Supporting Information

Macrophage-Engaging Peptidic Bispecific Antibody (pBsAb) for Immunotherapy via Facile Bioconjugation Strategy

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Contents

Experimental Section

General

N,N-Dimethylformamide (DMF) and dichloromethane (CH_2Cl_2) were dried using molecular sieves prior to use. Acetonitrile was of HPLC grade. All other solvents were of analytical grade and used as received without further purification.

Reverse-phase HPLC separation was performed on a XBridge BEH300 C18 column (5 μm, 4.6 mm × 150 mm) at a flow rate of 1 mL/min for analytical purpose or on a XBridge BEH300 Prep C18 column (5 μm, 10 mm × 250 mm) at a flow rate of 3 mL/min for preparative purpose using a Waters system equipped with a Waters 1525 binary pump and a Waters 2998 photodiode array detector. The condition used for the analysis was set as follows: solvent A = 0.1% trifluoroacetic acid (TFA) in acetonitrile and solvent B = 0.1% TFA in deionized water; gradient: 5% A + 95% B in the first 5 min, then changed to 15% A + 85% B in 10 min, further changed to 100% A + 0% B in 30 min, maintained under this condition for 5 min, changed to 0% A + 100% B in 5 min, maintained under this condition for further 5 min.

Electrospray ionization (ESI) mass spectra were recorded on an Agilent 6546 LC/Q-TOF mass spectrometer. The antibody was analyzed on a MAbPac Reversed Phase HPLC Column (4 μm, 3 mm × 50 mm) at a flow rate of 0.3 mL/min. The condition used for the analysis was set as follows: solvent A = 0.1% formic acid (FA) in deionized water and solvent B = 0.1% FA in acetonitrile; gradient: 80% A + 20% B in the first 0.5 min, then changed to 40% A + 60% B in 3 min, maintained under this condition for 5 min, further changed to 80% A + 20% B in 0.5 min, maintained under this condition for 1 min.

Size-exclusion chromatography analysis was performed on a Zenix-C SEC-300 Gel Filtration column (3 μm, 7.8 mm × 300 mm) at a flow rate of 1 mL/min using an Agilent 1260 Infinity II LC system equipped with an Agilent 1260 Quat Pump and an Agilent 1260 DAD WR. The solvent was sodium phosphate (150 mM, pH 7.0) and set as isocratic for 10 min.

Preparation of EGFR-binding peptide (EBP)

The peptide AcNH-CMYIEALDRYAC-$COHN_2$ (EBP) was synthesized by CEM Liberty Blue Automated Microwave Peptide Synthesizer via modified 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis protocol with the commercially available N-α-Fmoc-protected amino acids. The rink amide resin was used as the solid support. A solution of 20% piperidine in DMF was used to remove the Fmoc protecting group, and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) was used as the carboxyl group activating agent. An excess of the Fmoc-protected amino acid (4 equiv.), HATU (4 equiv.), and N,N-diisopropylethylamine (DIPEA) (8 equiv.) in DMF were used for each coupling at room temperature. CH_2Cl_2/pyridine/acetic anhydride (2/1/1 v/v/v) was used for N-terminal acetylation by stirring the mixture at room temperature for 30 min. After washing, the peptide was cleaved and deprotected by the solution containing 95% TFA, 2.5% triisopropylsilane (TIS), and 2.5% CH_2Cl_2 for 1 h. The resin was removed by filtration and the filtrate was precipitated by the addition of diethyl ether. After centrifugation, the supernatant was removed. Lyophilization of

the precipitated peptide afforded the crude peptide, which was purified by reverse-phase HPLC, followed by lyophilization.

Preparation of cyclic peptide-antibody conjugates

Human IgG (Sigma) or rabbit anti-SIRP-α monoclonal antibody (Sino Biological, China) was first dissolved in phosphate-buffered saline (PBS) (pH 7.4) to afford a 1 μg/μL stock solution. For the cyclic peptide-OPA conjugate (**cEBP-OPA**), the bifunctional linker **1** was firstly treated with a mixture of TFA and water (1:1 v/v) for 30 min at room temperature for the in situ deprotection of the *ortho*-phthalaldehyde unit, followed by neutralisation of the TFA and the reaction with 1 mM of EBP in borate buffer (pH 8.5) reacted with 1.1 mM of the bifunctional linker for 1 h at room temperature.[1] The product was characterized by ESI to confirm the cyclisation process. Without further purification, the **cEBP-OPA** was then added to the antibody solution in PBS at different mole ratios (antibody:**cEBP-OPA** = 1:5, 1:10, 1:20, 1:50). The mixture was stirred at room temperature for 30 min, and then filtrated through a Zeba Spin Desalting Column (40K MWCO) (Thermo Scientific, USA) at 1500 g for 2 min according to manual instruction to remove the excess unconjugated **cEBP-OPA** to form the **cEBP-IgG** or EGFR × SIRP-α **pBsAb**. The product was lyophilized as powder form for storage and further use.

Preparation of cyclic EBP peptide

The bifunctional linker 1 (1.1 mM) was dissolved in borate buffer (pH 8.5) and they were allowed to react with 1 mM of the linear EBP for 1 h at room temperature without the TFA treatment, which allow the OPA moiety remains protected. It was used as a control to demonstrate the cytotoxicity in the viability assay to compare with mAb and pBsAb. The cyclized EBP product was characterized by ESI to confirm the cyclization process.

Enzyme-linked immunosorbent assay (ELISA) for binding analysis of pBsAb to EGFR and SIRP-α and CD47-SIRP-α blockade

NUNC Maxisorp plates (Thermo Scientific) were coated with equimolar of EGFR or SIRP-α protein (Sino Biological, China) at 4 ˚C overnight. The plates were washed three times with PBS containing 0.05% Tween-20 and blocked with 2% bovine serum albumin in PBS containing 0.1% Tween-20 at room temperature for 2 hours. A five-fold serial dilution of pBsAb starting from 80 nM were added to the plates and incubated for 2 h at room temperature. For the study of CD47-SIRP-α blockade, a five-fold serial dilution of pBsAb starting from 20 nM were mixed with a fix amount of His-tag CD47 protein (40 nM) (Sino Biological, China) and then were added to the plates and incubated for 2 h at room temperature. After the incubation, the plates were washed three times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (ITK Southern Biotech) or HRP-conjugated anti-histag secondary antibody (Sino Biological, China) diluted in blocking buffer (1:2000) for 1 h at room temperature. Then, 100 μL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate (Biogene, China) was added into each well and incubated for 6 min and the reaction was stopped with HCl. The HRP activity was measured at 450 nm by plate reader. The half-

maximum effective concentration (EC_{50}) of the binding affinity was calculated by non-linear regression analysis on the binding curves using GraphPad Prism.

Cell lines and culture conditions

HT29 human colorectal adenocarcinoma cells (ATCC, no. HTB-38), HeLa human cervical carcinoma cells (ATCC, no. CCL-2), A549 lung carcinoma cells (ATCC, no. CCL-185), THP-1 human monocyte (ATCC, no. TIB-202) and RAW164.7 murine macrophage cells (ATCC, no. TIB-71) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, cat. no. 12100-046) supplemented with fetal bovine serum (FBS) (10%) and penicillin-streptomycin (100 unit mL^{-1} and 100 μg mL^{-1}, respectively). All the cells were grown at 37 ˚C in a humidified 5% CO_2 atmosphere.

Differentiation of THP-1 Monocytes into Macrophages:

THP-1 monocytes were seeded at a density of 2×10^5 cells/ml in 6-well plates. Phorbol 12-myristate 13-acetate (PMA) was added to the culture at a final concentration of 100 ng/ml. Cells were incubated with PMA for 48 hours to initiate differentiation. Following incubation, the medium was removed, and the cells were gently washed with phosphate-buffered saline (PBS) to remove any non-adherent cells and excess PMA. Fresh RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin was added to the differentiated cells, which were then further incubated for 24 hours to allow for the completion of differentiation into macrophages.

Confocal microscopic analysis of cellular binding of antibodies

Approximately 2×10^5 RAW264.7, HT29, A549, and HeLa cells in DMEM (2 mL) were seeded on a glass-bottom confocal dish and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. For the study of cell binding of **cEBP-IgG**, HT29 and HeLa cells were incubated with **cEBP-IgG** (20 nM) for 30 min at 4 °C. For the study of cell binding of anti-SIRP-α **mAb** or **pBsAb**, the four cells were incubated with the antibodies (10 nM) for 30 min in a complete medium at 4 ˚C. After rinsing twice with PBS containing 1% BSA, cells were post-incubated with 1:500 dilution of Alexa 647-conjugated anti-human secondary antibody (Abcam, USA) for detection of **cEBP-IgG** or Alexa 488-conjugated anti-rabbit secondary antibody (ITK Southern Biotech, China) for 30 min at 4 ˚C for detection of **pBsAb**. The solution was then removed, and the cells were rinsed with PBS twice before being examined using a Leica TCS SP8 high speed confocal microscope equipped with solid-state lasers. Alexa 488 was excited at 488 nm and its fluorescence was monitored at 500-530 nm. Alexa 647 was excited at 638 nm and its fluorescence was monitored at 660-690 nm. The images were digitized and analyzed using a Leica Application Suite X software.

Flow cytometric analysis of cellular binding of antibodies

Approximately 2×10^5 cells were placed in centrifuge tubes and rinsed with PBS containing 1% BSA. Then, they were incubated with different concentrations (0.8, 4.0, and 20.0 nM) of pBsAb or the native mAb in a medium at 4 ˚C for 30 min followed by rinsing with PBS containing 1% BSA twice. Alexa 488-conjugated anti-rabbit secondary antibody (ITK Southern Biotech, China) was incubated with the cells at 1:500 dilution for 30 min. After the reaction, the mixture was centrifuged at 1500 rpm for 3 min at room temperature. The pellet was washed with PBS (1.0 mL) and then subjected to centrifugation. The cells were then suspended in PBS (1.0 mL) and the intracellular fluorescence intensities were measured using a BD FACSVerse flow cytometer (Becton Dickinson) with 10^4 cells counted in each sample. The data collected were analyzed using the BD FACSuite. All experiments were performed in triplicate.

Cell-cell adhesion assay

Approximately 2×10^5 A549 cells per well were seeded on a 12-well cell culture plate and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere for 3 days until reaching above 90% confluency. RAW264.7/differentiated THP-1 macrophages (2×10^5 cells per well) were labelled with carboxyfluorescein succinimidyl ester (CFSE). After washing the macrophages, 20 nM of **pBsAb** or 20 nM of **mAb** were added to the macrophages. Then the mixture was added into the cells and allowed to interact for 15 min in full medium at 37 °C in a humidified 5% CO_2.[2] After 10 min, the confocal dish was extensively washed by PBS three times. Then the cells were examined using a ZEISS Avio Vert.A1 fluorescence microscope. The images were analysed by ImageJ to determine the number of CFSE-labelled macrophages remained on the confocal dish. The experiment was performed in quintuplicate.

Confocal microscopic analysis of antibody-dependent cellular phagocytosis

RAW264.7 macrophages were harvested by washing the adherent cells in 10 mL of cold PBS, incubating for 10 min, and then gently scraping to detach. Macrophages (2×10^5 cells) were labelled with carboxyfluorescein succinimidyl ester (CFSE) and the cancer cells (1×10^5 cells) were stained with CellTracker Red CMPTX as per the manufacturer's instructions. Two cells were then mixed together in a confocal dish in the presence of pBsAb or mAb at 20 nM for 24 h at 37 ˚C with the full medium. After 24 h, the solution was then removed, and the cells were rinsed with PBS twice before being examined using a Leica TCS SP8 high-speed confocal microscope equipped with solid-state lasers. Excitations at 488 nm and 577 nm were used to monitor the fluorescence inside the cells. The images were digitised and analysed using Leica Application Suite X software.

Flow cytometric analysis of antibody-dependent cellular phagocytosis

RAW264.7 macrophages were labelled with CFSE, while A549, HT29, and HeLa cancer cells were stained with CellTracker Red CMPTX according to the manufacturer's instructions. The macrophages (1×10^4 cells per well) and cancer cells (1×10^4 cells per well) were then

incubated together in a 96-well U-bottom plate in the presence of **pBsAb** or **mAb** (20 nM) for 2 h at 37 ˚C in a complete medium. After incubation, the cells were fixed with 4% paraformaldehyde and three times washed with PBS. The intracellular fluorescence was measured using a BD FACSVerse flow cytometer (Becton Dickinson) with $1x10^4$ cells counted in each sample.[3] The data collected were analyzed using the BD FACSuite. All experiments were performed in triplicate. The phagocytotic activity was calculated as the phagocytotic macrophages (double positive)/total macrophages × 100%.

Serum stability assay

A solution of 50% human serum (Sigma-Aldrich, USA) in phosphate-buffered saline (PBS) was prepared. Subsequently, then **pBsAb** or **mAb** at 50 nM were added into this serum-PBS solution and incubated at 37°C. At different time intervals (0-40 h), 100 µl of solution were taken from the samples and followed by rapid flash-frozen in liquid nitrogen to arrest any biochemical reactions. The samples were later thawed and analysed together through enzyme-linked immunosorbent assay (ELISA) to determine their binding affinity to target antigens.

Cell viability analysis

Approximately 1×10^4 cancer cells and 2×10^4 macrophages per well were mixed together in white 96-well plates in the presence of **pBsAb**, **mAb or Cyclic EBP** at 50 nM, they were allowed to co-culture overnight. At the same time, the wells with only cancer cells were prepared as controls. After overnight incubation, 50 µL of CellTiter Glo (Promega, USA) was added into each well. The luminescence signals were measured using Thermo Scientific Varioskan LUX Multimode Microplate Reader. All experiments were performed in triplicate. The cell viability was calculated as (1-(untreated co-culture sample - pBsAb or mAb treated co-culture sample)/ untreated cancer cells only sample) × 100%.[4]

Study of infiltration of macrophages into tumour spheroids

HT29 cells (5×10^4 cells per well) were seeded and cultured in ultra-low attachment U-bottom 96-well plate (ThermoFisher, USA) in a complete medium and at 37 ˚C in a humidified 5% CO_2 atmosphere. The spheroids of 100-200 µm in diameter were formed after 3 days. Then 1×10^5 CFSE-labelled RAW264.7 macrophages per well was added into the 96-well plate in the presence of **pBsAb** or **mAb** (50 nM). After 24 h, 5 µM of propidium iodide (PI) was added to detect dead cells for 1 h. The spheroids were gently washed and placed in confocal dishes. The Z-stack maximum projection images were taken by using Leica TCS SP8 high speed confocal microscope equipped with solid-state lasers. CFSE was excited at 488 nm and its fluorescence was monitored at 500-530 nm, while PI was excited at 552 nm and its fluorescence was monitored at 590-630 nm. The images were digitized and analyzed using a Leica Application Suite X software.

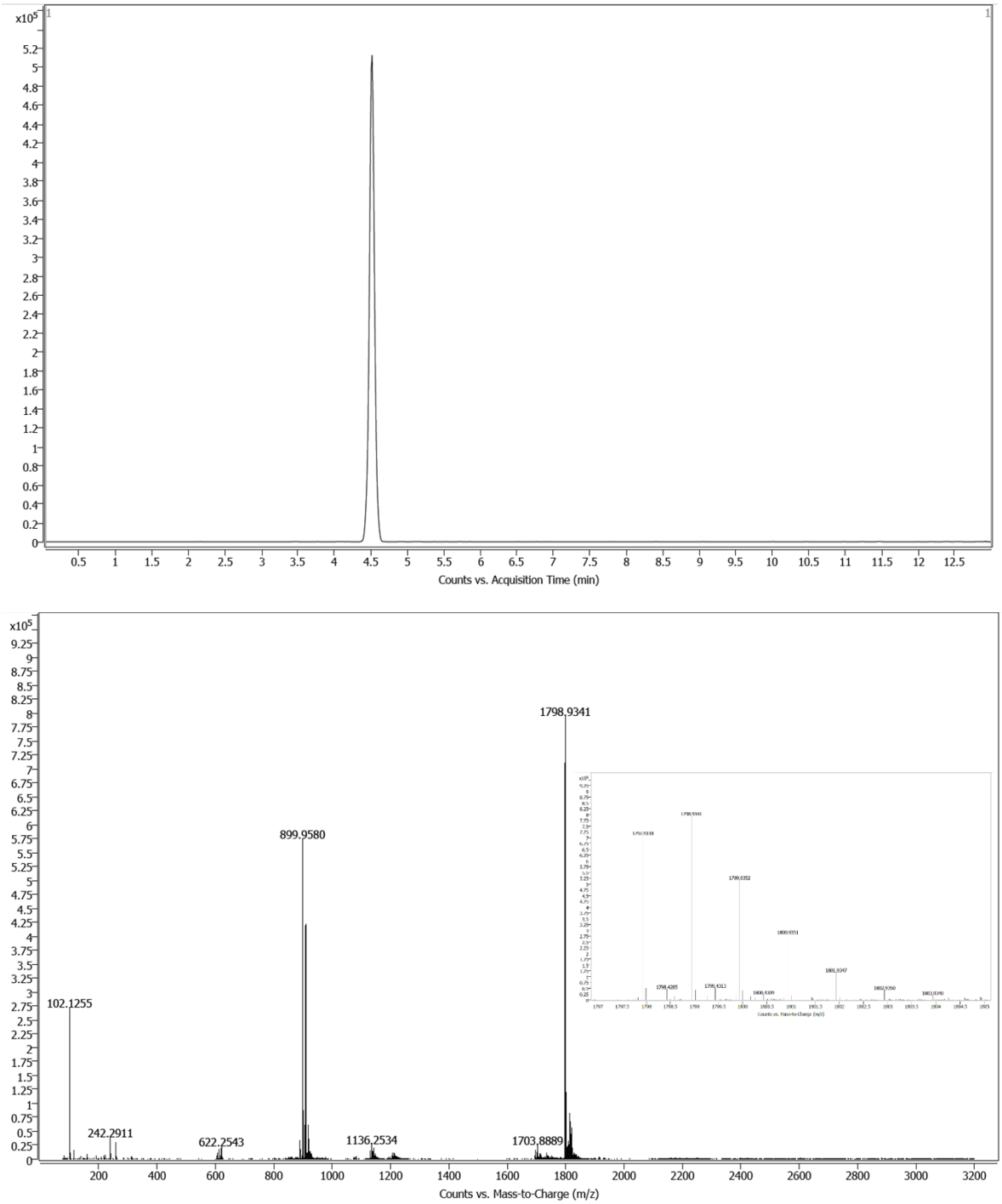

Figure S1. Reverse phase-HPLC chromatogram (Upper) and ESI mass spectrum (Lower) of **cEBP-OPA**. **cEBP-OPA**: HRMS (ESI): *m/z* calcd for $C_{84}H_{117}N_{16}O_{22}S_3$ $[M+H]^+$, 1798.7685; found, 1798.8341.

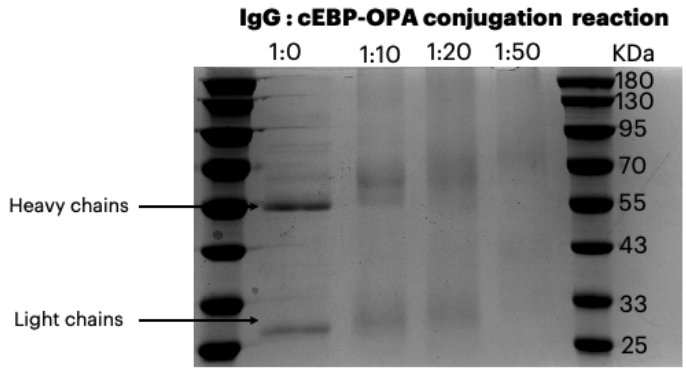

Figure S2. Reducing SDS-PAGE analysis of the conjugation of non-specific human IgG with different equivalents of **cEBP-OPA**. The SDS-PAGE image illustrated that increasing the ratio of **cEBP-OPA** resulted in an upward shift of the bands corresponding to the light-chain and heavy-chain. The smeared bands appeared when peptides were conjugated to the antibody. This indicated that different number of peptides were conjugated to the antibody at different positions, generating heterogeneous mixture of products.

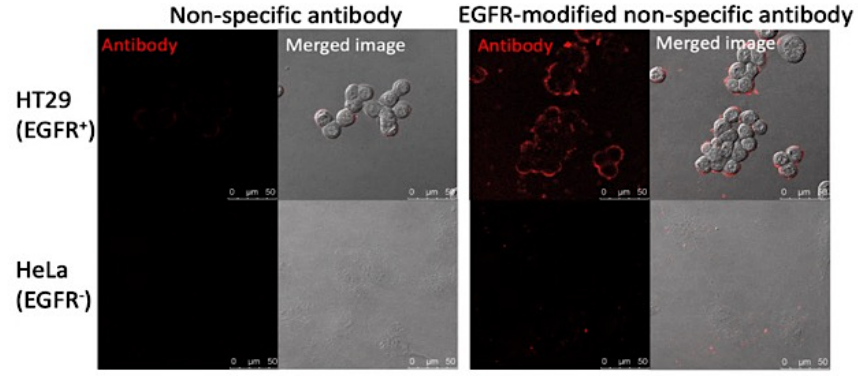

Figure S3. Figure 3. Confocal images of HT29 and HeLa cells after incubation with non-specific native IgG antibody (20 nM) or the modified IgG antibody with cyclic peptides cEBP-IgG (20 nM) for 1 h.

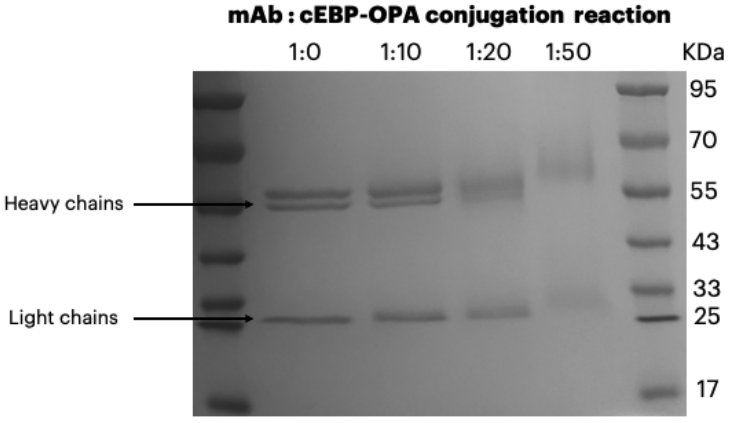

Figure S4. Reducing SDS-PAGE analysis of the conjugation of SIRP-α mAb with different equivalents of **cEBP-OPA**. The SDS-PAGE image showed that increasing the ratio of **cEBP-OPA** leaded to upward shift of the bands of light-chain and heavy-chain.

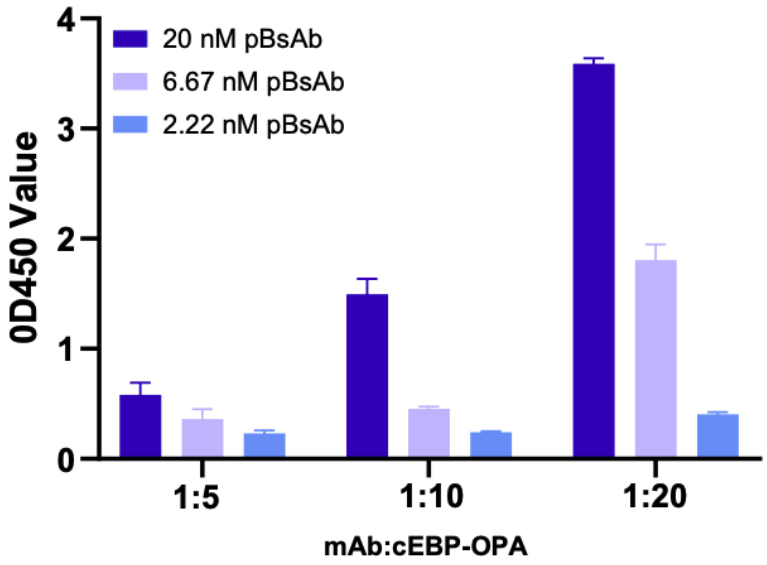

Figure S5 . ELISA binding experiment against EGFR recombinant protein using different pBsAbs produced by different reaction conditions with mole ratio of mAb:cEBP-OPA at 1:5, 1:10, and 1:20.

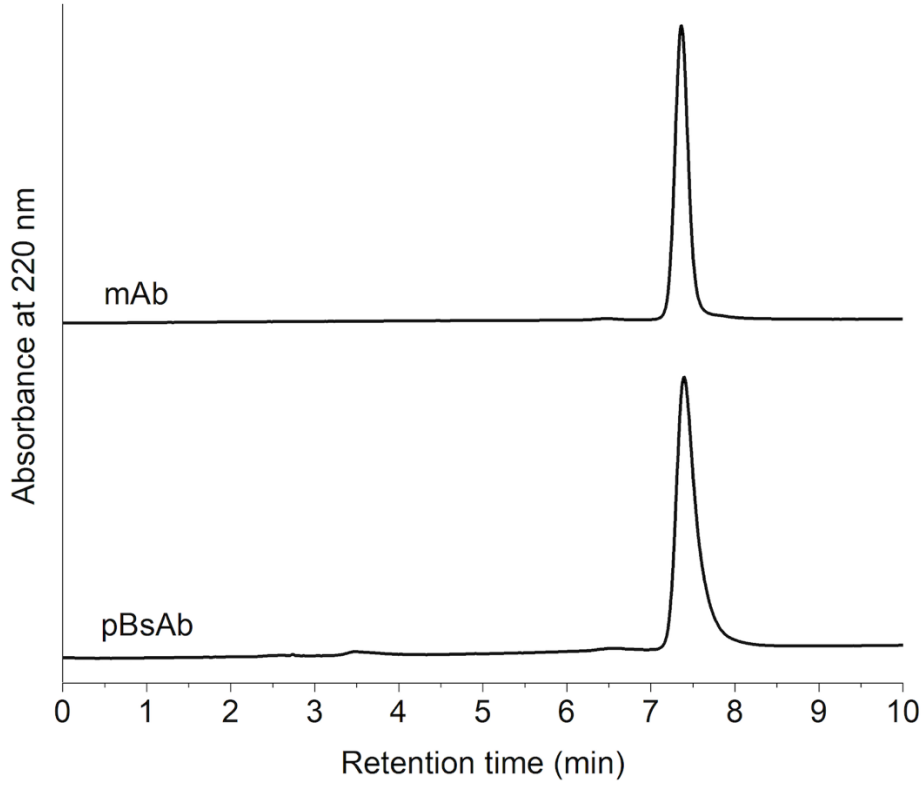

Figure S6. Size exclusion chromatograms of mAb and pBsAb. The data showed that no significant aggregation was observed for the pBsAb prepared with 20 equivalents of **cEBP-OPA** after overnight incubation. The retention time of mAb and pBsAb were determined to be 7.47 and 7.40 min, respectively.

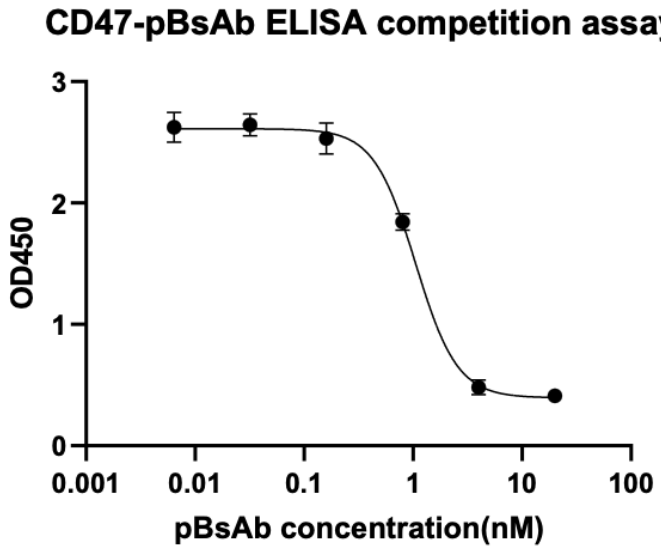

Figure S7. ELISA binding experiment of pBsAb CD47 blockage. The data showed that when the concentration of pBsAb >0.1 nM, the SIRP-α-CD47 interaction was inhibited. Data are expressed as the mean value ± standard error of the mean (SEM) of three independent experiments, each performed in triplicate

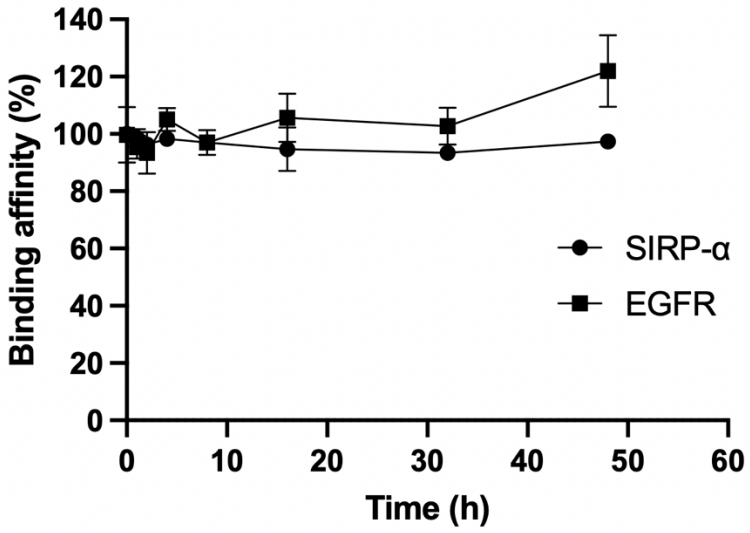

Figure S8. Serum stability experiment by ELISA binding assay. Percentage change of binding affinity of pBsAb against SIRP-α and EGFR at different time points in the presence of 50% serum.

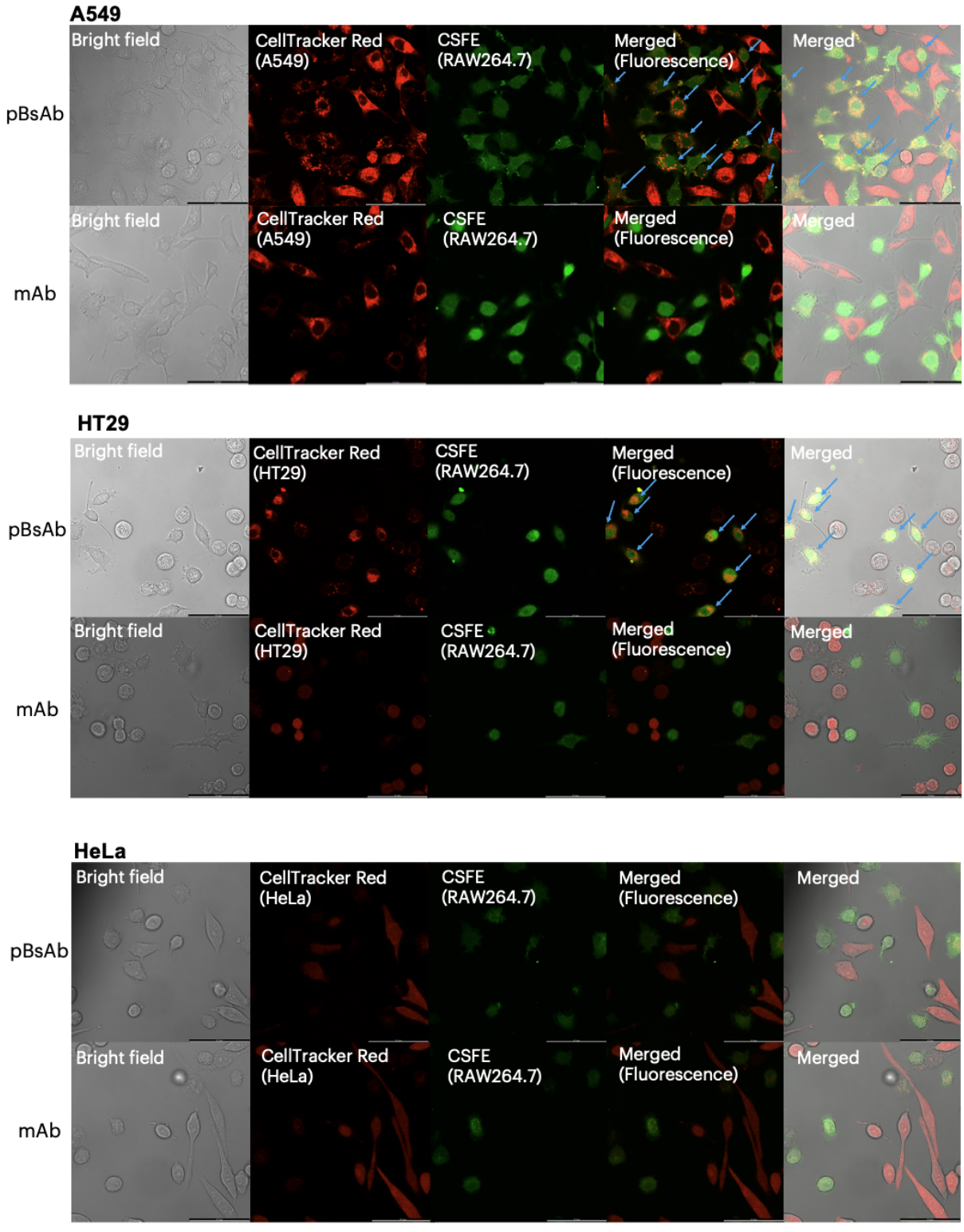

Figure S9. Confocal images of antibody-mediated cellular phagocytosis of macrophages against A549, HT29, and HeLa cells in the presence of pBsAb or mAb (20 nM) for 24 h. Blue arrows indicate the phagocytosed macrophages.

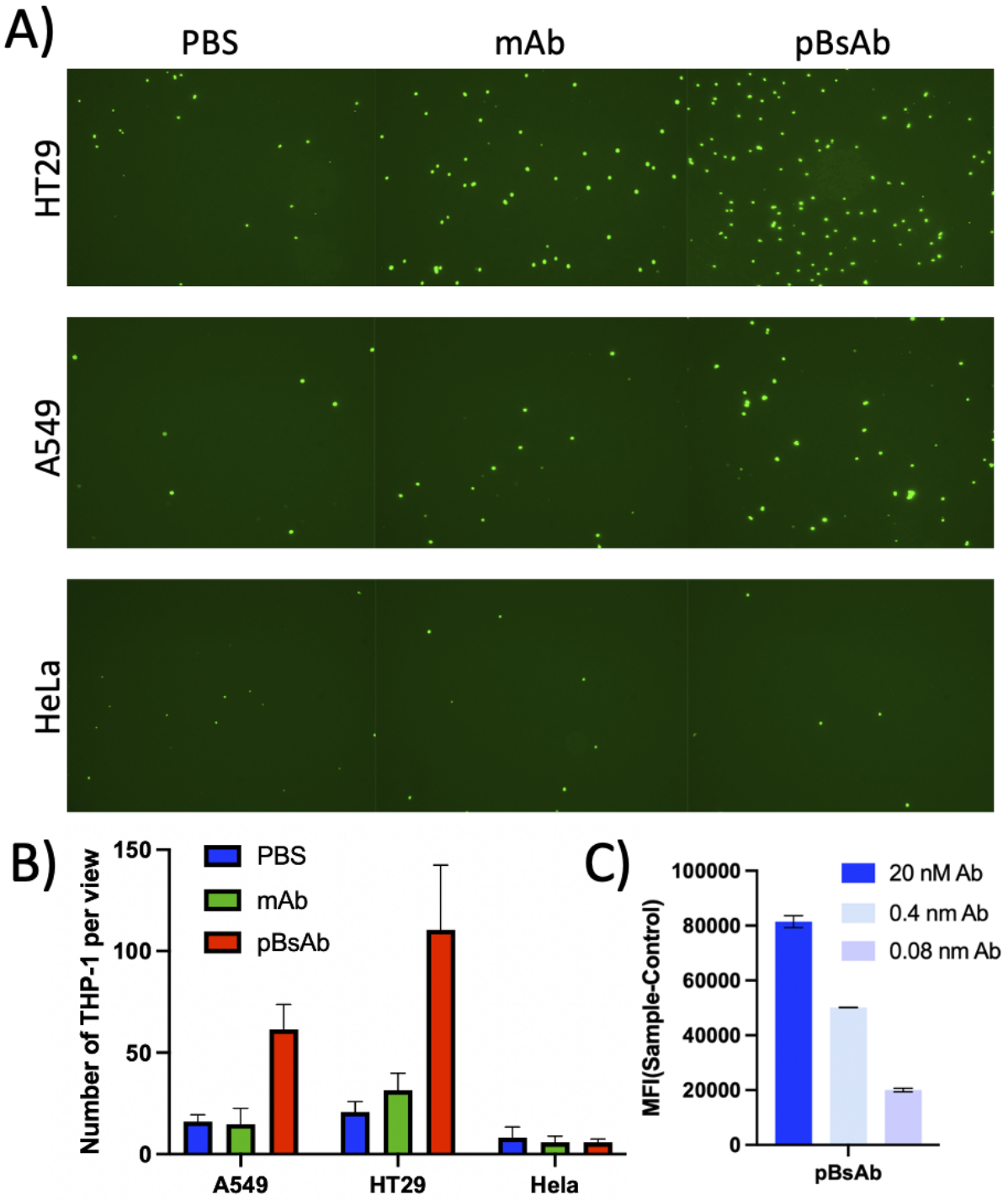

Figure S10. (A) Fluorescence microscope images of cell-cell adhesion experiment illustrating the adhesion of differentiated THP-1 macrophages (green) to the monolayer of EGFR-positive A549, HT29, and EGFR-low expressing HeLa cells in the presence of different antibodies at 20 nM or PBS. (B) Bar chart of quantified number of macrophages adhering to different cell lines. (C) Flow cytometric data of differentiated THP-1being treated with different concentration of pBsAb. Demonstrating that our pBsAb also has the ability to engage human SIRP-α to create cell-cell interaction and retaining SIRP-α receptor binding ability.

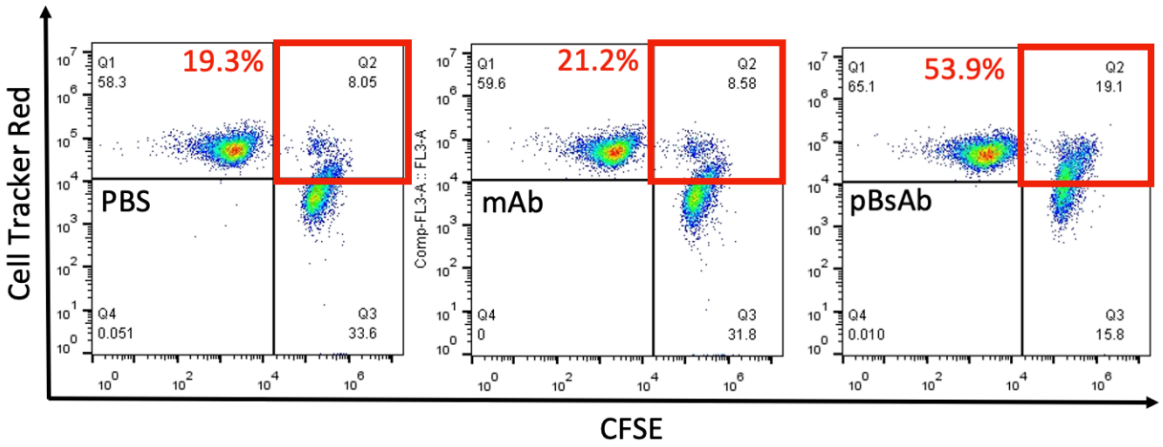

Figure S11. Flow cytometric quadrant analysis of the co-culture RAW264.7 macrophages with HT29 in the presence of pBsAb, mAb, and PBS. The addition of mAb compared to PBS suggesting the blockage of the SIRP-α could inhibit the "Don't eat me" signal and thus, enhanced the phagocytotic activity.

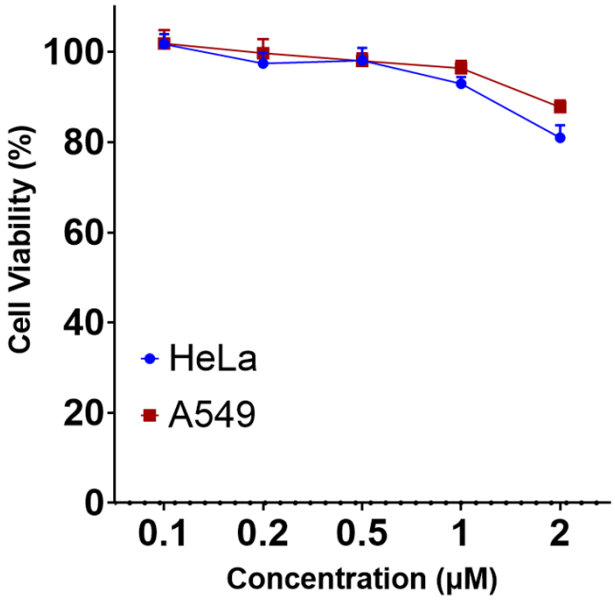

Figure S12. Cell viability assay of EBP cyclic peptide against A549 lung carcinoma. The data showed that the EBP cyclic peptide has no cytotoxicity against tested cell line.

References

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