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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⁺ 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*^{-/-} 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 NH₂ terminus to the COOH terminus of the human β_2m by a 15-amino acid peptide arm [10]. Probasin-*ERG* (*ERG*^{pb/pb}) 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*^{pb/pb} mice were generated by crossing HHD mice with the *ERG*^{pb/pb} 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⁺ 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 / 2.5×10^5 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 HBVcore₁₂₈₋₁₄₀ 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 2.5×10^5 splenocytes were seeded in each well in four replicates, and 2.5×10^5 peptide-loaded ($10 \mu\text{g}$ peptide/mL, for 2 h at 37°C) splenocytes pretreated with $50 \mu\text{g/mL}$ mitomycin C for 1 h were added to each well. To investigate the response of immunized mice to prostate cancer cell lines, 5×10^4 Splenocytes isolated from immunized mice were cultured with 5×10^4 tumor cells pretreated with 50 g/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- γ producing cells per million splenocytes.

Tetramer staining

AlexaFluor647-labeled HLA-A*0201 tetramers loaded with HA-M1₅₈ (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⁺ cells was determined by dividing the percentage of CD8⁺ cells that were ERG⁺ cells by the number of CD8⁺ cells that stained HA⁺.

Chromium release assay

Ten million target cells were suspended in 1ml of PBS and incubated with $200 \mu\text{Ci}$ of ^{51}Cr at 37°C for 2 hours. Target cells were washed 3 times with RPMI and 1×10^4 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 (sample reading-spontaneous release)/(maximum release – 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 $10 \mu\text{g/mL}$ of ERG or HA peptide for 4 hours at 37°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⁺ donors and aAPCs were mixed at a ratio of 20:1 at a cell density of 2×10^6 cells/mL in LGM-3 serum free media (Lonza, CC-3211) supplemented with 1% human AB serum (Atlanta Biologicals, 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. ERG₁₅₇ (366 ± 131 cells/ 10^6 splenocytes), ERG₂₉₅ (405 ± 119), and ERG₄₁₂ (272 ± 73) each induced a significant ERG-specific response compared to controls ($p < 0.05$), while ERG₆₃ (16) and ERG₂₇₈ (10) did not (**Figure 2A**). Confirmation of ERG₂₉₅ immunogenicity *in vivo* was undertaken using ERG₂₉₅-specific tetramer. ERG₂₉₅-immunized mice showed significant induction as detected by ERG₂₉₅ 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^{pb/pb} mice

To further characterize tolerance to the ERG antigen, we generated HHD $\times$ ERG^{pb/pb} mice that have human HLA-A*0201⁺ 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 HHD $\times$ ERG^{pb/pb} mice around 16 weeks old were immunized either 1 or 3 times at 7-day intervals with the ERG₂₉₅ peptide. Following 1 immunization, HHD mice had 275 ± 45 ERG₂₉₅ responsive cells per million splenocytes while the HHD $\times$ ERG^{pb/pb} mice had 177 ± 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 ± 120) and HHD $\times$ ERG^{pb/pb} mice (305 ± 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 ERG₁₅₇, ERG₂₉₅, or ERG₄₁₂ were co-cultured with PC3-A*0201⁺-ERG⁺ or PC3-A*0201⁺-ERG⁻ cells in an IFN γ 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⁺-ERG⁺ 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⁺-ERG⁺ 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⁺-ERG⁺ cells compared to PC3-A2.1⁺-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 ERG₂₉₅ 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⁺ haplotype were co-cultured with aAPCs loaded with either HA-M1₅₈₋₆₆ or ERG295 (See supplementary table 1 for details of patient characteristics). Induction of ERG-specific CTLs was then evaluated by IFN- γ 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⁺ 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⁺ T-cells to influenza derived HA-M1₅₈ 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-M1₅₈ 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^{pb/pb} 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⁺ 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⁺ 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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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 μ 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- γ 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^{pb/pb} 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- γ 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 Cr₅₁ labeled PC3 or PC3-ERG+ tumor cells and the specific lysis of the tumor cells was measured by the total Cr₅₁ released. Data shows mean $\pm$ 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⁺ 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- γ 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-M1₅₈ or ERG295, blood from patients was also analyzed by flow cytometry for HA-M1₅₈ or ERG295 tetramer positive cells. (C) Relative abundance of T-cells specific for

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