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Ectopic T-bet Expression Licenses Dendritic Cells for IL-12-Independent Priming of Type 1 T Cells In Vitro[1]

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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.   *The Journal of Immunology,* 2009, 183: 7250–7258.

D endritic cells (DC)[4] are professional APCs that capture, process, and present Ags to T cells in the form of peptides complexed with MHC molecules (1, 2). 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 (3–6).

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 (7). 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 (8, 9). 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 (10, 11). However, IL-12/STAT-4-independent mechanisms of type 1 T cell induction have also been reported (12, 13). 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 (14–16). 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 (17, 18). Conversely, T-bet expression suppresses Th2 differentiation by interfering with the type 2 *trans* activator GATA-3 (19, 20).

Intrinsic, low-level expression of T-bet in (at least a subset of) DC also appears crucial to the generation of type 1 immunity (15, 21–23). 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.

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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 (24). 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$_{α2b}$; Schering-Plough) plus IFN-γ (1000 U/ml; Endogen) plus polyinosinic-polycytidylic acid (20 μg/ml; Sigma-Aldrich) yielding αDC1 (25); 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 (26).

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.

Recombinant adenoviruses

Human T-bet (hT-bet) was PCR cloned from PBLs using the following primers: hT-bet: forward (Fwd) 5′-GTCGACGACGGCTACGGGAAG GTG-3′; reverse (Rev) 5′-GGATCCTTAGTCGGTGTCCTCCAACC-3′. The product was then digested with the restriction enzymes *Sal*I and *Bam*HI 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 (27). 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 (27, 28). 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 (27). 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 (24). 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 (24). Additional primer sequences included: T-bet, Fwd 5′-CCACCAGCCACTACAGGATG-3′ and Rev 5′-GGACGCCCCCTTGTTGTTT-3′; GATA-3, Fwd 5′-GT GCTTTTTAACATCGACGGTC-3′ and Rev 5′-AGGGGCTGAGATTC CAGGG-3′; Foxp3, Fwd 5′-GCACCTTCCCAAATCCCAGT-3′ and Rev 5′-TAGGGTTGGAACACCTGCTG-3′; and RORγt, Fwd 5′-AAATCT GTGGGGACAAGTCG-3′ and Rev 5′-TGAGGGTATCTGCTCCTTGG-3′. β-Actin primers were used as an internal positive control (24).

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; Ref. 29) 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 (6, 30). Briefly, DC. Tbet (ectopic T-bet-expressing DC) or control DC were pulsed with SEB (Sigma-Aldrich) at 0.1–10 ng/ml (with a standard dose of 1 ng/ml selected for standard use based on preliminary studies; supplemental Fig. 1)[5] 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.

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[5] The on-line version of this article contains supplemental material.

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 (31). 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 (28). 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 (EphA2$_{883-891}$, gp100$_{209-217}$; 10 μM each; Refs. 25 and 32) 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 (32). The HLA-A2-presented HIV-nef$_{190-198}$ peptide (32) 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 (33).
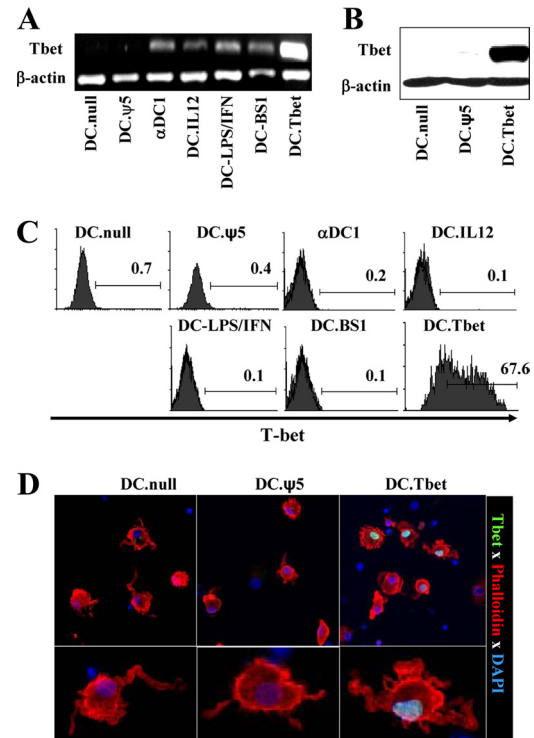
Statistical analyses

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 $p < 0.05$.

Results

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


FIGURE 1. Generation and characterization of DC.Tbets. Immature DC 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 DCsactivated 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 (green), phalloidin (red), and 4′,6′-diamidino-2-phenylindole (DAPI; blue). Data are representative of at least three independent assays performed for each panel.

(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 (24–27). 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*).

DC.Tbet selectively prime CD45RO$^-$ (naive) 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 (15, 21–23), we hypothesized that DC.Tbet cells might be enhanced in this capacity. We used a superantigen (SEB)
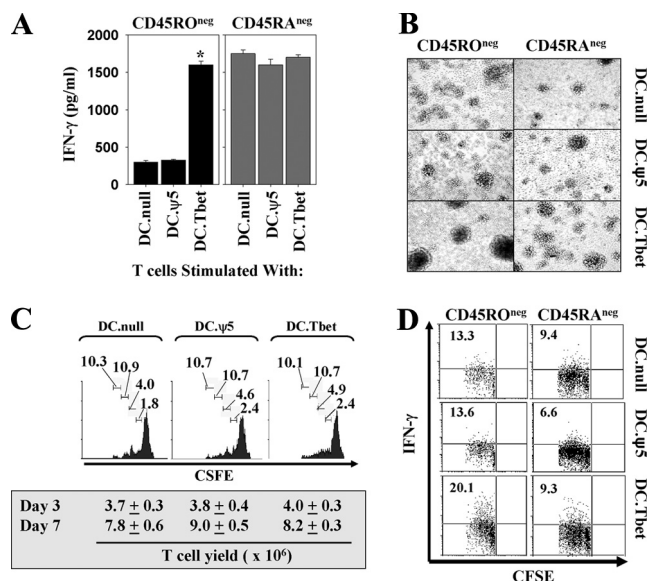
FIGURE 2. When compared with control DCs, DC.Tbets uniquely promote IFN-γ responses from naive, but not memory, bulk T cells in vitro. Bulk, naive (CD45RO⁻), or memory (CD45RA⁻) T cells were isolated by negative selection and cultured with autologous, SEB-pulsed DC.Tbets or control DCs at a ratio of 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.

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 (supplemental Fig. 1) 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. 2A, 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. 2B), 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. 2C), although the enhanced ability of daughter T cells to produce IFN-γ in DC.Tbet (+ CD45RO⁻ bulk T cell) cocultures was readily apparent (Fig. 2D). This latter increase was evident in both the percentage of IFN-γ⁺CSFE^low⁺ events (Fig. 2D) 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.

Since our initial bulk CD45RO^neg responder cell population also contained a subpopulation of ~15–18% CD4⁻CD56⁺ NK/NK T cells (Fig. 3A), which could serve as a direct source of IFN-γ and/or act as an intermediary for DC-induced type 1 T cell function (34), we MACS-isolated CD45RO⁻CD56⁻ T cells (Fig. 3A) and repeated our in vitro stimulation assays using autologous SEB-pulsed DC.Tbets vs control DCs as APCs. As shown in Fig. 3B, depletion of CD56⁺ cells from CD45RO⁻, bulk T cell responders did not inhibit the ability of DC.Tbets to promote superior IFN-γ

FIGURE 3. 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. 2A 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.ψ5. All data are representative of two independent assays performed.
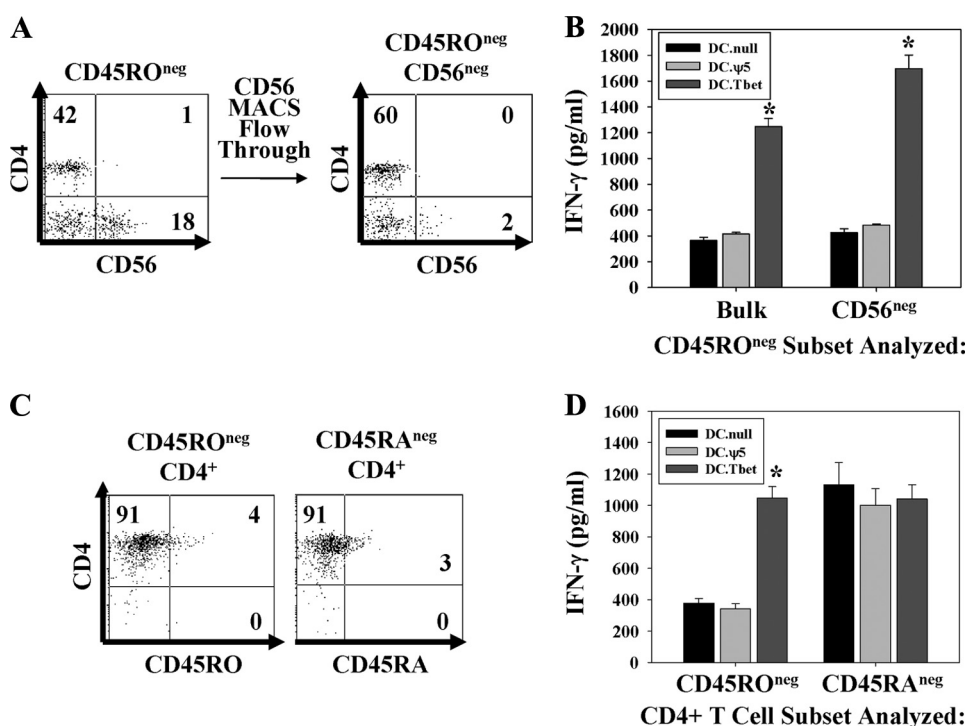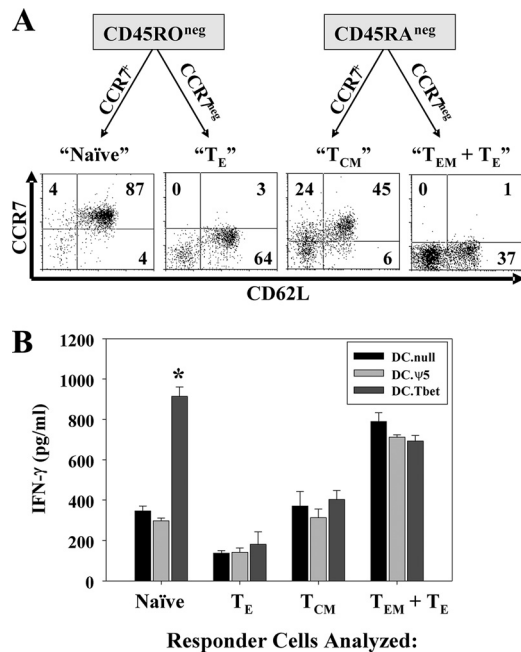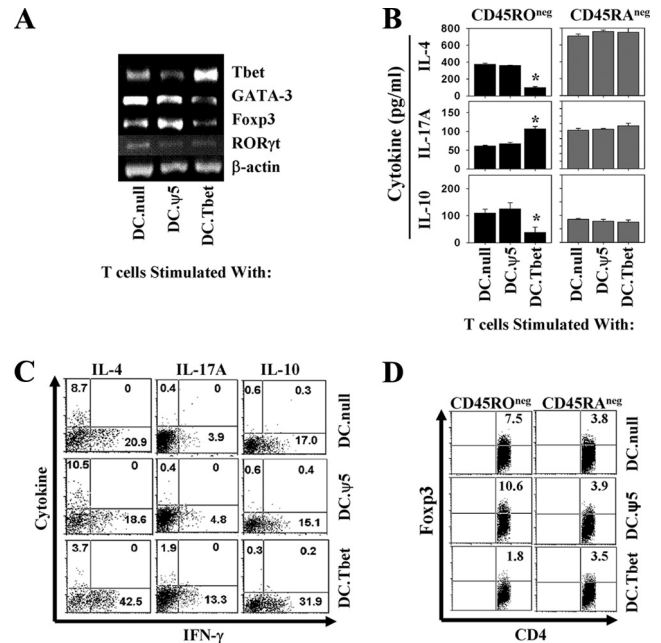
FIGURE 4. DC.Tbet selectively prime type 1 (IFN-γ) responses from CD45RO⁻CCR7⁺CD62L⁺ naive T cells in vitro. CD45RO⁻ or CD45RA⁻ cells were subsequently subdivided into CCR7⁺ and CCR7⁻ subpopulations using specific MACS beads and evaluated for their phenotypes by flow cytometry using mAbs against CCR7 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. 2*A*. 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.

FIGURE 5. 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. *, *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; Ref. 36). All data are representative of three independent assays performed.

production. This was further corroborated for CD4⁺ T responder cells positively isolated from the CD45RO⁻ and CD45RA⁻ bulk populations of cells (Fig. 3*C*), wherein SEB-pulsed, autologous DC.Tbets elicited superior IFN-γ production only from CD4⁺ CD45RO⁻ responder T cells (Fig. 3*D*).

Having discounted the importance of contaminant NK cells as a source of IFN-γ resulting from DC.Tbet priming of bulk, CD45RO⁻ responder cells, we next considered differential responsiveness of the various T cell functional subsets to DC.Tbet-based stimulation. Because T cell functional subsets may be discriminated 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 their composite phenotypes (35), we performed CCR7-MACS selection after first isolating CD45RO⁻ and CD45RA⁻ cells (Fig. 4*A*). These populations, enriched in the various T cell functional subsets, were then stimulated with autologous SEB-pulsed DC.Tbets vs control DCs for 72 h, and culture supernatants were analyzed for IFN-γ levels (Fig. 4*B*). Despite the lack of absolute purity for each of the T cell functional subsets, 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 (Fig. 4*B*).

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. 5*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. 5*A*). Furthermore, because T-bet directly binds to the IL-12Rβ2 promoter and enhances its expression in Th subsets (8), we performed flow cytometry analyses on CD4⁺ T cells harvested from DC.Tbet-driven cultures established with naive T cell responders. These analyses revealed that responder T cells were enriched in cells bearing the IL-12Rβ2⁺T-bet⁺ phenotype in DC.Tbet (vs control DC)-driven cultures (supplemental Fig. 2*A*). Corollary studies revealed that DC.Tbet differentially (vs control DC) induced naive (supplemental Fig. 2*B*), but not memory (supplemental Fig. 2*C*) Tc1 cell responses, based on CD8⁺ T cell expression of IFN-γ and granzyme B. Responder CD4⁺ and CD8⁺ T cell expression of the CXCR3 chemokine receptor (associated with type 1 T cell recruitment into (inflammatory) tumor sites; Ref. 37) was also increased ~2-fold (based on mean fluorescence intensity levels) if these T cells had been activated by DC.Tbets vs control DCs (supplemental Fig. 2*A* and data not shown).
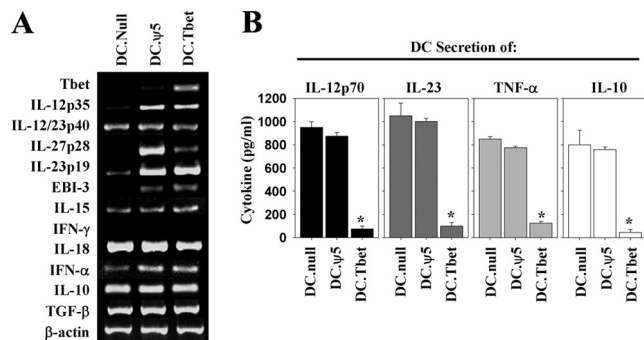
FIGURE 6. Impact of T-bet gene insertion on DC expression of cytokine mRNA and secreted cytokine levels. DC.Tbet vs control DC were analyzed for levels of the indicated cytokine mRNA (using RT-PCR in *A*) and secreted cytokines (using specific ELISA in *B*) after CD40 ligation as outlined in *Materials and Methods*. *, *p* < 0.05 vs DC.null and DC.ψ5 controls. All data are representative of three independent assays performed.

*DC.Tbet priming suppresses the generation of type 2 and Tregs from naive precursors*

Our preliminary analyses support large decreases in GATA-3 and Foxp3 (and little to no change in ROR-γt) mRNA expression levels in naive, bulk T cells primed using DC.Tbets vs control DCs (Fig. 5*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. 5*B* and CD3⁺ T cells assessed in Fig. 5*C*, 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. 5*D*). In slight contradiction to the RT-PCR data reported for ROR-γt in Fig. 5*A*, we noted a modest increase in IL-17A protein production from naive T cells primed using DC.Tbets vs control DCs (Fig. 5, *B* and *C*).

DC.Tbet induces type 1 polarization of naive T cells via a mechanism that is independent of IL-12 cytokine family members and requires DC-T cell contact/proximity

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 (8, 10, 11). 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. 6*A*). 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. 6*B* and supplemental Fig. 3). Consistent with the lack of expression of IFN-γ mRNA in any DC population analyzed in Fig. 6*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 (supplemental Fig. 4).

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. 7, *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. 7, *A–C*).

FIGURE 7. 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.
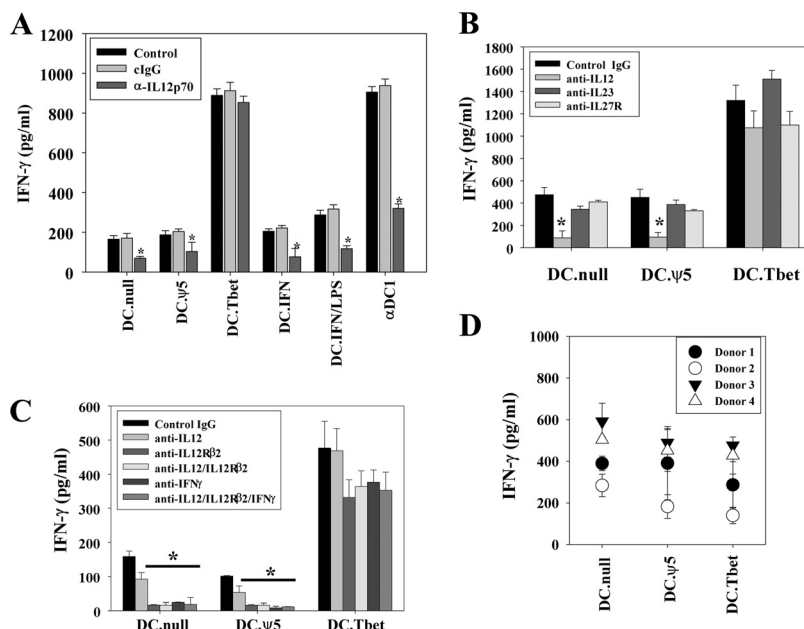
Because RT-PCR and ELISA analyses suggested the coordinate silencing of cytokine secretion by DC.Tbet, this implicated the likely dominant involvement of cell membrane interactions rather than soluble mediators in the differential ability of DC.Tbet to drive type 1 T cell responses in vitro. We confirmed 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. 7D), suggesting that physical separation of DC.Tbets from responder T cells mitigates their capacity to promote superior type 1 immunity in vitro.
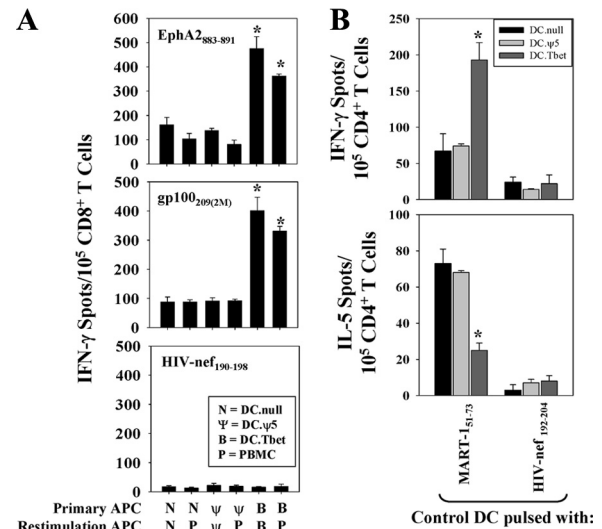
DC.Tbet promotes superior tumor Ag-specific, type 1 CD4⁺ and CD8⁺ T cell responses in vitro

To determine whether DC.Tbets were capable of promoting enhanced Tc1 immunity against tumor Ags (such as EphA2, as in Ref. 32, and gp100. as in Ref. 25), 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. 8A). 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. 8A). 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; supplemental Fig. 5) and then present the derivative HLA-DR4-presented $MART-1_{51-73}$ epitope (38) to a peptide-specific CD4⁺ T cell line (supplemental Fig. 6). 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. 8B, 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 ($p < 0.05$).

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 (39–41). In particular, Glimcher et al. (22, 23, 41) 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)

FIGURE 8. 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-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.ψ5.

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 (42). Furthermore, we did not detect Th17 cells coproducing both IFN-γ and IL-17A (Fig. 5C), 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 (43) and 2) signals mediated via the IL-23R are crucial for the development of T_H17 responses (44).

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 (supplemental Figs. 7, 8, and 9A). 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 (12, 13, 45), were not expressed (or expressed poorly) by DC.Tbet (supplemental Fig. 9A), 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 (supplemental Fig. 9B).

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 (46–49), 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 (50). 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 (51). 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 CD45RO$^-$CCR7$^+$CD62L$^+$ T cells.

Type 1 T cell responses appear most efficient in regulating disease development and progression in the cancer setting (7, 24, 25, 27, 28, 30, 34). 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 (7, 28, 52) 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 (52).

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 (25). 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 (36, 53) and to mediate robust antitumor activity within these sites (54). 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 (55) in the settings of cancer and infectious disease.

Acknowledgments

Disclosures

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