Video Article

Killer Artificial Antigen Presenting Cells (KaAPC) for Efficient *In Vitro* Depletion of Human Antigen-specific T Cells

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Abstract

Current treatment of T cell mediated autoimmune diseases relies mostly on strategies of global immunosuppression, which, in the long term, is accompanied by adverse side effects such as a reduced ability to control infections or malignancies. Therefore, new approaches need to be developed that target only the disease mediating cells and leave the remaining immune system intact. Over the past decade a variety of cell based immunotherapy strategies to modulate T cell mediated immune responses have been developed. Most of these approaches rely on tolerance-inducing antigen presenting cells (APC). However, in addition to being technically difficult and cumbersome, such cell-based approaches are highly sensitive to cytotoxic T cell responses, which limits their therapeutic capacity. Here we present a protocol for the generation of non-cellular killer artificial antigen presenting cells (KaAPC), which allows for the depletion of pathologic T cells while leaving the remaining immune system untouched and functional. KaAPC is an alternative solution to cellular immunotherapy which has potential for treating autoimmune diseases and allograft rejections by regulating undesirable T cell responses in an antigen specific fashion.

Video Link

The video component of this article can be found at http://www.jove.com/video/51859/

Introduction

Over the last two decades much progress has been made in understanding the pathogenic mechanisms of autoimmune diseases. However, the most common treatments still rely on corticosteroids as well as immunosuppressive drugs such as purine analogs, alkylating agents and calcineurin inhibitors[1]. The use of such drugs has greatly improved the prognosis of patients, yet the treatment does not provide a cure of the underlying immunological malfunction. Moreover, patients become vulnerable to infections and to the development of cancer.

Hence the ultimate goal for treatment of T cell mediated autoimmune diseases and allograft rejection is to specifically target only disease causing T cells while, at the same time, leaving the remaining immune system untouched and competent to fight immunological disorders. Utilizing antigen-presenting cells (APC) as a treatment to suppress peripheral T cell responses was first described as early as the 1970s by several groups. Since then many cell-based approaches have been developed (reviewed in Schütz *et al.*[2]). Strategies using FasL-expressing killer-APC as immunoregulatory cells showed promising results indicating therapeutic potential for treatment of autoimmunity, allograft rejection and chronic infections. However, there are significant limitations with strategies utilizing FasL Killer-APC that have ultimately limited their clinical applicability; (i) generation or isolation of APC is time, labor and cost intensive (ii) patients suffering from cytopenia due to immunosuppression provide limited amounts and quality of cells (iii) coating or transduction of APC with apoptosis inducing signals such as FasL results in highly variable phenotypes and (iv) Killer-APC are sensitive to cytotoxic effector functions of T cells consequently reducing their therapeutic potential. Therefore, it is difficult to guarantee a defined reproducible Killer-APC phenotype that can be utilized for antigen-specific modulation of T cell responses *in vivo*.

Based on our previous work with FasL expressing Killer-APC[3-5] and artificial antigen-presenting cells (aAPC)[6,7] we developed a bead-based approach (KaAPC) for antigen-specific inhibition or elimination of auto-reactive T cells *in vitro*. KaAPC consist of a HLA-A2-Ig dimer (signal 1) and an anti-Fas mAb (apoptosis inducing signal) covalently immobilized onto a surface of a 4.5 µm paramagnetic latex bead. They deplete antigen-specific T cells in a Fas/FasL mediated fashion from T cell cultures of multiple specificities in an antigen-specific manner and independent of the activation induced cell death (AICD) pathway. Dose dependent apoptosis in targeted T cells is induced rapidly already after 30 min[8].

KaAPC can be easily generated in a controlled fashion ensuring a defined off the shelf phenotype and overcome most of the shortcomings of cell based depletion strategies. Consequently, KaAPC display great potential for treatment of T cell mediated autoimmune diseases and allograft rejection, advancing the field of T cell specific treatment strategies.

Protocol

1. Generation of HLA-A2-Ig Based KaAPC

1. Prepare sterile borate buffer. Make a 0.1 M solution of boric acid in water and adjust the pH to 7.0. Sterile filter (0.22 µm) buffer and store at 4 °C.
2. Prepare sterile bead wash buffer. Use phosphate buffered saline (PBS) without magnesium and calcium. Add human AB serum for 3% final concentration. Add ethylenediaminetetraacetic acid (EDTA) for 2 mM final concentration. Add sodium azide (NaN_3) for 0.01% final concentration. Sterile filter (0.22 µm) buffer and store at 4 °C.
3. Transfer 250 µl of epoxy beads (about 100 x 10^6 beads) from stock into a sterile, screw top glass vial.
4. Add 250 µl of borate buffer.
5. Put glass vial on a magnet and let beads adhere for 2 min; aspirate buffer and remove glass vial from magnet.
6. Wash beads once with 1 ml borate buffer.
7. Resuspend beads into 550 µl of 0.018 µg/ml HLA-A2-Ig and 3.64 µg/ml anti-human CD95 mAb (clone CH11) and gently mix by shaking. (Please see discussion section for additional information.)
8. Incubate glass vial on a rotator end over end, at 4 °C for 24 hr.
9. Spin down beads at 300 x g for 2 min.
10. Place glass vial onto a magnet for 2 min and aspirate the “KaAPC loading solution”; remove glass vial from magnet, add 1 ml of bead wash buffer and resuspend carefully by shaking.
11. Repeat step 1.10 twice.
12. Incubate glass vial on a rotator at 4 °C for 24 hr in 1 ml bead wash buffer. At this point, all residual protein binding sites will be blocked by human AB serum contained in the bead wash buffer.
13. Spin down beads at 300 x g for 2 min.
14. Place glass vial onto a magnet for 2 min and aspirate bead wash buffer; remove glass vial from magnet and replace solution with 1 ml of PBS.

2. Quality Control, Peptide Loading, Washing, Storage, and Stability of KaAPC

1. Transfer 1.5 x 10^5 KaAPC into a FACS tube and wash with 1 ml FACS wash buffer.
2. Resuspend beads in 100 µl FACS wash buffer and add 1 µl of anti-mouse IgG1 PE (for detection of the Fc part of the HLA-A2-Ig) and 1 µl of anti-mouse IgM FITC (clone R6-60.2, for detection of the anti-human CD95 mAb).
3. Incubate for 15 min at 4 °C, wash with 1 ml FACS wash buffer, and resuspend in 250 µl FACS wash buffer and read staining by flow cytometry.

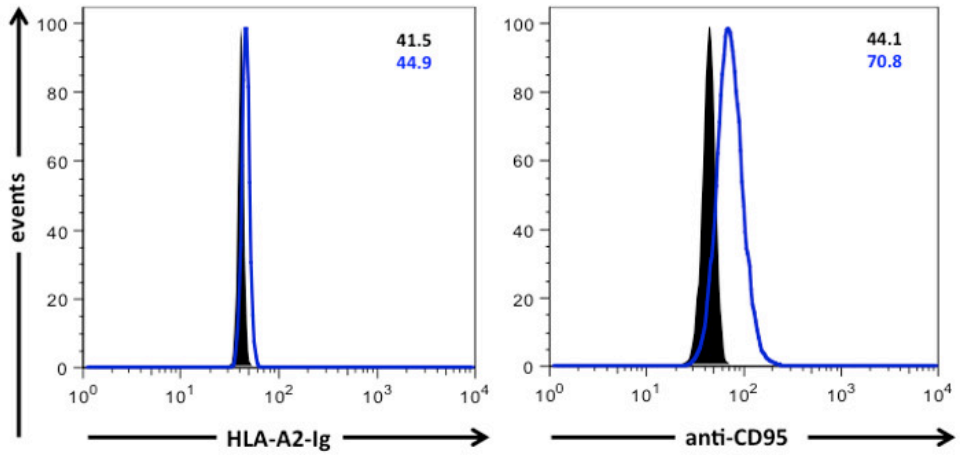

Figure 1. QC stain of a KaAPC batch made as stated in the protocol section. Staining was performed as described in protocol section 2. HLA-A2-Ig was detected using an anti-mouse-IgG1 PE mAb, whereas anti-human-CD95 (anti-Fas) was detected with an anti-mouse-IgM FITC mAb (see protocol section 2.2) (blue line). Filled black graphs represent empty beads stained with anti-mouse-IgG1 PE mAb or anti-mouse-IgM FITC mAb, respectively. Numbers in the upper right corner indicate the mean fluorescent intensity (MFI). Please click here to view a larger version of this figure.

4. For peptide loading transfer 20 x 10^6 KaAPC into a new sterile glass vial, place onto a magnet for 2 min, aspirate PBS and resuspend beads into 500 µl fresh PBS with 5 µg peptide (Keep in mind, only HLA-A2 restricted peptides will bind onto the dimer!).
5. Store loaded KaAPC at 4 °C until use, for at least 3 days to allow sufficient peptide loading onto the HLA-A2-Ig dimer.
6. Immediately prior to use, wash the peptide loaded KaAPC as indicated in 1.14; repeat this step at least 6x.
   NOTE: This is a crucial step to ensure that free peptide has to be removed completely to avoid false positive killing results; residual free peptide has been shown to induce apoptosis in antigen-specific T cell cultures[9]. To rule out a soluble peptide effect, run supernatant control samples of peptide loaded and washed KaAPC (see 3.8).
7. Count beads using hemocytometer and label with date, name of peptide and concentration.

NOTE: Loaded KaAPC remain functional at 4 °C for at least one month. After one month, re-loading with the same peptide allows KaAPC to be utilized for up to one year.

3. KaAPC *In Vitro* Killing Assay

1. Prepare co-culture media. Prepare complete RPMI medium supplemented with 3% T cell growth factor (made in the lab[6]) and 3% donor autologous plasma. If donor autologous plasma is unavailable, use heat-inactivated human AB serum.
2. Prepare AnnexinV binding buffer preparation. Dissolve 8.12 g sodium chloride (NaCl) and 0.12 g calcium chloride ($CaCl_2$) into 990 ml ddH_2O.Add 10 ml 1M HEPES buffer.
3. Generate human antigen-specific T cells. (For a detailed protocol please see a previous publication[7] utilizing artificial Antigen Presenting Cells (aAPC)). Ensure that peptide specificity is at least >75% as determined by tetramer staining.
4. For each sample incubate 2 x 10^5 antigen-specific T cells/well (in a 96 well round bottom plate) in 200 µl of co-culture medium at a 1:1 ratio with KaAPC for 48 hr in a humidified incubator at 37 °C and 5% CO_2.
5. Harvest samples and transfer into a FACS tube; wash with 1 ml AnnexinV binding buffer, spin down at 300 x g for 5 min, discard supernatant and resuspend in 100 µl of AnnexinV binding buffer
6. Stain samples with 1 µl AnnexinV FITC and 5 µl 7-AAD for 10-15 min at RT, wash with 1 ml AnnexinV binding buffer; spin down at 300 x g for 5 min, discard supernatant and resuspend in 250 µl AnnexinV binding buffer
7. Read samples immediately on flow cytometer.
8. Prepare the following samples to determine antigen-specific killing by KaAPC: T cells only; T cells + soluble anti-CD95; T cells + unloaded KaAPC; T cells + non-cognate peptide loaded KaAPC; T cells + cognate peptide loaded KaAPC; T cells + supernatant of cognate peptide loaded KaAPC.

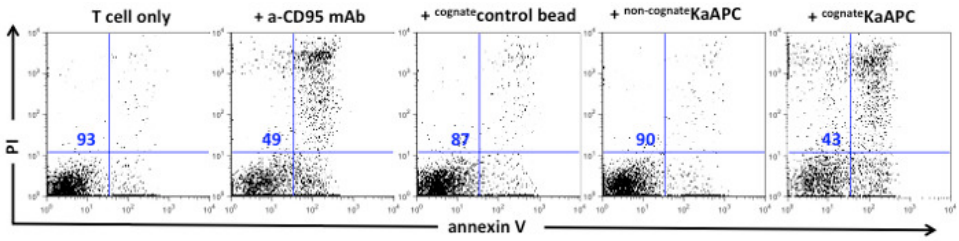

Figure 2. KaAPC killing assay. Peptide loaded KaAPC or control beads (HLA-A2-Ig only beads) were cultured for 48 hr at a 1:1 ratio with CMVpp65 specific T cells (~80%), harvested and subsequently stained with AnnexinV and propidium iodide (PI) to determine apoptosis by flow cytometry (see protocol section 3.3-3.7). "cognate" refers to beads loaded with CMVpp65 and "non-cognate" to beads loaded with Mart-1 peptide. Numbers indicate the percentage of viable T cells in the lower left quadrant of each plot. Please click here to view a larger version of this figure.

9. To perform crisscross-experiments, generate antigen-specific T cells of two different specificities.
10. Further evaluate the antigen-specific depletion-abilities of KaAPC with a simultaneous analysis of the antigen–specific elimination of T cells in heterogeneous T cell population. (For a detailed protocol please see Schütz *et al.*[10]).

Representative Results

The features of this protocol include the defined generation of a KaAPC phenotype that displays HLA-A2-Ig (signal 1) and anti-Fas mAb (apoptosis signal) at the same time, which in comparison to cell based approaches is not altered by coating or transduction efficacies and can be easily modulated during production (**Figure 1**). Furthermore, KaAPC, are not sensitive to cytotoxic effector functions of T cells and their functionality is not dependent on the quality of autologous antigen-presenting cells pre and post *in vitro* manipulation.

KaAPC are capable of depleting antigen-specific T cells *in vitro* when loaded with cognate peptide, while non-cognate loaded KaAPC will not deplete T cells (**Figure 2**). Therefore, KaAPC induce T cell apoptosis in an antigen-specific manner. Subsequently, we used KaAPC for selective depletion of antigen-specific T cells from a mixture of different T cell specificities, demonstrating that only minimal cytotoxicity was leaking into neighbor T cells (**Figure 3**). Thus, KaAPC represent an exquisite tool for in vitro antigen-specific depletion of human activated antigen-specific T cells with potential clinical applicability for the treatment of autoimmune diseases and allograft rejections.

Discussion

The most critical step in the protocol is to ensure the appropriate ratio of signal 1 (peptide MHC) and signal 2 (anti-Fas mAb). We have performed intensive titration experiments to define the perfect ratio for signal 1 and 2; it became evident that minimal variations due to differences in the HLA-A2 Ig dimer or anti-Fas mAb concentrations can interfere with the functionality of the KaAPC. Thus, the concentration of both signals should always be verified and quality of both proteins should be frequently tested even if it was ordered from a commercial source. Furthermore, the concentrations should always be determined by the same assay as different detection assays might result in different concentrations. Functionality of the HLA-A2-Ig can also be impaired due to incomplete refolding and/or inefficient peptide loading. Furthermore dimer molecules can aggregate to different degrees, which could impact on the functional activity of the protein. Therefore, the actual amount of dimer needed for the generation of a functional and specific KaAPC may vary. In addition, we found that the ideal range for the death inducing signal anti-Fas mAb on KaAPC is extremely tight. In our hands amounts greater 3.64 µg/ml lead to non-specific killing of Fas positive T cells while amounts below 3.64 µg/ml resulted in a dose-depending reduced killing activity. While these conditions seem quite tight, attention to these details will result in the generation of a functional and specific KaAPC.

While the current KaAPC phenotype is functional for depletion of activated antigen-specific T cells, initial experiments targeting naïve or resting antigen-specific T cells demonstrated no significant induction of antigen-specific apoptosis as these populations have reduced Fas expression. Therefore, one might envision adding a co-stimulatory signal onto the KaAPC to induce the up-regulation of Fas on resting T cells to make turn them into a target for the KaAPC. While this is feasible all three signals will have to be carefully titrated to ensure a functional KaAPC phenotype.

Adjusting the bead platform to a biocompatible or biodegradable matrix will enhance KaAPC *in vivo* applicability. Recently, Shen *et al.* have reported the successful *in vivo* usage of KaAPC utilizing 5 µm latex beads conjugated to anti-Fas (clone Jo2) and anti-His/H2-K^{b}-TRP2[11]. We have been able to show that the general concept of artificial antigen-presenting Cells (aAPC) can be successfully transferred from µm sized to nm sized particles[12] and that functionality is influenced by particle geometry[13]. Thus, by varying the size and/or shape of future KaAPC designs one might be able to impact on the *in vivo* functionality and bio-distribution, which could be key for successful treatment of organ specific autoimmune diseases.

KaAPC overcome the major shortcomings of cell based depletion strategies as an easy to use “off the shelf”, time and cost efficient approach that is independent of donor condition and pre-treatment. KaAPC are not targets of self- or paracrine killing signaling and not sensitive to cytolytic effector functions of CTL. KaAPC will require identification of the relevant disease antigen and rely on their specific HLA type. While currently numerous suitable HLA-A2 restricted antigens for type 1 diabetes, GvHD and multiples sclerosis have been identified[2], development of additional HLA class I dimer molecules as well as the ongoing identification of new autoimmune relevant antigens will further increase and broaden the applicability of KaAPC. Furthermore one could think of using KaAPC directed at different epitopes to target multiple antigens simultaneously.

In summary, KaAPC represent a flexible platform technology for antigen-specific T cell depletion. Their “lego-like” nature enables the inclusion of new signals such as additional co-stimulatory or inhibitory signals such as PD1 or TRAIL on various matrices, which could broaden and enhance their subsequent *in vitro* and *in vivo* applicability. We herein present the step-by-step protocol to generate KaAPC, the first bead based approach for the elimination of antigen-specific T cells from T cell mixtures with different specificities.

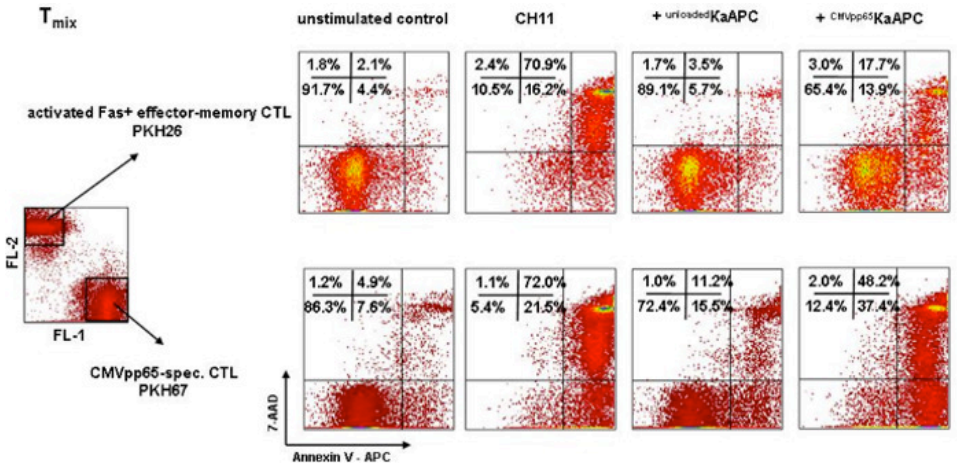

Figure 3. KaAPC eliminate CTL in an antigen-specific fashion. PKH26-labelled activated Fas^{+} effector-memory CTL and PKH67-labelled CMVpp65-specific CTL from the same donor were mixed to a 1:1 ratio and co-cultured with ^{CMVpp65}KaAPC for 48 h. Control cultures were treated with ^{unloaded}KaAPC, or 1 µg/ml of soluble anti-Fas-mAb (clone CH11). Minimal loss of viable cells was determined in untreated mixed CTL cultures (T_{mix}). Original FACS data for each CTL population of the T_{mix} are shown. Analysis of apoptosis was performed as previously published by separate gating on both T cell populations[10]. Numbers depicted in the upper left quadrants represents % of total cells. The illustrating figure is derived from one representative experiment out of three independent experiments. This research was originally published in Blood. Schütz, C. *et al.* Killer artificial antigen-presenting cells: a novel strategy to delete specific T cells. *Blood. 2008;* **111**, 3546–52. Copyright the American Society of Hematology. Please click here to view a larger version of this figure.

Disclosures

C.S. and M.F. have nothing to disclose.

Under a licensing agreement between NexImmune and the Johns Hopkins University, Drs. Schneck and Oelke are entitled to shares of royalty received by the University on sales of aAPC products described in this article. They also own NexImmune stock, which is subject to certain restrictions under University policy. Dr. Schneck is a member of the company"s Board of Directors and Scientific Advisory Board. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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