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Cells Expressing Indoleamine 2,3-Dioxygenase Inhibit T Cell Responses¹

Andrew L. Mellor,^{2*†} Derin B. Keskin,^{*†} Theodore Johnson,^{*†} Phillip Chandler,^{*†} and David H. Munn^{*†‡}

Pharmacological inhibition of indoleamine 2,3-dioxygenase (IDO) activity during murine gestation results in fetal allograft rejection and blocks the ability of murine CD8⁺ dendritic cells to suppress delayed-type hypersensitivity responses to tumor-associated peptide Ags. These observations suggest that cells expressing IDO inhibit T cell responses in vivo. To directly evaluate the hypothesis that cells expressing IDO inhibit T cell responses, we prepared IDO-transfected cell lines and transgenic mice overexpressing IDO and assessed allogeneic T cell responses in vitro and in vivo. T cells cocultured with IDO-transfected cells did not proliferate but expressed activation markers. The potency of allogeneic T cell responses was reduced significantly when mice were preimmunized with IDO-transfected cells. In addition, adoptive transfer of alloreactive donor T cells yielded reduced numbers of donor T cells when injected into IDO-transgenic recipient mice. These outcomes suggest that genetically enhanced IDO activity inhibited T cell proliferation in vitro and in vivo. Genetic manipulation of IDO activity may be of therapeutic utility in suppressing undesirable T cell responses. *The Journal of Immunology*, 2002, 168: 3771–3776.

The inability of T cells to eliminate certain chronic pathogens or to attack cancer cells displaying tumor-specific Ags suggests that natural immunoregulatory processes can suppress T cell-mediated immunity even when directed against neo Ags (1, 2). These natural immunosuppressive processes are a formidable barrier to immunotherapy of tumors and chronic viral infections. However, they represent therapeutic opportunities to moderate clinically undesirable T cell responses, such as those causing autoimmune diseases or rejection of tissue allografts (3, 4).

Recently, we have identified a natural immunosuppressive mechanism that prevents maternal T cell-mediated rejection of murine allogeneic fetuses (5, 6). These studies revealed that indoleamine 2,3-dioxygenase (IDO)³ activity normally contributes to maintenance of maternal T cell tolerance to fetal alloantigens because pregnant mice exposed to the IDO-specific inhibitor 1-methyl-tryptophan selectively rejected allogeneic fetuses, whereas syngeneic fetuses developed to term. The IDO mechanism is used by cultured human macrophages and dendritic cells to suppress in vitro T cell proliferation (7, 8). Several recent reports extend the potential biologic significance of the IDO mechanism to murine immunoregulatory CD8⁺ dendritic cells because their ability to suppress delayed-type hypersensitivity responses to tumor-associated peptides was blocked by exposure to 1-methyl-tryptophan (9–11). These data suggest that physiologic cells expressing

IDO inhibit the generation of T cell responses in vivo. To explain these phenomena we hypothesized that proximity to cells expressing IDO inhibits T cell activation, possibly due to localized depletion of the essential amino acid tryptophan (7, 12). However, pharmacological approaches using an inhibitor of IDO activity to moderate T cell responses in vivo do not permit unequivocal mechanistic interpretations of the observed functional outcomes.

To complement pharmacological studies and to further address relationships between IDO activity and inhibition of T cell responses, we used two molecular genetic strategies to enhance IDO activity in transfected cell lines and in new strains of transgenic mice. In the current study, we directly evaluated the hypothesis that enhanced IDO activity in cells or tissues inhibits T cell responses. We report that the potency of allogeneic T cell responses elicited by IDO-transfected cells in vitro and in vivo was reduced significantly and that allogeneic T cell responses elicited after T cell adoptive transfer were less potent in recipient transgenic mice that overexpressed IDO in tissue microenvironments.

Materials and Methods

DNA vectors and cloning

Full-length murine IDO cDNA was isolated from IFN- γ -stimulated RAW cells using standard RT-PCR and DNA cloning procedures. IDO cDNA was cloned into pGEM T-Easy (Promega, Madison, WI) and completely sequenced as described previously (13). A full-length (1.2-kb) IDO cDNA fragment was digested with *NotI* enzyme and cloned into *NotI*-cut pcDNA-3 cDNA expression vector containing CMV promoter elements.

Cell lines and transfection

A total of 2×10^7 MC57G (ATCC No. CRL2295; gift from Dr. D. Moskofidis, Immag, MCG) or MB49 (gift from Dr. J. Leonard, Genetics Institute, Cambridge, MA) tumor cells were electroporated in the presence of 20 μ g of linearized (*NruI*, *BglII*) pcDNA3-IDO or pcDNA-3 vector DNA using a setting of 320 mV/975 μ F on an electroporation machine (Bio-Rad, Hercules, CA). Cells were cultured in IMDM medium supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (IMDM complete) for 2 days to recover. Cells were harvested and plated in 96-well plates and cloned by limiting dilution in IMDM complete medium supplemented with 500 μ M tryptophan and 1200 μ g/ml G418 (Life Technologies, Rockville, MD). After 3 wk of selection, single clones were selected and analyzed.

*Program in Molecular Immunology, Institute of Molecular Medicine and Genetics, and Departments of ¹Medicine and ²Pediatrics, Medical College of Georgia, Augusta, GA 30912

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² Address correspondence and reprint requests to Dr. Andrew L. Mellor, Institute of Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th Street, CA 2006, Augusta, GA 30912-2000. E-mail address: amellor@mail.mcg.edu

³ Abbreviations used in this paper: IDO, indoleamine 2,3-dioxygenase.

Mice

All mice used for these studies were bred under specific pathogen-free conditions at the Medical College of Georgia. CBK transgenic mice express an H-2K^b transgene on the inbred CBA strain genetic background (14). BM3 (14) and A1 (15) transgenic mice are TCR-transgenic mice containing large cohorts of H-2K^b-specific CD8⁺ T cells or male (H-Y) Ag-specific CD4⁺ T cells on the inbred CBA strain genetic background. IDO-transgenic mice were generated by the staff of the Medical College of Georgia Transgenic Unit. Briefly, rDNA was microinjected into fertilized oocytes from inbred CBA/Ca strain parents using standard procedures. The DNA construct (MI) was prepared by ligating a murine full-length IDO cDNA into the cloning site of the cDNA expression cassette pDOI, which uses promoter elements from a murine MHC class II gene (16). Two transgenic (MI) founder mice were identified by Southern blot/in situ hybridization using a transgene specific probe, and mice were mated with CBA/Ca strain partners to establish two separate transgenic lineages, 31 and 33. Recipient mice for adoptive transfer experiments were generated by intercrossing MI transgenic (heterozygous) mice (line 33) with CBK transgenic (homozygous) mice and selecting double (MI × CBK) and single (CBK) transgenic littermates by genotypic analyses.

Western blotting

The pcDNA-3-IDO-transfected MC57G clones and vector-transfected clones were cultured to confluency. Total protein was extracted using RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 ng/ml PMSF, 66 ng/ml aprotinin). Samples were briefly sonicated (sonicator from Fisher Scientific, Pittsburgh, PA), avoiding heating and bubbling. Total protein was assayed using protein assay reagents (Pierce, Rockford, IL). Samples containing 50 μg of total protein lysate were separated on 12% SDS-PAGE. IDO protein was detected using a polyclonal Ab preparation from rabbits immunized with one of two synthetic murine IDO C-terminal peptides and anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Actin protein was detected using a mouse anti-actin mAb (Chemicon International, Temecula, CA) and anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) using standard blotting and detection techniques. In vivo IDO expression was assessed by removing spleens from MI transgenic and control CBA mice and placing them in 2 ml of ice-cold RIPA buffer. Samples were homogenized using a Powergen-125 homogenizer (Fisher Scientific). Homogenized samples were processed and blotted as described above.

HPLC analysis

A total of 10⁵ transfected MC57G or MB49 cells were seeded into 96-well plates in 200 μl of IMDM complete medium containing G418 (1 mg/ml). After 3 days, 75 μl of culture medium was removed and extracted with 1.4 ml of HPLC-grade methanol. Precipitated proteins were removed by centrifugation and supernatants were dried. Samples were reconstituted with 100 μl of HPLC-grade deionized water, and 20 μl of sample was injected into a C18 column (Luna C18(2), 250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA). Samples were eluted with a gradient of water:acetonitrile (0–80% acetonitrile) over 20 min. Standard concentration curves were prepared using mixtures of kynurenine and tryptophan. IDO activities in spleens of MI transgenic and CBA mice were assessed by culturing 10⁶ splenocytes in 200 μl of IMDM complete medium in 96-well plates for 3 days in the presence of IFN-γ (200 U/ml). Seventy-five microliters of culture medium was analyzed as described above.

Mixed lymphocyte cultures

Semiconfluent IDO-construct or vector-only transfected MC57G cells were harvested, washed twice to remove G418, and seeded (5, 2.5, and 1.2 × 10⁴ cells/well) into 96-well plates in 100 μl of IMDM complete medium with 2-ME (50 μM). Splenocytes from TCR transgenic BM3 mice (14) were stained with 1 μM CFSE (Molecular Probes, Eugene, OR) in PBS for 35 min at 37°C and washed twice with medium. A total of 10⁵ BM3 splenocytes in 100 μl/well were used as responders.

Immunizations

CBA/Ca mice received a total of two, four, or six i.p. injections at biweekly intervals of MC57G-V₀, MC57G-24, MC57G-26 (single cell suspensions of 10⁷ cells in 200 ul), or PBS (200 ul). Four days after the last injection, spleens were harvested. Lymphocytes were used as responders (5 × 10⁵ cells/well) in an MLR with irradiated C57BL/6 splenocytes (3000 rad, 1.25 × 10⁵ cells/well). Cultures were performed in triplicate wells using 96-well flat-bottom plates in 200 μl of IMDM complete medium with 2-ME (50 μM). Proliferation was assessed by adding [³H]thymidine (1 μCi/well) during the last 8–12 h of a 4-day culture. H-2^b-specific cytolytic

activity mediated by elicited T cells was assessed in chromium release assays using MC57 targets after coculture for 3 days with B6 splenocytes according to standard procedures (17).

Flow cytometric analyses

After coculture, cells were harvested, washed with PBS, and stained with PE-conjugated anti-mouse CD8α Ab (BD PharMingen, San Diego, CA) for 1 h. Cells were washed with PBS and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). CD69 expression by T cells was assessed by staining cocultures with PE-conjugated anti-mouse CD69 and APC-conjugated anti-mouse CD8α (BD PharMingen) and subjecting them to flow cytometry. Histograms and dot plots were produced from gated live lymphocyte populations using light scatter parameters. Semiconfluent MC57G IDO- and vector-transfected cells were harvested, stained with FITC-conjugated mAb to mouse H-2K^b (Caltag Laboratories, Burlingame, CA), and analyzed by flow cytometry as described above.

Apoptosis of T cells was assessed by flow cytometry 72 h after coculture. Cells were harvested and stained with CD8-PE, propidium iodide, and FITC-conjugated annexin V (BD PharMingen). Annexin V histograms were generated for gated CD8⁺ T cell populations. TCR expression by BM3 CD8⁺ T cells 72 h after coculture was assessed by staining cells with anti-TCR clonotypic Ab (Ti98 biotin), CyChrome-streptavidin (BD PharMingen).

T cell adoptive transfer

A total of 10⁷ splenocytes from BM3 mice in 200 μl of PBS were injected into the tail veins of recipient mice. Mice were sacrificed 96 h after adoptive transfer and single cell suspensions were prepared from spleen. RBCs were removed by osmotic lysis and cells were counted using a Coulter (Seattle, WA) counter. Donor T cells were detected by staining cell preparations with CD4-FITC, CD8-PE, and anti-clonotypic Ab (Ti98 conjugated with biotin) as described previously (14). Stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences) using CellQuest analysis software (BD Biosciences). A total of 2 × 10⁷ splenocytes from A1 female TCR-transgenic mice (15) were transferred into recipient male and female mice, which were sacrificed 90 h later. A1 donor T cells were detected using anti-CD4 and anti-Vβ8.1/2 mAbs (BD PharMingen) as described previously (15). Donor T cell numbers in spleen, mesenteric, and axillary lymph nodes were measured 20 h after adoptive transfer to assess whether enhanced IDO expression affected trafficking of donor splenocytes.

Results

IDO-transfected tumor cells

MC57G fibrosarcoma cell lines (H-2^b haplotype) were selected for this study because they elicit potent H-2K^b-specific T cell responses in vitro and do not express IDO constitutively (data not shown). After electroporation to introduce rDNA containing CMV promoter elements linked to murine IDO cDNA sequences, we isolated a series of IDO-transfected MC57G clones and screened them for IDO gene transcription, protein expression, and enzyme activity (Fig. 1). Cell lysates prepared from IDO-transfected MC57G clones contained a single band stained by rabbit polyclonal anti-murine IDO peptide-specific Abs, which was the same size as IDO protein detected in murine epididymis (Fig. 1A and data not shown). IDO-transfected cell lines catabolized tryptophan and produced kynurenine, a metabolite produced by oxidative catabolism of tryptophan (Fig. 1B). IDO-transfected and vector-transfected clones expressed comparable levels of surface H-2K^b Ag (Fig. 1C).

IDO-transfected cells inhibit in vitro T cell proliferation

IDO-transfected MC57G cells were cocultured with splenocytes from BM3 TCR-transgenic mice that were prestained with the tracking dye CFSE. BM3 mice contain large cohorts of H-2K^b-specific CD8⁺ T cells (14). After culture for 24–72 h, cocultures were stained with anti-CD8 mAbs and analyzed by flow cytometry to assess the total number of CD8⁺ T cells and the number of times they had divided (Fig. 2). The number of CD8⁺ T cells did not change after 72 h when cocultured with IDO-transfected MC57G

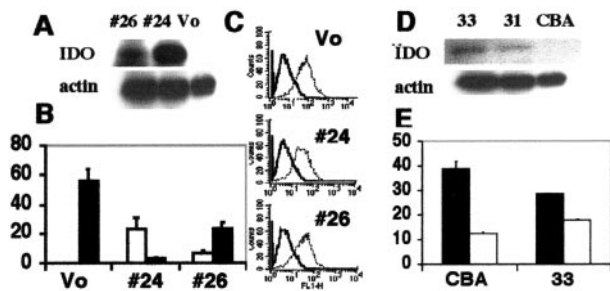


FIGURE 1. IDO expression and activity in IDO-transfected MC57G cell lines and IDO-transgenic mice. *A*, Western blot analyses of cell lysates from IDO-transfected clones 26 and 24 and a vector-transfected (V0) MC57G clone. IDO protein was detected using a polyclonal Ab from rabbits immunized with a synthetic IDO peptide. *B*, Concentrations (μM) of tryptophan (filled bars) and kynurenine (open bars) determined by HPLC analyses of medium used to culture transfected MC57G cells. *C*, Flow cytometric analyses of transfected MC57G cells stained with H-2K^b-specific mAb MC57G clones (dotted lines). Bold lines are staining profiles obtained in the absence of Ab. *D*, Western blot analyses of cell lysates prepared from splenocytes isolated from CBA and MI transgenic mice with high (line 33) or low (line 31) copies of integrated transgenes. *E*, HPLC analyses of medium used to culture splenocytes from MI transgenic (line 33) and CBA mice for 3 days with exogenous IFN- γ . Tryptophan concentrations (filled bars) were 29% less and kynurenine concentrations (open bars) were 44% more in medium used for culture of splenocytes from MI transgenic mice.

cells (Fig. 2A). In contrast, CD8⁺ T cell numbers increased 2.5-fold over the same period when cultured with vector-transfected MC57G cells after an initial lag phase. Flow cytometric analyses of CFSE staining profiles revealed that few CD8⁺ T cells still exhibited CFSE staining intensities comparable with undivided (naive) BM3 T cells (M1 marker; Fig. 2B), indicating that most CD8⁺ T cells had divided one to four times when cocultured with vector-transfected MC57G cells for 72 h. In contrast, most CD8⁺ T cells cocultured with IDO-transfected MC57G clones for the same time exhibited CFSE staining intensities comparable with undivided BM3 T cells. Based on these outcomes, we estimated that \sim 80% of naive T cells did not divide at all in the presence of IDO-transfected cells and that the rest (\sim 20%) divided once only. Similar outcomes were observed when BM3 T cells were cocultured with cloned IDO-transfected MB49 bladder carcinoma cells, another H-2^P-haplotype cell line (data not shown). Thus, T cell proliferation was limited in the presence of IDO-transfected tumor cells.

IDO-transfected cells stimulate expression of T cell activation markers

We evaluated whether incubation with IDO-transfected tumor cells induced expression of T cell activation markers. BM3 splenocytes were cocultured with IDO-transfected and vector-transfected tumor cells, stained with anti-CD69, anti-CD8, and anti-TCR (Ti98) mAbs, and analyzed by flow cytometry (Fig. 3). After 48 h, before proliferation began in control cultures, almost all CD8⁺ T cells coexpressed CD69, irrespective of whether they were cultured with IDO-transfected or vector-transfected MC57G cells (Fig. 3, *top panels*). Similarly, the ability of tumor cells to induce CD71 expression was not affected by IDO expression (data not shown). We also evaluated TCR expression levels on T cells using an anticolonotypic mAb (Ti98), because TCR down-regulation occurs when naive (resting) CD8⁺ T cells from BM3 transgenic mice are activated (14). The number of cells expressing high levels of TCR was reduced substantially and comparably after 72 h of co-

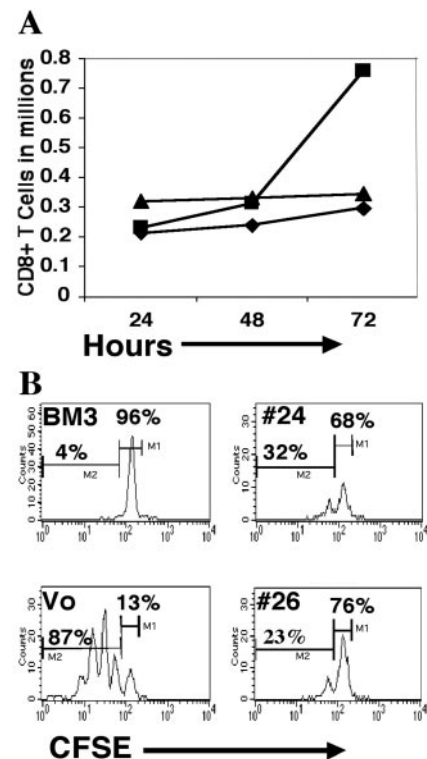


FIGURE 2. T cell proliferation in cocultures with IDO-transfected MC57 cells. *A*, Numbers of CD8⁺ T cells present in cultures containing IDO transfectants (\blacktriangle and \blacklozenge) or vector transfectants (\blacksquare) over time. *B*, CFSE staining profiles of viable CD8⁺ cells after coculturing BM3 splenocytes alone (*upper left panel*) or with MC57G vector-transfected cells (*lower left panel*) or IDO-transfected clones (*right panels*) for 72 h. Bars indicate percentage of undivided (M1, CFSE^{high}) and divided (M2, CFSE^{low}) CD8⁺ T cells. Data are representative of >10 experiments. Similar results were obtained when cocultures were analyzed at slightly earlier times (66 h).

culture with IDO-transfected and vector-transfected cells (Fig. 3, *middle panels*). These outcomes indicated that IDO expression by tumor cells expressing Ag had no effect on their ability to activate naive BM3 T cells by the criteria of inducing expression of activation markers or TCR down-regulation.

Next, we assessed annexin V staining of CD8⁺ T cells to detect early signs of undergoing apoptosis. After 48 h, comparable proportions (\sim 15–20%) of CD8⁺ T cells stained with annexin V in cocultures with IDO-transfected or vector-transfected MC57G tumor cells, whereas very few T cells incubated alone stained with annexin V (Fig. 3, *lower panels*). Thus, coculture with IDO-transfected MC57G tumor cells did not increase the proportion of T cells undergoing apoptosis. However, coculture with MC57G tumor cells enhanced annexin V staining of T cells before cell division occurred, probably due to tumor cell growth and nutrient consumption. After 72 h, the proportions of CD8⁺ T cells stained with annexin V were substantially higher in cocultures with MC57G tumor cells and showed wide variation in multiple experiments (40–70%, data not shown). However, increased annexin V staining of T cells was also observed at later times when BM3 splenocytes were incubated alone, showing that spontaneous T cell death rates were also higher. Similar results were obtained when T cells were stained with propidium iodide to assess T cell viability in cocultures (data not shown). These outcomes revealed that expression of IDO by Ag-presenting tumor cells did not cause T cells to die faster via apoptosis, especially before T cells started dividing

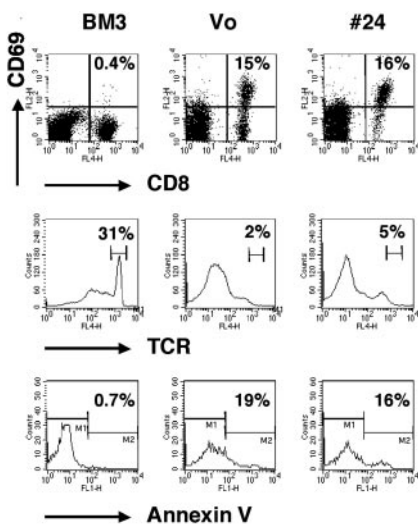


FIGURE 3. Phenotypic analyses of BM3 T cells cocultured with MC57G transfectants. *Upper panels*, Analyses of CD69 and CD8 expression by BM3 T cells cultured alone (*left panel*), with vector-transfected MC57G cells (*center panel*), or with IDO-transfected clone 24 cells (*right panel*). Percentages indicate overall proportions of CD69⁺CD8⁺ T cells detected in each cell population analyzed. *Middle panels*, Analyses of TCR expression by (gated) CD8⁺ T cells using Ti98 anti-clonotypic Ab after 72 h of coculture. Markers highlight T cells expressing high levels of TCR corresponding to naive CD8⁺ T cells in BM3 transgenic mice (14). *Lower panels*, Annexin V staining on gated CD8⁺ T cells after 48 h. Percentages indicate proportions of gated CD8⁺ T cells staining with annexin V Ab.

in the first 48 h of coculture. After prolonged coculture, it was difficult to detect specific effects of IDO-transfectants on T cell viability due to enhanced annexin V staining in all cultures.

IDO-transfected tumor cells inhibit alloreactive T cell responses in vivo

CBA (H-2^k haplotype) mice were injected twice weekly (for 1–3 wk) with allogeneic IDO-transfected (clone 24 or 26) or vector-transfected MC57G (H-2^b) tumor cells to assess whether IDO-transfected cells moderated *in vivo* T cell responses to H-2^b alloantigens. H-2^b-specific T cell responses were assessed by coculturing splenocytes from immunized mice (responders) with irradiated splenocytes from C57BL/6 (H-2^b stimulators) mice (Fig. 4). Elicited H-2^b-specific proliferative responses were significantly less potent when responders originated from mice injected with IDO-transfected clone 26 tumor cells, compared with responses from mice injected with PBS ($p \leq 0.02$). Similarly, IDO-transfectant clone 24 elicited less potent H-2^b-specific responses after two or four injections ($p \leq 0.02$). After six injections of clone 24 cells, proliferative responses were not significantly reduced relative to control mice injected with PBS, although the trend for reduced responses in IDO-transfectant-treated mice was still apparent in this group. Assays for T cell cytolytic function executed in parallel after 3 days of mixed lymphocyte culture (with irradiated B6 splenocytes) revealed similar trends in reduced killing of MC57 target cells when elicited T cells originated from mice exposed to IDO-transfected MC57 cells (data not shown). However, exposure to IDO-transfectants did not completely abrogate H-2^b-specific cytolytic functions, indicating that immunization with IDO-transfected MC57G tumor cells did not eliminate all H-2^b-specific cytolytic precursors in mice. These outcomes suggest that the pool of H-2^b-specific T cells was reduced when CBA mice were exposed to IDO-transfected MC57G cells before mixed lymphocyte culture. This effect was not an innate quality of MC57G cells

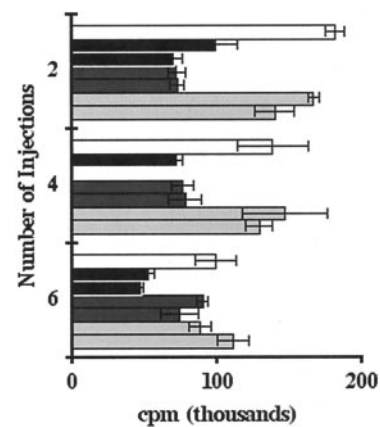


FIGURE 4. [³H]Thymidine incorporation in response to irradiated B6 splenocytes by responders isolated from CBA mice injected with PBS (open bars), IDO-transfected MC57G clones 26 (filled bars) or 24 (dark gray bars), or vector-transfected MC57G cells (light gray bars) as described in *Materials and Methods*.

per se because H-2^b-specific proliferative responses were comparable when responder splenocytes originated from naive (PBS-treated) CBA mice or from mice exposed to vector-transfected MC57G tumor cells. This suggested that IDO expression by MC57G cells was a critical factor and that MC57G cells, in common with other tumor cell lines, were not very immunogenic. These outcomes indicated that IDO-expressing H-2^b tumor cells reduced the potency of T cell responses to H-2^b alloantigens substantially below the level elicited when responders originated from naive CBA mice.

T cell responses are suppressed in IDO-transgenic mice

To further evaluate whether increased IDO activity inhibited *in vivo* T cell responses, we adapted an experimental system in which H-2K^b-specific CD8⁺ T cells from donor BM3 TCR transgenic mice mounted potent responses to recipient H-2K^b alloantigen after adoptive transfer (5, 14). For these experiments, we used IDO-transgenic (MI) mice expressing increased IDO activity due to expression of murine IDO cDNA linked to promoter elements derived from a murine MHC class II gene (see *Materials and Methods*). Phenotypic characterization of splenocytes isolated from MI transgenic mice lines 31 and 33 revealed increased amounts of IDO protein (Fig. 1D) and IDO enzyme activity (Fig. 1E). Recipient mice expressing H-2K^b alloantigen on the MI transgenic background were generated by intercrossing MI line 33, which expressed the highest amount of IDO protein in spleen (Fig. 1D), with H-2K^b (CBK) transgenic mice and identifying double- (MI × CBK) and single-transgenic (CBK) offspring by genotype analysis. BM3 splenocytes were injected into these recipients and numbers of donor T cells (Ti98⁺CD8⁺) present in recipient spleen were assessed by flow cytometry.

Ninety-six hours after adoptive transfer, numbers of donor BM3 CD8⁺ T cells detected in spleens of double-transgenic (MI × CBK) recipients were substantially less (~66%) than in spleens of CBK recipients (Fig. 5A). Comparable numbers of BM3 T cells (stained with the tracking dye CFSE) were detected in these lymphoid tissues from recipient CBA and MI transgenic mice, indicating that enhanced IDO expression did not alter the anatomical distribution of injected T cells (data not shown). As expected, adoptive transfer of BM3 splenocytes into CBA recipients elicited no T cell responses. Similar outcomes were obtained when male (H-Y) Ag-specific CD4⁺ T cells from A1 TCR transgenic donors

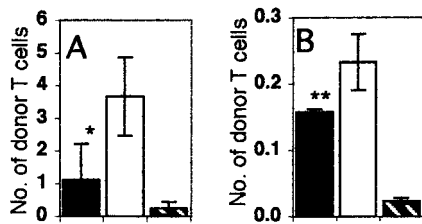


FIGURE 5. Numbers of donor T cells detected in spleens of recipient mice after adoptive transfer of allogeneic T cells from BM3 (A) or A1 (B) TCR-transgenic mice as described in *Materials and Methods*. A, BM3 (CD8⁺Ti98⁺) T cells detected in double-transgenic (MI × CBK; filled bars), CBK transgenic (open bars), and CBA (striped bars) recipient mice 96 h after adoptive transfer. B, A1 (CD4⁺Vβ8.1/8.2⁺) T cells detected in MI transgenic male (filled bars), CBA male (open bars), and CBA female (striped bars) recipient mice 90 h after adoptive transfer. *, $p < 0.001$; **, $p < 0.004$.

(15) were transferred into male and female MI transgenic mice (Fig. 5B). Approximately 33% fewer H-Y-specific donor CD4⁺ T cells were elicited in MI transgenic male mice expressing enhanced IDO activity. These outcomes indicated that enhanced IDO activity in MI transgenic mice inhibited alloantigen-specific T cell responses after T cell adoptive transfer in vivo.

Discussion

Data generated in this study directly address the hypothesis that cells expressing IDO inhibit T cell responses in vitro and in vivo. We show that IDO-transfected tumor cells and tissue microenvironments with enhanced IDO activity inhibited T cell proliferation and reduced the number of T cells elicited over time. These observations suggest that genetic or pharmacological manipulation of IDO activity may alter the susceptibility of cells and tissues to T cell responses. They also provide new insight into the physiologic role of APCs expressing IDO in vivo, such as immunoregulatory CD8⁺ dendritic cells in mice and human myeloid cells cultured from blood monocytes (9–11).⁴

The majority of T cells cultured with IDO-transfected MC57G tumor cells did not divide, even though they exhibited features of activated T cells, because expression of CD69, CD71 was induced and TCR levels were down-regulated. These outcomes suggest that the ability of MC57G tumor cells to deliver signals through the TCR/CD3 complex was not affected by IDO expression. This further implies that regulation of T cell proliferation in the presence of IDO-transfected tumor cells occurred after the majority of T cells entered the cell cycle and before completion of the first cell cycle. These outcomes with IDO-transfected cells recapitulate previous data showing that human macrophages expressing IDO blocked T cell cycle progression (7). However, previous studies with human myeloid cells expressing IDO relied exclusively on pharmacological inhibition of IDO activity. The studies reported here extend previous observations by showing that genetic manipulations leading to IDO expression in APCs also lead to inhibition of elicited T cells responses.

We could not assess whether T cells entered S-phase when cocultured with IDO-transfected MC57G tumor cells because background incorporation of thymidine by tumor cells was not reliably blocked by irradiation or use of mitomycin C in our experimental system (data not shown). However, data from experiments in which T cells were activated in the absence of the essen-

tial amino acids tryptophan or isoleucine/leucine showed that the ability of human and murine T cells to enter S-phase is exquisitely and selectively dependent on the presence of tryptophan midway through the G₀-S phase transition (data not shown). The precise mechanisms whereby T cell cycle progression depends on the availability of tryptophan remain obscure. Nevertheless, the data we report here are the first direct test of the hypothesis that genetic manipulations to enhance IDO expression in APCs lead to inhibition of T cell responses.

The eventual outcomes of physiologic immune responses depend critically on the functional status of dendritic cell subsets that either promote or suppress T cell responses (18, 19). Though much is known about mechanisms used by dendritic cells to generate effector T cells, less is known about critical processes they use to suppress or deviate T cell responses after Ag-specific activation. Many recent reports document a role for dendritic cells in immunoregulatory, rather than immunostimulatory, phenomena (3, 4, 20). However, details of the cellular, molecular, and biochemical mechanisms underlying these immunoregulatory phenomena remain obscure. Puccetti and colleagues (10, 11) have shown that murine splenic CD8⁺ dendritic cells are potent suppressors of delayed-type hypersensitivity responses to tumor-associated peptides presented by immunogenic CD8⁻ dendritic cells. The immunosuppressive properties of these CD8⁺ dendritic cells were blocked by 1-methyl-tryptophan, the same competitive inhibitor of IDO enzyme activity that blocked maternal T cell tolerance of fetal allografts during murine pregnancy (5, 6). More recently, this group has shown that murine macrophages and dendritic cells express IDO and that CD8⁺ dendritic cells, but not macrophages, express IDO constitutively (21). Moreover, CD8⁺ dendritic cells increased the rate of CD4⁺ T cell apoptosis, providing a potential mechanistic explanation for the immunosuppressive effects mediated by this dendritic cell subset in vivo (11, 21). In contrast, we observed no increase in the rate of T cell apoptosis when BM3 T cells were cocultured with IDO-transfected cells, although tumor cell growth in cocultures enhanced T cell apoptosis and precluded rigorous investigation at later times. Similarly, human T cells cultured with immunosuppressive macrophages or dendritic cells did not exhibit enhanced apoptosis (7, 8). These discrepancies might arise due to differences in the experimental systems used to assess the impact of IDO activity on T cell activation. For example, CD8⁺ T cells from BM3 TCR transgenic mice and human T cells may be inherently more resistant than CD4⁺ T cell clones to apoptosis. In addition, MC57G tumor cells, unlike CD8⁺ dendritic cells, may not deliver apoptotic signals to T cells. Nevertheless, the ability to inhibit T cell clonal expansion after activation, with or without subsequent apoptosis, provides a mechanistic explanation for the observed link between physiological expression of IDO and suppression of T cell responses in vivo. Clonal expansion of Ag-specific T cells is an obligatory step in generating effective physiologic immune responses due to the very low frequency of Ag-specific T cells present in naive T cell repertoires. Hence, the ability of APCs expressing IDO to block or inhibit T cell clonal expansion during the afferent phase would have a major impact on the potency of immune responses in vivo. In summary, data presented in this report suggest that genetic manipulations to force IDO expression may result in enhanced ability of cells and tissues to suppress T cell responses in vivo after transplantation.

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