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REROUTING PRE-EXISTING HOST VACCINE-INDUCED IMMUNITY TOWARDS BREAST CANCER

Bharat Kumar Reddy Chaganty

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**REROUTING PRE-EXISTING HOST VACCINE-INDUCED IMMUNITY TOWARDS
BREAST CANCER**

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TOWARDS BREAST CANCER**

**A
DISSERTATION**

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By
Bharat Kumar Reddy Chaganty, M.S.

Houston, Texas

May, 2016

Dedication

*To my parents, family and friends for all the love and support they
have provided.*

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I am always grateful to my mentor Dr. Zhen Fan, for his continuous support and guidance. He has been very encouraging and instrumental in my graduation process. I sincerely thank my thesis committee members Dr. Bingliang Fang, Dr. Gregory A. Lizee, Dr. Dean A. Lee, Dr. Gabriel Lopez Berestein and my previous committee member Dr. Kapil Mehta for their valuable inputs, guidance and discussion throughout my research work.

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REROUTING PRE-EXISTING HOST VACCINE-INDUCED IMMUNITY TOWARDS BREAST CANCER

Bharat Kumar Reddy Chaganty, M.S.

Advisory Professor: Zhen Fan, M.D.

For decades, investigators have attempted to activate the immune system to prevent cancer metastasis or recurrence; however, owing to host immune tolerance to cancer antigens and the immunosuppressive environment at tumor sites, many such attempts have failed. The recent success of anti-CTLA4, PD-L1 and PD-1 antibodies targeting immune checkpoint pathways and HPV vaccines has renewed hope that patient survival can be increased through enhancing T-cell responses. We propose to test a novel approach that may bypass host immune tolerance to cancer cells. We hypothesize that host T-cell immunity acquired through vaccination against or natural infection with infectious diseases—e.g., influenza—can be re-routed to breast cancer cells if the cancer cells also express the vaccine antigens and present the antigens in complex with MHC on the cell surface. We chose HER2-breast cancer as a model for proof-of-principle.

In our study, we first examined MHC-I expression, which is required for mediating T cell-mediated response, in a panel of breast cancer cell lines with low or high levels of HER2. A previous study in literature reported an inverse correlation between the levels of HER2 and MHC-I expression in breast cancer cells. In contrast to that finding, we found no significant direct inverse correlation between the levels of HER2 and MHC-I expression. In the presence of peripheral blood mononuclear cells (PBMC), trastuzumab treatment resulted in a significant increase not only in MHC-I expression but also CD86 expression in the panel

of breast cell lines. We demonstrated that this increase in MHC-I expression was correlated with an increase in IFN- γ in the co-culture of breast cancer cells and PMBC through trastuzumab-engaged PBMC. We further showed that trastuzumab treatment enhanced MHC-I expression *in vivo* in 4T1 mouse mammary tumors engineered to overexpress human HER2. To test our hypothesis of therapeutically redirecting preexisting non-cancer immunity developed through vaccination or contract with an infectious disease to cancer *in vivo*, we first immunized BALB/c mice with influenza PR8 virus to mimic flu vaccination, and then we challenged the mice with the highly aggressive 4T1 mouse mammary tumor cells or 4T1 cells lentivirally transduced to express hemagglutinin (HA) and nucleoprotein (NP) antigens of PR8 influenza virus, termed 4T1-HA+NP cells. We found a 70% rejection of 4T1-HA+NP tumors by day 12 and a significant reduction in tumor size and metastasis compared to mock (PBS) immunized mice. We also found that the anti-tumor responses in the influenza immunized group are associated with high percentage of memory CD8⁺ T cells, NK cells, mature DC's, and low percentage of Treg cells and MDSC infiltration to the 4T1-HANP tumors. We next developed a trastuzumab-based immunoliposome to test our hypothesis of redirecting host influenza-induced immunity to cancer by therapeutic delivering influenza antigens to HER2-overexpressing breast cancer cells. The HER2-targeting immunoliposome was confirmed to retain its high affinity binding to HER2 in HER2-overexpressing breast cancer cells *in vitro* and *in vivo*. The immunoliposome effectively delivered labelled antigenic MHC-I influenza antigens *in vivo* and induce tumor regression in HER2-overexpressing tumors in influenza pre-immunized mice but not in naïve mice. Our data confirm that pre-existing non-cancer immunity can be rerouted to cancer cells through therapeutic delivery of relevant antigens using an immunoliposome approach.

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Chapter 1: Introduction

Breast cancer is one of the most commonly diagnosed cancers in women and is the second leading cause of cancer deaths in women. About 15% of the newly diagnosed cancers are breast cancers. Breast cancers can be divided into several subtypes based on their gene expression profiles, including normal like, luminal A and B, basal type and HER2 overexpression (1). The HER2-overexpressing breast cancer makes up 20% to 25% of total breast cancer, in which the HER2 oncogene is amplified. Approximately 60% to 70% of breast cancers are the luminal subtypes (luminal A and B) that are positive for estrogen receptor (ER) and progesterone receptor (PR); whereas the basal subtype of breast cancer presents approximately 10% of total breast cancer and are mostly triple-negative (ER- and PR-negative and without HER2 overexpression) that are very hard to treat due to lack of targeted therapies (2). About 20%-30% of the treated breast cancer patients die due to metastasis (3).

HER2-overexpressing breast cancers

The human epidermal growth factor receptor family (HER family) has 4 members known as EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Among them, HER2 protein is strongly associated with breast cancers. Unlike its other family members, the ligand for HER2 is not known; however, homo dimerization with other HER2 molecules and hetero dimerization with EGFR, HER3, and HER4 leads to intracellular kinase activation of HER2 protein (4-6). HER2 kinase activation leads to activation of series of intracellular signaling events involving phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt), mitogen-activated kinase (MAPK) kinase (MEK)/MAPK pathways, etc. that regulate cell growth, differentiation and survival (7). Structural studies of HER2 molecule showed that HER2 is always in active confirmation and ready for interaction with other members of its family (4, 5).

About 20-25% of breast cancers overexpress HER2, which is casually associated with disease progression and metastasis (8). Apart from the standard chemotherapies, several HER2-targeted therapies are approved for treatment in the clinic, including monoclonal antibodies (trastuzumab, pertuzumab), antibody drug conjugate (trastuzumab emtansine), and a HER2/EGFR tyrosine kinase dual specific small molecule inhibitors (lapatinib) (6). The antibodies bind to the HER2 receptors on the extracellular domain and therefore prevent HER2 homo and hetero dimerization (4, 5), whereas, lapatinib inhibits the intracellular kinase activation of HER2 and EGFR molecules as shown in the Fig.1. (9-11). HER2 overexpressing cancers used to be very difficult to treat before the approval of trastuzumab (6), and trastuzumab is the only HER2 targeted adjuvant therapy approved in combination with chemotherapy (12).

Trastuzumab

Trastuzumab is a FDA-approved HER2-targeting antibody to treat breast cancers and some metastatic stomach cancers that overexpress HER2 (13, 14). It is currently approved standard of care as both adjuvant and neo-adjuvant therapy for HER2-overexpressing breast cancers (13, 14). Trastuzumab is given a single agent after the chemotherapy or in combination with docetaxel or paclitaxel (15). Several mechanisms of action for trastuzumab have been proposed upon trastuzumab binding to the extracellular portion of HER2, i) prevents HER2 homo dimerization, which prevents activation of HER2 kinase and other downstream signaling, leading to inhibition of cell survival, proliferation, and angiogenesis in HER2 overexpressing tumors (16, 17). ii) Antibody-dependent cell-mediated cytotoxicity (ADCC), which involves engagement of Fc-receptor-positive immune cells to the Fc region of IgG antibody and induce direct killing of HER2-overexpressing breast cancer cells through release of perforin and granzyme B, mostly by natural killer (NK) cells (18, 19). Trastuzumab is also shown to sensitize HER2-overexpressing breast cancer cells to radiation therapy and chemotherapy (20).

Moreover, in a recent preclinical study, it was shown that innate and adaptive immune responses, through CD8⁺T cells are also required for antitumor activity of trastuzumab-like anti-HER2 antibody in HER2/*neu*-overexpressing breast cancer cells (21). In clinical studies, patients who showed better response to trastuzumab treatment are found to have more tumor-infiltrating lymphocytes (TIL) present in the tumor stroma (22, 23). Trastuzumab-treated HER2 overexpressing cells are more susceptible to HER2-specific CD8⁺cytotoxic T cells *in vitro* (24). In addition, a recent study using mouse models has shown that trastuzumab-like anti-HER2 antibodies could be combined with immunotherapeutic agents, such as 4-1BB agonistic antibodies and antibodies targeting CTLA4 and PD1 for achieving better therapeutic response (25). Clinical trials of HER2-based tumor cell vaccines expressing growth factors and cytokines combined with trastuzumab are in progress (26-32). These promising preclinical and clinical data suggest that combination of trastuzumab with agents modifying the immune system could achieve better therapeutic outcomes than its currently approved treatment modality to treat or prevent breast cancer metastasis.

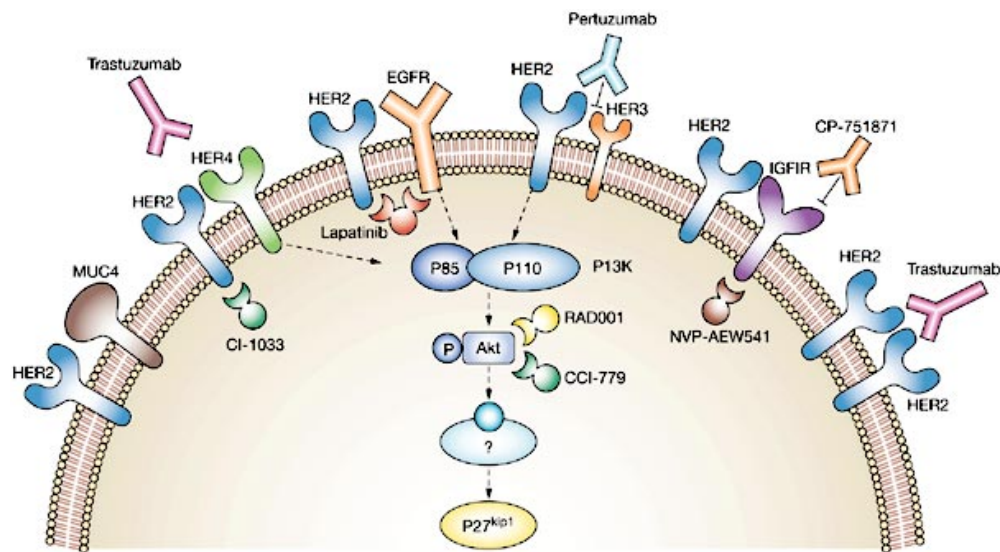


Figure1: Targeted therapies against HER2 signaling

As shown the figure, HER2 homo dimerization with other HER2 molecule and hetero dimerization with HER4 is targeted by trastuzumab. HER2 interaction with HER3 is targeted by pertuzumab, whereas lapatinib inhibits intracellular kinase activity of HER2 and EGFR. All these interactions lead to intracellular activation of PI3K-AKT signaling.

(Figure is taken with permission from Rita Nahta, Dihua Yu, MienChie Hung, Gabriel N Hortobagyi, Francisco J Esteva, Mechanisms of Disease: understanding resistance to HER2 targeted therapy in human breast cancer. Nature Clinical Practice Oncology (2006) 3, 269-280. License number: 3834410810205)

Innate and adaptive immune system for immunotherapy:

The immune system is a combination of well-structured cellular and molecular processes in our body to fight against foreign invaders like bacteria, virus, fungi and other pathogens (33). It is mainly divided into innate and adaptive immune system (33). Whenever a pathogen enters the body it is first recognized by innate immune system that consists of macrophages, dendritic cells, neutrophils, basophils, eosinophils, mast cells and NK cells (34). Pathogens express various conserved molecules on the surface called pathogen-associated molecular patterns (PAMPs) (35). Innate cells recognize these PAMPs through pattern recognizing receptors (PRRs) and produce pro-inflammatory cytokines and chemokines, activated through a series of intra cellular signaling molecules (35). Macrophages and dendritic cells are known as professional antigen-presenting cells; these cells phagocyte pathogens and present antigens to the T cells through major histocompatibility complex (MHC) (34, 35). NK cells are innate immune cells of lymphoid origin that kills cancer cells and virus infected cells based on their missing MHC-I expression on their surface (36). They produce various cytokines and are cytotoxic in nature and are majorly involved in the ADCC (36, 37).

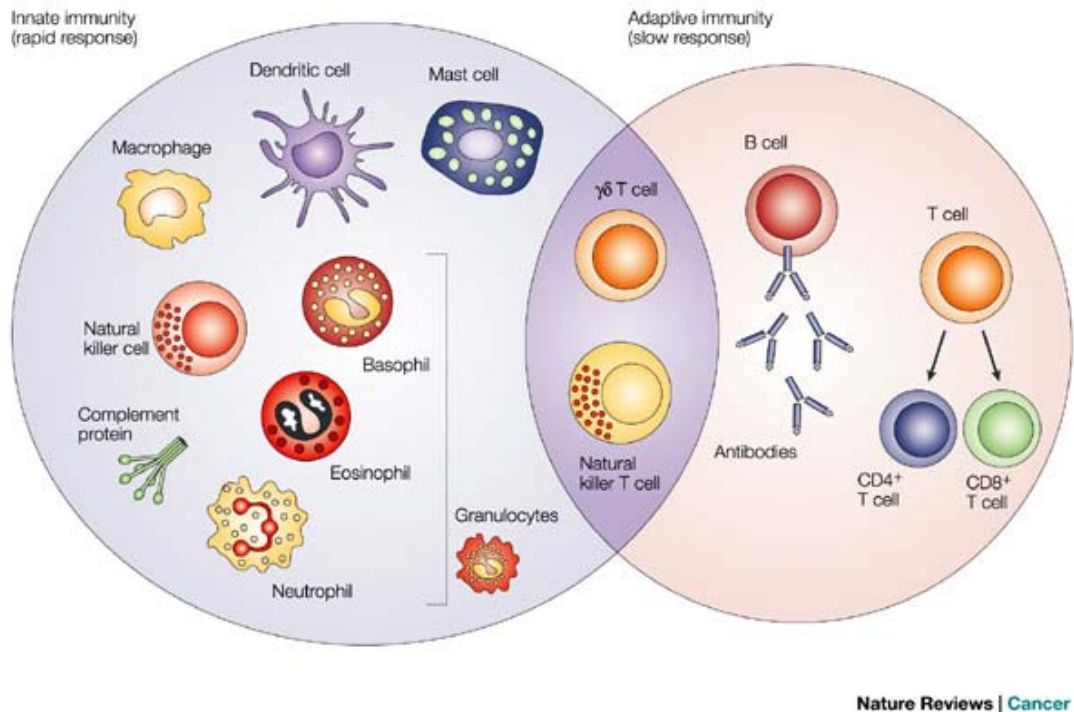


Figure 2: Innate and adaptive immune system in humans

Innate immune system consists of diverse cellular population like granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells that are quick in response and are not specific any antigen. The adaptive immune responses are however very specific to particular antigens and hence are slow in response. They consist of T cells (CD4+ T cells and CD8+ T cells), B cells and antibodies. Both T cells and B cells develop memory immune responses that reside in body for years

(Figure is taken with permission from Glenn Dranoff, Cytokines in cancer pathogenesis and cancer therapy. Nature Reviews Cancer 4, 11-22 (January 2004).License number: 3834481269719)

Adaptive immunity is comprised of T cells and B cells (34), which are often educated and activated by innate immune cells (36). T cells are divided into helper T cells (CD4⁺ T cells), cytotoxic T cells (CD8⁺ T cells) and regulatory T cells (Treg cells) (33). Antigen-presenting cells present foreign peptides to T cells through MHC, where CD8⁺ T cells detect MHC-I-bound endogenous peptides and CD4⁺ T cells detect only MHC-II-bound exogenous peptides (38, 39). T cell receptors recognizing MHC bound peptide is the first signal in the activation of T cells (38, 39). However, complete activation of T cell requires a secondary co-stimulatory signal through interaction of CD28 (on the T cell) and CD80/86 (on APC's) (38-40). Once activated naïve T cells rapidly divide into effector T cells to fight against pathogens. After the clearance of pathogens most of the effector cell population undergoes apoptosis except for a small percentage of population that are converted to memory T cells (41-43). The activity of T cells is very tightly regulated; after the clearance of pathogens the activated T cells are inhibited by Treg cells to avoid potential host tissue damage (41, 43). T cells are also regulated by immune checkpoint molecules CTLA-4, which bind to CD80/86, and PD-1, which binds to PD-L1/L2 on antigen-presenting to shut off the activated responses (44-48). Recently, these immune check point molecules are being successfully targeted by therapeutic interventions in cancer patients. In tumors, immune responses are often under suppression due to the presence of continuous self-antigens and lack of co-stimulation signal, which makes T cells often to become exhausted and anergic (49). These exhausted T cells often express high levels of immune check point molecules CTLA-4 and/or PD-1 on their surface, which can be targeted by therapeutic antibodies (47, 48, 50). Anti-CTLA-4 antibodies, such as ipilimumab, prevent the CTLA-4 and CD80/86 interaction, and anti-PD-1 antibodies, such as nivolumab and pembrolizumab, prevents PD-1 and PD-L1/L2 interaction, leading to activation and proliferation of the T cells (47, 51). Currently, ipilimumab, nivolumab and pembrolizumab antibodies are approved by FDA only for

treating metastatic melanoma (52). Combination of these checkpoint inhibitors showed better survival benefit than monotherapy in clinical trials of metastatic melanoma (52, 53). These anti-immune checkpoint antibodies are also being investigated in clinical trials through combination with other therapeutic antibodies, such as trastuzumab to treat HER2 overexpressing breast cancers (54, 55).

Immune responses in HER2-overexpressing breast cancers

Breast cancers are historically not considered immunogenic compared to melanomas or renal cell carcinomas (56, 57). However, in the case of HER2 overexpressing cancer cells, HER2-based vaccine therapies are in clinical trials owing to HER2 being dominantly expressed in the tumors (26-28, 30). In a series of HER2 peptide vaccine trials using E75 peptides alone or in combination with adjuvants or cytokines, antigen-specific T cell responses were found evident in patients with no high grade toxicities (26-28, 30). Currently, both HER2 whole protein-based and HER2 DNA-based vaccination strategies are also being tested in clinical trials (12). A recent evaluation of somatic mutations in several cancer types found that breast cancers are only partially mutated compared to melanomas (Fig.3) (57). Furthermore, a recent study showed that CD8⁺ T cells and FOXP3⁺ T cell (Treg cells) were infiltrated in large numbers of breast cancer patient samples, where it was found that CD8⁺ T cell infiltration was associated with 27-28% survival benefit in ER⁺/ER⁻-HER2⁺ patients (58, 59). This suggests a small number of HER2 overexpressing breast cancer tumors are immunogenic, due to which there is an infiltration of TIL's. Therefore utilizing immune therapy to the full extent by rendering these breast tumors immunogenic would increase the clinical benefit of patients.

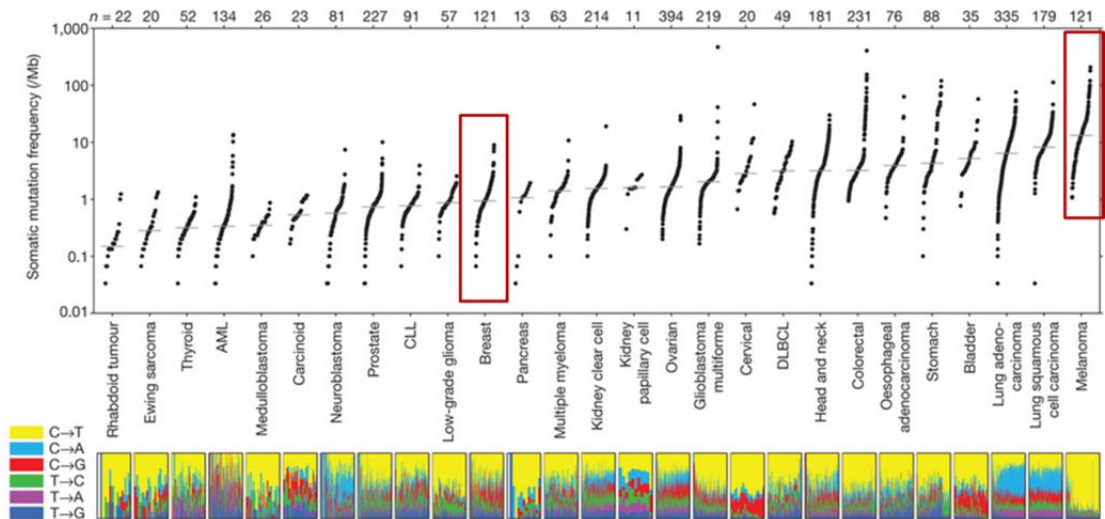


Figure 3: Analysis of somatic mutations in cancers

Somatic mutations in various cancer types ranging from low mutations (left) to high mutations (right), because of the exposure to carcinogens, ultraviolet light, virus etc. Highlighted in the figure are median mutation frequencies of breast cancers and melanoma that dictate the immunogenicity.

(Figure is taken with permission from Michael S. Lawrence, Petar Stojanov, Paz Polak, Gregory V. Kryukov, Kristian Cibulskis, Andrey Sivachenko, Mutational heterogeneity in cancer and the search for new cancer associated genes. Nature 499, 214–218 (11 July 2013). License number: 3834420694634)

Pre-existing immune memory and viral antigen specific immunity:

When a pathogen enters into the body, it triggers a primary innate and adaptive immune response; a portion of B cells and T cells of adaptive immunity later undergo phenotypic changes to become memory B and T cells (33, 35). These memory B and T cells are long lived and stay in the body for years after the infection is resolved (33, 35). Any re-encounter of the same antigens will trigger a massive secondary immune response that is much quicker and potent than the primary response (33, 35). Memory T cells are resistant to apoptosis and don't require re-priming in the lymphoid organs for mounting a response (60-62). Because cancer cells often grow aggressively, whereas primary immune responses are not potent enough to fight them, memory CD8⁺ and CD4⁺ T cell immune responses could then be ideally exploited for the purpose. However, the tumor microenvironments are often immune suppressive due to the presence of large number of Treg cells and cytokines that actively suppress the cytotoxic T cells in the tumors and lymph nodes. Moreover, tumor-associated antigens or cancer-specific antigens are often self-origin and the memory T cells for these antigens are often scant (63). For example, it has been reported that in metastatic melanoma patients where the tumor-specific T cells are found to be exhausted, CMV virus-specific immunity was still intact and functional (64). Therefore, adopting preexisting non-cancer memory T cells specific for foreign antigens could be a better option for mobilizing an immune response, if the tumors are “made” to express related foreign antigens through a therapeutic intervention. Virus- or bacteria-specific immunity already present in the patients could potentially be mobilized for targeting tumors, which could bypass the suppression of tumor microenvironment because they are memory T cells. Supporting this paradigm is some successes using intratumoral injection of bacteria or virus that has been previously tested in melanoma and other easy accessible tumors. The use of oncolytic virus has recently been appreciated evidence by recent approval by FDA of the use of first

oncolytic virus talimogene laherparepvec (T-vec) to treat local lesions in the skin and lymph nodes of melanoma patients (65).

Another example is a recent study reporting that presence of pre-existing bacillus Calmette-Guerin (BCG)-specific immunity in the bladder cancer patients has significantly enhanced the response rates of the patients to intravesical BCG immunotherapy (66). However, manufacturing of these viruses or bacteria in large scales and systemic administration of these microbial agents to patients posed significant challenge due to potential off target effects-induced adverse effects (67). These findings from these studies suggest that utilizing virus- or bacteria-specific pre-existing immunity and rerouting the immunity effectively to the tumor site through a targeted therapeutic intervention could be a viable option.

Targeted therapy using liposomes

Targeted cancer therapies utilize biological or chemical agents that specifically target the molecules of interest in cancer cells for activation or inhibition of a biological process (68, 69). Biologics are biological agents such as antibodies, recombinant proteins, whereas chemical agents for targeted cancer therapy include small molecule inhibitor drugs that achieve similar results (68, 69). There are vehicles for delivering both chemical and biological drugs to the tumors that are very small called nanocarriers (70). Nanocarriers or nanoparticles are particles of size less than 1000 nm; they are generally made of biodegradable materials such as lipids, gold, polymers, carbon nano tubes, quantum dots, etc.(70-74). Using liposomes to deliver chemotherapy drugs, siRNA, micro RNA and peptides to the tumors is well-investigated and appreciated. Currently, there are seven approved liposome formulated drugs in the clinic (70).

Liposomes can be either cationic or neutral, depending on the total charge present on them (71). These two types of liposomes have different bio distribution in the body that

significantly affects the kinetics, dynamics, efficacy and toxicity of the drugs delivered (71). Cationic liposomes are very efficacious in delivering drugs or siRNA to the cells; however, they are majorly trapped in the reticular endothelial system of the liver and significant toxicity has been reported in mouse studies (75, 76). To minimize the toxicities of cationic liposomes researchers have adopted neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) liposomes (77). DOPC liposomes were shown to successfully deliver siRNA targeting many oncogenic molecules to the tumors and achieved significant effect on inhibiting tumor growth in vivo with minimal toxicities (71, 77). However, these neutral nanoliposomes are not specifically targeted to cancers that make this delivery method solely depending on the leaky structure of tumor blood vessels. Polyethylene glycol (PEG)-lated liposomes showed partial improvement in the distribution and stability of the liposomes (78, 79). Hence to further enhance the delivery efficiency of liposomes, efforts were made to chemically conjugate antibodies or antibody fragments to the liposomes and this method was proven to improve the dynamics of liposomes with minor loss of antibody function (71). In addition to antibodies, natural ligands, peptides, aptamers, have also been explored (71, 80, 81). However, although these targeted delivery resulted in improvement than liposomes alone, they are still not effective enough to eradicate the tumors. Hence, a multifunctional antibody-based immune liposome that would combine several functions in one, such as blocking cell signaling, attracting immune cells and delivery payloads to the tumors to tumors sites without toxicity or with only minimal toxicity is highly desirable.

Objective and aims of the current work

Despite remarkable progress in past decade, HER2-overexpressing breast cancer remains a clinical problem as resistance to trastuzumab is common (8, 16). To effectively treat or prevent recurrence or metastasis of HER2-overexpressing breast cancer, a single treatment strategy is often not sufficient. Combining trastuzumab-induced therapeutic effects with powerful preexisting noncancer immunity would enhance clinical responses in cancer patients. However, as stated, breast cancers are generally not immunogenic due to being less frequently mutated and as per current evidence there is no evidence of virus or similar pathogens associated with this disease (56, 57). Hence, we propose to deliver influenza-related peptides via a trastuzumab-based immunoliposome that would render HER2-overexpressing breast tumors immunogenic so that they could be targeted by pre-existing influenza-specific memory CD8⁺T cells recruited to the tumor site. Of course, this strategy requires the expression of MHC-I on targeted cancer cells, a prerequisite that is critical for peptide presentation to the CD8⁺T cells (33, 36, 38, 39). On the basis of these considerations, we formulated the three specific aims of the study:

1. To examine the extent to which the expression of HLA-ABC in HER2-overexpressing breast cancer can be upregulated by trastuzumab through engaging immune effector cells.
2. To determine the extent to which pre-existing influenza immunity can effectively suppress the metastasis of aggressive mouse mammary tumors lentivirally transduced to express influenza antigens.
3. To develop a HER2-targeting immunoliposome for delivery of influenza antigenic peptides to HER2-overexpressing mouse mammary tumors and suppress the metastasis of aggressive mouse mammary tumors in mice pre-immunized with influenza virus.

Chapter 2: Correlation between HER2 and HLA-ABC in breast cancer cells

Contents of this chapter are based on Bharat K. R. Chaganty, Yang Lu, Songbo Qiu, Srinivas S. Somanchi, Dean A. Lee & Zhen Fan. Trastuzumab upregulates expression of HLA-ABC and T cell costimulatory molecules through engagement of natural killer cells and stimulation of IFN- γ secretion. OncoImmunology (Online). DOI: 10.1080/2162402X.2015.1100790

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Reference Number: P033016-04

Background:

Expression of MHC-I molecules also known as HLA-ABC in humans and H-2 antigens in mice are necessary and crucial for recognition by cytotoxic CD8⁺ T cells (82, 83). However, cancer cells are known to deploy multiple immunosuppressive mechanisms, including downregulation of MHC-I expression, to evade T cell responses (82). Few studies reported an inverse correlation between HER2 level and HLA-ABC expression in esophageal and breast cancer cells (84-86).

2.1 Lack of inverse correlation between HER2 and HLA-ABC in breast cancer cells

To determine if there is an inverse relationship between HER2 expression level and HLA-ABC expression level across multiple human breast cancer cell lines, we first examined expression of HLA-ABC in a panel of 10 breast cancer cell lines with different levels of HER2 expression using flow cytometry analysis after double-staining of the cells with trastuzumab plus fluorescein isothiocyanate (FITC)-conjugated anti-human IgG antibody and allophycocyanin (APC)-conjugated anti-HLA-ABC antibody. As shown in Figure 4A, in which

the 10 cell lines are arranged from low to high with respect to mean fluorescence intensity (MFI) value of HER2 expression, cell lines with low or undetectable levels of HER2 expression had MFI values of HLA-ABC expression ranging from very high (in MDA-MB-231 and T47D cells) to very low (in MCF7 and ZR75B cells). Cell lines with high levels of HER2 expression also had MFI values of HLA-ABC expression ranging from high (in MDA-MB-361, SUM190, and HCC1954 cells) to relatively low (in MDA-MB-453, SKBR3, and BT474 cells). Pearson correlation analysis showed no significant inverse correlation between HER2 expression and HLA-ABC expression in the cell line panel in terms of either MFI values ($p=0.21$) or percentages of positive cells ($p=0.44$) (Fig. 4B). Together, these data suggest that there is no definitive inverse correlation between the levels of HER2 and HLA-ABC expression among these human breast cancer cell lines

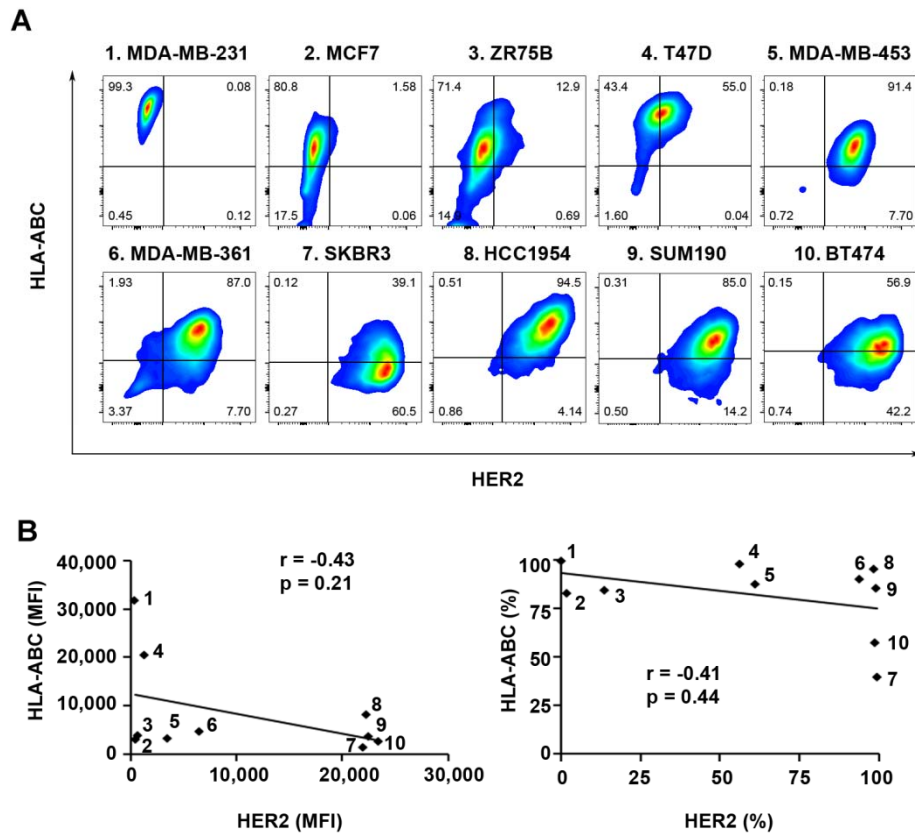


Figure 4: Expression of HLA-ABC in a panel of human breast cancer cell lines with and without overexpression of HER2

The indicated breast cancer cells were double-stained with APC-conjugated anti-HLA-ABC antibody and trastuzumab plus FITC-conjugated anti-human IgG antibody and subjected to flowcytometry analysis. **A.** Flow cytometric data on HLA-ABC expression versus HER2 expression in each cell line in contour plots. **B.** MFI values of HLA-ABC expression versus MFI values of HER2 expression (left) and percentages of HLA-ABC-positive cells versus percentages of HER2-overexpressing cells (right) in scatter plots. Pearson correlation coefficients (r) and p values are shown. The numbers next to the dots in the scatter plots correspond to the numbers next to the names of breast cancer cell lines in A.

2.1 Effect of altering HER2 level or kinase activity on the level of HLA-ABC expression in breast cancer cells

To determine whether there is a causal relationship between HER2 expression and HLAABC expression in HER2-overexpressing breast cancer cells within the same genetic context, we first compared the HLA-ABC expression between MCF7 breast cancer cells and MCF7-HER18 cells, a subline of MCF7 cells transfected to overexpress HER2 that effectively activates the signaling pathways downstream of HER2 in the cells (87, 88). As shown in Figure 5A, overexpression of HER2 does not lead to a decrease but rather a moderate increase in the MFI value of HLA-ABC expression in MCF7-HER18 cells compared to MCF7 cells. We next examined HLA-ABC expression in five HER2-overexpressing breast cancer cell lines (BT474, SKBR3, SUM190, MDA-MB-361, and HCC1954) after knockdown of HER2 expression by each of two different siRNAs. As shown in Figure 5B, the impact of HER2 knockdown on HLA-ABC expression differed by HER2-targeting siRNA and cell line. Knockdown of HER2 by siRNA#1 led to a significant increase in HLAABC expression (in terms of both MFI values and percentages of HLA-ABC-positive cells) in SUM190, MDA-MB-361, and HCC1954 cells, in which the baseline MFI and the baseline percentage of HLA-ABC-expressing cells were high, but a significant decrease in HLA-ABC expression in BT474 and SKBR3 cells, in which the baseline MFI and the baseline percentage of HLA-ABC-expressing cells were relatively low (Fig. 5B, lower panel). In contrast, siRNA#2, which produced slightly weaker HER2 knockdown than siRNA#1 did, had no significant effect on HLA-ABC expression except in BT474 cells, in which siRNA#2 lowered HLA-ABC expression as measured by both MFI and percentage of HLA-ABC-positive cells.

We found that in the three cell lines in which siRNA#1 led to an increase in HLA-ABC expression (SUM190, MDA-MB-361, and HCC1954; Figure 5A), siRNA#1 also led to a

significant increase in the levels of both total and Y-701- phosphorylated STAT1 (Figure 5C), which is a major transcription regulator of HLA-ABC expression (89), whereas siRNA#2 either had no effect on or led to a decrease in the level of Y-701-phosphorylated STAT1.

To determine whether there is an association between the level of HER2 kinase activity and HLA-ABC expression in HER2-overexpressing breast cancer cells, we examined whether treatment of HER2-overexpressing breast cancer cells with lapatinib, a HER2 kinase inhibitor, had an effect on HLA-ABC expression in such cells. As shown in Figure 6A, treatment with lapatinib at 0.5 or 1 μ M for 24 h inhibited HER2 kinase activity with different degrees in all five HER2-overexpressing breast cancer cell lines tested, as previously reported by others (90). However, we found no increase in HLA-ABC expression in any of the five cell lines; rather, we found decreases in the MFI value, the percentage of cells expressing HLA-ABC, or both, with particularly pronounced decreases in SKBR3 and SUM190 cells (Fig. 6B). Of note, as shown in Figure 3A, lapatinib, in addition to inhibiting the well-documented pathways downstream of HER2, including phosphorylation of PI3K/Akt and MEK/Erk, inhibited tyrosine phosphorylation of STAT1. This inhibition of activation-specific phosphorylation of STAT1 may be responsible for lapatinib-induced decrease in HLA-ABC expression.

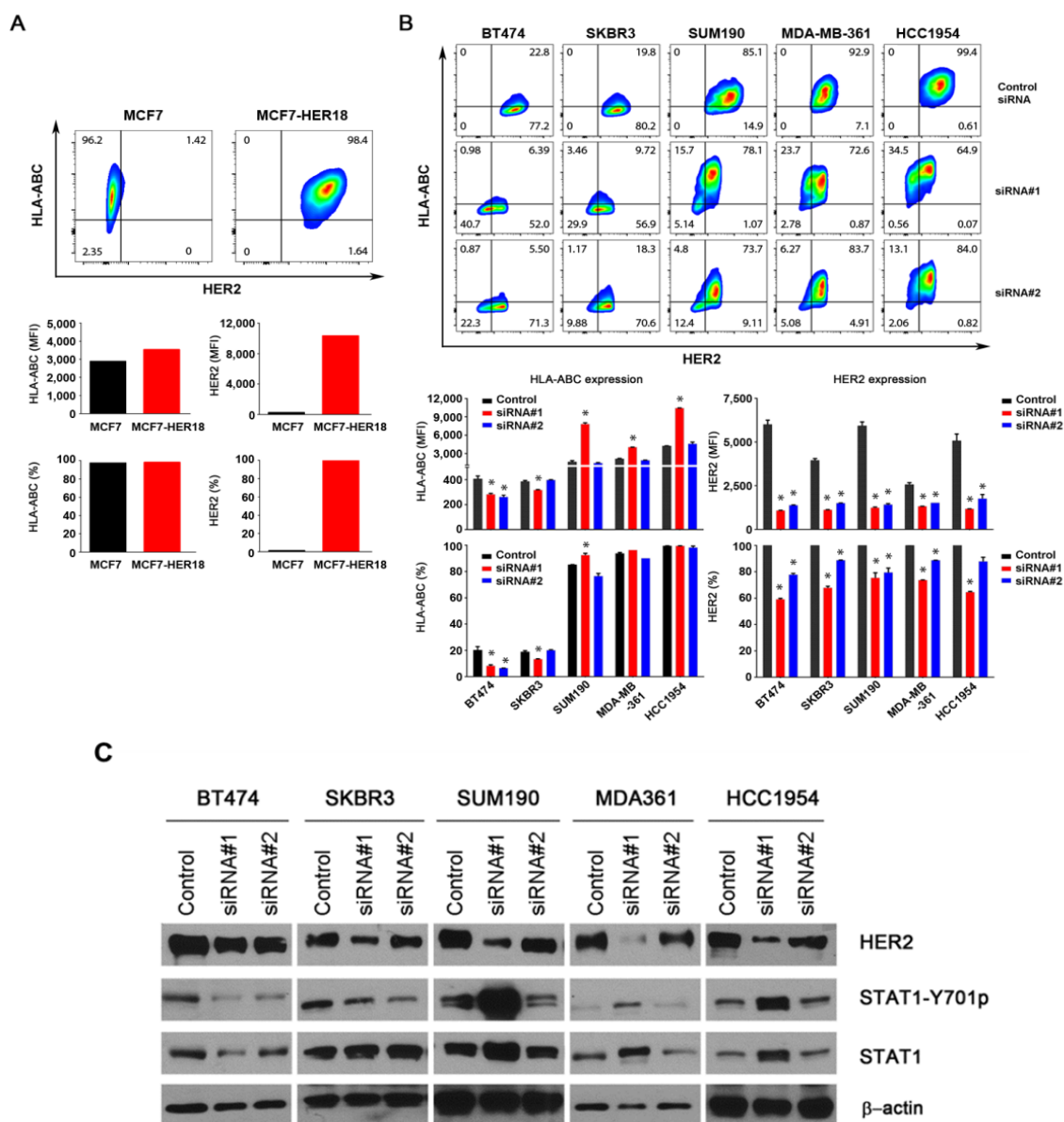


Figure 5: Effect of HER2 overexpression and knockdown on the level of HLA-ABC expression in breast cancer cells

A. MCF7 and MCF7-HER18 cells were double-stained with APC-conjugated anti-HLAABC antibody and trastuzumab plus FITC-conjugated anti-human IgG antibody and subjected to flow cytometry analysis. Upper panel, flow cytometric data on HLA-ABC expression versus HER2 expression in MCF7 and MCF7-HER18 cells in contour plots. Lower panel, MFI values of HLA-ABC and HER2 expression (left) and the percentages of cells expressing HLA-ABC and

HER2 (right) in MCF7 and MCF7-HER18 cells. B. The indicated HER2-overexpressing human breast cancer cells were transfected with one of two different HER2-specific siRNAs or control siRNA using Lipofectamine for 72 h in culture. The cells were double-stained for flow cytometry analysis of HLA-ABC and HER2 and the data were plotted as described in A. C. The cell lysates were subjected to Western blotting with the indicated antibodies.* $p < 0.05$ compared with corresponding control.

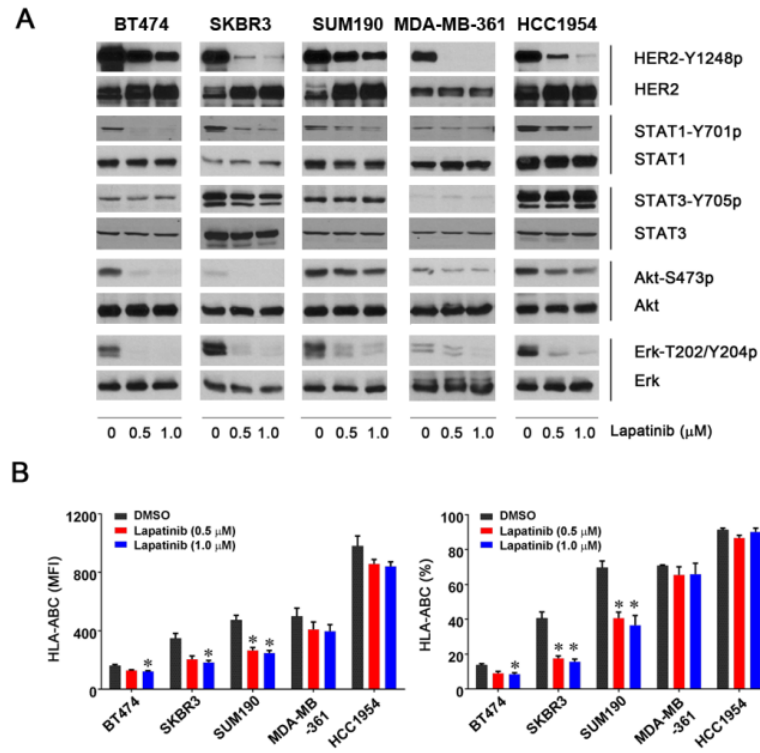


Figure 6: Effect of lapatinib on the level of HLA-ABC expression in HER2-overexpressing breast cancer cells

The indicated HER2-overexpressing human breast cancer cells were treated with vehicle (DMSO) or lapatinib (0.5 or 1.0 μM) for 24 h. After treatment, aliquots of cells were lysed for Western blot analysis with the indicated antibodies (**A**) or stained with APC-conjugated anti-HLA-ABC antibody for 30 min and subjected to flow cytometry analysis (**B**). Any cells undergoing apoptosis following treatment (Annexin-V-positive cells) were gated out before analysis of the flow cytometric data. Shown in (**B**) are the MFI values of HLA-ABC expression (left) and the percentages of HLA-ABC-positive cells in the cell lines treated with vehicle or lapatinib (right). * $p < 0.05$ compared with corresponding control.

Together, our data show that there is a lack of direct inverse correlation between the levels of HER2 expression and HLA-ABC expression in breast cancer cells. Although knockdown of HER2 expression by one particular siRNA (siRNA#1), which was associated with an increase in the level of STAT1, upregulated HLA-ABC expression in a cell type/context specific manner, knockdown of HER2 expression by other siRNAs or inhibition of HER2 kinase activity by lapatinib downregulated HLA-ABC expression in the cell line panel, suggesting that inhibition of HER2 signaling alone does not enhance and may even inhibit HLA-ABC expression in HER2-overexpressing breast cancer cells.

Chapter 3: Trastuzumab increases HLA-ABC and CD86 expression on breast cancer cells in the presence PBMC through IFN γ secretion

Contents of this chapter are based on Bharat K. R. Chaganty, Yang Lu, Songbo Qiu, Srinivas S. Somanchi, Dean A. Lee & Zhen Fan. Trastuzumab upregulates expression of HLA-ABC and T cell costimulatory molecules through engagement of natural killer cells and stimulation of IFN- γ secretion. OncoImmunology (Online). DOI: 10.1080/2162402X.2015.1100790

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Reference Number: P033016-04

Background:

MHC-I molecules present endogenous and intracellular peptides to CD8⁺ T cells, where the T cell receptor recognizes the MHC-I bound peptides i.e. called first signal of antigen presentation. For a T cell to be fully activated it needs a secondary signal through co-stimulatory molecules CD80 or CD86 binding to CD28 on the T cells (38-40). Cancer cells often down regulate their MHC-I expression to escape the recognition from T cells (82). Many therapeutic interventions are adopted to increase the MHC-I expression on cancer cells.

3.1 Trastuzumab increases HLA-ABC expression in HER2-overexpressing breast cancer cells in the presence of PBMC by engaging NK cells and thereby stimulating IFN- γ secretion

A previous study showed that trastuzumab had no impact on the expression of HLA-ABC or CD80 and CD86 T cell co-stimulatory molecules in non-breast cancer cells (24). Trastuzumab was recently reported to induce transcriptional inhibition of HER2 expression in HER2-overexpressing breast cancer cells by engaging Fc receptor-expressing immune effector

cells to stimulate IFN- γ secretion in the presence of PBMC through STAT1 activation (91). Because IFN- γ is a potent cytokine that stimulates HLA-ABC expression by activating STAT1 signaling (92), we decided to examine whether the presence of immune effector cells influences any effect of trastuzumab on HLA-ABC expression. We first cultured BT474, SKBR3, SUM190, MDA-MB-361, and HCC1954 cells for 48 h in the presence or absence of trastuzumab in culture medium. As shown in Figure 7, there were no significant changes in HLA-ABC expression level in these cells, and trastuzumab produced a decrease in the percentage of SUM190 cells expressing HLA-ABC. As expected, when these cells were co-cultured with PBMC, both the MFI value and the percentage of HLA-ABC-positive cells were markedly increased compared to the values in mono-culture. Notably, however, in all five cell lines, when cells were co-cultured with PBMC, the MFI value of HLA-ABC expression (but not the percentage of HLA-ABC-positive cells) was significantly higher in trastuzumab-treated cells than in control antibody-treated cells.

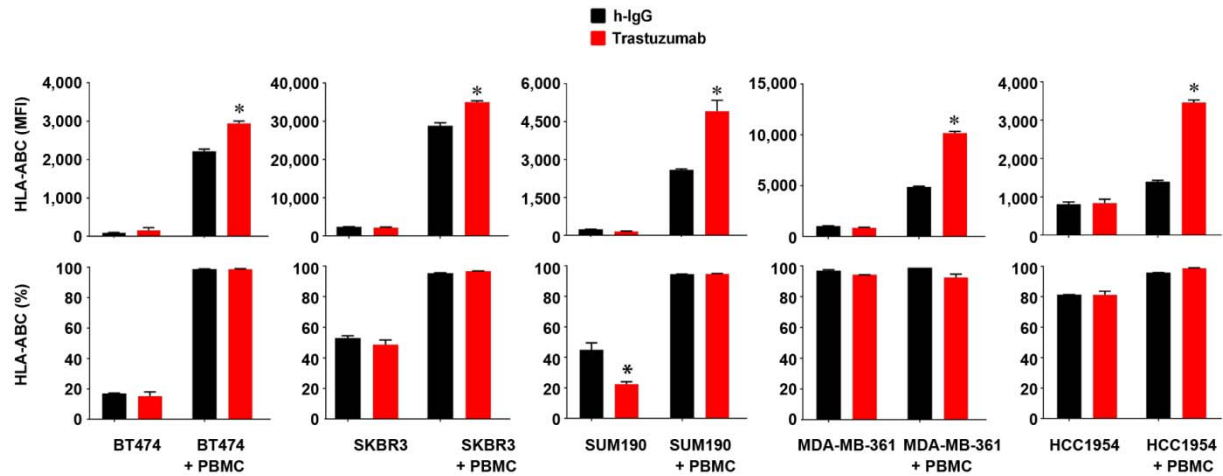


Figure 7: Increased HLA-ABC expression in HER2-overexpressing breast cancer cells by trastuzumab in the presence of PBMC

The indicated HER2-overexpressing human breast cancer cells were cultured in medium supplemented with 10% fetal bovine serum and were treated with 5 $\mu\text{g/ml}$ (~ 30 nM) trastuzumab or a control humanized IgG (h-IgG; bevacizumab) in mono-culture or co-culture with human PBMC at a ratio of 1:5 (cancer cells versus PBMC) for 48 h. After treatment, any PBMC grown in suspension were removed by washing the cells with PBS. The cells were then stained with APC-conjugated anti-HLA-ABC antibody and subjected to flow cytometry analysis. Any residual PBMC in the co-culture were gated out during flow cytometric analysis on the basis of the size difference between PBMC and the co-cultured breast cancer cells. Shown are the MFI values of HLA-ABC expression (upper) and the percentages of HLA-ABC-positive cells (lower). The data presented are from multiple experiments using PBMC from different donors. * $p < 0.05$ compared with corresponding control.

We used enzyme-linked immunosorbent assay (ELISA) to measure the level of IFN- γ in conditioned media from the co-culture experiment of BT474, SUM190, and HCC1954 cells with PBMC shown in Figure 7. We found that the level of IFN- γ was significantly higher in the conditioned media from trastuzumab-treated cell cultures than in the conditioned media from control antibody-treated cell cultures in the presence of PBMC, with particularly pronounced increases in BT474 and SUM190 cells (Fig. 8A). Because PBMC can secrete many cytokines, to confirm that the increase in IFN- γ was the cause of the trastuzumab-induced upregulation of HLA-ABC expression in breast cancer cells shown in Figure 7, we co-cultured BT474 and SUM190 cells with PBMC with and without an IFN- γ -neutralizing antibody. We found that the presence of an IFN- γ -neutralizing antibody abolished the trastuzumab-induced increase in the MFI of HLA-ABC in the co-cultures (Fig. 8B). This finding strongly indicates that IFN- γ secreted as a result of trastuzumab engagement with immune effector cells in PBMC plays a critical causal role in trastuzumab-mediated increase in HLA-ABC expression.

To identify the type of immune effector cell with the greatest contribution to the IFN- γ production in the conditioned media from the co-culture experiments, we sorted the NK cells (CD56+), monocytes/macrophages (CD14+), and T cells (CD3+) from the PBMC and subjected BT474 and SUM190 cells to co-culture with the individually sorted cells in the absence or presence of trastuzumab or a control antibody. The results (Fig. 8C) showed that NK cells were the main contributors to the increase in IFN- γ production in HER2-overexpressing breast cancer cells after trastuzumab treatment.

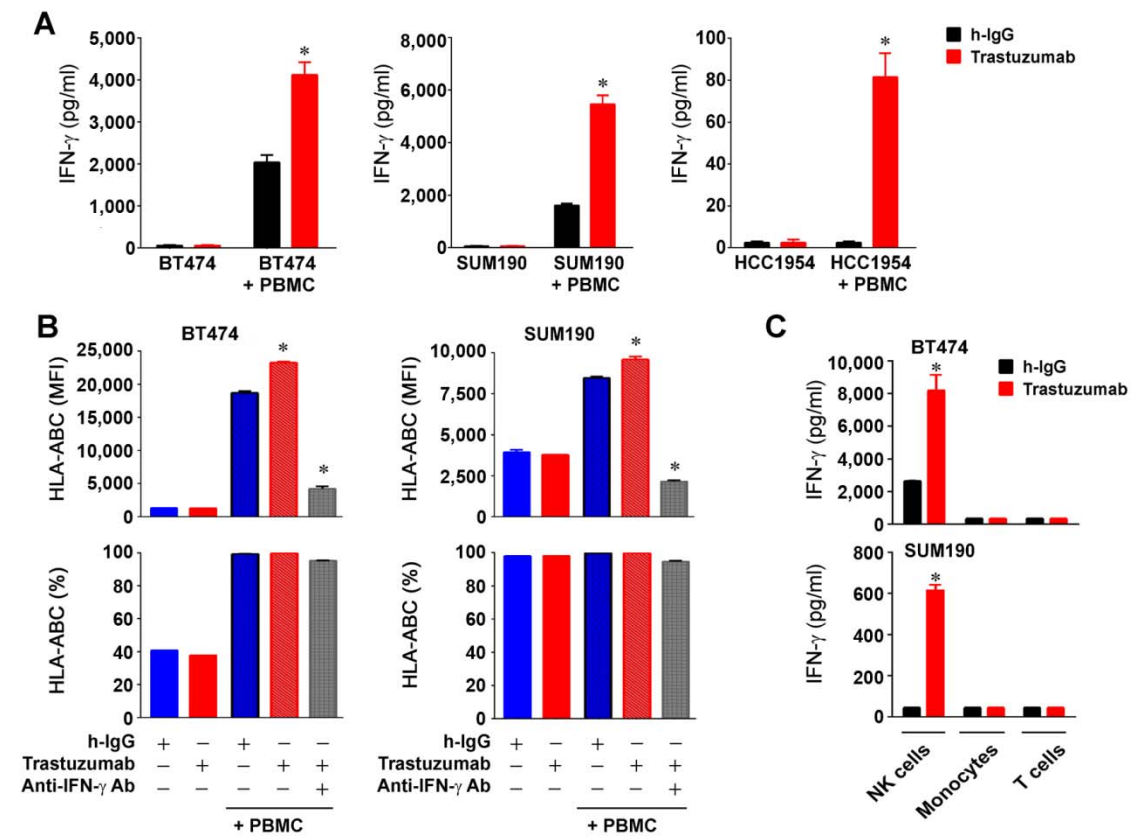


Figure 8: Increased HLA-ABC expression in HER2-overexpressing breast cancer cells by trastuzumab through engagement of NK cells and stimulation of IFN- γ secretion

A. BT474, SUM190, and HCC1954 cells were cultured in medium supplemented with 10% fetal bovine serum and were treated with 5 μ g/ml (~30 nM) trastuzumab or a control humanized IgG (h-IgG; bevacizumab) in mono-culture or co-culture with human 1.5×10^6 PBMC (at a ratio of 1:5, cancer cells versus PBMC) for 48 h. Cell-free conditioned medium from each culture was used for detecting the presence of human IFN- γ with an ELISA kit. * $p < 0.05$ compared with corresponding control.

B. BT474 and SUM190 cells were treated with the conditioned medium from the monoculture and co-culture described in (A) or with the conditioned medium plus an IFN- γ neutralizing antibody as indicated for 48 h. The cells were then stained with APC-conjugated anti-HLA-ABC antibody and subjected to flow cytometry analysis. Shown are the MFI values of HLA-

ABC expression (upper) and the percentages of HLA-ABC-positive cells (lower). * $p < 0.05$ compared with corresponding control or compared with the group treated with an IFN- γ neutralizing antibody.

C. BT474 and SUM190 cells were cultured in medium supplemented with 10% fetal bovine serum and were treated with 5 $\mu\text{g/ml}$ (~ 30 nM) trastuzumab or a control humanized IgG (h-IgG; bevacizumab) for 24 h in co-culture at a ratio of 1:5 (cancer cells versus immune effector cells) with 1.5×10^6 NK cells (CD56+), monocytes (CD14+), or T cells (CD3+). The immune effector cells were sorted by flow cytometry from PBMC after staining of PBMC with PE-Cy7-conjugated anti-CD56 antibody, APC-conjugated anti-CD14 antibody, and FITC-conjugated anti-CD3 antibody. Conditioned medium from each culture was used for detecting the presence of human IFN- γ with an ELISA kit. * $p < 0.05$ compared with corresponding control.

3.2 Trastuzumab increases the expression of CD86 co-stimulatory molecule in HER2-overexpressing breast cancer cells in the presence of PBMC

We also determined whether trastuzumab increased the expression of CD80 and CD86 co-stimulatory molecules. We first examined whether CD80 and CD86 are expressed in the breast cancer cell lines used in our study. We confirmed that HEK293 cells do not express CD80 or CD86 (Fig. 9A), as previously reported (93). Using these HEK293 cells as a negative control, we found that CD86 but not CD80 was expressed in BT474 and SUM190 cells (Fig. 9A). As shown in Figure 6B, both the MFI value and the percentage of CD86-positive cells were higher in BT474 cells co-cultured with PBMC than in BT474 cells in mono-culture. While trastuzumab marginally decreased CD86 expression in BT474 cells in mono-culture, trastuzumab significantly increased the MFI value of CD86 in co-culture. A similar pattern of results was found in SUM190 cells, although the results were less remarkable than in BT474 cells (Fig. 9B), which may be due to differences in genetic contexts between the cells.

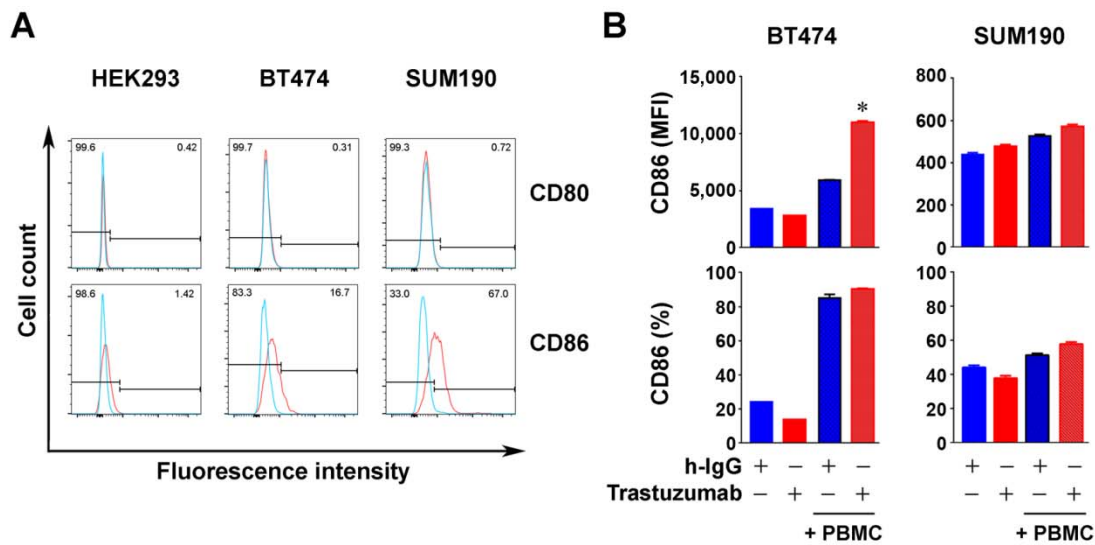


Figure 9: Increased expression of CD86 in HER2-overexpressing breast cancer cells by trastuzumab in the presence of PBMC

A. HEK293, BT474, and SUM190 cells were stained with PE-conjugated anti-human CD80 antibody and PE-Cy7-conjugated anti-human CD86 antibody and subjected to flow cytometry analysis.

B. BT474 and SUM190 cells were cultured in medium supplemented with 10% fetal bovine serum and were treated with 5 $\mu\text{g/ml}$ trastuzumab (~ 30 nM) or a control humanized IgG (h-IgG; bevacizumab) in mono-culture or co-culture with human PBMC at a ratio of 1:5 (cancer cells versus PBMC) for 48 h. The cells were then stained with PE-Cy7-conjugated anti-human CD86 antibody and subjected to flow cytometry analysis. Shown are the MFI values of CD86 expression (upper) and the percentages of CD86-positive cells (lower). * $p < 0.05$ compared with corresponding control.

3.3 Trastuzumab increases the expression of MHC-I molecules and CD80/CD86 in HER2-overexpressing breast cancer cells in vivo

Although co-culture of breast cancer cells with PBMC mimics the interaction of breast cancer cells with stromal cells in the tumor microenvironment following trastuzumab treatment, co-culture does not completely recapitulate that interaction in vivo. Thus, we further determined whether trastuzumab increased the expression of MHC-I molecules and CD80/CD86 in vivo in a mouse model. Because murine IFN- γ could not activate interferon receptor signaling in human breast cancer cells (as shown in SUM190 cells in Fig. 10A), whereas trastuzumab recognizes only human HER2, for the in vivo study we used 4T1 transplantable murine mammary tumor cells that were transduced to overexpress human HER2. We found that over 75% of 4T1 cells were positive for murine MHC-I (H-2Kd) expression; overexpression of human HER2 in the 4T1 cells did not have significant effect on the expression level of MHC-I (H-2Kd) (data not shown). When the 4T1/HER2 cells had grown into palpable tumors after transplantation in Swiss female nude mice, which are negative for H-2Kd expression, we treated the mice with trastuzumab or a control anti-human IgG. Analysis of H-2Kd expression in the tumors by flow cytometry showed a significant increase in the percentage of H-2Kd-expressing cells in the tumors from trastuzumab-treated mice compared with the tumors from control antibody-treated mice (Fig. 10B). Similarly, the percentages of CD80- and CD86-expressing cells were higher in the tumors from trastuzumab-treated mice than in the tumors from control antibody-treated mice (Fig. 10C). This experiment provides important in vivo evidence further supporting a role of trastuzumab in increasing expression of HLA-ABC/MHC-I and CD80/CD86 molecules in HER2-overexpressing breast cancer cells.

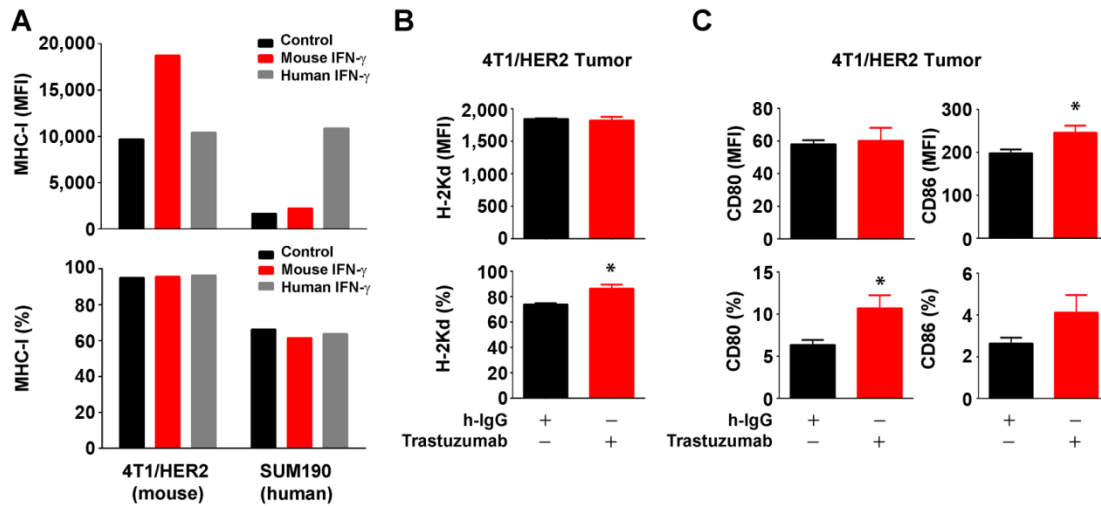


Figure 10: Increased expression of MHC-I and CD80/CD86 molecules by trastuzumab in HER2-overexpressing breast cancer cells in vivo

A. 4T1 mouse mammary tumor cells transduced to overexpress HER2 (4T1/HER2) and SUM190 human breast cancer cells were cultured in medium supplemented with 10% fetal bovine serum in the presence or absence of mouse IFN- γ (300 units/ml) or human IFN- γ (300 units/ml) for 24 h. The cells were then stained with APC-conjugated anti-H-2Kd (for 4T1/HER2) or APC-conjugated anti-HLA-ABC antibody (for SUM190) and subjected to flow cytometry analysis. Shown are the MFI values of MHC-I expression (upper) and the percentages of MHC-I-positive cells (lower).

B. 4T1/HER2 cells (2×10^6 cells) were implanted in the mammary fat pad of female Swiss nude mice (4-6 weeks old). Two weeks after the tumors were palpable, the mice were treated once with 200 μ g of trastuzumab or a control humanized IgG (rituximab) intraperitoneally. At 48 h after treatment, the mice were euthanized, the tumors were removed, and single cell suspensions were prepared and double-stained with APC-conjugated anti-H-2Kd antibody and trastuzumab plus FITC-conjugated anti-human IgG antibody. Cells were then subjected to flow cytometry analysis. Shown are the MFI values of H-2Kd expression (upper) and the percentages of H-2Kd

positive cells (lower) gated for HER2-positive cells. * $p < 0.05$ compared with corresponding control.

C. Single cell suspensions from 4T1/HER2 tumors described in **(B)** were triple-stained with APC-conjugated anti-mouse CD80 antibody, PE-conjugated anti-mouse CD86 antibody and trastuzumab plus FITC-conjugated anti-human IgG antibody, and subjected to flow cytometry analysis as described in A. Shown are the MFI values of CD80 and CD86 expression (upper) and the percentages of CD80- and CD86-positive cells (lower) gated for HER2-positive cells. * $p < 0.05$ compared with corresponding control.

Chapter 4: PD-L1 as a potential obstacle post trastuzumab treatment

Programmed cell death 1 (PD1) is a member of CD28 family of receptors and are expressed on the surface of T cells, B cells, NK cells, and other activated leukocytes. PD1 regulates the activity of T cells upon interaction with its ligand PD-L1 (51). PDL1 is expressed on immune cells and also in many cancer cells including breast cancers (94). Potential inducers of PD-L1 include IFN γ and pathogen-associated molecular patterns (PAMPs) etc., which triggers a series of intracellular events that are responsible for the translocation of transcription factors to the promoter site leading to PD-L1 expression (95). Currently, combination of trastuzumab and ant-PD1 therapy for treatment trastuzumab resistant tumors is active in clinical trials (54).

Trastuzumab treatment of HER2 overexpressing cells in the presence of PBMC induces IFN γ (Fig.8A). Because IFN- γ is a cytokine that stimulates PD-L1 expression through activation of STAT1 signaling, we tested the effect of trastuzumab treatment in the presence or absence of PBMC on PD-L1 expression on breast cancer cells. We treated the following breast cancer cell lines BT474, SKBR3, SUM190, and HCC1954 cells with IgG or trastuzumab for 48 h in the presence or absence of PBMC. As shown in Fig. 11, when treated with trastuzumab in the absence of PBMC there were no significant changes in PD-L1 expression in these cell lines. However, trastuzumab treatment significantly increased the expression levels of PD-L1 when these cells were co-cultured with PBMC, in three cell lines BT474, SKBR3, HCC 1954 and a marginal increase in SUM190 cells.

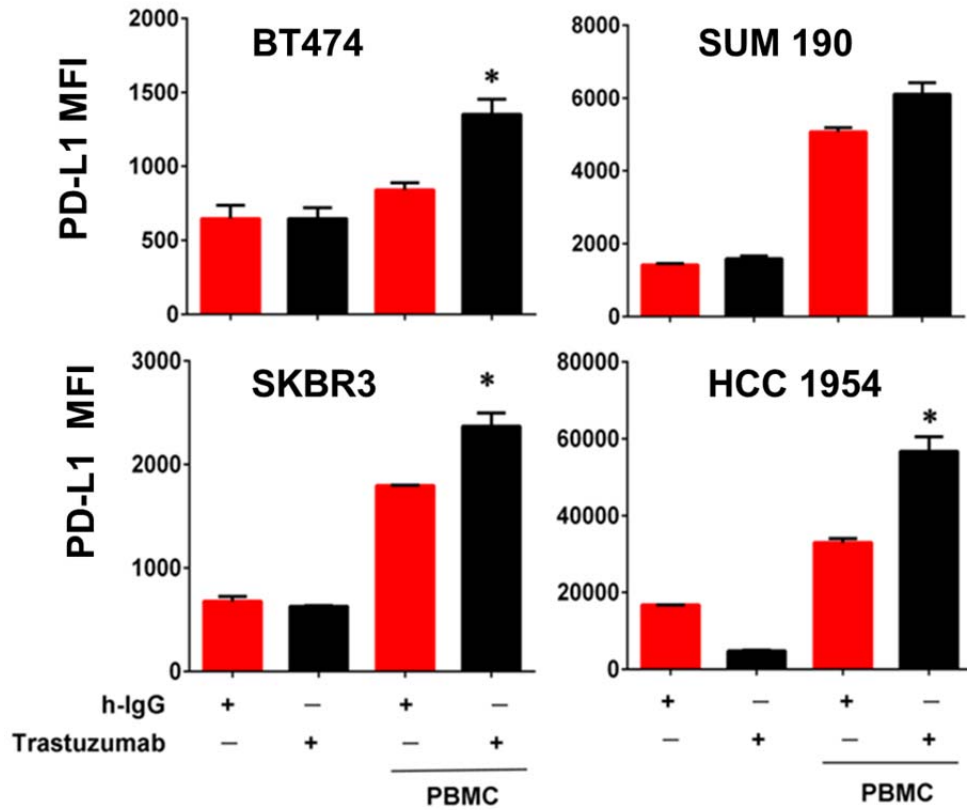


Figure 11: Trastuzumab treatment enhances PD-L1 expression in breast cancer cells

The above shown HER2-overexpressing breast cancer cell lines were cultured in complete medium and were treated with 5 $\mu\text{g/ml}$ trastuzumab or a control h-IgG (rituximab/bevacizumab) in co-cultured with human PBMC at a ratio of 1:5 or without PBMC. 48hr after the treatment, the PBMC in suspension removed by washing with PBS. The attached cells were then stained with APC-conjugated anti-PD-L1 antibody and subjected to flow cytometry analysis. Any residual PBMC in the co-culture were excluded out during flow cytometric analysis. MFI values of PD-L1 expression are shown. * $p < 0.05$ compared with corresponding control.

A recent study showed that treatment of HER2 overexpressing cells with trastuzumab in the presence of PBMC decreases the levels of HER2 through inhibition of HER2 transcription by IFN- γ (91). To determine if the increase in PD-L1 expression is not only through IFN- γ but also due to reduction in HER2 expression, we silenced the HER2 expression using three different HER2 specific SiRNA in four HER2-overexpressing breast cancer cell lines (BT474, SKBR3, SUM190 and HCC1954). After the knockdown of HER2 we found that the PD-L1 expression decreased in all the four cell lines as shown in Fig. 12A. We also over expressed HER2 in MCF cell line and found that HER2 expression significantly increased the PD-L1 expression in MCF7 cells (Fig.12B). This suggests that HER2 signaling could have a direct impact on the PD-L1 expression.

Finally, the underlying mechanism of trastuzumab, wherein the binding of trastuzumab to HER2 engages NK cells, results in secretion of IFN- γ , which upregulates HLA-ABC, CD80/CD86 and also PD-L1 expression. These activities of trastuzumab may contribute to the overall antitumor activity of trastuzumab through eliciting a cytotoxic T cell-mediated response to targeted cancer cells with PD-L1 being a potential obstacle.

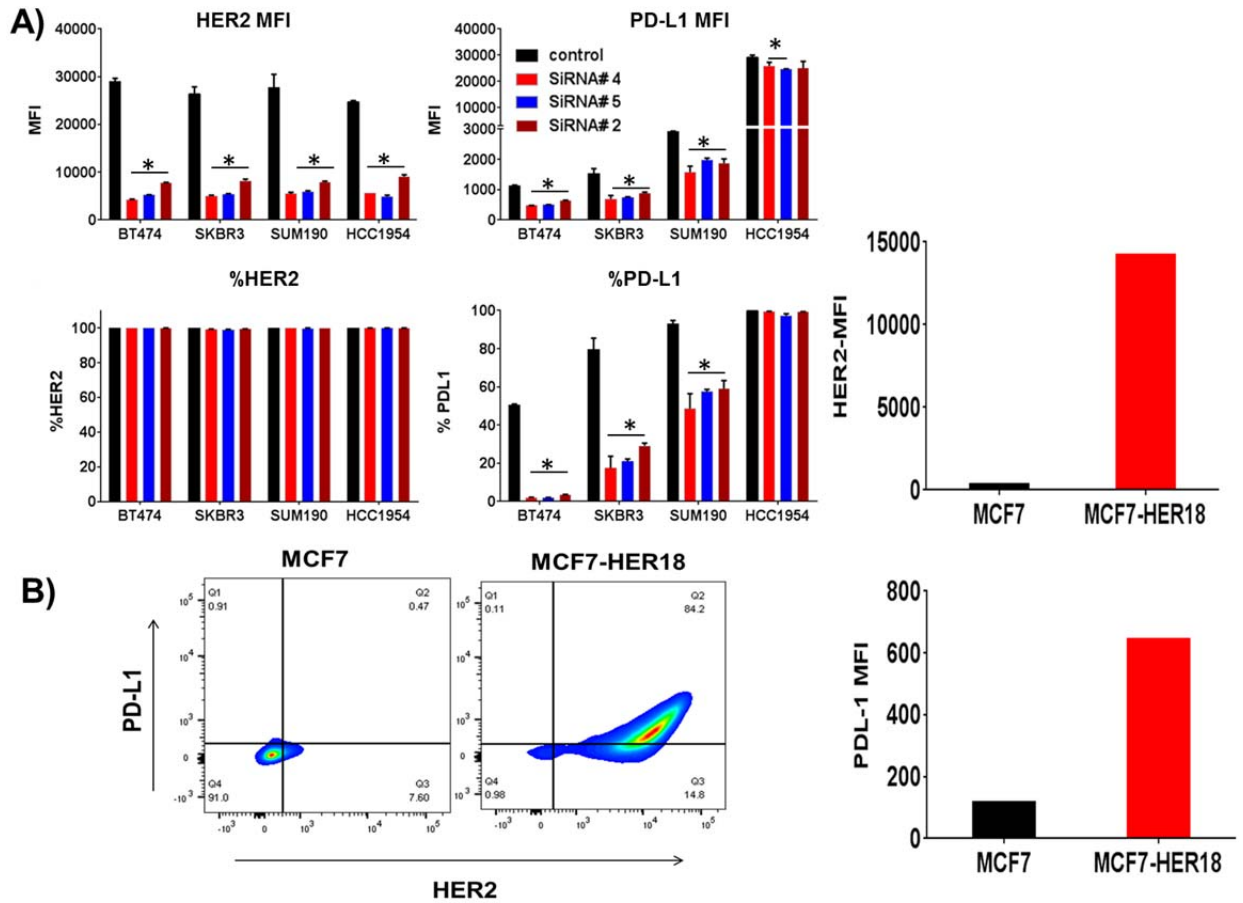


Figure 12: Effect of HER2 on the level of PD-L1 expression in breast cancer cells.

A. The above shown HER2-overexpressing breast cancer cells were transfected with two different siRNAs specific for HER2 or control siRNA using Lipofectamine for 72hr. The cells were detached and stained for PD-L1 and HER2 and analyzed by flow cytometry. Percentage cells positive and MFI are shown. B. MCF7 and MCF7-HER18 cells were stained with APC-conjugated anti-PD-L1 antibody and trastuzumab, followed by FITC-conjugated anti-human IgG antibody. Cells were analyzed by flow cytometry and MFI values of HER2 and PD-L1 are plotted as contour and bar graphs. $p < 0.05$ compared with corresponding control.

Chapter 5: Pre-existing influenza specific immunity is recruited to the tumors for regression of primary and metastatic mouse mammary expressing endogenous influenza antigens

Various studies have explored the importance of host immune responses in fighting cancers (35, 36, 39, 47, 63, 64). Cancer cells escape the immune responses through various mechanisms by down regulating antigen load, reducing the MHC expression and making tumors a suppressive environment (82). Cancer cells mainly express self-antigens and some mutated antigens, depending on the cancer type, that is correlated with exposure to the outside environment (57). For example the most immunogenic cancers are Melanomas, followed by lung carcinomas owing to the rate of occurrence of mutated antigens because of the exposure of the cancer prone cells to the carcinogens and other environmental factors (57). However breast cancers are not very immunogenic due to presence low rate of mutated antigens as shown in the Fig.3. Hence we hypothesized that if breast cancer cells are made to express foreign antigens (such influenza antigens), they could be targeted by preexisting T cell immunity of the host immune system. We used a well-established Influenza A virus (A/Puerto Rico/8/1934(H1N1)) model for immunization and immunodominant antigens nucleoprotein (NP), Hemagglutinin (HA) for expression in breast cancer cells using lentiviral system.

5.1 Analysis of influenza specific interferon γ responses and evaluation tumor regression in BALB/c mice

To test our hypothesis that expression of an influenza PR8 antigen NP in mouse mammary tumor cells (TUBO) could be targeted by the pre-existing Influenza specific immunity, we first stably expressed influenza PR8 NP antigen in TUBO cells using lentivirus named here as TUBO+NP. As shown in the Fig.13A, immunofluorescence was performed to

confirm the expression of NP antigen in the TUBO cells. We then analyzed the antigen specific immune responses ex vivo following influenza PR8 infection in BALB/c mice. The splenocytes from the PR8 or PBS immunized mice were collected and stimulated with TUBO or TUBO+NP cells for 72hrs. We found that PR8 immunization induced a greater amount of antigen specific IFN- γ responses when stimulated with TUBO+NP or TUBO+peptides, compared to PBS immunized mice. As expected we also saw a basal level of IFN- γ production in both the groups when stimulated with TUBO cells alone (Fig.13B). Further we tested our hypothesis in vivo, where we immunized mice either with PR8 or PBS on day 0 and challenged with TUBO cells expressing NP antigen in the mammary fat pad. Tumors were monitored every three days starting on day 9 i.e. when the tumors were palpable. We found that mice intranasally immunized with PR8 were able to significantly inhibit the growth of TUBO+NP cells compared to PBS immunized mice (Fig.13C). This suggests that a vaccine-induced immune response could be redirected to tumors effectively if the tumors express vaccine-related antigens.

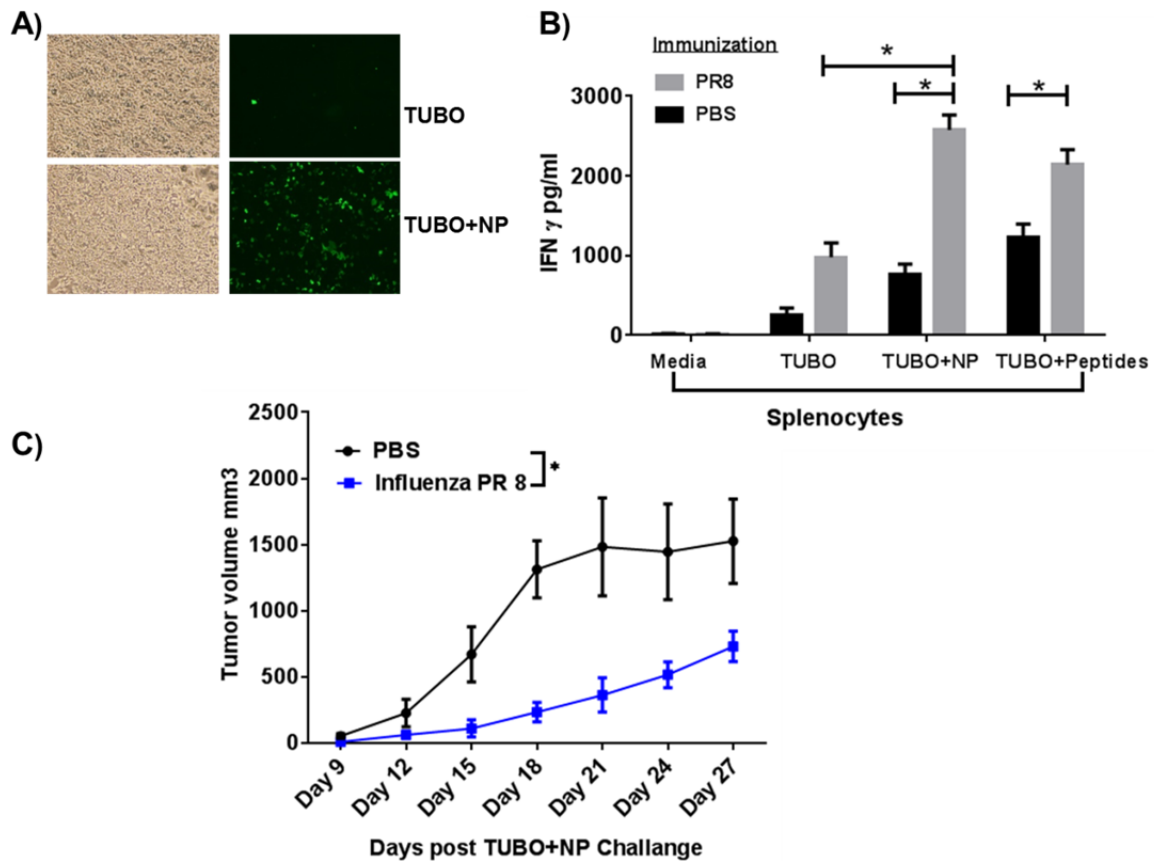


Figure 13: Antigen specific immune responses in Influenza PR8 immunized mice and primary tumor regression after TUBO+NP challenge:

A) TUBO cells or TUBO+NP cells were grown in monolayers and fixed with 2% paraformaldehyde and immunofluorescence for intracellular NP expression was performed using antisera from PR8 immunized mice. **B)** 4-5 week old BALB/c mice were immunized with PBS or influenza PR8 on day 0 and on day 30 splenocytes were isolated, 10^6 cells were plated in a 96 well plate along with the TUBO or TUBO+NP cells or TUBO with HA, NP peptides for 72hrs and supernatants were analyzed for IFN γ production by ELISA. **C)** On day 30 post immunizations with PBS or PR8 mice were challenged with 2.5×10^6 TUBO+NP cells. Tumors were measured using calipers and volume was enumerated by formula, volume $V = (\pi/6) * L * W^2$. * $p < 0.05$ compared with corresponding control.

5.2 Immuno-competent mouse model for testing metastatic breast cancer cell lines that express influenza PR8 antigens HA and NP

Cancer is a disease that is not treatable when they are metastasized to several organs in the body. Metastatic breast cancer often spreads to lungs, bones and brain causing severe complications and leading to death in about 90% patients (96). Hence we wanted to test our hypothesis of re-routing vaccine induced immunity to cancer cells in metastatic breast cancer model. To test this we adopted two different metastatic mouse mammary cell lines 4T1 and E0771. 4T1 cells forms primary tumors and metastases in BALB/c mice (97, 98), whereas E0771 cells forms tumors and metastases in C57BL/6 mice (98). As shown in Fig.13C, where we expressed a single influenza PR8 antigen NP in the TUBO cells and showed that firstly that the tumors can be formed in normal mice and could be regressed effectively if the mice were pre immunized with Influenza. However owing to the aggressive nature of the metastatic 4T1 and E0771 cells, we decided to express more than one antigen endogenously i.e. NP and HA. Therefore to test if there is any change in the tumor initiating properties of these cell lines after expressing NP and HA antigens, we first checked for the expressions of HA and NP in both 4T1 and E0771 cells by immuno-fluorescence using NP and HA specific antibodies (Fig. 14 A, B). We then injected both BALB/c and C57BL/6 mice with different amount of cells in 4T1 and E0771 cell lines respectively expressing NP, HA and luciferase, referred here after as 4T1-NP+HA-Luc and E0771-NP+HA-Luc respectively. These groups were compared to the parenteral cell lines 4T-Luc and E0771-Luc cells for tumor forming ability. We found that on day 17 in 4T1 tumor model almost all the mice developed tumors injected with 4T1-Luc or 4T1-NP+HA-Luc, except one mouse in 10^6 cells group where the tumor was not detectable by imaging. As expected all the tumors were actively growing in size by day 24. However in E0771 tumor model, on day 7 almost all the mice with different doses developed tumors

injected with E0771-Luc or E0771-NP+HA-Luc, except one mouse. But, surprisingly we saw a rejection of E0771-NP+HA-Luc cells by day 14 in all mice, contrary to E0771-Luc tumors that are actively growing. This confirms that using 4T1-NP+HA-Luc cells in BALB/c mice is a good model for testing our hypothesis in a metastatic setting.

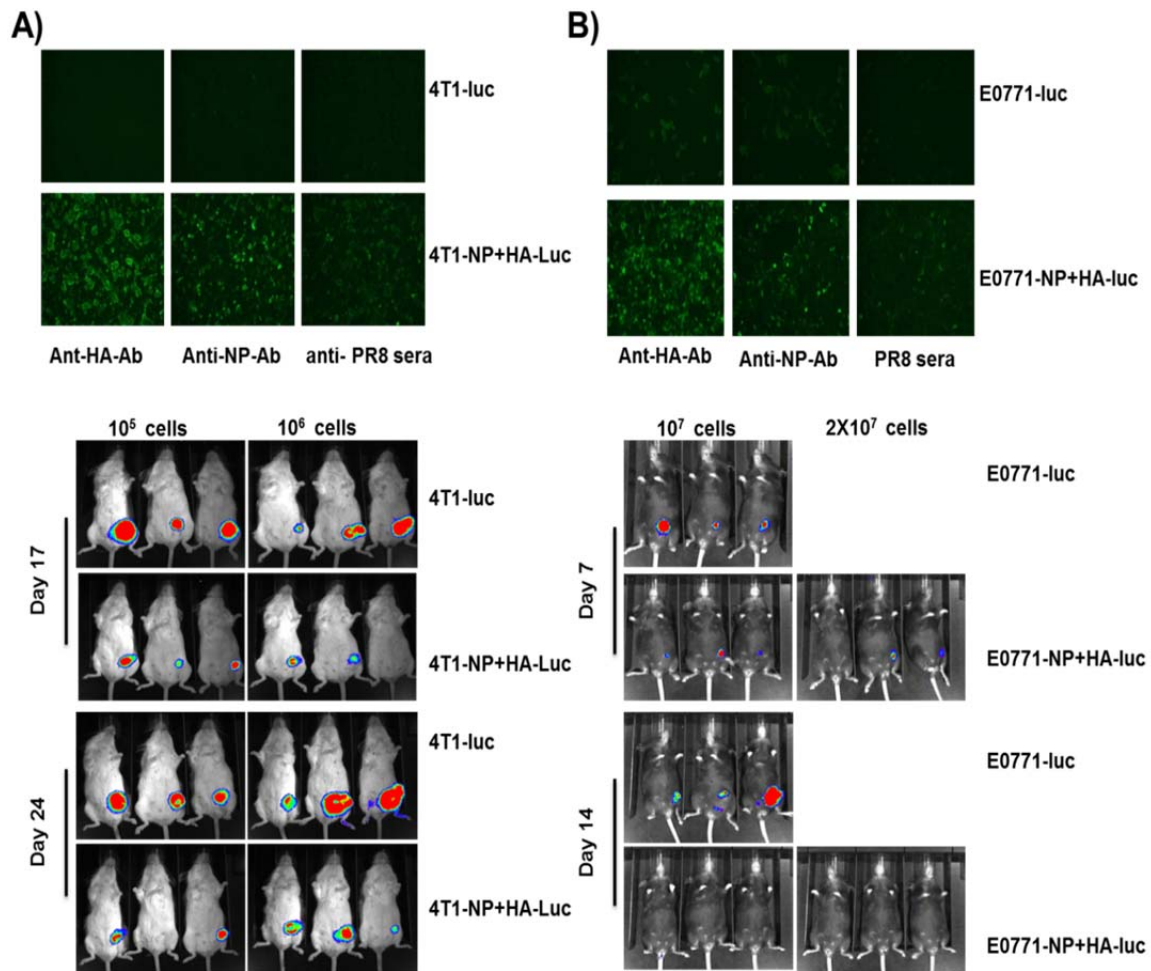


Figure 14: Establishment of metastatic mouse cell lines expressing influenza antigens for tumor development in mice

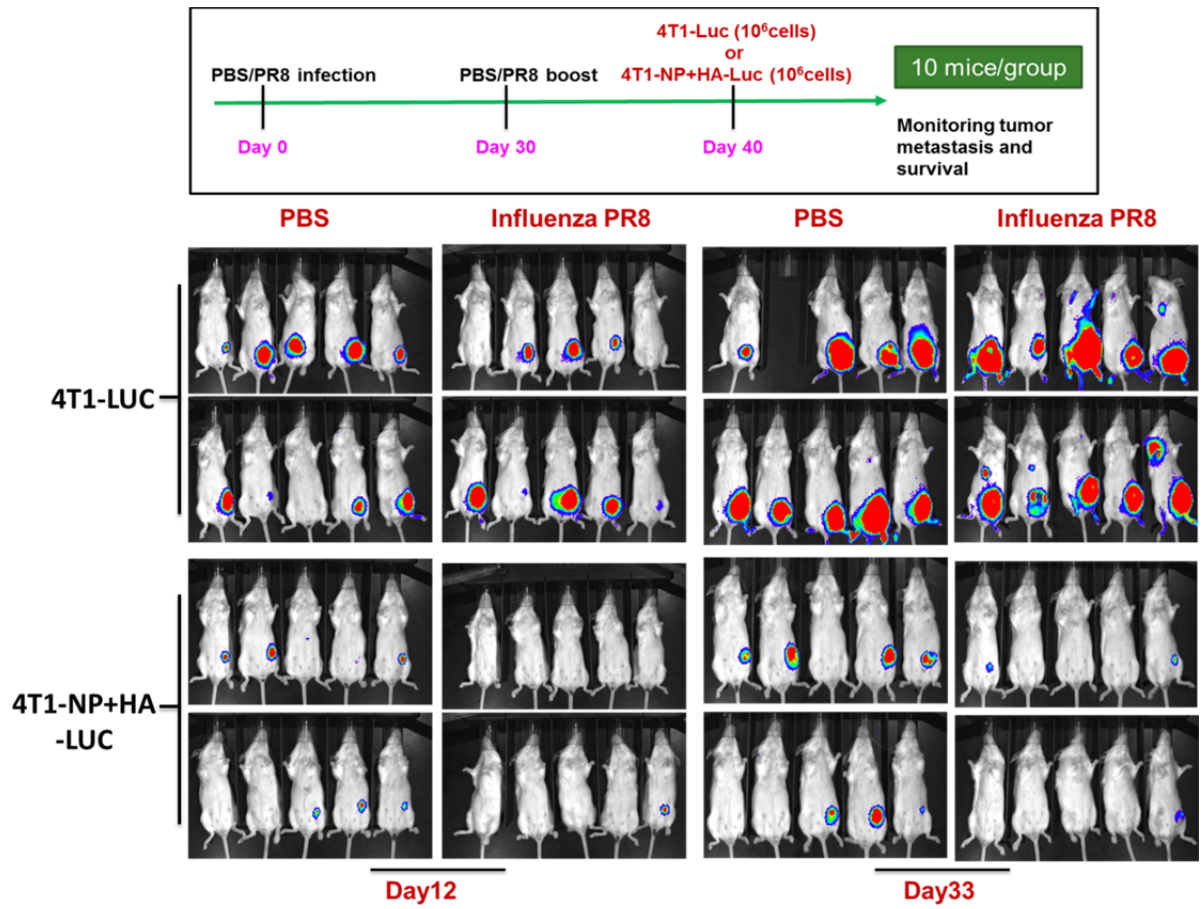
A) 4T1-Luc and 4T1-NP+HA-Luc were grown in monolayers and fixed with 2% paraformaldehyde and immunofluorescence for intracellular HA and NP expression was performed using HA, NP specific antibodies. 4-5 week old BALB/c mice were injected with 10^6 or 10^7 4T1-Luc or 4T1-NP+HA-Luc cells in the mammary fat pad and 3mg D-luciferin was injected i.p. before imaging by IVIS. **B)** E0771 and E0771-NP+HA-Luc cells were grown in monolayers and fixed with 2% paraformaldehyde and immunofluorescence for intracellular HA and NP expression was performed using HA, NP specific antibodies. 4-5 week old C57BL/6

mice were injected with 10^7 or 2×10^7 E0771 or E0771-NP+HA-Luc cells in the mammary fat pad and 3mg luciferin was injected i.p. before imaging by IVIS.

5.3 Regression of metastatic mammary tumors expressing influenza PR8 antigens by pre-existing Influenza immunity

BALB/c mice injected with 4T1 cells expressing NP and HA antigens is good model to testing the potency of pre-existing influenza specific immunity in a metastatic setting (Fig.14A). Hence we immunized 5-6 week old BALB/c mice on day 0 with either PBS or 20HA units of influenza PR8 intranasally. Mice were rested for 30 days for clearing the infection and development of adaptive immune responses. Mice were then boosted with PBS or 60HA units of influenza PR8 intranasally. Ten days after boosting, both the PBS and PR8 groups were further divided, so that each group was injected in the mammary fat pad with 4T1-Luc or 4T1-NP+HA-Luc. As shown Fig.15A tumors were palpable in both PBS and PR8 groups challenged with 4T1-Luc by day 12 and started to grow aggressively. However mice immunized with PBS and challenged with 4T1-NP+HA-Luc cells showed delayed growth. As expected, mice immunized with PR8 and challenged with 4T1-NP+HA-Luc showed better rejection of tumors by day 12 than any other group. By day 33, metastasis is evident in groups of mice challenged with 4T1-Luc, where the mice started to die. On day33 mice challenged with 4T1-NP+HA-Luc cells showed no signs of metastasis suggesting the immune dominant nature of NP and HA antigens. Also PR8 immunized mice rejected 7/10 tumors and became tumor free. The size of the tumors was measured till day 40 i.e. when most of the mice in 4T1-Luc challenged groups are dead. However, we monitored the survival of all the groups of mice till 180 days as shown in Fig.15B.

A)



B)

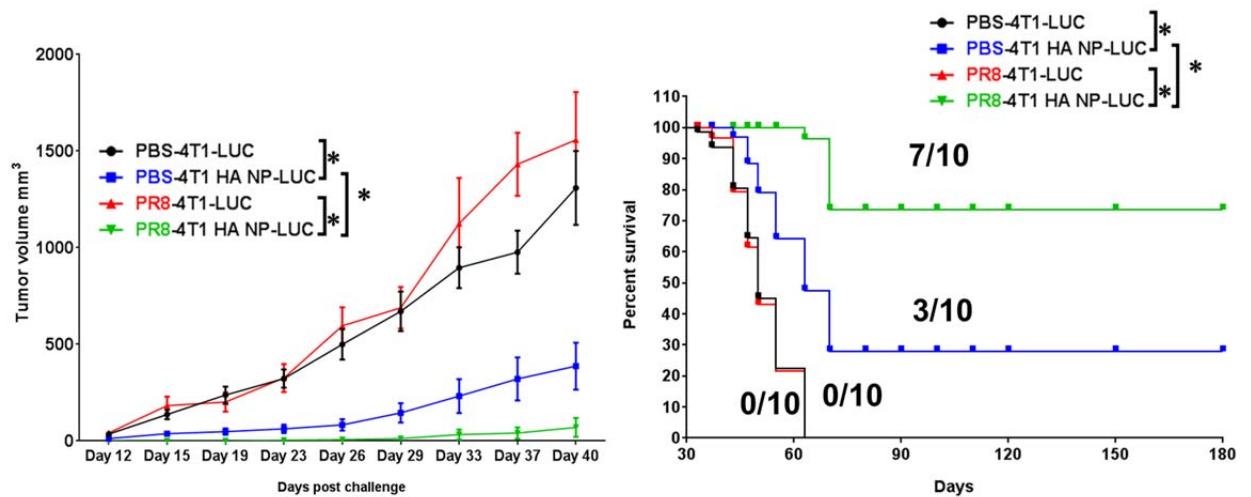


Figure 15: Pre-existing influenza specific immunity provides better protection against metastatic mammary tumors expressing influenza antigens NP and HA.

A) 5-6 week old BALB/c mice were immunized with PBS or 20HA units of influenza PR8 on day 0 and boosted on day 30 with same regimen. On day 40 mice were challenged with 10^6 cells of 4T1-Luc or 4T1-NP+HA-Luc in the mammary fat pad. Tumors were monitored by IVIS imaging by injecting luciferin i.p. B) Tumors were measured using calipers and volume was enumerated by formula, volume $V = (\pi/6) * L * W^2$. Survival of the mice was monitored till day 180. $p < 0.05$ compared with corresponding control.

5.4 Pre-existing influenza immunity activates innate immunity and attracts memory

CD8⁺Tcells at the tumor site

Tumors are comprised of cancer cells, stromal cells and immune cells (96). Cancer cells are deregulated body cells dividing uncontrollably and are self-origin (96). They mostly express self-antigens or mutated self-antigens that lead to induction of immune tolerance mediated by Treg cells (63, 64). It has been shown recently that memory Treg cells sense the tumor specific self-antigens very early (63). Activation of DC's and effector T cells is limited in the tumors because the effector T cells in the tumors are outnumbered by Treg cells contributing to an immune suppressive environment (63). Another cell type from myeloid lineage called myeloid derived suppressor cells (MDSC) effectively suppresses immune activation by producing several soluble molecules like TGF beta, VEGF, IL-10 etc. (96). To understand the immune cell repertoire that is responsible for better rejection of tumors in influenza PR8 immunized mice we analyzed the different innate and adaptive immune cell population in the tumor microenvironment by flow cytometry (Fig.15). 5-6 week old BALB/c mice were immunized on day 0 with either PBS or 20HA units of influenza PR8 intranasally. Mice were rested for 30 days for clearing the infection and development of adaptive immune responses. Mice were then boosted with PBS or 60HA units of influenza PR8 intranasally. Ten days after boosting, both the PBS and PR8 groups were further divided and challenged with 4T1-Luc or 4T1-NP+HA-Luc in the mammary fat pad. On Day 7 after the tumor challenge, the tumors were resected in all the four groups of mice and analyzed for immune cell infiltration. The innate immune cells infiltrating the tumors were characterized by various phenotypic markers for macrophages (CD45+CD11b+F4/80+), lymphoid DC (B220-CD45+CD11c+CD11b-), myeloid DC (B220-CD45+CD11c+CD11b+), NK cells (B220-CD45+DX5+), myeloid derived suppressor cells (CD11B+Ly6G (Gr1) +). As shown in Fig.16A the total number of DC's (CD11c+) that are

infiltrated in 4T1-Luc tumors or 4T1-NP+HA-Luc are not different in the PBS or PR8 immunized mice. However, in the tumors of PR8 immunized and 4T1-NP+HA-Luc challenged mice there is greater infiltration of DC's from lymphoid origin than myeloid origin compared to other groups. Also, DC's from both lymphoid and myeloid origin that infiltrated the tumors showed a higher expression of activation marker CD86 (Fig.16B) suggesting the DC maturation in the tumors for effective antigen presentation to the T cells. We also observed that PR8 immunized 4T1-NP+HA-Luc tumors have high NK cells infiltration (Fig.16C), and low tumor associated macrophages and low MDSC (Fig.16D) compared to tumors in the other groups. Together these results suggest that pre-existing immunity along with expression of immuno-dominant foreign antigens in the tumor activates innate immune responses that could lead to effective T cell responses.

We have also analyzed the T cell responses in tumor draining lymph nodes and tumors using various phenotypic markers in the cells such as CD45+CD3+ CD4+ (CD4+T cells), CD45+CD3+ CD8+ (CD8+T cells), CD45+CD3+ CD4+ CD44hi (CD4 memory T cells), CD45+CD3+ CD8+ CD44hi(CD8 memory T cells) and CD45+CD3+ CD4+ CD25+FOXP3+(Treg cells). As shown in Fig.17A the percentage of CD4+T cells and memory CD4+ T cells infiltrating in the tumors and tumor draining lymph nodes of PBS or PR8 immunized and 4T1-Luc or 4T1-NP+HA-Luc challenged mice are not significantly different. Also, the percentage of CD8+T cells in the tumors and tumor draining lymph nodes immunized with PBS or PR8 and challenged with 4T1-NP+HA-Luc are not significantly different either. However, in the tumors of PR8 immunized and 4T1-NP+HA-Luc challenged mice the percentage of memory CD8+ T cells are significantly greater compared to the other groups, suggesting that the pre-existing CD8+ T cell immunity of influenza is directed to the tumor site for targeting tumors expressing influenza antigens (Fig.17B).

Treg cells are a hindrance for the tumor targeting effector T cells; they suppress activation of CD4⁺ T cell and CD8⁺ T cells in the tumors leading to an immunosuppressive environment (60, 61). Hence, to test if there are any differences in the Treg population at the tumor site and tumor draining lymph nodes we stained for Treg in all the four groups of mice. We found that in PR8 immunized and 4T1-NP+HA-Luc challenged mice that had best protection against tumor challenge (Fig.15); the percentage of Treg population was significantly lower compared other groups (Fig.17C).

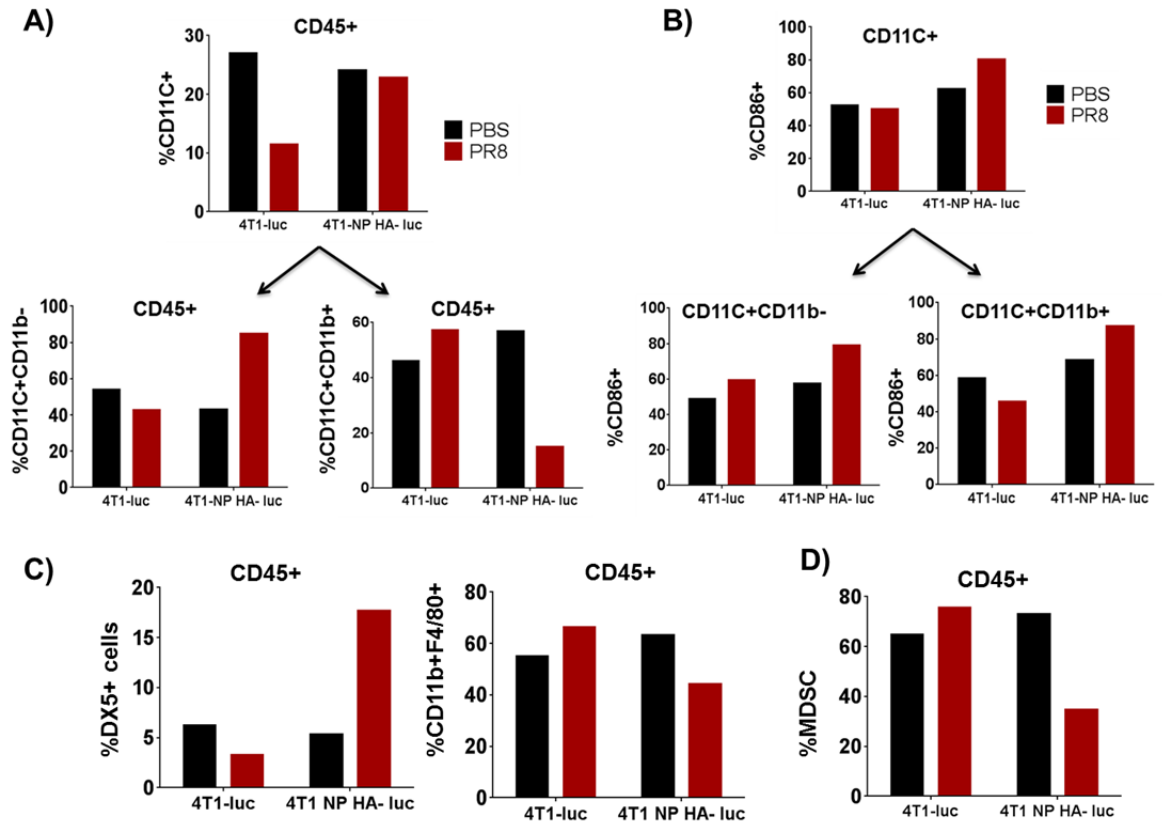


Figure 16: Activation of innate immune responses in PR8 immunized 4T1-NP+HA-Luc tumors

5-6 week old BALB/c mice were immunized with PBS or 20HA units of influenza PR8 on day 0 and boosted on day 30 with the same regimen. On day 40 mice were challenged with 10^6 cells of 4T1-Luc or 4T1-NP+HA-Luc in the mammary fat pad. After 7 days the tumors were collected and single cell suspensions were made and stained for various innate immune cell markers. Cells were stained with anti- CD45, B220, CD11c, CD11b, DX5, F4/80, CD86 antibodies and analyzed by flow cytometer. A) Percentage CD11c+ (dendritic cells) in CD45+ (leucocytes) and B220- (B cell marker) cells are shown and further characterized as CD11b- (lymphoid origin) and CD11b+ (myeloid origin). B) Percentage of cells positive for CD86 expression in total CD11c+, CD11c+CD11b- (lymphoid origin) and CD11c+CD11b+(myeloid

origin) are shown. C) Percentage DX5+ (NK cell marker), CD11b+F4/80+ (TAM marker) cells in CD45+ cells are shown. D) Percentage of cells positive for CD11B+Ly6G (Gr1) + (MDSC) in the tumors are shown.

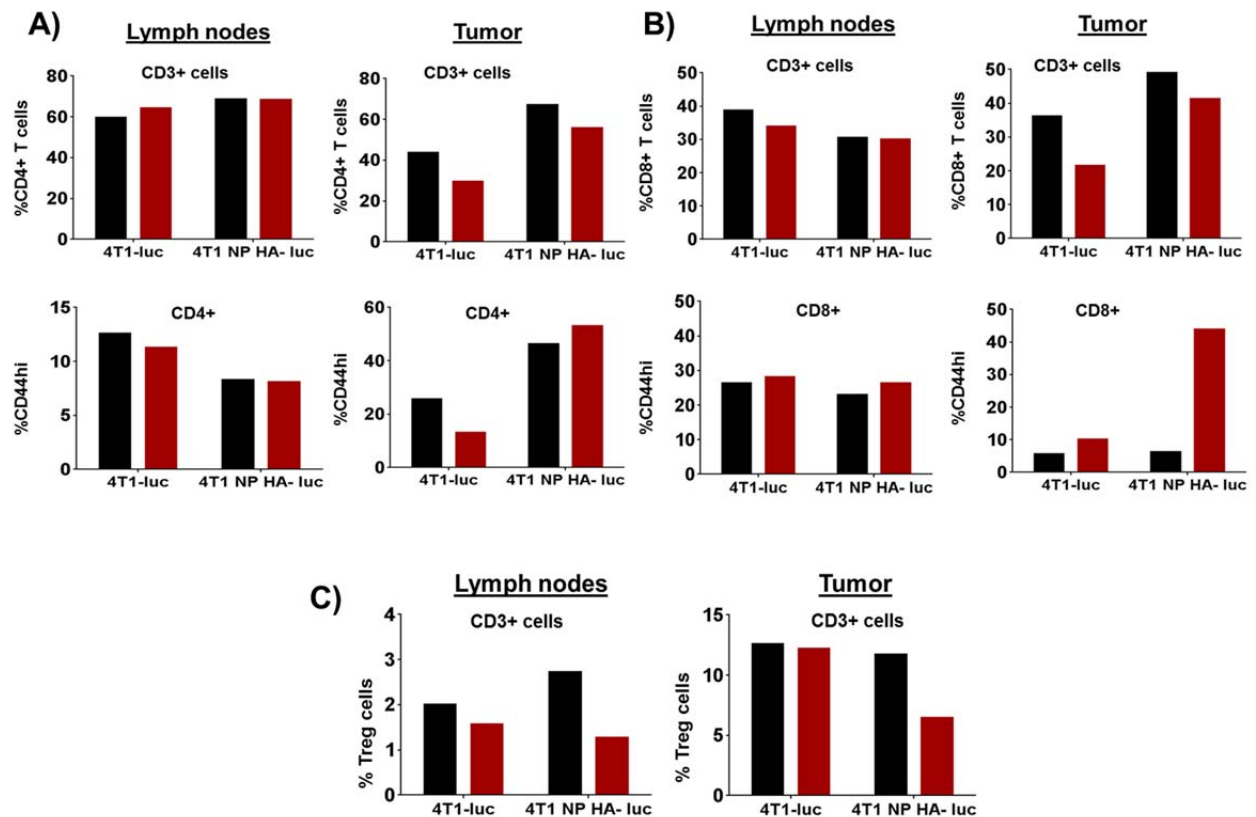


Figure17: Infiltration of memory CD8+T cells responses in PR8 immunized 4T1-NPHA-Luc tumors.

5-6 week old BALB/c mice were immunized with PBS or 20HA units of influenza PR8 on day 0 and boosted on day 30 with the same regimen. On day 40 mice were challenged with 10^6 cells of 4T1-Luc or 4T1-NP+HA-Luc in the mammary fat pad. After 7 days the tumors were collected and single cell suspensions were made and stained for various T cell markers. Cells were stained with anti- CD45, CD3, CD4, CD8, CD25, FOXP3, F4/80, CD44 expression and analyzed by flow cytometer. A) CD4+ T cells present in the tumor draining lymph nodes and tumor are shown as percentage of CD3+ cells along with CD4+ T cells that are CD44hi are shown. B) CD8+ T cells present in the tumor draining lymph nodes and tumor are shown as percentage of CD3+ cells along with CD8+ T cells, which are CD44hi are shown. C) Shown is the percentage of FOXP3+ cells in CD3+CD4+CD25+ cells that represent the Treg populations.

Chapter 6: Development of an anti-HER2 antibodies for delivery of immunogenic peptides to HER2 overexpressing tumors.

Due to antibody's specificity in targeting tumor-associated antigens, antibodies, specifically a single chain antibody scFv or an Fab fragment of antibody, have been commonly used to guide targeted delivery of liposomes with various types of payloads, such as siRNA, toxins or chemotherapeutic drugs (14, 18, 21, 24, 71, 91). Conventional approach to generating such immunoliposomes is to use a chemical conjugation method to link a complete antibody or an antibody fragment to the lipid bilayer of liposome in presence or absence of PEG chains (type I immunoliposomes) or to the distal end of the PEG chain (type II immunoliposomes). Such chemical methods inevitably will result in damage to the immune reactivity of an antibody. As stated in the introduction and previous chapters, anti-HER2 antibodies, by binding specifically to the extracellular domain of HER2 molecule on cell surface, interfere with HER2 homodimerization or heterodimerization, thereby downregulating HER2 tyrosine kinase activation-induced signaling. In addition, anti-HER2 antibodies can engage Fc-receptor expressing immune cells to induce innate and adaptive immune responses (14, 18, 21, 24, 91, 99, 100). To maximally retain the antitumor activity of trastuzumab, we propose to engineer an anti-HER2 antibody with a peptide fragment that could form complex with liposomes thereby sparing the chemical modification steps that can potentially cause damage to the antibody.

6.1 Construction of lentiviral vectors for expression of anti-HER2 antibody fused to a peptide domain.

Immunoglobulins are classified into IgG, IgA, IgD, IgE and IgM, IgG is the most abundant form of immunoglobulin in the body (101). Trastuzumab is a therapeutic IgG1 antibody (18). Trastuzumab is a therapeutic IgG1 antibody. We sub-cloned the cDNA of

trastuzumab heavy and light chains on the basis of public available sequence of trastuzumab and then genetically inserted a peptide fragment into the antibody sequence by PCR. Two types of peptide fragments were used, a short form called Δ Mb, and a long form called Mb, resulting two antibodies termed Δ Mb TZM and Mb TZM, respectively (Fig.18). A custom made fluorescence-tracked lentiviral transduction system was used to stably insert the antibody DNA sequence into the genome of CHO-S engineering cells (102, 103). CHO-S cells expressing both heavy chain and light chain are sorted using flow cytometry for enrichment of antibody production. Sorted CHO-s cells were propagated in suspension in shaker flasks. Conditioned medium containing antibodies are purified using Protein A beads (Fig.18A). As shown in Fig.18B, the engineered antibodies Mb-TZM and Δ Mb-TZM were similar to parental trastuzumab (TZM) with minor changes in migration when separated on a non-reducing and a reducing SDS gel.

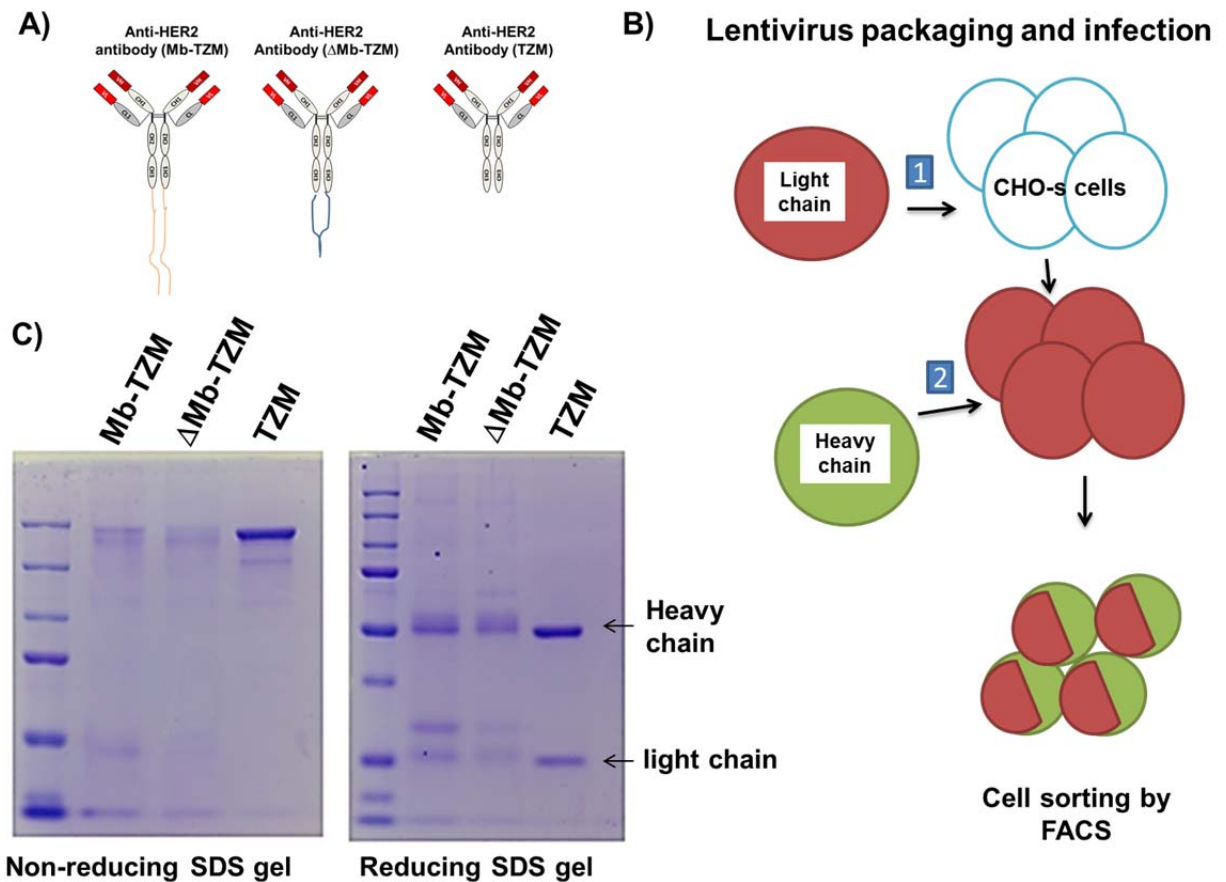


Figure 18: Production of Mb-TZM and ΔMb-TZM antibodies

A) Schematic representation of Mb-TZM, ΔMb-TZM and TZM (trastuzumab) antibodies. B) Light chain of trastuzumab is fused with mCherry and cloned into pLEX vector for lentiviral packaging and CHO-s cells were infected with light chain containing lentivirus and selected by Puromycin marker for high expression. The CHO-s cells expressing light chain are then infected with GFP fused heavy chain containing lentivirus and selected with Blasticidin marker. Cells expressing high light and heavy chains were sorted by selecting high mCherry and GFP expressing cells. C) CHO-s cells were lysed and antibodies are purified using Protein A beads. Purified antibodies were subjected to non-reducing and reducing SDS gel electrophoresis.

6.2 Expression of Mb-TZM and Δ Mb-TZM antibodies on the membrane of CHO-s cells

Mb-TZM and Δ Mb-TZM antibodies generated from the conditioned medium of CHO-S cells were purified by a Protein-A column. Purified antibodies were incubated with HER2-overexpressing breast cancer cells for testing its binding affinity. As shown in the Fig.19A, both Mb-TZM and Δ Mb-TZM antibodies bind to HER2-overexpressing BT474 cells but to UMSCC1, a human head and neck cancer cell line without overexpression of HER2. This finding indicated that the specificity of engineered antibodies is on par with trastuzumab. As note, we observed that the amount of antibody produced in the conditioned medium of CHO-S cells was limited, which was expected due to the hydrophobicity of Mb-TZM and Δ Mb-TZM that can cause stuck of antibody in the membrane of CHO-S cells. Indeed, we found that when the CHO-S cells were stained with goat anti-human IgG (H+L) antibody, followed by incubation with a fluorescence-labeled anti-goat IgG antibody, we found that both Mb-TZM and Δ Mb-TZM antibodies are expressed on the membrane of CHO-S cells. Such florescence positive staining was not seen in the CHO-S expressing trastuzumab (TZM). This finding supports our predication that both Mb-TZM and Δ Mb-TZM contain a peptide fragment that can interact with the lipid-laden membrane of the cells. This unique property of the engineered antibody meets our expected design that can potentially be utilized for forming complex with liposome.

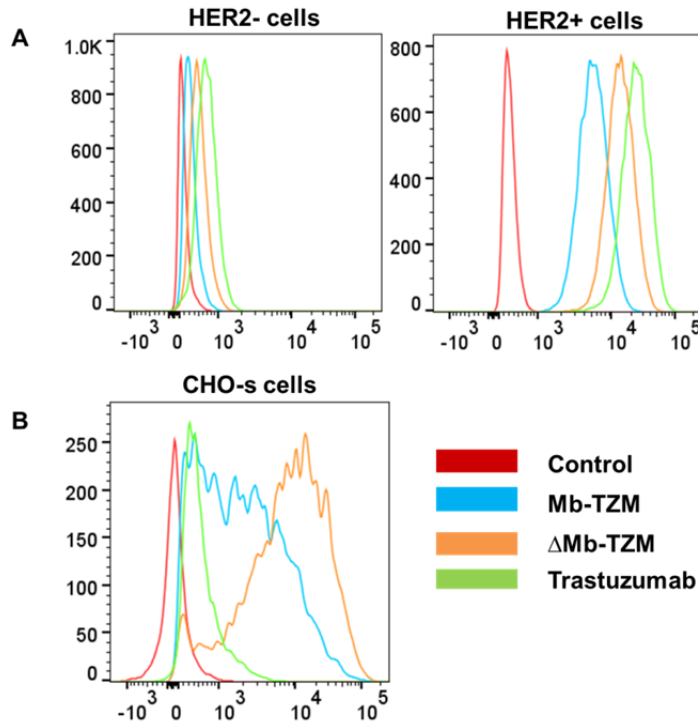


Figure 19: HER2 binding efficiency of engineered Mb-TZM or ΔMb-TZM antibodies

CHO-s cells were infected with lentivirus carrying heavy and light chains of Mb-TZM or ΔMb-TZM. **A)** Conditioned media from the CHO-s cells were purified for antibodies by Protein A purification. BT474 (HER2+) and UMSCC1 (HER2) cells were stained with Mb-TZM, ΔMb-TZM and trastuzumab (TZM), followed by secondary antibody: goat anti-human (H+L) FITC. Flow cytometry analysis of UMSCC1 and BT474 cells after staining. **B)** CHO-s cells infected with heavy and light chains of Mb-TZM or ΔMb-TZM or trastuzumab (TZM) were stained with goat anti human (H+L) Alexa Fluor 647 antibody and analyzed by flow cytometry. CHO-s cells alone were used as control.

6.3 Mb-TZM or Δ Mb-TZM antibodies conjugate with DOPC liposomes and deliver siRNA to HER2 overexpressing cancer cells to HER2 overexpressing cancer cells

We next tested to use Mb-TZM and Δ Mb-TZM for forming complex with DOPC liposomes encapsulated with fluorescence-labelled siRNA. Immunoliposomes were prepared by two different ways i) mixing fluorescence (Alexa Fluor 555)-labelled siRNA with DOPC followed by lyophilization and hydrating with a PBS solution containing antibodies or ii) mixing fluorescence-siRNA, the antibodies with DOPC, followed by lyophilization and hydrating with PBS. The immunoreactivity of either immunoliposomes was assessed by incubating with the HER2-overexpressing BT474 cells on a rocker for 30 min at 4°C. We found that both Mb-TZM and Δ Mb-TZM were integrated with liposome and delivered fluorescence-labelled siRNA to HER2-overexpressing cells but not to the control cells without HER2 overexpression. We observed Mb-TZM was slightly more efficacious in delivering the Alexa Fluor 555 siRNA than Δ Mb-TZM; whereas TZM had no such activity, similar to the background control without addition of antibody (Fig.20A). Comparing the two methods for generating the immunoliposomes, we found the second method shown in the rightmost bar in Fig.20A is the more efficient than the first method. Using this method, we further tested for a dose-dependent delivery of 6-FAM labelled siRNA in BT474 cells and found that immunoliposomes formed by mixing 2.5 μ g Mb-TZM with 2.5 μ g 6-FAM labelled siRNA and DOPC (shown in Fig.20 B,C) exhibited the greatest efficiency in delivering siRNA. Together, results from these pilot studies established an optimized protocol for generating a novel form of immune liposomes that were afterward tested for delivering siRNA and also peptides in vivo to HER2 overexpressing tumors in mice.

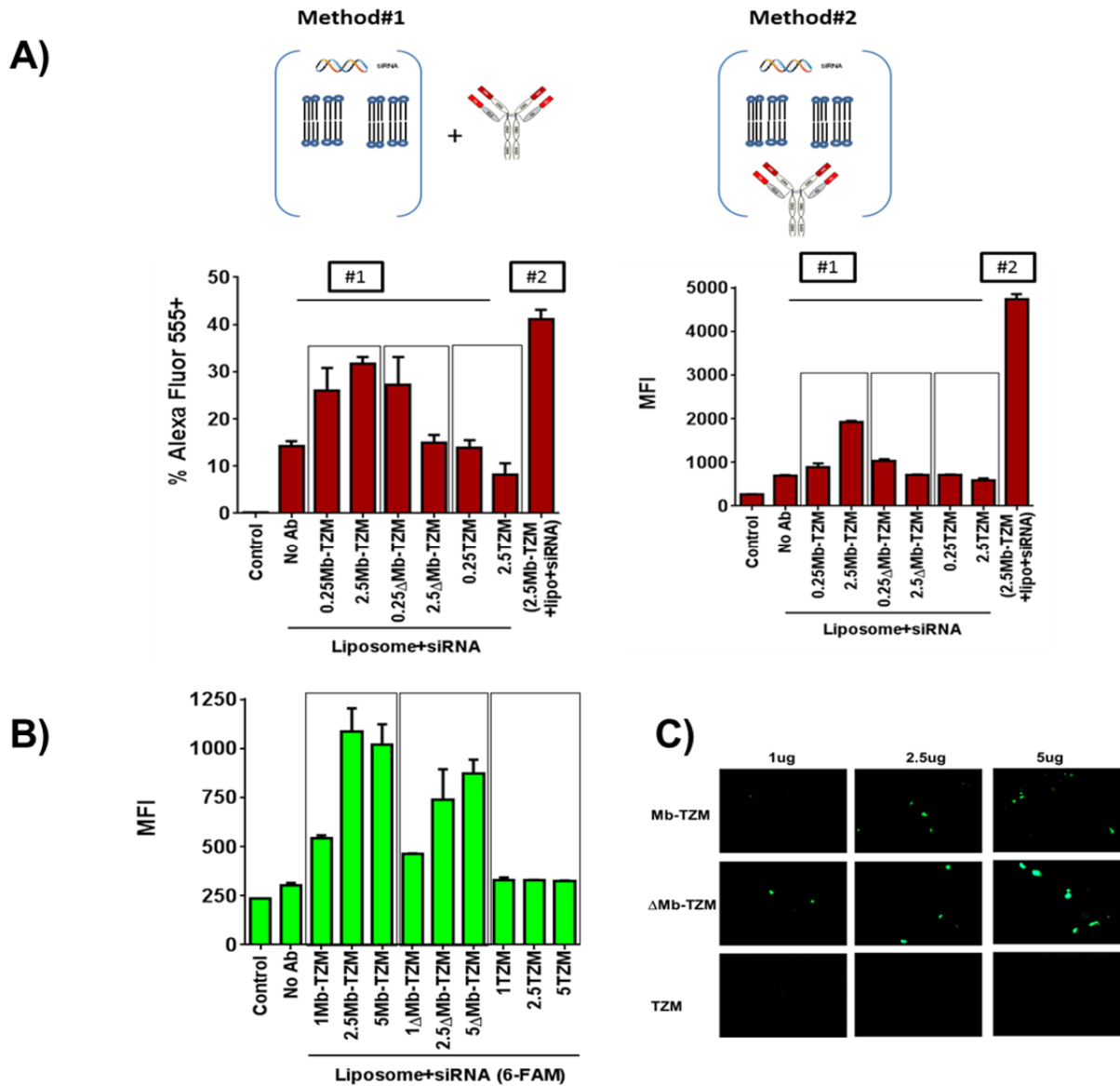


Figure 20: Mb-TZM or ΔMb-TZM antibody conjugated liposomes deliver labelled siRNA to the HER2 overexpressing cells in vitro

BT 474 cells (3×10^5) were incubated with immunoliposomes on a rocker at 4°C for 30 min and washed with FACS buffer; followed by incubation at 37°C for 30 min. Samples were analyzed by flow cytometer. Immunoliposomes were prepared as follows: **A)** 2.5μg Alexa Fluor 555 labelled siRNA were mixed with DOPC in the presence of tertiary butanol, followed by lyophilization and hydration with (0.25μg, 2.5μg) of Mb-TZM or ΔMb-TZM or TZM antibodies

or without antibody suspended in PBS. **B)** (1 μ g, 2.5 μ g, 5 μ g) Mb-TZM or Δ Mb-TZM or TZM antibodies or without antibody and 2.5 μ g 6-FAM labelled siRNA were mixed with DOPC in the presence of tertiary butanol, followed by lyophilization and hydration with PBS for formation of immune liposomes. **C)** As described above in B) the prepared immune liposomes were incubated with pre-seeded BT474 at 4°C for 30 min and washed with PBS; followed by incubation at 37°C for 8hr. The cells were then analyzed by for fluorescence by microscope.

6.4 In vivo delivery of labelled siRNA and peptides to HER2 overexpressing tumors by Mb-TZM and Δ Mb-TZM -immunoliposomes

DOPC neutral liposomes have been shown to effectively deliver siRNA, miRNAs in mouse with orthotopic tumor models (71, 77). However, these liposomes do not possess a tumor-specific recognizing potential, which negatively impacts this potential for being translated into clinical use because such approach may require a high dosage regimen which could be associated with systemic off target effects (71). As an approach of testing proof of principle, we examined the efficacy of Mb-TZM or Δ Mb-TZM in complex with DOPC liposomes to deliver the fluorescence-labelled siRNA and peptides encapsulated inside the liposome to HER2-overexpressing tumors in a mouse model. In this pilot study, we used bioluminescent 4T1 mouse mammary tumor cells transduced to overexpress human HER2 (hHER2). The bioluminescent 4T1-hHER2 cells were implanted into the mammary fat pad. One week after the implantation, mice were treated intravenously with Mb-TZM, Δ Mb-TZM or TZM in complex with DOPC liposomes that were encapsulated with Alexa Fluor 555-labelled siRNA as described. Tumor, liver, kidneys and lungs were resected and any organ-associated fluorescence was assessed ex vivo by IVIS imaging. As shown in Fig. 21A, Mb-TZM and Δ Mb-TZM immunoliposomes effectively delivered siRNA specifically to the tumors whereas TZM immunoliposomes did not. We next tested if these immunoliposomes could deliver peptides to the tumors and the peptides could be successfully presented with MHC-I on HER2-overexpressing tumor cells. In this context, we used E0771 cells overexpressing hHER2, which can present a known peptide in (SIINFEKL) in complex with MHC-I that can be detected by specific antibody using flow cytometry. We found that both Mb-TZM and Δ Mb-TZM conjugated immunoliposomes delivered SIINFEKL peptides to hHER2-overexpressing E0771 cells, whereas TZM immunoliposomes did not (Fig.21B). This important finding confirms our

prediction that Mb-TZM and Δ Mb-TZM antibodies can specifically and effectively deliver specific peptides encapsulated in the liposome to the hHER2-overexpressing tumors in vivo.

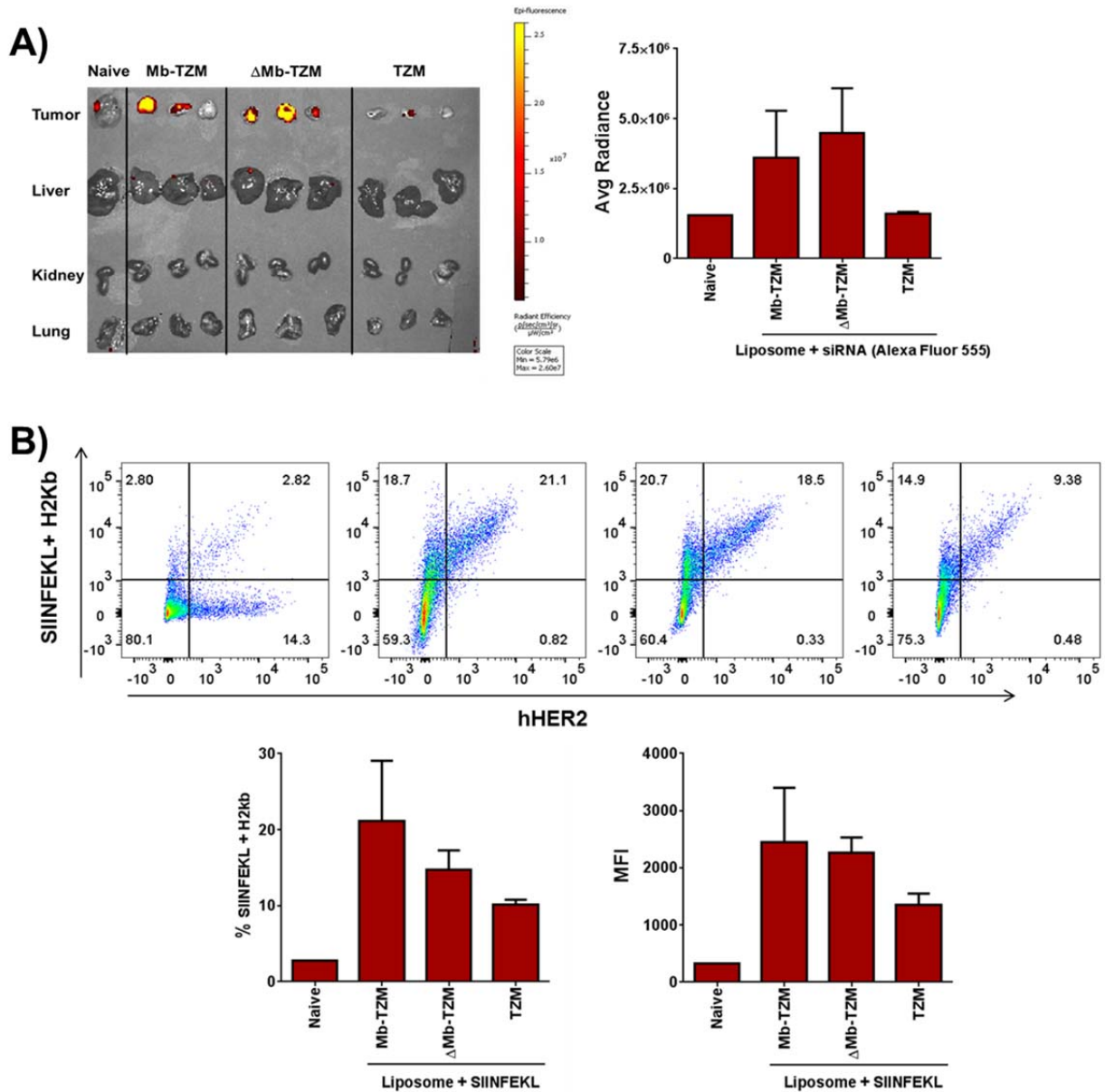


Figure 21: Mb-TZM and Δ Mb-TZM antibody conjugated liposomes deliver labelled siRNA and peptides to the HER2 overexpressing tumors

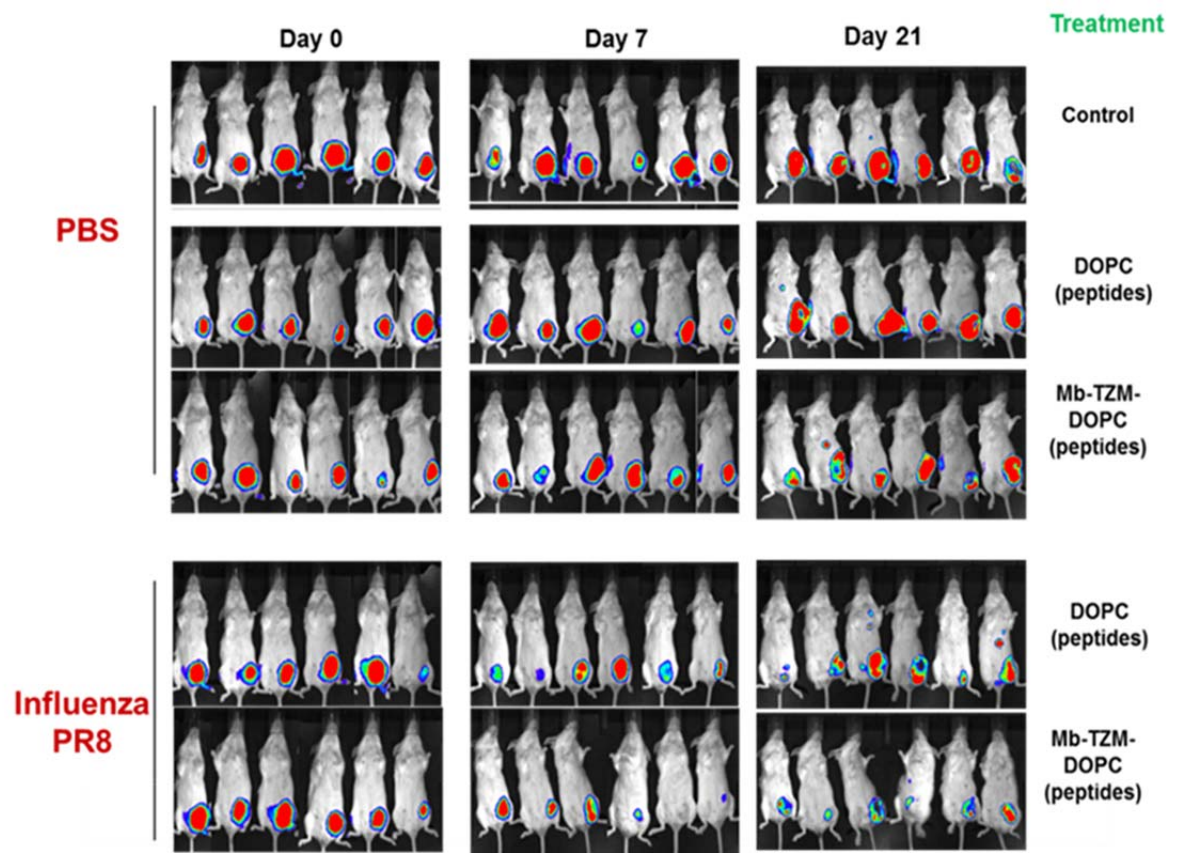
Mb-TZM or Δ Mb-TZM or TZM antibodies were mixed either with 5 μ g Alexa Fluor 555 labelled siRNA or 5 μ g SIINFEKL peptides with DOPC in the presence of tertiary butanol, followed by lyophilization and hydration with PBS for formation of immunoliposomes. **A)** 5-6 week BALB/c mice were injected with 5×10^6 4T1-HER2 cells in the mammary fat pad. Immunoliposomes were injected i.v. on day 8 after tumor implantation and 16 hr later the

tumors, liver, kidneys and lungs were analyzed for the presence of fluorescent siRNA by IVIS imaging. Average radiance collected from each tumor is plotted in the bar graphs. **B)** C57BL/6 mice were injected with 5×10^6 E0771-HER2 cells in the mammary fat pad. Immuno liposome were injected i.v. on day 8 after tumor implantation and 24 hr later the tumors were resected and single cells were made. Cells were stained for SIINFEKL bound MHC-I (H2-kb) using anti-(SIINFEKL+H2-kb) antibody and anti-HER2 antibody. Cells were analyzed by flowcytometry for SIINFEKL bound MHC-I in E0771- HER2 cells.

6.5 Re-routing pre-existing influenza T cell immunity to HER2 overexpressing mouse mammary tumors

Following our exciting finding that specific peptides can be delivered to hHER2-overexpressing tumors and can be successfully presented in complex with MHC-I in E0771-HER2 cells in vivo, we next examined whether Mb-TZM and Δ Mb-TZM antibodies conjugated with DOPC liposomes can deliver influenza antigenic peptides encapsulated in the immunoliposome to hHER2-overexpressing tumors in vivo (Fig.21). To prove the concept of re-routing pre-existing influenza PR8 immunity to 4T1-hHER2 tumors using Mb-TZM immune liposomes, Mb-TZM antibodies were mixed with DPOC encapsulated with two HA- and NP-related and MHC-I compatible peptides (IYSTVASSL and TYQRTRALV). Mice bearing 4T1-hHER2 tumor in mammary fat pads and pre-immunized with PBS or influenza PR8 received intravenous treatment with Mb-TZM-DOPC (containing the peptides) or DOPC only (containing the peptides). We have found that on day 7 after two doses of the treatment with the peptide-containing Mb-TZM-DOPC immunoliposome, the mice had less tumors burden compared to tumor burdens in mice either received no treatment or treatment with TZM (trastuzumab) alone or peptide-containing DOPC liposome alone, both in PBS- or PR8 immunized mice (Fig.22A, B). This encouraging preliminary finding indicates that Mb-TZM immunoliposomes are capable to deliver antigenic peptides to hHER2-overexpressing tumor cells in vivo that resulted in re-directing of pre-existing influenza-specific immune responses to the HER2-overexpressing tumors..

A)



B)

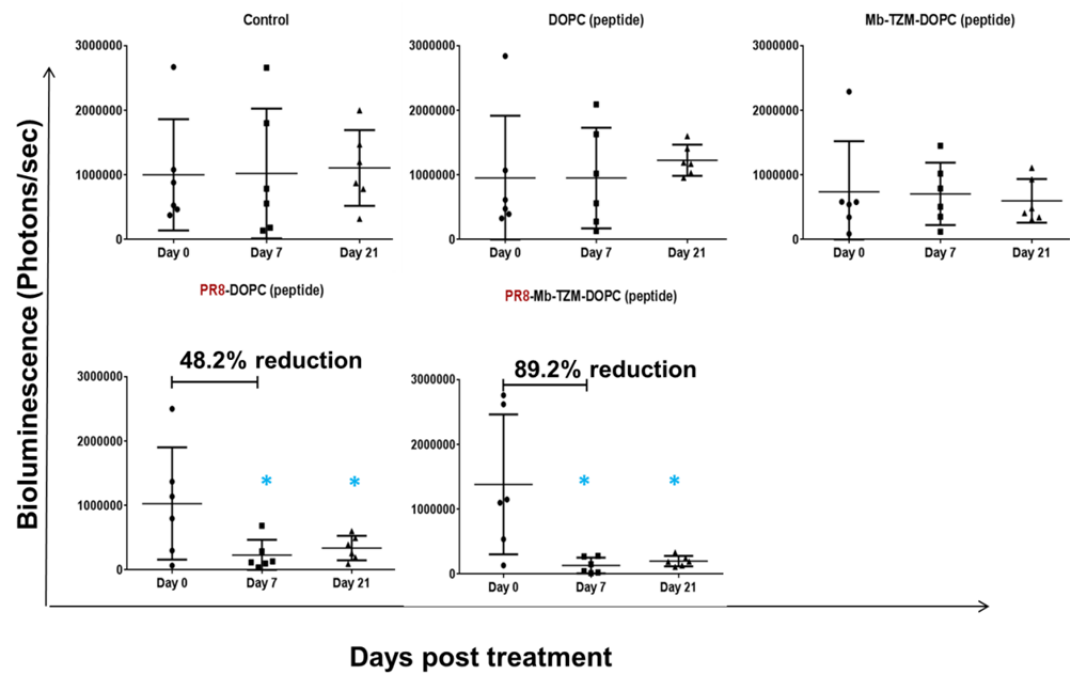


Figure 22: Mb-TZM-DOPC immunoliposomes deliver influenza peptides to the tumors and re-route pre-existing influenza specific immunity

5-6 week BALB/c mice were injected with 2×10^6 T1-HER2 cells in the mammary fat pad. One week later, when the tumors are formed Mb-TZM-DOPC (peptides) and DOPC (peptides) were injected i.v. into the mice every 3-4 days. A) Tumors were monitored by IVIS imaging on day 0 before the treatment and day 7, day 21 post treatment B) Average radiance efficiency of the tumors at day 0 before the treatment and day 7, day 21 post treatment were analyzed. * $p < 0.05$ compared with naive control on respective days.

Chapter 7: Discussion, conclusions and future plans:

HER2 overexpressing breast cancers are generally not immunogenic due to low mutation rate and there is no strong evidence of any virus or any pathogen associated to the cause of this disease (56, 57). We proposed to make HER2 overexpressing breast cancer cells immunogenic by delivering immunogenic peptides to the tumors through antibodies and make tumors susceptible to the pre-existing vaccine immunity. However, these peptides should be presented to CD8⁺ T cells through MHC-I. Few studies reported that an inverse correlation between the levels of HER2 and MHC-I expression in breast and esophageal cancers (85, 86, 104). We have tested MHC-I expression in a panel of HER2 overexpressing cell lines and found no correlation between HER2 and HLA-ABC expression. Also we found that trastuzumab significantly increased the expression of HLA-ABC, and co-stimulatory molecule CD86 in the presence of PBMC. Pre-existing memory immunity is very potent and rapid, however tumor antigen specific effector memory immune responses are very limited (33, 35, 63). Hence we tested our hypothesis of redirecting influenza virus specific immune responses to the tumors by endogenously expressing influenza virus antigens in primary and metastatic breast cancer cells. We found that these could be effectively targeted by pre-existing influenza specific immune responses (Fig.15). We observed this protection in mice was associated with dendritic cell activation and high memory CD8⁺ T cell infiltration to the tumors (Fig.16, 17). We then generated an anti-HER2 antibodies that conjugated with DOPC neutral liposomes and delivered labelled siRNA effectively (Fig.20, 21A). Additionally the antibody bond liposomes delivered peptides to the tumors and were presented on the MHC-I (Fig.21B). We also demonstrated that NP and HA peptides delivered to 4T1-HER2 tumors by antibody conjugated liposomes reduced tumor burden.

HLA-ABC expression in HER2 overexpressing breast cancer cells

In this aim we have shown that there is no significant correlation between HER2 and HLA-ABC, where silencing of HER2 expression in a panel breast cancer cell lines has no significant effect on HLA-ABC expression (Fig.5B). However, there are few exceptions, where we observed a marginal increase of HLA-ABC when HER2 was knocked down, but this is precisely cell specific. HER2 over expression was shown to have a negative effect on the expression of HLA-ABC in esophageal squamous carcinoma and breast cancers (86, 104). This could be due to the use of limited cell lines in the earlier studies study. Also, a HER2 targeting SiRNA had non-specifically increased STAT1 expression. Additionally, we also investigated the role of HER2 signaling on HLA-ABC expression and found that inhibition of HER2 signaling decreases HLA-ABC expression on breast cancer cells, which was accompanied by a decrease in STAT1 expression in the panel of cell lines (Fig.6B). However, this doesn't imply a positive relation between HER2 and HLA-ABC due to non-specific targeting of other molecules by small molecular inhibitors.

Small molecular inhibitors targeting MAPK pathway and some chemotherapy drugs could enhance the expression of HLA-ABC in cancer cells (105). Targeting HER2 signaling using trastuzumab and lapatinib (FDA approved small molecule inhibitor) targets several pro-oncogenic pathways including MAPK, AKT (9, 11, 90). Hence we examined if targeting HER2 signaling could increase the HLA-ABC expression in a panel of five HER2+breast cancer cell lines. Treatment of HER2+ breast cancer cell lines with 0.5uM, 1uM lapatinib for 24hr decreased the expression of HLA-ABC (Fig.6A). This decrease in HLA-ABC expression was accompanied by decrease in STAT1 expression in all five different cell lines (Fig.6B). Trastuzumab treated breast cancer patients showed higher infiltration of granzymeB expressing cytotoxic cells and T cells (24, 106, 107). Studies had shown the involvement of Fc

receptor+ immune cells in the mechanism of action of trastuzumab (14, 18, 91, 108) and also there is increasing evidence showing the importance of adaptive immunity in the efficacy of anti-neu/HER antibody treatment (21). Hence we proposed to test whether trastuzumab has any direct impact on HLA-ABC expression that is mandatory for T cells to recognize cancer cells. An important finding in our study is that trastuzumab by itself has no effect on HLA-ABC expression on breast cancer cells, but in the presence of immune cells (PBMC) trastuzumab treatment increased the HLA-ABC expression. We have shown from our experiments with conditioned medium that this increase in HLA-ABC was due to secreted cytokines from the immune cells. It is well known that IFN- γ increases HLA-ABC expression through activation of STAT1 signaling (89) and we found a significant amount of IFN- γ present in the conditioned medium from our PMBC co-culture experiments (Fig.8A). However there are several other cytokines, growth factors always present in the conditioned medium and we confirmed by neutralization of IFN- γ , where this increase in HLA-ABC is mostly abrogated(Fig.8B). The Fc portion of trastuzumab could interact with Fc receptors on NK cells, monocytes/macrophages, dendritic cells (36) .Here in this study, we also demonstrated that NK cells are the immune cells that are majorly involved in this n trastuzumab mediated up regulation of HLA-ABC. To further support our in vitro observations, we have also confirmed *in vivo* using transplantable murine 4T1-cells that express human HER2.Trastuzumab. Trastuzumab treatment significantly increased MHC-I on 4T1-HER2 cells compared to IgG treatment. It is well known that T cells are activated in two steps; in the first step antigenic peptides are recognized via MHC and in the second step they are activated via co-stimulation through CD28 - CD80 or CD86 interaction (40, 109, 110). In this study we also showed trastuzumab in the presence of PBMC increases the expression of both CD80 and CD86 in vivo.

The significance of activating exhausted T cells through various immunological approaches to target cancer cells is being well appreciated recently. Interaction of CTLA4 – CD80/86 or PD1-PD-L1 between T cells and cancer cells induces a stop signal for T cell activation. The usage of check point inhibitors anti-CTLA4 antibody, anti-PD1 and anti PD-L1 antibodies for targeting melanomas showed a tremendous success and these drugs are in clinical trials for other cancer types including breast cancers (46, 47, 50, 51, 54). We showed that trastuzumab in the presence of immune cells enhances the HLA-ABC, CD 80 and CD 86 expressions that good enough for mounting a T cell response. But response rates in patients for trastuzumab treatment are not absolute. There might be several factors for this limited response, one of them being fewer tumor infiltrating T cells or dysfunctional T cells expressing CTLA-4 and PD-1 (14, 54). IFN- γ is a well-known inducer of PD-L1 expression on the cancer cells (94, 95). Trastuzumab treatment induces a significant amount of IFN- γ in the micro-environment that might lead to increase in PD-L1 expression on cancer cells; we confirmed this by using PBMC co-culture experiments, where trastuzumab significantly increase PD-L1 expression (Fig.11). Recently it has been shown that HER2 down regulation by trastuzumab was observed in the presence of PBMC (91), and we observed similar result in our panel of cell lines too (data not shown). Hence we silenced HER2 using siRNA and found that HER2 knock down decreased PD-L1 expression significantly. This suggests that upon treatment with trastuzumab even though there is a reduction in HER2 levels, the PD-L1 is high due to effect of IFN- γ (Fig.23). In a recent study, combination of anti-ratErbB-2 mAb and anti-PD-1 mAb showed a synergistic effect on tumor reduction in immune-competent mice. Hence trastuzumab treatment enhances the HLA-ABC on cancer cells making them more visible to the T cells; this can be exploited for combination with anti-CTLA4 or anti-PD-1/PD-L1 mAb treatment owing for a better clinical response.

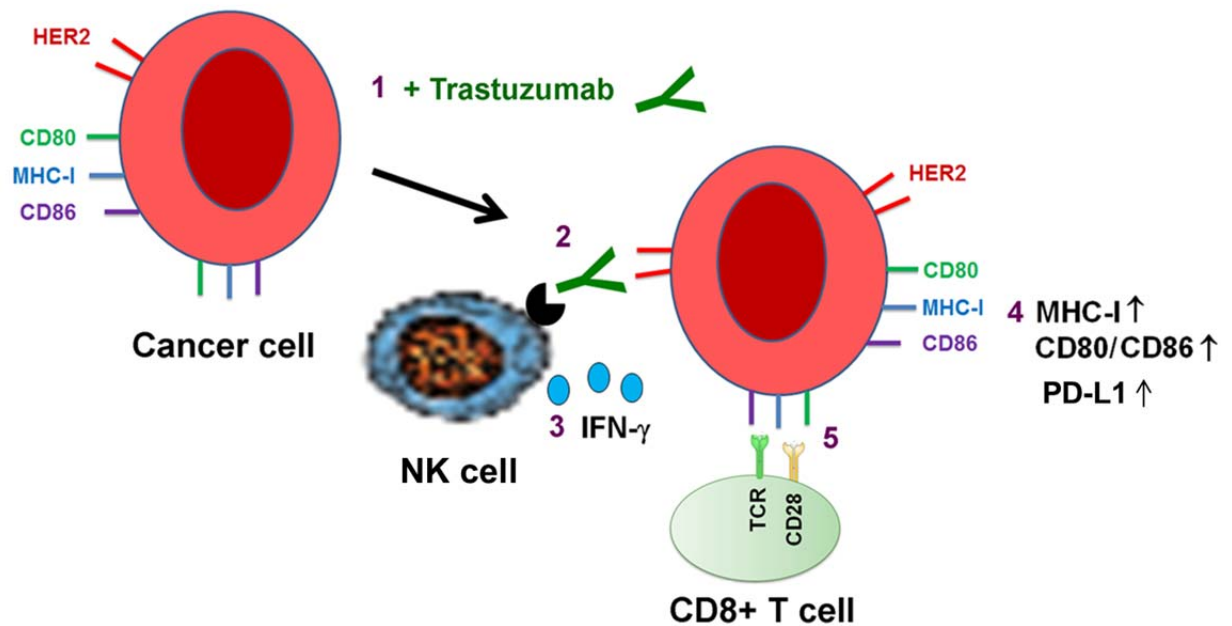


Figure 23: Proposed model of trastuzumab-induced expression of HLA-ABC and CD80/CD86 co-stimulatory molecules in cancer cells

Binding of trastuzumab to HER2 on breast cancer cell surface engages immune cells through interaction with the antibody's Fc fragment, leading to secretion of IFN- γ , which up regulates the expression of MHC-I and CD80/CD86 on targeted cells. The numbers show the sequence of events in the proposed model.

Virus specific memory immune responses for targeting mouse mammary tumors:

We have shown that trastuzumab treatment increases HLA-ABC expression that is necessary for peptide (antigen) presentation to the CD8+T cells. We further demonstrated that pre-existing viral immunity is effective enough in regression of mouse primary and metastatic mammary tumors endogenously expressing NP and HA antigens of influenza PR8.

The strategy of “tricking” the immune system to recognize the cancer cells as virus infected cells and eliminate them is well appreciated recently (65, 67). Intratumoral injection of accessible tumors with bacteria or virus in melanomas has been reported previously (65). A recent study has shown that vaccination of mice with adenoviral vectors expressing bacterial LacZ antigen and intratumoral injection of the same virus has had shown 50% reduction in the tumor burden (111). However, intratumoral virus injection used in this study could have led to lysis of cells and immune cell infiltration directly to the tumors. Here in our study, we endogenously expressed an immunodominant influenza PR8 antigen NP in TUBO cells and shown that pre-existing immunity against single antigen is sufficient enough for regression of tumors and this regression was congruent with greater IFN γ in the antigen specific recall assays. In some earlier studies, HA antigen was expressed in cancer cells and was used as a TAA in HA-transgenic mice and HA-T cell receptor transgenic mice for characterization T cell responses to tumor antigens (112-114). But none of these studies focused on targeting metastasis of breast cancer cells by the foreign antigens. As TUBO cells forms non-metastatic primary tumors, we further extended out studies to metastatic breast cancer models. We adopted two antigens HA and NP, for making metastatic 4T1 cells in BALB/c mice and E0771 cells in C57Bl/6 mice more immunogenic. We successfully engineered both cell lines to express influenza PR8 antigens NP and HA. We have shown that 4T1-Luc and 4T1-NP+HA-Luc cells form primary and metastatic tumors in BALB/c mice; surprisingly in C57Bl/6 only E0771-Luc

cells formed primary tumors and E0771-NP+HA-Luc cells were completely rejected (Fig.14). These suggest that antigen NP and HA are very immunogenic antigens and could be exploited for targeting tumors. We next proved our hypothesis of rerouting preexisting influenza specific memory immunity to tumors using 4T1-NP+HA-Luc metastatic model and shown that influenza PR8 immunized mice are significantly protected from 4T1-NP+HA-Luc tumors compared to non-immunized mice (Fig.15). As expected 4T1-NP+HA-Luc tumors are significantly less tumorigenic than 4T1-Luc due to NP and HA antigens expression suggesting the immunogenicity of the two antigens strong enough to regress tumors.

Tumors are generally infiltrated with immune cells, however majority of them are either dysfunctional or exhausted (49, 63, 64). In a recent study, early T effector and Treg cells responses after 4T1 tumor implantation were analyzed and found that tumor antigen specific Treg are stormed to the tumor site earlier than T effector cells (63). In our study we report that immunosuppressive cells Treg cells and MDSC are outnumbered by CD8⁺ memory T cells, mature dendritic cells, NK cells in 4T1-NP+HA-Luc tumors of mice with pre-existing influenza PR8 immunity. Suggesting both innate and adaptive immune responses are activated in the mice with pre-existing immunity. Because Treg cells are shown to be highly infiltrating normal tumors and suppress effector T cells; a study in transplantation immunology showed that memory T cells are resistant these Treg cells (60, 115). In our study we showed that 4T1-NP+HA-Luc tumors of influenza immunized mice have low Treg and high memory CD8⁺ T cell infiltration, that could have led to a synergistic effect for regression of tumors.

Hence, we confirm from our findings that exploiting memory CD8⁺T cell responses present in the patients specifically to foreign antigens and rerouting them to tumors will be best strategy for treating HER2⁺ breast cancers. The use oncolytic virus has recently been appreciated and the first oncolytic virus talimogene laherparepvec (T-vec) was approved by

FDA to treat local lesions in the skin and lymph nodes of melanoma patients. However, systemic usage of them is a challenge due to potential non-specific off target effects (71). Oncolytic virus herpes simplex virus (HSV-1) infects through herpesvirus entry mediator (HVEM) and measles virus through CD46 that are aberrantly expressed in tumors. These receptors are very commonly expressed on other normal cells, and also existing antibodies in the patients against oncolytic viruses reduces the infection rate drastically (65, 67). These challenges stimulated us to develop an antibody against HER2 molecule that could conjugate with DOPC liposomes for delivery of antigens to the tumors.

Anti-HER2 antibody for delivery of antigenic peptides to HER2 overexpressing tumors

We have developed anti-HER2 antibodies that could conjugate with DOPC liposomes for delivery of siRNA and peptides to HER2 overexpressing cancer cells. These engineered targeted therapy vehicles are expected to enhance the specific delivery of anti-cancer therapeutics significantly. Researchers have been using liposomes for delivery of drugs, anti-microbial agents, vaccines, nucleotides etc. (70, 71, 77). Over 12 different drug loaded liposomes have been approved in clinic for treating various diseases (70).

Recent studies have advanced the usage of liposomes to the next level by conjugating them with antibodies or antibody fragments (99, 100). Conventionally, three different strategies are adopted to conjugate the liposomes, where an antibody fragment is conjugated to the lipid bilayer of liposome in presence or absence of PEG chains (type I immunoliposomes) or it is conjugated to the distal end of the PEG chain (type II immunoliposomes). (71, 116). Coupling of these antibody fragments with lipid bilayer was previously done through chemical conjugation of head groups of the lipid with fragments of antibody, which could lead to loss of biological function (117). However in this study, we generated a biological way of conjugating lipid bilayer to the antibodies. We successfully cloned and expressed light chains and the two

engineered heavy chains of trastuzumab Mb-TZM and Δ Mb-TZM in CHO-s cells. We confirmed that these two antibodies are having same binding properties as trastuzumab. To our surprise, we observed that both the antibodies produced by CHO-s cells are expressed mostly on the surface, suggesting that Mb-TZM and Δ Mb-TZM could be capable of conjugating with lipid layered liposomes that are similar to cell membrane. Mb-TZM and Δ Mb-TZM antibodies were added directly to the DOPC lipids along with labelled SiRNA for liposome formation without the need for any chemical modifications. We report here that the Mb-TZM and Δ Mb-TZM delivered labeled SiRNA to HER2 overexpressing cancer cells both *in vitro* and *in vivo*. Surprisingly we didn't observe any accumulation of these siRNA in the liver, kidney and lungs; they are only present in the tumors, suggesting that the delivery of siRNA was very specific to the HER2+ tumors. Delivery of anti-oncogenic SiRNA's through DOPC liposomes were previously shown to have therapeutic benefit in ovarian, breast and other cancer types (71, 77). Our immunoliposomes (Mb-TZM-DOPC and Δ Mb-TZM-DOPC) showed better delivery efficiency than the DOPC liposomes alone (Fig.21A).

In this study, we also tested our hypothesis to immunogenic deliver peptides to the HER2 overexpressing tumors. Recent studies have shown therapeutic benefit by intratumoral injections of oncolytic viruses or bacterial components due to the storming of immune cells nonspecifically to the tumors (65, 67). However, systemic usage of them is a challenge due to potential non-specific off target effects. Hence, we here show that Mb-TZM-DOPC and Δ Mb-TZM-DOPC immunoliposomes successfully delivered ovalbumin peptides specifically to HER2+ tumor cells, additionally, these peptides were presented in the context of MHC-I that was detected by a specific antibody (Fig.21B). We further show the redirection of pre-existing immunity against influenza PR8 to 4T1-HER2 cells through Mb-TZM-DOPC loaded with NP and HA MHC-I peptides liposomes.

Significance and future plans of the study:

In summary, we have shown that anti-HER2 targeting antibody (trastuzumab) in the presence of PBMC can significantly increase the expression of MHC-I (HLA-ABC) and stimulatory molecules CD80 and CD86 in HER2 overexpressing cells. In this context we made an important observation that along with increase in MHC-I expression, the PD-L1 expression was also significantly increased due to trastuzumab treatment. Over expression of PD-L1 is a caveat to the T cells, it could act as a secondary target along with trastuzumab therapy. Combination of anti-PD-1 and PD-L1 antibodies and trastuzumab therapy are currently in clinical trials to treat various cancers (51, 54).

In this study we have shown that pre-existing immunity regress the mouse mammary tumors if the cancer cells express influenza immunodominant antigens or peptides. We have achieved this through delivery of immunogenic influenza MHC-I peptides through engineered antibodies. The antibodies Mb-TZM and Δ Mb-TZM could deliver any liposome loaded therapeutics to HER2 overexpressing cells, which could be siRNA, small molecule drugs, chemotherapeutic drugs, enzymes etc. Our current research focused on delivery of the labelled siRNA or peptides with minimal amount of antibody bound to the liposomes, which could be further increased to add an additive effect of HER2 signal inhibition. A more complex study using different liposome: antibody molar ratios for best tolerated dose and greater efficacy will be needed. We have used an immune competent mouse model that has immunogenicity towards human HER2 molecule; hence for the future studies we will be using transgenic mice that are tolerant human HER2 (118). Also, our current study was limited in using influenza specific immunity; however other vaccination models can be combined with influenza leading to more choice of peptides that could be loaded to the liposomes. MHC-I (HLA-ABC) are very

polymorphic in humans, this raises an issue of patient specific peptides generation or usage of longer peptides (119).

Finally, we propose the model shown in the Fig. 22 where the patients possess healthy pre-existing immunity towards several vaccines taken in childhood, even after the diagnosis of cancer. The patients will then be boosted with immunodominant peptides of the vaccines with appropriate adjuvants. The patients will then be treated with Mb-TZM-DOPC liposomes containing immunodominant peptides of the vaccines that used for boosting. These peptides will be presented by the cancer cell in the context of MHC-I and will be attacked by memory T cells.

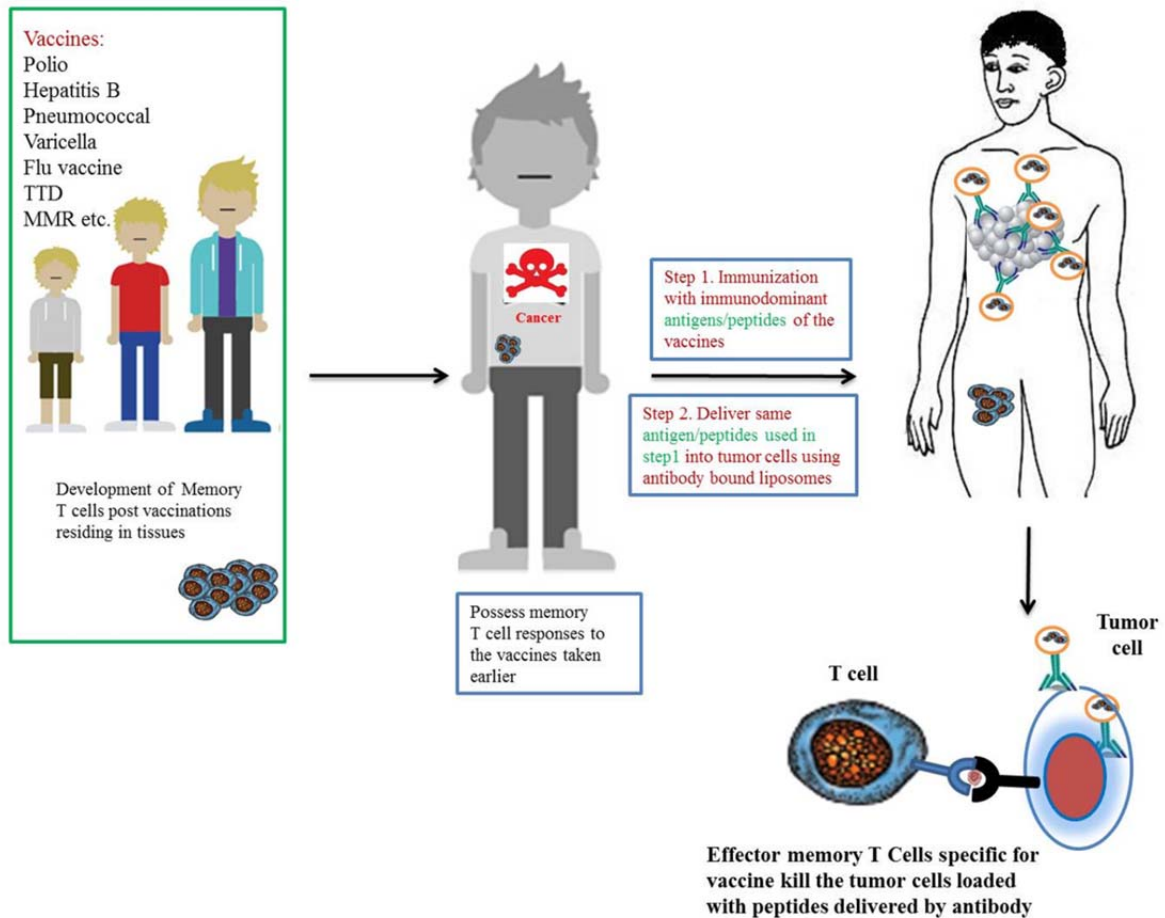


Figure 24: Re-routing vaccine induced T cell responses to HER2 overexpressing tumors

Several vaccinations are given from being a child to adult; the body develops memory immune responses against these vaccines that include T cell and antibody responses. Even after the diagnosis of cancer, these vaccine specific immune responses are still present and are potent enough in the body. Step: Boost with immunodominant antigens/peptides of the vaccines. Step2: Deliver same antigens/peptides to the tumors using immunoliposomes. Cancer cells present these peptides to vaccine specific memory CD8⁺ T cells and are prone to cytotoxicity.

Chapter 8: Materials and methods:

Contents of this chapter are based on Bharat K. R. Chaganty, Yang Lu, Songbo Qiu, Srinivas S. Somanchi, Dean A. Lee & Zhen Fan. Trastuzumab upregulates expression of HLA-ABC and T cell costimulatory molecules through engagement of natural killer cells and stimulation of IFN- γ secretion. OncoImmunology (Online). DOI: 10.1080/2162402X.2015.1100790

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Reference Number: P033016-04

Reagents: Trastuzumab, bevacizumab, and rituximab (Genentech) were obtained from the ambulatory pharmacy of The University of Texas MD Anderson Cancer Center.

Breast cancer cell lines:

BT474, HCC1954, MDA-MB-231, MDA-MB-361, MDA-MB-453, MCF7, MCF7-HER18, SKBR3, SUM190, T47D, ZR75B and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (50/50, v/v), 4T1 cells in (DMEM), E0771 cells in Roswell park memorial institute medium (RPMI-1640) and supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin in a 5% CO₂ atmosphere at 37°C as described previously (120, 121).

Western blot analysis: Cells were lysed in a lysis buffer containing 50 mM TrisHCl (pH 7.4), 150 mM NaCl, 0.5% Igepal CA-630, 50 mM NaF, 1 mM Na₃VO₄, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich) and kept on ice for 15 min. The lysates were cleared by centrifugation, and the supernatants were collected. Equal amounts of protein lysate, as determined using the Pierce Coomassie Plus colorimetric protein assay (Thermo Fisher Scientific), were separated by SDS–polyacrylamide

gel electrophoresis, blotted onto nitrocellulose, and probed with primary antibodies against HER2 (Calbiochem) and tyrosine 1248-phosphorylated HER2 (Cell Signaling Technology); STAT1 and Y701-phosphorylated STAT1 (Cell Signaling Technology); STAT3 and Y705-phosphorylated STAT3 (Cell Signaling Technology); Akt and S473-phosphorylated Akt (Cell Signaling Technology); Erk (Santa Cruz Biotechnology) and T202/Y204-phosphorylated Erk (Cell Signaling Technology); and β -actin (Sigma-Aldrich). The signals were visualized using the enhanced chemiluminescence detection kit (GE Healthcare).

Flow cytometric analysis of cells from *in vitro* assays:

Breast cancer cells were detached from culture dishes by incubation with a nonenzymatic citric acid-based buffer (0.135 M KCl, 0.15 M sodium citrate, 0.6 mM EDTA) or TrypLE reagent (Life Technologies) at 37°C until cells detached. Flow cytometric detection of HER2 in human breast cancer cells was performed by incubation of the cells with trastuzumab and FITC-conjugated anti-human IgG antibody (1-2 μ l in 100 μ l) for 30 min at 4°C. The fluorescently conjugated primary antibodies used in this study and the respective vendors were as follows: APC-conjugated anti-HLA-ABC (clone W6/32) antibody, (BioLegend), APC-conjugated anti-PD-L1 (clone 29E.2A3) antibody (BioLegend), PE-conjugated antihuman CD80 (clone 2D10) and PE-Cy7-conjugated anti-human CD86 (clone IT2.2) antibodies (BioLegend); for PBMC sorting phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD56 (clone MY31), APC-conjugated anti-CD14 (clone 61D3), and FITC-conjugated anti-CD3 antibodies (clone SK7, Tonbo Biosciences). IFN- γ -neutralizing antibody (clone NIB42, eBioscience) and FITC- or PE-labeled Annexin-V antibody was obtained from BD Biosciences Pharmingen.

Flow cytometric analysis of mouse tissues:

Cells from mouse tumor tissues or lymph nodes were prepared by mincing into small pieces and passing the samples into a fresh conical tube through a 70- μ m mesh cell strainer. Single cell

suspensions (0.5-1x10⁶) were prepared in 100 µl of fluorescence-activated cell sorting buffer (0.5% BSA in PBS) and stained with fluorescently conjugated primary antibodies or secondary antibodies (5-20 µl in 100 µl) for 30 min at 4°C. An isotype-matched antibody was used as a control. Excess antibodies were washed away twice with fluorescence activated cell sorting buffer prior to analysis under a BD Biosciences Canto II analyzer. The fluorescently conjugated primary antibodies used in this study and the respective vendors were as follows: APC-conjugated anti-mouse H-2Kd antibody (clone SF1-1.1.1, eBioscience); APC-conjugated anti-mouse CD80 (clone 16-10A1) and FITC/PE-conjugated anti-mouse CD86 (clone GL-1) antibodies (Tonbo Biosciences); PE-Cy7 Anti-Mouse CD3e (145-2C11) (Tonbo Biosciences), APC Anti-Mouse CD62L (L-Selectin) (MEL-14) (Tonbo Biosciences), VioletFluor™ 450 Anti-Human/Mouse CD44 (IM7) (Tonbo Biosciences), APC Anti-Mouse Foxp3 (3G3) (Tonbo Biosciences), PE Anti-Mouse CD25 (PC61.5) (Tonbo Biosciences) FITC Anti-Mouse CD4 (RM4-5) (Tonbo Biosciences), PE Anti-Mouse CD8a (53-6.7) (Tonbo Biosciences), PE-Cy7 Anti-Mouse CD3e (145-2C11) (Tonbo Biosciences), PE Anti-Mouse CD11c (N418) (Tonbo Biosciences), FITC Anti-Mouse Ly-6G (Gr-1) (RB6-8C5) (Tonbo Biosciences), FITC Rat IgG2a Isotype Control (2A3) (Tonbo Biosciences), Fc block (purified Anti-Mouse CD16 / CD32) (2.4G2). Pacific Blue™ anti-mouse CD49b (clone DX5) (pan-NK cells) (BioLegend), PE/Cy7 anti-mouse/human CD45R/B220 (clone RA3-6B2) (BioLegend), APC/Cy7 anti-mouse CD69 (H1.2F3) (BioLegend), PE/Cy7 anti-mouse H-2Kb/SIINFELK (BioLegend).

Knockdown of HER2 gene expression by siRNA: HER2-targeted siRNA (target DNA sequence #1, GGGAAACCTGGAACTCACC; #2, CATCAAAGTGTTGAGGGAA; #3, GGACATCTTCCACAAGAAC; #4, GCAGTTACCAGTGCCAATA; and #5,

CAGAATGGCTCAGTGACCT) and control siRNA were purchased from Sigma-Aldrich. The siRNA (200 pmol) and Lipofectamine 2000 (5 μ l, Life Technologies) were mixed in 100 μ l of minimal essential medium (Opti-MEM, Life Technologies) for 15 min, and the siRNA–Lipofectamine 2000 mixture was added into the culture medium of cells seeded at 3×10^5 cells/well in a 12-well plate. Six hours later, the medium was replaced with regular medium, and the cells were cultured for an additional 72 h before detection of HER2 expression knockdown, HLA-ABC and PD-L1 expression by flow cytometry or western blot.

Co-culture experiments with PBMC:

PBMC from healthy donors were isolated by Ficoll-Paque PLUS (GE Healthcare Life Sciences) gradient centrifugation as described previously 40. Briefly the buffy coats from healthy individuals were diluted to a final volume of 140 ml. In a 50ml tube add 15 ml Ficoll-Paque PLUS and layer 35 mL of buffy coat sample. Centrifuge for 20 minutes at 400g. The PBMC are isolated from the interface between plasma and Ficoll, wash the PBMCs with PBS and count the cells using hemocytometer. Cancer cells were incubated with PBMC at a ratio of 1:10 or 1:5 (cancer cells: PBMC) for 48 h. At the end of co-culture experiments, conditioned medium was collected, any PBMC in suspension were washed away with PBS, and the cancer cells from the co-culture were harvested, stained with the desired fluorescently conjugated primary antibody, and subjected to flow cytometry analysis.

ELISA: IFN- γ in conditioned medium from the co-culture experiments and antigen specific recall experiments was analyzed with a human IFN- γ ELISA kit from BioLegend and mouse IFN- γ ELISA kit from BD Biosciences. The procedure was performed according to the protocol provided by the vendor. In brief, anti- IFN- γ capture antibodies were coated to the wells in a 96-well plate in carbonate buffer and incubated overnight at 4°C. The wells were blocked

with PBS containing 10% fetal bovine serum for 1 h at room temperature, and then 100 μ l of conditioned medium (diluted if necessary) was added and the wells were incubated for an additional 2 h at room temperature. IFN- γ detection antibody was then added. Beginning 1 hour later, the wells were incubated for 30 min with HRP-conjugated biotin, and then TMB (3, 3',5,5'- tetramethylbenzidine) substrate was added. The wells were washed 3 to 5 times between each step with PBS containing 0.05% Tween-20. The 96-well plates were read at 450 nm for optical density using an Omega microplate reader.

Immunofluorescence assay:

Cells were grown in monolayers and fixed with 2% paraformaldehyde overnight at 4°C. Cells were washed with PBS and cells were blocked with 10% FBS in PBS containing 1% Triton X-100 for 1hr. Cells were washed with PBS. Primary antibodies against HA or NP or influenza PR8 were added in 10% FBS containing 1% goat serum and incubated at room temperature (RT) for 2hr. Cells were washed with PBS, and fluorescent tagged secondary antibodies against mouse or rabbit primary antibodies were incubated for 1hr at RT. Cells were washed with PBS and imaged by Olympus inverted microscope.

Antigen specific splenocyte recall assay:

Mouse splenocytes were collected and single cells were prepared by mashing with syringe plunger and passing the samples into a fresh conical tube through a 70- μ m mesh cell strainer. Single cell suspensions with (10^6 cells) per well were prepared in 100 μ l complete media and co-cultured with media, TUBO cells and TUBO+NP cells or TUBO+ NP peptides (TYQRTRALV) for 72hr. Cells were centrifuged and conditioned medium was collected and analyzed for IFN- γ by ELISA.

Recombinant antibody production:

Light chain of trastuzumab was cloned in to CHO-s cells using CMV promoter based pLEX expression system, where mCherry is fused to the light chain of trastuzumab by 2A sequence . pLEX-vector based recombinant lentivirus expressing light chain and mCherry were sorted by FACS. High mCherry expressing cells were transduced with Mb or Δ Mb modified heavy chains of trastuzumab fused with GFP using pLEX-based recombinant lentivirus. High mCherry and GFP expressing cells were sorted by FACS. CHO-s cells were expanded exponentially in shaker flasks. Cells were collected by centrifugation at 400g for 10 min. Cells were lysed with 50 mM TrisHCl (pH 7.4), 150 mM NaCl, 1% Igepal CA-630, 50 mM NaF, 1 mM Na₃VO₄, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich) and kept on ice for 15 min. Cells were centrifuged for 15 min at 13000rpm, supernatant was collected and 2X 0.1M glycine buffer (pH 8.0) was added to the supernatant to make 1x final concentration. Washed Protein A beads were incubated with supernatant overnight on a rolling shaker at 4°C. Antibodies were eluted from the beads using 0.1M glycine (pH 2.6) and immediately neutralized with 1MTrisHCl (pH 11). The antibodies are dialyzed against PBS using 10 KDa molecular size cutoff cassettes (Thermo scientific).

Preparation of immunoliposomes:

Neutral liposomes were prepared by mixing 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) with labelled siRNA or peptides with antibodies at different ratios. This mixture was added with Tween 20 at a ratio of 1:19 Tween 20: (siRNA or peptide/lipid+antibody) in the presence of excess tertiary butanol and vortexed before lyophilization using acetone/dry ice bath. Liposomes are reconstituted in PBS (without Ca and Mg) and vortexed for 3 seconds before use.

In vivo experiments:

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. Swiss female nude mice (5-6 weeks old) were obtained from the colony facility maintained by the Department of Experimental Radiation Oncology at MD Anderson Cancer Center. 5-6 weeks old BALB/c and C57BL/6 mice were obtained from Taconic, or NCI laboratories or Charles River laboratories. 4T1 mouse mammary cells were infected with a pLEX-based recombinant lentivirus containing human HER2. Stable 4T1 cells expressing human HER2 (4T1/HER2, 2×10^6 cells/mouse) were implanted in the mammary fat pad of Swiss nude mice or BALB/c mice.

4T1 or E0771 mouse mammary cells were infected with a pLEX-based recombinant lentivirus containing influenza PR8- HA or NP. Stable 4T1/E0771 cells expressing NP, HA and Luc (4T1/E0771-HANP-Luc) were implanted in the mammary fat pad of BALB/c (4T1) and C57BL/6 (E0771) mice.

In vivo Imaging:

Luminescence: 3mg D-luciferin was injected intraperitoneally in each mice and allowed to move freely for 10 min, and were anesthetized with 2-5% isoflurane in oxygen. Mice were imaged in IVIS 100 instrument for luciferase signal.

Fluorescence: Mice were euthanized in the CO2 chamber; tumors, liver, lungs and kidneys were resected and imaged for Alexa Fluor 555 signal using IVIS spectra. Fluorescence was calculated using region of interest (ROI) tool in Calipers Living Image software.

Statistical analysis:

Each experiment was repeated at least three times, and the mean values with standard error of the mean are presented. A two-tailed unpaired Student's t test was used to compare two groups of independent samples. For correlation analysis, Pearson's correlation coefficients were computed. $p < 0.05$ was considered statistically significant.

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Vita

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