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J Immunol. 2008 July 15; 181(2): 1063–1070.**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****Arvind Chhabra^{1,*}, Lili Yang², Pin Wang³, Begoña Comin-Anduix⁵, Raja Das¹, Nitya G. Chakraborty¹, Swagatam Ray¹, Shikhar Mehrotra⁶, Haiguang Yang³, Cinnamon L. Hardee⁴, Roger Hollis⁴, David I. Dorsky¹, Richard Koya⁵, Donald B. Kohn⁴, Antoni Ribas⁵, James S. Economou⁵, David Baltimore², and Bijay Mukherji^{1,*}**¹UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity. Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut²UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity. Division of Biology, California Institute of Technology, Pasadena, California³UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity. University of Southern California, Los Angeles, California⁴UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity. Children Hospital Los Angeles, Los Angeles, California⁵UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity. Department of Medicine and Surgery, University of California, Los Angeles, California⁶Department of Surgery, Medical University of South Carolina, Charleston, SC.**Abstract**

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 antigen specific manner -- "redirecting" CD4⁺ T cells to MHC class I-restricted epitopes through engineered expression of MHC class I-restricted epitope specific T cell receptors (TCR) 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 carried out 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-1₂₇₋₃₅ (M1), as a prototypic human tumor antigen system. We found that unpolarized CD4⁺CD25⁻ T cells engineered to express the M1 TCR selectively synthesize Th1 cytokines and exhibit a potent antigen-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.

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Introduction

“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 cytolytic T lymphocyte (CTL) response as most cancer cells express only MHC class I restricted epitopes. The task of generating a robust and long-lived CTL response to tumor-associated antigens – most of which are “self” antigens – faces many constraints. Among these is the lack of an effective strategy to engage CD4⁺ T helper (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 (TCR) 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 (TCR) 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 prior to 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 way 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. Here we show that human CD4⁺CD25[–] T cells engineered to express a TCR specific for the HLA-A2.1-restricted melanoma epitope, Melan-A/MART-1_{27–35} (hereafter to be referred to as M1 epitope) synthesize only Th1 cytokines IFN- γ and IL-2 and no 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, Maryland. 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 micro-well seeded with three MART-1_{27–35} peptide pulsed DC-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 (dC) tailing by RT; step 2 constituted the template switching and extension by RT, 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, 3 productively rearranged TCR alpha chain genes and three productively rearranged TCR beta chain genes were cloned. Using a TCR pairing assay, two TCR pairs, $\alpha 3\beta 15$ and $\alpha 2\beta 14$, were determined to be specific for the MART1₂₇₋₃₅ epitope based on the HLA-A2/MART-1 tetramer (Beckman Coulter) staining. To construct the FUW-M1-TCR/sr39tk lentivector, the alpha chain and beta 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 before (14). The vector supernatant was concentrated approximately 500-fold by ultrafiltration (Centricon plus 70 ultrafiltration filters; Millipore, Bedford, MA) followed by ultracentrifugation at $500 \times g$ 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 sub-confluent HT29 cells with serial dilutions of vector supernatant followed by extraction of genomic DNA after one week and quantification of integrated vector copy number by quantitative polymerase chain reaction (qPCR) using primers to the HIV-1 leader region (15). The numbers of transducing units per milliliter (TU/mL) were calculated by multiplying the vector copy number, which was extrapolated from a standard 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 Inc., USA) 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 antibody and cultured in the presence of 100 u/ml IL-2 for 5 days. Activated CD4 T cells ($0.5-1 \times 10^6$ cells) were transduced with the recombinant virus at 10 MOI. Transduction was carried out for 120 min. by slow shaking ($\sim 100\text{rpm}$), 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 u/ml IL-15. Transduction efficiency was quantified by staining transduced cells with M1 epitope specific tetramer (Beckman Coulter, USA). When needed, the TCR transduced populations were expanded by in-vitro stimulation with M1 peptide pulsed matured DC, 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). Briefly, they were stimulated by T2 cells pulsed with the cognate peptide (MART-1₂₇₋₃₅) or the control peptide (MAGE-3₂₇₁₋₂₇₉) at an effector T cells to target ratio of 10 for 16h. Supernatants were then analyzed for various cytokines. Melanoma cells were also used to stimulate the transduced cells to determine if they would respond to them.

Cytotoxicity assay

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

ELISA

IL-2, IL-4, IL-10 and IFN-gamma cytokines were measured by sandwich ELISA kit (Immunotech, Marseilles, France) according to the manufacturer's instruction. 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. 1A). The M1 epitope specific TCR positive T cells in circulation of the donor were predominantly V β 14 positive (Fig. 1B). Co-expression 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. 2A). Both of these TCR contained the V β 14 region and V α 2.1 and 2.2 regions respectively (Fig. 2A). Based on preliminary comparative studies, we elected to use the V α 2.2/ V β 14 TCR (to be referred as 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. 2B). A human ubiquitin-C promoter was used to drive the expression of three transgenes MART-1 TCR alpha, MART-1 TCR beta, 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. 2C). The functional integrity of the two TCR chains was confirmed by transducing Jurkat cells (Fig 2D). A dose-dependent increase in M1 specific tetramer and anti-V β 14 antibody stained Jurkat was observed in transduced populations (Fig 2D).

We next examined the functional integrity of the transgene in CD4⁺ T cells and characterized the nature of antigen-specific cytokine response exhibited by the M14.2 TCR transduced CD4 T cells. Fig. 3A shows that 12% of the non-polarized but pre-activated CD4⁺25⁻ 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 under-expression 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 no IL-4 or IL-10 in an epitope specific manner (Fig. 3B). 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 antigen 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 antigen (Fig. 3C and Fig. 5B). In contrast, they did not recognize the melanoma line JL that was HLA-A2.1 positive but did not express the MART-1 protein (Fig. 3C and Fig. 5B). Fig. 3D 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. 5A). 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 effector to target ratios (Fig. 6A–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. 7A & 7B) just as efficiently as the in vitro generated natural CD8+ CTL (Fig. 7C & 7D).

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. 8A, 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. Since 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. 8B). 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. 8C), similar to the M1 epitope specific CD8+ CTL (Fig. 8D & 8E).

Discussion

Most active specific and adoptive immunotherapeutic approaches for cancer center around cytolytic T lymphocyte (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+ T helper cells and CTL has turned out to quite difficult-- especially in an antigen 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 a co-receptor-dependent as well as co-receptor-independent manners (3–7). In 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 co-receptor-independent manner. Co-receptor-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 co-receptors. 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 (Fig. 3, Fig. 4 and Fig. 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 prior to 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 if 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 (Fig. 7 & Fig. 8), and they also elaborate a comparable amount of IFN- γ (Figs. 3C & 3D). 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. Although 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 co-receptor 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 CTLp also recognize resolves the problem in engaging the CTLp, 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 employed 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.

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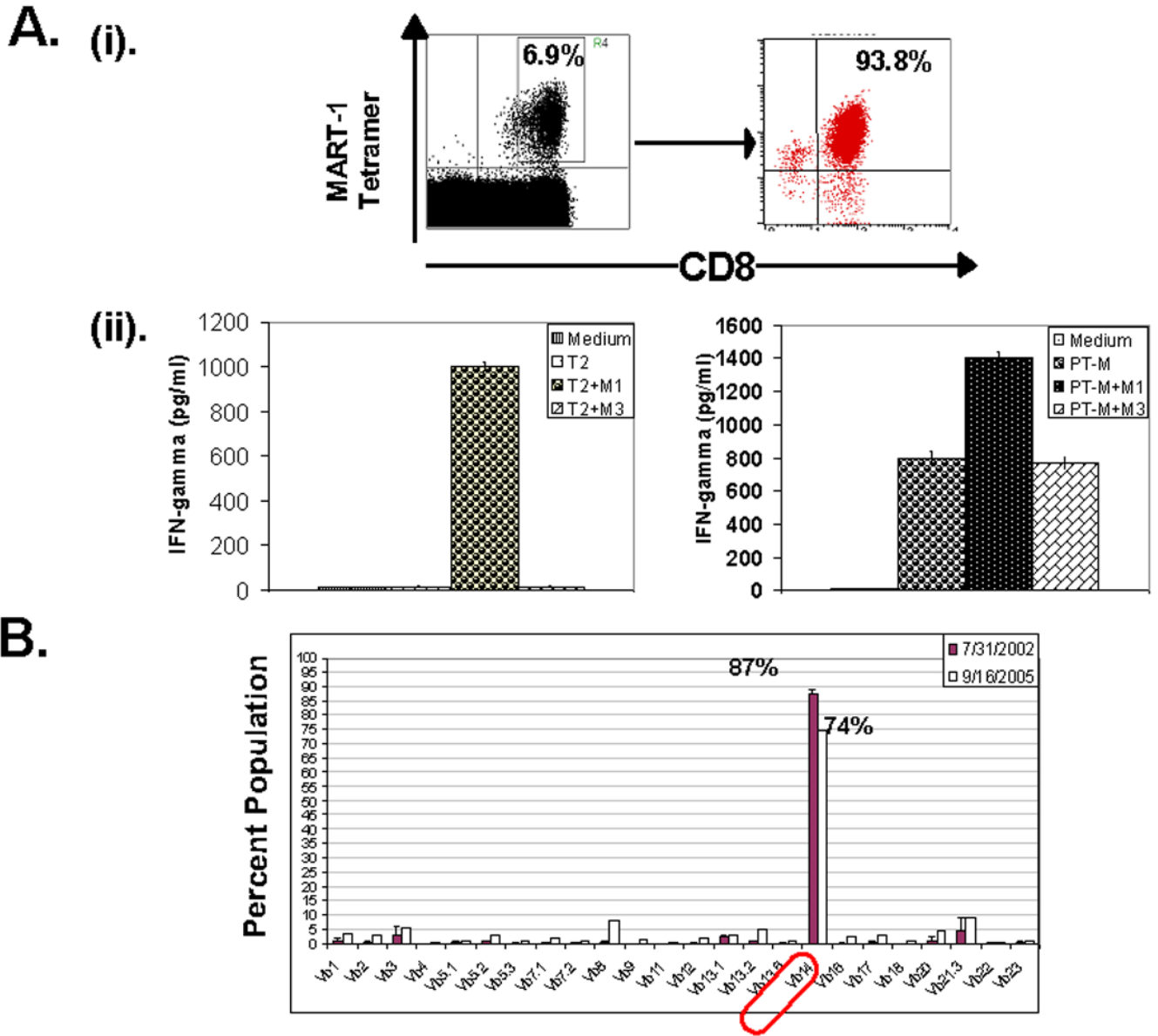


Figure 1. Generation of MART-1₂₇₋₃₅ epitope specific CTL line from the peripheral blood derived CD8 T cells of a melanoma patient. **(A)– (i).** An oligoclonal CTL line was generated from a melanoma patient’s peripheral blood derived CD8+ T cells, in a DC-T cell co-culture expansion protocol. **(ii).** The antigen specific effector function of the CTL line was tested by measuring IFN- γ release in the supernatant upon co-culture with either surrogate target cells, T2 (left panel) or MART-1 expressing human melanoma cells PT-M (right panel). CTL were co-cultured with target cells alone (T2/PT-M) or target cells pulsed with either cognate peptide, MART-1₂₇₋₃₅ (T2/PT-M +M1) or MAGE-3₂₇₁₋₂₇₉ 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-1₂₇₋₃₅ epitope specific CD8 T cells.

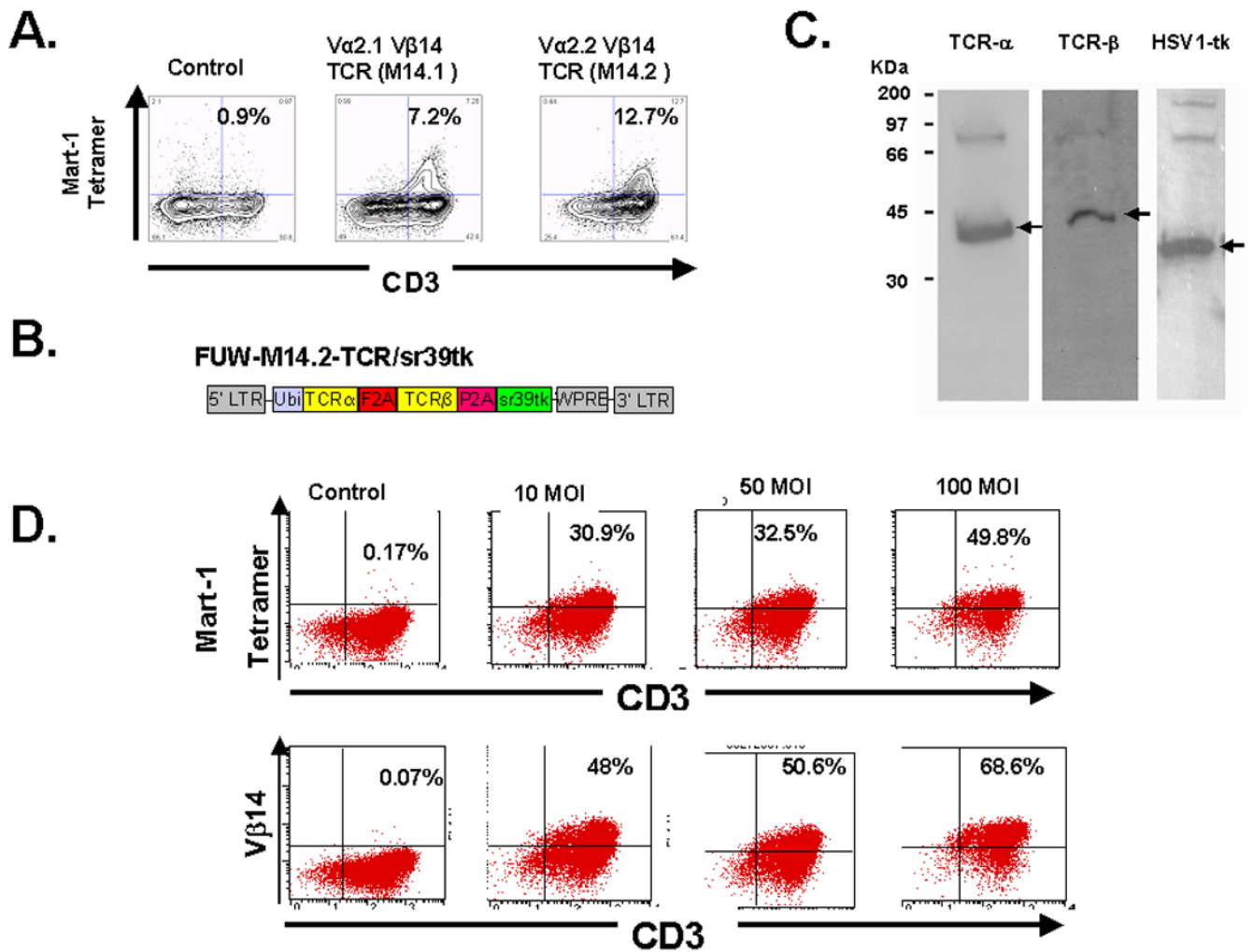


Figure. 2. Construction of a recombinant lenti virus vector encoding MART-1₂₇₋₃₅ epitope specific TCR. (A). Co-expression of PCR amplified α and β chains led to the identification of two functional M1 epitope specific TCRs. Both of these TCR consisted of Vβ14 region and Va2.1 and Va2.2 regions respectively. The Va2.1/ Vβ14 TCR was named as M14.1, while Va2.2/ 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 antibodies 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 weight bands correspond to uncleaved products. The cleavage efficiency of the linkers was estimated to be > 90%. (D). Transduction of Jurkat cells with transgenic TCR.

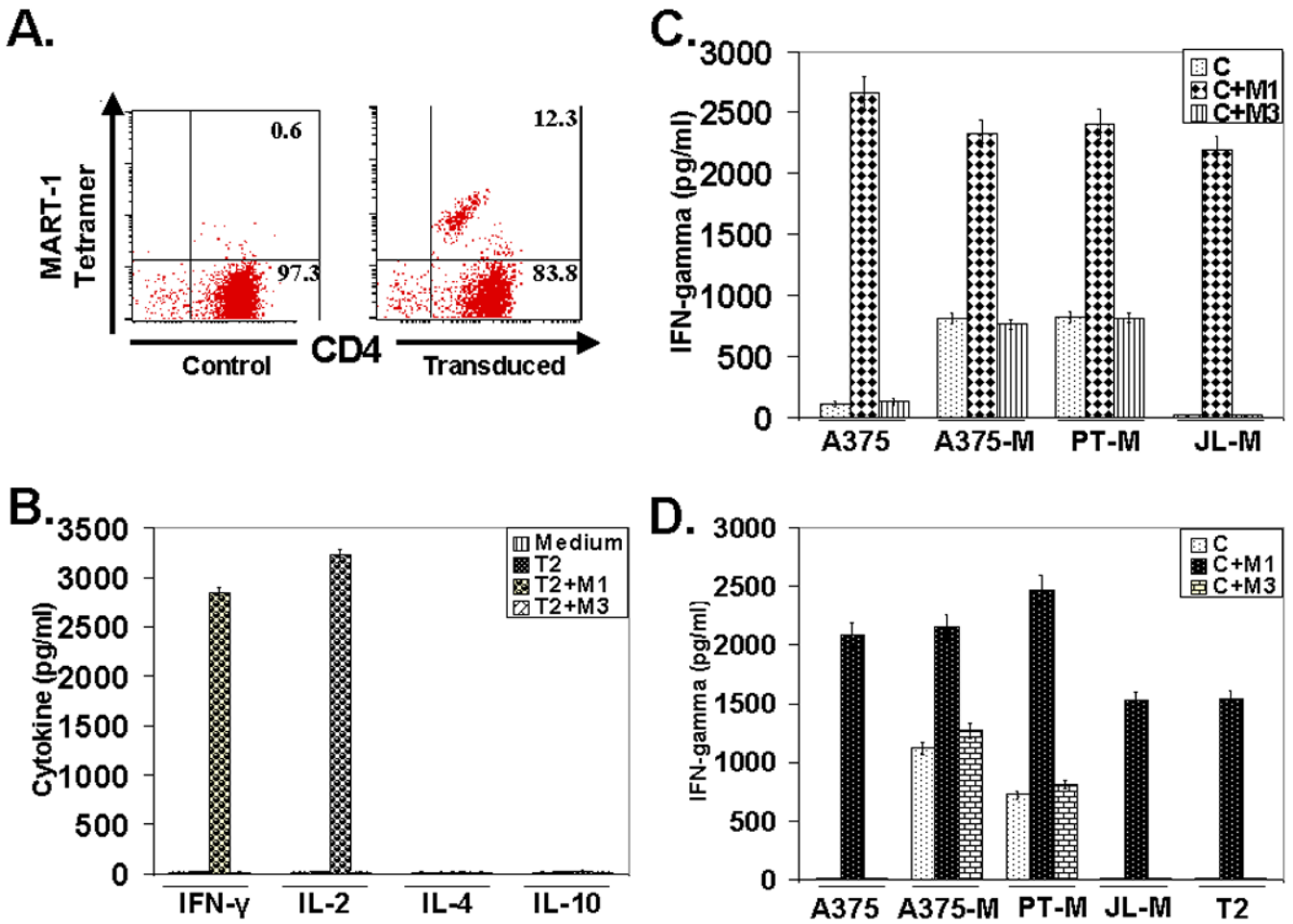


Figure 3.

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 co-cultured with T2 cells alone or T2 pulsed with MART-1₂₇₋₃₅ peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and cytokines released in the supernatant were quantified 16 hr following co-culture. (C). Effector cytokine response of TCR engineered CD4 T cells against human melanoma cells. Transduced CD4 T cells were co-cultured with human melanoma cell lines, A375 wild type & MART-1 expressing A375 (A375-M), PT-M and JL-M. Co-culture was set up either with cells alone or cells pulsed with MART-1₂₇₋₃₅ peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and cytokines released in the supernatant were quantified 16 hr following co-culture. (D). Effector cytokine response analysis of human peripheral blood derived MART-1₂₇₋₃₅ epitope specific CD8⁺ CTL against surrogate target, T2 or human melanoma cells. CTL were co-cultured 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 hr following co-culture, as done with TCR engineered CD4 T cells (Fig. 3C).

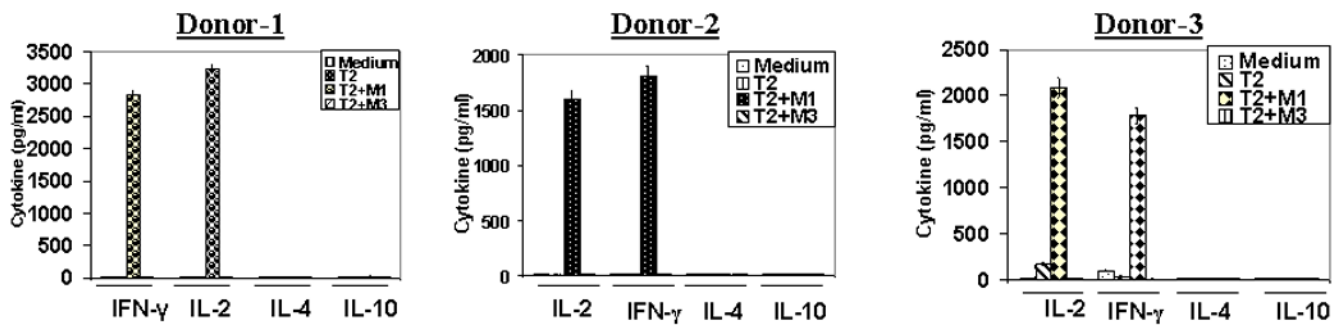


Figure 4.

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 co-cultured with T2 cells alone or T2 pulsed with MART-1₂₇₋₃₅ peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and cytokines released in the supernatant were quantified 16 hr following co-culture.

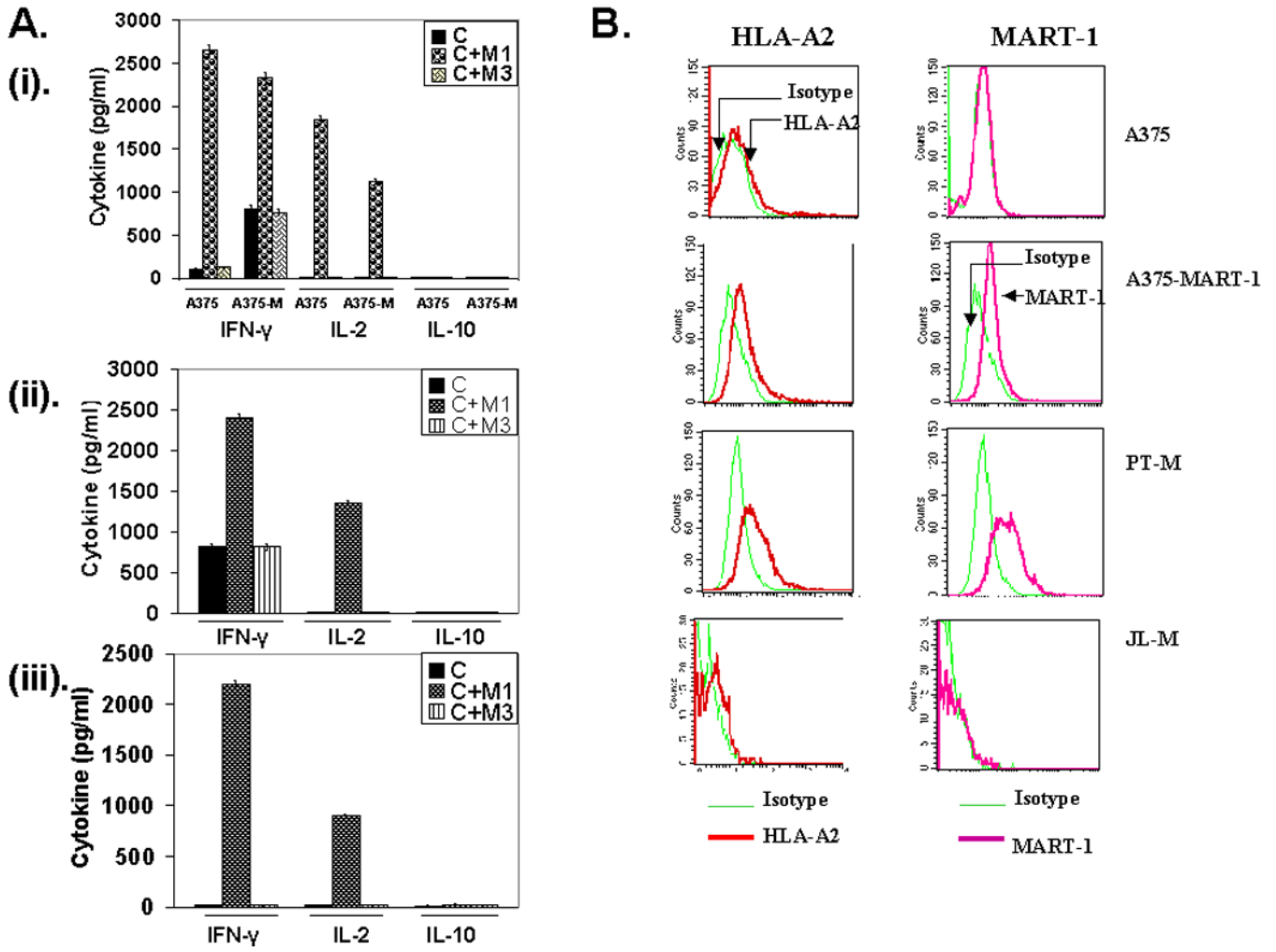


Figure 5. Characterization of cytokine response of TCR engineered CD4 T cells, in response to human melanoma cell lines. **(A)**, M14.2 TCR transduced CD4 T cells were co-cultured with human melanoma cell lines, A375 wild type & MART-1 expressing A375 **(i)**, PT-M **(ii)** and JL-M **(iii)**. Co-culture was set up either with cells alone or cells pulsed with MART-1₂₇₋₃₅ peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and cytokines released in the supernatant were quantified 16 hr following co-culture. **(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 antigen.

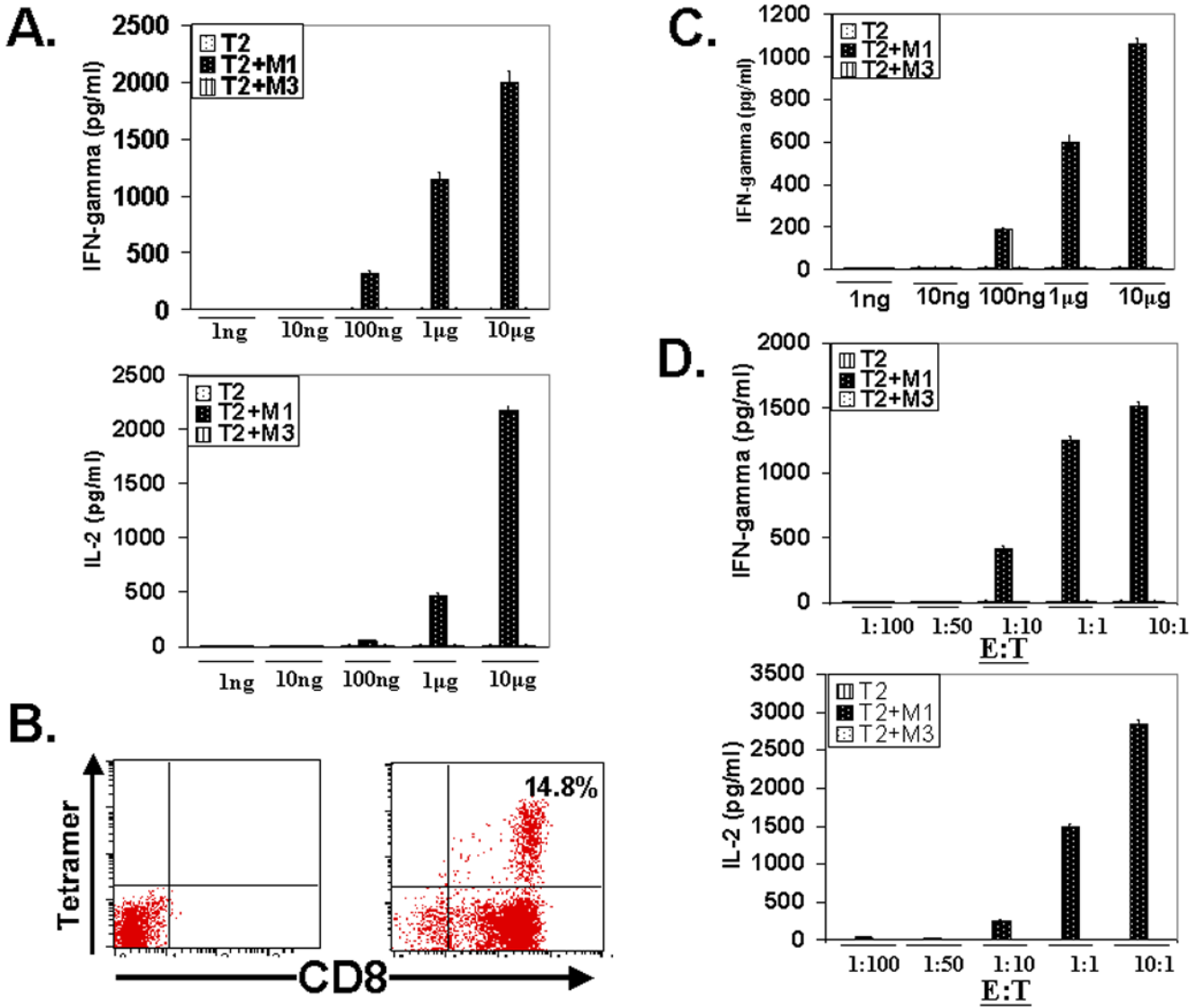


Figure 6. Transgenic TCR engineered CD4 T cells function as high avidity effector T cells. **(A).** Analysis of effector cytokine (IFN- γ and IL-2) release by transgenic TCR engineered CD4 T cells at a peptide dose range of 1ng-10 μ g. **(B).** Tetramer analyses of MART-1₂₇₋₃₅ antigenic epitope specific CTL population expanded from human peripheral blood, in a peptide pulsed DC-T cell co-culture expansion protocol. **(C).** Analysis of effector cytokine (IFN- γ) release by human peripheral blood derived CD8+ CTL precursor expanded MART-1₂₇₋₃₅ specific CTL population, at a peptide dose range of 1ng-10 μ g. **(D).** Analysis of effector cytokine (IFN- γ and IL-2) release by transgenic TCR engineered CD4 T cells at different effector to target ratios.

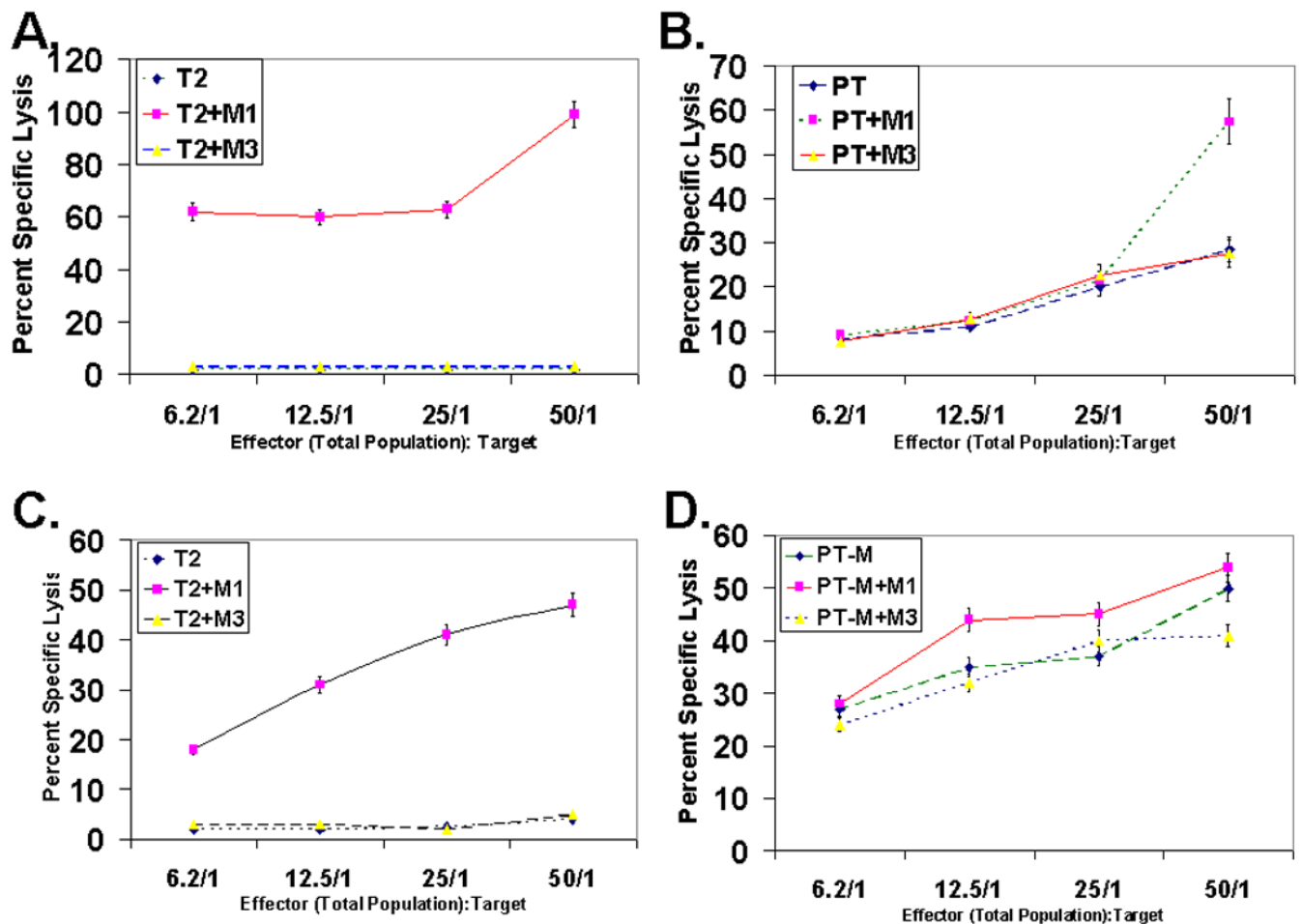


Figure. 7.

Cytolytic potential of MART-1₂₇₋₃₅ epitope specific TCR engineered CD4 T cells. (A & B). 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-1₂₇₋₃₅ peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and Cr⁵¹ release was quantified 6 hr post co-culture. Of note, lysis of the PT-M cells were 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 (7B). (C & D). Cytolytic potential analysis of MART-1₂₇₋₃₅ epitope specific human peripheral blood derived CD8⁺ T cells against T2 (C) or PT-M melanoma cells (D). Cytotoxicity assay was done by Cr⁵¹ release assay with Cr⁵¹ loaded T2 or PT-M melanoma cells, as done for MART-1₂₇₋₃₅ epitope specific M14.2 TCR engineered CD4 T cells (Fig. 7A & 7B).

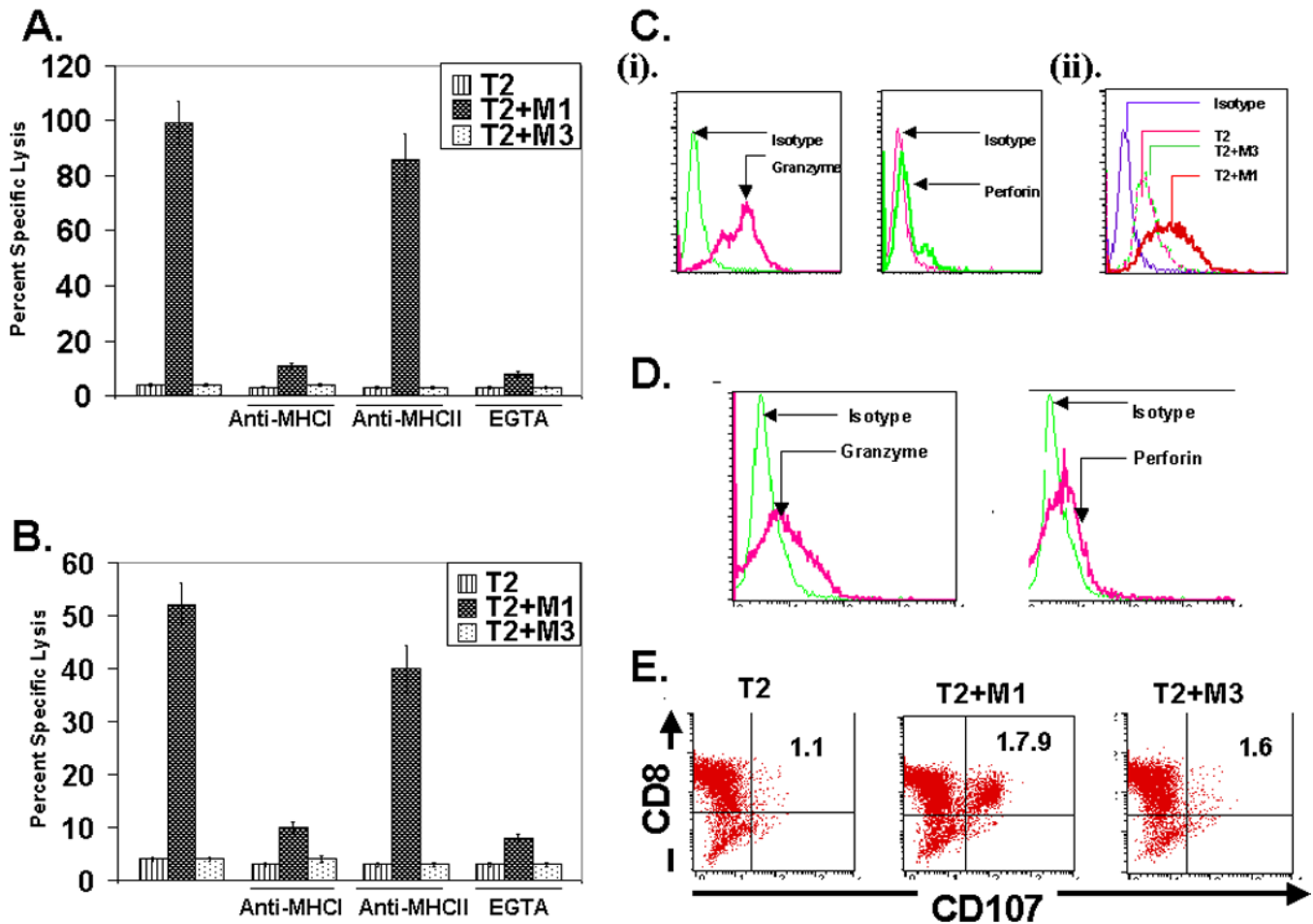


Figure.8

Figure 8.

MART-1₂₇₋₃₅ epitope specific TCR engineered CD4 T cells mediate cytolytic effector function in a MHC class I restricted and cytolytic granule exocytosis dependent manner. (A & B).

Analyses of cytolytic potential of MART-1₂₇₋₃₅ epitope specific M14.2 TCR engineered CD4 T cells (A) and MART-1₂₇₋₃₅ epitope specific human peripheral blood derived CD8+ CTL (B), in a Cr⁵¹ release assay, with Cr⁵¹ labeled T2 cells alone or T2 cells pulsed with MART-1₂₇₋₃₅ cognate peptide or MAGE-3₂₇₁₋₂₇₉ control peptide. Cytotoxicity assay were set up either in the absence or presence of anti-MHC class I, anti-MHC class II antibodies.

EGTA (4mM) pre-treatment 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 antigen encounter with T2 cells pulsed with MART-1₂₇₋₃₅ peptide epitope (ii). Transgenic CD4 T cells were co-cultured either with T2 cells alone or T2 cells pulsed with MART-1₂₇₋₃₅ cognate peptide or MAGE-3₂₇₁₋₂₇₉ control peptide and degranulation was measured by quantifying CD107 molecules, surface exposed upon antigen encounter. (D & E). Analyses of the expression of granzyme and perforin (D), and degranulation assay (E) were carried out with MART-1₂₇₋₃₅ epitope specific human peripheral blood derived CD8+ CTL, as done for TCR engineered CD4 T cells (Fig. 8C).