ImmunoTargets and Therapy

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

The Conjugation of Antibodies to Heat Shock Protein 70 Activates Immune Responses Against Tumor Cells

Mohammad Khosravi, Arezoo Rajaei, Babak Mohammadian, Masoudreza Seyfi Abad Shapouri

Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Khuzestan, Iran

Correspondence: Mohammad Khosravi, Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Khuzestan, Iran, Tel +989163803916, Email m.khosravi@scu.ac.ir

Purpose: The heat shock proteins (HSPs) of *Mycobacterium* have been identified as immunogenic antigens with chaperone functions. This study evaluated a method to enhance host immune responses against cancer cells by utilizing commercial antibodies conjugated with HSP70 derived from *Mycobacterium avium* subsp. *paratuberculosis*.

Methods: Recombinant HSP70, in conjugation with the monoclonal antibodies trastuzumab and bevacizumab, was employed for both in vivo and in vitro treatment of tumor cells. The cytotoxicity of the antibody-HSP70 complex was assessed on the MCF-7, LS174T, and HEK293T cell lines, after characterization of the cells for expression of the targets. The stimulation of complement activation pathways was examined using a complement fixation assay. The in vivo effects of the HSP70- antibodies were evaluated against xenograft LS174T and MCF-7 tumors in mice. The tumor tissues were evaluated through histopathological analysis.

Results: The cytotoxic effect of the antibody-HSP70 complex was significantly greater than that of other treatments against the MCF-7 and LS174T cell lines; this effect was confirmed in the presence of human plasma and leukocyte samples. Additionally, activation of the classical complement pathway has been demonstrated using HSP70 antibodies. Treatment of xenograft tumors with the antibody-HSP70 complex significantly reduced both tumor weight and growth rate, as well as decreased the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1). Histopathological analysis, along with the observed therapeutic efficacy, confirmed the safety of this approach.

Conclusion: Overall, stimulating the immune system against cancer cells by targeting innate immune responses could enhance the effectiveness of targeted therapies.

Keywords: heat shock protein 70, *Mycobacterium avium* subspecies *paratuberculosis*, cancer, antibody

Introduction

In recent decades, targeted therapies have been developed for cancer immunotherapy; however, their effectiveness, specificity, and accessibility remain limited. The targeted therapy approach faces challenges due to the presence of non-specific antigens on cancer cells. Antibodies have been employed to target tumor growth factors, ligands, and angiogenesis factors.[1] Monoclonal antibodies used in cancer treatment induce tumor cell cytotoxicity through various mechanisms. This collaboration between antibodies and the immune system is facilitated by processes such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Additionally, these antibodies can be used to deliver functional molecules, such as drugs and toxins, thereby enhancing cytotoxic effects.[2]

Trastuzumab, marketed under the trade name Herceptin, is a monoclonal antibody that specifically binds to the human epidermal growth factor receptor 2 (HER2) protein. This receptor could be found in various tissues, including breast and stomach tissue. HER2 stimulates the epidermal growth factor, promoting the growth and division of cancer cells. Additionally, it activates two primary signaling pathways that simultaneously sustain cell division and inhibit mechanisms of cell death.[3] Bevacizumab, known by the trade names Avastin and Stivant, is a recombinant monoclonal antibody

https://doi.org/10.2147/ITT.S565942
Received: 7 September 2025
Accepted: 3 March 2026
Published: 16 March 2026

that inhibits angiogenesis and have been used in cancer immunotherapy. By binding to vascular endothelial growth factor (VEGF), this antibody inactivates and impedes the angiogenesis process. Bevacizumab was employed in the treatment of colorectal, lung, kidney, and prostate cancers.[4]

HSPs of *Mycobacterium* have been identified as immunogenic antigens with chaperone functions.[5,6] Subsequent studies have confirmed the presence of these proteins in various bacteria and protozoan parasites. These proteins are highly immunogenic and stimulate innate immune responses. Heat shock proteins, which maintain stable structures across all living organisms, exhibit increased expression in response to adverse environmental conditions. HSP70, in particular, demonstrates significant immunogenic properties, showing over 50% sequence similarity between bacterial and eukaryotic organisms.[7] This protein has been characterized as a potent adjuvant that enhances both innate and adaptive immune responses.[8] Furthermore, immunization of mice with HSP70 conjugated to α-photoprotein has activated cytotoxic T cells against the associated antigen. Based on these findings, clinical trials were conducted for certain conjugate vaccines containing HSP70.[9]

This study aimed to develop a novel approach to enhance the host's immune response against targeted cancer cells. Innate immune cells and humoral components, particularly the complement system, efficiently recognize bacterial antigens. HSP70 from *Mycobacterium avium* subsp. *paratuberculosis* (MAP-HSP70) was utilized to stimulate immune responses. In addition, serum and leukocyte cytotoxicity against MCF-7, LS174T, and HEK293T cells was evaluated in the presence of MAP-HSP70 and a monoclonal antibody targeting surface markers or secretory proteins. Subsequently, the cytotoxicity of the antibody-HSP70 complex was assessed through in vivo evaluation of the xenografted tumors.

Materials and Methods

Preparation of HSP70

The recombinant protein of MAP-HSP70 was prepared in the previous research. Briefly, the HSP70 gene (AF254578) was cloned into the pET-24a plasmid. The recombinant plasmid was expressed in *E. coli* strain BL21 and purified using cobalt chelating resin and endotoxin removal kit (EtEraser, er0015).[10]

Ethics Statement

All steps of preparing rabbits hyperimmune serum and conducting animal testing on mice were carried out in accordance with the animal rights protection directive of Shahid Chamran University of Ahvaz, Ahvaz, Iran, with acceptance code EE1401.2.24.159941/scu.ac.ir. The human blood samples were collected between September 23 and November 15, 2023, and the study was approved by the Ethical Research Committee of Shahid Chamran University of Ahvaz, Ahvaz, Iran, under acceptance code EE1401.2.24.159941/scu.ac.ir and in complies with the declaration of HelsinkiThe temperature was maintained at 22°C, with a 12-hour cycle of light and darkness. Throughout the study, the animals were provided with adequate food and water.

Preparation of Rabbit Anti-HSP70 Antibody

Antibodies against the HSP70 protein of MAP were produced in two female New Zealand White rabbits, each weighing 2 ± 0.2 kg, obtained from the Razi Vaccine and Serum Research Institute, Karaj, Iran. These rabbits were hyperimmunized by injecting a mixture of antigen and Montanide adjuvant ISA763B VG (Seppic, France) in four steps with specific intervals. Briefly, 0.5 mL of HSP70 antigen at a concentration of 300 μg/mL was mixed with 0.5 mL of adjuvant and then injected into the muscle and at four points on the skin. The first injection was administered at day zero, the second injection was given 14 days later, the third injection was performed 12 days after the second injection, and the fourth injection was administered 10 days after the third injection. At the end of the immunization period, the animals were anesthetized via subcutaneous injection of a mixture containing 2% xylazine solution (10 mg/kg) and 10% ketamine solution (100 mg/kg). The hyperimmune serum samples were then collected from the animals. The purification of specific IgG from hyperimmune serum was achieved using ion exchange and affinity chromatography, as described by Khosravi et al.[11] The activity, purity and concentration of the antibodies were evaluated using enzyme-linked

immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Bradford assays, respectively.

Conjugation of HSP70 to Antibodies

The HSP70 (250 μg/mL) was mixed with 800 μL of MES buffer (100 mM, pH 6) (Sigma Aldrich, 69892) in a total volume of 1 mL. Subsequently, 100 μL of EDC (4 mg/mL) was added to the mixture and hand-shaken for 5 minutes. The mixture was then shaken at 90 rpm for 30 minutes. Following this, 100 μL of NHS (6 mg/mL) was incorporated into the sample and shaken manually for an additional 5 minutes. The samples were shaken for another 30 minutes. Next, Trastuzumab or Bevacizumab (AryoGen Pharmed, Iran) (100 μL, 1 mg/mL) was added to the mixture and shaken for 1 hour. The resulting conjugates were incubated overnight at 4°C. After incubation, Tris buffer was added to the samples as a blocking agent, achieving a final concentration of 50 mM, and the mixture was shaken for 1 hour on ice. The resulting conjugates were stored at 4°C.[12]

Purification of Antibody-HSP70

According to Berg and Fishman (2020),[13] 5 g of Sephadex G-150 (Sigma Alderich, G15050) was dissolved in double-distilled water at 4 °C for 24 hours. After this period, the Cefadex was soaked and rinsed twice with distilled water. The powder was then mixed with 100 mL of PBS and poured into a column. The column was washed three additional times with PBS, using a volume equal to that of the column. A sample containing trastuzumab conjugated to HSP, with a volume of 250 μL and a concentration of 100 μg/mL, was introduced into the column and allowed to flow through for 10 minutes. Subsequently, 30 fractions of 500 μL each were collected. The protein content of the isolated fractions was determined using the Bradford method, and related fractions were combined. The fractions containing the conjugated antibody were identified through SDS-PAGE analysis. Additionally, the conjugation of the antibodies to HSP70 was assessed using Western blotting and ELISA.

SDS-PAGE and Western Blotting Assay

SDS-PAGE was performed using an 11% resolving gel and a 4% stacking gel. Electrophoresis was conducted on samples including antibodies, HSP70-conjugated antibodies, HSP70, and a protein ladder (Sinaclone; Cat. No: SL7001). The pre-stained protein molecular weight marker contained proteins ranging from 11 to 245 kDa. The polyacrylamide gels were stained with Coomassie Blue staining solution for 20 minutes, followed by overnight destaining using a destaining solution. An unstained gel was used for Western blot analysis. Briefly, after electrophoresis, the gel was transferred to a PVDF membrane (Elabscience; Cat. No: E-BC-R266). Protein transfer was performed for 2 hours at 12 V using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was blocked by incubation in 5% skim milk solution for 2 hours. Subsequently, it was incubated with 10 mL of PBS containing rabbit anti-HSP70 antibody (10 μg/mL) for 1 hour at 37°C. The membrane was then washed three times and incubated with HRP-conjugated goat anti-rabbit antibody (ImmunoChemistry Technologies, USA) at a 1:5000 dilution for 60 minutes at room temperature. After four washing steps, the prepared substrate solution (25 mg of 4-chloro-1-naphthol (Sigma Aldrich, C8890) dissolved in 5 mL of cold methanol, 20 mL of cold PBS, and 25 μL of 30% H2O2) was added to the membrane. After 15 minutes, the membrane was washed three times.

Cell Culture

The cell lines including MCF-7 (HTB-22), LS174T (CL-188), and HEK293T (CRL-1573) were obtained from the Pasteur Institute of Iran. The cells were cultured in 50 cm^2 flasks using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, A5256701) and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively). The cells were incubated at 37°C with 5% CO2, and the medium was refreshed every two days.

Characterization of Cells

Quantification of Surface Expression Markers

The expression of HER2, VEGFR1, VEGFR2, and membrane-bound Hsp70 was quantified by flow cytometry using a MACSQuant Analyzer 9 flow cytometer (Miltenyi Biotec). Briefly, harvested and washed cells (2×10^5) were

incubated with the corresponding fluorochrome-conjugated monoclonal antibodies in flow cytometry buffer (PBS containing 2% v/v FBS) for 30 min on ice in the dark. The used antibodies were APC conjugated ErbB-2 (Her-2/CD340) Antibody, anti-human, REAfinity™ (130–124-474 Miltenyi Biotec), APC conjugated VEGFR-1 (Flt-1) Antibody, anti-human, REAfinity™ (130–120-776 Miltenyi Biotec), APC conjugated CD309 (VEGFR-2) Antibody, anti-human, REAfinity™ (130–117-984 Miltenyi Biotec), and FITC conjugated Anti-human cmHsp70.1 (multimmune GmbH). After incubation, cells were washed twice and resuspended in flow cytometry buffer containing SYTOX™ Blue (Invitrogen) to exclude dead cells. For each sample, at least 1×10^4 viable cells were acquired. The percentage of positive cells was determined by comparison with an isotype-matched antibody control, which was used to define the positive gates. The mean fluorescence intensity (MFI) fold change was calculated by dividing the MFI of positively stained cells by the MFI of the isotype control. Data were analyzed using FlowJo™ software (version 10.1).

Confocal Microscopy

The surface expression of HER2, VEGFR1, VEGFR2, and membrane-bound HSP70 was visualized by confocal microscopy. Cells (2×10^4 per well) were seeded overnight on μ-Slide 8-well chamber slides (ibidi GmbH). The following day, cells were washed twice with PBS and incubated on ice for 20 min with the respective fluorochrome-conjugated monoclonal antibodies diluted in PBS containing 2% v/v FBS. After staining, cells were washed gently with ice-cold PBS and subsequently fixed with 4% w/v paraformaldehyde (PFA) in PBS for 15 min at room temperature (RT). Nuclei were counterstained with DAPI (2 μg/mL; Thermo Fisher Scientific) for 5 min at RT in the dark. After final washing steps, cells were imaged using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems) equipped with Leica LAS X software.

Evaluation of Antibody and HSP70 Interaction with Cancer Cells

ELISA was used to determine the reactivity of the HSP70 and antibody to antigens of the tumor cell lines. Briefly, an indirect ELISA was performed by coating the wells of ELISA microtiter plates (Biofile, China) with 100 μL of whole cell lysate of MCF-7, LS174T or HEK293T at a concentration of 10 μg/mL. The antigens were diluted in a 0.1 M carbonate buffer with a pH of 9.6. After overnight incubation at 4°C, the coated plates were washed with 0.1 M PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) and blocked with 200 μL of 4% skim milk in PBS. The plate was incubated for 2 hours at 37°C and then washed with PBS-T. The prepared dilutions of the reagents, including Trastuzumab, Bevacizumab, and rabbit anti-HSP70, or HSP70 protein, in a volume of 100 μL (20 μg/mL), were added to their respective wells. The plates were incubated at 37°C for 1 hour. The rabbit anti-HSP70 antibody or the HRP-conjugated anti-human antibody was added to the wells that had been treated with HSP or HSP70-antibodies and antibodies, respectively. In the next step, the wells that were treated with rabbit anti-HSP70 antibody were incubated with HRP-conjugated anti-rabbit antibody. The plates were incubated for 1 hour, washed as described above, and then 60 μL of TMB substrate was added to each well. The plates were incubated in the dark for 10 minutes. The reaction was stopped by adding 60 μL of 2 M sulfuric acid. The optical density was measured using an spectrophotometer at 450 nm (AccuReader, Taiwan).

Preparation of the Human Serum and Leukocyte Samples

According to hypothesis of current study, the antibody-HSP70 conjugates could activate the cells and humoral components of the innate immune system adjacent to tumor cells more effectively than alone antibodies. The activation of these immune system components could lead to the destruction of tumor cells. To evaluate this hypothesis, we prepared human plasma samples. Two mL of blood were collected from six healthy adult participants, both male and female, aged 24 to 28 years, in accordance with ethical guidelines and with informed written consent for participation in the study. The plasma samples were separated and transferred into sterile microtubes. They were then stored in a freezer at −70°C for future experiments. The WBCs were separated from blood buffy coat samples. The remaining red blood cells (RBCs) were eliminated using sterilized RBC lysis buffer. The cell concentrations equal to 100,000 white blood cells per 1 mL of DMEM culture medium were prepared and used for experiments.

In-vitro Cytotoxicity Assay

When the cell density of cultured MCF-7, LS174T, and HEK293T flasks exceeds 75%, the cells were detached from the flask bottoms using trypsin. The collected cell pellet then resuspended in culture medium and centrifuged at 800 × g for 5 minutes. The resulting cell pellet was suspended in 10 mL of culture medium, and 100 µL of this cell suspension was added to each well of a microtiter plate. After 48 hours of incubation, the culture media was removed, and the wells were washed with PBS. Culture media containing varying concentrations of Bevacizumab, Trastuzumab, HSP70, Bevacizumab-HSP, and Trastuzumab-HSP (each at 10 µg/mL) were prepared. Subsequently, 100 µL of these samples were added to the designated treatment wells. Plasma samples, comprising 5% of the treatment volume, or leukocytes at a concentration of 10,000 cells/mL, were added to the designated test wells. The control wells receive culture medium containing either 5% human plasma or leukocytes at 10,000 cells/mL. Negative control wells contain cells supplemented with standard culture medium only. Four wells were allocated per treatment, and the experiment was repeated three times.

The MTT assay was performed 24 hours after the treatments were applied to the wells. To prepare for the assay, the culture media containing the treatments were removed by washing the wells twice with sterile PBS. An MTT solution (0.5 mg/mL) was prepared in culture medium. Then, 100 µL of the MTT solution was added to each well and incubated at 37°C with 5% CO2 for 3 hours. After incubation, the MTT solution was carefully aspirated from the wells. Subsequently, 100 µL of DMSO was added to each well, and the plate was incubated for an additional 30 minutes at 37°C. The optical absorbance of the wells was measured at 600 nm. Cell viability was calculated relative to the negative control wells.[11]

Complement Fixation Test

Three cell lines—LS174T, MCF-7, and HEK293T—were prepared as described. Each cell line was aliquoted into four microtubes, each containing 1×10^6 cells in 1 mL of PBS. These tubes were designated for treatment with 100 µL (20 µg/mL), of trastuzumab, bevacizumab, HSP70, antibodies conjugated with HSP70, and PBS as control samples. The treated microtubes were incubated for one hour at 37°C and washed twice with PBS before being resuspended in 1 mL of PBS. A prepared mixture of serum from six healthy human donors (100 µL) was added to each tube and incubated for one hour at 37°C. Sensitized sheep RBCs with IgG rabbit anti-RBC antibodies were then added to the tubes at a final concentration of 1%. The treated microtubes were incubated for one hour at 37°C and centrifuged at 800 × g for 4 minutes. The supernatant (100 µL) was removed from each microtube, and its optical density was measured using a spectrophotometer at 490 nm. Standard lysed RBC samples were prepared by diluting 100% lysed 1% RBCs used in the experiment.

In vivo Evaluation of the Anti-Tumor Effects of the Antibody-HSP70

Tumor Induction in Mice

Forty female immunocompressed BALB/C mice weighing 25±5 grams were underwent standard whole-body irradiation at a dose of 3 Gy (Elekta compact, X-6 MV, Elekta Solutions AB, Stockholm, Sweden) 24 hours prior to xenograft cell injection. The LS174T or MCF-7 cell line was cultured in T75 flasks, and the cells were harvested when they reached 70–80% confluence. After two washing steps of the flasks using PBS, 750 µL of 5% trypsin was added to each flask. After 3 minutes, 1.5 mL of FBS was added to each flask to neutralize the trypsin. Then, the detached cells were removed from the flask and centrifuged for 5 minutes at 800 g. The cell pellet was washed with PBS and counted using Neobar slides. Cell viability was evaluated using trypan blue staining. The mice were anesthetized with ketamine-xylazine at a concentration of 5–25 mg/mL and a dosage of 1 mL/kg of body weight. The cells were mixed with FBS-free medium, and a volume of 150 µL containing 2×10^6 MCF-7 or LS174T cells was injected subcutaneously into the dorsal flank area of twenty mice. After cell injection, the size of the tumors was measured using a digital caliper. The formula including Volume (mm3) = (A) × (B2)/2, was used for defining of the tumor sizes; where A represented the largest diameter (mm) and B represented the smallest diameter (mm) of tumors.

Treatment of Xenograft Tumors

Before conducting the main experiment, the optimal dosage and treatment schedules were determined in mice with xenograft tumors. The tumor tissues were removed from the animals during the pre-experiment phase and were evaluated by histopathological analyzed. As the tumor sizes reached 30 mm^3, a few days after cell injection, the mice were randomly divided into 4 groups (n=4). The LS174T xenografted mice were treated with bevacizumab, or bevacizumab-HSP, HSP or PBS as control groups at a dose of 250 µg/mL (100 µL), three times every other day. The MCF-7 xenograft tumor were treated with trastuzumab, or trastuzumab-HSP, or HSP or PBS as control groups at a dose of 250 µg/mL (100 µL), three times every other day. The changes in tumor size were evaluated three times in 2 weeks. After completing the treatment period, following euthanasia with high dose ketamine-xylazine, the size and weight of the tumor masses were measured in each mouse. According to ethical standards, mice bearing tumors reaching 20 mm in size or greater than 15% of their body weight were euthanized within 24 hours. The number of injected cells and the duration of the study were optimized to meet ethical criteria throughout the study period, with no animals dying before meeting the criteria for euthanasia.

Pathology Evaluation

Each group of mice was euthanized using a high-dose injection of ketamine-xylazine. Subsequently, the skin was removed and examined for the presence of tumors. The tumors were then isolated and evaluated for size and weight. The tumor samples were transferred to a 10% formalin solution. Following this, the organs within the peritoneal cavity—including the heart, lungs, stomach, liver, kidneys, and intestines—were excised and placed in a 10% formalin solution. After completing the standard procedures for preparing tissue sections were followed including the tissues cutting, embedding in paraffin, sectionning, and staining with hematoxylin and eosin (H&E). To assess the formation of collagen strands in the connective tissue, the tumor samples were stained using the trichrome staining method. The prepared pathological slides were analyzed in a blinded manner.

Immunohistochemistry for Analysis of CD31 Expression

The expression of CD31 was analyzed in prepared slides from three tumor tissue sections in each group to quantify blood vessels. The slides were immersed in boiled Tris-buffered saline (1X, Sigma, P5912) for 20 minutes. Following three washing steps with phosphate-buffered saline (PBS), a mixture of hydrogen peroxide and methanol (1:10) was applied to the slides for 10 minutes to neutralize endogenous peroxidase activity. The slides were washed again, and a 1:100 dilution of the primary antibody (ORB10314) was incubated on the tissues for 60 minutes at room temperature. After this incubation, the slides underwent three additional washing steps, and 100 µL of linker solution (Diagnostic BioSystems-PVP1000D) was added and incubated for 15 minutes. The slides were washed three more times, and 100 µL of polymer solution (Diagnostic BioSystems-PVP1000D) was applied for 30 minutes. After three washing steps, 100 µL of diaminobenzidine solution (ScyTek-ACV999) was added to the slides for 5 minutes, followed by a wash with distilled water for another 5 minutes. The slides were then immersed in hematoxylin staining solution for 10 minutes, washed, dehydrated, clarified, and coverslipped. The prepared immunohistochemistry slides were analyzed in a blinded manner. Imaging was performed using an optical microscope, and the positively stained brown cells were manually counted in 20 randomly selected microscopic fields.

Statistical Analysis

The data were compared among the groups using SPSS software version 22 and one-way ANOVA statistical tests. A $p<0.05$ value was considered statistically significant. Data are presented as mean ± standard deviations.

Results

HSP70 and Antibody Characterization

The molecular weight and reactivity of *Mycobacterium avium* subspecies *paratuberculosis* HSP70, trastuzumab, and bevacizumab conjugates were determined using SDS-PAGE and ELISA assays. Figure 1A shows HSP70 with a molecular weight of 70 kDa and the antibodies with a molecular weight of 170 kDa. The conjugation of HSP70 to the antibodies was confirmed by SDS-PAGE (Figure 1B) and Western blotting (Figure 1C). The ELISA assays

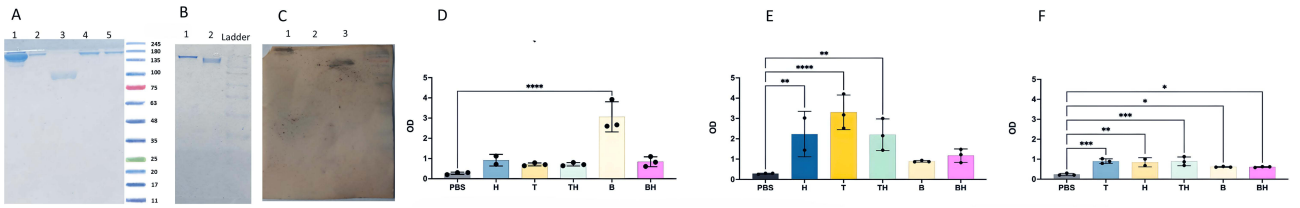

Figure 1 Reactivity of *Mycobacterium avium* subspecies *paratuberculosis* HSP70, bevacizumab, and trastuzumab with LS174T, MCF-7, and HEK293T cell lines. (**A**) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of: 1 and 2, rabbit anti-HSP70 purified by ion exchange and affinity chromatography; 3, HSP70; 4 and 5, bevacizumab and trastuzumab, respectively. (**B**) SDS-PAGE of: 1, antibody-HSP conjugates; and 2, unconjugated antibody. (**C**) Western blot assay including: 1, antibody-HSP conjugates; 2, unconjugated antibody; and 3, HSP70 for detection of HSP-antibody conjugation. (**D**–**F**) illustrate ELISA tests detecting antibody and HSP70 reactivity with the respective cell lines used: LS174T, MCF-7, and HEK293T. The reagents included PBS as a negative control (PBS), HSP70 (H), trastuzumab (T), HSP70-trastuzumab conjugate (TH), bevacizumab (B), and bevacizumab-HSP70 conjugate (BH), with wells coated by whole cell lysates from LS174T, MCF-7, and HEK293T cells. A single asterisk (*) denotes $p < 0.05$, two asterisks (**) indicate $p < 0.01$, three asterisks (***) represent $p < 0.001$ and four asterisks (****) represent $p < 0.0001$.

(Figures 1D–F) demonstrated the reaction of trastuzumab (T), bevacizumab (B), trastuzumab-HSP70 conjugate (TH), and bevacizumab-HSP70 conjugate (BH) to the coated LS174T, MCF-7, and HEK293T antigens. Additionally, the assays confirmed the interaction of HSP70 with the tumor antigen-coated wells.

Cell Characterizations

Flow cytometric analysis demonstrated robust surface expression of HER2 across all three cell lines, with LS174T cells exhibiting the strongest signal, followed by HEK293T and MCF-7. Membrane-bound Hsp70 was also detected on each cell type, though its abundance varied considerably, being most prominent on MCF-7 cells and less pronounced on HEK293T. VEGFR1 expression was detectable only at low levels in HEK293T cells, while VEGFR2 was not observed in this line. Collectively, these data highlight a heterogeneous distribution of tumor-associated antigens among the investigated cell lines. Confocal microscopy confirmed these findings, revealing plasma membrane localization of HER2, VEGFR1, VEGFR2, and mHsp70 in antigen-positive cells, with nuclear counterstaining by DAPI providing spatial context (Figure 2).

The Effect of HSP70-Antibodies on Cell Viability

According to Figure 3A, the LS174T, HEK293T, and MCF-7 cells treated with Trastuzumab-HSP70 plus serum (THS) exhibited significantly lower viability compared to the other groups. Additionally, the LS174T cells treated with Bevacizumab-HSP70 plus serum (BHS) also demonstrated significantly lower viability than the other groups. The toxicity of the antibody-HSP70 complexes on the normal HEK293T cell line was evaluated; unlike the significant cytotoxic effects observed with Trastuzumab-HSP70 plus serum, the Bevacizumab-HSP70 plus serum (BHS) exhibited lower toxicity against HEK293T cells compared to the other treatments. Furthermore, the LS174T cells treated with antibody-HSP70 and leukocytes (BHL and THL) showed significantly lower viability compared to the other groups (Figure 3A). The conjugation of HSP70 with Trastuzumab or Bevacizumab did not enhance the cytotoxicity of the antibodies against MCF-7 and HEK293T cells; however, Trastuzumab combined with leukocytes exhibited the greatest cytotoxic effects against these cells.

Complement Fixation Test (CFT)

The CFT test demonstrated significantly increased activation of the complement system against sensitized LS174T, MCF-7, and HEK293T cell lines when treated with antibody-HSP70 compared to alone antibodies. Additionally, HSP70-treated cells significantly utilized complement more effectively than those subjected to other treatments. Furthermore, trastuzumab mediated significantly greater complement activation than bevacizumab against MCF-7 and HEK293T cell lines, in contrast to the insignificant difference observed with the LS174T cell line (Figure 3B).

https://doi.org/10.2147/ITT.S565942

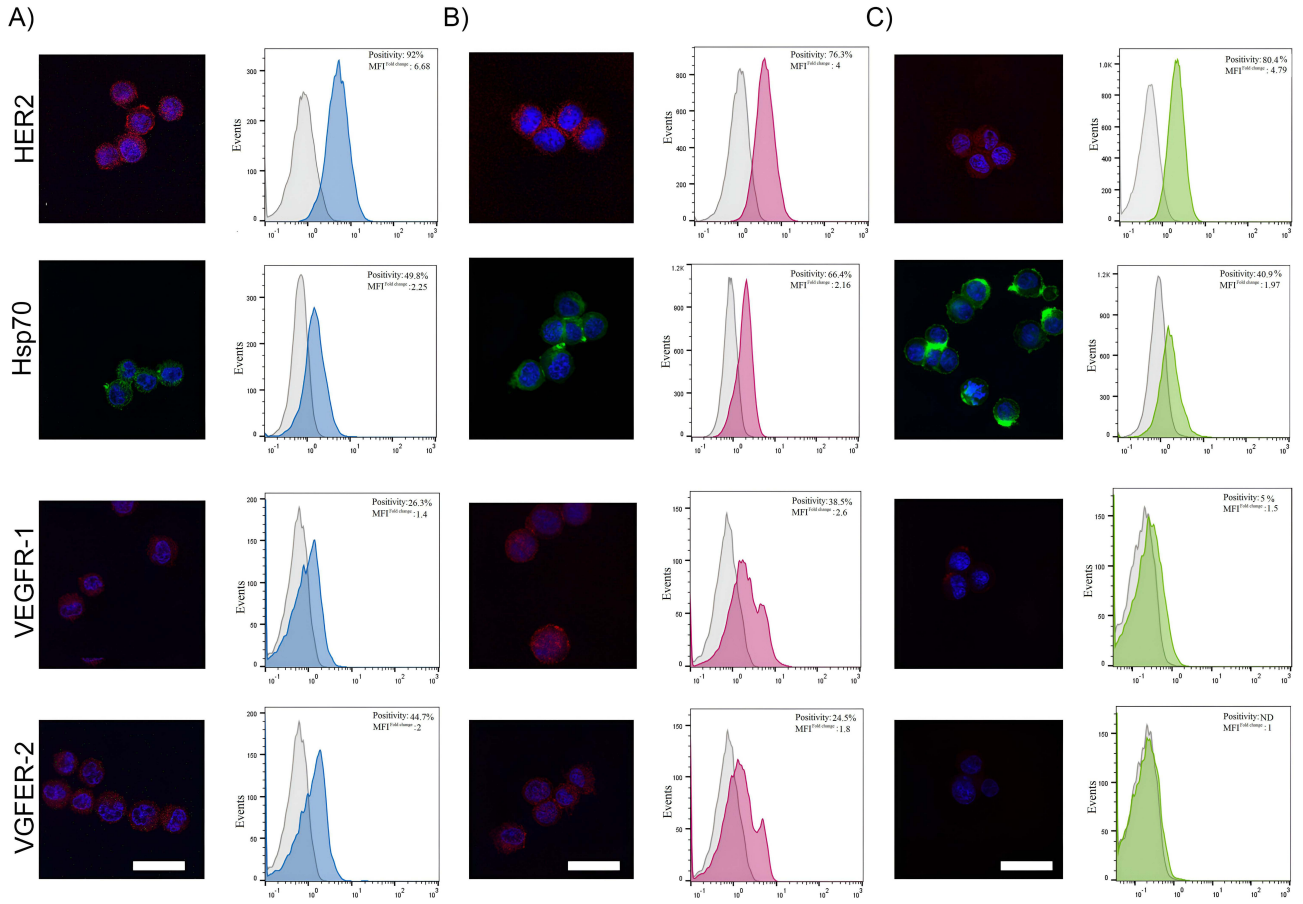

Figure 2 The surface expression of HER2, HSP70, and VEGFR1/2 on (**A**) LS174T, (**B**) MCF-7, and (**C**) HEK293T cell lines. The evaluated cell lines were positive for HER2 and HSP70 expression, whereas the HEK293T cell line showed negative expression of VEGFR2 and minimal expression of VEGFR1 (scale bar = 25 μm).

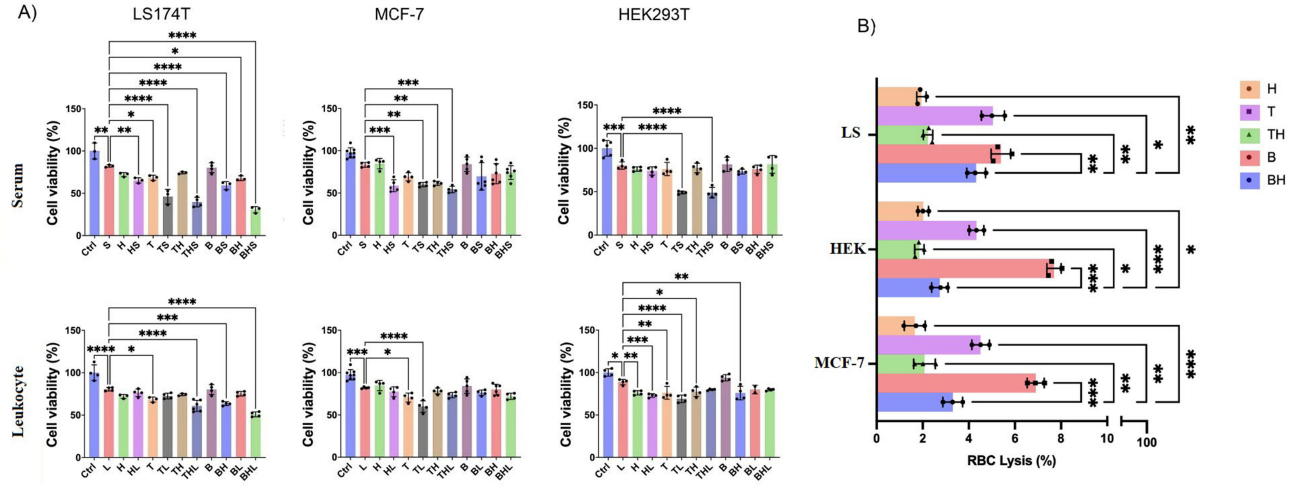

Figure 3 The cytotoxic effect of antibody-HSP70 plus serum or leukocyte against MCF-7, LS174T and HEK293T cell lines. (**A**) The cell viability results after treatment indicate the effectiveness of BH treatment in activating serum cytotoxicity against LS174T cells. The TH treatment also trigger serum cytotoxicity against three cell lines. Additionally, activation of leukocyte cytotoxicity was observed following TH and BH treatments of LS174T cells. (**B**) The complement fixation test demonstrated the effectiveness of TH and H in activating the complement system in the presence of all three cell lines. Furthermore, BH results show a significant difference compared to B in activating complement system. Trastuzumab (T), Serum (S), Bevacizumab (B), HSP70 (H), Trastuzumab (T), Leukocyte (L), the culture medium negative control (C). A single asterisk (*) denotes a significance level of less than 0.05, two asterisks (**) indicate a significance level of less than 0.01, and three asterisks (***) represent a significance level of less than 0.001 and and four asterisks (****) represent $p < 0.0001$.

https://doi.org/10.2147/ITT.S565942

The in vivo Results

Anti-Tumor Effects of the Antibody-HSP70

Four days after xenograft tumor induction with LS174T cells, the cancer-bearing mice were treated three times with HSP70, Bevacizumab (B), or Bevacizumab-HSP70 (BH) at a dose of 25 µg per mouse every other day (Figure 4A). As shown in Figures 4B and C, the tumor sizes in the B and BH groups on days 10, 14, and 21 after tumor induction were significantly smaller than those in the control group. Tumors were excised 21 days after induction, and tumor weights were measured in milligrams (mg). As illustrated in Figure 4D, the tumor weight in the Bevacizumab-HSP70 group, 14 days after the last treatment, was significantly lower than in the other groups. Similarly, xenografted MCF-7 tumor-

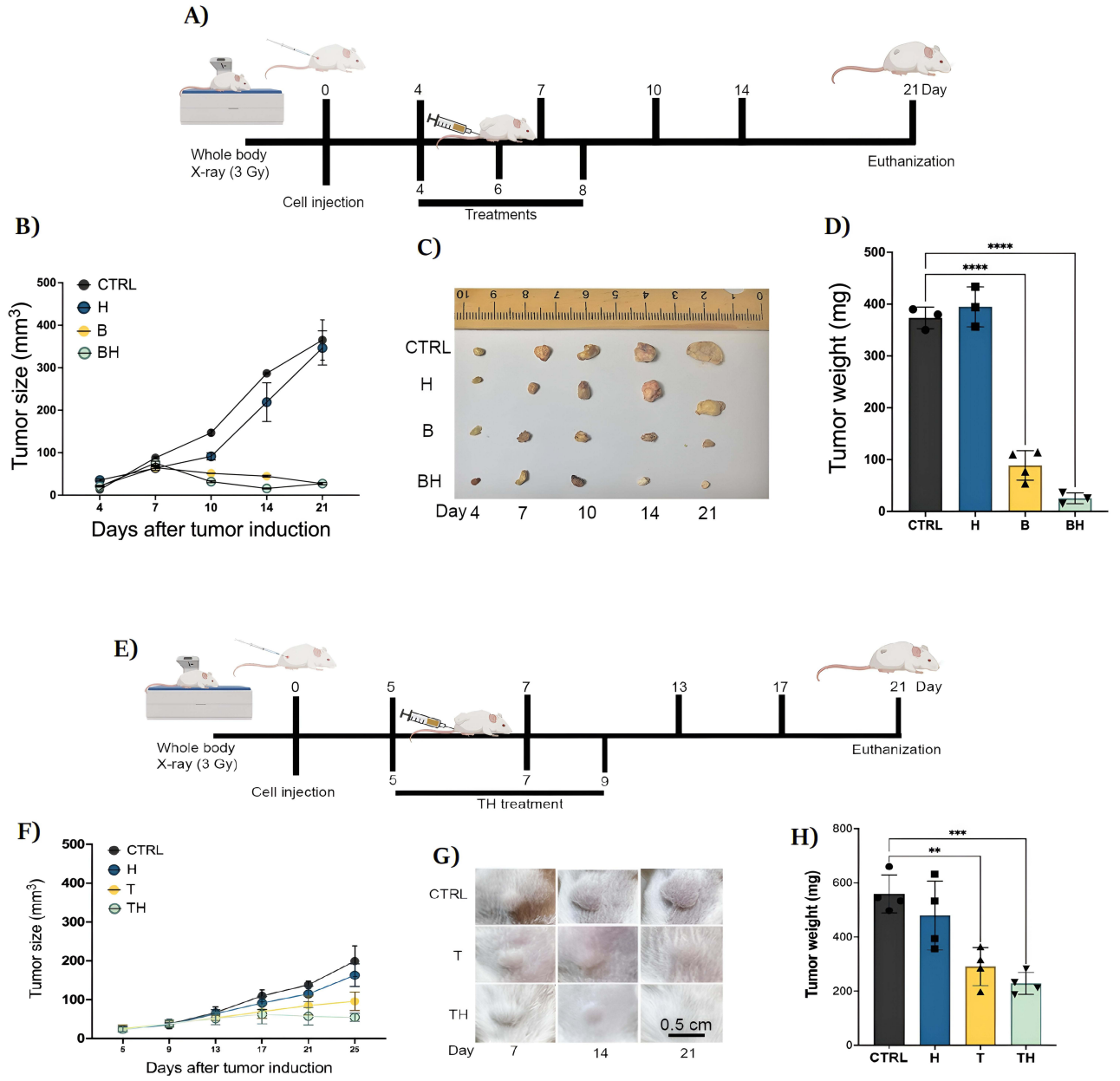

Figure 4 In vivo evaluation of the effects of antibody-HSP70 conjugates on the growth rate of xenograft tumors. (**A**) Four days after tumor induction with the LS174T cell line, cancerous mice were treated with three doses of Bevacizumab (B), Bevacizumab-HSP70 (BH), or HSP70 (H). (**B** and **C**) Size of LS174T tumors in each group is expressed as the mean ± standard error (mm^3). (**D**) Final LS174T tumor weight of the treated cancerous mice. (**E**) Five days after tumor induction with the MCF-7 cell line, cancerous mice were treated with Bevacizumab (B), Trastuzumab-HSP70 (BH), or HSP70 (H). (**F** and **G**) Size of MCF-7 tumors in each group is expressed as the mean ± standard error (mm^3). (**H**) Final MCF-7 tumor weight of the treated cancerous mice. Two asterisks (**) indicate $p < 0.01$, three asterisks (***) indicate $p < 0.001$, and four asterisks (****) indicate $p < 0.0001$.

https://doi.org/10.2147/ITT.S565942

bearing mice were treated three times with HSP70, Trastuzumab (B), or Trastuzumab-HSP70 (TH) at a dose of 25 µg per mouse, starting five days after tumor inoculation and administered every other day (Figure 4E). As shown in Figures 4F and G, tumor sizes in the B and TH groups on days 17, 21, and 25 after tumor induction were significantly smaller than those in the control group. Tumors were excised 25 days after induction, and tumor weights were recorded in milligrams (mg). As shown in Figure 4H, the tumor weight in the Trastuzumab-HSP70 group, 14 days after the last treatment, was significantly lower than in the other groups.

Pathological Evaluation of the Xenograft Tumors

On macroscopic examination, the organs of the cancerous mice including the intestine, stomach, lungs, kidneys, and liver appeared normal in color and size. Microscopic examination of the selected tissues revealed no tumor-related lesions in any of the samples. Furthermore, all prepared tissue sections appeared normal and free of lesions (Supplementary Figure 1). Tumor samples were collected on days 7, 10, and 14 following induction, and their microscopic characteristics were evaluated across all groups. The proliferation and mitotic figures were observed 7 days after tumor induction (Supplementary Figure 2). The presence of liquefactive necrosis may result from immune cell activation, while mitotic figures arise from the division of tumor cell nuclei. The varying sizes and shapes of tumor cells indicate their degree of malignancy. Liquefactive necrosis develops due to immune cell activity within 14 days following tumor induction (Figure 5A and B). A thin scaffold of connective tissue forms around the tumor tissue 7 days post-induction, accompanied by mitotic figures resulting from cell division. Additionally, a thick layer of connective tissue forms around the tumor 7 days after treatment with Bevacizumab (B), reflecting the immune response to the xenograft tumor. Fourteen days after treatment with Bevacizumab, liquefactive necrosis appears due to neutrophil activity in the tumor center and surrounding connective tissue.

The formation of collagen strands in the connective tissue surrounding the tumor was evaluated across different groups using the trichrome staining method. As shown in Supplementary Figures 2 and 3, the tumor connective tissue in the control group appeared purple. Additionally, the turquoise-colored scaffold was surrounded by the tumor within 7 days post-treatment and was located at the center of the necrotic tissue. Fourteen days after treatment with BH, caseous necrosis developed at the center, with connective tissue and collagen fibers forming around it (Supplementary Figure 3). Immunohistochemistry demonstrated the significant effects of HSP70 antibodies in reducing tumor blood vessels compared to control antibodies (Figures 5C–F). Furthermore, trastuzumab and bevacizumab significantly reduced blood vessel density compared to the control groups. However, HSP70 did not exhibit any significant effect on the reduction of CD31 expression (Supplementary Figure 4).

Discussion

Evading the host immune response is the initial step in cancer development. Tumor cells employ several mechanisms to escape surveillance by both innate and adaptive immunity. These include the secretion of immunosuppressive agents such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10), and prostaglandin E2 (PGE2); the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4); and the production of anti-apoptotic factors as Bcl-2 family or immune cell apoptotic factors like programmed death-ligand 1 (PD-L1).[14] These well-known strategies promote cancer progression. Pharmacological agents that can alter this tolerogenic tumor microenvironment, leading to metabolic reprogramming and pro-tumorigenic effects, could result in beneficial health outcomes for cancer patients.[15]

Previous research demonstrated the activation of immune responses against the MCF-7 cell line using antibodies conjugated to bacterial antigens.[11] The current study has developed a novel approach to stimulate the innate immune system within tumor tissue by utilizing the HSP70 protein from MAP. Cells of the innate immune system recognize bacterial antigens through pattern-recognition receptors. Additionally, humoral components of the innate immune system, such as the complement system, mannose-binding lectins, and C-reactive protein, can detect bacterial antigens. Natural antibodies also recognize bacterial antigens and may activate innate immune responses on the surface of tumor cells.

This study evaluated the cytotoxic effects of combination therapy involving HSP70 and the anticancer monoclonal antibodies Trastuzumab or Bevacizumab on the MCF-7, LS174T, and HEK293T cell lines. In vitro assays indicated that active serum destroys tumor cells more effectively when the antibodies were conjugated to HSP70. The role of HSP70 in

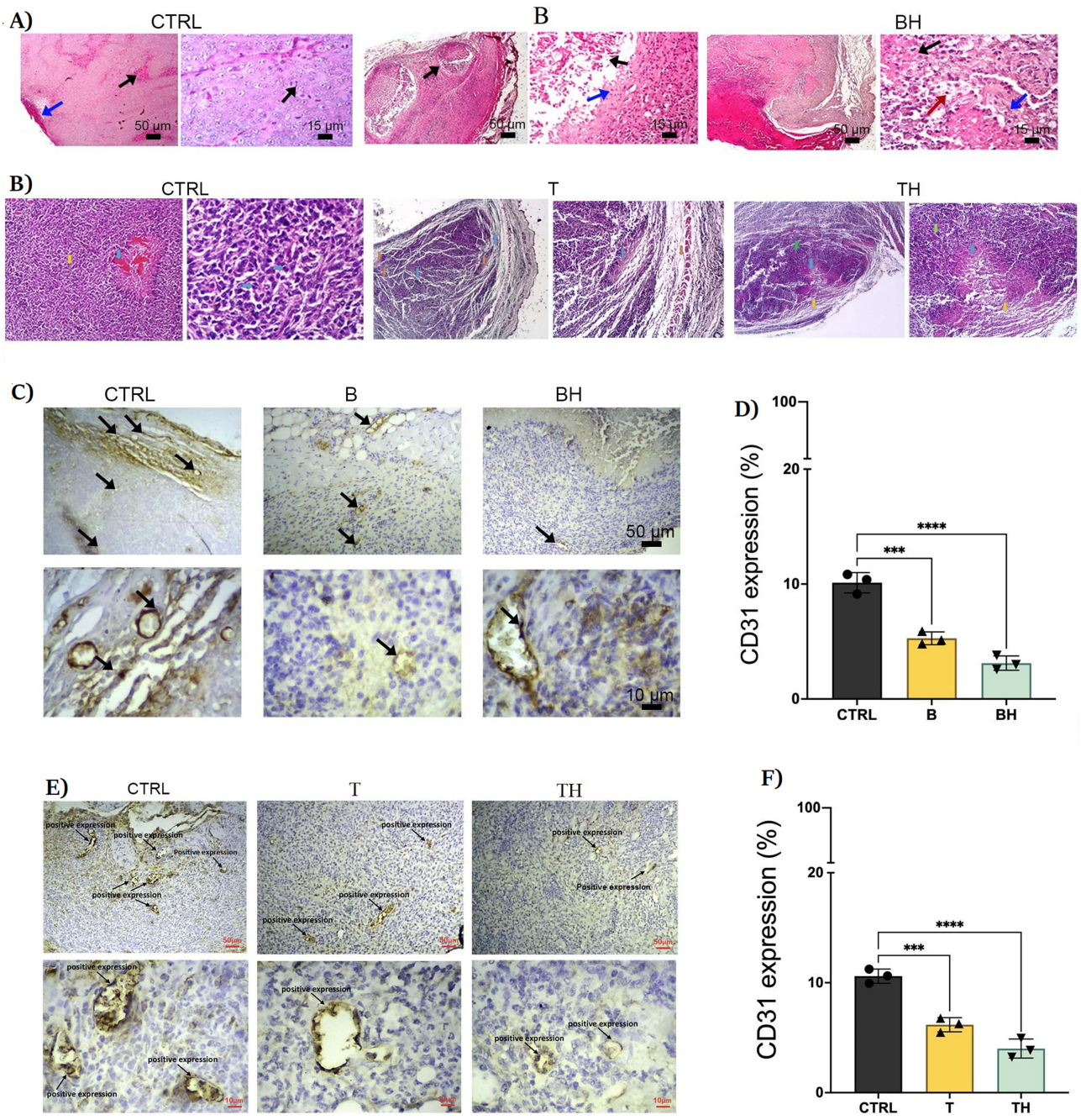

Figure 5 Pathological and immunohistochemical evaluation of the effects of antibody-HSP70 conjugates on xenograft tumors. (**A**) Histopathological analysis of LS174T xenograft tumors 14 days after tumor induction. In the left image of the control group, coagulative necrotic areas (black arrow) and liquefactive necrosis accompanied by hemorrhagic areas (blue arrow) are visible. The right image shows tumor cells of various shapes and sizes, along with mitotic figures (black arrow). In the bevacizumab group, the left image depicts liquefactive necrosis (black arrow), while the right image shows neutrophils (black arrow) within liquefactive necrosis (blue arrow). In the bevacizumab-HSP70 group, the left image reveals necrotic tumor cells in the center, surrounded by a thick layer of connective tissue. The right image shows fibroblasts (blue arrow) and lymphocytes (red arrow) around the area of liquefactive necrosis (black arrow). (**B**) Histopathological analysis of MCF-7 xenograft tumors was conducted 14 days after tumor induction. The control image shows tumor cells (yellow arrows) surrounding a central necrotic area (blue arrows) (left) and mitotic tumor cells (blue arrows) (right). The trastuzumab group displayed replacement of tumor cells with connective tissue (blue arrows) and a necrotic area (Orange arrows) in the left image; the necrotic area (blue arrows) and destruction of tumor tissue (orange arrows) are shown in the right image. The trastuzumab-HSP70 treated group exhibited tumor tissues with necrotic cells located at the margins (yellow arrows) and in the center (blue arrows). Recruitment of inflammatory cells is indicated by green arrows (left and right images). (**C**) Immunohistochemical analysis of CD31 expression in LS174T tumor samples was performed 14 days after treatment; the black arrows indicate differences in the expression of the CD31 marker. The significant effects of HSP70-antibody conjugates on reducing LS174T tumor blood vessels, compared to bevacizumab alone, are evident. (**D**) Comparison of CD31 expression 14 days after LS174T tumor induction among treated mice. (**E**) Immunohistochemical analysis of CD31 expression (black arrows) in MCF-7 tumor samples was conducted 14 days after treatment. (**F**) Comparison of CD31 expression 14 days following MCF-7 tumor induction among treated cancerous mice. Three asterisks (***) indicate $p < 0.001$, and four asterisks (****) indicate $p < 0.0001$.

https://doi.org/10.2147/ITT.S565942

activating immune responses against tumors was well established. These proteins serve as targets for the adaptive immune system and presented by antigen-presenting cells.[16] Anticancer antibodies typically enhance cancer treatment by activating both ADCC and CDC.[17] This study confirmed the cytotoxic effects of antibodies conjugated with HSP70 against cancer cells through activation of the classical complement pathway. Additionally, HSP70 demonstrated strong activation of the complement system through antibody-independent pathways; these findings are consistent with those of Prohaszka et al.[18] Also, Seguin-Devaux also describes a method for complement activation against HER-2–expressing tumor cells using a subunit of C4b-binding protein. The results of the mentioned study, in agreement, reveal the effectiveness of therapeutics for complement activation within the tumor microenvironment. It is important to note that, in addition to complement activation, as demonstrated by in vitro results, current method has the potential to directly activate leukocytes independently of the humoral components of the innate immune response.[19] Recognition of MAP-HSP70 by receptors such as Toll-like receptor 2 (TLR-2) can initiate pro-inflammatory pathways, resulting in activation of innate immune cells.[20] Shevtsov et al investigated the effects of conjugating HSP70 to iron oxide nanoparticles on glioma cancer in vitro and in vivo. They observed increased activity of cytotoxic T lymphocytes in the presence of dendritic cells, along with nanoparticles conjugated to HSP70 that specifically target cancer cells. Moreover, mice treated with this conjugate showed higher serum IFN-γ levels and a lower tumor growth rate compared to other groups.[21] Various studies have shown that the expression of HSP proteins was elevated in all types of cancers.[22] These proteins expressed during various stresses and significantly enhance cell survival under diverse conditions. More evidence indicates that HSP proteins actively interfere with the proliferation, differentiation, metastasis, and death of tumor cells.[23] The level of HSP70 expression is directly correlated with the progression of breast, bladder, and colon cancers; consequently, it is utilized as a target in cancer therapy.[24] In current study, we successfully confirmed the effects of immune system stimulation against tumor cells and tumor tissue destruction by conjugation of MAP-HSP70 and antibodies. Yuan et al,[25] developed a fusion protein for immunotherapy of ovarian cancer and mesothelioma, utilizing a single-chain antibody variable fragment (scFv) targeting mesothelin and *Mycobacterium tuberculosis* heat shock protein 70. They demonstrated stimulation of antigen-presenting cells and activation of cytotoxic T lymphocytes against tumors. Additionally, MacAry et al,[26] confirmed activation of cytotoxic T lymphocytes by human dendritic cells presenting mycobacterial HSP70. However, the current study employed the full antibody structure to activate the ADCC and CDC pathways.

The activation of immune cells against tumors using HSP subunits has been previously established.[21,27,28] Stangl et al,[29] utilized three injections of a monoclonal antibody targeting the TKD epitope on the surface of tumor cells. This approach effectively inhibited the growth of induced CT26 tumors and prolonged the survival of tumor-bearing mice. The study also revealed elevated levels of NK cells, macrophages, and granulocytes within the tumor tissue. The demonstrated mechanism was the induction of ADCC. Similarly, Lee et al,[30] confirmed the activation of human monocytes through the NF-κB and AP-1 signaling pathways by secretory extracellular HSP70. In contrast, some evidence suggests anti-inflammatory effects of secretory microbial HSP70 through activation of autophagy and regulatory T cells.[31] In the current study, we used MAP-HSP70 as a foreign immunogenic protein to activate innate immune responses. According to the results, the sensitivity of the evaluated cells to bevacizumab correlates with expression of the HER2 on the cell surface. LS174T cells showed greater sensitivity to both trastuzumab and bevacizumab conjugated to MAP-HSP70 in perecnce of serum and leukocyte compared to other cell lines. These findings support the application of trastuzumab antibody in the treatment of HER-2 positive colorectal cancers. Additionally, bevacizumab-HSP70 conjugates did not exhibit increased cytotoxic effects against MCF-7 and HEK293T cell lines, in contrast to the significant enhancement of cytotoxicity observed in LS174T cells. LS174T cells also demonstrated higher sensitivity to human leukocytes and serum than the MCF-7 cell line; these factors may contribute to the increased sensitivity of LS174T cells to BHS and BHL treatments. Beyond antibody binding to their targets on the cell surface, the expression of surface HSP70, which may be attributed to dimerization with MAP-HSP that was shown in ELISA results, further sensitizes cancer cells to HSP70-conjugated antibodies. Using other inducers of inflammatory responses, such as lipopolysaccharide, leads to failure in this targeting mechanism of HSP dimerization. In addition, HSPs and their substitutes are well known for potent activation of T cell immune responses, which are absent in most other immunogenic agents.[8,9]

The bacterial cell wall antigen of *Bacillus Calmette-Guérin* is a well-known agent used as an adjuvant in cancer immunotherapy.[32] Systemic injection of *Clostridium novyi* spores induces the production of inflammatory cytokines. It also promotes the circulation of inflammatory cells, including neutrophils, monocytes, and lymphocytes, which mount a response against cancer.[33] Furthermore, these inflammatory reactions trigger a cellular immune response that generates reactive oxygen species and proteolytic enzymes.[34] The present study, consistent with related research, confirms that bacterial antigens can be employed in a targeted manner as novel agents for cancer immunotherapy.

Current research, in agreement with the study by Milazzo et al,[35] has demonstrated the effectiveness of the HER2-specific antibody trastuzumab against LS174T and HEK293T cells. Additionally, Vadlamudi et al,[36] confirmed the expression of HER2 and its dimerization with HER3 in the LS174T cell line. They found that the expression of HER2 and its dimer form was influenced by treatment with an antibody reactive to HER3. Furthermore, treatment of LS174T cells with the probiotic *Bifidobacteria* resulted in a 6.7-fold reduction in HER2 expression.[37] The effects of the HER2-reactive antibody on LS174T cells have also been reported by Lin et al.[38] These studies are consistent with the expression of HER2 on the surface of the LS174T and HEK293T cell lines.

Based on the infiltration of immune cells including neutrophils, macrophages, and lymphocytes into the tumor microenvironment, necrosis of tumor tissues, particularly peripheral tumor necrosis, and the formation of connective tissue and collagen strands, pathological analysis confirms a reduction in the final size and weight of tumor cells. Given the presence of bacterial HSP70, activated complement proteins are expected to induce chemotaxis of neutrophils and monocytes. Activation of NK cells has previously been demonstrated using the TKD peptide derived from human HSP70 molecules[29] (which share 77% identity with MAP-HSP70), suggesting infiltration and activation of these immune cells. The foreign and immunogenic bacterial HSP70 is likely presented to T cells by antigen-presenting cells (APCs). Additionally, macrophages and B cells may be activated against MAP-HSP70 as a result of complement activation on the surface of tumor cells. The similarity between bacterial and human HSP70 (70% identity in the coding sequence of mRNA) may further contribute to these effects through molecular mimicry. Based on these observations, it is predicted that multifaceted pathways lead to tumor necrosis following treatment with antibody-HSP70 conjugates. Analyzing these pathways requires extensive and rigorous testing to elucidate the precise involved mechanisms. Therefore, the current method, which implies inspired anti-tumor effects, should be further investigated to clarify the actual mechanistic pathways. It is important to consider the off-target effects of antibody-based targeted therapy, as prolonged use beyond the duration of the current study may cause increased unwanted inflammation in healthy organs. Additionally, applying this method to healthy individuals, rather than the immunocompromised mice used in the current study, could theoretically result in more potent anti-tumor effects. However, membrane HSP70, a tumor-associated antigen not expressed on normal cells,[39] may still cause side effects related to excessive inflammation, necessitating optimization of the antibody-HSP70 dosage according to the immune response status.

Conclusion

According to the results, conjugating antibodies with HSP70 from *Mycobacterium avium* subspecies *paratuberculosis* effectively stimulates innate immune responses against MCF-7 and LS174T cancer cells. Tumor weight and size were significantly reduced in the group treated with the antibody-HSP70 conjugate compared to both the antibody-only group and the control group. Additionally, histopathological analysis revealed decreased CD31 expression in tumors treated with this conjugate therapy, along with a higher level of necrosis in tumor cells in the antibody-HSP70 group. Necrosis progressed more rapidly in this group than in the antibody-only group. Overall, stimulating the immune system against cancer cells by targeting innate immune responses could enhance the efficacy of targeted therapies.

Data Sharing Statement

All analyzed and raw data are available without restriction from the corresponding author upon request.

Consent to Participation and Publish

The human blood samples were collected with informed and written consent for participation in the study from six healthy adult participants, both male and female, aged 24 to 28 years. The samples were obtained under sterile

conditions, as approved by the ethical research committee of Shahid Chamran University of Ahvaz, with acceptance code EE1401.2.24.159941/scu.ac.ir.

Acknowledgment

The authors express their gratitude to AryoGen Pharmed in Iran for preparing the monoclonal antibodies and to the human participants, particularly Dr. Mohammad Vali Ch. Mohammad Pour, for their collaboration in providing human plasma samples.

Funding

This study was financially supported by Shahid Chamran University of Ahvaz, Ahvaz, Iran, under grant number SCU. VP1403.12470.

Disclosure

The authors declare that have no conflicts of interest in this work.

References

1. Slomovitz BM, Chelariu-Raicu A, Schmeler KM, et al. Phase 2 study of cetuximab (Erbitux) in patients with progressive or recurrent endometrial cancer. *Int J Gynecol Cancer*. 2020;30(11):1733–1737. doi:10.1136/ijgc-2020-001859
2. Singh S, Tank NK, Dwiwedi P, et al. Monoclonal antibodies: a review. *Curr Clin Pharmacol*. 2018;13(2):85–99. doi:10.2174/1574884712666170809124728
3. De P, Hasmann M, Leyland-Jones B. Molecular determinants of trastuzumab efficacy: what is their clinical relevance? *Cancer Treat Rev*. 2013;39(8):925–934. doi:10.1016/j.ctrv.2013.02.006
4. Foss A, Haydock R, Childs M, et al. TANDEM TRIAL: a factorial randomised controlled trial of dose and review schedule of bevacizumab (Avastin) for neovascular macular degeneration in the East Midlands. *BMJ Open Ophthalmol*. 2020;5(1):e000588. doi:10.1136/bmjophth-2020-000588
5. Dubaniewicz A. Immunogenic Mycobacterium tuberculosis heat shock protein in tuberculosis. *Pol Merkur Lekarski*. 2000;8(47):353–355. PMID: 10944961.
6. Wendling U, Paul L, van der Zee R, Prakken B, Singh M, van Eden W. A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. *J Immunol*. 2000;164(5):2711–2717. doi:10.4049/jimmunol.164.5.2711
7. Motta A, Schmitz C, Rodrigues L, et al. *Mycobacterium tuberculosis* heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation in vitro. *Immunology*. 2007;121(4):462–472. doi:10.1111/j.1365-2567.2007.02564.x
8. Harmala LA, Ingulli EG, Curtsinger JM, et al. The adjuvant effects of *Mycobacterium tuberculosis* heat shock protein 70 result from the rapid and prolonged activation of antigen-specific CD8+ T cells in vivo. *J Immunol*. 2002;169(10):5622–5629. doi:10.4049/jimmunol.169.10.5622
9. Wang XP, Wang QX, Lin HP, Xu B, Zhao Q, Chen K. Recombinant heat shock protein 70 functional peptide and alpha-fetoprotein epitope peptide vaccine elicits specific anti-tumor immunity. *Oncotarget*. 2016;7(44). doi:10.18632/oncotarget.12464
10. SheiniMehrabzade R, Siefi Abad Shapori M, Ghorbanpoor M, Gharibi D. Cloning of Mycobacterium aviumparatuberculosis hsp70 gene into pET-24a plasmid and its expression in Escherichia coli. *Vet Clin Pathol*. 2022;16(63):217–228. doi:10.30495/jvcp.2022.1954420.1362
11. Khosravi M, Khazaeil K, KhademiMoghadam F. Triggering of the immune response to MCF7 cell line using conjugated antibody with bacterial antigens: in-vitro and in-vivo study. *PLoS One*. 2022;17(10):e0275776. doi:10.1371/journal.pone.0275776
12. Scaria PV, Chen B, Rowe CG, et al. Protein-protein conjugate nanoparticles for malaria antigen delivery and enhanced immunogenicity. *PLoS One*. 2017;12(12):e0190312. doi:10.1371/journal.pone.0190312
13. Berg EA, Fishman JB. Purification of labeled antibodies using size-exclusion chromatography. *Cold Spring Harb Protoc*. 2020;10. doi:10.1101/pdb.prot099358
14. Germana E, Pepe L, Pizzimenti C, et al. Programmed cell death ligand 1 (PD-L1) immunohistochemical expression in advanced urothelial bladder carcinoma: an updated review with clinical and pathological implications. *Int J Mol Sci*. 2024;25(12):6750. doi:10.3390/ijms25126750
15. Strati A, Adamopoulos C, Kotsantis I, Psyrri A, Lianidou E, Papavassiliou AG. Targeting the PD-1/PD-L1 signaling pathway for cancer therapy: focus on biomarkers. *Int J Mol Sci*. 2025;26(3):1235. doi:10.3390/ijms26031235
16. Tamura Y, Torigoe T, Kukita K, et al. Heat-shock proteins as endogenous ligands building a bridge between innate and adaptive immunity. *Immunotherapy*. 2012;4(8):841–852. doi:10.2217/imt.12.75
17. Lundin J, Kimby E, Björkholm M, et al. Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). *Blood*. 2002;100(3):768–773. doi:10.1182/blood-2002-01-0159
18. Prohaszka Z, Singh M, Nagy K, et al. Heat shock protein 70 is a potent activator of the human complement system. *Cell Stress Chaperones*. 2002;7(1):17. doi:10.1379/1466-1268(2002)007
19. Seguin-Devaux C, Brandus B, Plesseria JM, et al. Directed-complement activation as a novel immunotherapeutic approach for HER2-breast cancer. *ImmunoTargets Ther*. 2025;Volume 14:979–995. doi:10.2147/ITT.S517584
20. Bulut Y, Michelsen KS, Hayrapetian L, et al. *Mycobacterium tuberculosis* heat shock proteins use diverse Toll-like receptor pathways to activate pro-inflammatory signals. *J Biol Chem*. 2005;280(22):20961–20967. doi:10.1074/jbc.M411379200

21. Shevtsov M, Stangl S, Nikolaev B, et al. Granzyme B functionalized nanoparticles targeting membrane Hsp70-positive tumors for multimodal cancer theranostics. *Small*. 2019;15(13):1900205. doi:10.1002/smll.201900205
22. Yi K, Sun C, Yuan Y, Luo Z, Luo H, Xie Y. A new weapon: the application of tumor vaccines based on extracellular exosomal heat shock proteins in immunotherapy. *Front Immunol*. 2025;16:1510650. doi:10.3389/fimmu.2025.1510650
23. Breuninger S, Stangl S, Werner C, et al. Membrane Hsp70—a novel target for the isolation of circulating tumor cells after epithelial-to-mesenchymal transition. *Front Oncol*. 2018;8:497. doi:10.3389/fonc.2018.00497
24. Tao Y, Messer JS, Goss KH, Hart J, Bissonnette M, Chang EB. Hsp70 exerts oncogenic activity in the Apc mutant Min mouse model. *Carcinogenesis*. 2016;37(7):731–739. doi:10.1093/carcin/bgw056
25. Yuan J, Kashiwagi S, Reeves P, et al. A novel *Mycobacterial* Hsp70-containing fusion protein targeting mesothelin augments antitumor immunity and prolongs survival in murine models of ovarian cancer and mesothelioma. *J hematol oncol*. 2014;7:1–4. doi:10.1186/1756-8722-7-15
26. MacAry PA, Javid B, Floto RA, et al. HSP70 peptide binding mutants separate antigen delivery from dendritic cell stimulation. *Immunity*. 2004;20(1):95–106. doi:10.1016/s1074-7613(03)00357-1
27. Multhoff G, Pfister K, Gehrmann M, et al. A 14-mer Hsp70 peptide stimulates natural killer (NK) cell activity. *Cell Stress Chaperones*. 2001;6(4):337. doi:10.1379/1466-1268(2001)006<337:AMHPSN>2.0.CO;2
28. Bashiri Dezfouli A, Yazdi M, Benmebarek MR, et al. CAR T cells targeting membrane-bound Hsp70 on tumor cells mimic Hsp70-primed NK cells. *Front Immunol*. 2022;13. doi:10.3389/fimmu.2022.883694
29. Stangl S, Gehrmann M, Riegger J, et al. Targeting membrane heat-shock protein 70 (Hsp70) on tumors by cmHsp70. 1 antibody. *Proc Natl Acad Sci U S A*. 2011;108(2):733–738. doi:10.1073/pnas.1016065108
30. Lee KJ, Kim YM, Kim DY, et al. Release of heat shock protein 70 (Hsp70) and the effects of extracellular Hsp70 on matric metalloproteinase-9 expression in human monocytic U937 cells. *Exp Mol Med*. 2006;38(4):364–374. doi:10.1038/emm.2006.43
31. Borges TJ, Wieten L, van Herwijnen MJ, et al. The anti-inflammatory mechanisms of Hsp70. *Front Immunol*. 2012;3:95. doi:10.3389/fimmu.2012.00095
32. Yuk JM, Shin DM, Song KS, et al. Bacillus calmette-guerin cell wall cytoskeleton enhances colon cancer radiosensitivity through autophagy. *Autophagy*. 2010;6(1):46–60. doi:10.4161/auto.6.1.10325
33. Diaz LA, Cheong I, Foss CA, et al. Pharmacologic and toxicologic evaluation of *C. novyi-NT* spores. *Toxicol Sci*. 2005;88(2):562–575. doi:10.1093/toxsci/kfi316
34. Xu J, Liu XS, Zhou SF, Wei MQ. Combination of immunotherapy with anaerobic bacteria for immunogene therapy of solid tumours. *Gene Ther Mol Biol*. 2009;13(1):36–52.
35. Milazzo FM, Vesci L, Anastasi AM, et al. ErbB2 targeted epigenetic modulation: anti-tumor efficacy of the ADC trastuzumab-HDACi ST8176AA1. *Front Oncol*. 2020;9:1534. doi:10.3389/fonc.2019.01534
36. Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R. Regulation of cyclooxygenase-2 pathway by HER2 receptor. *Oncogene*. 1999;18(2):305–314. doi:10.1038/sj.onc.1202307
37. Asadollahi P, Ghanavati R, Rohani M, Razavi S, Esghaei M, Talebi M. Anti-cancer effects of Bifidobacterium species in colon cancer cells and a mouse model of carcinogenesis. *PLoSOne*. 2020;15:e0232930. doi:10.1371/journal.pone.0232930
38. Lin L, Li L, Zhou C, et al. A HER2 bispecific antibody can be efficiently expressed in Escherichia coli with potent cytotoxicity. *Oncology Lett*. 2018;16(1):1259–1266. doi:10.3892/ol.2018.8698
39. Hachani K, Ghanem M, Pockley AG, Wollenberg B, Dezfouli AB, Multhoff G. Heat shock protein 70 (Hsp70) as a target for advancing immunotherapy in solid tumors. *Cytokine Growth Factor Rev*. 2025;86:83–95. doi:10.1016/j.cytogfr.2025.09.002