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Tumor Marker B7-H6 Bound to the Coiled Coil Peptide-Polymer Conjugate Enables Targeted Therapy by Activating Human Natural Killer Cells

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Supplementary Materials

Supplementary Methods

1. Preparation of PolCC+ Polymer-peptide Conjugate

1.1 Materials

Methacryloyl chloride, 1-aminopropan-2-ol, 3-aminopropanoic acid, 4,5-dihydrothiazole-2-thiol, dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N,N'-dicyclohexylcarbodiimide (DCC), 2,2'-azobis(isobutyronitrile) (AIBN), 4-cyano-4-thiobenzoylsulfanylpentanoic acid, N-(2-aminoethyl)biotinamide hydrobromide (biotin-NH2), N,N-dimethylacetamide (DMA), dimethyl sulfoxide (DMSO) and tert-butyl alcohol were purchased from Sigma-Aldrich, MT, USA. 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) was purchased from Wako Chemicals Europe GmbH, Germany. 2-Chlorotrityl chloride resin and protected amino acid derivatives were purchased from Iris Biotech, GmbH, Germany. [(1R,8S)-9-bicyclo[6.1.0]non-4-ynyl]methyl N-[2-[2-(2-aminoethoxy)ethoxy]ethyl]carbamate (Bcn-Peg2-NH2) was purchased from SiChem GmbH, Germany. 3-Amino-1-(11,12-didehydrodibenzo[b,f]azocin-5(6H)-yl)propan-1-one (Dbco-NH2) was purchased from Click Chemistry Tools, AZ, USA. All other reagents and solvents were purchased from Sigma-Aldrich, MT, USA. All chemicals and solvents were of analytical grade.

The contents of thiazolidine-2-thione (TT) groups, dithiobenzoate (DTB) end groups, and aza-dibenzocyclooctyne (Dbco) groups were determined spectrophotometrically on a Helios Alpha UV/VIS spectrophotometer (Thermospectronic, UK) using the following absorption coefficients: εTT = 10300 $L.mol^{-1}.cm^{-1}$ (305 nm, methanol), εDTB = 12100 $L.mol^{-1}.cm^{-1}$ (302 nm, methanol), εDbco = 13000 $L.mol^{-1}.cm^{-1}$ (292 nm, methanol).

Monitoring of the conjugation reactions of Dbco-NH_2, Bcn-Peg_2-NH_2, biotin-NH_2, and peptide azide EKE to the reactive polymer precursors was performed by HPLC using a 100 × 4.6 mm Chromolith Performance RP-18e column (Merck, Germany) and a linear gradient of water/acetonitrile (0–100 % acetonitrile) in the presence of 0.1 % TFA with a UV-Vis diode array detector (Shimadzu, Japan).

The determination of the molecular weights and polydispersity of the copolymers was performed by SEC on an HPLC system (Shimadzu, Japan) equipped with UV, differential refractive index, and multi-angle light scattering (LS) DAWN Heleos II (Wyatt Technology Corp., USA) detectors using a TSK 3000 SW_{XL} column (Tosoh Bioscience, Japan) (80 % methanol, 20 % 0.3 M acetate buffer pH 6.5) at a flow rate of 0.5 ml/min.

1.2 Preparation of Monomers

N-(2-Hydroxypropyl)methacrylamide (HPMA) and 3-methacrylamidopropanoylthiazolidine-2-thione (Ma-AP-TT) were prepared as described in references [86] and [87], respectively.

1.3 Preparation of Reactive Polymer Precursor

Copolymer poly(HPMA-co-Ma-AP-TT) (P-TT) was prepared by reversible addition–fragmentation chain transfer (RAFT) copolymerization of HPMA (1.0 g, 6.98 mmol, 90 mol. %) and Ma-AP-TT (200 mg, 0.78 mmol, 10 mol. %) using V70 (9.70 μmol, 2.99 mg) as an azo initiator and (1-cyano-1-methyl-ethyl) benzenecarbodithioate (19.4 μmol, 4.29 mg)

as a chain transfer agent (CTA). The molar ratio of monomers/CTA/initiator used was 800:2:1. The polymerization mixture was dissolved in tert-butyl alcohol with 15 % DMA (11.1 ml, 0.9 M solution of monomers), transferred into a glass ampule, bubbled with Ar, and sealed. After 16 h at 40 °C, the product was isolated by precipitation with acetone, washed with diethyl ether, and dried under vacuum. The copolymer was reacted with AIBN (10 molar excess) in DMA (15 % w/w solution of polymer) under Ar for 3 h at 70 °C in a sealed ampule to remove dithiobenzoate (DTB)ω-end groups [88]. The reaction mixture was isolated by precipitation with acetone; the precipitate was washed with diethyl ether and dried under vacuum to yield copolymer P-TT (0.8 g, 67 %). Molecular parameters of the product were M_w of 95500 g.mol^{-1} and M_w/M_n of 1.06. The content of the reactive TT groups was 7.0 mol. %.

1.4 Modification of Reactive Polymer Precursor with Biotin

Copolymer P-TT (50 mg, 23 μmol TT) in DMA (0.5 ml) and N-(2-aminoethyl)biotinamide hydrobromide (1.22 mg, 3.3 μmol) in DMA (0.2 ml) were mixed and reacted for 3 h at 25 °C. Then, Bcn-Peg$_2$-NH$_2$ (2.68 mg, 8.3 μmol) was dissolved in the reaction mixture, and DIPEA (5 μl, 32 μmol) was added. The reaction was completed after 3 h (as indicated by HPLC), the remaining TT groups were removed by adding 1-aminopropan-2-ol (1.8 μl). The polymer product was twice precipitated 5 min later into acetone/diethyl ether (1:1) to yield 48 mg of the desired copolymer P-biotin-Bcn. The biotin content was 1.0 mol. %, as determined by HABA assay [89].

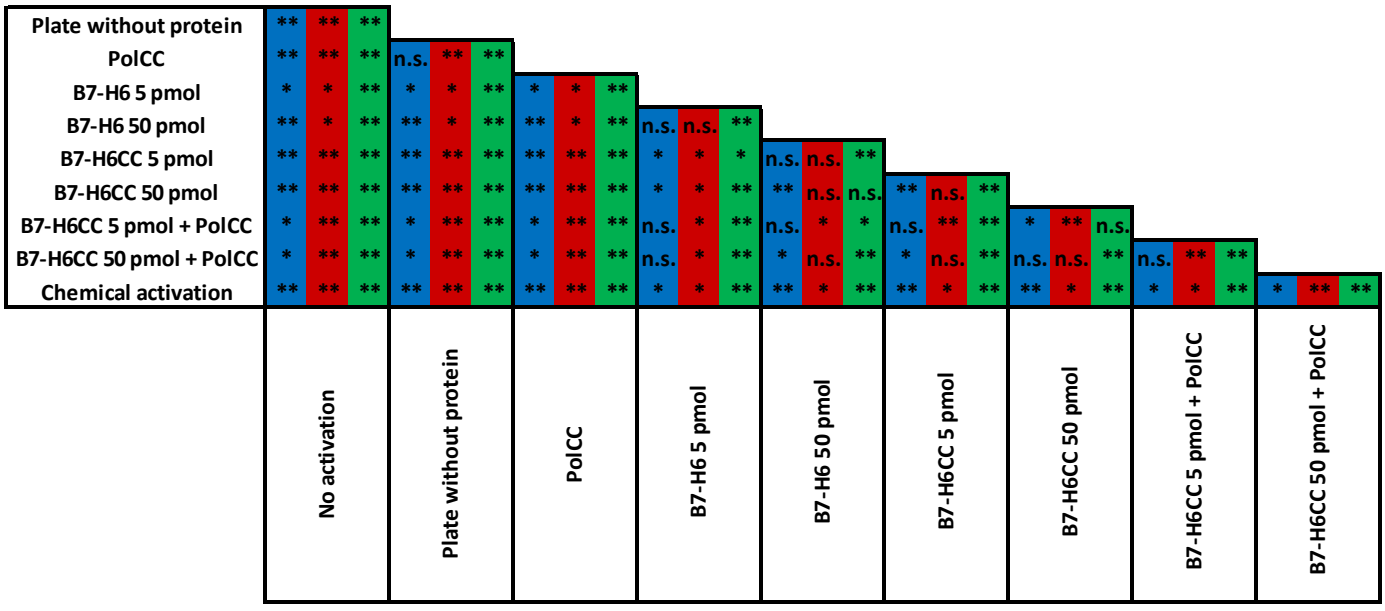
1.5 Attachment of Coiled Coil Peptides to the Polymer-biotin Precursor

Solution of copolymer P-biotin-Bcn (9.4 mg) in DMA (100 μl) and solution of the peptide azide (3.3 mg, 1 μmol) in DMA (50 μl) were mixed and reacted for 16 h at 25 °C. The progress of the cycloaddition was monitored by HPLC. Precipitation of the reaction mixture with acetone – ether (2:1) yielded 12.7 mg of the desired polymer-peptide conjugate. Molecular parameters of the product were M_w of 130000 g.mol^{-1} and M_w/M_n of 1.28. The content of the peptide was 26 % w/w.

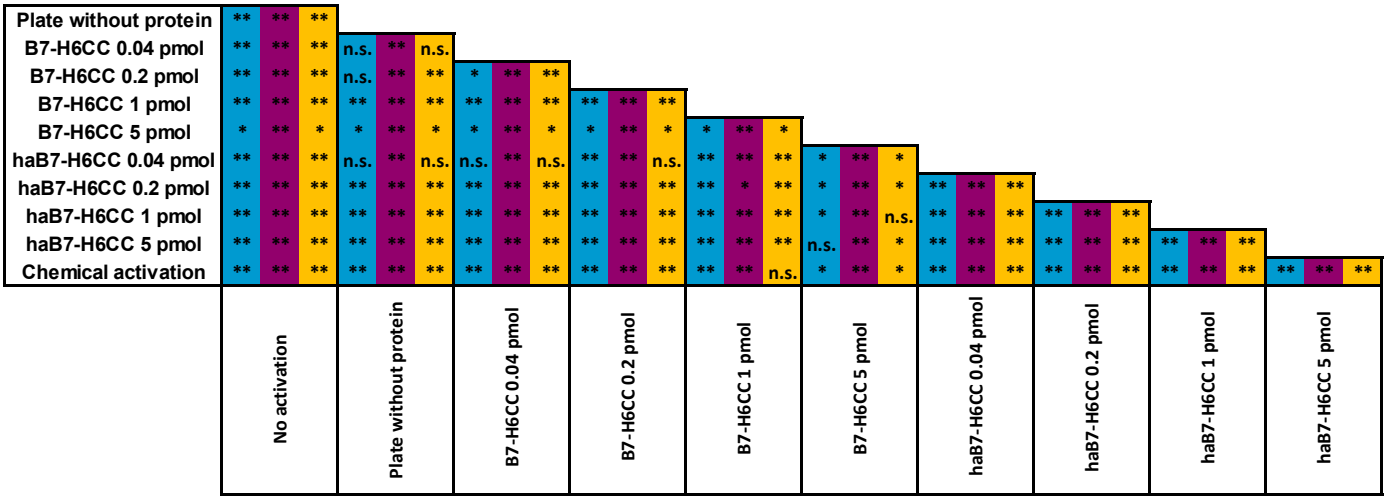
Supplementary data

2. Statistical analysis of NK cell activation with B7-H6CC and B7-H6CC:PolCC

The percentage of activated NK cells was assessed by the flow cytometry of NK cells stained with antiCD107a antibody and FlowJo software data analysis. The Mann-Whitney U test with a two-tailed hypothesis was used to compare the means. The result of all mean comparisons is shown in Supplementary Figure 1.



(a)

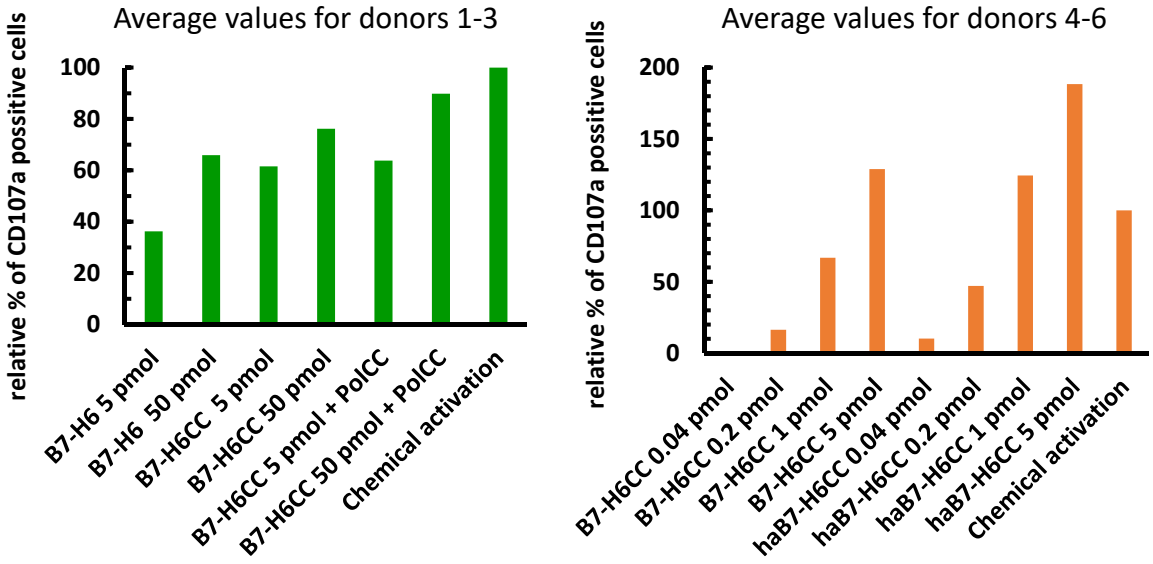


(b)

Supplementary Figure S1. Statistical analysis of NK cell activation with B7-H6 protein variants. (a) Statistical analysis of the first experiment comparing the ability of B7-H6, B7-H6CC, or B7-H6CC with PolCC to induce NK cell activation. (b) Statistical analysis of the second experiment comparing activation potential of B7-H6CC versus haB7-H6CC. n.s. stands for non-significant difference of the means, relevant p values are indicated with asterisks (** $p < 0.05$; * $p < 0.10$).

3. Global trends in NK cell activation with B7-H6CC and B7-H6CC:PolCC

To present global data analysis of NK cell activation instead of presenting results for individual donors, we constructed the graph representing a relative percentage of activated NK cells in each experiment. The baseline (activation in condition without protein) was subtracted from all conditions, and the chemical activation was considered as maximal activation (100 %). With this approach, we overcome the individual attributes of the donors, namely the different cellular sensitivity given by the magnitude between control conditions (negative control – plate without protein, positive control – chemical activation). The global trend in NK cell activation is represented in Supplementary Figure 2. The mean of technical triplicate was used in all calculations, and values from three independent donors were averaged.



(a) **(b)**

Supplementary Figure S2. Average values of NK cell activation present the global trend. **(a)** Global analysis with the data from the donor 1-3 comparing B7-H6 activation with B7-H6CC; **(b)** analysis comparing average values of B7-H6CC and haB7-H6CC activation in the experiment with donors 4-6.

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