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ORIGINAL ARTICLE

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 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

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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(210 M), 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 cancers [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 ERG-derived epitopes that are restricted to HLA-A*0201, 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 whether 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'Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France). These mice are β_2 $m^{-/-}$, $Db^{-/-}$ double knockout and express an HLA-A*0201 mono-chain composed of a chimeric heavy chain (α1 and α2 domains of HLA-A*0201 allele and the α3 and intracellular domains of Db allele) linked by its NH_2 terminus to the COOH terminus of the human β_2 m by a 15-amino-acid peptide arm [11]. 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 nonamer 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) [17] 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-week-old male HHD mice were injected subcutaneously 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$_{128–140}$ T helper epitope (TPPAYRPPNAPIL) [19]. Ten to 12 days 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 the 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 μg peptide/mL, for 2 h at 37 °C) splenocytes pretreated with 50 μg/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 μg/ml of mitomycin C for 1 h. ELISPOT was developed as described in the 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-M1$_{58}$ (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 1 ml of PBS and incubated with 200 μCi of ^{51}Cr at 37 °C for 2 h. 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 h. 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

Blood was collected as per our institutional IRB-approved protocol from a random pool of prostate cancer patients who did not undergo prostatectomy and a separate group of patients who had undergone this procedure. 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, donated by Marcus Butler at the Dana Farber Cancer Institute, Boston, MA) were used to expand HA or ERG295 antigen-specific T-cells as described in Butler et al. [20]. Briefly, aAPCs were cultured for 1 h in serum-free RPMI, washed 3 times with PBS, and then incubated in serum-free RPMI with 10 μg/ml of ERG or HA peptide for 4 h at 37 °C. aAPCs were then irradiated with 100 Gy X-rays or incubated with 50 μg mitomycin C for 30 min 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

A

Name	A.A Sequence	Score
ERG(11)	HIKEALSVV	23
ERG(63)	WLSQPPARV	23
ERG(131)	VIVPADPTL	24
ERG(157)	GLPDVNILL	22
ERG(194)	ILLSHLHYL	29
ERG(225)	LMHARNTDL	20
ERG(278)	ILGPTSSRL	24
ERG(295)	QLWQFLLEL	29
ERG(390)	SLYKYPSDL	24
ERG(412)	FVAPHPPAL	21

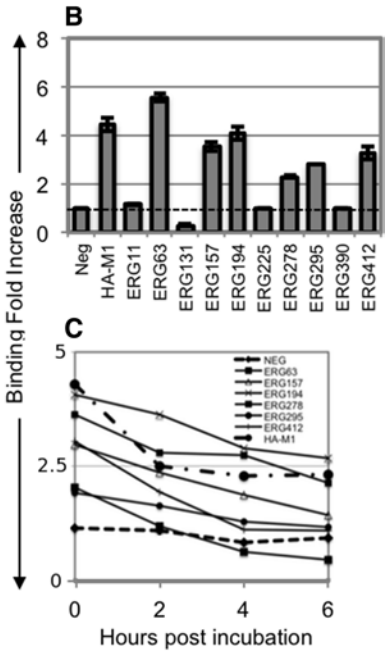

Fig. 1 Design and validation of immunogenic peptides derived from ERG. **a** Peptides selected based on SYFPEITHI algorithm. Predicted HLA-A0201-binding scores of peptide sequences from ERG using the SYFPEITHI algorithm. **b** Peptide binding assay. Candidate peptide binding to HLA-A0201 was assessed in an assembly assay on T2 cells by incubating T2 cells with 50 μg of peptide for 6 h and measuring surface expression of HLA-A0201. Media without peptide and HA-M1 peptides were used as negative and positive controls, respectively. Data show mean of 3 readings ± standard deviation and are 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 μg of peptide and HLA-A0201 expression measured by flow cytometry over 6 h by flow cytometry

1 day, cells were supplemented with 20 IU/ml of IL-2 and 10 ng/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 <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 Fig. 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 whether 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 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 (Fig. 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 h and strong stabilizing epitopes (ERG194 and ERG63) that significantly increased HLA-A*0201 expression for greater than 6 h (Fig. 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 mechanisms 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 β2 $m^{-/-}$, $D_b^{-/-}$ double

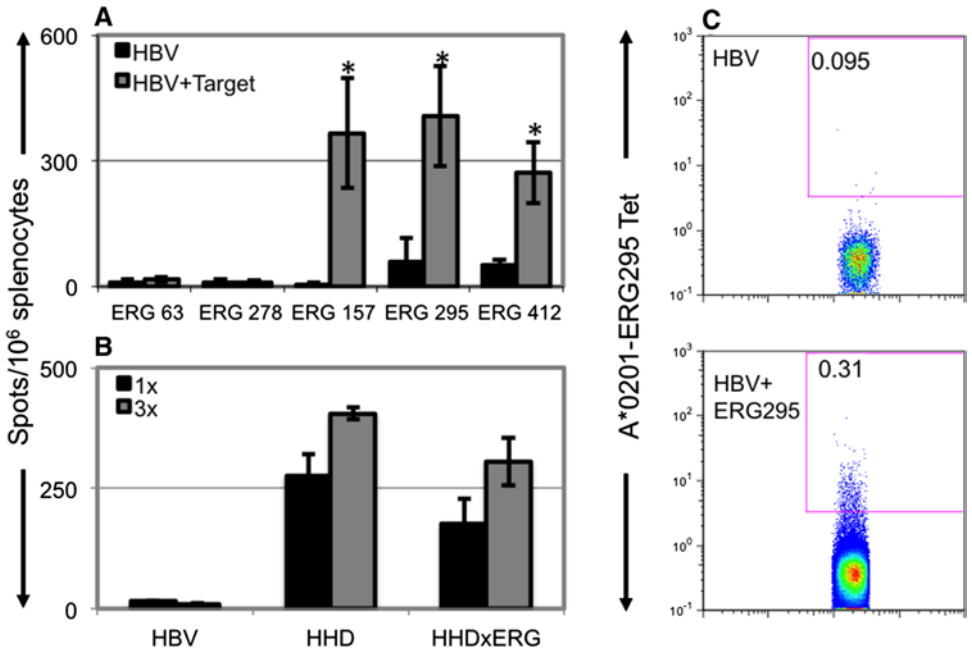

Fig. 2 In vivo immunogenicity of ERG-derived peptides. **a** In vivo immunogenicity of predicted ERG epitopes. HLA-A0201 transgenic HHD mice were immunized subcutaneously with ERG-derived peptides and HBV128 helper peptide. The recall response to the peptide was assessed by IFN-γ ELISPOT. Data are representative of 1 of 2 total experiments showing the mean from 3 mice ± standard deviation. **b** In vivo immunogenicity under tolerogenic conditions. ERGxHHD$^{pb/pb}$ mice were immunized with the ERG295 + HBV peptide 1 or 3 times. Data show mean from 3 mice ± 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 analyzed by flow cytometry. Tetramer data show plots from 3 mice combined into a single figure for each treatment

knockout and express the human HLA-A*0201 allele [11]. After immunization of HHD mice with candidate ERG peptides, the ERG-specific CTL responses were analyzed by ELISPOT. ERG_{157} (366 ± 131 cells/10^6 splenocytes), ERG_{295}(405 ± 119), and ERG_{412} (272 ± 73) each induced a significant ERG-specific response compared with controls ($p < 0.05$), while ERG_{63} (16) and ERG_{278} (10) did not (Fig. 2a). Confirmation of ERG_{295} immunogenicity in vivo was undertaken using ERG_{295}-specific tetramer. ERG_{295}-immunized mice showed significant induction as detected by ERG_{295} tetramer compared with control mice (Fig. 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 HHD × ERG$^{pb/pb}$ mice

To further characterize tolerance to the ERG antigen, we generated HHD × 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 × ERG$^{pb/pb}$ mice around 16 weeks old were immunized either 1 or 3 times at 7-day intervals with the ERG_{295} peptide. Following 1 immunization, HHD mice had 275 ± 45 ERG_{295} responsive cells per million splenocytes while the HHD × 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 × ERG$^{pb/pb}$ mice (305 ± 49) ($p = 0.26$); however, both had significantly more than the control mice ($p < 0.05$) (Fig. 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_{157}, ERG_{295}, or ERG_{412} were co-cultured with PC3-A*0201$^+$-ERG$^+$ or PC3-A*0201$^+$-ERG$^-$ cells in an IFNγ 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 with PC3-A2.1$^+$-Vector cells (Fig. 3a). Additionally, splenocytes from these mice caused specific lysis of PC3-ERG cells but not PC3-Vector cells (Fig. 3c). Similar results using LNCaP-Vector and LNCaP-ERG cells as targets showed ERG295 as the only epitope to induce ERG-specific targeting (Fig. 3b). These data indicate that an antigen-specific response can be generated against ERG-expressing HLA-A*0201 positive cells by immunization with ERG_{295} peptide.

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

Central and peripheral tolerance mechanisms inhibit T-cell activation against endogenous antigens such as 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-$M1_{58–66}$ or ERG295 (see Supplementary Table 1 for details of patient characteristics). Induction of ERG-specific CTLs was then evaluated by IFN-γ ELISPOT assay (Fig. 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 ± 0.63 % (Fig. 4b). To quantitate how patients responded to the autologous ERG antigen compared with a prototypical foreign epitope, the ratio of ERG295$^+$ T-cells to influenza-derived HA-$M1_{58}$-reactive cells was assessed by tetramer assay. Stimulation of T-cells from the blood of healthy HLA-A*0201 donors generated ERG295-reactive

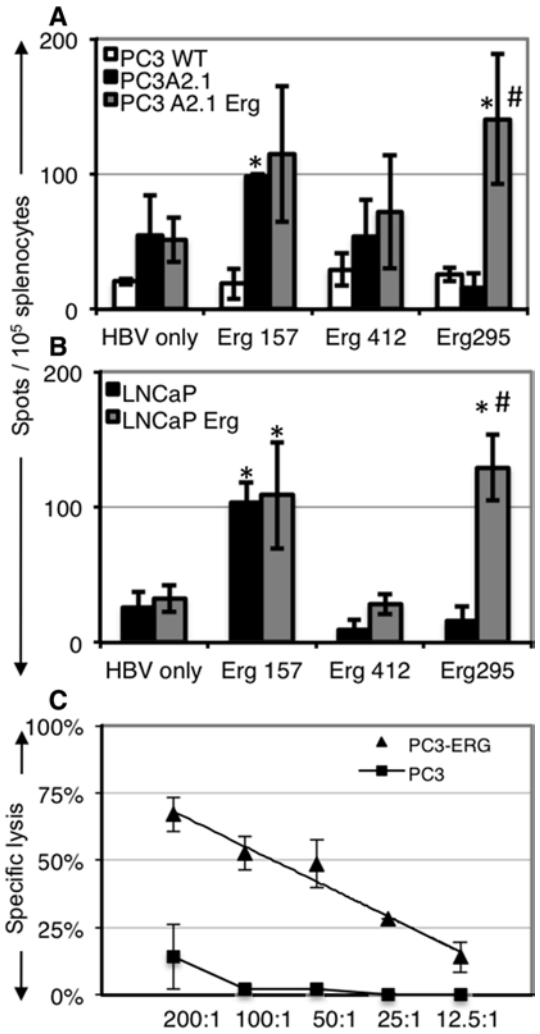

Fig. 3 T-cells isolated from ERG-immunized mice respond to human prostate cancer cell lines expressing ERG. **a**, **b** Reactivity of splenocytes from ERG-immunized mice against ERG-expressing human prostate cancer cell lines. Splenocytes from HHD mice immunized with HBV and various ERG-derived peptides 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 ± 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_{51} labeled PC3 or PC3-ERG+ tumor cells, and the specific lysis of the tumor cells was measured by the total Cr_{51} released. Data show mean ± standard deviation and are combined data from 2 separate experiments

T-cells at a frequency of 0.45 as often as HA-$M1_{58}$-positive cells. Prostate cancer patients' response to ERG295 antigen was significantly reduced compared with healthy

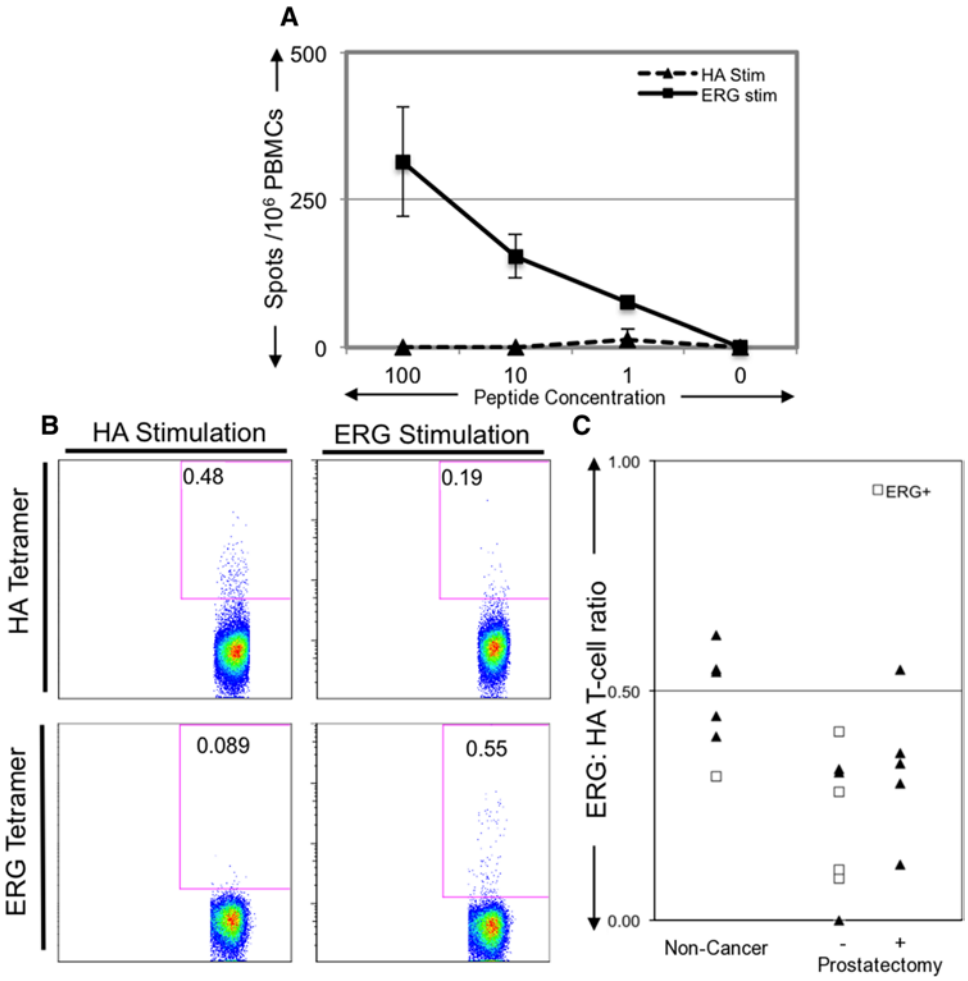

Fig. 4 ERG-reactive CD8 T-cells can be detected in the peripheral blood of HLA-A0201$^+$ subjects. **a** ELISPOT analysis of ERG295-reactive T-cells. PBMCs from HLA-A*0201 healthy donors were co-cultured with HA- or ERG-loaded aAPCs for 9 days. The recall response of healthy donor PBMCs to T2 cells loaded with ERG at various concentrations was measured by IFN-γ ELISPOT. Data show mean from 3 separate experiments ± standard deviation. **b** Tetramer analysis of ERG295-reactive T-cells. After stimulation with aAPCs loaded with HA-M1$_{58}$ or ERG295, blood from patients was also analyzed by flow cytometry for HA-M1$_{58}$- 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 denoted by white squares

patients ($p < 0.05$), but no significant difference was observed between patients pre- or post-prostatectomy (Fig. 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 (Fig. 4c). These data support what was observed in the mouse model (Fig. 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-A*0201 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 [23]. 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 with the strength of the in vitro stabilization [24]. 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 (Fig. 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 [25]. 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 [26, 27]. Nonetheless, together these data indicate that the ERG295 peptide is presented in an HLA-A*0201-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 support the observation that central deletion is imperfect, and results in detectable self-reactive T-cells in the periphery [28, 29]. 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 such as MART-1 in melanoma and PSA in prostate cancer [30, 31]. In addition to central tolerance, peripheral tolerance mechanisms such as 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 [32, 33]. ERG expression has been reported in endothelial cells in vitro [34]. 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 [35, 36]. 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 [21]. 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 indicate 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 [37], 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 [38], including PCa [39], 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 with non-oncogenic targets, such as 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.

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Conflict of interest The authors disclose no potential conflicts of interest.

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