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The ubiquitin-like protein, ISG15, is a novel tumor-associated antigen for cancer immunotherapy

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Abstract

The recent announcement of the first FDA-approved therapeutic vaccine for prostate cancer, Sipuleucel-T, is a watershed moment for the field of tumor immunotherapy. However, while Sipuleucel-T provides a powerful tool to clinicians for the most prevalent form of cancer in men, there remains an unmet need for a similar therapeutic strategy against breast cancer, the most prevalent cancer in women. While current breast cancer vaccines in development target several antigens, the most prevalent is the tumor-associated antigen, HER2. Initial results with HER2 vaccines appear promising in terms of efficacy; however, the lack of HER2 overexpression by a majority of breast tumors and the safety concerns associated with current HER2-targeted immunotherapy suggest that additional therapeutic strategies would be beneficial. Recently, several studies have identified ISG15 as a molecule highly expressed in numerous malignancies. ISG15 is a small ubiquitin-like protein regulated by type-I interferon and classically associated with viral defense. Elevated ISG15 expression in breast cancer is especially well documented and is independent of HER2, progesterone receptor, and estrogen receptor status. Additionally, high ISG15 expression in breast cancer correlates with an unfavorable prognosis and poor responses to traditional treatment strategies such as chemotherapy and radiation. To overcome these challenges, we employ a novel strategy to specifically target tumor-associated ISG15 expression with immunotherapy. We demonstrate that vaccination against ISG15 results in significant CD8-mediated reductions in both primary and metastatic mammary tumor burden. These results validate ISG15 as a tumor-associated antigen for cancer immunotherapy.

Keywords

ISG15; Breast cancer; *Listeria monocytogenes*; Vaccine

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Conflict of interest Yvonne Paterson wishes to disclose that she has a financial interest in Advaxis, a vaccine and therapeutic company that has licensed or has an option to license all patents from the University of Pennsylvania that concern the use of *Listeria monocytogenes* or listerial products as vaccines.

Introduction

The recent approval of Provenge, Sipuleucel-T, the first FDA-approved therapeutic vaccine for cancer, provides an additional therapeutic strategy for clinicians in the treatment of prostate cancer, the most common form of cancer in men [1]. However, there still remains an unmet need for a similar therapeutic strategy against breast cancer, the most common form of cancer in women [2]. Current breast cancer vaccines in development target several antigens, including the cancer/testis (CT) antigens [3]; however, the most prevalent vaccine target is the breast tumor-associated antigen (TAA), HER2, a member of the epidermal growth factor receptor family [4]. While initial results of studies targeting HER2 with immunotherapy for breast cancer appear promising, there remains safety concerns about this strategy due to myocardial HER2 expression and observed cardiac toxicity of current HER2-targeted immunotherapy [5, 6]. These safety concerns along with the fact that HER2 is overexpressed in only 15–20% of breast cancer cases [7] suggests that the discovery of additional TAAs would expand the usefulness of tumor immunotherapy as a therapeutic strategy in breast cancer treatment. The results presented in this manuscript suggest that interferon (IFN)-stimulated gene 15 (ISG15) is a novel breast TAA that can be successfully targeted with CD8-mediated tumor immunotherapy.

ISG15 is a small ubiquitin-like protein that is induced by type-I IFN and conjugated to target proteins by an enzymatic cascade [8–13]. ISG15 plays an important role in innate immunity by regulating anti-viral and anti-bacterial properties of type-I IFN [14–19]. However, new evidence is emerging that ISG15 may be involved in additional pathologies such as systemic lupus erythematosus [20] and cancer [21–27]. In fact, elevated expression of ISG15 is found in numerous human malignancies such as melanoma [21], endometrial cancer [22], bladder cancer [23], breast cancer [24], prostate cancer [25], oral cancer [26], and cervical cancer [27].

High ISG15 expression is particularly well documented in the case of breast cancer. In a large systematic analysis consisting of over 900 human breast carcinomas, ISG15 expression was significantly elevated in comparison with normal mammary tissue where expression was either absent or weak by immunohistochemical analysis [24]. Interestingly, there was no significant correlation between ISG15 expression and HER2 status and, unlike the CT antigens [28], no correlation between ISG15 expression and estrogen receptor or progesterone receptor status. Furthermore, high ISG15 expression in breast carcinomas was significantly correlated with unfavorable outcomes such as event-free survival, recurrence-free survival, and overall survival. These results are in accordance with a subsequent study that found high expression of interferon-related genes, including ISG15, in breast tumors correlates with greater resistance to traditional therapeutic approaches such as chemotherapy and radiation [29]. Due to the significant correlation between breast cancer and elevated ISG15 expression along with the resistance of these tumors to traditional therapeutic approaches, targeting ISG15 with tumor immunotherapy may provide clinicians with a novel and effective treatment strategy for breast cancer.

To determine whether ISG15 can be successfully targeted as a novel breast TAA, several models for elevated ISG15 expression in mouse mammary tumor cell lines and

autochthonous HER2/neu⁺ mammary tumor tissue were established. After construction of a *Listeria monocytogenes*-based CTL vaccine against ISG15, Lm-LLO-ISG15, administration of this vaccine elicited an adaptive immune response against two putative ISG15 H2K^d CTL epitopes. This immune response acts in a CD8-dependent manner to inhibit the growth of implanted tumors and reduce the spread of lung tumor metastases. We also demonstrate that therapeutic vaccination against ISG15 can significantly delay the progression of autochthonous mammary tumors and lead to the development of epitope spreading. These results establish a mouse model for elevated ISG15 expression in breast cancer and suggest that elevated levels of ISG15 can be effectively targeted by active tumor immunotherapy.

Materials and methods

Mice

BALB/c female mice (6–8 week old) from Charles River Laboratories (Wilmington, MA) were utilized for all experiments involving the 4T1-Luc mammary tumor cell line [30]. FVB/NJ female mice (6–8-week old) from Jackson Laboratories (Bar Harbor, ME) were utilized for all experiments involving the NT2 mammary tumor cell line [31]. A rat HER2/neu transgenic mouse strain in the FVB/N background [32], used in autochthonous mammary tumor formation studies, was housed and bred at the animal core facility at the University of Pennsylvania. All mouse experiments were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Cell lines

The metastatic mammary tumor cell line 4T1-Luc was received as a kind gift from Ellen Pure and utilized in tumor implantation studies in BALB/c mice [30]. The 4T1-Luc cell line was maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin. The NT2 mammary cancer cell line that expresses rat HER2/neu was utilized for tumor implantation studies in FVB/NJ mice [31]. NT2 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 20 µg/mL insulin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin. The non-transformed NIH-3T3 fibroblast cell line was obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin.

Detection of ISG15 expression in normal and tumor mouse mammary tissue

RNA was extracted from tissue or cells using the RNeasy RNA extraction kit (Qiagen, Valencia, CA) and converted to cDNA using a High Capacity Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA). The cDNA was then subjected to qPCR analysis with primers specific to ISG15 (qISG15.FOR 5'-ATGGCCTGGGACCTAAAG-3' and qISG15.REV 5'-TTAGGCACACTGGTCCCC-3'), 18S rRNA (18SRNA.FOR 5'-CGGCTACCACATCCAAG GAA-3' and 18SRNA.REV 5'-GCTGGAATTACCGCGG CT-3'), and β-actin (ACTIN.FOR 5'-GTGGGCCGCTC TAGGCACCAA-3' and

ACTIN.REV 5'-CTCTTTGATGT CACGCACGATTTC-3')-ISG15 expression was normalized to either β -actin (Fig. 1a) or 18S rRNA (Fig. 1c, d).

Western blot analysis of mammary tissue lysates

Normal mammary tissue from FVB/NJ mice ($n = 4$) and autochthonous mammary tumor tissue from HER2/neu transgenic mice in the FVB/N background ($n = 9$) were excised and processed into lysates. Briefly, tissue samples were snap-frozen in liquid nitrogen, pulverized, and solubilized in lysis buffer (PBS with 2% Triton X-100 and 0.02% saponin) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, Missouri). Lysates were mixed with 4 $\times$ LDS Sample Loading Buffer (Thermo Scientific, Rockford, IL) and subjected to SDS-PAGE. After transfer of separated proteins to a PVDF membrane, Western blot analysis was performed with either anti-mouse ISG15 antibody (eBioscience, San Diego, CA) or anti-GAPDH antibody (Sigma Aldrich, St. Louis, Missouri).

Listeria monocytogenes strains

To construct an attenuated *Listeria monocytogenes* (Lm)-based vaccine against ISG15, we first amplified the gene encoding mouse ISG15 (accession number NM_015783) from a construct containing mouse ISG15 cDNA with the following primers: Lm-LLO-ISG15.FOR 5'-TAAT-CTCGAG-ATGGCCTGGGACCTAAAG-3' and Lm-LLO-ISG15. REV 5'-ATTA-ACTAGT-TTAGGCACACTGGTCCCC-3'. The XhoI sequence underlined in the forward primer and the SpeI sequence underlined in the reverse primer were utilized for construction. The resulting amplicon was restriction-enzyme digested and ligated into the Lm expression plasmid, pGG34 [33]. The ISG15 sequence was genetically fused downstream to the sequence encoding truncated Listeriolysin O (tLLO) under the control of the *hly* promoter. Subsequently, pGG34-LLO-ISG15 was electroporated into the attenuated Lm strain, XFL7, and plasmid containing colonies were selected for resistance on Brain Heart Infusion (BHI)-chloramphenicol plates. A control vaccine, Lm-LLO-OVA, consisting of tLLO genetically fused to chicken ovalbumin (accession number NM_205152) was similarly constructed. To confirm proper construction of Lm-LLO-ISG15, the attenuated Lm-based vaccine and the control vaccine were each grown in BHI-chloramphenicol selection media and secreted proteins were precipitated with trichloroacetic acid (TCA). After boiling in SDS sample buffer, secreted proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Western blot analysis on the membrane was performed with anti-mouse ISG15 antibody (Santa Cruz Biotech, Santa Cruz, CA) to confirm the secretion of the tLLO-ISG15 fusion protein, anti-chicken ovalbumin antibody (clone 3 A11.2) to confirm the secretion of the tLLO-OVA fusion protein, and anti-LLO antibody (clone B3-19) to confirm the proper secretion of endogenous LLO. All Lm-based vaccines were administered intraperitoneally (i.p.) at either 2×10^8 or 5×10^8 CFU in 200 μ l of PBS.

ELISpot analysis

The 96-well filtration plates (Millipore, Bedford, MA) were coated with 15 μ g/ml rat anti-mouse IFN- γ antibody (clone AN18, MABTECH, Mariemont, OH) in 100 μ l of PBS. After overnight incubation at 4°C, the wells were washed and blocked with DMEM supplemented with 10% FCS. For Fig. 2c, splenocytes from each experimental group were added to the

wells along with HIV-gag H-2K^d CTL epitope peptide (AMQMLKETI) or predicted ISG15 H-2K^d CTL epitope peptides, pISG15-d1 (RGHSNIYEV), and pISG15-d2 (LGPSSTVML) (5 µg/ml) plus IL-2 (5U/ml). ISG15 H-2K^d CTL epitopes were predicted using RANKPEP prediction software available at <http://imed.med.ucm.es/Tools/rankpep.html>. For Fig. 4a, splenocytes from each experimental group were added to the wells along with HIV-gag H-2K^d CTL epitope peptide (AM-QMLKETI) or HER2/neu H-2K^d epitope peptides HER2-EC1 (PYNYLSTEY), HER2-EC2 (LFRNPHQALL), and HER2-IC1 (PYVSRLGI) [34]. Cells were incubated at 37°C for 24 h. The plate was washed with PBS followed by incubation with 1 µg/ml biotinylated IFN-γ antibody (clone R4-6A2, MABTECH, Mariemont, OH) in 100 µl PBS at 4°C overnight. After washing, 1:100 Avidin HRP (eBioscience, San Diego, CA) in 100 µl PBS was added and incubated for 1 h at room temperature. Spots were developed by adding 100 µl of substrate after washing and incubated at room temperature for 15 min. Color development was stopped by washing extensively in dH₂O, and spot-forming cells (SFC) were counted with an ELISpot reader.

Tumor immunotherapy with Lm-LLO-ISG15

For 4T1-Luc tumor studies, 1×10^5 tumor cells were implanted subcutaneously (s.c.) into the mammary tissue and mice were subsequently vaccinated i.p. on day 5, 12, and 19 with Lm-based vaccines. For NT2 tumor studies, 1×10^6 tumor cells were implanted s.c. into the hind flank and mice were subsequently vaccinated i.p. on day 7, 14, and 21 with Lm-based vaccines. Tumor volume was monitored by perpendicular caliper measurements throughout the course of the experiment. Tumor volume was calculated as $(\text{tumor diameter})^3 / 2$.

Tumor immunotherapy with ISG15 peptides

4T1-Luc tumor cells (1×10^5) were implanted s.c. into the mammary tissue of BALB/c mice, and mice were subsequently vaccinated on day 5, 12, and 19 with either 100 µl of PBS or 50 µg CpG oligodeoxynucleotides (ODN) mixed with control, HIV-gag H-2K^d CTL epitope peptide (AMQ MLKETI) (100 µg), or ISG15 peptides (100 µg), pISG 15-d1(RGHSNIYEV) and pISG15-d2(LGPSSTVML), in 100 µL of PBS s.c. proximal to the cervical lymph nodes. The immunostimulatory CpG ODN 1826 sequence, TC CATGACGTTTCCTGACGTT, is an adjuvant that was characterized by Davis et al. [35]. Tumor volume was monitored by perpendicular caliper measurements throughout the course of the experiment. Tumor volume was calculated as $(\text{tumor diameter})^3 / 2$. To determine the impact of ISG15 peptide vaccination on primary and challenge Lm infection, we adhered to a similar protocol. Briefly, to determine the impact of ISG15 peptide vaccination on primary Lm infection, 5 BALB/c mice were vaccinated s.c. with either HIV-gag H-2K^d CTL epitope peptide or pISG15-d1 along with CpG ODN on day 1 and day 7. Each group was subsequently infected i.p. on day 14 with 1×10^5 CFUs of wild-type Lm, strain 10403S [36]. Three days after primary infection, mice were processed to determine Lm CFUs in the spleen by selection on BHI-streptomycin plates. To determine the impact of ISG15 peptide vaccination on a challenge infection, three groups of 5 BALB/c mice were first vaccinated i.p. on day 1 with 1×10^3 CFUs of the attenuated Lm strain, DP-L 4029 [37]. Subsequently, mice were injected s.c. with either PBS, HIV-gag peptide vaccine, or pISG15-d1 peptide vaccine on days 30 and 37. Each of the treatment groups along with a naïve group were then

challenged on day 90 with 1×10^5 CFUs of 10403S and processed after 5 days to determine Lm CFUs in the spleen.

Metastatic tumor study

4T1-Luc tumor cells (1×10^5) were implanted s.c. into the mammary tissue, and mice were subsequently vaccinated on days 5, 12, and 19 with either peptide or Lm-based vaccines. Mice were killed on day 32 post tumor implantation and lungs were isolated and perfused with PBS. Lung surface metastatic nodules were then counted with a Nikon SMZ1B Zoom Stereomicroscope attached to a Fostec 8375 Illuminator and Ringlight.

Lymphocyte depletion experiments

CD8⁺ cells were depleted in 4T1-Luc tumor-bearing mice by injecting the mice with 0.5 mg of affinity-purified α -CD8 antibody (clone 2.43) on days 6, 7, 8, 10, 12, and 14 post tumor implantation. A control group of mice were also treated under the same conditions but with an isotype-matched, control antibody specific to beta-galactosidase. The concurrent tumor load study was adhered to as described in “Tumor immunotherapy with Lm-LLO-ISG15” in this methods section.

Winn assay for in vivo determination of effector cells

The Winn assay was performed as previously described with some modification [38]. Briefly, 4T1-Luc tumor cells (2×10^5) mixed with CD4-depleted splenocytes (depletion with mouse CD4 Dynabeads (Invitrogen, Carlsbad, CA) and confirmed by FACS analysis) from either twice control Lm-vaccinated or twice Lm-LLO-ISG15-vaccinated BALB/c mice (2×10^7) at a ratio of 1 tumor cell to 100 CD4-depleted splenocytes were implanted s.c. into the mammary tissue. Tumor development was then measured as described in “Tumor immunotherapy with Lm-LLO-ISG15” in this methods section.

Detection of HER2/neu-specific tumor infiltrating lymphocytes (TILs)

BALB/c mice were implanted with 4T1-Luc tumor cells (2×10^5) and immunized i.p. with control Lm or Lm-LLO-ISG15 and boosted 7 days later. Tumors were harvested 9 days after boosting and manually dissociated through a 100- μ m mesh filter into a single-cell suspension. The tumor cell suspension was then Ficoll-purified to remove dead cells and cellular debris by excluding the low-density fraction after centrifugation. The remaining tumor cells were then subjected to four-color flow cytometry for CD8 (53-6.7, FITC conjugated), CD62 ligand (CD62L; MEL-14, APC conjugated), CD11b (M1/70, PerCP-Cy5.5 conjugated), and HER2/neu-EC2 H-2D^q tetramer-PE conjugated (specific to PDSLRDLSVF) [39] using a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson, Mountain View, CA). Antibodies were purchased from eBioscience (San Diego, CA) and used at a 1:100 dilution. Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and used at a 1:200 dilution. Results were analyzed as described above to compare the ability of Lm-LLO-ISG15 to induce CD11b⁺, CD8⁺, CD62L⁺, and tetramer⁺ HER2/neu-specific TILs in comparison with control Lm vaccination.

Statistical analyses

One-tailed student's *t* tests were performed for all final tumor volume, metastatic load and immune response studies with Welch's correction applied for gene expression studies with autochthonous HER2/neu mammary tumors. Log rank test was performed for autochthonous HER2/neu mammary tumor progression studies. Statistical analyses were performed using GraphPad Prism version 4.0a for Macintosh (San Diego, CA, <http://www.graphpad.com>). Significant *P* values for all comparisons are depicted in figures as follows: **P* value < 0.05, ***P* value < 0.01, and ****P* value < 0.001.

Results

Since the initial studies documenting elevated expression of ISG15 in human endometrium tumor tissue by Desai et al. [22] and human melanoma tumor tissue by Padovan et al. [21], additional studies have confirmed this phenomenon in a multitude of human malignancies [23–27, 29]. While a recent study investigated the importance of ISG15 conjugation for tumorigenesis in mice [40], there is currently a lack of published data documenting elevated ISG15 expression in mouse tumors. To determine whether this phenomenon is conserved in mouse models of breast cancer, we assayed ISG15 expression in autochthonous mouse mammary tumors from HER2/neu transgenic mice [32], mouse mammary tumor cell lines, and a panel of normal and non-transformed mammary tissues and cell lines. As observed in human breast cancer, expression of ISG15 mRNA is significantly elevated in the autochthonous mouse mammary tumors in comparison with normal mouse mammary tissues (Fig. 1a). To confirm that the elevated ISG15 mRNA expression results in elevated protein production, Western blot analysis was performed with lysates of normal and HER2/neu tumor mouse mammary tissues. In comparison with normal mouse mammary tissue (Fig. 1b, top panel, lane 1), the conjugated form of ISG15 protein (bands above 20kD marker) is elevated in HER2/neu mammary tumor tissue (Fig. 1b, top panel, lanes 2–5). Elevated expression of the unconjugated form of ISG15 protein is also evident in mouse mammary tumor tissue (Fig. 1b, top panel, lanes 2, 4) in comparison with normal mammary tissue (Fig. 1b, top panel, lane 1). As a measure of total protein loading, the same lysates were probed for expression of the housekeeping protein, GAPDH (Fig. 1b, bottom panel, lanes 1–5). ISG15 mRNA expression was similarly elevated in mouse mammary tumor cell lines, 4T1-Luc and NT2, in comparison with normal mouse mammary tissues and a non-transformed mouse fibroblast cell line, NIH-3T3 (Fig. 1c). To alleviate concerns of elevated ISG15 expression in non-malignant tissues, ISG15 mRNA expression was analyzed in a panel of normal mouse tissues in comparison with HER2/neu mammary mouse tumor tissue. Significantly elevated expression of ISG15 mRNA in mammary tumor tissues was similarly observed when compared against each normal tissue type (Fig. 1d). This expression analysis confirms that ISG15 expression is significantly elevated in mouse models of breast cancer. Together with the finding that ISG15 mRNA is nominally expressed in a panel of normal tissues, these results suggest that ISG15 may be a promising novel TAA.

To assess the potential for ISG15 as a novel TAA, an Lm-based CTL vaccine, as previously described by Gunn et al. [33], was developed to target tumors with elevated ISG15 expression. Construction of the vaccine, Lm-LLO-ISG15, was accomplished by genetically

fusing the mouse ISG15 gene downstream of a gene encoding tLLO, already present in the Lm expression vector pGG34. To facilitate antigen processing and presentation, the tLLO gene contains a signal sequence that allows for secretion of the antigenic fusion protein into the host cytosol during infection. The pGG34-LLO-ISG15 construct was subsequently electroporated into the attenuated Lm strain, XFL7 (Fig. 2a). Proper secretion of the tLLO-ISG15 fusion protein was confirmed by Western blot analysis with anti-mouse ISG15 antibody against TCA-precipitated proteins from the media of an Lm-LLO-ISG15 growth culture (Fig. 2b, top panel). Similar production and secretion of a fusion protein of tLLO fused to chicken ovalbumin was observed from our control Lm when probed with anti-ovalbumin antibody (Fig. 2b, middle panel). Secreted proteins from Lm-LLO-ISG15 and the control Lm were also probed for endogenous LLO to confirm equivalent loading of secreted protein (Fig. 2b, bottom panel). Generation of ISG15-specific CD8⁺ T-cell responses was assayed by weekly administration of both Lm-LLO-ISG15 and a control Lm vaccine to groups of six-week-old female BALB/c mice. One week after the third vaccination, splenocytes from each group were subjected to ELISpot analysis to investigate IFN- γ responses against a control epitope and two putative ISG15 H2-K^d-restricted CD8⁺ T-cell epitopes predicted by RANKPEP. A significant increase in IFN- γ secreting SFCs was observed only in the splenocytes from the Lm-LLO-ISG15-vaccinated mice after stimulation with each predicted ISG15 CTL epitope peptide in comparison with control peptide stimulation (Fig. 2c). These results suggest that an ISG15-specific adaptive response can be generated by an attenuated Lm-based CTL vaccine against ISG15.

While ISG15 tissue expression is at low or undetectable levels under normal conditions, there is evidence for elevated ISG15 expression at the placental implantation site during pregnancy [41]. To determine whether an ISG15-specific immune response may severely impact fertility in Lm-LLO-ISG15-vaccinated female mice, a pregnancy study was performed. In comparison with control Lm-vaccinated female mice, the fertility of Lm-LLO-ISG15-vaccinated female mice was not significantly impaired as measured by litter size and pup weight (Fig. 2d, e, respectively). Generation of an ISG15-specific adaptive immune response with no obvious adverse effects encouraged examination of its efficacy in mouse models for breast cancer.

The therapeutic potential of an ISG15-specific adaptive immune response generated by Lm-LLO-ISG15 against breast cancer was initially investigated against implanted primary and metastatic mouse models of breast cancer. Implantation of NT2 tumor cells s.c. in the hind flank of FVB/NJ mice and subsequent vaccination with Lm-LLO-ISG15 resulted in significantly reduced tumor volume as compared to control vaccination (Fig. 3a). Similarly, Lm-LLO-ISG15 therapeutic vaccination significantly inhibited the growth of mammary tissue-implanted 4T1-Luc primary tumors (Fig. 3b). The ability of 4T1-Luc tumors to naturally metastasize after implantation in the mammary gland [30] allowed further investigation into the efficacy of an ISG15-specific CTL response against a more aggressive model for breast cancer. Significant reductions in the appearance of 4T1-Luc metastatic lung lesions were observed after Lm-LLO-ISG15 administration in comparison with control Lm (Fig. 3c).

To determine whether Lm-LLO-ISG15 could also provide therapeutic efficacy in a more clinically relevant model of human breast tumor development, we utilized a HER2/neu transgenic mouse model in the FVB/N background that, in the absence of therapeutic intervention, develops autochthonous mammary tumors after 4 months of age [32]. HER2/neu transgenic female mice were vaccinated every three weeks with Lm-LLO-ISG15 or a control Lm from week 6 to 21 after birth and subsequently monitored for mammary tumor incidence. In the mice administered Lm-LLO-ISG15, there was a significant delay to tumor progression in comparison with the control Lm-vaccinated group ($P < 0.0001$) (Fig. 3d). In fact, >80% of Lm-LLO-ISG15-vaccinated mice are still tumor-free by week 49 after birth while all control Lm-vaccinated mice have developed mammary tumors with a median time to progression of 31 weeks.

Recent studies demonstrate that the clinical efficacy of cancer vaccines significantly correlates with their ability to stimulate cross-priming and epitope spreading to additional TAAs [42–45]. Similar results were observed previously using Lm-based cancer vaccines where development of epitope spreading to additional TAAs was associated with vaccine efficacy [46, 47]. To assess whether epitope spreading develops after Lm-LLO-ISG15 vaccination, an ELISpot assay to detect HER2/neu-specific responses was performed with splenocytes from NT2 tumor-bearing mice after administration of either control Lm or Lm-LLO-ISG15. Splenocytes of Lm-LLO-ISG15-vaccinated mice contained significantly greater numbers of SFCs specific to known CTL epitopes within HER2/neu compared to control Lm-vaccinated mice (Fig. 4a). This result suggests that Lm-LLO-ISG15 vaccination results in epitope spreading to additional TAAs. In fact, evidence for epitope spreading was also observed after Lm-LLO-ISG15 vaccination against 4T1-Luc tumors, a tumor cell line that expresses HER2/neu very weakly [47]. 4T1-Luc tumors from Lm-LLO-ISG15-vaccinated mice contained a significantly higher percentage of HER2/neu-specific CD8⁺ 62L[−] TILs than 4T1-Luc tumors from control Lm-vaccinated mice (Fig. 4b). While epitope spreading to HER2/neu may provide some therapeutic efficacy, it is unclear whether this secondary response is robust enough to warrant cardiotoxicity safety concerns [6]. In summary, these tumor load studies demonstrate that vaccination against ISG15 can inhibit the growth of primary implanted mouse mammary tumors, inhibit metastatic spread, delay progression of autochthonous mammary tumors and generate epitope spreading to additional TAAs.

While the generation of robust IFN- γ responses and significant therapeutic tumor impact are suggestive of strong CTL responses, the dependence of ISG15-specific CD8⁺ T-cell function in Lm-LLO-ISG15 efficacy was investigated. Antibody-mediated depletion of CD8⁺ cells concurrent with vaccination in 4T1-Luc tumor-bearing mice completely abrogates the anti-tumor efficacy of Lm-LLO-ISG15 compared to mock depletion with a control antibody (Fig. 5a). As an in vivo measure of ISG15-specific CTL activity, we performed a Winn assay [38] to assess whether splenocytes enriched for CD8⁺ T cells from Lm-LLO-ISG15-vaccinated mice could directly inhibit 4T1-Luc tumor formation. Briefly, splenocytes from mice twice vaccinated with either Lm-LLO-ISG15 or a control Lm were depleted of CD4⁺ cells and placed in suspension together with 4T1-Luc tumor cells. The tumor cell and splenocyte suspension was then implanted into the mammary tissue of

BALB/c mice and tumor progression monitored. CD8⁺ T cell-enriched splenocytes from Lm-LLO-ISG15-vaccinated mice significantly inhibited tumor growth in comparison with those from control Lm-vaccinated mice (Fig. 5b). Additionally, all control Lm splenocyte-receiving mice developed tumors by day 21 post implantation while 40% of mice receiving ISG15-specific splenocytes were still tumor-free at day 43 (Fig. 5c). This result suggests that Lm-LLO-ISG15 induces a CD8-dependent adaptive immune response that results in direct lysis of tumor cells and is likely mediated by CD8⁺ T cells. To assess whether expansion of a single ISG15-specific CD8⁺ T-cell clone can result in anti-tumor efficacy, mice were implanted with 4T1-Luc tumor cells and vaccinated with either PBS alone or an adjuvant, CpG ODN [35], mixed with each ISG15 H2K^d epitope peptide or a control peptide. In mice vaccinated with CpG ODN and ISG15 H2K^d peptides, 4T1-Luc tumor volume and metastatic spread were significantly reduced in comparison with PBS alone and control peptide vaccination (Supplemental Fig. 1a and b, respectively). These data strongly suggest that expansion of ISG15-specific CD8⁺ T cells can directly inhibit the growth of tumors with elevated expression of ISG15.

Since ISG15 is known to play a significant role in the response to pathogens [14–19], we assessed whether ISG15 peptide vaccination had any impact on susceptibility to infection by wild-type Lm. After administration of an ISG15 peptide vaccine, no significant increase in susceptibility to primary Lm infection, as determined by splenic Lm CFUs, was observed in comparison with control peptide-vaccinated mice (Supplemental Fig. 1c). Similarly, in a challenge experiment, wherein mice are initially vaccinated with an attenuated strain of Lm, subsequent administration of an ISG15 peptide vaccine had no significant impact on the ability of mice to control a challenge Lm infection in comparison with control peptide-vaccinated mice (Supplemental Fig. 1d).

Discussion

The results presented in this manuscript support the hypothesis that targeting ISG15 with tumor immunotherapy is a promising and novel therapeutic strategy in the treatment of breast cancer. After establishing significantly elevated expression of ISG15 in mouse mammary tumor cell lines and autochthonous mammary tumor tissue, an Lm-based CTL vaccine against ISG15, Lm-LLO-ISG15, was constructed. Vaccination with Lm-LLO-ISG15 resulted in the generation of ISG15-specific IFN- γ responses and significant reductions in primary tumor burden, metastatic spread, and tumor incidence in three separate models of mouse breast cancer (Fig. 3). The therapeutic efficacy of Lm-LLO-ISG15 against primary mammary tumors was CD8-dependent and resulted in epitope spreading to an additional breast TAA, suggesting that vaccination against ISG15 is a promising strategy in breast cancer treatment.

While a previous study found that breast tumors expressing high levels of ISG15 and other interferon-related genes are resistant to traditional forms of therapy [29], immunotherapy that results in expansion of ISG15-specific CD8⁺ T cells may be a successful treatment alternative (Fig. 3a–c). In support of this notion, vaccination against ISG15 with Lm-LLO-ISG15 resulted in regression of tumors in a CD8-dependent manner (Fig. 5a), suggesting a pivotal role for CD8⁺ T cells. Furthermore, expansion of two separate ISG15-specific CD8⁺

T-cell clones by peptide vaccination resulted in tumor regression (Supplemental Fig. 1a) and significantly reduced metastatic tumor burden (Supplemental Fig. 1b). Finally, the results from the Winn assay, in which direct inhibition of tumor formation is assayed by implantation of tumor cells in a suspension with CD4-depleted splenocytes from vaccinated mice, demonstrate that Lm-LLO-ISG15 vaccination results in expansion of cell types with direct anti-tumor activity likely mediated by CD8⁺ T cells (Fig. 5b, c). Since attenuated Lm infection has been shown to stimulate a non-specific anti-tumor NK cell response [48, 49], it is possible that NK cells could be mediating tumor cell killing in the Winn assay. While we did not specifically deplete NK cells from the splenocytes, we think this unlikely since NK activity after Lm infection was limited to the liver and specific for liver metastases [48, 49]. In addition, splenocytes from animals receiving a control Lm vaccine failed to inhibit tumor formation (Fig. 5c), suggesting that induction of ISG15-specific responses is key to the efficacy of Lm-LLO-ISG15. Therefore, the results presented here demonstrate that expansion of ISG15-specific CD8⁺ T cells is an effective treatment strategy for breast cancer.

While direct lysis of tumor cells by ISG15-specific CD8⁺ T cells likely initiates the anti-tumor efficacy of ISG15 vaccination, there are other factors that may contribute to the overall mechanism of action. As Orntoft and colleagues demonstrated by immunohistochemical analysis of bladder tumors, elevated expression of ISG15 is found not just in tumor cells but also in the tumor stromal cells [23]. Therefore, the tumor stroma may also serve as a target for destruction by an ISG15-specific CTL response. In fact, due to their genetic stability in relation to tumor cells, tumor stromal cells are believed to present antigens more proficiently than tumor cells [50]. Additionally, previous studies demonstrate that specific destruction of tumor stroma can result in significant anti-tumor efficacy [51].

The observed HER2/neu-specific IFN- γ responses in the spleens of tumor-bearing animals and infiltration of HER2/ neu-specific TILs after Lm-LLO-ISG15 vaccination suggest that epitope spreading is also a likely factor in the efficacy of the vaccine. While we have not undertaken studies to determine the exact contribution of epitope spreading to the overall efficacy of Lm-LLO-ISG15, the magnitude of the responses (Fig. 4a, b) and previous studies [46] suggest it may play a significant role.

To address the possible safety concerns associated with an ISG15-specific CTL response, minimal expression of ISG15 in normal tissues was confirmed and no significant impact on mouse fertility was observed after Lm-LLO-ISG15 vaccination (Figs. 1, 2d, e, respectively). While ISG15 is also elevated in virus [52] or bacteria-infected [53] tissues, no observed impact on morbidity or mortality was found in Lm-LLO-ISG15-vaccinated mice after continual infection with the attenuated Lm vaccine (data not shown). In addition, mice administered an ISG15 peptide vaccine demonstrated no significant increase in susceptibility to either a primary or challenge infection with wild-type Lm in comparison with control vaccinated mice (Supplemental Fig. 1c and d). While further work to determine safety may be needed before an ISG15 vaccine is brought into the clinic, the results presented here suggest that generation of ISG 15-specific CTL responses has no obvious impact on general health, pregnancy, or response to pathogen.

The results presented in this paper demonstrate that targeting ISG15 is a therapeutic strategy that results in significant efficacy against mouse models for breast cancer. While many current therapeutic strategies for breast cancer are dependent on expression of HER2 and/or hormonal receptors, elevated ISG15 expression is independent of each [24] and suggests that targeting ISG15 may be an attractive treatment option for those that are HER2 and/or receptor-negative. Additionally, while previous studies suggest that ISG15-expressing tumors are resistant to traditional therapeutic strategies [29], their responsiveness to type-I IFN, as evidenced by elevated ISG15 expression, could be predictive of increased sensitivity to CD8⁺ T cell-mediated immunotherapy [54]. Therefore, we propose that targeting ISG15 with CTL-based tumor immunotherapy is a novel and promising therapeutic strategy in the treatment of breast cancer. In addition, given that ISG15 is also elevated in many other human malignancies such as melanoma [21], endometrial cancer [22], bladder cancer [23], prostate cancer [25], oral cancer [26], and cervical cancer [27], it is likely to be a broadly applicable target for cancer immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Elevated expression of ISG15 in mouse mammary tumors. **a** mRNA was extracted from autochthonous mouse mammary tumors ($n = 9$) excised from FVB/N HER2/neu transgenic mice and normal mammary tissues ($n = 4$) from FVB/NJ mice. After cDNA conversion, qPCR analysis was performed to determine mean ISG15 mRNA expression of each tissue relative to β -actin expression. **b** Western blot analysis of tissue lysates from representative normal mammary tissue and HER2/neu mammary tumor tissues from **a** with anti-ISG15 antibody, *top panel*, and with anti-GAPDH antibody to demonstrate equivalent protein loading, *bottom panel*. **c** qPCR analysis of cDNA from mammary tumor cell lines NT2 and 4T1-Luc were compared against normal mammary tissue and the non-transformed cell line NIH-3T3 for expression of ISG15 mRNA ($n = 3$). Mean expression and standard deviation of ISG15 mRNA from each cell line or tissue relative to 18S rRNA content are depicted, **d** qPCR analysis of ISG15 expression in a panel of normal tissues ($n = 3$) compared to a panel of autochthonous mammary tumors from HER2/neu transgenic mice consisting of three

samples from **a** and four additional tumor samples ($n = 7$). Mean expression and standard deviation of ISG15 mRNA from each tissue relative to 18S rRNA content are depicted

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Fig 2.

Construction of an Lm-based CTL vaccine against ISG15. **a** Illustration depicting the Lm expression vector, pGG34-LLO-ISG15, that was electroporated into the *prfA*⁻ XFL7 Lm strain to construct the attenuated Lm vaccine, Lm-LLO-ISG15. The plasmid, pGG34-LLO-ISG15, contains both the gene for the Lm transcription factor *prfA*, to complement for the lack of *prfA* in XFL7, and the gene for mouse ISG15 genetically fused downstream of tLLO under control of the *prfA* promoter, **b** Western blot analysis of TCA-precipitated proteins from the culture media of Lm-LLO-ISG15 and the control Lm vaccine, Lm-LLO-OVA. Precipitated proteins were subjected to SDS-PAGE and Western blot analysis with antibodies against mouse ISG15 (*top panel*), chicken ovalbumin (*middle panel*), and Listeriolysin O (*bottom panel*). **c** ELISpot analysis of ISG15-specific IFN- γ responses from splenocytes of 8-week-old BALB/c mice that were vaccinated three times i.p. with either Lm-LLO-ISG15 or control Lm. Briefly, splenocytes from each vaccination group were processed seven days after the last vaccination and stimulated with either control CTL epitope peptide or putative ISG15 CTL epitope peptides along with IL-2 in a 96-well plate coated with anti-IFN- γ antibody. After overnight incubation, plates were washed and processed for the detection of IFN- γ -secreting spot-forming cells (SFCs). Results are depicted as mean number IFN- γ -secreting SFCs per 2×10^6 splenocytes and standard deviation after peptide stimulation for each treatment group. **d** Mean number of pups per litter for female mice vaccinated twice with either a control Lm vaccine (2×10^8 CFU) or Lm-LLO-ISG15 (2×10^8 CFU). Three weeks after the last vaccination, female mice from each group were paired with male mice and followed until each female gave birth to at least

one litter. **e** Mean pup weight and standard deviation of 1-day-old littermates from each vaccinated group of females depicted in grams

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Fig 3.

Therapeutic impact on mouse mammary tumors after Lm-LLO-ISG15 vaccination. **a** Tumor load study to determine the effectiveness of Lm-LLO-ISG15 against implanted NT2 mammary tumors. NT2 tumor cells were implanted s.c. in the hind flank of FVB/ NJ mice and subsequently vaccinated with Lm-LLO-ISG15 or control Lm. Tumor size was monitored with calipers until experiment end and tumor volume calculated. Mean tumor volume and standard deviation of each treatment group over the course of the experiment are depicted. **b** Tumor load study to determine the ability of Lm-LLO-ISG15 vaccination to control the growth of implanted primary 4T1-Luc mammary tumors. 4T1-Luc tumor cells were implanted in the mammary tissue of BALB/c mice, and mice were subsequently vaccinated with Lm-LLO-ISG15 or control Lm. Mean tumor volume and standard deviation of each treatment group over the course of the experiment are depicted. **c** Metastatic tumor study to determine the ability of Lm-LLO-ISG15 vaccination to control metastatic spread of 4T1-Luc tumor cells after implantation in the mammary tissue. Briefly, 4T1-Luc tumor cells are implanted into the mammary tissue of BALB/c mice and mice are subsequently vaccinated with Lm-LLO-ISG15 or control Lm. After 32 days of implantation, lungs from vaccinated tumor-bearing mice are removed and perfused with PBS. Lung surface metastatic

nodules were then counted with a light microscope. Mean number of lung metastatic lesions and standard deviation for each treatment group are depicted at experiment end. **d** The FVB/N HER2/neu transgenic mouse model was used to determine whether Lm-LLO-ISG15 vaccination can delay autochthonous mammary tumor progression in comparison with control Lm vaccination. FVB/N HER2/neu transgenic mice were injected six times with either Lm-LLO-ISG15 (2×10^8 CFU) or the control Lm vaccine, Lm-LLO-OVA (2×10^8 CFU), starting at 6 weeks of age and continued every 3 weeks until week 21. Tumor incidence was monitored on a weekly basis. Percent tumor-free survival of each treatment group over the course of the experiment is depicted

Fig 4.

Tumor cell death induced by Lm-LLO-ISG15 results in epitope spreading. **a** ELISpot assay demonstrating epitope spreading to HER2/neu in splenocytes of Lm-LLO-ISG 15-vaccinated FVB/N HER2/neu transgenic mice. Mean number of IFN- γ -secreting SFCs and standard deviation after peptide stimulation for each group is depicted. **b** TIL tetramer analysis demonstrating a significantly higher percentage of HER2/neu-specific CD8⁺ 62L⁻ CD11b⁻ T cells in 4T1-Luc tumors after vaccination with Lm-LLO-ISG15 in comparison with tumors of control Lm-vaccinated mice

Fig 5.

Therapeutic impact of ISG15 vaccination is CD8-dependent. **a** CD8 depletion experiment of 4T1-Luc tumor-bearing mice. Briefly, BALB/c mice were implanted with 4T1-Luc tumor cells and depleted of CD8⁺ cells or mock depleted in addition to vaccination with Lm-LLO-ISG15 or control Lm. Mean tumor volume and standard deviation of each treatment group over the course of the experiment are depicted. **b** Winn assay performed to measure direct cytolytic activity of Lm-LLO-ISG15 CD8-enriched splenocytes. CD4-depleted splenocytes from Lm-LLO-ISG15- or control Lm-vaccinated mice were mixed with 4T1-Luc tumor cells and implanted in naïve BALB/c mice. Mean tumor volume and standard deviation of each group over the course of the experiment are depicted. **c** Graph depicting the percent tumor-free survival of BALB/c mice from the experiment depicted in Fig. 5b