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Tumor infiltrating lymphocytes from acute myeloid leukemia marrow can be reverted to CD45RA+ central memory state by reactivation in SIP (Simulated Infective Protocol)

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ABSTRACT

Simulated Infective Protocol (SIP) is an ex-vivo culture system modeled after the temporal changes of essential cytokines in an acute infection, and previously proven successful in converting T lymphocytes harvested and activated from peripheral blood of normal donors, to revertant CD45RA + Central Memory T lymphocytes (Tcmra) demonstrating properties akin to T Memory Stem Cells (Tscm). In this study, we applied similar SIP on tumor infiltrating lymphocytes (TIL) from bone marrow of patients diagnosed with acute myeloid leukemia (AML), and replicated the feasibility to convert activated TILs into Tcmra phenotype. These revertant Tcmra lymphocytes re-expressed CD45RA+, CCR7+, CD62L + and CD127+, shown improved survivability with longer telomere length, expressed memory properties including higher Eomes to Tbet ratio, and exhibited cytotoxicity against autologous AML blast cells.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with different prognostic factors including age, co-morbidity, cytogenetic changes and molecular biomarkers. In general, apart from acute promyelocytic leukemia, successful remission after chemotherapy induction is estimated to be ~70-80% but long term survival can be dismal for patients without any transplant option. Therefore new treatment modality is required to improve survival outcome and immunotherapy offers a promising option. Some of the recent key approaches to enhance efficacy of immunotherapy involve reversing T cell anergy, improving the target specificity or increasing memory T formation with better proliferative and cytotoxic capacity.

Classical T lymphocytic ontogeny is believed to progress along 2 different developmental pathways, ie (a) the Divergent Pathway of Naïve (Tnaive)→ Effector (Teff) → Effector Memory (Tem) or Central Memory (Tcm) separately, as well as (b) the Linear Pathway of either Naïve (Tnaive)→ Effector (Teff)→ Effector Memory (Tem)→ Central Memory (Tcm), or Naïve (Tnaive)→ Effector (Teff)→ Central Memory (Tcm)→ Effector Memory (Tem) (Wherry et al., 2003; Bouneaud et al., 2005). Consequently, CD45RA expression had traditionally been regarded as naive T or terminally differentiated Temra but recent evidence had suggested that some CD45RA revertants (Tcmra) were in fact memory T cells observed to develop after acute infection with stem cell-like properties (Wills et al., 1999; Carrasco et al., 2006; Gattinoni et al., 2011). Based on this observation that T cells can develop into long term memory after acute infection, we had previously formulated an ex vivo culture system SIP (Simulated Infective Protocol), which simulated the temporal changes of all essential factors identified during an acute murine CMV infection (Ho et al., 2013). When SIP was applied on T lymphocytes harvested from peripheral blood of normal donors, it was able to activate these lymphocytes to Teff, Tem and Tcm, and subsequently converted them to revertant Tcmra demonstrating characteristics supportive of long term memory development. Having demonstrated successful conversion of normal T lymphocytes, next we intend to assess the feasibility of SIP in modifying resident tumor infiltrating lymphocytes (rTIL).

A unified paradigm for rTIL is emerging which supports a common immune contexture within various cancers, comprising of three primary patterns ie. (a) Pattern 1 with minimal infiltrating lymphocytes, (b)

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Pattern 2 with adequate but predominantly suppressive lymphocytes & microenvironment, and (c) Pattern 3 with adequate and predominantly cytotoxic lymphocytes & microenvironment (Etienne et al., 2016). Overall outcome corroborated with best result in Pattern 3 with most cytotoxic TILs, intermediate for Pattern 1 and worst for Pattern 2 with highest suppressive TILs. Further analysis by other investigators supported the presence of different TIL subsets at various developmental stages, with majority arrested as T effector (Teff), T effector memory (Tem) and terminally differentiated T effector memory CD45RA+ (Temra). Those lymphocytes being more advanced further down differentiation pathway displayed lower proliferative and cytotoxic capacity. Moreover, a substantial number of these rTILs also expressed specificity to the associated cancer cells; in fact, adoptive cell therapy harnessing tumor specific TILs in metastatic melanoma, has achieved higher response rates in patients when compared to conventional chemotherapy (Rosenberg et al., 2011).

Therefore, we hypothesise that the anergised resident TILs with tumor specificity can be isolated from leukemia marrow at diagnosis, expanded and reverted to a Tcmra using SIP, thus providing enough cytotoxic lymphocytes with improved proliferative capacity to achieve sustainable tumor control and perhaps eradication in the long term. We proceeded in this study to apply similar SIP culture condition to lymphocytes recovered from marrow of patients with AML, and replicated the feasibility of generating Tcmra lymphocytes expressing similar Tscm-like characteristics with cytotoxicity against autologous blasts.

2. Materials and methods

2.1. Cells and reagents

Cryopreserved diseased (AML) human bone marrow mononuclear samples were kindly provided by the Haematology Repository of the Singapore General Hospital. These samples included AML subtype M1,M2, M3, M4 & M5, with average blast count of 63 ± 4.8% and were all collected prior to starting chemotherapy. The samples were thawed in a 37 °C water bath and washed using complete medium: ImmunoCult-XF T Cell Expansion Medium (Stemcell Technologies) and 1% Penicillin-Streptomycin (Gibco).The total number of viable cells was counted in trypan blue and an estimate of 3% of the cell count was taken as CD3 positive cells. Following our published Simulated Infective Protocol (SIP), the monocytes were activated by anti-CD3 stimulation with 2.5 μL/10^6 CD3+ cells anti-CD3 Dynabeads (Invitrogen), 15 μL /10^6 CD3+ cells anti-human NKG2D mAb (Clone 149810; R&D Systems), and 15 μL /10^6 CD3+ cells goat anti-mouse IgG microbeads (Miltenyi Biotec).

2.2. SIP protocol

The cells were cultured in complete medium with a milieu of cytokines that simulate an early acute infection: dexamethasone, INF-γ, TGF-β1, IL-6, IL-7, IL-12, and IL-15 (all mentioned cytokines were from Stemcell Technologies). The cytokine dose concentrations used were IL-6 (high dose 20 ng/ml, medium dose 2 ng/ml, low dose 0.04 ng/ml), IL-12 (high dose 2 ng/ml), (high dose 1000 U/ml)), dexamethasone (high dose 0.4 ug/ml), TGF-β1 (high dose 2.5 ng/ml, medium dose 0.2 ng/ml, low dose 0.01 ng/ml), IL-15 (high dose 10 ng/ml, low dose 1 ng/ml), and IL-7 (low dose 2 ng/ml). After 10 days, we ceased anti-CD3 stimulation, and provided a low dose of TGF-β1, IL-6, IL-7 and IL-15. At day 14, we conducted a CD8 negative selection sort (EasySep Human CD8 + T Cell Enrichment Kit, Stemcell Technologies), which involves the removal of all non-CD8 cells. Myeloid blast cells, which express CD33, CD34 and CD117, were removed from the culture to allow the remaining CD8 cells to go into complete resolution. The CD8 T cells then finally rest and begin conversion to central memory CD45RA + CD8 T cells (Tcmra). After Day 21 and beyond, cultured cells were harvested and Tcmra cells were sorted out via negative selection (EasySep Human CD45RO FITC Positive Selection Kit, Stemcell Technologies) for flow analysis, molecular analysis, telomere length measurement, and their cytotoxic capability against autologous leukaemic blast cells was assessed.

2.3. Flow cytometry analysis

Cultured cells were harvested at different time points and surface stained with mouse anti-human CD8 conjugated to PC5 (Clone B9.11; PC5, Beckman Coulter), CD45RA conjugated to FITC (Clone HI100; FITC, eBioscience), and various other antibodies: CCR7 (Clone 15053; PE, R&D Systems), CD62 L (Clone DREG-56; PE, BD Pharmingen), CD3 (Clone: UCHT1; PC5, Beckman Coulter), CD4 (Clone 13B8.2; PC5, Beckman Coulter), CD8 (Clone: B9.11; FITC, Beckman Coulter), CD13 (Clone: SJ1D1; FITC, Beckman Coulter), CD33 (Clone: D3HL60.251; FITC, Beckman Coulter), CD34 (Clone: 581; FITC, Beckman Coulter), CD45 (Clone: J.33; PE, Beckman Coulter). After staining, the cells are fixed using IOTest3 Fixative Solution (Beckman Coulter), followed by flow analysis on a Beckman Coulter FC500 flow cytometer. After Day 21, Tcmra cells were stained with additional extracellular surface markers CD27 (Clone M-T271; PE, BD Pharmingen), CD28 (Clone UCHL-1; PE, BD Pharmingen), CD127 (Clone R34.34; PE, Beckman Coulter) and CXCR3 (Clone 1C6/CXCR3; PE, BD Pharmingen). For intracellular staining, cultured cells were harvested and surface stained first with mouse anti-human CD8-PC5 and CD45RA-PE (Clone HI100; PE, eBioscience), fixed and permeabilized with IntraPrep Perm reagent kit (Beckman Coulter), and stained with the following mouse anti-human antibodies: BCL2 (Clone 124; FITC, DakoCytomation), Granzyme A (Clone CB9; FITC, BD Pharmingen), Granzyme B (Clone GB11; FITC, BD Pharmingen), Perforin (Clone dG9; FITC, eBioscience), INF-γ (Clone 4S.B3; FITC, BD Pharmingen), TNF-α (Clone MAb11; FITC, eBioscience), IL-2 (Clone MQ1-17H1; FITC, eBioscience) and Granulysin (Clone B-L38; FITC Acris).

2.4. Molecular analysis

Tcmra cells were harvested and washed once with 1X PBS and then RNeasy Mini Kit (Qiagen) was used for RNA extraction. The cultured lymphocytes were lysed in 350 μL of Buffer RLT. RNeasy spin columns were used, according to the manufacturer's instructions, to capture the RNA. RNase-free DNase I (Qiagen) was used to remove DNA contamination. RNA was eluted from the column with 40 μL of RNase-free water. RNA purity and concentration were measured with the Nanodrop 1000 Spectrophotometer at 260 and 280 nm. For each RNA sample, cDNA was amplified from 2 ug of total RNA by reverse transcription in 20 μL reactions with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured by using a 7500 Fast Real-Time PCR system with TaqMan real-time PCR assay (Applied Biosystems). The probe and primer set of TaqMan real-time PCR assay includes a pair of unlabeled PCR primers and a TaqMan probe with a FAM dye label on the 5′ end and minor groove binder on the 3′ end. TaqMan Universal PCR Master Mix and TaqMan probe and primers were used to quantify the two gene expressions separately (Eomesodermin and T-box transcription factor, T-bet). The TaqMan assay amplification consisted of an activation step (50 °C, 2 min and 95 °C, 10 min) followed by 50 cycles of denaturation (95 °C, 15 s), annealing, and elongation (60 °C, 1 min). The amount of mRNA was normalized to an internal control gene (HPRT1 gene). The comparative CT (22DCT) method was used to calculate the relative quantitation of gene expression for each sample.

2.5. Telomere length measurement

Total DNA was extracted from the cultured cells using a DNA extraction kit (Qiagen). Extracted DNA was digested with HphI and MnlIm, separated by 0.8% agarose gel electrophoresis with 1kb-DNA

ladder (Life Technologies), before being transferred to Hybond-XL membrane (Amersham) for Southern blot analysis. The blot was probed and imaged using a Typhoon FLA 9500. Data and measurement of the telomere length were analyzed by the Quantity One program (Bio-Rad), which converted the Tif image of the blots into a spectrum image for accurate determination of the midpoint of each band, back calculating against the DNA ladder, determining the telomere length in base pairs.

2.6. Cytotoxic assay

Patient bone marrow samples, containing high blast cell counts were thawed and used as targets in this assay. Purified autologous Tcmra cells from our cultures were used as effectors and plated together with the targets in a round bottom 96-well plate according to Effector:Target ratios of 0:1, 1:0, 1:1, 5:1, 10:1, 20:1 and 40:1. The cell mixtures were placed in a 5% CO^2 incubator at 37 °C for 4 h. After 4 h, the cell mixtures were first washed with PBS and subsequently with AnnexinV Binding Buffer (BD Pharmingen). Cells were then stained with blast markers (CD13-FITC, CD33-FITC, CD34-FITC), effector T cell marker (CD3-APC) and apoptosis markers, Annexin V-PE (PE; BD Pharmingen) and 7-aminoactinomycin D (7-AAD, BD Pharmingen) for flow cytometry analysis. Flow count fluorospheres were added to each tube before flow to obtain the absolute counts and analysis was done on the Becton Dickinson Accuri C6 Flow Cytometer. Autologous blasts were gated out using the blast markers and the expression of AnnexinV versus 7-AAD was plotted for the gated blasts, with quadrant 3 (bottom left quadrant) representing all living cells. The absolute (Abs) counts were calculated based on the number of recovered fluorospheres. The percentage cytotoxicity was determined by (M1 + M2)/M3 × 100, where M1 = Abs Erupted Dead Count (for targets in different E:T ratio), M2 = Abs Dead Count (for targets in different E:T ratio) and M3 = Abs Target Count (for control with targets alone).

3. Results

3.1. SIP culture and cell count

Our SIP protocol simulates an acute infective cycle, with CD8 cells activated and expanded from Day 0 to Day10, and allowed to rest thereafter. At Day3, Day10 and Day21, mean Tcmra cell counts were $0.046 \pm 0.033 \times 10^6$, $1.16 \pm 0.337 \times 10^6$ and $5.11 \pm 1.62 \times 10^6$ respectively, with fold increase comparing to Day3 as 26 ± 9X at Day10 and 115 ± 44X at Day21 (Fig. 1). All percentages data are presented as mean ± SEM.

3.2. Phenotypical development into Tcmra

From Day 0 to Day 10, both CD4 and CD8 T lymphocytes were expanded with predominant CD8 subtype, eg. CD4 (12.4 ± 2.1%) versus CD8 (75.7 ± 2.5%) on Day 10. Focusing on the CD8 lymphocytes, the majority on Day3 expressed CD45RA, gradually converted into an activated state of CD45RO + . On Day7, 77 ± 8.5% of CD8 cells were CD45RO+, with 66.3 ± 5.7% expressing CCR7 and 74.3 ± 9.4% expressing CD62L + . By Day10, CCR7+ expression was dim, and downregulated to only 38.9 ± % in CD45RO + cells. After Day10, all cytokines were washed away and the cells were subjected to a low dose of IL-6, TGF-β1, IL-15, and IL-7. From Day10 onwards, CD45RO + CD8 cells continued to proliferate, with little changes to CCR7 and CD62 L expression. On Day14, CD8 cells were sorted out via negative selection and allowed to go into complete resolution, gradually converting to central memory CD45RA + CD8 T cells (Tcmra). By Day21, we observed 67.6 ± 9.2% of CD8 cells expressing CD45RA and of these cells, CCR7 and CD62 L was expressed at 71.1 ± 5.8% and 91.0 ± 4.6% respectively. Up to Day31, 72.9 ± 6.9%of CD8 cells were converted into the stable Tcmra state which were CD8+ CD45RA + CCR7+ CD62L+ (Fig. 2A). Additional surface markers,

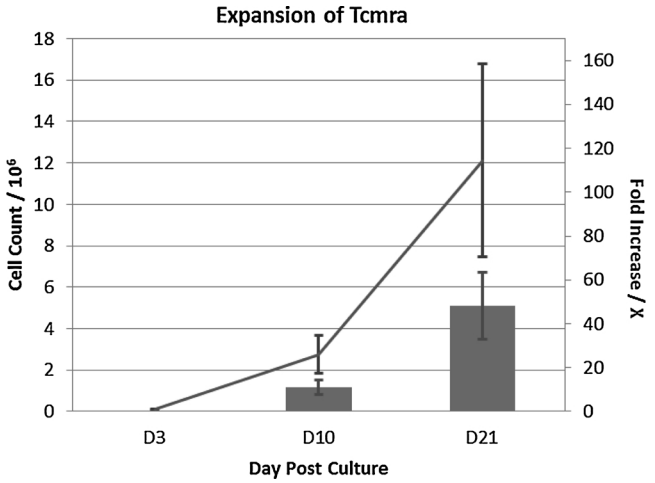

Fig. 1. Expansion of Tcmra. Absolute cell count is denoted by Bar chart while fold increase is represented by Line chart from D3 to D21. Data for absolute Tcmra cell count is presented as mean ± SEM, and these figures are representative of more than 3 independent experiments. D = number of days after commencement of SIP.

CD27 (99.3 ± 0.5%), CD28 (90.0 ± 9.2%), CD127 (89.0 ± 7.4%), CXCR3 (89.0 ± 7.4%) were also expressed on revertant Tcmra cells (Fig. 2B). All percentage data are presented as mean ± SEM.

3.3. Molecular Eomes/T-bet mRNA ratio analysis and telomere length

Other studies had established the expression of Eomes dominance over T-bet as a feature favouring memory T cell differentiation. In our study, Eomes/T-bet ratio of Tcmra were in fact higher than Tcmro, and we observed a mean value of 3.5 ± 1.2 versus 1.1 ± 0.4 from expanded Tcmra and Tcmro cells respectively (Fig. 3A). Tcmra were also found to have longer telomere length compared to Tcmro. The mean telomere length of Tcmra and Tcmro was 9.4 ± 0.8 kilo base pairs (kBP) and 8 ± 0.7 kBP respectively (Fig. 3B, **C**). All percentages data are presented as mean ± SEM.

3.4. Cytotoxic capacity against autologous leukemic blasts

Intracellular staining of the revertant Tcmra revealed that a fraction produced cytolytic enzymes, Granulysin and Perforin, at 69.8 ± 11.7% and 22.3 ± 6.7% respectively, though only a small population of 17.2 ± 6.7% expressed Granzyme A. Pro-inflammatory cytokines, TNF-α, INF-γ and IL-2 were found to be widely expressed, at 92.4 ± 5.4%, 90.2 ± 6.4% and 57.8 ± 10.2% respectively (Fig. 4A). Tcmra also demonstrated in vitro cytotoxic capacity against autologous AML myeloid blasts. AML myeloid blast cells were identified with CD13, CD33 and CD34 antibodies and the quantity of blast cell death was determined using apoptosis markers, AnnexinV and 7-AAD (Fig. 4B) Amount of cytotoxicity was found to increase, in concert to Tcmra versus AML myeloid blast ratios, with percent cytotoxicity at 6 ± 1.2%, 12 ± 1.4%, 18 ± 4.2%, 25 ± 7.3% and 45 ± 15.6% at 1:1, 5:1, 10:1, 20:1 and 40:1 Effector to Target ratios respectively (Fig. 4C). All percentages data are presented as mean ± SEM.

4. Discussion

In this study, CD8 lymphocytes harvested from marrow specimens of AML patients were activated and rested using our previously established SIP protocol, with an attempt to relieve their anergy by reverting to a latent state of Tcmra. Based on the flow immunophenotype, SIP was successful in activating and expanding CD8+ CD45RO + cells. These activated T cells downregulated CCR7 but maintained

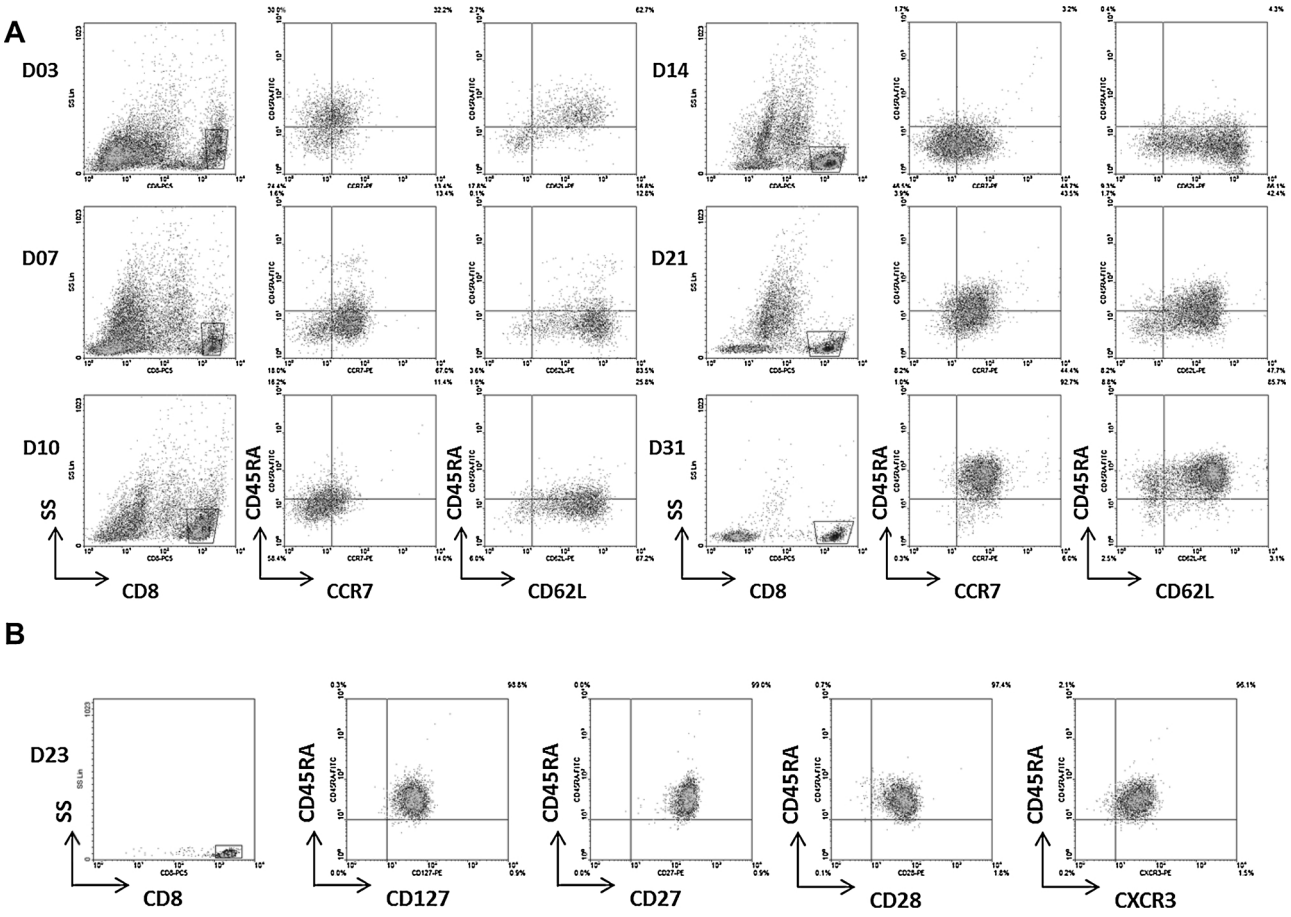

Fig. 2. Immunophenotypic development of TILs into Tcmra. (**A**) Flow cytometry plots showing phenotypic changes in CD45RA, CCR7 and CD62 L during the entire culture period up to Day31. (**B**) These plots demonstrated phenotypic results for additional surface markers, CD127, CD27, CD28 and CXCR3. D = number of days after commencement of SIP, and SS = side scatter.

CD62L + expression until Day14, and with subsequent removal of stimulatory antigen equivalent (ie. CD3 and myeloid blast cells), cultured CD8 T cells re-expressed CD45RA+, CCR7+, CD62L + and CD127+ and these phenotypic changes are supportive of successful reversion to a state of rest. Other studies had shown that CXCR3 potentially played a co-stimulatory role in T cell activation (Newton et al., 2009), and CXCR3 could also be a marker to predict the recall efficacy of CD8 + T cells, where CXCR3hi CD27hi CD8+ memory T cells mediate a stronger recall response compared to CXCR3lo CD27lo CD8+ (Hikono et al., 2007). The strong expression of memory markers CD27, CD28, CD127 and CXCR3 in our Tcmra thus supported the notion that TILs harvested from AML patients' marrow had recovered from their exhaustion and chronic repeated stimulation. According to our data, the removal of antigen presence at Day14 is crucial in the execution of resting phase in our cultured T cells, inducing the re-expression of CD45RA+, CCR7+ and CD62L+.

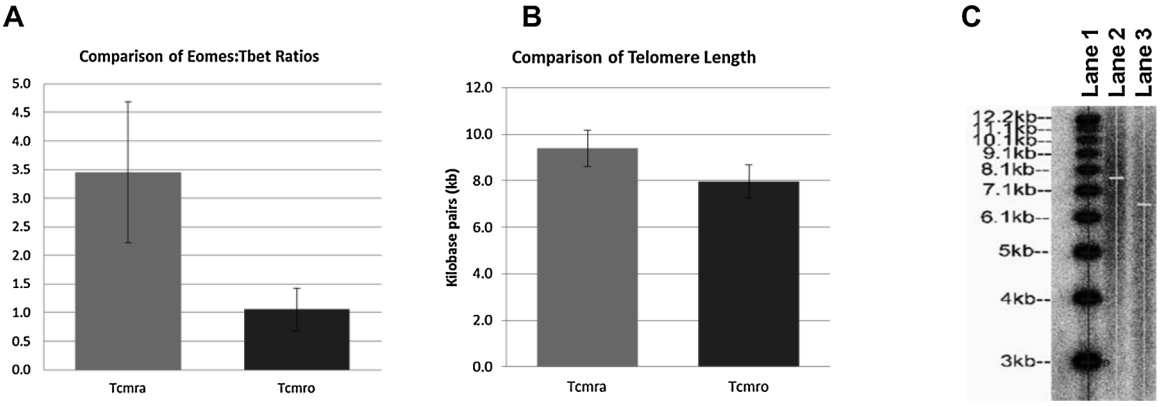

Fig. 3. Molecular and Telomere Length Comparisons. (**A**) This figure compared the expression of Eomes versus Tbet, and showed dominance of Eomes expression over Tbet in Tcmra cells beyond Day21. (**B**) This is graphical comparison of telomere length between Tcmra and Tcmro cells beyond Day21. (**C**) This figure is a representative example of the difference in telomere length between Tcmra and Tcmro cells from the same patient, measured in kb = kilobase pairs. *Lane 1* = kb-DNA Ladder from Invitrogen, *Lane 2* = Tcmra, *Lane 3* = Tcmro sample respectively. The peak intensity is marked with a black line across *Lane 2* at 7.9 kb and *Lane 3* at 6.6 kb. Data in Charts A and B are presented as mean ± SEM and is representative of at least 3 independent experiments.

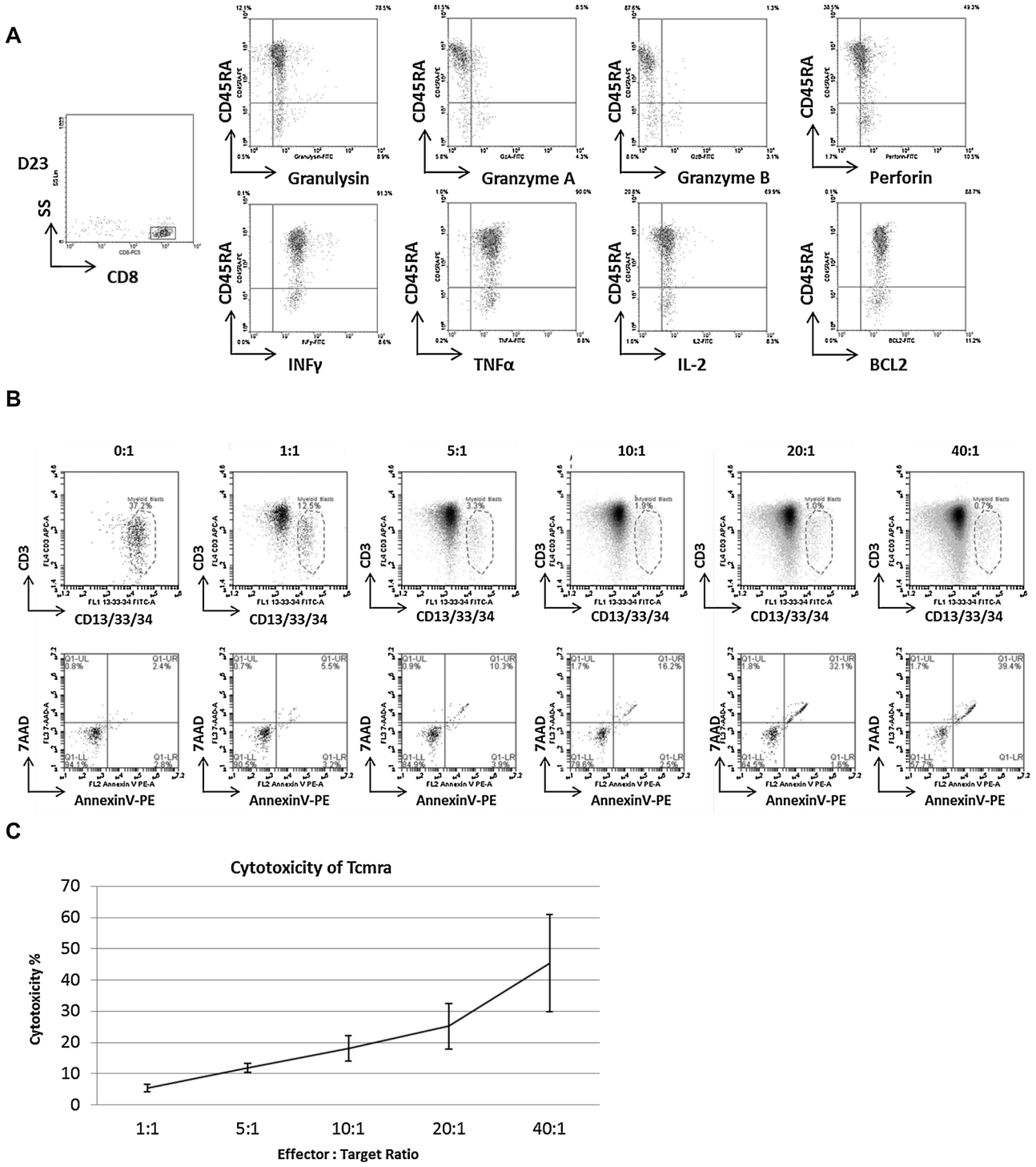

Fig. 4. Cytotoxic capacity of Tcmra (**A**) Panel A denoted the intracellular expression of Granulysin, Granzyme A, Granzyme B, Perforin, TNF-α, INF-γ, IL-2 and BCL2 on Day 23. (**B**) Panel B are flow cytometry plots showing the amount of cell death in the AML blast cell population, labeled with apoptosis markers 7AAD and AnnexinV-PE. AML blast cells were gated and identified using a combination of CD13, CD33 and CD34. (**C**) This graph showed the increasing cytotoxicity of Tcmra against autologous AML blast cells in increasing Effector to Target ratios of 1:1, 5:1, 10:1, 20:1 and 40:1. Data in this graph is presented as mean ± SEM, and these figures are representative of at least three independent experiments.

Molecular and telomere analysis on these cells exhibited increased Eomes/Tbet ratio and longer telomere length, which are all in keeping with stem cell memory like T and long term survivors. The increase in telomere length could be postulated to be due to expression of telomerase reverse transcriptase (TERT), which stabilizes and restores the length of the telomeres in cells, providing higher proliferative potential and longevity. Cell survival protein, BCL2, known for its suppressive role in apoptosis, was also found to be strongly expressed in the cultured Tcmra cells, indicating activation of survival mechanisms. Our SIP protocol not only induced reversion but also expanded Tcmra cells by a ˜115 fold increase on Day21 when compared to the absolute cell count at Day3. Our cultured Tcmra demonstrated long-term persistence, with a longest follow up to a maximum of 78 days *in vivo*. A large population of the revertant Tcmra cells demonstrated expression of

cytolytic enzymes, granulysin and perforin, and with the combined expression of inflammatory cytokines TNF-α, INF-γ and IL-2, these revertant Tcmra were shown to retain their cytotoxic capacities against autologous blast cells with their efficacy to kill blast cells in proportion to the Tcmra effectors versus blast ratios. Together, our findings supported the notion that CD45RA + central memory T lymphocytes can be generated in vitro from AML marrow at diagnosis, and capable of targeting and killing autologous blasts.

In conclusion, our current result has replicated the success of SIP protocol in rescuing and resting CD8 TILs recovered and expanded from AML patients' marrow. Thus further studies will be interesting to study the (a) feasibility to rest CD8 T lymphocytes recovered from other sources, e.g. leukapheresis product or TIL in solid tumors, and (b) feasibility of SIP to convert expanded lymphocytes from other activation protocols, e.g. CD3/CD28 stimulation, and Cytokine Induced Killers (CIK) production, before designing an appropriate animal model and translating into therapeutic modality for patients with AML. Hopefully SIP can ultimately be developed into a generic protocol to rescue anergised T lymphocytes in various tumours and invigorate them to battle more efficiently against cancer, and offer new avenue to engineer novel immunotherapeutic not just for AML patients but possibly other malignancies as well.

Declaration of conflict of interest

All authors have nothing to declare.

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