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Development of autologous C5 vaccine nanoparticles to reduce intravascular hemolysis *in vivo*

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Abstract

The complement system is emerging as a new target for treating many diseases. For example, Eculizumab, a humanized monoclonal antibody against complement component 5 (C5), has been approved for paroxysmal nocturnal hemoglobinuria (PNH) in which patient erythrocytes are lysed by complement. In this study, we developed vaccines to elicit autologous anti-C5 antibody production in mice for complement inhibition. Immunization of mice with a conservative C5 xenoprotein raised high titers of IgGs against the xenogenous C5, but these antibodies did not reduce C5 activity in the blood. In contrast, an autologous mouse C5 vaccine containing multiple predicted epitopes together with a tolerance-breaking peptide was found to induce anti-C5 autoantibody production *in vivo*, resulting decreased hemolytic activity in the blood. We further validated a peptide epitope within this C5 vaccine and created recombinant virus-like particles (VLPs) displaying this epitope fused with the tolerance breaking peptide. Immunizing mice with these novel nanoparticles elicited strong humoral responses against recombinant mouse C5, reduced hemolytic activity, and protected the mice from complement-mediated intravascular hemolysis in a model of PNH. This proof-of-concept study demonstrated that autologous C5-based vaccines could be an effective alternative or supplement for treating complement-mediated diseases such as PNH.

Graphical abstract

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AUTHOR CONTRIBUTIONS

L. Zhang., W. Qiu., S. Crooke, Y. Li and A. Abid performed experiments and analyzed data; B.Xu. constructed the mouse C5 homology model; M.Finn. supervised the preparation of VLPs, analyzed data and wrote the manuscript together with F.Lin.; F.Lin. conceived the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests

Introduction

The complement system is an important part of the innate immune system, consisting of multiple proteins present at varying levels in the blood(1). When complement is activated through any of three general pathways (by antibody-antigen complexes through the classical pathway, by certain carbohydrates through the lectin pathway, or spontaneously through the alternative pathway), C3 is activated to form C5 convertase and cleaves C5 into C5b and C5a. When C5 is activated in this way, membrane attack complexes (MACs) consisting of C5b-9 are assembled and form pores through the cell membrane, resulting in lysis or damage of the target cell. At the same time, the small fragment C5a is released into the fluid phase and binds to its receptor (C5aR) on nearby immune cells to promote inflammatory reactions. Activated complement cannot distinguish self from foreign cells, but self-cells express cell surface complement inhibitors, including CD55 and CD59, which confer protection from complement-mediated attack(1). Aberrant complement activation is integrally involved in many diseases, and many complement components are currently being tested as new targets for drug development(2). C5 is a promising example because its concentration in blood is relatively low ($\sim 100 \mu\text{g/mL}$ for C5 *v.s.* $\sim 1500 \mu\text{g/mL}$ for C3), and C5 inhibition should theoretically inhibit both the formation of MACs, which damage tissue cells, and suppress the release of C5a, a potent inflammation initiator that is involved in many pathological conditions.

Indeed, Eculizumab, a humanized anti-human C5 monoclonal antibody (mAb) has been successfully employed to treat complement-mediated diseases including paroxysmal nocturnal hemoglobinuria (PNH)(3) and atypical hemolytic uremic syndrome (aHUS)(4). In most patients with PNH, inactivation of the gene for phosphatidylinositol glycan A in the glycosyl phosphatidylinositol (GPI) pathway in hematopoietic stem cells results in red blood cells (RBCs) lacking the GPI-anchored cell surface complement inhibitors CD55 and CD59, rendering these cells highly susceptible to complement-mediated lysis(5). Eculizumab binds to C5 and inhibits the formation of MACs, thereby preventing complement-mediated hemolysis in PNH. Although highly effective, Eculizumab is the most expensive drug on the

market with an annual cost of more than \$400,000 USD/patient, and in most of the patients, life-long drug administration is required (6, 7). Clearly, the development of an effective and affordable C5-targeting therapy for treating PNH is a worthwhile goal.

We describe here our first exploration of the idea that eliciting a moderate immune response against the C5 protein may represent a cost-effective treatment of diseases such as PNH caused by excess complement activity. We have chosen to employ virus-like particles (VLPs) as the platform due to their immunogenic nature, well-defined structure, ability to present a wide variety of potential epitopes, and ease of production. While VLPs resemble viruses in most ways relevant to the generation of an immune response (repetitive structure, lymphatic trafficking, B cell recognition, T cell stimulation, packaged bacterial RNA)(8, 9), they are noninfectious and can act as self-adjuvants capable of breaking immune tolerance(10-12). The bacteriophage Q β is a highly effective and easily-modified VLP platform for these purposes(13). It has been reported that both the direct attachment of antigens to the surface of Q β VLPs using chemical crosslinkers and the production of recombinant Q β VLPs that display antigen epitopes on their surface using gene engineering techniques are effective in eliciting strong immune responses against various antigens for vaccine development. Such Q β VLP-based vaccines have been found to be safe in multiple Phase I and II clinical trials (14-18).

To explore the feasibility of eliciting anti-C5 antibody production from the host immune system to prevent complement-mediated hemolysis, we combined the identification of C5 epitopes using previously reported data and computational prediction with a modular strategy of VLP functionalization to produce candidate immunogens. These vaccine candidates were able to elicit anti-C5 autoantibodies and protect mice in a model of intravascular hemolysis. Our data suggest that autologous C5 vaccines could be developed as an alternative or supplement to Eculizumab for treating complement-mediated diseases such as PNH.

Results

Immunization with human C5 protein raises antibodies that do not reduce mouse C5 activity

Because 1) autoantigens are generally immune tolerized, 2) previous studies suggest that conservative antigens from different species could elicit the production of antibodies that cross-react with the autoantigens(19-23), and 3) mouse and human C5 protein share >80% homology(24), we initially immunized mice with purified human C5 protein, hoping that the developed polyclonal antibodies could cross-react with mouse C5, therefore inhibiting mouse C5 activity *in vivo*. Indeed, mice immunized with human C5 protein developed high titers of antibodies against human C5 (Fig. 1A), however, sera from these immunized mice had similar potency as sera from control mice in lysing sensitized sheep erythrocytes *ex vivo* (Fig. 1B). These results demonstrated that immunizing mice with human C5 did not develop cross-reacting antibodies to reduce mouse C5 activity *in vivo*.

Design and production of an autologous C5 vaccine with predicted protein surface epitopes together with the PADRE peptide

We then planned to immunize the mice with an autologous mouse C5 vaccine to develop mouse anti-mouse C5 autoantibodies with the goal of reducing or blocking C5 activity as in patients receiving Eculizumab. Analysis of the mouse C5 protein sequences identified 12 potential immunogenic epitopes that are likely to be on the surface of the protein (important so that the generated antibodies can have access to them under native conditions) (Table 1). We designed an artificial gene coding for all of these identified epitopes in a linear sequence together with three copies of a previously published tolerance-breaking peptide, PADRE(25, 26) (Fig. 2A). This polypeptide was expressed in *E. coli*, isolated from inclusion bodies, denatured, refolded, affinity purified via a 6 × His tag (Fig. 2B), and finally purified by reversed-phase HPLC (Fig. 2C).

Mice immunized with the autologous C5 vaccine develop C5-reactive autoantibodies and show decreased complement hemolytic activity

Mice that were immunized and boosted with the purified C5 multi-epitope polypeptide showed high IgG antibody titers against full-length recombinant mouse C5 protein by ELISA (Fig. 3A). Therefore, this C5 vaccine was able to break immune tolerance and develop a significant immune response to the autologous C5 epitopes. Furthermore, the mice receiving the C5-based polypeptide vaccine showed less complement-mediated hemolytic activity than the control mice (Fig. 3B, 3C). This suggests that the recombinant autologous C5 vaccine is effective in eliciting functional anti-C5 autoantibodies that have the intended outcome of inhibiting C5 activity.

Identification of the dominant epitopes within the autologous C5 vaccine using a synthesized overlapping peptide library

To identify which of the 12 C5 peptide sequences were the most effective in inducing the above immune response, we designed a library of overlapping 15-mer oligopeptides spanning the entire sequence of the recombinant C5 vaccine (Table 2). Using these individual peptides as ELISA probes, we assessed the IgG composition of the serum from the C5 vaccine-immunized mice and found that the sera contained high titers of IgGs against the peptide ACKPEASYKPSKEES, suggesting that this sequence contains a dominant surface epitope within the vaccine that elicited strong humoral responses.

Visualizing the C5 peptide containing the identified dominant epitope on mouse C5 three-dimensional structure model

The C5 peptide P2 (ASYKPSKEESTSGS) is originally predicted by the computer algorithms to be an immunogenic epitope exposed on the C5 protein surface and it contains the peptide sequence identified in the above-described epitope mapping experiments. To verify that this peptide is indeed on C5 protein surface for our future VLP-C5 vaccine development, we constructed a mouse C5 three-dimensional homology model based on the published human C5 crystal structure(27) using the Phyre2 protein recognition program following published protocols(28). Mouse C5 protein shares 89% homology with the human C5 sequence, providing a model with high confidence (100%) and high sequence coverage

(98%). Visualization of the location of the P2 peptide in the mouse C5 structure model showed it to be exposed on the solvent-exposed surface (Figure 4, in red), further supporting the hypothesis that it represents a suitable epitope for the development of VLP-C5 vaccines.

Preparation of a VLP-based C5 vaccine displaying the C5 epitope (P2)

A recombinant VLP bearing a linear peptide composed of the putative dominant C5 surface epitope P2 and the PADRE peptide (separated by a short spacer) was expressed as a C-terminal extension of the VLP coat protein (Fig. 5A). These particles were produced by a well-established method(29) in which a mixture of truncated and extended capsid proteins are coexpressed in *E. coli* and self-assemble into a hybrid nanoparticle in which an average of 50 copies of the extension were incorporated per capsid. The resultant VLP-C5 vaccine was characterized using dynamic light scattering, size-exclusion chromatography and gel electrophoresis (Fig. 5B,C&D), showing intact particles of the expected size (~ 30 nanometers) and composition.

Mice immunized with the VLP-C5 vaccine developed antibodies against the recombinant mouse C5 and showed reduced hemolytic activity

WT mice were immunized with either the VLP-C5 vaccine or the same amount of control VLP lacking the extended capsid component. Using the same analyses as described above, we found that mice receiving the control VLP produced no detectable recombinant mouse C5-reactive IgGs (Fig. 6A), whereas the VLP-C5 vaccine immunized mice developed high titers of IgG antibodies against intact recombinant mouse C5. Most importantly, the complement-mediated hemolytic activity of these VLP-C5 vaccine-immunized mice was also significantly reduced (Fig. 6B), indicating that this VLP-based vaccine is effective in eliciting C5-reactive autoantibody production and reducing C5 activity.

Mice immunized with the VLP vaccine are protected in a model of complement-mediated hemolytic anemia

To test whether the C5 vaccine approach is effective in protecting mice from complement-mediated intravascular hemolysis, as found in patients with PNH, we modified a previously established complement-mediated intravascular hemolytic anemia model(30). In brief, we introduced 200 μ L of C5-depleted human serum into each of the VLP-C5 vaccine-immunized mice and control mice by i.p. injection. After one hour, each mouse was bled retro-orbitally to collect sera to measure intravascular hemolysis, and urine was also collected to measure hemoglobinuria. We found that injection of C5-depleted human serum-induced much less severe hemolysis and hemoglobinuria in the VLP-C5 vaccine-immunized mice compared to the control mice (Fig. 7). This suggests that mouse C5 can replace human C5 to form functional MAC and that the VLP-C5 vaccine is effective in protecting mice from complement-mediated cell damage in vivo.

Discussion

Aberrant activity of the complement system is emerging as a target for treating certain inflammatory and autoimmune diseases(31). While excess complement activity can have many potential causes, a choke point of the system is the C5 protein, responsible for the

penultimate step in the induction of hemolytic function. Most of the complement-targeted reagents that have been approved or under development are mAbs(32); others are based on aptamers(33), peptides(34, 35), and small molecules(36). Given the life-long complement inhibition required in most of these patients, all currently available complement inhibition therapies suffer from limited half-life, high costs, and compliance issues. This is especially true in the case of the anti-C5 antibody Eculizumab, which many PNH patients have to receive by i.v. infusion every two weeks for life, at a current price of almost a half million dollars a year per patient(3, 37). Interest in C5 as a target has recently expanded to other diseases, with Eculizumab in late-stage clinical trials for conditions such as myasthenia gravis(38) and cold agglutinin disease(39) that involve excessive complement activation.

We show here proof-of-concept examples of C5 vaccine-based approaches for the production of autologous C5-reactive antibodies to reduce C5 activity for the treatment of complement-mediated diseases, mimicking the effect of Eculizumab. Vaccination with a homologous C5 from a different species (human) did not confer anti- mouse C5 function in mouse, although it did elicit a strong antibody response against the immunized human C5. This stands in contrast to many examples of the use of conservative xeno-antigens in antitumor vaccines, in which immunization elicited T- and/or B cell responses that cross-react with their mouse counterparts on tumor cells(20-23). In our case, we suggest that the overlapping epitopes between mouse and human C5 (which are more than 80% homologous) are well-tolerated in mice and that all the antibodies produced were likely against the epitopes that are unique in the human C5.

Instead, we were able to break tolerance in mouse using a linear polypeptide composed of all the computer-predicted strong immunogenic epitopes along with the PADRE sequence. We identified a potential dominant C5 peptide within these 12 computer-predicted immunogenic peptides by epitope mapping using an overlapping peptide library and constructed a mouse C5 structure model to ensure that this identified C5 peptide is exposed on the mouse C5 protein surface. We then developed VLP nanoparticles that display this dominant C5 surface peptide together with the PADRE sequence as a new autologous C5 vaccine. The VLP-based C5 vaccine proved to be especially effective at eliciting autologous anti-C5 antibody production, inhibiting C5 activity, and protecting the vaccinated mice from intravascular hemolysis and hemoglobinuria in a model that mimics PNH. Our results suggest that autologous C5 vaccines may hold potential as a supplement and/or alternative for Eculizumab for treating complement-mediated diseases. Of course, whether the human counterparts of the identified mouse C5 epitopes work in humans need to be confirmed in assays such as *in vitro* immunization using human peripheral blood mononuclear cells (40) followed by human C5 blocking experiments, and eventually, in human clinical studies.

Vaccination against C5 to have the immune system produce its own anti-C5 antibodies could represent a cost- and compliance-effective alternative to antibody infusion, with therapeutic effects prolonged by booster immunizations. Indeed, one publication has appeared testing the potential of a vaccination approach against complement in treating/preventing diseases (41). In that work, a peptide was designed to mimic one or more mouse C5a epitopes, and immunization of this peptide conjugated to carrier protein KLH elicited anti-C5a autoantibodies and protected the vaccinated mice from chronic neuroinflammation and

neuropathologic alterations in a model of Alzheimer's disease (41). This work supports the concept of complement-targeted vaccination for treating complement-mediated diseases, and it also significantly differs from the work presented here. The vaccine tested in the prior report was specific to C5a, which is a complement activation product released from activated C5, and therefore does not have an effect on C5b-9 (MAC) formation. In contrast, the C5 vaccines developed here do not target C5a, but are specific for the C5b part of C5 (the P2 epitope is within the C5b), which is the essential component to initiate the assembly of the MAC to lyse RBCs and to damage other tissues in pathological conditions.

Another important difference in these approaches is the relative concentrations of the target. The C5a protein exists in blood at very low levels (~0.5–50 ng/mL)(42, 43), whereas C5 concentration is approximately 100–200 µg/mL in the blood. It requires strong vaccines to break the tolerance to produce enough anti-C5 antibodies to significantly reduce C5 activity *in vivo*. By combining multiple epitopes and the PADRE peptide, and by using a VLP as a carrier, our approaches did indeed develop high titers of anti-C5 autoantibodies and decreased C5 activity. However, C5 activity was not completely eliminated in the immunized mice. This is significant because of the dangers inherent in the complete elimination of complement function. An eventual therapeutic application of this vaccine strategy would require a moderation, but not elimination, of C5 activity, which seems to be possible on the basis of this initial study.

Our use of the PADRE peptide was stimulated by its known ability to help break immune tolerance and to improve humoral responses against immunized antigens both in humans and in C57BL/6 mice (25, 26). PADRE works by strongly binding to 15 of 16 of the most common HLA-DR types tested to date in humans and I-A^b molecules in mice to boost T help responses to improve immune responses against both T cell- and B cell epitopes. Tests to support or refute the assumption that these mechanistic factors are important in the present application must await further studies.

It should also be noted that the use of human C5-depleted serum to induce intravascular hemolysis is a new model of situations found in PNH patients. Mouse complement is much weaker than human complement in hemolytic activity. The severe intravascular hemolysis and hemoglobinuria induced by injecting mice with C5-depleted serum, which does not have any hemolytic activity by itself, shows that mouse C5 can replace human C5 to form “hybrid” MAC to lyse mouse RBCs *in vivo*. We believe this model will be useful in other proof-of-concept studies testing mouse C5 targeted therapies in treating complement-mediated hemolysis or tissue damage.

In summary, we found that immunization of the conservative human C5 did not raise cross-reactive antibodies that reduced mouse C5 activity. We showed that immunization of a recombinant mouse C5 vaccine composed of multiple potential protein surface B cell epitopes and the PADRE peptides broke immune tolerance of C5 in mice and decreased C5 activity. Finally, we demonstrated that immunization of a VLP vaccine displaying one of the identified strong mouse C5 surface epitopes together with the PADRE peptide elicited strong humoral responses against mouse C5, reduced C5 activity and protected mice from complement-mediated hemolysis and hemoglobinuria in a model of intravascular hemolysis,

which closely mimic the situations found in PNH patients. Our results suggest that C5 auto-vaccines, especially the ones on the VLP platform, could be developed as a supplement and/or alternative for Eculizumab for treating complement-mediated diseases, especially those in which complement activation needs to be controlled for the rest of the life.

Materials and Methods

Mice and complement reagents

C57BL/6 WT mice were purchased from the Jackson Laboratory and maintained in the animal facility of Cleveland Clinic. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Cleveland Clinic. Purified human C5 protein and pooled human C5-depleted serum were purchased from Complement Technology Inc (Tylor, TX)

Mouse C5 vaccine design

The mouse C5 protein sequence was analyzed using Optimum Antigen Design™ software (Genscript, NJ) which utilizes an advanced antigen design algorithm based on several protein databases. The identified epitopes were compared with the published C5 protein crystal structure(27) to check for surface exposure. These steps provided twelve potential immunogenic epitopes that are likely present on the surface of the native mouse protein. An artificial gene was designed coding for a polypeptide comprised of these twelve potential epitopes interspersed in three places with a copy of the non-natural pan-DR epitope (PADRE) sequence (a linear peptide that improves humoral responses against antigens and helps break immune tolerance(25, 26)), and capped at the C-terminus with a 6XHis tag.

Mouse C5 vaccine expression and purification

The custom-synthesized artificial gene (Genscript, NJ) was cloned into the PET-21b expression vector and transformed into *E.coli* strain BL21. Expression of the desired protein, intended to be a recombinant mouse C5 vaccine, was induced using the Overnight Express™ Autoinduction System (EMD Millipore, MA) following manufacturer provided protocols. The protein formed inclusion bodies and was isolated using the B-PER® Bacterial Protein Extraction Reagent (Thermo Fisher, IL), denatured in 8 M urea, and refolded by dialysis against PBS containing gradually reduced concentrations of urea. The refolded recombinant C5 was then affinity-purified using HisPur™ Cobalt Resin (Thermal Fisher, IL) following our previously published and manufacturer-provided protocols. The purity of the resultant protein was checked by SDS-PAGE and then purified again using a C8 reversed-phase HPLC (Beckman, CA). The single major protein peak was collected for sequential experiments.

Mouse immunizations

C57BL/6 WT mice (8–12 weeks old) were used in all *in vivo* experiments. The amounts administered per mouse were as follows: human C5, 25 µg; recombinant mouse C5, 100 µg; VLP-based C5, 200 µg. For immunization, vaccines were emulsified with complete Freund's adjuvant (CFA) (Difco Laboratories, MI), then administered subcutaneously. For human C5 and recombinant mouse C5 vaccine studies, mice immunized with CFA alone were included

as controls; for VLP-based C5 vaccine studies, mice immunized with the same amount of wild-type (lacking the C5 epitope) VLPs were used as controls. The immunized mice were boosted 2 weeks after the initial immunization with the same amount of antigens in incomplete Freund's adjuvant (IFA) (Difco Laboratories, MI) once or twice after every other week.

Serum anti-C5 antibody titer measurements by ELISA

One week after the last boost, serum was collected from the tail vein and the anti-C5 IgG titers were measured by ELISA. Briefly, the ELISA plate was coated with 5 µg/mL purified full-length human C5 or recombinant mouse C5. After blocking, 1:1000 diluted serum samples were added to the plate and incubated for 2h. After washing, HRP-anti-mouse IgG was added as detective antibody. The absorbance was read at 450 nm using a microtiter plate reader (Molecular Devices, CA).

Ex vivo mouse complement-mediated hemolysis assay

Antibody-sensitized sheep erythrocytes (E^{shA}) were used in the *ex vivo* mouse complement-mediated hemolysis assay following previously published protocol with modifications(44). In brief, 15 µL mouse serum and 5 µL human C5 depleted serum were added to GVB⁺⁺ (5 mM Barbitol, 145 mM NaCl, 0.5 mM MgCl₂, 0.15 mM CaCl₂ and 0.1% Gelatin, pH 7.4) in a total volume of 90 µL, then 10 µL of E^{shA} (~ 5×10^6) suspended in GVB⁺⁺ were added to each sample and incubated in 37°C for 5mins. For negative control, 5 mM EDTA was added to the GVB⁺⁺ to inhibit the complement activation. After incubation, samples were centrifuged, and the absorbance of the supernatant was measured at 414 nm using a microtiter plate reader. To calculate the percentage of hemolysis rate, the following equation was used: Hemolysis rate (%) = $[(A-B)/(C-B)] \times 100\%$. A= OD₄₁₄ reading of sample in GVB⁺⁺, B= OD₄₁₄ reading of sample in GVB⁺⁺ with 5mM EDTA, C= OD₄₁₄ reading of maximum hemolysis induced by H₂O.

In vivo complement-mediated intravascular hemolysis and hemoglobinuria

Complement-induced intravascular hemolysis was induced followed by published protocols with modifications(30). In brief, each mouse was injected with 200 µL human C5-depleted serum intraperitoneally, and 1 hour later, blood was collected through retro-orbital bleeding (to minimize bleeding-related hemolysis). Sera were diluted at a 1:10 ratio in PBS in a total volume of 100 µL and hemolysis was quantitated by OD reading at 414 nm. At the same time of blood collection, the mouse abdomen was gently pressed to collect urine, which was diluted at a ratio of 1:10 in PBS before hemoglobin levels were measured by OD reading at 414 nm.

Epitope mapping of the recombinant mouse C5 vaccine

A library of 15-mer oligopeptides overlapping each other by 6 amino acids, spanning the entire sequence of the recombinant mouse C5 vaccine, was custom-synthesized (GenScript, NJ) and used to map the dominant epitope(s) by ELISA. In brief, each peptide (20 µg/mL) was used to coat a plate at 4°C overnight. After blocking with 1% BSA in PBS for two hours, the plate was incubated at room temperature for 2 h with 1:500 diluted sera collected

from the recombinant C5 vaccine-immunized mice. After washing, the plate was incubated with HRP-labeled anti-mouse IgG at RT for 1 h, followed by color development by adding the HRP substrate TMB and OD₄₅₀ reading using a microplate reader (Molecular Devices).

Mouse C5 protein structure modeling and peptides epitope localization

A structural homology model of mouse C5 was generated using the Phyre2 protein recognition server following established protocol³¹. The X-ray crystal structure of human C5 protein was used as the main template (PDB access code: 3cu7)¹⁹. Sequence homology between human and mouse C5 proteins is 89%. Normal modeling mode was used. Major steps of modeling include gathering homologous sequences, fold library scanning, loop modeling, and side-chain placement. The PyMOL visualization program (Schrödinger, LLC, MA) was used to display all the structural models in this work.

Construction of plasmids for VLP-C5 vaccine

The previously described pCDF-CP plasmid(29) has a multiple cloning site (containing NdeI and XhoI restriction sequences) immediately downstream of the viral coat protein (CP) gene. The DNA sequence for the PADRE peptide, codon optimized for *E. coli* expression, was inserted at this site to generate plasmid pCDF-CP-PADRE. This was further elaborated with the codon-optimized DNA for the identified C5 epitope (ASYKPSKEESTSGS, designated P2) downstream of the PADRE sequence, resulting in the plasmid pCDF-CP-PADRE-C5.

VLP-C5 vaccine production and characterization

The VLP-C5 vaccine was produced and characterized following previously published protocols(29). In brief, electrocompetent ClearColi BL21(DE3) *E. coli* cells (Lucigen) were cotransformed with both pCDF-CP-PADRE-C5 and pET28-CP to produce VLP-C5 particles. The control VLP particles were produced using only cells transformed with pET28-CP. Cells were plated on selective SOB agar. After 24 h, isolated colonies were selected into autoclaved SOB media (1% NaCl; 25 mL) containing appropriate antibiotics and grown overnight at 37°C. Cultures were diluted into autoclaved selective SOB media (1% NaCl; 500 mL) the following day. Cultures were grown at 37°C until the cultures reached mid-log phase (O.D.₆₀₀ ~ 0.9), at which time protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Cultures were maintained at 30°C for 16 h, after which cells were pelleted by centrifugation at 6,000 rpm for 10 min. Cells were lysed with a probe sonicator (10 min total, 5 s intervals) in an ice bath. Cell debris was removed by centrifugation at 14,000 rpm for 10 min, and supernatant collected. VLPs were precipitated by the addition of 30% (NH₄)₂SO₄ (w/v) at 4°C for 2 h, and samples were pelleted at 14,000 rpm for 10 min. The pellet was dissolved in 1x TBS and extracted with *n*-BuOH:CHCl₃ (1:1, v/v) to remove lipids and aggregates. The samples were centrifuged at 3,500 rpm for 10 min, and the aqueous phase was collected and subsequently loaded onto 10–40% sucrose density gradients. VLPs purified on gradients by centrifugation at 28,000 rpm for 4 h, and VLP bands were isolated via syringe. Particles pelleted by centrifugation at 68,000 rpm for 2 h, and subsequent pellets dissolved in 0.1M phosphate buffer and characterized.

The resulting VLPs were characterized by FPLC size-exclusion chromatography (Superose 6, monitored by absorbance at 280 nm), dynamic light scattering (Wyatt Dynapro plate reader), and microfluidic gel electrophoresis (Agilent 2100 Bioanalyzer with Series II Protein 80 chips). The average number of CP and CP-C5 subunits per particle was determined by integrating the electropherogram peaks in the instrument software. Levels of endotoxin contamination were found to be less than 0.1 EU/mL using Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher).

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Figure 1.

Immunization of mice with purified human C5 (hC5) generates anti-hC5 IgGs that do not reduce mouse C5 (mC5) activity. WT C57BL/6 mice were immunized with purified hC5 in CFA or CFA alone (control) and boosted 2 weeks later. One week after the boost, serum samples were collected. (A) serum samples were tested for anti-hC5 IgG antibody titers using a hC5 ELISA, showing high titers of anti-hC5 antibodies developed in the hC5 immunized (IM) mice (detectable at a 1:32000 dilution), but not control mice (not detectable even at a 1:500 dilution). (B) C5 activity in the serum samples were assessed by a modified E^{shA} hemolysis assay and no significant difference was found between the hC5-immunized mice and the control mice. n=5 in each group.

Figure 2.

Design, expression, and purification of the recombinant mouse C5 vaccine. (A) Amino acid sequence of the recombinant C5 vaccine, consisting of 12 computer-identified mouse C5 surface epitopes (non-highlighted amino acids), three copies of the PADRE peptide (highlighted in yellow), and a 6XHis-tag (highlighted in green). (B) Reversed-phase HPLC to further purify the recombinant C5P after affinity chromatography. (C) SDS-PAGE analysis of the purified recombinant C5 vaccine. M = markers; lys = whole *E. coli* lysate; ID = inclusion bodies; C5₁ = 60 µg of purified recombinant C5 vaccine; C5₂ = 20 µg of purified recombinant C5 vaccine.

Figure 4.

Verification of the P2 peptide on the surface of mouse C5 protein by homology modeling. A three-dimensional model of mouse C5 was constructed based on the published human C5 X-ray crystal structure. Mouse C5 protein shares 89% sequence homology with human C5 protein. The P2 epitope (red) was found to be exposed on mouse C5 surface based on this model. (A) Ribbon representation of the mouse C5 structure model. Amino (N)- and carboxy (C)-terminus of the protein are also labeled. (B) Surface representation of the mouse C5 structure model.

Figure 5.

Expression and characterization of hybrid Qβ VLPs. A) Schematic representation of plasmids used for particle expression. PADRE sequence highlighted in gold; C5 P2 peptide highlighted in blue. CP: coat protein. B) Electrophoretic analysis of VLPs displaying the P2 peptide. Bands for the wild-type CP and the CP-P2 fusion are indicated. Particle composition calculated using densitometry. C) FPLC elution profile for Qβ-P2 VLPs showing particles are intact and greater than 95% pure. D) Dynamic light scattering histogram of Qβ-P2 VLPs showing particles are monodisperse in size with a hydrodynamic radius of 16.9 nm.

Figure 6.

The VLP-C5 vaccine elicited high titers of anti-C5 autoantibodies and significantly decreased mouse C5 hemolytic activity. WT mice were immunized subcutaneously with the VLP-C5 vaccine or empty VLP as controls, and boosted once or twice after every other week. IgG titers of the anti-recombinant mouse C5 in the sera were measured before the immunization and after the last boost. (A) Before immunization, anti-C5 antibodies were not detectable in both two groups of mice. After the last boost, anti-mouse C5 IgGs were highly detectable in sera from VLP-C5 vaccine immunized mice but not in sera from the control mice. (B). In the modified *ex vivo* hemolysis assays, sera from the same VLP-C5 vaccine immunized mice and control mice were incubated with antibody-sensitized sheep RBCs (E^{shA}) to quantitate mouse C5 activity. Sera from C5 KO mice were used as negative controls. n=13 in each group.

Figure 7.

VLP-C5 vaccine immunization protects mice from complement-mediated hemolysis and hemoglobinuria in a model of PNH. (A) VLP-C5 vaccine immunized mice and empty VLP-immunized control mice were injected 200 μ L of human C5-depleted serum intraperitoneally. 1 hour later, blood was collected through venous sinus of the eye and the resultant sera were diluted at a ratio of 1:10 using PBS before OD values were measured at 414nm. $n=8$ in each group. (B) Urea samples were collected from the VLP-C5 vaccine immunized mice and empty VLP-immunized control mice, and hemoglobinuria were measured by reading OD₄₁₄ after 1:10 dilution with PBS. $n= 5$ in each group.

Table 1

Computer-predicted immunogenic C5 protein surface epitopes

Peptide No.	Start	Antigenic Determinant	Length	Antigenicity/Surface/Hydrophilicity
P1	1547	ISADSRKEKACKPE	14	2.66/0.79/1.14
P2	1410	ASYKPSKEESTSGS	14	2.56/0.86/0.69
P3	399	QETSDLETKRSITH	14	2.42/0.86/0.85
P4	1114	KCQLENGSFKENSQ	14	2.38/0.79/0.43
P5	657	NANADDSHYRDDS	14	2.32/0.79/0.86
P6	146	YSLGDDLKPAKRET	14	2.29/0.71/0.73
P7	442	LPEENQASKEYEAV	14	2.22/0.71/0.76
P8	1590	KTGEAADENSEVTF	14	2.19/0.86/0.65
P9	932	RVVPEGVKRESYAG	14	2.17/0.71/0.43
P10	630	KSDLGCGAGGGHDN	14	1.86/0.93/0.38
P11	129	HTDKPVYTPDQSVK	14	1.80/0.86/0.22
P12	1234	DTLKRPDSSVPSSG	14	1.73/0.86/0.58

Table 2

Peptide library for C5 epitope mapping

	Peptide
1	ISADSRKEKACKPEA
2	ACKPEASYKPSKEES
3	PSKEESTSGSAKFVA
4	SAKFVAAWTLKAQET
5	LKAQETSDLETKRSI
6	ETKRSITHKCQLENG
7	CQLENGSFKENSQNA
8	EN SQNANADDSHYRD
9	DSHYRDDSYSLGDDL
10	SLGDDLKPAKRETL
11	KRETLPEENQASKEY
12	QASKEYEAVAKFVAA
13	AKFVAAWTLKAKTGE
14	KAKTGEAADENSEVT
15	ENSEVTFRVVPEGVK
16	VPEGVKRESYAGAKF
17	YAGAKFVAAWTLKAH
18	WTLKAHTDKPVYTPD
19	PVYTPDQSVKKS DLG
20	KKSDLGCGAGGGHDN
21	GGGHDNAKFVAAWTL
22	VAAWTLKADTLKRPD
23	TLKRPDSSVPSSG
24	DTLKRPDSSVPSSG