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$CD4^+CD25^-$ T Cells Transduced to Express MHC Class I-Restricted Epitope-Specific TCR Synthesize Th1 Cytokines and Exhibit MHC Class I-Restricted Cytolytic Effector Function in a Human Melanoma Model[1]

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Cytolytic T cell-centric active specific and adoptive immunotherapeutic approaches might benefit from the simultaneous engagement of $CD4^+$ T cells. Considering the difficulties in simultaneously engaging $CD4^+$ and $CD8^+$ T cells in tumor immunotherapy, especially in an Ag-specific manner, redirecting $CD4^+$ T cells to MHC class I-restricted epitopes through engineered expression of MHC class I-restricted epitope-specific TCRs in $CD4^+$ T cells has emerged as a strategic consideration. Such TCR-engineered $CD4^+$ T cells have been shown to be capable of synthesizing cytokines as well as lysing target cells. We have conducted a critical examination of functional characteristics of $CD4^+$ T cells engineered to express the α- and β-chains of a high functional avidity TCR specific for the melanoma epitope, MART-$1_{27–35}$, as a prototypic human tumor Ag system. We found that unpolarized $CD4^+CD25^-$ T cells engineered to express the MART-$1_{27–35}$ TCR selectively synthesize Th1 cytokines and exhibit a potent Ag-specific lytic granule exocytosis-mediated cytolytic effector function of comparable efficacy to that of $CD8^+$ CTL. Such TCR engineered $CD4^+$ T cells, therefore, might be useful in clinical immunotherapy. ***The Journal of Immunology,*** **2008, 181: 1063–1070.**

Cancer vaccines and adoptive immunotherapy for cancer have achieved impressive clinical responses in some patients (1, 2), but the overall result of such treatments has been somewhat modest. Intense efforts are underway to improve the outcome of these forms of therapy. Most cancer vaccines and adoptive immunotherapeutic strategies seek to achieve a robust and long-lived CTL response, because most cancer cells express only MHC class I-restricted epitopes. The task of generating a robust and long-lived CTL response to tumor-associated Ags, most of which are self-Ags, faces many constraints. Among these is the lack of an effective strategy to engage $CD4^+$ Th cells.

Clinical trials with various forms of non-cognate help (universal class II epitope, tetanus toxoid, KLH, etc.) have been undertaken without clear evidence of benefit. A limited number of studies with MHC class II determined helper epitopes—as cognate help—has also been initiated. Unfortunately, only a few class II-restricted determinants have so far been defined, and only a limited number of patients can be matched for the appropriate MHC class I and class II alleles for which epitopes are available. The task of engaging cognate help targeting MHC class II-restricted epitopes in anti-tumor immunity, therefore, has turned out to be difficult. Lately, redirecting $CD4^+$ T cells to MHC class I-restricted epitopes through engineered expression of class I-restricted epitope-specific T cell receptors in $CD4^+$ T cells has emerged as a strategic consideration. It has been shown that $CD4^+$ T cells engineered to express relevant MHC class I-restricted epitope-specific T cell receptors can function as effector cells (3–7). They usually exhibit a mixed (i.e., Th1 as well as Th2/3) cytokine synthesis profile (3, 5, 6), unless before transduction they are polarized to Th1 phenotype (7). It has also been shown that MHC class I-restricted epitope-specific TCR-engineered $CD4^+$ T cells provide help toward the generation of memory CTL response in animal models (8, 9). These observations, and the pressing need for developing an effective method that would engage cognate help in tumor immunotherapy in humans, prompted us to undertake a critical examination of the biology of $CD4^+$ T cells engineered to express a relevant set of MHC class I-restricted human tumor epitope-specific TCR. In this study we show that human

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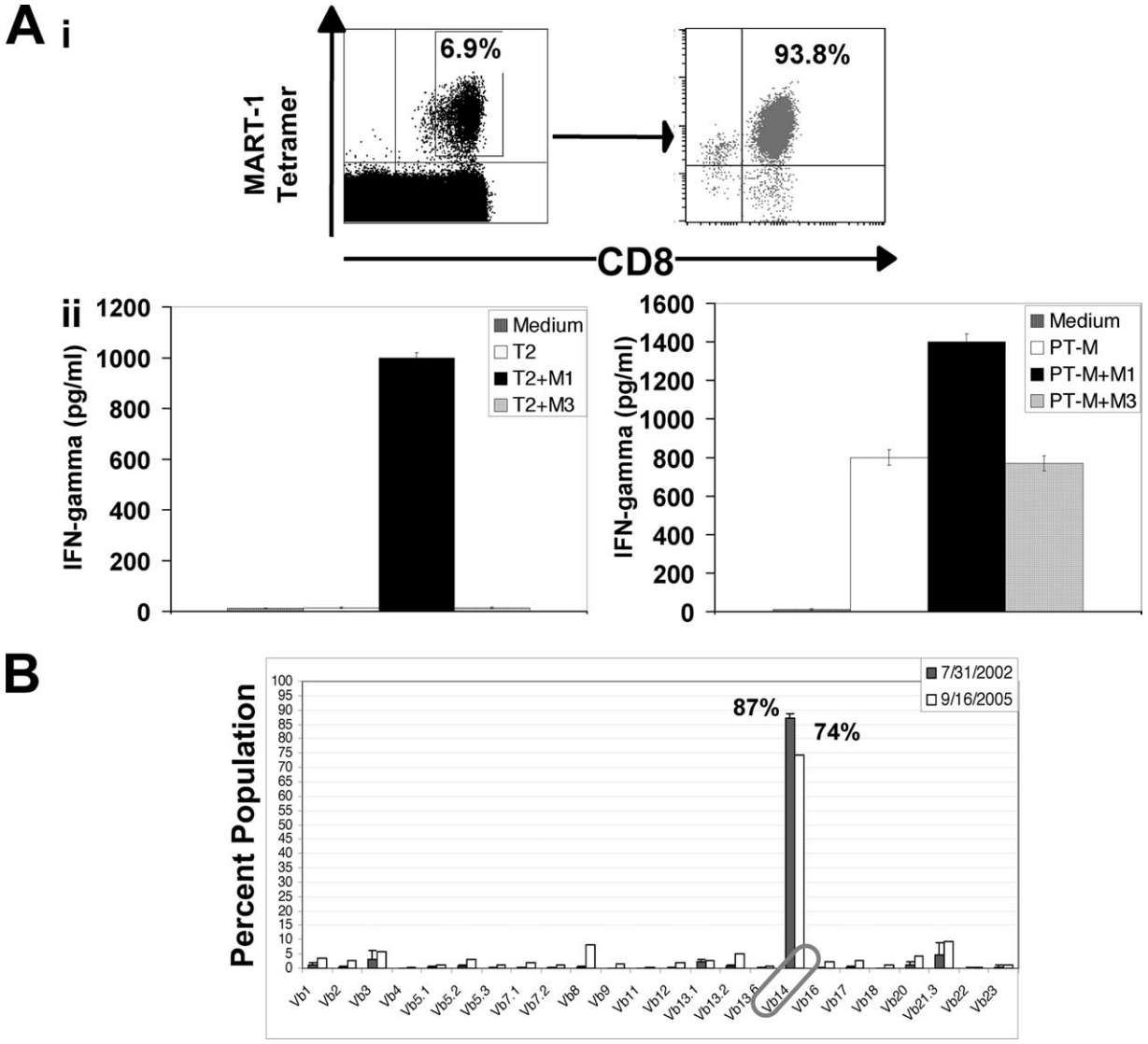

FIGURE 1. Generation of MART-$1_{27–35}$ epitope-specific CTL line from the peripheral blood-derived CD8 T cells of a melanoma patient. *Ai*, An oligoclonal CTL line was generated from a melanoma patient's peripheral blood-derived $CD8^+$ T cells, in a dendritic cell-T cell coculture expansion protocol. *Aii*, The Ag-specific effector function of the CTL line was tested by measuring IFN-γ release in the supernatant upon coculture with either surrogate target cells, T2 (*left panel*), or MART-1-expressing human melanoma cells PT-M (*right panel*). CTL were cocultured with target cells alone (T2/PT-M) or target cells pulsed with either cognate peptide, MART-$1_{27–35}$ (T2/PT-M $^+$M1), or MAGE-$3_{271–279}$ control peptide (T2/PT-M+M3), and cytokine released in supernatant was measured next day. *B*, TCR V-β clonotypic analysis of patient-derived MART-$1_{27–35}$ epitope-specific CD8 T cells.

$CD4^+CD25^-$ T cells engineered to express a TCR specific for the HLA-A2.1-restricted melanoma epitope, Melan-A/MART-$1_{27–35}$ (M1),[3] synthesize only Th1 cytokines IFN-γ and IL-2 and not IL-4 or IL-10. They also exhibit MHC class I-restricted and granule-mediated cytolytic activity in recognition of the M1 epitope presented on HLA-A2.1-positive surrogate target cells, as well as melanoma cells naturally displaying the M1 epitope.

Materials and Methods

Study population, cell lines, culture medium, and reagents

HLA-A2-positive melanoma patients and healthy donors were included in the study with informed consent. Culture medium and other reagents used to generate M1 epitope-specific T cells have been described previously (10). T2 cells, human melanoma cell line A375, was a gift of Steven A. Rosenberg (Surgery Branch, National Cancer Institute, Bethesda, MD). A375 cells engineered to express Melan-A/MART-1, A375-M have been described before (11). The melanoma cell lines PT-M and JL-M were established from two HLA-A2.1-positive melanoma patients.

Construction of recombinant lentivirus expressing MART-$1_{27–35}$ TCR

A MART-$1_{27–35}$ antigenic epitope-specific oligoclonal CTL line was generated from a limiting dilution microwell seeded with three MART-$1_{27–35}$ peptide-pulsed dendritic cell-activated $CD8^+$ T cells generated as described before (10, 11). Total RNA was extracted from the CTL line using a Qiagen RNeasy kit, and was used to clone out the TCR genes using the BD SMART™ RACE kit following the manufacturer's instructions. In brief, the first-strand cDNA was synthesized in two steps: step 1 constituted the first-strand synthesis coupled with deoxy cytidine tailing by reverse transcriptase; step 2 constituted the template switching and extension by reverse transcriptase, resulting in the 5′-RACE-Ready cDNA capped with a common primer at the 5′ end. The cDNA was then used to run 5′-RACE PCR to clone the TCR genes. From the oligoclonal CTL line, three productively rearranged TCR α-chain genes and three productively rearranged TCR β-chain genes were cloned. Using a TCR pairing assay, two TCR pairs, $\alpha3\beta15$ and $\alpha2\beta14$, were determined to be specific for the MART$1_{27–35}$ epitope based on the HLA-A2/MART-1 tetramer (Beckman Coulter) staining. To construct the FUW-M1-TCR/sr39tk lentivector, the

[3] Abbreviations used in this paper: M1, Melan-A/MART-$1_{27–35}$; M14.2, Vα 2.2/Vβ14.

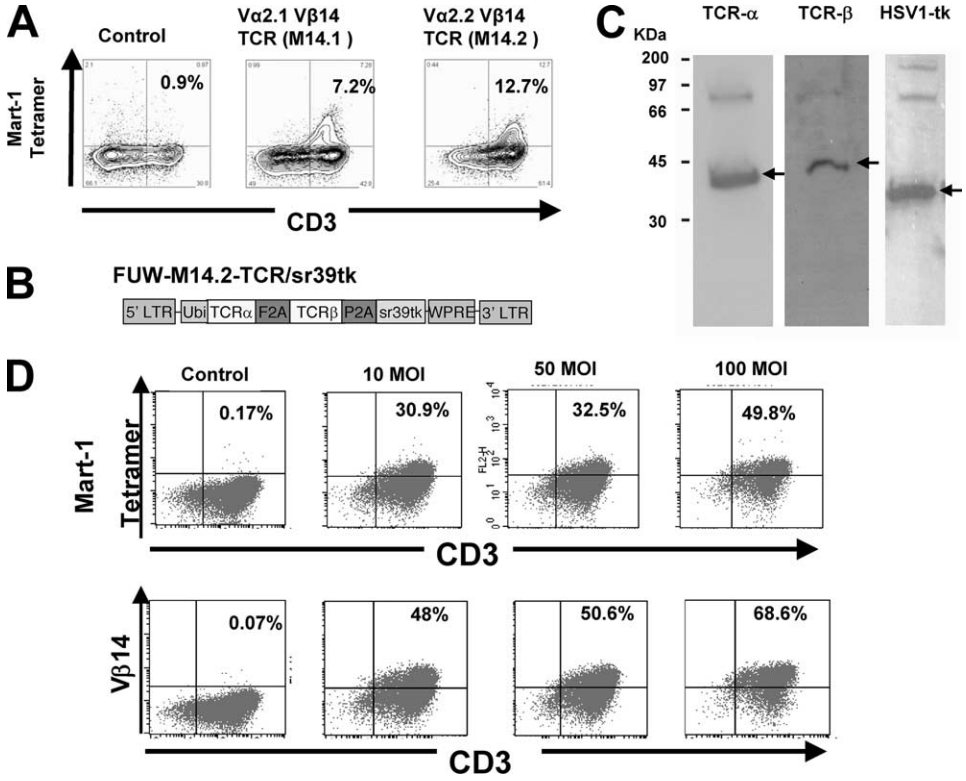

FIGURE 2. Construction of a recombinant lentivirus vector encoding MART-$1_{27–35}$ epitope-specific TCR. *A*, Coexpression of PCR amplified α- and β-chains led to the identification of two functional M1 epitope-specific TCRs. Both of these TCR consisted of Vβ14 region and Vα2.1 and Vα2.2 regions, respectively. The Vα2.1/Vβ14 TCR was named as M14.1, while Vα2.2/Vβ14 TCR was named as M14.2 TCR. M14.2 TCR was used in follow-up studies. *B*, Cloning of M14.2 TCR α and β-chains in a FUW-M1-TCR/sr39tk lentivirus vector. *C*, 293T cells were transduced with the lentiviral vector FUW-M14-TCR/sr39tk, lysed, and immunoblotted. Specific Abs were used to detect the constant region of the α- and β-chains of TCRs and the HSV1-tk. In the figure, arrows point to the expected size bands for each protein. Weaker high molecular weigh bands correspond to uncleaved products. The cleavage efficiency of the linkers was estimated to be ≥90%. *D*, Transduction of Jurkat cells with transgenic TCR.

α- and β-chains of M1 TCR and the sr39tk imaging/suicide gene were linked with F2A and P2A elements (12) using assembly PCR and were then cloned into the FUW lentivector (13).

The lentiviral vector was packaged by transient transfection of 293T cells with the vector plasmid, a plasmid expressing the HIV-1 gag and pol proteins (p8.9) and a plasmid encoding the VSV-G protein, as described

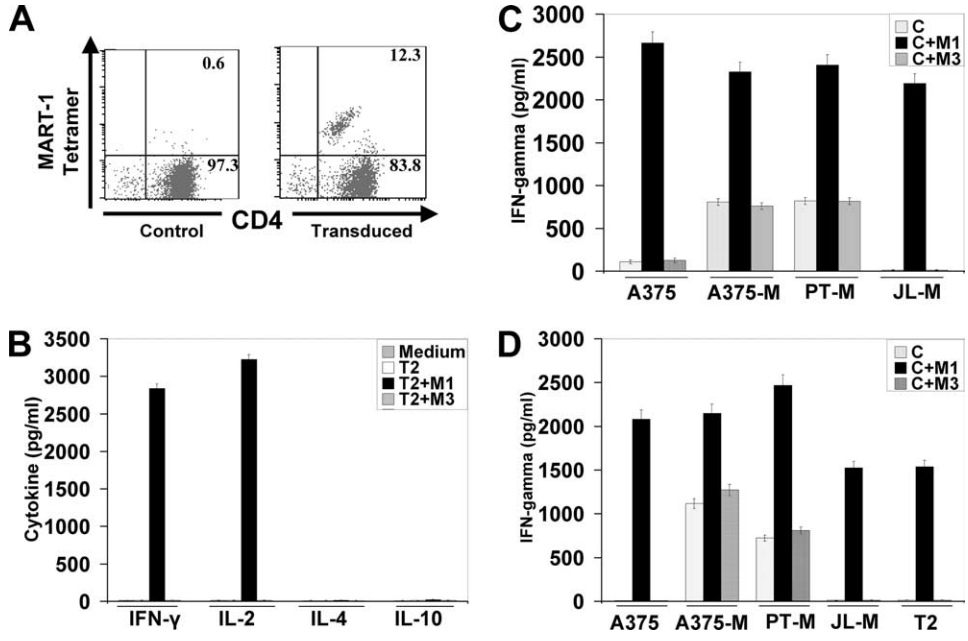

FIGURE 3. Transduction of T cells with transgenic TCR and analysis of the effector cytokine response. *A*, Transduction of activated human primary CD4 T cells with transgenic TCR. *B*, Characterization of effector cytokine response of TCR-engineered CD4 T cells. Transduced CD4 T cells were cocultured with T2 cells alone or T2 pulsed with MART-$1_{27–35}$ peptide or MAGE-$3_{271–279}$ control peptide, and cytokines released in the supernatant were quantified 16 h following coculture. *C*, Effector cytokine response of TCR-engineered CD4 T cells against human melanoma cells. Transduced CD4 T cells were cocultured with human melanoma cell lines, A375 wild type, and MART-1 expressing A375 (A375-M), PT-M, and JL-M. Coculture was set up either with cells alone or cells pulsed with MART-$1_{27–35}$ peptide or MAGE-$3_{271–279}$ control peptide, and cytokines released in the supernatant were quantified 16 h following coculture. *D*, Effector cytokine response analysis of human peripheral blood-derived MART-$1_{27–35}$ epitope-specific $CD8^+$ CTL against surrogate target, T2, or human melanoma cells. CTL were cocultured with T2 or melanoma cells alone or cells pulsed with cognate peptide (M1) or control peptide (M3), and IFN-γ released in the supernatant was measured 16 h following coculture, as done with TCR-engineered CD4 T cells (*C*).

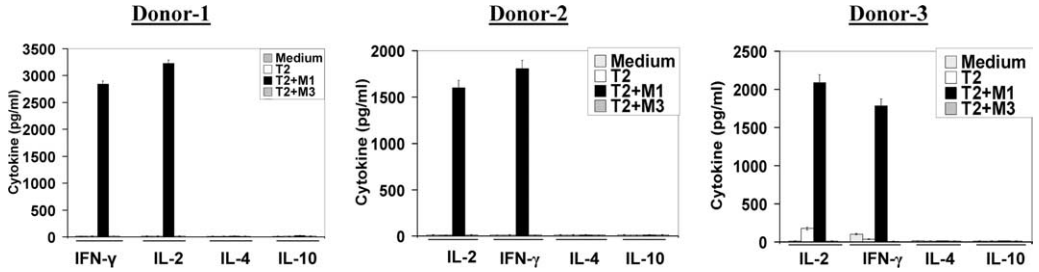

FIGURE 4. Characterization of cytokine response of TCR-engineered CD4 T cells. CD4 T cells derived from three different donors were transduced with M14.2 TCR and cocultured with T2 cells alone or T2 pulsed with MART-$1_{27–35}$ peptide or MAGE-$3_{271–279}$ control peptide, and cytokines released in the supernatant were quantified 16 h following coculture.

before (14). The vector supernatant was concentrated ~500-fold by ultrafiltration (Centricon plus 70 ultrafiltration filters; Millipore) followed by ultracentrifugation at 500 × *g* for 2 h. Concentrated vector supernatants were resuspended in X-Vivo15 serum-free medium and stored at −70°C until used. Titers were determined by transduction of subconfluent HT29 cells with serial dilutions of vector supernatant, followed by extraction of genomic DNA after 1 wk and quantification of integrated vector copy number by quantitative PCR using primers to the HIV-1 leader region (15). The numbers of transducing units per milliliter were calculated by multiplying the vector copy number, which was extrapolated from a standard

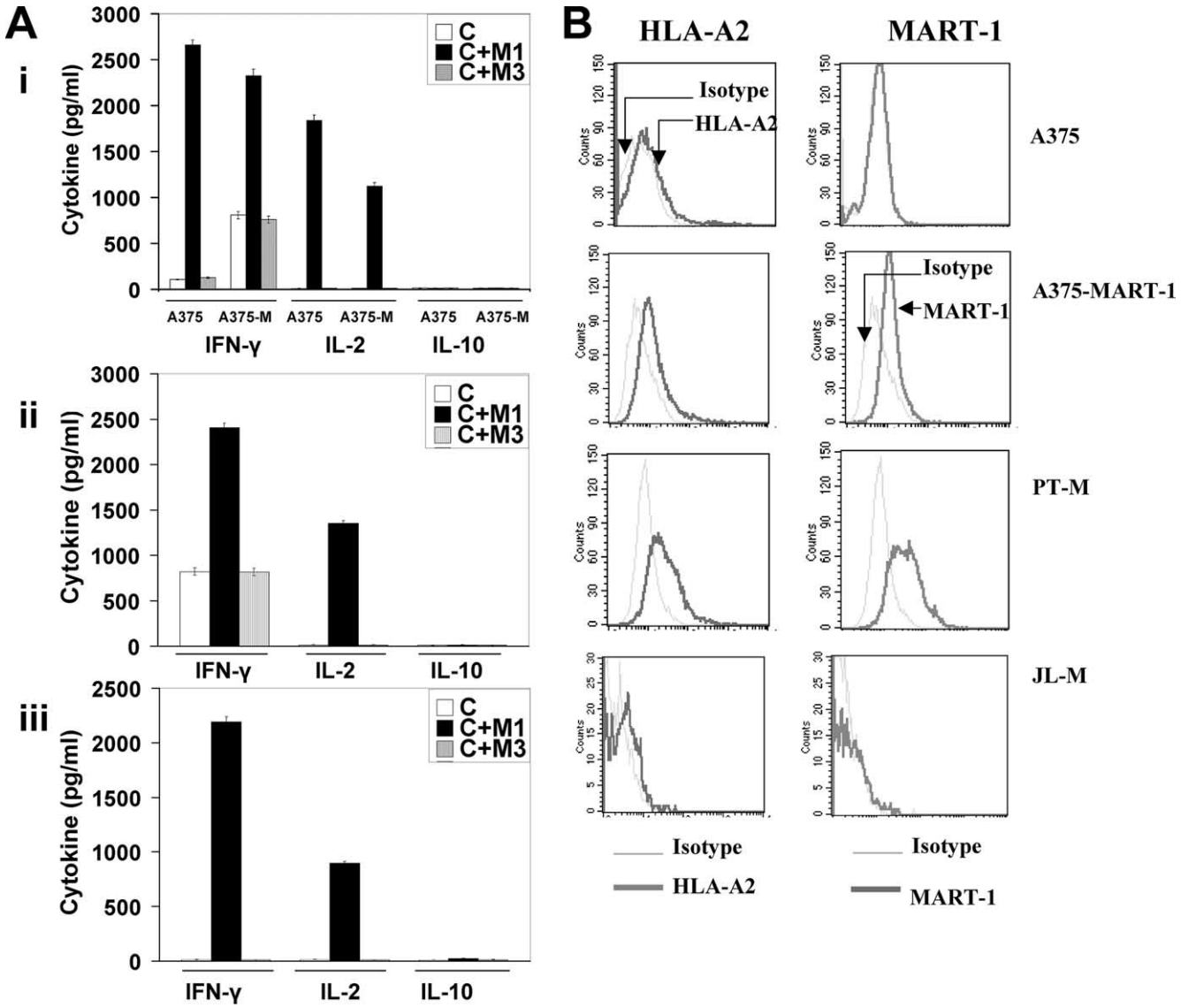

FIGURE 5. Characterization of cytokine response of TCR-engineered CD4 T cells, in response to human melanoma cell lines. *A*, M14.2 TCR transduced CD4 T cells were cocultured with human melanoma cell lines, A375 wild type, and MART-1 expressing A375 (*i*), PT-M (*ii*), and JL-M (*iii*). Coculture was set up either with cells alone or cells pulsed with MART-$1_{27–35}$ peptide or MAGE-$3_{271–279}$ control peptide, and cytokines released in the supernatant were quantified 16 h following coculture. *B*, Analysis of human melanoma cell lines, A375, A375-MART-1, PT-M, and JL-M, for the expression of HLA-A2 molecules and MART-1 Ag.

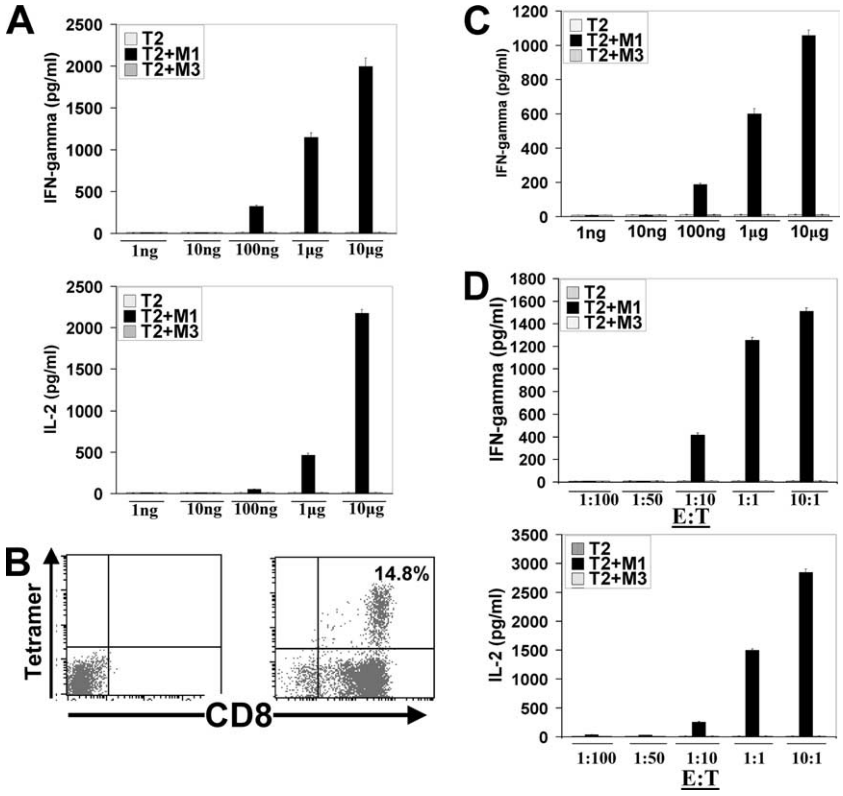

FIGURE 6. Transgenic TCR-engineered CD4 T cells function as high avidity effector T cells. *A*, Analysis of effector cytokine (IFN-γ and IL-2) release by transgenic TCR-engineered CD4 T cells at a peptide dose range of 1 ng-10 μg. *B*, Tetramer analyses of MART-$1_{27–35}$ antigenic epitope-specific CTL population expanded from human peripheral blood, in a peptide pulsed dendritic cell-T cell coculture expansion protocol. *C*, Analysis of effector cytokine (IFN-γ) release by human peripheral blood-derived $CD8^+$ CTL precursor-expanded MART-$1_{27–35}$-specific CTL population, at a peptide dose range of 1 ng-10 μg. *D*, Analysis of effector cytokine (IFN-γ and IL-2) release by transgenic TCR-engineered CD4 T cells at different E:T ratios.

curve of known vector copy number, the number of cells at the time of supernatant addition, and the supernatant dilution.

Transduction of $CD4^+$ T cells with lentiviral vector

Purified $CD4^+CD25^-$ subsets of $CD4^+$ T cells were isolated from $CD4^+$ T cells by magnetic beads purification method (Invitrogen) from Ficoll-Hypaque density gradients purified human peripheral blood lymphocytes as reported before (10, 11). Purified $CD4^+CD25^-$ T cells were then activated with 5 μg plate-bound anti-CD3 Ab and cultured in the presence of 100 μ/ml IL-2 for 5 days. Activated CD4 T cells (0.5–1 $X10^6$ cells) were transduced with the recombinant virus at 10 multiplicity of infection. Transduction was conducted for 120 min by slow shaking (~100 rpm) on a bench top mixer at room temperature. The next day, cells were transduced again under identical conditions and then cultured for 5 days in the presence of 1000 μ/ml IL-15. Transduction efficiency was quantified by staining transduced cells with M1 epitope-specific tetramer (Beckman Coulter). When needed, the TCR-transduced populations were expanded by in vitro stimulation with M1 peptide-pulsed matured dendritic cells, once or twice, as reported earlier (10, 11).

Cytokine synthesis by the transduced T cells

The ability of the TCR-transduced $CD4^+$ T cells to produce cytokines was determined in cytokine synthesis assay as previously described (10, 11). In brief, they were stimulated by T2 cells pulsed with the cognate peptide (MART-$1_{27–35}$) or the control peptide (MAGE-$3_{271–279}$) at effector T cells to a target ratio of 10 for 16 h. Supernatants were then analyzed for various cytokines. Melanoma cells were also used to stimulate the transduced cells to determine whether they would respond to them.

Cytotoxicity assay

The cytolytic ability of T cells was examined by the chromium release microcytotoxicity assay, done as described previously (16).

ELISA

IL-2, IL-4, IL-10, and IFN-γ cytokines were measured by sandwich ELISA kit (Immunotech) according to the manufacturer's instructions. The details have been published (10).

Results

An oligoclonal MART-$1_{27–35}$ epitope-specific CTL line was generated by limiting dilution culture from the in vitro activated/expanded $CD8^+$ T cells obtained from a patient who exhibited a remarkable high frequency of the M1 epitope-specific TCR-positive T cells in circulation (Fig. 1*A*). The M1 epitope-specific TCR-positive T cells in circulation of the donor were predominantly V β14 positive (Fig. 1*B*). Coexpression of the M1 epitope-specific TCR α- and -β-chains, derived from the M1 epitope-specific CTL line led to the identification of two M1 epitope-specific functional TCRs (Fig. 2*A*). Both of these TCR contained the Vβ14 region and Vα 2.1 and 2.2 regions, respectively (Fig. 2*A*). Based on preliminary comparative studies, we elected to use the Vα 2.2/Vβ14 (M14.2) TCR to study the biology of TCR-transduced CD4 T cells. A recombinant lentiviral vector encoding the M14.2 TCR was constructed (Fig. 2*B*). A human ubiquitin-C promoter was used to drive the expression of three transgenes: MART-1 TCR α, MART-1 TCR β, and suicide/imaging gene sr39tk. The suicide/imaging gene sr39tk was incorporated in our constructs for in vivo imaging studies in a future clinical trial. Two self-cleavage 2A-like liners (F2A, derived from food-and-mouth disease virus; P2A, derived from porcine teschovirus) (12) were used to link the three transgenes to achieve the optimal stoichiometric expression of these three proteins (Fig. 2*C*). The functional integrity of the two TCR chains was confirmed by transducing Jurkat cells (Fig. 2*D*). A dose-dependent increase in M1-specific tetramer and anti-V β14 Ab-stained Jurkat was observed in transduced populations (Fig. 2*D*).

We next examined the functional integrity of the transgene in $CD4^+$ T cells and characterized the nature of Ag-specific cytokine response exhibited by the M14.2 TCR-transduced CD4 T cells. Fig. 3*A* shows that 12% of the non-polarized but preactivated $CD4^+CD25^-$ T cells transduced with the FUW vector expressing the M14.2 TCR. A fraction of the M14.2 TCR-positive cells expressed CD4 molecules at lower density. The reason for this underexpression of CD4 molecules in the TCR-transduced population presently remains unclear. The M14.2 TCR-engineered $CD4^+$ T cells synthesized IFN-γ and IL-2, but not IL-4 or IL-10 in an

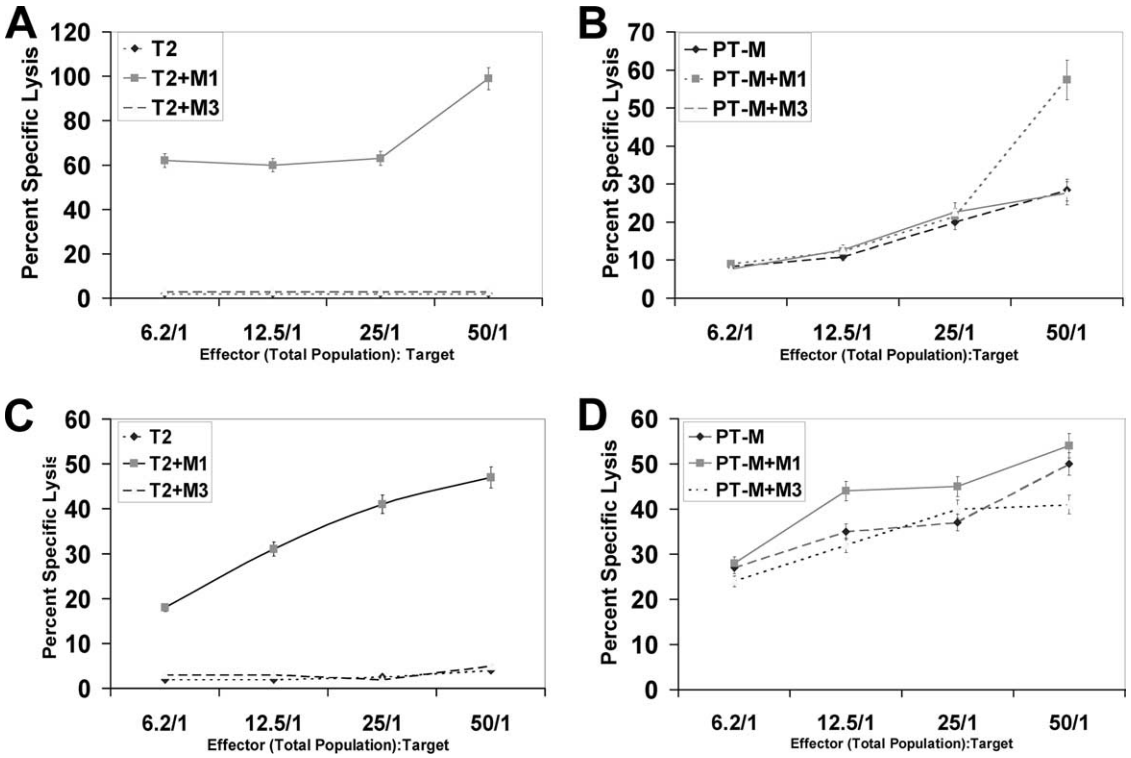

FIGURE 7. Cytolytic potential of MART-$1_{27–35}$ epitope-specific TCR-engineered CD4 T cells. Cytolytic potential of TCR-engineered CD4 T cells against peptide pulsed T2 cells (*A*) or MART-1 expressing melanoma cells, PT-M (*B*). Cytotoxicity assays were set up with target cells alone or pulsed with the MART-$1_{27–35}$ peptide or MAGE-$3_{271–279}$ control peptide, and Cr^{51} release was quantified 6 h post coculture. Of note, lysis of the PT-M cells was observed at all E:T ratios examined; the level of lysis was considerably higher at E:T of 50 when the PT-M cells were pulsed with the peptide (*B*). Cytolytic potential analysis of MART-$1_{27–35}$ epitope-specific human peripheral blood-derived $CD8^+$ T cells against T2 (*C*) or PT-M melanoma cells (*D*). Cytotoxicity assay was done by Cr^{51} release assay with Cr^{51}-loaded T2 or PT-M melanoma cells, as done for MART-$1_{27–35}$ epitope-specific M14.2 TCR-engineered CD4 T cells (*A* and *B*).

epitope-specific manner (Fig. 3*B*). Of note, this Th1 type bias was also seen with the M14.2 TCR-engineered $CD4^+$ T cells from three other donors in six separate experiments (Fig. 4). The TCR-transduced $CD4^+$ T cells also recognized M1 Ag on the HLA-A2.1-positive MART-1-transduced melanoma line A375, as well as the HLA-A2.1-positive melanoma cell line PT-M, naturally expressing the MART-1 Ag (Figs. 3*C* and 5*B*). In contrast, they did not recognize the melanoma line JL that was HLA-A2.1-positive but did not express the MART-1 protein (Figs. 3*C* and 5*B*). Fig. 3*D* shows the epitope recognition profile of autologous $CD8^+$ CTL. As can be seen, $CD8^+$ CTL, generated from the same donor in our standard in vitro CTL generation protocol (10), also exhibited essentially identical epitope recognition profile and comparable cytokine synthetic capacity. The M14.2 TCR-transduced $CD4^+$ T cells also exhibited Th1 type bias against the melanoma lines (Fig. 5*A*). Of note, the TCR-transduced $CD4^+$ T cells exhibited a comparable high functional avidity for the cognate peptide as displayed by the natural M1 epitope-specific high affinity TCR-expressing $CD8^+$ CTL (Fig. 6). The functional avidity of TCR-transduced CD4 T cells and $CD8^+$ CTL was examined at different peptide doses and at different E:T ratios (Fig. 6, *A–D*).

We next examined the cytolytic potential of transduced CD4 T cells (Fig. 7). As shown, the M14.2 TCR-engineered $CD4^+$ T cells lysed the M1 peptide loaded surrogate targets as well as lysed melanoma cells naturally expressing the epitope (Fig. 7, *A* and *B*) just as efficiently as the in vitro generated natural $CD8^+$ CTL (Fig. 7, *C* and *D*).

Next we compared the mechanism of the cytolytic effector function displayed by the transgenic TCR-transduced CD4 T cells with the MART-$1_{27–35}$ epitope-specific natural $CD8^+$ CTL (Fig. 8). As shown in Fig. 8*A*, the cytolytic effector function exhibited by TCR-engineered CD4 T cells was MHC class I-restricted and the calcium chelant EGTA blocked this cytolytic activity. Because calcium is required for release of perforin and granzyme B leading to cytotoxic death of target cells, these data suggest that the lytic activity of these cells was mediated by the release of lytic granules (Fig. 8*B*). The M14.2 TCR-engineered $CD4^+$ T cell expressed cytolytic effector molecules, granzyme and perforin, and they underwent degranulation after encountering the cognate epitope (Fig. 8*C*), similar to the M1 epitope-specific $CD8^+$ CTL (Fig. 8, *D* and *E*).

Discussion

Most active specific and adoptive immunotherapeutic approaches for cancer center around CTL-based responses generated through immunization or transferred adoptively with ex vivo-generated CTL. $CD4^+$ T cells might contribute in these strategies substantially (17–19), but the task of simultaneously engaging $CD4^+$ Th cells and CTL has turned out to be quite difficult, especially in an Ag-specific manner. Accordingly, redirecting $CD4^+$ T cells to recognize MHC class I-restricted epitopes through engineered expression of MHC class I-restricted epitope-specific TCR has gained attention. In animal models, it has indeed been shown that such TCR-engineered $CD4^+$ T cells can provide help (8, 9) as well as exhibit cytolytic effector function (20). In human systems, MHC class I-restricted epitope-specific TCR-engineered $CD4^+$ T cells have been shown to synthesize different cytokines in coreceptor-dependent as well as coreceptor-independent manners (3–7). In

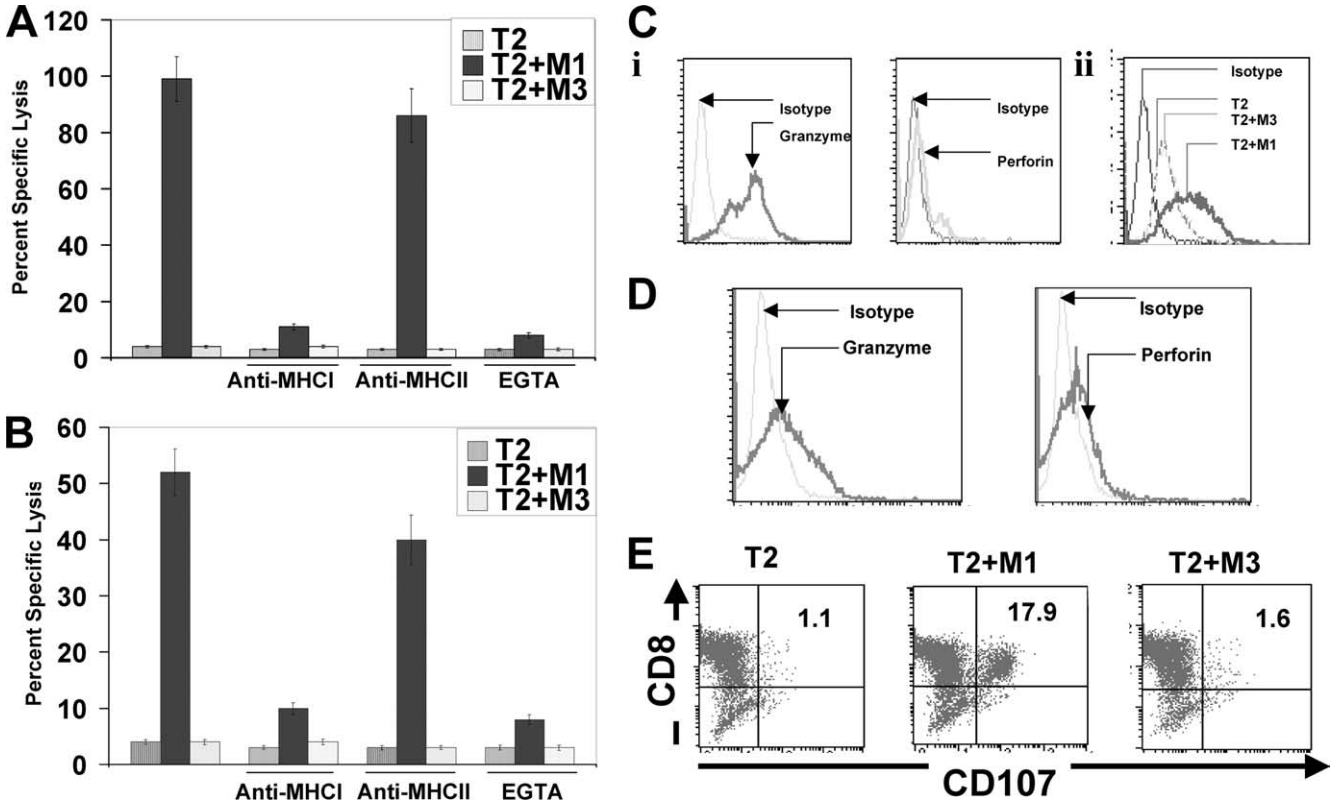

FIGURE 8. MART-$1_{27–35}$ epitope-specific TCR-engineered CD4 T cells mediate cytolytic effector function in a MHC class I-restricted and cytolytic granule exocytosis-dependent manner. Analyses of cytolytic potential of MART-$1_{27–35}$ epitope-specific M14.2 TCR-engineered CD4 T cells (*A*) and MART-$1_{27–35}$ epitope-specific human peripheral blood-derived $CD8^+$ CTL (*B*), in a Cr^{51} release assay, with Cr^{51} labeled T2 cells alone or T2 cells pulsed with MART-$1_{27–35}$ cognate peptide or MAGE-$3_{271–279}$ control peptide. Cytotoxicity assay was set up either in the absence or presence of anti-MHC class I, anti-MHC class II Abs. EGTA (4 mM) pretreatment was done to block the release of cytolytic granules. *C*, M14.2 TCR-engineered CD4 T cells express cytolytic effector molecules, granzyme, and perforin (*i*), and undergo degranulation following Ag encounter with T2 cells pulsed with MART-$1_{27–35}$ peptide epitope (*ii*). Transgenic CD4 T cells were cocultured either with T2 cells alone or T2 cells pulsed with MART-$1_{27–35}$ cognate peptide or MAGE-$3_{271–279}$ control peptide, and degranulation was measured by quantifying CD107 molecules, surface exposed upon Ag encounter. Analyses of the expression of granzyme and perforin (*D*), and degranulation assay (*E*) were conducted with MART-$1_{27–35}$ epitope-specific human peripheral blood-derived $CD8^+$ CTL, as done for TCR-engineered CD4 T cells (*C*).

general agreement with these studies, our results attest to the potential usefulness of such MHC class I-restricted epitope-specific TCR-engineered $CD4^+$ T cells in tumor immunotherapy. Our studies also reveal a number of additional interesting aspects of their biology.

First, our studies clearly show that the MHC class I-restricted epitope-specific TCR-engineered $CD4^+$ T cells can function in a coreceptor-independent manner. Coreceptor-dependent and -independent function by such TCR-engineered $CD4^+$ T cells has been described (21, 22). It is believed that a high affinity/avidity TCR might transmit productive signals without the participation of coreceptors. The affinity of the M14.2 TCR on $CD4^+$ T cells was found to be in the nanomolar range, and it was comparable to that of the naturally grown $CD8^+$ CTL (Fig. 6). These transgenic TCR-transduced CD4 T cells recognized the naturally expressed M1 epitope on melanoma cells, just as efficiently as the naturally grown $CD8^+$ CTL (Fig. 3).

Second, in contrast to previous reports of MHC class I-restricted epitope-specific TCR-engineered $CD4^+$ T cells synthesizing both type 1 and type 2/3 cytokines (3, 5, 6), the M14.2 TCR engineered $CD4^+$ T cells synthesize only Th1 type cytokines (Figs. 3, 4, and 5). The reason for this Th1/Tc1 bias in the M14.2 TCR engineered $CD4^+$ T cells remains unclear. It should be noted that we used only $CD4^+CD25^-$ subsets. Additionally, we did not polarize them in Th1 type condition before transduction, as was done in another study where Th1 type bias by this type of TCR-engineered $CD4^+$ T cells was reported (7). Given that $CD4^+$ T cells from three different donors engineered to express the M14.2 TCR elaborated only Th1/Tc1 cytokine (Fig. 4), it does not appear to be an isolated incidence. Careful studies will be needed to understand whether a given set of transgenic TCR dictates polarization or the functional polarization is an imprinted property acquired inherently during ontogeny or acquired in culture conditions. Whatever the explanation might turn out to be, one can envision a distinct advantage with the TCR-engineered $CD4^+$ T cells exhibiting Th1 bias from a translational viewpoint. TCR-engineered $CD4^+$ T cells exhibiting Th1 bias are likely to be true helper cells and more effective effector cells.

Third, the MHC class I-restricted and granule-mediated cytolytic property of the M14.2 TCR-engineered $CD4^+$ T cells provides a different understanding on the functional separation between a $CD4^+$ T cell and a $CD8^+$ T cell. Traditionally, $CD8^+$ T cells are viewed as quintessential cytolytic T cells and $CD4^+$ T cells as helper cells. Although a role of $CD4^+$ T cells in anti-tumor immunity is well documented in certain models (23–25), the mechanism by which $CD4^+$ T cells exhibit anti-tumor immunity in these models has never been fully clarified. The M14.2 TCR-engineered $CD4^+$ T cells appear to be just as good killer cells as their CD8 counterparts; they use granule-mediated lytic machinery (Figs. 7 and 8), and they also elaborate a comparable amount of

IFN-γ (Fig. 3, *C* and *D*). The mechanism(s) that re-directs $CD4^+$ T cells to perform as killer T cells presently remain unclear. Additional studies will be needed to address this issue. Though how $CD4^+$ T cells are re-directed to function as cytolytic T cells by signals through the transgenic CTL-derived TCR remains an open question, our data clearly show that the M14.2 TCR-engineered $CD4^+$ T cells can function as formidable effector cells. They could, therefore, expand the effector response repertoire substantially in an active specific or adoptive immunotherapy schema.

Finally, it should be pointed out that engaging such MHC class I-restricted epitope-specific and coreceptor-independent TCR-engineered $CD4^+$ T cells in tumor immunotherapy would have many advantages. For example, making CD4 T cells function through the same epitope that the CTL precursors also recognize resolves the problem in engaging the CTL precursors, Thp, and the APC presenting separate class I and class II epitopes simultaneously, a conundrum in orchestrating help in a three cell model. Second, the strategy does not rely on recruiting a rare Thp. By introducing a fairly large number of TCR-engineered $CD4^+$ T cells, the scope for providing cognate help could be vastly improved. Additionally, such $CD4^+$ T cells could do more than help. They could provide effector function of their own. The strategy can be used for active specific as well as in adoptive therapy models. Most importantly, as a large number of MHC class I-restricted tumor-associated epitopes and $CD8^+$ T cells capable of recognizing them are defined, a large number of patients could be matched for administration of MHC class I epitope-specific TCR-engineered $CD4^+$ T cells. As such, the translational potential of MHC class I epitope-engineered $CD4^+$ T cells in the setting of active specific or adoptive immunotherapy will be considerable.

Disclosures

The authors have no financial conflict of interest.

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