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

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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. Naïve 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.Tbet 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.Tbet promote improved type 1 functional polarization from naïve 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.

Dendritic cells (DC) 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 costimulatory 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-associatecd 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.

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-IFNα; Schering-Plough) plus IFN-γ (1000 U/ml; Endogen) plus polyinosinic-polycytidylic acid (20 μg/ml; Sigma-Aldrich) yielding dDC1 (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.BSI (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’-GTGACCCACCCGACCTACGGGAGGTG-3’; reverse (Rev) 5’-GGATCCTTAGTCGGTGTCCTCCAACC-3’.

The product was then digested with the restriction enzymes Sall and BamHI and the 1.7-kb fragment containing full-length hT-bet was ligated to pAdEasy-1 (pAd.Easy-1). Recombinant adenoviruses were generated, as previously described (27).

For mRNA analysis, DCs were harvested on day 2 (48 h posttransduction), fixed with 4% paraformaldehyde (PFA), and permeabilized with 0.2% saponin. For intracellular cytokine staining, DCs were cultured 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) in AIM-V media (Invitrogen) for 3 h at 37°C prior to washing and the addition of 100 μl of DC-T cell cocultures. 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% FBS) containing 1 μM monensin (all from Sigma-Aldrich), and hIL-17A (BioLegend) using commercial ELISAs (BD Biosciences, except for IL-23 ELISA from BenderMedSystems). Additional studies included DC stimulation for 24 h using agonists to TLR2 (HKLM; Invigene); TLR3 (polyinosin-polycytid acid; Sigma-Aldrich); TLR4 (LPS; Sigma-Aldrich), TLR5 (flagellin; Invivogen), TLR7 (miRNAmidoil; 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 (CFSE)

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) 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) in AIM-V media (Invitrogen) for 3 h at 37°C prior to washing and the addition of 100 μl of DC-T cell cocultures. 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, 1-glutamine, penicillin/streptomycin, and nonessential amino acids); all reagents from Invivogen 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 CFSE 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 cultured with rhIL-2 and 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 × 10^3) were plated in the bottom chamber of a 24-well transwell plate in 400 μl of TcMEM. After 24 h, 1 × 10^6 naïve T cells along with 1 × 10^5 SEB-pulsed-immature DC or 3 × 10^6 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.Δ5 (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 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, naïve 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_{151–73} peptide (10 μM). On day 14 of culture, T cells were harvested and assessed for their ability to recognize (in IFN-γ ELISPOT assays) autologous DC.null, DC.Δ5, or DC.Tbet cells pulsed for 48 h with freeze-thaw lysates (50 μg/ml) generated from Ad.MART1- vs Ad.Δ5-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 naïve CD8^+ T cells at a 10:1 T cell:DC ratio in the presence of 5 ng/ml rIL-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 rIL-2 and 5 ng/ml rIL-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_{90–198} 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.Tbet or control DCs were pulsed with 50 μg/ml freeze-thaw lysate generated from 293T cells infected with Ad.MART1 vs Ad.Δ5 for 24 h, 37°C and used to stimulate autologous MACS-isolated, naïve CD4^+ T cells as outlined above for CD8^+ T cell responses. On day 7 of cultures, responder CD4^+ T cells were restimulated with independently 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_{151–73} vs the HIV-nef_{90–208} (negative) control 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 adeno-virus encoding human T-bet (DC.Tbet) or control Ad.Δ5 (DC.Δ5) 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. 1A) 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.Δ5 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 non-viral culture methods, DC.Tbet were 63 ± 18% T-bet^+ over 15 independent experiments as determined by intracellular staining, as exemplified in Fig. 1C. Immunofluorescence microscopy revealed that T-bet protein was expressed predominantly in the nucleus of DC.Tbet cells (Fig. 1D).

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 (15, 21–23), we hypothesized that DC.Tbet cells might be enhanced in this capacity. We used a superantigen (SEB)
All data are representative of three independent assays performed.

Bulk, naive (CD45RO−) T cells were evaluated after 72 h of coculture with DC. Tbet vs control DCs. When compared with control DCs, DC.Tbet uniquely promoted type 1 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.Tbet 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.δ5), 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.Tbet vs control DCs. All data are representative of three independent assays performed. A repeated series of assays implementing bulk CD45RO− T cells were pulsed with 1 ng/ml SEB before coculture with autologous 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+ cells 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.δ5 cultures, respectively; data not shown). These data strongly suggest that DC.Tbet enhance type 1 responses from bulk, CD45RO− T cells via differential polarizing, rather than proliferative, signals.

Since our initial bulk CD45ROneg responder cell population also contained a subpopulation of ~15–18% CD4− CD56+ NK/NKT 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.Tbet 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.Tbet to promote superior IFN-γ (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 a 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). Since our initial bulk CD45RO− T cells were pulsed with 1 ng/ml SEB before coculture with autologous naive (MACS-isolated CD45RO−) cells or memory (MACS-isolated CD45RA−) T cells, it was necessary to verify the ability of DC.Tbet to promote superior type 1 responses from these cell populations as well. T cell responders were 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.Tbet vs DC.nulls or DC.δ5. All data are representative of two 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. 3C), wherein SEB-pulsed, autologous DC.Tbets elicited superior IFN-\(\gamma\) production only from CD4\(^+\) CD45RO\(^+\) responder T cells (Fig. 3D).

Having discounted the importance of contaminant NK cells as a source of IFN-\(\gamma\) 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\(^+\)), central memory (T\(_{CM}\); CD45RA\(^-\)CCR7\(^-\)CD62L\(^+\)), and effector memory (T\(_{EM}\); CD45RA\(^-\)CCR7\(^-\)CD62L\(^-\)) subpopulations based on their composite phenotypes (35), we performed CCR7\(^-\) central memory (T\(_{CM}\); CD45RA\(^-\)CCR7\(^-\)CD62L\(^+\)) and CCR7\(^-\) effector memory (T\(_{EM}\); CD45RA\(^-\)CCR7\(^-\)CD62L\(^-\)) MACS selection after first isolating CD45RO\(^+\) naive T cells, and subsequently analyzed these cells for their comparative expression of mRNAs encoding trans activator proteins (i.e., T-bet (Th1), GATA-3 (Th2), ROR\(\gamma\)T (Th17), and Foxp3, as Treg) linked to T cell function (Fig. 5A). 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), ROR\(\gamma\)T (~2-fold) and Foxp3 (~5-fold) transcripts when compared with T cells stimulated with control DCs (Fig. 5A). Furthermore, because T-bet directly binds to the IL-12R\(\beta\) 2 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-12R\(\beta\) 2 "T-bet" phenotype in DC.Tbet (vs control DC)-driven cultures (supplemental Fig. 2A). Corollary studies revealed that DC.Tbet differentially (vs control DC) induced naive (supplemental Fig. 2B), but not memory (supplemental Fig. 2C) Tc1 cell responses, based on CD8\(^+\) T cell expression of IFN-\(\gamma\) 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. 2A and data not shown).
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. 6A). 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. 6B and supplemental Fig. 3). Consistent with the lack of expression of IFN-γ mRNA in any DC population analyzed in Fig. 6A, 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, IL-27R, 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 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.Δ5 controls. All data are representative of three independent assays performed.

**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 aDC1 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-IL12p70, anti-IL23, or anti-IL27R 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<sup>+</sup><sub>B</sub>, 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<sup>+</sup> and CD8<sup>+</sup> T cell responses in vitro**

To determine whether DC.Tbets were capable of promoting enhanced Tc1 immunity against tumor Ags (such as EphpA2, as in Ref. 32, and gp100, as in Ref. 25), naive CD8<sup>+</sup> T cells were isolated from HLA-A2<sup>+</sup> normal donors and then cocultured with autologous DC.Tbets or control DCs pulsed with an equimolar mixture of the EphA2<sub>883–891</sub> and gp100<sub>209–217</sub> peptides. T cells were restimulated after 7 days of culture and then assessed for coproducing both IFN-γ- and IL-17-producing IFN-γ-producing CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 Th1 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<sub>151–73</sub> epitope (38) to a peptide-specific CD4<sup>+</sup> 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<sup>+</sup> T cell responders, DC.Tbets and control DCs were loaded with 293T.MART1 lysate for 24 h and then used to prime and boost (on day 7 of culture) autologous, naive CD4<sup>+</sup> T cells isolated from normal HLA-DR4<sup>+</sup> donors. As shown in Fig. 8B, CD4<sup>+</sup> T cells analyzed on day 14 of culture displayed superior levels of reactivity against the MART-1<sub>151–173</sub> 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<sup>+</sup> 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<sup>+</sup> 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) to license DC to preferentially support the in vitro development of type 1 (over type 2 and Treg) polarized responses from naive CD45RO<sup>−</sup>CGR7<sup>−</sup>Cd62L<sup>+</sup>, 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 (CXC3, 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<sup>+</sup> 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-27Rα 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 Tg17 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 DCs (or DC.EGF) 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 co-inhibitory 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., Epa2A and gp100) or protein (recombinant MART-I)-pulsed DCs 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 CXCRR3 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.

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Disclosures
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