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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*§,†,‡

CD8+ T cells and, to a lesser extent, asialoGM1+ NK cells. DC.mTbet-based therapy also promoted superior tumor-specific Tc1 responses in vitro (9). Such conditioned DC1s are now being translated into phase I clinical trial designs for the treatment of tumors that are syngenic to BALB/c mice (15). These cell lines were free of contaminating adventitious agents.

As previously described, MethA and CMS4 are chemically induced sarcomas that are syngenic to BALB/c mice (15). These cell lines were free of contaminating adventitious agents. 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.mThets) mediated 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 contamination. 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.mThets) mediated 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).

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Mycoplasmata 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 γ-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 pAdLxo 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 pCMVbeta proviral DNA plasmids. Adenoviral 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% trehalose at −80°C. Titers of viral particles were determined by optical densitometry. The empty E1/E3-substituted, replication-defective adenoviral vector (AdΔ5) 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 tibias/femurs of BALB/c mice, as previously described (5). Briefly, bone marrow precursors 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 AdΔ5 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 DCΔ5s) or DC.mTbets (104) were then injected intratumorally (i.t.) in a total 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 isotype control Ab (Sigma-Aldrich), 50 μg anti-CD4 mAb GK1.5 (American Type Culture Collection, Manassas, VA), 100 μg anti-CD8 mAb3B6-6.7 (provided by Dr. Zhanzhou You, University of Pittsburgh, PA), or 50 μl anti-asialoGM1 polyclonal Ab (pAb; to deplete NK cells, kindly provided by Dr. J. Wong, Japan). Adenoviral packaging cell line CRE8 (16) was used as a negative control for the targeted immune cell subset based on flow cytometry analysis of peripheral blood mononuclear cells obtained by tail venipuncture 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 Abs reactive against the mouse cell surface molecules H-2Kd, H-2-IAd, 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-aminoactinomycin 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-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 μU/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 MethA tumor cells (at 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 CD8+ MACS T cells were stimulated with irradiated (100 Gy) CMS4 cells for 5 d at a T cell-to-tumor cell ratio of 1:10 before being washed with PBS and then restimulated with irradiated CMS4 versus control (MethA; H-2b) tumor cells at a T cell-to-tumor cell ratio of 1:10 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+ J585 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-rabbit 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 Foxp3 Ab (eBioscience, San Diego, CA). For analysis of CD11b*Gr1+ MDCs, tissue sections were incubated with PE-conjugated rat 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 Ab from Millipore, followed by incubation at room temperature, then washed with 0.5% BSA and stained with Alexa Fluor 488-conjugated goat anti-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 μg/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-2d) DCs (DC.nulls or DC.p5s) 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-2d) 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 × 10^4) 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 10^5 H-2d 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.p5 (yielding DC.p5s) at an MOI of 250 for 24 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.p5s 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.p5s (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-2d) T responder cells were cocultured with H-2d DC.nulls, DC.p5s, 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 allogeneic 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 syngeneic H-2d 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.mTbets (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.mTbet 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.mTbet 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.mTbet (p < 0.05 versus cultures supplemented with isotype control pAb). In contrast, when DC.mTbet (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.hTbet (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.

DC.mTbet injected i.t. promote protective type 1 anti-tumor immunity: dependence on CD4+ T cells, CD8+ T cells, and NK cells in vivo

Based on the reported ability of i.t. injected DC populations to support effective cross-priming of therapeutic anti-tumor T cell responses in vivo (16, 22, 23), we next analyzed whether DC.mTbet were superior to control DCs in this regard. Mice harboring established (day 7) s.c. CMS4 sarcomas were treated i.t. with 1 × 106 DC.mTbet or control DCs, with an identical treatment applied 1 wk later (on day 14 posttumor inoculation). As shown in Fig. 5A, therapy using DC.mTbet, but not control DCs, resulted in the prolonged suppression of tumor growth (p < 0.05 versus control DCs beginning on day 14 posttumor inoculation). DC.mTbet-based therapy yielded an approximate 3-wk extension in overall survival versus control therapy (Fig. 5B; p = 0.0012 versus DC.nulls and p = 0.0014 versus DC.ϕ5s). Repeat experiments in which mice were depleted of T cell subsets or NK cells beginning just prior to the application of therapy revealed a major dependency of treatment efficacy on both CD4+ and CD8+ T cells (Fig. 5C). A minor dependency was also noted for NK cells, as injection of the depleting anti-asialoGM1 Ab partially reduced the anti-tumor protection afforded by i.t. administered DC.mTbet (Fig. 5C).
or CD4+ (CD8+ T cells from control-treated mice only rarely produced enhanced levels of IFN-γ). As shown in Fig. 7, large numbers of CD4+ (B, D) T cells as outlined in Materials and Methods. T cells were placed in the upper transwell chamber, with DCs placed in the upper and lower chambers of the transwell as indicated. After 72 h of coculture in the absence or presence of 2 μg per well of control Ig or neutralizing anti-IL-12p70 pAb, cell-free supernatant was harvested from the upper well and analyzed using an mIFN-γ-specific ELISA. Data are reported as the means ± SD of triplicate determinations. *p < 0.05 versus control DC.nulls or DC.ψ5s. **p < 0.05 versus isotype control Ig.

**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 DCs or control therapy (i.e., day 21 posttumor inoculation). After specific MACS purification, CD8+ T cells were stimulated in vitro with irradiated CMS4 tumor cells, CMS4-5s, and DC.mTbets) and H-2d MACS-isolated, splenic CD8+ (A, C) or CD4+ (B, D) T cells as outlined in Materials and Methods. T cells were placed in the upper transwell chamber, with DCs placed in the upper and lower chambers of the transwell as indicated. After 72 h of coculture in the absence or presence of 2 μg per well of control Ig or neutralizing anti-IL-12p70 pAb, cell-free supernatant was harvested from the upper well and analyzed using an mIFN-γ-specific ELISA. Data are reported as the means ± SD of triplicate determinations. *p < 0.05 versus control DC.nulls or DC.ψ5s. **p < 0.05 versus isotype control Ig.

**FIGURE 4.** The optimal type 1-polarizing capacity of DC.mTbet DCs requires intimate DC–T cell contact and is partially dependent on IL-12p70. Transwell cultures were established using H-2d DCs (i.e., DC.nulls, DC.ψ5s, and DC.mTbets) and H-2d MACS-isolated, splenic CD8+ (A, C) or CD4+ (B, D) T cells as outlined in Materials and Methods. T cells were placed in the upper transwell chamber, with DCs placed in the upper and lower chambers of the transwell as indicated. After 72 h of coculture in the absence or presence of 2 μg per well of control Ig or neutralizing anti-IL-12p70 pAb, cell-free supernatant was harvested from the upper well and analyzed using an mIFN-γ-specific ELISA. Data are reported as the means ± SD of triplicate determinations. *p < 0.05 versus control DC.nulls or DC.ψ5s. **p < 0.05 versus isotype control Ig.

**FIGURE 5.** I.t. injection of DC.mTbet DCs provides therapeutic benefit that is dependent on CD4+ T cells, CD8+ T cells, and NK cells. A, BALB/c mice bearing day 7 s.c. CMS4 tumors were treated with i.t. injection of 1 × 10^6 DC.mTbet or control DCs (DC.nulls or DC.ψ5s). An identical retreatment was provided 1 wk later. Tumor growth was monitored every 3–5 d and is reported as means ± SD for five animals per cohort. *p < 0.05 when compared with mice treated with DC.nulls or DC.ψ5s. B, Kaplan-Meier survival plot for the overall survival of mice in the various treatment groups; *p = 0.0012 and 0.0014 for DC.mTbet-treated mice versus DC.null- and DC.ψ5-treated animals, respectively. C, The experimental plan in Fig. 5A was modified to include cohorts of CMS4-bearing mice in which CD4+ T cells, CD8+ T cells, or asialoGM1† NK cells were depleted (beginning on day 6 [i.e., 1 d prior to i.t. delivery of DC.mTbet]) by specific Ab administration as described in Materials and Methods. *p < 0.05 when compared with mice treated with DC.nulls or DC.ψ5s. Data are representative of three independent experiments performed in all cases.

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 DCs (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. mTbets versus control therapy (Supplemental Fig. 4).

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

CD11bGr1+ 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 costained 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.

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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.mTbet therapy 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+, and CD8+ T cells from the 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/function 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 sunitinib; 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.Tbets 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 activate 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.mTbet therapy would 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-2b) 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 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**

The authors have no financial conflicts of interest.
References


Supplemental Fig. 1. Phenotype of DC.mTbet and control DC. DC expression of the indicated surface markers and intracellular T-bet were analyzed by two-color FCM as described in Materials and Methods. Data are representative of three independent experiments performed.
Supplemental Fig. 2. Intratumoral delivery of DC.T-bet enhances CD4⁺ and CD8⁺ T cell expansion and the development of tumor-reactive Tc1 effector cells in the tumor draining lymph node. Established day 7 CMS4 tumors were injected with 1 x 10⁶ DC.mTbet or control DC.ψ5, with an identical re-treatment one week later. On day 21 (7 days after the second intratumoral injection of DC), tumor draining lymph nodes were harvested, and total numbers of CD4⁺ (A) and CD8⁺ (B) T cells determined by FCM. In C, MACs-purified CD8⁺ T cells from tumor draining lymph nodes were co-cultured with irradiated CMS4 tumor cells for 5 days and washed, then restimulated with CMS4 tumor cells or unrelated MethA (H-2d) sarcoma cells for 48h. Supernatants from these restimulation cultures were then analyzed for mIFN-γ content by specific ELISA. Data are representative of those obtained in 3 independent experiments. Results were shown as reported as mean ± SD. *p < 0.05, **p < 0.01, for DC.mTbet versus DC.ψ5.
Supplemental Fig. 3. Ectopic expression of T-bet does not negatively affect the survival of DC.mTbet. DC null, DC.ψ5 and DC.mTbet were generated as outlined in the Materials and Methods. Forty-eight hours after the initiation of adenoviral infections, cells were harvested and stained with 7-AAD and Annexin-V FITC prior to analysis using flow cytometry. Insert numbers reflect the percentage of total cells in each quadrant. The presented data are representative of information obtained in three independent experiments performed.
Supplemental Fig. 4: DC,mTbet exhibit enhanced persistence in the CMS4 TME and promote increased tumor infiltration by host CD11c+ DC. (A) Established day 7 CMS4 tumors were injected with 1 x 10^7 DC,mTbet or control DC.v5 cells that had been pre-labeled with CFSE (Sigma-Aldrich, 0.5 mM for 30 min at 37°C). Three days after injection, tumor lesions were harvested, fixed, frozen, sectioned, and stained with anti-CD11c-PE mAb (BD-Biosciences). Tumor sections were also counterstained with Hoechst to detect nuclei before analysis by fluorescence microscopy. After analysis of 10 high-power fields (HPF), the mean ± SD was calculated for total CD11c+ cells (B), CD11c-PE+ only (i.e. not co-expressing CFSE) cells (C) and CD11c-CFSE+ cells (D). *p < 0.05, **p < 0.01 for DC,mTbet vs. DC.v5 injected tumors.
Supplemental Fig. 5. DC.mTbet exhibit control level capacity to uptake CMS4 tumor-associated antigenic material and to mediate the apoptotic death of CMS4 tumor cells in vitro. In (A), DC.mTbet or control DC were co-cultured with CMS4 tumor cells that had been previously infected for 48h with a recombinant adenovirus encoding EGFP provided by the University of Pittsburgh Cancer Institute’s Vector Core Facility (Shared Resource). MOI ~ 50 for 12h at 37°C at a DC:CMS4-EGFP ratio of 5:1. Cells were then stained with APC-conjugated anti-CD11c antibodies (eFluor® 450) and analyzed by flow cytometry. Successful DC uptake of tumor material was interpreted as CD11c+EGFP+ events. Inset numbers reflect the percentage of CD11c+EGFP+ among total CD11c+ DC in co-cultures. In (B), the DC.mTbet or control DC were co-cultured with uninfected CMS4 tumor cells at a DC:CMS4 ratio of 5:1 for 12h. Harvested cells were then co-stained with APC-conjugated anti-CD11c antibodies and FITC-conjugated anti-Annexin-V (BD PharMingen) antibodies and analyzed by flow cytometry. The calculated percentage of apoptotic tumor cells (Annexin-V+CD11c+ events) is indicated in the upper left quadrant of each panel. All data are representative of results obtained in 3 independent experiments.