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Molecular characterization of single-chain antibody variable fragments (scFv) specific to Pep27 from *Streptococcus pneumoniae*

Dongho Kim [a], ShinA Jang [a], Jihye Oh [a], Seungsu Han [a], Soobin Park [a], Prachetash Ghosh [b], Dong-Kwon Rhee [b], Sangho Lee [a, *]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon, 16419, South Korea
[b] School of Pharmacy, Sungkyunkwan University, Suwon, 16419, South Korea

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ABSTRACT

Pep27 from *Streptococcus pneumoniae* is reported to initiate pneumococcal autolysis, thereby constituting a major virulence factor. Although a few antisera recognizing Pep27 have been reported, no monoclonal, well-characterized antibody for Pep27 has been developed. Here we screened two single-chain antibody variable fragments (scFv) using a phage display from a large human synthetic scFv library to select clones E2 and F9. Dissociation constants (K_d) of E2 and F9 were 1.1 μM and 0.50 μM, respectively. E2 and F9 did not cross-react with other pneumococcal and unrelated proteins. The epitopes of Pep27 were localized to residues 24, 26 and 27 by alanine scanning. Molecular docking analysis supported the experimentally investigated epitope. The E2 and F9 clones specifically detected Pep27 in an environment mimicking *in vivo* conditions, demonstrated in human serum. The scFv clones characterized here represent molecular tools for the detection of pneumococcal diseases with potential for further improvement in affinity.

1. Introduction

Pep27 from *Streptococcus pneumoniae* is a small secretory, autolytic peptide containing 27 amino acid residues. Originally reported as a "death signal peptide" in the *vex123-pep27-vncRS* locus that encodes the Vex ABC transporter and the VncRS two-component system as well as the signal peptide Pep27 in vancomycin tolerance [1] the exact function of Pep27 is still disputed. However, recent studies elucidated the biological roles of Pep27 in pneumococcal infection. Pep27 exhibits antibacterial activities by activating phosphatase activity [2]. Analogues of Pep27 with enhanced hydrophobicity exhibit anticancer activities by penetrating the plasma membrane of cultured cells [3]. The loss of Pep27 renders pneumococci avirulent and results in decreased levels of capsular polysaccharide, establishing that Pep27 is a pneumococcal virulence factor [4]. Solution NMR studies reveal that the N-terminal half of Pep27 is a helix while the C-terminal half is virtually a random coil [3].

Loss of pneumococcal virulence caused by Pep27 loss promoted the development of *pep27* mutant strain as a live vaccine for pneumococcal diseases. Intranasal immunizations with the *pep27* mutant strain protect mice against pneumococcal infection in a serotype-independent manner [4] and also against influenza virus [5]. The pep27 mutant vaccine apparently promotes cytokine secretion and boosts secretory-IgA levels in a mouse model [4]. These findings strongly suggest that Pep27 plays a crucial role in pneumococcal and influenza virus infection prompting the development of antibody-based therapeutics. We therefore initiated the screening of antibodies in the form of single-chain variable fragments (scFv) and characterized them at the molecular level.

2. Results and discussion

2.1. Selection of an scFv specific to pneumococcal Pep27

Two versions of Pep27 with sequences differing at amino acid residues 12 and 16 have been reported (Fig. 1A). The "R6" sequence (UniProt accession number Q8DQS1) is derived from the genomic sequence of R6 strain. The "p28" sequence (UniProt accession number Q9S4I9) is derived from the same R6 strain [1] although it is modified as D12G and T16A when treated as a "mutant" of the "R6" sequence. The "p28" sequence reportedly initiates the cell

* Corresponding author.
E-mail address: sangholee@skku.edu (S. Lee).

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A

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R6      1  MRKEFHNVLSSDQLLTDKRPARDYNRK
p28     1  MRKEFHNVLSSGQLLADKRPARDYNRK
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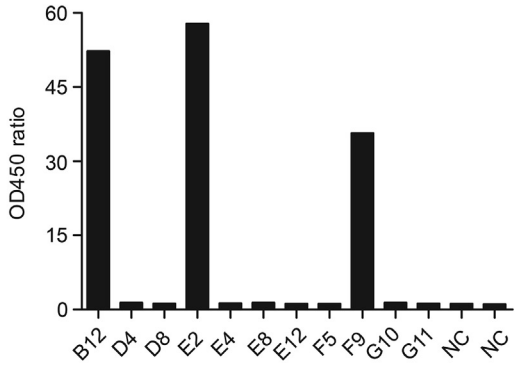
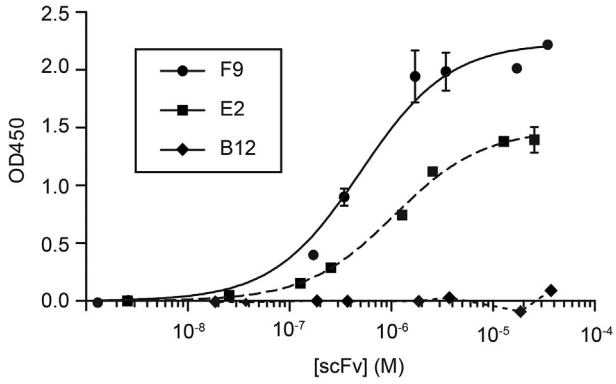
B



C



K_d (E2) = 1.08 ± 0.2μM
K_d (F9) = 0.50 ± 0.2μM

D

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                                                CDR-H1                                  CDR-H2
E2      1  EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYYMSWVRQAPGKGLEWVSGISPDDSSTYY
F9      1  EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYDMSWVRQAPGKGLEWVSSISPSSGSIYY

                                                                        CDR-H3
E2     61  ADSVKGRFTISRDNSKNTLYLQMNSLRAEDAAVYYCARDVRMCTSMSCSYYYGMDVWGQG
F9     61  ADSVQGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAIIKCAPDACYYDDGMDVWGQG

                                                                CDR-L1
E2    121  TLVTVSSGGGGSGGGGSGGGGSQSVLTQPPSASGTPGQRVTISCSGSSSNIGSNSVSWYQ
F9    121  TLVTVSSGGGGSGGGGSGGGGSQSVLTQPPSAPGTPGQRVTISCSGSSSNIGSNAVTWYQ

                        CDR-L2                                          CDR-L3
E2    181  QLPGTAPKLLIYYNSHRPSGVPDRFSGSKSGTSASLAVSGLRSEDEADYYCGTWDSSLNA
F9    181  QLPGTAPKLLIYANSHRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCGSWDASLNA

E2    241  YVFGGGTKLTVLG
F9    241  YVFGGGTKLTVLG
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Fig. 1. Amino acid sequences of the two Pep27 variants and biopanning of scFv clones specific to pneumococcal Pep27. (A) Amino acid sequences of the two Pep27 variants used as antigens in biopanning are shown in single-letter codes. For explanation of the designations "R6" and "p28", see the main text. (B) The best 11 colonies from the final biopanning are displayed with their OD^{450} ratios (OD^{450} of His_6-MBP-Pep27/OD^{450} of His_6-MBP), along with negative controls (labeled "NC"). Each well in the two enzyme-linked immunosorbent assay (ELISA) plates was coated with His_6-MBP-Pep27 and His_6-MBP. A total of 94 colonies from the final round of biopanning were subjected to ELISA analysis. (C) ELISA-based affinity analysis of anti-Pep27 scFv E2, F9. The K_d values of E2 and F9 were estimated by kinetic analysis. (D) Amino acid sequences of the two scFv candidates (E2 and F9) selected from biopanning are shown in single-letter codes. Six CDR regions are labeled. Identical residues are shown in the black background.

death program of *S. pneumoniae* via VncRS two-component system. To select scFvs responsive to both Pep27 sequences, we employed two types of Pep27 as antigens: His_6-MBP-$Pep27^{R6}$ and synthetic $Pep27^{p28}$. We conducted five rounds of biopanning with the first four rounds employing the synthetic $Pep27^{p28}$ and the final one using the recombinant His_6-MBP-$Pep27^{R6}$ (hereafter referred to as "His_6-MBP-Pep27") (Supplementary Table 1). Three clones - B12, E2 and F9 - exhibited significantly high signal compared with the negative controls (Fig. 1B). However, the clone B12 turned out to be a false positive based on ELISA (Fig. 1C). Dissociation constants (K_d) of the clones E2 and F9 were 1.1 μM and 0.5 μM, respectively (Fig. 2B). Sequence alignment revealed that these two positive clones differ predominantly in the CDR-H3 with a few minor variations in CDR-H2 (Fig. 1D).

2.2. Cross-reactivity of E2 and F9

We examined the cross-reactivity of E2 and F9 against other pneumococcal and unrelated proteins by immunoblotting (Fig. 2). Both E2 and F9 specifically recognized MBP-Pep27 but not the three pneumococcal proteins (GST-PspA, ClpL and IlvC) nor the two unrelated proteins (MBP and BSA). These results demonstrate that both E2 and F9 are scFvs specifically binding to Pep27.

2.3. Epitope and paratope mapping

To establish that E2 and F9 are a scFv highly specific to pneumococcal Pep27 with a moderate affinity, we analyzed the epitope region in Pep27. We subsequently constructed a series of deletion mutants of His_6-MBP-Pep27. Immunoblot analysis revealed residues 18-27 as the minimal epitope involved in the recognition by E2 and F9 (Fig. 3A), demonstrating a successful selection strategy covering both sequence variants of Pep27 because the variations were localized at the amino acid positions 12 and 16. Since the "R6" sequence is associated with serotype 2 and the "p28" with serotypes 4 and 14 based on sequence matching, the two scFvs selected including E2 and F9 in principle detected both variants of Pep27, resulting in a broader coverage.

To unveil the roles of each residue in the minimal epitope region, we performed alanine scanning to identify Y24, R26 and K27 of Pep27 as the key residues in the epitope region (Fig. 3B). We analyzed the immunoblot results in lieu of structural contexts. The *ab initio* model for Pep27 derived by the *Robetta* server [6], featuring the N-terminal helix and the C-terminal loop harboring residues 18-27 (Fig. 3C and E), was consistent with NMR findings suggesting that residues 15-27 assume random-coils [3]. Homology models for E2 and F9 were generated using the *SWISS-MODEL* server [7]. Molecular docking between scFv (E2 or F9) and Pep27 was performed using the *HADDOCK* server [8] with restraints ensuring that only residues 15-27 of Pep27 bind with an scFv. The *HADDOCK*-derived structural model of Pep27:E2 suggests that N25 of Pep27 form a hydrogen bond with Y112 of E2 and Pep27 R26 of Pep27 forms hydrogen bonds with Y33 and D54 of E2 (Fig. 3D). The importance of R26, possibly forming two hydrogen bonds and electrostatic interface with E2, is consistent with the alanine scanning results (Fig. 3B). Although N25 is implicated in the interaction with E2 in the structural model, the alanine scanning data shows no such evidence possibly attributed to a different rotamer dominating in N25 in the real structure. The docked model of Pep27:E2 also suggests a possible hydrogen bond between D12 of Pep27 and T58 of E2. Since D12 of Pep27 is located in the N-terminal helix with unknown effect in binding by E2 and F9, we attribute this potential interaction to a false positive result possibly due to an alternate rotameric arrangement or alignment of the N-terminal helix of Pep27. The structural model of Pep27:F9 suggests

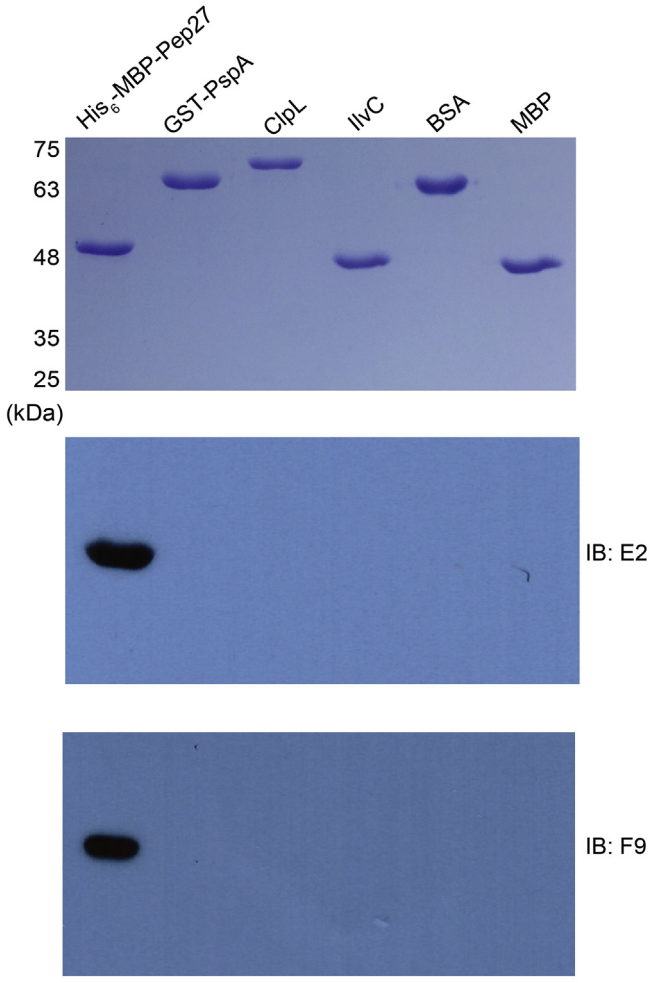

Fig. 2. Immunoblotting analysis of cross-reactivity of E2 and F9. Analysis of cross-reactivity of E2 and F9 against three pneumococcal proteins (GST-PspA, ClpL and IlvC) and two unrelated proteins (BSA and MBP) by immunoblotting is shown. Primary antibodies (E2 and F9 at 0.2 mg/mL, dilution 1:100) in the immunoblots are E2 and F9, and the secondary antibody is the anti-HA-HRP (0.2 mg/mL, dilution 1:3000) conjugate.

that K27 of Pep27 forms a hydrogen bond with T129 of F9, consistent with the more severe effect following binding by K27A of F9 compared with similar E2 mutant (Fig. 3B and D). In both docked models, we found no clear indication of the interaction between Y24 of Pep27. A closer look at the microenvironment centered on Y24 of Pep27 suggests that Y24 may mediate the binding to either E2 or F9 provided that the C-terminal loop of Pep27 adopts a configuration from the current docked model. Paratopes of E2 and F9 are located in the CDR-H and CDR-H3 region, respectively. Notably, the two docked models suggest that E2 and F9 contain non-overlapping paratopes. Taken together, our structural analysis using the docked model showcasing the binding between Pep27 and two scFvs (E2 and F9) reinforces alanine scanning results for epitope mapping of Pep27.

2.4. Recognition of Pep27 by E2 and F9 in human serum

To evaluate whether both E2 and F9 scFvs work in an environment mimicking *in vivo* conditions, we tested the recognition of Pep27 by E2 and F9 in human serum background (Fig. 4). Despite the appearance of weak non-specific bands, E2 and F9 recognized Pep27 equally well in the background of human serum, establishing

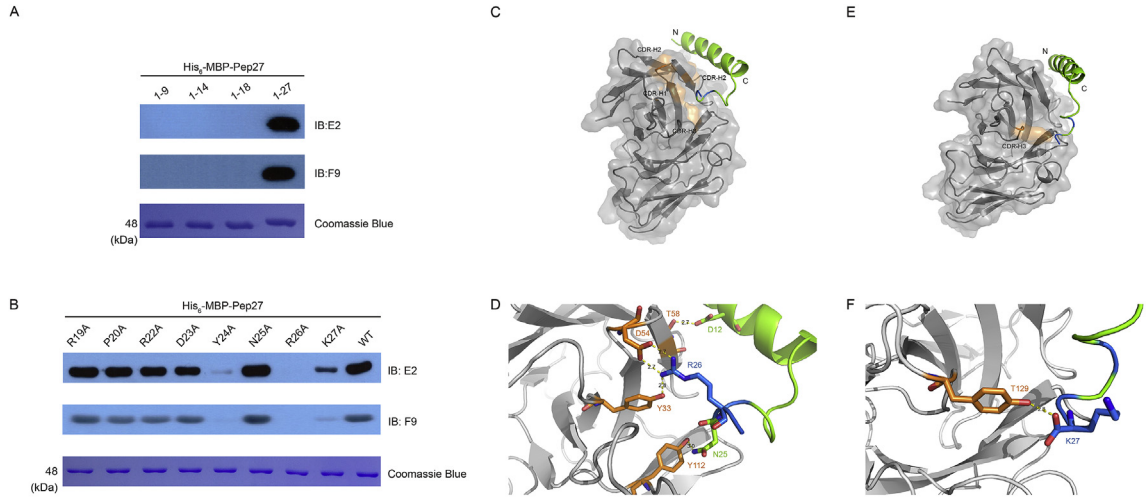

Fig. 3. Epitope and paratope mapping of E2 and F9. (A) Four His_6-MBP-Pep27 deletion mutants were used to map the epitope on Pep27 recognized by E2 and F9. The residue number of each deletion mutant is shown. (B) Each of the eight His_6-MBP-Pep27 residues was mutated to alanine to map the epitope recognized by E2 and F9 (0.2 mg/mL, dilution 1:100) at residue level. The residue number of each deletion mutant is shown. A single-letter amino acid code was used. Amount of the loaded protein per lane was 1 μg. Anti-HA-HRP (0.2 mg/mL, dilution 1:3000) was used as a secondary antibody. (C, E) The *HADDOCK*-derived structural model for scFv:Pep27. Pep27 is depicted as a ribbon representation and scFv as a surface model. The epitope in Pep27 identified experimentally is colored in blue. The N and C-termini are labeled along with the helix of Pep27. Predicted paratope of scFv is colored in orange and the rest in gray. (D, F) The *HADDOCK*-derived structural model for scFv:Pep27. Coloring and representation schemes are the same as in panels C and E. This figure was generated using *PyMOL* (Schrödinger). (C, D) E2. (E, F) F9. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the diagnostic role of E2 and F9 in principle. E2 apparently shows fewer non-specific bands than F9, indicating that E2, despite slightly worse affinity, may be more appropriate for *in vivo* use.

3. Materials and methods

3.1. Cloning, expression and purification of Pep27

DNA encoding Pep27 (amino acid sequence designated as "R" in Fig. 1A) was cloned into a parallel His_6-MBP vector that was modified based on the parallel vector system [9] in *Bam*HI and *Xho*I sites for the expression of His_6-MBP-Pep27 fusion protein. The identity of the construct was confirmed by DNA sequencing. The resulting plasmid was transformed into *E. coil* strain BL21 (DE3). A single colony was inoculated into 10 mL Luria-Bertani (LB) broth supplemented with 50 μg/mL of ampicillin overnight. The next day, the culture was transferred into 500 mL LB media containing 50 μg/mL of ampicillin, and grown until OD_{600} reached 0.6–1.0. A 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture, and grown at 16 °C for 16 h with shaking. The culture was harvested by centrifugation. The pellet was resuspended in phosphate-buffered saline (PBS), and lysed by sonication. The cell debris was cleared and the supernatant was added to Ni-NTA Agarose resin (Qiagen). The resin was washed with wash buffer (PBS pH 7.4 and 20 mM imidazole). The His_6-MBP-Pep27 was eluted in the elution buffer (PBS pH 7.4 and 300 mM imidazole) and dialyzed at 4 °C overnight. The resulting Pep27 was concentrated and loaded to a Superdex 70 16/60 size exclusion chromatography column (GE HealthCare) pre-equilibrated with PBS. Synthetic Pep27 (amino acid sequence designated as "p28" in Fig. 1A) was purchased from Anygen.

3.2. Biopanning using phage display

A synthetic human scFv library encoding HA-tagged scFv clones was used for biopanning [10]. Phages were prepared by infecting *E. coli* ER2537 cells containing the synthetic library with a VCSM13 helper phage as described previously [11]. The first four rounds of biopanning were performed in immunotubes coated with the synthetic Pep27 ("p28" sequence; Fig. 1A) conjugated with gradually decreased levels of biotin concentrations (20, 15, 10 and 7 μg/mL). The final fifth round of biopanning was performed with 5 μg/mL of recombinant His_6-MBP-Pep27 ("R6" sequence; Fig. 1A). Since the first four rounds of biopanning were performed with the synthetic Pep27, no negative selection was made in the fifth round using His_6-MBP only. The bound phages were eluted by adding 1 mL of 10 mM triethylamine to the tubes. The eluted phages were immediately neutralized with 0.5 mL of 1 M Tris-HCl pH 7.4. The phage-infected *E. coli* ER2537 cells were plated on LB broth/ampicillin/2% glucose agar plates to estimate the phage output from the biopanning. The remaining ER2537 cells were used for input titer and contamination test. To select scFv clones specifically binding to Pep27, single colonies from output titer plate were grown in a 96-well plate until an OD_{600} of 0.6–1.0 was reached and induced with 1 mM IPTG. The recombinant His_6-MBP-Pep27 at 1–10 mg/mL in PBS was coated on the wells of an ELISA plate. The cells in each well were centrifuged and the cell pellets resuspended in cold 1× TES buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA and 20% w/v sucrose) for 30 min on ice and then subsequently transferred to cold 0.2× TES for 1 h on ice. An ELISA assay was performed using an HRP-conjugated anti-HA antibody (Santa Cruz Biotechnology, sc-7392; 0.2 mg/mL, 1:3000 dilution) as the secondary antibody and the final signals were recorded at 450 nm. A total of 94 colonies bound to His_6-MBP-Pep27 and His_6-MBP by ELISA. The OD^{450} value of His_6-MBP-Pep27 was divided by the OD^{450} value of His_6-MBP and the ratio was compared.

3.3. Single chain antibody purification

A single colony of scFv-expressing *E. coli* was selected to inoculate 10 mL of LB/ampicillin and grown overnight at 37 °C with shaking. Next day, the overnight culture was added to 500 mL of SB/ampicillin media and grown at 37 °C with vigorous shaking until OD600 reached 0.5–0.8. IPTG was added to the mid-log phase culture at 1 mM final concentration. The culture was grown overnight at 30 °C with vigorous shaking. Next day, the overnight

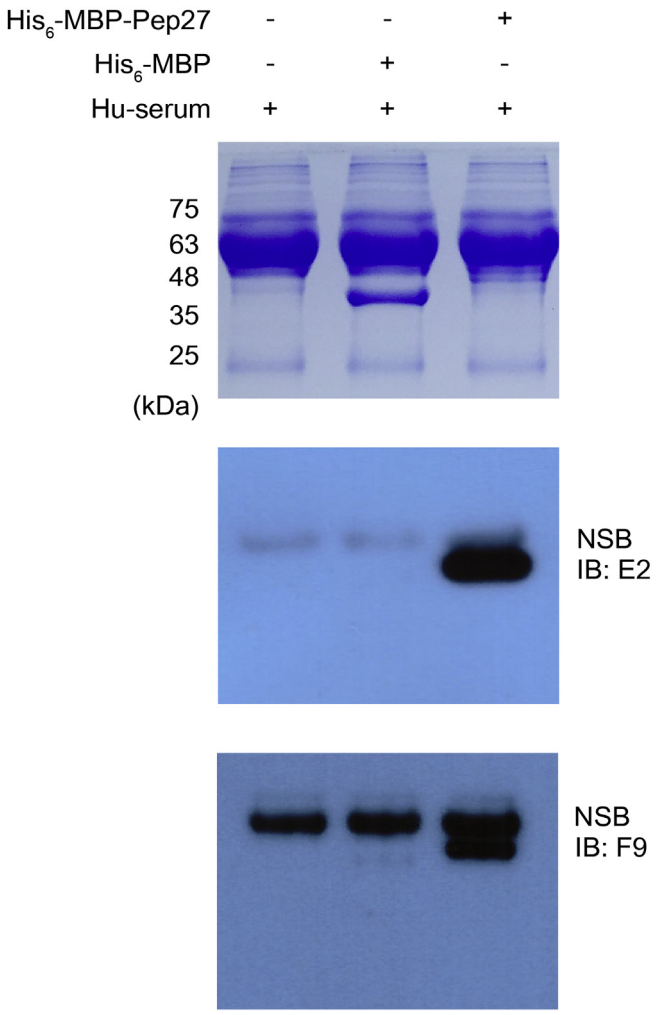

Fig. 4. Recognition of Pep27 by E2 and F9 in human serum. Recombinant His_6-MBP and His_6-MBP-Pep27 (1 μg each) was added to a pre-diluted human serum (1/40), followed by SDS-PAGE analysis. Primary antibodies included E2 and F9 (0.6 mg/mL, dilution 1:100) with anti-HA-HRP as the secondary antibody (0.2 mg/mL, dilution 1:1000).

culture was centrifuged and the supernatant was discarded. The pellet was resuspended in the cold 1xTES buffer for 30 min on ice and subsequently transferred to the cold 0.2xTES buffer for 1 h on ice. $MgCl_2$ was added at 5 mM final concentration and mixed well to block EDTA. The resuspended cell was centrifuged and the supernatant added to Ni-NTA agarose resin. The resin was washed with wash buffer (PBS pH 7.4, 20 mM imidazole and 0.5 mM DTT) and eluted with elution buffer (PBS pH 7.4, 300 mM imidazole and 0.5 mM DTT). The eluted fraction was concentrated to 5 mL and loaded to a Superdex 70 16/60 size exclusion chromatography column (GE HealthCare) pre-equilibrated with PBS.

3.4. Immunoblot

SDS-PAGE for the purified His_6-MBP-Pep27 was performed using 12% gel and transferred onto a PVDF membrane (45 V for 60 min). Membrane was blocked with 5% skim milk in TBSt overnight. Anti-Pep27-scFv-E2 and F9 was used for primary antibody. Anti-HA-HRP (Santa Cruz Biotechnology, sc-7392) was used as a secondary antibody (0.2 mg/mL, 1:3000 dilution).

3.5. Enzyme-linked immunosorbent assay

His_6-MBP-Pep27 was coated on half the total area of Corning® 96 well in the clear flat bottom polystyrene high binding microplate (Costar) and incubated overnight at 4 °C. Next day the overnight culture was washed 3 times with a wash buffer (PBS supplemented with 0.1% tween-20). The plate was blocked with a blocking solution (5% skim milk in PBS containing 0.1% tween-20) followed by purification with scFv as the primary antibody (0.3 ng/mL ~ 1 mg/mL). The plate was washed 3 times with a wash buffer (PBS supplemented with 0.1% tween-20) and diluted with HRP-conjugated anti-HA antibody (Santa Cruz Biotechnology, sc-7392; 0.2 mg/mL, 1:3000 dilution) representing the secondary antibody. The plate was washed 3 times with a wash buffer (PBS supplemented with 0.1% tween-20) and added SureBlue Reserve™ Substrate (KPL) and the reaction was discontinued by adding a stop solution (1 M NaOH). The final signal readings were recorded at 450 nm.

3.6. Pep27:scFv modeling

To determine the Pep27 structure, we performed a *de novo* structure prediction modeling with *Robetta* server (http://robetta.bakerlab.org) using its amino acids sequence [6]. We obtained five final models from the *Robetta* server and selected the best one based on the protein secondary structure consistent with that of a Pep27 solution structure reported previously [3]. For homology modeling of scFvs E2 and F9, we used a *SWISS-MODEL* server (https://swissmodel.expasy.org) [7] with one of the scFv homology models (PDB ID: 4BUH, chain A) as a template [7]. Protein-protein docking was performed using the *HADDOCK* server (https://haddock.science.uu.nl) [12]. To comply with the solution structure of Pep27 [3], we considered N-terminal (1-5) and C-terminal (16-27) regions of Pep27 as fully flexible during *HADDOCK* modeling. We also considered each of the six CDR regions of scFv structure as semi-flexible. The *HADDOCK* server facilitated Pep27:scFv docking with the random patches option containing flexible regions of interaction. A total of 12 and 32 docked structures of E2 and F9 were clustered respectively. The Z-score of Cluster is listed in Supplementary Table 2. We analyzed the results to select the most appropriate docking model consistent with the experimental results for Pep27 epitope mapping.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.05.054.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.05.054.

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