Published in final edited form as:

Clin Immunol. 2010 September ; 136(3): 338–347. doi:10.1016/j.clim.2010.04.013.

MHC-I restricted Melanoma Antigen Specific TCR Engineered Human CD4⁺ T Cells Exhibit Multifunctional Effector and Helper Responses, In Vitro

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Abstract

MHC class I-restricted human melanoma epitope MART-1_{27–35} specific TCR engineered CD4⁺CD25[–] T cells synthesize Th1 type cytokines and exhibit cytolytic effector function upon cognate stimulation. A detailed characterization of such TCR-engineered CD4⁺CD25[–] T cells now reveals that they are multifunctional. For example, they undergo multiple rounds of division, synthesize cytokines (IFN- γ , TNF- α , IL-2, MIP1 β), lyse target cells, and “help” the expansion of the MART-1_{27–35} specific CD8⁺ T cells when stimulated by the MART-1_{27–35} peptide pulsed DC. Multiparametric analyses reveal that a single TCR-engineered CD4⁺ T cell can perform as many as five different functions. Nearly 100% MART-1_{27–35} specific TCR expressing CD4⁺ T cells can be generated through retroviral vector-based transduction and one round of *in vitro* stimulation by the peptide pulsed DC. MHC class I-restricted tumor epitope specific TCR-transduced CD4⁺ T cells, therefore, could be useful in immunotherapeutic strategies for melanoma or other human malignancies.

Keywords

Cancer Immunotherapy; TCR; Multi functional CD4 T Cells

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Conflict of Interest Statement: The authors have no financial interest.

Introduction

A role for CD4⁺ T cells in immune response in tumor immunity, in general, and in tumor immunotherapy, in particular, is widely acknowledged [1;2;3]. However, CD4⁺ T cells recognize epitopes on MHC class II molecules and most solid tumor cells do not express MHC class II molecules. These facts impose considerable difficulty in developing a way to incorporate them in therapeutic designs. As such, how to engage CD4⁺ T cells in tumor immunotherapy has become a critical strategic issue. Lately, the use of CD4⁺ T cells transduced to express the α/β chains of a relevant MHC class I-restricted tumor epitope specific TCR has emerged as a mechanism to achieve that goal [4;5;6;7;8]. That such MHC class-I restricted epitope specific TCR-engineered CD4⁺ T cells recognize the epitope on MHC class-I molecules with or without the requirement of co-receptor (i.e., CD8 molecules) engagement and exhibit effector function has been described by several groups in animal models [4;5] as well as in human systems [9;10;11;12]. We have shown that human CD4⁺CD25⁻ T cells transduced to express the α/β TCR chains specific for the Melan-A/MART-1₂₇₋₃₅ epitope, express type I cytokines and exhibit cytolytic function in a co-receptor-independent fashion [12]. Considering the potential of such tumor epitope specific TCR-engineered CD4⁺ T cells in human tumor immunotherapy, we undertook an extended examination of their biology. Here we show that the MHC class I-restricted MART-1₂₇₋₃₅ epitope specific TCR-transduced CD4⁺CD25⁻ T cells undergo multiple rounds of division and exhibit multifunctional effector function (synthesize IFN- γ , TNF- α , IL-2, mobilize lytic granules and exhibit cytolytic effector function against melanoma targets) in a cognate manner without requiring co-receptor-mediated additional signal. They also amplify the expansion of the MART-1₂₇₋₃₅ epitope specific CD8⁺ T cells in an epitope specific CTL generation assay, in vitro.

Materials and Methods

Study population, cell lines and reagents

The study population consisted of HLA-A2-positive healthy donors. The study was approved by Institutional Review Board and written consents were taken from all participants. Separation of CD4⁺CD25⁻, CD8⁺ T cells and culture conditions were described previously [12]. T2 cells- a lymphoblastoid cell line with mutated TAP, was a gift from Peter Cresswell, Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut and the MART-1 negative melanoma line A375 cells engineered to express the MART-1 protein (A375-M) have been described before [13]. The melanoma cell lines PT-M and M-202 were established from two HLA-A2.1 positive melanoma patients. MART-1₂₇₋₃₅ (M1) and MAGE-3₂₇₁₋₂₇₉ (M3) peptides were purchased from NeoMPS (USA).

Retroviral vector construction

MART-1₂₇₋₃₅ epitope specific DMF5 TCR was isolated from a high avidity tumor infiltrating lymphocyte (TIL) clone has been previously described [10]. The PG13 packaging cell line [14] to produce DMF5 retrovirus was cultured in DMEM (Hyclone, USA) supplemented with 10% FBS. The cultures were grown to 70% confluence. Fresh medium was added and the supernatant containing the virus was harvested 16 h later.

Generation of MART-1 TCR transduced CD4⁺CD25⁻ and CD8⁺ T cells

CD4⁺CD25⁻ and CD8⁺ T cells were activated by plate-bound anti-CD3 (5 μ g) and anti-CD28 (1 μ g/ml) antibodies in presence of 100U/ml IL-2. After 48hr, the cells were infected with DMF5 TCR retrovirus containing supernatant in the presence of Retronectin (Takara, Japan) as per manufacturer protocol. 48hr after infection cells were stained with MART-1

specific tetramer (Beckman Coulter, USA) and analyzed by flow cytometry in FACScalibur (BD Biosciences, USA). The transduced cells were rested in culture for 7–10 days with medium changes and used for functional analyses or used after being frozen in FBS with 10% DMSO. The viability of thawed cells was always in excess of 90% and no significant difference in functional profile was observed with the frozen cells.

CFSE labeling and proliferation assays

CD4⁺CD25[−] and CD8⁺ T cells transduced with MART-1 specific DMF5-TCR were labeled with 1mM CFSE (Invitrogen, USA) in PBS according to published protocol [15]. Autologous DC were matured in LPS as published [12;13] and pulsed with 50mM of peptide. The CFSE labeled CD4F5 or CD8F5 cells were added to the peptide pulsed DC or tumor cell lines in 1:10 (Target:T cell) ratio. After 4 days, cells were analysed by flow cytometry.

Cytokine secretion assay

T2 cells were pulsed with different dilutions of peptide in complete medium for 30min. 2×10^4 peptide pulsed T2 were incubated overnight (approximately 16h) with 2×10^5 CD4F5 or CD8F5 T cells in 500 μ l final volume in each well of a 48 well plate. The supernatants were collected and cytokines (IL-2, IFN γ , TNF α , IL-10, IL-4 and TGF- β) were measured in ELISA using an ELISA kit (R&D Systems, USA).

Intracellular cytokine staining

Reagents for flow cytometric analysis (anti-CD107a-FITC, anti-IL-2-PerCP-Cy5.5, anti-IFN γ -V450, anti-TNF α -PE-Cy7, anti-MIP-1 β -PE, anti-CD3-APC) were purchased from BD Biosciences (USA) and used as directed by the manufacturer. FoxP3 was stained using anti-FoxP3-APC and FoxP3 staining buffer from Miltenyi Biotec Inc (CA, USA) according to manufacturer protocol. For intracellular cytokine assays, 5×10^5 T cells were incubated with 5×10^4 T2 pulsed with peptides. After 6 hr of incubation, the cells were stained and analyzed with LSR-II flow-cytometer (BD Biosciences). The data were analyzed with FlowJo (TreeStar, USA) and polyfunctionality was assessed with PESTLE and SPICE softwares (provided by Mario Roederer, NIH, Bethesda, MD).

Cytotoxicity assay

1×10^3 melanoma target cells were labeled with ^{51}Cr and cytotoxicity was examined by 4 hr ^{51}Cr release assay [16] in presence of 50 fold excess K-562 as cold target competitors.

Co-culture to assess putative helper function of the TCR-engineered CD4⁺ T cells

Previously published MART-1_{27–35} specific *in vitro* CTL generation protocol [16] was used as the basic CTL generation assay to assess helper function of the MART-1_{27–35} specific TCR transduced CD4⁺CD25[−] T cells. Briefly, co-cultures were set up with freshly isolated CD8⁺ T cell and DMF5 TCR engineered CD4⁺CD25[−] T cells (with mock transduced CD4⁺ T cells as control) against the MART-1_{27–35} peptide loaded matured autologous DC. After 8–10 days, the numbers of MART-1_{27–35} epitope specific CD8⁺ T cells were determined by tetramer staining.

Results

MART-1_{27–35} epitope specific TCR-engineered CD4⁺ T cells proliferate upon cognate stimulation

It is now quite clear that α/β TCRs that are restricted to MHC class I determinants, when expressed on to CD4⁺ T cells, are functional -- i.e., they send productive signal

[9;12;17;18]. We have also previously shown that human CD4⁺CD25⁻ T cells engineered to express the MHC class-I-restricted MART-1₂₇₋₃₅ epitope specific TCRs synthesize type 1 cytokines and exhibit cytolytic function [11;12]. Although MHC class I-restricted epitope specific TCRs work on CD4⁺ T cells, such MHC class I-restricted TCR-engineered CD4⁺ T cells are yet to be fully characterized especially in the context of their potential usefulness in human tumor immunotherapy. Using a different set of MART-1₂₇₋₃₅ epitope specific TCR, DMF5, with improved transduction efficiency [10], we undertook a more detailed characterization of the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4⁺CD25⁻ T cells, *in vitro*. We first examined the robustness of the transduction and the efficiency in generating large numbers of MHC class-I restricted melanoma epitope specific TCR-expressing CD4⁺CD25⁻ T cells. As shown in Fig.1, a large fraction of CD4⁺CD25⁻ T cells could be transduced with the DMF5 TCR retroviral vector to express the MART-1₂₇₋₃₅ epitope specific TCR and a substantially larger fraction expressing the MART-1₂₇₋₃₅ epitope specific TCR could be obtained after a single *in vitro* stimulation with the MART-1₂₇₋₃₅ peptide-loaded DC. A nearly homogenous population of MART-1₂₇₋₃₅ epitope specific TCR expressing populations could be obtained after a second stimulation (data not shown). Fig. 2A shows the proliferative potential of the TCR transduced CD4⁺CD25⁻ T cells in comparison with similarly engineered CD8⁺ T cells (Fig. 2B) assessed in CFSE dilution assay. As shown, the TCR-engineered CD4⁺ as well as CD8⁺ T cells exhibit multiple rounds of division when they encounter the epitope on autologous DC (Figs. 2A & 2B). Of considerable interest, they also undergo multiple rounds of division when stimulated by melanoma cells (Fig. 2C).

MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4⁺ T cells are multifunctional

We then carried out a more extended functional characterization of DMF5 TCR-engineered CD4⁺CD25⁻ T cells and found that these TCR-engineered CD4⁺ T cells are multifunctional (Figs. 3 & 4). Figure 3A shows the cytokine synthetic ability (composite data) of the DMF5 transduced CD4⁺ and CD8⁺ T cells from 5 different donors. As shown, they synthesize IFN- γ , TNF- α , IL-2, MIP-1 β . They also expose CD107a (Figs. 3B & 4A) upon cognate stimulation and exhibit cytolytic function (Fig. 4B). Of interest, intracytoplasmic staining revealed that a significant fraction of them exhibit more than one function – a sizeable fraction exhibiting multiple cytokine synthesis as well as exposing CD107a (Fig. 3B & C). Importantly, our analysis showed that a cell that makes IL-2 can also synthesize TNF- α and that both IL-2- and IFN- γ -secreting cells expose CD107a, i.e., LAMP (Fig. 3B). Of further interest, the DMF5 TCR-engineered CD4⁺ T cells do not express FoxP3 and TGF- β , even when stimulated by the appropriate ligand (Fig 3D). The cytolytic function of the TCR-engineered CD4⁺CD25⁻ T cells is not precisely comparable to that of the CD8⁺ T cells against all target cells in chromium release assay (Fig 4B). Nonetheless when taken with their ability to expose CD107a (Fig 4A), as well as to lyse melanoma cells (Fig. 4B), the cytolytic function of the TCR-engineered CD4⁺ T cells is a distinct bonus. Given that the melanoma epitope specific TCR-engineered CD4⁺ T cells did not express CD8 co-receptors and as CD8 co-receptor expression by TCR engineered CD4⁺ T cells have been shown to enhance effector function of MHC class-I TCR-engineered CD4⁺ T cells [11;19], it is possible to argue that they might be made far better lytic effector cells if they were made to co-express CD8 molecules. Co-expressing CD8 molecules has not been a particularly difficult task [11].

MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4⁺ T cells provide “help” during the activation/expansion of CD8⁺ T cells bearing endogenous MART-1₂₇₋₃₅ epitope specific TCR, *in vitro*

Given that CD4⁺ T cells provide important helper functions to CD8⁺ T cells in cell-mediated immune responses, we examined if the MART-1₂₇₋₃₅ epitope specific TCR

expressing CD4⁺CD25⁻ T cells could “help” the CD8⁺ T cells in CTL generation/expansion process. In our years of experience with *in vitro* CTL generation studies in the human melanoma model (i.e., co-culturing CD8⁺ T cells with peptide loaded matured DC), we have seldom found optimum functional activation and good expansion of the epitope specific CD8⁺ T cells in the absence of exogenous cytokines such as IL-2 or IL-15. Accordingly, we addressed whether or not the CD4⁺CD25⁻ T cells could help the CTL activation/expansion process (CTL burst) in our *in vitro* CTL generation co-cultures in the absence of exogenous IL-2. Figure 5A shows the result of a representative co-culture experiment demonstrating that the expansion of the epitope specific CD8⁺ T cell population was substantially amplified by the DMF5 epitope specific TCR expressing CD4⁺ T cells, when they were stimulated by the MART-1₂₇₋₃₅ peptide in the assay. Figure 5B shows the composite data from 5 separate experiments. The DMF5-transduced CD4⁺ T cells from 5 HLA-A2 positive donors were co-cultured with the untransduced CD8⁺ T cells against peptide pulsed DC. The enhancements of MART-1₂₇₋₃₅ peptide specific CD8⁺ T cell expansion (fold expansion of MART-1₂₇₋₃₅ tetramer positive populations), if any, was then determined by flowcytometry. As can be seen, the number of MART-1₂₇₋₃₅ epitope specific CD8⁺ T cells were considerably higher at all three CD4: CD8 ratios and that the increases were observed only when the co-cultures were performed with the MART-1₂₇₋₃₅ peptide. No enhancement was observed with the mock-transduced CD4⁺ T cells or with the DMF5-transduced CD4⁺ T cells in co-culture with CD8⁺ T cells against the HLA-A2 binding control MAGE-3₂₇₁₋₂₇₉ peptide, M3. Of interest, they expanded the relevant TCR expressing CD8⁺ T cells in the CTL generation cultures without any exogenous cytokine supplementation. While it is possible that IL-2 synthesized by the CD4⁺ T cells provided the help, additional work will be needed to determine the underlying mechanism(s) behind the helper effect of the TCR-transduced CD4⁺ T cells.

Discussion

Active specific immunotherapy have produced remarkable clinical responses in some melanoma patients at times [20;21] and adoptive cell therapy with tumor-reactive tumor infiltrating lymphocytes (TIL) have produced more impressive results in selected patients with metastatic melanoma [22]. Melanoma reactive TIL, however, can be generated in about half of melanoma patients and TIL can only rarely be obtained from patients with other histologic cancer types. New strategies are needed to improve the outcome of these types of therapeutic approaches. Given that CD4⁺ T cells do positively influence the priming phase of CD8⁺ CTL response generation as well as facilitate the CTL memory generation process [23;24;25], it is widely acknowledged that among other strategies that could be useful to improve the results of tumor immunotherapy, figuring out a way that would simultaneously engage CD8⁺ as well as CD4⁺ T cells would be very helpful. Additionally, although the need for “antigen specific” CD4⁺ T cells as “helper” cells is not yet clearly established, it is also believed that a strategy that would engage CD4⁺ helper cells and CD8⁺ CTL recognizing a relevant tumor epitope is likely to be highly effective. In this context, the data presented here are noteworthy.

When collectively taken in the context to the question of how to engage cognate CD4⁺ T cells -- along with CD8⁺ T effector cells -- in tumor immunotherapy, several interesting points emerge from the data. First, our data clearly show that a large number of melanoma epitope specific TCR-engineered CD4⁺CD25⁻ T cells can be obtained through viral vector-based transduction and *in vitro* stimulation and that these MHC class I-restricted TCR transduced CD4⁺CD25⁻ T cells exhibit essentially all the effector functions that are normally expected from CD8⁺ T cells. Additionally, the data show that they are truly “multifunctional” – i.e., they synthesize multiple cytokines, they are cytolytic, and they provide “help” during CTL burst *in vitro* CTL activation/expansion protocol. Given that the

value of multifunctional effector function [26] is increasingly apparent in HIV immunity [27] as well as in tumor immunity [28], the multifunctional nature of the MHC class I-restricted TCR-engineered CD4+CD25- T cells would be useful in tumor immunotherapy. In this context, the robust proliferative potential of the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells after encountering the epitope on DC or after encountering target cells suggests that they could not only be made to proliferate through traditional DC and peptide-based stimulation – a widely used practice in active specific immunization research – they could also be driven to undergo expansion at tumor sites – a highly desirable goal. Tumor antigen-driven proliferation and functions of CD4+ T cells at tumor sites could have many positive effects. It should be, however, acknowledged that although our past study [12] have shown and present observations also show that the TCR-engineered CD4+ T cells exhibit Th1 type phenotype, that CD4+ T cells engineered to express such MHC class I-restricted TCR (at least, a fraction of them) may have T regulatory (Treg) activities under certain conditions. Additional work will be needed to determine condition(s) that would consistently generate Th1 type CD4+ effector T cells and condition(s) that could lead to the generation of epitope specific Treg cells through TCR transgenesis of CD4+ T cells.

Our data showing that they amplify the burst size of the CD8+ CTL add another dimension to their functional repertoire. Given that a role for CD4+ T cells in the CTL priming and in CTL memory generation is well established [23;24;25], the potential usefulness of such MHC class I-restricted epitope specific CD4+ T cells in active specific immunization can be easily envisioned. We have not addressed if the MART-1₂₇₋₃₅ epitope specific TCR-engineered CD4+ T cells could be made to transition into CD4+ memory T cells themselves or if they could also “help” CD8+ T cells to become memory CTL, the possibility exists that under appropriate conditions (*in vitro* and/or *in vivo*) they could serve such purposes. While additional work will be needed to figure out conditions that would make such MHC class I-restricted and epitope specific TCR-engineered CD4+ T cells to serve such purposes, the successful use of this strategy for the induction of T cell memory response in an animal tumor model [4] and the relationship between multifunctional effector response and clinical benefit observed in a recent peptide and CTLA-4 antagonist-based immunization trial [28] strongly argue for moving these types of MHC class-I-restricted tumor epitope specific TCR-engineered CD4+ T cells to the clinic. Although our previous study [12] and this study have been carried out with a single (but two different) set of tumor epitope specific TCRs, the remarkably concordant results do not seem to be a reflection of a particular set of TCR. Of note, MHC class-I-restricted α/β TCRs specific for other human tumor-associated epitopes have also been found to function when grafted onto CD4+ T cells [9;12;17;18].

The concept of adoptive immunotherapy with TCR-gene transduced T cells has moved to clinical trial [8]. While it is too early to assess its overall effectiveness, this type of cancer immunotherapeutic approach would have limitations. Virtually all the well recognized reasons underlying failures of T cell-based immunotherapy [29] are likely to frustrate adoptive immunotherapy with TCR-transduced T cells. Nonetheless, it should be pointed out, that most approaches to adoptive immunotherapy or active specific immunotherapy for that matter have, so far, been based on strategies designed to harnessing the effector functions of CD8+ CTL as most human cancer cells express only MHC class I molecule-associated epitopes. Two groups of investigators have recently shown that MHC class II-restricted epitope specific TCR-engineered CD4+ T cells exhibit superb anti-tumor effector responses including cytolytic function and that they could be activated and expanded through immunization, *in vivo*, in animal models [30;31]. The data presented here show a potentially novel way to directing MHC class I associated antigen specific CD4+CD25- T cells – as effector cells and as “helper” cells --to human tumors that do not express MHC class II molecules [32;33]. Accordingly, these types of tumor epitope specific and MHC class I-restricted CD4+CD25- T cells -adoptively transferred in patients followed by

immunization (to expand both CD4⁺ T cells and native CD8⁺ T cells bearing endogenous TCR for the given epitope) or adoptively transferred with similar TCR engineered CD8⁺ T cells followed by immunization – could be novel and valuable strategies in active specific or adoptive T cell-based tumor immunotherapy.

Acknowledgments

We thank Dr Mario Roederer (Vaccine Research Center, NIAID, NIH, Bethesda, MD) for providing multifunctional FACS analysis software.

The work was supported by PHS grants CA 83130 (BM), CA 88059 (BM), CA 129816 (JSE) and grants from the Dowling Foundation (BM), Samuel Waxman Cancer Research Foundation, W.M. Keck Foundation, Joy and Jerry Monkarsh Fund (JSE for UCLA-CALTECH-CHLA-USC-UCONN Consortium on Translational Program in Engineered Immunity), Breast Cancer Alliance, Connecticut (AC) and MO 1RR06192 from GCRC, UCHC.

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Fig.1.

Transduction of CD4⁺CD25⁻ and CD8⁺ T cells with DMF5 TCR expressing retrovirus and further enrichment of the TCR expressing T cells. CD4⁺CD25⁻ (**A**) and CD8⁺ (**B**) T cells were transduced with the DMF5 retroviral vector, then stimulated by the MART-1₂₇₋₃₅ peptide pulsed autologous DC, and analyzed for MART-1₂₇₋₃₅ epitope specific population by tetramer staining flowcytometry. Representative of six separate experiments is shown.

Fig.2.

Proliferative potential of the TCR engineered CD4+CD25⁻ and CD8⁺ T. The DMF5 TCR transduced CD4+CD25⁻ (A) or CD8⁺ (B) cells were labeled with CFSE (panel a-day 0) and incubated for 4 days alone (panel b), with autologous mature DC (panel c), with DC pulsed with control peptide MAGE-3 (panel d) or with DC pulsed with MART-1₂₇₋₃₅ peptide (panel e). (C). Proliferation of the CFSE labeled DMF5 TCR transduced CD4+CD25⁻ T cells when stimulated by melanoma cells. CD4F5 cells at day 0 (a) and at day 4 (b-f) incubated alone (b) or with PT-M (c), wild type A375 (d), A375 cells pulsed with exogenous MART-1 peptide (e), A375M1 (MART-1 transfected A375) (f). Representative of four separate experiments is shown.

Fig.3.

Functional analyses of the DMF5 TCR transduced T cells. **(A).** Cytokine synthesis by the DMF5 TCR transduced CD4+CD25⁻ **(i)** and CD8⁺ **(ii)** T cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cytokine secreted in the supernatant were quantified 16 hr post co-culture set up. Data represents composite analysis of results (mean $\pm$ SEM) of 5 separate experiments with TCR-transduced T cells from 5 different donors. **(B).** Multiparametric intra-cellular cytokine staining of DMF5 TCR bearing CD4+CD25⁻ **(left column)** and CD8⁺ **(right column)** cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cells were stained for IL-2, IFN- γ , TNF- α , MIP-1 β and CD107a by intra-cellular staining. Selected combinations are shown as indicated. **(C).** Analysis of polyfunctional response exhibited by the TCR-transduced CD4+CD25⁻ and CD8⁺ T cells. The TCR-transduced T cells were stimulated with either the MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control

peptide (M3) and analyzed for multiple functional parameters shown on the X-axis by flowcytometry. The data were analyzed with FlowJo (TreeStar, USA) and polyfunctionality was assessed with PESTLE and SPICE softwares (provided by Mario Roederer, NIH, Bethesda, MD). Each slice of the pie chart shows fraction of total responsive cells were positive for given number of functions (color coded groups below the bar plot). **(D).** **(i)** CD4+CD25⁻ TCR transduced T cells were stimulated with MART-1₂₇₋₃₅ cognate peptide (M1) or MAGE-3₂₇₁₋₂₇₉ control peptide (M3) and cells were stained with anti-FoxP3-APC by intracellular staining. **(ii)** Freshly isolated human peripheral blood CD4+CD25⁺ T cells were stained for FoxP3 as a positive control. **(iii)** TCR transduced CD4+CD25⁻ T cells were stimulated for 16hr with either T2 alone, M3 or M1 pulsed T2 and supernatant were tested for TGF- β and TNF- α by ELISA. Representative of three separate experiments is shown.

Fig.4.

Cytolytic function of the DMF5 TCR transduced T cells show cytotoxicity against melanoma cells. **(A)**. Staining for surface exposure CD107a by CD4+CD25- (**upper**) and CD8+ (**lower**) T cells, when stimulated by A375, MART-1₂₇₋₃₅ peptide pulsed A375 cells (A375+M1) and the HLA-A2/MART-1 positive line, PT-M. The filled area represents isotype; black line shows interaction with the targets alone; red line shows the interaction with the targets pulsed by the control peptide MAGE-3₂₇₁₋₂₇₉; and blue line shows the interaction with the targets pulsed with the MART-1₂₇₋₃₅ peptide. **(B)**. Cytotoxicity by the DMF5 TCR transduced CD4+CD25- **(i)** and CD8+ **(ii)** T cells in 4hr ⁵¹Cr release assay (PT-M & M202: naturally MART-1 expressing human melanoma lines, A375: HLAA2+/MART-1neg. line; and A375+M1: MART-1₂₇₋₃₅ peptide pulsed A375 cells). Representative of three separate experiments is shown.

Fig.5.

Amplification of the CD8⁺ CTL expansion by the DMF5 TCR transduced CD4⁺CD25⁻ T cells. **(A).** A representative experiment is shown where untransduced CD8 T cells were incubated with autologous mature DC pulsed with MART-1 peptide in the presence or absence of CD4F5 T cells without any exogenous cytokine. A representative of four separate experiments is shown. **(B).** Composite data from similar co-culture experiments with TCR transduced CD4⁺CD25⁻ T cells and untransduced CD8⁺ T cells from 4 different donors. Fold increase of MART-1₂₇₋₃₅ specific population (mean $\pm$ SEM) was determined by counting the MART-1 tetramer positive cells in flowcytometry with CD8 gating. Of note, no expansion was observed with the mock-transduced CD4⁺ T cells or with the TCR-transduced CD4⁺CD25⁻ T cells when set up against the MAGE-3₂₇₁₋₂₇₉ control peptide, M3. Results from 4 experiments with 4 different donors are shown.