

Characterization of a unique conformational epitope on free immunoglobulin kappa light chains that is recognized by an antibody with therapeutic potential.

Andrew T. Hutchinson^{a,b*}, Ralitzia Alexova^{a*}, Vanessa Bockhorni^{a*}, Paul A. Ramsland^{c,d,e}, Darren R. Jones^a, Cameron V. Jennings^a, Kevin Broady^{a,b}, Allen B. Edmundson^f and Robert L. Raison^{a,b}.

^aDepartment of Medical and Molecular Biosciences, University of Technology Sydney, Ultimo, NSW 2007, Australia

^bThe iThree Institute, University of Technology Sydney, Ultimo, NSW 2007, Australia

^cCentre for Immunology, Burnet Institute, Melbourne, VIC 3004, Australia

^dDepartment of Immunology, Monash University, Alfred Medical Research and Education Precinct, Melbourne, VIC 3004, Australia.

^eDepartment of Surgery Austin Health, The University of Melbourne, Heidelberg, VIC 3084, Australia

^fProtein Crystallography Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104.

Email Addresses:

ATH:andrew.hutchinson@uts.edu.au, RA:ralitzia.alexova@alumni.uts.edu.au,
VB:vanessa_bockhorni@hotmail.com, PAR:pramsland@burnet.edu.au, DRJ:jones@cellmid.com.au,
CVJ:cam_jennings@hotmail.com, KB:kevin.broady@uts.edu.au, ABE:sedmund608@aol.com,
RLR:robert.raison@uts.edu.au

Correspondence: Andrew T. Hutchinson
Department of Medical and Molecular Biosciences
University of Technology Sydney
PO Box 123, Ultimo, NSW 2007, Australia
Phone: +61 2 9514 1789
Fax: +61 2 9514 8206
Email: andrew.hutchinson@uts.edu.au

*Equal contribution to this work

ABSTRACT

The murine mAb, K-1-21, recognizes a conformational epitope expressed on free Ig kappa light chains (FκLCs) and also on cell membrane-associated FκLCs found on kappa myeloma cells. This has led to the development of a chimeric version of K-1-21, MDX-1097, which is being assessed in a Phase II clinical trial for the treatment of multiple myeloma. The epitope recognized by K-1-21 is of particular interest, especially in the context that it is not expressed on heavy chain-associated light chains such as in an intact Ig molecule. Using epitope excision techniques we have localized the K-1-21 epitope to a region spanning residues 104-110 of FκLC. This short strand of residues links the variable and constant domains, and is a flexible region that adopts different conformations in FκLC and heavy chain-associated light chain. We tested this region using site-directed mutations and found that the reactivity of K-1-21 for FκLC was markedly reduced. Finally, we applied *in silico* molecular docking to generate a model that satisfied the experimental data. Given the clinical potential of the Ag, this study may aid the development of next generation compounds that target the membrane form of FκLC expressed on the surface of myeloma plasma cells.

Key Words: Immunoglobulin Light Chain, Immunotherapy, Antibody, Epitope, Molecular Modelling

Non-Standard Abbreviations: C, constant; FLC, free immunoglobulin light chain; FκLC, free immunoglobulin kappa light chain; κLC, kappa light chain; LC, light chain; V, variable.

1. INTRODUCTION

Therapeutic mAbs represent the largest class of biological agents with widespread use in oncology and autoimmunity. Each Ab is unique, and can function through many mechanisms such as inducing apoptosis, recruitment of effector cells and complement, and blocking interactions between receptors and ligands that are vital for activation of a target cell (Piggee, 2008). Understanding the modes by which Abs interact with their cognate Ags is invaluable for future developments in Ab based therapeutics. Unfortunately, many Ab-Ag complexes fail to crystallise and their structures cannot be determined by high resolution X-ray diffraction. Complicating the matter further, most protein-binding Abs recognize targets through conformational epitopes, and conventional mapping techniques are limited (Caoili; Chen et al., 2009; Kiselar and Downard, 1999; Saul and Alzari, 1996). One such Ab is the murine mAb, K-1-21, which recognizes a conformational epitope on human free Ig kappa light chains (FκLC), but does not recognise kappa light chains (κLC) that are in association with Ig heavy chain (HC) such as in an intact Ig molecule (Boux et al., 1983; Raison and Boux, 1985).

In humans, there are two isotypic variants of light chain (LC), kappa and lambda with 65% of B cells expressing the former (Barandun et al., 1976; Bradwell et al., 2008). LCs are composed of two beta-sheet rich Ig domains, the variable (V) and constant (C) domains that are connected by a short strand referred to as the 'switch' region (Schiffer et al., 1973). In an Ab molecule, the V domain of LC, in conjunction with the V domain of the associated heavy chain (HC), forms the Ag binding site. Diversity in the LC V domain is achieved by shuffling and recombination of individual germline gene segments, the variable and joining genes, during B cell development. The recombined V domain is then fused to the C domain which does not undergo recombination events (Abbas et al., 2007).

During the process of Ab synthesis, LCs are produced in excess of HCs which causes the secretion of FLCs by plasma cells (Bradwell et al., 2008; Hopper and Papagiannes, 1986). FLCs exist in three forms: as covalent and non-covalent homodimers, formed via interactions of adjacent V and C domains, and as free monomers (Bradwell et al., 2008). High levels of FLC in serum can be indicative of an underlying plasma cell malignancy such as multiple myeloma (Bradwell et al., 2008; Mead et al., 2004). Furthermore, analysis with mAb K-1-21 has shown that FκLCs are bound to sphingomyelin on the cell membrane of myeloma plasma

cells (Goodnow and Raison, 1985; Hutchinson et al., 2010; Walker et al., 1985). This has led to the development of a chimeric form of K-1-21, termed MDX-1097, that is currently undergoing assessment in a Phase II clinical trial for the treatment of multiple myeloma (Australian New Zealand Clinical Trials Registry Number: 12610000700033).

Although early studies on the characterization of K-1-21 suggested that the Ab recognizes a conformational epitope located in the C domain of F κ LC (Raison and Boux, 1985), it remains to be elucidated how the mAb interacts with F κ LC but fails to recognize HC-associated forms of κ LC as found in an Ig molecule or an Fab fragment (Boux et al., 1983) (see Supplementary Fig. 1). In this study, we utilized a range of techniques to identify regions on F κ LC that may comprise the epitope recognized by K-1-21. Using the experimental data, we then applied *in silico* molecular docking to arrive at a suitable model for the Ab-Ag complex formed between K-1-21 and F κ LC.

2. MATERIALS AND METHODS

2.1 General reagents

K-1-21 was produced on a fee for service basis by the Australian Commonwealth Scientific and Industrial Research Organization. F κ LC were purified from the urine of MM patients by ammonium sulfate precipitation as described previously (Raison and Boux, 1985). Monomers and dimers of F κ LC in these preparations were separated by size exclusion chromatography.

2.2 K-1-21 affinity purification of *in situ* derived F κ LC peptides

F κ LC peptides that had undergone proteolysis whilst being stored in urine were affinity purified by passage over a K-1-21 CNBr-activated Sepharose column (GE Biosciences). Purified peptides were subjected to two-dimensional electrophoresis. Briefly, samples were separated on a 3-10 pI immobilized gradient strip (BioRad), then size separated by SDS-PAGE in a 4-12% Criterion XT gel (BioRad). Gels were stained with Flamingo fluorescent stain (BioRad) and imaged on a Pharos FX Plus Molecular Imager (BioRad). Spots containing relevant peptides were excised from the gel and trypsinised for nanoLC/MS-MS analysis which was performed by the Australian Proteome Analysis Facility (APAF), at Macquarie University, NSW.

2.3 Epitope excision

Epitope excision was performed essentially following the protocol described by Parker and Tomer (Parker and Tomer, 2000). Briefly, K-1-21 was immobilized on CNBr-Sepharose then incubated with 50 μ g F κ LC for 2.5 h at room temperature. Unbound Ag was washed away and trypsin was added at a 1:20 w/w enzyme-substrate ratio. The digestion was carried out at 37°C for 24 h with slow rotation. Trypsin and unbound peptides were removed by washing the columns with PBS prior to performing the MS analysis. In addition, F κ LC was incubated with trypsin in solution for use as a positive control for tryptic digestion.

MALDI-MS was performed on a Voyager-DE STR (PerSeptive Biosystems) instrument equipped with a nitrogen laser (337 nm). The accelerating voltage was set at 20 kV and spectra were acquired by averaging data of 100 laser shots. Sepharose beads (15 μ L) were loaded on a C18 ZipTip (Millipore), washed three times with 0.1% TFA and bound peptides were eluted with 2 μ L of matrix solution. Only peptides in the detection range of 600-5000 Da were analysed. Peptides present in the mass spectra were identified by a Mascot search for

tryptic peptides with the same mass against a human or mouse protein database, allowing ± 3 Da error and up to 3 missed cleavage sites.

2.4 Site-directed mutagenesis and expression of F κ LC constructs

The κ LC gene was amplified from JJN-3 cell line cDNA with forward and reverse primers comprising the sequence 5'-CACCATGGAAACCCCAGCG-3' and 5'-CTAGCACTCTCCCCTGTTG-3' respectively. Using this as a template, internal mutagenic primers were designed (see Supplementary Table 2), and mutated constructs were amplified using κ LC specific primers with the various mutagenic primers. PCR products were cloned into the entry vector pENTR/D-TOPO then subcloned into the pT-Rex/DEST30 vector according to the manufacturer's recommendations (Invitrogen). Truncated κ LC constructs were synthesized by GENEART, Germany. Purified expression plasmids were transfected into HEK-293 cells using the Freestyle Max expression system (Invitrogen). At Day 3, supernatants were collected and analysed by surface plasmon resonance.

2.5 Testing the reactivity of K-1-21 to mutated F κ LC constructs

The binding reactivity of K-1-21 to F κ LC constructs was assessed by surface plasmon resonance on a Biacore 2000 (GE Biosciences). K-1-21, polyclonal anti- κ LC (Sigma) and polyclonal anti- λ LC (isotype control; Sigma) were covalently coupled to the surface of a CM5 sensor chip (GE Biosciences). Cell supernatants from κ LC transfected HEK-293 cells were injected for 10 min at a flow rate of 20 μ L/min, followed by a stabilization step for 10 min. The surface was regenerated by injecting 10 mM glycine, pH 1.8 for 30 s at a flow rate of 100 μ L/min. K-1-21 and anti- κ LC arbitrary response units for each F κ LC construct were standardized with reference to a F κ LC standard curve, then expressed as a percentage of the F κ LC control transfectant.

2.6 Derivation of molecular models and docking studies

A homology model of the K-1-21 Fv (VL and VH domains) was constructed using the Homology module of the Insight II program suite, version 98 (Accelrys, San Diego, CA, USA). Templates for the models were selected from the Protein Data Base (PDB) on the basis of sharing high amino acid similarities and CDRs of the same lengths and canonical structures to the sequences being predicted (Ramsland et al., 1999; Ramsland et al., 1997; Ramsland et al., 2001). Templates used to generate the K-1-21 Fv were from PDB codes:

12E8 for VL and 2RCS for VH (coordinates for the CDR3 of the HC were from 1BFV). The initial template-based K-1-21 Fv model was generated using the Modeller algorithm (Sali and Blundell, 1993), which was subjected to conjugate-gradient energy minimization until convergence to an energy gradient of 0.001 kcal/mol using the Crystallography and NMR System (Brunger et al., 1998). Harmonic restraints of 10.0 kcal/(mole Å²) were imposed on all main chain heavy atoms during the energy minimization simulations.

A homology model of a covalent κLC dimer was generated using procedures similar to those outlined above, however, the Discovery Studio package, version 2.5 (Accelrys, USA) was used to prepare and optimize the model. Briefly, the V and C domains from the Del κLC (PDB: 1B6D) were superimposed onto the 1.9 Å resolution structure of the Sea covalent λLC dimer, PDB: 1JVK (Bourne et al., 2002; Terzyan et al., 2003). An interchain disulfide linkage was introduced linking the two κLC monomers and the model was optimized by the Modeller algorithm and energy minimization within Discovery Studio.

Rigid body molecular docking was performed with the ClusPro 2.0 algorithm (<http://cluspro.bu.edu/>) using K-1-21 as the receptor and Chain B of the FκLC non-covalent dimer, PDB:1B6D, as the ligand (Comeau et al., 2007). Attraction regions were set on the κLC comprising the residues 105-109 in the switch region, and the algorithm was run in Ab mode with the non-CDR regions masked in K-1-21. An appropriate model was chosen based on having a low energy 'best fit' score and whether κLC switch residues made contact with K-1-21 CDRs (therefore satisfying the experimental data). The derived model was then optimized by the RosettaDock algorithm (<http://rosettadock.graylab.jhu.edu/>) which takes into account sidechain flexibility (Lyskov and Gray, 2008; Schueler-Furman et al., 2005).

2.7 Kinetic analysis and determination of affinity constants

For kinetic studies, we utilised an anti-Fc mAb capture method to determine affinity constants. The advantage of this approach is that it prevents the mAb surface from degradation between cycles due to the low pH in the regeneration buffer. As such, new mAb is captured onto the chip before each run. In brief, goat anti-mouse Ab (Sigma) was immobilized to the surface of a CM5 chip (GE Biosciences). K-1-21 at 10 µg/mL was injected for 1.5 min at 30 µL/min and the surface was stabilized for 10 min. This was followed by triplicate injections of FκLC monomers, covalent and non-covalent dimer at

concentrations ranging from 500 nM to 3.9 nM for 5 min. Dissociation for 15 min was followed by regeneration with 10 mM glycine pH 1.8 for 45 s at 100 μ L/min flow rate. The data was fitted to a 1:1 Langmuir binding model using the BiaEvaluation 3.1 software (GE Biosciences) and the calculated kinetic values were regarded as acceptable at $\text{Chi}^2 < 2$ and residuals in the range of -2 to +2.

3. RESULTS

3.1 The K-1-21 epitope comprises part of the C domain of F κ LC

We had previously identified a kappa Bence Jones protein (F κ LC) that had undergone proteolytic digestion whilst being stored in urine (presumably by proteases present in the sample). Analysis of this material using a K-1-21 affinity column yielded a number of κ LC fragments that were reactive with K-1-21. In order to identify these peptides, K-1-21 affinity purified κ LC fragments were subject to 2D electrophoresis. Two groups of peptides were present in the K-1-21 purified material; a monomeric F κ LC population migrating at approximately 25 kDa and peptides with apparent MW between 10-15 kDa (Fig. 1A). Of these lower MW peptides, four spots were selected for further analysis and following gel extraction and tryptic digestion, were analysed by LC/MS.

Alignment of the tryptic peptides from the four samples revealed that the smallest peptide within which the epitope was retained yielded a sequence that commenced at R108 and terminated at K183 (Fig. 1B & Supplementary Table 1). This ruled out the C-terminal region from K183 onwards as being involved in the epitope. However, the N-terminal residue of this peptide could not be unequivocally assigned because the amino acid immediately preceding R108 is K107. Furthermore, in all κ LCs there is a primary amine at 103 (either lysine for JK1, JK2, JK3 and JK4 gene segments, or an arginine for the JK5 gene segment). Since the peptide samples were trypsinized before identification by LC/MS, cleavage at residues 103 and 107 is unlikely to have yielded detectable peptides in the LC/MS analysis. In support of this, we note that a F κ LC peptide spanning residues 108-183 would have an estimated mass of 8kDa which is smaller than the actual mass of the peptide as shown by SDS-PAGE (between 10-15kDa; Fig. 1B). As such, it is likely that N-terminal residues preceding R108 were present but not identified as a result of tryptic digest.

3.2 The switch region in F κ LC is protected by K-1-21 during trypsin digest

Epitope excision was undertaken in order to further define the location of the epitope. The nature of this experiment relies on the protection of the antigenic determinant from enzymatic proteolysis when an Ag-Ab complex is formed. The success of such epitope protection assays is determined by the resistance of the Ab to digestion (Parker and Tomer, 2000).

F κ LC bound to K-1-21 immobilised on Sepharose were digested with trypsin and, after washing, peptides bound to the Ab on the beads were identified by MALDI-MS and Mascot database search. The smallest peptide (656.09 Da; LEIKR) was identified as part of the switch region (residues 104-108, the junction between V and C domains). This sequence is identical in both human and murine κ LC, thus it was possible that this peptide originated from either the Ab or the Ag, or both (Fig. 1B and Supplementary Fig. 2). However, exhaustive washing of the Sepharose-immobilized complex subsequent to proteolysis and prior to MALDI-MS, should eliminate any peptides originating from the Sepharose-bound K-1-21. Furthermore, this peptide was not present in the mass spectrum of F κ LC digested in the absence of K-1-21 (see Supplementary Fig. 2), suggesting that the cleavage site between K107 and R108 was protected when F κ LC is bound to K-1-21. A slightly larger peptide (862.14 Da) was also observed to be bound to the immobilized Ab and was identified as an overlapping sequence, LEIKRTV (residues 104-110). This sequence is unique for human κ LC and probably resulted from non-specific cleavage after V110 (Fig. 1B and Supplementary Fig. 2). Again, this result suggested that epitope protection had taken place. Numerous other peptides were present at much lower peak intensities and were assigned as murine (K-1-21) HC and LC V region sequences (see Supplementary Fig. 2).

3.3 Comparisons of κ LC structures reveal a conformational change at the switch region in F κ LCs

The unique specificity of K-1-21 to F κ LC rather than HC-associated κ LC suggests there are conformational differences between the two forms of the molecule. We compared the structures of a F κ LC non-covalent dimer, PDB: 1B6D, with a HC-associated κ LC derived from the Fab, PDB: 2FGW. Interestingly, chain A of 1B6D dimer overlaid remarkably well with 2FGW κ LC indicating they are very similar in conformation (Fig. 2A). In contrast, the backbone of chain B in 1B6D dimer markedly deviated from the Fab κ LC in two places: at the switch region comprising the residues 104-109 and at a loop, spatially adjacent to this

region, consisting of residues 165-171 (Fig. 2B). Fig. 3 highlights these regions in more detail. As shown, residues 104-109 and 165-171 of chain B of 1B6D dimer differ greatly in conformation in comparison to the corresponding regions found in 2FGW κ LC and chain A of 1B6D. Moreover, when looking at the position of the V and C domains in chain B, it becomes apparent that much of this conformational change can be attributed to a more acute angle formed between the two domains. As regions 104-109 and 165-171 in chain B are the only sites on F κ LC that are conformationally different to HC-associated κ LC, it reinforces the possibility that this region comprises the epitope bound by K-1-21.

3.4 Mutation and truncation of F κ LC residues confirms the switch region as an essential component of the epitope

Epitope excision and structural comparisons of κ LC highlighted the switch region and the adjacent loop as a putative epitope bound by K-1-21. In order to further define the epitope, we used site-directed mutagenesis and polypeptide truncation to identify critical residues involved in the binding of K-1-21 to F κ LC. The κ LC from the JN3 myeloma cell line was cloned and the product expressed transiently in HEK-293 cells. Importantly, the expressed product retained the epitope bound by K-1-21 and also reacted with a polyclonal anti- κ LC Ab as determined by Biacore biosensor analysis.

The κ LC constructs outlined in Table 1 were expressed and tested for reactivity with K-1-21 and polyclonal anti- κ LC. Arbitrary response units of the recombinant proteins to K-1-21 and polyclonal anti- κ LC on the sensor chip were standardized with reference to a F κ LC standard curve then expressed as a percentage of the control full length recombinant κ LC (Table 1). The C domain construct showed reactivity to K-1-21 and polyclonal anti- κ LC of 43% and 44%, respectively. These values were consistent with this fragment being approximately half the mass of the full length F κ LC, and thus resulting in values that are approximately half the full length F κ LC control. This interpretation was confirmed by the fact that the ratio of the reactivity of K-1-21 and the polyclonal Ab to this fragment (0.98) was similar to that of the full length F κ LC control. This confirmed that the K-1-21 epitope is retained in the peptide commencing at residue V104 and extending to the penultimate residue 214. To further define the K-1-21 epitope we generated and expressed another truncated κ LC construct beginning at residue K107 for comparison with the construct described above that contained the entire switch region (Table 1). Interestingly, removal of just the first three residues (104-106)

almost completely abolished the reactivity of K-1-21 to the truncated κ LC (K-1-21 to polyclonal anti- κ LC binding reactivity ratio of 0.12; Table 1) confirming the critical role of these residues in defining the K-1-21 epitope.

Mutation of either E105 or T109 to alanine in the full length κ LC construct resulted in significant reduction in the ability of K-1-21 to recognize the polypeptide. The reactivity of K-1-21 was 36% and 28% for the E105A and T109A mutations respectively when compared to the non-mutated control. These mutations had significantly less effect on reactivity with polyclonal anti- κ LC (78% for E105A and 67% for T109A). Although these lower values could be attributed to low F κ LC expression levels by the transfected mutants, the large discrepancy in reactivity values between K-1-21 and anti-F κ LC suggest that part of the K-1-21 epitope had been affected. This was highlighted in the F κ LC reactivity ratio of K-1-21 to the polyclonal anti- κ LC was reduced to 0.46 for E105A and 0.42 for T109A indicating that the effect of the mutations was largely restricted to the epitope recognized by K-1-21.

It was noted that κ LCs derived from the JK3 segment contain an aspartic acid at 105 (found in approximately 5% of κ LC sequences; Kabat et al., 1992) rather than a glutamic acid. As this residue was shown to be a key component of the epitope for K-1-21, it may have an impact on the clinical utility of MDX-1097 (the human chimeric equivalent of K-1-21) for a subset of myeloma patients. Therefore it was necessary to confirm that K-1-21 can still interact with κ LCs that have an aspartic acid at 105. Interestingly, mutation of E105 to aspartic acid resulted in enhanced binding of K-1-21 to F κ LC as indicated by a high reactivity ratio of 1.70 for K-1-21 versus the polyclonal anti- κ LC (Table 1). Thus we can conclude that MDX-1097 will retain reactivity to myeloma cells expressing κ LCs with D105.

We also tested the role of the region around residues 165 to 171 by shuffling the native sequence DSKD (residues 167-170) to KDDS. For this construct the ratio of K-1-21 binding reactivity to anti- κ LC reactivity was 0.79 which indicated that the epitope was only partially affected (Table 1). To further define this region, we mutated Q166 to a proline in order to locally disrupt the conformation of the peptide chain. Interestingly, this mutation had no effect on the ability of K-1-21 to bind F κ LC (Table 1). Although this data suggests that the loop comprising the residues 165 to 171 does not play a definitive role in the K-1-21 epitope,

it is still possible this region may be part of the conformational epitope, but plays little role in the specificity of K-1-21.

3.5 Model derivation and analysis

To test the proposed role of the switch region in the K-1-21 epitope, we used *in silico* molecular docking to probe this region, and to derive an appropriate model for the K-1-21 – F κ LC complex. Initially we derived a structure of the K-1-21 Fv based on homology modelling. The K-1-21 Fv model was then docked onto the non-covalent F κ LC dimer 1B6D with the ClusPro 2.0 algorithm (Comeau et al., 2007).

We found that K-1-21 could be docked onto the proposed epitope in Chain B of 1B6D. In this model, T109 is found buried deep in a hydrophobic binding pocket of K-1-21 formed by multiple tyrosine residues. In addition, a potential hydrogen bond (H-bond) occurs between T109 and V100 in K-1-21 HC. K107 side chain atoms interact *via* van der Waals forces with N32 and Y91 of K-1-21 LC, and the aromatic ring of Y104 in K-1-21 HC is found in close association with backbone atoms of K107. Main chain atoms between K107 and R108 form H-bonds with Y91 of K-1-21 LC. R108, in the proposed epitope, forms another H-bond with the side chain Y33 in K-1-21 HC, which is stabilized by van der Waals contacts between the side chains of Y101 in K-1-21 HC. Additionally, Y101 of K-1-21 HC also forms an H-bond with backbone atoms of K169 (Fig. 4A & B).

In contrast to this model, K-1-21 failed to dock onto the same region in Chain A. As Chain A shares a similar conformation to HC-associated κ LC (Figs. 2 & 3), we also tested whether κ LC Fabs could be docked into the K-1-21 Fv. Similar to Chain A of 1B6D, none of these Fabs were able to dock via their switch regions to K-1-21. This further highlights the unique conformation of the proposed epitope in Chain B of 1B6D F κ LC.

In addition, we attempted docking K-1-21 to a model of a covalent F κ LC dimer with both LCs in an extended conformation compared to Chain B of 1B6D. Again, the algorithm failed to dock K-1-21 Fv to either of the κ LC switch regions. This was an unexpected result, since K-1-21 has been shown to bind to covalently associated F κ LC dimers (Raison and Boux, 1985). In this model, the switch region of Chain A is similar to HC-associated κ LC (and Chain A of 1B6D dimer). However, Chain B of the covalent dimer is different to Chain B

from the non-covalent dimer 1B6D. This is partly due to a more extended conformation of the V and C domains in the covalent dimer (Fig. 4C). Thus K-1-21 binding to a covalent κ LC may require a conformational change in the switch region inducing a more acute angle between the V and C domains. Access to the K-1-21 epitope on covalent κ LC is still predicted to be feasible since, in the model, one LC monomer displays a conformation closer to the acutely bent κ LC of the non-covalent dimer than to the extended κ LC associated with the HC in an Fab (Fig. 4C).

Subsequently, we have measured the affinity of K-1-21 binding to monomers, covalent dimers and non-covalent dimers of F κ LC by plasmon resonance. We found that K-1-21 has higher affinity for non-covalent dimers than monomers and covalent dimers (Table 2). This indicates that the conformation of the K-1-21 epitope in non-covalent dimers is more accessible for binding to K-1-21. In addition, these data support the concept that induced fit plays a dominant role in K-1-21 binding to covalent κ LC dimers. This is highlighted by the finding that the rigid-body docking program was unable to generate complexes of the K-1-21 Fv with the switch regions of the covalent κ LC dimer model. It is therefore possible that a small conformational change in the switch region may be necessary to allow the binding of covalent dimers to K-1-21.

4. DISCUSSION

Previous studies have shown that K-1-21 recognizes a conformational epitope located in the C domain of F κ LC. Interestingly, it does not recognize HC-associated κ LC (in the form of an Ab molecule or Fab), which suggests there are conformational differences between the two forms of the molecule (Raison and Boux, 1985). In this work, we initially used epitope excision with tryptic digestion to further define the region recognized by K-1-21. This yielded two overlapping peptides that were protected from tryptic digestion. These peptides encompassed residues L104 to V110 that make up the switch region connecting the V and C domain of κ LC.

By analysing structures of HC-associated κ LC and F κ LC, we noted that the switch region and a spatially adjacent loop (residues 165-171) were conformationally different in the two forms of the molecule. Mutagenesis was then utilized to further examine the potential role of these regions in the epitope. It was found that alanine mutations at switch residues E105 and T109, reduced the reactivity of K-1-21 to F κ LC by more than 50% (based on the ratio of K-1-21 reactivity to the polyclonal anti- κ LC reactivity). Furthermore, a partial switch deleted mutant (C domain commencing at K107) showed minimal binding to K-1-21, thus confirming that the switch region contains residues critical to the integrity of the epitope. To analyse the role of the adjacent loop, a shuffle mutant was expressed (residues 167-170 shuffled from DSKD to KDDS) that showed a small but definitive reduction in the ability to bind to K-1-21. However, mutating Q166 to proline, in order to disrupt the peptide chain, showed no effect on K-1-21 reactivity. Together these results suggest this region is not critical to the epitope, but may provide a supporting role in the K-1-21 interaction with F κ LC.

In the absence of an X-ray crystal structure, we derived a model of K-1-21 Fv based on previously solved structures of homologous mAbs. We next utilized the rigid-body molecular docking algorithm ClusPro 2.0 to determine whether the switch region could be accommodated in the binding site of K-1-21 (Comeau et al., 2007). A best-fit model was selected based on a combination of having low energy (highly ranked docking poses) and whether it was supported by the experimental data. The semi-rigid RosettaDock algorithm, which allows side chain flexibility (Schueler-Furman et al., 2005), was then used to further refine the model. The model we derived was consistent with our experimental data. T109,

one of the key residues in the mutagenesis study, was found buried deep in the tyrosine-rich binding pocket of K-1-21, with an H-bond formed between the side chain of T109 and the backbone of V100 in the K-1-21 HC. Other important contact residues in the epitope were K107 and R108. Notably, these residues were protected by tryptic digest in the epitope excision experiment, which suggests they are located in the binding site of K-1-21. It is interesting to note that E105, which was thought to be a key component of the epitope based on the mutagenesis study, makes no contact with K-1-21 in the model. However, this residue is close to 107-109 (KRT residues) and may provide part of the scaffold for appropriate presentation of the contact residues to K-1-21. This is supported by the finding that a truncated form of κ LC, which begins at K107, and therefore does not have E105, fails to bind to K-1-21 indicating the loss of the epitope. Interestingly, K169 was found H-bonded with Y101 in K-1-21 HC. Seeing this interaction was mediated by main chain atoms rather than the side chain of K169, this suggests that a number of different residues may be substituted into this region without compromising the interaction with K-1-21. In support of this is the observation that various mutations in the loop comprising residues 166-170 showed little effect on K-1-21 binding. As such, it cannot be ruled out that this region does not play a definitive role in the epitope, however the interaction may be via an induced fit mechanism allowing for a range of different amino acids to be substituted into the peptide loop.

In addition, we also tried docking K-1-21 Fv with HC-associated κ LCs, to which the mAb does not bind (Boux et al., 1983) (see Supplementary Fig. 1), and did not find any model in which K-1-21 bound to the κ LC via similar mechanisms. This further highlights the conformational nature of the epitope. Molecular docking also failed to find an appropriate model for K-1-21 bound to covalent F κ LC dimer. Experimental evidence shows that covalent F κ LCs can bind to K-1-21, albeit with lower affinity than non-covalent dimers. Combined with the docking results we suggest that conformational changes are required for covalent F κ LC dimers to interact with K-1-21. Perhaps the κ LC require a bent or acute conformation (with respect to the V and C domains) before they can bind K-1-21, which explains the unique specificity of this Ab for human F κ LC.

In addition to soluble F κ LC, K-1-21 is highly specific for a cell surface determinant called KMA (kappa myeloma Ag) that consists of a complex of aggregated F κ LC bound to sphingomyelin (Hutchinson et al., 2010). KMA is expressed on a range of B cell

malignancies, and the chimeric version of K-1-21, MDX-1097, is currently being assessed in a Phase II clinical trial for multiple myeloma (Australian New Zealand Clinical Trials Registry Number: 12610000700033). Given KMA's therapeutic potential as a tumour-specific Ag, our findings are of interest in that the identified epitope region could be used for the rationale design of 'next generation' anti-KMA therapeutics. Developing new therapeutic agents targeting KMA is not straightforward given that κ LCs are also part of intact Ig molecules that are a major component of the B cell receptor that is expressed on most B cell subsets. Thus our determination of the K-1-21 epitope, which is unique to F κ LC rather than HC-associated κ LC, may be of great benefit in future screening or immunisation strategies for the development of anti-KMA compounds.

In summary, we have utilized a number of experimental techniques to locate the epitope on F κ LC that is recognised by K-1-21. Using this information, we then applied *in silico* molecular docking to arrive at a suitable model for the K-1-21 mAb – F κ LC complex. In the absence of an empirically solved molecular structure, this approach may be suitable for the derivation of models for many other Ab-Ag complexes.

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CONFLICT OF INTEREST

A.T.H., D.R.J., C.V.J., and R.L.R. are previous employees of Immune System Therapeutics Ltd. K.B. and A.B.E. have served in an advisory role to Immune System Therapeutics Ltd. A.T.H., D.R.J., C.V.J., K.B., A.B.E., and R.L.R. own stock in Immune System Therapeutics Ltd.

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FIGURE LEGENDS

Fig. 1. Identification of K-1-21 affinity purified F κ LC peptides. F κ LC that had undergone some proteolytic digestion *in situ*, was affinity purified by Sepharose-conjugated K-1-21. (A) The eluted fraction was subject to 2D electrophoresis then spots corresponding to fragments of κ LC (approximately 10-15 kDa) were cut out for analysis by nanoLC/MS-MS. (B) κ LC sequence showing the C domain and the switch region (residues 104-214). Underlined residues represent the smallest K-1-21 specific *in situ* derived peptide as identified by nanoLC/MS-MS. Residues in bold correspond to switch region fragments identified by epitope excision (see Table 1). Note residue 104 can be either a leucine or valine due to variability in κ LC joining gene segments.

Fig. 2. Comparison of peptide backbones in F κ LC and HC-associated κ LC C domains. (A) C domain overlay of chain A 1B6D F κ LC (grey) with κ LC of 2FGW Fab (black). (B) C domain overlay of chain B 1B6D F κ LC (grey) with κ LC of 2FGW Fab (black).

Fig. 3. Surface and ribbon diagrams of the proposed epitope region on κ LC. (A-C) Ribbon diagrams, (D-F) surface diagrams. Chain B 1B6D dimer: A and D. Chain A 1B6D dimer: B and E. LC 2FGW Fab: C and F. Red highlighted residues constitute the switch region (residues 104-109) and blue highlighted residues are the adjacent loop (residues 165-171).

Fig. 4. K-1-21 Fv bound to F κ LC model. (A) Surface and ribbon diagrams of the model. Magenta and cyan structures denote the K-1-21 HC and LC respectively. Grey denotes Chain B of 1B6D non-covalent dimer. (B) Key interactions taking place between K-1-21 Fv and the switch region of 1B6D non-covalent dimer. Atoms are colored by type (1B6D carbon, grey; K-1-21 HC carbon, magenta; K-1-21 LC carbon, cyan; nitrogen, blue; oxygen, red). H-bonds are shown as green dashed lines. (C) Carbon-alpha trace V domain overlays of Chain B κ LC 1B6D (magenta), Chain B covalent dimer model (cyan) and κ LC chain of 2FGW Fab (black).

TABLES**Table 1**Reactivity of recombinant F κ LC constructs to K-1-21 and polyclonal anti- κ LC.

Construct	Description	Reactivity (% of κ LC control)		Ratio*
		K-1-21	anti- κ LC	
κ LC	Full length κ LC control	100	100	1
VEIKRTV + C domain	Residues 104 to 214	43	44	0.98
KRTV + C domain	Residues 107 to 214	5	43	0.12
E105A	E105 mutated to A	36	78	0.46
T109A	T109 mutated to A	28	67	0.42
E105D	E105 mutated to D	135	79	1.70
KDDS	DSKD (167-170) shuffled to KDDS	67	85	0.79
Q166P	Q166 mutated to P	91	92	0.99

* Defined as the ratio of K-1-21 and anti- κ LC reactivity to the various κ LC constructs

Table 2

Kinetic constants for the interaction between K-1-21 and FkLCs.

	$k_a(\text{M}^{-1}\text{s}^{-1})$	$k_d(\text{s}^{-1})$	$K_A(\text{M}^{-1})$	$K_D(\text{M})$
Non-covalent dimers	15.3×10^4	1.05×10^{-3}	14.5×10^7	6.9×10^{-9}
Covalent dimers	9.98×10^4	1.96×10^{-3}	5.09×10^7	19.6×10^{-9}
Monomers	3.64×10^4	0.74×10^{-3}	4.87×10^7	20.6×10^{-9}

Figure 1
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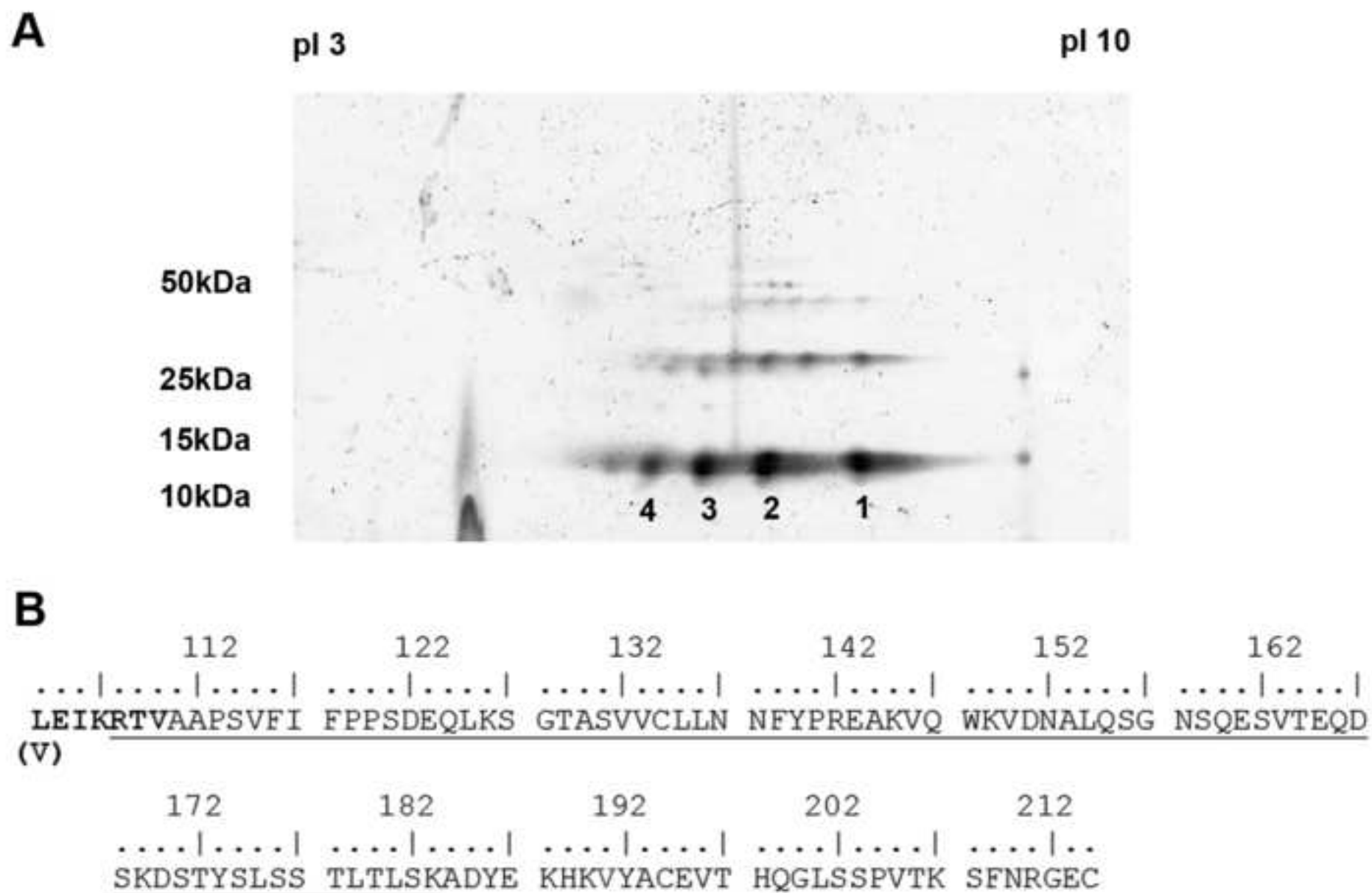


Figure 2
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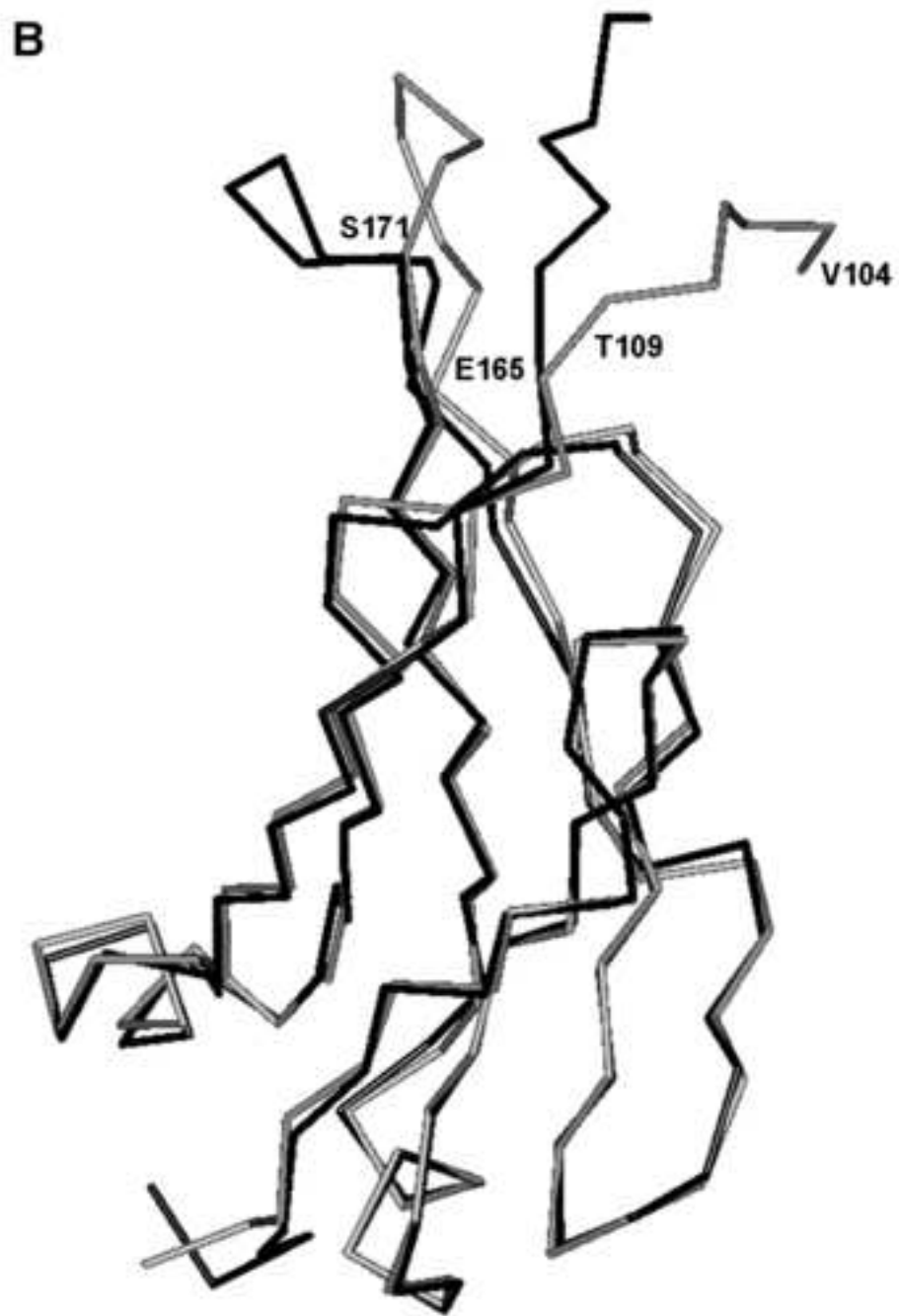


Figure 3
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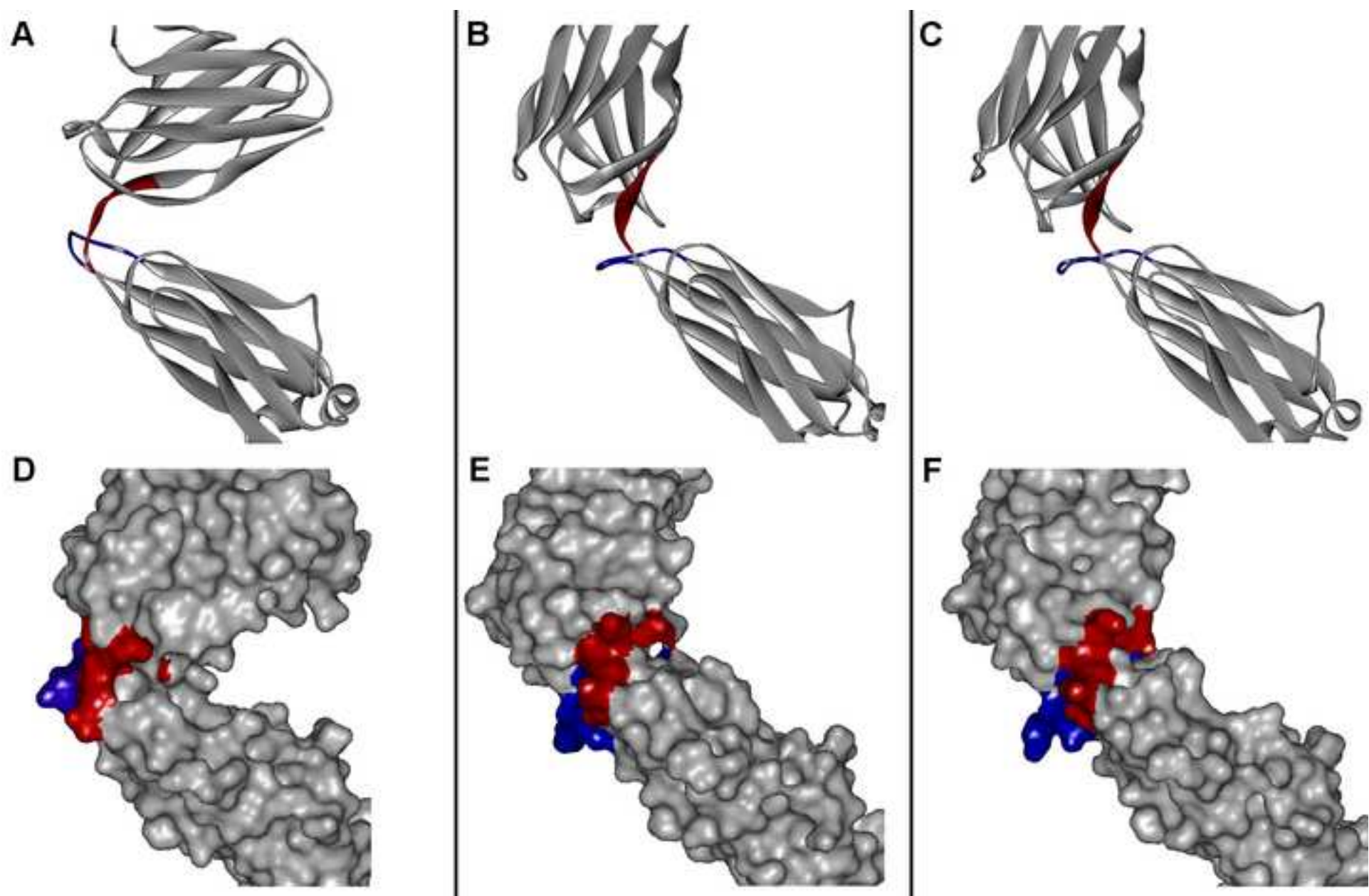


Figure 4
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