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Effective suppression of C5a-induced proinflammatory response using anti-human C5a repebody

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ABSTRACT

The strongest anaphylatoxin, C5a, plays a critical role in the proinflammatory responses, causing the pathogenesis of a number of inflammatory diseases including sepsis, asthma, and rheumatoid arthritis. Inhibitors of C5a thus have great potential as therapeutics for various inflammatory disorders. Herein, we present the development of a high-affinity repebody against human C5a (hC5a), which effectively suppresses the proinflammatory response. A repebody scaffold composed of leucine-rich repeat (LRR) modules was previously developed as an alternative protein scaffold. A repebody specifically binding to hC5a was selected through a phage display, and its affinity was increased up to 5 nM using modular engineering. The repebody was shown to effectively inhibit the production of C5a-induced proinflammatory cytokines by human monocytes. To obtain insight into a mode of action by the repebody, we determined its crystal structure in complex with hC5a. A structural analysis revealed that the repebody binds to the D1 and D3 regions of hC5a, overlapping several epitope residues with the hC5a receptor (hC5aR). It is thus likely that the repebody suppresses the hC5a-mediated immune response in monocytes by blocking the binding of hC5a to its receptor. The anti-hC5a repebody can be developed as a potential therapeutic for C5a-involved inflammatory diseases.

1. Introduction

The complement system plays a crucial role in the immune response, especially in a host defense system [1]. Upon activation of the complement system, anaphylatoxins such as C3a and C5a are generated through their cleavage at a specific site, and act directly on neutrophils and monocytes, triggering proinflammatory responses against bacterial pathogens and foreign cells [2]. However, the dysregulation of such complement activation has been implicated in numerous clinical states, including sepsis [3,4], asthma [5,6], chronic obstructive pulmonary disease [7,8], and age-related macular degeneration [9,10], which are mainly caused by the strongest anaphylatoxin, C5a. Most of the functional effects by C5a proceed through interaction with its receptor (C5aR; same as CD88) which belongs to a G protein-coupled receptor [11,12]. C5a binds to its receptor through three distinct regions, inducing intracellular signal transduction to proinflammatory responses such as cytokine production [12]. In this regard, the three regions on C5a are considered an effective target for preventing the C5a/C5aR interactions, and accordingly, the downregulating inflammatory response. Over the past decade, significant effort has been focused on the development of C5a-targeting therapeutics for inflammatory diseases [13,14].

We previously developed a repebody scaffold composed of leucine-rich repeat (LRR) modules [15]. The repebody was shown to offer distinct advantages as a non-antibody scaffold, including a high bacterial expression level, high stability, and easy engineering [15,16]. A modular engineering approach was revealed to be effective for increasing the binding affinity of a target-specific repebody [17–19]. Herein, we present the development of a high-affinity repebody specific for human C5a (hC5a) that effectively inhibits the proinflammatory response. An anti-hC5a repebody was selected through a phage-display, and its binding affinity was

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increased up to a low nanomolar range through a modular evolution approach. The resulting repebody was shown to remarkably suppress the hC5a-induced cytokine production from human monocytes. We determined the crystal structure of the repebody in complex with hC5a, and investigated its mode of action. Details are reported herein.

2. Materials and methods

2.1. Selection of repebodies specific for hC5a

The selection of repebodies specific for hC5a was carried out through a phage display, as described in our previous work [15,17].

2.2. Protein expression and purification

hC5a with an N-terminal His_6-tag and repebodies with C-terminal His_6-tag were cloned into a pET21a vector. The constructs were transformed into an Origami B (DE3). The cells were grown in an LB medium and induced with 0.5 mM IPTG. After incubation for 20 h at 18 °C, the cells were disrupted through sonication. After 1 h of centrifugation, the supernatant was loaded onto a Ni-NTA column (Qiagen). The protein was eluted using a buffer containing 250 mM imidazole. The eluted protein was further purified through a gel permeation chromatography (Superdex 75, GE Healthcare) with PBS (pH 7.4).

2.3. Phage enzyme-linked immunosorbent assay (Phage-ELISA)

hC5a (10 μg/ml) was immobilized on a 96-well maxisorp plate (Nunc). After blocking with TPBSA, phage-displayed repebodies were added. The wells were washed three times using TPBS, followed by incubation with an HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare) in TPBSA. After the removal of the antibody, a 3,3′,5,5'-tetramethylbenzidine (TMB) solution (Sigma-Aldrich) was added, and the reaction was stopped by adding 1 N H_2SO_4. The signal was scanned using an Infinite M200 plate reader (Tecan) at 450 nm. The specificity of the repebody was tested as described above.

2.4. Isothermal titration calorimetry (ITC)

The binding affinity of the repebodies was measured through ITC (iTC 200; MicroCal) in PBS (pH 7.4) at 25 °C. 0.2 mM of a repebody in a syringe was injected into 0.02 mM hC5a in a cell. The dissociation constant was calculated using the Origin program (OriginLab, Northampton, MA).

2.5. Crystallography and structure determination

The repebody (r-3E8) and hC5a without N-terminal His_6-tag cut using thrombin were prepared. Crystals of the repebody in complex with hC5a (38 mg/mL) were grown in 1200 different conditions through a sitting-drop method using a Mosquito (TTP Labtech, UK). The crystals obtained through the first screening process were grown using a hanging-drop method. The optimized condition for crystallization was 0.1 M sodium acetate (pH 5.1–5.3), 2% (w/v) PEG 400, and 1.8–2.0 M ammonium sulfate. The diffraction data of the crystals were collected using an X-ray source at the PAL5C beam line of the Pohang Accelerator Laboratory (Pohang, Korea). The data were processed using a HKL2000 package. The crystal belonged to space group $P222_1$. The program PHENIX was utilized for molecular replacement (MR) using the structure of a repebody (PDB ID: 3RFS) as the search model. The electron density of hC5a was found and modeled after obtaining the initial solution using the search model. All figures were produced using a PyMOL program. The structure of the r-3E8/hC5a complex was deposited into the Protein Data Bank (PDB ID: 5B4P).

2.6. Isolation and culture of human monocytes

Venous blood was drawn from healthy subjects into sterile blood collection tubes, and peripheral blood mononuclear cells (PBMC) were isolated through density sedimentation over Histopaque-1077 (Sigma-Aldrich). All of the healthy control subjects provided their informed consent before enrolling in the study. The cells were incubated for 1 h at 37 °C, and nonadherent cells were removed by pipetting off the supernatant. Adherent monocytes were collected as previously described [20]. The recovered cells were >95% $CD14^+$, as determined through the flow cytometry with an anti-CD14 antibody. The cells were then incubated at 37 °C in a humidified, 5% CO_2 atmosphere until used in the experiments. All experimental procedures were approved by the Chungnam National University Institutional Research and Ethics Committee.

2.7. Quantitative cytokine ELISA

Human monocytes were seeded at 1×10^6 cells/mL and serum-deprived overnight in an incubator. Following pre-incubation with varying amounts of repebody (0.1, 1, 5, and 10 μg/mL) for 1 h, the cells were stimulated with lipopolysaccharides (LPS, 100 ng/ml; Sigma-Aldrich L3024) and hC5a (10 μg/mL). After incubation for 18 h, the supernatants were collected, and their cytokine levels were determined using individual ELISA kits from Duo-Set Ab pairs (BD Pharmingen) according to the manufacturer's instructions.

3. Results

3.1. Selection of repebodies specifically binding to hC5a

To select repebodies specifically binding to hC5a, we constructed a repebody library by introducing random mutations into six hypervariable sites of two adjoining modules, LRRV2 and LRRV3 (Fig. 1A), as described in our previous work [15,18]. The repebody library, which included approximately 10^8 clones, was displayed on a pIII M13 phage coat protein. After five rounds of a standard panning process applied to hC5a, nine repebodies specific for hC5a were selected. A sequence analysis of the selected repebodies revealed three distinct amino acid sequences at variable sites. We determined their binding affinities through isothermal titration calorimetry (ITC), and finally chose r-1G7 which had the highest binding affinity ($K_d = 2.5$ μM) for the affinity maturation (Figs. 1B and 2A).

3.2. Affinity maturation through modular evolution approach

C5a is known to trigger numerous immune responses by binding to its receptor, activating intracellular signal pathways. The dissociation constant of hC5a against its receptor has been reported to be around 1–5 nM, which is much lower than that of r-1G7. We thus intended to increase the binding affinity of r-1G7 up to a low nanomolar range to effectively block the binding of hC5a to its receptor using a modular evolution approach, as described in our previous work [17]. A repebody library was constructed by introducing random mutations into four hypervariable sites of a nearby module (LRRV5), and three sites of the module LRRVe on r-1G7 (Fig. 1A). At the same time, we removed the C-terminal loop (PDB ID: 3RFS, residues 239 through 246), which appears to interfere with the binding of hC5a to r-1G7 owing to a steric hindrance. After five rounds of the standard panning process, 15 clones were