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TITLE: Development of Artificial Antigen Presenting Cells for Prostate Cancer Immunotherapy

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14. ABSTRACT While adoptive immunotherapy holds promise as a treatment for cancer, development of adoptive immunotherapy has been impeded by the lack of a reproducible and economically viable method for generating therapeutic numbers of antigen-specific CTL. The issues of reproducibility and cost, in large part, relate to the use of cellular dendritic cells (DC) for expansion of CTL. Underlying disease and pretreatment often affect the number of and efficacy of DC. Induction of DC takes time and is dependent on costly cytokine mixtures. Our preliminary data indicates that HLA-Ig complexes coupled to beads (HLA-Ig based artificial Antigen Presenting Complexes, aAPC) can induce and expand antigen-specific T cells and possibly be used to replace standard DC-based ex vivoexpansion of CTL. Potential advantages of aAPC over cellular DC not only relate to the variability in function and viability of DC, but also using aAPC one can load all HLA complexes with the specific antigenic peptide(s) of choice, modulate the costimulatory signals, and enrich/sort for the antigen-specific cells of interest.

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Introduction:

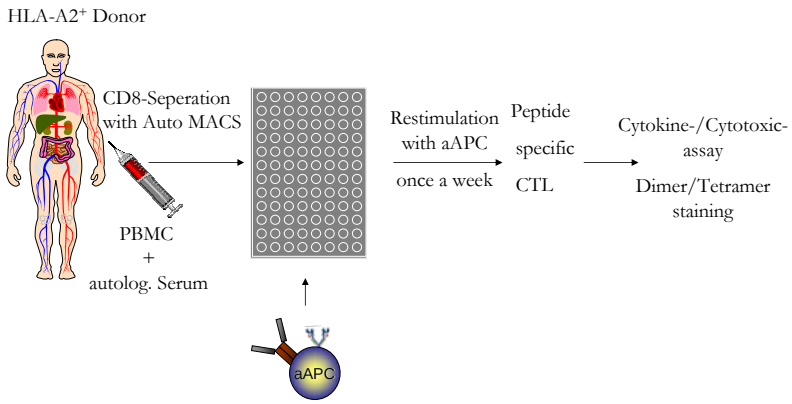
Our goals were the induction of prostate-specific CTL and development of appropriate in vivo animal models for testing prostate-specific CTL. In year one we reported on preliminary data on development of an animal model, as described in the original Specific Aim #2, to test the in vivo efficacy of aAPC induced prostate cancer specific CTL. In year two we reported on initial attempts to grow anti-PSA-3A and PSA-1 prostate-specific CTL, initial Specific Aim #1. In this final report we report on further development of human/SCID model for in vivo efficacy testing and more data on only limited expansion of prostate specific CTL.

Background: An Overview of Adoptive Immunotherapy

A major goal in the field of immunotherapy is to generate an effective cell-mediated anti-tumor immune response. Adoptive immunotherapy involves induction and expansion of antigen-specific T cells, *ex vivo*, followed by transfer of autologous antigen-specific T cells back into patients [1-3]

The development of an artificial Antigen-Presenting Cell (aAPC) has opened the gateway for *ex vivo* stimulation and expansion of tumor-specific T cells to clinically relevant numbers. Initially, June and colleagues developed approaches for non-specific expansion of CTL derived either from TIL-cultures or tetramer-based sorting for enrichment of antigen-specific CTL. By coupling beads to anti-CD3 and anti-CD28 mAbs, they have been able to expand CD4+ T cells. However, this non-specific system is limited in two ways. First, such anti-CD3/anti-CD28 beads failed to support long-term growth of CD8+ CTL, even when T cell growth factor (e.g. IL-2) was added. Second, it was not possible to maintain the antigen-specificity during expansion [4, 5], both significant requirements for induction and expansion of tumor-specific T cells to clinically relevant quantities. Our preliminary data demonstrate that an artificial Antigen Presenting Cell (aAPC), made by coupling HLA A2-Ig and anti-CD28 to beads, can reliably induce and expand antigen-specific CTL from healthy donors. This approach promises to be a facile, cost-effective, and excellent alternative to the more time consuming and expensive dendritic cell based expansion. In our preliminary data, which supported funding of the grant, we showed that this approach can be used to expand multiple clinically relevant CTL populations including CTL specific for CMV or for melanoma antigens. In the current application we proposed to demonstrate functional efficacy of HLA-Ig complexes conjugated to beads as artificial Antigen Presenting Cells (aAPC) for inducing and expanding anti-PSA-3A and PSA-1 prostate-specific CTL. The specific aims are to *1) optimize aAPC structure and duration of stimulation, and 2) analyze the in vivo function of aAPC-induced CTL.*

Schematic for Induction and Expansion of peptide-specific CTL using aAPC

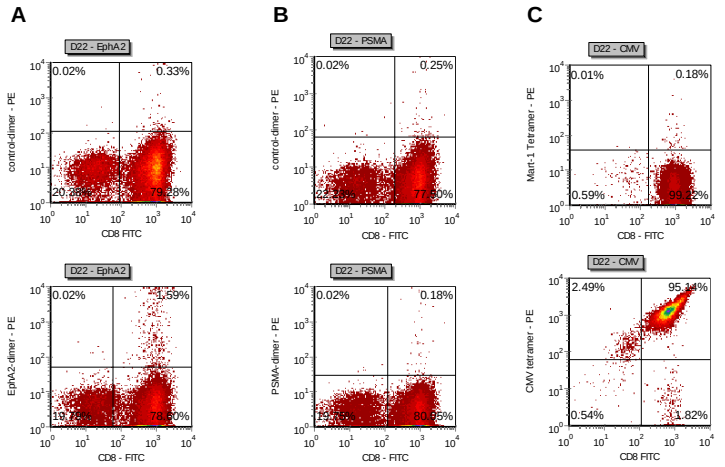


Body:

For evaluating the efficacy of HLA-A2-Ig based artificial Antigen Presenting Complexes (aAPC) approach for treatment of prostate, we proposed to study in vitro expansion of CTL from blood and looked at the response to several candidate antigens. We initially focused on our goals, as identified in the Specific Aims of the application, on induction of anti-PSA-3A and PSA-1 prostate-specific CTL.

CD8+ T-cells were isolated from healthy donors and co-cultured with aAPC as described in the schematic above for a 4-10 week period. PBMCs were obtained from healthy donors by Ficoll centrifugation and separated for CD8+ t-cells. After separation CD8+ T-cells were cocultured with peptide pulsed aAPCs and harvested after one week. The old beads were removed; cells were counted and restimulated in 96 well plates with fresh aAPC. This procedure was normally performed until cell number was reduced to less than $5x10^5$ cells.

Figure 2: Dimer/tetramer analysis of aAPC induced CTL after 3 weeks of culture



We performed the stimulation as shown in the schematic for 8 donors with PSA-3A and PSA-1 peptide pulsed aAPC. As a positive control we additionally pulsed aAPC with Mart-1 peptide. After 4 weeks no specific CTL could be seen for the experimental peptides whereas the culture co-incubated with the Mart-1 pulsed aAPC showed specific cells in a tetramer stain after 2 weeks. Further investigation was restricted by the limited CTL number obtained in the aAPC stimulated culture.

Legend: Figure 2 shows tetramer staining of T cells which were stimulated for 3 weeks with A) EphA2 loaded aAPC, B) PSMA loaded aAPC and C) CMV loaded aAPC. Only small numbers using the EphA2 peptide and no specific T cells using the PSMA peptide.

Secondary to the limited expansion of PSA-3A and PSA-1-specific CTL, we also analyzed the ability of aAPC to mediate expansion of two additional prostate specific antigens, EphA2 and PSMA specific CTL. We performed aAPC stimulation as described in Figure 1 for multiple donors with PSMA, EphA2. We could only detect EphA2 specific T cells from 3 donors and no PSMA specific T cells were generated. Figure 2 shows a representative example. The specificity and functionality of the EphA2-specific CTL was confirmed by specific lyses of peptide pulsed target cells in a ^{51}Cr-release assay (data not shown).

Based on these studies we realize that additional studies will be required utilizing newer versions of aAPC and possibility T cell with different antigenic-specificity to ensure that enough

prostate antigen-specific CTL can be cultured reliably from donors. Additional potential second generation aAPC were discussed in the year two report and could serve as a basis additional studies.

Specific Aim 2) Development of model for in vivo activity of CTL We focused further in vivo efforts on development of a model of antigen-specific human CTL in a related system, Mart-1 specific CTL that recognize a human melanoma cell line. In this system we found that we can expand those human antigen-specific CTL and after four rounds of *in vitro* stimulation, 55.4% of total CD8+ T cells were Mart-1 specific. Starting with a population of 10 x 10^6 total CD8+ T cells that were less than 0.1% specific, approximately 50 x 10^6 Mart-1 specific CTL could be generated within a month. This rapid expansion of Mart-1 specific CTL was seen consistently in several donors tested (data not shown). We have also characterized the phenotype of aAPC expanded CTL. After 4 weeks, the aAPC generated T cells were largely $CD3^+CD8^+$ cells with >93% of the cells expressed the memory cell marker CD45RO, but negative for CD45RA. Further analysis showed that 22% of the cells within total CD8+ T lymphocytes expressed CD62 ligand, and only 3% of the cells expressed CCR7. Thus, the aAPC expanded CTL were $CD45RA^-$, $CD45RO^+$, $CCR7^-$ and $CD62L^{+/-}$, characteristics of effector memory T cells. The CD8+ T cells before culture were mostly $CD45RA^+$, $CD45RO^-$, $CCR7^-$ and $CD62L^+$.

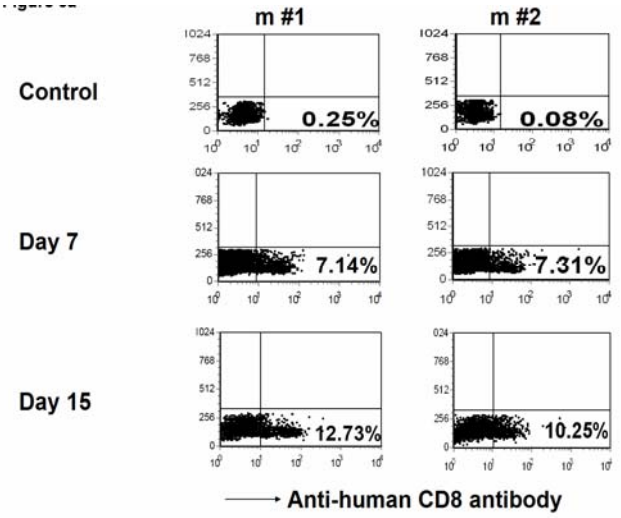
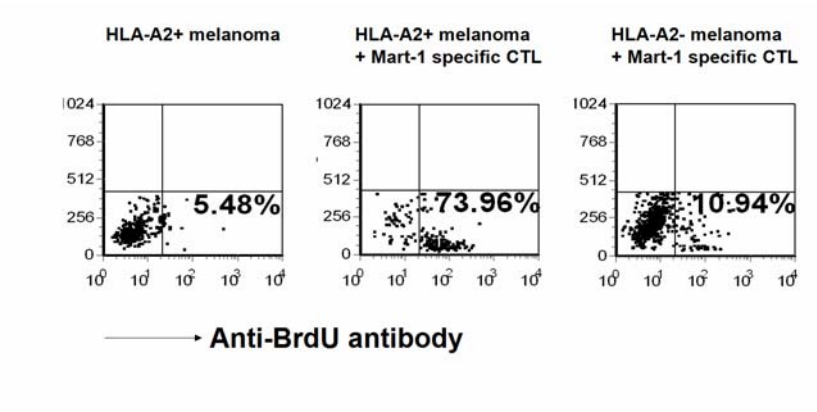


Figure 3. Survival of aAPC expanded Mart-1 specific CTL *in vivo*. (a) SCID mice were injected s.c. with HLA-A2+ melanoma cells for tumor induction. After 2 weeks, these mice were transferred i.v. with Mart-1 specific CTL (5 x 10^6 cells/mouse) and also injected i.p. with recombinant human IL-2 (rhIL-2) (2 x 10^5 IU/mouse) on days 0 and 2. Blood was drawn serially before and on 7, 15 and 21 days after T cell transfer. The peripheral blood lymphocytes were stained with anti-human CD8 antibody and analyzed by flow cytometry. Data is shown for 2 individual mice analyzed at different time points as mentioned. The percentage of human CD8+ T cells within total peripheral blood lymphocytes is given in the lower right corner. A representative of two independent experiments is shown.

Survival of aAPC generated specific CTL *in vivo*

Cell viability in vivo is an important consideration in adoptive CTL transfer. We therefore monitored the fate of adoptively transferred Mart-1 specific CTL in the peripheral blood at different time points after transfer to tumor bearing mice. SCID mice were injected subcutaneously (s.c.) with HLA-A2+ melanoma cells to induce tumor growth and after 2 weeks Mart-1 specific CTL were infused intravenously (i.v.). PBMC obtained immediately before and on 7, 15 and 21 days after infusion were analyzed for the presence of the transferred CTL. Our results (Figure 3, above) show that before transfer the frequency of CD8+ T cells within peripheral blood lymphocytes was <0.3%. 7 days after transfer, 7% of the transferred CTL could be detected in the blood. A significant increase in the number of CD8+ T cells (10-12%)

was observed on day 15 after infusion. However, the number of transferred CTL had decreased indicating the disappearance of transferred CTL in the blood after 2 weeks of transfer. As expected, these cells did not stain with anti-mouse CD8 antibody (data not shown). Moreover, 73% of the transferred Mart-1 specific CTL were found to be proliferating on day 7 after transfer as revealed by BrdU incorporation (Figure 4), showing that the increase in the number of transferred CTL in the blood is most likely due to proliferation of the transferred cells, as suggested by previous studies. Together these results clearly demonstrate survival of the transferred Mart-1 specific CTL for several days *in vivo*.



To summarize these experiments show that in a related human/SCID model for adoptive immunotherapy we see survival and expansion of adoptively transferred CTL. These systems therefore show great promise for future studies evaluating the potential role of adoptive immunotherapy in the treatment of prostate cancer.

Bulleted List of Key Accomplishments:

1) Development of two models for in vivo survival of human antigen-specific CTL in SCID mice.
2) Worked with human PBMC for expansion of various prostate antigen-specific CTL.
3) Use of various different accessory molecules detailed in Specific Aim #1.
4) Tested additional donors for expansion of anti-PSA-3A, anti-PSA1, anti-PSMA, and anti-EphA2 specific CTL. Limited expansion of specific CTL was seen.
5) Tested second generation aAPC in systems for expansion of Mart-1 specific CTL from multiple donors.
6) Tested in vivo tracking of antigen-specific CTL in an in vivo human/SCID model.

Reportable Outcomes:

1) Currently two reportable outcome are anticipated. The first is a manuscript describing the in vivo efficacy of aAPC expanded CTL as described above. Currently the manuscript is in preparation and can be supplied if requested.
2) Another manuscript on the second generation aAPC is being discussed. This will require additional experiments for completion.

Summary:

We have highlighted studies that demonstrate the importance of exploring adoptive immunotherapy as a logical method for treatment of prostate cancer. To summarize, the proposed experiments investigated expansion of prostate-specific CTL using aAPC-mediated T cell induction to various prostate antigen-specific CTL. In this application, we proposed to evaluate varying parameters essential to optimize aAPC function. These included altering the ratios of signal 1 to signal 2 and the types of signal 2 on the aAPC. We had limited success in growth of prostate-specific CTL. We were however able to establish new models looking at persistence of human CTL in SCID models of in vivo persistence of human CTL.

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