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Intralesional Delivery of Dendritic Cells Engineered to Express T-bet Promotes Protective Type 1 Immunity and the Normalization of the Tumor Microenvironment

Yanyan Qu,* Lu Chen,† Angela D. Pardee,† Jennifer L. Taylor,* Amy K. Wesa,* and Walter J. Storkus*‡,‡

T-bet (Tbx21), a T-box transcription factor, has been previously identified as a master regulator of type 1 T cell polarization. We have also recently shown that the genetic engineering of human dendritic cells (DCs) to express human T-bet cDNA yields type 1-polarizing APCs in vitro (1). In the present study, murine CD11c+ DCs were transduced with a recombinant adenovirus encoding full-length murine T-bets (DC.mTbets) and analyzed for their immunomodulatory functions in vitro and in vivo. Within the range of markers analyzed, DC.mTbets exhibited a control DC phenotype and were indistinguishable from control DCs in their ability to promote allogenic T cell proliferation in MLR in vitro. However, DC.mTbets were superior to control DCs in promoting Th1 and Tc1 responses in vitro via a mechanism requiring DC–T cell cell interaction or the close proximity of these two cell types and that can only partially be explained by the action of DC-elicited IL-12p70. When injected into day 7 s.c. CMS4 sarcoma lesions growing in syngenic BALB/c mice, DC.mTbets dramatically slowed tumor progression (versus control DCs) and extended overall survival via a mechanism dependent on both CD4+ and CD8+ T cells and, to a lesser extent, asialoGM1+ NK cells. DC.mTbet-based therapy also promoted superior tumor-specific Tc1 responses in the spleens and tumor-draining lymph nodes of treated animals, and within the tumor microenvironment it inhibited the accumulation of CD11b+Gr1+ myeloid-derived suppressor cells and normalized CD31+ vascular structures. These findings support the potential translational utility of DC.Tbets as a therapeutic modality in the cancer setting.


Dendritic cells (DCs) play important roles in regulating the magnitude and nature of specific T cell responses that underlie effective cancer immunotherapy (2–5). In particular, the state of DC polarization may determine, in turn, the biased polarization of functional T cell responses (6), with type 1 T cell-mediated immunity commonly linked with superior anti-tumor efficacy in vivo (7). Recent attention has been focused on defining means by which to condition or engineer DCs to attain so-called DC1s that license type 1 T cell-mediated immunity (6, 8).

In this regard, combinations of proinflammatory cytokines and TLR ligands have been demonstrated to yield DC1-like APCs producing high levels of IL-12p70 and eliciting robust Tc1/T1 T cell responses in vitro (9). Such conditioned DCs are now being translated into phase I clinical trials designs for the treatment of patients with various forms of cancer (10, 11).

Alternatively, DCs have been engineered with cDNA encoding type 1-polarizing cytokines, such as type 1 and type 2 IFNs, IL-12p70 and IL-18 (5, 12–14), among others, again yielding APCs with improved competence to drive and sustain Th1 and Tc1 immunity in vitro and/or in vivo. We have also recently demonstrated that infection of human DCs with a recombinant adenovirus encoding human T-bet, a transactivator protein associated with type 1 polarity in T effector cells, results in these APCs attaining DC1 status in vitro (1). Interestingly, these human DCs promote polarized type 1 T cell responses via a mechanism that appears independent of secreted cytokines (including IL-12p70), but dependent on the DC–T cell interaction or the close approximation of these two cell types.

The present studies were performed to assess whether murine DCs engineered to express murine T-bet (mT-bet) exhibited comparable DC1 functionality in vitro and in vivo when injected directly into tumor lesions in a murine sarcoma model. We report that DCs infected with recombinant adenovirus encoding mT-bet (DC.mTbets) mediate anti-tumor activity in vivo via the enhanced activation of anti-tumor Tc1 cells and the normalization of myeloid-derived suppressor cell (MDSC) levels and the vasculature within the tumor microenvironment (TME).

Materials and Methods

Mice

Female 6- to 8-wk-old C57BL/6 (H-2b) and BALB/c (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were handled under aseptic conditions per an Institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines and culture

As previously described, MethA and CMS4 are chemically induced sarcomas that are syngenic to BALB/c mice (15). These cell lines were free of...
Msxplasma contamination and were maintained in complete medium (CM; RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10 mmol/l L-glutamine; all reagents were purchased from Invitrogen, Carlsbad, CA) at 5% CO2 tension in a 37°C humidified incubator. For T cells culture, 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO) was added to CM.

Adenoviral vectors

E1/E3-substituted, replication-defective (adenoviral vector [Ad5-derived]) adenoviruses were constructed through Cre-lox recombination (16). For recombinant adenovirus encoding mT-bet (Ad.mT-bet) construction, cDNA encoding full-length mouse T-bet (amino acids 1–530) was excised from the pcDNA3.1-mT-bet plasmid (provided by Dr. L. Glimcher, Harvard University, Boston, MA) (17) using the restriction enzyme EcoRI, with the isolated cDNA then ligated into an EcoRI cloning site in the pAdLox shuttle vector (16), yielding pAdLox.mT-bet. After sequence validation of the plasmid, recombinant Ad.mT-bet was generated by cotransfection of pAdLox.mT-bet and Ad5 helper virus DNA in the packaging cell line CRE8 (16). Ad.mT-bet was purified from specific CRE8 lysates by cesium chloride density-gradient centrifugation and subsequent dialysis before storage in 3% threahole at ~80°C. Titters of viral particles were determined by optical densitometry. The empty E1/E3-substituted, replication-defective adenoviral vector (Ad.Δ5) was used as negative control vector in all studies, as previously described (16, 18).

Generation of bone marrow-derived DCs and transduction with adenoviral vectors in vitro

DCs were generated from the tibia/femurs of BALB/c mice, as previously described (5). Briefly, bone marrow precursor cultures were cultured for 7 d in CM supplemented with 1000 U/ml recombinant murine (rm)GM-CSF and 1000 U/ml rIL-4 (both from Peprotech, Rocky Hill, NJ). CD11c+ DCs were then purified using specific MACS beads (Miltenyi Biotec, Auburn, CA) and infected with recombinant adenovirus (either Ad.Δ5 or Ad.mT-bet) at a multiplicity of infection (MOI) of 250 for 48 h. Intracellular staining and flow cytometry were used to document expression of mT-bet in Ad-infected DCs.

CMs4 therapy model

BALB/c mice received s.c. injection with 5 × 105 CMS4 tumor cells in the right flank on day 0. On day 7, mice were randomized into treatment cohorts (five mice each) exhibiting comparable mean tumor sizes (i.e., ~40 mm3). Control DCs (DC-nulls or DC.Δ5s) or DC.mTbets were then injected intratumorally (i.t.) (in a volume of 50 μl (in PBS) on days 7 and 14 posttumor inoculation. Tumor size was assessed every 3 or 4 d and recorded in mm3 by determining the product of the largest perpendicular diameters measured by vernier calipers.

In vivo immune cell subset depletion

On days 6, 13, and 20 after tumor inoculation, mice were injected i.p. with purified Abs: 50–100 μg rat isoform control Ab (Sigma-Aldrich), 50 μg anti-CD4 mAb GK1.5 (American Type Culture Collection, Manassas, VA), 100 μg anti-CD8 mAb3-6.7 (provided by Dr. Zhaoyang You, University of Pittsburgh), or 50 μl anti-asialoGM1 polyclonal Ab (pAb; to deplete NK cells) (Wako Pure Chemicals, Japan). Adenoviral packaging cell line CRE8 (16) was used as an infiltrative target for the targeted immune cell subset based on flow cytometry analysis of peripheral blood mononuclear cells obtained by tail vein puncture from treated mice 24 h after Ab administration (data not shown).

Flow cytometry

Control and Ad-infected DCs were stained with the following Abs and their corresponding isotype controls: PE- or FITC-conjugated mAbs reactive with the mouse cell surface molecules H-2Kd, H-2-IAa, CD11c, CD40, CD54, CD80, CD86 (all mAbs from BD Biosciences, San Diego, CA). After incubation for 30 min at 4°C in the dark, DCs were washed twice with FACS buffer (0.1% BSA and 0.05% sodium azide in PBS) before being analyzed by flow cytometry. Control and DC.mTbets viability was also analyzed after staining cells with 7-aminactinomycin D and annexin V-FITC (both from BD Biosciences, San Jose, CA) as previously described (1). All flow analysis was performed using a FACScan flow cytometer and CellQuest software (BD Biosciences).

Western blotting

DC-nulls and gene-modified DCs (DC.Δ5s or DC.mTbets) were harvested after 48 h of transduction by adenovirus vectors. Western blotting was then performed as previously described (19). Briefly, harvested cells were incubated with lysis buffer (1% Triton X-100, 10 mmol/l Tris-HCl [pH 7.4], 1 mmol/l EDTA, 150 mmol/l NaCl, 0.2 mmol/l sodium orthovanadate, 0.5% Nonidet P-40 in PBS; all reagents from Sigma-Aldrich) containing a protease inhibitor mixture (Complete Mini; Roche Diagnostic Systems, Indianapolis, IN) for 30 min on ice. After centrifugation at 13,500 × g for 30 min, the supernatant was mixed 5:1 with SDS-PAGE running buffer, and proteins were separated on 10% PAGE gels. mAbs against T-bet and HRP-conjugated anti-mouse anti-mouse Ab (both from Santa Cruz Biotechnology, San Diego, CA) were used to detect the expression of T-bet. β-actin was subsequently detected with rabbit-anti-Ab (Abcam, Cambridge, MA) and HRP-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology) as an internal control. Proteins were visualized by a Western Lightning chemiluminescence detection kit (PerkinElmer, Waltham, MA) and exposed to X-Omat film (Eastman Kodak, Rochester, NY).

Evaluation of CD8+ T cell responses against CMS4 tumors ex vivo

For in vitro stimulation cultures, spleens were harvested from two mice per cohort 7 after the second i.t. injection with adenoviral-transduced DCs (i.e., day 21 after tumor inoculation), and pooled splenocytes (2 × 106 cells/well) were stimulated with irradiated (100 Gy) CMS4 cells (2 × 105 cells/well) in the presence of 30 U/ml recombinant human IL-2 (Chiron, Emeryville, CA) for 5 d in 24-well culture plates. Responder CD8+ T cells were then isolated using magnetic bead cell sorting (Miltenyi Biotec), then cocultured with CMS4 tumor cells or irrelevant control M eth A tumor cells (a T cell-to-tumor cell ratio of 10:1) in 96-well round-bottom plates in a humidified incubator at 37°C and 5% CO2 for 48 h. Cell-free supernatants were then stored at ~80°C until analysis with cytokine-specific ELISA. For evaluation of tumor-specific T cell responses within the tumor-draining lymph nodes (TDLNs), these lymphoid organs were harvested on day 21 after tumor inoculation, and CMS4+ MACS T cells were stimulated with irradiated (100 Gy) CMS4 cells for 5 d at a T cell-to-tumor cell ratio of 10:1 before being washed with PBS and then restimulated with irradiated CMS4 versus control (MethA; H-2d) tumor cells at a T cell-to-tumor cell ratio of 10:1 for 48 h. Cell-free supernatants were then stored at ~80°C until analysis with cytokine-specific ELISA.

ELISA

In some experiments, control and transduced DCs were stimulated with CD40L+ J558 cells (8) at a 1:1 ratio for 24 h, with supernatants then harvested for determination of secreted levels of TNF-α and IL-12p70 using specific ELISAs purchased from BioLegend (San Diego, CA) and BD Biosciences, respectively. Supernatants harvested from T cell cultures were analyzed for IL-10 and IFN-γ content using specific OptEIA ELISA sets (BD Biosciences) according to the manufacturer’s instructions. Triplicate determinations were used in all instances, with data reported as the means ± SD.

Imaging of tumor tissues

Tumor samples were prepared and sectioned as previously reported (20). Briefly, tumor tissues were harvested and fixed in 2% paraformaldehyde (Sigma-Aldrich) at 4°C for 1 h, then cryoprotected in 30% sucrose for 24 h. Tumor tissues were then frozen in liquid nitrogen and 6-mm cryosections prepared. For analysis of T cell subsets, sections were first stained with purified rat anti-mouse CD8α or purified rat anti-mouse CD4 mAbs (both from BD Pharmingen, San Diego, CA) for 1 h. After washing, sections were stained with Alexa Fluor 488-conjugated goat anti-rat secondary Ab (Jackson Immunoresearch Laboratories, West Grove, PA). To detect DCs in tumor tissue, tissue sections were stained with FITC-conjugated anti-CD11c Ab (BD Biosciences). For coanalysis of CD4 and Foxp3 expression, sections were washed and incubated with FITC-conjugated anti-mouse CD4 Ab and PE-conjugated anti-mouse anti-Foxp3 Ab (eBioscience, San Diego, CA). For analysis of CD11b+Gr1+ MDCs, tissue sections were incubated with PE-conjugated anti-mouse CD11b and FITC-conjugated anti-mouse Gr1 (both from BD Pharmingen). For analysis of CD31 and NG2 markers, the tissue sections were first incubated with rat anti-mouse CD31 mAb (clone 3C7; from Millipore, Bedford, MA) and anti-NG2 rabbit Ab (Santa Cruz Biotechnology) at 37°C; 5% CO2. The sections were then incubated with 0.5% BSA and stained with Alexa Fluor 488-conjugated goat anti-rat Ab and Cy3-conjugated goat anti-rabbit Ab (both from Invitrogen). To determine in situ cell death, an in situ cell death detection kit (Roche Diagnostics Systems) was used. After staining with primary and secondary Abs, the slides were washed and counterstained with 2 mg/ml Hoechst 33258 (Sigma-Aldrich) for 30 s. After washing, sections were then covered in Gelvatol (Monsanto, St, Louis, MO) and a coverslip was applied. Slide images were acquired using an Olympus 5000 scanning confocal microscope (Olympus America, Center Valley, PA). The positively stained cells were quantified by analyzing...
the images at a final magnification of ×20. Cells number and vascular area were analyzed using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA).

**MLR and transwell assays**

To evaluate the allostimulatory function of control versus engineered DCs, MLR were performed as previously described, with minor modification (16). Control C57BL/6 (H-2b) DCs (DC.nulls or DC.φ5s) or DC.mTbets were seeded (2 × 10^4 cells/well) in round-bottom 96-well plates. CD4+ MACS (Miltenyi Biotec) splenic T cells from wild-type BALB/c (H-2b) mice were labeled with 0.5 μM CFSE (Sigma-Aldrich) for 15 min at room temperature, after which T cells were washed three times with CM, and 2 × 10^5 cells were added to control wells or wells containing DCs in a total volume of 200 μl CM per well. After 72 h of culture, cells were harvested and analyzed by flow cytometry for dilution of CFSE signal. Triplicate determinations were used in all instances, with data reported as the means ± SD.

Transwell assays were performed as previously reported (1). Briefly, DC.mTbets or control DCs (5 × 10^5) derived from C57BL/6 mice were plated in the bottom chamber of a 24-well transwell plate in 400 μl CM, and, 24 h later, 10^5 BALB/c splenic T cells (MACS-isolated CD4+ T cells or CD8+ T cells) along with 10^3 H-2b DC.nulls were placed in the upper chamber of the transwell plate, bringing the total volume to 600 μl CM. As positive controls, cultures were established with C57BL/6 DC.mTbets or control DCs and BALB/c T cells in the upper chamber and no cells in the lower chamber. Where indicated, replicate wells received saturating levels of neutralizing goat anti–mIL-12p70 pAb or isotype control pAb (both from R&D Systems, Minneapolis, MN). Cell supernatants were collected from the upper chamber after 72 h of coculture for performance of IFN-γ ELISA. Triplicate determinations were used in all instances, with data reported as the means ± SD.

**Statistical analysis**

A two-tailed Student t test was used for data analysis. Null hypothesis was rejected, and differences were assumed to be significant at a value of p < 0.05.

**Results**

**Phenotypic characterization of DC.mTbets**

Cultured day 7 bone marrow-derived DCs were left uninfected (DC.nulls) or they were infected with Ad.mTbet (generating DC.mTbets) or control Ad.φ5 (yielding DC.φ5s) at an MOI of 250 for 48 h. DCs were then assessed for expression of T-bet protein using immunofluorescence microscopy (Fig. 1A), intracellular staining as monitored by flow cytometry (Fig. 1B), and Western blotting (Fig. 1C). More than 50% of DC.mTbets expressed elevated levels of T-bet protein, which was predominantly localized to the cell nucleus. DC.nulls and DC.φ5s expressed little or no detectable T-bet protein.

We next analyzed DC.mTbets versus control DCs for expression of cell surface molecules associated with their ability to (co) stimulate T cells (i.e., MHC class I, MHC class II, CD40, CD54, CD80, CD86) or to traffic to secondary lymph nodes (i.e., CCR7) to cross-prime T cells in vivo. As shown in Supplemental Fig. 1, when compared with DC.φ5s (thereby controlling for effects associated with adenoviral infection) or DC.nulls, we noted no significant differences in expression of any of these markers in DC.mTbets. All DC (>90% CD11c+) populations expressed an MHC class I+, MHC class II+, CD40+, CD54+, CD80+, CD86+, CCR7+ phenotype.

An analysis of cytokine production by the various DC populations after CD40 ligation (21) revealed that DC.mTbets produced slightly more IL-12p70 and TNF-α than did control DCs (Fig. 2A). There were no significant differences between the DC cohorts with regard to IL-10 or IL-18 production (Fig. 2A), and none of the evaluated DC populations produced detectable intracellular levels of IFN-γ (Fig. 2B). DC.mTbets also failed to secrete discernable levels of IFN-γ based on ELISA (data not shown).

Although not differentially affecting T cell proliferation, DC.mTbets promote superior type 1 T effector cell induction in vitro

To determine the impact of transgenic T-bet on the ability of DCs to drive T cell responses in vitro, we used MLR. CFSE-labeled CD4+ (H-2b) T responder cells were cocultured with H-2b DC.nulls, DC.φ5s, or DC.mTbets for 3 d, at which time flow cytometry was used to analyze T cell proliferation based on dilution of CFSE fluorescence intensity. As shown in Fig. 3A and 3B, there was no significant difference between the various DC cohorts in their ability to stimulate most allogenic T cells to enter into proliferative cycling. To investigate the potential type 1-polarizing effects of DC.mTbets on T cell responders, a Staphylococcus enterotoxin B (SEB) model was employed. DC.mTbets or control DCs were pulsed with SEB in vitro for 3 h, then washed and cocultured with MACS-purified CD4+ or CD8+ T cells (from syngenic H-2b splenocytes) for 72 h. Specific ELISA performed on cell-free supernatants revealed that DC.mTbets were superior to control DCs in their capacity to elicit IFN-γ production from responder CD4+ (Fig. 3C) and CD8+ (Fig. 3D) T cells. Hence, consistent with our previous findings for human DC.Tbets (1), murine DC.mTbets appear to promote improved type 1
immune responses by contributing differential polarizing, rather than proliferative, signals to responder T cells.

To determine whether DC.mTbets mediate superior type 1 T cell activation via dominant mechanisms involving cell-to-cell contact or secreted mediators, we established transwell MLR cultures in which H-2b DCs were either cocultured with responder H-2d CD4+ or CD8+ T cells, or the two populations were separated from one another. As shown in Fig. 4A and 4B, coculture conditions permissive for intimate DC.mTbets and (CD8+ or CD4+) T cell contact yielded increased IFN-γ production from responder T cells when compared with cocultures established using DC.nulls or DC.ϕ5s. Inclusion of saturating doses of neutralizing anti–IL-12p70 pAb partially reduced the allostimulatory activity of DC.mTbets (p < 0.05 versus cultures supplemented with isotype control pAb). In contrast, when DC.mTbets (lower chamber) were separated from T cells cocultured with control DC.nulls (upper chamber), we observed no increased production of IFN-γ by responder CD8+ (Fig. 4C) or CD4+ (Fig. 4D) T cells versus cocultures established with control DC populations in the lower transwell chamber. As expected, in these “separated” (DC.null plus T cell) cocultures, IL-12p70 appeared to play a dominant role in the activation of type 1 alloresponder T cells (Fig. 4C, 4D). Therefore, as was the case for human DC.hTbets (1), direct DC.mTbet–T cell interaction or close proximity appears crucial for the ability of these APCs to promote superior type 1 T cell activation in vitro via a mechanism that is largely IL-12–independent.

**FIGURE 2.** Impact of T-bet gene insertion on DC expression/secretion of cytokines in vitro. A, DC.Tbet versus control DCs were analyzed by ELISA for secretion of IL-12p70, TNF-α, IL-10, and IL-18 24 h after CD40 ligation, as outlined in Materials and Methods. Results were reported as the means ± SD of triplicate determinations. *p < 0.05 for DC.mTbets compared with either DC.nulls or DC.ϕ5s. B, Expression of intracellular IFN-γ was evaluated in DC.mTbets versus control DCs by flow cytometry. All data are representative of three independent experiments performed in each instance.

**FIGURE 3.** DC.mTbets promote superior type 1 T cell responses in vitro, without affecting T cell proliferation. DC.nulls, DC.ϕ5s, and DC.mTbets were generated from C57BL/6 (H-2b) mice and used to stimulate CFSE-labeled, MACS-purified CD4+ T cells isolated from BALB/c (H-2d) in MLR cultures as outlined in Materials and Methods. After 72 h, T cell proliferation (based on CFSE dilution) was analyzed by flow cytometry (A), with the total percentage (mean ± SD) of proliferating T cells reported in B. To assess the differential capacity of DC.mTbets to polarize type 1 T cell responses in vitro, an SEB model was applied. SEB-pulsed DC.mTbets or control DCs were cocultured with MACS-purified CD4+ (C) or CD8+ (D) T cells for 72 h. Cell-free supernatants from these cultures were then analyzed using mIFN-γ ELISA. Data are reported as the means ± SD of triplicate determinations. All experiments were performed three times, with comparable data obtained in all cases. *p < 0.05 for DC.mTbets versus DC.nulls or DC.ϕ5s.
I.t. injection of DC.mTbet DCs promotes improved activation of anti-tumor Tc1 cells in the periphery and increased frequencies of tumor-infiltrating lymphocytes in vivo

Splenocytes were harvested from CMS4 tumor-bearing animals 7 d after the second i.t. injection of DC.mTbet or control therapy (i.e., day 21 posttumor inoculation). After specific MACS purification, CD8^+ T cells were stimulated in vitro in response to stimulation with CMS4 tumor cells, CMS4-specific ELISA. Data are reported as the means ± SD of triplicate determinations. *p < 0.05 versus control DC.nulls or DC.φ5s. **p < 0.05 versus isotype control Ig.

The ability of DC.mTbet-based therapy to sponsor robust type 1 anti-tumor T cell responses in the spleen and TDLNs is a minimal criterion for successful immunotherapy. Optimal efficacy would be vested from treated animals suggest increased numbers of both CD4^+ and CD8^+ T cells in the secondary lymphoid organs of DC.mTbet- versus control-treated mice (day 21), with CD8^+ T cells producing enhanced levels of IFN-γ in vitro in response to stimulation with CMS4 tumor cells, CMS4-reactive Tc1 effector cells were enriched (comprising more than a fourth of the splenic CD8^+ T cell population) in mice treated with DC.mTbet (Fig. 6). Similar analyses performed on TDLNs harvested from treated animals suggest increased numbers of both CD4^+ and CD8^+ T cells in the secondary lymphoid organs of DC.mTbet- versus control-treated mice (day 21), with CD8^+ T cells producing enhanced levels of IFN-γ in vitro in response to stimulation with CMS4 tumor cells versus control H-2^d MethA sarcoma cells (Supplemental Fig. 2).

The ability of DC.mTbet-based therapy to sponsor robust type 1 anti-tumor T cell responses in the spleen and TDLNs is a minimal criterion for successful immunotherapy. Optimal efficacy would be presumed to occur only if such Ag-experienced T cells were recruited into the TME where they may regulate tumor growth/progression. As a consequence, we next evaluated whether i.t. delivery of DC.mTbet resulted in increased frequencies of tumor-infiltrating lymphocytes (TIL). As shown in Fig. 7, large numbers of CD4^+ and CD8^+ T cells were readily imaged in day 21 tumor sections generated from DC.mTbet- but not control DC-treated mice (Fig. 7).

We also observed that the frequency of CD11c^+ DCs in the TME was very sparse in day 21 tumor sections isolated from mice treated with control DCs, but these levels were increased >10-fold if the mice had been treated using i.t. delivered DC.mTbet (Fig. 7). This large increase in TIDC appeared to result from two processes: (1) increased longevity of the injected DC.mTbet versus control DCs at the time of injection; Supplemental Fig. 3), and (2) improved recruitment of non-injected host CD11c^+ DCs into the TME posttreatment with DC.mTbet versus control therapy (Supplemental Fig. 4).

DC.mTbet-based therapy normalizes the TME: effects on MDSCs, regulatory T cells, and angiogenesis

CD11b^+Gr1^+ MDSCs are both necessary and sufficient to mediate the suppression of T and B cell responses in the TME (24). To determine whether i.t. delivery of DC.mTbet alters the prevalence of MDSCs in the TME, tumor sections were stained with anti-CD11b and anti-Gr1 mAb and analyzed by fluorescence micro-
scopy. As shown in Fig. 8, treatment with DC.mTbets, but not control DCs, resulted in a significant decrease in the numbers of Gr1+CD11b+ MDSCs in the day 21 CMS4 TME.

Similarly, CD4+CD25+ Tregs are commonly enriched in the periphery and, even more so, in the TME of patients with cancer (20, 25, 26), where they may compromise anti-tumor T effector cells (25, 27, 28). As shown in Fig. 8, the prevalence of CD4+ Foxp3+ TILs was significantly decreased as a consequence of DC.mTbet delivery into the TME. Additionally, based on substantive treatment effects on CD8+ TIL numbers, the ratio of CD8+ T cells to Tregs in the TME of mice treated with DC.mTbets versus control DCs was dramatically increased (data not shown).

Effective immunotherapy has also been posited to result in vascular normalization, a phenomenon in which leaky microvessels in the TME are eradicated (29). This results in larger (diameter), more stable blood vessels in association with decreased interstitial fluid pressure, and the improved deliverability of pharmacologic agents and/or immune effector cells into the TME, leading to corollary increases in tumor cell apoptosis (30). Given the profound increases in TILs observed after treatment with DC.mTbets, we next analyzed the surface area and morphologic complexity of CD31+ vascular structures in the TME of treated mice. As shown in Fig. 8, NG2+ pericyte-decorated CD31+ blood vessels, and apoptotic cells (by TUNEL) (original magnification ×20). In all cases, sections were counterstained with Hoechst dye to detect nuclei. The mean ± SD number of each parameter is reported based on the imaging of 10 high-powered fields per slide in B. *p < 0.01 for DC.mTbets versus DC.nulls or DC.ψ5s. Three independent experiments were performed, with each yielding comparable data. HPF, high-powered field.
therapy, the weblike network of branched microvessels was eradicated, leaving behind simple vascular tube structures.

**DC.mTbet-based therapy results in increased apoptosis in the TME**

A corollary expectation for improved recruitment of Tc1 TIL effector cells and for the anti-angiogenic effects associated with i.t. delivery of DC.mTbets would be increased (tumor) cellular apoptosis in the TME of this treatment cohort. As shown in Fig. 8, the number of TUNEL + events within the CMS4 TME was increased by >10-fold after two treatment cycles with DC.mTbets versus control DCs.

**Discussion**

Although T-bet is commonly considered as a master regulator of the type 1 T cell responses (31–34), it also clearly plays a permissive role in supporting proinflammatory responses from cells of the innate immune system (35, 36). Indeed, the capacity of DCs to promote type 1 immunity has been reported to be highly dependent on the low levels of T-bet protein constitutively expressed by at least some subsets of DCs (33). Hence, we hypothesized that the reinforcement of T-bet expression in DCs using rAd.mTbet viral transfection would yield robust DC1-type APCs that were competent to (re)polarize type 1 anti-tumor T cell responses in vitro and in vivo.

The major findings in the present study are that DCs engineered to express the T cell transactivator T-bet serve as an effective therapeutic agent (compared with control DCs) when delivered into the TME, based on improved (1) cross-priming of systemic anti-tumor type 1 T cell responses, (2) frequencies of type 1 TILs and CD11c + DCs in the TME, (3) normalization of the TME (based on reductions in MDSC frequencies and vascular complexity), and (4) frequencies of apoptotic (TUNEL +) tumor cells in TME. Ab depletion studies support the required action of both CD4 + and CD8 + T cells (in addition to asialoGM1 + NK cells) in the treatment effectiveness of DC.mTbets. Notably, CD8 + T cells isolated from the spleens and TDLNs of DC.mTbet-treated mice directly recognized (based on IFN-γ production) MHC class I +, MHC class II + CMS4 tumor cells in vitro. These effector cells are likely activated in vivo as a consequence of APCs that have acquired apoptotic/necrotic tumor debris and then emigrated from the therapy-normalized TME to the TDLNs and spleen. The therapy-induced tumoricidal process within the TME does not appear to involve the differential, direct tumoricidal activity of injected DC.mTbets, as these APCs exhibited only low, control-level capacity to promote the apoptotic death of CMS4 tumor cells in vitro (Supplemental Fig. 5).

Interestingly, i.t. delivery of DC.mTbets significantly reduced levels of Tregs (based on a CD4 + Foxp3 + phenotype) in the TME when compared with control treatment groups. Furthermore, the anti-tumor responses associated with this approach appear due to the “overrunning” of a limited Treg suppression pathway via the influx of large numbers of therapy-induced, type 1 effector T cells into the TME. This reversal in CD8 + T cell versus Treg numbers/frequency within the TME may be facilitated or sustained due to therapy-associated changes in CD11b+Gr1 + MDSC content (reduced by ~60–70% in the TME). MDSCs have been reported to inhibit T effector cell function via a range of mechanisms, including the depletion of amino acids [arginine, tryptophan, or cysteine/cystine (37, 38)], the production of ROS and peroxynitrite (39) and the uncoupling of TCR-ζ-chain signaling (40), among others. How DC.mTbet therapy limits MDSC numbers in the tumor remains unknown, but given the suggested normalization in vascular structures in the TME postinjection of DC.mTbets, one could consider that reductions may occur in hypoxia-sensitive chemokines (such as CCL2 and CCL5) that are known to recruit MDSCs (41, 42). Alternatively, or additionally, early type 1 T cell recruits into the TME may limit the development of MDSCs from precursor myeloid cells (43). Regardless of such potential mechanisms, one could consider inhibition of residual MDSC numbers/function in the TME (using drugs such as sunsitib; Ref. 44) as a means to further improve the efficacy of i.t. delivered DC.mTbets in combinational treatment.

The present findings confirm and extend our previous human in vitro studies (1), where DC.hTbets were found to promote the differentiation of type 1 T effector cells without significantly altering responder T cell proliferation. As with human DC.Tbets (1), we observed that DC.mTbets were minimally altered with regard to their cell-surface expression of MHC, costimulatory, or integrin molecules. A slight point of variance with human DC.Tbets that failed to produce increased quantities of cytokines upon activation (1), murine DC.Tbets (versus control DCs) secreted higher levels of IL-12p70 and TNF-α (but not IL-10, IL-18, or IFN-γ) after CD40 ligation in vitro. Human DC.Tbets were determined to mediate their type 1-polarizing effects on T cells in a largely contact-dependent manner, which did not appear to involve key cytokines such as IL-12p70 or IFN-γ itself (1). In the present study, we have also observed that the superior ability of DC.mTbets to promote type 1 CD4 + and CD8 + T cell responses in vivo requires intimacy between APCs and T cells. However, unlike the human model, murine DC.mTbets elaborated IL-12p70 appears to play at least a minor role in the resultant development of Tc1 and Th1 responses. The in vivo relevance of DC-secreted products in the therapeutic mechanism of action associated with DC.mTbets will be best determined in models using DCs prepared from specific cytokine-deficient strains of animals. Given the breadth of available knockout strains (including IL-12p35−/− and IL-12p40−/− mice) on the H-2b (but not H-2d) background, we are presently developing a MCA205 sarcoma model in syngenic C57BL/6 mice to resolve how specific cytokine production competency by DC.Tbets relates to the therapeutic efficacy of this biologic agent. Should the in vivo role of IL-12p70 be shown to be minimal in the setting of DC.mTbet-based therapeutic efficacy, it would then be intriguing to evaluate the impact of combined T-bet plus IL-12p70 gene therapy, given the potentially synergistic/complementary mechanisms of action associated with these agents in sponsoring protective, type 1 anti-tumor immunity.

In summary, our results suggest that genetic engineering of DCs to express the Th1 transcription factor T-bet yields an APC that is competent to cross-prime protective type 1 anti-tumor immunity after delivery into the TME in vivo. DC.Tbets also appear to mediate a range of locoregional effects (i.e., MDSC reduction, vascular normalization) that may improve the delivery/function of therapy-induced T effector cells into/within the TME. Despite potential minor differences between human and mouse DC.Tbets with regard to secretion of IL-12p70 and TNF-α (and possibly additional cytokines) and the role played by IL-12p70 in supporting the type 1-polarizing activity of DC.Tbets, we think that our preclinical studies advocate the implementation of DC.Tbets as a therapeutic agent in the management of patients with cancer.

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**Disclosures**

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