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Complementation of antigen presenting cells to generate T lymphocytes with broad target specificity

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Abstract

Antigen-specific T-cells provide a therapy for cancer that is highly specific, self-replicating and potentially devoid of toxicity. Ideally, tumor-specific T-cells should recognize multiple epitopes on multiple antigens to prevent tumor immune-escape. However the large-scale expansion of such broad-spectrum T-cells has been limited by the availability of potent autologous antigen-presenting cells that can present antigens on the polymorphic array of each patient's HLA allotype. We evaluated a novel antigen-presenting complex (KATpx) in which antigens in the form of peptide libraries can be presented by autologous activated T-cells while co-stimulation is complemented *in trans* by an HLA-negative K562 cell line genetically modified to express CD80, CD83, CD86 and 4-1BBL (K562cs). The additional co-stimulation provided by K562cs significantly enhanced T-cell expansion in culture over autologous activated T-cells alone while maintaining antigen specificity. We validated this antigen-presenting system by generating Epstein Barr Virus (EBV) antigen-specific T-cells from healthy donors and from patients with EBV-positive malignancies including nasopharyngeal carcinoma (NPC) and multiply relapsed EBV-positive lymphoma. These T-cells were specific for EBNA1, LMP1 and LMP2, the viral antigens expressed in these type 2 latency EBV-associated malignancies. The KATpx system consistently activated and expanded antigen specific T-cells both from healthy donors and from 5 of 6 patients with lymphoma and 6 of 6 with NPC, while simplifying the process for generating APCs by eliminating the need for live virus (EBV) or viral vectors to force expression of transgenic EBV

antigens. Hence KATpx provides a robust, reliable, and scalable process to expand tumor-directed T-cells for the treatment of virus-associated cancers.

Keywords

Immunotherapy; T-cell therapy; cancer; lymphoma; nasopharyngeal carcinoma

INTRODUCTION

T-cells whose native receptors are directed to tumor-associated antigens have proved effective in the treatment of several human cancers.¹⁻³ In order to expand sufficient numbers of these cells for human use it has been necessary to use “professional” antigen presenting cells (pAPC), which can effectively present weak tumor-associated antigens and provide essential co-stimulatory signals. Unfortunately, these pAPC are usually unavailable in sufficient numbers to match the needs of culture expansion, and investigators have attempted to substitute alternative cell types to provide APC function.

DCs are the most potent known APCs and have been widely used in clinical trials to activate weak tumor antigens.^{4,5} At least three cell types other than dendritic cells (DCs) have been used as APCs for clinical studies, but individually all have mechanistic or practical limitations that preclude broader use. For example, autologous EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) can induce T-cell responses to subdominant virus-associated tumor antigens such as LMP1 and LMP2 if they are forced to express the target at high level, for example by adenovirus transduction, but they also express strong endogenous antigens.⁶ Therefore, T-cells with specificity for antigens derived from the transducing adenovirus or the more immunogenic EBV-LCL antigens that are absent on the tumor cells may come to dominate the cultures. Moreover, the lengthy preparation time to make an EBV-LCL and the need for live viruses/viral vectors introduce regulatory problems during progression to later phase, large-scale clinical application. Alternatively, artificial APCs (aAPCs) such as the HLA-negative K562 cell line expressing transgenic HLA molecules have been used to expand antigen-specific T-cells directed against epitopes expressed by single HLA alleles,⁷ but this strategy is limited to patients sharing the HLA antigens expressed by the APC. Moreover, a single HLA molecule can express only a limited range of potential epitopes and so cannot expand T-cells with the range of specificities induced by autologous pAPC, thereby facilitating immune escape by antigen loss variants of the tumor. We and others have shown that autologous activated T-cells (aATCs) can also substitute as APC, presenting antigens associated with the patients entire complement of HLA Class I and Class II antigens. Unfortunately, however, such aATCs often fail to adequately support the expansion of patient T-cells, likely due to lack of appropriate co-stimulation.^{8,9}

To investigate whether the deficiencies of individual substitute-APCs can complement one another in trans to produce a fully functional antigen-presenting complex, we combined the broad HLA-associated antigen presentation functions of aATCs with an HLA-negative K562 cell line genetically modified to express critical costimulatory ligands (K562cs).¹⁰ This combination of pepmix-pulsed aATCs and K562cs cell line (KATpx) was validated in

generating T-cells specific for EBV type 2 latency proteins (including EBNA1, LMP1 and LMP2) from healthy donors and from patients with EBV-positive lymphoma and nasopharyngeal cancer (NPC), since such T-cells have been effective in the treatment of both types of EBV-associated malignancy.^{1, 11, 12} We describe how this use of cellular complementation is robust, scalable, and GMP compliant and circumvents many of the obstacles to the creation of tumor-directed T-cells for clinical use.

MATERIALS AND METHODS

Blood donors and cell lines

After informed consent to Baylor College of Medicine Institutional Review Board-approved protocols, peripheral blood was obtained from EBV seropositive healthy volunteers, and peripheral blood mononuclear cells (PBMCs) isolated on lymphoprep (Axis Shield, Oslo, Norway). PBMCs from 6 patients with multiply relapsed or refractory lymphoma, and 6 patients with multiply-relapsed NPC were obtained from cryopreserved samples from patients on clinical trials. PBMCs were used to generate EBV-LCLs, aATCs, DCs, and effector T-cell lines.

EBV-LCLs were established by infecting PBMC with the B95-8 strain of EBV in the presence of cyclosporin A, as previously described.¹³ The K562 cell line genetically modified to express CD80, CD83, CD86, 4-1BBL and CD32 (K562cs) was a gift from Dr. Carl June (University of Pennsylvania, Perelman School of Medicine) and was maintained in culture medium (RPMI-1640, Hyclone, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Waltham, MA) and 2mM/l GlutaMAX TM-I (Invitrogen, Carlsbad, CA). Stable expression of co-stimulatory molecules CD80, 83, 86, and 4-1BBL over at least 6 months of culture was validated by flow cytometry. All cell lines tested negative for mycoplasma.

Generation of antigen-presenting cells (APCs)

Activated T-cells—aATCs for antigen presentation were generated by stimulation of PBMCs (5×10^5 cells per well) on non-tissue culture treated 24-well plates coated with a CD3 antibody produced by the OKT3 hybridoma (ATCC #CRL 8001, Manassas, VA) and CD28 antibody (Becton Dickinson BD, Franklin Lakes, NJ) (CD3/28 MAbs, each at 1 μ g/ml). ATCs were maintained in T-cell medium (TCM) (RPMI 1640, (Hyclone, Waltham, MA) supplemented with 45% Click's medium (Irvine Scientific, Santa Ana, CA), 2 mmol/l GlutaMAX TM-I (Invitrogen, Carlsbad, CA), and 5% Human AB Serum (Valley Biomedical, Winchester, VA)] and supplemented with IL2 (50U/ml, NIH, Bethesda, VA), which was replenished every 3 days. Two days before antigen-specific T-cell restimulation, aATCs were re-activated on CD3/28 MAb-coated plates to upregulate costimulatory molecules and then irradiated and peptide-pulsed immediately before being used as APCs.

DC generation—Monocytes were isolated from fresh or frozen PBMCs by CD14 selection using MACS Beads (Miltenyi, Bergisch Gladbach, Germany), and cultured in DC media (CellGenix supplemented with 2 mmol/l GlutaMAX TM-I) (CellGenix USA, Antioch, IL) 800 U/ml GM-CSF (Sargramostim Leukine; Immunex, Seattle, WA) and

1,000U/ ml IL4 (R&D Systems, Minneapolis, MN) for 5 days. IL4 and GM-CSF were replenished on day 3. On day 5, immature DCs were matured using a cytokine cocktail containing 10 ng/ml IL6, 10 ng/ml IL1 β , 10 ng/ml TNF- α (R&D Systems, Minneapolis, MN), 1 μ g/ml PGE2 (Sigma, St Louis, MO), 800 U/ml GM-CSF and 1,000 U/ml IL4 for 48 hours.¹³

Transduction of DCs and LCLs with adenovirus vector—To generate Ad-DC/ LCL-stimulated T-cells, DCs and LCLs were transduced with Ad5f35- LMP1-IRES-LMP2 (Ad-LMP) at a multiplicity of infection (MOI) of 5000 in a small volume (~100 μ L) for 90 minutes at 37°C, 5% CO₂ two days before stimulation, as previously described.² After incubation DCs or LCLs were washed, and resuspended in TCM. Transduced DCs were used to stimulate PBMCs and transduced LCLs were used to restimulate effector T-cells at a 4:1 ratio of T-cells to LCL.

Effector T-cell generation using adenovector-transduced DCs and LCLs (Ad-DC/LCL method)—CD14-negative PBMCs or whole PBMCs were stimulated with Ad-LMP transduced DCs at stimulator:responder (S:R) ratios between 1:10 to 1:20. IL15 (5 ng/mL, Peprotech, Rocky Hill, NJ) was added to cocultures generated from patients with lymphoma. On day 9, and weekly thereafter, effector T-cells were restimulated with transduced LCLs at an S:R ratio of 1:4. Cultures were supplemented with IL2 (50U/ml, NIH, Bethesda, VA) twice weekly from day 12.

Loading antigen-presenting cells (APCs) with pepmixes—Overlapping peptide libraries (pepmixes) comprised peptides of 15 amino acids (aa) in length, overlapping by 11aa and spanning the entire protein sequences of LMP1, LMP2 and EBNA1, and were purchased from JPT Technologies (Berlin, Germany). Pelleted PBMCs, aATCs or mature DCs were pulsed with a cocktail containing all 3 pepmixes (1ng per peptide per 10⁶ PBMCs, ATCs or DCs) for 30 minutes at 37°C, in 5% CO₂. PBMCs were directly resuspended in TCM, while LCLs, ATCs and DCs were first washed with 10ml of phosphate-buffered saline (PBS) (Sigma, St Louis, MO) and subsequently resuspended in TCM.

Preparing ATCs and K562cs cells for use as APCs—ATCs were re-activated by plating on CD3/28 MAb-coated plates for 2 days before they were used to stimulate effector T-cells. On the day of stimulation, ATCs were harvested, irradiated at 30Gy using a RS2000 X-ray irradiator (RadSource, Suwanee, GA). We then pulsed the cells with pepmixes, as described above, and resuspended them in TCM. K562cs cells were irradiated at 100Gy, washed, recounted and combined with pepmix-pulsed ATCs to stimulate effector T-cells.

Generation of antigen-specific effector T-cells using the DC(px) and PBMC(px) methods—For the DC(px) method, CD14-depleted or whole PBMCs were stimulated with pepmix-pulsed autologous DCs at S:R ratios between 1:10 to 1:20. For the PBMC(px) method, whole PBMCs were pulsed directly with pepmixes. Stimulated cells were cultured in TCM supplemented with 1000U/ml IL4 (R&D Systems, Minneapolis, MN) and 10 ng/ml IL7 (PeproTech, Rocky Hill, NJ), as previously described.¹⁴ On day 9, and weekly thereafter, effector T-cells were restimulated with KATpx at a responder T-cell : pepmix-

pulsed autologous ATC: K562cs ratio of 1:1:5 (unless otherwise indicated). Cultures were supplemented with IL2 (50U/ml) twice weekly from day 12.

T-cell analysis

Prior to each restimulation, we analyzed T-cells for phenotype, specificity and function using flow cytometry, ELISpot and ^{51}Cr cytotoxicity assays.

Phenotyping T-cells were phenotyped with antibodies to lymphoid subsets (CD4, CD8, CD14, CD16, CD56) and effector/memory subsets (CD28, CD45RA, CD45RO, and CD62L) (Becton Dickinson BD, Franklin Lakes, NJ). Approximately 200,000 cells were washed once with PBS (Sigma, St Louis, MO) containing 1% FBS (HyClone; Thermo Fisher Scientific, Hudson, NH), pelleted, and antibodies added in saturating amounts (5-10ul). After 30 minutes incubation at 4°C in the dark, cells were washed with PBS, fixed with CytoFix (BD, Franklin Lakes, NJ) and analyzed. Data from 10,000 to 20,000 live cells from each population were acquired on a FACSCalibur flow cytometer and analyzed using Cell Quest software (BD, Franklin Lakes, NJ), with the exception of costimulatory expression phenotyping performed in Figure 2, in which we used Gallios flow cytometer, analyzed using Kaluza software (Beckman Coulter, Inc, Indianapolis, IN), and normalized the MFI to that of Cell Quest.

Enzyme-linked Immunospot (ELISpot) assay—*IFN- γ* ELISpot analysis was used as a semi-quantitative measure of antigen-specific effector T-cells as previously described.¹⁴ Briefly 2.5×10^4 to 10^5 effector T-cells were seeded in triplicate wells and stimulated with individual pepmixes spanning EBNA1, LMP1, LMP2 of EBV, and Hexon or Penton of adenovirus at 0.1ug per peptide per well, or 10^5 autologous LCL per well. A pepmix derived from the sequence of the cancer testis antigen, NY-ESO-1, at 0.1ug per peptide per well, and PHA, at 2ug per well, were used as negative and positive controls, respectively. After 18 hours of incubation, plates were developed and sent to Zellnet Consulting (NJ) for quantification. Spot forming cell (SFC) counts and input cell numbers were plotted, and a linear regression calculated after excluding plateau data points. The frequency of T-cells specific to each antigen was expressed as specific SFC per input cell numbers.

Cytotoxicity assay—The cytotoxic specificity of effector T-cells was measured in a standard 6-hour ^{51}Cr release assay, using effector:target (E:T) ratios from 40:1 to 5:1. We used autologous or allogeneic LCLs or ATCs alone or pulsed with pepmixes as the labeled target cells. Percent specific release was determined from the mean of triplicates as [experimental release – spontaneous release] ÷ [maximum-release (with triton X-100) – spontaneous release].

Statistical analysis

We used Prism (GraphPad Software, Inc. La Jolla, CA) for parametric and non-parametric analyses as appropriate.

RESULTS

Replacement of adenovirus vector with peptides

To replace the Ad-LMP1/2 vector used in our standard LMP-specific T-cell manufacturing protocol¹⁵ (Supplementary Figure 1A), we used peptide mixtures (pepmixes) spanning the protein sequences of the three EBV tumor-associated antigens LMP1, LMP2 and EBNA1 that are expressed by EBV latency type 2-associated malignancies and are only weakly immunostimulatory. We first determined whether antigen-specific T-cells could be activated by the addition of pepmixes directly to PBMCs, within which monocytes and B-cells have APC function, or whether pepmix-pulsed DCs would produce improved T-cell activation. After pepmix loading, DCs were combined with PBMCs at a 20:1 ratio of PBMCs to DCs, while directly pulsed PBMCs were cultured alone (Supplementary Figure 1B). The characteristics of the responder T-cells from 14 healthy donors on day 9 were compared with those of T-cells from the same donors stimulated with “conventional” Ad-LMP-transduced DCs (Ad-DC method).

The expansion of PBMCs was similar, regardless of the source of antigen/antigen-presenting cell, with a mean expansion of 1.6 fold (range 0.8 to 4.5) in the Ad-DC condition, 1.7 fold (range 0.5 to 6.5) in the DC(px) condition and 1.0 fold (range 0.5 to 2.4) in the PBMC(px) condition (Figure 1A). This modest but comparable overall expansion masked substantial differences in the enrichment of EBNA1-, LMP1- and LMP2-antigen-specific T-cells (Figure 1B). We were unable to detect T-cell responses to these antigens using IFN- γ ELISpot assays on day 0 (not shown), but by day 9 we were able to detect a mean of 97 (range 0-255) spot forming cells (SFC) per 10^5 cells in response to Ad-DCs; 1294 (range 149-2103) SFC per 10^5 cells in response to DC(px); and 1086 (range 144-2489) SFC per 10^5 cells in response to pepmixes alone (PBMC(px)). Hence, both DC(px) and PBMC(px) produced significantly higher frequencies of antigen specific T-cells than did Ad-DC (Figure 1B, $p < 0.001$ for EBNA1, ns for LMP1, and $p < 0.01$ and $p < 0.05$ for LMP2). The ratios of CD4:CD8 T subsets and their expression of effector/memory markers such as CD62L and CD28 were similar in all cultures with a mean at around 75% (data not shown). Thus, peptide-pulsed DCs and PBMCs could reactivate T-cells specific for EBNA1, LMP1 and LMP2 as good as or better than Ad-LMP-transduced DCs.

Pepmix-pulsed aATCs produce limited expansion of pepmix-activated T-cells

To further expand antigen-specific T-cells, high ratios of APC to responder T-cells (1:4) are required,¹⁶ so that effective, autologous APCs must be available in large numbers. We and others previously determined that HLA class I and II and co-stimulatory molecules are upregulated on T-cells by activation with CD3/CD28 MAbs and that aATCs could be used as APCs, potentially replacing EBV-LCL for second and subsequent rounds of T-cell stimulation and expansion.^{8,9} We therefore analyzed the kinetics of costimulatory molecule expression after T-cell activation with CD3/CD28 MAbs between days 0 and day 16, and found that expression of MHC class I and II molecules as well as second tier (CD80 and CD86) and third tier (4-1BB ligand and CD40 ligand) co-stimulatory molecules was greatest 2 to 3 days after a second stimulation on day 10 (Figure 2A). Hence, aATCs were used as APCs 2-3 days after their second CD3/28 activation to present pepmixes to responder T-

cells. However, as shown in Figure 2B, aATCs produced a mean of only 4.2 fold expansion after the second stimulation between days 9 and 16 and no expansion after subsequent stimulations.

K562 costimulatory cells (K562cs) complement pepmix-pulsed aATCs in inducing expansion of antigen-specific T-cells

We therefore asked whether the HLA-negative K562cs cell line engineered to express CD80, CD83, CD86 and 4-1BB ligand could complement pepmix-pulsed aATCs and continue to support antigen-specific T-cell proliferation over multiple rounds of stimulations (Supplementary Figure 1C). In preliminary experiments we determined the optimal ratio of T-cell: aATC: K562cs ratio in KATpx to be 1:1:5 (data not shown). At this ratio, KATpx induced exponential expansion of responder T-cells that could be sustained over at least 3 stimulations (Figure 2B) while maintaining antigen specificity (Supplementary Figure 2). To understand why K562cs induced better T-cell expansion than ATCs alone, we compared the levels of expression of costimulatory molecule on each cell type. While the percentage of cells expressing co-stimulatory molecules were similar between K562cs and aATC with the exception of 4-1BBL (Figure 2C), the mean fluorescence intensity (MFI) of each of the positive populations was greater on K562 cells ($p < 0.001$) (Figure 2D). The expression of costimulatory molecules on mature DCs is shown for comparison (Figure 2C and 2D).

Comparable function and specificity of healthy donor T-cells expanded using KATpx or Ad-DC/LCL

After establishing that KATpx could expand antigen specific T-cells, we compared this method to our original Ad-DC/LCL method (Figure 3A). In 14 healthy donors, KATpx produced significantly greater expansion of antigen-specific T-cells than Ad-LCLs, with a mean expansion between days 9 and 16 of 7.1 fold (range 1.5 to 16.5) using Ad-DC/LCL; 36.3 fold (range 6.0 to 114.0) using DC(px)/KATpx; and 19.0 fold (range 6.7 to 33.7) using PBMC(px)/KATpx (Figure 3B). The difference between Ad-LCL and KATpx expanded T-cells was maintained over two additional stimulations until cultures were cryopreserved on day 30 (Figure 3C). ELISpot analysis of T-cell antigen-specificity on day 16 showed similar frequencies of LMP1 and LMP2-specific T-cells produced by all three methods (Figure 3D), while as expected, the frequency of EBNA1-specific T-cells was significantly higher in both DC(px) and PBMC(px) culture conditions. Phenotypic analysis showed that the ratios of CD4:CD8 T-cells were similar in all cultures, with average percentage of CD4 of culture day 16 using Ad-DC/LCL method was at $48.3\% \pm 5.8$; DC(px)/KATpx at $46.4\% \pm 6.3$; and PBMC(px)/KATpx at $51.1\% \pm 6.2$; and average percentage of CD8 of culture day 16 using Ad-DC/LCL method was at $41.9\% \pm 6.7$; DC(px)/KATpx method at $41.9\% \pm 5.8$; and PBMC(px)/KATpx at $45.5\% \pm 7.0$.

To ensure that pepmix induced T-cells could recognize tumor cells that naturally processed and presented these tumor-associated antigens, we compared the ability of pepmix and Ad-DC/LCL-generated T-cells to recognize and kill autologous LCLs that naturally express EBNA1, LMP1 and LMP2. At a T-cell to LCL ratio of 20:1, the mean LCL killing by T-cells from 6 healthy donors on day 23 was 46.67% (range 24.7 to 62.8%) for Ad-DC/LCL cultures; 33.1% (7.2 to 85.7%) from DC(KATpx) cultures and 50% (22.5 to 62.3%) from

PBMC(KATpx) cultures (Figure 3E). Thus T-cells activated using all three conditions could recognize and lyse target cells naturally expressing the targeted antigens.

To determine which T-cell components contributed to the cytotoxic function of the effector population, we activated and expanded T-cells specific for each antigen individually. EBNA1, LMP1 and LMP2 monospecific T-cells could all lyse autologous LCLs (Supplementary Figure 3A), and no consistent differences were detected between the avidity of LMP1 and LMP2 monospecific T-cell lines in IFN- γ ELISpot assays and bulk Ad-DC/LCL lines using limiting dilutions of peptide (Supplementary Figure 3B).

The KATpx strategy can expand T-cells specific for EBNA1, LMP1 and LMP2 from patients with multiple relapsed or refractory lymphoma

After the first stimulation of PBMCs from lymphoma patients, little expansion was seen on day 9 irrespective of the source of the APC. By day 16, however, expansion was greatest in DC(px)/KATpx cultures, intermediate in PBMC(px)/KATpx cultures and least in Ad-DC/LCL cultures (Figure 4A). T-cell surface phenotype was unaffected by the APCs used to generate them (Figure 4B), but DC(px) and PBMC(px) expanded higher frequencies of EBNA1-, LMP1- and LMP2- specific T-cells (Figure 4C). Even though KATpx-expanded cultures had higher frequencies of T-cells specific for tumor-associated antigens, LCL expanded cultures had higher *in vitro* cytotoxicity against autologous LCLs (Figure 4D), most likely due to recognition of non type-2 latency antigens expressed by LCL APCs. Although there appeared to be no consistent differences in cultures initiated with PBMC(px) and DC(px) from healthy donors, with lymphoma patients DCs appear to provide advantages to effector T-cells both in expansion and specificity.

KATpx more effectively reactivated antigen-specific T-cells than Ad-DC/LCLs in patients with advanced NPC

In contrast to lymphoma patients, we saw no significant difference in expansion of T-cells from NPC patients by day 16 using the three culture conditions (Figure 5A). However, by day 30, after 2 more stimulations, DC(px)/KATpx and PBMC(px)/KATpx cultures had all continued to expand at similarly rapid rates, while Ad-DC/LCL stimulated cells ceased to expand (Figure 5A). The ratios of CD4:CD8 T subsets and levels of the effector/memory markers CD62L and CD28 were similar in all cultures (Figure 5B), but KATpx produced higher frequencies and absolute numbers of EBNA1, LMP1 and LMP2-specific T-cells by day 23 than Ad-DC/LCLs (Figure 5C). T-cells generated from NPC patients using all three strategies could kill LCLs (Figure 5D).

Discussion

We have shown complementation between autologous T-cells pulsed with pepmixes and a costimulatory cell line (K562cs) for the production of antigen-specific CD4+ and CD8+ T-cells. This combination forms a potent antigen-presenting complex (KATpx) that can produce several logs of expansion of tumor-directed T-cells, while maintaining broad antigen specificity and sustaining T_H1 activity, including target-antigen dependent IFN- γ release and cytotoxicity to cells that naturally express the tumor antigens. The approach is

effective both for healthy donors and for patients with lymphoid and epithelial malignancies associated with EBV, and can shorten the time taken to reach the numbers of T-cells required for clinical doses (range 10^7 to 10^9 cells/ m^2) by at least one week.

Tumor-specific T-cells for adoptive immunotherapy should ideally recognize multiple antigens and epitopes on each tumor, be capable of proliferating extensively in patients and be able to enter the memory compartment in order to provide long-lasting protection. It is important therefore to have a strategy for the large-scale expansion of polyclonal T-cells with both cytotoxic and helper functions containing T-cell subpopulations with memory potential. Such a strategy must be robust, scalable and capable of reactivating T-cells that may have been rendered anergic by tumors, even if these cells come from patients who have received multiple cycles of chemoradiotherapy.

The reactivation and expansion of anergic memory T-cells specific for weak or subdominant antigens requires potent APCs. Ideally these should be autologous so that they can present the widest possible range of antigens and epitopes against the complex MHC background of each patient. They must also be abundant, since the ratio of antigen-specific T-cell to APC should be at least 1:4 and preferably 5:1.¹⁷ Finally they should provide appropriate costimulatory molecules.¹⁸ DCs are the most potent APC, and are practical for the first stimulation of PBMCs when the frequency of tumor-specific T-cells is low. Even though we did not find a clear benefit to the use of DCs as APCs for the first stimulation in healthy donors, DCs were superior for T-cell expansion in some patients. Thus, they may provide a more robust and reliable source of APC for the first stimulation.

For subsequent stimulations, large numbers of DCs may be obtained by apheresis, but this adds greatly to the expense and complexity of manufacturing. Another possible source of autologous APC is the EBV-transformed B-cell line (EBV-LCL), which can be expanded to large numbers from most individuals.^{19, 20} EBV-LCLs were able to expand clinically effective T-cells specific for viral tumor antigens expressed as transgenes from adenovirus vectors.^{21, 22} However, both LCLs and adenovirus vectors contain strong viral antigens that compete with weaker tumor-associated transgenic antigens. For example some CTL lines used in our current clinical trials of LMP-specific T-cells for the treatment of lymphoma and NPC, were dominated by unwanted T-cell specificities directed to the hexon and penton proteins of adenovirus and to the immunodominant early lytic or type 3 latency antigens of EBV that are absent on the tumor targets (Supplemental Figure 4). Furthermore, the establishment of EBV-LCLs for each patient takes a minimum of 6 weeks and longer in patients who have received lymphotoxic chemotherapy. Finally, EBV-LCLs cannot be generated from patients within 6 months of receiving the B-cell depleting antibody Rituximab, now a standard treatment for B-cell lymphoma.

Other groups have circumvented the requirement for autologous APCs by selecting antigen-specific T-cells prior to non-specific expansion, for example by capturing T-cells that secrete IFN- γ in response to antigenic stimulation.²³ This strategy can be effective, but increases the complexity and expense of the procedure, may select only a fraction of the functional antigen-specific T-cell response and is difficult for low-frequency T-cells. Antigen-specific T-cells can also be selected using tetramers or streptamers, but this strategy

cannot identify T-cells specific for all possible epitopes presented by all possible HLA phenotypes.(Busch D refs) Tumor-infiltrating lymphocytes (TILs) are often tumor-specific and can be expanded non-specifically *ex vivo*. This strategy has been highly successful for the expansion of melanoma-specific T-cells, but has been difficult to extend to other tumor types that do not have TILs or that are surgically inaccessible.

We have addressed each of these mechanistic and practical limitations in turn using a model system that produced clinically relevant T-cells directed to virus-associated tumor antigens. We used aATCs that express HLA class I and class II molecules to present overlapping peptides libraries containing all possible HLA class I-restricted epitopes for each antigen and many class II-restricted epitopes, and complemented them using HLA-negative artificial costimulatory cells (K562cs) *in trans*. This antigen-presenting complex (KATpx) effectively expanded antigen-specific T-cells that had initially been activated either by pepmixes alone or by DCs pulsed with pepmixes. We have validated each component of this complex (peptides, aATCs and K562cs), individually, and in combination.

Peptides can be presented by virtually any cell type as they require minimal processing by endonucleases to produce the 8-10mer peptides required to be presented by HLA class I molecules. Peptides have previously been used individually to stimulate T-cells both *in vitro* and *in vivo*, but this requires prior knowledge of epitopes recognized by each individual²⁴ and cannot produce the broad range of CD4+ and CD8 T-cells induced by peptide libraries. Two major concerns with any peptide approach to antigenic stimulation of T-cells are that non-physiological peptides (those not naturally produced by intracellular processing) may be presented and that low affinity T-cells may be activated by high peptide concentrations. However, we have shown that EBNA1, LMP1 and LMP2 pepmix stimulated healthy donor T-cells have similar specificity and avidity to those generated using full length antigens expressed by the Adenoviral vector, likely because the combined pepmixes contain about 300 peptides, and they can recognize and lyse EBV-infected target cells that naturally express the tumor associated antigens.

We and others have evaluated aATCs alone as APCs for the activation of tumor-specific T-cells.^{8,9} They express HLA class I and II molecules and an array of co-stimulatory molecules that increased significantly after a second stimulation with CD3 and CD28 antibody (Figure 2A). While aATCs are able to reactivate T-cells specific for weak tumor antigens with the additional signals provided by cytokines,^{8,9} they were unable to sustain sufficient levels of effector T-cell expansion (Figure 2B), likely because of inadequate levels of co-stimulation (Figure 2D). Artificial APCs derived from the HLA-negative K562 cell line by genetic modification with an array of cytokines and/or costimulatory molecules can non-specifically expand NK cells and TILs or if modified with surface antigens, can expand T-cells genetically modified to express chimeric antigen receptors (CARs).²⁵⁻²⁸ However, even if K562 cells, are engineered to express one or more HLA molecules, there are all-but insuperable practical difficulties in matching all polymorphisms at all loci to enable these K562 to present the full range of tumor directed T-cell specificities in all donors.

The KATpx approach we describe overcomes the limitations of each strategy by combining two cellular components with complementary properties. In all phenotypic and functional

assays, KATpx expanded T-cells proved to be comparable to those generated from traditional APC while providing an alternative that is both more robust and more readily scalable. Since T-cells can be used as a source of antigen-specific T-cells, a clinical product can be produced from less than 60 mLs of blood in about 3 weeks. Our approach should be readily adapted for other tumor-associated viral antigens simply by changing the pepmixes and is compliant with good manufacturing practices (GMP) allowing this platform to be applied to late phase clinical trials of T-cell immunotherapies for a range of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Activation of T-cells specific for EBV latency type II malignancy associated antigens using Ad-DC, DC(px) or PBMC(px) methods

A. On day 9, bulk culture cell expansion was evaluated by counting viable cells using trypan blue exclusion. **B.** The frequency of T-cells specific for EBNA1, LMP1 and LMP2 was evaluated by IFN- γ ELISpot assays; spot forming cells (SFC) per 10⁵ cells are shown. The frequencies of T-cell secreting IFN- γ in response to EBNA1 and LMP2 pepmix was significantly higher in the conditions stimulated with pepmix than with Ad-DC. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant)

Figure 2. Basic design of KATpx and functional evidence of KATpx expanded T-cells
A. aATCs from 4 healthy donors were non-specifically activated on CD3/CD28 mAbs coated plates on day 0 and day 10. Surface markers of co-stimulatory molecules were measured every 48 hours using flow cytometry (n=4). **B.** PBMCs from 4 healthy donors were activated using PBMC(px) method with EBNA1, LMP1, and LMP2 pepmixes on day 0 (see Methods). On day 9 and weekly thereafter cells were stimulated with aATC pulsed with pepmix or KATpx at T-cells:aATC:K562cs ratio of 1:1:5. Bulk culture expansion was compared using trypan blue exclusion (n=4). By day 23, fold-expansion of KATpx-

stimulated T-cells culture was significantly higher than cultured expanded with pepmix pulsed aATCs. **C-D.** Expression level of CD80, CD83, CD86 and 4-1BBL on K562cs vs aATC 2 days post stimulation, vs mature DC. (** $p < 0.01$; *** $p < 0.001$; ns= non-significant)

Figure 3. Application of KATpx to expand T-cell specific for EBNA1, LMP1 and LMP2 from healthy donors

A-B. Schematic of activation of EBNA1-, LMP1- and LMP2-specific T-cells using the Ad-DC method, then subsequently expanded with Ad-LCL (A), or with DC(px) or PBMC(px) methods, then subsequently expanded with KATpx (B). **C-E.** PBMCs from 14 healthy donors were activated and expanded either with Ad-DC, DC(px) or PBMC(px) methods. **C.** On day 9, 16, 23 and 30, bulk culture expansion was compared by counting using trypan blue exclusion. **D.** EBNA1-, LMP1- and LMP2-specific T-cell frequencies were compared

in IFN- γ ELISpot assays in with spot forming cells (SFC) per 10^5 cells are shown (n=9). **E.** cytotoxicity against autologous LCLs was measured in ^{51}Cr release assays. (*p<0.05; **p<0.01; ***p<0.001; ns: non-significant)

Figure 4. Activation and expansion of EBNA1-, LMP1-, and LMP2-specific T-cells from lymphoma patients

EBNA1-, LMP1- and LMP2-specific T-cells were generated from 6 EBV-positive lymphoma patients using Ad-DC/LCL, or DC(px)/KATpx or PBMC(px)/KATpx approaches. **A.** Bulk culture expansion was evaluated by counting weekly using trypan blue exclusion. **B.** Lymphocyte subsets were compared on Day 23 using flow cytometry. **C.** EBNA1-, LMP1- and LMP2-specific T-cell frequencies were compared on day 23 using

IFN- γ ELISpot. **D.** Cytotoxicity against autologous LCLs was measured in ^{51}Cr release assays. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant)

Figure 5. Activation and expansion of EBNA1-, LMP1-, and LMP2-specific T-cells from NPC patients
EBNA1-, LMP1- and LMP2 –specific T-cells were generated from 6 EBV-positive NPC patients using Ad-DC/LCL, or DC(px)/KATpx or PBMC(px)/KATpx approaches. **A.** Bulk culture expansion was evaluated by counting weekly using trypan blue exclusion. **B.** lymphocyte subsets were compared on Day 23 using flow cytometry. **C.** EBNA1-, LMP1- and LMP2-specific T-cell frequencies were compared on day 23 using IFN- γ ELISpot. **D.**

Cytotoxicity against autologous LCLs was measured in ^{51}Cr release assays. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant)