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14. ABSTRACT
The hormonal milieu influences immune tolerance as well as the immune response against viruses and cancer, but the direct effect of androgens on cellular immunity remains largely uncharacterized. We therefore sought to evaluate the effect of androgens on murine and human T cells in vivo and in vitro. We found that murine androgen deprivation in vivo elicited RNA expression patterns conducive to interferon signaling and T cell differentiation. Interrogation of mechanism showed that testosterone regulates Th1 differentiation by inhibiting IL-12 induced Stat4 phosphorylation: in murine models, we determined that androgen receptor binds a conserved region within the phosphatase, Ptpn1, and consequent up-regulation of Ptpn1 then inhibits IL-12 signaling in CD4 T cells. The clinical relevance of this mechanism, whereby the androgen milieu modulates CD4 T cell differentiation, was ascertained as we found that androgen deprivation reduced expression of Ptpn1 in CD4 cells from patients undergoing androgen deprivation therapy for prostate cancer. Our findings, that demonstrate a clinically relevant mechanism by which androgens inhibit Th1 differentiation of CD4 T cells, provide rationale for targeting androgens to enhance CD4-mediated immune responses in cancer or, conversely, for modulating androgens to mitigate CD4 responses in disorders of autoimmunity.

15. SUBJECT TERMS
Testosterone; T cell differentiation; Ptpn1

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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 Prostvac 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.

Body

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

The 2 models to be established were a transgenic mouse model and a cell line to test the anti-tumor efficacy of the $ERG_{319-327}$ epitope. The transgenic mouse model we made spontaneously develops prostate cancer that expresses ERG as well as the human HLA-A0201 allele. This ERG/TRAMP/HLA-A0201 mouse has been generated and is currently being used to test the in vivo anti-tumor activity of the $ERG_{319-327}$ epitope.

Generation of the second model, the TRAMPC2-ERG-HLA-A0201 cell line is ongoing. Viability of the cells was poor after transfection with 2 different plasmids. We are currently constructing a single plasmid that contains both HLA-A0201 and ERG.

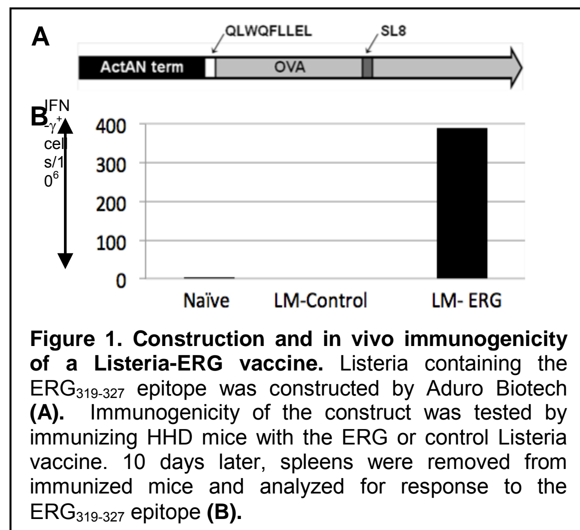


Figure 1. Construction and in vivo immunogenicity of a Listeria-ERG vaccine. Listeria containing the $ERG_{319-327}$ epitope was constructed by Aduro Biotech **(A).** Immunogenicity of the construct was tested by immunizing HHD mice with the ERG or control Listeria vaccine. 10 days later, spleens were removed from immunized mice and analyzed for response to the $ERG_{319-327}$ epitope **(B).**

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

To deliver the $ERG_{319-327}$ epitope in vivo, we constructed a listeria-based delivery system in partnership with Aduro Biotech. Aduro Biotech are currently undertaking phase I and II clinical trials using this delivery platform for a range of different cancers. In vivo testing of the Listeria-ERG construct has been performed, and a strong $ERG_{319-327}$ response is generated (Figure 1). Using the in vivo models developed above, we are currently testing the anti-tumor efficacy of this listeria vaccine construct, however, these experiments are incomplete and no data is presented here.

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

These cells were not viable after transfection with the HLA-A0201 and we have discontinued this approach. As described in the attached paper, we have measured the presence of ERG

reactive cells using a tetramer assay.

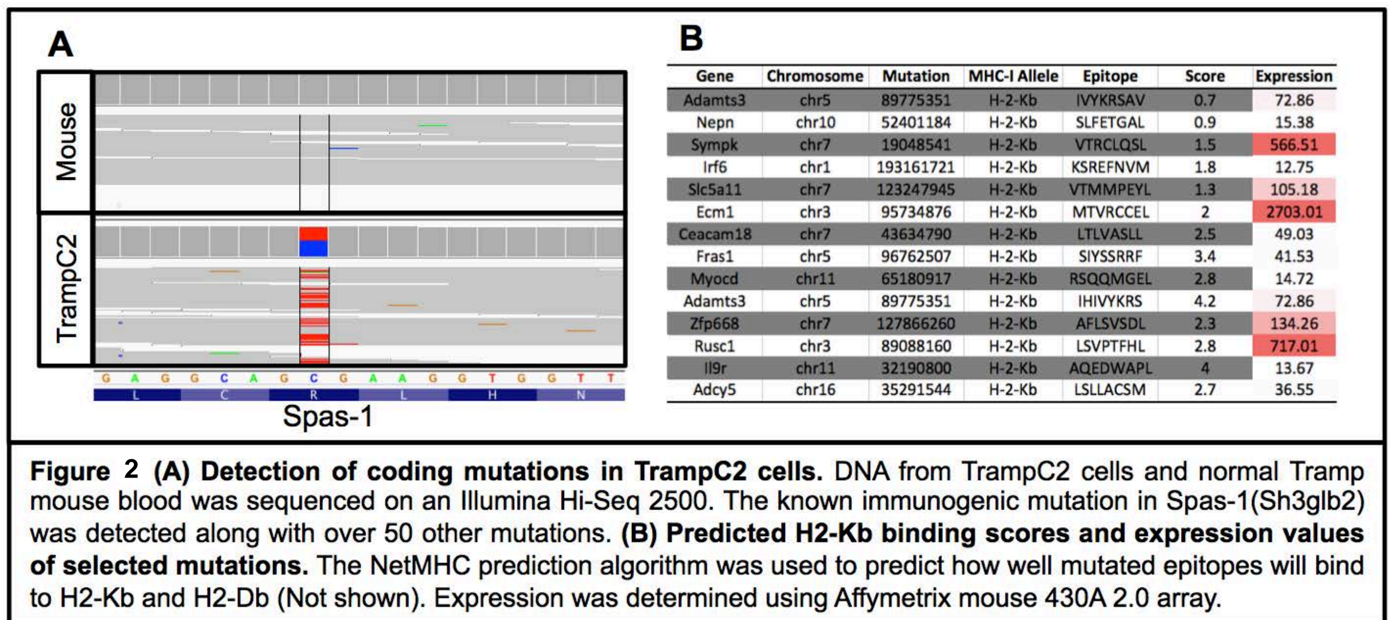
2b) Analyze the number and activity of ERG reactive T-cells against LNCaP/ERG+, PC3/HLA-A0201+/ERG+ and VCaP HLA-A0201+ cells stratifying for ERG status

Instead of this approach, we decided to quantification of ERG reactive T-cells using a tetramer assay. Details of this assay can be found in the attached paper (Kissick et al., 2013). We found that patients possess $ERG_{319\text{-}327}$ reactive T-cells. These data indicate that the $ERG_{319\text{-}327}$ epitope could potentially be used in a vaccine for HLA-A0201 prostate cancer patients.

3. Sequencing RNA from TRAMPC1 and TRAMPC2 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 2).



Gene	Chromosome	Mutation	MHC-I Allele	Epitope	Score	Expression
Adamts3	chr5	89775351	H-2-Kb	IVYKRSAV	0.7	72.86
Nepn	chr10	52401184	H-2-Kb	SLFETGAL	0.9	15.38
Sympk	chr7	19048541	H-2-Kb	VTRCLQSL	1.5	566.51
Irf6	chr1	193161721	H-2-Kb	KSREFNVM	1.8	12.75
Slc5a11	chr7	123247945	H-2-Kb	VTMMPEYL	1.3	105.18
Ecm1	chr3	95734876	H-2-Kb	MTVRCCEL	2	2703.01
Ceacam18	chr7	43634790	H-2-Kb	LTLVASLL	2.5	49.03
Fras1	chr5	96762507	H-2-Kb	SIYSSRRF	3.4	41.53
Myocd	chr11	65180917	H-2-Kb	RSQQMGEL	2.8	14.72
Adamts3	chr5	89775351	H-2-Kb	IHIVYKRS	4.2	72.86
Zfp668	chr7	127866260	H-2-Kb	AFLSVSDL	2.3	134.26
Rusc1	chr3	89088160	H-2-Kb	LSVPTFHL	2.8	717.01
Il9r	chr11	32190800	H-2-Kb	AQEDWAPL	4	13.67
Adcy5	chr16	35291544	H-2-Kb	LSLLACSM	2.7	36.55

Figure 2 (A) Detection of coding mutations in TrampC2 cells. DNA from TrampC2 cells and normal Tramp mouse blood was sequenced on an Illumina Hi-Seq 2500. The known immunogenic mutation in Spas-1(Sh3glb2) was detected along with over 50 other mutations. **(B) Predicted H2-Kb binding scores and expression values of selected mutations.** The NetMHC prediction algorithm was used to predict how well mutated epitopes will bind to H2-Kb and H2-Db (Not shown). Expression was determined using Affymetrix mouse 430A 2.0 array.

In addition to the many mutations present in the TRAMP-C2 cell line, we found a number of frame-shift mutations that generated large neo-antigen sequences. One of these frame shift deletions occurred in p53

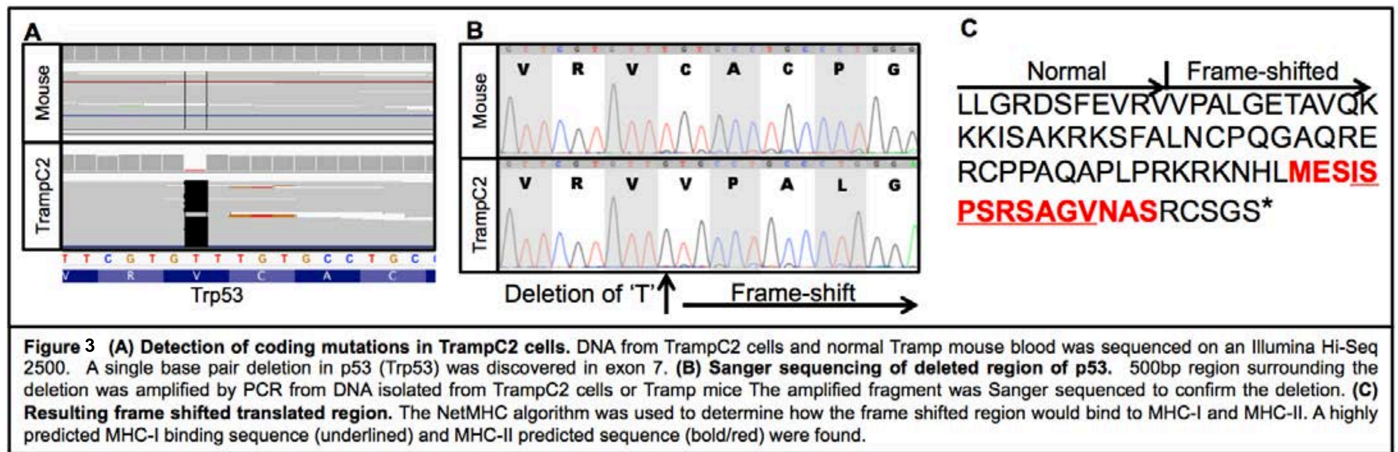


Figure 3 (A) Detection of coding mutations in TrampC2 cells. DNA from TrampC2 cells and normal Tramp mouse blood was sequenced on an Illumina Hi-Seq 2500. A single base pair deletion in p53 (Trp53) was discovered in exon 7. **(B) Sanger sequencing of deleted region of p53.** 500bp region surrounding the deletion was amplified by PCR from DNA isolated from TrampC2 cells or Tramp mice The amplified fragment was Sanger sequenced to confirm the deletion. **(C) Resulting frame shifted translated region.** The NetMHC algorithm was used to determine how the frame shifted region would bind to MHC-I and MHC-II. A highly predicted MHC-I binding sequence (underlined) and MHC-II predicted sequence (bold/red) were found.

Immunogenicity of the peptides targeting mutations and frame shift deletions are currently being tested *in vivo*. If this approach is successful, we will sequence the TRAMP-C1 cell line and perform the same analysis.

3b) Treat subcutaneous TRAMP-C1 and TRAMP-C2 tumors with peptides derived from mutations

These experiments will begin once the previous aim has been completed.

Additional data collected

In addition to our work investigating the ERG derived epitopes, we have investigated epitopes from the prostate cancer antigen, SIM2. This work was recently published and is attached to this report (Kissick et al., 2014). Also, our previous work has found that tolerance to tumor antigens occurs during the development of prostate tumors in TRAMP mice, and that castration can partially reverse this tolerance (Arredouani et al., 2010). We have investigated the mechanisms of this effect and found that testosterone regulates expression of the phosphatase Ptpn1, and this inhibits CD4 T-cell differentiation. This work was recently accepted for publication in Proceedings of the National Academy of Science and is currently in press (Submission attached). We plan to investigate immunization with ERG in combination with castration and Ptpn1 inhibition to determine if this novel mechanism can enhance vaccine regimes in TRAMP mice.

Key Research Accomplishments

1. Validation that the $ERG_{319-327}$ epitope effectively targets cell lines expressing ERG
2. Discovery of $ERG_{319-327}$ reactive T-cells in HLA-A0201 prostate cancer patients
3. Identification of epitopes derived from the prostate cancer antigen SIM2.
4. Discovery of potential neo-antigens derived from mutations and genomic changes in prostate cancer cell lines.
5. Discovery that Ptpn1 is regulated by testosterone, and that this phosphatase inhibits CD4 T-cell differentiation.

Reportable Outcomes

Publications

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, Dunn LK, Pellegrini KL, On ST, Noel JK, Arredouani MS. Androgens alter T-cell immunity by inhibiting T helper 1 differentiation. **Proc Natl Acad Sci USA**, In Press, 2014

Funding

Dr Kissick successfully applied for a Young Investigator Award from the Prostate Cancer Foundation using data acquired from this grant. This highly competitive award facilitates the transition of a young investigator to a faculty position and is worth $75,000/year for 3 years.

Employment

Based on the data produced from this grant, Dr Kissick has been employed at Emory University in the department of Urology as a Senior Associate for the remainder of this fellowship, and transition to the position of Assistant Professor at the completion of this award in 2015.

Conclusion

The work presented here demonstrates that the $ERG_{319-327}$ epitope can generate an anti-tumor effect, and that prostate cancer patients possess cells reactive to this epitope (Kissick et al., 2013). We have reported similar findings for epitopes derived from another prostate cancer antigen, SIM2 (Kissick et al., 2014). We are currently testing the in vivo anti-tumor effect of these epitopes in vivo using a Listeria based delivery platform. Additionally, we have found that the TRAMP-C2 cell line has numerous mutations and frame shift deletions that generate neo-antigens that could be potential targets for immunotherapy. Currently, the anti-tumor effect of these peptides is being investigated in *in vivo* models. Finally, we have investigated how androgen deprivation affects T-cell function and identified a novel mechanism that may enhance vaccine efficacy in toleragenic tumor conditions (Kissick et al., In Press). Together, this work identifies novel epitopes to treat prostate cancer, and a mechanism by which testosterone regulates tolerance to host antigens.

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Appendices

Cancer Immunol Immunother
DOI 10.1007/s00262-013-1482-y

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

Electronic supplementary material The online version of this article (doi:10.1007/s00262-013-1482-y) contains supplementary material, which is available to authorized users.

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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

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

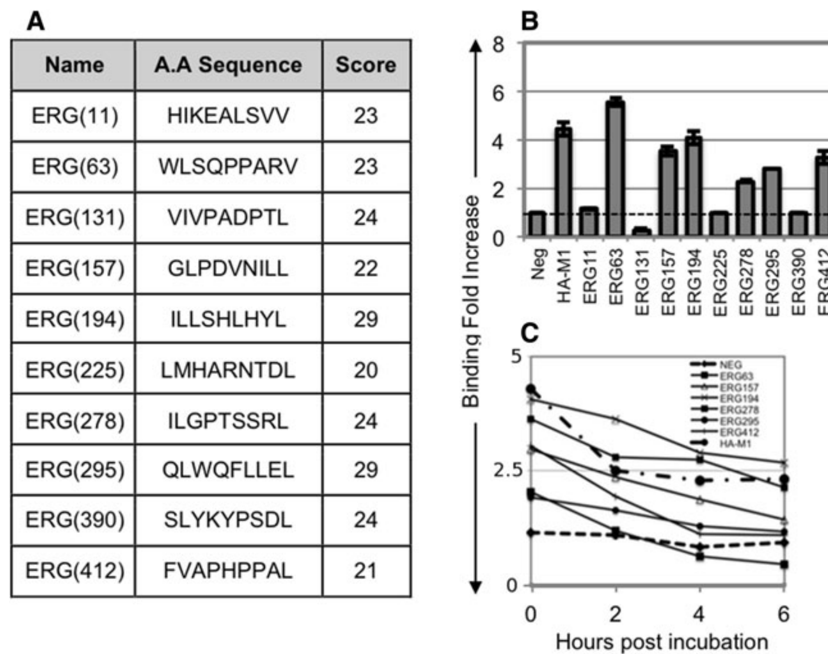
AlexaFluor647-labeled HLA-A*0201 tetramers loaded with $HA\text{-}M1_{58}$ (GILGFVFTL) or ERG295 (QLWQFLLEL) were produced by the NIH tetramer facility at Emory University (Atlanta, GA). Splenocytes from HHD mice were stained with anti-CD8-FITC and 7-AAD. Cells were gated for positive expression of CD8 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 $\beta 2\ m^{-/-}$, $D_b^{-/-}$ double

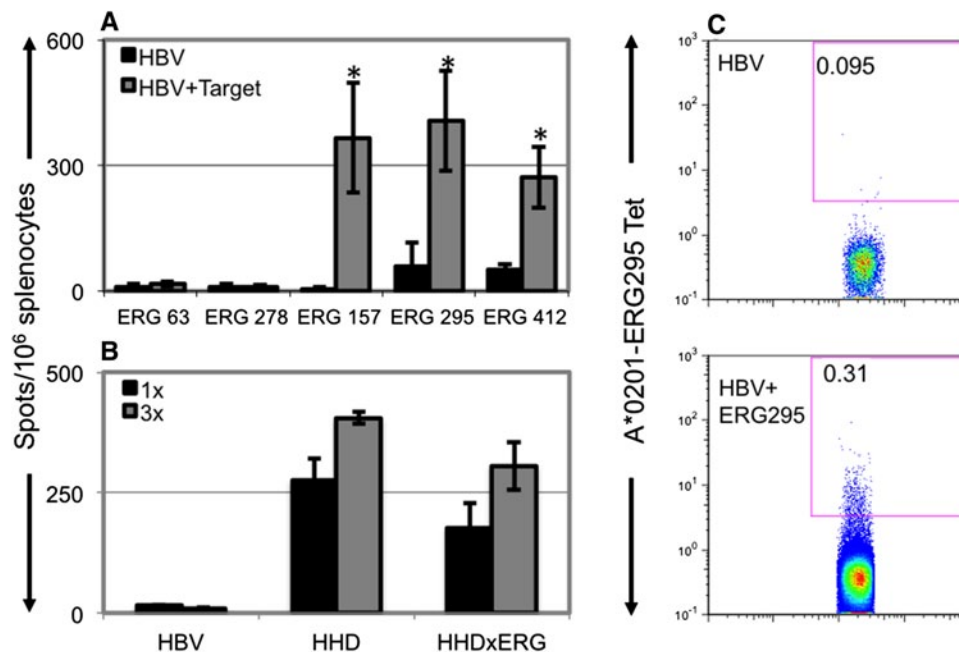


Fig. 2 In vivo immunogenicity of ERG-derived peptides. **a** In vivo immunogenicity of predicted ERG epitopes. HLA-A0201 transgenic HHD mice were immunized subcutaneously with ERG-derived peptides and HBV128 helper peptide. The recall response to the peptide was assessed by IFN-γ ELISPOT. Data are representative 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

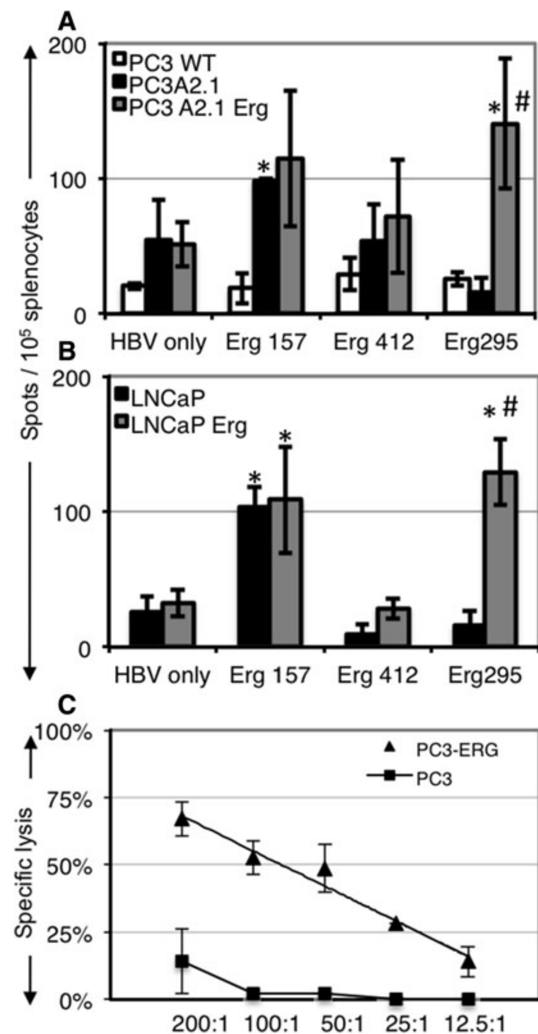


Fig. 3 T-cells isolated from ERG-immunized mice respond to human prostate cancer cell lines expressing ERG. **a**, **b** Reactivity of splenocytes from ERG-immunized mice against ERG-expressing human prostate cancer cell lines. Splenocytes from HHD mice immunized with HBV and various ERG-derived peptides 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

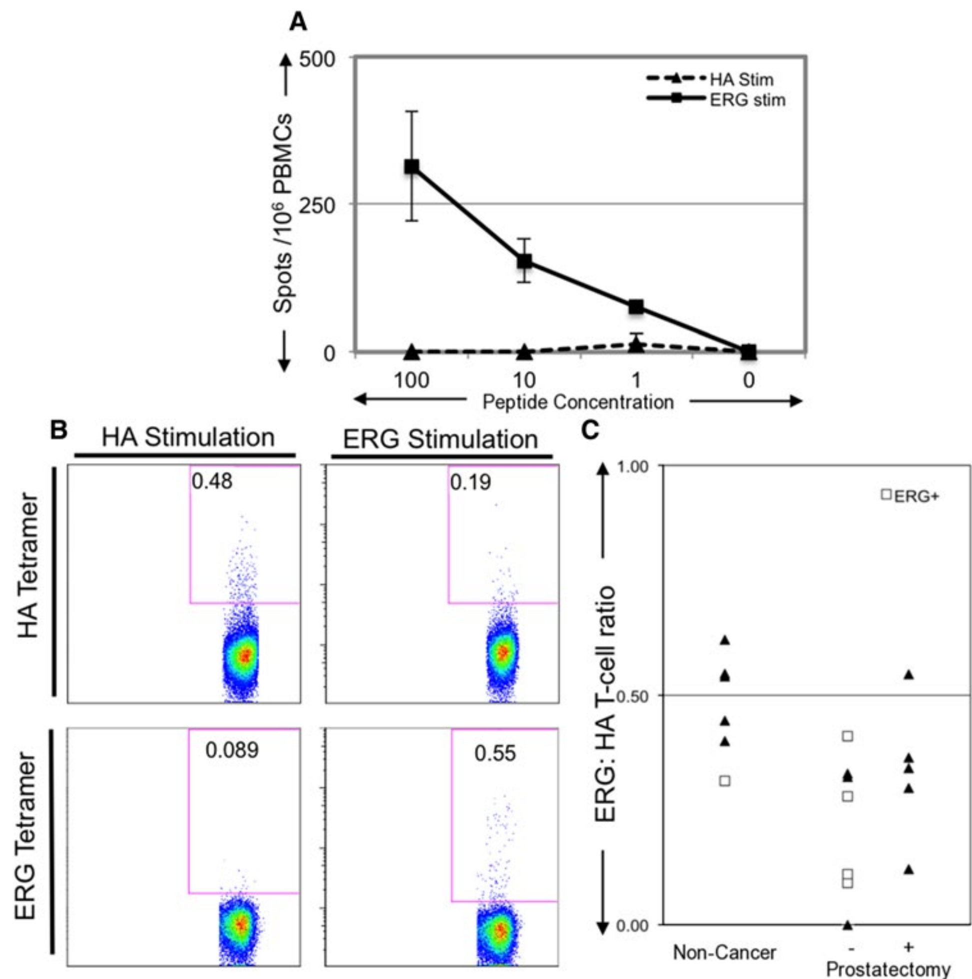


Fig. 4 ERG-reactive CD8 T-cells can be detected in the peripheral blood of HLA-A0201$^+$ subjects. **a** ELISPOT analysis of ERG295-reactive T-cells. PBMCs from HLA-A*0201 healthy donors were co-cultured with HA- or ERG-loaded aAPCs for 9 days. The recall response of healthy donor PBMCs to T2 cells loaded 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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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

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Abstract

Here, we sought to determine whether peptide vaccines designed harbor both class I as well as class II restricted antigenic motifs could concurrently induce CD4 and CD8 T cell activation against autologous tumor antigens. Based on our prior genome-wide interrogation of human prostate cancer tissues to identify genes over-expressed in cancer and absent in the periphery, we targeted SIM2 as a prototype autologous tumor antigen for these studies. Using humanized transgenic mice we found that the 9aa HLA-A*0201 epitope, $SIM2_{237-245}$, was effective at inducing an antigen specific response against SIM2-expressing prostate cancer cell line, PC3. Immunization with a multi-epitope peptide harboring both MHC-I and MHC-II restricted epitopes induced an IFN-γ response in CD8 T cells to the HLA-A*0201-restricted $SIM2_{237-245}$ epitope, and an IL-2 response by CD4 T cells to the $SIM2_{240-254}$ epitope. This peptide was also effective at inducing $CD8^+$ T-cells that responded specifically to SIM2-expressing tumor cells. Collectively, the data presented in this study suggest that a single peptide containing multiple SIM2 epitopes can be used to induce both a CD4 and CD8 T cell response, providing a peptide-based vaccine formulation for potential use in immunotherapy of various cancers.

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Introduction

Defined epitope vaccines elicit an immune response by immunization with a synthetic fragment derived from the target protein. This synthetic fragment is most commonly a 9–10aa long peptide selected to bind human leukocyte antigen (HLA) class I. In the case of cancer vaccines, epitopes that are restricted to a particular MHC-I haplotype are designed and used to stimulate the immune system against tumor-associated antigens (TAAs) [1]. In recent years, this approach for vaccine development has delivered many immunogenic epitopes derived from known TAAs [1–4]. With the advent of high throughput methodologies, the TAA-derived immunogenic epitope portfolio has been significantly enriched due to comprehensive profiling of TAAs of all cancer types.

Peptide-based vaccines for cancer therapy have been developed and subjected to preclinical and clinical testing in numerous studies. Most notably, vaccination with the gp100-209:217(210M), an HLA-A*0201-restricted epitope derived from the melanoma antigen gp100, significantly improved the clinical response and median overall survival of stage IV melanoma patients receiving IL-2 therapy [2]. While peptide-based cancer vaccines had limited success through the years, the survival benefit gained from the gp100-209:217(210M) melanoma epitope vaccine trial was received with much enthusiasm, and has reinvigorated interest in peptide vaccines for cancer immunotherapy. Clinical trials in numerous cancers including melanoma, mesothelioma, colorectal and cervical cancer have been completed and shown this could be an effective strategy for inducing a clinically beneficial immune response against TAAs [1]. Recent studies suggest the inclusion of multiple MHC class I restricted epitopes and addition of MHC class II epitopes in a single longer peptide to improve vaccine outcome [5–8]. Longer multi-epitope peptides targeting p53 have been shown to induce a p53-specific CD4 and CD8 T-cell response in early stage clinical trials against colorectal cancer [9]. Similarly, long peptide immunization against the mesothelioma antigen WT1 induced antigen-specific, CD4 and CD8 T cell response in 6 out of 9 patients [10]. Most impressively, a multi-epitope vaccine against the Human Papillomavirus (HPV) oncogenic E6 and E7 proteins to treat HPV-induced vulvar intraepithelial neoplasia resulted in reduction in symptoms in 60% of patients and complete clearance of disease in 25% of them [11]. These clinical findings support the idea that multi-epitope vaccines can induce effective CD4 and CD8 anti-TAA responses resulting in measurable clinical benefit.

Using a genome-wide interrogation strategy to identify genes that are expressed abundantly in human prostate cancer but sparsely in non-cancerous adult tissues, we previously identified numerous putative prostate TAAs including ETS related gene (ERG) and Single-minded homolog 2 (SIM2) [3,4]. Additionally, we have identified SIM2-derived, HLA-A*0201–restricted, immunogenic epitopes with potential anti-cancer activity [3,12]. Here we aimed to further investigate the immunogenicity of SIM2-derived peptides using humanized mice and human prostate HLA-A*0201-positive cell lines expressing this antigen. We also designed and tested longer peptides harboring multiple MHC-I and MHC-II-restricted epitopes to evaluate whether peptide vaccines that deliver both class-I and class-II restricted epitopes could concurrently induce CD4 and CD8 T cell activation responsiveness *in vivo* with a single peptide.

Methods

Mice and animal ethics statement

HHD mice were obtained from Dr. Francois Lemonnier (Unite d'Immunité Cellulaire Antivirale, Institut Pasteur, Paris, France). These mice are β_2m−/−, 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 β2m by a 15–amino acid peptide arm [13]. 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 line

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* [14]. The HLA-A*0201-containing plasmid was a gift from Dr. Gordon Freeman at Dana Farber Cancer Institute.

In silico analysis of gene expression data

SIM2 gene expression data were obtained through the Oncomine Research Edition (www.oncomine.org). The database was queried for microarray datasets that show a 2-fold change in SIM2 expression and a p value<.01 between cancer and control groups.

Peptide design

The SIM2 protein sequence was downloaded from the NCBI protein database (NP_005060.1). The IEDB (http://www.iedb.org/) epitope prediction algorithm (Available at http://tools.immuneepitope.org/main/html/tcell_tools.html) was then used to predict regions of the protein that may bind MHC-I and MHC-II molecules [15,16].

SIM2-derived peptide immunogenicity in transgenic mice

Eight- to 12-wk-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) [17] or the SIM2 derived I-Ab epitope, LKLIFLDSRVTEVTG. Mice immunized with the long SIM2 peptide received 150 ug total under the same conditions. 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 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 manufacturer's instruction (Mabtech, Murine IFN-γ ELISPOT kit). Spots measured in these experiments were multiplied by the appropriate dilution factor to express IFN-γ producing cells per million splenocytes.

To measure the IL-2 response of CD4 T-cells, pure CD4$^+$ T-cells were isolated using the EasySep mouse CD4 T-cell enrichment kit from StemCell Technologies (Cat: 19752). IL-2 ELISPOT was performed as described by the manufacturer's instruction (eBioscience; 88-7824). CD4$^+$ cells were co-cultured with splenocytes loaded with various peptides (10 µg peptide/mL, for 2 h at 37°C) and treated with 50 µg/mL mitomycin C for 1 hr. ELISPOT plates were developed after 24 hours.

Intracellular flow cytometry

Splenocytes were isolated from immunized HHD mice and co-cultured at a 1:1 ratio with T2 cells loaded with 10 µg of peptide/mL, for 2 h at 37°C. Cells were incubated overnight with Brefeldin A. Cells were stained for surface antigens and then permeabilized using eBioscience permeabilization buffers (eBioscience; 88-8824-00), and then stained intracellularly for IFN-γ. Cells was analyzed with a BeckmanCoulter Galios flow cytometer.

Statistical analysis

Statistical analysis was performed using the Student's T-test. P values of less than 0.05 were considered significant and are denoted by an asterisk in figures.

Results

SIM2 is overexpressed in various cancers

Previously, we reported that SIM2 was an ideal target for prostate cancer immunotherapy, being a protein overexpressed in prostate cancer with little expression in peripheral tissue [3]. To further investigate the suitability of this gene as a target for immunotherapy, we used the Oncomine database to examine the expression of SIM2 in other cancers (**Figure 1A**). Our initial findings in prostate cancer were replicated in other prostate cancer datasets within the Oncomine database (**Figure 1B**). Additionally, we found that many other cancers overexpressed SIM2. In particular, colon cancer had more than a 4-fold increase (**Figure 1C**) in SIM2 expression, and breast cancer had more than a 2-fold increase (**Figure 1E**). Significant increases were also

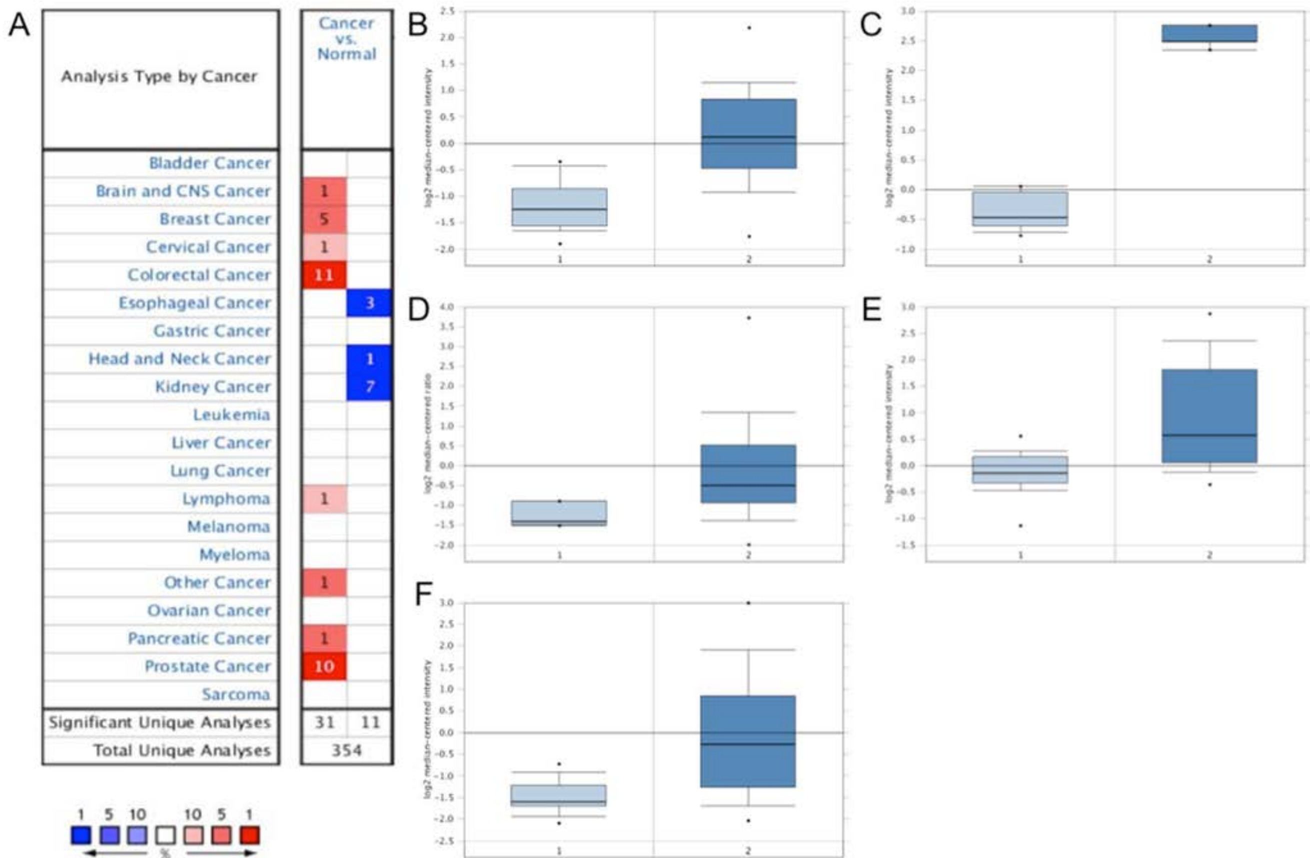

Figure 1. Human SIM2 gene expression analysis in various cancers. SIM2 gene expression data were extracted from the Oncomine Research Edition. Microarray datasets that show a 2-fold change in SIM2 expression between cancer and control groups and a p value <0.01 are highlighted. (**A**) Comparison of SIM2 gene expression between cancer and control specimens. Red color indicates SIM2 overexpression and the blue color indicates SIM2 down-regulation in cancer. Numbers in the boxes indicate the number of datasets showing statistical significance. Box plots were obtained from the datasets selected in (**A**) to highlight significant overexpression of SIM2 in Prostate Carcinoma (1. Prostate Gland (n = 23), 2. Prostate Carcinoma (n = 65); $P = 2.41\times10^{-14}$, [40]) (**B**); Colon Carcinoma (1. Colon (n = 10), 2. Colon Carcinoma (n = 5); $P = 1.65\times10^{-12}$ [41]). (**C**); Breast Carcinoma (1. Breast (n = 4), 2. Invasive Breast Carcinoma (n = 154); $P = 2.25\times10^{-4}$, [42]) (**D**); Oligodendroglioma (1. Brain (n = 23), 2. Oligodendroglioma (n = 50); $P = 3.31\times10^{-9}$ [43]) (**E**); and Pancreatic Carcinoma (1. Pancreas (n = 16), 2. Pancreatic Carcinoma (n = 36); $P = 3.01\times10^{-7}$ [44]) (**F**).
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found in pancreatic cancer and oligodendroglioma (**Figure 1D and 1F**). Together, these data indicate that SIM2 is an attractive immunotherapeutic target for a wide range of cancers.

$SIM2_{237}$ is naturally processed and presented on HLA-A*0201 in prostate cancer cells

Previously, we had identified a number of 9aa long immunogenic HLA-A*0201-restricted epitopes derived from SIM2 [3]. To determine if any of these immunogenic peptides were processed and presented by human prostate cancer cells expressing SIM2, we investigated the activity of splenocytes from HLA-A*0201 transgenic HHD mice immunized with the $SIM2_{205}$ (YQIVGLVAV), $SIM2_{237}$ (SLDLKLIFL), $SIM2_{241}$ (KLIFLDSRV), or control peptide against PC3 and LNCaP cells stably expressing HLA-A*0201. From our previous work, we have identified SIM2 expression in PC3 cells but not LNCaP cells [12]. We found that a significantly increased number of splenocytes isolated from $SIM2_{237}$ produced IFN-γ ($198/10^5$ cells) in response to PC3-A2.1 cells compared to control mice ($55/10^5$ cells), indicating that SIM2-expressing cells process and present this epitope (**Figure 2A**). In contrast, splenocytes from $SIM2_{241}$ and $SIM2_{205}$ immunized mice had no increased activity against the PC3 cells compared to control immunized mice. Additionally, splenocytes from all SIM2 immunized mice had no increased response against PC3 cells that did not express HLA-A*0201, indicating that this effect was dependent on the MHC-I complex. Splenocyte activity of SIM2 peptide immunized mice was also tested against LNCaP cells, a cell line that does not express SIM2. Splenocytes from all SIM2-immunized mice had no increased activity against these cells compared to controls (**Figure 2B**). These data suggest that $SIM2_{237}$ is the immune-dominant epitope in an HLA-A*0201 restricted setting and could be a potential epitope to target prostate cancer.

In silico design and validation of a multi-epitope vaccine containing the prostate cancer epitope $SIM2_{237}$

While immunization with $SIM2_{237}$ generated an antigen specific response against tumor cells, the immunization regimen required the addition of an HBV-derived I-Ab-restricted epitope (HBV_{128}) to induce this response. While providing CD4 stimulation using an HBV-derived peptide is efficient, it does not generate tumor antigen specific CD4 cells. Because CD4 T cells can directly kill tumor cells, expanding cells specific for the target tumor antigen may be beneficial. To eliminate the need of the HBV

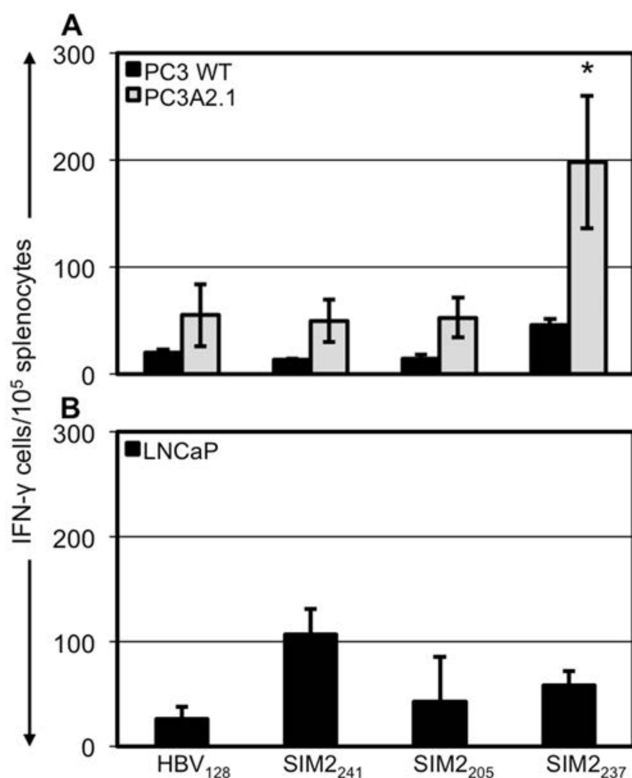

Figure 2. T-cells isolated from SIM2 immunized mice respond to human Prostate Cancer cell lines expressing ERG. Reactivity of splenocytes from SIM2 peptide immunized mice against human prostate cancer cell lines PC3 (**A**) and LNCaP (**B**). Splenocytes from HHD mice immunized with HBV and various SIM2-derived peptides or HBV alone were co-cultured with PC3 or LNCaP. 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 SIM2-immunized mice against the cell lines was repeated in 2 separate experiments.
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helper peptide, we hypothesized that CD4 and CD8 T-cells could be stimulated by a single peptide derived from SIM2 containing both MHC-I and MHC-II binding epitopes. To design this multi-epitope long peptide, we extended the amino acids around the $SIM2_{237}$ core and used prediction algorithms to determine if the longer peptides had MHC-II binding potential. Following this approach, we found that this peptide was predicted to bind many human MHC-II molecules (**Table 1**). This peptide also included an I-Ab-restricted epitope, allowing us to test whether the long SIM2 peptide could induce a $SIM2_{237}$ response in the HHD mice. To determine the *in vivo* immunogenicity of the longer peptide, we immunized HHD mice with the $SIM2_{237}$ CD8 epitope and CD4 HBV helper peptide or the long peptide containing both the MHC-I and MHC-II peptide (**Figure 3A**). Mice immunized with $SIM2_{237}$ peptide alone had no significant recall response to the peptide. In contrast, mice immunized with both $SIM2_{237}$ and HBV_{128} had a significantly increased IFN-γ recall response to the $SIM2_{237}$ antigen. We found that replacing the HBV_{128} peptide with the SIM2 derived MHC-II, $SIM2_{240-254}$ (LKLIFLDSRV-TEVTG) still generated a recall response to the $SIM2_{237}$ epitope. Similarly, when mice were immunized with $SIM2_{230-256}$ (NMFMFRASLDLKLIFLDSRVTEVTGYE) containing both MHC-I and MHC-II epitopes, a significant CD8 recall response to the $SIM2_{237}$ epitope was elicited. Intracellular flow cytometry for IFN-γ confirmed these findings, showing that splenocytes from mice immunized with either the $SIM2_{237}$+HBV_{128} combination, $SIM2_{237}$+$SIM2_{240-254}$ combination or the longer $SIM2_{230-256}$ peptide all generated a significant IFN-γ recall response to the $SIM2_{237}$ epitope ($p<0.05$) (**Figure 4**). Additionally, the IL-2 response of CD4 cells to the helper peptides was measured by ELISPOT. Mice immunized with the HBV_{128} generated a significantly greater recall response to HBV_{128} compared to controls. Similarly, mice immunized with $SIM2_{240-254}$ or $SIM2_{230-256}$ generated a significantly greater IL-2 response to the MHC-II-restricted $SIM2_{240-254}$ epitope compared to controls. These data support our hypothesis that the longer $SIM2_{230-256}$ peptide could simultaneously generate both a CD4 IL-2 response against the $SIM2_{240-254}$ epitope, as well as an IFN-γ response against the $SIM2_{237}$ epitope.

The multi-epitope $SIM2_{230-256}$ peptide induces an antigen-specific response against human SIM2-expressing prostate cancer cell lines

We further tested the effectiveness of the $SIM2_{230-256}$ peptide by measuring the IFN-γ recall response against SIM2-expressing prostate cancer cells. HHD mice were immunized with either the long peptide or HBV_{128}. Splenocytes were co-cultured with PC3 or PC3-A2.1 cells and recall activity was measured by IFN-γ ELISPOT. Splenocytes isolated from mice immunized with the long-SIM2 peptide had significantly increased activity against PC3-A2.1 cells. Additionally, the number of splenocytes isolated from long-SIM2 immunized mice responding to the PC3-A2.1 cells was significantly higher than those responding to the PC3-WT cell line, indicating that this recall response was dependent on expression of HLA-A*0201 (**Figure 5A**). Cells from $SIM2_{230-256}$-

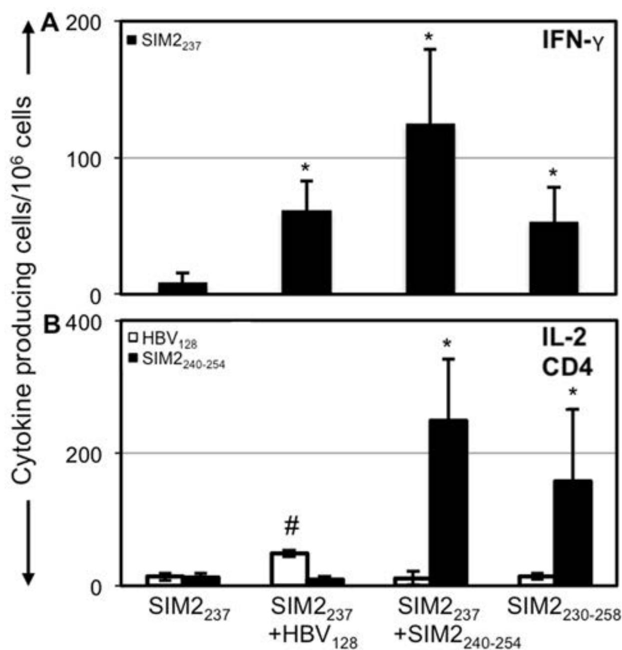

Figure 3. $SIM2_{230-256}$ induces an IFN-γ and CD4 IL-2 response. IFN-γ production by splenocytes in mice immunized with various treatments. Mice were immunized with either the 9aa $SIM2_{237}$ epitope combined with HBV or $SIM2_{240-254}$, or the $SIM2_{230-256}$ peptide alone. IFN-γ production was measured by ELISPOT. IL-2 production by CD4 T-cells. CD4 T-cells were sorted from the spleens of immunized mice and tested for reactivity to HBV_{128} and $SIM2_{240-254}$ by IL-2 ELISPOT.
doi:10.1371/journal.pone.0093231.g003

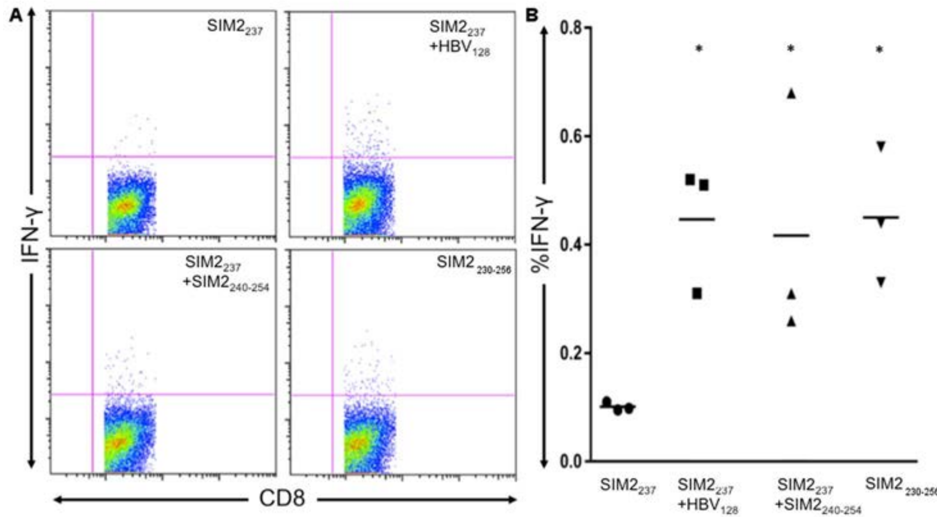

Figure 4. IFN-γ production by CD8 T-cells from SIM2-immunized mice. Mice were immunized with either $SIM2_{237}$, $SIM2_{237}$+HBV_{128}, $SIM2_{237}$+$SIM2_{240–254}$ or $SIM2_{230–256}$. Splenocytes were harvested and incubated overnight with T2 cells loaded with the $SIM2_{237}$ peptide. IFN-γ was measured by flow cytometry. FACS plots show the median IFN-γ production for each group (A) and replicate data obtained from each group (B).
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immunized mice were also tested for activity against the SIM2 negative cell line LNCaP. No increased IFN-γ response was detected against this cell line (**Figure 5B**). Together these data suggest that a single peptide containing both MHC-I and MHC-II epitopes derived from SIM2 can induce T-cell activity against SIM2-expressing prostate cancer cells.

Discussion

Peptide vaccines have been traditionally designed to elicit CTL responses against tumor antigens [18–21] resulting in some, but limited clinical benefit, mainly due to the transience and low magnitude of the immune responses they induce [22]. However, recent evidence suggests the importance of CD4 T helper cells in the anti-tumor immune process [23–25]. The contribution of CD4 T cells to antigen-specific immunity is well appreciated in mounting immune responses to pathogens, a well-orchestrated process whereby both class I and class II MHC-mediated epitope presentation takes place [26]. Activated CD4 T cells secrete many cytokines that stimulate dendritic cells, leading to enhanced antigen presentation and potentiated anti-tumor immunity [27,28]. In addition, CD4 T cell-mediated responses are suspected to contribute to the establishment of memory responses [29]. CD4 cells have also been found to develop cytotoxic activity and be able to eradicate melanoma tumors in lymphopenic hosts [30]. Collectively, these findings provide rationale for induction of

Table 1. Human MHC-II-restricted epitopes predicted from the SIM2 long peptide using IEDB tool.

Allele	Sequence	Percentile Rank[a]	Comb.Lib. IC50(nM)[b]
HLA-DRB1*07:01	MFMFRASLDLKLIFL	0.31	57.04
HLA-DPA1*02:01/DPB1*01:01	MFMFRASLDLKLIFL	0.92	7
HLA-DRB1*09:01	MFMFRASLDLKLIFL	1.78	1.18
HLA-DPA1*02:01/DPB1*05:01	FMFRASLDLKLIFLD	1.83	0.19
HLA-DRB4*01:01	LDLKLIFLDSRVTEV	1.98	130.98
HLA-DPA1*03:01/DPB1*04:02	MFMFRASLDLKLIFL	2.08	3.31
HLA-DPA1*01/DPB1*04:01	NMFMFRASLDLKLIF	3.22	138.55
HLA-DQA1*01:01/DQB1*05:01	MFMFRASLDLKLIFL	3.91	0.48
HLA-DPA1*01:03/DPB1*02:01	NMFMFRASLDLKLIF	4.69	0.72
HLA-DRB3*01:01	NMFMFRASLDLKLIF	5.31	113.08
HLA-DRB1*01:01	LKLIFLDSRVTEVTG	7.88	0.67
HLA-DQA1*04:01/DQB1*04:02	MFRASLDLKLIFLDS	9.51	190.23
HLA-DQA1*05:01/DQB1*02:01	NMFMFRASLDLKLIF	11.5	2.95
HLA-DQA1*03:01/DQB1*03:02	RASLDLKLIFLDSRV	15.99	58.07

Only the top epitopes having the lowest percentile score and lowest IC50 are selected. One epitope is shown for each HLA allele out of 137 predicted binders.
[a]Percentile Rank – Percentage of all peptides binding with this efficacy or lower.
[b]CombLib IC40 – Predicted peptide concentration required to bind 50% of MHC molecules.
doi:10.1371/journal.pone.0093231.t001

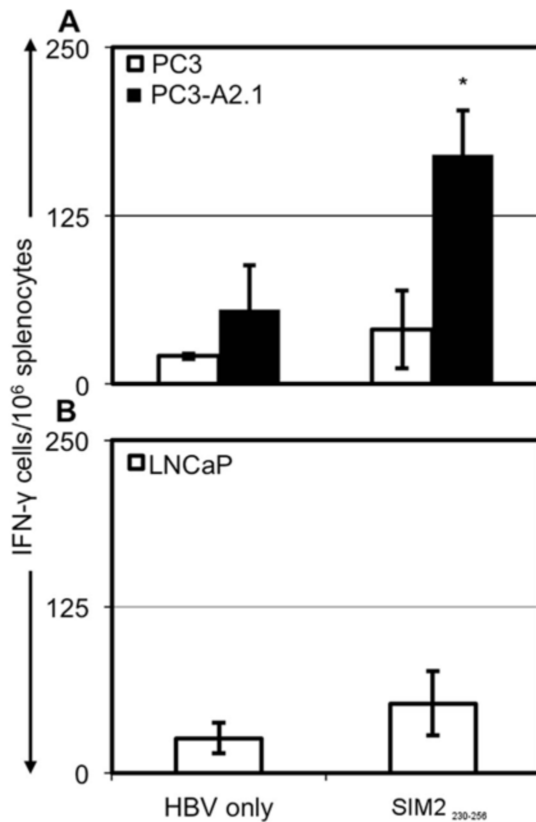

Figure 5. Splenocytes from $SIM2_{230-256}$-immunized mice response to PC3-A2.1 cells. Splenocytes from HHD mice immunized with HBV and various $SIM2_{230-256}$ peptides or HBV alone were co-cultured with PC3, PC3-A2.1 (**A**) or LNCaP (**B**). Production of IFN-γ by splenocytes in response to these tumor cell lines was assessed by ELISPOT. Data is representative of 2 experiments and shows mean ± standard deviation.
doi:10.1371/journal.pone.0093231.g005

CD4 T cell responses with cancer vaccines, either alone, or in combination with MHC-I-restricted epitopes.

We and others have previously demonstrated overexpression and specificity of SIM2 in prostate cancer patients [31,3,12]. Additionally, we have identified SIM2-derived, HLA-A2.1-restricted epitopes that exhibit the ability to break immune tolerance to SIM2 in mice, and identified SIM2-specific auto-antibodies in sera from patients with PCa [3]. Our work has subsequently suggested a biological role for SIM2 in PCa [12]. However, we have not determined whether the HLA-A2.1-restricted epitopes we identified are naturally processed and presented in tumor cells, nor have we identified longer epitopes that could also trigger CD4 T cell responses. In the present work, we show that overexpression of SIM2 is not limited to PCa. SIM2 is similarly overexpressed in several other malignancies, including colon cancer, breast cancer, cervical cancer, pancreatic cancer and oligodendroglioma, suggesting SIM2 may be an attractive target for immunotherapy of a wide range of cancers. Interestingly, while an overexpression of SIM2 in cancer might suggest a tumorigenic role for SIM2, its frequent down-regulation in other cancers such as oesophageal, kidney, and head and neck cancers (**Figure 1**) might suggest a tumor suppressive role. In fact, SIM2 has been shown to suppress breast cancer growth and invasion in a xenograft model [32]. More intriguing is the observation that Down's syndrome patients are prone to acute leukemia, including acute lymphoblastic leukemia (ALL), while solid tumors, especially breast cancer, is rare [33]. SIM2 is among many transcription factors encoded by genes located on the human chromosome 21. Together, these studies suggest that SIM2 is an attractive immunotherapeutic target for a range of different cancers.

Our data showed that a $SIM2_{237}$-specific response could be elicited against the SIM2-expressing PC3, but not against the SIM2 negative LNCaP cell line (**Figure 2**). However, $SIM2_{241}$ and $SIM2_{205}$ could not induce a CTL response against these same cells, despite both of these peptides showing antigen-specific CTL responses in HLA-A*0201 transgenic mice [3]. In this study we were unable to test the response to another SIM2 expressing prostate cancer cell line, VCaP, due to the cell lines failure to grow after transfection with HLA-A0201. However, The findings that not all immunogenic peptides generate a response against SIM2 expressing cell lines 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 [34,35]. Nonetheless, together these data indicate that the $SIM2_{237}$ peptide is presented in an HLA-A*0201-restricted manner on cells expressing the SIM2 molecule.

Algorithms that predict MHC-II-restricted epitopes indicate that all proteins, native and mutated, harbor multiple potential MHC-II-restricted epitopes. Compared to MHC-I epitopes, MHC-II-restricted epitopes exhibit a much wider specificity and cross-reactivity, as exemplified by the ability of the PADRE (Pan DR epitope) peptides to recognize a high number of MHC-II alleles in both human and mouse [36]. In the case of SIM2 protein, it is clear many of the epitopes we predicted to bind HLA-DR/DP/DQ would target large populations of patients because of their wide specificity. However, while targeted clinical use of these epitopes would necessitate HLA typing of patients, our mouse immunogenicity tests suggest the long SIM2 peptide harbors an IA-b-restricted epitope(s), as evidenced but the ability of the long peptide to elicit a $SIM2_{237}$-specific CTL response in the absence of the HBV_{128} helper peptide. This response is equal in magnitude to that induced with the combination of HLA-A2.1-restricted $SIM2_{237}$ and the I-Ab-restricted HBV_{128} epitopes. Because long peptides are internalized and processed by dendritic cells, our results indicate a successful internalization and processing of the long peptide and an optimal presentation in the context of both MHC-I and MHC-II complexes to T lymphocytes. The ability of dendritic cells to successfully achieve these steps implies cancer vaccines could be made that contain one single peptide, thus dramatically reducing the cost and regulatory procedures on the path to clinical application. Previous studies have elegantly demonstrated that an increase in the length of the peptide used for vaccination strongly affects the magnitude of the induced CTL response [6,37]. Comparative experiments showed vaccination with long peptides containing a CTL epitope outperformed vaccination with the CTL peptide alone at inducing effective anti-tumor CTL responses [38]. The low effectiveness of CTL epitopes was shown to be due to the transient nature of the response they can elicit and their failure to induce CTL memory [39].

Together the findings of this study suggest prostate tumor cells expressing SIM2 present the $SIM2_{237}$ epitope in an HLA-A*0201-dependent fashion. Additionally, the multi-epitope peptide $SIM2_{230-256}$ can provide TCR stimulation to both CD4 T cells and CD8 T cells simultaneously. Furthermore, this peptide contains numerous epitopes predicted to bind to various human MHC-II molecules, suggesting that this peptide could induce a CD4 T-cell response in individuals with many different HLA-DR/DP/DQ alleles. Collectively, these data indicate that an effective

antigen-specific response can be augmented by concurrent inclusion of class-I and class-II restricted epitopes in peptide vaccine formulations targeting autologous human tumor antigens.

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Author Contributions

Conceived and designed the experiments: HTK MGS LKD MSA. Performed the experiments: HTK LKD. Analyzed the data: HTK MGS LKD MSA. Contributed reagents/materials/analysis tools: HTK MGS LKD MSA. Wrote the paper: HTK MGS LKD MSA.

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Androgens alter T cell immunity by inhibiting T helper 1 differentiation

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Abstract

The hormonal milieu influences immune tolerance as well as the immune response against viruses and cancer, but the direct effect of androgens on cellular immunity remains largely uncharacterized. We therefore sought to evaluate the effect of androgens on murine and human T cells in vivo and in vitro. We found that murine androgen deprivation in vivo elicited RNA expression patterns conducive to interferon signaling and T cell differentiation. Interrogation of mechanism showed that testosterone regulates Th1 differentiation by inhibiting IL-12 induced Stat4 phosphorylation: in murine models, we determined that androgen receptor binds a conserved region within the phosphatase, Ptpn1, and consequent up-regulation of Ptpn1 then inhibits IL-12 signaling in CD4 T cells. The clinical relevance of this mechanism, whereby the androgen milieu modulates CD4 T cell differentiation, was ascertained as we found that androgen deprivation reduced expression of Ptpn1 in CD4 cells from patients undergoing androgen deprivation therapy for prostate cancer. Our findings, that demonstrate a clinically relevant mechanism by which androgens inhibit Th1 differentiation of CD4 T cells, provide rationale for targeting androgens to enhance CD4-mediated immune responses in cancer or, conversely, for modulating androgens to mitigate CD4 responses in disorders of autoimmunity.

Significance Statement

Testosterone has been implicated as a regulator of the immune response to viruses, vaccines, host tissue and cancer. Despite this pleiotropic effect on the immune system, the mechanisms underlying this effect are not well understood. In this study, we investigated how testosterone altered gene expression and signaling mechanisms in CD4 T cells in mouse models, and prostate cancer patients undergoing androgen deprivation therapy. We found that testosterone inhibited Th1 differentiation by up-regulating the phosphatase, Ptpn1, in both mice and humans. Additionally, the androgen receptor bound a highly conserved region of the Ptpn1 gene, suggesting an evolutionarily important purpose of this mechanism. This study provides a mechanism to explain recent discoveries regarding the role of testosterone-mediated inhibition of the immune response.

\body

Introduction

The gender specific hormones, testosterone and estrogen, have a number of immuno-modulatory effects. This is demonstrated in a many immunological settings. Women almost universally respond as well or better than men to antibody inducing vaccinations (1). For example, healthy women treated with the trivalent inactivated influenza vaccine generate a greater antibody titer than men (2). Findings like these have led to the suggestion that estrogen promotes Th2 differentiation and antibody production (3). Further supporting an increased antibody response caused by estrogen, over 80% of patients suffering from antibody driven auto-immunities such as Systemic Lupus Erythematosus, Sjörgren's syndrome and Hashimoto's thyroiditis are women (4). In contrast to estrogen, how testosterone affects the immune system is less clear, but its role in immunity against viruses and host antigens is certainly immunosuppressive.

Recently, it was reported that testosterone levels negatively correlated with the antibody response to the trivalent inactivated seasonal influenza vaccine by interfering with lipid metabolism (5). Testosterone levels are also positively correlated with the viral load of Venezuelan equine encephalitis virus in macaques (6). In addition to the response to viruses, testosterone regulates the response to host antigens in many biological systems. Elevated levels of testosterone following colonization with commensal microbes correlated with reduced islet inflammation and protection from type-1 diabetes in NOD mice (7). Also, tolerance to tumor antigens is regulated by testosterone, as androgen ablation in a mouse model of prostate cancer reversed $CD4^{+}$ T cell tolerance to a prostate restricted tumor antigen (8). Similarly, castration of male mice prior to vaccination with prostate-specific antigens enhanced $CD8^{+}$ T cell vaccine response in many studies (9, 10). Patients undergoing androgen deprivation in prostate cancer had increased infiltration of T cells into benign and malignant prostate tissue (11). Based on these findings, clinical trials are currently underway to test the combinatorial efficacy of androgen deprivation and immunotherapy in prostate cancer patients (12). Together, these observations suggest a critical role for testosterone in maintaining T cell tolerance towards not only viruses, but also host and tumor antigens.

Despite these observations that testosterone inhibits immunity, the precise molecular mechanisms by which testosterone achieves this effect are poorly understood. Here, we sought to address this question by performing gene expression profiling of CD4 T cells isolated from castrated mice. Gene expression analysis revealed a critical effect of testosterone on CD4 T cell differentiation and identified the tyrosine phosphatase Ptpn1 as a mediator of androgen-induced suppression of CD4 T cell differentiation. The research presented here highlights a novel molecular mechanism by which testosterone suppresses immunity, and allows a better understanding of gender differences in the response to viruses, autoimmunity, and immune escape in prostate cancer.

Results

Gene expression profiling of CD4 T cells isolated from castrated mice reveals altered differentiation and signaling

To investigate the molecular mechanisms by which androgen withdrawal affects the T cell response to self-antigens, gene expression of $CD4^+$ T cells isolated from the spleens of surgically castrated or control mice was profiled using Affymetrix micro-arrays. Of the 1037 2-fold differentially expressed genes, 637 were down regulated, and 400 up-regulated following castration. Using the Ingenuity Pathway Analysis (IPA) software package, we found that the two most significantly altered pathways within these differentially regulated were interferon signaling and T-helper cell differentiation (**Figure 1A**). About half of the genes that constitute these two pathways were differentially expressed. In particular, numerous genes related to Th1 differentiation such as T-bet, IL-12R, and IFN-γ were upregulated (**Figure 1B**). To confirm these micro-array findings, expression of key $CD4^+$ T cell differentiation genes was measured by RT-PCR. Consistent with the microarray analysis, a significant increase in expression of T-bet and IFN-γ was observed, whereas no change was observed for the other master transcription factors involved in $CD4^+$ T cell differentiation, Gata-3, Foxp3, and Ror-γt (**Figure 1C**), suggesting that differentiation of CD4 T cells into the T_h1 phenotype was preferentially affected. Expression of the Th1 surface markers CXCR3, CCR5, and IL-12Rb2 were analyzed. CXCR3 was significantly increased in CD4 cells in castrated mice, while the other 2 markers were unchanged (supplementary figure 1). Additionally, analysis of upstream transcription factors responsible for the observed gene changes predicted significant activation of the Th1-related transcription factors IRF-1, -3 and -7, and STAT1 in castrated CD4 T cells (Supplementary table 2). These data suggest that important signaling pathways and gene expression regulatory networks orchestrating Th1 differentiation are altered following castration.

Testosterone inhibits Th1 differentiation in vitro

T-helper cell differentiation is a critical junction of an immune response and Th1 differentiation is particularly important in the case of the response to self and tumor antigens (13). Following the *in silico* identification of the major disruptions to T_h1 differentiation by androgen deprivation, we sought to further investigate the effect of androgen on this differentiation pathway *in vitro*. $CD4^+$ T cells isolated from spleens of male mice were incubated overnight in androgen-free culture medium. These cells were then cultured under Th1 polarizing conditions in the presence of the androgen analogue R1881 or vehicle control and then re-stimulated with PMA/Ionomycin 3 days later. We found that $CD4^+$ T cells treated with androgen produced significantly less IFN-γ as measured by intracellular flow cytometry (**Figure 2A & B**). To determine whether this was due to inhibition of Th1 differentiation or the recall response, IFN-γ production was measured in cells differentiated without androgen but re-stimulated in its presence. These cells produced the same amount of IFN-γ as cells that were not exposed to androgen, indicating that androgen inhibited IFN-γ production before re-stimulation. Similarly, IFN-γ production by cells differentiated for 3 days in the presence of androgen, but re-stimulated overnight in androgen-free media produced the same

amount of IFN-γ as cells both differentiated and re-stimulated in the presence of androgen, suggesting that androgen inhibition of IFN-γ production by $CD4^+$ T cells occurs during the T_h1 differentiation phase. To further investigate the effect of androgen on T cell differentiation, RNA was isolated from cells differentiated in androgen or vehicle control prior to PMA/Ionomycin stimulation. Consistent with the *in silico* analysis, $CD4^+$ T cells differentiated in the presence of androgen had significantly reduced levels of T-bet and IFN-γ (**Figure 2C**). Together, these data suggest that androgen exerts its effect on T cells during the early events involved in T_h1 differentiation.

Testosterone inhibits IL-12-induced STAT4 phosphorylation and downstream gene expression

Since T_h1 differentiation critically relies on IL-12-induced phosphorylation of STAT4, we analyzed this protein in CD4 T cells treated with IL-12. STAT4 phosphorylation was reduced in $CD4^+$ T cells treated with androgen 30 minutes following IL-12 exposure. The androgen treated cells still showed a slight reduction in STAT4 phosphorylation 60 minutes following IL-12 treatment. However, after 24 hours cells treated with androgen or vehicle had equal levels of pSTAT4 (**Figure 2D**). Since insufficient induction of pSTAT4 during $CD4^+$ T cell activation can lead to T cell anergy or induction of regulatory T cells (14), we hypothesized that testosterone may increase the threshold of IL-12 required to induce Th1 differentiation. $CD4^+$ T cells were cultured under Th1 polarizing conditions with increasing concentrations of IL-12 either in the presence of 1ng/ml R1881 or vehicle control, and the expression of T-bet was assessed 3 days later. R1881 treatment resulted in significantly less T-bet expression in $CD4^+$ T cells receiving 0, 3.125, and 12.5 ng/ml of IL-12 (**Figure 2E**). However, at the higher concentrations of IL-12 (25-100ng/ml), androgen had no significant effect on T-bet expression. These data support the notion that Th1 differentiation is inhibited in the presence of androgen, and also, that $CD4^+$ Th1 polarization is specifically impaired in low IL-12 conditions via a reduction in STAT4 phosphorylation.

Castration of mice leads to increased T cell infiltration in lung, prostate, and intestine

Androgen deprivation therapy increases lymphocyte infiltration in the prostate gland of both mice and men and this infiltration is predominantly CD4+ T-cells (10, 11). This observation was made in the context of prostate cancer and omitted evaluating non-prostatic tissue inflammation. Given our finding that testosterone reduced the amount of IL-12 required to induce CD4 cell differentiation, we hypothesized that testosterone's effect might extend beyond the prostate. To investigate this, liver, lung, prostate, and intestine samples were collected from male mice one month following surgical castration, and stained for CD3 expression. We found a significant increase in the number of $CD3^+$ cells in the prostate gland (4 fold) and lungs (2.5 fold). Infiltration in the prostate was mostly found in the peripheral zone (supplementary figure 2). Additionally, a non-significant increase was observed in the small intestine (p=0.056) (**Figure 2F**). The presence of some sparse and sporadic T cell infiltrates in tissues of sham castrated mice indicates the onset of a minimal, spontaneous "autoimmune" phenotype, and the significantly increased presence of T cells in non-prostatic tissues of castrated animals suggests that testosterone may limit the reactivity of T cells to host tissue.

Testosterone up-regulates the tyrosine phosphatase Ptpn1 and inhibits Tyk2 phosphorylation

Many molecules regulate phosphorylation of STAT4 following IL-12 exposure. Therefore, we sought to examine the expression of signal transduction molecules upstream of STAT4 in an attempt to understand how testosterone inhibits IL-12 signaling. To identify signal transduction components that may be regulated by testosterone, we searched for genes with opposite expression in $CD4^+$ T cells exposed to high or low testosterone conditions by comparing $CD4^+$ T cells isolated from castrated mice to testosterone treated $CD4^+$ T cells *in vitro*. We found that the genes encoding protein tyrosine phosphatase non-receptor 1 and 11 (Ptpn1 and Ptpn11) were significantly decreased in castrated mice and significantly up regulated in androgen treated $CD4^+$ T cells, suggesting that the expression of these phosphatases is regulated by testosterone (**Figure 3A & B**). Candidate genes from the SOCS, PIAS, and Jak family of molecules were affected in one condition but not the other (Supplementary data 1). These data suggest that while androgen seems to exhibit diverse effects on T cell signaling, only Ptpn1 and Ptpn11 were directly controlled by the presence of androgen under our experimental conditions.

To investigate whether androgen directly affects expression of Ptpn1 and Ptpn11, CD4 T cells were treated overnight with androgen and Ptpn1 and Ptpn11 gene expression was analyzed. We found that Ptpn1 was significantly up regulated (1.7 fold), whereas no significant difference in Ptpn11 expression was seen (**Figure 4C**). To further understand how androgen regulates expression of Ptpn1, we investigated publically available gene expression profiling datasets involving testosterone treatment to find how Ptpn1 expression was affected in other cell types and conditions. Ptpn1 was found to be unchanged or up-regulated by testosterone exposure in all datasets examined, including human prostate cancer cell lines, mouse adipose tissue, and mouse lacrimal and submandibular glands (**Figure 3D**). These data show that Ptpn1 expression is regulated by androgen in numerous cell types, including lymphocytes.

Ptpn1 is a phosphatase that dephosphorylates IGFR, Jak2 and Tyk2, thereby inhibiting downstream signals triggered by these molecules (15). Our IPA analysis of $CD4^+$ T cells from castrated mice predicted that the kinase Tyk2 was significantly activated in castrated mice (Supplementary table 2). The up-regulation of Ptpn1 by testosterone, leading to the dephosphorylation of Tyk2 could explain how androgens inhibit Th1 differentiation. To investigate this, $CD4^+$ T cells were treated overnight with androgen and Tyk2 phosphorylation was measured following administration of IL-12. Strong Phospo-Tyk2 signal was detected 5 minutes after IL-12 treatment under androgen free conditions. In contrast, androgen treated cells had a much lower level of Tyk2 and STAT4 phosphorylation (**Figure 3E**). To investigate the contribution of Ptpn1 to this inhibition, cells were treated with a specific Ptpn1 inhibitor for two hours before the administration of IL-12. The inhibitor restored the phosphorylation of Tyk2 and STAT4, suggesting that Ptpn1 induction by testosterone inhibits IL-12 signaling (**Figure 3E**). This finding supports our hypothesis that up regulation of Ptpn1 by testosterone leading to the inhibition of Tyk2 phosphorylation is a mechanism by which testosterone inhibits Th1 polarization.

Androgen receptor binds a conserved region within intron 3 of Ptpn1

Following testosterone binding, the androgen receptor is translocated to the nucleus where it associates with androgen response elements (ARE) in the DNA. When bound to DNA, androgen receptor (AR) can regulate gene expression by directly initiating transcription or indirectly altering the epigenetic environment of the target gene (16). Androgen receptor was detectable in CD4 cells isolated from both sham and castrated mice (supplementary figure 3). To investigate how androgen affects Ptpn1 expression, we created a search algorithm to identify potential AR binding sites in the Ptpn1 gene and upstream promoter region. Five putative AR-binding sites were found in and around the Ptpn1 gene (**Figure 4A**). To determine whether AR bound any of these regions, chromatin from $CD4^+$ T cells treated with R1881 or vehicle was immunoprecipitated (ChIP) with an anti-AR antibody and potential binding sites were tested by PCR. Interestingly, no significant enrichment for the predicted AR binding site in the promoter region was observed. However, the predicted binding site in the intron between exon 3 and 4 (intron 3) was significantly enriched after R1881 treatment (**Figure 4A)**. Because we have observed androgen specific down regulation of Ptpn1 expression in both human and mouse cells, we assessed the homology of mouse intron 3 amongst human, mouse and three other species. While the exonic regions of Ptpn1 were highly conserved across many species, human and mouse introns rarely showed homology above 50% (**Figure 4B**). However, regions of micro-homology, defined as 20-50bp regions of very high homology (>80%) conserved across multiple species, were present in some introns (highlighted in blue in **Figure 4C**). One of these regions contained the AR binding site that we identified in intron 3, and a similar site in the same intron was also present in Rat, Macaca Monkey, and Elephant, suggesting that an evolutionarily conserved role of androgen regulation of Ptpn1 might exist (**Figure 4C**). Together, these data show that androgen regulates expression of Ptpn1 in numerous cell types, in both human and mouse, and that a conserved androgen binding site in the third intron of the Ptpn1 gene may be the critical regulator of this effect.

CD4 cells isolated from patients undergoing ADT have decreased Ptpn1 expression

Prostate cancer patients undergoing androgen derivation therapy (ADT) have increase T cell infiltration into the prostate gland, and castrated mice have an improved response to vaccination against tumor antigens (8-10). Given the homology of the AR binding site between the mouse and human Ptpn1 gene, we investigated whether Ptpn1 was regulated in a similar way in human T cells *in vivo*. To do this, we isolated $CD4^+$ T cells from the peripheral blood of prostate cancer patients undergoing androgen deprivation therapy (ADT). $CD4^+$ T cells from patients on ADT showed a 2-fold reduction in Ptpn1 expression compared to control patients (**Figure 5**). In comparison to mice, there was no significant change in IFN-γ or T-bet expression in the human CD4 T cells. Additionally, there was no difference in any other CD4 T cell transcription factors (Supplementary data 1). These data support the conserved function of androgen receptor regulation of Ptpn1 and may explain the improved T cell response to the tumor in ADT patients.

Discussion

Here, we have investigated the effect of testosterone on CD4 T cell function. Our data suggests that testosterone inhibits CD4 T cell differentiation by up-regulating the phosphates Ptpn1. Ptpn1 is an enzyme that is expressed in many cell types, including lymphocytes and importantly, it dephosphorylates both Jak2 and Tyk2, the upstream kinases responsible for Stat4 phosphorylation (15, 16). Since Ptpn1 is known to dephosphorylate Tyk2, our data showing testosterone reduced Tyk2 phosphorylation supports this role of Ptpn1. Additionally, Tyk2 phosphorylation could be partially restored by co-administration of a specific Ptpn1 inhibitor, further suggesting that androgen inhibition of Stat4 phosphorylation and Th1 differentiation is mediated through up regulation of Ptpn1. This role of Ptpn1 has not been previously reported, however, other tyrosine phosphatases, Ptpn6 and Ptpn11, inhibit Th1 differentiation (17-19). Inhibited Th1 differentiation by Ptpn1 has clear implications to the immune response, and helps explain previous observations that testosterone limits viral vaccine effectiveness, viral clearance, the response to host antigens, and the T cell response to cancer (5-9).

Beyond de-phosphorylating Tyk2, Ptpn1 has many other known roles, and likely contributes to suppression of the immune system beyond what we have reported. For example, we found that androgens could also inhibit Th17 differentiation (supplementary figure 4). Stat5 is also a substrate for Ptpn1, and Ptpn1 deficiency increases Stat6 phosphorylation in B cells (20, 21). Additionally, Ptpn1 deficient mice suffer from systemic inflammation, increased leukocyte migration, and is a potential molecule in regulating the allergic response (22). In addition to regulating known pathways affecting immunity, Ptpn1 has a critical role in regulating metabolism. Ptpn1 regulates both IGF signaling by inhibiting receptor kinases associated with the receptors of these molecules (23, 24). In addition, there are a number of Single Nucleotide Polymorphisms (SNPs) found in Ptpn1 that are associated with hypertension and obesity (25). One of the critical findings of a recent report demonstrating the negative correlation of testosterone and influenza vaccine efficacy was that testosterone altered lipid metabolism in the immune system (5). Given that Ptpn1 is a regulator of IGF signaling, it is rational to expect that testosterone induced expression of Ptpn1 may be associated with regulation of lipid metabolism, and therefore, could be one of the mechanisms that leads to reduced influenza vaccine efficacy by testosterone.

The mechanism of androgen induced regulation of Ptpn1 expression was also addressed in this work. Various studies have demonstrated the presence of androgen receptor (AR) both in the cytosol and on the membrane of T lymphocytes (26, 27). We found that there was an AR binding site in the intron between exon 3 and 4 of the Ptpn1 gene. However, how the binding of AR to this region increased Ptpn1 expression of the gene is unclear. One possibility is chromatin modification of this region by factors that associate with the androgen receptor (28). The effect of this has been shown recently in genome wide investigations where AR binding to DNA has been found to be associated with increased accessibility of the location and this could be how AR regulates Ptpn1 expression (29). Others have recently found that AR binds Ptpn1 in human prostate cancer cells leading to Ptpn1 expression although the binding was at a different location

from the one we found (30). While these observations implicate AR mediated expression of Ptpn1, the question remains of why androgen regulates Ptpn1. We found that the AR binding site was highly conserved amongst numerous species within a region of very low homology, indicating that the regulation clearly has some evolutionary importance. Why males need to be more protected from autoimmunity is unclear. Others have offered plausible speculation that the evolutionary role of testosterone mediated immune suppression may be to protect men from pathogen exposure during traumatic injury, for which in many species the males are more likely to suffer (5). Our data suggests an additional possibility; that since the role of Ptpn1 is important in metabolism regulation and IGF-1 signaling (32), testosterone regulation of Ptpn1 is more important for these processes and the effect of testosterone on the immune system either directly or by altered lipid metabolism may be a bystander effect. Nonetheless, testosterone does contribute to expression of Ptpn1 in multiple cell types and species, and in T cells this increase in Ptpn1 expression reduces the response of the cells to IL-12.

The findings presented here show a potential mechanism to explain how testosterone alters CD4 T cell function. We found that down regulation of Ptpn1 following castration increased Th1 differentiation in sterile inflammatory conditions. Further investigation in vitro found that testosterone limited Stat4 phosphorylation and Th1 differentiation. We propose that these findings may help explain why testosterone helps alleviate Th1 driven autoimmune conditions. Furthermore, these findings support the idea of using ADT as an adjuvant for immunotherapeutic intervention in prostate cancer and suggest that Ptpn1 targeting could be investigated as an adjuvant for cancer vaccine against tumor antigens.

Methods

Surgical castration

C57Bl/6J mice were castrated as previously described (9). Mice were used in experiments 28 days after castration, the earliest time point we have previously found to be when androgen dependent gene expression is lost (9).

Histology

Trained pathologists at the BIDMC histology core performed tissue sections and immunohistochemistry staining. Slides were stained using anti-CD3 (Abcam, 134096) at a 1:500 dilution and a secondary Biotin SP conjugated anti-Rabbit IgG diluted 1:250 (Jackson ImmunoResearch, 711-065-152). A microscope operator blinded to the experimental design and staining procedure acquired 10 random fields of view from each slide. CD3+ cells were counted using an automated counting algorithm in ImageJ.

Microarray analysis

$CD4^{+}Foxp3^{GFP}$ cells were isolated from mice using FACS. RNA was extracted and gene expression was assessed using Affymetrix's (Santa Clara, CA) GeneChip Mouse Genome 430 PM arrays over 2 separate experiments. Data can be accessed from the GEO database, accession number GSE54945.

Pathways and network based analysis

Interactive networks, pathways and functions analysis was performed on this list of genes using commercial system biology oriented package Ingenuity Pathways Analysis (IPA) (www.ingenuity.com).

T cell isolation and T_h1 differentiation

The CD4 T cells isolated from human and mouse samples used for gene expression analysis were sorted by FACS. A detailed gating strategy to isolate these cells is shown in supplementary figure 5. CD4 T cells used for in vitro experiments were isolated from the spleen of male C57Bl/6J mice using StemCell Technologies CD4 T cell isolation kit (Cat: 19765). To eliminate the effect of endogenous testosterone, T cells were incubated overnight in charcoal stripped media. For Th1 differentiation experiments, CD4 cells were incubated with anti-CD3/anti-CD28 stimulation beads (Life Technologies), 5ng/ml IL-12 (Peprotech) and 10ng/ml IL-2 (Peprotech). To investigate the role of androgen, T cells were treated with either 2ng/ml R1881 (PerkinElmer) or DMSO as a vehicle control. Re-stimulation of T cells was performed using eBioscience cell stimulation cocktail (PMA/Ionomycin, Cat#: 00-4979-93). IFN-γ production by CD4 T cells was assessed by flow cytometry using eBioscience antibodies.

Western blot

CD4 cells were isolated as described above and incubated overnight in charcoal-stripped androgen free media. T cells were treated with 5ng/ml IL-12, 2ng/ml R1881 or vehicle control. Experiments investigating the role of Ptpn1 used the inhibitor "3-(3,5-Dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide" at a concentration of 4μM (Millipore, Cat # 539741).

Protein was extracted using cell lysis buffer (Cell Signaling) as described by manufacturer's instruction. Western blot analysis of pSTAT4 (D2E4) and PTyk2 (Cell Signaling #9321) was performed following the manufacturer's protocol. Figures shown are representative of at least 3 separate experiments

Androgen receptor binding site prediction

Putative androgen receptor-binding sites in the Ptpn1 gene were determined by searching the mouse Ptpn1 gene for regions of high similarity to the ARE-I sequence, AGAACANNNAGTGCT.

Ptpn1 homology

Homology between species was determined by comparing the similarity of the mouse Ptpn1 gene to the human Ptpn1 gene using a custom python script. Briefly, the mouse and human Ptpn1 genes were subdivided into 300bp frames. These fragments of the mouse gene were then paired with the fragments derived from the human gene based on their homology. Within the 300bp matching frames, 50bp regions from the mouse gene were compared to 50bp regions in the human gene and the location with the highest percentage homology reported. The highest percentage homology of a region is reported in Figure 4B. The same approach was used for comparing the Mouse and Rat copies of Ptpn1.

Chromatin immunoprecipitation

CD4 T cells were isolated as described above and incubated overnight in testosterone free media. T cells were then treated in triplicate with 2ng/ml R1881 or DMSO as a vehicle control for 12 hours. Samples were pooled after 12 hours and chromatin immunoprecipitation was performed using the Cell Signaling enzymatic preparation kit (Cat# 9003) (See supplemental table 1 for specific information).

Statistical analysis

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

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Figure Legends

Figure 1. Th1 differentiation is enhanced in castrated mice. (A) Significantly altered pathways in CD4 cells isolated from castrated mice. Genome-wide gene expression profiling of CD4 T cells isolated from the spleen of male mice 1 month following castration was performed and significantly altered signaling pathways determined using the Ingenuity Pathway Analysis package. (B) Genes changed in CD4 T cell differentiation pathways. CD4 T cell differentiation pathway was found to be significantly altered in castrated mice and differentially expressed genes associated with these pathways are shown. (C) PCR confirmation of important differentiation genes. Gene expression of important differentiation regulators/markers in CD4 T cells isolated from sham (•) or castrated mice (■) was measured by RT-PCR. Data combined from 2 separate experiments is shown.

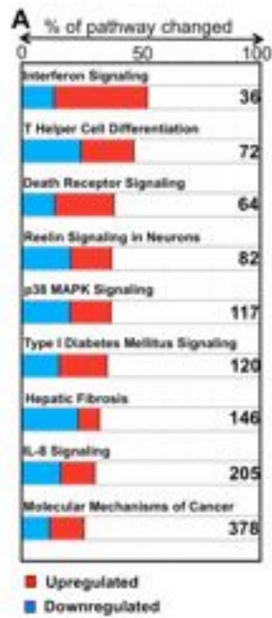
Figure 2. Th1 differentiation is inhibited by testosterone *in vitro*. (A and B) IFN-γ production by CD4 cells differentiated with testosterone. Spleen CD4 T cells were cultured under Th1 polarizing conditions and treated with the androgen receptor agonist R1881 or vehicle control. Representative plots of IFN-γ production measured by flow cytometry after re-stimulation with PMA/Ionomycin. Mean ± SD of IFN-γ production after Th1 differentiation and re-stimulation in the presence of R1881 or vehicle for 5 samples. (C) Gene expression in testosterone treated Th1 cells. Fold change of gene expression of Th1-related genes from CD4 T cell differentiation (n=3). (D) Testosterone effect on Stat4 phosphorylation. CD4 T cells were analyzed for levels of phospho-STAT4 at different time points following treatment with 5 ug/ml IL-12 in the presence or absence of R1881. (E) Testosterone effect on T-bet expression. T-bet expression in CD4 T cells after 4 days of culture under Th1 polarizing conditions in the presence or absence of R1881 (n=5)..(F) Castration induces T cell infiltration into various tissues in mice. Prostate, lung, and gut specimens were collected from castrated male mice 1 month following surgical castration and stained for CD3. Data shows mean counts from 10 random fields of view from each sample ± SD. Each sample has 3-4 mice per group

Figure 3. Expression of Ptpn1 is regulated by androgens. (A). Gene expression of various cell signaling components in Th1 CD4 T cells. CD4 cells were cultured in the presence or absence of androgen under Th1 conditions for 3 days. Gene expression was measured by qPCR. (B) Effect of testosterone on Ptpn1 and Ptpn11 in vivo. Expression of Ptpn1 and Ptpn11 in CD4 T cells isolated from spleens of control or surgically castrated male mice 4 weeks post-surgery (n= 6 and 7 respectively from 2 separate experiments). (C) Direct effect of testosterone on Ptpn1. Expression of Ptpn1 in murine CD4 T cells treated with R1881 overnight (n=3). (D) Effect of testosterone on Ptpn1 expression in other cell types. Expression of Ptpn1 from various datasets available on the Gene Expression Omnibus (GEO) where cells had been treated with androgen. Numbers in parentheses indicate dataset GEO identification numbers. (E) Effect of Ptpn1 on Tyk2 and STAT4 phosphorylation after IL-12 exposure. CD4 T cells were treated with 5µg/ml IL-12 in the presence or absence of R1881, with and without treatment with a Ptpn1-specific inhibitor. CD4 cells were analyzed for levels of phosph-Tyk2 5 minutes after treatment. Blots are representative of 2 experiments.

Figure 4. Androgen receptor binds to a region in intron 3 of the Ptpn1 gene. (A) Chromatin Immunoprecipitation and qPCR quantification of AR binding to Ptpn1. Male mouse spleen CD4 T cells treated overnight with R1881 or vehicle control. Chromatin immunoprecipitation for Androgen Receptor was performed and fold enrichment was determined by qRT-PCR of putative AR binding regions within the Ptpn1 gene. (B) Homology between species of Intron 3 of Ptpn1. Homology of Ptpn1 between mouse and human or rat. Exon is shown in red and regions of high homology conserved between species are shown in blue. (C) DNA sequence of AR binding region in different species.

Figure 5. Ptpn1 expression is reduced in CD4 cells isolated from prostate cancer patients undergoing androgen deprivation. CD4 cells were isolated from patients undergoing ADT by

FACS. Ptpn1 expression in CD4 T cells isolated from PBMC of prostate cancer patients on androgen deprivation therapy and control patients; n=13 and 7.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% of pathway changed
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50
100
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T Helper Cell Differentiation
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Death Receptor Signaling
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Reelin Signaling in Neurons
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Type I Diabetes Mellitus Signaling
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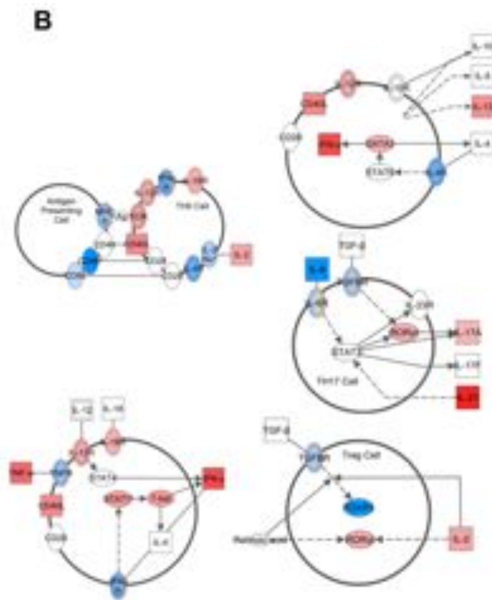
Upregulated
Downregulated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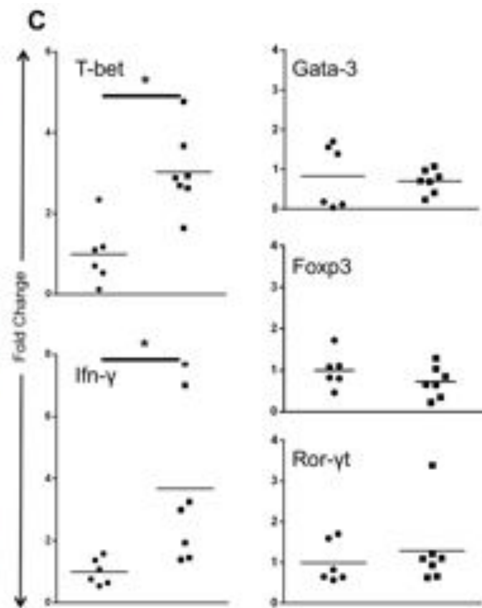
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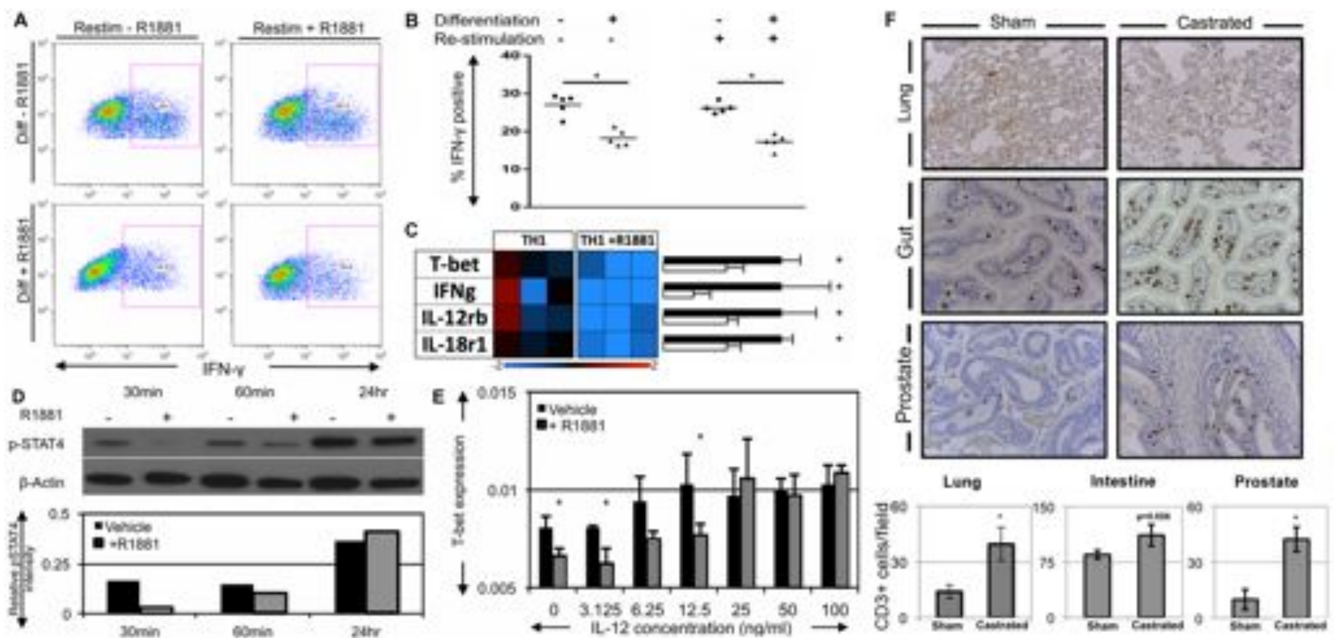
Gata-3
Foxp3
Ifn-γ
Ror-γt
Fold Change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Restim + R1881
Diff - R1881
Diff + R1881
IFN-γ
B
Differentiation
Re-stimulation
% IFN-γ positive
C
TH1
TH1 +R1881
T-bet
IFNg
IL-12rb
IL-18r1
D
30min
60min
24hr
R1881
p-STAT4
β-Actin
Vehicle
+R1881
Relative pSTAT4 intensity
E
Vehicle
+ R1881
T-bet expression
IL-12 concentration (ng/ml)
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Castrated
Lung
Gut
Prostate
Lung
Intestine
Prostate
CD3+ cells/field
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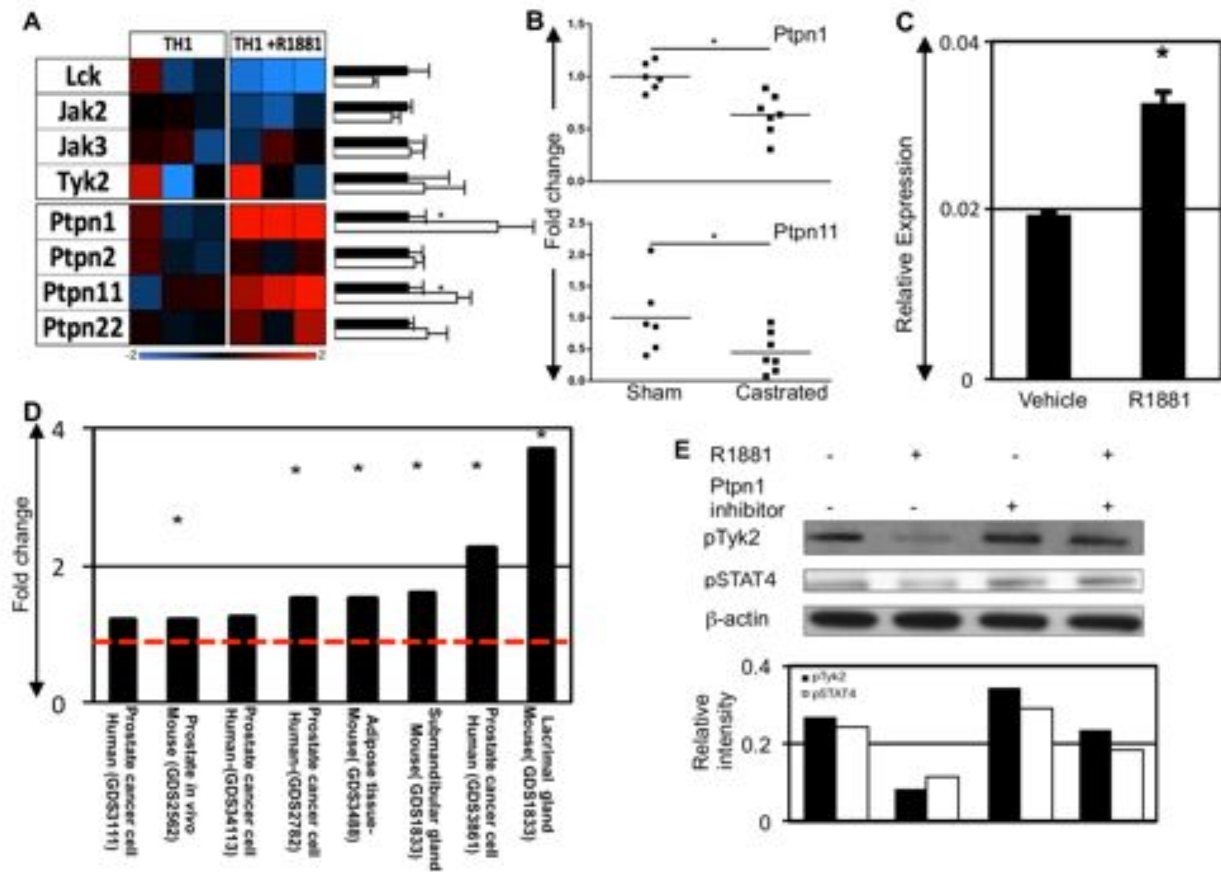
Castrated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TH1
TH1 +R1881
Lck
Jak2
Jak3
Tyk2
Ptpn1
Ptpn2
Ptpn11
Ptpn22
B
Ptpn1
Ptpn11
Fold change
Sham
Castrated
C
Relative Expression
0.04
0.02
0
Vehicle
R1881
D
Fold change
4
2
0
Prostate cancer cell Human (GDS3111)
Prostate in vivo Mouse (GDS2562)
Prostate cancer cell Human-(GDS3413)
Prostate cancer cell Human-(GDS2782)
Adipose tissue-Mouse(GDS3488)
Submandibular gland Mouse(GDS1833)
Prostate cancer cell Human (GDS3861)
Lacrimal gland Mouse(GDS1833)
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pSTAT4
β-actin
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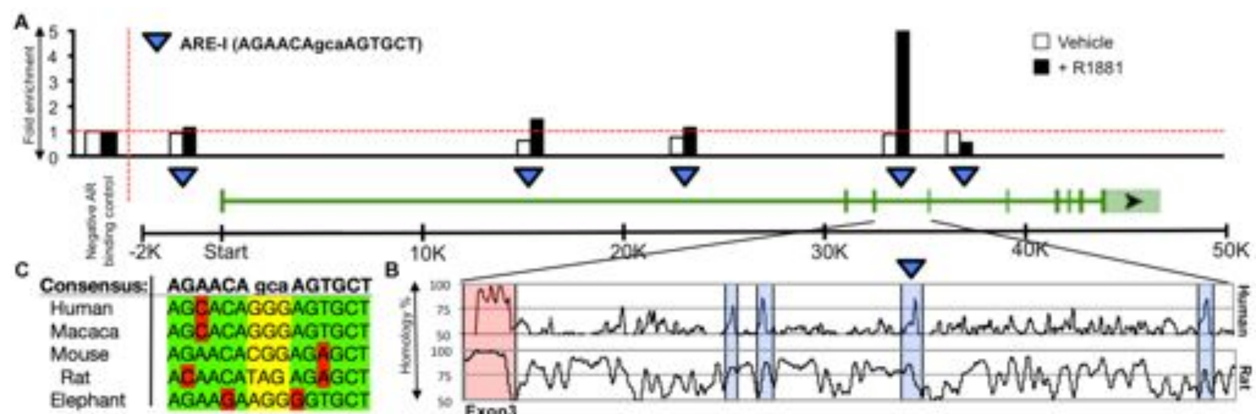
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ARE-I (AGAACAgcaAGTGCT)
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+ R1881
Fold enrichment
Negative AR binding control
-2K
Start
10K
20K
30K
40K
50K
B
Homology %
Human
Rat
Exon3
C
Consensus:
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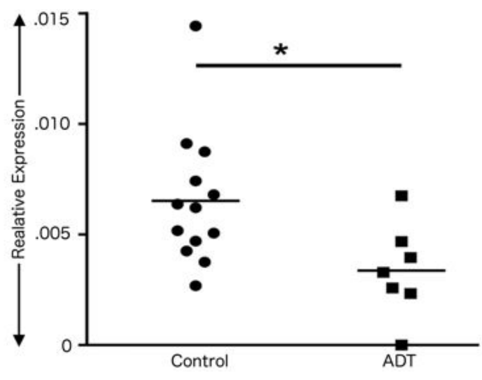

Realative Expression
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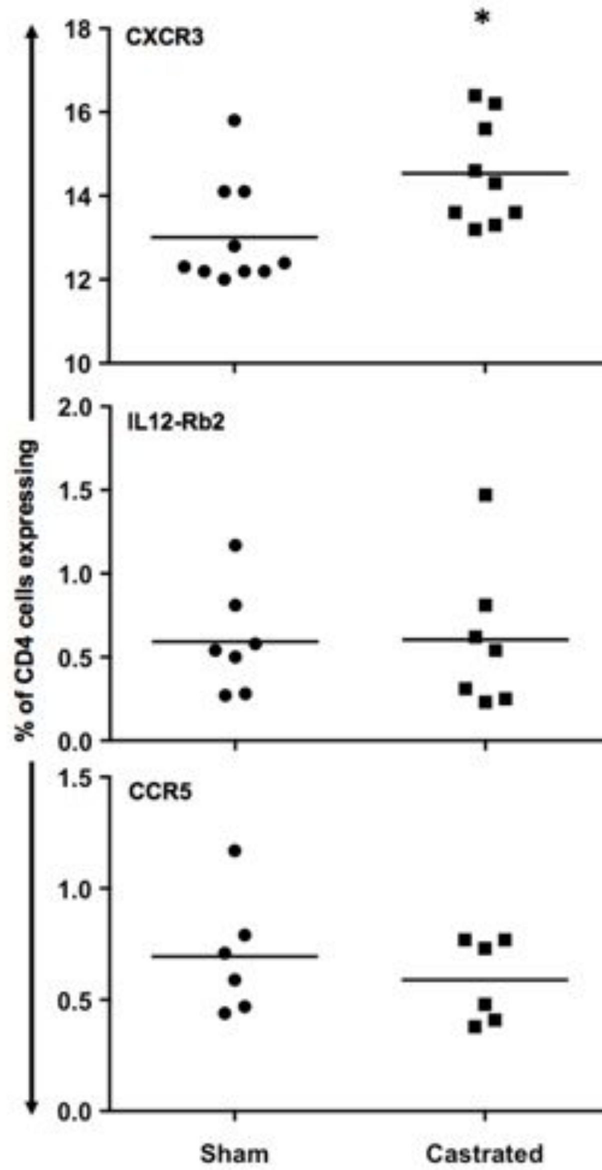
.010
.005
0
*
Control
ADT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16
14
12
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IL12-Rb2
2.0
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CCR5
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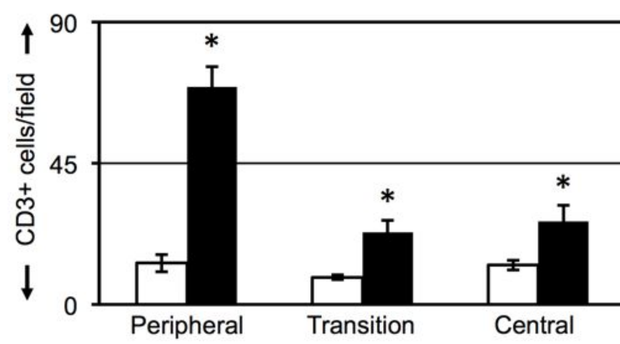
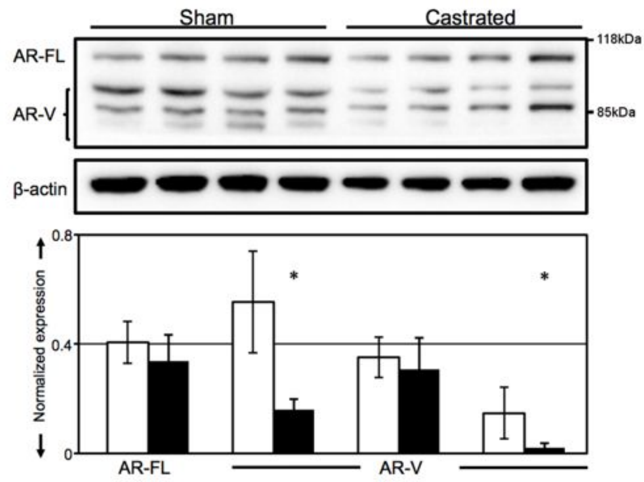
1.0
0.5
0.0
% of CD4 cells expressing
Sham
Castrated



90
45
0
CD3+ cells/field
*
*
*
Peripheral
Transition
Central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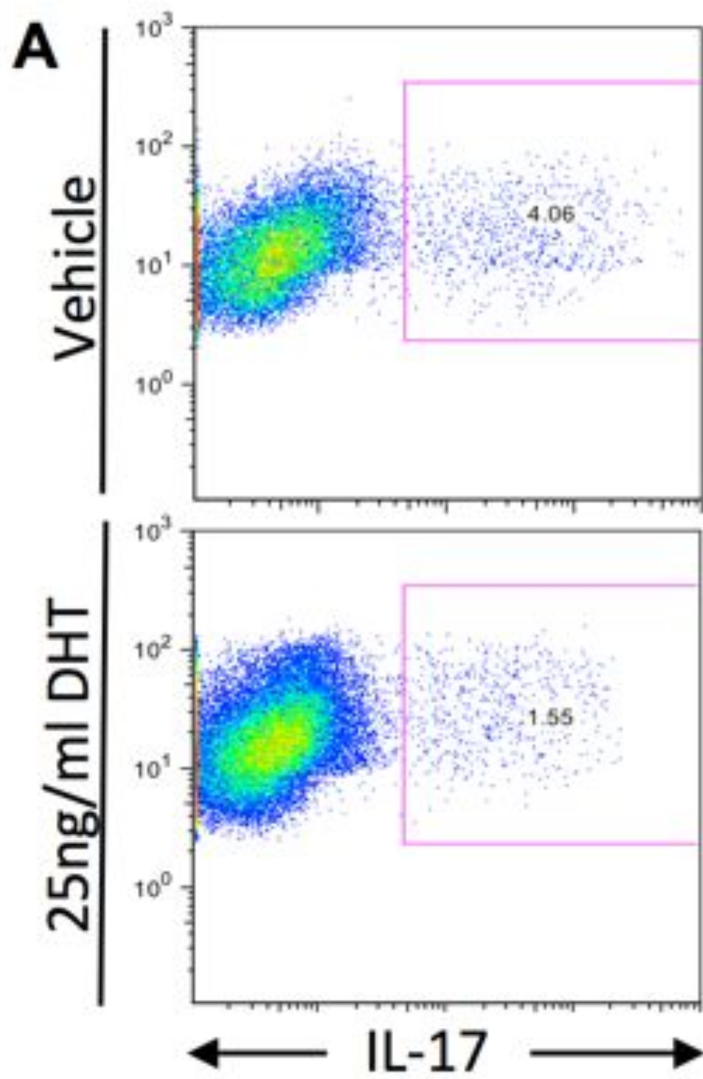
*
AR-FL
AR-V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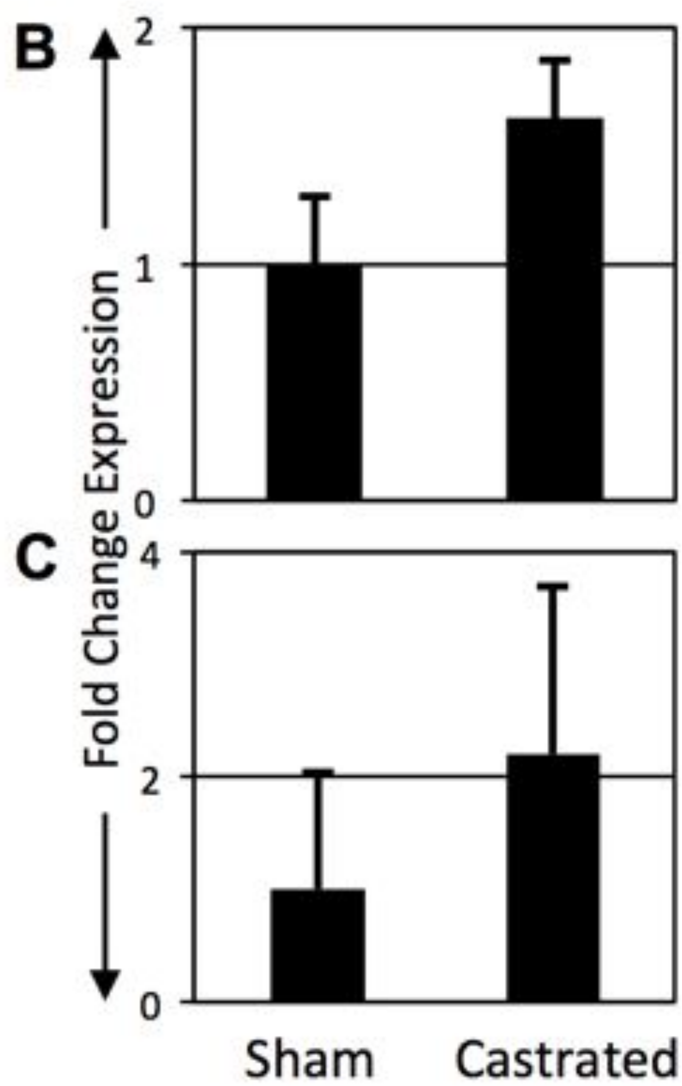
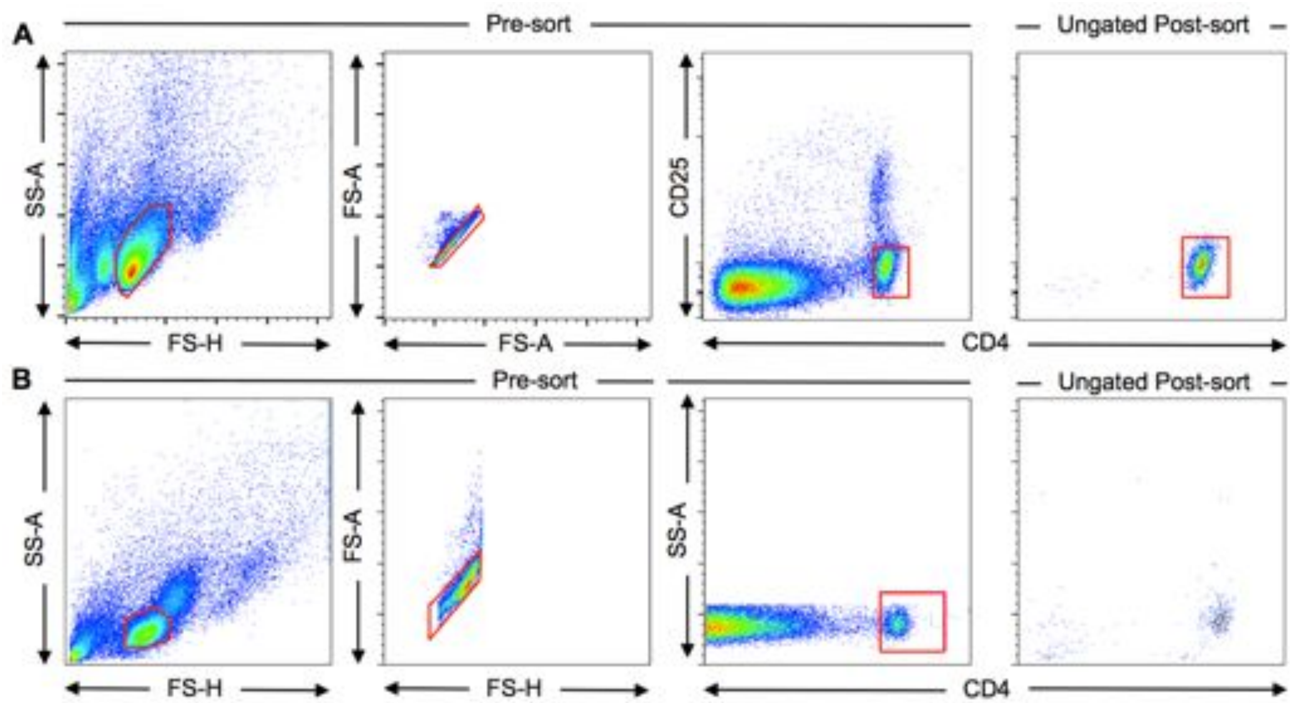
1.55
IL-17


B
C
Fold Change Expression
Sham
Castrated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Pre-sort
Ungated Post-sort
SS-A
FS-H
FS-A
FS-A
CD25
CD4
B
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Ungated Post-sort
SS-A
FS-H
FS-A
FS-H
SS-A
CD4