SUPPLEMENTAL INFORMATION

Tumor-expressed IDO recruits and activates MDSCs in a Treg-dependent manner

Rikke B. Holmgaard, Dmitriy Zamarin, Yanyun Li, Billel Gasmi, David H. Munn, James P. Allison, Taha Merghoub, and Jedd D. Wolchok

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and Balb/c mice (6-8 weeks old) as well as $IDO^{-/-}$ and $Rag^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratory. Pmel-1 TCR transgenic mice have been reported (Muranski et al., 2008; Overwijk et al., 2003) and were provided by N. Restifo (National Cancer Institute (NIH), Bethesda, MD). $Foxp3^{GFP}$ knock-in mice were a gift from Dr. A. Rudensky (Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY), and were previously described (Schaer et al., 2013). $Foxp3^{DTR}$ mice were generated in the laboratory of Dr. G. J. Hammerling (German Cancer Research Center (DKFZ), Heidelberg, Germany), and previously described (Li et al., 2010). All mice were maintained in microisolator cages and treated in accordance with the NIH and American Association of Laboratory Animal Care regulations. All mouse procedures and experiments for this study were approved by the MSKCC Institutional Animal Care and Use Committee.

Cell lines

The murine cancer cell lines for melanoma (B16F10, referred to as B16) and breast cancer (4T1) were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and penicillin with streptomycin (complete RPMI media). B16-IDO was generated by transduction of B16F10 with *GFP* plus the *IDO* gene as previously described (Holmgaard et al., 2013). B16F10 transduced with *GFP* alone were used as control cells (B16-WT).

Detection of IDO protein by western blotting

For detection of IDO expression, tumor cells were harvested and washed twice with PBS. Cells were then lysed in lysis buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 10% glycerol, and protease inhibitor cocktail (Sigma-Aldrich)). Cell lysate was centrifuged at 12,000 RPM for 15 minutes. The protein concentration of supernatnats was measured by Bradford Protein Assay (New England BioLabs). Equal amounts of the total protein were separated by 4-12% Bis-Tris Gel with MOPS SDS Running Buffer (Invitrogen) and immunoblotted with rabbit polyclonal IDO antibody at a final concentration of 1:200 (0.001 ug/ml) (Santa Cruz Biotechnology). Horseradish peroxidase conjugated goat-anti-rabbit IgG served as secondary antibody (Jackson ImmunoResearch).

Intracellular staining for IDO protein

For detection of IDO expression, tumor cells were harvested and washed twice with PBS. After fixation and permeabilization using FoxP3 Fixation and Permeabilization Kit (eBioscience), cells were stained with a rabbit polyclonal anti-IDO antibody (Santa Cruz Biotechnology). FITC conjugated anti-rabbit IgG served as secondary antibody (Jackson ImmunoResearch).

IDO activity

The biological activity of IDO was evaluated by measuring the level of tryptophan and L-kynurenine in tumor and blood sera from B16-IDO and B16-WT tumor-bearing mice. Mouse blood was obtained by retro-orbital puncture when tumors

were 500 to 1000 mm in diameter. The conditioned medium related to the same number of cells was centrifuged at 14,000 RPM for 5 minutes to remove debris. Blood was allowed to clot for 30 minutes on ice and centrifuged at 3,600 RPM for 5 minutes and serum was aspirated. Tumors were weighed, placed in 350 µl PBS, and homogenized with bead homogenizer for 5 minutes. Debris were removed by 2 rounds of centrifugation at 14,000 RPM. Tryptophan and L-kynurenine concentrations were quantified by ELISA, according to the manufacturer's instructions (LDN Labor Diagnostika Nord and Immundiagnostik). Samples were read with a SpectraMax 340PC (Molecular Devices) at 450 nm.

Tumor challenge and treatment experiments

On day 0 of the experiments, tumor cells were injected intradermally (i.d.) in the right flank. For the B16 model, $2.5x10^5$ B16-WT or B16-IDO cells were injected into C57BL/6 mice and for the 4T1 model, $5x10^5$ cells were used in Balb/c mice. Bilateral flank tumor models were established by injection of $2.5x10^5$ B16-WT cells i.d. in one flank and $2.5x10^5$ B16-IDO cells i.d. in the contralateral flank.Treatments were given as single agents or in combinations with the following regimen for each drug. The IDO inhibitor drug indoximod/D-1MT (IDO*i*) was either dissolved in methylcellulose and administered in drinking water (2mg/ml, mice drank 4.5-5.5ml/day, Sigma-Aldrich) or formulated and administered in implantable subcutaneous pellets (140mg/pellet, 14-day-release, Innovative Research of America). Treatment with IDO*i* was initiated on day 1 ending on day 15 post tumor challenge. Control groups received placebo pellets without the active product (Innovative Research of America). Anti-CTLA-4 antibody (100ug/mouse, clone 9H10, BioXcell) and anti-PD-1 antibody (250ug/mouse, clone RPM1-14, BioXcell) was injected intraperitoneal (i.p.) on days 3, 6 and 9. Control groups received a corresponding dose of isotype antibody i.p. Tumors were measured every second or third day with a caliper, and the volume (length x with x height) was calculated. The animals were euthanized for signs of distress or when the total tumor volume reached $1000mm^3$.

Isolation of tumor-infiltrating cells and lymphoid tissue cells

Tumors and spleens were harvested 2 weeks after tumor cell inoculation. Tumor samples were finely chopped and treated with 1.67 U/ml Liberase (Roche) and 0.2 mg/ml DNase (Roche) in RPMI for 30 minutes at 37°C. Tumor samples were then mashed through a 100-µm cell strainer (BD Biosciences) to generate single-cell suspensions in RPMI supplemented with 7.5% FCS. Cells from mouse spleens were isolated by grinding spleens through 100-µm filters. After red blood cell (RBC) lysis (ACK Lysing Buffer, Lonza) when required, all samples were washed and re-suspended in FACS buffer (PBS/2%FCS).

Flow cytometry and morphology analysis

Cells isolated from mouse tumors and spleens were pre-incubated (15 minutes, 4°C) with anti-CD16/32 monoclonal antibody (Fc block, clone 2.4G, BD Biosciences) to block unspecific binding and then stained (30 minutes, 4°C) with appropriate dilutions of various combinations of the following fluorochrome-conjugated antibodies: anti-CD3-eFluor 450 (clone 17A2), anti-MHC Class II-eFluor 450 (clone M5/114.15.2), anti-IL-4Rα-PE (clone mIL4R-M1), anti-CXCR3-PE (clone CXCR3-173), anti-CD19-PE (clone MB19-1), anti-NK1.1-PE (clone PK136), anti-CSF-1R-PE (clone AFS98), anti-CD8-PE Texas Red (clone 5H10), anti-Gr1-PerCP-Cy5.5 (clone R86-8C5), anti-CD69-PerCP-Cy5.5 (clone H1.2F3), anti-CD4-PE-Cy7 (clone RM4-5), anti-CD62L-PE-Cy7 (clone MEL-14), anti-CD45-APC (clone 104), anti-F4/80-APC (clone BM8), anti-CD44-APC (clone IM7), anti-CD11c-Alexa Fluor 700 (clone N418), anti-CD25-APC eFluor 780 (clone PC61.5), and anti-CD11b-APC eFluor 780 (clone M1/70) antibodies, all purchased from BD Biosciences, eBioscience or Invitrogen. The cells were further permeabilized using Foxp3 Fixation and Permeabilization Kit (eBioscience) and stained for Foxp3 (clone FJK-16s, Alexa-Fluor-700-conjugated, eBioscience) and Ki67 (clone SolA15, eFluor-450-conjugated, eBioscience). The stained cells were acquired on a LSRII Flow Cytometer using BD FACSDiva software (BD Biosciences) and the data were processed using FlowJo software (Treestar). Dead cells and doublets were excluded on the basis of forward and side scatter.

Purification of MDSCs

Mouse tumor and spleen single-cell suspensions were generated as described in the previous section. Tumor cells were subsequently separated from debris over a Ficoll gradient (Sigma-Aldrich). B cells were depleted from splenocytes using CD19 microbeads and LD columns according to manufacturer's instructions (Miltenyi Biotec) to enrich the myeloid fractions. Cells were stained with anti-CD45.2-Alexa-Fluor-700, anti-CD11b-APC-Cy7 and anti-Gr1-PerCP-Cy5.5 antibodies for flow sorting on a FACSAria™ II Cell Sorter (BD Biosciences). Dead cells were excluded using DAPI (Invitrogen). Purity of flow-sorted populations was above 90%.

T cell suppression assay with MDSCs

Spleens and lymph nodes from naive mice were isolated and grinded through 100-µm filters to generate a single cell suspension. After RBC lysis, $CD8^+$ cells were purified using anti-CD8 (Ly-2) microbeads (Miltenyi Biotech) according to manufacturer's protocol and labeled with 1 mM CFSE (Invitrogen) in pre-warmed PBS for 10min at 37°C. The CFSE-labeled $CD8^+$ T cells were then plated in complete RPMI media supplemented with 0.05 M β-mercaptoethanol onto round bottom 96-well plates ($1x10^5$ cells per well) coated with 1 µg/ml anti-CD3 (clone 1454-2C11) and 5 µg/ml anti-CD28 (clone 37N). Purified MDSCs were added in indicated ratios and plates were incubated at 37°C. Percent $CD8^+$ T cell proliferation was measured by assessing CFSE dilution by flow cytometry after 48-72 hours of culture (LSRII flow cytometer, BD Biosciences). Controls were wells without $CD11b^+$ cells (Stim+) and wells without $CD11b^+$ cells and anti-CD3/CD28 antibody (Stim-). In some experiments a trans-well system was used, where MDSCs were plated in cell culture inserts with polyethylene terephthalate (PET) membranes (0.4 µm pore size, BD Falcon). When indicated, the inducible nitric oxide synthase (iNOS) inhibitor L-*N*-monomethyl arginine (L-NMMA, 500 µM, Calbiochem), the arginase-specific inhibitor N-hydroxy-nor-arginine (nor-NOHA, 500 µM, Calbiochem), the IDO inhibitor indoximod/D-1MT (200 µM, Sigma-Aldrich), anti-TGFβ (50 µg/ml, clone

1D11.16, eBioscience) or an anti-PD-L1 (50 μg/ml, clone 9G2, BioXCell) blocking antibody was added to the cultures.

Cell migration

Dissociated tumor cells were placed in the bottom chamber of a 24-well cell culture plate in complete RPMI media supplemented with 0.05 M β-mercaptoethanol (BME). MDSCs ($2x10^5$) purified from spleens of tumor-bearing mice were labeled with 1 mM CellTrace™ Far Red DDAO-SE (Thermo Fisher Scientific) in pre-warmed PBS (10 minutes, 37°C) and place in the upper chamber (3 μm cell culture inserts with PET membranes, BD Falcon). Inserts were pre-coated with 0.1 ml collagen gel solution (400 μg/ml rat tail collagen I (BD Bioscience) in PBS containing 1 mM Mg^{2+} and 1 mM Ca^{2+}, neutralized with 1M Sodium Hydroxide) for 24 hours at 4°C. MDSCs were allowed to migrate to the bottom well for 24 hours at 37°C, 5% CO_2. Migrated cells were then analyzed by flow cytometry on a LSRII Flow Cytometer (BD Bioscience) and quantified using Count Bright Absolute Counting Beads (Invitrogen). Migrated cells were distinguished by CellTrace™ Far Red DDAO-SE labeling. Percentage migration was calculated as: (number of migrated cells) x 100/(total cells added per well). IL-6, IL-10, MCP-1, IFNγ, TNF, and IL-12p70 recombinant proteins (500 pg/ml, BD Bioscience) (+) or media alone (-) were used for control chambers. In some experiments, tumors were pre-incubated with anti-CD25 antibody (clone PC-61, BioXcell) for 2 hours at 37°C. Where indicated, $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ and/or $CD8^+Foxp3^-$ cells sorted from tumors of $Foxp3^{GFP}$ mice were added to the bottom chamber.

Cytokine measurements

Tumors were weighed, placed in 350 μl PBS, and treated in a bead homogenizer for 5 minutes. Debris were removed by 2 rounds of centrifugation at 14,000 RPM to obtain cytokines in solution. Cytokine levels were measured using Luminex Mouse Cytokine Magnetic 20-Plex Panel (Invitrogen) according to the manufacturer's

instructions. Where indicated, supernatants from *in vitro* cell culture experiments were collected and cytokine levels were measured as described above.

Arg1 activity

FACS-sorted MDSCs were washed in PBS. Pellets were resuspended in 100 µl lysis buffer (0.4% Triton X-100 with 10 mM Tris-HCL and protease inhibitors) for 30 minutes at 4°C. Arg1 activity in the supernatants was measure using the QuantiChrom Arginase Assay Kit (BioAssay Systems).

NO production

A total of 10^5 FACS-sorted MDSCs were cultured in 200 µl in complete RPMI media with or without 1 µg/ml LPS. Supernatants were collected after 24 hours, and nitrite concentrations were determined using the Nitrite Colorimetric Assay Kit according to manufacturer's protocol (Cayman Chemical).

ROS production

Single-cell suspensions from mouse tumor and spleens were incubated at 37°C in PBS containing 2.5 µM oxidative-sensitive dye DCFDA (Invitrogen) with or without 30 ng/ml PMA for 30 minutes. Subsequently, cells were stained with anti-CD45.2-Alexa-Fluor-700, anti-CD11b-APC-Cy7 and anti-Gr1-PerCP-Cy5.5 antibodies and analyzed for DCFDA signal by flow cytometry.

Taqman gene expression analysis

RNA from FACS-purified MDSCs was isolated using the RNeasy kit (QIAGEN). cDNA was then produced from RNA using the Superscript II Reverse Transcription kit (Invitrogen). Relative gene expression was then determined using the ABI 7500 RT-PCR System using VIC- and FAM-conjugated primer probes (Applied Biosystems). Primers for amino acids, synthases, cytokines and receptors presented here included Arg1, NO, TGFβ, PD-L1, and IL-4Rα. *hprt* was used as the endogenous

control. Relative gene expression was calculated using Taqman primers by the ΔΔCt method.

Isolation of Pmel lymphocytes and adoptive transfer

Spleens and lymph nodes from pmel-1 TCR transgenic mice were isolated and grinded through 100-µm filters. After RBC lysis, $CD8^+$ cells were purified by positive selection using Miltenyi magnetic beads. The isolated cells were loaded with CellTrace™ Far Red DDAO-SE (Thermo Fisher Scientific) and injected into recipient animals via tail vein at indicated numbers. Activated $CD8^+$ T cells were generated by culturing splenocytes with soluble anti-CD3 (1 ug/ml, clone 145-2C11, eBioscience) and anti-CD28 (2ug/ml, clone 37.51, eBioscience) for 72 hours. Recombinant mouse IL-2 (30 U/ml, Chiron) was added for the final 24 hours of culture. $CD8^+$ T cells were subsequently positively selected with Miltenyi magnetic beads prior to injection via tail vein, as described above. The frequency and proliferation of pmel cells were measured 2 weeks after tumor challenge and 7 days after adoptive transfer of $1x10^6$ *in vitro* activated $CD8^+$ pmel T cells using Thy1.1 antibody and by assessing CellTrace™ Far Red DDAO-SE dilution by flow cytometry, respectively.

Purification of Tregs

$Foxp3^+$ Tregs were purified from tumors of $Foxp3^{GFP}$ mice. Tumor single-cell suspensions were generated as described previously. $CD4^+$ T cells were enriched by Ficoll gradient (Sigma-Aldrich) and CD4-negative microbeads (Miltenyi Biotec), before sorting for GFP expression on a FACSAria™ II Cell Sorter (BD Biosciences). Dead cells were excluded using DAPI (Invitrogen). Purity of flow-sorted populations was above 90%.

T cell suppression assay with Tregs

Spleens and lymph nodes from naive mice were isolated and grinded through 100-µm filters to generate a single cell suspension. After RBC lysis, $CD8^+$ cells were purified using anti-CD8 (Ly-2) microbeads (Miltenyi Biotech) according to

manufacturer's protocol and labeled with 1 mM CellTrace™ Violet Stain (Life Technologies) in pre-warmed PBS for 20min at 37°C. The labeled $CD8^+$ T cells were then plated in complete RPMI media supplemented with 0.05 M β-mercaptoethanol onto round bottom 96-well plates ($5x10^4$ cells per well). Plates were pre-coated with 1 μg/ml anti-CD3 (clone 1454-2C11) and 5 μg/ml anti-CD28 (clone 37N). Purified $Foxp3^+$ Tregs were seeded in indicated ratios and plates were incubated at 37°C. Percent $CD8^+$ T cell proliferation was determined by assessing CellTrace™ Violet dilution by flow cytometry after 48-72 hours of culture (LSRII flow cytometer, BD Biosciences). Controls were wells without $Foxp3^+$ Tregs (Stim+) and wells without $Foxp3^+$ Tregs and anti-CD3/CD28 antibody (Stim-).

Patient material

Surgical specimens from patients with metastatic melanoma were processed within 3 hours. Single cell suspensions were prepared using the human tumor dissociation kit (Miltenyi Biotec) according to the manufacturer protocol. Briefly cells were obtained by mincing the tumors into small pieces ($1mm^3$) and dissociated using enzymatic digestion with 1 hour incubation at 37°C with constant shaking in gentleMACS™ Octo Dissociator. Cell viability was assessed by trypan blue exclusion. Cells were frozen at a concentration of $5x10^6$/ml in 90% FBS with 10% DMSO until experiments were performed.

Whole blood was drawn in cell preparation tubes (CPT) containing sodium heparin (BD Vacutainer). PBMCs were isolated from whole blood by centrifuging the CPT tubes at 800g for 25 min. The plasma was collected and retained for other experiments. The interface cells were harvested and washed twice with PBS with 10% FCS at 500g and 450g for 10 minutes respectively. PBMCs were then resuspended in complete RPMI 1640 with 10% autologous plasma or PHS. For cryopreservation, PBMCs were resuspended in FCS with 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO), frozen at -80°C for 2-3 days and then stored in liquid nitrogen until tested.

Staining and flow analysis of TILs from cancer patients

Patient TIL samples were washed and stained with appropriate dilutions of various combinations of the following fluorochrome-conjugated antibodies: anti-CD14-FITC (clone M5E2), anti-HLA-DR-PE Texas Red (clone G46-6), anti-CD11b-PerCP-Cy5.5 (clone M1/70), anti-CD33-PE-Cy7 (clone P67.6), anti-CD45-APC eFluor 780 (clone 2D1), anti-CD8- PE Texas Red (clone 3B5), anti-CD4-PerCP-Cy5.5 (clone OKT4), and anti-CD69-PE-Cy7 (clone F950) antibodies, all purchased from BD Biosciences, eBioscience or Invitrogen. The cells were further permeabilized using FoxP3 Fixation and Permeabilization Kit (eBioscience) and stained for Foxp3 (clone 259D/C7, Alexa-Fluor-700-conjugated, eBioscience), IDO (PE-conjugated, R&D Systems), and Arginase (APC-conjugated, R&D Systems), or with matched isotype control antibodies. The stained cells were acquired on a LSRII Flow Cytometer using BD FACSDiva software (BD Biosciences) and the data were processed using FlowJo software (Treestar). Dead cells and doublets were excluded on the basis of forward and side scatter.

Proliferation assay with PBMCs and TILs from cancer patients

$CD45^+$ cells were positively selected from patient PBMC or TIL samples by lymphoprep isolation (Sigma-Aldrich) and MACS separation (Miltenyi Biotec) according to manufacturer's recommendations. $HLA\text{-}DR^{-/low}CD14^+$ effector cells were obtained first by depleting $HLA\text{-}DR^+$ cells followed by enrichment of $CD14^+$ cells from the $HLA\text{-}DR^{-/low}$ fraction, using antibody labeled microbeads (Miltenyi Biotec). Purity of sorted cells populations was assessed by flow cytometry using antibodies for HLA-DR and CD14 (eBiosciences) and LIVE/DEAD Fixable Aqua Dead Cell Stain-Amcyan (Invitrogen). The target cell population was obtained by $CD14^+$ depletion (Miltenyi Biotec). Target cells were labeled with CFSE as described above, and resuspended in complete RPMI media supplemented with 0.05 M β-mercaptoethanol and 50 U/ml IL-2. Target cells were plated onto round bottom 96-well plates ($5x10^4$ cells per well) coated with 5 μg/ml anti-CD3 (clone OKT3), and $CD14^+HLA\text{-}DR^{-/low}$ and $CD14^+HLA\text{-}DR^+$ cells were added at effector to target ratio

1:1. The plate was incubated for 6 days at 37°C. Proliferation was assessed by flow cytometry using antibodies for CD4 and CD8 (eBioscience).

Immunohistochemistry

Tissue sections (5 μm) were prepared from formalin-fixed, paraffin-embedded material and collected on Superfrost®/plus microscope slides (Fisher Scientific). After deparaffinization and rehydration, the slides were boiled in 50 mM citrate buffer (pH 6) for 30 minutes to retrieve the antigens. After the slides were allowed to cool to room temperature, immunohistochemistry (IHC) was performed. Before applying primary antibodies, the sections were immersed in antigen retrieval solution (Leica or Ventana Medical Systems) and blocked with Background Buster (Innovex) and avidin/biotin blovk (Ventana Medical Systems). Mouse anti-human monoclonal antibody against IDO (2.5 μg/ml, CalBioreagent) and rabbit anti-human monoclonal antibody against CD14 (1:4 dilution, Ventana) were then applied at RT for 1 hour, while mouse anti-human monoclonal antibody against Foxp3 (5 μg/ml, Abcam) were applied at 4°C overnight. Secondary antibodies (Leica Bond Polymer Refine Red Detection Kit, Leica or biotinylated horse anti-mouse IgG, Vector Labs) were then added and incubated for 60 or 30 minutes at RT, respectively. A tertiary reagent was applied according to the manufacturer's instructions. Antigen detection was performed by Bond Polymer Refine Red Detection (Leica) or by a color reaction with DAB detection Kit (Ventana Medical Systems). The sections were counterstained with hematoxylin (Ventana Medical Systems) and mounted with Permount™ media (Fisher Scientific). The slides were scanned with Discovery XT processor (Ventana Medical Systems) or Mirax® scanner (Carl Zeiss) and images were acquired with Mirax® viewer 1.11 software.

Statistics

Differences between groups were calculated by 2-tailed Student's *t* test or by two-way ANOVA. Values of $p < 0.05$ were considered to be statistically significant. Evaluation of survival patterns in tumor-bearing mice was performed by the

Kaplan-Meier method, and results were ranked according to the Mantel-Cox Log-Rank test. Survival was defined as mice with tumors < 1.000 cm^3. The strength of a relationship or correlation was calculated using Pearson's and Spearman's Correlations.

FIGURE LEGENDS

FIGURE S1: Positive correlation between IDO and CD14 mRNA expression in human melanoma, Related to Figure 1

Relationship between IDO and CD14 mRNA in human melanoma. Data are retrieved from the TCGA database (http://www.cbioportal.org).

FIGURE S2: Expression of IDO promotes tumor growth and resistance to immune checkpoint blockade, Related to Figure 2

(**A**) Western blot analysis of IDO expression. B16-IDO and B16-WT cells were harvested and cell lysates were fractionated using the Bis-Tris Gel System. IDO protein was detected with a polyclonal IDO antibody. Arrows show 45kDa and 72kDa bands corresponding to IDO and IDO-GFP proteins, respectively. (**B**) GFP signal in B16 cells transduced with either *GFP* (B16-WT) or *IDO-GFP* (B16-IDO) constructs. Non-transduced B16 cells were used for comparison. (**C**) Growth curves for the transduced cell lines *in vitro*. (**D**) Percentage of pmels of total $CD8^+$ T cells in LN, TDLN, spleen, and tumor of B16-WT and B16-IDO tumor-bearing mice 2 weeks after tumor challenge and 7 days after adoptive transfer of *in vitro* activated $CD8^+$ pmels, and proliferation of pmels from TDLNs and tumors in each group. Data are represented as mean ± SEM.

FIGURE S3: IDO promotes expansion of myeloid $CD11b^+$ cells in tumors, Related to Figure 3

(**A**) B16-IDO and B16-WT tumors were harvested 2wks after tumor cell inoculation and analyzed for infiltration with various immune cell subsets. Absolute numbers of $CD45^+$ cells per gram of tumor. (**B**) Tumor growth and weight of IDO*i* and vehicle treated B16-IDO tumors measured after adoptive transfer of activated pmel as indicated. Data represents mean +/- SEM (***A***) or one representative experiment (***B***).

FIGURE S4: Phenotype and suppressive capabilities of tumor-infiltrating myeloid $CD11b^+$ cells, Related to Figure 4

(**A**) ROS production in $CD11b^+Gr1^{int}$ and $CD11b^+Gr1^{high}$ cells from B16-IDO tumors depict as MFI of DCFDA after PMA stimulation. (**B**) Relative expression of Arg1, NO, TGFβ, PD-L1, and IL-4Rα mRNA in $CD11b^+Gr1^{int}$ cells purified of B6-IDO tumors compared with their B16-WT counterparts. Data are represented as mean ± SEM.

FIGURE S5: Activation of MDSCs is dependent on IDO expression, Related to Figure 5

Flow analysis of IDO protein in 4T1 tumors 2wks after tumor cell inoculation. Cells were stained with a polyclonal anti-IDO antibody (open histograms) against a matched isotype control (filled histogram). Data are represented as mean ± SEM.

FIGURE S6: Local and systemic effects of tumor IDO expression, Related to Figure 6

(**A**) $CD11b^+Gr1^{int}$ subsets enriched from spleens of B16-IDO and B16-WT tumor-bearing mice were evaluated for expression of F4/80, CD11c, MHC class II and Ly6C markers (open histograms) against their matched isotype controls (filled histograms). (**B**) Arginase-1 activity in $CD11b^+Gr1^{int}$ splenocytes from B16-IDO and B16-WT tumor-bearing mice compared to arginase-1 activity in $CD11b^+Gr1^{int}$ TILs. (**C**) Nitrite/NO concentrations in supernatants of LPS stimulated $CD11b^+Gr1^{int}$ splenocytes compared to nitrite concentrations in $CD11b^+Gr1^{int}$ TILs. (**D**) IL-4Rα expression within the $CD11b^+Gr1^{int}$ gated population of splenocytes from B16-IDO

tumor-bearing mice. (**E**) Relative percentages of $CD11b^+$ of total $CD45^+$ cells and frequencies of $Gr1^{high}$ and $Gr1^{int}$ cells within $CD11b^+$ gates in splenocytes from B16-IDO tumor-bearing mice treated with IDO*i* or vehicle control. (**F**) B16-IDO tumor–bearing mice were injected i.p. with BrdU on day 18 after tumor cell inoculation. After 1 hr, BrdU uptake by $CD11b^+Gr1^{int}$ cells from BM, blood, spleen, and tumor was analyzed by flow cytometry. Values represents mean +/- SEM.

FIGURE S7: Accumulation of MDSCs in tumors is dependent on the adaptive immune response, Related to Figure 7

$Foxp3^{DTR}$ mice inoculated with B16-IDO tumors and injected with DT or PBS. Frequency of $Foxp3^+$ Tregs of total $CD3^+$ T cells in TDLNs, spleens and tumors of mice injected with DT or PBS, and representative flow plots. Values represents mean +/- SEM.

REFERENCES

Holmgaard, R.B., D. Zamarin, D.H. Munn, J.D. Wolchok, and J.P. Allison. 2013. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med 210:1389-1402.

Li, X., E. Kostareli, J. Suffner, N. Garbi, and G.J. Hammerling. 2010. Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. Eur J Immunol 40:3325-3335.

Muranski, P., A. Boni, P.A. Antony, L. Cassard, K.R. Irvine, A. Kaiser, C.M. Paulos, D.C. Palmer, C.E. Touloukian, K. Ptak, L. Gattinoni, C. Wrzesinski, C.S. Hinrichs, K.W. Kerstann, L. Feigenbaum, C.C. Chan, and N.P. Restifo. 2008. Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood 112:362-373.

Overwijk, W.W., M.R. Theoret, S.E. Finkelstein, D.R. Surman, L.A. de Jong, F.A. Vyth-Dreese, T.A. Dellemijn, P.A. Antony, P.J. Spiess, D.C. Palmer, D.M. Heimann, C.A. Klebanoff, Z. Yu, L.N. Hwang, L. Feigenbaum, A.M. Kruisbeek, S.A. Rosenberg, and N.P. Restifo. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med 198:569-580.

Schaer, D.A., S. Budhu, C. Liu, C. Bryson, N. Malandro, A. Cohen, H. Zhong, X. Yang, A.N. Houghton, T. Merghoub, and J.D. Wolchok. 2013. GITR pathway activation abrogates tumor immune suppression through loss of regulatory T cell lineage stability. Cancer immunology research 1:320-331.

Figure S1

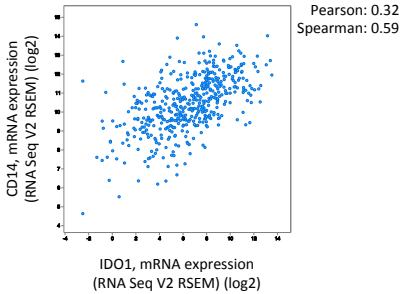

CD14, mRNA expression
(RNA Seq V2 RSEM) (log2)
IDO1, mRNA expression
(RNA Seq V2 RSEM) (log2)
Pearson: 0.32
Spearman: 0.59

Figure S2

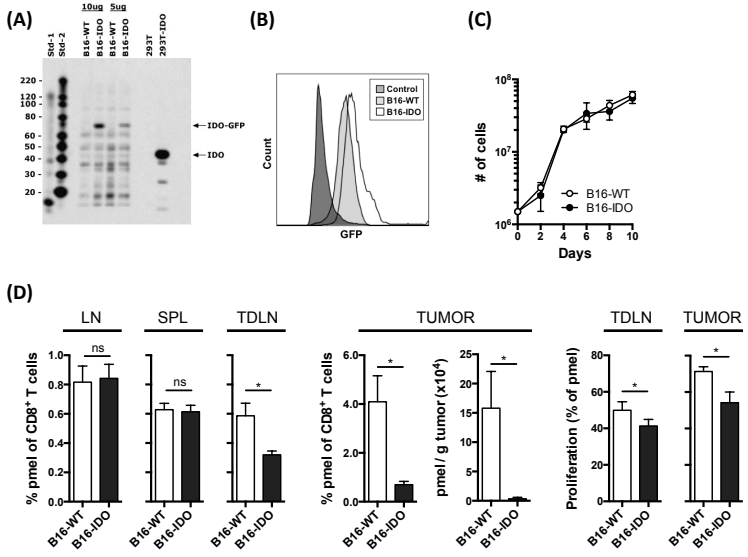

(A)
10ug
5ug
Std-1
Std-2
B16-WT
B16-IDO
B16-WT
B16-IDO
293T
293T-IDO
220
120
100
80
60
50
40
30
20
IDO-GFP
IDO
(B)
Control
B16-WT
B16-IDO
Count
GFP
(C)
# of cells
B16-WT
B16-IDO
Days
(D)
LN
SPL
TDLN
TUMOR
TDLN
TUMOR
% pmel of CD8+ T cells
pmel / g tumor (x10^4)
Proliferation (% of pmel)
ns
*
B16-WT
B16-IDO

Figure S3

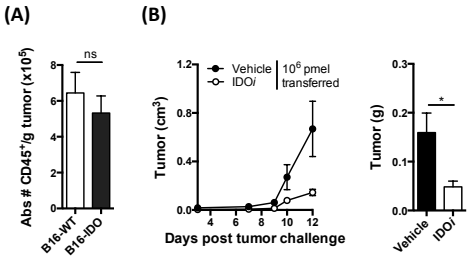
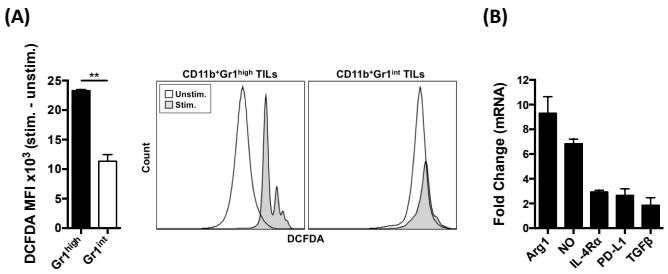

(A)
(B)
ns
Abs # CD45+/g tumor (x10^5)
B16-WT
B16-IDO
Vehicle
IDOi
10^6 pmel transferred
Tumor (cm^3)
Days post tumor challenge
*
Tumor (g)
Vehicle
IDOi

Figure S4


(A)
DCFDA MFI x10³ (stim. - unstim.)
**
Gr1high
Gr1int
CD11b+Gr1high TILs
CD11b+Gr1int TILs
Unstim.
Stim.
Count
DCFDA

(B)
Fold Change (mRNA)
Arg1
NO
IL-4Rα
PD-L1
TGFβ

Figure S5

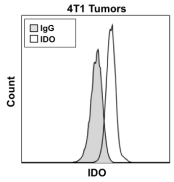


Figure S6

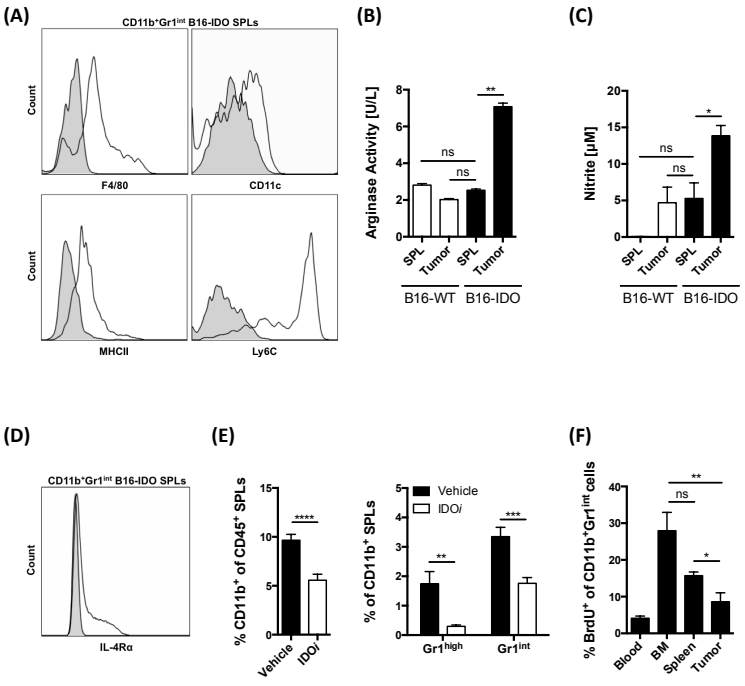
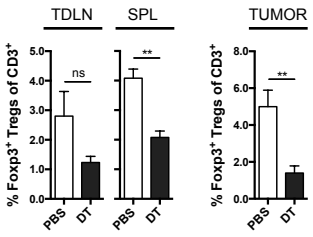

(A)
CD11b+Gr1int B16-IDO SPLs
Count
F4/80
CD11c
MHCII
Ly6C
(B)
Arginase Activity [U/L]
ns
ns
**
SPL
Tumor
SPL
Tumor
B16-WT
B16-IDO
(C)
Nitrite [µM]
ns
ns
*
SPL
Tumor
SPL
Tumor
B16-WT
B16-IDO
(D)
CD11b+Gr1int B16-IDO SPLs
Count
IL-4Rα
(E)
% CD11b+ of CD45+ SPLs
****
Vehicle
IDOi
% of CD11b+ SPLs
Vehicle
IDOi
**
***
Gr1high
Gr1int
(F)
% BrdU+ of CD11b+Gr1int cells
**
ns
*
Blood
BM
Spleen
Tumor

Figure S7


TDLN
SPL
TUMOR
% Foxp3+ Tregs of CD3+
5.0
4.0
3.0
2.0
1.0
0.0
ns
**
8.0
6.0
4.0
2.0
0.0
**
PBS
DT
PBS
DT
PBS
DT