Title: T-cell targeted immunotherapy against the tumor associated antigen survivin (BIRC5) as a potential neoadjuvant immunotherapy for triple negative breast cancer.

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Abstract

Background

Triple negative breast cancer (TNBC) cells are, by definition, estrogen and progesterone receptor negative and do not produce high levels of the HER2 protein. Checkpoint inhibitor immunotherapy has been approved for patients with TNBC, but the prognosis for these patients is poor compared with patients presenting with other common forms of breast cancer. Survivin (BIRC5) is a tumor-associated antigen (TAA) previously described to be overexpressed in triple negative breast cancer, but not expressed, or expressed at very low levels, in normal tissue, representing targets for cytotoxic T lymphocyte (CTL) immunotherapy.

Study Design

A BALB/c TNBC mouse model using the 4T1 cell line was used to explore the effect of an adjuvanted microsphere synthetic vaccine containing survivin peptides on tumor growth rate. The vaccine was administered via intraperitoneal injection at study start with a second dose given 14 days later. An orthotopic injection of 4T1 cells into mammary tissue was performed on the same day as the administration of the second vaccine dose. The mice were followed for up to 41 days with subcutaneous measurements of tumor volume made every three days (Figure 1).

Results

Vaccination with survivin peptide antigens resulted in a statistically significant diminution of tumor-takes and a deceleration of primary tumor growth volume in BALB/c mice challenged with 4T1 cells versus control. This effect was seen in both the 250 4T1 cell and the 500 4T1 cell challenge groups. The mice produced an ELISpot immune response to one of the survivin peptide antigens used in the vaccine and this response was only seen after the adjuvanted microsphere vaccine was administered.

Conclusion

The synthetic, adjuvanted microsphere peptide vaccine given 14 days before challenge, and on the day of challenge, was able to diminish tumor-takes and decelerate tumor growth in BALB/c mice challenged with 250 or 500 4T1 cells injected into mammary tissue. This model suggests that T-cell immunotherapy specifically targeting survivin might be an applicable neoadjuvant immunotherapy therapy for triple negative breast cancer. More pre-clinical studies and clinical trials will be needed to explore this concept further.

Keywords: Breast Cancer, Immunotherapy, Bioinformatics, Next-Generation Sequencing, Neoadjuvant Therapy, tumor-associated antigen, survivin, BIRC5, TNBC, NGS, TAA

Abbreviations

mRNA = messenger RNA

NGS = next-generation sequencing

RNA-seq = RNA-Seq

SNP = single nucleotide polymorphisms

TAA = tumor-associated antigen

TPM = transcripts per million

WES = whole exome sequencing

APC = antigen presenting cells

CTL = cytotoxic T lymphocyte

ELISpot = enzyme-linked immunosorbent spot

MHC = major histocompatibility complex

TNBC = triple negative breast cancer

IACUC = Animal Care and Use Committee

AAALAC = Association for Assessment and Accreditation of Laboratory Animal Care

1. Introduction

A subset of immunotherapy involves stimulating CD8+ cytotoxic T lymphocytes (CTLs) to selectively kill cancer cells (1) thus avoiding damage to normal tissue.

CTLs recognize non-self-peptides presented in the context of MHC class I molecules present on pathogen infected cells or on the surface of antigen presenting cells (APC) specialized in pathogen-derived protein processing. Peptide/MHC class I complexes on the surface of such specialized APC are powerful stimulators of CTL responses. The evoked peptide/MHC class I specific CTL cell population expands, seeks out and lyses cells presenting those targets (2). In the context of cancer, the immune system recognizes non-self protein sequences (e.g. resulting from mutation or transformation) naturally without pharmaceutical intervention (1) with mixed success. Immunotherapy approaches have been shown to help T-cells recognize certain non-self-peptides and mount an immune response if a target is identified correctly and delivered effectively (3).

One potential class of non-self-antigens for targeted immunotherapy in cancer patients is represented by tumor-associated antigens (TAAs) that are differentially expressed in tumor tissue compared to normal tissue (4). Survivin, also known as BIRC5, is an attractive TAA because it is expressed in a wide range of cancer types, including breast cancer, but is nearly absent in normal, mature cells (5–8). Survivin (BIRC5) is an inhibitor of apoptosis that is normally expressed during fetal development, but also allows cancer cells to grow without regulation due to its effects on multiple signaling pathways (9). While self-proteins are often not targetable by the immune system's cytotoxic T-cells due to tolerance, or are avoided therapeutically due to the possibility of provoking harmful autoimmunity (e.g. type 1 diabetes and myocarditis), successful targeting of survivin has been shown to be safe in multiple clinical trials with varying levels of efficacy (10). TAAs such as survivin have also been shown to be expressed throughout the tumor during multiple cell cycle phases and in metastatic tissue (11,12).

Technology used to produce stimulation of CD8+ T-cells to attack specified targets on cancer cells has evolved over the past two decades with various vaccination strategies having been described to improve immune responses to administered peptide antigens. The development of peptide vaccine platforms for delivery of antigens to produce a robust immune response to peptide antigens, as well as techniques for the identification of the correct peptide antigens required to give a specific immune response using those platforms, have been described (13).

2. Materials and Methods

These studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of GemPharmatech Co (Wilmington, DE). The care and use of animals was conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the National Institutes of Health guidelines.

In order to test the efficacy of an adjuvanted microsphere synthetic peptide vaccine specific for survivin in BALB/c mice, we identified survivin peptide antigens potentially capable of stimulating MHC Class I and Class II restricted, tumor-specific T-cell response in BALB/c mice. These peptide sequences were determined by reviewing publications outlining cytotoxic responses using various peptide vaccine formulations, including phages and ex-vivo dendritic cell preparations (14,15).

In addition, NetMHCIIpan and NetMHCII were utilized to identify QP19, a region predicted to bind to class II MHC to stimulate CD4 helper T-cells (16,17). The sequences of these murine

class I H-2 K,D,L binding peptides and murine class II H-2 I-E and I-A binding peptides are given in Table 1.

In order to fully characterize the TNBC model mouse 4T1 cell line (ATCC, Manassas, VA), frozen 4T1 cell samples were sent to Complete Genomics (Beijing, China) for genetic sequencing. DNA was extracted from the cryopreserved 4T1 cell line, and BALB/c mouse tails (taken as a source of normal tissue DNA) that were snap-frozen on dry ice after removal. The DNA from the samples was library-prepared using the Agilent SureSelect XT Mouse All Exon Kit (Agilent Technologies, Santa Clara, CA). RNA was extracted from the frozen 4T1 cell line and from also from snap-frozen, normal mammary tissue samples harvested from BALB/c mice. RNA samples were prepared for mRNA sequencing via poly-A tail capture with MGI Tech Company reagents (MGI, Shenzhen, China). Samples were sequenced by Complete Genomics on the BGISEQ-500 (BGI, Beijing, China) at 100 base pair paired end reads. DNA read coverage for normal tissue and tumor was 100x and 300x respectively, with RNA read count at 80 million paired end reads. To process the data, FASTP was used to perform quality control and adapter trimming and BWA-MEM aligned reads to the GRCm38 reference mouse genome (18–20). The germline sequences were confirmed to match the peptides identified for vaccine administration. RNA expression of survivin was examined by adapter trimming and quality control with FASTP (18), followed by pseudoalignment with Kallisto on the GENCODE v25 mouse transcriptome for quantification in Sleuth expressed as transcripts per million (21–23).

A blend of PLGA microspheres, prepared as described in a previous publication, was manufactured containing individual synthetic peptides selected from the primary amino acid

sequence of the survivin protein ranging in length from 40 to 140 amino acids depending on the isoform (Table 1) (24–27). The adjuvanted microsphere vaccine formulation contained 3 μg/mg of survivin-specific class 1 and class 2 peptides and 0.5 μg/mg of the TLR-9 oligonucleotide agonist CpG (ODN-1018). The microspheres were delivered in a 200 μl volume of PBS / polysorbate 80 injectate solution containing the TLR-4 agonist MPLA at a concentration of 100 μg/ml.

Enzyme-linked immunosorbent spot for interferon gamma (ELISpot) assays were performed with BALB/c splenocytes obtained from control and vaccinated surviving tumor bearing mice on day 41 after 4T1 tumor inoculation. Splenocytes were prepared as previously described (28). The peptide antigens used in the ELISpot assays were the same as those used in the peptide vaccine and each peptide antigen was tested individually, loaded into ELISpot wells at 10 μg/ml final concentration. ELISpot assay plates were prepared and processed as per the manufactures instructions (3321-4HPT-10, Mabtech Inc., Cincinnati, OH). ELISpots were enumerated by machine (CTL S6 Entry M2, Shaker Heights, Cleveland, OH) to calculate the frequency of gamma interferon producing T-cells in the splenocyte populations.

Previous studies with this adjuvanted microsphere platform have shown that T-cell expansion capable of providing protection against viral challenge is present 14 days in mouse models (25,26). A 4T1 inoculation dose ranging study was undertaken to find the maximum 4T1 cell count that would show limited tumor growth for 14 days as shown in the study design schematic in supplementary materials Figure S1. Based on the 4T1 dose ranging data shown in supplementary materials Figure S2, the challenge study was designed with one cohort receiving a

250 4T1 cell inoculation dose and the other receiving 500 cells of orthotopically injected 4T1 breast cancer cells. Ten of the animals in each cohort received two doses of intra-peritoneally delivered adjuvanted peptide microspheres, and the control mice were given two doses of blank microspheres (control; without peptide antigen and adjuvants) given both fourteen days before 4T1 cell inoculation, and again at the same time as orthotopic injection of 4T1cells. Subcutaneous tumor volumes were measured every three days with a 41-day endpoint after implantation as shown in the study design schematic in Figure 1. Mouse tumor size was measured non-invasively every three days through the use of calipers applied to the tumor located in the subcutaneous space. The tumor volume (TV) was expressed in mm^3 using the modified ellipsoid formula: $TV = ½(length \times width^2)$ (29,30). This data was used to calculate the tumor growth rate expressed as mm^3/day. Tumor-take frequencies were defined as the number of mice with measurable tumors on the indicated day divided by the total number of mice inoculated with 4T1 cells.

3. Results

Survivin was highly expressed in all 4T1 cell line samples and was seen at very low levels in the BALB/c normal mammary tissue samples as illustrated in Figure 2. Of the six peptides loaded into the adjuvanted microsphere formulation shown in Table 1, only QP19 (QIWQLYLKNYRIATFKNWP), produced a positive ELISpot response as shown in Figure 3. The mice who were not vaccinated did not produce a detectable response to the survivin QP19 peptide antigen. Although published studies suggested that the administered MHC class I peptide epitopes were immunogenic in BALB/c mice, we observed that only QP19 produced a T-cell response as measured by ELISpot.

The inoculation dose of 4T1 at both the 250 and 500 cell levels did not result in a tumor-take frequency of 100% (Figure 4). However, vaccination with survivin peptide antigens was associated with statistically significant diminution of tumor-take frequencies when control and vaccinated mice were compared. In addition, animals dosed with 4T1 tumor cells receiving the adjuvanted microsphere peptide vaccine had statistically significant slower tumor growth rates compared with controls as illustrated in Figure 5.

4. Discussion

CD8+ and CD4+ lymphocytes typically target foreign proteins, such as those from viruses and bacteria. Because cellular immunity is finely tuned by the thymus to target foreign invaders such as viruses, self-proteins are generally ignored or tolerated by the immune system. A notable exception to this is in the setting of auto-immune diseases (31). The targeting of survivin peptides that are HLA matched to the host for a cell-mediated immune response to kill tumor cells is of particular interest. MHC-restricted responses to peptides located on the survivin protein have been shown to elicit an immune response, including immunotherapies targeting survivin in a number of clinical trials (10). A comprehensive list of various approaches used clinically to elicit a host immune response against survivin-expressing tumors is shown in Table 2. A collection of HLA-restricted survivin peptide antigens identified across these various studies also raises the possibility of a broadly applicable immunotherapy for tumors that express survivin. As these studies also illustrate, eliciting a reliable, clinically significant immune response to peptide antigens is challenging. Ensuring that the correct peptide sequence is selected

and delivered effectively for T-cell expansion to occur have been obstacles to the development of safe and effective targeted immunotherapies.

In this study, we found that multiple putative peptide antigens, derived from the primary sequence of survivin and predicted to bind to MHC Class I molecules of BALB/c mice, did not elicit a detectable *ex-vivo* immune response. The QP19 peptide antigen, predicted to bind to I-A^d/I-E^d, the MHC Class II molecules of BALB/c, was able to elicit an *ex-vivo* gamma-IFN T-cell ELISpot response. Overall, vaccination with adjuvanted microspheres containing the mixture of both MHC Class I and Class II survivin peptides resulted in significant deceleration of tumor growth and lower tumor-take frequencies. This later observation suggests that protective T-cell responses were vaccine-induced and operant prior to tumors growing to measurable volumes. One possible explanation for these observations would be the presence of one or more CD8+ epitopes co-localized within QP19, producing the observed T-cell response and anti-tumor growth activity associated with vaccination. Analysis of all possible overlapping peptides of 8-9 amino acids in length within QP19 using NetMHC and NetMHCpan found 4 potential BALB/c MHC matched peptide antigens as listed in Table 3 (17,32). Further studies to identify possible survivin-protective CD8+ T cell epitopes demonstrated by *ex-vivo* ELISpot response, and the formal demonstration of CD4+ T cell helper activity evoked by vaccination with QP19 await further experimentation.

4.1 Vaccine Platforms

The history of clinical studies involving peptide-specific targeted immunotherapy for survivin has shown that peptide delivery system optimization is required to elicit a strong immune

response (10). A small peptide injected on its own, even when combined with adjuvants known to enhance a T-cell response, has been shown not to trigger a robust response (26). As we describe here, microspheres can be manufactured that encapsulate peptides and the TLR-9 agonist CpG in a biodegradable PLGA polymer, delivered after reconstitution in a saline solution with the TLR-4 agonist MPLA to produce a cellular immune response to the administered peptide antigens as demonstrated by ELISpot (26). We have previously demonstrated the delivery system to immunize C57BL/6 mice against the Ebola virus nucleocapsid protein with prevention of mortality and morbidity after a single intraperitoneal injection (25). An additional study showed that *rhesus macaques* receiving pre-exposure prophylaxis with adjuvanted microspheres from the same platform described here, loaded with peptides directed against the SARS-CoV-2 nucleocapsid protein, did not have radiographic evidence of pulmonary infiltrates characteristic of COVID-19 seen in unvaccinated controls (33). As we show here, peptides delivered using this adjuvanted microsphere technology can create a TAA-specific immune response capable of reducing tumor growth rate using the aggressive 4T1 triple negative murine breast cancer model.

4.2 Usage

In immuno-oncology, various pharmaceuticals are often administered with in combination with checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 (34). These drugs have made a considerable contributions to a wide range of cancer treatments (35). However, usage of immune checkpoint inhibitors can be associated with severe immune related adverse events (e.g. myasthenia gravis and acute autoimmune myocarditis) as a result of dysregulated T-cell attack (36)

Targeted immunotherapy directed against peptide sequences within the survivin protein, or other tumor associated antigens, may be an effective neoadjuvant therapy that could be administered before or after breast cancer tumor excision. Survivin is expressed in a wide range of cancers making survivin a TAA potentially applicable to a wide range of cancer types, as shown in Table 2. Although targeting neoantigens using personalized immunotherapy developed to target a specific patient's tumor may provide benefits, the complexities associated with routinely obtaining high quality DNA and RNA gene sequencing data and the need to then rapidly manufacture and administer a personalized immunotherapy are significant barriers interfering with widespread adoption of this approach (37–39).

4.3 Conclusion

In this study, we found that no detectable *ex-vivo* immune response to the survivin peptide QP19 was generated in BALB/c controls, unvaccinated and inoculated with the 4T1 TNBC cell line. A statistically significant response to QP19 in study animals that were dosed with the vaccine was however observed. Although we did not use the two adjuvants in the blank microsphere control group, we have previously shown in another immunotherapy model that adjuvanted microspheres alone did not result in a statistically significant T-cell response to target antigens (25).

Various studies in literature and clinical trials have shown varying degrees of efficacy for identified peptide targets applicable to a wide range of cancers (10). The use of immunotherapy for breast cancer has gained attention recently (40). TAAs provide the opportunity to develop

immunotherapy targeting a fixed set of peptides with collective HLA restrictions predicted to provide broad population coverage that could be administered to breast cancer patients without the need for patient-specific tumor gene sequencing and manufacturing of personalized immunotherapy. Targeted T-cell immunotherapy triggering a T-cell immune response against survivin as neoadjuvant therapy has the potential to reduce recurrence after surgical excision of breast tumor tissue if the number of cells remaining after tumor debulking is low enough to result in CTL attack sufficient to blunt tumor-take and tumor growth rate. Previous studies have seen mixed efficacy with unprotected peptides used as immunotherapy (10). A delivery system such as the adjuvanted microsphere encapsulation described herein, may be able to effectively deliver peptides to produce T-cell expansion against TAA targets such as survivin expressed by various tumors in cancer patients.

Acknowledgements

A pre-print of this manuscript was posted on BioRxiv (41).

Contribution to the Field Statement

Immunotherapy has helped many patients with cancer by harnessing their immune systems to kill cancer cells. Currently available immunotherapy has been of limited benefit for some types of cancer. In this paper, we describe the use of a vaccine to blunt the growth of a mouse breast cancer tumor. The mice were injected with cells from a triple negative breast cancer tumor which is a type of breast cancer with no known cure. The data from this study suggests that it may be possible to give this type of vaccine before breast surgery to improve a patient's outcome by making it harder for cancer cells left behind after the surgery to grow again. Existing immunotherapy for triple negative breast cancer works by rallying support from the patient's immune system broadly and has not been shown to be curative. This study used a vaccine immunotherapy to train the immune system to attack targets usually only found on tumor cells which may produce more benefit than traditional immunotherapy.

Figures, Figure Legends and Tables

Figure 1

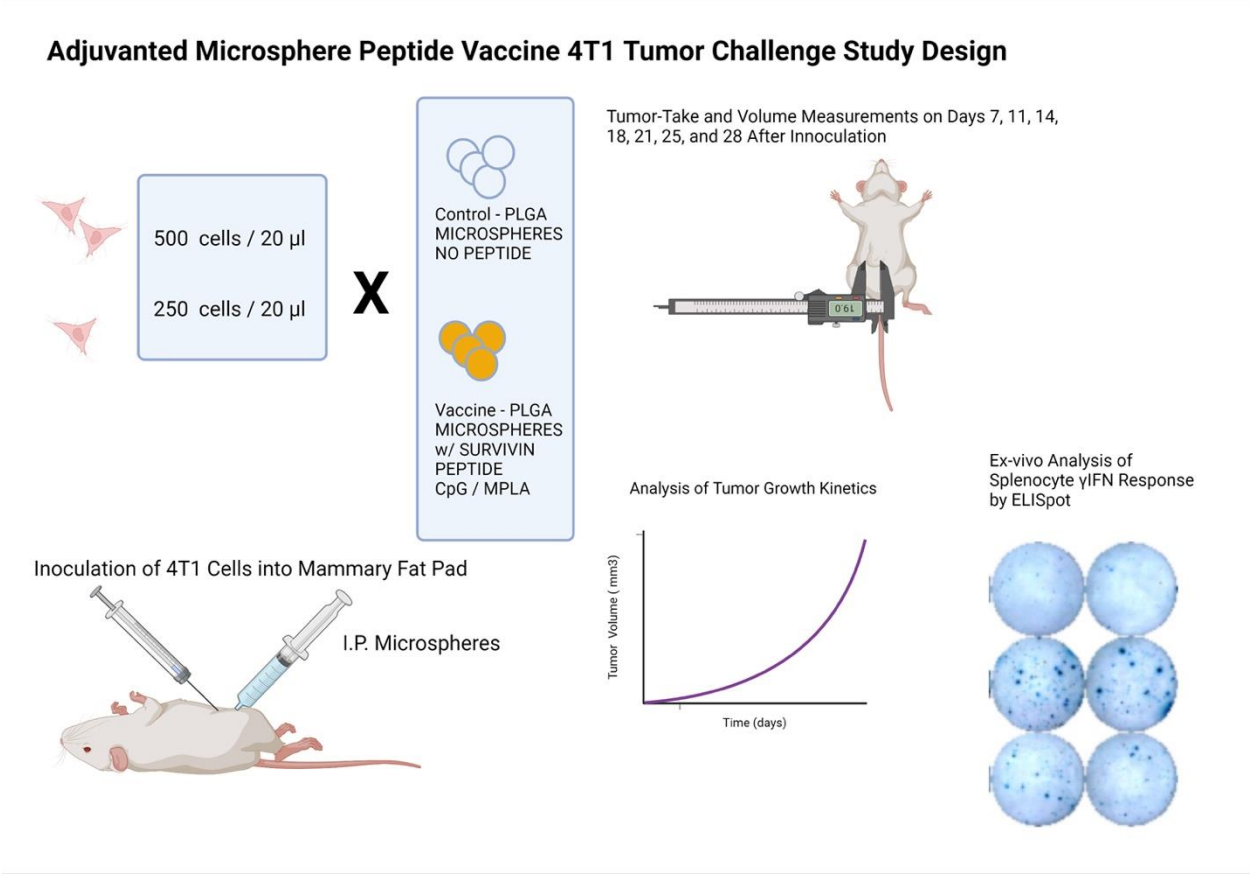

Figure 1 legend. Study design schematic for a BALB/c mouse model 4T1 TNBC tumor challenge microsphere peptide vaccine efficacy study.

Figure 2

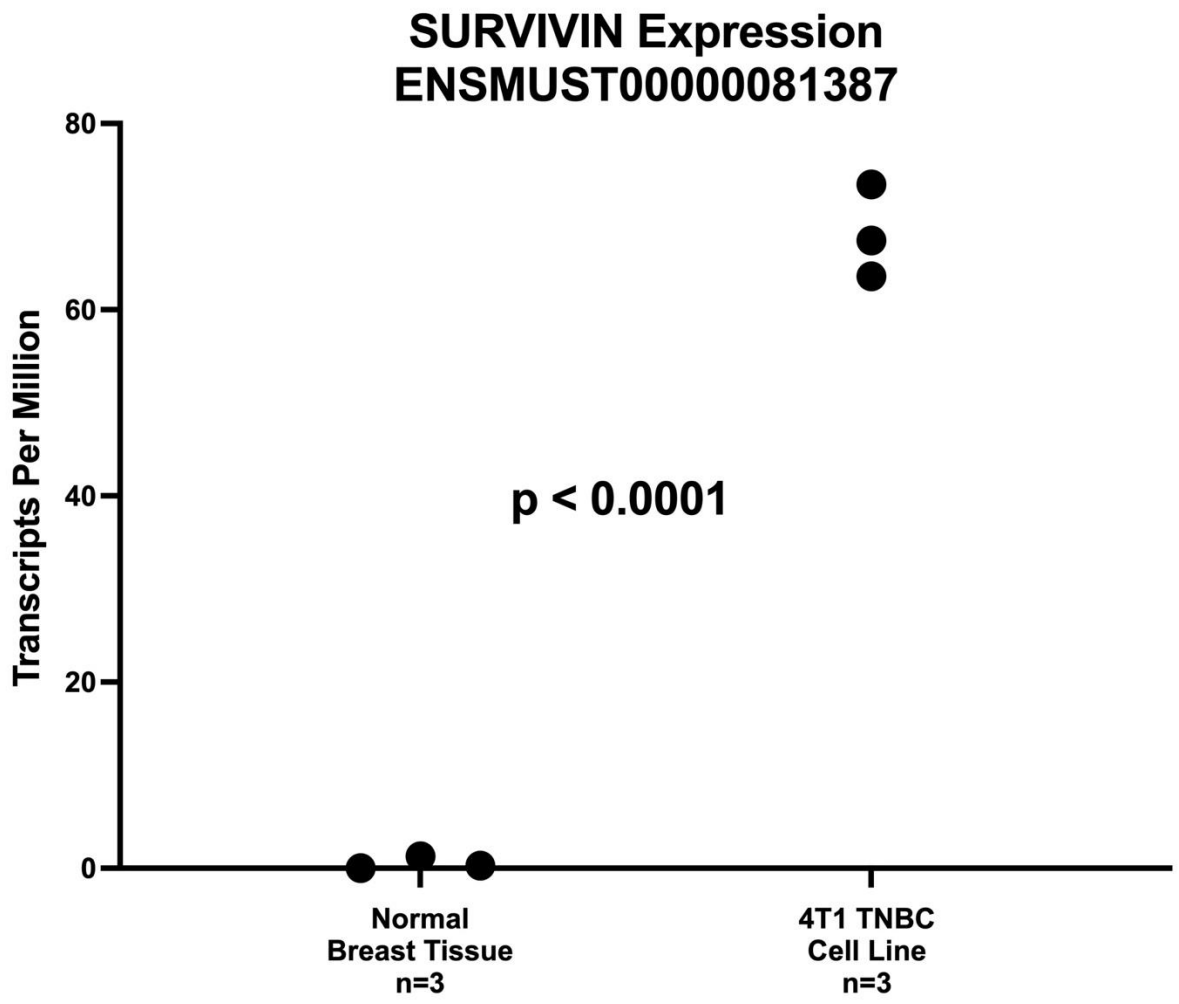

Figure 2 legend. Expression level from mRNA-Seq of mouse wild-type Survivin transcript (ENST0000081387) in normal breast tissue and the 4T1 cell line used in this study.

Figure 3

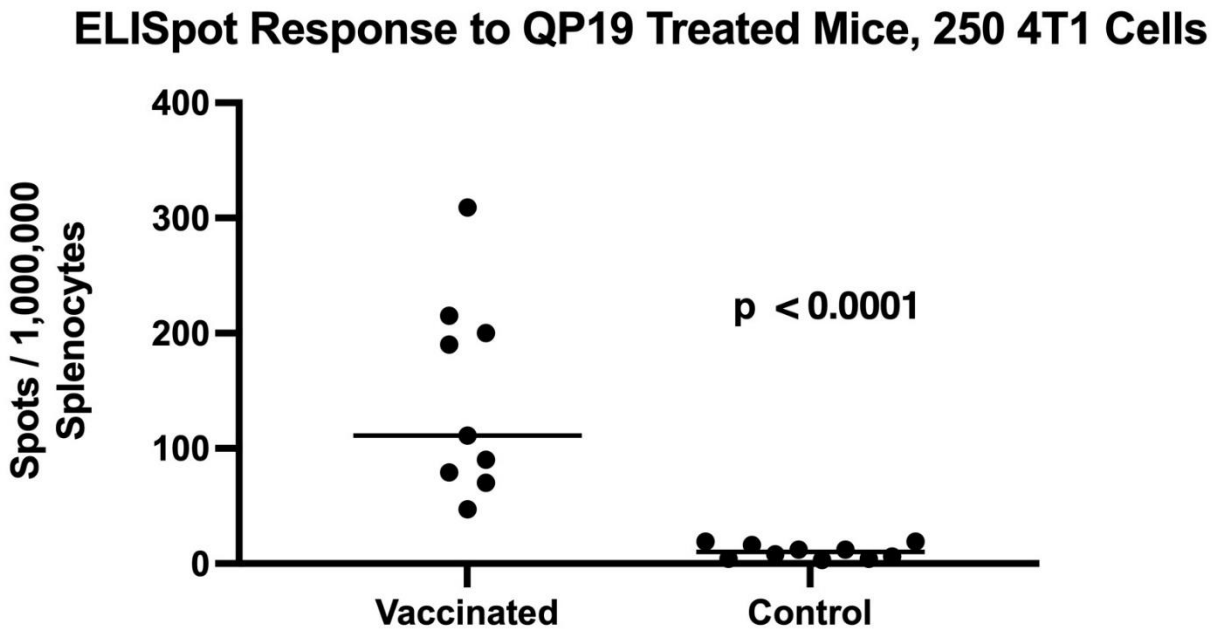

Figure 3 legend. ELISpot response to QP19 in vaccinated and unvaccinated mice that received 250 cells of 4T1. Statistical significance was determined using the unpaired, non-parametric, Mann-Whitney T-test.

Figure 4

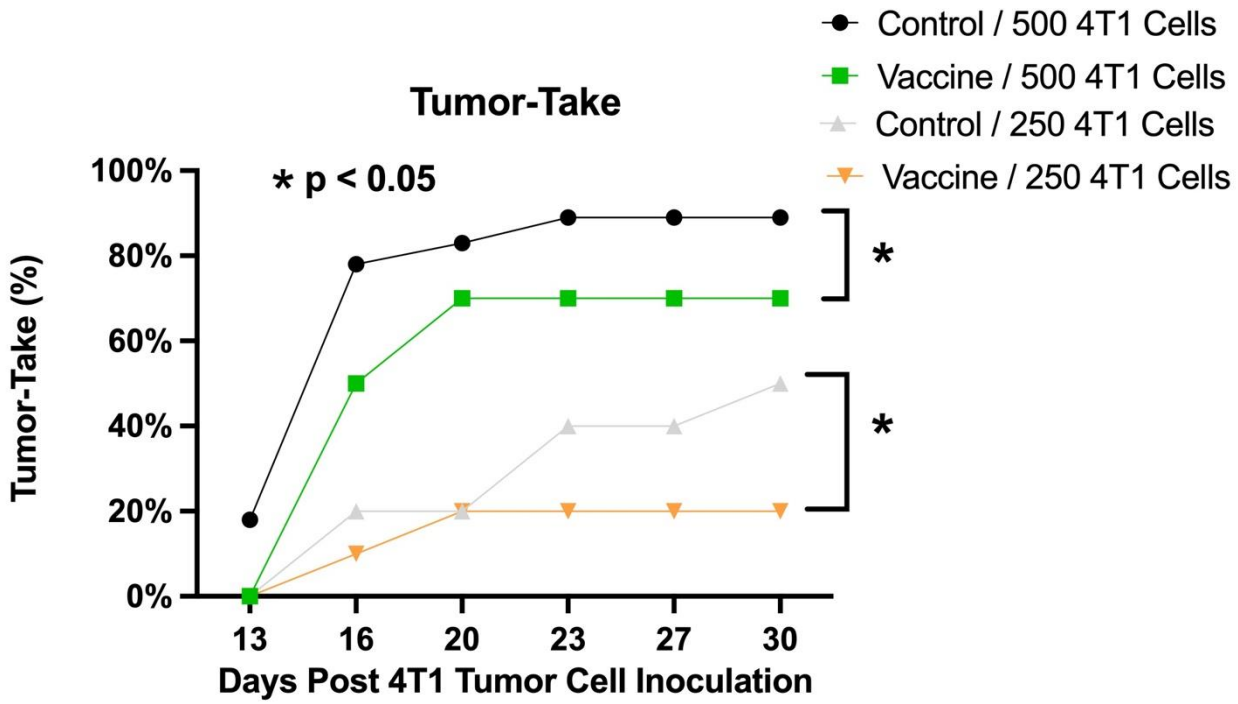

Figure 4 Legend. Percent tumor-take as a function of time after orthotopic injection of the 4T1 tumor cell inoculation dose into mammary tissue for vaccinated and control groups. Tumor-take was defined as the number of mice with measureable tumors on the indicated day divided by the total number of mice inoculated with 4T1 cells. For the 500 cells group: Control n=18; Vaccine n=10. For the 250 cells group: Control n=10, 250; Vaccine n=10. Statistical significance was determined by ANOVA (Prizm Graphpad software).

Figure 5

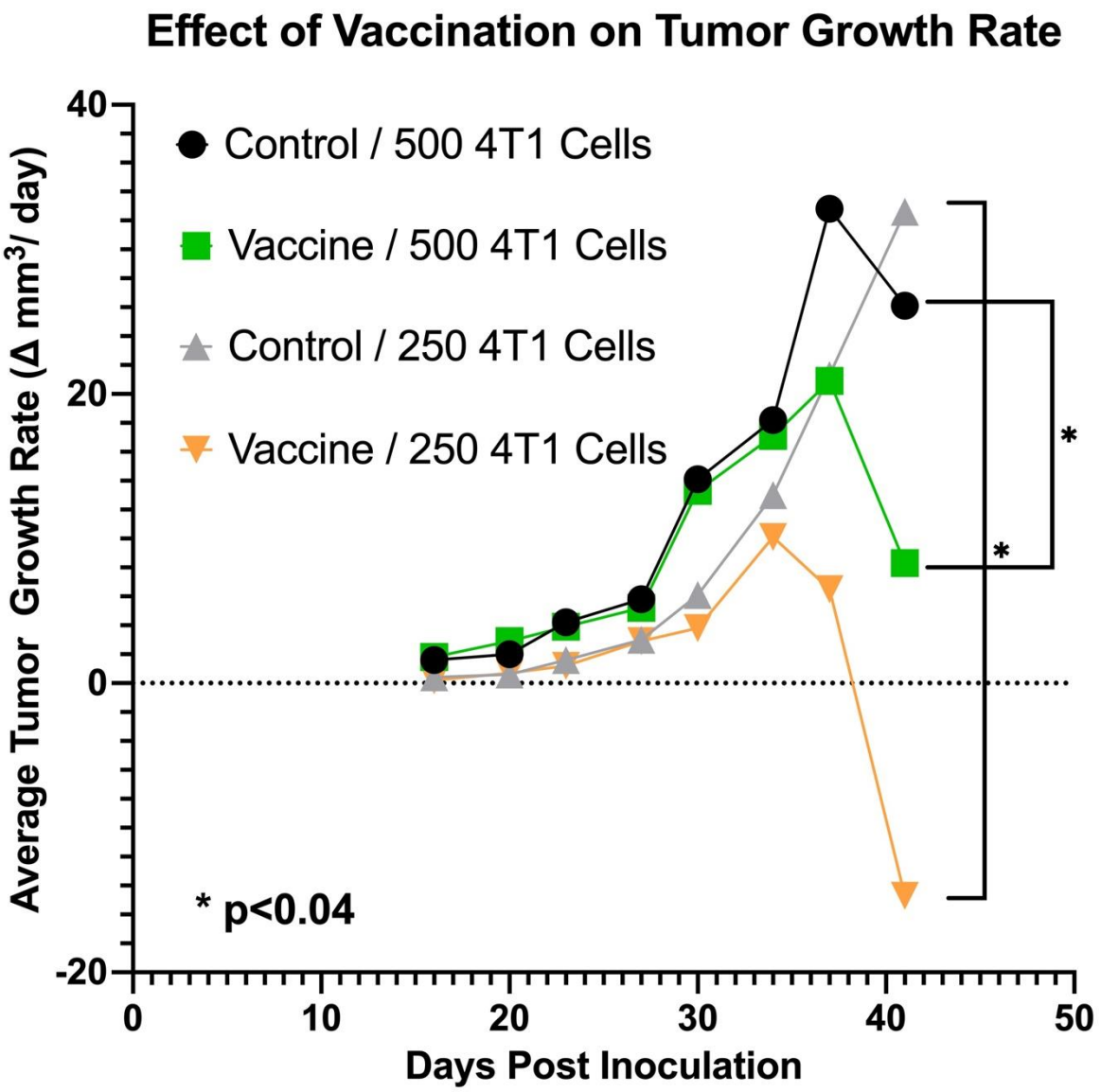

Figure 5 legend. Comparison of average tumor growth rates (vaccinated versus control) for the 250 cell and 500 cell 4T1 tumor inoculation challenge groups. The sample size was n=10 for each group. Statistical significance was assessed using ANOVA and Prism Graphpad software.

Table 1

Epitope Name	Peptide Sequence	Class	Position	MHC Match Screening Method
AL9	ATFKNWPFL	1	20-28	(17,32)
AM9	AFLTVKKQM	1	85-93	(15)
GI9	GWEPDDNPI	1	66-74	(14,15)
TI9	TAKTTRQSI	1	127-135	(Siegel et al., 2003)
QP19	QIWQLYLKNYRIATFKNWP	2	8-26	(16,42)(16,42)
PADRE	AKFVAAWTLKAAA	2	N/A	(43)

Table 1 legend. Literature references and prediction tools supporting a BALB/c MHC match to each of the six peptides microencapsulated into the adjuvanted microsphere vaccine platform described here.

Table 2

Cancer Type	Clinical Stage	Peptide	HLA	Delivery	Results	Citations
Glioblastoma	Phase II	DLAQCFFMFKELEGW	A*02, A*03, A*24	A peptide mutated from the wild type sequence conjugated to the adjuvant keyhole limpet haemocyanin, injected subcutaneous 4 times biweekly along with Montanide ISA 51 with sargramostim.	Increased survivin-specific IgG antibodies and CD8+ T-cells generated in majority of patients with some efficacy for PFS and OS along with temozolomide.	(44,45)
Multiple	Phase II	AYACNTSTL	A*24:02	Subcutaneous injection of peptide at multiple intervals, 14 days apart; addition of IFN-α or IFN-β in later studies; addition of IFA in later studies	Increased levels of peptide specific CTL in a portion of patients, but adjuvants are added due to lack of clinical efficacy, however, progression free survival was not increased.	(7,46–51)
Ovarian	Phase I	FTELTLGEF, LMLGEFLKL, RISTFKNWPK, STFKNWPFL, LPPAWQPFL, EPDLAQCFY	A*01, A*02, A*03, A*24, B*7, B*35	Multiple subcutaneous injection of peptides used I previous studies, where a portion are mutated from wild-type, multiple times with Montanide ISA 51 with metronomic cyclophosphamide in some cohorts.	All patients showed increased survivin-specific T-cells, including both CD4+ and CD8+, more so with metronomic cyclophosphamide. Clinical results not evaluated.	(52)
Melanoma	Phase II	LMLGEFLKL, and a peptide from IDO	A*02	Multiple subcutaneous injection of peptide mutated from survivin and indoleamine 2,3-dioxygenase with Montanide ISA 51. Topical 5% imiquimod cream and GM-CSF to assist in delivery and response.	The majority of patients had an immune response, with an increase of memory CD4+ and CD8+. No significant results seen, but tumor regression in one individual.	(53)
Solid Tumors	Phase I	FTELTLGEF, LMLGEFLKL, RISTFKNWPK, STFKNWPFL, LPPAWQPFL	A*01, A*02, A*03, A*24, B*7	Subcutaneous injection of peptide, some mutated from wild-type, multiple times with Montanide ISA 51.	Survivin-specific T-cells in the majority of patients, with most of those not detected before vaccination. Clinical results not evaluated.	(54)
Melanoma	Phase II	FTELTLGEF, LMLGEFLKL, EPDLAQCFY	A*01, A*02, B*35	Deep Subcutaneous injection of 3 peptides differing from wild type sequence along with Montanide ISA 51. Different regimen used for vaccination frequency and addition of cyclophosphamide.	Increase survivin-specific CD8+ T-cells in the majority of individuals. Significant extension seen in overall survival.	(55)
Prostate	Phase I/II	TLGEFLKLDRERAKN, TLPPAWQPFL, ELTLGEFLKL, and multiple non-survivin class 1 and class 2 peptides	DRB1*0X, A*02	Peptides subcutaneously injected or loaded onto autologous DC at intervals with one of the following; Imiquimod, GM-CSF, local hyperthermia or the TLR-7/8 agonist mRNA/protamine complex.	Several HLA-DRB1 present this peptide for a Th1 CD4+ response, however, one patient had an anaphylactic reaction to this peptide after multiple doses.	(56,57)

Table 2 legend. Clinical trial list detailing survivin peptide immunotherapy studies conducted over the last two decades for a variety of tumor types.

Table 3

Epitope Name	Peptide Sequence	Class	Position
LI8	LYLKNYRI	1	12-19
LA9	LYLKNYRIA	1	12-20
KF8	KNYRIATF	1	15-22
LF9	LKNYRIATF	1	14-21

Table 3 legend. NetMHC and NetMHCpan were used to scan all possible overlapping peptides of 8-9 amino acids in length within QP19 (32,42). The four potential BALB/c MHC matched class I peptide antigens found within QP19 are listed here.

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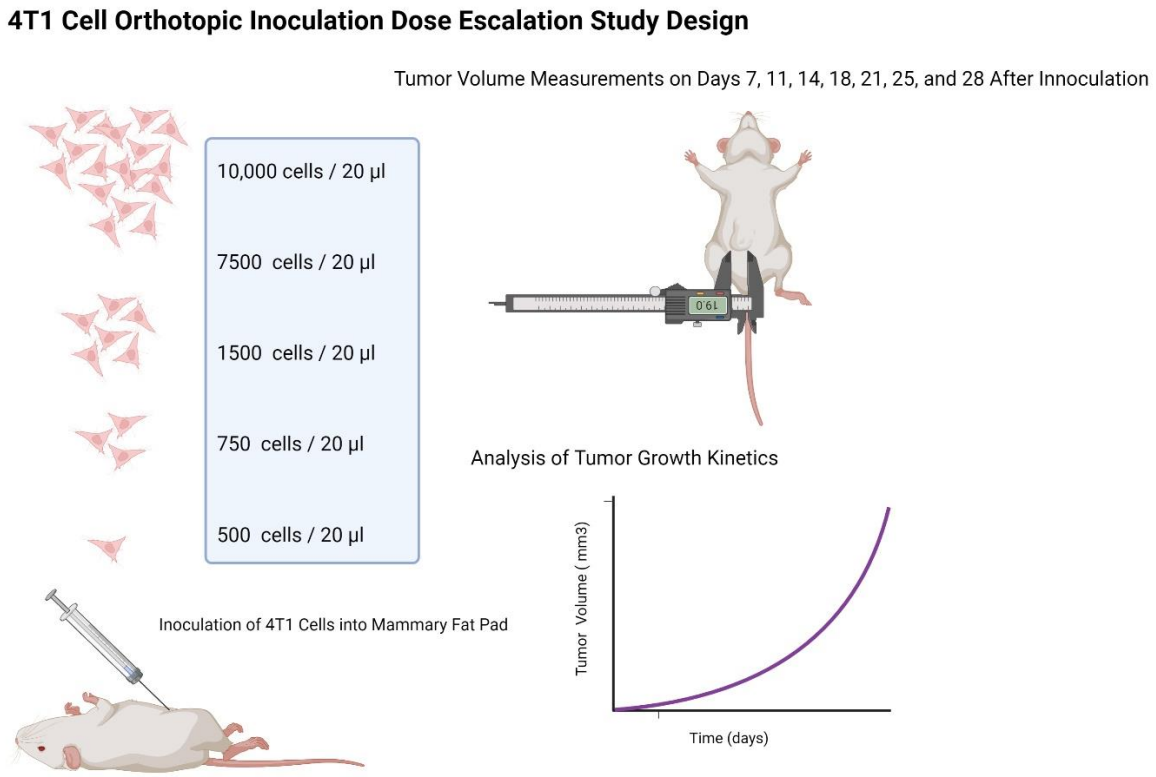
7 Supplementary Figures and Tables

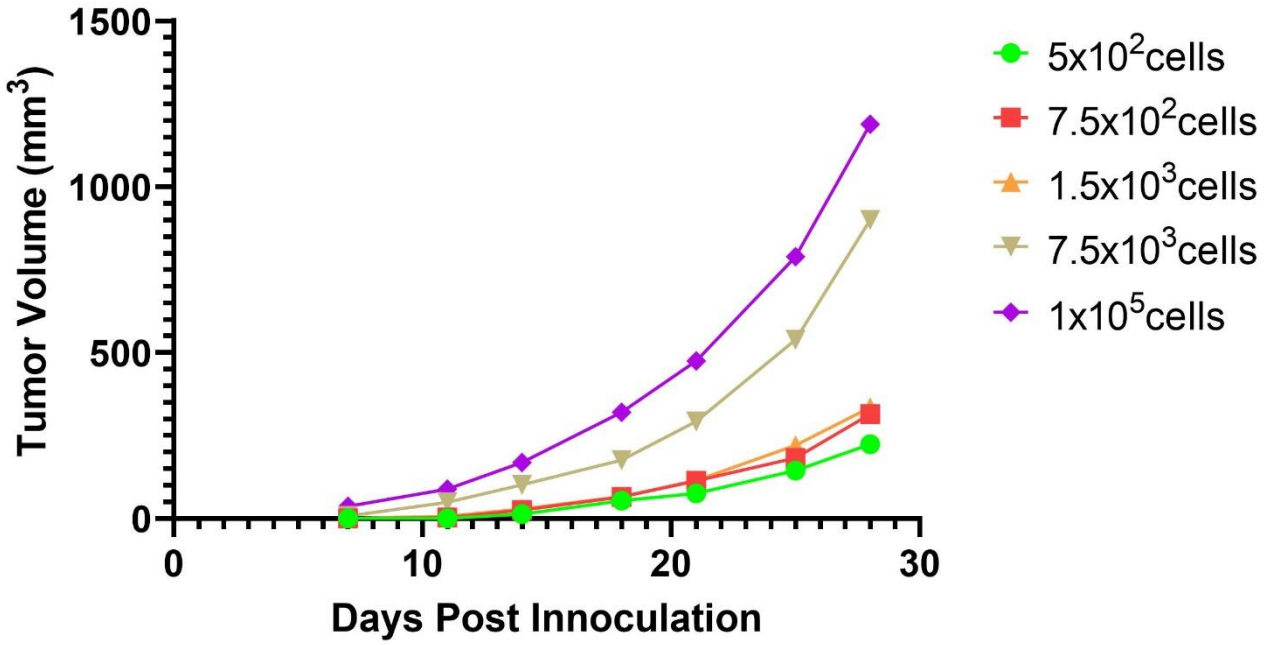
7.1 Supplementary Figures

Figure S1



Supplementary Figure 1 legend. Study design schematic for 4T1 TNBC cell line inoculation dose escalation study. Varying numbers of 4T1 tumor cells were injected into mouse mammary tissue.

Figure S2



Supplementary Figure 2 legend. Average growth rate curves shown after injection of escalating numbers of 4T1 TNBC cancer cells into BALB/c mouse mammary tissue at T0. Sample size was n=10 mice per group.