Efficacy of Levo-1-Methyl Tryptophan and Dextro-1-Methyl Tryptophan in Reversing Indoleamine-2,3-Dioxygenase–Mediated Arrest of T-Cell Proliferation in Human Epithelial Ovarian Cancer

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Abstract
It has been reported that levo-1-methyl tryptophan (L-1MT) can block indoleamine-2,3-dioxygenase (IDO) expressed by human dendritic cells (DC), whereas dextro-1-methyl tryptophan (D-1MT) is inefficient. However, whether L-1MT or D-1MT can efficiently reverse IDO-induced arrest of human T-cell proliferation has not been clarified. Here, we show a marked immunosuppressive effect of IDO derived from INDO-transfected 293 cell, IDO+ ovarian cancer cells, and monocyte-derived DCs on CD4+ Th1 cells, CD8+ T cells, and natural killer cells derived from peripheral blood, ascites, and tumors of ovarian cancer patients. We found that, whereas L-1MT and D/L-1MT can restore proliferation of tumor-derived and peripheral blood T-cell subsets, D-1MT does not effectively restore IDO-induced arrest of T-cell proliferation. Although D-1MT inhibited kynurenine production at high concentrations, L-1MT was more effective in abrogating kynurenine generation and tryptophan depletion, whereas tryptophan was completely depleted by IDO even in the presence of high amounts of D-1MT. Together, the results indicate that, whereas the generation of tryptophan metabolites (kynurenines) by IDO is important in mediating suppression of T-cell proliferation, the degree to which tryptophan depletion is restored by 1MT is also critical in overcoming IDO-induced arrest of T-cell proliferation. [Cancer Res 2009;69(13):5498–504]

Introduction
The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) catalyzes tryptophan metabolism and plays a key role in the immune tolerance of semiallogeneic fetus (1), an effect possibly through tryptophan depletion and via the generation of proapoptotic metabolites (2, 3). Several lines of evidence also suggest a link between IDO enzyme activity and tumoral immune tolerance (3). IDO+ human antigen presenting cells (DC) in preclinical models (16). A potential explanation for the discrepant biochemical and antitumor effects was provided by the discovery of the IDO2 gene, which seems to be preferentially inhibited by dextro-1MT (D-1MT; ref. 17). However, recent human studies indicate that levo-1MT (L-1MT) inhibits IDO activity in human DCs via blockade of IDO1 (18). Thus, whereas the IDO2 gene seems to be functional in murine models, it was not found to be functional in humans (18) and may not adequately explain the effects of D-1MT.

To determine which of the 1MT stereoisomers should be tested in human ovarian cancer immunotherapy trials, we assessed susceptibility of lymphocytes obtained from peripheral blood, ascites, and tumor tissues of EOC patients to IDO-expressing cells and examined the ability of different isomers of 1MT in overcoming T-cell suppression. Our data indicate that T cells derived from EOC patients are suppressed by IDO-expressing cells. Although human IDO activity can be inhibited by D-1MT, L-1MT, and racemic (D/L-1MT), only L-1MT and D/L-1MT can reverse T-cell suppression. These findings have significant implications in selecting IDO inhibitors for testing in clinical trials.
Materials and Methods

Patients, cell lines, and reagents. Peripheral blood, ascitic fluid, and tumor specimens were obtained from patients undergoing debulking surgery for EOC at Roswell Park Cancer Institute. Blood samples were also obtained from normal healthy volunteers as controls. All specimens were collected under an approved protocol from the institutional review board. Human embryonic kidney cell line 293 and IDO1 (encoded by IDO)–transfected 293 cell line (293IDO) were gifts from Dr. Benoît J. Van den Eynde (3). Primary ovarian cancer cell lines were generated in our laboratory from patients undergoing surgery at our institution. Established ovarian cancer cells were purchased from American Type Culture Collection. Monocyte-derived DCs were generated as previously described (2, 19). Briefly, CD14+ cells were purified from normal donor peripheral blood lymphocytes (PBL) by monocyte isolation kit II (Miltenyi Biotec) and cultured in the presence of 50 ng/ml recombinant human interleukin-4 (IL-4; R&D Systems) and 50 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (R&D Systems) in complete media. On day 6 of culture, immature DCs (iDC) were treated for 36 h with a combination of 500 ng/ml CD40 ligand (CD40L; Sigma-Aldrich) and 5 μg/ml lipopolysaccharide (Sigma-Aldrich). IDO synthesis was induced by incubating iDCs with 50ng/ml IFN-γ (Becton Dickinson Pharmingen) along with maturation factors. IDO activity was measured by kynurenine production from the supernatant.

Isolation of PBL, tumor-associated lymphocytes, tumor-infiltrating lymphocytes, and lymph nodes. Peripheral blood samples were collected in heparinized tubes, and mononuclear cells were isolated by density gradient separation using Ficoll/Hypaque (Sigma-Aldrich). Tumor-associated lymphocytes (TAL) were obtained from centrifuged cell pellets of ascitic fluid. The pellets were washed twice in PBS, placed on Ficoll-Hypaque density gradients, and centrifuged again to harvest TALs and tumor cells. For the isolation of tumor-infiltrating lymphocytes (TIL) or lymphocytes from draining lymph nodes (LN), tumor specimens or lymph nodes were finely minced in cell culture medium and single-cell suspensions were washed twice in PBS followed by Ficoll-Hypaque purification. Enriched TAL and TIL preparations were washed twice in PBS and frozen in media containing 90% fetal bovine serum and 10% DMSO at −80°C until use.

Assessment of cellular proliferation and suppression. Magnetic bead separation technology (Invitrogen Dynal AS) was used to generate CD8+ (CD4+, or CD25-depleted CD4+ (CD4+CD25–) T-cell subsets. CFSE-labeled T cells were separately cultured with IDO+ suppressive cells with or without 1 μM/L D-IMT, L-IMT, or D/L-IMT (Sigma-Aldrich) with plate-bound anti-CD3 (UCHT1 clone, 5 μg/ml) and soluble anti-CD28 (CD82B clone, 1 μg/ml) monoclonal antibodies (mAbs; BD Biosciences). The ability of IDO to suppress the growth of responder T cells was assessed by fluorescence-activated cell sorting (FACS) after coculture for 3 to 6 d.

Antibodies and FACS analysis. Anti–CD3-APC H7, CD4-APC, CD8-PE-Cy7, CD25-PE-Cy5, CD56-PE, and Annexin V-FITC apoptosis detection kit I were purchased from Becton Dickinson Pharmingen. LIVE/DEAD Fixable Dead Cell Stain kits were from Invitrogen. Surface staining was performed for 15 to 20 min at 4°C in FACS tubes containing 1 to 2 million total cells in 100 μl staining buffer (PBS with 1% FCS and 0.05% NaN₃ Sigma-Aldrich). For intracellular staining for cytokines expression, CD4+ T cells were incubated with 1 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μM/L ionomycin (Sigma-Aldrich) for 2 h. Brefeldin-A (Sigma-Aldrich) was added to the samples, and the cells were incubated for an additional 4 h. After that, cells were stained with PE-Cy7–labeled CD4 mAb and LIVE/DEAD, fixed, and stained intracellular cytokine with allophycocyanin-labeled IFN-γ mAb and PE-labeled IL-4 mAbs (BD Pharmingen) in permeabilizing solution (CALTAG) containing normal mouse IgG (CALTAG) at room temperature for 30 min. Anti-IDO antibodies were from Chemicon International and used as described (20). Stained samples were acquired on an LSR II flow cytometer (BD Biosciences) using FACSDiVa software, and listmode data were analyzed with WinList (Verity Software House).

High-performance liquid chromatography measurement of tryptophan and kynurenine. The high-performance liquid chromatography (HPLC) method reported for the separation and measurement of tryptophan and kynurenine in cell extracts (21) was adapted to ultra-performance liquid chromatography (Waters Associates). Cell culture supernatant was extracted by adding 1.3 mL of ice-cold methanol to 150 μl of sample. The precipitates were removed by centrifugation, and the

Figure 1. Immunosuppressive effect of IDO on T cells and NK cells derived from PBMCs, TILs, and TALs of ovarian cancer patients. A, CFSE-labeled CD4+ (top) and CD8+ (bottom) T cells isolated from PBMCs of EOC patients were cocultured with 293IDO cells. Proliferation of T cells was determined by FACS. B, proliferation of CD4+ and CD8+ cells from EOC TALs or TILs was suppressed by 293IDO cells. Columns, mean of three independent experiments; bars, SD. C, IDO suppresses NK cell proliferation. CFSE-labeled EOC TALs were cocultured with 293 cells as described for 4 d. NK (CD56+CD3–) cell proliferation was detected by FACS after gating on CD56+ cells. *, P < 0.05 compared with T cells cocultured with 293 cells without IDMT.

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supernatant was dried under vacuum. Samples were reconstituted in 150 μL of mobile phase, and a 20-μL injection was made. Standards (ranging from 0.1 to 5 μg/mL) and quality control samples prepared in mobile phase were extracted, as described above along with the cell supernatant samples. The separation of tryptophan and kynurenine was carried out on an Acquity BEH C18 column (2.1 × 100 mm 1.7 μm, Waters Associates). Mobile phase consisted of (a) 10% acetonitrile with 10% of 10 mmol/L formic acid (pH 3) and (b) 10% of 10 mmol/L formic acid in 100% acetonitrile (pH 3). Flow rate was set at 0.533 mL/min. Separation was achieved using a gradient from 0 to 80% (b) in 3 min. Detection was at 225.1 nm. Retentions for tryptophan and kynurenine were 1.1 and 0.75 min respectively.

**Immunohistochemistry.** Tumor specimens were fixed with buffered formalin and embedded in paraffin. Sections (5 μm) were placed on glass slides, heated at 60°C for 20 min, and then deparaffinized with xylene and ethanol. For antigen retrieval, tumor specimens mounted on glass slides were immersed in preheated antigen retrieval solution (DAKO high-ph solution; DAKO) for 20 min and allowed to cool for 20 min at room temperature. After the inactivation of endogenous peroxidase, purified IDO-specific mAb (Abcam; 1:20 dilution or 75 μg/mL) were then added and incubated overnight at 4°C. The primary antibody was detected with a biotinylated antimouse IgG (DAKO). Diaminobenzidine tetrahydrochloride was then added for development for 10 min and then followed by counterstaining with hematoxylin solution. Immunohistochemical reactivity was graded based on percentage of tumor cell positivity and intensity as follows: negative (no staining), 6% to 25% of cells stained (+); 26% to 50% of cells stained (++); 51% to 75% of cells stained (+++); and >75% of cells stained (+++). The intensity was graded as negative (n), weak (w), moderate (m), and strong (s). Negative control slides omitting the primary antibody were included in all assays.

**Statistical analysis.** Comparison between paired or unpaired groups was performed using the appropriate Student’s t test. A P value of <0.05 was defined as statistically significant.

**Results**

L-1MT, but not D-1MT, restores proliferation of T cells suppressed by IDO in peripheral blood mononuclear cells, TILs, and TALs of ovarian cancer patients. To investigate the effect of IDO on T-cell proliferation and cytokine production, we first showed that human CD4+ T-cell proliferation was inhibited by IDO-expressing cells, and this inhibition was most significant in the Th1 subset and can be reversed by the IDO inhibitor L-1MT (Supplementary Figs. S1–S3, available online). To determine the efficiency of 1MT stereoisomers in inhibiting human IDO function, (Supplementary Figs. S1–S3, available online). To determine the efficiency of 1MT stereoisomers in inhibiting human IDO function, the effects of D-1MT, L-1MT, and D/L-1MT on T-cell proliferation were tested. CFSE-labeled CD4+ T cells isolated from peripheral blood of ovarian cancer patients were cocultured with IDO-expressing cells. As shown in Fig. 1A, >70% of cells were divided when CD4+ T cells were cocultured with 293 cells with or without different isoforms of 1MT, suggesting that none of the isoforms affect CD4+ T-cell proliferation by themselves. In contrast, CD4+ T-cell proliferation was significantly reduced to <2% when they were cocultured with 293IDO. Notably, CD4+ T-cell proliferation was restored to 59% and 54%, respectively, when L-1MT and D/L-1MT were added. However, the addition of D-1MT did not restore T-cell proliferation.

Next, we examined the different isoforms of 1MT in relation to CD8+ T cells derived from peripheral blood of EOC patients. Consistent with the results from CD4+ T cells, proliferation of CD8+ T cells was also suppressed by 293IDO cells and the suppression was restored by L-1MT and D/L-1MT, but not by D-1MT (Fig. 1A).

To determine the direct role of IDO in suppressing T-cell proliferation in the human ovarian tumor environment, we isolated TILs and TALs from ovarian cancer patients and cocultivated these lymphocytes with 293 or 293IDO cells with or without 1MT stereoisomers. Similar to the results obtained from healthy donor and ovarian cancer PBMCs, CD4+ and CD8+ T cells derived from TILs or TALs proliferated significantly when cocultured with 293 cells with and without 1MT. On the other hand, CD4+ and CD8+ T-cell proliferation was inhibited when cocultured with 293IDO cells. However, T-cell proliferation was significantly restored when L-1MT and D/L-1MT were added to 293IDO cocultures. D-1MT was inefficient in reversing T-cell suppression (Fig. 1B). Interestingly, we also observed NK (CD56+CD3+) cell suppression by 293IDO cells in PBMCs, TILs, and TALs of ovarian cancer patients (Fig. 1C). Proliferation of NK cells was suppressed by IDO, and L-1MT, but not D-1MT, was able to restore NK cell proliferation.

**IDO+ ovarian cancer cells suppress T-cell proliferation.** Because of the profound suppressive effects of IDO-expressing cells on tumor-derived and peripheral blood CD4+, CD8+ T cells, and NK cells, we tested a tissue microarray of 201 EOC specimens. IDO expression was observed in 83 of 192 (43%) of ovarian cancer specimens and tumor expression of IDO resulted in lower ratio of tryptophan to kynurenine (T/K) in the tumor environment (Supplementary Fig. S4). To investigate the role of IDO expressed by ovarian tumor cells on T-cell suppression, we generated primary ovarian cancer cell lines from patients undergoing surgery for ovarian cancer. We tested the effects of primary ovarian cancer cell lines generated in our laboratory (e.g., RP-OV-17534) and an

**Figure 2.** A, T cells are suppressed by conditioned media derived from ovarian cancer cells. Primary (RP-OV-17534) and established (OV2774) ovarian cancer cell line supernatants after 6 d of culture were collected, and T cells from normal donor PBLS were cultured under anti-CD3 mAb stimulation. At 4 d later, CD4+ T-cell proliferation was tested by CFSE dilution. B, L-1MT and D/L-1MT are more efficient in suppressing IDO functional activity of both primary ovarian cancer cells and established ovarian cancer cell lines. Primary (RP-OV-17534) and established (OV2774) cancer cells were treated or not with IFN-γ with or without different 1MTs for 6 d. Supernatants were collected, and kynurenine concentrations were measured.
established ovarian cancer line (OV2774) on T-cell proliferation \((n = 3\) for each group; Fig. 2A). Because it has been reported that IFN-\(\gamma\) induces IDO expression, we also treated tumor cells with IFN-\(\gamma\) before T cells were cultured in media conditioned by the tumor cells. T cells were cultured for 4 days in conditioned media derived from culturing the ovarian cancer cell lines. T-cell proliferation was higher in ovarian cancer cell line OV2774 and RP-OV-17534 conditioned media compared with RP-OV-17534 IFN-\(\gamma\)-treated and OV2774 IFN-\(\gamma\)-treated conditioned media (Fig. 2A). These findings indicate that conditioned media derived from IFN-\(\gamma\)-treated primary and established ovarian cancer cells resulted in more T-cell suppression compared with non–IFN-\(\gamma\)-treated ovarian cancer cells. Next, we tested whether the observed effects of the culture conditions on T-cell proliferation rates were related to functional IDO enzyme activity as measured by kynurenine levels in each of the cell cultures. As shown in Fig. 2B, primary ovarian cancer cells (RP-OV-17534) had IDO activity without IFN-\(\gamma\) treatment whereas established ovarian cancer cells (OV2774) did not show IDO enzyme activity. After IFN-\(\gamma\) treatment, OV2774 showed significant IDO enzyme activity whereas RP-OV-17534 showed further increase in IDO activity. Functional IDO activity from both cells was inhibited by L-1MT and D/L-1MT, whereas D-1MT was less efficient in inhibiting IDO activity. Together with the T-cell suppression results (Fig. 2A), the data indicate that IDO+ ovarian cancer cells also suppress T-cell proliferation and that L-1MT and D/L-1MT are more efficient than D-1MT in inhibiting functional IDO enzyme activity from human ovarian cancer cells.

D-1MT inhibits IDO activity but does not reverse T-cell proliferation. To verify whether 293IDO showed IDO enzyme activity, concentrations of tryptophan and kynurenine were measured by HPLC in culture supernatants (Fig. 3A). In cell-free media, there was no detectable tryptophan degradation whereas 293 cells cultures resulted in 50% reduction in tryptophan levels after 4 days. Importantly, the culture supernatants from 293IDO cells showed that tryptophan was completely degraded into kynurenine. These results are consistent with the data from our T-cell suppression assays and provided additional confirmation that IDO enzyme activity resulted in T-cell suppression in our coculture systems.

To determine the potential reasons for the failure of D-1MT to effectively restore IDO-mediated suppression of human T-cell proliferation, concentrations of tryptophan and kynurenine from 293IDO supernatant cultured for 4 days in the presence of various amounts of different 1MT isomers were measured by HPLC (Fig. 3B). Both L-1MT and D/L-1MT were very efficient in inhibiting kynurenine production by 293IDO cells. Although D-1MT was effective, it achieved less inhibition of kynurenine production even at high concentrations. This is contrary to a previous report wherein D-1MT was shown to be completely inefficient in inhibiting human IDO enzyme activity (18). However, even at this higher concentration, D-1MT could still not restore CD8\(^+\) and CD4\(^+\) T-cell proliferation from normal donors and EOC patients (Fig. 3C). An examination of tryptophan catabolism by IDO in these cultures revealed that, even when L-1MT and D-1MT inhibited kynurenine production to the same degree, they differed significantly with respect to suppression of tryptophan consumption (Fig. 3C). We noted about the same amount of tryptophan in the supernatant of both 293 cells and 293IDO cells cultured in the presence of L-1MT. In contrast, there was almost no detectable tryptophan in the supernatant of 293IDO cells with and without D-1MT, suggesting that tryptophan was completely depleted by 293IDO cells even in the presence of 1mmol/L of D-1MT. Together, the results indicate that, whereas the generation of tryptophan metabolites

![Figure 3](image-url)

Figure 3. Effect of different 1MT isomers on IDO activity and T-cell proliferation. A, concentrations of tryptophan and kynurenine were measured in supernatants of cultures on day 4. Columns, mean of three independent experiments; bars, SD. B, D-1MT inhibits IDO activity. IDO enzyme activity (measured as kynurenine production in culture supernatants) by 293IDO cells was inhibited by different isomers of 1MT. 293IDO cells without 1MT were used for comparing percentage of inhibition of maximal kynurenine production. C, T-cell suppression correlates with tryptophan deprivation. Proliferation of CFSE-labeled CD8\(^+\) or CD4\(^+\) cells purified from EOC TALs and concentrations of tryptophan and kynurenine were measured. \(* P < 0.05\) compared with 293 cells cultures without 1MT. D, exogenous tryptophan was able to reverse Th1 cells suppressed by 293IDO cells supernatant. Supernatant from 293 cells and 293IDO cells cultured in the presence or absence of different 1MTs for 4 d were collected, and healthy donor T cells were cultured in these conditioned media with or without exogenous 100 \(\mu\)mol/L tryptophan under anti-CD3 stimulation. At 6 d later, T-cell proliferation and cytokines IFN-\(\gamma\) and IL-4 expression were analyzed by FACS.
(kynurenine) by IDO is important in mediating suppression of T-cell proliferation, the degree to which tryptophan depletion is abrogated by 1MT is also critical in overcoming IDO-induced arrest of T-cell proliferation.

To confirm the significant role of tryptophan depletion in IDO-dependent T-cell suppression, supernatant from 293 cells or 293IDO cells cultured in the presence or absence of different 1MTs for 4 days were collected and healthy donor T cells were cultured in these conditioned media with or without exogenous 100 µmol/L tryptophan under anti-CD3 stimulation. Six days later, T-cell proliferation and cytokine expression were analyzed by FACS. As expected, CD4+ T-cell proliferation was suppressed in 293IDO supernatant compared with those cultured in 293 cell supernatant and L-1MT and DL-1MT were more efficient than D-1MT in restoring T-cell proliferation. Importantly, this reversal of T-cell suppression could also be achieved by culturing T cells in 293IDO supernatant supplemented with tryptophan (Fig. 3D). Our data from Supplementary Fig. S1B indicate that 293IDO inhibited IFN-γ CD4+ cells whereas there was an increase in IL-4+ CD4+ cells. As shown in Fig. 3D, this IDO-dependent suppression of Th1 cells was reversed by both L-1MT and D/L-1MT, as well as exogenous tryptophan. Again D-1MT was less efficient in reversing suppression of Th1 cell proliferation (Fig. 3D).

IDO+ DC suppresses T cells whereas L-1MT and DL-1MT, but not D-1MT, reverse this suppression. To further confirm the potential in vivo implications of our findings in human ovarian cancer, we extended our analysis to IDO+ DCs. In previous reports, IDO+ DCs were characterized by coexpression of CD123 and CCR6 (20). Single-cell suspensions from TILs, tumor draining pelvic/paraortic lymph nodes (TDLN) of ovarian cancer patients, normal donor pelvic lymph nodes (NDLN), and PBMCs were stained with antibodies specific for CD123 and human IDO, as described. As shown in Supplementary Fig. S5, the frequencies of CD123+IDO+ cells in TILs were higher than in PBMCs of ovarian cancer patients and normal donors, but PBMCs of patients had more CD123+IDO+ cells than normal donor PBMCs. In addition, there was a significant increase in the frequency of IDO+DCs in TDLNs compared with NDLNs. These observations suggest that a population of IDO+DCs are generated in secondary lymphoid organs of ovarian tumor-bearing hosts and at the tumor microenvironment.

Next, we generated monocyte-derived iDCs from healthy donor PBLs, induced iDC to mature DCs (mDC) by CD40L and LPS. Some of the DCs were also treated with IFN-γ (hereafter called IFN-DC) to induce IDO production. Kynurenine analyses from supernatant enzyme activity (12.8 ± 0.26 µmol/L, 5.86 ± 0.10 µmol/L, 5.86 ± 0.10 µmol/L, P = 0.02 compared with iDC). This is a significant increase in the frequency of IDO+ DCs in TDLNs compared with NDLNs. These observations suggest that a population of IDO+DCs are generated in secondary lymphoid organs of ovarian tumor-bearing hosts and at the tumor microenvironment.

To determine their regulation of T-cell proliferation, DCs were coincubated with autologous PBLs and stimulated with anti-CD3 antibody in the presence or absence of different 1MTs. Without 1MT, both mDC and IFN-DC suppressed CD4+ (Fig. 4B) and CD8+ (Fig. 4C) T-cell proliferation (P < 0.05). Whereas L-1MT and D/L-1MT reversed the IDO+ DC-mediated T-cell suppression, D-1MT could not reverse the suppression of T cells (Fig. 4B and C). Proliferation of CD4+ and CD8+ T cells in mDC with D-1MT were 54.5 ± 1.81% (P = 0.01 compared with iDC no MT condition) and 66.05 ± 2.26% (P = 0.02), respectively.

Discussion
In the present study, we have shown that IDO is expressed in human ovarian tumors and that IDO is functional within the tumor environment, as evidenced by lower tryptophan/kynurenine ratios in ascites of IDO+ve compared with IDO-ve tumors. Thus, expression of IDO in ovarian cancer could be associated with immune tolerance via suppression of T-cell proliferation, leading to significant constraints on ovarian tumor immunity. Although we found no correlation of IDO tumor expression with clinicopathologic characteristics (supporting on-line text), this may reflect the
fact that the majority of patients in our study (92%) had advanced stage (III and IV) disease. In a previous study, whereas advanced stage EOC patients with noIDO-specific staining by immunohistochemistry survived without relapse, the survival of patients with diffuse tumor expression of IDO was only 11 months (8). Therefore, it is critical to identify IDO inhibitor drugs that may enhance the efficacy of spontaneous and vaccine-induced immune responses against human ovarian cancer.

We have also shown IDO-dependent inhibition of CD4+ Th1 and CD8+ T cells from peripheral blood, ascites, and tumors of EOC patients using 293IDO cells. This model allowed us to obtain reproducible results by coculturing IDO expressing cells with EOC lymphocytes. We observed IDO-specific inhibition of T-cell pro-

fission from all the anatomic compartments, and this was concomitant with tryptophan degradation and kynurenine generation. The IDO-mediated arrest of T-cell proliferation from EOC patients was associated with increased cell death via apoptosis, reduction in CD3, CD4, CD69 expression, and seemed to preferentially affect IFN-γ-producing Th1 cells rather than IL-4-producing Th2 cells (Supplementary Fig. S1–S3). Interestingly, another group also reported that IDO inhibited Th1 clones, but not Th2 clones (22). Because IDO does not affect up-regulation of CD25 expression on T cells, we propose that the inhibitory effects of IDO on human CD4+ T-cell proliferation are unlikely to be mediated via inability of T cells to respond to IL-2. Indeed, we observed suppression of T cells by 293IDO, in the presence or absence of IL-2 (data not shown).

To determine the potential in vivo physiologic relevance of our findings, we showed that primary ovarian carcinoma cells constitutively show functional IDO enzymatic activity and IDO-dependent suppression of T-cell proliferation. We also showed the presence of IDO+ DCs at ovarian tumor sites and TDLNs and the capacity of IDO+ DCs to mediate suppression of T-cell proliferation. Because kynurenic acid is a metabolite that is not subject to rapid subsequent metabolism, it is conceivable that persistently high concentrations in ascitic fluid of EOC patients could evoke three potential IDO-mediated mechanisms for suppression of T cells and NK cells in EOC. First, the direct effects of tumor expression of functional IDO leading to low tryptophan to kynurenine ratio in the tumor environment acts on naive or antigen-experienced cells and leads to arrest of proliferation. Second, IDO competent DCs in the tumor environment may also act directly (or indirectly via tryptophan catabolites) to suppress T-cell and NK cell proliferation. Third, at the time of antigen presentation to naive, resting T cells in ovarian cancer draining lymph nodes, IDO-competent DCs might suppress the initiation of immune responses to tumor-derived antigens.

To select the most efficient IDO inhibitor for future clinical testing in EOC patients, we examined two stereoisomers, along with a racemic mixture of 1MT. We showed that L-1MT was able to reverse the IDO-mediated arrest of T-cell proliferation in all of the in vitro model systems (293IDO, IDO+ ovarian cancer cells, IDO+ DCs). We also observed that L-1MT and D/L-1MT were more efficient than D-1MT in restoring T-cell proliferation arrest by abrogating tryptophan depletion. Whereas previous reports (17, 18) indicated that L-1MT, but not D-1MT, inhibited human IDO activity, we found that both L-1MT and D-1MT suppressed IDO enzyme activity when D-1MT was used at high concentration (≥800 μmol/L). Although L-1MT was able to almost completely inhibit 293IDO enzyme activity and restored tryptophan concentrations to levels comparable with cells cocultured with 293 alone, D-1MT (even at high concentrations) was less efficient in inhibiting kynurenine production and did not restore tryptophan. We consistently found that the generation of tryptophan catabolites was accompanied by very low concentrations of tryptophan (<1 μmol/L) when IDO+ cells were cocultured with T cells in the presence or absence of D-1MT. On the other hand, lower amounts of kynurenine were generated when IDO+ cells were cocultured with T cells in the presence of L-1MT, and significantly higher amounts of tryptophan (>10 μmol/L) were detectable, indicating the critical need for (a) preventing on-going tryptophan depletion and (b) reducing the amount of tryptophan catabolites to efficiently abrogate IDO-mediated T-cell suppression. In support of this notion, our data indicate that exogenous tryptophan restored 293IDO-mediated arrest of T-cell proliferation. Although we have not examined the role of additional tryptophan catabolites, it is possible that D-1MT also does not efficiently reverse the generation of 3-hydroxykynurenine and 3-
hydroxyanthranilic acid and, when combined with tryptophan depletion, limits the ability of D-1MT from restoring IDO-mediated arrest of T-cell proliferation.

Whereas our results indicating L-1MT but not D-1MT reverse IDO-dependent suppression of T cells are consistent with previous reports (18), a recent study (23) indicated at least equivalent activity (enzyme and inhibition of T-cell suppression) of D-1MT and L-1MT. The discrepancy in results might be related to the distinct biochemical and structural differences between mouse and human IDO (24). Moreover, IDO2 (25), which is strongly inhibited by D-1MT, was recently described and shown to be the most active form of IDO in mice (18). In contrast, whereas IDO2 is expressed by human DCs (18) and tumors (26), it is functionally inactive and consequently not affected by 1MT. Indeed, we failed to detect any IDO activity from human IDO2-transfected 293 cells (data not shown). Therefore, we could not analyze the effects of different 1MTs on human IDO2 activity and any IDO2-mediated T-cell suppression. These observations are consistent with the report by Lob and colleagues (18) wherein human DCs were shown to express both IDO1 and IDO2, but only IDO1-mediated tryptophan catabolism. Furthermore, IDO1 activity was blocked by L-1MT, whereas D-1MT was inefficient. In a more recent study by the same authors (26), although IDO2 was found to be expressed in human tumors, tryptophan degradation was entirely provided by IDO1 and D-1MT did not inhibit the IDO activity of malignant cells. Although these reports did not address the effect of the different isomers of 1MT on T-cell proliferation, it is clear from these studies and our data that human IDO2 has little or no functional activity and may not be critical for human tumor immunity. Taken together with the recent results by Lob and colleagues (18, 26), we conclude that L-1MT is more efficient than D-1MT in abrogating IDO-mediated arrest of T-cell proliferation in human ovarian cancer. The in vivo effects of D-1MT and L-1MT will still need to be tested in clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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L-1MT Reverses IDO-Mediated T-Cell Arrest

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Efficacy of Levo-1-Methyl Tryptophan and Dextro-1-Methyl Tryptophan in Reversing Indoleamine-2,3-Dioxygenase–Mediated Arrest of T-Cell Proliferation in Human Epithelial Ovarian Cancer

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