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Artificial antigen-presenting cells expressing CD80, CD70, and 4-1BB ligand efficiently expand functional T cells specific to tumor-associated antigens

Wanyong Zeng[a], Mei Su[a], Karen S. Anderson[a], Tetsuro Sasada[a,b,*]

[a] *Cancer Vaccine Center, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA*
[b] *Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Japan*

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ABSTRACT

Professional antigen-presenting cells (APCs), notably dendritic cells (DCs), are the most potent for expanding antigen-specific T cells *ex vivo*. However, the labor-intensive and expensive procedure for customized preparation of autologous APCs has hampered their broad clinical application. Artificial APC (aAPC) systems, which can be readily prepared from off-the-shelf components, have been proposed as a promising alternative to custom-made professional APCs. Here, in order to develop a novel aAPC system, we established K562 erythroleukemia cells expressing different combinations of co-stimulatory molecule ligands, CD80, CD70, and/or 4-1BB ligand (4-1BBL). When nucleofected with *in vitro*-generated mRNA encoding a tumor-associated antigen, MART-1, the K562 cells expressing all of CD80, CD70, and 4-1BBL were the most efficient for expansion of functional T cells specific to an HLA-A2-restricted immunodominant epitope, MART-$1_{26–35}$. In addition, only the K562 cells expressing all three of these co-stimulatory molecule ligands could clearly expand T cells specific to other less immunogenic antigen epitopes, $gp100_{154–162}$ and $Cyp1B1_{239–247}$, through transfection with *in vitro* generated gp100 and Cyp1B1 mRNA, respectively. These results indicated that non-redundant and synergistic effects of co-stimulation *via* CD28/CD80, CD27/CD70, and 4-1BB/4-1BBL might be critical for eliciting efficient expansion of T cells; co-stimulation *via* the 4-1BB/4-1BBL interaction might expand antigen-specific T cells by preventing apoptotic cell death triggered by specific antigens in the presence of the CD28/CD80 and CD27/CD70 signaling. Taken together, our findings suggested that this K562-based aAPC system expressing CD80, CD70, and 4-1BBL would be useful for efficiently stimulating functional antigen-specific T cells *ex vivo*, in particular when detailed information on the epitope specificities is unavailable.

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Introduction

Professional antigen-presenting cells (APCs), particularly dendritic cells (DCs), have been reported to be the most potent stimulant for expanding antigen-specific T cells *ex vivo* (Melief, 2008; O'Neill et al., 2004; Steinman and Banchereau, 2007). However, isolation and/or generation of autologous APCs is labor intensive and expensive, since large volumes of blood need to be processed to obtain sufficient numbers of APCs. In addition, disease conditions often affect the quality and quantity of APCs, which may have a significant impact on the efficiency of priming and/or expansion of antigen-specific T cells (Gabrilovich et al., 1997; Satthaporn et al., 2004). Artificial APCs (aAPCs), which can be readily prepared from "off-the-shelf" components, thus have been proposed as a promising alternative to custom-made autologous APCs to efficiently stimulate antigen-specific T cells *ex vivo* (Kim et al., 2004; Turtle and Riddell, 2010).

Several types of aAPCs for the *ex vivo* expansion of antigen-specific T cells have been developed with promising results (Kim et al., 2004; Turtle and Riddell, 2010). For example, cell-based aAPCs have been studied in several different cell lines, such as *Drosophila melanogaster* cells (Sun et al., 1996), NIH 3T3 murine fibroblasts (Latouche and Sadelain, 2000), and K562 human erythroleukemia

Abbreviations: APCs, antigen-presenting cells; DCs, dendritic cells; aAPCs, artificial antigen-presenting cells; 4-1BB, ligand (4-1BBL); MHC, major histocompatibility complex; TCR, T cell receptors; mAb, monoclonal antibody.

* Corresponding author at: Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan.
Tel.: +81 942 31 7551; fax: +81 942 31 7699.
E-mail address: tsasada@med.kurume-u.ac.jp (T. Sasada).

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cells (Butler et al., 2007; Maus et al., 2002). Acellular aAPCs have also been created from various materials, such as microcon-size latex, polyglycolide, magnetic beads, and lipid-base vesicles (Oelke et al., 2003; Prakken et al., 2000; Steenblock and Fahmy, 2008). However, it should be noted that, in most of the currently available aAPC systems, synthetic antigenic peptides need to be loaded onto major histocompatibility complex (MHC) molecules expressed on aAPCs to stimulate antigen-specific T cells. These aAPC systems are thus well suited to expand T cells specific to well-known antigen epitopes. However, only limited information is currently available regarding the detailed epitope specificities and/or HLA restrictions for most tumor-associated antigens. Therefore, novel aAPCs, which efficiently process whole tumor antigens that are exogenously loaded in the form of proteins, mRNAs, or DNAs, and present immunogenic epitopes to antigen-specific T cells, would be useful for stimulating and expanding antigen-specific T cells when detailed information on the epitope specificities are unavailable. However, only a few studies on the development of such aAPCs have been reported thus far.

Professional APCs express ligands for various co-stimulatory molecules on their surface to enhance T cell activation, differentiation, and/or proliferation, and to prevent apoptotic cell death of activated T cells (Melief, 2008; O'Neill et al., 2004; Steinman and Banchereau, 2007). For optimal stimulation, T cells require interactions between co-stimulatory molecules and their ligands, in addition to engagement of T cell receptors (TCR) (Williams and Bevan, 2007). One of the most potent and well-studied co-stimulatory molecules for early activation of naïve T cells is CD28, which interacts with its ligands, CD80 or CD86, on APCs (Rudd et al., 2009). However, additional interactions have been reported to be essential for optimal T cell stimulation, since the CD28–CD80/CD86 interaction alone cannot fully explain the long-lasting, effective T cell responses. In particular, members of the tumor necrosis factor (TNF)/TNF receptor (TNFR) family, including 4-1BB/4-1BB ligand (4-1BBL), CD27/CD70, ICOS/ICOS ligand and OX40/OX40 ligand, are reported to play a crucial role in efficient T cell activation by enhancing T cell proliferation at the initial phase of stimulation and preventing cell death at the later phase (Croft, 2009; Watts, 2005). Although the roles of these molecules have been increasingly clarified, especially in mice, it still remains to be elucidated which combinations of co-stimulatory molecules/ligands would be desirable for the efficient *ex vivo* expansion of antigen-specific T cells in humans.

In the current study, we employed K562 erythroleukemia cells as the scaffold to generate a cell-based aAPC that could stimulate antigen-specific T cells through exogenous loading of whole antigens in the form of mRNAs. K562 cells were chosen because they have the following characteristics: (1) an absence of endogenously expressed HLA molecules, with the possible exception of HLA-C, which would minimize the possibility of unintended allogeneic T cell responses; (2) an abundant cell surface expression of adhesion molecules, including CD54 (ICAM-1) and CD58 (LFA-3), which are known to play important roles in T cell stimulation; and (3) easy handling for cell expansion and transgene expression (Butler et al., 2007; Maus et al., 2002). In addition, we and others have recently demonstrated that K562 cells can efficiently process tumor and/or viral antigens exogenously loaded in the form of mRNAs (Anderson et al., 2011; Britten et al., 2005). Here, we established K562-based aAPCs that express different combinations of co-stimulatory molecule ligands, CD80, CD70, and/or 4-1BBL, and examined their efficiency in stimulating T cells through exogenous loading of whole-antigen mRNAs. Our results suggest that the K562-based aAPC expressing all three of these co-stimulatory molecule ligands could provide a novel approach for the efficient *ex vivo* expansion of functional antigen-specific T cells. In particular, this aAPC system would be useful for immune monitoring and identification of novel 'real' antigenic epitopes that are naturally processed and presented, when detailed information on the epitope specificities is unavailable.

Materials and methods

Peptides, plasmids, and RNA synthesis

The HLA-A*0201 (HLA-A2)-restricted peptide epitopes, MART-$1_{26–35}$ (EAAGIGILTV), $gp100_{154–162}$ (KTWGQYWQV), $Cyp1B1_{239–247}$ (SLVDVMPWL), and $HER2_{369–377}$ (KIFGSLAFL), were synthesized by New England Peptide (Fitchburg, MA). A series of 15-mer peptides overlapping by 11 amino acids, derived from the MART-1 sequence, was purchased from Mimotopes (Victoria, Australia). The expression plasmids for CD80 (pORF-hCD80), 4-1BBL (pORF-h41BBL), and CD70 (pORF-hCD70) were from Invivogen (San Diego, CA). The pOBT7-MART-1 (Melan-A) and the pOBT7-Cyp1B1 plasmids were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The pCMV6-XL5-gp100 was from OriGene (Austin, TX). The full-length HLA-A*0201 (A2) cDNA under control of the CMV promoter (pCMV-HLA-A2) was a kind gift of Dr. Gordon Freeman (Dana-Farber Cancer Institute). For RNA production, mRNAs encoding full-length MART-1, gp100, and Cyp1B1 genes were prepared from the linearized endotoxin-free plasmids by *in vitro* transcription using an mMESSAGE mMACHINE kit and *in vitro* polyadenylation system (Ambion, Austin, TX). After treatment with DNase (Ambion) to remove the template plasmids, synthesized RNAs were purified by RNeasy columns (Qiagen, Valencia, CA). The size and quality of *in vitro*-generated mRNAs were confirmed using the Agilent 2100 Bioanalyzer (Palo Alto, CA).

Cell lines and establishment of aAPCs

MART-1-positive melanoma cell lines, K008 (HLA-A2-negative) and K029 (HLA-A2-positive), were obtained from Dr. Glenn Dranoff (Dana-Farber Cancer Institute) (Soiffer et al., 1998). The TAP-deficient cell line T2 was obtained from Dr. Peter Cresswell (Yale University School of Medicine) (Anderson et al., 1993). K562 cells were from ATCC. To establish K562-based aAPCs stably expressing different combinations of co-stimulatory molecule ligands, CD80, CD70, and 4-1BBL, K562 cells were nucleofected with 10 μg of pORF-hCD80, pORF-hCD70, and/or pORF-h41BBL plasmids by Amaxa Nucleofector technology (Lonza, Walkersville, MD), and cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Lonza), 2 mM glutamine (Cellgro, Manassas, VA), 20 mM HEPES (Invitrogen), and 15 μg/ml gentamicin (Invitrogen). The transfected cells were sorted by using PE-conjugated anti-CD80 (Beckman Coulter, Miami, FL), anti-CD70 (BD Biosciences, San Jose, CA), or anti-4-1BBL monoclonal antibodies (mAbs) (BD Biosciences) in combination with anti-PE Micro-Beads (Miltenyi Biotec Inc., Auburn, CA) to isolate and enrich the cells stably expressing the transduced molecules. The expression of each co-stimulatory molecule ligand was examined by flow cytometry after staining with the specific antibodies. Sorting was repeated several times, until more than 95% of the cells became positive for expression of the transduced genes.

Nucleofection of mRNA and DNA

For nucleofection, 2×10^6 cells were washed twice with PBS, resuspended with 100 μl of PBS/HEPES buffer, and transfected with the *in vitro*-generated mRNAs (20 μg) and pCMV-HLA-A2 (10 μg) using Amaxa Nucleofector technology (program T-016; Lonza) (Anderson et al., 2011). After nucleofection, the cells were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, and 15 μg/ml gentamicin, and then

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