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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-elaborated 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 with the tumor microenvironment 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 antitumor 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/Tf1 T cell responses in vivo (9). Such conditioned DCs are now being translated into phase I clinical trial designs for the treatment of patients with various forms of cancer (10, 11).

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Abbreviations used in this paper: Ad, adenovirus; Ad.dQ5, empty E1/E3-substituted, replication-defective adenoviral vector; Ad.mTbet, recombinant adenovirus encoding mT-bet; CM, complete medium; DC, dendritic cell; DC.mTbet, DCs infected with recombinant adenovirus encoding mT-bet; HPF, high-powered field; i.t., intratumoral(ly); MDSC, myeloid-derived suppressor cell; MOI, multiplicity of infection; mT-bet, murine T-bet; pAb, polyclonal Ab; rm, recombinant murine; SEB, Staphylococcus enterotoxin B; TDNL, tumor-draining lymph node; TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment; Treg, regulatory T cell.

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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...
MSCs were cultured in 10% fetal bovine serum and 10% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 4°C. After centrifugation at 13,500 g for 20 min, the cell pellet was resuspended in FBS-free media containing 10% dimethyl sulfoxide (DMSO) and stored at −80°C.

Adenoviral vectors

The generation of adenoviral vectors for gene therapy involved the packaging of the adenovirus genome into viral capsids. This was achieved by using a Cre-lox recombination system to substitute the adenovirus genome with the desired genetic material. The process involved the removal of the adenovirus genome from the viral capsids, followed by the insertion of the therapeutic gene of interest, and then the reassembly of the adenovirus particles.

Western blotting

The protein expression of the therapeutic gene was analyzed using Western blotting. This involved the separation of proteins by size using SDS-PAGE, followed by transfer to a membrane and the detection of the protein of interest using specific primary and secondary antibodies. The band intensity was quantified using densitometry.

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

In vitro stimulation cultures were prepared by stimulating responder CD8+ T cells with irradiated CMS4 tumor cells. The response was measured by the production of cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-2 (IL-2) using specific ELISAs.

Imaging of tumor tissues

Tumor tissue imaging was performed using various techniques such as fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) to detect the expression of the therapeutic gene of interest. Imaging was performed using confocal microscopy and digital imaging systems to visualize the expression patterns and localization of the therapeutic gene within the tumor tissues.

Conclusion

In conclusion, the gene therapy approach described in this study demonstrated the potential for using adenoviral vectors to target and inhibit tumor growth. Further studies are needed to optimize the therapeutic gene delivery and to evaluate the clinical efficacy of this approach in treating specific tumor types.
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-2\textsuperscript{b}) DCs (DC.nulls or DC.\(\phi\)5s) or DC.mTbets were seeded (2 \times 10\textsuperscript{4} cells/well) in round-bottom 96-well plates. CD4\textsuperscript{+} MACS (Miltenyi Biotec) splenic T cells from wild-type BALB/c (H-2\textsuperscript{b}) mice were labeled with 0.5 \mu M CFSE (Sigma-Aldrich) for 15 min at room temperature, after which T cells were washed three times with CM, and 2 \times 10\textsuperscript{5} cells were added to control wells or wells containing DCs in a total volume of 200 \mu l 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 \times 10\textsuperscript{5}) derived from C57BL/6 mice were plated in the bottom chamber of a 24-well transwell plate in 400 \mu l CM, and, 24 h later, 10\textsuperscript{5} BALB/c splenic T cells (MACS-isolated CD4\textsuperscript{+} T cells or CD8\textsuperscript{+} T cells) along with 10\textsuperscript{3} H-2\textsuperscript{d} DC.nulls were placed in the upper chamber of the transwell plate, bringing the total volume to 600 \mu l 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-\(\gamma\) 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.\(\phi\)5 (yielding DC.\(\varphi\)5s) 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.\(\varphi\)5s 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.\(\varphi\)5s (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.\(\text{mTbets}\). All DC (\(\geq 90\%\) CD11c\textsuperscript{+}) populations expressed an MHC class I\textsuperscript{+}, MHC class II\textsuperscript{+}, CD40\textsuperscript{+}, CD54\textsuperscript{+}, CD80\textsuperscript{+}, CD86\textsuperscript{+}, CCR7\textsuperscript{+} 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-\(\alpha\) 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-\(\gamma\) (Fig. 2B). DC.mTbets also failed to secrete discernable levels of IFN-\(\gamma\) 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\textsuperscript{+} (H-2\textsuperscript{b}) T responder cells were cocultured with H-2\textsuperscript{b} DC.nulls, DC.\(\varphi\)5s, 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\textsuperscript{+} or CD8\textsuperscript{+} T cells (from syngeneic H-2\textsuperscript{b} 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-\(\gamma\) production from responder CD4\textsuperscript{+} (Fig. 3C) and CD8\textsuperscript{+} (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 responsive 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-2d 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. 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.dT5s. Inclusion of saturating doses of neutralizing anti–IL-12 p70 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-12 p70 appeared to play a dominant role in the activation of type 1 allosresponder T cells (Fig. 4C, 4D). Therefore, as was the case for human DC.hTbet1 (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.mTbets 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.mTbets 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.mTbets or control DCs, with an identical treatment applied 1 wk later (on day 14 posttumor inoculation). As shown in Fig. 5A, therapy using DC.mTbets, 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.dT5s). 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.mTbets (Fig. 5C).
or CD4+ (CD8+ T cells from control-treated mice only rarely produced producing enhanced levels of IFN-γ by ELISA (24-h assay; Fig. 6B).) Whereas splenic CD8+ T cells from control-treated mice only rarely produced IFN-γ 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-2d 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 are recruited into the TME where they may regulate tumor growth/progression. As a consequence, we next evaluated whether i.t. delivery of DC.mTbet DCs (i.e., DC.nulls, DC.ϕ5s, and DC.mTbets) and H-2d MACS-isolated and 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.ϕ5s. **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 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-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). 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-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. mTbets versus control therapy (Supplemental Fig. 4).

**FIGURE 4.** The optimal type 1-polarizing capacity of DC.mTbet requires intimate DC–T cell contact and is partially dependent on IL-12p70. Transwell cultures were established using H-2b DCs (i.e., DC.nulls, DC.ϕ5s, and DC.mTbets) and H-2d MACS-isolated CMS4 tumor cells and analyzed for levels of intracellular IFN-γ in response to stimulation with CMS4 tumor cells versus control H-2d MethA sarcoma cells (Supplemental Fig. 2). 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.ϕ5s. **p < 0.05 when compared with mice treated with DC.nulls or DC.ϕ5s.

**FIGURE 5.** I.t. injection of DC.mTbet 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.mTbets or control DCs (DC.nulls or DC.ϕ5s). 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.ϕ5s. 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.ϕ5-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.ϕ5s. Data are representative of three independent experiments performed in all cases.
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.c5s. 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.mTbet therapies 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.mT-bet 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 therapeutic 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 effects 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.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 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.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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