Research Article

Curtailed T-cell activation curbs effector differentiation and generates CD8⁺ T cells with a naturally-occurring memory stem cell phenotype

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Human T memory stem (TSCM) cells with superior persistence capacity and effector functions are emerging as important players in the maintenance of long-lived T-cell memory and are thus considered an attractive population to be used in adoptive transfer-based immunotherapy of cancer. However, the molecular signals regulating their generation remain poorly defined. Here we show that curtailed T-cell receptor stimulation curbs human effector CD8⁺ T-cell differentiation and allows the generation of CD45RO⁻CD45RA⁺CCR7⁺CD27⁺CD95⁻-phenotype cells from highly purified naïve T-cell precursors, resembling naturally-occurring human TSCM. These cells proliferate extensively in vitro and in vivo, express low amounts of effector-associated genes and transcription factors and undergo considerable self-renewal in response to IL-15 while retaining effector differentiation potential. Such a phenotype is associated with a lower number of mitochondria compared to highly-activated effector T cells committed to terminal differentiation. These results shed light on the molecular signals that are required to generate long-lived memory T cells with potential application in adoptive cell transfer immunotherapy.

Keywords: Adoptive cell transfer • CD8⁺ • Effector T cells • T-cell activation • T memory stem cells

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Introduction

Specific antigen recognition by the T-cell receptor initiates a program of clonal expansion and effector differentiation in naïve T (T naïve) cells that allows the migration to peripheral tissue and removal of infected targets [1]. Following resolution of the infection, most of these effector T cells die, leaving a population of long-lived memory T cells that is capable of more rapid and enhanced response following a second encounter with the pathogen [1]. The memory T-cell compartment is highly heterogeneous [2], comprising subsets that are different at the gene expression, metabolic and epigenetic level [3]. Current models suggest that circulating memory T cells are maintained in a stem cell-like fashion, were less differentiated cells self-renew while generating more differentiated progeny with enhanced killing capacity [4]. Such heterogeneity
is important for anti-tumor immunity, specifically in the context of adoptive cell transfer (ACT) immunotherapy [4]. Indeed, preclinical and clinical evidence demonstrated that T cells committed to terminal differentiation, such as effector memory (TEM) or terminal effector T (TIE) cells, are poorly effective in mediating tumor regression following ACT [5]. Rather, less differentiated T cell memory (TSCM) and central memory T (TCM) are more potent in this regard because capable to persist in the long-term [6–10].

The clinical use of these memory T cells has been hampered by their limited number in the peripheral blood and at the tumor site, thus methods have been proposed to expand the pool of existing TSCM by homeostatic cytokines [11] or to arrest differentiation of the more abundant T N precursors [8, 12–15]. T N activation with αCD3/2/28 antibody-conjugated beads in the presence of the glycogen synthase kinase-3β (GSK-3β) inhibitor TWS119 blocked differentiation and generated CD45RO–CCR7–CD95+ TSCM-like cells with improved anti-tumor immunity [8]. However, TWS119 decreases cell viability [12] and inhibits T-cell proliferation [8, 13]. The addition of IL-7 and IL-21 to the stimulation cocktail expanded the initial cell number by ∼6-fold and allowed to generate naturally-occurring TSCM [14]. Similarly, IL-7 and IL-15 in combination with strong αCD3/28 stimulation (3 beads: 1 cell) induced TSCM-like cells with a hybrid CD45RO–CD45RA+ phenotype that is rarely found in vivo [13]. Nevertheless, they were endowed with superior persistence capacity in xenogenic models compared to TCM and TEM. In summary, the use of specific combinations of cytokines or inhibition of specific signaling pathways have been demonstrated to arrest T-cell differentiation, but the specific role of potency of TCR stimulation as regards to the generation of TSCM cells remains ill-defined.

Here we show that decreased TCR stimulation, achieved either by using a low αCD3/28 bead:cell ratio or by limiting the stimulation through the CD3 receptor, in the presence of IL-7 and IL-15 favors the generation of TSCM cells with a naturally-occurring CD45RA–CD45RO+ phenotype. Such differentiation is associated with decreased T-cell activation and expression of effector-associated transcripts and transcription factors.

### Results

Curtailed T-cell receptor stimulation in combination with IL-7 and IL-15 generates TSCM cells

We first evaluated the effect of different cytokines, either alone or in combination, and potency of TCR stimulation on the differentiation of purified human peripheral blood T N cells (Supporting Information Fig. 1) as assessed by surface expression of CD45RO and CCR7. We decided to use a low (i.e., 1:2) bead:cell ratio, as a previous report using a 3:1 ratio was associated with the acquisition of CD45RO by differentiating T N [13]. Overall, IL-7 and IL-15 combined with αCD3/28 was the best combination to maintain a CD45RO–CCR7+ phenotype (Fig. 1A) and to generate bona fide CD45RO–CD45RA+CCR7+CD27+CD95+ TSCM-like cells (Fig. 1B; hereafter referred to as iTSCM). By contrast, T-cell activation with αCD3/2/28 in the presence of IL-7/IL-15 or IL-2 preferentially generated CD45RO–CCR7+ TEM-like cells (Fig. 1A and C). The addition of IL-18 to IL-2 stimulation had limited effect on T-cell phenotypes compared to IL-2 alone, while the addition of IL-12 generated bona fide CD45RO–CD45RA–CCR7+CD27hiCD95+ T effectors (iTEff) (Fig. 1A–B). Besides differential expression of known T-cell differentiation markers, iTSCM also displayed lower CD38 and HLA-DR (indicative of activation) and Ki-67 (proliferation) compared to iTEff (Fig. 1B) and had lower forward scatter (FSC, indicative of cell size). A summary of the T-cell differentiation phenotypes (defined in Materials and methods) induced by selected culture conditions is shown in Fig. 1C. Overall, more potent stimulation due to triggering of CD2 significantly increased the frequency of non-viable cells at the end of the culture period, irrespectively of the cytokine cocktail (Fig. 1D). In iTEff condition, this was compensated by increased proliferation (Fig. 1E). Despite the lower proliferation and activation compared to iTEff in vitro, iTSCM cells expanded robustly over time in vivo following adoptive transfer into NSG mice (Fig. 1F) and infiltrated multiple organs, including the spleen, the liver and, at a lesser extent, the lung (Fig. 1G).

### Polyclonal and antigen-specific TSCM cells are early-differentiated T cells

The capability to produce effector cytokines following TCR stimulation is shaped by the degree of memory differentiation, where less differentiated T cells mainly produce IL-2 and TNF while more differentiated ones mainly produce IFN-γ [16]. We therefore reasoned that iTSCM and iTEff had different patterns of cytokine production. To test this, day 12 iTSCM were re-stimulated with PMA/ionomycin (P/I) for 3 h. Figures 2A and D show that iTSCM mainly produced TNF and IL-2, either in combination or alone, while little IFN-γ, while iTEff were mostly characterized by cells producing the 3 cytokines together. To confirm that the same pattern of functionality could be observed at the antigen-specific level, we took advantage of an accelerated DC maturation protocol [17] and of the elevated frequency of Mart-1-specific CD8+ T cells to generate antigen-specific TSCM cells from T N precursors. To this end, we stimulated PBMCs from HLA-A∗02+ donors with the native, 10-mer Mart-1 peptide EAAGIGILTV, or its heteroclitic variant ELAGIGILTV, that is capable to bind cognate TCRs with higher avidity (hereafter named EA and ELA, respectively). Both peptides primed a CD8+ proliferative response, as assessed by CFSE dilution and tetramer staining (Fig. 2B), although ELA generated a higher frequency of antigen-specific TSCM cells as well as a higher total count of antigen-specific TSCM cells compared to EA (Fig. 2C). Subsequent ELA-peptide restimulation 7 days post initial priming induced antigen-specific cytokine production in both CD8+ populations (Supporting Information Fig. 3). Among these, CD45RO–CCR7+CD95+ TSCM-phenotype cells had a similar pattern of IFN-γ, IL-2, and TNF expression, irrespectively of the
Figure 1. Generation of iTSCM cells. (A) Representative expression of CD45RO and CCR7 in ex vivo PBMCs and in sorted CD8<sup>+</sup> T<sub>N</sub> cells as evaluated by flow cytometry following culture with different cytokine cocktails and T-cell receptor dependent stimulation for 11 days. Similar data were obtained from a second donor. (B) Sorted CD8<sup>+</sup> T<sub>N</sub> cells were cultured in αCD3/28+IL-7/15 or αCD3/2/28+IL-2/12 for 11 days and the expression of naïve and memory-associated markers in the two conditions (black line histogram and dashed line histogram, respectively; n = 4 for all parameters, except n = 2 for Ki-67, CD38, and HLA-DR) was evaluated as in (A), by flow cytometry. Total CD8<sup>+</sup> T cells from a healthy donor were used as control staining (ex vivo CD8<sup>+</sup>; filled gray histogram). (C) The proportion of CD8<sup>+</sup> T cells with the TSCM, TCM, TEM, and TTE phenotypes (gated as in Supporting Information Fig. 1) after culture in the indicated conditions (αCD3/28+IL-7/15: n = 22; αCD3/2/28+IL-7/15: n = 9; αCD3/2/28+IL-2/12: n = 12) for 11 days was determined by flow cytometry and shown as mean ± SEM. (D) Mean ± SEM of the percentage of viable cells and (E) fold expansion in cell number, compared to baseline, of sorted CD8<sup>+</sup> T<sub>N</sub> cells cultured as in C. (F, G) Human CD8<sup>+</sup> T<sub>N</sub> cells cultured in αCD3/28+IL-7/15 were transferred into NSG mice and at the indicated days after transfer, the (F) absolute numbers of CD8<sup>+</sup> T cells in the circulation, and (G) those cells infiltrating different organs at necropsy 32 days post transfer were evaluated by flow cytometry (see Supporting Information Fig. 2 for the gating strategy). Data shown as mean ± SEM of 5 replicate mice, one single experiment.* p < 0.05, ** p < 0.01, and *** p < 0.001, Wilcoxon test.
Figure 2. Polyclonal and antigen-specific T<sub>SCM</sub> cells are early-differentiated T cells. (A) CD8<sup>+</sup> T<sub>N</sub> cells were activated as in Fig. 1C to generate iT<sub>SCM</sub> and iT<sub>Eff</sub>. Representative IL-2, IFN-γ and TNF expression in iT<sub>SCM</sub> cells and iT<sub>Eff</sub> cells following PMA/ionomycin (P/I) stimulation was evaluated by intracellular flow cytometry staining; ns: nonstimulated. (B) HLA-A*02<sup>+</sup> PBMCs from healthy donors were stimulated with FLT-3, IL-1β, PGE<sub>2</sub> and TNF (DC maturation/activation mix, day 0) and Mart1 peptide and IL-7 and IL-15 (day 1). Representative CFSE dilution and frequency of Mart1-specific CD8<sup>+</sup>T cells (top) and CD45RO and CCR7 expression in Mart-1<sup>+</sup> cells (black) superimposed to total CD8<sup>+</sup>T cells from the same culture (bottom) as assessed by flow cytometry. Data shown are representative of nine other measurements from four independent experiments. (C) The frequency of Mart-1<sup>+</sup> among CD8<sup>+</sup> cells (left; ELA: n = 6; EAA: n = 9) and the relative T<sub>SCM</sub> count (right; ELA: n = 6; EAA: n = 5) following peptide stimulation is shown as mean ± SEM. Relative T<sub>SCM</sub> count was calculated only when the total number of cells within the Mart-1<sup>+</sup> fraction was >15. (D) The production of IL-2, IFN-γ and TNF following PMA/ionomycin (P/I) stimulation of cells obtained as in (A) or following ELA Mart-1 peptide stimulation of cells obtained as in (B) was measured by flow cytometry. Data shown as mean ± SEM; 5 independent experiments. The number of independent donors tested is indicated in the figure. The legend refers to the initial stimulation at day 0 and to the restimulation at day 7 (either ELA peptide or P/I). In all flow cytometry plots, values indicate percentage of cells within the gate. *p < 0.05 versus iT<sub>SCM</sub>, Wilcoxon test.

original peptide used for their generation, that was mostly comparable to that of iT<sub>SCM</sub> generated by αCD3/28 beads and restimulated with P/I (Fig. 2D).

iT<sub>SCM</sub> cells undergo limited activation and effector differentiation

To gain more mechanistic insights into the differentiation of purified T<sub>N</sub> cells toward the iT<sub>SCM</sub> phenotype, we quantified the level of molecules, either at the protein or mRNA level, that are responsible for effector differentiation. Specifically, transcription factors T-bet and Eomes control effector T-cell function [18, 19] and increase progressively with human peripheral differentiation [8], while IRF4 [20], IRF8 [21], and miR-155 [22] are mostly regulated by the potency of TCR activation. Accordingly, T-bet and Eomes increased progressively with the duration of the culture period (Fig. 3A), while IRF4, IRF8, and miR-155 were mostly detected during the first week of culture, then their expression waned (Fig. 3B). Overall, these molecules were generally
overexpressed in iTEff vs. iTSCM throughout the stimulation period, thus recapitulating the differential activation status observed in Fig. 1B and indicating limited activation and effector differentiation of iTSCM cells. It has also been proposed that subsets of T cells at different stages of differentiation harbour different numbers of mitochondria, where the mitochondrial content increases with progressive differentiation of T cells isolated ex vivo (T(N)<T(CM)<T(EM)) [23]. Single cell analysis by confocal microscopy revealed that iTSCM harbored fewer mitochondria and appeared to have little cytoplasm compared to iTEff (Fig. 3C), thus corroborating the lower FSC levels observed in Fig. 1B.

Despite using a low bead:cell ratio to generate TSCM with the naturally-occurring CD45RO⁻ CD45RA⁺ phenotype, it is difficult to evaluate the specific contribution of CD3 and CD28 molecules in this regard. To this aim, we stimulated TN cells in TSCM-promoting conditions (i.e., with IL-7 and IL-15) and in the presence of titrated amounts of plate-bound αCD3 and soluble αCD28, then assessed CD45RA and CD45RO expression (Fig. 3D; Supporting Information Fig. 4). Phenotypic dynamics depend on the level of proliferation [24]. As different strengths of TCR stimulation result in differential proliferation, analysis of specific markers was performed according to the level of CFSE dilution (i.e., generations).
Naturally-occurring TSCM are known to preferentially self-renew in proliferating cells toward a CD45RO– CD45RA phenotype, while decreasing expression of CCR7 and CD27 and de novo expressed CD95 following stimulation, stained with CFSE and induced to proliferate as in Fig. 1C was measured by flow cytometry. Data are shown as mean ± SEM of n = 4 samples as in B. (D) CFSE dilution (top) and CD45RO and CCR7 expression following αCD3/2/28 + IL-2 and IL-12 stimulation (bottom) of iTSCM cells generated as in Fig. 1C was measured by flow cytometry. Data shown are representative of measurements made for three samples from two independent experiments.

Self-renewing and multipotent capacity of iTSCM cells

When >50 cells were available in each gate. Changing the concentration of αCD28 had no impact on T-cell phenotypes (not shown), while decreasing αCD3 resulted in the progressive shift of proliferating cells toward a CD45RO+ CD45RA− phenotype (Fig. 2D–E, Supporting Information Fig. 4). These cells also maintained the expression of CCR7 and CD27 and de novo expressed CD95 (Supporting Information Fig. 4), confirming that curtailed TCR stimulation favors the generation of TSCM cells.

Figure 4. Self-renewing and multipotent capacity of iTSCM cells. (A) Ex vivo sorted Tn, TSCM, and total memory T (TMEM; defined as CD45RO−) stimulation. (Supporting Information Fig. 1) CD3+ T cells as well as iTSCM (generated as in Fig. 1C) were incubated with IL-15 for 12 days (black line histogram). CFSE dye dilution was measured by flow cytometry. Filled gray histogram indicates unstimulated control. (B) The percentage of CFSE-diluting cells in (A) is shown as mean ± SEM of n = 4 samples from two independent experiments (n = 2 each). *p < 0.05 versus CTRL, Wilcoxon test. (C) The frequency of TSCM-phenotype cells (defined as in Supporting Information Fig. 1) out of total CD8+ T cells after stimulation of Tn cells for 6 days in iTSCM polarizing conditions (pre IL-15) or after stimulation of the same iTSCM with IL-15 for 12 days (post IL-15) was measured by flow cytometry. Data are shown as mean ± SEM of n = 4 samples as in B. (D) CFSE dilution (top) and CD45RO and CCR7 expression following αCD3/2/28 + IL-2 and IL-12 stimulation (bottom) of iTSCM cells generated as in Fig. 1C was measured by flow cytometry. Data shown are representative of measurements made for three samples from two independent experiments.

Discussion

We show that curtailed T-cell receptor stimulation favors the generation of early-differentiated CD45RO+ CD45RA+ TSCM cells from naive precursors, and that this is associated with restrained T-cell activation and upregulation of transcriptional regulators that are otherwise responsible for effector differentiation. Similar phenotypes could be obtained by using Dynabeads from Thermo Fisher (data not shown), that are currently being used to expand T cells in ACT clinical trials. Such molecular dynamics were evident since the very first hours following stimulation in vitro. These data are important as increasing evidence in preclinical models and in humans indicate that T cells at earlier stages of differentiation, such as TSCM and TEM cells are better suited to persist long-term in vivo following adoptive transfer and are endowed with more potent activity, such as that directed toward tumors [26].

By separately testing molecules involved in T-cell activation and by titrating the amount of signal delivered to T cells, we found a role for CD3, but not for CD28 in defining the acquisition of the CD45RO+ CD45RA+ TSCM phenotype. These iTSCM were both self-renewing and multipotent and had features of early-differentiated T cells in terms of cytokine production profile in response to polyclonal and antigen specific stimulation. Indeed, in vitro priming of Tn cells with the native self/tumor antigen Mart-1 EAA, and its heteroclitic variant ELA, results in the generation of a subset of TSCM cells with functional capacity that is similar to iTSCM generated by polyclonal stimulation. Despite priming less Tn precursors due to its decreased binding strength to the TCR, which results in a lower total number of Mart-1-specific CD8+ T cells (Fig. 2B), EAA selects those TCRs with the highest avidity that is in turn associated with more pronounced anti-tumor activity [27]. It has been therefore speculated that EAA is preferable to ELA in vaccine regimens. ELA/EAA peptide vaccination in combination with CpG and Montanide in melanoma patients resulted in the early (<3 months) generation of Mart-1-specific TSCM cells that were capable to persist in the long term [27]. However, antigen-specific TSCM generation was shown to occur much earlier, such as following infection with simian immunodeficiency virus (SIV) in rhesus macaques, where SIV-specific TSCM cells developed as early as 7 days after infection, expanded clonally and coincidentally with the differentiation of bona fide Teff. Interestingly, a much lower proportion of TSCM displayed expression of HLA-DR and Ki-67 in iTSCM polarizing condition. According to their memory properties, iTSCM proliferated in response to IL-15 similarly to ex vivo TSCM and bulk memory T cells (TMEM; sorted as CD45RO+), while ex vivo Tn were non-proliferating (Fig. 4A), as previously described [8, 9, 11]. A summary of CFSE dilution from 4 donors is shown in Fig. 4B. Similar proliferation could be observed following iTeff-polarizing condition. While T cells in IL-15 largely maintained their original phenotype (Fig. 4C), those in iTeff-polarizing condition differentiated to CD45RO+ CCR7+ TEM and CD45RO+ CCR7− TEM cells (Fig. 4D), thus indicating self-renewal capacity and multipotency, respectively.
human CD8+ T-cell subsets sorted ex vivo by transmission microscopy and revealed that mitochondrial content increases progressively in human TCM and TEM compared to TN [23]. Differently from previous studies [14], Van der Windt et al., reported a higher mitochondrial content in cells kept in IL-15 compared to those kept in IL-2 [28]. Differences in protocols used to generate memory precursors could explain these contrasting results. In fact, Van der Windt et al. generated memory T cells by initially activating TN in the presence of IL-2 for 3 days, followed by a switch in IL-15 for 2 additional days, thus leading to a higher activation of IL-2 stimulation, although transient, has long term effect on T-cell metabolism. While initial exposure to IL-2 might better mimic the in vivo condition during effector differentiation, IL-2 stimulation in vitro should be avoided when generating T cells for ACT, because inducing more committed differentiation (Fig. 1A) [29], and loss of anti-tumor capacity [14].

Inhibition of effector differentiation of TN precursors has been achieved in multiple ways, such as pharmacological intervention, cytokine cocktails and genetic manipulation [26]. More recently, inhibition of bromodomain and extra-terminal motif (BET) proteins has been shown to limit BATF expression and favor the generation of TSCM from human TN [30]. The same group reported similar findings when stimulating bulk T cells with cell-based artificial antigen-presenting cells expressing ligands for CD3 and CD28 only transiently [31]. Of note, BATF is part of a multiprotein DNA-binding complex comprising Jun and IRF4 or IRF8 and is involved in the generation of effector T cells [32]. Our observations suggest that low but detectable levels of IRF4 and IRF8 are necessary for initial T-cell activation induced by the TCR but are dispensable for the generation of iTSCM. We propose that limiting TCR stimulation during priming, and so the expression of transcriptional regulators of effector differentiation can be exploited as an additional strategy to generate iTSCM for ACT immunotherapy.

Materials and methods

Mice

All animal experiments were conducted upon the approval of the Humanitas IACUC and the Italian Ministry of Health (protocol 256/2015-PR). NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NSG) mice (Jackson Laboratories), bred in SPF conditions, were used for adoptive transfer experiments. Briefly, 1–2 × 10^6 CD8+ T cells, expanded in vitro with αCD3/28-conjugated beads (T-cell activation/expansion kit, Miltenyi; 1 bead:2 cells), were co-transferred by retroorbital injection with 8 × 10^6 PBMCs depleted of CD8+ T cells. For mononuclear cell isolation, tissues were minced and filtered through a 100 μM cell strainer.

Cells

All experiments were approved by the Humanitas Research Hospital IRB. Peripheral blood mononuclear cells (PBMCs) were isolated fromuffy coats and frozen in liquid nitrogen according to standard procedures.

Flow cytometry and cell sorting

Fluorochrome-conjugated monoclonal antibodies were purchased from BD Biosciences, Biolegend and eBioscience (see Supporting Information Table 1 for details), and titrated to determine optimal concentration. Either fresh or frozen cells were used. Frozen cells were thawed and stained for flow cytometry as described [11]. HLA-A*0201/MART-126-35 ELAGIGILTV tetramer staining (conjugated to streptavidin-BV421) was performed at 37°C for 20 min. Intracellular transcription factors were detected following fixation of cells with the FoxP3/transcription factor staining buffer set (eBioscience). T-cell subsets were defined as follows: TN, CD45RO+CD45RA+CCR7+CD27+CD95+; TSCM, CD45RO+CD45RA–CCR7+CD27+CD95+; TCM, CD45RA+CCR7+; TEM, CD45RO–CCR7+; TIL, CD45RO–CCR7–; bulk memory T (TMBM) cells: CD45RO+. TNs were FACSc-sorted as depicted in Supporting Information Fig. 1. Samples were acquired on a Fortessa flow cytometer or separated via a FACS Aria III cell sorter (all from BD Biosciences). Flow cytometry data were compensated with FlowJo (FlowJo LLC) by using single-stained controls prepared with antibody-capture beads (BD Biosciences).

Cell culture

PBMC or sorted T cells (0.25–1 × 10^6 cells/mL) were cultured in complete RPMI medium (10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine), and stimulated with αCD3/2/28 antibody-coated beads. New wells were set daily with beads or 10 ng/mL PMA/ionomycin stimulation. When preparing beads bound to αCD3/28 only, the amount of αCD2 in the mix was replaced by PBS (without calcium and magnesium, referred to as PBS−/−). Alternatively, cells were stimulated with plate-bound αCD3 (clone OKT3) plus soluble CD28 (clone CD28.2) in 96-flat-bottomed wells for 9 days. Human cytokines (Peprotech) were pre-titrated, then used at 10 ng/mL unless specified. Cell proliferation was determined by the analysis of CFSE (5 μM; Life Technologies) dilution [11]. Cells were subsequently stimulated with IL-15 (Peprotech) for 12 days at 50 ng/mL, or left in 1 ng/mL IL-15 (non-proliferating control). Cell number was determined by Trypan blue dye exclusion.

To induce cytokine production by PMA/ionomycin stimulation and to measure intracellular cytokine production, cells were treated as described [33]. Antigen-specific T cells were grown by using an accelerated DC maturation protocol, as described [17]. Briefly, 3.5 × 10^6 CFSE-stained PBMCs from HLA-A*02+ donors were plated in 48-well plates in AIM-V medium (Life Technologies) with 50 ng/mL (DC maturation) on day 0 and with IL-1β, PGE-2, TNF (DC activation mix) and the Melan-A/Mart1 peptide antigens ELAGIGILTV or EAAGIGILTV (2 μg/mL; peptide chemistry facility, University of Lausanne) on day 1. IL-7 and IL-15 (both...
at 10 ng/mL) were included in each well on day 1. Cells were collected at day 7 for FACS analysis, otherwise restimulated with 2 μg/mL ELAGIGLTV for 18 h in the presence of GolgiPlug (BD Biosciences), according to manufacturer’s instructions.

**Real-time PCR (qPCR)**

Total RNA, purified with RNeasy Micro Kit (Qiagen), was retro-transcribed using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) and analyzed by qPCR with hydrolysis probes: IRF8 (Hs00175238_m1) and 18S (Hs99999901_s1) as reference gene (Applied Biosystems) using the ABI 7900HT Sequence Detection System (Applied Biosystems). For Micro-RNA (miR) expression analysis, RNA was isolated with mirVana kit (Ambion). Mature miR-155 and RNU44 small nuclear RNA were reverse transcribed with specific primers provided by Applied Biosystems and TaqMan RT MicroRNA Kit (Applied Biosystems). qPCR was performed with miR-155 and RNU44 specific TaqMan primers (Applied Biosystems) and Universal PCR Master Mix, No AmpErase® UNG (Roche) in MICROAMP® Fast Optical 96-Well Reaction Plate (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Expression levels were normalized (ΔCt) to RNU44 or 18S endogenous controls and expression fold change relative to CD8+ T<sub>NI</sub> cells were calculated using 2<sup>−ΔΔCt</sup> sample/ΔCt naive formula.

**Confocal microscopy**

CD8<sup>+</sup> T cells were washed in PBS<sup>−/-</sup> and incubated with 1 mL of pre-warmed Mitotracker Green (25nM prepared in PBS<sup>−/-</sup>) for 30 min at 37°C. To allow T-cell adhesion, slides were previously incubated for 30 min with 0.02% polylisin and coated for 3 h at 37°C with αCD3 (OKT3 clone, BD Biosciences; 10 μg/mL in PBS<sup>−/-</sup>) and αCD28 (CD28.2 clone, BD Biosciences; μg/mL in PBS<sup>−/-</sup>) followed by 3 washes in PBS<sup>−/-</sup>. T cells (0.15 × 10<sup>6</sup>) were then layered on slides and incubated for 15 min at 37°C. After incubation, cells were fixed with 4% PFA for 10 min, washed twice with 2% BSA in PBS<sup>−/−</sup> and once with 2% BSA, 0.05% tween in PBS<sup>−/−</sup>. To identify nuclei, cells were counterstained with DAPI (Invitrogen) by incubating for 10’ at RT. Slides were acquired with an FV1000 confocal microscope (Olympus). Images were analyzed with ImageJ (NIH).

**Statistical analysis**

Analysis was performed using GraphPad PRISM (6.0b) and SPICE 5.22 software. Non-parametric paired or unpaired Wilcoxon rank test were used to compare two groups. P values are two-sided and were considered significant when ≤0.05.

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**References**


Abbreviations: ACT: adoptive cell transfer · TCR: T-cell receptor · TEff: naive T cell · TSCM: T stem cell memory · TCM: central memory T cell · TEM: effector memory T cells · TEF: effector T cell

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