IMMUNOTHERAPEUTIC ACTION OF T-BET GENE MODIFIED DENDRITIC CELLS

by

Lu Chen

B.S., Wuhan University, 2007

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This dissertation was presented

by

Lu Chen

It was defended on

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and approved by

Lisa H. Butterfield, Ph.D., Professor, Department of Medicine

Binfeng Lu, Ph.D., Associated Professor, Department of Immunology

Pawel Kalinski, MD, Ph.D., Professor, Department of Surgery

Charles R. Rinaldo Jr, Ph.D., Professor, Department of Pathology

Todd A. Reinhart, Sc.D., Professor, Department of Infectious Diseases & Microbiology

Dissertation Advisor: Walter J. Storkus, Ph.D., Professor, Departmental of Dermatology

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T-bet is a Type-1 transcription factor that regulates the development of Type-1 T cell and Type-1 anti-tumor immunity. T-bet expression in Dendritic Cells (DC) is required for the ability of these antigen-presenting cells (APC) to prime Type-1-polarized T cell responses. Since T-bet is typically expressed by very low frequencies of activated DC (< 1%), we hypothesized that ectopic expression of T-bet as a consequence of recombinant adenovirus (rAd).T-bet transfection would yield a robust population of DC that were capable of (re)polarizing Type-1 anti-tumor T cell responses *in vitro* and *in vivo*. Indeed, human DC engineered to express high levels of T-bet strongly potentiated the development of Type-1 T cells from naïve T cells and concomitantly suppressed Th2 and Regulatory T cell (Treg) development *in vitro*. Interestingly, the superior Type-1 functionality of DC.Tbet seems to be largely independent of intrinsic Interleukin-12 (IL-12) production, as IL-12 neutralizing antibody failed to affect the Type-1 T cell development driven by DC.Tbet. Furthermore, murine DC.Tbet displayed strong anti-tumor function when delivered into tumor sites as a cancer therapeutic modality. The therapeutic efficacy of mDC.Tbet requires the participation of host NK cells and $CD8^+$ T cells, whose induction was independent of the ability of injected mDC.Tbet to produce IL-12 family member cytokines or IFN-γ, or to migrate to tumor-draining lymph nodes (TDLN) based on CCR7 ligand chemokine recruitment. However, optimal therapeutic protection afforded by i.t. delivered mDC.Tbet did require that the injected DC express MHC class I molecules. Analysis of effectively treated

tumors revealed early recruitment/activation of NK cells and naïve T cells in the tumor microenvironment within 2 days of intratumoral delivery of DC.Tbet. Hence, my data support a model in which the injected mDC.Tbet serve as dominant drivers for the extranodal (cross)priming of therapeutic immunity within the tumor microenvironment (TME).

Gene array analyses performed on human DC.Tbet and control DCs (DC.ψ5) derived from 5 healthy donors revealed numerous differentially expressed genes between DC.Tbet and DC.ψ5 that might associate with the Type-1 polarizing function of DC.Tbet.

When taken together, my data suggest that DC.Tbet promote Type-1 anti-tumor immunity through multifaceted cellular and molecular mechanisms.

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PREFACE

I would like to first thank my mentor, Dr. Walter Storkus, for sharing his considerable scientific knowledge and being always so supportive and encouraging. Thanks to the previous and current members of the Storkus lab for sharing technical support, scientific ideas, encouragement, laughter and friendship.

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1.0 INTRODUCTION

The immune system constantly monitors for and eliminates cancerous/precancerous cells through the process of immunosurveillance, at the same time, tumor cells also develop concerted mechanisms to promote immune tolerance and to escape immunosurveillance [1]. The goal of cancer immunotherapy is to break a state of immune tolerance to tumor cells, thereby promoting their eradication *in vivo*. Successful anti-tumor immunity typically requires the generation of antigen-specific Type-1 CD8+ cytotoxic T cells (CTL/Tc1) that can directly kill cancerous cells, and CD4+ T helper 1 (Th1) cells that sustain CTL response(s) and memory CD8+ T cell expansion. The induction of anti-tumor T cell responses depends on the cross-presentation capability of dendritic cells which are the most potent antigen presenting cells (APCs) [2]. Ex vivo generated, antigen (Ag)-loaded DCs used in immunotherapies may enhance otherwise insufficient anti-tumor immunity in mice and humans. However, the limited success of DC-based cancer vaccines in the clinical setting suggests the necessity for further improvements in such therapies.

T-bet (TBX21), a T-box transcription factor family member, is a key regulator of the Type-1 immunity. It plays a central role in the development and maintenance of effector function of Th1 cells and CTLs [3, 4]. Intrinsic T-bet expression in dendritic cells is directly associated with the T cell priming function of DCs and is important for the development of anti-tumor Type-1 immunity driven by DCs. Based on these notions, DCs engineered to express high level

of T-bet (DC.Tbet) would be presumed to possess improved Type-1 polarizing function, with an enhanced potential when used in cancer immunotherapy approaches.

1.1 CANCER IMMUNOLOGY

The theory that the immune system scans for and eliminates nascent transformed cells that continuously arise in our body was first proposed by Ehrlich in 1909. Later it was recognized that the interplay between the immune system and cancer not only restricts tumor growth, but that it might also ultimately facilitate tumor progression by modifying the antigenic nature of cancer cells and by activating counter-regulatory immune mechanisms that allow a cohort of tumor cells to escape immunologic control [1]. The term immunoediting has been used to describe this dual host-protective and tumor-promoting action of immunity. There are three phases involved in the immunoediting process: elimination, equilibrium and escape (the three "E" s) [5]. Advancement in the understanding of the immunoediting process should improve the efficacy of immunotherapeutic strategies.

1.1.1 Immunosurveillance

In the mid-20th century, Burnet and Thomas postulated that nascent cells could be recognized and eliminated by host immune system through the process of immunosurveillance [1, 6]. This theory was contentious until 1990s when experimental gene knock-out mouse models became available for evaluating the role of immune cellular components in monitoring and eliminating tumor growth. The critical role of host lymphocytes in limiting tumor growth was first proved in

the $Rag2^{-/-}$ mouse model, in which $Rag2^{-/-}$ mice defective in mature immune components T, B and NKT cells displayed an increased carcinogen-induced tumor incidence [2]. NK cells were also shown to restrict tumor growth, as depletion of these cells in C57BL/6 mice using either anti-NK1.1 (depletes both NK and NKT cells) or anti-asioGM1 (depletes NK cells and activated macrophages) antibodies rendered these mice 2-3 times more susceptible to spontaneous and chemically-induced tumor formation when compared to control wild-type mice [7, 8]. Accelerated tumor growth was observed in mouse models defective in mature immune effector mechanisms such as interferon-γ (IFN-γ) signaling ($IFN\gamma R^{-/-}$mice) and perforin ($pfp^{-/-}$ mice), suggesting important roles of these soluble immune factors in controlling tumor growth [9, 10]. Furthermore, the elevated incidence of non-oncoviral cancers in immunosuppressed transplant recipients when compared to the general population supported the important role of host immunity in tumor monitoring in human [11]. Such data suggest that immune system actively monitors and eliminates tumor development and progression via a range of effector cells and pro-inflammatory factors.

1.1.2 Immune suppression and escape

Immunosurveillance is not always successful as tumor develops counter-regulatory mechanisms to suppress protective immunity and evade immunosurveillance. First, the loss of surface antigen expression on tumor cells renders them invisible to CTLs. This occurs partially through the down-regulation of HLA class I expression, which is noted in many tumors including melanoma, colorectal carcinoma and breast carcinoma [12, 13]. The mechanism underlying the loss of HLA class I expression includes dysregulation in antigen processing and presentation machinery (i.e. β2-microglobulin and TAP1, TAP2) which have been reported in progressor tumors [14, 15].

Second, defective death receptor signaling and lack of costimulation on tumor cells surface contribute to the survival of tumor cells. Two critical death receptors that control tumor development are Fas ligand and TRAIL. Fas ligand has cytoplasmic death domains which transmit apoptotic signals through caspase signaling, the expression of one caspase inhibitor (cFLIP) is believed to enhance the resistance to death receptor-mediated apoptosis in tumor cells [16]. Lack of costimulation molecules on tumor cell surface may also assist with tumor evasion as it could lead to T cell anergy and suboptimal activation of NK cells [17, 18]. Third, tumor cells produce immunosuppressive cytokines and chemokines to counter-regulate the function of immune effector cells and support the recruitment/development of immune suppressive cells such as Tregs and Myeloid Derived Suppressor Cells (MDSCs). Vascular endothelial growth factor (VEGF), for example, is over-expressed in many tumors and it supports tumor angiogenesis and mestastasis while suppresses DC maturation [19, 20]. Another inhibitory cytokine, IL-10, is also frequently increased in the serum of cancer patients, IL-10 suppresses the differentiation of DC from stem cell precursors and inhibits antigen presentation and the development of Type-1 T helper cell response *in vivo* [21, 22]. Similarly, high concentrations of TGF-β, Prostaglandin E2 (PGE2) are also found in cancer patients and are associated with disease progression [23] [24].

1.1.3 Cancer immunotherapy strategies

Advancement in our understanding of tumor immunology facilitated the design of novel immunotherapies. One promising strategy is the vaccine-based therapy that aimed at boosting host anti-tumor immune responses by enhancing the tumor associated antigen (TAA) presentation in tumor microenvironment (TME). Various modalities/adjuvants were explored in

this strategy, such as DC pulsed with TAA, DC fused to tumor cells, gene modified tumor cells and DNA based therapy using plasmid or viral vector. Systemic anti-tumor T cell responses including some complete responses were reported in several DC-based vaccine trials [25]. Another promising treatment strategy is the adoptive cell-based therapy, in which autologous lymphocytes were isolated from patients, expanded and modified ex vivo and then transferred back into patients to kill tumor cells. Recently, the chimeric antigen receptor (CAR) engineered T cell adoptive transfer strategy achieved great success in acute lymphoid leukemia (ALL) patients, with complete remissions observed in patients who had previously relapsed with aggressive B-cell malignancies [26]. Besides, recombinant cytokines and monoclonal antibodies were also used in cancer immunotherapy, with the intent to directly inhibit tumor growth and/or to indirectly restrict tumor growth through the action of these factors on angiogenesis and anti-tumor immune function. Many cytokines such as GM-CSF, IL-2, IL-7, IL-12, IL-15, IL-18, IL-21 and IFN-α were explored for their potential to treat patients in clinical trials [27]. For instance, IL-2 has been firmly established as an effective treatment for advanced-stage melanoma/renal cell carcinoma [28], as is also the case for IFN-α in the treatment of chronic myeloid leukemia [29]. In addition, monoclonal antibodies have been designed to directly target tumor associated antigens/inhibitory costimulatory molecules in order to enhance anti-tumor immune responses [30]. For instance, the monoclonal antibody (BMS-936558) which blocks inhibitory PD-1 signaling (thereby enhancing anti-tumor T effector cell survival) and trastuzumab which binds to extracellular domain of HER2 and inhibits the survival of HER2-dependent breast cancer cells have each yielded objective responses in a fraction of treated patients [31, 32].

1.2 DENDRITIC CELLS AS CANCER IMMUNOTHERAPEUTIC AGENTS

DCs are the most potent antigen presenting cells that play a key role in the initiation and modulation of immune responses [33]. Upon encountering foreign antigens or antigens released from tissue damages, DCs capture and process Ags into peptides that could bind to MHC class I or MHC class II molecules, and subsequently migrate to the lymphoid organs, where they present MHC-peptide molecules to $CD8^+$ or $CD4^+$ T-helper cells. Ag-loaded DCs provide 3 signals to T cells via: 1) engagement of MHC-peptide complex with appropriate TCR; 2) interaction of costimulatory and accessory molecules that stabilize peptide MHC/TCR interactions at the immune synapse and sustain enhanced T cell proliferation and maturation; and 3) secretion of the polarizing cytokines which determine the differentiation direction of the T cell (Type-1, Type-2, Th3 or Th17, Treg) [34, 35]. In the cancer setting, DCs orchestrate innate and adaptive anti-tumor immune responses. DCs promote tumor cell apoptosis through enhancement of cytotoxic functions mediated by NK cells, NKT cells and DCs [36, 37]. Locoregionally-released tumor-associated Ags may be captured by DCs and (cross) presented to TAA-specific T cells [38]. Effective homing of TAA-specific T cells into the tumor site and cognate recognition of tumor cells by specific effector cells may lead to the elimination of tumor cells *in vivo* [39]. It is important to keep in mind that DCs exhibit considerable plasticity in their ability to skew Th responses in the tumor microenvironment. For example, DCs that normally inducing Th1 profiles can be subverted to Th2-skewing DCs when treated with anti-inflammatory cytokines such as IL-10 and TGF-β or with PGE2 [21, 40], which are commonly found in abundance in the TME. Hence, host DC may be subverted by the TME to be ineffective promoters of protective immunity.

The unique ability of DCs to induce and sustain primary immune responses makes them prime candidates for vaccination protocols in the cancer setting [41]. DC-based cancer vaccines are one of the most attractive anti-tumor immunotherapies that aim to induce both therapeutic and protective T cell immunity (tumor specific memory T cells that can control tumor relapse) in cancer patients. The efficacy of DC-based cancer vaccine depends on numerous DC- or host-related factors, including subsets, the maturation and activation status, and migratory potential of applied DCs, Ag dose loaded onto DC, vaccination schedule, route of injection and the disease stage of the patients, among others [36, 41]. DCs capable of eliciting effective anti-tumor Type-1 immunity are considered as desirable candidates for cancer vaccination [42]. Clinical trials using DC-based cancer vaccine have now been conducted in a broad range of cancer types, including melanoma, lymphoma, myeloma, prostate and renal cell cancer [41]. All published trials have demonstrated the safety of such approaches. In clinical trials using monocyte-derived, peptide-pulsed DCs fully-matured under rigorous quality control parameters (GM-CSF/IL-4 followed by IL-1β, TNF-α, PGE2, IL-6), two groups have reported clinical and immunological responses in melanoma or renal cancer patients , especially in those patients with a low tumor burden [43, 44]. Overall, tumor regression has only occurred in a very minor subpopulation of treated patients, suggesting the necessity to further optimize DC-based therapies in order to maximize the prophylactic or therapeutic anti-tumor immunity.

1.3 T-BET AND ITS MULTIFACETED ROLE IN PROTECTIVE TYPE-1 IMMUNITY

T-bet (T-box expressed in T cells, Tbx21) is a T-box family transcription factor that regulates Type-1 immunity [45]. It controls T helper 1(Th1) lineage commitment, and the development and/or maintenance of effector cell fates in T, B, DCs and NK cells [46]. T-bet controls the production of IFN-γ and regulates the expression of chemokines and chemokine receptors which controls the recruitment/trafficking of Th1 cells.

1.3.1 T-bet in T lymphocytes

T-bet was first identified by the Glimcher group as a Th1-specific transcription factor [45]. It controls IFN-γ, IL12Rβ and CXCR3 expression in Th1 cells and directs Th1 commitment [47-49]. Retroviral transduction of T-bet into polarized Th2 or Tc2 (CD8 T cells that producing IL-4) cells redirected these cells to become Th1/Tc1 cells by inducing IFN-γ production while repressing IL-4, IL-5 production in these cells [45]. Mice lacking T-bet (T-bet$^{-/-}$) failed to develop Th1 cells and displayed a dramatic reduction of IFN-γ production by CD4^{+} T cells and NK cells, with an increase in Th2 responses *in vivo* in association with airway hyperactivity [4]. Such data support the dominant and non-redundant role of T-bet in the development/commitment of Th1-polarized T cells.

In CD8 T cells, T-bet plays a important role in the development and function of the effector CD8^{+} T cell (CTL) by controlling the production of IFN-γ, perforin and granzyme of CTLs [50-52]. However in contrast to its role in CD4^{+} T cells, T-bet, together with another T-box transcription factor Eomes, function in a cooperative and partially redundant way to regulate

effector gene expression in $CD8^+$ T cells. It is reported in several studies that a substantial portion of $CD8^+$ T effector function was preserved in the absence of T-bet [52-54], while mice with compound mutations in the gene encoding Eomes and T-bet displayed additive defects in effector gene expression by $CD8^+$ T cells and NK cells. Further studies suggested that T-bet and Eomes were closely associated with the expression of CD122 (the receptor specifying IL-15 responsiveness) which was required to drive proliferative renewal of memory $CD8^+$ T cells in the absence of pathogen stimulation/attack [55-58]. Therefore, in $CD8^+$ T cells T-bet collaborate with Eomes to control the effector CTL responses and long-term renewal of memory $CD8^+$ T cells to their effector potency.

1.3.2 T-bet in dendritic cells

T-bet is expressed in human and mouse DCs. In human DCs, T-bet can be induced through an ERK-dependent pathway [59]. In unstimulated, murine splenic DCs, T-bet is expressed at low constitutive levels. However, upon stimulation with IFN-γ, T-bet expression is rapidly elevated (and comparable to level found in Th1 cells [4]). T-bet is required for IFN-γ production from DCs, but is not essential for the development or migration of DCs. T-bet also regulates the T cell priming/polarizing function of DCs, as T-$bet^{-/-}$ dendritic cells fail to activate Th1 programming in adoptively transferred antigen-specific T cells [4]. Wang et al. reported the essential role of T-bet in inflammatory arthritis (CAIA model in mice), and T-$bet^{-/-}$ mice were largely resistant to the development of inflammatory arthritis [60]. They also observed that T-$bet^{-/-}$ DCs were defective in activating T cells, as adoptively transfer of KLH-pulsed T-$bet^{-/-}$ DCs failed to prime Ag-specific T cells *in vivo*. In the same study, T-bet was found to control the production of several proinflammatory cytokines and chemokines by DC (such as IL-1α, macrophage

inflammatory protein-1α (MIP-1α) and thymus- and activation-related chemokine (TARC)) after stimulation with LPS [60]. These studies support an important role for T-bet in DCs, by linking both innate and adaptive immunity in order to effectively promote Type-1 immunity *in vivo*.

1.3.3 T-bet in other immune cells

T-bet has been implicated in controlling the maturation and effector function of NK cells. T-bet$^{-/-}$ NK cells do not fully mature and are sensitive to apoptosis. T-bet deficiency also results in reduced NK cell numbers and diminished egress of NK cells from the bone marrow and lymph nodes into the blood circulation [61]. As in the case of CD8^{+} T cells, T-bet acts together with Eomes in a spatial-temporal manner to control NK cell development, with T-bet stabilizing the immature TRAIL^{+} NK pool, and Eomes supporting the maturation of TRAIL^{+} NK cells into mature DX5^{+} NK cells while T-bet and Eomes are both required for the later stages of NK maturation into DX5^{+}CD11b^{+} NK cells [61]. With regard to NK effector function, T-bet controls Granzyme B production in NK cells, while Eomes upregulates IFN-γ and Granzyme C expression in NK cells. T-bet expression in NK cells is associated with the enhanced ability of these effector cells to inhibit cancer metastasis (due at least in part to the enhanced longevity and functionality of NK cells), and T-bet$^{-/-}$ mice are susceptible to melanoma development, potentially due to impaired NK-driven innate immune responses which serve as a prelude to the development of an effective adaptive anti-tumor immune response [62]. In B cells, T-bet appears to be required to control Type-1-polarized effector cell fate [63]. These data suggest that T-bet exerts functional immunomodulatory pressure within a broad range of immune cell lineages.

1.4 TYPE-1 IMMUNITY AND TYPE-1 T CELL DEVELOPMENT

1.4.1 The critical role of Type-1 immunity in cancer immunology

Type-1 innate and adaptive immunity are the major components of host anti-tumor protective responses. The main effector cells shown to mediate anti-tumor function are Type-1 $CD8^+$ cells (CTLs) and $CD4^+$ helper (Th1) cells. Tc1 cells are IFN-γ secreting $CD8^+$ T cells that directly kill tumor cells [33, 64]. Development of Tc1 cells requires assistance from Th1 cells, which activate (i.e. licensing) APC and enhance the expression of both MHC and costimulatory molecules on APC to cross-prime $CD8^+$ T cell responses. Th1 also may directly activate CTLs by secreting IFN-γ and IL-2 to support CTL function and the secondary expansion of memory $CD8^+$ T cells [Ref]. Type-1 DC produce high levels of IL-12p70 and efficiently promotes Th1 and Tc1 development from naïve T cells, hence the therapeutic upreulation of IL-12 production by DC *in vivo* represents a preferred clinical endpoint of many cancer immunotherapies [42, 65-68]. Besides the adaptive Type-1 immune effectors, Type-1 macrophages (M1) have also been shown to be proinflammatory in nature, allowing for the enhanced priming of Type-1 anti-tumor T cell responses [69]. In conclusion, both Type-1 innate and adaptive immunity play critical roles in preventing and eliminating tumors *in vivo*.

1.4.2 Type-1 T cell development

The differentiation of naïve CD4 T cells starts when TCR encounters MHC II-peptide complex presented on an APC. Upon TCR engagement, 3 major signals direct T helper (Th) differentiation: the TCR signal (strength and duration), signals from ligation of co-stimulatory

molecules and from local cytokine environment [70]. Among these signals, the cytokine milieu is considered as the most potent factor that determines Th cell fate, with IL-12p70 inducing Th1 lineage commitment and IL-4 directing Th2 lineage development. Th1 lineage is characterized by the production of proinflammatory cytokine IFN-γ and is known to control intracellular infection or tumor growth; while Th2 lineage produce cytokines IL-4, IL-5 and IL-23, and eliminates parasitic infections [71], [72].

T-bet expression in Th1 cells is induced by TCR signaling and strongly elevated by the IFN-γR/STAT1 signal transduction pathways [73, 74]. T-bet transactivates IFN-γ gene transcription, upregulates the expression of IL-12Rβ on newly-activated T cells and stabilizes its own expression through the autocrine effect of IFN-γ in an autocatalytic loop [47],[75]. Concomitantly, T-bet suppresses Th2 cell commitment by repressing the expression of IL-4 and GATA-3 which controls Th2 cell fate [76, 77]. Other transcription factors that are involved in Th1 development include: Runx3 which together with T-bet binds to enhancers and the promoters of IFN-γ gene thereby promoting IFN-γ expression; Hlx, a Th1-specific homeobox gene that appears to interact with T-bet to enhance the transcription of a group of T-bet regulated genes [77]; also T-bet interacts with Bcl-6 to repress genes that are critical in opposing Th cell fates [78, 79]. Hence, T-bet cooperates with other transcription factors to fortify Th1 cell fate.

Historically, IL-12 has been considered the most potent Th1 initiating cytokine, and one that is indispensable for Th1 development, however, more recent studies suggest that IL-12 is not essential at least under certain stage/circumstances of Th1 differentiation, and there are other cytokines such as IFN-γ, IL-23, IL-27, IL-18 that may promote Th1 differentiation. As described by Schulz et al., there are at least two sequential phases of T-bet expression and activation of IFN-γ gene transcription during Th1 development [80]. In the first phase, TCR

signaling collaborates with IFN-γ signaling to induce T-bet expression while TCR signaling restricts IL-12Rβ expression on the T cell surface; in the second phase, diminished TCR engagement derepresses IL-12Rβ which increases the responsiveness of T cells to IL-12. IL-12 at this stage is the principle driver for T-bet expression and reinforces Th1 commitment. These data suggest that IL-12 is dispensable at least in initiation phase of Th1 development and it serves more to stabilize the Th1 cell fate than initiating Th1 differentiation.

Th1 lineage commitment is also controlled by epigenetic mechanisms. For example, T-bet recruits chromatin remodeling complexes including Jmjd3 (H3K27-demethylase) to target the removal of the repressive H3K27me3 modification in order to activate Th1 signature genes [81]. These complexes help to establish a more permissive chromatin state which retains the capacity of opposing Th lineages to re-express T-bet target genes. The epigenetic regulation of Th1 gene expression leads to the flexibility of Th cells that are amenable to repolarization signals as opposed to terminally polarized T cell subsets [80, 82, 83].

1.5 THE DEVELOPMENT AND FUNCTION OF TERTIARY LYMPHOID ORGAN

In the classical model of peripheral T cell activation, tissue resident DCs capture antigens (such as foreign pathogens, tumor cell debris, etc.) in an inflammatory microenvironment, leading to the migration of antigen-laden $CCR7^+$ DCs to regional draining lymph nodes [LN; aka secondary lymphoid organs (SLO)], where activation of cognate T cells occurs [84, 85]. After appropriate proliferative expansion and maturation, T effector cells may then enter the blood circulation and be recruited into tissue sites where they are competent to recognize and react against relevant APCs, such as virally infected host cells or tumor cells [86]. Recent evidence obtained in a range

of translational and clinical models suggests, however, that this classical/conventional paradigm may be operationally overly simplistic, and that extranodal (cross)priming of antigen-specific T cells can occur in peripheral tissues, often times in conditionally established tertiary lymphoid organs (TLO) [87-89].

1.5.1 The Role of Tertiary Lymphoid Organ (TLO) in diseases

While classical SLO are encapsulated structures that develop in predictable locations as a consequence of normal immune system development, under pathologic conditions, ectopic lymphoid tissues (aka TLO) may develop in peripheral tissue sites of chronic inflammation. Similar to SLO, TLOs have typical lymphoid features including segregated B cell follicals and T cell enriched areas, HEV, germinal center and follicular DCs [90]. TLOs in inflammation sites are believed to promote immune responses against self-protein (autoimmune diseases) or neoantigens (cancer) through concentrating antigens and lymphocytes, and thus increasing the efficiency of interactions between antigen-specific lymphocytes and antigen presenting cells locally in the inflammation site. TLO formation has been reported within inflamed organs of patients with rheumatoid arthritis [91], psoriatic arthritis [92], autoimmune gastritis [AIG; Ref. [93]], juvenile dermatomyositis [94], and Sjögren's syndrome [95], among others. TLO formation has also been identified in the lungs of influenza virus-infected mice [96], the livers of hepatitis C virus (HCV)-infected patients [97] and in the stomachs of patients infected with *Helicobacter pylori* [98]. "Dysfunctional" human lung allografts exhibiting chronic inflammatory responses have also been found to commonly contain TLO [99].

Furthermore, a burgeoning literature supports tumor associated TLO as important sites of extranodal T cell priming and epitope spreading in the responder T cell repertoire [90, 100]. TLO have been identified in a subset of human melanoma lesions, in which mature DC were found to maintain intimate contact with recruited T cell populations, consistent with the notion of operational extranodal (cross)priming within the tumor microenvironment (TME) [101, 102]. Similar results have been reported for murine melanoma models [88, 103]. In line with this model, naïve lymphocytes have been identified in TLO within pulmonary lesions of patients with lung cancer, making it likely that these immune cells encountered their cognate antigen for the first time and developed into antigen-specific T effector cells within the TME *in vivo* [104, 105]. TLO featuring DC/Type-1 T cell clusters proximal to B cell "nests" have been identified in human non-small cell lung cancer specimens [106]. In such instances, the density of mature DC found in TLO appeared to be associated with improved long term survival [106, 107]. Furthermore, Mulé and colleagues have recently performed a metagene analysis on human (Stage IV, non-locoregional) melanoma metastases and identified a 12-chemokine gene signature (i.e., CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, CXCL13) correlating with the presence of TLO (containing $CD20^+$ B cell follicles with prominent areas of $CD4^+$and $CD8^+$ T cells, but not $FoxP3^+$ Treg cells), with better overall survival noted in the TLO^+ subset of patients [101].

1.5.2 The developmental formation of SLO and TLO

The developmental formation of SLO is believed to require the interaction of so-called lymphoid tissue inducer cells [14] bearing a CD3-CD4+CD45+IL-7R+c-Kit+ phenotype that produce lymphotoxin α/β [LT α/β; Ref. [108, 109]] with LTβR+ stromal "organizer" cell populations that

may derive from adipocyte precursors [110], leading to corollary stromal cell elaboration of the SLO homeostatic chemokines CCL19, CCL21, and CXCL13 [87, 90, 103, 111, 112]. These chemokines sustain recruitment of LTi and other lymphocytes into SLO, resulting in the development of a mature lymphoid organ architecture [i.e., based on the formation of follicular structures containing B cells and surrounding "cortical" zones that are diffusely populated by $CD4^+$ and $CD8^+$ T cells, antigen-presenting cells (including CD11c+ DC), and $PNAd^+$ high-endothelial venules (HEV] [99, 103, 105] .

The conditional formation of TLO in peripheral tissues appears to require the collaboration of a similar cast of cellular participants, soluble mediators, and signaling pathways associated with the orchestration of SLO development [111, 112]. Ectopic delivery of LTα/β or LIGHT (aka TNFSF-14 or CD258) promotes $PNAd^+$ HEV, CCL19/CCL21 production, massive naïve T cell infiltration, and (tumor-specific) cross-priming in the context of TLO structures [87, 96, 109, 113-115]. Consistent with these findings noted for pro-TLO immunobiology of LTβR ligands, blockade of the LTβR precludes formation of TLO *in vivo* [116]. Unlike SLO organogenesis, the induction of TLOs doesn't seem to require LTi [117]; instead, the interaction between mature $CD3^+CD4^+$ T cells and dendritic cells are believed to trigger the development of TLOs [118], [119].

1.5.3 Therapeutic Promotion of TLO

The potential role of TLOs in increasing local anti-tumor immune responses prompted immunotherapy strategies aimed at enhancing TLO development in TME to improve the clinical outcome. Perhaps the most strategically simple means to apply this paradigm in the cancer setting reflects the implantation of SLO/TLO directly into the TME. Recently, scaffold-based

lymphoid tissue engineering has been developed as a means to transplant “intact” TLO directly into tumors in order to affect clinical benefit [120]. A previously mentioned alternative to this strategy is clearly the delivery of the LTβR ligands (LTα, LTβ, or LIGHT), agonist anti-LTβR antibodies or downstream TLO-associated chemokines (CCL19, CCL21, CXCL13) via protein-based or genetic therapy in order to instigate the locoregional development of TLO in the TME leading to inhibition of tumor growth *in vivo* and extended overall survival [87, 103, 114, 121, 122].

It also appears that the administration of appropriately activated/engineered DC is sufficient to nucleate and/or maintain the development of TLO *in vivo* [97, 123]. For instance, mice vaccinated sub-cutaneously with syngenic DC loaded with apoptotic/necrotic B16 melanoma cell debris developed operational TLO [pseudocapsule; $PNAd^{+}$ vascular endothelial cells (VEC), T cell/DC infiltrates] at sites of injection, leading to the activation of protective anti-tumor immunity [123]. DC genetically engineered to secrete high-levels of CCL21 (DC.CCL21) and injected directly into B16 murine melanomas promoted strong extranodal T cell crosspriming/recruitment into the TME, even in $LT\alpha^{-/-}$ mice that lack SLO [103, 123, 124]. The superiority of DC.CCL21 in enhancing the cross-priming of protective Type-1 anti-tumor T cell responses has also been confirmed in alternate murine models [125].

1.6 STATEMENT OF THE PROBLEM

DC-based cancer immunotherapies have been shown to be safe and capable of inducing Ag-specific immune responses in clinical trials, however, the overall clinical benefits were still statistically insignificant [126, 127]. This is partly due to the suppressive TME that restricts DC-mediated functions (including the ability to prime antigen-specific T cells and to produce pro-inflammatory cytokines/chemokines), leading to adaptive immune polarization towards non-protective Type-2 or regulatory responses [128-130]. T-bet is a Type-1 immune cell-associated transcription factor that is required for the development in anti-tumor immunity at the level of NK cells, DC and T cells [131]. One aim in my study was to generate a strongly Type-1 polarized population of DC through highly expressing T-bet and would be capable of promoting robust Type-1 T cell priming from naïve responder cell populations (including $CD8^+$ CTLs), while coordinately suppressing Type-2 and Treg responses *in vitro*. When translated to tumor-bearing mice, these gene-modified DC would be envisioned to serve as a cellular therapy designed to overcome existing immune suppression in the TME, thereby allowing for renewed promotion of effective anti-tumor immune responses *in vivo*.

Animal models are important tools for the investigation of the therapeutic benefits of cancer immunotherapies prior to their assessment in randomized clinical trials, as these models allow for the in-depth study of locoregional and systemic immune responses/mechanisms relevant to effective treatment (i.e. in the TME, TDLN and Spleen). In my study, C57BL/6 mice bearing established s.c. MCA205 sarcomas were used to evaluate the therapeutic efficacy of DC.Tbet-based therapy, with the possibility of subsequently resolving the underlying mechanism(s) of action using an extensive panel of syngenic gene knock-out mice.

IL-12 is an important pro-inflammatory cytokine that promotes anti-tumor immunity by enhancing Type-1 T cell function, promoting effector CTL differentiation and inhibiting angiogenesis [132]. Many DC-based cancer vaccines have been based on the engineering of DC to produce high levels of IL-12p70 in hopes of augmenting the Type-1 cross-priming function of these APCs [133, 134]. Interestingly, I observed that DC.Tbet promoted Type-1 T cell development and anti-tumor Type-1 immunity independent of their intrinsic capacity to produce IL-12p70, suggesting that DC.Tbet utilize a novel anti-tumor mechanism that is distinct from what known to be employed by conventional DC. Understanding this unique mechanism will be necessary for the rational design and improvement of DC.Tbet-based (combinational) therapies for use in prospective human clinical trials targeting cancer management and eradication. Based on gene array analyses and transgenic mouse models, I was able to identify molecular and cellular components associated with the potent Type-1 polarizing function and anti-tumor benefits linked to DC.Tbet.

2.0 ECTOPIC T-BET EXPRESSION LICENSES DENDRITIC CELLS FOR INTERLEUKIN-12 INDEPENDENT PRIMING OF TYPE-1 T CELLS *IN VITRO*

Michael W. Lipscomb[1], Lu Chen1, Jennifer L. Taylor[2], Christina Goldbach[3], Simon C. Watkins[3], Pawel Kalinski[1,3,5], Lisa H. Butterfield[1,3,5], Amy K. Wesa[1,2], and Walter J. Storkus[1,2,5]

From the Departments of [1]Immunology, [2]Dermatology, [3]Surgery, and [4]Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, and the [5]University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213

These data were reported in *Journal of Immunology (December 1, 2009 vol. 183).* Lu Chen contributed to the DC.Tbet-driven T cell polarization function and mechanism study, and generated the responder T cell proliferation assay data and transwell assay data for this study. All authors contributed to the scientific discussion of this manuscript.

2.1 ABSTRACT

T-bet (TBX21) is a transcription factor required for the optimal development of Type-1 immune responses. Although initially characterized for its intrinsic role in T cell functional polarization, endogenous T-bet may also be critical to the licensing of Type-1-biasing APCs. Here, we investigated whether human dendritic cells (DC) genetically engineered to express high levels of T-bet (i.e., DC.Tbet) promote superior Type-1 T cell responses *in vitro*. We observed that DC.Tbet were selective activators of Type-1 effector T cells developed from the naive pool of responder cells, whereas DC.Tbet and control DC promoted Type-1 responses equitably from the memory pool of responder cells. Naive T cells primed by (staphylococcal enterotoxin B or tumor-associated protein-loaded) DC.Tbet exhibited an enhancement in Type-1- and a concomitant reduction in Th2- and regulatory T cell-associated phenotype/function. Surprisingly, DC.Tbets were impaired in their production of IL-12 family member cytokines (IL-12p70, IL-23, and IL-27) when compared with control DC, and the capacity of DC.Tbet to preferentially prime Type-1 T cell responses was only minimally inhibited by cytokine (IL-12p70, IL-23, IFN-γ) neutralization or receptor (IL-12Rβ2, IL-27R) blockade during T cell priming. The results of transwell assays suggested the DC.Tbet-mediated effects are predominantly the result of direct DC-T cell contact or their close proximity, thereby implicating a novel, IL-12-independent mechanism by which DC.Tbets promote improved Type-1 functional polarization from naive T cell responders. Given their superior Type-1 polarizing capacity, DC.Tbet may be suitable for use in vaccines designed to prevent/treat cancer or infectious disease.

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2.2 INTRODUCTION

Dendritic cells (DC) are professional APCs that capture, process, and present Ags to T cells in the form of peptides complexed with MHC molecules. heath [ref] DCs support the activation and functional maturation of Th1, Th2, Th17, and regulatory $CD4^+$ T cells, as well as $CD8^+$ T cells, NK cells, and innate myeloid immune cells [135-137].

In a diverse array of infectious disease states and in the cancer setting, host protection is largely afforded via the generation of Type-1 immunity. Type-1 T cell induction is believed to require DC presentation of cognate Ag, in addition to costimulator molecules, such as B7 and TNF family member molecules, and polarizing cytokines, such as IL-12, IL-23, and IL-27 [138, 139]. Functional polarization of Type-1 T cells can be augmented by IL-12 and IL-27, which act through STAT4 and STAT1, respectively, to promote IFN-γ and Type-1-associated accessory molecules [140, 141]. However, IL-12/STAT-4-independent mechanisms of Type-1 T cell induction have also been reported [142, 143]. In such cases, Type-1 polarization requires intrinsic expression of the T-bet transcription factor in T cells which is regulated in a TCR- and STAT1-dependent manner [144-146]. Silencing of T-bet in T cells suppresses IFN-γ and STAT1 expression levels during Ag-specific T cell differentiation, resulting in the unbalanced development of IL-4-secreting Th2 cells [147, 148]. Conversely, T-bet expression suppresses Th2 differentiation by interfering with the type 2 trans activator GATA-3 [149, 150].

Intrinsic, low-level expression of T-bet in (at least a subset of) DC also appears crucial to the generation of Type-1 immunity [144, 151-153]. Our results suggest that human DCs, engineered using recombinant adenovirus to express high levels of T-bet protein in a high percentage of DC, selectively prime and expand Type-1 T cells from naive precursors *in vitro*, while concomitantly restricting Th2 and regulatory T cell (Treg) polarization profiles. Human

DCs genetically engineered to express high levels of T-bet (i.e., DC.Tbet) pulsed with tumor Ag-derived protein or peptide epitopes proved to be superior activators of melanoma Ag-specific Th1 and Tc1 effector cells *in vitro*, thereby supporting the potential utility of these APCs in vaccines against infectious disease or cancer.

2.3 MATERIALS AND METHODS

Preparation of DC and T cells. DCs (>98% $CD11c^+CD14^-$) were generated from normal donors with written consent under an Institutional Review Board-approved protocol, as previously described [65]. Where indicated, day 5 immature DCs were activated for 24 h by incubation with inflammatory stimuli including 1) IL-1β (25 ng/ml; Sigma-Aldrich) plus TNF-α (50 ng/ml; Sigma-Aldrich) plus IFN-α (3000 U/ml; intron A-$IFN_{\alpha 2b}$; Schering-Plough) plus IFN-γ (1000 U/ml; Endogen) plus polyinosinic-polycytidylic acid (20 μg/ml; Sigma-Aldrich) yielding αDC1; 2) LPS (250 ng/ml; Sigma-Aldrich) yielding DC.LPS; 3) LPS (250 ng/ml) plus IFN-γ (1000 U/ml) yielding DC.LPS/IFN; or 4) 1 nM bryostatin-1 (Sigma- Aldrich) yielding DC.BS1 [59]. Plastic-nonadherent cells, enriched in T cells, were collected and stored at −80°C for 5–7 days during the DC culture period. After thawing, naive or memory T cells were negatively isolated using CD45RO or CD45RA MACS microbeads (Miltenyi Biotec), respectively, per the manufacturer's protocols. Isolated cell populations were >98% pure based on corollary flow cytometry analyses. In some cases, $CD4^+$ or $CD8^+$ naive or memory T cell subsets could then be further isolated by positive selection using specific MACS beads as indicated. In additional experiments, $CD45RO^-$ and/or $CD45RA^-$ cells were depleted of $CD56^+$ cells, or they were

separated into their $CCR7^+$ vs $CCR7^-$ subpopulations using specific MACS beads (Miltenyi), as indicated in appendix Fig. 1, 2.

Recombinant adenoviruses. Human T-bet (hT-bet) was PCR cloned from PBLs using the following primers: hT-bet: forward (Fwd) 5′-GTCGACGACGGCTACGGGAAGGTG-3′; reverse (Rev) 5′-GGATCCTTAGTCGGTGTCCTCCAACC-3′. The product was then digested with the restriction enzymes SalI and BamHI and the 1.7-kb fragment containing full-length hT-bet was ligated into the adenoviral-Cre-Lox (Ad.lox) vector. After sequence validation, recombinant adenoviruses were generated, as previously described [67]. The mock (empty) adenoviral vector Ad.ψ5 and/or Ad.EGFP (encoding the enhanced GFP) were used as negative controls, as indicated. Adenoviral vectors encoding full-length human IL-12p70 or MART-1 protein (Ad.MART1) have been previously described [67, 154]. All adenoviruses were expanded, purified, and provided by the University of Pittsburgh Vector Core Facility (Shared Resource).

Adenoviral infection of cells. Day 5 immature DC were infected with adenoviruses at a multiplicity of infection (MOI) of 300 at 37°C for 48 h as previously described [67]. 293T human kidney epithelial cells (American Type Culture Collection; ATCC) were infected with Ad.MART1 or Ad.ψ5 at an MOI of 20 for 48 h before being used to generate freeze-thaw cell lysates.

Abs. Abs reactive against T-bet (Santa Cruz Biotechnology), CCR7, CD3, CD4, CD8, CD25, CD45RA, CD45RO, CD54, CD70, CTLA-4, IFN-γ, IL-17A, MHC class I, MHC class II (BD Biosciences), CD80, CD86, B7-H1, CXCR3, granzyme B, Foxp3, IL-4 (eBioscience), CD212, Jagged-1, TGF-βRII (R&D Systems), IFN-γ, IL-4, IL-10 (Miltenyi Biotec), CD11c, GITR, GITR-L (BioLegend), DLL4 (Novus Biologicals), MART-1 (Vector Laboratories), or β-

actin (Invitrogen) were used in flow cytometry, immunofluorescence microscopy, and Western blot experiments, as indicated. Anti-HLA-A2 mAbs BB7.2 and MA2.1 (ATCC) and anti-HLA-DR4 mAb 359-13F10 (a gift from Dr. Janice Blum (Indiana University, Bloomington, IN) were used to determine the HLA phenotype of normal donors and melanoma patients based on flow cytometry analysis of PBMCs. Neutralizing/blocking anti-human (h) IL-12p70 polyclonal Ab (pAb; R&D Systems), anti-IL12Rβ2 pAb (R&D Systems), anti-hIL-23 pAb (R&D Systems), anti-IL-27R pAb (TCCR/WSX-1; R&D Systems), anti-hIFN-γ pAb (R&D Systems), anti-hIFN-γR1 mAb (R&D Systems), anti-CD70 (Ancell), and recombinant human (rh) Notch-1/Fc chimera (R&D Systems) were used at final concentrations of 10 μg/ml, per the manufacturers' recommendations.

Flow cytometry and fluorescence microscopy analyses. Cell surface and intracellular staining of cells was performed and monitored by flow cytometry, as previously described [65]. For immunofluorescence microscopy, 1×10^5 DCs were cytospun and fixed onto slides. Cells were permeabilized and stained with T-bet primary Ab (Santa Cruz Biotechnology) and conjugated with goat anti-mouse Alexa Fluor 488 secondary Ab (Invitrogen). The counterstains used included Hoechst nuclear dye (Sigma-Aldrich) and F-actin-binding rhodamine phalloidin (Invitrogen). Fluorescence images were then captured using an Olympus BX51 microscope (Olympus America).

RT-PCR. For mRNA analysis, DCs were harvested on day 2 (48 h posttransduction), and MACS-isolated naive or memory $CD4^+$ T cells were harvested on day 3 after initial priming by DCs. RNA was isolated with Trizol (Invitrogen). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Applied Biosystems) and random hexamers (Applied Biosystems). Semiquantitative PCR was used to amplify cDNA for

expression of gene-specific products. Specific primers were used for IL-12p35, IL-12/23p40, IL-23p19, IL-27p28, EBI-3, IL-15, IL-18, IL-10, TGF-β, IFN-α, and IFN-γ as previously described [65]. Additional primer sequences included: T-bet, Fwd 5′-CCACCAGCCACTACAGGATG-3′ and Rev 5′-GGACGCCCCCTTGTTGTTT-3′; GATA-3, Fwd 5′-GTGCTTTTTAACATCGACGGTC-3′ and Rev 5′-AGGGGCTGAGATTCCAGGG-3′; Foxp3, Fwd 5′-GCACCTTCCCAAATCCCAGT-3′ and Rev 5′-TAGGGTTGGAACACCTGCTG-3′; and RORγt, Fwd 5′-AAATCTGTGGGGACAAGTCG-3′ and Rev 5′-TGAGGGTATCTGCTCCTTGG-3′. β-Actin primers were used as an internal positive control [65].

Analysis of cytokine production from DCs. To estimate the profile of cytokines produced by DCs after cognate DC-T cell interaction, 6×10^4 DCs were cocultured with J558 CD40L (i.e., CD40L contributes signals normally provided by newly activated T cells) expressing fibroblasts for 24 h at a DC:J558 ratio of 1:2 in 96-well flat-bottom plates in 200 μl of AIM-V culture medium. Supernatants were collected and stored at −80°C before analysis using commercial ELISAs for human IL-12p70, IL-23, TNF-α, and IL-10 (BD Biosciences, except for IL-23 ELISA from BenderMedSystems). Additional studies included DC stimulation for 24 h using agonists to TLR2 (HKLM; Invivogen) TLR3 (polyinosinic-polycytidylic acid; Sigma-Aldrich), TLR4 (LPS; Sigma- Aldrich), TLR5 (flagellin; Invivogen), TLR7 (imiquimod; Invivogen), as well as a trimeric form of CD40L (a gift from Dr. Andrea Gambotto, University of Pittsburgh, Pittsburgh, PA), as indicated.

Responder T cell proliferation studies (CSFE). The superantigen staphylococcus enterotoxin B (SEB) model for priming autologous T cells was used in these studies [155]. Briefly, DC.Tbet (ectopic T-bet-expressing DC) or control DC were pulsed with SEB (Sigma-

Aldrich) at 0.1–10 ng/ml in AIM-V media (Invitrogen) for 3 h at 37°C prior to washing and the addition of 10^4 DCs to 96-well round-bottom plates. Sorted $CD45RO^-$ (naive) or $CD45RA^-$ (memory) T cells were labeled with 0.5 μM CFSE (Invitrogen) in PBS for 15 min at 37°C, before being washed twice, with 10^5 T cells (resuspended in TcMEM (IMDM supplemented with 10% heat-inactivated human AB serum, L-glutamine, penicillin/streptomycin, and nonessential amino acids); all reagents from Invitrogen with the exception of serum (Sigma-Aldrich) added to wells containing DCs along with 100 U/ml rhIL-2 (Peprotech). Responder T cells were evaluated for CSFE dilution by flow cytometry on day 3 of cocultures.

Responder T cell polarization studies. T cells were plated with SEB-pulsed DC.Tbet or control DC at an E:T ratio of 1:10 in TcMEM. Supernatant of DC-T cell cocultures were collected on day 3 and analyzed for hIFN-γ production using a commercial ELISA (BD Biosciences). Additionally, on day 3, $CD4^+$ T cells were MACS isolated from DC cocultures. Total RNA was isolated for RT-PCR analysis or T cells were costained with mAbs to CD4, CD212 (IL-12Rβ2), and T-bet for flow cytometric analysis. In additional studies, T cells cultured with SEB-pulsed DC.Tbet or control DC on day 0 were restimulated on day 5 with identically prepared DC and supplemented with 20 U/ml rhIL-2 (Peprotech) and 5 ng/ml rhIL-7 (Sigma-Aldrich) every other day. On day 12 or 14 of coculture, T cells were collected and assayed for cytokine (IFN-γ, IL-4, IL-17A, and IL-10), cell surface (CXCR3), and intracellular (Foxp3 and granzyme B) protein expression by flow cytometry. To evaluate intracellular cytokine expression, T cells were stimulated with PMA (1 μg/ml) and ionomycin (50 ng/ml) for 4 h, with 2 μM monensin (all from Sigma-Aldrich) added over the final 2 h of culture. Where indicated, cell culture supernatants were analyzed for secreted levels of hIFN-γ, hIL-4, hIL-10 (all from BD Biosciences), and hIL-17A (BioLegend) using commercial ELISAs.

Neutralizing/blocking studies. Briefly, DC.Tbet or control DCs were plated with T cells at a DC:T cell ratio of 1:10 in triplicate in 96-well flat-bottom plates, in the presence or absence of neutralizing/blocking Abs or recombinant fusion protein. On day 3, cell-free supernatants were collected and evaluated using a hIFN-γ-specific ELISA. Alternatively, T cells were restimulated on day 5 with SEB-pulsed DC and supplemented with rhIL-2, rhIL-7, and neutralizing Abs, with T cells harvested on day 14 for analysis of intracellular IFN-γ production by flow cytometry.

Transwell assays. DC.Tbet or control DC (5×10^5) were plated in the bottom chamber of a 24-well transwell plate in 400 μl of TcMEM. After 24 h, 1×10^6 naive T cells along with 1×10^5 SEB pulsed-immature DC or 3×10^5 anti-CD3/CD28 microbeads (Invitrogen) were placed in the upper chamber of the transwell plate bringing total volume to 600 μl of TcMEM. Cell supernatants were collected from the upper chamber on day 3 for IFN-γ ELISA analyses.

Generation of lysates containing rMART-1 protein. 293T human kidney epithelial cells (ATCC) were infected with Ad.MART1 at an MOI of 20 for 48 h, at which time freeze-thaw lysates were generated as previously described [156]. 293T cells infected with Ad.ψ5 (MOI 20) were used to generate a negative control lysate. Expression of MART-1 mRNA/protein in transduced 293T cells was determined using RT-PCR and immunohistochemistry, and MART-1 protein in lysates confirmed by Western blot, as previously described [154]. Total lysate protein content was estimated by OD_{280} (1.2 mg = 1.0 OD units full scale at 280 nm), and lysates were stored at −80°C until being used to load DCs for T cell induction and recognition assays.

Analysis of DC processing of recombinant MART-1 protein for recognition by specific $CD4^+$ T cells. MACS-isolated, naive $CD4^+$ T cells were isolated from HLA-$DR4^+$ (based on monocyte staining with anti-HLA-DR4 mAb 359-13F10 as monitored by flow cytometry)

normal donors as outlined above, and stimulated twice on a weekly basis with control DC (DC.null) pulsed with the MART-$1_{51–73}$ peptide (10 mM). On day 14 of culture, T cells were harvested and assessed for their ability to recognize (in IFN-γ ELISPOT assays) autologous DC.null, DCψ5, or DC.Tbet cells prepulsed for 48 h with freeze-thaw lysates (50 μg/ml) generated from Ad.MART-1- vs Ad.ψ5-infected 293T cells.

Ag-specific T cell responses. PBMCs were isolated from healthy, normal donors with written consent under an Institutional Review Board-approved protocol. For $CD8^+$ T cell responses, DCs were generated from HLA-$A2^+$ normal donors (i.e., lymphocytes staining with both the anti-HLA-A2 mAbs BB7.2 and MA2.1 as monitored by flow cytometry), as outlined above, and DC.Tbet or control DC were pulsed with HLA-A2-restricted peptide epitopes ((EphA$2_{883–891}$, gp$100_{209–217}$; 10 μM each) ref. [157]) for 3 h at 37°C before culturing with MACS-isolated naive $CD8^+$ T cells at a 10:1 T cell:DC ratio in the presence of 5 ng/ml rhIL-7. $CD8^+$ T cell cultures were expanded by a second stimulation on day 7 with identically prepared DCs or with peptide-pulsed autologous, irradiated PBMCs. Restimulated cultures were supplemented with 20 U/ml rhIL-2 and 5 ng/ml rhIL-7, with cytokines replenished every other day. On day 14, the frequency of peptide-specific $CD8^+$ T cells was analyzed in IFN-γ ELISPOT assays (Mabtech) using HLA-$A2^+$ T2 cells as APCs that were performed as previously described [157]. The HLA-A2-presented HIV-nef$_{190–198}$ peptide [157] served as a (negative) specificity control in these assays. For $CD4^+$ T cell responses, DCs were generated from HLA-$DR4^+$ normal donors (based on monocyte staining with anti-HLA-DR4 mAb 359-13F10 as monitored by flow cytometry) as outlined above. DC.Tbets or control DCs were pulsed with 50 μg/ml freeze-thaw lysate generated from 293T cells infected with Ad.MART1 vs Ad.ψ5 for 24 h, 37°C and used to stimulate autologous MACS-isolated, naive $CD4^+$ T cells, as outlined above for $CD8^+$ T cell

responses. On day 7 of cultures, responder $CD4^+$ T cells were restimulated with identically prepared, Ag-loaded DCs, and cultures were supplemented with rIL-2 and rIL-7 as noted above. On day 14, the frequency of MART-1-specific $CD4^+$ T cells was analyzed in IFN-γ and IL-5 ELISPOT assays (Mabtech) using autologous control DC pulsed with the MART-$1_{51–73}$ vs the HIV-$nef_{192–204}$ (negative) control HLA-DR4-presented peptide epitopes as target cells [158].

Statistical analyses. 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 $\mathbf{p} < 0.05$.

2.4 RESULTS

2.4.1 *In vitro* modulation of T-bet expression in human DCs

Human DCs were generated from peripheral blood monocytes and transduced with recombinant adenovirus encoding human T-bet (DC.Tbet) or control Ad.ψ5 (DC.ψ5) for 48 h. DCs were also generated using known Type-1 polarizing culture conditions, yielding DC1. Harvested DCs were analyzed for T-bet mRNA (via RT-PCR; Fig. 1**A**) and protein expression (via Western blot and flow cytometry; Fig. 1, **B** and **C**). As shown in Fig. 1, **A**–**C**, T-bet expression in untreated immature DC (DC.null) and DC.ψ5 was very low (at both the transcript and protein levels), with expression levels augmented in DC.null cells by 24 h of culture in the presence of inflammatory stimuli [42, 59, 65]. However, in marked contrast to the <1% frequency of T-bet^+, DCs developed using nonviral culture methods, DC.Tbet were 63 ± 18% T-bet^+ over 15 independent experiments as determined by intracellular staining, as exemplified in Fig. 1**C**.

Immunofluorescence microscopy revealed that T-bet protein was expressed predominantly in the nucleus of DC.Tbet cells (Fig. 1**D**).

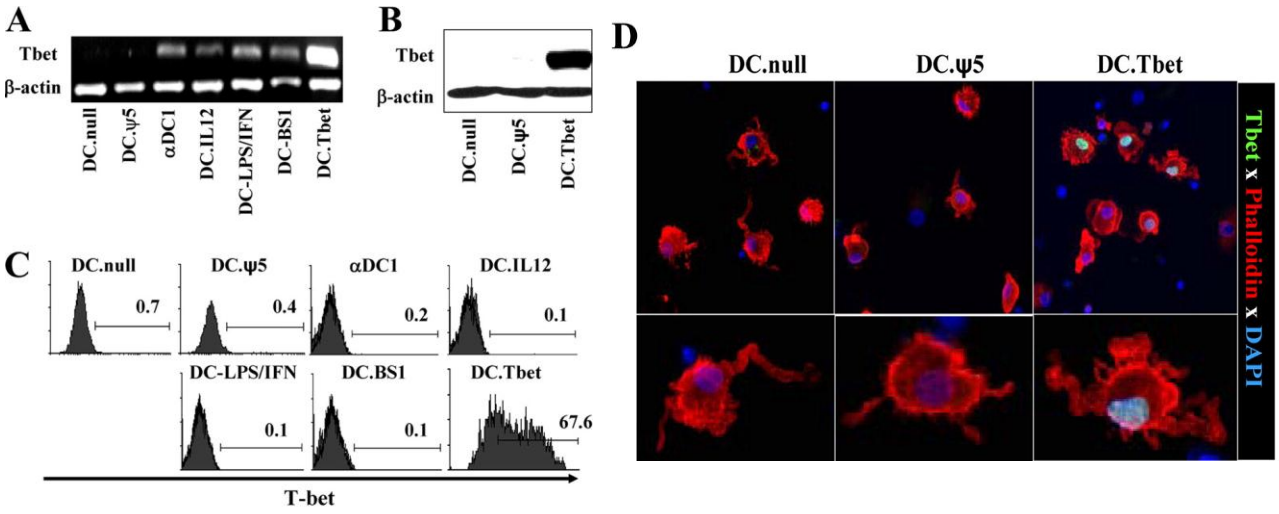


Figure 1. Generation and characterization of DC.Tbets

Immature DCs were generated from peripheral blood monocytes by culturing with IL-4 and GM-CSF for 5–6 days. DC were left untransduced (DC.null) or transduced with recombinant adenoviral vectors containing an empty cassette (Ad.ψ5), human T-bet (Ad.Tbet), or hIL-12p70 (Ad.IL12), yielding DC.ψ5, DC.Tbet and DC.IL12 cells, respectively. **A**, DC.null, DC.ψ5 , DC.Tbet, and DC.IL12, as well as DCs activated for 24 h in the presence of inflammatory stimuli (i.e., yielding αDC1, DC-LPS/IFN, and DC.BS1 as described in Materials and Methods) were analyzed for T-bet vs control β-actin mRNA expression by RT-PCR. In **B** and **C**, the indicated DC populations were analyzed for T-bet protein expression by Western blotting and intracellular immunofluorescence staining monitored by flow cytometry, respectively. **D**, Confocal immunofluorecence microscopic images of the indicated DC populations stained for expression of T-bet [147], phalloidin (Red), and 4′,6′-diamidino-2-phenylindole (DAPI; blue). Data are representative of at least three independent assays performed for each panel.

2.4.2 DC.Tbet selectively prime CD45RO$^-$ (naïve) T cells toward Type-1 polarization *in vitro*

Given previous reports that intrinsic (low-level) expression of T-bet in DC is crucial to the ability of these APC to promote Type-1 T cell responses [144, 151-153], we hypothesized that DC.Tbet cells might be enhanced in this capacity. We used a superantigen (SEB) model to investigate DC.Tbet-induced specific responses from naive vs memory T cell populations *in vitro*. Briefly, DC.Tbet or control DCs were pulsed with 1 ng/ml SEB before coculture with autologous naive (MACS-isolated CD45RO$^-$ cells) or memory (MACS-isolated CD45RA$^-$) bulk (CD4$^+$ and CD8$^+$) T cells at a DC:T cell ratio of 1:10 for 72 h. These conditions were chosen based on dilutional analyses in which optimal IFN-γ was observed from responder T cells within both the control DC- and DC.Tbet-stimulated cohorts at an SEB dose of 1 ng/ml.

As shown in Fig. 2**A**, IFN-γ production from activated naive, but not memory, bulk (CD4$^+$ and CD8$^+$) T cells was significantly up-regulated when primed by SEB-pulsed DC.Tbet vs SEB-pulsed control DC (**p** = 0.004). Macroscopically, DC.Tbet-activated cultures developed from naive, bulk T cell precursors contained very large cellular clusters (Fig. 2**B**), suggestive of differentially enhanced T cell proliferation within such cultures. However, a repeated series of assays implementing bulk CD45RO$^-$ vs CD45RA$^-$ T cells that were prelabeled with 0.5 μM CFSE before coculture with control DCs or DC.Tbet, revealed no significant changes in the frequencies of daughter cell generations (CD45RO$^-$ T cells; Fig. 2, **C** and **D**) or T cell yields on day 3 or 7 of culture (Fig. 2**C**), although the enhanced ability of daughter T cells to produce IFN-γ in DC.Tbet (+ CD45RO$^-$ bulk T cell) cocultures was readily apparent (Fig. 2**D**). This latter increase was evident in both the percentage of IFN-γ$^+$CSFE^{low+} events (Fig. 2**D**) and the approximate doubling in mean fluorescence intensity levels for IFN-γ expression in responder T

cells (157 for DC.Tbet cultures vs 73 or 71 for DC.null or DC.ψ5 cultures, respectively; data not shown). These data strongly suggest that DC.Tbets enhance Type-1 responses from bulk, $CD45RO^-$ T cells via differential polarizing, rather than proliferative, signals.

To exclude the potential impact of contaminant NK/NKT cells on the IFN-γ level observed in the *in vitro* stimulation assay, we MACS-isolated $CD45RO^-CD56^-$ T cells (appendix Fig. 1) and repeated our the assay using autologous SEB-pulsed DC.Tbets vs control DCs as APCs. As shown in appendix Fig. 1**B**, depletion of $CD56^+$ cells from $CD45RO^-$, bulk T cell responders did not inhibit the ability of DC.Tbets to promote superior IFN-γ production. Next, we further define T cell functional subsets into naive ($CD45RO^-CCR7^+CD62L^+$), effector (T_E; $CD45RO^-CCR7^-CD62L^{dim+}$ or $CD45RA^-CCR7^-CD62L^{dim+}$), central memory ($T_{CM}$; $CD45RA^-CCR7^+CD62L^+$) and effector memory (T_{EM}; $CD45RA^-CCR7^-CD62L^{dim+}$) subpopulations based on MACS isolation and flow staining. Only naive T cells that were highly enriched (~90% pure) for the $CCR7^+CD62L^+$ phenotype exhibited differential responsiveness to DC. Tbets (vs control DCs) based on a substantial up-regulation in their production of IFN-γ (appendix Fig. 2**B**). This result proved that DC.Tbet preferentially support type 1 development of naive ($CD45RO^-CCR7^+CD62L^+$), but not memory, T cell precursors.

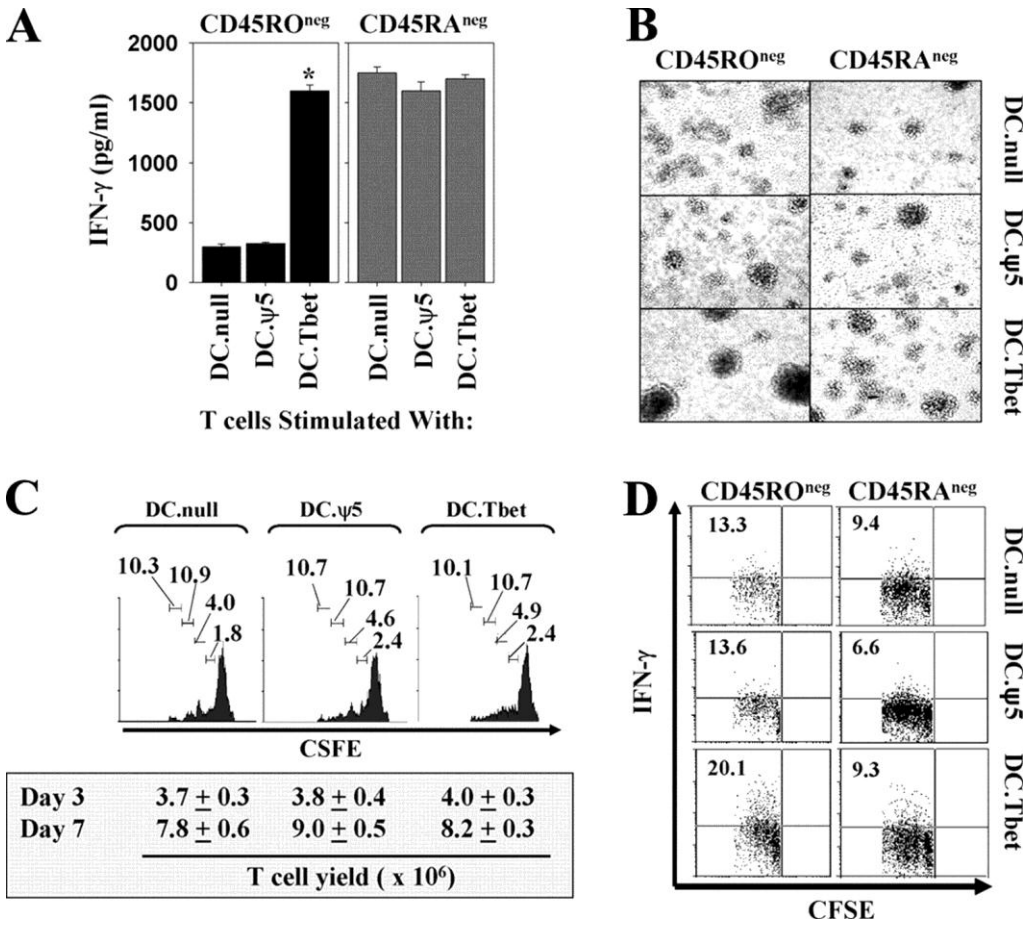


Figure 2. DC.Tbets uniquely promote IFN-γ responses from naïve bulk T cells *in vitro*

Bulk, naive ($CD45RO^-$), or memory ($CD45RA^-$) T cells were isolated by negative selection and cultured with autologous, SEB-pulsed DC.Tbets or control DCs at a ratio of 10:1, respectively. After 72 h, supernatants were collected for analysis using IFN-γ ELISA (***A***; ∗, $p < 0.05$ vs DC.null or DC.ψ5), and the cocultures were assessed under bright-field microscopy (×10; ***B***). Identical cultures using CFSE-labeled T cells (gated on $CD3^+$ cell populations) were analyzed for CFSE dilution based on daughter-cell generation by flow cytometry and quantitated for cell yield (on days 3 and 7 of culture) in ***C. D***, Intracellular expression of IFN-γ in CFSE-labeled T cells was evaluated after 72 h of coculture with DC.Tbets vs control DCs. All data are representative of three independent assays performed.

2.4.3 DC.Tbet priming suppresses the generation of type 2 and Tregs from naïve precursors

To further assess responder T cell polarization status, we isolated $CD3^+$ T cells from DC-bulk T cell cocultures after 72 h and analyzed these cells for their comparative expression of mRNAs encoding **trans** activator proteins (i.e., T-bet (Th1), GATA-3 (Th2), RORγt (Th17), and Foxp3, as Treg) linked to T cell function (Fig. 3**A**). We observed that naive T cells stimulated with DC.Tbet cells were enriched (~5-fold as assessed by densitometry analysis of gel bands; data not shown) in T-bet, and reduced in GATA-3 (~4-fold), RORγt (<2-fold) and Foxp3 (~5-fold) transcripts when compared with T cells stimulated with control DCs (Fig. 3**A**). To corroborate these findings at the protein level, we assessed the polarization state of responding $CD4^+$ T cells by analyzing their cytokine production profiles. We confirmed reductions in the levels of IL-4 and IL-10 produced by naive (but not memory) T cells stimulated with autologous SEB-pulsed DC.Tbet (vs control DC; $p < 0.05$) as analyzed in ELISA and intracellular staining protocols (bulk cells analyzed in Fig. 3B and $CD3^+$ T cells assessed in Fig. 3C, respectively). Also, the frequency of responder $CD4^+Foxp3^+$ T cells was reduced after activation of naive (but not memory T cells) with SEB-pulsed DC.Tbets vs control DCs (Fig. 3**D**). In slight contradiction to the RT-PCR data reported for ROR-γt in Fig. 3**A**, we noted a modest increase in IL-17A protein production from naive T cells primed using DC.Tbets vs control DCs (Fig. 3**B** and **C**).

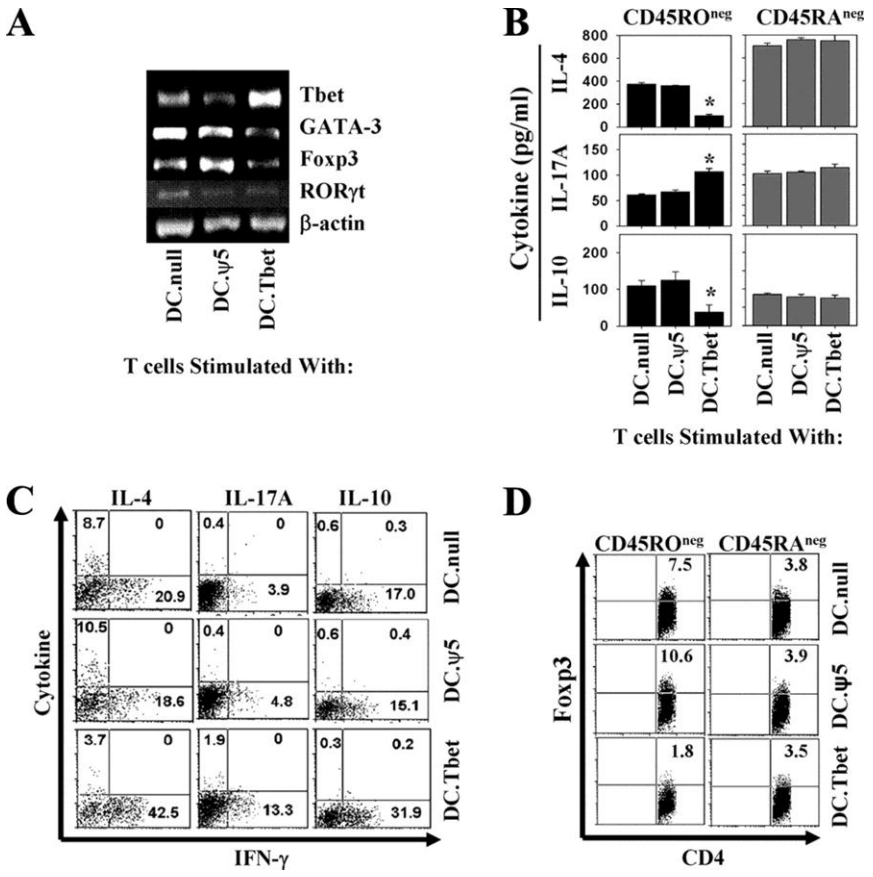


Figure 3. DC.Tbets selectively prime naive, bulk T cells toward Type-1 polarization *in vitro*

Bulk $CD45RO^-$ or $CD45RA^-$ T cells were stimulated with autologous, SEB-pulsed DC.Tbets vs control DCs as described in Fig. 2**A**. **A**, $CD3^+$ T cells were MACS isolated from the $CD45RO^-$ cocultures and RT-PCR performed to analyze relative Tbet, GATA-3, Foxp3, RORγt, and β-actin transcript levels. **B**, Day 3 coculture supernatants harvested from bulk T cell-DC cocultures were analyzed for levels of IL-4, IL-17A, and IL-10 by specific ELISA. ∗, $\mathbf{p} < 0.05$ for DC.Tbet vs DC.null or DC.ψ5. **C**, Day 3 responder $CD3^+$ T cells MAC isolated from cocultures initiated using $CD45RO^-$ responder cells were analyzed for intracellular IFN-γ, IL-4, IL-17A, and IL-10 by flow cytometry as outlined in **Materials and Methods**. **D**, Cocultures were restimulated on day 5 with DC.Tbets or control DCs (as outlined in **Materials and Methods**) and $CD4^+$ T cells MACS isolated for analysis of intracellular expression of Foxp3 by flow cytometry on day 14 (when Foxp3 expression is expected to be retained only in Treg and nonactivated, non-Treg cells). All data are representative of three independent assays performed.

2.4.4 DC.Tbet are suppressed in the secretion of IL-12 family cytokines and IFN-γ

The ability of DC.Tbets to selectively augment Type-1 responses from naive T cells initially suggested the likely involvement of DC-produced IL-12 family members such as IL-12p70, IL-23, and IL-27 [139-141, 159]. We found that although DC.Tbets expressed reduced levels of IL-27p28 mRNA, transcript levels for all other IL-12- and IL-23-associated mRNAs, as well as a number of alternate DC-associated cytokines were unchanged in DC.Tbets vs control DCs (Fig. 4A). Strikingly, despite DC.Tbet exhibiting an essentially control DC cytokine mRNA profile, these APCs were profoundly suppressed (vs control DCs) in their capacity to secrete any cytokine evaluated (i.e., IL-12p70, IL-23, TNF-α, and IL-10) either spontaneously or in response to CD40 ligation or TLR stimulation (Fig. 4**B** and 4**C**). Consistent with the lack of expression of IFN-γ mRNA in any DC population analyzed in Fig. 4**A**, IFN-γ was not produced at detectable levels by any of the DC cohorts (i.e., <4.7 pg/ml as determined by specific ELISA; data not shown). Additional analyses suggest that the inability of DC.Tbets to produce these cytokines was not the result of reduced DC vitality or enhanced sensitivity of these APCs to apoptosis vs control DC.

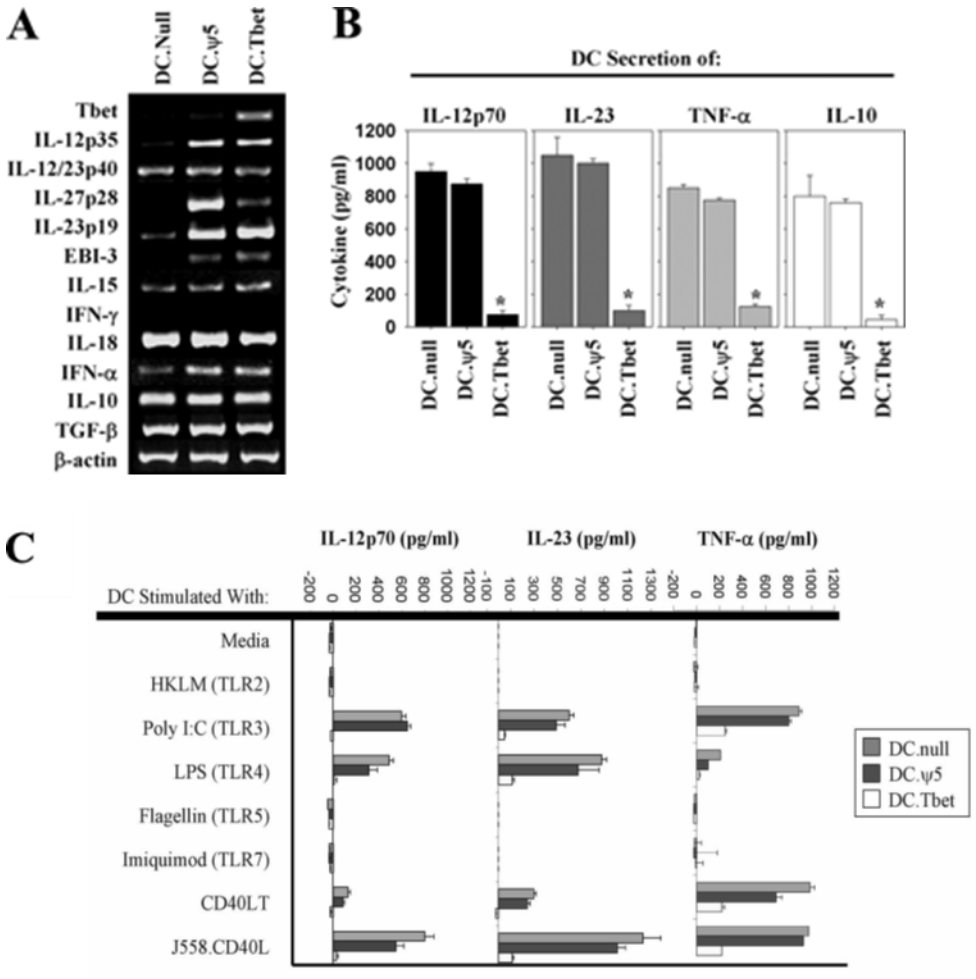


Figure 4. DC.Tbet are suppressed in their capacity to secrete cytokines in response to CD40 ligation or TLR ligands when compared with control DCs

DC.Tbet vs control DCs were analyzed for levels of the indicated cytokine mRNA (using RT-PCR in **A**) and secreted cytokines (using specific ELISA in **B, C**) upon CD40 ligation or TLR ligands stimulation. As outlined in *Materials and Methods*. ∗, $p < 0.05$ vs DC.null and DC.ψ5 controls. All data are representative of three independent assays performed.

2.4.5 DC.Tbet promote Type-1 T cell development via a novel mechanism that is largely independent of IL-12 while requires close DC-T cell proximity

Despite low levels of cytokine production by DC.Tbets, we evaluated whether IL-12 family member cytokines (or IFN-γ itself) were involved in the priming of Type-1-polarized T cell responses by SEB-pulsed DC.Tbets vs control DCs. *In vitro* stimulations of naive, bulk T cells were recapitulated in the absence or presence of neutralizing/blocking Abs reactive against IL-12p70, IL-23, IL12Rβ2, IL27R, and/or IFN-γ (Fig. 5, **A–C**). IFN-γ production by T cells primed by all control DC populations (including DC/IFN, DC/IFN plus LPS, and anti-DC1) was clearly dependent on IL-12p70 and/or IFN-γ itself, as well as a functional IL-12Rβ2-dependent signaling pathway. However, this was not the case for naive, bulk T cells activated using DC.Tbet cells. Indeed, antagonism of these cytokines/cytokine receptors did not significantly affect the ability of DC.Tbets to prime Type-1 T cell responses *in vitro* (Fig. 5, **A–C**).

Because RT-PCR and ELISA analyses suggested the coordinate silencing of cytokine secretion by DC.Tbet, this implicated the likely dominant involvement of cell membrane interactions rather than soluble mediators in the differential ability of DC.Tbet to drive Type-1 T cell responses *in vitro*. We confirmed this hypothesis by coculturing $CD45RO^{-}$, bulk T cells with anti-CD3/CD28 mAb-coated beads or with SEB-pulsed control DC in the upper chambers of transwell plates, with DC.Tbets or control DCs placed in the lower chambers. After 72 h of culture, supernatants harvested from the various T cell cultures were all found to contain comparable levels of IFN-γ (Fig. 5**D**), suggesting that physical separation of DC.Tbets from responder T cells mitigates their capacity to promote superior Type-1 immunity *in vitro*.

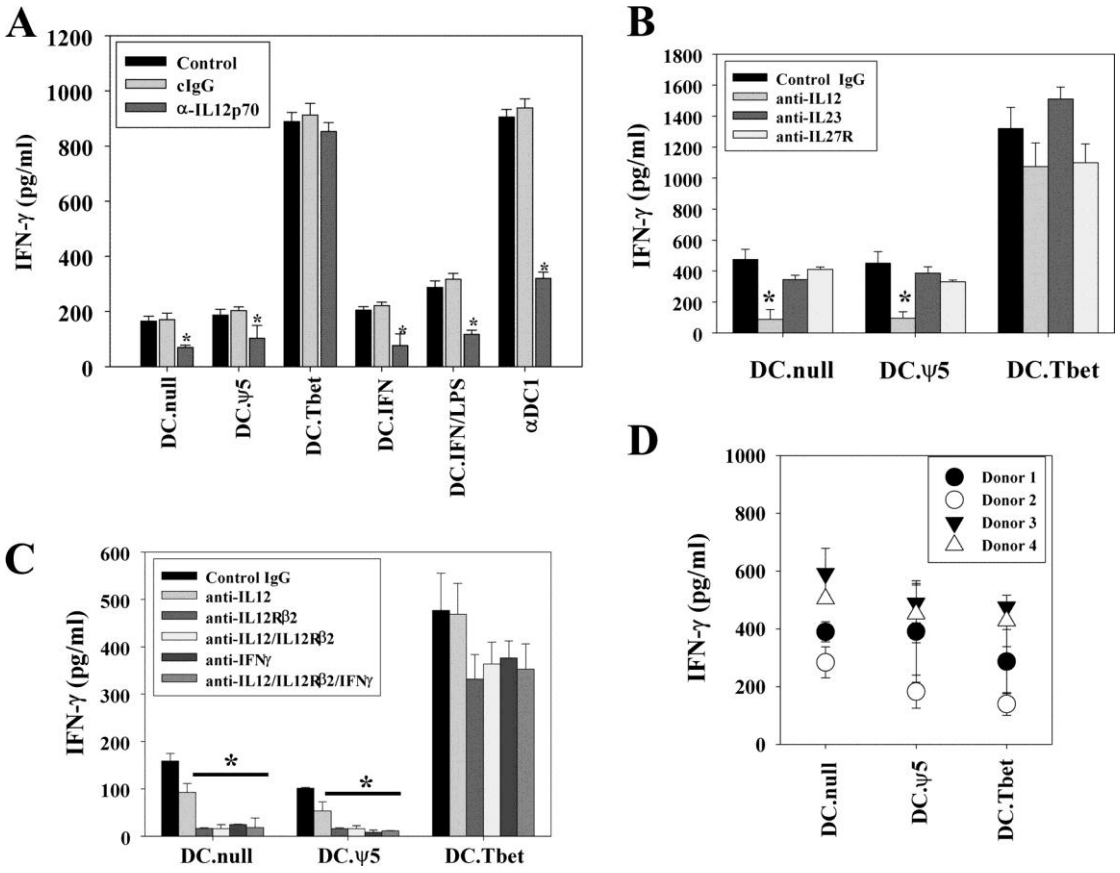


Figure 5. DC.Tbet induction of Type-1 immunity from naive T cell responders is independent of IL-12 family member cytokines and requires DC.Tbet-T cell contact or their close proximity

DC-naive T cell cocultures were established as outlined in Fig. 2 using SEB-pulsed, autologous DC.Tbet, culture-conditioned DC (i.e., DC.IFN, DC.IFN/LPS or αDC1 as described in Fig. 1 and *Materials and Methods*) or control DC as APC. Cocultures were developed in the absence or presence of control IgG or neutralizing/blocking anti-IL12p70 (***A***), anti-IL-12p70, anti-IL23 or anti-IL-27R pAbs (***B***), or anti-IL12p70, anti-IL12Rβ2 and/or anti-IFN-γ pAbs (***C***). In *A–C* experiments, cell-free supernatants were harvested after 72 h of DC-T cell coculture and levels of IFN-γ determined using a specific ELISA. ∗, $p < 0.05$ vs control IgG. ***D***, Transwell assays were performed as described in *Materials and Methods*, with culture supernatants analyzed for levels of IFN-γ via ELISA. All data are reported as the mean ± SD of triplicate well determinations and are representative of at least three independent assays performed using different donors.

2.4.6 DC.Tbet promotes superior tumor Ag-specific, Type-1 $CD4^+$ and $CD8^+$ T cell responses *in vitro*

To determine whether DC.Tbets were capable of promoting enhanced Tc1 immunity against tumor Ags (such as EphA2 and gp100. as in Ref.[42]), naive $CD8^+$ T cells were isolated from HLA-$A2^+$ normal donors and then cocultured with autologous DC.Tbets or control DCs pulsed with an equimolar mixture of the $EphA2_{883–891}$ and $gp100_{209–217(2M)}$ peptides. T cells were restimulated after 7 days of culture and then assessed for populational frequencies of peptide-specific, IFN-γ-producing $CD8^+$ T cells on day 14. We observed that T cell cultures primed using DC.Tbets (vs control DCs) contained significant increases in their frequencies of Type-1 $CD8^+$ T cells reactive against both the EphA2 and gp100 peptides, but not a negative control HIV-nef peptide epitope (Fig. 6**A**). We observed elevated Ag-specific responses for the DC.Tbet-primed cohort of $CD8^+$ T cells regardless of whether peptide-pulsed autologous DC.Tbets or PBMCs were used as APCs in the restimulation phase of this experiment (Fig. 6**A**). This supports the likelihood that the dominant impact of DC.Tbet on specific Tc1 responses occurs during the priming phase.

To address whether DC.Tbet were similarly capable of promoting improved T_H1 responses against a tumor Ag, we initially showed that these APCs were fully competent to uptake and process exogenous recombinant MART-1 protein (in the form of a freeze-thaw lysate of 293T previously transduced with a recombinant adenovirus encoding hMART-1) and then present the derivative HLA-DR4-presented $MART\text{-}1_{51–73}$ epitope [160] to a peptide-specific $CD4^+$ T cell line. To determine whether MART-1 protein-pulsed DC.Tbet cells were competent to preferentially prime Type-1 responses from naive $CD4^+$ T cell responders, DC.Tbets and control DCs were loaded with 293T.MART1 lysate for 24h and then used to prime and boost (on

day 7 of culture) autologous, naive $CD4^+$ T cells isolated from normal HLA-$DR4^+$ donors. As shown in Fig. 6**B**, $CD4^+$ T cells analyzed on day 14 of culture displayed superior levels of reactivity against the MART-$1_{51–73}$ peptide epitope in IFN-γ (and reduced specific responses in IL-5) ELISPOT assays using autologous DC.nulls as APCs if they had been developed using MART-1^+ lysate-pulsed DC.Tbet vs control DC ($\mathbf{p} < 0.05$).

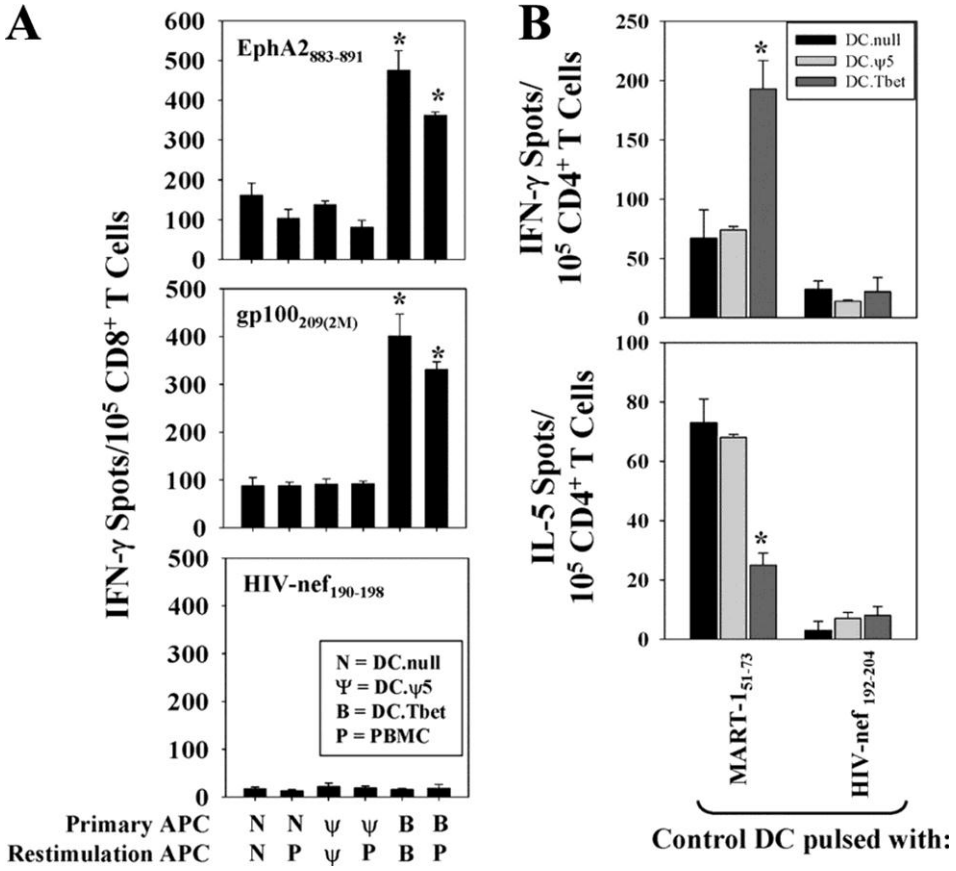


Figure 6. DC.Tbet promotes superior tumor Ag-specific priming of Type-1 T cell responses from naive $CD8^+$ T cell precursors *in vitro*

A, HLA-$A2^+$ DC.Tbet or control (untreated of DC.ψ5) DC were pulsed with the HLA-A2-presented tumor-associated peptides $EphA2_{883–891}$ and $gp100_{209–217(2M)}$ and used to stimulate autologous, MACS-isolated, naive $CD8^+$ T cells. Responder T cell cultures were restimulated with identically prepared (peptide-pulsed, autologous) DC or control PBMC on day 7, with restimulated cultures supplemented with rhIL-2 and rhIL-7 (as outlined in *Materials and Methods*). On day 14 of culture, Ag-specific T_C1 responses were assessed in IFN-γ ELISPOT assays using HLA-$A2^+$ T2 cells as APC for relevant (EphA2, gp100) vs irrelevant (HIV-$nef_{190–198}$ negative control) peptides.

Data are representative of one of three independent normal HLA-$A2^+$ donors evaluated. ***B***, Naive, $CD4^+$ T cells were isolated from the peripheral blood of HLA-$DR4^+$ normal donors and stimulated on days 0 and 7 with autologous DC.Tbet or control DC that had been pre-pulsed for 24 h with a freeze-thaw lysate generated from HLA-$DR4^-MART\text{-}1^-$ 293T human kidney epithelial cells infected with either Ad.MART-1 or Ad.ψ5 control virus (MOI 20 for 24 h at 37°C). On day 14 of cultures, responder $CD4^+$ T cells were analyzed in 24-h IFN-γ ELISPOT assays for reactivity against autologous DC.null cells pulsed with either the HLA-DR4-presented MART-$1_{51–73}$ peptide epitope or the negative control HLA-DR4-presented HIV-$nef_{192–204}$ peptide epitope. Representative data is depicted for 1 of 2 independent normal HLA-$DR4^+$ donors evaluated. In both *A* and *B*, data are reported as the mean ± SD of triplicate assay determinations; ∗, $p < 0.05$ vs DC.null or DC.ψ*5*.

2.5 DISCUSSION

The transcription factor T-bet was originally identified as a master regulator of Th1 development but has since been found to differentially regulate genes in CD8+ effector T cells, B cells, and NK and NKT cells [50, 51, 53]. In particular, Glimcher et al. [4, 50] have shown that endogenous expression of T-bet in DCs is necessary for optimal induction of Type-1 T cell responses. A major finding in the current studies is that ectopic (over)expression of T-bet (as a result of recombinant adenoviral T-bet cDNA delivery) to license DC to preferentially support the *in vitro* development of Type-1 (over type 2 and Treg) polarized responses from naive (CD45RO−CCR7+CD62L+), but not memory, T cell precursors. Preferential enhancement in Type-1 T cell development was reflected at the level of differential trans activator molecule mRNA expressed (with T-bet increased and GATA-3, as well as Foxp3 being decreased) and cytokines secreted (with IFN-γ increased, and IL-4 as well as IL-10 being decreased). Furthermore, levels of cell surface (CXCR3, IL-12Rβ2) and effector (granzyme B, IFN-γ)

molecules associated with Type-1 functionality were increased in naive T cells after specific activation with DC.Tbets vs control DCs. Although, ROR-γt mRNA transcripts appeared unaffected or, in some cases, somewhat reduced in naive T cells primed with DC.Tbets vs control DCs, we found that the level of IL-17A secreted by these responder T cells tended to be modestly increased ($p < 0.05$ vs control DC-stimulated T cells). This may not be too surprising due to the mutual functional exclusivity between Foxp3+ Treg (suppressed after DC.Tbet stimulation) and Th17 T cells (potential compensatory enhancement), as previously reported by others [161]. Furthermore, we did not detect Th17 cells coproducing both IFN-γ and IL-17A (Fig. 3C), suggesting that IFN-γ analyzed in our studies is stringently associated with bona fide Type-1 T cell responses.

A second major finding in our work relates to the IL-12 cytokine family-independent mechanism(s) involved in DC.Tbet activation of Type-1 $CD4^+$ and $CD8^+$ effector cells from naive T cells. Indeed, we noted that 1) production of IL-12p70, IL-23, and IL-27, as well as all other cytokines evaluated, was suppressed in DC.Tbets vs control DCs and 2) neutralizing Abs against IL-12p70, IL-23p19, IL-12Rβ2, and IL-27R all failed to attenuate DC.Tbet-mediated induction of Type-1 responses from naive T cells. It remains formally possible that the absence of cytokine (i.e., IL-23 and IL-27)-mediated signaling into T cells could reinforce their Type-1 functional polarization, as others have previously shown that 1) IL-27 mediates the differentiation of naive T cells into IL-10 producing Tr1 cells [162] and 2) signals mediated via the IL-23R are crucial for the development of T_H17 responses [163].

Results obtained in transwell assays support the critical importance of DC.Tbet-T cell interaction or proximity in order for Type-1 polarizing signals to be conveyed during the T cell priming event. Yet a survey of DC surface molecules for expression levels revealed no striking

differences between DC.Tbet and control DC.ψ5 (or DC.EGFP) for MHC molecules, integrins, co-stimulatory/inhibitory molecules or modulatory receptors (appendix Fig. 3). CD70 and NOTCH ligands δ-like-4 and Jagged-1 which have been previously shown to contribute to the functional polarization of responder T cells by DCs [142, 143, 164], were not expressed (or expressed poorly) by DC.Tbet (appendix Fig. 4**A**), and appeared functionally irrelevant in our model system since the inclusion of specific blocking reagents had no perceptible impact on the ability of DC.Tbet to support enhanced Type-1 responses from $CD45RO^{-}$, bulk T cells (appendix Fig. 4**B**).

Overall, our data appear to support a novel mechanism by which DC.Tbet preferentially prime Type-1 T cell responses from naive T cell precursors. This is manifest in enhanced DC-naive T cell clustering at early phases of the induction process (i.e., day 3) via a process that was not correlated with T cell proliferation/expansion based on CFSE dilution analyses *in vitro*. These data could suggest that DC.Tbet-naive T cell interactions may be uniquely prolonged due to the sustained interfacing of key MHC/TCR and costimulatory/integrin/adhesion molecules and/or to the abbreviated impact of coinhibitory or intercellular repulsion molecules [165-168], resulting in a reinforced commitment of newly primed T cells toward a state of Type-1 functional polarization. It is also possible that DC.Tbets may be refractory to dissociating signals, such as those contributed via newly activated T cell-expressed CTLA-4 [169]. If such interactions underlie the observed selective priming of Type-1 immunity by DC.Tbets, this could explain the inability of DC.Tbet to affect superior Type-1 responses from the activated, memory T cell population, given that memory T cells are known to exhibit a lower activation threshold requirement for both signal 1 (MHC/peptide) and signal 2 (costimulation) when compared with naive T cells [170]. We are currently pursuing a further characterization (genomic, proteomic) of

changes occurring in DC.Tbet that may be implicated in the selective priming Type-1 responses from naive T cells.

Type-1 T cell responses appear most efficient in regulating disease development and progression in the cancer setting [65, 67, 154, 155, 171, 172]. Hence, the ability to predictably generate tumor-specific Type-1 immunity is a major target for cancer immunotherapy-based approaches. A means to accomplish this goal includes the use of vaccines that may selectively and predictably augment the development of Tc1 and Th1 effector T cell populations. Although such vaccines have commonly integrated autologous DCs as a biological adjuvant [127, 154, 171] over the past decade, significant heterogeneity in DC subsets and variable states of maturation have yielded equivocal results in both preclinical tumor models and clinical trials applying DC-based modalities [127].

In this context, methods to condition or engineer DC1 that are particularly competent to expand and develop Type-1 T cell-mediated antitumor immunity may improve clinical efficacy of DC-based cancer vaccines. In this regard, (IL-12p70-independent) DC.Tbets promote at least equitable Type-1 T cell responses to (IL-12p70-dependent) αDC1, a current gold standard for clinically applied DC1 [42]. Given the apparent non-overlapping mechanism of Type-1 immune induction by DC.Tbets and IL-12p70, it might be envisioned that these two agents might act synergistically in promoting Tc1 and Th1 responses. We are currently evaluating this possibility *in vitro*.

Our *in vitro* stimulation experiments using tumor peptide (i.e., EphA2 and gp100) or protein (recombinant MART-1)-pulsed DC.Tbets clearly support the improved capacity of this vaccine to promote specific Tc1 and Th1 responses *in vitro* from naive $CD8^+$ and $CD4^+$ T cells, respectively. Such Type-1 T cells would be predicted to be competent to both infiltrate tumor

lesions (as associated increases in CXCR3 expression are observed) *in vivo* [64, 159] and to mediate robust antitumor activity within these sites [173]. Furthermore, because DC.Tbets retain their capacity to uptake whole (tumor) proteins and to process and then prime tumor Ag-specific, Type-1 $CD4^+$ and $CD8^+$ T cell responses *in vitro*, they may also be envisioned as a therapeutic modality to be injected directly into tumor lesions *in vivo* (where they may acquire and then preferentially prime Type-1 antitumor T cell responses). Overall, the potent capacity of DC.Tbet to promote Ag-specific Type-1 T cell responses while coordinately minimizing type 2/Treg functional responses suggests that (DC.Tbet-based) vaccines may yield enhanced therapeutic efficacy *in vivo* [174] in the settings of cancer and infectious disease.

3.0 INTRALESIONAL DELIVERY OF DENDRITIC CELLS ENGINEERED TO EXPRESS T-BET PROMOTES PROTECTIVE TYPE-1 IMMUNITY AND THE NORMALIZATION OF THE TUMOR MICROENVIRONMENT

Yanyan Qu[2], Lu Chen[1],Angela Pardee[1], Jennifer L. Taylor[2], Amy K. Wesa[1,2], and Walter J. Storkus[1,2,5]

From the Departments of [1]Immunology, [2]Dermatology, and [4]Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, and the [5]University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213

These data were reported in *Journal of Immunology (Sep. 2010* vol. 185*).* Lu Chen contributed to the study of the *in vitro* T cell polarizing function of mouse DC.Tbet. All authors contributed to the scientific discussion of this manuscript.

3.1 ABSTRACT

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* [175]. 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 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 within the tumor microenvironment it inhibited the accumulation of CD11b+Gr1+ myeloid-derived suppressor cells and normalized CD31+ vascular structures. These findings support the potential translational utility of DC.Tbets as a therapeutic modality in the cancer setting.

3.2 INTRODUCTION

Dendritic cells (DCs) play important roles in regulating the magnitude and nature of specific T cell responses that underlie effective cancer immunotherapy [34, 36, 176, 177]. In particular, the state of DC polarization may determine, in turn, the biased polarization of functional T cell responses [42], with Type-1 T cell-mediated immunity commonly linked with superior anti-tumor efficacy *in vivo* [178]. 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 [178, 179].

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/Th1 T cell responses *in vitro* [180]. Such conditioned DC1s are now being translated into phase I clinical trial designs for the treatment of patients with various forms of cancer [65, 181].

Alternatively, DCs have been engineered with cDNA encoding Type-1-polarizing cytokines, such as Type-1 and type 2 IFNs, IL-12p70 and IL-18 [66, 177, 182], among others, again yielding APCs with improved competence to drive and sustain Th1 and Tc1 immunity *in vitro* and/or *in vivo*. We have also recently demonstrated that infection of human DCs with a recombinant adenovirus encoding human T-bet, a transactivator protein associated with Type-1 polarity in T effector cells, results in these APCs attaining DC1 status *in vitro* [175]. Interestingly, these human DC.Tbets promote polarized Type-1 T cell responses via a mechanism that appears independent of secreted cytokines (including IL-12p70), but dependent on the DC–T cell interaction or the close approximation of these two cell types.

The present studies were performed to assess whether murine DCs engineered to express murine T-bet (mT-bet) exhibited comparable DC1 functionality *in vitro* and *in vivo* when

injected directly into tumor lesions in a murine sarcoma model. We report that DCs infected with recombinant adenovirus encoding mT-bet (DC.mTbets) mediate anti-tumor activity *in vivo* via the enhanced activation of anti-tumor Tc1 cells and the normalization of myeloid-derived suppressor cell (MDSC) levels and the vasculature within the tumor microenvironment (TME).

3.3 MATERIALS AND METHODS

Mice. Female 6- to 8-wk-old C57BL/6 (H-2^b) and BALB/c (H-2^d) 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 [183]. These cell lines were free of Mycoplasma contamination and were maintained in complete medium (CM; RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10 mmol/l L-glutamine; all reagents were purchased from Invitrogen, Carlsbad, CA) at 5% CO_2 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 adenoviruses were constructed through Cre-lox recombination [184]. 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) [3] using the restriction enzyme EcoRI, with the isolated cDNA then ligated into an EcoRI cloning site in the pAdLox shuttle vector [184], yielding pAdlox.mT-bet. After sequence validation of the plasmid, recombinant Ad.mT-bet was generated by cotransfection of pAdLox.mT-bet and ψ5 helper virus DNA into the adenoviral packaging cell line CRE8 [184]. Ad.mT-bet was purified from specific CRE8 lysates by cesium chloride density-gradient centrifugation and subsequent dialysis before storage in 3% threalose at −80°C. Titers of viral particles were determined by optical densitometry. The empty E1/E3-substituted, replication-defective adenoviral vector (Ad.ψ5) was used as negative control vector in all studies, as previously described [184, 185].

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 [177]. Briefly, bone marrow precursors were cultured for 7 d in CM supplemented with 1000 U/ml recombinant murine (rm)GM-CSF and 1000 U/m rmIL-4 (both from Peprotech, Rocky Hill, NJ). $CD11c^+$ DCs were then purified using specific MACS beads (Miltenyi Biotec, Auburn, CA) and infected with recombinant adenovirus (either Ad.ψ5 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 mTbet in Ad-infected DCs.

CMS4 therapy model. BALB/c mice received s.c. injection with 5×10^5 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 mm^2). Control DCs (DC.nulls or DC.ψ5 s) or DC.mTbets (10^6) 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 mm^2 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 mAb3-6.7 (provided by Dr. Zhaoyang You, University of Pittsburgh), or 50 μl anti-asialoGM1 polyclonal Ab (pAb; to deplete NK cells; Wako, Osaka, Japan). Ab-mediated depletion was >95% effective for the targeted immune cell subset based on flow cytometry analysis of peripheral blood monuclear 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 mAbs reactive against the mouse cell surface molecules H-$2K^d$, H-2-IA^d, 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 [175]. All flow analysis was performed using a FACScan flow cytometer and CellQuest software (BD Biosciences).

Western blotting. DC.nulls and gene-modified DCs (DC.ψ5 s or DC.mTbets) were harvested after 48 h of transduction by adenovirus vectors. Western blotting was then performed as previously described [186]. 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 goat anti-mouse Ab (both from Santa Cruz Biotechnology, San Diego, CA) were used to detect the expression of T-bet. β-actin was subsequently detected with rabbit anti-actin Ab (Abcam, Cambridge, MA) and HRP-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology) as an internal control. Probed 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 d after the second i.t. injection with adenoviral-transduced DCs (i.e., day 21 after tumor inoculation), and pooled splenocytes (2×10^6 cells/well) were stimulated with irradiated (100 Gy) CMS4 cells (2×10^5 cells/well) in the presence of 30 IU/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% CO_2 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 10:1 before being

washed with PBS and then restimulated with irradiated CMS4 versus control (MethA; H-2^d) tumor cells at a T cell-to-tumor cell ratio of 10:1 for 48 h. Cell-free supernatants were then stored at −80°C until analysis with cytokine-specific ELISA.

ELISA. In some experiments, control and transduced DCs were stimulated with $CD40L^+$ J558 cells [179] 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 [187]. Briefly, tumor tissues were harvested and fixed in 2% paraformaldehyde (Sigma-Aldrich) at 4°C for 1 h, then cryoprotected in 30% sucrose for 24 h. Tumor tissues were then frozen in liquid nitrogen and 6-mm cryosections prepared. For analysis of T cell subsets, sections were first stained with purified rat anti-mouse CD8α or purified rat anti-mouse CD4 mAbs (both from BD Pharmingen, San Diego, CA) for 1 h. After washing, sections were stained with Alexa Fluor 488-conjugated goat anti-rat secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). To detect DCs in tumor tissue, tissue sections were stained with FITC-conjugated anti-mouse 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+ MDSCs, 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 and rabbit anti-mouse NG2 (both from Millipore, Bedford, MA) for 1 h at room temperature, then washed with 0.5% BSA and stained with Alexa Fluor 488-conjugated goat anti-rat Ab and Cy3-conjugated goat anti-rabbit Ab (both from Invitrogen). To determine in situ cell death, an in situ cell death detection kit (Roche Diagnostics Systems) was used. After staining with primary and secondary Abs, the slides were washed and counterstained with 2 mg/ml Hoechst 33258 (Sigma-Aldrich) for 30 s. After washing, sections were then covered in Gelvatol (Monsanto, St. Louis, MO) and a coverslip was applied. Slide images were acquired using an Olympus 500 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 [184]. Control C57BL/6 (H-2^b) DCs (DC.nulls or DC.ψ5 s) 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-2^d) 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. Briefly, DC.mTbets or control DCs (5×10^5) derived from C57BL/6 mice were plated in the bottom chamber of a 24-well transwell plate in 400 μl CM, and, 24 h later, 10^6 BALB/c splenic T cells (MACS-isolated $CD4^+$

T cells or $CD8^+$ T cells) along with 10^5 H-2^b 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 $\mathbf{p} < 0.05$.

3.4 RESULTS

3.4.1 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.ψ5 (yielding DC.ψ5 s) at an MOI of 250 for 48 h. DCs were then assessed for expression of T-bet protein using immunofluorescence microscopy (Fig. 7**A**), intracellular staining as monitored by flow cytometry (Fig. 7**B**), and Western blotting (Fig. 7**C**). 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.ψ5 s expressed little or no detectable T-bet protein.

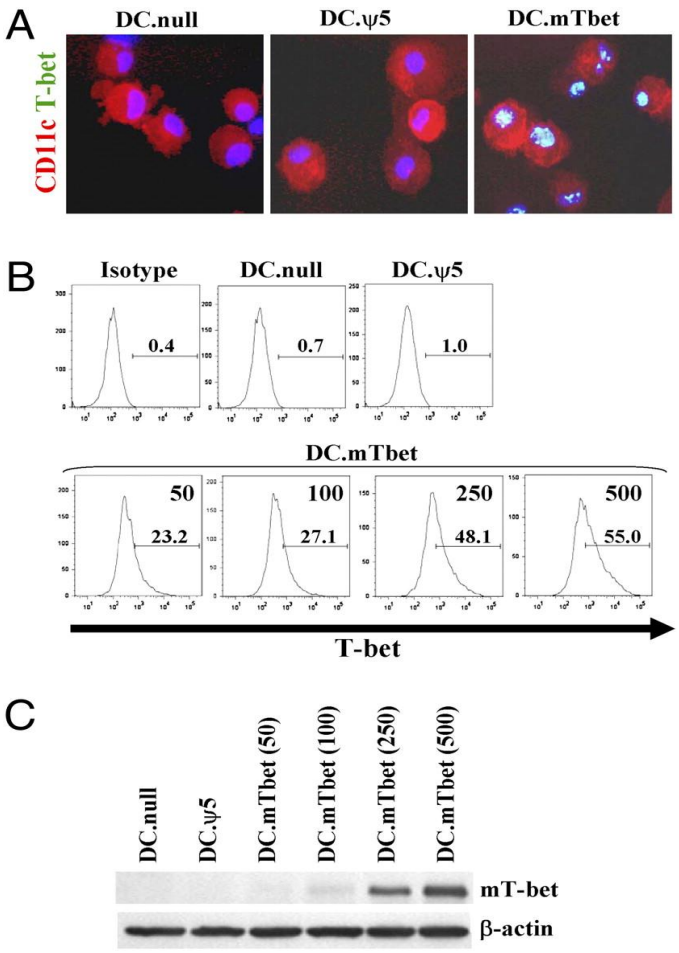


Figure 7. Ectopic expression of mT-bet in mouse CD11c$^+$ DCs

Murine DC.Tbets were generated from bone marrow CD11c$^+$ DCs. Bone marrow DCs were cultured in RPMI 1640 complete medium supplemented with rmGM-CSF and rmIL-4 at 37°C, 5% CO_2 for 7 d as previously described [188], prior to being transduced with adenovirus containing mT-bet cDNA. **A**, T-bet protein expression was localized to the nucleus in DC.mTbets (MOI of 250) when analyzed by immunofluorescence intracellular staining, with little to no expression of T-bet observed in control DCs (DC.nulls and DC.ψ5 s) (original magnification ×120). Intracellular staining and flow cytometry (**B**) and Western blot (**C**) analyses similarly supported differential expression of mT-bet protein in DC.mTbets versus control DCs. In **B**, inserted numbers in the upper right corner of DC.mTbet panels reflect the MOI used for adenoviral infection. Control Ad.ψ5 infections performed at MOI of 500. Numbers above gating region reflect the percentage of mT-bet$^+$ cells. In **C**, numbers in parentheses reflect MOI used for Ad.mT-bet infection of DCs. DC.ψ5 s were generated using an MOI of 500. All data are representative of information obtained in two independent experiments.

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 traffick to secondary lymph nodes (i.e., CCR7) to cross-prime T cells *in vivo*. When compared with DC.ψ5 s (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. (Data not shown) 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 revealed that DC.mTbets produced slightly more IL-12p70 and TNF-α than did control DCs (Fig. 8**A**). There were no significant differences between the DC cohorts with regard to IL-10 or IL-18 production (Fig. 8**A**), and none of the evaluated DC populations produced detectable intracelluar levels of IFN-γ (Fig. 8**B**). DC.mTbets also failed to secrete discernable levels of IFN-γ based on ELISA (data not shown).

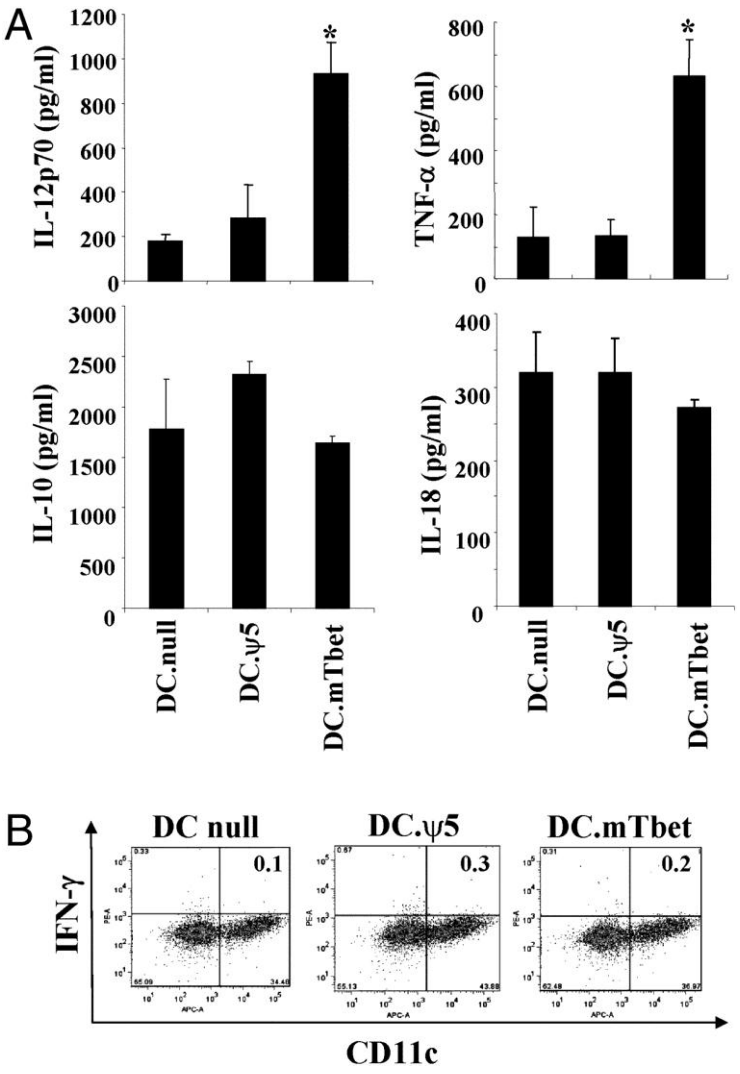


Figure 8. Impact of T-bet gene insertion on DC expression/secretion of cytokines *in vitro*

A, DC.Tbets versus control DCs were analyzed by ELISA for secretion of IL-12p70, TNF-α, IL-10, and IL-18 24 h after CD40 ligation, as outlined in *Materials and Methods*. Results were reported as the means ± SD of triplicate determinations. **p* < 0.05 for DC.mTbets compared with either DC.nulls or DC. ψ5s. **B**, Expression of intracellular IFN-γ was evaluated in DC.mTbets versus control DCs by flow cytometry. All data are representative of three independent experiments performed in each instance.

3.4.2 DC.mTbets promote superior Type-1 T effector cell induction *in vitro* without differentially affecting T cell proliferation compare to control DCs.

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 $CD4^+$ ($H\text{-}2^d$) T responder cells were cocultured with $H\text{-}2^b$ DC.nulls, DC.ψ5s, 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. 9**A** and 9**B**, there was no significant difference between the various DC cohorts in their ability to stimulate most allogenic T cells to enter into proliferative cycling. To investigate the potential Type-1-polarizing effects of DC.mTbets on T cell responders, a Staphylococcus enterotoxin B (SEB) model was employed. DC.mTbets or control DCs were pulsed with SEB *in vitro* for 3 h, then washed and cocultured with MACS-purified $CD4^+$ or $CD8^+$ T cells (from syngenic $H\text{-}2^b$ 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-γ production from responder $CD4^+$ (Fig. 9**C**) and $CD8^+$ (Fig. 9**D**) T cells. Hence, consistent with our previous findings for human DC.Tbets [175], murine DC.mTbets appear to promote improved Type-1 immune responsives by contributing differential polarizing, rather than proliferative, signals to responder T cells.

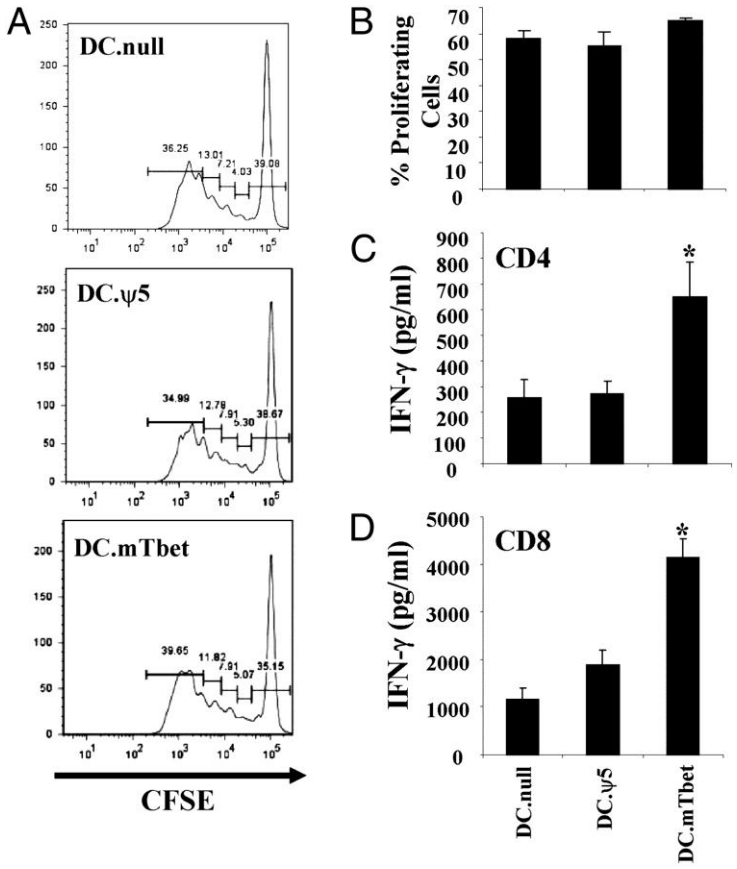


Figure 9. DC.mTbets promote superior Type-1 T cell responses *in vitro*, without affecting T cell proliferation

DC.nulls, **,** DC.ψ5s, and DC.mTbets were generated from C57BL/6 (H-2^b) mice and used to stimulate CFSE-labeled, MACS-purified $CD4^+$ T cells isolated from BALB/c (H-2^d) in MLR cultures as outlined in *Materials and Methods*. After 72 h, T cell proliferation (based on CFSE dilution) was analyzed by flow cytometry (**A)**, with the total percentage (mean ± SD) of proliferating T cells reported in **B**. To assess the differential capacity of DC.mTbets to polarize Type-1 T cell responses *in vitro*, an SEB model was applied. SEB-pulsed DC.mTbets or control DCs were cocultured with MACS-purified $CD4^+$ (**C**) or $CD8^+$ (**D**) T cells for 72 h. Cell-free supernatants from these cultures were then analyzed using mIFN-γ ELISA. Data are reported as the means ± SD of triplicate determinations. All experiments were performed three times, with comparable data obtained in all cases. *$p < 0.05$ for DC.mTbets versus DC.nulls or DC.ψ5s.

3.4.3 DC.mTbets promote superior Type-1 T effector cell induction *in vitro*

To determine whether DC.mTbets mediate superior Type-1 T cell activation via dominant mechanisms involving cell-to-cell contact or secreted mediators, we established transwell MLR cultures in which H-2b DCs were either cocultured with responder H-2d $CD4^+$ or $CD8^+$ T cells, or the two populations were separated from one another. As shown in Fig. 10**A** and 10**B**, 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.ψ5s. Inclusion of saturating doses of neutralizing anti–IL-12p70 pAb partially reduced the allostimulatory activity of DC.mTbets ($p < 0.05$ versus cultures supplemented with isotype control pAb). In contrast, when DC.mTbets (lower chamber) were separated from T cells cocultured with control DC.nulls (upper chamber), we observed no increased production of IFN-γ by responder CD8+ (Fig. 10C) or CD4+ (Fig. 10**D**) 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. 10**C**, 10**D**). Therefore, as was the case for human DC.hTbets [175], direct DC.mTbet–T cell interaction or close proximity appears crucial for the ability of these APCs to promote superior Type-1 T cell activation *in vitro* via a mechanism that is largely IL-12–independent.

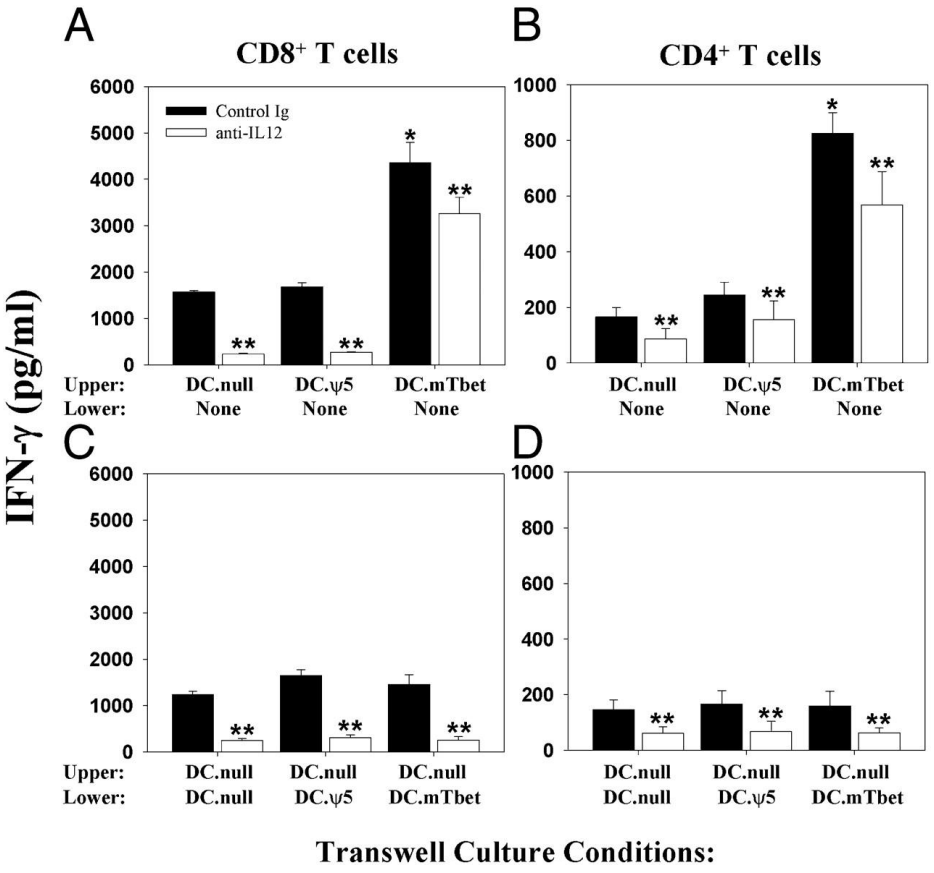


Figure 10. The optimal Type-1-polarizing capacity of DC.mTbets requires intimate DC–T cell contact and is partially dependent on IL-12p70

Transwell cultures were established using H-2^b DCs (i.e., DC.nulls, DC.ψ5s, and DC.mTbets) and H-2^d 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 2μg 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.ψ5 s. **$p < 0.05$ versus isotype control Ig.

3.4.4 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* [41, 67, 184], 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. 11**A**, 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. 11**B**; $p = 0.0012$ versus DC.nulls and $p = 0.0014$ versus DC.ψ5 s). 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. 11**C**). 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. 11**C**).

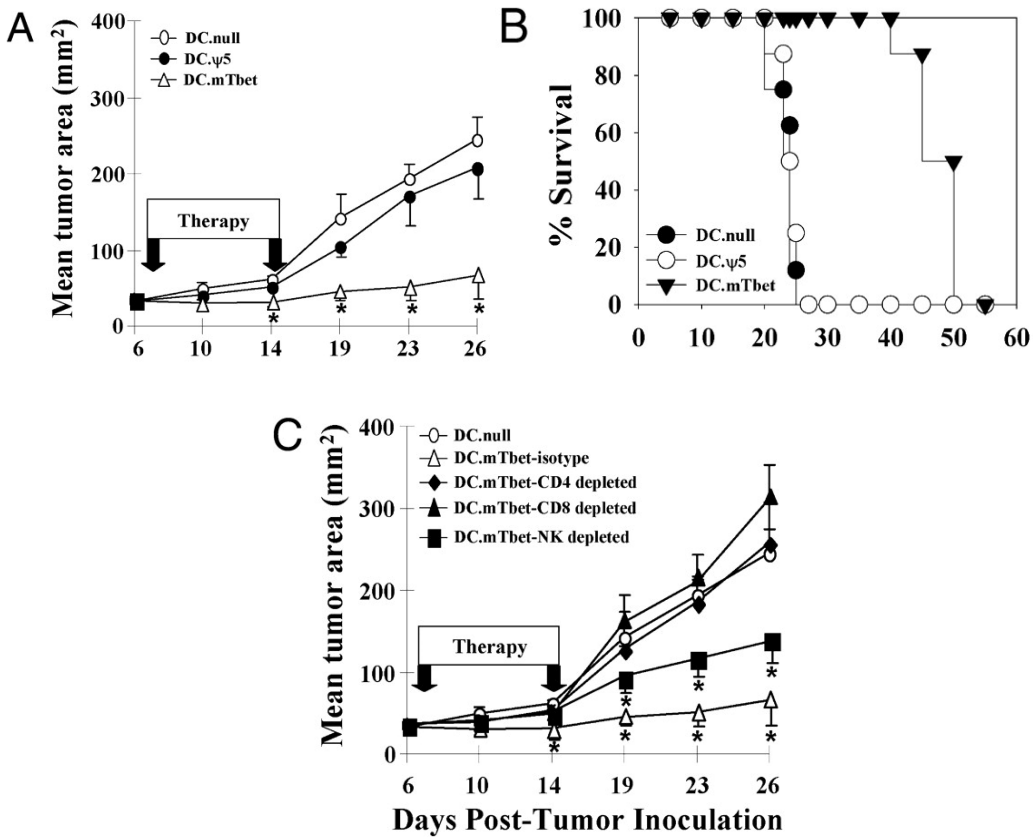


Figure 11. I.t. injection of DC.mTbets provides therapeutic benefit that is dependent on $CD4^+$ T cells, $CD8^+$ T cells, and NK cells

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.ψ5 s). 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.ψ5 s. **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. 11**A** 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.mTbets]) by specific Ab administration as described in **Materials and Methods**. ***p** < 0.05 when compared with mice treated with DC.nulls or DC.ψ5 s. Data are representative of three independent experiments performed in all cases.

3.4.5 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.mTbets or control therapy (i.e., day 21 post tumor inoculation). After specific MACS purification, $CD8^+$ T cells were stimulated *in vitro* with irradiated CMS4 tumor cells and analyzed for levels of intracellular IFN-γ as monitored by flow cytometry (6-h assay; Fig. 12**A**) or for secreted levels of IFN-γ by ELISA (24-h assay; Fig. 12**B**). 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.mTbets (Fig. 12). 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 $H\text{-}2^d$ MethA sarcoma cells (appendix Fig 5).

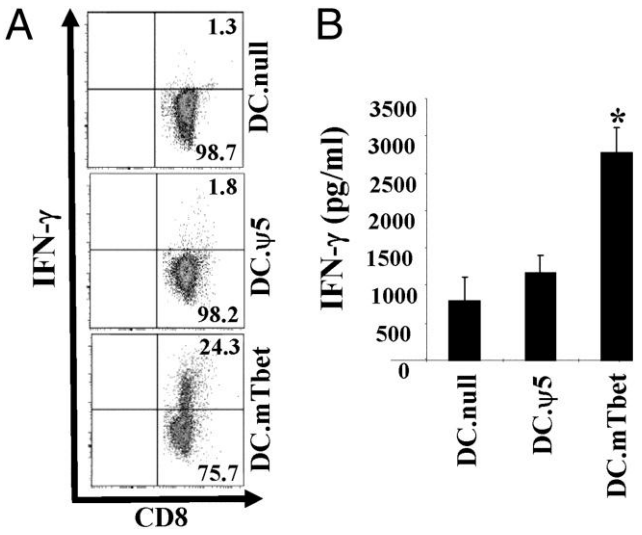


Figure 12. I.t. delivery of DC.mTbets promotes the systemic activation of Type-1 anti-tumor T cells

Mice bearing day 7 CMS4 tumors were treated as outlined in Fig. 10**A**. On day 21 posttumor inoculation, splenocytes were harvested and $CD4^+$ and $CD8^+$ T cells were purified by specific MACS. T cells were then stimulated in vitro with irradiated CMS4 tumor cells, and levels of intracellular IFN-γ were assessed by flow cytometry (**A**) and secreted levels of IFN-γ were determined by specific ELISA (**B**). ELISA values are reported as the means ± SD of triplicate determinations. ***p** < 0.05 for DC.mTbets versus DC.nulls or DC.ψ5 s. All data were confirmed in three independent experiments.

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.mTbets resulted in increased frequencies of tumor-infiltrating lymphocytes. As shown in Fig. 13, 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. 13). 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.mTbets (Fig. 13). This

large increase in TIDC appeared to result from two processes: (1) increased longevity of the injected DC.mTbets versus control DCs in situ (even though we observed no difference in the viability of DC.mTbets versus control DCs at the time of injection), and (2) improved recruitment of noninjected host $CD11c^+$ DCs into the TME post treatment with DC.mTbets versus control therapy (appendix Fig. 6).

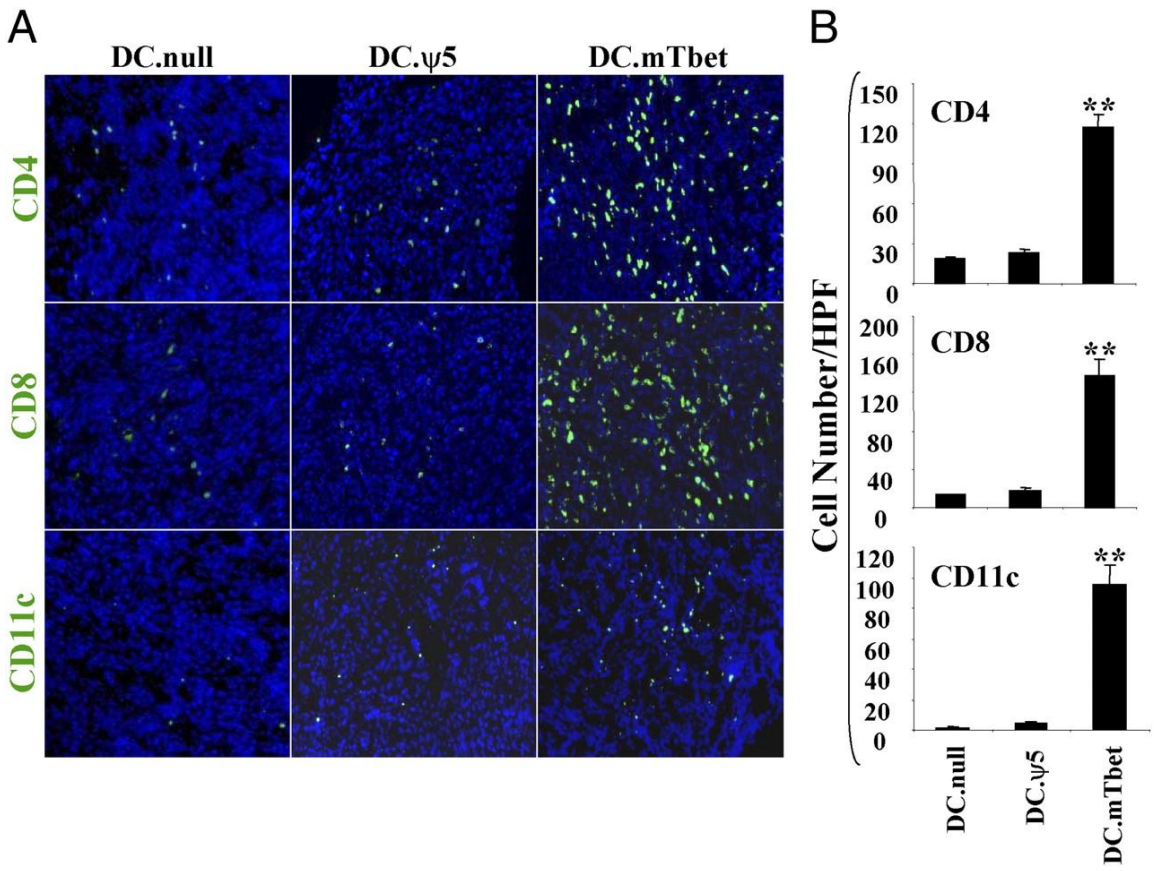


Figure 13. I.t. delivery of DC.mTbets promotes increased infiltration of T cells and DCs into the TME

BALB/c mice bearing day 7 CMS4 tumors were treated as described in Fig. 11**A**. On day 21, tumors were resected, fixed, frozen, and sectioned. Tissue sections were then stained with anti-CD4, anti-CD8, or anti-CD11c mAb, counterstained with Hoescht (nuclear) dye, and then analyzed by fluorescence microscopy as described in **Materials and Methods** (**A**) (original magnification ×20). We also report the means ± SD values for the number of tumor-

infiltrating $CD4^+$ T cells (**B**), $CD8^+$ T cells (**C**), and $CD11c^+$ cells (**D**) based on the imaging of 10 high-powered fields per slide. ****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.

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

$CD11b^+Gr1^+$ MDSCs are both necessary and sufficient to mediate the suppression of T and B cell responses in the TME [189]. To determine whether i.t. delivery of DC.mTbets alters the prevalence of MDSCs in the TME, tumor sections were costained with anti-CD11b and anti-Gr1 mAb and analyzed by fluorescence microscopy. 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 [187, 190, 191] where they may compromise anti-tumor T effector cells [190, 192, 193]. As shown in Fig. 14, 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 [194]. 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 [195]. 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. 14, $NG2^+$ pericyte-decorated $CD31^+$ vessels were dramatically reduced in their complexity (based on morphology and quantitated by calculating total $CD31^+$ cell surface area) in tumors treated with DC.mTbets versus control DCs. Indeed, as a consequence of DC.mTbet-based therapy, the weblike network of branched microvessels was eradicated, leaving behind simple vascular tube structures.

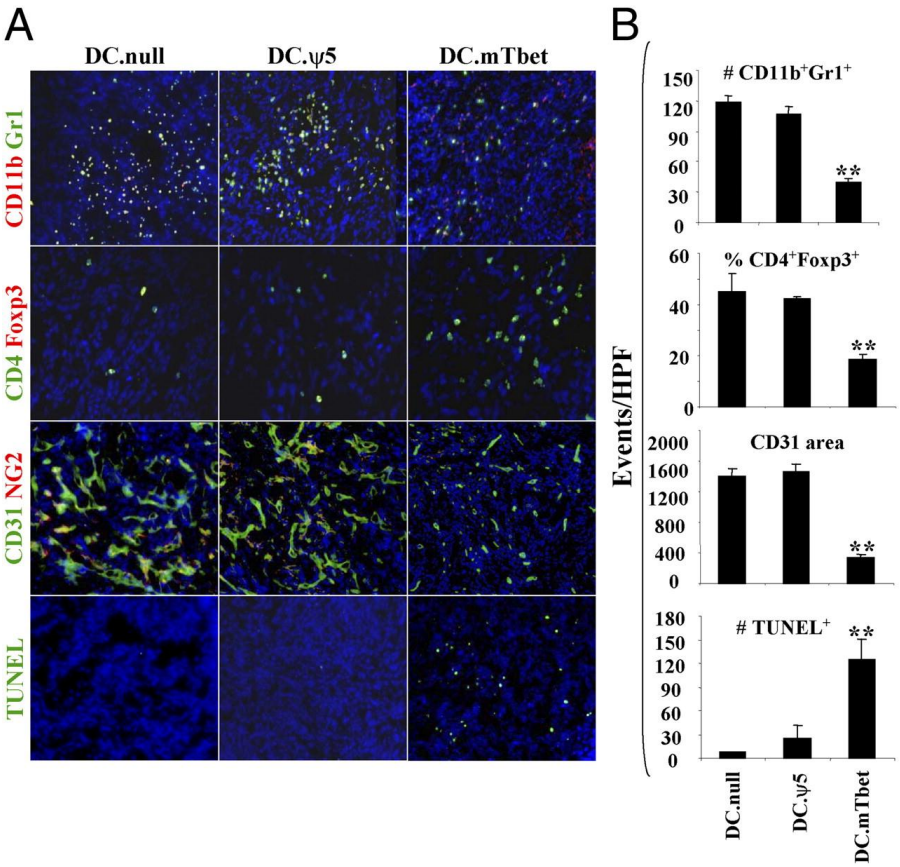


Figure 14. I.t. delivery of DC.mTbet reduces MDSC and Treg numbers, normalizes blood vessels, and enhances tumor cell apoptosis in the TME

A, Day 21 tumor sections prepared as described in the Fig. 13 legend and **Materials and Methods** were analyzed by fluorescence micrscopy to detect $CD11b^+Gr1^+$ MDSCs, $CD4^+Foxp3^+$ Tregs, $CD31^+NG2^+$ 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.ψ5 s. Three independent experiments were performed, with each yielding comparable data. HPF, high-powered field.

3.4.7 DC.mTbets-based therapy results in increased apoptosis in the TME

A corollary expectation for improved recruitment of Tc1 TIL effector cells and for the anti-angiogenic effects associated with i.t. delivery of DC.mTbets would be increased (tumor) cellular apoptosis in the TME of this treatment cohort. As shown in **Fig. 14**, the number of $TUNEL^+$ events within the CMS4 TME was increased by >10-fold after two treatment cycles with DC.mTbets versus control DCs.

3.5 DISCUSSION

Although T-bet is commonly considered as a master regulator of the Type-1 T cell responses [50, 196, 197], it also clearly plays a permissive role in supporting proinflammatory responses from cells of the innate immune system [198, 199]. 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 [50]. 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 asialo$GM1^+$ NK cells) in the treatment effectiveness of DC.mTbets. Notably, $CD8^+$ T cells isolated from the spleens and TDLNs of DC.mTbet-treated mice directly recognized (based on IFN-γ production) MHC class I^+, MHC class II^- CMS4 tumor cells *in vitro*. These effector cells are likely activated *in vivo* as a consequence of APCs that have acquired apoptotic/necrotic tumor debris and then emigrated from the therapy-normalized TME to the TDLNs and spleen. The therapy-induced tumoricidal process within the TME does not appear to involve the differential, direct tumoricidal activity of injected DC.mTbets, as these APCs exhibited only low, control-level capacity to promote the apoptotic death of CMS4 tumor cells *in vitro* (Data not shown).

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 cystein/cystine [200, 201]], the production of ROS and peroxynitrite [202] and the uncoupling of TCR-ζ–chain signaling [203], 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 [204, 205]. Alternatively, or additionally, early Type-1 T cell recruits into the TME may limit the development of MDSCs from precursor myeloid cells [206]. Regardless of such potential mechanisms, one could consider inhibition of residual MDSC numbers/function in the TME (using drugs such as sunitinib) 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 [175], 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 [175], 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 [175], 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 [175]. 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 cyotokine-deficient strains of animals. Given the breadth of available knockout strains (including IL-12p35$^{-/-}$ and IL-12p40$^{-/-}$ mice) on the H-2^{b} (but not H-2^{d}) 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.

4.0 EXTRANODAL INDUCTION OF THERAPEUTIC IMMUNITY IN THE TUMOR MICOENVIRONMENT AFTER INTRATUMORAL DELIVERY OF TBET GENE-MODIFIED DENDRITIC CELLS

Lu Chen, B.S.[1], Jennifer L. Taylor, Ph.D.[2], Nina Chi Sabins, B.S.[1], Devin B. Lowe, Ph.D.[2], Yanyan Qu, Ph.D.[3] , Zhaoyang You, Ph.D.[1,2] and Walter J. Storkus, Ph.D.[1,2,4]

From the Departments of [1]Immunology, [2]Dermatology and [3]Pediatrics, University of Pittsburgh School of Medicine and the [4]University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213.

These data were published in *Cancer Gene Therapy (*12 July 2013*)*. Lu Chen generated majority of the data. All authors contributed to the discussion part of this manuscript.

4.1 ABSTRACT

Murine dendritic cells (DC) transduced to express the Type-1 transactivator T-bet (i.e. mDC.Tbet) and delivered intratumorally (i.t.) are superior to control wild-type DC in slowing the growth of established subcutaneous (s.c.) MCA205 sarcomas in vivo. Protective immunity required the participation of both NK cells and $CD8^+$ T cells, whose induction was independent of the ability of injected mDC.Tbet to produce IL-12 family member cytokines or IFN-γ, or to migrate to tumor-draining lymph nodes (TDLN) based on CCR7 ligand chemokine recruitment. However, optimal therapeutic protection afforded by i.t. delivered mDC.Tbet did require that the injected DC to express MHC class I molecules and was associated with the acute recruitment of (naïve) T cells and NK cells into the treated TME. 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. When taken together, our data suggest that protective anti-tumor $CD8^+$ T cell priming resulting from mDC.Tbet gene therapy predominantly occurs extranodally within the tumor site. Consistent with such a paradigm, we observed that mDC.Tbet (versus control mDC.Null) intrinsically produce elevated levels of the chemokines and promoted the acute infiltration of NK cells and naïve $CD45RB^+$ T cells into the TME.

4.2 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 [207-209]. 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 [210, 211]. 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 [209, 212]. 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 [213-216]. 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 [217].

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 [218], and that when injected directly into established CMS4 sarcoma lesions in Balb/c ($H\text{-}2^d$) mice, that protective immunity results [219]. 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.

4.3 MATERIALS AND METHODS

Mice. Female 6-8 week old wild-type C57BL/6 (H-2b) mice, as well as, IL-12p35$^{-/-}$, IL-12p40$^{-/-}$, IFN-$\gamma^{-/-}$, β2M$^{-/-}$ and CCR7$^{-/-}$ mice (all on the B6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). Female CD11c-DTR (H-2b) conditional DC-deficient mice were kindly provided by Dr. Adrian Morelli (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 according to 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. 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 <u>in vitro</u>. DC were generated from the tibias/femurs of mice, and infected with recombinant adenovirus (either empty, control Ad.ψ5 or Ad.mT-bet) at an MOI of 250 for 48h, as previously described [219]. 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 x 10^5 MCA205 sarcoma cells in the right flank on day 0. On day 7 or 8 post-tumor inoculation, 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 or 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.

<u>In vivo</u> depletion of $CD8^+$ T cells, NK cells and $CD11c^+$ DC. In selected experiments where indicated, mice were injected i.p. with 100 µg anti-CD8 mAb3-6.7 (ATCC) or 50 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 [220]. 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, and pooled splenocytes stimulated with irradiated (100 Gy) MCA205 cells (2 x 10^5 cells/well) in the presence of 30 IU/ml recombinant human IL-2 (Chiron Corp., Emeryville, CA) for 5 days in 24 well culture plates. Responder CD8$^+$ T cells were then isolated using magnetic bead cell sorting (Miltenyi Biotec, Auburn, CA), then cocultured with MCA205 tumor cells or irrelevant control tumor cells (T cells: tumor cells = 10:1) in 96-well round bottom plates in a humidified incubator at 37°C and 5% CO_2 for 48h. Cell-free supernatants were stored at -80^{o}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 $\pm$ SD.

Imaging of tumor tissues. Tumor samples were prepared and sectioned as previously reported [219]. 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, followed by Cy3-conjugated donkey

anti-goat pAb (both from 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 [219]. 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 RT-PCR array. Total RNA was isolated from mDC.Tbet and mDC.Null using Trizol reagents (Invitrogen). Total RNA was further purified using the RNeasy Plus Mini 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 the expression of 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.

4.4 RESULTS

4.4.1 Therapeutic benefits of intratumoral delivery of mDC.Tbet are both 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. 15**A**) but not **B** and T cell-deficient $RAG1^{-/-}$ mice (Fig. 15**B**). 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. 15**C**, 15**D**). 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. 15**D**).

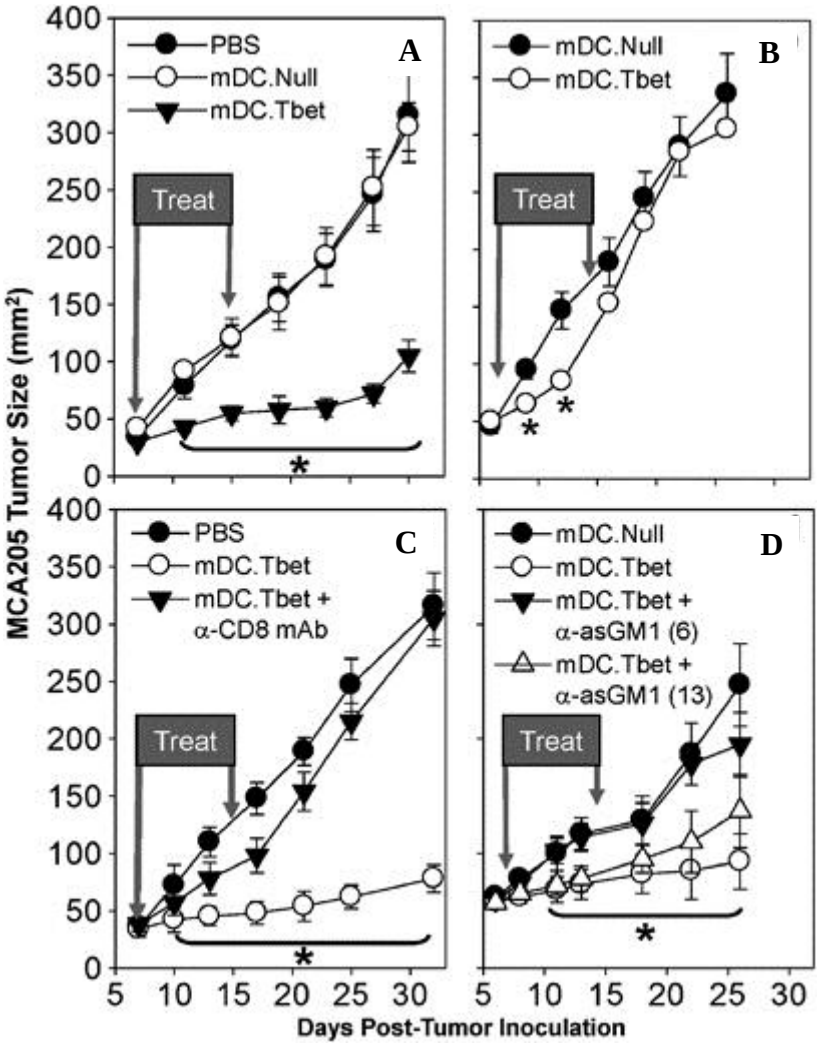


Figure 15. Intratumoral delivery of mDC.Tbet into established subcutaneous MCA205 sarcoma slows tumor growth via a mechanism involving innate and adaptive immunity

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. Phosphate-buffered saline or 10^6 mDC (as indicated) were then injected directly into subcutaneous MCA205 sarcomas established in syngenic wild-type (**A**) or RAG1$^{-/-}$ (**B**) mice on days 7 and 14 after tumor inoculation. The experiment described in a was then repeated, with cohorts of mDC.Tbet-treated mice also receiving intraperitoneal injections of depleting anti-CD8 (**C**) or anti-NK (i.e. anti-asGM1 on days 6, 13 and 20; or on days13 and 20; **D**) antibodies. In all cases, tumor growth was monitored every 3–4 days and is reported in mm^2 (mean±s.d. of five animals per group). Data are representative of three independent experiments performed. *P<0.05 versus phosphate-buffered saline or mDC.Null on the indicated days of analysis (ANOVA).

4.4.2 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 [218]. However, in contrast to hDC.Tbet that are poor cytokine (including IL-12p70 and IFN-γ) secretors when compared with their untransfected counterparts [218], mDC.Tbet actually produce significantly more IL-12p70 than control DC [219] 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. 16, 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. 16**A**), or IFN-γ$^{-/-}$ (Fig. 16**B**) 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.

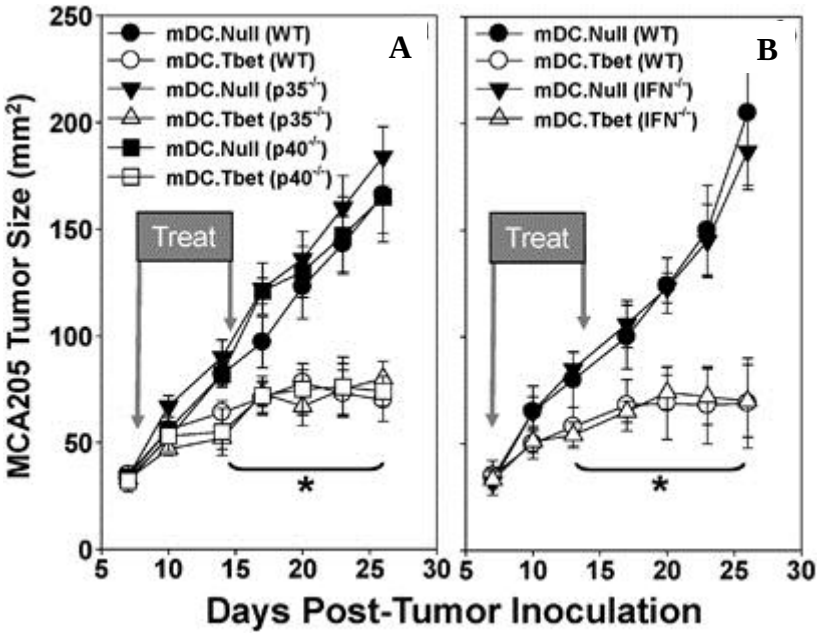


Figure 16. Delivery of mDC.Tbet into tumors mediates antitumor activity independent of the intrinsic capacity of the injected DCs to produce IL-12 family member cytokines or IFN-γ

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 (**A**) or IFN-γ$^{-/-}$ (IFN$^{-/-}$) mice (**B**). The various DC (10^6) were then injected directly into subcutaneous MCA205 sarcomas established in wild-type C57BL/6 mice on days 7 and 14 after tumor inoculation. Tumor growth was then monitored every 3–4 days and is reported in mm^2 (mean±s.d. of five animals per group). Data are representative of three 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).

4.4.3 Intratumoral delivery of DC.Tbet generated from β2M$^{-/-}$ 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 [218, 219]. 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 $\beta 2M^{-/-}$ 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 $\beta 2M^{-/-}$ (i.e. mDC.Tbet ($\beta 2M^{-/-}$)) 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 ($\beta 2M^{-/-}$)-treated cohort re-established accelerated growth kinetics versus tumors in mice treated with mDC.Tbet (WT) cells (Fig. 17**A**). 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 ($\beta 2M^{-/-}$)-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. 17**B**). Indeed, anti-MCA205 responses among $CD8^+$ T cells harvested from mice treated with mDC.Tbet ($\beta 2M^{-/-}$) 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. [229], but that it is likely needed for the sustained function and 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 ($\beta 2M^{-/-}$) 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. 17C**, all therapies using mDC.Tbet (from either WT or $\beta 2M^{-/-}$ 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 followed by mDC.Tbet ($\beta 2M^{-/-}$), followed by mice receiving mDC.Tbet ($\beta 2M^{-/-}$) on day 7 regardless of which secondary treatment was applied. These results suggest that durability of protective immunity ($\geq$ 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 ($\beta 2M^{-/-}$) 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 ($\beta 2M^{-/-}$) cells rather than DC.Tbet (WT) cells are delivered on day 14 post-inoculation.

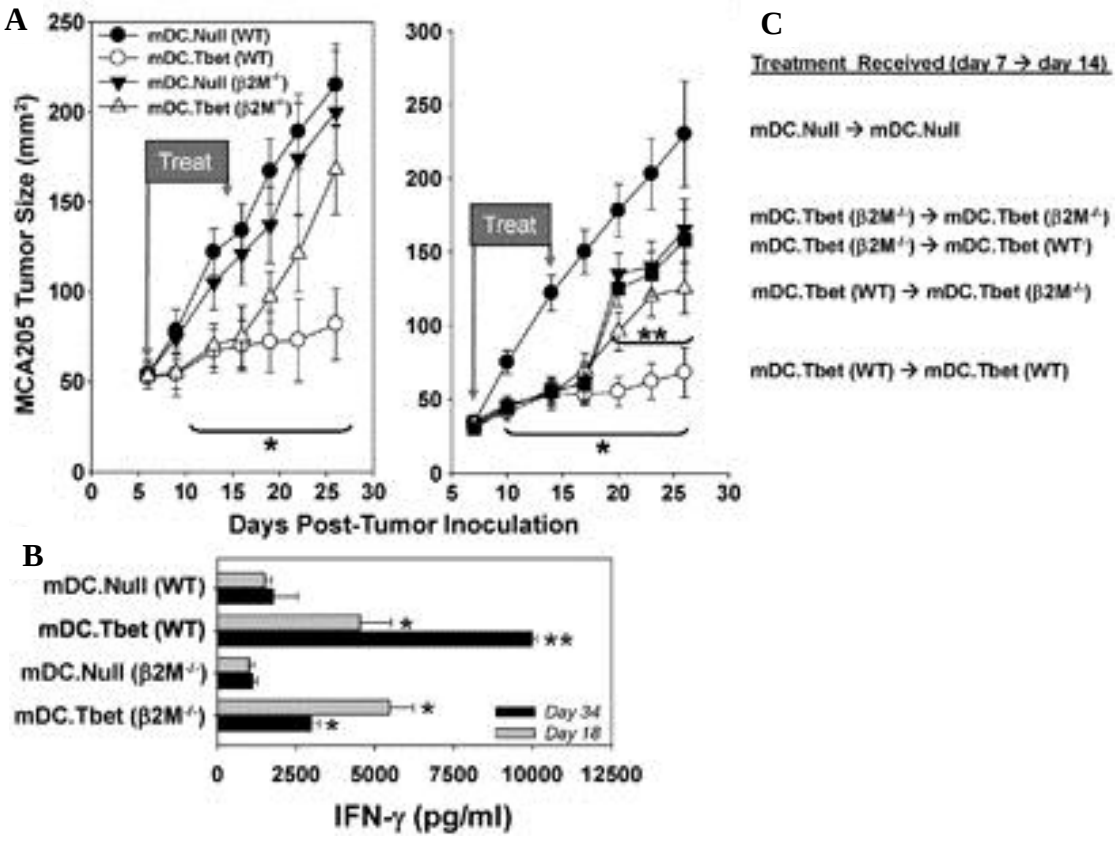


Figure 17. Intratumoral therapy with DC.Tbet developed from β2M$^{-/-}$ mice promotes a transient phase of antitumor benefit that ultimately fails, leading to the re-establishment of progressive tumor growth which cannot be 'rescued' by booster injections of mDC.Tbet

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 subcutaneous MCA205 sarcomas in C57BL/6 mice on days 7 and 14 after tumor inoculation. Tumor growth was then monitored every 3–4 days and the tumor size reported in mm^2 (mean±s.d. of five animals per group). In **B**, on day 18 or 34 after 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 cocultured in the absence or presence of irradiated MCA205 tumor cells at a 10:1 (T cell-to-tumor cell ratio) for 48 h, 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, a study design was repeated, with the exception that the second intratumoral injection of mDC.Tbet (day 14 after tumor inoculation)

was either mDC.Tbet (WT) or mDC.Tbet ($\beta 2M^{-/-}$). Data are representative of three 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 three independent experiments performed.

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

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) [221, 222] activate host DC populations known to effectively crossprime T cells, such as $CD8\alpha^+CD11c^+$ DC or $CD103^+CD11b^{neg}$ DC [223-225], 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 $CD8\alpha^+CD11c^+$ and $CD103^+CD11b^{neg}$ DC; ref. [224]) as a therapy on days 7 and 14 post-tumor inoculation. In both instances, mDC.Tbet-based treatment provided substantial and sustained anti-tumor protection (Fig. 18A) suggesting that these important host cross-priming DC populations are not critically required in this process. Since additional host $CD11c^+CD8\alpha^{neg}$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. 18B, 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 [226-229]. 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 days7 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. 18C).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.

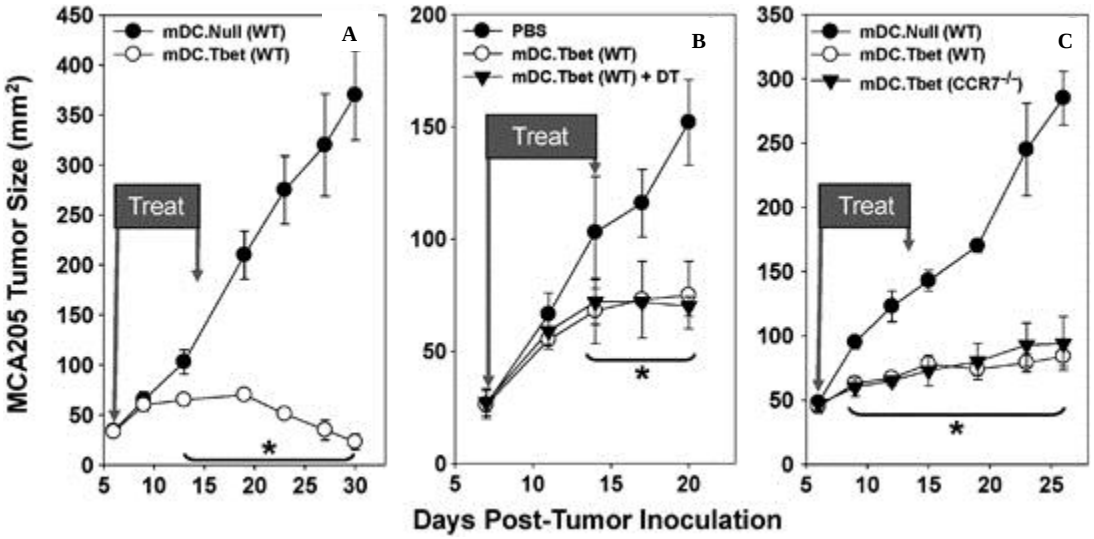


Figure 18. Host crosspresenting DC and the ability of intratumorally delivered mDC.Tbet (WT) to traffick to tumor-draining lymph nodes are not required for the antitumor efficacy of this therapeutic approach

MCA205 tumors were established subcutaneous in the flanks of syngenic BATF3$^{-/-}$ (**A**) or CD11c-DTR (**B**) mice. On days 7 and 14 after tumor inoculation, 10^6 mDC.Null (WT) or mDC.Tbet (WT) were injected intratumorally, with tumor growth monitored every 3–4 days and the tumor size reported in mm^2 (mean±s.d. of five animals per group). In b, host CD11c$^+$ DC were depleted *in vivo* by intraperitoneal administration of DT as described in Materials and methods section. In **C**, established subcutaneous MCA205 tumors in wild-type C57BL/6 mice were treated with days 7 and 4 intratumoral 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 three 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 b for mDC.Tbet (WT) versus mDC.Tbet (WT)+DT or in c for mDC.Tbet (CCR7$^{-/-}$) versus mDC.Tbet (WT) (ANOVA). Panel data are representative of three independent experiments performed.

4.4.5 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 suggest that vast majority of DC delivered into a tumor lesion *in vivo* fail to migrate out of the tumor lesion [219, 230, 231], suggesting that their predominant therapeutic impact likely occurs within the TME. Although somewhat unconventional, previous reports suggest that the crosspriming of naïve, antigen-specific T cell responses can occur in extranodal tissue sites including bone marrow, skin, lungs and even tumors [228, 232-234] 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-2b) Tbet-ZsGreen reporter mice that encode ZsGreen protein driven-off a genomic Tbet/TBX21 promoter [235]. 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 (Tbet-ZsGreen) green fluorescence of these lymphoid subsets [235]. As shown in Fig. 19A/19B, within a 48h period of administering mDC.Tbet into tumors, a dramatic increase in Type-1 (T-bet+) NK cells as well as CD4+ and CD8+ T cells within the TME was observed when compared to MCA205 tumors in control treated mice. Consistent with many of these recruits representing "naïve" cell populations, fluorescence microscopy revealed a preponderance of $CD45RB^+CD3^+$ TIL in mDC.Tbet (WT) but not control-treated mice (Fig. 19C; appendix Table I), with 60% of $CD45RB^+CD3^+$ T cells coexpressing Tbet-$ZsGreen^+$ (appendix Table 1).

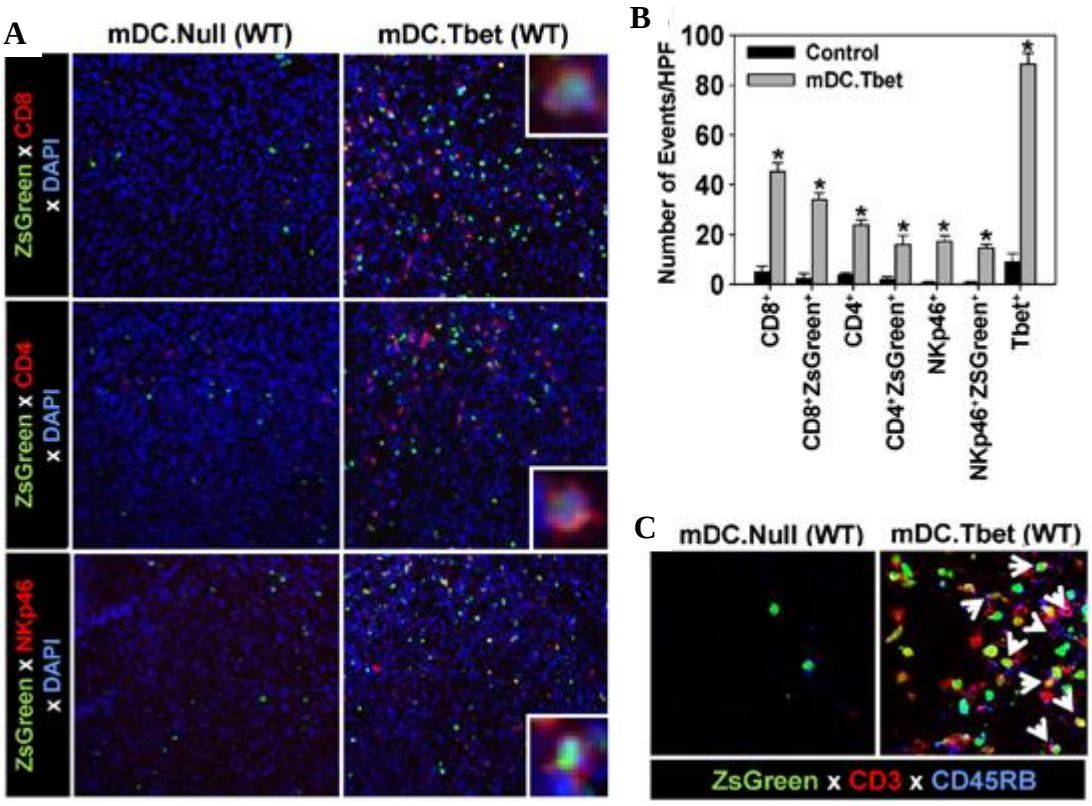


Figure 19. Early recruitment and activation of Type-1 T cells and NK cells in the TME after intratumoral delivery of mDC.Tbet versus mDC.Null

In **A**, MCA205 tumors were established subcutaneous in the flanks of syngenic (H-2^b) Tbet-ZsGreen reporter mice. On day 7 after tumor inoculation, 10^6 mDC.Null (WT) or mDC.Tbet (WT) were injected intratumorally. After 2 days, (i.e. days 9 after 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 **A** images was performed using the Metamorph software and is reported as the mean±s.d. of 10 high-power fields per specimen. In c, tumor sections from **A** were analyzed by fluorescence confocal microscopy for the presence of 'naïve' T cells based on coexpression of CD45RB (blue) and CD3 [236], 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 appendix Table 1. Panel data are representative of three independent experiments performed.

Since such acute recruitment (and "priming") 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. 20**, mDC.Tbet intrinsically produced delevated levels of the chemokines CCL1, CCL4, CCL6, CCL12, CCL17, CXCL12 and CXCL15 when compared with control DC.

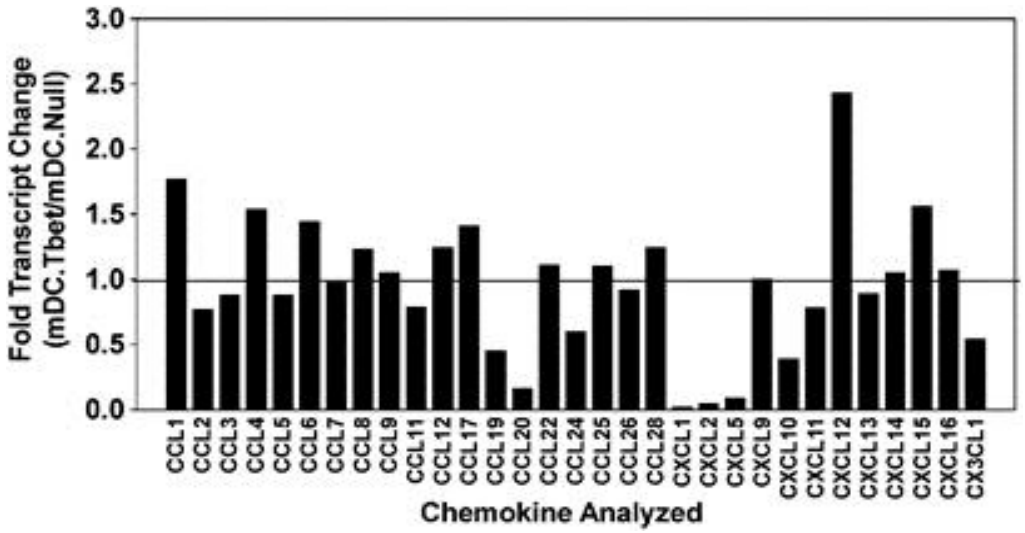


Figure 20. Differential expression of chemokine transcripts by mDC.Tbet versus control mDC.Null

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 reverse transcription-polymerase chain reaction 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.

4.5 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 slows 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, which allows extranodal priming of protective immunity in the TME. Our hypothetical model of this paradigm does not preclude additional (more conventional) cross-priming 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;) [237-239]. 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 [228, 232, 234, 240-242]. Notably, the (cross)priming of protective T cells can be leveraged by the selective production of recruiting chemokines, as a consequence of gene therapies applied to the TME [242, 250-252] [87, 233, 243]. NK cells, which can mediate tumoricidal activity as well as the "licensing" of DC for improved T cell crosspriming capacity [253, 278] [244, 245], 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 [246-248]. Also of significant interest, a recent report by Messina *et al.* [247] 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 the NK (CCL1, CCL4, CCL6, CXCL12) and naïve T cell (CCL4, CCL17, CXCL12) recruiting chemokines **Fig. 6;** refs. [238]). 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) [68] and CCL4 were also overexpressed at the transcript level by human DC.Tbet versus

control human DC (appendix Table 2). Future experiments employing neutralizing antibodies or shRNA 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 [249]. 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 [250]. Alternatively or additionally, soluble β2M (found in microgram/ml quantities in serum) may be taken up along with tumor antigens by mDC.Tbet ($\beta 2M^{-/-}$), allowing for the stabilization of sufficient MHC I/tumor peptide complexes [251] on the injected DC cell surface to allow for at least a limited degree of specific T cell induction in the TME. It is also conceivable that mDC.Tbet $\pm$ 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 inefficient APC for this purpose [178].

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 [228]. When challenged with aerosolized live mycobacteria, $CCR7^{-/-}$ animals crossprime specific T cells in the lungs rather than the mediastinal lymph nodes [228]. 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 [228]. 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 early evidence of Type-1 polarization (Fig. 19**C**, appendix Table **1**).

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 [179, 252, 253]. Thus DC engineered to produce IFN-γ enhanced recruitment of i.v. administered Type-1 anti-tumor $CD8^{+}$ T cells in a CXCL10/IP-10 chemokine-dependent manner [178]. Also in this light, several recent reports [179, 252] 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 [178, 219], thereby reinforcing the recruitment and/or sequestration of protective anti-tumor $CD8^{+}$ T cells into/within the TME and extending the window of therapeutic tumor management.

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.

5.0 SUMMARY AND INTERPRETATION

5.1 PROPOSED MODEL FOR THE ANTI-TUMOR ACTION OF DC.TBET

Based on the notion that T-bet expression in DC subsets controls the Type 1 priming function of these cells [4, 153], we hypothesized that the reinforcement of T-bet expression in DCs using rAd.T-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*. We first demonstrated that gene-modified human dendritic cells that highly express T-bet protein (DC.Tbet) strongly promoteD Type-1 T cell polarization from naïve cell responders while concomitantly suppressing Th2 and Treg development [175]. ref. Interestingly, the Type-1 polarizing function of human DC.Tbet seems to be largely independent of IL-12 family cytokines as these DCs were suppressed in their production of IL-12p70, IL-23, and IL-27, as well as all other cytokines evaluated; and neutralizing Abs against IL-12p70, IL-23p19, IL-12Rβ2, and IL-27R all failed to attenuate DC.Tbet-mediated induction of Type-1 responses from naive T cells [175]. Similar results were observed in our analyses of murine DC.Tbet which displayed potent Type-1 T cell polarizing function that was not affected by the provision of IL-12 neutralizing antibody. Murine DC.Tbet also mediated potent anti-tumor activity when injected intratumorally into MCA205 (H-2^b) sarcoma tumor bearing mice (C57BL/6 mice), 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 the TME.

Using the MCA205 ($H\text{-}2^b$) sarcoma model and transgenic mice as sources of injected DC.Tbet into tumor-bearing mice, we characterized a series of molecular and cellular events that are likely responsible for the anti-tumor function of DC.Tbet. Consistent with the IL-12 independent Type1-polarizing function observed in DC.Tbet *in vitro*, the protective effects of i.t.-delivered DC.Tbet were demonstrated to be independent of the intrinsic production of IL-12 family cytokines (IL-12, IL-23, and IL-27) and IFN-γ. Since IL-12 family cytokines are important anti-tumor mediators, these data indicate that DC.Tbet employ a unconventional therapeutic mechanism(s) that is less dependent on the cytokines secreted by DCs. Interestingly, depletion of either NK cell or $CD8^+$ T cell populations completely ablated anti-tumor benefit afforded by DC.Tbet suggesting that both NK cells and $CD8^+$ T cells were required for the anti-tumor immunity driven by DC.Tbet. NK cells were needed throughout the whole treatment as late depletion of NK cells (beginning on day 13 post tumor inoculation) still affected the therapeutic benefit of DC.Tbet. In this regard, DC.Tbet might work through NK cells to optimally activate tumoricidal $CD8^+$ T cells, which would explain the nearly complete ablation of therapeutic effect of DC.Tbet upon NK depletion. Indeed, it has been reported that the cross-talk between DCs and NK cells in the TME or SLO facilitated the development of anti-tumor immune responses [254, 255]. NK-DC crosstalk promotes DC1 (Type-1 DC) development and the corollary induction of Th1 and CTL responses [256, 257]. On another hand, DC-based vaccination promotes NK cell activation which may also be required for subsequent inhibition of tumor metastasis [258]. DCs can activate NK cells through cytokines such as IL-12, IL-15 and

Type-1 IFN which are known to enhance NK cell production of IFN-γ, proliferation and cytolytic activity [256]. Cell-cell interactions between DC and NK cells might also activate NK cells through surface receptors on DCs (such as NKG2D) [259, 260]. In our study, the mechanisms by which DC activate NK cells may involve the direct transfer of T-bet protein from DC.Tbet to NK cells, as indicated by the transfer of T-bet protein from DC.Tbet (but not control DC) to T cells *in vitro* (Appendix Fig. 8), and T-bet as an important transcription factor controls the maturation and effector function of not only T cells, but also NK cells [61].

MHC class I expression on DC.Tbet is required for their optimal anti-tumor function as $\beta 2m^{-/-}$DC.Tbet used as either the first or the second treatment modality failed to induce efficient anti-tumor immunity when compared to tumor-bearing mice treated i.t. with wild-type (WT) DC.Tbet. It appears that although MHC class I expression on injected DC.Tbet may not be required for the initial induction of anti-tumor immunity, but that it is likely needed for the sustained function and therapeutic action of T effector cells in treated mice on or after day 16 of treatment. Interestingly, despite the expected requirement of MHC class I for direct cross-presentation of antigen to T cells by injected DC, $\beta 2m^{-/-}$ DC.Tbet were still able to induce WT-level tumor-specific $CD8^+$ T cell responses in the first 10 days post–treatment. Several mechanisms might explain this result: 1) $\beta 2m^{-/-}$ DC.Tbet might pick up soluble β2m from the TME or they might acquire intact MHC-peptide complexes from tumor cells upon cell-cell contact; 2) $\beta 2m^{-/-}$ DCTbet may recondition the TME so that tumor cells/other innate immune cells such as macrophages could serve as APCs to prime tumor-specific CTL responses; 3). $\beta 2m^{-/-}$ DC.Tbet may activate NK cells to facilitate the T cell priming in the TME.

$CCR7^{-/-}$ DC.Tbet that are incapable of migrating to lymph node still provided a similar level of anti-tumor protection as WT DC.Tbet, indicating that the therapeutic action mediated by

DC.Tbet likely occurred principally in the TME, with minimal required involvement of SLO. As early as day 2 post-treatment, a dramatic increase in Type-1 (T-bet^{+}) NK cells as well as CD4^{+} and CD8^{+} T cells were observed within the TME when compared to MCA205 tumors in control treated mice; naïve T cells (CD3^{+}CD45RB^{+}) were also observed in the TME within 48hrs post treatment with around 60% of these TIL expressing T-bet (T-bet-ZsGreen^{+}). A corroborative staining with CD44 (CD3^{+}CD45RB^{+} CD44^{-} or CD3^{+}CD62L^{+} CD44^{-}) should be used in future experiments to better discern the naïve T cell phenotype. It is likely that DC.Tbet support the extranodal priming of naïve T cells in the TME, possibly in the tertiary lymphoid organs (TLOs) induced by intratumorally injected DC.Tbet. The formation of TLOs has been reported in the TME of many tumor types (lung cancer, melanoma, colon cancer etc), and the presence of TLOs in TME usually predicted better clinical outcome [247, 259, 260]. Immunohistochemichal staining of tumor sections isolated 5 days post DC.Tbet treatment revealed lymphoid-like structures featuring condensed T cells areas in the TME. Further staining for B cells (B220, CD19) and HEV markers (MECA-79, HECA-452, G72, and G152) are needed to define these structures.

Normally, CCR7 expression is required for the migration of DC into draining SLO [261, 262]. Although DC.Tbet lacking CCR7 (generated from CCR7$^{-/-}$ mice) provided wild type therapeutic protection, it was unclear whether a very small (but highly functional) portion of injected DC.Tbet trafficked into LN leading to the priming of anti-tumor immune responses in the TDLN. Thus, in future studies, DC.Tbet traffick *in vivo* should be analyzed in depth using intravital microscopy tracing of fluorescently-labeled DC.Tbet or immunofluorescence staining of DC.Tbet (labeled or origins from T-betZsGreen^{+} mice) in LN at various longitudinal time points. Mice lacking functional SLO such as Lymph Toxin α-deficient (LTα$^{-/-}$) mice would

serve as a useful tool to study if SLOs including TDLN is at all needed in DC.Tbet mediated anti-tumor Type-1 immunity development.

It will also be important to identify the "pioneer" cell types that are recruited into the TME immediately post treatment, as these cells might play crucial roles in the establishment of TLOs in the TME. It is possible that extranodal priming of anti-tumor T cell responses is not just restricted to DC.Tbet based therapy but is a common mechanism associated with other DC-based vaccine strategies (with variation based on the magnitude of TLO formation elicited). Analysis of extranodal T cell priming and the formation of TLOs in the TME of mice treated with other DC vaccines will be necessary to establish the generalizable impact of extranodal T cell priming on the anti-tumor efficacy of DC-based cancer immunotherapies.

Based on these data, I would propose a hypothetical model for the anti-tumor action of DC.Tbet (Figure 21). In this model, DC.Tbet upon i.t. delivery into TME, promotes rapid recruitment of naïve T cells and NK cells through: 1) chemokines secreted by DC.Tbet 2). chemokines produced by other immune cells (Macrophage, NK cells etc) or tumor cells reconditioned by DC.Tbet. Once arrive in the TME, these immune effector cells further enhance chemokines production in the TME through interacting with the tumor cells or other immune components in the TME, which continue to recondition the TME and lead to the recruitment of subsequent immune cells. It is likely that $CD4^+$ T cells are among the early recruited cell populations as the mature $CD4^+$ T cells interact with DCs to promote the formation of TLOs. The majority of anti-tumor T cell (cross)priming happens extranodally in the TME, with DC.Tbet strongly promoting the development of Th1 and tumor killing $CD8^+$ T cells; these primed $CD8^+$ T cells remain in the TME where they are sustained by secondary i.t. injection of mDC.Tbet, although some peripheral (tumor-draining lymph nodes, spleen) expansion of the

antitumor $CD8^+$ T-cell may also be therapeutically potentiated over time. On another hand, DC.Tbet reduce the number of immunosuppressive cells MDSC and Tregs, and promote the normalization of tumor vasculature which facilitate the anti-tumor immunity in the TME. The early tumor regression observed in 7-10 days post the first DC treatment are not heavily dependent on the direct cross-priming of $CD8^+$ T cells by DC.Tbet, rather it may result from multiple alternative mechanisms: 1) NK cells are recruited and activated by DC.Tbet through DC-NK crosstalk which enhances the NK cytolytic activity and promotes the helper activity of NK cells; 2) DC.Tbet recondition the TME to recruit immune cells and to foster the non-DC mediated tumor antigen presentation to anti-tumor $CD8^+$ TIL.

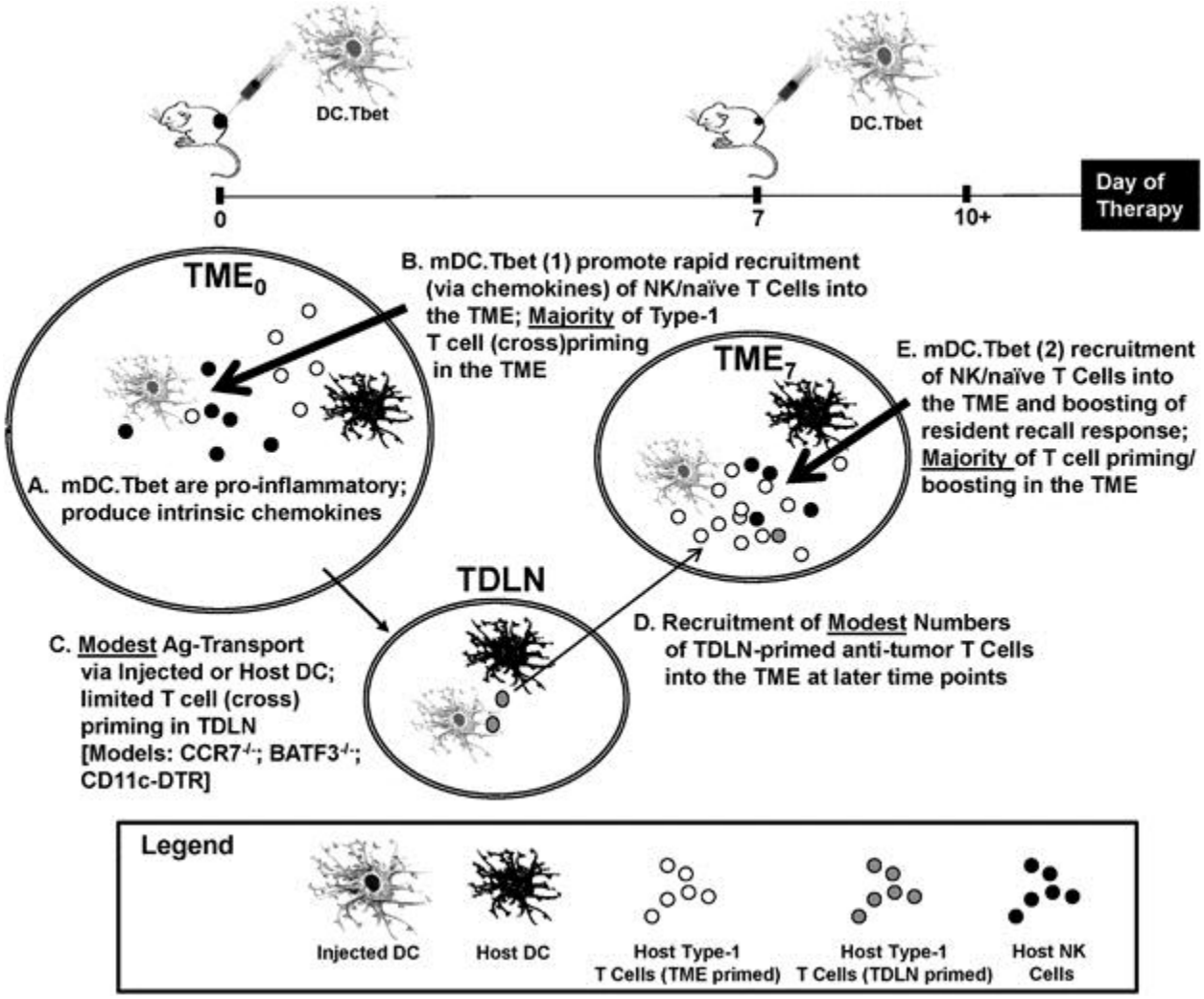


Figure 21. A hypothetical model for extranodal priming of therapeutic immunity in the TME after intratumoral delivery of mDC.Tbet

In this model, delivery of mDC.Tbet leads to the acute recruitment of NK cells and naive $CD8^+$ T cells, based on injected DC production of chemokines. Such recruitment into the TME fosters the crosspriming and polarization of antitumor $CD8^+$ 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 intratumorally delivered DC.Tbet ($\beta 2M^{-/-}$). Host DC populations do not have dominant roles in the therapeutic benefits associated with intratumoral delivery.

5.1 SUMMARY OF STUDIES RELATED TO THE MOLECULAR MECHANISMS UNDERLYING THE TYPE-1 POLARIZING FUNCTION OF DC.TBET

Gene array analysis were performed on human DC.Tbet vs. control DCs (DC.ψ5) in order to study differentially expressed gene sets that might contribute to the superior Type-1 polarizing action mediated by DC.Tbet. In these studies, DC.Tbet and DC.ψ5 were generated from 5 unrelated healthy donors, and treated with or without IFN-γ and LPS in the medium for 24 hrs. All 20 samples were then analyzed by gene expression profiling using an oligonucleotide microarray with more than 44,000 probes (Agilent) (appendix Fig. 7). 1563 genes were differentially expressed between these 4 groups of DCs (F test, p less than 0.05). Hierarchical clustering analysis of these differentially expressed genes suggest that differences between IFN-γ and LPS treated DC.Tbet and treated DC.ψ5 are greater than those between untreated DC.T-bet and untreated DC.ψ5 (appendix Fig. 7A, B), suggesting that upon activation DC.Tbet further changes the expression of many genes that might contribute to the immunostimulatory function of DCs. Comparison of untreated DC.Tbet with untreated DC.ψ5 revealed 343 up-regulated genes and 560 down-regulated genes in DC.Tbet. Genes up-regulated in untreated DC.Tbet were most likely to belong to the following functional groups: Cellular Movement, Inflammatory Response, Organismal Functions, Hematopoiesis, and Immune Cell Trafficking. Genes down-regulated in untreated DC.Tbet were most likely to belong to the following functional groups: Cardiovascular Disease, Genetic Disorder, Skeletal and Muscular Disorders, Cellular Movement and Immunological Disease. Genes up-regulated in LPS and IFN-γ treated DC.Tbet were most

likely to belong to the following functional groups: Inflammatory Response, Organismal Injury and Abnormalities, Respiratory Disease, Cancer and Infection Mechanism (data not shown). Genes down-regulated in treated DC.Tbet were most likely to belong to the following functional groups: Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response and Inflammatory Disease (data not shown). Up-regulate genes in treated DC.Tbet seemed to associate with immune functions, including inflammatory response, cancer and infection mechanism, cellular movement, hematological system development and function, and immune cell trafficking.

Pathway analysis suggest that genes in the hepatic fibrosis/hepatic stellate cell activation and LXR/RXR activation pathways were downregulated in both treated and untreated DC.Tbet. The LXR/RXR related pathways are interesting since LXR pathway activation was shown to interfere with DC maturation and to negatively regulate T cell simulation driven by DCs [263].

Several genes that are highly up-regulated (2 fold or more) in microarray were evaluated by RT-PCR (to confirm the expression; data not shown) and are studied for their function. RRAD is a Ras-related GTPase that may be involved in cytoskeleton reorganization (important for cosimulatory activity and signal transduction) [264] and insulin resistance in diabetes [265]; it may also act as a functional tumor suppressor [266]. Another two Ras-related GTPase, Rac1 and Rac2 were reported to regulate the T cell priming function in DCs [267]. To better understand the role of RRAD in DC.Tbet functionality, a recombinant adenovirus carrying RRAD (rAd.RRAD) cDNA was generated by the Vector Core Facility of the UPCI and used to transduce DCs in gain-of-function studies. To date, the transduction of DCs with rAd.RRAD was not successful in generating DC expressing high levels of RRAD protein. This might be due to poor adenovirus vector quality or to unknown regulatory mechanisms in DCs that restricted

ectopic over-expression of RRAD under a viral promoter (this is less likely as successful upregulation of RRAD in cultured fat and muscle cells has been previously achieved) [265]. Several transmembrane proteins of the tetraspanin superfamily were be upregulated in expression by DC.Tbet when compared with DC.ψ5 (Tetraspanin12, Tetraspanin2 and Tetraspanin17). Tetraspanins regulate cell migration and fusion [268], signaling transduction, immune response and protein trafficking [269, 270]. Tetraspanins are capable of associating with each other and partner/signaling proteins and forming lateral organization of membrane proteins – Tetraspanin Enriched Domains (TEM) [271]. TEM usually contains integrins, adhesion molecules [ICAM-1, VCAM-1 etc], signaling molecules such as GPCR [271], and MHC molecules, CD4, CD8 and CD19 in immune cells [272]. TEM on DCs are believed to preorganize MHC-peptide complexes before encountering T cells to facilitate the activation of cognate T cells, and the adhesion and proliferative function of leukocytes [273]. Tetraspanins are also preferentially found in exosomes/microvesicles and might contribute to the immunostimulatory function of these cell-free structures [274]. To assess the impact of transgenic Tsp12, I generated rAd.Tsp12 vectors and transduced DC. DC.Tsp12 displayed only weak T cell priming capacity *in vitro* when pulsed with SEB and cocultured with naïve T cells (data not shown). These data suggest that high levels of Tsp12 may be a biomarker asociated with the superior Type-1 polarizing potential of DC.Tbet, but that this molecule is not intimately involved in this immunologic process. Another interesting factor found to be highly-overexpressed in treated DC.Tbet was IL-36γ, a newly identified IL-1 family cytokine that promoted Th1 cell responses from naïve T cell precursors *in vitro* [275]. The study of DC.IL36γ and the impact of neutralizing IL-36γ in DC.Tbet via the use of siRNA knock-down or the application of neutralizing antibody is an active area of future work by our laboratory.

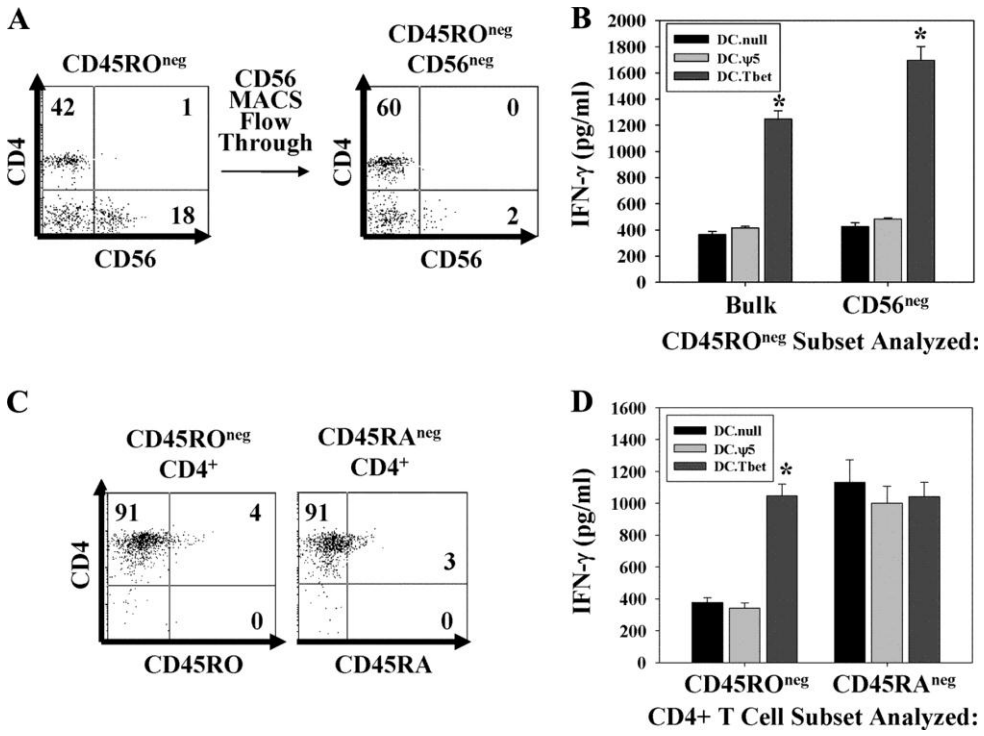
Among the down-regulated genes observed in DC.Tbet vs. control DC were CCL2 and CCL7. These chemokines are of special interest as they may be associated with the inhibition of accumulation of MDSC in tumors treated with DC.Tbet [276]. In addition, MARCH-I (Membrane-associated RING-CH-1) is an ubiquitin ligase that regulates the ubiquitination of MHC class II and B-7 molecules on DC surface [277, 278]. The down-regulation of MARCH-I in mature/activated DCs lead to the upregulation and stabilization of MHC class II and may enhance the immunostimulatory function of DCs [279, 280]. The over-expression and down-regulation of MARCH-I in DCs by adenoviral-mediated gene transduction and/or RNA interference manipulation, respectively, could be used to study the role of differential MARCH-I expression in DC.Tbet-mediated function and anti-tumor efficacy.

Several proteins such as IL-1β and Wnt that participate in non-classical secretion (microvesicle/exosome mediated secretion) pathways [281, 282] were also determined to be upregulated in DC.Tbet when compared to control DCs. Microvesicles (MVs) are membrane fragments secreted from the endosomal membrane compartment with a size range from 30nm-1000nm (exosome 30-100nm, MVs 100-1000nm) [283]. MVs may carry and transfer mRNA and proteins between cells, and these structures are capable of directly stimulating immune cells or to serve as immune suppressors depending on their cellular source [283-285]. I believe that DC.Tbet upregulate their secretion of MV/exosomes leading to: 1) directly activation of T effector cells; 2) the transfer of T-bet protein/mRNA from DC.Tbet to nearby T cells and other immune cells. To test this hypothesis, I harvested microvesicles from the day 2-cultured supernatants of DC.Tbet and DC.ψ5 by sequential supercentrifuge and then analyzed their protein content by western blot (appendix Fig. 8**B**). DC.Tbet seem to produce more MVs as a bigger pellet size was consistently associated with the centrifuged product isolated from

DC.Tbet's culture supernatant (from 3 different experiments). Transmission electron microscopy revealed a heterogeneous composition of MVs with vesicles' size spanning from 60-200 nm in diameter. Western-blotting analysis of these vesicles suggested these MVs contained high levels of MHC molecules and Tsp12 (data not shown). Interestingly, MVs derived from DC.Tbet, but not control DC.ψ5, also contained abundant concentrations of T-bet protein. When directly adding these MVs into naïve T cell cultures, DC.Tbet-derived MVs displayed stronger Type-1 T cell priming capability when compared to MVs derived from control DCs (appendix Fig. 8**C**). Overall, these data suggest that DC.Tbet-derived MVs contain T-bet protein, and these MVs are capable of promoting Type-1 T cell responses over short distances *in vitro*, which may partially explain the superior Type-1 polarizing function of DC.Tbet.

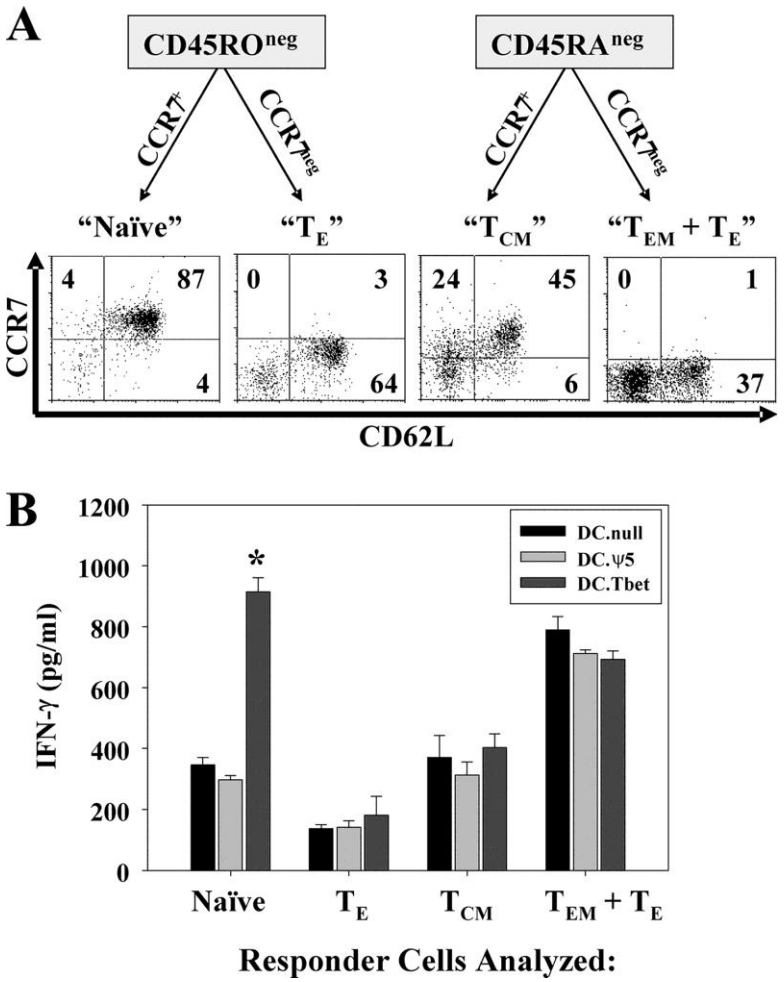
The fact that MVs derived from DC.Tbet contains T-bet protein indicates the possibility of T-bet protein/mRNA transferring from DC.Tbet to T cells or other cells in the proximal microenvironment. To test this hypothesis, I co-cultured SEB -pulsed DC.Tbet with T cells derived from T-bet$^{-/-}$ splenocytes, after 2-4 hrs T-bet content in these T cells was analyzed by flow cytometry. As shown in Appendix Fig. 8**D**, about 0.5% T-bet positive events were observed amongst intrinsically T-bet$^{-/-}$ T cells, suggesting that T-bet protein were actively transferred from DC.Tbet to T-bet$^{-/-}$ T cells. Repeating this experiment again using SIINFEKL (OVA)-pulsed DC and OT-I (anti-SIINFEKL) TCR transgenic T cells demonstrated that Ag-specific contact between DC.Tbet and T cells led to the enhanced transfer of T-bet from DC.Tbet to T cells. In future experiments, MVs from DC.Tbet will be evaluated for their potential in mediating T-bet protein transfer to T cells in the therapeutic setting.

APPENDIX



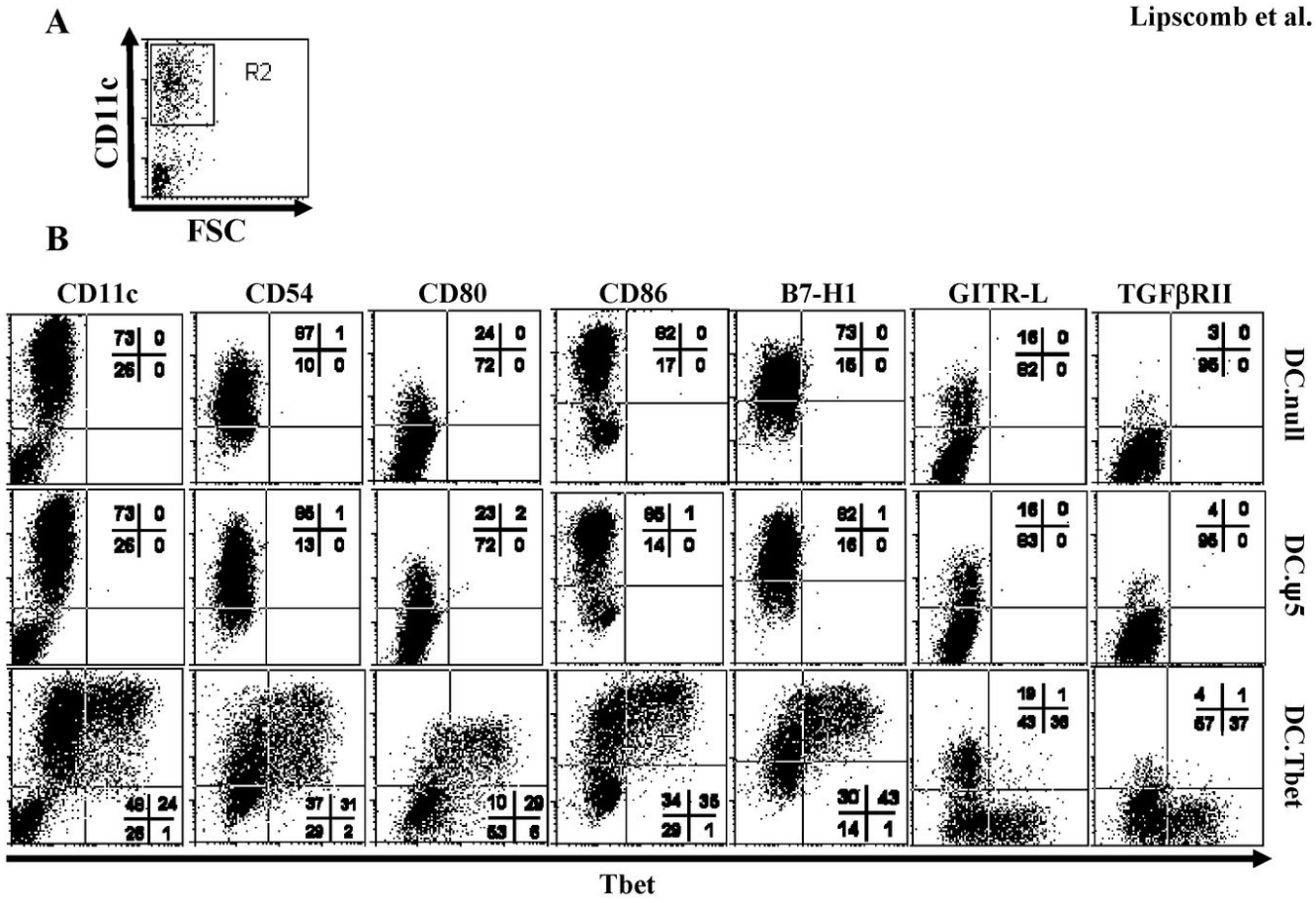
Appendix Figure 1. DC.Tbets promote Type-1 (IFN-γ) responses from CD45RO$^-$CD56$^-$ and CD45RO$^-$CD4$^+$T cells *in vitro*.

Cultures were established as outlined in Fig. 2*A* using bulk CD45RO$^-$ cells or CD45RO$^-$ cells depleted of contaminant CD56$^+$ cells using MACS (*A*) as responders. Culture supernatants were evaluated for IFN-γ production after 72 h of coculture using a specific ELISA (*B*). Alternatively, MACS-isolated CD45RO$^-$CD4$^+$ and CD45RA$^-$CD4$^+$ T cells (*C*) were used as responders, with day 3 culture supernatants evaluated for IFN-γ levels (*D*). ∗, $p < 0.05$ for DC.Tbets vs DC.nulls or DC.ψ*5*. All data are representative of two independent assays performed.



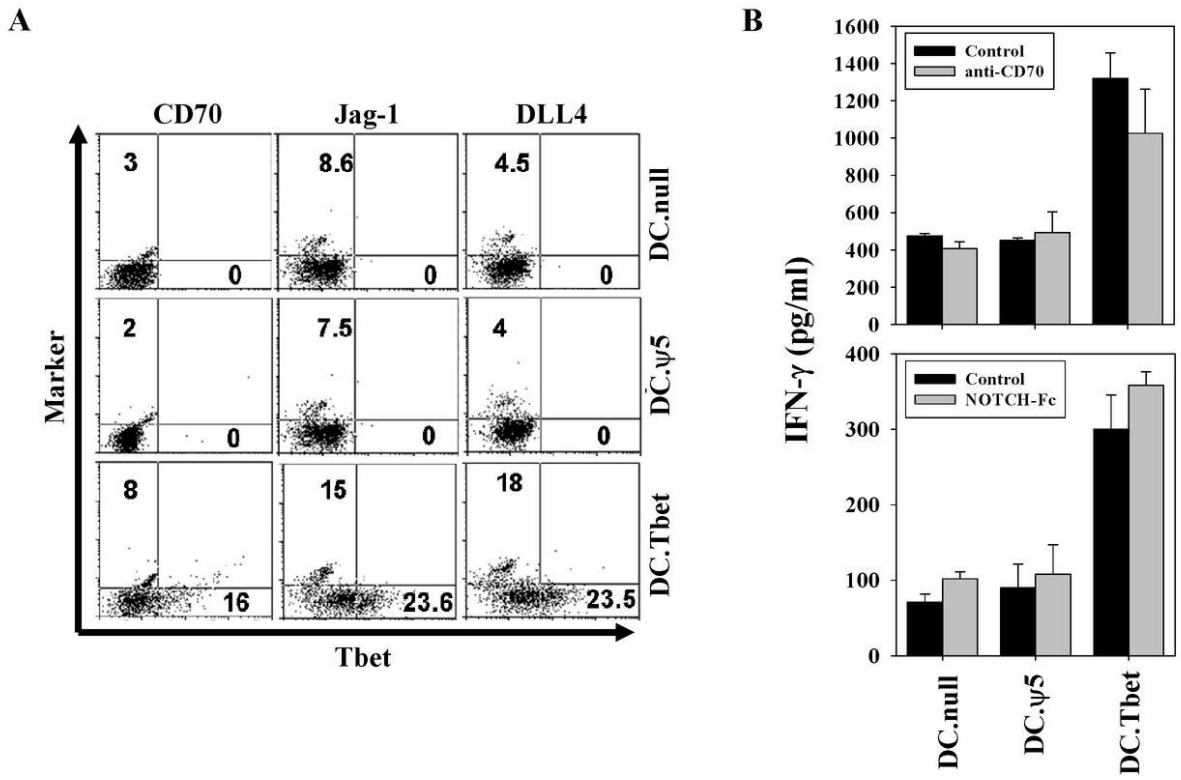
Appendix Figure 2. DC.Tbet selectively prime Type-1 (IFN-γ) responses from CD45RO$^-$CCR7$^+$CD62L$^+$ naive T cells *in vitro*.

CD45RO$^-$ or CD45RA$^-$ cells were subsequently subdivided into CCR7$^+$ and CCR7$^-$ subpopulations using specific MACS beads and evaluated for their phenotypes by flow cytometry using mAbs against CCR7 and CD62L (A). These four cell populations were then used as responders against autologous, SEB-pulsed DC.Tbet or control DC as described in Fig. 2A. Day 3 coculture supernatants were evaluated for IFN-γ content by ELISA (B). ∗, $p < 0.05$ for DC.Tbet vs DC.null or DC.ψ5 . All data are representative of two independent assays performed. T_E, Effector T cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell.



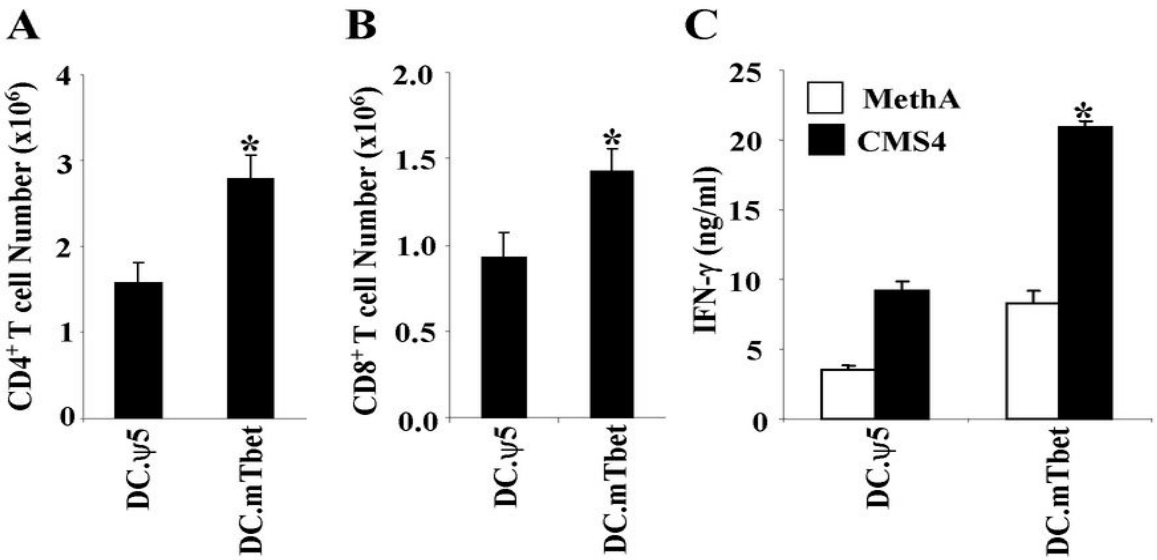
Appendix Figure 3. Phenotypes of DC.Tbet vs. control DC.

DC.Tbet and control DC were generated as outlined in Materials and Methods and phenotyped by flow cytometry. With the exception of the CD11c cohort itself, in all other populations, cells were initially gated on CD11c+ events and then assessed for expression of the indicated markers using specific antibodies/ Data are representative of 6 independent experiments performed, each using a different donor.



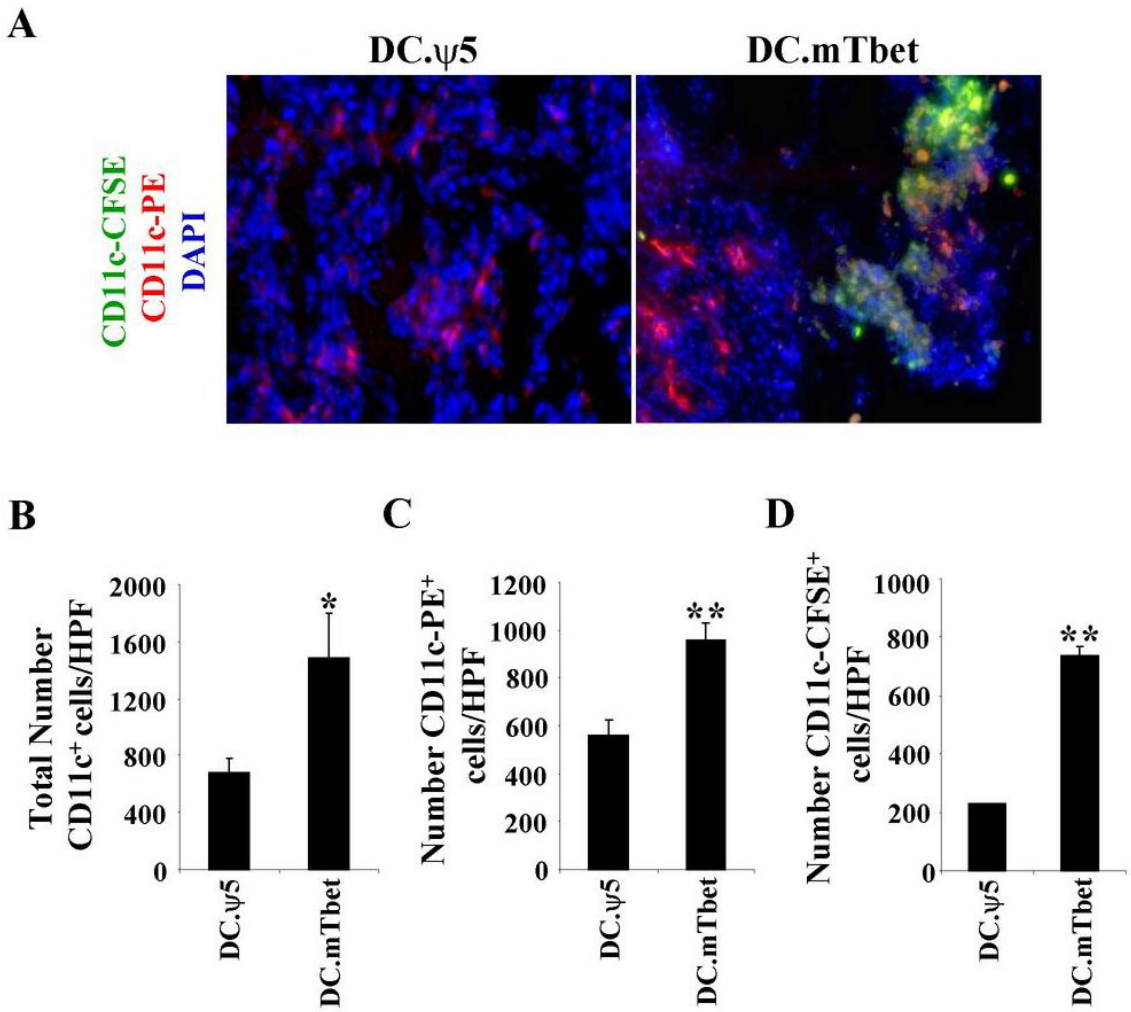
Appendix Figure 4. The ability of DC.Tbet to promote superior Type-1 T cell responses *in vitro* is not inhibited by blockade of DC-expressed CD70 or NOTCH ligands.

A. DC.Tbet were initially analyzed for cell surface expression of CD70 or the NOTCH ligands Jagged-1 (Jag-1) and Delta-Like 4 (DLL4) by flow cytometry. **B**. Naïve bulk T cells were then stimulated with SEB-pulsed DC.Tbet or control DC in the absence or presence of 10 μg/ml of the blocking anti-CD70 mAb or NOTCH-Fc fusion protein as indicated. After 3 days, culture supernatants were analyzed for levels of IFN-γ by ELISA



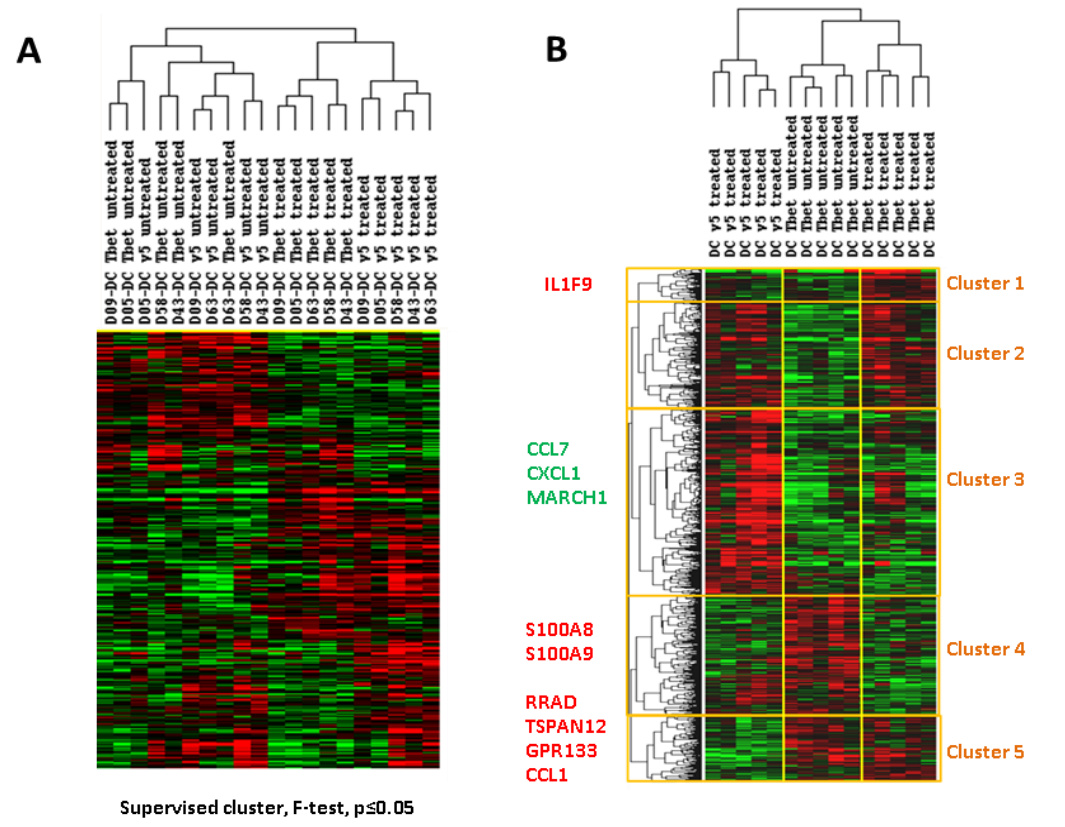
Appendix Figure 5. Intratumoral delivery of DC.Tbet enhances $CD4^+$ and $CD8^+$ T cell expansion and the development of tumor-reactive Tc1 effector cells in the tumor draining lymph node.

Established day 7 CMS4 tumors were injected with $1X10^6$ DC.mTbet or control DC.ψ5 with an identical re-treatment one week later. On day 21, tumor draining lymph nodes were harvested and total numbers of $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells were 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 48 h. 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. $*p < 0.05$ for DCm.Tbets vs. DC.ψ5.



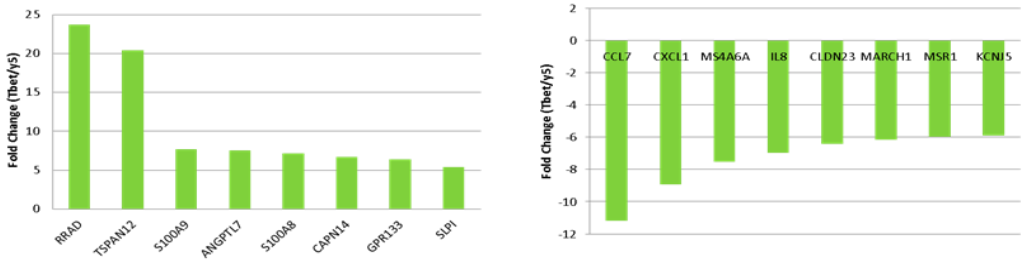
Appendix Figure 6. DC.mTbet exhibit enhanced persistence in the CMS4 TME and promote increased tumor infiltration by host CD11c$^+$ DC.

A. Established day 7 CMS4 tumors were injected with DC.mTbet or control DCs that had been pre-labeled with CFSE. Three days after the injection, tumor lesions were harvested, 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 cells **C**, and CD11c-CFSE$^+$ cells **D**. Results were shown as reported as mean ± SD. $*p < 0.05$ $**p$ for DC.mTbet vs. DC.ψ5 injected tumors.



C

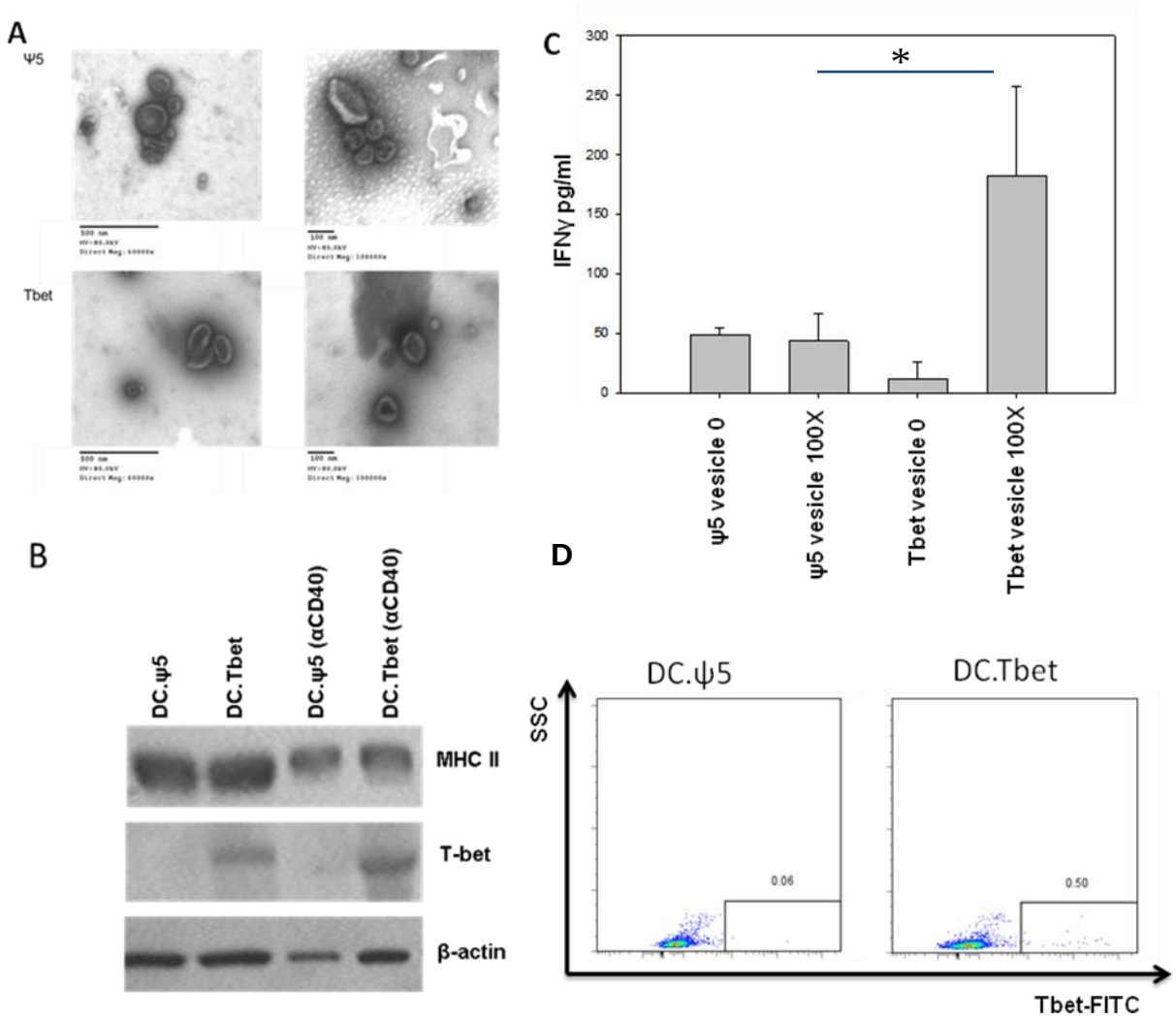
Gene Symbol	RRAD	TSPAN12	S100A9	ANGPTL7	CAPN14	GPR133	SLPI
Tbet/Ψ5 fold change	23.73344	20.40831	7.731656	7.574634	6.674362	6.374894	5.375249
Gene Symbol	**CCL7**	**CXCL1**	**MS4A6A**	**IL8**	**MARCH1**	**MSR1**	**KCNJ5**
Tbet/Ψ5 fold change	-11.1736	-8.92048	-7.51318	-6.95552	-6.14831	-5.96987	-5.8813



Appendix Figure 7. Gene array analysis of DC.Tbet vs. control DCs.

DC.Tbet and control DCs (DC.ψ5) were generated from PBMCs derived from 5 healthy donors, treated with or without LPS and IFN-γ for 24hrs. Total RNA from these cells were extracted from each cell line using the miRNeasy mini kit (Qiagen, Westburg, Leusden, NL). Gene expression experiments were performed on 4 × 44 K Whole Human Genome Microarray (Agilent technologies). Results were analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute and Cluster and TreeView software. **A,** Hierarchical Clustering Analysis of 1563 Differentially Expressed Genes among all 20 DC samples (F-test, p≤0.05); **B**.

Hierarchical clustering analysis of 1485 genes differentially expressed among IFN-γ and LPS treated DC.Tbet, IFN-γ and LPS treated DC.ψ5 and untreated DC.Tbet (F-test, $p \leq 0.05$) **C**. Highly up- or down-regulated genes in DC.Tbet (5 folds or more compared to control DCs).



Appendix Figure 8. Microvesicles derived from DC.Tbet contain T-bet protein and induce higher level of IFN-γ from naïve allo-T cells compare to DC.ψ5.

Microvesicles secreted from DC.Tbet or DC.ψ5 were collected by sequential high speed centrifugations. The phenotype of these MVs were analyzed by electron microscopy (**A**), and the protein content were analyzed by western blotting (**B**). These vesicles were evaluated for their T cell priming efficacy in a coculture system with MVs and naïve T cells ($CD45RA^{+}$) (**C**). In (**D**) SEB pulsed DC.Tbet were cocultured with $T\text{-}bet^{-/-}$ T cells for 2-4 hrs, after which CD3, CD4 and T-bet were stained by immunofluorescence staining and analyzed by flow cytometry. Events shown in D were gated on $CD3^{+}$ T cells. ∗, $p < 0.05$ for DC.Tbet vs DC.ψ5 [t-test].

Appendix Table 1. 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 per HPF	mDC.Null-treated number of events per HPF
$CD3^+$	64.3±9.8*	6.0±1.6
$CD45RB^+CD3^+$	40.0±5.4*	2.7±0.9
$CD45RB^{neg}CD3^+$	24.3±10.1*	3.3±0.5
$CD45RB^+CD3^+ZsGreen^+$	25.0±6.4*	2.0±0.8
$CD45RB^+CD3^+ZsGreen^{neg}$	15.0±3.4*	0.6±0.5

Fluorescence confocal microscopy images obtained in Fig. 19**C** 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.

Appendix Table 2. Chemokine transcripts in hDC.Tbet versus hDC.Null (> 1.5 fold).

Chemokine Analyzed	Fold Change (hDC.Tbet/hDC.Null)	p-value
CCL1	4.814	0.009
CCL27	1.677	0.012
CXCL16	1.625	0.015
CXCL11	1.581	0.028
CCL4	1.518	0.031
CXCL2	-2.315	0.025
CCL2	-4.500	0.005
CXCL1	-8.920	0.001

Resting human DC were generated from the peripheral blood of 5 normal donors, with DC (both > 98% $CD11c^{+}CD14^{neg}$) then infected with empty adenovirus (hDC.Null) or hDC.Tbet for 48h. Message RNA was then extracted and gene profiling were performed as described in Appendix Fig. **7**. Results are reported as mean fold differences.

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