Article

Establishment and Characterization of a Cell Based Artificial Antigen-Presenting Cell for Expansion and Activation of $CD8^+$ T Cells *Ex Vivo*

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Artificial antigen-presenting cells are expected to stimulate the expansion and acquisition of optimal therapeutic features of T cells before infusion. Here CD32 that binds to a crystallizable fragment of IgG monoclonal antibody was genetically expressed on human K562 leukemia cells to provide a ligand for T-cell receptor. CD86 and 4-1BBL, which are ligands of co-stimulating receptors of CD28 and 4-1BB, respectively, were also expressed on K562 cells. Then we accomplished the artificial antigen-presenting cells by coupling K32/CD86/4-1BBL cell with OKT3 monoclonal antibody against CD3, named K32/CD86/4-1BBL/OKT3 cells. These artificial modified cells had the abilities of inducing $CD8^+$ T cell activation, promoting $CD8^+$ T cell proliferation, division, and long-term growth, inhibiting $CD8^+$ T cell apoptosis, and enhancing $CD8^+$ T cell secretion of IFN-γ and perforin. Furthermore, antigen-specific cytotoxic T lymphocytes could be retained in the culture stimulated with K32/CD86/4-1BBL/OKT3 cells at least within 28 days. This approach was robust, simple, reproducible and economical for expansion and activation of $CD8^+$ T cells and may have important therapeutic implications for adoptive immunotherapy. *Cellular & Molecular Immunology*. 2007;5(1):47-53.

Key Words: artificial antigen-presenting cell, expansion, activation, CD86, 4-1BBL

Introduction

Antigen-specific lymphocytes can be primed and amplified *in vivo* by active immunization or expanded *ex vivo* before their infusion for adoptive cell therapy. The immunological synapse between T cell and antigen-presenting cell (APC) is a critical event for the activation and expansion of T cells. The professional APCs, which include dendritic cells, macrophages and B cells, are better equipped than other cell types to maximize T-cell stimulation. However, isolation and expansion of these cells on an autologous basis, which is necessary to match the T cell's HLA restriction, are expensive and time-consuming processes that hinder the broad implementation of therapeutic T cell expansion (1). Ideally, we hope to get a ready-to-use APC, applicable to any patient, that permits selective expansion of T cells specific for any viral or tumor antigens, yielding large numbers of therapeutically potent cells (2).

Owing to K562 cell line negative for HLA-A, B and DR, they would not promote an allogeneic response in clinical trials, and can easily be killed by natural killers. The K562 cells have been engineered to stably express the human low-affinity Fcγ receptor, CD32, and termed K32. After K32 cells are coated with anti-CD3 and anti-CD28 mouse IgG monoclonal antibodies (K32/CD3/28), they could stimulate the expansion of polyclonal $CD4^+$ T lymphocytes, but not $CD8^+$ T cells. When the K32 cells were transfected with 4-1BBL which can preferentially activate $CD8^+$ T cells, $CD8^+$ T cells showed long-term expansion and survival (3). In addition, K32 cells also show constitutive expression of B7-H3, ICAM-1, and LFA-3 (3, 4), all of which are ligands for co-stimulatory receptors other than CD28.

Although K32/4-1BBL/CD3/28 cells could activate and rapidly expand polyclonal and antigen-specific $CD8^+$ T cells, the cost of CD3 and CD28 antibodies is relatively high for clinical therapy especially in developing countries. CD28 is expressed on resting and activated T cells, binds to B7.1 (CD80) and B7.2 (CD86), and is the most potent co-stimulatory molecule described (5). Despite strong structural similarities, B7-1 and B7-2 exhibit different biochemical

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features, and their binding to the co-stimulatory receptors results in distinct T cell functional outcomes. CD80 is a bivalent dimer (two binding sites) and CD86 is a monomer (single binding site). CTLA-4 (CD152) is also a bivalent dimer (two binding sites) whereas CD28 is a monovalent (single binding site) dimer. Based on the differential affinities of these interactions, it has been inferred that CD80 interactions favour CD152 whereas CD86 interactions are biased towards CD28 (6, 7). In addition, although the cross-linking of CD28 by a specific mAb averts concomitant ligation of CTLA-4, these mAbs do not recapitulate the conformational integrity of a physiological immune synapse. Here we developed a K32 cell-based universal artificial APC system through co-transfecion of CD86 and 4-1BBL for expansion and activation of polyclonal cytotoxic T lymphocytes effectively *ex vivo*.

Materials and Methods

Bacterial strains, plasmids and cell lines

Escherichia coli strain DH5α was utilized for the construction and preparation of plasmids. *Escherichia coli* strain M15 (QIAGEN) was used for the production of recombinant proteins. Plasmid pVITRO2-mcs that contain two separate multiple cloning sites, recombinant 4-1BBL vector of pORF-h4-1BBL v16 were both from Invivogen. Other plasmids were pGEMT vector (Promega), pcDNA3.1 vector (Invitrogen), and plasmid pQE3.1 neo (QIAGEN). K562 cells (ATCC), U937 cells (ATCC) and colon cancer cell line LS-174-T (HLA-A2 positive) from cell bank of Chinese Academy of Sciences were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin, at 37°C in a 5% CO_2 incubator. Subcultures were carried out every 3-4 days.

Cloning and construction of cell-based aAPCs

Human CD32a was cloned from U937 cells into pcDNA3.1 neo vector (8, 9) with primers (CGC AAG CTT GAT GGC TAT GGA GAC CCA AAT G, CCG GAA TTC AGC AAG CTG AGA GTA TGA CCA) according to its cDNA sequence (Genbank accession No. M31932). Human CD86 was cloned from peripheral blood mononuclear cells (PBMC) with primers (CGC GGA TCC TTT GTG ACA GCA CTA TGG GAC T, CCG GTC GAC GGA AAG GGT AGA AAA AAT GAA T) based on the cDNA sequence (Genbank accession No. NM006889). The CD86 and 4-1BBL gene fragments were inserted into separate multiple cloning sites of pVITRO2-mcs. K562 cells were transfected with the recombinant pV-CD86-4-1BBL expression vector with liposome (X-tremeGene Q2, Roche). After selection by hygromycin, K562/CD86/4-1BBL cells were sorted by fluorescence-activated cell sorting (FACS). Then recombinant pcDNA3.1-CD32 vector was transfected into K562/CD86/4-1BBL cells with liposome (Roche). After co-selection by neomycin and hygromycin, K32/CD86/4-1BBL cells were established by FACS sorting and kept in culture of neomycin (200 μg/ml) and hygromycin (100 μg/ml).

Cell stimulations using aAPCs and long-term culture

PBMC from HLA-A2 positive healthy donors were isolated by Ficoll gradient. In CD69 expression tests, we mixed the PBMC with the various stimulatory cells and detected $CD8^+$ T cells with PE-Cy5-anti-CD8 mAb (eBioscience) by flow cytometry. In other experiments, $CD8^+$ T cells were sorted by positive selection labeled with PE-Cy5-anti-CD8 mAb, and the resulting population consisted of > 95% $CD8^+$ T cells. K32/CD86/4-1BBL cells were loaded with OKT3 (anti-CD3 monoclonal antibody, eBioscience) at 0.5 μg/ml for 30 min at room temperature. Stimulatory cells (K562, K562/CD86/4-1BBL, K32/CD86/4-1BBL/OKT3) were lethally irradiated with 100 Gy and washed 2× in RPMI 1640 prior to stimulation. With exogenous IL-2 (20 IU/ml, Peprotec) in the medium, all stimulators were mixed with $CD8^+$ T cells at a 1:1 ratio of three rounds at days 1, 8, and 15. The $CD8^+$ T cell concentration was maintained at 0.5×10^6 cells/ml throughout the culture, and up to 1×10^8 was cultured in flasks. Cultured $CD8^+$ T cells were enumerated on a counting plate with typan blue exclusion of dead cells every 2-3 days.

Flow cytometry and FACS sorting

Cells were stained with antibodies (and/or MHC tetramers) at 4°C, analyzed and sorted on a FACSAria. Fluorochrome-conjugated monoclonal antibodies of CD32, CD86, CD8, CD69 and isotype control antibodies were purchased from eBioscience. Anti-4-1BBL antibody was bought from Biolegend. When $CD8^+$ T cell were co-cultured with indicated stimulatory cells for 6 days, apoptotic assay based on Annexin V and propidium iodide was conducted according to the manufacturer's protocol (Jingmei Biotech, China). All flow cytometric data were analyzed with FACSDiva software.

Proliferation of $CD8^+$ T cells

MTS/PMS assay (Promega) was used to detect the proliferation of $CD8^+$ T cells. The lymphocytes were grown in 96-well plates and stimulated with K562, K562/CD86/4-1BBL, and K32/CD86/4-1BBL/OKT3 cells, respectively. A sample of $CD8^+$ T cells cultured alone was kept as a control and samples were run in triplicates. At day 5 and day 8, MTS/PMS was added to each well. Four hours later, the absorbent data were collected at 490 nm. For CFSE (carboxyfluorescein diacetate succinimidyl easter, Molecular Probes) labeling, $CD8^+$ T cells were washed twice in PBS and incubated with 5 μM CFSE for 15 min at room temperature. After labeling, cells were washed 3× in culture medium and mixed with lethally irradiated stimulatory cells. At days 3 and 5, samples were analyzed with a FACSDiva software gating on viable (forward/side scatter) cells.

Assays of IFN-γ secretion and intracellular perforin

Six days after the second stimulation with the different genetically modified K562 cells or no stimulation, the culture supernatants were collected to detect IFN-γ contents. The concentrations of IFN-γ were assayed by ELISA (Jingmei Biotech, China) according to the manufacturer's instructions. Meanwhile, we observed perforin secretion of the cultured CTLs *ex vivo*. The LS-174-T cells were treated by mitomycin

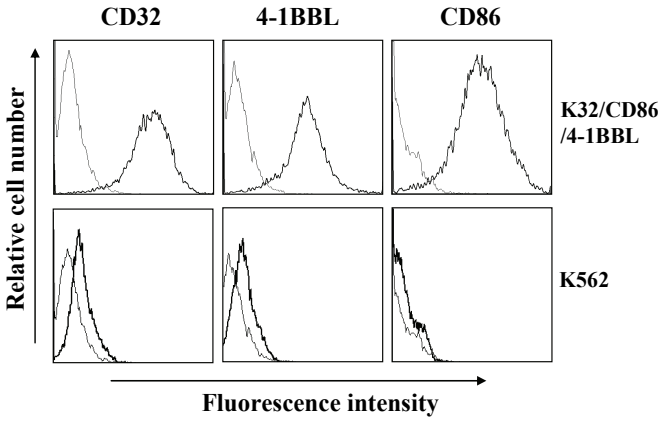

Figure 1. CD32a, CD86, and 4-1BBL were stably co-expressed on the surface of K32/CD86/4-1BBL cells. K32/CD86/4-1BBL cells were stained with FITC-anti-CD32 antibody, PE-anti-4-1BBL antibody or PE-Cy5-anti-CD86 antibody (up row), and analyzed by flow cytometry (bold lines). Weak lines represented irrelevant isotype-matched fluorochrome-conjugated antibodies. K562 cells were also analyzed for corresponding expression of these molecules (low row).

C (10 μg/ml, Sigma) for 2 h, and then washed 3× in culture medium. Effectors and targets were mixed at the ratio of 10:1. Four hours later, fixation buffer was added to block perforin secretion. In the following 20 min, cells were harvested, washed, permeabilized, and stained with Alexa Fluor 647-perforin antibody (Biolegend). All samples were analyzed using the software of FACSDiva.

Generation of sHLA-A2/fluMP tetramer and flow cytometric analysis

HLA-A2 tetramers were produced as described (10). Briefly, human β_2m and the extracellular domain of the HLA-A2 heavy chain linked at its carboxyl terminus to a BirA substrate peptide were expressed separately in *Escherichia coli* and isolated as insoluble aggregate. The expressed HLA-A2-BirA substrate peptide and β_2m subunits were solubilized and refolded together *in vitro* in the presence of peptide (fluMP 58-66 GILGFVFTL, synthesized by Shanghai Biotech Bioscience and Technology Company). Folded material was then subjected to enzymatic biotinylation by BirA enzyme. HLA-A2/peptide complexes were purified twice on sephacryl S-300 high resolution column by gel filtration chromatography. Tetrameric complexes of biotinylated HLA-A2/peptide were produced by mixing purified, biotinylated heterodimer with Streptavidin-phycoerythrin (Vector, Germany) at a molar ratio of 10:1. For peptide-specific $CD8^+$ T cell detecting, CTLs were stained with the tetramers at the optimum concentration (5-10 μg/ml). For staining, 5×10^5 CTLs were incubated at 37°C for 30 min in the dark with PE-labeled tetramer, then with combinations of the following CD8-FITC for 30 min at 4°C. Data were collected on the FACSAria within 1 h after staining and analyzed using the FACSDiva software.

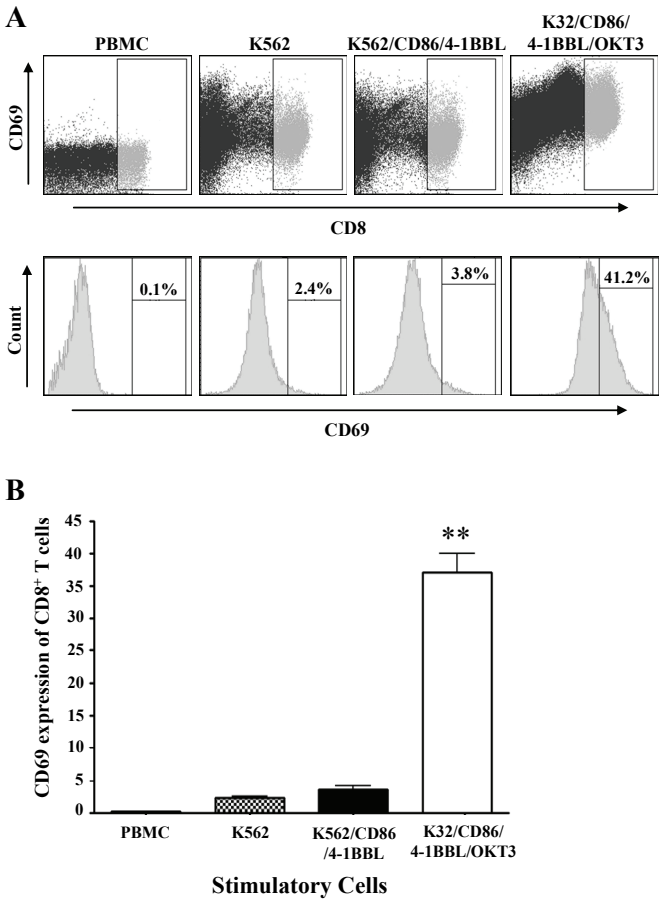

Figure 2. Activation of $CD8^+$ T cells in stimulation with K32/CD86/4-1BBL/OKT3 cells. (A) The lethally irradiated aAPCs were incubated with freshly isolated PBMC for 24 h, and the expression of CD69 on $CD8^+$ T cells was detected by flow cytometry. PBMC stained with PE-Cy5-CD8 and PE-CD69 were shown at the up row, while the low row showed CD69 expression on $CD8^+$ T cells. (B) The percentages of $CD69^+$ cells in $CD8^+$ T cells co-cultured with various aAPCs were calculated. $^{**}p < 0.01$, compared with PBMC alone, in stimulation with K562, or K562/CD86/4-1BBL cells. Results were shown as mean ± SD of three replicates.

Statistical analysis

The differences between various treatment groups were analyzed by two-tailed Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Construction of aAPCs (K32/CD86/4-1BBL)

K562 cells were transfected with the recombinant pV-CD86-4-1BBL expression vector, in which genes of CD86 and 4-1BBL were inserted into each separate multi-cloning site. After hygromycin selection, we got the genetically modified K562 cells with stable expression of CD86 and 4-1BBL firstly, which was termed K562/CD86/4-1BBL. Then pcDNA3.1-CD32 vector was transfected into K562/CD86/

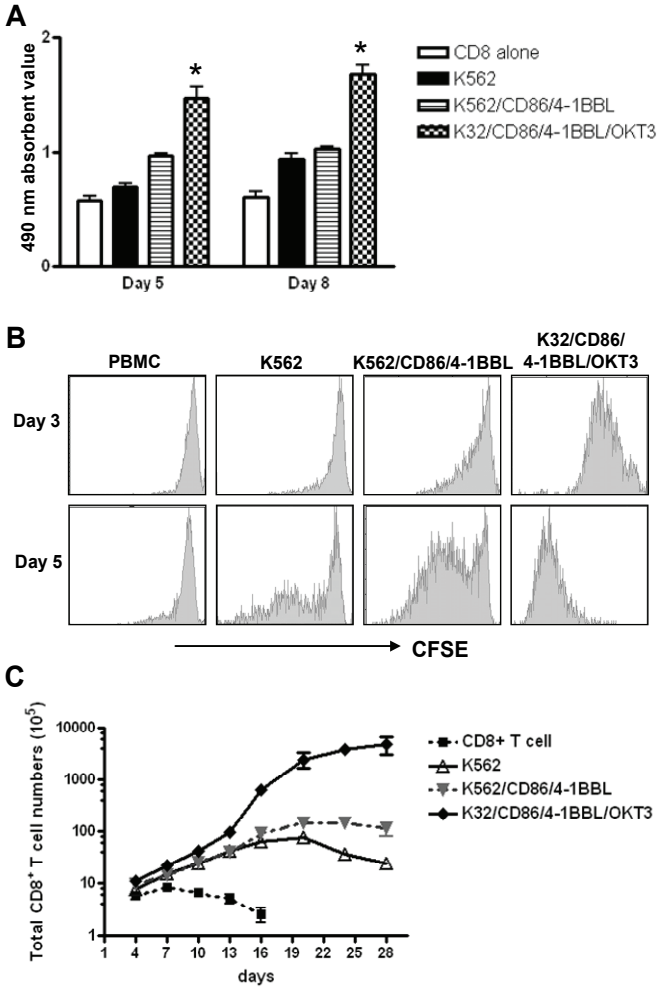

Figure 3. K32/CD86/4-1BBL/OKT3 cells promoted proliferation, division and long-term growth of CD8+ T cells. (A) Proliferation of $CD8^+$ T cells in stimulation with the indicated aAPCs were measured by MTS/PMA method. *$p < 0.05$, compared with $CD8^+$ T cells alone, in stimulation with K562, or K562/CD86/4-1BBL. Results were shown as mean ± SD from triplicate cultures. (B) The devision of $CD8^+$ T lymphocytes in stimulation with the indicated aAPCs at days 3 and 5 was detected by CFSE staining. (C) Growth curve of $CD8^+$ T cells in culture with no stimulating cells, irradiated K562 cells, irradiated K562/CD86/4-1BBL cells and irradiated K32/CD86/4-1BBL/OKT3 cells. The irradiated cells were added to the culture three times at days 1, 8, 15, respectively.

4-1BBL cells. With the co-selection of hygromycin and neomycin, CD32 was expressed on K562/CD86/4-1BBL cells, termed K32/CD86/4-1BBL cells. The co-expression of CD32, CD86 and 4-1BBL on K562 cells was verified by flow cytometery with antibodies of FITC-CD32, PE-Cy5-CD86 and PE-4-1BBL (Figure 1). It was expected that through CD32 ligation with crystallizable fragment from OKT3 monoclonal antibody against CD3, the artificial APCs may provide both the first signal (OKT3-CD3) and the co-stimulatory signal (CD86-CD28, 4-1BBL-4-1BB) to activate T lymphocytes.

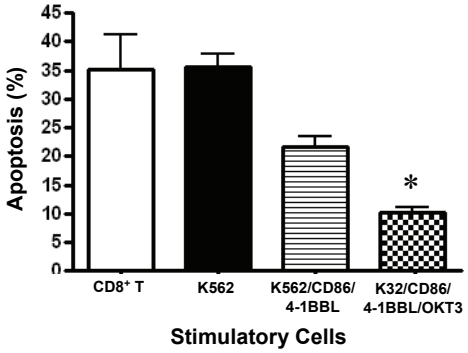

Figure 4. K32/CD86/4-1BBL/OKT3 inhibited apoptosis of CD8+ T cells. The apoptosis of $CD8^+$ T cells stimulated with the indicated aAPCs for 6 days were measured by Annexin V and propidium iodide staining. *$p < 0.05$, compared with $CD8^+$ T cells alone, in co-culture with K562, or K562/CD86/4-1BBL cells. Results were shown as mean ± SD of three replicates.

K32/CD86/4-1BBL/OKT3 cells efficiently activated polyclonal $CD8^+$ T cells

After K32/CD86/4-1BBL/OKT3 cells were obtained, we observed whether K32/CD86/4-1BBL/OKT3 cells could activate $CD8^+$ T cells. The lethally irradiated K562 cells, K562/CD86/4-1BBL cells, and K32/CD86/4-1BBL/OKT3 cells were incubated with freshly isolated PBMC for 24 h, and the expression of CD69 was tested as the marker of cell activation. K32/CD86/4-1BBL/OKT3 cells could efficiently up-regulate the expression of CD69 on $CD8^+$ T cells, while CD69 positive T cells had no variation in stimulation with K562/CD86/4-1BBL cells, K562 cells, and $CD8^+$ T cells alone (Figure 2).

K32/CD86/4-1BBL/OKT3 cells promoted proliferation, division and long-term growth of $CD8^+$ T cells

The ability of K32/CD86/4-1BBL/OKT3 to stimulate the proliferation of $CD8^+$ T cells was tested by MTS/PMA method. At days 5 and 8, the total $CD8^+$ T cell number in culture stimulated with K32/CD86/4-1BBL/OKT3 was higher than in cultures stimulated with K562, K562/CD86/4-1BBL and no stimulation ($p < 0.05$) with the same original number of 1×10^5/ml according to absorbent value at 490 nm (Figure 3A). Next we testified whether K32/CD86/4-1BBL/OKT3 cells could induce the $CD8^+$ T cell division by labeling freshly isolated $CD8^+$ T cells with CFSE and tracking cell division at days 3 and 5. The division rate of $CD8^+$ T cells stimulated with K32/CD86/4-1BBL/OKT3 was faster than those stimulated with K562, K562/CD86/4-1BBL and no stimulation. Especially at day 5, most $CD8^+$ T cells in culture with no stimulation were in the primary generation whereas most $CD8^+$ T cells in culture stimulated with K32/CD86/4-1BBL/OKT3 were offspring of about 3-6 generations (Figure 3B). Then we determined whether the K32/CD86/4-1BBL/OKT3 cells were sufficient to maintain long-term propagation of $CD8^+$ T cells. After three rounds of stimulation, they remained the exponential growth at least within 35 days.

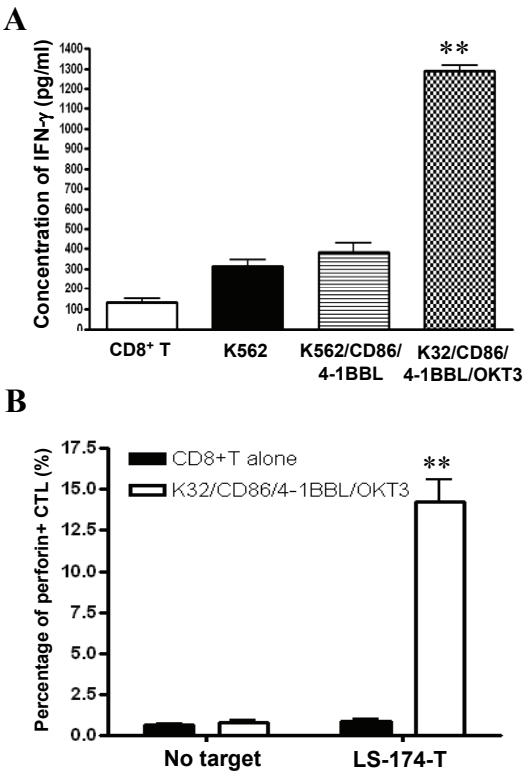

Figure 5. K32/CD86/4-1BBL/OKT3 cells could promote the biological function of CD8$^+$ T cells. (A) Concentrations of IFN-γ from culture supernatants in stimulation with indicated sAPCs were measured by ELISA. $^{**}p < 0.01$, compared with CD8$^+$ T cells alone, in co-culture with K562, or K562/CD86/4-1BBL cells. (B) Intracellular perforin expression in CTLs was analyzed by flow cytometry according to the manufacturer's instructions after CD8$^+$ T cells incubated with LS-174-T cells at 10:1 ratio for 4 hours. $^{**}p < 0.01$, compared with CTLs cultured alone. Results were shown as mean ± SD of three replicates.

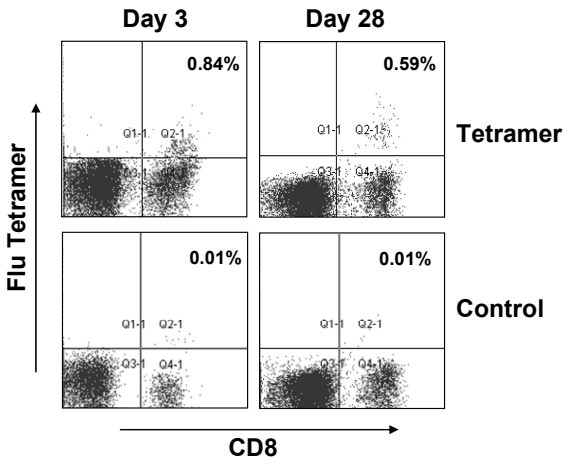

Figure 6. The maintenance of antigen-specific CD8$^+$ T cells in stimulation with K32/CD86/4-1BBL/OKT3 cells. The cells were stained with FITC-labeled anti-CD8 antibody and HLA-A*0201 tetrameric MHC loaded with influenza matrix protein peptide. Left panels, initial CD8$^+$flu$^+$ T cells at day 3. Right panels, CD8$^+$flu$^+$ T cells at day 28 after three rounds of stimulation. PE-Streptavidin was stained as control. Results were representative of three experiments with different donors.

The total CD8$^+$ T cell number reached 5×10^8 at day 28 with the initial number of 5×10^5. Although low concentration of exogenous IL-2 (20 IU/ml) was added to the medium, all the CD8$^+$ T cells which were cultured alone resulted in apoptosis within 2 weeks (Figure 3C).

K32/CD86/4-1BBL/OKT3 cells inhibited apoptosis of CD8$^+$ T cells

Although the CD8$^+$ T cell number could be arrived for clinical therapy, one would expect the activated and expanded lymphocytes *ex vivo* would not enter the state of apoptosis after infusion into the body. Next we assessed the viability of CD8$^+$ T cells stimulated by the various K562 cells and no stimulating cells after 6 days' culture by fluorescent staining with Annexin V and propidium iodide. In the T-cell culture with K32/CD86/4-1BBL/OKT3 cells, apoptotic cells were fewer than other 3 culture conditions (Figure 4). In addition, the survival of CD8$^+$ T cells stimulated with K32/CD86/4-1BBL/OKT3 was observed. With a long time of 20 days and 40 days after the third stimulation, we found about 80% cells and 70% cells were viable, respectively.

K32/CD86/4-1BBL/OKT3 cells enhanced IFN-γ and perforin secretion from CD8$^+$ T cells

In order to test the biological function alteration of polyclonal CD8$^+$ T cells in culture with K32/CD86/4-1BBL/OKT3 cells *ex vivo*, we detected the IFN-γ and perforin secretion 6 days after the second stimulation. IFN-γ from supernants of cultured CD8$^+$ T cells stimulated with K32/CD86/4-1BBL/OKT3 was higher than those from CD8$^+$ T cells which were cultured alone and cultured with K562 cells ($p < 0.01$) (Figure 5A). For MHC limitation, we selected HLA-A2 positive colon cancer cell line (LS-174-T) as target cell. The CD8$^+$ T cells in culture with stimulatory cells were sorted to be mixed with targets at the ratio of 10:1 for 4-hour incubation. Then CTLs were labeled with intracellular perforin antibodies to evaluate the cytotoxicity. In comparion with CD8$^+$ T cells cultured alone, the percentage of perforin positive CTLs stimulated with K32/CD86/4-1BBL/OKT3 cells was significantly higher ($p < 0.01$) (Figure 5B). So K32/CD86/4-1BBL/OKT3 cells not only promoted CD8$^+$ T cells to secrete IFN-γ, but also enhanced their cytotoxicity.

Antigen-specific CTLs could be retained by K32/CD86/4-1BBL/OKT3 cells

In clinical immunotherapy we likely require cells with antigen-specific cytolytic functions which were expanded efficiently *ex vivo*. Next we generated soluble HLA-A*0201/flu tetramer to test the antigen-specific CTLs at day 3 and 28. The tetramer-positive population was present at an initial frequency of 0.84%. After 3 rounds of stimulation with K32/CD86/4-1BBL/OKT3 cells, the percentage of tetramer

positive cells was 0.59% (Figure 6). During the whole procedure, no specific peptides of flu were added into the culture system. The proliferative capacity of the antigen specific $CD8^+$ T cells that remained after 28-day culture suggested that these CTLs could have long-term engraftment potential after adoptive transfer.

Discussion

We have developed a ready-to-use non-specific artificial APC based on K562 cell to efficiently stimulate the activation and expansion of purified human polyclonal $CD8^+$ T cells *ex vivo*. Furthermore, the aAPC (K32/CD86/4-1BBL/OKT3) could promote $CD8^+$ T cell long-term growth, inhibit apoptosis, enhance its activity such as secretion of IFN-γ and perforin, and retain the proliferation of antigen-specific CTLs.

Artificial antigen-presenting systems encompass both cell-based and acellular technologies. Several diverse scaf-foldding systems such as genetically engineered insect cells, mouse fibroblasts, human tumor cell lines (3, 4, 11-13), microbeads, artificial liposomes, and biomembrane derivatives (exosomes, immunosomes) (14, 15) have been developed. However, the self-limitation of insect cells at 37°C (the viable temperature for *D. melanogaster* cells is 27°C) could lead to massive release of *D. melanogaster* antigens and limit the duration of the contact between the T cell and the aAPC (16, 17). Mouse fibroblasts yielded on average 30×10^6 specific CTLs, however much higher T-cell doses may be required in the clinic (18-20). The bead-based systems also have some drawbacks, including high cost of the beads, the labor-intensive process of removing the beads from the culture before infusion, and the inability of these beads to expand $CD8^+$ T cells (21-24). The artificial liposomes and exsomes show some potential for T-cell expansion, but the high cost, long-time procedure and biosafety should be considered for clinical therapy (25, 26).

The K562 cell-based aAPCs have several advantages over other aAPCs based on beads, insect cells, or biomembrane derivatives, such as negative MHC expression, mycoplasma free, better formation of the immunological synapse as a result of the fluidity of its membrane, well growth in serum-free medium (3, 4, 11, 13). Due to the preferential binding of B7-2 to CD28 and B7-1 to CTLA-4, and both CD28 and B7-2 constitutively expressed on T cells and APCs, respectively, relatively week interaction between CD28 and B7-2 may suffice as an early co-stimulatory interaction to initiate T cell activation (6, 7). Furthermore, the study of fibroblast aAPCs showed that the low level of CD80 expression induced tolerance versus activation and high-level CD80 expression was necessary for efficient activation of T cells (18, 27). Compared with K32/4-1BBL/CD3/28 cells, CD86 on K32/4-1BBL/OKT3 cells can not only prefer to bind with CD28 receptor to transmit positive signal, avert concomitant ligation of CTLA-4, but also act as a more natural ligand in a physiological immune synapse. In addition, the substitution of anti-CD28 antibody with CD86 which was stably expressed on K32/4-1BBL cells would decrease the cost dramatically in the potential clinical adoptive immuno-therapy.

To induce $CD8^+$ T cell activation and proliferation efficiently, CD86 was an indispensable ligand besides CD3-TCR ligation. CD28 mediates a primary co-stimulatory signal that promotes IL-2 secretion, T-cell expansion, Th1 differentiation and short-term T-cell survival. However, CD28 signaling may also facilitate T-cell death under certain conditions. In addition, K32/CD3/28 cells promoted the expansion of $CD4^+$ T cells whereas failed to support long-term growth of $CD8^+$ T cells. 4-1BB stimulation preferentially activated $CD8^+$ T cells *in vitro* and amplified generation of CTL responses *in vivo* (28, 29). It was shown that the addition of 4-1BB co-stimulation not only could promote $CD8^+$ T cell proliferation, but also overcome activation-induced non-responsiveness to increase IL-2 and Bcl-xL expression and survival of $CD8^+$ T cells (3). In our study, although low concentration of IL-2 (20 IU/ml) was added to the culture medium, $CD8^+$ T cells with no stimulation of aAPCs undergone apoptosis about 2 weeks, which confirmed that IL-2 could induce T cell death after activation (30).

Antigen-specific cytotoxic T cells could be maintained by culture with K32/CD86/4-1BBL/OKT3 cells *ex vivo* about a month. Although we did not sorted out the fluMP-peptide specific T-cell populations to study the proliferation, after three rounds of stimulation with the aAPCs and no fluMP peptide or no heterodimer of HLA-A*0201/fluMP were added to the culture system, about 70% of the $CD8^+$FluMP $tetramer^+$ cells were still detected. It is worthy noting that it is usually very hard to sort out 10^5 cells with tetramer staining by FACS for the low percentage of a particular antigen specific CTLs in 10 ml fresh peripheral blood. We inferred that antigen-specific CTLs could be expanded more efficiently if they were cultured in the system of irradiated K32/CD86/4-1BBL/OKT3 cells combining with soluble HLA-A*0201/fluMP coated on culture-plate. Meanwhile, soluble HLA-A*0201/fluMP can be fused to the crystallizable fragment of immunoglobulin. The recombinant fusion protein would be produced with eukaryotic expression system such as Bac-to-Bac baculovirus expression system (Invitrogen). Protein of HLA-A*0201/fluMP-IgFc as a dimer will be coupled with CD32 on the aAPCs to transmit first signal for specific $CD8^+$ T cell activation. The aAPCs based on beads coupling with HLA-Ig and CD28-specific antibody supported expansion of both a high-affinity antiviral CMV-specific CTL and a low-affinity Mart-1-specific CTL (21). We might expect that if K32/CD86/4-1BBL/ HLA-A*0201/fluMP-IgFc cells are established, the induction of antigen-specific CTLs for expansion was at least as good as K32/CD86/4-1BBL/OKT3 cells.

For clinical adoptive immunotherapy for patients with cancer and viral diseases, we still need to observe the activity of the CTLs *in vivo* which were expanded and activated *in vitro*. Considering the wide range of diseases that would be amenable to treatment, K32/CD86/4-1BBL/OKT3 cells would be a robust, easy-to-use, comparatively economic, no MHC limitation, and reproducible approach to expansion and

activation of $CD8^+$ T cells and antigen-specific CTLs *ex vivo*. In addition, the artificial antigen-presenting cells may be useful for the *in vitro* propagation of CTLs for experiment.

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