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A Human ErbB2-Specific T-Cell Receptor Confers Potent Antitumor Effector Functions in Genetically Engineered Primary Cytotoxic Lymphocytes

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Abstract

The ErbB2 protein is a member of the tyrosine kinase family of growth factor receptors that is overexpressed in cancers of the breast, ovary, stomach, kidney, colon, and lung, and therefore represents an attractive candidate antigen for targeted cancer immunotherapy. Cytotoxic T lymphocytes specific for various immunogenic ErbB2 peptides have been described, but they often exhibit both poor functional avidity and tumor reactivity. In order to generate potent $CD8^+$ T cells with specificity for the $ErbB2_{369–377}$ peptide, we performed one round of *in vitro* peptide stimulation of $CD8^+$ T cells isolated from an HLA-$A2^+$ patient who was previously vaccinated with autologous dendritic cells pulsed with HLA class I ErbB2 peptides. Using this approach, we enriched highly avid ErbB2-reactive T cells with strong ErbB2-specific, antitumor effector functions. We then stimulated these ErbB2-reactive T cells with $ErbB2^+$ HLA-$A2^+$ tumor cells *in vitro* and sorted tumor-activated $ErbB2_{369–377}$ peptide T cells, which allowed for the isolation of a novel T-cell receptor (TCR) with $ErbB2_{369–377}$ peptide specificity. Primary human $CD8^+$ T cells genetically modified to express this ErbB2-specific TCR specifically bound $ErbB2_{369–377}$ peptide containing HLA-A2 tetramers, and efficiently recognized target cells pulsed with low nanomolar concentrations of $ErbB2_{369–377}$ peptide as well as nonpulsed $ErbB2^+$ HLA-$A2^+$ tumor cell lines *in vitro*. In a novel xenograft model, ErbB2-redirected T cells also significantly delayed progression of $ErbB2^+$ HLA-$A2^+$ human tumor *in vivo*. Together, these results support the notion that redirection of normal T-cell specificity by TCR gene transfer can have potential applications in the adoptive immunotherapy of ErbB2-expressing malignancies.

Introduction

The *ERBB2* (HER-2/NEU) PROTO-ONCOGENE encodes a member of a group of epithelial tyrosine kinase receptors involved in the initiation and progression of diverse malignancies, including breast, ovarian, and gastric cancers (Wong *et al.*, 1995; Engel and Kaklamani, 2007). *ErbB2* gene amplification and overexpression leads to uncontrolled cell growth and survival, increased colony formation (Bartsch *et al.*, 2007), and impaired DNA repair (Pietras *et al.*, 1994). Several different immunotherapeutic approaches directed against ErbB2-expressing breast and ovarian tumors have been developed to date. Anti-ErbB2 antibody-based immunotherapies, such as the monoclonal antibody trastuzumab, can treat breast cancer patients with ErbB2 overexpression, but this approach has not been as efficacious in ovarian cancer patients (Bookman *et al.*, 2003). Additionally, cancer vaccines have been utilized to induce specific antitumor immunity, but produced only weak T-cell responses and did not induce objective tumor regression (Knutson *et al.*, 2002; Disis *et al.*, 2004; Peoples *et al.*, 2005).

T-cell receptor (TCR) gene transfer has been developed over the last decade as a reliable method to generate large numbers of T cells of a given antigen specificity for adoptive cellular therapy of viral infectious diseases, virus-associated malignancies, and cancer (Engels and Uckert, 2007). The

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clinical feasibility of TCR gene therapy was first demonstrated in melanoma using a TCR specific for MART1, a commonly expressed melanoma antigen (Morgan *et al.*, 2006). Adoptive transfer of MART1 TCR-transduced $CD8^+$ T cells in 15 patients resulted in durable engraftment of the transferred population and significant tumor regression in two patients, demonstrating a proof of concept of adoptive T-cell transfer (Morgan *et al.*, 2006). We later identified a higher affinity MART-1-specific TCR that conferred improved functional avidity and clinical efficacy in melanoma, although with greater incidence of vitiligo, uveitis, and hearing loss resulting from collateral destruction of normal melanocytes (Johnson *et al.*, 2006, 2009). ErbB2-directed TCR gene therapy would appear to hold significant promise for common epithelial cancers; however, isolation of highly avid ErbB2-specific TCRs directly from cancer patients has been challenging and not clinically tested.

One promising strategy to generate ErbB2-specific T cells relies on vaccination of patients bearing $ErbB2^+$ tumors with powerful immune regimens that can overcome immunological ErbB2 self-tolerance and prime preexisting T-cell immunity. We previously demonstrated that administration of an autologous, matured dendritic cell (DC) vaccine pulsed with ErbB2-derived HLA class I and II peptides to $HLA\text{-}A2^+$ patients with $ErbB2^+$ breast tumors efficiently primed ErbB2-specific T cells, increased their frequency, and resulted in measurable tumor regression in some patients in an ErbB2/DC vaccine study (Czerniecki *et al.*, 2007). In this report, we characterize a novel ErbB2-specific TCR that was isolated from $ErbB2_{369–377}$-specific $CD8^+$ T cells expanded from an $HLA\text{-}A2^+$ patient who was previously vaccinated on the ErbB2/DC study. The isolated TCR conferred transduced $CD8^+$ T cells with high specificity and avidity for the HLA-A2-restricted $ErbB2_{369–377}$ epitope, demonstrating reactivity against peptide-loaded targets and tumor cells expressing endogenous antigen.

Materials and Methods

Cells

Retroviral packaging was performed in immortalized normal fetal renal 293GP cells kindly provided by Dr. Paul Robbins (Center of Cancer Research, National Cancer Institute, Bethesda, MD). Human cell lines used in immune-based assays include the ovarian cancer cell lines SKOV3, OVCAR3, OVCAR-2, and OV55-2; the human breast cancer cell line MDA231; the human melanoma cell lines 624 and 938 (Marincola *et al.*, 1994; Rivoltini *et al.*, 1995); the human T-cell lymphoblastic lymphoma cell line SupT1; and the T2 lymphoblastoid cell line. Cell lines were maintained in RPMI-1640 (Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 2 m*M* L-glutamine, 100 μg/ml penicillin, and 100 U/ml streptomycin. All cell lines were routinely tested for mycoplasma contamination.

Preparation of ErbB2 peptide-loaded monocyte-derived DCs

All patients underwent pretreatment leukapheresis on a Baxter CS3000 using monocyte enrichment settings in the Apheresis Unit at the Hospital of the University of Pennsylvania under an Institutional Review Board (IRB)–approved protocol (Czerniecki *et al.*, 2007). Patient peripheral blood monocytes were enriched from the leukapheresis product by elutriation. Monocytes were washed, counted, and cultured at 3×10^6/ml in sterile 24-well plates in RPMI medium supplemented with 10% FBS, 500 IU/ml of recombinant research-grade human granulocyte-macrophage colony stimulating factor (GM-CSF), and 250 IU/ml of interleukin-4 (IL-4) for 4 days. On day 5, about 1,000 units/ml of IFN-γ was added in the culture followed by overnight incubation at 37°C. On day 6, lipopolysaccharide (LPS) was added at 10 ng/ml for 6 hr to complete maturation of the DCs. The dendritic cells' DC1 phenotype was analyzed by flow cytometry using monoclonal antibodies against CD80, CD86, CD83, and CD40. The dendritic cells matured to the DC1 phenotype were subsequently pulsed with HLA-A2 class I-binding $ErbB2_{369–377}$-specific peptide at 10 μg/ml, for 2 hr as described previously (Czerniecki *et al.*, 2007). DCs were harvested 2 hr later, washed, counted, and assessed for viability before coculture with $CD8^+$ T cells.

In vitro *$CD8^+$ T-cell priming with ErbB2 peptide-pulsed DCs (DC1)*

For collection of vaccine-primed T cells, patients underwent posttreatment leukapheresis on a Baxter CS3000 under an IRB-approved protocol for human subjects research 2 weeks after last vaccine dose (Czerniecki *et al.*, 2007). Autologous ErbB2 peptide-loaded DCs were cocultured with column-purified postvaccination $CD8^+$ T cells at a ratio of 10:1 in 48-well plates. IL-2 (50 IU/ml) was added to the cultures on day 2. After 10 days of sensitization, the $CD8^+$ T cells were harvested and restimulated with T2 cells pulsed with either relevant or irrelevant peptides or tumor cell lines. Supernatants were harvested after 24 hr and analyzed by enzyme-linked immunosorbent assay (ELISA).

Cytokine release assays

Cytokine release assays were carried out by coculture of 1×10^5 T cells with 1×10^5 tumor cells or peptide-loaded T2 cells per well in triplicate in 96-well round-bottom plates in 200 μl complete medium. For the preparation of peptide-loaded T2 antigen presenting cells (APCs), the latter were resuspended at 1×10^7/ml and loaded with ErbB2 or MART1 peptides at various peptide concentrations (1 ng/ml to 10 μg/ml) at 37°C for 2 hr. T2 cells were then washed twice with phosphate buffered saline (PBS) and resuspended at 1×10^6/ml with RPMI-1640 supplemented with 10% heat-inactivated FBS. After 20–24 hr, cell-free supernatants were assayed for the presence of IFN-γ using the BioLegend ELISA MAX Deluxe kit.

Construction of retroviral vectors

To identify the sequences of the TCR genes, a 5′-RACE-PCR (Kit) amplifying the variable regions of the TCRα and TCRβ chains including CDR3 was performed with RNA isolated from the T-cell clones. RACE-PCR products were sequenced. TCRα and TCRβ chains were linked by 2A peptide linker (TCRb-P2A-TCRa), and the complete constructs were cloned into the retroviral vector plasmid pMSGV1 vector backbone, a derivative of the vector pMSGV (murine stem cell virus [MSCV]-based splice-gag vector) that utilizes an MSCV long terminal repeat (LTR) (Cohen *et al.*, 2005).

Recombinant retrovirus production

Replication-defective retroviral vectors were produced as previously described (Wargo *et al.*, 2009). Briefly, 1×10^6 of 293-GP cells (transient viral producer cells) in a 6-well plate were cotransfected with 1.5 μg of retroviral vector DNA from each of the constructs and 0.5 μg of envelope DNA (RD114) using the Lipofectamine 2000 reagent (Invitrogen) and Optimem medium (BD Biosciences). The medium was changed to Dulbecco's modified Eagle's medium with 10% FBS after 18 hr, and viral supernatants were harvested at the 48-hr time point.

Human T-cell transduction

Primary human $CD8^+$ T cells were purchased from the Human Immunology Core at University of Pennsylvania and were isolated from healthy volunteer donors following leukapheresis by negative selection. All specimens were collected under a University IRB–approved protocol, and written informed consent was obtained from each donor. T cells were plated at 1×10^6/ml in 24-well plates (Costar) in the complete medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 10 m*M* HEPES) and stimulated with beads coated with anti-CD3 and anti-CD28-mAbs as described by the manufacturer (Invitrogen) (Levine *et al.*, 1997) for 18–24 hr before transduction. For retroviral transduction, non–tissue culture-treated 12-well plates (Becton Dickinson Labware) were treated with 25 μg/ml of recombinant retronectin at 4°C as directed by the manufacturer (RetroNectin; Takara). After an overnight incubation, the retronectin was removed and wells were blocked with 2% bovine serum albumin in PBS at room temperature for 30 min. The retroviral vector supernatant (2–3 ml) was then applied by centrifugation ($2000\times g$ for 2 hr) and removed by aspiration. About 5×10^5 of stimulated T cells were added to each well in a final volume of 1 ml RMPI growth medium. Plates were centrifuged for 10 min at $1000\times g$ and incubated overnight. The transduction process was repeated the following day. After transduction, the cells were grown in RPMI with 10% FBS, and human recombinant interleukin-2 (IL-2) (Novartis) was added every other day to 100 IU/ml final concentration. Cell density of $0.5–1\times10^6$ cells/ml was maintained.

Flow cytometry

To determine T-cell antigen specificity, $CD8^+$ T cells were stained with anti-CD8-FITC and APC-labeled $ErbB2_{369–377}$ or $MART1_{27–35}$ tetramer (Becton Dickinson). To assess T-cell activation phenotype, T cells were stained with the above reagents plus a PerCPCy5.5-labeled anti-human CD69 mAb. DC phenotype was assessed using CD14-PerCPCy5.5, CD11c-APC, HLA-DR-PE, CD80-FITC, CD86-FITC, CD83-FITC, and CD40-FITC. All antibodies were purchased from BD Biosciences.

Real-time PCR

Real-time PCR (RT-PCR) was used to analyze the expression of human TAP1, TAP2, tapasin, and LMP2 (antigen processing machinery [APM] components) in tumor cell lines. RNA was first isolated from tumor cells using the RNA easy kit (Qiagen). cDNA was then generated from 1 μg of RNA using First Strand Ready-To-Go beads (GE Healthcare). RT-PCR was then performed in triplicates using Applied Biosystem's TaqMan primers specific for TAP1, TAP2, tapasin, LMP2, and β-actin. mRNA levels were normalized to β-actin and compared with mRNA levels of APM-deficient T2 cells. Data are presented as fold mRNA level.

Xenograft model of breast cancer

All animals were obtained from the Stem Cell and Xenograft Core of the Abramson Cancer Center, University of Pennsylvania. Mice were bred, treated, and maintained under pathogen-free conditions in-house under University of Pennsylvania's Institutional Animal Care and Use Committee–approved protocols. For *in vivo* T-cell functional assessment, 6–12-week-old female NSG mice were subcutaneously injected on the flank with 1×10^6 MDA231 cells previously mixed with 1×10^6 ErbB2-specific T cells in 0.2 ml PBS. Control mice were injected with MDA231 tumor cells mixed with 1×10^6 MART1-specific T cells. Each group consisted of five mice. Tumor growth was determined by caliper measurement over time and tumor volumes calculated using the formula $V=½$ (length$\times$ width^2), where length is the greatest longitudinal diameter and width is the greatest transverse diameter. Mice were terminated after 40 days or earlier if they became distressed and moribund. Following termination, tumors were resected, photographed, and weighted.

Statistical analysis

GraphPad Prism 4.0 (GraphPad Software) was used for statistical analysis.

Results

Induction of ErbB2-specific $CD8^+$ T cells with ErbB2 peptide-loaded DCs

Peripheral blood monocytes and peripheral blood T cells were obtained from an HLA-$A2^+$ patient (M10) who had previously been vaccinated with autologous DCs pulsed with a cocktail of HLA class I and class II peptides, including the HLA class I-restricted $ErbB2_{369–377}$ peptide (Czerniecki *et al.*, 2007). This patient's postvaccination $CD8^+$ T cells demonstrated a robust IFN-γ response (>350 ng/ml) against autologous DCs pulsed with $ErbB2_{369–377}$ peptide following one round of *in vitro* stimulation. Furthermore, postvaccination $CD8^+$ T cells secreted 73-fold higher IFN-γ levels in response to the HLA-$A2^+$/$ErbB2^+$ breast cancer cell line MDA231 compared with IFN-γ secreted against control cell lines (Czerniecki *et al.*, 2007; Koski *et al.*, 2012). Of note, the patient's prevaccination $CD8^+$ T cells showed low levels of IFN-γ production in response to either target, establishing evidence of a strong, vaccine-induced anti-ErbB2 response.

The patient's peripheral blood monocytes were matured into DCs utilizing an *in vitro* protocol and showed relatively high expression levels of CD80, CD86, CD83, and CD40 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hum). The matured DCs were then pulsed with $ErbB2_{369–377}$ peptide and used for the *in vitro* stimulation of $CD8^+$ T cells purified from the patient's postvaccination peripheral blood. Following 7 days of *in vitro* stimulation, nearly 3% of the viable $CD8^+$ T cell population

recognized the stimulating ErbB2$_{369–377}$ peptide as assessed by binding of an HLA-A2/ErbB2$_{369–377}$ tetramer (Fig. 1). This represented a 17-fold increase over 1 week, relative to the starting percentage of ErbB2-specific T cells observed in the blood of the postvaccinated patient. ErbB2-specific T cells did not bind to MART-1$_{26–35}$ tetramer complexes, demonstrating their specificity for ErbB2$_{369–377}$ peptide. In contrast, MART-1 TCR-transduced T cells did not bind to the ErbB2$_{369–377}$ tetramer complex, but exhibited strong binding to MART-1$_{26–35}$ tetramer complexes (Fig. 1). Collectively, ErbB2 peptide-loaded DCs were capable of boosting the frequency of ErbB2$_{369–377}$ peptide-specific T cells.

ErbB2-specific CD8$^+$ T cells exert potent effector functions against ErbB2 peptide-loaded target cells and ErbB2-expressing cancer cells

To evaluate their effector functions, ErbB2-specific T cells were initially exposed to HLA-A2$^+$ T2 cells preloaded with the ErbB2$_{369–377}$ peptide. ErbB2-specific T cells displayed high peptide-specific IFN-γ production upon coculture with antigen presenting cells (T2 cells) loaded with the relevant ErbB2 peptide. As expected, no IFN-γ was produced upon exposure to T2 cells pulsed with the irrelevant MART-1$_{26–35}$ peptide. As a positive control for functionality, MART-1-specific T cells recognized and reacted against MART-1$_{26–35}$ peptide-loaded T2 cells (Fig. 2A).

We further evaluated the functional avidity of these T cells by analyzing the production of IFN-γ in response to incubation with T2 target cells pulsed with titered amounts of the ErbB2$_{369–377}$ peptide. ErbB2$_{369–377}$-specific T cells exerted high functional avidity, as they were capable of secreting high amounts of IFN-γ even at low concentrations (1 n*M*) of the specific peptide (Fig. 2B). We therefore investigated if the ErbB2-specific T cells were able to recognize endogenously processed and presented ErbB2$_{369–377}$ peptide. Coculture assays were performed utilizing ErbB2-specific T cells with HLA-A2-matched or HLA-A2-mismatched ovarian, breast, and melanoma cancer cells that express different levels of the ErbB2 protein (Lanitis *et al.*, 2012). ErbB2$_{369–377}$-specific CD8$^+$ T cells specifically recognized and secreted IFN-γ upon interaction with ErbB2$^+$ HLA-A2$^+$ ovarian or breast cancer cells, while no recognition of HLA-A2$^-$ or ErbB2$^-$ tumors was observed (Fig. 2C). There was no correlation between the intensity of ErbB2 surface expression by tumor cell lines and the IFN-γ secretion by T cells (data not shown). To this end, we investigated the expression of various components of APM by tumor cells, including TAP1, TAP2, tapasin, and LMP2, via RT-PCR to determine if deficiencies existed in the peptide-processing pathway of these tumor cells. ErbB2$^+$ tumor cell lines that were recognized to a lesser extent by the ErbB2$_{369–377}$-specific T cells (SKOV-3 and OVCAR-3) (Fig. 2C) displayed a reduced mRNA expression of tested APM molecules (Fig. 2D). Tumor cell lines that were well recognized by the ErbB2$_{369–377}$-specific T cells (OVCAR-2, OV55-2, and MDA231) (Fig. 2C) displayed a higher level of expression in most of the APM molecules investigated (Fig. 2D). Therefore, lack of recognition of some ovarian tumors by ErbB2$_{369–377}$-specific T cells may be attributed, in part, to a lack of necessary APM components in the tumor cells, as observed elsewhere (Han *et al.*, 2008). This observation highlights that both ErbB2 and HLA-A2 molecules are required, but not sufficient, for optimal immune recognition. Together, we conclude that vaccine-primed ErbB2$_{369–377}$-specific T cells exert potent effector

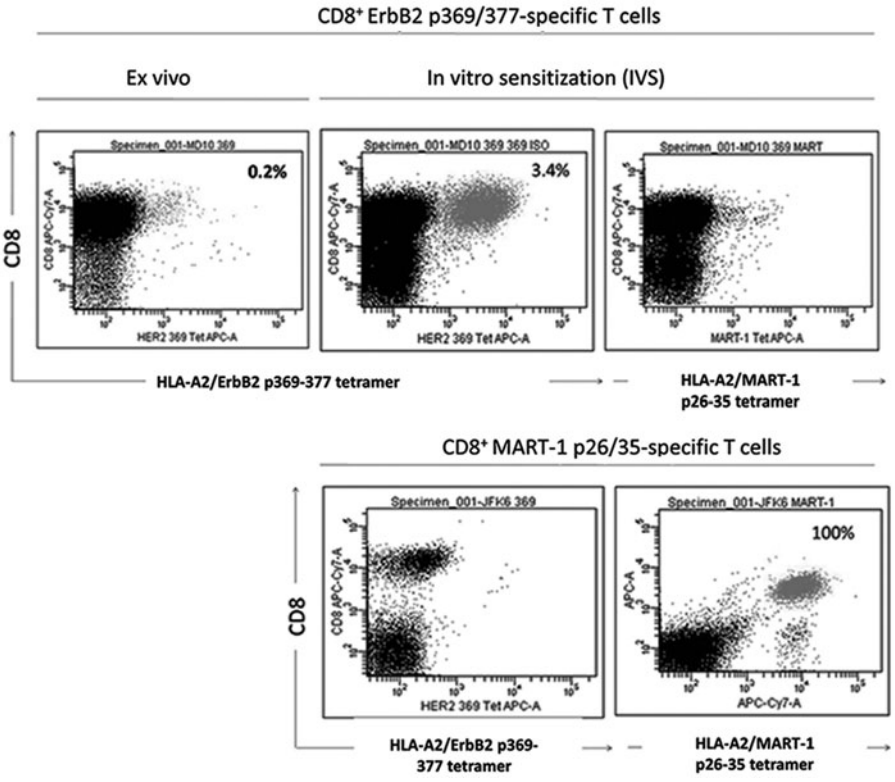

FIG. 1. ErbB2-pulsed DC1 increase the frequency of ErbB2-directed T cells. CD8$^+$ T cells were purified from a patient with ductal carcinoma *in situ* (DCIS) post-administration of the ErbB2-pulsed-DC1 vaccine and cocultured for 7 days with ErbB2$_{369–377}$ peptide-pulsed autologous dendritic cells. After 1 week, CD8$^+$ T cells were harvested and analyzed via flow cytometry with labeled tetramer bound to ErbB2$_{369–377}$ or MART1$_{26–35}$. MART1 T cells served as negative control effector cells. The percentage of positive cells for CD8 and ErbB2 are indicated on the dot plot.

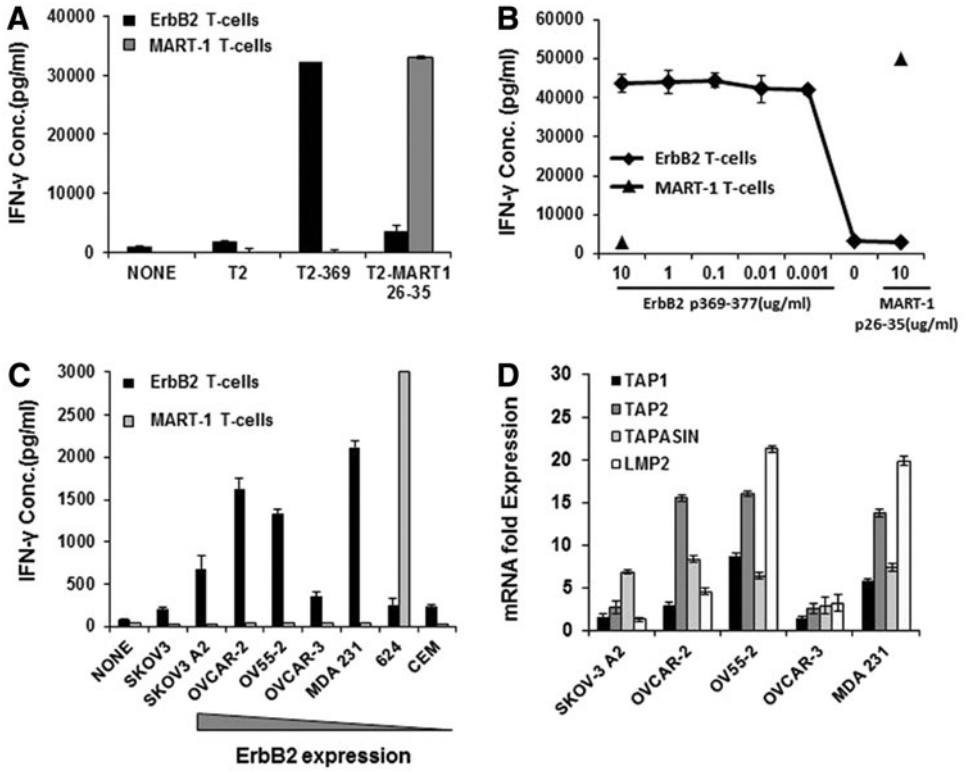

FIG. 2. ErbB2$_{369–377}$-specific T cells strongly recognize peptide-pulsed T2 cells and differentially recognize HLA-A2-restricted ErbB2-expressing tumor cells. **(A)** IFN-γ production of ErbB2$_{369–377}$-specific T cells in response to peptide-pulsed targets. ErbB2- or MART1-specific T cells were cocultured with T2 cells loaded with HLA-A2-restricted ErbB2$_{369–377}$ or MART1$_{26–35}$ peptide for 18 hr. **(B)** ErbB2$_{369–377}$-specific T cells exhibit high avidity against the relevant peptide. ErbB2$_{369–377}$-specific T cells were incubated for 18 hr with T2 cells pulsed with a range of concentrations of ErbB2$_{369–377}$ peptide or 10 ug/mL control (MART-1) peptide. MART1 T cells served as negative control effector T cells. **(C)** ErbB2 or MART1-specific T cells were cultured alone (none) or stimulated overnight with human HLA-A2-restricted ErbB2$^+$-established cancer cell lines. SKOV-3 (HLA-A2$^-$ ErbB2$^+$) and CEM (HLA-A2$^-$ ErbB2$^-$) served as negative control tumor targets. **(D)** APM expression of HLA-A2-restricted ErbB2-expressing tumor cell lines. The mRNA levels of human TAP1, TAP2, TAPASIN, and TAP2 were quantified by real-time PCR. mRNA levels are expressed as fold increase over the APM-negative T2 cell line. β-Actin was used as an endogenous gene control. Results depict the mean ± SD of triplicate wells. For all assays, IFN-γ was quantified from cell-free supernatants by ELISA and is reported as the mean concentration (pg/ml) ± SEM of duplicate wells. APM, antigen processing machinery; ELISA, enzyme-linked immunosorbent assay.

Table 1. T Cell Receptor α and β DNA Constructs

TCR α and β chain sequencing results[a]				*TCR α/β retroviral constructs*[b]	
TRAV	*Number of clones*	*TRBV*	*Number of clones*	*Construct number*	*TCR construct*
AV1-1	1	BV2(22s1)	1	1	AV12-1 BV3-1(CB1)
AV1-2	1	BV3-1(9S1)	2,3	2	AV3 BV3-1(CB-1)
AV2	1	BV4-1(7S1)	1	3	AV12-2a BV3-1(CB-1)
AV3	2	BV4-3(7S2)	1	4	AV12-2b BV3-1(CB-1)
AV10	1	BV5-1(5S1)	1	5	AV12-2a BV3-1(CB-2)
AV12-1	2,1	BV5-4(5S6)	1	6	AV12-2b BV3-1(CB-2)
AV12-2	1,1,1,1	BV5-6(5S2)	1	7	AV3 BV3-1(CB-2)
AV17	1	BV20-1(2S1)	1,1,1	8	AV12-1 BV3-1(CB-2)
AV21	1,1				
AV38-1	1				
AV38-2	1,1,1,1,1				

[a]TCR V α/β usage of HLA-A2/ErbB2 multimer$^+$ CD69$^+$ CD8$^+$ T-cells. Twenty-three TCR α chain clones and 14 TCR β chain clones were isolated from ErbB2-specific CD8$^+$ T-cells. The TRAV and TRBV repertoire was determined by sequencing. The number of repeats for each clone is shown on the right side of the table.

[b]Eight different retroviral backbones encoding eight different TCR α/β combinations were constructed for the propagation of retroviral particles. TCR α and β chains that were presented more than once in the TCR repertoire were subcloned into the MSGV-1 retroviral backbones.

TCR, T-cell receptor; TRAV, T cell receptor α chain variable; TRBV, T cell receptor β chain variable.

functions against peptide-loaded targets and HLA-A2-matched ErbB2-expressing tumor cells.

Identification and isolation of ErbB2-specific TCR α/β genes

Tumor recognition by T cells is often accompanied with specific upregulation of T-cell activation surface antigens such as the early activation marker CD69. In order to capture $ErbB2_{369–377}$-specific T cells with high avidity for the tumor-presented $ErbB2_{369–377}$ peptide, we cocultured the ErbB2-specific T cells with HLA-$A2^+$ $ErbB2^+$ MDA231 cells for 24 hr. ErbB2-specific T cells that upregulated CD69 (Supplementary Fig. S2) and bound HLA-A2/$ErbB2_{369–377}$ tetramer were then isolated via fluorescence-activated flow sorting. In order to determine the TCR variable (TCRV) α-chain and TCRVβ-chain repertoire of the captured ErbB2-specific T cells, total RNA was isolated from the sorted cells and subjected to 5′ RACE. Twenty-three individual α-chain cDNA clones and 14 individual β-chain cDNA clones were fully sequenced from 2 independent PCRs. Sequence data demonstrated two relatively dominant sequences in the TCRVβ repertoire that belonged to the BV3-1(9S1) family of β chains. More heterogeneity was observed in the TCRVα repertoire, with two repeats each for the AV3 and the AV12-1 α chains (Table 1).

TCR α and β chains that presented more than once in the TCR repertoire were subcloned into the MSGV-1 retroviral backbone. A total of eight retroviral vectors harboring the α- and β-chain cDNAs were constructed (Table 1). Retroviruses encoding the eight different TCR α/β combinations were produced and utilized for the transduction of SupT1 cells. Subsequently, the genetically modified SupT1 cells were stained with HLA-A2/$ErbB2_{369–377}$ tetramer and assessed via flow cytometry to identify TCRs with specificity for the $ErbB2_{369–377}$ peptide. One out of eight TCR combinations exhibited specific

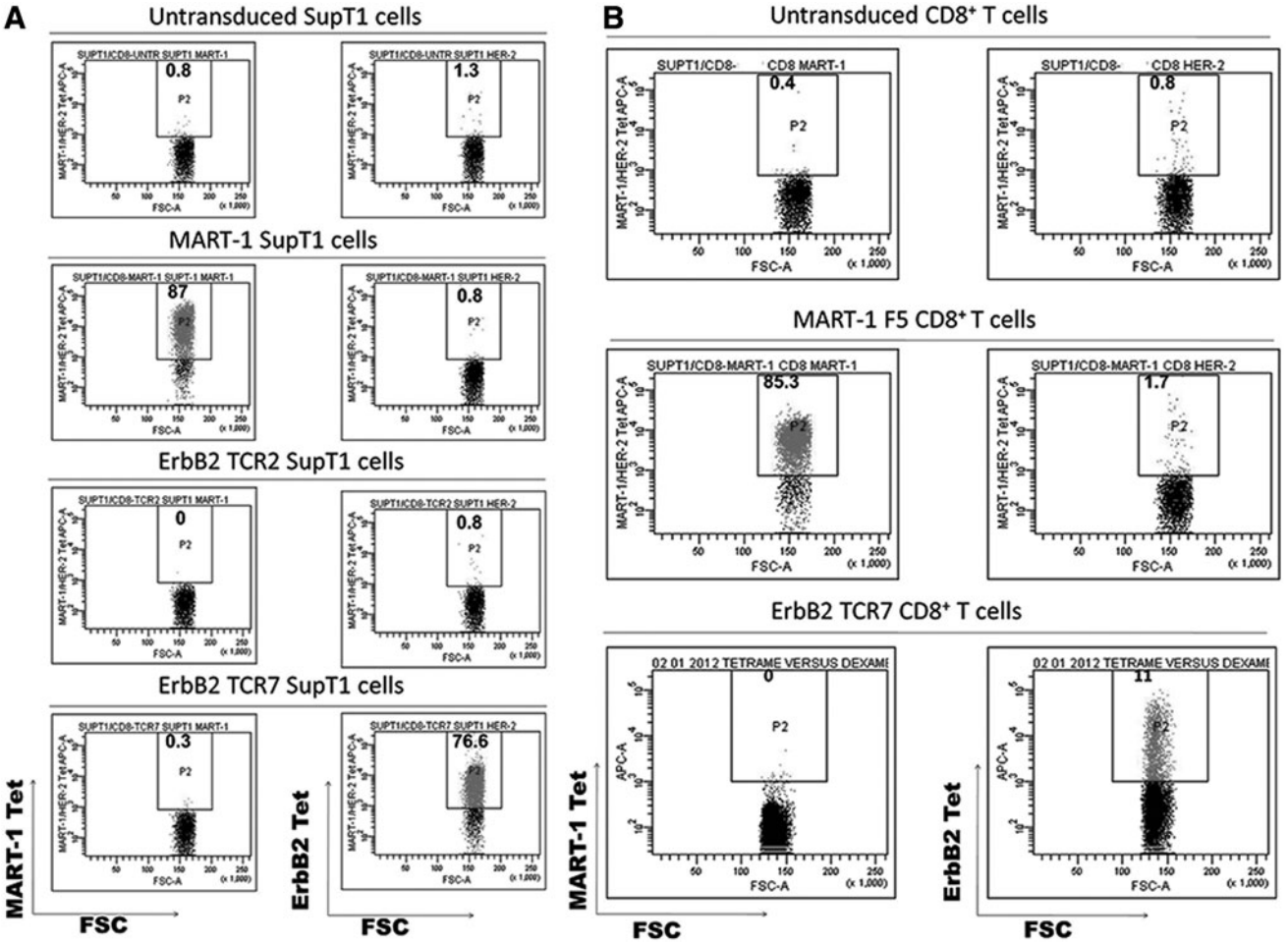

FIG. 3. Expression of the ErbB2 TCR on retrovirally transduced SupT1 cells and $CD8^+$ T cells. **(A)** Screening of TCR α/β pairs by retroviral transduction of SupT1 cells. Retroviruses encoding eight different TCR combinations were screened for $ErbB2_{369–377}$ specificity by transduction of SupT1 cells. HLA-A2/$ErbB2_{369–377}$ tetramer staining of the genetically modified SupT1 cells was performed 5 days after transduction and analyzed by flow cytometry. Two representative SupT1 populations are shown, each bearing different TCRs whose α and β chains were isolated from the ErbB2-specific polyclonal $CD8^+$ T cells. Untransduced (NV) and MART1 SupT1 cells served as negative controls for HLA-A2/$ErbB2_{369–377}$ tetramer binding. **(B)** HLA-A2/$ErbB2_{369–377}$ tetramer staining of primary TCR-transduced $CD8^+$ T cells. $CD8^+$ T cells transduced with either the ErbB2 TCR7 or the MART1 TCR and untransduced $CD8^+$ T cells (NV) were stained with the indicated HLA-A2/peptide tetramers. Numbers represent the percentage of $tetramer^+$ cells. TCR, T-cell receptor.

and strong binding to the HLA-A2/ErbB2$_{369–377}$ tetramer (Fig. 3A). Hence, this paired TCR harboring the AV3 α chain and the BV3-1 β chain was chosen for further characterization (herein referred to as ErbB2$_{369\text{-}377}$-specific TCR7 or ErbB2$_{369\text{-}377}$ TCR).

Retroviral transfer of ErbB2$_{369–377}$-specific TCR7 into CD8$^+$ T cells confers antigen specificity

We next investigated the functional properties that TCR7 confers upon expression in primary human T cells. Retroviral TCR gene transfer into CD8$^+$ T cells resulted in specific HLA-A2/ErbB2$_{369–377}$ tetramer binding (Fig. 3B). However, the percentage of tetramer$^+$ cells was low ($\sim$10%) when compared with SupT1 cells, suggesting that transduced TCRs may not be assembled well enough to be detected or that mispairing with endogenous α chains may occur. Importantly, however, even at low tetramer binding frequencies, the ErbB2 TCR7-transduced T cells demonstrated specific, robust reactivity against peptide-pulsed APC targets (Fig. 4A). ErbB2 TCR T cells demonstrated high peptide avidity, as they secreted high IFN-γ levels at peptide concentrations as low as 1 ng/ml (Fig. 4C). Upon analyzing the tumor reactivity of the ErbB2 TCR CD8$^+$ T cells, we observed IFN-γ secretion in response to HLA-A2$^+$ ErbB2$^+$ OVCAR-2 and MDA231 tumor cells at levels similar to that produced by the initial ErbB2 polyclonal T-cell population (Fig. 4B/Fig. 2C). No reactivity was observed against tumors lacking HLA-A2 or ErbB2 expression or HLA-A2$^+$ 624 melanoma cells expressing very low levels of ErbB2 (Fig. 4B).

T cells expressing ErbB2$_{369–377}$-specific TCR7 delay tumor growth in vivo

To determine the antitumor efficacy of T cells expressing ErbB2$_{369–377}$-specific TCR7 *in vivo*, we subcutaneously coinjected equal numbers of TCR7- or control MART-1$_{26–35}$ TCR-transduced CD8$^+$ T cells and MDA231 tumor cells into NOD/SCID/IL2-γ_c^{null} (NSG) mice and monitored tumor outgrowth. MDA231 tumors grew aggressively with palpable tumors evident 14 days after injection. Compared with MART-1 TCR-specific T cells, ErbB2 TCR7-transduced T cells were capable of significantly delaying tumor burden over time (Fig. 5A). At the termination of the study, mice were euthanized and tumors were excised. Consistent with the measured tumor volume (Fig. 5A), resected tumors from the ErbB2 TCR7 group were visibly smaller (Fig. 5B) and weighed significantly less compared with those in mice treated with the MART-1 TCR (Fig. 5C).

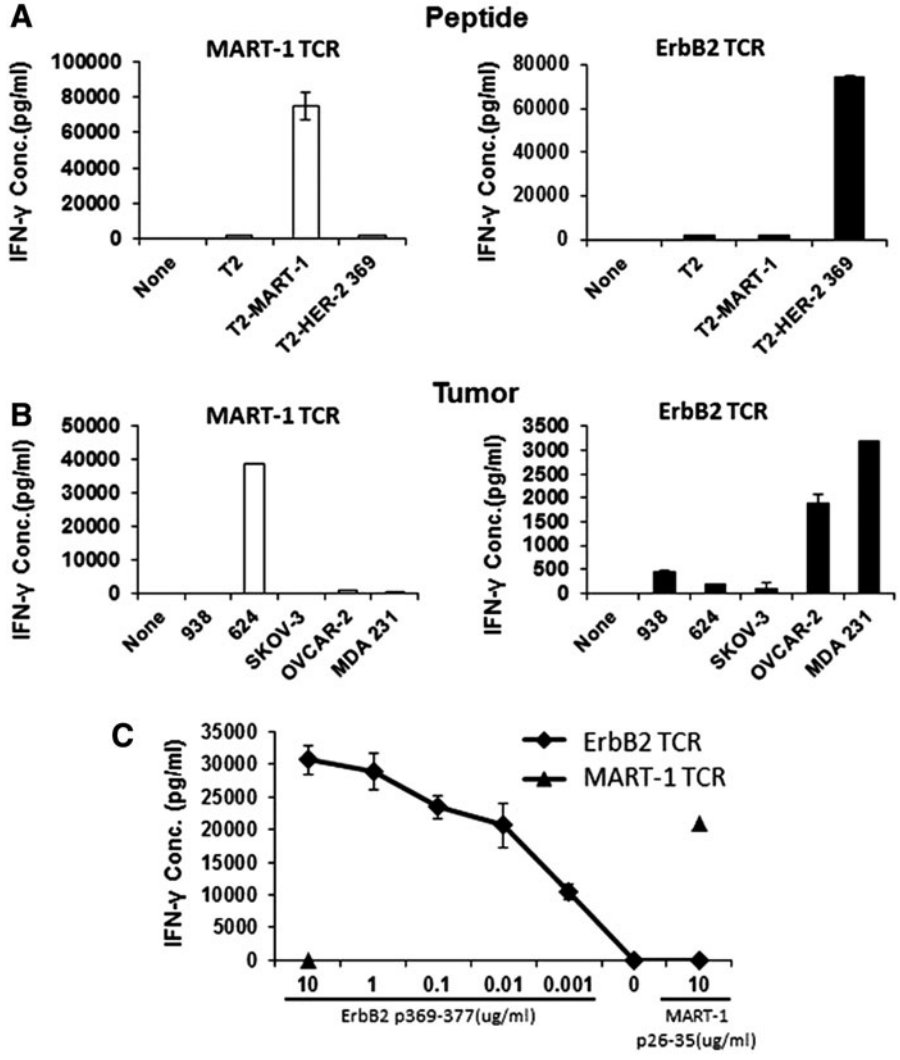

FIG. 4. ErbB2$_{369–377}$-specific T cells show potent IFN-γ production in response to ErbB2-peptide-loaded targets and ErbB2-expressing cancer cell lines *in vitro*. **(A)** ErbB2 or MART1 TCR-transduced T cells were cocultured with T2 cells loaded with HLA-A2-restricted ErbB2$_{369–377}$ or with MART1$_{26–35}$ for 18 hr. **(B)** ErbB2 or MART1 TCR-transduced T cells were cultured alone (none) or stimulated overnight with human HLA-A2-restricted ErbB2$^+$-established cancer cell lines. SKOV-3 (HLA-A2$^-$ ErbB2$^+$) and CEM (HLA-A2$^-$ ErbB2$^-$) served as negative control tumor targets. **(C)** CD8$^+$ T cells transduced with the ErbB2$_{369–377}$-specific TCR as well as the control MART1 TCR were incubated 11 days after transduction for 18 hr with T2 cells pulsed with a range of titrated concentrations of ErbB2$_{369–377}$ peptide. T2 pulsed with MART1$_{26–35}$ peptide served as negative control target T cells. For all assays, IFN-γ was quantified from cell-free supernatants by ELISA and is reported as the mean concentration (pg/ml) $\pm$ SEM of duplicate wells.

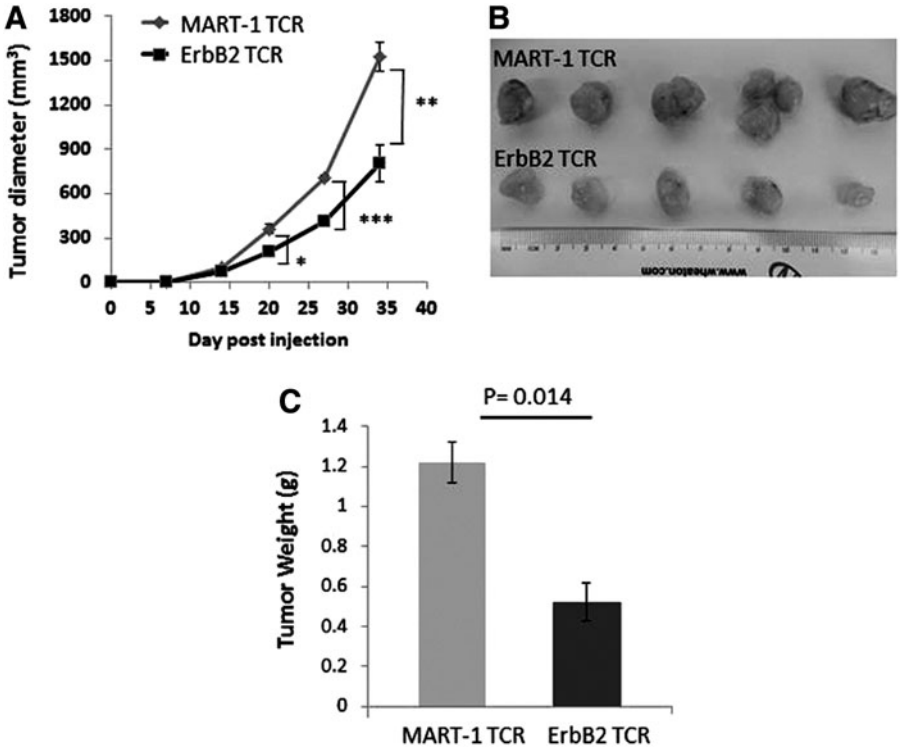

FIG. 5. T cells expressing $ErbB2_{369–377}$-specific TCR7 delay tumor growth *in vivo*. T cells expressing $ErbB2_{369–377}$-specific TCR7 delay tumor growth *in vivo*. Retrovirally transduced ErbB2 TCR7 $CD8^+$ T cells and the breast cancer cell line MDA231 were co-injected subcutaneously into the flank of NSG mice on day 0. MART1-specific F5 TCR-transduced T cells co-injected with MDA231 were used as controls. **(A)** Tumor growth was determined by caliper measurement over time. Results are expressed as mean tumor volume ($mm^3 \pm SEM$) with $n=5$ for all groups. Statistical significance of $p<0.05$ is reported as $*p=0.0495$, $**p=0.0075$, and $***p=0.0029$. After 35 days, tumors were resected, photographed **(B)**, and measured for tumor weight **(C)**. NSG, NOD/SCID/γ-chain$^{-/-}$.

Discussion

Introduction of tumor-specific TCR genes has been proposed as a method to produce *de novo* antitumor lymphocytes for cancer immunotherapy without the need to isolate tumor-reactive T cells (Cordaro *et al.*, 2002; Schumacher, 2002; Sadelain *et al.*, 2003; Willemsen *et al.*, 2003). This proposition requires the existence of tumor antigens common to divergent human cancers and the isolation of a tumor-reactive TCR from the appropriate T-cell population that recognizes these natural tumor antigens.

Since its discovery, the synthetic $ErbB2_{369–377}$ peptide has been widely investigated for the *ex vivo* and *in vivo* generation of ErbB2-specific cytotoxic T-lymphocytes following stimulation *in vitro* (Brossart *et al.*, 1998; Rongcun *et al.*, 1999; Anderson *et al.*, 2000; Seliger *et al.*, 2000; Keogh *et al.*, 2001; zum Buschenfelde *et al.*, 2002; Liu *et al.*, 2004) or vaccination (Zaks and Rosenberg, 1998; Brossart *et al.*, 2000; Knutson *et al.*, 2002; Murray *et al.*, 2002; Peoples *et al.*, 2005). Some ErbB2-specific T cells exert high reactivity against the ErbB2 peptide, but fail to recognize endogenously processed peptide presented by $ErbB2^+$ tumors (Zaks and Rosenberg, 1998; Conrad *et al.*, 2008). Recent work demonstrates that $ErbB2_{369–377}$-specific T cells cross react with the overlapping HLA class I-restricted $ErbB2_{373–382}$ peptide (Henle *et al.*, 2013). Importantly, $ErbB2_{373–382}$ is naturally processed and $ErbB2_{373–382}$-specific T cells also cross react with the $ErbB2_{369–377}$ peptide (Henle *et al.*, 2013), suggesting continued clinical importance for the $ErbB2_{369–377}$ peptide though controversy of its natural processing exists.

We sought to isolate and test ErbB2-reactive T cells from $HLA\text{-}A2^+$ patients with $ErbB2^+$ breast tumors who had been vaccinated with autologous preconditioned DCs (DC1) pulsed with ErbB2 HLA class I and II peptides (Czerniecki *et al.*, 2007). DCs polarized toward the DC1 phenotype produce cytokines and chemokines critical for maximizing antitumor immunity (Xu *et al.*, 2003) and therefore may enhance the efficacy of antitumor vaccines and offer a strong approach to induce and expand tumor-reactive T cells *in vivo* and *ex vivo*. After one round of *ex vivo* stimulation with DC1 cells loaded with $ErbB2_{369–377}$ peptide, the frequency of $ErbB2_{369–377}$ peptide-specific T cells increased to a level (~3.4%) sufficient for robust downstream functional analysis. Of note, these T cells were capable of recognizing peptide loaded onto T2 cells at nanomolar levels, but also $HLA\text{-}A2^+$ ErbB2-expressing tumors. Fluorescence-activated cell sorting allowed us to maximize the purity of ErbB2-specific T cells (~95%), and molecular analysis of the TCR repertoire and subsequent testing of various TCR α and β combinations led us to identify and isolate a novel $ErbB2_{369–377}$-specific TCR (TCR7 AV3/BV3-1).

Retroviral particles encoding the ErbB2 TCR were propagated and utilized for the genetic engineering of primary T cells. We routinely observed nearly a 10% TCR expression efficiency by transduced T cells, as measured by binding to $ErbB2_{369–377}$ multimers. Although the percentage of $multimer^+$ cells was low in primary human T cells, we observed high expression of ErbB2 TCR in SupT1 cells (~80%) that lack endogenous TCR α and β chains, suggesting the possibility that mispairing with endogenous TCR α/β chains impairs proper paired assembly of the exogenous TCR chains on the surface of the transduced T cells. Nevertheless, transduced T cells demonstrated HLA-A2-restricted, ErbB2-specific effector T cells functions, as measured by cytokine release against peptide-pulsed targets and $HLA\text{-}A2^+$ $ErbB2^+$ ovarian and breast cancer tumor cells lines. Similar to the starting ErbB2-specific T-cell population, high functional avidity of the $ErbB2_{369–377}$ TCR-transduced T cells was

demonstrated by their ability to recognize T2 cells pulsed with very low amounts of the cognate peptide (1 ng/ml) and their ability to significantly delay tumor outgrowth in a human breast cancer xenograft model.

Our finding that tumor growth was only moderately delayed *in vivo* suggests that further preclinical refinement of this TCR gene approach is warranted. Proper pairing of exogenous TCR α/β chains may represent one maneuver to augment TCR-transduced T cell function *in vivo*. Multiple approaches exist that lessen mixed dimer formation and increase the expression of properly dimerized exogenous TCR on the T-cell surface. This can be achieved by replacing the constant region of the human TCR chains by their murine counterparts (Cohen *et al.*, 2006), the introduction of additional cysteine residues within the constant region of the TCR α and β chains (Cohen *et al.*, 2007; Voss *et al.*, 2008), the provision of exogenous CD3 molecules (Ahmadi *et al.*, 2011), and/or the inclusion of small interfering RNA (siRNA) to specifically downregulate the endogenous TCR (Okamoto *et al.*, 2009). We anticipate that the application of the above maneuvers will augment the expression of the $ErbB2_{369–377}$ TCR on the surface of transduced T cells. In addition, we expect that the refinement of $ErbB2_{369-377}$ TCR expression will lead to enhanced antitumor efficacy *in vivo* and robust tumor eradication. Alternatively, tumor intrinsic effects may also limit T-cell activity. For instance, the reactivity of ErbB2 TCR T cells can be potentiated by immune checkpoint blockade via the co-administration of recombinant human antibodies specific for negative immunoregulatory molecules, such as B7-H4, which is often expressed by tumor cells (Dangaj *et al.*, 2013).

In summary, the $ErbB2_{369–377}$-specific TCR described here represents an ''off-the-shelf'' reagent that can be utilized to generate autologous tumor antigen-specific T cells without the need to identify antitumor T cells unique for each patient. This approach can yield sufficient numbers of T cells with high avidity and specificity for the $ErbB2_{369–377}$ peptide for the treatment of a variety of common epithelial malignancies.

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Author Disclosure Statement

No competing financial interests exist.

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