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Targeting and liposomal drug delivery to CD40L expressing T cells for treatment of autoimmune diseases

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ABSTRACT

CD40L is considered as an important target for the treatment of autoimmune diseases. There have been many efforts devoted to the development of antibodies and other molecules to disrupt CD40/CD40L interaction for therapeutic benefits. In this study, we designed a CD40L specific peptide ligand — A25 based on CD40L crystal structure and molecular docking studies. Its binding affinity and specificity to CD40L were confirmed by Surface Plasmon Resonance (SPR) measurements. The peptide A25 was then conjugated on the surface of liposomes and shown to be able to mediate specific liposomal drug delivery to CD40L+ cells. Loaded with the cytostatic drug methotrexate (MTX), the A25 modified liposome could significantly reduce the CD40L+ cell ratios in the experimental autoimmune encephalomyelitis (EAE) mice, resulting in great improvement in clinical scores. Since CD40L+ cells are involved in the pathological development of many auto-immune diseases, A25 conjugated drug targeting systems may be useful for developing therapies that are more efficacies and with less side effects.

1. Introduction

CD40L (CD154) is a 39KD type II transmembrane protein that belongs to the TNF superfamily [1]. It is mainly expressed on activated T lymphocytes, activated platelets, activated B lymphocytes etc. [2,3]. It mainly interacts with CD40 and plays prominent role in many immunological and pathophysiological processes [4]. In patients suffering from autoimmune diseases such as rheumatoid arthritis (RA) [5], multiple sclerosis (MS) [6,7], and systemic lupus erythematosus (SLE) [8], CD40L expression was found to be elevated in certain T cell populations. Therefore, it is considered as a valuable target for drug development to treat autoimmune diseases, as well as related symptoms such as allograft rejection and atherosclerosis [9–12].

Most studies up to date have been focusing on developing antibodies against CD40L to block CD40-CD40L interaction to intercept the abnormal T/B cell activation. Anti-CD40L bindings were shown to result in depletion of CD40L+ activated T cells [13]. But the anti-CD40L antibodies that were developed and tested in clinic studies [14,15] (Ruplizumab and Toralizumab) were both found to lead to thromboembolic incidences [16,17], presumably due to the vascade effect involving antibody Fc domain [18,19].

Smaller antibody fragments such as the F(ab′)2 from CD40L mAb hu5c8 were proposed to block CD40/CD40L interaction but avoid the thromboembolic effects. However, the therapeutic efficacy was lower without the Fc mediated cytotoxicity and effector functions [20,21]. Even smaller antagonist against CD40/CD40L binding was also reported. Fournel S., et al. designed a peptidomimetic molecule based on the CD40L trimer assembly [22,23]. It was found to be able to block the binding of CD40L to CD40 in cell based assays and did not have any thromboembolic effect [24].

Based on these findings, we designed a peptide ligand that can specifically bind to the trimeric CD40L structure and developed a liposome carrier system containing the peptide for the cytostatic drug methotrexate (MTX). We showed that such a ligand–drug–carrier combination could bind to CD40L+ positive cells and specifically delete CD40L+ activated T cells. Such an effect could be highly desirable in treating various autoimmune diseases.

2. Materials and methods

2.1. Materials and reagents

All the peptides used including MOG35-55 (MEVGWYRSPFSRVVH LYRNGK), A25 (ESEEEDGGGC), A25c (DSDDDEGGGC) and other peptide library components were synthesized by Chengdu Kaijie Biopharm Co. Ltd. (Chengdu, CHN). They were all HPLC purified with purity >95%.

Abbreviations: hCD40L, human CD40L; TEM, transmission electron microscopy; PEG, polyethylene glycol; MTX, methotrexate; PDI, polydispersity index; TPA, phorbol ester 12-O-tetradec-anoylphorbol-13-acetate; EAE, experimental autoimmune encephalomyelitis; mCD40L, mouse CD40L

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Table 1
The list of peptide sequences by Autodock energy ranking.

Label	Sequence	Lowest docking energy (kcal/mol)
A25	ESEEED	−19.99
C40	TSEKYE	−19.42
C20	TSEEYE	−19.22
A84	ESEKQD	−19
C15	TSEELE	−18.95
A117	ESEYQD	−18.67

The docking energies of peptides were generated by Autodock software, based on crystal structure of CD40L. Genetic Algorithm-Local Search was used to calculate, the lowest docking energy was ranked, the peptide with lowest docking energy was selected as candidate.

Recombinant human CD40 and CD40L were purchased from R&D Systems (Minneapolis, US).

1,2-Distearoylsn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (DSPE-PEG2000), 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide(Polyethylene-Glycol)2000] (DSPE-PEG2000-Maleimide), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids (AL, US). EggPC was from Japan NOF Corporation (Tokyo, Japan). N-(fluorescein-5-thiocarbamoyl-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine) (fluorescein-DHPE) was from Invitrogen Corporation (USA).

PE labeled anti-hCD40L, anti-mCD40L, anti-mCD80 and FITC labeled anti-mCD3 antibodies were purchased from eBioscience (San Diego, US). Recombinant mouse-soluble CD40L kit (rmsCD40L plus enhancer) was from Enzo (US). Methotrexate (MTX), ionomycin, hyaluronidase, and Complete Freund's Adjuvant were from Sigma (US). Tert-butanol, trifluoroacetic acid (TFA, HPLC grade), acetonitrile, (ACN, HPLC grade) and pertussis toxin (PT) were all from Merck Chemicals (Darmstadt, GER). Phorbol ester 12-O-tetradec-anoylphorbol-13-acetate (TPA) was purchased from Cell Signaling Technology (US). All other chemicals of analytical grade were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, CHN).

2.2. Peptide–PEG–DSPE synthesis

Various peptides were conjugated to DSPE-PEG2000 via the maleimide functional group. Specifically, DSPE–PEG2000–Maleimide was suspended in 50 mM HEPES buffer (pH 6.5, degassing) containing the peptides. The molar ratio of peptide to lipid was 3:2. They were mixed well and incubated overnight at 10 °C under nitrogen protection. After the reaction, the solution was dialyzed (Thermo Fisher Scientific, MWCO 3.5K, Rockford, US) against distil H_2O overnight to remove excess peptide. The conjugation efficiency was confirmed to be over 99% by HPLC analysis. HPLC analysis used an Agilent 1100 Series HPLC system with G1314A variable wavelength detector (Agilent Technologies, CA, USA) and the HPLC column used was ZORBAX 300SB C3 chromatographic column (5 μm, 4.6 × 150 mm, Agilent Technologies). The mobile phase linear gradient was changed from 5% A (95% B) to 95% A (5% B). A = 0.1% TFA in ACN and B = 0.1% TFA in water. The flow rate was 1 ml/min, and detection was set at 215 nm.

2.3. Peptide docking

Various peptide structures were docked onto a CD40L dimer structure using the AUTODOCK3.0.5 program on the Shanghai Supercomputer Center Shuguang A4000. The CD40L dimer structure was obtained based on the A&C chain of hCD40L trimer crystal structure from PDB (ID: 1I9R) and optimized by SPDBV3.7. Genetic Algorithm-Local Search was used to calculate the docking energy. The population size was set at 50, and 3000 generations were evaluated. The maximum number of energy valuation was set at 100,000,000 times. The lowest docking energy was ranked for the selection of candidate peptide sequences.

2.4. SPR analysis

The bindings between various peptides with hCD40L trimer were evaluated using BIAcore X100 (GE healthcare, US). The inhibition assay (solution competitive binding strategy) was employed. Specifically, the CM5 sensor chip (GE healthcare, US) was activated with 0.2 M EDC and 0.05 M NHS for 7 min at 5 ml/min flow rate. Then CD40 solution was added containing 10 mM NaAc (pH 4.5) for 10 min (30 μg/min flow rate), followed by 1 M ethanolamine hydrochloride (GE healthcare, US) to block the chip. For the binding experiment, hCD40L were mixed with the peptides at different ratios in HBS-EP buffer (GE healthcare, US) and injected (flow rate hCD40L 20 ng/min). The SPR signal changes were calculated to evaluate the peptide inhibition abilities.

2.5. Preparation of the peptide–drug–liposome system

Peptide–PEG2000–DSPE was synthesized as described. They were combined with eggPC, cholesterol and fluorescein–DHPE at the molar ratio of 2:70:30:1 to make fluorescence labeled peptide–liposomes. For the peptide–drug–liposomes, peptide–PEG2000–DSPE was mixed with DPPC and cholesterol at the ratio of 2:56:42. The lipid mixtures were dissolved in tert-butanol, lyophilized thoroughly, and then rehydrate with PBS (with or without 1 mg/ml of methotrexate). The resulted liposome solutions were freeze–thaw 5 times and extruded through

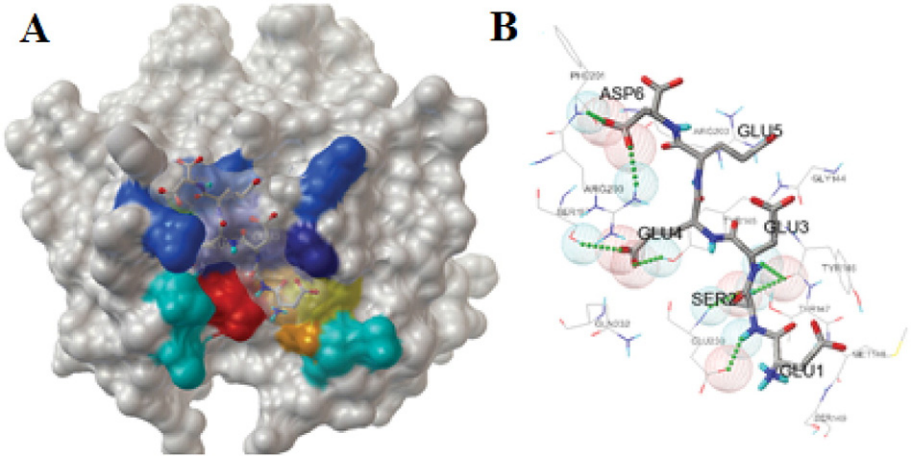

Fig. 1. The model of the interaction between A25 and hCD40L. (A): Steric complementarity between the peptide A25 and hCD40L. (B): The lowest energy docked conformation of A25 inside the hCD40L pocket.