# Supporting Information

## Pilotte et al. 10.1073/pnas.1113873109

### SI Materials and Methods

**Cell Lines and Tumor Samples.** P815B (a gift from P. Chen, Harvard Medical School) is a subline of mastocytoma P815 previously used as a vector-transfected control (1, 2). P815B cells were transfected with expression vector pEF6/V5-His (Invitrogen) containing the mouse *TDO2* ORF and selected with 5 μg/mL Blasticidin (Invitrogen). Control cells were transfected with plasmid pEF6/V5-His-LacZ. Cell lines P1.istA-B-, P815B-mIDO cl6, and L1210. P1A.B7-1 have been described (3–6). Human HEK-293-EBNA cells (Invitrogen) were transfected with expression vector pEF6/V5-His containing the human *TDO2* ORF and were similarly selected. TDO-positive clones were selected and used in all experiments. The human *TDO2* ORF was amplified from liver RNA by RT-PCR using sense primer 5′-AACCTCCGTGCTTCTCA-3′ and antisense 5′-TCATCTTAATGCTTAGTCTTG-3′ primer. The mouse *TDO2* ORF was amplified from liver RNA using sense primer 5′-ACCTGTGTGGTCCTGAGA-3′ and antisense primer 5′-TCCTCCTAATCGCTCACC-3′. Samples of neoplastic lesions were selected from archival material.

**Cellular Assay for TDO and IDO Activity.** The assay was performed in 96-well flat bottom plates seeded with $2 \times 10^5$ cells in a final volume of 200 μL for mouse cell lines and in 48-well flat bottom plates seeded with $2.5 \times 10^5$ cells in a final volume of 500 μL for human cell lines. To determine TDO or IDO activity, the cells were incubated overnight at 37 °C in HBSS (Hanks Balanced Salt Solution, Invitrogen) supplemented with 80 μM L-tryptophan (mouse cell lines) or in IMDM (Invitrogen) (human cell lines). The plates were then centrifuged 10 min at 300 × *g*, and 150 μL of the supernatant were collected. The supernatant was analyzed by HPLC to measure the concentration of tryptophan and kynurenine, based on the retention time and the UV absorption (280 nm for tryptophan, 360 nm for kynurenine). For the HPLC analysis, 55 μL of supernatant were mixed with 55 μL of 6% (wt/vol) trichloroacetic acid to precipitate the proteins. After centrifugation, 100 μL of supernatant were collected and injected onto an Onyx Monolithic C18 column (Phenomenex).

**Enzymatic Assay for TDO Activity on Crude Extracts.** Enzymatic assays of the recombinant human TDO (hTDO) expressed in *Escherichia coli* were conducted as described in ref. 7. Dry pellets of frozen tumor cells were lysed in 50 mM potassium phosphate buffer (pH 7.5), 150 mM KCl, 250 mM sucrose, 1 mM L-tryptophan, 10 μM bovine hemin and protease inhibitors (complete EDTA free, Roche Applied Science). The extract was first centrifuged at 4 °C for 5 min at 700 × *g*, and the supernatant was centrifuged for 15 min at 20,000 × *g*. The buffer of the clarified extract was then exchanged to 50 mM potassium phosphate buffer (pH 7.5) using a HiTrap desalting column (GE Healthcare), and aliquots were frozen in liquid nitrogen and kept at −80 °C until use. TDO activity was measured as follows. The reaction mixture contained (final concentrations) 50 mM potassium phosphate buffer (pH 7.5), protease inhibitors, 20 mM ascorbic acid, 10 μM methylene blue, 500 units/mL catalase (catalase from bovine liver, Sigma), and L-tryptophan as indicated in the absence or presence of 1-methyl-L-tryptophan or TDO inhibitor 680C91 as indicated. The reaction was initiated by the addition of 100 μL of cell extract to 100 μL of reaction mixture prewarmed at 37 °C. The reaction was conducted at 37 °C for 10, 30, and 60 min and stopped by addition of 40 μL trichloroacetic acid 30% (wt/vol). To convert *N*-formylkynurenine to kynurenine, the reaction mixture was incubated at 65 °C for 30 min. Then, 125 μL of the reaction mixture were mixed with 125 μL of 2% (wt/vol) 4-(Dimethylamino)benzaldehyde in acetic acid and incubated for 10 min at room temperature. Kynurenine concentrations were determined by measuring the absorbance at 480 nm. A standard curve was made with pure kynurenine. The rate of catalysis was calculated from the increase of kynurenine concentration between 10 min and either 30 or 60 min, as required to stay within the linear phase of *N*-formylkynurenine production.

**Lysis Assay.** Chromium release assay was performed in 4 h as described (4), using CTLs derived from splenocytes of mice expressing a transgenic P1A-specific TCR (8). A 50-fold excess of unlabeled P1.ist A-B- cells was added to all targets as competitor cells (4).

**Tetramer Staining and Fluorescence-Activated Cell Sorting Analysis.** H-2L$^d$/P1A tetramers were produced as described (9). Peritoneal cells were stained for 15 min at room temperature in PBS buffer containing 1% BSA, 10 nM phycoerythrin-labeled H-2L$^d$/P1A tetramer, and FITC-conjugated antibody to Fc-γ receptor III. Peridinin chlorophyll protein-conjugated antibody to CD8 and FITC-conjugated antibodies to CD4, CD11b, and CD19 (all from BD PharMingen) were added for an additional incubation of 15 min. Cells were analyzed on a FACScan flow cytometer (BD Biosciences). The CD8-positive and FITC-negative cells were gated.

**Quantitative RT-PCR Analysis of mRNA Expression.** RNA purification and cDNA synthesis were performed as described (10). Quantitative PCR amplifications were performed with the PCR core kit (Eurogentec). 1/40 of the cDNA produced from 2 μg of total RNA was amplified in a final volume of 25 μl in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). For *IDO1*, we performed as described (5). For human *TDO2*, we used sense primer 5′-CATGGCTGGAAAGAACTC-3′, antisense primer 5′-CTGAAGTGCTCTGTATGAC-3′ and probe FAM-5′-TTTAGAGCCACATGGATTTAACTTCTGGG-3′-TAMRA. For mouse TDO2, we used sense primer 5′-CATGGCTGGAAAGAACAC-3′, antisense primer 5′-GGAGTGCACGGTATGAC-3′ and probe FAM-5′-TTTAGAGCCAAATGGATTTAATTTCTGGG-3′-TAMRA. Conditions for PCR were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Specificity of the primers was assessed by cloning and sequencing PCR products. *TDO2* and *IDO1* expression levels were normalized to the β-actin expression level and expressed as number of mRNA per cell, based on our previous estimation of 2,000 actin mRNA molecules per tumor cell, as described (11).

For classical RT-PCR (Table 4), RNA purification and cDNA synthesis were performed as described (10), and PCR amplification was performed with Takara Taq (Takara). 1/100 of the cDNA produced from 2 μg of total RNA was amplified in a final volume of 25 μL in a Biometra UNO-thermoblock. For human *IDO1*, we used sense primer 5′-GAGGAGCAGACTACAAGAATG-3′ and antisense primer 5′-GCATACAGATGTCTCTGCTATG-5′ in the following conditions: 5 min at 94 °C, 31 cycles (94 °C for 1 min, 61 °C for 1 min and 72 °C for 2 min), and 10 min at 72 °C. For human *TDO2*, we used sense primer 5′-TTGGACTTCAATGACTTCAGAGA-3′ and antisense primer 5′-TGCCCAGCATTCTGTGC-3′ in the following conditions: 5 min at 94 °C, 31 cycles (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min), and 10 min at 72 °C.

**Cellular Assay for TDO Inhibition.** To determine the IC50, $2 \times 10^5$ cells from P815B-mTDO cl12, from P815B-mIDO cl6 (5) or

from 293E-hTDO cl119 were incubated 8 h at 37 °C in 200 µl HBSS supplemented with 80 µM L-tryptophan and a titration of the compound ranging from 0.156 to 80 µM (680C91) or from 0.78 to 400 µM (LM10). The concentration of tryptophan and kynurenine was measured by HPLC-UV. In the conditions used for IC50 estimation, about 50% of the initial amount of tryptophan was degraded in the absence of inhibitor, and an equimolar amount of kynurenine was produced, whereas the reaction rate remained constant (Fig. S4). The percentages of inhibition of tryptophan degradation and kynurenine production by the compounds were calculated in reference to this maximal activity. Dose–response curves for the inhibition of tryptophan degradation and the inhibition of kynurenine production were usually superimposable. The initial wells containing the cells in the remaining volume of 50 µL were used to estimate cell viability in a classical MTT assay. To that end, 50 µL of culture medium (Iscove medium with 10% FCS and amino acids) were added to the wells together with 50 µL of MTT. After 3–4 h of incubation at 37 °C, 100 µL of SDS/DMF were added to dissolve the crystals of formazan blue and the absorbance at 570 nm/650 nm was measured after overnight incubation at 37 °C.

**Compounds Synthesis and Characterization.** All chemical reagents and solvents were used as obtained from commercial sources (Sigma Aldrich, Acros). All reactions were performed under an inert argon (Alphagaz 2) atmosphere. Melting points were determined with a Büchi B-540 capillary melting point apparatus in open capillaries and are uncorrected. TLC was performed on silica gel plates ($60F_{254}$, 0.2 mm thick, Merck) with visualization under UV light (254 nm and 365 nm). $^{1}$H-NMR spectra were recorded in $d_6$-DMSO solution on a Jeol JNM EX 400 spectrometer at 400 MHz with tetramethylsilane (TMS) as internal standard. $^{13}$C- and $^{19}$F-NMR spectra were recorded on the same spectrometer in $d_6$-DMSO solution at 100 MHz and 376.2 MHz, respectively. Chemical shifts (δ) are expressed in ppm downfield from TMS. Elemental analyses (C, H, N) were performed on a Thermo Finnigan FlashEA 1112 apparatus. Analytical LC/MS analyses were performed on an Agilent 1100 Series LC/MSD Trap system using UV detection at 254 and 361 nm. Mass spectra were recorded using electron spray ionization (ESI) operating in positive mode. The following two methods were applied: *method 1*: injection of 10 µL of a 20 µg.mL$^{-1}$ acetonitrile solution onto a $C_{18}$-3.5µm Zorbax SB column (100 × 3 mm); separation using a gradient (flow rate: 0.5 mLmin$^{-1}$) of acetonitrile in acetic acid (0.1% vol/vol in water) from 5 to 95% acetonitrile over 5 min, kept for 3 min, then reversed to 5% acetonitrile within 0.1 min and hold for an additional 5.4 min. *Method 2*: injection of 10 µL of a 2 µg.mL$^{-1}$ acetonitrile solution onto a $C_{18}$-5µm Agilent LiChrospher 100 column (250 × 4 mm); separation using a gradient (flow rate: 1 mLmin$^{-1}$) of increasing concentration of acetonitrile in a mobile phase acetonitrile and acetic acid (0.1% vol/vol in water) from 20 to 80% over 20 min. At 20 min, the gradient was kept at 80% acetonitrile for 20 min. HRMS were obtained by direct infusion of a solution at 400 pg·µL$^{-1}$ (acetonitrile/formic acid 0.1% in water; 50/50) on an ESI-TOF MSMS spectrometer, maXis (UHR-TOF, Bruker). The acquisition was made with micrOTOFcontrol (Bruker) in positive mode from 50 to 690 *m/z* in 1 s with the "focus mode" activated to increase the resolution. Calibration was made with sodium acetate. The spectra were processed and deconvoluted with Data analysis (Bruker). Automated flash chromatography was performed on a Biotage AB (Uppsala, Sweden) SP1 system equipped with prepacked flash KP-Sil silica cartridges. A gradient of ethylacetate/cyclohexane was used for elution. The elution started with an ethylacetate/cyclohexane ratio of 12/88 for one column volume (CV), the ratio increased to 62/38 over 5 CV, kept for 1 CV, then increased to 100/0 over 7 CV, and finally kept at this value over 5 CV. The synthesized molecules were detected by UV absorption at 254 and 320 nm. All new compounds were determined to be at least >95% pure by LC/MS. The yields reported refer to chromatographically and spectroscopically pure (>95%) product fractions.

The lead compound, 680C91, was prepared from commercially available 6-fluoro-1*H*-indole-3-carbaldehyde as described in the literature (12). The *trans*-6-Fluoro-3-[2-(1*H*-tetrazol-5-yl)vinyl]-1*H*-indole, LM10, was prepared from 6-fluoro-1*H*-indole-3-carbaldehyde via the corresponding *trans*-3-(6-fluoro-1*H*-indol-3-yl) acrylonitrile.

**Synthesis of *trans*-3-(6-fluoro-1*H*-indol-3-yl)acrylonitrile.** 6-Fluoro-1*H*-indole-3-carbaldehyde (12.4 g, 76 mmol) was dissolved in dioxane (400 mL). Triethylamine (26 mL, 185 mmol) and cyanoacetic acid (11 g, 128 mmol) were added and the resulting clear bright orange solution was stirred for 15 min at room temperature. Piperidine (16.5 mL, 165 mmol) was added, and the mixture was heated at reflux temperature for 18 h. Upon cooling down, the reaction mixture was concentrated under reduced pressure to 200 mL, ethyl acetate (500 mL) and water (500 mL) were then added, and vigorously stirred. The organic layer was collected, and the aqueous phase was extracted with ethyl acetate (1 × 200 mL). The organic layers were combined, washed with brine (1 × 200 mL) and dried over $MgSO_4$. After filtration, silica (30 g) was added to the organic phase, and the solvent evaporated under reduced pressure. The crude residue was deposited on the top of the column for automated flash chromatography purification. The product was eluted with ~50% ethyl acetate. The pooled column fractions were concentrated under reduced pressure, and cyclohexane (300 mL) was added to precipitate the title compound. The precipitate was collected, washed twice with cyclohexane (2 × 30 mL), and dried at 40 °C under reduced pressure to afford the title compound as a yellow powder (4.6 g, yield = 33%), m.p.: 170–172 °C; TLC (EtOAc:cyclohexane, 2:1 vol/vol): RF = 0.64; LC-MS $t_R$ 5.8 min (method 1), 14.0 min (method 2), *m/z* [MH+] 187; $^{1}$H-NMR (400 MHz, $d_6$-DMSO): δ 11.85 (1H, bs), 7.90 (1H, dd, $J$ = 8.8, 5.4 Hz), 7.85 (1H, s), 7.68 (1H, d, $J$ = 16.7 Hz), 7.23 (1H, dd, $J$ = 9.9, 2.5 Hz), 6.98 (1H, dt, $^{3}J$ = 9.3 Hz, $^{4}J$ = 2.3 Hz), 6.05 (1H, d, $J$ = 16.7 Hz); $^{13}$C-NMR (100 MHz, $d_6$-DMSO); δ 159.8 (d, $J_{CF}$ = 235.5 Hz), 144.5, 137.9 (d, $J_{CF}$ = 12.4 Hz), 132.5, 121.8, 121.6 (d, $J_{CF}$ = 10.5 Hz), 121.0, 112.6, 109.8 (d, $J_{CF}$ = 24.8 Hz), 99.2 (d, $J_{CF}$ = 25.7 Hz), 89.6; $^{19}$F-NMR (376.2 MHz, $d_6$-DMSO): δ -119.7; UV/vis: $\lambda_{max}$ 320 nm; HRMS (*m/z*): $[M]^+$ calcd for $C_{11}H_7FN_2$, 186.0593; found, 186.0597; analysis (% calcd, % found for $C_{11}H_7FN_2$): C (70.96, 71.18), H (3.79, 3.70), N (15.05, 14.83).

**Synthesis of *trans*-6-Fluoro-3-[2-(1*H*-tetrazol-5-yl)vinyl]-1*H*-indole (LM10).** The *trans*-3-(6-fluoro-1*H*-indol-3-yl)acrylonitrile was subjected to a [3+2] cycloaddition with azide according to a general procedure described in the literature (13): anhydrous aluminum chloride (4.1 g, 31 mmol) was suspended in dry tetrahydrofuran (THF; 20 mL) at 0 °C. Sodium azide (5.4 g, 83 mmol) was added and the suspension was heated at reflux temperature for 2 h and then allowed to cool down for 10–15 min. The starting material 4 (4.3 g, 23 mmol) was added at this point along with additional 5 mL of THF (used for rinsing the flask). The dark vermilion-colored suspension was heated at reflux temperature for 18 h. Upon cooling down to room temperature, the reaction mixture was poured into 1M aqueous citric acid (180 mL). This phase was extracted three times with ethyl acetate (3 × 250 mL). The combined organic layers were washed with brine (1 × 150 mL) and dried over $MgSO_4$. After filtration, silica (10 g) was added to the organic phase, and the solvent evaporated under reduced pressure. The crude residue was deposited on the top of the column for automated flash chromatography purification. The product was eluted with ~75% ethyl acetate. The pooled column fractions were processed as described above for *trans*-3-(6-fluoro-1*H*-indol-3-yl)

acrylonitrile to afford the title product as a pale yellow powder (1.7 g, yield = 32%), m.p.: 228–230 °C; TLC (EtOAc:cyclohexane, 2:1 vol/vol): RF = 0.04; LC-MS $t_R$ 5.1 min (method 1), 11.2 min (method 2), *m/z* [MH+] 230; $^1$H-NMR (400 MHz, $d_6$-DMSO): δ 11.69 (1H, bs), 7.89–7.87 (2H, m), 7.78 (1H, d, $J$ = 16.7 Hz), 7.22 (1H, dd, $J$ = 9.8, 2.3 Hz), 7.02 (1H, d, overlapped), 7.01 (1H, overlapped); $^{13}$C-NMR (100 MHz, $d_6$-DMSO); δ 159.7 (d, $J_{CF}$ = 235.5 Hz), 155.2, 137.7 (d, $J_{CF}$ = 12.39 Hz), 132.2, 130.5, 122.2, 121.1 (d, $J_{CF}$ = 9.53 Hz), 112.7, 109.4 (d, $J_{CF}$ = 23.8 Hz), 104.9, 99.0 (d, $J_{CF}$ = 25.8 Hz); $^{19}$F-NMR (376.2 MHz, $d_6$-DMSO): δ -120.2; UV/vis: $\lambda_{max}$ 326 nm; HRMS (*m/z*): $[M]^+$ calcd for $C_{11}H_8FN_5$, 229.0764; found, 229.0769; analysis (% calcd, % found for $C_{11}H_8FN_5$): C (57.64, 57.35), H (3.52, 3.63), N (30.55, 30.31).

**Statistics.** The statistical analyses were performed using SPSS software (SPSS). We used the two-tailed Student *t* test for statistical analysis of the proportions of P1A-specific T cells (Fig. 1*D*). In the experiments reported on Figs. 1*A* and 2*B*, tumor occurrence was monitored from the day of tumor challenge (day 0). Times to tumor appearance were plotted according to the Kaplan Meier method and were compared with the logrank test.


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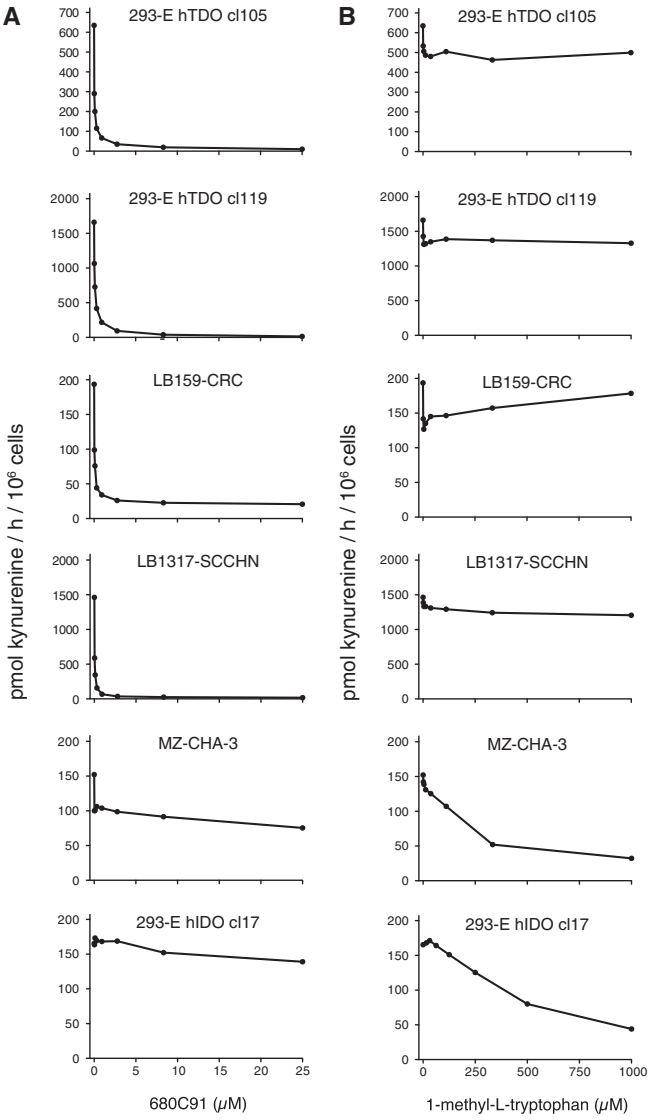


**Fig. S1.** Full dose–response curves for TDO inhibition by 680C91 (*A*) and 1MT (*B*) in the cellular assay of cell lines listed in Table 2. Cell lines 293-E hTDO cl105, 293-E hTDO cl119 and LB159-CRC express only TDO. Cell lines LB1317-SCCHN and MZ-CHA-3 express both TDO and IDO, TDO expression being prominent in the former line and IDO in the latter line. Cell line 293-E hIDO cl17 (1) expresses only IDO. The cellular assay was performed as described in Materials and Methods, under linear conditions with no more than 50% reduction of the initial tryptophan concentration (80 μM). The data illustrate the respective contribution of TDO and IDO in cell lines expressing both enzymes. The activity values may differ from those reported in Table 2 because the experiments were performed with different batches of cells collected many months apart, and because of slight differences in the experimental setting.

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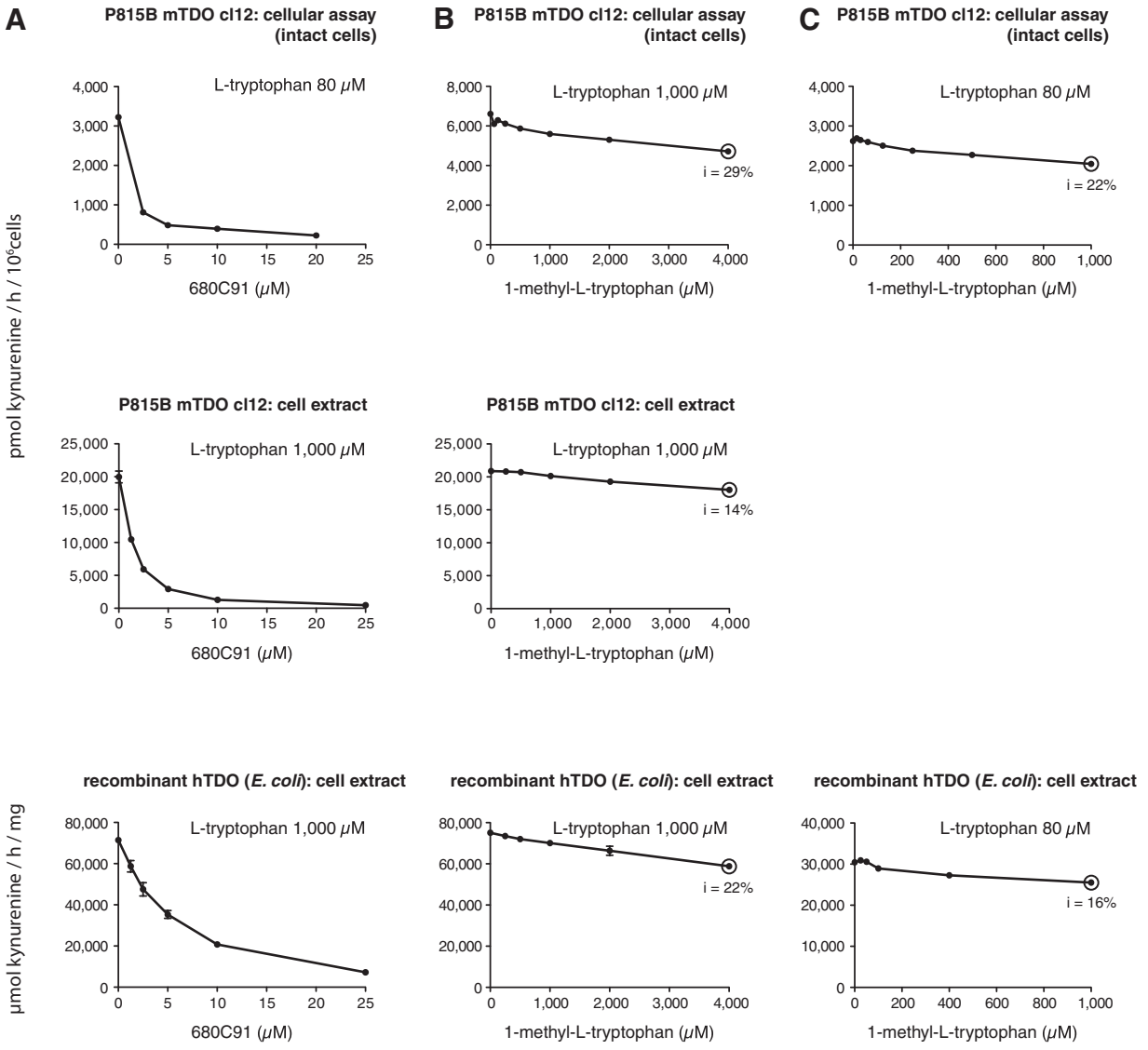


**Fig. S2.** Comparison of dose–response curves for TDO inhibition tested in the cellular assay (*Top*), the assay on cell extracts (*Middle*), and the recombinant TDO (*Bottom*). (*A*) TDO inhibitor 680C91 was tested at the indicated concentrations (0–25 μM) in a cellular assay performed with P815B mTDO cl12 cells (*Top*) or in an in vitro assay performed with cell extracts from the same cells (*Middle*) or in an in vitro assay performed with recombinant human TDO (*Bottom*). The initial tryptophan concentration is indicated. (*B*) IDO inhibitor 1-methyl-L-tryptophan (1MT) was tested at the indicated concentrations (0–4000 μM) in a cellular assay performed with P815B mTDO cl12 cells (*Top*) or in an in vitro assay performed with cell extracts from the same cells (*Middle*) or in an in vitro assay performed with recombinant TDO (*Bottom*). A tryptophan initial concentration of 1 mM, suited for the in vitro assay, was used in all three assays to allow comparisons. The percentage of inhibition (i) is indicated for the highest dose of 1MT. (*C*) IDO inhibitor 1MT was tested at the indicated concentrations (0–1000 μM) in a cellular assay performed with P815B mTDO cl12 cells (top) or in an in vitro assay performed with recombinant TDO (bottom). A physiological initial concentration of tryptophan (80μM) was used. The percentage of inhibition (i) is indicated for the highest dose of 1MT. Kynurenine production was measured as indicated in Materials and methods. The data indicate a weak inhibitory effect of 1MT at high doses in the cellular assay, which is likely due partly to inhibition of tryptophan transport by 1MT as reported (1) and partly to direct inhibition of TDO activity by 1MT at high doses, as observed in the in vitro assays with cell extract and with recombinant human TDO.

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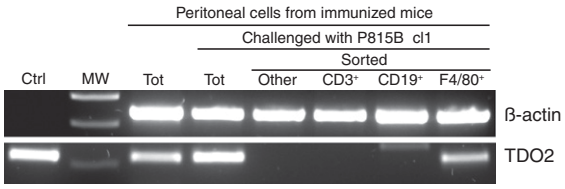
A




B

| | Sorted peritoneal cells | TDO2 mRNA/1,000 cells |
|---|---|---|
| Naive mice | $CD3^+$<br>$CD19^+$<br>$F4/80^+$<br>$CD3^-$, $CD19^-$, $F4/80^-$ | nt<br>0<br>0.5<br>0 |
| Immunized mice | $CD3^+$<br>$CD19^+$<br>$F4/80^+$<br>$CD3^-$, $CD19^-$, $F4/80^-$ | 0<br>0<br>12<br>0 |
| Immunized mice challenged with tumor cells 3 days before the experiment | $CD3^+$<br>$CD19^+$<br>$F4/80^+$<br>$CD3^-$, $CD19^-$, $F4/80^-$ | 0<br>0<br>1.3<br>0 |
| Immunized mice challenged with tumor cells 7 days before the experiment | $CD3^+$<br>$CD19^+$<br>$F4/80^+$<br>$CD3^-$, $CD19^-$, $F4/80^-$ | 0<br>0<br>8<br>0 |

**Fig. S3.** Expression of TDO in peritoneal macrophages. (*A*) RT-PCR amplification of TDO2 mRNA in cells isolated from the peritoneal cavity of mice immunized (or not) against P1A and challenged or not with TDO-negative P815B cells. Cells were sorted (or not) by FACS into $CD3^+$, $CD19^+$ or $F4/80^+$ cells. TDO2 cDNA was used as positive control (Ctrl). (*B*) Quantitative assessment of TDO2 expression in sorted peritoneal cells. Expression was measured by real-time RT-PCR (Taqman) and normalized to β-actin. Mice were immunized (or not) by i.p. injection of living L1210.P1A.B7-1 cells ($10^6$ cells) and challenged (or not) 4 wk later by intraperitonal injection of TDO-negative P815B cl1 ($4 \times 10^5$ cells). After 3 d (*A* and *B*) or 7 d (*B*), peritoneal cells were collected (six or seven mice per group) by washing the peritoneal cavity with 4 mL of EDTA-PBS (4 °C). Mononuclear cells were isolated with Lymphoprep (Axis-Shield), and sorted on a FacsAria III (BD Biosciences) after triple staining with FITC-conjugated anti-CD3 (cl17A2), APC-conjugated anti-CD19 (cl6D8) and PE-conjugated anti-F4/80 (clA3-1) (Biolegend). Total RNA was isolated from frozen pellets of sorted cells with TriPure isolation Reagent (Roche). Taqman was performed as described in Materials and Methods. Classical RT-PCR was performed for TDO2 using the same primers as for the Taqman at the same temperature for 40 cycles; and for β-actin (25 cycles) as described in ref. 1.

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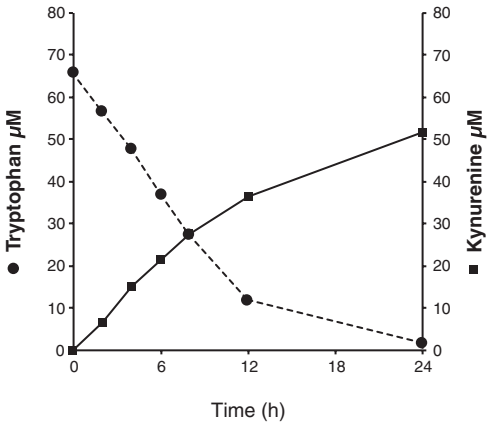


**Fig. S4.** Tryptophan and kyurenine concentration in supernatants of TDO-expressing cells plated in the conditions used for the cellular assay. P815B mTDO cl12 ($2 \times 10^5$ cells) were incubated in 200 μL of HBSS containing 80 μM tryptophan. Tryptophan and kynurenine concentrations were measured by HPLC at the indicated times. The data show that the reaction remains linear until about 50% tryptophan is degraded. These conditions were used to test the inhibitors and calculate IC50 (Fig. 2, Fig. S1, and Fig. S2). The data also show that most of the tryptophan is degraded into kynurenine, indicating that the assay indeed reliably measures TDO activity.