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Research Article

Tumor-Infiltrating Regulatory Dendritic Cells Inhibit CD8+ T Cell Function via L-Arginine Metabolism

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Abstract

Dendritic cells (DC) have a critical effect on the outcome of adaptive immune responses against growing tumors. Whereas it is generally assumed that the presence of phenotypically mature DCs should promote protective antitumor immunity, evidence to the contrary does exist. We describe here a novel mechanism by which tumor-infiltrating dendritic cells (TIDC) actively contribute to the suppression of protective $CD8^+$ T-cell–based antitumor immunity. Using the BALB/NeuT model of spontaneously arising mammary carcinoma, we found that canonical MHC $II^+/CD11b^+/CD11c^{high}$ TIDCs act as regulatory DCs to suppress $CD8^+$ T-cell function, resulting in diminished T-cell–based antitumor immunity *in vivo*. Stimulation of naive T cells with regulatory TIDCs resulted in an altered cell fate program characterized by minimal T-cell expansion, impaired IFNγ production, and anergy. Suppression by regulatory TIDCs overcame stimulatory signals provided by standard DCs, occurred in the absence of cognate interactions with T cells, and was mediated primarily by arginase metabolism of L-arginine. Immunosuppressive TIDCs were found in every murine tumor type examined and were phenotypically distinct from tumor-infiltrating $CD11c^{int\text{-}low}/CD11b^+/Gr\text{-}1^+$ myeloid-derived suppressor cells. Thus, within the tumor microenvironment, MHC II^+ TIDCs can function as potent suppressors of $CD8^+$ T-cell immunity. [Cancer Res 2009;69(7):3086–94]

Introduction

The development of protective T-cell–based antitumor immunity is critically dependent on the normal function of dendritic cells (DC). This has led to a great interest in using DC-based therapies for the treatment of progressively growing tumors. However, clinical results to date have yielded minimal success (1), and the reasons for this remain unclear.

Many tumors, including human breast cancers, contain appreciable numbers of tumor-infiltrating DCs (TIDC; 2–4), but the stimulatory capacity of these DCs is often compromised (reviewed in refs. 5–7). For example, tumor-derived cytokines such as vascular endothelial growth factor can block DC maturation, leading to failed T-cell priming in tumor-draining lymph nodes (6, 8, 9). However, blocked DC maturation is not the only obstacle to be overcome. In mice, DC maturation is characterized phenotypically by up-regulation of surface markers such as MHC II, CD80, and CD86. Although it was once thought that phenotypically mature DCs exclusively promoted T-cell activation, this paradigm is now known to be incorrect (10). For example, disruption of E-cadherin adhesion induces DCs to up-regulate MHC II and costimulatory molecules, yet simultaneously programs them to acquire a tolerogenic function (11). In breast carcinomas, MHC II^+ DCs can aid tumor progression by generating interleukin (IL)-13–producing $CD4^+$ T cells (12). MHC II^+ DCs in ovarian cancer patients have also been shown to express inhibitory molecules such as B7-H1 that can down-modulate T-cell effector responses via ligation of PD-1 (13). Clearly, a more thorough understanding of the ways in which DCs participate in immunosuppression is essential if T-cell– and DC-based tumor immunotherapies are to achieve greater clinical efficacy.

In nontumor model systems, DCs have been generated *in vitro* that function as regulatory cells to suppress $CD4^+$ T-cell function even in the presence of stimulatory DCs (14–18). This ability to dominate over signals provided by standard DCs differentiates such regulatory DCs from simply immature or tolerogenic DCs. We hypothesized that regulatory DCs would develop naturally during the outgrowth of tumors, and we used the BALB/NeuT model of spontaneous murine mammary carcinoma to explore this possibility.

We found that NeuT tumors are infiltrated by fully committed, $CD11b^+$ $CD11c^{high}$ DCs that express MHC II, CD80, and CD86, yet which impair $CD8^+$ T-cell antitumor immunity *in vivo* and T-cell function *in vitro*. Thus, tumors can induce matured, canonical TIDCs to act as potent suppressors of T-cell–based antitumor immunity. Our work adds to the growing body of evidence indicating that phenotypically "mature" MHC II^+ TIDCs may function primarily to impair protective T-cell immunity, rather than promote it.

Materials and Methods

Mice. BALB/c NeuT mice, kindly provided by Jay Berzofsky (National Cancer Institute, Bethesda MD), and DUC18 TCR transgenic mice have been described previously (19–22). BALB/c mice were purchased from the National Cancer Institute. All animal studies were done in accordance with institutional guidelines at the Washington University School of Medicine.

Immunofluorescence. Tumors were suspended in optimum cutting temperature compound, frozen in 2-methylbutane cooled with liquid nitrogen, sectioned, fixed with acetone, and stored at −20°C until use. Before staining, sections were blocked with 5% normal goat serum, Fc receptor blocking 2.4G2 antibody (BD Biosciences), and 0.1% Tween 20. Staining was done with anti-CD11c (clone HL3, BD Biosciences), followed by antirat Cy3 and anti–$I\text{-}A^d$-FITC (BD Biosciences). Immunofluorescence was visualized under a fluorescent microscope and pictures were taken with a

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Nikon DXM1200 digital camera. Final image processing was done using Photoshop 7.0 software (Adobe).

DC isolation. DCs were harvested from NeuT mammary carcinomas, CMS5 fibrosarcomas, or 4T1 mammary carcinomas when tumors were >10 mm in diameter. Control splenic DCs (spDC) were isolated from spleens of tumor-free BALB/c mice. Organs were dissociated as described (20). For enrichment of spDCs, cells were incubated with Miltenyi CD11c microbeads and purified over two sequential Macs LS columns according to the manufacturer's protocol (Miltenyi Biotec) to achieve >93% $CD11c^+$ DCs. Unless otherwise noted, TIDCs were sort-purified based on their coexpression of CD11c and CD11b and exclusion of Gr-1 using a MoFlo high-speed flow cytometer (DakoCytomation). Fc receptors were blocked with 2.4G2 (BD Biosciences) and normal mouse serum before surface staining.

T-cell proliferation assays. T cells were harvested from DUC18 transgenic mice and purified over two sequential Macs LS columns using CD8 microbeads. The percentage of DUC18 T cells was determined via flow cytometry based on coexpression of CD8 and Vβ8.3. Naive DUC18 T cells were cultured as described (20) at 5×10^4 per well in flat-bottomed 96-well plates with the indicated numbers of spDCs, TIDCs, or both. Before plating, DCs were pulsed with 0.5 μmol/L of tumor extracellular signal–regulated kinase (tERK) peptide (22). On day 3, wells were pulsed with [^{3}H]thymidine for 18 h, harvested, and counted on a beta counter. The following were used where indicated: 10 mmol/L L-norvaline (Sigma), 100 μmol/L L-N^6-(1-iminoethyl)lysine (L-nil; Sigma), and 1 mmol/L N^G-nitro-L-arginine (L-NNA; Sigma), 2 mmol/L L-arginine (Sigma). In some experiments, T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; 20) and plated at 2×10^6 DUC18 T cells per well with 2×10^5 DCs in six-well plates for 96 h before harvest and analysis.

L-Arginine assays. L-Arginine incorporation was done by culturing 5×10^5 DCs/mL in a 200-μL volume in 96-well flat-bottomed plates in complete medium made with RPMI 1640 containing 100 μmol/L L-arginine. L-[^{3}H]Arginine (5 μCi/well) was added at time 0 and plates were harvested at the indicated times. Arginase activity in cell lysates was determined by measuring the production of L-ornithine as described (23) after culturing 2×10^6 Miltenyi Macs-purified TIDCs or spDCs overnight with or without the addition of 100 μg/mL rmIL-4 (Peprotech). Nitric oxide (NO) production was evaluated by measuring the total concentration of nitrate/nitrite in culture supernatant after culturing cells overnight in the absence or presence of 250 ng/mL IFNγ (kindly provided by Dr. Robert Schreiber, Washington University School of Medicine, St. Louis, MO) using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical).

Cell staining for flow cytometry. T cells were routinely stained with anti-CD8 allophycocyanin (APC; BioLegend), 7-amino-actinomycin D (7-AAD; Sigma), and Vβ8.3 phycoerythrin (PE) or FITC (BD Biosciences) to allow gating on live, DUC18 T cells. CD3ζ staining was done after addition of 500 μg/mL digitonin and anti-TCRζ (Abcam). Intracellular cytokine staining was done as described (24) using anti-IFNγ APC (BioLegend), anti-CD8 PE/Cy5 (BioLegend), and anti-Vβ8.3 FITC.

DCs were routinely stained with anti-CD11c biotin (clone HL3, BD Biosciences) plus streptavidin APC (Caltag Laboratories), 7-AAD, anti-CD11b PE (clone M1/70, BioLegend), and one of the following: anti–Gr-1 (RB6-8C5, BD Biosciences), H-$2K^d$ FITC (SF1-1.1, BD Biosciences), I-A^d FITC (39-10-8, BD Biosciences), CD40 FITC (3/23, BD Biosciences), CD80 FITC (16-10A1, BioLegend), CD86 FITC (GL-1, BioLegend), or CD54 FITC (3E2, BD Biosciences).

Tumor studies. The CMS5 fibrosarcoma cell line (25) was cultured as described (20) and injected s.c. at 3×10^6 cells per mouse in the right hind flank, with or without the addition of 1×10^6 Macs-purified TIDCs. For mice receiving T cells, 1×10^6 naive DUC18 T cells were injected i.v. 4 d after tumor cell challenge. Recipient mice were either BALB/c controls or NeuT mice at ~14 to 15 wk of age, when spontaneous mammary tumors were just becoming palpable (26). The 4T1 mammary carcinoma cell line was cultured as described (27) and injected s.c. into mammary gland #4 at 7.5×10^5 cells per mouse. For all tumors, areas (product of orthogonal diameters) were measured every other day for 40 d or until tumors were >20 mm in diameter. Progressively growing tumors were defined as those tumors that displayed a continuous increase in cell size during the course of the observation period.

Statistical analyses. Statistics were done using a two-tailed Student's *t* test for unpaired observations, with correction for unequal variations as needed, via GraphPad software.

Results

NeuT mammary tumors are heavily infiltrated by phenotypically mature DCs. To determine whether TIDCs could actively suppress $CD8^+$ T-cell function, we used BALB/c NeuT mice (hereafter referred to as "NeuT"). Progressively growing NeuT mammary tumors contained numerous $CD11c^+$ cells that infiltrated tumor beds beyond the $CD31^+$ vasculature (Fig. 1*A*) and showed positive coexpression of I-A^d along with characteristic DC morphology.

Flow cytometric analysis of NeuT tumors revealed a striking accumulation of $CD11c^{high}/CD11b^+$ cells with advancing tumor progression, such that they accounted for more than 10% of the live cells present by the time NeuT mice had reached 20 weeks of age (Fig. 1*B*; Supplementary Fig. S1*A*). $CD11c^{high}$ cells from NeuT tumors seemed to be conventional myeloid DCs, with low to negative coexpression of CD4, CD8, B220, CD45RB, and the lineage markers CD3, CD19, and DX5 (Supplementary Fig. S1*B* and data not shown). Tumor-infiltrating $CD11c^{high}$ cells expressed higher levels of I-A^d, CD80, and CD86 than did $CD11c^{high}/CD11b^+$ spDCs from tumor-free mice (Fig. 1*B*), yet displayed a phenotype that was distinct from that of spDCs that were matured *in vivo* in response to lipopolysaccharide (LPS) administration (Fig. 1*B*). *Ex vivo* stimulation of $CD11c^{high}/CD11b^+$ cells with CpG 1826 led to increased expression of CD40 and CD86, suggesting that these cells could be matured further and were not a terminally differentiated "end point" population (Supplementary Fig. S1*C*). Collectively, these data illustrate that tumor-infiltrating $CD11c^{high}/CD11b^+$ cells are canonical, committed DCs, and we refer to them henceforth as tumor-infiltrating dendritic cells (TIDC).

To determine if NeuT TIDCs were phenotypically distinct from previously described $CD11b^+/Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSC), we analyzed tumor-infiltrating cells from multiple mice for CD11c, CD11b, and Gr-1 expression. MDSCs are heterogeneous populations of suppressor cells defined phenotypically in mice by their coexpression of CD11b and Gr-1 and by their immature state in the presence of tumor-derived factors (6, 28–31). $CD11c^{high}$ NeuT TIDCs do not coexpress Gr-1 (Fig. 1*C*), differentiating them from $CD11b^+/Gr\text{-}1^+$ MDSCs that exist in the peripheral blood of NeuT mice (32) and which also infiltrated NeuT mammary tumors (Fig. 1*C*). NeuT TIDCs expressed MHC II and CD86 (Fig. 1*B*), whereas tumor-infiltrating NeuT MDSCs did not (data not shown). A minor, third population of $CD11c^{high}/CD11b^{high}$ cells was identified that had bimodal expression of Gr 1 (Fig. 1*C*); because these cells could represent immature DCs, they were excluded from further analysis.

NeuT TIDCs promote tumor outgrowth *in vivo*. We next wanted to determine whether NeuT TIDCs supported or inhibited $CD8^+$ T-cell–mediated tumor immunity. We began by examining TIDC function in an adoptive transfer model *in vivo* and used the CMS5 fibrosarcoma model, in which small established tumors are rejected following transfer of naive, tumor antigen–specific $CD8^+$ TCR transgenic DUC18 T cells (20, 22, 26). The kinetics of DUC18 T-cell–mediated CMS5 rejection are well characterized and highly reproducible, making this an ideal system with which to examine the *in vivo* effects of TIDC function.

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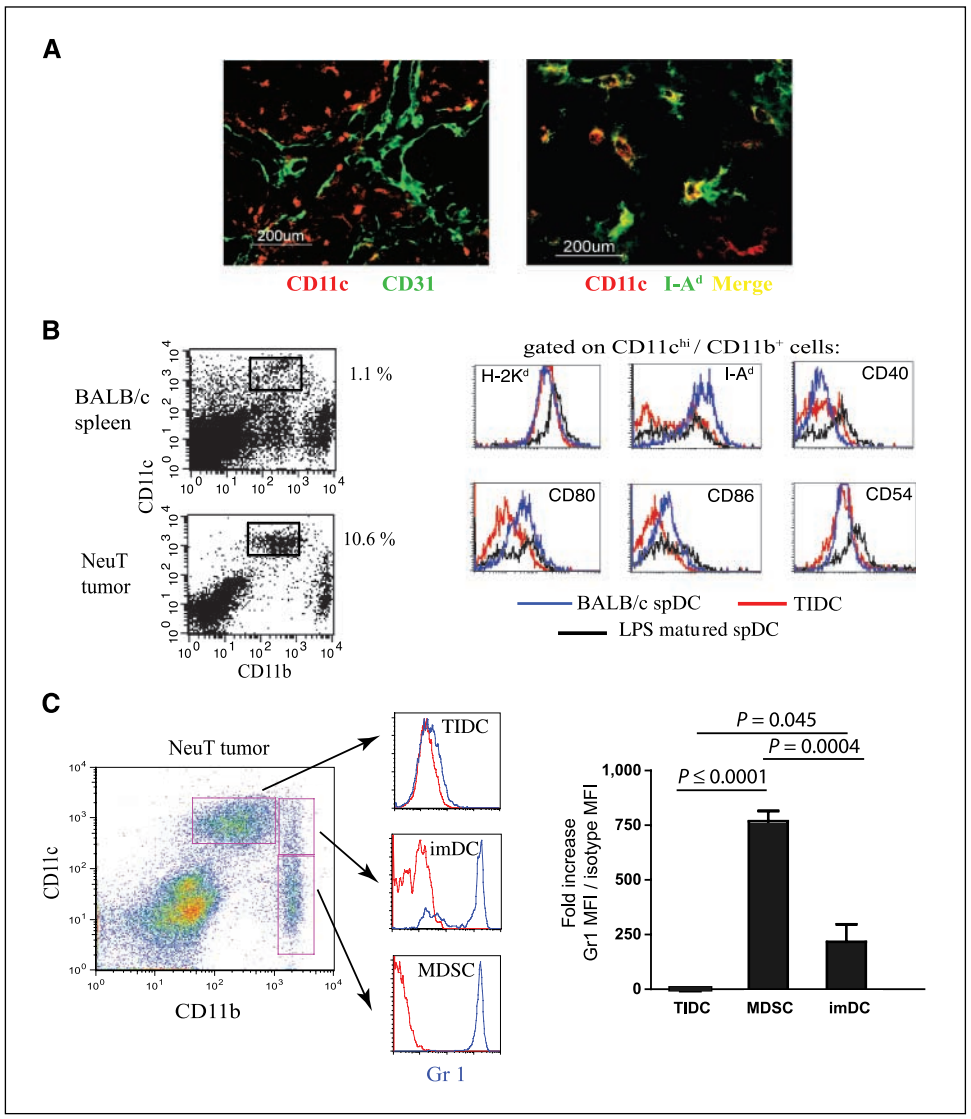

Figure 1. Canonical CD11c^{+}/MHC II^{+} DCs infiltrate NeuT tumors. *A,* NeuT tumors were stained for CD11c-Cy3 (*red*) and CD31 FITC or I-Ad FITC (*green*). Cells expressing both appear in yellow. One tumor, representative of five, is shown. *B,* spleens from tumor-free BALB/c mice and mammary tumors from NeuT mice were harvested and stained. The percentage of live, gated CD11c^{high} cells is shown, along with maturation marker expression on these cells. LPS-treated BALB/c mice received 70 μg of *Salmonella abortus* LPS 1 d before harvest and staining of splenocytes. *C,* gates for CD11c^{high} TIDCs and CD11c$^{int\text{-}low}$ MDSCs are shown, along with surface expression of Gr 1. *Blue lines,* specific antibodies; *red lines,* isotype controls. *Right, columns,* mean fold increase of Gr1 MFI over isotype MFI for six individual mice from three experiments; *bars,* SE.

CMS5 fibrosarcoma cells were either injected into recipient BALB/c mice alone or as a 3:1 mixture with purified CD11c^{+} TIDCs from NeuT tumors. Purified TIDC preparations contained <2% contaminating CD11b^{+}/Gr-1^{+} MDSCs (Supplementary Fig. S2*A*). In the absence of tumor antigen–specific DUC18 T cells, both groups of subcutaneous CMS5 tumors grew progressively and with similar kinetics through day 16 post-challenge, at which time animals were sacrificed due to the presence of unacceptably large tumors in some mice (Fig. 2*A*).

The effects of TIDCs on CD8^{+} T cells were examined by injecting CMS5 cells alone or with enriched TIDCs as above, then adoptively transferring 1×10^{6} naive DUC18 T cells 4 days later. In mice that received CMS5 cells plus DUC18 T cells, a short period of tumor outgrowth occurred before tumor rejection (Fig. 2*A*). Only 7% of these mice experienced any tumor regrowth through day 40 (1 of 15 mice in four experiments; Fig. 2*B*). A reversal of this trend was seen when TIDCs were admixed with CMS5 cells, such that 100% of mice (15 of 15 mice in three experiments) ultimately experienced progressive tumor outgrowth (Fig. 2*A* and *B*). Inhibition of antitumor immunity was specific to TIDCs because sort-purified CD11c^{high}/CD11b^{+} spDCs from tumor-free BALB/c mice failed to similarly affect late tumor outgrowth (Fig. 2*B*). Thus, it seemed that TIDCs promoted eventual tumor outgrowth but did not noticeably affect initial T-cell priming and tumor regression.

We were surprised that coinjection of TIDCs with CMS5 cells affected tumor outgrowth in such a delayed manner. To determine if TIDCs could suppress initial T-cell–mediated tumor regression if recipient mice already bore established tumors, we repeated the above experiments using as recipients either tumor-free BALB/c mice (as above) or NeuT mice at ~14 to 15 weeks of age, when mammary tumors were just becoming palpable. At this age, relatively few myeloid DCs are present endogenously within developing NeuT tumors (Supplementary Fig. S1*A*). Previous work in our laboratory had shown that distal NeuT mammary tumors do not affect the ability of DUC18 T cells to reject small, transplanted CMS5 flank tumors (26). However, when CMS5 + TIDC were injected into NeuT mice and T cells were transferred 4 days later, nearly 40% of recipient mice showed uncontrolled CMS5 tumor outgrowth from day 2 onward, and this increased steadily to 85% by day 20 (Fig. 2*C*). Therefore, progressive CMS5 outgrowth occurred in NeuT mice despite the presence of tumor antigen–specific DUC18 T cells. This suggested that when transferred into

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mice already bearing distal mammary tumors, TIDCs exerted a stronger suppressive effect *in vivo*.

To further examine the functional effects of TIDCs on DUC18 T cells, tumor-infiltrating DUC18 T cells from NeuT mice were examined for their ability to produce IFNγ on restimulation *ex vivo* with antigen-pulsed spDCs. As shown in Fig. 2*C*, the percentage of IFNγ$^+$ DUC18 T cells from CMS5 + TIDC tumors was decreased 43% relative to DUC18 T cells from CMS5 control tumors. Collectively, these results suggested that NeuT TIDCs act primarily to inhibit CD8$^+$ T-cell–mediated antitumor immunity and promote tumor outgrowth. However, because these experiments were unable to distinguish between TIDC suppression of T-cell–mediated antitumor immunity and TIDC promotion of tumor outgrowth via enhanced angiogenesis or other non–immune-mediated mechanisms, we next turned to examining the direct effects of TIDCs on CD8$^+$ T cells *in vitro*.

NeuT TIDCs anergize naive CD8$^+$ T cells. Previous reports on *in vitro* generated regulatory DCs described them as being poor stimulators of naive CD4$^+$ T cells, inducing minimal T-cell proliferation and IFNγ production (14, 15). To determine whether NeuT TIDCs functioned similarly, TIDCs were sort-purified, pulsed with tERK peptide, and used to stimulate naive CD8$^+$ DUC18 T cells. This resulted in diminished T-cell proliferation compared with controls (Fig. 3*A*). Decreased T-cell proliferation in the presence of TIDCs was not due to increased apoptosis (Fig. 3*B*) but seemed to result from fewer T cells progressing normally through the cell cycle (Supplementary Fig. S2*B*). Similar to what had been reported by Zhang and colleagues (14), TIDC-stimulated DUC18 T cells showed impaired up-regulation of CD25 (Fig. 3*C*), suggesting an impaired ability of T cells to respond to IL-2. Diminished T-cell proliferation could be due to a decrease in the quantity or quality of antigen being presented by TIDCs or a shortened duration of antigen presentation. Gating on CD11c$^+$ cells from the T-cell + DC cocultures shown in Fig. 3*B* revealed that a similar percentage of viable, Annexin V$^-$ spDCs and TIDCs were present after 96 hours of culture (12.5% viable spDCs versus 18.9% TIDCs). Additionally, expression of CD69 on T cells was equivalent at 96 hours, indicating that the duration of antigen presentation and the quantity of antigen being presented were comparable between spDCs and TIDCs (Fig. 3*C*). In contrast, IFNγ production was dramatically impaired in TIDC-stimulated T cells (Fig. 3*C*). Thus, it seems that TIDC-stimulated T cells were receiving a qualitatively different priming signal than were spDC-stimulated T cells.

The combined lack of sustained T-cell proliferation and INFγ production, coupled with intact CD69 up-regulation, was reminiscent of cells becoming programmed for anergy. To investigate this possibility, enriched naive CD8$^+$ T cells from DUC18 mice were cultured with either tERK-pulsed spDCs or TIDCs for 4 days, then purified and rested. Then both sets of T cells were restimulated with tERK-pulsed spDCs. T cells that had undergone primary stimulation with TIDCs produced virtually no IFNγ on secondary stimulation (Fig. 3*D*). Therefore, primary encounter of naive CD8$^+$ T cells with TIDCs initiates a cell fate program that leads to aborted proliferation, minimal cytokine production, and anergy.

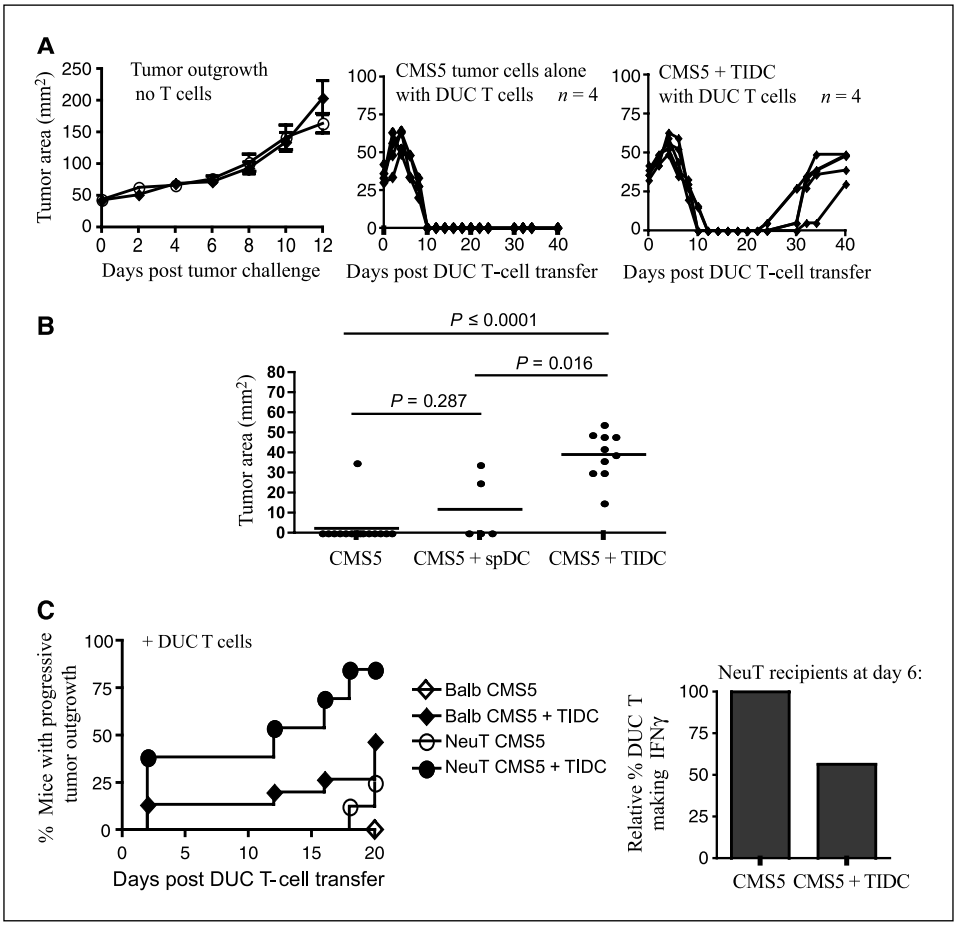

Figure 2. TIDCs from NeuT mammary tumors promote tumor outgrowth *in vivo*. *A,* CMS5 fibrosarcoma cells were injected s.c. alone or together with NeuT TIDCs on day 0 into BALB/c mice. *Points,* mean tumor outgrowth through day 16 (n = 11 mice for CMS5 alone and n = 10 for CMS5 + TIDC); *bars,* SE. *Closed circles,* CMS5 alone; *closed diamonds,* CMS5 + TIDC. Data are cumulative from three independent experiments. *Center,* individual tumor regression data for four mice receiving CMS5 on day −4 with naive DUC18 T cells transferred i.v. on day 0. *Right,* individual tumor regression data for four mice receiving CMS5 + TIDC on day −4, with naive DUC18 T cells transferred i.v. on day 0. *B,* cumulative scatter plots of tumor sizes at day 40 from all individual mice used in multiple experiments. *C,* progressive tumor outgrowth in NeuT recipient versus BALB/c recipient mice after DUC18 T-cell transfer on day 0. For BALB CMS5 versus BALB CMS5 + TIDC, P = 0.0202; for BALB CMS5 versus NeuT CMS5, P = 0.1208; for BALB CMS5 versus NeuT CMS5 + TIDC, P = 0.0001; for NeuT CMS5 versus NeuT CMS5 + TIDC, P = 0.0014 (all, two-tailed t test with Welch's correction). For NeuT recipients only, the relative percentage of gated tumor-infiltrating DUC18 T cells making IFNγ at day 6 post-transfer is shown as the mean from four to five individual mice.

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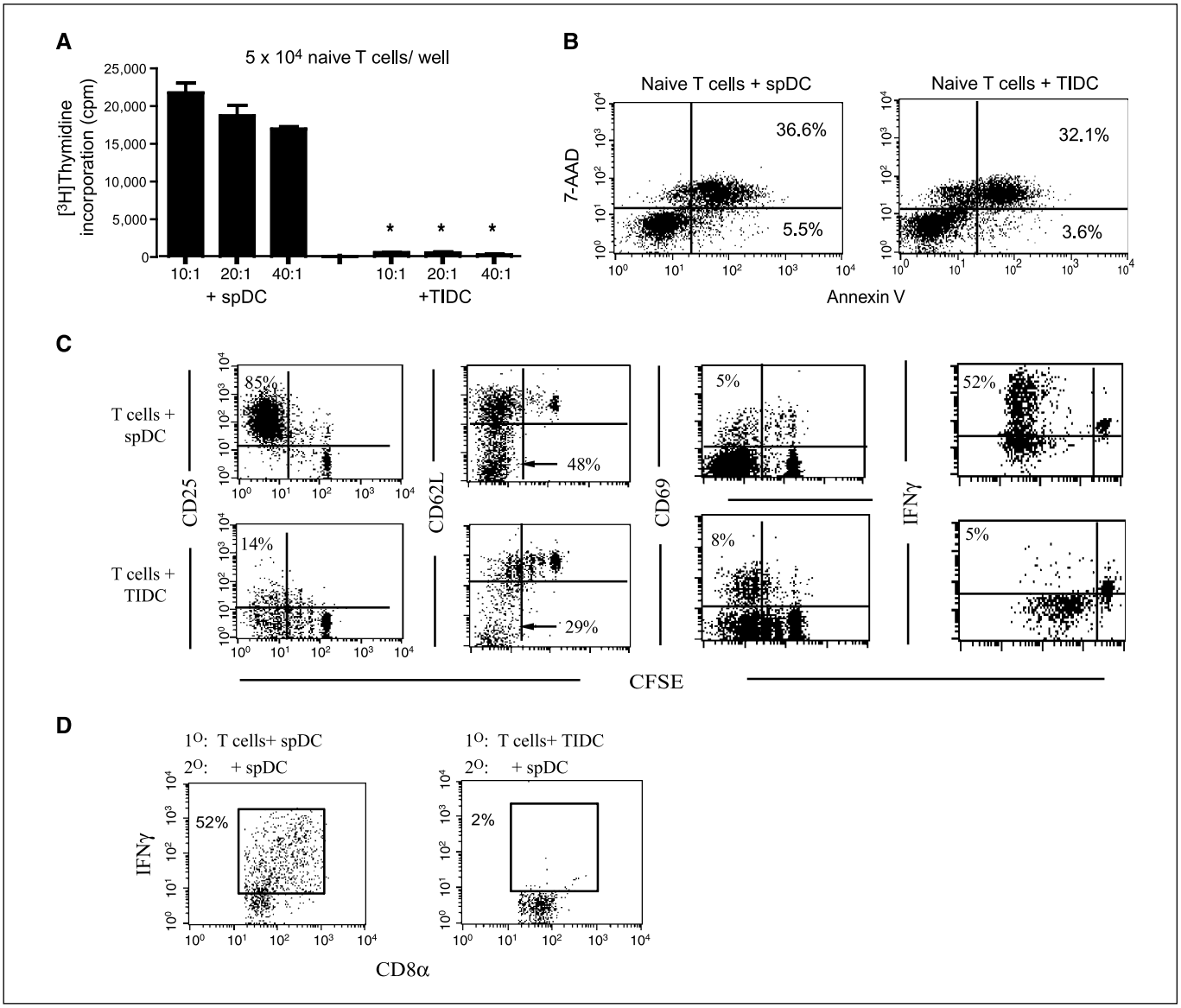

Figure 3. TIDC stimulation of DUC18 T cells leads to impaired proliferation and anergy. *A,* proliferation of naive DUC18 T cells cultured with tERK peptide-pulsed spDCs or NeuT TIDCs for 96 h at the indicated T-cell/DC ratios. Representative data of more than 15 experiments. *, $P < 0.001$, versus spDCs at each ratio. *B,* Annexin V staining on gated Thy1.1$^+$ T cells after 96 h of culture. *C,* naive DUC18 T cells were CFSE labeled and then cultured at 10:1 ratio with spDCs or NeuT TIDCs. Gated on live, CD8$^+$ DUC18 T cells at 96 h, with percentages of divided cells in specific quadrants indicated. Representative data of more than five experiments. *D,* following primary stimulation with TIDCs, DUC18 T cells become unresponsive to secondary stimulation with spDCs as evidenced by a lack of IFNγ production at 48 h.

NeuT TIDCs act as regulatory cells to suppress T-cell proliferation in the presence of stimulatory spDCs. A defining hallmark of *in vitro* generated regulatory DCs is their ability to act in a dominant fashion to suppress naive CD4$^+$ T-cell proliferation in the presence of standard, stimulatory DCs. We therefore asked whether NeuT TIDCs could actively suppress CD8$^+$ T-cell proliferation as evidence of their regulatory nature. To this end, NeuT TIDCs were sort-purified, according to the gate shown in Fig. 4*A*, and pulsed with tERK peptide. As before, T-cell proliferation was robust in the presence of tERK-pulsed spDCs. When this 10:1 T-cell to spDC ratio was maintained and increasing numbers of TIDCs were titrated into the culture, T-cell proliferation steadily dropped (Fig. 4*A*). Overall, the mean level of TIDC suppression was 43% when equal numbers of spDCs and TIDCs were present in cocultures with T cells (Fig. 4*B*; $n = 15$ experiments). To determine if the decrease in T-cell proliferation was due to active suppression by TIDCs or passive interference with access to stimulatory DCs, we combined equal numbers of spDCs, one set pulsed with tERK and the other with an irrelevant but H2-K^d-binding influenza peptide (22), rather than adding TIDCs to spDCs. Minimal inhibition of T-cell proliferation ensued (Fig. 4*B*). Therefore, NeuT TIDCs were acting as regulatory TIDCs to actively, not passively, impede T-cell expansion.

We then asked if cognate interactions between DUC18 T cells and TIDCs were required for suppression to occur by using TIDCs that were pulsed with the irrelevant but H2-K^d–binding influenza

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peptide. As with tERK-pulsed TIDCs, the mean level of suppression exceeded 40% (Fig. 4*B*; n = 8 experiments). Therefore, inhibitory signals from TIDCs dominate over positive signals provided by standard DCs, and TIDC presentation of cognate antigen is not required for suppression to occur.

We then asked whether suppressive TIDCs were present in other murine tumors. Whereas small transplanted CMS5 fibrosarcomas can be rejected by DUC18 T cells (Fig. 3*B*), large CMS5 tumors cannot (22, 33). Therefore, $CD11c^{high}/CD11b^{+}$ TIDCs were sort-purified from large, nonrejectable CMS5 tumors and used in suppression assays, as were $CD11c^{high}/CD11b^{+}$ TIDCs from primary 4T1 mammary carcinomas. Whereas the mean level of suppression varied among the three tumor models, $CD11c^{high}/CD11b^{+}$ TIDCs from all tumors were able to suppress T-cell proliferation in the presence of stimulatory spDCs from tumor-free mice (Fig. 4*C*). Therefore, regulatory TIDCs are not unique to NeuT tumors.

TIDC regulatory function depends on arginase metabolism of L-arginine. To determine the mechanism by which NeuT TIDCs suppressed T-cell proliferation, we began by evaluating well-known soluble inhibitors such as vascular endothelial growth factor, indoleamine 2,3-dioxygenase (34), and IL-10. Blockade of these factors with antibodies or pharmacologic agents only minimally affected T-cell proliferation (data not shown). We then examined the involvement of L-arginine metabolism in TIDC-mediated suppression. During a 24-hour culture, NeuT TIDCs incorporated five times more L-arginine than did spDCs (Fig. 5*A*). After uptake into cells, L-arginine is metabolized through two main pathways by inducible nitric oxide synthase (iNOS) and arginase (29). Culture of TIDCs with IL-4 led to increased arginase I protein expression (Supplementary Fig. S2*C*) and arginase activity (Fig. 5*B*). In contrast, NO production was not elevated in NeuT TIDCs versus spDCs (Fig. 5*B*), even after an overnight stimulation with IFNγ that led to increased iNOS protein expression (Supplementary Fig. S2*D*). As further evidence for the role of L-arginine metabolism in TIDC-mediated suppression, we tested T cells for evidence of phosphorylated eukaryotic translation initiation factor 2a, the initial sensor of cellular stress caused by amino acid starvation (35, 36), and found it to be increased (Fig. 5*C*). Additionally, culture of T cells with regulatory TIDCs led to decreased CD3ζchain expression, as had been reported in other models of L-arginine deprivation (37). However, T cells cultured with TIDCs showed no evidence of tyrosine nitrosylation (data not shown), and 2′,7′-dichloro-fluorescein diacetate staining showed negligible production of reactive oxygen species by NeuT TIDCs in response to coculture with DUC18 effector T cells (Supplementary Fig. S2*E*). Collectively, these data suggested a role for arginase, but not iNOS, metabolism of L-arginine during TIDC suppression of T-cell proliferation.

We then directly addressed whether L-arginine metabolism by arginase was required for NeuT TIDC inhibition of $CD8^{+}$ T-cell proliferation. Pharmacologic inhibitors were selected for their specific activity on either iNOS or arginase pathways, and titrations were done to determine the optimal doses for use in our model (data not shown). The addition of 2 mmol/L L-arginine, to prevent its depletion from culture medium, resulted in an average 40% restoration in T-cell proliferation (n = 10 experiments; Fig. 6*A*). Use of either L-nil or L-NNA, both iNOS inhibitors, did not significantly increase T-cell proliferation, supporting our functional data that showed no increase in NO production by TIDCs relative to spDCs. Norvaline is a commonly used arginase inhibitor (38) and addition of this compound restored T-cell proliferation to nearly 80% of that seen for T cells cultured with spDCs alone (mean, 79%; n = 6 experiments). Taken together, these data indicate that TIDCs inhibit T-cell proliferation primarily via arginase-mediated metabolism of L-arginine.

Studies in other models have shown that limited L-arginine availability can lead to T-cell hyporesponsiveness and cell cycle arrest (39). DUC18 T cells cultured with spDCs in the absence of L-arginine

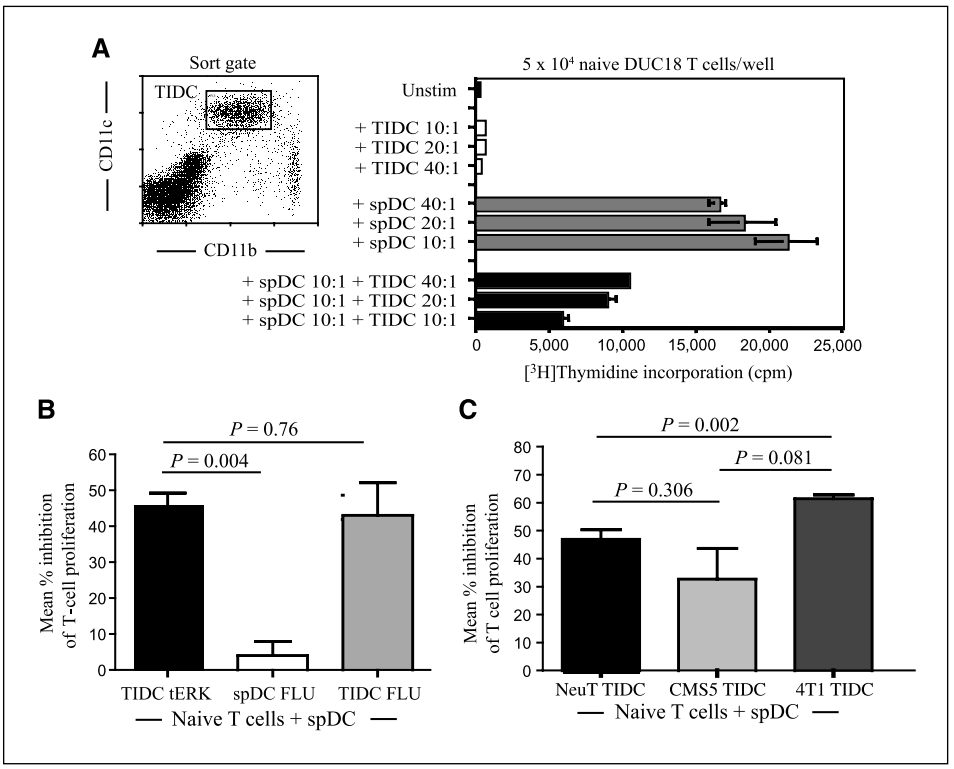

Figure 4. NeuT TIDCs act as regulatory DCs to dominantly suppress naive $CD8^{+}$ T-cell proliferation. *A,* the gate used to sort-purify NeuT TIDCs is indicated (*left*). Naive DUC18 T cells were incubated alone or in the presence of tERK-pulsed NeuT TIDCs, BALB/c spDCs, or both, at the indicated T-cell/DC ratios. Representative of 17 assays. *B,* T cells, spDCs, and NeuT TIDCs were cultured at 10:1:1 ratio. *Columns,* mean percent inhibition of T-cell proliferation as compared with T cells plus spDCs alone; *bars,* SE. For TIDCs pulsed with tERK (*black columns*), n = 17 experiments; for spDCs pulsed with K^{d} FLU peptide (*white columns*), n = 5 experiments; for TIDCs pulsed with K^{d} FLU peptide (*gray columns*), n = 8 experiments. *C,* T cells were cultured as in *B*, with NeuT TIDCs, CMS5 TIDCs, or 4T1 TIDCs. *Columns,* mean percent inhibition of T-cell proliferation from 4 to 15 independent experiments; *bars,* SE.

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showed deficient cell cycle progression in comparison with controls cultured with L-arginine (Fig. 6*B*). A nearly identical cell cycle profile was observed when DUC18 T cells were cultured with TIDCs and spDCs in the presence of L-arginine. The addition of norvaline completely reversed this trend and restored normal cell cycle progression. Therefore, arginase-expressing TIDCs from NeuT mice caused T cells to undergo cell cycle arrest equivalent to that observed when T cells were cultured in the absence of L-arginine.

Discussion

Tumor-derived immunosuppression is a major impediment to successful clinical applications of immunotherapy. Recently, intense research efforts have focused on identifying populations of immunosuppressive tumor-associated cells and the intracellular pathways that direct their functions, with the goal of ultimately improving the clinical efficacy of immune-based antitumor therapies (5, 7, 40, 41).

Our results provide clear evidence that tumors can induce canonical, MHC II$^+$ TIDCs to acquire regulatory functions and support tumor outgrowth by suppressing CD8$^+$ T-cell function. Previous studies had shown that MHC II$^+$ DCs contributed to tumor outgrowth by subverting CD4$^+$ T-cell differentiation toward IL-13 production (12) or by down-regulating effector T-cell function through B7-H1 ligation of the inhibitory receptor PD-1 (13). We now show that MHC II$^+$ TIDCs can actively suppress CD8$^+$ T-cell priming and expansion via metabolism of L-arginine and can counteract CD8$^+$ T-cell–mediated tumor rejection *in vivo*. Collectively, these data suggest that within the tumor microenvironment, MHC II$^+$ DCs act primarily to inhibit protective antitumor immunity. Therefore, therapeutic strategies aimed at increasing the numbers of mature DCs within tumors may fail due to TIDC acquisition of regulatory functions.

Currently, much research into tumor-associated suppressor cell populations focuses on MDSCs. MDSCs are a heterogeneous population of immature myeloid cells that share a "lack or reduced expression of markers of mature myeloid cells, expression of both Gr-1 and CD11b molecules in mice, inability to differentiate into mature myeloid cells in the presence of tumor-derived factors, high levels of reactive oxygen species, and activation of arginase I and other molecules" (30). According to this definition, and the results presented here, CD11c^{high}/CD11b$^+$/Gr-1$^-$ regulatory TIDCs constitute a distinct population of myeloid-lineage suppressor cells within solid tumor masses. Thus, it is possible that regulatory TIDCs complement the suppressive actions of MDSCs and tumor-associated macrophages *in vivo*, and that the relative contribution of each population is dictated by both local cell numbers and suppressive capacity on a per-cell basis.

During our investigation into the functional capacity of regulatory TIDCs, we found that the magnitude and timing of TIDC suppression were critically linked to the host environment. In tumor-free BALB/c mice, coinjection of CMS5 fibrosarcoma cells with regulatory TIDCs had no apparent effect on tumor antigen–specific T-cell priming, and initial tumor regression occurred with normal kinetics (Fig. 3*A*). During the equilibrium phase of the antitumor immune response (42), coinjection of regulatory TIDCs 30 to 40 days earlier shifted the balance so that tumor outgrowth ensued. Inhibition of T-cell effector/memory responses occurred in 100% of BALB/c mice receiving CMS5 cells plus regulatory TIDCs (Fig. 3*B*). One explanation for this is that, similar to what we observed *in vitro*, the quality of initial T-cell priming was altered so

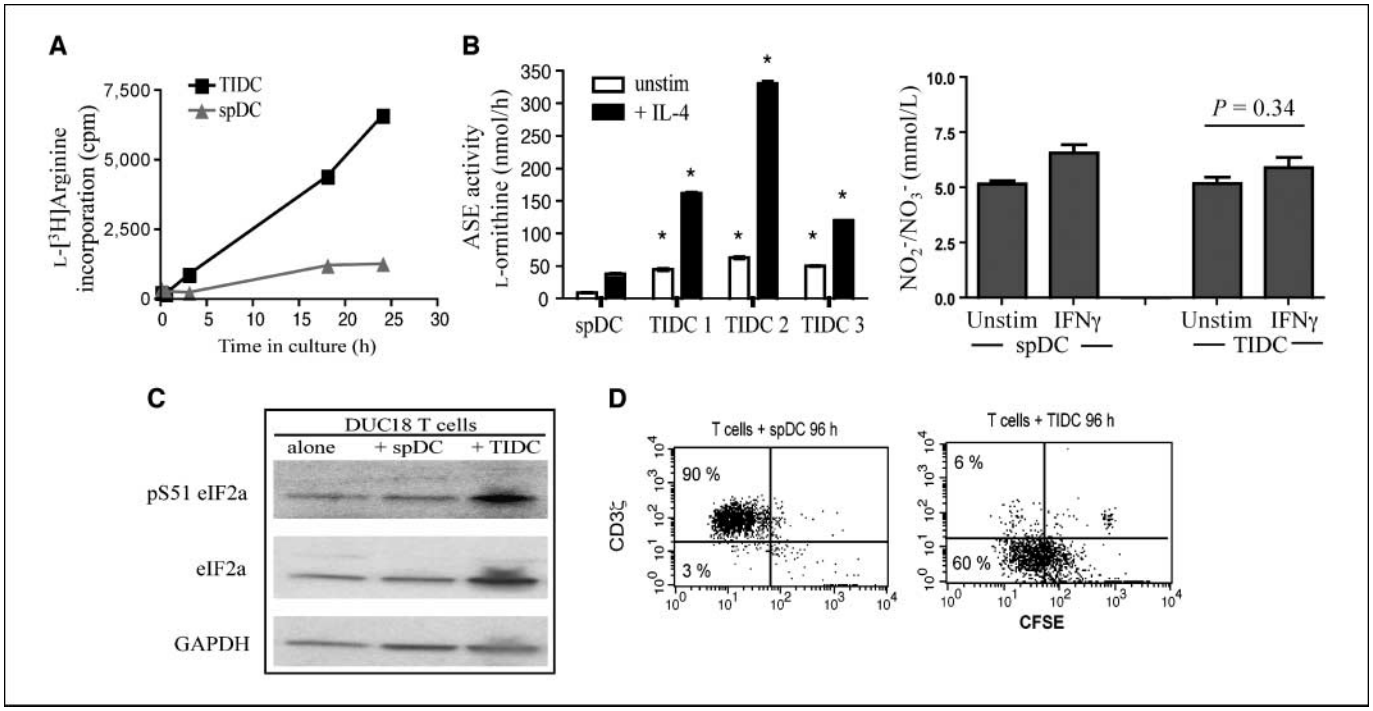

Figure 5. NeuT TIDCs use arginase to metabolize L-arginine. *A,* L-[^{3}H]arginine incorporation of purified TIDCs and spDCs was measured at 0, 0.5, 3, 18, and 24 h. *Points,* means for triplicate wells; *bars,* SD (error bars are too small to be seen). *B,* arginase activity measured in lysates from spDCs pooled from 12 mice or TIDCs taken from 3 individual NeuT mice. Cells were cultured in medium alone or with IL-4 before harvest and lysis. *, $P < 0.001$, TIDC versus spDC controls. NO production as measured by the concentration of total nitrite/nitrate after culturing spDCs or TIDCs for 24 h, with or without IFNγ. Cumulative data from four experiments are shown. *C,* increased expression of the phosphorylated form of eukaryotic translation initiation factor 2a (*eIF2a*) when T cells are cultured with TIDCs. *D,* down-regulation of surface CD3ζ chain expression on DUC18 T cells stimulated with TIDCs.

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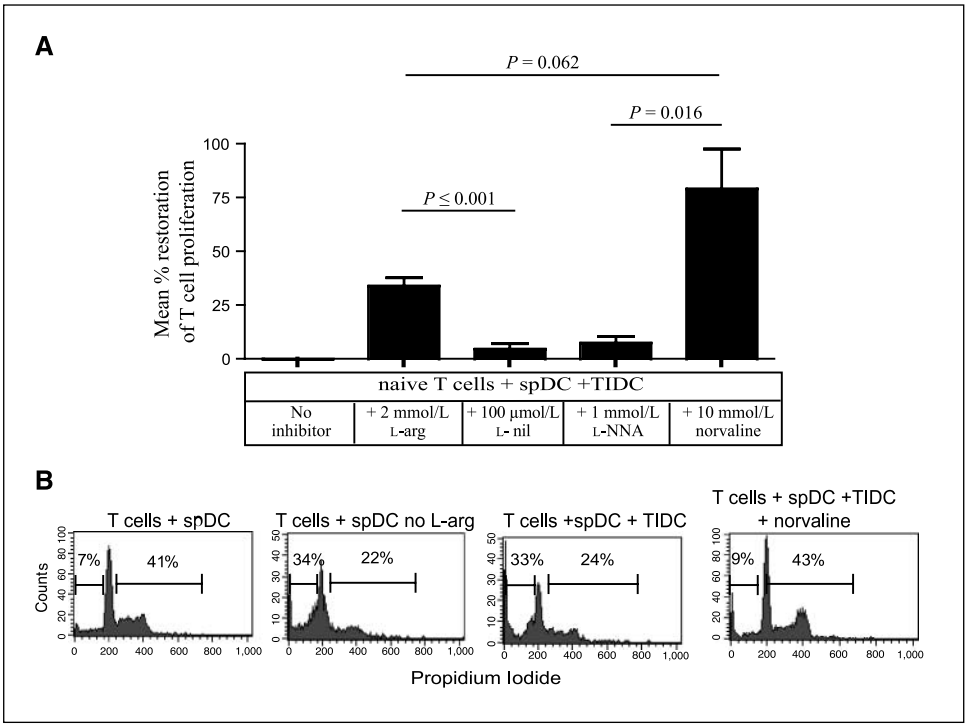

Figure 6. NeuT TIDCs use arginase metabolism of L-arginine to suppress CD8 T-cell proliferation. *A,* the mean percent restoration in T-cell proliferation, relative to T cells plus spDC controls, is shown for T cells cultured with spDCs plus TIDCs +/− L-arginine, L-nil, L-NNA, or norvaline. *Columns,* mean of 9 to 17 independent experiments; *bars,* SE. *B,* propidium idodide cell cycle analysis on naive DUC18 T cells cultured as indicated for 96 h. The percentages of both dying and replicating cells are given for each condition.

that downstream effector and memory cells were defective in their abilities to respond to secondary or tertiary antigenic encounters. In contrast, when regulatory TIDCs were coinjected into a recipient in which spontaneous mammary tumors were already developing, suppression of antitumor immunity was apparent more rapidly. Thus, it seems that systemic changes caused by developing mammary carcinomas created an environment that was more conducive to regulatory TIDC suppression, and in this situation, regulatory TIDCs had a pronounced effect on T-cell priming. It is important to consider, however, that regulatory TIDCs could also be promoting tumor outgrowth independent of any effects on the antitumor immune response. For example, DCs in other systems have been shown to promote tumor angiogenesis (43, 44), and it is possible that regulatory TIDCs are exerting similar effects in our model system.

L-Arginine is a conditionally required amino acid, and as with indoleamine 2,3-dioxygenase metabolism of tryptophan, elevated metabolism of L-arginine by suppressor cells can lead to diminished T-cell function *in vitro* and *in vivo* (23, 29, 34, 45, 46). Limited L-arginine availability results in blocked cell cycle progression of *in vitro* stimulated T cells (39). We found that coculture of $CD8^+$ T cells with regulatory TIDCs also resulted in impaired cell cycle progression, even in the presence of stimulatory spDCs (Supplementary Fig. S2; Fig. 6). However, it does not seem that decreased L-arginine availability is the sole reason for diminished T-cell expansion in our model system. Providing an excess of L-arginine restored T-cell proliferation by only 40%, whereas inclusion of the arginase inhibitor norvaline restored T-cell proliferation to 79% of that seen in positive controls. From this, we conclude that a separate mechanism, downstream of arginase activity, is equally responsible for the induction of anergy and cell cycle arrest in DUC18 T cells cultured with regulatory TIDCs. The nature of this arginase-dependent mechanism remains to be determined.

Our study shows a role for MHC II^+, arginase-expressing TIDCs in the suppression of antitumor immunity. We predict that a reevaluation of "mature" MHC II^+ TIDCs in other systems will reveal the presence of DCs functioning in a regulatory capacity. Although we identified regulatory TIDCs in every murine tumor model examined, the extent to which matured TIDCs contribute to the impairment of antitumor immunity in cancer patients remains to be seen. If present, this would suggest that strategies aimed at specifically eliminating TIDCs with regulatory functions could enhance the efficacy of T-cell–based immunotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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