

# Reinstalling Antitumor Immunity by Inhibiting Tumor-Derived Immunosuppressive Molecule IDO through RNA Interference<sup>1</sup>

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Tumor-derived immune suppression is a major impediment to successful immune/gene cancer therapy. In the present study, we describe a novel strategy to disrupt tumor-derived immune suppression by silencing a tolerogenic molecule of tumor origin, IDO, using small interfering RNA (siRNA). Silencing of IDO in B16F10 cells in vitro using IDO-siRNA prevented catabolism of tryptophan and inhibited apoptosis of T cells. IDO-siRNA treatment of B16F10 cells in vitro inhibited subsequent growth, tumor formation, and the size of tumor formed, by those cells when transplanted into host mice. In vivo treatment of B16F10 tumor-bearing mice successfully postponed tumor formation time and significantly decreased tumor size. Furthermore, in vivo IDO-siRNA treatment resulted in recovery of T cells responses and enhancement of tumor-specific killing. Thus, silencing IDO may break tumor-derived immune suppression. These data indicate that RNA interference has potential to enhance cancer therapy by reinstalling anticancer immunity. *The Journal of Immunology*, 2006, 177: 5639–5646.

**T**he ability of cancer cells to evade or escape immune detection and destruction has been recognized as a hallmark of carcinogenesis and cancer progression (1). Essential to this process is the ability of tumor cells to create a state of immune tolerance or nonresponsiveness toward otherwise immunogenic tumor Ags. Unfortunately, our understanding of genetic and pathological events involved in immune tolerance remains largely incomplete. However, recent developments have helped to shed light into at least one of the underlying mechanisms by which tumor cells achieve tolerance through local suppression.

IDO, an enzyme that specifically catabolizes tryptophan, is an amino acid essential for T cell viability and proliferation. IDO is found under basal conditions in the epididymis, thymus, gut, lung, placenta, and some subsets of dendritic cells (DC)<sup>3</sup> (2). IDO was originally discovered in 1967 in the rabbit intestine (3) and is the object of renewed attention by immunologists in view of its capacity to act as an inducible negative regulator of T cell viability, proliferation, and activation during inflammation. In addition to potential in direct effects by IDO on APC function, IDO has been proposed to suppress T cells by degrading tryptophan (4) and in-

creasing the level of tryptophan degradation products (kynurenic acid and quinolinic acid) (5). Both of these activities suppress T cell response by inducing T cell apoptosis.

Recently, IDO has been found in human cancers of variable origin (6) and is implicated in tumor evasion in several murine models (6, 7). Due to its ability to suppress immune response endogenous tumor Ags, IDO represents an ideal target for immunomodulatory drugs.

Small interfering RNA (siRNA) technology represents a highly specific and efficient mechanism of gene silencing (8). As an endogenous viral defense mechanism, RNA interference (RNAi) uses a specific subset of enzymes that can bind and cleave homologous transcripts within mammalian and plant cells. We have reported successful use of siRNA silence immune-associated genes to induce immunomodulation for the purpose of transplantation (9–11). In view of the therapeutic potential of IDO suppression, we hypothesized that efficient silencing of IDO would be achieved by siRNA and could inhibit tumor-induced T cell suppression, reinstall antitumor immunity, and reduce tumor progression.

In the present study we applied this potent gene silencing method to reduce tumor-derived IDO. Using a murine melanoma model, we found that silencing IDO reduced catabolism of tryptophan and suppressed T cell apoptosis in vitro and inhibited tumor growth in vivo. Treatment of mice bearing B16F10 melanoma tumor with IDO-siRNA successfully postponed tumor onset and significantly decreased tumor size. Immune recovery was achieved primarily by rescue of T cell proliferation and through the enhancement of tumor-specific lysis. To our knowledge, this is the first report of use of RNA interference as a potential cancer therapeutic functioning through the inhibition of tumor-derived immune suppression and the reinstallation of anticancer immunity.

## Materials and Methods

### Animal and cell lines

Male C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory. A murine melanoma cell line established from a C57BL/6 mouse and designated B16F10 was obtained from the American Type Culture

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; KLH, keyhole limpet hemocyanin; 1-MT, 1-methyl-tryptophan; pDC, plasmacytoid DC; RNAi, RNA interference; siRNA, small interfering RNA.

Collection and was maintained in RPMI 1640 medium (Sigma-Aldrich) with 10% FBS, L-glutamine, penicillin, and streptomycin at 37°C in 5% CO<sub>2</sub>.

#### *IDO-specific siRNA expression vectors*

The target sequence in endogenous IDO mRNA (GUUCUAGAAGGAUC CUUGA) was selected on the basis of unique sequence and optimal GC content. Expression vectors directing synthesis of siRNA complementary to that sequence were constructed using the pQuiet plasmid (Welgen). Specific IDO-siRNA insert sequences were as follows: sense, 5'-CGCGGT TCTAGAAGGATCCTTGATTCTAGAGATCAAGGATCCTTCTAGAA CTTTTTA-3', and antisense, 5'-CAAGATCTTCTAGGAAGTAAG ATCTCTAGTTCCTAGGAA GATCTTGAAAAATGATC-3'. The insert sequences contained a 19-mer hairpin sequences specific to the IDO mRNA target, a loop sequence separating the two complementary domains, a two nucleotide overhang at the 3' end, a polythymidine region to terminate transcription, and a 5' single-stranded overhang for ligation into the pQuiet vector using the *MluI* and *SpeI* restriction sites. Both sense and antisense hairpin siRNA-encoding oligonucleotides were annealed and inserted into pQuiet plasmid. IDO-expressing vectors were amplified in *Escherichia coli* and were purified using a Qiagen kit (Qiagen).

#### *Gene silencing*

Transfection of siRNA into B16F10 cells was conducted using LipofectAMINE 2000 reagent (Invitrogen Life Technologies). Briefly, cells were plated into either 12-well plates ( $2 \times 10^5$  cells/well) or 6-well plates ( $5 \times 10^5$ ) and allowed to grow overnight in 1 or 2 ml of complete medium without antibiotics. Four micrograms of IDO-siRNA-containing plasmid was incubated with 10  $\mu$ l of LipofectAMINE 2000 reagent in 250  $\mu$ l of Optimal serum-reduced medium (Invitrogen Life Technologies) at room temperature for 20 min. The mixture was then added to B16F10 cell cultures grown to 90–95% confluence. Control cells were treated with transfection reagent alone, nonsense siRNA, or were untreated. To test the potency and efficacy of gene silencing, IFN- $\gamma$  (200 U/ml) was added to the medium 4 h after the start of transfection to up-regulate IDO expression. Twenty-four to 48 h later, transfected B16F10 cells were washed with PBS and used immediately for subsequent experiments.

Transfection of siRNA into DC was conducted as described previously (9). Briefly, IDO-siRNA-containing plasmid (4 or 6  $\mu$ g) was incubated with 20  $\mu$ l of GeneSilencer reagent (Gene Therapy Systems) in 50  $\mu$ l of serum-free RPMI 1640 medium at room temperature for 30 min. The mixture was then added to 400  $\mu$ l of DC cultured in 6-well plates. Control cells were treated with transfection reagent alone, nonsense siRNA, or were untreated. After 4 h of incubation, an equal volume of RPMI 1640 supplemented with 20% FCS, 20 ng/ml recombinant murine GM-CSF (PeproTech), and 20 ng/ml IL-4 (PeproTech) was added to cell suspensions, and IFN- $\gamma$  (200 U/ml) was added to the medium. Twenty four to 48 h later, transfected DC were washed and used for subsequent experiments.

#### *RT-PCR*

Total RNA from IDO-silenced, nonsense-siRNA-silenced, or mock-transfected B16F10 cells was isolated using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized using an RNA PCR kit (Invitrogen Life Technologies) with the supplied oligo d(T)16 primer. Reverse transcription reaction product (1  $\mu$ mol) of was used for the subsequent PCR. The primers used for IDO flanked the IDO-siRNA target sequences (forward primer, 5'-GGGCTTTGCTCTACCACATCCACT-3', and reverse primer, 5'-ACATCGTCATCCCCTCGGTTC-3'). GAPDH (internal negative control) primers were used as described previously (9). PCR conditions used were as follows: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s (30 cycles). PCR products were visualized using gel electrophoresis by staining with ethidium bromide in a 1.5% agarose gel.

#### *Western blot analysis*

Cytoplasmic extracts were prepared from IDO-silenced and control B16F10 cells mechanically released from tissue culture plates by scraping in cold PBS. Cells were collected by centrifugation (800  $\times$  g) and then resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1% NP40, 1 mM DTT, and 0.5 mM PMSF) with Complete protein inhibitor (Roche Diagnostics). Protein content was determined (Bio-) and 100  $\mu$ g of each cell lysate was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), blocked with 5% fat-free milk (Carnation) and 3% BSA in TBS-T (0.25% Tween 20), probed with a mouse anti-human IDO mAb that binds to both human and mouse IDO (clone 10.1, catalog no. MAB5412; Chemicon International) and goat anti-

mouse  $\beta$ -actin Ab (Sigma-Aldrich) according to the manufacturer's instructions, and visualized by an ECL assay (Amersham Biosciences).

#### *HPLC*

Twenty-five microliters of 15% perchloric acid was added to 50  $\mu$ l of culture supernatant to precipitate proteins. After incubation at room temperature for 10 min the samples were centrifuged at 12,000 rpm for 3 min. Sample pH was raised to ~4 by addition of 20  $\mu$ l of a citrate buffer (containing 200  $\mu$ l of 2 M citric acid with 280  $\mu$ l of 10 M sodium hydroxide and 480  $\mu$ l of reverse osmosis water). The samples were then mixed and centrifuged at 12,000 rpm for an additional 3 min. Seventy-five microliters of clear supernatant was loaded into a HPLC column (Hewlett-Packard 1090). Analytes were separated using a Spherisorb C8. Tryptophan was detected fluorometrically using excitation and emission wavelengths of 253 and 313 nm, respectively, whereas kynurenine was detected with the UV-VIS detector set to 360 nm. Peak area and quantitation was determined using Agilent Chemstation software.

#### *Bone marrow-derived DC*

Bone marrow-derived DC was obtained as previously described (9). In brief, bone marrow cells were flushed from the femurs and tibias of tolerant, rejective, and naive mice, washed in ice-cold PBS, and cultured at  $2 \times 10^6$  cells/well in 12-well plates (Corning) in 2 ml of RPMI 1640 (Invitrogen Life Technologies) medium supplemented with 10% FCS (Invitrogen Life Technologies), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ M 2-ME (Invitrogen Life Technologies), 10 ng/ml recombinant murine GM-CSF (PeproTech), and 10 ng/ml IL-4 (PeproTech). Nonadherent cells were removed after 48 h of culture, and fresh medium was added every 48 h. DC were used for in vitro experiments after 7 days of culture.

#### *Flow cytometry*

Phenotypic analysis of isolated or cultured DC was performed on a FACScan (BD Biosciences). All Abs were purchased from BD Pharmingen. T cell subsets were analyzed by staining with PE-conjugated CD4 or CD8 mAbs. Apoptosis of T cells was determined by double staining with above Abs and FITC-conjugated annexin V. All flow cytometric analyses were performed using appropriate isotype controls.

#### *CTL-derived lysis assay*

Cytotoxic activity was measured using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega) and the manufacturer's instructions. Briefly, target B16F10 cells were plated in triplicate in a U-bottom 96-well tissue culture plate (5000 cells/well) and incubated for 4 h with CD8<sup>+</sup> T cells isolated from B16F10 tumor-bearing mice at various ratios of effectors to target cells. Maximal release of LDH was recorded by incubating the target cells with lysis solution. Target cells without effector cells were used as a comparator to control for spontaneous LDH release. Released LDH in culture supernatants was detected after a 30-min incubation using a coupled enzymatic assay. The intensity of the color formed is proportional to the number of lysed cells. Cytotoxicity was calculated using the following formula: percent cytotoxicity = ((absorbance) - (spontaneous effector cell LDH release) - (spontaneous target cell LDH release))/((maximal LDH release) - (spontaneous target cell LDH release))  $\times$  100.

#### *Immunization of mice with peptide-pulsed DC*

After maintenance for 7 days in vitro, bone marrow-derived DC were transfected with IDO-siRNA or transfection reagent alone as described above, and pulsed with 10  $\mu$ g/ml keyhole limpet hemocyanin (KLH) Ag (Sigma-Aldrich) for 24 h. DC were then activated with LPS plus TNF- $\alpha$  for 24h, washed extensively with cold PBS, and used for subsequent experiments. Ag-pulsed DC ( $2 \times 10^6$  cells/mouse) were injected subcutaneously into syngeneic mice. Mice were sacrificed after 10 days and cell suspensions were prepared from the spleen and lymph nodes. These cells were cultured in 96-well plates at a concentration of  $4 \times 10^5$  cells/well in the presence or absence of Ag for 72 h. Cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine for the last 18 h. The cultures were harvested onto glass fiber filters (Wallac). Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter and the data was analyzed with UltraTerm 3 software.

#### *Mixed lymphocyte reaction*

T cells were purified from BALB/c splenocytes using nylon wool columns and were used as responders ( $1 \times 10^6$ /well). Allogeneic 9-day cultured and activated DC ( $5\text{--}40 \times 10^3$  cells, C57BL/6 origin) were used as the stimulators. A 72-h MLR was performed and the cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine for the last 18 h. The cultures were harvested onto glass

fiber filters (Wallac). Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter and the data was analyzed using UltraTerm 3 software.

#### Animal experiments

B16F10 cells were transfected with IDO-siRNA *in vitro*. Twenty four hours after transfection, the cells were harvested and washed twice in PBS and viable, trypan blue-excluding cells were counted. Viable cells ( $2 \times 10^5$ ) in a volume of 0.2 ml PBS were injected s.c. into the upper hind leg of each mouse. Mice were checked for tumor formation on alternate days. When tumors appeared, tumor size was measured by caliper and the volumes were estimated using the following formula: tumor volume =  $0.5 \times (\text{tumor width})^2 \times (\text{tumor length})$ .

For *in vivo* treatment,  $2 \times 10^5$  cells were suspended in 200  $\mu$ l of PBS and injected s.c. into the upper hind leg of each mouse. When the tumor size reached  $\sim 5$ –7 mm in diameter, six mice in each group received the first intratumoral or systemic injection of siRNA, or nonsense siRNA, followed by twice-weekly injections until the termination of the experiment.

#### Statistics

The unpaired Student's *t* test, assuming equal variances was used to determine the statistical significance of the difference in mean cell number or mean percentage in flow cytometry. This test was also used to analyze data when two groups were compared. MLR data were analyzed using a one-way ANOVA followed by the Newman-Keuls test. Differences with *p* values  $< 0.05$  were considered significant.

## Results

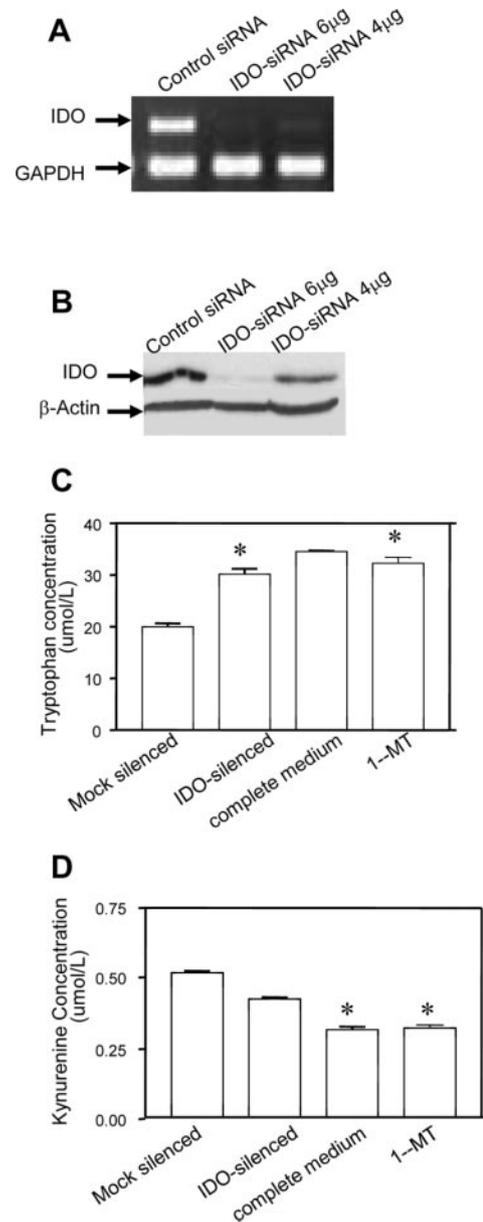
### IDO is efficiently silenced by siRNA in B16F10 melanoma cells

IDO has been shown to be expressed in B16F10 melanoma cells (6). To test the efficacy of gene silencing in these cells, we used liposome transfection to deliver our IDO-siRNA-containing pQuiet plasmid into B16F10 cells *in vitro*. Twenty-four hours after transfection, potent gene silencing was observed at the transcriptional level as detected by RT-PCR (Fig. 1A). The protein level of IDO was also significantly decreased as demonstrated by Western blot analysis (Fig. 1B), indicating that expression of IDO in B16F10 cells can be effectively inhibited by IDO-siRNA.

One of the substrates of IDO is tryptophan (12). Thus, to characterize the effects of silencing on enzyme efficacy, we examined the change in levels of tryptophan within B16F10 culture medium upon silencing. B16F10 cells silenced by siRNA displayed significantly lower IDO functionality ( $p < 0.05$ ) than B16F10 cells transfected with nonsense siRNA. Using the medium of nonsense siRNA-treated B16F10 cells as the negative control, HPLC analysis of cell culture medium from B16F10 cells with control and silenced IDO expression revealed higher levels of tryptophan (Fig. 1C) and lower levels of kynurenine, a degradation product of tryptophan (Fig. 1D) after IDO silencing. Culture medium from silenced cells had levels of tryptophan (Fig. 1C) and kynurenine (Fig. 1D) similar to fresh, unused culture medium used to establish baseline tryptophan and kynurenine levels. Addition of 1-MT (a chemical inhibitor of IDO) to the culture medium had an effect similar to treatment with IDO siRNA treatment (Fig. 1D). Taken together, these data suggest that IDO enzyme function was efficiently reduced by siRNA-based silencing.

### Silencing IDO in B16F10 cells before inoculation inhibits tumor growth

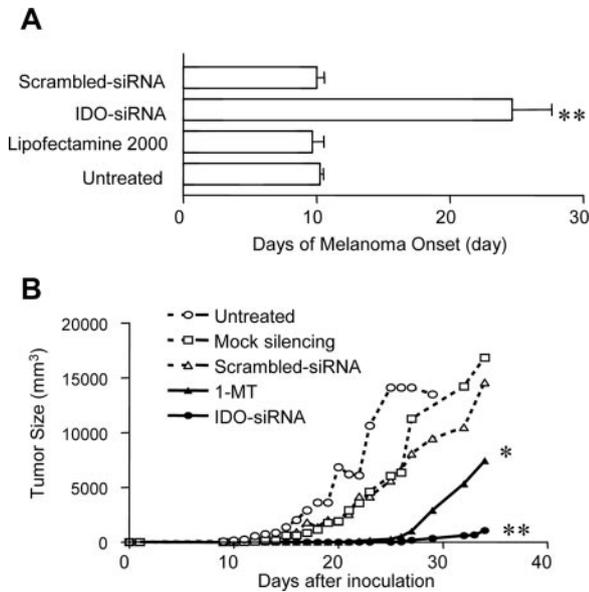
It has been reported that IDO expression is correlated with tumor progression (13). Tumor cells expressing IDO produce larger and more aggressive tumors than those with IDO expression depleted by treatment with small molecules capable of inhibiting IDO enzyme activity and level (6, 14). We therefore postulated that down-regulation of IDO in B16F10 cells before inoculation would substantially restrain tumor growth. To test this hypothesis, we transfected B16F10 cells with IDO-siRNA using *in vitro* liposome-mediated transfection. IDO-silenced B16F10 cells were then



**FIGURE 1.** IDO is efficiently silenced by siRNA. B16F10 cells ( $5 \times 10^5$ ) were seeded into 6-well plates and transfected with 4–6  $\mu$ g of IDO-siRNA or nonsense siRNA (negative control) using LipofectAMINE 2000. *A*, Silencing efficiency at the transcriptional. GAPDH (negative control) and IDO mRNA were detected by RT-PCR 24 h after transfection to assess the efficacy of silencing IDO transcripts. *B*, Silencing efficiency at the protein level. Forty-eight hours after transfection, cells were harvested, and total protein was extracted for Western blotting. The blot was labeled with anti-IDO Ab (upper) and anti- $\beta$ -actin Ab (lower). *C*, Silencing IDO preserves tryptophan in B16F10 culture medium. Tryptophan levels in B16F10 culture medium were measured by HPLC to assess the efficiency of silencing on enzyme functionality. *D*, Silencing IDO reduces tryptophan catabolism into kynurenine. B16F10 culture medium was assessed for kynurenine by HPLC. The data are presented as means  $\pm$  SEM and are representative of three repeat experiments (\*,  $p < 0.01$  as comparing with mock-silenced group).

subcutaneously injected into syngeneic C57BL/6 mice. Tumor onset time in IDO-siRNA-treated mice was substantially postponed ( $p < 0.05$ ) compared with onset time in mice injected with non-silenced or nonsense-siRNA-treated B16F10 cells (Fig. 2A).

In addition to tumor onset time we measured the size of tumors derived from IDO-silenced and control B16F10 cells. Parental,



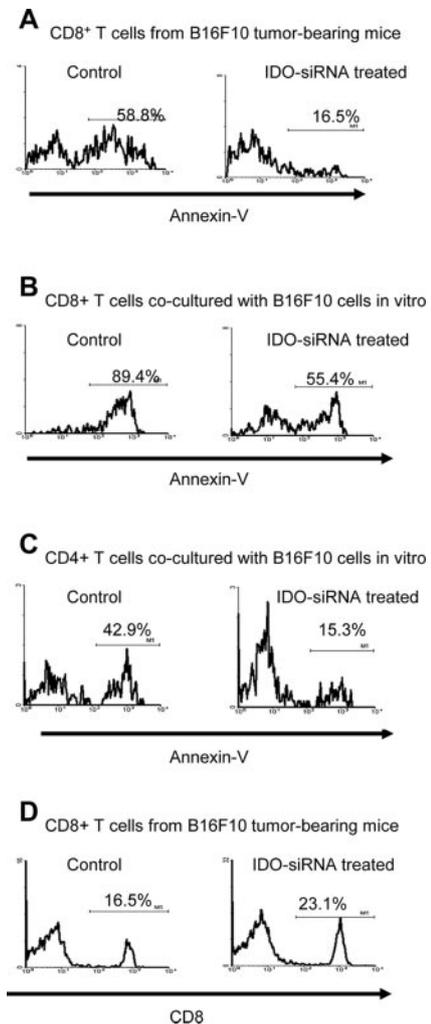
**FIGURE 2.** Silencing IDO hinders tumor progression and tumor onset. *A*, Silencing IDO delays tumor onset. B16F10 cells ( $2 \times 10^5$ ) were injected s.c. into the upper hind legs of C57BL/6 mice. B16F10 cells were either transfected in vitro with IDO-siRNA or nonsense siRNA before injection. Alternatively, wild-type B16F10 cells were injected as an additional negative control. Tumor presence and growth was assessed and measured every other day. Tumor onset (day 0) was established as the point in time when tumors reached 5 mm in diameter. *B*, Silencing IDO slows tumor growth. C57BL/6 mice were injected s.c. with  $2 \times 10^5$  B16F10 cells into their upper hind legs as described above or with B16F10 cells pretreated with 400  $\mu$ M 1-MT. Tumor presence and growth was assessed and measured every other day. The data are presented as means  $\pm$  SEM and are representative of four repeat experiments (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$  compared with mock-silenced, nonsense siRNA-treated and untreated groups).

mock-silenced (reagent alone), and nonsense-siRNA-treated B16F10 cells grew vigorously in syngeneic C57BL/6 mice. Treatment with 1-MT (a chemical inhibitor of IDO) partially inhibited tumor growth, and IDO-siRNA treatment of B16F10 cells before s.c. challenge led to suppression in tumor growth resulting in tumors  $\sim$ 15 times smaller than all negative controls 34 days post-challenge (Fig. 2*B*). It is not possible to directly compare the effects of siRNA down-regulation of IDO mRNA with responses to small molecule IDO inhibitors. However, it is clear that IDO-siRNA was effective in inhibiting tumor growth.

#### *Silencing IDO in B16F10 cells suppresses tumor-induced T cell apoptosis*

As indicated earlier, high IDO activity is believed to substantially inhibit T cells due to the extreme sensitivity of T cells to local tryptophan levels (15) and to downstream metabolites of the kynurenine pathway (16, 17). It is therefore believed that IDO inhibition occurs through two distinct mechanisms: one targeting the proliferation of locally responding T cells, and the other inducing T cell apoptosis. We therefore hypothesized that silencing IDO using siRNA would reduce tumor-induced T cell apoptosis. We observed a much smaller proportion of T cells undergoing apoptosis in C57BL/6 mice receiving intratumoral IDO-siRNA treatment (Fig. 3*A*).

We further characterized T cell apoptosis in vitro and observed B16F10 cells inducing widespread apoptosis in both CD4<sup>+</sup> (Fig. 3*B*) and CD8<sup>+</sup> T cells (Fig. 3*C*) as detected by FACS detection of FITC-conjugated annexin V staining. As expected, the silencing of



**FIGURE 3.** Silencing IDO suppresses B16F10-induced T cell apoptosis. *A*, Silencing IDO reduces CD8<sup>+</sup> T cell apoptosis in tumor-bearing mice. B16F10 cells ( $2 \times 10^5$ ) were injected s.c. into the hind flank of C57BL/6 mice. Tumor presence and growth was assessed and measured every other day. When tumor size reached 5 mm in diameter, mice were administered 50  $\mu$ g of IDO-siRNA or 0.2 ml of PBS (negative control). When tumor size reached 2000 mm<sup>3</sup>, mice were sacrificed. CD8<sup>+</sup> T cells were isolated from the spleen and lymph nodes using CD8<sup>+</sup> MACS beads. Apoptosis was determined by FITC-conjugated annexin V followed by subsequent FACS analysis. *B*, Silencing IDO reduces the apoptosis of splenic CD8<sup>+</sup> T cells in vitro. C57BL/6 spleen cells ( $1 \times 10^6$ ) were cultured with  $1 \times 10^5$  B16F10 cells. B16F10 cells were either wild-type (negative control) or had been transfected previously with IDO-siRNA for 24 h in vitro. Eighteen hours after coculture, spleen cells were collected and double-stained with PE-labeled anti-CD8<sup>+</sup> Ab and with FITC-conjugated annexin V and then analyzed by FACS. *C*, Silencing IDO reduces the apoptosis of splenic CD4<sup>+</sup> T cells in vitro. Spleen cells were cocultured with B16F10 cells as described in *B*. Eighteen hours after coculture, spleen cells were collected and double-stained with PE-labeled anti-CD4<sup>+</sup> Ab and FITC-conjugated annexin V and analyzed by FACS. *D*, Silencing IDO increases the proportion of CD8<sup>+</sup> T cells in tumor-bearing C57BL/6 mice. B16F10 cells ( $2 \times 10^5$ ) were injected s.c. into the upper hind legs of C57BL/6 mice. Tumor presence and growth was assessed and measured every other day. When tumor size reached 5 mm in diameter, mice were treated with 50  $\mu$ g of IDO-siRNA in 0.2 ml of PBS intratumorally twice a week, or 0.2 ml of PBS (negative control). When tumor size reached 2000 mm<sup>3</sup>, the mice were sacrificed, and CD8<sup>+</sup> cells from spleen and lymph nodes were isolated using CD8<sup>+</sup> MACS beads. CD8<sup>+</sup> cells were subsequently stained with FITC-labeled anti-CD8 Ab and analyzed by FACS.

IDO led to a significant reduction in apoptosis in both subsets of T cells, but most prominently in the CD8<sup>+</sup> fraction (Fig. 3C). These observations led us to postulate that decreasing the ratio of CD8<sup>+</sup> T cells (the primary antitumor effector) through tumor-induced apoptosis represents a means of tumor evasion and can be subsequently reversed by IDO-siRNA treatment. In support of this notion, we found that the overall proportion of CD8<sup>+</sup> T cells was higher in mice treated intratumorally with IDO-siRNA (16.5% (Fig. 3A) vs 23.1% (Fig. 3D)) in comparison with untreated control mice.

#### Treating melanoma by in vivo IDO-siRNA administration

Suppression of IDO using the chemical antagonist 1-MT has been reported to inhibit tumor growth in the P815 murine mastocytoma model (6). We attempted to explore the feasibility of intratumoral treatment of melanoma using IDO-siRNA. We established a melanoma by injecting B16F10 cells into the upper hind legs of syngeneic recipient C57BL/6 mice. Fifty micrograms of IDO-siRNA in liposomal carrier was injected intratumorally into tumor-bearing recipients at three time points after tumors had reached 5–7 mm in diameter. Intratumoral IDO-siRNA treatment significantly restricted tumor growth in comparison with nontreated mice (transfection reagent alone; Fig. 4A). Similar results were observed in allogeneic recipient BALB/c mice (Fig. 4B).

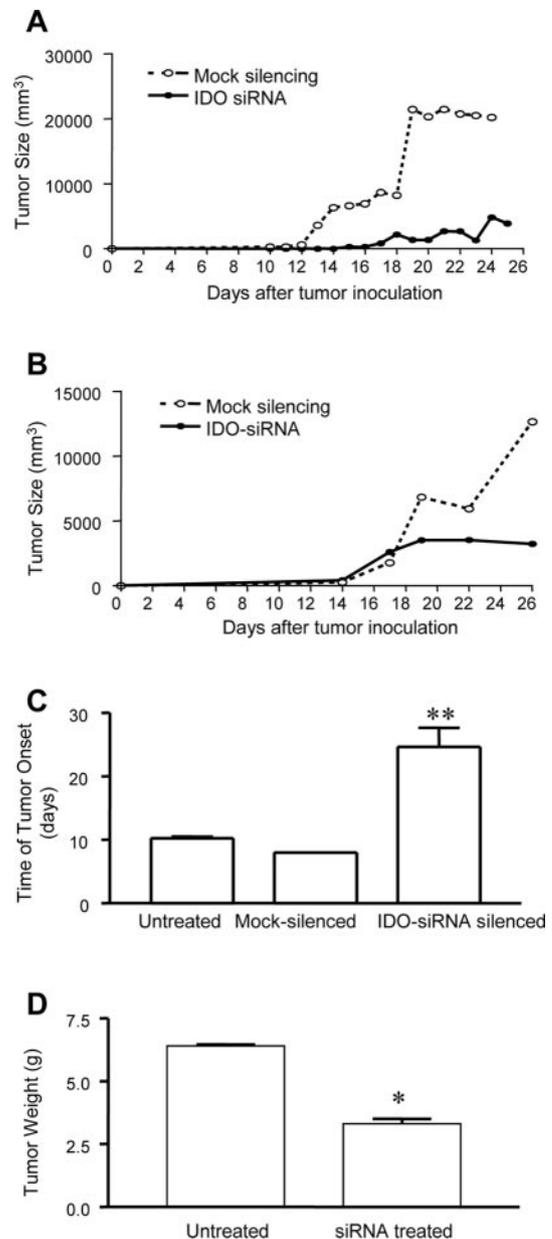
In vivo administration of IDO-siRNA not only postponed the onset of tumor formation, but also dramatically decreased tumor size in syngeneic C57BL/6 (Fig. 4C) and BALB/c (Fig. 4D) recipients, respectively. These results implied a novel anticancer therapy through inhibition of tumor-derived immune suppressive IDO using siRNA.

#### Reinstalling antitumor immunity through targeted silencing of IDO

The mechanisms responsible for the immunosuppressive actions of IDO have been proposed to be: 1) depletion of tryptophan (2, 15); and/or 2) generation of toxic downstream metabolites of the kynurenine cascade, including picolinic and quinolinic acids (17–19). Both of these activities may suppress T cell response through a decrease in proliferation and effector function; or, alternatively and more pertinent to mechanism through induction of apoptosis. It was hypothesized that disruption of these potential mechanisms of immunosuppression and immune evasion would lead to recovery of antitumor T cell activity and antitumor immunity. Based on the potential of IDO-siRNA treatment from our previous in vitro and in vivo experiments we proposed that siRNA-derived treatment could rescue the immune response allowing for recovery of a potent and directed antitumor immune response driven primarily by CTL.

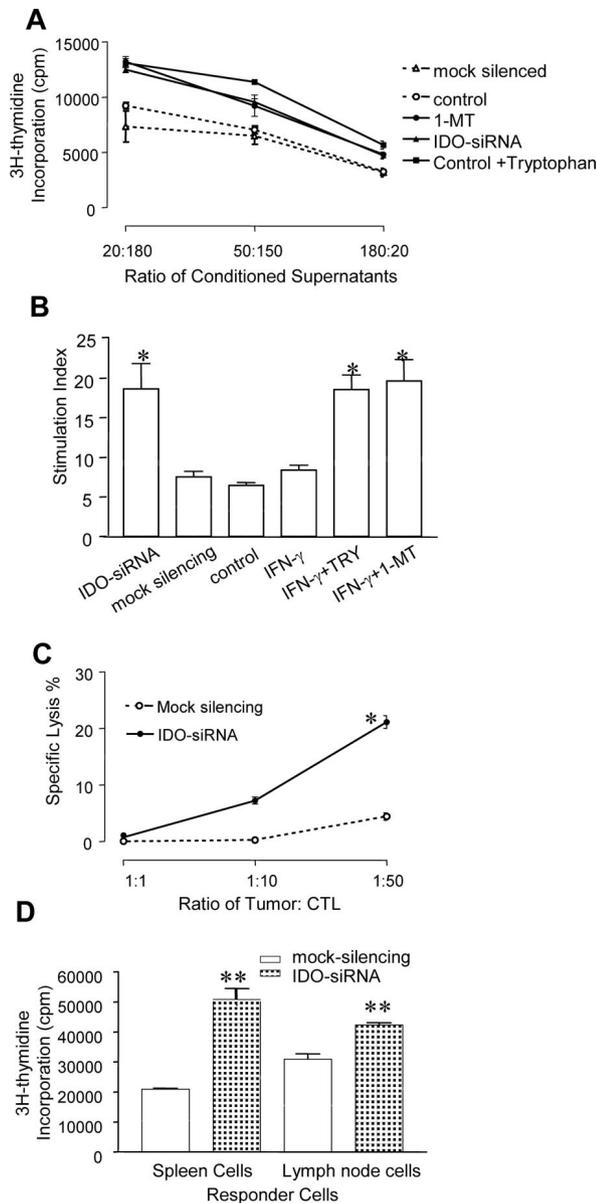
Because tryptophan reduction has been implicated in subsequent suppression of T cell suppression (6, 14), we examined the anti-proliferative effects imposed by B16F10 culture supernatant, in which we observed substantially reduced tryptophan levels (Fig. 1C). As expected, culture supernatant from IDO-siRNA-treated B16F10 cells had substantially less capacity to suppress proliferation of anti-CD3 mAb-stimulated T cells, compared with supernatant from mock-silenced B16F10 cells (Fig. 5A). Addition of tryptophan to the medium rescued the inhibition of T cell proliferation induced by tryptophan depletion. (Fig. 5A).

To confirm that the inhibitory effect of B16F10-conditioned medium on T cell proliferation was related primarily to the expression of IDO, we performed a MLR in which allogeneic BALB/c T cells were cocultured with C57BL/6-derived DC that were made to express lower IDO levels by virtue of treatment with IDO-siRNA or 1-MT, or higher IDO levels by virtue of IFN- $\gamma$  treatment (20) (Fig. 5B). After silencing IDO in DC using siRNA, T cell proliferation



**FIGURE 4.** Intratumoral IDO-siRNA treatment inhibits tumor growth in C57BL/6 and BALB/c mice. *A*, Mean tumor volumes in C57BL/6 mice challenged with s.c. injection of  $2 \times 10^5$  B16F10 cells. IDO-siRNA or nonsense siRNA bound to liposomes was injected intratumorally when tumors reached a size of 5 mm. *B*, Mean tumor volumes in BALB/c mice challenged with s.c. injection of  $2 \times 10^5$  B16F10 cells. IDO-siRNA and control siRNA was delivered as described in *A*. *C*, Mean time for tumor onset in syngeneic C57BL/6 mice challenged with s.c. injection of  $2 \times 10^5$  B16F10 cells. B16F10 cells were wild-type, pretreated with IDO-siRNA, or pretreated with LipofectAMINE 2000 transfection medium alone in vitro. *D*, Mean weight of tumor (g) at end point of observation (death or time elapse) in BALB/c mice challenged with s.c. injection of  $2 \times 10^5$  B16F10 cells. B16F10 cells were pretreated with IDO-siRNA or nonsense siRNA in vitro. The data are presented as means  $\pm$  SEM (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$  as comparing with mock silenced and untreated groups).

was significantly elevated in comparison to the proliferation observed when nonsilenced (transfecting reagent treatment) or mock-silenced (vector treatment) DC were used as stimulators. This enhanced T cell response was also seen in an MLR using DC in which IDO was inhibited by 1-MT. On the other hand, when using DC in which IDO expression was up-regulated by IFN- $\gamma$



**FIGURE 5.** Rescue of antitumor immunity by treatment with IDO-siRNA. *A*, Silencing IDO reduces tumor-induced suppression of T cell proliferation. A total of  $2 \times 10^5$  IDO-siRNA-transfected or wild-type B16F10 cells was cultured in complete medium for 48 h. The media were used Con A ( $5 \mu\text{g/ml}$ ) activated T cell culture in a 96-well plate in varying ratios relative to complete RPMI 1640 medium. T cell proliferation was measured by a [ $^3\text{H}$ ]thymidine incorporation assay. *B*, IDO-silenced DC enhance DC-induced T cell proliferation. BALB/c splenic T cells ( $5 \times 10^5$ ) were cocultured with  $5 \times 10^4$  IDO-silenced C57BL/6 DC, mock-silenced DC, or nonsilenced DC for 72 h. Nonsilenced DC was stimulated by  $200 \mu\text{g/ml}$  IFN- $\gamma$  overnight and washing out of IFN- $\gamma$  for twice before coculture with splenic cells. Cultures containing nonsilenced DC and stimulated with IFN- $\gamma$  were further separated into three groups treated with media containing  $200 \mu\text{M}$  1-MT,  $400 \mu\text{M}$  tryptophan, or complete medium only. T cell response was assessed by thymidine incorporation. *C*, IDO-siRNA treatment improves CTL-induced tumor lysis. Naive T cells from C57BL/6 mice were primed with DC previously pulsed with tumor lysate to generate B16F10-specific CTL. CTL were incubated with IDO-siRNA-treated or wild-type B16F10 (target) cells at different E:T cell ratios for 4 h. Target cell lysis was determined by LDH release. *D*, IDO-siRNA treatment enhances Ag-specific T cell response. In vitro DC were transfected with IDO-siRNA or treated with reagent alone and were pulsed with  $10 \mu\text{g/ml}$  KLH for 24 h. DC were then activated with LPS + TNF- $\alpha$  for 24 h. Ag-pulsed DC ( $2 \times 10^6$  cells/mouse) were injected s.c. into C57BL/6 mice. Ten days after immunization, T cells from draining lymph nodes were

treatment, T cell proliferation was even lower than observed in mock-silenced or untreated controls (Fig. 5*B*). Taken together, these data suggest a strong mechanistic link between IDO (as a single agent) and T cell suppression, which can be subsequently overcome by targeted silencing of IDO. To further demonstrate the direct involvement of tryptophan in IDO-mediated immune regulation, we added tryptophan to the T cell culture after IFN- $\gamma$  stimulation. As shown in Fig. 5*B*, the addition of tryptophan rescued T cell response that was inhibited by IDO.

Since the silencing of IDO led to substantial reduction in both tumor-induced T cell apoptosis (Fig. 3) and in the suppression of T cell proliferation (Fig. 5*A*), we decided to further examine whether this recovery of immune response could lead to a subsequent rescue of directed tumor lysis. To achieve this, we isolated CD8 $^+$  T cells from B16F10 tumor-bearing C57BL/6 mice, and cocultured these cells with B16F10 cells either silenced by IDO-siRNA or nonsilenced. Tumor-specific lysis was significantly enhanced when T cells had been cocultured with IDO-silenced B16F10 cells (Fig. 5*C*), indicating that IDO plays a critical role in the evasion of targeted CTL-derived lysis, an event that can be enhanced by siRNA-based treatment.

Although tumor-derived expression of IDO is a well-characterized mechanism of tumor cell immune evasion, the notion of APC-derived immunosuppression through IDO expression has been only weakly explored and remains poorly understood. Our observations indicate that silencing of IDO in DC stimulates an increase in T cell response (Fig. 5*B*). We therefore hypothesized that IDO may also disrupt Ag presentation (a common mechanism in oncogenic immune evasion). To address this notion we extracted DC from C57BL/6 mice, silenced IDO using siRNA, and pulsed our DC with KLH, a well-recognized model Ag. After immunization of mice with IDO-silenced or nonsilenced DC, a KLH-specific response assay was performed. IDO-silenced DC were indeed able to function more effectively in Ag presentation than IDO-expressing DC (Fig. 5*D*).

## Discussion

Evidence continues to accumulate in support of the notion that tumor-derived immunosuppression is an essential process in tumor evasion and general tumor acceptance (1). Immunosuppression and evasion can be achieved through a variety of mechanisms, many of which are well-characterized, experimentally supported, and at the disposal of cancer cells. These include secretion of Th2-associated cytokines such as IL-10 or TGF- $\beta$  leading to Th2 polarization (17, 21–23), over-expression of Fas ligand/TRAIL (24, 25), overexpression of complement inhibitors (DAF and CD55) (26), and overexpression of HLA-G protecting against NK-induced lysis (27). A novel mechanism of tumor-derived immunosuppression through IDO has gained attention recently and has stimulated research in cancer immunology (6, 7, 13, 28, 29).

IDO is expressed by a variety of cells, including different subsets of both murine and human tumor cells (6). Recently, IDO has been implicated in tumor evasion in several murine tumor models. Many cancer cell lines, primary tumors and host APC express IDO concurrent with reduced or abolished antitumor T cell responses (13, 30). P815 murine mastocytoma cells that express IDO were

harvested, and placed in 96-well plates ( $4 \times 10^5$  cells/well). After culture 72 h in the presence or absence of KLH ( $50 \mu\text{g/ml}$ ), T cell proliferations were determined by [ $^3\text{H}$ ]thymidine incorporation assay. The data are presented as means  $\pm$  SEM and are representative of two repeat experiments (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$  as comparing with mock silenced, scrambled siRNA-treated and untreated groups).

able to form large, stable tumors when introduced into preimmunized hosts which would normally reject the tumors outright (6). The immunosuppressive effect of IDO was completely reversed by the introduction of 1-MT leading to renewed tumor rejection, an observation similar to that achieved by studies on fetal rejection whereby 1-MT administration led to renewed fetal rejection (31).

Due to its association with suppressed immune responses to endogenous tumor Ags, IDO represents an ideal target for immunomodulatory drugs. Such drugs can be used to safely enhance the efficacy of standard chemotherapeutic agents. Experimental treatment of cancer using tryptophan inhibitors, such as 1-MT (6) and MTH-trp (7), have been reportedly successful in several tumor models. Although therapeutic effects are significant and promising, treatments with these inhibitors did not completely prevent tumor outgrowth, perhaps because of incomplete inhibition of IDO by these inhibitors (6). In addition, synergistic therapeutic effects in cancer treatment has been achieved using IDO inhibitor in combination with other chemotherapeutic drugs (7, 32). However, promotion of inflammatory conditions by these small chemical inhibitors remains a valid concern (28).

To explore therapeutic potential of IDO suppression, we set out to use a method with greater potential for safety and effectiveness (siRNA) to silence IDO. We hypothesized that efficient IDO silencing would be achieved by siRNA transfection leading to a decrease in tumor-induced T cell suppression and an increase in T cell-induced tumor apoptosis resulting in a reduction in tumor progression. IDO siRNA-pretreated B16F10 cells generated s.c. tumors in BALB/c mice dramatically more slowly than B16F10 cells treated with appropriate control siRNA. The resulting tumors were ~15 times smaller than all negative controls 34 days post-B16F10 cell inoculation (Fig. 2B). In addition, tumors derived from B16F10 cells pretreated with IDO-siRNA progressed more slowly than those pretreated with 1-MT, indicating the potential superiority of IDO-siRNA treatment. We further characterized the *in vivo* potential of IDO-siRNA treatment by measuring tumor progression after intratumoral injection of IDO-siRNA. Intratumoral IDO-siRNA treatment significantly slowed tumor growth in both syngeneic (Fig. 4A) and allogeneic (Fig. 4B) recipients compared with tumor growth in nontreated mice.

Gene silencing by siRNA has been identified as a very promising therapeutic strategy for cancer treatment (33). siRNA silencing of K-Ras in human pancreatic cancer cells has been demonstrated to inhibit the s.c. growth of these cells in immune compromised mice (34). It has already been reported that the *in vivo* administration of siRNA targeting  $\beta$ -catenin mRNA results in tumor regression in a murine model of colon cancer (35). Similarly, *in vivo* silencing of vascular endothelial growth factor in fibrosarcoma cells by siRNA led to decreased angiogenesis and decreased tumor growth (36).

Although use of siRNA to treat cancer by silencing tumor cell oncogenes directly would be ideal, a clinically applicable protocol is yet to be developed since complete gene silencing and *in vivo* siRNA delivery is still in development. Our strategy, demonstrated in this study, is to silence immune suppressive genes. This may not require "complete" gene silencing (technically unavailable at present) to exert a clinically advantageous effect. Inhibiting tumor growth through effective but not less-than-total silencing of IDO, rather than silencing expression of tumor cell oncogenes where complete inhibition in all cells is likely to be necessary for effect, may be a useful antitumor strategy that negates the potential disadvantage of the incomplete nature of siRNA target down-regulation. Furthermore, suppression of immune evasion through IDO suppression may have the advantage of being useful in treatment of a broad range of tumor types.

Realizing the promise of *in vivo* siRNA administration we sought to underline some of the key mechanisms explaining anti-cancer effects upon IDO silencing. Initially, we observed a much smaller proportion of T cells undergoing apoptosis in mice treated with IDO-siRNA (Fig. 3A). Silencing of IDO led to a significant reduction in apoptosis in both subsets of T cells. It is formally possible that IDO could inhibit T cell proliferation as well as increase T cell apoptosis, and such an inhibition of proliferation led to decreased numbers of T cell numbers similar to that caused by apoptosis. However, we observed that IDO-siRNA treatment also increased the total number of T cells *in vivo* (Fig. 3). Such an increase in T cell number may be partially due to IDO-siRNA-mediated inhibition of T cell apoptosis. These results reveal the therapeutic potential of silencing IDO, indicating an apparent IDO-siRNA-mediated rescue from apoptosis of T cells after exposure to B16F10 tumor cells.

In addition, we set out to characterize the effect of silencing on the rescue of directed immunity, which required the development of tumor-specific T lymphocytes. Strategies have been developed to "prime" T cells with Ags presented by prepulsed DC (37, 38). Using a tumor lysate-based DC vaccine as previously described (37), we generated sets of B16-specific T cells to characterize CTL-derived lysis of B16F10 cells. Antitumor immunity involves a complex interaction between both tumor cells and CTL, with lysis being induced by both cell types and targeted against each other. We sought to characterize the rescue response of siRNA on the targeted destruction of tumor cells, an optimal situation for cancer therapy representing a natural and nondetrimental removal of tumor cells. We observed a dramatic increase in the specific lysis of B16F10 cells when cocultured with B16-specific cytotoxic CD8<sup>+</sup> T lymphocytes (Fig. 5C), indicating the rescue of directed immunity. Taken together, these mechanistic data suggest profound potential for IDO-siRNA derived therapy at various levels. It was observed that IDO-siRNA treatment reduced tumor-derived T cell apoptosis and tumor-derived inhibition of T cell proliferation.

Immunotherapy has generally aimed at up-regulating antitumor immunity. Since DC are the most potent APC (39), they were used as an "adjuvant" for tumor Ags. Pulsing DC with tumor Ags or fusing tumor cells with DC resulted in a more potent immunogenic vaccine (39, 40). However, the limited data testing clinical effectiveness of such vaccines is less than promising (41). The inefficiency of immunotherapy in clinicals may be due to tumor-derived immune suppression that attenuated or countered antitumor effects.

On the other hand, the role of IDO-expressing DC in tumors has also been recently explored (42). One mechanism that is believed to contribute to the lack of an immune response against tumors may be the presentation of tumor Ags by tolerogenic host APCs that express IDO (15, 43). Since it is now clear that DC may be either activating or tolerizing, it is tempting to consider the role they might play in tumor tolerance. Recent melanoma studies have found that large and clearly abnormal numbers of IDO-positive cells in tumor-draining nodes (44). Furthermore, mouse tumor-draining lymph nodes contained a small subset of plasmacytoid DC (pDC) that constitutively expressed immunosuppressive levels of IDO (42). *In vitro*, these pDC demonstrated suppressed T cell responses against Ags presented by the pDC themselves, and also suppressed T cell responses to third-party Ags presented by non-suppressive APC. Adoptive transfer of DC from tumor-draining nodes into naive hosts caused profound local T cell anergy, especially toward Ags expressed by the transferred DC (42). Therefore, silencing IDO in DC may enhance the effects of tumor vaccines. In support of this, IDO-silenced DC functioned more effectively in Ag presentation than IDO-expressing DC as indicated through a KLH-specific recall test (Fig. 5D). Such an observation, however,

could have arisen due to the IDO-induced reduction of tryptophan and hence IDO may not specifically and exclusively target Ag presentation. Therefore, inhibitory factors such as IDO may have a negative impact on the effectiveness DC-mediated cancer vaccines. Silencing IDO in both DC and tumor may achieve greater-than-addition anticancer effects by inhibiting tumor-derived immune suppression and increasing antitumor immunity.

In summary, we demonstrated the potential of a novel strategy of cancer immunotherapy to silence tumor-expressed IDO using siRNA. Silencing IDO resulted in the inhibition of tumor cell growth. Furthermore, treating tumor-bearing mice with siRNA inhibited tumor formation. These results therefore support a therapeutic potential for RNAi through silencing tumor-derived immunosuppressive genes.

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## Disclosures

The authors have no financial conflict of interest.

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