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

Da-Eun Hwang a, Jung-Min Choia, Chul-Su Yang b, Joong-jae Lee a, Woosung Heua, Eun-Kyeong Joc, Hak-Sung Kim a, *

a Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 305-701, Republic of Korea
b Department of Molecular and Life Sciences, Hanyang University, Ansan, 15588, Republic of Korea
c Department of Microbiology, Chungnam National University College of Medicine, Daejeon, Republic of Korea

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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.

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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
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 His6-tag and repebodies with C-terminal His6-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%,5%,5%-tetramethylbenzidine (TMB) solution (Sigma-Aldrich) was added, and the reaction was stopped by adding 1 N H2SO4. 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 His6-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 P2_2_2_1. The program PHENIX was utilized for molecular replacement (MR) using the structure of a repebody (PDB ID: 3FRS) 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% CO₂ 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 × 10⁶ 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 Pharmsingen) 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 10⁸ clones, was displayed on a pIIIL 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 (Kₐ = 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 LRRV6 on r-1G7 (Fig. 1A). At the same time, we removed the C-terminal loop (PDB ID: 3FRS, 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...
selected, and their sequences were determined. We measured their binding affinities, and selected r-2F5 (Fig. 1B), which showed the lowest dissociation constant of 33.4 nM (Fig. 2A), which corresponds to about a 75-fold increase compared to r-1G7. To check whether the C-terminal loop of r-2F5 interferes with the binding to hC5a, we constructed an r-2F5 variant containing the loop. As a result, the binding affinity of the r-2F5 variant was shown to decrease to 76.3 nM (Supplementary Fig. S1), which indicates that the C-terminal loop of r-1G7 intervenes with an interaction between r-1G7 and hC5a. To further increase the binding affinity, we conducted a second affinity maturation based on the clone r-2F5.

We constructed a third library by introducing mutations into four residues on the module LRRV4 of r-2F5 (Fig. 1A). To select repabodies with a higher affinity than r-2F5, we used r-2F5 as a competitor and increased its level up to 800 μg/mL during the panning process. As a result, we finally selected r-3E8, which showed a binding affinity of 5.0 nM (Figs. 1B and 2A), which corresponds to about a 500-fold and a 7-fold increase compared to r-1G7 and r-2F5, respectively. It is interesting to note that the selected r-3E8 has a comparable binding affinity for C5a to the C5a receptor. This result also demonstrates the general applicability and effectiveness of the modular engineering approach to the affinity maturation of a target-specific repobody.

3.3. Characterization of selected repabodies

We next characterized the selected repabodies (r-1G7, r-2F5 and r-3E8) in terms of their secondary structure and specificity. The repabodies were expressed in E. coli Origami B (DE3) cells, and purified using a C-terminal His6-tag and size-exclusion chromatography. A circular dichroism (CD) analysis of the repabodies revealed that the selected repabodies have almost the same secondary structure as a template repobody scaffold (Fig. 2B), which indicates that the selected repabodies retain the intact structure despite numerous mutations. We examine the specificity of the finally selected r-3E8 using a phage-ELISA. Three complement anaphylatoxins (C3a, C4a, and C5a) are known to share a similar fold, and hC5a possesses 43.6% and 38.5% of the amino acid sequence similarity with hC3a and hC4a, respectively. We tested hC3a, which shows a higher sequence similarity, and observed that r-3E8 had a negligible binding activity against hC3a (Fig. 2C). Furthermore, the repobody showed a negligible binding affinity against mouse C5a (mC5a) (Fig. 2C), indicating the high specificity of the selected repobody toward hC5a.

3.4. Suppression of hC5a-induced cytokine production by the repobody

We assessed the inhibitory effect of r-3E8 on the immune response in vitro. The binding of hC5a to its receptor on the monocytes leads to the production of various cytokines such as TNF-α, IL-6, and IL-1β through activation of relevant signaling pathways, thereby resulting in the upregulation of immune responses against foreign invaders [21,22]. However, the excessive amounts of proinflammatory cytokines are known to cause severe pathological development including sepsis, asthma, and rheumatoid arthritis [1]. To investigate whether r-3E8 inhibits C5a-induced immune response in vitro, we isolated human primary monocytes from PBMC and cultured them under serum-deprived conditions. The monocytes were pre-incubated with varying repobody concentrations for 1 h, followed by stimulation with hC5a (10 μg/mL). After incubation for 18 h, the levels of three cytokines (TNF-α, IL-6, and IL-1β) in the culture medium were analyzed using ELISA. As shown in Fig. 3, r-3E8 effectively inhibited the production of proinflammatory cytokines. LPS is a major component of a bacterial outer membrane, and is known to elicit an immune response...
through binding to a toll-like receptor [23]. It has been reported that C5a synergistically enhances the LPS-induced cytokine production by immune cells in vitro [22]. To check whether the repebody can also suppress the synergistic effect of C5a and LPS, monocytes were pre-incubated with varying concentrations of r-3E8, and stimulated with *E. coli*-derived LPS (100 ng/mL) and hC5a (10 μg/mL). As a result of a cytokine analysis in a culture medium, r-3E8 was shown to significantly inhibit the synergistic effect of hC5a and LPS on the production of the three cytokines (TNF-α, IL-6, and IL-1β) by the monocytes in a dose-dependent manner (Fig. 3). On the other hand, r-2F5 exhibited no significant effect on hC5a-induced inflammatory response mainly owing to its weak binding affinity compared to the hC5a receptor (Supplementary Fig. S2). Based on the result, it is clear that the hC5a-specific repebody (r-3E8) inhibits the immune response of monocytes triggered by hC5a alone, as well as by hC5a and bacterial LPS, implying the potential of the repebody as a therapeutic for the C5a-mediated inflammatory diseases.

3.5. Crystal structure of r-3E8 in complex with hC5a

To obtain insight into a mode of action by anti-hC5a repebody, we solved the crystal structure of r-3E8 in a complex with hC5a at a resolution of 2.4 Å (Table S1). The crystal structure was determined through a molecular replacement method using the structure of hC5a (PDB ID: 1KJS) and the model structure of r-3E8. In a complex crystal structure, the repebody was shown to retain a horseshoe-like structure, and hC5a preserves four α-helices connected by three loops (D1, D2, and D3) (Fig. 4A), as described elsewhere [12]. The repebody binds to hC5a through the concave region where the libraries were generated, and hC5a interacts with the repebody mainly through the loop regions, D1 (residues 28 through 33) and D3 (residues 38 through 46). In particular, Arg46 of the D3 region in hC5a creates a hydrogen bonding network with Asp93, Leu115, and Asp93 of the repebody, which comprises six possible hydrogen bonds, residing Arg46 in the center of the network (Fig. 4B). Thus, Arg46 of hC5a is considered to be the key residue of the binding
interface. Interestingly, Arg46 of hC5a is equivalent to Leu49 in mC5a, and the repebody showed no cross-reactivity to mC5a despite a high sequence similarity between human and mouse C5a. To investigate the importance of a hydrogen bonding network and Arg46 of hC5a in a repebody/hC5a complex, we conducted a mutational analysis for hC5a. We mutated Arg46 of hC5a into Leu, and measured the binding affinity of hC5aR46L mutant toward the repebody. As a result, the binding affinity was difficult to determine through ITC owing to the overly weak interaction between them, which indicates that the hydrogen bonding network centered by Arg46 of hC5a is particularly crucial for interactions between the repebody and hC5a (Supplementary Fig. S3). Moreover, it was elucidated that the high specificity of the repebody toward hC5a predominantly arises from the key residue Arg46, equivalent to Leu49 in mC5a, and the repebody showed no cross-reactivity to mC5a despite a high sequence similarity between human and mouse C5a. To investigate the importance of a hydrogen bonding network and Arg46 of hC5a in a repebody/hC5a complex, we conducted a mutational analysis for hC5a. We mutated Arg46 of hC5a into Leu, and measured the binding affinity of hC5aR46L mutant toward the repebody. As a result, the binding affinity was difficult to determine through ITC owing to the overly weak interaction between them, which indicates that the hydrogen bonding network centered by Arg46 of hC5a is particularly crucial for interactions between the repebody and hC5a (Supplementary Fig. S3). Moreover, it was elucidated that the high specificity of the repebody toward hC5a predominantly arises from the key residue Arg46 in hC5a, which is equivalent to Leu49 in mC5a and Ala48 in hC3a. Because Leu49 in mC5a and Ala48 in hC3a are unable to generate hydrogen bonds with the repebody, a negligible interaction was observed between the repebody and either mC5a or hC3a. In addition to Arg46, the repebody shades the D1 region of hC5a through weak interactions (Supplementary Fig. S4). The D1 region of hC5a and Arg46 of hC5a are primarily binding sites for an hC5a receptor [12]. Our structural analysis revealed that the repebody creates a strong hydrogen bonding network with Arg46, and has weak interactions with the D1 region. Based on this result, it is likely that the repebody effectively causes a steric hindrance to the interaction between hC5a and its receptor, thereby inhibiting the hC5a-mediated immune response in monocytes.

4. Discussion

We demonstrated that the hC5a-specific repebody effectively suppresses proinflammatory response in vitro by blocking the binding of hC5a to its receptor. C5a is known to play a key role in the host defense system against foreign invaders. However, the elevated level of C5a in the plasma is implicated in the pathogenesis of numerous inflammatory diseases. Previous studies have suggested that hC5a is a promising target for the development of therapeutics against inflammatory diseases. The developed repebody with a binding affinity of about 5 nM was shown to strongly inhibit the production of proinflammatory cytokines from human monocytes such as TNF-α, IL-6, and IL-1β, which have been known to be involved in a number of causes of inflammatory pathogenesis [24–26]. A structural analysis of the repebody in complex with hC5a revealed that the repebody effectively interferes with the binding of hC5a to its receptor, demonstrating the mode of action by the repebody in the hC5a-mediated signaling process in monocytes. Taken together, it is likely that an anti-hC5a repebody can be developed as a potential therapeutic for inflammatory diseases.

Construction of a repebody library on the concave region followed by a phage display enabled the selection of hC5a-specific repebodies. A modular evolution approach was shown to be efficient and straightforward for increasing the binding affinity of a
receptor up to a low nanomolar range, allowing for easy development of a repebody with desired binding affinity for its target. The third repeated cycles led to an increase in the binding affinity of a repebody by around 500-fold compared to the initially selected r-1G7, which indicates the efficiency and utility of the modular evolution approach. This approach involves the optimization of the binding interface between the repebody and target in a module-by-module manner, and seems to be applied to repeat proteins composed of consensus modules. Our previous studies also demonstrated the utility and efficiency of the modular engineering approach in the affinity maturation of a repebody. It is noteworthy that the developed repebody showed high specificity toward hC5a, exhibiting a negligible cross-reactivity against other human anaphylatoxins and mC5a. Such specificity reflects the distinct feature of a repebody, considering that diverse repeat proteins composed of LRR modules are involved in many protein-protein interactions in nature. The developed repebody effectively suppressed the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β from human monocytes stimulated with C5a.

A structural analysis revealed that Arg46 of hC5a creates six hydrogen bonds with Asp93, Leu115, and Asp140 in r-3E8, providing detailed information on the binding interface between the repebody and hC5a. A mutational study showed that the R46L mutation of hC5a results in a loss of interaction with the repebody, indicating that such hydrogen bonds contribute significantly to the binding of the repebody to hC5a. This result also implies that the high specificity of the repebody stems from the hydrogen bonding networks, considering that the repebody cannot generate strong interactions with Leu49 in mC5a, and Ala48 in hC3a, which are equivalent to Arg46 of hC5a. In addition, the repebody was shown to constitute the binding interface with the D1 and D3 regions in hC5a, which are overlapping epitope sites with the hC5aR, demonstrating the mode of action by the repebody in the hC5a-mediated signaling process. It is thus plausible that the repebody has an inhibitory effect on the hC5a-induced immune response by blocking hC5a from binding to its receptor through a steric clash with the hC5aR. Taken together, the repebody with a high affinity for hC5a exhibited a remarkable inhibitory effect on the proinflammatory response in vitro by blocking the binding of hC5a to its receptor through a steric hindrance. It is expected that an anti-hC5a repebody can be developed as a potential therapeutic agent for numerous inflammatory diseases.

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Appendix A. Supplementary data

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Transparency document

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