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Adoptive T-cell therapy for malignant disorders

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Adoptive cell transfer (ACT) using *ex vivo* manipulated T lymphocytes has emerged as an important advance in cancer immunotherapy, allowing for re-education and re-setting of the host immune system. Recent technological advances, particularly the development of artificial antigen presenting cells (aAPCs) for *ex vivo* stimulation and cell expansion that improve upon nature, can re-educate T lymphocytes, enhancing their potency and function. These technologies have ushered in a new generation of cell-based immunotherapeutics.

T-cell sources and flavors

The diversity of T cells for ACT is vast given that T cells may be derived from various anatomic locations, separated into different lymphocyte subsets, enriched based on phenotypic or functional characteristics such as antigen specificity, *ex vivo* activated by numerous methods, and genetically modified to change their inherent specificity, homing capacity, function, and survival *in vivo*. Ideally, T cells for ACT would possess the following properties: i) demonstrated potency and specificity against the tumor or infectious organism, ii) efficient engraftment enabling a high effector to target ratio, iii) long-term persistence *in vivo* and memory establishment, and iv) be easily obtained and efficiently manufactured.

Naive $CD4^+$ and $CD8^+$ T cells enter developmental programs after activation that ultimately result in the generation of effector memory (TEM) and long-lived central memory T cells (TCM). Understanding the mechanism underlying memory generation is accordingly critical to the development of culture systems that optimally produce populations of TEM and TCM cells *in vitro* to establish strong antitumor responses and long-lived memory for continued immune surveillance after infusion. $CD8^+$ T cells are well-established as potent effectors of anti-tumor and -viral immune responses *in vivo*; however, for the generation and/or maintenance of $CD8^+$ T-cell memory, $CD4^+$ T-cell help is required. Co-transfer of $CD4^+$ T cells can augment tumor immunity by enhancing the survival and function of transferred $CD8^+$ T cells through the secretion of cytokines such as IL-2 and the expression of CD40L, which increases antigen-presenting cell (APC) activation. Human $CD4^+$ T cells can differentiate into multiple subsets but the potential roles of these subsets in antitumor immunity are only beginning to be understood. $CD4^+$ T-helper (Th) cells were classically separated into two different subsets, Th1 and Th2, based on their pattern of activation induced cytokine production. Another subset, $CD4^+CD25^+$ regulatory/suppressor T cells (Tregs), can suppress anti-tumor immunity and were found to be associated with poor survival in human malignancies, implying that Tregs should be depleted from T-cell populations for adoptive transfer. Recently, a newly identified inflammation-associated $CD4^+$ T-cell subset (Th17) has been shown to mediate greater destruction of large tumors in mice after ACT than both Th1 and Th2 subsets. With the broad array of T cells with distinct phenotypic and functional qualities for potential use in adoptive immunotherapy, there is a need to develop novel and specific *ex vivo* culture methods for each of these T-cell subsets.

T cells for therapy: general approaches

Two broad T-cell preparatory approaches are utilized for the *ex vivo* activation and expansion of T cells for ACT therapy, namely specific and polyclonal stimulation. The former approach relies upon the isolation and activation *in vitro* of antigen-specific T cells harvested from the selected anatomic site, followed by repetitive antigen stimulation *in vitro* to preferentially expand antigen-specific T-cell clones. In the latter approach, polyclonal *ex vivo* activation of the T cells is performed using a non-specific T-cell stimulus, such as anti-CD3 antibody, with or without anti-CD28 antibody or IL-2, which preserves the polyclonal repertoire *in vitro*. When reinfused into the patient, polyclonal T cells then respond directly to antigens presented directly on

transformed or virally infected cells, or indirectly on APCs such as dendritic cells, B cells and macrophages. The polyclonal approach is predicated on the following three assumptions: i) the patient harbors antigen-specific T cells; ii) the antigen-specific T cells have been primed in the patient; and iii) the antigen-specific T-cell population is functionally impaired *in vivo*. Polyclonal T-cell expansion may thus be desirable when expanding T cells with unknown or manifold specificities, such as tumor-infiltrating lymphocytes or T cells from patients receiving polyvalent cancer vaccines. The therapeutic efficacy of immunomodulatory agents, such as IL-2 and anti-CTLA-4 antibodies, supports the notion of pre-existent tumor antigen-specific T-cell dysfunction *in vivo*. While repetitive stimulation with antigen can provide specificity, it is costly in materials and labor, an expansion period of many weeks is needed, and this may result in a population of T cells with limited capability to engraft and persist. Comparatively, the polyclonal approach is technically more rapid, feasible, and generally less expensive, making it more widely applicable for clinical use. Polyclonal cells may also be redirected towards particular antigen specificities via gene transfer.

First generation artificial antigen presenting cells (aAPCs) for T-cell tuning

Dendritic Cells (DCs) deliver a constellation of antigen-specific and costimulatory signals to T cells. *Ex vivo* approaches using autologous DCs to expand T cells for adoptive immunotherapy have been hampered by difficulties in obtaining large numbers of these terminally differentiated, short-lived cells. Major obstacles to the use of DCs in adoptive immunotherapy include the expense of preparing DCs, batch-to-batch variation among donors, and poor yields from *in vitro* cultures. Furthermore, the reported dysfunctional nature of DCs from cancer patients further complicates their use.[1] The rapid expansion method developed by Riddell and co-workers uses irradiated allogeneic peripheral blood mononuclear cells as APCs (also known as feeder cells) to expand CTLs for adoptive transfer.[2] The main limitation of this approach is in clinical scale-up because conforming to FDA-mandated requirements for the validation and qualification of allogeneic feeder cells can be tedious and expensive. Therefore, a current priority is to develop alternative approaches, such as aAPCs, that can support the randomized large-scale trials to definitively demonstrate clinical efficacy and which are required for regulatory agency approval.

In recent years, a greater understanding has emerged of the receptor signaling pathways for T-cell activation, particularly the recognition that both a primary specificity signal via the T-Cell Receptor (TCR) (Signal 1) and a costimulatory/regulatory signal via the CD28 receptor (Signal 2) are simultaneously required for the generation of full T-cell effector function and a long-lasting immune response.[3] In cancer or infectious disease, antigen-specific T cells may have been deleted or tolerized due to suboptimal T-cell activation.

With this knowledge, a first generation of aAPCs was developed to provide efficient and reproducible methods of mimicking the signal provided to T cells by dendritic cells, but without delivering a negative costimulatory signal. These *off-the-shelf* aAPCs consist of clinical grade anti-human CD3 and anti-human CD28 monoclonal antibodies covalently linked to magnetic beads, which serve to cross-link the endogenous CD3 and CD28 receptors on the T cell. This bead-based aAPC enables the most efficient reported growth of human polyclonal naïve and memory $CD4^+$ T cells.[4] A 50 to 1000 fold T-cell expansion can be achieved following an initial stimulation and 10-14 days of *ex vivo* culture. In terms of cell function, the expanded cells retain a highly diverse TCR repertoire and, by varying the culture conditions, can be induced to secrete cytokines characteristic of T helper 1 (Th1) or T helper 2 (Th2) cells.[5] One important advantage of this bead-based system is that it does not cross-react with CTLA4 and, therefore, provides unopposed CD28 stimulation for more efficient expansion of T cells. Another, unanticipated discovery was that cross-linking of CD3 and CD28 with bead-immobilized antibody renders $CD4^+$ T lymphocytes highly resistant to HIV infection.[6] This is due to the down-regulation of CCR5, a necessary co-receptor for the internalization of HIV, and the induction of high levels of β-chemokines, the natural ligands for CCR5, and allows for the efficient culture of $CD4^+$ T cells from HIV-infected study subjects. *Ex vivo* expansion may also indirectly enhance T-cell activity by removing T cells from a tumor-induced immunosuppressive milieu.[7,8] Other key features of these aAPCs are that exogenous growth factors or accessory cells are not needed to enable the T-cell stimulation and expansion, as with previous methods.

These aAPC methods allow for T cells to be grown rapidly *ex vivo* to clinical scale for therapeutic applications. The technology enables direct T-cell activation, instead of indirect activation via vaccines, which can be modulated by the nature of cell dose as necessary to achieve a clinical response.[4] These observations provided the pre-clinical data for the first use of GMP-compliant anti-CD3/CD28 beads in a Phase I adoptive immunotherapy trial.[9] As discussed below, antibody CD3/CD28-coated beads have since been extensively used to expand T cells and $CD4^+$ T cells for use in multiple clinical trials.[5,10-13]

Second generation cell-based artificial antigen presenting cells

While bead-based aAPCs continue to be used in both clinical and pre-clinical studies, they suffer from certain limitations, such as dependence on the availability of clinical grade antibodies and less efficient extended proliferation of $CD8^+$ T cells. Recently, aAPC lines derived from the chronic myelogenous leukemia line K562 and transduced to express an array of T-cell stimulatory ligands have been described.[14] K562 cells do not express Major Histocompatibility Complex (MHC) or T-costimulatory ligands, and these cells retain many other attributes that make DCs such effective APCs, such as cytokine production, expression of the adhe-

sion molecules ICAM and LFA-3 that enhance T-cell-APC interactions, and macropinocytosis. These cells have been transduced with a library of lentiviral vectors for the customized expression of stimulatory and costimulatory molecules that can be used to activate and expand different subsets of T cells, and can also be further modified to amplify antigen specific T cells in culture.[14] This approach allowed for the generation of K562-based aAPCs capable of expressing multiple gene inserts, including human lymphocyte antigen (HLA)-A2, CD32 (the low-affinity Fc receptor), CD64 (the high-affinity Fc receptor) CD80, CD83, CD86, CD137L (4-1BBL) and CD252 (Ox40L),[14] among others. These cell-based aAPCs have proved to be more efficient at activating and expanding $CD8^+$, $CD28^-$ TEM, and antigen-specific T cells, than the magnetic bead-based aAPC.[14] In addition, the cells are capable of stimulating CD4 cells efficiently. Antibody-loaded CD64-expressing K562 cells can be cryopreserved, thawed, and used, with no loss of function, thus permitting even greater standardization of aAPC lots.

The flexibility of the K562-based aAPC system also allows for selective expansion of antigen specific cells from a polyclonal population. Peptide pulsed aAPC expressing exogenous HLA molecules through genetic introduction or HLA-Ig fusion protein coupling can be used to preferentially expand T cells with natural receptors and reactivity for MHC-restricted tumor or viral epitopes.[15] Further, T cells engineered to express specific chimeric antigen receptors that recognize cell surface proteins such as CD19 can be selectively expanded using aAPC genetically modified to the cognate protein.[16] Thus, K562 cells represent flexible scaffolds to which the desired MHC molecules, costimulatory ligands, and cytokines can be introduced without any of the disadvantages of DC (i.e. the need to derive natural DCs from either G-CSF mobilized $CD34^+$ cells or monocytes, patient specific expansion, limited life span, and limited replicative capacity). Moreover, these cells have been injected into humans as part of a tumor vaccine, signifying that these cells can be used in a GMP manner. Bead or cell-based aAPCs have also been developed that are optimized for Th2 cells,[5] and for T-regulatory cells.[17]

Emerging artificial antigen presenting cell technologies

Additional approaches for the *ex vivo* T-cell expansion include acellular liposomes, exosomes, and cell-based mouse fibroblast and insect cell systems.[18] Recently reported aAPC technologies include biodegradable micro- and nanoparticles constructed of poly(lactide-co-glycolide) (PLGA) that incorporate avidin.[19] These aAPCs are designed to present ligands and costimulatory molecules, and encapsulate immune enhancing cytokines that are released after T-cell contact, resulting in preferential $CD8^+$ T-cell expansion. Alternatively, synthetic microbead-based aAPC displaying peptide bound MHC class II and costimulatory molecules can induce antigen-specific $CD4^+$ T cells, which may help generate and/or maintain $CD8^+$ T-cell memory *in vivo*.[20] Interestingly, T cells themselves may serve as a cellular aAPC platform for transmission of costimulatory signals. T cells with natural recognition ligands that are genetically engineered to express CD28 and 4-1BBL costimulatory molecules can engage their respective receptors either in cis to elicit autocostimulation or in trans to other T cells through bystander costimulation to induce robust expansion.[21] Optimizing the next generation aAPC systems for T-cell activation and expansion may depend on elucidating the spatial organization of the TCR and accompanying costimulatory molecules within the immune synapse. The recent use of patterned multi-round microcontact printing of anti-CD3 and anti-CD28 antibodies on a planar substrate has provided a first glimpse into the impact of TCR and CD28 ligand geometry on T-cell activation, and provides a basis upon which current 3-dimensional aAPC systems may be improved.[22]

In this issue of the Journal, Zappasodi *et al.*[23] report on the generation of highly flexible and scalable aAPCs that could be suitable for use in clinical trials. Rather than an inert surface or cell, the authors have used *membrane microdomains* based on ganglioside GM1 enriched-liposomes as a scaffold for ligand stimulation of T cells. These microdomains have the potential to be manufactured at a reasonable cost and in large batches. The authors modified aAPCs that were initially developed as tools to explore the immune synapse and T-cell signaling by the addition of anti-CD3 mAb to trigger the TCR complex, anti-CD28 mAb to provide a costimulatory signal, and anti-LFA-1 to provide an additional signal and strengthen the immune synapse through this adhesion receptor. The results indicate that these engineered microdomain aAPCs can effectively stimulate and expand polyclonal T cells. The expanded cells retained phenotypic and functional attributes consistent with effector T cells rather than regulatory/suppressor T cells. The microdomain aAPC could also expand antigen-specific T cells for the melanoma antigen MART-1 following pre-stimulation with peptide loaded HLA-A*0201+ T2 cells. Since the expression of 4-1BB on K562-based aAPC has recently been shown to expand Ag-specific memory CD8 T cells,[15] the addition of anti-4-1BB or 4-1BB ligand to microdomain aAPC would also be expected to allow expansion of these T cells without the necessity for T2 cell pre-stimulation. The next steps in evaluating these

Table 1. Points to consider for artificial antigen presenting cells in *ex vivo* engineering of an effective T-lymphocyte therapy.

- Presentation of appropriate signals for particular T-cell subset and conservation/enhancement of T-cell function, proliferative capacity, engraftment ability
- Consistent and stable potency of aAPC from lot to lot
- Cost effectiveness and efficiency of aAPC manufacture and T-cell expansion
- Need/method for removal prior to T-cell infusion if residual carryover on to T cells during stimulation may result in toxicity or confer immunogenicity

novel aAPC described by Zappasodi would be *in vivo* experiments infusing aAPC expanded T cells in animal models to track homing, engraftment and function. This could be accomplished in a humanized mouse model and provide the necessary pre-clinical data for human trials. Some considerations for the evaluation of aAPC for use in clinical trials are listed in Table 1.

Clinical trials of aAPC engineered T lymphocytes

In addition to clinical trials conducted using variations of the rapid expansion method for T-cell expansion described above, which has cost, efficiency and functional drawbacks for wide scale application,[2] a 10 patient clinical trial in melanoma has been conducted using insect cells as aAPCs to produce antigen-specific T cells.[24] To date, several hundred infusions of anti-CD3/anti-CD28 bead aAPC stimulated T cells have been safely administered in clinical trials to treat hematologic cancers and HIV, in clinical trials at several sites in the U.S. For clinical trials in lymphoma,[10] chronic myelogenous leukemia (CML),[11] and myeloma,[25] patients were administered activated autologous T cells. In the first trial, patients with high risk lymphoma were given one infusion of the cells on day 14 post CD34 selected hematopoietic stem cell transplantation (HCT), and demonstrated accelerated reconstitution of lymphocyte numbers and function.[10] In the CML trial, rapid recovery of lymphocyte counts following T-cell infusion and complete cytogenetic remissions were observed.[11] The randomized phase I/II study in subjects with advanced myeloma was designed to examine the relative benefits of pre- and post- transplant vaccine immunizations in combination with adoptive T-cell transfer. Post-stem cell transplant lymphocyte reconstitution and Prevnar vaccine response were evaluated in 42 subjects. Similar to the lymphoma trial, it appears as though the infusion of activated autologous T cells by day 14 post-transplant resulted in the induction of homeostatic T-cell proliferation in the first few weeks following transplantation, which may prove to be a useful way to generate and/or enhance protective anti-tumor immunity.[25] In addition, only those subjects that received antigen experienced T cells made appropriate antibody responses. A follow-on trial is now open in which the potency of a putative myeloma specific vaccine is being tested to lead to a myeloma-directed T-cell mediated *graft vs. myeloma* effect. In a completed trial in an allogeneic setting, activated donor leukocyte infusions (aDLI) were administered to treat relapsed advanced hematologic malignancies after allogeneic bone marrow transplantation and standard DLI.[13] Of the 17 subjects evaluable for response, 8 achieved a complete remission (CR) with 6 still alive in CR a median of 17 months after aDLI. This trial suggests that adoptive transfer of activated allogeneic T cells is associated with durable CR in a subset of subjects without excessive graft versus host disease. From these early trials, promising results in heavily pre-treated patients have led to the initiation of a second series of multiple dose and randomized trials in hematologic malignancies and other cancers, and also in HIV, that are ongoing to address the efficacy of aAPC engineered T-cell therapies. At the same time, trials with the cell based K562 aAPC described above are in pre-clinical development for the expansion of tumor-specific T lymphocytes and Tregs. GMP-compliant master cell banks of K562 aAPCs are being evaluated and characterized and will soon enter phase I clinical trials.

Conclusion

Ex vivo manipulation may serve to activate, re-educate or endow T cells with enhanced or novel functions in a way that was not possible *in vivo*, perhaps due to masking of Ag or disease-induced immunosuppression. Central to the success of future clinical trials of engineered T lymphocytes is the determination of whether and how vaccine therapy in combination with adoptive transfer should be employed, and how to engineer and manufacture the respective T lymphocytes for human testing in a variety of disease settings. The ideal *ex vivo* culture process in a research setting must be adapted to clinical scale with clinically compatible reagents to achieve regulatory compliance and yet still be robust enough to support large scale trials of potent engineered T cells.

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