Addition of an Fc-IgG induces receptor clustering and increases the *in vitro* efficacy and *in vivo* anti-tumor properties of the thrombospondin-1 type I repeats (3TSR) in a mouse model of advanced stage ovarian cancer

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HIGHLIGHTS

- Addition of a Fc IgG generates a construct with a ½ life of approximately 8 days in serum.
- Fc3TSR has significantly enhanced anti-tumor activity *in vitro*, compared to native 3TSR.
- Fc-induced clustering of the CD36 receptor mediates the enhanced efficacy of Fc3TSR.
- Fc3TSR normalizes ovarian tumor vasculature.
- Fc3TSR induces potent regression of advanced stage epithelial ovarian cancer in an orthotopic, syngeneic mouse model.

ABSTRACT

Objectives. Tumor vasculature is structurally abnormal, with anatomical deformities, reduced pericyte coverage, and low tissue perfusion. As a result of this vascular dysfunction, tumors are often hypoxic, which is associated with an aggressive tumor phenotype, and reduced delivery of therapeutic compounds to the tumor. We have previously shown that a peptide containing the thrombospondin-1 type I repeats (3TSR) specifically targets tumor vessels and induces vascular normalization in a mouse model of epithelial ovarian cancer (EOC). However, due to its small size, 3TSR is rapidly cleared from circulation. We now introduce a novel construct with the 3TSR peptide fused to the C-terminus of each of the two heavy chains of the Fc region of human IgG1 (Fc3TSR). We hypothesize that Fc3TSR will have greater anti-tumor activity *in vitro* and *in vivo* compared to the native compound.

Methods. Fc3TSR was evaluated *in vitro* using proliferation and apoptosis assays to investigate differences in efficacy compared to native 3TSR. In light of the multivalency of Fc3TSR, we also investigate whether it induces greater clustering of its functional receptor, CD36. We also compare the compounds in vivo using an orthotopic, syngeneic mouse model of advanced stage EOC. The impact of the two compounds on changes to tumor vasculature morphology was also investigated.

Results. Fc3TSR significantly decreased the viability and proliferative potential of EOC cells and endothelial cells in vitro compared to native 3TSR. High-resolution imaging followed by image correlation spectroscopy demonstrated enhanced clustering of the CD36 receptor in cells treated with Fc3TSR. This was associated with enhanced downstream signaling and greater *in vitro* and *in vivo* cellular responses. Fc3TSR induced greater vascular normalization and disease regression compared to native 3TSR in an orthotopic, syngeneic mouse model of advanced stage ovarian cancer.

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1. Introduction

Epithelial ovarian cancer (EOC) is the most common and the most lethal gynecological malignancy, characterized by late detection and low five-year survival rates. Over the past 30 years, the five-year survival rate has seen only modest improvement as current treatments have limited efficacy and are prone to resistance [1].

As with other solid tumors, progression of disease is dependent on the process of angiogenesis for nutrient delivery and waste removal. The process of angiogenesis is closely regulated by a balance between stimulatory and inhibitory angiogenic factors. Thrombospondin (TSP)-1 is an endogenous matricellular glycoprotein with natural anti-angiogenic effects [2]. TSP-1 is a 450 kDa homotrimeric glycoprotein with six distinct types of modular domains [3] giving the protein multiple functions. TSP-1 regulates a multitude of processes, such as angiogenesis, cell proliferation, apoptosis, adhesion, migration and immunity [4-6]. While native TSP-1 exerts several functions that inhibit cancer progression, its large size and multiple biological activities pose considerable challenges to its development as a therapeutic compound. While some regions of TSP-1 inhibit angiogenesis, the N-terminal domain reportedly has pro-angiogenic activity [7]. Thus, fragments of TSP-1, with smaller size and more defined function, could be important inhibitors of tumorogenesis.

The anti-angiogenic functions of TSP-1 that are mediated by the membrane protein CD36 have been predominantly localized to the three type 1 thrombospondin repeat (TSPR) region [8,9]. In addition to its anti-angiogenic effects on endothelial cells, TSPR has also been shown to directly induce apoptosis in ovarian cancer cells through a CD36-dependent mechanism [10-12]. In vivo, TSPR has potent antitumor efficacy in a host of preclinical models of cancers including pancreatic [13,14], liver and lung [15], glioblastoma [10] and ovarian [16,17]. An important function of 3TSR is its ability to specifically target tumor vasculature, resulting in pruning and normalization. By reducing tumor hypoxia and enhancing perfusion through vascular normalization, 3TSR has increased the uptake and efficacy of chemotherapy drugs [17] and oncolytic viruses and immune cells [18] in preclinical models of advanced stage ovarian cancer. However, due to its small size of approximately 18 kDa, 3TSR is susceptible to rapid clearance from circulation, necessitating daily injections to maintain biological activity. To overcome this deficiency, we have generated a construct with one TSPR peptide fused to the C-terminus of each of the two heavy chains of the Fc region of human IgG1 [19,20]. Therapeutic agents that are modified to include a Fc domain have demonstrated improved pharmacokinetic properties, due to recycling by the neonatal Fc receptor (FcRn) in circulation, which plays a central role in slowing IgG catabolism [21]. IgG-based therapeutics also allow for safe and scalable purification procedures, making them ideal for clinical use [22,23]. As seen in other angiogenesis inhibitors such as Angiobec, addition of an Fc domain increases the half-life of the protein in circulation from hours to weeks [24,25]. When the anti-angiogenic protein endostatin was linked to an Fc domain, it demonstrated biphasic activity, allowing for dosing up to 100-fold lower than for endostatin not linked to Fc [26].

The concept of multivalency is frequently found physiologically. With multivalency, multiple ligands on a single construct bind to multiple receptors on another construct, which enhances binding avidity and localizes signaling components [27,28]. Dimeric protein interactions have been shown to enhance receptor clustering, the grouping of membrane receptors into nano structures that are imperative for the activation of several signaling pathways [27]. Given that CD36 receptor clustering is crucial for optimal downstream effector Fyn activation, we hypothesized that engineering a single Fc fusion protein with two 3TSRs in dimeric configuration may promote clustering of CD36 [29]. It was anticipated that improved stability, avidity, and delivery of 3TSR would lead to a potent compound for clinical trials.

2. Materials and methods

2.1. Expression of Fc3TSR

3TSR was generated as described previously [14]. The DNA encoding Fc3TSR was codon-optimized for expression in Human Embryonic Kidney (HEK293) cells and cloned into transient expression vector pIT5 (Novoprolabs). Transient transfection was performed using the Expifectamine™ 293 Transfection Kit in Expifectamine Expression Medium (ThermoFisher). Recombinant Fc3TSR was purified from the cell culture supernatant by protein A chromatography.

2.2. Cell lines and cultures

Spontaneously transformed murine ovarian epithelial ID8 cells (generously donated by Drs. K. Roby and P. Terranova, Kansas State University, Manhattan, KS, USA), murine microvascular endothelial cells (mEC; American Type Culture Collection, Manassas, VA, USA) and human ovarian cancer CAOV3 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% l-glutamine and 1% antibiotic/antimycotic (ABAM). Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Wisent, Saint-Jean-Baptiste, QC) and supplemented with 20% FBS, 2% l-glutamine and 1% ABAM. Human dermal microvascular endothelial cells (HDMECs) were isolated by the cell biology core at Beth Israel Deaconess Medical Center (Department of Pathology) by the method of Richards and coworkers [30]. The cells were cultured on dishes coated with 50 μg/mL collagen (Inviogen) and maintained in Endothelial Cell Growth media (EBM-2) (Clonetics Corp., San Diego, CA, USA) containing 20% FBS, 1 μg/mL hydrocortisone acetate, 5 x 10^5 dibutyryl-cAMP, 200 units/mL penicillin, 100 units/mL streptomycin and 250 μg/mL amphotericin. Cell cultures were maintained on 100 mm cell culture plates (Corning Inc., NY, USA) by passaging cells once they reached 70-80% confluency.

2.3. CD36 knockdown

CD36 was knocked down in ID8 cells as described previously [31] in which numerous CD36 siRNA constructs were tested and the one with the most potent CD36 inhibition was chosen. Briefly, ID8 cells were transfected with 200 nmol/L CD36 siRNA (Sigma-Aldrich Canada Ltd., Oakville, ON) diluted in reduced serum media (Gibco, Opti-MEM) for 48 h. Immunofluorescence and western blotting were performed as described below on control ID8 cells and CD36-knockdown ID8 cells.

2.4. Migration assay

All cell migration assays were performed using a Boyden’s chamber and polycarbonate filters with an eight μm pore size (Corning Inc.,
Corning, NY, USA). Briefly, each side of the filter was coated with 50 μg of collagen type I/mL in phosphate-buffered saline (PBS) for 1 h at room temperature. HDMEC cells were washed, counted, and resuspended in 0.1% Bovine Serum Albumin (BSA) in PBS. 8.0 × 10^3 cells/400 μL were seeded in the upper chamber in triplicate and incubated at 37 °C for 90 min. Twenty ng/mL of recombinant VEGF-A (R&D Systems, Minneapolis, MN, USA) in 0.1% BSA in PBS was added to the lower chamber and incubated for an additional five hours. In these experiments 0.1% BSA was used in the lower chamber as a negative control. After five hours, the upper filter was scraped with cotton-tipped swabs, and cells in the lower chamber were fixed in methanol for 2 min and stained in Giemsa (Richard-Alan Scientific, Kalamazoo, MI, USA) for 30 min. For three separate wells per treatment group, four fields in the lower filter were counted at 20× magnification, and the percentage of migrated cells was calculated considering the highest average of migrated cells as 100%.

For ID8 cell migration experiments, 1.0 × 10^5 ID8 cells were seeded in the upper chamber with serum free DMEM and either 3TSR or Fc3TSR at 10 nM. Upper chambers were coated with 20% Matrigel (100 μL/well; BD Biosciences, San Diego, CA, USA). The bottom chamber contained 0.75 mL of complete growth medium with 10% FBS. After incubation for 24 h (37 °C, 5% CO₂), cells were washed from the upper chamber of three separate wells and the cells on the bottom side of the three chambers were fixed and stained. Cells were counted in five random fields at 100× magnification per well and the mean of the five counts was taken. Data represents the mean count of each chamber, for three chambers.

The standard deviation was calculated considering each transwell as an independent experiment. The error bars indicate the standard error of the mean. P-values are based on the unpaired Student’s t-test with two-tailed distribution.

2.5. Resazurin sodium salt cell viability assay

Human EOC cells, CAOV3 and OVCAR3, were seeded in 96-well plates (1.0 × 10^4 cells/well; 3 technical replicates). Cells were then cultured in media (untreated), or were exposed to 3TSR and Fc3TSR at various doses (0.1, 1.0, 10, 100, 1000 nM) for 48 h. Following treatment incubation, resazurin sodium salt (Millipore-Sigma, Oakville, ON) was added to a final concentration of 20 μg/mL. This is a metabolic agent whereby resazurin is metabolized into resorufin leading to a colour change detectable by fluorescence [32]. After a 4 h incubation, the fluorescence was read at excitation and emission wavelengths of 535/25 nm and 590/35 nm, respectively. This assay was repeated in triplicate. Data was graphed as a percent of cell viability (following treatment) normalized to untreated, control cells.

2.6. Receptor clustering

1.0 × 10^4 ID8 cells were cultured on 24 mm glass coverslips (ThermoFisher Scientific, Massachusetts, USA) until 80% confluent and left untreated (n = 4 slips) or were treated with 100 nM of 3TSR or Fc3TSR (n = 4). In accordance with the assumption that a constant number of cell membrane receptors must be present between treatment groups to meet conditions for image correlation spectroscopy (ICS) analysis [33], cells were treated for 30 min (Fig. 4D). Following completion of incubation time, cells were washed twice with PBS and fixed using 4% paraformaldehyde in PBS for 10 min. Paraformaldehyde was removed and cells were washed twice with PBS, followed by blocking with 5% BSA in PBS at 4 °C overnight. Cells were labeled with the primary antibody (1:500, anti-CD36, Novus Biologicals, Colorado, USA) for 2 h at room temperature. Following two more washing steps, cells were blocked with 5% BSA in PBS for 30 min at room temperature. BSA was removed and cells were incubated with fluorescein labeled secondary antibody (1:1000, AlexaFluor 488 anti-IgG, Invitrogen, California USA) for 1 h. Cells were washed twice with PBS and affixed onto microscope slides (ThermoFisher Scientific, Massachusetts, USA) using an anti-fade mountant containing DAPI nuclear counterstain (ThermoFisher Scientific, Massachusetts, USA). Slides were dried for 2 h at room temperature and imaged using the Olympus Fluoview FV1200 laser scanning microscope. The apical membrane of cells was captured using a 60× objective under oil emersion, allowing for a pixel size of 0.062 μm/pix and using a 200 μs/Pixel scan speed to allow for optimal resolution. Olympus Image Binary (OIB) images (n = 12 cells per treatment group) were obtained and analyzed using the Fiji Plug-in of Imagej software (National Institutes of Health, Maryland USA) following ICS as described [34].

2.7. In vitro immunofluorescence

The ability of 3TSR and Fc3TSR to induce apoptosis and inhibit proliferation in endothelial and epithelial cells was evaluated by immunofluorescent staining for cleaved caspase-3 and phosphorylated histone H3. Endothelial and ovarian tumor cells were cultured on glass coverslips in serum-reduced (1% FBS in DMEM) or serum-free media (DMEM alone) with or without 3TSR or Fc3TSR (using a 10-fold dilution series ranging from 0.1–1000 nM) or vehicle control (50 mM NaPO₄, 150 mM NaCl) for 24 h. Cells were then fixed with 10% neutral buffered formalin for 1 h, rinsed with PBS and permeabilized with 0.2% (v/v) Triton X 100 (Sigma-Aldrich Canada Ltd., Oakville, ON) for 5 min. Cells were blocked with 5% BSA in PBS and incubated with primary antibodies: anti-phosphorylated histone H3 (1:2000 dilution; Abcam, Massachusetts USA) or anti-cleaved caspase-3 (1:500 dilution; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. AlexaFluor-594-conjugated secondary antibodies (1:100 dilution; Life Technologies Inc., Burlington, ON) were then added for 1 h at room temperature followed by 4′,6-diamidino-2-phenylindole (DAPI) (0.05 μL/mL; Sigma-Aldrich, Canada Ltd., Oakville, ON) staining for 1 min. Cell images were captured using an Olympus inverted epifluorescence microscope and Metamorph integrated morphometry software (Molecular Devices, Sunnyvale, CA).

For downstream effector colocalization, 1.0 × 10^4 ID8 cells were cultured on 24 mm glass coverslips (Fisher Scientific) until 80% confluent and left untreated (n = 4 slips) or were treated with 100 nM of 3TSR or Fc3TSR (n = 4) for various time points ranging from 10 min to 45 min of treatment. Following treatment incubations, cells were fixed with 10% neutral buffered formalin for 1 h, rinsed with PBS and permeabilized with 0.2% (v/v) Triton X 100 (Sigma-Aldrich Canada Ltd., Oakville, ON) for 5 min. Cells were blocked with 5% BSA in PBS and incubated overnight at 4 °C with either i) anti-CD36 (1:500, Novus Biologicals, Colorado USA) and anti-Fyn (1:1000, Santa Cruz Biotechnology Inc., Texas USA) or ii) anti-Fyn (1:1000, Santa Cruz Biotechnology Inc., Texas USA) and anti-jNK 1,2,3 (1:1000, Abcam, Massachusetts USA). Following two more washing steps, cells were incubated with species specific Alexa-Fluor fluorescently labeled secondary antibodies (1:500 dilution; Life Technologies Inc., Burlington, ON). Cells were washed twice with PBS and affixed onto microscope slides (ThermoFisher Scientific, Massachusetts, USA) using an anti-fade mountant containing DAPI nuclear counterstain (ThermoFisher Scientific, Massachusetts, USA). Slides were dried overnight at room temperature and imaged using an Olympus inverted epifluorescence microscope and Metamorph integrated morphometry software (Molecular Devices, Sunnyvale, CA).

Human ovarian cancer cells (CAOV3 and OVCAR3) were analyzed for expression of CD36 using immunofluorescence. Briefly, cells were cultured on 24 mm glass coverslips (Fisher Scientific) until 80% confluent and left untreated or were treated with 100 nM of 3TSR or Fc3TSR. After treatment on coverslips, cells were fixed in 10% neutral buffered formalin for 1 h at room temperature and rinsed in PBS. Cells were permeabilized by adding 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature, blocked for 10 min in 5% BSA in PBS, and exposed to primary antibody (anti-CD36; 1:400 dilution; Novus Biologicals, Colorado, USA) overnight at 4 °C. AlexaFluor–488 conjugated antirabbit antibody (1:100 dilution; Life Technologies Inc., Burlington, ON)
was added for 2 h at room temperature, followed by incubation with DAPI (ThermoFisher Scientific, Massachusetts, USA) for 10 min and then affixed onto microscope slides (ThermoFisher Scientific, Massachusetts, USA). Slides were dried at room temperature overnight and imaged with an Olympus inverted fluorescence microscope and Metamorph integrated morphometry software (Molecular Devices, Sunnyvale, CA).

2.8. Western blots

ID8 cells were cultured in serum-free medium with 3TSR or Fc3TSR or vehicle control as previously described for timepoints ranging from 10 min to 24 h. Following treatment (10 nM or 100 nM as indicated), cells were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. Protein concentrations were determined using a Bio-Rad DC Protein Assay kit (Bio-Rad, Mississauga, ON).

All western blots were performed using a Bio-Rad wet-transfer western blot apparatus. Twenty or forty μg of protein was denatured and reduced before being separated on a gradient by SDS-PAGE. Amersham Hybond ECL nitrocellulose blotting membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) were used for protein transfer, and membranes were then blocked in 5% (w/v) skim milk or BSA in Tris-buffered saline plus 0.1% tween 20 (TBST) for one hour. Membranes were probed overnight at 4 °C with anti-VEGF (1:500 dilution, Abcam, Massachusetts USA), anti-phospho-VEGFR-2/Flk-1 (1:1000 dilution; Millipore Canada Ltd., Etobicoke, ON), anti-VEGF receptor 2 (1:1000 dilution; Cell Signaling Technology, Danvers, MA), anti-Fas receptor (1:500 dilution; Novus Biologicals, Oakville, ON), anti-Fas ligand (1:500 dilution; Santa Cruz Biotechnology Inc.), anti-cleaved caspase-3 (1:500 dilution; Cell Signaling Technology, Massachusetts USA), anti-phospho-Jnk (1:1000 dilution; Abcam, Massachusetts USA), anti-Jnk 1,2,3 (1:2000 dilution, Abcam, Massachusetts USA), anti-Fyn (1:2000 dilution, Santa Cruz Biotechnology Inc., Texas USA), anti-phospho-Fyn (1:1000 dilution, Abcam, Massachusetts USA), anti-CD36 (1:2000 dilution, Novus Biologicals, Colorado USA) or anti-β-actin (1:5000 dilution; Cell Signaling Technology, Massachusetts USA) primary antibodies. Membranes were then washed with TBST and incubated with horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Massachusetts USA) for one hour at room temperature. Membranes were washed with TBST and proteins of interest were detected with Clarity Western ECL Substrate (Bio-Rad, Mississauga ON) and imaged using a BioRad Gel Doc. Densitometry analysis was performed using the tools in ImageLab software (Bio-Rad) and reported as relative densitometric units relative to β-actin or stain-free protein.

2.9. Orthotopic mouse model

Wild-type, syngeneic 10 week old C57BL/6 female mice (Charles River Laboratories, Boston, MA, USA) were housed under standard conditions at the University of Guelph as per the Canadian Council on Animal Care guidelines. Our orthotopic, syngeneic model has been described previously [17,18,35]. In brief, 1.0 × 10^6 ID8 cells suspended in 6 μL of sterile PBS were injected directly under the ovarian bursa of post-pubescent immunocompetent mice. In this model, large primary tumors are formed 60 days after tumor cell injection, followed by the development of abdominal ascites and secondary peritoneal lesions at which point the model has disease characteristics consistent with women with stage III EOC [31]. Weekly intraperitoneal injections with low-, mid-, or high doses of 3TSR and Fc3TSR were initiated at 60 days post-tumor cell injection. Initial intravascular injection of Fc3TSR was conducted for PK analysis. For therapeutic testing, mice received intraperitoneal injections to provide direct exposure in the primary and metastatic tumor environment. The dosing schedule for 3TSR and Fc3TSR was created to allow for equimolar concentrations in vivo, based on the predicted clearance rates of native 3TSR and the larger Fc constructs. Experimental groups are outlined in Table 1.

Mice were euthanized at 90 days post-tumor cell injection following 4 weeks of treatment. Primary tumors were excised and weighed to determine the effect of treatment on tumor size and fixed in 10% neutral buffered formalin. Metastatic lesions in the peritoneal cavity were assessed using a previously published scoring system to determine the extent of secondary disease [16,17,31,36]. Ascites fluid was aspirated and quantified following euthanasia.

2.10. VEGF ELISA

At 90 days post-tumor cell injection in the orthotopic ID8 model, whole blood was collected via cardiac puncture. Clotted whole blood was centrifuged (2000 × g for 15 min) and serum was collected, aliquoted and stored at -80 °C for subsequent quantification of VEGF concentrations by ELISA (R&D Systems, Minneapolis, MN, USA).

2.11. In vivo immunofluorescence

To determine the effects of 3TSR or Fc3TSR on proliferation and apoptosis of tumor tissue, cryosections of primary tumor tissue collected at 90 days post-tumor cell injection were prepared. Tissue sections were incubated with anti-phosphorylated histone H3 (1:200 dilution, Abcam, Cambridge, MA) or anti-cleaved caspase-3 (1:100 dilution, Cell Signaling Technologies, Massachusetts USA) primary antibodies. Membranes were then washed with TBST and incubated with horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Massachusetts USA) for one hour at room temperature. Nuclear staining was achieved through incubation with DAPI (0.05 μg/mL; Sigma-Aldrich) for 10 min to 24 h. Following treatment (10 nM or 100 nM as indicated), tissues were incubated for 1 h with a secondary antibody cocktail of tail of rabbit anti-CD31 (1:50 dilution; Abcam, Massachusetts USA) for 60 days post-tumor cell injection. Initial intravascular injection of Fc3TSR was conducted for PK analysis. For therapeutic testing, mice received intraperitoneal injections to provide direct exposure in the primary and metastatic tumor environment. The dosing schedule for 3TSR and Fc3TSR was created to allow for equimolar concentrations in vivo, based on the predicted clearance rates of native 3TSR and the larger Fc constructs. Experimental groups are outlined in Table 1.

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2.12. Vessel normalization and density

Evaluation of microvessel density in tumors of treated mice was performed as discussed above. Vessel maturity was evaluated through colocalization of endothelial cell marker CD31 and pericyte marker alpha smooth muscle actin (SMA) staining in tissues from mice treated with 3TSR or Fc3TSR for 4 weeks. Cryosections of tumor tissue were blocked with 5% BSA in PBS for 10 min, and then incubated with a cocktail of rabbit anti-CD31 (1:50 dilution; Abcam, Massachusetts USA) and mouse anti-SMA (1:1000 dilution; Santa Cruz Biotechnology Inc., Texas USA) overnight at 4 °C. Following the overnight incubation, tissues were incubated for 1 h with a secondary antibody cocktail of AlexaFluor-488 conjugated anti-mouse and AlexaFluor-594 conjugated anti-rabbit IgG secondary antibodies (1:100 dilution; Life Technologies Inc.). After rinsing with PBS, tissues were stained with DAPI (0.05 μg/mL; Sigma-Aldrich, Canada Ltd.) for 1 min to counterstain nuclei blue. Quantification of vessel maturity was performed as a
percentage of vessels exhibiting SMA-positive staining to total CD31-positive vessels.

2.13 Data analysis

All data collected is expressed as a mean ± the standard error of the mean. In vitro experiments contained three replicates, and in vivo experiments used 6 animals per group. Means, standard errors of the means and statistical analyses were calculated using GraphPad Prism 6.0c statistical software (GraphPad Software Inc., La Jolla, CA, USA). Data obtained from p-H3 proliferation and cleaved caspase-3 apoptosis assays were analyzed with two-way analysis of variance (ANOVA) and significant differences in the means were determined using Tukey’s post-hoc multiple comparisons test. Data collected from invasion assays and western blots were analyzed using one-way ANOVA followed by Tukey’s post-hoc test to identify significance between means. One-way ANOVA was performed on all in vivo data, followed by Tukey’s post-hoc analysis. Data was analyzed relative to vehicle controls for each experiment. Differences between means were considered significant when p < 0.05.

3. Results

3.1 Recombinant Fc3TSR has prolonged serum half-life

3TSR was expressed as a Fc3TSR fusion protein composed of the Fc domain of human IgG1 fused to the N-terminus of 3TSR via a flexible (Gly4Ser)4 linker. The monomeric Fc3TSR polypeptide has a molecular weight of 46 kDa based on its amino acid sequence and migrated with an apparent molecular weight of approximately 51 kDa on Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) under reducing and non-reducing conditions.
Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) under reducing conditions; under non-reducing conditions, it ran as a homodimer (Fig. 1A). The pharmacokinetics of Fc3TSR was determined in C57BL/6 mice injected intravenously with 1 mg/kg or 10 mg/kg of Fc3TSR ($n = 6$ mice/group). The concentrations of Fc3TSR in the plasma at different time points were determined by human IgG enzyme-linked
immunosorbent assay (ELISA). The concentration of Fc3TSR in the plasma was found to be dose-proportional and the plasma concentrations declined according to a half-life of approximately 5 days (Fig. 1B). Therefore, it is anticipated that near-optimal levels of Fc3TSR can be sustained in circulation for at least 1 week in humans following a single dose. Furthermore, an anti-human Fc western blot showed that Fc3TSR remained intact in circulation throughout the time-course of 10 days (Fig. 1C).

3.2. Fc3TSR increases the apoptotic response and decreases proliferation of ovarian cancer cells in vitro compared to native 3TSR

To assess the effect of 3TSR and Fc3TSR on ovarian cancer cell function, spontaneously transformed murine ovarian surface epithelial cells (ID8) were treated with increasing concentrations of 3TSR or Fc3TSR in vitro. At nanomolar concentrations, Fc3TSR induced significantly (p < 0.05) higher levels of the apoptotic marker cleaved caspase-3 compared to 3TSR via immunofluorescence and western blotting (Fig. 2A&C). In addition, Fc3TSR significantly (p < 0.05) increased expression of other apoptotic cascade factors such as Fas ligand, as well as decreased expression of the survival factor vascular endothelial growth factor receptor (VEGFR)-2 in ID8 cells (Fig. 2C). Both 3TSR and Fc3TSR inhibited VEGF signaling in ID8 cells by reducing the phosphorylation and activation of VEGFR-2 (Fig. 2C). The ability of the type I repeats of TSP-1 to modify cell proliferation was also assessed following treatment with a range of concentrations of 3TSR and Fc3TSR. Immunofluorescence staining for nuclear proliferative factor phosphorylated histone H3 (pH3) demonstrated significantly (p < 0.05) reduced proliferation of Fc3TSR-treated ID8 cells at higher nanomolar concentrations (Fig. 2B). The effect of 3TSR and Fc3TSR on ID8 cell migration was also evaluated. While 3TSR treatment reduced migration of ID8 ovarian cancer cells, compared to vehicle (p < 0.05), Fc3TSR reduced migration of these cells compared to both vehicle-treated controls (p < 0.01) and 3TSR-treated cells (p < 0.05) (Fig. 2D). Human ovarian cancer cells were confirmed to express the CD36 receptor (Fig. 2E). In vitro viability assay demonstrated that Fc3TSR induced a significant reduction in viability in human EOC cells, and this reduction in viability was greater than that seen with native 3TSR (Fig. 2E).

3.3. Fc3TSR increases the apoptotic response and decreases proliferation of endothelial cells in vitro compared to native 3TSR

Next, we assessed the apoptotic and proliferative responses of murine microvascular endothelial cells (mECs) to 3TSR and Fc3TSR following treatment with a concentration gradient of 3TSR or Fc3TSR in vitro to further investigate anti-angiogenic potential between the compounds. At nanomolar concentrations, Fc3TSR significantly (p < 0.05) increased cleaved caspase-3 expression compared to vehicle-treated control or 3TSR via immunofluorescence analysis and western blot quantification (Fig. 3A&C). Similar to protein analysis of ID8 cells following treatment with 3TSR or Fc3TSR, mECs in both treatment groups exhibited an increase in expression (p < 0.05) of apoptotic pathway factors such as Fas; however, a decrease in Fas ligand was observed in Fc3TSR-treated cells (Fig. 3C). VEGF signaling was also modulated in mECs following treatment with either 3TSR or Fc3TSR, with Fc3TSR significantly (p < 0.05) reducing VEGF and VEGFR-2 expression in vitro (Fig. 3C). There was minimal effect of 3TSR or Fc3TSR on cell proliferation, with no differences between 3TSR and Fc3TSR on expression of pH3 (Fig. 3B). Human dermal microvascular endothelial cell (HDMEC) migration was also inhibited by 10 nM 3TSR or Fc3TSR (p < 0.001), with the two reagents having comparable activity (Fig. 3D). Similar to the response of ID8 cells, the most efficacious dosage of 3TSR or Fc3TSR fell within the range of 1.0 to 1000 nM. These data indicate that the Fc3TSR fusion protein has increased apoptotic activity in vitro compared to 3TSR.

3.4. Fc3TSR enhances CD36 receptor clustering and regulates ovarian cancer cell apoptosis and proliferation through CD36-dependent mechanisms

The role of CD36 in mediating the effects of Fc3TSR was evaluated using small interfering (si)-RNA-mediated knockdown of the receptor. Wild-type ID8 cells or CD36-knockdown ID8 cells were treated with 3TSR or Fc3TSR at 10 nM for 24 h and subjected to immunofluorescent staining and western blot analysis. In native cells, both 3TSR or Fc3TSR increased expression of cleaved caspase-3 and Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1). However, Fc3TSR induced a more potent increase in expression than 3TSR (p < 0.05) (Fig. 4A). Conversely, 3TSR and Fc3TSR inhibited expression of p-H3, VEGF and phosphorylated VEGFR-2 (p < 0.05) (Fig. 4A). Knockdown of CD36 by siRNA abrogated these effects of both 3TSR and Fc3TSR in vitro (Fig. 4AB&B). Western blot analysis demonstrated an increase in cleaved caspase-3 expression following 3TSR treatment (p < 0.01), and this was further increased (p < 0.01) by Fc3TSR (Fig. 4C). Both 3TSR and Fc3TSR reduced expression of VEGF (p < 0.01) (Fig. 4A), with Fc3TSR having a more potent inhibitory effect than 3TSR on phospho-VEGFR-2 expression (p < 0.05) (Fig. 4C). Again, CD36 knockdown abolished these effects, and expression in treated cells did not differ from controls (Fig. 4C). In order to follow conditions for Image Correlation Spectroscopy (ICS), CD36 protein expression levels in ID8 cells at acute treatment timepoints were tested to select a timepoint with consistent CD36 expression across treatments (30 min) (Fig. 4D). To determine whether the more potent downstream effector activation of Fc3TSR compared to 3TSR was as a result of enhanced CD36 receptor clustering following Fc3TSR treatment, ICS was performed (Fig. 4E). ICS revealed significantly increased aggregation of CD36 on the apical membrane of ID8 cells treated with Fc3TSR compared to untreated cells (p < 0.0001) and cells treated with equal concentrations of native 3TSR (p < 0.001) (Fig. 4E). CD36 aggregation on the apical membrane of mEC cells treated with 3TSR or Fc3TSR was statistically significant compared to untreated controls (p < 0.05; Fig. 4E).

3.5. 3TSR and Fc3TSR acutely induce expression of CD36 signaling molecules

In order to assess activation of the CD36 receptor, downstream expression and phosphorylation of proto-oncogene tyrosine-protein kinase Fyn (Fyn) and c-Jun NH(2) terminal kinase (JNK) was investigated. ID8 cells were left untreated or treated with 3TSR or Fc3TSR to induce CD36 clustering. Western blot analysis of total protein revealed an increase in expression of phosphorylated Fyn in Fc3TSR-treated cells compared to untreated cells (p < 0.01) or cells treated with an equimolar concentration of 3TSR (p < 0.05) following 15 min of treatment (Fig. 5A). Levels of phosphorylated JNK were significantly increased in cells treated for 15 min with 3TSR or Fc3TSR compared to untreated cells. Concentrations
of inactive FYN and JNK protein remained relatively unchanged compared to untreated controls, but spiked at 24 h post-treatment (p < 0.05), when phosphorylated counterparts decreased (Fig. 5A). Expression of VEGF was significantly decreased at 30 min post-treatment with 3TSR (p < 0.05) and Fc3TSR (p < 0.01) compared to untreated controls. Levels of VEGF continued to decline up to 24 h post-treatment (Fig. 5A). Similarly, expression of VEGFR2 was significantly decreased at 30 min post-treatment for both 3TSR (p < 0.0001) and Fc3TSR (p < 0.0001) compared to untreated controls (Fig. 5A). Immunofluorescence of CD36/FYN revealed colocalization of these downstream factors as early as 10 min after treatment with 3TSR or Fc3TSR (Fig. 5B). Immunofluorescence staining of FYN/JNK 1,2,3 revealed colocalization and nuclear localization of these effectors at acute timepoints following clustering events (Fig. 5B).

3.6. Fc3TSR inhibits ovarian tumor growth in vivo

Sixty days post-orthotopic ID8 cell injection, mice developed disease characteristics consistent with stage III EOC, with respect to the primary tumor, abdominal ascites, and proliferative secondary disease lesions. Intervention with 3TSR or Fc3TSR treatments at low-, mid- or high-doses was initiated at 60 days post-tumor cell injection (n = 6 mice/group) and lasted for 4 weeks until mice were sacrificed. By 90 days post-tumor cell injection, administration of 3TSR or Fc3TSR at all doses resulted in tumors that were significantly (p < 0.05) smaller than PBS-treated controls (Fig. 6A). Administration of Fc3TSR at low- or mid-doses (Table 1; Methods) significantly (p < 0.05) reduced primary tumor size compared to both PBS- and 3TSR-treated mice (Fig. 6A). In both 3TSR and Fc3TSR groups, the high-dose treatment was less effective than either the low- and mid-dose treatments, although it still suppressed tumor growth (p < 0.05) compared to PBS-treated mice (Fig. 6A). Secondary disease was measured by extent of lesions throughout the peritoneal cavity at euthanasia. Fc3TSR treatment at the low- or mid-dose significantly (p < 0.05) reduced the number of peritoneal metastatic lesions compared to all other treatment groups (Fig. 6B). In both 3TSR- and Fc3TSR-treated animals, the mid-range dosage exhibited the most anti-tumor activity and reduced (p < 0.05) the extent of secondary disease compared to the low- and high-dose treatment in each group (Fig. 6B). Nevertheless, all dosages of 3TSR and Fc3TSR significantly (p < 0.05) reduced the number of peritoneal lesions when compared to mice treated with PBS alone (Fig. 6B). Ascites accumulation was also measured and both 3TSR and Fc3TSR significantly (p < 0.05) decreased malignant ascites accumulation compared to PBS-treated controls. Mice treated with mid-dose Fc3TSR had the greatest reduction in ascites volume among all groups studied (Fig. 6C).

3.7. Fc3TSR reduces the expression of immunosuppressive cytokines in vivo

Serum VEGF concentrations of mice treated with PBS or low-, mid- or high-doses of 3TSR or Fc3TSR were quantified by ELISA. Both agents significantly (p < 0.05) decreased systemic VEGF concentrations compared to control mice. Once again, mice treated with Fc3TSR had the greatest reduction in serum VEGF concentrations at all dose regimens compared to all other treatments (Fig. 6D). Moreover, we assessed orthotopic ID8 tumor tissues collected from mice treated with 3TSR or Fc3TSR (both at mid-dose regimen) for additional immunosuppressive cytokines. As TSP-1 has been shown to modulate the tumor microenvironment, we previously showed that 3TSR has the ability to reduce anti-tumor and vascular normalizing properties of native 3TSR. Not only does Fc3TSR increase the apoptotic response and decreases proliferation of endothelial cells in vitro, compared to native 3TSR. A. mEC cells were treated with increasing concentrations of 3TSR and Fc3TSR (0.1 nM to 1000 nM) for 24 h, and then fixed and stained for cleaved caspase-3 (magnification 100×; Scale bar – 100 μm). B. Cells were treated with increasing concentrations (0.1 nM to 1000 nM) of 3TSR and Fc3TSR for 24 h, fixed and stained for phosphorylated histone H3 (magnification 100×; Scale bar – 100 μm). C. mEC cells were treated for 24 h with 10 nM 3TSR or Fc3TSR and lysed for protein collection. Western blot analysis was performed for apoptotic and angiogenic factors. Densitometry was performed and the graph shows the amount of protein relative to β-actin controls. D. HDMEC cells were either treated with VEGF (50 ng/mL) or without VEGF in the presence of increasing concentrations of Fc3TSR. For all bar graphs, bars with different letters are statistically different from one another (p < 0.05).
clinical perspective, this enhanced stability in circulation would likely allow for weekly administrations rather than requiring daily injections. In vivo, Fc3TSR reduced ovarian tumor volume and metastatic disease to a greater extent than native 3TSR.

Interestingly, in addition to the improved efficacy in vivo with increased half-life, Fc3TSR also exhibited greater pro-apoptotic effects in vitro, compared to native 3TSR. TSP-1 binds its cell surface receptor CD36 [41,42]. Upon activation of

**Fig. 4.** Fc3TSR regulates ovarian cancer cell apoptosis and proliferation through CD36-dependent mechanisms. A. Murine ID8 ovarian cancer cells were subjected to siRNAi to knockdown expression of the CD36 receptor and were treated with 10 nM 3TSR or Fc3TSR for 24 h. Cells were fixed and stained for downstream apoptotic, proliferative, and angiogenic factors (magnification 200×; Scale bar = 20 μm). B. CD36 expression following siRNA knockdown in 3 biological replicates (magnification 200×; Scale bar = 20 μm). C. After 24 h of treatment, ID8 cells were lysed and subjected to western blot analysis for cleaved caspase-3 and phosphorylated Vascular Endothelial Growth Factor Receptor (VEGFR-2). Densitometry was performed and presented as amount of protein relative to β-actin controls. D. Expression of CD36 protein in cells treated with 100 nM of 3TSR or Fc3TSR was performed at acute timepoints to determine ICS treatment timepoint (30 min). E. After 30 min of treatment with either 3TSR (100 nM) or Fc3TSR (100 nM), ID8 cells were fixed and subject to CD36 staining. Images were obtained at 60× magnification and subject to image correlation spectroscopy (ICS) (Scale bar 20 μm). For bar graphs in A, bars with different symbols are statistically different from one another (p < 0.05). For bar graphs C-E, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**Fig. 5.** Fc3TSR enhances expression of factors downstream of CD36 in ID8 cells. A. Total protein was collected from murine ID8 ovarian cancer cells left untreated or treated with 100 nM of 3TSR or Fc3TSR for a range of acute timepoints surrounding receptor clustering events. Samples were subject to western blot analysis using antibodies against downstream factors vital for CD36 effector functions, as indicated. Expression was calculated relative to protein from untreated cells *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. B. Immunofluorescence colocalization was performed on ID8 cells treated with 100 nM of 3TSR or Fc3TSR at various timepoints using markers against i) Fyn (red) and CD36 (green) and ii) Fyn (red) and JNK 1,2,3 (green) to determine localization (magnification 600×; Scale bar - 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CD36, the Src-family kinase Fyn is activated, which leads to increased activation of caspase-3 and apoptosis [8,43]. 3TSR is able to retain this activity of TSP-1 by recruiting Fyn to CD36 [44]. Although the mechanisms of CD36 expression and protein synthesis are understudied, upregulation of CD36 phosphorylation correlates with high levels of CD36 gene transcription and protein synthesis. Other groups [45] have proposed that beyond basal levels of CD36 phosphorylation, phosphorylation of CD36 in response to a stimulus (in our case, 3TSR or Fc3TSR) only occurs on newly synthesized protein. Phosphorylation of CD36 has been shown to be blocked by inhibiting CD36 protein synthesis. This may contribute to the enhanced expression of CD36 seen following 3TSR/Fc3TSR treatment. There is evidence of enhanced downstream

**Fig. 6.** Fc3TSR inhibits ovarian tumor cell survival and modifies blood vessel density and maturity in vivo. A. Tumors were induced in syngeneic mice and allowed to progress without intervention for 60 days. At 60 days post-tumor induction, mice received PBS or 3TSR or Fc3TSR in low, medium, or high doses (refer to Table 1) until 90 days post-tumor induction. At 90 days post-tumor induction, tumors were collected and weighed (n = 6 mice/group). B. Metastatic tumors were scored (1: 1–3 tumors in the abdomen, 2: 4–10 tumors in the abdomen, 3: >10 tumors in the abdomen). C. Ascites was collected and the volume measured (n = 6 mice/group). D. Concentrations of Vascular Endothelial Growth Factor (VEGF) in serum were quantified by Enzyme Linked Immunosorbent Assay (ELISA). E. At 90 days post-tumor induction, primary tumors were collected and lysed for western blot analysis of various cytokines. For all graphs, bars with different letters are statistically different from one another (p < 0.05).
Fig. 7. Fc3TSR inhibits ovarian tumor cell survival and modifies blood vessel density and maturity in vivo. Tumors collected at 90 days post-tumor induction, following 30 days of treatment, were fixed and subjected to immunofluorescent staining for A. phosphorylated histone H3, B. cleaved caspase-3, and C. colocalization of CD31/alpha smooth muscle actin (n = 6 mice/group). For all graphs, bars with different letters are statistically different from one another (p < 0.05). Scale bars for histology images are 100 μm.
signaling of CD36 following ligand-induced compaction and aggregation of this receptor [29]. In this study, we show that the dimeric configuration of Fc3TSR enables enhanced activity in vitro compared to native 3TSR due to greater clustering of CD36. Multiple 3TSR ligands in our Fc fusion protein induced CD36 to form nanoclusters, enhancing ligand-receptor interactions and downstream signaling in ID8 cells (see schematic diagram of Fc3TSR functions, Fig. 8). We demonstrate that CD36/Fyn colocalization is evident 10 min after treatment with 3TSR or Fc3TSR. Further, the expression of pFyn is significantly enhanced after 15 min of treatment of Fc3TSR compared to untreated cells and an equimolar concentration of 3TSR. Throughout our in vitro experiments in which CD36 was knocked down, the apoptotic and proliferative effects of Fc3TSR were abrogated, confirming that the effects of the 3TSR fusion protein were at least partly mediated through this receptor. Interestingly, we found that there was a spike in CD36 expression in ID8 cells following acute treatment (15 min) with either 3TSR or Fc3TSR. Loss of function studies have shown that CD36 overexpression is vital to initiate a senescent state in a variety of mammalian cells [46]. A handful of chemotherapeutic agents have been shown to exploit cellular senescence to suppress tumorigenesis [47]. This data lends merit to investigating Fc3TSR as a potential senescence stimulus, further emphasizing its multi-modal potential.

Similar to our in vitro findings, it is likely that the greater anti-angiogenic and anti-tumor effects seen following Fc3TSR treatment in vivo were due to clustering and enhanced Fyn activation in endothelial and tumor cells, in addition to the increased efficacy associated with prolonged half-life in circulation. The increased expression of Fas ligand, thought to mediate the induction of endothelial cell apoptosis [43]. In this study we did not observe increased Fas ligand expression in a murine microvascular endothelial cell line. Since the initial studies were performed with primary endothelial cells, it is possible that the endothelial cell line used in the present study has lost this response. By contrast, the ID8 cell line did have increased expression of Fas ligand after treatment with 3TSR or Fc3TSR.

In this study, we demonstrated that 3TSR was effective at inducing tumor regression when used at 0.05 mg/kg/week, making it the first time efficacy has been shown at this low of a dose. The efficacy at this dose was striking given that previously published studies used

Fig. 8. Schematic model for the multi-modal mechanisms of 3TSR and Fc3TSR in Epithelial Ovarian Cancer. A. The Fc domain facilitates the in vitro manufacturing process, producing high yields of protein in mammalian cells. B. Fc3TSR includes contact sites for the Neonatal Fc Receptor (FcRn), a systemic receptor known to delay lysosomal degradation, allowing for enhanced protein half-life in circulation. C. The Transforming Growth Factor Beta (TGF-β) activation sequence has been shown to inhibit angiogenesis and ovarian tumor growth. D. Binding of surface-expressed CD36 has anti-angiogenic effects on endothelial cells and has been shown to induce apoptosis in ovarian cancer cells. E. The 7-mer active sequence inhibits microvascular endothelial cell motility toward Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (FGF-2). F. Sequence inhibits FGF-2-induced angiogenesis. G. Sequence inhibits FGF-2- and VEGF-induced angiogenesis. H. Obligatory dimeric configuration of two 3TSR peptides promotes nanoclustering of CD36 on the target cell membrane, leading to better apoptotic and anti-angiogenic efficacy.
milligram quantities of 3TSR on a daily basis [17]. We hypothesize that perhaps 3TSR binds to another molecule within the peritoneal cavity and is gradually released into the ascites fluid over time. A wide range of molecules, including proteoglycans and extracellular matrix proteins, have been reported to bind to the TSRs and may sequester 3TSR to mediate this depot effect [48]. A number of reports show that antiangiogenic molecules such as endostatin, inhibit angiogenesis with a U-shaped dose response [49,50]. Fc3TSR demonstrated a U-shaped dose response, with the most efficacious dose being the mid-range concentration (0.155 mg/kg), both in vitro and in vivo. Recombinant TSP-1 peptides have also been shown to elicit a U-shaped dose response in endothelial cell migration assays [51]. This biphasic effect of therapy should be considered in future studies for optimal clinical dose finding.

In vivo, treatment with 3TSR or Fc3TSR resulted in an increased proportion of mature pericyte-covered vessels. We speculate that activation of CD36 induces endothelial cell apoptosis specifically in blood vessels in which pericytes are absent. As tumor angiogenesis occurs rapidly, blood vessels are often formed in the absence of perivascular smooth muscle cells [52,53], and as such are poorly-organized with increased fenestrations and leakiness [54]. In addition to providing structural support, pericytes have direct cellular communication with endothelial cells and provide protection against apoptosis [55]. In retinal vascular disease, loss of pericyte coverage of retinal vessels facilitates TSP-1 mediated endothelial cell death, vascular destabilization and capillary loss [56]. Fc3TSR induced a higher degree of vascular normalization, possibly again due to increased CD36 clustering and Fyn activation with multiple 3TSR peptides (Fig. 8).

In our preclinical model of advanced stage ovarian cancer, Fc3TSR induced tumor regression, decreased metastatic disease and increased vascular normalization to a greater extent than native 3TSR. In addition to enhanced efficacy, Fc3TSR offers a clinically relevant intervention due to its prolonged stability in circulation and reduced administration frequency. Aside from its direct effect on ovarian cancer cells, Fc3TSR induced potent vascular normalization and as such may be an important translational tool to increase the uptake and efficacy of a host of other therapies when used in combination.

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Author contributions
KM, StK, MP, LAS, and DP were responsible for methodology, investigation, and writing. BBW and SKW were responsible for methodology, supervision, project administration, and writing. JL and JP were responsible for conceptualization funding acquisition, project administration, supervision, and writing.

Declaration of Competing Interest
J.P. and J.L. are co-inventors on patent US20140271641A1 for the treatment of ovarian cancer with 3TSR.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2021.11.006.

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