


Vaccines targeting *ESR1* activating mutations elicit anti-tumor immune responses and suppress estrogen signaling in therapy resistant ER+ breast cancer

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ABSTRACT

ER+ breast cancers (BC) are characterized by the elevated expression and signaling of estrogen receptor alpha (*ESR1*), which renders them sensitive to anti-endocrine therapy. While these therapies are clinically effective, prolonged treatment inevitably results in therapeutic resistance, which can occur through the emergence of gain-of-function mutations in *ESR1*. The central importance of *ESR1* and development of mutated forms of *ESR1* suggest that vaccines targeting these proteins could potentially be effective in preventing or treating endocrine resistance. To explore the potential of this approach, we developed several recombinant vaccines encoding different mutant forms of *ESR1* (*ESR1mut*) and validated their ability to elicit *ESR1*-specific T cell responses. We then developed novel *ESR1mut*-expressing murine mammary cancer models to test the anti-tumor potential of *ESR1mut* vaccines. We found that these vaccines could suppress tumor growth, *ESR1mut* expression and estrogen signaling in vivo. To illustrate the applicability of these findings, we utilize HPLC to demonstrate the presentation of *ESR1* and *ESR1mut* peptides on human ER+ BC cell MHC complexes. We then show the presence of human T cells reactive to *ESR1mut* epitopes in an ER+ BC patient. These findings support the development of *ESR1mut* vaccines, which we are testing in a Phase I clinical trial.

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Introduction


Approximately 75% of breast cancers (BC) are estrogen receptor α (ER+) and treated with anti-estrogen (endocrine) therapies, such as tamoxifen and aromatase inhibitors. While these therapies are often highly effective, ~25% of patients with localized cancers (Stage I–III) and nearly all with metastatic cancers (Stage IV) develop resistance to these therapies.^{1–7} A common resistance mechanism in these heavily treated patients occurs through gain-of-function mutations in *ESR1*, found in 20–40% of patients with metastatic ER+ BC who received endocrine therapies, but rarely in primary tumors.^{5,8,9} These gain-of-function mutations are clustered within the ligand-binding domain (LBD) of *ESR1* at one of three neighboring amino acids, all of which lead to ligand-independent ER activity and neomorphic activation of various pathways as a major mechanism of acquired endocrine resistance.^{1,8,10–12}

ESR1 is highly expressed in ER+ breast cancer and its mutations cause amino acid alterations, which generate potential neoantigens that can be recognized and targeted immunologically. Neoantigens are formed by mutations to self-proteins that render them immunologically non-self, which many studies have suggested to be a central factor in generating anti-tumor immunity.^{13,14} While most neoantigens are formed by

randomly occurring mutations,¹⁴ emergence of *ESR1* activating mutations in ER+ BC after prolonged endocrine therapy occurs in a predictable manner as a mechanism of therapeutic resistance. In addition to their ligand-independent estrogen signaling, studies have also documented that mutated *ESR1* confers neomorphic oncogenic properties, making it a driver of tumor progression and escape.^{10–12} We have previously demonstrated the critical importance of targeting oncogenic pathways, in comparison to non-essential tumor associated antigens, for the efficacy of anti-tumor vaccines.^{15–17} Thus, given the critical importance of *ESR1* mutations in sustaining ER+ BC oncogenic signaling, their predictable emergence as neoantigens, and the elevated expression of *ESR1* in ER+ BC, we hypothesized that a vaccination strategy targeting mutant forms of *ESR1* (*ESR1mut*) could be an effective therapeutic approach to immunologically prevent or treat endocrine resistant cancers.

In our study, we developed vaccines to several mutant forms of human *ESR1* and determined that these vaccines could all elicit robust *ESR1*-specific T cell responses. We then developed immune competent models of human *ESR1mut* expressing murine mammary cancer and demonstrated that different human *ESR1mut* vaccines could elicit cross-protective anti-tumor responses that suppressed *ESR1mut*

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expression and estrogen signaling. We then validated the presentation of *ESR1* peptides on human ER+ BC MHC complexes. These results led to the initiation of a clinical trial targeting *ESR1*mut using peptide vaccines. Analysis of one of the trial participants documents the existence of *ESR1*mut-specific responses even prior to vaccination. This result suggests the potential to augment these responses with *ESR1*mut vaccination to prevent the clinical emergence of *ESR1*mut+ BC and prevent the development of *ESR1*mut-mediated endocrine resistance.

Results

Development of an adenoviral vaccine targeting *ESR1* mutants in vivo

To test the potential of vaccination against mutant forms of *ESR1*, we developed a series of 1st generation [E1,E3-] adenoviral vectors expressing wild-type *ESR1* or one of the three dominant *ESR1* mutant genes (comprising >75% of *ESR1* mutations) to immunologically target wild-type or endocrine resistance-associated mutant ER^{18,19} (Figure 1a). While *ESR1* is highly homologous between mice and human (~90%),²⁰ we found that immunizing mice with any of these vaccines

sufficient to break immune tolerance and induce human *ESR1*-specific T cell immunity that was comparable between *ESR1*-WT and various *ESR1*mut vaccines in C57BL/6 mice, without signs of obvious autoimmunity (Figure 1b). Given the parity of responses, we next wanted to determine if *ESR1*-specific responses in mice were concentrated against particular regions of *ESR1*, so we constructed vaccines encoding different subunits of *ESR1* to ascertain if certain regions of *ESR1* harbored more immunodominant epitopes.^{21,22} We constructed three roughly equal (~200 amino acid) *ESR1* subunits, comprised of an N-terminal, a middle, and C-terminal domain (containing the neopitope site), which were all incapable of stimulating estrogen signaling (Figure S1). To determine if these vaccines could induce presentation on human HLA, we vaccinated human HLA-A2+ transgenic mice. These studies revealed that N-terminal and C-terminal vaccines yielded potent *ESR1*-specific T cell responses, but that the Y537N mutant form of the C-terminal vaccine had the greatest response (Figure 1c). We then expanded our vaccination of HLA-A2+ transgenic mice to include additional *ESR1* C-terminal mutant vaccines (*ESR1*-Y537S and D538G). In these studies, we again observed that C-terminal vaccines elicited stronger T cell responses compared to full-length versions, but no significant differences were observed between different *ESR1*-WT and *ESR1*mut

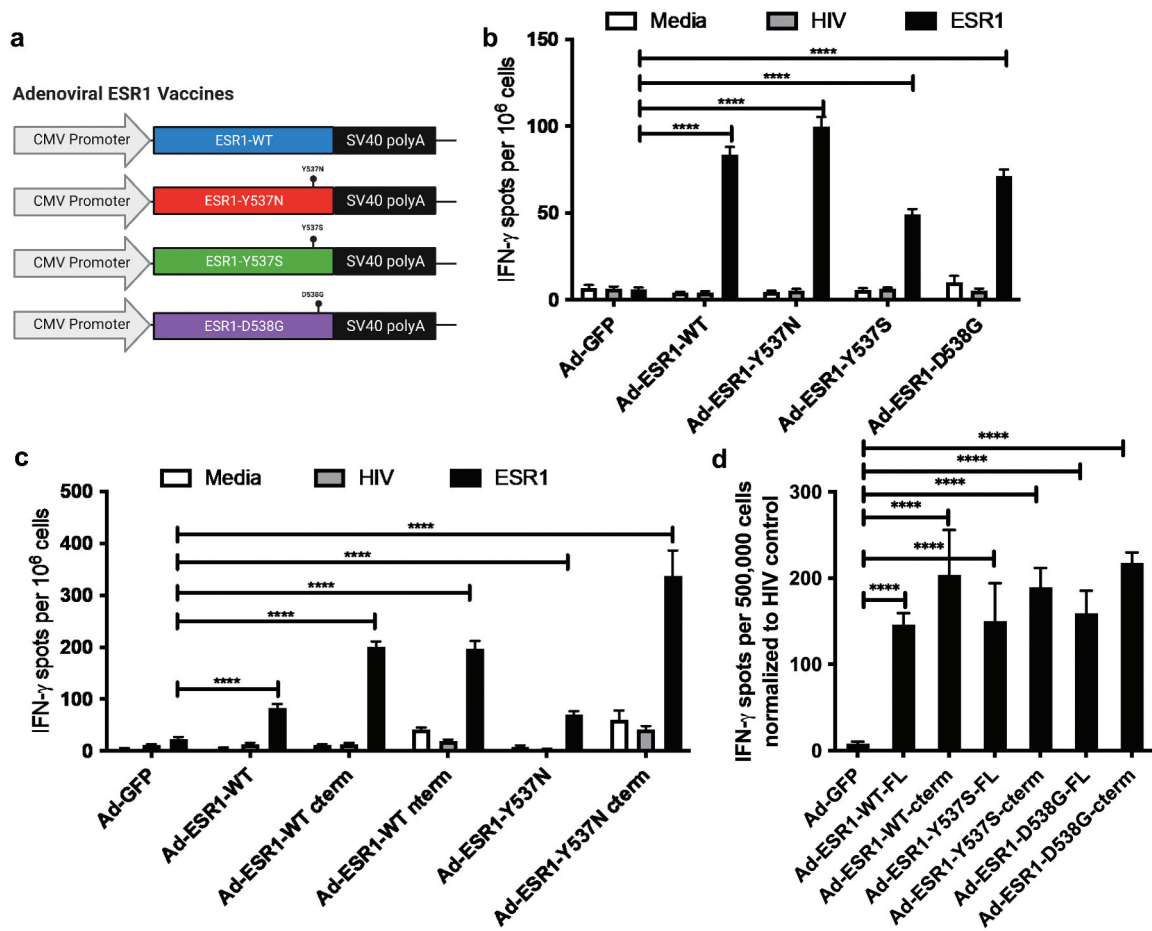


Figure 1. Development and testing of human *ESR1* targeting vaccines. (a) Diagram of E1 region for [E1,E3-] adenoviral *ESR1* vaccines. (b,c) C57BL/6J mice (b) and HLA-A2-Tg (c) were vaccinated with indicated viruses and assessed for *ESR1*-specific T cell responses at 2 weeks post vaccination through IFN γ ELISPOT ($n = 5$ mice/group). (d) HLA-A2 Tg mice were vaccinated with the indicated viruses and assessed for *ESR1*-specific T cell responses at 2 weeks post vaccination through IFN γ ELISPOT ($n = 3-4$ mice/group). Error bars represent SEM. Significance determined via T test with Bonferroni correction for multiple comparisons. **** $p < .0001$.

vaccines (Figure 1d). Given the large amount of overlap between the sequences encoded by *ESR1*-WT and *ESR1*mut vaccines, splenocytes from mice vaccinated with the c-terminal *ESR1* vectors were assessed by IFN-gamma ELISPOT after stimulation with peptides from the region that forms the neoepitope. This included the vaccine matched neoepitope peptides (peptides specific for the mutation, for instance a peptide containing Y537N for an Ad-*ESR1*-Y537N vaccine) and stimulation using peptides from non-matched mutants (Figure S2A). These assays revealed weak induction of neoepitope specific peptide responses in individual *ESR1*mut vaccinated mice in comparison to control Ad-GFP or Ad-*ESR1*-WT-cterm mice.

As peptide specific responses are highly sensitive to MHC haplotypes for presentation and binding, we also utilized Ad-*ESR1*Y537N to vaccinate a cohort of diversity outbred mice (Figure S2B). While we found strong responses against adenoviral epitopes, we found far more variable responses against *ESR1*-specific and *ESR1*mut neoepitope peptides, with some mice having almost no response and other having a robust response. However, we did observe a positive correlation between *ESR1*-specific and *ESR1* neoepitope responses and found evidence for cross-reactivity between *ESR1*-Y537N and Y537S neoepitopes. These results suggest that in mice, systemic *ESR1* neoepitope-specific T cell responses can occur but are a minor population in the spleen, with the response dominated by the large number of shared epitopes between *ESR1*-WT and *ESR1*mut.

Oncogenicity of *ESR1* mutants and development of immune competent *ESR1*mut cell line model

As no immune competent mouse cell line models of ERmut+ BC exist, we sought to develop one to test the impact of *ESR1*-WT and *ESR1*mut vaccination on tumor growth. As an initial step, we validated the ability of our *ESR1*mut genes to confer ligand-independent estrogen signaling (Fig S3A) and transduced an ER+ BC line (MCF-7) with *ESR1*-WT or *ESR1*-Y537N to assess their oncogenic functional capacity. In the absence of estrogen, RNAseq analysis of these tumors revealed that *ESR1*-Y537N cells have activation of hormonal signaling pathways, dysregulation of apoptotic and cytokine pathways, with upregulation of proliferative pathways (Figure S3B). More critically, we found that

in mice without exogenous estrogen supplementation in vivo, MCF7/*ESR1*-Y537N permitted robust estrogen-independent growth, in comparison to MCF7/*ESR1*-WT cells (Figure S3C). Subsequent studies confirmed this for an *ESR1*-Y537S mutant (Figure S3D) and validated the oncogenic nature of different *ESR1*mut expression in ER+ BC, supporting that activating *ESR1* mutant expression may enhance the growth of murine mammary cells.

To determine if these mutants also confer estrogen-independent activation to facilitate the growth of mouse mammary cells, we first expressed human *ESR1* and various human *ESR1* mutants in a mouse mammary line (MM3MG) and confirmed the robust stimulation of estrogen independent signaling pathways by *ESR1*mut expression with comparable levels of *ESR1* and *ESR1*mut expression (Figure 2a,b). Next, we tested the in vivo growth of stably transduced MM3MG-*ESR1*-Y537N cells in comparison to previously generated stably transduced MM3MG-HER Δ 16 (strong HER2 oncogene), which we found capable to transforming MM3MG cells.^{16,23} These studies revealed that an *ESR1* mutant expression conferred a comparable growth advantage in the mammary fat pad (Figure 2c). We then compared MM3MG-*ESR1*-Y537N cells with MM3MG cells expressing either *ESR1*-WT or *ESR1*-Y537S and found that both *ESR1* mutants elicited more robust growth in non-estrogen supplemented mice in comparison to *ESR1*-WT mice (Figure 2d). Collectively, these results suggested that *ESR1*mut expressing MM3MG cells represented a potential model of *ESR1*mut expressing cancers that could be interrogated by vaccination.

Vaccination to prevent and treat *ESR1*mut+ BC

Given the ability of different Ad-*ESR1*mut vaccines to elicit *ESR1*-specific responses, we next wanted to determine if these vaccines could prime the immune system to prevent the outgrowth of tumors expressing an *ESR1* mutant gene using this new model. To test this concept, we vaccinated mice with Ad-*ESR1*-Y537N and 2 weeks later implanted murine mammary tumor cells expressing *ESR1*-Y537N (Figure 3a). In this setting, preventative vaccination had a significant anti-tumor effect and elicited *ESR1*-specific T cell responses, suggesting the potential of vaccination to prevent the outgrowth of *ESR1*mut cancers (Figure 3b). Notably, when mice were vaccinated and implanted with *ESR1*-WT expressing cells, we observed no impact from vaccination, suggesting strong on-

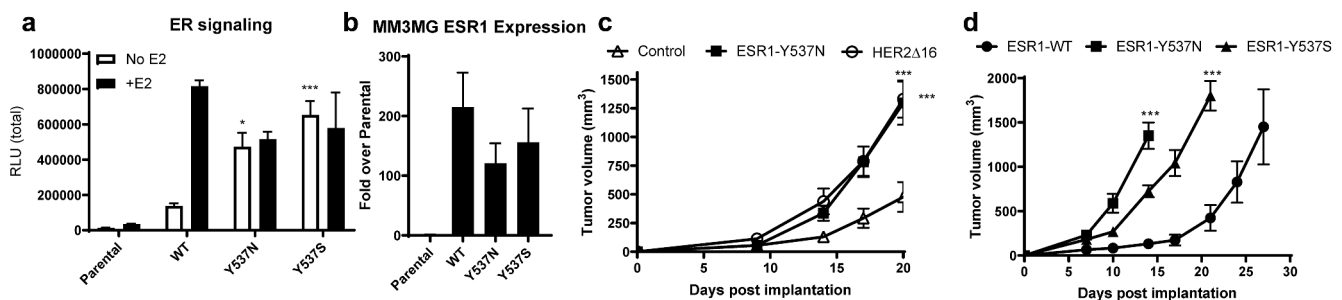


Figure 2. Development of ERmut+ mouse mammary tumor model. (a) MM3MG cell lines expressing Y537N or Y537S doxycycline inducible *ESR1* mutants were assessed at 24 hours post-dox/estrogen stimulation for the indicated pathway ($n = 3$ /group). (b) QRT-PCR assessment of *ESR1* expression in MM3MG lines. (c) Indicated MM3MG cells were implanted into BALB/c mice and allowed to grow, measured bi-weekly ($n = 5$ mice/group). (d) Indicated MM3MG cells were implanted into BALB/c mice and allowed to grow, measured bi-weekly ($n = 5$ mice/group).

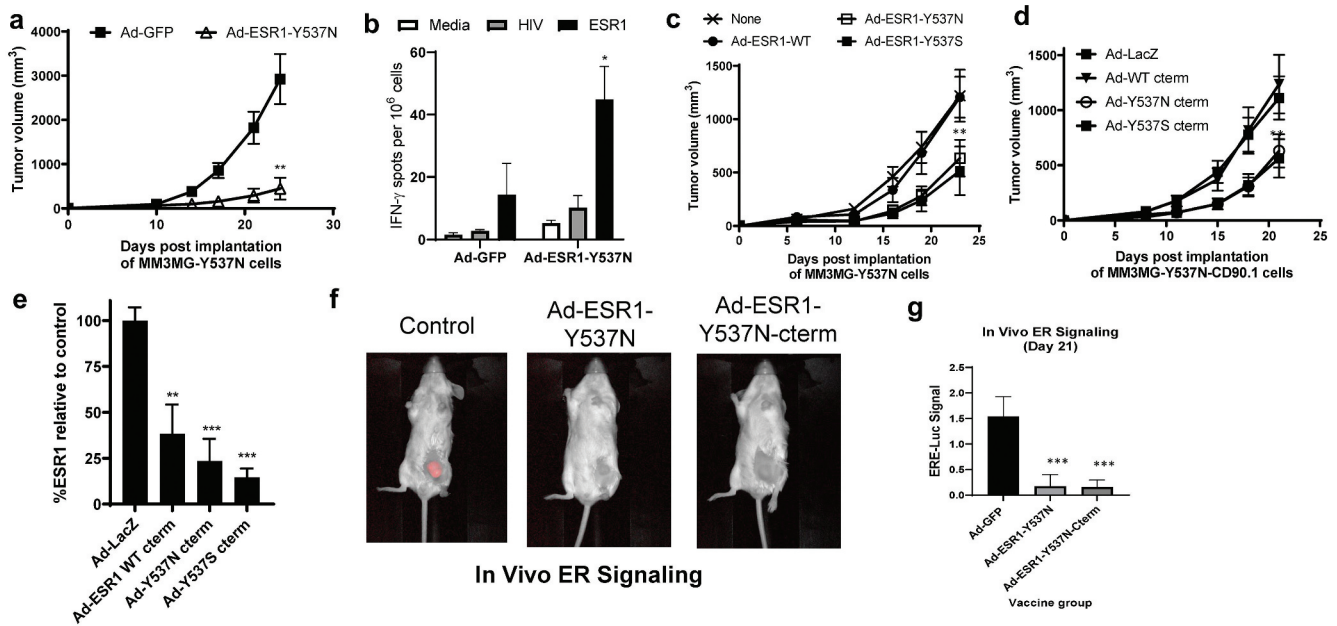


Figure 3. Anti-tumor impact of ESR1mut vaccines in an ERmut+ mouse mammary tumor model. (a) BALB/c mice were vaccinated with indicated virus and MM3MG-ESR1-Y537N cells were implanted two weeks post-vaccination and tumor growth measured biweekly. (b) spleens from mice in (a) were assessed by IFN γ ELISPOT assay ($n = 5$ mice/group). (c,d) BALB/c mice were implanted with MM3MG-ESR1-Y537N (c) or MM3MG-ESR1-Y537N-CD90.1 (d) expressing cells and vaccinated the following day with tumor growth measured bi-weekly ($n = 5$ mice/group). (e) Tumors from (d) were removed and CD90.1 expression was measured ex vivo as a surrogate of ESR1 expression. Shown as % of expression seen in control treated cells. Error bars represent SEM. * $p < .05$; ** $p < .05$; *** $p < .001$. (f,g) Representative images and analysis (g) of MM3MG-ESR1-Y537N-CD90.1/ERE-Luc tumors vaccinated with ad-GFP control or ad-ESR1-Y537N as in (c,d).

target anti-*ESR1*mut effects from vaccination (Fig S4A). Given that many patients have existing ER+ BC containing *ESR1* mutations and that multiple types of mutations can occur, we next tested the impact of different *ESR1*mut and *ESR1*-WT therapeutic vaccinations in tumor-bearing mice. In this study we implanted *ESR1*-Y537N tumors and subsequently vaccinated tumor bearing mice with Ad-*ESR1*-wild-type, Y537N, and Y537S vaccines (Figure 3c). We found that both Ad-*ESR1*-Y537N and Ad-*ESR1*-Y537S were able to slow the growth of *ESR1*-Y537N expressing tumors, whereas we surprisingly observed that Ad-*ESR1*-WT had no effect. These data could suggest an anti-tumor role for *ESR1*mut reactive T cells in the tumor microenvironment, possible neoantigen immunodominance in the context of a developing tumor, antigen spreading following vaccination for *ESR1*mut, or cross-protection between different *ESR1* mutants. To validate that this effect was specific for C-terminal subunit vaccines, we repeated this experiment using C-terminal truncated vaccines (for *ESR1*-WT, Y537N and Y537S), as well as with the inclusion of an Ad-LacZ control (Figure 3d). Here we used an MM3MG line expressing *ESR1*-Y537N linked to CD90.1 by a T2A self-cleaving peptide tag. This allows for assessment of the levels of *ESR1* expression by staining for surface expression of CD90.1. This experiment again demonstrated that enhanced anti-tumor responses were afforded by both mutant vaccines, in contrast to control or *ESR1*-WT c-terminal vaccination. However, analysis of tumor outgrowths demonstrated that all *ESR1* vaccines significantly suppressed *ESR1*-Y537N expression. Loss of the target antigen by tumors from vaccinated mice confirms the potency of all *ESR1* vaccines to elicit a suppression of *ESR1* expression, but with stronger anti-tumor responses by *ESR1*mut vaccines (Figure 3e). To

determine if selection against *ESR1*mut expression suppressed ER signaling, we transduced MM3MG-*ESR1*-Y537N cells with a stable Estrogen signaling pathway reporter lentivirus (ERE-Luc) and repeated a vaccination with Ad-*ESR1*-Y537N, Ad-*ESR1*-Y537-cterm, or an Ad-GFP control. This experiment revealed a significant suppression of ER signaling in tumors in vivo from both *ESR1*mut vaccines (Figure 3f,g), which was again associated with reduced growth of Ad-*ESR1*-Y537N vaccinated mice in comparison to control (Figure S4B). Collectively, these experiments demonstrate that *ESR1*mut vaccines are capable of eliciting anti-tumor immune responses that suppress *ESR1*mut expression and signaling, thus negating the impact of *ESR1*mut expression and depriving cancer cells of this endocrine resistance mechanism.

Presentation and immunogenicity of ESR1 epitopes against human T cells

Having demonstrated some efficacy in *ESR1*mut vaccination in mouse models, we wanted to further explore its potential as a clinical immunotherapeutic target in humans. To first validate that peptides encoded by *ESR1* were presented by major histocompatibility complexes (MHC), we utilized proteomic analysis of *ESR1*mut+ MCF7 cells through HLA class I immune-precipitation and peptide HPLC. These analyses identified multiple *ESR1* peptides as being potentially presented by HLA-A2 complexes, including an Y537N mutant neoepitope (Table S1), thus supporting the potential *ESR1*-specific T cell recognition of ER+ BC.

Given our ability to elicit *ESR1* and *ESR1*mut-specific T cell responses, we subsequently initiated a Phase I clinical trial to determine if we could elicit or enhance

T cell responses to *ESR1* and *ESR1mut* through peptide vaccination, using a mix of four 10-mer *ESR1mut* and one 10-mer *ESR1*-WT peptide in Montanide with GM-CSF (NCT04270149). While ongoing, our preliminary studies document more *ESR1*-reactive T cells in PBMCs from ER+ BC patients (prior to vaccination, Wk 0), in comparison to levels observed from normal donors following *ESR1*-peptide stimulation (Figure 4a–c). Expansion and re-stimulation with a pool of the five vaccine containing peptides showed similar levels of IFN γ +TNF α + double producing CD8 T cells pre- and post-vaccine (Figure 4b). Notably, we observe greater responses against an overlapping peptide pool spanning the entire *ESR1* protein in ER+ patient PBMCs after the completion of vaccination, suggesting some ability to enhance *ESR1*-specific immunity beyond just the vaccination epitopes (Figure 4c). While incomplete, these responses suggest the presence of *ESR1*-reactive T cells in ER+ BC patients, which may be augmented through vaccination. Our ongoing trial and future trials will

determine if the vaccine strategies utilized will be sufficient to expand these populations to elicit a therapeutic effect and potentially prevent or delay the development of endocrine resistance.

Discussion

The predictable resistance to endocrine therapy in metastatic ER+ breast cancer creates an opportunity to target the outgrowth of predictably arising ER-LBD-mutant cells with a preventative vaccine to block progression and treatment failure for 20–40% of patients. The ability of vaccines to prevent diseases is well-established, and is based on their ability to generate potent immunity against pathogen xenoantigens capable of eradicating cells expressing the antigen target, through the induction of T cell-mediated immunity to limit expression of these genes and minimize downstream signaling, or reduce the number of cells highly expressing these genes. Based on this premise, we demonstrated the ability of multiple *ESR1mut*

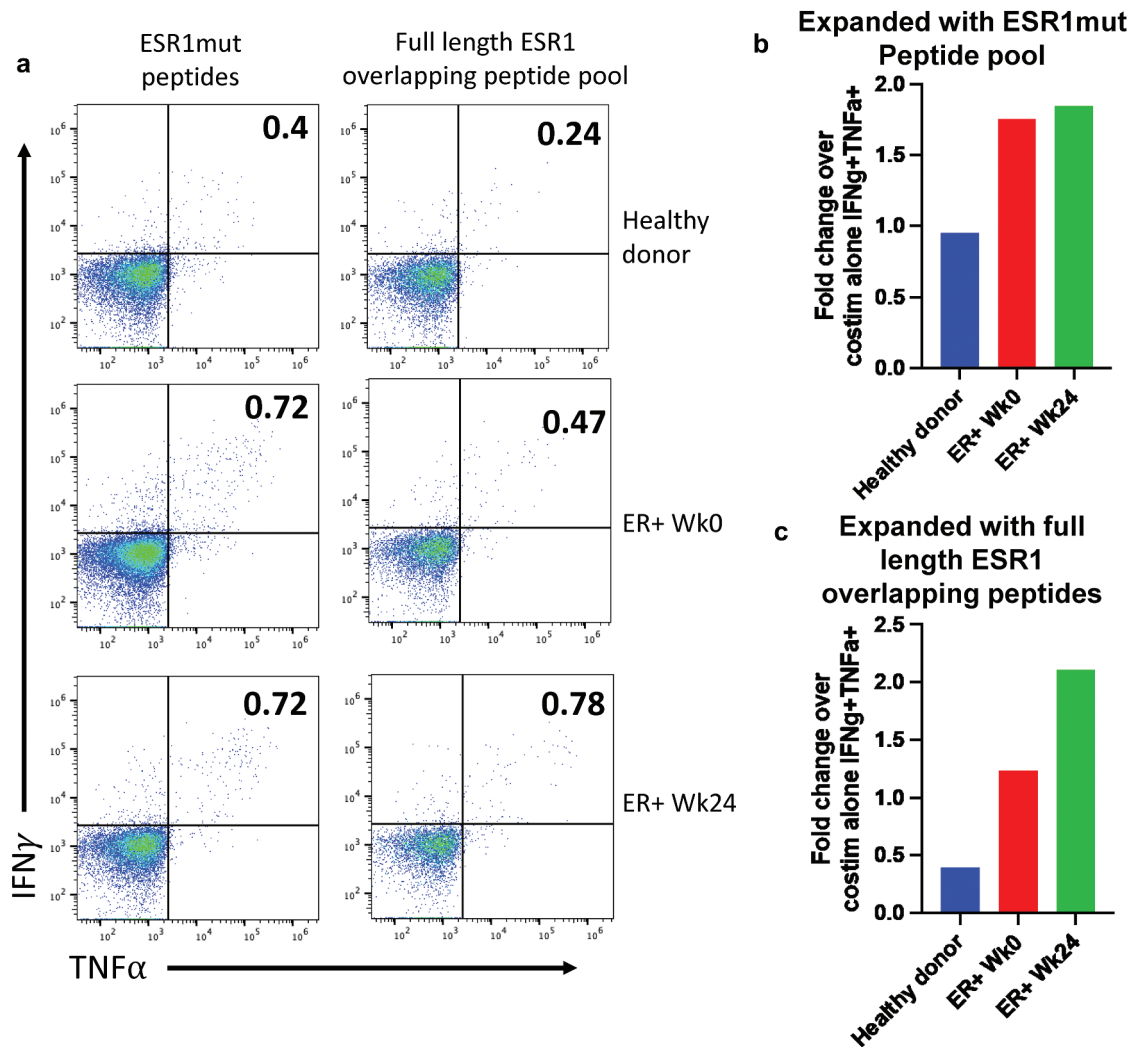


Figure 4. Expansion of *ESR1*-reactive human T cells. (a) PBMCs from a healthy donor and a patient enrolled in NCT04270149 at week 0 and week 24 after completing 6 vaccinations. Cells were expanded in vitro for 9 days with the vaccine containing *ESR1mut* peptides (left) or an overlapping peptide pool that encompassed the entire *ESR1* protein (right). Cells were then restimulated, stained, and analyzed by spectral flow cytometry for IFN γ and TNF α . Cells shown are pregated on live, singlets, CD3+, CD4-, CD8+, CD45RA-, CD45RO+. (b,c) the fold change in expression percent of *ESR1mut* peptides (b) or full length *ESR1* (c) compared to a negative control that received costimulation but no peptide is shown for each sample.

vaccines to elicit human *ESR1*-specific T cell responses and stimulate anti-tumor responses against a novel immune competent model of *ESR1*mut+ mammary cancer.

In our development of this novel *ESR1*mut+ model, we independently validated work from multiple groups that *ESR1* LBD mutants induce estrogen-independent ER signaling and neomorphic signaling changes^{2,8,10-12} by demonstrating the capacity of *ESR1* mutants to elicit enhanced estrogen oncogenic signaling in human and mouse cells, which promotes growth in vivo and suppression of interferon genes (such as *IFI27* and *SOCS2*, Figure S3, Figure 2). These findings are consistent with other groups that have documented the capacity of estrogen signaling to suppress immune and inflammatory responses,²⁴⁻²⁶ as well as the suppression of Stat1 downstream of *ESR1* targets, similar to the progesterone receptor.²⁷

Using this *ESR1*mut+ model, we found that *ESR1*-WT and *ESR1*mut vaccination can elicit anti-tumor responses, critically reducing *ESR1*mut expression and suppressing estrogen signaling in tumor outgrowths (Figures 1 and 3). As mutated *ESR1* confers intrinsic endocrine resistance and to neighboring cells through paracrine mechanisms,¹⁰ the reduction of *ESR1*mut expression through vaccination may be a critical means to eliminate the development of this type of resistance. In our studies, we found that prophylactic vaccination against *ESR1*mut could be highly effective in slowing the development of *ESR1*mut+ cancers (Figure 3a), as well as reducing ER expression and signaling in *ESR1*mut+ cancers (Figure 3e-g), thus suggesting the potential of vaccinating patients prior to the development of *ESR1* mutations. It is important to note that while estrogen signaling was seen in our *ESR1*mut expressing cell lines, these tumors do not appear addicted to ER expression in the same manner as human ER+ tumors (Fig S3). This could partially explain the moderate anti-tumor responses observed despite the induction of a robust anti-*ESR1* immune response. While the loss of antigen represented a viable escape mechanism for our tumor model, it may be less likely to occur in patients with ER+ breast cancer suggesting that these vaccines could be more impactful clinically. However, our data also suggest that local immune suppressive mechanisms are likely to play a key role in mitigating anti-tumor responses in established cancers, which has been repeatedly observed in preclinical and clinical studies.¹⁶⁻²⁸⁻³¹ Strategies to overcome these limitations will be critical in extending the therapeutic efficacy of these types of cancer vaccines. As we observed anti-tumor cross-protection between vaccines targeting different *ESR1* mutations (Figures 1 and 3c,d), our study also suggests the potential of *ESR1*mut vaccines to induce T cells with TCR capable of cross-reacting to multiple different mutations. This is likely to be critically important in a prophylactic setting where several different potential mutations might arise in ER+ BC cells.

The *ESR1* vaccines described here encoded the human *ESR1* gene and immunogenicity was measured in mice, making human *ESR1* a potential foreign antigen. While this does represent a lower threshold for breaking immune tolerance with

a vaccine than would typically be seen in patients, the human and mouse *ESR1* genes share ~90% sequence homology.³² This, together with the evidence that tumor cells expressing human *ESR1* were able to reliably grow without rejection in mice, suggests a level of tolerance to human *ESR1*. Further testing in a transgenic animals expressing human *ESR1* is planned to better assess our vaccines ability to break immune tolerance, as we have performed with other human oncogenes.^{16,17}

In our examination of human ER+ BC models, we found evidence for the presentation of different ER peptides, including mutated peptides (Table S1). Given this evidence of antigen presentation of ER peptides, we predicted that there would be a higher prevalence of T cells capable of recognizing *ESR1*-WT and *ESR1*mut peptides in patients, which we confirmed in the initial assessments of a single ER+ BC patient in our ongoing Phase I clinical trial (Figure 4). However, the potential clinical impact of *ESR1*-specific T cell responses remains unknown, as ER+ BC has been reported to have lower levels of HLA expression that may limit T cell responsiveness.³³⁻³⁵ This may be interpreted as evidence for immune surveillance, as TILs have been found to positively associate with outcome with survival in ER+ BC, with Foxp3+ T regulatory cells having an inverse association with survival.³⁴⁻³⁶ These studies suggest that ER+ BC may yet be responsive to anti-tumor T cells, which may explain clinical responses observed in trials testing PD-1 immune checkpoint inhibitors in ER+ BC.²⁰ In this context, the existence of *ESR1*-specific responses in an ER+ BC patient at higher levels than those seen in non-tumor patients may suggest that vaccination could augment these responses. As such, tolerance to *ESR1* may be 'broken' during the development of ER+ tumors to induce a small subdominant population of *ESR1*-reactive T cells, which may play a role in early immune surveillance.³⁷ Supporting this notion, *ESR1*-specific IgG autoantibodies have been detected in ~50% of Systemic Lupus Erythematosus patients and in ~40% of patients with Systemic Sclerosis.³⁸⁻⁴⁰ However, this may also suggest that responses to ER readily occur but are effectively suppressed, which may set a high threshold for achieving ER-specific anti-tumor vaccine efficacy. While ER signaling plays a key role in multiple tissues for different physiologic processes, the systemic use of ER antagonists, inhibitors and degraders demonstrates that systemic inhibition of ER function is not overly toxic, which is corroborated by case reports of men and women with genetic loss of function mutations in *ESR1*.^{41,42}

The importance in *ESR1* in ER+ BC coupled with its non-essential nature and the ability to break immune tolerance to *ESR1* in patients collectively make it an ideal immunotherapeutic target to combat resistance in 20-40% of ER+ BC patients. Moreover, a focus on the mutated, neoepitope region of *ESR1*mut may allow for a more potent induction of T cell immunity due to a lower level of immune tolerance and local immunodominance that might translate into a more impactful prevention of endocrine resistance for a subsets of patients. Despite this, we would note that the clinically efficacy of vaccine-induced ER-specific T cell responses may be offset by a reduction of HLA expression

or local immune suppression, as is the case in many types of tumors. These insights and preclinical data support our ongoing clinical trial testing the safety and immunologic efficacy of a mutant *ESR1* multi-peptide vaccine in patients with metastatic ER+, HER2- BC (NCT04270149). This general approach of vaccines targeting oncogenic neoantigens may also find utility in other cancers as a therapy or preventative strategy to subvert resistance.

Methods

Cell line and adenoviral vector construction

Tumor cell lines 293T, MCF7, and MM3MG were obtained from and maintained as recommended by the American Tissue Culture Collection (ATCC). Cells were modified by stable lentivirus transduction⁴³ and selection for expression of indicated genes. LeGO vectors (Addgene) were used to track MCF7-*ESR1*-WT and MCF7-*ESR1*-Y537N cells. Adenoviral vectors were generated using standard cloning techniques as previously described.⁴⁴ *ESR1* mutants were generated through site-directed mutagenesis and cloned into various lentiviral constructs (previously established in our laboratory) using Gateway cloning techniques (Invitrogen). Lentiviral vectors encoding *ESR1*-Y537N-T2A-CD90.1 were generated using Geneblocks (IDT) and assembled using Gibson Isothermal Assembly reactions (NEB) into lentiviral vectors. A Greenfire ER signaling reporter was purchased from SBI and utilized to infect MM3MG-*ESR1*-Y537N-CD90.1 cells to generate ER-Luc signaling reporter cells. Adenoviral vectors were generated using standard techniques utilized in our previous studies.^{16,17,45} All cloning details, plasmid maps, and sequences are available upon request.

Quantitative rt-PCR

Real-time PCR was performed using an ABI 7300 system using standard methods and intron spanning primers for *ESR1*, and control housekeeping genes. TaqMan probes for *ESR1* specific mutations were generated (PrimeTime 5' 6-FAM/ZEN/3' IBFQ 18 bases; IDT). Expression differences were assessed using the ddCT method against GAPDH control gene and a control treatment group

Next-generation RNA sequencing

MCF7 cells expressing *ESR1*-WT or *ESR1*-Y537N were cultured in media supplemented with charcoal-dextran treated FBS (Omega Scientific) with or without [20 nM] estrogen. Total RNA was extracted using a RNeasy Kit (Qiagen) and sequencing libraries prepared using the Illumina TruSeq Stranded mRNA Library Prep Kit according to the manufacturer's protocol. Sequencing was performed on the Illumina HiSeq 4000 (50bp/SR/~300 M reads), then aligned to the hg19 reference genome (STAR aligner) and annotated using Partek® Genomics Suite® software (version 9.0.20, Copyright©2018 Partek Inc). The data was normalized using the TMM method.⁴⁶ Significance was defined as genes with

a differential gene expression of <0.05 FDR and >|2| fold change.

Mice, vaccination and tumor cell implantation

SCID-beige (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7; Taconic Biosciences, CBSCBG), BALB/c and DO (Jackson Labs, stock 000651), DO (009376) and C57BL/6-*Mcph1*^{Tg(HLA-A2.1)1Enge/J} (Jackson Labs, stock 003475) were purchased and bred at Duke University. Mice were used between 6 and 12 weeks of age. MCF7 and MM3MG cells were injected subcutaneously into the mammary fat pad or flank of mice (1×10^5 – 1×10^6 cells per animal) and measured biweekly. Tumor measurements were made using calipers and volumes calculated using the formula ($v = \text{width} \times \text{width} \times (\text{length}/2)$). Assessments of luciferase activity were performed by IP injection of 2.9 mg of D-luciferin (Gold Biotechnology) and imaging on a Pearl Imager (LiCOR) with analysis of Region of Interest (ROI) determined using ImageStudio v5.2. Mice were vaccinated with a single dose of Ad-*ESR1* and Ad-*ESR1*-MUT constructs via footpad injection of 2×10^9 particles/mouse (20 uL per footpad/40uL per mouse) while anesthetized with isoflurane. All mouse experiments were done in accordance with Duke Institutional Animal Care and Use Committee – approved protocol (A080–20-04). In vaccine experiments to assess immunogenicity, mice were vaccinated and immune responses assessed 2 weeks post-vaccination (Figures 1 and 2). In our preventative vaccine experimental strategies, mice were vaccinated 2 weeks prior to implantation of tumors (Figure 3a, S4A). Tumor treatment vaccine experiments were performed by implantation of tumor cells with vaccination 1 days post-implantation (Figure 3c,d, S4B).

Ethical use of lab animals

All mice were maintained, bred, and utilized experimentally in accordance with guidelines set out by the Animal Care and Use Program through Duke University's Division of Laboratory Animal Resources (DLAR). All animal procedures, protocols and studies were approved by the Institutional Animal Care and Use Committee at Duke University (A115-17-05 and A043-23-02).

ESR1 signaling

In signaling assays, 293T cells stably expressing dox-inducible *ESR1* mutants were transfected with dual luciferase reporter constructs (Signal Reporter Assay Kit 336841, Qiagen) and harvested at 24–48 hrs for measurement of luciferase activity. Each condition was plated in quadruplicate and GFP control vectors were used as negative controls.

Flow cytometry of mouse samples

For flow cytometry, cells were isolated from cell lines or ex vivo tumors. Unless indicated, all flow cytometry was done on tumors from mice when tumors reached a terminal

endpoint volume (~2000 mm³). Prior to staining, tumors were digested using a mix of collagenase (1 mg/mL), DNase (20 U/mL), and hyaluronidase (100 µg/mL) for 90 minutes at 37°C. Digested tumors were mechanically dissociated by smashing through a 40-µm cell strainer (Greiner Bio-One). Red blood cells were lysed with RBC lysing buffer (Sigma). Fixable Aqua dye (Invitrogen) was added to assess cell viability. Cells were incubated with fluorochrome-conjugated antibodies and fixed with 1% formalin (Sigma). Antibodies used include: CD45 (30F11), CD8β (YTS156.7.7), CD4 (GK1.5), CD11b (M1/70), CD90.1 (OX-7) (all Biolegend). Data were collected using an LSR II flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star).

Peptide elution/mass spectrometry

10⁹ MCF7 cells were washed with PBS to remove serum proteins and resuspended in lysis buffer (1% NP40, 150 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, protease inhibitors, Sigma-Aldrich). Cell lysates were subjected to two rounds of immunoprecipitation using 1 mg pan HLA class I-specific antibody and 1 mL of Protein A/G beads (Pierce Biotechnology). The sample solution was heated to 85°C and after cooling to room temperature, peptides were separated from the antibody and HLA molecules by size-exclusion centrifugation (Amicon Ultra-3 10 kDa molecular mass cutoff membrane filters, Millipore). The filtrate was concentrated using vacuum centrifugation and subjected to HPLC (high performance liquid chromatography) and MS (mass spectrometry) analyses. Lastly, synthetic peptides were obtained (New England Peptide) for the MHC class I-bound peptides that were identified by HPLC-MS/MS analyses, and the sequences was confirmed under identical conditions of collision used to identify the MHC class I bound peptides. The SYFPEITHI prediction algorithm (<http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm>) was used to check binding affinity for HLA-A2 and all listed peptides were predicted to have medium-high binding affinity.

Expansion of human *ESR1* peptide specific CTLs

PBMCs were thawed and expanded as previously described.⁴⁷ Briefly, cells were plated (Day 0) with GM-CSF, IL-4, and Flt3-L overnight to mature antigen presenting cells. On Day 1, LPS, R848, and IL-1b were added with peptides (1 uM each). Starting on Day 2 and every 2–3 days after that IL-2, IL-7, and IL-15 were added. On Day 9 cells were washed, counted, and replated with anti-CD28, anti-CD49d, anti-CD107a and desired peptides for 8 hours prior to staining for flow cytometry.

Peptide sequences used:

| | |
|-----------------------------------|------------|
| <i>ESR1</i> WT _{535–544} | PLYDLLLEML |
| <i>ESR1</i> _{Y535S} | PLSDLLEML |
| <i>ESR1</i> _{Y573N} | PLNDLLEML |
| <i>ESR1</i> _{D538G} | PLYGLLEML |
| <i>ESR1</i> _{L544V} | PLYDLLLEMV |

Spectral flow cytometry on human PBMCs

Restimulated cells were stained with ViaDye Red (Cytek Biosciences) for viability and then stained for surface markers indicated (Sup Table S2) in BrilliantViolet buffer (BD Biosciences). Cells were fixed, permeabilized, and stained for intracellular antibodies using the FoxP3 fix-perm kit (Tonbo Biosciences) according to manufacturer's instructions. Samples were acquired using a Cytek Northern Lights spectral cytometer and analyzed using FlowJo V10 (Tree Star).

ELISPOT

Mouse (Mabtech Inc.) or human (Endogen Inc.) IFN-γ ELISPOT assays were performed according to manufacturer's instructions. Briefly, splenocytes (500,000 cells/well) or CTLs from PBMCs (50,000 cells/well) were stimulated with *ESR1* peptide pool (1 µg/ml/peptide, JPT) or irrelevant HIV-gag peptide mix (1 µg/ml/peptide, JPT) for 24 hours. PMA (50 ng/ml) and Ionomycin (1 µg/ml) (Sigma) were used as positive controls.

Statistical analysis

Data are presented as mean ± SEM. Data from experiments with 3 or more treatment groups were analyzed by 1-way ANOVA or 2-way ANOVA with Bonferroni's multiple comparisons test. A 2-tailed, unpaired Student's t test was used for experiments with only 2 groups. Tumor volumes were analyzed at the terminal endpoint only, unless otherwise indicated. Statistical analysis was performed using Prism (GraphPad). *p* values of .05 or less were considered statistically significant. Not all significant differences are shown in every graph. **p* < .05; ***p* < .01; ****p* < .001

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Disclosure statement

ZCH and HKL are both named inventors on patents for *ESR1*mut vaccination and both founders, equity holders, and on the scientific advisory board of Replicate Biosciences, which holds a license on these patents.

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Data availability

All sequencing data that support the findings of this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE153033. All other relevant data are available from the corresponding author upon request.

Author contributions

Conception and study design: GPD, EJC, JD, AH, MM, HKL, ZCH; Data generation: GPD, EJC, JD, CAR, GJL, JW, XYY, AS, TW, ZCH; Analysis and interpretation of data: GPD, EJC, JD, ZCH; Drafting of manuscript: EJC, ZCH; Critical revision of manuscript: all authors; Statistical analysis: EJC, ZCH; Supervision and funding: HKL, EJC, ZCH. All authors reviewed and approved the final manuscript.

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