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Efficient amplification of melanoma-specific CD8$^+$ T cells using artificial antigen presenting complex

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Abbreviations: aAPCs, artificial antigen presenting complex; ACT, adoptive cell transfer; rAd, recombinant adenovirus; TRP-2, tyrosinase-related protein-2

Abstract

***In vitro* large amplification of tumor-specific cytotoxic T lymphocytes (CTLs) and adoptive transfer of these cells is one of the most promising approaches to treat malignant diseases in which an effective immune response is not achieved by active immunization. However, generating sufficient numbers of tumor-specific CTLs stimulated with autologous antigen presenting cells (APCs) *in vitro* is one of the most problematic steps in the adoptive cell transfer (ACT) therapy. To circumvent this problem, we have developed an artificial antigen presenting complex (aAPCs) using MHC class I molecules loaded with a melanoma-specific TRP-2 peptide epitope. Our results show that TRP-2-specific CD8$^+$ T cells elicited by immunization with recombinant adenovirus expressing the mini-gene epitope are efficiently stimulated and amplified *in vitro* to a greater extent by aAPCs than by natural splenic APCs. These aAPC-induced CTLs recognized endogenously processed antigens present on B16F10 melanoma cells. Efficient stimulation and proliferation of antigen-specific T cells was also confirmed using ovalbumin peptide-loaded aAPCs and OT-I TCR transgenic cells. These results demonstrate that prior *in vivo* immunization, which increases the precursor frequency, simplifies posterior expansion of tumor-specific CD8$^+$ T cells, and aAPCs is superior to autologous APC for *in vitro* amplification. This "prime and expand" regimen can be an alternative method for large amplification of rare tumor-specific CTLs and aAPCs should be a useful tool for ACT immunotherapy.**

Keywords: antigen-presenting cells; antigen presentation; epitopes, T-lymphocyte; melanoma; T-lymphocytes, cytotoxic

Introduction

The clinical goal of cancer immunotherapy is to provide either active or passive immunity against malignancy. However, many tumors have developed strategies to prevent generation of effective immune responses *in vivo* and/or escape a host's immune surveillance. Advances in cellular immunology and molecular biology have elucidated mechanisms of immune regulation and effector functions against tumors as well as many target antigens recognized by tumor-specific cytotoxic T lymphocytes (CTLs). The ultimate effector cells that mediate the destruction of tumor cells in animal models are the cytotoxic T cells. Most tumor cells express tumor-specific and/or tumor-associated antigens that can be loaded onto MHC molecules. T cell lines can be generated that specifically recognize these MHC-restricted tumor antigens. However, the existence of tumor antigen-specific CD8 T cells in patients and experimental animals is not sufficient for the rejection of established tumors (Wick *et al.*, 1997; Prevost-Blondel *et al.*, 1998; Rosenberg *et al.*, 1998). The failure of antigen-specific CD8 T cells to eliminate antigen-expressing tumor cells *in vivo* can be explained by many possible mechanisms. For example, tumor-specific CTLs themselves might be functionally deficient, anergic, or not fully differentiated in the immunosuppressive tumor environment (Ochsenbein *et al.*, 2001). Alternatively, regulatory CD4$^+$-CD25$^+$ T cells and/or suppressive cytokines can impede development of effective immune reactions and/or effector functions against cancer cells (Shimizu *et al.*, 1999). This feature of tumor evasion of a host immune system has engendered many strategies for immunotherapeutic methods against cancers.

Adoptive cell transfer (ACT) immunotherapy with antigen-specific CTLs is a promising approach for the treatment of various malignant tumors and hematologic malignancies (Dudley and Rosenberg, 2003), since ACT therapy provides advantages and opportunities that are not available with other im-

munotherapeutic approaches. First, highly-avid, highly tumor-reactive CTLs can be selected and rapidly expanded *ex vivo*, circumventing suppressive immunoregulatory mechanisms induced by the tumor environment. Secondly, other therapeutic regimens, such as immunodepleting chemotherapy and/or vaccination with tumor antigens, can be combined with ACT therapy without compromising the activity of anti-tumor lymphocytes. The combined strategy of immunodepleting chemotherapy and ACT has led to complete or partial responses in melanoma and lymphoma patients (Rooney *et al.*, 1998; Dudley *et al.*, 2002).

Amplification of antigen-specific CTLs involves the use of patient-derived, autologous antigen presenting cells (APCs), usually monocyte-derived dendritic cells (DCs) that have been loaded with tumor antigens to stimulate peripheral blood lymphocytes or tumor infiltrating lymphocytes. However, one major limitation is that a large amount of blood is required to obtain enough autologous DCs and there is variability in the quality and quantity of DCs obtained that presumably effects the quality of *ex vivo* expanded CTLs. In addition, the low frequency of precursor cells specific to self-antigens, such as shared tumor-associated antigens, makes it difficult to perform isolation and rapid expansion of desired antigen-specific CTLs (Houghton, 1994). Other approaches for the expansion of CTLs involve anti-CD3 antibodies. However, stimulation with CD3-specific antibodies is associated with a decrease in antigen specificity even when starting with highly enriched antigen-specific CTL populations (Maus *et al.*, 2002).

Soluble forms of MHC molecules loaded with specific epitope peptides are able to directly stimulate cognate CD8 T cells *in vitro* (Greten *et al.*, 1998) and can be used as artificial APCs when immobilized on solid supports (Curtsinger *et al.*, 1997; Oelke *et al.*, 2003). Thus, artificial APCs based on immobilized soluble MHC molecules have the potential capability of overcoming the limitations associated with autologous DCs and antibody-based stimulation. Here, we immobilized soluble H-2K^b molecules loaded with specific peptides onto 6 μm latex beads to produce an artificial antigen presenting complex (aAPC). Using these aAPCs, we show that TRP-2-specific CD8 T cells elicited *in vivo* by priming with a recombinant adenovirus are efficiently and reproducibly expanded to a greater extent than by spleen-derived syngeneic APCs.

Materials and Methods

Recombinant viruses

Replication-defective adenoviruses were generated *via* insertion of foreign sequences by homologous

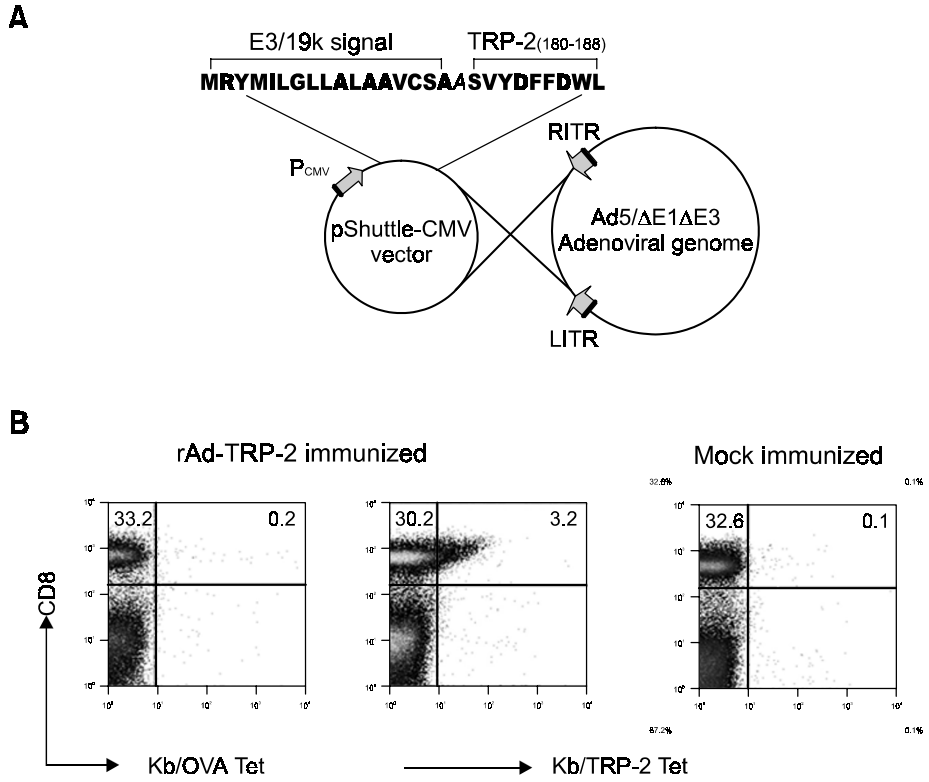

Figure 1. (A) Schematic diagram showing construction of the rAd-TRP-2 replication-defective adenovirus expressing the codon-optimized E3/19k signal-TRP-2 epitope as a mini-gene. The amino acid sequences of the E3/19k signal, including the initiating methionine and the TRP-2 epitope, are indicated in bold. An additional alanine residue resulting from insertion of a restriction endonuclease site is in shown in italics. (B) C57BL6 mice were immunized with the virus and 3 weeks later splenocytes from the immunized mice were stimulated *in vitro* with the peptide for 6 days. T cells were surface stained with CD8-PE/Cy5 and cognate Kb/TRP-2 tetramer-PE conjugate or irrelevant Kb/OVA tetramer-PE. The percentages of tetramer-positive cells and negative cells among lymphocyte-gated cells are indicated in each plot.

recombination and subsequent purification of recombinant progeny as described previously (He *et al.*, 1998). The rAd-TRP-2 recombinant adenovirus encoding the mouse TRP-2 epitope as a minigene contains the codon-optimized signal sequence of the adenovirus E3/19k protein followed by the codon-optimized TRP-$2_{180\text{-}188}$ epitope (SVYDFFDWL) sequence (Figure 1A). The rAd-E3/19k control virus contained only the E3/19k signal sequence. Correct integration of the sequences was confirmed by PCR-based viral genome analysis and direct sequencing using primers flanking the CMV promoter.

Mice and immunization

C57BL/6j and OT-I TCR transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-I TCR-transgenic mice were maintained by breeding heterozygous OT-I mice to wild-type C57BL/6j mice. All mice were housed and bred under SPF conditions. For immunization, 6-week-old female C57BL/6j mice were injected intraperitoneally with 1×10^8 pfu of replication-defective adenovirus.

Preparation of soluble MHC I monomer and artificial APC

Kb/TRP-2 and Kb/OVA monomer complexes were generated using a procedure described by D. Busch and E. Pamer (Yale University, New Haven, CT). Briefly, H-$2K^b$ heavy chain-biotinylation site fusion and human β2-microglobulin were expressed in *E. coli* and purified from inclusion bodies. Soluble H-$2K^b$ monomers were generated in the presence of high concentrations of relevant peptides. Complexes were then biotinylated using BirA ligase (Avidity, Denver, CO) and purified through Superdex-75 gel filtration and Mono-Q anion exchange chromatography.

To prepare artificial APC, 6 μm latex beads (Interfacial Dynamics, Portland, OR) were washed twice with sterile 25 mM MES buffer. The beads were first incubated with a saturated amount of Neutravidin (Pierce) for 12 h at room temperature on a rotator as recommended by the manufacturer, then washed and blocked with PBS/1% BSA, and finally incubated with an excess amount of biotinylated Kb/TRP-2 or Kb/OVA monomers (approximately 300 μg purified monomer/10^8 beads) to saturate the biotin-binding sites. The resulting artificial APCs were resuspended in 1 ml of PBS/10^8 beads and stored at 4°C until use. The artificial APCs were stored for 3 months without any noticeable loss of activity.

Stimulation of T cells

$CD8^+$ T lymphocytes were enriched from spleen cells of immunized mice by depletion of $CD8^-$ cells using a CD8 isolation kit (Miltenyi Biotec). The resulting CD8 T cells were usually >90% purity based on flow cytometric analysis. For preparation of splenic APCs, spleens from naïve mice were removed, teased gently apart, and passed through a sterile steel mesh screen. Cells were then separated by loading onto a Percoll density gradient and centrifuging at 400 g for 30 min at 25°C. Cells at the interface were collected, washed twice with the medium, and further purified by positive selection using anti- CD11c magnetic beads (Miltenyi Biotec). The typical purity of $CD11c^+$ cells was approximate 80% to 90% as determined by flow cytometry. Purified CD8 T cells were stimulated with either 1 μM peptide-pulsed $CD11c^+$ APCs or artificial APCs in the presence of anti-CD28 antibody (0.5 μg/ml; clone 37.51 NA/LE grade, BD Pharmingen) at a stimulator: responder ratio of 1:5 in 6-well plates in 5 ml of complete IMDM supplemented with a high concentration of recombinant human IL-2 (40 U/ml; R&D systems, Minneapolis, MN). For costimulation experiments (Figure 3), the same amount of anti- CD137 antibody (clone 1AH2; BD Pharmingen) was used together with or instead of anti-CD28 antibody. On day 7 and weekly thereafter, cells were collected by density gradient centrifugation, counted, and restimulated with either syngeneic APCs or artificial APCs at the same ratio. Absolute numbers of antigen-specific $CD8^+$ T cells were calculated by multiplying the percentage of tetramer-positive cells by the total number of mononuclear cells.

Flow cytometric analysis

Cells were resuspended in FACS buffer (1% FBS, 0.03% sodium azide in PBS) at 10^7 cells/ml. Approximately 5×10^5 cells were stained with antibodies against CD8, CD11a, Vα2, Vβ5, and PE-conjugated Kb/TRP-2 or Kb/OVA tetramer for 40 min on ice. Tetramers were generated as described by D. Busch and E. Pamer (Yale University, New Haven, CT), and used at optimal dilutions determined by titration. To enumerate the number of cytokine-producing cells, intracellular cytokine staining was performed. Approximately 10^6 lymphocytes were cultured in a culture tube in a volume of 1 ml with 5×10^5 EL4 cells pulsed with peptides. Cells were incubated for 5 h at 37°C in 5% CO_2. Brefeldin A (10 μg/ml; Sigma, St. Louis, MO) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. The antibodies used were anti-IFN-γ (clone XMG1.2) or its control isotype antibody (rat IgG1).

Results and Discussion

The main goal of this study was to investigate whether artificial APCs composed of immobilized MHC I

molecules on the surface can substitute for autologous APCs for the enrichment of tumor antigen-specific polyclonal CTLs. We used tyrosinase-related protein-2 (TRP-2) antigen expressed by murine B16 melanoma cells as a model antigen. The TRP-$2_{180-188}$ epitope (SVYDFFVWL) is recognized by both murine and human melanoma-reactive CTLs in the context of H-$2K^b$ and HLA-A2, respectively (Parkhurst *et al.*, 1998). Thus, TRP-2 antigen should be useful in both preclinical and clinical settings for tumor immunotherapy studies. As the frequency of naïve CTL precursors for the self-associated TRP-2

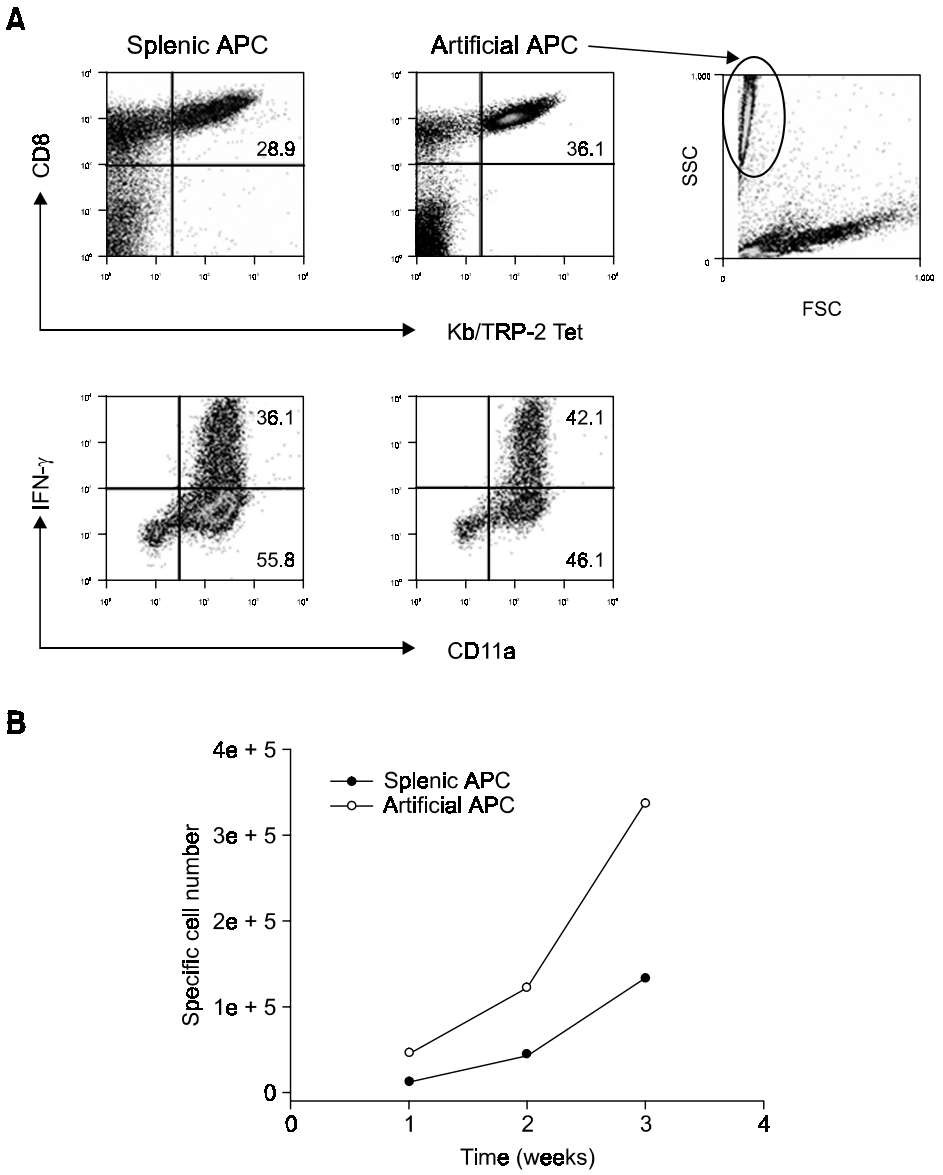

Figure 2. Enhanced amplification of melanoma antigen-specific CTLs using aAPCs. (A) Tetrameric analysis of *in vitro* expanded CD8 T cells with either splenic APCs or aAPCs. Purified CD8 T cells from rAd-TRP-2 immune mice were stimulated three times with either peptide-pulsed splenic $CD11c^+$ APCs or aAPCs in the presence of 0.5 μg/ml of anti-CD28 antibody at a stimulator: responder ratio of 1:5. Cells were stained with CD8-PE/Cy5 and cognate tetramer-PE and the percentage of tetramer-positive cells among lymphocyte-gated cells is indicated in each plot. For intracellular IFN-γ staining, Ficoll-purified cells were stimulated with peptide-loaded EL4 cells, surface stained, permeabilized, and then stained with anti-IFN-γ antibody. The percentages of $CD11a^+$ cells and IFN-γ-producing cells among $CD8^+$ cells are indicated in each plot. (B) Absolute TRP-2-specific cell numbers were calculated from the total live cell numbers in the culture after each round of stimulation. Results are representative of three different experiments, each with different groups of mice.

epitope was expected to be very low, we used a prior immunization method to increase the number of precursors. For the TAP-independent presentation of the endogenously synthesized peptide, the ER insertion/signal sequence of adenovirus E3/19k was fused at the N-terminus of the peptide epitope (Bacik *et al.*, 1994). This mini-gene cassette was inserted into a shuttle vector to generate a recombinant adenoviral genome (Figure 1A). Mice were immunized with recombinant adenovirus expressing the TRP-2 epitope (rAd-TRP-2) to induce a polyclonal TRP-2-specific CTL response. As shown in Figure 1B, melanoma TRP-2-specific $CD8^+$ T cells were induced by priming mice with the rAd-TRP-2 virus and *in vitro* restimulation with synthetic TRP-$2_{180\text{-}188}$ peptide, as detected by Kb/TRP-2 tetramer staining. After mock immunization, TRP-2-specific $CD8^+$ T cells were barely detected after one or two rounds of amplification (Figure 1B). The specificity of the tetramer staining was confirmed by control Kb/OVA tetramer staining.

The ability of artificial APCs to stimulate TRP-2-specific polyclonal CTLs was analyzed and compared with that of spleen-derived natural APCs. Purified CD8 T cells from rAd-TRP-2-primed mice were stimulated for 7 days with either peptide-pulsed syngeneic $CD11c^+$ APCs or artificial APCs plus anti-CD28 antibody. Tetramer staining and intracellular cytokine staining assays were then performed. As shown in Figure 2A and B, improved enrichment of TRP-2-specific CD8 T cells was consistently observed during repeated stimulation with artificial APCs over three weeks when compared to a splenic APC-mediated enrichment. The control artificial APCs containing either the Kb/OVA peptide or the Db/hgp100 peptide complex on the surface did not stimulate any response (data not shown). Artificial APCs were easily distinguished from live cells by their distinct forward and side scatter profiles, as shown in Figure 2A (circled region), facilitating analysis of cell phenotypes.

An important parameter in evaluating the function of CTL is recognition and killing of target cells expressing a cognate antigen-MHC complex. We, therefore, tested the ability of aAPC-stimulated CTLs to recognize endogenously processed antigen on B16F10 melanoma cells and TRP-2 epitope-pulsed EL4 target cells. The aAPC-stimulated bulk CTL culture showed preferential recognition of B16F10 melanoma cells *in vitro,* as measured by IFN-γ release (Figure 3A). It has been reported that some cells induced by aAPC produced IL-4 when stimulated with target tumor cells (Oelke *et al.*, 2003). However, we were unable to detect Th2 cytokines, such as IL-4, after stimulation of TRP-2-specific cells with melanoma target cells in our setting (data not shown). Since difference in the cytotoxic capabilities of Tc1 and Tc2 subsets is a controversial issue (Maggi *et al.*, 1994; Sad *et al.*, 1995), it has to be determined whether Tc1 type CTLs are more efficient than the Tc2 subset for ACT therapy. We also tested the killing of target cells by aAPC-induced CTLs. TRP-2-specific cells mediated a dose-dependent lysis of cognate peptide-loaded EL4 targets but not of control MHC-matched, OVA peptide-loaded targets (Figure 3B). Thus, aAPC-induced CTL populations from immunized mice recognized endogenously processed, cognate antigen-MHC complexes.

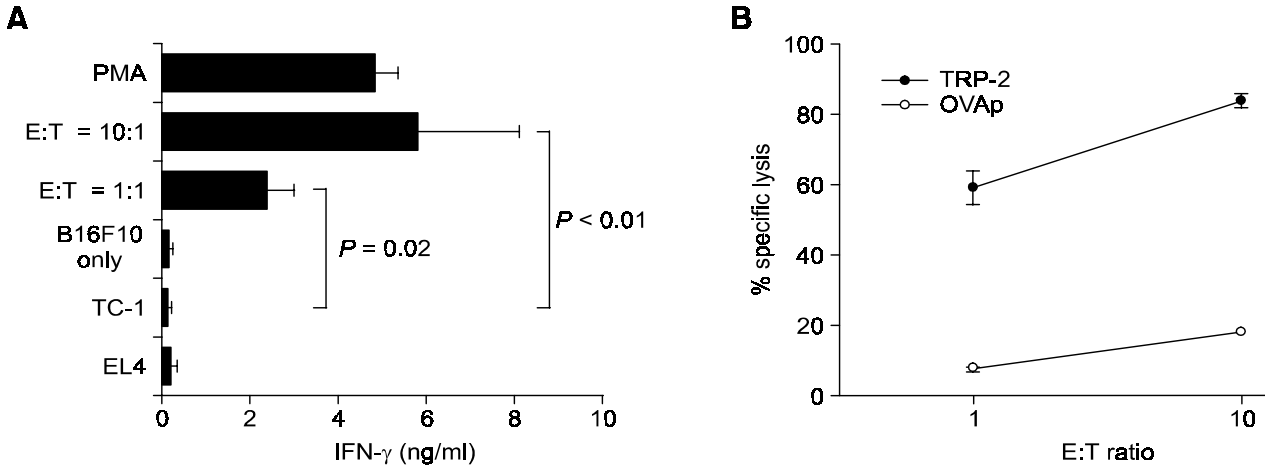

Figure 3. Recognition of endogenously processed melanoma antigen on target cells by aAPC-amplified T cells. (A) IFN-γ production of TRP-2-specific CTLs amplified with aAPCs was determined after incubation for 24 h with B16F10 melanoma cells, EL4, or TC-1, irrelevant tumor cells. The data are representative of two separate experiments at the effector:target ratios of 10:1 and 1:1. (B) Percent specific lysis by aAPC-stimulated TRP-2-specific CTLs is shown for peptide-pulsed EL4 target cells and control H-$2K^b$-restricted OVA peptide-pulsed targets. Values represent quadruplicate experiments at the effector:target ratios of 10:1 and 1:1.

These results demonstrate that artificial APCs can be successfully used to selectively enrich tumor-reactive CTLs for adoptive immunotherapy.

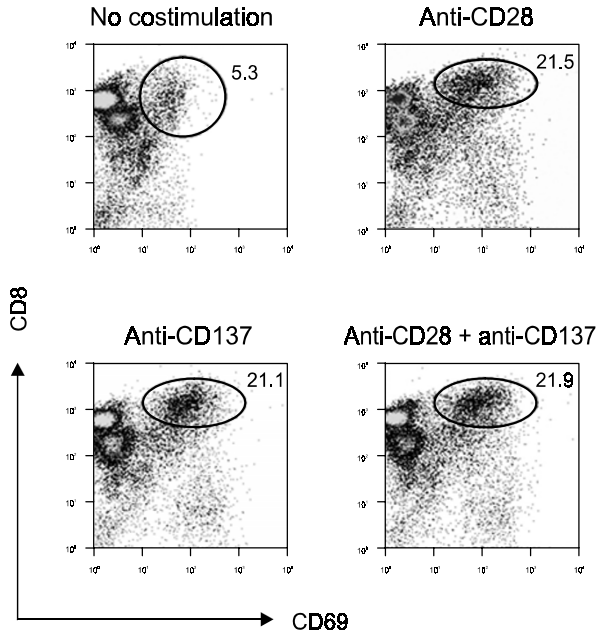

Figure 4. Frequency of activated TRP-2-specific CD8 CTLs after stimulation with aAPCs in the presence of costimulatory antibodies. Purified CD8 T cells from *in vitro* cultures were stimulated with Kb/TRP-2-coated aAPCs alone or with anti-CD28, anti-CD137, or anti-CD28 plus anti-CD137. After 2 days of stimulation, cells were surface stained with anti-CD8 and anti-CD69. The percentage of CD69 expressing cells in the circle region is shown in the upper right corner.

We also tested whether artificial APCs with or without antibodies to costimulatory molecules, such as CD28 and CD137 (4-1BB), can further stimulate the initial activation and proliferation of TRP-2-specific CD8 T cells. The T cells were stimulated with or without antibodies to co-stimulatory molecules in the presence of the same number of artificial APCs. We found that the initial activation and proliferation of TRP-2-specific CD8 T cells that were stimulated with artificial APCs and costimulatory antibody were enhanced when compared to cells activated by aAPC alone, as judged by CD69 expression (Figure 4) and CFSE dilution (data not shown). However, the initial activation and proliferation of TRP-2-specific CD8 T cells were almost equivalent when anti-CD28 antibody was replaced with anti-CD137 costimulatory antibody (Figure 4). The simultaneous addition of two costimulatory antibodies to the culture showed no further enhancement of aAPC-mediated CD8 T-cell activation. These results indicate that the requirement for costimulation during initial aAPC-induced activation was satisfied by either anti-CD28 or anti-CD137 antibody and different costimulatory signals did not have an additional benefit.

To further confirm the better efficacy of artificial APCs for the enrichment of antigen-specific CTLs, we used OT-I TCR transgenic T cells for *in vitro* stimulation with either artificial APCs or spleen-derived APCs. OT-I cells were isolated and MACS-purified from the spleen and lymph nodes of transgenic mice and subsequently stimulated with either $OVA_{257-264}$ (SIINFEKL) peptide-pulsed APCs or Kb/OVA monomer-coated artificial APCs. During the first 3 days, OT-I cells proliferated more efficiently

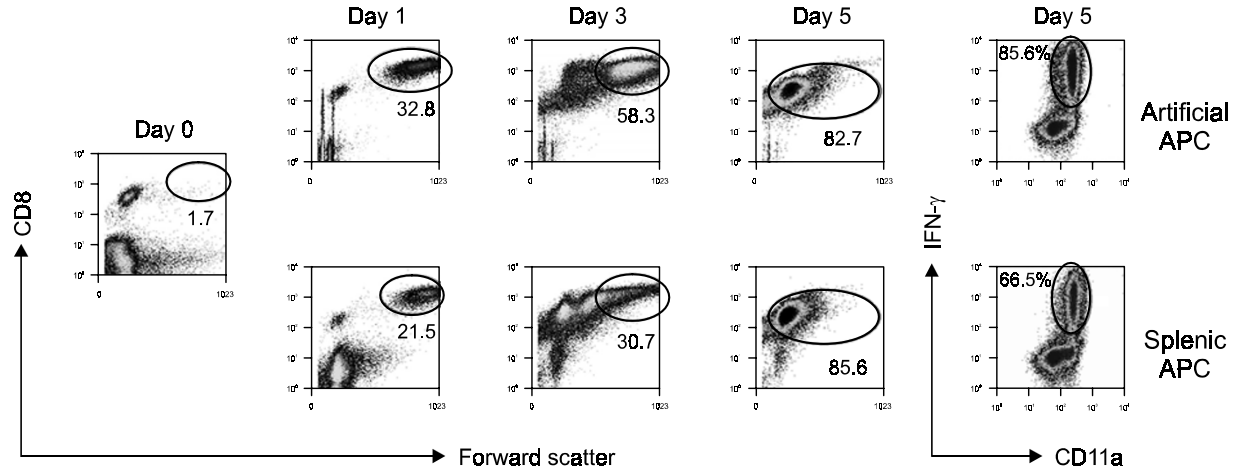

Figure 5. Enhanced proliferation of OVA-specific OT-I cells stimulated with Kb/OVA-coated aAPCs. MACS-purified CD8 T cells from OT-I TCR transgenic mice were stimulated with artificial APCs or with splenic APCs. During stimulation, cells were stained with anti-CD8 at each indicated time point. The percentage of lymphoblast-sized $CD8^+$ T cells in each plot is shown below the circle region. For intracellular IFN-γ staining, cells were stimulated with OVA peptide-loaded EL4 cells, surface stained, permeabilized, and then stained with anti-IFN-γ antibody. The percentages of $CD11a^+$ cells and IFN-γ-producing cells among $CD8^+$ cells are indicated in each plot.

when stimulated with artificial APCs than with peptide-pulsed APCs, as indicated by the number of lymphoblast-sized OT-I $CD8^+$ T cells (Figure 5). At day 5 after stimulation, OT-I cells returned to the resting size and the numbers of surviving cells in the culture were almost equal in both groups, as shown in CD8-FSC plots (Figure 5). However, the surface staining of cells with some activation markers, such as CD11a and intracellular IFN-γ staining at day 5, revealed that the actual numbers of activated, IFN-γ-producing OT-I cells were higher in the group stimulated with artificial APCs than in peptide-stimulated cells (Figure 5, 85.6% vs. 66.5% of total live cells, respectively). Thus, these results confirmed that artificial APCs loaded with a specific peptide have a better capacity for *ex vivo* activation and initial expansion of antigen-specific CD8 T cells.

For practical reasons, the artificial APCs described in this study have several advantages over natural cellular APCs. The easy and inexpensive preparation of aAPC is a major benefit when compared to the difficult, tedious, and high-cost DC preparation. The easy preparation and relatively long stability (we observed no detectable loss of specificity and capacity to stimulate responders within two to three months at 4°C) permits additional rounds of aAPC-mediated CTL expansion. We used recombinant MHC I molecules expressed and purified from *E. coli* to construct artificial APCs loaded with a specific peptide, whereas others used MHC-Ig fusion proteins from transfected mammalian cells (Oelke *et al.*, 2003). Even though it was not possible to directly compare the stimulation efficiencies of the two different aAPC constructs, higher protein yields and lower costs are significant advantages for the *E. coli* expression system over mammalian one.

aAPCs can be constructed to effectively present any desired ligand to CTLs, and the surface densities of the ligands on artificial APCs can be readily controlled. Thus, aAPCs can be easily adapted using other MHC class I-Ag complexes for other specific responses, including HLA-restricted CTLs. Also, artificial APCs can be easily removed from the culture by a one-step density gradient centrifugation for subsequent application, such as infusion into the body. In addition, it has been previously shown that tumor antigen-MHC complexes immobilized on cell size microspheres not only augment tumor-specific CTL activity, but reduce tumor growth when injected *in vivo* (Goldberg *et al.*, 2003), demonstrating the potential for using artificial class I/tumor peptide complexes for active immunization. If aAPC constituents are prepared in clinical grade, they can be used for the primary induction of antigen-specific CTLs both *in vivo* and *in vitro*.

The high frequency of CTL precursors greatly simplifies the subsequent expansion of antigen-specific T cells for adoptive transfer (Pittet *et al.*, 1999; Oelke *et al.*, 2003). Thus, if the precursor frequencies of naive and/or memory T cells for the target antigens in the body are too low, a priming immunization might be helpful for rapid expansion of desired antigen-specific CTLs later. In this regard, our immunization regimen with the recombinant adenovirus should be considered as a priming method performed before large scale amplification of rare tumor-specific CTLs *in vitro* and subsequent adoptive transfer therapy. Future studies in preclinical and clinical settings will evaluate the efficacy of this "prime and expand" method and appropriate application of aAPC technology should significantly advance the field of adoptive immunotherapy.

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