Take Immune Cells Back on Track: Glycopolymer-Engineered Tumor Cells for Triggering Immune Response

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Table of Contents

1. Experimental Procedures

1.1 Chemical Materials and Methods
1.1.1 Chemical Reagents
1.1.2 Characterization.
1.1.3 Synthesis of chloroalkane-conjugated chain transfer agent
1.1.4 Preparation of glycopolymers containing chloroalkane end groups

1.2 Biological Reagents and Biological Assays
1.2.1 Biological Reagents and Methods
1.2.2 Cell Culture
1.2.3 Construction of HeLa cells stably expressing HTP
1.2.4 Engineering of HeLa cell surfaces with synthetic well-defined glycopolymers
1.2.5 Co-culture of U937/DC2.4 and glycopolymer-engineered HeLa cells
1.2.6 Lactate dehydrogenase (LDH) release assay
1.2.7 Analysis of CD86 and CD206 expression on macrophages by flow cytometry
1.2.8 Detection of inflammatory cytokine secretion levels

2. References
Supporting information

1. Experimental Procedures
1.1 Chemical Materials and Methods

1.1.1 Chemical Reagents.
4-Cyanopentanoic acid dithiobenzoate (CPADB) was from Sigma-Aldrich Chemical Co. 2-(2-Boc-aminoethoxy) ethanol was from Adamas Co., Ltd (Shanghai, China). N-Hydroxysuccinimide (NHS), 1-chloro-6-iodohexane (stabilized with copper chips), sodium hydride (60%, dispersion in paraffin liquid), dicyclohexylcarbodiimide (DCC) and anhydrous tetrahydrofuran (THF) (stabilized with BHT) were purchased from TCI Co., Ltd (Shanghai, China). 2,2′-Azisobutyronitrile (AIBN) from Sinopharm Chemical Reagent Co. (Shanghai, China) was recrystallized from ethanol and dried under vacuum before use. Anhydrous N,N-dimethylformamide (DMF) was from Aladdin Co., Ltd (Shanghai, China). 2-Methacrylamido glucopyranose (MAG) and 2-methacrylamide mannose (MAM) were synthesized as reported previously. Other organic solvents used were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China) and distilled prior to use. Deionized water (DIW), purified to a minimum resistivity of 18 MΩ·cm by a Millipore water purification system, was used in all experiments.

1.1.2 Characterization.
All synthetic products were analyzed by $^1$H NMR (Bruker, 300 MHz, tetramethylsilane (TMS) as internal standard at 293 K) to verify their chemical structure and composition. Spectra were recorded in CDCl$_3$ or D$_2$O solution ($^1$H NMR: CDCl$_3$ = 7.26 ppm, D$_2$O = 4.79 ppm; for $^{13}$C NMR: CDCl$_3$ = 77.0 ppm). Mass spectra (MS) were acquired using a Bruker micrOTOF-Q III instrument. Fourier transform infrared (FT-IR) spectra were recorded using a Nicolet 6700 spectrometer with 32 scans per sample. The number-average molecular weights (M$_n$) and polydispersity indices (PDI) of the polymers were determined by size exclusion chromatography (SEC) using a Waters 1515 gel permeation chromatograph with polyethylene glycol standards.

1.1.3 Synthesis of chloroalkane-conjugated chain transfer agent. 
$\textit{t-Butyl (2-(2-(6-chlorohexyl)oxy)ethoxy)ethyl} \textit{carbamate (B).}$
To a solution of 2-(2-Boc-aminoethoxy) ethanol (4.30 g, 20.95 mmol) in anhydrous THF (40 mL) and DMF (20 mL) at 0°C NaH (60% dispersion in paraffin liquid, 1.12 g, 28.00 mmol) was added portionwise. After stirring at 0°C for 30 min, 1-chloro-6-iodohexane (4.80 mL, 31.60 mmol) was added to the mixture at 0°C. The reaction mixture was stirred at 0°C for 20 min, at room temperature for 16 h, then quenched with saturated aqueous NH$_4$Cl solution. The mixture was extracted twice with ethyl acetate and the combined extracts were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. The residue was chromatographed on silica gel using 3:1 hexane/ethyl acetate to afford tert-butyl (2-(2-(6-chlorohexyl)oxy)ethoxy)ethyl carbamate (B). $^1$H NMR (300 MHz, CDCl$_3$) (Figure S1): δ 3.61-3.50 (m, 8H), 3.47-3.43 (t, J=6 Hz, 2H), 3.32-3.28 (t, J=6 Hz, 2H), 1.81-1.72 (m, 2H), 1.65-1.55 (m, 2H), 1.49-1.33 (m, 13H).
2-(2-(Chloromethoxy)ethoxy)ethan-1-amine (C).
To a solution of B (1.35 g, 4.17 mmol) in 30 mL of CH₂Cl₂ at 0°C was added 5 mL of TFA. After stirring for 2.5 h at 0°C, TFA and solvent were removed and the residue was diluted with 30 mL of CH₂OH. The solution was cooled to 5°C and K₂CO₃ (1.65 g, 11.93 mmol) was added to the mixture. The mixture was stirred at 5°C for 10 min, filtered and evaporated. The residue was diluted with H₂O (20 mL) and the mixture was extracted four times with ethyl acetate. The combined extracts were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel chromatography to afford 2-(2-(chloromethoxy)ethoxy)ethan-1-amine (C).

1H NMR (300 MHz, CDCl₃) (Figure S2): δ 3.63-3.50 (m, 8H), 3.48-3.44 (t, J=6 Hz, 2H), 2.92-2.88 (t, J=6 Hz, 2H), 2.66 (s, 2H), 1.81-1.72 (m, 2H), 1.64-1.55 (m, 2H), 1.50-1.30 (m, 4H).

5-((2-(2-((6-Chlorohexyl)oxy)ethoxy)ethyl)amino)-2-cyano-5-oxopentan-2-yl benzodithioate (A).
CPADB (0.50 g, 1.91 mmol), NHS (0.33 g, 2.87 mmol) and DCC (0.74 g, 3.82 mmol) were dissolved in 10 mL of DMF. The reaction was followed by thin layer chromatography. 2-(2-(chloromethoxy)ethoxy)ethan-1-amine (C) (387 mg, 1.73 mmol, dissolved in 10 mL of DMF) was added to the reaction mixture when the spot from CPADB had disappeared. The mixture was stirred overnight at 30°C. The mixture was washed with water, then concentrated. Purification by silica gel chromatography using 1:100 CH₃OH/CH₂Cl₂ gave A. 1H NMR (300 MHz, CDCl₃) (Figure S3): δ 7.92-7.89 (m, 2H), 7.59-7.54 (m, 1H), 7.42-7.37 (m, 2H), 6.21 (brs, 1H), 3.64-3.52 (m, 8H), 3.50-3.45 (m, 4H), 2.68-2.51 (m, 2H), 2.47-2.38 (m, 2H), 1.94 (s, 3H), 1.81-1.72 (m, 2H), 1.66-1.57 (m, 2H), 1.48-1.33 (m, 4H). 13C NMR (300 MHz, CDCl₃) (Figure S4): δ 205.92, 170.25, 144.57, 132.99, 128.56, 126.67, 118.71, 118.71, 71.32, 70.34, 70.03, 69.61, 46.08, 45.05, 39.43, 34.14, 32.51, 31.82, 29.44, 26.66, 25.39, 24.21. MS for C₂₃H₃₃ClN₂O₃S₂ [M + H]⁺ m/z: 485.1600 (calculated); 485.1663 (found). MS for C₂₃H₃₃ClN₂O₃S₂ [M + Na]⁺ m/z: 507.1600 (calculated); 507.1490 (found).

1.1.4 Preparation of glycopolymers containing chloroalkane end groups.
Glycopolymers containing chloroalkane end groups were synthesized via RAFT polymerization of MAG or MAM using the synthesized chain transfer agent A. Briefly, MAG or MAM (0.1240 g, 0.5 mmol), A (0.0024 g, 5.0 μmol), and AIBN (0.0004 g, 2.5 μmol) were dissolved in DMF. The solution was then de-oxygenated by bubbling with nitrogen for 30 min. The polymerization was carried out at 70°C for 12 h under a nitrogen atmosphere. The reaction mixtures were dialyzed for 2 days against DIW to remove unreacted monomer. The glycopolymer solutions were lyophilized to give fluffy solid products. The biotin-labeled glycopolymer pMB was generated via RAFT polymerization of MAG and biotin monomer (a custom synthesized compound from Lianke Biotechnology Service Department, Suzhou, China). Briefly, MAG (0.1112 g, 0.45 mmol), biotin monomer (0.0257 g, 0.05 mmol), A (0.0024 g, 5.0 μmol), and AIBN (0.0004 g, 2.5 μmol) were dissolved in DMF. The polymerization was carried out by the same methods as described above. SEC traces of the products are shown in Figure S9.
1.2 Biological Reagents and Biological Assays

1.2.1 Biological Reagents and Methods.
High-glucose Dulbecco’s modified Eagle’s medium (DMEM) was obtained from HyClone (Logan, USA). RPMI-1640 medium, penicillin-streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Plasmocin™ was purchased from InvivoGen Co., Ltd. (San Diego). Bovine serum albumin (BSA), paraformaldehyde and Triton X-100 were from Sigma-Aldrich. DAPI (4’, 6-diamidino-2-phenylindole) was purchased from Invitrogen (Waltham, MA). FITC-conjugated avidin (FITC-avidin) was purchased from Sigma-Aldrich. Primary antibody hemagglutinin (HA) and fluorescein isothiocyanate (FITC)-conjugated secondary antibody were from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Anti-CD68 antibody was obtained from Abcam Co., Ltd. FITC anti-human CD68 antibody was purchased from BioLegend (San Diego, CA). Human IL-10, IL-12p70 and TNF-α and mouse IL-12p70 enzyme linked immunosorbent assay (ELISA) kits were purchased from Fcmacs Biotech (Nanjing, China). Inducible nitric oxide synthase (iNOS) assay kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). U937 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. DC2.4 cells were from Millipore. Fluorescence images were captured using an SP5II confocal microscope (Leica Microsystems). The images were overlapped using Image-Pro Plus 6.0 software. Flow cytometry was performed on a BD FACSVerse™ system and the data were analyzed using FlowJo with 10000 events during collection.

1.2.2 Cell Culture.
HeLa cells were maintained in DMEM medium. U937 and DC2.4 cells were maintained in RPMI-1640 medium. All media were supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated at 37°C with 5% (v/v) CO₂ in a water-saturated chamber and the medium was replaced every one to two days as required.

1.2.3 Construction of HeLa cells stably expressing HTP.
The hemagglutinin (HA)-tagged HTP gene inserted between the sequence encoding the Ig κ-chain leader and the PDGFR transmembrane domain³ (Figure S10) was a customized plasmid from Genewiz Co., Ltd. (Suzhou, China). HeLa cells were plated and transfected with the HTP plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. HeLa cells that had been transfected with HTP were selected using complete DMEM medium supplemented with G418 solution. The medium was replaced every 2 days for 3 weeks. The concentration of G418 in the medium was 0.4 mg/mL in the first week and was decreased to 0.2 or 0.1 mg/mL for the following two weeks. After 3 weeks, the HA-immunofluorescence staining assay of cells was carried out to demonstrate the display of HTP on the cell membranes. In detail, the HeLa cells were plated and cultured on the chamber slides in complete DMEM medium overnight. The cells were then washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min, and
rinsed three times in PBS. The cells were permeabilized with 0.1% Triton X-100 for 5 min. After being washed twice with PBS, cells were blocked using blocking solution (3% BSA/PBS) for 30 min. Then the cells were incubated with primary antibody of HA overnight at 4°C. After washing three times with PBS, the cells were incubated with FITC-conjugated secondary antibody at room temperature for 1 h, followed by two washes with PBS. Representative images were captured using a laser scanning confocal microscope. The fluorescence images demonstrated that the HeLa cells stably expressing membrane-bound HTP were obtained (Figure S11).

1.2.4 Engineering of HeLa cell surfaces with synthetic well-defined glycopolymers. HeLa cells stably expressing HTP were seeded on chamber slides at a density of 2×10⁴ cells per well in complete DMEM medium. After incubation overnight at 37°C, 5% CO₂, the cells were washed with 200 μL of PBS twice and incubated with 150 μL of pMB solution (0.1 mg/mL) in serum-free DMEM at 37°C for 1 h. Then, 200 μL of PBS/well was added to wash away excess polymers and the cells were incubated with FITC-avidin solution for 1 h at 4°C. Images were captured using a laser scanning confocal microscope.

1.2.5 Co-culture of U937/DC2.4 and glycopolymer-engineered HeLa cells. HeLa cells stably expressing HTP were seeded on chamber slides or on 6-well tissue culture plates and incubated for 12 h at 37°C (5% CO₂) in complete DMEM medium. The cells were then washed twice with PBS and incubated with pMAG or pMAM solution (0.1 mg/mL) in DMEM, or in normal medium without glycopolymers at 37°C for 1 h, respectively. The cells were then washed three times with PBS to remove unbound glycopolymers. U937/DC2.4 cells were seeded on HeLa-adhered plates (U937/DC2.4 cells: HeLa cells, 1:1 or 2:1) and incubated for appropriate times. For visualizing cell-cell interactions between U937 and modified HeLa cells, U937 cells were seeded on HeLa-adhered plates and the cell images were captured by IncuCyte ZOOM (Essen Bioscience) every two hours.

1.2.6 Lactate dehydrogenase (LDH) release assay. Measurement of cytotoxicity of unmodified HeLa or glycopolymers-engineered HeLa cells upon with U937 was carried out using a Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific). The day previous to the experiment 3×10⁴ HTP-HeLa cells per well were seeded into a 24-well plate in 1 mL of complete DMEM medium. The cells were allowed to adhere to the plate overnight in a 37°C incubator supplemented in 5% CO₂. The following day the adhered HTP-HeLa cells were incubated in pMAG or pMAM solution (0.1 mg/mL) in DMEM, or in normal medium without glycopolymers at 37°C for 1 h, respectively. The cells were then washed three times with PBS to remove unbound glycopolymers. Then U937 cells were added to each well at a ratio of 1:1 (U937: HeLa). The cells are allowed to incubate for 2 days at 37°C with 5% CO₂. Forty-five minutes prior to testing 100 μL of lysis buffer was added to three wells that correspond to the max lysis of only HTP-HeLa cells at a density of 3×10⁴ cells. At the end of the two-day incubation, the samples were centrifuged at 2000 rpm for 3 min.
Following centrifugation, 50 μL of supernatant was added into a new 96-well plate. An additional 50 μL of reaction mixture was added to each well and the plate was allowed to incubate at room temperature for 30 min in the dark. The reaction was stopped by adding 50 μL of the stop buffer included in the kit, and the absorbance of each well was read at 490 nm. Data was corrected for the culture medium background control, and values were determined according to the following equation:

\[
\text{%Cytotoxicity} = \frac{\text{Experimental value} - \text{Effectector Cells Spontaneous Control}}{\text{Target Cells Maximum Control} - \text{Target Cells Spontaneous Control}} \times 100.
\]

1.2.7 Analysis of CD86 and CD206 expression on macrophages by flow cytometry.
After co-culture of U937 cells with natural HeLa cells or glycopolymer-engineered HeLa cells for 4 days in a 6-well tissue culture plate, the cells were washed three times with PBS, detached using trypsin, and centrifuged at 1000 rpm for 5 min. Subsequently, the cells were washed twice with PBS and labeled with PE anti-human CD86 and CD206 antibody for 30 min. The cells were then resuspended in 0.5 mL of PBS for flow cytometry analysis to determine the numbers of CD86+ and CD206+ cells.

1.2.8 Detection of inflammatory cytokine secretion levels by ELISA.
Supernatants from co-culture of U937/DC2.4 cells and glycopolymer-engineered HeLa cells were collected to determine the concentrations of IL-10, IL-12p70 and TNF-α using ELISA kits. Owing to the instability of NO, it’s easier and more accurate to quantify the iNOS production responsible for generating NO. The detailed processes for detecting iNOS activity are shown as follows. 100 μL of co-culture supernatants was added into EP tubes, while 100 μL of double-distilled water was added in a new EP tube as the blank group. Then 100 μL Reagent Six in the kit was added to each tube and mixed with co-culture supernatants or water. After that, 200 μL of Reagent One, 10 μL of Reagent Two and 100 μL of Reagent Three were added into tubes, respectively. The mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 100 μL of Reagent Four and 2000 μL of Reagent Five included in the kit, and the absorbance of solution in each tube was read at 530 nm. Values were determined according to the following equation:

\[
iNOS \text{ Activity (U/mL)} = \frac{OD \text{ in experimental group} - OD \text{ in blank group}}{38.3 \times 10^{-6}} \times \frac{2.51 + 0.1}{0.1} \times \frac{1}{1 \times 15} \times 1000.
\]
**Scheme S1.** Synthesis procedure for chloroalkane-conjugated chain transfer agent.

**Figure S1.** $^1$H-NMR spectrum of tert-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate (B) in CDCl$_3$. 
**Figure S2.** $^1$H-NMR spectrum of 2-(2-(chloromethoxy)ethoxy)ethan-1-amine (C) in CDCl$_3$.

**Figure S3.** $^1$H-NMR spectrum of 5-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)-2-cyano-5-oxopentan-2-yl benzodithioate (A) in CDCl$_3$. 
Figure S4. $^{13}$C-NMR spectrum of 5-((2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)-2-cyano-5-oxopentan-2-yl benzodithioate (A) in CDCl$_3$.

Figure S5. FT-IR spectrum of A, showing peaks at 1653 cm$^{-1}$, 1443 cm$^{-1}$ and 3318 cm$^{-1}$ attributed to C=O, C–N and N–H, respectively, in amide bonds; and peaks at 2931 cm$^{-1}$ and 2851 cm$^{-1}$ attributed to methylene groups. The peak at 1227 cm$^{-1}$ is attributed to C=S bonds in thioesters.
Figure S6. $^1$H-NMR spectrum of synthesized pMAG in D$_2$O.

Figure S7. $^1$H-NMR spectrum of the synthesized pMAM in D$_2$O.
Figure S8. $^1$H-NMR spectrum of synthesized pMB in D$_2$O. The molar content of biotin groups in the glycopolymers is 2.5%.

Figure S9. SEC traces of synthetic glycopolymers using 70% TFA/H$_2$O (0.1%) and 30% TFA/CAN (0.1%) as the mobile phase.

Table S1. Molecular weights of the synthesized glycopolymers.

| Glycopolymers | [M]: I: CTA | $M_n$ (1H NMR) | $M_n$ (Theoretical) | $M_n$ (SEC) | $M_n$/$M_n$ (SEC) | Conversion (%) |
|---------------|-------------|----------------|---------------------|-------------|-----------------|---------------|
| pMB          | 100:0.5:1   | 9900           | 9970                | 6010        | 1.25            | 37.3          |
| pMAG         | 100:0.5:1   | 18500          | 13400               | 5740        | 1.17            | 52.2          |
| pMAM         | 100:0.5:1   | 20300          | 13500               | 5220        | 1.21            | 52.5          |

[a][MAG]: [biotin monomer] = 9:1.
Figure S10. Composition of the HTP plasmid. The Hemagglutinin A (HA) epitope was added for labeling, and the PDGFR transmembrane domain was inserted to achieve cell membrane-bound HTP.

Figure S11. Confocal fluorescence imaging of HeLa cells stably expressing HTP using immunofluorescence staining of HA.
**Figure S12.** Confocal microscope images of HeLa cells expressing HTP stably, incubated for 1 h with 0.1 mg/mL of pMB at 37°C. The cells were stained with FITC-avidin (10 μg/mL) before imaging.

**Figure S13.** HeLa cells lacking HTP expression do not incorporate glycopolymers into their membranes. HeLa cells were incubated with pMB for 1 h at 37 °C, and stained with FITC-avidin (green). No appreciable biotin signal (green) was observed. Also no green fluorescence signal was observed in the cells stably expressing HTP without pMB incubation.
Figure S14. Cell lysis upon incubation U937 with HTP-HeLa or glycopolymers-engineered HeLa cells over 2 days by LDH cytotoxicity assay (** p < 0.01 by t-test). Natural or glycopolymers-modified HeLa cells with U937 were co-cultured at 1:1 ratio.
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