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Stem cell-like properties of memory T cells in human immune reconstitution

PhD student:

Veronica Zanon

(Matr.R10726)

Under the supervision of:

Prof. Domenico Mavilio

Dr. Enrico Lugli

Head of PhD Programme:

Prof. Massimo Locati

A.A.

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A Mauro

ABSTRACT

The memory T cell compartment comprises a plethora of subsets that are diverse at the functional, transcriptional and metabolic level. T memory stem cells (T_{SCM}) are the least differentiated subset endowed with superior immune reconstitution capacity, anti-tumor activity and long-term persistence compared to more differentiated progeny in preclinical models. However, their role in human T cell recovery remains undetermined.

In the first part of my work, I investigate the dynamics of T cell reconstitution in human haploidentical bone marrow transplantation (haplo-BMT) with post-transplant cyclophosphamide (pt-Cy) and provide evidence that donor antigen-specific naïve (T_N)-derived T_{SCM} cells survive pt-Cy and are the most abundant circulating T cell population in the early days after transplantation. I further demonstrate that these cells later contribute to peripheral reconstitution by differentiating into effector cells. With these experiments, I define the cellular basis of human T cell reconstitution following haploidentical transplantation with pt-Cy and propose to employ naive-derived T_{SCM} cells in the clinical setting to overcome immunodeficiency. However, the clinical use of these cells is limited by their paucity *ex vivo*. Therefore, I dedicate the second part of this work to investigate the biological and molecular mechanisms involved in the generation and expansion of T_{SCM} cells from $CD8^+$ T_N precursors. I show that curtailed T-cell receptor stimulation curbs effector $CD8^+$ T-cell differentiation and allows the generation of $CD45RO^-$ $CD45RA^+CCR7^+CD27^+CD95^+$ -phenotype cells from highly purified T_N precursors, resembling naturally-occurring T_{SCM}. 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 high numbers of long-lived memory T cells with potential application in adoptive cell transfer immunotherapy.

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1 INTRODUCTION

1.1 Adaptive Immunity: maturation, differentiation and homeostasis of T cells

T cells belong to the adaptive arm of the immune system. They derive from committed lymphoid progenitors cells that arise the bone marrow (BM) and infiltrate the thymus via the blood[1]. These progenitors, or thymocytes, maturate and generate both αβ and γδ T cells[2]. The maturation consists in the re-arrangement of variable (V), diversity (D) and joining (J) segments of the T cell receptor (TCR) loci[3]. αβ double negative (DN) T cells first express a pre-non-rearranged TCR-α chain which pairs later with the TCR-β rearranged chain (pre-TCR-αβ)[1]. At the cell surface, the pre-TCR-αβ is associated with the intracellular CD3/ζ-complex-which is involved in the intracellular transduction of the signal[4]. The first active signal provided by cortical epithelial cells induces biochemical changes in the cytoplasmic portions of CD3 complex which includes the phosphorylation of the tyrosine-based activation motifs (ITAMs) by the Src family tyrosine kinase LCK and FYN[1]. These signals are fundamental for the selection of correct β-chain and further maturation of T cells[2]. This first active signal induces the proliferation of thymocytes, the rearrangement of α-chain and the generation of a fully mature TCR-αβ[2]. At these stage almost the 90% of thymocytes are double positive (DP) for CD4 and CD8 costimulatory proteins[2]. DP cells are located in the cortex region of the thymus where they interact with self-peptides presented by major histocompatibility complex (MHC). The interaction with “self” is essential for thymocytes survival and for the commitment of DP cells towards $CD4^+$ or $CD8^+$ single positive (SP) T cell lineage (called positive

selection). The commitment towards the $CD4^+$ or $CD8^+$ T cell lineage depends on the interaction with two different classes of MHC expressed by cortical antigen presenting cells (APCs): MHC class I (MHC-I) induces the maturation of $CD8^+$ T cells while MHC-II induces the maturation of $CD4^+$ T cells[5]. T cells capable to survive positive selection migrate into the cortico-medullary junction of the thymus. Here, they encounter APCs such as macrophages, dendritic cells, bone-marrow derived APCs expressing tissue-restricted peptides:MHC. T cells which bind MHC with high affinity are harmful as they could lead to unwanted self-directed activities, thus are negative selected and die by apoptosis[6]. Those cells that have avoided negative selection maturate into naïve T cells (T_N)[6]. Mature T_N cells leave the thymus and reach secondary lymphoid organs (*i.e.* spleen and lymphnodes) where they continuously scan APCs in search of their cognate-antigen (Ag). After Ag recognition, T_N cells undergo activation and generate more differentiated effector cells (expansion phase) whose function is to eliminate the pathogen or cancer cells[1]. After infection, the vast majority of T cells (95%) dies (contraction phase), while a small fraction survives and generates memory cells, which persist in the long term[7]. Memory cells are clonally expanded and activate more rapidly than T_N cells, thus ensuring protection in case of re-infection[8].

1.1.1 **Models of T cell memory formation**

After the resolution of primary infection, around 90-95% of $CD8^+$ effector T cells die, while a small fraction survives and generates memory T cells with long-term persistence. The mechanisms that regulate T cell fate after activation remain poorly defined. In this regard, two main models of differentiation have been proposed. This first model, also called the “linear model”, proposes that memory cells are generated

from effector cells during the contraction phase after initial infection[9]. The second, alternative model is the “developmental model” according to which memory cells arise directly from T_N cells that never experienced a full-activation and differentiation toward an effector stage[9]. The two models show different predictions about the proliferation capacity, self-renewal and multipotency of effector and memory subsets. The “linear model” predicts that memory cells have proliferated at least the same as effector cells from which they are generated[9], although this is somehow confuted by the finding that the length of telomeres[10,11,12] and activity of telomerase[13] are both reduced in effector compared to memory cells[9]. Instead, these findings support the “developmental model” which predicts that memory cells proliferate less than effector cells during the acute phase. The predicted multipotency in the “linear model” is that effector cells are able to generate memory cells. On the other side, in the “developmental model” the effector cells are not expected to generate memory cells, as they are in a terminal developmental stage and are committed to die. In line with the “developmental model”, experiments of repeated Ag stimulation of T cells *in vitro*, showed an increased effector function but impaired memory formation[14]. Conversely, memory $CD8^+$ T cells were able to generate more differentiated effector cells while generating a pool of cells maintaining their original phenotype[15].

In support to the “linear model”, Rafi Ahmed and colleagues recently showed that memory $CD8^+$ T cells are generated from effector cells through a process of cellular de-differentiation. They investigated the epigenetic modifications (*i.e.* methylation associated with gene repression) of T_N and effector-associated genes in terminal effectors (TE) and memory precursors (MP) during viral infection *in vivo*. The methylation profile of T_N-associated genes and de-methylation of effector genes was

similar in MP and TE cells during infection. Longitudinal phenotypic and epigenetic analysis showed that at the end of infection, Ag-specific MP but not TE cells were able to de-methylate and re-express (both at the mRNA and protein level) T_N associated genes, thus suggesting that MP cells generate memory through a mechanism of T cell de-differentiation[16].

With the improvement of single cell technologies (*i.e.* single cell adoptive transfer and tracking, single-cell quantitative PCR, RNA and DNA sequencing), researchers were recently able to provide a deep characterization of the $CD8^+$ T comparment and its developmental potantial at the single cell level. Experiments based on the adoptive transfer of individually labeled T cells by the means of unique, inheritable DNA barcodes revealed the heterogeneity of T_N response during infection[9]. In particular, Gerlach et al. tracked the progeny of individual mouse $CD8^+$ T_N cells and demonstrated that, even for T cells expressing the same TCR, both differentiation capacity and clonal expansion are heterogeneous. Specifically, T_N cells that experienced massive proliferation during the primary response preferentially generated KLRG-1^+ effector cells, while those proliferating less preferentially differentiated into long-lived $CD62L^+CD27^+$ cells[17]. Similar findings were also confirmed by an independent group[18]. Using imaging techniques, Steven Reiner and colleagues reported that a T_N cell undergoing its first cell division during Ag response segregates unequal amounts of molecules that are involved in fate specification, such as the IFN-γ receptor, T-bet and others, to its daughter cells and subsequently demosntrated that the progeny has differential capacity to generate effector and memory cells[19]. All these findings are in support of the “developmental model” where differentiation and proliferation are tightly linked.

1.1.2 **The complexity of the T cell memory compartment**

The T cell compartment is not merely composed by naïve and memory cells, but it is highly heterogeneous in terms of phenotypic composition, functional activity and capability to respond to homeostatic or antigenic stimulation[20]. Using monoclonal antibodies (mAbs) and flow cytometry, which allow the identification of lymphocytes at the single cell level, the heterogeneity of this compartment has been revealed[21]. In 1999, Sallusto and Lanzavecchia identified two subsets on the basis of different migratory capability in tissues and organs: the central memory (T_{CM}) and the effector memory (T_{EM}) T cells[10]. The latter preferentially migrate to peripheral tissues and show immediacy of effector functions in response to Ag re-exposure[10]. On the other hand, T_{CM} migrate preferentially to secondary lymphoid organs, and show little immediate effector functions, although possess the capability to proliferate and differentiate rapidly upon Ag exposure[10]. Transitional memory cells (T_{TM}) cells are more differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells in terms of phenotype and expansion capability in response to IL-15 *in vivo*[22]. The most differentiated memory subset is represented by the terminal effector cells (T_{TE})[21]. These cells, which are more frequent in the $CD8^+$ than the $CD4^+$ population, possess potent effector functions but are poorly proliferative and tend to die rapidly after activation, compared to less differentiated memory cells[7,8,22].

Almost 10 years ago, studies in mice and later in humans and rhesus macaque identified a new T cell subset, less differentiated than the T_{CM} cells: the T stem cell memory, or T_{SCM} cells[15,23,24]. This population represent 2-3% of total circulating peripheral blood mononuclear cells (PBMCs). Like T_N cells, T_{SCM} maintain the capability to self-renew, to generate more differentiated subsets, to undergo massive proliferation and to infiltrate secondary lymphoid organs[15,23,24]. On the other

hand, like memory cells, T_{SCM} have undergone multiple cell divisions, as suggested by their lower content of T cell receptor excision circles (TREC, indicative of T cell divisions) compared to T_N cells, proliferate in response to IL-15 and rapidly produce effector cytokines such as IFN-γ, IL-2 and TNF-α after super-Ag stimulation[15]. Collectively, these data suggest that less differentiated T cells are able to generate more differentiated ones according to the relationship $T_N \rightarrow T_{SCM} \rightarrow T_{CM} \rightarrow T_{TM} \rightarrow T_{EM} \rightarrow T_{TE}$, where less differentiated cells are endowed with superior multipotent, self-renewal, proliferative capacities and anti-tumor abilities[24], while more differentiated cells preferentially infiltrate tissues and acquire rapidly cytotoxic and effector functions upon stimulation (**Figure 1**)[15,22,24].

1.1.3 **Markers to define T cell subsets**

T_N and memory subsets can be identified by the expression of a specific combination of surface and intracellular markers (**Figure 1**), as revealed by flow cytometry[21,23,25,26].T_N cells are characterized by the expression of different markers: the member of the tumor necrosis factor receptor CD27, the CC chemokine receptor-7 (CCR7) and the cell adhesion molecule L-selectin (CD62L), both necessary for the homing to secondary lymphoid organs, the long isoform of the tyrosine phosphatase CD45R (CD45RA), which is involved in T cell activation, the lymphocyte function-associated antigen-1 LFA-1 (CD11a) and LFA-3 (CD58)[21,22]. Moreover, T_N cells express the α-chain of IL-7 Receptor (IL-7Rα or CD127), which has important implication for T_N homeostasis by mediating their survival[21,22].

As regards the memory compartment, T_{SCM} maintain a phenotype similar to T_N cells ($CCR7^+$, $CD27^+$, $CD45RA^+$, $CD62L^+$, $CD127^+$), however they upregulate the death receptor CD95 and the β-chain of the Interleukin (IL)-2/IL-15 receptor complex

(CD122), which are expressed by memory cells[21]. Similarly, T_{CM} maintain the expression of naïve markers CD62L, CD127, CCR7 and CD27, but are $CD45RA^-$ while upregulate the CD45R shortest isoform, CD45RO[8,20,22,27]. Human T_{EM} downregulate CCR7, are heterogeneous for CD62L and upregulate receptors necessary for the homing to inflamed tissues like CCR3, CCR5 and C-X-C Motif Chemokine Receptor-6 (CXCR6). CCR3, CCR5 and CXCR6 are largely expressed by T_{CM}, T_{TM} and T_{EM} but not by T_N and T_{SCM}[8,22,27]. Like T_{CM}, T_{EM} retain the expression of CD45RO, CD95 and CD122[21]. T_{TE} cells acquire CD45RA expression, however they are distinguished from T_N cells by the absence of CCR7 and CD62L[22]. Moreover, T_{TE} cells express exhaustion and senescence markers like the marker of terminal differentiation CD57, the killer cell lectin-like receptor -1 (KLRG-1) and display phosphorylation of histone H2AX (γH2AX), indicative of DNA damage[8,27].

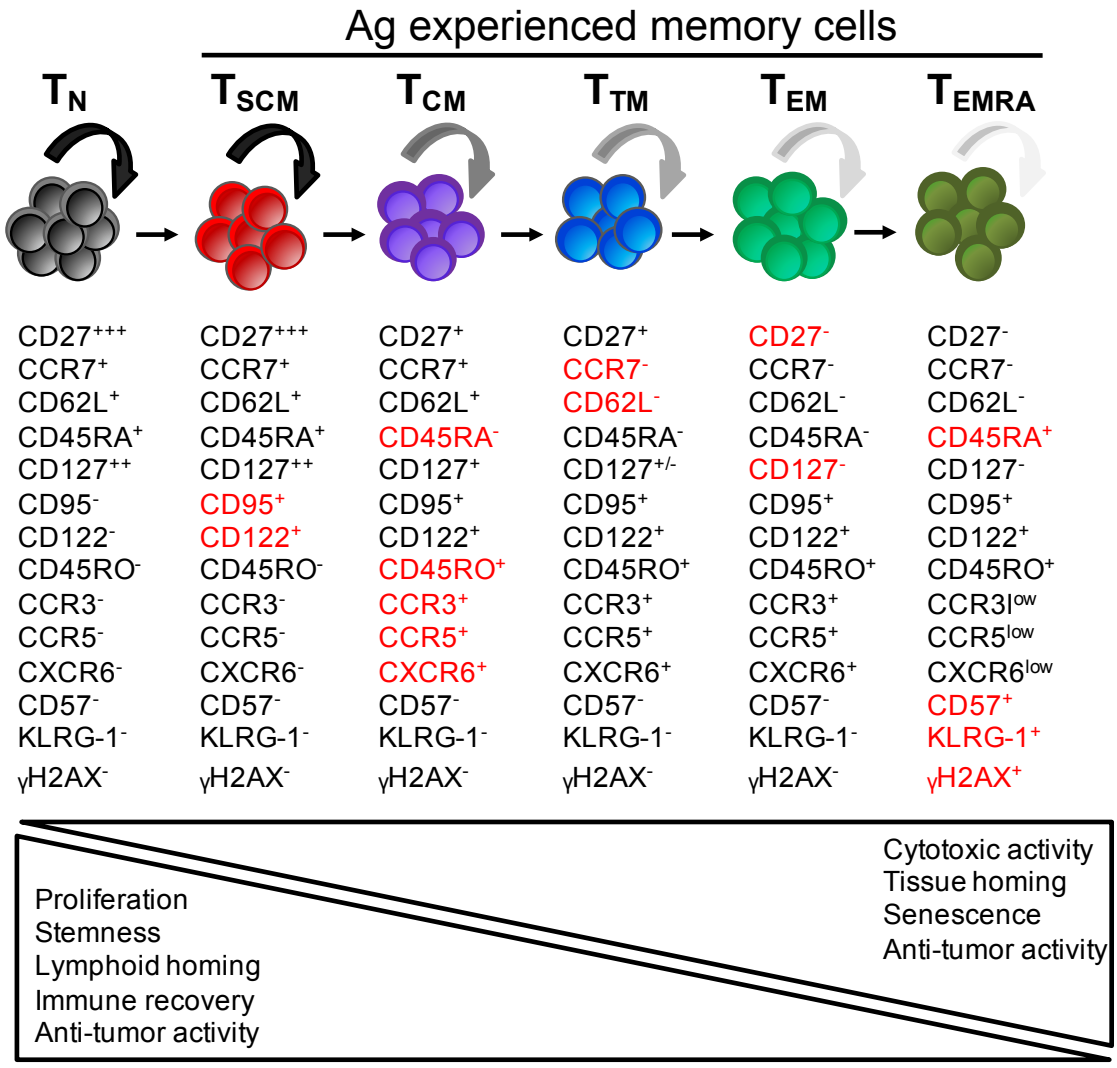

Figure 1. T cell differentiation process, functions and phenotype of T cells. Ploychromatic flow cytometry analysis allows the identification of T cells at the single cell level and permits the identification of different T cell subsets on the basis of specific combination of markers. In red, the markers that change during differentiation from the previous subsets. Going from less differentiated T cells to more differentiated ones, functional abilities also change.

1.1.4 **Homeostasis of human T cells**

The number of lymphocytes in the body is tightly controlled by homeostatic mechanisms that is the slowly turnover in the absence of Ag stimulation[28,29]. The maintenance of T cell numbers involves both common gamma chain (γc) cytokines (IL-2, IL-4, especially IL-7 and IL-15) and contact with self-pMHC expressed by APCs[30]. Sprent and coworkers established that under steady-state conditions, memory T cells turnover at a relatively higher rate compared to T_N cells[31,32]. On the

other hand, homeostatic proliferation of T_N cells is more dependent on TCR-self pMHC interaction[30]. Indeed, they survive less than memory cells if deprived of TCR-self pMHC contact[33 34].

An important role in homeostatic proliferation is held by common γc cytokines[33-35]. Both T_N and memory cells need common γc cytokines signals to undergo homeostatic proliferation[34]. It is now well known that T_N cells are more dependent on IL-7 to self-renew compared to more differentiated cells[34]. IL-7 is produced in the bone marrow, thymus, secondary lymphoid tissues, liver and intestine by non-hematopoietic stromal and epithelial cells[36]. The production of IL-7 is not dependent from external stimuli and is stable[36]. T cells respond to IL-7 via IL-7R[36]. This receptor is composed of 2 subunits: the common γ-chain CD132 (shared with IL-2, IL-4, IL-9, IL-15 receptors), and the α-chain (CD127), which confers cytokine specificity[36]. CD127 is expressed by all T cell subsets and is downregulated in T_{TE} cells[33]. The essential role of IL-7 was discovered several years ago by the finding that blocking contact with IL-7, either by blocking IL-7R in normal mice or by adoptive transfer of T cells into IL-7 deficient mice, T_N cells were not able to survive or to persist in the host[36]. At low levels, IL-7 activates the downstream IL-7R pathway[33]. Binding of IL-7 to IL-7R induces the activation of Janus Kinase 1 (Jak1) and Janus Kinase 2 (Jak2), which are bound to CD127 and CD132, respectively[36]. This binding recruits the Signal Transducer and Activator of Transcription 5a/b (STAT5a/b), which migrates to the nucleus and upregulates the transcription of the antiapoptotic molecule Bcl-2[36]. In lymphopenic conditions, the concentration of IL-7 increases because of the reduced number of T cells[34]. The increased amount of IL-7 amplifies the weak signal of TCR-self pMHC resulting in a gradual differentiation

towards memory cells[37]. However, the presence of memory cells reduces this mechanism, as they compete with T_N cells for IL-7 signals[30,32].

Other cytokines are involved in T cell proliferation during lymphopenia, in particular IL-2 and IL-15. These cytokines are structurally and functionally related and share two of their receptor chains, the IL-2/15Rβ (CD122) and the common γc CD132. IL-15 is produced primarily by DCs, monocytes and epithelial cells in the presence of inflammatory signals[38]. Initially IL-15 was identified as a soluble molecule. Later it was shown that IL-15 also exists as a membrane-bound form complexed to the α-chain of its receptor (IL-15Rα) with high affinity. The membrane-bound form, which is the prevalent form *in vivo*, binds target cells through the so-called trans-presentation[38]. Trans-presentation is mediated by the membrane-bound complex IL-15/IL-15Rα. If non-complexed with IL-15Rα, IL-15 binds to IL-15Rβγ complex with lower affinity[38], inducing activation of Src family molecules including Lymphocyte Cell-Specific Protein-Tyrosine Kinase (Lck) and Proto-oncogene Tyrosine-protein Kinase Fyn. These molecules subsequently induce Phosphoinositide 3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) signaling pathway[39].

Because IL-2 and IL-15 share receptor subunits (CD122, CD132), both these cytokines activate similar downstream pathways including activation of MAPK, the induction of Bcl-2, the phosphorylation of Lck and spleen tyrosine kinase (Syk)[39]. All these signals lead to maturation and proliferation of T cells[39]. IL-2Rα (CD25) and CD122 are progressively upregulated with T cell differentiation[10,27] thus suggesting an important role of IL-15 in the homeostasis of memory, but not T_N cells. The importance of IL-15 was demonstrated in studies where memory cells were transferred in IL-15 deficient mice. In these hosts, memory cells failed to undergo

homeostatic proliferation and died rapidly[20]. Similar results were obtained in mice deficient in one of three chains of IL-15R and IL-2R: IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132)[40,41]. In these lymphopenic hosts donor T_N cells proliferated and differentiated rapidly into memory and effector cells. Importantly, it was observed that IL-2 induced the generation of effector cells, while IL-15 sustained memory differentiation[40,41]. To determine the role of γc-cytokines in T cell homeostasis, Geginat and coworkers investigated the acquisition of functional and phenotypic features of purified T_N,T_{SCM}, T_{CM}, and T_{EM} subsets isolated from human PBMCs[20]. Purified T cell subsets were activated *in vitro* via cytokine and antigenic stimulation. Cytokine stimulation was provided by IL-2, IL-7 and IL-15, while Ag activation was provided by mature DCs[20]. In the absence of antigenic stimulation, the response to IL-7 and IL-15 was low in all subsets, but proliferation increased when IL-2 was added to the cytokine cocktail[20]. Concerning the antigenic stimulation, the authors observed that T_N activation was more dependent on costimulation compared to more differentiated T cells. Moreover, after T cell activation the capability to produce IFN-γ increased over T cell differentiation, while less differentiated T_N, T_{SCM} and T_{CM} cells produced mainly IL-2[10,20]. Finally, they observed that all subsets generated $CCR7^-CD45RA^-$ effector cells after Ag stimulation, while a fraction maintained the original phenotype. Similarly, T_{SCM} cells were capable to generate more differentiated T_{CM} and T_{EM} upon TCR triggering, while 60% of them maintained the original phenotype. Conversely, only 30% of stimulated T_{CM} were able to self-renew and they could only to differentiate into T_{EM} subsets, thus indicating that at least *in vitro*, “de-differentiation” does not occur[15].

1.1.5 **Hematopoietic stem cell transplantation (HSCT)**

HSCT following either marrow-ablative or non-myeloablative condition regimen is widely used in the clinic for the treatment of multiple diseases, including lymphoma, acute and chronic leukemias, multiple myeloma, myelodysplastic syndromes, myeloproliferative disorders and autoimmune diseases. Several clinical protocols have been developed over the years, and they differ in terms of:

- stem cells source (*e.g.*, bone marrow, umbilical cord blood, peripheral blood G-CSF mobilized stem cells);
- type of transplant: autologous (transplanted cells derive from the patient), allogeneic (cells are isolated from a third-party donor), haploidentical (donor and patient share half of their HLA haplotype system) and HLA-identical;
- type of conditioning regimen, classified on the basis of the duration of cytopenia: myeloablative conditioning (MA), causing irreversible cytopenia and in the need of stem cell (SC) support; reduced-intensity conditioning (RIC), causing cytopenia for a variable duration and for which SC support is useful, and non myeloablative conditioning (NMA), causing minimal cytopenia and for which SC support is not necessary[42];
- depletion of T cells from donor graft in order to eliminate allo-reactive T cells. This approach is used to avoid the activation of donor T cells against recipient's tissues that are recognized as non-self, a reaction collectively referred to as graft-versus-host-disease (GVHD), characterized by selective damage to the skin, liver, gastrointestinal tract and mucosa of patients[43]. The severity of GVHD is correlated to HLA mismatch between the donor and the recipient, and is defined as

acute (aGVHD) when observed in the first 100 days after BMT[44], otherwise it is defined as chronic (cGVHD)[45]. *Ex vivo* T cell depletion, may be employed to prevent GVHD without the morbidity associated with immunosuppressive drugs[46,47]. This procedure reduces significantly the incidence and severity of GVHD, but does not improve overall survival. Moreover, donor T cells also play an important role in the prevention of infections and mediate malignancy eradication (graft-versus-tumor effect)[43]. Thus, the absence of donor T cells in the graft is associated with increased risk of serious infections, relapse and death due to prolonged immune deficiency in the recipients[43]. For these reasons, other methods are required to selectively inhibit alloreactivity, while preserving immunity against pathogens and tumor cells.

1.1.6 **Unmanipulated HLA haplo-BMT with high dose cyclophosphamide**

Generally, the employment of HLA-identical relatives for allogeneic transplantation represent the best choice for HSCT or BMT. However, donor availability represents a major problem as only one-third of candidates for allogeneic BMT (allo-BMT) have HLA-matched donors[45]. To increase the number of potential donors for almost all patients, the group of Leo Luznik at the John's Hopkins University in Baltimore, USA, developed an HLA-haploidentical BMT with high dose, post-transplant cyclophosphamide (Cy), given eraly after bone marrow infusion[48-50]. Cy, an alkylating agent employed since 1960s to modulate allograft responses[51], selectively targets proliferating cells and induces apoptosis. In the contest of haploidentical transplantation, both donor and recipient cells proliferate quickly in response to allo-Ags, thus becoming susceptible to the effect of Cy[51]. Several

studies performed in mice showed that the administration of Cy early after allo transplantation prevents GVHD, thus increasing overall survival[44]. The administration of Cy early after transplant allows the elimination of allogeneic T cells, while non-allogeneic T cells are spared, respond to increased concentrations of homeostatic cytokines, mainly IL-2, IL-15 and IL-7, inflammatory cytokines (induced by pre-transplant conditioning) and exogenous Ags, thereby contributing to immune reconstitution[48-50].

1.1.7 **T cell recovery after allogeneic stem cell transplantation**

Immunodeficiency after stem cell transplantation represents a crucial and inevitable condition for patients[48]. The chemotherapy, the intensity of transplant preparative regimen, the age of patients, the occurrence of aGVHD, the onset of CMV and opportunistic infections and the administration of immune-suppressive drugs all affect the recovery and function of T cells[52]. After transplantation, T cell numbers can be restored via two different pathways: peripheral expansion of residual oligoclonal T cells in response to Ags, allo-Ags or cytokines and generation of new T_N cells from progenitors via thymopoiesis[53]. The former occurs early but can last up to 1 year after transplant due to the increased availability of cytokines (mainly IL-7 and IL-15), exogenous Ags or allo-Ags which induce the rapid expansion of the T cell pool[52], while the latter occurs several months after transplantation and it is characterized by the generation of new T_N cells. This “thymic dependent” pathway is capable to ideally restore a polyclonal T cell receptor repertoire[52]. The thymus is most productive in the first 6 months of life, but remains active during the first 20 years of life. In adults and in elderly the thymic output declines progressively, hence resulting in a reduction of T cell repertoire and

oligoclonal expansion of T cells[52]. For this reason, young individuals undergoing BMT are generally characterized by better T cell recovery compared to older individuals. In this regard, multiple strategies have been conceived to independently improve the functionality of thymus, such as by altering the numbers of infused T progenitors, the intensity of conditioning regimen or administration of immune-suppressive drugs [52]. A clear understanding of the molecular mechanisms that govern the generation, the functionality and maintenance of T cells are therefore important to predict the immune-recovery after transplantation.

1.2 Immunotherapy as alternative/additional approaches to HSCT and BMT

Immunotherapy approaches based on vaccines, drugs or autologous T cell sources represent a powerful alternative for patients with large-state haematological malignancies, solid tumors and refractory diseases[54]. These approaches have several advantages compared to traditional therapies based on radiotherapy, chemotherapy and transplantation, as they are in general more specific for the cancer and, in the case of vaccines, have little or no side effects after immunization, high specificity and good safety profiles[55]. In this thesis, anti-cancer vaccines will not be discussed in detail. Despite a major effort of scientific research in the past two decades, induction of anti-tumor immunity by vaccination led to poor clinical results in terms of overall response and prolonged survival. The causes of such failure have to be ascribed to multiple reasons, including the difficulty in identifying cancer-specific antigens and the poor immunogenicity of such antigens, among others. Below, I discuss a series of different immunotherapy approaches based on immune checkpoint inhibitors, tumor-specific neoantigens, adoptive cell transfer of *ex vivo*

expanded TILs, T cells expanded by Ag-pulsed APCs and T cells modified with Ags specific T cell receptors (TCR) or chimeric antigen receptors (CARs). These are only a minority of the enormous efforts made by scientists and clinicians to explore the complexity of cancer immunotherapy.

1.2.1 **Immune checkpoint inhibitors**

Humanized monoclonal antibodies (mAbs) directed to antigens expressed on tumor cells have now been employed in the clinic for nearly 20 years thanks to their potent anti-tumor effect. Initially, the use of mAbs was restricted to treat haematological malignancies, but recent developments on the so-called “immune checkpoint inhibitors” allow to treat solid tumors as well[56]. Immune checkpoints molecules are expressed on the surface of tumors or immune effector cells and their role is to counterbalance the positive effect of costimulation. Thus, these inhibitory molecules are required to achieve an appropriate immunological response[57]. In the absence of costimulation, activation of T cells is blunted and generates immune tolerance[57]. In this regard, immune checkpoint molecules limit effector responses, thus preventing harmful and unwanted self-directed activities[58]. Usually, tumor cells are capable to overexpress inhibitory molecules such as the cytotoxic T lymphocyte activation antigen 4 (CTLA-4) and the programmed death-1 (PD-1) receptor or ligand (PD-L1) which inhibit T cell activation and function[55,59]. Overcoming these mechanisms of peripheral tolerance by mAbs mediate tumor regression by unleashing anti-tumor immunity, that is mostly exerted by effector $CD4^+$ and $CD8^+$ T cells[58]. The anti-tumor activity of CTLA-4 inhibition have been investigated in numerous murine tumor models, such as renal cell carcinoma, prostate carcinoma and lymphoma[60]. In less immunogenic mice models such as mammary carcinoma

SM1 or melanoma B16, anti-CTLA-4 therapy did not demonstrate efficacy in tumor eradication. However, the combination of anti-CTLA-4 with other therapies such chemotherapy, radiation and a variety of vaccines with tumor antigens increased anti-tumor activity[61]. A similar study reported that treatment of metastatic melanoma patients with anti-CTLA4 mAb Ipilimumab improved overall survival, and demonstrated for the first time that the immune system can reject solid tumors[62,61]. Subsequent studies focused the attention on the PD-1/PD-L1 molecules, both overexpressed by tumor infiltrating T cells and tumor cells (*e.g.*, breast, kidney, lung ovary, colon as well as non epithelial tumors such as melanoma, T cell lymphoma, multiple myeloma and various types of lymphomas)[63]. Mice tumor models of multiple myeloma, melanoma and mammary carcinoma showed that antibody-mediated blockade of PD-L1 promoted cancer regression[60]. Recent clinical trials using anti-PD1[61] or anti- PD-L1[64,61] showed an enhanced T cell response in patients with late-stage melanoma[61]. Anti-PD-1 is particularly effective in refractory or relapsed lymphomas[65]. However, despite reaching complete remission, patients are treated with allogeneic BMT to increase chances of cure. Immune checkpoint blockade is currently being tested in multiple cancer types. Despite significant clinical gains are observed in the setting of treatment with these molecules, the responses to this form of therapy are not effective in all cancer types, especially those with low mutational burden and/or low immunogenicity. In order to increase the anti-tumor activity of immune cells, additional tumor-specific molecules called neoantigens have been exploited.

1.2.2 **Neoantigens**

In the last years a novel class of cancer-targets have been studied: the so called cancer neoantigens. These molecules represent a class of antigens derived from somatic DNA mutations (*e.g.* nonsynonymous point mutations, insertion-deletions, gene fusion and frameshift mutations) in cancer cells[66]. Thus, neoantigens are considered important targets for immunotherapy approaches because they are tumor-specific and lack of expression in normal tissues. Advances in next generation sequencing (NGS) technologies enabled the identifications of candidate neoantigens with relatively high accuracy . It is important to note, however, that the vast majority of mutations are not translated (*i.e.* nonsense mutations, non coding mutations)[66]. Instead, those mutated proteins that are translated must be processed into short peptide fragments and complexed with MHC-I/MHC-II molecules prior to the presentation on the cell-surface[66]. A number of computational tools have been developed in order to predict the structure, the binding affinity, the proteasomal processing and intracellular transportation of these hypothetical neoantigens[66]. Recent studies have demonstrated that approaches based on neoantigen vaccines are able to induce a robust anti-tumor activity in mice. Among these, Castel *et al.*[67] vaccinated the B16F10 melanoma mice with 50 different predicted neoantigens. 16 of candidate neoantigens were immunogenic as assessed by IFN-γ and ELISPOT-assay, and 2 of them induced a marked anti-tumor activity *in vivo*. The identification of neoantigen-specific $CD8^+$ and $CD4^+$ T cells in TILs from melanoma patients and promising results from pre-clinical studies have induced a great interest in the generation of neoantigen-based vaccines. In a clinical trial performed by Ott *et al.*, patients were vaccinated with neoantigens identified through NGS data from cancer and normal cells. The authors showed no disease recurrence in four of six

vaccinated melanoma patients at 20-30 month after surgical resection of the tumor. The remaining two patients achieved a complete remission after treatment with anti-PD-1 antibody[69]. Similarly, Sahin *et al.* created a synthetic RNA-based vaccine encoding for neoantigens. Such RNA molecules were previously demonstrated to be taken up by DCs resident in lymph-nodes[70]. 8 of 13 vaccinated melanoma patients showed a complete tumor regression after vaccination during the entire follow-up period. The other 5 patients had cancer relapse. However, after anti-PD-1 treatment, cancer regression was observed in one of these patients[70]. These works indicates that neoantigen-specific anti-tumor response occurs spontaneously in cancer patients and that neoantigens have the potential to be employed in vaccines to increase this pre-existing anti-tumor immunity.

1.2.3 **Adoptive cell transfer**

An alternative, effective immunotherapy approach based on the use of immune system to mediate tumor regression relies on adoptive T cell transfer (ACT), where anti-tumor T cells, either isolated from the patient or genetically-modified to confer tumor specificity, are activated and expanded *in vitro* (to increase their numbers) prior to infusion in the patient. The first ACT-based immunotherapy was used in the 1980s, using lymphokine-activated killer (LAK) cells to treat tumors in mice models and humans[71]. Later, cytokine-induced killer (CIK) cells isolated and expanded from patient's PBMCs were tested[55]. However, the low specificity of CIK and LAK-based therapies showed a limited efficacy[55]. Thus, more specific ACT-based methods were developed. One relies on the isolation, activation, expansion and re-infusion of tumor-infiltrating lymphocytes (TILs)[55]. The first TIL-based therapy was performed by Rosenberg *et al.* in 1988 for the treatment of melanoma patients[72]. In this trial, T

cells were isolated from patient's tumor tissues, were expanded *in vitro* for two weeks using high-dose IL-2 (6000 U/mL) and then infused back into patients[73]. The treatment showed a significant objective response (cancer regression up to 29% of treated patients)[73]. However, the tumor regression was transient due to the low capability of T cells to persist in the host. More recently, clinical trials using a lymphodepleting preparative regimen followed by TIL infusion along with high-dose IL-2 showed an increase of anticancer responses (ranging from 49% to 72%) in melanoma patients[55,74,75]. It is thought that lymphodepletion eliminates immunosuppressive cells, in particular $CD4^+$ regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and autologous lymphocytes which compete for IL-7 and IL-15. The depletion of these cells results in the amplification of anti-tumor activity and persistence of infused TILs[76]. Despite the positive clinical outcome in melanoma patients, TIL-based therapies show some limitations. First, transferred T cells do not persist in the long term, thus limiting the anti-tumor effect. This is due to the initial quality of the isolated T cells, that are terminally-differentiated in origin. Moreover, IL-2, that is used for expansion, primes T cells for apoptosis. Second, TILs are generally specific for tumor-associated antigens that are also shared with normal cells, therefore ACT often results in autoimmune reactions[77].

Strategies are being developed in order to increase the persistence and the capability of T cells to specifically recognize and eliminate cancer cells. These methods include: T cells expanded by Ag-pulsed APCs and T cells modified with Ag-specific T cell receptors (TCR) or chimeric antigen receptors (CAR)[55]. In regard to the first method, T cells enriched from TILs or whole PBMCs are exposed to APCs previously pulsed with peptides derived from cancer cells, so that only tumor-specific T cells are able to expand[78,79]. This method can ideally be employed in

nearly all patients as cancer-specific T cells can be obtained by stimulating autologous PBMCs. However, as mentioned above, the vast majority of such tumor Ags are generally self Ags expressed by normal cells, hence capable to trigger autoimmune reactions. Clinical trials using MelanA specific CD8 T cells showed tumor regression in 8 of 10 patients with refractory metastatic disease[80]. In a more recently clinical trial 11 patients with metastatic melanoma were treated with Cy as conditioning regimen, before the infusion of Ag-specific T cell additioned with low-dose IL-2. This study showed that 5 of 11 patients had a stable disease, while 1 patient had complete remission that lasted for 3 years after treatment[81].

The identification of tumor-specific peptides/epitopes has been a major effort of scientific research for decades, and has been recently exploited in the use of ACT where T cells are modified with antigen-specific TCRs or CARs. In this regard, autologous or third-party T cells are genetically modified in order to redirect T cells to recognize and eliminate cencer cells[82]. TCR-transduced T cells specific for Ags including cancer-testis antigen NY-ESO-1, Glycoprotein-100 (gp100), Carcinoembryonic antigen (CEA), Melanoma Antigen Recognized By T-Cells 1 (MART-1) and Melanoma-Associated Antigen 3 (MAGEA3) have been tested in several clinical trials[82]. The first clinical trial using autologous T cells genetically modified to express the MART-1 TCR was reported by Rosenberg *et al.* in 2002 at the NIH[83]. In this study, melanoma patients showed cancer regression without autoimmune activity. Conversely, subsequently studies using MART-1-specific T cells with higher affinity for the Ag had little tumor regression and increased side effects such as autoimmune toxicity, as these cells mediated keratinocyte destruction. Immunotherapy approaches using engineered-TCR represent a promising strategy for anti-cancer therapy as anti-tumor activity is enhanced in the

presence of high affinity-TCR. However, clinical trials suggest that TCR with intermediate affinity are preferable, as high affinity T cells may induce severe autoimmunity disease.

CARs are a valuable alternative to TCRs. CARs are hybrid receptors where the extracellular portion is a hybrid light chain immunoglobulin, bound to a spacer element, a transmembrane domain and an intracellular domain involved in T cell activation pathways[73]. CAR expression allows for redirection of T cell specificity towards a tumor-antigen independent of the MHC[84]. CARs are generally classified into three generations according to the number of signaling domains[77]. In the first-generation, CARs contain the CD3ξ or the FC-γ receptor domain. In the second and third generation, these receptors have additional costimulatory molecules such as CD28, CD27, Inducible T-Cell Costimulator (ICOS), 4-1BB (CD137), OX-40 (CD134), which have been shown to improve T cell persistence, anti-tumor activity and proliferation, compared to first generation CARs[54,77]. Manufacturing of CAR T cells is a complex method involving multiple steps including: apheresis collection of T cells from peripheral blood of patient, T cell engineering via a CAR-expressing lentiviral or retroviral vector and expansion *in vitro* prior to infusion[77]. However, CARs have several advantages over TCRs. First, CARs are not MHC restricted, therefore they can be ideally employed in any patient. Second, tumor activity is not affected by tumor-escape mechanisms that are generally observed with the use of TCRs, such as HLA downregulation. Third, CARs could ideally target any protein, supposing antibodies against that protein have been generated[77]. The most promising results from CAR-based therapy was shown in patients affected by B cell malignancies treated with anti-CD19 CAR T cells in 2003[85-88]. The authors showed that CD19 specific CAR T cells expanded and persisted following infusion, allowing

both tumor eradication and long-term surveillance[85-88]. CAR-T19 products were recently approved by the FDA in 2017 for the treatment of pediatric, young and adult relapsed/refractory B-cell ALL. Unfortunately, the vast majority of CAR-based therapies showed limited efficacy with serious side effects[89]. CAR-T cell based immunotherapy can result in unwanted toxicity in different ways. First, the infusion of large numbers of activated lymphocytes which recognize their target on tumor cells can induce the release of a high amount of pro-inflammatory cytokines (IL-6, IL-10, GM-CSF, TNF-α, IFN-γ), thus generating a so-called "cytokine storm"[90]. This side effect can be controlled using steroids which in turn may limit the efficacy of CAR treatment[91]. Second, CARs can recognize molecules which are expressed by both normal and cancer cells, or they can cross-react with Ags expressed by normal cells. In both cases, CAR treatment can result in on-target off-tumor toxicity[90].

Despite different targets are currently being explored both at the preclinical and clinical level, CAR T cell therapy is still far from being used for the majorit of cancers. This is due to the lack of ideal cancer Ags, the short term persistence of T cells, the inefficient trafficking of T cells in tumor sites and presence of an immunosuppressive environment[90]. The limitations of CAR-based therapy are currently being studied. Recently, Yang *et al*. showed that the stimulation of Ag-specific TCR expressed by CAR-T cells impairs the ability of CAR-CD8 (CAR8) to eliminate leukemic cells *in vivo*[84]. The authors generated CAR-CD4 (CAR4) and CAR8 cells with defined endogenous TCR specificity for male minor histocompatibility antigen HY. They transferred HY-specific CAR4 and CAR8 cells from female donors into leukemic-bearing male (HY^+) and female (HY^-) mice and observed that despite HY-specific CAR4 T cells were able to eradicate tumor, when infused in both male and female mice, HY-specific CAR8 cells were not able to

eliminate leukemic cells, when infused in male mice. The simultaneous activation of TCR and CAR completely abolished the capacity of CAR8 cells to infiltrate the bone marrow. Moreover, these cells were more prone to apoptosis and showed an exhausted phenotype which is associated to a poor effectiveness of immunotherapy[84]. These observations shed light on the complex biological aspects of CAR T cell immune biology, indicating the necessity to rationally design CAR constructs in order to improve clinical efficacy.

1.2.4 **T cell differentiation status and ACT**

All the ACT based immunotherapies described previously show critical points which are poor expansion, low anti-tumor activity and low persistence of infused T cells due to the extended *ex vivo* manipulation. As described before, different T cell subsets are endowed with different capabilities to infiltrate inflamed tissues, to proliferate and to differentiate into potent effectors. In the scenario of a tumor, more differentiated $CD62L^-$ memory T cells would be the preferred cells for ACT[92]. However, clinical data clearly indicate that the infusion of less differentiated cells correlates with a better clinical outcome. Several experiments were conducted in order to determine the correlation between differentiation status of T cells and anti-tumor immune response[92]. In mice models of solid tumors it was demonstrated that T_{CM} were expanded, persisted and eradicated tumors more efficiently than infused T_{EM}[93]. According to these data, experiments conducted in immunodeficient mice and non-human primates showed that T_{Eff} originated from T_{CM} persisted better than T_{EM}-derived T_{Eff} following ACT *in vivo*[94,95].

To date, the least differentiated memory T cells identified in humans, *i.e.* the T_{SCM} mentioned in paragraph 1.1.1, are the preferred T cell subset to be used in ACT,

owing to their enhanced self-renewal and their capability to simultaneously derive potent, more differentiated effectors[15,24,96]. However, their paucity in the peripheral blood and at the tumor site limits their clinical use. For this reason, methods have been proposed to generate and expand the pool of existing T_{SCM} from less differentiated naïve precursors[15,23,37,97,98].

1.2.5 **Methods to generate T_N-derived T_{SCM} *in vitro***

Immunotherapy approaches used in clinical trials largely employ TCR or CAR-modified T cells derived from TILs or whole PBMCs. These methods simplify the manufacturing process that is, however, not standardized among patients as the PBMCs composition largely vary among individuals as a consequence of Ags exposure[99], age [100] and systemic treatments[101]. Moreover, more differentiated subsets such as T_{EM} and T_{TE} , which are abundant in PBMCs (especially in the $CD8^+$ population), are not able to generate viable T cell products *in vitro*[102]*.* Rather, they could inhibit the expansion and function of less differentiated T cells with enhanced capacity[103].

Effector and memory T cell differentiation is a complex process that involves the interaction of different molecules and pathways. Intense investigation over the past two decades led to the identification of the major molecular mechanisms that are involved in the generation of short-lived effectors and long-lived memory T cells. Memory T cell differentiation can be regulated at different levels, which in turn can result in the differential activation of signalling molecules, pathways and transcription factors (TFs). Schematically, 3 major levels of regulation can be identified:

- At the TCR signalling level, *e.g.*, strength of the signal and type of costimulation;
- At the intracellular level, *e.g.* TFs, signalling and metabolic pathways;
- At the environmental (extracellular) level, *e.g.* cytokines, type of APC, presence of suppressive cell types, molecules, metabolites, etc.

These regulatory checkpoints have been exploited in the recent years with the final aim to arrest T cell differentiation while promoting long-term memory development, an important correlation of functionality in ACT immunotherapy of cancer. I will not discuss the molecular mechanisms of memory T cell differentiation in detail, but will focus on those pathways whose manipulation has been show to induce T_{SCM} cells.

Following peptide recognition by the TCR, signal transduction is integrated at the level of PI3K/serine-threonine protein kinase (Akt) signalling pathway, which contributes to orchestrate downstream effector molecules and transcription factors such as Mammalian Target Of Rapamycin (mTOR), Nuclear Factor Kappa B (NF-κB), JAK/ STAT and Forkhead Box O (FOXOs) transcription factor familiy. Since the amplitude of Akt stimulation correlates with effector cell formation, Kim *et al.* investigated the Akt blockade to increase $CD8^+$ T cell memory formation[104]. Treatment of mice with pan-Akt inhibitor A-443654 reduced mTOR function during the expansion phase after infection and correlated with increased memory formation[104]. Along this line, human $CD8^+$ T cell activation in the presence of Akt inhibitors allowed T cell proliferation while inhibiting differentiation, increased persistence following ACT in immunodeficient mice and improved anti-tumor responses in a xenogeneic model of myeloma[105] and melanoma[106].

Similarly, the Wnt/β-catenin signalling pathway has been shown to have a role in the generation and maintenance of $CD8^+$ memory T cells[107]. In particular, studies involving constitutive expression or loss of function of β-catenin suggested that the survival and maintenance of memory $CD8^+$ T cells are Wnt/β-catenin dependent. Studies by Restifo group showed that augmented Wnt signalling induced by glycogen synthase kinase-3β (GSK3β) inhibition favoured the generation of $CD8^+$ T_{SCM} from T_N precursors both in mice[54] and humans[15] and showed increased anti-tumor capability compared to memory T cells upon adoptive transfer. However, it is worth noting that GSK3β inhibition also impaired T cell expansion and decreased cell viability[15,108]. To improve the protocol, cytokines such as IL-7 and IL-21 were add to the stimulation cocktail. These cells expanded by ~6-fold and generated naturally-occurring T_{SCM}[97]. *In vitro* generated human T_{SCM} resulted transcriptomically, functionally and phenotypically similar to naturally occurring T_{SCM}[97]. After activation, these cells were genetically modified to express a CD19-CAR and were shown to possess potent anti-leukemic functions in immunodeficient mice[97].

Similarly, Cieri *et al*. stimulated $CD62L^+CD45RA^+$ T_N cells with IL-7 and IL-15 combined with strong αCD3/28 stimulation (3 beads:1 cell). The induced T_{SCM}-like cells displayed a hybrid $CD45RO^+CD45RA^+$ phenotype that is rarely found *in vivo*[37]. Nevertheless, the cells expanded ~50-fold *in vitro* and persisted longer than T_{CM} and T_{EM} when infused in xenogeneic models compared to T_{CM} and T_{EM}[37].

Recently, it has been shown that epigenetic manipulation of T cell memory subsets via histone modification or DNA methylation influences T cell differentiation[109,110]. In particular, Kagoya *et al.* showed that $CD3^+$ T cells activated with APCs expressing αCD3 mAb, CD80 and CD83 in the presence of JQ1, a

specific inhibitor of extra-terminal motif (BET) proteins and bromodomain, supported *in vitro* expansion of T_{SCM} and T_{CM} cells by suppressing Basic Leucine Zipper ATF-Like Transcription Factor (BATF), an important regulator of $CD8^+$ T cell effector differentiation[111]. These cells maintained functional and transcriptional properties of naturally occurring T_{SCM} and T_{CM}, respectively. When infused in NSG mice, JQ1 treated cells were able to expand, to infiltrate tissues and to reduce tumor burden significantly more than cells grown in the absence of JQ1[111].

Collectively, these reports show that it is possible to generate T_{SCM} *in vitro* starting from whole PBMCs or naïve precursors. However, all these protocols have limitations. The identification of the molecular signals that are required for the differentiation of T_{SCM} cells may have important practical implications for improved protocols of ACT.

2 AIM OF THE STUDY

Haplo-BMT combined with pt-Cy is changing the perspective of allogeneic transplantation because it allows the identification of suitable donors for patients who lack an HLA-matched donor. The mechanisms of T cell recovery following this type of successful transplant remain poorly understood. Moreover, it is not clear whether pt-Cy spares non-alloreactive T cells and thus favors immune reconstitution. The first aim of this work was the identification of the biological mechanisms that govern human T cell recovery after haplo-BMT followed by high doses pt-Cy.

We found that T_{SCM} cells with increased immune reconstitution capacity were the predominant T cell subet during the early phase of transplantation and demonstrated that these cells contribute to the reconstitution of the host by generating more differentiated effector cells. As the adoptive transfer of large numbers of these cells would be beneficial for immunodeficient patients, including HSCT patients, I focused the second part of my work on elucidating the molecular mechanisms at the basis of T_{SCM} generation in humans. We focused on the extrinsic signals and on the potency of TCR stimulation that are required to block T cell differentiation of T_N cells while allowing proliferation.

3 MATERIALS AND METHODS

3.1 Cells

PBMCs isolated from buffy coats and from patient's peripheral blood were stored in liquid nitrogen according to standard procedures.

3.2 Patients and transplantation procedures

Clinical and experimental protocol were approved by the IRB of Humanitas Research Hospital (Prot. Nr Humanitas 222/14) and Istituto Nazionale Tumori and both patients and donors signed consent forms in accordance with Declaration of Helsinki. A total of 51 patients were enrolled for haplo-BMT and treated with the protocol established by Luznik *et al.*[48] as shown in **Figure 2**. All experiments were performed using randomly selected patients, unless specified (*i.e.* Ag-specific stimulation experiments).

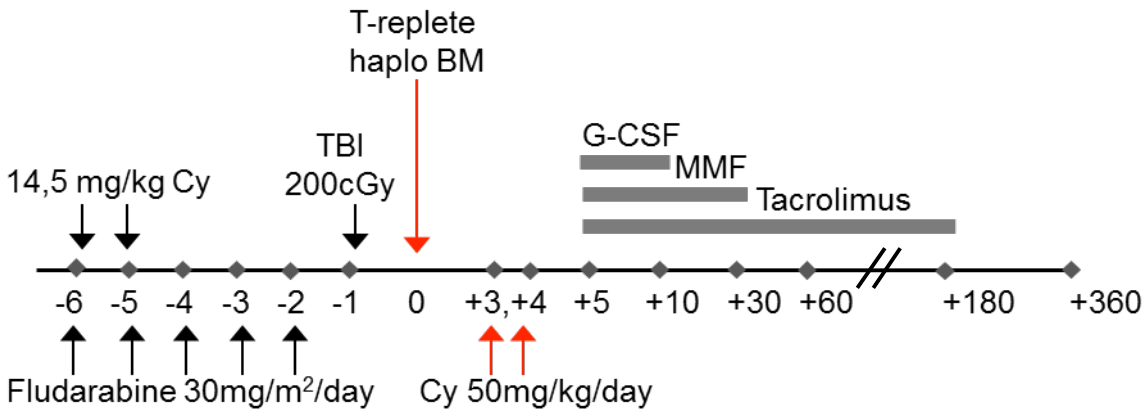

Figure 2. Schematic representation of Luznik's haploidentical transplantation protocol.

3.2.1 **Luznik's transplantation protocol**

The transplant procedures used to treat patients enrolled in this study consist in a non-myeloablative T cell replete haplo-identical bone marrow transplantation. Briefly, as reported in **Figure 2**, the patients were prepared with a conditioning regimen consisting in 14,5 mg/kg Cy at day -6, -5; Fludarabine from day -6 to day -2 and low dose total body irradiation (TBI, 200 CentiGray) at day -1. The GVHD was controlled by 50 mg/kg Cy administred at days +3, +4 after BMT, Tacrolimus (FK506) administred at 1 mg continuously from day+5 until day +180 and Mycophenolate Mofetil (MMF) administred at 15 mg/kg 3 times/day from day+5 until day +35 after transplant. Granulocyte-colony stimulating factor (G-CSF) was administred from day +5 in all patients.

HLA typing was perfomed in all patients and patient's relatives. The trasplant was considered haploidentical when at least one of the two alleles from donor's HLA -A, -B, -Cw, -DRB1 and DBQ1 loci matched the recipient. The details of the protocol can be find in the web-site www.clinicaltrials.gov, protocol numbers NCT02049424 and NCT02049508. Patients characteristics are listed in **Table 1.**

No.	Disease	Donor	Sex D/R	Age D/R	CMV D/R	Disease status before transplant	Indication for transplant	aGVHD (grade, localization)	aGVHD (therapy)	cGVHD (grade, localization)	cGVHD (therapy)	Infections/Virus reactivations	Follow-up (weeks)	Disease status	Reason for stopping follow-up
1	HL	Father	M/M	52/27	+/+	PR	RAA	NA		NA		H1N1	5	NA	Deceased
2	HL	Son	M/F	24/46	+/+	CR	TAA	-		-		*Non albicans candida*	5	CR	Graft failure
3	HL	Mother	F/F	51/19	+/+	CR	RAA	-		-		EBV	53	CR	End of the study
4	HL	Father	M/F	52/22	-/+	CR	RAA	2, Skin	Methylprednisolone	Mild, GI tract	Budesonide	Parainfluenza virus, CMV, EBV	55	CR	End of the study
5	NHL	Sister	F/M	64/53	+/+	PR	RAA	-		-		JC	9	NA	Deceased
6	HL	Brother	M/M	47/45	-/+	CR	RAA	2, Skin	ECP	Mild, Skin	Prednisone	*S. epidermidis,* BK, EBV	36	CR	End of the study
7	NHL	Sister	F/F	49/49	+/+	PR	RAA	AS		-		CMV	6	PR	Consent withdrawal
8	HL	Mother	F/F	61/33	+/+	CR	RAA			-		-	25	CR	Other*
9	NHL	Brother	M/M	47/45	+/+	CR	HR	AS		NA		*ESBL E. Coli*	5	NA	Consent withdrawal
10	NHL	Brother	M/M	62/57	+/-	CR	RAA	2, Skin	Methylprednisolone	-		*S. aureus, S.epidermidis*	13	CR	Consent withdrawal
11	HL	Brother	M/F	29/34	-/-	CR	TAA	2, Skin	ECP	-		-	50	PD	Deceased
12	NHL	Son	M/F	23/44	+/+	CR	TAA	NA		NA		*E. faecalis, A. fumigatus*	6	NA	Deceased
13	NHL	Brother	M/M	46/54	+/+	PR	TAA	1, Skin	Tacrolimus	-		*ESBL E. coli,* CMV	27	CR	Other*
14	HL	Son	M/F	24/57	-/+	CR	TAA	-		-		*ESBL E.coli,* HHV6	6	CR	Graft failure
15	NHL	Brother	M/F	62/57	+/+	CR	RAA	AS		-		*C. difficile,* CMV, *E. coli*	9	CR	Consent withdrawal
16	HL	Mother	F/F	60/24	+/+	CR	TAA	NA		NA		RSV	4	NA	Deceased
17	HL	Mother	F/M	45/24	+/+	PD	RAA	-		-		CMV	8	PD	Consent withdrawal
18	NHL	Father	M/M	51/25	+/+	CR	TAA	1, Skin	-	-		-	53	CR	End of the study
19	HL	Sister	F/F	38/47	+/+	CR	RAA	1, Skin	-	-		-	36	CR	Other*
20	NHL	Brother	M/M	50/51	-/+	PR	Ref	-		-		*ESBL E. coli,* CMV, BK	23	SD	Other*
21	HL	Sister	F/F	32/28	+/-	CR	RAA	-		-		-	3	CR	Other*
22	HL	Sister	F/M	21/25	+/-	CR	RAA	AS		AS		-	4	CR	Consent withdrawal
23	HL	Mother	F/F	54/26	+/+	PR	Ref	NA		NA		-	3	NA	Deceased
25	NHL	Son	M/M	35/62	+/-	PR	RAA	2, Skin	ECP	Mild, Skin	-	*S. maltophilia,* CMV	37	CR	End of the study
26	NHL	Sister	F/F	49/42	+/+	PR	RAA	1, Skin	-	-		-	33	CR	End of the study
27	HL	Cousin	M/F	48/40	+/+	PR	RAA			-		CMV, EBV	25	CR	Other*
28	HL	Father	M/F	51/21	-/-	PR	TAA	1, Skin	-	NA		-	6	PD	Consent withdrawal
29	HL	Father	M/M	51/24	+/+	SD	RAallo	-		-		-	4	NA	Deceased
31	HL	Brother	M/F	24/31	+/+	PR	RAA	-		-		HSV	12	NA	Other
32	HL	Father	M/M	57/32	+/+	CR	RAA	-		NA		CMV, *S. epidermidis, P. aeruginosa*	9	NA	Deceased
33	HL	Brother	M/M	47/37	-/-	SD	RAA	-		NA		*E. coli*, HHV6, Coronavirus, *Aspergillus*, *E. coli*	14	PD	Deceased
34	AML	Mother	F/M	63/33	+/+	CR	HR	-		-		*C. difficile*, CMV, BK, H1N1, EBV	51	CR	End of the study
35	HL	Brother	M/M	22/30	+/+	CR	RAallo	-		-		CMV	13	CR	Consent withdrawal
36	HL	Brother	M/F	71/66	+/+	CR	RAA	NA		NA		CMV	3	CR	Deceased
37	NHL	Brother	M/M	55/54	+/+	CR	RAA	1, Skin	ECP	-		*E.coli*, HSV	51	CR	End of the study
38	NHL	Cousin	F/M	27/35	+/+	CR	HR	-		-		HHV6, CMV	20	PD	Other*
39	AML	Cousin	F/M	43/50	+/+	AD	Ref	NA		NA		*Stenotrophomonas*, HHV6	3	AD	Deceased
48	ALL	Mother	F/M	51/24	+/+	CR	Ref	1, Skin	-	NA		EBV	9	CR	Ongoing
51	NHL	Brother	M/M	59/55	+/+	CR	TAA	NA		NA		-	3	NA	Consent withdrawal

Table 1. Characteristics of the patients enrolled in the study. Abbreviations: HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; D/R, donor/recipient; CMV, Cytomegalovirus; M, male; F, female; PR, partial remission; CR, complete remission; PD, progressive disease; SD, stable disease; RAA, Relapse after autologous; TAA, Tandem autologous-allogeneic; HR, High-risk; Ref, Refractory; RAallo, relapse after allogeneic transplantation; NA, not applicable; AS, developed after sampling was stopped; ECP, Extracorporeal photochemotherapy; GI, Gastrointestinal; *EBV, Epstein-Barr virus; JC, John Cunningham polyomavirus; S. Epidermidis, Staphylococcus Epidermidis; BK, BK polyomavirus; ESBL E. Coli, Extended-spectrum beta-lactamase-producing Escherichia Coli; S. aureus, Staphilococcus aureus; E. faecalis, Enterococcus faecalis; A. Fumigatus, Aspergillus Fumigatus; HHV6, Human Herpes Virus 6; C. Difficile, Clostridium Difficile; RSV, Respiratory Syncytial Virus S. Maltophilia, Stenotrophomonas Maltophilia; HSV, Herpes Simplex Virus; P. aeruginosa, Pseudomonas aeruginosa.* **Notes**: *: the patient stopped coming to the clinic.

3.3 Sample collection

Peripheral blood (25-100mL) and donor BM (5-10mL) samples were collected in heparinized tubes in the Haematology and Bone Marrow Transplantation Unit of Humanitas Cancer Center. Samples were collected at -d6, d0, d3 (before Cy administration), d7, every week until month +3, then every month until one year after transplantation (**Figure 3**). BM was collected during transplant procedures. PBMCs were isolated from blood and BM sample using a density gradient centrifugation after blood stratification on Ficoll-Paque Premium (GE Healthcare).

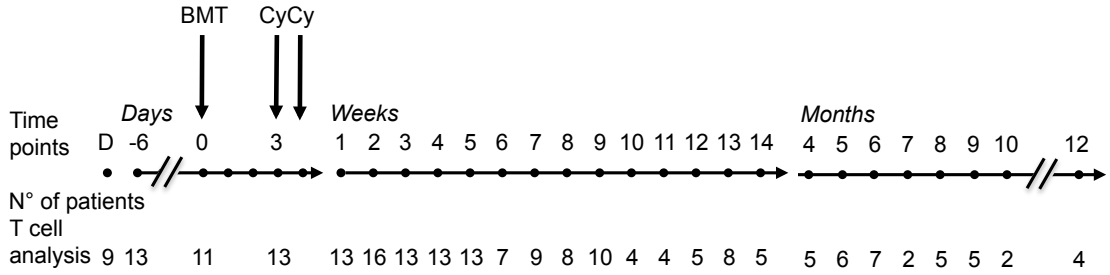

Figure 3. Schematic representation of sample collection after haplo-BMT. Blood samples were collected at the time points here indicated. The number of patients analysed at each time point is shown in the bottom row.

3.4 Flow cytometry and cell sorting

Samples were analysed or sorted by flow cytometry and cell sorter using the following fluorochrome-conjugated monoclonal antibodies (mAbs, **Table 2**):

specificity	Ab	Clone
human	CD8	RPA-T8
human	CD3	UCHT1
		HIT-3A
		SP-34.2
		OKT-3
human	CD4	SK3
		M-T477
		RPA-T4
		OKT-4
human	CD45RA	HI100
human	CD14	M5E2
human	CD45RO	UCHL1
human	CD27	O323
		1A4CD27
human	CCR7	150503
human	CD95	DX2
human	CD31	WM59
human	Ki-67	B56
human	CD127	A019D5
human	CD25	M-A251
human	HLA-A*02	BB7.2
human	HLA-A*B07	BB7.1
human	CD57	HNK-1
human	HLA-DR	G46-6
human	IFN-γ	B27
human	IL-2	MQ1-17H12
human	TNF	Mab11
human	CD38	HIT2
human	T-bet	4B10
human	Eomes	WD1928
human	IRF8	V3GYWCH
human	CD45	H-130
mouse	CD45	30-F11

Table 2. Fluorochrome conjugated mAbs used for flow cytometry analysis and T cell sorting. The specificity (human, mouse), the marker identified by each mAb and the clone are shown.

The following peptide:MHC class I tetramers, were used for Ag-specific experiments[112]: HLA-A*0201/MART$_{26-35}$ ELAGIGILTV and HLA-A*0201/MART$_{26-35}$ EAAGIGILTV, HLA-A*0201/WT$_{37-45}$ VLDFAPPGA, HLA-A*0201/WT$_{126-134}$ RMFPNAPYL, HLA-A*0201/WT$_{235-243}$ CMTWNQMNL.

Fluorochrome-conjugated mAbs were purchased from BD, BioLegend and BD Bioscences. Some antibodies were conjugated in the laboratory of Dr. Mario Roederer from purified unlabelled mAbs (BD). These antibodies were conjugated following the protocols reported in the website: http://www.drmr.com/abcon. All antibodies were titrated on human PBMCs and used at the concentration giving the best signal-to-noise ratio, as described[21]. For staining and cell sorting, either frozen and fresh cells were employed. Frozen cells were thawed in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine (all from Life Technologies) containing 50 U/mL Benzonase nuclease (EMD Biochemicals), followed by staining for flow cytometry.

In all stainings, cells were stained for 15 min at room temperature (RT) with a live/dead fixable dead cell stain kit (Life Technologies) to eliminate dead cells. After that, cells were stained with for 20 min at RT with a combination of surface mAbs, as described[21].

Fluorochrome-conjugated peptide-MHC-I tetramers and chemokine receptor were stained by incubating cells at 37°C for 20 min. The Cytofix/Cytoperm kit (BD Biosciences) allowed to detect intracellular Ki-67. Intracellular transcription factors were detected following fixation of cells with the FoxP3/transcription factor staining buffer set (eBioscience). Samples were acquired on a Fortessa flow cytometer as shown in **Figure 4** and **Figure 6** or separated via a flow cytometry Aria III cell sorter as depicted in **Figure 5** (all machines are from BD Biosciences). Single-stained

controls prepared with antibody-capture beads (BD) were used for compensation. T cell subsets were defined as shown in **Figure 4**: recent thymic emigrants (T_{RTE}) were $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}CD31^{+}$; T_N $CD4^{+}$ cells were identified as $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}CD31^{-}$ (for $CD8^{+}$ T cells, T_N were defined as $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}$, irrespective of CD31 expression); T_{SCM}, $CD45RO^{-}CD45RA^{+}CCR7^{+}CD27^{+}CD95^{+}$; T_{CM}, $CD45RO^{+}CCR7^{+}$; T_{EM}, $CD45RO^{+}CCR7^{-}CD27^{+}$; T_{TM}, $CD45RO^{+}$ $CCR7^{-}$ $CD27^{-}$; bulk memory T (T_{MEM}) cells: $CD45RO^{+}$ [23].

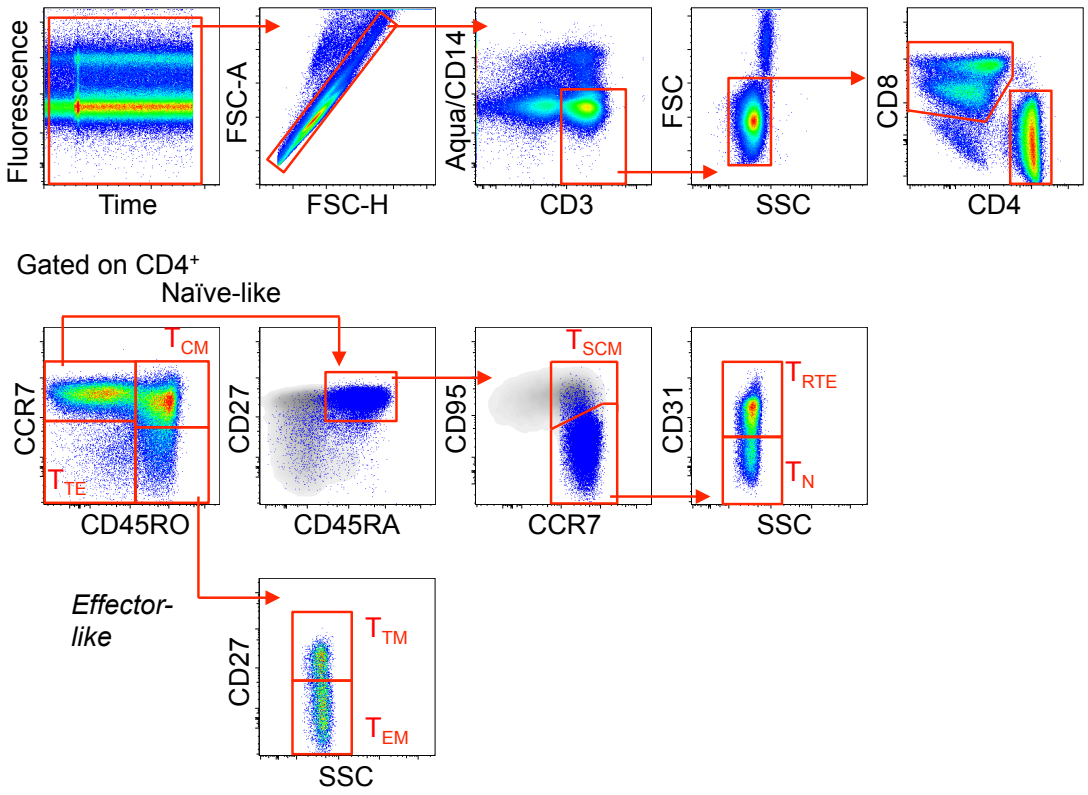

Figure 4. Gating strategy adopted to identify T cell subsets by flow cytometry analysis. Fluorescence stability was evaluated over time. Using forward scatter-area (FSC-A) and forward scatter height (FSC-H) gate doublets and aggregates were excluded from analysis. $CD4^{+}$ and $CD8^{+}$ T cells were identified on live/dead^{-} $CD14^{-}CD3^{+}$cells. Gating on $CD4^{+}$ T cells, T_{TE} were identified as $CD45RO^{-}CCR7^{-}$; T_{CM} were $CD45RO^{+}CCR7^{+}$; effector-like cells were $CD45RO^{+}CCR7^{-}$ and further distinguished on the basis of CD27 expression as T_{TM} ($CD27^{+}$) and T_{EM} ($CD27^{-}$); T_{SCM} were $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{+}$; T_{RTE} were $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}CD31^{+}$; T_N were $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}CD31^{-}$. For $CD8^{+}$ T cells, T_N were defined as $CD45RO^{-}CCR7^{+}CD27^{+}CD45RA^{+}CD95^{-}$, irrespective of CD31 expression. A similar gating strategy was used for $CD8^{+}$ T cells.

T_N were purified from whole PBMCs as depicted in **Figure 5**.

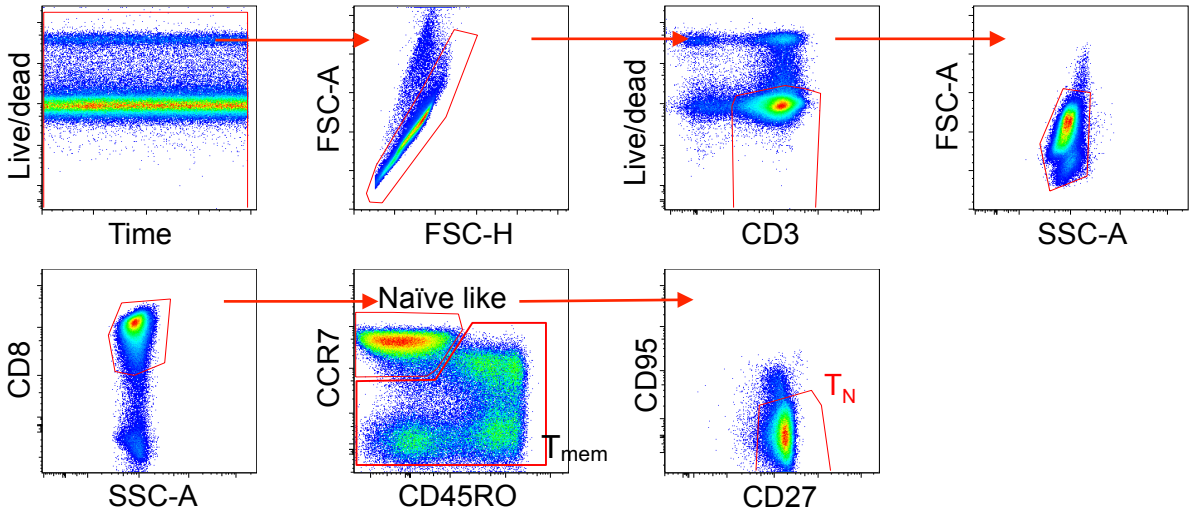

Figure 5. Gating strategy used to purify CD8$^+$ T_N and bulk T_{MEM} subsets. CD8$^+$ T cells were enriched by negative selection from thawed or fresh PBMCs. Time-gated cells were selected as singlets on the basis of FSC-A and FSC-H parameters. Live T cells were identified as live/dead$^-$CD3$^+$ and lymphocytes on the basis of FSC-A and SSC-A physical parameters. Identification of naïve and memory T cell subsets in CD8$^+$ T cells was performed by the use of 4 markers. CCR7 and CD45RO allowed the discrimination of naïve-like T cells (*i.e.*, CCR7$^+$CD45RO$^-$) from bulk-memory cells (T_{MEM}). CD27 and CD95 markers were used to discriminate T_N (CD27$^+$CD95$^-$) from T_{SCM} (CD27$^+$CD95$^+$) cells. A similar gating strategy was used to purify T cell subsets from CD4$^+$ population.

For ACT experiments in mice, CD8$^+$ T cells were identified as shown in **Figure 6.**

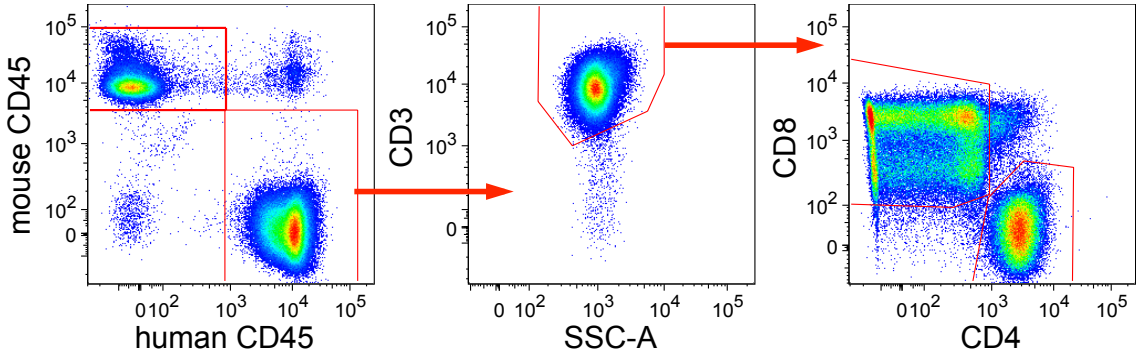

Figure 6. Gating strategy used to identify human CD8$^+$ iT_{SCM} infused in NSG mice. The gating strategy here shown was used to identify human T cells in peripheral blood and PBMCs isolated from mice organs.

3.5 Analysis of donor/patient mixed chimerism

We analysed three patients at the following time points post haplo-BMT: d63, d91 and d121. DNA was isolated from purified T cell populations (sorted as live/dead$^-$ CD3$^+$CD56$^-$CD20$^-$CD14$^-$ T cells) using the Qiamp Mini Kit (Qiagen), according to the manufacture's instructions. The isolated DNA was amplified using a multiples PCR fluorescent approach (AmpFISTR Identifiler Plus, Applied Biosystems) on an ABI 9700 thermal cycler (Applied Byosistem). The reaction was performed to analyse at least 3000 cells as described previously[113]. At least five of these HLA loci were amplified using a singleplex Quantitative Fluorescent PCR approach. Primer sequence were obtained from the database UniSTS (website: www.ncbi.nlm.nih.gov).

PCR products were run on Capillary Electrophoresis System (ABI310, Applied biosystems) and analysed using GENESCAN 3.1.2 software. Single PCR products were analysed in electropherograms as peaks variable in length and color. Residual mixed chimerism was calculated on the basis of the relative length of donor and recipient alleles as describerd previously[113].

3.6 Enumeration of self Ag-specific T cells By MHC class I tetramers

The MHC-I tetramers described in section **Methods 2.4** were used to recognize T cells with TCRs specific for MART-1 or WT-1 Ags. Given the large numbers of cells needed for the quantification of Ag-specific T cells, PBMCs isolated from buffy-coats instead of donor's BM or peripheral blood, were used. Briefly, CD8$^+$ T cells were enriched by negative selection (Stem Cell Technologies) and stained with tetramers for 15 min at 37°C. Cells were then washed with PBS without calcium and

magnesium (referred to as $PBS^{-/-}$) and stained with mAbs as previously described. For each sample, at least $3*10^6$ events were acquired by flow cytometry.

The threshold of positivity (0.003926 and 0.003705 for MART-1 and WT-1 positive fraction respectively; **Figure 14**) was calculated by analyzing the 75th percentile of distributions obtained analyzing tetramer-binding in the $CD4^+$ population (negative control).

3.7 Cell cultures and stimulation of T cells

3.7.1 *In vitro* T cell differentiation

Purified T cells or PBMCs ($0.25x10^6$ cells/mL, unless otherwise indicated) were cultured in complete RPMI medium (10% FBS, 1% penicillin/streptomycin, 2mM L-glutamine), and stimulated with αCD3/2/28 antibody-coated beads (Miltenyi). When preparing beads bound to αCD3/28 only, the amount of αCD2 in the mix was replaced by $PBS^{-/-}$. Otherwise, cells were activated with plate-bound αCD3 (1μg/mL unless indicated; clone OKT3; BioLegend) plus soluble CD28 (1ug/mL unless specified; clone CD28.2; BD) in 96 flat-bottomed wells for 9 days. Human cytokines (IL-2, IL-12, IL-7, IL-15, IL-18; Peprotech) were pre-titrated, then used at 10 ng/mL, unless otherwise indicated.

3.7.2 Proliferation of T cell subsets

Cell proliferation was determined by the analysis of 5-(and6)-carboxyfluorescein diacetate-succinimidyl ester (CFSE; Life Technologies) dilution[23]. After CFSE staining (5μM, unless indicted), cells were stimulated with IL-15 (Peprotech) for 8 days at 50 ng/mL, or left in 1 ng/mL IL-15 (non-proliferating control). At the end of culture period, cells were harvested and analysed by FACS. Cell number was

determined by Trypan blue dye exclusion. When stimulating patient's PBMCs, different time points (n=5; d41, d53, d56, d57 and d65) were used to generate **Figure 13B**.

3.7.3 PMA/Ionomycin stimulation and T cell cytokine production

To induce cytokine production, PBMCs were cultured in 96-well plate and stimulated with phorbol 12-myrisate 13-acetate (PMA, 10 ng/mL; Sigma Aldrich) and Ionomycin (1 µg/mL; Sigma Aldrich) in the presence of Golgi Plug (1 µg/mL; BD Bioscences). 4h after stimulation, cells were harvested and analysed by flow cytometry. When stimulating patient's PBMCs, different time points (n=5; d41, d53, d56, d57 and d65) were used to generate **Figure 13C.**

3.7.4 Co-cultures of purified T cells with auto/allo APCs

For mixed lymphocyte reaction (MLR) experiments, sorted T_N and bulk memory T cell subsets were cultured at a ratio of 1:1 with autologous APCs (auto-APCs) or MHC-mismatched APCs (allo-APCs) for the times indicated in the text. APCs (sorted as live/dead$^-$CD3$^-$CD56$^-$) were purified from PBMCs. Anti-MHC-I (G46-2.6, 10 µg/mL) and MHC-II (Tu39, 10 µg/mL) blocking antibodies were employed to inhibit APC:T cell interactions.

3.7.5 Stimulation of Ag-specific T cells

Self Ag-specific T cells were grown by using an accelerated DC maturation protocol, as described[114]. Briefly, at d0, $3.5x10^6$ CFSE-stained PBMCs from HLA-A*02$^+$ donors were seeded in 48-well plates in AIM-V medium (Life Technologies) additioned with FLT-3 (50 ng/mL; DC maturation; R&D) and the Melan-A/MART-1 peptide variants ELAGIGLTV or EAAGIGILTV (2 µg/mL; peptide chemistry facility,

University of Lausanne). 24h after stimulation, IL-1β (10 ng/mL; R&D), PGE-2 (1 μM; TOCRIS), TNF-α (1U/μg; DC activation mix, R&D) and IL-7 and IL-15 (both at 10 ng/mL) were included in each well. Cells were collected at day 7 for flow cytometry analysis, otherwise restimulated with 2 μg/mL ELAGIGLTV for 18h in the presence of Golgi Plug (1 μg/mL; BD Biosciences).

3.8 Real-time PCR (qPCR)

Total RNA was isolated from cells with RNeasy Micro Kit (Qiagen) and retrotranscribed using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed with hydrolysis probes: *IRF8* (Hs00175238_m1) and *18S* (Hs99999901_s1) as reference gene (Applied Biosystems) using the ABI 7900HT Sequence Detection System (Applied Biosystems). miRNA were isolated with mirVana kit (Ambion). Mature miR-155 and RNU44 small nucleolar 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_N cells were calculated using $2^{-(\Delta Ct\ sample - \Delta Ct\ naive)}$ formula.

3.9 Confocal microscopy

iT_{SCM} or iT_{Eff} $CD8^+$ T cells washed in $PBS^{-/-}$ were incubated with 500 μl of pre-warmed Mitotracker Green (25 nM prepared in $PBS^{-/-}$) for 30 min at 37°C. In the

meantime slides were treated to allow T cell adhesion: first incubated with 0.02% polylisin (Sigma) for 30 min at RT, than incubated for 3h at 37°C with αCD3 (OKT3 clone, BD Biosciences; 10 µg/mL in $PBS^{-/-}$) and αCD28 (CD28.2 clone, BD Biosciences; 10 µg/mL in $PBS^{-/-}$). $0.15x10^6$ cells were attached on slides and incubated for 15 min at 37°C. After incubation, cells were immediately fixed with 4% Formalin for 10 min, washed twice with 2% BSA in $PBS^{+/+}$ and once with 2% BSA, 0.05% tween in $PBS^{+/+}$.

Finally, cells were incubated with DAPI (dilution 1:25000 in H_2O) for 10 min at RT. Slides were acquired with an FV1000 confocal microscope (Olympus). Images were analysed with ImageJ (NIH).

3.10 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-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratories), bred in SPF conditions, were used for adoptive transfer experiments. For all experiments mice were grouped for age (5-7 weeks). Briefly sorted $CD8^+$ T_N cells were expanded *in vitro* with αCD3/28-conjugated beads (T cell activation/expansion kit, Miltenyi; 1 bead:2 cells) in the presence of IL-7 + IL-15 (Peprotech; 10 ng/mL each). At d7-8 cells were harvested counted and washed twice with sterile saline solution. $1-2x10^6$ $CD8^+$ iT_{SCM} (100 µl/mouse) were co-transferred by retro-orbital injection with $8x10^6$ PBMCs depleted of $CD8^+$ T cells (using anti-APC MicroBeads; Miltenyi). Tail bleeding was performed at d16 and d26. Mice were sacrified 1 month after transplantation to avoid GVHD. Spleen, lung and liver were collected, tissues were minced and filtered through a 40 µM cell strainer.

3.11 Statistical analysis

Analysis was performed using GraphPad PRISM (6.0b) and SPICE 5.22 software. Non-parametric paired or unpaired Wilcoxon rank test and unpaired Mann-Whitney test were employed to compare two groups. For more than three groups, One-Way Analysis of Variance (ANOVA) was used. Spice software was used to analyse pie chart differeces (permutation test). P values are two-sided and were considered significant when ≤ 0.05.

4 RESULTS

4.1 T_N-derived T_{SCM} contribute to immune recovery after haploidentical-BMT

4.1.1 T cell numbers normalize one year after BMT

The T cell recovery after BMT represents a crucial point for the positive outcome of transplant in terms of overall survival, as T cells are the mainly players of GVT and host protection from opportunistic infections. We initially evaluated the absolute counts of $CD3^+$, $CD4^+$ and $CD8^+$ T cells (**Figure 7**) as well as of $CD4^+$ and $CD8^+$ T cell subsets (**Figure 8**) over time.

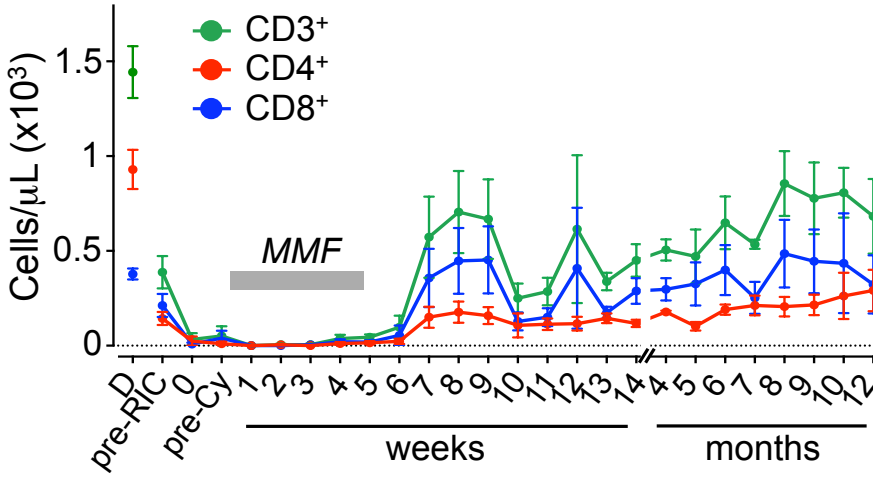

Figure 7. T cell recovery in BMT patients over time. Mean±SEM of absolute counts (cells/µl) of $CD3^+$, $CD4^+$and $CD8^+$ populations in the first year after haplo-BMT. Abbreviations: MMF, mycophenolate mofetil. The number of patients analysed at each time point was previously described in section 2.3, **Figure 3**.

T cell numbers increased from weeks 6 post haplo-BMT, when the MMF immunosuppressive drug was discontinued. After that, cell numbers declined equally in $CD4^+$ and $CD8^+$ populations at week 9. Subsequently, T cells numbers

progressively increased until 1 year after transplantation (when the sample collection stopped). Such increases paralleled the proliferation rate of T cell subsets (**Figure 9)**. During the first year, $CD4^+$ and $CD8^+$ memory phenotypes predominated, while at the end of the first year post transplantation T_N reappeared, probably due to restored thymic-output, as assessed by the increase of T_{RTE} cells in CD4 population (**Figure 8**).

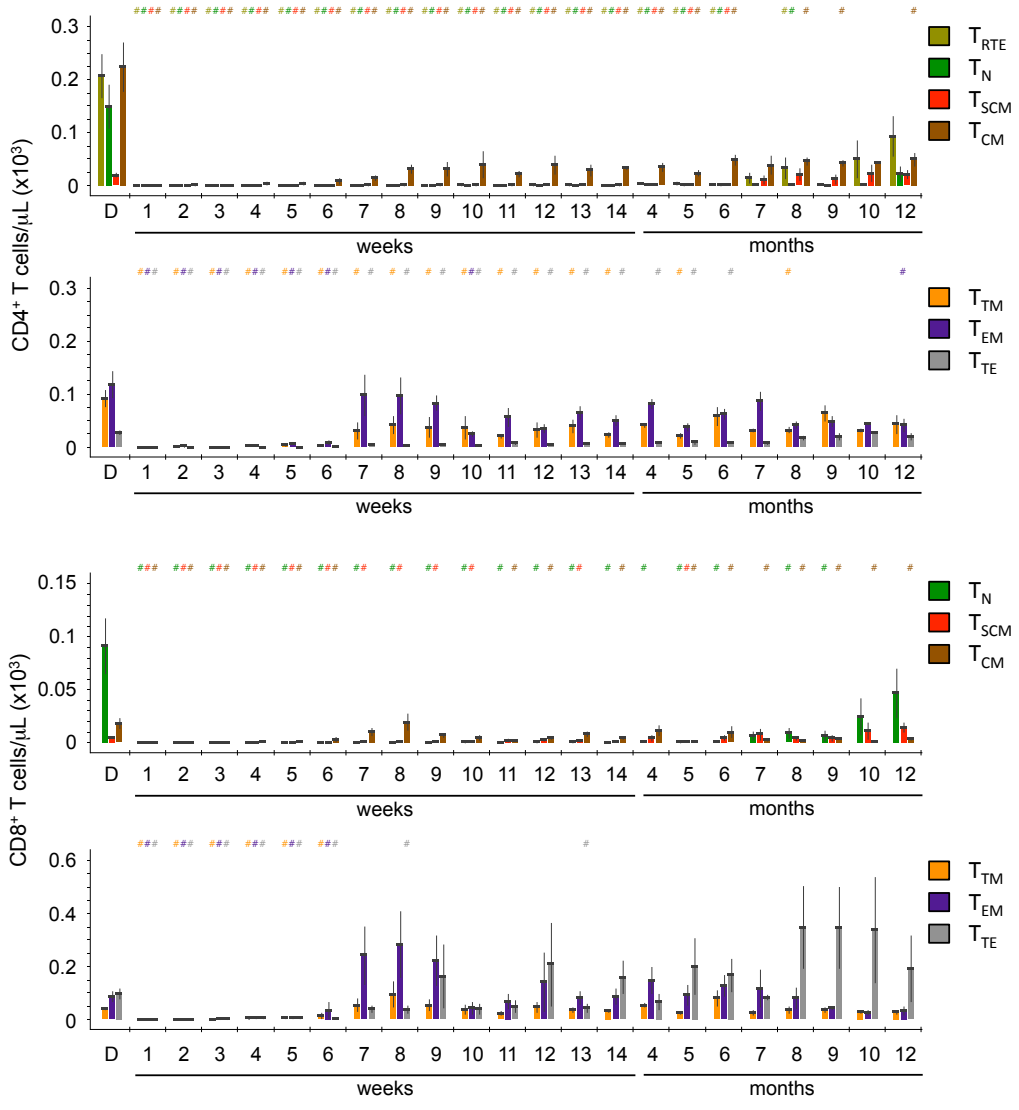

Figure 8. T cell subsets dynamics following haplo-BMT. Mean±SEM of absolute counts (cells/µl) of T cells subests (both $CD4^+$ and $CD8^+$ populations) of patients and donors after transplantation. The number of patients analysed in each time point is shown in section 2.3, **Figure 3**. #=P<0.05 vs Donor; Mann-Whitney test.

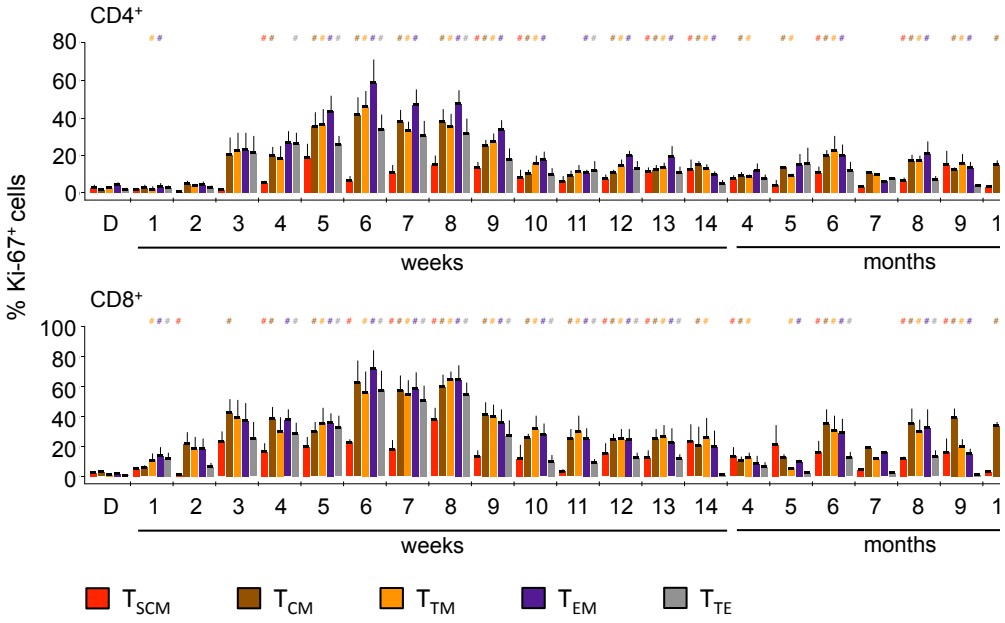

Figure 9. Absolute counts of proliferating CD4$^+$ and CD8$^+$ T cell subsets. Mean±SEM expression of Ki-67 by memory T cell subsets during T cell recovery. T_{RTE} and T_N cells were not considered as they expressed little Ki-67. Week 6 represent the peak of proliferation for all subsets. The numbers of patients analysed at each time point is shown in section 2.3, **Figure 3**. # = P<0.05 vs. Donor; Mann-Whitney test.

4.1.2 T cell recovery is donor dependent

In Luznik's protocol, patients are prepared with a non-myeloablative conditioning regimen and undergo T cell replete haplo-BMT. This means that both donor T cells and chemo-resistant host T cells could contribute to immune-reconstitution[115]. Thus, in collaboration with Dr. G. Bulfamante and Dr. B. Cassani we first analysed donor-recipient chimerism in patients PB within 120 days after transplantation. This analysis revealed that more than 98% of cells were of donor origin (data not shown). To exclude a possible bias due to a presence of high numbers of donor cells, we performed chimerism analysis on sorted live CD3$^+$CD56$^-$CD20$^-$CD14$^-$CD4$^+$ or CD8$^+$ from three patients at different time points after BMT (d63, d91 and d126) and confirmed that all circulating T cells were of donor origin (**Figure 10A**).

In addition, using flow cytometry and HLA-specific mAbs, we were able to detect donor and patient's T cells over time (HLA-A*02 or HLA-B*07 mismatch; **Figure 10B-C**). We observed that, during the first month after BMT, donor and recipient T cells coexisted in the patient (**Figure 10C**), while at 1 month after BMT patients displayed 100% donor chimerism (**Figure 10B-C**). Overall these results suggest that only donor T cells contribute to immune recovery, at least in the circulation.

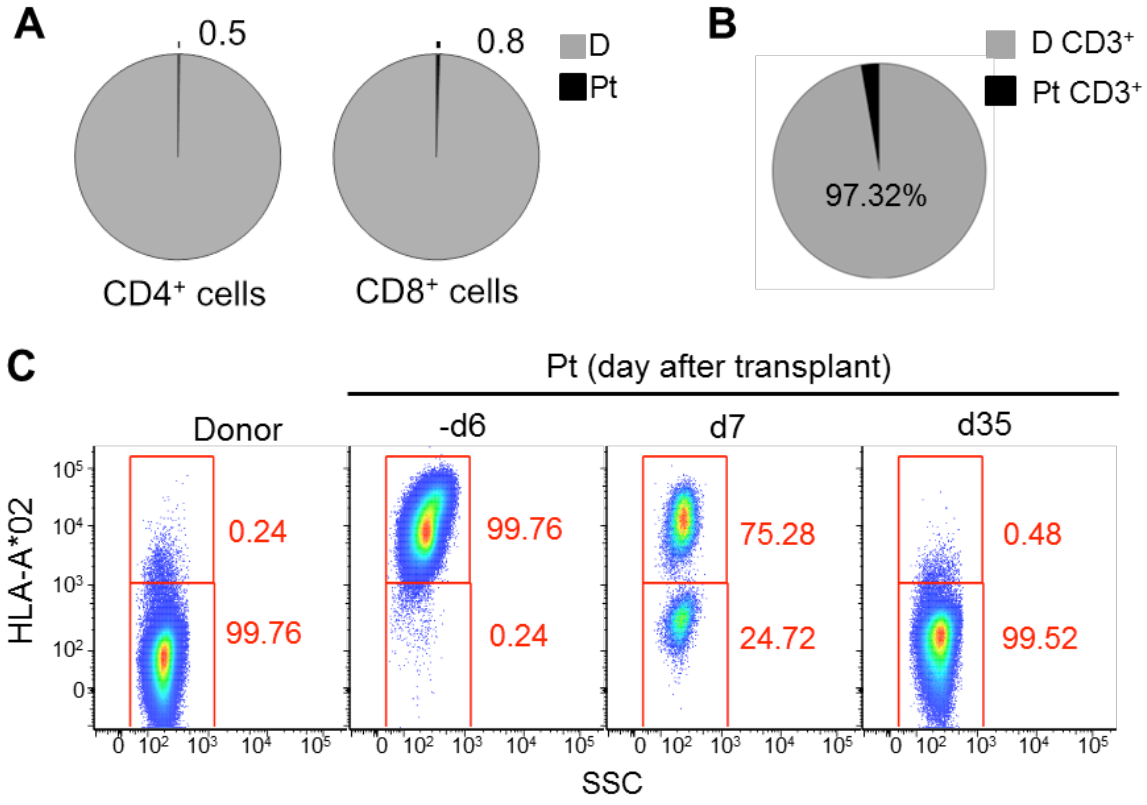

Figure 10. T cell recovery is donor dependent. (A) Proportions of Pt (black) and D (gray) chimerism in purified $CD4^+$ and $CD8^+$ T cells. Pies show the median of 3 Pt analysed at d60 after haplo-BMT**. (B)** D (gray) ad Pt (black) chimerism in $CD3^+$ population was confirmed by flow cytometry in additional 4 subjects. **(C)** D and Pt residual chimerism over time. Taking advantage from the HLA mismatch between D and Pt $CD3^+$ cells were discriminated using fluorochrome-conjugated mAbs directed to a specific HLA (here D HLA-A*02^- and Pt HLA-A*02^+). flow cytometry analysis were performed at indicated time points.

4.1.3 **Donor T_N cells preferentially survive to Cy**

It is well known that in response to Ags or lymphopenia, T cells undergo massive proliferation and generate effector cells characterized by the lack of CCR7, CD45RA and acquisition of CD45RO expression[22,52]. Thus, we analysed if this was also the case in our cohort and we observed that, at d3 after BMT, the vast majority of circulating $CD3^+$ T cells expressed activation (HLA-DR) and proliferation (Ki-67) markers (**Figure 11A-B**). Moreover, these cells acquired a $CCR7^-CD45RO^+$ effector phenotype, irrespectively of their original differentiation status (*i.e.* T_N, T_{SCM}, T_{CM} or T_{EM} phenotype) (**Figure 11B**). As described before[44], Cy preferentially depletes proliferating cells. Following the administration of Cy *in vivo* at d3 and d4 after transplantation, we observed that Ki-67^+ cells disappeared after Cy administration (**Figure 11A**). Only a proportion of relatively quiescent cells was spared by Cy (**Figure 11A**). Fine analysis of Ki-67 expression along with the differentiation status of T cells revealed that the vast majority of circulating $CD4^+$ and $CD8^+$ T_N cells were quiescent before Cy administration, indirectly suggesting that they are less sensitive to Cy (**Figure 11C**). In contrast, memory T cells expressed high levels of proliferating Ki-67 marker (**Figure 11C**). Given that only donor T cell contribute to immune-recovery, we extensively analysed the phenotype and maturation status of recipient and donor T cells by 18-color flow cytometry before the initiation of reconstitution (*i.e.*, at day 7). Patients T cells preferentially expressed a memory phenotype (with some differences between $CD4^+$ and $CD8^+$ populations in terms of T_{CM}, T_{EM}, T_{TE} subsets distribution) with high levels of activation (HLA-DR) and senescence (CD57) markers (**Figure 11D**). In contrast, donor T cells displayed a T_{SCM} phenotype ($CCR7^+$, $CD45RA^+$, $CD27^+$, $CD45RO^-$, $CD95^+$) and expressed low levels of activation (HLA-DR) and senescence (CD57) markers (**Figure 11D**). The

frequency of T_{SCM} among the donor $CD4^+$ and $CD8^+$ populations largely exceeded that observed in the infused grafts (**Figure 11E**) and in peripheral blood of healthy controls (2-3%)[15]. Collectively, these results suggest a possible contribution of $CD4^+$ and $CD8^+$ T_{SCM} in T cell recovery after BMT.

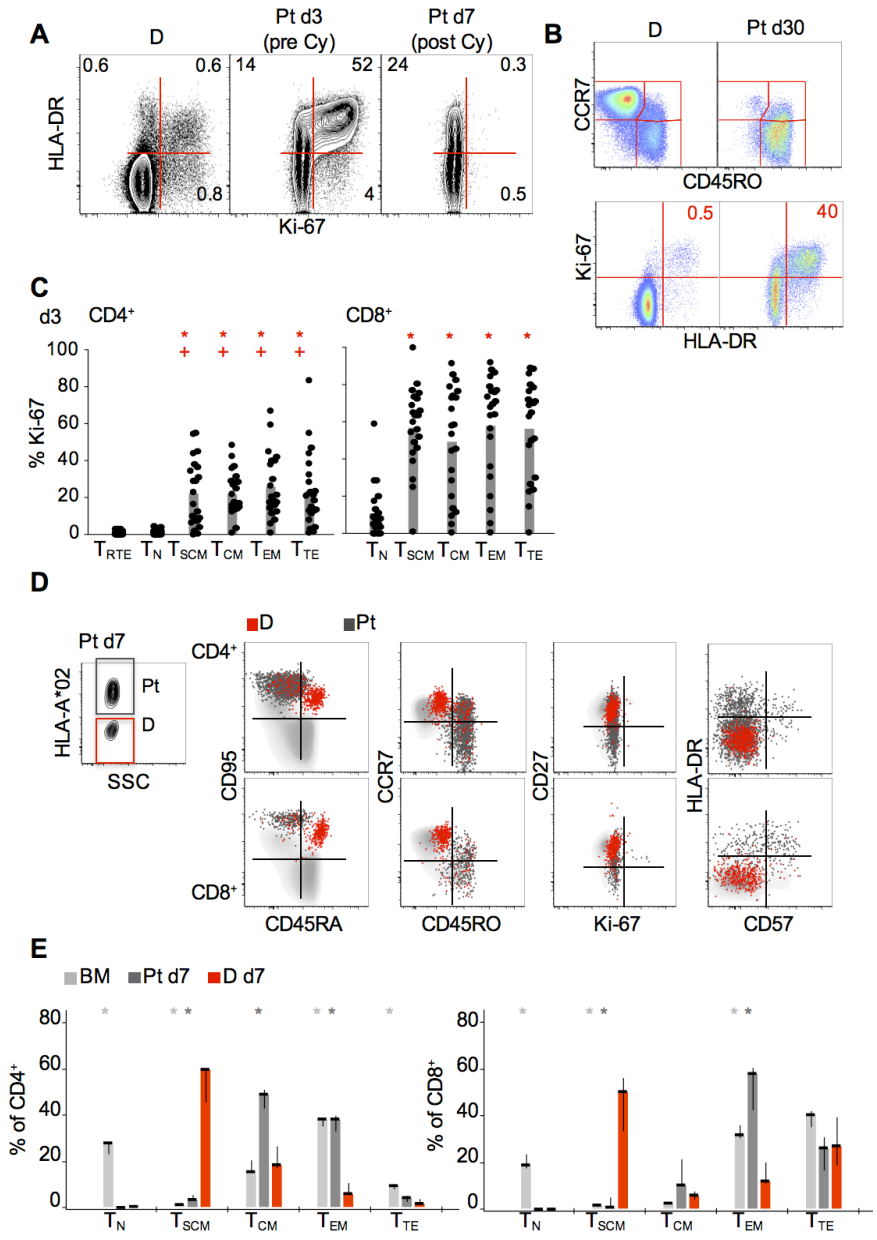

Figure 11. Donor T_N cells preferentially survive to Cy. **(A)** Representative (out of 10) activation (HLA-DR) and proliferation (Ki-67) marker expression in donor (D) and patient (Pt) $CD3^+$ T cells at the time point indicated. **(B)** Surface markers CCR7/CD45RO, HLA-DR and Ki-67 expression in donor (D) and patient (Pt) $CD8^+$ T cells 1 month after transplantation.

(C) Mean±SEM of frequency (n=22, each dot represent a patient) of proliferating Ki-67$^+$ T cell subsets at d3 after haplo-BMT in CD4$^+$ and CD8$^+$ population. +, *: P<0.05 vs T_{RTE} and T_N respectively; Wilcoxon test. **(D)** Donor derived (D, red, HLA-A*02$^-$) and patient (Pt, dark grey, HLA-A02$^+$) CD4$^+$ and CD8$^+$ T cells were identified in patient's peripheral blood by flow cytometry. Then a simultaneous flow cytometry analysis of differentiation (CCR7, CD45RA, CD27, CD45RO, CD95), activation (HLA-DR), senescence (CD57) and proliferation (Ki-67) markers was performed. Light grey cells in the background are CD4$^+$or CD8$^+$ T cells from peripheral blood of healthy controls.

4.1.4 **Post-transplant T_{SCM} originate from infused donor T_N cells**

All memory T cells, including T_{SCM}, identified in the circulation of patients were proliferating before Cy administration. Thus, because Cy depleted preferentially proliferating T cells, it seems unlikely that the high numbers of T_{SCM} at d7 originated from T_{SCM} that were transferred with the graft and that survived Cy **(Figure 11C)**. We then supposed that T_{SCM} could originate from T_N infused with the graft. To confirm this hypothesis, we focused our attention on a specific T_N subset of CD4$^+$ population, the T_{RTE}, identified by the surface expression of CD31 along with naïve markers. These are early differentiated T_N cells that recently egressed from the thymus. Upon T cell activation and differentiation into memory, CD31 is rapidly downregulated, therefore T_{SCM} and memory T cells do not express CD31[15]. CD8$^+$ T cells were not studied in this regard, as CD31 does not identify CD8$^+$ T_{RTE}. After transplantation almost all patients' T cells displayed a memory phenotype, thus obviating the necessity to discriminate donor's from patient's CCR7$^+$CD45RO$^-$ naïve-like (NL) T cells to follow CD4$^+$ T_{RTE} (**Figure 11D**). These NL cells also expressed naïve markers like CD45RA and CD27 (data not shown). NL-CD4$^+$ T cell in the graft were predominantly CD95$^-$, but acquired progressively CD95 expression both in CD31$^-$ and CD31$^+$ fraction, over time (**Figure 12A**). This differentiation was not due to the presence of Cy, as we demonstrated that Mafosfamide (the active form of cyclophosphamide, used *in vitro*) did not affect T cell phenotypes *in vitro* (data not shown). At d7 after BMT, the percentage of CD31$^+$ T_{SCM} was similar to

$CD31^+$ T_N fraction adoptively transferred with the graft (**Figure 12B**). We supposed that the high amount of cytokines and inflammatory molecules due to the lymphopenic environment and transplantation conditions (*i.e.* non myeloablative preparative regimen), triggered T_N cell differentiation.

Previous reports performed in mice showed that memory cells are more resistant to Cy treatments[49,116]. To better clarify the mechanism involved in the resistence to Cy activity we performed *in vitro* experiments using MLR cultures of highly purified T_N or memory T cells with allo/auto-APCs. In the presence of allo- but not auto-APCs, T_N cells were highly activated ($CD25^+$), underwent proliferation (*i.e.*, diluted CFSE) and upregulated CD45RO expression within 3 days after activation (**Figure 12C**). These data suggest that Ki-67^+ memory fraction depleted by Cy administration in patients contained also activated allo-reactive T_N cells. Finally, we investigated whether T_N differentiation was a consequence of the lymphopenic or allogeneic environment. As expected, *in vitro* incubation of T_N with allo- but not auto-APCs induced CD95 upregulation in the non-alloreactive fraction (defined as negative for both CD25 and CD69 activation markers, here not shown). Moreover, CD95 expression was curtailed when using MHC-I or MHC-II blocking antibodies indicating that allogeneic environment could play an important role in the acquisition of the T_{SCM} phenotype (**Figure 12D-E**).

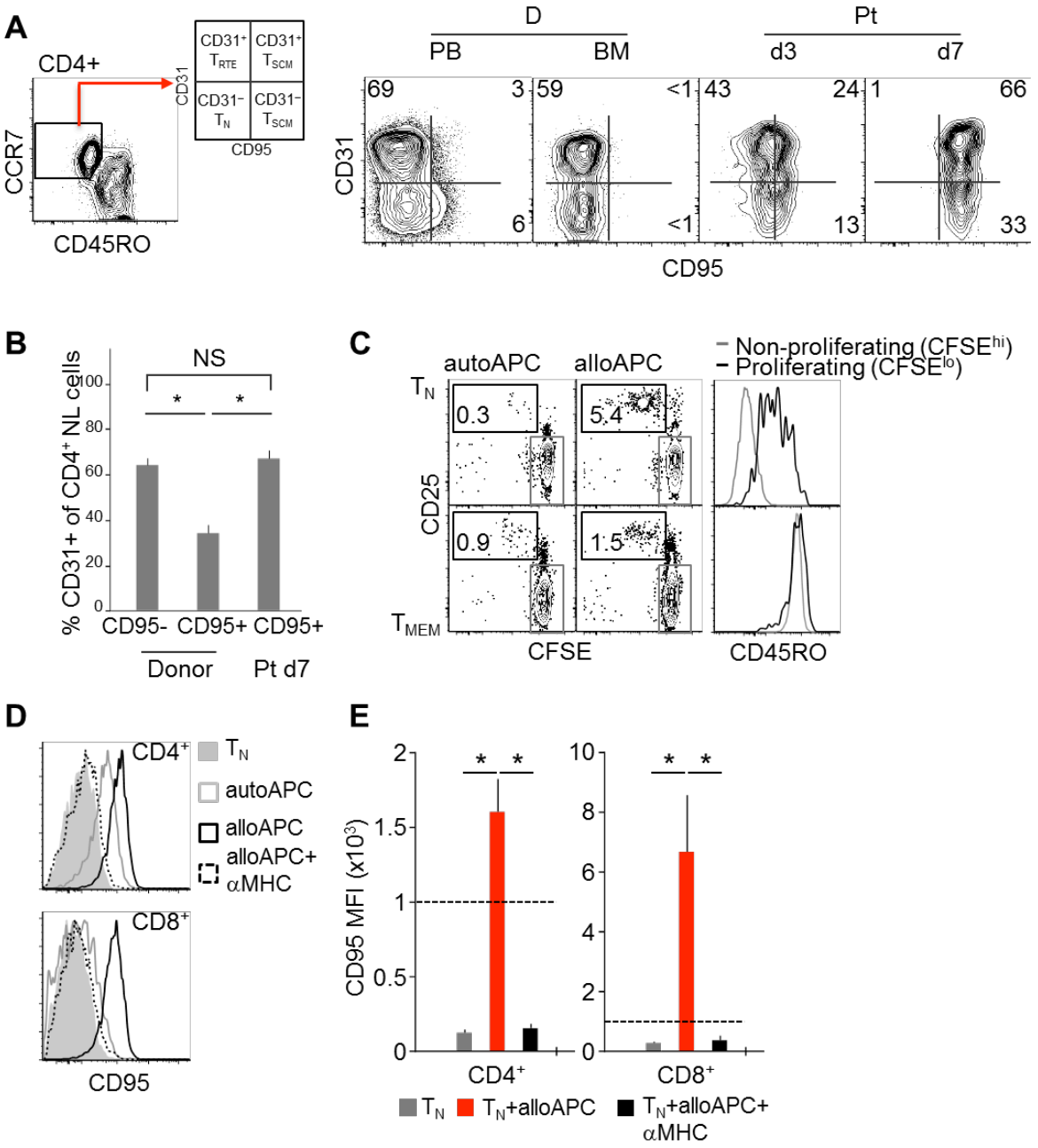

Figure 12. Post-transplant T_{SCM} originate from infused donor T_N cells. **(A)** Representative (out of 12) CD31 and CD95 expression on naïve-like ($CCR7^+CD45RO^-$, NL) $CD4^+$ T cells from donor's (D) bone marrow (BM) and peripheral blood (PB) and patient's (Pt) PB at indicated time points after haplo-BMT. **(B)** Mean±SEM of CD31 expression in circulating $CD4^+$ T_N and T_{SCM} from donor's BM and patient's PB at d7 after transplantation. *:P<0.05, Wilcoxon test.**(C)** CFSE dilution, memory marker CD45RO and activation marker CD25 expression by $CD8^+$ T_N and T_{MEM} after incubation with auto/allo-APCs for 3 days. Black gate and black line identify proliferating $CFSE^{low}$ T cells. Grey gate and line identify non proliferating $CFSE^{high}$ T cells.**(D)** Representative analysis of CD95 expression on $CD4^+$ and $CD8^+T_N$ cells incubated with different stimuli indicated in the legend. **(E)** Summary of the data obtained in (D). CD95 MFI is normalized against T_N + autoAPC culture condition (dotted line=1); n=8, from 4 independent experiments. *=P<0.05.

4.1.5 **Post-transplant T_{SCM} are true memory cells**

In order to understand whether T_N-derived T_{SCM} possess functional capabilities of memory T cells, we tested the ability of T_{SCM} to proliferate in response to IL-15 and to produce effector cytokines after PMA/Ionomycin stimulation. We first tested these approaches on T cells isolated from blood samples collected early after transplantation. However, cells were not able to respond to IL-15 or PMA/Ionomycin until d40 post BMT (data not shown). This was probably due to the immunosuppressive therapy (MMF + Tacrolimus) administered to patients until d35. To overcome this problem, we repeated experiments with cells isolated from blood samples collected at later time points (*i.e.* from d41 to d65). At these time points, the cell numbers were too low for FACS sorting. In previous experiments, we showed that IL-15 stimulation does not affect the phenotype of T_{SCM} cells[15]. This allowed us to perform experiments by stimulating whole PBMCs *in vitro*. After IL-15 stimulation, T_{SCM} from patients (d41-d65 post BMT) diluted CFSE similarly to memory T cells (**Figure 13A-B**). In line with their identity, T_N cells were not able to proliferate in response to IL-15 (**Figure 13A-B**).

To further demonstrate that donor T_N-derived T_{SCM} possess memory features, we stimulated PBMCs from haplo-BMT (d41-d65) with PMA/Ionomycin and evaluated their cytokine profile by flow cytometry analysis. Upon PMA/Ionomycin stimulation the proportion of T cell subsets among $CD4^+$ and $CD8^+$ population did not change (data not shown). T_{SCM} produced a combination of IFN-γ, IL-2 and TNF-α similarly to naturally occurring T_{SCM}, but distinct from the cytokine profile of T_N and memory T cells from healthy controls (**Figure 13C**). Collectively these data show that donor T_N-derived T_{SCM} acquire memory properties after haplo-BMT *in vivo*.

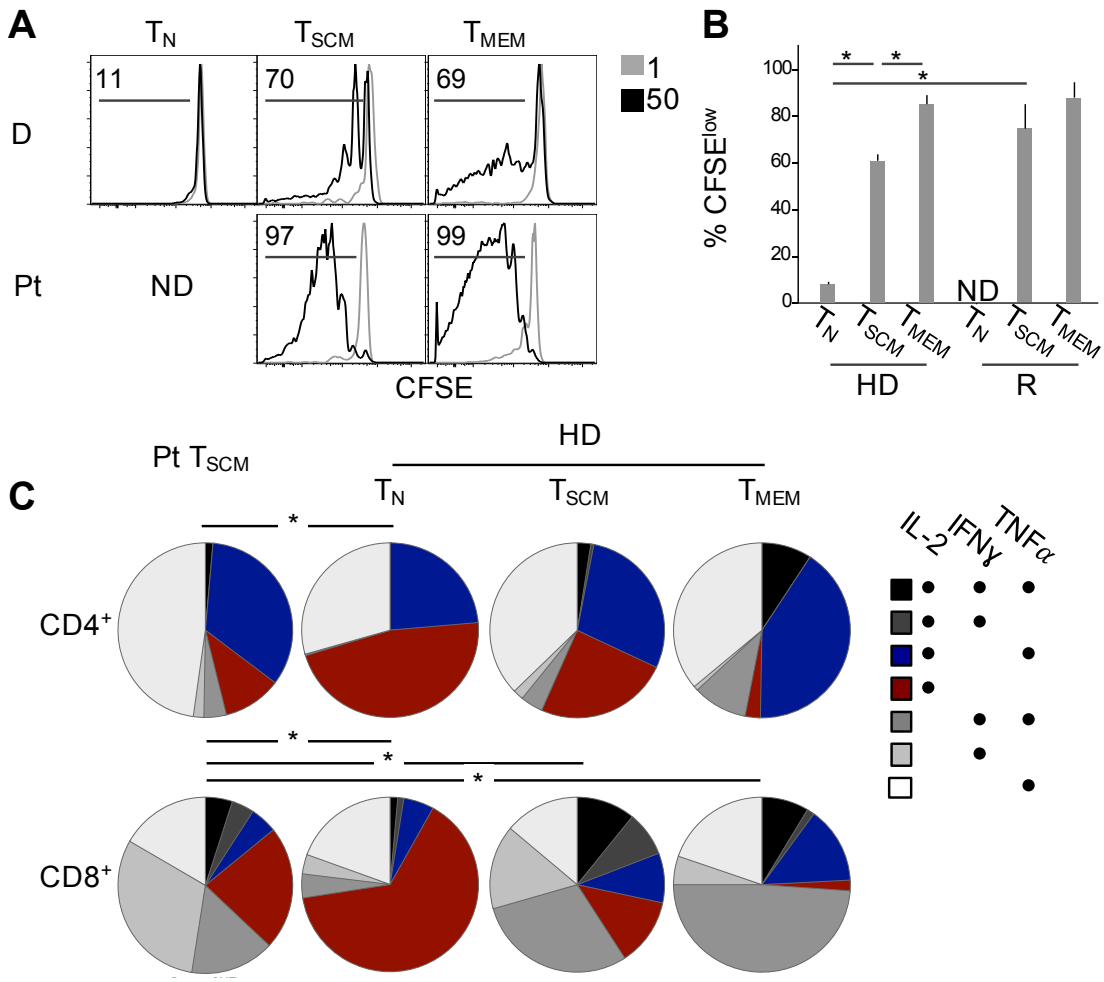

Figure 13. Post-transplant T_{SCM} are true memory cells. **(A)** Frequencies of $CFSE^{low}$ cells in each $CD8^+$ T subsets from a representative healthy donor (HD) and patient (Pt) at d41 post haplo-BMT. Whole PBMCs from HD and Pt were cultured with 1 ng/mL (grey histogram, non proliferating control) or 50 ng/mL (black histogram) of IL-15 for 8 days. In Pt, $CD95^-$ T_N were not detected (N/D) *ex vivo* or after stimulation with IL-15. T_{MEM}: $CD45RO^+$ memory T cells. **(B)** Summary of 3 independent experiments (n=6) as described in A. The mean±SEM of $CFSE^{low}$ $CD8^+$ T cell subsets is shown. *= P<0.05; Mann-Whitney test. **(C**) Pies show the intracellular cytokine profile (IFN-γ, IL-2 and TNF-α) following PMA/ionomycin stimulation for 4 hr in gated T_{SCM} from patients (n=3; at d35, d42 and d49 post haplo-BMT) and in T cell subsets from healthy donors (HD; n=4). *=P<0.05; permutation test.

4.1.6 **Persistence and memory differentiation of adoptively-transferred T_N**

Next, we investigated the capability of donor T_N-derived T_{SCM} that survived to Cy treatment to contribute to immune recovery. Should this be the case, antigen-specific T_N cells infused with the graft that are non allogenic would be found in the memory compartment during the recovery phase. The frequency of antigen-specific T cells in the T_N comparment is usually very low, *i.e.* between $1/10^5$ and $1/10^6$ cells, however T cells specific for some self/tumor-associated antigens including MART-1 and WT-1 are relatively high because they can escape negative selection in the thymus[117,118], thus allowing their measurement with standard flow cytometry and p:MHC-I tetramers. We first excluded that new generation of T_N cells by the thymus could confuse these data, as TREC levels in a subset of individuals receiving h-HSCT was undetectable up to 6 months post transplantation (not shown). As expected, MART-1 and WT-1 specific $CD8^+$ had a naïve phenotype in healthy donors (**Figure 14A-C**). After infusion, both MART-1 and WT-1 specific T cells were able to persist in the host up to d90 after BMT (**Figure 14B**) and acquired a $CD45RO^+CCR7^-CD95^+$ phenotype as soon as d45 after BMT for MART-1 (**Figure 14C**). These data suggest that Ag-specific T_N cells survive to pt-Cy treatment and contribute to immune recovery of the T cell compartment.

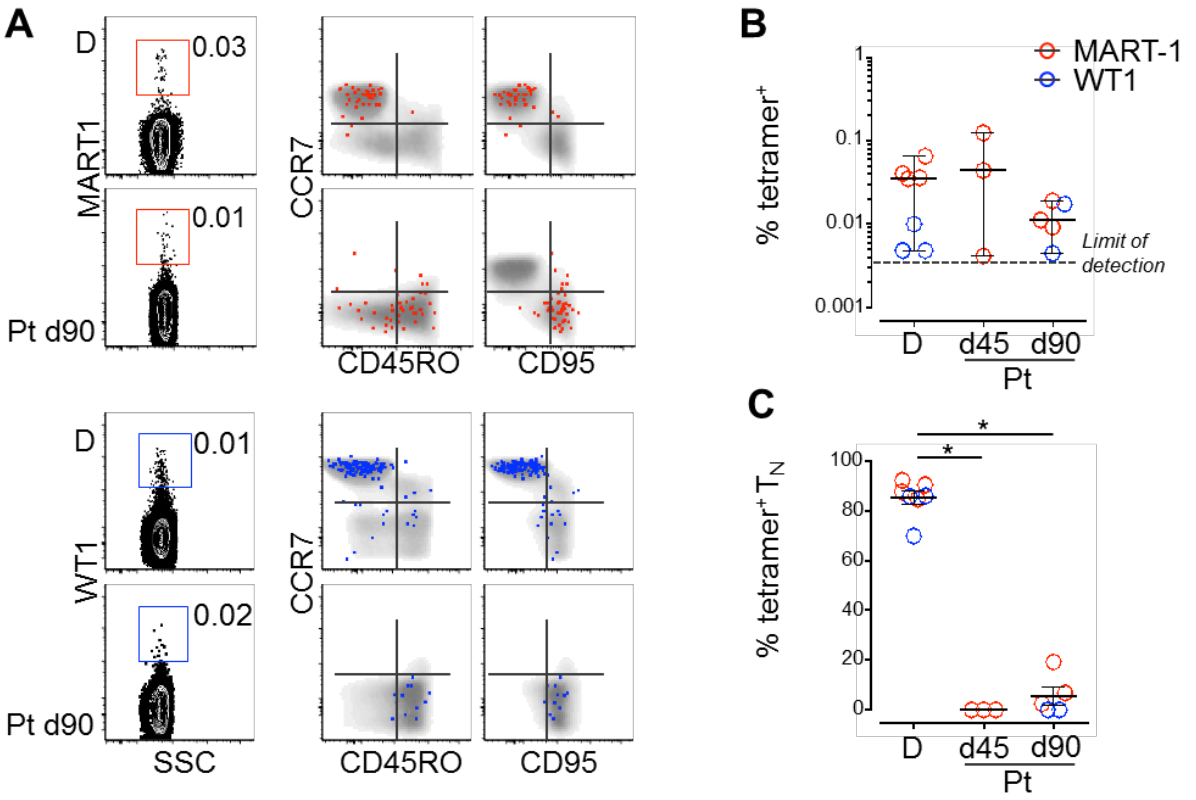

Figure 14. Persistence and memory differentiation of adoptively-transferred T_N. (A) Phenotype and percentage of MART-1 (red dots) and WT-1 (blue dots)-specific $CD8^+$ T cells identified by MHC class I tetramers in 1 representative donor (D) and the corresponding patient (Pt). Numbers indicate the frequency of cells in each gate. In light grey total $CD8^+$ T are shown. **(B)** Summary of the mean±SEM of the MART-1 and WT-1 specific $CD8^+$ T cells shown in (A). **(C)** Frequency of MART-1 and WT-1 specific $CD8^+$ T cells with a T_N-phenotype in Ds and Pts at time points indicated after transplantation. *=P<0.05, Mann-Whitney test.

4.2 Generation of T_N-derived T_{SCM} (iT_{SCM}) for ACT

Results obtained in the first part of this work suggest the non-redundant role of donor T_N-derived T_{SCM} in immune reconstitution. Moreover, in previous reports, adoptive transfer of selected T cell subsets in mice and non-human primates showed that less differentiated T cells are endowed with superior anti-tumor, self-renewal, multipotency and persistence capabilities compared to more differentiated subsets (see chapter 1.1)[119,120,93,92,54]. Because the low numbers of T_{SCM} in the peripheral blood limits their clinical application, the possibility to expand this population *in vitro* represents a promising approach for the improvement of ACT. For this reason, in the last part of this work, we optimized a method to generate high numbers of fully functional T_{SCM} from naïve $CD8^+$ precursors *in vitro*, and at the same time, investigated the molecular mechanisms involved in their generation.

4.2.1 **Curtailed T cell receptor stimulation in combination with IL-7 and IL-15 generates T_{SCM} cells.**

We first evaluated the effect of different combination of cytokines, and potency of TCR activation on the differentiation of highly purified human $CD8^+$ T_N cells (isolated from peripheral blood) as assessed by surface expression of CD45RO and CCR7. We decided to stimulate cells with a low bead:cell ratio (*i.e.* 1:2), as previous report showed that higher bead:cell ratio (3:1) induced the acquisition of CD45RO expression in T_N cells [37]. Overall, IL-7 and IL-15 combined with αCD3/28 was the best combination to maintain a $CD45RO^-CCR7^+$ phenotype (**Figure 15A**) and to generate bona fide $CD45RO^-CD45RA^+CCR7^+CD27^+CD95^+$ T_{SCM}-like cells (**Figure 15B**; hereafter referred to as iT_{SCM}).

By contrast, a stronger TCR stimulation with αCD3/2/28 in the presence of IL-7/IL-15 or IL-2 preferentially generated $CD45RO^{+}CCR7^{+}$ T_{CM}-like cells (**Figure 15A-C**).

IL-2 alone or in combination with IL-18 had limited effects on T cell differentiation, while the addition of IL-12 to IL-2 induced the generation of bona fide $CD45RO^{+}CD45RA^{-}CCR7^{-}CD27^{int}CD95^{+}$ T effectors (iT_{Eff}) (**Figure 15A-B**). Compared to iT_{Eff}, iT_{SCM} displayed lower expression of Ki-67 (indicative of proliferation), CD38 and HLA-DR (indicative of activation) markers and showed a lower size (indicated by forward-scatter, FSC-A) (**Figure 15B**). A summary of the phenotypes induced by selected culture conditions is shown in **Figure 15C**. Overall, increasing TCR stimulation through the addition of CD2 to the culture conditions, resulted in an increase of the frequency of dead cells at the end of culture period (**Figure 15D**). In iT_{Eff} condition, this was compensated by increased proliferation (**Figure 15E**). Despite the lower activation and proliferation status compared to iT_{Eff} *in vitro*, iT_{SCM} cells expanded robustly over time *in vivo* following adoptive transfer into NSG mice (**Figure 15F**) and infiltrated multiple organs, including the spleen, the liver and, at a lesser extent, the lung (**Figure 15G**).

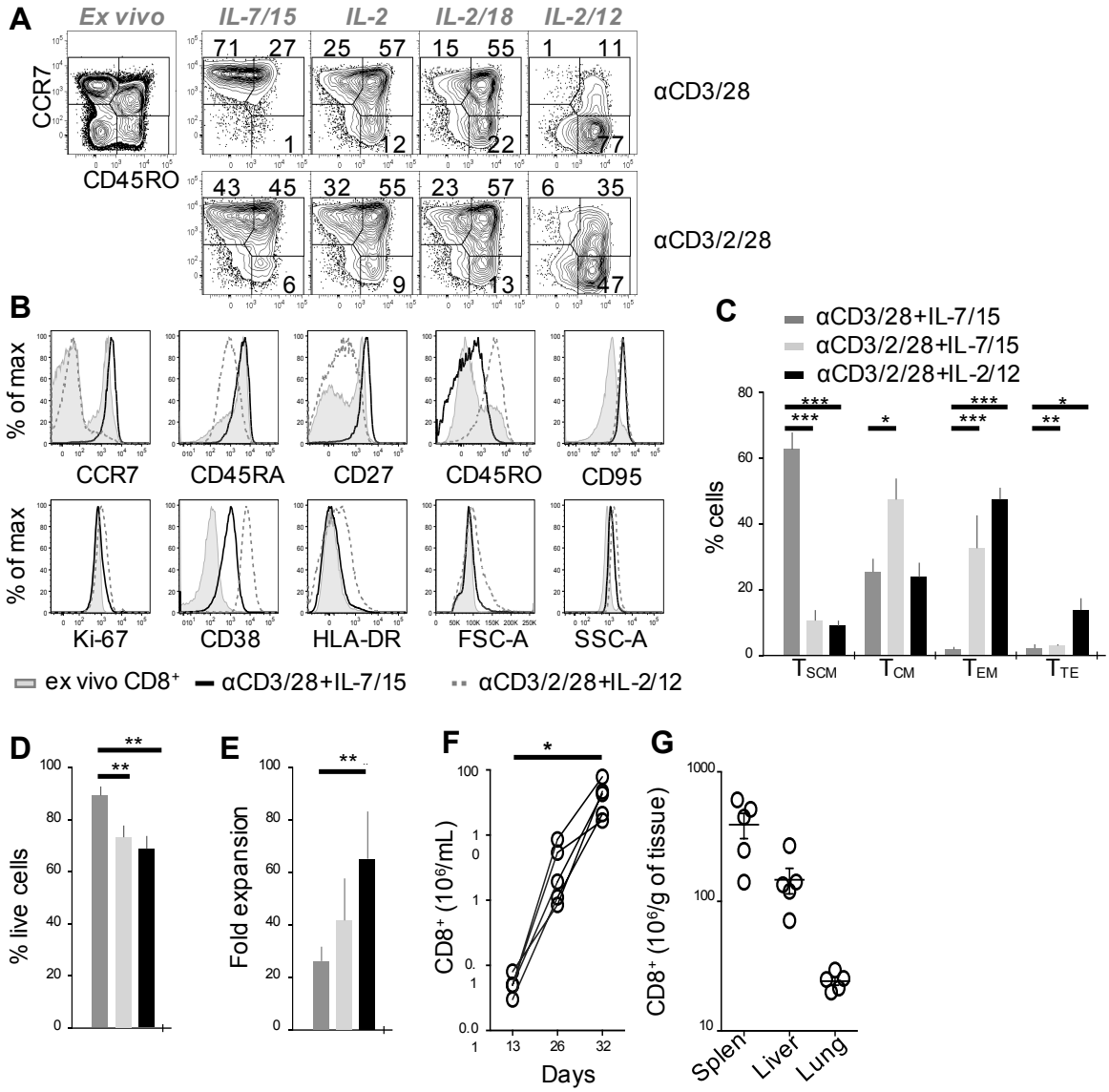

Figure 15. Curtailed T cell receptor stimulation in combination with IL-7 and IL-15 generates T_{SCM} cells. (A) Representative flow cytometry analysis of surface marker expression (CD45RO, CCR7) in *ex vivo* PBMCs and in sorted $CD8^+$ T_N cells following culture with different combination of cytokines and T cell receptor (TCR) stimulations for 11 days. (**B)** Sorted $CD8^+$ T_N cells were cultured in αCD3/28+IL-7/15 (iT_{SCM}) or αCD3/2/28+IL-2/12 (iT_{Eff}) for 11 days as in (A). The expression of naïve and memory markers in the two conditions (black line=iT_{SCM}; dotted line= iT_{Eff}; light grey full histogram=total $CD8^+$ T cells, used as control) is shown; n=4 for all parameters with the exception of n=2 for Ki-67, CD38 and HLA-DR markers. (**C)** Mean±SEM of frequencies of $CD8^+$ T cells with the T_{SCM}, T_{CM}, T_{EM} and T_{TE} phenotypes, 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. (**D)** Mean + SEM of the frequencies of live cells and (**E)** fold expansion in cell number, compared to baseline, of sorted $CD8^+$ T_N cells cultured as in C. (**F, G)** Human $CD8^+$ T_N stimulated with αCD3/28+IL-7/15 for 8 days were transferred into NSG mice. **(F)** Tail bleeding was performed at indicated time points, human $CD8^+$ infused cells were identified as described in **Figure 6** and absolute counts were evaluated by flow cytometry analysis. **(G)** The absolute counts described in (F) were determined also in tissues and organs isolated from sacrificed mice. Data shown as mean±SEM of 5 replicate mice, one single experiment. * P<0.05, ** P<0.01 and *** P<0.001, Wilcoxon test.

4.2.2 **Polyclonal iT_{SCM} cells are early-differentiated memory cells.**

The capability to produce specific combination of cytokines upon PMA/Ionomycin stimulation depends by the degree of memory differentiation[22]. Less differentiated T cells mainly produce IL-2 and TNF-α while more differentiated ones mainly produce IFN-γ[22]. We therefore reasoned that iT_{SCM} and iT_{Eff} had different patterns of cytokine production. We stimulated iT_{SCM} and iT_{Eff} (d12) with PMA/Ionomycin (P/I) for 4 hours in the presence of Glolgi Plug. iT_{SCM} produced mainly TNF-α and IL-2, either alone or in combination but little IFN-γ, while iT_{Eff} produced the 3 cytokines together (**Figure 16**), thereby indicating that iT_{SCM} are early differentiated memory cells.

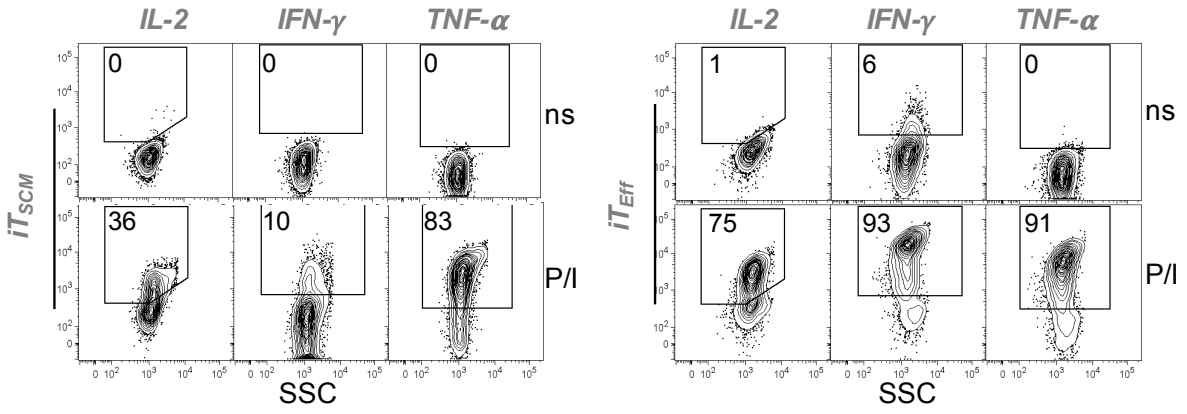

Figure 16. Polyclonal iT_{SCM} cells are early-differentiated memory cells. (A) highly purified $CD8^+$ T_N cells were stimulated as in Figure 15C to generate iT_{SCM} and iT_{Eff}. After 7 days, cells were harvested and stimulated with PMA/Ionomycin (P/I) for 4h in the presence of Golgi Plug. Unstimulated cells (ns) were included as negative control. IL-2, IFN-γ and TNF-α production was evaluated by flow cytometry.

4.2.3 **Ag-specific iT_{SCM} are functionally and phenotypically similar to polycolonal iT_{SCM}**

To understand whether the same pattern of cytokine production shown in **Figure 16**, could be observed at the Ag-specific level, we used an accelerated DC maturation protocol[114] to generate high numbers of $CD8^+$ MART-1 specific iT_{SCM} from T_N precursors. To do this, we stimulated whole 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 EAA and ELA, respectively). Both peptides induced proliferation of the Ag-specific $CD8^+$ cells, as assessed by CFSE dilution (**Figure 17A**). However, ELA induced either higher frequencies and total numbers of MART-1 specific T_{SCM} cells compared to EAA stimulation (**Figure 17B**). Restimulation of ELA- or EAA-primed T cells with ELA-peptide (2 μg/mL) at d7, induced the production of cytokines shown in **Figure 17C**. The profile was similar to that produced by iT_{SCM} generated by αCD3/28 beads and restimulated with P/I (**Figure 17D**). These results suggest that the generation of Ag-specific T_{SCM} is comparable to polyclonal iT_{SCM} both at the phenotypical and functional level.

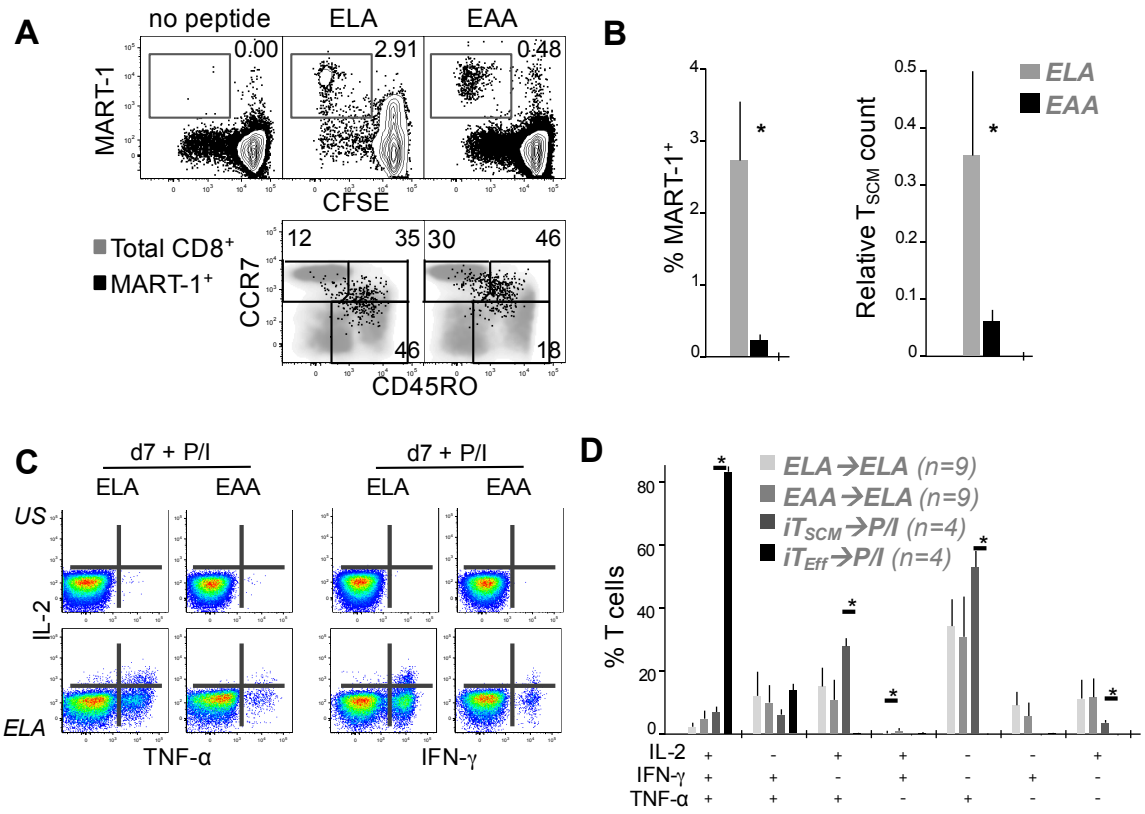

Figure 17. Ag-specific iT_{SCM} are functionally and phenotypically similar to polycolonal iT_{SCM}. **(A)** HLA-A*02$^+$ PBMCs from healthy controls were stimulated with a cocktail of cytokines (including FLT-3, IL-1β, PFE2 and TNF-α) to induce maturation of dendritic cells (DC). ELA or EAA peptides were added to induce activation and expansion of MART-1 specific T cells. The presence of IL-7 and IL-15 sustained differentiation through T_{SCM} phenotype. Representative frequencies (assessed by flow cytometry) of CFSE dilution of MART-1 specific T cells at different culture conditions (top; no peptide= ELA or EAA were not added to the culture) and CD45RO/CCR7 expression in MART-1 specific T cells (black dots) superimposed to total CD8$^+$ T cells from the same culture (bottom) are shown. Data are representative of n=9 donors from 4 independent experiments. **(B)** Mean+SEM of the percentage of MART-1 specific on total CD8$^+$ cells (left; ELA: n=6; EAA: n=9) and the relative T_{SCM} count (right; ELA: n=6; EAA: n=5) following peptide stimulation. Relative T_{SCM} count was calculated only when the total number of cells within the MART-1 positive fraction was >15. **(D)** Summary of the results of cells stimulated as in (A) with PMA/Ionomycin (P/I) and in (B) with ELA/EAA peptides. For all conditions IL-2, TNF-α and IFN-γ production was evaluated by flow cytometry. Data are shown as mean+SEM. The number of healthy controls used for each experiment is shown in the legend and derive from 5 independent experiments. 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 vs. iT_{SCM}, Wilcoxon test.

4.2.4 **iT_{SCM} cells undergo limited activation and effector differentiation.**

In order to better understand the molecular mechanisms involved in T_{SCM} formation, we quantified effector molecules production, both at the protein and mRNA level. Specifically, we investigated two transcription factors (TF): T-bet and Eomes. These TF increase progressively with T cell differentiation and control effector functions of T cells [121,122]. Moreover we investigated also IRF4[123], IRF8[124] and miR-155[125]. The expression of these molecules is regulated by the potency of TCR activation. Accordingly, T-bet and Eomes increased progressively with the duration of the culture period (**Figure 18A**), while IRF4, IRF8 and miR-155 were mostly detected during the first days of culture, then their expression waned (**Figure 18B**). All these molecules showed a higher expression in iT_{Eff} compared to iT_{SCM} throughout the stimulation period, thus recapitulating the differential activation status observed in **Figure 15B**.

It has also been proposed that the content of mitochondria increases along the differentiation of T cells[126]. Accordingly, confocal microscopy analysis revealed that iT_{SCM} harboured fewer mitochondria and appeared to have little cytoplasm compared to iT_{Eff} (**Figure 18C**), thus corroborating the lower size observed in **Figure 15B**.

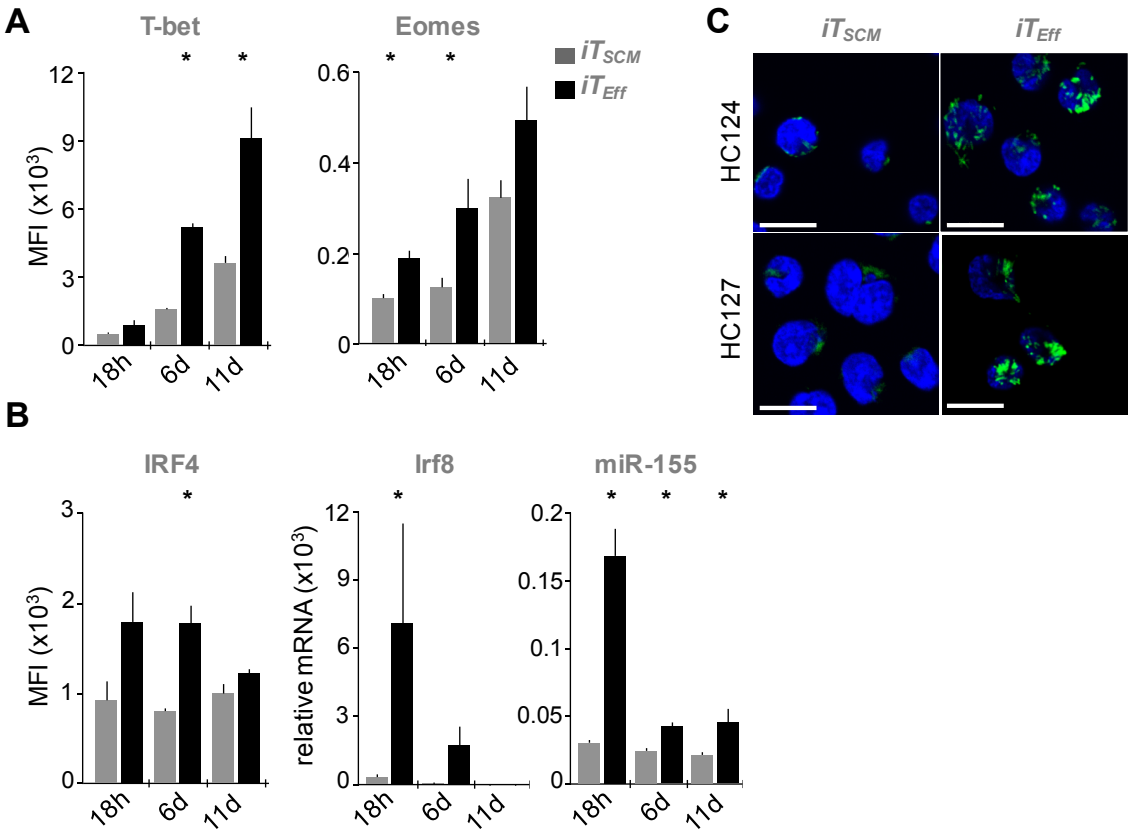

Figure 18. iT_{SCM} cells undergo limited activation and effector differentiation. (**A, B)** highly purified $CD8^+$ T_N cells were activated with αCD3/28+IL-7/15 (iT_{SCM} condition) or αCD3/2/28+IL-2/12 (iT_{Eff} condition). The expression (shown as mean + SEM) of effector molecules was evaluated by flow cytometry (n=3; T-bet, Eomes, IRF4; expressed as median fluorescence intensity or MFI) or by RT-qPCR (n=4; Irf8, miR-155 expression reative to S18 house keeping gene). h:hours; d, days. * P<0.05 vs. iT_{SCM}, Wilcoxon test. (**C)** Confocal microscopy analysis (100x magnification) of mitotracker green (green=mitochondria) and DAPI (blue=Nuclei) in iT_{SCM} cells and iT_{Eff} cells generated from $CD8^+$ T_N cells as in (A). Picture shows cells from 2 different healthy controls. Similar data were obtained from 2 additional individuals. Scale bars, 10 μm.

4.2.5 **Curtailed CD3 but not CD28 stimulation contributes to iT_{SCM} formation**

Using a low bead:cell ratio T_N were less activated and did not upregulate the memory marker CD45RO. However, it is difficult to determine the specific contribution of CD3 or CD28 molecules to T cell differentiation in this model. For this aim, we stimulated CFSE labeled T_N cells in T_{SCM}-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 we assessed naïve (CCR7, CD45RA, CD27) and memory (CD45RO, CD95) markers expression (**Figure 19A-C**). It is well known that T cell differentiation strongly correlates with T cell proliferation[127]. 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). Analyses were performed when >50 cells were available in each gate (**Figure 19A-C**). Different amounts of αCD28 had no impact on T cell differentiation (not shown), while decreasing concentrations of αCD3 resulted in the progressive shift of proliferating cells towards a $CD45RA^{+}CD45RO^{-}$ phenotype (**Figure 19A-B**). These cells also maintained the expression of CCR7 and CD27 and de novo expressed CD95 (**Figure 19C**), confirming that curtailed TCR stimulation limits T cell differentiation and favors the generation of T_{SCM} cells.

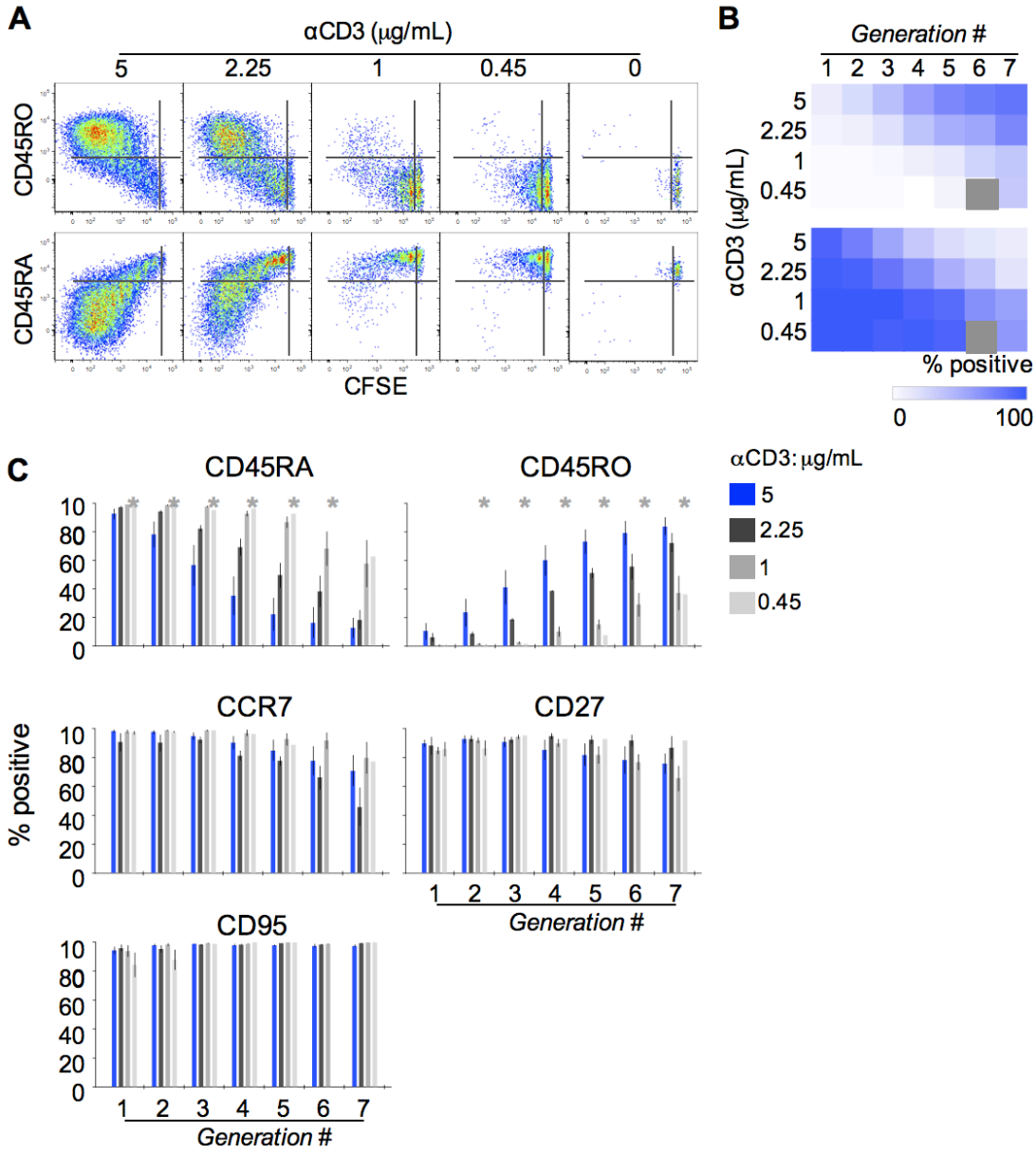

Figure 19. Curtailed CD3 but not CD28 stimulation contribute iT_{SCM} formation. (**A)** Representative flow cytometry analysis of CD45RO (top) and CD45RA (bottom) in CFSE-diluting $CD8^+$ T_N cells following stimulation with different concentrations of plate-bound αCD3 and 5 μg/mL soluble αCD28. (**B)** Heat map of data as in (A) according to CFSE generation (n=1-4, gates were not take into consideration when count/cycle was<50; grey box: data not available due to the low cell count). **(C)** Frequencies of marker expression of human $CD8^+$ T_N cells stimulated with different concentrations of plate-bound αCD3 (indicated in the legend) and soluble aCD28 (5 μg/mL). The expression of the indicated markers was evaluated in different CFSE peaks (generation #) by flow cytometry. * P<0.05 vs. 5 μg/mL aCD3, Wilcoxon test.

4.2.6 **Self-renewing capability and multipotency of iT_{SCM} cells.**

Naturally-occurring T_{SCM} are known to preferentially self-renew *in vitro* and *in vivo* compared to T_{CM} and T_{EM}, while simultaneously capable to generate more differentiated cells [128,24,15]. To assess both these aspects respectively, iT_{SCM} were collected at d7 after stimulation, stained with CFSE and induced to proliferate in response to the homeostatic cytokine IL-15 (50ng/mL, while 1ng/mL IL-15 was used as negative control) or to αCD3/2/28 beads + IL-2 and IL-12 (iT_{Eff}-polarizing condition). Similarly to *ex vivo* T_{SCM} and bulk memory cells (sorted as $CD45RO^+$, T_{MEM}), iT_{SCM} proliferated in response to IL-15, while T_N remained quiescent as expected (**Figure 20A**). A summary of CFSE dilution from 4 healthy controls is shown in **Figure 20B**. While the vast majority of iT_{SCM} maintained their original phenotype upon in IL-15 stimulation (**Figure 20C**), those in iT_{Eff}-polarizing condition differentiated to $CD45RO^+CCR7^+$ T_{CM} and $CD45RO^+CCR7^-$ T_{EM} cells (**Figure 20D**), thus indicating self-renewal capacity and multipotency, respectively.

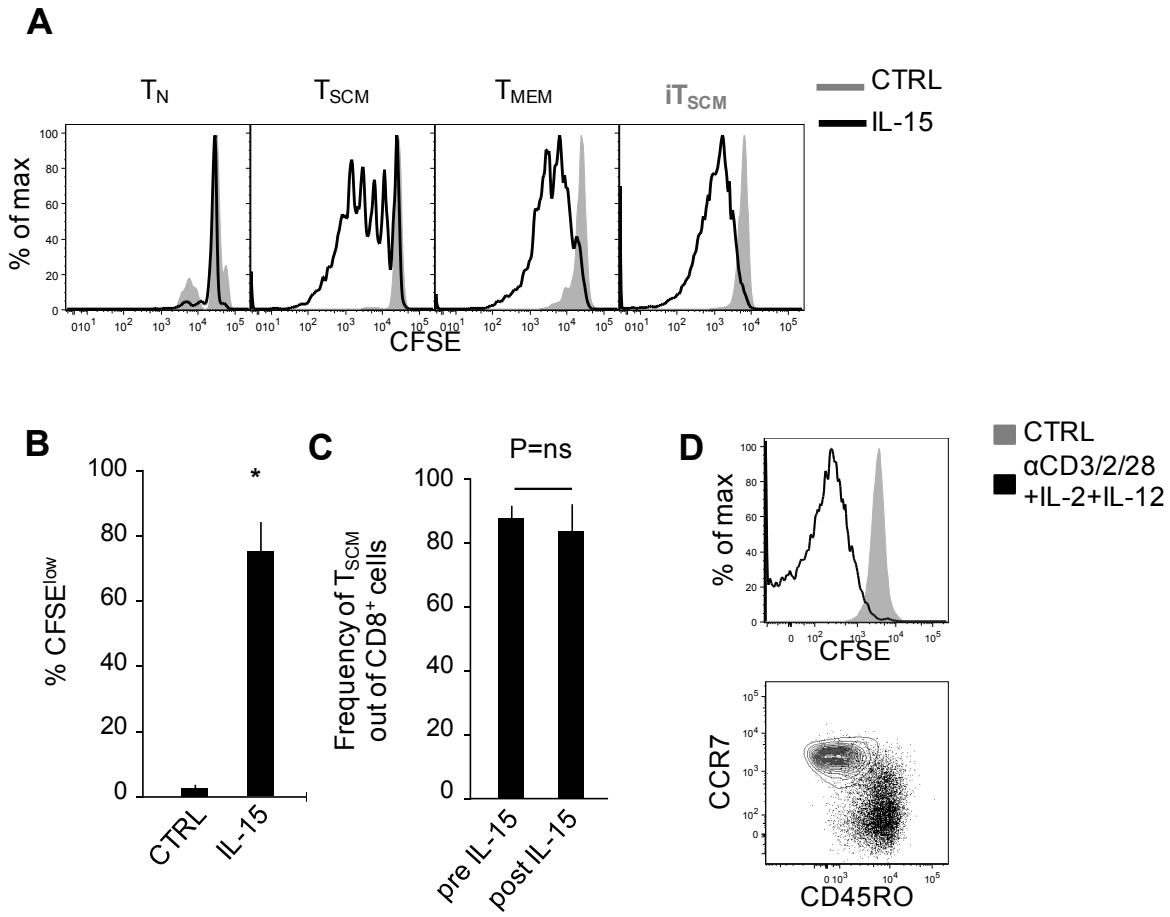

Figure 20. Self-renewing capability and multipotency of iT_{SCM} cell. (**A)** iT_{SCM} (generated as in Figure 15C) and purified T_N, T_{SCM} and total memory T (T_{MEM}; defined as $CD45RO^+$) $CD8^+$ T cells were incubated with IL-15 for 12 days (50ng/mL, black line histogram). CFSE dilution, indicative of cell proliferation, was evaluated by flow cytometry. Filled grey histograms indicates unstimulated control (IL-15 1ng/mL). **(B)** Summary of the frequencies of proliferating iT_{SCM} cells ($CFSE^{low}$) shown in (A) are depicted as mean±SEM (n=4 individuals from two independent experiments). * P<0.05 vs. control (CTRL), Wilcoxon test. (**C)** Percentage of T cells with a T_{SCM} phenotype (out of total $CD8^+$ T cells), after stimulation of T_N in iT_{SCM} condition cultures (described in Figure 15C; pre-IL-15) and after additional 12 days of stimulation with IL-15 (post-IL-15; IL-15 50ng/mL). Frequencies are shown as mean±SEM of n=4 healthy individuals from 2 independent experiments. (**D)** CFSE dilution (top) and CD45RO/CCR7 expression following αCD3/2/28 + IL-2 and IL-12 stimulation (iT_{Eff} conditions; bottom) of iT_{SCM} cells generated as in Figure 15C was measured by flow cytometry. Data shown are representative of measurements made for 3 samples from two independent experiments.

5 DISCUSSION AND CONCLUSIONS

$CD8^+$ T_{SCM} cells are rare, antigen-experienced cells. Among memory cells, T_{SCM} represent the subset endowed with superior immune-recovery, anti-tumor, self-renewal, multipotency and persistence capabilities, in mice and non-human primates[54,92,93,119,120]. However, their role in human settings it has not been demonstrated. Thus, we investigated the immune recovery of T cell subsets in the contest of Haplo-BMT followed by pt-Cy. Haplo-BMT with pt-Cy has recently been introduced as an approach that provides a donor for nearly all patients lacking an HLA-matched donor. On one hand, the presence of T cells infused with the graft enhance graft versus tumor (GVT) effects[50]. On the other hand, *in vivo* T cell depletion by pt-Cy and immunosuppressive regimen adopted to reduce GVHD, can lead to severe infections[52]. It is unclear how pt-Cy works as regards the transfer of immunity from donor to recipient. Here, we showed a nonredundant role of donor T_N-derived T_{SCM} in human T cell reconstitution after haplo-BMT and pt-Cy administration, both at the polyclonal and Ag-specific level. A proposed model of the events occurring in this scenario is depicted in **Figure 21.**

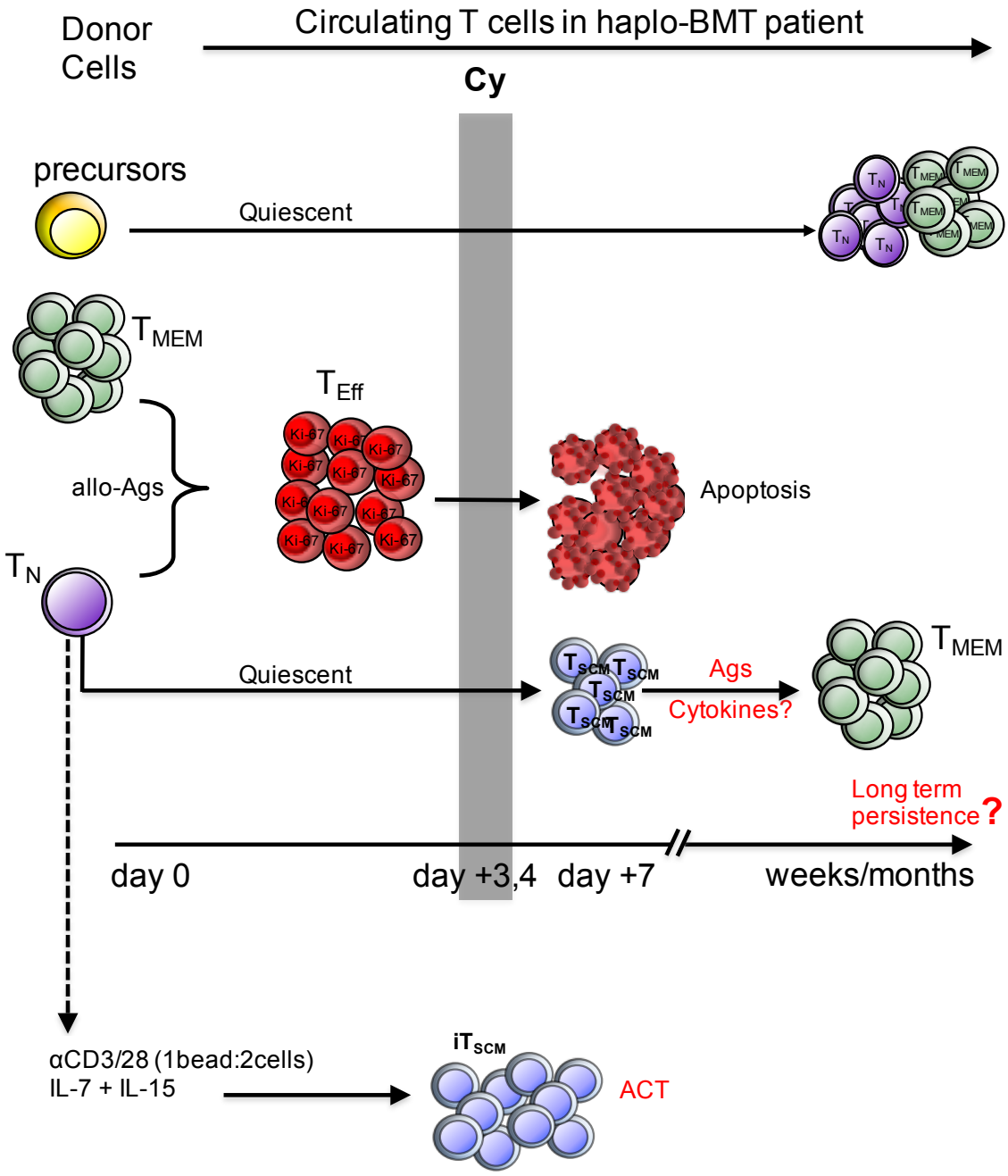

Figure 21. Proposed mechanism for immune reconstitution following haploBMT with non-myeloablative conditioning regimen and high-dose Cy after transplant, and *in vitro* T_{SCM} generation from T_N precursors for ACT. Donor BM–derived haematopoietic stem cells, lymphoid precursors, T_N and T_{MEM} are infused with the graft. Haematopoietic stem cells and lymphoid precursors survive pt-Cy and contribute to T cell reconstitution months after BMT. Conversely allo-reactive, activated and proliferating (HLA-DR$^+$, Ki-67$^+$)T_{MEM} and T_N cells are depleted by pt-Cy. Quiescent T_N spared by Cy rapidly differentiate into T_{SCM} as soon as d7 after haplo-BMT. In the following weeks, naïve-derived T_{SCM} are able to expand in response to self/tumor antigens (Ags) or homeostatic expansion, thus generating T_{MEM}. Donor T_N cells could be employed for ACT after *in vitro* T_{SCM} formation and expansion. This could contribute to faster immune recovery, protection of patients from opportunistic infections and increased GVT.

It is important to note that only donor T cells are responsible for immune recovery. It can be speculated, therefore, that the composition of the graft in terms of presence of different T cell subsets shapes subsequent immune recovery. Multiple studies have been conducted in this regard in clinical protocols over that the one investigated here, with somewhat contrasting results. I demonstrated with my work that both antigen-specific T_N (**Figure 14**) and memory T cells (published in Roberto *et al.*[129]; here not shown) survive pt-Cy and contribute to immune recovery, thus justifying future analysis of the influence of graft composition on adverse event development and disease progression. It should be noted however, that the rate of alloreactivity between donor and recipient, and thus the depletion of infused cells by pt-Cy cannot be predicted *ex vivo*. Therefore, pt-Cy treatment may play a confounding role in this regard.

Extensive analysis of T cell subsets during the first week after transplantation revealed that nonalloreactive T_N preferentially survived to pt-Cy, which were unexpectedly outnumbered by T_{SCM} -phenotype cells cells at d7 (**Figure 11**). Conversely, as much as 70% of memory/effector T cells were proliferating at d3 after transplantation and were subsequently depleted by pt-Cy (**Figure 11**). Our experiments showed that both T_N and memory T cells proliferated when stimulated with allo-APCs *in vitro* and that T_N rapidly upregulated the memory marker CD45RO, thus suggesting that the proliferating memory/effector cells detected at d3 *in vivo* also include T cells that originated from both compartments (**Figure 12**).

T_{SCM} cells represented the dominant donor T cell subset in the circulation after pt-Cy and we hypothesized they could originate from the T_N infused with the graft. Recently Cieri *et al.* suggested that the posttransplant lymphopenic environment

may favour the generation of T_{SCM} from naive precursors[37]. In line with these results, we observed that the acquisition of CD95 expression by T_N could be induced in a culture condition mimicking the allogeneic stimulation occurring *in vivo* (**Figure 12)**. A weak point of the current study is that the differentiation of T_{SCM} from T_N precursors could not be demonstrated *in vivo* at the clonal or Ag-specific level due to the paucity of T cells that could be recovered in the post-transplant setting. However, experiments conducted with polyclonal T cell populations (**Figure 12**) supported the concept that T_N can generate T_{SCM} cells in the context of allo-recognition. In line with our observations Cieri *et al*. showed that the TCRB sequences of T_N sorted from leukapheresis product (LP) were found in all memory subsets isolated from peripheral blood of Pts at d30 after transplantation, suggesting that T_N are able to generate all subsets of more differentiated cells, including T_{SCM}. Moreover, the authors observed that sequences present in LP-T_{CM} were found preferentially in T_{CM} and T_{EM}/T_{EFF} repertoires at d30 after transplant, whereas less than 10% were found in the T_{SCM} repertoire. Sequences retrieved in LP-$T_{EM/EFF}$ were found mainly within d30 T_{EM}/T_{EFF}, although conversion into T_{CM} was also observed (27%). Again, less than 10% of TCRB sequences identified in LP-T_{EM}/T_{EFF}were retrieved in the T_{SCM} compartment at d30. These and our data support a progressive model of differentiation according to the relationship $T_N \rightarrow T_{SCM} \rightarrow T_{CM} \rightarrow T_{EM} \rightarrow T_{EFF}$. Although only T cells isolated from PB could be analysed for ethical reasons, expansion or redistribution of pre-existing donor T_{SCM} from lymphoid tissues seems unlikely, given the preferential depletion of proliferating cells by Cy administration and the virtual absence of T_{SCM} in the infused BM, respectively.

Our experiments indicate that MART-1 and WT-1 specific T_N cells infused with the graft survived pt-Cy, persisted in the patient and subsequently generated memory

cells (**Figure 14**). Self Ag-specific memory cells are rarely observed in the peripheral blood of healthy individuals. As regards to MART-1, specific-memory cells were observed in metastatic melanoma patients, but not in healthy individuals[15,130], thus suggesting a role of Ag, inflammatory cytokines and costimulation in their generation[131]. Similar results were obtained in the context of tumors over-expressing WT-1[132]. it has also been shown that T_N cells can differentiate into memory cells in the absence of antigenic stimulation, rather in response to the lymphopenic environment (*i.e.*, increased levels of homeostatic cytokines IL-2, IL-7 and IL-15)[40,133]. Further studies are required to clarify whether increased availability of homeostatic cytokines rather than cognate Ag stimulation are the major mechanism involved in self/tumor antigen-specific differentiation of T_N into effectors and memory cells. Our data suggest that, in the lymphopenic environment that is observed post-transplant, pt-Cy-resistant donor T_N cells generates T_{SCM} both at the polyclonal and Ag–specific level, and that they subsequently contribute to immune recovery.

Despite the molecular mechanisms that are at the basis of long-term memory formation have been in part elucidated in the past decade, it is still unclear whether the same signals regulate T_{SCM} cell differentiation in humans. The expansion of high numbers of T_{SCM} with enhanced multipotency, self-renewal, anti-tumor and long-term persistence capacity has important practical implications for conditions where potent and long-lasting T cell responses are needed, including HSCT and ACT. Therefore, in the second part of this work we concentrated our effort in optimizing a method to generate high numbers of $CD8^+$ T_{SCM} derived from unprimed T_N precursors. We showed that curtailed TCR activation induced the generation of early-differentiated $CD45RA^+CD45RO^-$ T_{SCM} cells from T_N cells (**Figure 15**), and

that this was associated with a weak upregulation of transcription factors and molecules involved in effector differentiation and T cell activation (**Figure 18**). Similar phenotypes could be observed by using beads other than Miltenyi beads, *i.e.*, Dynabeads (Thermo Fisher, data not shown), that are currently used in clinic trials for the expansion of T cells in ACT.

Previous reports demonstrated that T_N stimulated with a high bead:cell ratio (*i.e.* 3:1) induced an unexpected double positive $CD45RO^+CD45RA^+$ phenotype that is rarely found *in vivo*[37]. It has been previously demonstrated that the strength of TCR signaling can influence effector and memory formation[134]. Therefore, we hypothesized that lowering the amount of signals to be delivered by beads to T cells could influence T_{SCM} cell formation. Indeed, by using a 1:2 αCD3/28 bead:cell ratio, we could generate T_{SCM} cells with a naturally-occurring $CD45RO^-CD45RA^+$ phenotype. To better define the role of TCR stimulation in this regard, we delivered differential signals via CD3 and CD28 by titrating antibodies capable to activate these signalling molecules and we found a role for CD3, but not for CD28 in the generation of the $CD45RO^+CD45RA^-$ T_{SCM} phenotype (**Figure 19**). Thus, the strength of TCR signal via CD3 can influence the type of progeny derived from T_N cells, while costimulation plays no role in this regard, at least in this *in vitro* model. The generated T_{SCM} showed similar features to naturally-occuring T_{SCM} in terms of proliferation, self-renewal capabilities, multipotent abilities and cytokine profile in response to Ag-specific and polyclonal stimulation (**Figures 15, 16** and **19**). Interestingly, we could not identify differences in the quality of the T_{SCM} response following priming with low and high affinity MART-1 peptides, EAA and ELA respectively. EAA binds TCR with a decreased strength compared to his heterolitic form ELA, and resulted in lower total number of MART-1 specific $CD8^+$ T cells. EAA

selects those TCRs with the highest avidity that is in turn associated with increased anti-tumor activity[135]. For this reason, it has been supposed that vaccines delivering the EAA peptide would induce a better response compared to ELA containing vaccines[135]. Vaccination regimens with ELA rather than EAA, coupled with CpG and Montanide in melanoma patients, resulted in early generation (< 3 month) of MART-1 specific T_{SCM}. These cells showed long term persistence capabilities[135]. Differently, studies conducted in rhesus macaques infected with simian immunodeficiency virus (SIV), showed that Ag-specific T_{SCM} appeared much earlier, *i.e.*, 7 days after infection[24]. These cells expanded clonally and coincidentally with the formation of T_{Eff} cells, but showed higher expression of self-renewal and persistence molecules such as BCL2, MCL1, LEF1 and lower expression of activation (HLA-DR) and proliferation (Ki-67) markers compared to T_{Eff}, thereby suggesting that they have higher capacity to develop into stable memory cells[24]. Overall, this could reflect limited TCR stimulation occurring *in vivo*.

iT_{SCM} also showed lower mitochondrial content compared to iT_{Eff}. (**Figure 18**) In line with these results, a previous report analysed the mitochondrial content of sorted $CD8^+$ T cell subsets by transmission microscopy and observed that mitochondrial content increases in memory subsets (T_{CM} and T_{EM}) compared to T_N cells[126]. In contrast, Van de Windt wt al., showed that T cells activated in the presence of IL-2 for 3 days and switched in IL-15 for 2 additional days, an approach to mimic the generation of memory T cells *in vitro*, had an increased mitochondrial content compared to those kept in IL-2 (and thus resembling effecor cells)[136]. Differences in protocols used to differentiate memory and effector T cells could explain these contrasting results. When generating T_{SCM} cells from T_N, we avoid exposure to IL-2 which could prime T cells for effector differentiation and apoptosis.

The general feeling among laboratories generating cells for ACT is that IL-2 should be avoided in order to limit excessive differentiation[137] and loss of the anti-tumor capacity[97].

Inhibition of effector differentiation of T_N precursors has been achieved in multiple ways, such as pharmacological intervention, cytokine cocktails and genetic manipulation[138]. More recently it has been demonstrated that T_{SCM} formation can be induced by the use of bromodomain and extra-terminal motif (BET) inhibitors, which in turn limit BATF expression[111]. Of note, BATF is part of a multiprotein DNA-binding complex comprising Jun and IRF4 or IRF8 and is involved in the formation of effector T cells[139]. Our experiments suggest that a mild IRF4 and IRF8 upregulation are necessary for the activation of T_N cells but dispensable of T_{SCM} formation.

In conclusion, we show that adoptively-transferred T_N cells generate T_{SCM} cells in the context of h-HSCT, and that these cells play a non-redundant role in subsequent T cell reconstitution. Given the relative polyclonality of these T_N-derived T_{SCM}, adoptive cell transfer of such cells generated in the laboratory could be conceived. We further show that T_{SCM} cells can be generated from T_N precursors by curtailed TCR stimulation during priming. Current effort in the laboratory is devoted to identify further downstream molecular signals that can be further exploited in the context of T_{SCM} differentiation and ACT.

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