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Targeting of the non-mutated tumor antigen HER2/neu to mature dendritic cells induces an integrated immune response that protects against breast cancer in mice

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Abstract

Introduction: Vaccines in a protein format are attractive for breast and other cancers given their relative simplicity of manufacture and ability to be injected repeatedly. However, soluble human epidermal growth factor receptor (HER2)/neu protein as a vaccine has not been immunogenic. When protein is directly targeted to antigen uptake receptors, such as DEC205 (DEC), efficient processing and presentation of antigen takes place. The aim of this study was to determine the immunogenicity of a HER2 protein vaccine that directly targets to DEC^+ dendritic cells (DCs) in a mouse breast cancer model.

Methods: We genetically engineered the HER2 extracellular domain into a monoclonal antibody specific for DEC (DEC-HER2). Mice of various genetic backgrounds were immunized with DEC-HER2 in combination with DC maturation stimuli (poly IC ± CD40 Ab). Vaccine-induced T cell immunity was determined by analyzing the ability of $CD4^+$/$CD8^+$ T cell to produce interferon (IFN)-gamma and proliferate upon antigen rechallenge. Sera were assessed for the presence of antigen specific antibody (Ab). For vaccine efficacy, FVB/N mice were immunized with DEC-HER2 in combination with poly IC and protection against neu-expressing mammary tumors was assessed. Protection mechanisms and tumor-specific T cell responses were also evaluated.

Results: We demonstrate that DEC-HER2 fusion mAb, but not Ctrl Ig-HER2, elicits strong, broad and multifunctional $CD4^+$ T cell immunity, $CD8^+$ T cell responses, and humoral immunity specific for HER2 antigen. Cross-reactivity to rat neu protein was also observed. Importantly, mice xeno-primed with DEC-HER2 were protected from a neu-expressing mammary tumor challenge. Both $CD4^+$ and $CD8^+$ T cells mediated the tumor protection. Robust anti-tumor T cell immunity was detected in tumor protected mice.

Conclusions: Immunization of mice with HER2 protein vaccine targeting DEC^+ DCs *in vivo* induced high levels of T- and B-cell immunity. Non-targeted HER2 protein was poorly immunogenic for $CD4^+$ and $CD8^+$ T cells. This vaccination approach provided long-term survival benefit for mice challenged with neu-expressing tumor following as little as 2.7 μg of HER2 protein incorporated in the vaccine. Vaccine-induced $CD4^+$ and $CD8^+$ T cells were both essential for tumor protection. This immunization strategy demonstrates great potential towards the development of vaccines for breast cancer patients.

Introduction

Despite recent diagnostic and therapeutic advances, breast cancer remains the second leading cause of female cancer mortality in affluent countries. Targeted therapy for breast cancer has focused on receptor tyrosine kinases of the epidermal growth factor receptor (EGFR and ErbB) family, which provide critical checkpoints of cell fate decisions [1, 2]. Aberrations in some members of this gene family rank among the most frequent oncogenic insults in breast cancer. The HER2/*neu* proto-oncogene encodes a tyrosine kinase growth factor receptor (p185) of the ErbB family. It is overexpressed in about 20-40% of invasive breast carcinomas, and in approximately 70% of in situ ductal carcinomas. HER2/neu overexpression is usually associated with a poor clinical prognosis [3, 4].

HER2/neu has been an attractive target for another distinct type of targeted therapy, immune therapy. Although HER2/neu is expressed by malignant cells as a non-mutated self-antigen, immune tolerance is not absolute. Both HER2/neu-specific T cell and antibody responses have been detected in patients with HER2/neu-expressing cancers [5-9]. Additionally, HER2-specific cytolytic T lymphocyte response has been generated *in vitro* with T cells from patients with HER2-expressing tumors [6, 10-12].

Vaccines in a protein format are attractive for breast and other cancers given their relative simplicity of manufacture and ability to be injected repeatedly. However, soluble HER2/neu protein as a vaccine has not been immunogenic and has usually failed to confer protection against HER2/neu-expression tumors [13-15]. Anti-tumor immunity can be enhanced when HER2 extracellular domain is fused to cytokines or combined with antibodies fused to cytokines [15]. Other efforts to improve immunogenicity include mannosylation of the HER2 protein by producing the recombinant protein in yeast [16]. On the other hand, when antigen is directly targeted to antigen uptake receptors, efficient processing and presentation takes place. HER2/neu protein has been incorporated into different vaccine platforms that directly target to antigen-presenting cells (APC). Recently, several receptors have been tested for the delivery of HER2 antigen, including B7-1/2 [17, 18], CD11c [19], CD40 [20], mannose [21] and Fcγ receptors [22]. Together, these studies suggest that compared to non-targeted vaccinations, targeting HER2 to receptors expressed on APCs can improve HER2-specific T cell responses and anti-tumor immunity against HER2-expressing tumor challenge in mouse models.

One of the dendritic cell (DC) specific receptors that have not been explored for HER2 vaccination is the DEC-205 (“DEC”, CD205) receptor, a type I C-type lectin [23]. Expression of DEC in mice is abundant on $CD8\alpha^+$ DCs, which have a superior capacity of cross-presentation

[24, 25]. Although other receptors on DCs can be targeted [26, 27], so far DEC is the only receptor that has been visualized on the numerous DCs within the T cell areas of human lymphoid organs [28]. Targeting the DEC receptor leads to efficient endocytosis of antigens into endocytic vesicles containing MHC class II molecules. This results in a hundred-fold more efficient antigen uptake and T cell stimulation than fluid phase or solute pinocytosis [29-31]. Delivery of antigen to $DEC^+CD8\alpha^+$ DCs *in vivo* improves cross-presentation to $CD8^+$ T cells [31, 32]. Increased antigen delivery efficiency through DEC significantly reduces the amount of protein required for the induction of T cell immunity. Vaccine induced T cells have cancer-resisting features, such as combined $CD4^+$ and $CD8^+$ T cell immunity, production of Th1 type cytokines, and the ability to proliferate upon antigen rechallenge.

Previous studies have shown that ligation of DEC receptor by targeting Ab conjugated to antigen does not mature DCs but induce tolerance [30, 33]. To overcome immune tolerance mediated by steady state DCs, DC maturation adjuvants need to be included in the vaccine. Examples of potent adjuvants are synthetic double strand RNA, polyinosinic:polycytidylic acid (poly IC) and its more RNase-resistant analogue stabilized with poly-L-lysine (poly ICLC). Both preclinical and clinical studies demonstrate that poly IC and poly ICLC are superior adjuvants for induction of potent T cell immunity. Longhi et al has shown that compared to other TLR agonists, the TLR3 ligands poly IC and poly ICLC stand out as the most potent adjuvants for T cell immunity when combined with DEC-gag mAb immunization in mouse model [34]. A recent clinical study by Caskey et al demonstrated that poly ICLC can be a reliable and authentic viral mimic for inducing innate immune response and for use as a vaccine adjuvant in humans [35]. Poly IC is under clinical investigation in combination with a DEC-targeted HIV protein vaccine in our lab (Caskey et al. unpublished results).

The aim of this study was to determine the immunogenicity of HER2 protein vaccine that targeted to DEC^+ DCs in a preclinical mouse breast cancer model. To deliver HER2 protein to DEC^+ DCs in situ, we genetically engineered the HER2 extracellular domain into monoclonal antibodies (mAb) specific for DEC and tested the immunogenicity of this fusion mAb in mice in combination with DC maturation stimuli. For the tumor vaccine study, we xeno-primed mice with HER2 protein followed by a neu-expressing tumor challenge.

Materials and methods

Mice

Animal experiments were designed to fulfill the ethical and scientific principles that provided by Institutional Animal Care and Use Committee of The Rockefeller University (approved protocol #08117). Mice were maintained under specific pathogen-free conditions and used at 6-8 weeks of age. C57BL/6, BALB/c, FVB/N and HLA-A2.1 transgenic mice in the C57BL/6 background [C57BL/6-Tg(HLA-A2.1)1Enge/J] were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). $DEC^{-/-}$ mice were generated and provided by Dr. M. Nussenzweig (The Rockefeller University, New York, NY, USA) and are available from The Jackson Laboratory. At least three mice per group were used in immunization experiments.

Cell lines

The neu-expressing mammary tumor cell line NT2.5 was derived from a spontaneous mammary tumor in female *neu*-N mice (FVB/N background). The cell line was established and kindly provided by Dr. E.M. Jaffee (Johns Hopkins University School of Medicine, Baltimore, MD). NT2.5 tumor cells were grown in a previously defined Breast Media, which consisted of RPMI (Gibco, Invitrogen, Carlsbad, CA, USA) with 20% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids, 1% Na pyruvate, 0.5% penicillin/streptomycin, 0.02% gentamicin (Gibco, Invitrogen), and 0.2% insulin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37℃ in 5% CO2. The HER2 stably transfected tumor cell line, E0771/E2, was generously provided by Dr. W.Z. Wei (Karmanos Cancer Institute, Wayne State University, Detroit, MI). Anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) hybridoma cells were obtained from ATCC (Manassas, VA, USA) and maintained according to ATCC protocols.

Construction and production of fusion mAb

DNA coding HER2 extracellular domain (amino acid 22-653) was cloned in frame into the COOH terminus of anti-DEC (DEC-HER2) or control IgG heavy chain (Ctrl Ig-HER2) as described previously [30]. Fusion mAb was expressed by transient transfection in 293T cells and purified on protein G columns (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Purified mAb was characterized by SDS-PAGE and Western blot using anti-mouse IgG-HRP (SouthernBiotech, Birmingham, AL, USA) or anti-HER2 mAb (clone 42, BD Transduction Laboratories, San Jose, CA, USA). Specific binding of the fusion mAb was verified using Chinese hamster ovary (CHO) cells stably transfected with mouse DEC receptor. Binding was detected by flow cytometry using phycoerythrin-conjugated goat anti-mouse IgG mAb (Jackson

ImmunoResearch Laboratories, Inc. West Grove, PA, USA) and Alexa Fluor 488-conjugated mouse-anti-HER2 mAb (clone 24D2, BioLegend, San Diego, CA, USA). All Abs had <0.125 endotoxin units/mg in a Limulus Amebocyte Lysate assay, QCL-1000 (Bio Whittaker, Walkersville, MD).

Peptides

Overlapping (staggered by four amino acids) 15-mer peptides covering the HER2 and neu extracellular domain and HIV gag p24 protein were synthesized by H. Zebroski in the Proteomics Resource Center of The Rockefeller University. The use of peptides overcomes, in large part, the need for antigen processing by antigen-presenting cells during the immune assays. The 161- and 147-member HER2 and neu peptide libraries were divided into seven and six pools respectively.

Immunization

Mice were immunized intraperitoneally (i.p.) with 5 μg DEC-HER2 or Ctrl Ig-HER2 fusion mAb in combination with 50 μg poly IC (polyinosinic:polycytidylic acid) (InvivoGen, San Diego, CA, USA). When indicated, a combination of 50 μg poly IC and 25 μg agonistic anti-CD40 mAb (clone 1C10) was used to mature DCs.

Intracellular cytokine staining

Bulk splenocytes were stimulated with specific peptide pools (2 μg/mL or indicated concentration) or medium alone in the presence of a costimulatory anti-CD28 mAb (clone 37.51) for 6 h. Brefeldin A (10 μg/mL) (Sigma-Aldrich) was added for the last 5 h to accumulate intracellular cytokines. Anti-CD28 mAb was only used in 6 h intracellular cytokine staining assay, but not in other T cell immune assays, including ELISA, ELISPOT and CFSE dilution assays. For functional avidity analysis, graded doses (10–0.0016 μg/mL) of peptides were used to restimulate splenocytes. After stimulation, cells were washed, incubated with anti-CD16/CD23 mAb (clone 2.4G2) to block Fcγ receptor for 15 min at 4°C. Cells were stained with Live/Dead Fixable Aqua vitality dye (Invitrogen), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone RM4–5), PerCP-Cy5.5-conjugated anti-CD8 (clone 53–6.7), and Pacific Blue-conjugated anti-CD3 (clone 17A2) (eBioscience, San Diego, CA, USA) for 20 min at 4°C. Cells were fixed, permeabilized (Cytofix/Cytoperm Plus; BD Biosciences, USA) and stained with allophycocyanin (APC)-conjugated anti-IFNγ, phycoerythrin (PE)-conjugated anti-IL-2, and PECy7-conjugated anti-tumor necrosis factor (TNF)-α mAbs for 20 min at 4°C (BD Biosciences, San Diego, CA, USA) and resuspended in stabilizing fixative (BD Biosciences). Data were

collected using a BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

CFSE dilution assay

A CFSE (5,6-carboxy fluorescein diacetate succinimidyl ester) dilution assay was used to assess the proliferative capacity of T cells. Bulk splenocytes ($2x10^7$ cells/mL) were labeled with 2.5 μM CFSE (Invitrogen) in a 37℃ water bath for 10 min. CFSE-labeled T cells were restimulated with pools of peptide (0.2 μg/ml) for 4 days, often in combination with intracellular cytokine staining of cells restimulated for the last 6 h of culture.

Mouse IFNγ ELISPOT

Multiscreen-HA MAHA 54510 (Millipore, Billerica, MA, USA) plates were coated with 10 μg/mL of purified rat-anti-mouse-IFNγ mAb (clone R46A2; BD Biosciences) in PBS overnight at 4℃. Plates were washed and blocked with PBS/1% BSA for 1 h at 37℃. 3×10^5 MACS-purified $CD8^+$ or $CD4^+$ T cells were cultured for 2 d with 1×10^5 purified $CD11c^+$ spleen DCs pulsed with the peptide mix (1 μg/mL) or NT2.5 tumor lysate (10 μg/mL). Biotin-conjugated rat-anti-mouse-IFNγ mAb (clone XMG 1.2, 2 μg/mL, BD Biosciences) was used as the detection antibody. After 2 h incubation with detection antibody, spots were visualized with VECTASTAIN ABC kit (VECTOR Laboratories, INC., Burlingame, CA, USA), followed by diaminobenzidine as the substrate (Invitrogen). Spots were counted in an ELISPOT reader (Autoimmun Diagnostika GmBH, Germany).

Mouse cytokine ELISA

Splenic $CD4^+$ and $CD11c^+$ cells were purified by MACS. $3x10^5$ $CD4^+$ cells were incubated with $1x10^5$ $CD11c^+$ cells with peptide mix (2 μg/mL) in 96-well U-bottomed plates for 48 h. Concentrations of IFNγ, IL-4, IL-10 and IL-17 in supernatant were measured by Ready-SET-Go ELISA Sets (eBioscience).

ELISA for anti-HER2/neu antibodies

To detect HER2-specific Ab response, we produced FLAG-HER2 soluble protein by transient transfection of 293T cells and purification with anti-FLAG affinity gel (Sigma). The quality of FLAG-HER2 protein was verified by SDS-PAGE gel under non-reducing condition (Additional File 1, Figure S1). We coated high-binding ELISA plates (Nunc; Thermo Fisher Scientific Inc., Rochester, NY, USA) with 500 ng/mL (50 ng/well) of FLAG-HER2 overnight at 4°C. Plates were washed with PBS/0.1% Tween-20 and blocked with PBS/0.1% Tween 20/5% BSA for 1 h at

37°C. Serial dilutions of serum were added to the plates and incubated for 1 h at 37°C. Secondary goat anti-mouse IgG-specific Abs conjugated with HRP (Southern Biotech) were added and visualized with tetramethylbenzidine (TMB) (eBioscience) at room temperature for 5–10 min. To determine the IgG isotype, anti-mouse IgG1 or IgG2a Abs were used. The reported titers represent the highest dilution of sample showing an OD_{450} higher than 0.1. The data were presented as the $\log_{10}$ antibody titer. To determine whether serum IgG can bind to HER2/neu-expressing tumor cells, serial diluted serum were incubated with E0771/E2 ($HER2^+$) or NT2.5 (neu^+) tumor cells for 15 min at 4°C. Anti-mouse IgG-PE mAb were used to detect the binding of serum IgG to tumor cells. Data were acquired using a BD LSR II flow cytometer (BD Biosciences).

Tumor protection

FVB/N mice were immunized i.p. with 5 μg DEC-HER2 or Ctrl Ig-HER2 mAb together with 50 μg poly IC on days 0 and 28. 50 μg poly IC alone was injected as a negative control. Ten days after the boost immunization, mice were inoculated subcutaneously with 1×10^6 NT2.5 tumor cells in the shaved right flank. Tumor size was measured three times every week using a caliper. Tumor volumes were estimated according to the formula: length × $(\text{width})^2$ × 0.5. For survival analysis, tumor sizes ≥ 500 mm^3 were defined as the experimental endpoint. For antibody depletion, 200 μg CD4 or CD8 or both mAb were given to mice i.p. after boost immunization, on 9, 6, and 3 days before tumor challenge. Isotype control rat IgG was given as a negative control. Efficiency of depletion was confirmed by FACS analysis of peripheral blood cells.

Statistical analysis

All analysis was performed using Prism 4.0 GraphPad software (San Diego, CA, USA). A two-sided Student's *t*-test (between two groups or conditions) was applied to compare statistical significance between peptide-specific responses and treatment groups of immunized mice. Survival studies were analyzed by Kaplan-Meier survival cures and log rank test. Results were considered statistically significant when $P<0.05$.

Results

HER2 extracellular domain can be introduced into a functional DEC antibody

To deliver HER2 protein to mouse DCs directly *in vivo*, we cloned the extracellular domain of HER2 (aa22-653) in frame into the heavy chain of the anti-mouse DEC mAb (DEC-HER2) (Figure 1A). The heavy chain of an isotype matched non-reactive control IgG was also engineered as a non-targeting control (Ctrl Ig-HER2). The fusion mAbs were composed of a 140~150 kDa heavy chain, consistent with a predicted mass of 90 kDa for the HER2 ECD fused to the ~50 kDa heavy chain of unconjugated mouse IgG1 (Figure 1B). To verify whether the fusion mAb bound to the mouse DEC receptor, a stable CHO cell transfectant, expressing mouse DEC on the surface, was stained with indicated concentration of mAb. As analyzed by FACS, DEC-HER2, but not Ctrl Ig-HER2 mAb, demonstrated proper DEC binding activity (Figure 1C).

DEC targeting of HER2 induces strong and broad HER2 specific $CD4^+$ T cells

To determine the immunogenicity of the DEC HER2 fusion mAb, we immunized C57BL/6 ($H\text{-}2^b$) mice with DEC-HER2 or Ctrl Ig-HER2 i.p. at 5 μg dose (equivalent to 14 pmol or 2.7 μg of HER2 protein). Poly IC (50 μg) and agonistic anti-CD40 mAb (25 μg) were used to mature DCs. In C57BL/6 mice, $CD4^+$ T cells responded strongly to epitopes present in HER2 peptide pool 4 and 5 (Figure 2A). We also found a weak response against HER2 epitopes in pool 3 and 7 (Figure 2A). When CD28 mAb was removed from culture, T cell responses were reduced but remained significantly higher than background signals (Additional File 2, Figure S2A). The T cell response was specific to HER2, since there was no reactivity to HIV gag peptides. Although Ctrl Ig-HER2 vaccination induced a significant $CD4^+$ T cell response against peptide pool 5, its magnitude was significantly weaker than that induced by DEC-HER2 (Figure 2A). The breadth of the response was limited to pool 5. We did not observe significant $CD4^+$ T cell responses against other pools.

In addition to effector T cell pool size, we also determined the proliferative capacity of vaccine induced $CD4^+$ T cells upon antigen restimulation by CFSE dilution assays. Immunized T cells were stimulated with HER2 or control peptides *in vitro* for 4 d. When recalled with HER2 peptide pools for the last 6 h, the proliferating $CD4^+$ T cells produced IFNγ specifically against HER2 (Figure 2B).

To determine whether vaccine induced $CD4^+$ T cells are multifunctional, as defined by combination of IFNγ, IL-2 and TNFα production at single cell level [36], we measured the

relative proportion of potential combinations of cytokines as depicted by pie charts and shown in Additional file 3, Figure S3 (Left panel). The mean percentage of $CD4^+$ T cells producing IFNγ/IL-2/TNFα was ~57%. Thus, DEC-HER2 vaccination elicited multi-functional $CD4^+$ T cell responses.

To determine the types of T helper responses induced by vaccination, we measured the production of the Th1/Th2/Th17 cytokines (IFNγ, IL-4, IL-10 and IL-17), by ELISA. $CD4^+$ T cells from immunized mice were cocultured with $CD11c^+$ cells in the presence of HER2 peptide pool 5 or HIV gag peptide mix for 48 h. As shown in Figure 2C, in mice immunized with DEC-HER2, we detected a dominant Th1 response with little Th2 or Th17 cytokine secretion from mice immunized with DEC-HER2. T cells from DEC-HER2 immunized mice produced significantly higher amount of IFNγ, but similar levels of IL-4/IL-10/IL-17 compared to T cells from mice immunized with Ctrl Ig-HER2 mAb. These data demonstrate that targeting DC via DEC primed strong Th1 responses against HER2.

Poly IC adjuvants strong and broad $CD4^+$ T cell responses to DEC-targeted HER2

As a monotherapy, Poly IC has been tested at high doses in cancer patients with a favorable safety profile [37-41]. To assess the adjuvant activity of poly IC, we primed and boosted mice with DEC-HER2 in combination with 50 μg poly IC, without anti-CD40 mAb. We found that poly IC as the only adjuvant mediated significant $CD4^+$ T cell responses in mice immunized with DEC-HER2 mAb, but not in mice treated with Ctrl Ig-HER2. We identified four reactive HER2 peptide pools, namely pools 3, 4, 5 and 7 (Figure 3A), which are the same immunogenic pools when poly IC and CD40 Ab were used together as the adjuvants. The mean proportion of $CD4^+$ T cells producing IFNγ/IL-2/TNFα was ~50% (Additional file 3, Figure S3, right panel), which is similar to the response when poly IC and CD40 Ab were co-administrated. Thus, DEC-HER2 vaccination with poly IC can elicit multi-functional $CD4^+$ T cell responses.

To our knowledge, HER2-specific CD4 T cell epitopes have not been identified in C57BL/6 mice. To identify the individual reactive CD4 epitopes, we restimulated immune $CD4^+$ T cells with single peptides from pool 3, 4, 5, and 7. Seven peptides were able to induce significant IFNγ production by $CD4^+$ T cells from DEC-HER2 immunized mice (Table 1 and Additional file 4, Figure S4). The dominant CD4 T cell epitopes in C57BL/6 mice locate within the $HER2_{284\text{-}302}$ region (NPEGRYTFGASCVTACPYN).

To determine whether DEC-HER2 can induce $CD4^+$ T cell responses in different MHC backgrounds, we immunized BALB/c ($H\text{-}2^d$) and FVB/N ($H\text{-}2^q$) mice. Significant $CD4^+$ T cell

response was induced with DEC-HER2 was targeted to the DEC receptor, although the magnitude was lower than that observed in C57BL/6 mice (Figure 3B and C). The dominant epitope(s) presented in BALB/c mice were located in pool 2 (Figure 3B). The amino acid sequence spanning HER2 peptide pool 2 is shown in Additional file 5, Table S1 and the predicted I-A^d-restricted epitopes are shown in Additional file 6, Table S2. $CD4^+$ T cell responses can also be induced in the FVB/N mice, with the dominant epitope(s) located in HER2 peptide pool 4 (Figure 3C and Additional file 5, Table S1).

To determine whether the DEC receptor is essential for vaccination, we immunized $DEC^{-/-}$ mice. DEC-HER2 did not induce HER2-specific T cell responses in $DEC^{-/-}$ mice (Figure 3D). The dependence of DEC expression in this model is consistent with our previous studies using other immune antigens [32, 42].

To determine the pattern recognition receptor required for poly IC, we tested $TLR3^{-/-}$ and $TLR3^{-/-}MDA5^{-/-}$ mice. Although significantly reduced, half of the adjuvant effect of poly IC remained intact $TLR3^{-/-}$ mice. In contrast, poly IC completely lost its adjuvant effect in $TLR3^{-/-}$ $MDA5^{-/-}$ mice (Figure 3E). These data suggest both endosomal TLR3 and the cytosolic sensor MDA5 are required for the maximal adjuvant effect of poly IC.

Thus, targeting HER2 to DCs activated by poly IC induced potent, broad and multi-functional $CD4^+$ T cell immunity in mice, which are important features of a successful vaccination. Induction of T cell immunity was dependent on the expression of DEC receptor for antigen uptake and TLR3/MDA5 for DC maturation.

HER2 protein targeted to DCs can cross-prime $CD8^+$ T cells

To assess cross-priming of HER2 protein by DEC targeting, we vaccinated FVB/N (H-2^q) mice with DEC-HER2 or Ctrl Ig-HER2 in combination with poly IC. We found that HER2-specific $CD8^+$ T cell responses were elicited in mice vaccinated with DEC-HER2 but not in mice vaccinated with Ctrl Ig-HER2 mAb (Figure 4A). The dominant epitope(s) are located in peptide pool 5 (Figure 4A) and the amino acid sequence spanning pool 5 is shown in Additional file 5 Table S1. We also found that HER2-specific $CD8^+$ T cells can proliferate and produce IFNγ when restimulated with HER2 peptides (200 ng/ml) (Figure 4B).

To test whether DEC targeting can enhance cross-priming in a human HLA haplotype, we administered DEC-HER2+poly IC to HLA-A2 transgenic mice on the C57BL/6 background. Although we did not detect cross-priming in wild type C57BL/6 mice, the HLA-A2 transgenic mice developed HER2-specific $CD8^+$ T cell responses, as analyzed by ELISPOT assay (Figure

4C). We identified that peptide pool 5 contained the reactive CD8 epitope(s). The amino acid sequence spanning HER2 pool 5 is shown in Additional file 5, Table S1. Two previously identified HLA-A2-restricted epitopes are located in peptide pool 5. They are $HER2_{435-443}$ (ILHNGAYSL) and $HER2_{466-474}$ (ALIHHNTHL) [43, 44]. Thus, targeting HER2 to DCs not only enhanced Th1 responses, but also cross-presentation to $CD8^+$ T cells.

DEC targeted HER2 vaccination xeno-primes neu-specific $CD4^+$ T cells

To determine if T cells induced by human HER2 protein vaccination are able to cross-react with the homologous rat neu protein, we restimulated T cells with neu peptides. Despite amino acid sequence differences between human HER2 and rat neu, we found that $CD4^+$ T cells induced by DEC-HER2 vaccination were able to secrete IFNγ upon restimulation with the neu peptide pool (Figure 5A). To estimate the functional avidity of the vaccine-induced $CD4^+$ T cells, we restimulated splenocytes from mice immunized with DEC-HER2+poly IC with graded concentrations of HER2 peptide pool 5 or neu peptide pool 4 (from 10–0.0016 μg/ml or 6.67–0.001 μM). As shown in Figure 5B, HER2- and neu-specific T cells have a similar EC_{50} (i.e., concentration of peptide that leads to 50% of the maximal responses). Thus, xeno-priming with HER2 protein can induce homologous rat neu-specific $CD4^+$ T cell responses, which is important for tumor vaccination studies using rat neu-expressing tumor models.

DEC targeting leads to HER2/neu-specific humoral immunity

To determine whether targeting HER2 to DEC can generate humoral responses, we measured serum anti-HER2 total IgG by ELISA. In FVB/N mice, both DEC-HER2 and Ctrl Ig-HER2 mAb induced HER2-specific Ab responses with a similar titer of ≥ 1800 in total IgG, and ≥ 600 in IgG1 or IgG2a (**Figure 6A**).

To investigate whether the serum IgG can recognize natural HER2/neu protein presented on tumor cells, we performed flow cytometry-based assays. Serum IgG bound to the HER2-expressing tumor cell line E0771/E2 up to a 1:400 dilution (Figure 6B). Immune sera also bound to neu protein on the surface of NT2.5 tumor cells at a 1:100 dilution, although the binding was much weaker (~10-fold) than to HER2 protein (Figure 6C). These data indicate that cross-reactivity of serum HER2-specific IgG to neu protein is weak, unlike what we observed with T cell cross-reactivity (Figure 5). The baseline Mean Fluorescence Index (MFI) without immune serum was similar between two tumor cells lines.

DEC-HER2 vaccination significantly delays tumor growth

To assess whether the vaccine induced HER2/neu-specific T cell immunity can mediate protective anti-tumor immunity, we immunized FVB/N mice with DEC-HER2 or Ctrl Ig-HER2 in combination with poly IC. Ten days after boost immunization, mice were challenged with NT2.5 tumor cells in the mammary fat tissue. Vaccination with only 5 μg of DEC-HER2 protein with poly IC significantly delayed tumor growth (Figure 7A). Although Ctrl Ig-HER2 vaccination was able to induce anti-HER2 Ab responses (Figure 6), it was unable to protect mice from tumor outgrowth. As expected, administration of poly IC alone did not delay tumor growth. Although not all of the mice that received DEC-HER2+poly IC vaccinations were tumor-free, the survival rate (up to 80 d) of DEC-HER2-immunized mice was significantly greater than that of Ctrl Ig-HER2 treated or untreated mice.

To determine the underlying tumor protection mechanism, we depleted $CD4^+$, $CD8^+$ or both populations at the effector phase, the time of tumor challenge. Depletion efficiency was verified by FACS analysis of peripheral blood lymphocytes the day before tumor challenge (Additional file 7, Figure S5). We found that both $CD4^+$ and $CD8^+$ T cells were required for tumor protection in the vaccinated mice as determined by tumor growth kinetics (Figure 7C). Survival analysis (up to 60 d) shows that depletion of $CD4^+$ T cells did not completely abrogate survival benefit of DEC-HER2 vaccination. However, depletion of $CD8^+$ T cells completely abrogated the tumor protection effect (Figure 7D). These data suggest that $CD8^+$ T cells play a more critical role for long-term survival than $CD4^+$ T cells.

To determine the induction of anti-tumor T cell responses, we harvested the splenocytes 14 d after tumor challenge and restimulated purified $CD4^+$ or $CD8^+$ T cells with purified splenic $CD11c^+$ cells pulsed with peptides mix (HIV gag, HER2 or neu) or NT2.5 tumor lysate. Strong HER2 and neu-specific $CD4^+$ (Figure 7E) and $CD8^+$ (Figure 7F) T cell responses against HER2/neu were induced by DEC-HER2, but not by Ctrl Ig-HER2. $CD4^+$ and $CD8^+$ T cells also produced IFNγ upon restimulation with NT2.5 tumor lysate-pulsed DCs. Thus, DEC-HER2 xeno-priming significantly increased anti-tumor immunity, which led to a significant delay in tumor outgrowth. Thus, vaccine-induced $CD4^+$ and $CD8^+$ T cells were both essential for tumor protection and long-term survival.

Discussion

In this study, we have found that delivery of the HER2 tumor antigen within DEC mAb allowed for efficient immunization of HER2/neu specific T cells at a low dose (5 μg chimeric Ab, or 2.7 μg HER2 protein). We have also demonstrated that vaccine-induced T cell immunity significantly delayed neu-expressing tumor growth in mice.

To overcome the weak T cell immunity that is typically elicited by HER2 protein vaccines, we delivered HER2 to the DEC^+ DCs *in vivo*. High efficiency of targeting tumor antigen to DEC^+ DCs allows a significantly lower dose of protein to achieve potent $CD4^+$ and $CD8^+$ T cell responses. We found that only a single dose of 5 μg chimeric mAb (equivalent to 2.7 μg of HER2 protein) was able to induce strong $CD4^+$ T cell immunity in mice (Figure 2). On the other hand, at the same dose, linking HER2 protein to an isotype control mAb was inefficient in inducing T cell immunity. Similarly, studies from other groups have shown that usually a much higher dose of soluble HER2/neu protein is required to induce detectable T cell immunity [13-15, 17, 45]. Even at two doses of 50 μg, soluble HER2 protein only induces marginal T cell immunity [14]. The robust $CD4^+$ T cell responses induced by vaccination is Th1 dominant, as measured by high IFNγ, but low IL-4/IL-10/IL-17 production (Figure 2C). Increased Th1 immunity is usually associated with a better outcome in cancer patients [46, 47].

The efficient induction of $CD4^+$ T cell responses by our vaccine approach is dependent on the expression of DEC receptor. In $DEC^{-/-}$ mice, we did not detect significant HER2-specific T cell immunity (Figure 3D). This result is consistent with our previous vaccination studies using other antigens [32, 42]. The T cell tolerance induced by steady-state immature DCs was overcome by administration of TLR3 agonist poly IC with the protein vaccine. We demonstrated that recognition of poly IC by its cellular receptors TLR3 and MDA5 are essential for the induction of HER2 immunity (Figure 3E), which is consistent with our previous findings [48]. We observed a slightly increased background response to HER2 when poly IC was combined with DEC-HER2 vaccination, but the background response significantly reduced at later time points (>3 weeks) after immunization (unpublished results). The observed temporary general immune stimulation is most likely due to the activation of bystander T cells caused by the high amount of IFNγ, IL-2 and TNFα secreted by the vaccine-induced T cells (Additional file 3, Figure S3). These bystander activated T cells might in turn amplify vaccine-induced immune response against the tumor antigen, which would represent a beneficial outcome of our vaccine strategy.

Targeting HER2 to activated DCs not only enhanced the magnitude, but also the quality of the $CD4^+$ T cell responses in four ways. First, broad T cell responses were developed in three MHC haplotypes ($H\text{-}2^d$, $H\text{-}2^b$ and $H\text{-}2^q$) tested here (Figure 3A and Table 1). Second, the vaccine-induced T cells produced multiple cytokines (IFNγ, TNFα and IL-2), which are important in regulating the expansion of $CD4^+$ and $CD8^+$ T cells (Additional file 3, Figure S3). Third, the vaccine induced HER2-specific $CD4^+$ T cells proliferated rigorously and secreted IFNγ upon antigen challenge (Figure 2B). Fourth, the HER2-specific $CD4^+$ T cells cross-reacted to rat neu antigen with similar functional avidity (Figure 5), despite the sequence differences between the two homologs.

The high quantity and quality of vaccine-induced $CD4^+$ T cell responses have several implications for tumor immunotherapy. First, they can enhance the magnitude and longevity of $CD8^+$ T cell immunity and promote infiltration of $CD8^+$ T cells into the tumor milieu [49-51]. This is supported by vaccine studies in breast cancer patients reported by Knutson et al. Their clinical data indicates that immunization with a peptide vaccine designed to stimulate $CD8^+$ T cells alone only generates low and short lived immune responses [52]. Second, HER2/neu-specific Th1 cells can home to tumor site, secrete IFNγ and other inflammatory cytokines in tumor microenvironment and boost the function of macrophages and DCs [53, 54]. Activation of APCs may increase processing and presentation of endogenous tumor antigen from dying cells resulting in “epitope spreading”, which refers to the development of immunity to tumor antigens other than HER2/neu that could halt the progression of HER2/neu negative variants [55]. Third, $CD4^+$ T cells are also cytotoxic directly against tumor cells [56-58], although the tumor cells that we evaluated here lack MHC II (unpublished result).

Immunotherapy approaches that induce integrated $CD4^+$ and $CD8^+$ T cell responses are desirable. We show here that DEC-HER2 not only induced strong $CD4^+$ T cells responses, but also significant $CD8^+$ T cells responses at a low dose (Figure 4). These $CD8^+$ T cells proliferate rigorously upon restimulation with HER2 peptide *in vitro* (Figure 4B). Importantly, ,using HLA-A2 transgenic mice we found that $CD8^+$ T cell responses can be induced in different MHC haplotypes (Figure 4C). The responding HER2 peptide pool 5 indeed contains two A2-restricted HER2 epitopes that have been described previously [43, 44]. This result is consistent with previous findings that targeting protein to activated DCs, especially $CD8\alpha^+$ DCs, can significantly enhance antigen cross-presentation to $CD8^+$ T cells [27, 31, 59, 60].

Targeting HER2 to DEC^+ DCs not only induced integrated $CD4^+$ and $CD8^+$ T cell responses, but also serum Ab response (Figure 6). Importantly, HER2/neu-specific IgG

induced by immunization can recognize naturally derived HER2/neu epitopes that are expressed on HER2/neu-expressing tumor cells (Figure 6B, C). Although HER2-specific Ab responses between DC-targeted or non-targeted HER2 protein were similar as assessed by titer and isotypes, the breadth and functional qualities of the Ab could be different.

Strong HER2-specific immunity induced by DEC-targeting immunization is translated into significant anti-tumor responses in a transplantable tumor model in FVB/N mice. Xeno-priming mice with 5 μg of DEC-HER2 protein in combination with poly IC significantly delayed the development of transplantable neu-expressing tumor (Figure 7). Vaccination not only delayed the growth of the tumors (Figure 7A), but also improved the long-term overall survival of the mice (Figure 7B). These results indicate that an integrated $CD4^+$ and $CD8^+$ T cell immunity is the major tumor protection mechanism in neu-expressing tumor challenged FVB/N mice (Figure 7C, D). These results are consistent with previous reports [61].

We found that $CD8^+$ T cells are playing a more dominant role in tumor protection as depletion of $CD8^+$ T cells had a more dramatic effect on tumor growth and overall survival. Induction of HER2/neu-specific T cell immunity was also confirmed by *in vitro* T cell assays (Figure 7E, B). Interestingly, we observed a robust $CD4^+$ T cell responses when neu-expressing tumor-lysate pulsed DCs were used as the antigen source. The response is even stronger than that using HER2/neu-peptide pulsed DCs as stimuli. There are three explanations for this unexpected finding. First, the quantity of neu-epitopes presented on DCs may be higher with tumor-lysate pulsed DCs. Second, epitope spreading could be induced with DEC-HER2 vaccination. Therefore, the higher responses could represent a cumulative response against HER2/neu and other tumor antigens. Third, posttranslational modifications (glycosylation and phosphorylation etc.) presented on naturally derived neu-epitopes, but not on synthetic peptides, may boost T cell recognition and enhance TCR signal strength resulting a stronger $CD4^+$ T cell activation.

In summary, our results show that targeting HER2 protein to activated DCs in situ significantly enhances anti-tumor T cell immunity and we propose that this strategy provides a feasible approach for immunotherapy in cancer patients.

Conclusions

We demonstrated that immunization of mice with a HER2 protein vaccine targeting DEC^+ DCs *in vivo* induced high levels of T- and B-cell responses as determined by various *in vitro* immune assays. Non-targeted HER2 protein was poorly immunogenic for $CD4^+$ and $CD8^+$ T cells. Analysis of the quality of vaccine induced Th1 cells revealed multiple features that favor anti-tumor immunity, such as breadth, proliferative capacity and multiple cytokine production. This vaccination approach protected mice from neu-expressing tumor outgrowth following as little as 2.7 µg of HER2 protein in the vaccine. Vaccine-induced $CD4^+$ and $CD8^+$ T cells were both essential for tumor protection. This vaccine approach is feasible and cost-efficient in clinical settings. Since a fully humanized DEC mAb has been brought into phase I clinical trials with HIV antigens, this vaccine strategy would seem logical to pursue in the development of vaccines for breast cancer patients.

Abbreviations

Ab: antibody; Ag: antigen; APC: antigen-presenting cell; DC: dendritic cell; ECD: extracellular domain; ELISA: enzyme-linked immunosorbent assay; ELISPOT: enzyme-linked immunosorbent spot; FACS: fluorescence-activated cell sorting; Ig: immunoglobulin; MACS: magnetic-activated cell sorting; HER: human epidermal growth factor receptor; IFN: interferon; IL: interleukin; mAb: monoclonal antibody; MHC: major histocompatibility complex; TNF: tumor necrosis factor

Competing interests

Ralph M. Steinman had financial interests in Celldex, which is developing DEC antibodies for human use. Li-Zhen He and Tibor Keler are current employees of Celldex.

Authors' contribution

The authors' contributions to this research are reflected in the order shown with the exception of RMS, who supervised all aspects of this research and the preparation of this manuscript. BW performed designed and performed most of the immune assays, animal experiments and laboratory analysis and wrote the original manuscript; NZ performed flow cytometry assays, animal experiments and helped draft and edit the manuscript; LZH carried out molecular cloning and prepared HER2 soluble protein; LZ was responsible for fusion antibody production, animal tumor experiments and mouse colony maintenance; JMYK was responsible for hybridoma cell culture and mAb purification; TK participated in data interpretation and was involved in drafting, critical reviewing and revising the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Characterization of HER2 fusion mAb. (**A**) Structure of HER2 fusion mAb. N', amino-terminus; C', carboxyl-terminus; V region, variable region; C region, constant region; ECD, extracellular domain (amino acid 22-653). (**B**) Fusion mAb (DEC-HER2 or Ctrl Ig-HER2) was produced by transient transfection of 293T cells with the appropriate vector and purified on a protein G column. Imperial protein staining (left panel) and Western blotting of the fusion mAb with the anti-mouse IgG1-HRP (αmIgG) or anti-HER2-Biotin/SAv-HRP (αHER2) Ab as indicated below the figures. M, Molecular weight standards (kDa). Lane 1, empty DEC mAb; lane 2, DEC-HER2 mAb; lane 3, Ctrl Ig-HER2 mAb. (**C**) FACS staining data showing the binding capacity of graded doses (0, 0.05, 0.5 and 5 μg/mL) of the indicated fusion mAb to the DEC or Neomycin (Neo) stably transfected CHO cells. Binding was detected by fluorescent-labeled secondary Ab specific for mouse IgG1 or HER2 antigen.

Figure 2. A single dose of DEC-HER2 fusion mAb vaccine immunizes HER2 specific $CD4^+$ T cells *in vivo*. (**A**) Groups of C57BL/6 mice were vaccinated i.p. with 5 μg of DEC-HER2 or Ctrl Ig-HER2 in combination with poly IC (50 μg) and anti-CD40 Ab (25 μg). Additional groups received adjuvants alone or left untreated. Two weeks after immunization, splenocytes were restimulated without added peptides (medium alone), or with 2 μg /ml HER2 peptide pools 1-7 or irrelevant HIV gag peptides. Intracellular cytokine staining was determined by FACS after 6 h *in vitro* stimulation. Percentage of $IFN\gamma^+CD4^+$ T cells is shown as mean ± SE with 3-4 mice per group. One of three independent experiments. (**B**) CFSE dilution assay, mice were immunized as in (**A**), bulk splenocytes were labeled with 2.5 μM CFSE and restimulated with 0.2 μg HER2 peptide pool 1-7 or HIV gag peptide mix or medium alone for 4 d. T cell proliferation was analyzed in combination with intracellular cytokine staining of the cells restimulated for the last 6 h. Percent of proliferating ($CFSE^{low}$) and $IFN\gamma^+CD4^+$ T cells is shown with 3-4 mice per group. One of two independent experiments. (**C**) Th1/Th2/Th17 cytokine ELISA. Mice were immunized as in (**A**). Two weeks after boost, splenic $CD4^+$ T cells and $CD11c^+$ cells were isolated. $3x10^5$ $CD4^+$ cells were incubated with $1x10^5$ $CD11c^+$ cells in 96-well U-bottomed plate in the presence of 2 μg/mL HER2 peptide pool 5 or HIV gag peptides for 48 h. Concentrations of IFNγ/IL-4/IL-10/IL-17 in culture supernatant were measured by ELISA. One of two independent experiments (n=4 mice per group). (* $P<0.05$; ** $P<0.01$, *** $P<0.001$; NS, not statistically significant)

Figure 3. Poly IC as a mono-adjuvant for strong and broad HER2-specific $CD4^+$ T cell responses. C57BL/6 (**A**) or BALB/c (**B**) or FVB/N (**C**) mice were primed with 5 μg DEC-HER2 +

50 μg poly IC and boosted 4 weeks later. Two weeks after the boost, HER2 specific IFNγ production was quantified by ELISPOT assay. All experiments were performed with at least three mice per group and one of two to three independent experiments is shown here. (**D**) Requirement for DEC expression. $DEC^{-/-}$ or wild type C57BL/6 mice were primed and boosted four weeks apart with DEC-HER2+poly IC, Ctrl Ig-HER2+poly IC or none. Two weeks after boost, splenocytes were harvested and restimulated with medium alone or 2 μg/ml HIV gag or HER2 peptide pool 5 for 6 h. IFNγ production was measured by intracellular cytokine staining. FACS blot were shown in the figure. Three to four mice per group for one experiment, total two experiments. (E) Requirement for pattern recognition receptors. Wild type, TLR3 KO or TLR3/MDA5 DKO C57BL/6 mice were immunized with DEC-HER2+poly IC. Two weeks after the boost vaccination, splenocytes were harvested and restimulated with medium alone, or 2 μg/ml HIV gag or HER2 peptide pool 5 for 6 h. IFNγ production was measured by intracellular cytokine staining. Three mice per group. (*** $P<0.001$)

Figure 4. Cross-presentation of HER2 protein by DEC-HER2 immunization. (**A**) FVB/N mice were primed and boosted with DEC-HER2 or Ctrl Ig-HER2 (5 μg) in combination with poly IC (50 μg). Two weeks after the boost, spleen $CD8^+$ T cells were purified by MACS and restimulated with spleen $CD11c^+$ DCs in the presence of medium alone or 1 μg/mL HIV gag peptide or HER2 peptide pools. IFNγ production was measured by ELISPOT assay. (**B**) Proliferative capacity of HER2-specific $CD8^+$ T cells. Mice were immunized as in (**A**), bulk splenocytes were labeled with CFSE and restimulated with medium or 200 ng/mL HIV gag or HER2 peptide pool 1-7 for 4 d. Cells were restimulated for the last 6 h and IFNγ production was measured by intracellular cytokine staining. (**C**) HLA-A2 transgenic mice were vaccinated as in (**A**) and HER2 specific $CD8^+$ T cell responses were measured by ELISPOT assay. Purified $CD8^+$ T cells were restimulated with medium alone or 1 μg/ml HIV gag peptide mix or HER2 peptide pool 1-7. All experiments were performed with at least three mice per group and one of three experiments is shown. (*** $P<0.001$)

Figure 5. HER2 immunization primes strong neu-specific $CD4^+$ T cell responses in mice. (**A**) C57BL/6 mice were primed with DEC-HER2 in combination with poly IC. Two weeks after the boost, splenocytes were restimulated with medium alone, HIV gag peptides, HER2 peptide pool 5 or corresponding neu peptide pool 4 (2 μg/ml). IFNγ production was measured by intracellular cytokine staining. (**B**) Functional avidity of HER2/neu-specific $CD4^+$ T cells. Titrated dose of HER2 or neu peptide pool were used to restimulate splenocytes and IFNγ production was

quantified. Data depicts the percentage of maximum response at each concentration. (*** P<0.001). Two experiments, with total six mice per group.

Figure 6. Induction of anti-HER2/neu humoral immunity. FVB/N mice were vaccinated with DEC-HER2 or Ctrl Ig-HER2 with poly IC as the adjuvant. Sera were collected two weeks after the boost. (**A**) Titers of anti-HER2 IgG were quantified by ELISA as described in Methods. (**B-C**) Binding of HER2/neu-expressing tumor cells with immune sera measured by flow cytometry. Sera were diluted 1:100, 1:400 or 1:1600 and incubated with HER2-expressing E0771/E2 (**B**) or rat neu-expressing NT2.5 (**C**) tumor cells for 15 min at 4°C. Binding of antibody to tumor cells were detected by staining with anti-mouse IgG-PE antibody and FACS analysis. Mean fluorescence index (MFI) are shown. (** P<0.01, *** P<0.001; NS, not statistically significant)

Figure 7. Targeting HER2 protein to DCs protects mice from a challenge with HER2/neu-expressing mammary tumor cells. (**A-B**) FVB/N mice were immunized with DEC-HER2 or Ctrl Ig-HER2 with poly IC, or poly IC alone or left untreated. Mice were challenged with one million NT2.5 tumor cells in the mammary fat tissue ten days after the boost immunization. Tumor growth was monitored by caliper measurement three times a week. Tumor volume (mm^3) is shown in (**A**). Survival analysis is shown in (**B**). Three independent experiments (n=5 per group). (**C-D**) To examine the mechanism of tumor protection, $CD4^+$, $CD8^+$ or both types of T cells were depleted after immunization (before tumor challenge) as described in Methods. Mice were challenged with one million NT2.5 tumor cells and tumor growth curve are shown in (**C**). Survival analysis is shown in (**D**). Two independent experiments (n=10 mice per group). (**E-F**) Anti-tumor immunity was determined fourteen days after tumor challenge. Splenic $CD4^+$ and $CD8^+$ cells were purified by MACS isolation. Splenic $CD11c^+$ cells were purified from naïve FVB/N mice. $CD4^+$ (**E**) or $CD8^+$ (**F**) T cells were restimulated with $CD11c^+$ cells pulsed with 1 µg/ml of HIV gag, HER2 peptide pool 5 or neu peptide pool 4 or 10 µg/ml NT2.5 tumor lysate at T:DC ratio of 3:1 for three days. IFNγ production was measured by ELISPOT assay. Four mice per group, one of two independent experiments. (* P<0.05; ** P<0.01, *** P<0.001; NS, not statistically significant)

Additional files

Additional file 1. Figure S1. Characterization of FLAG-tagged HER2 recombinant protein. Extracellular domain of HER2 was cloned into FLAG-His-tagged expression vector (pS:FLAG-His). FLAG-HER2 protein was produced by transient transfection of 293T cells and further purified with anti-FLAG column (Sigma). The quality of FLAG-HER2 protein was checked with SDS-PAGE gel under non-reducing condition. 2 μg protein was loaded in indicated well.

Additional file 2. Figure S2. Effect of agonistic CD28 mAb on intracellular cytokine staining assay. (**A**) C57BL/6 mice were immunized with DEC-HER2+poly IC. Two weeks after boost, splenocytes were restimulated with medium alone, HIV gag peptides, or HER2 peptide pool 1-7 with or without CD28 mAb (2 μg/mL) during the 6 h simulation. IFNγ production was measured by intracellular cytokine staining. (**B**) Functional avidity of $CD4^+$ T cells. Mice were immunized as in (**A**), splenocytes were restimulated with titrated dose of HER2 peptide pool 4 and IFNγ production was measured by intracellular cytokine staining. Data depicts the percentage of maximum response at each concentration.

Additional file 3. Figure S3. Functional characterization of IL-2-, IFNγ- or TNFα-producing $CD4^+$ T cells by multiparameter flow cytometry. C57BL/6 mice were immunized with DEC-HER2+poly IC. Two weeks after boost immunization, splenocytes were restimulated with 2 μg/mL of HER2 peptide pool 5 and analyzed for cytokines production by FACS. The pie charts show the quality of the cytokine response, comprised of seven functionally distinct populations producing IL-2, IFNγ- and TNFα, individually or in any combination. The percentages are based on the production of the respective cytokines within the live $CD3^+CD4^+$ population.

Additional file 4. Figure S4. Identification of HER2-specific $CD4^+$ T cell epitopes in C57BL/6 mice. Mice were immunized with DEC-HER2+poly IC. Two weeks after the boost immunization, splenic $CD4^+$ and $CD11c^+$ cells were isolated and cocultured in the presence of 2 μg/mL indicated individual HER2 peptide from pool 3, 4, 5, and 7. IFNγ production was quantified by ELISPOT assay. The ID of responding HER2 peptide is indicated above the

Additional file 5. Table S1. Immunogenic peptide sequences.

Additional file 6. Table S2. Prediction of $I\text{-}A^d$ restricted HER2 epitopes.

corresponding bar.

Additional file 7. Figure S5. Depletion efficiency of $CD4^+$ and $CD8^+$ T cells in peripheral blood analyzed by flow cytometry. The day before tumor challenge, peripheral blood cells were harvested by submandibular bleeding and depletion efficiency was analyzed by flow cytometry. Live $CD3^+$ cells were gated for $CD4^+$/$CD8^+$ population analysis. Shown FACS dot plot from one representative mouse.

Table 1: Identification of $CD4^+$ T cell responding peptides in C57BL/6 mice.

Peptide Pool	Responding 15-mer peptide	Position	Sequence	IFNγ spots/$3x10^5$ $CD4^+$ T cells
3	p72	284-298	NPEGRYTFGASCVTA	206 ± 20.8
4	p73	288-302	RYTFGASCVTACPYN	503 ± 183.5
5	p104	405-423	EITGYLYISAWPDSL	34 ± 7.5
	p105	409-427	YLYISAWPDSLPDLS	27 ± 13.6
	p108	421-435	DLSVFQNLQVIRGRI	25 ± 7.5
	p109	425-438	FQNLQVIRGRILHN	24 ± 7.3
7	p148	571-585	NGSVTCFGPEADQCV	43 ± 12.3

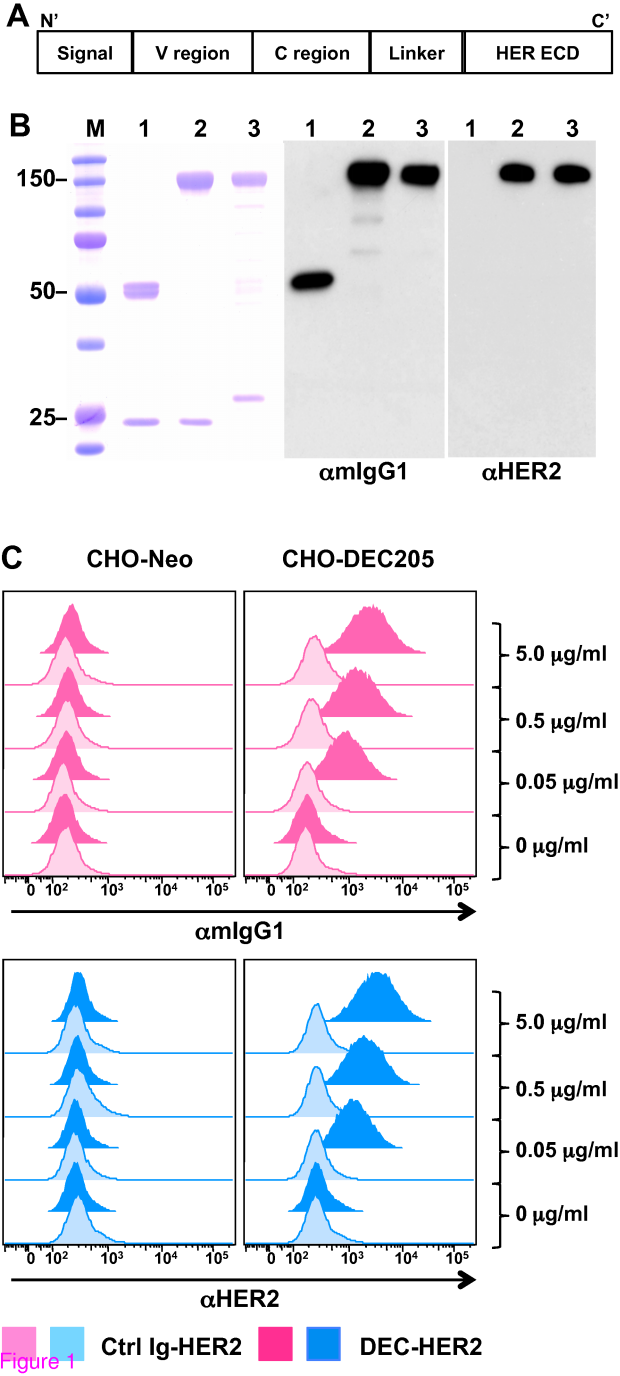
A
N'
C'
Signal
V region
C region
Linker
HER ECD
B
M
1
2
3
1
2
3
1
2
3
150–
50–
25–
αmIgG1
αHER2
C
CHO-Neo
CHO-DEC205
5.0 μg/ml
0.5 μg/ml
0.05 μg/ml
0 μg/ml
0
10²
10³
10⁴
10⁵
αmIgG1
αHER2
Ctrl Ig-HER2
DEC-HER2

Figure 1

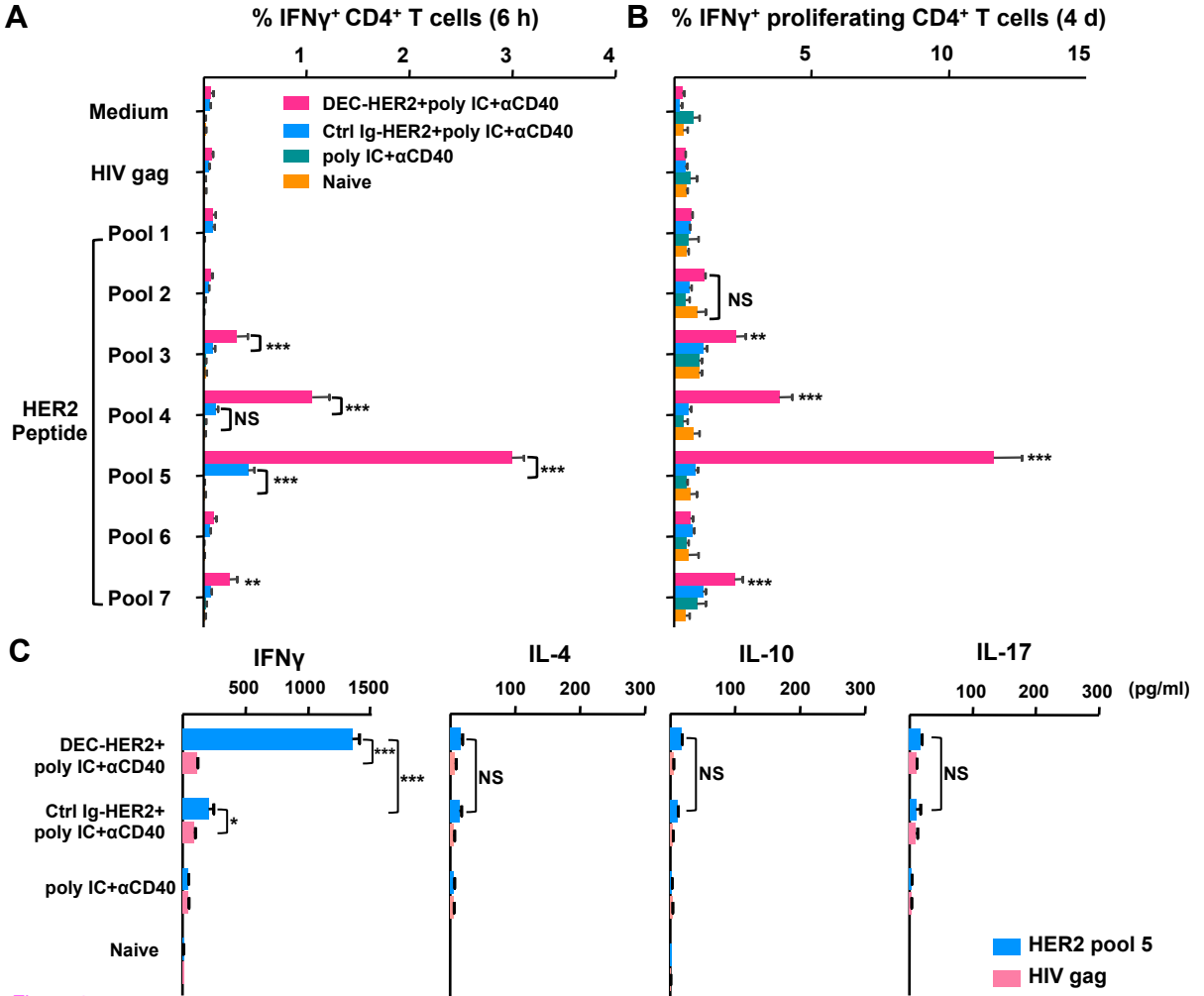
A
% IFNγ+ CD4+ T cells (6 h)
1
2
3
4
Medium
HIV gag
HER2 Peptide
Pool 1
Pool 2
Pool 3
Pool 4
Pool 5
Pool 6
Pool 7
DEC-HER2+poly IC+αCD40
Ctrl Ig-HER2+poly IC+αCD40
poly IC+αCD40
Naive
***
***
NS
***
***
**
B
% IFNγ+ proliferating CD4+ T cells (4 d)
5
10
15
NS
**
***
***
***
C
IFNγ
500
1000
1500
IL-4
100
200
300
IL-10
100
200
300
IL-17
100
200
300
(pg/ml)
DEC-HER2+ poly IC+αCD40
Ctrl Ig-HER2+ poly IC+αCD40
poly IC+αCD40
Naive
***
***
*
NS
NS
NS
HER2 pool 5
HIV gag

Figure 2

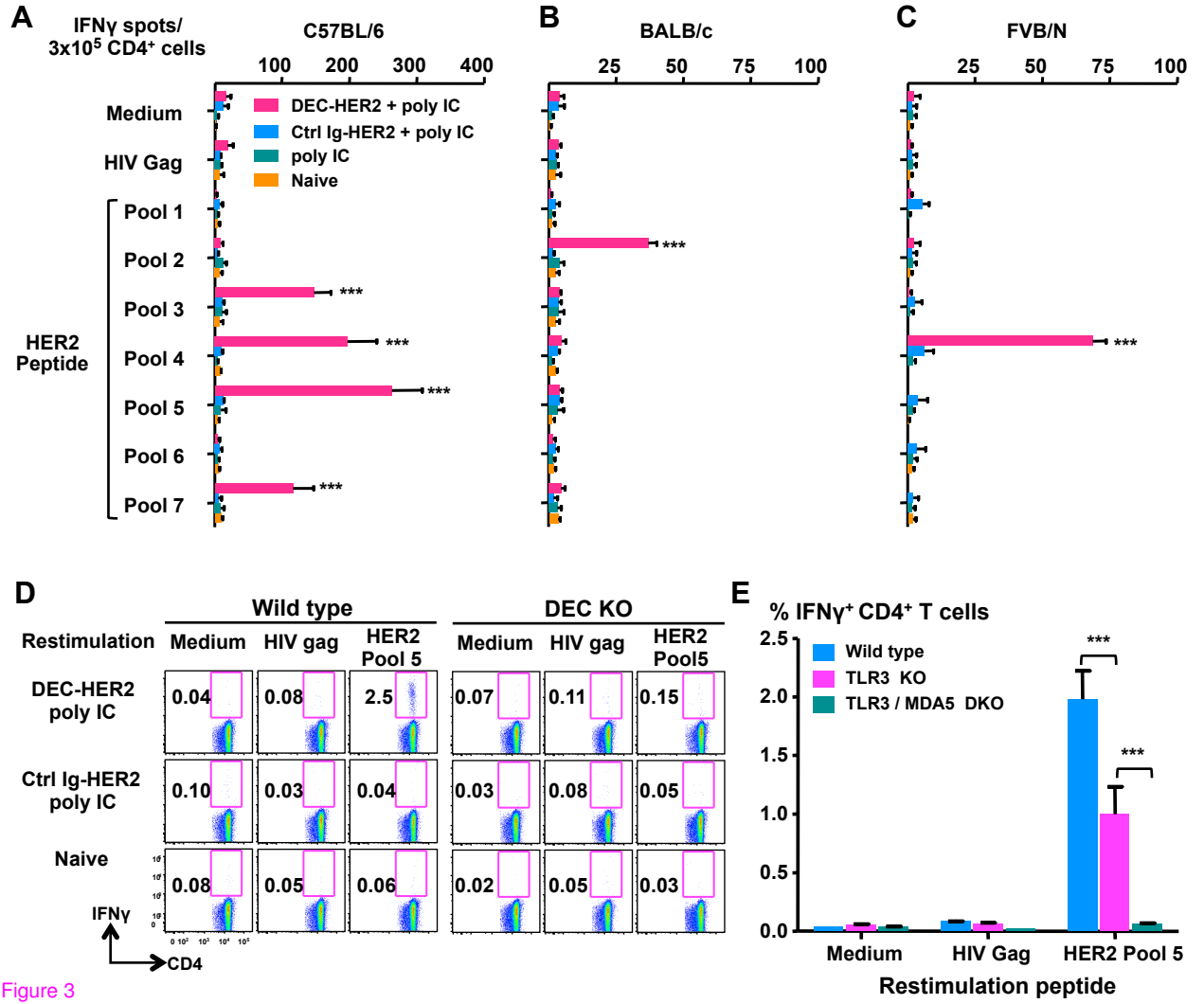
A
IFNγ spots/
3x10^5 CD4+ cells
C57BL/6
100
200
300
400
Medium
HIV Gag
Pool 1
Pool 2
Pool 3
Pool 4
Pool 5
Pool 6
Pool 7
HER2
Peptide
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
poly IC
Naive
***
B
BALB/c
25
50
75
100
C
FVB/N
D
Wild type
DEC KO
Restimulation
Medium
HIV gag
HER2
Pool 5
HER2
Pool5
DEC-HER2
poly IC
Ctrl Ig-HER2
poly IC
Naive
0.04
0.08
2.5
0.07
0.11
0.15
0.10
0.03
0.04
0.03
0.08
0.05
0.08
0.05
0.06
0.02
0.05
0.03
IFNγ
CD4
E
% IFNγ+ CD4+ T cells
Wild type
TLR3 KO
TLR3 / MDA5 DKO
2.5
2.0
1.5
1.0
0.5
0.0
Medium
HIV Gag
HER2 Pool 5
Restimulation peptide

Figure 3

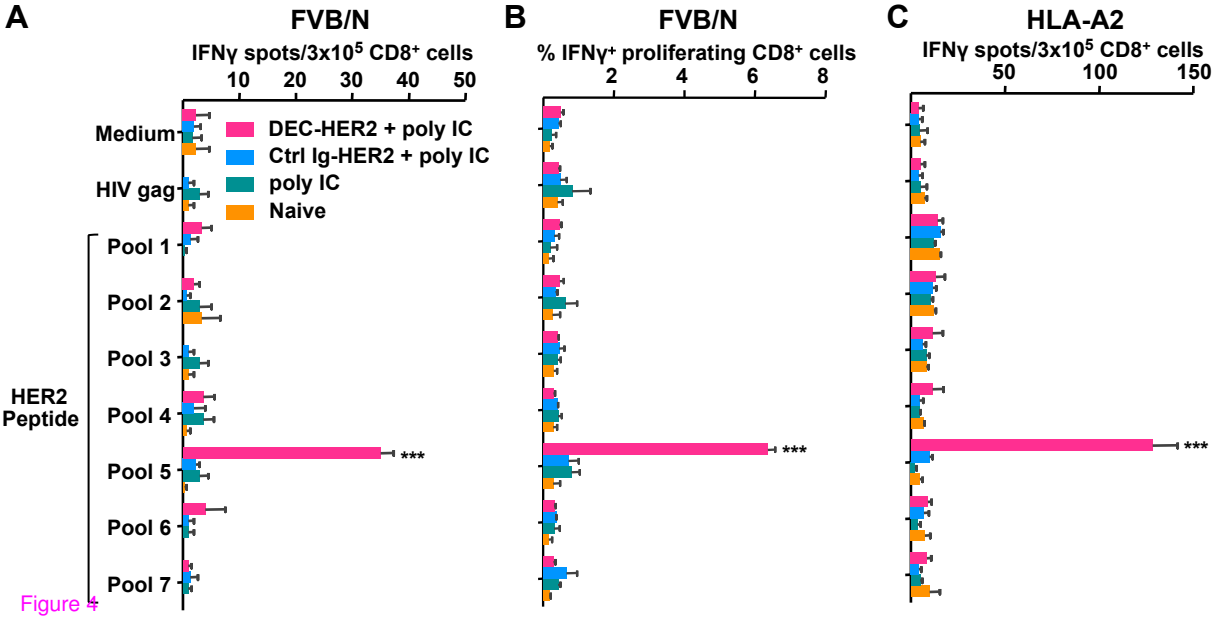
A
FVB/N
IFNγ spots/3x10^5 CD8+ cells
10
20
30
40
50
Medium
HIV gag
HER2 Peptide
Pool 1
Pool 2
Pool 3
Pool 4
Pool 5
Pool 6
Pool 7
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
poly IC
Naive
***
B
FVB/N
% IFNγ+ proliferating CD8+ cells
2
4
6
8
***
C
HLA-A2
IFNγ spots/3x10^5 CD8+ cells
50
100
150
***

Figure 4

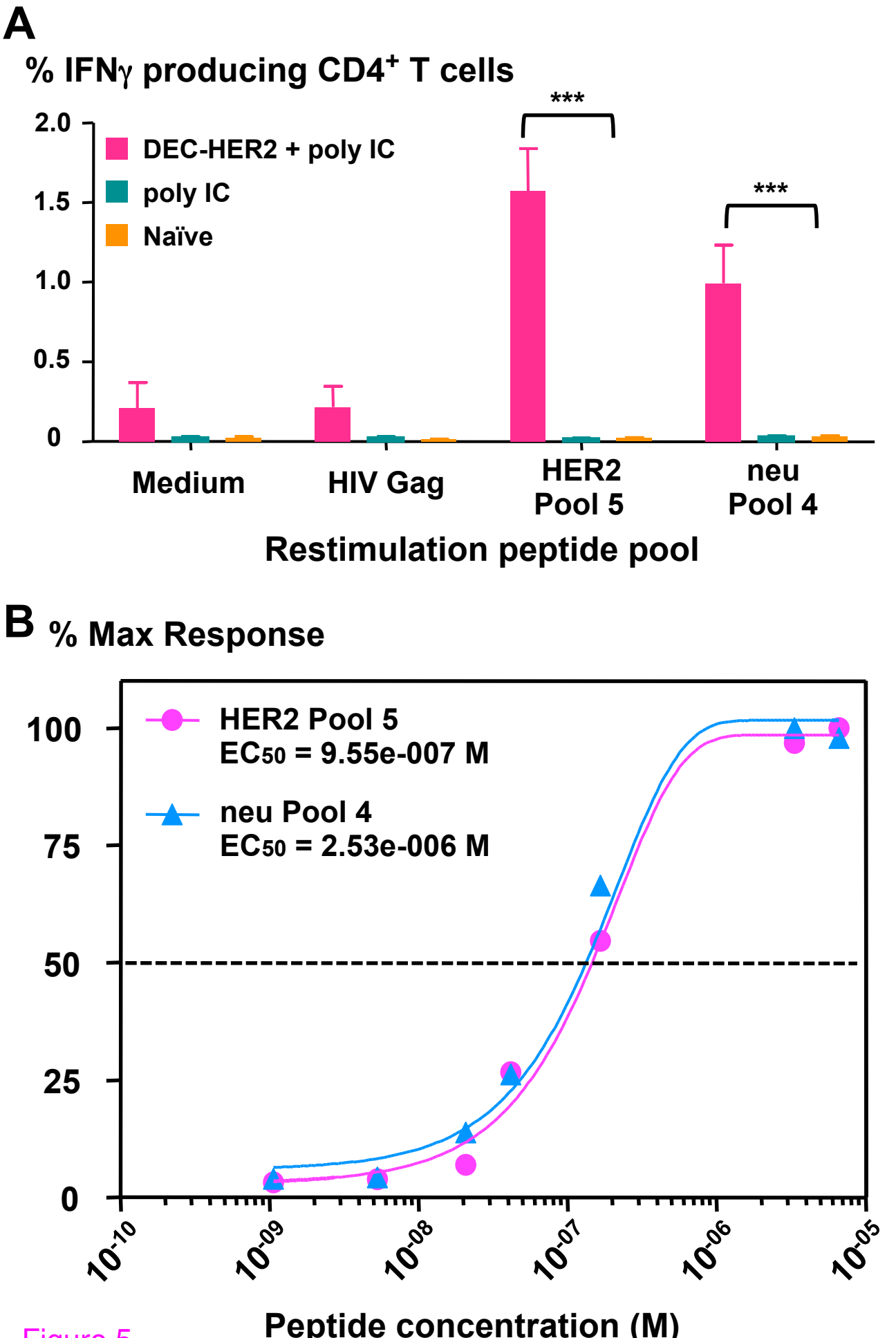
A
% IFNγ producing CD4+ T cells
2.0
1.5
1.0
0.5
0
DEC-HER2 + poly IC
poly IC
Naïve
***
***
Medium
HIV Gag
HER2 Pool 5
neu Pool 4
Restimulation peptide pool
B
% Max Response
100
75
50
25
0
HER2 Pool 5
EC50 = 9.55e-007 M
neu Pool 4
EC50 = 2.53e-006 M
10-10
10-09
10-08
10-07
10-06
10-05
Peptide concentration (M)

Figure 5

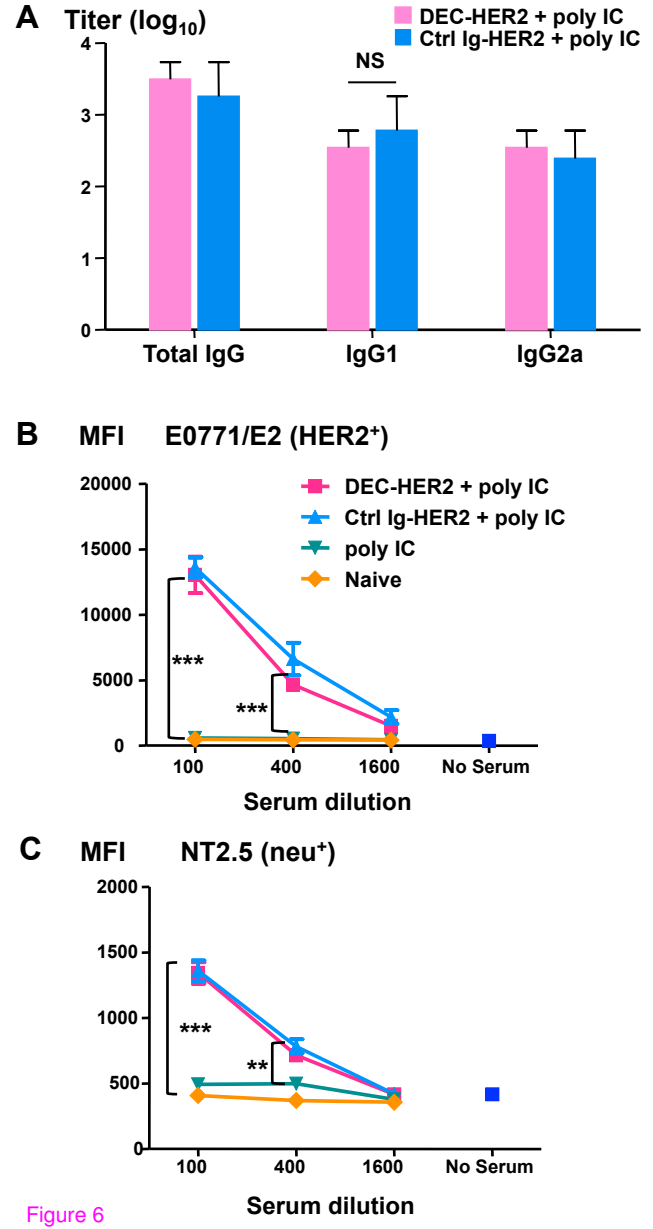
A
Titer (log_{10})
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
NS
Total IgG
IgG1
IgG2a
B
MFI E0771/E2 (HER2+)
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
poly IC
Naive
***
***
100
400
1600
No Serum
Serum dilution
C
MFI NT2.5 (neu+)
***
**
100
400
1600
No Serum
Serum dilution

Figure 6

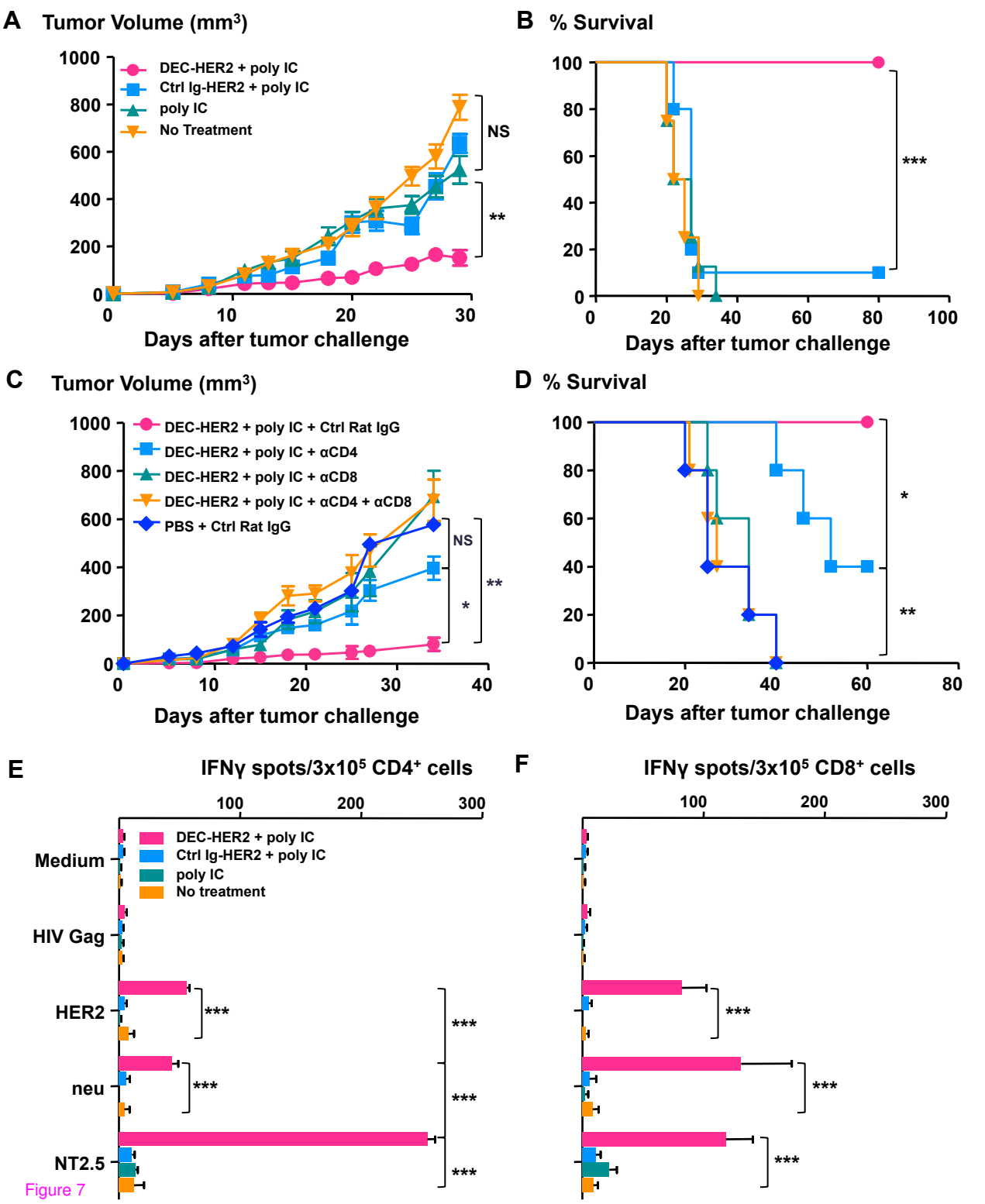
A
Tumor Volume (mm3)
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
poly IC
No Treatment
NS
**
Days after tumor challenge
B
% Survival
***
Days after tumor challenge
C
Tumor Volume (mm3)
DEC-HER2 + poly IC + Ctrl Rat IgG
DEC-HER2 + poly IC + αCD4
DEC-HER2 + poly IC + αCD8
DEC-HER2 + poly IC + αCD4 + αCD8
PBS + Ctrl Rat IgG
NS
*
**
Days after tumor challenge
D
% Survival
*
**
Days after tumor challenge
E
IFNγ spots/3x10^5 CD4+ cells
DEC-HER2 + poly IC
Ctrl Ig-HER2 + poly IC
poly IC
No treatment
Medium
HIV Gag
HER2
neu
NT2.5
***
F
IFNγ spots/3x10^5 CD8+ cells
***

Figure 7

Additional files provided with this submission:

Additional file 1: Figure S1.pdf, 53K
http://breast-cancer-research.com/imedia/2109681813687572/supp1.pdf
Additional file 2: Figure S2.pdf, 51K
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Additional file 3: Figure S3.pdf, 62K
http://breast-cancer-research.com/imedia/1390726550687572/supp3.pdf
Additional file 4: Figure S4.pdf, 67K
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Additional file 5: Table S1.doc, 30K
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Additional file 6: Table S2.doc, 36K
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