Supplemental online material

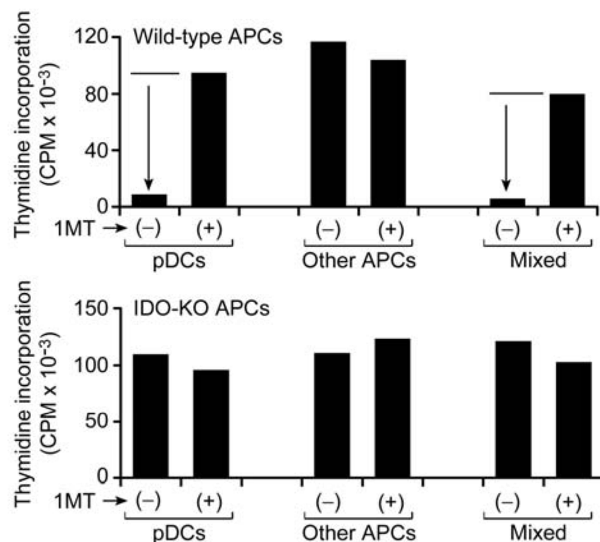

Figure S1. The D-isomer of 1MT is specific for IDO.
Tumors were grown in either IDO-deficient (IDO-KO) hosts or WT hosts, both on the B6 background. The pDC fraction ($CD11c^{+}B220^{+}$) containing the IDO^{+} DCs was sorted from TDLNs. The balance of TDLN cells, which included all the other APCs, was also collected in each sort. MLRs were performed using the two populations (pDCs and all other cells) as stimulators for BM3 T cells, separately and mixed together. The upper panel demonstrates suppression by the pDC fraction, which was reversed by D-1MT. There was no suppression by the other-APC fraction, and no effect of D-1MT. Mixing of the two stimulator populations demonstrated that suppression by IDO was dominant, as previously described (1). However, when the pDC fraction was derived from IDO-KO mice, there was no suppression by pDCs, and no effect of D-1MT on T cell proliferation. Thus, IDO-KO DCs lacked the target for D-1MT, confirming that the D isomer acted on IDO, not via some off-target effect.

Additional detailed methods

Mice

Animal studies were approved by the institutional animal use committee of the Medical College of Georgia or the Lankenau Institute for Medical Research. C57BL/6 (B6) mice and rag1-KO mice (B6 background) were from Jackson Laboratory (Bar Harbor, ME). FVB/N-Tg(MMTVneu)202Mul/J mice, homozygous for a rat cNeu transgene under the mouse mammary tumor virus promoter (MMTV-*Neu* mice) (2) were from Jackson Laboratory. BALB/cAnNCr

mice were from Charles River Laboratories (Frederick, MD). IDO-KO mice have been previously described (3).

Sources of 1MT isomers

Studies of the various 1MT isomers in human DCs were performed on at least two different sets of each compound, supplied in blinded fashion by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD), and results confirmed using multiple different commercial lots of each isomer obtained from Sigma-Aldrich. All experiments gave similar results. For in vivo studies, 1-methyl-D-tryptophan was supplied by the Developmental Therapeutics Program, National Cancer Institute and confirmed using commercially prepared D and DL isomers (Sigma-Aldrich). 1MT was prepared as a 20 mM stock solution in 0.1 N NaOH and adjusted to pH 7.4; solutions were stored at 4° C and protected from light.

Administration of 1MT and chemotherapy agents

Administration of 1MT by implantable subcutaneous pellets was performed as described (4, 5). Pellets tended to release higher amounts during the early period of the infusion, so the treatment periods stated are the nominal release times.

To prepare 1MT for oral gavage, 1 g of 1MT (Sigma) was added to a 15 ml conical tube with 7.8 ml Methocel/Tween [0.5% Tween 80/0.5% Methylcellulose (v/v in water; both from Sigma)]. The mixture was bead milled overnight by adding 1-2 ml by volume of 3 mm glass beads (Fisher) and mixing by inversion. The next day, the 1MT concentration was adjusted to 85 mg/ml by adding an additional 4 ml Methocel/Tween and mixing again briefly. The 1MT slurry was administered by oral gavage at 400 mg/kg/dose (0.1 cc/20 g mouse) using a curved feeding needle (20 G x 1 1/2 in; Fisher). For bid (twice a day) dosing, 1MT was administered once in the morning and once in the evening.

For administration in drinking water, D-1MT was prepared at 2 mg/ml in water as described above, supplemented with a small amount of aspartame (2 envelopes per liter) to improve acceptance by the mice, and filter sterilized. The solution was delivered in standard autoclaved drinking-water bottles. Mice drank 4.5-5.0 ml/day (similar to consumption of water without drug). Plasma levels of D-1MT at the end of 6 days were 33-40 uM.

Paclitaxel (Hanna Pharmaceuticals, Wilmington, DE), 6 mg/ml in 50% Cremphor EL / 50% ethanol, was diluted in saline delivered i.v. Cyclophosphamide was from Bristol-Myers Squibb (Princeton, NJ) or Hanna Pharmaceuticals, and gemcitabine from Eli Lilly (Indianapolis, IN).

In vivo bioluminescence imaging of 4T1-luc tumors

For bioluminescence imaging experiments, luciferase-expressing derivative of the 4T1 cell line (4T1-luc) was prepared by stable transfection of pCAG-luc (generously provided by Dr. J Sawicki), which expresses the firefly luciferase gene under the control of the β-actin promoter and the CMV IE enhancer. Transfection was followed by three rounds of single cell cloning to establish a cell line with stably expressed luciferase activity. Prior to imaging, tumor-bearing mice were anesthetized by intramuscular injection of a mixture of 25 mg/kg ketamine/5 mg/kg xylazine hydrochloride (Hanna Pharmaceuticals, Wilmington, DE). Anesthetized mice were injected intraperitoneally with 150 mg/kg firefly luciferin (Xenogen, Alameda, CA). At 5 min after administration of the substrate, *in vivo* images were acquired with an IVIS charge-coupled-device camera system (Xenogen). Data analysis was performed with the LivingImage 2.5

software package (Xenogen).

IDO enzyme assays

Purification of recombinant human His6-tagged IDO produced by E. coli strain BL21DE3pLys, and the 96-well plate-based spectrophotometric assay to monitor enzymatic activity, were performed essentially as described (6). Briefly, 1MT enantiomers were solubilized in dimethylsulfoxide (DMSO) containing 0.1N HCl and added at concentrations of 100, 50, and 0 µM (but maintaining constant DMSO and HCl dilutions of 1:1000) to wells containing the reaction mixture (6) in which the tryptophan concentration was varied from 0-200 µM, followed by addition of IDO enzyme. Plates were sealed with plastic wrap and incubated 1 hr in a humidified 37˚C incubator, after which the reactions were terminated by addition of 12.5 µl 30% TCA per well. Plates were then resealed in plastic wrap, incubated 30 min at 50˚C to hydrolyze the reaction product N-formylkynurenine to kynurenine, and centrifuged 10 min at 2400 rpm in a Sorvall tabletop centrifuge. Supernatants were transferred to a flat-bottom 96-well plate, mixed with 100 µl Ehrlich reagent (2% p-dimethylamino benzaldehyde w/v in glacial acetic acid), and incubated 10 min at room temperature. Absorbance at 490 nm was read on a Bio-Tek Synergy NT plate reader to quantitate the reaction product.} Global nonlinear regression analysis and computation of best fit Ki values was performed using the Prism4 software package (GraphPad).

For HeLa cell assays, HeLa human tumor cells (ATCC) were seeded at 4.0 x 10^4 cells per well in DMEM/phenol red free media supplemented with 10% FBS (Hyclone) and penicillin-streptomycin (Gibco). The following day, 1-MT enantiomers or the racemic mixture were solubilized in DMSO/0.1 N HCl and serially diluted in assay wells while maintaining the DMSO/HCl dilution constant at 1:1000. 100 ng/ml of human recombinant IFN-γ (R&D Systems, Minneapolis, MN) was then added per well to stimulate IDO expression. Following IFN-γ addition, plates were incubated 20 hr at 37˚C in a humidified CO2 incubator. Supernatants (200 µl media/well) were harvested and analyzed for kynurenine as described (5).

For measurement of kynurenine production by human DCs in allo-MLRs, culture supernatants were harvested on day 5 of MLR and analyzed by high-performance liquid chromatography as previously described (1). Similar patterns were also obtained at days 2 and 3 of MLR.

Human monocyte-derived APCs and allo-MLRs.

Work with human materials was performed under protocols approved by the Institutional Review Board of the Medical College of Georgia. Our systems for culturing IDO^+ human monocyte-derived DCs and macrophages have been previously described (1, 7). For DCs, the features of the culture system relevant to maximizing IDO expression included the use of leukocytapheresis followed by counterflow elutriation for preparation of monocytes; culture for 7 days in serum-free X-vivo15 medium (BioWhitaker, Walkersville, MD) supplemented with GMCSF + IL4; maturation during the final 48 hrs of culture with TNFα, IL1β, IL6 and PGE2 (but without IFNγ, CD40-ligand or TLR-ligands); and harvesting of only the non-adherent cell fraction. The PGE2 reagent was found to be labile, and so was frozen in aliquots and mixed fresh for each experiment. Monocyte-derived macrophages were cultured as previously described (7) using recombinant human macrophage colony-stimulating factor (R&D Systems), with IFNγ (100 U/ml) added for the last 24 hrs.

For human allo-MLRs, 2.5×10^4 nonadherent DCs were mixed with 5 x 10^5 allogeneic lymphocytes in 250 ul of medium (10% fetal calf serum in RPMI-1640) in "V"-bottom culture

wells (Nalge-Nunc, Rochester, NY). V-bottom wells gave superior IDO activity, as previously described (1). For mitogen-activated T cell proliferation, purified lymphocytes were activated with immobilized anti-CD3 antibody plus soluble anti-CD28 (1). After 5 days, proliferation was measured by 4 hr [^{3}H]thymidine-incorporation assay.

Mouse tumor-draining lymph node pDCs and MLRs

Mouse IDO-expressing DCs from tumor-draining lymph nodes, as used in our previous publications (8, 9). Tumors were implanted using 1 x 10^{6} B78H1·GM-CSF cells injected subcutaneously in the anteriomedial thigh of syngeneic wild-type B6 mice, or mice with a targeted disruption of the IDO gene (IDO-KO mice) (3). After 11 days, inguinal LNs were removed for cell sorting to enrich for IDO^{+} DCs, which were contained in the plasmacytoid DC fraction (CD11c^{+}B220^{+} cells). These sorted B220^{+}CD11c^{+} DCs contained all of the IDO-mediated suppressor activity, and were used as stimulators in allo MLRs as described (8). Responder cells were 1×10^{5} nylon-wool enriched BM3 TCR-transgenic BM3 T cells, recognizing the -H2K^{b} allo-antigen expressed on the B6-background DCs, as described (8). After 3 days, proliferation was measured by 4-hr thymidine incorporation assay. All MLRs were performed in V-bottom culture wells (Nalge-Nunc, Rochester, NY), and the IDO^{+} DCs were not irradiated.

Western blots

Affinity-purified polyclonal rabbit antibody was raised against the peptide sequence DLIESGQLRERVEKLNMLC, from the N-terminal portion of the published human IDO sequence (NM002164) (10), conjugated to KLH, and has been previously described (11). Affinity-purified polyclonal rabbit antibody against the C-terminal peptide sequence LKTVRSTTEKSLLKEG conjugated to ovalbumin was prepared similarly. 2D-Western blots were performed using a Protean IEF cell and Mini-Protean blotting system (BioRad, Hercules, CA) as described (11). Both antibodies detected single bands consistent with the predicted molecular weight for one or more splice-variants of IDO (12), and the bands were fully neutralized in Western blots by their respective immunizing peptides.

To rule out the possibility that the N-terminal epitope (which was constitutively expressed) might be a spurious cross-reacting band, we conducted a BLAST search of the Genbank protein database using the immunizing peptide. This peptide sequence matched only IDO. The N-terminal band was constitutive in macrophages, but validation studies showed that the band was not found in other cell types (e.g., B cells), and the levels of the N-terminal band was regulated in DCs by maturation status. Reactivity of the N-terminal antibody was fully neutralized by the immunizing peptide sequence.

Statistical analysis

For analysis of variance, the comparison was performed on the two arms that of interest in order to test for an effect of 1MT when combined with chemotherapy: thus, in all analyses, the chemotherapy+vehicle arm was compared to chemotherapy+1MT arm in each experiment. Because the group sizes were small in each individual experiments, 3 identical experiments were performed and analyzed together where possible. If needed, a log transformation was used on tumor size to render tumor growth to experiment end a linear trend with homogeneous variance across time. A regression of tumor size on day was performed for each mouse. The resulting slopes were interpreted as the rate of growth of tumor size to end-point (euthanasia). Where

indicated, identical experiments were pooled and analyzed in a 3 Experiment × 2 Group analysis of variance (ANOVA) where an interaction between experiment and group was investigated. When comparing the effect of chemotherapy+vehicle and chemotherapy+1MT on the rate of tumor growth for two types of mice (WT vs. IDO-KO) a 2 Group x 2 Type ANOVA with interaction was used.

In Fig. 2 and Fig. 6 comparisons of interest were fold-change in tumor size, and age at death, respectively. Since all mice were alive at the end of the experiments the survival data were not censored and the mean was an unbiased representation of μ. However, for experiments using both survival and fold-change measurements, the SDs between groups varied considerably, and the fold-change measurements were likely not normally distributed; therefore, data were analyzed using a two group Wilcoxon exact test, due to the distributional issues and the small sample size. Significance was determined at $p < [0.05/(\text{number of comparisons})]$ for each experiment. SAS version 9.1.3 was used for all analyses

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