Award Number: W81XWH-08-1-0660

TITLE: TPD52: A Novel Vaccine Target for Prostate Cancer

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REPORT DATE: September 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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**1. REPORT DATE**
SEP 2011

**2. REPORT TYPE**

**3. DATES COVERED**
01-09-2010 to 31-08-2011

**4. TITLE AND SUBTITLE**
TPD52: A Novel Vaccine Target For Prostate Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**

**5c. PROGRAM ELEMENT NUMBER**

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**6. AUTHOR(S)**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Texas Tech University Health Sciences Center, Lubbock, TX, 79430

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

**10. SPONSOR/MONITOR’S ACRONYM(S)**

**11. SPONSOR/MONITOR’S REPORT NUMBER(S)**

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

**15. SUBJECT TERMS**

**16. SECURITY CLASSIFICATION OF:**

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**17. LIMITATION OF ABSTRACT**
Same as Report (SAR)

**18. NUMBER OF PAGES**
19

**19a. NAME OF RESPONSIBLE PERSON**

Standard Form 298 (Rev. 8-98)
Prepared by ANSI Std Z99-18
**TPD52: A Novel Vaccine Target for Prostate Cancer**

Dr. Robert Bright  
E-Mail: robert.bright@ttuhsc.edu

**ABSTRACT**

Tumor protein D52 (D52) is a novel self-onco-antigen involved in cellular transformation, proliferation and metastasis that is over-expressed in prostate cancer cells. The overall goal of this Award is to test the efficacy of D52-based vaccines in the TRAMP murine model of prostate cancer, and to characterize vaccine induced mechanisms of tumor immunity. Due to unforeseen circumstances during this funding period primarily involving the animal vendor and maternity leave for funded personnel we lost over 6 months of productivity. Consequently, we requested and were granted a no cost extension through 8/31/2012. Despite this unfortunate loss of time, over the past 12 months we made the following significant findings: Heterologous prime-boost vaccination induced more than 80% protection from primary tumor challenge the best we have observed thus far, but only 30% protection from secondary challenge about 8 months later. Similarly, if animals were immunized and rested for 8 months before challenge only about 50% were protected from significant tumor growth suggesting that induction of a more durable response may require modulation of peripheral mechanisms of immune suppression as we previously reported. Finally, we demonstrated for the first time in vivo the critical importance of both CD4+ and CD8+ effector T cells in tumor protection following D52 immunization.

**SUBJECT TERMS**

Human TPD52 (hD52); murine TPD52 (mD52); transgenic adenocarcinoma mouse prostate (TRAMP); xenogeneic antigen; T regulatory cells (Treg); transforming growth factor beta 1 (TGF-β1)

**Security Classification:**

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<thead>
<tr>
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<td>19</td>
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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>5</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>7</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>7</td>
</tr>
<tr>
<td>Conclusion</td>
<td>8</td>
</tr>
<tr>
<td>References</td>
<td>10</td>
</tr>
<tr>
<td>Figures and Tables</td>
<td>11</td>
</tr>
<tr>
<td>Appendices</td>
<td>NA</td>
</tr>
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INTRODUCTION

In 2009, the National Cancer Institute sponsored a pilot project to prioritize cancer vaccine target antigens for translational research. The study involved developing a set of nine “ideal” tumor antigen characteristics, with therapeutic function, immunogenicity and a role in oncogenicity deemed as having the greatest weight or importance [1]. From a representative list of antigens, a group defined as over-expressed self-proteins stands out as the group with the largest number of candidate target antigens to be developed as future vaccines to treat or prevent cancer. It is generally accepted that over-expressed self-tumor associated antigens represent viable candidate vaccine target antigens, and it is largely understood what constitutes an effective vaccine formulation or delivery platform. It is also clear that peripheral tolerance mechanisms are playing a role in diminishing vaccine induced tumor immunity. However, it is not clear what peripheral mechanisms are involved in inducing tolerance to this understudied group of self-tumor associated antigens. Limited knowledge in this area is an important problem that will inhibit future development of effective vaccines for many life-threatening cancers to include prostate cancer.

Tumor protein D52 (D52) is a novel over-expressed self-onco-protein involved in cellular transformation, proliferation and metastasis. D52 over-expression has been demonstrated in several human malignancies including prostate [2, 3, 4], breast [5, 6, 7, 8], and ovarian [9] carcinomas. Recent studies have identified D52 as one of 12 important markers, along with MUC-1 and PSA, that could be used as a molecular fingerprint of human prostate cancer enabling more accurate and sensitive diagnosis and prognosis of aggressive disease [10]. Our laboratory independently identified and cloned D52 from human prostate cancer cells, isolated from patients undergoing radical prostatectomy, using differential gene expression analysis of our novel paired cancer and normal human prostate epithelial cell cultures. The murine orthologue of D52 (mD52) naturally mirrors human D52 (hD52) with respect to known function and over-expression in tumor cells, and shares ~86% protein identity with the human orthologue [11]. Recently we demonstrated that transfection and stable expression of mD52 cDNA in mouse 3T3 fibroblasts (3T3.mD52) induced increased proliferation, anchorage independent cell growth, the ability to form subcutaneous tumors and spontaneous lethal lung metastases in vivo when 3T3.mD52 cells were inoculated subcutaneously into naïve, syngeneic, immune-competent mice [12]. Together, these data strongly suggest that D52 expression may be important for initiating and perhaps maintaining a tumorigenic and metastatic phenotype and thus may be important for tumor cell survival. We demonstrated for the first time that mD52 induces protection in mice against sarcoma challenge when administered as recombinant protein-based vaccine with CpG-ODN [13]. We also reported that mD52 DNA vaccination induced an immune response that rejected tumors in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer. The T cell cytokine secretion patterns suggested a Th1-type cellular immune response was involved [14]. Others reported that an mD52 overlapping peptide vaccine was effective in a murine breast cancer model [15].

Our goal is to determine the best modulation measures needed with vaccination to effectively target D52. The Transgenic Adeno-carcinoma of the Mouse Prostate (TRAMP) model has been employed to study D52 as a vaccine antigen. Naïve mice have been immunized with either mD52 or hD52 plasmid DNA or recombinant protein or with both in a heterologous prime-boost regimen. In some experiments, modulation of regulatory T cells or TGF-β1 accompanied vaccination. Following immunization, mice are typically challenged with a tumorigenic dose of mD52 positive, autochthonous TRAMP tumor cells. To test vaccine durability immunized mice that survive primary tumor challenge are rechallenged 6-8 months later. In some experiments mice are challenged up to 8 months after the final immunization. Significant tumor protection has been observed more than ten months post tumor challenge. The T cell cytokine secretion patterns from tumor challenge survivors indicate that a memory, cellular immune response is involved in tumor rejection, and that both CD4+ CD25+ Tregs and a unique subset of regulatory CD8 T cells may play a role in inhibiting vaccine induced tumor immunity.
Although our research accomplishments described here address the specific funding period from September 1, 2010 through August 31, 2011, due to unforeseen circumstances during this funding period we lost over 6 months of experimental productivity. Consequently, we requested from CDMRP and were granted a no cost extension through 8/31/2012. Despite this unfortunate loss of time, we made the following significant findings over the past 12 months. Heterologous prime-boost vaccination induced more than 80% protection from primary tumor challenge the best we have observed thus far. However, only 30% protection from secondary challenge given about 8 months later was observed. Similarly, when animals were immunized and rested for 8 months before challenge only about 50% were protected from significant tumor growth suggesting that induction of a more durable response may require modulation of peripheral mechanisms of immune suppression as we previously reported. Finally, we demonstrated for the first time in vivo the critical importance of both CD4+ and CD8+ effector T cells in tumor protection following D52 immunization. In addition, we began studies to further assess the role of both CD4+ and CD8+ Treg cells in suppressing D52-vaccine induced tumor immunity. To this end, we immunized mice with mD52 DNA as this regimen provided moderate protection only as compared to hD52 DNA or heterologous prime-boost. This would allow us to assess whether modulation of CD4+CD25+ Tregs or CD8+ CD122+ Tregs or both together would result in greater than 50% tumor protection following mD52 DNA immunization as we reported previously. Moreover, it is critical that we address a true auto-antigen scenario by immunizing mice with mouse D52 since translational studies and perhaps early human studies may not allow for the administration of the xenogeneic mouse gene or protein to human patients.

Research Accomplishments

The following research discussion contains unpublished data that should be protected.

For our continued efforts to optimize our vaccine strategy to try and reach 100% protection for primary tumor challenge we immunized groups of male C57BL/6 mice using a heterologous prime-boost regimen. Priming consisted of plasmid DNA encoding human D52 (hD52) for three injections at 14-day intervals followed by a single boost with mD52 protein. Primary and secondary tumor challenges were administered on day 44 and 181 post immunization, respectively. In some vaccine groups CD4 or CD8 depleting antibodies were injected with vaccination and primary tumor challenge (Figure 1). Following immunization and prior to tumor challenge tail vein blood was collected into heparin treated tubes from 5 randomly selected mice from each group. Peripheral blood lymphocytes were analyzed by flow cytometry to determine T cell depletion efficacy. Dual staining with fluorochrome labeled antibodies specific for CD4 (FITC) or CD8 (PE) revealed we had obtained 80 to near 100% depletion of the targeted T cell subsets indicating that our efforts to remove either CD4 or CD8 effectors in vivo were successful (Figure 2). Approximately 80% (8/10) of the immunized mice that were simultaneously depleted of CD4 T cells developed tumors. Nearly 90% (8/9) of the CD8 T cell depleted and immunized mice developed tumors compared to < 20% (1/6) of immunized and mock depleted (non-specific IgG) mice developed significant tumor growth by day 126 (Figure 3). This demonstrated for the first time that T cell-mediated immunity is critical for tumor protection following D52-based vaccination in vivo. Somewhat surprising was the tremendous impact helper CD4 T cells have on D52-vaccine induced tumor immunity, clearly help for CTL-mediated killing is necessary. Given that D52 is a small (~27kD) intracellular protein and in light of our previous in vitro data the critical role CD8 effector T cells (CTLs) play was expected. A summary of tumor growth in control immunized mice (represented by empty vector DNA, irrelevant protein, adjuvant alone or no immunization) compared to immunized mice that were mock depleted, CD4 T cell depleted or CD8 T cell depleted is depicted in figure 4. As
we have reported previously the cytokine profile of T cells from immunized and protected mice continues to be dominated by a Th1-type cellular immunity phenotype defined by production of IFN-\(\gamma\) in the absence of detectable Th2 or Th17 cytokines IL-4 and IL-17, respectively. Interestingly, we continue to see detectable production of IL-10 in a subset of what appear to be MHC-restricted, tumor specific CD8 T cells (summarized in Table 1). Together, these data provide ongoing support for our hypothesis that CD8+ CD122+ Treg cells that produce IL-10 along with CD4+ CD25+ Treg cells may suppress D52-specific vaccine-induced immune responses.

To address the issue of T regulatory cell (Treg) suppression of vaccine induced tumor immunity further we began studies targeting either CD25 or CD122 Treg cells with D52 immunization in vivo. Treg cells, whether CD4+ CD25+ or more recently CD8+ CD122+, have been best described in models of autoimmunity. This is expected given that the presumed role of Tregs in the periphery is to control responses to self-tissue antigens and prevent subsequent autoimmune pathologies. Since D52 is a non-mutated self-protein that is simply over-expressed in tumor cells, a presumed role for Tregs in suppressing D52-based vaccination is logical. We have observed varying degrees of tumor protection following D52-vaccination, depending on the route of injection, adjuvant employed and composition of the vaccine (DNA vs. protein or both). Protection has ranged from 0 to 80%. In order to see the most dramatic effect on protective immunity with in vivo depletion of Treg cell subsets we chose a vaccine approach that has been consistently in the middle of the protection range. We previously reported that vaccination with mD52-DNA resulted in ~50% protection from tumor challenge in the TRAMP model. We chose mD52-DNA delivered i.m. to begin our in vivo evaluation of the role of Treg cells. Groups of male CD57BL/6 mice were immunized i.m. with 50 \(\mu\)g of mD52-plasmid DNA at 14 day intervals for a total of 4 injections. Tumor challenge consisted of an inoculation (s.c.) with 5 \(\times 10^5\) TRAMP-C2 cells 14 days after the final immunization. Groups of immunized mice were either mock depleted (non-specific IgG) or depleted of CD25+ or CD122+ cells at the time of each immunization and finally at the time of tumor challenge (Figure 5). Following immunization and prior to tumor challenge tail vein blood was collected into heparin treated tubes from 3-5 randomly selected mice from each group. Peripheral blood lymphocytes were analyzed by flow cytometry to determine Treg depletion efficacy. Dual staining with fluorochrome labeled antibodies specific for CD4 (FITC) and CD25 (PE) revealed we had obtained over 70% depletion of the targeted CD4+ CD25+ Treg cell subset (Figure 6A). Similarly, dual staining with fluorochrome labeled antibodies specific for CD8 (FITC) and CD122 (PE) revealed we had obtained over 70% depletion of the targeted CD8+ CD122+ Treg cell subset (Figure 6B). Together, these data indicate that our efforts to remove either CD4+ CD25+ or CD8+ CD122+ Treg cells in vivo were successful (Figure 6). It is important to note that it has been reported that CD4+ CD25+ Treg cells typically make up <5% of the gated lymphocytes analyzed and CD8+ CD122+ Treg cells typically make up 1 to 2%. A TRAMP tumor cell challenge experiment takes 6-9 months to complete depending on whether or not the animals are re-challenged to test immune memory and vaccine durability. Our efforts to deplete immunized animals of CD4+ CD25+ Tregs or CD8+ CD122+ Tregs are at the 42 day post tumor challenge as such this experiment is ongoing and is expected to conclude in 4 to 5 months from the time of writing this report. However, some interesting observations have emerged thus far. Approximately 100% (8/8) of the immunized mice that were simultaneously depleted of CD25 Tregs are tumor free by day 42 (Figure 7B). Whereas, only 33% (3/9) of immunized CD122 depleted mice remain tumor free by day 42 (Figure 7C). And nearly 85% (6/7) of immunized and mock depleted (non-specific IgG) mice remain tumor free (Figure 7A). Though interesting, it is far too soon to come to any conclusions. Six weeks is early in tumor development and as indicated by the tumor volume on the Y-axis of figure 7, these tumors are quite small compared to those in figure 3 for day 126 where the tumors are an order of magnitude greater in volume. In our experience as the experiment approaches 5 to 6 months the tumors could continue to grow or regress. Finally, table 2 is an attempt to summarize our vaccine efforts to date, in line with our original statement of work.
**Future Work**

In the coming weeks and months we will perform T cell function assays and determine tumor volume on mice immunized with or without modulation of Treg cells (CD25+ or CD122+ cells depleted) *in vivo*. We will also have direct *in vivo* have data on the role of CD4+ CD25+ and CD8+ CD122+ Treg cells from our ongoing experiments to deplete these T cell subsets with mD52-DNA vaccination. We plan to perform immune assays for IFN-γ production, ELISA based cytokine capture assays for the production of IL-4, IL-10, IL-17 and IFN-γ to assess the role of various T Helper cell subsets, and CTL-mediated killing assays. For the upcoming year, we will further evaluate the role of T reg cells with *in vitro* assays. We will also further our studies of TGFβ-1 to assess its role in suppression of tumor immunity following D52-based vaccination.

**KEY RESEARCH ACCOMPLISHMENTS**

*(The research accomplishments outlined below were completed by a 50% FTE associate and myself at 30% effort)*

- We demonstrated that heterologous prime-boost vaccination comprised of priming with 3 injections of hD52-DNA followed by mD52-protein boost resulted in 80% protection from tumor challenge in the TRAMP model of prostate cancer. This is the most protection from primary challenge we have observed to date.
- We demonstrated that our heterologous prime-boost vaccine approach was not as effective at preventing tumor growth on rechallenge 8 months after vaccination. Similarly, immunized mice that were rested 8 months then challenged for the first time were only 50% protected compared to 80% for primary challenge at 14 days, suggesting this approach does not induce as durable response as we have observed previously.
- We demonstrated for the first time *in vivo* the critical importance of both CD4+ and CD8+ effector T cells in tumor protection following D52 immunization by depleting the T cell subsets in conjunction with heterologous prime-boost vaccination.
- As we have seen with various other vaccine approaches the response appears to be dominated by a TH1-type cellular immune response where CD8+ CTLs are critical.
- We initiated studies to evaluate the role of regulatory T cells in suppressing D52-based vaccination by *in vivo* depletion of CD25+ or CD122+ T cells, representing CD4+ CD25+ Treg cells and CD8+ CD122+ Treg cells, respectively.

**REPORTABLE OUTCOMES**

**Abstracts:**


**Manuscripts:**

Several relevant manuscripts are in preparation or submitted. None pertaining to work supported by this award are in print or in press at present. We expect to have 3-4 publications relevant to this funding award printed in the next 2 years.

**Presentations:**

CDMPR CRPR IMPaCT 2011 Investigator Highlights online research video. This was 1 of 2 highlighted investigators.

• American Cancer Society, Man to Man, prostate cancer education and support group. Lubbock, Texas. “Prostate Cancer Vaccination” Spring 2011.

• Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Science, Grand Rounds. Bright RK. “Vaccination Targeting Over-Expressed Self-Oncoantigens: the TPD52 story” September, 2010

**Funding applied for:**

• Cancer Prevention Research Institute of Texas (CPRIT); submitted 05/31/2011; Robert Bright (PI); “Therapeutic Targeting of a Novel Onco-protein Shared by Multiple Human Cancers”; total direct costs: $1,223,741

• National Cancer Institute; NIH 1 R01A1 CA157465-01; submitted 04/08/11; Robert Bright (PI); “Shared Over-Expressed Self-Cancer Antigens: Novel Targets for Vaccination”; 30% effort; total direct costs: $1,250,000

**CONCLUSIONS**

Overall our efforts to induce tumor immunity following heterologous DNA-prime, protein-boost immunization have been more than promising. It is becoming clear that hD52 is a more immuno-potent antigen in mice resulting in superior tumor immunity despite that fact the these are highly conserved genes phylo-genetically and the human and mouse proteins share nearly 86% identity. We predicted this to be so given the success of others involving the application of murine or rodent tumor associated antigens as xenogeneic vaccines in human clinical trials. It is reasonable to speculate that murine D52 could be developed in the future for human use as a xenogeneic vaccine formulation. Clearly some form of prime-boost will likely result in the most potent immune response. Although we did see the greatest protection against primary tumor challenge to date with this approach, durability was not as strong and the goal is 100% protection before we move into treatment models. Perhaps, hD52-DNA followed by hD52-protein instead of using mD52-protein to boost will be even more effective, with increased durability for recurrent disease.

Previously we determined that CD25+ Treg cells and TGF-β1 likely play a critical role in inhibiting D52-based immunity following vaccination. This is not surprising given that D52 is a non-mutated self-protein that is over-expressed in tumors. These data are critical for guiding and driving our efforts to optimize vaccination with immune modulation. In addition, we previously uncovered a novel and understudied subset of CD8+ IL-10 secreting regulatory T cells. These CD8 Tregs have been described in models of infectious disease and autoimmunity but have lacked investigation in cancer immunity. Deeper understanding of the role of these novel Treg cells along with CD25 Treg cells could lead to the development of more potent and effective vaccines targeting over-expressed self-antigens like D52, resulting in more effective immune therapies for many malignancies to include prostate cancer. To this end, we have begun studies to assess the role of CD25 and CD122 Treg cells in our D52-vaccine TRAMP model of prostate cancer, with our initial studies depleting these T cells subsets in vivo in conjunction with vaccination and tumor challenge.
RELEVANCE “So What”:

D52 is involved in the induction of transformation and metastasis and has been shown by us and other investigators to be over-expressed in human prostate, breast, lung, ovarian and colon cancers. Recent studies have identified TD52 as one of twelve important protein markers, along with MUC-1 and PSA that can be used as a molecular fingerprint of human prostate cancer enabling more accurate and sensitive diagnosis and prognosis of aggressive disease. Our ongoing studies suggest that D52 vaccination induces immunity capable of rejecting tumors in vivo without the induction of autoimmunity or other harmful side effects. **We believe the results generated by our research will demonstrate that D52 is capable of inducing tumor immunity when administered as a vaccine, and that this immunity will specifically destroy prostate tumor cells without autoimmune side effects.**

D52-based vaccines defined and characterized by this study could be clinically developed to treat advanced cancer or for preventing progression and metastasis. DNA and protein-based vaccines have already proven to have little to no toxicity in patients when studied in clinical trials, making these approaches very safe. Our results are expected to have an important positive impact on cancer vaccine development by providing a deeper understanding of how to induce tumor immunity with vaccines targeting non-mutated self-proteins that are over-expressed in tumors. Our vaccine data, together with increased knowledge on how to manipulate Treg cell-mediated mechanisms of peripheral tolerance to self-tumor associate antigens, will likely lead to the next generation of prostate cancer vaccines with greater potency.
REFERENCES


Figure 1

**500 ug of control or depleting mAb i.p. with all 4 immunizations and with tumor challenge**

**Figure 1. Heterologous Prime-Boost Immunization and T cell Depletion Schedule:** All three hD52-DNA priming immunizations were administered in alternating quadricep muscles. A boost immunization with mD52 protein was administered subcutaneously (s.c.). Primary and secondary tumor challenge doses were $5 \times 10^5$ TRAMP-C2 tumor cells administered s.c. in opposite flanks. T cell depletion: 500 ug of specific mAb (anti-CD4 or anti-CD8) i.p. on the days the vaccine was administered for a total of 4 injections. Mock depletion was IgG isotype matched non-specific mAb.
**Figure 2.** Flow Cytometric Analysis of T cell Subsets Following In Vivo Depletion. The data show the percent of either CD4+ or CD8+ T cells in the peripheral blood of mice injected with subset specific mAb (CD4 depleted or CD8 depleted) or control mAb (mock depleted) based on the gated lymphocyte population.
Figure 3.

Figure 3. Tumor Growth Following Heterologous Prime Boost Vaccination: Male C5/Black/6 mice, age 6-8 weeks old were vaccinated every 10 days beginning with human D52.pcDNA3.1 DNA (hD52-DNA) i.m. for three injections ending with recombinant mouse D52 protein (mD52-protein) + 1826 ODN/CpG s.c. Tumor challenge was administered 14 days later with D52 expressing TRAMP-C2 cells. A role for CD4+ or CD8+ T cells was assessed in response to vaccination and tumor challenge by in vivo depletion of the specific T cell subsets. A) Immunized, mock depleted. B) Immunized, CD4 T cell depleted. C) Immunized, CD8 T cell depleted.
Figure 4. T Cell Depletion with hD52-DNA-mD52-Protein Heterologous Prime Boost Vaccination. The data show the percent of tumor free mice more than 120 days post tumor inoculation following immunization with mock depletion (center bar) or T cell depletion (right bar). The vaccine was ineffective if either A) CD4+ or B) CD8+ T cells were depleted further underscoring their importance in D52 induced tumor immune rejection. Control is represented by vector DNA and irrelevant protein immunization, incomplete Freunds adjuvant alone (IFA) or no immunization followed by tumor challenge.
Table 1

**T Cell Cytokine Profile Summary**

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<tr>
<th>Cytokine Production</th>
<th>Vaccine hD52 DNA</th>
<th>mD52 DNA</th>
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<tr>
<td>IL-4</td>
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<td>negative</td>
<td>not done</td>
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<tr>
<td>IL-17</td>
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<td>TGF-β1</td>
<td>negative</td>
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<td>IL-10</td>
<td>positive</td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>positive</td>
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Representative results from ELISA analyses of culture supernatants from *in vitro* stimulated T cells

**Figure 5**

*Figure 5. mD52-DNA Immunization and Treg Depletion Schedule:* All four mD52-DNA priming immunizations were administered in alternating quadriceps muscles. Primary tumor challenge dose was 5x10⁵ TRAMP-C2 tumor cells administered s.c. Treg cell depletion: 300 ug of specific mAb (anti-CD25 or anti-CD122) given i.p. on the day of the first and third vaccinations, and a final injection of 600 ug was administered at the time of tumor challenge. Mock depletion was IgG isotype matched non-specific mAb.
Figure 6A. Flow Cytometric Analysis of Treg cell Subsets Following In Vivo Depletion.
The data show the percent of either CD4+CD25+ (Fig. 6A) or CD8+CD122+ (Fig. 6B) Treg cells in the peripheral blood of mice injected with subset specific mAb (CD25 depleted or CD122 depleted) or control mAb (mock depleted) based on the gated lymphocyte population.
Figure 6B. Flow Cytometric Analysis of Treg cell Subsets Following In Vivo Depletion. The data show the percent of either CD4+CD25+ (Fig. 6A) or CD8+CD122+ (Fig. 6B) Treg cells in the peripheral blood of mice injected with subset specific mAb (CD25 depleted or CD122 depleted) or control mAb (mock depleted) based on the gated lymphocyte population.
Figure 7. Tumor Growth Following mD52-DNA Vaccination: Male C57Black/6 mice, age 6-8 weeks old were vaccinated every 14 days beginning with mouse D52 (mD52)-DNA as described in figure 5. Tumor challenge was administered 14 days later with D52 expressing TRAMP-C2 cells. A role for CD25+ or CD122+ Treg cells was assessed in response to vaccination and tumor challenge by in vivo depletion of the specific Treg cell subsets. A) Immunized, mock depleted. B) Immunized, CD25 Treg cell depleted. C) Immunized, CD122 Treg cell depleted.
Table 2

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</tr>
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<td>hD52-protein +ODN in IFA, deplete TGF-B1</td>
<td>subcutaneous</td>
<td>80%</td>
<td>Not tested</td>
<td>Black6/TRAmp</td>
</tr>
<tr>
<td>hD52-DNA prime / mD52-protein boost</td>
<td>DNA- intramuscular Protein- subcutaneous</td>
<td>80%</td>
<td>incomplete</td>
<td>Black6/TRAmp</td>
</tr>
</tbody>
</table>

Summary of our general vaccine findings under this award to date