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Sorting through subsets: Which T cell populations mediate highly effective adoptive immunotherapy?

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Abstract

CD8$^+$ T cells have been described as being naïve (T_N) or one of four antigen-experienced subtypes representing a continuum of differentiation and maturation: stem cell memory (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector T cells (T_{EFF}). In mice, adoptive cell transfer (ACT) of less differentiated T_N, T_{SCM} and T_{CM} subsets have consistently demonstrated superior *in vivo* expansion, persistence, and antitumor capacities relative to the more differentiated T_{EM} and T_{EFF} cells. Retrospective analyses from human ACT trials have confirmed that transfer of less differentiated T cell subsets is highly correlated with objective clinical responses. These findings, combined with the recent ability to convey *de novo* antigen reactivity with high efficiency through genetic engineering of exogenous T cell or chimeric antigen receptors, now challenge the field with three important questions: 1) how should less differentiated T cell subsets be isolated for human clinical trials?; 2) what is the best means of expanding T cells *ex vivo* in such a way as to not corrupt the beneficial traits of the younger subsets?; and 3) is it necessary to physically separate younger subsets from their more differentiated counterparts? Answering these questions will allow for the rational development of the next generation of highly effective and potentially curative T cell therapies for the treatment of cancer.

Introduction

Adoptive cell transfer (ACT), the *ex vivo* expansion and re-infusion of antigen (Ag)-specific T cells to patients, represents a highly effective and potentially curative systemic therapy for patients with advanced solid and hematologic cancers[1,2,3,4,5], recurrent viral diseases[6] and post-transplantation lymphoproliferative disease.[7] Historically, the most pressing technical issue in ACT therapies has been the generation of a sufficient quantity of Ag-specific T cells for transfer.[8,9] For some solid cancers such as melanoma, an elegantly simple solution to this problem was achieved through the discovery that T lymphocytes infiltrating tumor deposits (or TIL cells) frequently possess specific reactivity against autologous or human leukocyte antigen (HLA)-matched tumor lines.[10] When TIL cells are obtained from

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surgically resected tumor masses, expanded non-specifically *ex vivo*, and subsequently re-infused into patients in conjunction with a lymphocyte depleting pre-conditioning regimen that includes total body irradiation[11], cancer regression can be observed in the majority of cases.[12,13]

Beyond Ag specificity, T cells are heterogeneous with respect to a myriad of other parameters, including anatomic localization, proliferative and engraftment potentials, as well as cytokine secretion, metabolic, and gene expression profiles[14,4,15,16]. In principle, each of these parameters may independently influence a T cell's ability to mediate cancer regression following ACT. As a matter of course, however, many of these attributes tend to cluster together in discrete, easily discernible populations defined by a characteristic pattern of cell surface markers detectable through the use of fluorescent-activated cell sorting (FACS)[17] or, more recently, mass spectrometry[18]. A straightforward and functionally significant means of classifying T cell subsets can be accomplished by assessing for the co-expression of the lymphoid homing molecules L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7). T cells which display these 2 molecules have a propensity to home to secondary lymphoid structures where they can actively survey professional antigen presenting cells for the presence of cognate Ag. Cells in this category include naïve T cells (T_N) as well as two Ag experienced memory T cell populations: the recently identified T memory stem cell (T_{SCM})[19,20,21] population and central memory T cells (T_{CM})[22]. Although in humans both T_N and T_{SCM} express the RA isoform of CD45, T_{SCM} can be distinguished from T_N based on the expression of the IL-2/IL-15β chain receptor (CD122) and Fas (CD95). Central memory T cells, on the other hand, have acquired the expression of the prototypical human Ag experienced T cell marker, CD45RO. In addition to their anatomic localization in lymphoid organs, all three of these T cell subsets possess robust proliferative and engraftment capacities.[21]

By contrast, effector memory (T_{EM}) and effector T cells (T_{EFF}) are Ag experienced T cells that have strongly down-regulated CD62L and CCR7 and therefore preferentially reside in peripheral rather than lymphoid tissues. Effector memory and T_{EFF} are poised to rapidly execute effector functions upon activation, as evidenced by their capacity to release large amounts of inflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) and their ability to rapidly lyse Ag-expressing targets. However, these subsets also tend to possess a relatively limited proliferative and engraftment potential compared with their $CD62L^+$ counterparts[23,24,25,21,26,27], properties which correlate with their shortened telomere lengths.[28,22]

In most clinical trials performed to date using TIL cells, the phenotypic and functional attributes of the transferred T cells were subject to natural variation and therefore were largely outside of investigational control. Despite this fact, detailed retrospective analyses from TIL trials have uncovered multiple, cell-intrinsic parameters which significantly correlate with the ability of T cells to mediate objective cancer regression in patients. For example, early studies revealed that properties such as a short duration in culture[29,30,31,32] or a relatively rapid doubling time[29,30] were correlated with clinical responses. More recently, additional parameters such as clonotypic persistence[33,13], telomere length[34,13], CD27 expression by $CD8^+$ T cells upon IL-2 withdrawal[35,13], and the frequency of cells with a central memory (T_{CM}) phenotype in the infusion product[36] have also been correlated with responses. In addition, it has also been noted that highly differentiated tumor Ag-specific effector $CD8^+$ T cell (T_{EFF}) clones generally engraft poorly and are inefficient at mediating cancer regression relative to results seen when a more heterogeneous population of T cells is transferred in TIL trials.[37,38,39,40,41] Taken together, these observations in humans have led to the hypothesis that less differentiated T cells may confer superior antitumor efficacy relative to T_{EM} and terminally differentiated T_{EFF} cells.

Causal relationships between T cell differentiation and antitumor efficacy

The question of which T cell subsets should be targeted for ACT has remained a point of contention and controversy for many years.[2,42,43,44] Because the ultimate goal of adoptive immunotherapy is to generate T cells capable of patrolling sites throughout the body in search of metastatic cancer deposits to destroy, it was initially presumed that the most desirable T cells for transfer should possess a natural propensity to infiltrate peripheral tissues and demonstrate strong, immediate, cytolytic capabilities. In effect, this would mean that the CD62L$^-$ T_{EM} and T_{EFF} populations would be the preferred cell types for transfer. However, this assumption stood at odds with empiric clinical data suggesting that transfer of less differentiated T cells were correlated with tumor responses. Systematic investigations were therefore undertaken in pre-clinical animal models to determine whether a causal relationship exists between a T cell's differentiation status at the time of infusion and antitumor efficacy (Figure 1).

In one set of experiments, murine CD8$^+$ T cells derived from the pmel-1 T cell receptor transgenic mouse[45] were re-iteratively stimulated *in vitro* to generate cells occupying progressively more advanced stages of differentiation termed early, intermediate, or late effectors.[25] Consistent with their greater maturational state, intermediate and late effector T cells acquired strong IFNγ-releasing and cytolytic capacities and up-regulated the expression of key transcription factors (TFs) associated with effector-differentiation, including *Eomes*[46] and *Id2*[47,48], as well as the replicative senescence marker killer cell lectin-like receptor G1 (KLRG-1).[49] Reciprocally, as T cells became more differentiated, they lost the ability to release IL-2 and down-regulated the expression of multiple cell surface markers, including CD62L, CCR7, CD27, and IL-7Rα (CD127). Additionally, re-stimulated T cells reduced the expression of naïve-associated TFs such as *Id3*.[47,48] When the intermediate and late effector T cells were transferred *in vivo*, these cells expanded and persisted poorly and, most importantly, were significantly impaired in their ability to cause tumor regression relative to naïve and early effector T cells.

In parallel, experiments were also conducted appraising the ability of conventional CD8$^+$ T cell memory subsets to mediate cancer regression following ACT (Figure 2). Initially, the antitumor efficacy of tumor-reactive T_{CM} relative to T_{EM} CD8$^+$ T cells was compared.[24] Similar to results observed in viral challenge models[23,26,27], T_{CM} exhibited far greater proliferative and survival capacities *in vivo* following vaccination with cognate tumor Ag relative to T_{EM} cells. While both T cell memory subsets could mediate cancer regression following ACT, only T_{CM} induced complete responses at the cell dose tested while mice receiving T_{EM} ultimately succumbed to uncontrolled tumor growth. Subsequently, the ability of T_{SCM} to mediate cancer regression relative to the T_{CM} and T_{EM} populations was directly compared at limiting cell doses roughly 2 orders of magnitude less than administered in prior experiments.[20,50] Consistent with earlier results, T_{CM} mediated superior *in vivo* expansion, persistence, and antitumor efficacy compared with T_{EM}. However, T_{SCM} CD8$^+$ T cells were even more potent than T_{CM} cells on a per-cell basis. When the potency of tumor regression was evaluated as a function of the input population of T cells, a significant linear correlation between T cell differentiation status and anti-tumor efficacy was found in the order T_{SCM}>T_{CM}>T_{EM}.[50] These results were confirmed in a separate, vaccine-independent tumor treatment model system where human T cell subsets genetically engineered to express an anti-mesothelin chimeric antigen receptor (CAR) were used to treat human mesothelioma xenografts in immune-deficient mice.[21]

The ability of naturally occurring Ag-specific and genetically engineered T_{EFF} derived from different CD8$^+$ T cell subsets has also been evaluated. Initially, the relative engraftment efficiencies of T_{EFF} derived from conventional memory subsets was investigated (Figure 2).

In both an immune-deficient mouse model receiving transfer of human T cells[51] as well as in non-human primates[52], T_{EFF} derived from T_{CM} precursors demonstrated superior persistence following ACT relative to T_{EM}-derived T_{EFF}. Remarkably, these differences were observed despite the fact that both memory derived T_{EFF} subsets possessed a highly differentiated phenotype at the time of cell transfer, characterized by the low expression of CD62L, CCR7, CD28, and CD127 and high expression of granzyme B and perforin. These data suggest that currently used panels of cell surface markers used to characterize T cell subsets is missing important heterogeneity, possibly as a result of differences in the genetic[53], epigenetic[54] or metabolic profiles[55] of otherwise phenotypically indistinguishable T cells on a single-cell level.

While T_{CM} cells often represent a minor population in humans, T_N are generally the predominant population present in the peripheral circulation.[28,21,56,57] Moreover, T_N possess longer telomeres and therefore have a greater replicative capacity compared with the Ag experienced subsets.[28,58] For these reasons, the phenotypic, functional, and anti-tumor capacities of T_{EFF} derived from a naïve rather than T_{CM} population has been evaluated in both mice[59] and humans.[56] Unlike the results obtained comparing memory-derived T_{EFF}[51,52], effector cell derived from T_N remained phenotypically and functionally distinguishable from the T_{CM}-derived T_{EFF}. Naive-derived T_{EFF} retained the ability to release IL-2 while withholding the acquisition of the senesce marker KLRG-1. By contrast, T_{EFF}-derived from T_{CM} lost the ability to secrete IL-2 and significantly up-regulated the expression of KLRG-1. Additionally, human naïve-derived $CD8^+$ T_{EFF} exhibited superior retroviral transduction efficiencies for an exogenous T cell receptor (TCR) and maintained significantly longer telomere lengths compared with both T_{CM}- and T_{EM}-derived T_{EFF} following *ex vivo* expansion.[56] When transferred into tumor-bearing mice, T_{EFF} derived from naïve cells exhibited superior *in vivo* expansion, persistence, and antitumor efficacy relative to T_{CM}-derived T_{EFF}[59] Collectively, these data confirm that T_{EM} represent an inferior T cell population for adoptive immunotherapies and demonstrate that among the $CD62L^+$ subsets, the RA^+ fraction should be retained rather than focusing solely on isolating, expanding, and re-infusing cells derived exclusively from the T_{CM} subset.

Finally, it should be noted that the conclusion that less differentiated T cells are superior to their more differentiated counterparts in mediating antitumor immunity is not restricted solely to $CD8^+$ T cells but appears to be generalizable to $CD4^+$ T cells as well. Using gene set enrichment analysis based on the gene expression profiles of memory $CD8^+$ T cells which had undergone re-iterative stimulations *in vivo* using heterologous vaccine constructs[60], Th17 cells were found to have a gene expression profile highly enriched in genes associated with primary $CD8^+$ memory T cells.[61] By contrast, Th1 cells had an expression profile that was enriched in late memory $CD8^+$ T cells obtained after multiple rounds of *in vivo* stimulation. Across multiple model systems, adoptively transferred Th17 cells proliferated, persisted, and mediated superior antitumor immunity relative to Th1 cells.[61,62,63] In conclusion, findings made in mice, non-human primates, and humans have established a causal inverse relationship between T cell differentiation status and the relative capacities of transferred T cells to engraftment, proliferate, and mediate antitumor immunity. These data strongly support the use of the less differentiated $CD62L^+$ T_N, T_{SCM}, and T_{CM} subsets over the $CD62L^-$ T_{EM} and T_{EFF} for adoptive immunotherapies.

How should T subsets be isolated for human trials?

Having established in pre-clinical models that younger T cell subsets possess superior traits for adoptive immunotherapy, cellular therapists are now confronted with a practical but critical challenge: generating simple, reproducible, high throughput, and economically feasible means of isolating defined T cell populations under good manufacturing (GMP)

conditions for incorporation into human clinical trials. Most approaches to cellular isolation are both conceptually and technically straightforward. Despite this fact, the ability to isolate and expand defined cellular products represents a major regulatory hurdle as evidenced by the fact that only two autologous cell therapies, cultured chonodrocytes (Carticel)[64] and a monocyte culture sensitized with the prostate cancer-associated antigen PAP (sipuleucel-T, Provenge)[65], have been approved for use by the United States Food and Drug Administration.

Magnetic bead isolation

Magnetic bead isolation has for many years been used for the isolation of defined immune cell populations in animal models[66] and increasingly is also being applied for T cell isolation and manipulation protocols in early phase human clinical trials. This system makes use of specific monoclonal antibodies targeting different cell surface associated Ags conjugated to super-paramagentic particles colloquially referred to as beads[67] (Figure 3, middle panel). By incubating a bulk population of immune cells with monoclonal antibodies conjugated to beads followed by passage through a magnetic column, T cell populations can be enriched either by directly pulling out labeled cells by positive selection or, alternatively, depleting non-target cells through negative selection. Negative and positive selections can be performed serially to increase the purity of the final cell product. Moreover, with the recent development of reversible antibody conjugated beads, the potential exists to perform serial positive selections, thus allowing for the isolation of rare subsets defined by expression of multiple cell surface markers with high purity[68].

Using magnetic bead isolation, highly enriched $CD8^+$ T cells have been transferred in melanoma TIL clinical trials[69]. Similarly, magnetic beads also have been used to isolate $CD4^+$ T cells for transfer to human immunodeficiency virus infected patients[70] or patients with hematologic malignancies following allogeneic stem cell transplantation (SCT).[71] The isolation and transfer of defined T cell subsets need not be limited simply to the separation of bulk T cell populations based on the expression of either of the CD8 or CD4 co-receptors, however. For example, IL-2Rα (CD25) positive selection has been used alone or in combination with $CD8^+/CD19^+$ lymphocyte depletion to isolate and transfer naturally occurring T regulatory cells (T_{regs}) to patients following allogeneic SCT as graft versus host disease prophylaxis.[72,73]

More recently, Wang *et al.* in this issue of the *Journal of Immunotherapy* and Terakura *et al.* in *Blood* have describe optimized protocols for the isolation and genetic modification of human $CD8^+$ T_{CM} under GMP conditions.[74] These advances were made possible through the introduction of a new, GMP-quality monoclonal antibody targeting human CD62L. Both of these studies underscore what is currently a limiting factor for magnetic bead isolation strategies: the relative paucity of GMP-quality monoclonal antibodies. It is presently estimated that when all available products from manufacturers are counted together, only a dozen or so GMP-quality antibodies for bead isolation are currently available, including antibodies targeting markers not expressed by T cells. As T cell subsets become defined with increasing precision using multiple cell surface markers, such as the recently described human T_{SCM} population[21] rapid translation of promising pre-clinical discoveries will become increasingly bottlenecked by this limitation.

In addition to isolation of T cell subsets based on the expression of surface phenotypic markers, magnetic bead isolation may also be used to isolate Ag-specific T cells. This can be accomplished either directly through the isolation of T cells reactive against HLA-peptide multimer complexes[75] or indirectly using a cytokine capture reagent to label and subsequently enrich cytokine-secreting T cells following *in vitro* stimulation of a bulk

population of lymphocytes.[76] Proof of principle that labeling of cells using HLA-peptide tetramers followed by enrichment with magnetic beads can be used clinically was demonstrated by Cobbold *et al.* who successfully isolated and then re-infused CMV-reactive $CD8^+$ T cells.[77] Following ACT of cells prepared in this manner, patients in this study had a demonstrable increase in the frequency of CMV-specific T cells in the circulation, exhibited TCR Vβ clonotypic persistence, and contemporaneously exhibited decreases in CMV viral titers. More recently, similar results were obtained when CMV-specific $CD8^+$ T cells were isolated and transferred using GMP-grade reversible binding HLA-peptide streptamers.[78]

While magnetic bead isolation of HLA-peptide multimer bound T cells allows for the isolation and transfer of Ag-specific T cells, alone this approach does not provide for the selection of specific T cell subsets. The pairing of Ag and subset specificity may be accomplished using cytokine capture. In principle, by pairing capture of cytokines associated with less differentiated $CD8^+$ T cells, such as IL-2, with stimulation with HLA-defined epitopes, one can isolate younger Ag-specific T cells. Pre-clinically, this approach has successfully been used to isolate and expand Ag-specific IFNγ-producing T cells with specificity for adenovirus[79,80], cytomegalovirus (CMV)[81], Epstein-Bar virus (EBV)[82], aspergillus[83], as well as Ags associated with certain myeloid and lymphoid leukemias.[84] Clinically, the isolation and transfer of viral-specific T cells using cytokine capture has been effective in reducing viral titers in immune-compromised patients with adenovirus[85] or CMV[86] viremia following SCT. Cytokine capture has also been used to isolate and transfer EBV-specific T cells to patients for the treatment of post-transplant lymphoproliferative disease.[87]

Preparative FACS-sorting and microfluidic chips

Complimenting magnetic bead isolation strategies are procedures which isolate cells stained for multiple cell surface markers which are carried in a fluidic stream (Figure 3, right panel). These approaches include preparative scale (FACS)-sorting[88] and the recently developed use of microelectromechanical systems (MEMS) chips[89] in combination with a FACS-based detection system. In both cases, cells can be labeled with multiple markers, allowing for the isolation of well-defined T cell subsets at high purity. FACS-sorting enables the simultaneous use of up to 18 different labels, however this capability comes at the cost of a relatively low throughput and cell yield compared with bead isolation protocols. Moreover, given the limited number of GMP quality antibodies which are currently available, flow- and bead-based isolation protocols suffer from similar limitations with respect to the number of markers which can be used.

Compared with the use of magnetic bead-based purification strategies, the use of preparative FACS-sorting to isolate T cell subsets for clinical trials is relatively underdeveloped. To date, most studies using FACS-sorted T cell populations have been pre-clinical in nature only. For example, preparative scale FACS-sorting with GMP-adaptable reagents has been used to isolate T_{regs} from healthy volunteers[90] and patients with diseases such as type 1 diabetes.[91] To date, only one clinical trial has been published using FACS-sorted T_{regs}. In this study, naturally occurring T_{regs}, characterized by the surface expression pattern $CD4^+CD25^+CD127^-$, were isolated by flow-sorting, expanded *ex vivo*, and subsequently re-infused into patients with GVHD[92]. Other clinical studies using flow-sorted T_{regs} are either underway or planned, and results are anticipated in the near future. In principle, MEMS-based sorting has the potential to dramatically improve sorting speeds compared with conventional FACS-sorters while at the same time minimizing the risk of electrically-mediated cell damage. However, this technology remains very nascent and clinical applications using this technology have yet to receive approval by the F.D.A.

Functional isolation using PCR-based screening

Most recently, the ability to isolate Ag-specific T cell clones with defined functional attributes characteristic of different T cell subsets has been demonstrated using a high throughput PCR-based screening method (Figure 3, left panel).[93,94] In this approach, microcultures of patient-derived peripheral blood mononuclear cells undergo *in vitro* sensitization with Ags of interest, such as the shared melanoma/melanocyte Ags MART-1 or gp100, and cells are subsequently screened for their ability to express specific cytokines. Initially this technique was developed with the goal of isolating $CD8^+$ T_{EFF} with a high capacity to express IFNG following stimulation.[94] However, based on pre-clinical data demonstrating the superior ability of $CD8^+$ T_{CM} to engraft and mediate antitumor efficacy relative to T_{EM}[24], it has subsequently been adapted to select microcultures enriched in IL-2 producing T cells.[95] As noted above, the ability to release IL-2 is a functional characteristic of the less differentiated T_{CM} subset.[24,14] In this manner, Wang *et al.* have recently reported the ability to isolate, expand, and transfer rare human melanoma-specific $CD8^+$ T_{CM} cells. Consistent with their ability to express IL2 following stimulation with an MHC class I-restricted epitope, microcultures with a high IL2:IFNG ratio were enriched in Ag-specific $CD8^+$ T cells with a $CD45RO^+CD62^+$ T_{CM} phenotype. By contrast, microcultures with a low IL2:IFNG ratio were enriched in Ag-specific T_{EM} cells. Following subsequent expansion and re-infusion, T cell clones generated in this manner engrafted and persisted at high frequencies in four of the five patients 1 month after transfer. Although objective tumor regression was not observed in this cohort of patients, all five patients developed a cutaneous $CD8^+$ T cell infiltration associated with autoimmune dermatitis. Thus, PCR-based functional screening for Ag-specific T cells provides a valuable new tool for the isolation of specific T cell subsets.

How should T cells be expanded to minimize corruption?

In mice[50,96] and humans[29,31,32], the absolute number of transferred T cells has often (although not universally) been correlated with tumor responses. However, vigorous *ex vivo* expansion to generate large numbers of T cells inexorably drives T cell differentiation[25,97] and a loss of *in vivo* anti-tumor efficacy.[25,38,40,39] For this reason, culture strategies must be developed which do not corrupt the beneficial attributes of the less differentiated T cell subsets after they have been isolated.

For most pre-clinical and ACT trials, the basic manner in which T cells are propagated to therapeutic levels has not significantly changed in more than 20 years. This methodology combines high doses of IL-2 to establish the initial T cell cultures[98,99,100] followed by rapid expansion of cells using potent antigenic stimulation using agonistic antibodies against CD3 in conjunction with allogeneic feeder cells.[101] However, it is now recognized that the combination of TCR stimulation and strong IL-2 signaling drives cells to become terminally differentiated T_{EFF} with a compromised ability to successfully enter the long-lived memory pool.[102,103] Therefore, efforts have been made to explore whether the use of common gamma chain signaling (γ_c) cytokines besides IL-2 may produce less differentiated and more therapeutically potent anti-tumor T cells.

For example, it has been shown in mice that expansion of naïve $CD8^+$ T cells in the presence of IL-15 generates cells with the phenotypic[104,105,106], functional[104,105], and metabolic properties[107] of naturally occurring T_{CM} cells. Accordingly, when IL-15 expanded T_{CM}-like cells were transferred into tumor-bearing hosts, they exhibited superior proliferative and antitumor responses compared with IL-2 expanded cells which possessed a T_{EM}-like phenotype (Figure 1).[105] These findings were recently extended to human patients where IL-15 was combined with a novel artificial antigen presenting cell to generate a

polyclonal population of tumor-reactive T cells by *in vitro* sensitization of peripheral lymphocytes.[108] T cells expanded using this system exhibited a T_{CM} phenotype and demonstrated clonotypic persistence and the ability to mediate objective clinical responses following cell transfer.[109] Likewise, when the alternative γ_c cytokine IL-21 is used to expand tumor-reactive $CD8^+$ T cells, the resultant population retains a minimally differentiated phenotypic and functional profile[110,111,112,113]. In mice, IL-21 expanded tumor-reactive T cells demonstrate augmented proliferative and antitumor capacities relative to cells expanded in either IL-2 or IL-15.[110]

Complementing the use of alternative γ_c cytokines, the ability of small molecule modulators of key metabolic and developmental pathways to restrict T cell differentiation has also been evaluated[114]. It is increasingly being recognized that a T cell's commitment between memory and effector cell fates is governed by transitions between different metabolic states.[15,55] These states, in turn, are dictated by inputs from multiple sources, including the TCR, co-stimulatory, and cytokine receptors. Signals from these receptors tend to converge at common developmental and differentiation signal transduction pathways, such as the PI3K-AKT-mTOR and Wnt-β-catenin pathways.[4,115] As such, modulation of these pathways can potentially withhold the acquisition of full effector function and maintain T cells in a less differentiated memory-like state. Inhibition of the mTOR pathway using the inhibitor rapamycin, for example, limits the acquisition of key effector associated TFs such as Tbet and promotes memory $CD8^+$ T cell formation.[116] Upon ACT, rapamycin-sensitized $CD8^+$ T cells exhibited superior antitumor functions compared with control cells. Similarly, it has also been shown that promotion of the canonical Wnt-β-catenin pathway using the GSK3β inhibitor TWS119 or recombinant Wnt proteins promotes the formation of T_{SCM} and T_{CM} $CD8^+$ T cells while limiting the formation of T_{EM} cells.[20] Upon ACT, TWS119 expanded $CD8^+$ T cells are significantly better at mediating tumor regression compared with cells expanded in a vehicle control. In contrast with both mTOR and GSK3β inhibitors, which both limit T cell proliferation while also restraining T cell differentiation, inhibition of the AKT isoforms AKT1 and AKT2 has recently been shown to promote the formation of T_{CM}-like cells without limiting the yield of cells.[117] Future experiments will need to be conducted to determine whether direct inhibition of the AKT pathway can successfully uncouple the processes of T cell expansion from cellular differentiation in the generation of therapeutic T cells.

In conclusion, it is now clear that the standard means of expanding T cells to therapeutic levels, namely the use of high dose IL-2 and strong antigenic stimulation, can corrupt the favorable phenotypic and functional attributes of the differentiated T cells. For this reason, the use of alternative γ_c cytokines alone or in combination small molecules which modulate critical signal transduction pathways will need to be adopted to preserve the benefits of the isolated younger subsets.

Is it necessary to separate younger cells from their mature counterparts?

The ability of subsets of lymphocytes to interact with one another to influence their differentiation status or effector functions has been well characterized. For example, it has been shown that effector and memory $CD8^+$ T cells can kill Ag-bearing dendritic cells, thereby indirectly restricting the differentiation of naïve $CD8^+$ T cells by removing their ability to be primed.[118] T cells may also directly regulate the differentiation of other lymphocytes either in a positive or negative direction. The ability of $CD4^+$ T_{regs} to suppress the differentiation and execution of effector functions by both T_{EFF} $CD4^+$ and $CD8^+$ T cells has been well characterized.[119,120,121] Conversely, the ability of T cells to augment the differentiation and effector functions of B cells[122] or other T cells[123] via the super TNFα-family member CD40L has also been shown. However, whether naïve and memory $CD8^+$ T

cell subsets can physically interact with one another to influence one another's differentiation status and ultimately antitumor function remains unknown.[124] Resolution of this question remains a critical priority. If older T cell subsets do not have a deleterious impact on the antitumor function of the less differentiated subsets, the need for T cell subset isolation would be less of an imperative than generating strategies to expand and transfer large numbers of cells which contain at least some younger populations. If, on the other hand, older T cells corrupt the beneficial functional attributes of the younger T cell subsets, efficient methods of large scale subset isolation would become a requisite.

Conclusions

It is now clear from relevant pre-clinical animal models and detailed retrospective analyses of human clinical trials that infusion of the less differentiated T_N, T_{SCM} and T_{CM} subsets is associated with superior T cell engraftment, persistence, and antitumor immunity compared with T_{EM} and T_{EFF} cells. With the recent advent of genetic engineering technology and the ability to reliably confer tumor-Ag reactivity with high efficiency[125], it is now possible to generate tumor-reactive $CD8^+$ T cells of any memory subset. In order to facilitate clinical experiments in humans where the functional characteristics of the input population of T cells, beyond tumor-reactivity, can be carefully tested and dissected, robust isolation strategies which efficiently and reliably purify desired T cell subsets will be required. Concurrently, modification of culture conditions in order to prevent the corruption of isolated younger T cell subsets during *ex vivo* expansion must be routinely introduced into practice. Finally, the question of whether younger and older T cell subsets may interact with one another in a way that influences their differentiation status and ultimately antitumor function must be resolved. Accomplishing these goals will allow for the development of the next generation of highly effective and potentially curative T cell therapies for the treatment of cancer.

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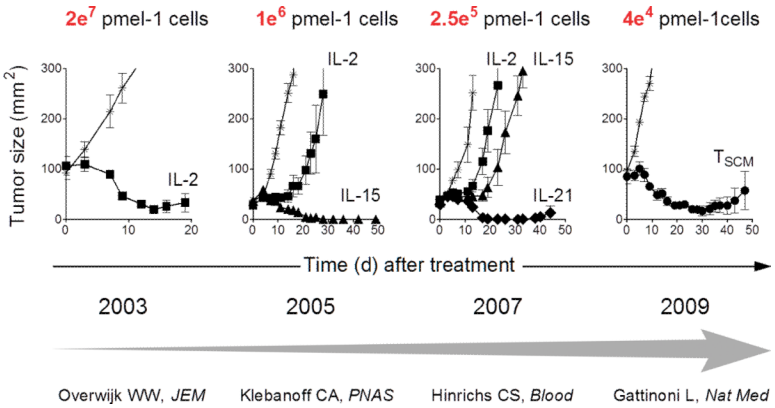


Figure 1. Re-iterative progress in enhancing the efficacy of adoptive CD8$^+$ T cell therapy for the treatment of melanoma using the pmel-1 mouse model

The use of alternative γ_c cytokines or less differentiated T cell subsets has resulted in progressive improvements in the therapeutic efficacy of tumor-reactive CD8$^+$ T cells, thus providing for effective tumor destruction with the transfer of limited numbers of cells. IL, interleukin; T_{SCM}, T memory stem cell.

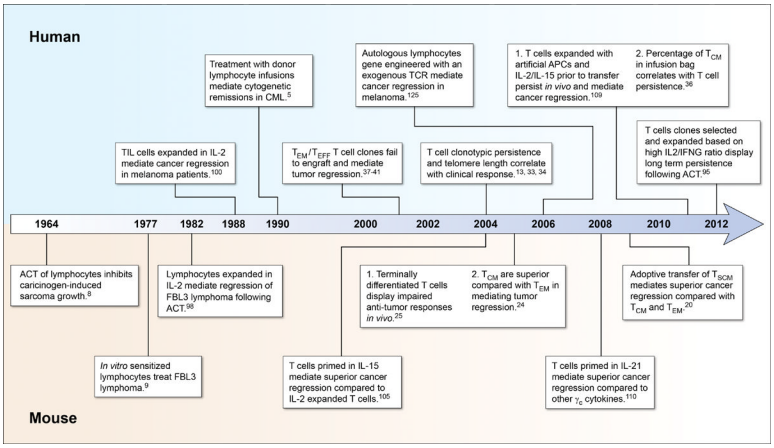
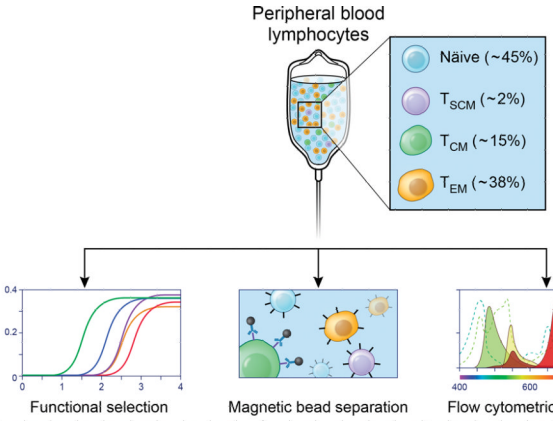


Figure 2. A timeline of progress in the understanding of T cell qualities associated with effective adoptive immunotherapies for the treatment of cancer in mice and humans

ACT, adoptive cell transfer; T_{SCM}, T memory stem cell; T_{CM}, T central memory; T_{EM}, T effector memory; T_{EFF}, T effector cell; γ_c, common γ-chain receptor; CML, chronic myelogenous leukemia; TCR, T cell receptor; APC, antigen presenting cell.



Purity	+	++	+++
Cell yield	+	+++	+
Time of labor	+++	+	++
Number of parameters	+	++	+++
Cost	+	++	+++

Figure 3. Clinical strategies to isolate specific T cell subsets for adoptive cell transfer

Peripheral blood lymphocytes can be sorted in specific T cell subsets by employing diverse strategies, including functional isolation by PCR-based screening (left panel), magnetic bead isolation (center panel) and flow cytometric cell-sorting (right panel). Each technique has relative benefits and limitation in terms of cell purity and yield, complexity of the parameters used for isolation, labor and cost which are represented as low (+), intermediate (++) or high (+++).