

Engineering Artificial Antigen-presenting Cells to Express a Diverse Array of Co-stimulatory Molecules

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To facilitate the therapeutic application of antigen-presenting cells (APCs), we have developed a cell-based artificial APC (aAPC) system by engineering K562 cells with lentiviruses to direct the stable expression and secretion of a variety of co-stimulatory molecules and cytokines. Here we report the use of a combinatorial lentiviral gene transfer approach to achieve long-term stable expression of at least seven genes in the K562 parental cell line. Expression of various combinations of genes on the aAPC enables the precise determination of human T-cell activation requirements, such that aAPCs can be tailored for the optimal propagation of T-cell subsets with specific growth requirements and distinct functions. The aAPCs support *ex vivo* growth and long-term expansion of functional human CD8 T cells without requiring the addition of exogenous cytokines, in contrast to the use of natural APCs. Distinct populations of T cells can be expanded with aAPCs expressing CD137L (4-1BBL) and/or CD80. Finally, the aAPCs provide an efficient platform to expand genetically modified T cells and to maintain CD28 expression on CD8 T cells. Therefore, K562-based aAPCs have therapeutic potential for adoptive immunotherapies and vaccinations.

Received 31 October 2006; accepted 6 February 2007; advance online publication 20 March 2007. doi:10.1038/mt.sj.6300134

INTRODUCTION

Professional antigen-presenting cells (APCs), such as dendritic cells (DCs) and lymphocytes, are composed of diverse subsets with specific effector functions. Two main lineages of DCs are recognized: the myeloid lineage, which includes Langerhans cells and interstitial DCs, and the plasmacytoid lineage.¹ According to current concepts, DCs undergo stepwise activation/maturation.² Immature DCs are essential to the maintenance of peripheral tolerance to self antigens and to the development of active immunity.³ In tissue, DCs become activated in response to microbial signals (toll-like receptor ligands) and other micro-environmental signals, triggering migration and differentiation

into a mature antigen-loaded DC. The ability to generate DCs from blood monocytes or other precursors has enabled studies testing adoptive transfer of antigen-bearing DCs in humans. Injection of mature DCs can boost T-cell immunity in healthy volunteers,⁴ and injection of immature DCs can lead to the inhibition of T-cell responses via the induction of regulatory T cells.⁵ Therefore, DC immunization is of considerable interest for immunotherapy of cancer and auto-immunity. However, generation of DCs is laborious and expensive. The culture process is resource intensive and utilizes a cocktail of cytokines, and there is variability among donors.⁶ In addition, there have been reports of dysfunctional DCs in cancer patients.⁷

We and others have demonstrated the successful expansion of human lymphocytes using artificial APCs (aAPCs) in place of natural APCs.^{8–12} We have chosen the K562 cell line as a scaffold because the cells do not express major histocompatibility complex molecules, which therefore prevents allogeneic responses. The cells also contain adhesion molecules that enhance T cell–aAPC interactions. In this study, we have created an improved aAPC system to express a diverse array of co-stimulatory and human lymphocyte antigen (HLA) molecules. Previous studies demonstrated expression of up to three genes of interest in aAPCs using retroviral transduction methods.^{12,13} Here we use lentiviral vector technology to demonstrate stable surface expression of at least seven genes in the K562 cell-based aAPCs. The aAPCs have an efficiency comparable to that of natural DCs for driving T-cell expansion; they are especially efficient for activation of human CD8 T cells, for maintaining CD28 surface expression, and for expansion of genetically modified T cells. Finally, the co-stimulatory ligands on the aAPCs enable efficient proliferation and expansion of CD8 T cells without the use of exogenous cytokines or feeder cells as used in current cell culture processes.

RESULTS

K562 aAPCs with CD32 or CD64

The parental K562 line was first stably transduced with the CD32 intermediate-affinity Fc receptor or the CD64 high-affinity Fc receptor. Single-cell clones were obtained by flow-sorting, and surface expression of the molecules was assessed by flow

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cytometry (**Figure 1a**). After incubation with a mouse IgG1 or mouse IgG2a antibody and washing, only the CD64 aAPC retained expression of mouse IgG2a, and no detectable antibody remained on the CD32 aAPC. These results are consistent with reported properties of human CD32 and CD64.¹⁴ To test the relative binding of anti-CD3, a mouse IgG2a monoclonal

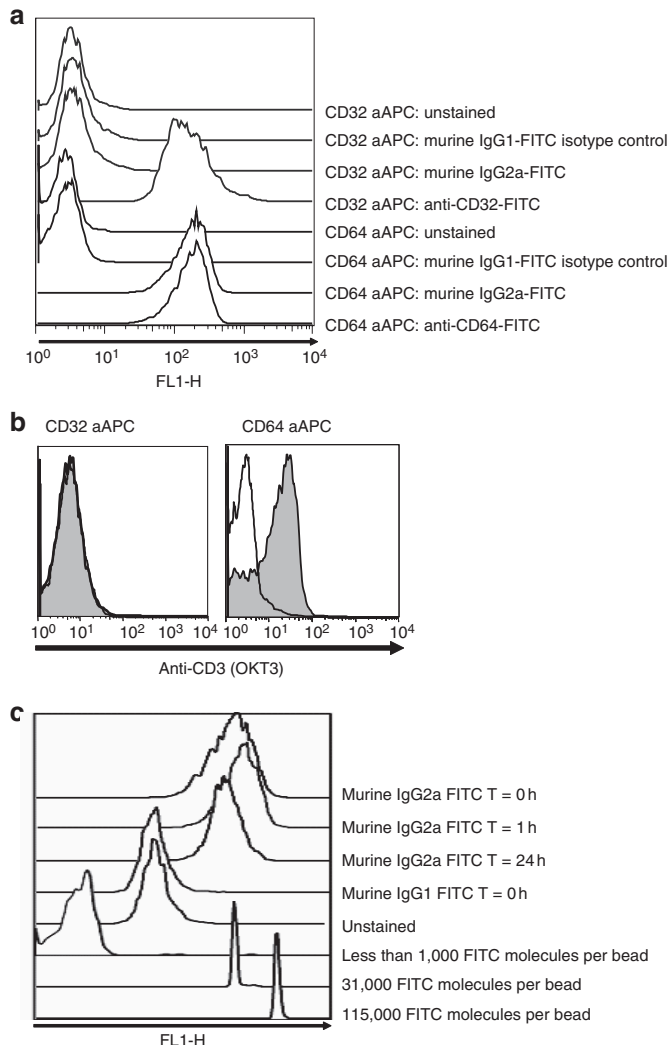


Figure 1 Lentiviral-mediated expression of CD64 and CD32 in K562 artificial antigen-presenting cells (aAPCs). **(a)** Expression of CD64 and CD32 in K562 cells. The parental K562 cell line was transduced with a lentiviral vector expressing CD64 or CD32, and the cells were sorted and cultured as described in Materials and Methods. **(b)** Relative binding of anti-CD3 to CD32- and CD64-transduced K562 cells. CD32 and CD64 aAPCs were washed into serum-free Roswell Park Memorial Institute 1640 medium and cultured overnight at 37 °C in 5% CO₂. The aAPCs were loaded with 0.5 μg/ml anti-CD3 and stained with secondary antibody. The open histogram represents cells stained with secondary detection antibody only; the shaded histogram represents cells pre-loaded with anti-CD3 and then stained with secondary detection antibody. **(c)** Prolonged surface presentation of Fc-bound antibody on CD64-transduced K562 aAPCs. CD64 aAPCs were incubated in serum-free medium overnight at 37 °C in 5% CO₂, then loaded with 1 μg/ml per 1 × 10⁶ of either murine IgG1 or murine IgG2a fluorescein isothiocyanate (FITC)-conjugated antibodies for 30 minutes at 4 °C. FITC-conjugated Immuno-brite fluorospheres (Beckman Coulter, Fullerton, CA) were used to calculate the number of molecules bound to the CD64-transduced aAPCs.

antibody, CD64 and CD32 aAPCs were incubated with anti-CD3 (OKT3) and washed. We found that only the CD64 aAPCs had detectable surface expression of anti-CD3 (**Figure 1b**). In previous studies using plasmid-transfected K562 cells, we found that CD32-expressing aAPCs were able to stimulate T cell proliferation, but only when the anti-CD3 was left in the culture medium, which is consistent with the low-level binding of the anti-CD3 to CD32.⁹ When CD64 aAPCs were loaded with anti-CD3, washed, and then placed back into culture, quantitative flow cytometry demonstrated that the binding and surface expression of the antibody were stable and remained at high levels for at least 24 hours (**Figure 1c**).

To determine the efficiency of the CD32 and CD64 aAPCs in activating T cells, the aAPCs were loaded with various concentrations of anti-CD3 and anti-CD28 antibodies (murine IgG2a isotype). The antibody-loaded aAPCs were lethally irradiated and added to cultures of T cells in a 1:2 ratio of aAPCs to T cells (**Figure 2a**). The aAPCs were efficient at inducing T cell proliferation, with most T cells exhibiting three or four cell divisions on the day of analysis. The dose-response of the antibody loading level revealed that only nanogram concentrations of antibody were required for loading, which is consistent with the efficient binding of the antibodies to CD64.

Efficiency of K562 aAPCs compared with standard culture systems

In the experiments reported above, the aAPCs were used as a standalone T cell culture system, in that no exogenous cytokines were added to the culture medium. We next carried out experiments to compare the relative efficiencies of the K562 aAPCs and conventional modes of CD8 T cell stimulation. When examined on day 5 of culture, CD8 T cell cultures containing phytohemagglutinin and interleukin-2 (IL-2) or anti-CD3 and IL-2 had significant cell division; however, there remained a substantial number of T cells in the culture that had failed to divide (**Figure 2b**). Similarly, anti-CD3/CD28-conjugated beads were efficient at inducing CD8 T cell proliferation, but a substantial number of CD8 T cells also failed to divide, and this was not due to a lack of IL-2. In contrast, the anti-CD3/CD28-loaded CD64 aAPCs recruited a greater number of the input CD8 T cells into cell division, and this was more efficient than the anti-CD3/CD28-conjugated beads. This was not due to sub-optimal activation by the antibody-coated beads, because the anti-CD3/CD28-conjugated beads were added at a ratio previously determined to be optimal (3 beads per T cell). Finally, we determined that the anti-CD28 monoclonal antibody was not required, because CD64-86 aAPCs (*i.e.*, K562 cells engineered to express CD64 and the CD28 ligand CD86) were also highly efficient at inducing CD8 T cell proliferation.

For potential therapeutic applications, it would be useful to have off-the-shelf aAPCs that could simply be added as feeder cells to T cell cultures. Therefore, CD64-86 aAPCs were loaded with OKT3, irradiated, and cryopreserved. To test the efficacy of the pre-loaded aAPCs, the cells were thawed and added to carboxy fluoroscein succinimidyl ester-labeled T cells. The efficiency of these pre-loaded aAPCs was similar to that of freshly prepared OKT3-loaded CD64-86 aAPCs (**Figure 2c**).

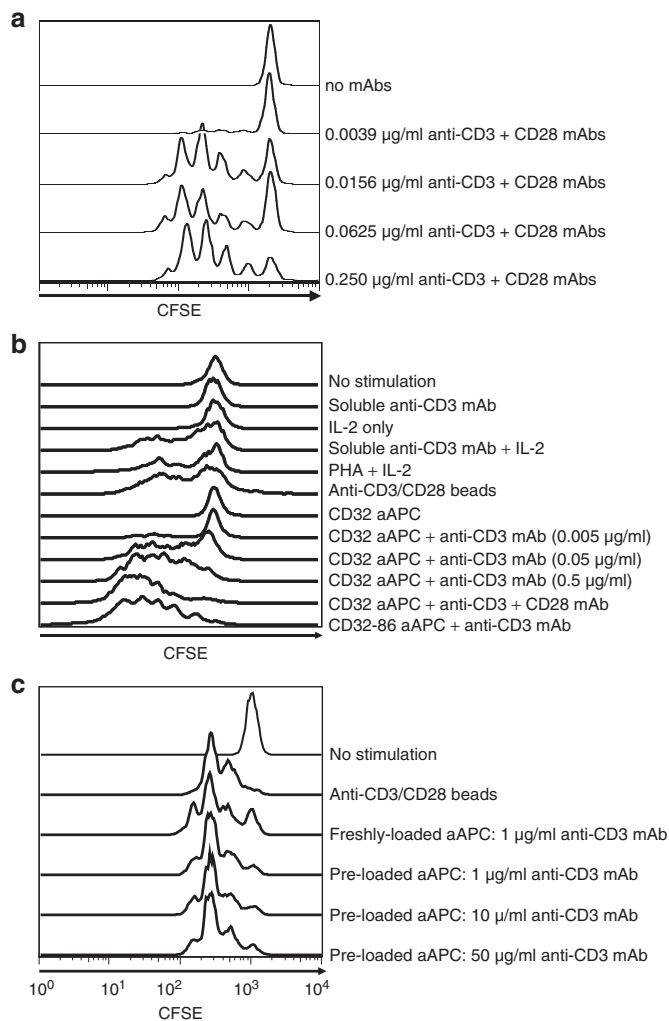


Figure 2 Efficient T-cell activation and proliferation using lentiviral-transduced K562 artificial antigen-presenting cells (aAPCs). **(a)** CD64-transduced K562 aAPCs were incubated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) at the indicated concentrations and then washed. The antibody-loaded aAPCs were lethally irradiated and placed in culture with carboxyfluorescein succinimidyl ester (CFSE)-labeled CD8 T cells at a 1:2 ratio of aAPCs to T cells. T-cell proliferation was assessed on day 4 of culture by flow cytometry. **(b)** Efficiency of CD8 T cell activation and proliferation after various modes of stimulation. CD8 T cells from the peripheral blood of a normal donor were labeled with CFSE and stimulated with soluble anti-CD3 (10 µg/ml), phytohemagglutinin (PHA) (3 µg/ml), anti-CD3/CD28-conjugated beads (3:1 ratio of beads to T cells), and irradiated CD32-transduced or CD32- and CD86-transduced aAPCs (2:1 ratio of T cells to K562 aAPCs) in the presence of the indicated amount of anti-CD3, or anti-CD3 plus anti-CD28 at 0.5 µg/ml, or else as otherwise noted. On the first day of stimulation, 300 IU/ml recombinant interleukin-2 (IL-2) was added. CD8⁺ T cell proliferation was assessed on day 4 of stimulation by flow cytometry. Histograms represent CFSE profiles of gated CD8⁺ T cells. **(c)** T cell proliferation induced by an "off-the-shelf" aAPC. Anti-CD3-loaded, lethally irradiated fresh or cryopreserved CD64-86-transduced K562 cells mediate efficient activation and growth of CD4 T cells. K562 cells expressing CD64 and CD86 were cryopreserved with or without pre-loaded anti-CD3 (OKT3) at 1, 10, or 50 µg/ml. Pre-loaded aAPCs were thawed for immediate use, and freshly prepared OKT3-loaded aAPCs were added as indicated to CFSE-labeled CD4⁺ T cells (2:1 ratio of T cells to K562 aAPCs). T cells were also stimulated using anti-CD3/CD28 beads and proliferation was assessed on day 4 of culture by flow cytometry.

Stable expression of multiple genes using lentiviral vectors

From the above experiments, we concluded that CD32- or CD64-transduced aAPCs could efficiently activate primary T cell cultures. Our previous work used plasmid-mediated expression of CD32 and CD137L in the K562 cell to create the aAPC, and antibiotic selection was required to maintain expression of the introduced genes.⁹ Following a month in culture, the lentiviral-transduced CD32 aAPC remained uniformly positive, whereas CD32 was lower in magnitude and had a wider distribution of expression in the plasmid-transfected cells, despite antibiotic-mediated selection pressure (**Supplementary Figure S1**).

We next stably transduced a variety of co-stimulatory molecules into K562 cells to determine the feasibility of expressing complex arrays of co-stimulatory molecules. We found that multiple genes could be stably expressed in the K562-based aAPCs after lentiviral transduction. Parental K562 cells do not express co-stimulatory molecules or show HLA class I and HLA class II surface expression. To determine the ability of K562 cells to express multiple genes after lentiviral transduction, K562 cells were transduced and shown to express a variety of molecules, including CD32, CD64, CD40, CD40L, CD70, CD80, CD83, CD86, ICOSL, GITRL, CD137L (4-1BBL), CD252 (OX40L), HLA-A201, and HLA DR-0401 (**Figure 3a**). Consistent with previous reports,^{15,16} HLA A2-expressing aAPCs pulsed with peptides induced flu-specific CTL activity in CD8 T cells, indicating functional antigen presentation (**Figure 3b**). Thus, in principle, given the efficacy of lentiviral vector transduction technology, this approach has the potential to create aAPCs that display combinatorial arrays of co-stimulatory molecules along with Fc molecules or HLA alleles to achieve polyclonal or antigen-specific stimulation.

aAPC–T cell cluster formation

The first visible event in T cell activation is the formation of clusters between the APCs and T cells.¹⁷ Cultures of aAPCs alone, or T cells in the presence or absence of soluble anti-CD3, remained free of cluster formation (**Figure 4a, b, and e**). In addition, mixtures of irradiated aAPCs and CD8 T cells remained free of clusters in the absence of anti-CD3 antibody (**Figure 4c**). In contrast, when aAPCs and T cells were mixed in the presence of anti-CD3 antibody, large clusters of aAPCs and T cells formed (**Figure 4f and g**). The clusters grew in size between 48 and 72 hours (**Figure 4h**), at which point nearly all T cells in the culture were contained in large clusters. The irradiated aAPCs began to disintegrate on approximately day 4 of culture (data not shown), so that by day 6 the cultures contained only viable T cells that could be easily disaggregated into single cells.

aAPC-mediated expansion of CD28[−]CD8 T cells

Our earlier experiments indicated that the K562-based aAPCs were more efficient than anti-CD3/CD28-coated beads and other conventional culture systems at inducing CD8 T cell proliferation (**Figure 2a**). Previous studies from several laboratories have shown that the CD28[−] T cell subset is relatively hypoproliferative¹⁸ and has eroded telomeres, suggesting a more extensive replicative history.^{19,20} To test the hypothesis that recruitment of CD28[−] cells into division accounted for the enhanced proliferation, CD8 T cells

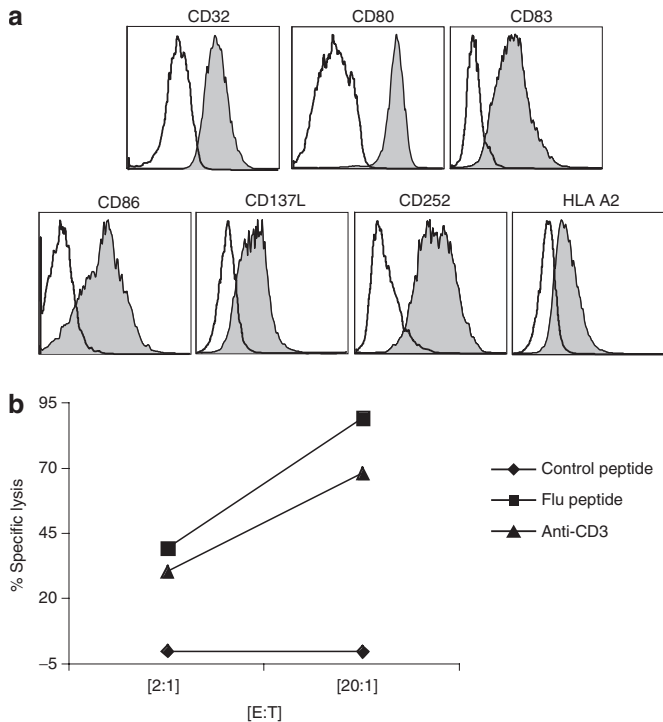


Figure 3 Lentiviral-mediated expression of multiple genes and functional peptide presentation in K562-based artificial antigen-presenting cells (aAPCs). **(a)** K562 cells were transduced with lentiviral vectors containing gene inserts for human lymphocyte antigen (HLA)-A2, CD32, CD80, CD83, CD86, CD137L (4-1BBL), and CD252 (OX40L) and sorted with MOFLO to isolate single-cell clones. Clones were cultured for 2 months without antibiotics, and expression was detected by flow cytometry. Histograms represent protein expression in the CD32-80-83-86-137L-252-A2 cell line (shaded) compared with parental K562 cells (open). **(b)** K562 cells were transduced with lentiviral vectors for CD32 and HLA-A*0201 and sorted based on surface expression. The K562 aAPC targets (T) were pulsed overnight with peptides and labeled with ^{51}Cr as described in **Supplementary Materials** and **Methods**. Peptide-pulsed aAPCs or anti-CD3-loaded aAPCs were cultured with a flu-specific CD8 $^{+}$ effector clone (E) isolated from an HLA-A2 $^{+}$ donor. Cultures were harvested after 4 hours, and CTL activity, measured by percentage specific lysis, was calculated for each target: flu-peptide pulsed (squares), HTLV-1 tax control peptide (diamonds), and anti-CD3-loaded (triangles).

were sorted by flow cytometry into CD28 $^{+}$ and CD28 $^{-}$ subsets. Sorted T cell subsets were then labeled with carboxy fluoroscein succinimidyl ester (CFSE) and stimulated with aAPCs expressing CD32, CD80, CD83, CD86, and CD137L, or with conventional anti-CD3/CD28 beads. In addition, T cells were cultured in medium containing soluble anti-CD3 only. On day 8 of the culture, proliferation was assessed by dilution of CFSE. Contrary to previous reports, we found that the CD28 $^{-}$ T cells could be efficiently stimulated. All cells divided when stimulated with aAPCs, whereas anti-CD3/CD28 bead stimulation left a substantial fraction of undivided T cells (**Figure 5**). In contrast, stimulation by both aAPCs and bead cultures could induce all of the CD28 $^{+}$ T cells to divide.

Helper-independent proliferation of human CD8 T-cell subsets

Recent reports have indicated that previous strategies for *ex vivo* expansion of T cells often resulted in cells that were terminally

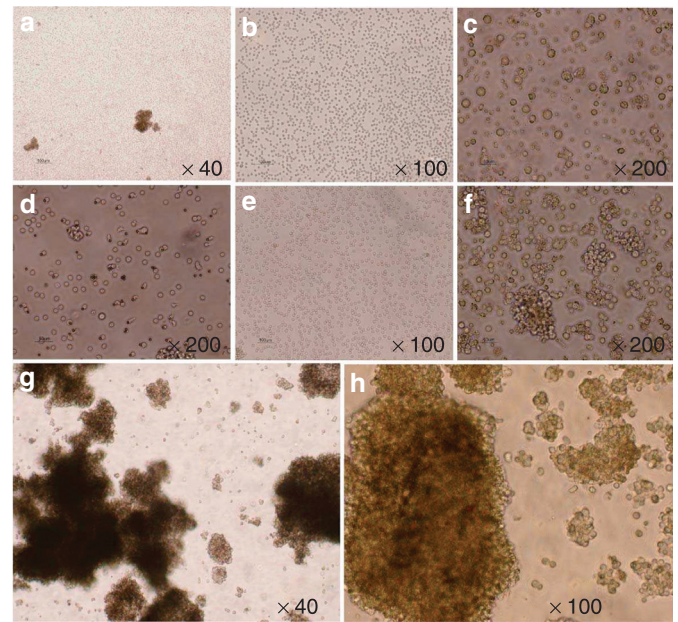


Figure 4 Formation of artificial antigen-presenting cell (aAPC)-T cell clusters as viewed with an inverted phase-contrast microscope. The relative capacity of aAPCs to aggregate T cells is shown in the presence and absence of anti-CD3 (OKT3, 0.5 $\mu\text{g}/\text{ml}$). K562 cells transduced with CD32, CD80, CD83, CD86, and CD137L (4-1BBL) were lethally irradiated and mixed with CD8 T cells at a 1:2 ratio. **(a)** CD32-80-83-86-137L aAPC only; **(b)** CD8 T cells only; **(c)** CD8 T cells and aAPC only; **(d)** CD8 T cells and anti-CD3/CD28 beads; **(e)** CD8 T cells and anti-CD3; **(f-h)** CD8 T cells, aAPC, and anti-CD3. Micrographs **a-g** and **h** depict days 2 and 3 of culture, respectively.

differentiated with poor proliferative potential, and that adoptively transferred T cells that maintained CD28 expression and had longer telomeres correlated with improved engraftment and enhanced anti-tumor effects.²¹ We assessed the cell surface phenotype for CD8 T cells that had been expanded using the K562 aAPC system. The expanded population retained expression of CD28, CD27, and CD62L compared with day-0 phenotype (**Figure 6a**). The CD8 T cells were also able to produce cytokines IL-2 and interferon- γ after activation with phorbol myristate acetate and ionomycin (**Figure 6b**), suggesting that the cells retained their replicative capacity as well as effector function.

For some forms of adoptive immunotherapy, such as the use of tumor-infiltrating lymphocytes,²² it would be useful to expand all CD8 T cells to retain a balance of effector functions and to preserve the input T cell receptor repertoire. To determine which co-stimulatory molecules would facilitate this, we cultured CD8 T cells with anti-CD3-loaded CD32 aAPCs expressing CD137L (4-1BBL) with or without CD80. To maximize the ability to detect independent co-stimulatory effects, the cultures were carried out with no exogenous cytokines or helper cells. The T cell cultures were re-stimulated between days 8 and 12 in culture, as described in Materials and Methods. For the first week of culture, growth of T cells with anti-CD3/CD28 beads was equivalent to that with the K562 aAPCs (**Figure 7a**). However, after 2 weeks of culture, the bead-stimulated cultures reached a plateau, consistent with previous reports demonstrating that anti-CD3/CD28 beads are less efficient for sustained CD8 T cell proliferation.^{9,23}

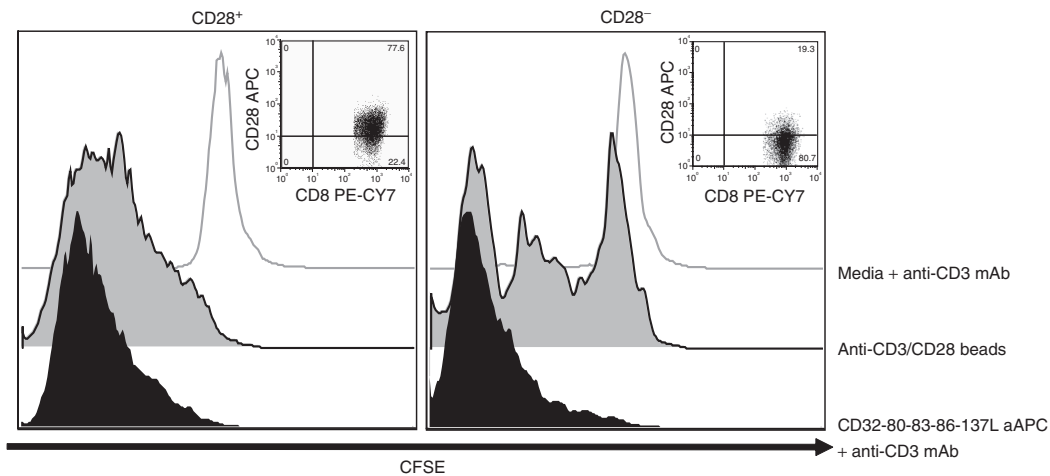


Figure 5 Artificial antigen-presenting cells (aAPCs) mediate helper-independent expansion of CD8 T-cell subsets. Efficient induction of CD8 T-cell subset proliferation using anti-CD3-loaded CD32-80-83-86-137L aAPCs. CD8 T cells were divided into CD28⁺ and CD28⁻ subsets by flow sorting. The T-cell subsets were carboxyfluorescein succinimidyl ester (CFSE)-labeled and cultured with media and soluble anti-CD3 (OKT3, 0.5 μ g/ml), anti-CD3/CD28 beads (3:1 ratio of beads to T cells), or CD32-80-83-86-137L loaded with anti-CD3 (2:1 ratio of T cells to aAPCs). Exogenous cytokines were not added to the culture. Overlay of CFSE histograms on day 8 of culture is shown. Inset dot plots represent expression of CD28 (y-axis) and CD8 (x-axis) on cells recovered on day 8 after stimulation with anti-CD3-loaded CD32-80-83-86-137L aAPC. mAb, monoclonal antibody.

In contrast, the T cell cultures stimulated with K562 aAPCs continued to exhibit exponential CD8 T cell expansion for more than 3 weeks. The aAPCs expressing both CD80 and CD137L were superior to those expressing CD137L alone: the CD32-CD137L aAPCs permitted approximately 1,000-fold expansion, whereas the CD32-80-CD137L aAPCs stimulated approximately 10,000-fold expansion after a single re-stimulation. Monitoring of cell size indicated that the T cells stimulated with the CD32-80-137L aAPCs had a more prolonged increase in cell volume than the other cultures (**Figure 7b**). However, CD8 T cells stimulated with aAPCs containing CD80 alone tended to rest down earlier than those stimulated with aAPCs that expressed CD137L by itself or with CD80, resulting in a reduced number of T cells at the end of stimulation (**Figure 7c**). A remarkable feature of the aAPC cultures is that exogenous cytokine addition was not required. The helper-independent growth of the CD8 T cells is likely due to more vigorous co-stimulation, as the aAPCs themselves do not secrete cytokines that support T cell growth (**Supplementary Table S1**).

aAPC-mediated expansion of lentiviral-engineered CD8 T cells

Genetically engineering T cells for adoptive transfer is another strategy being tested for immunotherapy. In preliminary experiments, we determined that the optimal strategy to transduce and expand CD8 T cells was a sequential anti-CD3/CD28 bead and aAPC culture approach (**Figure 8a**). The K562 aAPCs were able to maintain uniform and bright CD28 expression, whereas the T cells in the bead culture had down-regulated their CD28 expression. This may be important, as other studies have shown that maintained CD28 expression correlates with improved engraftment and anti-tumor efficacy.²¹ Furthermore, the maintenance of CD28 expression may account for the previous observation of prolonged helper-independent growth of CD8 T cells in aAPC

cultures and not in bead cultures (**Figure 7a**). Re-stimulation with the K562 aAPCs resulted in efficient expansion of the green fluorescent protein-expressing CD8 T cells, even in the absence of exogenous cytokines (**Figure 8c** and **d**).

DISCUSSION

Lentiviral vector technology has been used to engineer K562 cells to create aAPCs that more closely replicate the features of DCs. In this work, we have shown that at least seven genes of interest can be stably expressed in the K562-based aAPC. Using this approach, numerous combinations of co-stimulatory molecules and cytokines can be expressed in the aAPC scaffold to be used for immunotherapy. We have shown that the K562 aAPCs expressing ligands for CD28 and CD137 (4-1BB) are superior for long-term propagation of CD8 T cells, and that the addition of other co-stimulatory molecules such as CD83 can support the expansion of CD28⁻ T-cell subsets. We have also shown that the K562 aAPCs achieve efficient expansion of genetically modified T cells and promote the maintenance of CD28 on the surface of the cultured T cells. We demonstrate in this work the potency of the K562 aAPC system in anti-CD3-mediated polyclonal stimulation; in addition, the aAPCs can also be engineered to express HLA molecules, either class I or II, for antigen-specific enrichment and activation of both CD8 and CD4 T cells.

The results indicate that the K562-based aAPCs were more efficient at inducing all CD8 T cells to divide than conventional T-cell culture approaches. It is possible that CD83 stimulation may further increase the effects of CD28 and 4-1BB stimulation.¹² The expanded cells could produce IL-2 and interferon- γ after re-stimulation, suggesting that they are not terminally differentiated effector cells.

In some states of immunopathology, often characterized by persistent antigen, human T cells may down-regulate the expression of CD28 on their surface.^{18,19} The loss of CD28 is often correlated

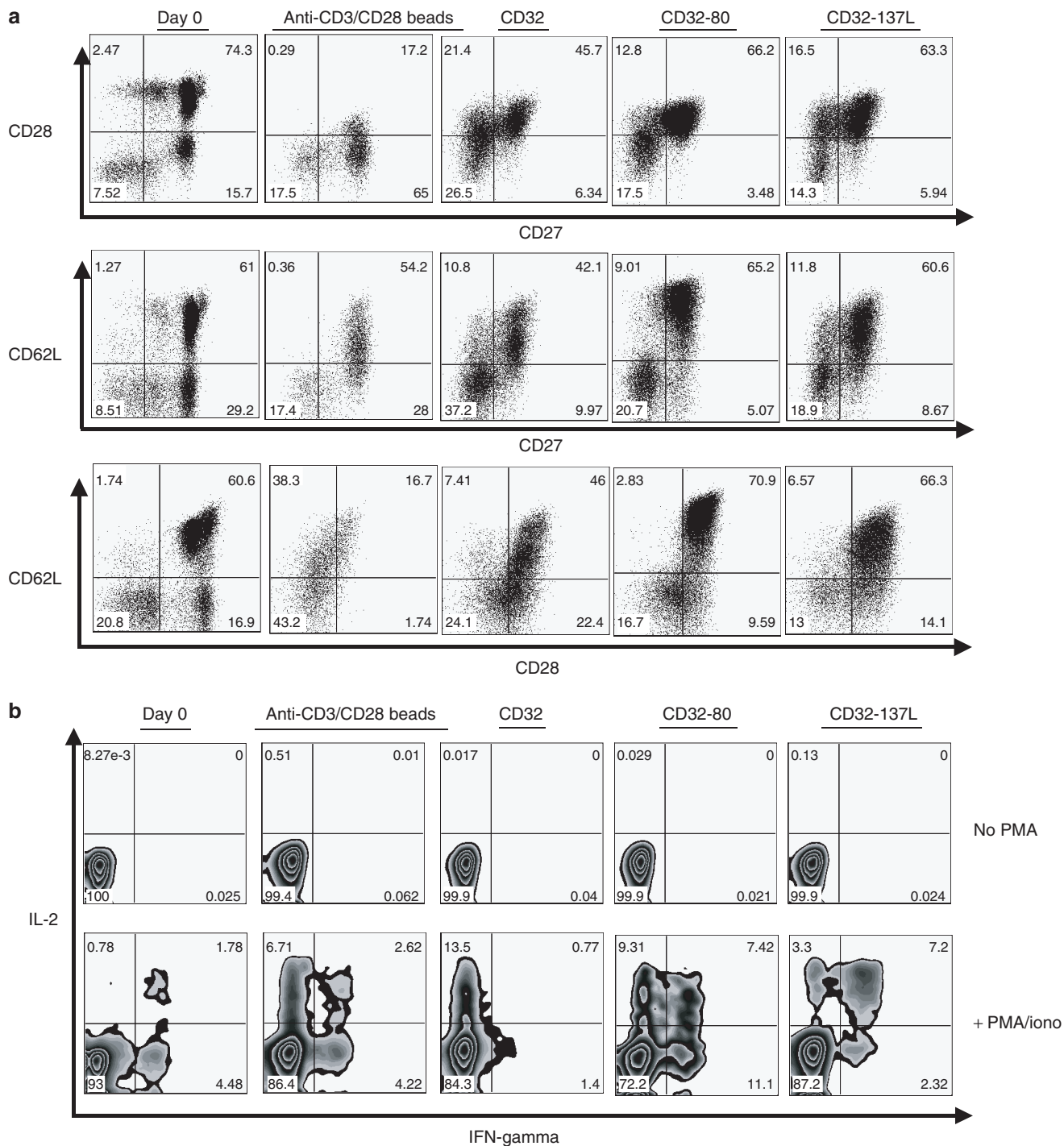


Figure 6 K562 artificial antigen-presenting cell (aAPC)-expanded CD8 T cells retain cell surface phenotype and ability to produce cytokines. Bulk CD8 T cells were expanded for two rounds of stimulation with anti-CD3/CD28 beads or the indicated aAPCs loaded with anti-CD3 as described in the Materials and Methods. When the cells returned to resting state, their phenotype was analyzed using flow cytometry and compared with CD8 T cells from day 0 or cryopreserved for future characterization. **(a)** Cell surface expression of CD28, CD27, and CD62L was measured for each condition. Quadrants were designed based on negative isotype control, and cells were gated on live CD8⁺ cells. **(b)** Induction of cytokine production in K562 aAPC-expanded CD8 T cells. Cryopreserved CD8 T cells from day 0 or expanded with the K562 aAPCs indicated were thawed and incubated for 5 hours in medium alone (top panel) or with phorbol myristate acetate (PMA) and ionomycin (iono) (bottom panel) and assessed for intracellular interleukin-2 (IL-2) and interferon- γ (IFN- γ) production.

with telomere degradation and impaired telomerase induction, decrease in IL-2 production, and replicative senescence.²⁰ Nevertheless, CD28⁻ T cells accumulate *in vivo*, leading to the notion that they are able to divide. Our results using K562-based aAPCs

reveal that nearly all CD28⁻ T cells have the potential to undergo at least several rounds of proliferation *in vitro*.

Generating aAPCs that express the high-affinity Fc receptor CD64 permits the removal of soluble anti-CD3 antibodies

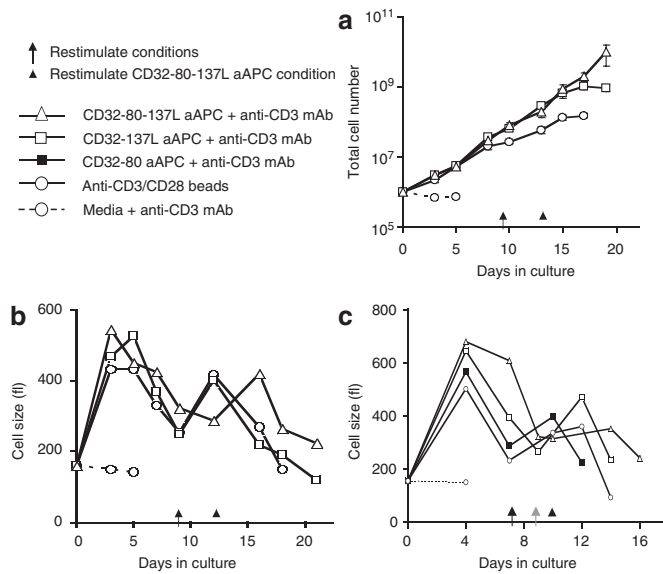


Figure 7 Non-overlapping roles of CD80 and CD137L (4-1BBL) in helper-independent expansion of human CD8 T cells with artificial antigen-presenting cells (aAPCs). CD8 T cells (1×10^6) were stimulated with 0.5×10^6 irradiated aAPCs pre-loaded with anti-CD3 expressing CD32-CD137L (open squares) or CD32-80-137L (triangles), or stimulated with 3×10^6 anti-CD3/28 beads (circles), or cultured in media plus anti-CD3 (dotted line). CD8 T cells are re-stimulated when cells returned to resting volume (250 fl–300 fl) on day 8–12 in culture with re-addition of aAPCs or beads, as indicated by the arrow or arrowhead. No exogenous cytokines were added to the T-cell cultures. Figures are representative of at least three independent experiments using multiple normal donor CD8 T cells. **(a)** Total number of CD8 T cells in culture. **(b)** T-cell volume (fl) as determined by electronic cell sizing. **(c)** T-cell volume (fl) of CD8 T cells expanded with CD32 aAPCs expressing CD80 alone (shaded squares) or CD137L alone (open squares) or CD80 and CD137L (open triangles). Cultures were re-stimulated when they returned to resting volume, as indicated by the arrow or arrowhead. Re-stimulation of cells stimulated with anti-CD3-loaded CD32-137L aAPCs occurred on day 9 of culture (gray arrow).

from the aAPCs, and lymphocyte expansion using the anti-CD3-loaded CD64 aAPCs after washing is comparable to the CD32 aAPCs loaded and co-cultured in the presence of soluble antibody. In addition, precise amounts of antibodies can be added to generate distinct T cell receptor signal strengths to propagate T cell subsets that may exhibit different activation thresholds.²⁴ Finally, by pre-loading the CD64 aAPCs with anti-CD3 and then cryopreserving aliquots of cells for future use, we have generated an “off-the-shelf” reagent that can be conveniently used to grow human T cell sub-populations for research or clinical applications.

MATERIALS AND METHODS

Generation and culture of lentiviral-transduced K562 aAPCs. Human cDNA molecules were amplified: CD32 (primary neutrophils, GenBank accession no. U90939.1), CD64 (Open Biosystems, GenBank accession no. BC032634), CD80 (GeneBank accession no. BC042664.1), CD83 (Raji, American Type Culture Collection, Manassas, VA, GenBank accession no. BC030830.1), CD86 (mature primary DCs, GenBank accession no. NM_006889), CD137L (4-1BBL, activated primary B cells, GenBank accession no. NM_003811), CD252 (OX40L, mature primary DCs, Genbank accession no. X79929), HLA-A*0201 (The International Cell and Gene Bank, <http://www.ihwg.org>) were cloned into a self-inactivating

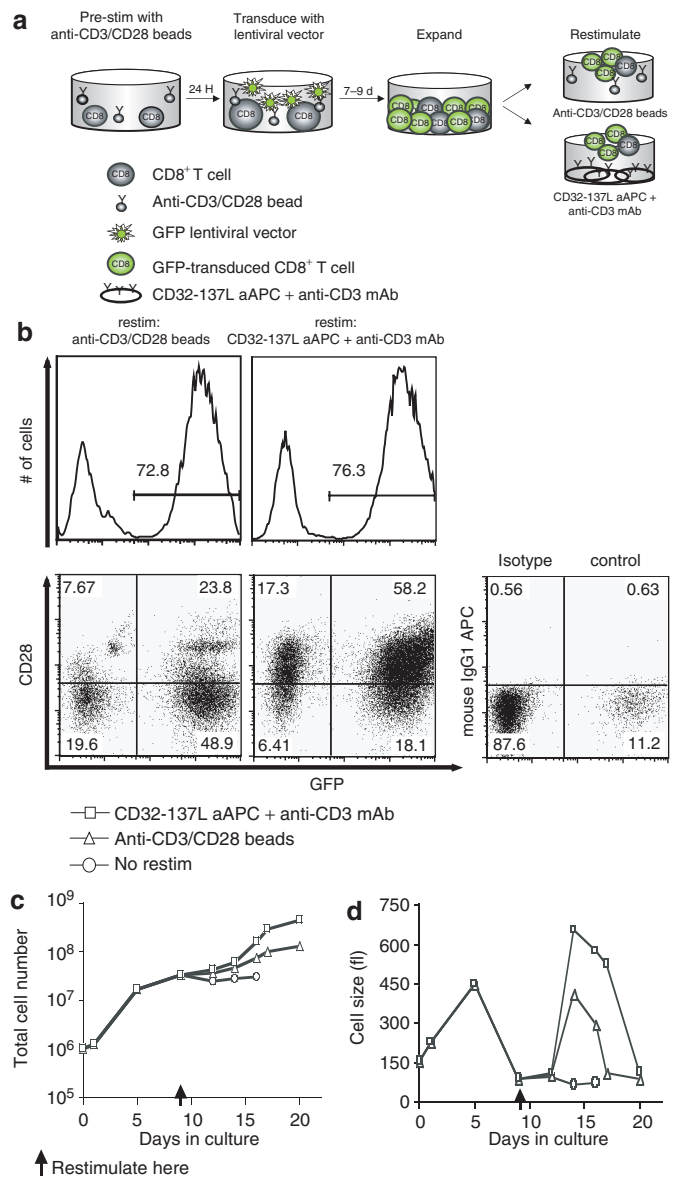


Figure 8 K562 artificial antigen-presenting cell (aAPC) supports expansion of lentivirally transduced CD8 T cells. **(a)** Schematic of the experimental protocol. **(b)** Phenotype of cells assessed on day 17 in culture by flow cytometry. Histograms in top panel represent green fluorescent protein (GFP) expression. Bottom panel shows quadrants of GFP (x-axis) and CD28 (y-axis) compared with isotype control on far right. **(c)** Growth curve of cell counts and **(d)** mean cell volume graphs represent first stimulation (beads and 24-hour transduction) followed by re-stimulation indicated by the arrow with anti-CD3/CD28 beads (triangles) or anti-CD3-loaded CD32-137L aAPCs (squares), or without re-stimulation (circles).

lentiviral vector designated pCLPS. All vector gene expression was directed under a cytomegalovirus promoter. Lentivirus was generated via “split genome” transfection of packaging cell line 293T cells with lentiviral transfer vector, gag/pol, rev, and vsv.g plasmids as described elsewhere.^{25,26} Viral supernatant was harvested and concentrated through ultracentrifugation 24 and 48 hours after transfection. K562 cells (American Type Culture Collection, Manassas, VA) were transduced with the lentiviral vector CD32-pCLPS or CD64-pCLPS, and cells with high expression were cloned using high-speed MOFLO sorting (Cytomation, Fort Collins, CO) to generate CD32-transduced or CD64-transduced aAPCs. The cells were engineered

to express multiple genes through sequential transduction and sorting and named after each gene was introduced. K562 aAPCs were cultured in AIM V (Gibco BRL/Life Technologies, Grand Island, NY) containing 3% human AB serum (Valley Biomedical, Winchester, VA). Routine assays for gene expression via flow cytometry and for mycoplasma (Cambrex Bio Science, Walkersville, MD) were conducted on a monthly basis. Transfected K562 aAPCs were cultured in AIM V supplemented with antibiotics G418 (Cellgro/Mediatech, Herndon, VA) and hygromycin B (Roche, Indianapolis, IN) to maintain gene expression as described.⁹

Polyclonal stimulation and long-term culture of CD8 T cells. Fresh peripheral blood lymphocytes were obtained by leukopheresis and elutriation, and CD8 T cells were purified by negative selection using the OKT4 antibody (American Type Culture Collection, Manassas, VA) as described elsewhere,²⁷ the Rosette CD8 T cell purification kit using negative selection (Stem Cell Technologies, Vancouver, Canada), or the MACs CD8 T Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA). Lymphocytes were used immediately or cryopreserved for future use in medium containing 90% human AB serum plus 10% dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO). CD8 T cells were cultured in medium designated R-5 containing Roswell Park Memorial Institute-1640 medium (Gibco/Invitrogen, Carlsbad, CA) plus 5% human AB serum (Valley Biomedical, Winchester, VA), 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (Gibco/Invitrogen, Carlsbad, CA), and incubated at 37 °C in 5% CO₂. Before stimulation of 1 × 10⁶ CD8 T cells, 0.5 × 10⁶ CD32-transduced aAPCs were lethally irradiated with 100 Gy, washed, and resuspended at 1 × 10⁶ cells/ml into R-5 culture medium. The aAPCs were then added to a 24-well plate and loaded with anti-CD3 (OKT3; Orthoclone, Bridgewater, NJ) monoclonal antibody at 0.5 µg/ml for 10 min at room temperature. CD8 T cells were resuspended at 2 × 10⁶ cells/ml, and 0.5 ml of cells was added dropwise to the antibody-loaded aAPCs. Before the CD64-transduced aAPCs were used, the cells were washed and resuspended in serum-free media for 24 hours to free up the Fc receptors for optimal antibody loading for stimulation. After anti-CD3 loading, extra antibody was removed by washing the aAPCs at least twice and resuspending in R-5 before coculture with T cells. Initial T cell concentration was 1 × 10⁶ cells/ml total volume. Anti-CD3/28 bead stimulation was performed at a ratio of three beads to one T cell, as described elsewhere.²⁸ T cell stimulation cultures were monitored for cell volume and enumerated on a Coulter Multisizer 3 (Beckman Coulter, Fullerton, CA) every 2–3 days. Cells were re-stimulated at 8–11-day intervals, when the expansion rate plateaued and/or the mean lymphocyte volume decreased to 250 fl.

See also **Supplementary Materials and Methods**.

ACKNOWLEDGMENTS

We would like to thank William DeMuth III (Abramson Family Cancer Research Institute) for MOFLO-sorting technical assistance and Coral Haas (Abramson Family Cancer Research Institute) for administrative assistance. This work was supported in part by National Institutes of Health awards CA105216 and AI060477 and Immunobiology of Normal and Neoplastic Lymphocytes training grant T32 CA 09140. The authors have declared a potential conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. Lentiviral-mediated expression results in increased expression and stability compared with the transfection-based approach.

Table S1. Constitutive cytokine secretion by a K562 aAPC transduced with CD32.

Materials and Methods.

REFERENCES

- Banchereau, J and Palucka, AK (2005). Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**: 296–306.
- Reis e Sousa (2001). Dendritic cells as sensors of infection. *Immunity* **14**: 495–498.
- Steinman, RM, Hawiger, D and Nussenzweig, MC (2003). Tolerogenic dendritic cells. *Annu Rev Immunol* **21**: 685–711.
- Dhodapkar, MV, Steinman, RM, Sapp, M, Desai H, Fossella, C, Krasovsky, J *et al.* (1999). Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* **104**: 173–180.
- Dhodapkar, MV, Steinman, RM, Krasovsky, J, Munz, C and Bhardwaj, N (2001). Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* **193**: 233–238.
- Nestle, FO, Banchereau, J and Hart, D (2001). Dendritic cells: on the move from bench to bedside. *Nat Med* **7**: 761–765.
- Almand, B, Resser, JR, Lindman, B, Nadaf, S, Clark, JJ, Kwon, ED *et al.* (2000). Clinical significance of defective dendritic cell differentiation in cancer. *Clin Cancer Res* **6**: 1755–1766.
- Latouche, JB and Sadelain, M (2000). Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells. *Nat Biotechnol* **18**: 405–409.
- Maus, MV, Thomas, AK, Leonard, DG, Allman, D, Addya, K, Schlienger, K *et al.* (2002). *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* **20**: 143–148.
- Oelke, M, Maus, MV, Didiano, D, June, CH, Mackensen, A and Schneck, JP (2003). *Ex vivo* induction and expansion of antigen-specific cytotoxic T cells by HLA-Ig-coated artificial antigen-presenting cells. *Nat Med* **9**: 619–624.
- Thomas, AK, Maus, MV, Shalaby, WS, June, CH and Riley, JL (2002). A cell-based artificial antigen-presenting cell coated with anti-CD3 and CD28 antibodies enables rapid expansion and long-term growth of CD4 T lymphocytes. *Clin Immunol* **105**: 259–272.
- Hirano, N, Butler, MO, Xia, Z, Ansen S, von Bergwelt-Baildon, MS, Neuberger, D *et al.* (2006). Engagement of CD83 ligand induces prolonged expansion of CD8⁺ T cells and preferential enrichment for antigen specificity. *Blood* **107**: 1528–1536.
- Numberjapon, T, Serrano, LM, Singh, H, Kowolik, CM, Olivares, S, Gonzalez, N *et al.* (2006). Characterization of an artificial antigen-presenting cell to propagate cytolytic CD19-specific T cells. *Leukemia* **20**: 1889–1892.
- Ravetch, JV and Bolland, S (2001). IgG Fc receptors. *Annu Rev Immunol* **19**: 275–290.
- Britten, CM, Meyer, RG, Kreer, T, Drexler, I, Wolfel, T and Herr, W (2002). The use of HLA-A*0201-transfected K562 as standard antigen-presenting cells for CD8⁺ T lymphocytes in IFN-gamma ELISPOT assays. *J Immunol Methods* **259**: 95–110.
- Yuan, J, Gallardo, HF, Rasalan, T, Ranganathan, R, Wang, J, Zhang, Y *et al.* (2006). *In vitro* expansion of Ag-specific T cells by HLA-A*0201-transfected K562 cells for immune monitoring. *Cytotherapy* **8**: 498–508.
- Inaba, K and Steinman, RM (1987). Monoclonal antibodies to LFA-1 and to CD4 inhibit the mixed leukocyte reaction after the antigen-dependent clustering of dendritic cells and T lymphocytes. *J Exp Med* **165**: 1403–1417.
- Lewis, DE, Tang, DS, Adu-Oppong, A, Schober, W and Rodgers, JR (1994). Energy and apoptosis in CD8⁺ T cells from HIV-infected persons. *J Immunol* **153**: 412–20.
- Monteiro, J, Batiwalla, F, Ostrer, H and Gregersen, PK (1996). Shortened telomeres in clonally expanded CD28⁺ CD8⁺ T cells imply a replicative history that is distinct from their CD28⁺ CD8⁺ counterparts. *J Immunol* **156**: 3587–3590.
- Effros, RB, Dagarag, M, Spaulding, C and Man, J (2005). The role of CD8⁺ T-cell replicative senescence in human aging. *Immunol Rev* **205**: 147–157.
- Zhou, J, Shen, X, Huang, J, Hodes, RJ, Rosenberg, SA and Robbins, PF (2005). Telomere length of transferred lymphocytes correlates with *in vivo* persistence and tumor regression in melanoma patients receiving cell transfer therapy. *J Immunol* **175**: 7046–7052.
- Dudley, ME, Wunderlich, JR, Robbins, PF, Yang, JC, Hwu, P, Schwartztruber, DJ *et al.* (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* **298**: 850–854.
- Laux, I, Khoshnan, A, Tindell, C, Bae, D, Zhu, X, June, CH *et al.* (2000). Response differences between human CD4⁺ and CD8⁺ T-cells during CD28 costimulation: implications for immune cell-based therapies and studies related to the expansion of double-positive T-cells during aging. *Clin Immunol* **96**: 187–197.
- Iezzi, G, Karjalainen, K and Lanzavecchia, A (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8**: 89–95.
- Reiser, J (2000). Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Ther* **7**: 910–913.
- Parry, RV, Rumbley, CA, Vandenberghe, LH, June, CH and Riley, JL (2003). CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-xL, and IL-2 expression in primary human CD4 T lymphocytes. *J Immunol* **171**: 166–174.
- June, CH, Ledbetter, JA, Gillespie, MM, Lindsten, T and Thompson, CB (1987). T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol Cell Biol* **7**: 4472–4481.
- Levine, BL, Bernstein, WB, Connors, M, Craighead, N, Lindsten, T, Thompson, CB, June, CH *et al.* (1997). Effects of CD28 costimulation on long term proliferation of CD4⁺ T cells in the absence of exogenous feeder cells. *J Immunol* **159**: 5921–5930.