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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 discovered that Bin1 loss can promote tumorigenesis through an immune escape mechanism and that this correlated with the negative regulatory impact that Bin1 can exert on the important immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO). We had also demonstrated that, 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. As a result of our work on this project, we have obtained direct evidence that in the MMTV-Neu model IDO activity in plasmacytoid dendritic cells from the tumor draining lymph nodes may be more relevant than in the tumor cells themselves, a finding that appears to be of general relevance to breast cancer. Furthermore, we have found that 1-methyl-D-tryptophan (D-1MT), the presumptive IDO inhibitor which is in early phase clinical trials, may instead be directly targeting IDO2, an IDO-related enzyme that we recently discovered. Our data argue that genetic evaluation of patients for known IDO2 polymorphisms may be critically important to interpreting trial outcomes with D-1MT.

15. SUBJECT TERMS
Bin1, IDO, indoleamine 2,3-dioxygenase, tryptophan, 1-methyl-tryptophan, immune escape, tolerance, transgenic mice

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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]. In this context, we have identified the immunomodulatory gene *IDO* as a negatively-regulated downstream target of Bin1. *IDO* encodes indoleamine 2,3-dioxygenase, 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 was the first to connect the *IDO* gene to a known cancer suppression pathway, and dovetails 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 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 [3] (see Appendices). Based on this knowledge, our proposed studies were aimed at addressing the links between *Bin1* loss, *IDO* dysregulation, and host immunity in mouse breast cancer models.

BODY

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

In year 1 of the grant period, 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 (**Fig. 1**) [4]. Technical difficulties had initially precluded similar evaluation of IDO levels in these tumors as proposed in the grant. We overcome this roadblock through the establishment of 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 [5] and they were willing to evaluate tissues from our breast tumor-bearing mice. In an initial experiment, reported in year 2 on the evaluation of IDO in tumors formed by 4T1 breast carcinoma isografts implanted ectopically into the mammary fatpad, no evidence of IDO expression was observed in the actual tumor but rather was observed in tumor draining lymph nodes (TDLNs). In year 3, we successfully stained specimens from tumor-bearing MMTV-*Neu* mice and a similar staining pattern has emerged (**Fig. 2**). Again, although there was no evidence of IDO expression in the tumor, IDO expression was detected in TDLNs in what appear morphologically to be plasmacytoid dendritic cells (pDCs). This is the same sort of staining pattern that the Munn group previously reported in a melanoma isograft model [6], which has led to the hypothesis that, in the case of some tumors, immune escape can be mediated by IDO expression in the TDLNS. Data from human breast cancer patients, which Dr, Munn's group has collected, is consistent with this being the more common mechanism of immune escape for breast cancer and we are currently writing up these findings for publication.

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

In year 1, we reported that, although *Bin1* gene expression 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 (**Fig. 3**). We also reported 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 (**Fig. 4; Table 1**). These data were published as part of an article on the role of Bin1 loss in breast cancer progression published in the journal *Cancer Research* [4] (see Appendices). To specifically address how *Bin1* loss affects tumorigenesis driven by lactation-dependent expression of the c-*Neu* proto-oncogene in the mammary gland as described in the grant proposal, we crossed 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 had 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 targeted 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 (**Fig. 5**). In order to circumvent this problem, we 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. 6**) to produce mice with all of the requisite genetic elements in place. We have since generated the necessary cohorts of FVB-strain MMTV-*Neu*$^{+/-}$Wap*Cre*$^{+/-}$ *Bin1*$^{flox/KO}$ experimental mice and MMTV-*Neu*$^{+/-}$Wap*Cre*$^{+/-}$*Bin1*$^{flox/wt}$ control mice to perform the study. Preliminary outcome analysis indicates that mammary gland targeted Bin1 loss does not demonstrably affect either tumor multiplicity or latency in this model. So far these data are consistent with outcomes from the carcinogenesis studies and we are currently waiting for histopathological analysis of these samples to determine if Bin1 loss promotes malignant progression in a similar manner as well. Because Bin1 loss in this model is targeted to the mammary gland epithelium and IDO appears to be expressed in dendritic in the tumor draining lymph nodes, we no longer expect there to be a direct connection between Bin1 loss and a contribution of IDO to tumor development in the breast cancer context.

Task 3. Investigate the chemopreventative activity of IDO inhibitor treatment in relation to *Bin1* status
AND
Task 4. Profile tumor-associated immune cell populations and functionally characterize the involvement of specific T cell populations.

The rationale for these final two Tasks was predicated on the prediction that loss of Bin1 in developing tumors of MMTV-*Neu* mice would promote tumor development through dysregulated elevation of IDO directly in the breast cancer cells. Data obtained during the course of this project has now led us to call this particular model into question. Since beginning work on this project, we have become increasingly convinced that the relative importance of tumor-expressed IDO may be contextual and that breast cancer may instead be a tumor type in which IDO activity in the normal

stroma, particularly in antigen presenting dendritic cells (DCs) in the tumor draining lymph nodes (TDLNs), is most relevant to tumor outgrowth. This idea is based on our findings in the MMTV-*Neu* model that IDO inhibition cooperates with chemotherapy to produce regression of primary tumors [2] even though no evidence of IDO expression in the primary tumor is apparent. As described below, this new way of thinking about IDO in breast cancer has been further corroborated in studies using the highly metastatic 4T1 breast carcinoma cell line [7], which 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 [8].

Interestingly, we have discovered that the particular compartment in which IDO activity is relevant, be it tumor or stroma, may have significant bearing on the development of potential therapeutic agents targeting IDO. Specifically, the two different stereoisomers of the IDO inhibitor 1MT appear to behave quite differently when targeting tumoral versus stromal IDO activity. The D isomer of 1MT (D-1MT) is currently being developed by NewLink Genetics Corp. in conjunction with the National Cancer Institute (NCI). Based in part on preclinical data generated through this funding mechanism, D-1MT has received Investigational New Drug (IND) approval from the Food and Drug Administration (FDA) and has recently entered into early phase clinical testing. Therefore it is particularly critical to be cognizant of how this specific inhibitor may be predicted to behave in a particular therapeutic context. We have pursued this question as part of the collaboration established with Drs. David Munn and Andrew Mellor, who initiated the preclinical testing of D-1MT through the NCI RAID program.

Initially we became interested in the discrepancy between *in vitro* and *in vivo* findings with the two different isomers of 1MT (D and L), where L is in the same conformation as the naturally occurring form of the amino acid tryptophan. Based on published reports, we had expected that the L isomer of 1MT would be a more potent IDO inhibitor than the D isomer and thus would show greater cooperativity against tumors. In order to directly examine this issue biochemically, we tested the ability of the different 1MT isomers to inhibit IDO activity in a cell-free, purified enzyme assay as well as in cancer cells induced to express IDO. As has been previously reported, the L isomer had a substantially lower Ki for inhibiting activity of the purified IDO enzyme than did the D isomer **(Fig. 7**). Likewise, when IDO was induced in the HeLa human cervical cancer cell line by interferon-γ treatment, EC50 determinations again revealed L-1MT to be a more potent inhibitor than D-1MT (**Fig. 8**). However, Dr. David Munn's laboratory has found that, in the case of toleragenic dendritic cells (DCs), D-1MT is at least as good an inhibitor of cellular IDO activity as L-1MT [9]. Furthermore, when tested for their ability to relieve IDO-mediated suppression of T cell activation in a mixed lymphocyte response (MLR) assay, D-1MT was found to be superior to L-1MT as well as to the racemic form DL-1MT [9]. It has been proposed that, for at least some types of cancer, IDO activity associated with toleragenic DCs in the tumor draining lymph nodes may be particularly relevant to immune escape by the tumor. Data from Dr. Munn's laboratory demonstrating D-1MT efficacy in targeting IDO-dependent, DC-mediated immune tolerance and our own data showing the superiority of D-1MT in cooperating with chemotherapeutic agents in two mouse models of breast cancer, the MMTV-Neu transgenic model and the 4T1 mammary carcinoma isograft model (**Fig. 9A,B**), are consistent with this idea of IDO-expressing, toleragenic DCs being important to tumoral immune escape in the context of breast cancers. A manuscript incorporating these findings has now been published in the journal *Cancer Research* [9] (see Appendices).

Our recent work has gone on to uncover a possible explanation for the conundrum surrounding the 1MT stereoisomers in the discovery of a second IDO related enzyme that is specifically inhibited by D-1MT. BLAST searches of the publicly available human genome database for *INDO*-related sequences, led us to come across a second predicted gene directly adjacent to *INDO* at 8p12. Identified by the locus designator *LOC169355*, (which has since been changed to *INDOL1* (*INDO-like-1*)), the predicted gene sequence corresponded to only a fragment of the *INDO* gene. This, however, turned out to be a misannotation. Searching the human genomic sequence identified a complete set of putative exons encoding a full length gene, termed here *IDO2*, and a complete set of exons could be found in the syntenic region of the mouse genome as well. By RT-PCR, we have confirmed expression of the predicted full length human *IDO2* transcript as well as at least four truncated splice variants [10]. The full-length *IDO2* transcript is comprised of 11 exons (**Fig. 10**). An additional exon 1a in humans, encoding 8 N-terminal amino acids, has not yet been found in the mouse. The human and mouse IDO2 proteins are more highly conserved (72% identical) than their IDO counterparts (62% identical). Although the IDO and IDO2 proteins do not share a high degree of homology (43% identical), amino acids determined by crystallographic analysis and mutagenesis studies to be critical for IDO to catabolize tryptophan are highly conserved in IDO2 suggesting that it may be catalytically active as well. Indeed, the ability of IDO2 to catabolize tryptophan was confirmed using recombinant V5 epitope-tagged IDO2 ectopically expressed in a human embryonic kidney cell line (**Fig. 11**). Of particular interest, however, was the finding that in contrast to IDO, IDO2 was preferentially inhibited by the D isomer of 1MT. The differential was quite striking, with no evidence of inhibition by the L isomer at 50 μM at which concentration the maximal inhibition of kynurenine production by the D isomer had been achieved (**Fig. 11**). A manuscript incorporating these findings has now been published in the journal *Cancer Research* [10] (see Appendices).

Two single nucleotide polymorphisms (SNPs) producing non-synonymous codon changes within the coding sequence for the *IDO2* gene, which are predicted to severely impact enzymatic function, have been identified through evaluation of the public human NCBI SNP database (**Fig. 12**). One, a T to A transition in exon 10, changes a tyrosine at position 359 to a stop codon. This results in premature termination of the protein immediately prior to a conserved histidine residue that in IDO is essential for catalytic activity [11]. The other, a C to T transversion in exon 8, changes an arginine at position 248 to a tyrosine. This residue is located at a position equivalent to R231 in IDO, which has been demonstrated by site directed mutagenesis to be critical for catalytic activity and, from the crystal structure, is postulated to be involved in substrate recognition through hydrophobic interactions [12]. This residue is predicted to reside near the entrance to the active site and the presence of the bulky tryptophan side chain may hinder substrate access as well (J. Lalonde, personal communication). Both polymorphisms have been confirmed by site directed mutagenesis to reduce the activity of ectopically expressed IDO2 to undetectable levels. In both cases, the protein product was found to be destabilized in the cells (unpublished results), and so the actual impact of these polymorphisms on enzymatic activity as opposed to expression still remains to be formally evaluated. Remarkably, both of these inactivating polymorphisms are highly represented in the general population. Data from 339 individuals in the public database suggests that there may be some ethnic variation in the frequency of occurrence of these polymorphisms with the R248W most prevalent in individuals of European descent, the Y359Stop most prevalent in individuals of Asian descent, and a lower frequency both inactivating alleles in individuals of African descent. This evaluation is based on relatively small groups and the numbers should be expanded to confirm any trends, but still, the overall frequency at which both *IDO2* alleles are potentially inactivated appears to be remarkably high, ranging from up to 25% of individuals of African descent to possibly as high

as 50% of individuals of either European or Asian descent (**Fig. 12**). This raises questions regarding how important the functional role of IDO2 actually is and whether there might be counterbalancing selective pressures on its expression due to both advantages and disadvantages that it might provide the host. IDO, for instance, has been implicated as being both protective against inflammatory pathology associated with infection as well as promoting tumoral immune escape. Along these lines, an interesting question to explore will be how these *IDO2* polymorphisms track with susceptibility and outcomes for different types of cancers. NewLink Genetics Corp. has initiated Phase-I clinical trials of the presumptive IDO inhibitor, 1-methyl-D-tryptophan (D-1MT), with breast cancer as a lead indication. Our recently published finding of the previously unrecognized IDO2 gene product being the preferential target for D-1MT, rather than IDO, has clear ramifications for genetic screening of individuals enrolled in such a trial due to loss-of-function polymorphisms in the *IDO2* gene that are present in the general population [10]. Therefore, future studies of the role of IDO2 in breast cancer development will have immediate bearing on how data from current clinical trials of D-1MT are interpreted as well as on how new inhibitors are designed (specifically targeting IDO2, IDO or both enzymes) in order to achieve maximum therapeutic benefit.

4T1 is an aggressively metastatic breast cancer model. Mortality in this model results from the development of disseminated metastases, particularly pulmonary metastases. The increased survival achieved with combination therapy data in the 4T1 model (**Fig. 13A**) suggested to us the possibility that IDO might be important to the establishment of metastases in this model. Because no detectable IDO expression was observed in 4T1 tumors, it also seemed likely that the relevant compartment for IDO expression was in the stroma. 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. David Munn and Andrew Mellor. Acquiring their IDO knockout mouse strain has allowed us to perform experiments aimed at dissecting the role of IDO in tumor development more directly than would have been possible with just the use of small molecule IDO inhibitors as we had originally proposed. Based on our IDO inhibitor treatment data published in *Cancer Research* [9], 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, showed significantly improved survival over wild type mice that was comparable to what was achieved with the combination of 1MT + cyclophosphamide in the wild type mice even though primary tumor outgrowth was unaffected (**Fig. 13A,B**). A dramatic reduction in lung nodules, (indicative of pulmonary metastases), was observed in lungs from IDO knockout mice as compared to lungs from wild type mice (**Fig. 13C**). The impact of IDO loss on metastasis was quantitatively assessed using a colony formation assay to compare tumor burden in the lungs. At 5 weeks post-challenge wild type mice had, on average, a 10-fold higher tumor burden in their lungs than did IDO knockout mice (**Fig. 13D**). This was not due to an intrinsic difference in the ability of metastatic 4T1 cells to escape from the site of the primary tumor in the context of the IDO knockout host as the number of 4T1 cells found in the bloodstream was equivalent between the wild type and IDO knockout mice. Metastatic disease is the primary cause of mortality in cancer patients and these are the first data to demonstrate that IDO may be an important therapeutic target to interfere with this critical aspect of breast cancer development.

KEY RESEARCH ACCOMPLISHMENTS

- 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*.

- Collaborated 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 immunohistochemical staining data consistent with IDO expression being predominantly associated with plasmacytoid dendritic cells in the tumor draining lymph node rather than in the tumor itself in this model.

- 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. Outcomes data from this experiment, consistent with previously reported carcinogenesis results, have been collected and we are currently awaiting histopathological analysis.

- Demonstrated that the D isoform of the IDO inhibitor 1-methyl-tryptophan, which selectively targets stromal rather than tumoral IDO activity, effectively combines with chemotherapy in two different mouse breast cancer models. These data were published in *Cancer Research*.

- Discovered a new IDO-related gene product, IDO2, that is specifically inhibited by the D isomer of 1MT, providing a possible explanation for the biological activity attributable to this compound. These data were also published in *Cancer Research*.

- Utilized an IDO knockout mouse, provided through our ongoing collaboration with Drs. David Munn and Andrew Mellor, to demonstrate that the absence of stromal IDO is sufficient to effectively delay the development of pulmonary metastases in an orthotopic breast carcinoma isograft model.

REPORTABLE OUTCOMES

Manuscripts

Muller, A.J., W.P. Malachowski, and G.C. Prendergast. IDO in cancer: Targeting pathological immune tolerance with small molecule inhibitors. *Expert Opin. Ther. Targets* **9**:831-849 (2005).

Muller, A.J. and G.C. Prendergast. Marrying immunotherapy with chemotherapy: Why say IDO? *Cancer Research* **65**:8065 (2005).

Malachowski, W.P., R. Metz, G.C. Prendergast, and **A.J. Muller**. A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition and therapeutic application. *Drugs of the Future* **30**:897 (2005).

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).

Metz, R., J.B. DuHadaway, U. Kamasani, L.L. Kleintop, **A.J. Muller**, and G.C. Prendergast. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor IDO inhibitory compound D-1MT. *Cancer Res.* **67**:7082-7087 (2007).

Chang, M.Y., J. Boulden, J.B. Katz, L. Wang, T.J. Meyer, A.P. Soler, **A.J. Muller**, and G.C. Prendergast. Bin1 ablation increases susceptibility to cancer during aging, particularly lung cancer. *Cancer Res*. **67**:7605-7612 (2007).

Muller, A.J., R. Metz, and G.C. Prendergast. Differential targeting of tryptophan catabolism in tumors and in tumor-draining lymph nodes by stereoisomers of the IDO inhibitor 1-methyl-tryptophan. *International Congress Series* 1304C pp. 250-261 (2007).

Kumar, S., W.P. Malachowski, J.B. DuHadaway, J.M. LaLonde, P.J. Carroll, D. Jaller, R. Metz, G.C. Prendergast, and **A. J. Muller**. Indoleamine 2,3-dioxygenase is the anticancer target for a novel series of potent naphthoquinone-based inhibitors. *J. Med. Chem.* **51**: 1706-1718 (2008).

Katz, J.B., **A.J. Muller** and G.C. Prendergast. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. *Immunol. Rev.* **222**:206-21 (2008).

Ramalingam, A., J.B. Duhadaway, E. Sutanto-Ward, Y. Wang, J. Dinchuk, M. Huang, P.S. Donover, J. Boulden, L.M. McNally, A.P. Soler, **A.J. Muller**, M.K. Duncan, and G.C. Prendergast. Bin3 deletion causes cataracts and increased susceptibility to lymphoma during aging. *Cancer Res.* **68**:1683-1690 (2008).

Banerjee, T., J.B. DuHadaway, P. Gaspari, E. Sutanto-Ward, D.H. Munn, A.L. Mellor, W.P. Malachowski, G.C. Prendergast and **A.J. Muller**. A key *in vivo* antitumor mechanism of action of natural product-based brassinins is inhibition of indoleamine 2,3-dioxygenase. *Oncogene* **27**:2851-7 (2008).

Kumar, S., D. Jaller, P. Bhumika, J.M. LaLonde, J.B. DuHadaway, W.P. Malachowski, G.C. Prendergast, and **A. J. Muller**. Structure based development of phenyl-imidazole-derived inhibitors of indoleamine 2,3-dioxygenase. *J. Med. Chem.* (*in press*).

Muller, A.J., M.D. Sharma, P.R. Chandler, J.B. DuHadaway, M.E. Everhart, B.A. Johnson III, D.J. Kahler, J. Pihkala, A.P. Soler, D.H. Munn, G.C. Prendergast, A.L. Mellor. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. *Proc. Natl. Acad. Sci.* (*submitted*).

Abstracts/Presentations

American Association for Cancer Research 96th Annual Meeting. Anaheim, CA. April 16-20, 2005.
Abstract presented: "Transcriptional control of *IDO* by the cancer suppression gene *Bin1*, a key mechanism for restraining tumor immune escape."
(Minisymposium talk selected from Abstract)

NCI Conference on Translational Immunology Related to Cancer. Bethesda, MD. September 22-23, 2005
Abstract presented: "Pharmacological inhibition of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) cooperatively leverages cytotoxic chemotherapy"
(Poster)

International Society for Biological Therapy of Cancer (iSBTc) 20th Annual Meeting. Alexandria, VA. November 10-13, 2005.
Abstract Presented: "Pharmacological inhibition of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) cooperatively leverages cytotoxic chemotherapy."
(Plenary session talk selected from Abstract)
AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics. Philadelphia, PA. November 14-18, 2005
Abstract Presented: "Development of Brassinin Derivatives as IDO Inhibitors for Combinatorial Cancer Treatment"
(Poster)

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)

Centro Nacional de Investgaciones Oncologicas/Nature. Madrid, Spain. October 3-6, 2007.
Abstract presented: "IDO inhibition: an emerging therapeutic strategy targeting immune escape by tumors"
(Poster)

American Association for Cancer Research 98th Annual Meeting. Los Angeles, CA. April 14-18, 2006.
Abstract presented: "Brassinin compounds exhibit anti-cancer activity mediated through inhibition of the immunotolerogenic enzyme Indoleamine 2,3-dioxygenase"
(Poster)

NewLink Genetics Corporation, Ames, IA. January 10, 2007
"Evaluation And Development Of IDO Inhibitors To Defeat Tumoral Immune Tolerance"
(Invited Speaker)

29th Annual Induction Ceremony of Sigma Xi
Saint Joseph's University, Philadelphia, Pa. April 25, 2007
"Turning the Immune System Against Cancer: New Developments on an Old Idea"
(Keynote Speaker)

Farmingdale State College Campus-Wide Bioscience Seminar Series
Farmingdale State College, Farmingdale, NY. September 17, 2007
"Development of Small Molecule Inhibitors to Defeat Tumoral Immune Tolerance by Targeting IDO"
(Invited Speaker)

Nature-CNIO Conference on Oncogenes and Human Cancer: The Next 25 Years
Madrid, Spain October 3-6, 2007
Abstract presented: "IDO inhibition: an emerging therapeutic strategy targeting immune escape by tumors"
(Poster)

International Symposium: Immunoregulation and translational research: Immunology at the crossroads
Centro de Investigacion Medica Aplicada (CIMA), Universidad de Navarra
Pamplona, Spain April 8-9, 2008
Abstract presented: "Targeting tumoral immune tolerance with IDO inhibitors"
(Invited Speaker)

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 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 cell with elevated IDO in the tumor draining lymph node appears to be associated with the outgrowth of autochthonous MMTV-*Neu* breast carcinomas as well as orthotopic breast carcinoma isografts as we had shown previously.

The observation that IDO-activity is differentially targeted by the two isoforms of the IDO inhibitor 1-methlyl-tryptophan (1MT), so that the L form is more effective against IDO-activity expressed in tumors while the D form is more effective against IDO-activity 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 may be answered by our discovery of a novel IDO related enzyme, IDO2, that is specifically inhibited by D-1MT. D-1MT has just entered into early phase clinical trials, so 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 the activity of stromal expressed IDO2 where D-1MT is active rather than tumor expressed IDO where it is not . Furthermore, our data suggest that targeting IDO/IDO2 may be an effective approach to impair breast cancer metastasis. Our studies are the first to demonstrate that IDO/IDO2 inhibition can be effective against tumor metastasis, and clinically this may be even more relevant than the data that have been generated regarding the activity of IDO/IDO2 inhibitory compounds against primary tumors.

REFERENCES

[1] K. Ge, J. Duhadaway, D. Sakamuro, R. Wechsler-Reya, C. Reynolds, G.C. Prendergast. Losses of the tumor suppressor BIN1 in breast carcinoma are frequent and reflect deficits in programmed cell death capacity. Int J Cancer. 2000;85(3):376-83.
[2] A.J. Muller, J.B. Duhadaway, P.S. Donover, E. Sutanto-Ward, G.C. Prendergast. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med. 2005;11(3):312-9.
[3] A.J. Muller, G.C. Prendergast. Marrying immunotherapy with chemotherapy: why say IDO? Cancer Res. 2005;65(18):8065-8.
[4] M.Y. Chang, J. Boulden, E. Sutanto-Ward, J.B. Duhadaway, A.P. Soler, A.J. Muller, et al. Bin1 ablation in mammary gland delays tissue remodeling and drives cancer progression. Cancer Res. 2007;67(1):100-7.
[5] M. Friberg, R. Jennings, M. Alsarraj, S. Dessureault, A. Cantor, M. Extermann, et al. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer. 2002;101(2):151-5.

[6] D.H. Munn, M.D. Sharma, D. Hou, B. Baban, J.R. Lee, S.J. Antonia, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest. 2004;114(2):280-90.
[7] C.J. Aslakson, F.R. Miller. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. 1992;52(6):1399-405.
[8] B.A. Pulaski, S. Ostrand-Rosenberg. Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines. Cancer Res. 1998;58(7):1486-93.
[9] D.Y. Hou, A.J. Muller, M.D. Sharma, J. DuHadaway, T. Banerjee, M. Johnson, et al. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res. 2007;67(2):792-801.
[10] R. Metz, J.B. Duhadaway, U. Kamasani, L. Laury-Kleintop, A.J. Muller, G.C. Prendergast. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. Cancer Res. 2007;67(15):7082-7.
[11] T.K. Littlejohn, O. Takikawa, R.J. Truscott, M.J. Walker. Asp^{274} and His^{346} are essential for heme binding and catalytic function of human indoleamine 2,3-dioxygenase. J Biol Chem. 2003;278(32):29525-31.
[12] H. Sugimoto, S.I. Oda, T. Otsuki, T. Hino, T. Yoshida, Y. Shiro. Crystal structure of human indoleamine 2,3-dioxygenase: Catalytic mechanism of O2 incorporation by a heme-containing dioxygenase. Proc Natl Acad Sci USA. 2006;103(8):2611-6.

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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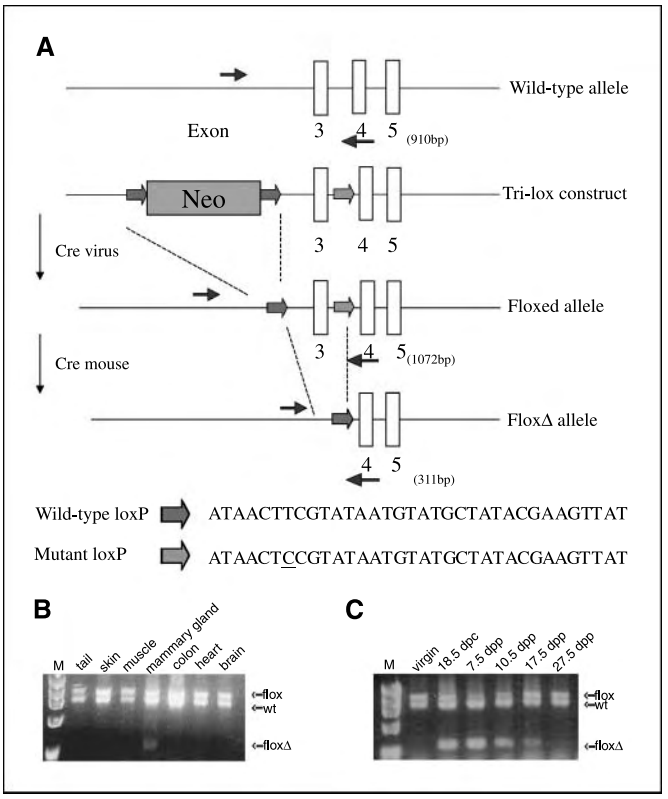

Figure 1. Tissue-specific deletion of *Bin1* in murine mammary gland. *A,* Bin1 flox targeting construct. *White boxes,* exons. *Colored arrows,* the WT (*solid*) and variant mutant (*hatched*) loxP sites; *thin arrows,* location of PCR primers with the size of the predicted amplification products given 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.

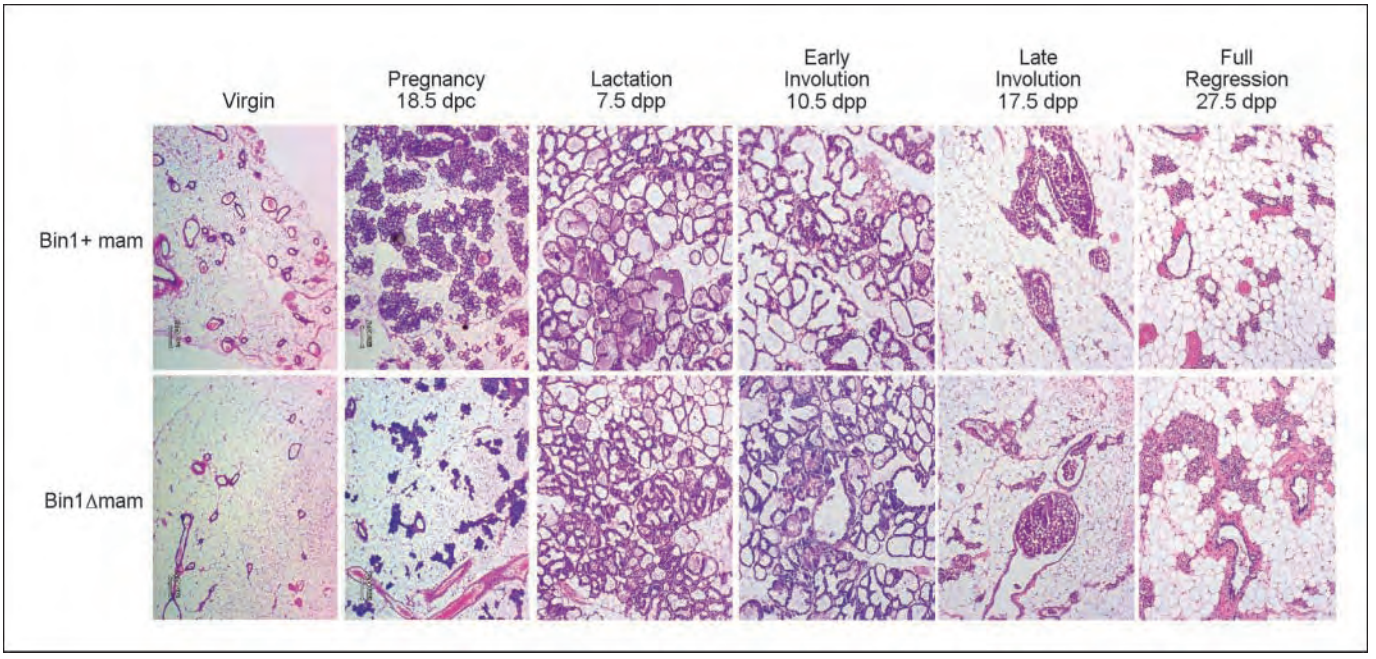

Figure 2. *Bin1* ablation delays mammary ductolobular remodeling during pregnancy. At the times indicated, mammary gland tissue sections were prepared and processed from Bin1+mam and Bin1Δmam mice for histologic analysis.

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.

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.

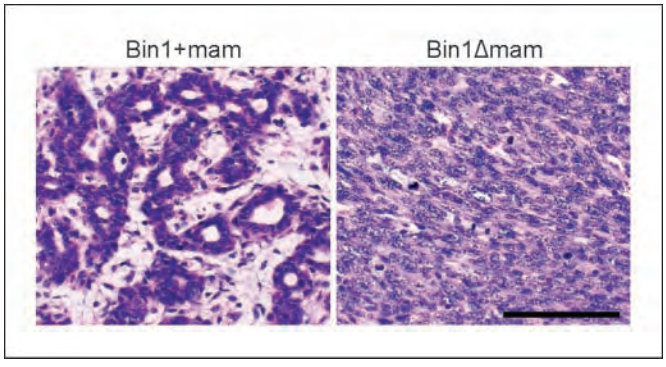

Figure 3. Increased histopathologic grade of DMBA-induced mammary tumors lacking Bin1. DMBA-induced mammary gland tumors excised at necropsy from Bin1+mam and Bin1Δmam mice were fixed in paraffin, and tissue sections were processed for H&E staining.

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

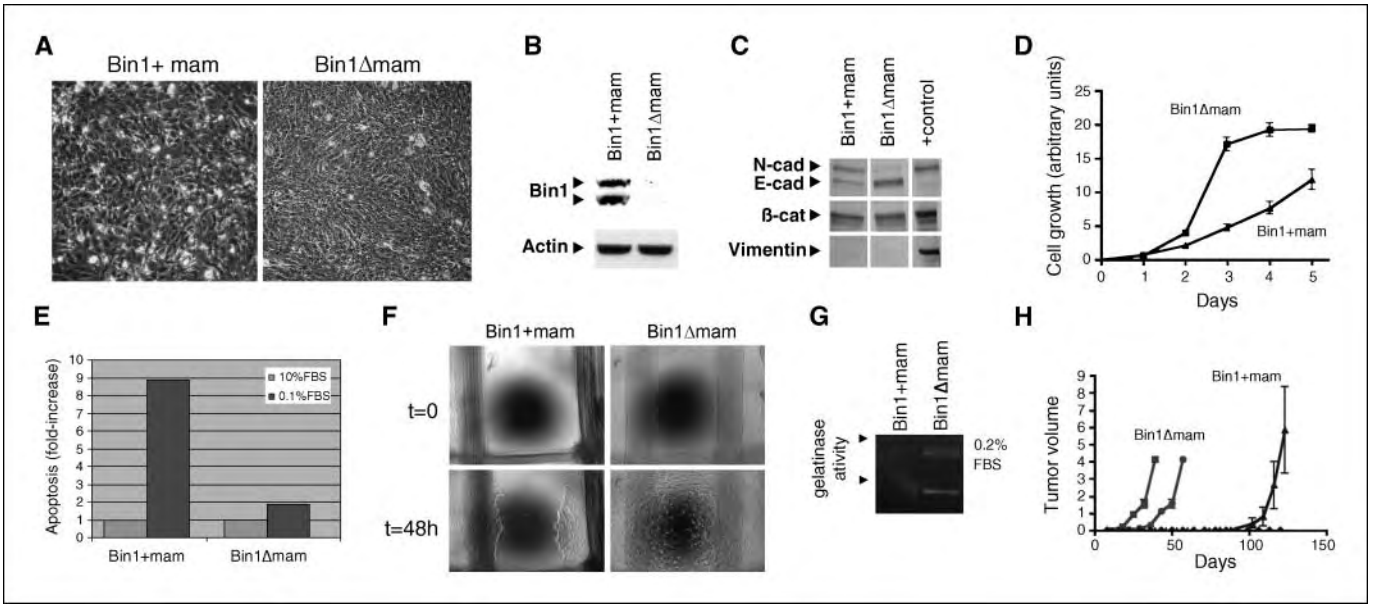

Figure 4. Advanced neoplastic character of MMECs established from DMBA-induced mammary tumors lacking Bin1. *A,* morphology. Cells were photographed using phase optics. *B,* Bin1 status in representative pair of MMEC tumor cell lines. Total cellular protein was extracted MMEC tumor cell lines and Bin1 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

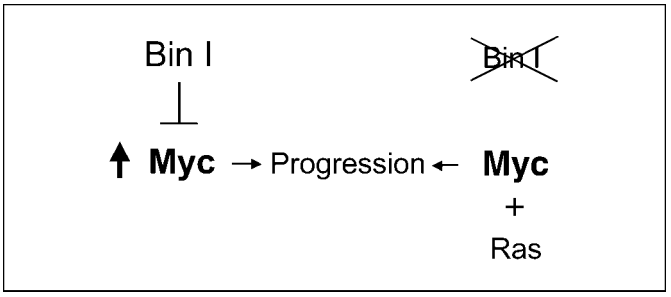

Figure 5. Model. Maintaining Myc expression is sufficient to drive cell division and tumorigenesis, yet in many human cancers, Myc is both deregulated and overexpressed, implying the existence of a selection for high Myc levels to provide a progression benefit. The model proposes that acquisition of 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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References

1. Jackson-Grusby L. Modeling cancer in mice. Oncogene 2002;21:5504–14.
2. Dragani TA. 10 years of mouse cancer modifier loci: human relevance. Cancer Res 2003;63:3011–8.
3. Sakamuro D, Elliott K, Wechsler-Reya R, Prendergast GC. BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. Nat Genet 1996;14:69–77.
4. Elliott K, Sakamuro D, Basu A, et al. Bin1 functionally interacts with Myc in cells and inhibits cell proliferation by multiple mechanisms. Oncogene 1999;18:3564–73.
5. Pineda-Lucena A, Ho CS, Mao DY, et al. A structure-based model of the c-Myc/Bin1 protein interaction shows alternative splicing of Bin1 and c-Myc phosphorylation are key binding determinants. J Mol Biol 2005; 351:182–94.
6. Deming SL, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br J Cancer 2000; 83:1688–95.
7. Butler MH, David C, Ochoa G-C, et al. Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/RVS family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of Ravier in brain and around T tubules in skeletal muscle. J Cell Biol 1997;137: 1355–67.
8. Ramjaun AR, Micheva KD, Bouchelet I, McPherson PS. Identification and characterization of a nerve terminal-enriched amphiphysin isoform. J Biol Chem 1997;272: 16700–6.
9. Tsutsui K, Maeda Y, Tsutsui K, Seki S, Tokunaga A. cDNA cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. Biochem Biophys Res Commun 1997;236:178–83.
10. Wechsler-Reya R, Sakamuro D, Zhang J, Duhadaway J, Prendergast GC. Structural analysis of the human BIN1 gene: evidence for tissue-specific transcriptional regulation and alternate RNA splicing. J Biol Chem 1997; 272:31453–8.
11. Peter BJ, Kent HM, Mills IG, et al. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science 2003;303:495–9.
12. Ren G, Vajjhala P, Lee JS, Winsor B, Munn AL. The BAR domain proteins: molding membranes in fission, fusion, and phagy. Microbiol Mol Biol Rev 2006;70:37–120.
13. Miaczynska M, Christoforidis S, Giner A, et al. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell 2004;116:445–56.
14. Elliott K, Ge K, Du W, Prendergast GC. The c-Myc-interacting adapter protein Bin1 activates a caspase-independent cell death program. Oncogene 2000;19: 4669–84.
15. Ge K, DuHadaway J, Du W, Herlyn M, Rodeck U, Prendergast GC. Mechanism for elimination of a tumor suppressor: aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. Proc Natl Acad Sci U S A 1999;96:9689–94.
16. Ge K, DuHadaway J, Sakamuro D, Wechsler-Reya R, Reynolds C, Prendergast GC. Losses of the tumor suppressor Bin1 in breast carcinoma are frequent and reflect deficits in a programmed cell death capacity. Int J Cancer 2000;85:376–83.
17. Ge K, Minhas F, DuHadaway J, et al. Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma. Int J Cancer 2000;86:155–61.
18. DuHadaway JB, Sakamuro D, Ewert DL, Prendergast GC. Bin1 mediates apoptosis by c-Myc in transformed primary cells. Cancer Res 2001;16:3151–6.
19. Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Targeted deletion of the suppressor

gene Bin1/amphiphysin2 enhances the malignant character of transformed cells. Cancer Biol Ther 2004;3:1236–42.

20. Muller AJ, DuHadaway JB, Sutanto-Ward E, Donover PS, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunomodulatory target of the tumor suppressor gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.

21. Mao NC, Steingrimsson E, DuHadaway J, et al. The murine Bin1 gene functions early in myogenesis and defines a new region of synteny between mouse chromosome 18 and human chromosome 2. Genomics 1999;56:51–8.

22. Muller AJ, Baker JF, DuHadaway JB, et al. Targeted disruption of the murine Bin1/amphiphysin II gene does not disable endocytosis but results in embryonic cardiomyopathy with aberrant myofibril formation. Mol Cell Biol 2003;23:4295–306.

23. Aldaz CM, Liao QY, LaBate M, Johnston DA. Medroxyprogesterone acetate accelerates the development and increases the incidence of mouse mammary tumors induced by dimethylbenzanthracene. Carcinogenesis 1996;17:2069–72.

24. Lebowitz P, Casey PJ, Prendergast GC, Thissen J. Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 1997;272:15591–4.

25. Routhier EL, Donover PS, Prendergast GC. hob1^{+}, the homolog of Bin1 in fission yeast, is dispensable for endocytosis but required for the response to starvation or genotoxic stress. Oncogene 2003;22:637–48.

26. Wagner KU, Wall RJ, St-Onge L, et al. Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 1997;25:4323–30.

27. Stewart TA, Pattengale PK, Leder P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell 1984;38:627–37.

28. Buters JT, Sakai S, Richter T, et al. Cytochrome *P*450 CYP1B1 determines susceptibility to 7,12-dimethylbenz[*a*]anthracene-induced lymphomas. Proc Natl Acad Sci U S A 1999;96:1977–82.

29. Bauer F, Urdaci M, Aigle M, Crouzet M. Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. Mol Cell Biol 1993;13:5070–84.

30. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev 2000;14:163–76.

31. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004;114:280–90.

32. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269–74.

33. Galderisi U, Di Bernardo G, Cipollaro M, et al. Induction of apoptosis and differentiation in neuroblastoma and astrocytoma cells by the overexpression of Bin1, a novel Myc interacting protein. J Cell Biochem 1999;74:313–22.

34. Tajiri T, Liu X, Thompson PM, et al. Expression of a MYCN-interacting isoform of the tumor suppressor BIN1 is reduced in neuroblastomas with unfavorable biological features. Clin Cancer Res 2003;9:3345–55.

35. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-*myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. Cell 1987;49:465–75.

36. Nesbit CE, Tersak JM, Prochownik EV. MYC oncogenes and human neoplastic disease. Oncogene 1999;18:3004–16.

37. Moreno E, Basler K. dMyc transforms cells into super-competitors. Cell 2004;117:117–29.

38. Moreno E, Basler K, Morata G. Cells compete for decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. Nature 2002;416:755–9.

39. Kajiho H, Saito K, Tsujita K, et al. RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway. J Cell Sci 2003;116:4159–68.

40. Hehir DJ, McGreal G, Kirwan WO, Kealy W, Brady MP. c-*myc* oncogene expression: a marker for females at risk of breast carcinoma. J Surg Oncol 1993;54:207–9.

41. Watson PH, Safneck JR, Le K, Dubik D, Shiu RP. Relationship of c-*myc* amplification to progression of breast cancer from *in situ* to invasive tumor and lymph node metastasis. J Natl Cancer Inst 1993;85:902–7.

42. Kreipe H, Feist H, Fischer L, et al. Amplification of c-*myc* but not of c-erbB-2 is associated with high proliferative capacity in breast cancer. Cancer Res 1993;53:1956–61.

43. Borg A, Baldetorp B, Ferno M, Olsson H, Sigurdsson H. c-*myc* amplification is an independent prognostic factor in postmenopausal breast cancer. Int J Cancer 1992;51:687–91.

44. Berns EM, Klijn JG, van Putten WL, van Staveren IL, Portengen H, Foekens JA. c-*myc* amplification is a better prognostic factor than HER2/*neu* amplification in primary breast cancer. Cancer Res 1992;52:1107–13.

45. DuHadaway JB, Lynch FJ, Brisbay S, et al. Immunohistochemical analysis of Bin1/amphiphysin II in human tissues: diverse sites of nuclear expression and losses in prostate cancer. J Cell Biochem 2003;88:635–42.

46. Wechsler-Reya R, Elliott K, Herlyn M, Prendergast GC. The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. Cancer Res 1997;57:3258–63.

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

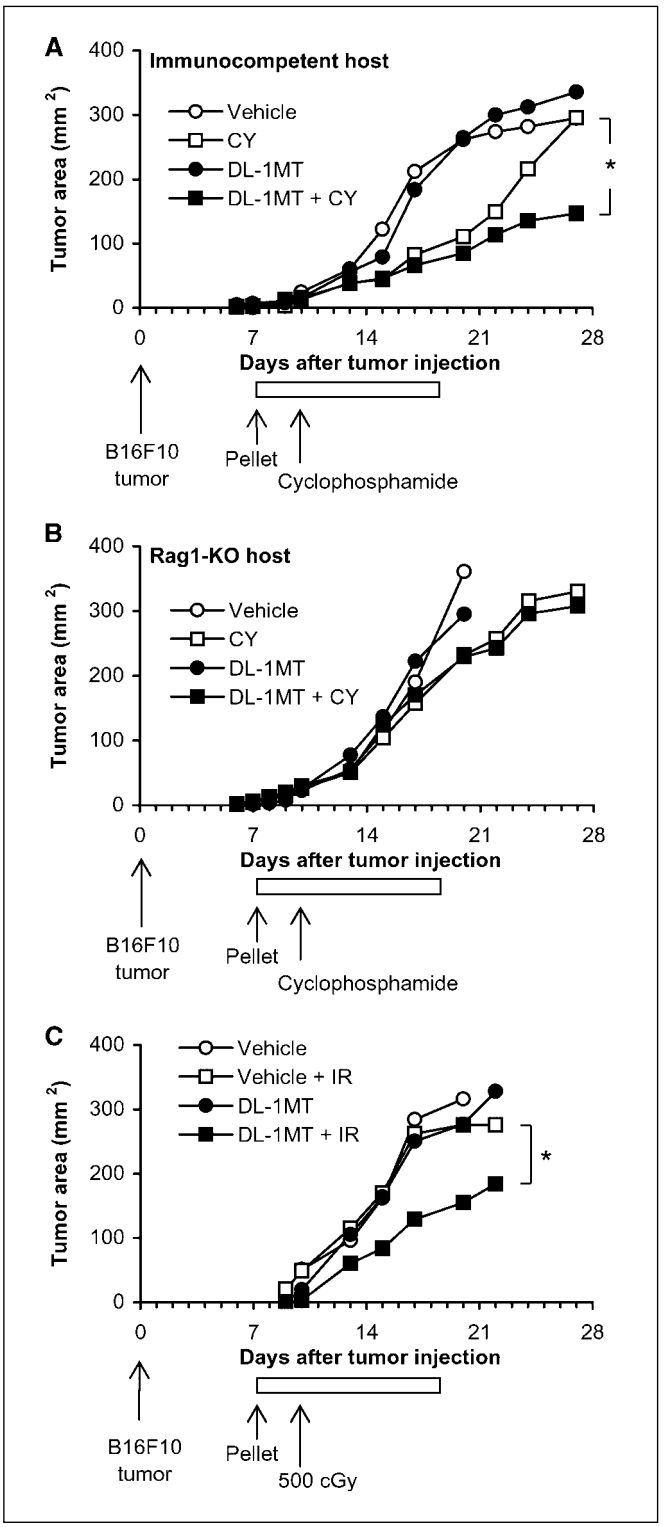

Figure 1. Effect of parenteral DL-1-methyl-tryptophan (*DL-1MT*) in B16F10 tumors. *A,* B16F10 tumors were implanted in syngeneic C57BL/6 mice. Beginning on day 7, mice were treated as shown with timed release s.c. pellets of DL-1-methyl-tryptophan (20 mg/d) plus cyclophosphamide (*CY*; 150 mg/kg i.p. × 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

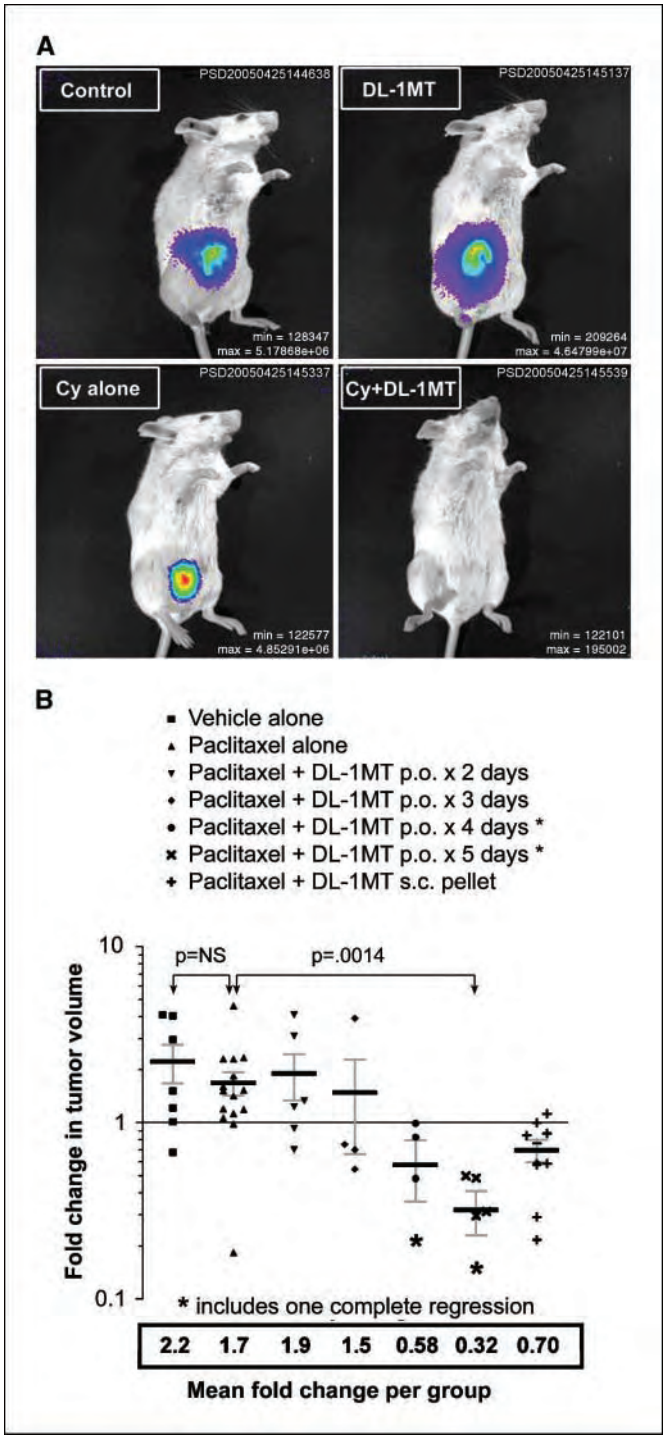

Figure 2. Oral DL-1-methyl-tryptophan in orthotopic 4T1 and autochthonous MMTV-*Neu* tumors. *A,* orthotopic tumor isografts were established in the mammary fat pad. Treatment was initiated concurrent with tumor challenge, using cyclophosphamide i.p. at 100 mg/kg, once a week and DL-1-methyl-tryptophan 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.

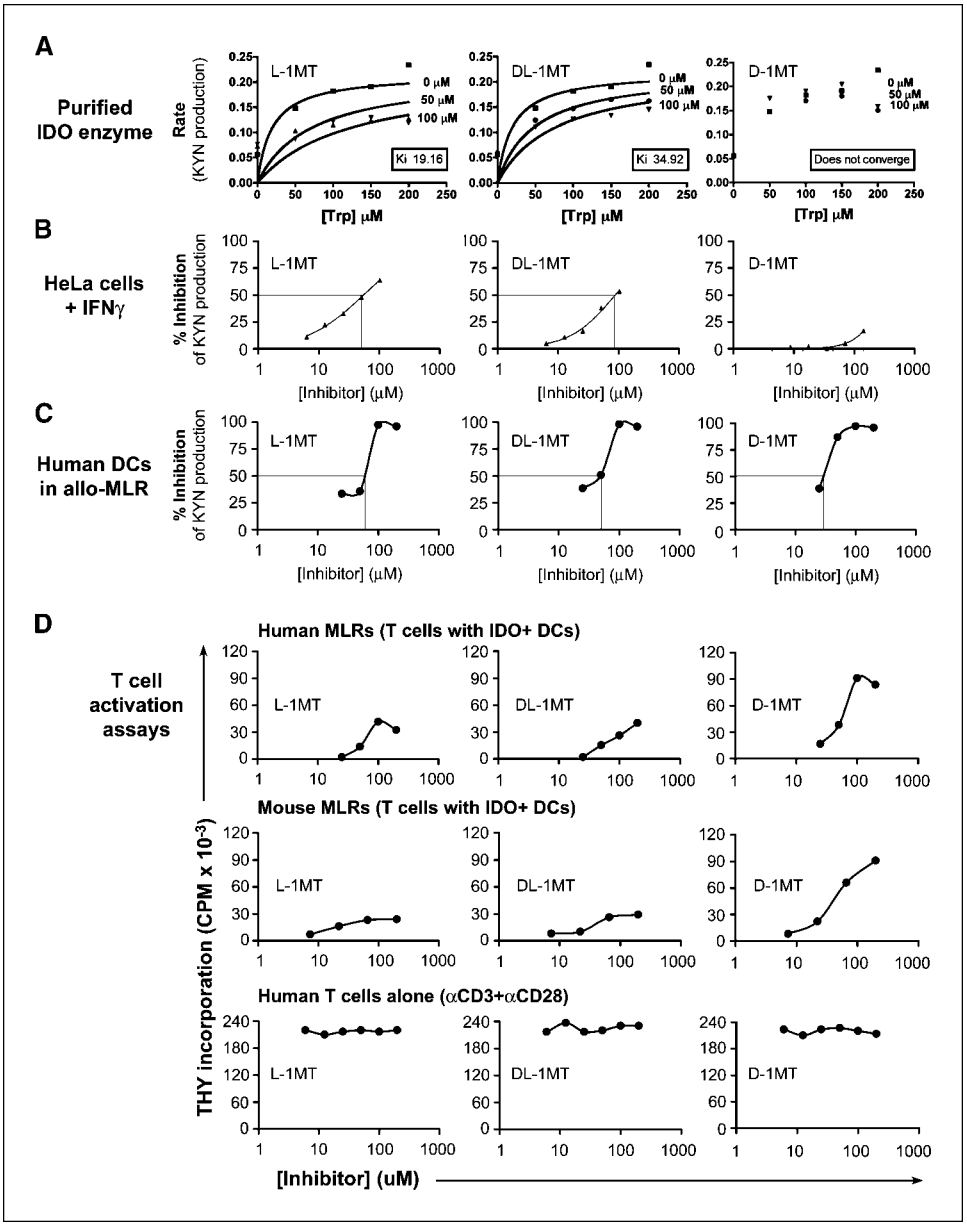

Figure 3. Effect of different isomers on *in vitro* enzyme assays and T-cell proliferation. *A,* enzyme kinetics, measured as kynurenine (*KYN*) production in cell-free assay, for purified recombinant human IDO, showing the effect of the L, DL, and D forms of 1-methyl-tryptophan in the presence of varying 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*.

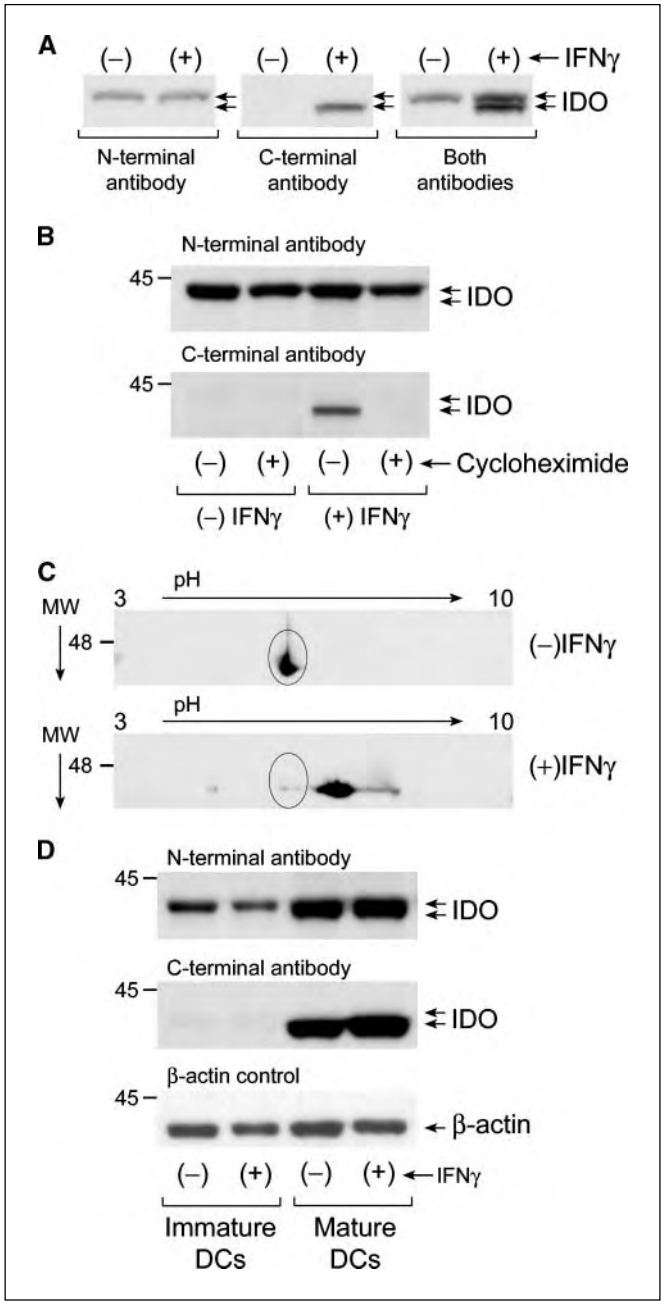

Figure 4. Evidence for two possible isoforms of human IDO. *A,* human monocyte–derived macrophages were prepared as described (11), with or without IFNγ treatment for the final 24 h. Lysates were analyzed by Western blot using antibodies against the NH_2-terminal portion of IDO, the COOH-terminal portion, 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

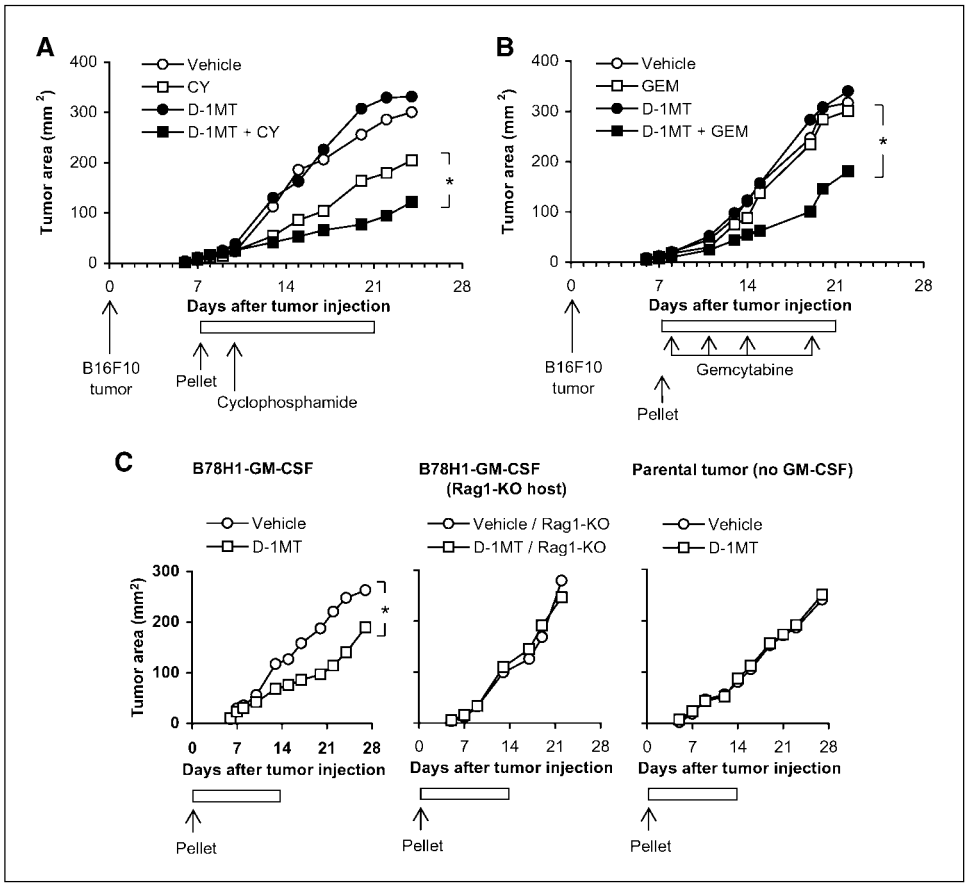

Figure 5. Effect of parenteral D-1-methyl-tryptophan in the B16F10 model. *A,* mice with B16F10 tumors were treated in a design similar to Fig. 1*A*, except using the D isomer of 1-methyl-tryptophan at a 4-fold lower dose (5 mg/d by timed release pellets). Cyclophosphamide was given at 150 mg/kg i.p. 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

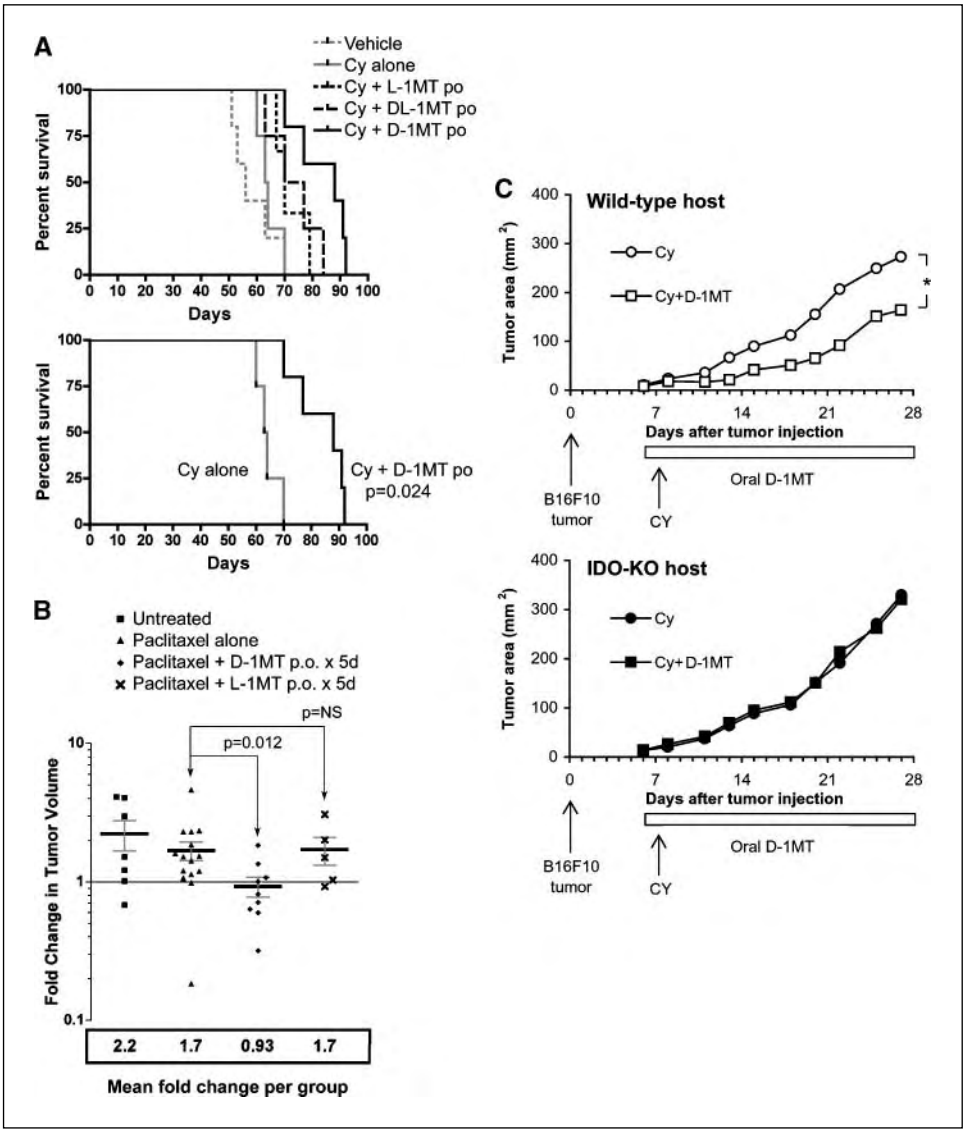

Figure 6. D-1-methyl-tryptophan provides greater survival benefit in combination therapy, in an IDO-dependent fashion. *A,* 4T1-luc orthotopic isografts were established in the mammary fat pad. Cyclophosphamide was given at 25 mg/kg orally once a week, and 1-methyl-tryptophan (D, L, or DL) given at 400 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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References

1. Munn DH, Zhou M, Attwood JT, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281:1191–3.
2. Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG, Stenson WF. Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. Gastroenterology 2003;125:1762–73.
3. Kwidzinski E, Bunse J, Aktas O, et al. Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. FASEB J 2005;19:1347–9.
4. Hayashi T, Beck L, Rossetto C, et al. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. J Clin Invest 2004;114:270–9.
5. Grohmann U, Fallarino F, Bianchi R, et al. A defect in tryptophan catabolism impairs tolerance in nonobese diabetic mice. J Exp Med 2003;198:153–60.
6. Grohmann U, Orabona C, Fallarino F, et al. CTLA-4-Ig regulates tryptophan catabolism *in vivo*. Nat Immunol 2002;3:1097–101.
7. Swanson KA, Zheng Y, Heidler KM, Mizobuchi T, Wilkes DS. $CDllc^+$ cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. Am J Respir Cell Mol Biol 2004;30:311–8.
8. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269–74.
9. Okamoto A, Nikaido T, Ochiai K, et al. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. Clin Cancer Res 2005;11:6030–9.
10. Brandacher G, Perathoner A, Ladurner R, et al. Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells. Clin Cancer Res 2006;12:1144–51.
11. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999; 189:1363–72.
12. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000;164:3596–9.
13. Munn DH, Sharma MD, Lee JR, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002;297:1867–70.
14. Munn DH, Sharma MD, Mellor AL. Ligation of B7–1/B7–2 by human $CD4^+$ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. J Immunol 2004; 172:4100–10.
15. Tan PH, Beutelspacher SC, Xue SA, et al. Modulation of human dendritic-cell function following transduction with viral vectors: implications for gene therapy. Blood 2005;105:3824–32.
16. Tan PH, Yates JB, Xue SA, et al. Creation of tolerogenic human DC via intracellular CTLA4: a novel strategy with potential in clinical immunosuppression. Blood 2005;106:2936–43.
17. Braun D, Longman RS, Albert ML. A two step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic cell maturation. Blood 2005;106: 2375–81.
18. Vacca C, Fallarino F, Perruccio K, et al. CD40 ligation prevents onset of tolerogenic properties in human dendritic cells treated with CTLA-4-Ig. Microbes Infect 2005;7:1040–8.
19. Orabona C, Puccetti P, Vacca C, et al. Toward the identification of a tolerogenic signature in IDO-competent dendritic cells. Blood 2006;107:2846–54.
20. Grohmann U, Fallarino F, Bianchi R, et al. IL-6 inhibits the tolerogenic function of $CD8\alpha(+)$ dendritic cells expressing indoleamine 2,3-dioxygenase. J Immunol 2001;167:708–14.
21. Mellor AL, Chandler P, Baban B, et al. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. Int Immunol 2004;16:1391–401.
22. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004; 114:280–90.
23. Baban B, Hansen A, Chandler P, et al. A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type 1 interferon-signaling following B7 ligation. Int Immunol 2005;17:909–19.
24. Mellor AL, Baban B, Chandler PR, Manlapat A, Kahler DJ, Munn DH. Cutting edge: CpG oligonucleotides induce splenic $CD19^+$ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN type 1 signaling. J Immunol 2005; 175:5601–5.
25. Munn DH, Sharma MD, Baban B, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005;22:633–42.
26. Lee JR, Dalton RR, Messina JL, et al. Pattern of recruitment of immunoregulatory antigen presenting cells in malignant melanoma. Lab Invest 2003;83: 1457–66.

27. von Bergwelt-Baildon MS, Popov A, Saric T, et al. CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells *in vivo*: additional mechanisms of T-cell inhibition. Blood 2006;108:228–37.
28. Lee JH, Torisu-Itakara H, Cochran AJ, et al. Quantitative analysis of melanoma-induced cytokine-mediated immunosuppression in melanoma sentinel nodes. Clin Cancer Res 2005;11:107–12.
29. Muller AJ, Malachowski WP, Prendergast GC. Indoleamine 2,3-dioxygenase in cancer: targeting pathological immune tolerance with small-molecule inhibitors. Expert Opin Ther Targets 2005;9:831–49.
30. Cady SG, Sono M. 1-methyl-DL-tryptophan, b-(3-Benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and b-[3-benzo(b)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys 1991;291:326–33.
31. Mellor A, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4:762–74.
32. Munn DH. Indoleamine 2,3-dioxygenase, tumor-induced tolerance and counter-regulation. Curr Opin Immunol 2006;18:220–5.
33. Muller AJ, Duhadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.
34. Lake RA, Robinson BW. Immunotherapy and chemotherapy-a practical partnership. Nat Rev Cancer 2005;5:397–405.
35. Klebanoff CA, Khong HT, Antony PA, Palmer DC, Restifo NP. Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. Trends Immunol 2005;26:111–7.
36. Muller AJ, Prendergast GC. Marrying immunotherapy with chemotherapy: why say IDO? Cancer Res 2005;65:8065–8.
37. Peterson AC, Migawa MT, Martin MJ, et al. Evaluation of functionalized tryptophan derivatives and related compounds as competitive inhibitors of indoleamine 2,3-dioxygenase. Med Chem Res 1994;3:531–44.
38. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science 1994;264:961–5.
39. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399–405.
40. Pulaski B, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. In: Coligan J, Kruisbeek A, Margulies D, Shevach E, Strober W, editors. Current protocols in immunology. New York: John Wiley & Sons; 2000. p. 20.2.1–.2.16.
41. Dummer W, Niethammer AG, Baccala R, et al. T cell homeostatic proliferation elicits effective antitumor autoimmunity. J Clin Invest 2002;110:185–92.
42. Odemuyiwa SO, Ghahary A, Li Y, et al. Cutting Edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. J Immunol 2004;173:5909–13.
43. Fallarino F, Vacca C, Orabona C, et al. Functional expression of indoleamine 2,3-dioxygenase by murine CD8α(+) dendritic cells. Int Immunol 2002;14:65–8.
44. Borrello I, Sotomayor EM, Cooke S, Levitsky HI. A universal granulocyte-macrophage colony-stimulating factor-producing bystander cell line for use in the formulation of autologous tumor cell-based vaccines. Hum Gene Ther 1999;10:1983–91.
45. Bronte V, Chappell DB, Apolloni E, et al. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits $CD8^+$ T cell responses by dysregulating antigen-presenting cell maturation. J Immunol 1999;162:5728–37.
46. Rutella S, Bonanno G, Procoli A, et al. Hepatocyte growth factor favors monocyte differentiation into regulatory interleukin (IL)-10++IL-12low/neg accessory cells with dendritic-cell features. Blood 2006;108:218–27.
47. Potula R, Poluektova L, Knipe B, et al. Inhibition of Indoleamine 2,3-dioxygenase (IDO) enhances elimination of virus-infected macrophages in animal model of HIV-1 encephalitis. Blood 2005;106:2382–90.
48. Belladonna ML, Grohmann U, Guidetti P, et al. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. J Immunol 2006;177:130–7.
49. Agaugue S, Perrin-Cocon L, Coutant F, Andre P, Lotteau V. 1-methyl-tryptophan can interfere with TLR signaling in dendritic cells independently of IDO activity. J Immunol 2006;177:2061–71.
50. Ghiringhelli F, Larmonier N, Schmitt E, et al. $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34:336–44.
51. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of $CD4^+25^+$ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;105:2862–8.
52. Munn DH, Mellor AL. The tumor-draining lymph node as an immune-privileged site. Immunol Rev 2006;213:146–58.

Novel Tryptophan Catabolic Enzyme IDO2 Is the Preferred Biochemical Target of the Antitumor Indoleamine 2,3-Dioxygenase Inhibitory Compound D-1-Methyl-Tryptophan

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Abstract

Small-molecule inhibitors of indoleamine 2,3-dioxygenase (IDO) are currently being translated to clinic for evaluation as cancer therapeutics. One issue related to trials of the clinical lead inhibitor, D-1-methyl-tryptophan (D-1MT), concerns the extent of its biochemical specificity for IDO. Here, we report the discovery of a novel IDO-related tryptophan catabolic enzyme termed IDO2 that is preferentially inhibited by D-1MT. IDO2 is not as widely expressed as IDO but like its relative is also expressed in antigen-presenting dendritic cells where tryptophan catabolism drives immune tolerance. We identified two common genetic polymorphisms in the human gene encoding IDO2 that ablate its enzymatic activity. Like IDO, IDO2 catabolizes tryptophan, triggers phosphorylation of the translation initiation factor eIF2α, and (reported here for the first time) mobilizes translation of LIP, an inhibitory isoform of the immune regulatory transcription factor NF-IL6. Tryptophan restoration switches off this signaling pathway when activated by IDO, but not IDO2, arguing that IDO2 has a distinct signaling role. Our findings have implications for understanding the evolution of tumoral immune tolerance and for interpreting preclinical and clinical responses to D-1MT or other IDO inhibitors being developed to treat cancer, chronic infection, and other diseases. [Cancer Res 2007;67(15):7082–7]

Introduction

Tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO) mediates a protolerogenic mechanism that suppresses T cells, providing balance or feedback control in immune reactions (1, 2). This role for IDO was first established with the demonstration that the specific bioactive IDO inhibitor 1-methyl-tryptophan (1MT; 3) can trigger T cell–mediated rejection of allogeneic mouse concepti (4, 5). More recently, IDO has become recognized as a central mediator of immune tolerance in many settings. In cancer, IDO expression in tumor cells and antigen-presenting cells present in tumor-draining lymph nodes mediates an important mechanism of immune escape (6). IDO inhibitors trigger antitumor immunity (7, 8) and act synergistically with conventional or experimental chemotherapies (9, 10). Based on preclinical efficacy studies, the D stereoisomer of 1MT has emerged as a clinical lead inhibitor that is entering human trials. D-1MT has superior antitumor activity relative to the L stereoisomer in most preclinical models, and IDO is genetically required for the activity of D-1MT (11). However, at the level of biochemical specificity, the distinction between the two isomers is complicated, with the D isomer exhibiting little biochemical activity as an IDO inhibitor relative to L isomer (11). In dendritic cells, both isomers block tryptophan catabolism comparably but the D isomer is again relatively more active biologically (11). Two possible resolutions to this disparity in results are that D-1MT targets either an undefined cellular isoform of IDO, for example, an alternate spliced or modified isoform, or a different target. Here, we corroborate the latter possibility with the discovery of a novel IDO-related enzyme that is a preferential target for biochemical inhibition by D-1MT.

Materials and Methods

All materials and methods are included as online Supplementary Material.

Results

IDO2 is a novel tryptophan-catabolizing enzyme that is preferentially inhibited by D-1MT. We discovered IDO2 by Basic Local Alignment Search Tool searches of the human genome using IDO sequences as probes, identifying a new gene on chromosome 8p12 just downstream of the IDO gene *INDO*. At the time of discovery, genome annotation in this region referred to an anonymous gene termed LOC169355 that was changed later to a misannotated partial gene termed *INDOL1* (IDO like-1; Hs.122077). By trial and error, we identified exons permitting assembly of a full-length IDO-related gene termed *IDO2*. This nomenclature was chosen to distinguish it from *INDOL1*, which remains misannotated as incomplete gene in the database. By homology searching, we also identified the mouse orthologue *Ido2*.

Oligonucleotide primers specific to murine and human coding regions were used to amplify cDNAs by reverse transcription-PCR (RT-PCR) from total RNA isolated from various tissues (Supplementary Figs. S1 and S2). In this manner, we obtained full-length cDNAs with complete coding regions including four alternatively spliced variants of each gene. The primary human transcript is derived from 11 exons encompassing a 74 kb region of chromosome 8p12 (Fig. 1*A* and Supplementary Fig. S3). In three of the five splice isoforms of *IDO2* mRNA we identified, introduction of an out-of-frame stop codon causes a premature truncation of IDO2 protein. Transcripts are initiated only 5 to 7 kb

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

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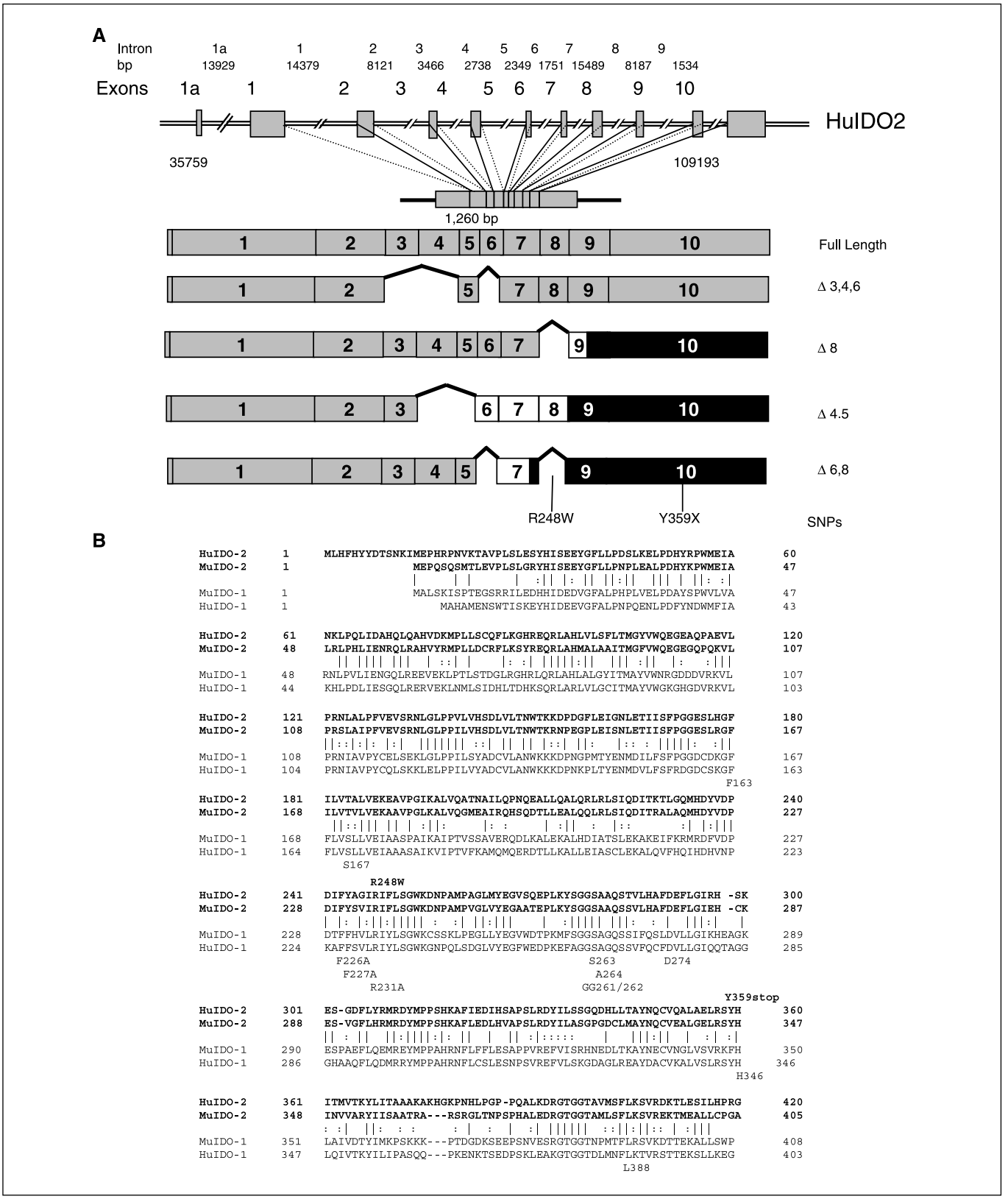

Figure 1. IDO2 structure and similarities to IDO. *A,* structure of human *IDO2* gene and transcripts. Complete coding region is 1,260 bp encoding a 420-amino-acid polypeptide. Alternate splice isoforms lacking the exons indicated are noted. *White boxes,* a frameshift in the coding region to an alternate reading frame leading to termination. *Black boxes, 3′* untranslated regions. Nucleotide numbers, intron sizes, and positioning are based on IDO sequence files NW_923907.1 and GI:89028628 in the Genbank database. *B,* amino acid alignment of IDO and IDO2. Amino acids determined by mutagenesis and the crystal structure of IDO that are critical for catalytic activity are positioned below the human IDO sequence. Two commonly occurring SNPs identified in the coding region of human IDO2 are shown above the sequence that alter a critical amino acid (R248W) or introduce a premature termination codon (Y359stop).

downstream of the *INDO* gene. The mouse gene seems to differ in its lack of the alternate exon 1a found in the human gene; otherwise, exon positions are conserved, indicating gene duplication during evolution of this region of the genome. Human and mouse IDO2 proteins are 420 and 405 amino acids, respectively, and are more conserved (72% identical, 84% similar) than IDO proteins (62% identical, 77% similar). Alignments between IDO and IDO2 sequence reveal highly conserved features that mediate heme and substrate binding (Fig. 1*B*), although the overall level of sequence conservation is not particularly high (43% identical, 63% similar for human). Significantly, residues determined by IDO mutagenesis and crystallographic analysis to be critically important for catalytic activity are highly conserved in IDO2 (Fig. 1*B*).

To confirm the expectation that IDO2 catabolizes tryptophan, we expressed it in a doxycycline-regulated T-REX cell system where formation of the enzymatic product *N*-formyl-kynurenine (Kyn) was monitored. Stable cell lines expressing V5 epitope–tagged or untagged proteins with similar levels of doxycycline-induced expression were used for analysis (Fig. 2*A*; Supplementary Fig. S4). As expected, both human and murine IDO2 catabolized tryptophan effectively as measured by Kyn production (Fig. 2*B*). Based on IDO-IDO2 similarity, we compared the ability of known IDO inhibitors to block the activity of IDO2 in T-REX cells. For reasons mentioned above, the IDO inhibitor D-1MT was of particular interest based on uncertainties about its biochemical target (9, 11). Therefore, we evaluated how IDO1 or IDO2 activity was affected by the D or L stereoisomers of 1MT, or by a third inhibitor MTH-trp (9). Consistent with previous observations (11), we found that IDO activity was modestly inhibited by L-1MT but not D-1MT. In contrast, IDO2 activity was inhibited by D-1MT but not L-1MT. This pattern of inhibition was specific to these 1MT isomers insofar as MTH-trp inhibited the activity of both enzymes (Fig. 2*C*). These results identify IDO2 as a relevant target for biochemical inhibition by D-1MT, which may explain its well-documented antitumor effects.

IDO2 expression is more restricted than IDO but includes dendritic cells. By RT-PCR analysis, we found *IDO2* is expressed in a subset of tissues expressing IDO. Primers spanning the complete human coding region detected full-length mRNAs only in placenta and brain, whereas primers specific to exon 10 (found to be common to all human *IDO2* cDNAs) detected *IDO2* mRNAs in human liver, small intestine, spleen, placenta, thymus, lung, brain, kidney, and colon (Fig. 3*A* and *B*). Although RT-PCR reactions spanning exons 1 to 8 might not have been sensitive enough to detect low-level transcripts, exon 1a–specific primers gave similar results (data not shown), implying that other transcription start

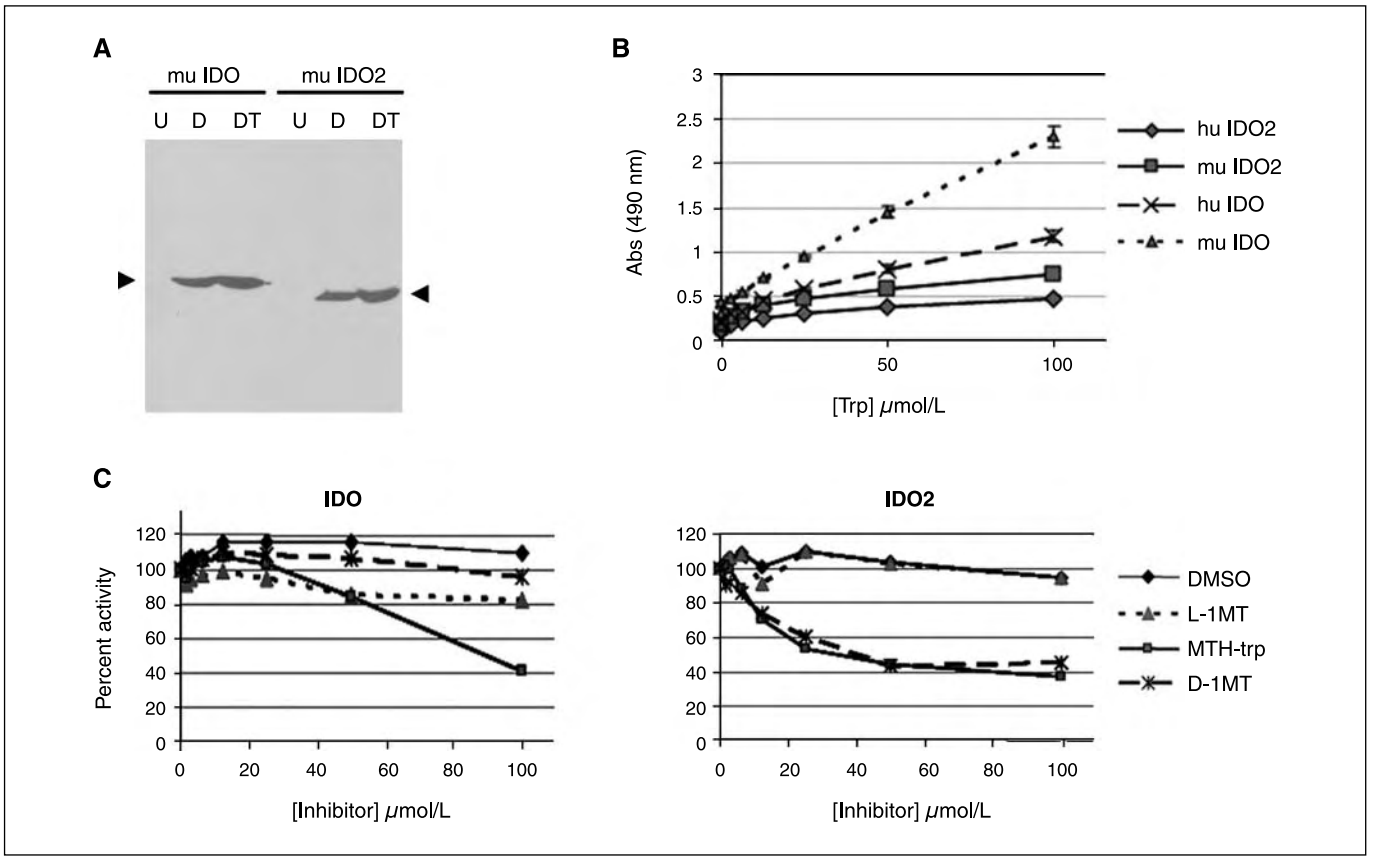

Figure 2. Tryptophan catabolic activity of IDO2 and inhibition by D-1MT. *A,* inducible expression of IDO and IDO2 in representative T-REX cells. Western blot analysis of the V5 epitope–tagged proteins indicated was done with a horseradish peroxidase–conjugated anti-V5 antibody (Invitrogen) in cells that were untreated (*U*), treated with 20 ng/mL doxycycline (*D*), or treated with doxycycline and 100 μmol/L tryptophan (*DT*). *B,* tryptophan catabolism. T-REX cells were seeded at 60% to 70% confluence in 96-well dishes in medium supplemented with 0 to 100 μmol/L tryptophan. Kyn production was determined 48 h later and normalized to protein levels as determined by sulforhodamine B assay. Each enzyme was catalytically active, based on increased Kyn levels with increasing substrate concentrations, although IDO2 seemed to be 2- to 4-fold less active than IDO when normalized to protein levels as determined by sulforhodamine B assay. *Points,* mean of values determined in triplicate and normalized to cellular protein levels. *Abs,* absorbance. *C,* effect of IDO inhibitors on IDO2 catalytic activity. T-REX cells were seeded and processed as above except for the addition to the medium of 0 to 100 μmol/L of the IDO inhibitors MTH-trp, L-1MT, D-1MT, or vehicle control (DMSO). *Points,* mean of values determined in triplicate and normalized to cellular protein levels as before.

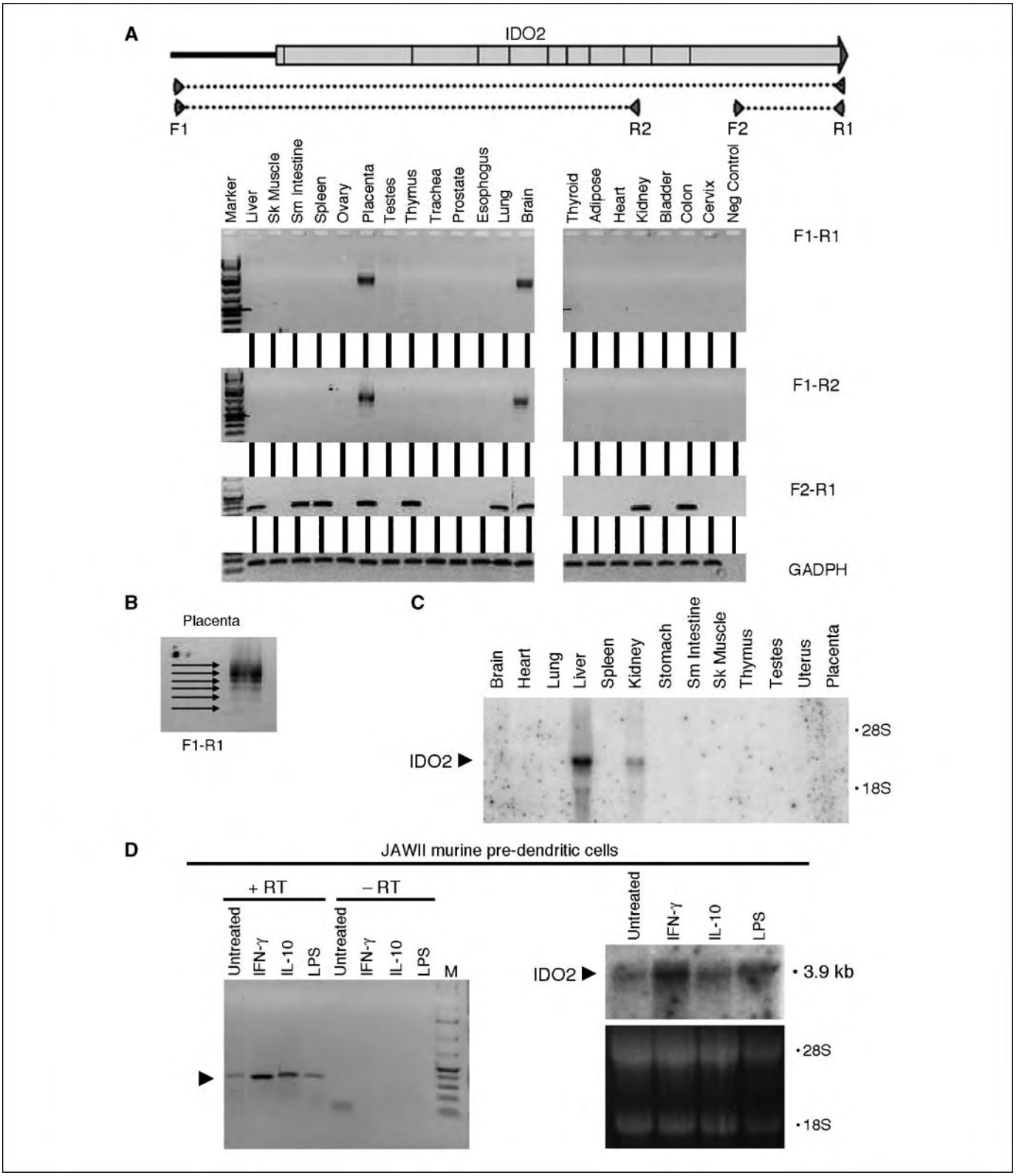

Figure 3. Tissue-specific and dendritic cell expression of IDO2. *A,* human tissues. A panel of total RNAs (Ambion) was analyzed by RT-PCR and agarose gel electrophoresis. *Cartoon above the figure,* location of primer pairs used for RT-PCR as indicated next to the gel photos. Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. *B,* human placenta. Expression of splice variants characterized as detected by the F1-R1 primer pair spanning the full-length cDNA. A similar pattern of expression was observed with primers extending through exons 8 to 10 as F2-R1. *C,* murine tissues. A commercial Northern blot (Seegene) was hybridized to murine IDO2 cDNA probe before washing and autoradiography using standard methods. *D,* murine JAWII predendritic cells. *Top,* RT-PCR analysis. Total RNA isolated from cells that were unstimulated or stimulated 24 h with IFN-γ, IL-10, or lipopolysaccharide was analyzed using primers F6 and R5 for murine IDO-2 (Supplementary Fig. S2). *Bottom,* Northern analysis. RNAs were fractionated on an agarose gel, blotted to nitrocellulose, and hybridized with a murine IDO2 cDNA probe. *Top,* ethidium-stained gel photograph showing intact 28S and 18S rRNAs. *LPS,* lipopolysaccharide.

sites may exist in human *IDO2*. Northern analysis of mouse tissue RNAs confirmed a more narrow range of expression, revealing detectable *IDO2* transcripts only in liver and kidney (Fig. 3*C*). In a query of the NCBI SAGEmap database with a sequence tag to *IDO2*, the top four hits in terms of tag count prevalence were all identified as bone marrow–derived dendritic cell libraries. Because D-1MT inhibits kynurenine production in dendritic cells and block their ability to activate T cells (11), we examined *IDO2* expression in an established predendritic mouse cell line (JAWII) that matures to dendritic cells after treatment with IFN-γ. *IDO2* mRNA was expressed in unstimulated JAWII cells, and IFN-γ treatment and, to a lesser extent, IL-10 or lipopolysaccharide treatment increased levels modestly (Fig. 3*D*). Using an IDO2-specific monoclonal antibody, we confirmed expression of IDO2 protein in JAWII cells by Western blotting and indirect immunofluorescence microscopy, the latter of which revealed a generally cytoplasmic pattern of expression like IDO (data not shown). Although we could not detect Kyn production in JAWII cells, we confirmed that full-length cDNAs cloned from these cells encoded a fully active enzyme in T-REX cells (data not shown). Together, these observations defined a pattern of expression for IDO2 that includes dendritic cells.

Common genetic polymorphisms in human IDO2 compromise or abolish enzymatic activity. During characterization of *IDO2* cDNAs, we identified two single nucleotide polymorphisms (SNP) that abolished enzymatic activity. One C-T SNP affecting R248 in human IDO2 was structurally analogous to R231 in human IDO, which makes a critical contact with the indole ring of tryptophan (12). The nonsynonymous substitution (R248W) reduced catalytic activity ~90% in T-REX cells (Supplementary Fig. S5). A second T-A SNP affecting Y359 generated a premature stop codon (Y359X), which completely abolished activity (Supplementary Fig. S5). Strikingly, both SNPs were commonly found in human genomic DNAs in public databases, with the C-T SNP being highly represented in individuals of European descent, the T-A SNP being highly represented individuals of Asian descent, and neither SNP being as prevalent in individuals of African descent (Supplementary Fig. S6). Thus, as many as 50% of individuals of European or Asian descent and 25% of individuals of African descent may lack functional IDO2 alleles. This analysis implicates these SNPs as having a broad effect on IDO2 activity in human populations, which may have a significant bearing on the interpretation of clinical responses to drug-like inhibitors of IDO2 like D-1MT.

IDO2 and IDO each activate LIP, an inhibitory isoform of immune regulatory transcription factor NF-IL6, but IDO2 produces a tryptophan-independent signal. Tryptophan catabolism by IDO triggers GCN2-dependent phosphorylation of the translation initiation factor eIF-2α (13). Activation of this pathway inhibits translation of most messages with the exception of certain messages essential for stress-related functions. Additional contributions of IDO to tolerogenesis are imparted by Kyn and other downstream catabolites (14–16). We evaluated the ability of IDO2 to activate this pathway in T-REX cells. In IDO-expressing cells, Kyn production was constant for 4 days postinduction after which cell growth rate slowed appreciably. This effect related to tryptophan depletion rather than Kyn elevation, because supplementing the culture medium with tryptophan rescued the effect (Supplementary Fig. S7). In IDO2-expressing cells, tryptophan consumption was slower such that cell growth was not affected (Supplementary Fig. S7). Nevertheless, induction of IDO2 caused GCN2-dependent phosphorylation of eIF-2α like IDO (data not shown). To compare downstream effects, we examined how IDO or IDO2 activation affected translation of LIP, an inhibitory isoform of the transcription factor NF-IL6/CEPBβ that is up-regulated by amino acid deprivation by a switch to an alternate translational start site (17). Both enzymes up-regulated LIP strongly, however, restoring tryptophan to culture medium reversed LIP induction only when stimulated by IDO (Fig. 4). Thus, IDO2 produced a distinct signal for LIP activation that was independent of tryptophan availability. This signal required catalytic activity because it was inhibited by D-1MT (Fig. 4). These findings implied that IDO2 has a distinct signaling role in cells compared with IDO.

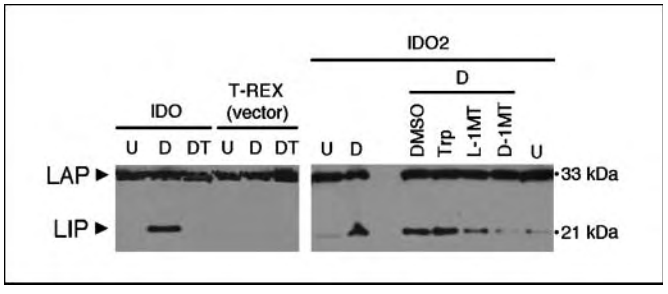

Figure 4. Distinct role of IDO2 in tryptophan catabolic signaling to transcription factor LIP. Western analysis of LIP and LAP isoforms of NF-IL6/CEBPβ was done using lysates isolated from T-REX cells seeded into 12-well dishes that were uninduced (*UI*), treated with 20 ng/mL doxycycline (*Dox*), or treated with doxycycline and 100 μmol/L tryptophan (*Tryp*). In the lanes indicated, cells were also treated with 100 μmol/L L-1MT or D-1MT.

Discussion

The findings of this study are significant and timely regarding how tryptophan catabolism suppresses T-cell immunity, how immune escape evolves during cancer progression, and how the D stereoisomer of the widely studied IDO inhibitor 1MT, presently entering phase I clinical trials, acts to elicit antitumor responses in animals. Given the striking therapeutic effects of D-1MT in preclinical models of cancer and other diseases (6), our findings point to IDO2 as an important therapeutic target and genetic modifier for understanding disease susceptibility. The existence of widely dispersed genetic polymorphisms in human populations that ablate catalytic activity argues that knowing the genetic status of IDO2 of individuals enrolled in D-1MT trials may be important for understanding clinical responses. Given the likelihood that IDO2 may contribute to immune tolerance, two implications are that individuals heterozygous or homozygous for catalytically inactive alleles may be (*a*) less susceptible to developing diseases driven by immune suppression, and (*b*) less susceptible to manifesting clinical responses to D-1MT or other IDO2 inhibitory compounds. Due to deficiencies in IDO2 activity, such individuals may be relatively less prone to immune escape and malignant progression of oncogenically initiated lesions, but relatively more prone to autoimmune disorders. Given differences in the antitumor responses seen in various preclinical cancer models to L-1MT versus D-1MT (11), it may also be interesting to evaluate the murine *IDO2* gene for related polymorphisms.

In LIP, we have defined a novel component of the tryptophan catabolism signaling pathway triggered by IDO or IDO2, using it here to reveal a mechanistic difference in how translational control by these enzymes may modulate immune tolerance. As a downstream reporter, LIP could provide a useful biomarker for genetic and biochemical pathways activated by IDO1 or IDO2 in cells that express

NF-IL6 (also known as CEBPβ). In essence, LIP is a dominant inhibitory isoform composed of only the DNA binding region of NF-IL6 (17). By interfering with target genes that control stress signaling, cell growth, and immune modulation, LIP is well positioned to mediate stable effects of IDO or IDO2 on immune tolerance generated by antigen-presenting cells or other cell types. Using LIP, we found that transient activation of IDO2 generates a stable signal that persists independently of tryptophan availability. The potential significance of this mechanism is that it could be used to propagate tolerance from a local to a peripheral immune environment, away from an initial site of tryptophan catabolism (18), for example, to support cancer metastasis. Differences in LIP response argue that the functions of IDO and IDO2 may be distinct, even if outcomes for eliciting immune tolerance are similar.

IDO2 may address key questions about how 1MT manifests its antitumor activity. Previous studies indicated that D-1MT can inhibit tryptophan catabolism in human dendritic cells and that the *IDO* gene is needed for antitumor activity, implicating IDO in the D-1MT mechanism at some level (11). Our findings do not rule out the possibility that 1MT may target an endogenous IDO protein differing at some level, for example, due to posttranslational modification (11); however, identifying IDO2 addresses a key gap in knowledge concerning the biochemical target of D-1MT. In most models, D-1MT displays much better antitumor activity than L-1MT prompting the choice made for clinical development. One implication is that compounds with dual specificity for IDO and IDO2 may exert more potent antitumor efficacy, and MTH-trp fulfills this expectation (9). Based on genetic knockout studies supporting a role for IDO in the response to D-1MT at some level (11), our findings strongly suggest cross-talk or cooperation between the functions of IDO and IDO2 in immune regulation. Consistent with this idea, IDO activity may be supported by other elements involved in tryptophan catabolism (16, 19). In future work, it will be important to examine IDO-IDO2 cooperation as well as how catabolites of tryptophan catabolism may figure into IDO2 action.

Addendum

Recently we became aware of another group reporting the identification of this gene (20).

Acknowledgments

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We apologize to investigators whose work was not cited due to size restrictions for publication.

References

1. Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4:762–74.
2. Puccetti P. On watching the watchers: IDO and type I/II IFN. Eur J Immunol 2007;37:876–9.
3. Cady SG, Sono M. 1-Methyl-DL-tryptophan, β-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and β-[3-benzo(*b*)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys 1991;291:326–33.
4. Munn DH, Zhou M, Attwood JT, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281:1191–3.
5. Mellor AL, Sivakumar J, Chandler PKS, Molina H, Mao D, Munn DH. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. Nat Immunol 2001;2:64–8.
6. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest 2007;117:1147–54.
7. Friberg M, Jennings R, Alsarraj M, et al. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer 2002;101:151–5.
8. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269–74.
9. Muller AJ, DuHadaway JB, Sutanto-Ward E, Donover PS, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunomodulatory target of the tumor suppressor gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.
10. Muller AJ, Prendergast GC. Indoleamine 2,3-dioxygenase in immune suppression and cancer. Curr Cancer Drug Targets 2007;7:31–40.
11. Hou DY, Muller AJ, Sharma MD, et al. Inhibition of indoleamine 2,3-dioxygenase in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with antitumor responses. Cancer Res 2007;67:792–801.
12. Sugimoto H, Oda SI, Otsuki T, Hino T, Yoshida T, Shiro Y. Crystal structure of human indoleamine 2,3-dioxygenase: Catalytic mechanism of O_2 incorporation by a heme-containing dioxygenase. Proc Natl Acad Sci U S A 2006;103:2311–6.
13. Munn DH, Sharma MD, Baban B, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005;22:633–42.
14. Fallarino F, Grohmann U, Vacca C, et al. T cell apoptosis by tryptophan catabolism. Cell Death Diff 2002;9:1069–77.
15. Fallarino F, Grohmann U, You S, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor ζ-chain and induce a regulatory phenotype in naive T cells. J Immunol 2006;176:6752–61.
16. Belladonna ML, Grohmann U, Guidetti P, et al. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. J Immunol 2006;177:130–7.
17. Descombes P, Schibler U. A liver-enriched transcriptional activator protein, LAP, a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 1991;67:569–79.
18. Grohmann U, Bianchi R, Belladonna ML, et al. IFN-γ inhibits presentation of a tumor/self peptide by $CD8\alpha^-$ dendritic cells via potentiation of the $CD8\alpha^+$ subset. J Immunol 2000;165:1357–63.
19. Manlapat AK, Kahler DJ, Chandler PR, Munn DH, Mellor AL. Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory $CD19^+$ dendritic cells. Eur J Immunol 2007;37:1064–71.
20. Ball HJ, Sanchez-Perez A, Weiser S, et al. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. Gene 2007;396:203–13.

Marrying Immunotherapy with Chemotherapy: Why Say IDO?

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Abstract

Activation of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) in cancer cells facilitates immune escape. A recent study now shows how small-molecule inhibitors of IDO can be used to leverage the efficacy of traditional chemotherapeutic drugs that are used to treat cancer in the clinic. By promoting antitumor immune responses in combination with cytotoxic chemotherapy, IDO inhibitors may offer a drug-based strategy to more effectively attack systemic cancer. (Cancer Res 2005; 65(18): 8065-8)

Background

During the breakdown in cellular physiology that accompanies malignant tumor development, cancer cells develop certain emblematic characteristics that include inherent cellular properties (cell intrinsic) as well as properties defined through interaction with the host environment (cell extrinsic). Fundamental cell-intrinsic characteristics of cancer cells include immortalization, growth signal self-sufficiency, insensitivity to growth inhibitory signals, and apoptosis resistance, whereas fundamental cell-extrinsic characteristics include the capacity for angiogenesis, invasion, metastasis, and immune escape. Establishment of the importance of immune escape to malignant progression has been relatively recent (1). Indeed, studies of the cell-extrinsic traits of cancer have, in general, tended to lag behind studies of the cell-intrinsic traits, because the former can not be easily evaluated in simple tissue culture systems. Moreover, these processes are generally associated more with epigenetic changes and modifier effects than with mutation of the classically defined oncogene and tumor suppressor pathways that have, until recently, been the major focus of research in molecular cancer biology.

The interactions between developing tumors and the immune system are complex and dynamic. On the one hand, inflammation provides a host of protumorigenic factors and suppression of immune responses can actually promote tumor regression in some model systems (2). On the other hand, cancer cells are also subject to immune surveillance with pressure on tumors to evade or subvert the immune response that tumor antigens should elicit (3). The development of immunotherapeutic strategies has focused predominantly on stimulating or supplementing immune effector cells. It is becoming increasingly apparent, however, that immune tolerance may be dominant in cancer patients and that it will be essential to breach established immune suppressive mechanisms for immunotherapy to be effective (1).

One strategy of immune escape that is used by cancer cells (Fig. 1) has been adapted from a mechanism that normally exists to prevent maternal immune response to paternal fetal antigens that are present during gestation (4). An inescapable consequence of sexual reproduction among histoincompatible individuals is that some means to circumvent maternal immunity must be hardwired into the system to protect the allogeneic fetus. The catabolic enzyme indoleamine-2,3 dioxygenase (IDO; EC 1.13.11.42) has been implicated in providing immune protection to the developing conceptus. IDO catalyzes the initial step in the degradation of tryptophan in the pathway leading to biosynthesis of NAD^+. Activation of IDO in placental trophoblast cells has been proposed to lead to the establishment of immune tolerance through either localized depletion of tryptophan or accumulation of toxic catabolites. This process is immune suppressive because T cells undergoing antigen-dependent activation are exquisitely sensitive to local tryptophan catabolism, which can cause them to arrest in G_1, become anergic, or die (5–7). In a key experiment, treatment of pregnant female mice with 1-methyl-tryptophan, a small-molecule inhibitor of IDO, has been shown to promote T cell–mediated destruction of allogeneic but not syngeneic concepti (4). IDO has also been more generally implicated in CTL-associated protein-4 (CTLA-4)–induced immune tolerance mediated through reverse B7 signaling *in vivo* (8).

Immune Escape in Cancer: Modulation of Indoleamine-2,3 Dioxygenase Expression by Bin1

A connection between elevated urinary tryptophan catabolites and bladder cancer was first reported in the 1950s (9). Since then, elevated levels of IDO-generated catabolites have been associated with a number of malignancies. This phenomenon was initially thought to be a tumoricidal consequence of IFN-γ, which stimulates expression of IDO in cells (10). However, a radical rethinking of the significance of IDO in cancer has been engendered by its implication in the prevention of allogenic conceptus rejection and by the evidence that IDO is overexpressed in most tumors and/or tumor-draining lymph nodes (11–13). How does IDO become deregulated in cancer cells? One possible answer has emerged from studies of a gene called *Bin1*, a cancer suppressive gene that seems to limit cancer to a large extent by limiting immune escape.

Bin1 was initially identified in a two-hybrid screen for c-Myc-interacting proteins (14). Along with the *Bin3* gene, *Bin1* is one of two related genes that are conserved through evolution to yeast and that define a family of adapter proteins characterized by a unique fold termed the BAR domain (14, 15). Frequent loss or attenuation of Bin1 occurs in advanced breast cancer, prostate cancer, melanoma, astrocytoma, neuroblastoma, and colon cancer (16–19).[1] At least 10 different Bin1 splice isoforms exist in mammalian cells of which two are ubiquitously expressed, whereas the remainder are restricted to specific terminally differentiated tissues including neurons and skeletal muscle cells. The different splice isoforms exhibit different patterns of subcellular localization and cancer suppressive activity, arguing that they have different functions. A precedent for BAR adapter proteins with dual trafficking and transcriptional functions

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[1] K. Xie et al., unpublished observations.

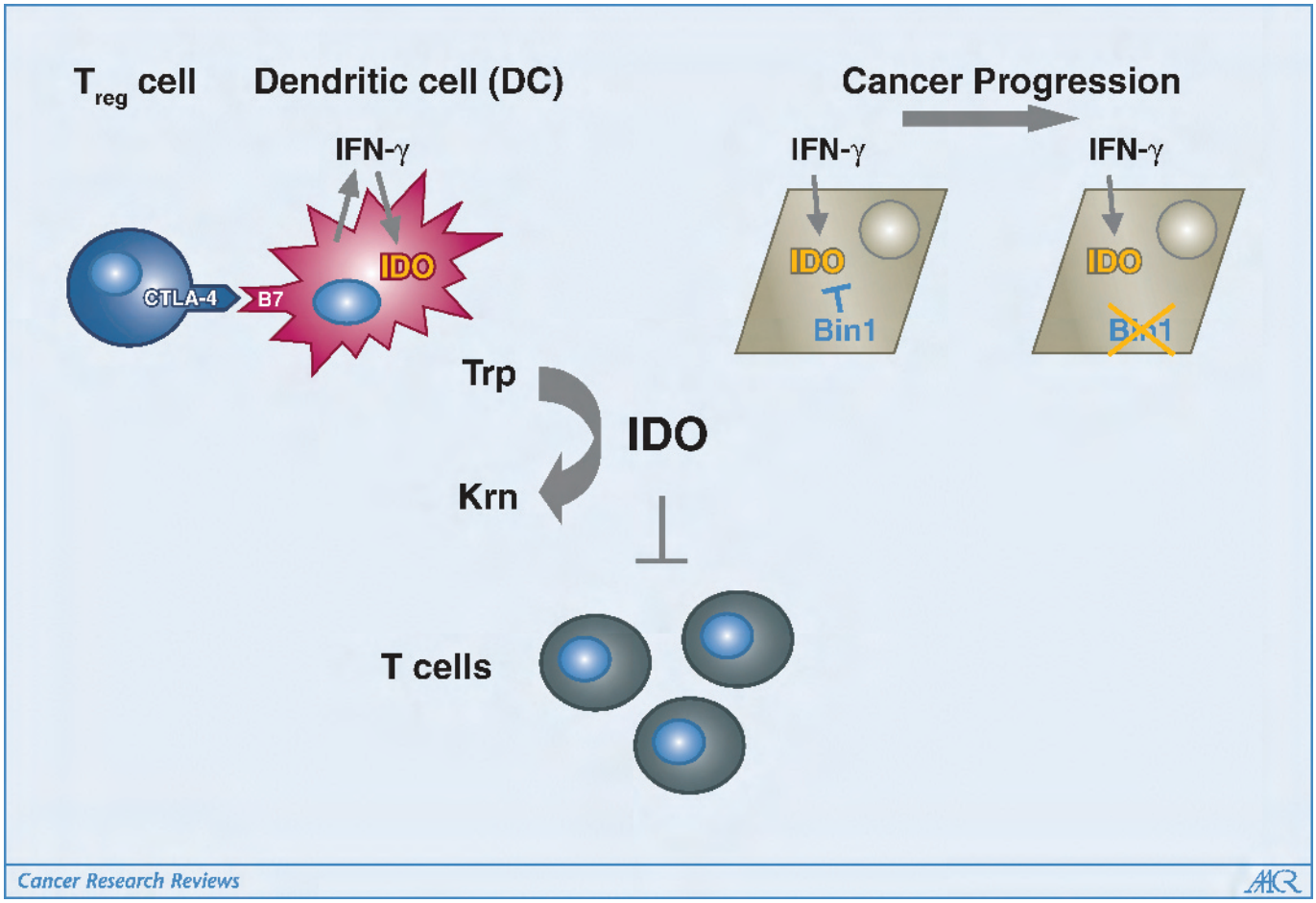

Figure 1. Mechanisms of IDO-induced tumoral immune escape. IDO expression in local immune stroma and directly in tumor cells has been implicated in promoting immune tolerance. IDO is upregulated in antigen-presenting dendritic cells (*DC*) by autocrine IFN-γ released as a result of T_{reg} cell–induced CTLA-4/B7-dependent cell-cell signaling. Local tryptophan catabolism limits the proliferation and survival of T cells that would otherwise be activated by tumor antigens on the APC. This mechanism may operate in tumor-draining lymph nodes. In tumor cells, attenuation of Bin1 leads to superactivation of IDO expression by IFN-γ, directly suppressing activation of T cells in the local tumor environment. Blocking IDO activity systemically with small molecule inhibitors (e.g., 1-methyl-tryptophan) reverses T-cell suppression that occurs as a result of tryptophan catabolism in both settings.

has been established through studies of APPL, a Rab5-binding endosomal protein that translocates to the nucleus upon epidermal growth factor stimulation to associate with the NuRD/MeCP1 nucleosome remodeling and transcriptional repression complex (20). Likewise, the ubiquitously expressed Bin1 splice isoforms, which encode its anticancer properties, have been implicated in both endosomal trafficking and transcriptional repression (21, 22). The possibility that Bin1 adapter proteins may affect pathways leading to the nucleus has garnered additional support based on possible involvement in the trafficking of signal transducer and activator of transcription (STAT) and nuclear factor-κB (NF-κB) transcription factors (23, 24).

Studies aimed at understanding how Bin1 restricts tumor outgrowth identified immune tolerance established through IDO deregulation as a likely mechanistic explanation (25). Deleting the *Bin1* gene from cells resulted in superinduction of IDO gene expression by IFN-γ. *In vitro* transformation of *Bin1*-null and *Bin1*-expressing primary mouse embryo keratinocytes with c-*myc* and mutant *Ras* oncogenes produced cell lines with similar *in vitro* growth properties. However, when these cells were grafted s.c. into syngeneic animals, the *Bin1*-null cells formed large tumors, whereas the *Bin1*-expressing cells formed only indolent nodules. This dichotomy reflected a difference in immune response to the cells, as *Bin1*-expressing cells produced rapidly growing tumors when introduced into either athymic nude mice or syngeneic mice depleted of $CD4^+/CD8^+$ T cells. Treatment of mice with the small-molecule IDO inhibitor 1-methyl-tryptophan suppressed the outgrowth of *Bin1*-null MR KEC tumors in syngeneic mice, but had no effect on tumor growth in mice lacking T cells (either nude mice or immunodepleted syngeneic animals). Taken together, these findings indicated that the deregulation of IDO, which accompanies *Bin1* loss in these cells, promotes tumorigenicity by enabling immune escape. The frequent Bin1 attenuation and IDO overexpression observed in human cancers warrants further evaluation of the relationship between these two events.

Cooperation of Indoleamine-2,3 Dioxygenase Inhibitors with Chemotherapy

The Bin1-IDO studies prompted us to evaluate IDO inhibitors as potential anticancer agents. This effort revealed that immune modulation via IDO inhibition can significantly increase the efficacy of a variety of traditional chemotherapeutic drugs. In several preclinical models of cancer, single-agent therapy with an IDO inhibitor is only marginally efficacious, at best slowing tumor growth

(11, 12, 25). In contrast, regression of established tumors can be achieved by combining an IDO inhibitor with a cytotoxic chemotherapeutic drug (25). In the MMTV-*neu* transgenic mouse model of breast cancer (harboring the c-*neu* proto-oncogene controlled by the mouse mammary tumor virus promoter), which closely resembles human ductal carcinoma *in situ*, established tumors refractory to single-agent therapy underwent regression when enrolled on the combination regimen. This response could not be explained by drug-drug interactions that might raise effective exposure to the cytotoxic agent, and it was dependent on T-cell immunity because depletion of $CD4^+$ T cells abolished the efficacy of the combination therapy. These results offer an initial step in validating IDO as a drug development target in the context of a cytotoxic combination treatment modality.

As a possible drug development target, IDO has a number of appealing features. First, as a single-chain catalytic enzyme with a well-defined biochemistry, IDO is highly tractable for developing small-molecule inhibitors compared with most other therapeutic targets in cancer. Second, the only other enzyme that catalyzes the same reaction, TDO2, has a more restricted expression and substrate specificity, mitigating "off-target" issues posed by novel agents. Third, bioactive and orally bioavailable "lead" inhibitors exist that serve as useful tools for preclinical validation studies. Fourth, an *Indo* gene "knockout" mouse has been reported to be viable and healthy (26), indicating that IDO inhibitors will be unlikely to produce unmanageable mechanism-based toxicities (although promotion of inflammatory conditions would remain a valid concern). Fifth, pharmacodynamic evaluation of IDO inhibitors can be done easily by examining the blood serum levels of tryptophan and kynurenine, the chief substrate and downstream product of the IDO reaction, respectively. Lastly, small-molecule inhibitors of IDO likely offer substantial logistical and cost advantages relative to biological or cell-based therapies that aim at modulating immunity. IDO inhibitors may be useful not only in cancer but also in other pathologic settings, where it is desirable to relieve immune suppression and/or break immune tolerance (e.g., chronic viral infections).

Future Perspective

One general question raised by the work on combining IDO inhibitors with cytoxic agents is how an immunotherapy can effectively enhance the efficacy of chemotherapy. As detailed elsewhere (27), there are at least six critical factors for inducing an antitumor immune response that might be augmented by cytotoxic chemotherapy including antigen threshold, antigen presentation, T-cell response, T-cell traffic, target destruction, and generation of memory. Consensus is lacking as to whether chemotherapy affects immune responsiveness through direct disruption of toleragenic mechanisms or indirectly through tumor cell killing. In some experimental settings, tumor cell killing by cytotoxic agents has been shown to be critical for cooperativity with no evidence of direct effects on cross-presentation by antigen-presenting cells (APC) or on endogenous immune responsiveness (27). The finding that tumor cells killed by alkylating agents such as cyclophosphamide are more effective at activating APCs, when compared with tumor cells killed by antimetabolites or freeze thaw (28), suggests some specificity to this mechanism of immune stimulation. IFN-γ can reportedly sensitize resistant tumor cell lines to apoptosis induction by cytotoxic agents independent of their p53 status (29). In this way, immunotherapy might cooperate with chemotherapy to augment tumor cell killing and indirectly generate additional proinflammatory signals. On the other hand, there is a long history of cyclophosphamide treatment preferentially neutralizing the suppressor arm of the immune system to enhance antitumor responses (30), and such a mechanism of action has been suggested for other cytotoxic agents as well (31). Recently, there has been a growing realization that it is precisely these tolerizing mechanisms that must be overcome for an immunotherapeutic strategy to be successful (1). In this context, both an IDO inhibitor and a cytotoxic agent might be acting as complimentary immunotherapies. Studies have indeed shown that when enhancement of antitumor T-cell responses by immunotherapy with CTLA-4 antibodies (CTLA-4 blockade) was combined with subtherapeutic doses of chemotherapy that shifted the cytokine profile to that of a Th1 response, this potentiated the treatment of established tumors in a mouse model and correlated with enhanced Th1 responsiveness in the treated mice (31). In this context, it is interesting to note that IDO has been proposed to be a downstream effector for the induction of CTLA-4-mediated immune tolerance (8).

IFN-γ may provide a key to understanding how the complex interplay between tumor and stroma is affected by IDO activity and inhibition. A number of reports argue that IFN-γ suppresses tumor outgrowth. Likewise, IDO activity can have antitumor consequences and its up-regulation by IFN-γ may significantly contribute to the negative effect of IFN-γ on tumors (10). These observations seem to run counter to the idea that IDO contributes positively to tumorigenesis, but this interpretation ignores the inherently complex and evolving nature of the interaction between developing tumors and the host immune system. IFN-γ has been directly implicated in the process called immune editing, whereby the immunogenic environment of the host provides positive selection for reduced tumoral immunogenicity (3). Specifically, IFN-γ signaling contributes to an immune-based host environment that suppresses tumor incidence but which can also drive formation of tumors that are more highly aggressive within an immune context (33). At early stages of tumor development, IDO up-regulation by IFN-γ may be detrimental. However, if tumor cells can adapt to the tryptophan poor environment, then keeping IDO under IFN-γ control could give tumor cells the flexibility of turning IDO off and thereby mitigating its negative consequences in the absence of elevated IFN-γ levels that would signal an active Th1 response.

Alternatively, because IDO acts as the rate-limiting enzyme in NAD^+ biosynthesis, one can also envision scenarios in which constitutive expression of IDO in cancer cells is intrinsically beneficial (e.g., under hypoxic conditions that tend to confer drug resistance). Notably, poly(ADP-ribose) polymerase (PARP)–mediated NAD^+ consumption drives "programmed necrosis" independent of the major apoptotic effectors p53, Bax, Bak, and caspases in cancer cells that have become dependent on glycolysis to maintain ATP levels (34). If tumor cells turn on the NAD^+ biosynthesis pathway, they may be able to override sensitization to PARP. In targeting the rate-limiting step for NAD^+ biosynthesis, IDO inhibitors would be expected to cooperate with chemotherapeutic drugs by reestablishing the sensitivity of tumor cells to PARP activation by these drugs. Unlike apoptosis, this necrotic form of cell death is highly proinflammatory potentially incorporating an immune component into the therapeutic response as well. By raising these issues, studies of IDO inhibitor cooperativity with chemotherapy should not only provide insights into the mechanistic basis for this new therapeutic approach but may also afford a deeper understanding of the complex contextual relationship between cancer cells and the multifaceted immune/stromal environment.

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References

1. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5:263–74.

2. Erdman SE, Sohn JJ, Rao VP, et al. $CD4^+$ $CD25^+$ regulatory lymphocytes induce regression of intestinal tumors in ApcMin/$^+$ mice. Cancer Res 2005;65:3998–4004.

3. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21:137–48.

4. Munn DH, Zhou M, Attwood JT, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 1998;281:1191–3.

5. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999; 189:1363–72.

6. Fallarino F, Grohmann U, Vacca C, et al. T cell apoptosis by tryptophan catabolism. Cell Death Differ 2002;9: 1069–77.

7. Munn DH, Sharma MD, Baban B, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005;22:633–42.

8. Grohmann U, Orabona C, Fallarino F, et al. CTLA-4-Ig regulates tryptophan catabolism *in vivo*. Nat Immunol 2002;3:1097–101.

9. Boyland E, Williams DC. The metabolism of tryptophan. 2. The metabolism of tryptophan in patients suffering from cancer of the bladder. Biochem J 1956; 64:578–82.

10. Ozaki Y, Edelstein MP, Duch DS. Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon γ. Proc Natl Acad Sci U S A 1988;85:1242–6.

11. Friberg M, Jennings R, Alsarraj M, et al. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer 2002;101:151–5.

12. Uyttenhove C, Pilotte L, Theate I, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 2003;9:1269–74.

13. Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004; 114:280–90.

14. Sakamuro D, Elliott KJ, Wechsler-Reya R, Prendergast GC. BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. Nat Genet 1996;14:69–77.

15. Peter BJ, Kent HM, Mills IG, et al. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science 2004;303:495–9.

16. Ge K, Duhadaway J, Sakamuro D, Wechsler-Reya R, Reynolds C, Prendergast GC. Losses of the tumor suppressor BIN1 in breast carcinoma are frequent and reflect deficits in programmed cell death capacity. Int J Cancer 2000;85:376–83.

17. Ge K, Minhas F, DuHadaway J, et al. Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma. Int J Cancer 2000;86:155–61.

18. Ge K, DuHadaway J, Du W, Herlyn M, Rodeck U, Prendergast GC. Mechanism for elimination of a tumor suppressor: aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. Proc Natl Acad Sci U S A 1999;96:9689–94.

19. Tajiri T, Liu X, Thompson PM, et al. Expression of a MYCN-interacting isoform of the tumor suppressor BIN1 is reduced in neuroblastomas with unfavorable biological features. Clin Cancer Res 2003; 9:3345–55.

20. Miaczynska M, Christoforidis S, Giner A, et al. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell 2004;116:445–56.

21. Leprince C, Le Scolan E, Meunier B, et al. Sorting nexin 4 and amphiphysin 2, a new partnership between endocytosis and intracellular trafficking. J Cell Sci 2003; 116:1937–48.

22. Elliott K, Sakamuro D, Basu A, et al. Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms. Oncogene 1999;18: 3564–73.

23. Muller AJ, DuHadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Targeted deletion of the suppressor gene Bin1/Amphiphysin2 accentuates the neoplastic character of transformed mouse fibroblasts. Cancer Biol Ther 2004;3:1236–42.

24. Bild AH, Turkson J, Jove R. Cytoplasmic transport of Stat3 by receptor-mediated endocytosis. EMBO J 2002; 21:3255–63.

25. Muller AJ, Duhadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med 2005;11:312–9.

26. Baban B, Chandler P, McCool D, Marshall B, Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase expression is restricted to fetal trophoblast giant cells during murine gestation and is maternal genome specific. J Reprod Immunol 2004;61:67–77.

27. Lake RA, Robinson BW. Opinion: immunotherapy and chemotherapy: a practical partnership. Nat Rev Cancer 2005;5:397–405.

28. Rad AN, Pollara G, Sohaib SM, Chiang C, Chain BM, Katz DR. The differential influence of allogeneic tumor cell death via DNA damage on dendritic cell maturation and antigen presentation. Cancer Res 2003;63:5143–50.

29. Ossina NK, Cannas A, Powers VC, et al. Interferon-γ modulates a p53-independent apoptotic pathway and apoptosis-related gene expression. J Biol Chem 1997;272: 16351–7.

30. Polak L, Turk JL. Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. Nature 1974;249:654–6.

31. Machiels JP, Reilly RT, Emens LA, et al. Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001; 61:3689–97.

32. Mokyr MB, Kalinichenko T, Gorelik L, Bluestone JA. Realization of the therapeutic potential of CTLA-4 blockade in low-dose chemotherapy-treated tumor-bearing mice. Cancer Res 1998;58:5301–4.

33. Shankaran V, Ikeda H, Bruce AT, et al. IFNg and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;410:1107–11.

34. Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB. Alkylating DNA damage stimulates a regulated form of necrotic cell death. Genes Dev 2004; 18:1272–82.

CURRICULUM VITAE

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Education

1992 Ph.D. in Molecular Biology
University of California, Los Angeles CA
Doctoral Dissertation – Determinants for the activation of the BCR/ABL oncogene

1985 B.A. in Chemistry/Biology
Pomona College, Claremont CA

Professional Experience

5/06-Present **Associate Professor**
Lankenau Institute for Medical Research, Wynnewood, PA

5/02-5/06 **Assistant Professor**
Lankenau Institute for Medical Research, Wynnewood, PA
Primary Projects
- Small molecule immunotherapeutics targeting IDO for cancer treatment
- Genetic and molecular characterization of the *pgct1* locus
- Conditional Bin1 gene disruption in the mouse to directly investigate Bin1 tumor suppressor activity

Supervisory responsibilities
Two technicians and one postdoctoral fellow

11/01-5/02 **Senior Research Investigator**
Inflammatory Diseases
Bristol-Myers Squibb Company, Wilmington, DE
(acquired DuPont Pharmaceuticals Company)
Primary Projects
- *In vivo* pharmacological profiling of small molecule TACE inhibitors for treatment of rheumatoid arthritis

Supervisory responsibilities
Two staff scientists

1999-2001 **Senior Research Scientist**
Department of Cancer Research
DuPont Pharmaceuticals Company, Glenolden, PA
Primary Projects
- Investigation of the physiological function of the putative tumor suppressor gene Bin1
- *In vivo* evaluation of the efficacy of cdk inhibitory compounds in an ovarectomized-mouse uterine proliferation assay

Supervisory responsibilities
2000-01 Three staff scientists and one Ph.D. level scientist
1999-00 One staff scientist

1992-1999 **Post-Doctoral Fellow**
Molecular Biology Department, Princeton University, Princeton NJ
Preceptor: **Dr. Arnold J. Levine**
Primary Projects:
- Genetic mapping of a male germ cell tumor susceptibility locus of 129 strain mice that demonstrates enhanced penetrance in the absence of functional p53.
- Genetic mapping of the transplantation resistance locus *Gt(B6)* of the embryonal carcinoma cell line F9.

Supervisory responsibilities
1997-99 Supervision of one technician
1997-98 Undergraduate thesis advisor to Katherine Heiden
1996 Undergraduate summer research advisor to Brian Jacobs
1995 Undergraduate summer research advisor to Alexandra Koprowski
1994-95 Undergraduate thesis advisor to Bethany Freeman
1993-94 Undergraduate summer research advisor to Maxellede Ezin

1985-1992 **Pre-Doctoral Fellow**
Molecular Biology Institute, University of California, Los Angeles, Los Angeles CA
Preceptor: **Dr. Owen N. Witte**
Primary Project:
- Determination of the role of BCR sequences in the oncogenic activation of the chimeric BCR/ABL gene product of chronic myelogenous leukemia.

1982-1985 (summers) **Research Associate**
Veterans Administration Medical Center, Sepulveda CA
Preceptor: **Dr. Lajos Piko**
Primary Project:
- Analysis of intracisternal A particles and their expression in early mouse embryos

Adjunct Appointments

2004-Present	Saint Joseph's University. Philadelphia, PA
2006-Present	Department of Pathology and Laboratory Medicine, Drexel University, Philadelphia, PA
2006-Present	Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

Honors and Awards

1992-94	Amgen Postdoctoral Fellowship
1985-88	Cellular and Molecular Biology Training Grant
1984-85	ARCS Foundation Scholarship

Scientific Society Memberships

American Association for Cancer Research
Sigma Xi (Saint Joseph's University Chapter)
American Association for the Advancement of Science
European Society for Cancer Immunology and Immunotherapy

Institutional Committees

2003-Present	Member, Lankenau Institutional Animal Care and Use Committee
2003-05	Member, LIMR Library Committee (Chair 04-05)
2003-05	Member, LIMR Computer Committee
2004-05	Member, LIMR Seminar Committee
2005-07	Member, LIMR Patent Committee
2005-Present	Member, LIMR Equipment Committee (Chair since 05)

Lankenau Hospital Committees

2004-06	Member, Leukemia and Lymphoma Working Group

Study Sections/Grant Reviews

1999-2001	Scientific Reviewer, Molecular Genetics Panel USAMRMC Breast Cancer Research Program
2007	Israel Science Foundation
2008	Association for International Cancer Research

Editorial Board Membership

2008-Present	International Journal of Tryptophan Research

Ad Hoc Reviewer

Cancer Research
Nature Genetics
Proceedings of the National Academy of Sciences
Oncogene
Blood
Cancer Detection and Prevention
Cancer Immunology and Immunotherapy
EMBO Journal
Clinical Chemistry
Cancer Letters

Consulting

2003-05	OncoRx, Inc., Scientific Co-founder and Biological Research Consultant
2005-Present	NewLink Genetics Corp., Consultant (following acquisition of OncoRx)

PUBLICATIONS

Original Research

1. **Muller, A.J.** and O.N. Witte. The 5'-noncoding region of the human leukemia-associated oncogene *BCR/ABL* is a potent inhibitor of *in vitro* translation. *Mol. Cell. Biol.* **9**: 5234-5238 (1989).

2. Lugo, T.G., A.-M. Pendergast, **A.J. Muller,** and O.N. Witte. Tyrosine kinase activity and transforming potency of *bcr-abl* oncogene products. *Science* **247**: 1079-1082 (1990).

3. **Muller, A.J.,** J.C. Young, A.-M. Pendergast, M. Pondell, N.R. Landau, D.R. Littman, and O.N. Witte. BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome positive human leukemias. *Mol. Cell. Biol.* **11**: 1785-1792 (1991).

4. Pendergast, A.-M., **A.J. Muller,** M.H. Havlik, R. Clark, F. McCormick, and O.N. Witte. Evidence for regulation of the human ABL tyrosine kinase by a cellular inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 5927-5931 (1991).

5. Pendergast, A.-M., **A.J. Muller,** M. H. Havlik, Y. Maru, and O.N. Witte. BCR sequences essential for transformation by the BCR/ABL oncogene bind to the ABL SH2 regulatory domain in a nonphosphotyrosine-dependent manner. *Cell* **66**: 161-171 (1991).

6. **Muller, A. J.,** A. M. Pendergast, M. H. Havlik, L. Puil, T. Pawson, and O. N. Witte. A limited set of SH2 domains binds BCR through a high-affinity, phosphotyrosine-independent interaction. *Mol. Cell. Biol.* **12**: 5087-5093 (1992).

7. **Muller, A. J.,** A. M. Pendergast, K. Parmar, M. H. Havlik, N. Rosenberg, and O. N. Witte. *En bloc* substitution of the SH2 domain activates the transforming potential of the c-Abl protein tyrosine kinase. *Proc. Natl. Acad. Sci. U.S.A.* **90**: 3457-3461 (1993).

8. Goga, A., J. McLaughlin, A. M. Pendergast, K. Parmar, **A. Muller,** N. Rosenberg, and O. N. Witte. Oncogenic activation of c-*ABL* by mutation within its last exon. *Mol. Cell. Biol.* **13**: 4967-4975 (1993).

9. **Muller, A.J.,** S. Chatterjee, A. Teresky, and A.J. Levine. The *gas5* gene is disrupted by a frameshift mutation within its longest open reading frame in several inbred mouse strains and maps to murine Chromosome 1. *Mamm. Genome* **9**: 773-774 (1998).

10. **Muller, A.J.,** K.B. Heiden, A.K. Teresky, and A.J. Levine. Genetic mapping of the embryonal carcinoma transplantation resistance locus *Gt(B6)* to mouse Chromosome 8. *Immunogenetics* **49**: 949-956 (1999).

11. **Muller, A.J.,** A.K. Teresky, and A.J. Levine. A male germ cell tumor susceptibility determining locus, *pgct1*, identified on murine chromosome 13. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 8421-8426 (2000).

12. DuHadaway, J.B., W. Du, S. Donover, J. Baker, A. Liu, D.M. Sharp, **A.J. Muller,** and G.C. Prendergast. Transformation-selective apoptotic program triggered by farnesyltransferase inhibitors requires *Bin1*. *Oncogene* **22**:3578-3588 (2003).

13. **Muller, A.J.,** J.F. Baker, J.B. DuHadaway, K. Ge, G.E. Farmer, P.S. Donover, R. Meade, C. Reid, R. Grzanna, A.H. Roach, N. Shah, A.P. Soler, and G.C. Prendergast. Targeted disruption of the murine *Bin1/Amphiphysin II* gene does not disable endocytosis but results in embryonic cardiomyopathy with aberrant myofibril formation. *Mol. Cell. Biol.* **23**:4295-4306 (2003).

14. **Muller, A.J.,** J.B. DuHadaway, P.S. Donover, E. Sutanto-Ward, and G.C. Prendergast. Targeted deletion of the suppressor gene Bin1/Amphiphysin2 accentuates the neoplastic character of transformed mouse cells. *Cancer Biol. Ther.* **3**:1236-1242 (2004).

15. **Muller, A.J.,** J.B. DuHadaway, P.S. Donover, E. Sutanto-Ward, and G.C. Prendergast. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy. *Nat. Med.* **11**:312-319 (2005).

16. 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).

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

18. 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).

19. Metz, R., J.B. DuHadaway, U. Kamasani, L.L. Kleintop, **A.J. Muller**, and G.C. Prendergast. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor IDO inhibitory compound D-1MT. *Cancer Res.* **67**:7082-7087 (2007).

20. Chang, M.Y., J. Boulden, J.B. Katz, L. Wang, T.J. Meyer, A.P. Soler, **A.J. Muller**, and G.C. Prendergast. Bin1 ablation increases susceptibility to cancer during aging, particularly lung cancer. *Cancer Res*. **67**:7605-7612 (2007).

21. Kumar, S., W.P. Malachowski, J.B. DuHadaway, J.M. LaLonde, P.J. Carroll, D. Jaller, R. Metz, G.C. Prendergast, and **A. J. Muller**. Indoleamine 2,3-dioxygenase is the anticancer target for a novel series of potent naphthoquinone-based inhibitors. *J. Med. Chem.* **51**: 1706-1718 (2008).

22. Ramalingam, A., J.B. Duhadaway, E. Sutanto-Ward, Y. Wang, J. Dinchuk, M. Huang, P.S. Donover, J. Boulden, L.M. McNally, A.P. Soler, **A.J. Muller**, M.K. Duncan, and G.C. Prendergast. Bin3 deletion causes cataracts and increased susceptibility to lymphoma during aging. *Cancer Res*. **68**:1683-1690 (2008).

23. Banerjee, T., J.B. DuHadaway, P. Gaspari, E. Sutanto-Ward, D.H. Munn, A.L. Mellor, W.P. Malachowski, G.C. Prendergast and **A.J. Muller**. A key *in vivo* antitumor mechanism of action of natural product-based brassinins is inhibition of indoleamine 2,3-dioxygenase. *Oncogene* **27**:2851-7 (2008).

24. Kumar, S., D. Jaller, P. Bhumika, J.M. LaLonde, J.B. DuHadaway, W.P. Malachowski, G.C. Prendergast, and **A. J. Muller**. Structure based development of phenyl-imidazole-derived inhibitors of indoleamine 2,3-dioxygenase. *J. Med. Chem.* (*in press*).

25. **Muller, A.J.**, M.D. Sharma, P.R. Chandler, J.B. DuHadaway, M.E. Everhart, B.A. Johnson III, D.J. Kahler, J. Pihkala, A.P. Soler, D.H. Munn, G.C. Prendergast, A.L. Mellor. Chronic inflammation that facilitates tumor progression creates local immune suppression by inducing indoleamine 2,3 dioxygenase. *Proc. Natl. Acad. Sci.* (*submitted*).

Peer-Reviewed Review Articles

1. **Muller, A.J.**, W.P. Malachowski, and G.C. Prendergast. IDO in cancer: Targeting pathological immune tolerance with small molecule inhibitors. *Expert Opin. Ther. Targets* **9**:831-849 (2005).

2. **Muller, A.J.** and G.C. Prendergast. Marrying immunotherapy with chemotherapy: Why say IDO? *Cancer Res.* **65**:8065-8068 (2005).

3. Malachowski, W.P., R. Metz, G.C. Prendergast, and **A.J. Muller**. A new cancer immunosuppression target: indoleamine 2,3-dioxygenase (IDO). A review of the IDO mechanism, inhibition and therapeutic application. *Drugs of the Future* **30**:897-910 (2005).

4. **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).

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

6. Katz, J.B., **A.J. Muller** and G.C. Prendergast. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. *Immunol. Rev.* **222**:206-21 (2008).

Book Chapters and Conference Proceedings

1. Witte, O.N., M. Keliher, **A.J. Muller,** A.M. Pendergast, M. Gishizky, J. McLaughlin, C. Sawyers, Y. Maru, N. Shah, C. Denny, and N. Rosenberg. Role of the *BCR/ABL* oncogene in the pathogenesis of Philadelphia chromosome positive leukemias. In: Origins of Human Cancer: A Comprehensive Review. J. Brugge, T. Curran, E. Harlow, F. McCormick (Ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor , NY. pp. 521-526 (1991).

2. **Muller A.J.** and G.C. Prendergast. Indoleamine 2,3-dioxygenase in immune escape: regulation and therapeutic inhibition. In: Cancer Immunotherapy: Immune Suppression and Tumor Growth. G.C. Prendergast, E.M Jaffee (Ed.) Academic Press, Burlington, MA. pp. 347-368 (2007).

3. **Muller, A.J.**, R. Metz, and G.C. Prendergast. Differential targeting of tryptophan catabolism in tumors and in tumor-draining lymph nodes by stereoisomers of the IDO inhibitor 1-methyl-tryptophan. *International Congress Series* 1304C pp. 250-261 (2007).

Citations (*h*-graph)
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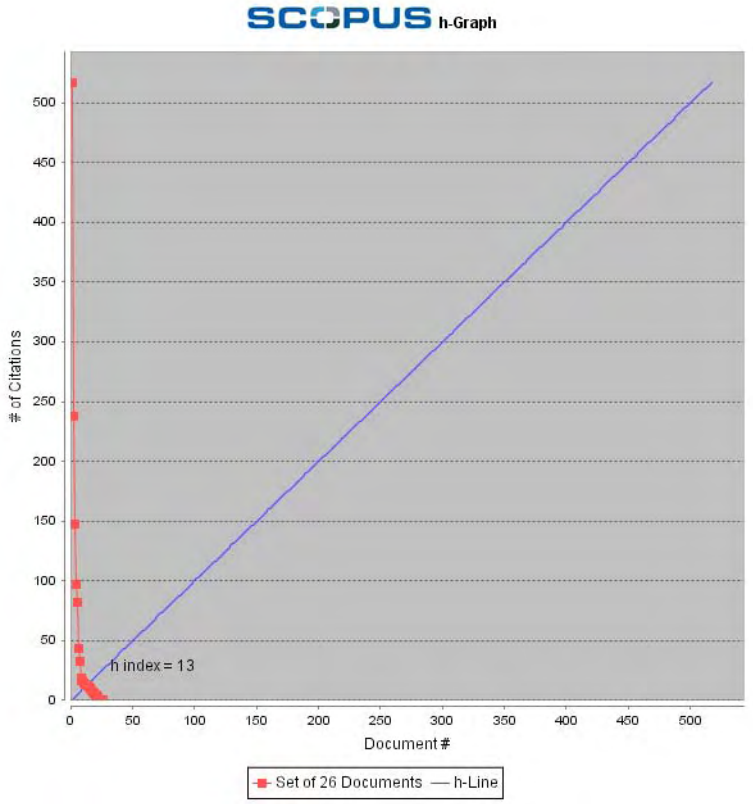

Patent Filings

Application No.: PCT/US2004/005155
Title: "Novel Methods for the Treatment of Cancer"
Inventors: Prendergast, **Muller,** DuHadaway, Malachowski
Jurisdiction Filed: International
Filing Date: 27 March 2003

Application No.: PCT/US2004/005154
Title: "Novel IDO Inhibitors and Methods of Use"
Inventors: Prendergast, **Muller,** DuHadaway, Malachowski
Jurisdiction Filed: International
Filing Date: 27 March 2003

Database Contributions

Mouse Phenome Database Solicited contribution
Accession # MPD:12 Year: 1998
Corresponding investigators: Arnold J. Levine, **Alexander J. Muller**

RECENT LECTURES AND SYMPOSIA (2002-06)

18th Annual Meeting on Oncogenes
La Jolla, CA. June 21-24, 2002
"The Murine *Bin1* Gene is Dispensable for Endocytosis but Critically Involved in Normal Development and the Suppression of *Myc*-driven Tumorigenesis."
(Talk selected from Abstract)

20th Annual Meeting on Oncogenes
Frederick, MD. June 16-20, 2004
"The Cancer Suppression Gene Bin1 Genetically Controls Indoleamine 2,3 Dioxygenase (IDO), an Immunomodulatory Enzyme Identified as a Target for Enhanced Combinatorial Cancer Therapy."
(Talk selected from Abstract)

American Association for Cancer Research 96th Annual Meeting
Anaheim, CA. April 16-20, 2005
"Transcriptional Control of *IDO* by the Cancer Suppression Gene *Bin1*, a Key Mechanism for Restraining Tumor Immune Escape."
(Talk selected from Abstract)

Bryn Mawr Hospital Medical Grand Rounds
Bryn Mawr Hospital, Bryn Mawr, PA. July 7, 2005
"Leveraging Chemotherapy With Drugs Targeting Tumoral Immune Escape"
(Invited Speaker)

International Society for Biological Therapy of Cancer (iSBTc) 20th Annual Meeting
Alexandria, VA. November 10-13, 2005
"Pharmacological Inhibition of the Immunomodulatory Enzyme Indoleamine 2,3-Dioxygenase (IDO) Cooperatively Leverages Cytotoxic Chemotherapy."
(Talk selected from Abstract)

Drexel University, Department of Pathology and Laboratory Medicine, Philadelphia, PA. March 1, 2006
"From Bad to Worse: Investigating How Tumors Escape Extrinsic Host Control Mechanisms"
(Invited Speaker)

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

Kimmel Cancer Center/Lankenau Institute for Medical Research Joint Research Symposium
Thomas Jefferson University, Philadelphia, PA. November 27, 2006
"From Bad to Worse: Investigating How Tumors Escape Extrinsic Host Control Mechanisms"
(Invited Speaker)

NewLink Genetics Corporation, Ames, IA. January 10, 2007
"Evaluation And Development Of IDO Inhibitors To Defeat Tumoral Immune Tolerance"
(Invited Speaker)

29th Annual Induction Ceremony of Sigma Xi
Saint Joseph's University, Philadelphia, PA. April 25, 2007
"Turning the Immune System Against Cancer: New Developments on an Old Idea"
(Keynote Speaker)

Farmingdale State College Campus-Wide Bioscience Seminar Series
Farmingdale State College, Farmingdale, NY. September 17, 2007
"Development of Small Molecule Inhibitors to Defeat Tumoral Immune Tolerance by Targeting IDO"
(Invited Speaker)

International Symposium: Immunoregulation and translational research: Immunology at the crossroads
Centro de Investigacion Medica Aplicada (CIMA), Universidad de Navarra
Pamplona, Spain April 8-9, 2008
"Targeting tumoral immune tolerance with IDO inhibitors"
(Invited Speaker)

SUPPORTING DATA

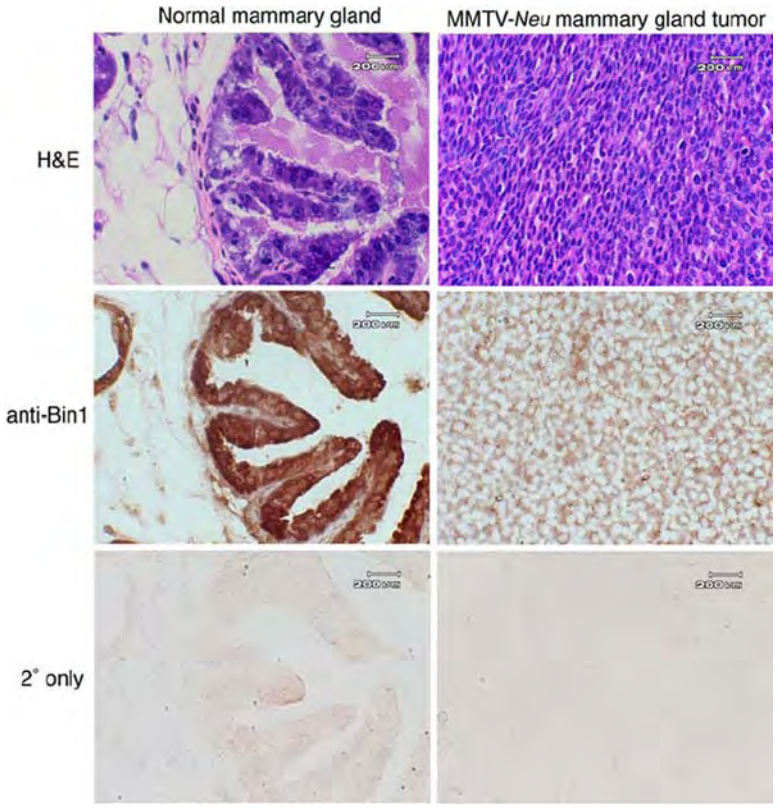

Figure 1. Immunohistochemical analysis of reveals an abnormal Bin1 staining pattern in MMTV-*Neu* mouse mammary gland tumor cells.

Panels on the right are from a representative autochthonous MMTV-*Neu* mouse tumor, panels on the left are from an adjacent normal mammary gland. Formalin-fixed sections were stained with; hematoxalin + eosine (top panels), α-Bin1 antibody 2F11 (middle panels), or secondary antibody alone (bottom panels).

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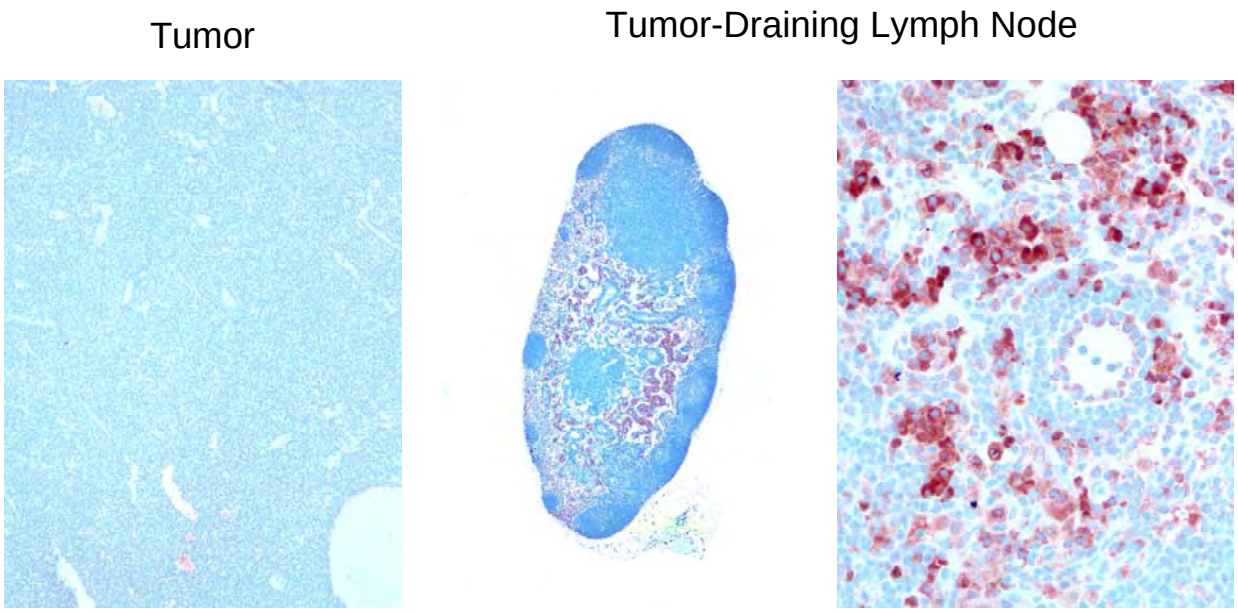

Figure 2. IDO expression is evident in the tumor draining lymph nodes but not in the autochthonous primary tumors formed in MMTV-*Neu* transgenic mice.
Immunohistochemical staining with rabbit polyclonal antibody to mouse IDO. Left: Primary MMTV-*Neu* mammary gland tumor stained for IDO (red, x100). Right: Draining inguinal lymph node stained for IDO (red, x100 & x400).

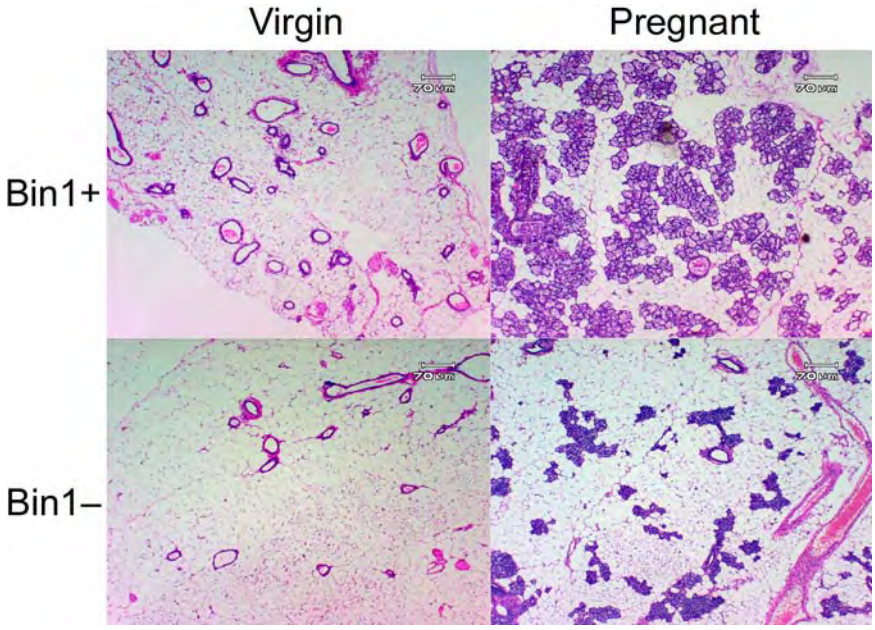

Figure 3. Aberrant mammary gland development associated with Bin1 loss. H&E stained sections of representative formalin-fixed mammary gland tissues from nonparous (left panels) and 18.5 dpc late-term pregnancy (right panels) female mice. Bin+ and Bin1– indicate WapCre$^{+/-}$ Bin1$^{flox/wt}$ and WapCre$^{+/-}$ Bin1$^{flox/KO}$ genotypes respectively.

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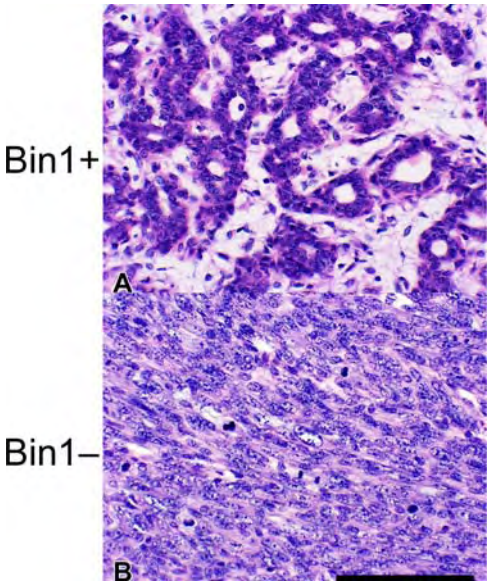

Figure 4. Bin1 loss is associated with poorly differentiated tumor histopathology.
H&E stained sections of DMBA-induced mammary gland tumors. Bin+ and Bin1– indicate Wap*Cre*(+/–), *Bin1*(flox/wt) and Wap*Cre*(+/–),*Bin1*(flox/KO) genotypes respectively.

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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.

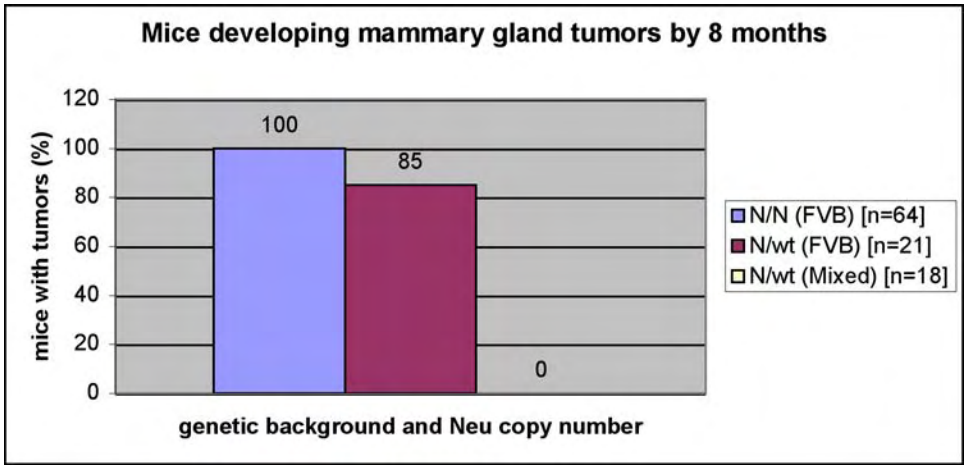
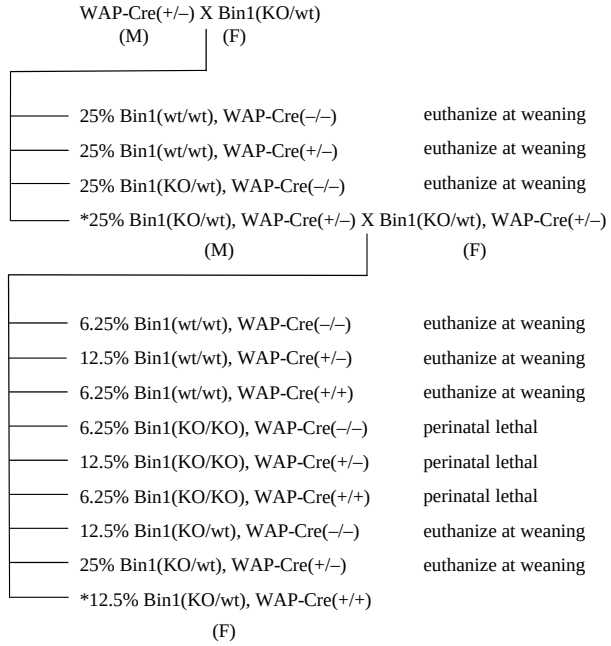


Figure 5. Impact of Neu transgene copy number and strain background on mammary gland tumor development.
Following two rounds of pregnancy and nursing initiated at 2-3 months of age, mice were monitored for the appearance of mammary gland tumors. N/N = homozygosity and N/wt = hemizygosity for the MMTV-Neu transgene. The mixed background includes C57BL/6, 129SvIm/J, and FVB strain alleles.

A WAP-Cre Transgenic Mouse Breeding Flowchart



B MMTV-cNeu Transgenic Mouse Breeding Flowchart

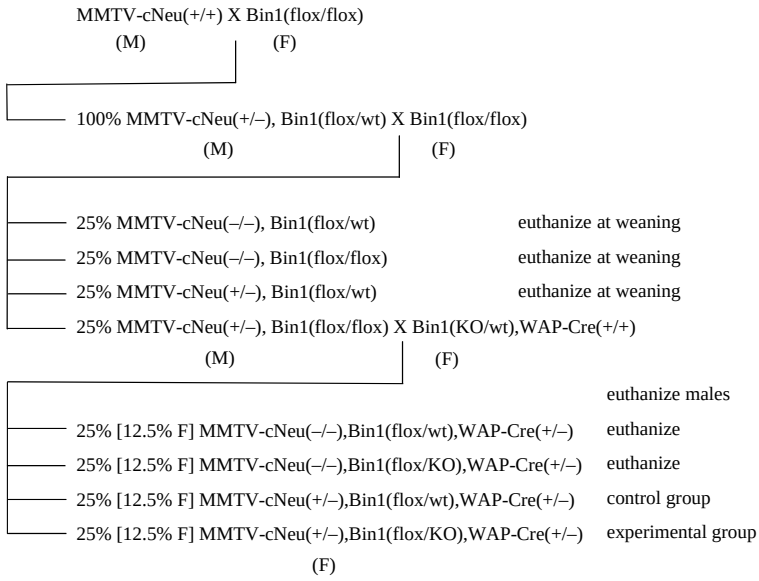


Figure 6. Breeding strategy for producing FVB-strain mammary gland targeted Bin1-null mice. The alleles for the Cre recombinase transgene controlled by the whey acidic protein promoter [WAP-Cre], the constitutive Bin1 knockout [Bin1(KO)], and the conditional Bin1 knockout [Bin1(flox)] were all individually 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.

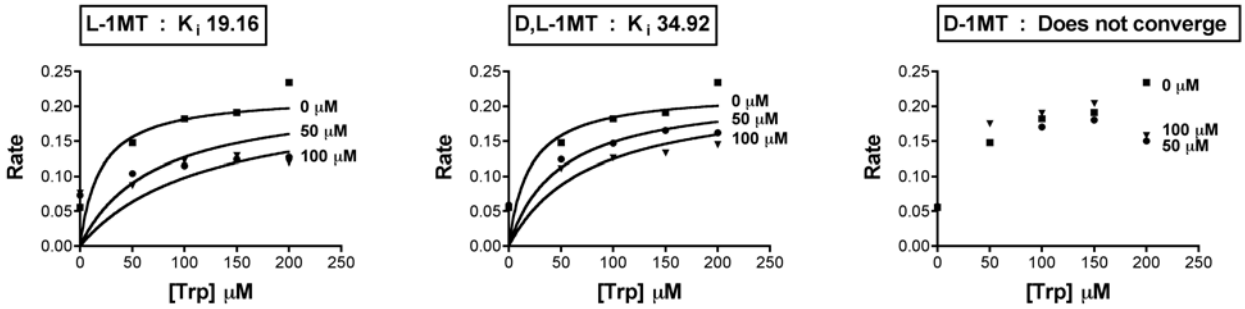


Figure 7. L-MT inhibits purified IDO enzyme activity more effectively than D-1MT.
Enzyme kinetic data demonstrating the impact of the L and D 1MT isomers and the D,L racemate on purified, recombinant IDO enzyme activity in the presence of varying concentrations of L-tryptophan substrate. Global nonlinear regression analysis and computation of best fit Ki values, (shown for each compound), was performed using the Prism4 software package (GraphPad).

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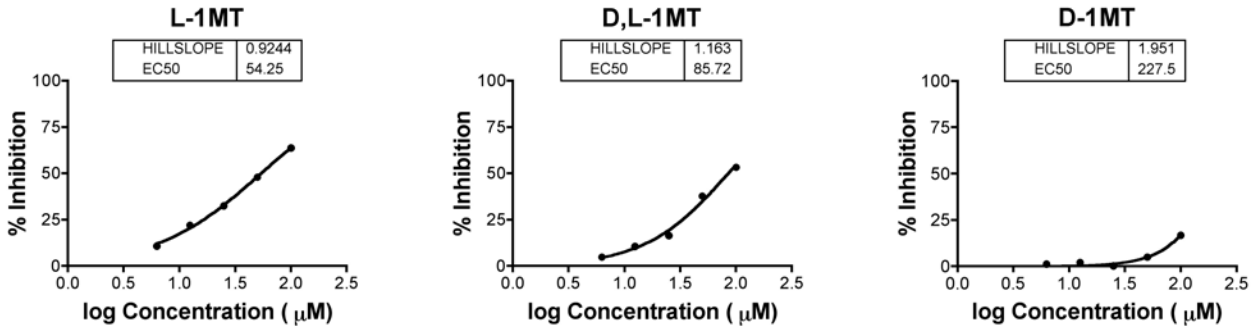

Figure 8. L-MT inhibits IDO enzyme activity in HeLa cells more effectively than D-1MT.
The impact of dose escalation of the L and D 1MT isomers and the D,L racemate on IDO activity was evaluated in HeLa cells stimulated for 24h with IFN-γ. Nonlinear regression analysis and calculation of EC50 and Hillslope values, (shown for each compound), was performed using the Prism4 data analysis program (GraphPad).

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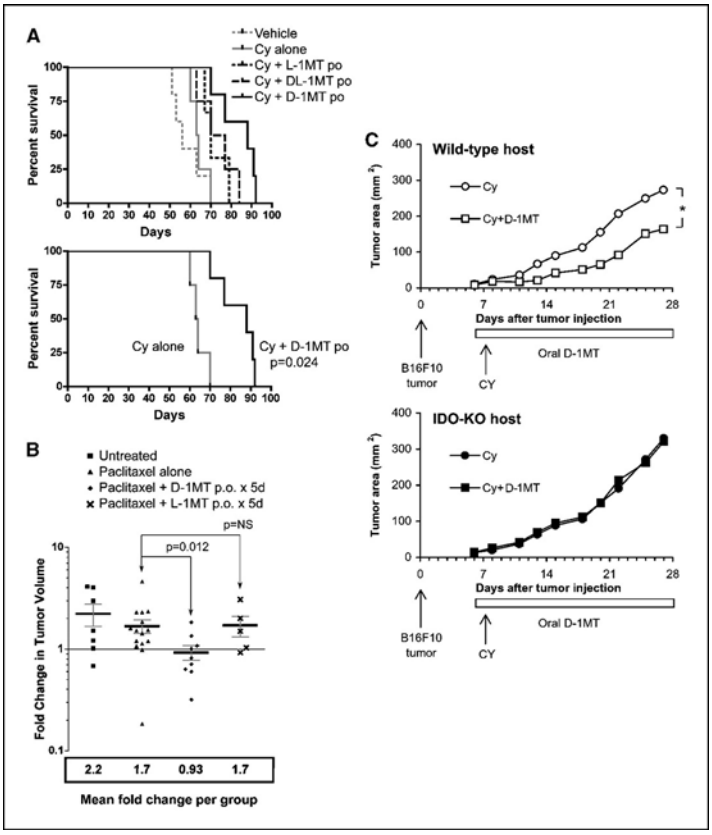

Figure 9. D-1MT provides greater survival benefit in combination therapy, in an IDO-dependent fashion.

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.

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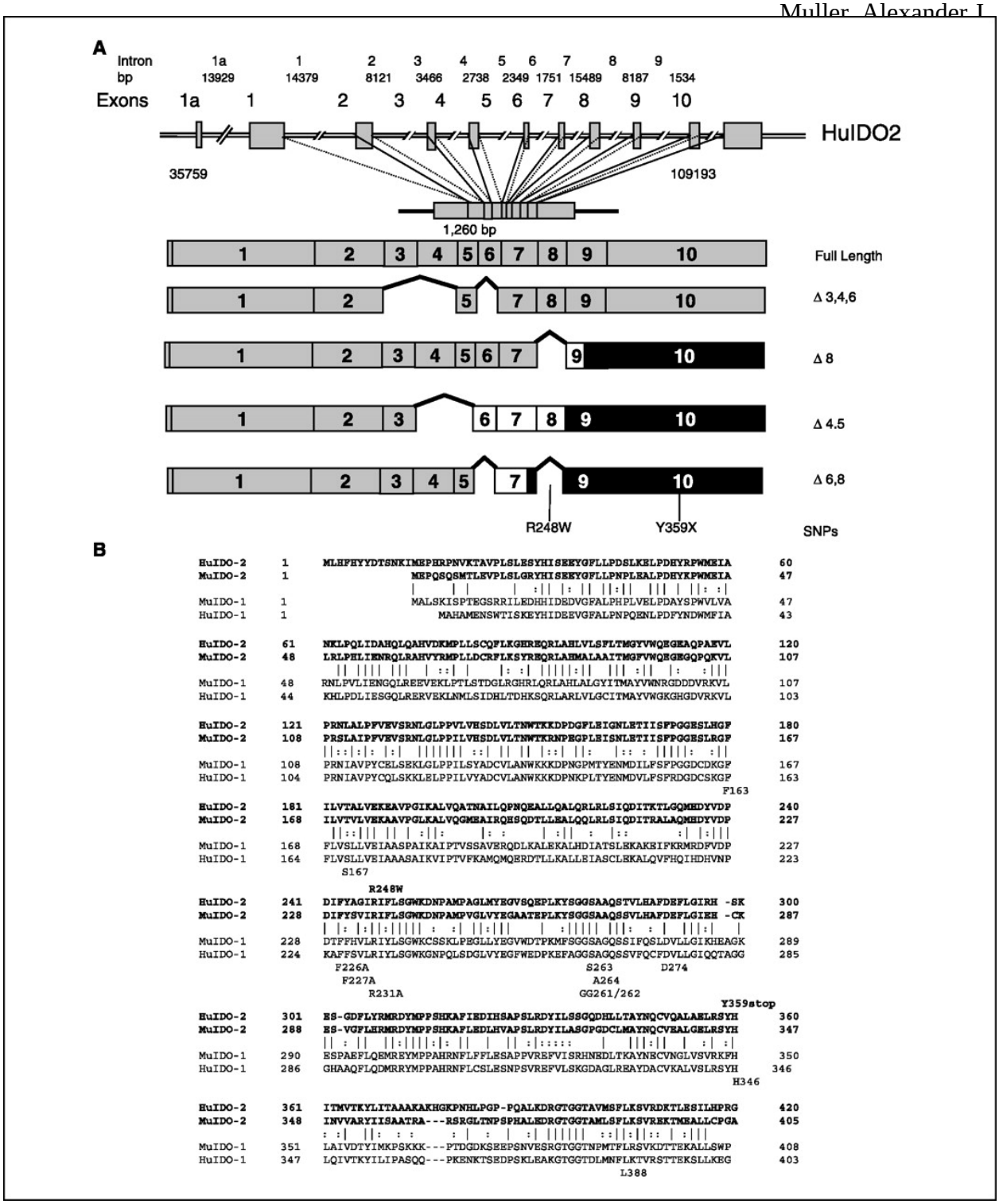

Figure 10. IDO2 structure and similarities to IDO.

A*)* structure of human *IDO2* gene and transcripts. Complete coding region is 1,260 bp encoding a 420-amino-acid polypeptide. Alternate splice isoforms lacking the exons indicated are noted. *White boxes,* a frameshift in the coding region to an alternate reading frame leading to termination. *Black boxes,* 3' untranslated regions. Nucleotide numbers, intron sizes, and positioning are based on IDO sequence files NW_923907.1 and GI:89028628 in the Genbank database. **B)** amino acid alignment of IDO and IDO2. Amino acids determined by mutagenesis and the crystal structure of IDO that are critical for catalytic activity are positioned below the human IDO sequence. Two commonly occurring SNPs identified in the coding region of human IDO2 are shown above the sequence that alter a critical amino acid (R248W) or introduce a premature termination codon (Y359stop).

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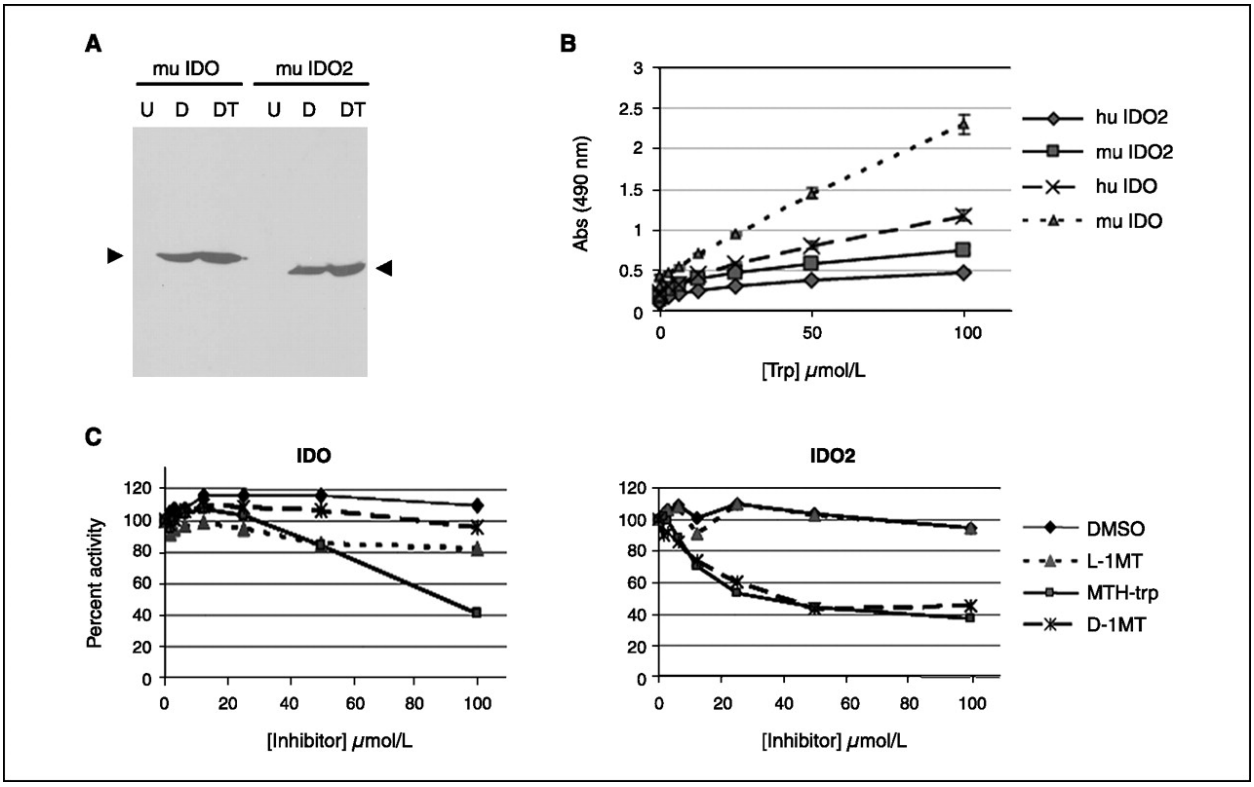

Figure 11. Tryptophan catabolic activity of IDO2 and inhibition by D-1MT.
A) inducible expression of IDO and IDO2 in representative T-REX cells. Western blot analysis of the V5 epitope–tagged proteins indicated was done with a horseradish peroxidase–conjugated anti-V5 antibody (Invitrogen) in cells that were untreated (*U*), treated with 20 ng/mL doxycycline (*D*), or treated with doxycycline and 100 μmol/L tryptophan (*DT*). **B)** tryptophan catabolism. T-REX cells were seeded at 60% to 70% confluence in 96-well dishes in medium supplemented with 0 to 100 μmol/L tryptophan. Kyn production was determined 48 h later and normalized to protein levels as determined by sulforhodamine B assay. Each enzyme was catalytically active, based on increased Kyn levels with increasing substrate concentrations, although IDO2 seemed to be 2- to 4-fold less active than IDO when normalized to protein levels as determined by sulforhodamine B assay. *Points,* mean of values determined in triplicate and normalized to cellular protein levels. *Abs,* absorbance. **C)** effect of IDO inhibitors on IDO2 catalytic activity. T-REX cells were seeded and processed as above except for the addition to the medium of 0 to 100 μmol/L of the IDO inhibitors MTH-trp, L-1MT, D-1MT, or vehicle control (DMSO). *Points,* mean of values determined in triplicate and normalized to cellular protein levels as before.

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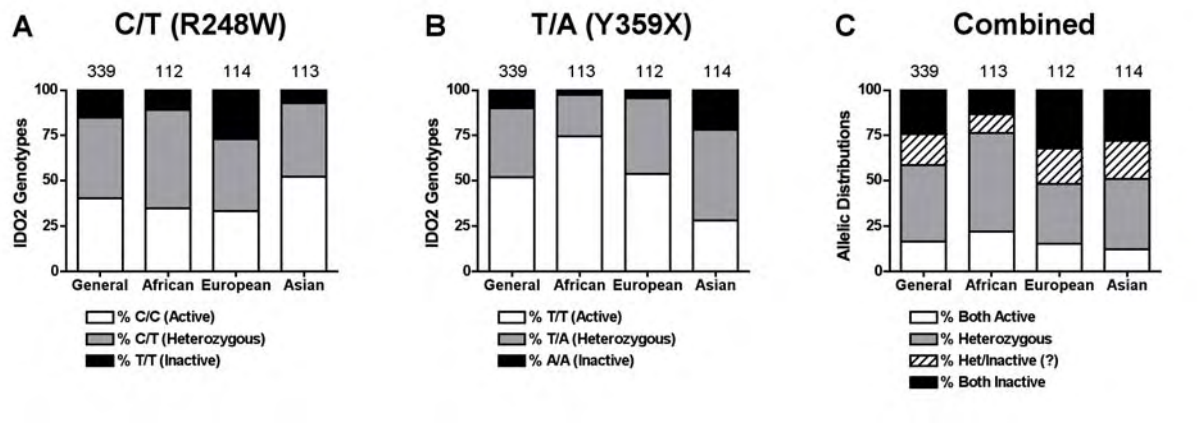

Figure 12. Non-functional *IDO2* SNPs are highly represented in the human population.
A SNP database from 341 individuals was evaluated for the frequency of alternate alleles of the two functional SNPs identified in the human *IDO2* gene, rs10109853 (C/T; codon change R248W) and rs4503083 (T/A; codon change Y359Stop). Of the individuals represented in the databse, 114 were categorized as European, 114 as Asian, 23 as African American and 90 as Sub-Saharan African. The percent distribution of the different allelic pairs are graphed for **A)** the C/T alleles, **B)** the T/A alleles and **C)** the active vs. inactive alleles combining data from both allelic variants. The distribution pattern for the entire dataset is represented by the first bar on the left followed by separate analyses for the African, European, and Asian groups. The N for each analysis is listed at the top of the bar. In a few instances, the SNP analysis was uninformative resulting in the N values being somewhat lower than the total number of individuals evaluated. In panel C it was sometimes impossible to unequivocally determine whether two inactivating SNPs were located in the same or different alleles, and these instances are represented separately in the distribution pattern by a hatched segment on the graph. However, sequence data presented in this report indicating that the two polymorphism tend to be independently segregating alleles suggests that the distribution of these equivocal alleles is likely to be skewed toward both being inactive. The SNP datasets are available through the NCBI ENTREZ SNP web site at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snp.

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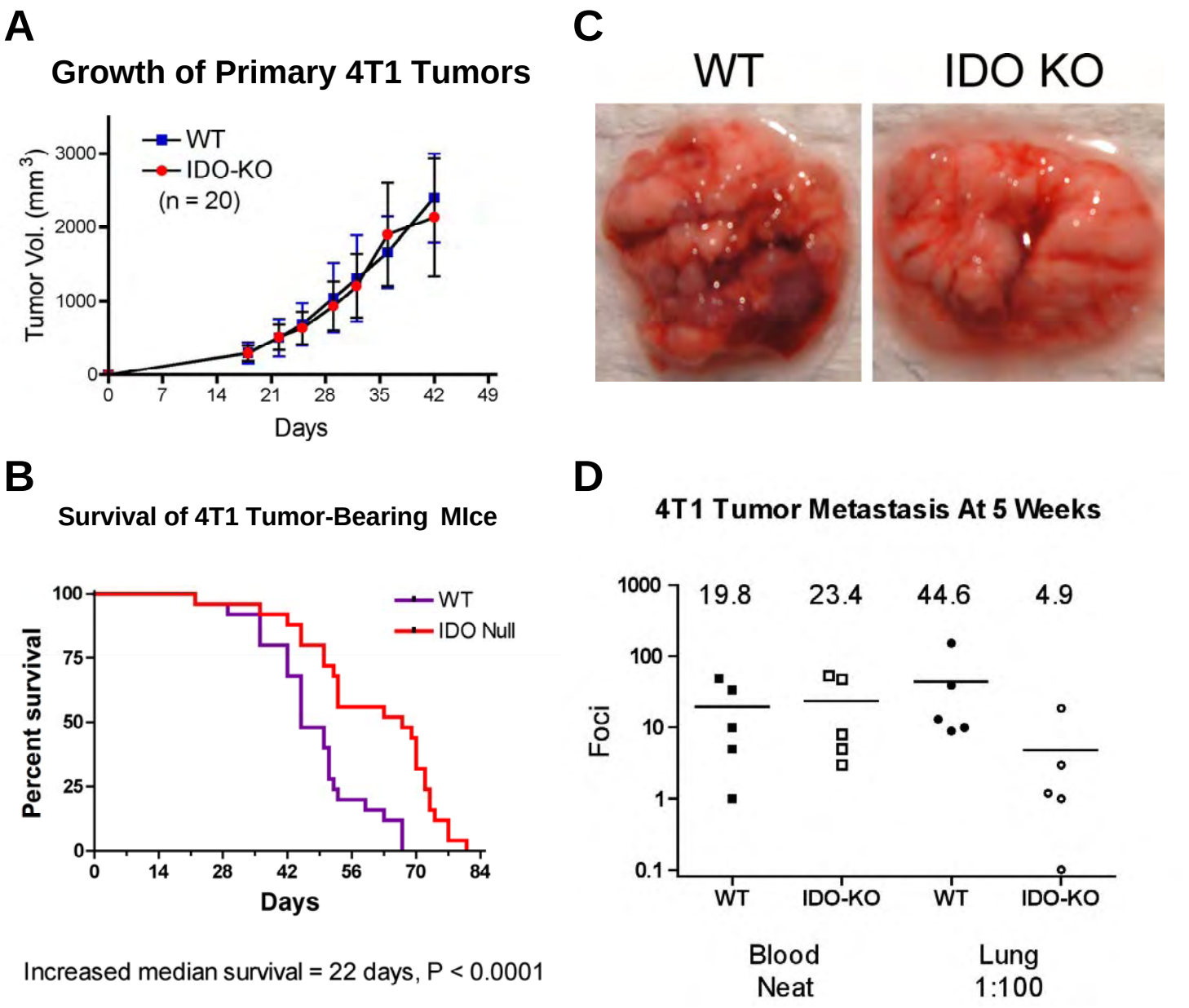

Figure 13. Loss of IDO in the stroma prolongs the survival of mice bearing 4T1 breast tumor isografts. by impairing metastasis.
4T1-luc orthotopic isografts were established by injection of $1x10^4$ cells into the mammary fatpad of wild type and IDO knockout BALB/c strain mice. **A**) Growth of primary tumors as determined by caliper measurements. Graphed as the mean ± SEM. **B**) Survival of mice bearing 4T1 tumors. 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 $p < 0.05$. **C**) Lungs from mice taken at 7 weeks following challenge with 4T1 cells. **D**) Number of 6-thioguanine-resistant colonies formed from blood and lung biopsies taken from mice at 5 weeks following challenge with 4T1 cells.