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Extranodal induction of therapeutic immunity in the tumor microenvironment after intratumoral delivery of *Tbet* gene-modified dendritic cells

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Abstract

Murine dendritic cells (DC) transduced to express the Type-1 transactivator T-bet (i.e. mDC.Tbet) and delivered intratumorally (i.t.) as a therapy are superior to control wild-type DC in slowing the growth of established subcutaneous (s.c.) MCA205 sarcomas *in vivo*. Optimal anti-tumor efficacy of mDC.Tbet-based gene therapy was dependent on host NK cells and $CD8^+$ T cells, and required mDC.Tbet expression of MHC class I molecules, but was independent of the capacity of the injected mDC.Tbet to produce pro-inflammatory cytokines (IL-12 family members or IFN-γ) or to migrate to tumor-draining lymph nodes (TDLN) based on CCR7 ligand chemokine recruitment. Conditional (CD11c-DTR) or genetic ($BATF3^{-/-}$) deficiency in host antigen crosspresenting DC did not diminish the therapeutic action of i.t.-delivered wild-type mDC.Tbet. Interestingly, we observed that i.t delivery of mDC.Tbet (versus control mDC.Null) promoted the acute infiltration of NK cells and naïve $CD45RB^+$ T cells into the tumor microenvironment (TME) in association with elevated expression of NK- and T cell-recruiting chemokines by mDC.Tbet. When taken together, our data support a paradigm for extranodal (cross)priming of therapeutic Type-1 immunity in the TME after i.t. delivery of mDC.Tbet-based gene therapy.

Keywords

Dendritic Cell; Gene Therapy; T-bet; Sarcoma; Immunotherapy; Chemokines

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Dendritic cells (DC) are key antigen-presenting cells (APC) that serve as qualitative and quantitative rheostats for developing T cell responses in immune competent hosts (1–3). By carefully manipulating the conditions under which DC acquire, process and cross-present antigens, the resulting cognate T cell-mediated immunity may be modulated with regard to its magnitude, functional polarity and effector/memory status (4, 5). When taken in the context of intrinsic or therapeutically-induced immunity, DC can profoundly impact T cell-mediated protection versus pathogenesis in the setting of infectious disease, autoimmunity or cancer (6, 7). In the cancer setting, Type-1 $CD8^+$ T cell (aka Tc1) responses have been most commonly associated with endogenous host protection or therapeutic benefit to immunotherapy (8–11). The ability to generate Type-1-polarized immunity has in turn been shown to depend on intrinsic expression of the transactivator protein T-bet (aka TBX21) by T cell responders, but also perhaps more intriguingly, by DC (12).

We have recently reported that DC transduced to express high levels of ectopic T-bet (DC.Tbet) are superior activators of Type-1 $CD8^+$ T cells from naïve T cell precursors in human *in vitro* experiments (13), and that when injected directly into established CMS4 sarcoma lesions in Balb/c ($H\text{-}2^d$) mice, that protective immunity results (14). It remains unclear as to how DC.Tbet promote superior protective immunity, particularly *in vivo*. Using a s.c. MCA205 sarcoma model in C57BL/6 recipients and a range of informative syngenic ($H\text{-}2^b$) mutant strains of mice serving as sources of mDC.Tbet cell for injection or as model hosts, we observed that i.t. delivery of mDC.Tbet promotes the rapid recruitment and (cross)priming of polarized Type-1 NK and $CD8^+$ T cell-mediated immunity within the TME that protects against tumor progression. These events were associated with a differential chemokine profile produced by injected DC.Tbet versus control DC, with the subsequent Type-1 polarization of $CD8^+$ T effector cells proving to be independent of mDC.Tbet production of IL-12 family member cytokines or IFN-γ. Overall, our data support a model in which the injected mDC.Tbet serve as dominant drivers for the extranodal (cross)priming of therapeutic immunity within the TME.

MATERIALS AND METHODS

Mice

Female 6–8 week old wild-type C57BL/6 ($H\text{-}2^b$) mice, as well as, IL-12p35$^{-/-}$, IL-12p40$^{-/-}$, IFN-γ$^{-/-}$, β2M$^{-/-}$ and CCR7$^{-/-}$ mice (all on the B6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Female CD11c-DTR ($H\text{-}2^b$) conditional DC-deficient mice were kindly provided by Dr. Louis Falo (University of Pittsburgh). Female BATF3$^{-/-}$ mice deficient in cross-presenting DC were generated from 129-Batf3$^{-/-}$ mice kindly provided by Dr. Ken Murphy (Washington University-St. Louis) after backcrossing with C57BL/6 mice for 5 generations. Tbet-ZsGreen reporter mice were kindly provided under an MTA by Dr. Jinfang Zhu (NIH/NIAID) via the NIAID repository maintained at Taconic (Hudson, NY). All animals were handled under aseptic conditions per an Institutional Animal Care and Use Committee (IACUC)-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines and culture

The MCA205 sarcoma (H-2^b) cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA), was free of *Mycoplasma* contamination and was maintained in complete medium (CM: RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 10 mmol/L L-glutamine, all reagents were purchased from Invitrogen, Carlsbad, CA) at 5% CO_2 tension in a 37°C humidified incubator.

Generation of BM-derived DC and transduction with adenoviral vectors in vitro

DC were generated from the tibias/femurs of mice, and infected with recombinant adenovirus (either empty, control Ad.ψ5 to produce mDC.Null or Ad.mT-bet to produce mDC.Tbet) at an MOI of 250 for 48h, as previously described (14). Intracellular staining and flow cytometry was used to document expression of mTbet in Ad-infected DC (mDC.Tbet) as previously reported (14).

Therapy model

Recipient wild-type, mutant or transgenic (H-2^b) mice received s.c. injections of 5×10^5 MCA205 sarcoma cells in the right flank on day 0. On day 7 or 8 post-tumor inoculation as indicated, mice were randomized into treatment cohorts of 5 mice each exhibiting comparable mean tumor sizes (i.e. approximately 40 mm^2). Control DC (mDC.Null) or mDC.Tbet (10^6) developed from wild-type C57BL/6 or syngenic mutant mice were then injected i.t. in a total volume of 50 μl (in PBS) on days 7–8 post-tumor inoculation and again 1 week later. Mean tumor size (± SD) was then assessed every 3–4 days and recorded in mm^2 by determining the product of the largest orthogonal diameters measured by vernier calipers. Mice were sacrificed when tumors became ulcerated or if they reached a size of 400 mm^2, in accordance with IACUC guidelines.

In vivo depletion of $CD8^+$ T cells, NK cells and $CD11c^+$ DC

In selected experiments as indicated, mice were injected i.p. with 100 μg anti-CD8 mAb3-6.7 (ATCC) or 25 μl anti-asialoGM1 pAb (anti-asGM1; WAKO, Osaka, Japan) on days 6, 13 and 20 after tumor inoculation. In some experiments, anti-asGM1 antibody was administered on days 13 and 20 post-tumor inoculation. To deplete $CD11c^+$ DC from CD11c-DTR mice, diphtheria toxin (DT; Sigma-Aldrich, St. Louis, MO) was provided i.p. at a dose of 4 μg DT/kg beginning on day 6 post-tumor inoculation, as previously described (15). Specific cell depletion was > 95% effective *in vivo* based on flow cytometry analysis of peripheral blood monuclear cells obtained by tail venipuncture from treated mice 24–48h after Ab or DT administration (data not shown).

Evaluation of $CD8^+$ T-cell responses against MCA205 tumor cells ex vivo

For *in vitro* stimulation cultures, spleens were harvested from 2 mice per cohort at various indicated timepoints after the intratumoral injection of PBS, mDC.Null or mDC.Tbet. Splenic $CD8^+$ T cells (4×10^5) were isolated using specific magnetic bead cell sorting (MACS; Miltenyi Biotec, Auburn, CA), cultured in the absence or presence of irradiated (100 Gy) MCA205 cells (4×10^4 cells/well) for 2 days in 96-well flat bottom plates in a

humidified incubator at 37°C and 5% CO_2 Cell-free supernatants were then harvested and stored at −80°C prior to analysis using cytokine-specific OptEIA ELISA sets (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Triplicate determinations were used in all instances, with data reported as the mean ± SD.

Imaging of tumor tissues

Tumor samples were prepared and sectioned as previously reported (14). Briefly, tumor tissues were harvested and fixed in 2% paraformaldehyde (Sigma-Aldrich) at 4°C for 1h, then cryoprotected in 30% sucrose for 24 hours. Tumor tissues were then frozen in liquid nitrogen and 6 micron cryosections prepared. For analysis of T cell subsets, sections were first stained with purified rat anti-mouse CD8α or purified rat anti-mouse CD4 (both from BD-Pharmingen, San Diego, CA) mAbs for 1h. After washing, sections were stained with PE-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, West Grove, PA). To detect NK cells and naïve leukocytes, tissue sections were first stained with goat anti-mouse NKp46 antibody (R&D Systems, Minneapolis, MN), followed by Cy3-conjugated donkey anti-goat pAb (Invitrogen). To detect naïve leukocytes, tissue sections were stained with Cy5-conjugated rat anti-mouse CD45RB antibody (Abcam, Cambrideg, MA). Cell nuclei were then stained with DAPI as previously described (14). After washing, sections were then covered in Gelvatol (Monsanto, St. Louis, MO) and a coverslip applied. Slide images were acquired using an Olympus 500 scanning confocal microscope (Olympus America). The positively stained cells were quantified by analyzing the images at a final magnification of ×20. The number of cells in sections with a given fluorescence phenotype was quantitated using Metamorph Imaging software (Molecular Devices, Sunnyvale, CA).

RNA purification and PCR array analyses

Total RNA was isolated from mDC.Tbet and mDC.Null using Trizol reagents (Invitrogen). Total RNA was further purified using the RNeasy Plus Micro Kit (Qiagen) including the gDNA Eliminator spin column. The purity and quantity of the total RNA was assessed using Nanodrop ND-1000 (CelBio SpA, Milan, Italy). Total RNA (1 μg) was reversed transcribed into cDNA using the RT2 First Strand Kit (Qiagen) and the cDNA added to RT2 SYBR Green ROX™ qPCR Mastermix (Qiagen) and used for quantitative PCR using the RT2 Profiler PCR Array (96-well) for Mouse Chemokines and Receptors (Qiagen) all according to the manufacturer's instructions. Reactions were performed on a StepOnePlusTM Real-Time PCR thermocycler (Applied Biosystems) using the recommended cycling conditions. All mRNA expression levels were normalized to expression of control GAPDH mRNA.

Statistical analysis

Comparisons between groups were performed using a two-tailed Student's *t* test or one-way Analysis of Variance (ANOVA) with *post-hoc* analysis, as indicated. All data were analyzed using SigmaStat software, version 3.5 (Systat Software, USA). Differences with a p-value < 0.05 were considered as significant.

RESULTS

Therapeutic benefits of intratumoral delivery of mDC.Tbet are T and NK cell-dependent

To assess the requirement for both innate and adaptive immunity in a successful therapeutic response to i.t. mDC.Tbet-based treatment (provided on days 7 and 14 post-tumor s.c. MCA205 sarcoma inoculation), we employed wild-type C57BL/6 or syngenic RAG1$^{-/-}$ mice as hosts, without or with co-treatment of anti-asialoGM1 pAb or anti-CD8 mAb to depleted NK cells and CD8^{+} T cells, respectively. We observed that intratumoral delivery of mDC.Tbet, but not control mDC.Null slowed MCA205 tumor growth in wild-type C57Bl/6 mice (Fig. 1A) but not B and T cell-deficient RAG1$^{-/-}$ mice (Fig. 1B). Selective depletion of either CD8^{+} T cells or NK cells (beginning on day 6 post-tumor inoculation) also completely ablated protection against tumor growth afforded by intratumoral delivery of mDC.Tbet (Fig. 1C, 1D). Interestingly, even late depletion of NK cells (beginning on day 13 post-tumor inoculation) resulted in a blunting of anti-tumor protection suggesting the continued importance of NK function in the "booster" phase of mDC.Tbet-based i.t. therapy (Fig. 1D).

Intratumoral delivery of T-bet gene transduced DC (DC.mTbet) generated from wild-type or IL-12p35$^{-/-}$, IL-12p40$^{-/-}$ or IFN-γ $^{-/-}$ mice provide similar therapeutic benefit against MCA205 sarcomas

We have previously reported that human DC engineered to express ectopic Tbet (i.e. hDC.Tbet) promote superior Type-1 T cell polarization *in vitro* via a mechanism that is poorly antagonized by neutralizing anti-IL12 or anti-IFN-γ antibodies (13). However, in contrast to hDC.Tbet that are poor cytokine (including IL-12p70 and IFN-γ) secretors when compared with their untransfected counterparts (13), mDC.Tbet actually produce significantly more IL-12p70 than control DC (14) which could underlie their improved therapeutic potency in tumor-bearing mice. To definitively address the role of intrinsic IL-12p70 and IFN-γ production from mDC.Tbet in their therapeutic efficacy, we generated control DC (DC.Null) and DC.Tbet from the bone marrow of wild-type C57BL/6 mice or IL-12p35$^{-/-}$, IL-12p40$^{-/-}$ or IFN-γ$^{-/-}$ mice (on a C57BL/6 background), and injected these cells directly into s.c. MCA205 sarcomas that had been established for 7 days in C57BL/6 mice. An identical treatment was provided again one week later (i.e. on day 14 post-tumor injection). As shown in Fig. 2, untreated MCA205-bearing mice or tumor-bearing mice treated with control DC (regardless of their source) displayed indistinguishable progressive tumor growth. In contrast, therapies integrating i.t. delivery of wild-type DC.Tbet or DC.Tbet developed from wild-type C57BL/6 mice or IL-12p35$^{-/-}$, IL-12p40$^{-/-}$ (Fig. 2A), or IFN-γ$^{-/-}$ (Fig. 2B) mice resulted in similarly prolonged suppression of tumor growth. These data suggest that (optimal) therapeutic efficacy of this approach is not dependent upon intrinsic production of IL-12 family member cytokines (i.e. IL-12p70, IL-23 or IL-35; ref. 16) or IFN-γ by mDC.Tbet.

Intratumoral delivery of DC.Tbet generated from $\beta_2M^{-/-}$ mice provides an initial wild-type level of therapeutic protection that later becomes sub-optimal, which correlates with anti-tumor $CD8^+$ T cell responsiveness in treated mice

Our previous work suggested that the improved ability of DC.Tbet to elicit protective Type-1 $CD8^+$ T cell responses required the intimate contact or close proximity of these two cell populations during the antigen crosspresentation process (13, 14). To investigate the requirement of MHC class I/peptide-presentation by injected mDC.Tbet in the therapeutic benefits associated with this cellular therapy, we delivered control or mDC.Tbet generated from wild-type versus $\beta2M^{-/-}$ mice into s.c. MCA205 tumors on days 7 and 14 post-tumor inoculation and analyzed tumor growth and anti-tumor $CD8^+$ T cell function over time. We observed that treatment with mDC.Tbet developed from $\beta2M^{-/-}$ (i.e. mDC.Tbet ($\beta2M^{-/-}$)) or wild-type C57BL/6 (i.e. mDC.Tbet (WT)) mice provided a comparable degree of protection against tumor growth through day 16–19 post-tumor inoculation, at which time tumors in the mDC.Tbet ($\beta2M^{-/-}$)-treated cohort re-established accelerated growth kinetics versus tumors in mice treated with mDC.Tbet (WT) cells (Fig. 3A). An analysis of splenic $CD8^+$ T cells harvested from the treated animals on day 18 and 34 revealed that although T cell production of IFN-γ in response to *in vitro* stimulation with MCA205 tumor cells was elevated to a comparable degree in the mDC.Tbet (WT) and mDC.Tbet ($\beta2M^{-/-}$)-treated cohorts on day 18, only the mice receiving mDC.Tbet (WT) cells exhibited boosted anti-tumor Tc1 responses on day 34 of the study (Fig. 3B). Indeed, anti-MCA205 responses among $CD8^+$ T cells harvested from mice treated with mDC.Tbet ($\beta2M^{-/-}$) had dramatically eroded to essentially control levels by day 34. These data tentatively suggest that MHC class I expression by the injected mDC.Tbet may not be required for the initial induction of anti-tumor $CD8^+$ T cells (despite its expected requirement for direct crosspresentation of antigen to T cells by injected DC; ref. 17), but that it is likely needed for the sustained function and optimal therapeutic action of T effector cells in treated mice on or after day 16 of treatment.

To test this directly, we performed a “criss-cross” study design in which MCA205 tumor-bearing mice first received i.t. delivery of mDC.Tbet (WT) or mDC.Tbet ($\beta2M^{-/-}$) on day 7 post-tumor inoculation, followed by either population of mDC.Tbet cells one week later. Control therapy consisted of i.t. delivered DC.Null on both days 7 and 14. As depicted in Fig. 3C, all therapies using mDC.Tbet (from either WT or $\beta2M^{-/-}$ mice) exhibited indistinguishable anti-tumor protection benefits through day 17 post-MCA205 inoculation. Thereafter, the extended therapeutic efficacy was greatest in mice receiving 2 injections of mDC.Tbet (WT), followed by mice receiving mDC.Tbet (WT) on day 7 then mDC.Tbet ($\beta2M^{-/-}$) on day 14, followed by mice receiving mDC.Tbet ($\beta2M^{-/-}$) on day 7 regardless of which secondary treatment was applied. These results suggest that durability of protective immunity (≥ day 17) activated by mDC.Tbet-based treatment is determined by whether the injected mDC.Tbet express MHC class I at the time of priming (with the most robust responses involving day 7 delivery mDC.Tbet (WT) cells). Therapies initiated with mDC.Tbet ($\beta2M^{-/-}$) cells atrophy over time and are not salvaged by secondary treatment with DC.Tbet (WT) cells. Furthermore, therapy initiated with DC.Tbet (WT) cells deteriorates more quickly if DC.Tbet ($\beta2M^{-/-}$) cells rather than DC.Tbet (WT) cells are delivered on day 14 post-inoculation.

Therapeutic benefits provided by intratumoral delivery of mDC.Tbet is independent of host CD11c$^+$ and CD103$^+$CD11b$^-$ DC populations and does not require CCR7-dependent mDC.Tbet trafficking to secondary lymphoid tissues

Our therapeutic results using mDC.Tbet ($\beta 2M^{-/-}$) suggested the enhanced early priming of anti-MCA205 CD8$^+$ T cells in a system where the injected APC were conceptualy not competent in crosspriming capacity. This suggested a possible paradigm in which the injected mDC.Tbet might directly or indirectly (via NK cell cross-licensing, refs. 18, 19) activate host DC populations known to effectively crossprime T cells, such as CD8α^+CD11c$^+$ DC or CD103$^+$CD11b^{neg} DC (20–23), in the TME or tumor-draining lymph node. To address this issue, mDC.Tbet were injected into s.c. MCA205 tumors established in BATF3$^{-/-}$ mice (deficient in CD8α^+CD11c$^+$ and CD103$^+$CD11b^{neg} DC; ref. 23) as a therapy on days 7 and 14 post-tumor inoculation. In this model, mDC.Tbet-based treatment provided substantial and sustained anti-tumor protection (Fig. 4A) suggesting that these important host cross-priming DC populations are not critically required in the therapeutic response. Since additional host CD11c$^+$CD8α^{neg}DC populations might also participate in the therapeutic crosspriming of adaptive immunity in our model, we also delivered mDC.Tbet (WT) into MCA205 tumors established in CD11c-DTR mice to which DT could then be administered in order to selectively deplete CD11c$^+$ host DC *in vivo*. As shown in Fig. 4B, we observed that this maneuver had no detrimental impact on the protection of tumor-bearing mice treated with mDC.Tbet (WT), suggesting that host CD11c$^+$ DC did not play a dominant role in the anti-tumor benefits associated with this immunotherapy.

Expression of CCR7 by crosspresenting DC is required for their migration from peripheral tissue sites to secondary lymphoid organs in response to ligand chemokines, CCL19 and CCL21 (24–27). To address the requirement of i.t.-delivered mDC.Tbet to migrate to tumor-draining lymph nodes in order to crossprime therapeutic T cells that provide protection against tumor progression, we generated these APC from wild-type or CCR7$^{-/-}$ mice and injected them into MCA205 sarcomas established in wild-type C57BL/6 mice on days 7 and 14 post-tumor inoculation. We observed that intratumoral therapy incorporating mDC.Tbet (CCR7$^{-/-}$) provided a level of anti-tumor protection that was indistinguishable from that seen in MCA205-bearing mice treated with wild-type mDC.Tbet (Fig. 4C). This suggests that the therapeutic action mediated by i.t. administration of mDC.Tbet likely occurs principally in the TME, with minimal required involvement of secondary lymphoid tissues.

Intratumorally-delivered mDC.Tbet promote superior early recruitment/activation of Type-1 CD8$^+$ T cells and NK cells within the therapeutic TME in association with enhanced production of chemokines

Several recent publications indicate that the vast majority of DC delivered into a tumor lesion *in vivo* fail to migrate out of the tumor lesion (14, 28, 29), suggesting that their predominant therapeutic impact likely occurs within the TME. Although somewhat unconventional, the crosspriming of naïve, antigen-specific T cell responses can occur in extranodal tissue sites including bone marrow, skin, lungs and even tumors (27, 30–32). To evaluate whether early recruitment and activation of Type-1 T cell and NK cell responses were occurring in the MCA205 microenvironment, we established tumors s.c. in syngenic (H-2^b) Tbet-ZsGreen reporter mice that encode fluorescent ZsGreen protein driven off a

genomic Tbet/TBX21 promoter (33). Tumor-bearing mice were left untreated, or they were treated with i.t. delivered mDC.null or mDC.Tbet generated from wild-type C57BL/6 mice. Two days later, tumors were harvested and tissue sections evaluated by fluorescence microscopy for Type-1-polarized $CD4^+$ T cell, $CD8^+$ T cell and NK ($NKp46^+$) cell responses based on the conditional (ZsGreen) fluorescence of these lymphoid subsets (33). As shown in Fig. 5A/5B, within a 48h period of administering mDC.Tbet (versus control mDC.Null) into tumors, a dramatic increase in Type-1 ($ZsGreen^+$) NK cells, $CD4^+$ T cell and $CD8^+$ T cells was observed within the TME. Many of these T cell recruits appeared to represent "naïve" or newly-primed cell populations, since fluorescence microscopy revealed a preponderance of $CD45RB^+CD3^+$ TIL in mDC.Tbet (WT) but not control-treated mice (Fig. 5C; Table I), with 60% of $CD45RB^+CD3^+$ T cells coexpressing ZsGreen protein (Table I).

Since such acute recruitment and priming/activation of T cells and NK cells into/within the TME would be most simply explained based on chemokines elaborated from i.t.-delivered mDC.Tbet, we performed chemokine gene expression profiling of mDC.Tbet (WT) versus mDC.Null (WT). As shown in Fig. 6, mDC.Tbet intrinsically expressed elevated transcript levels (≥ 1.2 fold) for the chemokines CCL1, CCL4, CCL6, CCL8, CCL12, CCL17, CCL28, CXCL12 and CXCL15 when compared with control DC.

DISCUSSION

The major finding of this work is that DC engineered to express high levels of the Type-1 transactivator protein T-bet (aka TBX21) and injected into established MCA205 sarcomas promote therapeutic immunity via an unconventional mechanism in an unconventional location in tumor-bearing mice. Our data suggest that mDC.Tbet slow tumor growth via an immune-mediated mechanism involving the activation of effector $CD8^+$ T cells and NK cells, that the (acute) cross-priming of anti-tumor $CD8^+$ T cells does not qualitatively require intrinsic expression of MHC class I molecules on the cell surface of injected DC, nor does it involve the critical participation of host ($CD8\alpha^+CD11c^+$ or $CD103^+CD11b^{neg}$) DC populations classically known to promote the robust crosspriming of T cells. Furthermore, the injected DC need not have the intrinsic capacity to produce IL-12 family cytokines (i.e. IL-12, IL-23, IL-35) or IFN-γ, or to migrate to TuDLN based on responsiveness to CCR7 ligand chemokines. Interestingly, the injection of mDC.Tbet directly into the tumor lesion appears to support the rapid recruitment and activation of Type-1 ($Tbet^+$) T cells and NK cells within the TME, allowing for extranodal priming of protective immunity in this system. Our hypothetical model of this paradigm (Fig. 7) does not preclude additional (more conventional) crosspriming of protective $CD8^+$ T cells in TuDLN, however, the treatment-associated benefits of effector cells elicited in this manner appear modest in comparison with those promoted within the TME as a consequence of mDC.Tbet administration.

Naive T cells (including recent thymic emigrants; RTE) are believed to circulate through non-lymphoid tissues as part of their normal migratory pathway, and can also be recruited or retained in peripheral (non-nodal) sites based on locoregional production of chemokine ligands for CCR7 (i.e. CCL19, CCL21), CCR9 (i.e. CCL25) and CXCR4 (CXCL12/SDF-1α; 34–37). Under such (unconventional) conditions, extranodal priming of naïve T

cells has been reported to occur in a range of tissues including the bone marrow, liver, lungs, skin and even tumors (27, 30, 31, 36, 38, 39). Notably, the (cross)priming of protective T cells can be leveraged by the selective production of recruiting chemokines as a consequence of certain gene therapies applied to the TME (32, 40, 41). NK cells, which can mediate tumoricidal activity as well as the "licensing" of DC for improved T cell crosspriming capacity (42, 43), may also be recruited into extranodal tissue sites based on DC-produced chemokines such as CCL1/I-309, CCL2/MCP-1, CCL4/MIP-1, CCL5/RANTES, CCL7/TARC, CCL22/MDC, CXCL8/IL-8 or CXCL10/IP-10 (44–46). Also of significant interest, a recent report by Messina *et al.* (47) suggests that a 12 chemokine (i.e. CCL2-CCL5, CCL8, CCL18-CCL21, CXCL9-CXCL11 and CXCL13) gene signature may be associated with the presence of lymph node-like stuctures within the TME of advanced-stage melanomas and be predictive of patient responsivenss to immunotherapy and overall survival. In this regard, our transcriptional profiling of murine DC.Tbet suggest the differential ability of DC.Tbet versus control DC to produce NK (CCL1, CCL4, CCL6, CCL8, CXCL12) and naïve T (CCL4, CCL17, CXCL12) cell-recruiting chemokines (Fig. 6; refs. 35, 44, 48–53) in support of extranodal induction of protective immunity in the TME. Of these, CCL1 (which along with CCL17 promotes extended cognate interaction of naïve T cells with DC and consequent Type-1 T effector cell polarization; ref. 54) and CCL4 were also overexpressed at the transcript level by human DC.Tbet versus control human DC.Null (Table SI). Future experiments employing neutralizing antibodies or siRNA knock-down of these chemokines in injected DC.Tbet may allow us to determine the intrinsic importance of one or more of these soluble recruiting molecules in the anti-tumor efficacy of our DC-based gene therapy and to discern whether this treatment approach supports the establishment of lymph node-like structures in the TME over time in cases of stabilized disease.

Once recruited into the TME as a consequence of i.t.-delivered mDC.Tbet, optimal crosspriming of anti-tumor $CD8^+$ T cells is likely mediated predominantly by the injected mDC.Tbet that have acquired, processed and presented tumor-associated antigens in the MHC class I complexes *in situ*, based on results obtained in our studies employing therapeutic mDC.Tbet generated from $\beta2M^{-/-}$ mice. However, even though mDC.Tbet ($\beta2M^{-/-}$) support the induction of some degree of protective $CD8^+$ T cell-mediated immunity, the resultant anti-tumor protection associated with this immunity appears to be of a lower quality/durability than that developed in therapies using mDC.Tbet (WT). Such lower therapeutic efficiency/efficacy linked to administration of mDC.Tbet ($\beta2M^{-/-}$) could be the result of one or more limitations, including but not limited to; i.) the provision of suboptimal activation signals by mDC.Tbet ($\beta2M^{-/-}$) to cognate T cell responders based on the limited crosspresenting ability of these MHC class I-deficient APC; ii.) the modest ability of these APC to crossprime/boost protective $CD8^+$ T cells in secondary lymphoid organs; and/or iii.) premature demise or NK-mediated eradication of these MHC class-deficient APCs *in vivo* thereby limiting the functional duration of adaptive immune stimulation (55). With regard to the first point, it is conceivable that despite a genetic deficiency in β2M expression, that mDC.Tbet ($\beta2M^{-/-}$) may acquire a limited capacity to activate tumor-specific $CD8^+$ T cells in the TME via the pirating of tumor membrane components or the uptake of tumor-derived exosomes, both of which contain MHC class I/peptide complexes (56). Alternatively or additionally, soluble β2M (found in microgram/ml

quantities in serum; ref. 57) may be taken up along with tumor antigens by mDC.Tbet ($\beta 2M^{-/-}$), allowing for the stabilization of sufficient MHC I/tumor peptide complexes (58) on the injected DC cell surface to permit at least a limited degree of specific T cell induction in the TME. It is also conceivable that mDC.Tbet ± NK cells condition the TME to allow for the priming/activation of $CD8^+$ T cells by $CD11c^{neg}$ tumor-associated macrophages or even tumor cells themselves, although these would likely be considered as comparatively inefficient APC for this purpose (59).

That extranodal (cross)priming of T cell responses in peripheral tissues versus secondary lymphoid organs may yield a responding T cell repertoire that differs in overall magnitude or quality has been previously suggested in infectious disease models in $CCR7^{-/-}$ mice (27). When challenged with aerosolized live mycobacteria, $CCR7^{-/-}$ animals crossprime specific T cells in the lungs rather than the mediastinal lymph nodes (MDLN; ref. 27). The resulting immunity protects against only low doses of bacterial rechallenge, in contrast to wild-type mice vaccinated in a similar manner, in which case the animals withstand far greater doses of bacterial challenge (27). This paradigm could underlie the inability of anti-tumor T cells crossprimed by mDC.Tbet ($\beta 2M^{-/-}$) to regulate the growth of MCA205 tumors after they reach a certain size (i.e. tumor load), resulting in lethal, progressive disease. Our data also suggest that the inherent quality (and anti-tumor efficacy) of anti-tumor $CD8^+$ T cells is dominantly imprinted at the time of first i.t. delivery of mDC.Tbet, since a booster injection of mDC.Tbet (WT) did not dramatically reinforce the anti-tumor protection initiated by mDC.Tbet ($\beta 2M^{-/-}$). This interpretation is further supported by our findings that within 2 days of i.t. delivery of mDC.Tbet more than ½ of the enriched population of $CD45RB^+CD3^+$ TIL already expresses evidence of Type-1 polarization based on Zs-Green reporter protein expression (Fig. 5C, Table I).

The theoretical locoregional impact of “booster” mDC.Tbet delivered i.t. on day 14 post-tumor inoculation on protective $CD8^+$ T cells resulting from the initial administration of mDC.Tbet into the TME may be extrapolated from previous studies in which genetically-modified DC were injected i.t. in concert with the adoptive transfer of pre-activated tumor antigen-specific $CD8^+$ T cells (60–62). Thus DC engineered to produce IFN-α enhanced recruitment of i.v. administered Type-1 anti-tumor $CD8^+$ T cells in a CXCL10/IP-10 chemokine-dependent manner (60). Also in this light, several recent reports (61–63) are intriguing since i.t. delivery of DC loaded with tumor antigens (versus unloaded DC) was observed to maximally enhance the accumulation and anti-tumor efficacy of adoptively-transferred or vaccine-induced tumor antigen-specific $CD8^+$ T cells. This suggests that the crosspresentation of cognate antigen by injected mDC.Tbet (rather than or in addition to tumor cells themselves) to $CD8^+$ T cells in the TME may be critical for optimal and sustained anti-tumor T effector cell function *in vivo*. Booster i.t.-delivered mDC.Tbet would also be expected to intrinsically produce or promote the IFN-γ-dependent (from Type-1 T cells and NK cells) elaboration of CXCR3 ligand chemokines (14, 60), thereby reinforcing the recruitment and/or sequestration of protective anti-tumor $CD8^+$ T cells into/within the TME and extending therapeutic benefit.

When taken together, our data support the ability of i.t. delivered mDC.Tbet (WT) to recruit, prime and sustain superior Type-1 anti-tumor immunity within the TME. Intratumoral

delivery of hDC.Tbet would be predicted to have translational merit in the context of vaccines and as a co-therapy with adoptive cellular therapy in patients with accessible (i.e. injectable) forms of solid cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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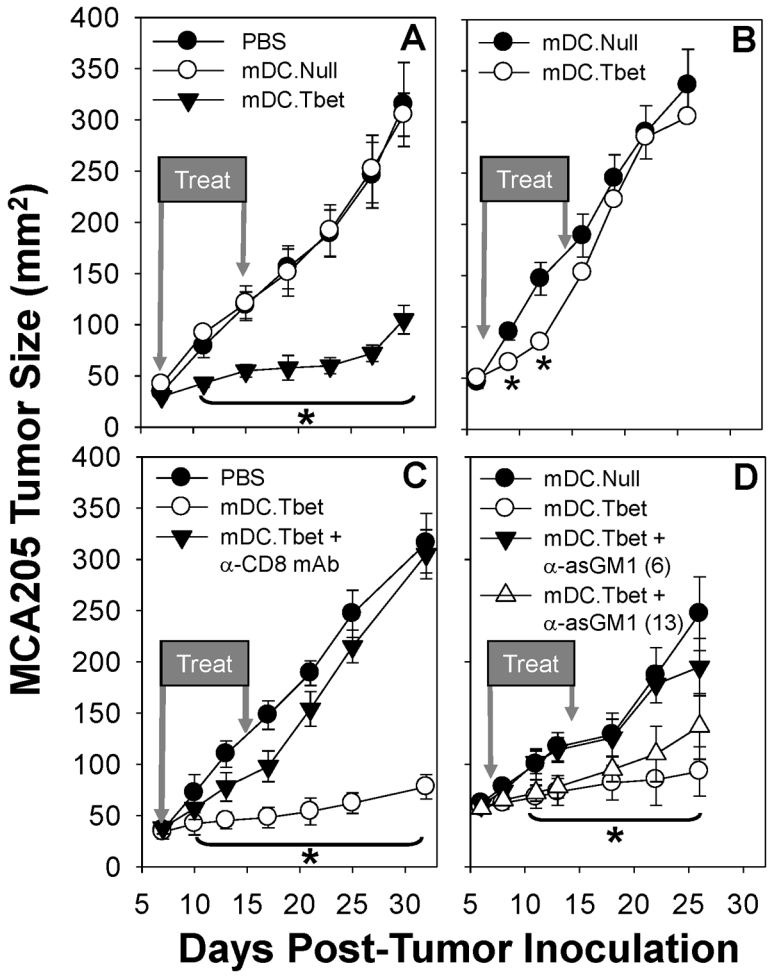

Figure 1. Intratumoral delivery of mDC.Tbet into established s.c. MCA205 sarcoma slows tumor growth via a mechanism involving innate and adaptive immunity

Control DC (mDC.Null) or mDC.Tbet were generated from the bone marrow of wild-type C57BL/6 mice as outlined in Materials and Methods. PBS or 10^6 mDC (as indicated) were then injected directly into s.c. MCA205 sarcomas established in syngenic wild-type (panel **A)** or RAG1$^{-/-}$ (panel **B**) mice on days 7 and 14 post-tumor inoculation. The experiment described in panel **A** was then repeated, with cohorts of mDC.Tbet-treated mice also receiving i.p. injections of depleting anti-CD8 (panel **C**) or anti-NK (i.e. anti-asGM1 on days (6), 13 and 20; or on days (13) and 20; panel **D**) antibodies. In all cases, tumor growth was monitored every 3–4 days and is reported in mm^2 (mean +/− SD of 5 animals/group). Data are representative of 3 independent experiments performed. *$p < 0.05$ versus PBS or mDC.Null on the indicated days of analysis [ANOVA].

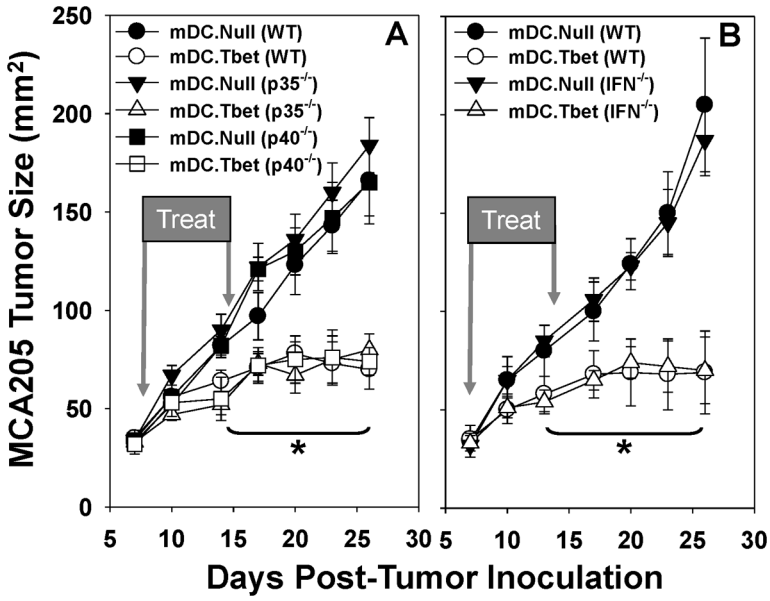

Figure 2. Delivery of mDC.Tbet into tumors mediates anti-tumor activity independent of the intrinsic capacity of the injected DCs to produce IL-12 family member cytokines or IFN-γ

Control mDC.Null or mDC.Tbet were generated from the bone marrow of C57BL/6 wild-type (WT) mice or from syngenic IL-12p35$^{-/-}$ (p35$^{-/-}$) or IL-12p40$^{-/-}$ (p40 $^{-/-}$) mice (panel **A**) or IFN-γ$^{-/-}$ (IFN$^{-/-}$) mice (panel **B**). The various DC (10^6) were then injected directly into s.c. MCA205 sarcomas established in wild-type C57BL/6 mice on days 7 and 14 post-tumor inoculation. Tumor growth was then monitored every 3–4 days and is reported in mm^2 (mean +/− SD of 5 animals/group). Data are representative of 3 independent experiments performed in each case. *$p < 0.05$ for mDC.Tbet (WT), mDC.Tbet (p35$^{-/-}$), mDC.Tbet (p40$^{-/-}$) and mDC.Tbet (IFN$^{-/-}$) versus control mDC.Null-treated animals on the indicated days of analysis [ANOVA].

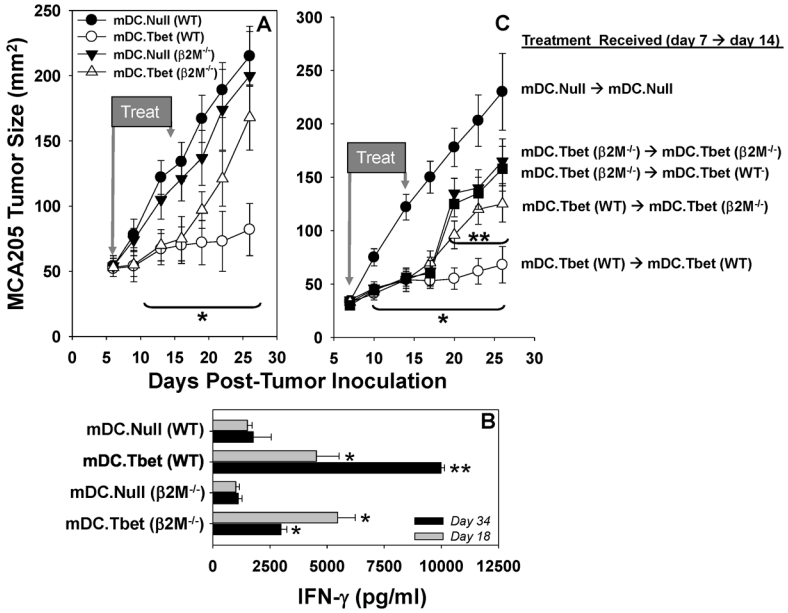

Figure 3. Intratumoral therapy with DC.Tbet developed from β2M$^{-/-}$ mice promotes a transient phase of anti-tumor benefit that ultimately fails, leading to the reestablishment of progressive tumor growth which cannot be "rescued" by booster injections of mDC.Tbet (WT)

A, Control mDC.Null or mDC.Tbet were generated from the bone marrow of wild-type C57BL/6 mice (WT) or syngenic (H-2^b) mice deficient in MHC class I expression (based on β_2m-deficiency; β2M$^{-/-}$) as outlined in Materials and Methods, and (10^6) of a given APC population injected directly into established s.c. MCA205 sarcomas in C57BL/6 mice on days 7 and 14 post-tumor inoculation. Tumor growth was then monitored every 3–4 days and the tumor size reported in mm^2 (mean +/− SD of 5 animals/group). In **B**, on day 18 or 34 post-tumor inoculation (i.e. 4 or 20 days after the second DC injection, respectively), CD8$^+$ splenocytes were (MACS) isolated for functional analysis. CD8$^+$ T cells were co-cultured in the absence or presence of irradiated MCA205 tumor cells at a 10:1 (T cell-to-tumor cell ratio) for 48 hours, at which time cell-free supernatants were analyzed for IFN-γ content by ELISA. Reported data have deducted values obtained for T cell only cultures. *p < 0.05 [t-test] versus mDC.Null (WT); **p < 0.05 versus all other cohorts [ANOVA]. In **C,** Fig. 3A study design was repeated, with the exception that the second i.t. injection of mDC.Tbet (day 14 post-tumor inoculation) was either mDC.Tbet (WT) or mDC.Tbet (β2M$^{-/-}$). Data are representative of 3 independent experiments performed. *p < 0.05 versus mDC.Null (WT) [t-test]; **p < 0.05 versus the DC.Tbet (WT) → DC.Tbet (WT) treated cohort [ANOVA]. Panel data are representative of 3 independent experiments performed.

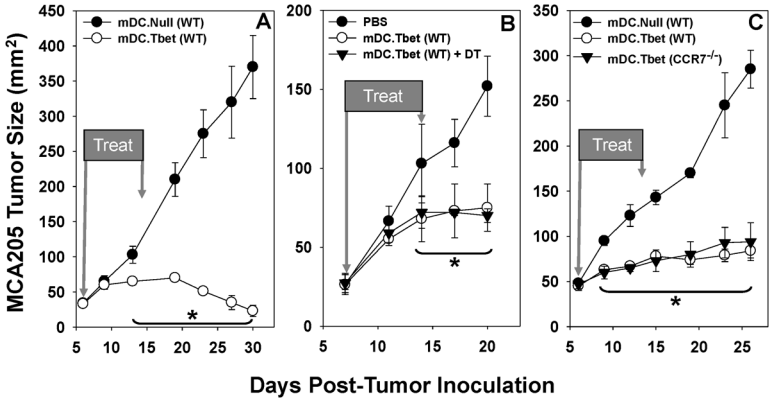

Figure 4. Host crosspresenting DC and the ability of i.t. delivered mDC.Tbet (WT) to traffick to TDLN are not required for the anti-tumor efficacy of this therapeutic approach

MCA205 tumors were established s.c. in the flanks of syngenic BATF3$^{-/-}$ (panel **A**) or CD11c-DTR (panel **B**) mice. On day 7 and 14 post-tumor inoculation, 10^6 mDC.Null (WT) or mDC.Tbet (WT) were injected i.t., with tumor growth monitored every 3–4 days and the tumor size reported in mm^2 (mean +/− SD of 5 animals/group). In **B**, host CD11c$^+$ DC were depleted in vivo by i.p. administration of DT as described in Materials and Methods. In **C**, established s.c. MCA205 tumors in wild-type C57BL/6 mice were treated with day 7 and day 14 i.t. injections of 10^6 mDC.Null or mDC.Tbet generated from either C57BL/6 WT mice or syngenic CCR7$^{-/-}$ mice and tumor size monitored longitudinally. Data are representative of 3 independent experiments performed. *$p < 0.05$ for mDC.Tbet (WT or CCR7$^{-/-}$) versus mDC.Null (WT) [ANOVA] at the indicated time points; Not significant difference (NS) in panel **B** for mDC.Tbet (WT) versus mDC.Tbet (WT) + DT or in panel **C** for mDC.Tbet (CCR7$^{-/-}$) versus mDC.Tbet (WT) [ANOVA]. Panel data are representative of 3 independent experiments performed.

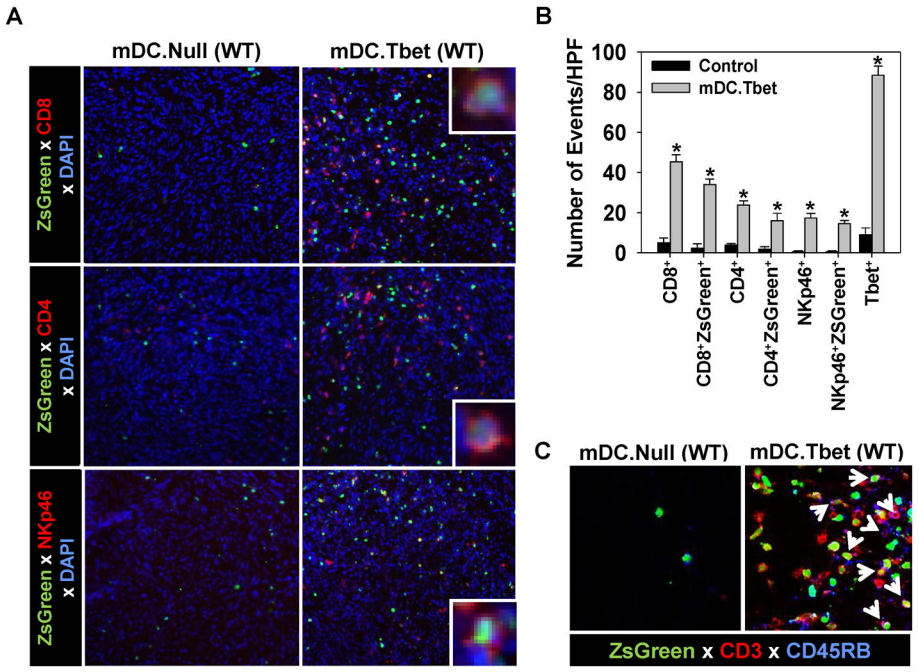

Figure 5. Early recruitment and activation of Type-1 T cells and NK cells in the TME after i.t. delivery of mDC.Tbet versus mDC.Null

In **A**, MCA205 tumors were established s.c. in the flanks of syngenic (H-2^b) Tbet-ZsGreen reporter mice. On day 7 post-tumor inoculation, 10^6 mDC.Null (WT) or mDC.Tbet (WT) were injected intratumorally. Two days later (i.e. days 9 post-tumor inoculation), animals were euthanized and tumor isolated for fluorescence microscopy analysis of infiltrating $CD4^+$ T cells, $CD8^+$ T cells and $NKp46^+$ NK cells as described in Materials and Methods. In situ activated Type-1 host cells express ZsGreen protein as a consequence of transcription driven off the mTbet promoter. In **B**, quantitation of events in Fig. 5A images was performed using Metamorph software and is reported as the mean ± SD of 10 high-power fields (HPF)/specimen. In **C**, tumor sections from Fig. 5A were analyzed by fluorescence confocal microscopy for the presence of "naïve" T cells based on co-expression of CD45RB (blue) and CD3 (red), with intrinsic Tbet-ZsGreen expression indicated in green. White arrows indicate $CD45RB^+CD3^+$ T cells (red/blue overlay yielding a fuschia pseudocolor). Quantitation of fluorescence images was performed using Metamorph software and is reported in Table 1. Panel data are representative of 3 independent experiments performed.

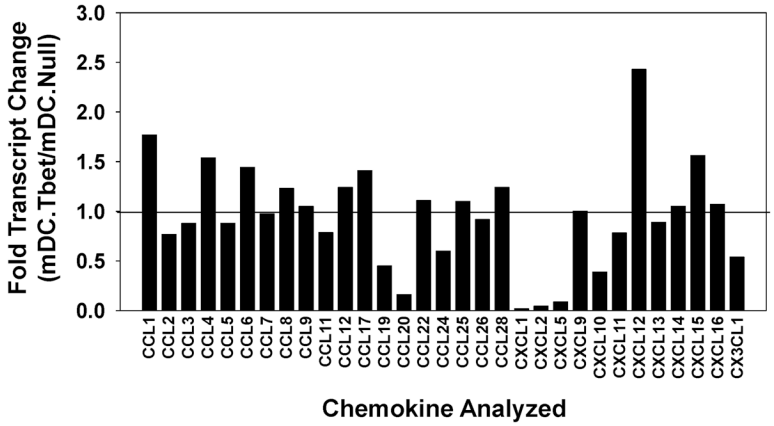

Figure 6. Differential expression of chemokine transcripts by mDC.Tbet versus control mDC.Null

mDC.Tbet (WT) and mDC.null (WT) were prepared as outlined in Materials and Methods and allowed to incubate for an additional 48h after infection with recombinant adenovirus. After extracting mRNA from both cell populations, chemokine/chemokine receptor transcripts were then analyzed using a commercial real-time RT-PCR array as described in Materials and Methods. The ratio of transcript levels for a given gene product among total tumor mRNA isolated from mDC.Tbet versus mDC.Null is reported.

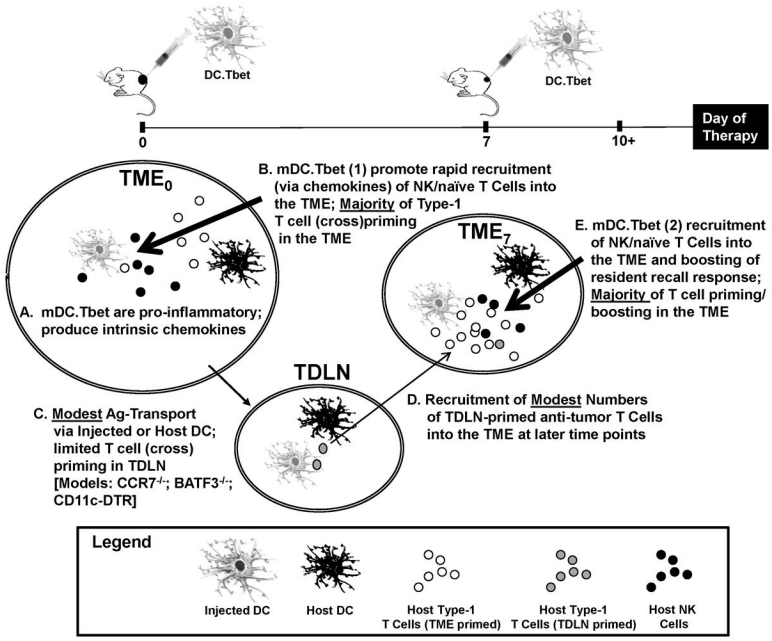

Figure 7. A hypothetical model for extranodal priming of therapeutic immunity in the TME after i.t. delivery of mDC.Tbet

In this model, i.t. delivery of mDC.Tbet leads to the acute recruitment of NK cells and naïve $CD8^+$ T cells, based on injected DC production of chemokines. Such recruitment into the TME fosters the crosspriming and polarization of anti-tumor $CD8^+$ T cells in a manner independent of IL-12 family member cytokine or IFN-γ production by the injected mDC.Tbet cells. Optimal induction of protective immunity requires MHC class I expression by the injected DC based on our observations for the inferior quality/durability of protective $CD8^+$ T cells developed in therapies using i.t. delivered DC.Tbet ($\beta 2M^{-/-}$). Host DC populations do not play dominant roles in the therapeutic benfits associated with i.t. delivery of mDC.Tbet. Activation of specific anti-tumor T cells in the TDLN is comparatively weak. The majority of extranodally-primed $CD8^+$ T cells remain in the TME and is boosted/ sustained by the second i.t. injection of mDC.Tbet, although some peripheral (TDLN, spleen) expansion of the anti-tumor $CD8^+$ T cell may also be therapeutically potentiated over time.

Table I

MCA205 tumors in mice treated with i.t. delivered mDC.Tbet contain abundant levels of $CD45RB^{+}CD3^{+}$ TIL.

Cell Phenotype	mDC.Tbet-Treated Number of Events/HPF	mDC.Null-Treated Number of Events/HPF
$CD3^{+}$	$64.3 \pm 9.8^{*}$	6.0 ± 1.6
$CD45RB^{+}CD3^{+}$	$40.0 \pm 5.4^{*}$	2.7 ± 0.9
$CD45RB^{NEG}CD3^{+}$	$24.3 \pm 10.1^{*}$	3.3 ± 0.5
$CD45RB^{+}CD3^{+}ZsGreen^{+}$	$25.0 \pm 6.4^{*}$	2.0 ± 0.8
$CD45RB^{+}CD3^{+}ZsGreen^{NEG}$	$15.0 \pm 3.4^{*}$	0.6 ± 0.5

Fluorescence confocal microscopy images obtained in Fig. 5C were analyzed using Metamorph software as described in Materials and Methods, with data as the mean ± SD of 10 HPF/specimen.

$^{*}p < 0.05$ for mDC.Tbet (WT) versus mDC.Null (WT) [t-test]. Data are representative of those obtained in 3 independent experiments performed.