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Improvement and characterisation of CHIPS' affinity towards the C5a receptor

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Abstract Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS) is a protein that inhibits migration of neutrophils to the site of infection by binding to and blocking signalling from the C5a receptor. CHIPS may function as an anti-inflammatory therapeutic compound. Screening for CHIPS variants with increased affinity for the C5aR was performed using phage display, followed by sequencing of interesting clones.	
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Improvement and characterisation of CHIPS' affinity towards the C5a receptor

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Sammanfattning

Hos proteinläkemedel utnyttjar man naturliga proteiners egenskaper och i vissa fall vill och kan man förändra dessa för att de skall fungera i kroppen på önskat sätt. Det finns många sjukdomar som skapar ett mycket aktivt immunförsvar. Detta är inte alltid positivt då en kraftig respons ibland kan skada den drabbade individen. Man vill därför ta fram läkemedel som motverkar den inflammatoriska reaktionen.

Chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) är ett nyligen upptäckt protein som kan hämma delar av immunförsvaret. Tanken är att det här proteinet ska fungera som ett anti-inflammatoriskt läkemedel. Proteinet verkar genom att binda till en receptor som finns uttryckt på ytan av vissa celler i immunförsvaret och som därmed hindrar dessa celler att skapa ett inflammatoriskt respons.

Syftet med det här projektet har varit att selektera fram varianter av CHIPS som har stark inbindning till receptorn ur ett bibliotek av muterade varianter och analysera dessa. De varianter som starkt bundit till receptorn har kunnat samlas upp och analyserats. Intressanta proteinvarianter har sekvenserats och jämförts med en icke-muterad variant. Detta resulterade i ett antal utbyten av aminosyror, varav ett var frekvent förekommande. Dessa utbyten kan spela en viktig roll i inbindningen av CHIPS till receptorn på immuncellerna och därmed vara intressanta att bevara i en terapeutisk CHIPS-variant.

Examensarbete 20p inom Civilingenjörsprogrammet Molekylär Bioteknik

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Abbreviations

aa	amino acid
Ab	antibody
BSA	bovine serum albumine
C5aR	C5a receptor
cfu	colony forming units
CHIPS	Chemotaxis Inhibitory Protein of *Staphylococcus aureus*
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence-activated cell sorting
HA	hemagglutinin
HEK293	human embryonic kidney 293
HPR	horseradish peroxidase
IPTG	isopropyl-b-thiogalactoside
mAb	monoclonal antibody
PBS	phosphate buffered saline
RPMI	developed at Roswell Park Memorial Institute, hence the acronym RPMI
RT	room temperature
SA	streptavidin
TMB	3, 3', 5, 5' tetramethylbenzidene
wtCHIPS	wild type CHIPS

1 Introduction

1.1 The immune system

The immune system is the defence mechanism against invading pathogens and consists of two different parts; the innate and the adaptive immune system, both with populations of specified cells. To recognise pathogens, the immune cells have a set of cell-surface receptors that discriminate between self and non-self fragments. The adaptive immune system is very specific and has a memory in a set of antibodies against pathogens from earlier encounters. Its mechanisms function very efficient but it takes several days to obtain a full response. Until the adaptive immune response is established the innate immune system is the primary defence mechanism.

1.1.1 The innate immune system

The skin or mucosa barrier is the first line of defence. The early recognition of the invading pathogens is generic and unspecific and does not change during the individual's lifetime. The innate immune system recognises structures from the pathogen that are conserved throughout the evolution. This recognition triggers a response that produces chemokines and cytokines, which are substances that affect the cell itself or other cells. Phagocytes are a family of innate immune cells that deplete invading pathogens by phagocytosis. Via the phagocyte membrane they engulf the pathogen, followed by a killing mechanism inside the cell. Macrophages and neutrophils are common phagocytes that are activated rapidly after an infection. The first cells to be recruited for phagocytosis are macrophages, which resides in all tissue. The macrophages in turn produce chemokines that attract neutrophils by a chemoattractant gradient. Neutrophils are a major family of phagocytes that reside in the blood, from which they easily migrate through the vasculature to the infected tissue.

1.1.2 The complement system

The complement system is a biochemical cascade of proteins that acts in several ways as a complement to the innate immune system. This biochemical cascade can cause a rapidly growing immune response. The complement response can be triggered by infection of bacteria in three different ways that has in common that they generate C3 convertases. The convertase will form C3a and C3b form C3. An important function of the early complement system is opsonisation of the pathogens. C3 convertases bind to the surface of the pathogen and trigger cleavage of C3 to C3a and C3b. C3a mediates inflammation while C3b is an

opsonin that binds covalently to the pathogen, targeting it for phagocytosis by phagocytes expressing the C3b receptor. Further down the enzymatic cascade, C5a will be a product. C5a initiates the membrane attack complex that can mediate lysis of bacteria. C5a also activate phagocytes and trigger an inflammatory response in the infected area. The small C5a is stable and has a high biological activity acting as a chemoattractant. It attracts neutrophils strongly but it also has chemotactic activity for monocytes and macrophages. C5a binds to its specific cell surface receptor, the C5a receptor (C5aR) and thereby activates multiple intracellular signalling pathways. The C5aR belongs to a family of G protein-coupled receptors with seven transmembrane regions. (Janeway, 2001)

A two-site binding model is proposed for C5a binding to the receptor. The N-terminal part of C5a attaches to the extracellular N-terminal part of the receptor, and the C-terminal part interacts with the transmembrane bundle of the receptor. This latter interaction is crucial for activation of the receptor (see figure 1) (Postma *et al*., 2005). The immune system can be strongly activated by C5a. When produced in excess, a circulatory collapse, similar to an allergic reaction may occur. This can cause inflammatory diseases, like rheumatoid arthritis, systemic inflammatory response syndrome and many others (Pellas *et al*., 1999).

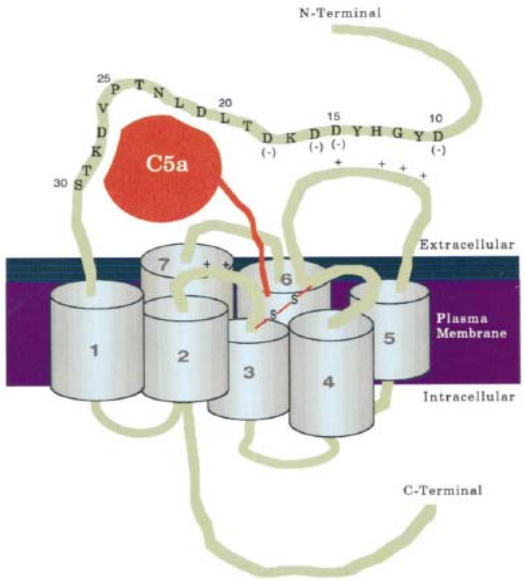

Figure 1. A proposed model of the seven-transmembrane C5a-receptor and the binding of C5a (Chen *et al.* 1998) illustrated with permission from Journal of Biological Chemistry. The N-terminal part of the receptor is extracellular and the C-terminal part is intracellular.

1.1.3 Chemotaxis Inhibitory Protein of *Staphylococcus aureus*

Many pathogens have developed ways to escape the immune system and thereby have the ability to colonise the host. The majority of *Staphylococcus aureus* (*S. aureus*) strains are known to possess an activity to delay the migration of neutrophils to the infected area in various ways. One important feature of *S. aureus* is to counteract the killing by macrophages and neutrophils by secretion of toxins that damage the membrane of the host cells. This mechanism makes it possible for the bacteria to obtain essential nutrition at the same time as the immune cells are killed (Dinges *et al*., 2000).

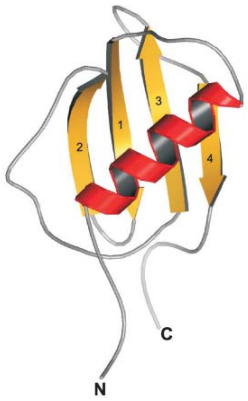

Figure 2. NMR structure of CHIPS $_{31\text{-}121}$ showing the α-helix and the four stranded β-sheets illustrated with permission from de Haas (2005).

The complement system is the major line of defence against an infection by *S. aureus*. The membrane attack complex from the complement system cannot kill *S. aureus* directly. Therefore, opsonisation of the bacteria is the most efficient host defence followed by phagocytosis by neutrophils and macrophages that are attracted by the opsonins (Moore, 2004). A very efficient way for *S. aureus* to escape killing by phagocytes is to keep them away from the infected area. As described earlier, the phagocytes migrate towards a gradient of chemoattractants. One defence mechanism from the bacteria is to secrete a substance that bind to the receptors on the neutrophils and thereby inhibit signalling mechanisms. CHemotaxis Inhibitory Protein of Staphylococcus aureus (CHIPS) is a 14.1 kDa exoprotein which is found in >60% of clinical *S. aureus* isolates. CHIPS binds the C5aR with high affinity and thereby inhibits binding of C5a to its receptor. This mechanism inhibits the migration and activation of neutrophils (de Haas *et al*., 2004). CHIPS does not mimic C5a in binding to the C5aR. Instead of binding like C5a with a two-site binding model, CHIPS binds only the extracellular N-terminal part of the C5aR. It has been shown that a short sequence in

the N-terminal C5aR of nine amino acids (aa 10-18) is the shortest sequence to which CHIPS can bind (Postma *et al*., 2005).

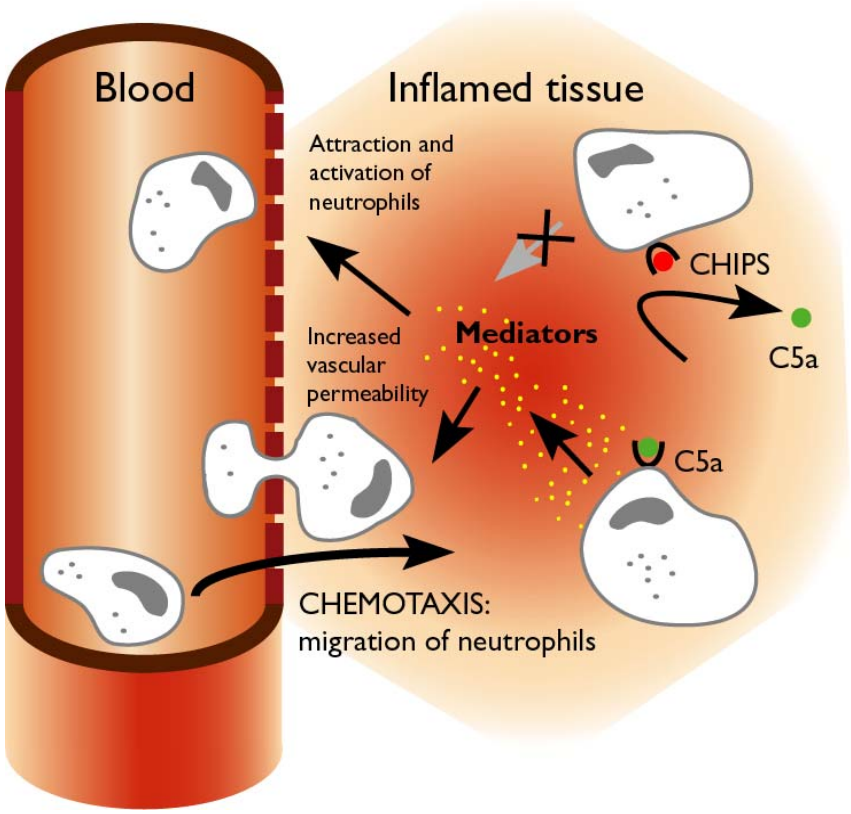

Figure 3. CHIPS blocks the binding of C5a to the C5a receptor on neutrophils and thereby inhibits migration of the immune cells to the site of infection. Illustrated with permission from Alligator Bioscience.

1.2 Phage display

To extract proteins, polypeptides or antibodies with desired properties from a large collection of variants, phage display is a common in vitro selection technique. A phage is a bacterial virus that can infect gram-negative bacteria by attaching to pili on the outer cell wall of the bacteria. The phage has a circular ssDNA genome with up to twelve genes, named pI, pII, pIII etc. The genes in the phage genome code for proteins necessary for replication and encapsidation of the phage particle within the host. The structure is tube formed with an outer shield composed of thousands of copies of a small coat protein (pVIII). Assembly of the phage particle takes place in the cytoplasmic membrane starting on the tip of the phage and building it up until the other end is reached. A phage infection does not kill the host bacteria since the phages replicate within the host cell and are thereafter secreted. There are five different coat proteins that may be used to display proteins, though the pIII and pVIII are by

far the most commonly used. pIII is the most popular since it is surface exposed at one tip of the phage, while pVIII is present all over the outer shield.

In a phage selection, the gene of the protein of interest is fused with the gene of the chosen phage coat protein. Thereafter, the promoter is triggered and the proteins encoded in the genome are expressed. The coat protein will be displayed, linked to the protein of interest. The displayed protein will then be able to interact with other molecules. The phage particles can be used for selection in many different ways, for example against other molecules bound to beads or ELISA plates or to receptors expressed on cell surfaces. There are two possible ways to construct the phages for the selections. The proteins can be displayed using natural phage vectors where all genes needed are on the same circular genome and the gene of interest is inserted upstream the coding sequence for the coat protein. The other method is to use plasmid-based phagemid vectors. The small phagemid vector is much easier to work with than the entire phages since there is less genetic material that can be disrupted in the cloning. These plasmids contain the gene for the coat protein fused with the gene of interest and a weak promoter. *Escherichia coli* bacteria that contain the plasmid can then be transfected with a helper phage. Without the helper phage, the plasmid is not infectious. All proteins, from both the helper phage and the plasmid will then be expressed and assembled and thereafter secreted as complete phage particles.

In phage display there is a direct link between the phenotype and the genotype which is very useful. Phages carrying the phenotype also have the genotype in its own genome or as a plasmid. Figure 4 shows a schematic figure of how phage libraries can be used. The phage library can first be selected against peptides that can be bound to beads, followed by collection, amplification and purification. Thereafter, the amplified phage stock can be used for additional rounds against the same target peptide to create at phage stock with amplified strong binders or against new targets to obtain more specific binders. (“Phage Display: A Practical Approach”, Russel), (Paschke, 2006).

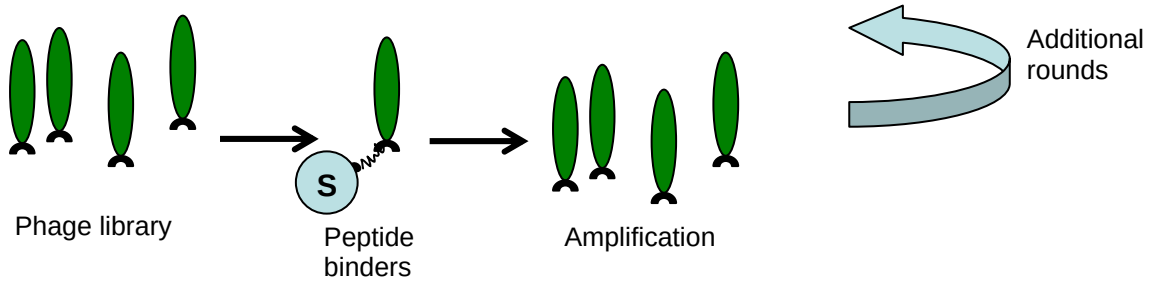

Figure 4. Schematic figure of phage display selections and amplification. The entire library is first selected against peptide and thereafter eluted, collected and amplified. The new amplified phage stock can then be used in additional rounds to obtain a library of amplified strong binders.

1.3 The current project

C5a appears early in the inflammatory cascade thus, blocking the C5aR signalling mechanism is a very promising target for anti-inflammatory therapy. Most people have encountered CHIPS expressing *S. aureus* strains since it is a common bacterium. Therefore, the majority of the population harbour antibodies towards CHIPS which causes problems in using CHIPS as an anti-inflammatory therapeutic compound. To make CHIPS function as an anti-inflammatory drug a strong retained inhibition of the C5aR combined with depletion of antibody epitopes is necessary. Phage libraries consisting of CHIPS variants with high mutation rates have been used as starting material. Previously, the CHIPS libraries have been screened for variants with decreased interaction with CHIPS specific human IgG to find variants that are not recognised by the human immune system. When the antigenic epitopes in CHIPS are deleted the protein may have decreased the binding to the C5aR.

The aim of this project was to find variants with high affinity for the receptor. This was followed by analysis of what amino acids are involved in the binding so that the blocking function of C5aR by CHIPS can be retained. It has been shown that CHIPS binds to the N-terminal part of the C5aR (aa 10-18) (Postma *et al*., 2005). Therefore, fragments of the N-terminal part of the C5aR could be used as binding targets as well as the entire receptor. What remains is to combine variants lacking antigenic epitopes with strong binders to the C5aR in one CHIPS protein.

2 Material and methods

2.1 Cloning of the N-terminal part of the human C5aR

A pcDNA 3.1 vector (Invitrogen) containing the human C5aR was used as DNA template to create the N-terminal C5aR fragment. The sequence (aa 1-38) was amplified by PCR using 0.4µM of each primer (forward 5'-tagtgaggatccgaactccttcaattataccac-3' and reverse 5'-agtaccagatctgatgtctggaacacgc-3', with restriction sites for BamHI and BglII underlined), 0.2mM dNTP, 1x DyNAzyme buffer, 0.5U DyNAzyme DNA polymerase (Finnzymes) with 1/50 Phusion proof reading enzyme (Finnzymes) and 1ng DNA template in a total volume of 50µl. The PCR program consisted of a denaturing step at 95ºC for 2 min followed by 30 cycles of 95ºC for 30s, 45ºC for 30s and 72ºC for 30s and further elongation at 72ºC for 7 min. For digestion of the PCR product and the vector, pRSET (Invitrogen), 20kU BamHI and 40kU BglII restriction enzymes were used, in a total volume of 50µl. The reactions were incubated overnight at 37ºC. After cleavage, the PCR product was purified using JetQuick purification kit (Genomed). To avoid self ligation, the vector was dephosphorylated with 1.36U SAP (shrimp alkaline phosphatase, USB). The reaction was incubated at 37ºC for 1h and the enzyme was deactivated at 65ºC for 15 min and thereafter the vector was purified with JetQuick purification kit (Genomed). Ligation was performed with 40U/µl T4 DNA ligase (NEB) at 16ºC overnight. 5µl ligation reaction were transformed into 50µl E. coli DH5α chemically competent cells (Invitrogen) by heat-shock pulse at 37ºC for 20s followed by addition of 950µl S.O.C (Invitrogen) and incubation with shaking at 37ºC for 1h. Transformed DH5α were plated on LB plates with 50µg/ml ampicillin and incubated overnight at 37ºC. 82 colonies were analysed in colony-PCR for correct inserts with forward vector primer (5'-CTA GTT ATT GCT CAG CGG T-3') and a reverse primer from the inserted sequence (5'-TAATACGACTCACTATAGGG-3'). The PCR program consisted of a denaturing step at 95ºC for 5 min followed by 35 cycles of 94ºC for 30s, 57ºC for 30s and 72ºC for 30s and further elongation at 72ºC for 7 min. Positive clones were sent for sequence analysis at MWG Biotech, Germany.

To create a stop codon in the end of the gene, a mutation was inserted by the use of Quickchange II site-directed mutagenesis kit (Stratagene) following manufacturer's descriptions. An A at position 310 was changed to a T, thereby forming a TGA stop codon. Briefly, a reaction of PfuUltra High Fidelity DNA polymerase (0.05U/µl), dNTP mix, reaction buffer, DNA template (2.2ng) and 0.2µM of each primer

A310T (5'-gtgttccagacatcTGATCTGCAGCTGGTAC-3') and A310T-antisense (5'-GTACCAGCTGCAGATCAgatgtctggaacac-3') in a total volume of 50µl. The PCR program consisted of a denaturing step at 95ºC for 30s followed by 12 cycles of 95ºC for 30s, 55ºC for 1 min and elongation at 68ºC for 3 min (1min/kb). 10U of DpnI was added to the reaction and incubated at 37ºC for one hour. 4µl of DpnI treated PCR-product was transformed into 50µl *E. coli* XL1-blue Supercompentent cells (Stratagene) by heat-shock pulse at 42ºC for 45s followed by addition of 950µl S.O.C (Invitrogen). XL1-blue cells were plated on LB plates with 50µg/ml ampicillin and incubated at 37ºC overnight. Colonies were used to inoculate LB medium with 50µg/ml ampicillin overnight and plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen). The sequence was confirmed by DNA sequence analysis at MWG Biotech, Germany.

2.2 Expression of the N-terminal C5aR

pRSET containing N-terminal C5aR (aa 1-38) was transformed by heat-shock into chemically competent E. coli BL-21 Star (DE3) pLys cells (Invitrogen) for expression of the 1-38 peptide. Heat shock pulse was carried out at 42ºC for 40s followed by addition of 950µl S.O.C (Invitrogen) and incubation at 37ºC for 1h. Transformed cells were plated on LB plates with 50µg/ml ampicillin and 34µg/ml chloramphenicol at 37ºC overnight. Colonies were then used to inoculate LB medium supplemented with 50µg/ml ampicillin and 34µg/ml chloramphenicol and grown at 37ºC overnight. The overnight culture was used to inoculate fresh medium and grown until OD_{600} reached 0.5. Exponentially growing cells were induced with 0.5mM IPTG (isopropyl-b-thiogalactoside) and incubated at 37ºC for 3h. 1ml of culture was centrifuged at maximum speed for 5 min and pelleted cells were lysed in 100µl buffer consisting of Benzonase (Sigma, 0.025U/ml) and rLysozyme (Novagen, 1U/ml) diluted in 0.05% Tween 20 (Bio-Rad) in PBS (phosphate buffered saline) and incubated at RT for 10 min. 10µl of the lysed cells was taken out for analysis on SDS-PAGE. The remains of the lysed cells were centrifuged and 10µl of the supernatant was analysed on SDS-PAGE. The two fractions were denatured for 10 min at 70^{o}C after addition of Novex Tricine SDS 2x Sample Buffer. Thereafter, the samples were separated on a 10-20% Tricine gel (Invitrogen) at 200V followed by staining with Coomassie Simply Blue safe stain (Invitrogen) or exposed to immunoblotting. The separated proteins were transferred to Immun-blot PVDF 0.2µm membrane (BioRad) for 15 min at 150mA and the membrane was then blocked in PBS with 3% BSA (Bovine Serum Albumin) for one hour at RT and washed in 0.05% Tween 20 in PBS for 3x5min. The membrane was then incubated for one hour at RT with the detection

antibody, anti-His (Novagen, 0.2ng/ml) diluted in 1.5% PBS-BSA. Thereafter the membrane was washed 3x5min in 0.05% Tween 20 in PBS. The secondary antibody, polyclonal HRP (horseradish peroxidase)-conjugated goat anti-mouse antibody (Dako Cytomation) was diluted 1/1000 in 1.5% PBS-BSA and the membrane was incubated with the antibody for 45 min at RT. The membrane was washed 3x5min in PBS 0.05% Tween 20 and thereafter in distilled water. TMB stabilized substrate for HRP (Promega) was used to develop his-tagged proteins on the membrane.

2.3 Purification of the N-terminal C5aR

Exponentially growing E. coli BL-21 star DE3 pLysS transformed with plasmid pRSET containing N-terminal C5aR were grown in 4x200ml until OD_{600} ~ 0.5, followed by induction of IPTG as described above. Bacteria were centrifuged at 4500rpm, at 4ºC for 15 min. The pellets were frozen at -20ºC. Pelleted cells were lysed in lysis buffer pH 7.8 (6M Guanidine HCl, 20mM Sodium Phosphate, 0.5M NaCl) and stored on ice. Bacteria were sonicated for 3x5s and thereafter centrifuged at 5000rpm, at 4ºC, for 1h. The supernatant was filtered through a 0.45µm membrane (Millipore) and pH was adjusted to 7.8. Expressed peptides were purified on a 1ml HisTrap Ni-column (GE Healthcare) according to manufacturer's protocol. Briefly, the column was washed with five column volumes H_2O and thereafter equilibrated with five column volumes lysis buffer. The sample was transferred to the column at 1ml/min and the column was then washed with five column volumes binding buffer (8M urea, 20mM Sodium Phosphate, 0.5M NaCl, 40mM Imidazole, pH 7.8) followed by five column volumes wash buffer (8M urea, 20mM Sodium Phosphate, 0.5M NaCl, 100mM Imidazole, pH 7.8). His-tagged peptide was eluted with five column volumes elution buffer (8M urea, 20mM Sodium Phosphate, 0.5M NaCl, 500mM Imidazole, pH 7.8). Fractions of 0.5ml were collected continuously during all steps in the purification and analysed at 280nm. 10µl from fractions with high absorbance were separated on SDS-PAGE 10-20% Tricine gel (Invitrogen) and analysed with immunoblotting and gel staining as described above. Fractions giving positive results in immunoblotting were pooled and buffer was exchanged to PBS on a 2.5ml PD-10 column (GE Healthcare).

2.4 Coupling of the N-terminal C5aR to Dynabeads M-270 Epoxy

Dynabeads M-270 Epoxy (Invitrogen) were washed in 0.1M sodium phosphate buffer, pH 7.4, according to manufacturer's protocol. Thereafter, $2x10^8$ beads were coated with 60µg of purified N-terminal C5aR, by slow tilt rotation at 4ºC for 48 hours. Beads were washed four

times in PBS. To test functionality of the coated beads, wtCHIPS was captured at RT with shaking for 1h. Supernatant was collected and analysed by ELISA.

Greiner 96 plates were coated with monoclonal antibody 2H7 (3µg/ml) at 4ºC overnight. Wells were washed three times with 0.05% Tween 20 in PBS and thereafter blocked with 4% PBS-BSA with 0.05% Tween 20 with shaking at RT for one hour. After washing three times, 50µl of supernatant wtCHIPS from beads was added and incubated in the plate with shaking at RT for one hour. A standard curve of input wtCHIPS, with concentrations from 1.56-800ng/ml was made for comparison of signals. Wells were washed three times with 0.05% Tween 20 in PBS and primary antibody, rabbit-anti-CHIPS-N-PEP IgG, was added at 3µg/ml followed by incubation at RT with shaking for one hour. After three times of washing, HRP-conjugated goat-anti-rabbit IgG (Dako Cytomation, 1/20 000) diluted in 0.05% Tween 20 in PBS-BSA was added and the plate was further incubated at RT, for one hour. Wells were washed six times in 0.05% Tween 20 in PBS. Then, Chemilum substrate PICO (Pierce) was added and incubated for one minute and the luminescence was analysed in a luminescence reader.

2.5 Cell free expression of the N-terminal C5aR

Cell free expression was performed using Expressway Cell-Free E. coli Expression System (Invitrogen) following manufacturer's protocol. Briefly, 10µl E. coli slyD- extract, IVPS E. coli Reaction Buffer (-A.A), 1.25mM Amino Acids (-Met), 1.5mM methionine and T7 Enzyme mix were mixed, per reaction. Plasmid DNA (500ng) was diluted in 21.6µl of the mix above and DNase/RNase-free Distilled H_2O was added to a final volume of 25µl. The reaction was transferred to a sterile 96-well U-shaped plate. Surrounding wells were filled with H_2O. The reaction was incubated in a MultitronII plate shaker (Infors AG) with shaking at 600 rpm at 30ºC, for 30 min. Thereafter, 25µl feeding buffer (DNAse/Rnase-free Distilled Water, IVPS Feed Buffer (-A.A), 1.25mM Amino Acids (-Met) and 1.5mM Methionine) was added. The reaction was further incubated at 600rpm at 30ºC for 4.5h followed by centrifugation for 5 min at maximum speed. The supernatant was separated and analysed on SDS-PAGE 10-20% Tricine Gel (Invitrogen) as described above.

2.6 Transfection and expression of the N-terminal C5aR and the entire C5aR in HEK293 cells

HEK293 cells were seeded in 3 ml RPMI-1640 (Invitrogen) in 6-well plates (Greiner) the day before transfection to obtain 70% confluence on the day of transfection. 4µg N-terminal C5aR

(aa 1-38) in pDISPLAY and the entire C5aR in pcDNA 3.1 vector, respectively were transfected with Lipofectamin 2000 (Invitrogen) reagent, following manufacturer's protocol. Medium was replaced by 1.5 ml Optimem (Invitrogen) and 0.5ml DNA-lipofectamin-mix was added drop-wise to the cells. Cells were then incubated at 37ºC for 3.5h and the medium was thereafter replaced by RPMI-1640. 48 hours after transfection, cells were analysed for receptor expression by flow cytometry.

A direct labelling of transfected cells was performed with anti-HA (hemagglutinin, 5µg/ml) for N-terminal C5aR expression and anti-FLAG (10µg/ml) for the entire C5a receptor expression. 250 000 transfected cells were washed in PBS, followed by addition of antibody diluted in 0.05% PBS-BSA to a total volume of 50µl for one hour on ice with shaking. Thereafter, cells were washed in 0.05% PBS-BSA and fixed with 0.5% paraformaldehyde/PBS and analysed by flow cytometry. To test the functionality of the receptors on the transfected cells, binding of CHIPS was analysed. 1µg/ml (50ng) wtCHIPS was added to 250 000 transfected HEK293 cells and incubated with shaking on ice for 30 min. To wash cells, 1ml 0.05% PBS-BSA was added and cells were centrifuged at 1200 rpm for 10 min. Cells were then incubated with 50µl 5µg/ml 2H7 monoclonal anti-CHIPS antibody on ice for 30 min followed by washing as described above. Thereafter, cells were resuspended in 50µl fluorescently (RPE) labelled goat-anti-mouse Ab (Dako) diluted 1/50 in 0.05% PBS-BSA and incubated with shaking on ice for 30 min. Cells were then washed in 0.05% PBS-BSA and fixed with 0.5% paraformaldehyde-PBS and analysed by flow cytometry.

2.7 Preparation of phage stock from a library

Starting material were two randomly mutated CHIPS libraries with a mutation frequency of 2.5–3.6 aa/sequence cloned in the phagemid vector pFAB75 (Engberg et al, 1995) and transformed into E. coli TOP10F' (Invitrogen). The two different libraries were grown in LB with 1µg/ml tetracycline, 50µg/ml ampicillin and 1% Glucose at 37ºC until OD_{600} ~ 0.5. Helper phage VCSM13 was added in 20x excess to infect the exponentially growing culture. Cells were then incubated for 30 min at 37ºC without shaking, thereafter pelleted by centrifugation and resuspended in LB with tetracycline (10µg/ml), ampicillin (50µg/ml), kanamycin (10µg/ml) and IPTG (1mM). The cultures were then further incubated at 30ºC with shaking overnight. After pelletation by centrifugation at 3500rpm for 30 min, the supernatant was collected and 0.25 volumes of 20% PEG6000 2.5M NaCl was added to

precipitate the phages. The precipitated phages were then resuspended in 0.1% PBS-BSA. To titrate the phage stock, phages were allowed to infect E. coli TOP10F' at 37ºC for 30 min and thereafter plated on LB plates with 34µg/ml tetracycline and 50µg/ml ampicillin. Colony forming units (cfu)/ml were calculated.

2.8 Phage selection on C5aR peptide (aa 7-28)

To remove any potential streptavidin binders, a selection was performed by adding phage stock (10^{11} cfu/ml) to 10^7 Streptavidin Coated Dynabeads M-280 (Invitrogen). The phage stock (500µl) was incubated with the beads for 30 min in 200µl 5x selection buffer (3% PBS-BSA with 0.05% Tween 20), and 300µl 0.1% PBS-BSA on rotator at RT. Then, beads were separated from the phage stock on a magnet and sulfonated and biotinylated N-terminal C5aR peptide aa 7-28 (AnaSpec) was added to a final concentration of 10^{-7}M. Then, the mix was incubated on a rotator at RT for 1h. After incubation, 10^7 Streptavidin Coated Dynabeads M-280 were added to interact with the biotinylated peptide on a rotator at RT for 15 min. Beads were then collected and washed five times in selection buffer and three times in PBS. Elution of bound phages was performed by incubation with 450µl 0.1M glycine with 0.1% BSA, pH 2.2 for 10 min, with gentle shaking, followed by neutralisation with 50µl 1M Tris pH 9.0. The eluted phages were titrated by infection of exponentially growing E. coli TOP10F' and cfu/ml were calculated.

2.9 Phage selection on cells

Phages, prepared as above, were blocked in 3% PBS-BSA on rotation, for one hour. To remove potential unspecific binders, a primary selection against 10^7 untransfected cells, blocked with 2% PBS-BSA for 30 min on ice, was performed by incubation for four hours at 4^oC. The supernatant was collected and used in a specific selection on 10^6 HEK293 cells transfected with either N-terminal C5aR or the entire C5aR or U937 cells with stable expression of the entire C5aR. Cells were incubated with phages for one hour at 4ºC or 37ºC, thereafter cells were collected by centrifugation and washed two to four times in 1% PBS-BSA. Elution of bound phages was performed by incubation with 450µl 0.1M glycine with 0.1% BSA, pH 2.2 for 10 min, with gentle shaking, followed by neutralisation with 50µl 1M Tris pH 9.0. The eluted phages were titrated by infection of exponentially growing *E. coli* TOP10F' and cfu/ml were calculated.

2.10 ELISA analysis of phage binding to N-terminal peptide

Phage stocks of phages selected on peptide or cells were prepared as described above and titrated in E. coli TOP10F'. Cfu/ml were calculated. A NUNC maxisorp 96 plate was coated with streptavidin (Sigma, 5µg/ml) in PBS and incubated at 4ºC, overnight. The wells were washed three times in 0.05% Tween 20 in PBS. Thereafter, the plate was blocked with 4% PBS-BSA at RT with shaking for one hour, followed by three washings with 0.05% Tween 20 in PBS. Sulfonated and biotinylated N-terminal C5aR peptide (aa 7-28, AnaSpec), was incubated with the coated plate at RT with shaking for one hour, followed by three times of washing with 0.05% Tween 20 in PBS. Prepared phage stocks were diluted to 10^7 -10^9 cfu/ml and incubated with the plate for one hour, at RT with shaking. Standard curves of input phage stocks with concentrations from 10^7-10^{11}cfu/ml were made for comparison of signals.

Bound phages were detected with anti-M13 (1µg/ml, Amersham) antibodies with shaking at RT for one hour, followed by three times of washing with 0.05% Tween 20 in PBS. An HRP-conjugated rabbit-anti-mouse-Ig antibody (0.65µg/ml, Dako Cytomation) was used as secondary antibody and incubated with shaking at RT for one hour, followed by six times of washing with PBS 0.05% Tween 20. OPD fast (Sigma) was used as HRP substrate following manufacturer's protocol and the plate was analysed at 492nm.

2.11 Analysis of phage binding to U937/C5aR cells

U937 cells with stable expression of C5aR were washed in PBS. Unselected phages and phages from selections were prepared as above and added to 250 000 cells per reaction followed by incubation on ice for 30 min. Cells were pelleted at 1200 rpm at 4ºC, for 10 min and buffer was aspirated. Mouse-anti-M13 (5µg/ml, Amersham) in 0.05% PBS-BSA was added to the cells in a total volume of 50µl and incubated with shaking on ice for 30 min, followed by washing as above. A secondary antibody, goat-anti-mouse-RPE (1/100, Dako) diluted in 0.05% PBS-BSA was added and incubated with shaking on ice for 30 min. Thereafter, cells were washed as described above and resuspended in 0.5% paraformaldehyde-PBS followed by flow cytometry analysis.

3 Results and discussion

3.1 Selection strategy

There are various ways to perform successful phage selections to find stronger binders to a certain target. The target can be peptides (Huang *et al*, 2005) or proteins (Jestin *et al*, 2001) bound to beads or microtiter wells. Cells expressing the protein of interest have also been successfully used in phage selections (Fransson *et al*, 1995). G-protein coupled receptors are proteins that are difficult to produce since they contain a transmembrane region (Wagner et al, 2006). Therefore, combining different selection strategies may increase the possibility to find variants with strong affinity for the G-protein coupled receptor, C5aR.

To find CHIPS variants with higher affinity for the C5aR, the strategy was to first select CHIPS variants against shorter soluble peptides and thereafter against cells expressing either the N-terminal part of the C5aR or the entire C5aR (figure 5). The selection against the short soluble peptide was performed since we were not sure of how specifically CHIPS would bind to the receptors on the cells. A longer peptide of the N-terminal C5aR (aa 1-38) was intended to be used as a complement to the shorter synthesised peptide (aa 7-28) in the phage selections to soluble target. The shorter peptide is known to be the minimum part of the C5aR to which CHIPS can bind (Postma *et al*., 2005). Though, it was not known if the shorter peptide was long enough to find variants of CHIPS that also bind the entire receptor. We are in the end interested in variants that bind the entire receptor and not only peptide binders. The variants that bind only the smaller part of the receptor may although give useful information of what amino acids that are involved in the binding of CHIPS to the receptor. Therefore, efforts were made to clone the longer peptide into a pRSET vector followed by expression in *E. coli* and purification on a Ni-column.

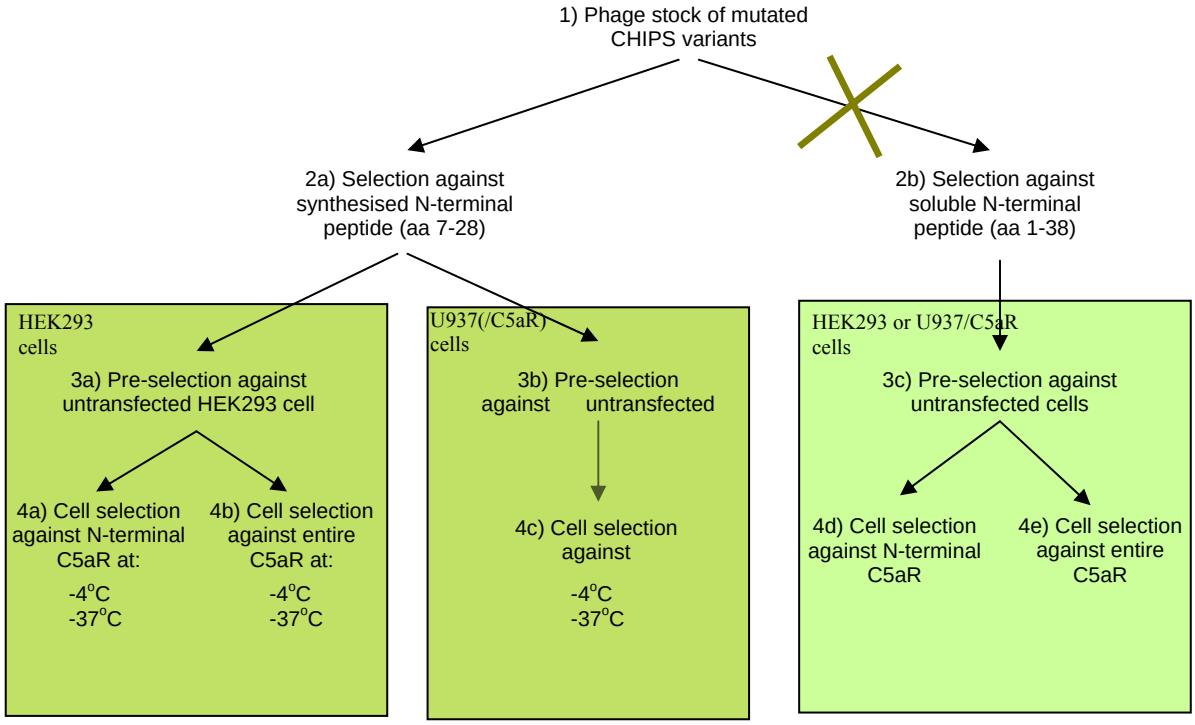

Figure 5. Schematic figure of phage selections where starting material was phage stock libraries of mutated CHIPS variants **(1)**. The phage stock was intended to be used in selections against synthesised N-terminal peptide (aa 7-28) **(2a)** and a longer soluble N-terminal peptide (aa 1-38) **(2b)**. (Because of difficulties with expression of the longer peptide this selection was not performed.) After selections against the shorter peptide (aa 7-28), selections against untransfected HEK293 **(3a)** or U937 cells **(3b)** was performed to eliminate unspecific binders. The phages were then used in selections against cells transfected with either the N-terminal C5aR or the entire receptor at incubation temperatures 4°C and 37°C **(4a-c)**.

3.2 Cloning, expression and purification of the N-terminal C5aR

Cloning of the N-terminal C5aR gene into the pRSET vector was successfully performed. To create a TGA stop codon in the end of the gene, a point mutation was inserted at position 310 where an A was exchanged to a T. To confirm the expected sequence it was analysed by sequencing.

Expression of the gene was performed but with low yield of the protein. The molecular weight of the peptide, with a linker and a His_{6x}-tag is 7.5 kDa. Separation of the expressed peptide on SDS-PAGE revealed weak bands of approximately the correct size in both the soluble fraction and the insoluble lysed fraction. Since the bands were weak no figure can be shown here. The identification of the correct protein was strengthened by immunoblotting, using antibodies against the His_{6x}-tag.

The soluble peptide was then attempted to be purified on a HisTrap Ni-Column. Fractions from the washing step showed absorbance at 280nm but there was no detection of proteins in

the elution fractions. The washing buffer contained imidazole. This may have caused the early elution of the bound protein since imidazole often is used for elution in Ni-columns. The fractions absorbing light at 280nm were pooled and separated on SDS-PAGE gel, showing bands of approximately 22kDa. This band size indicates that the peptide may appear as trimers. To test the functionality of the putative purified peptide, coupling to epoxy beads followed by binding of CHIPS was carried out. This resulted in a decreased signal in ELISA, indicating that CHIPS may bind to the peptides which were bound to the ELISA plate, though the background was high. The decreased signal can also be a result of unspecific binding of CHIPS to the beads. Therefore, we were not sure of what protein had been purified. An attempt to obtain higher expression of the peptide using a cell free expression system was also performed but no expression of the peptide could be detected. It is possible that the shorter peptide may have undergone degradation by proteases during the expression and thereof the low yield. To obtain a higher yield of product in the expression there are various things to do. The short peptide could have been expressed fused with a larger protein to evade degradation. The induction time could have been reduced and addition of protease inhibitors like PMSF or aprotinin could also have increased the yield (Qiagen). Since no expression of this longer peptide (aa 1-38) was possible, the selections had to be based on the shorter peptide (aa 7-28) followed by cell selections.

3.3 Transfection of HEK293 cells

The transfection rate and the functionality of the N-terminal part of the C5aR and the entire receptor in transfected HEK293 cells were analysed simultaneously on transfected cells.

HEK293 cells were successfully transfected with either the pDISPLAY vector containing the N-terminal C5aR or the pcDNA3.1 vector containing the entire C5aR. The transfection rate was analysed in flow cytometry. The pDISPLAY vector with the shorter peptide contains a hemagglutinin (HA) epitope allowing direct labelling of FITC conjugated anti-HA antibody. The pcDNA3.1 vector with the entire receptor contains a FLAG-tag for direct labelling of FITC conjugated anti-FLAG antibody. The direct labelling (figure 6a-c) indicates successful expression of both N-terminal C5aR and the entire C5aR, though with a lower signal for the entire C5aR (figure 6c). The expression of the entire receptor might be low but the low signal can also be a result of poorly expressed FLAG-tag or weak binding of the anti-FLAG antibody to the tag. To test the functionality of the shorter peptide and the entire receptor, CHIPS was incubated with the transfected cells, followed by staining with 2H7 anti-CHIPS

antibody and thereafter with RPE conjugated anti-mouse antibody. The significant shifts in the histograms indicate functional N-terminal C5aR (figure 6e) as well as entire C5aR (figure 6f). This strengthens the theory that the direct labelling of the FLAG-tag failed. To evaluate the binding of the anti-FLAG antibody towards its tag, the antibody could be titrated and an optimal concentration could possibly give an increased response in flow cytometry as compared to the data shown in figure 6c.

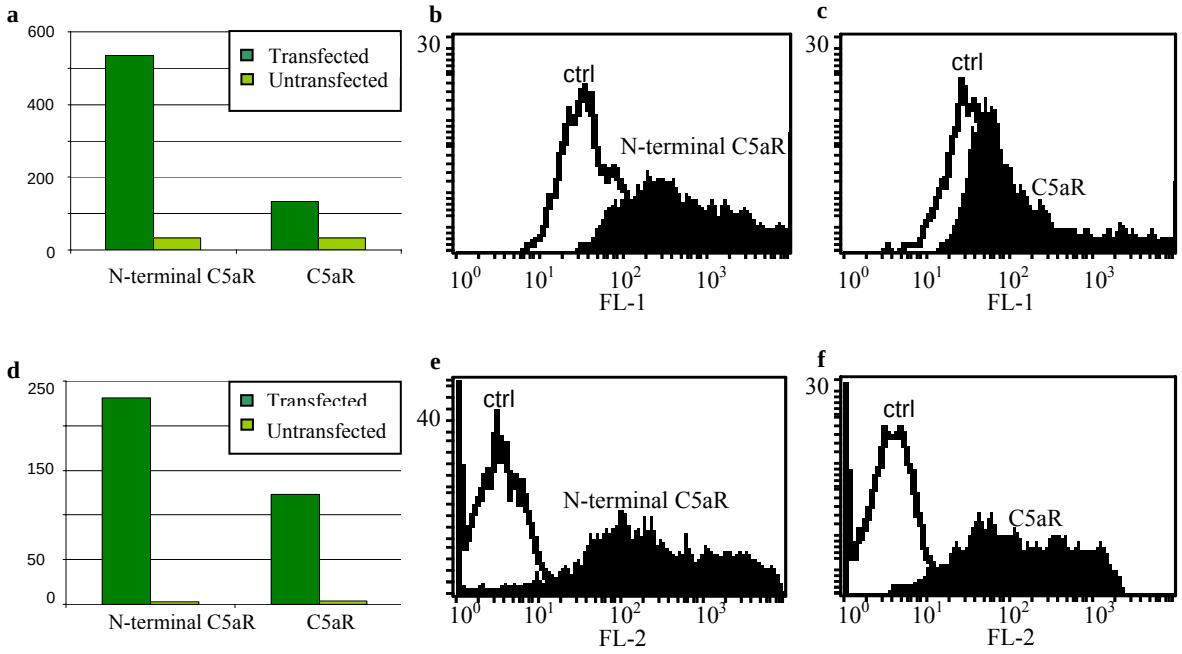

Figure 6. FACS analysis of HEK293 cells transfected with either the N-terminal C5aR or the entire C5a receptor. Upper panel showing direct labelling of cells transfected with either N-terminal C5aR incubated with anti-HA antibody **(a, b)** or C5aR transfected cells incubated with anti-FLAG antibody **(a, c)**. The result indicates successful labelling of N-terminal C5aR but failure of labelling the entire C5aR. Lower panel showing transfection and functionality test of the receptor by CHIPS binding **(d-f)**. Testing functionality of N-terminal C5aR **(d, e)** and the entire receptor **(d, f)**. The products of the two transfections are both functional. Comparing geometric mean on **a** and **d** y-axis.

3.4 Phage selections toward higher affinity for the C5a receptor

In the phage selection the library with all the different variants of the CHIPS was first screened for binders to the biotinylated short peptide, which in turn can be attached to streptavidin coated beads. The beads can be collected and the phages bound to the peptide can be eluted and is called output. The number of colony forming units per millilitre (cfu/ml) can then be compared to the unselected phage stock, called input. The eluted phages can then be amplified and used as input in a negative selection against untransfected cells to remove phages that bind unspecifically to the surface of the cells. Thereafter the non-binding phages can be collected and used in a positive selection against transfected cells expressing either the N-terminal C5aR or the entire C5aR (figure 7). Binding phages in this selection are referred to as output. As a comparison of the result from different phage selections, an output/input ratio can be calculated. A low ratio indicates that few phages have bound to the target. Hence, a

higher ratio indicates that many phages have bound to the target. Ratios that are increasing after each round of selection, indicate an enrichment of binders.

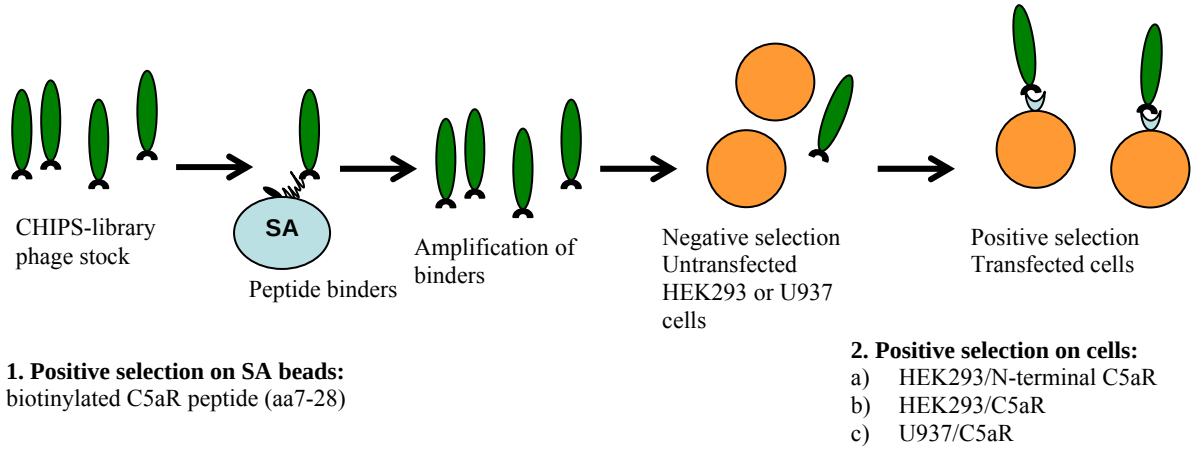

Figure 7. Schematic figure of phage selection of C5aR binders. First, the library of CHIPS variants is selected against a biotinylated peptide that can be attached to streptavidin beads. The bound phages can be collected, eluted and thereafter amplified. The phage stock with the functional variants is then used in a negative selection against untransfected cells to remove variants of CHIPS that bind unspecificly to the surface of the cells. Thereafter, non-binding phages are collected and used in a positive selection against transfected cells, expressing either the N-terminal part of the C5aR or the entire C5aR.

A selection against the soluble short peptide (aa 7-28) was performed to collect the functional CHIPS variants from the chips library phage stock (figure 5). Two libraries consisting of randomly mutated CHIPS variants with 2.5-3.6 mutations/gene were pooled prior to the selections. The phages were first incubated with streptavidin coated beads to eliminate unspecific binders, followed by incubation with the peptide. Since the peptide was biotinylated, it could be collected using the streptavidin coated beads. Phages bound to the collected peptides were then eluted and the cfu/ml were calculated (table 1). The collected phages were then used as input in cell selections.

The first selection against the soluble short peptide resulted in a very low output/input ratio (table 1a). This indicates that many of the phages from the library did not bind to the peptide and thereby they were eliminated. This is expected since the library may contain many variants that are not functional and lack the ability to bind to parts of the receptor or the entire receptor. Thereafter, all selections started with incubation of phages with untransfected HEK293 or U937 cells to eliminate phages with unspecific binding to the cell surface (figure 5). A selection against empty tubes could also have been performed, to avoid phages attaching to the plastic material. It was considered that it would not be necessary since tubes never were empty during the selections. Thereafter, selections against HEK293 cells transfected with either the N-terminal part of the receptor or the entire receptor were carried out. Incubation of

the phages with the different cells was performed at 4ºC and 37ºC. U937 cells have a stable expression of the entire C5aR (Kew et al, 1997) and were used in phage selections at 4ºC and 37ºC (figure 5). Cfu/ml was calculated after all selections and compared with the cfu/ml for the corresponding input phage stock. Among the cell selections, the selection at 37ºC against N-terminal C5aR or the entire receptor expressed on HEK293 cells resulted in the lowest ratio of output/input (table 1c, e) indicating that these selections resulted in few phages binding to the receptor. Since the selection was performed at 37ºC with two more washing steps than selections at 4ºC, this condition seem to have been more stringent. The selections against the U937 cells with stable expression of C5aR resulted in higher ratio, indicating a higher amount of bound phages at both 4ºC and 37ºC.

Table 1. Comparison of the ratio of output/input phage stocks (cfu/ml) for all selections. The selection against the small peptide gave a very low ratio **(a)**. Cell selections at higher stringency, at 37ºC, resulted in the lowest ratio **(c, e)**. A low ratio indicates few binders.

Selection against:	Input (cfu/ml)	Output (cfu/ml)	Ratio (output/input)
a) peptide (aa 7-28)	1.9×10^{11}	3.4×10^{5}	1.79×10^{-6}
b) N-terminal C5aR at 4ºC	1.0×10^{10}	5.5×10^{7}	5.50×10^{-3}
c) N-terminal C5aR at 37ºC	1.3×10^{11}	2.0×10^{6}	1.54×10^{-5}
d) C5aR at 4ºC	1.0×10^{10}	2.7×10^{7}	2.70×10^{-3}
e) C5aR at 37ºC	1.3×10^{11}	1.2×10^{7}	9.23×10^{-5}
f) C5aR at 4ºC on U937	1.9×10^{11}	5.0×10^{8}	2.63×10^{-3}
g) C5aR at 37ºC on U937	1.9×10^{11}	2.6×10^{8}	1.37×10^{-3}

To evaluate the increase of the binding capacity of the phage stocks to the receptor from the above selections was analysed in ELISA and by flow cytometry. It is expected that the phage stocks with the highest improvements contain the most interesting mutations for strong binding to the receptor. In ELISA, the short biotinylated peptide was bound to the streptavidin coated ELISA plate, followed by incubation with the phage stock from the above selections. The signals from bound phages relative the signal from the standard curves from the input phages resulted in an improvement of the phages binding capacity to the receptor (figure 8). Only a minor improvement in binding capacity was obtained in the first selection, against the short synthesised peptide. Which was expected since the output/input ratio was low. The other selections (figure 8b-g) gave an improvement of the binding capacity up to 34 times (figure 8c). The selection against the entire C5aR resulted in lower improvement than the selections against the N-terminal peptide. Since the phage itself is bulky and the entire receptor has a big complex structure it can be difficult for the phage to attach to the entire receptor. Since the shortest sequence necessary for CHIPS to be able to bind the receptor is isolated on the shorter N-terminal peptide, this may facilitate phage binding. The binding to the U937 cells with the entire receptor gave a low signal at both selection temperatures. One reason for the

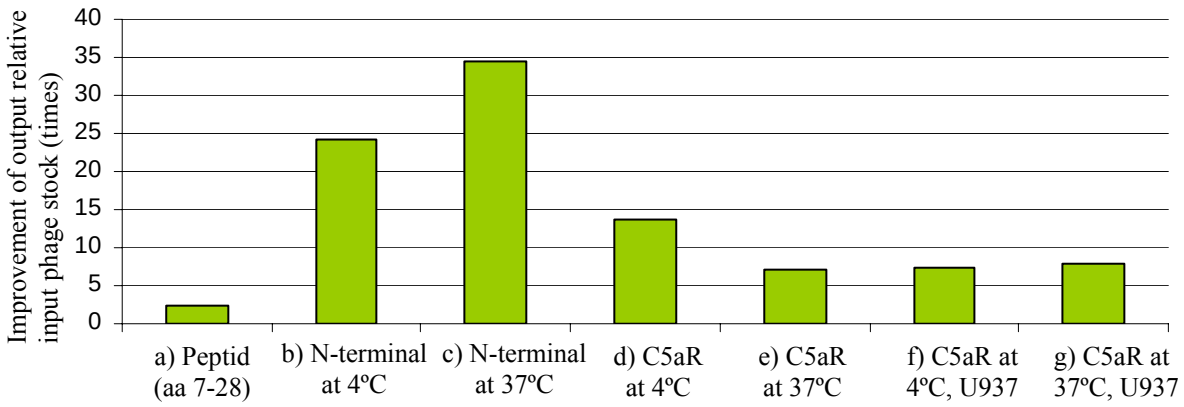

Figure 8. Relative improvement of phage selections analysed in ELISA. The labelling on the x-axis **(a-g)** indicates from what selection the phage stocks are obtained. Selections against the N-terminal C5aR resulted in the highest improvement **(b, c)**.

different results between the two cell types could be the differences in the number of receptors expressed on the cell surface. The HEK293 cells are transiently transfected with either the N-terminal C5aR or the entire receptor while the U937 cells are stably transfected with the entire receptor and possibly the transiently transfected cells express a higher number of receptors on the surface. To compare the expression rate between the different cell types, one could incubate CHIPS with the transfected cells and analyse the binding by flow cytometry. The values for the increased binding capacities was analysed by flow cytometry and showed similar results as the ELISA, but with lower signal for the selection against HEK293/C5aR cells compared to the ones selected against U937/C5aR cells (figure 9). However the experiments were only performed once and no standard deviation has therefore been calculated. Small differences might not be significant.

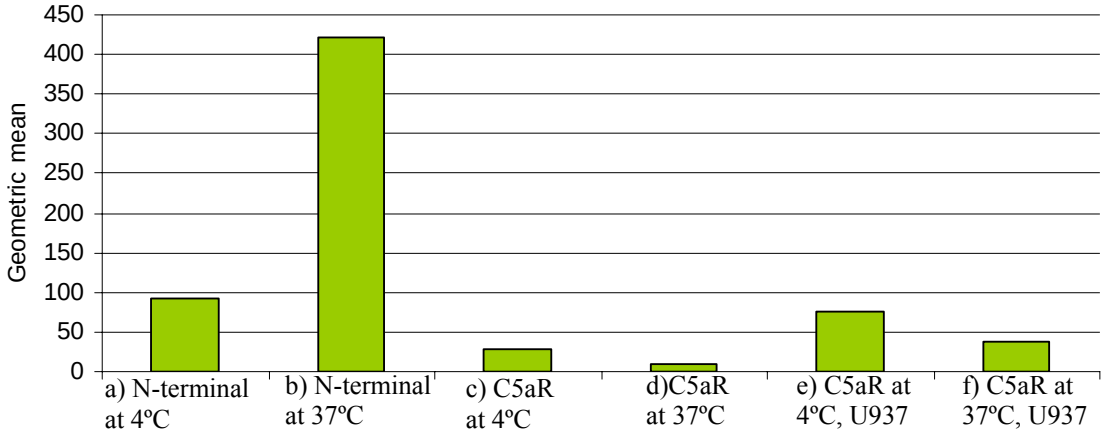

Figure 9. Analysis of phages from different selections, binding to U937/C5aR cells, analysed by flow cytometry. The phages were selected against HEK293 cells transfected with either N-terminal C5aR or the entire C5aR or U937 cells with stable expression of C5aR. The labelling indicates from what selection the phage stocks are obtained **(a-f)**.

The values for the improvements were calculated in comparison to the background of untransfected cells and unspecific labelling. A high value from the ELISA assay indicates that the binding capacity of the entire phage stock to the receptor has improved, but it does not tell if the affinity of the phages is higher or not. The selection against the N-terminal peptide at the higher stringency (37°C) was the only selection where CHIPS variants with significantly higher affinity for the C5aR could be found by flow cytometry. This selection was not only performed at 37°C, it also contained two more washing steps than selections at 4°C. This could have contributed to the higher stringency which resulted in a set of CHIPS variants with higher affinity for the receptor.

3.5 Characterisation of binders

We chose to sequence clones selected against both the N-terminal receptor and the entire receptor. Based on the ELISA results, we chose the N-terminal C5aR at 37°C and the C5aR at 4°C in HEK293 cells. 24 colonies from each selection were sequenced, (table 2). Failed sequencings are not shown. The big number of failed sequences may depend on problem with the preparations of samples that were sent for sequencing. One frequent amino acid change was revealed which may be important for the binding to the receptor. At position 69 in the amino acid sequence, a lysine was changed to an alanine by base exchanges AAA to GCA in 14 clones out of 26. Eight of the 14 clones had additional changes. The other substitutions were scattered with no obvious clustering, though they can be important for the binding. The combination of the K69A with another amino acid change could be an answer to the question of what amino acids are involved in the binding to the receptor. Six of the clones harboured no changes. This may indicate that the original sequence has a high affinity for the receptor. Here, the sequences of single clones were analysed, while in ELISA and flow cytometry a pool of phages was analysed. Therefore, to better understand the contribution of each amino acid change, the single clones need to be analysed for binding in ELISA and flow cytometry.

The structure of the CHIPS protein is visualised in figure 10 with the lysine (K) at position 69. The amino acid number 69 is surface exposed and directed outwards from the protein. This strengthens the theory that an amino acid at this position could be important for the binding to the receptor. Changing from a long positively charged lysine to a smaller neutral alanine seems to improve the binding to the entire C5a receptor as well as to the shorter N-terminal peptide. The lysine has a bigger space filling volume than the alanine. The smaller uncharged alanine may facilitate a tighter binding to the C5aR. Although the wtCHIPS with the lysine at position 69 functions well, it may not be the optimal construction.

Table 2. Amino acids changes in the sequenced clones, either from N-terminal C5aR (N) or C5aR (C) selections. 14 out of 26 clones had an amino acid change from a lysine at position 69 to an alanine. Eight of these clones had additional mutations within the sequence. Six of the clones had no mutation at all in the gene.

Clone	F3	P5	P7	E10	E20	K30	N31	N47	K51	K61	N68	K69	Y71	T73	N77	T78	N86	L90	M93
N-2																			
N-8										A								P	
N-14																			
N-16	Y											A		I					
N-18											D	A							
N-20		R										A							
N-23																R			
N-24			H			I		Y											
C-25									R								D		
C-26												A							
C-27												A							L
C-28																			
C-29			S									A							
C-30																			
C-31															S			P	
C-37																			
C-38												A							
C-39												A							
C-40				D								A							
C-41							D					A					S		
C-42								D											
C-43												A							
C-44												A							
C-45					K							A	F						
C-47																			
C-48												A							

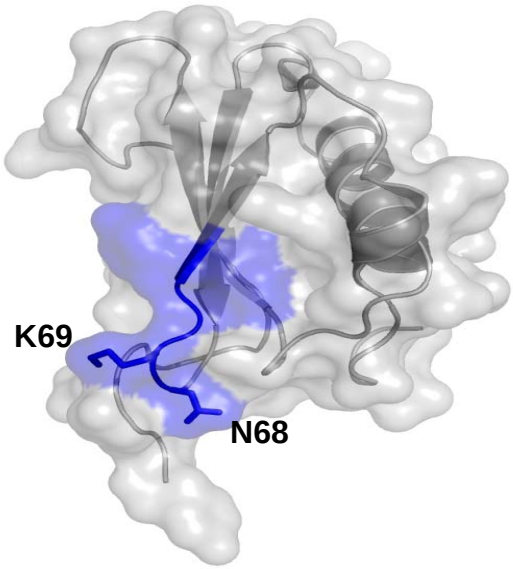

Figure 10. A structure model of CHIPS from Alligator Bioscience where the lysine at position 69 is labelled K69.

Conclusion and further project

A protocol for affinity maturation of CHIPS towards the C5aR has been established. Successful phage selections were performed on cells and the selection strategy to use a longer soluble peptide was not necessary in order to obtain CHIPS variants with higher affinity for the C5aR. Many different amino acid changes and one in particular have been found that may be important for the binding to the N-terminal part of the C5aR. Further, the sequenced clones of CHIPS have to be analysed individually in ELISA and FACS in a similar way to what have been done here. So far, no result indicated that a certain clone has higher affinity for the receptor, but a pool of phages with stronger binders has been identified. It could also be interesting to dock CHIPS proteins with different combinations of mutations to the N-terminal part of the entire receptor *in silico* to see what interactions that are proposed.

When amino acids in CHIPS involved in the binding to the C5aR are identified these properties have to be combined with the non-antigenic properties to create a functional CHIPS variant. The CHIPS variant with the new properties should then bind the receptor strongly at the same time as it is not recognised by the human immune system.

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References

Chen, Z., Zhang X., Gonnella N. C., Pellas T. C., Boyar W. C., Ni F. (1998). ”Residues 21-30 within the extracellular N-terminal region of the C5a receptor represent a binding domain for the C5a anaphylatoxin.” J Biol Chem **273**:10411-10419.

de Haas, C. J., Veldkamp, K. E., Peschel A., Weerkamp F., Van Wamel WJ, Heezius EC, Poppelier MJ, Van Kessel KP, van Strijp JA.. (2004). "Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent." J Exp Med **199**(5): 687-95.

Dinges, M. M., P. M. Orwin, Schlievert P. M. (2000). "Exotoxins of Staphylococcus aureus." Clin Microbiol Rev **13**(1): 16-34, table of contents.

Engberg, J., Andersen P. S., Nielsen L. K., Dziegiel M., Johansen L. K., Albrechtsen B. (1995) "Phage-display libraries of murine and human antibody Fab fragments." Methods Mol. Biol. **51**:355-376.

Fransson, J., Tornberg U. C., Borrebaeck C. A., Carlsson R., Frendeus B. (2006). “ Rapid induction of apoptosis in B-cell lymphoma by functionally isolated human antibodies.” International Journal of Cancer **119**(2):349-358.

Huang, L., Sato, A. K., Sachdeva M., Fleming, T., Townsend, S., Dransfield, D. T. (2005). “Discovery of human antibodies against the C5aR target using phage display technology.” J Mol Recognit. **18**(4):327-33.

Janeway, C., Immunobiology, 5th ed. (2001), New York and London, Garland Science Publishing.

Jestin, J.-L., Volioti G., Winter G. (2001). "Improving the display of proteins on filamentous phage." Research in Microbiol. **152**(2): 187-191.

Kew, X., Peng T., DiMartino S. J., Madhavan D., Weinman S. J., Cheng D., Prossnitz E. R. (1997). "Undifferentiated U937 cells transfected with chemoattractant receptors: a model system to investigate chemotactic mechanisms and receptor structure/function relationships." J. Leukoc. Biol. **61**:329-337.

Paschke, M. (2006) “Phage Display systems and their applications.” Appl Microbiol Biotechnol **70**:2-11.

Pellas, T. C. and L. P. Wennogle (1999). "C5a receptor antagonists." Curr Pharm Des **5**(10): 737-55.

Postma, B., W. Kleibeuker, Poppelier MJ, Boonstra M, Van Kessel KP, Van Strijp JA, de Haas CJ., (2005). "Residues 10-18 within the C5a receptor N terminus compose a binding domain for chemotaxis inhibitory protein of Staphylococcus aureus." J Biol Chem **280**(3): 2020-7.

Qiagen, “The QIAexpressionist”-handbook. 5th ed., 2003. http://www1.qiagen.com/literature/, (6 Mar, 2007).

Russel, M., T. Clarkson, H. B. Lowman. “Phage Display: A Practical Approach” (The Practical Approach Series, 2004).

Wagner, S., Bader M. L., Drew D., de Gier J. W.(2006). “Rationalizing membrane protein overexpression.“ Trends Biotechnol. **24**:364-371.