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Genetically engineered fixed K562 cells: potent “off the shelf” antigen presenting cells for generating virus specific T cells

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Abstract

Background aims—The human leukemia cell line K562 represents an attractive platform for creating artificial antigen presenting cells (aAPC). It is readily expandable, does not express HLA class I and II and can be stably transduced with various genes.

Methods—In order to generate CMV antigen-specific T cells for adoptive immunotherapy, we transduced K562 with HLA-A*0201 in combination with costimulatory molecules.

Results—In preliminary experiments, irradiated K562 cells expressing HLA-A*0201 and 4–1BBL pulsed with CMV pp65 and IE-1 peptide libraries failed to elicit antigen-specific CD8⁺ T cells in HLA-A*0201⁺ peripheral blood mononuclear cells (PBMC) or isolated T cells. Both wildtype K562 and aAPC strongly inhibited T cell proliferation to the bacterial superantigen staphylococcus enterotoxin B (SEB), OKT3, and in mixed lymphocyte reaction (MLR). Transwell experiments suggested that suppression was mediated by a soluble factor; however, MLR inhibition was not reversed using TGF- β blocking antibody or PGE2 inhibitors. Full abrogation of the suppressive activity of K562 on MLR, SEB, and OKT3 stimulation was only achieved by brief

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Authorship

Contribution: K.T. designed the research, performed experiments, analyzed data, and drafted the manuscript; P.M. designed experiments and commented on the manuscript; S.M. performed experiments, analyzed data, and commented on the manuscript; H.F. designed experiments and commented on the manuscript; S.K. performed experiments and analyzed data; K.K. performed experiments and analyzed data; N.F.H. commented on the manuscript; A.J.B. designed and supervised research, analyzed data, and drafted the manuscript; and J.J.M. designed and supervised research, performed experiments, analyzed data, and drafted the manuscript.

Disclosure of Interest

The authors declare no potential conflicts of interest in this study.

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fixation with 0.1% formaldehyde. Fixed, pp65 and IE-1 peptide-loaded aAPC induced robust expansion of CMV-specific T cells.

Conclusions—Fixed gene-modified K562 cells can serve as effective aAPC to expand CMV-specific CTL for therapeutic use in patients after stem cell transplantation. Our findings have implications for broader understanding of the immune evasion mechanisms used by leukemia and other tumors.

Keywords

Artificial APC; Cytomegalovirus; Cytotoxic T cell; Fixation

Introduction

The treatment of viral infection and malignant diseases by adoptive transfer of *in vitro* expanded antigen-specific T lymphocytes is an emerging approach with promising clinical efficacy (1–3). Professional antigen-presenting cells (APC) such as dendritic cells (DC) are key elements in the generation of virus or tumor antigen-specific T cells in sufficient numbers for clinical use from naive CD8 and CD4 lymphocytes (4). DC express MHC class I and II (5) together with costimulatory molecules. Critically, 4–1BBL (CD137L) plays an important role in expanding antigen-specific CD8 T cells (6–9). While DC are highly effective in stimulating T cells, they need to be matured in culture for up to 7 days before they can function as APC (10–12). In addition, the generation of DC is associated with high costs, and DC themselves cannot be expanded. These constraints have motivated a number of investigators to make artificial APC (aAPC) with comparable ability to engage and costimulate CD4 and CD8 lymphocytes. The mouse NIH3T3 fibroblast lines (13) and the chronic myeloid leukemia K562 line have been used for this purpose (14). In contrast to DC, such aAPC have the advantage of being infinitely renewable and expandable “off-the-shelf” products. With the potential to be distributed worldwide, such aAPC would improve the standardization, speed, and reliability of generating T cell products. Several investigators have used genetically engineered K562 aAPC to generate tumor-specific T cells for adoptive immunotherapy (15, 16). We set out to generate a library of K562 cells transduced with common MHC class I and II antigens and costimulatory molecules for use as aAPC. We identified an inherent antiproliferative property of both wildtype and transduced K562 lines, which could be eliminated by fixation in formaldehyde. Here we describe how such fixed K562 lines transduced with HLA-A*0201 and 4–1BBL can induce robust CD8 T cell responses to CMV IE-1 and pp65 peptide libraries.

Methods

Blood samples

Leukapheresis cells collected from healthy donors were obtained under Institutional Review Board-approved protocols for allogeneic stem cell transplantation. Peripheral blood mononuclear cells (PBMC) were isolated from leukapheresis product by Ficoll-Hypaque and cryopreserved to standard procedure. Cells were thawed and rested overnight at 37°C, 5% CO₂ in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10%

human AB serum (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (T cell medium).

Cell lines

K562 cells were cultured in RPMI 1640 supplemented 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies) (Complete medium). 293FT cells were cultured with DMEM (Life technologies) supplemented 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

Vector construction

4-1 BBL was amplified from mature DC cDNA and cloned into pCI to yield pCI-4-1BBL (CD137L expression vector). 4-1BBL was subsequently subcloned into pMSCV-IRES-GFP (17). The CD137L open reading frame (ORF) was amplified from pCI-4-1BBL and subcloned into pCR4-TOPO vector with TOPO TA Cloning Kit (Life Technologies) to yield pCR4-4-1BBL. The CD137L ORF was subsequently excised from the pCR4 construct using BamHI. The IRES-GFP cassette was removed from pMSCV using XhoI and SalI, and the 4-1BBL ORF was cloned into this site to yield pMSCV-4-1BBL. Codon optimized CD80, CD83, and CD64 ORFs were synthesized by GeneArt (Life Technologies) and cloned into the lentiviral vector pRRSIN-cPPT-MSCV-WPRE (18), kindly provided by Dr. Morgan (Surgery Branch, NCI, NIH, Bethesda, MD) to yield pRRL-CD80, -CD83, and -CD64. Codon-optimized HLA-DRB1*1501 and -DRA were cloned into pRRL as a fusion gene separated by T2A, a spacer sequence and a furin recognition sequence (19). HLA-DM and Invariant chain (Ii) were similarly designed and cloned into pRRL.

Generation of artificial APC (aAPC)

The pMSCV-4-1BBL vector and helper plasmid pCI-Ampho were transfected into 293FT cells using Lipofectamin 2000 (Life Technologies) according to manufacturer's instruction. The pRRL-CD80, -CD83, -CD64, -HLA-DR15-DRA, and -HLA-DM-Ii were cotransfected with the helper plasmids pMG, pRSV-Rev, and pMDL/pRRE into 293FT cells using Lipofectamin 2000. All cell-free supernatants were harvested on day 4 and day 5 post-transfection and immediately frozen at -80°C. Retroviral transduction was performed with Retronectin (Takara Bio Inc., Otsu, Shiga, Japan) coated plates as described previously (17). After adding thawed virus supernatant to the plates, the plates were centrifuged 1,700g- for 90 minutes at room temperature. After centrifugation, virus supernatants were discarded, then either K562 or HLA-A*0201 transduced K562 cell line (K-A2, kindly provided by Dr. Griffioen, Leiden University Medical Center, Leiden, the Netherlands), suspended in complete medium, were added to the wells and incubated at 37°C, 5% CO₂. The next day, fresh complete medium was added. After 4 or 5 days, cells brightly expressing HLA-A*0201, HLA-DR, CD137L, CD80 and CD83 were sorted on a Becton Dickinson FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA). HLA-DM/Ii was transduced into HLA-DR positive cells and Ii-bright cells were similarly sorted (Supplemental Fig. 1).

Monoclonal antibodies

The following commercially available fluorochrome-conjugated monoclonal antibodies (mAb) were used: (1) α -HLA-A2-fluorescein isothiocyanate (FITC), α -HLA-DR-allophycocyanin (APC), α -CD137L-phycoerythrin (PE), α -CD80-PE-Cy5, α -CD83-APC, α -CD4-V500, α -CD8-APC-H7, α -CD107a-FITC, and α -tumor necrosis factor (TNF)-PE-Cy7 (BD Biosciences, San Jose, CA, USA); (2) α -CD3-eFluor®605NC, α -CD74-PE, and α -interferon- γ (IFN- γ)-PE (eBioscience, San Diego, CA, USA); (3) α -CD14-Pacific Blue and α -CD19-Pacific Blue (Life Technologies); (4) α -CD3-Brilliant Violet 605™ (Biolegend, San Diego, CA, USA).

Flow cytometric analysis

To assess T cell reactivity to CMV peptide libraries, the cells were stimulated with CMV pp65 and IE-1 peptide libraries in the presence of GolgiStop, GolgiPlug, and CD107a FITC mAb (BD Biosciences) in T cell medium for 6 hours as described previously (22). After stimulation, cells were stained as previously outlined (20, 21). First, cells were washed with phosphate-buffered saline (PBS) and stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Vivid) (Life Technologies) for 15 minutes at room temperature, then washed with FACS Buffer (PBS containing 0.5% Bovine Serum Albumin and 0.05% Sodium Azide). Next, the cells were surface stained with anti-CD3, -CD4, -CD8, -CD14 and -CD19 mAbs. Following surface staining, the cells were fixed and permeabilized with BD Cytotfix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), and stained intracellularly with anti-IFN- γ and -TNF mAbs. After staining, the cells were fixed with 4% formaldehyde (Tousimis Research Corp., Rockville, MD, USA) for 10 minutes at room temperature and washed with FACS buffer. Cells were acquired on a LSRII Fortessa (BD Biosciences).

Proliferation assay

T cells, isolated by negative selection with MACS Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) according to manufacturer's instruction, or PBMC were labeled with 0.4 μ M carboxyfluorescein diacetate, succinimidyl ester (CFSE; Life Technologies) as described previously (22). CFSE stained responder PBMC were cocultured with irradiated (25 Gy) HLA-mismatched donor PBMC in a mixed lymphocyte reaction (MLR) in either the presence or absence of K562, or K562 transduced CD137L (K-A2-CD137L) for 6 days in 200 μ L/well of T cell medium at a responder:stimulator:aAPC ratio of 2:1:2. Alternatively, these responders were stimulated with SEB or OKT3, with/without K562, or HLA-DR15, HLA-DM-Ii transduced K562 (KR15), or CD64 transduced K562 (K64) in 200 μ L/well of T cell medium at a 1:4 responder:aAPC ratio. K562, aAPC, and irradiated PBMC were stained with CellTrace™ Violet Cell Proliferation Kit (Life Technologies). Data was analyzed using FlowJo (version 8.8.6; Treestar, Ashland, OR, USA). % proliferation inhibition was calculated by: $(\% \text{ of CFSE dim/low, no aAPC} - \% \text{ of CFSE dim/low, with K562 or aAPC}) / (\% \text{ of CFSE dim/low, no aAPC}) \times 100$.

Formaldehyde fixation

K562 and aAPCs were fixed for 10 min at room temperature using various concentrations of formaldehyde, washed first with PBS, and then with serum-containing medium before use in experiments.

Generation of CMV-specific T cell lines

Immature DC were generated from elutriated monocytes of HLA-A*0201⁺ donors cultured in the presence of GM-CSF (800 IU/mL) and IL-4 (1,000 IU/mL) for 3 to 4 days, and then matured with 1,000 IU/mL of IFN- γ (InterMune Inc. Brisbane, CA, USA) and 30 ng/mL of LPS (Calbiochem, Darmstadt, Germany) for 2 to 3 days. Mature dendritic cells (mDC) were prepared 5 to 7 days prior to T cell stimulation. mDC and K-A2, K-A2-CD137L, and K-A2-CD137L transduced CD80 and CD83 (K-A2-CD137L-CD80/83) were pulsed in T cell medium with 1 μ g/mL of CMV pp65 and IE-1 peptide libraries at 37°C for 30 minutes, irradiated (75 Gy for DC, and 100 Gy for aAPC), and then used to stimulate T cells in T cell medium supplemented with IL-7 (10 ng/mL, CellGenix, Portsmouth, NH, USA), IL-12 (10 ng/mL, Peprotech, Rocky Hill, NJ, USA), and IL-15 (5 ng/mL, CellGenix). On day 3, T cell medium with IL-15 was added with half medium change. Subsequently, cells were split and fresh T cell medium was added every other day.

Cytotoxicity assay

K562, K-A2, and T2 cells were stained with CFSE, pulsed with 1 μ g/ml of pp65 and IE-1 peptide libraries, and washed twice with T cell medium. Pulsed cells were then used to stimulate T cell lines at various effector:target ratios (E:T ratios) for 16 hours, after the incubation, a viability indicator, Ethidium Homodimer-1 (EthD-1; Life Technologies) was added prior to sample acquisition. Wildtype K562 was used as a negative control.

Statistics

All data was analyzed by PRISM 5 (GraphPad Software, Inc., La Jolla, CA, USA). P values were calculated using either one-way ANOVA followed by a Newman-Keuls multiple comparison test, or a two-way ANOVA followed by a Bonferroni multiple comparison test.

Results

K562 cells inhibit T cell proliferation

When wildtype or transduced K562 cells were cocultured with 0.2×10^6 PBMC, T cell proliferation, measured by CFSE dilution, was strongly inhibited in a dose-dependent manner (Figure 1A, B). Wildtype and gene-modified, irradiated (100 Gy) K562 cells inhibited both SEB- and OKT3- induced T cell proliferation (representative data shown in Figure 1C, 1D).

Inhibitory effect of K562 on T cell proliferation is independent of cell-cell contact

To further explore the inhibitory effect of K562 cells, wildtype K562 and aAPC were cultured for 6 days in a transwell plate separated by a 0.4 μ m pore membrane from PBMC which were stimulated with HLA-disparate PBMC at a responder:K562 (or aAPC) ratio of

1:1. PBMC alone was used as a negative control and PBMC in the MLR without K562 or aAPC cells were used as a positive control (Figure 2A, B). Again, both wildtype K562 and transduced K562 cells, whether irradiated or not pretreated, inhibited T cell proliferation (Figure 2C, D, E, F).

Formaldehyde fixation abrogates the inhibitory effect of K562

To prevent the secretion of soluble inhibitory factors from aAPC, cells were treated with varying concentrations of formaldehyde. In the MLR, aAPC pretreated with 0.1% formaldehyde increased T cell proliferation (Figure 3A) while not pretreated or 100 Gy irradiated aAPC retained their inhibitory effect ($P<0.0001$; Figure 3B). We next assessed antigen presenting capacity of the aAPC after formaldehyde fixation. OKT3 or SEB were loaded onto K64 and KR15 respectively, then either irradiated (100 Gy), or fixed with 0.1% formaldehyde. Once again, irradiated aAPC blocked T cell proliferation, but proliferation was enhanced when stimulated with fixed aAPC (Figure 3C, D).

Generation of CMV-specific T cells with fixed aAPC

To generate CMV pp65 and IE-1-specific cytotoxic T cells, T cells from three HLA-A*0201⁺, CMV seropositive healthy donors were cultured for 14 days with pp65 and IE-1 peptide library pulsed, irradiated or fixed, K-A2 or K-A2-CD137L in the presence of IL-7, IL-12, and IL-15. After 14 days coculture, T cells were tested for their response to pp65 and IE-1 peptide libraries. All T cells were gated with CD3⁺, CD14⁻, CD19⁻, and cytotoxicity was analyzed on the pure CD8⁺ positive population (Supplemental Fig. 2). In all cases, peptide pulsed, fixed aAPC induced more CD107a⁺, IFN- γ ⁺, and TNF⁺ CD8⁺ T cells than irradiated aAPC. An anti-CMV response was only detected in 1/3 of donors primed with irradiated peptide pulsed aAPC (Table I). The cytokine production of T cells stimulated with irradiated aAPC or fixed aAPC for this donor showed statistic significance ($P<0.05$, Figure 4A). In addition, CMV-specific CD8⁺ T cells generated with fixed aAPC were polyfunctional (23). Fixed aAPC produced statistically significant increases in the percentage of CD107a⁺IFN- γ ⁺ and TNF⁺IFN- γ ⁺ cells (representative data shown in Figure 4B, C). In order to evaluate the antigen-dependent cytolytic activity of the T cell responses, specific lysis was measured after 16 hours exposure to peptide pulsed and unpulsed K-A2 and the HLA-A*0201 expressing T2 cell line at various E:T ratios. Wildtype K562 was used as a negative control. T cell lines generated with peptide pulsed, fixed aAPC demonstrated clear HLA-A*0201 restricted CMV-specific cytotoxicity (Figure 5A, B).

Formaldehyde fixation of K562 facilitates generation of CMV-specific T cells

Four HLA-A*0201⁺, CMV seropositive healthy donors were tested to generate CMV-specific cytotoxic T Lymphocytes (CTL). Purified T cells were stimulated with various aAPC. To study the ability of aAPC to expand *in vitro* antigen-primed T cells, peptide pulsed DC were used for the first stimulation and either peptide pulsed DC or peptide pulsed, fixed aAPC were used for the second stimulation in T cell medium supplemented with cytokines. To evaluate the capability of aAPC to prime and expand CTL, peptide pulsed, fixed aAPC were used for both the first and second stimulation in T cell medium supplemented with cytokines. T cell stimulation by peptide pulsed DC followed by pulsed, fixed aAPC stimulation consistently demonstrated the induction of equal or greater

expansion of CMV-specific CD8⁺ T cells when compared to two stimulations with pulsed DC (Figure 6A). In this culture condition, we expanded the absolute number of CMV specific T cells after 14 days priming (Supplemental Figure 3). All donors stimulated with peptide pulsed, fixed CD137L transduced aAPC in both the first and second stimulation, notably K-A2-CD137L, induced a significantly greater frequency of CD107a⁺/IFN- γ ⁺CD8⁺ T cells than K-A2 (Figure 6B, increments of double positive cells are listed on Table II). With the exception of one donor, double stimulation with peptide pulsed aAPC was not able to expand absolute numbers of T cells without transduced co-stimulatory molecules (Supplemental Figure 4).

Discussion

Here we demonstrate the feasibility of using peptide pulsed aAPC to induce the expansion of CMV antigen-specific T cells. Our initial attempts to induce CMV-specific T cell expansion were disappointing. This led us to explore the apparent antiproliferative effect of the aAPC on antigen-stimulated T cells. We considered a number of potential mechanisms for the inhibitory effect. First, we established that the effect was not exclusively a property of transduced K562 lines, since wildtype K562 equally inhibited T cell proliferation. In addition, we found that aAPC inhibited a T cell response across a semipermeable membrane, which suggests that inhibition does not require cell-cell contact. Although K562 cells are known to produce the anti-proliferative cytokine TGF- β (24), we were unable to block inhibition with TGF- β antibody. In addition, PGE2 inhibitor could not suppress the inhibitory effect (data not shown). We have also added culture supernatant from aAPC's to T cells stimulated by MLR, OKT3, and SEB but did not identify the inhibitory mechanism. This is the subject of ongoing study. However, modification of the aAPC by fixation with formaldehyde at concentrations as low as 0.1% enhanced the proliferation of T cells stimulated with allogeneic PBMC, OKT3, and SEB. Other investigators have not reported inhibition of T cell proliferation by K562 aAPC. However in their studies aAPC were used only to expand already initiated T cell responses, whereas we used aAPC to initiate the culture of antigen specific T cells at high stimulator:responder ratios. aAPC loaded with anti-CD3 and -CD28 monoclonal antibodies were capable in our hands to stimulate T cell proliferation as others have reported (24, 27). However fixation greatly improved T cell proliferation under all culture conditions. We have found that K562 cells share the ability to inhibit T cell proliferation with other myeloid, but not lymphoid, cell lines suggesting they myeloid leukemia derived cell lines may have properties similar to those of myeloid derived suppressor cells (data not shown).

Using fixed aAPC, we were able to induce strong antigen-specific proliferative responses to CMV pp65 and IE-1. Furthermore, expanded T cells were shown to be polyfunctional and exhibited antigen-specific cytotoxicity.

While the CD137L transduced, fixed aAPC would be effective for eliciting viral-specific T cells from responders with large T cell memory pools (25, 26), expansion with the aAPC is likely to be less efficient when the antigen is presented to naïve T cells, as would occur with CMV seronegative donors, or when eliciting T cell responses to tumor antigens. From the work presented here, the expansion of antigen-specific CD8⁺ T cells using DC for the first

stimulation and fixed aAPC for the second stimulation nearly equaled that of two stimulations with DC. While we showed that a primary stimulation with aAPC could induce CTL expansion, in order to stimulate a predominantly naïve T cell population, stimulation with DC followed by fixed aAPC would be an effective method to expand CTL.

Having optimized antigen presentation by aAPC, we now plan to generate a library of CD137L aAPC transduced with common individual HLA-A and -B molecules, as well as HLA-DR and HLA-DQ molecules. A library of approximately 20 cell lines would provide an appropriate aAPC for most individuals who share at least one HLA-A, -B, or -C and one -DR or -DQ with the responder (27–29). Future studies exploring the nature of the inhibitor(s) produced by K562 lines and further optimization of APC function through the insertion of genes coding for other costimulatory molecules and cytokines are planned (30–33). The erythroleukemic origin of the K562 line confers both opportunities and disadvantages for application as an aAPC. K562 expresses a variety of tumor and leukemia associated antigens (34, 35) including WT1(36), PRAME (37), and proteinase 3 (35), which are overexpressed in myeloid leukemia cell [enref_6_enref_2s](#). Compared with NIH 3T3 cell, which are also used for aAPC cells, a potential advantage of the K562 aAPC is the possibility that it can naturally present a variety of myeloid leukemia specific antigens through the inserted MHC molecules, making it an attractive candidate for inducing leukemia-specific APC. Indeed, irradiated K562 cells have been used for vaccination in CML patients (38, 39). Our aAPC could also be modified to express other leukemia associated antigens by gene transduction. Fixation of irradiated K562 cells would provide a further safety level to prevent accidental transfer of live leukemia cells into the patient. We chose formaldehyde because we thought it might block export of inhibitory molecules without damaging antigen presentation by its fixative properties on cell membranes. In virus vaccination, formaldehyde fixation is a common method to safely inactivate viruses (40) while conserving APC function of EBV-LCL (41). Our results indicate that even sublethally irradiated K562 apoptose and no longer proliferate after fixation.

In conclusion our results show that fixed genetically modified K562 cell lines can serve as effective aAPC and could be used to expand virus-specific CTL and possibly leukemia-specific CTL, in order to prevent or treat viral infection or leukemic relapse in patients after stem cell transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Barrett AJ, Le Blanc K. Immunotherapy prospects for acute myeloid leukaemia. Clin Exp Immunol. 2010;161:223–32. [PubMed: 20529084]
2. Bollard CM, Rooney CM, Heslop HE. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. Nat Rev Clin Oncol. 2012;9:510–9. [PubMed: 22801669]

3. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol.* 2012;12:269–81. [PubMed: 22437939]
4. Takamizawa M, Rivas A, Fagnoni F, Benike C, Kosek J, Hyakawa H, et al. Dendritic cells that process and present nominal antigens to naive T lymphocytes are derived from CD2+ precursors. *J Immunol.* 1997;158:2134–42. [PubMed: 9036958]
5. Pardoll DM. Spinning molecular immunology into successful immunotherapy. *Nat Rev Immunol.* 2002;2:227–38. [PubMed: 12001994]
6. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, et al. 4–1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J Exp Med.* 1997;186:47–55. [PubMed: 9206996]
7. Bukczynski J, Wen T, Ellefsen K, Gaudie J, Watts TH. Costimulatory ligand 4–1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses. *Proc Natl Acad Sci U S A.* 2004;101:1291–6. [PubMed: 14745033]
8. Zhang H, Snyder KM, Suhoski MM, Maus MV, Kapoor V, June CH, et al. 4–1BB is superior to CD28 costimulation for generating CD8+ cytotoxic lymphocytes for adoptive immunotherapy. *J Immunol.* 2007;179:4910–8. [PubMed: 17878391]
9. Wolf M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, et al. Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. *Blood.* 2007;110:201–10. [PubMed: 17371945]
10. Comoli P, Basso S, Azzi A, Moretta A, De Santis R, Del Galdo F, et al. Dendritic cells pulsed with polyomavirus BK antigen induce ex vivo polyoma BK virus-specific cytotoxic T-cell lines in seropositive healthy individuals and renal transplant recipients. *J Am Soc Nephrol.* 2003;14:3197–204. [PubMed: 14638918]
11. Li J, Melenhorst J, Hensel N, Rezvani K, Sconocchia G, Kilical Y, et al. T-cell responses to peptide fragments of the BK virus T antigen: implications for cross-reactivity of immune response to JC virus. *J Gen Virol.* 2006;87:2951–60. [PubMed: 16963754]
12. Nair S, Archer GE, Tedder TF. Isolation and generation of human dendritic cells. *Curr Protoc Immunol.* 2012;Chapter 7:Unit7 32.
13. Dupont J, Latouche JB, Ma C, Sadelain M. Artificial antigen-presenting cells transduced with telomerase efficiently expand epitope-specific, human leukocyte antigen-restricted cytotoxic T cells. *Cancer Res.* 2005;65:5417–27. [PubMed: 15958591]
14. Maus MV, Thomas AK, Leonard DG, Allman D, Addya K, Schlienger K, et al. Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4–1BB. *Nat Biotechnol.* 2002;20:143–8. [PubMed: 11821859]
15. Hirano N, Butler MO, Xia Z, Berezovskaya A, Murray AP, Ansen S, et al. Efficient presentation of naturally processed HLA class I peptides by artificial antigen-presenting cells for the generation of effective antitumor responses. *Clin Cancer Res.* 2006;12:2967–75. [PubMed: 16707591]
16. Ye Q, Loisiou M, Levine BL, Suhoski MM, Riley JL, June CH, et al. Engineered artificial antigen presenting cells facilitate direct and efficient expansion of tumor infiltrating lymphocytes. *J Transl Med.* 2011;9:131. [PubMed: 21827675]
17. Melenhorst JJ, Solomon SR, Shenoy A, Hensel NF, McCoy JP, Jr., Keyvanfar K, et al. Robust expansion of viral antigen-specific CD4+ and CD8+ T cells for adoptive T cell therapy using gene-modified activated T cells as antigen presenting cells. *J Immunother.* 2006;29:436–43; discussion 365–6. [PubMed: 16799339]
18. Yang S, Rosenberg SA, Morgan RA. Clinical-scale lentiviral vector transduction of PBL for TCR gene therapy and potential for expression in less-differentiated cells. *J Immunother.* 2008;31:830–9. [PubMed: 18833004]
19. Yang S, Cohen CJ, Peng PD, Zhao Y, Cassard L, Yu Z, et al. Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. *Gene Ther.* 2008;15:1411–23. [PubMed: 18496571]
20. Sloan EM, Melenhorst JJ, Tucker ZC, Pfannes L, Brenchley JM, Yong A, et al. T-cell immune responses to Wilms tumor 1 protein in myelodysplasia responsive to immunosuppressive therapy. *Blood.* 2011;117:2691–9. [PubMed: 21097671]

21. Scheinberg P, Melenhorst JJ, Brenchley JM, Hill BJ, Hensel NF, Chattopadhyay PK, et al. The transfer of adaptive immunity to CMV during hematopoietic stem cell transplantation is dependent on the specificity and phenotype of CMV-specific T cells in the donor. *Blood*. 2009;114:5071–80. [PubMed: 19776383]
22. Melenhorst JJ, Scheinberg P, Lu J, Ambrozak DR, Sosa E, Zhao L, et al. Regulatory T-cell depletion does not prevent emergence of new CD25+ FOXP3+ lymphocytes after antigen stimulation in culture. *Cytotherapy*. 2008;10:152–64. [PubMed: 18368594]
23. Precopio ML, Betts MR, Parrino J, Price DA, Gostick E, Ambrozak DR, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med*. 2007;204:1405–16. [PubMed: 17535971]
24. Butler MO, Lee JS, Ansen S, Neuberg D, Hodi FS, Murray AP, et al. Long-lived antitumor CD8+ lymphocytes for adoptive therapy generated using an artificial antigen-presenting cell. *Clin Cancer Res*. 2007;13:1857–67. [PubMed: 17363542]
25. Lin GH, Sedgmen BJ, Moraes TJ, Snell LM, Topham DJ, Watts TH. Endogenous 4–1BB ligand plays a critical role in protection from influenza-induced disease. *J Immunol*. 2009;182:934–47. [PubMed: 19124736]
26. Sluijter BJ, van den Hout MF, Stam AG, Loughheed SM, Suhoski MM, van den Eertwegh AJ, et al. 4–1BB-mediated expansion affords superior detection of in vivo primed effector memory CD8+ T cells from melanoma sentinel lymph nodes. *Clin Immunol*. 2010;137:221–33. [PubMed: 20708974]
27. Tiercy JM, Nicoloso G, Passweg J, Schanz U, Seger R, Chalandon Y, et al. The probability of identifying a 10/10 HLA allele-matched unrelated donor is highly predictable. *Bone Marrow Transplant*. 2007;40:515–22. [PubMed: 17646842]
28. Pedron B, Guerin-El Khourouj V, Dalle JH, Ouachee-Chardin M, Yakouben K, Corroyez F, et al. Contribution of HLA-A/B/C/DRB1/DQB1 common haplotypes to donor search outcome in unrelated hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:1612–8. [PubMed: 21536142]
29. Tiercy JM. Analysis of 250 HLA-B44 genotypes in European Caucasoids: high diversity and preferential ABCDRB1 associations in B*4402, B*4403, and B*4405 haplotypes. *Tissue Antigens*. 2005;65:429–36. [PubMed: 15853897]
30. O'Connell RM, Balazs AB, Rao DS, Kivork C, Yang L, Baltimore D. Lentiviral vector delivery of human interleukin-7 (hIL-7) to human immune system (HIS) mice expands T lymphocyte populations. *PLoS One*. 2010;5:e12009. [PubMed: 20700454]
31. Kerkar SP, Muranski P, Kaiser A, Boni A, Sanchez-Perez L, Yu Z, et al. Tumor-specific CD8+ T cells expressing interleukin-12 eradicate established cancers in lymphodepleted hosts. *Cancer Res*. 2010;70:6725–34. [PubMed: 20647327]
32. Steenblock ER, Fadel T, Labowsky M, Pober JS, Fahmy TM. An artificial antigen-presenting cell with paracrine delivery of IL-2 impacts the magnitude and direction of the T cell response. *J Biol Chem*. 2011;286:34883–92. [PubMed: 21849500]
33. Hsu C, Hughes MS, Zheng Z, Bray RB, Rosenberg SA, Morgan RA. Primary human T lymphocytes engineered with a codon-optimized IL-15 gene resist cytokine withdrawal-induced apoptosis and persist long-term in the absence of exogenous cytokine. *J Immunol*. 2005;175:7226–34. [PubMed: 16301627]
34. Luzzio BB, Luzzio CB. Properties and usefulness of the original K-562 human myelogenous leukemia cell line. *Leuk Res*. 1979;3:363–70. [PubMed: 95026]
35. Greiner J, Ringhoffer M, Taniguchi M, Li L, Schmitt A, Shiku H, et al. mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies. *Int J Cancer*. 2004;108:704–11. [PubMed: 14696097]
36. Inoue K, Ogawa H, Sonoda Y, Kimura T, Sakabe H, Oka Y, et al. Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. *Blood*. 1997;89:1405–12. [PubMed: 9028964]
37. Yan M, Himoudi N, Basu BP, Wallace R, Poon E, Adams S, et al. Increased PRAME antigen-specific killing of malignant cell lines by low avidity CTL clones, following treatment with 5-Aza-2'-Deoxycytidine. *Cancer Immunol Immunother*. 2011;60:1243–55. [PubMed: 21553146]

38. Smith BD, Kasamon YL, Kowalski J, Gocke C, Murphy K, Miller CB, et al. K562/GM-CSF immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate. *Clin Cancer Res.* 2010;16:338–47. [PubMed: 20048335]
39. Brusic A, Hainz U, Wadleigh M, Neuberg D, Su M, Canning CM, et al. Detecting T-cell reactivity to whole cell vaccines: Proof of concept analysis of T-cell response to K562 cell antigens in CML patients. *Oncoimmunology.* 2012;1:1095–103. [PubMed: 23170257]
40. Amanna IJ, Raue HP, Slifka MK. Development of a new hydrogen peroxide-based vaccine platform. *Nat Med.* 2012;18:974–9. [PubMed: 22635006]
41. Hammer MH, Brestrich G, Mittenzweig A, Roemhild A, Zwinger S, Subklewe M, et al. Generation of EBV-specific T cells for adoptive immunotherapy: a novel protocol using formalin-fixed stimulator cells to increase biosafety. *J Immunother.* 2007;30:817–24. [PubMed: 18049333]

Figure 1. K562 and K562 based aAPC inhibit T cell proliferation.

Responder PBMC were stimulated in MLR, with OKT3, or with SEB to induce proliferation. Experiments were performed in triplicate and proliferation was measured by CFSE dilution using flow cytometry. (A) Responder PBMC (0.2×10^6 cells) were stimulated in MLR, with irradiated HLA-mismatched PBMC (0.1×10^6 cells) with various concentrations K562 or aAPC. When concentration of K562 or aAPC exceeded 0.2×10^6

cells, T cell proliferation was strongly inhibited. (B) Responder cells were stimulated with 10 ng/mL of SEB for 6 days. Greater than 0.4×10^6 cells of K562 or aAPC inhibited T cell proliferation. (C) 10 ng/mL OKT3 and (D) 10 ng/mL SEB were added to responder PBMC with or without K562 at a 1:4 ratio, PBMC:K562. T cells were cultured for 6 days with T cell medium. In both stimulations, T cell proliferation was inhibited in the presence of K562.

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Figure 2. Antiproliferative effect on T cells occurs independent of cell-cell contact.

A mixed lymphocyte culture was separated from K562 and aAPC by a 0.4 μ m pore membrane in a transwell plate. (A) Negative control with PBMC alone. (B) Responder PBMC proliferation in the absence of K562 or aAPC. (C) Inhibition of responder PBMC proliferation in the presence of irradiated wildtype K562. (D) Inhibition of responder PBMC proliferation in the presence of irradiated K562 transduced HLA-A*0201 and CD137L (K-A2-CD137L). (E) Not pretreated wildtype K562, and (F) not pretreated K-A2-CD137L similarly inhibited proliferation of responder PBMC.

Figure 3. Formaldehyde fixation abrogates the inhibitory effect of K562.

Fixation of aAPC with formaldehyde abrogated their inhibitory effect on MLR, OKT3 and SEB stimulation. (A) Representative data of CFSE dilution. K562 and aAPC were pretreated with formaldehyde from 1% to 0.01% concentration, and then added to MLR. Greater than 0.1% concentrations of formaldehyde restored T cell proliferation in MLR. (B) 1% and 0.1% formaldehyde fixation restored T cell proliferation ($P < 0.0001$). 0%: 100 Gy irradiated, not formaldehyde fixation. NT: Not irradiation, not formaldehyde fixation. (C) CD64 transduced K562 (K64) pulsed with OKT3, and (D) HLA-DR15, HLA-DM, and Ii

transduced K562 (KR15) pulsed with SEB were pretreated with either 100 Gy irradiation or 0.1% formaldehyde fixation, and added to responder PBMC. Coculture with fixed aAPC permitted T cell proliferation in both settings.

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Figure 4. Fixed aAPC induce HLA-A*0201 restricted CMV-specific CTL.

HLA-A*0201⁺ healthy donor T cells were stimulated with irradiated or fixed K-A2 or K-A2-CD137L after pulsing with CMV pp65 and IE-1 peptide libraries. T cells were cocultured with aAPC for 14 days in the presence of IL-7, IL-12, and IL-15. In order to evaluate CMV pp65 and IE-1 specificity, CTL were incubated for 6 hours with pp65 and IE-1 peptide libraries with no further addition of APC. After this incubation, cytokine production was measured by flow cytometry. (A) Fixed K-A2 and K-A2-CD137L induced more CD107a⁺, IFN-γ⁺, and TNF⁺ CD8⁺ T cells compared to irradiated aAPCs (*: $P < 0.05$). (B), (C) Fixed aAPC induced not only single cytokine producing cells, but CD107a⁺/IFN-γ⁺ or TNF⁺/IFN-γ⁺, polyfunctional CD8⁺ T cell lines better than irradiated aAPC (*: $P < 0.05$). Ir: irradiated, Fixed: pretreated with 0.1% formaldehyde, No aAPC: MLR with no aAPC.

Figure 5. HLA-A*0201 specific killing of fixed aAPC induced CTL.

In order to test the antigen-dependent cytolytic activity, antigen-specific lysis was measured after 16 hours exposure to peptide pulsed or unpulsed K-A2 and T2 cell line at 10:1 to 1.25:1 E:T ratios. T cell lines generated with peptide pulsed, fixed K-A2 (A) and K-A2-CD137L (B) demonstrated clear HLA-A*0201 restricted CMV-specific cytotoxicity.

● : Wildtype K562, ■: K-A2, ▲: Peptide pulsed K-A2, ○: Peptide pulsed T2.

A

B

Figure 6. Efficacy of formaldehyde fixation to generate CMV-specific CTL.

Four HLA-A*0201⁺ healthy donors were used to generate CMV-specific CTL. (A) T cells first stimulated with peptide pulsed autologous DC for 7 days in the presence of cytokines, then restimulated with either peptide pulsed autologous DC or peptide pulsed, fixed aAPC for another 7 days. Stimulation with peptide pulsed, fixed aAPC generally elicited CMV-specific T cell responses equal to, or greater than two stimulations with DC. (B) After stimulating twice with pulsed, fixed aAPC, CD137L transduced aAPC generally induced greater frequency of CMV-specific CTL than did K-A2. ns; not significant, ***: $P < 0.0001$, **: $P < 0.01$, *: $P < 0.05$.

Table I.
CMV-specific T cell generation with irradiated or fixed, peptide pulsed aAPC

CMV-specific T cell generation of three HLA-A*0201⁺ individuals for 14 days.

Percentages are mean of triplicate experiments.

NP: Not Proliferated

% of CD107a ⁺ /IFN- γ ⁺ cells						
aAPC	Irradiated K-A2	Fixed K-A2	Irradiated K-A2- CD137L	Fixed K-A2- CD137L	Irradiated K-A2- CD137L-CD80/83	Fixed K-A2- CD137L-CD80/83
Donor 1	1.20%	19.2%	6.4%	17.8%	4.6%	5.7%
Donor 2	NP	13.8%	NP	22.5%	NP	10.8%
Donor 3	NP	31.1%	NP	15.5%	7.8%	15.9%

Table II.
CMV-specific CTL generation with DC and/or aAPC stimulation from HLA-A*0201⁺ healthy donors

Four HLA-A*0201⁺ healthy donor T cells are stimulated weekly for two weeks with DC or formaldehyde fixed aAPC in presence of cytokines. Mean of triplicate experiments was used to determine the % of CD107a⁺/IFN- γ ⁺ cells. Increment of CD107a⁺/IFN- γ ⁺ cells are calculated by % of positive cells stimulated with DC or aAPC / % of positive cells stimulated by DC twice (DC/DC). DC; dendritic cells, K-A2; K562 transduced HLA-A2, K-A2-CD137L; K562 transduced HLA-A*0201 and CD137L.

CD107a ⁺ /IFN- γ ⁺ cells							
1st stimulation / 2nd stimulation	DC / DC (%)	K-A2-CD137L / K-A2-CD137L (%)	Increment of CD107a ⁺ / IFN- γ ⁺ cells	DC / K-A2 (%)	Increment of CD107a ⁺ / IFN- γ ⁺ cells	DC / K-A2-CD137L (%)	Increment of CD107a ⁺ / IFN- γ ⁺ cells
Donor 4	8.66	10.5	1.21	7.8	0.9	19.7	2.27
Donor 5	48	26.2	0.55	49.9	1.03	46.6	0.97
Donor 6	19.7	20.8	1.05	29.7	1.51	28.6	1.45
Donor 7	32.2	4.1	0.13	36.0	1.12	35.1	1.1
Mean $\pm$ SD	27.1 $\pm$ 16.9	15.4 $\pm$ 10.6		30.9 $\pm$ 17.5		32.5 $\pm$ 11.3	