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<b>14. ABSTRACT</b> Vaccines against prostate cancer are one of the few immunotherapies that have shown success for this cancer. IN this project we studied the potential of vaccines targeting the ERG gene and mutations in the tumors. We found that we could generate an anti-tumor T-cell response with peptides against the ERG gene or mutations specific to the tumors. However, we also found that the T-cell response was greatly limited in mice with ERG expressing tumors. We hypothesize that tolerance mechanisms in the cancer bearing mice limit the vaccine responsiveness.					
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## **1. Introduction**

Cancer vaccines aim to direct the immune system against proteins specifically expressed by the tumor. Ideal targets for immunotherapy are proteins expressed highly in the tumor and expressed very low levels or not at all in other tissues. Early studies identified these antigens by co-culturing tumor explants with patient lymphocytes and analyzing the antigen specificity of T-cells that responded to the tumor. The melanoma antigen, MAGE-1, was identified using this approach, and similar experiments identified a range of antigens in other cancers (Traversari et al., 1992; van der Bruggen, 1991). Antigens discovered using these methods are now approved for use or are currently in clinical trials for many different cancers. In the case of prostate cancer, Sipuleucel-T targets the prostate cancer antigen prostatic acid phosphatase (PAP) and is currently the best available treatment for metastatic castration resistant prostate cancer (CRPC) (Kantoff et al., 2010). Similar treatments targeting other known prostate cancer antigens such as Prostavac are also under investigation (Kantoff et al., 2010b, NCT01322490). While this initial approach to antigen discovery is showing excellent results, new technology has advanced vaccine design. We have used a novel genomics based approach using micro-array profiling to analyze tumor gene expression to discover potential prostate cancer antigens such as Erg and Sim2 (Arredouani et al., 2009). This project aims to validate this method of antigen discovery by investigating the efficacy of these epitopes in *in vitro*, and *in vivo* models, and to expand upon this method by using next generation sequencing to identify coding mutations in cancer that could be novel targets for immunotherapy.

## **2. Key Words**

Immunotherapy

Prostate Cancer

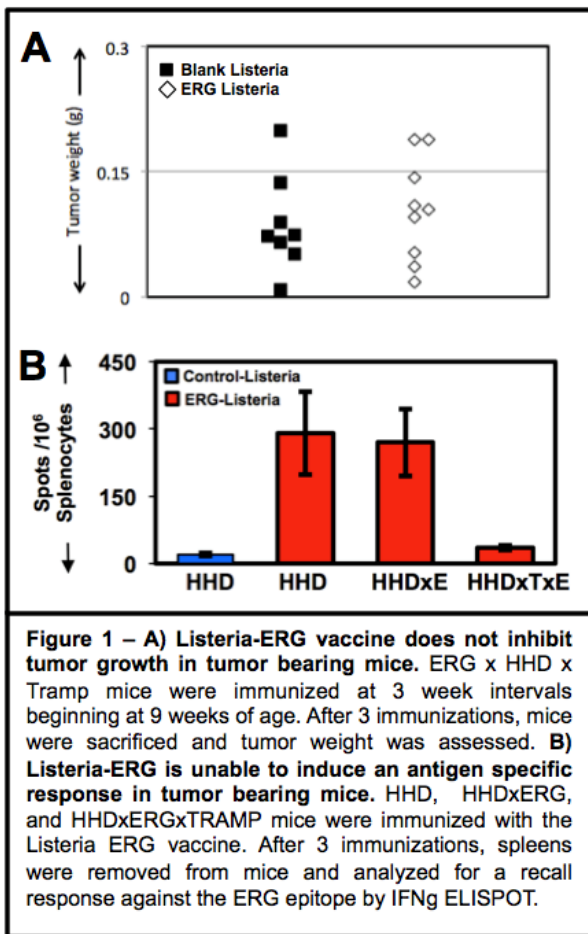
T Cell Exhaustion

Vaccines

### 3. Accomplishments

### 3. Overall Project Summary

Outcomes of the SOW goals are listed below:



1. Determine the *in vivo* anti-tumor effect of the ERG-derived short and long peptides in a transgenic ERG/TRAMP/A2.1 mouse model and a TRAMP-C2/A2.1/ERG subcutaneous model

1a) Establish 2 *in vivo* ERG expressing PCa models to investigate the anti-tumor effect of ERG319-327 derived peptides

To determine if the ERG-319-327 epitope could be used to elicit an anti tumor response we bred transgenic ERG/TRAMP/A2.1 mice. We also generated HHD/ERG mice to determine immunization against a lower grade cancer. These mice developed tumors at a comparable rate to Tramp mice.

1b) Investigate the anti-tumor activity of the ERG peptides against the ERG expressing transgenic tumor model and the subcutaneous ERG tumor model (Months 4-12)

Instead of using peptides, we generated a listeria-based vaccine that expressed the ERG319-327 epitope (Figure 1A). Listeria-ERG generated a strong T-cell response against the ERG epitope was detectable in both HHD and HHD-ERG mice. However, when the ERG/TRAMP/A2.1 mice were immunized, there was a significant reduction in the number of T-cells that responded to the ERG319-327 epitope (Figure 1B). In addition, no changes tumor mass

between immunized and un-immunized mice was observed. These data suggest that while a strong immune response can be generated against ERG, this is lost in mice where ERG is expressed as a tumor antigen.

2. Investigating the number and functionality of ERG reactivity of T-cells in PCa patient blood based on ERG fusion status

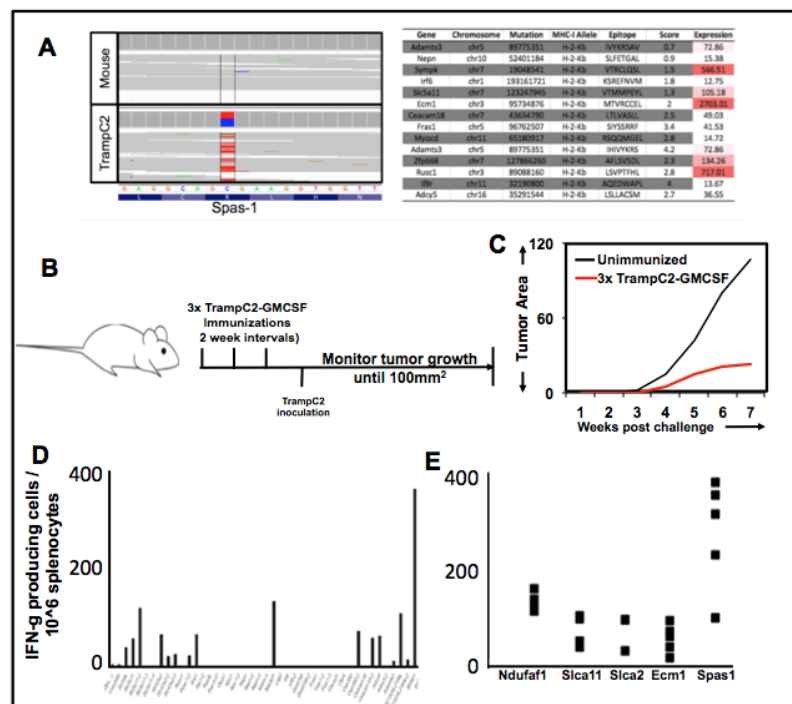
2a) Establish VCaP HLA-A0201 expressing cells (Months 1-4)

This cell line failed to grow and we did not complete this aim

2b) Analyse the number and activity of ERG reactive T-cells against LNCaP/ERG+, PC3/HLA-A0201+/ERG+ and VCaP HLA-A0201+ cells stratifying for ERG status (Months 4-24)

This aim was completed without the VCaP addition and details are outlined in the attached published manuscript (Kissick, 2013).

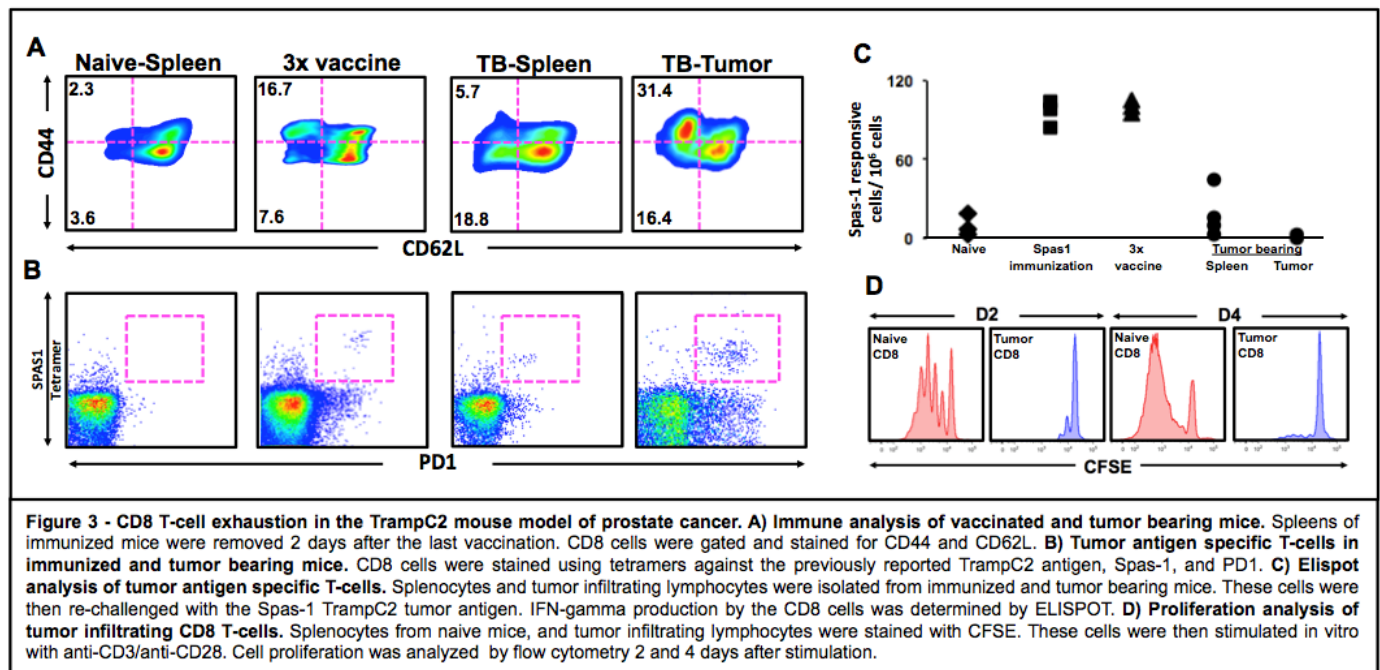
3. Sequencing RNA from TRAMP1 and



**Figure 2 – A) Detection of coding mutations in TrampC2 cell.** DNA from TrampC2 cells and normal tramp mouse blood was sequenced. The known immunogenic mutations in Spas-1 was detected along with over 50 other mutations. Binding of MHC epitopes to H2KB was then predicted using the NetMHC algorithm. **B) Experimental design.** Mice are immunized 3 times with  $1.5 \times 10^6$  irradiated TrampC2-GMCSF cells at 2 week interval. 2 weeks after the last immunization, mice are challenged subcutaneously with  $2 \times 10^6$  live TrampC2 cells. **C) Tumor growth and mouse survival.** Tumor area was measured twice weekly and mice were sacrificed when tumors reached  $100 \text{mm}^2$ . **D and E) Protected mice have detectable immune response against coding mutations.** Spleens from immunized mice that had not developed tumors after 12 weeks were removed and analyzed for responsiveness to the 50 highest predicted immunogenic mutations in the Tramp C2 tumors using IFN- $\gamma$  ELISPOT. In repeat experiments, only the top 5 epitopes were tested and found to generate a similar response.

## TRAMP2 to determine the presence of coding, immunogenic mutations and whether immunization against these mutations can elicit an anti-tumor response

3a) Sequence the RNA from TRAMP-C1 and TRAMP-C2 cells and identify the coding mutations that generate potentially immunogenic epitopes in TRAMP-C1 and TRAMP-C2 cells



We instead performed DNA sequencing to detect mutations in these cell lines due to the ease of sample preparation. Using this technique, sequencing of TRAMP-C2 cells was performed and numerous potentially immunogenic epitopes were discovered (**Figure 2A**).

### 3b) Treat subcutaneous TRAMP-C2 tumors with peptides derived from mutations

To test if these epitopes were recognized by we immunized mice with irradiated Tramp-C2 GMCSF tumor cells. After 3 immunizations, mice were challenged with a subcutaneous inoculation of TrampC2 tumor cells (**Figure 2B**). The vaccine successfully slowed tumor growth, and 4/5 mice had no detectable tumor 7 weeks after tumor challenge (**Figure 2C**). We then tested the recall response against 50 immunogenic epitopes encoding tumor specific mutations in these immunized mice that had rejected live TrampC2 tumors. Of the 50 tested, 5 generated a detectable T-cell recall response suggesting that numerous mutations are recognized by the T-cells (**Figure 2D and E**). Further analysis of the vaccine regime found that it induced a large population of CD44<sup>+</sup>CD62L<sup>-</sup> effector CD8 T-cells (**Figure 3A**). Additionally, CD8 cells recognizing the previously reported SPAS-1 antigen were detectable in the spleen of the immunized mice (**Figure 3B**). We next tested the recall response against the Spas-1 antigens in these tumor bearing mice. Similar to what we found in the ERG-Tramp mice, mice with large tumors had significantly reduced number of T-cells responding to the tumor epitope (**Figure 3C**) and had reduced ability to respond to anti-CD3 stimulation (**Figure 3D**). Based on this finding that T-cells specific for mutations are present in tumors and tumor bearing mice but have lost the ability to respond to antigen we are now focused on identifying the mechanisms that control T-cell exhaustion in prostate cancer (See **Additional Data Collected** section).

We also used individual peptides to investigate responsiveness, but only vaccination with the SPAS-1 eptopes generated an anti-cancer effect. As this had been reported by others previously, we did not publish these data. We believe that this is an important observation, and highlights that relatively few mutations actually generate an anti-cancer effect. For this reason our work now focuses on mechanisms that inhibit the ongoing response, rather than looking for

### What was accomplished under these goals?

Aim 1 was completed, and we found that the expression of ERG in the context of tumors resulted in a reduced antigen specific response. This was reported in Figure 1 of the 2015

Aim 2 found that human patients have ERG reactive T-cells. This work was published in Kissick et al., 2013 (See section 6)

Aim 3 found that mutations exist in tumors and a T-cell response could be detected against some of these mutations. However, these cells were ultimately exhausted in a similar manner as other epitopes. This work was reported in figure 2 and 3 of the 2015 progress report.

**What opportunities for training and professional development has the project provided?**

Dr. Kissick received a K99/R00 award from the NCI as a direct result from this work. This project has allowed Dr. Kissick to continue his post-doctoral training and take a tenure track faculty position at Emory University.

**How were the results disseminated to communities of interest?**

Data from this project was published in peer reviewed journals (see section 6 for products)

**What do you plan to do during the next reporting period to accomplish the goals?**

Final report – Nothing for the next period

#### **4. Impact**

##### **What was the impact on the development of the principal discipline(s) of the project?**

This work showed that the transcription factor ERG is a possible vaccine target for prostate cancer. It also highlighted some of the challenges of using mutations as vaccine targets. Specifically, we found that mutation specific T-cells exist in tumors, however, only a few of the many mutations actually generate a T-cell response. Furthermore, these cells have a similar exhausted phenotype as self-epitopes. For this reason we have moved the focus of our research towards looking at the mechanisms that control T-cell exhaustion in cancer.

##### **What was the impact on other disciplines?**

None.

##### **What was the impact on technology transfer?**

None.

##### **What was the impact on society beyond science and technology?**

We have continued to develop a vaccine targeting ERG, and we anticipate that a DNA vaccine targeting this protein may be used to treat patients with prostate cancer.

#### **5. CHANGES/PROBLEMS:**

##### **Changes in approach and reasons for change**

None

**5. CHANGES/PROBLEMS:**

**Changes in approach and reasons for change**

None

**Actual or anticipated problems or delays and actions or plans to resolve them**

None

**Changes that had a significant impact on expenditures**

None

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

None

## **6. Products**

### **Publications, Abstracts, and Presentations**

**Kissick HT**, Sanda MG, Dunn LK, Arredouani MS. Development of a peptide-based vaccine targeting TMPRSS2:ERG fusion-positive prostate cancer. *Cancer Immunol Immunother*. 2013 Dec;62(12):1831-40. doi: 10.1007/s00262-013-1482-y.

**Kissick HT**, Sanda MG, Dunn LK, Arredouani MS. Immunization with a peptide containing MHC class I and II epitopes derived from the tumor antigen SIM2 induces an effective CD4 and CD8 T-cell response. *PLoS One*. 2014 Apr 1;9(4):e93231. doi: 10.1371/journal.pone.0093231.

**Kissick HT**, Sanda MG. The Role of Active Vaccination in Cancer Immunotherapy: Lessons from Clinical Trials. *Current Opinions in Immunology*, 2015 Aug;35:15-22. doi:10.1015/j.coi.2015.05.004

**Kissick HT**. Is It Possible to Develop Cancer Vaccines to Neoantigens, What Are the Major Challenges, and How Can These Be Overcome? Neoantigens as Vaccine Targets for Cancer. *Cold Spring Harb Perspect Biol*. 2017 Dec 18. pii: a033704.

### **Website(s) or other Internet site(s)**

None

### **Technologies or techniques**

None

### **Invention, Patents, and Licenses**

None

### **Other Products**

None

## **7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

**What individuals have worked on the project?**

No Change

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

No Change

**What other organizations were involved as partners?**

No Other Organizations



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*Cancer Immunol Immunother.* 2013 December ; 62(12): . doi:10.1007/s00262-013-1482-y.

## Development of a peptide-based vaccine targeting TMPRSS2:ERG fusion positive prostate cancer

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### Abstract

Identification of novel vaccine targets is critical for the design and advancement of prostate cancer (PCa) immunotherapy. Ideal targets are proteins that are abundant in prostate tumors while absent in extra-prostatic tissues. The fusion of the androgen-regulated TMPRSS2 gene with the ETS transcription factor ERG occurs in approximately 50% of prostate cancer cases and results in aberrant ERG expression. Because expression of ERG is very low in peripheral tissue, we evaluated the suitability of this protein as an antigen target in PCa vaccines. ERG-derived HLA-A\*0201-restricted immunogenic epitopes were identified through a 3-step strategy that included *in silico*, *in vitro*, and *in vivo* validation. Algorithms were used to predict potential HLA-A\*0201-binding epitopes. High scoring epitopes were tested for binding to HLA-A\*0201 using the T2-based stabilization assay *in vitro*. Five peptides were found to bind HLA-A\*0201 and were subsequently tested for immunogenicity in humanized HLA-A\*0201 transgenic mice. The *in vivo* screening identified three immunogenic peptides. One of these peptides, ERG295, overcame peripheral tolerance in HLA-A\*0201 mice that expressed prostate restricted ERG. Also, this peptide induced an antigen specific response against ERG-expressing human prostate tumor cells. Finally, tetramer assay showed detectable and responsive ERG295-specific cytotoxic lymphocytes in peripheral blood of HLA-A\*0201<sup>+</sup> prostate cancer patients. Detection of ERG-specific CTLs in both mice and the blood of prostate cancer patients indicates that ERG-specific tolerance can be overcome. Additionally, these data suggest that ERG is a suitable target antigen for PCa immunotherapy.

### Keywords

Prostate Cancer; Vaccine; ERG; Epitope

### Introduction

Numerous vaccine therapies for PCa are currently in various phases of clinical trials or clinical use. Tumor antigen-specific vaccines have been shown to improve PCa survival in Phase III (Provenge) [1] and Phase II (Prostvac) [2] clinical trials. These studies provide proof-of-principal that prostate cancer is responsive to immunotherapy with antigen-specific cancer vaccines. While these treatments are promising, there is significant room for

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**Conflict of Interest**

The authors disclose no potential conflicts of interest

improvement. Recent trials in advanced renal cell carcinoma found that the clinical outcome of patients receiving a multi-peptide vaccine, IMA901, correlated with the number of vaccine epitopes the patient responded to [3]. With this in mind, immunotherapy to treat prostate cancer may be improved by defining new epitopes targeting novel prostate cancer antigens that could be used alongside current targets.

Defined epitope vaccines use minimal protein sequences to direct the humoral or cellular immune response against the desired target. Epitope vaccines have the advantage of allowing precise immune control and the capability to direct the immune response against the most antigenic regions of the target. These vaccines can be short 9 amino-acid long peptides that bind a particular MHC-I molecule, or longer peptides that contain multiple class I epitopes. Inclusion of CD4 epitopes in defined epitope vaccines also enhances B cell, and CD8 T cell function. In the case of cancer, defined epitope vaccines elicit a response against proteins expressed specifically in the tumor. Treatment of patients with gp100:209:217(210M), an HLA-A\*0201 restricted melanoma epitope, in combination with IL-2 significantly improved median overall survival of metastatic melanoma patients [4]. Multi-epitope vaccines targeting the E6 and E7 oncoproteins of human papilloma virus type 16 have also been used to treat high-grade vulvar intraepithelial neoplasia. This treatment resulted in complete regression of the lesions in 25% of women [5]. Numerous defined epitope vaccines are also in phase I and phase II clinical trials for PCa (Clinical Trial Numbers: NCT00616291, NCT00694551, NCT01784913). One potential limitation of these current PCa epitope vaccines is that they target antigens with little or no functional role. This may allow for selection of antigen negative variants with no fitness cost to the tumor. Targeting an antigen with oncogenic function may be more suitable for cancer vaccines because the selection of antigen negative variants could have additional anti-tumor benefits.

Recently, fusion between the androgen-regulated *TMPRSS2* and the *ETS* transcription factor *ERG* has been described in PCa. This fusion leads to *TMPRSS2* promoter-driven regulation of *ERG* expression and is present in approximately 50% of prostate cancer [6]. Given that low levels of *ERG* are found in the periphery and that the fusion product promotes tumor progression, we aimed to develop a defined epitope vaccine to induce CTLs specific for *ERG* [7-9]. In the present study, we sought to identify HLA-A\*0201-restricted epitopes derived from human *ERG*, the most common HLA allele in Caucasians [10]. These 9-residue peptides were predicted using different algorithms and tested for their ability to bind and stabilize the HLA-A\*0201 complex *in vitro*. Also, we investigated whether these *ERG* derived epitopes could overcome peripheral tolerance by investigating immunogenicity in both humanized HLA-A\*0201 (HHD) and HLA-A\*0201/probasin-*ERG* hybrid mice. Finally, to determine if epitope reactive T cells were present in prostate cancer patients PBMCs, *ERG* fusion positive and *ERG* fusion negative patients were tested for reactivity to the epitopes.

## Methods

### Mice

HHD mice were obtained from Dr. Francois Lemonnier (Unite d'Immunité Cellulaire Antivirale, Institut Pasteur, Paris, France). These mice are  $\beta_2m^{-/-}$ , *Db*<sup>-/-</sup> double knockout and express an HLA-A\*0201 mono-chain composed of a chimeric heavy chain ( $\alpha 1$  and  $\alpha 2$  domains of HLA-A\*0201 allele and the  $\alpha 3$  and intracellular domains of *Db* allele) linked by its NH<sub>2</sub> terminus to the COOH terminus of the human  $\beta_2m$  by a 15-amino acid peptide arm [10]. Probasin-*ERG* (*ERG*<sup>pb/pb</sup>) mice on the B57BL/6 background were obtained from Dr. Pier Paolo Pandolfi (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA) and were generated as described in Carver *et al* [8]. HHD × *ERG*<sup>pb/pb</sup> mice were generated by crossing HHD mice with the *ERG*<sup>pb/pb</sup> mice. Offspring were genotyped

for expression of both molecules. All mice were housed in pathogen-free conditions, and all experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center.

### Cell lines

T2 cells used in HLA-A\*0201 binding assays and as targets in ELISPOT assays were obtained from ATCC and cultured as described in the accompanying product protocol. PC3 and LNCaP lines were obtained from ATCC. PC3-A\*0201<sup>+</sup> cells were produced by transfecting wild type PC3 cells with an HLA-A\*0201-puromycin containing retrovirus produced as described in Maeurer *et al* [12]. ERG-RFP or RFP expression was induced in the PC3 and LNCaP cells using a lentiviral transduction system provided by Dr. Owen Witte (UCLA, Los Angeles, CA) as described in Zong *et al* [13] (See supplemental figure 1).

### Prediction of epitopes derived from ERG

To predict potential ERG-derived nonamers epitopes that bind HLA-A\*0201, the most frequent haplotype in Caucasians, the ERG protein sequence was processed using SYFPEITHI, RankPep, and NetMHC prediction algorithms [14-16]. The 10 highest scoring peptides that were predicted by all algorithms were selected for further screening.

### Peptide binding and stabilization of HLA

All peptides were acquired from Chi Scientific (Maynard, MA). Peptide purity was tested by HPLC and was greater than 95% in all instances. Peptides were dissolved in either water or DMSO. HLA stabilization assay using T2 cells was used to assess binding of peptides to the HLA-A2.1 complex. Briefly, T2 cells were cultured for 6 h in serum-free Iscove's modified Dulbecco's medium (American Type Culture Collection) before the addition of candidate peptides at a concentration of 50 µg /  $2.5 \times 10^5$  cells/ml and further overnight incubation at 37°C. Cells surface HLA-A2.1 expression was analyzed by flow cytometry. A negative peptide (NEG) [16] and the Flu matrix peptide M1 binder peptide [18] served as controls. The relative binding affinity of a given peptide was calculated as MFI (peptide)/MFI (negative peptide). Only relative binding affinities of 1.5 or higher were considered for further testing. To test stabilization over time, T2 cells were incubated overnight with 50 µg/mL of each candidate peptide at 37°C in serum-free Iscove's modified Dulbecco's medium. Cells were then incubated with brefeldin A (Sigma) at 10 µg/mL for 1 h, washed, and incubated at 37°C for 0, 2, 4, or 6 h in the presence of brefeldin A (50 ng/mL). At each time point, cells were then stained with anti-HLA-A\*0201 mAb (BB7.2). For each time point, peptide-induced HLA-A\*0201 expression was calculated as follows: (mean fluorescence of peptide-loaded T2 cells) / (mean fluorescence of negative peptide-loaded T2 cells). The rate of dissociation is reflected by the loss of A2.1 expression over time.

### ERG-derived peptide immunogenicity in transgenic mice

Eight- to 12-wk-old male HHD mice were injected sub-cutaneously on the right flank with 100 µg of each candidate peptide emulsified in 50 µL of incomplete Freund's adjuvant and 50 µL PBS in the presence of 150 µg of the I-Ab-restricted HBVcore<sub>128-140</sub> T helper epitope (TPPAYRPPNAPIL) [19]. Ten to 12 d after immunization, spleens were harvested and splenocytes were tested for peptide-induced specific release of IFN-γ by enzyme-linked immunospot (ELISPOT) assay.

### ELISPOT assay

ELISPOT was performed as described by manufacturer's instruction. Briefly, 96-well Millipore Immobilon-P plates were coated with 100 µL/well mouse IFN-γ-specific capture mAb (AN18; Mabtech, Inc.) at a concentration of 10 µg/mL in PBS overnight at 4°C. To

investigate the recall response to immunization with various peptides, a total of  $2.5 \times 10^5$  splenocytes were seeded in each well in four replicates, and  $2.5 \times 10^5$  peptide-loaded ( $10 \mu\text{g}$  peptide/mL, for 2 h at  $37^\circ\text{C}$ ) splenocytes pretreated with  $50 \mu\text{g}/\text{mL}$  mitomycin C for 1 h were added to each well. To investigate the response of immunized mice to prostate cancer cell lines,  $5 \times 10^4$  Splenocytes isolated from immunized mice were cultured with  $5 \times 10^4$  tumor cells pretreated with  $50 \text{ g}/\text{ml}$  of mitomycin C for 1h. ELISPOT was developed as described in manufacturer's instruction (Mabtech, Murine IFN-gamma ELISPOT kit). Spots measured in these experiments were multiplied by the appropriate dilution factor to express IFN- $\gamma$  producing cells per million splenocytes.

### Tetramer staining

AlexaFluor647-labeled HLA-A\*0201 tetramers loaded with HA-M1<sub>58</sub> (GILGFVFTL) or ERG295 (QLWQFLLEL) were produced by the NIH tetramer facility at Emory University (Atlanta, GA). Splenocytes from HHD mice were stained with anti-CD8-FITC and 7-AAD. Cells were gated for positive expression of CD8 and negative staining with 7-AAD. PBMCs that had been stimulated with aAPCs were stained with tetramer-AlexaFluor647, anti-CD8-FITC and 7-AAD. Relative expression of ERG tetramer<sup>+</sup> cells was determined by dividing the percentage of CD8<sup>+</sup> cells that were ERG<sup>+</sup> cells by the number of CD8<sup>+</sup> cells that stained HA<sup>+</sup>.

### Chromium release assay

Ten million target cells were suspended in 1ml of PBS and incubated with  $200 \mu\text{Ci}$  of  $^{51}\text{Cr}$  at  $37^\circ\text{C}$  for 2 hours. Target cells were washed 3 times with RPMI and  $1 \times 10^4$  cells were placed in 96 well V-bottom plates. Splenocytes isolated from mice were co-cultured at ratios between 200:1 and 12.5:1 for 4 hours. Spontaneous radiation release was determined by incubating target cells without any effector cells and maximal release was determined by incubating cells with 0.5% SDS instead of effector cells. Specific target lysis was determined using the formula  $(\text{sample reading} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ .

### In vitro Expansion of Erg-specific CTL from Prostate Cancer Patients and Control Men screening

Blood was collected from a random pool of prostate cancer patients who did not undergo prostatectomy and a separate group of patients who had undergone this procedure as per our institutional IRB-approved protocols. Peripheral blood mononuclear cells (PBMC) were isolated using BD Tigertop tubes, washed three times with PBS and stained with anti-HLA-A\*0201-FITC (BB7.2) or isotype control. Among patients with newly diagnosed (untreated) prostate cancer, TMPRSS2:ERG status was determined using a urine TMPRSS2:ERG fusion assay (performed by Gen-Probe, CA) as previously described [22]. Artificial antigen presenting cells (aAPCs) were used to expand HA or ERG295 antigen specific T-cells as described in Butler *et al* 2009 [20]. Briefly, aAPCs were cultured for 1 hour in serum free RPMI, washed 3 times with PBS and then incubated in serum free RPMI with  $10 \mu\text{g}/\text{ml}$  of ERG or HA peptide for 4 hours at  $37^\circ\text{C}$ . aAPCs were then irradiated with 100Gy X-rays or incubated with  $50 \mu\text{g}$  mitomycin-C for 30 minutes and washed 5 times with PBS. PBMCs isolated from HLA-A\*0201<sup>+</sup> donors and aAPCs were mixed at a ratio of 20:1 at a cell density of  $2 \times 10^6$  cells/ml in LGM-3 serum free media (Lonza, CC-3211) supplemented with 1% human AB serum (Atlanta Biologics, S40110). After one day, cells were supplemented with 20IU/ml of IL-2 and 10ng/ml IL-15 every second day for 9 days. After 9 days cells were analyzed for tetramer binding by flow cytometry.

## Statistical Analysis

Statistical analysis was performed using the Student's T-test. P values of less than 0.05 were considered significant.

## Results

### In silico prediction and in vitro validation of ERG derived HLA-A\*0201- restricted peptides

For peptides to be immunogenic, they must bind and stabilize the MHC complex. The SYFPEITHI, RankPep, and NetMHC algorithms were used to select potential MHC-I binding sequences from the ERG protein. Predictions covered areas downstream of the fusion location of human ERG with TMPRSS2 and focused on the HLA-A\*0201 haplotype. Shown in Figure 1A are the top 10 peptides derived from the ERG protein that were predicted to bind to HLA-A\*0201 by the SYFPEITHI algorithm and confirmed by RankPep and NetMHC. To validate the *in silico* analysis and determine if these peptides physically bound to HLA-A\*0201, a T2 assembly assay was performed. In this assay peptide binding to MHC-I is quantified by stabilization of HLA-A\*0201 transit/expression on the cell surface as measured by FACS. Peptide binding to HLA-A\*0201 was validated for six of the 10 screened peptides that stabilized the HLA-A\*0201 complex (**Figure 1B**). Peptide-HLA-A\*0201 dissociation rate correlated with time and showed weak stabilizing epitopes (ERG157, ERG412, ERG295) that did not significantly increase HLA-A\*0201 expression after 6 hours and strong stabilizing epitopes (ERG194 and ERG63) that significantly increased HLA-A\*0201 expression for greater than 6 hours (**Figure 1C**).

### Autologous, ERG derived, HLA-A\*0201-restricted peptides are immunogenic in humanized HHD mice

*In vivo* immunogenicity requires the presence of a CD8 T-cell that recognizes the specific peptide/MHC complex. Additionally, peripheral tolerance mechanism may inhibit T-cells recognizing a peripherally expressed antigen like ERG. Given that human ERG is 99% homologous to murine ERG, the HHD mouse is an appropriate model to determine whether the identified peptides could elicit an *in vivo* T-cell response. These mice are  $\beta 2m^{-/-}D_b^{-/-}$  double knockout and express the human HLA-A\*0201 allele [10]. After immunization of HHD mice with candidate ERG peptides, the ERG-specific CTL responses were analyzed by ELISPOT. ERG<sub>157</sub> ( $366 \pm 131$  cells/ $10^6$  splenocytes), ERG<sub>295</sub> ( $405 \pm 119$ ), and ERG<sub>412</sub> ( $272 \pm 73$ ) each induced a significant ERG-specific response compared to controls ( $p < 0.05$ ), while ERG<sub>63</sub> (16) and ERG<sub>278</sub> (10) did not (**Figure 2A**). Confirmation of ERG<sub>295</sub> immunogenicity *in vivo* was undertaken using ERG<sub>295</sub>-specific tetramer. ERG<sub>295</sub>-immunized mice showed significant induction as detected by ERG<sub>295</sub> tetramer compared to control mice (**Figure 2C**, 0.31% vs 0.095% CD8 T-cells,  $p < 0.05$ ). These results demonstrate that, despite low endothelial expression of ERG, immunization with select autologous peptides can elicit an ERG-specific CTL response in HHD mice.

### The capacity to induce ERG-specific CTL persists despite increased prostate-specific ERG expression in HHDx ERG<sup>pb/pb</sup> mice

To further characterize tolerance to the ERG antigen, we generated HHD x ERG<sup>pb/pb</sup> mice that have human HLA-A\*0201<sup>+</sup> and over-express human ERG specifically in the prostate. As human ERG shares 99% homology with the mouse counterpart, this is an appropriate model to investigate tolerance to the self-antigen. Male HHD or HHDxERG<sup>pb/pb</sup> mice around 16 weeks old were immunized either 1 or 3 times at 7-day intervals with the ERG<sub>295</sub> peptide. Following 1 immunization, HHD mice had  $275 \pm 45$  ERG<sub>295</sub> responsive cells per million splenocytes while the HHDxERG<sup>pb/pb</sup> mice had  $177 \pm 51$ , both significantly more than the control immunized mice. Similarly, following 3 immunizations, no significant

difference in the number of ERG295 responsive T-cells was observed between HHD ( $405 \pm 120$ ) and HHD $\times$ ERG<sup>pb/pb</sup> mice ( $305 \pm 49$ ) ( $p=0.26$ ), however, both had significantly more than the control mice ( $p < 0.05$ ) (**Figure 2B**). Together these findings indicate that the potentially tolerizing effects of prostate restricted ERG expression can be overcome with selected ERG derived peptides.

### ERG is naturally processed and presented by HLA-A2.1+ human prostate tumor cells

Proteasomal digestion of cellular proteins and presentation of the peptide products on the HLA molecules is a restricted process, and not all constituents are presented. Therefore, we next investigated whether the ERG derived HLA-A\*0201-restricted peptides were endogenously processed and presented by human prostate cancer cells. To this aim, PC3 and LNCaP cells that stably expressed HLA-A\*0201 and ERG or a vector control were constructed (see supplementary Figure 1). Splenocytes harvested from mice immunized with control or ERG<sub>157</sub>, ERG<sub>295</sub>, or ERG<sub>412</sub> were co-cultured with PC3-A\*0201<sup>+</sup>-ERG<sup>+</sup> or PC3-A\*0201<sup>+</sup>-ERG<sup>-</sup> cells in an IFN $\gamma$  ELISPOT assay. Splenocytes isolated from mice immunized with the ERG412 peptide had no significant differences in activity against WT PC3, PC3-A2.1-Vector or PC3-A2.1<sup>+</sup>-ERG<sup>+</sup> tumor cells when compared to controls. In contrast, splenocytes from ERG157-immunized mice had significantly increased activity against both PC3-A2.1-Vector and PC3-A2.1<sup>+</sup>-ERG<sup>+</sup> cells when compared to controls suggesting that this peptide may increase T-cell activity against the PC3 cells regardless of ERG expression. Finally, an ERG specific response was generated in mice immunized with ERG295 as shown by a significantly increased response to the PC3-A2.1<sup>+</sup>-ERG<sup>+</sup> cells compared to PC3-A2.1<sup>+</sup>-Vector cells. Additionally, splenocytes from these mice caused specific lysis of PC3-ERG cells but not PC3-Vector cells (**Figure 3D**). Similar results using LNCaP-Vector and LNCaP-ERG cells as targets showed ERG295 as the only epitope to induce ERG specific targeting (**Figure 3B**). These data indicate that an antigen specific response can be generated against ERG expressing HLA-A\*0201 positive cells by immunization with ERG<sub>295</sub> peptide.

### Detection and expansion of ERG295 reactive CTL in prostate cancer patients

Central and peripheral tolerance mechanisms inhibit T-cell activation against endogenous antigens like ERG. Additionally, presentation of antigens in a tumor-specific context can result in antigen-specific T-cell anergy [21]. To investigate how patients may respond to the ERG295 epitope, PBMCs from healthy and prostate cancer patients with the HLA-A\*0201<sup>+</sup> haplotype were co-cultured with aAPCs loaded with either HA-M1<sub>58-66</sub> or ERG295 (See supplementary table 1 for details of patient characteristics). Induction of ERG-specific CTLs was then evaluated by IFN- $\gamma$  ELISPOT assay (**Figure 4A**), and a significant recall response was generated at all peptide concentrations. In addition, the number of antigen-specific T-cells was assessed using an ERG295-HLA-A\*0201 tetramer. Following stimulation with the aAPC-loaded ERG295 peptide, the mean percentage of CD8<sup>+</sup> T-cells from all patients tested that were ERG295 tetramer positive was  $0.95\% \pm 0.63\%$ . To quantitate how patients responded to the autologous ERG antigen compared to a prototypical foreign epitope, the ratio of ERG295<sup>+</sup> T-cells to influenza derived HA-M1<sub>58</sub> reactive cells was assessed by tetramer assay. Stimulation of T-cells from the blood of healthy HLA-A\*0201 donors generated ERG295 reactive T-cells at a frequency of 0.45 as often as HA-M1<sub>58</sub> positive cells. Prostate cancer patient's responses to ERG295 antigen was significantly reduced compared to healthy patients ( $p < 0.05$ ) but no significant difference was observed between patients pre- or post prostatectomy (**Figure 4C**). To investigate the patients' failure to respond to the ERG295 epitope, we assessed eligible patients' ERG status by urine PCR [22]. Interestingly, the *in vitro* response to the ERG295 antigen was evident in patients with TMPRSS2:ERG fusion as well as in those lacking the fusion (**Figure 4C**). These data support what was observed in the mouse model (**Figure 2B**) and indicates that prostate

specific over-expression of ERG does not necessarily abrogate the response of ERG-specific CTLs.

## Discussion

In this study, we sought to design a defined epitope vaccine targeting the transcription factor ERG. Toward this goal we used a multi-step approach involving *in silico*, *in vitro*, and *in vivo* investigation to determine which portions of the ERG protein are presented by HLA-A0201 to the immune system. Also, we investigated whether tolerance to the auto-antigen was a factor limiting its use as an immunotherapy target. Peptides were selected to not include regions close to the N-terminus of the protein as the fusion to TMPRSS2 occurs somewhat randomly and may result in loss of the region targeted by the vaccine. From 10 *in silico* identified potential HLA-A\*0201 binding epitopes, 6 were found to bind efficiently. This rate of success is consistent with what has previously been reported for this approach [24]. Interestingly, the peptides found to be the least stable *in vitro* had the highest immunogenicity *in vivo*. This is in contrast to previous findings showing that *in vivo* immunogenicity correlates to the strength of the *in vitro* stabilization [25]. *In vivo* testing of the epitopes in humanized HHD mice revealed that 3 of the 6 induced a CD8 T-cell response as detected by ELISPOT. Our data showed that an ERG295 specific response could be generated against ERG expressing cell lines (Figure 3). However, ERG157 and ERG412 could not induce a response against these same cells, despite both of these peptides having a higher binding affinity to HLA-A0201. While it was unexpected that the lower binding peptide resulted in a better anti-tumor response, investigation of any links between *in vitro* peptide/MHC class I interactions and *in vivo* immunogenicity has found no correlation exists [26]. Also, this finding supports the notion that peptide presentation is more complex than MHC-I binding affinity and that numerous factors contribute to peptide presentation including affinity for the TAP molecule and cytosolic half-life [27-28]. Nonetheless, Together these data indicate that the ERG295 peptide is presented in an HLA-A0201 restricted manner on cells expressing the ERG molecule.

A significant consideration for any tumor vaccine is tolerance to the epitope. We found that ERG specific T-cells from patients and both HHD mice and HHD×ERG<sup>pb/pb</sup> mice were present. Our data supports the observation that central deletion is imperfect, and results in detectable self-reactive T-cells in the periphery [29, 30]. Further supporting the idea that central tolerance in humans is imperfect, numerous other groups have been able to expand self antigen specific T-cells against TAAs like MART-1 in melanoma and PSA in prostate cancer, [31, 32]. In addition to central tolerance, peripheral tolerance mechanisms like T-cell anergy, deletion and induction of peripheral antigen specific regulatory T-cells (Tregs) would be expected to inhibit the expansion of ERG295 specific T-cells. One possibility is that anergy and peripheral deletion require persistent antigen exposure and may be limited if the antigen is only expressed at very low levels [33, 34]. ERG expression has been reported in endothelial cells *in vitro* [35]. However, our previous work selected ERG as a potential antigen for immunotherapy based on data from the Gene Expression Atlas (BioGPS) of the Genomics Institute of the Novartis Research Foundation showing that ERG expression was absent or expressed very low in all peripheral tissues [36-37]. A further consideration regarding peripheral tolerance in cancer is that antigens presented in the context of a tumor can induce tolerance in TAA specific T-cells [20]. We did not investigate how ERG being presented as a TAA altered the response to the antigen in mouse models, which is potentially a limitation of this work. However, our findings showing that ERG295 reactive cells could be expanded and detected in TMPRSS2:ERG positive prostate cancer patients indicates that prostate tumor ERG expression does not always limit expansion of ERG295 positive cells. Why a variable response to the antigen occurred in patients is unclear from this study. However, moving forward to clinical investigation of this epitope, pre-screening

of patients for reactivity to the ERG295 epitope should be performed so that later correlation to vaccine efficacy can be made. Nonetheless, these data clearly show that in some cases the potential recipients of this vaccine, i.e. prostate cancer patients with ERG<sup>+</sup> prostate tumors, possess ERG295 reactive T-cells.

Together, our findings indicate that vaccines targeting the transcription factor ERG may elicit a CTL response in patients and that prostate cancer cells expressing ERG will be potential targets of these induced CTL. In addition, because of the sequence homology between ERG and the ETS factor FLI1, a gene that fuses with the EWS gene in the majority of Ewing sarcoma cases [38], FLI1 harbors the same QLWQFLLEL sequence and could therefore be a potential target of vaccines developed using this epitope for HLA-A\*0201<sup>+</sup> patients with Ewing sarcoma. Also, the ETS1 factor, a proto-oncogene that is present in several malignancies [39], including PCA [40] also harbors the ERG295 epitope, hence offering the possibility to use this epitope as a vaccine component to target melanoma, lymphoma, liver, kidney, brain and CNS, and esophageal cancers. Additionally we hypothesize that immunizing against tumor oncogenes, such as ERG, compared to non-oncogenic targets like PSA could create a conditional lethality where the tumor must remain a target of the immune system or discard the target oncogene. Following this premise and the data presented in this paper, further investigation of vaccines targeting ERG for the treatment of prostate cancer is justified.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## References

1. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010; 363(5):411–22. doi: 10.1056/NEJMoa1001294. [PubMed: 20818862]
2. Kantoff PW, Schuetz TJ, Blumenstein BA, Glode LM, Bilhartz DL, Wyand M, et al. Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol*. 2010; 28(7):1099–105. doi: 10.1200/JCO.2009.25.0597. [PubMed: 20100959]
3. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med*. 2012; 18(8) doi: 10.1038/nm.2883.
4. Schwartztruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med*. 2011; 364(22):2119–27. doi: 10.1056/NEJMoa1012863. [PubMed: 21631324]

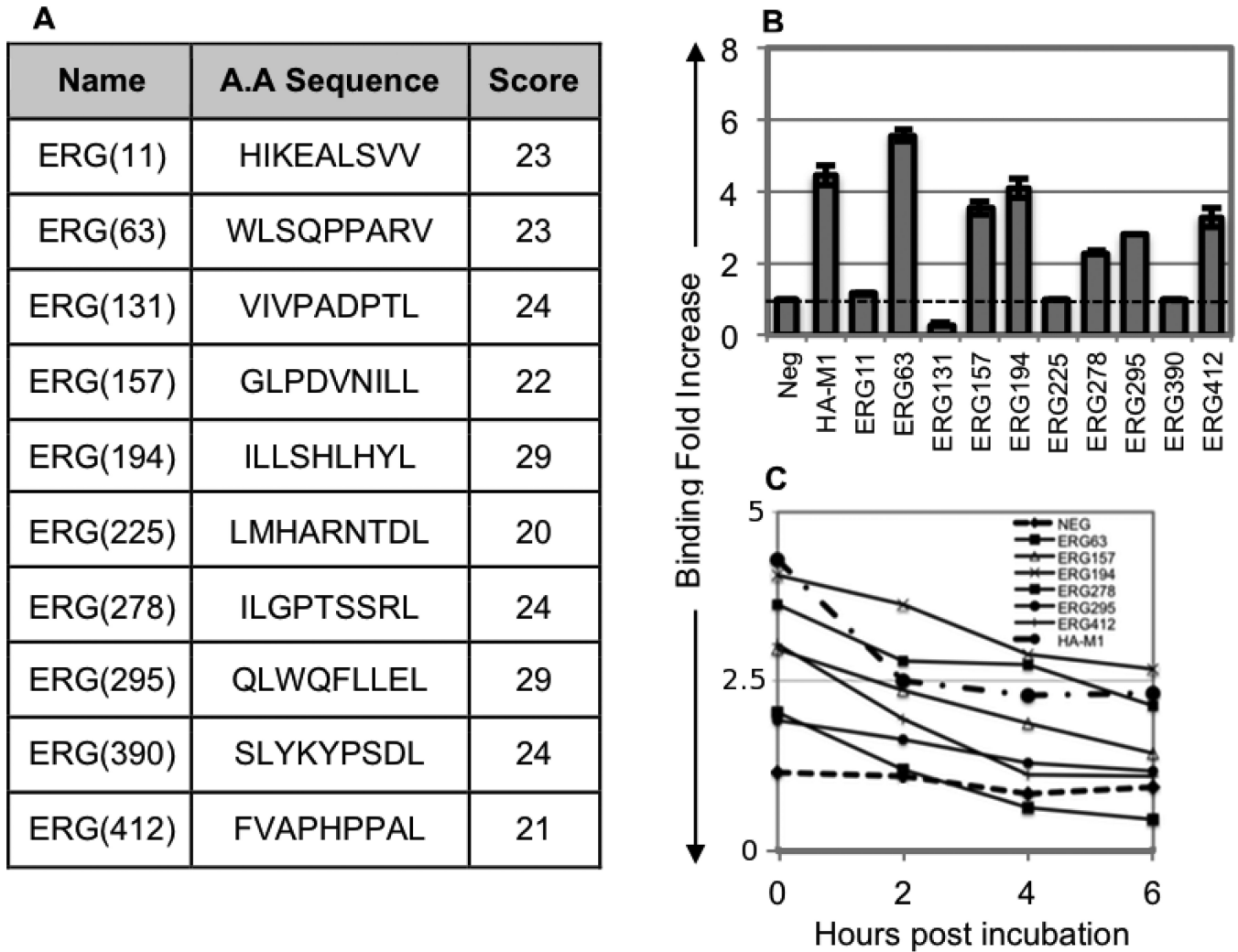
5. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*. 2009; 361(19):1838–47. doi: 10.1056/NEJMoa0810097. [PubMed: 19890126]
6. Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science*. 2005; 310(5748):644–8. doi: 10.1126/science.1117679. [PubMed: 16254181]
7. Yu J, Mani RS, Cao Q, Brenner CJ, Cao X, Wang X, et al. An integrated network of androgen receptor, polycomb, and TMPRSS2-ERG gene fusions in prostate cancer progression. *Cancer Cell*. 2010; 17(5):443–54. doi: 10.1016/j.ccr.2010.03.018. [PubMed: 20478527]
8. Carver BS, Tran J, Gopalan A, Chen Z, Shaikh S, Carracedo A, et al. Aberrant ERG expression cooperates with loss of PTEN to promote cancer progression in the prostate. *Nat Genet*. 2009; 41(5):619–24. doi: 10.1038/ng.370. [PubMed: 19396168]
9. King JC, Xu J, Wongvipat J, Hieronymus H, Carver BS, Leung DH, et al. Cooperativity of TMPRSS2-ERG with PI3-kinase pathway activation in prostate oncogenesis. *Nat Genet*. 2009; 41(5):524–6. doi: 10.1038/ng.371. [PubMed: 19396167]
10. Middleton D, Menchaca L, Rood H, Komerofsky R. New allele frequency database. *Tissue Antigens*. 2003; 61(5):403–7. <http://www.allelefrequencies.net>. [PubMed: 12753660]
11. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J Exp Med*. 1997; 185(12):2043–51. doi: 10.1084/jem.185.12.2043. [PubMed: 9182675]
12. Maeurer MJ, Gollin SM, Storkus WJ, Swaney W, Karbach J, Martin D, et al. Tumor escape from immune recognition: loss of HLA-A2 melanoma cell surface expression is associated with a complex rearrangement of the short arm of chromosome 6. *Clin Cancer Res*. 1996; 2(4):641–52. [PubMed: 9816214]
13. Zong Y, Xin L, Goldstein AS, Lawson DA, Teitell MA, Witte ON. ETS family transcription factors collaborate with alternative signaling pathways to induce carcinoma from adult murine prostate cells. *Proc Natl Acad Sci U S A*. 2009; 106(30):12465–70. doi: 10.1073/pnas.0905931106. [PubMed: 19592505]
14. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999; 50(3-4):213–9. doi: 90500213.251. [PubMed: 10602881]
15. Reche PA, Glutting JP, Reinherz EL. Prediction of MHC class I binding peptides using profile motifs. *Hum Immunol*. 2002; 63(9):701–9. doi: S0198885902004329. [PubMed: 12175724]
16. Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, Nielsen M. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. *Nucleic Acids Res*. 2008; 36:W509–12. doi: 10.1093/nar/gkn202. [PubMed: 18463140]
17. Bhasin M, Singh H, Raghava GP. MHCBN: a comprehensive database of MHC binding and non-binding peptides. *Bioinformatics*. 2003; 19(5):665–6. doi: 10.1093/bioinformatics/btg055. [PubMed: 12651731]
18. Moss PA, Moots RJ, Rosenberg WM, Rowland-Jones SJ, Bodmer HC, McMichael AJ, et al. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. *Proc Natl Acad Sci U S A*. 1991; 88(20):8987–90. doi: 10.1073/pnas.88.20.8987. [PubMed: 1833769]
19. Milich DR, Hughes JL, McLachlan A, Thornton GB, Moriarty A. Hepatitis B synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope. *Proc Natl Acad Sci U S A*. 1988; 85(5):1610–4. [PubMed: 2449694]
20. Butler MO, Lee JS, Ansen S, Neuberg D, Hodi FS, Murray AP, et al. Long-lived antitumor CD8+lymphocytes for adoptive therapy generated using an artificial antigen-presenting cell. *Clin Cancer Res*. 2007; 13(6):1857–67. doi: 10.1158/1078-0432.CCR-06-1905. [PubMed: 17363542]
21. Getnet D, Maris CH, Hipkiss EL, Grosso JF, Harris TJ, Yen HR, et al. Tumor recognition and self-recognition induce distinct transcriptional profiles in antigen-specific CD4 T cells. *J Immunol*. 2009; 182(8):4675–85. doi: 10.4049/jimmunol.0803400. [PubMed: 19342643]

22. Tomlins SA, Aubin SM, Siddiqui J, Lonigro RJ, Sefton-Miller L, Miick S, et al. Urine TMPRSS2:ERG fusion transcript stratifies prostate cancer risk in men with elevated serum PSA. *Sci Transl Med*. 2011; 3(94):94ra72. doi: 10.1126/scitranslmed.3001970.
23. Mani RS, Iyer MK, Cao Q, Brenner JC, Wang L, Ghosh A, et al. TMPRSS2-ERG-mediated feed-forward regulation of wild-type ERG in human prostate cancers. *Cancer Res*. 2011; 71(16):5387–92. doi: 10.1158/0008-5472.CAN-11-0876. [PubMed: 21676887]
24. Lin HH, Ray S, Tongchusak S, Reinherz EL, Brusic V. Evaluation of MHC class I peptide binding prediction servers: applications for vaccine research. *BMC Immunol*. 2008; 9:8. doi: 10.1186/1471-2172-9-8. [PubMed: 18366636]
25. Harndahl M, Rasmussen M, Roder G, Dalgaard Pedersen I, Sorensen M, Nielsen M, et al. Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. *Eur J Immunol*. 2012; 42(6):1405–16. doi: 10.1002/eji.201141774. [PubMed: 22678897]
26. Assarsson E, Sidney J, Oseroff C, Paschetto V, Bui HH, Frahm N, Brander C, et al. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J Immunol*. 2007; 178(12):7890–901.
27. Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, van Veelen P, et al. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity*. 2003; 18(1):97–108. doi: 10.1016/S1074-7613(02)00511-3. [PubMed: 12530979]
28. Neeffjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol*. 2011; 11(12):823–36. doi: 10.1038/nri3084. [PubMed: 22076556]
29. Zehn D, Bevan MJ. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity*. 2006; 25(2):261–70. doi: 10.1016/j.immuni.2006.06.009. [PubMed: 16879996]
30. Bouneaud C, Kourilsky P, Bousso P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity*. 2000; 13(6): 829–40. doi: S1074-7613(00)00080-7. [PubMed: 11163198]
31. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, et al. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci U S A*. 1994; 91(14):6458–62. [PubMed: 8022805]
32. Thomas-Kaskel AK, Zeiser R, Jochim R, Robbel C, Schultze-Seemann W, Waller CF, et al. Vaccination of advanced prostate cancer patients with PSCA and PSA peptide-loaded dendritic cells induces DTH responses that correlate with superior overall survival. *Int J Cancer*. 2006; 119(10):2428–34. doi: 10.1002/ijc.22097. [PubMed: 16977630]
33. Rocha B, von Boehmer H. Peripheral selection of the T cell repertoire. *Science*. 1991; 251(4998): 1225–8. [PubMed: 1900951]
34. Rocha B, Grandien A, Freitas AA. Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. *J Exp Med*. 1995; 181(3):993–1003. [PubMed: 7869056]
35. McLaughlin F, Ludbrook VJ, Cox J, von Carlowitz I, Brown S, Randi AM. Combined genomic and antisense analysis reveals that the transcription factor Erg is implicated in endothelial cell differentiation. *Blood*. 2001; 98(12):3332–9. doi: 10.1182/blood.V98.12.3332. [PubMed: 11719371]
36. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol*. 2009; 10(11):R130. doi: 10.1186/gb-2009-10-11-r130. [PubMed: 19919682]
37. Arredouani MS, Lu B, Bhasin M, Eljanne M, Yue W, Mosquera JM, et al. Identification of the transcription factor single-minded homologue 2 as a potential biomarker and immunotherapy target in prostate cancer. *Clin Cancer Res*. 2009; 15(18):5794–802. doi: 10.1158/1078-0432.CCR-09-0911. [PubMed: 19737960]
38. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature*. 1992; 359(6391):162–5. doi: 10.1038/359162a0. [PubMed: 1522903]

39. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Varambally R, Yu J, Briggs BB, et al. OncoPrint 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia*. 2007; 9(2):166–80. doi: 10.1593/neo.07112. [PubMed: 17356713]
40. Shaikhibrahim Z, Wernert N. ETS transcription factors and prostate cancer: the role of the family prototype ETS-1 (review). *Int J Oncol*. 2012; 40(6):1748–54. doi: 10.3892/ijo.2012.1380. [PubMed: 22366814]

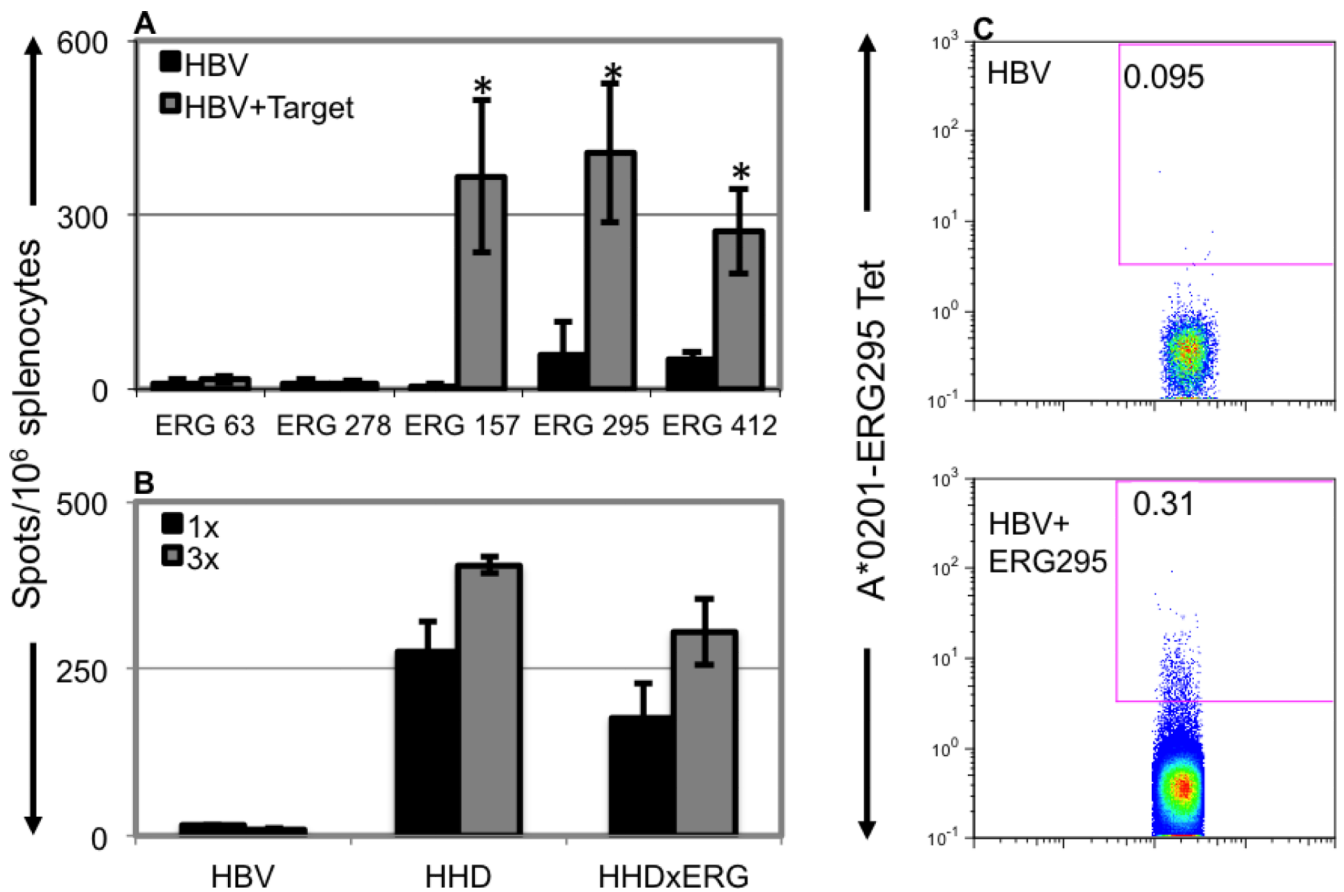
**Précis**

This work identifies an immunogenic, naturally processed epitope derived from ERG, a transcription factor that is over-expressed in about 50% of prostate cancer patients as a result of its fusion with the androgen-regulated gene TMPRSS2.



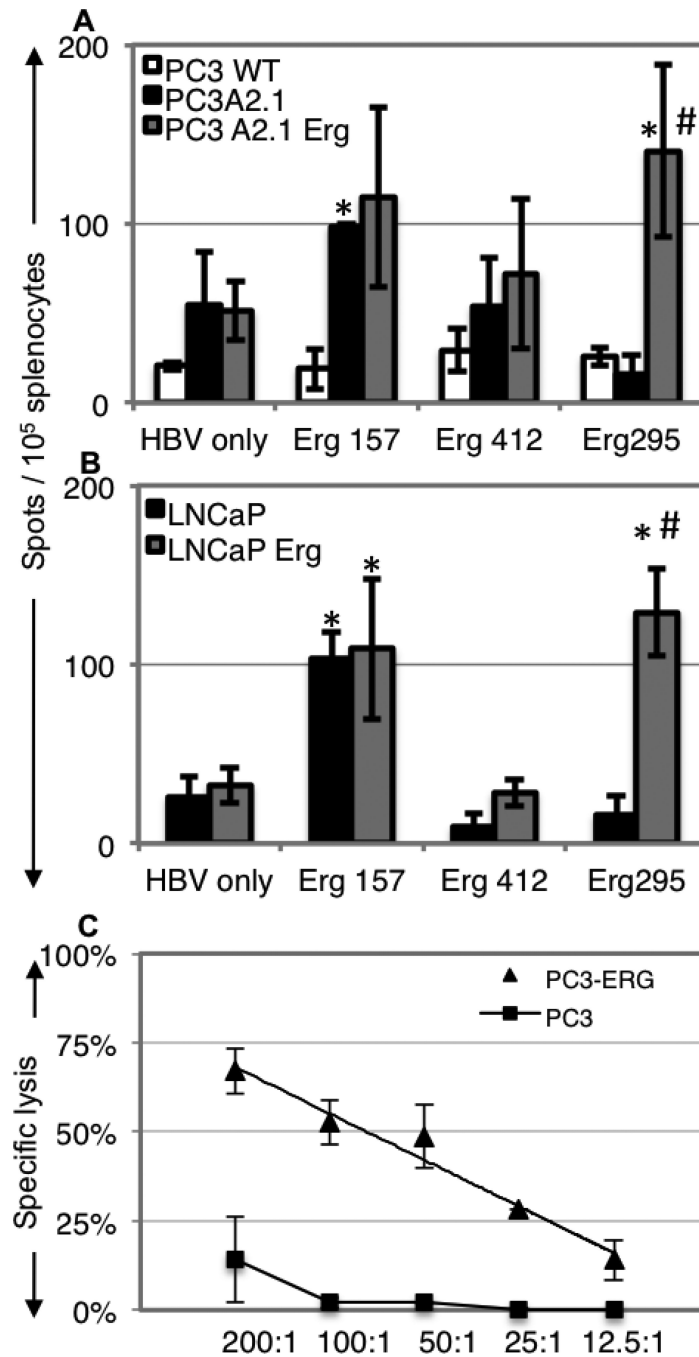
**Figure 1. Design and validation of immunogenic peptides derived from ERG**

(A) Peptides selected based on SYFPEITHI algorithm. Predicted HLA-A0201 binding scores of peptide sequences from ERG using the SYFPEITHI algorithm. (B) Peptide binding assay. Candidate peptide binding to HLA-A0201 was assessed in an assembly assay on T2 cells by incubating T2 cells with 50ug of peptide for 6 hours and measuring surface expression of HLA-A0201. Media without peptide and HA-M1 peptides were used as negative and positive controls respectively. Data shows mean of 3 readings  $\pm$  standard deviation and is representative of 2 total experiments. All peptides showing binding above the threshold level (dotted line) were significantly increased over the negative control. (C) Stabilization Assay. T2 cells were incubated with 50 $\mu$ g of peptide and HLA-A0201 expression measured by flow cytometry over 6 hours by flow cytometry.



### Figure 2. In vivo immunogenicity of ERG derived peptides

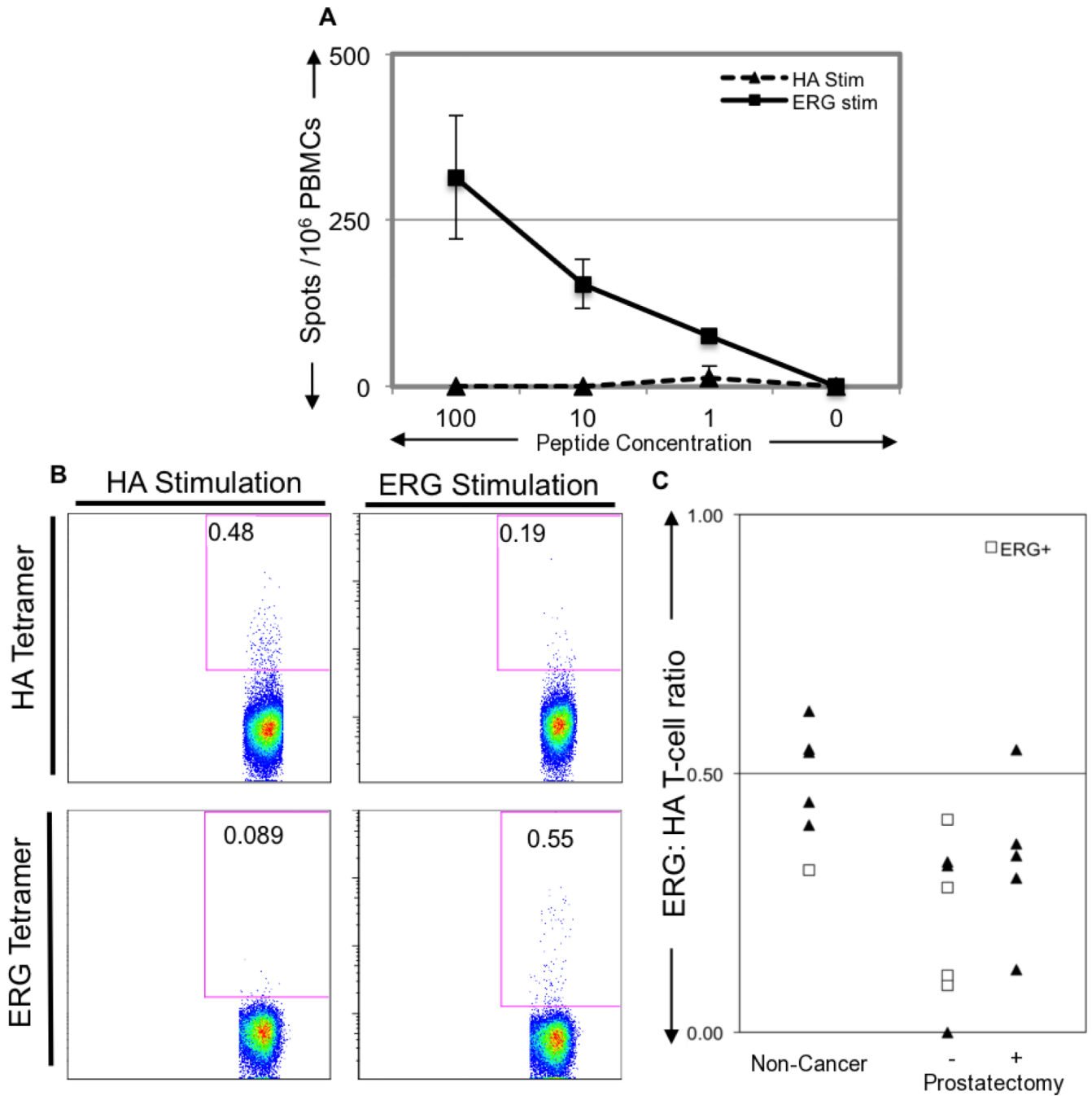
(A) In vivo immunogenicity of predicted ERG epitopes. HLA-A0201 transgenic HHD mice were immunized subcutaneously with ERG-derived peptides and HBV128 helper peptide. The recall response to the peptide was assessed by IFN- $\gamma$  ELISPOT. Data is representative of 1 of 2 total experiments showing the mean from 3 mice  $\pm$  standard deviation. (B) In vivo immunogenicity under toleragenic conditions. ERGxHHD<sup>pb/pb</sup> mice were immunized with the ERG295 + HBV peptide 1 or 3 times. Data shows mean from 3 mice  $\pm$  standard deviation from 1 experiment. (C) Tetramer analysis of ERG295 immunized HHD mice. Splenocytes isolated from ERG295 mice were stained with an ERG295 loaded HLA-A0201 tetramer and analysed by flow cytometry. Tetramer data shows plots from 3 mice combined into a single figure for each treatment



**Figure 3. T-cells isolated from ERG immunized mice responds to human Prostate Cancer cell lines expressing ERG**

(A and B) Reactivity of splenocytes from ERG-immunized mice against ERG expressing human prostate cancer cell lines. Splenocytes from HHD mice immunized with HBV and various ERG-derived peptides or HBV alone were co-cultured with PC3, LNCaP, PC3-ERG or LNCaP-Erg tumor cell lines. Production of IFN- $\gamma$  by splenocytes in response to these tumor cell lines was assessed by ELISPOT. Figures show mean  $\pm$  standard deviation of 3 mice from one experiment. The effect of splenocytes from ERG295 immunized mice against the various cell lines was repeated in 3 separate experiments. Significant ( $p < 0.05$ ) difference between the ELISPOT response to the same cell line and mice receiving the control

immunization is represented by \*, while # represents a significant difference in response between ERG+ or ERG- cell lines in mice receiving the same immunization. (C) Anti-tumor cell activity of splenocytes isolated from ERG295 immunized mice. Splenocytes from ERG295 immunized mice were cultured with Cr<sub>51</sub> labeled PC3 or PC3-ERG+ tumor cells and the specific lysis of the tumor cells was measured by the total Cr<sub>51</sub> released. Data shows mean ± standard deviation and is combined data from 2 separate experiments.



**Figure 4. ERG reactive CD8 T-cells can be detected in the peripheral blood of HLA-A0201<sup>+</sup> subjects**

(A) ELISPOT analysis of ERG295 reactive T-cells. PBMCs from HLA-A0201 healthy donors were co-cultured with HA or ERG-loaded aAPCs for 9 days. The recall response of healthy donor PBMCs to T2 cells loaded with ERG at various concentrations was measured by IFN- $\gamma$  ELISPOT. Data shows mean from 3 separate experiments  $\pm$  standard deviation.

(B) Tetramer analysis of ERG295 reactive T-cells. After stimulation with aAPCs loaded with HA-M1<sub>58</sub> or ERG295, blood from patients was also analyzed by flow cytometry for HA-M1<sub>58</sub> or ERG295 tetramer positive cells. (C) Relative abundance of T-cells specific for

ERG. The ratio of ERG<sup>+</sup> CD8<sup>+</sup> T-cells to HA<sup>+</sup> T-cells for healthy and prostate cancer patients. Patients positive for ERG are by the denoted by white squares.