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Human cell-based artificial antigen-presenting cells for cancer immunotherapy

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Summary

Adoptive T-cell therapy, where antitumor T cells are first prepared *in vitro*, is attractive since it facilitates the delivery of essential signals to selected subsets of antitumor T cells without unfavorable immunoregulatory issues that exist in tumor-bearing hosts. Recent clinical trials have demonstrated that antitumor adoptive T-cell therapy, i.e. infusion of tumor-specific T cells, can induce clinically relevant and sustained responses in patients with advanced cancer. The goal of adoptive cell therapy is to establish antitumor immunological memory, which can result in life-long rejection of tumor cells in patients. To achieve this goal, during the process of *in vitro* expansion, T-cell grafts used in adoptive T-cell therapy must to be appropriately educated and equipped with the capacity to accomplish multiple, essential tasks. Adoptively transferred T cells must be endowed, prior to infusion, with the ability to efficiently engraft, expand, persist, and traffic to tumor *in vivo*. As a strategy to consistently generate T-cell grafts with these capabilities, artificial antigen-presenting cells have been developed to deliver the proper signals necessary to T cells to enable optimal adoptive cell therapy.

Keywords

artificial antigen-presenting cell; cytotoxic T cell; cytokine; adoptive therapy; K562; memory

Introduction

Cancer immunotherapy utilizes the power and specificity of the immune system to treat cancer (1–6). The goal of cancer immunotherapy is to build long-lasting tumor-specific immunologic ‘memory’ in patients that enables the lifelong rejection of tumors. The molecular determination of human melanoma-specific antigens by Boon and colleagues (7, 8) more than 20 years ago sparked the development of antigen-specific antitumor immunotherapy. There are two prominent types of antigen-specific cancer immunotherapy. One is adoptive T-cell therapy where tumor antigen-specific T cells are expanded *in vitro* and then infused back into patients (9, 10). The other is through vaccination, which is the administration of a cancer antigen in concert with an adjuvant to elicit antitumor T cells *in vivo* (11–13).

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The daunting challenges of inducing clinically relevant sustained antitumor specific immunity following vaccination has led several groups to explore the potential of adoptive T-cell therapy (14). At least theoretically, there are several advantages to the use of adoptive cell therapy over vaccination as a treatment of cancer. First, tumor-specific T cells can be activated and expanded to large numbers *in vitro* avoiding the tenacious tolerogenic and immunosuppressive properties of the tumor microenvironment (15, 16). Secondly, a subset of T cells with desired functional and phenotypic qualities can be specifically selected and infused to patients (17, 18). In fact, adoptive T-cell therapy has recently been shown to have the potential to induce clinically relevant antitumor responses in patients suffering from advanced cancer. For example, the adoptive transfer of *in vitro* activated tumor-infiltrating lymphocytes to lymphodepleted melanoma patients and subsequent high dose IL-2 treatment are capable of producing clinically significant responses (19, 20). Adoptive therapy of melanoma-specific T cells has also showed clinical activity (21, 22). Demonstration that adoptively transferred anti-Epstein Barr virus (EBV)-specific T cells can induce clinical responses in patients with Hodgkin's disease and nasopharyngeal carcinoma is similarly compelling (23, 24). Furthermore, administration of anti-CD19 chimeric antigen receptor (CAR)-transduced T cells resulted in impressive clinical responses in patients with $CD19^+$ B-cell lymphoma and leukemia (25–30). Taken all together, these encouraging clinical results suggest that adoptive transfer of large numbers of functional antitumor T cells might become effective treatment for cancer patients.

Sufficient numbers of *in vitro*-generated, tumor-specific cells are necessary for effective therapeutic infusion. *In vitro*-expanded T cells must also possess the capacity to engraft, proliferate, and persist *in vivo* with sufficient antitumor function to induce sustained antitumor activity. Originally, autologous antigen-presenting cells (APCs) such as dendritic cells, monocytes, and activated B cells have been employed to generate tumor-specific T cells *in vitro*. However, the requirement to access cancer patients' blood to prepare autologous APC from each patient in a timely manner is cumbersome. Furthermore, the quantity and quality of prepared autologous APC varies between individuals. To overcome these issues inherent to patient-derived autologous APC, investigators have recently proposed using cell-based artificial APCs (aAPCs) as an off-the-shelf, standardized, and renewable reagent to reliably expand antitumor T cells *in vitro* for adoptive therapy. Several excellent general reviews of the history of the aAPC concept have already been published (31, 32). In this article, therefore, we focus on recent advances in the development of K562, human leukemic cell line-based aAPCs that are being exploited to generate T-cell grafts for effective adoptive cell therapy for cancer.

Phenotypic and functional attributes of T-cell grafts desired for optimal antitumor adoptive therapy

T cells can be classified into naive or one of three major antigen-experienced subtypes: central memory T cell, effector memory T cell, and terminally differentiated effector T cells. New data are emerging regarding the putative human T memory stem cell population, and readers are directed to several excellent papers covering this topic (18, 33–36). There has been an active debate on whether memory T cells develop from naive or terminally differentiated effector T cells and on the relationship between central and effector memory T cells (37). However, it is clear that these four subgroups represent a continuum of T-cell differentiation and maturation (38, 39). Both naive and antigen-experienced central memory T cells coexpress the lymphoid homing molecules L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7). These two subsets of T cells that display CD62L and CCR7 have a predisposition to home to secondary lymphoid structures where they can actively survey professional APCs, i.e. dendritic cells, for the presence of cognate antigen. While, in

humans, naive T cells are positive for CD45RA, central memory T cells lose the expression of CD45RA and instead acquire the expression of the archetypal human antigen-experienced T-cell marker CD45RO. In addition to their preferential anatomic localization in lymphoid organs, these two T-cell subsets retain a strong replicative capacity. In contrast, effector memory and terminally differentiated effector T cells are both antigen-experienced T cells and have strongly downregulated CD62L and CCR7 expression. Accordingly, these two subsets of T cells preferentially reside in peripheral tissues rather than secondary lymphoid tissues. Upon activation by T-cell receptor engagement, both effector memory and terminally differentiated effector T cells are poised to exert robust effector functions; they can release large amounts of inflammatory cytokines such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) and rapidly kill antigen-expressing targets using perforins, granzymes, and Fas ligand. However, these two subsets with potent effector functions generally bear shortened telomere lengths and a limited proliferative potential compared with naive or central memory T cells (40, 41). The conundrum to solve here is which subset is the best used to achieve the goal of adoptive cell therapy, which is to establish antitumor immunological memory resulting in life-long rejection of tumor cells in patients.

Using TCR-transgenic mice, Restifo and his group (42, 43) have elegantly demonstrated that antigen-specific naive and central memory T cells are more effective than effector memory and terminally differentiated effector T cells in the eradication of large, established tumors. Paradoxically, $CD8^+$ T cells that acquired complete effector properties and exhibited enhanced antitumor reactivity *in vitro* were less effective in triggering tumor regression and tumor eradication *in vivo*. These results suggest that T-cell grafts do not necessarily need to be armed with full effector function prior to infusion for optimal antitumor activity *in vivo* (44). Instead, naive T cells used in adoptive transfer could engraft, undergo priming, and acquire effector functions *in vivo* (45).

At present, however, adoptive transfer of large numbers of antigen-specific, naive T cells is not feasible in humans. Therefore, in a non-human primate model, antigen-specific central memory and effector memory T cells were compared in terms of their capacity to engraft and proliferate. Using T cells specific for cytomegalovirus, Riddell and his colleagues (46) demonstrated that antigen-specific $CD8^+$ T-cell clones derived from central memory T cells, but not effector memory T cells, persisted for a long period of time *in vivo*, reacquiring phenotypic and functional attributes of memory T cells. These results suggest that clonally derived $CD8^+$ T cells isolated from central memory T cells are distinct from those derived from effector memory T cells and retain an intrinsic capacity that enables them to survive after adoptive transfer.

Jensen and his group (47) analyzed the engraftment of adoptively transferred human antigen-specific central memory and effector memory T cells in an immunodeficient mouse model. The engraftment of central memory T cells was superior in magnitude and duration to effector memory T cells. Importantly, persisting T cells were polyclonal, suggesting that the ability to engraft is a general feature of central memory T cells. Although effector memory T cells could proliferate extensively after transfer, they underwent rapid apoptosis. In contrast, central memory T cells were resistant to apoptosis and able to establish a persistent pool of functional T cells *in vivo* characterized by higher CD28 expression.

Previous clinical trials demonstrated that the use of less-differentiated, minimally cultured ‘young’ TILs, which have longer telomeres and higher levels of the costimulatory molecules CD27 and CD28, may be an important factor for successful antitumor adoptive cell therapy (48–50). Therefore, in humans the best T-cell grafts currently available seem be those that are minimally cultured and contain less differentiated T cells and possess a ‘young phenotype’ such as $CD27^+$ $CD28^+$ $CD45RO^+$ $CCR7^+$ $CD62L^+$ central memory T cells (34,

51, 52). As described above, unlike terminally differentiated effector T cells, central memory T cells are not yet fully equipped with effector functions such as cytotoxicity and IFNγ secretion. Accordingly, the best culture conditions for preparing T cells for adoptive therapy cannot be chosen simply by comparing the potency of the expanded T-cell effector functions. Instead, this selection should be based on several factors including the retention of a young phenotype, telomere length, and/or other markers that would predict for better persistence *in vivo* in contrast to *in vitro* effector function. It should be noted that the capacity for IL-2 secretion is one of the functions that central memory T cells do exhibit and that it diminishes along with maturation. Therefore, one of the goals of adoptive therapy may be to infuse T cells with a preserved ability for antigen-specific IL-2 secretion.

In early clinical trials where cancer patients were treated with large numbers of antitumor T-cell clones, clinical efficacy was usually limited and there was little persistence of infused T-cell clones in most cases even when high does IL-2 was administered (53–55). This could be at least partly attributed to the fact that the T cells used for these adoptive immunotherapy trials were selected for their capacity to produce high levels of IFNγ and for their ability to efficiently and specifically lyse relevant target cells, which reflects a matured phenotype instead of the potential to survive *in vivo* following transfer. The mere generation of large numbers of highly differentiated T cells appeared insufficient to trigger tumor regression in adoptive T-cell transfer.

Principles of stimulation to generate T-cell grafts for antitumor adoptive therapy

For the purpose of effective adoptive T-cell therapy, the critical question is how to generate, within a short period of time, large numbers of ‘young’ antitumor T cells with a central memory phenotype and avidity sufficient to recognize tumor. It has been demonstrated that the fate of antigen-specific T cells are largely determined by how they are stimulated during the priming phase (56–58). When naive T cells are suboptimally primed, they may become hyporesponsive or anergic and show a lack of proliferation and/or effector function in response to antigen. To avoid this anergic state induced by suboptimal priming, it is critical that naive T cells are initially primed by APCs in an immunologically productive environment. Further expansion of primed antigen-specific T cells requires restimulation within an optimal immunologic context that includes exposure to γ chain receptor cytokines such as IL-2, IL-7, IL-15, and IL-21 (59–61). On the other hand, repeated or over stimulation using highly immunogenic professional APCs, such as dendritic cells and CD40L-stimulated B cells, unavoidably incurs T-cell activation-induced cell death (AICD), especially for T cells that possess high antigen-specific avidity. As a result, ‘maximal’ stimulation with professional APCs can result in the accumulation of low avidity T cells *in vitro*. Also, maximal T-cell activation is inevitably accompanied by T-cell differentiation, which should be minimized for T-cell grafts used in optimal adoptive therapy as discussed in the previous section. Therefore, it is undoubtedly true that professional APCs such dendritic cells are critical for the generation of productive immunity *in vivo*, especially during the priming phase. Because of their highly potent immunogenicity, however, professional APCs may not be the best cellular APC to generate *in vitro* T-cell grafts for optimal adoptive cell therapy of cancer patients.

The signal strength that is delivered to T cells by APCs for the purpose of generating T-cell grafts must be strong enough for their proliferation but not so strong that terminal differentiation occurs or that AICD of high avidity T cells is induced. Since there exists no naturally occurring, readily available APC that can deliver such a signal, researchers have developed artificial APC (aAPCs) that that have been engineered to provide optimal control over the signals provided to T cells used for adoptive therapy.

K562 as a backbone of cell-based aAPCs

To serve as a backbone of cell-based and gene-engineered aAPCs, several requirements must be satisfied. First, the induction of allospecific T cells by aAPCs should be absent or minimal if any does occur. Therefore, the backbone cells should not express any HLA class I or II molecules. Second, they should be easily gene-manipulated and also the expression of the manipulated genes must be stable. Third, a track record of previously using the backbone cell line safely in human beings would be preferable. Since K562 cells satisfy all three of these requirements, they have been widely utilized as a backbone cell line for a series of aAPCs by many investigators (31, 32). K562 is a human erythroleukemic cell line that was derived from a patient with chronic myelogenous leukemia in blastic crisis (62, 63). K562 cells do not express endogenous HLA class I, II, or CD1d molecules but do express ICAM-1 (CD54) and LFA-3 (CD58), which are adhesion molecules required to form an effective immunological synapse (64). The expression of K562's endogenous HLA class I and II molecules can be induced by treatment with chemicals and/or cytokines (65, 66). Although they endogenously express CD80 at a low level, they do not express other costimulatory molecules such as CD86, CD83, 4-1BBL, OX40L, ICOSL (B7H2, B7RP1), or CD40L (64). They also lack the expression of inhibitory molecules such as PDL1 (B7H1), PDL2 (B7DC), B7H3, and B7H4 (B7X, B7S1). K562 secretes M-CSF, IL-6, IL-8, TGF-β, MIP-1α but not IFN-γ, GM-CSF, IL-10 or any common γ-chain receptor cytokines tested (64). Below, we review K562-based aAPCs gene-engineered to expand $CD8^+$, $CD4^+$, $CD3^+$, CD19 CAR-transduced T cells, invariant natural killer T (iNKT) cells, and NK cells (Table 1).

aAPCs for antigen-specific $CD8^+$ T cells

To deliver signal 1 to antigen-specific $CD8^+$ T cells, HLA class I molecules were transduced into K562 cells. Since K562 endogenously express β2-microglobluin, transduction of HLC class I gene alone is sufficient for its surface expression. Overexpression of β2-microglobulin did not lead to the upregulated expression of ectopically expressed HLA class I molecules (Naoto Hirano, unpublished data). In addition, virtually all previously reported K562-derived aAPCs have been engineered to express B7 molecules, CD80 and/or CD86 (67) to deliver the signal 2, costimulatory signal. Note that K562 endogenously express intermediate levels of CD80 but not CD86 (64, 65).

K562 endogenously expresses a high level of CD32 but not CD16 or CD64 (68). In fact, K562 is one of the first cell lines in which CD32 was identified (69). June's group transduced K562 with CD32 and 4-1BBL which was coated with anti-CD28 monoclonal antibody (mAb), and used it to expand $CD8^+$ T cells in the presence of soluble anti-CD3 mAb in an antigen-independent manner (70). CD32 was later replaced by CD64 to enable coating with both anti-CD3/28 mAbs and the removal soluble anti-CD3 mAb from the cultures. These aAPCs were able to expand not only $CD8^+$ $CD28^+$ T cells but also, to some extent, $CD8^+$ $CD28^-$ cells in the absence of $CD4^+$ T-cell help (67).

In addition to B7 molecules such as CD80 and CD86, additional immunoaccessory molecules such as 4-1BBL and CD83 have been transduced to the aAPCs depending on the attributes with which investigators wanted to endow the expanded T cells (64, 67, 71). CD83 is a type I membrane glycoprotein belonging to the immunoglobulin superfamily and is highly expressed by professional APCs such as mature dendritic cells (72–74). It has been shown that engagement of CD83 ligand induces prolonged expansion of $CD8^+$ T cells and preferential enrichment for antigen specificity (75). CD83 can enrich for the expansion of T cells from both naive and memory precursor T cells in an antigen-specific manner. Also, a study using knockout mice revealed that CD83 is required for B cells and $CD4^+$ T-cell longevity (76). We introduced HLA class I, CD80, and CD83 molecules to K562 aiming for

the expansion, enrichment, and longevity of antigen-specific $CD8^+$ T cells (75). Using K562-derived aAPC expressing HLA-A2*02:01, CD83, and CD83 and no other feeder cells, we were able to expand and maintain tumor-antigen specific $CD8^+$ T cells up to 1 and a half year *in vitro* (64). Long-lived $CD8^+$ T cells also lacked the expression of exhaustion markers such as PD1, LAG-3, CTLA-4, and Tim-3 and instead expressed a high level of CD25.

As described above, other groups transduced 4-1BBL for the purpose of bulk expansion of cultured T cells. In our system, however, 4-1BBL did not enable optimal enrichment of naive-derived antigen-specific $CD8^+$ T cells (71). One explanation for this is that 4-1BBL can stimulate memory T cells without signal 1 through the T-cell receptor engagement (77). Therefore, it can stimulate any memory T cells in the cultures in an antigen-independent manner. In fact, 4-1BBL was excellent in increasing the total number of cultured T cells. Whether 4-1BBL-transduced aAPC can generate long-lived antigen-specific $CD8^+$ T cells like CD83-transduced aAPC is unknown.

K562 cells are equipped with conventional proteasome machinery and can process and present $CD8^+$ T-cell epitopes derived from endogenous proteins via ectopically expressed HLA class I molecules (78). Furthermore, IFN-γ treatment can induce immunoproteasome machinery while downregulating conventional proteasome machinery. K562 is also capable of cross-presentation albeit less efficiently compared to monocyte-derived *in vitro* generated dendritic cells (Tanaka M, Hirano N, unpublished data).

aAPCs for antigen-specific $CD4^+$ T cells

HLA class I-restricted $CD8^+$ T cells are primary mediators of adaptive immunity due to their potent direct effector function. However, many studies have demonstrated that $CD4^+$ T cells provide help which is critical for generating optimal antitumor $CD8^+$ T-cell responses and long-lasting memory (79–81). In addition to HLA class molecules, K562 lacks the expression of invariant chain (Ii, CD74) and HLA-DM (68, 82). In the endoplasmic reticulum (ER), the nascent MHC class II molecule has its peptide-binding cleft blocked by Ii to prevent class II molecules from binding endogenous peptides derived from intracellular proteins (83). Following export to endosomes/lysosomes, Ii is then broken down in stages, leaving only a small fragment called class II-associated invariant chain peptide (CLIP) which continues to block the peptide binding cleft. Then, HLA-DM catalyzes the removal of CLIP which is replaced by peptides derived from exogenous proteins which have been imported into endosomes/lysosomes (84, 85). To mimic this antigen processing and presentation pathway of HLA class II-restricted $CD4^+$ T-cell antigenic peptides, we produced aAPC for antigen-specific $CD4^+$ T cells by transducing HLA-DR or DP α and β chains, Ii, and DM α and β chains in conjunction with CD80 and CD83 (68, 82). When pulsed with HLA-restricted peptide, these aAPCs were able to expand long-lived HLA-restricted antigen-specific $CD4^+$ T cells.

Monocytes and DCs can process and present HLA class II-restricted peptides derived from exogenous protein taken up via pinocytosis, receptor-mediated endocytosis, or phagocytosis. K562 cells do not have measurable phagocytic capability but do possess pinocytotic activity comparable to monocytes or DCs (68). Although strongly positive for CD32 expression, K562's CD32-mediated endocytotic activity is relatively weak. When CD64 was introduced along with the FcR common γ subunit, however, K562 acquired efficient endocytotic activity mediated via CD64, enabling an uptake of a soluble antigen/antibody complex and processing and presentation of $CD4^+$ T-cell epitopes derived from the antigen (68). Antigen-specific $CD4^+$ T cells expanded using these aAPCs were also long-lived and could have both Th1 and Th2 phenotypes. Long-lived $CD4^+$ T cells also lacked the expression of Foxp3 and induced no or very low expression of exhaustion markers such as PD1, LAG-3, and

Tim-3. It has yet to be shown whether K562-derived aAPCs can expand antigen-specific Foxp3-positive thymus-derived and/or induced $CD4^+$ regulatory T (Treg) cells. This demonstration may be difficult, since K562 cells secrete IL-6, which may compromise the expansion of $Foxp3^+$ Treg cells (86, 87).

aAPCs for polyclonal $CD3^+$ T cells

We also developed a genetically engineered K562-based aAPC, named aAPC/mOKT3, which expresses a membranous form of the anti-CD3 mAb, clone OKT3, in addition to CD80 and CD83 (88). Without requiring the addition of allogeneic feeder cells, aAPC/mOKT3 was able to expand both peripheral and tumor-infiltrating T lymphocytes (TILs). Stimulation with aAPC/mOKT3 did not expand $Foxp3^+$ regulatory T cells, and expanded TILs predominantly secreted Th1-type cytokines, IFN-γ and IL-2 but not IL-4 or IL-10. There was no obvious clonal skewing in the T cells expanded. Interestingly, while the number of both $CD4^+$ and $CD8^+$ T cells increased, $CD8^+$ T cells expanded substantially better than $CD4^+$ T cells, and therefore dominated cultures from every donor tested. This is in stark contrast to other pan T-cell expansion systems such as anti-CD3/CD28 mAb-coated beads or other aAPC-based cell system (see below), which invariably favor the expansion $CD4^+$ T cells over $CD8^+$ T cells (89). Similar fold expansion of $CD3^+$ T cells was obtained with the aAPC/mOKT3-based and antibody-coated bead-based expansion systems.

In this aAPC-based system, the presence of autologous $CD4^+$ T cells was associated with significantly improved $CD8^+$ T-cell expansion *in vitro*. The $CD4^+$ T-cell-derived cytokines IL-2 and IL-21 were necessary but not sufficient for this effect. $CD4^+$ T-cell help of $CD8^+$ T-cell proliferation was partially recapitulated by both adding IL-2/IL-21 and by upregulation of IL-21 receptor on $CD8^+$ T cells. These results are intriguing, since IL-2 and IL-21 are mediators of $CD4^+$ T-cell help *in vivo*. IL-2, one of the few effector cytokines made by naive $CD4^+$ T cells, expands activated T cells and is necessary for the development of $CD8^+$ T-cell memory responses against pathogens (90). While $CD8^+$ T-cell responses during acute viral infections are relatively independent of IL-2, the development of protective $CD8^+$ T-cell memory responses required IL-2 exposure during priming phase (91–93). *In vivo* models also indicate that IL-21 is critical for containing chronic viral infections and preventing the deletion of high affinity antiviral $CD8^+$ T cells. IL-21 secretion by $CD4^+$ T cells enables the generation, sustained proliferation, and maintenance of polyfunctional $CD8^+$ T cells during chronic infection (94–96). These observations also provide important base for the use of IL-2 and IL-21 in the $CD8^+$ T-cell cultures to make T-cell grafts for antitumor adoptive therapy.

Another group applied the aAPC, K562/CD32/4-1BBL, originally developed to expand $CD8^+$ T cells in an antigen-independent way, for the expansion of polyclonal $CD3^+$ T cells in the presence of IL-2 (97). This aAPC was able to numerically expand TILs in a manner similar to an established rapid expansion method with less requirement for allogeneic feeder cells. However, the aAPC-expanded TILs possessed low CD4/CD8 ratios unlike the ones grown using the aAPC-mOKT3 described above. To fix this issue, the same group modified the aAPCs to secrete IL-21 (98). This IL-21-secreting aAPC induced the expansion of $CD8^+$ T cells with a $CD27^+CD28^+$ young phenotype and superior functional cytotoxic effector characteristics without collateral expansion of Treg cells. These results suggest that when expanding a mixture of $CD4^+$ and $CD8^+$ T cells such as peripheral $CD3^+$ T cells or TILs, to avoid the outgrowth of $CD4^+$ T cells and sustain the expansion of $CD8^+$ T cells, IL-21 derived from $CD4^+$ T cells or provided exogenously is pivotal. Since $CD8^+$ T cells play often play an indispensable role in TIL-based adoptive cell therapy, this is critically important, because some of the TILs prior to expansion possess very few $CD8^+$ T cells compared to $CD4^+$ T cells.

aAPCs for chimeric antigen receptor-transduced T cells

The use of antitumor T cells that can kill tumor cells without HLA restriction holds great promise to extend the applicability of cellular immunotherapy to more cancer patients. Chimeric antigen receptor (CAR)-transduced T cells are where T cells are transduced with synthetic chimeric receptors with an extracellular antigen-binding domain, a spacer domain, and an intracellular signaling module, most commonly the CD3ζ chain and one or more costimulatory domains such as CD28 or 4-1BB (99–101). In theory, K562 expressing a cognate target surface antigen for any given CAR and costimulatory molecules should be able to serve as an APC to stimulate the CAR-transduced T cells.

Cooper's group (102–104) has intensively studied K562-based APCs designed to propagate CD19 CAR-transduced T cells. Based on their work, Cooper and June (105) collaborated to generate clinical grade K562-derived aAPCs expressing CD64, CD86, 4-1BBL, truncated CD19, and mbIL-15/IRES-EGFP to expand CD19-transduced T cells for adoptive therapy clinical trials. They opened a new Phase I protocol at the MD Anderson Cancer Center (Houston, TX) to examine the safety and feasibility of administering autologous genetically modified T cells expressing a CD19-specific CAR (capable of signaling through chimeric CD28 and CD3ζ) into patients with CD19 expressing lymphoid malignancies (see below). The T cells are genetically modified by nonviral gene transfer of the Sleeping Beauty system and CAR^+ T cells selectively propagated in a CAR-dependent manner on designer aAPCs (106).

aAPCs for invariant natural killer T cells

One of the major limitations in using conventional T cells as a T-cell graft in adoptive cell therapy is their HLA restriction (107). The utilization of iNKT cells, which are restricted by monomorphic CD1d, as a graft for adoptive transfer may extend immune cell therapy to more cancer patients regardless of HLA allele expression. Human iNKT cells express invariant TCR Vα24, which is paired with TCR Vβ11, and recognize glycolipids presented on monomorphic CD1d (108–110). Therefore, tumor-specific iNKT cells can attack $CD1d^+$ tumor cells such as leukemia and lymphoma cells without HLA restriction. We molecularly engineered aAPCs to expand iNKT cells by introducing CD1d, CD80, and CD83 to K562 cells (111). Using these aAPCs pulsed with synthetic ligands such as α-galactosyl ceramide, we were able to expand highly polyclonal iNKT cells harboring various CDR3β sequences. The expanded iNKT cells secreted IFN-γ and/or IL-4 in a CD1d-dependent manner and their subset were autoreactive recognizing endogenous ligands presented by CD1d.

aAPCs for natural killer cells

Like iNKT cells, NK cells can also target cancer cells irrespective of their HLA alleles (107). K562 is a well-known target of NK cells. Therefore, K562-derived aAPCs can also be excellent stimulators of NK cells. To generate K562-derived aAPCs to expand NK cells, K562 was transduced with a membrane-bound form of IL-15 along with 4-1BBL (112). The membrane-bound form of IL-15 consisted of CD8α signal peptide, IL-15 mature peptide, and the transmembrane domain of CD8α. This system has been clinically adapted to grow large numbers of NK expansion *in vitro* (113).

Another group used K562-aAPC expressing CD64, CD86, 4-1BBL, truncated CD19, and mutated membrane-bound IL-15 (mbIL-15) originally intended to expand anti-CD19-CAR-transduced T cells (see above) to expand NK cells (105). mbIL-15 is composed of human IL-15 peptide fused to modified human IgG4 Fc region and CD4 transmembrane domain. It was found, however, that *in vitro* proliferation of NK cells stimulated by IL-15 expressing aAPC was limited by telomere shortening. To address this issue, the same group transduced

mbIL-21 (membrane-bound human IL-21) instead of mbIL-15 onto K562 expressing CD64, CD86, 4-1BBL, truncated CD19 (114). In contrast to mbIL-15, mbIL-21-expressing aAPCs promoted log-phase NK cell expansion without evidence of senescence for up to 6 weeks of culture. NK cells expanded with mbIL-21 were similar in phenotype and cytotoxicity to those expanded with mbIL-15, with retained donor KIR repertoires and high expression of natural cytotoxicity receptors (NCRs), CD16, and NKG2D, but had superior cytokine secretion. mbIL-21-expanded NK cells had significant cytotoxicity against all tumor cell lines tested, retained responsiveness to inhibitory KIR ligands, and demonstrated enhanced killing via antibody-dependent cell cytotoxicity (104, 115).

Cytokines used for aAPC-based T-cell expansion

It has become clear that $CD8^+$ T-cell fate is regulated by three signals: TCR engagement (signal 1), costimulation (signal 2), and an inflammatory stimulus (signal 3) via cytokines such as IL-12 or type I interferons (type I IFN). Direct signaling via IL-12 and type I IFN receptors favors terminal differentiation of effector $CD8^+$ T cells with potent cytotoxic activity and IFNγ production but with limited survival. In contrast, memory precursor $CD8^+$ T cells producing IL-2 and exhibiting a phenotypically more naive status are more likely to survive and establish memory. Therefore, the fact that K562 cells do not secrete proinflammatory cytokines such as IL-12 or type I IFNs might be beneficial in the selective expansion of central memory T cells with a young phenotype. It should be reiterated again that effector function such as cytotoxicity or IFNγ secretion is not a prerequisite for antitumor T cells used in adoptive cell therapy. Instead, as discussed above, when we expand antitumor T cells, we should employ a culture protocol which induces less *in vitro* differentiation and maturation, enables engraftment, and sets the stage for expansion *in vivo* as effective antitumor T cells.

To date, IL-2 has been predominantly used as a T-cell growth factor in the generation of antitumor T cells for adoptive transfer (116). IL-2, which is a prototypic member of the common γ chain family of cytokines, has been shown *in vitro* to promote T-cell activation, proliferation and survival, and the expanded T cells lysed tumor (116). However, IL-2 exposure can also lead to activation-induced cell death (AICD), progressive differentiation and maturation, and to the induction of immunosuppressive Treg cells (117). Therefore, it is obvious that IL-2 possesses both a positive and negative impact on the induction of an effective antitumor immunological response.

When expanding T cells using K562-based aAPC, IL-2 is most efficient to enrich antigen-specific T cells compared to other common γ chain cytokine family members such as IL-7, IL-15, and IL-21. This is probably because the difference between antigen-specific and non-specific T cells' sensitivity to IL-2 is greater than their sensitivity to other cytokines. Therefore, almost all current protocols using K562-aAPC as a T-cell stimulator require the addition of IL-2 to the cultures as a base cytokine.

IL-7 is important for the maintenance of both $CD4^+$ and $CD8^+$ matured T cells *in vivo* (118–120). It has yet to be reported whether IL-7 plays a unique role that cannot be replaced by other cytokines including IL-2, IL-15, or IL-21, in K562-aAPC-based T-cell expansion systems.

IL-15 and IL-21 also belong to the common γ chain cytokine family and play a role in T-cell proliferation, survival, and function (121). Both cytokines are involved in the maintenance and expansion of memory $CD8^+$ T cells, NK, NKT, and the function of effector $CD8^+$ T cells. Mouse studies have shown that adoptively transferred T cells demonstrated superior *in vivo* persistence and tumor elimination when pre-treated with either IL-15 or IL-21 (122–

124). Furthermore, it has been suggested that T cells exposed to IL-15 or IL-21 become resistant to immunosuppressive activity of Treg cells. Based on these data, the use of IL-15 and/or IL-21 has been incorporated into the generation of antitumor T-cell grafts for optimal adoptive cell therapy multiple investigators (98, 104, 106, 125).

K562 cells can be gene-engineered to secrete cytokines and also express them as a membrane-bound form (98, 104, 125). However, the cytokine level provided by K562-based aAPC is usually not sufficient for the sustained expansion of T cells, since K562-based aAPC needs to be γ-irradiated before mixed with the T cells and are quickly eliminated from cultures. Therefore, it is almost always mandatory to also exogenously add cytokines at least once a week or usually every few days. It is also critical to limit the concentrations of cytokines added to the *in vitro* T-cell cultures (51, 64, 75). This helps to avoid cytokine addiction where the cultured T cells become dependent on supraphysiologic, high concentrations of cytokines for survival and function *in vivo*. Otherwise, when adoptively transferred, the T cells may die quickly and engraft poorly from the paucity of cytokines available *in vivo*.

Generation of clinical grade aAPC-A2, clone 33

Based on our preclinical work, we developed an aAPC-based T-cell generation system suitable for use in clinical trials (64). We generated aAPC-A2, clone 33, under cGMP conditions to expand MHC class I-restricted, antigen-specific $CD8^+$ T cells. This clinical grade version of aAPC-A2 was produced by transfecting K562 simultaneously with four non-retroviral plasmids that encode for HLA-A*02:01 (A2), CD80, CD83, and a puromycin resistance gene. Drug selection and limiting dilution were performed to obtain over 71 individual aAPC-A2 clones for characterization. aAPC-A2, clone 33 was selected for high expression of HLA-A2, CD80, and CD83 which was stable for more than two months of *in vitro* culture. aAPC, clone 33 was also chosen since it demonstrated a superior capacity to induced proliferative allogeneic T-cell responses, and it consistently showed the ability to expand clinically relevant numbers of antigen-specific T cells. It expanded T cells specific for the melanoma-associated antigen MART1 in multiple, different HLA-$A2^+$ healthy donors with multimer staining between 10% and 66%. We noted that aAPC-A2, clone 33 expanded a similar number of MART1-specific T cells as mature DCs *in vitro*. But more importantly, we found that using aAPC-A2, clone 33 we successfully generated MART1-specific T cells from patients with metastatic melanoma (19% to 49% tetramer positive) within a short, three week time period. Moreover, we found that such T-cell lines were not terminally differentiated and displayed a mixture of central memory and effector memory phenotypes as we observed in preclinical experiments in healthy donors. Expanded T cells also possessed antitumor effector function as demonstrated by antigen-specific cytotoxicity and IFNγ secretion. T cells could kill MART1 expressing, HLA-$A2^+$ tumor cell lines but not HLA-$A2^-$MART1^+ or HLA-$A2^+$MART1^- tumor lines. Also, generated T cells were unable to kill HLA-$A2^+$ non-malignant fibroblasts further confirming that killing was peptide specific and suggesting a low risk for inducing off-target autoimmunity.

To produce a standardized supply of aAPCs, a master cell bank and clinical lots of aAPC, clone 33 were generated under current GMP conditions. Testing to satisfied scrutiny by FDA and NIH regulations for clinical use included sterility and mycoplasma testing, electron microscopy for virus like particles, adventitious virus testing, and screening by PCR for a panel of human pathogenic viruses. With the aAPC-A2, clone 33 cell bank in hand, we possessed a standardized, renewable, and unlimited source of aAPC for use in clinical trials. Our approach for generating clinical grade tumor antigen-specific T cells for adoptive therapy was informed by the principles outlined in this review (64, 75). To expand sufficient numbers of T cells for adoptive therapy without prolonged cell culture and repetitive

stimulations, our large-scale cultures began with peripheral blood mononuclear cells obtained by leukapheresis. We then purified $CD8^+$ T cells from the pheresis product by positive selection using the CliniMACS system (Miltenyi Biotec). By doing this, we avoided contamination of T cell cultures with immunosuppressive Treg cells or monocytes. Large scale cultures were then incubated at 37°C in large volume, static gas permeable bags where T cells were stimulated with MART1 peptide-pulsed, irradiated aAPC-A2, clone 33 every 7 days for a total of three stimulations. Low dose IL-2 and IL-15 were added to T-cell cultures to avoid cytokine addiction as discussed above. Within 3 weeks, we typically generated more than 10^9 total expanded $CD8^+$ T cells in both healthy donors and melanoma patients. Functional characteristics such as multimer positivity, effector function by cytotoxicity and IFNγ secretion, and central memory/effector memory phenotype were similar for these large scale cultures as with our studies performed at a smaller scale.

Adoptive therapy clinical trials using aAPC-A2, clone 33

The design of our first-in-human clinical trial using aAPC-A2, clone 33 was influenced by the intriguing preclinical data that T cells educated *in vitro* with aAPC-A2 could be maintained *in vitro* for a surprisingly long period of time. We hypothesized that aAPC-A2, clone 33 generated antigen-specific T cells, with their central memory/effector memory phenotype, could establish antitumor memory in patients upon adoptive transfer. To induce effective antitumor memory, these *in vitro* educated T cells would need to engraft, expand *in vivo*, persist, and traffic to sites of tumor. To test whether aAPC-A2 generated T cells had an intrinsic capacity to accomplish these tasks, we infused patients with T cells alone. No other therapy or immunomodulation was given, including prior lymphodepleting conditioning, cytokine therapy such as IL-2 administration, or vaccine therapy. We chose the melanoma-associated antigen MART1 as our target since necessary immune assessment technologies were available. This allowed our study to carefully evaluate persistence and localization of MART1 T cells following infusion to patients (22, 54). In this phase I study, we met our primary endpoints to demonstrate safety and feasibility, and also found that infused T cells could function as memory T cells, persist long-term, traffic to tumor sites, and induce antitumor clinical and immunological effects.

A total of nine HLA-A2 positive, advanced melanoma patients between the ages 49–80 were enrolled onto the study. T-cell grafts were successfully generated and infused to every patient participating on the study according to the protocol outline, without any production delays or failures to generate grafts. A total of 17 infusions of autologous MART1-specific CTL were administered. The second graft was generated from $CD8^+$ T cells obtained by leukapheresis performed two weeks after the first infusion. All patients received two infusions with the exception of one who was taken off study early due to disease progression. Even for this patient, a second CTL graft would have been available for administration. All infusions were administered in the outpatient setting and were well tolerated, with no treatment related severe adverse events. Grade 1 events possibly related to infusions were limited to fatigue, pruritis, and pain at sites of tumor. One patient experienced asymptomatic peripheral blood eosinophilia three weeks following the second infusion that spontaneously resolved within one week. No other patients experienced unexpected or treatment related laboratory abnormalities. It is important to note that we were able to enroll patients with co-morbid diseases such coronary artery disease, history of myocardial infarction, and diabetes mellitus onto this study since the treatment involved no pre-infusion lymphodepleting chemotherapy nor high-dose IL-2 administration.

In every patient on the study, we were able to successfully generate greater than 1.8×10^9 MART1-specific CTL using aAPC-A2, clone 33. The first infusion (28.0% MART1 multimer positivity, mean) was given on day 0, and the second infusion (30.7% MART1

multimer positivity, mean) was given 35 days later. As we observed in our preclinical studies with healthy volunteers and with melanoma patients, aAPC-A2 generated MART1 T cells were polyclonal, displayed an central memory/effector memory phenotype ($CD45RA^{-}CD27^{+}CD28^{+/-}CD62L^{+/-}$), and possessed antigen-specific effector functions, i.e. cytotoxicity and IFN-γ secretion. An ability to recognize both peptide pulsed targets and tumor cells demonstrated that aAPC-A2, clone 33-generated T cells possessed sufficient specificity and avidity to mediate antitumor effects.

After infusion, we immediately observed an increase in the frequency of peripheral MART1-specific $CD8^{+}$ T cells in all patients (range 4–384 fold). An increase in circulating MART1-specific $CD8^{+}$ T cells was observed over several weeks even though previous reports indicated that persistence of transferred T cells for more than a week required either pre-infusion lymphodepletion or IL-2 administration (22, 53, 54). At days 14 and 49, we observed a statistically significant increase in MART1-specific T cells in the patients infused (P=0.01). In three patients who did not receive other therapy, we continued to observe increases of MART1-specific T cells on days 102, 258, and 358.

These engrafted T cells were phenotypically and functionally memory T cells. Since many of our patients' baseline MART1 precursor T cells possessed a naive phenotype ($CD45RA^{+}CD62L^{+}$), we were able to distinguish infused MART1 CTL from pre-existing MART1 T cells by monitoring differences in the phenotype. Immediately after infusion, we detected infused MART1 T cells with a central memory/effector memory phenotype in all patients, and sustained increases in circulating MART1-specific T cells with a central memory phenotype were observed at 5 weeks post infusion in 7 of the 9 treated patients. Moreover, in the patients with prolonged increases of circulating MART1 T cells at days 102–358, we found that these persisting T cells continued to possess a central memory phenotype ($CD45RA^{-}CD62L^{+}$) and were positive for CD27, CD28, and CD127. These data indicated that persisting T cells had a memory phenotype and were not terminally differentiated. It is interesting to note that the increase in $CD45RA^{-}CD62L^{+}$ central memory MART1-specific T cells occurred after transfer even though some of the infused T cell grafts contained a minority of CD62L expressing MART1 T cells. This suggested that aAPC-A2 *in vitro* educated $CD45RA^{-}CD62L^{+}$ central memory T cells did indeed behave as memory T cells and preferentially persisted *in vivo*. In contrast, effector memory $CD45RA^{-}$ $CD62L^{-}$ and terminally differentiated $CD45RA^{+}$ $CD62L^{-}$ T cells infused may not have engrafted or expanded.

Functional analysis confirmed that adoptive transfer of MART1 T cells resulted in long term enhancement of MART1-specific memory responses. We documented MART1-specific recognition by persisting MART1 T cells 56–70 days after infusion by IFN-γ ELISPOT using fresh PBMC directly *ex vivo*, without any *in vitro* stimulation. Direct *ex vivo* recognition by enzyme-linked immunospot assa7 (ELISPOT) of $HLA\text{-}A2^{+}MART1^{+}$ tumor cells but not $HLA\text{-}A2^{+}MART1^{-}$ tumor cells was also measured. To test delayed type hypersensitivity (DTH) reactions, we performed intradermal injections of MART1 peptide three weeks prior to and three weeks after the first T-cell infusion. Enhanced DTH reactions characterized by induration and erythema were observed, and histologic examination of biopsies of DTH sites showed recruitment of mononuclear cells admixed with eosinophils accumulating around blood vessels. Recruitment of $CD3^{+}$ $CD8^{+}$ lymphocytes was present, consistent with memory recall response mediated by the infused MART1-specific T cells. Together, these data showed that persisting MART1 T cells not only possessed a memory phenotype, but also functioned as memory T cells. It is important to note that these observations were made in patients who received no further treatment other than the initial T-cell infusion.

Clinically, antitumor activity of infused aAPC-A2 clone 33 educated MART1 T cells was observed. One patient experienced a complete response by PET/CT after CTL infusion that has been continuous and ongoing for more than 54 months. Four of the eight remaining patients had stable disease, including one mixed response, at the first assessment time point on day 70. Two of these patients benefited from prolonged clinical stabilization, with radiographic mixed responses documented by PET/CT, and additional anti-cancer therapy was not required for 11 and 12 months. These clinical findings were consistent with reports for other immune based therapies where prolonged disease control is considered to be a clinically beneficial, immunologically mediated outcome (126).

This antitumor clinical activity suggested that infused aAPC-A2, clone 33 generated CTL trafficked to and attacked tumor. In patients with accessible tumor, we obtained pre- and post-infusion excisional biopsies for analysis of the tumor microenvironment. In the patient who experienced a complete response, a post infusion tumor biopsy on day 5 demonstrated a strong, overwhelming antitumor immune response with local brisk infiltration of lymphocytes into peripheral and central areas of tumor ($225/mm^2$) compared to a pre-infusion biopsy ($4/mm^2$). Lymphocytes were predominantly $CD8^+$ T cells, and near complete tumor cell destruction was present with tumor/lymphocyte satellitosis, hemorrhagic cell necrosis, and fibrosis. While fewer lymphocytes stained for CD4, Foxp3 staining was absent, suggesting a lack of regulatory T cells. In samples from other patients who experienced stable disease or progression, we saw variable degrees of lymphocytic infiltration with the presence of $Foxp3^+$ lymphocytes.

In fresh tumor biopsy digests, we also demonstrated the presence of MART1-specific T cells within the tumor microenvironment by direct multimer staining of tumor infiltrating lymphocytes without any *in vitro* expansion. We could not exclude the possibility that the MART1-specific T cells found in the tumor were derived endogenously and not from the infused grafts since the infused CTL were not gene-marked. However, as a surrogate, we molecularly tracked the MART1 T cell clones to determine whether clones found in the grafts were also present within the tumor biopsies. To do this, we performed clonotypic analysis using the CDR3 sequences of isolated MART1-specific T cells obtained from the tumor as a molecular marker. In the two patients with complete or mixed clinical responses, we identified MART1-specific CTL clonotypes that existed in both the CTL grafts and tumor biopsies. This data provided strong evidence that aAPC-A2 educated MART1 T cells engrafted, persisted, and expanded in patients upon transfer, trafficked to tumor sites, and mediated clinically meaningful antitumor destruction. Establishment of antitumor memory by engraftment of infused CTLs appeared to shift the balance between tolerance and antitumor immunity.

Combination of aAPC-based adoptive cell therapy with other therapies

Adoptive transfer of aAPC-A2 generated T cells alone had antitumor clinical activity in our proof-of-concept, phase I clinical trial. While some patients had prolonged stable disease, all but the one patient with a durable complete response ultimately progressed and required further therapy. To improve the clinical utility of our approach, it is possible that the antitumor immunological memory established by aAPC-A2, clone 33 generated T cells could be combined with other therapies to induce synergistic clinical effects. Data from prospectively designed combination therapy trials are not yet available. However, effects of sequential therapies can be examined for hypothesis generating purposes since seven of the nine patients in our study received additional therapy for disease progression. While only single patients received RAF265, an oral multi-kinase inhibitor or high dose IL-2, five patients were treated with the anti-CTLA-4 mAb, ipilimumab, for their next therapy at a dose of either 3 mg/kg or 10 mg/kg. In contrast to the historical response rate of ~15% to

ipilimumab (127, 128), a high rate of clinical benefit was seen with three achieving durable partial responses lasting 16, 41+, and 59+ months when treated at the 10 mg/kg dose (Table 2). Two patients had stable disease lasting 5 and 6 months when treated at the 3 mg/kg dose. Importantly, prior K562-based immunotherapy with exposure to potential hematopoietic stem cell antigens did not induce unexpected autoimmune hematologic toxicity such as hemolytic anemia or aplastic anemia with ipilimumab therapy. As expected, immune related adverse events consistent with ipilimumab therapy were observed such as rash, vitiligo, and autoimmune-mediated colitis. One patient with Graves' ophthalmopathy was observed. All toxicities were successfully managed with standard corticosteroids and supportive care approaches. These clinical data suggested that a synergistic interaction with no additional toxicity could exist when antitumor immunity was established by aAPC-A2, clone 33-based T-cell therapy followed by CTLA-4 blockade.

One advantage of prior T-cell adoptive transfer was that we could continue to monitor engrafted MART1 T cells in patients after further treatment with CTLA-4 blockade. *Ex vivo* analysis of peripheral T cells in the five patients treated with ipilimumab revealed that the percentage of circulating MART1 $CD8^+$ T cells increased by an average of 1.8 fold (range, 1.3–3.2, P=0.03) 2–3 months after the initiation of ipilimumab treatment. Furthermore, analysis revealed that these MART1 specific T cells had a consistent increase in the $CD45RA^-$ memory phenotype (P=0.03). As an example, we intensively tracked at the cellular and molecular levels the persisting MART1 T cells in a patient who received maintenance ipilimumab every three months for over a year. In this patient, we observed a massive expansion of MART1 specific T cells by ~100 fold so that ~8% of all circulating $CD8^+$ T cells on day 537 after T-cell infusion stained with the MART1 multimer. In this patient, at baseline prior to CTL infusion, rare, but detectable at 0.04%, MART1 multimer positive $CD8^+$ T cells had a naive phenotype ($CD45RA^+$ $CD62L^+$). Then, after transfer, not only was there an increase in the MART1 T-cell frequency but there was also a shift in the phenotype of detected MART1 T cells so that about half the circulating cells had a central memory phenotype ($CD45RA^-CD62L^+$). With administration of ipilimumab therapy, the expanding MART1 T cells displayed a central memory/effector memory phenotype and possessed memory function. These circulating T cells were able to recognize $MART1^+$ tumor cells directly *ex vivo* without *in vitro* sensitization using an IFNγ ELISPOT.

At the molecular level, we tracked the MART1 T cells by CDR3-specific RT-PCR of persisting $MART1^+$ T cells. We identified 7 MART1 T-cell clonotypes that were present in both infused T-cell grafts and in the circulation on day 56 in the $CD45RA^-CD8^+$ T-cell memory fraction. Three of these clones continued to be detected within the memory T-cell fraction on day 167. Intriguingly, on day 537 after CTLA-4 blockade therapy when 8% of $CD8^+$ T cells were MART1 specific, one of these clones, 02Vβ14B, was again detected exclusively in the $CD45RA^-$ memory fraction of circulating $CD8^+$ T cells. These data indicated that CTLA-4 blockade induced a striking expansion *in vivo* of adoptively transferred antitumor CTL with memory phenotype and function.

As single therapies, adoptive cell transfer and ipilimumab may not be able to induce clinical benefit in the majority of advanced melanoma patients. However, our data suggest that a powerful treatment strategy could involve first establishing antitumor memory responses with adoptive transfer, which could be further augmented by anti-CTLA-4-induced immune activation to induce clinically beneficial biological responses. Our data provided strong cellular and molecular evidence that aAPC-A2, clone 33 generated T cells could be adoptively transferred, engraft, and then persist and expand as memory T cells. Thus primed, antitumor immune responses could be enhanced by immune manipulation with agents such as ipilimumab, resulting in augmented immunological memory and the observed tumor regressions in patients with treatment refractory disease.

To examine the potential synergy of combination approaches, our clinical program is proceeding with the following strategies to enhance engraftment, expansion, and antitumor activity of aAPC-A2, clone 33 generated CTL after transfer: (i) pre- or post-treatment with immune checkpoint blockade using ipilimumab and/or anti-PD-1 agents, (ii) combination with mutant BRAF inhibitors, which has been reported to increase the expression of tumor associated antigens such as MART1, (iii) lymphodepletion with cyclophosphamide to eliminate immune suppressive cells such as Treg cells, remove cytokine sinks, and provide space for homeostatic expansion, and (iv) localized antitumor radiotherapy as a way to induce enhanced local antitumor effects and distant abscopal effects. The establishment of antitumor memory with aAPC-A2, clone 33 educated T cells can then serve as a platform for combination strategies.

aAPCs for T-cell expansion following TCR gene transfer

HLA-A2 donors have a high frequency of precursor T cells able to recognize MART1, and this facilitates the timely generation of MART1 T-cell cultures for adoptive cell transfer. However, for other tumor antigens, the precursor frequency is lower and the *in vitro* generation of tumor-specific T cells with aAPC-A2, clone 33 may require additional rounds of stimulation for some patients to achieve the desired T-cell dose. To overcome this hurdle, we have shown that the TCR gene transfer technology can be combined with aAPC-based stimulation. In preclinical studies, we generated tumor-specific T cells by TCR gene transfer against NY-ESO-1 in HLA-A2 donors and against Wilms tumor 1 in HLA-A24 donors. Then, antitumor T cells were further expanded with peptide-pulsed aAPC-A2 or aAPC-A24, respectively, resulting in the enrichment of antigen-specific T cells with high functional avidity. By combining with TCR gene transfer, it is possible to make T-cell grafts with properties similar to MART1 T cells expanded from peripheral T cells using aAPC-A2, clone 33. T-cell grafts generated in this way are minimally cultured and are not terminally differentiated. To generate highly avid T cells, the effects of peptide concentration on pulsed aAPC on functional avidity, including antigen-specific IL-2 secretion and recognition of tumor cells endogenously expressing antigen are being evaluated. Efforts to translate this work to the clinic are ongoing. We are defining the standard operating procedures for large scale expansion of NY-ESO-1 specific TCR transduced T cells in HLA-A2 donors using aAPC-A2, clone 33. In addition, we are generating clinical grade aAPC-A24, where instead of HLA-A2, HLA-A24 will be expressed in conjunction with CD80 and CD83.

Other K562-based aAPCs for clinical use

Several other groups are currently conducting clinical trials of adoptive cell transfer that utilize aAPC derived from K562, further confirming the cell line's versatility and utility (Table 3A,B). To provide both TCR stimulation and optimal costimulation, Rosenberg's group at the National Cancer Institute (NCI) in Bethesda, MD, successfully treated two metastatic melanoma patients with TIL expanded using the K562-based aAPC, 7F11ECCE (NCT00512889; Mark Dudley, personal communication). Using a lentiviral system, this aAPC was engineered to express CD64 for loading the anti-CD3 mAb OKT3 and to express 4-1BBL to provide costimulation. 7F11ECCE was used during the rapid expansion protocol (REP) of established antitumor TIL cultures to expand high numbers (10^9–10^{10}) of therapeutic cells. While allogeneic PBMC feeder cells from healthy donors where not entirely eliminated from the cultures, the number of allogeneic PBMC feeder cells needed for cell expansion was lower than that required in the standard REP. The investigators also indicated that, compared to standard TIL cultures, 7F11ECCE-expanded cultures differed by containing fewer $CD4^+$ and more NK cells. In contrast to our aAPC-A2, clone 33-based antigen-specific T cell study, aAPC-expanded TIL in this study were infused into patients after receiving high dose chemotherapy for lymphodepletion, and high dose IL-2 was

administered after TIL infusion. No responses were seen for the two patients on this particular study, which is currently closed and has not been reopened for accrual of additional patients. However, the use of a standardized, unlimited supply of aAPC for T-cell expansion that would replace allogeneic feeder cells remains of great interest and would help to enable the dissemination of TIL-based adoptive cell therapy.

Our group is currently manufacturing a clinical grade version of a second-generation aAPC, aAPC-mOKT3, which expresses a membranous form of OKT3 on the cell surface in order to enable the expansion of both $CD4^+$ and $CD8^+$ T cells. While clinical grade aAPC-A2, clone 33 was generated by transfecting K562 with four non-retroviral plasmids, we have successfully achieved stable expression by simultaneously transfecting with 5 plasmids. Plasmids encoding the membranous forms of the heavy and light chains of the murine anti-human $CD3^+$ monoclonal antibody, OKT3, were exchanged for the HLA-A2 encoding plasmid, and like aAPC-A2, aAPC-mOKT3 was also produced using plasmids encoding for CD80, CD83 and a puromycin resistance gene. To create a standardized supply of aAPC-mOKT3, we are isolating a series of clones with stable expression of mOKT3, CD80, and CD83, endogenous expression of adhesion molecules CD54 and CD58, and lack of HLA class I and II expression. Our selection strategy to choose clones for the master cell bank is based on the ability of aAPC-mOKT3 clones to induce the unbiased expansion of peripheral $CD3^+$ T cells. aAPC-mOKT3-expanded $CD3^+$ T cells are required to show a lack of TCR Vβ subtype skewing and contain functionally competent antitumor and anti-viral antigen specific T cells. The clinical grade aAPC-mOKT3 clone is to be evaluated on its ability to induce T-cell expansion, especially $CD8^+$ T cells, while not inducing the expansion of Foxp3 regulatory T cells. Our final evaluation includes pre-clinical modeling of aAPC-mOKT3 candidate clones to expand TIL with effector memory/central memory, non-terminally differentiated phenotype. In keeping with our prior studies, we will also confirm that expanded TIL secrete Th1 cytokines such as IL2 and IFN-γ upon stimulation rather than suppressive cytokines such as IL-10. These results serve as the basis for clinical trials where aAPC-mOKT3 is used to expand TIL and transgenic $CD3^+$ for adoptive immunotherapy regardless of patient HLA subtype.

The group at Baylor College of Medicine (Houston, TX) is currently using the clinical grade aAPC, K562cs in a protocol to treat patients with therapy resistant EBV-related lymphomas. K562cs was engineered to express CD32, CD80, CD83, CD86, and 4-1BBL. Instead of loading K562cs with a mAb such as OKT3 to provide signal 1, the investigators use the aAPC to provide antigen-specific T cells with costimulatory signals (NCT01555892; Cliona Rooney, personal communication). EBV-specific T cell cultures are first generated using dendritic cells or monocytes pulsed with overlapping EBV-derived peptides derived from LMP1, LMP2 and EBNA1. Then, in a second stimulation, peptide pulsed autologous T cells are used to present antigen and the K562cs is used as a feeder cell to expand cultures for therapeutic use. If this approach is successful, the cumbersome and time-consuming requirement to generate autologous EBV transformed B cells as autologous APC for anti-EBV therapy would be avoided. This strategy, termed KATpx by the investigators, holds promise for T-cell specific therapy of EBV, VZV, vaccinia virus, cytomegalovirus, and adenovirus related diseases.

Cooper's group at MD Anderson Cancer Center is using their GMP K562-based aAPC (clone #4) expressing CD64, CD86, 4-1BBL, truncated CD19, and membrane bound IL-15 to expand clinical grade CAR^+ T cell grafts (129, 130). Using the *Sleeping Beauty* system established in their preclinical studies described above, CD19 CAR-transduced T cells were expanded by repeated stimulation using irradiated aAPC (clone#4). Optimal culture conditions were recently defined to include the additional supplementation of T-cell cultures with soluble recombinant human IL-2 and IL-21. Within one month, clinically relevant

numbers of functional CD19-specific CAR^+ T cells were generated from both peripheral and umbilical cord blood. Clinical studies using this aAPC have undergone successful FDA review and are open for the treatment of patients with CD19 expressing hematologic malignancies (Table 3A). In these studies, all patients will first receive lymphodepleting chemotherapy, either specifically for the purposes of the CAR^+ T cell infusion or within the context of standard autologous, allogeneic, or umbilical cord hematopoietic stem cell transplantation.

Several groups are using K562-based aAPCs to expand large numbers of NK cells for the treatment of hematologic malignancies (Table 3B). Investigators in multiple myeloma at the University of Arkansas (Little Rock, AR) are conducting a series of studies with aAPC-expanded NK cells. In these studies, the aAPC, K562-mbIL15-41BBL, initially reported by Campana and colleagues (113, 131) at St. Jude Children's Research Hospital was manufactured and characterized by the Center for Cell and Gene Therapy at Baylor College of Medicine. Using this aAPC, clinical grade NK cells are being expanded to large numbers in the gas permeable G-Rex system and are being infused to multiple myeloma patients. NK cell infusions are being administered as a single therapy to asymptomatic, but high risk, multiple myeloma patients or in combination with bortezomib to relapsed patients with high risk disease.

In another set of NK studies at MD Anderson, clinical grade aAPC (clone #9) expressing CD64, CD86, 4-1BBL, truncated CD19, and membrane-bound IL-21 was repurposed for the expansion of NK cells. While aAPC (clone #9) also expresses engineered CD19 and CD64, the expanding NK cells presumably do not respond to these molecules. The MD Anderson group using this aAPC is focusing on the use of NK cells for the treatment of CLL, multiple myeloma and myeloid leukemia in the transplant setting 5 to 8 days prior to stem cell infusions. Therefore, all infusions, in contrast to the Arkansas studies, will be given to patients after lymphodepleting chemotherapy. Additionally, in the CLL and multiple myeloma studies lenalidomide is being given on days −8 and −2 prior to stem cell infusion in order to enhance NK effector functions *in vivo*. As mentioned previously, investigators found that the phenotype and cytotoxicity of NK cells expanded with aAPC (clone #9) were similar to their aAPC that expressed IL-15 instead of IL-21, but had superior cytokine secretion and proliferation (104, 132). Data is not yet available on which aAPC generates NK cells with superior engraftment or antitumor function in patients with cancer.

Blazar and colleagues (133) at the University of Minnesota (Minneapolis, MN) have generated clinical grade KT64/86, which is a cell line engineered to express CD86 and CD64 by lentiviral gene transfer. KT64/86 was loaded with anti-CD3 mAb and used to expand purified, polyclonal, human natural Treg cells in the presence of IL-2 and rapamycin. While repeated stimulations could increase the yield of expanded Treg cells over mAb coated beads, purity and suppressive function was dependent on continued use of rapamycin in culture. Rapamycin prevented the initial outgrowth of IL-2 and IFNγ secreting T cells following expansion with KT64/86. Importantly, in a xenogeneic model of GVHD, KT64/86-expanded natural Treg cells were effectively shown to ameliorate disease. These results support the investigators' ultimate goal to use KT64/86 to expand nTreg cells from multiple HLA-typed donors to create nTreg banks for an off-the-shelf therapy to prevent and treat GVHD and transplant graft rejection. Their current study (NCT00602693) involves the administration on day +1 following umbilical cord blood stem cell transplantation of expanded Treg cells derived from umbilical cord blood. In this study, the aAPC-generated Treg cells are being given to a cohort of patients that can be compared to an earlier cohort of patients previously treated with CD3/CD28 bead-expanded Treg cells on the same protocol.

Conclusions

We and others have found that K562-derived aAPCs provide a versatile platform for generating therapeutic grafts for cellular therapies that include antigen-specific $CD8^+$ T cells, antigen-specific $CD4^+$ T cells, polyclonal $CD3^+$ T cells, CAR-expressing T cells, TCR engineered T cells, Treg cells, iNK cells, and NK cells. As listed in Table 3A,B, our completed clinical protocol and the number and diversity of other ongoing studies are testaments to the cell line's ease of use in the GMP setting, susceptibility to genetic manipulation, and lack of expression of immunologically deleterious molecules. These characteristics have enabled K562 to be used to create an impressive variety of aAPC specifically tailored to generate a particular cell product. In our experience, with aAPC-A2 we were able to develop a T-cell culture system that was specifically designed to expand antigen-specific $CD8^+$ T cells that could persist and be long-lived in humans. This aAPC-based culture system enabled us to follow the appropriate principles of stimulation to generate T cell grafts for effective adoptive therapy. We engineered K562 to ectopically express costimulatory molecules CD80 and CD83 with HLA-A2 in order to provide optimized stimulation of antigen-specific T cells, but not excessive stimulation that would lead to AICD of highly avid T cells. We also included the addition of low doses of soluble cytokines to avoid over dependence and included IL-15, which had been reported to favor the expansion of antigen-specific T cells with a central memory phenotype and to enable *in vivo* persistence in animal models. These aAPC-generated T cells could be maintained *in vitro* for surprisingly long periods suggesting a capacity to engraft and establish antitumor memory.

In our completed proof-of-concept clinical study, we examined whether *in vitro*-educated T cells generated with GMP grade aAPC-A2, clone 33, and low dose IL-2 and IL-15 could persist in humans. Without any further treatment such as in lymphodepletion or IL-2 administration, adoptively transferred T cells were shown to establish antitumor immunologic memory, traffic to the tumor, mediate biological responses. Alone or in combination with other immune modulators, these adoptively transferred T cells promoted antitumor immunity and induced antitumor clinical responses. This aAPC-based approach generated T-cell grafts easily and consistently in every patient enrolled onto the study within a short time frame. This is pertinent, not only from a clinical utility point of view but also from the point of view of generating antitumor T cells that are not exhausted by prolong *in vitro* stimulation. This human cell line aAPC-based approach is technically feasible in its simplicity and reproducibility, can be easily transported to other institutions, and can serve as a platform for combining with other immune modulators and standard treatments. To demonstrate this fact, we are now conducting a series of investigator-initiated combination studies designed to enhance antitumor memory established by the transfer of aAPC-A2, clone 33-generated T cells.

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Table 1

K562-based artificial APCs to expand human Natural Killer and T cells.

NK/T cells	Restriction	Antigen-specificity	Transduced molecules	Trials
$CD8^+$ T cells	HLA class I	Yes	HLA class I, CD80, CD83	Yes
$CD8^+$ T cells	CD3	No	CD32 or 64, 4-1BBL	No
$CD4^+$ T cells	HLA class II	Yes	HLA class II α and β, CD80, CD83, invariant chain, HLA-DM α and β	No
$CD3^+$ T cells	CD3	No	Membranous form anti-CD3 mAb, CD80, CD83	No
$CD3^+$ T cells	CD3	No	CD32 or 64, 4-1BBL	Yes
CD19-CAR-transduced T cells	CD19	Yes	CD64, CD86, 4-1BBL, truncated CD19, membrane-bound IL-15	Yes
iNKT cells	CD1d	Yes	CD1d, CD80, CD83	No
NK cells	NA	NA	4-1BBL, membrane-bound IL-15	Yes
NK cells	NA	NA	CD64, CD86, 4-1BBL, truncated CD19, membrane-bound IL-15 or IL-21	Yes

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Table 2

Update to clinical responses to infused MART1 T cells generated with aAPC-A2, clone 33.

No.	Age/sex	Metastatic disease sites at entry	Notable comorbid conditions	Best overall response to MART1 T cell infusions	Time to next therapy	Response and duration to next therapy (months)
1	74/M	Liver, spleen, adrenal, lung, skin, brain	HTN, h/o bowel obstruction	PD	Death on day 51	--
2	69/M	Lung, skin	h/o DVT on warfarin	PD	Day 103 ipilimumab (10 mg/kg)	PR, 16
3	49/M	Lung, adrenal	--	SD (MR)	Day 146 ipilimumab (10 mg/kg)	PR, 59+
4	68/M	Skeletal muscle, lung, mediastinum, cardiac	Asbestosis, cardiac metastasis	SD	Day 140 RAF265	SD, 3
5	66/M	Lymph nodes	DM, HTN, mild renal insufficiency	CR, 54+	No other therapy needed	--
6	55/M	Lung	HTN	SD	Day 287 HD IL-2	Death due to IL-2 therapy
7	70/M	Lung, skin	DM, CAD, h/o MI	PD	Day 335 ipilimumab (3 mg/kg)	SD, 6
8	80/M	Lung, mediastinum	CAD, h/o MI, s/p CABG, pacemaker for sick sinus syndrome	SD	Day 372 ipilimumab (3 mg/kg)	SD, 5
9	64/M	Lung, skin	HTN	PD	Day 146 ipilimumab (10 mg/kg) + bevacizumab	CR, 41+

Abbreviations: CAD, coronary artery disease; CABG, coronary artery bypass graft; DM, diabetes mellitus; DVT, deep vein thrombosis; h/o, history of; HTN, hypertension; s/p, status post.

Table 3a

K562-based artificial APCs for anti-tumor T cell clinical studies.

K562-based aAPC and transduced molecules	Target Disease	Target Cell for Expansion and Notes	Phase	Status	ClinicalTrials.gov Identifier.
aAPC-A2, clone 33: HLA class I, CD80, CD83	Advanced melanoma	Autologous aAPC-generated MART1 T cells	I	Closed	NCT00512889; DFCI; future studies at Princess Margaret
7F11ECCE: CD64, 4-1BBL	Advanced melanoma	TIL for transfer after lymphodepletion using aAPC loaded with OKT3	II	Closed	NCT01369875; NCI
K562cs: CD32, CD80, CD83 CD86, 4-1BBL	EBV-positive lymphoma	EBV-specific T cell lines, aAPC used for costimulation, not antigen-specific stimulation	I	Open	NCT01555892; Texas Children's and Methodist Hospitals
aAPC (clone #4): CD64, CD86, 4-1BBL, truncated CD19, membrane- bound IL-15	B-Lineage Lymphoid Malignancies After Auto SCT	Autologous CD19-specific CAR T cells	I	Open	NCT00968760; MDACC
	B-Lineage Lymphoid Malignancies After Umbilical Cord SCT	Allogeneic CD19-specific CAR T cells derived from cord blood	I	Open	NCT01497184; MDACC
	B-Lineage lymphoid malignancies after allo-SCT	Allogeneic CD19-specific CAR T cells	I	Open	NCT01362452; MDACC
	B-cell chronic lymphocytic leukemia	Autologous CD19-specific CAR T cells	I	Open	NCT01653717; MDACC

Table 3b

K562-based artificial APCs for NK cell and Treg clinical studies.

K562-based aAPC and transduced molecules	Target Disease	Target Cell for Expansion and Notes	Phase	Status	Ref.
K562-mbIL15- 41BBL;	Relapsed high risk multiple myeloma	Autologous NK cells plus bortezomib	I	Open	NCT01212897; University of Arkansas
4-1BBL, membrane- bound IL-15	Asymptomatic multiple myeloma post standard therapy	Autologous NK cells	I	Open	NCT01884688; University of Arkansas
aAPC (clone #9): CD64, CD86, 4-1BBL, truncated CD19, membrane- bound IL-21	B-cell chronic lymphocytic leukemia undergoing umbilical cord SCT	Allogeneic NK cells derived from cord blood	I	Open	NCT01619761; MDACC
	Multiple myeloma undergoing umbilical cord SCT	Allogeneic NK cells derived from cord blood	I	Open	NCT01729091; MDACC
	Myeloid leukemia undergoing SCT	Allogeneic NK cells derived from donor	I	Open	NCT01823198; MDACC
KT64/86: CD64, CD86	Advanced hematologic malignancies with umbilical cord SCT	Natural Treg from umbilical cord cells expanded with aAPC loaded with OKT3	I	Open	NCT00602693; University of Minnesota

Abbreviations: aAPC, artificial antigen presenting cell; CAR, chimeric antigen receptor; DFCI, Dana-Farber Cancer Institute; GFP, green fluorescence protein; EBV, Epstein-Barr Virus; mIL-15; membrane bound IL-15; mIL-21, membrane bound IL-21; MDACC, M.D. Anderson Cancer Center; MTD, maximum tolerated dose; NCI, National Cancer Institute; PMCC, Princess Margaret Cancer Centre; SCT, stem cell transplant.