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Adoptive Therapy with Chimeric Antigen Receptor Modified T Cells of Defined Subset Composition

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Abstract

The ability to engineer T cells to recognize tumor cells through genetic modification with a synthetic chimeric antigen receptor has ushered in a new era in cancer immunotherapy. The most advanced clinical applications are in targeting CD19 on B cell malignancies. The clinical trials of CD19 CAR therapy have thus far not attempted to select defined subsets prior to transduction or imposed uniformity of the CD4 and CD8 cell composition of the cell products. This review will discuss the rationale for and challenges to utilizing adoptive therapy with genetically modified T cells of defined subset and phenotypic composition.

Introduction

A significant advance in the field of adoptive T cell therapy (ACT) is the ability to confer upon T cells specificity for tumor antigens or tumor cell surface molecules by the introduction of genes that encode high affinity tumor-targeting T cell receptors (TCRs) or synthetic chimeric antigen receptors (CARs) respectively^{1,2}. The most impressive clinical results have been achieved with the adoptive transfer of T cells modified to express a CAR that targets the B cell lineage CD19 molecule, where patients with refractory B cell malignancies including acute lymphoblastic leukemia, chronic lymphocytic leukemia, and lymphoma have been induced into a complete remission after receiving an infusion of autologous T cells modified with a CD19-specific CAR³⁻⁸. However, not all patients have responded to therapy and the basis for lack of a therapeutic effect in some patients has not been fully elucidated. Moreover, serious, life-threatening toxicities have been observed in a subset responding patients treated with CD19 CAR-modified T cells, and follow-up is too short to determine what proportion of patients will have durable responses³⁻⁸. Thus, challenges remain to make ACT with CD19 CAR T cells a reproducibly effective and safe therapy. Additionally, to what extent ACT with CAR (or TCR) modified T cells can be applied to targets that have been identified in common epithelial tumors remains uncertain, and will depend both on the and validation of current candidate target molecules and on defining optimal T cell products in different settings^{9,10}. Our group has focused on developing a platform for ACT in which T cell products of defined composition can be engineered with tumor-targeting receptors, and this review will discuss the rationale, development, and implementation of this approach.

T cell subsets and pathways of differentiation

It is apparent from the graft versus leukemia effect of allogeneic stem cell transplant, and earlier studies of adoptive T cell therapy with unmodified tumor-infiltrating lymphocytes in melanoma that T cells have the potential to eradicate large, refractory human tumors¹¹⁻¹³. However, a detailed mechanistic understanding of the requirements for T cells to eradicate tumors in different settings has remained elusive. The ability to engineer T cell to recognize tumor cells provides a unique opportunity to uncover principles that might be broadly applied to many cancers.

The intrinsic properties and regulation of T cells that are isolated and expanded for adoptive transfer are important aspects to consider in the design of cancer immunotherapeutics. The T cell arm of adaptive immunity consists of phenotypically distinct naïve antigen-experienced cells that provide a large diversity of T cell receptors to enable effective immune responses to new foreign antigens; and subsets of antigen-experienced memory cells that are clonally expanded following antigen recognition and provide for rapid, life-long recall responses to previously encountered antigens¹⁴⁻¹⁶. Naïve and memory T cell subsets vary in function, expression of cell surface molecules, and their frequency in the peripheral blood in normal individuals and patients depends on age, prior pathogen exposure, and the type, intensity and duration of cytotoxic therapies that may have been previously administered. Naïve T cells are CD45RA⁺ and CD95⁻, and express CD62L and CCR7 molecules for lymph node homing, and CD28 and CD27 molecules to engage

costimulatory ligands. By contrast, memory T cells are CD45RO⁺ and CD95⁺, and contain both CD62L⁺ CCR7⁺ central (T_{CM}) and CD62L⁻ CCR7⁻ effector memory (T_{EM}) subsets¹⁴. T_{CM} typically express CD28 and CD27 costimulatory molecules uniformly, whereas T_{EM} are heterogeneous for expression of these molecules. Recently, a CD45RA⁺, CD62L⁺, CD95⁺, CD122⁺ subset with a phenotype intermediate between that of T_N and T_{CM} has been identified and proposed as a memory stem cell (T_{SCM})^{17,18}. Definitive evidence for “stemness” within this subset would require the demonstration of the capacity to both self renew and differentiate to all effector and memory phenotypes, and these key features have not yet been demonstrated experimentally. Nevertheless, this intermediate CD45RA⁺, CD95⁺, CD62L⁺ T cell subset has important attributes, such as long telomere length, high proliferative capacity and expression of costimulatory molecules, that may be advantageous in ACT¹⁷.

There has been controversy as to the lineage relationships of effector and memory T cell subsets. Mouse models of viral infection have provided insights into how effector and memory CD8⁺ T cell subsets arise from naïve precursors during the course of infection, and into the basis for longevity of T cell memory¹⁹. Recent fate mapping studies of the differentiation of individual naïve T cells after antigen stimulation are consistent with a model in which naïve T cells differentiate in a linear fashion to long-lived T_{CM}, and to rapidly expanding, but shorterlived T_{EM} and T_E cells^{15,16} (Figure 1). An important finding in the studies that analyzed individual cell fates was that large T_{EM} subsets formed after a primary immune response typically failed to dominate the secondary response to antigen rechallenge¹⁵. This is consistent with the observation that T_{EM}, which express higher levels of granzyme B than T_{CM} and have a greater capacity for immediate effector function, have lower proliferative potential in response to restimulation, and suggest these cells are further along in the differentiation pathway²⁰.

Gene marking to track the fate of T_E cells derived from different subsets

The marked differences in phenotypic composition and differentiation states of T cells in the peripheral blood suggested that distinct populations might have different utility in adoptive therapy. Thus, we developed preclinical models to determine how the differentiation state of T cells from which genetically modified effector cells might be derived affected their ability to persist long-term, migrate to lymph nodes and bone marrow, and proliferate to rechallenge with antigen. The initial model we selected to study was the non-human primate *Macaca nemestrina*. Macaques like humans, are commonly infected with cytomegalovirus (CMV), making it possible to isolate virus-specific T cells from the T_{CM} and T_{EM} subsets. Moreover, the culture methods that are used to activate, propagate, and transfer genes into macaque T cells are similar to those employed in human adoptive therapy^{21,22}. In these experiments, we purified CD8⁺ T cells from the T_{CM} and T_{EM} subsets by flow sorting, derived T_E clones specific for cytomegalovirus (CMV) from each of these subsets, and used retrovirus mediated gene transfer to introduce a B cell lineage surface marker to facilitate tracking their in vivo after adoptive transfer^{21,22}. We sequentially infused the gene-marked T cells back to the same animals, without administering any lymphodepleting chemotherapy before T cell infusions, or growth and survival promoting cytokines after T cell transfer (Figure 2A,B). These studies demonstrated that gene-marked, antigen-specific CD8⁺ T_E

clones derived from the T_{CM} subset persisted in significant frequencies in the blood after adoptive transfer, migrated to lymph nodes and bone marrow, and reconstituted both T_{CM} and T_{EM} phenotypes, whereas those derived from the T_{EM} subset survived in the blood for less than 7 days after adoptive transfer and were not detected in lymph nodes, bone marrow, or tissue sites²². The transferred T_{CM}-derived effector cells were long-lived and capable of responding to viral antigen challenge. Animals that were followed for > 4 years retained large numbers of gene marked cells in the memory pool, demonstrating that the progeny of a single T_{CM} can provide long-lasting immunity in a primate²³. Subsequent experiments examined the ability of T_N-derived T_E cells to persist after ACT, and showed that T cell products derived from T_N also survived at high levels in non lymphodepleted animals, however it was not possible to evaluate the ability of these cells to respond to antigen challenge (Berger C, Riddell SR, unpublished data). These studies demonstrate that lymphodepleting chemotherapy prior to adoptive transfer and post infusion cytokines are not essential to establish long-lived T cell immunity, and reveal marked cell intrinsic differences in the ability of in vitro derived T_E cells to persist and function in vivo depending on their subset derivation. Notwithstanding these insights, it is important to note that the non-human primate model has many limitations including the fact that the transferred T cells did not express a CAR and the studies were not performed in a tumor-bearing host.

To address these limitations, subsequent studies examined the persistence, proliferation and therapeutic efficacy defined subsets of human T cells that were genetically modified with marker genes or tumor-reactive CARs in ACT using immunodeficient Nod/Scid/gc^{-/-} (NSG) mice alone, or engrafted with human tumors^{10,24}. These experiments have confirmed the distinct behavior and therapeutic efficacy of individual T cell subsets, and defined compositions of naïve and memory CD4⁺ and CD8⁺ T cells modified with a CD19-specific CAR that mediate potent tumor eradication using remarkably small cell doses (Sommermeier D, Hudecek M, Riddell SR, unpublished data). Unfortunately, these xenograft models do not recapitulate the toxicities that have been observed in clinical trials, and it is as yet unknown whether cell products of different composition exhibit distinct toxicity profiles.

Preparing clinical gene-modified T cell products of defined composition

Gene transfer makes it possible to rapidly obtain tumor-reactive T cells for cancer therapy, and provides the opportunity to utilize T cell products in which the composition and function are precisely defined. Despite this opportunity, none of the reported clinical trials of CD19 CAR therapy have attempted to select defined subsets prior to transduction, or ensure uniformity of the T cell product in different patients, even at the level of CD4 and CD8 composition^{3-8,25,26}. As a consequence, the T cell products administered to patients vary widely in phenotype and subset derivation, and the implications of this variation for therapeutic efficacy, toxicity, and cell persistence are uncertain. The reasons for simply transducing whatever T cells can be harvested from the patient for ACT are well founded. First, selecting defined T cell subsets for transduction adds complexity and expense to a cell manufacturing and culture process that is already complex and costly. Second, many patients have a relative lymphopenia making it challenging to isolate specific subsets such as CD8⁺ T_{CM} that may be present in the blood at very low frequencies. Third, robust,

validated, clinical grade methods for selecting T cells of different phenotypes from peripheral blood or leukapheresis products obtained from patients are not readily available. Finally, an argument can be made that unselected cells have already shown therapeutic efficacy in some patients and further refinement in cell product composition is unnecessary, despite the in vitro and preclinical data that suggests potency and efficacy can be improved.

Clinical cell therapy applications in immunotherapy and other fields of medicine are increasing dramatically, and it seems likely that this trend will continue. It is also likely that regulatory agencies will require more stringent characterization of cell products that are administered to patients. Although the technologies used for cell separation have been advancing, they have not kept pace with the rapid expansion of clinical applications. Unmet or incompletely met needs are the requirements for improvements in cell processing speed, an increase in the number of clinical grade antibodies and fluorochromes, strategies for ensuring sterility during processing, and technologies that enable complete removal of selection reagents from the final product that is to be administered to the patient. In the case of genetically engineered T cell therapy, it can be particularly challenging with current technologies to isolate rare cell subsets that require a constellation of cell surface markers for purification, such as CD8⁺ T_{CM} that typically comprise <2% of peripheral blood cells, and CD8⁺ T_{SCM} that are present in an even lower frequency.

Methods for purifying T cell subsets for clinical adoptive therapy

Our lab has investigated several approaches for obtaining T cell subsets for manufacturing CAR T cell products including clinical grade fluorescence activated cell sorting (FACS) and immunomagnetic enrichment technologies. Flow sorting provides optimal purity but is cumbersome when dealing with large starting cell populations, and is limited by the lack of clinically approved antibody/fluorochrome conjugates and by the potential for alterations in cell viability and function during high speed sorting. Novel microfluidic technologies that are in development are eagerly awaited since these methods promise to greatly improve the speed of cell sorting in entirely closed, aseptic devices.

We reported the development of clinical grade immunomagnetic selection methods for isolating human CD8⁺ T_{CM} that rely on the use of several commercially available clinical grade antibody bead conjugates using the CliniMACS device^{27,28}. With this approach CD4⁺, CD14⁺ and CD45RA⁺ cells are removed from peripheral blood mononuclear cells by depletion with antibody conjugated paramagnetic beads, and then the CD62L⁺ fraction from the remaining cells is positively selected with an anti CD62L labeled bead to enrich CD45RO⁺, CD62L⁺, CD8⁺ T_{CM}. The enriched T_{CM} can be activated with anti CD3/CD28 beads or with antigen, modified with tumor-specific CARs using retroviral or lentiviral vectors, and expanded for use in adoptive therapy^{27,28}. This method is effective for enriching CD8⁺ CD62L⁺ CD45RO⁺ T cells, but substantial numbers of contaminating myeloid cells remain in the cell product in some patients. These contaminating cells do not persist during culture, but their presence at the time of gene transfer complicates the transduction procedure with viral vectors, since these myeloid cells can adsorb vector particles and reduce the efficiency with which the T cells are modified²⁷. Moreover, the yield of the target CD8⁺ T_{CM} population could be improved. Despite these limitations, this

approach is currently being used successfully and reproducibly in the cell manufacturing process for our clinical trial of autologous CD19 CAR T cell therapy for refractory B cell malignancies.

A new technology that allows separation of T cells based on a constellation of cell surface markers is the use of low affinity Fab-fragments fused to *Strep*-tag II. As monomers, the Fab fragments do not have sufficient affinity for stable binding to the target molecule on the cell surface. However, when multimerized on a StrepTactin bead, these reagents stably bind to the cell and facilitate selection based on marker specificity. A key element of this technology is that Fab multimer binding can be rapidly reversed by the addition of excess D-biotin, which because of a higher affinity for StrepTactin competes for binding with StrepTag II and dissociates the Fab multimer complexes into Fab monomers that cannot stably bind to the cell²⁹. Thus, this methodology allows for serial positive enrichment of T cells based on multiple markers, and can select virtually any desired subset of T cells, including CD8⁺ T_{CM} that can be distinguished by the combination of CD45RO, CD62L, and CD8 markers²⁹. The inherent advantages of this system are high purity and the ability to remove the bound reagent prior to further manipulation or infusion of the cell product into the patient. This method is currently being advanced for clinical applications with CD19 CAR T cells.

Clinical Studies

Preclinical data supports the potential benefits of selecting specific T cell subsets for genetic modification for enhancing the potency and reproducibility of cancer immunotherapy, and potentially reducing toxicity if lower cell doses are effective. The development of cGMP applicable methods for selecting defined T cell subsets has led to the initiation at the Fred Hutchinson Cancer Research Center of the first study of CD19 CAR T cell therapy for advanced CLL, ALL, and lymphoma in which the cell products are formulated in a defined composition that is uniform for each patient (www.clinicaltrials.gov NCT-01865617). The results of this study will provide insights into how the composition of genetically modified T cells that target CD19 might affect toxicity, and the potency and durability of therapy.

Summary

Immunotherapeutic approaches to cancer in the past decade have advanced based on new understanding of the interactions between tumors and host immunity, the identification of antigens and pathways that can be targeted. T cell therapy targeting CD19 on B cell malignancies with cells engineered to express a CD19-specific CAR is an exciting advance that has the potential to improve outcomes for many patients. A deeper understanding of the potential utility of distinct subsets of T cells, and determining synergism between subsets may assist in realizing the full potential of engineered T cells both for therapy of B cell malignancies and more broadly for other malignancies.

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Figure 1. Model for linear differentiation of T cell subsets

The phenotype of naïve, memory, and effector T cell subsets is shown as a linear pathway of differentiation based on fate mapping studies in murine models^{15,16}. The putative memory stem cell is indicated by the hatched rectangle, since the stemness of this phenotype has not been definitively demonstrated.

A. Cell Derivation

CD8⁺CD62L⁻ T_{EM}

CD8⁺CD62L⁺ T_{CM}

1 cell

Gene Marking
CD19 (◀) or CD20 (▶)

>10⁹ T_E cells

B. Cell Transfer and Fate Determination

Figure 2. Non-human primate model for evaluating fate of adoptively transferred T cells from defined memory subsets

A. Cell derivation. CD8⁺ T cells were sorted into central and effector subsets based on surface marker expression, and single cells specific for cytomegalovirus antigens were derived, genetically marked with a B cell lineage surface marker (truncated CD19 or CD20), and expanded in vitro. B. Cell transfer and fate determination. T cells from T_{CM} and T_{EM} precursors were adoptively transferred to lymphoreplete animals. Migration to lymph nodes and bone marrow was assessed between 4 and 7 days after transfer, and long term persistence, phenotype and function was assessed by flow cytometry, PCR and viral antigen challenge as described^{21,22}.