Supplementary Materials and Methods

Cells

Mouse MSCs were cultured in α-MEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen, Grand Island, NY). Non-adherent cells were removed after 24 hr, and adherent cells were maintained with medium replenishment every 3 days. To obtain MSC clones, cells at confluence were harvested and seeded into 96-well plates by limiting dilution. Individual clones were then picked and expanded. Human MSCs were maintained at low glucose α-MEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen). The "stemness" of all MSCs was determined by examining their potential to differentiate into adipocytes, osteoblasts, and chondrocytes, and by staining for specific cell surface markers (data not shown). B16-F0 melanoma tumor cells were expanded in vitro in complete DMEM medium. EL4 lymphoma cells were expanded in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Cell proliferation assay

T cell blasts were generated from freshly isolated mouse splenic lymphocytes. Cells (1×10^6 /ml) were activated by soluble anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) for 48 hr, washed and cultured with IL-2 (200 U/ml) alone for another 24 hr. All T cell cultures were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 μM β-ME (RPMI complete medium). Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (New Jersey Blood Center, New Brunswick, NJ) by gradient density centrifugation over 60% isotonic Percoll. Cell counts and viability were

assessed by trypan blue staining. PBMC were activated by plastic-bound anti-human CD3 (OKT3, 1 μg/ml). These human T cell blasts (1 x 10^5) were harvested, washed and co-cultured at various ratios with iNOS$^{-/-}$ MSCs transfectants expressing constitutive or inducibe IDO in 96-well plates for the indicated times. Freshly-isolated splenocytes (1 x 10^5) were also co-cultured with inducible IDO-expressing MSCs at graded ratios in the presence of soluble anti-CD3 and anti-CD28. Resultant cell proliferation was assessed by a standard ^{3}H-thymidine (Tdr) incorporation assay. Briefly, 0.5 μCi of ^{3}H-Tdr (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ) was added to each well 8 hr before termination of the cultures by freezing at -80°C. After thawing, cells in plates were harvested and the incorporated ^{3}H-Tdr was assessed by using a Wallac Microbeta scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

Detection of kynurenine

Two hundred microliters of cell culture supernatant was mixed with 100 μl of 30% trichloroacetic acid and incubated at 50°C for 30 min. After centrifugation (15,400 × *g* for 1 min), 125 μl supernatant was mixed with an equal volume of Ehrlich's reagent (100 mg p-dimethylbenzaldehyde in 5 ml glacial acetic acid) in a 96-well plate. Samples were read against a reagent blank with a 490-nm filter in a microplate spectrophotometer (Multiskan MS, Lab Systems, CA). The change in kynurenine concentration was obtained by subtracting the control value from the sample values, normalized against commercially-obtained kynurenine as concentration standards.

Real-time PCR

Total RNA was isolated by using an RNeasy mini kit (Qiagen, Valencia CA). Genomic DNA was removed from total RNA using the RNase-Free DNase kit (Qiagen). First-strand cDNA was generated for each RNA sample using the Sensiscript

RT Kit (Qiagen). Random hexamers were then used to prime cDNA synthesis. mRNA was quantitated by real-time PCR (MX-4000 from Stratagene) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The relative copy number of mRNA was normalized against endogenous β-*actin* mRNA.

Western blotting analysis

Cell pellets were washed three times with PBS and then resuspended in 100 μl protein lysis buffer (62.5 mM Tris-HCl, pH 6.9, 2% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.04% bromphenol blue). Whole cell protein was separated on a 10% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane by electroblotting (Roche Molecular Biochemicals, Laval, QC) and probed with monoclonal antibodies against IDO or β-actin. Bound specific antibodies were revealed by chemiluminescence (Amersham ECL, GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

Flow cytometric analysis

To analyze lymphocytes in tumors, tumor tissue was ground between the frosted surfaces of two glass microscopic slides. Released cells were harvested in complete RPMI medium, filtered through nylon mesh (90 microns), washed twice with PBS, and resuspended in PBS buffer containing 5% FBS. For surface staining, cells were first blocked with anti-CD16/32 (clone 93; eBioscience) for 30 mins. After washing twice, cells were then stained with fluorescence-conjugated antibodies. For intracellular staining, cells were fixed with Fix/Perm buffer (BD Biosciences) for 30 mins. After washing twice, cells were stained with PE-conjugated anti-Foxp3 (clone NRRF-30; eBioscience, San Diego, CA). All stained cells were examined by flow cytometry on a FACScan flow cytometer (BD Biosciences, San Jose, CA), using CellQuest software for

data acquisition and analysis. Non-specific binding was excluded by using isotype control antibodies.

DNA content assay

Splenocytes were co-cultured with MSCs in the presence of anti-CD3 and anti-CD28 in 24-well plates. At different time points (24 hr and 48 hr) cells were harvested, washed with PBS, and then resuspended in staining buffer (PBS containing 0.2% saponin, 50 μg/ml of propidium iodide, and 10 μg/ml RNase A). After incubating for 30 min at 37 °C in dark, the cells were analyzed by flow cytometry to determine total cellular DNA content. The percentage of cells with S+G2/M phase DNA content was calculated on histogram analysis.

Preparation and visualization of cryosections

Mice bearing B16-F0 melanoma tumors, with administration of inducible IDO-expressing MSCs (with GFP in the transfection vector) or $iNOS^{-/-}$ MSCs (without GFP), were euthanized and perfused with freshly prepared 4% paraformaldehyde in PBS. The tumor samples were then removed and soaked in 4% paraformaldehyde for additional 2 hr, and then transferred to 20% sucrose and incubated at 4°C overnight. After another 6 hr in 30% sucrose, the tumor samples were then frozen in OCT/ dry ice/isopentane for frozen sectioning and evaluation. Frozen glass slides with tissue sections were mounted with Vectashield Mounting Medium containing DAPI for nuclear identification. The processed slides were then visualized under a confocal microscope (Zeiss LSM 700).

Immunohistochemistry

Frozen microscopic slides with 5-μm cryosections were air-dried for 1 hr at room temperature, and then fixed in ice-cold methanol at −20°C for 10 min and blocked in 10%

normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) for 90 min, followed by a 5-min wash in PBS. IDO was detected by using a monoclonal mouse anti-human IDO antibody (Abcam, Cambridge, MA), diluted 1:50 in 1% NDS. After incubation overnight at 4°C, the slides were washed 4 times with PBS, air-dried, and incubated for 1 hr at room temperature with donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Reactions were developed using diaminobenzidine (DAB) as a chromogenic substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted. Microophotographic images were obtained with a 40× objective.