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Knockdown of T-bet expression in Mart-$1_{27–35}$-specific T-cell-receptor-engineered human $CD4^+$ $CD25^-$ and $CD8^+$ T cells attenuates effector function

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Summary

Gene transfer to create tumour epitope-specific cytolytic T cells for adoptive immunotherapy of cancer remains an area of active inquiry. When the Mart-$1_{27–35}$-specific DMF5 T-cell receptor (TCR) is transferred into peripheral human $CD4^+$ T cells, the reprogrammed cells exhibit a T helper type 1 (Th1) phenotype with significant multifactorial effector capabilities. The T-bet transcription factor plays an important role in determination of the Th1 differentiation pathway. To gain a deeper understanding of how T-bet controls the outcome of human T-cell reprogramming by gene transfer, we developed a system for examining the effects of short hairpin RNA-mediated T-bet gene knockdown in sorted cell populations uniformly expressing the knockdown construct. In this system, using activated peripheral human $CD4^+$ $CD25^-$ and $CD8^+$ T cells, T-bet knockdown led to attenuation of the interferon-γ response to both antigen-specific and non-specific TCR stimulation. The interleukin-2 (IL-2) antigen-specific response was not attenuated by T-bet knockdown. Also, in TCR-reprogrammed $CD8^+$ cells, the cytolytic effector response was attenuated by T-bet knockdown. T-bet knockdown did not cause redirection into a Th2 differentiation pathway, and no increased IL-4, IL-10, or IL-17 response was detected in this system. These results indicate that T-bet expression is required for maintenance of the $CD4^+$ $CD25^-$ and $CD8^+$ effector phenotypes in TCR-reprogrammed human T cells. They also suggest that the activation protocol necessary for transduction with retrovectors and lentivectors may commit the reprogrammed cells to the Th1 phenotype, which cannot be altered by T-bet knockdown but that there is, nevertheless, a continuous requirement of T-bet expression for interferon-γ gene activation.

Keywords: knockdown; Mart1; T-bet.

Introduction

Early clinical trials of adoptive immunotherapy for human cancers such as melanoma and lymphoma indicate that it is a promising approach.[1,2] The discovery of ever-increasing numbers of human tumour-specific epitopes has led to the use of T-cell receptor (TCR) gene transfer to create tumour epitope-specific cytolytic T cells (CTL) for adoptive immunotherapy. Therefore, consideration has been given to the redirection of both $CD8^+$ and $CD4^+$ T cells to recognize MHC class I determinants by engineered expression of MHC class I-restricted TCR genes, which has been accomplished by several groups in human cells[1,3–8] and mouse cells.[9–11] Although the use of TCR-engineered $CD8^+$ as anti-tumour CTL has been the focus of studies directed at bringing them into clinical practice, recent work on TCR-engineered $CD4^+$ cells has raised interest because of their effector properties that suggest they may play a role in the development of anti-tumour adoptive immunotherapy. Such MHC class I epitope-specific $CD4^+$ cells exhibit mixed [T helper type 1 (Th1) and Th2] cytokine profiles,[4,5,7] may function as CTL-type effectors,[3,6,12] and may provide cognate help toward the generation of memory CTL in mice.[9–11] Human $CD4^+$

cells reprogrammed with MHC class I-restricted TCR directed against the MART-$1_{27–35}$ epitope display a Th1 phenotype with potent antigen-stimulated release of interferon-γ (IFN-γ).[3] These TCR-engineered $CD4^+$ cells exhibit antigen-induced cell proliferation, display a multifunctional effector phenotype, and can provide a cognate helper function for generation of MART-$1_{27–35}$ $CD8^+$ natural CTL.[12] Since the generation of inflammatory Th1 effectors and type 1 CTL appears to be imperative for the development of effective adoptive immunotherapy, it would be useful to better understand the control of the determination of these phenotypes.

Little is known about how T cells reprogrammed by TCR gene transfer differ from anti-tumour T cells derived from patients, whether peripheral or tumour-infiltrative. Furthermore, the optimum conditions for creating potent anti-tumour T cells by TCR gene transfer are not understood and are presently subject to considerable technical constraints. In contrast with transgenic murine systems, human T cells may only be manipulated *in vitro*, creating hurdles for understanding processes such as events in early activation, but there is considerable knowledge available about certain human anti-tumour T-cell responses, especially for melanomas.[3,13,14] However, there are significant differences between mice and humans that must be addressed experimentally to refine adoptive immunotherapy with TCR-engineered human T cells. Lack of persistence of efficacy appears to be associated with problems of exhaustion, loss of proliferation potential, activation induced cell death, and lack of a cognate 'help' function for the adopted TCR-transgenic T cells.[15] Although these problems have been studied in murine systems, there is a clear requirement for focused analysis that is human-specific.

Antigen-specific anti-tumour Th1 and type 1 cytotoxic T cells (Tc1) are the principal components of adoptive immunotherapy for cancers. Th1 cells help Tc1 cells by producing interleukin-2 (IL-2) and IFN-γ,[6,16–18] essential for a potent antigen-specific $CD8^+$ CTL response.[12,19,20] In this sense, the role of the transcription factor T-bet, a principal determinant of both Th1 and Tc1 differentiation, becomes important. Ectopic expression or small interfering RNA-mediated knockdown of either GATA-3 or T-bet can also affect polarization in mouse $CD4^+$ T cells.[21] The transcription factor T-bet directs the Th1 lineage commitment and is required for IFN-γ expression in naive $CD4^+$ T cells but not $CD8^+$ T cells.[22,23] In T-bet knockout mice, $CD4^+$ cells fail to generate Th1 responses and default to the Th2 pathway.[23] Interferon-γ can, as well, induce the expression of T-bet through the signal transduction and activation of transcription 1 (STAT1) pathway,[24] whereas IL-12 drives Th1 commitment through the STAT4 pathway.[25] Th2 lineage commitment appears to be driven by GATA-3 through down-regulation of STAT4 and Th2 lineage commitment is suppressed by T-bet through STAT4 induction.[26] Recent studies indicate that T-bet cooperates with the transcription factor Runx during $CD4^+$ Th1 differentation to activate the IFN-γ gene and silence IL-4 expression.[27,28] GATA-3 is expressed in $CD4^+$ T cells committed to the Th2 lineage[29] through specifying the transcriptional competence of the Th2 cytokine gene cluster, which encodes IL-4, IL-5 and IL-13.[30,31] Therefore, T-bet primarily acts by opposing GATA-3 action, suggesting that Th2 polarization is really the default mode.

We examined the effects of knocking down T-bet gene expression using lentivector-expressed T-bet short hairpin RNA (shRNA) in TCR-engineered human peripheral $CD4^+$ $CD25^-$ and $CD8^+$ T cells. T-bet knockdown in both $CD4^+$ $CD25^-$ and $CD8^+$ cells caused attenuation of IFN-γ expression in response to TCR stimulation either non-specifically with anti-CD3 antibody or with antigen, without affecting IL-2 expression or causing stimulated release of Th2 or Th17 cytokines. Furthermore, cytotoxic effector function of TCR-engineered $CD8^+$ cells was attenuated by T-bet knockdown. This system for stable shRNA-mediated knockdown of gene expression in TCR-engineered human T cells should allow the further dissection of factors influencing the differentiation and anti-tumour potency of these cells.

Materials and methods

Study population, cell lines, culture medium and reagents

Healthy adult donors were enrolled and consented with Institutional Review Board approval. Culturing and separation of $CD4^+$ $CD25^-$, $CD8^+$ T cells using magnetic beads were described previously.[3,12] The antigen-presenting T2 cell line deficient for transporter for antigen presentation (TAP-deficient) was a gift from P. Cresswell, Department of Immunobiology, Yale University.[32] Mart-$1_{27–35}$ and MAGE-$3_{271–279}$ peptides were purchased from NeoMPS (San Diego, CA). Culture medium consisted of Iscove's modified Dulbecco's medium (IMDM; Invitrogen Life Technologies, Grand Island, NY) supplemented with 10%fetal bovine serum (FBS; Gemini Bioproducts, Calabasas, CA). Recombinant human IL-2 (rIL-2), rIL-4 and rIL-15 were purchased from R&D Systems (Minneapolis, MN). Primary antibodies anti-hCD3 and anti-hCD28 were purchased from eBiosciences (San Diego, CA) and anti-T-bet (sc-21749), anti-lamin-B1 (sc-377000) and anti-perforin-1 (sc-33655) were purchased from Santa Cruz Biotechnology (Dallas, TX). Purified mouse anti-human IFN-γ monoclonal antibody (554548) was procured from BD Biosciences (San Jose, CA). For FACS staining anti-T-bet (561265), anti-hIFN-γ (560371), anti-hIL-4 (560672), anti-hIL-10 (554707), anti-hCD107a (555801) and anti-granzyme-B (560213) antibodies were purchased from BD Biosciences; anti-hIL-2 antibody (500322) was procured from Biolegend (San Diego, CA).

T-bet shRNA lentivector construction

Lentivector pLLU2G to be used for RNAi, employing a U6 RNA polymerase III promoter to drive shRNA expression and a human Ubc promoter to drive enhanced green fluorescent protein (eGFP) expression,[33] was obtained from Addgene (Cambridge, MA). Three candidate target sequences (shT-bet2–4) for silencing human T-bet (Gene bank: BC039739.2) were selected by using the shRNA selection tool available at http://sirna.wi.mit.edu/ while the fourth sequence (shT-bet1) was previously described.[34] The T-bet shRNA target sequences (Table 1) were inserted into pLLU2G vector between unique *Hpa*I and *Xho*I sites and DNA sequencing of inserts and surrounding vector sequences was carried out for confirmation.[35] Lentivector stocks were prepared by transient co-transfection of 293T cells with lentiviral vector plasmid, helper plasmid Δ8.9 expressing pol proteins, a plasmid expressing VSV-G protein and a plasmid expressing Rev protein simultaneously using Lipofectamine 2000 reagent purchased from Invitrogen (Carlsbad, CA) as per the manufacturer's protocol.[36] The culture medium containing lentivirus was harvested 48 hr post transfection, filtered, then concentrated 40-fold by centrifugation at 56 600 ***g*** for 90 min. Concentrated virus suspended in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS was frozen at −80° until use. Lentivector stocks were titered by measuring eGFP$^+$ focus-forming units on 293T cells and stocks of approximately 1×10^7 focus-forming units/ml were consistently obtained.

Mart-1$_{27–35}$ epitope TCR retrovector

The Mart-1$_{27–35}$ epitope-specific retrovector TCR, named DMF5, was obtained from a constitutive GaLV envelope PG13 packaging cell line generously provided by Dr Steven Rosenberg (National Institutes of Health, Bethesda, MD).[12,13,37] Viral supernatants were harvested from subconfluent cells in DMEM with 10% FBS.

Transduction of CD4$^+$ CD25$^-$ and CD8$^+$ T cells with lentivirus, retrovirus, culture and sorting

CD4$^+$ CD25$^-$ and CD8$^+$ T cells were activated by culturing with plate-bound anti-CD3 and anti-CD28 antibodies (5 μg each) with 100 U/ml IL-2. After 48 hr, the cells were transduced with lentivector using Polybrene (8 μg/ml) and centrifugation at 1200 ***g*** for 90 min at room temperature. The lentivector-transduced infected cells were then cultured for 24 hr in IMDM with 10% FBS and IL-2 before infecting them with DMF5 TCR retrovector as described previously.[12] After 48 hr the infected cells were stained with Mart-1$_{27–35}$ epitope-specific tetramer (Beckman Coulter, Brea, CA) and analysed for both GFP and tetramer expression with the FACScalibur (BD Biosciences). The GFP$^+$ lentivector-transduced cells were sorted on the FACS Aria (BD Biosciences) after culturing the cells in IMDM with 10% FBS and IL-15 (100 U/ml) for 7–10 days.

Table 1. Four target sequences for knock-down of T-bet gene expression in primary human T cells

Name	Target sequence
shTbet1_FP	5′ CGCTTCCAACACGCATATCTT 3′
shTbet2_FP	5′ GCCTGGACCCAACTGTCAATT 3′
shTbet3_FP	5′ GCCTACCAGAATGCCGAGATT 3′
shTbet4_FP	5′ GTCCAACAATGTGACCCAGAT 3′

Cytokine release assays

T2 cells (5000) were pulsed with either control Mage-3$_{271–279}$ peptide or Mart-1$_{27–35}$ peptide at 25 μg/ml in complete medium for 30 min. To the peptide-pulsed T2 cells a 10-fold excess of engineered CD4$^+$ CD25$^-$ or CD8$^+$ T cells was added in a volume of 200 μl/well and incubated for 16 hr. The supernatants were collected and cytokines (IL-2, IL-4, IL-10, IL-17 and IFN-γ) were measured by ELISA (R&D Systems).

Intracellular staining of T cells

Engineered T cells co-cultured with peptide-pulsed T2 cells for 1 hr or 5 hr. These cultures were treated with Brefeldin A and incubated for 5 hr or overnight, respectively. The cells were then permeabilized, stained with fluorescent antibodies, and analysed with a MACSQuant (Miltenyi Biotech, Bergisch, Gladbach, Germany). Flow cytometric data were analysed with FlowJo (TreeStar, Ashland, OR).

Cytotoxic T-cell degranulation assay

The cytotoxic effector function of engineered CD8$^+$ T cells was assessed by an antigen-induced degranulation assay, measuring antigen-induced surface exposure of lysosomal-associated membrane glycoprotein-1 or CD107a.[38] The CTL degranulation assay was performed by incubating 1×10^5 CD8$^+$ T cells and phycoerythrin-conjugated anti-CD107a antibody with either non-specific plate-bound anti-CD3 antibody (1 μg) or antigen-pulsed T2 cells for 5 hr. Then Brefeldin A was added and the cells were incubated overnight before harvesting the cells for FACS.

Results

Silencing T-bet gene expression in Jurkat human T cells

Jurkat human CD4$^+$ T cells were transduced with control lentivector pLLU2G and four vectors specifying different

human T-bet shRNA. For each vector, two million GFP-positive transduced cells were sorted and nuclear protein extracts were analysed by immunoblotting with anti-T-bet antibodies; anti-Lamin B1 was used as a loading control. Of the four T-bet shRNA constructs, two (T-bet2 and T-bet 3) showed effective silencing of T-bet expression in nuclear extracts, and the T-bet2 construct was chosen for further studies (Fig. 1a).

T-bet silencing in Mart-$1_{27–35}$ TCR-engineered primary human $CD4^+$ $CD25^-$ and $CD8^+$ T cells

Flow cytometric analysis of $CD4^+$ $CD25^-$ and $CD8^+$ cells transduced with either control or T-bet shRNA lentivectors and the F5M1 retrovirus expressing a Mart-$1_{27–35}$-specific TCR. In cells transduced with the control vector, T-bet expression following anti-CD3 antibody restimulation was demonstrated by intracellular staining and flow cytometry. Mart-$1_{27–35}$ TCR-engineered $CD4^+$ $CD25^-$ and $CD8^+$ cell populations transduced with the T-bet shRNA construct showed significantly reduced numbers of cells expressing T-bet, compared with the cells transduced with the control lentivector, when the cells were restimulated with anti-CD3 antibody (Fig. 1b,c; central panel).

Non-specific stimulation of T-bet silenced Mart-$1_{27–35}$ TCR-engineered human T cells

Mart-$1_{27–35}$ TCR-engineered $CD4^+$ $CD25^-$ and $CD8^+$ cells transduced with either control or T-bet knockdown lentivectors were stimulated non-specifically with anti-CD3 antibody, and the IFN-γ response was studied by intracellular staining and flow cytometry. Interferon-γ expression was found to be significantly reduced in the cells transduced with the T-bet shRNA construct (Fig. 1b, c; lowest panel).

Mart-$1_{27–35}$ antigen-specific stimulation of T-bet silenced TCR-engineered human T cells

$CD4^+$ $CD25^-$ were transduced with the F5M1 Mart-$1_{27–35}$ TCR retrovector and lentivectors expressing control or T-bet shRNA and were sorted for GFP expression and exposed to the cognate peptide (Mart-$1_{27–35}$) or a control peptide (MAGE-$3_{271–279}$) with T2 antigen-presenting cells (Fig. 2a, b). The cognate Mart-$1_{27–35}$ peptide on an appropriate antigen-presenting cell triggered a potent release of both IFN-γ and IL-2 from the TCR-engineered $CD4^+$ $CD25^-$ cells transduced with the control lentivector (Fig. 2c,d).

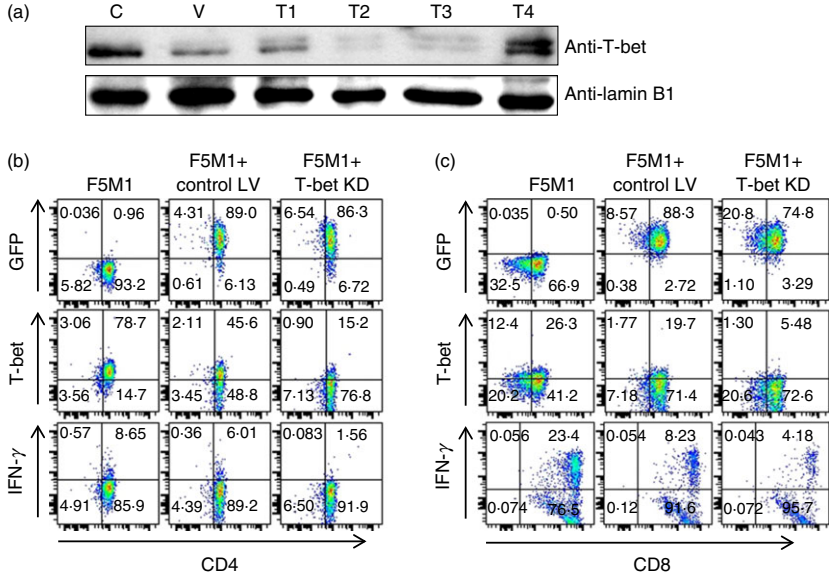

Figure 1. Knockdown of T-bet in Mart-$1_{27–35}$ T-cell receptor (TCR) -engineered primary human T cells causes reduced interferon-γ (IFN-γ) expression. Nuclear protein fractions were isolated from Jurkat cells transduced with or without control lentivirus or lentivirus engineered to express four different short hairpin (sh) RNA sequences to silence T-bet gene expression. The green fluorescent protein (GFP) positive lentivirus-infected cells were sorted to a purity of > 95%. The nuclear protein fractions were resolved on SDS–PAGE and immunoblotted with Anti-T-bet and Anti-Lamin-B antibodies. (a) Nuclear protein fraction: lane C, uninfected Jurkat cells; lane V, Jurkat cells infected with lentivirus harbouring empty vector pLLU2G; lanes T1 to T4, Jurkat cells transduced with lentivirus harbouring pLLU2G vector engineered to express shT-bet1, shT-bet2, shT-bet3 and shTbet4, respectively. (b, c) Primary human $CD4^+$ $CD25^-$ and $CD8^+$ cells were activated by plate bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2). These cells were then transduced with F5M1 retrovirus and/or lentivirus with control or shRNA to silence T-bet. These GFP-positive lentivirus infected cells were sorted (top panel) and restimulated with plate bound anti-CD3 antibody (overnight) before intracellular staining for T-bet and IFN-γ. The data shown here are representative of five (for $CD4^+$ $CD25^-$) and three (for $CD8^+$) independent experiments.

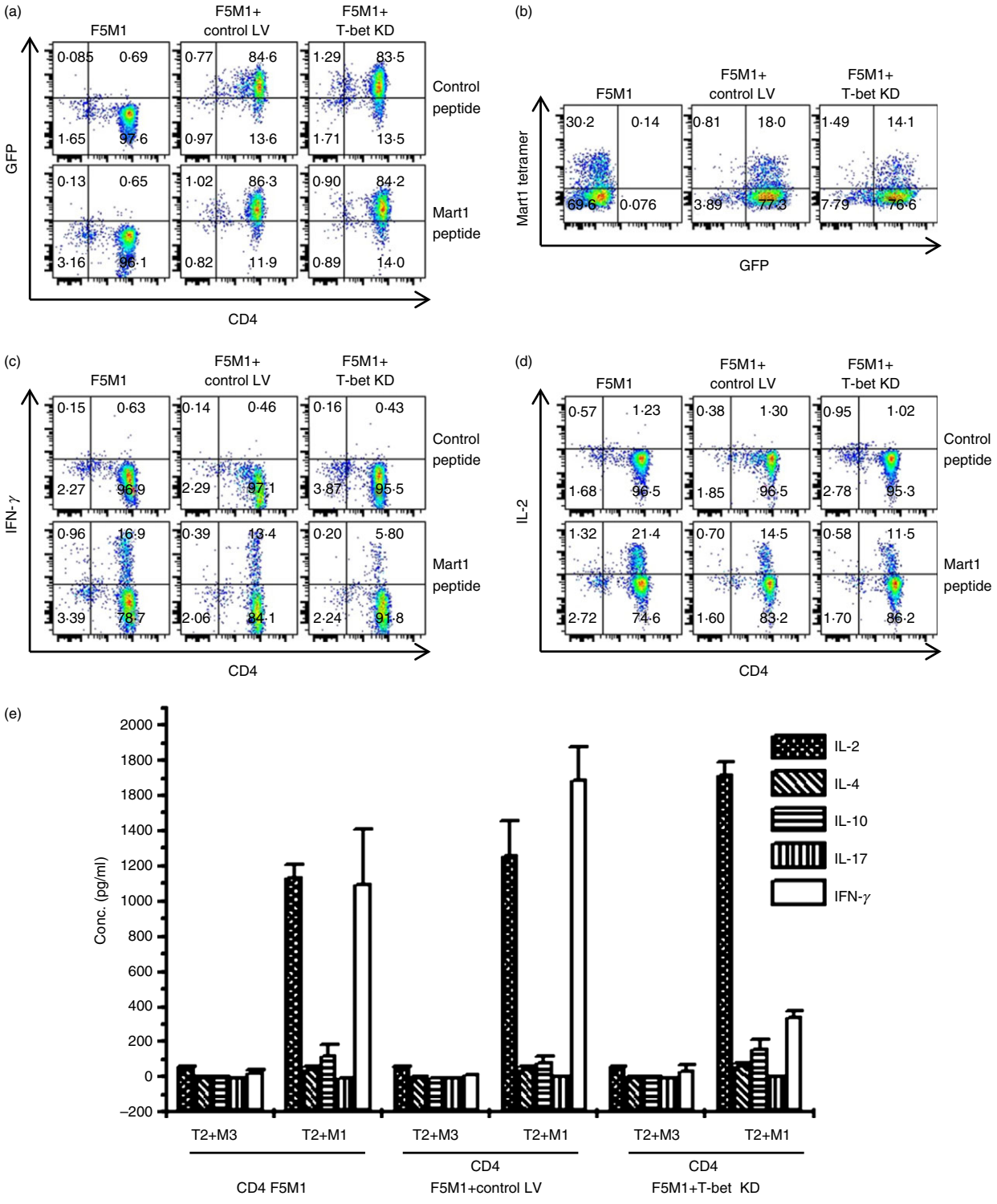

Figure 2. Decline in interferon-γ (IFN-γ) expression on Mart-$1_{27–35}$ antigen-specific stimulation of T-bet knocked down primary human $CD4^+$ $CD25^-$ cells engineered to express Mart-$1_{27–35}$ T-cell receptor (TCR). Primary human $CD4^+$ $CD25^-$ cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2). These cells were then engineered to express Mart-$1_{27–35}$ TCR infecting them with F5M1 retrovirus and/or lentivirus with control or T-bet short hairpin (sh) RNA. The green fluorescent protein (GFP) -positive lentivirus-infected cells were sorted (a) and Mart-$1_{27–35}$ TCR-engineered cells were estimated by tetramer staining (b). Intracellular IFN-γ (c) and IL-2 (d) expression levels were estimated by co-culture of T2 cells pulsed with either control peptide (MAGE-$3_{271–279}$) or cognate peptide (Mart-$1_{27–35}$) with the T-bet knocked-down $CD4^+$ $CD25^-$ T cells. Antigen-specific cytokine release of IFN-γ, IL-2, IL-4, IL-10 and IL-17 in supernatant was estimated ~16 hr following co-culture by ELISA (e). The data shown here are representative of five independent experiments.

However, the Mart-$1_{27–35}$ TCR-engineered $CD4^+$ $CD25^-$ cells transduced with the T-bet shRNA lentivector exhibited an IL-2 response similar to cells transduced with the control lentivector, but a significantly attenuated IFN-γ response (Fig. 2c,d). The attenuated IFN-γ response to antigen due to T-bet silencing was not only evident from intracellular staining of IFN-γ and IL-2 (Fig. 2c,d) but also by ELISA of culture supernatants (Fig. 2e). Besides confirming the results of the intracellular staining, the cytokine release assay failed to detect any differences in the release of IL-4, IL-10, or IL-17 in the antigen-stimulated $CD4^+$ $CD25^-$ cells due to T-bet silencing (Fig. 2e).

In a similar manner, activated human $CD8^+$ cells were transduced with Mart-$1_{27–35}$ TCR F5M1 retrovirus and T-bet knockdown or control lentiviruses. The transduced GFP^+ $CD8^+$ cells were then sorted and subjected to antigen-specific stimulation with T2 antigen-presenting cells pulsed with either Mart-$1_{27–35}$ or control MAGE-$3_{271–279}$ peptides (Fig. 3a,b). Antigen stimulation elicited a potent IL-2 response from the TCR-engineered $CD8^+$ cells that was not affected by T-bet silencing, but the IFN-γ response was significantly attenuated by T-bet silencing when studied by intracellular staining and flow cytometry (Fig. 3c,d). The effect of T-bet silencing on antigen-stimulated release of IFN-γ and IL-2 showed a similar pattern when studied in an antigen-specific cytokine release assay, using ELISA (Fig. 3e). The cytokine release assay failed to detect any differences in the release of IL-4, IL-10 and IL-17 in the antigen-stimulated $CD8^+$ cells due to T-bet silencing (Fig. 3e).

T-bet knockdown with Th2 polarizing conditions in human $CD4^+$ $CD25^-$ T cells

In primary human $CD4^+$ $CD25^-$ T cells engineered to express Mart-$1_{27–35}$ TCR cultured in the presence of IL-15, knockdown of T-bet did not lead to antigen-triggered elaboration of Th2 cytokines (IL-4 and IL-10). Because the activation and transduction protocol was carried out under slightly Th1-polarizing conditions, we asked if the imposition of Th2-polarizing culture conditions (IL-4 and anti-IFN-γ) after the engineering with TCR and T-bet knockdown vectors would lead to a different outcome. The human $CD4^+$ $CD25^-$ T cells engineered with TCR and T-bet knockdown vectors, with appropriate controls, were cultured under Th2 polarizing conditions for 48 hr before exposure to antigen, then analysed for intracellular levels of T-bet, IFN-γ, IL-4 and IL-10 by FACS (Fig. 4). No antigen-induced intracellular expression of IL-4 (Fig. 4c) or IL-10 (Fig. 4d) was observed in TCR-engineered $CD4^+$ $CD25^-$ T cells knocked-down for T-bet expression.

Effect of T-bet silencing on $CD8^+$ cell cytoxicity

Mart-$1_{27–35}$ TCR-engineered $CD8^+$ cells exhibit antigen-specific cytotoxicity in addition to antigen-triggered cytokine release accompanied by surface display of CD107a, which is a surrogate assay for the detection of cytotoxicity mediated by release of perforin and granzyme into the immune synapse formed between $CD8^+$ effectors and their targets.[38] T-bet silencing significantly attenuated the CD107a surface display in TCR-engineered $CD8^+$ cells stimulated non-specifically with anti-CD3 antibody (Fig. 5a). Also, Figure 5(b) shows that T-bet silencing of Mart-$1_{27–35}$ TCR-engineered $CD8^+$ cells significantly attenuated antigen-stimulated CD107a surface display.

Effect of T-bet knockdown on expression of granzyme-B and perforin-1 in TCR-engineered human $CD4^+$ $CD25^-$ and $CD8^+$ T cells

Antigen-specific cytolytic function has been described for TCR-engineered human $CD4^+$ and $CD8^+$ T cells,[39] and has been associated with both perforin and granzyme-B expression.[3] We looked at the affect of T-bet knockdown on antigen-induced expression of granzyme-B and perforin-1 in human $CD4^+$ $CD25^-$ and $CD8^+$ T cells engineered with the Mart-$1_{27–35}$ TCR (Fig. 6). Intracellular granzyme-B and perforin-1 expression in TCR-engineered human $CD4^+$ $CD25^-$ and $CD8^+$ cells were analysed by FACS with and without T-bet knock-down (Fig. 6). First, we observed no effect of Mart-$1_{27–35}$ antigen stimulation on granzyme-B-positive $CD4^+$ T cells, but the percentage of perforin-1-positive cells increased (Fig. 6a,b). Knockdown of T-bet in these $CD4^+$ $CD25^-$ cells did not affect the number of granzyme-B-positive cells but impaired the increase of perforin-1-positive cells on antigen-specific stimulation (Fig. 6a,b). In TCR-engineered $CD8^+$ cells, T-bet knockdown had no significant effect on the number of granzyme-B-positive cells, even though there were significantly more in $CD8^+$ than in $CD4^+$ $CD25^-$ cells (Fig. 6c). The antigen-stimulated increase in the perforin-1-positive Mart-$1_{27–35}$ TCR-engineered $CD8^+$ T cells were attenuated by T-bet knockdown (Fig. 6d).

Discussion

Adoptive immunotherapy with TCR-engineered T cells appears to offer an attractive approach to the treatment of human cancers. The prospect, however, is fraught with limitations, some of which derive from an incomplete understanding of the fundamental processes involved in TCR gene transfer to peripheral human lymphocytes. As TCR gene transfer using lentivectors and/or retrovectors requires activation of these cells to effect the transduction, the effect of activation appears to affect the subsequent differentiation and effector functions of the transduced cells. Human peripheral $CD4^+$ T cells engineered to express an MHC class I-restricted MART-$1_{27–35}$ epitope-specific TCR consistently exhibited a Th1 effector phenotype.[3,12] This observation led us hypothesize that either

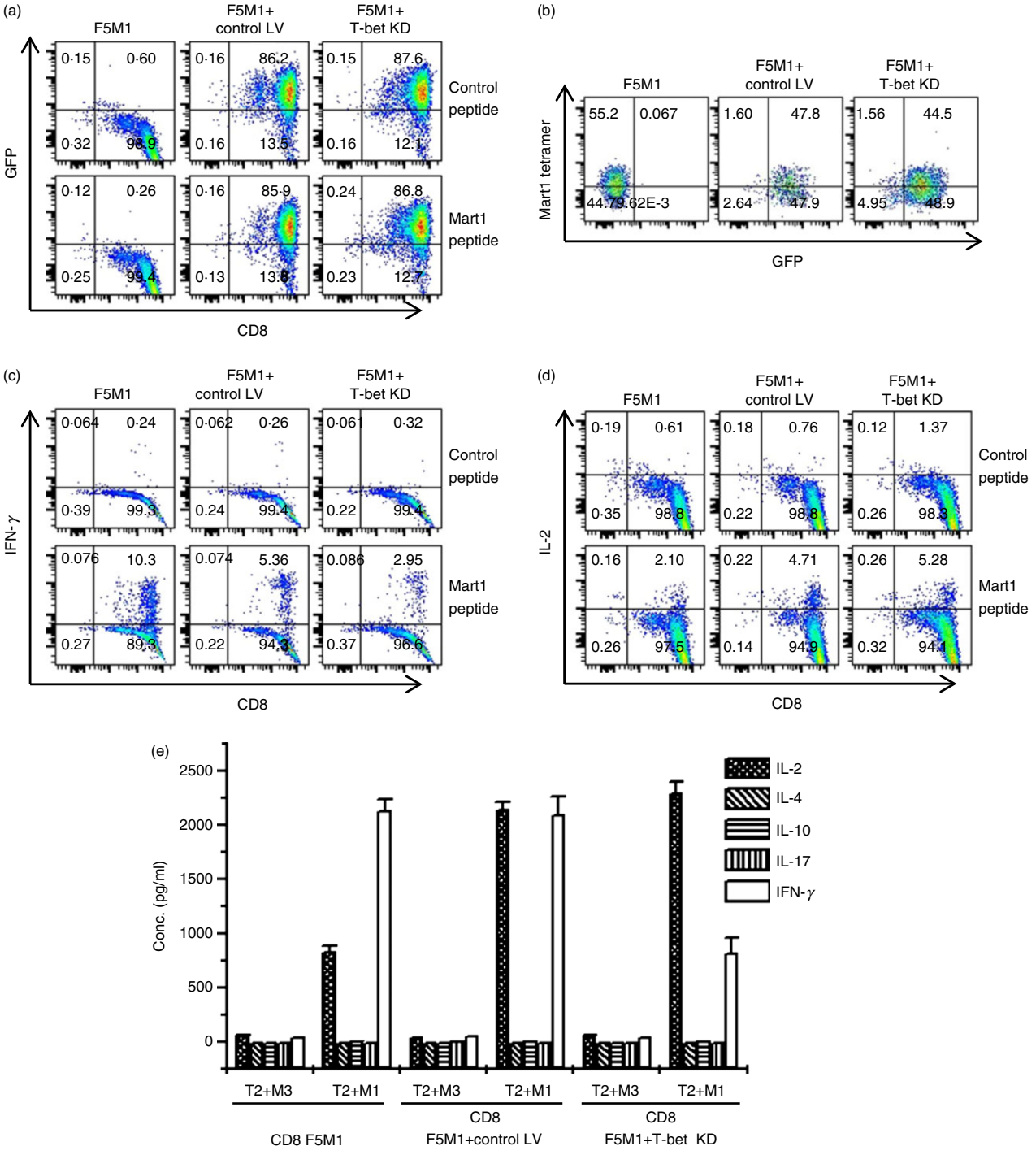

Figure 3. Decrease in interferon-γ (IFN-γ) expression on Mart-$1_{27–35}$ antigen-specific stimulation of T-bet knocked down primary human $CD8^+$ T cells engineered to express Mart-$1_{27–35}$ T-cell receptor (TCR). Primary human $CD8^+$ T cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2). These cells were then engineered to express Mart-$1_{27–35}$ TCR by infecting them with F5M1 retrovirus and/or lentivirus with control or T-bet short hairpin (sh) RNA. The green fluorescent protein (GFP) -positive lentivirus-infected cells were sorted (a) and Mart-$1_{27–35}$ TCR engineered cells were estimated by tetramer staining (b). Intracellular IFN-γ (c) and IL-2 (d) expression levels were estimated by co-culture of T2 cells pulsed with either control peptide (MAGE-$3_{271–279}$) or cognate peptide (Mart-$1_{27–35}$) with the T-bet knocked down $CD8^+$ T cells. Antigen-specific cytokine release of IFN-γ, IL-2, IL-4, IL-10 and IL-17 in supernatant was estimated ~16 hr following co-culture by ELISA (e). The data shown here are representative of three independent experiments.

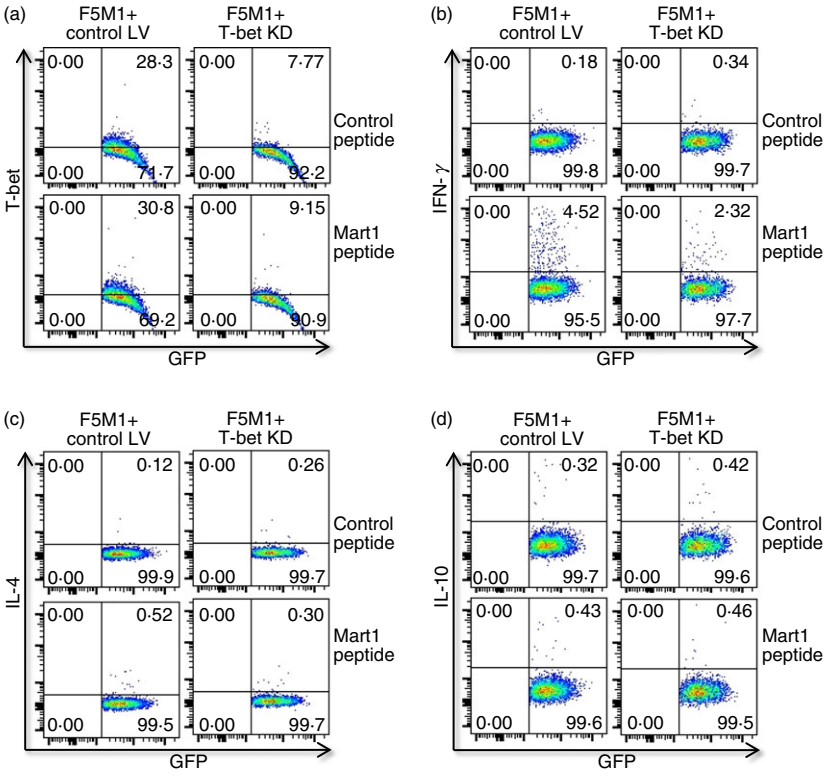

Figure 4. Effect of T helper type 2 (Th2) polarizing culture conditions on T-bet knocked-down primary human T-cell receptor (TCR) -engineeered $CD4^+$ $CD25^-$ T cells. Primary human $CD4^+$ $CD25^-$ T cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2) and then engineered with the F5 Mart-$1_{27–35}$ TCR retrovector and control or T-bet short hairpin (sh) RNA lentivectors. These cells were cultured under Th2 polarizing condition [IL-2 at 100 U/ml + IL-4 at 1000 U/ml + anti-human interferon-γ (IFN-γ) at 1 μg/ml] for 48 hr. The green fluorescent protein (GFP) -positive $CD4^+$ $CD25^-$ cells were gated to determine intracellular expression of T-bet (a), IFN-γ (b), IL-4 (c) and IL-10 (d) in T cells co-cultured with T2 cells pulsed with either control peptide (MAGE-$3_{271–279}$) or cognate peptide (Mart-$1_{27–35}$).

the activation conditions or the TCR, itself, were responsible for the polarization and we further hypothesized that by interfering with the expression of a principal determinant of Th1 differentiation, namely T-bet, that we could affect the downstream effector function of the engineered T cells. Also, as T-bet mouse knockouts lack Th1 cells and exhibit a preponderance of Th2 cells,[22,23] we also hypothesized that by interfering with T-bet expression and Th1 differentiation, we might find that TCR-engineered $CD4^+$ T cells might express a new phenotype with down-regulated IFN-γ production and up-regulation of Th2 cytokines such as IL-4 or IL-10.

To test these hypotheses we developed a robust system for studying the effects of shRNA-mediated gene knockdown in TCR-engineered human T cells. The system allowed the generation of near-homogeneous populations of shRNA-expressing cells by live sorting for transduced cells that could be further characterized for effector function and stimulated cytokine release in culture and by FACS. We were able to obtain information about the role of T-bet in TCR-engineered human T lymphocytes that allowed comparison with results obtained in mice that could have important implications for the design of more sophisticated approaches to the use of TCR-engineered T cells in cancer therapy.

The evidence shows that we generated stable, nearly homogeneous populations of human $CD4^+$ $CD25^-$ and $CD8^+$ T cells with reduced T-bet expression achieved through shRNA-mediated knock-down. The T-bet target sequence proving most effective for shRNA-mediated knockdown of expression is distinct from previously reported T-bet target sequences. The shRNA expression vector chosen expressed GFP driven by the Ubi-C promoter, which is active in human T lymphocytes in early stages of activation, enabled us to perform live sorting and analysis after transduction. A similar approach to achieving gene expression knockdown in human T cells has recently been described.[40] The effect of the activation methods employed before transduction may have contributed to the differentiated state of the engineered cells, therefore anti-CD3 stimulation of TCR signalling in human T cells would tend to promote Th1 differentiation. Since lentivector and retrovector transduction does not occur in quiescent human T cells, some activation method must be employed for this technical approach, and it seems likely that activation methods other than the one we employed may allow generation of transduced populations with different effector phenotypes.

Previous reports had shown differences in the effects of T-bet knockdown on effector function triggered by polyclonal or antigen-specific TCR stimulation,[23,41] we chose to study the effects of T-bet knockdown in a system that could be stimulated through the TCR by either method. $CD8^+$ T cells from T-bet knockout mice responded normally to polyclonal TCR stimulation,[23] whereas $CD8^+$ T cells from OT-1 T-bet knockout mice showed attenuated effector responses to stimulation by the OT-1 cognate $OVA_{257–264}$ epitopic peptide.[41] In our human experimental

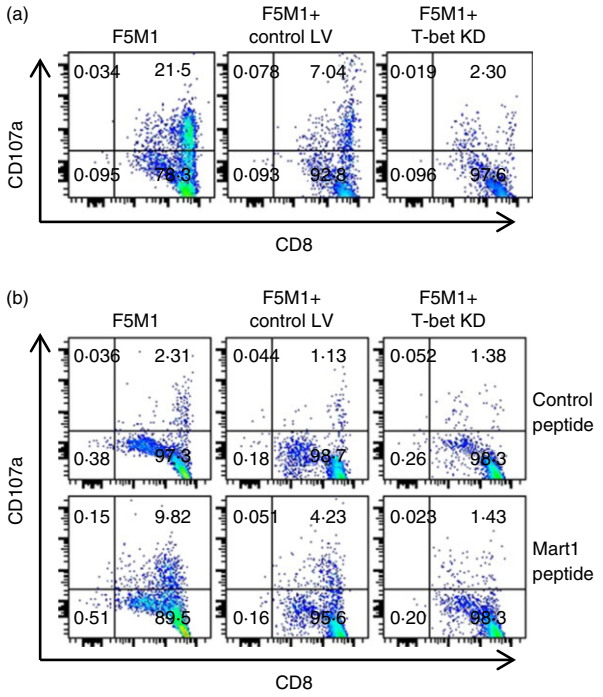

Figure 5. Attenuation of cytotoxic effector function in T-bet knocked down primary human $CD8^+$ T cells engineered to express Mart-$1_{27–35}$ T-cell receptor (TCR). Primary human $CD8^+$ T cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2). These cells were then engineered to express Mart-$1_{27–35}$ TCR by infecting them with F5M1 retrovirus and/or lentivirus with control or T-bet short hairpin (sh) RNA. The green fluorescent protein (GFP) -positive lentivirus-infected cells were sorted. These cells were either restimulated with anti-CD3 antibody or co-cultured with T2 cells pulsed with either control peptide (MAGE-$3_{271–279}$) or cognate peptide (Mart-$1_{27–35}$). Cytotoxicity or degranulation of these engineered human $CD8^+$ T cells was measured by surface exposure of CD107a either on non-specific anti-CD3 antibody stimulation. (This result is from a single experiment) (a) or Mart-$1_{27–35}$ antigen-specific stimulation (the data shown here are representative of three independent experiments) (b).

system, both forms of TCR stimulation yielded similar results, suggesting that we have identified a distinction between the roles of T-bet in murine and human $CD8^+$ effector function.

We found that in primary human Mart-$1_{27–35}$ TCR-engineered T cells ($CD4^+$ $CD25^-$ and $CD8^+$), knock-down of T-bet expression did not interfere with IL-2 release with Mart-$1_{27–35}$ antigen-specific TCR stimulation. This finding was not consistent with results obtained in murine systems and served as a valuable internal experimental control.[22,41,42] Although IL-2 is a pleotropic regulator of T-cell differentiation, its expression is not known to be directly controlled by T-bet.[43]

Interferon-γ gene expression, in contrast, is positively regulated, in part, by T-bet by virtue of the presence of several T-bet binding sites in the IFN-γ gene regulatory region. As there are numerous other transcription factor binding sites within that region, the positive regulatory effect of T-bet cannot be the sole determinant of IFN-γ expression, but appears to have a prominent role.[44–48] Furthermore, the present study indicates that, in the case of IFN-γ expression, T-bet does not operate as a permissive determinant, or differentiation switch, but is required for an effective MART-$1_{27–35}$ antigen-specific anti-tumour response.

T-bet knockout mice lack Th1 $CD4^+$ T cells but have prominent Th2 $CD4^+$ T-cell responses to antigen,[23] and the mechanism underlying this phenomenon appears to be that GATA-3 drives Th2 differentiation as a default pathway, but T-bet antagonizes the effect of GATA-3, driving Th1 differentiation.[23,47,49] The generation of antigen-specific Th2 or Treg cells by the manipulation of TCR-engineered cells is certainly a worthy goal with translational implications, and we hypothesized that knock-down of T-bet expression in naive human $CD4^+$ cells might lead to Th2-type differentation due to unopposed GATA-3 action. In our system, the effect of knocking-down T-bet expression in TCR-engineered $CD4^+$ cells did not yield Th2 cells, as no antigen-stimulated release of IL-4 or IL-10 was detected. As the pre-transduction activation step in our system involved TCR stimulation with anti-CD3 antibody, it may be that the initial period of activation was sufficient to oppose GATA-3 action to activate the Th2-specific regulatory loci. Furthermore, culturing the T-bet knockdown Mart-$1_{27–35}$ TCR-engineered $CD4^+$ $CD25^-$ cells in Th2 polarizing conditions (IL-4 plus anti-IFN-γ) following transduction (Fig. 4) still did not skew these cells towards the Th2 phenotype. Also, previous work with this system examined the effects of varying the intensity of the antigenic stimulus on differentiation, showing that reduction by four orders of magnitude diminished the intensity of the response but did not skew toward the Th2 phenotype.[12] Our results may reflect important differences between the roles of T-bet in human and mouse T-cell differentiation. A better understanding of the interplay of the various regulatory factors early after activation and T-bet knockdown may be obtained by examination of the state of chromatin remodelling in these engineered cells.

Although T-bet is a principal determinant of $CD4^+$ differentiation, and appears to be required for IFN-γ gene expression, the present study shows that it plays a role in the maintenance of human $CD8^+$ T-cell effector function. CD107a is a marker expressed on the cell surface following activation-induced degranulation, a necessary precursor of cytolysis by $CD8^+$ T cells[38] that has been used to characterize TCR-engineered human T cells.[3,12] Using this assay, we found that knocking down T-bet expression in TCR-engineered $CD8^+$ T cells attenuated their cytolytic effector function, which is distinct from IFN-γ or IL-2

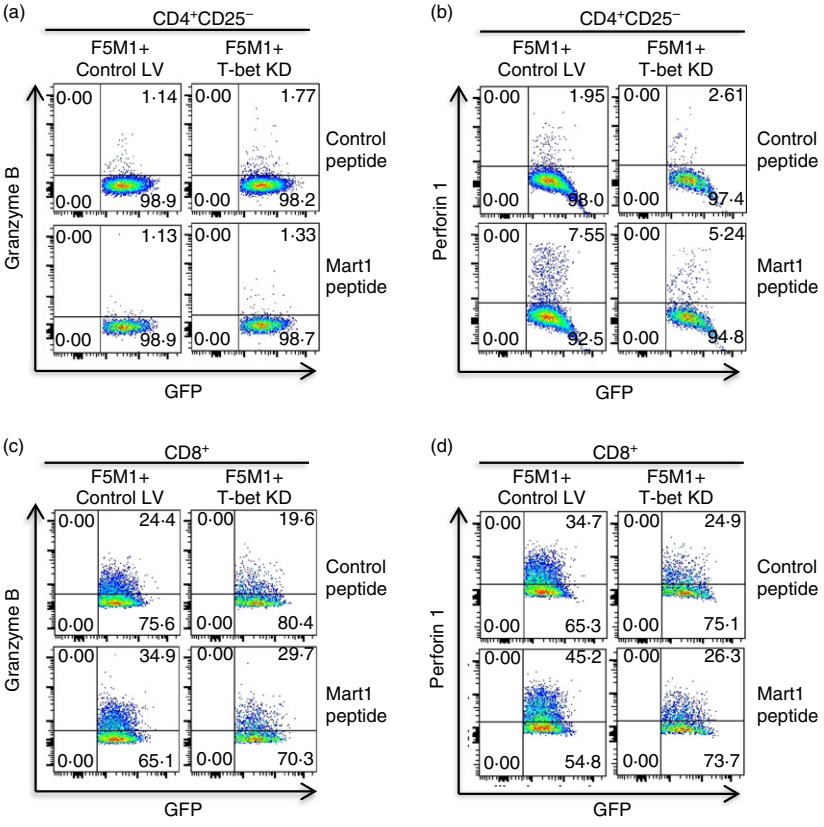

Figure 6. Effect of T-bet knockdown on intracellular expression of granzyme-B and perforin-1. Primary human $CD4^+$ $CD25^-$ and $CD8^+$ T cells were activated by plate-bound anti-CD3 and anti-CD28 antibodies in the presence of interleukin-2 (IL-2). These cells were then engineered to express Mart-$1_{27–35}$ T-cell receptor (TCR) by infecting them with F5M1 retrovirus and lentivirus with control or T-bet short hairpin (sh) RNA. The green fluorescent protein (GFP) positive cells were gated to determine intracellular expression of granzyme-B and perforin-1 in cells co-cultured with T2 cells pulsed with either control peptide (MAGE-$3_{271–279}$) or cognate peptide (Mart-$1_{27–35}$). (a) and (c), intracellular granzyme-B expression in engineered $CD4^+$ $CD25^-$ and $CD8^+$ T cells. (b) and (d), intracellular perforin-1 expression in engineered $CD4^+$ $CD25^-$ and $CD8^+$ T cells.

expression. Cytotoxic granules containing perforin and granzyme-B are found in cytotoxic T lymphocytes and natural killer cells,[50] but perforin and granzyme-B have been found in human Mart-$1_{27–35}$ TCR-engineered $CD4^+$ $CD25^-$ T lymphocytes.[3] Activated natural killer cells and $CD8^+$ T cells have coordinately induced levels of Eomes, perforin and granzyme-B; however, activated $CD4^+$ T cells express lower levels of Eomes, perforin and granzyme-B.[51] We observed that the expression of perforin-1 and granzyme-B in $CD8^+$ cells was greater than the $CD4^+$ $CD25^-$ T cells, when both were engineered for Mart-$1_{27–35}$ TCR expression. We found that there was no significant effect of T-bet knockdown on granzyme-B expression in human $CD4^+$ $CD25^-$ and $CD8^+$ T cells; however, we found that T-bet knockdown attenuated perforin-1 expression in activated TCR-engineered $CD4^+$ $CD25^-$ and $CD8^+$ T cells. Differences between mice and humans in this regard have not been reported, but different studies in mouse models have shown that T-bet-deficient mice show reduced expression of either perforin or granzyme-B.[51–54] Eomes and T-bet jointly regulate cytotoxicity by causing progressive loss of perforin mRNA in bone marrow natural killer cells from compound mutant Eomes and Tbx21 mice.[54] Perforin and granzyme-B levels are not appreciably regulated by T-bet in $CD8^+$ T cells from P14 TCR transgenic mice activated either with antigen or non-specifically; Runx3 and Eomes (but not T-bet) appear to co-operate in perforin expression.[53]

Our studies indicate that T-bet expression is necessary for Th1/Tc1 effector function involving IFN-γ in both $CD4^+$ $CD25^-$ and $CD8^+$ Mart-$1_{27–35}$ TCR-engineered peripheral human T cells and also appears to be necessary for robust cytotoxicity of TCR-engineered $CD8^+$ cells stimulated specifically or non-specifically. Engineering human peripheral T cells with both shRNA-expressing lentivectors and TCR-expressing retrovectors appears to offer a valid approach for generating and manipulating antigen-specific effector cells that may have translational value. By expanding the repertoire of genes that can be subjected to shRNA-mediated knockdown in this context, it may be possible to develop a deeper understanding of the factors influencing the differentiation of TCR-engineered cells and to offer the possibility of generating T-lymphocyte populations with a variety of effector and regulatory properties.

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Disclosure

None.

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