Targeting Tissue Factor for Immunotherapy of Triple-Negative Breast Cancer Using a Second-Generation ICON

Zhiwei Hu¹, Rulong Shen², Amanda Campbell³, Elizabeth McMichael³, Lianbo Yu⁴, Bhuvaneswari Ramaswamy⁵, Cheryl A. London⁶, Tian Xu⁷, and William E. Carson III¹

Abstract

Triple-negative breast cancer (TNBC) is a leading cause of breast cancer death and is often associated with BRCA1 and BRCA2 mutation. Due to the lack of validated target molecules, no targeted therapy for TNBC is approved. Tissue factor (TF) is a common yet specific surface target receptor for cancer cells, tumor vascular endothelial cells, and cancer stem cells in several types of solid cancers, including breast cancer. Here, we report evidence supporting the idea that TF is a surface target in TNBC. We used in vitro cancer lines and in vivo tumor xenografts in mice, all with BRCA1 or BRCA2 mutations, derived from patients’ tumors. We showed that TF is overexpressed on TNBC cells and tumor neoangiogenesis in 50% to 85% of TNBC patients (n = 161) and in TNBC cell line–derived xenografts (CDX) and patient-derived xenografts (PDX) from mice, but was not detected in adjacent normal breast tissue. We then describe the development of a second-generation TF-targeting immunoconjugate (called L-ICON1, for lighter or light chain ICON) with improved efficacy and safety profiles compared with the original ICON. We showed that L-ICON1 kills TNBC cells in vitro via antibody-dependent cell-mediated cytotoxicity and can be used to treat human and murine TNBC CDX as well as PDX in vivo in orthotopic mouse models. Thus, TF could be a useful target for the development of immunotherapeutics for TNBC patients, with or without BRCA1 and BRCA2 mutations.

Introduction

Breast cancer is the most common newly diagnosed cancer and second leading cause of cancer death among women in the United States in 2016 (1). Breast cancer deaths are largely the result of recurrent and metastatic disease that typically does not respond well to current treatments (2). Triple-negative breast cancer (TNBC), so-called because the tumor cells lack expression of three targetable proteins, the estrogen receptor, the progesterone receptor, and the epidermal growth factor receptor HER2, is difficult to treat malignancies and is usually considered incurable (3–10). TNBC comprises approximately 15% of globally diagnosed breast cancer (4, 11, 12). At present, there are no molecularly targeted therapies approved for TNBC.

TNBC is often associated with mutations in breast cancer predisposition genes BRCA1 and BRCA2 (13). The identification of BRCA1 (14) and BRCA2 (13) in the mid-1990s has led to a better understanding of the molecular pathogenesis of hereditary breast cancer. BRCA1 and BRCA2 are human genes encoding tumor suppressor proteins that ensure the stability of DNA. When either of these genes is mutated, or altered, such that its protein product either is not made or does not function correctly, DNA damage may not be repaired properly. As a result, BRCA1 and BRCA2 mutations are associated with an increased risk for female breast and ovarian cancer. Approximately 20% of TNBC patients harbor a mutation in either the BRCA1 or BRCA2 genes (15). Thus, identification of biomarkers and oncotargets for BRCA1- and 2-associated hereditary breast cancer and development of corresponding targeted immunotherapy could provide efficacious treatment regimens for TNBC, with or without BRCA1 and BRCA2 mutation, and reduce the mortality associated with the malignancy.

To identify a therapeutic target in TNBC, we focused on tissue factor (TF), based on previous findings in breast cancer patients (16) and human breast tumor xenografts from animal studies (17–20). TF is a 47-kDa membrane-bound cell surface receptor that initiates the extrinsic coagulation cascade pathway upon injury to vessel wall integrity (21–23) and modulates angiogenesis (24–27). It is highly expressed in many types of solid cancers (28, 29), including non-TNBC. In breast cancer, TF expression can be detected on both the breast cancer cells and the tumor vascular endothelial cells (VECs) in breast cancer patients (16) and in a mouse model of human breast cancer (17), but not in adjacent normal breast tissues (16). We reported that TF is an angiogenic-specific receptor in VEGF-stimulated angiogenic vascular endothelial models (30) and is
also could be a target for cancer stem cells (CSC) in several types of solid cancer (31), including breast cancer. Other studies have also connected TF with TNBC (18, 19). However, it is still not known whether TF is expressed in TNBC cells and tumor neovasculature, and if so, to what extent. IFTF is indeed highly and selectively expressed in TNBC, it could serve as a target for immunotherapy of TNBC.

TF expression has been detected previously on other types of solid cancers, leukemia, and sarcomas (28, 29), including melanoma (32, 33), prostate cancer (34), and head and neck cancer (35), suggesting that it might be a useful therapeutic target. Pursuing that approach, we developed the TF-targeting immuno-conjugate agent named ICON, which consists of full-length factor VII peptide (406 amino-acid residues, aa) fused to the Fc region of IgG1 (32–35), in which VII, the TF-targeting domain, binds TF with specificity (30) and high affinity [picomolar dissociation constant (Kd); ref. 36], and the IgG1 Fc binds complement and Fc receptors (CD16) on immune cells such as natural killer (NK) cells. After binding to the Fc domain, NK cells and the complement cascade kill ICON-bound target cancer cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), respectively (35). Intraleisonal ICON therapy of experimental murine and human melanoma (32, 33), prostate (34), and head and neck tumors (35) with an ICON adenooviral vector leads to growth inhibition of tumors with minimal effects on normal tissues (33). However, ICON is large (210 kDa; ref. 35). In addition, hypercoagulation is documented in virtually all cancer types (37), albeit at different rates, and is a second leading cause of death in cancer patients (38). It is partially the result of TF produced by and released from cancer cells (39, 40). To eliminate the coagulation activity of VII in the ICON, a coagulation-active lysine reside at position 341 (K341A) was mutated to an alanine in VII peptide by site-directed mutagenesis (34). With this mutation (K341A), the procoagulant effects of ICON were reduced but not eliminated (34). In the current work, we developed a strategy to eliminate the procoagulant effects of ICON, reduce its molecular mass, and retain its binding activity to TF so that ICON could be more effective for treatment of cancer patients when administered systemically.

To identify TF as a surface target in TNBC, we investigated TF expression on TNBC cells and tumor VECs in TNBC cell–derived xenografts (CDX) and patient-derived xenografts (PDX) from mice as well as in a total of 161 TNBC tumor tissues from patients with TNBC, including 14 cases of paraffin-embedded whole tumor tissues and 147 cases of TNBC tumors on tissue microarray (TMA) slides. Because TNBC is associated with BRCA1 and BRCA2 mutations (41), we also investigated TF expression on human TNBC cell lines and in CDX and PDX tumors with or without BRCA1 or BRCA2 mutations.

To develop a TF-targeting ICON with improved safety for targeted immunotherapy of TNBC, we modified ICON by removing the heavy chain (153–406 aa) of VII. The resulting construct contains the noncoagulating VII light chain (1–152 aa) fused to human IgG1 Fc. We named this construct light chain ICON or lighter ICON (LICON1). We compared molecular weights, binding activities, coagulation activities of L-ICON1 with ICON in vitro and compared the in vivo efficacy of L-ICON1 and ICON for immunotherapy of TNBC in orthotopic CDX and PDX mouse models.

Materials and Methods

Cell lines and reagents

Chinese hamster ovary cells (CHO-K1) were purchased from the ATCC in 2004 and were grown in F12K complete growth medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Sigma) and 1 × penicillin and streptomycin (Invitrogen). Human TNBC cell lines MDA-MB-231 (BRCA1-wt, BRCA2-mutant), BT20 (BRCA1-wt, BRCA2-wt), and MDA-MB-468 (BRCA1-wt), non-TNBC lines MCF7/MDR and MCF7/MDR/TF (BRCA1-wt, BRCA2-mutant), murine breast cancer cells 4T1 and EM16 were purchased from ATCC in 2001 to 2009 except for murine TNBC line 4T1 (kind gift from Dr. William Carson at The Ohio State University (OSU), 2013) and human non-TNBC lines MCF7 and MCF7/MDR (kind gift of Dr. Zping Lin at Yale University, 2008). The cancer cells were grown in DEMEM or RPMI1640 complete growth medium supplemented with 10% HI-FBS and 1 × penicillin and streptomycin (Invitrogen). MCF7/MDR line is a low TF-expressing MCF7 line with multidrug resistance (MCF7/MDR; ref. 17) and MCF7/MDR/TF is a high TF-expressing MCF7/MDR (MCF7/MDR/TF) derived from MCF7/MDR by being infected with retroviral vector encoding TF (kind gift of Dr. Michael Bromberg; ref. 42). MDA-MB-231 and 4T1 lines were transfected using xtremeGene HP transfection reagent (Roche, μL transfection reagent: 1 μg plasmid DNA; Roche) with plasmid vectors (1 μg ACT PBase and 1 μg PB Transposon, constructed and kindly provided by Dr. Tian Xu’s laboratory at Yale University) encoding luciferase (Luc) and green fluorescent protein (GFP). The transfected cell lines were selected and maintained for stable expression of Luc and GFP by supplementing growth media with blasticidin (Invitrogen; at a final concentration of 10 μg/mL for selection and 5 μg/mL for maintaining the cell culture). Following the manufacturer’s instructions, ADCC effector cells, a Jurkat-based cell line transfected with CD16 and N-FAT/Luciferase, were purchased from Promega (Cat. No. G7102) in 2014 and were grown in RPMI 1640 complete growth medium supplemented with 100 μg/mL G418, 250 μg/ml hygromycin, 10% HI-FBS, 1 × nonessential amino acids, 1 × sodium pyruvate and 1 × penicillin and streptomycin (Invitrogen). The 293AD cell line was purchased from Cell Biolabs (Cat. No. AD-100, 2014) and was grown in DEMEM (high glucose) supplemented with 10% HI-FBS, 0.1 mmol/L MEM Non-Essential Amino Acids, 2 mmol/L l-glutamine and 1 × penicillin and streptomycin (Invitrogen). All cell cultures were maintained mycoplasma free by routinely supplementing with Plasmocin (Invivogen, Cat. No. Ant-mpt, 25 μg/mL for treatment and 5 μg/mL for maintenance) or Ciprofloxacin HCl (Enzo, Cat. No. 380-288-G025, 10 μg/mL) in cell culture medium. Cultures were tested annually for mycoplasma contamination. The latest date they were tested by using MycoAlert Mycoplasma Detection Kit (Lonza, Cat. No. L107-118) was June 26, 2017. Authenticity of cancer cell lines was determined by validating TF expression by flow cytometry, Western blots, and/or cell ELISA, as described below. Thawed cancer cells were maintained in culture for 1 month. CHO-K1 producer cells for L-ICON1 protein production were maintained for 2 months.

IHC for staining TF expression

The use of patients’ tissues with nonidentifiable information was reviewed and approved by OSU Cancer Institutional Review Board and was conducted following the OSU Human Research
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Protection Program in accordance with the ethical principles and guidelines summarized in the Belmont Report (https://web.archive.org/web/20111017133845/http://www.hhs.gov/ohrp/archive/documents/19790418.pdf). Fourteen TNBC tumor tissues, confirmed by pathology diagnosis to lack detectable estrogen receptor, progesterone receptor and epidermal growth factor receptor Her2, were obtained from The Cooperative Human Tissue Network of OSU. Generation of OSU TNBC TMs was previously described (43). TMA slides with 147 cases of TNBC tumor tissues and 147 cases of matched normal breast tissues (usually adjacent to the tumor tissues) were obtained from OSU Pathology. For each patient, TMA slides had two cores of tumor tissues and two cores of matched normal tissue adjacent tumor. Some samples were lost as samples detached from the slides during IHC staining. Some tumor tissues that did not contain tumor cells were excluded. TF expression was stained with a mouse monoclonal antibody (HTF1, kind gift of Dr. William Konigsberg at Yale University) and/or with a commercial goat anti-human TF (Sekisui Diagnostics, REF 4501), both of which we showed bind to human TF, the same antigen/receptor that is recognized by ICONs and fVII peptides (30). The detailed procedures have been similarly described in our previous publications (17, 44).

The IHC procedures for staining TF and CD31 in TNBC TMA slides were optimized and performed by the OSU Pathology Core Facility. Paraaffin-embedded 4-μm TMA slides of patients’ TNBC tumors were placed on a 60°C oven for 1 hour, cooled, deparaffinized, and rehydrated through xylene and graded ethanol solutions to water. All slides were quenched for 5 minutes in a 3% hydrogen peroxide aqueous solution to block for endogenous peroxidase. Antigen retrieval was performed in a 1× solution of Target Retrieval Solution pH 6.0 (Dako) by Heat-Induced Epitope Retrieval for 25 minutes at 96°C using a vegetable steamer (Black & Decker). Slides were stained with the Intellipath Autostainer Immunostaining System using mouse monoclonal anti-human TF antibody (HTF1, 1:100, 1 mg/mL stock) for staining TF or using mouse monoclonal anti-CD31 (1:80, culture supernatant; Dako, Cat. M0823, Clone JC70A) for staining endothelial cells at room temperature for 30 minutes, followed by MACH3 Mouse HRP-Polymer Detection (Biocare Medical) for 20 minutes at room temperature and then by 5-minute incubation with liquid DAB+ Chromagen (Dako). TF staining was verified by using a commercial polyclonal antibody against human TF (Sekisui Diagnostics, REF 4501). The slides were counterstained in Richard Allen hematoxylin (Thermo Fisher Scientific), dehydrated through graded ethanol solutions, cleared with xylene, and coverslipped.

The IHC procedure for CDX and PDX tumors was performed by the Comparative Pathology and Mouse Phenotyping (CPMPSR) Laboratory of the OSU Veterinary School. Human TNBC CDX (MDA-MB-231) and PDX (JAX TM00089, breast tumor markers: Laboratory of the OSU Veterinary School. Human TNBC CDX the Comparative Pathology and Mouse Phenotyping (CPMPSR) solutions, cleared with xylene, and coverslipped.

Facility. Paraf
slides were optimized and performed by the OSU Pathology Core publications (17, 44).

Constructions of plasmid DNA vectors encoding murine and human L-ICON1

Construction of an expression plasmid vector pcDNA3.1(+) encoding cDNA for the wild-type (WT) human fVII fused to human IgG1 Fc has been described (32, 34). Plasmid vectors pcDNA3.1(+) encoding mouse and human L-ICON1 were similarly constructed using recombinant DNA techniques. Briefly, the cDNA for the first 152 amino acids of mouse and human fVII light chain were PCR amplified from the plasmid vectors encoding their parental ICONs. We used primers as follows. The cDNAs of human and mouse fVII light chain were subcloned in frame in pcDNA3.1(+) containing human IgG1 Fc between EcoRI and BamHI. The cDNA sequences of ICON and L-ICON1 were verified by Sanger-based DNA sequencing at the Genomic Shared Resources at Yale University and at OSU. The cDNA sequence of L-ICON1 is deposited with accession no. KX760097 at GenBank. To make recombinant L-ICON1 proteins, the plasmid vectors encoding mouse and human L-ICON1 cDNAs were transfected into CHO-K1 cells (ATCC) using xtremeGene HP transfection reagent (Roche). The stably transfected L-ICON1 producer CHO-K1 cells were selected under 1 mg/mL G418 (Gibco) and were cultured in suspension at a start density of 2.5 × 10⁷ cells/mL in CHO serum-free medium (SMF4CHO, Hyclone) supplemented with 1 μg/mL vitamin K1 (Sigma). The SFM was collected twice a week, and L-ICON1 protein was purified from the supernatants of SFM by a 5 mL HiTrap rProtein A FF affinity column (GE Healthcare) following the manufacturer’s instructions, as described (35). Molecular weights of L-ICON1 and ICON proteins were analyzed by 8% to 16% SDS-PAGE nonreducing gel (Bio-Rad Laboratories). The concentration of L-ICON1 protein was determined by Protein Assay Reagent (Bio-Rad Laboratories) using bovine serum albumin standards (Pierce).

Construction and production of adenoviral vectors encoding L-ICON1

The adenoviral system we used to make adenoviral vector encoding L-ICON1 was RAPAD CMV Adenoviral Expression System (Cell Biolabs, Cat. No. VPK-252). Briefly, the cDNA of L-ICON1 was subcloned into the pacAd5 CMV-K-Npa shuttle vector between EcoRI and NotI. Pac I–digested shuttle vector pacAd5 CMV-L-ICON1 and the Ad backbone DNA (pacAd59.2-100) were separated by Agarose gel electrophoresis, recovered from the gel slices, and cotransfected of the Pac I–digested shuttle
vector encoding L-ICON1 and the backbone DNA (at a ratio of 4 μg:1 μg) into adenoviral packaging cell line 293AD (Cell Biolabs) using xtremeGene HP transfection reagent (Roche) following the manufacturer’s instructions. A blank control vector (AdBlank) was similarly produced using the pacAd5 CMV-K-Npa vector without encoding a transgene and pacAd5 9.2-100 backbone DNA. The adenoviral vectors were amplified by infecting 293AD cells (Cell Biolabs) and purified by CsCl (Sigma) ultracentrifugation, as described (32–35). The titer of infectious viral particles was determined by measuring Optical density (OD) of 260 nm of 20-fold diluted viral particles in 0.1% sodium dodecyl sulfate (SDS) and calculated using the following formula: 1 OD260 nm = 1 × 10^{12} viral particles per mL, as described (34). OD260 nm was assessed on Spectrophotometer (Beckman, Model DU650).

To verify the production of L-ICON1 protein by AdL-ICON1 vectors, MDA-MB-231 cancer cells in 6-well plate were infected with CsCl-purified AdL-ICON1 (GenBank KX760097), AdhICON (human ICON, hfVII/hlgG1Fc; GenBank AF272774), AdmICON (mouse ICON, mfVII/hlgG1Fc, GenBank AF272773), and AdBlank (as negative control) vectors at an MOI of 3,000:1 by incubating viral vectors with cancer cells in serum-free medium at 37°C and 5% CO₂ for 2 hours. Next morning the cells were washed once with 1× DPBS and grown in SFM4CHO supplemented with 1 μg/mL Vitamin K1 (Sigma) for 4 days. The supernatants of SFM from viral vector-infected cancer cells were assayed in MDA-MB-231 cancer cell ELISA above for determining the concentration and binding activity of L-ICON1 and ICONs. The L-ICON1 and ICON proteins in SFM were further confirmed by immunoprecipitation-Western blot (IP-WB), as described below.

Factor VII coagulation activity assay
Following the manufacturer’s instruction, the retained coagulation activity of FVII light chain peptide in the L-ICON1 protein was determined by Factor VII Human Chromogenic Activity Kit (Abcam), which was developed to determine human FVII coagulation activity, specifically the ability of FVII to bind and form a complex with TF and then to activate factor X (fX) to fXa. The amidolytic activity of the TF/fVIIa complex was quantitated by the amount of fXa produced using an fXa substrate that releases a yellow para-nitroaniline (pNA) chromophore. The change in absorbance of the pNA at 405 nm was quantitated by an FXa Assay Kit (Abcam), which was developed to determine factor VII light chain activity in the L-ICON1 and ICON proteins. After L-ICON1 concentration was determined by paired factor VII antibody Sandwich ELISA, cancer cells (MDA-MB-231, BT20, MCF7/MDR/TF, 4T1, and EM16) were seeded at 1 × 10⁴ cells per well in 96-well flat microplates. When the cells reached about 95% confluence, they were washed once with warm PBS, fixed in 4% paraformaldehyde at room temperature for 20 minutes, blocked with 1% BSA in calcium containing buffer (1 × TBS/Ca, 10 mmol/L Tris–HCl pH8.0, 150 mmol/L NaCl, 10 mmol/L CaCl₂) and then incubated with L-ICON1, ICON, and an isotype control (human IgG, Sigma) proteins in 1 × TBS/Ca buffer followed by incubation with HRP-conjugated anti-human IgG1Fc (human specific, 1:10,000; Sigma) for detection of human IgG1Fc portion of ICON and L-ICON1 proteins. After final wash, OPD was added as substrate and the absorbance at 490 nm (A490 nm) was read on SpectraMax i3 microplate reader (Molecular Devices).

Sandwich ELISA assays for measuring the concentration of L-ICON1 in cell culture medium and mouse plasma samples
L-ICON1 concentration was determined by paired factor VII antibody Sandwich ELISA (Cedarlane Laboratories) following the manufacturer’s instructions with modifications as follows. Anti-human FVII capture antibody (1:200) was coated in EIA 96-well strip plates, was blocked with 1% BSA, and then incubated with cell culture supernatants in serum-free medium (SF4CHO) or 1:5 diluted mouse plasma samples in PBS followed by HRP-conjugated anti-human IgG1Fc (human specific, 1:10,000) for detection of full-length peptides of ICON and L-ICON1, as described above. The standard proteins in Sandwich ELISA were Hitrap Protein A affinity purified-ICON and L-ICON1 proteins, as described above and earlier (35).

ADCC effector assay
The effect of L-ICON1-mediated ADCC to TNBC cells was determined using an ADCC effector assay (Promega; ref. 46) following the manufacturer’s instruction with a modification to the assay medium by using DMEM medium, instead of RPMI 1640. DMEM medium contains a higher calcium concentration (1.8 mmol/L) than RPMI 1640 (0.42 mmol/L) per the information on calcium concentrations in cell culture medium on the Sigma-Aldrich website (calcium is required for ICON and FVII binding to its receptor TF; ref. 32). Briefly, human TNBC (MDA-MB-231 and BT20) and non-TNBC (MCF7/MDR/TF) cells (1 × 10⁴ cells per well in 100 μL growth medium) were seeded in duplicate into the 60 inner wells and grown overnight in 96-well white assay plate (Corning) at 37°C and 5% CO₂. Then, 95 μL of growth medium was removed from each well. Prewarmed 25 μL
ADCC effector assay medium (DMEM supplemented with 0.5% super low IgG fetal bovine serum [HyClone]) was added to 60 inner wells, whereas 75 µL ADCC effector assay medium was added to 36 outer wells. Then, 25 µL of ICON protein at serial dilutions of 3 x 10^5 or 5 x 10^5 concentrations was added in duplicate wells to the cancer cells, whereas human IgG (Sigma) was used as an IgG isotype control. After 15-minute incubation at 37 °C, 25 µL ADCC effector cells [Promega; a T cell–derived leukemia cell line Jurkat transfected with Fc receptor CD16 and NFAT-Luciferase under the control of CD16 binding followed by NFAT activation] were added (2.5 x 10^5 cells per well) to achieve a ratio of effector to target (E:T) at 25:1 and incubated for 6 or 19 hours at 37 °C and 5% CO2. At the end of 6-hour or 19-hour incubation, the plate and Bio-Glo assay reagent (Promega) were equilibrated at room temperature for 15 minutes and then equal volume (75 µL) of Bio-Glo reagents was added to 60 inner wells and two outer wells with medium only as background control. Raw bioluminescence (RLU) was read on SpectraMax i3 (Molecular Devices).

Generation of orthotopic mouse models of TNBC CDXs and PDXs

The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of OSU. To generate orthotopic mouse models of tumor line–derived xenografts, 5 x 10^5 human TNBC MDA-MB-231/Luc+GFP cells in 50 µL of 1:1 mixed PBS/Matrigel (BD Biosciences) or 5 x 10^5 murine TNBC 4T1/Luc+GFP cells in 50 µL PBS were injected into the fourth left mammary gland fat pad in 4 to 6-week-old, female CB-17 SCID (Taconic Farms) or Balb/c mice (The Charles River Laboratory), respectively. Both CB-17 SCID and Balb/c mice have intact NK cells and are crucial for mediating ICON and antibody therapy (35). To generate an orthotopic TNBC PDX model, we purchased a TNBC PDX donor NSG mouse (NOD SCID gamma, no mature B, T, NK cells and no complement) with BRCA1 mutation from The Jackson Laboratory (JAX TM00089, breast tumor markers: TNBC ER+/PR+/HER2−, BRCA1 V757fs). Surgeries were performed in accordance with the OSU IACUC Rodent Surgery Policy. Mice were anesthetized with isoflurane. For the donor NSG mouse (JAX, TM00089), an incision was made over the flank of tumor to extract the tumor tissue, which was washed once in RPMI medium supplemented with 1 x penicillin and streptomycin and was cut in sterile PBS into ~3-mm pieces in diameter for implantation into the recipient mice. CB-17 SCID mice (Taconic Farms) were used as recipient mice. For recipient mice, an incision was made over the fourth right mammary gland, for direct implantation of one piece of ~3-mm PDX tumor tissues from the donor tumor tissue into it. One drop of tissue adhesive (Vetbond) and/or 1 wound clip (Sigma) was used to close the incision site in routine fashion. Animals were monitored for recovery from anesthesia before returning to routine husbandry. Animals were checked daily until wounds were healed, and analgesics (buprenorphine, s.c. 0.1 mg/kg in 100 µL sterile PBS) were administered to provide pain relief for up to 72 hours after surgery.

Adenoviral gene therapy using AdL-ICON1 in vivo in mouse models of human and murine TNBC CDX and PDX

When tumor size reached 150 to 200 mm3, the mice were randomized into control, L-ICON1–treated or ICON-treated groups. The control mice were intratumorally (i.t.) injected with 1 or 4 x 10^10 VP of AdBlank and the treated mice were injected with 1 or 4 x 10^10 VP of AdL-ICON1 or AdICON vectors weekly for orthotopic human TNBC CDX and PDX or every 4 days for orthotopic 4T1 CDX, as specified in figure...
In vivo mouse models and bioluminescence imaging of breast cancer xenografts

To image tumor cells that stably express Luc and GFP, the mice were i.p. injected with 100 μL of luciferin solution. Ten minutes after injection of luciferin, the mice were imaged for quantitatively measuring tumor bioluminescence intensity under anesthesia using inhalation of isoflurane under IVIS Bioluminescence Imaging System (Caliper). The results from IVIS imaging were presented as RLU.

Statistical analysis

The data in vitro and in vivo are presented as mean ± SEM (or mean ± SD as specified) and analyzed by ANOVA and t test for statistical significance using Prism software (GraphPad) and SAS 9.4 software. Fisher exact test was used to test IHC score percentage difference between whole tumor tissues and TMA tissues. For analyses of statistical significance, duplicate or triplicate wells in each group were used for in vitro assays in tissue culture plates and 5 mice per group were used in vivo in animal studies. Statistical significance is presented as *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 and “ns” stands for no (statistical) significance.

Results

TF is expressed by TNBC cancer cells and tumor VECs

To examine TF expression in TNBC with WT and mutated BRCA1 and BRCA2, we first determined TF expression on TNBC lines. Supplementary Fig. S1 showed that TF was expressed by TNBC cell lines, either with WT or mutated BRCA1 and BRCA2 (47, 48), including MDA-MB-231 (BRCA1-wt, BRCA2-mutant; Supplementary Fig. S1A), BT20 (BRCA1-wt, BRCA2-wt; Supplementary Fig. S1B), and MDA-MB-468 (BRCA1-wt; Supplementary Fig. S1C). To verify the binding specificity for TF of the monoclonal anti-human TF (HTF1) used in the flow cytometry, we infected a non-TNBC human breast cancer MCF7/MDR line (Supplementary Fig. S1D), which was derived from the MCF7 line (BRCA1-WT, BRCA2-mutant) with multidrug resistance (MDR) and both are known for low TF expression (17), with a...
retroviral vector encoding human TF (42). We showed that the resulting MCF7/MDR/TF line expressed TF as detected by anti-TF (Supplementary Fig. S1E).

To translate the findings from TNBC cell lines to preclinical mouse models of TNBC, we examined TF expression in human TNBC CDX and PDX tumor tissues from severe-combined immunodeficiency (SCID) mice. With IHC, TF expression was detected on the TNBC cells (Supplementary Fig. S2A) in TNBC CDX tissues, but not in adjacent normal tissues (Supplementary Fig. S2A). In addition, TF expression was also detected on the TNBC cells (Supplementary Fig. S2C) and tumor VECs (Supplementary Fig. S2C) in TNBC PDX tumors, whose vascular origin was confirmed by positive staining for endothelial marker CD31 (Supplementary Fig. S2D). Isotype Ab control (Supplementary Fig. S2B) and second Ab alone (Supplementary Fig. S2E) were used as negative controls. Mitosis was observed in PDX tumor tissues (Supplementary Fig. S2C–S2F).

To translate the findings from mouse models to TNBC patients, we then examined TF expression in primary TNBC tumor tissues from 14 patients. By IHC, 12 of 14 (85.7%) TNBC tumor tissues expressed TF on the surface of TNBC cells (Fig. 1A; Supplementary Fig. S2C). Nine patients who expressed TF on tumor cells also expressed TF on the surface of tumor VECs (64.3% of 14 cases; Fig. 1B). The endothelial origin of tumor VECs was verified through expression of endothelial marker CD31 (Supplementary Fig. S3A). Normal breast gland cells and normal VECs in adjacent normal breast tissues or adenosis (a benign inflammation with enlarged milk-producing glands but not a cancer) were negative for TF expression (Fig. 1C). Mouse IgG isotype was used as a negative control (Fig. 1D).

In light of these results, and to determine statistical significance, we investigated TF expression in 147 additional cases of TNBC tumors and 147 matched normal breast tissues from TNBC patients via TMA. Of TNBC tumor samples, 71 of 147

Figure 3.

L-ICON1 has improvements over the first-generation ICON. A, Diagrams of L-ICON1 and ICON. FVII light chain: 1-152 aa and heavy chain: 153-406 aa; IgG1 Fc: Hinge region followed by two constant regions (CH2 and CH3). -S-S:- Disulfide bonds in the hinge region to form homodimer of ICON and L-ICON1. B, SDS-PAGE analysis shows a 50% reduction in molecular weight of L-ICON1 as compared with ICON. C, Depletion of coagulation activity of L-ICON1 as compared with those in ICON (K341A) and ICON (WT). FVIIa: Active form of FVII (American Diagnostica) as a positive coagulation control; FVIIa-FFR: Active-site inhibited FVIIa (American Diagnostica) as coagulation-inactive control. All FVII proteins were at FVII physiological plasma concentration (10 nmol/L). Assays were in duplicate wells and repeated once with consistent observations. Representative results (mean ± SEM) from two independent experiments. D, Binding activity of L-ICON1 and ICON to MDA-MB-231 cells by flow cytometry analysis. Second Ab-FITC alone or unstained cells were controls. E, Binding activity of L-ICON1 and ICON to MDA-MB-231 cancer cells using ELISA analysis. Human IgG was an isotype control. Representative results (mean ± SEM) in D and E from two or three independent experiments. NS (not significant), *, **, ***, and **** indicate statistical significance: P values >0.05, <0.05, <0.01, <0.001, and <0.0001 by ANOVA model, respectively.

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(48.3%) were positive for TF expression on the surface of TNBC cancer cells (Fig. 2A, B and D) and 11 of 147 (7.5%) were positive for TF on tumor VECs (Supplementary Fig. S3B and S3C), whereas normal breast tissues \( n = 147 \) were negative for TF expression (Fig. 2A and C; Supplementary Fig. S3D). There was a significant difference of IHC score percentage of TF expression on TNBC cancer cells between whole tumor tissues and TMA tissues \( (P = 0.0002474, \text{Fisher exact test; Fig. 2D}) \).

Modification in L-ICON1 eliminated its procoagulant effects

To target TF, an oncotarget for TNBC, we developed a second-generation ICON by modifying the first-generation TF-targeting ICON (Fig. 3A). Following the tradition of making mouse and human ICONs (32, 34) so that the mouse version can be tested in mouse studies and the human version can be used in future clinical trials, mouse and human L-ICON1s were constructed (Supplementary Fig. S4A). DNA sequencing analysis confirmed that both the mouse (mL-ICON1) and human L-ICON1 (hL-ICON1 or L-ICON1; GenBank accession no. KX760097) have the same sequence of human IgG1 Fc as those in mouse and human ICONs (GenBank accession nos. AF272773 and AF272774; i.e., a 16-amino acid residue hinge region followed by constant heavy chain domains 2 and 3; Supplementary Fig. S4A). SDS-PAGE nonreducing gel analysis showed that the molecular weight of Protein A-affinity purified L-ICON1 protein was approximately 100 kDa (Fig. 3B). Thus, L-ICON1 is less than half the molecular weight of ICON (210 kDa; Fig. 3B).

To verify that the coagulation activity of FVII light chain in L-ICON1 was eliminated, the coagulation activity of L-ICON1 was measured and compared with those of FVII coagulation active-site mutated ICON (i.e., ICON K341A), WT-FVII ICON (i.e., ICON WT), activated form of FVII (FVIIa; as a positive control), and active-site inhibited FVIIa (FFR-FVIIa; as a negative control). For this assessment, L-ICON1 was used at 10 nmol/L, a physiological concentration of zymogen FVII in blood circulation. Compared with the coagulation activity (100%) of FVIIa, L-ICON1 at the same concentration (10 nmol/L) had undetectable coagulation activity \((-6.061 \pm 0.000\%\)\), comparable with FFR-FVIIa \((-4\%; \text{P} = 0.0026 \text{for } L-ICON1 \text{vs. FFR-FVIIa})\), whereas the first-generation ICON (K341A) and the WT ICON (WT) exhibited approximately 5% and 58% coagulation activity \((\text{P} = 0.0104 \text{for } L-ICON1 \text{vs. ICON K341A} \text{and } \text{P} = 0.0179 \text{for } L-ICON1 \text{vs. ICON WT})\), respectively (Fig. 3C; Supplementary Fig. S5; Supplementary Table S1).

Figure 4. L-ICON1 can mediate ADCC for TNBC and non-TNBC cancer cells. A, L-ICON1 binding to TNBC cancer lines (MD-MB-231 and BT20) and non-TNBC line (MCF7/MDR/TF). hlgG: Human IgG isotype control. B, L-ICON1 mediates ADCC effector assay for TNBC and non-TNBC cancer lines. A and B. \(P\) values were analyzed by the ANOVA model. Representative results (mean \(\pm\) SEM) from three independent experiments.
ICON and L-ICON1 similarly bound TNBC and non-TNBC cancer cells

To compare L-ICON1 and ICON binding activity on cancer cells, we used flow cytometry and cancer cell ELISA assays. L-ICON1 and ICON bound similarly to TNBC MDA-MB-231 cells in flow cytometry assay (Fig. 3D). By cancer cell ELISA, L-ICON1 showed an increased activity to MDA-MB-231 cells compared with ICON ($P < 0.01$ to $0.0001$, L-ICON1 vs. ICON; Fig. 3E). The results showed that the modifications in L-ICON1 did not reduce its binding activity for TF-expressing TNBC.

Murine and human L-ICON1s could bind both the human (MDA-MB-231; Supplementary Fig. S4B) and the murine cancer cells (4T1 and EMT6; Supplementary Fig. S4C and S4D). However, hL-ICON1 had significantly stronger binding activity to those cancer cells than mL-ICON1 ($P < 0.0001$; Supplementary Fig. S4B–S4D). Thus, hL-ICON1 (L-ICON1 for simplicity) was used in vitro and in vivo experiments thereafter.

L-ICON1 induced ADCC

To elucidate the mechanism of action of L-ICON1, we performed an ADCC effector assay (Fig. 4). L-ICON1 binds to two TNBC lines (MDA-MB-231 and BT20) and one non-TNBC chemoresistant line (MCF7/MDR/TF as a TF-positive control; Supplementary Fig. S1E; Fig. 4A). Furthermore, L-ICON1 could induce ADCC to those TNBC lines and non-TNBC line (Fig. 4B), whereas the human IgG isotype-negative control had no binding (Fig. 4A) and had no ADCC effect (Fig. 4B) on those breast cancer cells. The results demonstrate that L-ICON1 can mediate ADCC effect on killing human TNBC and non-TNBC cells.

L-ICON1 is more effective than ICON in vivo for treatment of TNBC xenografts

To compare the efficacy of L-ICON1 and ICON, we constructed adenoviral vectors encoding L-ICON1 (AdL-ICON1) and verified that AdL-ICON1 vectors could produce L-ICON1 protein after infecting MDA-MB-231 cells in vitro, and that cells so treated could secrete L-ICON1 protein into the cell culture medium. Cancer cell ELISAs showed that the concentrations (mean $\pm$ SD) of L-ICON1, murine ICON (mICON, murine fVII K341A fused to human IgG1Fc), and human ICON (hICON, human fVII K341A fused to human IgG1Fc) were $15,842.0 \pm 234.4$ ng/mL, $90,28.4 \pm 1,388.0$ ng/mL and $9,611.7 \pm 1,111.1$ ng/mL in serum-free culture media 4 days after infection (Supplementary Table S2; $P < 0.001$ for AdL-ICON1 vs. AdmICON and AdhICON; no statistical
L-ICON1 is effective and safe for treatment of murine TNBC

To examine the efficacy of L-ICON1 for murine TNBC in immunocompetent mice, we transfected the murine breast cancer line 4T1 for stably expressing GFP and luciferase (Supplementary Fig. S6). 4T1 cells serve as a model of murine TNBC (49) for generating an orthotopic mouse model in syngeneic Balb/c mice. L-ICON1 showed a decrease (65.8% reduction as compared with the control tumor on day 10 after initiation of treatment) in tumor size in this murine TNBC model (P < 0.001; Fig. 6A). The average tumor volume was 192 mm³ at the time of initiation of treatment (day 0) in the AdL-ICON1–treated mice, which was significantly bigger (68.4% bigger) than the average tumor volume (114 mm³) in the control group at the same time (P < 0.05; Fig. 6A). Mice were humanely sacrificed when evidence of extreme sickness was present, per IACUC protocol. On day 10 after treatment, only 1 of 5 L-ICON1–treated mice had to be sacrificed (Fig. 6A), compared with 5 of 5 (100%) control mice by the same time point (P = 0.0203 for AdL-ICON1 vs. AdBlank control; Fig. 6B). Under in vivo bioluminescence imaging, no metastasis was observed in both L-ICON1 and control groups at the time of sacrifice of these animals (day 10 after treatment, i.e., day 18 after 4T1 cell implantation).

L-ICON1 treated TNBC PDXs in an orthotopic mouse model

To translate L-ICON1 therapy for TNBC to the clinic, we tested its efficacy in an orthotopic TNBC PDX (with BRCA1 mutation) model in CB-17 SCID mice. The results demonstrated that significance for AdmICON vs. AdhICON by ordinary one-way ANOVA), whereas AdBlank did not produce any L-ICON1 or ICON protein (0.0 ± 0.0 ng/mL).

After having verified the production of L-ICON1 and ICON proteins by AdL-ICON1 and AdICON vectors in vitro, we compared the in vitro effects of ICON and L-ICON1, as intratumoral injection of adenoviral vectors encoding L-ICON1 or ICON, for immunotherapy of human TNBC xenografts in an orthotopic mouse model using MDA-MB-231/Luc + GFP cell line. The results demonstrated that L-ICON1 showed a significantly increased therapeutic effect (80% reduction as compared with control tumor on day 21) in vivo than ICON treatment (60% reduction as compared with control tumor on day 21; P = 0.0042; Fig. 5A). Furthermore, the results in Fig. 5 showed that L-ICON1 inhibited orthotopic TNBC growth in mice (11% smaller on day 21 than its starting volume on day 0; P < 0.0001), as measured by tumor size (83.6% reduction as compared with control tumor on day 23; Fig. 5A) and by in vivo bioluminescence (83.6% reduction as compared with control tumor on day 23; Fig. 5B–D), compared with control adenoviral vector (AdBlank). Tumor weights also showed a significant difference ex vivo (Fig. 5E).

Body weight was assessed as an indicator of safety, per previously recorded models (17, 44). There was no difference in whole mouse body weights between L-ICON1 and control groups (Fig. 5F). At the time of sacrifice, which was 2 days after the last injection of AdL-ICON1 (4 × 10¹⁵ VP per injection), the L-ICON1 protein was detected in mouse plasma from the AdL-ICON1–treated mice at a concentration of 794.8 ± 156.4 ng/mL (~8 nmol/L; ~200 ng/mL L-ICON1 protein produced/10¹⁵ VP intraleisonally injected), whereas no L-ICON1 protein was detected in the plasma samples from control vectored mice (Supplementary Table S2; P < 0.0001 for AdL-ICON1 vs. AdBlank).

L-ICON1 is inhibited orthotopic PDX growth in mice as measured by tumor size (P < 0.0001, 93.7% reduction as compared with control tumor on day 43; Fig. 7A). PDX tumors in control mice grew quickly; these mice had to be sacrificed on day 43 after the first i.t. injection of control vectors. Tumor weights also showed a significant difference ex vivo (P = 0.0044, L-ICON1–treated tumors on day 58 vs. control tumors on day 43; Fig. 7B).

Discussion

TNBC not only is a significant clinical malignancy lacking molecularly defined surface targets, but also is a molecularly heterogeneous disease associated with BRCA1 and BRCA2 mutations. To identify TF as a surface target in TNBC, we investigated TF expression on human TNBC lines with or without BRCA1 and BRCA2 mutations. To translate the findings from in vitro to in vivo and ultimately to the clinic, here we also investigated TF expression on the TNBC cancer cells and tumor VECs in human TNBC CDX and PDX models from mice and in TNBC patients’ tumors. PDX models are considered a better cancer xenograft model than the standard CDX model for evaluating anticancer drug response;
results from PDX models better predict the clinical outcome of anticancer agents (50). The percentage (48%) of TF-positive TNBC on TMA slides \((n = 147)\) was lower than that (85%) found by examination of regular paraffin-embedded whole TNBC tumor tissues \((n = 14)\). This was possibly due to the fact that tumors are heterogeneous and that only small tissue samples are taken for making TMA slides, which is one of the limitations of the TMA technique (31). In future studies, we would suggest that multiple specimens from each tumor should be used for IHC screening for enrollment of patients into clinical trials of L-ICON1 therapy. TF is expressed by cancer stem cells isolated from TNBC cell lines, whereas L-ICON1-treated tumors were measured at the end of experiment on day 58. Intratumoral injections of adenoviral vectors for control (AdBlank) and L-ICON1 (AdL-ICON1) were done on days 0, 4, 7, 14, 21, 28, 35, and 42. The L-ICON1 treated mice were additionally i.t. injected on days 49 and 56. Data are presented as mean \(\pm\) SEM from one experiment. \(P\) values were analyzed by two-way ANOVA (A) and by \(t\) test (B, GraphPad).

L-ICON1 has several advantages over ICON. First, the MW of L-ICON1 is reduced more than 50% compared with ICON. Second, L-ICON1 has no detectable coagulation activity, whereas the coagulation activities in ICON (K341A) and ICON (WT) are about 5% and 58% of that for FVIIa, respectively. L-ICON1 may therefore be a safer product than ICON. Third, human L-ICON1 binds both mouse and human breast cancer cells, whereas human ICON bound strongly to a human melanoma line, but weakly to a mouse melanoma line (34). These features of L-ICON1 will allow us to test human L-ICON1 in preclinical mouse models of human and murine cancers and translate the findings directly to clinical trials. Lastly, the current study showed that L-ICON1 was more effective than ICON in treating TNBC tumor xenografts in the orthotopic mouse model, and \(in vivo\) studies demonstrated that L-ICON1 immunotherapy via intraskeletal injections of AdL-ICON1 was effective and safe for the treatment of human TNBC (MDA-MB-231 with \(BRC\)A2 mutation) and murine TNBC (4T1; ref. 49) in orthotopic CDX mouse models. L-ICON1 could arrest the PDX tumor growth of TNBC with \(BRC\)A1 mutation in an orthotopic mouse model.

Figure 7.
L-ICON1 is effective for the treatment of patient’s TNBC in an orthotopic PDX mouse model in CB-17 SCID mice. A, Therapeutic efficacy of L-ICON1 was determined \(in vivo\) by measuring tumor volume using calipers as compared with a control vector (AdBlank)-treated PDX \((n = 5\) in each group). B, Tumor weights of control mice and L-ICON1-treated mice \((P = 0.0044)\). Tumor weights from the control mice were measured when the control mice had to be sacrificed on day 43, whereas L-ICON1-treated tumors were measured at the end of experiment on day 58. Intratumoral injections of adenoviral vectors for control (AdBlank) and L-ICON1 (AdL-ICON1) were done on days 0, 4, 7, 14, 21, 28, 35, and 42. The L-ICON1 treated mice were additionally i.t. injected on days 49 and 56. Data are presented as mean \(\pm\) SEM from one experiment. \(P\) values were analyzed by two-way ANOVA (A) and by \(t\) test (B, GraphPad).
ICON is efficacious and safe in preclinical animal (mouse, rat, pig, and non-human primate) models of cancer (32–35), age-related macular degeneration (AMD; refs. 53, 54) and endometriosis (55, 56). ICON is being tested in phase I and II clinical trials in patients with AMD (wet form) and ocular melanoma. Indeed, as in angiogenic endothelial cells in cancers, TF is aberrantly expressed by the angiogenic VECs in vitro (30) and in vivo in nonmalignant diseases that are angiogenesis dependent, such as AMD (53) and endometriosis (55). As an improved neovascular-targeting agent, L-ICON1 might have therapeutic potential to treat pathologic angiogenesis-dependent malignancy and nonmalignant diseases.

To target TF as part of cancer immunotherapy, in addition to the ICON and L-ICON molecules that are designed to bind to TF by using its natural ligand VII, either full-length peptide with mutated procoagulation active site (K341A) or light-chain peptide lacking procoagulation activity, several humanized monoclonal antibodies (TF-HuMab) and/or antibody–drug conjugates (TF-ADC) are also being studied in preclinical and clinical studies (19, 57). ICON and L-ICON have several advantages compared with anti-TF and TF-ADC: (i) the $K_d$ for VIII binding to TF is up to $10^{-12}$ M (58), in contrast to anti-TF that has a $K_d$ in the range of $10^{-8}$ to $10^{-9}$ M for TF (59); (ii) ICON and L-ICON are produced by recombinant DNA technology, allowing these TF-targeting protein agents to be made from human sources for clinical trials without need for humanization process that is required for monoclonal antibodies (57); (iii) because ADC is made by covalently conjugating drugs to antibodies, most ADCs exist as heterogeneous mixtures and require site-specific conjugation technologies (60). Nevertheless, these studies of TF-targeting Ab and ADC support the idea that TF-targeted therapies have the potential to treat a range of solid cancers (28, 29).

We report that TF is a useful target in treatment of TNBC models, and immunotherapy with TF-targeting L-ICON1 showed better efficacy and safety than the original ICON. Immunotherapy targeted at TF may warrant further investigation for TNBC patients. If such immunotherapy proves effective and safe in clinical trials, such treatment regimens might reduce the mortality associated with TNBC. TF is highly expressed on cancer cells on non-TNBC breast cancer as well as in many other solid cancers, acute myeloid leukemia, acute lymphocytic leukemia, and sarcomas (28, 29): it is expressed in 80% to 100% of breast, 40% to 80% of lung, 84% of ovarian, 95% of primary melanoma, 100% of metastatic melanoma, 53% to 90% of pancreatic, 57% to 100% of colorectal, 63% to 100% of hepatocellular carcinoma, 60% to 78% of primary and metastatic prostate, and 47% to 75% of glioma tumors. Thus, TF-targeting immunotherapy may have the potential to treat a variety of TF-positive solid cancers, leukemias, and sarcomas (28, 29).

**Disclosure of Potential Conflicts of Interest**

Z. Hu is co-inventor of U.S. patents on the original neovascular-targeted immunconjugates (ICON) and is the inventor of U.S. patent application on the second-generation ICON (L-ICON1). No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: Z. Hu, C.A. London, W.E. Carson III Development of methodology: Z. Hu Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Hu, R. Shen, A. Campbell, E. McMichael Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Hu, R. Shen, A. Campbell, E. McMichael, L. Yu, B. Ramaswamy Writing, review, and/or revision of the manuscript: Z. Hu, A. Campbell, L. Yu, B. Ramaswamy, C.A. London, W.E. Carson III Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Hu Study supervision: Z. Hu Other (provided plasmid DNAs): T. Xu

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Targeting Tissue Factor for Immunotherapy of Triple-Negative Breast Cancer Using a Second-Generation ICON


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