading to the postulated existence of an alternative maturation pathway that produces a professional class of pro-toleragenic DCs, which are characterized by their ability to induce IDO[26]. How such cells come to be present in the TDLNs is not yet known.

The evaluation of small-molecule inhibitors of IDO, as reported so far, has centred primarily around indole-containing compounds[27]. Of these, 1MT — which was identified in 1991 as a competitive inhibitor of IDO[28] — is the compound that has been used almost exclusively for experimental studies. 1MT is not particularly soluble, nor is it a potent IDO inhibitor, particularly in

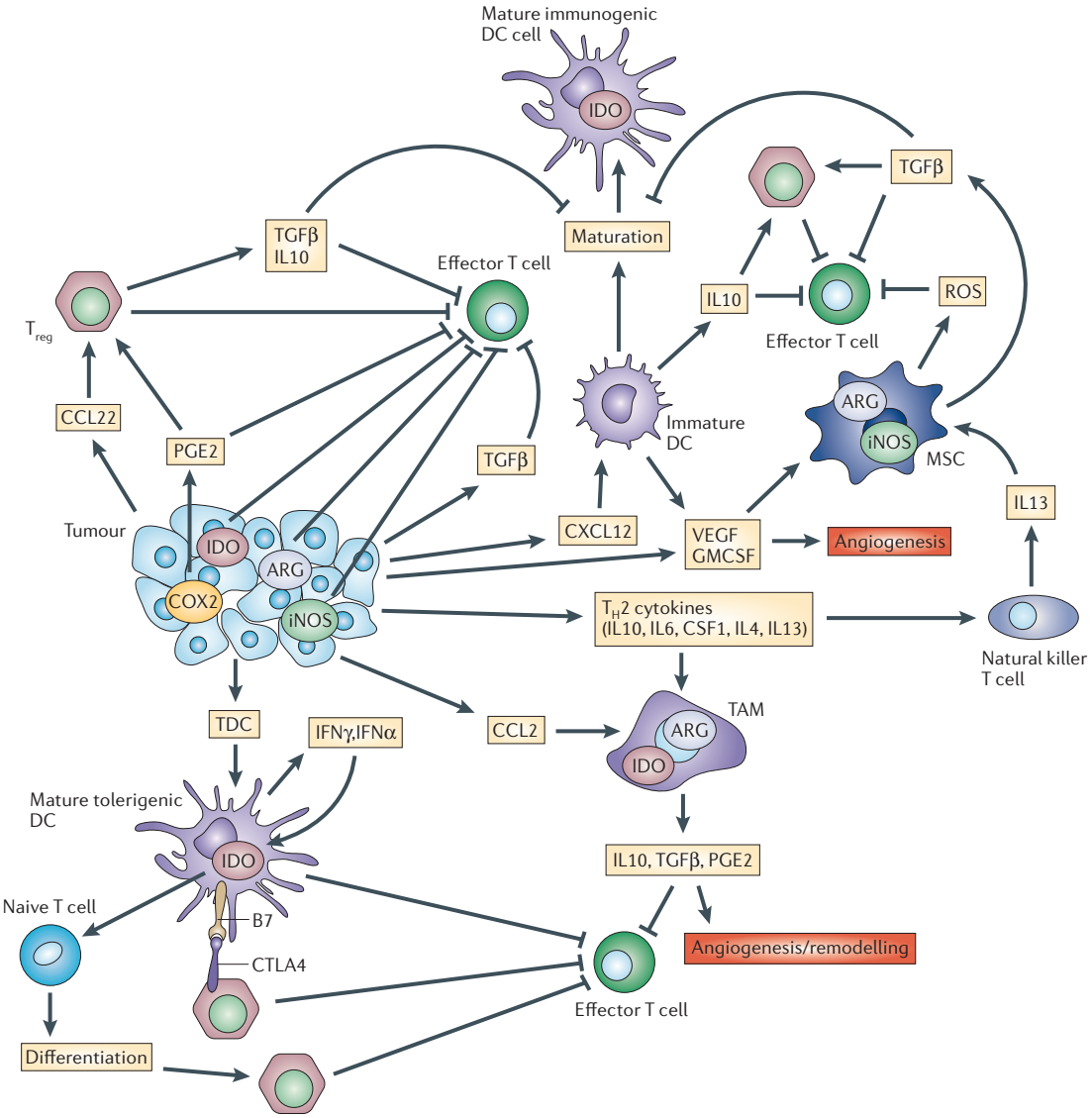

Figure 1 | **Interactions between the tumour and cells of the immune system that foster an immunosuppressive microenvironment.** Through the production of cytokines (such as interleukin 10 (IL10), transforming growth factor β (TGFβ), IL13 and vascular endothelial growth factor (VEGF)) and chemokines (such as CCL22, CCL2 and CXCL12), tumours can promote the migration and expansion of cells that negatively regulate the immune system. T-regulatory (T_{reg}) cells, which block T-effector cells through both direct interaction and the production of immunosuppressive cytokines such as TGFβ and IL10, can be recruited as well as myeloid suppressor cells (MSCs), immature dendritic cells, natural killer T cells (NKTs) and tumour-associated macrophages (TAMs). These cells act to suppress the proliferation of effector-T cells (such as $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells), the production of cytokines (such as interferon γ (IFNγ) and IL2), and cytolytic activity. Both tumour cells and host cells can express enzymes that are involved in immune suppression (such as arginase (ARG), indoleamine 2,3-dioxygenase (IDO), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS)). In addition, mature, toleragenic dendritic cells can also accumulate in tumour-draining lymph nodes. Induction of IDO activity in toleragenic DCs, through interaction with T_{reg} cells expressing cytotoxic-T-lymphocyte-associated antigen-4 (CTLA4), can promote further T_{reg} expansion and differentiation. CSF1, colony-stimulating factor-1; GMCSF, granulocyte-macrophage-CSF; PGE2, prostaglandin E2; ROS, reactive oxygen species; TDC, tumour-derived cytokine.

cell-based assays. However, it has favourable pharmacokinetic attributes, such as oral bioavailability, low clearance and low protein binding[11,15]. Also, there have been no reports of toxicity being directly attributable to 1MT, even when administered at high levels, except for dehydration when 1MT was delivered to mice in their drinking water[11]. However, this might have been simply due to the mice drinking insufficient quantities of water, as our own unpublished studies have shown no evidence of dehydration when comparably high levels of 1MT were administered by oral gavage over an extended period. The direct *in vivo* experimental evidence for the role of IDO in immune tolerance has come from studies in mice, and evidence that IDO is involved in pathological

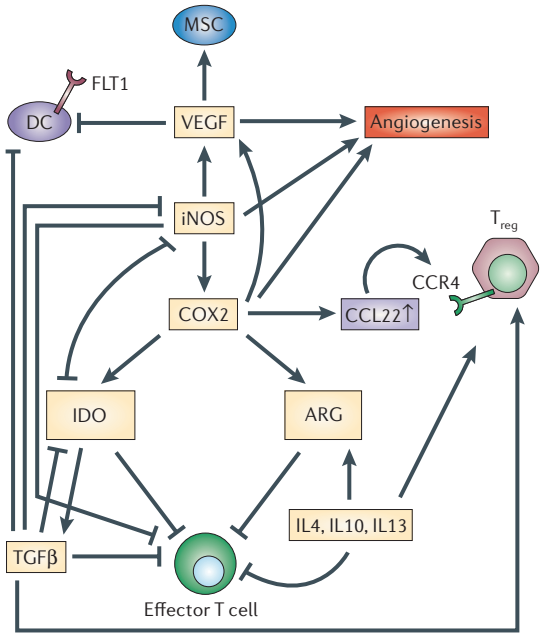

Figure 2 | **Inter-regulation of the various cytokines and enzymes associated with immune suppression.** Cyclooxygenase 2 (COX2), through the production of prostaglandin E2 (PGE2), has a central role in regulating the expression of other immunosuppressive modulators, including the enzymes arginase (ARG) and indoleamine 2,3-dioxygenase (IDO), the cytokine vascular endothelial growth factor (VEGF) and the chemokine CCL22. The type II cytokines interleukin (IL)10, IL13 and IL4, also induce ARG expression, and inducible nitric-oxide synthase (iNOS) regulates both the expression of VEGF and IDO as well as the activity of COX2. In addition to its known immunosuppressive effects, transforming growth factor β (TGFβ) might also have positive effects on the immune system by inhibiting IDO and iNOS expression. DC, dendritic cell; FLT1, FMS-related tyrosine kinase-1; MSC, myeloid suppressor cell; pDC, plasmacytoid dendritic cell; T_{reg}, regulatory T cell.

immune tolerance associated with human tumours is so far limited to epidemiological observations and *in vitro* tissue-culture studies[20]. Carefully designed clinical trials of small-molecule IDO inhibitors might afford a unique opportunity to probe its role in normal and pathophysiological mechanisms of immune tolerance directly in humans. Based on accumulated preclinical data, the (D)-isomer of 1MT is currently being developed by the US National Cancer Institute (NCI) Rapid Access to Intervention Development (RAID) programme and NewLink Genetics Corporation for clinical evaluation (TABLE 1; D. Munn, personal communication). (D)-1MT was chosen because it has been reported to be more biologically active than (L)-1MT[29]. In combination with cytotoxic agents, we have also found that the (D)-isomer is more effective than the (L)-isomer for treating tumour-bearing mice in two different breast cancer models (A.M. unpublished observations). However, tryptophan occurs naturally as the (L)-isomer and, consistent with this but in contrast to the biological data, (L)-1MT inhibits the purified IDO enzyme more potently than (D)-1MT[30].

This discrepancy between *in vitro* and *in vivo* results raises the concern that the mechanism of action of 1MT might include possible off-target effects that should be borne in mind as clinical-trial results are evaluated.

The demonstration that other IDO inhibitory compounds have biological activity has helped to alleviate concerns about the mechanism of action of 1MT. Similar to 1MT, methylthiohydantoin-tryptophan (MTH-Trp) cooperates with paclitaxel to regress autochthonous mouse breast carcinomas[15] (TABLE 1). More recently, the plant-derived indole-containing phytochemical brassinin has been found to have moderate inhibitory activity against the IDO enzyme *in vitro*[31]. This translates to biological activity — we have found that brassinin shows cooperation with paclitaxel in the treatment of autochthonous mouse breast carcinomas (A.M. unpublished observations). Structure–activity relationship information has been gleaned from testing a series of brassinin analogues[31] and rational drug design of new IDO inhibitors should be greatly aided by the publication of the IDO crystal structure[32]. High-throughput screening, however, remains the most effective way to identify completely new structural series, and a number of new IDO-inhibitory compounds from diverse structural classes have recently been reported from an innovative yeast screen[33]. Targeting the GCN2 kinase (also known as eukaryotic translation initiation factor 2α kinase 4; EIF2AK4) with a small-molecule inhibitor might be considered as a possible alternative to direct IDO inhibition, based on recent evidence that IDO mediates T-cell suppression through activation of the GCN2 kinase pathway[34]. This promotes downregulation of the T-cell receptor (TCR) CD3ζ chain in cytotoxic T lymphocytes (CTLs) as well as TGFβ-dependent, peripheral induction of regulatory T cells (T_{regs})[142].

Arginase and nitric-oxide synthase

As with IDO, a complex relationship exists between catabolism of the amino acid (L)-arginine, immunity and tumorigenesis[35,36], which involves both direct effects on tumour growth and survival as well as indirect effects involving tumour-associated immune cells (BOX 3). The two types of enzyme that are responsible for arginine catabolism are the arginase (ARG; encoded by *ARG1* and *ARG2*) and nitric-oxide synthase (NOS; encoded by *NOS1*, *NOS2* and *NOS3*) enzymes, and products of both pathways have been implicated in the modulation of tumour growth. Ornithine, a downstream product of ARG catabolism of (L)-arginine, is the precursor for the biosynthesis of polyamines, which serve as important endogenous tumour promoters[37]. Increased polyamine levels are frequently observed in tumours because of increased ornithine decarboxylase (ODC) activity[38], and it has been proposed that ARG is also increased in tumour cells to feed into this biosynthetic pathway[39]. Indeed, evidence of increased ARG activity has been detected in patients with NSCLC, colorectal, breast, skin and prostate cancer[35]. Other studies, however, have indicated that infiltrating myeloid suppressor cells (MSCs) or tumour-associated macrophages (TAMs) (BOX 1) might be a more important primary source of

Autochthonous
Originating where found. Distinguishes mouse cancer models bearing *de novo*-formed tumours from more commonly used tumour-cell graft models.

Box 2 | **Tryptophan catabolism**

Indoleamine 2,3-dioxygenase (IDO), which is encoded by the *INDO* gene, is a haem-containing, monomeric oxidoreductase that catalyses the degradation of the essential amino acid tryptophan to *N*-formyl-kynurenine. The expression and activity profiles of IDO are distinct from those of tryptophan dioxygenase (TDO2), a predominantly liver-based enzyme that catalyses the same enzymatic reaction as IDO and maintains proper tryptophan balance in response to dietary uptake. IDO, by contrast, is expressed in various tissues, with particularly high levels found in the epididymis, the placenta of pregnant females and across large mucosal surfaces such as the gut and lung epithelia. IDO activity is induced at sites of inflammation by the type II interferon IFNγ, and less so by the type I interferons IFNα and IFNβ.

IDO-knockout mice are viable with no evidence of susceptibility to developing spontaneous autoimmunity or alterations in immune-system development, which suggests that IDO inhibition, in a therapeutic setting, might produce minimal side effects. IDO has been mechanistically implicated in 'cross tolerization', whereby dendritic cells (DCs) pick up antigens from the periphery and present them in a toleragenic context within the draining lymph node. Of particular note has been the finding that IDO activity in DCs can function as an important downstream effector for the immunoregulatory molecule cytotoxic-T-lymphocyte-associated antigen-4 (CTLA4). B7 ligands on dendritic cells have been implicated in a new 'reverse-signalling' mechanism that is required for the autocrine production of IFNγ, and which drives induction of IDO through a signal transducer and activator of transcription-1 (STAT1)-dependent mechanism. $CD4^+CD25^+$ T_{reg} cells, which constitutively express CTLA4 on their surface, have been shown to promote IDO activity in dendritic cells through this mechanism. IDO is integrated within the complex milieu of regulatory factors that promote immune tolerance. Cyclooxygenase 2 is a positive driver of IDO expression, which is consistent with its role in mediating immune suppression. IDO has conversely been reported to affect prostaglandin synthesis, which suggests a possible feedback interaction. Inducible nitric-oxide synthase and IDO seem to be mutually antagonistic in cell-based studies. Transforming growth factor β (TGFβ) has also been reported to antagonize IFNγ-mediated induction of IDO expression in human fibroblasts. This seems to run counter to the immunosuppressive activity that is ascribed to TGFβ, but it is consistent with the ability of TGFβ to antagonize positively regulated targets of IFNγ.

tumour-associated ARG activity than the tumour cells themselves[40–42]. In support of this concept, macrophages transfected with *ARG1* were shown to promote tumour-cell proliferation through increased production of ornithine[43].

In addition to its direct effects on tumour proliferation, ARG activity has also been shown to be associated with myeloid-cell-mediated immune suppression[36] (FIG. 1). Treatment with the ARG inhibitor *N*-hydroxy-nor-L-Arg (nor-NOHA) impaired subcutaneous tumour formation by Lewis lung carcinoma cells in syngeneic animals but not in severe combined immunodeficient mice (SCID mice), which is indicative of an immune-mediated effect[41]. In this model, ARG expression was associated with tumour-infiltrating TAMs and, consistent with the proposed mechanism of ARG action[44], the infiltrating T cells showed reduced expression of the CD3ζ chain, which is a key signalling component of the TCR. Decreased expression of the CD3ζ chain has also been observed in patients with many types of cancer[36,42]. In patients with renal-cell carcinoma (RCC), increased ARG activity was detected in peripheral blood mononuclear cells, and was accompanied by decreased expression of the CD3ζ chain and diminished production of IL2 and IFNγ[42]. In these studies, ARG1 expression was localized to the $CD11b^+CD14^-$ immature MSC subpopulation, and depletion of these cells restored T-cell proliferative capacity, cytokine production and expression of the CD3ζ chain, further highlighting the potential clinical relevance of ARG-mediated immune suppression.

In general, ARG and NOS2 (commonly referred to as inducible NOS: iNOS) are reciprocally regulated[35]. Macrophage production of nitric oxide (NO) through iNOS catabolism of arginine is associated with increased tumoricidal activity[45], and a shift from NO to ornithine production by intra-tumoral myeloid cells has been correlated with tumour progression[46], which indicates that the development of ARG-expressing MSCs might be an important stage in tumour progression and not just a tumour-associated phenomenon. However, in response to certain stimuli such as lipopolysaccharide (LPS) or IL4, MSCs have been shown to upregulate both ARG and iNOS pathways simultaneously[47,48]. Furthermore, $CD11b^+Gr1^+$ MSCs that are isolated from *iNos*-null mice do not inhibit T-cell proliferation, which indicates that NO is also an essential component of MSC-mediated immune suppression[49]. NO blocks signalling through the IL2 receptor expressed by T lymphocytes by impeding phosphorylation of the intracellular-signalling proteins signal transducer and activator of transcription-5 (STAT5, also known as STAT5B), AKT, and extracellular signal-regulated kinase (ERK)[49]. In addition to the anti-proliferative effects of NO, MSC production of reactive oxygen species, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), has been linked to both ARG and iNOS[47,50]. Finally, it has been shown that $F4/80^+$ TAMs that are derived by adoptive transfer of $Gr1^+$ MSCs from tumour-bearing mice into tumour-bearing recipients promoted T-cell apoptosis through a mechanism that was both ARG and iNOS-dependent and that required STAT1, but not STAT3 or STAT6, signalling[40]. This indicates that ARG and/or iNOS might have a role in TAM-mediated immune suppression as well.

Based on these studies, it could be predicted that inhibition of arginine metabolism by simultaneously blocking ARG and iNOS would be synergistic in promoting anti-tumour immunity and, in fact, it has been shown that combination of the ARG inhibitor, *N*-hydroxy-L-Arg (NOHA), with the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA), can restore the expansion and cytolytic function of tumour-infiltrating T cells in pre-clinical models[51]. DeSanto *et al.*[52] also demonstrated that nitroaspirin (NCX-4016) restored T-cell proliferation in the presence of MSC by reducing both ARG and NOS activity in the $CD11b^+$ subpopulation, whereas neither the NO donor nor aspirin alone had this effect. Using a series of analogues, they were able to demonstrate that the NO group was essential for inhibiting NOS activity and the aspirin spacer was important for modulating ARG activity. Treatment of tumour-bearing animals with nitroaspirin increased the number of tumour-specific cytotoxic T-lymphocytes (CTLs) and, when combined with a tumour vaccine, increased survival.

A number of arginine analogues that block ARG activity are available as tool compounds (TABLE 1). Relative to NOHA and nor-NOHA, which are known to inhibit ARG1 at micromolar concentrations[53], the boronic-acid derivatives 2(S)-amino-6-boronohexanoic acid (ABH) and S-(2-boronoethyl)-L-cysteine (BEC) show improved

Severe combined immunodeficient mice
SCID mice are homozygous for the spontaneous $Prkdc^{scid}$ mutation in the protein kinase, DNA-activated, catalytic polypeptide encoding gene. These mice are characterized by an absence of functional T cells and B cells and are excellent hosts for allografts and xenografts.

Table 1 | **Representative small-molecule inhibitors of proposed immunotherapy targets**

Target	Inhibitor	Company/institution	1° indications	Development status	References
IDO	1MT, MTH-Trp	NewLink	Oncology/HIV	Discovery	15,28
ARG	BEC, ABH	University of Pennsylvania	Oncology	Discovery	54
iNOS	L-NMMA	Fujisawa	Sepsis	Discontinued	51,133
	PS-891169	Pharmacopeia	Inflammation	Discovery	IDDB*
ARG/iNOS	NCX-4016	NicOx SA	Oncology/Cardio	Phase II	52
COX2	Celebrex	Pfizer	Inflammation	Launched	134
			Oncology	Phase II	63,66
	Rofecoxib	Merck	Inflammation/Oncology	Withdrawn	62,135
EP2/EP4	CP-533536	Pfizer	Osteoporosis	Phase I	IDDB
TGFβRI	SB-505124	GlaxoSmithKline	IInflammation/Oncology	Discovery	136
	SD-208	J&J/Scios	Inflammation/Oncology	Discovery	96
	LY580276	Lilly	Inflammation/Oncology	Discovery	137
JAK/STAT	JSI-124	H. Lee Moffitt Cancer Center	Oncology	Discovery	101–103
	CPA-7	H. Lee Moffitt Cancer Center	Oncology	Discovery	100,138
VEGFR1(FLT1)	SU5416	Pfizer	Oncology	Discontinued	116,139
	AG-13736	Pfizer	Oncology	Phase II	IDDB
CCR4	IC-487892	ICOS	Inflammation	Discovery	IDDB
CXCR4	AMD3100	AnorMed	Oncology/HIV	Discovery	140
CCR2	INCB3344	Pfizer	Inflammation	Discovery	141

*Investigational drugs database. ABH, 2(S)-amino-6-boronohexanoic acid; ARG, arginase; BEC, S-(2-boronoethyl)-L-cysteine; COX2, cyclooxygenase 2; EP2, prostaglandin E receptor 2; FLT1, FMS-like tyrosine kinase 1; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric-oxide synthase; JAK, Janus kinase; L-NMMA, N^G-monomethyl-L-arginine; 1MT, 1-methyl-tryptophan; MTH-Trp, methylthiohydantoin-tryptophan; NCX-4016, nitroaspirin; STAT, signal transducer and activator of transcription; TGFβRI, transforming growth factor-β receptor type I; VEGFR1, vascular endothelial growth factor receptor 1.

potency, with ABH being the most potent ARG inhibitor reported so far[54]. None of these compounds seem to be selective for ARG1, the isoform that is implicated in mediating immune suppression[55]; however, it is unclear whether greater selectivity will afford any advantage, as inhibiting ARG1 might produce toxicity associated with its role in the urea cycle[56]. The most promising agent at this point seems to be NCX-4016 as it targets both ARG and iNOS simultaneously. NCX-4016 is currently in clinical trials for cardiovascular disorders and colon cancer because of its reduced gastrointestinal toxicity relative to aspirin[57]. This agent seems to be well tolerated, and based on preclinical studies, might increase the efficacy of both chemotherapeutic and immune-based approaches in cancer. Finally, it should be noted that certain tumour types are auxotrophic for arginine due to a deficiency in arginosuccinate-synthetase enzyme activity, and that a pegylated form of the arginine-degrading enzyme, arginine deiminase, is in clinical trials having been shown to inhibit tumour growth in preclinical models[58]. Therefore, prevention of arginine catabolism through arginase inhibition could be contraindicated for certain tumour types.

Cyclooxygenase 2 (COX2)

The cyclooxygenase enzyme, COX2, is overexpressed in several tumour types, including colon, breast and gastric tumours, and NSCLC[59]. Through the production of the arachidonic-acid catabolite, prostaglandin E2 (PGE2), COX2 affects various processes that are relevant to tumorigenesis and malignant progression, including apoptosis, angiogenesis, invasiveness, and inflammation/immunosuppression[60]. The importance of COX2 in countering anti-tumour immunity (FIG. 1) has been highlighted by *in vivo* studies. Using the Lewis lung carcinoma model, Stolina *et al.*[61] demonstrated that inhibition of *COX2* expression with antisense oligonucleotides or inhbition of COX2 activity with the selective COX2 inhibitor, SC-58236, led to a significant reduction in tumour burden and prolonged survival. In these studies, reduced tumour burden was associated with increased lymphocyte infiltration, increased IL12 and IFNγ levels, and decreased IL10 levels, which is consistent with a role for COX2 in supporting T-helper-cell-2 responses (T_H2 responses). Interestingly, the effects on tumour growth were reversed by the transfer of lymphocytes from IL10 transgenic mice, which is consistent with a crucial role for IL10 in this process. In addition to evidence of single-agent activity, studies in several animal models have shown that COX2 inhibition can significantly increase the ability of anti-tumour vaccines to inhibit tumour growth and prolong survival[62–66]. The potential clinical relevance of these results has been demonstrated in breast cancer patients who have impaired DC and T-cell function that correlates with COX2 and PGE2 expression in the tumour and with reduced T_H1 and increased T_H2 cytokine levels in the serum[67]. Together these results indicate that clinical trials combining COX2 inhibitors with anti-tumour vaccines are warranted.

T_H2 responses
A T-helper-2 response involves production of cytokines, such as IL4, which stimulate antibody production. T_H2 cytokines promote secretory immune responses of mucosal surfaces to extracellular pathogens as well as allergic reactions.

Box 3 | **Arginine catabolism**

Mammals express two distinct arginase isoforms that are encoded by the independent genes *ARG1* and *ARG2*. The two isoforms are structurally similar and have very similar enzymatic properties, catalysing the breakdown of L-arginine to L-ornithine and urea. However, ARG1 and ARG2 are controlled quite differently in terms of tissue distribution, regulation of expression and subcellular localization.

The ARG1 isoform that is implicated in promoting immune tolerance is a cytosolic enzyme that is also highly expressed in the liver as a component of the urea cycle. In mouse myeloid cells, ARG1 expression can be induced by T_H2-type cytokines and anti-inflammatory signals, including interleukin 4 (IL4), IL13, transforming growth factor β, and granulocyte-macrophage colony stimulating factor. ARG2, on the other hand, is constitutively expressed in the mitochodria of various cell types, where its precise function remains unclear.

The phenotypes of the *Arg1*- and *Arg2*-knockout mice confirm their distinct functional roles. *Arg1*-knockout mice die by 2 weeks of age due to hyperammonaemia, which is consistent with the role of ARG1 as the final enzyme of the urea cycle. *Arg2*-knockout mice, however, show no gross phenotype except for increased serum arginine levels. In humans, ARG1 deficiency is associated with hyperargininaemia, progressive mental impairment, growth retardation and occasional episodes of hyperammonaemia. These symptoms appear later in development and are less severe than those observed with other disorders of the urea cycle, presumably due to partial compensation by ARG2.

The alternative pathway through which L-arginine is catabolized uses the nitric-oxide synthase (NOS) enzymes to generate L-citrulline and nitric oxide. The family of NOS enzymes includes endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2). iNOS is expressed in both myeloid cells and malignant cells, and is an important mediator of inflammation and tumorigenesis through the production of reactive nitrogen species. In turn, NOS enzymes modulate the expression and activity of several key growth factors and enzymes, including poly ADP ribose polymerase, vascular endothelial growth factor, matrix metalloproteinases and cyclooxygenase 2.

In addition to the effects of COX2 on DC and T-cell function, a role in the generation of immune-suppressing T_{reg} cells has also been proposed. PGE2 was shown to increase the inhibitory capacity of $CD4^+CD25^+$ T_{reg} cells and to induce a suppressor phenotype in $CD4^+CD25^-$ cells that correlated with the induction of FOXP3 — a cell-lineage specification factor that is crucial for the development of T_{reg} cells[68,69]. This effect was dependent on PGE2 signalling through its receptors, EP2 and EP4 (also known as prostaglandin E receptor 2 (PTGER2) and PTGER4), as the induction of T_{reg} cells was reduced in *Ep4*-null mice and completely ablated in *Ep2*-null mice[69]. COX2 inhibition reduced the intra-tumoral levels of $CD4^+CD25^+FOXP3^+$ T_{reg} cells by 60%, which correlated with increased T_H1 cytokines, decreased T_H2 cytokines and increased tumour-specific T-cell release of IFNγ[65]. In addition, PGE2 has also been shown to upregulate the expression of both IDO and ARG1 (REFS 70,71).

The selective COX2 inhibitors, celecoxib and rofecoxib (TABLE 1), have both been tested in phase II clinical trials in combination with chemotherapeutic agents in patients with NSCLC, pancreatic, breast and colorectal cancers and, for the most part, have shown additional clinical benefit beyond that observed with chemotherapy alone[72–76] although the mechanisms responsible for these effects remain undetermined. With the recent concern regarding the safety of selective COX2 inhibitors[77], targeting the family of prostaglandin receptors (EP1–EP4) might serve as an alternative means to affect the PGE2 pathway in cancer, and several small-molecule antagonists of EP2 and/or EP4 are currently in development for various indications (TABLE 1). In this regard, tumour growth was significantly attenuated and survival prolonged in *Ep2*-null mice[78] and this effect did not seem to be due to alterations in angiogenesis, but rather, to improved DC function resulting in increased levels of infiltrating $CD4^+$ and $CD8^+$ T cells and improved CTL function. It should be noted, however, that *Ep2*-null mice show reduced fertility and hypertension[79], so it is unclear what the safety profile of an EP2 antagonist would be. In addition, the relative contribution of EP2 versus the three other EP-receptor subtypes through which PGE2 can signal remains to be defined.

Transforming growth factor β

TGFβ is expressed by most malignant tumours and probably functions as a tumour suppressor early in tumour development by directly suppressing the proliferation of cancer cells[80,81]. However, as cancer cells evolve, they frequently become refractory to TGFβ-mediated growth inhibition through mutations in the TGFβ receptor (TGFβR) and/or downstream signalling proteins such as Smad proteins, and this can promote epithelial–mesenchymal transition (EMT) and a metastatic phenotype. Although these cancer cells are no longer responsive to TGFβ they continue to overexpress it, and this characteristic is thought to contribute to the profound immunosuppression that is observed in patients with advanced or metastatic tumours[80,81].

Several studies have examined how TGFβ regulation of T-cell function affects tumour immunity. Early studies focused on the direct effect of TGFβ on $CD8^+$ CTLs, as it was shown that TGFβ could suppress IL2 production and CTL activation[82]. Tumour cells transfected with TGFβ were shown to suppress CTL function both *in vitro* and *in vivo*[83], and to significantly attenuate the efficacy of DC-based tumour vaccines[84]. Transgenic mice expressing a dominant-negative form of the receptor, dnTGFβRII, likewise failed to develop tumours on inoculation of EL-4 thymoma or B16 melanoma cells due to an increased tumour-specific CTL response[85]. TGFβ suppression of tumour-specific CTL responses seems to be multifactorial, as TGFβ also affects the differentiation of $CD4^+$ T_H cells[86,87]. In addition, TGFβ has also been shown to antagonize T_H1 responses through suppression of IFNγ production by natural killer cells (NKs)[88], and to block the maturation and activation of DCs by reducing their ability to present antigen and to stimulate T-cell proliferation[89].

The role of TGFβ in the context of T_{reg} cells has been controversial. Although neutralizing antibodies to TGFβ have been shown to reverse immune suppression by T_{reg} cells in several models of inflammation[90,91], most studies have concluded that this suppression is not directly mediated by TGFβ, as neutralization of TGFβ with either antibodies or a soluble TGFβRII-Fc does not reverse the T_{reg}-mediated suppression of T-cell proliferation *in vitro*. Furthermore, $CD4^+CD25^+$ T_{reg} cells from *Tgfb*-null mice are as effective as cells from wild-type animals in blocking T-cell growth[92].

T_H1 responses
A T-helper-1 cell-mediated immune response is mediated by pro-inflammatory cytokines such as IFNγ, IL1 and TNFα. It promotes cellular immune responses against intracellular infections and malignancy.

Recent work has revealed a prominent role for TGFβ in the expansion of T_{reg} cells in the periphery. The size of the peripheral but not the thymic compartment of T_{reg} cells was significantly reduced in young *Tgfb*-null mice and this was associated with decreased FOXP3 expression[93]. In addition, exposure of $CD4^+CD25^-$ cells to TGFβ *in vitro* induced FOXP3 expression, which caused them to differentiate towards a T_{reg} phenotype that is capable of suppressing $CD4^+$ T-cell activation and cytokine production[94].

Based on the direct effects of TGFβ on tumour growth, as well as its effects on the immune system, several approaches to develop inhibitors of the TGFβ pathway have been investigated. These include antisense oligonucleotides and monoclonal antibodies to TGFβ, as well as small-molecule inhibitors of the TGFβRI kinase[81,95] (TABLE 1). AP-12009, an antisense oligonucleotide to *TGFβ*, is in Phase I/II trials in patients with glioma, and seems to be well tolerated and to significantly increase survival time[81]. In addition, a vaccine that contains allogeneic tumour cells that are modified to express antisense *TGFβ* is in Phase I/II clinical trials[81]. Using this approach, a response rate of ~30% has been reported in NSCLC, with no serious toxicities observed[95]. In preclinical models, small-molecule inhibitors of TGFβRI, which are exemplified by SD-208, have been shown to block TGFβ signalling in both tumour cells and peripheral blood mononuclear cells, and to prolong the survival of tumour-bearing animals. Interestingly, efficacy was associated with increased immune-cell infiltration in the tumours and not with direct effects on tumour proliferation, apoptosis or angiogenesis[96].

Cytokine signalling

Historically, small-molecule inhibition of cytokine receptor–ligand interactions has not proven fruitful. However, downstream signalling pathways might be more tractable alternative targets. In particular, most tumour-associated immunosuppressive cytokines, including IL10, IL4, IL13, CSF1 and IL6, signal through the Janus kinase (JAK)–STAT pathway[97]. IL23, which was recently shown to be overexpressed by tumour cells and to both promote inflammation and reduce CTL infiltration, also signals through this pathway[98]. It should be noted, however, that a number of immune-enhancing cytokines (IL2, IL7, IL15, IL12 and IFNγ) also signal through the JAK–STAT pathway. So, in order to maintain the immune response without blunting the desired early expansion of antigen-specific T cells, inhibitors of JAK–STAT signalling might have to be given at a distinct time point, either before or after vaccine therapy. Despite this caveat, many studies specifically focusing on the STAT3 protein have been carried out. Wang *et al.*[99] showed that tumour-cell expression of constitutively active STAT3 inhibits the production of several pro-inflammatory cytokines and chemokines, and induces the release of factors that suppress DC maturation. In these studies, *STAT3* antisense oligonucleotides, or a dominant-negative form of STAT3 (STAT3β), led to the increased production of IFNβ, tumour-necrosis factor α (TNFα), IL6, CCL5 (also known as RANTES) and CXCL10 (also known as IP-10) in the absence of an inductive signal. In addition, exposure of DCs to media from STAT3β-transfected tumour cells led to increased expression of IL12, MHC class II and CD40, and concomitantly increased the ability of the DCs to stimulate T-cell proliferation and cytokine production. Further studies have shown that STAT3-expressing K1735 melanoma sublines were capable of forming tumours in immunocompetent mice, whereas sublines that lacked STAT3 either failed to form tumours, or the tumours that formed were rejected and displayed heavy T-cell infiltration[100]. The STAT3-negative sublines were, however, capable of forming tumours in SCID mice, consistent with the role of immunity in suppressing their outgrowth.

These initial studies exploring the therapeutic potential of targeting STAT3 have been confirmed and expanded through the use of small-molecule inhibitors of the STAT3 pathway (TABLE 1). JSI-124 (cucurbitacin I) has been shown to selectively inhibit the activation of JAK2 and STAT3 *in vitro*[101]. Although it does not block JAK2 kinase activity directly, and the actual biochemical target remains unknown. Nevertheless, using JSI-124 *in vitro* and *in vivo*, Nefedova *et al.*[102,103] have demonstrated that inhibition of STAT3 activation significantly reduced the $Gr1^+CD11b^+$ MSC population with a concomitant increase in the $CD11c^+$ DC population that was capable of effectively stimulating T-cell responses. Experiments to assess the effect of JSI-124 inhibition of STAT3 on anti-tumour responses *in vivo* were conducted in the MethA sarcoma model. This tumour model does not have hyperactivated STAT3, so JSI-124 does not affect tumour growth directly. In this model, immunization with DCs that expressed exogenous p53 as a tumour-specific antigen decreased tumour growth transiently but, in combination with JSI-124, this treatment markedly prolonged the inhibition of tumour growth and significantly increased the tumour-antigen-specific CTL response without any obvious signs of toxicity. Studies with a different inhibitor, CPA-7 (a platinum (IV) compound that specifically disrupts STAT3 signalling), have generated similar anti-tumoral effects and have implicated many immune components, including macrophages, NKTs and T cells, as important effectors[100,104].

In addition to STAT3, STAT6 has also been implicated in tumour-mediated immune suppression[105]. *Stat6*-null mice are capable of rejecting both transplantable tumour cells and spontaneously arising tumours[106,107] through the action of tumour-specific $CD8^+$ CTLs[108]. A reduction in ARG-producing, immunosuppressive MSCs in combination with an increase in NO-producing, immunostimulatory M1 macrophages results in the increased CTL responses observed in these mice[109]. This further highlights the central role of the Stat pathways in tumour-mediated immune escape. It should be noted that, as with STAT3, it is difficult to develop small-molecule inhibitors that directly target DNA-binding proteins such as STAT6, and none are currently reported to be in development. The kinase activity of the upstream JAK proteins are, however, quite amenable to small-molecule

blockade and a number of JAK inhibitors have been disclosed[110]. The caveat remains, however, that because JAKs also regulate cytokine-mediated immune activation, the net outcome will be difficult to predict.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is well known for stimulating endothelial-cell proliferation and formation of tumour neovasculature, and a number of therapeutic agents, both biological agents and small molecules, have been developed to target this activity. The most advanced of these is the humanized monoclonal antibody bevacizumab, which, in combination with chemotherapy, has been approved by the US Food and Drug Administration (FDA) as a first-line therapy for the treatment of metastatic colorectal cancer[111]. In addition, VEGF has been implicated in promoting defective APC function in DCs from both tumour-bearing mice and cancer patients[112–114]. Treatment of tumour-bearing mice with an anti-VEGF antibody at a dose that did not block tumour growth directly but decreased serum VEGF levels by 90%, led to an increase in mature DC numbers, improved DC function and resulted in a pronounced decrease in tumour growth that was associated with an enhanced tumour-specific CTL response[115]. Inhibition by VEGF of DC maturation from progenitor cells seems to be mediated predominantly through FMS-related tyrosine kinase 1 (FLT1; also known as VEGFR1) signalling, rather than signalling through kinase-insert-domain receptor (KDR; also known as VEGFR2 or fetal liver kinase (FLK1) in the mouse). This is because VEGF was able to inhibit the development of mature DCs from *Kdr*-null embryonic stem cells but not from *Flt1*-null cells[116]. However, the necessity for the kinase activity of FLT1 in modulating DC maturation has come into question. SU5416, a small-molecule inhibitor of the FLT1 and KDR kinases, could only partially reverse the inhibition of DC maturation that is elicited by VEGF treatment *in vitro*[116]. This indicates that there are both kinase and non-kinase-dependent functions for the FLT1 receptor in blocking DC maturation. The development of small-molecule FLT1 inhibitors (TABLE 1) to overcome this effect of VEGF might therefore be problematic, as such compounds typically target the kinase activity of the receptor. Diminished induction of the nuclear factor κB (NFκB) signalling pathway seems to be a downstream effect of VEGF binding by FLT1, which is closely associated with inhibition of DC maturation[116,117] and might offer another point of intervention.

Chemokine receptors

Chemokines, a family of low-molecular-weight cysteine-containing cytokine-like proteins, are generally regarded as the key determinants of leukocyte migration[118]. The composition of the cellular infiltrate in solid tumours is clearly influenced by the specific chemokines that are produced by both the tumour and the stroma. From the standpoint of identifying targets to alleviate the immunosuppressive environment, the chemokine receptor CCR4 has emerged as one potential candidate. CCR4 binds specifically to the chemokine CCL22 (also known as macrophage-derived chemokine), and has been shown to be expressed on T_{reg} cells[119–121]. In epithelial ovarian carcinomas, it was reported that tumour cells and tumour-infiltrating macrophages produce CCL22, which attracts T_{reg} cells into the tumour and malignant ascites. Interestingly, in these studies there were significantly fewer T_{reg} cells in the TDLNs, and a significant trend towards accumulation in the tumour, especially in patients with late-stage cancer. This indicates that T_{reg} cells are actively recruited, and can suppress T-cell effector functions directly in the tumour in addition to suppressing T-cell priming in the TDLNs. These studies also revealed a significant correlation between the presence of T_{reg} cells in the tumour and decreased survival in ovarian cancer patients, independent of disease stage. Although these studies were conducted in individuals with ovarian cancer, the observation that PGE2 can induce CCL22 expression[122] indicates that trafficking of T_{reg} cells into tumours through CCR4 might be a phenomenon that can be generalized to several types of cancer. Small-molecule inhibitors of CCR4 are in development by several pharmaceutical companies for the treatment of asthma (TABLE 1). Based on the data discussed above, these compounds might also have a use in cancer.

The migration of MSCs and plasmacytoid DCs into tumours and TDLNs is also mediated by chemokine receptors. It has been reported that ovarian carcinomas produce high levels of CXCL12 (also known as SDF1) which induces the migration of plasmacytoid DC precursors (pre-DC2 cells) into the tumour through the chemokine receptor, CXCR4 (REF. 123). These pre-DC2 cells express low levels of co-stimulatory molecules and suppress T-cell responses through the secretion of IL10. Munn *et al.* also reported the expression of CCR6 on the subpopulation of plasmacytoid DCs that produces high levels of IDO[29]. In addition, tumour production of CCL2 (also known as MCP1) was associated with TAM accumulation and was a significant indicator of early relapse in breast cancer patients[124]. These studies indicate that agents that block these receptors might also be therapeutically useful. Finally, it should be noted that inhibitors of CCR2 and CCR5 show direct anti-tumour effects that are postulated to be mediated through anti-angiogenic mechanisms, and inhibitors of CXCR4 have been shown to be anti-metastatic. These additional functions of chemokines in cancer are covered in much greater detail in other recent reviews[118,125].

Development considerations

Given the complexities of the cellular and molecular mechanisms involved in tumoral immune tolerance (FIG. 1), it is fair to question how inhibiting any one specific target could be effective. The broad context in which these mechanisms are presented might be misleading, however, as individual tumours might rely only on discreet subsets of these mechanisms. Furthermore, as illustrated in FIG. 2, the targets being considered do not function in isolation. Rather, there is a reasonable amount of cross-talk, such that a specific inhibitor might have the potential to broadly affect the network. At the other extreme, there is the concern that abrogating tolerance too effectively will

Cytotoxic T lymphocyte-associated antigen-4
CTLA4 is a co-stimulatory-type molecule that antagonizes effector-T-cell responses.

produce severe autoimmune reactions beyond the limits that can be adequately managed. It will require careful preclinical and clinical evaluation to identify those agents that strike the right balance between outcomes that are neither insufficiently, nor overly, robust.

As shown for highly mutable infectious agents, such as HIV, successful targeting of tumour cells might require the application of many agents that target different mechanisms that are important to their survival. Compounds that target toleragenic effector mechanisms will probably be most effective in conjunction with other therapeutic modalities. A particularly interesting, and perhaps counterintuitive, combinatorial regimen that can elicit cooperative or even synergistic anti-tumour activity is the combination of immunotherapy with conventional cytotoxic chemotherapy[126]. It has generally been assumed in the cancer field that chemotherapy would probably contravene approaches that are aimed at increasing anti-tumour immunity given their cell-based nature. However, various agents with possible anti-toleragenic activity, including monoclonal antibodies against cytotoxic-T-lymphocyte-associated antigen-4 (CTLA4) and VEGF, and small-molecule inhibitors of COX2 and IDO, have been found to cooperate with cytotoxic chemotherapy in experimental models, and such combinations are now being tested in the clinic[15,127–129]. The ability to cooperate with standard chemotherapy is distinctly advantageous in that trial design should be more straightforward for a combination therapy that involves one approved agent as opposed to two untested agents. It might even be possible to incorporate such anti-toleragenic agents as adjuncts to standard therapeutic regimens, which could further facilitate their adoption into clinical practice.

The tumoricidal activity of cytotoxic agents is likely to be a central feature of the cooperative response, as the massive cell death that is induced by these agents can be pro-immunogenic[126] and probably contributes to immune activation through increased cross-presentation of tumour antigens[130]. The concept of combining discreet cytotoxic and immunomodulatory agents might, however, be overly simplistic for fully understanding the therapeutic benefit provided by these agents. For instance, cytotoxic chemotherapeutic agents might, in some cases, directly modulate the immune response[131]. The therapeutic role of the immunomodulatory agent in a combinatorial treatment regimen might be subject to more nuances as well. In particular, the targets of such agents might have important consequences for the viability of tumour cells apart from increasing immune escape, as genes that provide a survival advantage on several levels offer an efficient way for tumours to respond to different selective pressures. Clearly this is the case for targets such as VEGF, which has a crucial role in angiogenesis, and COX2, which has been implicated in angiogenesis and protection against apoptosis. Likewise, it has been suggested that ARG1 might promote tumour growth by feeding into the production of polyamines, and we have speculated that IDO could potentially provide a direct survival benefit to tumour cells in its capacity as the crucial first step in the NAD^+ biosynthetic pathway[132]. Given all these complexities, the therapeutic ramifications achieved with any particular combination should not be overgeneralized. However, early indications are that clinical outcomes could be affected for the better by incorporating agents targeting pathological immune tolerance into the armamentarium of anticancer treatment modalities.

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Competing interests statement
The authors declare no competing financial interests.

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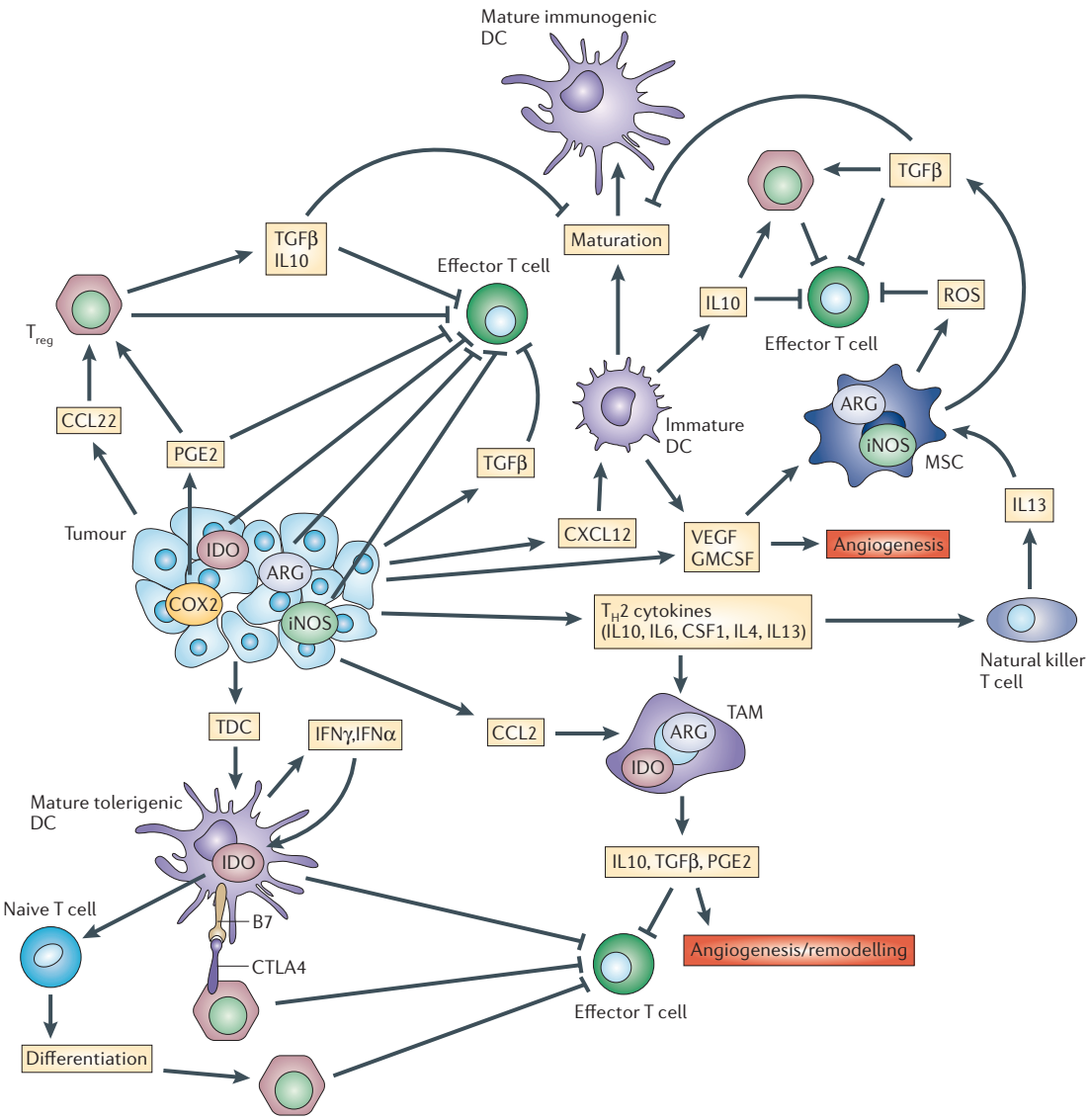
ERRATUM

Targeting the mechanisms of tumoral immune tolerance with small-molecule inhibitors

Alexander J. Muller and Peggy A. Scherle

Nature Rev. Cancer **6**, 613–625 (2006)

There is an error in Figure 1 of this article on page 616. The mature immunogenic dendritic cell at the top of the figure should not be expressing IDO. The correct version of the figure is shown below.



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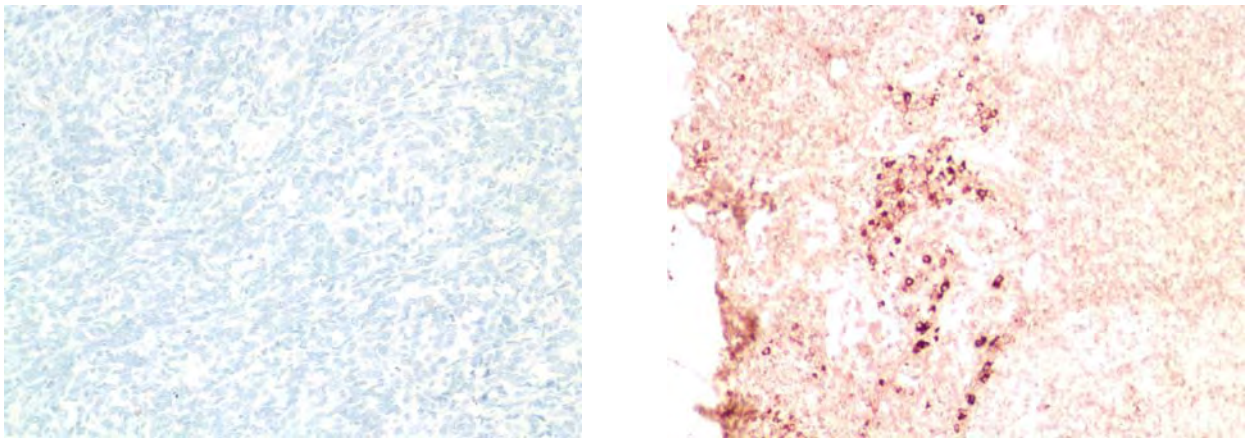

Figure 1. IDO expression is evident in the tumor draining lymph nodes but not in the primary tumors formed by 4T1 breast carcinoma orthotopic isografts.
Immunohistochemical staining with rabbit polyclonal antibody to mouse IDO. Left: Primary 4T1 tumor stained for IDO (red, x100). Right: Draining inguinal lymph node stained for IDO (red, x100).

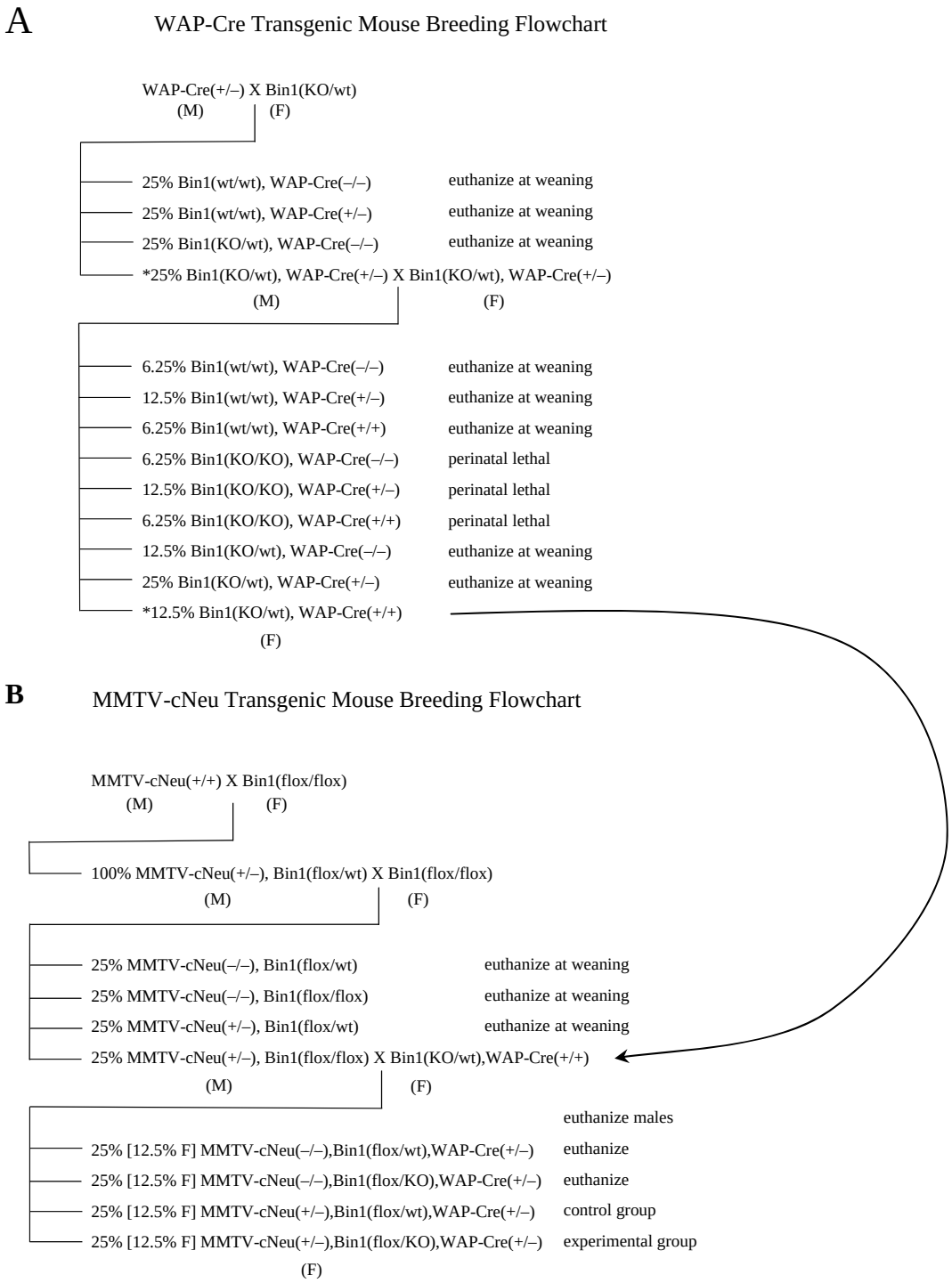

Fig. 2. Breeding strategy for producing FVB-strain mammary gland targeted Bin1-null mice. The alleles for the Cre recombinase transgene controlled by the whey acidic protein promoter [WAP-Cre], the constitutive Bin1 knockout [Bin1(KO)], and the conditional Bin1 knockout [Bin1(flox)] were all individually introduced onto the FVB strain background by performing 5 or more generations of backcrossing prior to initiating these crosses. The two flowcharts diagram the breeding steps that were followed to generate experimental and control groups mice with the desired genotypes. This required (A) two generations of breeding to obtain Bin1(KO/wt),WAP-Cre(+/+) mice that were used in breeding scheme B where indicated by the arrow and (B) three generations of breeding to obtain experimental MMTV-cNeu(+/-), Bin1(flox/KO),WAP-Cre(+/-) and control MMTV-cNeu(+/-),Bin1(flox/wt),WAP-Cre(+/-)mice.

Table 1. Bin1 loss drives progression of DMBA-induced mouse mammary carcinomas

Genotype	Regimen	No. mice w/ tumors (%)	Tumors per mouse	Latency (d)*	Grade† T,N,M, (sum)	Differentiation status	Lung metastasis (%)
Bin1+	Nonparous	0/6 (0)	0	NA	ND	NA	0/0 (0)
Bin1+mam	Parous	1/19 (5)	1	NA	ND	WD	0/1 (0)
Bin1Δmam	Parous	2/24 (8)	1	NA	ND	PD	0/2 (0)
Bin1+mam	DMBA	8/8 (100)	2.3 ± 1.3	128 ± 47	1.6,1.5,2.3 (5.4)	WD	4/8 (50)
Bin1Δmam	DMBA	14/14 (100)	2.2 ± 1.4	115 ± 36	2.3,2.5,2.9 (7.8)	PD	7/14 (50)

NOTE: Nonparous and parous mice not treated with DMBA were monitored for their full life span for breast tumor formation. In these groups, the small number of tumors that arose was all seen in elderly animals of >1 year of age. Uniparous animals treated with DMBA exhibited similar latencies for mammary tumor formation regardless of genotype that were not significantly longer than 99 days reported in CD2F1 mice (23). All DMBA-treated animals were carefully examined at necropsy for lung metastases, other neoplasms, and other pathologic lesions in major organs (see text), with any suspected lesions confirmed by histologic analysis.
Abbreviations: NA, not applicable; ND, not determined; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
*Number of days after last DMBA treatment.
†Grade according to the Nottingham modification of the Bloom-Richardson system (three variables): T (tubule formation), 1-3; N (nuclear pleomorphism—nuclear variation in size and shape), 1-3; M (mitotic count—mitoses), 1-3.

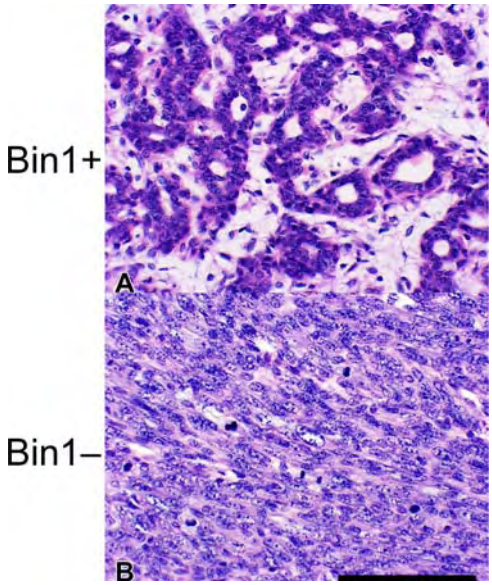

Figure 3. Bin1 loss is associated with poorly differentiated tumor histopathology.
H&E stained sections of DMBA-induced mammary gland tumors. Bin+ and Bin1– indicate Wap*Cre*(+/–), *Bin1*(flox/wt) and Wap*Cre*(+/–),*Bin1*(flox/KO) genotypes respectively.

Table 2. Enforced c-Myc expression supercedes the impact of Bin1 on breast tumor differentiation status

Genotype	Regimen	Breast tumor incidence	Grade[a] T	N	M	(sum)	Lung mets	Differentiation status[b]
cMyc;Bin1+	Parous	7/7 (100%)	1.4	2.7	2.9	(7)	5/7 (71%)	MD-PD
cMyc;Bin1Ęmam	Parous	7/7 (100%)	1.7	2.9	2.9	(7.4)	6/7 (86%)	MD-PD
cMyc;Bin1+	DMBA	**6/6 (100%)**	2.7	3	3	(8.7)	6/6 (100%)	**PD**
cMyc;Bin1Ęmam	DMBA	**3/7 (43%)**	3	3	3	(9)		

[a] Grade according to the Nottingham modification of the Bloom-Richardson system (3 parameters):
T (tubule formation): 1-3
N (nuclear pleomorphism - nuclear variation in size and shape): 1-3
M (mitotic count - mitoses): 1-3
[c] MD, moderately differentiated; PD, poorly differentiated

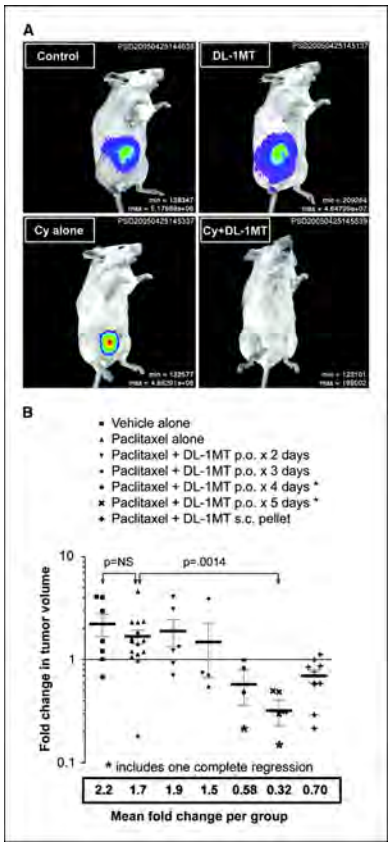

Figure 4. Oral DL-1MT in orthotopic 4T1 and autochthonous MMTV-*Neu* tumors.

A. Orthotopic tumor isografts were established in the mammary fatpad. Treatment was initiated concurrent with tumor challenge, using cyclophosphamide (Cy) i.p. at 100 mg/kg qd, 1×/week; and DL-1MT oral gavage at 400 mg/kg per dose, twice daily, 5×/week. Bioluminescence imaging of 4T1 tumor cell line transfected with luciferase, showing the impact of each treatment on tumor burden. Treatment received by each mouse is indicated on the figure. Images were produced at 4 weeks following the initiation of treatment.

B. MMTV-*Neu* mice bearing 0.5-1.0 cm spontaneous tumors were treated for 2 weeks with either vehicle alone, paclitaxel (PTX) alone (13.3 mg/kg i.v. q. M/W/F), or paclitaxel plus oral DL-1MT (400 mg/kg i.v. twice daily, administered for up to 5 days during the first week, as indicated in the legend). Paclitaxel was administered i.v. at over the two week treatment period. The last group received subcutaneous pellets of 1MT, as in Fig. 1. Fold changes in individual tumor volumes over the 2 week period are plotted for each group. Bars indicate the mean fold change for each group (also listed in the box below the graph) with SEM. Fully regressed tumors are noted by an asterisk in the legend and are included in the calculation of the mean and SEM. For the statistical analyses shown by the arrows, the two comparisons of interest were [vehicle alone vs. paclitaxel alone] and [paclitaxel alone vs. paclitaxel+D-1MT × 5 days]. Significance was determined at $p<0.025$ using a two-group Wilcoxon exact test.

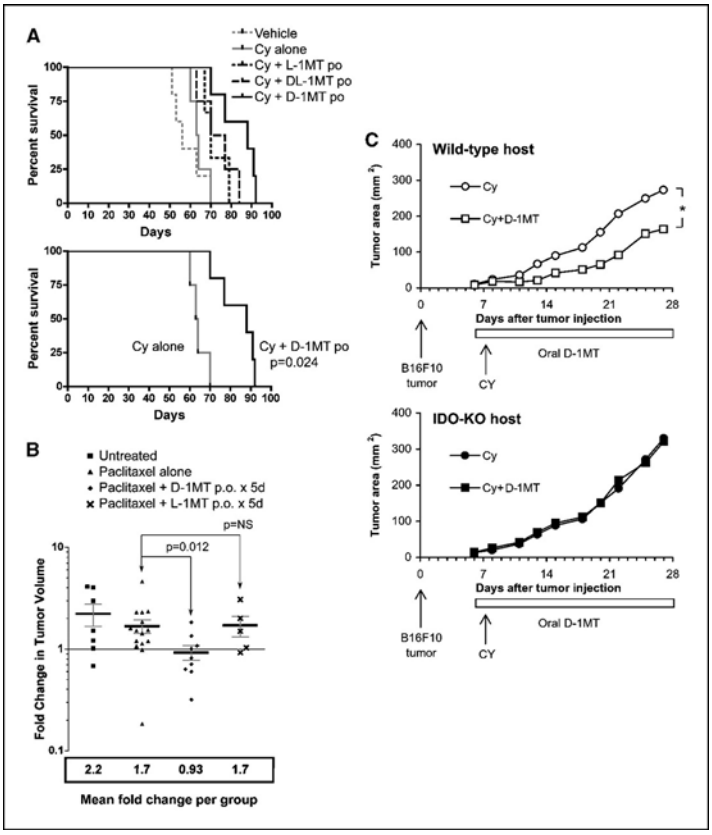

Figure 5. D-1MT provides greater survival benefit in combination therapy, in an IDO-dependent fashion.
A. 4T1-luc orthotopic isografts were established in the mammary fatpad. Cy was administered at 25 mg/kg orally qd 1×/week, and 1MT (D, L or DL) administered at 400 mg/kg by oral gavage twice daily 5×/week by gavage, beginning at the time of tumor implantation. The upper graph shows time to endpoint for all groups; the lower graph shows only the Cy vs Cy+D-1MT groups, for clarity. The comparisons of interest were between [D-1MT+CY vs CY] and [L-1MT+CY vs CY]. Since survival data were not censored, groups were analyzed using a two-group Wilcoxon exact test; statistical significance was determined at $p<0.025$. The combination of D-1MT+CY showed a significant survival benefit over CY alone ($p=0.024$), while L-1MT+CY was not different from CY alone ($p=0.14$).
B. MMTV-*Neu* mice with tumors were treated for 2 weeks, receiving either vehicle alone, paclitaxel alone or paclitaxel (13.3 mg/kg q. MWF) plus oral D-1MT or L-1MT for 5 days, as indicated. For statistical analysis, the comparisons of interest were [D-1MT+paclitaxel vs paclitaxel alone] and [L-1MT+paclitaxel vs paclitaxel alone]. Significance was determined at $p<0.025$ using a two-group Wilcoxon exact test. The fold change of the D-1MT+paclitaxel group was significantly smaller than that of paclitaxel alone ($p=0.012$), whereas paclitaxel+L-1MT was not different from paclitaxel alone ($p=0.85$).
C. The effects of the D isomer of 1MT require an intact host IDO gene. B16F10 tumors were grown in either wild-type B6 hosts or IDO-KO hosts on the B6 background, as shown. All groups received Cy, with or without oral D-1MT (2 mg/ml in drinking water). Analysis by ANOVA showed that Cy+D-1MT was significantly different (* $p<0.05$) than Cy alone for the wild-type hosts, but there was no effect of D-1MT when tumors were grown in IDO-KO hosts.

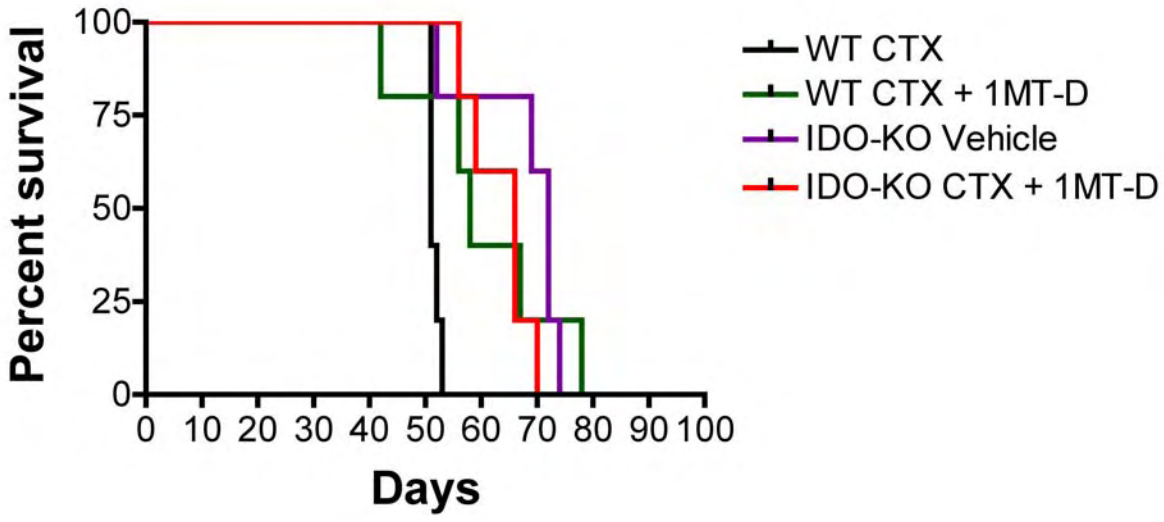

Figure 6. Loss of IDO in the stroma prolongs the survival of mice bearing metastatic 4T1 breast tumor isografts.

4T1-luc orthotopic isografts were established by injection of $1x10^4$ cells into the mammary fatpad of wild type and IDO knockout BALB/c strain mice. Cy was administered at 25 mg/kg p.o. qd 1×/week, and D-1MT administered at 400 mg/kg p.o. bid 5×/week by gavage, beginning at the time of tumor implantation. Since survival data were not censored, groups were analyzed by a two-group logrank test (equivalent to the Mantel-Haenszel test) using GraphPad Prism4 statistical analysis software; statistical significance was determined at $p < 0.05$.

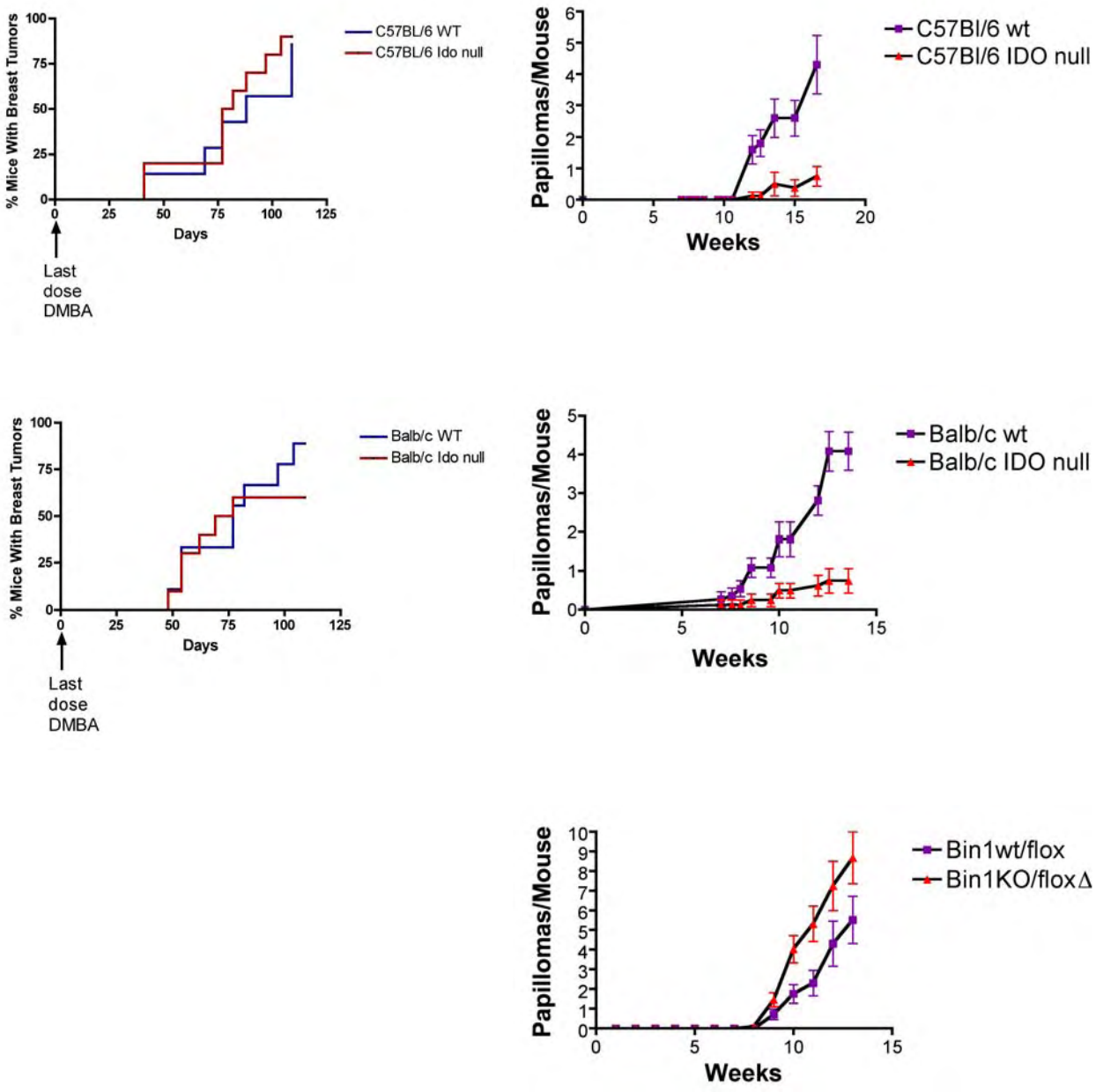

Figure 7. In carcinogenesis models, development of breast tumors is not affected by the absence of IDO while development of skin tumors is substantially suppressed.
Left side: Comparison of the breast tumor appearance in IDO knockout versus wild type mice in the C57BL/6 (top) and BALB/c (bottom) strain backgrounds. Uniparous mice received two s.c. 20 mg MPA time release pellets and 4 doses of DMBA at 50 mg/kg p.o. to preferentially induce mammary gland tumors.
Right side: Comparison of skin papilloma appearance in IDO knockout versus wild type mice in the C57BL/6 (top) and BALB/c (middle) strain backgrounds. (Bottom) Comparison of skin papilloma appearance in Bin1 mosaic knockout versus wild type mice (mixed strain background). In all cases a single s.c. administration of 400 nmol DMBA was followed by twice weekly s.c. administration of 10 µg TPA for the remainder of each study.

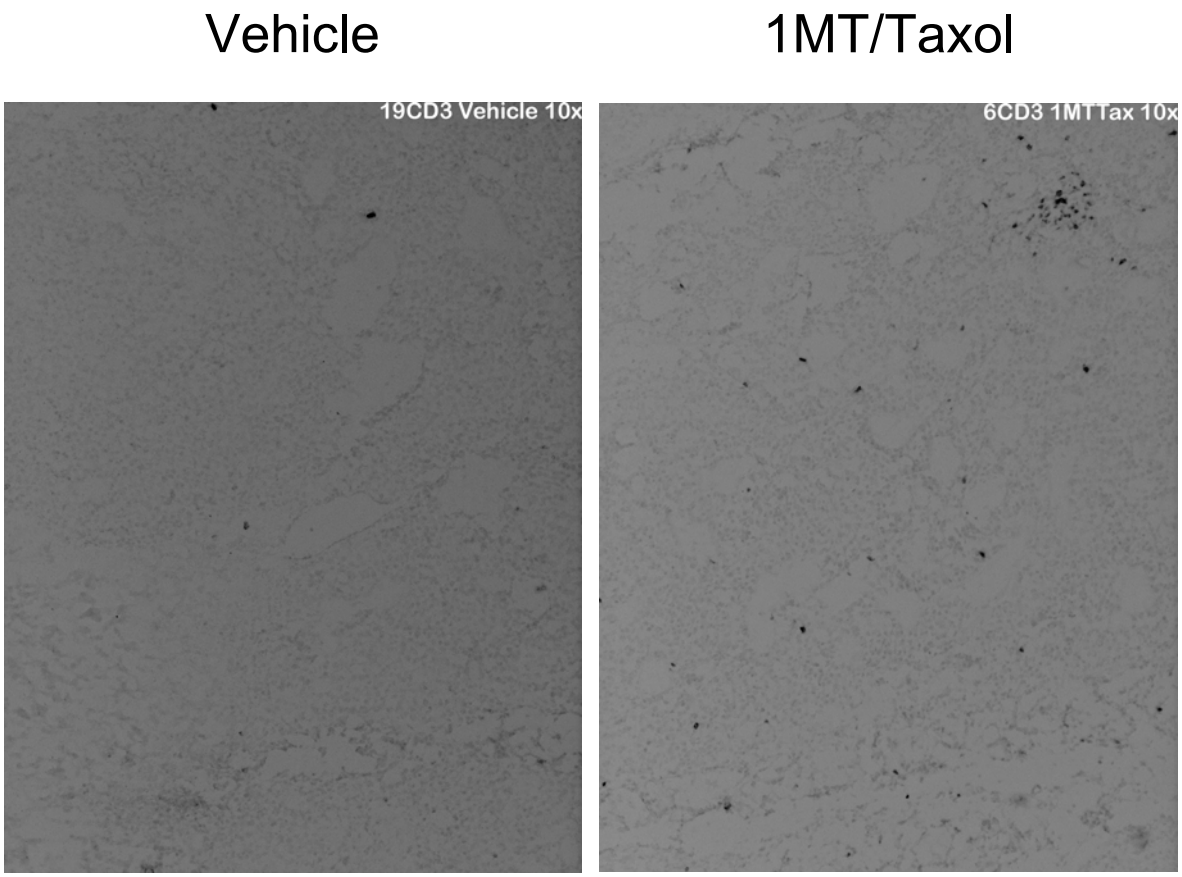

Figure 8. MMTV-*Neu* mice treated with paclitaxel and 1MT have elevated numbers of intratumoral T cells.
Immunohistochemical staining of paraffin fixed sections anti-CD3 monoclonal antibody. Left: Mammary gland tumor stained for CD3 (black, x100) from vehicle treated animal. Right: Mammary gland tumor stained for CD3 (black, x100) from an animal treated for 2 weeks with paclitaxel (13.3 mg/kg i.v. q. M/W/F) and timed release s.c. pellets of DL-1-methyl-tryptophan (20 mg/d).