Preferential T cell Expansion by Artificial Antigen Presenting Cells Expressing 4-1BBL Alone or in Combination with CD80 or CD86

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ABSTRACT

Background: Varieties of artificial antigen presenting cells (aAPCs) with different efficiencies have been introduced to expand whole T cell population or antigen specific ones for the purpose of T cell therapy. From antibody coated beads to gene modified dendritic cells each has some advantages and disadvantages. However, no one can ignore the importance and the necessity of costimulation interaction during T cell activation. **Objective:** This study was designed to compare the effectiveness of CD80/CD86 and 4-1BBL, two major costimulatory families, in costimulation of autologous T cell responses. **Methods:** We used recombinant non-replicative adenoviral vectors and transferred genes of these ligands to autologous blood monocytes and skin fibroblasts to create aAPCs system. T cell response to anti-CD3 pan stimulation and some viral peptide Ags, in co-culture with gene modified monocytes and fibroblasts were studied using CFSE and HLA tetramers, respectively. **Results:** Over-expression of ligands was able to expand the T cell population significantly higher than normal cells with no interference with antigen stimulation. Presence of 4-1BBL alone or in combination with B7 members enhanced T cell expansion and promoted more Ag-specific cells to accumulate in these culture systems. **Conclusion:** Considering the inhibitory proportion of B7 costimulation route, 4-1BBL, as an alternative signaling pathway, in combination with B7 will promote T cell proliferation and expansion.

Keywords: CD80, CD86, 4-1BBL, Antigen-Presenting Cells

INTRODUCTION

Cancer adoptive cell therapy (ACT) uses patients' own cells to expand, activate and re-infuse back to the patients in order to fight tumors (1). Proper ex-vivo conditions should be provided to select and expand the right Ag reactive cells with optimal phenotypic and functional

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characteristics and permit their activation away from host or tumor regulatory constraints. Several laboratory methods have been used to activate and expand human T cells to the numbers required for ACT trials. Results of beads coated with anti-CD3 and anti-CD28 antibodies have shown promising potentials to expand bulk of T lymphocytes (2). However, due to the nature of stimulation, these cells lack any tumor antigen specificity and no objective tumor responses are seen. Moreover, using bead-based system is incapable of expanding CD8 T cells and a labor-intensive process is needed to remove the beads from the cultures before infusion (3). Alternative methods have utilized cell based systems such as autologous dendritic cells, $CD40^+$ B cells and EBV-LCLs. These cells usually are pulsed with synthetic peptides or peptides derived from tumors and challenged to activate and expand T cells in an antigen-specific manner (4-6). Successful expansion of human lymphocytes using these systems instead of natural APCs has been shown frequently (7-10). In spite of their potentials to present specific peptides to T cells in conjunction with costimulatory molecules, they have limitations due to the technical difficulties in generating large numbers of cells, the lot-to-lot variation and high cost. Therefore, artificial antigen presenting cells (aAPCs) based on cellular carriers, which are more available and uniform could be more advantageous. This system allows testing the ability of natural ligands per se rather than antibodies to stimulate T cells. In addition to antigenic peptides, aAPCs may express suitable costimulatory molecules to confer T cells' need for proper activation. Among costimulatory molecules, CD80/86 of B7 family are two well known costimulants which supply the first costimulatory contact during antigen stimulation. However, multiple mechanisms may prevent their action afterward (11,12). On the other hand, there are other costimulatory interactions which may sustain T cell activation process and continue their proliferation and expansion. The costimulatory function of 4-1BBL (CD137L) of TNF super-family which can inhibit activation induced cell death (AICD) and prolong cell proliferation have been shown in different systems (13,14). In several attempts, B7 members and 4-1BBL costimulatory pathways have been recruited successfully together to expand T cells in singular or in combination after expression on different cell lines which show some advantages of one to the other and consider cooperation or even synergistic relations between these two pathways (15-18). More evaluations are needed for a consensus regarding to the interactions of these two systems and possible practical use of them. Here, we employed recombinant non-replicative adenovirus vectors to transfer natural ligands for CD28 and CD137 into blood adherent mononuclear cells, precursors to dendritic cells (DCs), to over-express CD80 and 4-1BBL and compared their efficacy for anti-CD3 stimulation in an autologous culture system. Furthermore, in order to study the role of these two major costimulatory pathways for Ag-specific approach, cultured fibroblasts were forced to express costimulatory ligands after infection with corresponding viruses and loaded with synthetic viral peptide antigens and challenged to activate and expand cognate T cells. Using Ag-MHC-I tetramers, we showed different patterns of enhanced proliferation and accumulation of Ag-specific T cells. Such results emphasize on the importance and the possible prospective of aAPC for ex-vivo antigen specific T cell expansion using costimulatory ligands.

MATERIALS AND METHODS

Recombinant Non-Replicating Adenoviruses Expressing Co-Stimulatory Ligands. Recombinant E1- and E3-deleted Ad-5 adenoviruses expressing either human CD80, CD86

(Ad-CD80/CD86) or 4-1BBL (Ad-4-1BBL) or enhanced green fluorescent protein (Ad-GFP, used as a control) were described previously (19, 20). Briefly, coding regions of each gene was cloned through reverse transcription polymerase chain reaction (RT-PCR) on total RNA from cultured LCL cell line or mature cultured DC. Selected genes were initially cloned into the plasmid pxLNCX (21) and the correct sequences confirmed by sequencing, before insertion into adenovirus vector contained large E1 deletion. Recombinant viruses were rescued in HEK293 cells, and purified by CsCl density gradient ultracentrifugation and plaque purification. The concentration of virus particles was determined by DNA assay using the fluorescent dye PicoGreen (Molecular Probes, Invitrogen, Paiseley, UK).

Costimulatory Ligand Over-Expression. Low passage primary cultured fibroblasts were prepared from frozen stocks or established in culture by using skin biopsy from healthy laboratory volunteers with proper informed consent. The growing fibroblasts were expanded in culture and used to express one or two of CD80 or CD86 with or without 4-1BBL after infection with corresponding viruses. Ad-GFP infected cells also were used as control in all experiments. Fibroblasts were infected in suspension with recombinant viruses at multiplicity of infection (MOI) of 3000 virus particles (vp)/cell singly or in combination (of either Ad-CD80, Ad-CD86 with Ad-4-1BBL) or 6000vp/c of control virus, 48-72 hr in advance. During the infection time, 300U/ml rh-IFN-γ was added to the media in order to up-regulate MHC expression. The MHC up-regulation could help efficient loading of the peptide on the fibroblasts for Ag presentation to the T cells.

Similarly, 3000 and 6000 MOI of recombinant viruses were used to infect normal blood monocytes to express excess of costimulatory molecules without undergoing the DC maturation. All infected cells were checked for the costimulatory ligand expression by flow cytometry using commercial antibodies anti-CD80-FITC, anti-CD86-PE and anti-CD137L-PE from BD, Pharmingen (Oxford, UK). Appropriate isotype controls were used to indicate levels of background staining.

Viral Peptide Antigen. Peptide antigens (10ng/ml stock in DMSO) (Alta bioscience Birmingham University, UK) were chosen from BZLF1 protein of EBV (^{190}RAKFKQLL^{197}) (22), IE1 protein of CMV (^{199}ELKRKMIYM^{207}) (18) and nucleoprotein of influenza (^{380}ELRSRYWAI^{388}) (23) viruses, which all were HLA-B8 specific. As an irrelevant peptide control, if possible, HLA matched antigen of H-Y (UTY), (^{566}LPHNHTDL^{573}) (24) and/or anti-CD3 stimulation was used instead of peptide stimulation in replicates of dual costimulation.

Lymphocyte Proliferation against Artificial Antigen Presenting Cells. The ability of the infected cells to costimulate T lymphocytes in response to sub-optimal soluble anti-CD3 antibody stimulation at 100ng/ml or viral peptide Ag stimulation was compared with uninfected cells. Co-cultures of vector infected, peptide Ag loaded fibroblasts with peripheral blood mononuclear cells (PBMCs) of HLA-B8 donors were re-stimulated at weekly intervals by transferring the growing PBMCs to the fresh infected, and peptide loaded fibroblasts. Proliferation of Ag specific T cells was evaluated at different time points using peptide-MHC class I tetramers and flow cytometry.

In case of monocytes, freshly purified cells were plated into 24-well plates and used 48 hrs post-infection with adenoviral vectors for co-culture with autologous CFSE labeled lymphocytes stimulated with soluble anti-CD3. Lymphocyte proliferation was evaluated at different time points during 14 days culture by flow cytometry.

Tetramer Staining. The expansion of antigen specific memory T cells against viral antigenic peptides was checked at different time points using PE-labeled HLA-B8 specific tetramer reagent for each peptide, kindly provided by Dr. A. Hislop and Dr. N. Khan (Birminghamm

University, UK). First, 5×10^5 cultured or fresh PBMCs were incubated with 0.1-0.5μg of tetramer reagent at 37°C for 15 min. After washing with cold PBS/FCS (2%) buffer, cells were re-suspended and incubated with CD8 mAb (Beckman Coulter, High Wycombe, UK) for 30 min at 4°C. The stained cells were washed and analyzed by flow cytometry.

CFSE Lymphocyte Labeling. After washing PBMCs in PBS, cells were re-suspended at 2×10^7cells/ml in PBS. The stock of 10mM CFSE (Molecular Probes) in DMSO was diluted in PBS to achieve 5 μM concentrations. Diluted CFSE was added to the cells in a ratio of 1:1 (vol:vol) and vortexed gently. The cell suspension then incubated for 15 minutes at 37°C, agitating periodically. An equal volume of complete RPMI culture medium was added to the cells and left for one minute at room temperature to stop the labeling process. The cells were washed 3 times and re-suspended in culture media to put into culture with gene modified monocytes while stimulating the culture with 100ng/ml anti-CD3.

RESULTS

Over-Expression of Costimulatory Ligands after Adenovirus Gene Transfer. Blood monocyte cells were partially purified by plastic adherence depletion (up to 80% $CD14^+$, data not shown) and infected with recombinant Ad-GFP_{6000}, Ad-$CD80_{3000}$, Ad-$CD86_{3000}$ and Ad-4-$1BBL_{3000}$ viruses to express GFP as control, CD80, CD86 and 4-1BBL, respectively. Figure 1a-c show typical profiles of ligand expression by infected monocyte cells compared to the original non infected cells. More than 10% of the non-infected adherent blood mononuclear cells expressed CD80 after two days culture. Infection with Ad-CD80 raised that up to 70% after two days incubation. On the other hand, there was a significant level of CD86 expression in non-infected monocytes (60%), which was up regulated both in percentage and mean fluorescent intensity (MFI) after the infection (95% and 2000, respectively). Cultured monocytes did not express significant level of 4-1BBL, however infection of these cells with Ad-4-1BBL resulted after two days in expression of this ligand on almost all (95%) cultured monocytes. Similarly Figure 1d-f show ligand expression by infected fibroblasts in relation to un-infected cells. As shown, non-infected cultured fibroblasts express neither CD80 and CD86 nor 4-1BBL normally. Infection of fibroblasts with Ad-$CD80_{3000}$, Ad-$CD86_{3000}$ and Ad-4-$1BBL_{3000}$ results in expression of the ligands in 96%, 90% and 68% of the fibroblasts infected with the corresponding viruses, respectively. Inclusion of IFN-γ in the culture of fibroblasts resulted in up-regulation of HLA-I and -II in these cells which made them more equipped to load peptide antigens (data not shown).

Enhancement of Sub-Optimal Anti-CD3 Stimulation. In order to study the role of the costimulatory molecules individually or in combination in autologous experimental conditions, blood adherent mononuclear cells were used to over-express different costimulatory molecules after infection with corresponding viruses. Lymphocytes derived from the same donor, labeled with CFSE and co-cultured with gene modified monocytes in presence of anti-CD3 and cultures evaluated during 14 days. Results show (Figure 2), that some lymphocytes underwent multiple cell divisions in all culture conditions even with GFP infected monocytes by day 4 of culture. However, by single CD80 or CD86 costimulation, up to 5 cell divisions with significantly increased number of replicated cell was observed. At this early time point, 4-1BBL costimulated cultures also showed comparable number of cell divisions; nevertheless, the overall cell proliferation was lower than with CD80 or CD86 alone and dual costimulation.

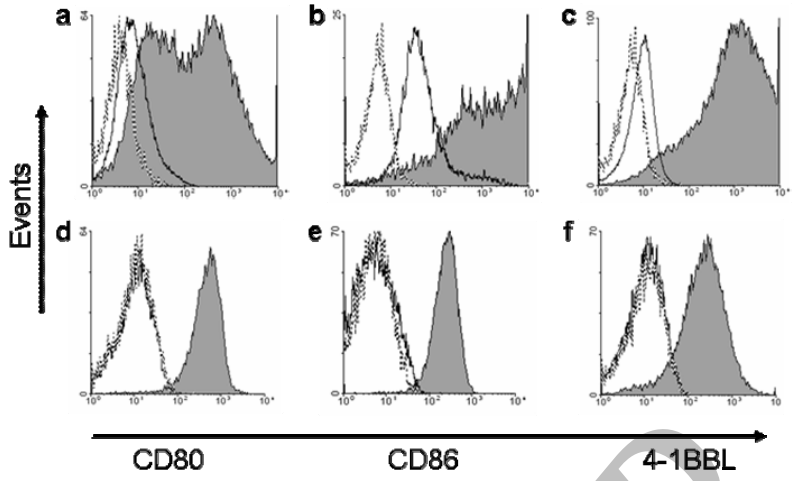

Figure 1. Typical surface expression of different costimulatory molecules in cultured blood monocytes and tissue fibroblasts before and after infection with different adenovirus vectors. Normal cultured human monocytes (top row) and tissue fibroblast cells (bottom row) were infected with Ad-$CD80_{3000}$, Ad-$CD86_{3000}$ and Ad-4-$1BBL_{3000}$ viruses in suspension, 48-72 hr in advance. Infected cells harvested and analyzed for expression of CD80 (a and d), CD86 (b and e) and 4-1BBL (c and f) by flow cytometry (shaded areas). Dotted and solid lines show isotype staining antibody and expression of corresponding markers by non-infected cells, respectively.

After 8 days CD80 and CD86 costimulated cultures showed up to 7-8 cell divisions. At this time significantly greater increase in lymphocyte proliferation was observed in the cultures with 4-1BBL costimulation and its combination with CD80 or CD86 resulted in the greatest increase in lymphocyte proliferation. At day 15, the overall growth of GFP control culture was less than the others and there was perhaps a very modest further growth in CD80 costimulated cultures. However, cultures with monocytes expressing 4-1BBL showed more persistent proliferation of the lymphocyte population. Dual costimulation by infected monocytes had still greater effect, giving the largest number of divided cells, and a very small residual in un-divided peak. Thus, although, the monocytes alone had significant costimulatory activity, shown most clearly for the GFP control, this could be greatly enhanced by infection with Ad-CD80 and Ad-4-1BBL singly and in combination.

Expansion of Antigen-Specific CD8 T Cells. Checking the peripheral blood of HLA-B8 donors showed that 7 or 2.3% of their CD8 lymphocytes were either ELK or RAK specific before culturing the cells. No apparent influenza ELR reactive CD8 T cell in the peripheral blood of that particular donor was seen at time zero (Figure 3a). In case of ELK and RAK, examining the cultures at day 3 showed a significant decrease in those frequencies in most test culture conditions during that time (data not shown) which could be due to TCR down regulation post Ag stimulation. Lymphocyte stimulation with different peptides presented by normal fibroblasts resulted in a brief and transient accumulation of recognizing cells in some cultures, however those cells did not survive long enough and a significant cellular loss happened during the time (Figure 3b, c and d). Frequency of antigen specific cells in the GFP control cultures had only a transient increase in some cultures (up to 25% of CD8 cells at day 18 by ELK peptide stimulation). Nevertheless, it diminished and cultures did not expand or survive. As depicted in Figure 3, re-stimulation of lymphocytes with H-Y peptide antigen in conjunction

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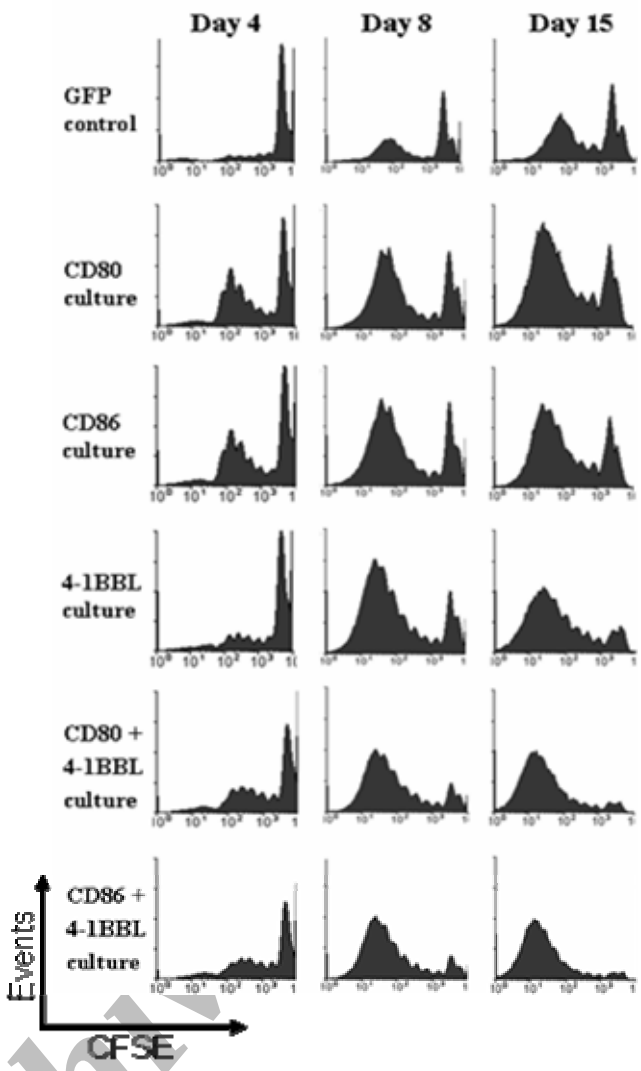

Figure 2. Typical analysis of lymphocyte proliferation by CFSE labeling and flow cytometry in cultures costimulated with vector infected monocytes.
Blood adherent monocytes were purified from healthy donors and infected as described in Figure 1 with Ad-CD80, Ad-CD86, Ad-4-1BBL and the combinations of Ad-CD80 or Ad-CD86 with Ad-4-1BBL. Ad-GFP_{6000} infection was also considered as control. Autologous non-adherent PBMCs were kept in complete media until the infected cells were ready. Cells were labelled with CFSE and co-cultured with infected monocytes. Lymphocyte cell division was analysed at day 4, 8 and 15 of culture by flow cytometry. Peaks show successive cellular division of labeled lymphocytes by halving of cellular fluorescence.

with double costimulation (Figure 3b and c) was not effective either and Ag-specific CD8 cells did not show a significant expansion at any time point. Likewise, presence of both costimulatory molecules but in the absence of peptide or anti-CD3 stimulation was not very efficient to increase the Ag reactive populations and after multiple re-stimulations only minority of cells were recognized with peptide-tetramers in those cultures. After re-stimulation of cultures with ELK peptide by passaging the cells into the corresponding culture conditions at days 7, 15 and 21, presence of Ag-specific CD8 lymphocytes were analyzed at days 10, 18 and 30 of the culture. By CD80 costimulation, a significant increase in the frequency of ELK specific cells was observed during that period of time. In fact, up to 50%, 81% and 55% of CD8 cells in CD80 costimulated culture revealed to be ELK specific

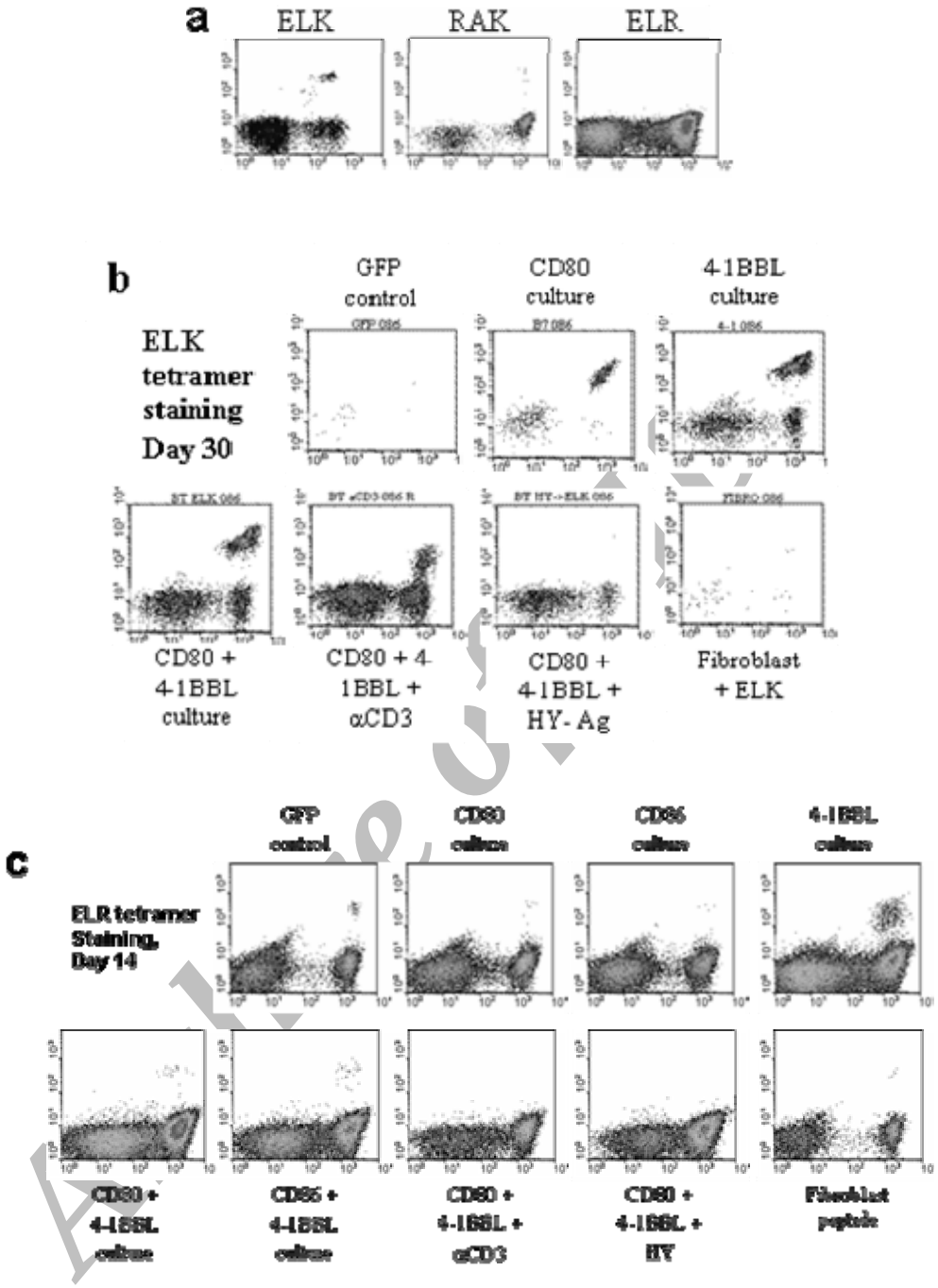

Figure 3. Typical examples of peptide specific $CD8^+$ T cell populations in cultures with different costimulation.
Cultured fibroblasts of male HLA-B8 donors were infected and prepared as explained in Figure 1. Infected fibroblasts were loaded with different peptides during few hr by incubation of infected fibroblasts in serum free medium containing 10µg/ml peptide. Autologous non-adherent PBMCs were added to the cultures at $1\text{-}1.5X10^6$ cells and the cultures were maintained by an almost weekly passage of the lymphocytes to the corresponding fresh infected fibroblasts loaded with antigen. Presence of ELK, RAK and ELR peptide Ag specific CD8 T cells were monitored using peptide-HLA tetramer with CD8 counter-staining at zero time (a). Panels b and c show results of day 17 for ELK and day 14 for ELR, respectively. Anti-CD3 at 100ng/ml was used as non-specific T cell stimulator in conjunction with double infected fibroblasts.

at days 10, 18 and 30, respectively. However, T cells did not show a considerable proliferation in those conditions and overall cell count reduced gradually. 4-1BBL costimulated cultures also showed increase in frequency of ELK specific cells and 30%, 50% and 37% of CD8 cells at the mentioned days were ELK specific, respectively. Providing double costimulation by CD80 and 4-1BBL resulted in accumulation of 14%, 43% and 31% ELK specific cells in the culture with massive proliferation (Figure 3b).

In the same way re-stimulation/costimulation of the cultures with RAK peptide at day 7 resulted in significant expansion of the peptide specific CD8 lymphocytes in dual or single costimulated cultures at day 10. At this time point, single CD80 and 4-1BBL costimulated cultures had 12% and 6.3% CD8 lymphocytes stained with tetramers, respectively. In cultures with dual costimulation, more than 13% of the CD8 cells were recognizing RAK tetramer. Following another round of passage on day 14, cultures were re-analyzed on day 17 of the culture. At this time point, significant proportions of CD8 lymphocytes in the cultures with CD80, 4-1BBL or dual costimulation were showing reactivity with RAK tetramers (30%, 21% and 25%, respectively, data not shown).

To investigate costimulatory effect of different ligands in costimulation of an old memory immune response, ELR influenza peptide antigen was selected and activation of recognizing T cells were studied in an elderly HLA-B8 male donor with no history of Flu vaccination. Figure 3c illustrates examples of flow cytometry density plots of ELR tetramer staining in different culture conditions at day 14. Even in the absence of evident ELR specific CD8 cells at the beginning, providing single or dual costimulation with peptide sensitization, resulted in relative proliferation of lymphocytes and appearance of reactive cells after seven days. At this time point, in CD80 and CD86 costimulated cultures, only 0.6% and 0.4% of the growing CD8 cells were ELR specific, respectively. On the other hand, up to 1.5% of the CD8 lymphocytes in 4-1BBL costimulated cultures were reacting with ELR tetramer. ELR Ag presentation to the lymphocytes in conjunction with both costimulatory ligands resulted in considerable lymphocyte proliferation however, the frequency of Ag specific cells in these cultures was around 0.5% of CD8 lymphocytes. By day 14, after a round of passage to the fresh corresponding costimulation condition, frequency of ELR specific lymphocytes in CD80 and CD86 costimulated cultures or in the cultures with dual costimulation did not increase substantially and there was only 1% Ag specific lymphocyte in these cultures. On the other hand, compared to day 7, frequency of ELR specific CD8 cells in 4-1BBL costimulated cultures, with up to 4-fold increase, reached to 5.7%.

Cell Enumeration of Responding Ag-Specific Lymphocytes. In spite of the high frequency of ELK and RAK peptide specific CD8 cells in the cultures with CD80 costimulation, the actual increase in the cell number of Ag recognizing cells in these cultures were lower than 4-1BBL and dual costimulated cultures. As an example, Figure 4 plots the typical distribution of the actual number of RAK specific and non-specific CD8 cells in the cultures with different costimulations at day 17 post-culture. There were significantly lower total and antigen specific cell number in the cultures with CD80 costimulation. On the other hand, cultures with 4-1BBL and dual costimulation had an accumulation of antigen specific lymphocytes in the culture 2 to 5.7 times higher than single CD80 costimulation, respectively. Dual costimulation in conjunction with anti-CD3 activation also resulted in massive proliferation in the lymphocytes; however the percentage and therefore the actual number of antigen specific cells in those cultures were low as well. Similar results were found in Ag-specific CD8 cell count for ELK stimulated cultures.

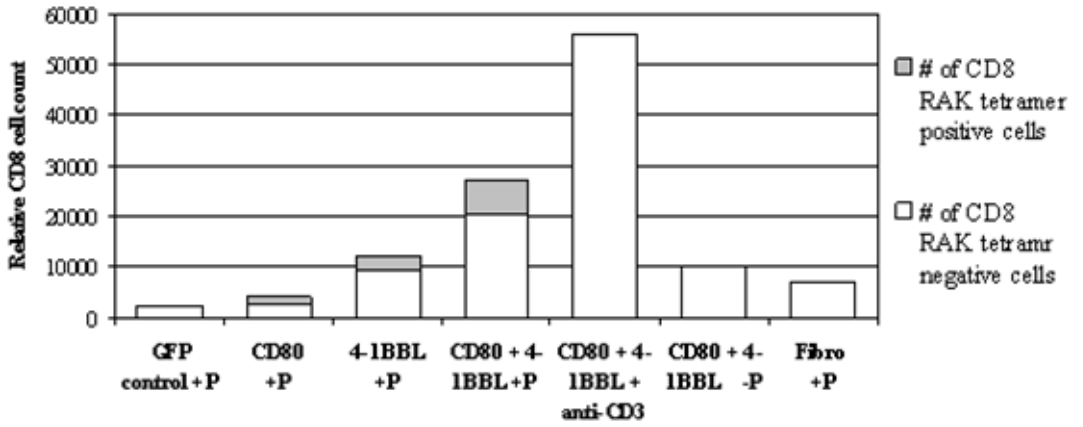

Figure 4. Relative cell count of RAK specific and non-specific CD8 cells in cultures with different costimulation treatments on days 17 postculture.
Viable cell counts of different cultures were determined and relative cell number of RAK specific CD8 cells calculated according to the CD8 ratio in every culture condition.
+/-: with or without peptide.

Due to the higher frequency of the ELR specific cells in 4-1BBL costimulated cultures, greater numbers of CD8 lymphocytes with ELR specificity were generated in 4-1BBL costimulated cultures. 4-1BBL costimulation results in 3 and 14 times more Ag specific CD8 T lymphocyte expansion than dual and CD80 costimulated cultures, respectively. This shows the direct effect of 4-1BBL costimulation for costimulation of ELR specific lymphocyte to proliferate. In dual costimulation, the whole lymphocyte population expands, however increase in ELR specific CD8 lymphocytes is not prominent in this culture in spite of ELR Ag stimulation (data not shown).

DISCUSSION

The ex-vivo priming and expansion of human CTLs need proper Ag stimulation and costimulation. Among the varieties of aAPCs that have been introduced for this job so far, acellular beads, expressing anti-CD3/anti-CD28 efficiently, expand $CD4^+$, but not $CD8^+$ T cells. On the other hand, cell-based aAPCs can effectively expand cytolytic $CD8^+$ cells. However, optimal costimulatory signals and more technical, immunologic, practical and regulatory aspects of manufacturing these T cell products should be considered to guarantee their safety, survival and function (25). In this study we investigated and compared the effectiveness of CD80/86-CD28 interaction, as the first line costimulatory pathway, with a molecular pair of TNF/TNFR superfamily, 4-1BB and 4-1BBL. First, over-expression of costimulatory ligands on the surface of autologous blood monocytes in co-culture of T cells was studied while T cells were stimulated by sub-optimal soluble anti-CD3. Adherent blood monocytes are one of the possible sources to make dendritic cells, the best known APC. However, monocytes themselves are inadequate to fully help T cells during the activation process. Nevertheless, occurrence of some T cell divisions of control Ad-GFP infected cultures in CFSE profiles of lymphocyte proliferation (Figure 2) was not unexpected, considering the significant background level of endogenously expressed costimulatory molecules in monocytes (Figure 1). Much higher cell division was found in cultures with over-expression of costimulatory ligands. In fact, pattern of anti-CD3 lymphocyte activation with delayed

response to 4-1BBL and higher response with dual costimulation, was found with infected monocytes. In a comparable study, Watt and her colleagues have used similar Ad-4-1BBL to convert autologous cultured monocytes into efficient APC in Ag-specific set up (26). They found the Ad-4-1BBL modified monocytes to be highly effective for expanding and activating T cell memory response to some tested viral antigens. In that work Ad-4-1BBL modified monocytes showed faster kinetics than B7.1 costimulation to expand influenza specific CD8 cells. However, in the first part of our work, suboptimal anti-CD3, rather than specific Ag, was used and Ad-4-1BBL consistently (at least in three separate experiments) had a slight delay compared to CD80 or CD86 costimulation. Unlike 4-1BBL costimulation, the efficiency of CD80 or CD86 costimulation did not last long and suppressed at the later time points. At the end of experiments (day 15), cultures with single 4-1BBL costimulation or cultures with dual costimulation had smaller amounts of undivided cells compared to cultures with CD80 or CD86 costimulation. This finding is well in accordance with the fact that 4-1BB/4-1BBL interactions could control the number of surviving $CD8^+$ effector memory cells, late in the primary response (27). Results of other investigators also show that combination of anti-CD3/4-1BBL complex preferentially expand memory cells, resulting in superior enrichment of Ag-reactive T cells and recognition of previously primed CD8 T cells (28).

In order to reduce the effect of background expression of costimulatory molecules in experiments with monocytes, and to study the sole costimulatory effect of CD80, CD86 and 4-1BBL in an autologous Ag-specific system, singly or in combination, low passage primary cultured fibroblasts were adapted as aAPC to express viral Ags. Fibroblasts have been used frequently to express different transgenes with least amount of interference (29). Known viral peptide antigens of CMV, EBV and influenza viruses, specific for HLA-B8, were selected to be presented by these gene-modified fibroblasts in co-culture with lymphocytes. Normal cultured fibroblasts do not express major costimulatory ligands for T cell activation (Figure 1) but do express HLA-I. Therefore, it is possible to load peptide fragments on to these cells, especially after IFN-γ treatment, to form a surrogate type antigen presenting cell with defined interacting ligands after gene transfer. In our study non-infected fibroblsts were able to express peptide fragments through surface HLA. However, in all tested subjects, their activity was significantly lower than cultures with costimulations. Expression of costimulatory molecules on the surface of fibroblasts made these cells able to help a major fraction of T cells to expand in response to anti-CD3 stimulation (Figure 3). Moreover, peptide antigen presentation by GFP-vector infected fibroblasts to T cells did not result in significant proliferation of specific T cells and T cell proliferation was not much different compared to peptide loaded, non-infected fibroblasts. This clearly proves the role of costimulatory ligand transgene expression by fibroblasts. Loading dually infected fibroblasts with HLA-matched peptide fragment of HY antigen did not induce any significant activation in CD8 T cells of tested male donors, proving lack of interference of adenovirus vector specific response in that Ag presentation.

Reaction of ELK-specific T cells from tested donors was known to be TCR vβ14 restricted (unpublished data). Analyzing the ELK-specific cells showed that this restriction does not change in different conditions of costimulation and majority of tetramer positive T cells express the same β chain (data not shown). It means antigen specificity remained preserved during expansion in response to Ag and different costimulation in our experimental condition.

It has been proposed recently that IL-15 transpresentation and anti-CD3 stimulation by aAPC is well capable to proliferate and enhance effector molecule expression by $CD8^+$

T cells in the absence of other costimulations (30). Besides, literature shows that IL-15 efficiently cause 4-1BB expression by T cells (31,32). Therefore, greater proliferation and longer survival of CD8 T cells in response to IL-15 could be attributed to 4-1BB signal transduction. Comparing the relative cell numbers of peptide specific CD8 cells in our results showed that the net product of peptide specific cells and their survival is much higher in cultures with 4-1BBL costimulation alone (in case of ELR) or in combination with CD80 or CD86 costimulation (in cases of ELK and RAK). As mentioned earlier, results of Buckz et al. also showed faster kinetics of influenza Ag specific T cell proliferation by 4-1BBL than CD80 costimulation. Lower T cell proliferation by CD80/86 could be due to the activation of inhibitory mechanisms against B7 costimulation or loss of CD28 expression in effector memory cells (34).

Emergence of $CD28^-$ cells during immune response or accumulation of these cells during life (35), reinforces the importance of costimulatory pathways rather than B7/CD28 (36). Therefore 4-1BBL could be one of the potential costimulatory molecules for activation of $CD28^-$ T cells. Almost only $CD28^+$ T cells were reactive to ELR tetramer (Data not shown); even though the overall expansion of ELR specific CD8 cells in CD80 and CD86 costimulated cultures were significantly lower than in the culture with 4-1BBL costimulation. In experiments using RAK and ELK, the percentages of peptide specific cells increased by CD80 or CD86 costimulation with time and a significant proportion of cultured CD8 cells were peptide specific. However, in these cultures the actual peptide-specific CD8 cells were fewer than 4-1BBL costimulated cultures. Combination of these two costimulation with 4-1BBL could expand peptide specific cells and there was a cooperation of the two costimulations for expansion and survival of specific cells.

In overall, results of this study showed that CD80, CD86 and 4-1BBL can function efficiently to costimulate the expansion of Ag-specific memory T cells in an ex-vivo setup. More important, single 4-1BBL expression by fibroblasts was able to make a CD80 independent costimulation for Ag specific cells and that costimulation maintained the reactive population longer than CD80 or CD86 costimulation.

Therefore, 4-1BBL and possibly more alternative costimulatory ligands, rather than those for CD28 pathway, are available to be tested for different purposes of ex-vivo T cell expansion.

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