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Original Article

Tyrosine sulfation in N-terminal domain of human C5a receptor is necessary for binding of chemotaxis inhibitory protein of *Staphylococcus aureus*

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Aim: *Staphylococcus aureus* evades host defense through releasing several virulence proteins, such as chemotaxis inhibitory protein of staphylococcus aureus (CHIPS). It has been shown that extracellular N terminus of C5a receptor (C5aR) forms the binding domain for CHIPS, and tyrosine sulfation is emerging as a key factor in determining protein-protein interaction. The aim of this study was to evaluate the role of tyrosine sulfation of N-terminal of C5aR in its binding with CHIPS.
Methods: Expression plasmids encoding C5aR and its mutants were prepared using PCR and site-directed mutagenesis and were used to transfect HEK 293T cells using calcium phosphate. Recombinant CHIPS protein was purified. Western blotting was used to examine the binding efficiency of CHIPS to C5aR or its mutants.
Results: CHIPS exclusively binds to C5aR, but not to C5L2 or C3aR. A nonspecific sulfation inhibitor, sodium chlorate (50 nmol/L), diminishes the binding ability of C5aR with CHIPS. Blocking sulfation by mutation of tyrosine to phenylalanine at positions 11 and 14 of C5aR N terminus, which blocked sulfation, completely abrogates CHIPS binding. When tyrosine 14 alone was mutated to phenylalanine, the binding efficiency of recombinant CHIPS was substantially decreased.
Conclusion: The results demonstrate a structural basis of C5aR-CHIPS association, in which tyrosine sulfation of N-terminal C5aR plays an important role. Our data may have potential significance in development of novel drugs for therapeutic intervention.

Keywords: *Staphylococcus aureus;* chemotaxis inhibitory protein; tyrosine sulfation; post-translational modification; chemotactic receptor; C5aR

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Introduction

Staphylococcus aureus is a causative agent of pulmonary infections in immunocomponent normals as well as in immunocompromised individuals. In particular, the prevalence of highly virulent methicillin-resistant strains is increasingly becoming a public health challenge in hospital or community environments[1]. One possible mechanism underlying this may be that staphylococci release chemotaxis inhibitory protein of staphylococci (CHIPS) encoded by chp that contributes to the evasion of these bacteria from the immune system of the host. CHIPS has been shown to be necessary for inhibition of the early immune response by blocking the initial activation and recruitment of neutrophils and monocytes into the site of infection[2, 3].

The cellular receptor for CHIPS is C5aR with a nanomolar binding affinity[4]. C5aR is a seven-transmembrane segment belonging to the family member of rhodopsin-like G-protein coupled receptors (GPCRs)[5]. A previous study with chimeras and point mutations indicated that CHIPS exclusively bound to the N-terminal C5aR, in which acidic aspartic acid at positions 10, 15 and 18 and glycine at position 12 were specifically important for binding, whereas tyrosine at positions 11 and 14 did not involve CHIPS binding[6]. It should be pointed out that these authors used a FLAG tag fused into the N-terminus of the whole C5aR. FLAG tag is not applicable for studies regarding tyrosine sulfation because it contains a sulfatable tyrosine (DYKDDDDK) which probably provides the additional binding energy[7]. A subsequent study of the association

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between CHIPS and a sulfated peptide based on N-terminus of the human C5aR using nuclear magnetic resonance (NMR) demonstrated that sulfated tyrosine at positions 11 and 14 of N-terminal C5aR significantly contributed to the tight binding[8].

Tyrosine sulfation is a post-translational modification occurring in a variety of secreted and integral membrane proteins and, in many cases, enhances the interactions between these proteins and their corresponding ligands, or invading pathogens[9, 10]. This modification tends to occur in acidic regions of proteins, usually containing multiple tyrosines[9, 11]. Several receptors for chemokines and hormones, including CCR5, CXCR3, C3XCR1, and thyroid-stimulating hormone receptor, have been shown to be sulfated on tyrosines in their amino terminal extracellular domains and some of this sulfation is critical for ligand binding[12–16]. Amino-terminal sulfation of tyrosines in the chemotactic receptor for C5a also contributes to formation of the docking site for the C5a anaphylatoxin[17]. We have previously demonstrated that sulfated tyrosine 174 in second extracellular loop of C3aR is essential for binding and signaling with native C3a[18]. Sulfotyrosine in the amino terminal sequence of CCR5 is important for binding of the natural ligands [macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES] and certain HIV-1 gp120/CD4 complexes, and facilitates the entry of CCR5-using strains of HIV-1[13, 19]. Duffy binding protein of plasmodium vivax (*P vivax*) utilized sulfotyrosines of the Duffy antigen/receptor for chemokines for erythrocyte invasion by *P vivax*[20]. Thus, tyrosine sulfation represents a critical modification for conferring the natural function of a number of 7TMS receptors.

Here we report that C5aR, not C5L2 and C3aR, is an exclusive receptor for CHIPS binding. Furthermore, we show that two sulfotyrosines of the C5aR N-terminus are critical for CHIPS binding, and sulfated tyrosine at position 14 of N-terminal C5aR has a greater role in an association between CHIPS and C5aR. These data underscore that tyrosine sulfation plays an important role in the determining C5aR-CHIPS association and provides useful insights into how to block the binding of CHIPS to C5aR.

Materials and methods

Cells, plasmids, and constructs

HEK 293T cells were cultured in Dulbeco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. An expression plasmid encoding the human C5aR fused with a myc tag at its amino terminus was generated by PCR amplification of human genomic DNA, and subcloned into the pcDNA 3.1 expression vector (Invitrogen). All the C5aR variants YFY, YYF, YFF in which one or two tyrosines were mutated to phenylalanine were made by the PCR-based QuickChange method (Stratagene) and confirmed by sequencing the entire reading frame. The expression plasmids encoding the wild-type C5L2, C3aR, CXCR3, and platelet activating factor receptor (PAFR) were constructed as above described.

The plasmids encoding human tyrosyl protein sulfotransferases (TPST) 1 and 2, and small hairpin RNA (shRNA) targeting nucleotides 259-276 of TPST1 and nucleotides 73−94 of TPST2 were provided by Dr H Choe[18, 19].

Purification of recombinant CHIPS protein

Expression and purification of recombinant CHIPS was as previously described with minor modification[3, 21, 22]. Briefly, chp, with the exclusion of its signal sequence, was amplified by PCR on genomic DNA extracted from methicillin-resistant staphylococcus aureus (MRSA) ATCC 29213, cloned into pET32a (+) vector, then transformed into *Escherichia coli* BL21(DE3) plays (Invitrogen). In order to measure the expression of CHIPS, a His tag was fused into N-terminus of CHIPS. Purified inclusion bodies obtained from bacteria were induced with 1 mmol/L of isopropyl-1-thio-β-*D*-galactopyranoside (Invitrogen) overnight at 20°C and solubilized in 6 mol/L guanidine HCl. The protein was dialyzed against 10 mmol/L Tris-HCl, pH 8.0 and purified on HiTrap column (GE Healthcare Biosciences), using 10 mmol/L Tris-HCl, pH 8.0, 1 mol/L NaCl for elution. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). The concentration of purified CHIPS was 96 mg/L.

Assay of chp in Clinical *S aureus* isolates in patients

Thirteen clinical MRSA and 13 MSSA strains were isolated from sputum or bloodstream in patients diagnosed with pulmonary infection. PCR was carried out on genomic DNA of using the forward primer 5′-ATGAAAAA-GAAATTAGCAACAACAG-3′ and the reverse primer 5′-TTAGTATGCATATTCATTAGTTTTTCC-3′. The size of PCR product was 450 bp.

The study protocol was reviewed and approved by the Peking Union Medical College Hospital Human Research Ethics Committee and all subjects gave informed written consent to participate in the study.

Western blotting analysis

The whole protein was prepared as previously described[23]. After removing the culture medium, cells were extensively washed three times, harvested and lysed in solubilization buffer containing a protease inhibitor cocktail (Sigma). Cell debris was removed by centrifugation at 12 000×*g* for 15 min at 4°C. The supernatants were eluted by adding an equal volume of sample loading buffer, then run on 12% SDS-PAGE gels before transferring to PVDF membranes. Memebranes were incubated in the presence of the indicated antibodies. Antibodies were as follows: anti-myc (Signal Chem), anti-His (Signal Chem). Primary antibody application was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse IgG) (Santa Cruz). Blots were developed using an enhanced chemiluminescence detection system (ECL) (Amersham) as per the manufacturer's instructions.

Analysis for binding of CHIPS to C5aR

Binding experiments were performed with HEK 293T cells

transfected with wild type C5aR or its variants using calcium phosphate[14, 17, 18]. One day later, cells were washed with PBS, and split into two separate aliquots, one used for binding assay and one for receptor expression analyses. CHIPS 3 μg/mL was incubated with cells expressing wild-type C5aR or C5aR variants for 30 min on ice. Western blotting was used to assay the binding efficiency and receptor expression.

Sodium chlorate treatment

Transfected cells were cultured in medium containing the various concentrations of sodium chlorate for at least 48 h at 37 °C[14, 24].

Results

Prevalence of clinical *S aureus* isolates

The gene encoding for CHIPS, chp, lies at a bacteriophage-encoded immune cluster, seen in greater than 60% of *S aureus* isolates[3]. To examine the prevalence of chp in clinical *S aureus* isolates in our hospital, one of the major referral medical centers in China, we screened 13 methicillin-resistant strains and 13 methicillin-sensitive isolates (MSSA) from blood stream of infected individuals by PCR. Our data showed that chp was present in more than 30% of *S aureus* strains. Of note, more than 30% of MSSA was PCR-positive for chp.

Production and characteristics of recombinant CHIPS

A truncated version of CHIPS that did not contain a signal sequence at the N-terminus showed a similar C5aR blocking activity compared to the CHIPS[21]. Recombinant CHIPS was produced in *E coli* with a His tag fused into N-terminus of CHIPS to facilitate the recognition by anti-His antibody.

To demonstrate a direct interaction between CHIPS and the receptors for anaphylatoxins and chemokines, we performed transfection studies.

For this purpose, HEK cells were transfected with the expressing plasmids encoding C5aR, C5L2, C3aR, CXCR3, and PAFR. Figure 1 demonstrates the binding of CHIPS to C5aR overexpressed in HEK 293T cells. There was no binding of CHIPS to C5L2, C3aR, CXCR3, and PAFR observed even after longer exposure (Figure 1). These results indicate a direct binding of CHIPS to the C5aR.

The ATP sulfrylase inhibitor sodium chlorate decreases the binding affinity of CHIPS to C5aR but not C5aR expression

Sulfate can be added to proteins at sites of N- or O-linked glycosylation or on tyrosines via post-translational modifications[25]. C5aR has been shown to be modified by N-linked glycosylation. We treated C5aR-transfected-cells with various concentrations of sodium chlorate, a nontoxic inhibitor of ATP sulfrylase activity[14, 26], for at least 48 h in sulfate-free media. We identified a direct inhibition of the binding of CHIPS to wild-type C5aR by sodium chlorate in a concentration-dependent manner. Figure 2 shows that the binding of CHIPS to cellular C5aR was completely abolished in presence of 50 nmol/L of sodium chlorate, providing an independent evidence for the requirement of sulfation for CHIPS binding.

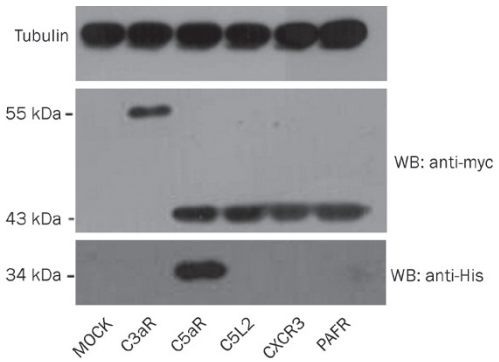

Figure 1. CHIPS exclusively binds to C5aR, but not to C5L2. HEK 293T cells were transfected with plasmids encoding the wild-type C5aR, C5L2, C3aR, CXCR3, and PAFR with N-terminal epitope tag. After 24 h they were divided into two aliquots, one was retained for analysis of receptor expression, one was used for binding experiments. N-terminally His-tagged CHIPS 3 μg/mL was incubated with transfected cells for 30 min on ice. The samples were subjected to 12% SDS-PAGE gels, transferred to PVDF membranes, detected by the indicated primary antibodies, followed by horseradish peroxidase-conjugated anti-mouse IgG. All samples were run under reducing condition. Numbers at the left indicate the positions of molecular weight markers.

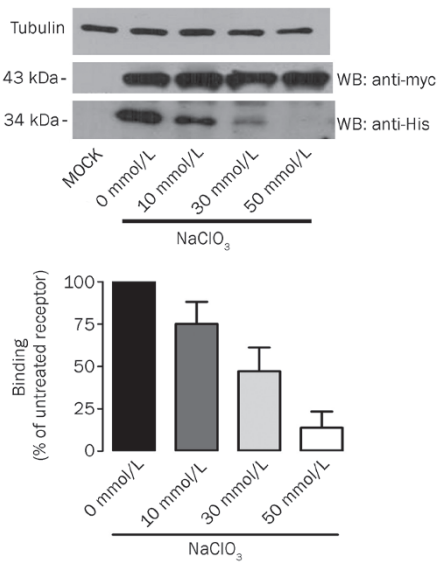

Figure 2. Effect of tyrosine sulfation of C5aR on CHIPS binding. HEK 293T cells were transfected with plasmids encoding the wild-type C5a receptor with N-terminal myc tag. After 24 h, cells were divided into two aliquots, one was retained for analysis of receptor expression, one was treated with the various concentrations of sodium chlorate for at least 48 h and then incubated with 3 μg/mL of N-terminally His-tagged CHIPS for 30 min on ice. The binding efficiency of C5aR-CHIPS was checked by Western blotting. Shown is representative of 3 independent experiments. The data represent the mean±SD of three experiments.

Sulfated tyrosines in the N-terminus of the C5aR contribute to CHIPS binding

Previous work has shown that sulfation of tyrosines on the extracellular amino terminal domain of the C5a anaphylatoxin receptor is a critically important post-translational modification for ligand recognition[17]. Given the importance of the sulfotyrosines in ligand binding, we examined whether a similar post-translational modification might be important for CHIPS as well. Tyrosines at positions 11 and 14 in N terminus were individually and doubly mutated to phenylalanine. Constructs were transfected into HEK 293T cells and tested for expression and binding.

In order to facilitate the determination of relative levels of cell surface expression by Western blotting, expression plasmids encoded for the wild-type C5aR or its mutants were fused in frame with an amino-terminal myc-tag, recognized by the antibody, 9E10. Simultaneously, cells from the same transfection were incubated with 3 μg/mL of CHIPS for 30 min on ice. Cells were extensively washed three times, lysed, and then transferred to PVDF membrane. Finally the PVDF membrane containing CHIPS-C5aRs complexes were incubated with the anti-His antibody, which is specific for the His-tag fused to the N-terminus of CHIPS. Western blot analysis of the binding of CHIPS to the wild-type C5aR or its mutants expressed in HEK 293 T cells was accomplished using the anti-His antibody.

As shown in Figure 3, when two tyrosines at positions 11 and 14 were changed to phenylalanine, the ability that C5aR YFF variant bound CHIPS was completely lost. We then investigated the specific contribution of each of the two tyrosines of the C5aR to CHIPS binding by assaying receptor variants (YFY and YYF) in which tyrosines 11 and 14 had been altered individually to phenylalanine. Figure 3 shows that cells transfected with either the YFY variant efficiently associated with CHIPS compared with cells expressing wild-type C5aR. By contrast, YYF variant bound CHIPS less efficiently than wild-type C5aR and the YFY variant, suggesting a greater role of sulfotyrosine 14 in binding CHIPS. Cell surface expression of this mutant receptor was essentially same as the wild type receptor as indicated by their fluorescence intensity of anti-His-tagged antibody staining assessed by FACS analysis (data not shown). Taken together, these data show that both tyrosine sulfate moieties of the C5aR N terminus contribute to the formation of CHIPS binding site.

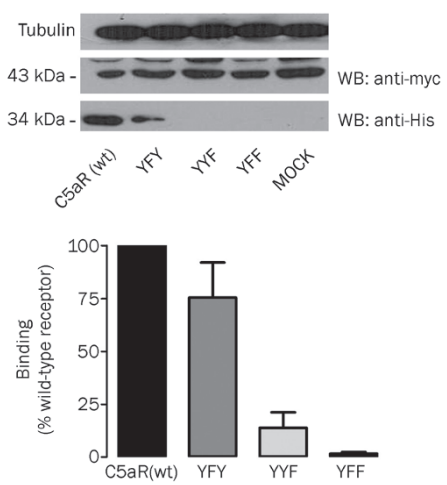

Figure 3. Tyrosine sulfation significantly contributes to the binding of CHIPS to C5aR. (A) HEK 293T cells were transfected with plamids encoding wild-type C5aR or the C5aR variants YFY, YYF or YFF. Cells were incubated with 3 μg/mL of N-terminally tagged-His CHIPS as described in Materials and Methods. Cells were extensively washed, and CHIPS bound by C5aR was determined by Western blotting using anti-His antibody. Aliquots of the same transfections were analyzed for C5aR expression levels by Western blotting using the anti-myc-tag antibody 9E10. Shown is representative of three independent experiments. The data represent the mean±SD of three experiments.

Effect of enzymatic modulation of sulfation on the binding ability of C5aR to CHIPS

One possible explanation for blockade of CHIPS binding to C5aR variant YFY is due to the absence of sulfate moiety on tyrosine 14. We cannot exclude the possibility that the expression of phenylalanine at this site changed receptor conformation due to the increase in hydrophobicity caused by the lack of the hydroxyl group[14, 26]. We assessed these possibilities by using tyrosine protein sulfotransferases 1 and 2 (TPSTs), which have been shown to increase the extent of tyrosine sulfation of GPCRs for ligand binding[14, 18, 19]. We co-transfected the plasmid encoding C5aR variant YFY with constructs for TPST1 and 2. Cells transfected with C5aR YFY alone showed the same receptor expression level compared to cells co-transfected with the plasmids encoding C5aR YFY variant and TPSTs as indicated by their fluorescence intensity of anti-His-tagged antibody staining assessed by FACS analysis (data not shown). The cells co-transfected with C5aR variant YFY and TPSTs displayed the significant increase in binding efficiency with CHIPS (Figure 4). Thus, sulfation of tyrosine 14 on the C5aR N-terminus is a critical determinant for recognition by CHIPS.

The activity of TPSTS can be modified by transfection with constructs encoding shRNAs targeting the TPSTs to decrease them. In the case of C3aR and CXCR3, tyrosine sulfation was reduced by shRNAs against TPSTs, and maximal binding was significantly decreased accordingly[14, 18]. However, blockade of the activity of TPSTs by shRNAs partially inhibited the binding of CHIPS to C5aR variant YFY (Figure 4).

Discussion

The molecular cloning of the C5a receptor places this molecule in the superfamily of G-protein coupled 7TMS receptors[27, 28]. C5a, a key component of complement system, is particularly important for recruitment of leukocytes and effective clearance of invading pathogens. C5a exerts its effect via the binding and activation of C5aR[5, 29]. More recently, we, and others have demonstrated the existence of sulfated tyrosines in amino terminal sequences of several chemoattractant receptors that play a critical role in receptor function[13, 14]. The C5a anaphylatoxin receptor has been shown to contain sulfotyrosines in

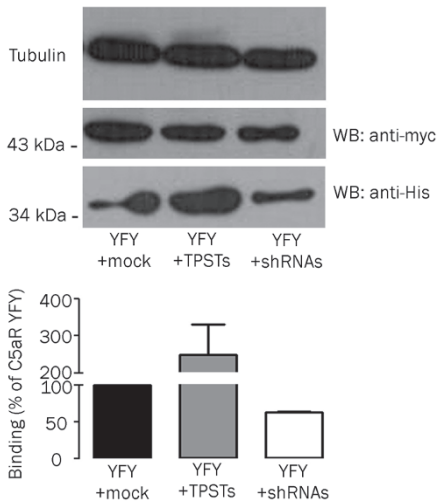

Figure 4. Effect of enzymatic modulation of tyrosine sulfation on the association between CHIPS and C5aR YFY. HEK 293T cells were co-transfected with the plasmid encoding C5aR YFY or with plasmids encoding TPSTs or with shRNAs targeting TPSTs as indicated. The binding efficiency of CHIPS to C5aR YFY was determined by Western blotting identical to Figure 2. Results are expressed as means±SD. *n*=3 independent experiments.

its amino terminus that are involved in formation of the docking site[17]. Our data showed that more than 30% of MSSA was PCR-positive for the gene encoding for CHIPS, chp. The present study shows that C5aR, but not its second receptor C5L2 and other G-protein coupled receptor, is a unique receptor for CHIPS. Furthermore, our data validate and extend the results from the previous studies on association between C5aR and CHIPS by demonstrating that inhibition of sulfation effectively interferes with the ability of CHIPS to bind cells overexpressing C5aR, and thus tyrosine sulfation appears to contribute to the proper binding of CHIPS to this receptor. Even though there are two sulfatable tyrosines in N-terminus of C5aR, sulfated tyrosine 14 is significantly involved in CHIPS binding. Our data are consistent with the previously published results showing the inability of CHIPS to bind to the second C5a receptor, C5L2, although C5L2 was also sulfated on N-terminal tyrosine residues in a very similar pattern to C5aR[30, 31]. Taken together, these data suggest the very high specificity of CHIPS binding.

A study with mutation and chimeras reported that the C5aR lacking residues 1-18 of N-terminus showed a significant decrease in binding to CHIPS compared to wild-type C5aR[6]. Nikiforovich *et al* further demonstrated that CHIPS only bound to the first binding site of C5a to C5aR 10-18 fragment containing two sulfrotyrosines[32]. Similarly, alteration of aspartic acids 10, 15, and 18 and the glycine at position 12 had a profound effect on CHIPS binding. By contrast, the NMR study demonstrated that a tyrosine-sulfated peptide at positions 11 and 14 based on C5aR N-terminus has (about 400 times) stronger affinity in binding CHIPS than the non-sulfated peptide[8]. The presence of the two sulfate moieties on C5aR that contribute to its association with CHIPS may be helpful in explaining these conflicting data. Sulfate is a charged, highly polarizable moiety that may provide significant energy to the binding[10]. This tyrosine sulfation may contribute to the association of the negatively charged receptor N-terminus of C5aR with the positively charged CHIPS. Consistently, we demonstrate the critical role of tyrosine sulfation of C5aR N-terminus in CHIPS binding. Our data raise the possibility that the C5aR N-terminal aspartic acids are perturbed by CHIPS binding probably due to interfering with acidic domain necessary for tyrosine sulfation, like C5a-C5aR interaction[17].

Tyrosines at 11 and 14 of N-terminal C5aR, which are flanked by acidic amino acids indicative of sulfation, have been demonstrated to be sulfated. Sulfated tyrosine 11 had a greater role in the C5a binding[17], whereas sulfotyrosine 14 was shown to be important for CHIPS binding in this current study. These data are in agreement with the predicted substrate requirement for TPSTs[11]. To ensure that loss of CHIPS binding commensurate with mutation of tyrosine 14 to phenylalanine reflected the loss of sulfate and not indirect structural alterations of the receptor, we attempted to first co-express the C5aR variant YFY with TPST1 and 2. We showed a greater increase in binding efficiency of CHIPS to the cells co-transfected with C5aR variant YFY and TPST than the cells transfected with the YFY variant alone, which supports the importance of the sulfate moiety for enhancement of CHIPS binding. However, when the co-transfect ions were performed using shRNA constructs targeting TPST1 and 2, binding efficiency was partially inhibited with essentially no change in receptor expression (as assessed by FACS analysis, data not shown). The observation might be reflective of the enzymatic activity of TPSTs synthesized prior to shRNA transfection[33, 34]. In addition, the partial inhibition of CHIPS to the wild-type C5aR or its mutants caused by shRNAs might suggest that optimal binding requires the participation of other component of the receptor in CHIPS binding, such as posttranslational tyrosine phosphorylation[35]. Our data are in agreement with a very recent observation using a phosphorylated analog of C5aR N-terminus that the phosphate group at tyrosine 14 was involved in stronger binding of CHIPS to C5aR[35].

Tyrosine sulfation may be important for the initial capture of C5aR-expressing cells by CHIPS and less important for other potential downstream functions. A recent study showed that a tyrosine-sulfated peptides based on the N-terminus of C5aR interacted with CHIPS and could inhibit C5a-induced calcium influx[8]. Thus, the present study might suggest that CHIPS competitively associates with the primary binding site of C5a located at the N-terminus of the C5aR, thereby preventing the C-terminal tail of C5a from contacting the activation domain of the C5aR and blocking downstream signaling. Tyrosine sulfation may represent a general mechanism utilized by the virulence proteins secreted by invading pathogens that mediate the rapid capture of circulating leukocytes.

In summary, the current study has not only defined the structural requirement for the CHIPS binding, but also demonstrated that tyrosine sulfation has a wider biological sig-

nificance. Since *S aureus* continually poses a threat to human health, a precise knowledge of the structural basis for the binding of CHIPS to C5aR as we have shown should have great significance in developing potent drugs to reduce the pathogeneticity of this infection.

Abbreviations

CHIPS, Chemotaxis inhibitory protein; C3aR, C3a receptor; C5aR, C5a receptor; CCR5, CC chemokine receptor 5; CXCR3, CXC receptor 3; PAFR, platelet activating factor receptor; 7TMS, 7 transmembrane segment; GPCR, G-protein coupled receptor. PBS, phosphate-buffered saline. WB: Western Blotting.

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Author contribution

Zhen-jia LIU and Yan-juan YANG performed the whole experiment; Lei JIANG helped and did some experiments; Ying-chun XU and Ai-xia WANG provided the MRSA strain and helped the experiment; Guan-hua DU supervised the experiment; Jin-ming GAO designed and supervised the experiments, and drafted the manuscript.

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