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Role of memory T cell subsets for adoptive immunotherapy

Dirk H. Busch^{1,2,5,§}, Simon P. Fräßle^{1,2}, Daniel Sommermeyer^{3,4}, Veit R. Buchholz¹, and Stanley R. Riddell^{2,3,4,§}

¹ Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München (TUM), Munich 81675, Germany

² Focus Group “Clinical Cell Processing and Purification”, Institute for Advanced Study, TUM, Munich 81675, Germany

³ Program in Immunology, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

⁴ Department of Medicine, University of Washington, Seattle, WA 98109, USA

⁵ National Center for Infection Research (DZIF), Munich 81675, Germany

Abstract

Adoptive transfer of primary (unmodified) or genetically engineered antigen-specific T cells has demonstrated astonishing clinical results in the treatment of infections and some malignancies. Besides the definition of optimal targets and antigen receptors, the differentiation status of transferred T cells is emerging as a crucial parameter for generating cell products with optimal efficacy and safety profiles.

Long-living memory T cells subdivide into phenotypically as well as functionally different subsets (e.g. central memory, effector memory, tissue-resident memory T cells). This diversification process is crucial for effective immune protection, with probably distinct dependencies on the presence of individual subsets dependent on the disease to which the immune response is directed as well as its organ location.

Adoptive T cell therapy intends to therapeutically transfer defined T cell immunity into patients. Efficacy of this approach often requires long-term maintenance of transferred cells, which depends on the presence and persistence of memory T cells. However, engraftment and survival of highly differentiated memory T cell subsets upon adoptive transfer is still difficult to achieve. Therefore, the recent observation that a distinct subset of weakly differentiated memory T cells shows all characteristics of adult tissue stem cells and can reconstitute all types of effector and memory T cell subsets, became highly relevant. We here review our current understanding of memory subset formation and T cell subset purification, and its implications for adoptive immunotherapy.

§ to whom correspondence should be addressed: dirk.busch@tum.de and sriddell@fredhutch.org.

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1.1 Introduction

Antigen-specific T cells can provide highly efficient and long-lasting immunity against infections. Furthermore, T cell immune protection can be targeted towards some cancers [1]. Physiological antigen-specific T cell responses originate from a small number of naïve precursor cells that are vigorously expanded upon the initial priming process [2]. During this expansion phase, most activated T cells acquire effector functions. Following this effector phase most T cells die, and only a small fraction survives beyond the contraction phase and stably persist as memory T cells even in the absence of antigen [3]. Technologies allowing multi-parameter detection on single cell level have revealed a high degree of phenotypic and functional diversity within epitope-specific T cell populations both during the effector as well as during the memory phase [4-6]. These patterns of diversification generated during infection or in response to vaccination seem to be important for the quality of antigen-specific immunity [7,8].

Adoptive T cell therapy aims at the therapeutic transfer of antigen-specific T cells. According to the concept of memory T cell subset diversification and the specific role of individual subsets for protective immunity, this approach relies on effective engraftment or regeneration of effector and memory T cell populations after cell transfer [9]. Therefore, a deeper understanding of the generation and maintenance of T cell subsets will become key for the generation of highly effective T cell products.

1.2 Memory T cell subsets

The relevance of diversification in the context of immunological memory first became apparent with the observation that memory T cells can be subdivided by distinct patterns of adhesion molecules and chemokine-receptors expressed on their cell surface [10]. These phenotypic differences translate into migratory differences: 'Central memory T cells' (TCMs) continuously recirculate – like naïve T cells (TNs) – via the blood stream to lymphoid organs whereas 'effector memory T cells' (TEMs) preferentially migrate to nonlymphoid tissues [11]. The recent identification of tissue-resident memory T cells (TRMs) [12,13], which might be further subdivided depending on the respective organ they reside in [14], further adds to the complexity and diversity of the memory T cell compartment.

Beyond phenotypical subset diversification and distinct tissue distribution or migration patterns, T cells can develop into lineages producing characteristic patterns of effector cytokines. This was first described for CD4⁺ T cells by Tim Mosmann and colleagues with the identification of T helper 1 (Th1) and Th2 cells [15], and has been expanded over the past years to other lineages encompassing Th17 cells, follicular T helper cells and regulatory T cells [16]. Similar effector cytokine patterns have been described for CD8⁺ memory T cells as well as innate lymphocytes [17]. Although there seems to be a degree of plasticity between different effector cytokine lineages, they can be maintained for long periods of time ('cytokine memory') [18].

The identification and classification of distinct memory T cell subsets by surface markers is still challenging, as combinations of different markers are necessary to narrow down more or less homogeneous subsets. Results from different studies have to be interpreted with caution since different antibodies and marker combinations are used for the identification of similar subsets, and also due to differences in markers between animal models and humans. Typical marker combinations for the identification of major subsets in humans are CD45RO⁺ / CD62L⁺ / CD127⁺ / CD27⁺ / CCR7⁺ for TCMs, CD45RO⁺ / CD62L⁻ / CD127⁺ / CD27⁺ / CCR7⁻ for TEMs and CD45RO⁺ / CD62L⁻ / CD103⁺ / CD69⁺ for TRMs; in mice CD44⁺ / CD62L⁺ / CD127⁺ / CD27⁺ / KLRG1⁻ for TCMs, CD44⁺ / CD62L⁻ / CD127⁺ / CD27⁺ / KLRG1⁺ for TEMs and CD44⁺ / CD62L⁻ / CD103⁺ / CD69⁺ for TRMs [14,19] (Fig. 2). However, various other surface markers (e.g. CX3CR1) [20] have been proposed to refine the identification of memory T cell subsets.

1.3 Memory T cell Ontogeny and Stemness

It has become increasingly accepted in the field that specific patterns of effector and memory T cell subset diversification influence the quality, efficacy and longevity of T cell immunity [7,8]. Therefore, many groups are currently developing strategies to skew *in vivo* T cell differentiation towards defined subsets. Although different infections or vaccination protocols indeed can lead to distinct T cell subset distributions [21,22], the underlying differentiation processes are still incompletely understood and controversially discussed. In the past at least three different models for the *in vivo* generation of effector and memory T cell subsets have been proposed [23]. These differ mainly with respect to the proposed timing of memory T cell development during the expansion phase of the immune response.

The so-called ‘linear differentiation model’ (Fig. 1, upper graph) postulates that upon priming all recruited T cells vigorously proliferate and only after reaching peak expansion, does a small fraction of responding cells further develop into long-living subsets, like TCM, TEM and TRM while the remaining T cells die. This model predicts that T cell clones undergoing excessive proliferation during the expansion phase will be overrepresented in the memory T cell pool [24,25].

Through implementation of KLRG1 surface staining the group of Susan Kaech identified that memory and effector subset diversification occurs well before peak expansion of CD8⁺ T cell responses and proposed the so-called ‘decreasing potential model’ [26,27] (Fig. 1, middle graph). According to this model early effector T cells undergo 8-10 cell division and then diversify into short-lived effectors (SLECs) that continue to proliferate toward the tail end of the response and memory precursor effector cells (MPECs) that proliferate less but are more likely to persist into the memory phase.

An even earlier determination of memory and effector T cell fates is proposed by the so-called ‘progressive differentiation model’ (Fig. 1, lower graph). Originally developed by Federica Sallusto and Antonio Lanzavecchia this model suggests that memory T cells are derived directly from naïve T cells and further differentiate into shorter-lived effector subsets [28,29]. In this model TCMs maintain a high proliferative capacity and are capable to generate progeny with effector characteristics. Towards the end of the progressive

differentiation pathway more differentiated subsets arise, which have largely lost their proliferative capacity and are maintained as TEM or TRM or as SLECs are deleted from the T cell repertoire.

In line with the progressive differentiation model, especially the group of Nicholas Restifo identified similarities in between the generation of T cell immune responses and the maintenance of tissues with high cell turnover (like skin, mucosa or bone marrow) [30]. Maintenance of such tissues crucially relies on a small subset of adult tissue stem cells, which are defined by their capacity to differentiate into functionally diverse daughter cells (multipotency) as well as to self-renew into further tissue stem cells [31,32]. In analogy, it was proposed that the T cell system contains within the memory T cell compartment a specialized subset with stem cell-like characteristics [33]. This hypothesis suggests that in an adoptive transfer setting only a defined subset of stem cell-like T cells will be capable of reconstituting immunity for extended periods of time. Over the past years researchers have searched intensively for this enigmatic stem cell-like subset of T memory cells. The Restifo group identified a $CD45RA^+ / CD45RO^- / CCR7^+ / CD62L^+ / CD28^+ / CD127^+ / CD27^+ / CD95^+$ subset of weakly differentiated T cells and coined the term T memory stem cell (TSCM) for it [34] (Fig. 2). This subset is detectable in very low numbers among circulating human PBMCs [34,35]. In addition, an antigen-experienced but $CD44^{low}$ memory T cell subset has been suggested as the murine homologue of human TSCMs [36,37]. Indeed, T cells with a TSCM phenotype are maintained long-term, are weakly differentiated and show a molecular signature (e.g. high sca-1 expression) that is found in other tissue stem cells as well [30].

Although TSCM and TCM are phenotypically distinct, gene expression profiling and functional analyses demonstrate that differences are quite small as compared to the differences with TEM or terminally differentiated effector T cells [30] (Fig. 2). Concerning the *in vivo* persistence of these relatively undifferentiated memory subsets in humans the group of Daniel Speiser recently provided impressive data showing that T cells specific to yellow fever virus could be found in individuals up to 35 years after vaccination [38]. Another recent study used sequencing of specific integration sites to follow the long-term persistence of genetically modified T cell subsets after donor lymphocyte infusion [39]. All these studies strongly support the ‘adult tissue stem cell’ model, suggesting that recall expansion and immune reconstitution are dependent on a specialized compartment of weakly differentiated T cells. In fact, one might speculate that TCMs and TSCMs belong to the same memory stem cell pool (Fig. 2).

The recent development of sophisticated *in vivo* single cell fate mapping technologies has allowed a deeper understanding of the dynamic processes underlying memory and effector differentiation [40]. Studies utilizing genetic barcoding [41,42] or single cell transfer experiments [43-45] and advanced mathematical modeling based on these datasets [44,46] provided strong support for a strictly progressive differentiation pathway during priming, expansion and subset diversification of antigen-specific $CD8^+$ T cells. Transgenerational single cell transfer experiments recently demonstrated unequivocally that individual TCM can both self-renew and generate a diverse offspring *in vivo* [45]. For these experiments, so-called ‘clonogenic’ assays were performed in which single resting TCMs were transferred

into recipient mice to demonstrate their potency during *in vivo* rechallenge through *Listeria monocytogenes* (*L.m.*) infection. Single TCM derived from this first rechallenge were then transferred into naïve recipients and exposed to *L.m.* infection again. Such experiments were found to be impossible with single TEM and even populations of 100 transferred TEMs re-expanded weakly and were lost upon repetitive transfer. In stark contrast, single secondary TCM demonstrated virtually identical potency as the primary TCM they had descended from and thereby, proved their self-renewal capacity.

1.4 Clinical purification of T cell subsets

As summarized above, defined T cell subsets have different qualities in terms of effector function, proliferative capacity as well as immune reconstitution and memory formation. Therefore, it is believed that T cell subset purification can be used to generate cell products with more predictable efficacy and safety profiles for adoptive immunotherapy. Especially subsets derived from the memory stem cell pool (such as TCMs or TSCMs) have gained substantial attention, as the adoptive transfer of even very low numbers of T cells from these subsets can reconstitute robust and long term maintained immune responses [45,47]. Interestingly, it has recently been suggested that the additional presence of other, more differentiated memory T cell subsets can even disturb the establishment of long-term immunity [48] – emphasizing the relevance of subset purification for successful adoptive T cell therapy. However, clinical purification of defined T cell subsets is hampered by the fact that multiple parameters are required for their identification.

Flow cytometry using fluorochrome-conjugated antibodies targeting combinations of subset defining surface molecules is most commonly used for multi-parameter T cell subset purification in basic research. However, the relatively complicated machinery makes it difficult to transfer this approach to clinical cell selection under GMP conditions and/or in completely closed systems. Nevertheless, first jet-in-air flow cytometers with disposable nozzles and sterile fluidics have been transferred to GMP facilities for clinical cell sorting (like the Influx from Becton Dickinson; [49]). Several microfluidics-based systems are currently under development to allow clinical fluorochrome-conjugated antibody-based multi-parameter purification even in fully closed systems (like the “Owl” from Miltenyi Biotec). The use of paramagnetic bead-conjugated antibodies for cell labeling and subsequent purification represents currently the gold standard in clinical cell purification (like the CliniMACSystem from Miltenyi Biotec). However, this approach usually doesn't allow positive selection of defined cell populations over multiple parameters. Recently developed reversible staining technologies [50,51], which enable complete removal of cell-labels upon selection for serial enrichment strategies, overcome this problem and further facilitate clinical cell selection of defined cell subsets over multiple parameters. In addition, removal of cell selection reagents before clinical application might help to reduce negative impacts derived from the cell label itself (e.g. reduced cell viability/functionality, label-mediated toxicity or immunogenicity). Therefore, reversible labeling or innovative label-free cell selection systems [52] are receiving increasing attention for clinical cell sorting.

1.5 Relevance of T cell subsets for adoptive transfer of primary (unmodified) T cells

There is strong scientific rationale for evaluating the adoptive transfer of unmodified T cells for clinical medicine based on current understanding of the function of adaptive T cell immunity in human health. An exciting application being pursued by many laboratories is the use of adoptive T cell transfer to augment an inadequate endogenous immune response to tumors. This can be accomplished by selecting T lymphocytes that have the requisite antigen specificity from the patient's blood or tumor infiltrate (TIL), expanding the cells numerically *in vitro*, and re-infusing them to mediate a therapeutic effect. A notable success of adoptive therapy with unmodified autologous T cells pioneered by Steven Rosenberg is in the treatment of metastatic melanoma, where tumor reactive T cells can be expanded from tumor infiltrates as polyclonal populations, and administered in large cell doses ($10^{10} - 10^{11}$ cells) followed by high dose IL-2 to mediate partial or complete tumor regression [53,54]. Tumor regression was also observed with the transfer of *in vitro* expanded melanoma reactive T cell clones that were derived from the peripheral blood, although durable responses were less frequent than observed with TIL [55]. In both of these settings, the persistence of transferred tumor-reactive T cells in the blood measured by analysis of clonotypic T cell receptor gene sequences present in the infused population or by MHC tetramer staining correlated with antitumor activity [53,55]. Dudley and colleagues showed that T cell persistence is improved by administering lymphodepleting chemotherapy prior to the infusion of T cells to increase the levels of cytokines such as IL15 and IL7 that function to maintain normal lymphocyte survival and homeostasis [56,57]. However, even with lymphodepletion prior to cell transfer, the *in vivo* persistence of infused T cells is unpredictable in individual patients. Tumor-reactive T cells that are present in tumor bearing patients are prone to be chronically stimulated by antigen, which can result in increased expression of negative regulatory molecules such as PD-1 [58], and the acquisition of a differentiated effector phenotype that may affect their ability to proliferate and survive after adoptive transfer. This is supported by analysis of the phenotype of TIL after *in vitro* expansion, which suggested that cell products that retain markers such as CD27 and CD28 that correspond with a less differentiated phenotype were more efficacious in adoptive therapy [59]. Thus, improving the outcome of TIL therapy may require techniques for isolating T cells that are selected for specificity for tumor associated antigens and for expanding these cells in ways that preserve or impose a less differentiated phenotype to provide superior proliferation, survival, and sustained effector function *in vivo*.

T cells specific for tumor-associated antigens have also been isolated and expanded from naïve precursors in the blood and used for adoptive therapy. A challenge with this approach, particularly for self-antigens where the available naïve T cell repertoire has undergone thymic selection, is the outgrowth of T cells that express TCRs with low avidity for their target antigen. One advantage of starting with TN is that their differentiation can be controlled *in vitro* by the use of cytokines such as IL-7, IL15, and IL21, and/or small molecules that act e.g. via the WNT-beta-catenin [34,36,37] pathway and promote the acquisition of TSCM or TCM phenotypes. In murine models of ACT for cancer, TSCM and TCM derived from TN under such culture conditions show improved potency in adoptive

therapy [30,60-64]. To optimize the culture of TN, it may be important to purify these cells prior to their stimulation it has recently shown that the presence of memory T cells during stimulation of TN can accelerate their differentiation through non-apoptotic Fas signaling and provide less effective cell products for adoptive transfer [48]. This unanticipated interaction between T cells from different subsets during *in vitro* culture highlights potential benefits of clinical cell selection methods that allow enrichment of specific T cell subsets that have desired properties.

The reconstitution of T cell responses to pathogens in patients that have undergone allogeneic hematopoietic stem cell transplant is a special setting where primary T cells can be derived from the quiescent memory pool of T cells in the donor and used as a starting source for adoptive therapy. In early studies, T cell clones or polyclonal T cells specific for cytomegalovirus or Epstein Barr virus were isolated and expanded from virus seropositive stem cell donors, and shown to persist and mediate antiviral activity after transfer to their respective recipients [65,66]. In the CMV studies, multiple T cell clones were transferred and analysis of persisting T cells using clonal TCR genes to distinguish individual clones demonstrated that only a subset of the transferred virus-specific T cells were detected in the blood late after adoptive transfer [65]. Studies in a non-human primate model in which gene-marked CMV-specific T cell clones were derived from sort-purified TCM or TEM subsets demonstrated that those derived from TCM were uniquely capable of persisting for years after transfer and establishing diverse memory subsets, showing that even after *in vitro* activation and culture therapeutic T cells derived from TCM have a superior capacity to persist *in vivo*, [67,68].

The capacity of pathogen-specific memory T cells to proliferate *in vivo* in response to infection and differentiate to effector cells, suggested that refined approaches that rely on the isolation and direct adoptive transfer of even small numbers of pathogen-specific memory T cells from the blood of the donor might be effective in restoring protective immunity in patients. This has now been evaluated using reversible HLA Streptamers to enrich small numbers (total $\sim 3 \times 10^4$ T cells or $< 4 \times 10^3$ T cells per kg of recipient body weight) of CMV-specific T cells directly from the blood of the allogeneic hematopoietic stem cell donor, and the immediate transfer of these cells without *in vitro* culture resulted in vigorous *in vivo* expansion in response to virus reactivation and control of infection [47].

1.6 Relevance of T cell subsets for genetically engineered T cell products

Gene transfer technologies using viral or non-viral delivery can now reliably introduce transgenes encoding TCRs or synthetic chimeric antigen receptors (CARs) that confer antigen specificity and retarget primary T cells to recognize tumors or pathogens [69]. Dramatic evidence for the therapeutic potential of genetically redirected T cells comes from studies targeting CD19 with CAR-T cells in patients with acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, and non-Hodgkin's lymphoma (NHL), where long-term remissions have been achieved in a significant subset of patients [70-74]. A majority of studies are genetically modifying T cells without *a priori* subset selection resulting in phenotypically and functionally heterogeneous cell products in individual patients. One potential problem with transducing T cells without regard to composition is that patients

with previous exposure to lymphocytotoxic chemotherapy to treat the malignancy are often very lymphopenic and have higher frequencies of TEM in both CD4 and CD8 subsets [75]. Some centers have excluded nearly one quarter of potentially eligible patients due to failure to expand CD19 CAR-T cell products after stimulation and transduction of unselected T cell populations [76].

An advantage of genetic modification to derive therapeutic cell products is that T cells from subsets that can proliferate *in vitro* and are more effective in preclinical models can be selected for genetic modification, cultured under specific conditions that retain these properties, formulated into defined compositions, and studied for therapeutic potency (Fig. 3). Preclinical human tumor xenograft models have been used to examine the potency of genetically modified T cells derived from different subsets that are sort-purified before transduction. These results have shown that CAR-T cells manufactured from CD8⁺ and CD4⁺ TN and TCM subsets are readily produced and more potent than those derived from the TEM subset, and demonstrated synergistic antitumor activity of combining the optimal CD8⁺ and CD4⁺ T cell subsets in defined ratios [75]. Subsets with superior antitumor activity display more rapid and sustained *in vivo* proliferation and *in vivo* persistence that is necessary to achieve eradication of large established tumors. The principles established in these models are now being tested in clinical trials and the preliminary results show that selecting T cell subsets and manufacturing CAR-T cell products is feasible in even severely lymphopenic patients and the CAR-T cells exhibit reproducible *in vivo* expansion and marked antitumor activity when administered in very low cell doses [75]. Improvements in clinical cell selection methodology and use of cell culture conditions that promote retention of TSCM and TCM properties in a larger fraction of the transduced T cells may further enhance activity and facilitate large scale application of these new therapeutic modalities.

1.7 Conclusions

Adoptive T cell therapy relies on effective engraftment or regeneration of effector and memory populations from primary (unmodified) or genetically engineered T cells. According to the concept of subset diversification with distinct functions and characteristics of subpopulations, it becomes increasingly acknowledged that pre-selection of defined subsets before *in vitro* gene modification and/or expansion or for direct adoptive cell transfer of (uncultured) primary T cells is a powerful strategy for the generation of most effective cell products for immunotherapy.

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Highlights

- Differentiation status of transferred T cells is a crucial parameter for effective adoptive immunotherapy.
- A distinct subset of weakly differentiated memory T cells resembles all characteristics of adult tissue stem cells.
- Pre-selection of defined subsets is a powerful strategy for more effective clinical T cell products.

Figure 1. Proposed models of memory T cell differentiation

The three major models for memory T cell differentiation differ mainly concerning the time point at which fate decisions into long-living memory T cells and short-living terminally differentiated effector T cells occur. In the linear differentiation model memory T cells differentiate only after clonal expansion has reached its peak and thereby originate from heavily proliferated effector T cells. In the decreasing potential model memory T cells separate from short-lived effector T cells before the peak of expansion and are thereby derived from a population of less proliferated early effector cells. In the progressive

differentiation model memory T cells are derived directly from naïve T cells and proliferate slowly but generate short-living, rapidly expanding effector progeny throughout the expansion phase.

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Figure 2. Proposed ontological relationships of memory and effector T cell subsets in human and mouse

T naïve-like memory cells, T memory stem cells (T_{SCM}) and central memory T cells (T_{CM}) that recirculate through blood and lymphoid organs are thought to be capable of self-renewal. T_{CM} are however, the only subset for which this capacity has been proven through clonogenic (i.e. single cell-based) assays. Effector memory T cells (T_{EM}) and localized tissue resident memory T cells (T_{RM}) are situated in peripheral organs, recirculate to a lesser extent or not at all and are thought to re-expand weakly upon renewed antigen challenge. Terminally differentiated effector T cells (T_{EFF}) are short-lived and incapable of re-expansion. T_{RM} may be derived from T_{CM} or T_{EM} into the tissue. Whether or not T_{RM} cells are capable to generate further T_{EFF} cells upon antigen challenge is currently unclear. Memory T cell subsets are distinguished by the indicated surface markers. Marker expression is indicated below the corresponding subset. These markers also serve as potential targets for clinical enrichment strategies.

Figure 3. Scheme of the steps involved in selecting and/or engineering T cells of defined specificity and phenotype for adoptive therapy

A. Patients undergo leukapheresis or blood collection to provide heterogeneous populations of T cells that contain variable frequencies of CD4 and CD8 T cells and TN, TSCM, TCM and TEM subsets. B. Clinical selection methods capable of selecting on multiple parameters are used to select desired antigen specificity and subset based on cell surface phenotype. C. In the case of genetic modification, selected T cells are activated and transduced with gene delivery vectors to express a T cell or chimeric antigen receptor. D. Selected or transduced cells may be further expanded using cytokines or small molecules that retain differentiation states that facilitate therapeutic efficacy. E. T cells are reinfused to patients in cell doses that achieve the desired therapeutic effect.