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14. ABSTRACT We have developed a novel strategy that combines tumor immunotherapy targeting PAP and targeted immune modulation of CTLA4 and have generated a lead cellular therapy that will safely enhance vaccine-mediated immunity. This lead cellular therapy, called DC-PAPvac-C, consists of dendritic cells (DCs) co-transfected with prostate tumor antigen, PAP RNA and anti-CTLA4 RNA. In this study we will establish the preclinical efficacy and safety of our cellular therapy product, DCs transfected with RNA that encodes PAP and anti-CTLA4 and generate data required for an Investigational New Drug (IND) application. In this report we have demonstrated that local CTLA4 modulation in combination with PAP-specific immunization using RNA-transfected DCs elicits superior antigen-specific T cell responses in TRAMP mice. We have now demonstrated that PD1/PDL1 blockade synergizes with our novel vaccine strategy that combines induction of tumor-specific T cells with local blockade of the inhibitory T cell receptor, CTLA4. DC vaccine with local CTLA4 blockade in combination with systemic PD1/PDL1 blockade is more effective at induction of antigen-specific T cells than DC vaccine and local CTLA-4 blockade alone. We have completed the three cGMP production runs including lot release testing of the final DC-PAPvac-C cellular vaccine product.					
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Novel immune modulating cellular vaccine for prostate cancer immunotherapy (PC121288)

Principal Investigator: Smita Nair, PhD

INTRODUCTION:

The goal of immunotherapy is to stimulate T cells that recognize and destroy tumor cells; however, a major challenge to greater vaccine efficacy is immune suppression mediated by inhibitory receptors on activated T cells, specifically cytotoxic T lymphocyte antigen 4 (CTLA4). Systemic administration of anti-CTLA4 blocking antibodies has demonstrated clinical effectiveness in melanoma patients, but, consistent with its mode of action, anti-CTLA4 antibody causes significant immune-related adverse events. Strategies for delivering anti-CTLA4 to the site of T cell activation while limiting systemic exposure are needed. Therefore, our objective is to design a prostate cancer immunotherapy strategy that will 1] Enhance the function of tumor antigen-specific T cells by targeted modulation of immune receptor function and 2] Lead to the development of a clinically effective prostate cancer immunotherapy, without inducing severe autoimmunity. We have designed an innovative approach for targeted delivery of antibodies to sites where anti-tumor T cells are induced, using dendritic cell (DCs) transfected with antibody-encoding RNA. When we immunized mice with DCs transfected with tumor antigen RNA and anti-CTLA4 RNA, we observed enhanced anti-tumor immunity, without autoimmunity. Specific to the prostate cancer antigen PAP, we activated more potent anti-PAP cytotoxic T lymphocyte (CTL) responses *in vitro* using DCs modified to express PAP and secrete the anti-CTLA4 antibody. We hypothesize that a vaccine consisting of DCs modified with RNA encoding PAP and anti-CTLA4 will result in increased immunogenicity toward PAP over PAP alone. As described in our proposal, we will conduct preclinical studies with our lead cellular therapy - DCs modified with RNAs encoding PAP and anti-CTLA4 antibody - DC-PAPvac-C. Our intention is to advance this product into clinical trials.

KEYWORDS:

dendritic cell vaccine, dendritic cells electroporated with RNA, immune checkpoint blockade, local CTLA4 modulation, prostate cancer immunotherapy, prostatic acid phosphatase (PAP), and RNA-based vaccines

OVERALL PROJECT SUMMARY:

Protocols specific to this proposal and approval dates:

Mouse studies, Aim 1:

Protocol 1 Aim 1:

Duke University IACUC Approval: 03-28-2013 for 3 years (Protocol A082-13-03)

Duke University IACUC Annual Renewal Approval: 03-27-2015 (Protocol A082-13-03)

ACURO Approval: 07-16-2013 (Protocol reference number PC121288)

Mouse Protocol Rewrite after expiration of Protocol A082-13-03

Duke University IACUC Approval: 02-25-2016 for 35 months and 3 weeks until 02-17-2019 (Protocol A023-16-02)

ACURO Approval: 04-04-2016 (Protocol reference number PC121288)

Human Studies, Aims 2 and 3:

Protocol 1 Aim 2:

Duke University IRB Approval, IRB DECLARATION OF RESEARCH NOT INVOLVING HUMAN SUBJECTS: approved on 09-25-2014 and does not expire (Protocol number Pro00057900, Title: Tumor infiltration with immune effectors)

HRPO Research Not Involving Human Subjects Determination Memorandum Approval: 10-07-2014 (HRPO Log Number A-17872.1)

Protocol 2 Aim 3:

Duke University IRB Approval: 04-11-2013 (Protocol number Pro00044351)

Duke University IRB Continuing Review Approval: 04-11-2014 (approved on 03-14-2014)

Duke University IRB Continuing Review Approval: 04-11-2015 (approved on 03-16-2015)

HRPO Approval: 08-15-2013 (HRPO Log Number A-17872.2)

Duke University IRB Final Report Approval: 03-04-2016

Final Report submitted to HRPO for approval: 04-12-2016 (HRPO Log Number A-17872.2)

This report is being written on the heels of getting approval for a 12-month (1-year) Extension Without Funds Request (EWOFF) to September 29 2017 to finish work associated with award number W81XWH-13-1-0423.

Reason for 1-year Extension Without Funds Request (EWOFF) request:

1] Work associated with Major Task 1: Determine the in vivo systemic and tumor-associated immune response that correlates with anti-tumor activity of the lead cellular therapy, DC-PAPvac-C (months 1-30) and Subtask 7: Monitor tumor burden (time to palpable tumor) and monitor survival. Harvest prostate complex/tumor and analyze tumor weight, tumor grade, tumor apoptosis and harvest mouse organs for analysis of inflammatory infiltrates and autoimmunity.

Details:

The experiments in the TRAMP transgenic mice are taking much longer than we anticipated. This is because the experiments start when mice are 12 weeks old and terminate when mice are 40-42 weeks old (based on our ongoing experiments). Also it takes longer to get these mice from the vendor, as they are harder to breed. Importantly, we cannot always stagger the experiments, because data from the preceding experiment is used to plan the next analysis. Finally the endpoint involves a thorough analysis of each mouse, isolation of the urogenital tract (UGT) and analysis of prostate by immunohistochemistry to get the tumor score. The histologic grade/tumor score is our primary endpoint to determine if our therapy is working.

We now have 80 mice on order and plan to order another 100-120 mice. This will allow us to conduct rigorous experiments that are reproducible (we plan to repeat these experiments 3 times) as we outlined in the original proposal. So with the mice in hand our experiments extend into Jan/March of 2017 and the subsequent experiments will run into Spring/Summer of 2017. In Summer/Fall 2017, we will collect all the data and write up the

manuscript. Based on our preliminary analysis we anticipate that we will complete all these tasks.

2] Work associated with Major Task 1: Determine the in vivo systemic and tumor-associated immune response that correlates with anti-tumor activity of the lead cellular therapy, DC-PAPvac-C (months 1-30) and Subtask 8: Monitor autoimmune prostatitis in normal BL/6 mice using DC-mPAPvac-mC.

Details:

The explanation is similar to above. Experiments will be conducted in Spring and Summer 2017. Experimental description is below.

Monitoring autoimmune prostatitis (lymphomononuclear cell infiltrates in prostate gland): Non-transgenic male mice (BL/6) will be immunized with the regimen that successfully eliminates tumors in TRAMP mice. Prostates will be isolated, embedded in paraffin and 25-50 cryostatic sections/mouse covering the entire prostate will be stained with H&E for the presence of discrete intraprostatic lymphomononuclear cell infiltrates organized in nodules.

3] Work associated with Major Task 2: Using human prostate cancer tissue, determine the presence of immunologic markers that were identified in Aim 1 as correlated with vaccine efficacy. Develop assays to be used in subsequent human clinical trials of DC-PAPvac-C as markers of an effective immune response (months 1-30) and Subtask 1, 2 and 3: Obtain pre- and post-vaccine treatment human prostate tissue. Analyze immune infiltrates using immunohistochemistry. Analyze for the presence of tumor apoptosis and apoptotic proteins.

Details:

We plan to analyze 20 patient tumor samples under this protocol. As of September 2016, we have obtained human prostate tissue from 3 patients and preliminary data was submitted as part of Year 2 report. In April we acquired 4 additional samples. We will continue to collect the remaining samples over the next year so that our analysis is robust and can be applied to future clinical trials.

Major Task 1: Determine the in vivo systemic and tumor-associated immune response that correlates with anti-tumor activity of the lead cellular therapy, DC-PAPvac-C (months 1-30)

Subtask 1 (1-3 months, completed): Generate mouse PAP RNA, anti-mouse CTLA4 RNA, mouse actin RNA and control IgG RNA for murine immunotherapy studies in TRAMP mice

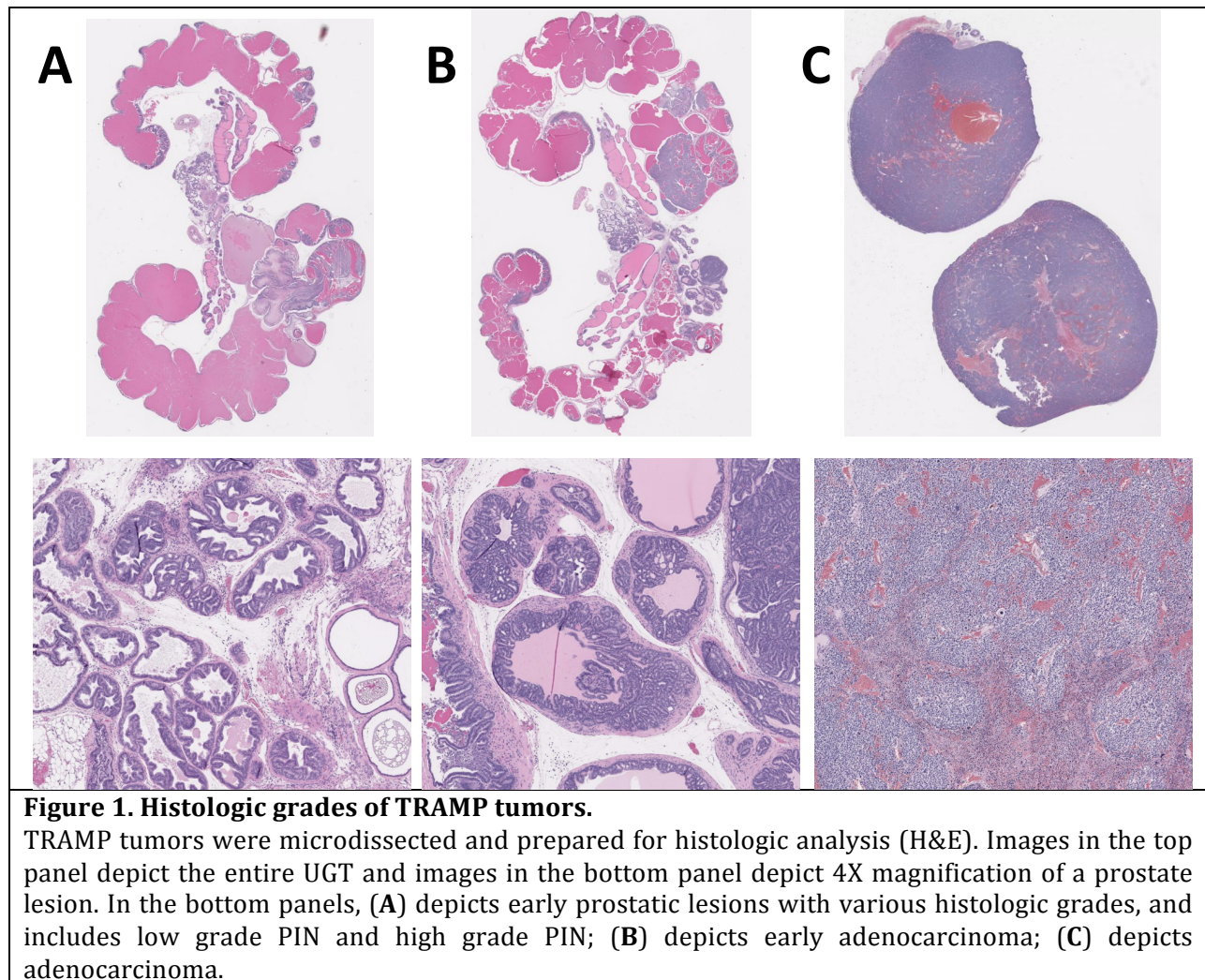
Subtask 2 (3-6 months, completed): Start first experiment with TRAMP mice, immunize mice using DC-mPAPvac-mC and controls

Subtask 3 (6-12 months, completed): Analyze immune responses in the periphery: T cell analysis, ELISpot analysis anti-PAP antibody analysis

Subtask 4 (8-20 months, ongoing): Harvest tumors from 30-week old mice to analyze tumor weight, tumor grade, tumor apoptosis and immune infiltrates and harvest mouse organs (lymph nodes, lungs, kidney, testis, colon, liver, muscle) for analysis of

inflammatory infiltrates and autoimmunity

Preliminary analysis demonstrates T cell infiltration in subcutaneous TRAMP-C1 tumors (Year 2 report). Analysis of tumor grade and tumor weight in TRAMP transgenic mice is pending. Below we present some preliminary analysis of TRAMP prostate tumor histological analysis (**Figure 1**). This analysis was not optimal and a second set of experiment is currently ongoing (part of Subtask 7).



Subtask 5 (12-15 months, completed): Start second experiment with TRAMP mice, immunize mice using DC-mPAPvac-mC and controls

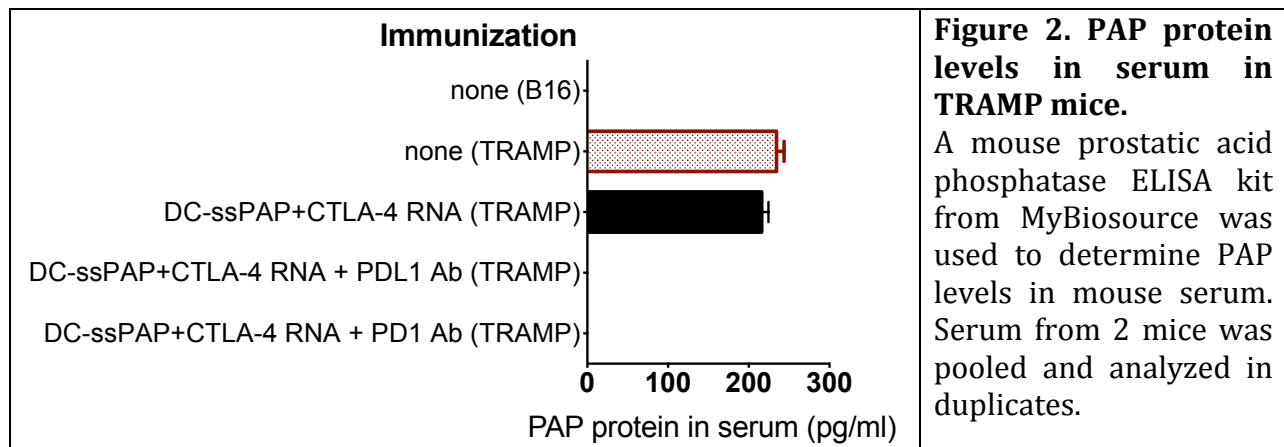
Subtask 6 (15-21 months, completed): Analyze immune responses in the periphery: T cell analysis, ELISpot analysis and anti-PAP antibody analysis

Microscopic lesions of prostate cancer can be observed in the TRAMP mice between ages 18 and 24 weeks. In a new experiment, TRAMP mice (16-18 weeks old) were vaccinated **once** subcutaneously with 4×10^5 DCs that had been transfected with mRNA encoding ssPAP and anti-CTLA-4 (DC-mPAPvac-mC). Where indicated, mice received 3 doses of 200

µg of anti-PDL1 or anti-PD1 antibody intraperitoneally on the day of vaccination, and weekly thereafter. The rationale for PD1/PDL1 blockade was to determine if our novel vaccine strategy that combines induction of tumor-specific T cells with local blockade of the inhibitory T cell receptor, CTLA4, synergizes with systemic PD1/PDL1 blockade. PD1/PDL1 blockade can be easily incorporated into clinical trials of DC-based vaccination in men with prostate cancer.

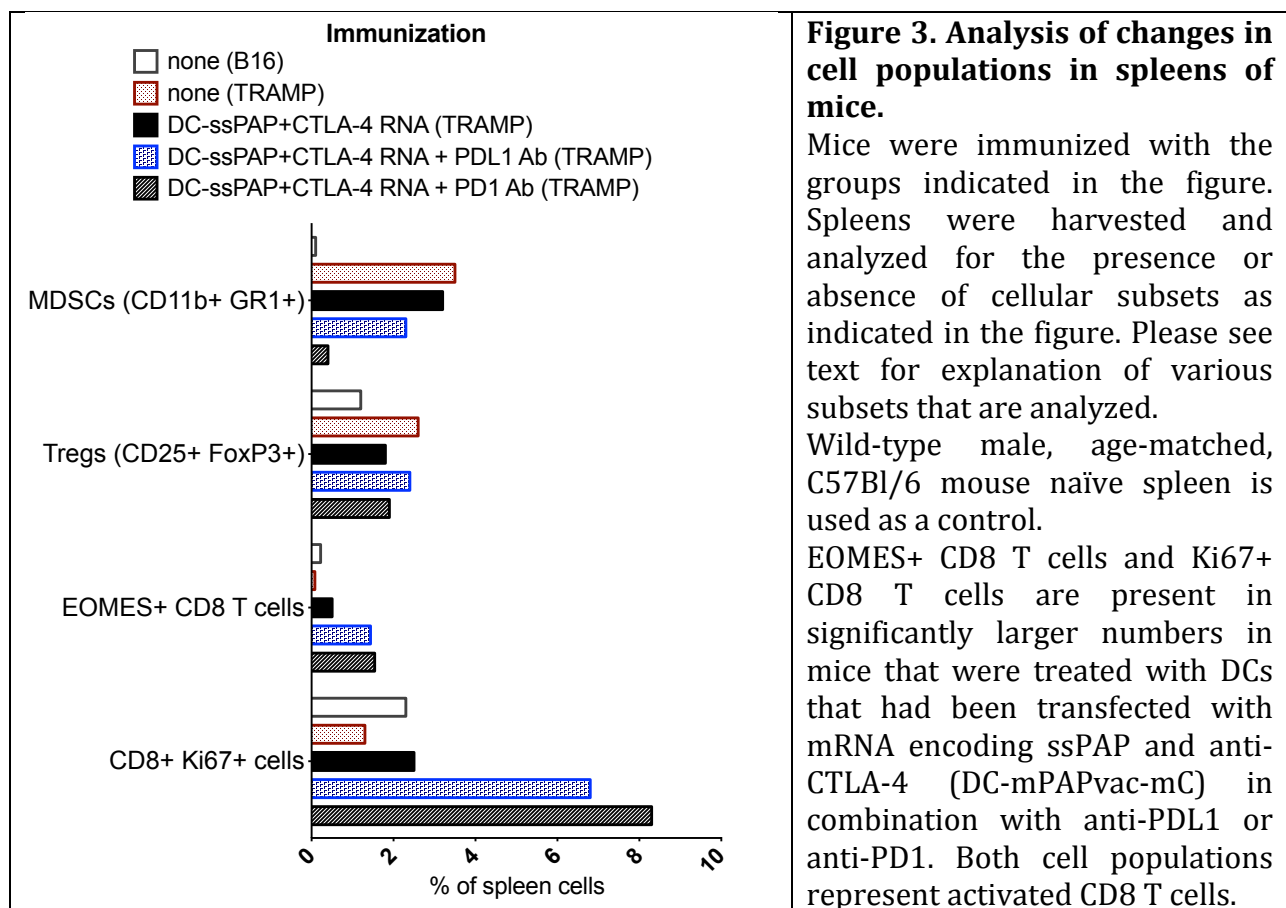
Mice were sacrificed 1 week after the last treatment and prostate, spleen, and blood were harvested. Analysis of prostate tumors is ongoing. In addition, half of the animals are still alive and the prostates will be harvested at a later date and will be examined by a pathologist for the presence or absence of prostate cancer metastases and for progression of localized cancer.

PAP ELISA: PAP levels in TRAMP mice or B16 mice have never been published, and low levels in TRAMP transgenic mice could indicate induction of an anti-PAP response. Also included was serum from age matched B16 male mice, age matched TRAMP mice, and serum obtained from immunized groups as indicated in **Figure 2**. Our data suggests that combining a single immunization of DCs that had been transfected with mRNA encoding ssPAP and anti-CTLA-4 (DC-mPAPvac-mC) with anti-PDL1 or anti-PD1 eliminates the level of PAP protein in mouse serum. As such some of our experimental values are below the detection limit of the ELISA, which is 150 pg/ml.



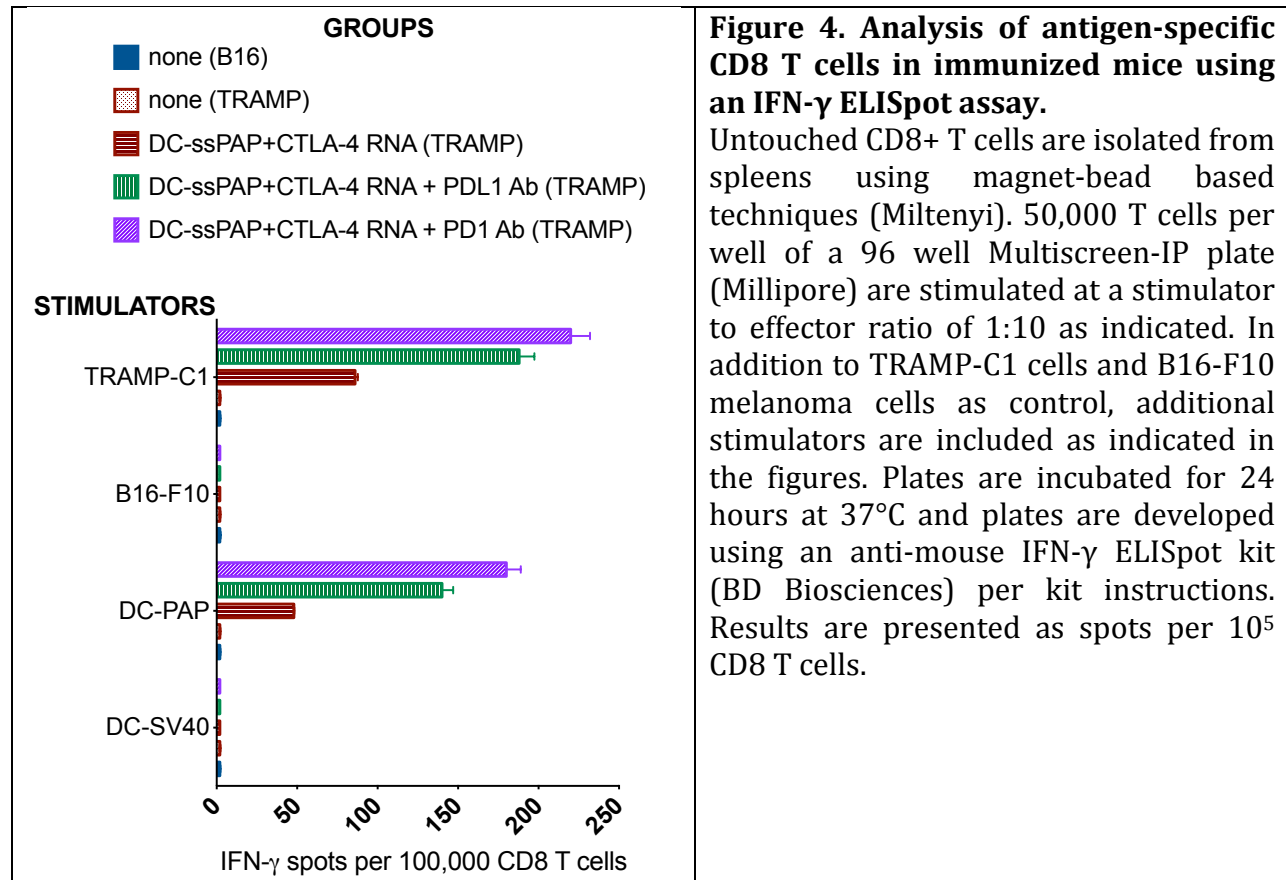
Phenotypic analyses: Splenocytes from treated or untreated animals were analyzed by flow cytometry. There were major differences in cell numbers using lineage markers CD4, CD8, CD20, or NK1.1 (not shown). Please refer to **Figure 3** for analysis. Numbers of myeloid-derived cells (also referred to as immature myeloid cells or myeloid-derived suppressor cells (MDSCs) characterized as CD11b+ and Gr1+) were significantly reduced in spleens of anti-PD-1 treated animals. Myeloid cells are an important component of cancer and inflammation. Changes in the myeloid compartment include inhibition of differentiation of DCs, polarization of macrophages toward M2 suppressive state, and expansion of MDSCs. MDSCs represent a heterogeneous population of pathologically activated myeloid cells (includes neutrophils, macrophages and DCs). In mice, these cells are defined as CD11b+Gr1+ cells. MDSCs are considered immunosuppressive and promote tumor angiogenesis, tumor cell invasion, and metastases. Expansion of MDSCs is considered a consequence of tumor progression.

Frequencies of EOMES+, a marker of CD8 T cell activation, and CD8+Ki67+ cells were significantly increased in mice that were immunized with DCs that had been transfected with mRNA encoding ssPAP and anti-CTLA-4 (DC-mPAPvac-mC) in combination with anti-PDL1 or anti-PD1. Treg frequencies did not significantly vary between treatment groups.



IFN- γ ELISpot assay: Untouched CD8 T cells were harvested from spleens and were tested for IFN- γ production using an ELISpot assay. Data is depicted in **Figure 4**.

All CD8 T cell responses were TRAMP-C1 specific and also specific for DC-PAP mRNA transfected cells. Surprisingly, no autoimmunity was observed in any of the treatment groups, and we could not detect any responses against the SV40 large T antigen.



Subtask 7 (17-30 months, ongoing): Monitor tumor burden (time to palpable tumor) and monitor survival. If mice have tumors then sacrifice and harvest prostate complex/tumor and analyze.

We have 2 ongoing experiments:

Experiment 1 Groups:

1. DC-control RNA + systemic control antibody
2. DC-prostate tumor antigen RNA + systemic control antibody
3. DC-prostate tumor antigen RNA/CTLA-4 RNA + systemic control antibody
4. DC-prostate tumor antigen RNA/CTLA-4 RNA + systemic anti-PD-1
5. DC-prostate tumor antigen RNA + systemic anti-CTLA-4 + systemic anti-PD-1

In Year 1 and 2 reports we demonstrated the expression of PAP, PSCA, PSMA and STEAP in the prostate cell lines TRAMP-C1 and TRAMP-C2. Both are cell lines that have been generated by in vitro propagation of tumors isolated from TRAMP transgenic mice. One of the main reasons for examining other prostate antigens was to determine if we could

generate a vaccine that targets all of these antigens, instead of PAP antigen alone. We also demonstrated that we are indeed able to generate antigen-specific CD4 and CD8 T cells that target each of the antigens used, namely PAP, PSCA, PSMA and STEAP. Indeed, PAP specific responses seem to be lower than the responses induced against the other prostate tumor antigens.

So we are immunizing TRAMP transgenic mice with DCs transfected with all prostate tumor antigen RNAs. Instead of co-transfecting a single DC prep with all four RNAs, we transfected DCs with each RNA and then combined the DC-RNA preparations prior to immunization. Therefore our DC vaccine consisted of a combination of 4 different DC formulations with 200,000 cells of each of the following: DC-PAP + DC-PSCA + DC-PSMA + DC-STEAP to give a combined dose of 8×10^5 DCs per vaccination.

Experiment 2 Groups:

1. DC-prostate tumor antigen RNA + systemic control antibody
2. DC-prostate tumor antigen RNA + systemic anti-CTLA-4
3. DC-prostate tumor antigen RNA + systemic anti-CTLA-4 + systemic anti-PD-1
4. DC-prostate tumor antigen RNA + systemic control antibody + non-adherent splenocytes
5. DC-prostate tumor antigen RNA + systemic anti-CTLA-4 + non-adherent splenocytes
6. DC-prostate tumor antigen RNA + systemic anti-CTLA-4 + systemic anti-PD-1 + non-adherent splenocytes

In our published study in the TRAMP model (Hess et al. 2006), we were unable to break tolerance to the tumor antigen (SV40 T antigen) using an RNA-lipid formulation as our vaccine strategy. We were however able to induce an immune response and break tolerance when we combined the RNA-lipid vaccine with adoptive transfer (AT) of syngeneic naïve splenocytes from non-transgenic male BL/6 mice. We are therefore testing adoptive transfer of syngeneic naïve non-adherent splenocytes in combination with our DC vaccine (Hess et al. 2006).

Hess PR, Boczkowski D, Nair SK, Snyder D, Gilboa E. Vaccination with mRNAs encoding tumor-associated antigens and granulocyte-macrophage colony-stimulating factor efficiently primes CTL responses, but is insufficient to overcome tolerance to a model tumor/self antigen. Cancer Immunol Immunother. 2006 Jun;55(6):672-83. PMID: 16133108

Major Task 2: Using human prostate cancer tissue, determine the presence of immunologic markers that were identified in Aim 1 as correlated with vaccine efficacy. Develop assays to be used in subsequent human clinical trials of DC-PAPvac-C as markers of an effective immune response (months 6-30)

Subtask 1 (6-30 months, ongoing): Obtain pre- and post-vaccine treatment human prostate tissue and embed them in paraffin and generate sections for immunohistochemistry.

We now have a protocol that has been determined not human subjects research that has been approved by both Duke and HRPO.

We plan to analyze 20 patient tumor samples under this protocol. As of September 2016, we have obtained human prostate tissue from 3 patients and preliminary data was submitted as part of Year 2 report. In April we acquired 4 additional samples. We will continue to collect the remaining samples over the next year.

Subtask 2 (8-30 months, ongoing): Analyze immune infiltrates

Major Task 3: Perform FDA mandated validation of DC-PAPvac-C to confirm anti-CTLA4 mAb expression and PAP presentation by human DCs transfected with mRNA encoding hPAP and anti-human CTLA4 (months 1-30)

Subtask 1 (1-9 months, completed): Characterize and optimize human PAP (hPAP) expression and anti-CTLA4 secretion by human dendritic cells (DCs)

Subtask 2 (10-15 months, completed): Evaluate the function of optimized DC-PAPvac-C

Subtask 3 (8-12 months, completed): Generate, validate, test and vial clinical-grade RNA for cGMP production of DC-PAPvac-C

Subtask 4 (12-30 months, completed): Perform three cGMP production runs including lot release testing of the final DC-PAPvac-C cellular vaccine product in the GMP-certified cellular processing laboratory

KEY RESEARCH ACCOMPLISHMENTS:

1. In murine studies, we have demonstrated that local CTLA4 modulation in combination with PAP-specific immunization using DCs transfected with PAP RNA and CTLA4 RNA is superior to DC PAP RNA immunization in T cell function assays and in the TRAMP-C1 subcutaneous tumor model.
2. In murine studies, we have demonstrated that DCs transfected with mPAP-SS RNA and CTLA4 RNA is superior to DC PAP RNA and CTLA4 RNA immunization in T cell function assays and in the TRAMP-C1 subcutaneous tumor model. We have also demonstrated the induction of anti-PAP antibodies in immunized mice.
3. We have conducted experiments to evaluate prostate tumor antigen expression in TRAMP-derived cell lines. Based on our data we have cloned additional antigens, PSCA and STEAP, for testing in DC-RNA vaccines. We show that among the 4 antigens used as vaccines in TRAMP transgenic mice, anti-PAP T cell response was the weakest as compared to T cell responses directed towards PSMA, PSCA and STEAP.
4. We have now demonstrated that PD1/PDL1 blockade synergizes with our novel vaccine strategy that combines induction of tumor-specific T cells with local blockade of the inhibitory T cell receptor, CTLA4.
5. Using human cells, we have performed FDA mandated validation of DC-PAPvac-C to confirm anti-CTLA4 mAb expression and PAP presentation by human DCs transfected with mRNA encoding hPAP and anti-human CTLA4.
6. We have completed the three cGMP production runs including lot release testing of the final DC-PAPvac-C cellular vaccine product.

CONCLUSION:

Recently, two forms of immunotherapy have demonstrated clinical benefit in patients: active immunotherapy, in which subjects are immunized with antigen presenting cells activated against tumor antigens ex vivo (e.g. sipuleucel-T in prostate cancer) and

treatment with systemic immune modulators, such as an antagonistic anti-CTLA4 mAb (e.g. ipilimumab in melanoma). However the use of anti-CTLA4 was associated with adverse events. We have developed a novel strategy that combines tumor immunotherapy targeting PAP and targeted immune modulation of CTLA4 and have generated a lead cellular therapy that will safely enhance vaccine-mediated immunity. This lead cellular therapy, called DC-PAPvac-C, consists of autologous monocyte-derived DCs co-transfected with prostate tumor antigen, PAP RNA and anti-CTLA4 RNA. Thus, targeted delivery of anti-CTLA4 antibody to sites where anti-tumor T cells are induced by tumor antigen-presenting DCs will potentially eliminate adverse effects associated with systemic administration of anti-CTLA4, while also enhancing vaccine-induced immune responses and expanding the potential role for immunotherapy in patients with cancer. In this study we will establish the preclinical efficacy and safety of our cellular therapy product, DCs transfected with RNA that encodes PAP and anti-CTLA4 and generate data required for an IND application. Importantly and relevant to our planned clinical trial implementation, we will develop a biomarker of therapeutic efficacy and demonstrate the feasibility of measuring these biomarkers.

In this report we have demonstrated that local CTLA4 modulation in combination with PAP-specific immunization using RNA-transfected DCs elicits robust and superior functional T cell responses in TRAMP mice. We have now demonstrated that PD1/PDL1 blockade synergizes with our novel vaccine strategy that combines induction of tumor-specific T cells with local blockade of the inhibitory T cell receptor, CTLA4. DC vaccine with local CTLA4 blockade in combination with systemic PD1/PDL1 blockade is more effective at induction of antigen-specific T cells than DC vaccine and local CTLA-4 blockade alone. Using human cells, we have performed FDA mandated validation of DC-PAPvac-C to confirm anti-CTLA4 mAb expression and PAP presentation by human DCs transfected with mRNA encoding hPAP and anti-human CTLA4. Finally, we have completed the three cGMP production runs including lot release testing of the final DC-PAPvac-C cellular vaccine product.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Nothing to report

INVENTIONS, PATENTS AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

Nothing to report

OTHER ACHIEVEMENTS:

Nothing to report

REFERENCES:

None

APPENDICES:

None