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

Andrew L. Mellor, 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 overexpressing CD8+ dendritic cells to suppress in vitro T cell proliferation (7, 8). Several recent reports extend the potential biologic significance of the IDO mechanism to moderate 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 × 10⁶ 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 μg of linearized (NotI, BglI) pcDNA3-IDO or pcDNA3 vector DNA using a setting of 320 mV/975 μF on an electroporation machine (Bio-Rad, Hercules, CA). Cells were cultured in IMDM medium supplemented with 10% FCS, 1-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/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 μM tryptophan and 1200 μg/ml G418 (Life Technologies, Rockville, MD). After 3 wk of selection, single clones were selected and analyzed.

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

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

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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-2Kb 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-2Kb-specific CD8+ T cells or male (H-Y) Ag-specific CD8+ 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 (MO) founder mice were identified by Southern blot hybridization using a transgene specific probe, and mice were mated with CBA/Ca strain partners to establish two separate transgenic lines, 33 and 33. Recipient mice for adoptive transfer experiments were generated by intercrossing transgenic (heterozygous) mice (line 33) with CBK (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 107 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 1 ml of HPLC-grade methanol. Samples containing 50 μg of total protein lysate were separated on 12% SDS-PAGE. IDO protein was detected in murine epididymis (Fig. 1), and 33. Recipient mice for adoptive transfer experiments were generated by intercrossing transgenic (heterozygous) mice (line 33) with CBK (homozygous) mice and selecting double (MI × CBK) and single (CBK) transgenic littermates by genotypic analyses.

IDO-transfected tumor cells

MC57 fibrosarcoma cell lines (H-2b haplotype) were selected for this study because they elicit potent H-2Kb-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 catalyzed 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-2Kb 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-2Kb-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
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 ~80% of naive T cells did not divide at all in the presence of IDO-transfected cells and that the rest (~20%) divided once only. Similar outcomes were observed when BM3 T cells were cocultured with cloned IDO-transfected MB49 bladder carcinoma cells, another H-2b-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 anticoncanotypic 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 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 (~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.
IDO-transfected tumor cells inhibit alloreactive T cell responses in vivo

CBA (H-2k haplotype) mice were injected twice weekly (for 1–3 wk) with allogeneic IDO-transfected (clone 24 or 26) or vector-transfected MC57G cells (H-2b) to assess whether IDO-transfected cells mediated in vivo T cell responses to H-2b alloantigens. H-2b-specific T cell responses were assessed by coculturing splenocytes from immunized mice (responders) with irradiated splenocytes from C57BL/6 (H-2b stimulators) mice. Elicited H-2b-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 < 0.02). Similarly, IDO-transflectant clone 24 elicited less potent H-2b-specific responses after two or four injections (p < 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-transflectant-treated mice was still apparent in this group. Assays for T cell proliferative 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 clone 26 tumor cells (data not shown). However, exposure to IDO-transflectants did not completely abrogate H-2b-specific cytolytic functions, indicating that immunization with IDO-transfected MC57G tumor cells did not eliminate all H-2b-specific cytolytic precursors in mice. These outcomes suggest that the pool of H-2b-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 per se because H-2b-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-2b tumor cells reduced the potency of T cell responses to H-2b 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-2Kb-specific CD8+ T cells from donor BM3 TCR transgenic mice mounted potent responses to recipient H-2Kb 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-2Kb 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-2Kb (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<sup>+</sup>/Ti98<sup>+</sup>) 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<sup>+</sup>/V8.1/8.2<sup>+</sup>) 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.

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 cell 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 essential 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 G<sub>0</sub>-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<sup>+</sup> dendritic cells are potent suppressors of delayed-type hypersensitivity responses to tumor-associated peptides presented by immunogenic CD8<sup>+</sup> dendritic cells. The immunosuppressive properties of these CD8<sup>+</sup> 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<sup>+</sup> dendritic cells, but not macrophages, express IDO constitutively (21). Moreover, CD8<sup>+</sup> dendritic cells increased the rate of CD4<sup>+</sup> 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<sup>+</sup> T cells from BM3 TCR transgenic mice and human T cells may be inherently more resistant than CD4<sup>+</sup> T cell clones to apoptosis. In addition, MC57G tumor cells, unlike CD8<sup>+</sup> 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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