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Combination of a therapeutic cancer vaccine targeting the endogenous retroviral envelope protein ERVMER34-1 with immune-oncology agents facilitates expansion of neoepitope-specific T cells and promotes tumor control

Maria del Mar Maldonado, Maria Gracia-Hernandez, Loc Huu Le, Masafumi Iida, James L Gulley, Renee N Donahue, Claudia Palena, Jeffrey Schlom, Duane H Hamilton

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Center for Immuno-Oncology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA

Correspondence to
Dr Jeffrey Schlom;
schlomj@mail.nih.gov

ABSTRACT

Background Endogenous retroviruses (ERVs) are remnants of retrovirus germline infections that occurred over the course of evolution and constitute between 5% and 8% of the human genome. While ERVs tend to be epigenetically silenced in normal adult human tissues, they are often overexpressed in carcinomas and may represent novel immunotherapeutic targets. This study characterizes the ERV envelope protein ERVMER34-1 as a target for a therapeutic cancer vaccine.

Methods The expression of ERVMER34-1 in multiple healthy adult and cancer tissues was assessed, as was its immunogenicity, to ascertain whether specific T cells could lyse human carcinoma cell lines expressing ERVMER34-1. Furthermore, the ability of a rationally designed ERVMER34-1-targeted therapeutic vaccine to induce tumor clearance in two murine carcinoma models expressing ERVMER34-1 was examined either as a monotherapy or in combination with anti-programmed cell death protein-1/programmed death-ligand 1 monoclonal antibody (mAb) or the interleukin-15 superagonist N-803.

Results The ERVMER34-1 protein was shown to be overexpressed in 232/376 of human carcinomas analyzed while being absent in most healthy adult tissues. High levels of ERVMER34-1 RNA expression associate with decreased survival in uveal melanoma, adenoid cystic, and head and neck carcinomas. ERVMER34-1-specific T cells were detected in peripheral blood mononuclear cells (PBMCs) of patients with cancer but not healthy donors following an overnight stimulation. However, reactive T cells are readily expanded from both healthy donor and patient with cancer PBMCs following a 7-day in vitro stimulation. Furthermore, ERVMER34-1-specific T cells selectively kill human carcinoma cell lines expressing ERVMER34-1. A novel, rationally designed, therapeutic cancer vaccine targeting ERVMER34-1 mediated tumor control in established syngeneic murine tumors expressing the full-length ERVMER34-1 protein. When combined with

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Endogenous retroviral proteins are aberrantly expressed in human cancers and represent a pool of tumor-associated antigens that could be targeted therapeutically.

⇒ ERVMER34-1 is an endogenous retroviral envelope protein overexpressed in human carcinomas.

WHAT THIS STUDY ADDS

⇒ This is the first report characterizing the immunogenicity of ERVMER34-1 in both patients with cancer and healthy donors.

⇒ This is the first study demonstrating that ERVMER34-1-reactive $CD8^+$ T cells can specifically kill target cells expressing the antigen.

⇒ This is the first study of a therapeutic cancer vaccine targeting ERVMER34-1.

⇒ This is the first report observing that a therapeutic vaccine targeting ERVMER34-1 can induce tumor regression in a syngeneic murine model engineered to express the human ERVMER34-1 protein.

⇒ This is the first report to demonstrate that ERVMER34-1 vaccination stimulates the expansion of neoepitope-reactive T cells that, when combined with programmed cell death protein-1/programmed death-ligand 1 blockade, facilitates tumor control, which is further enhanced by the addition of the interleukin-15 superagonist N-803.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study characterizes the ERVMER34-1 protein as an immunotherapeutic target for human carcinomas and provides the preclinical rationale for clinical study of an ERVMER34-1-targeted therapeutic cancer vaccine representing a potential first-in-class immunotherapy for cancer.

checkpoint blockade, the vaccine promoted expansion of neoepitope-reactive T cells whose function was further enhanced when combined with N-803. This expansion of neoepitope-reactive T cells was associated with tumor control.

Conclusions This study reveals the potential of a vaccine that targets the retroviral envelope protein ERVMER34-1 and supports its continued development toward clinical testing as a new class of therapeutic cancer vaccine.

BACKGROUND

Retroviruses require integration into the chromosome of their host to allow for the successful completion of their life cycle. While most of these integrations occur in somatic tissues, in rare instances, they occur in germline cells.[1] This viral endogenization results in the production of gametes harboring the provirus, which can be transmitted vertically to future generations. Although a relatively infrequent event, endogenization of proviral sequences has occurred many times over the course of vertebrate evolution, and it is estimated that 5–8% of the modern human genome is composed of these endogenous retrovirus (ERV)-like sequences.[1–3] However, in the absence of a positive selective pressure, over the course of evolution there is an accumulation of substitutions, deletions, and insertions within the provirus. As a result, within the modern human genome, the majority of ERVs consist of highly fragmented remnants of the original provirus,[1 2 4 5] with only a few having an intact, or nearly complete proviral sequence.[3 6 7] The majority of these intact proviral sequences belong to the ERV-K family of retroviruses, which are thought to be among the most recently endogenized retroviruses.[8] However, it should be noted that despite the presence of these intact proviruses, numerous studies have failed to detect infectious ERV-derived particles in humans.[9 10]

ERVs that encode complete or near-complete open reading frames (ORFs) for viral proteins are often epigenetically silenced, and it is estimated that only 7–30% of ERV sequences in the human genome are transcriptionally active in healthy adult tissues.[11–18] As epigenetic dysregulation is known to be a hallmark of all cancers,[19] it is thought that ERV proteins may be aberrantly expressed by cancer cells.[20] This atypical expression of ERVs in tumors may represent a family of proteins that can be targeted using therapeutic cancer vaccines. In support of this idea, several studies have identified ERV-reactive T cells in patients diagnosed with breast, ovarian, kidney, melanoma, gastrointestinal and hematological cancers.[21–27] Furthermore, vaccines targeting ERVs have demonstrated efficacy in various preclinical models.[28–31] The ERVs targeted in these preclinical studies are often chosen because of high expression in tumors as compared with healthy tissues, without an appreciation of the basal expression levels observed in healthy tissues, which is essential when selecting an immunological target.

In the present work, RNA expression libraries were evaluated to identify ERV transcripts highly expressed in human carcinoma tissues while being expressed at low levels or absent across histologically normal, tumor-adjacent tissues via analysis of published gene expression data sets. Based on this screen, ERVMER34-1 was identified as a potential novel target for a therapeutic cancer vaccine. ERVMER34-1 is the envelope protein for the human endogenous medium-reiteration-frequency-family-34 ORF, which was initially characterized as a protein expressed in normal human placenta and in carcinomas of ovarian, endometrium, colon, head and neck, and lung origin.[32 33]

In this study, we generated a specific monoclonal antibody (mAb) and extensively characterized ERVMER34-1 as a target for a therapeutic cancer vaccine. We demonstrate that (1) ERVMER34-1-reactive T cells are present in the blood of some patients with cancer at a higher precursor frequency than healthy donors; (2) ERVMER34-1-reactive T cells can be efficiently expanded from human peripheral blood mononuclear cells (PBMCs) collected from either patients with cancer or healthy donors; (3) ERVMER34-1-reactive $CD8^+$ T cells can efficiently and selectively lyse human carcinoma cell lines expressing the target antigen; (4) a rationally designed ERVMER34-1 vaccine, devoid of the membrane-trafficking, immunosuppressive and cleavage domains, was able to expand reactive T cells and enhance tumor control in two murine carcinoma models; and (5) the combination of ERVMER34-1 vaccine and programmed death-ligand 1 (PD-L1)/programmed cell death protein-1 (PD-1) blockade promotes enhanced tumor control and associates with the increased expansion of neoepitope-reactive T cells whose antitumor function is increased with the addition of the interleukin (IL)-15 superagonist N-803 (nogapendekin alfa inbakicept; ANKTIVA). These studies thus provide the rationale for the continued development of a therapeutic cancer vaccine targeting the ERVMER34-1 protein in combination with checkpoint blockade and/or other immune-modulating agents toward clinical testing.

METHODS

Cell lines

The C57BL/6-derived colon carcinoma MC38 cell line was obtained and cultured as previously described.[34] The BALB/c-derived breast carcinoma EMT6 cell line was purchased from American Type Culture Collection (ATCC). All human carcinoma cell lines SW620 (colon), SW480 (colon), HCT 116 (colon), MDA-MB-231 (breast), HTB1 (bladder), and K562 (lymphoblast) were purchased from the ATCC and cultured in their recommended media. Cell lines were determined to be *Mycoplasma*-free by using a MycoAlert Mycoplasma Detection Kit (Lonza) and used at low passage number from the date of acquisition. To generate the SW620 ERVMER34-1 CRISPR cell line, cells were co-transfected with a recombinant Cas9 protein version V.2 and a TrueGuide synthetic guide RNA targeting human ERVMER34-1 (ID number CRISPR924929_SGM) using the Lipofectamine

CRISPRMAX transfection reagent (Thermo Fisher). SW620 ERVMER34-1 knockout CRISPR cells were single-cell sorted and ERVMER34-1 negative cells were identified by immunoblot. For the generation of MC38 and EMT6 lines that overexpress ERVMER34-1, the human ERVMER34-1 gene was synthesized (Thermo Fisher) and cloned into the pNGFR expression plasmid,[35] which was kindly provided by Dr Warren S Pear (Perelman School of Medicine at the University of Pennsylvania; Addgene plasmid # 27489). MC38 and EMT6 cells were transfected with pERVMER34-1-NGFR, and single-cell sorted based on surface expression of NGFR using an MA900 cell sorter (Sony Biotechnology). ERVMER34-1 expression was confirmed by western blot.

Generation of ERVMER34-1 antibody

The ERVMER34-1 mAb was used here to assess the expression of the protein in human tumor biopsies via immunohistochemical analysis and in protein lysates via western blot. The antibody was generated against and recognizes the human ERVMER34-1; there is no mouse homologue for this protein. A recombinant His8-tagged human ERVMER34-1 protein spanning amino acids 27–432 was produced via an expression system in SF21 insect cells. BALB/c mice (n=5) were immunized with 100 µg of the purified protein (GenScript), and the resulting hybridomas were screened for reactivity against the recombinant protein via ELISA. Following the selection of 20 clones, a murine IgG1 anti-ERVMER34-1 monoclonal antibody was selected for further studies based on its ability to specifically bind ERVMER34-1 via western blot and detect the protein in tissues via immunohistochemistry. Five clones were selected and five subclones of each were subsequently screened and ranked by their ability to specifically bind ERVMER34-1 via western blot and detect the protein in tissues via immunohistochemistry, with the 14F3 clone being selected for use in these studies.

Mice

Female C57BL/6 and BALB/c mice were obtained from the NCI Frederick Cancer Research Facility. Mice were between 5 and 6 weeks old at the start of experiments and were maintained under pathogen-free conditions in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines, using ARRIVE2 reporting guidelines[36] that are described in the the online supplemental materials. Tumors were induced by implanting 3×10^5 MC38 tumor cells or 1×10^6 MC38 pERVMER34-1 tumor cells subcutaneously into the animal flank. 10^6 EMT6 pERVMER34-1 tumor cells were implanted subcutaneously into the mammary fat pad.

Vaccination and treatments

Human adenovirus type 5, in which the E1/E3 genes have been deleted, was used in this study. Both control adenoviral and a modified ERVMER34-1 sequence (designated ERVMER34-1 vaccine) particles were produced by VectorBiolabs. Mice were injected subcutaneously with either 10^{10} viral particles of control adenovirus, or an ERVMER34-1 vaccine diluted in phosphate-buffered saline (PBS) following tumor establishment in mice at time points indicated in respective figure legends. Anti-PD-L1 (10F.9G2, Bio X Cell, 200 µg) or anti-PD-1 (RecombiMAb anti-mouse PD-1, RMP1-14-CP151, Bio X Cell, 200 µg) was administered intraperitoneally and diluted in PBS. 1 µg of the IL-15 superagonist N-803 (nogapendekin alfa inbakicept; ANKTIVA) was administered subcutaneously and was supplied by ImmunityBio via a Cooperative Research and Development Agreement with the NCI.

Other methods are described in the online supplemental materials and methods.

RESULTS

ERVMER34-1 as a potential therapeutic target

To identify ERVs that could potentially be targeted by a therapeutic cancer vaccine, we assessed RNA expression levels of ERVs annotated in publicly available data sets where we observed no or relatively low ERVMER34-1 expression in tumor-adjacent tissues, compared with higher levels of expression in multiple tumor types (figure 1A). To confirm expression at the protein level, a novel monoclonal antibody was developed and used to investigate the expression of ERVMER34-1 protein across normal tissues and various tumor types using commercially available tissue microarrays. As reported in online supplemental table S1, most normal tissues had no detectable ERVMER34-1 protein expression, except for very low levels detected in isolated cells in the cerebellum, small intestine and colon, breast, bronchus, uterus, kidney and testis. Examples of ERVMER34-1 staining in normal adult tissues are presented in figure 1B.

Unlike in normal tissues, the expression of ERVMER34-1 protein was high in several tumor types, including colon (23/39 cases), head and neck (50/69 cases) and bladder cancer (8/17 cases), lung adenocarcinoma (21/49 cases), lung squamous cell carcinoma (33/50), breast carcinoma (65/93), and endometrial cancer (32/59 cases) (figure 1C and online supplemental tables S2-8). In all cases, ERVMER34-1 expression was restricted to the tumor cells as it was found to co-localize with the tumor marker cytokeratin (figure 1D). Interestingly, while the ERVMER34-1 protein was not detectable in normal lung tissues, expression was sometimes observed in histologically normal lung adjacent to the tumor (online supplemental figure S1A and tables S5 and S6), particularly in those cases where the matched tumor was also positive for ERVMER34-1 (online supplemental tables S5 and S6).

To further investigate this phenomenon, we next assessed ERVMER34-1 expression by immunoblot in protein lysates from tumors, normal adjacent, and healthy tissues. As shown in figure 1E, ERVMER34-1 expression was higher in lung and colon adenocarcinoma samples (tumor) as compared with matched tumor-adjacent

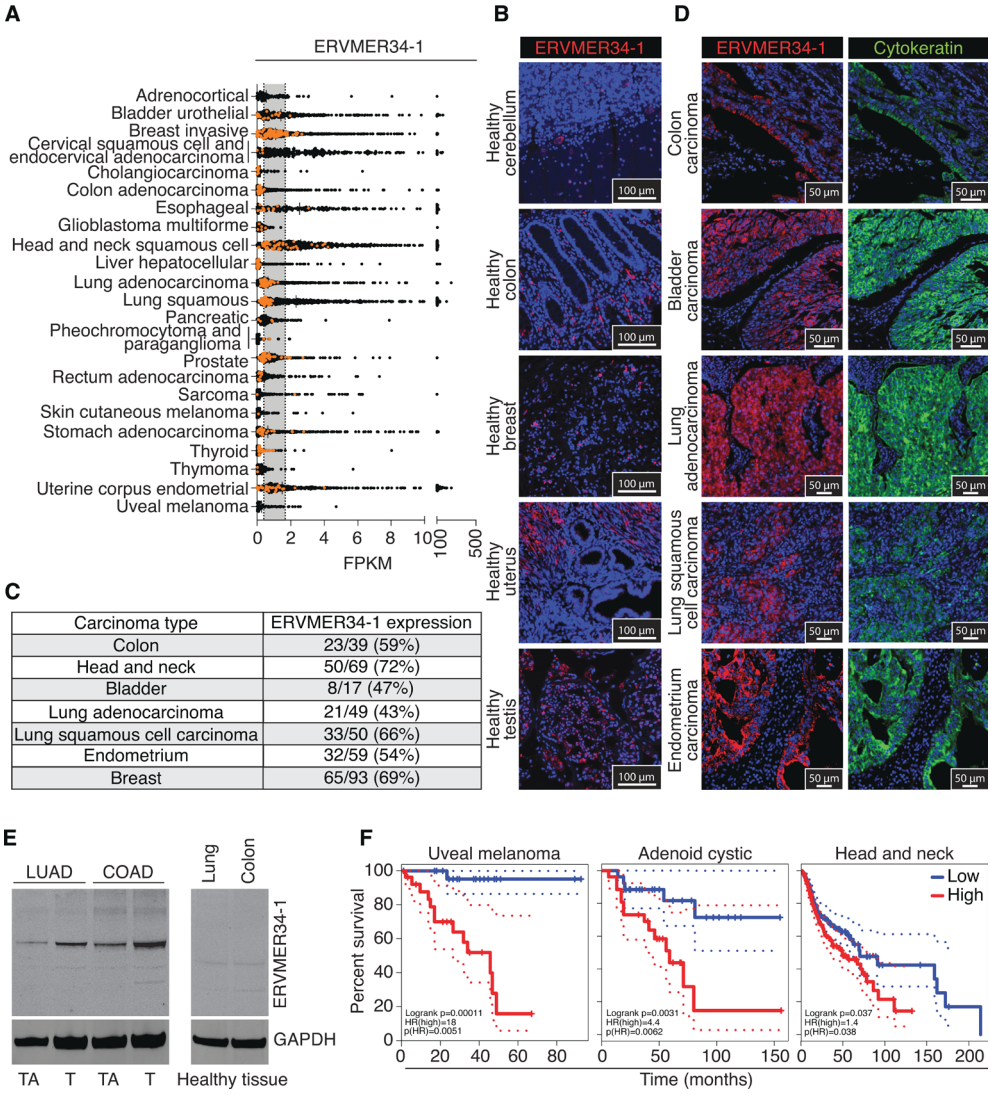

Figure 1 Identification of ERVMER34-1 as a potential therapeutic target. (A) RNA expression levels of ERVMER34-1 in multiple human carcinomas (black dots) along with histologically normal tissues adjacent to the tumor when available (orange dots). The shaded area indicates the median expression of ERVMER34-1 in normal tissues adjacent to the tumor plus 2 SD. The number of samples included in the analysis for each tumor type and corresponding adjacent normal tissues, respectively, were: adrenocortical (79, 0), bladder urothelial (411, 19), breast invasive (1,095, 112), cervical squamous cell and endocervical adenocarcinoma (304, 3), cholangiocarcinoma (35, 9), colon adenocarcinoma (469, 41), esophageal (161, 11), glioblastoma multiforme (155, 5), head and neck squamous cell (500, 45), liver hepatocellular (371, 51), lung adenocarcinoma (525, 59), lung squamous (501, 49), pancreatic (177, 4), pheochromocytoma and paraganglioma (178, 3), prostate (499, 52), rectum adenocarcinoma (166, 10), sarcoma (259, 2), skin cutaneous melanoma (103, 1), stomach adenocarcinoma (375, 32), thyroid (502, 58), thymoma (119, 2), uterine corpus endometrial (547, 35), uveal melanoma (80, 0). (B) Representative images of immunohistochemical (IHC) staining of ERVMER34-1 protein expression (red) in histologically normal human cerebellum, colon, breast, uterus, and testis. Scale bars indicate 100 µm. Blue signal represents DAPI staining. (C) The table shows the number of colon, head and neck, bladder, lung, endometrium, and breast carcinoma tissues demonstrating ERVMER34-1 expression in greater than 10% of tumor cells as assessed by IHC, relative to the total number of tissues evaluated for each tumor type. The percent positive is indicated in parentheses. (D) Representative images of immunohistochemical staining of ERVMER34-1 protein expression (red) in human colon carcinoma, bladder carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, and endometrial carcinoma. Cytokeratin (green) is included as a tumor marker. Scale bars indicate 50 µm. Blue signal represents DAPI staining. (E) Western blot of ERVMER34-1 expression in protein lysates of matched tumor (T) and tumor adjacent (TA) tissues collected from patients diagnosed with either lung (LUAD) n=1 or colon (COAD) adenocarcinomas n=1 (left panel). ERVMER34-1 expression in lung n=1 and colon n=1 protein lysates collected from healthy donors is shown in the right panel. GAPDH is used as a loading control. (F) Survival analysis of patients with cancer was performed using Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) for patients diagnosed with uveal melanoma (n=26 high; n=26 low), head and neck squamous carcinoma (n=170 high; n=169 low) and adenoid cystic carcinoma (n=26 high; n=26 low) based on high (red) and low (blue) ERVMER34-1 RNA expression in tumor tissues. Cut-offs for high and low ERVMER34-1 expression were set to the top 66% and lower 33%, respectively. DAPI, 4',6-diamidino-2-phenylindole; FPKM, Fragments Per Kilobase per Million mapped fragments; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

normal tissues, while lung and colon tissues obtained from healthy donors had no ERVMER34-1 protein expression.

Analysis of data in the Gene Expression Profiling Interactive Analysis database also demonstrated an association between high levels of ERVMER34-1 expression and decreased survival in patients with uveal melanoma, adenoid cystic carcinoma, and head and neck squamous cell carcinoma, suggesting that ERVMER34-1 expression may play a role in tumor progression in some cancer types (figure 1F). Based on these results, the hypothesis was developed that ERVMER34-1 might be a promising immunotherapeutic target.

ERVMER34-1-specific T cells in patients with cancer and healthy donors

To assess ERVMER34-1 immunogenicity in humans, a 15-mer overlapping peptide library was chemically synthesized, spanning the entire ERVMER34-1 protein sequence minus a 170-amino acid sequence (highlighted in online supplemental figure S2A) corresponding to a fragment with high homology with other human proteins, which was removed to increase the specificity of our assays and that of a potential vaccine targeting ERVMER34-1. The resulting peptides in the library are listed in online supplemental table S9. Using this ERVMER34-1 peptide library, immune reactivity in healthy donor and patient with cancer PBMCs was assessed using an overnight (16-hour) interferon (IFN)-γ Enzyme-Linked ImmunoSpot (ELISpot) assay. As shown in figure 2A and online supplemental table S9, there was a significantly higher magnitude of immune responses to ERVMER34-1 in PBMCs collected from patients diagnosed with breast, lung and colon cancer compared with healthy donors. Interestingly, no difference was observed when responses in healthy donors were compared with those observed with PBMCs from patients with prostate cancer, which is in line with the lack of ERVMER34-1 messenger RNA overexpression in prostate cancer. To further assess the immunogenicity of ERVMER34-1 in healthy donors, PBMCs collected from donors expressing a diverse array of HLA-A alleles were either stimulated with each individual ERVMER34-1 peptide for 16 hours in an IFN-γ ELISpot assay, or stimulated for 7 days in vitro in the presence of the pooled peptide library, followed by stimulation with each individual peptide in a 16-hour IFN-γ ELISpot assay (figure 2B). While healthy donors did not demonstrate detectable ERVMER34-1-reactive T cells when evaluated in a 16-hour assay, immune responses could be detected against multiple 15-mer peptides of ERVMER34-1 following a 7-day in vitro stimulation (online supplemental figure S2C) indicative of either a low frequency or naïve status of reactive T cells in healthy donors. Using PBMCs from patients with breast, colon, and lung cancer, we observed a similarly diverse ERVMER34-1 immune response following a 7-day stimulation (online supplemental figure S2D). A selected IFN-γ ELISpot image from PBMCs of a patient with breast cancer is presented in figure 2C.

To directly compare the immunogenicity of ERMVER34-1 to other tumor-associated antigens, PBMCs collected from healthy donors were incubated with overlapping peptide libraries of ERVMER34-1, CEACAM5, MUC1 and PSA using the 7-day in vitro stimulation assay followed by a 16-hour restimulation. Using an intracellular cytokine flow cytometric assay as an immunological readout, we observed the ERVMER34-1 library was more efficient at expanding reactive T cells from the blood of healthy donors than other tumor-associated antigens assayed (online supplemental figure S2E).

Expansion of ERVMER34-1–specific CD8$^+$ cytotoxic T cells

To expand ERVMER34-1-reactive T cells, the HLA-deficient, human chronic myeloid leukemia cell line K562 was modified to generate an artificial antigen-presenting cell line (termed "aAPC"), which was engineered to express the full-length ERVMER34-1 protein (online supplemental figure S3A) along with HLA-A2 and the costimulatory molecule CD80 (online supplemental figure S3B). ERVMER34-1-reactive T cells were expanded from healthy HLA-A2 donors by incubating purified CD8$^+$ T cells with irradiated aAPCs. Following three weekly stimulation cycles, the specificity of the CD8$^+$ T-cell cultures was assessed by an ELISpot assay using individual peptides comprising the 15-mer ERVMER34-1 peptide library. Analysis of T cells expanded from a healthy donor (Donor A) demonstrated primary reactivity against a single, apparently immunodominant, 15-mer peptide, MGSLSNYALLQLTLT (figure 2D). In subsequent studies using 9-mer peptides contained within this 15-mer peptide sequence, YALLQLTLT was identified as the epitope recognized by the ERVMER34-1-reactive T cells (figure 2E). Similarly, several immunodominant peptides were recognized by T cells expanded from a second healthy donor (Donor B), including 15-mer and 9-mer peptide sequences (figure 2F and G).

The ability of the expanded ERVMER34-1-reactive CD8$^+$ T cells to lyse human carcinoma cells was then evaluated using the HLA-A2 positive human SW620 colon carcinoma cell line, which naturally expresses the ERVMER34-1 protein. To define the antigen specificity of the lysis, an SW620 cell line was generated in which the expression of ERVMER34-1 was silenced by using a CRISPR-based strategy (figure 2H). With this SW620 isogenic cell pair, it was demonstrated that ERVMER34-1 CD8$^+$ cytotoxic T cells lysed the parental SW620 cells but failed to lyse SW620 targets silenced for ERVMER34-1 (Donor C; figure 2I). The specific killing of SW620 cells was also demonstrated with ERVMER34-1-reactive CD8$^+$ T cells expanded from three additional healthy donors (online supplemental figure S3C).

We also assessed ERVMER34-1 protein expression in additional HLA-A2 expressing human carcinoma cell lines expressing differing levels of ERVMER34-1 (figure 2J) and subsequently evaluated their susceptibility to lysis by ERVMER34-1-reactive CD8$^+$ T cells expanded from Donor B. As shown in figure 2K, there was a direct

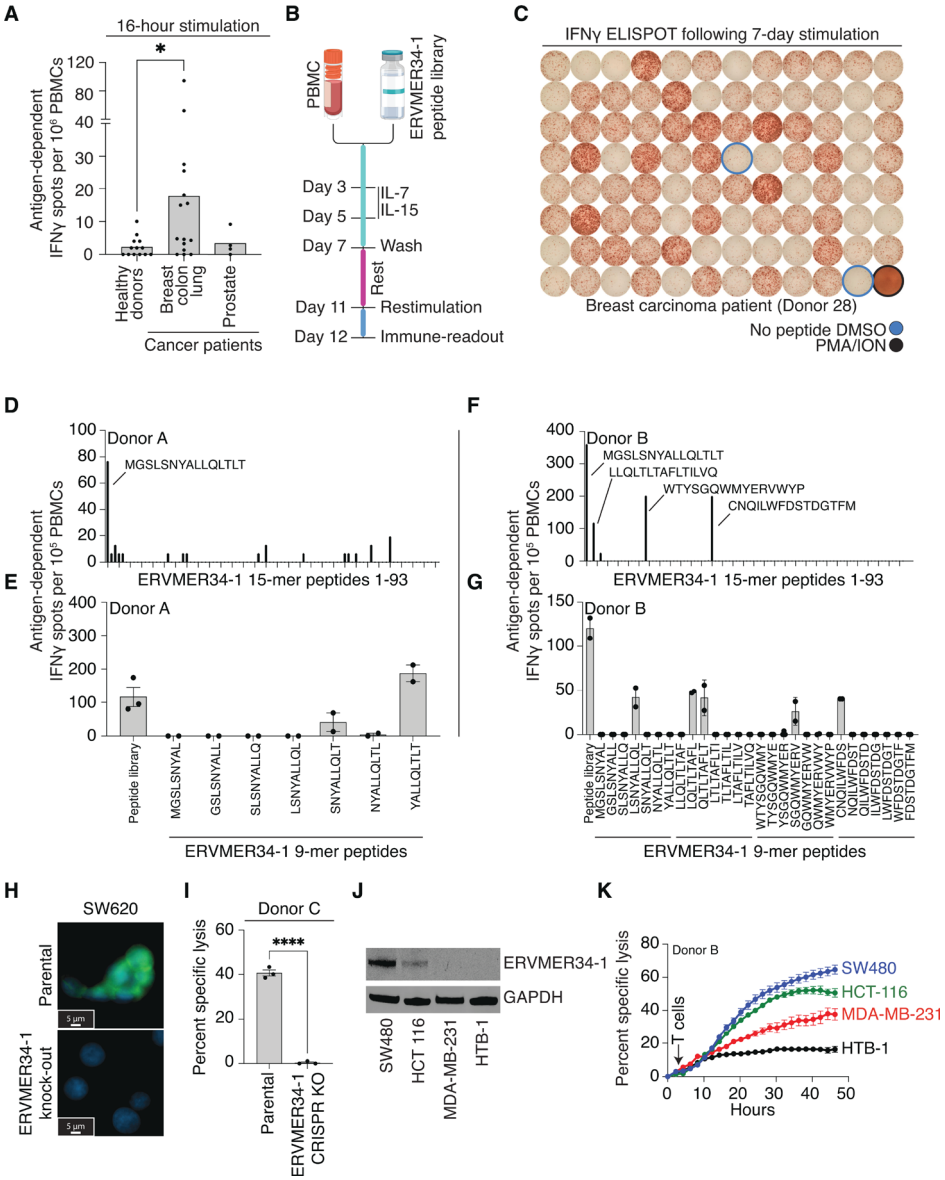

Figure 2 ERVMER34-1-specific immunity can be expanded through in vitro stimulation. (A) Immune reactivity of healthy donor PBMCs (n=13) and patient with cancer PBMCs (patients with breast, n=4; colon, n=4; lung, n=7; and prostate carcinoma, n=4) against the 15-mer ERVMER34-1 peptide library, as assessed using a 16-hour IFN-γ ELISpot assay. *p≤0.05 by an unpaired t-test. (B) Graphical representation of 7-day in vitro expansion of reactive T cells using the ERVMER34-1 peptide library. (C) Representative image of IFN-γ ELISpot plate using PBMCs collected from a patient with breast cancer using the stimulation protocol described in panel B. Each well contains a single 15-mer peptide comprising our ERVMER34-1 peptide library for a total of 93 peptides. Wells outlined in blue are no-peptide DMSO negative control. The well outlined in black is a PMA/ION positive control. Data of responses from an additional 11 donors are presented in online supplemental figure 2B. (D) Mapping the diversity of ERVMER34-1-reactive $CD8^+$ T cells expanded from PBMCs from healthy Donor A using individual 15-mer ERVMER34-1 peptides that comprise our peptide library. (E) Identifying epitopes of ERVMER34-1-reactive $CD8^+$ T cells expanded from healthy Donor A using 9-mer peptides. (F) Mapping the diversity of ERVMER34-1-reactive $CD8^+$ T cells expanded from PBMCs from healthy Donor B using individual 15-mer ERVMER34-1 peptides that comprise our peptide library. (G) Identifying epitopes of ERVMER34-1-reactive $CD8^+$ T cells expanded from healthy Donor B using 9-mer peptides. (H) Immuno-fluorescent analysis of ERVMER34-1 expression (green signal) in either parental SW620 or SW620 ERVMER34-1 CRISPR knock-out cell line. Blue signal represents DAPI staining. Scale bars indicate 5 µm. (I) Percent specific lysis of the parental SW620 and SW620 ERVMER34-1 CRISPR knockout cell lines following overnight incubation with ERVMER34-1-specific $CD8^+$ T cells expanded from PBMCs collected from healthy Donor C and using an effector-to-target ratio of 5:1. ****p≤0.0001 by an unpaired t-test. (J) Western blot of ERVMER34-1 protein expression in multiple human carcinoma cell lines; GAPDH used as a protein loading control. (K) Lysis of indicated human carcinoma cell lines over time using ERVMER34-1-specific $CD8^+$ T cells expanded from healthy Donor B using an effector-to-target ratio of 10:1. DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ELISpot, Enzyme-Linked ImmunoSpot; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; PBMCs, peripheral blood mononuclear cells; PMA/ION, phorbol 12-myristate 13-acetate/ionomycin.

correlation between the level of ERVMER34-1 expression in the tumor cells and the magnitude of lysis mediated by ERVMER34-1-reactive CD8$^+$ T cells. Efficient lysis was observed even in the presence of low levels of ERVMER34-1 protein, as was the case with the MDA-MB-231 cell line (figure 2K).

Generation of a therapeutic cancer vaccine targeting ERVMER34-1

In an effort to target ERVMER34-1, a recombinant adenoviral vector was generated encoding a rationally modified ERVMER34-1 sequence (hereinafter referred to as ERVMER34-1 vaccine). Figure 3A shows the sequence and various domains of the native ERVMER34-1 protein. In creating the ERVMER34-1 vaccine, the membrane trafficking signal peptide (highlighted in blue in the sequence shown in figure 3A) was eliminated to prevent the protein from being expressed on the surface of an infected cell. In addition, a 170-amino acid region characterized by a high degree of homology to other human proteins was removed; this deletion also eliminated the cleavage and immunosuppressive domains of the ERVMER34-1 protein (highlighted in green and pink, respectively, in the sequence shown in figure 3A). The ERVMER34-1 vaccine, with the above sequences removed, was capable of inducing ERVMER34-1 protein expression following the in vitro infection of human dendritic cells (online supplemental figure S4A). As the surface domain of the ERVMER34-1 protein can be shed from the cell surface,[32] an ELISA assay was performed to detect ERVMER34-1 expression in the sera of mice prior to and 3 days following the administration of the ERVMER34-1 vaccine. As predicted, removal of the signal peptide from the ERVMER34-1 sequence contained in the vaccine prevented shedding of ERVMER34-1 in the sera of vaccinated mice (online supplemental figure S4B).

To assess the ability of the vaccine to expand ERVMER34-1-reactive T cells in vivo, non-tumor-bearing BALB/c and C57BL/6 mice were primed on day 0 and boosted on day 7 with either a control adenovirus or the ERVMER34-1 vaccine. ERVMER34-1 immunity was assessed 2 weeks following the second dose of vaccine using a 16-hour IFN-γ ELISpot assay. As shown in figure 3B, vaccination of either BALB/c or C57BL/6 mice with ERVMER34-1 vaccine expanded T cells recognizing a diverse array of individual 15-mer ERVMER34-1 peptides. In performing intracellular cytokine assays, we also observed that the vaccine expanded primarily ERVMER34-1-reactive CD8$^+$ T cells that are multifunctional, producing both IFN-γ and tumor necrosis factor (TNF)-α following overnight exposure to the ERVMER34-1 peptide library (figure 3C and online supplemental figure S4C). Based on these results, subsequent experiments were performed using the ERVMER34-1 vaccine in tumor-bearing mice.

Vaccination against ERVMER34-1 mediates tumor control against MC38 tumors overexpressing ERVMER34-1

To evaluate the ability of the vaccine to mediate antitumor activity in preclinical models in vivo, the murine MC38 colon carcinoma cell line was engineered to express the full-length ERVMER34-1 protein (MC38 pERVMER34-1) (figure 3D). Mice bearing 100 mm^3 MC38 pERVMER34-1 tumors were treated with a single dose of either a control adenovirus or the ERVMER34-1 vaccine. As shown in figure 3E, tumor clearance was observed in 4/9 mice treated with the ERVMER34-1 vaccine as compared with 1/10 mice treated with the control adenovirus, resulting in a significant increase in survival for mice treated with the ERVMER34-1 vaccine (figure 3F).

To evaluate the potential mechanisms involved in the antitumor efficacy of the vaccine, tumors collected 6 days following administration of the vaccine were evaluated for the presence of immune subsets. The ERVMER34-1 vaccine induced an increase in tumor infiltration with CD8$^+$ T cells; these cells were more proliferative (CD8$^+$ Ki-67$^+$) and tended to be of the effector memory phenotype compared with those in mice receiving the control adenovirus (figure 3G). No increase in tumor-infiltrating CD4$^+$ T cells following vaccination was observed (data not shown). A marked expansion of T cells reactive against the ERVMER34-1 pooled peptide library was observed within the tumor microenvironment (TME) in mice that had received the ERVMER34-1 vaccine (figure 3H). When reactivity was assessed using individual 15-mer ERVMER34-1 peptides, we observed that the diversity of the immune response seen within the tumor-infiltrating T cells was similar to that observed in splenocytes (figure 3I). Interestingly, vaccination induced only a trend in the expansion of T cells with reactivity against a pool of neoepitopes known to be expressed in the MC38 tumor model. Furthermore, we did observe a significant expansion of T cells reactive against the murine endogenous retroviral peptide p15e, which is highly expressed in MC38 cells (figure 3J).

Mice that had resolved their tumors following treatment with the ERVMER34-1 vaccine were rechallenged with the parental MC38 cells on their left flank, and the MC38 pERVMER34-1 cells on their right flank. All mice (16/16) were able to control rechallenge with the MC38 pERVMER34-1 cell line demonstrating memory against ERVMER34-1; however, roughly half of the mice (9/16) were protected against challenge with MC38 parental cells, an effect likely caused by MC38 antigen spreading in some of the cured mice (online supplemental figure S4D). Interestingly, we observed an increase in IFN-γ within the TME (figure 3K), which associates with an increased expression of PD-L1 by both tumor cells (CD45 negative cells) and CD11b$^+$ myeloid cells in mice treated with the ERVMER34-1 vaccine (figure 3L).

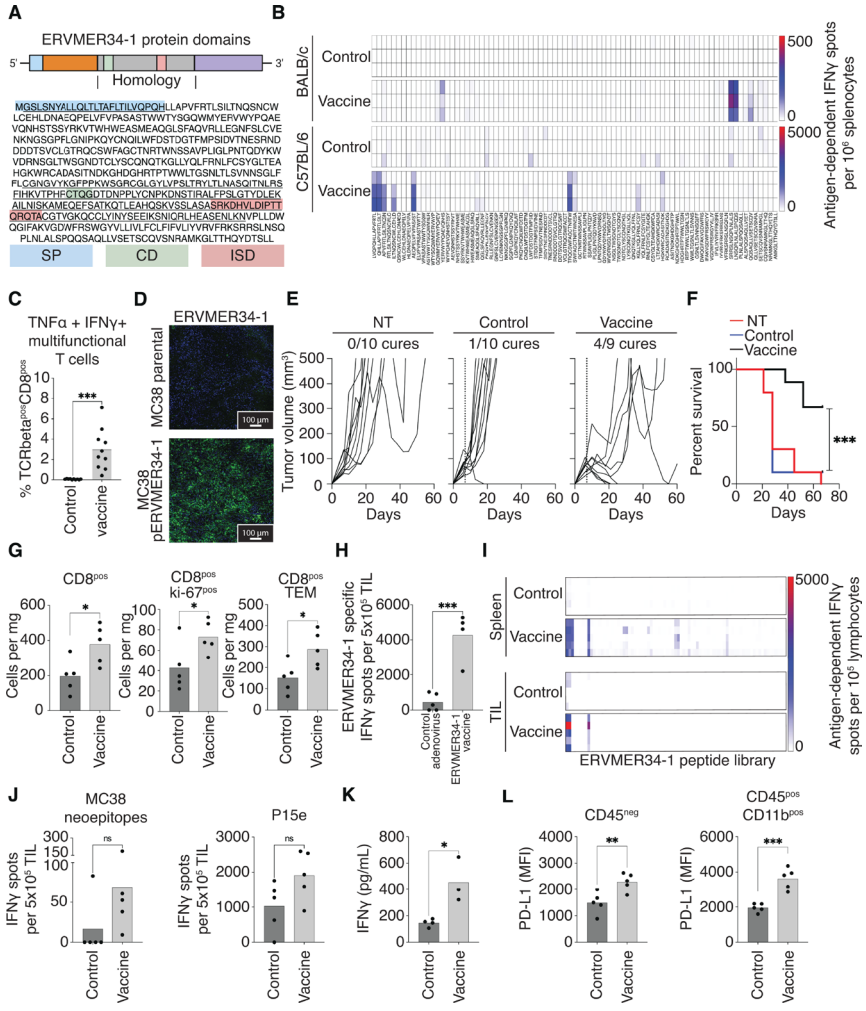

Figure 3 Generation of a therapeutic cancer vaccine targeting ERVMER34-1. (A) Amino acid sequence of ERVMER34-1 with identified protein regions highlighted. The sequences removed from the ERVMER34-1 vaccine are underlined. (B) Enumeration of ERVMER34-1-reactive T cells in splenocytes harvested from non-tumor-bearing BALB/c and C57BL/6 mice vaccinated on days 0 and 7 with either control adenovirus or ERVMER34-1 vaccine using a 16-hour IFN-γ ELISpot assay performed 2 weeks following the last vaccine dose. Each row is an individual animal, and each column is a single peptide comprising the 15-mer peptide library (n=3 mice per group). (C) Enumeration of ERVMER34-1-specific multifunctional T cells in non-tumor-bearing C57BL/6 mice following vaccination with either control adenovirus or the ERVMER34-1 vaccine as assessed by an ex vivo intracellular cytokine production assay. Number indicates the percentage of total TCR_{beta}+ CD8+ T cells secreting both TNF-α and IFN-γ in response to ERVMER34-1 peptides (n=10 mice per group). Mice were treated as described above. Data representative of two independent experiments. (D) Immunofluorescence staining of ERVMER34-1 protein expression (green signal) in MC38 parental and MC38 pERVMER34-1 tumors. Blue signal represents DAPI staining; scale bars indicate 100 µm. (E) Tumor growth curves of MC38 pERVMER34-1 tumors in mice that received either no treatment (NT) or a single dose (10^{10} viral particles, subcutaneously) of either control adenovirus or ERVMER34-1 vaccine. Mice were randomized and treated when tumors reached a size of 50–100 mm^3 (n=9–10 mice per group). Data representative of two independent experiments. (F) Survival curves of mice bearing MC38 pERVMER34-1 tumors that received no treatment or one dose of either control adenovirus or ERVMER34-1 vaccine. (G) Flow cytometry analysis of tumor-infiltrating lymphocytes in mice bearing MC38 ERVMER34-1 tumors treated with control adenovirus or ERVMER34-1 vaccine (n=5 mice per group). Immune readout was performed 6 days post-vaccination. (H) Enumeration of specific ERVMER34-1-reactive cells present in expanded tumor-infiltrating lymphocytes as assessed by an IFN-γ ELISpot assay using the ERVMER34-1 peptide library as a source of antigen. (I) ERVMER34-1 immune reactivity of splenocytes and expanded tumor-infiltrating lymphocytes harvested from mice vaccinated with control adenovirus or ERVMER34-1 vaccine as assessed by an IFN-γ ELISpot assay using individual 15-mer ERVMER34-1 peptides that comprise our library (n=5 mice per group). (J) Comparison of immune reactivity of MC38 neoepitopes, and p15e of tumor-infiltrating T cells in mice bearing MC38 pERVMER34-1 tumors treated with either control adenovirus or ERVMER34-1 vaccine (n=4–5 mice per group). (K) Assessment of IFN-γ present within the tumor microenvironment in mice treated with ERVMER34-1 vaccine (n=3–4 mice per group). (L) Expression of PD-L1 on the surface of $CD45^-$ cells (left panel) and $CD45^+CD11b^+$ cells (right panel) within the MC38 pERVMER34-1 tumors of mice treated with either control adenovirus or ERVMER34-1 vaccine. *p≤0.05; **p≤0.01; ***p≤0.001 by an unpaired t-test. DAPI, 4',6-diamidino-2-phenylindole; ELISpot, Enzyme-Linked ImmunoSpot; IFN, interferon; PD-L1, programmed death-ligand 1; TCR, T-cell receptor; TIL, tumor-infiltrating T cell; TNF, tumor necrosis factor.

Combination of immune-oncology agents with ERVMER34-1 vaccination enhances antitumor immunity by promoting the expansion of neoepitope-reactive T cells

Based on the observed increases in PD-L1 expression both in tumor and myeloid cells from tumors of mice treated with the ERVMER34-1 vaccine, we then assessed the impact of the addition of PD-L1 blockade to ERVMER34-1 vaccination in mice bearing large MC38 pERVMER34-1 tumors. A single dose of ERVMER34-1 vaccine was administered to animals with relatively large (~300 mm^3) tumors, followed by three doses of anti-PD-L1 (figure 4A). While the ERVMER34-1 vaccine alone was unable to control the growth of these large tumors, the addition of PD-L1 blockade was able to induce remarkable tumor control,

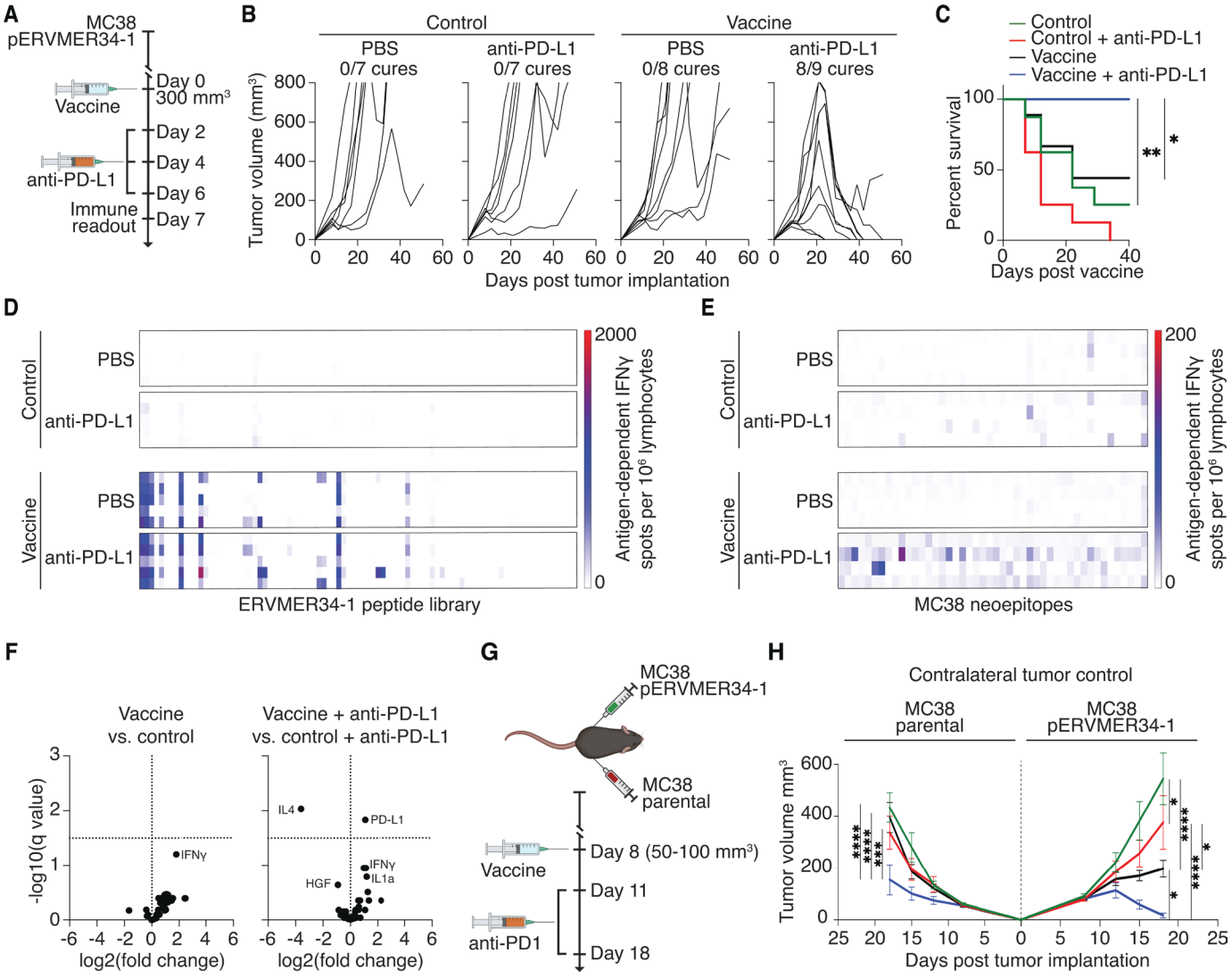

Figure 4 Combination of immune checkpoint blockade with ERVMER34-1 vaccine enhances its antitumor efficacy. (A) Graphical representation of tumor treatment schedule. (B) Tumor growth curves of MC38 pERVMER34-1 tumors treated with either control adenovirus or ERVMER34-1 vaccine with or without the addition of anti-PD-L1 as indicated in panel A (n=9–10 mice per group). Data representative of two independent studies. (C) Survival curves of mice treated as indicated in panel A. (D) Immune reactivity of splenocytes harvested from mice treated with either control adenovirus or ERVMER34-1 vaccine with or without the addition of anti-PD-L1 as indicated in panel A using a 16-hour IFN-γ ELISpot assay with individual 15-mer ERVMER34-1 peptides in the assay (n=5 mice per group). (E) Immune reactivity of splenocytes harvested from mice treated with either control adenovirus or ERVMER34-1 vaccine with or without the addition of anti-PD-L1 as indicated in panel A using a 16-hour IFN-γ ELISpot assay with individual MC38 neoepitope peptides in the assay (n=5 mice per group). (F) Volcano plot of changes in cytokines and chemokines present within the tumor microenvironment in mice treated as indicated in panel A as assessed by the Olink Target 48 Cytokine assay (n=5 mice per group). (G) Graphical representation of tumor treatment schedule. (H) Growth curves of contralateral MC38 and MC38 pERVMER34-1 tumors in mice treated as indicated in panel G. Green line: control adenovirus, red line: control adenovirus+anti-PD-1, black line: ERVMER34-1 vaccine, and blue line: ERVMER34-1 vaccine+anti-PD-1 (n=9 mice per group). Data representative of two independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001 by a two-way analysis of variance followed by a Tukey's multiple comparisons test when comparing ≥3 groups. ELISpot, Enzyme-Linked ImmunoSpot; IFN, interferon; PBS, phosphate-buffered saline; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1.

with tumor clearance observed in 8/9 mice as compared with 0/8 mice treated with the ERVMER34-1 vaccine alone (figure 4B), leading to significantly enhanced survival (figure 4C). Interestingly, the addition of PD-L1 blockade to vaccine did not further enhance the expansion of ERVMER34-1reactive T cells as compared with vaccine alone (figure 4D); rather, it promoted the expansion of T cells reactive against neoepitopes expressed in the MC38 model (figure 4E) that were not observed in mice treated with the ERVMER34-1 vaccine alone. We also observed that while vaccine monotherapy enhanced IFN-γ production in the TME, treatment of these large tumors with the combination of ERVMER34-1 vaccine and PD-L1 blockade associated with increased soluble PD-L1, IFN-γ, and IL-1a in the TME, along with a concordant reduction of IL-4 (figure 4F).

To assess the importance of the expansion of neoepitope-reactive T cells in the antitumor efficacy of the combination therapy, we performed a contralateral tumor study (figure 4G) where we observed that the combination of ERVMER34-1 vaccine with anti-PD-L1 not only induced clearance of MC38 pERVMER34-1 tumors, but also resulted in enhanced control of MC38 tumors, implanted on the opposite flank, that did not express the antigen ERVMER34-1 targeted by the vaccine (figure 4H).

The syngeneic EMT6 breast carcinoma has been shown to be a relatively immunologically cold tumor model.[34] To assess the antitumor efficacy of the potential therapeutic cancer vaccine targeting ERVMER34-1 in this model, we engineered the EMT6 cell line to express the full-length ERVMER34-1 protein (EMT6 pERVMER34-1) (figure 5A). When administered as a monotherapy, the vaccine induced a modest reduction in the rate of tumor growth, while a more robust and significant antitumor response was achieved when the ERVMER34-1 vaccine was combined with PD-L1 blockade (figure 5B and C). Similar to our observations in the MC38 pERVMER34-1 model, the addition of anti-PD-L1 to the treatment regimen did not enhance the magnitude of the ERVMER34-1 immune response as compared with mice treated with ERVMER34-1 vaccine alone (figure 5D). However, we observed that some mice (6/10) treated with the combination of ERVMER34-1 vaccine and PD-L1 blockade had an enhanced expansion of T cells reactive to neoepitopes expressed in the EMT6 model (NXF1 and EIF3B) (figure 5E). This high neoepitope immune response following therapy was associated with improved infiltration of $CD8^+$ T cells into the TME (figure 5F) and a coordinated increase of inflammatory cytokines such as TNF-α, IFN-γ and IL-1a/b within the TME (figure 5G). Furthermore, when the level of tumor control was compared between mice that had high versus low numbers of neoepitope-reactive T cells, tumor control was seen only in mice with high neoepitope immune responses as compared with similarly treated animals with low neoepitope immune responses (figure 5H). Interestingly, we observed a significant inverse correlation between tumor size and the magnitude of the neoepitope immune response only in animals treated with the combination of ERVMER34-1 vaccine plus PD-L1 blockade (figure 5I).

N-803 is an IL-15 superagonist designed to activate natural killer (NK) cells and $CD8^+$ T cells that has recently been approved by the Food and Drug Administration for the treatment of Bacillus Calmette-Guerin (BCG)–unresponsive non-muscle invasive bladder cancer when administered in combination with BCG.[37] We sought to ascertain whether the addition of N-803 to the treatment regimen of ERVMER34-1 vaccine plus checkpoint inhibition could further facilitate tumor control by promoting the expansion and antitumor activity of neoepitope-reactive T cells in mice treated with the ERVMER34-1 vaccine. Using the triplet treatment regimen in the EMT6 model illustrated in figure 5J, we observed 7/14 mice responding to therapy at the time of sacrifice (14 days following the administration of the first dose of vaccine) (figure 5K). We did not observe an enhancement of the ERVMER34-1 peptide immune response in mice responding to therapy (figure 5L); however, we observed a significant enhancement in the number of T cells reactive against neoepitopes expressed by the EMT6 carcinoma cell line in splenocytes collected from responding as compared with non-responding mice receiving the triplet therapy (figure 5M). Furthermore, within the tumor-draining lymph nodes of treated animals, we observed that combining N-803 with the ERVMER34-1 vaccine resulted in an increased presence of CD8+ T cells along with NK cells, but not CD4+ T cells within the tumor-draining lymph node. Interestingly, the triplet combination of N-803+vaccine+PD-1 blockade increased the frequency of lytic NKG2D-expressing NK cells as compared with the vaccine+N-803 doublet (online supplemental figure S5).

DISCUSSION

ERVs constitute a class of immunologically relevant tumor-associated antigens that can potentially be targeted. In this study, we use a therapeutic cancer vaccine targeting the ERVMER34-1 envelope protein; however, there is the potential to use ERVMER34-1 therapeutic antibodies, antibody-drug conjugates or CAR-T cells to specifically target ERVMER34-1 expressed on the surface of carcinoma cells and will be the subject of future studies. At present, to our knowledge, only one ERV-targeted therapy is being assessed clinically, consisting of adoptively transferred T cell-receptor transduced T cells targeting an HLA-A*11:01 restricted epitope of HERV-E in patients with clear cell renal cell carcinoma.[24 38 39] Over the past two decades, several preclinical studies have targeted various ERVs using therapeutic cancer vaccines.[21–31] Most of this preclinical work, however, has focused on the targeting of the ERV-K (HML2) family of retroviruses; while highly expressed in tumors, ERV-K encoded proteins are also overexpressed in multiple normal adult tissues.[40] Because of the potential for these ERV-K-targeted therapies to induce off-tumor toxicities, there has been a hesitancy to develop any ERV-targeted therapies for clinical use.

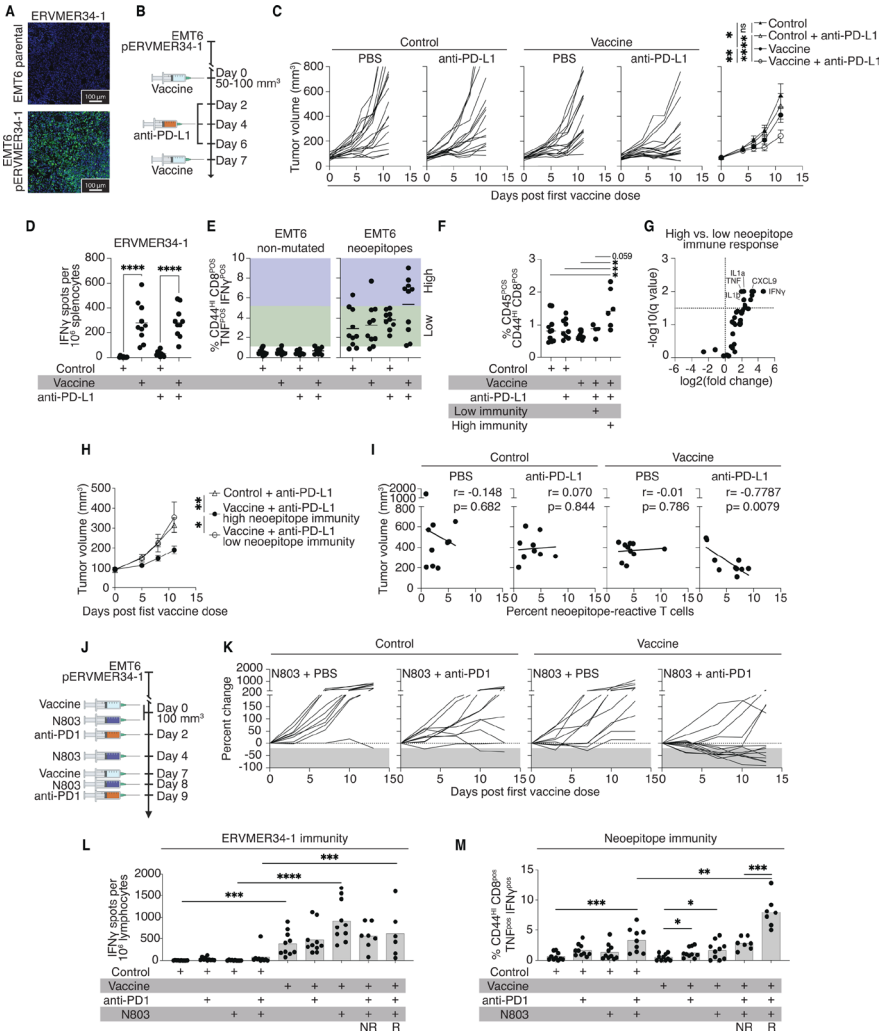

Figure 5 Vaccine targeting ERVMER34-1 in combination with immune-oncology agents enhances tumor control. (A) Immunofluorescence staining of ERVMER34-1 protein expression (green signal) in EMT6 parental and EMT6 pERVMER34-1 tumors. Blue signal represents DAPI staining. Scale bars indicate 100 µm. (B) Treatment scheme for panels C–I with mice being treated with either control adenovirus or the ERVMER34-1 vaccine. (C) Tumor growth curves of mice treated as indicated in panel B (n=16–18 mice per group). Data representative of two independent studies. *p≤0.05; **p≤0.01; ****p≤0.0001 by a two-way ANOVA followed by a Tukey's multiple comparisons test. (D) ERVMER34-1 immune reactivity of splenocytes harvested from mice vaccinated with control adenovirus or ERVMER34-1 vaccine as assessed by a 16-hour IFN-γ ELISpot assay (n=10 mice per group). ****p≤0.0001 by an unpaired t-test. (E) Enumeration of multifunctional T cells in splenocytes specific for a pool of neoepitopes expressed in the EMT6 tumor model (right panel). Control non-mutated neoepitope immune responses are presented in the left panel. (F) Flow cytometry assessment of CD8 effector cells in the tumor of mice treated as indicated. Low and high neoepitope immunity mice are segregated as indicated in panel E by an unpaired t-test. (G) Volcano plot of differences in cytokines and chemokines present within the TME assessed in high versus low neoepitope immune responses by the Olink Target 48 cytokine assay. (H) Tumor growth curves of mice treated with ERVMER34-1 vaccine+anti-PD-L1, which had been identified in panel E as having either a high or low neoepitope immune response as compared with mice treated with control adenovirus+anti-PD-L1 using a two-way ANOVA followed by a Tukey's multiple comparisons, *p≤0.05; **p≤0.01. (I) Correlation analysis of neoepitope-reactive T cells in the EMT6 pERVMER34-1 tumor model with tumor volume of mice treated as indicated in panel B. (J) Treatment scheme for panels K–M with mice being treated with either control adenovirus or the ERVMER34-1 vaccine. (K) Spider plots of changes in tumor volume of EMT6 pERVMER34-1 tumors treated as indicated in panel J (n=10–14 mice per group). Shaded region indicates greater than 20% decrease in tumor size. Data representative of two independent studies. (L) Enumeration of ERVMER34-1-reactive T cells in splenocytes from mice treated as indicated in panel J as assessed by a 16-hour IFN-γ ELISpot assay using the ERVMER34-1 peptide library. Mice that received the triplet regimen that were unresponsive to treatments are labeled as non-responders (NR), whereas those that showed tumor shrinkage greater than 20% are labeled as responders (R). (M) Enumeration of neoepitope reactive T cells in splenocytes from mice treated as indicated in panel J as assessed by intracellular cytokine staining. Mice that received the triplet regimen that were unresponsive to treatments are labeled as NR, whereas those that showed tumor shrinkage greater than 20% are labeled as R. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001 by an unpaired t-test by comparing two groups. ANOVA, analysis of variance; ELISpot, Enzyme-Linked ImmunoSpot; IFN, interferon; PBS, phosphate-buffered saline; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; TNF, tumor necrosis factor.

However, there are thousands of ERVs, each having a unique pattern of expression, that could potentially be targeted using a therapeutic cancer vaccine. Thus, careful selection of an appropriate target is essential for the successful development of immunotherapies targeting ERVs. Herein, we demonstrate the ERV envelope protein ERVMER34-1 to be a promising, tumor-specific novel therapeutic target.

ERVMER34-1 has not been broadly studied, and its function both in healthy and tumor tissues is largely unknown. In fact, only two publications have described the ERVMER34-1 protein, both of which have identified it as being aberrantly expressed in several solid tumor types as assessed by RNA and immunohistochemistry.[32 33] Expanding on those observations, a novel ERVMER34-1 monoclonal antibody was generated for this study, its specificity defined, and used to demonstrate the overexpression of ERVMER34-1 protein in multiple tumor types, while being absent or expressed at low levels in most adult healthy tissues.

Immunogenicity is an important requirement of any potential cancer vaccine target. In this study, ERVMER34-1-reactive T cells were detected in a 16-hour assay in PBMCs collected from patients with cancer, suggesting that patients may naturally be developing an immune response against the ERVMER34-1 protein expressed in tumor cells. Such ex vivo immune responses were not detectable in healthy donors. However, ERVMER34-1-specific T cells could be expanded from PBMCs collected from both healthy donors and patients with cancer following a 7-day in vitro stimulation.

One common criticism of targeting tumor-associated antigens using therapeutic cancer vaccines is the thought that because reactive T cells undergo negative selection during T-cell development, T cells within the periphery tend to be present at low frequencies and be composed of T-cell receptors with low avidity to their target antigen. Here we reported on the expansion of reactive T cells following stimulation with an ERVMER34-1 peptide library using both patients with cancer and healthy donor PBMCs. This expansion of ERVMER34-1-reactive T cells was more efficient than what was observed for other tumor-associated antigens such as CEACAM5, MUC1 and PSA. Furthermore, these T-cell responses were generated in healthy donors with diverse HLA-A alleles with reactivity against a broad array of ERVMER34-1 peptides comprising the peptide library. Importantly, we demonstrated that ERVMER34-1-reactive $CD8^+$ T cells were capable of specifically lysing human carcinoma cell lines that endogenously express differing amounts of the target antigen, but not tumor cells in which the target was silenced.

In designing an appropriate therapeutic cancer vaccine, it is essential to consider the biology of the protein being targeted. For example, when designing a therapeutic cancer vaccine targeting the transcription factor brachyury, researchers removed 25 amino acids involved in DNA binding from the antigen encoded within the vaccine to ensure that the targeted antigen could not function as a transcription factor.[41] In the case of targeting ERV envelope proteins, similar precautions should also be taken. It has been well established that the envelope protein of many retroviruses can inhibit the proliferation of immune cells and possesses immune modulatory activity able to counteract the host's antiviral immune response.[42 43] This modulatory activity has also been observed with some ERV envelope proteins,[43–46] where the expression of ERV envelope protein allows allogenic tumor engraftment in mice.[47] As such, the vaccine described here has been modified to remove these potential immunosuppressive properties; in addition, the signaling and cleavage domains were also removed to prevent shedding of the ERVMER34-1 surface unit.

When used as a monotherapy, the ERVMER34-1 vaccine administered to mice was able to induce the expansion of ERVMER34-1 peptide reactive T cells and the regression of established MC38 pERVMER34-1 tumors. This regression was associated with an influx of $CD8^+$ T cells and the induction of an inflammatory TME as defined by an increase in the presence of IFN-γ and PD-L1 expression, suggesting the vaccine may enhance susceptibility of tumors to PD-1 blockade. We subsequently observed that vaccination did indeed enhance the sensitivity of EMT6 pERVMER34-1 tumors to PD-1/PD-L1 blockade. Furthermore, treatment of large MC38 pERVMER34-1 tumors with the combination of vaccine and PD-L1 blockade promoted robust tumor clearance. In both the EMT6 and MC38 models, this enhanced tumor control observed with the combination of vaccine and anti-PD-1/PD-L1 associates with the expansion of neoepitope-reactive T cells. These neoepitope-reactive T cells appear to be responsible for tumor control in contralateral tumors which did not express ERVMER34-1 and in cases where vaccine alone is unable to mediate tumor regression. The antitumor function of these neoepitope-reactive T cells is further enhanced with the addition of the IL-15 superagonist N-803. Furthermore, in animals responding to combination therapy, there was a demonstrated increase in inflammatory cytokines and chemokines and enhanced infiltration of effector CD8+ T cells within the TME. Cancer-associated fibroblasts, myeloid-derived suppressor cells, tumor-associated macrophages and endothelial cells can all play an active role in suppressing the generation of effective antitumor immunity. Future studies will examine the roles of each of these cell types that are required to promote tumor regression as well as facilitate the expansion of neoepitope-reactive T cells. Additional studies will be necessary to provide more detailed mechanistic insights into how the ERVMER34-1 vaccine modifies the TME and whether it has any effects on tumor angiogenesis or metastasis. Moreover, future studies with other tumor models with a higher degree of heterogeneity are also needed to further strengthen the broader applicability of the ERVMER34-1 vaccine and to further evaluate its safety and efficacy. Numerous published studies demonstrate the benefit of combining vaccine

with both chemotherapy and radiotherapy.[48 49] Although such studies are beyond the scope of this manuscript, we plan to assess this combination in future studies.

The antitumor effects observed in mice to an exogenously expressed antigen demonstrate that the modifications made to the ERVMER34-1 gene within the vaccine can function as an antitumor entity, but do not confirm anti-human tumor immunity. That would require clinical study. The demonstration that patients with carcinomas have higher levels of anti-ERVMER34-1 T-cell responses as compared with healthy volunteers, and that human T cells generated in vitro with reactivity against ERVMER34-1 can lyse human carcinoma cell lines provides the rationale for the analysis of this vaccine in clinical studies, first as a monotherapy, and subsequently in combinations with anti-PD-1 and immune-modulating agents such as N-803.

X James L Gulley @gulleyj1

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Contributors DHH is responsible for the overall content. DHH, JS, CP and RND conceived the study. MdMM, MG-H, LHL, MI and DHH carried out the experiments. JLG was responsible for acquiring clinical samples. DHH, MdMM, MG-H, LHL, MI, CP, RND and JS wrote and/or revised the manuscript. All authors reviewed the manuscript, interpreted data, and approved the final version. JS is the guarantor.

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Competing interests The authors have no competing interests to disclose.

Ethics approval All animal experimental studies were performed under the approval of the NIH Intramural Animal Care and Use Committee. All mice were housed and maintained in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines: NIH AALAC approval: ClO-2 and LTIB-057. This study involves the use of de-identified peripheral blood mononuclear cells that were obtained from healthy human volunteers who provided written informed consent at the NIH Clinical Center Blood Bank (NCT00001846). Peripheral blood mononuclear cells were obtained from carcinoma patients enrolled onto the following clinical studies at the NIH Clinical Center: NCT00179309, NCT01772004, NCT03050814, NCT00088413, NCT02179515, NCT01519817, NCT00923741, NCT00924092, NCT02840994, or NCT00113984. All protocols were approved by the Institutional Review Board of the Center for Cancer Research at the National Institutes of Health, and each study was conducted according to the principles of the Declaration of Helsinki and was performed in compliance with Good Clinical Practice guidelines. Written informed consent was obtained from all donors.

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Data availability statement Data are available upon reasonable request. Values for all data points shown in graphs and values behind the reported means are available upon request with a data transfer agreement.

ORCID iDs
Maria del Mar Maldonado http://orcid.org/0000-0001-6847-2707
Maria Gracia-Hernandez http://orcid.org/0000-0001-9334-8177
Loc Huu Le http://orcid.org/0000-0002-3177-8662
Masafumi Iida http://orcid.org/0000-0003-0095-9767
James L Gulley http://orcid.org/0000-0002-6569-2912
Renee N Donahue http://orcid.org/0000-0002-6828-3073
Claudia Palena http://orcid.org/0000-0002-0445-4486
Jeffrey Schlom http://orcid.org/0000-0001-7932-4072
Duane H Hamilton http://orcid.org/0000-0002-9730-8209

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