Formation of Assemblies on Cell Membranes by Secreted Proteins: Molecular Studies of Free Lambda Light Chain Aggregates Found on the Surface of Myeloma Cells

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Abbreviations: AFM, atomic force microscopy; AL, light chain amyloidosis; APC, allophycocyanin; DMPS, 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPPA, 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; FLC, free immunoglobulin light chain; LC, light chain; LCDD, light chain deposition disease; LUV, large unilamellar vesicle; MM, multiple myeloma; PA, phosphatidic acid; PC, phosphocholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; PS, phosphatidylserine.

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SYNOPSIS

We have described the presence of cell membrane-associated κ free immunoglobulin light chains (FLC) on the surface of myeloma cells. Notably, the anti-κFLC mAb, MDX-1097, is being assessed in clinical trials as a therapy for κ light chain isotype multiple myeloma. Despite the clinical potential of anti-FLC mAbs, there have been limited studies on characterizing membrane-associated FLCs at a molecular level. Furthermore, it is not known if λFLCs can associate with cell membranes of myeloma cells. In this study, we describe the presence of λFLCs on the surface of myeloma cells. We found that cell surface-associated λFLC are bound directly to the membrane and in an aggregated form. Subsequently, membrane interaction studies revealed that λFLCs interact with saturated zwitterionic lipids such as phosphatidylcholine and phosphatidylethanolamine, and using automated docking, we characterize a potential recognition site for these lipids. Atomic force microscopy confirmed that membrane-associated λFLCs are aggregated. Given our findings, we propose a model whereby individual FLCs show modest affinity for zwitterionic lipids, with aggregation stabilizing the interaction due to multivalency. Notably, this is the first study to image FLCs bound to phospholipids and provides important insights into the possible mechanisms of membrane association by this unique myeloma surface antigen.
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INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy of clonal plasma cells in the bone marrow. MM plasma cells secrete free immunoglobulin light chains (FLC), and indeed, serum FLC levels are a prognostic marker disease [1, 2]. FLCs are inherently unstable molecules and can show a propensity to aggregate, even at physiological conditions [3]. Consequently, patients with an underlying plasma cell dyscrasia such as MM can present with an associated light chain aggregation/deposition disorder. Two major forms of light chain (LC) aggregation disorders include the formation of amyloid fibrils or amorphous aggregates of FLCs, which are deposited within tissues and can eventually lead to organ failure and death [4, 5].

FLC aggregation is thought to be dependent on partial unfolding into an intermediate structure. This unfolding exposes previously buried hydrophobic regions within β-strands that promote the association of similarly unfolded β-strands on adjacent FLCs, leading to aggregation [6]. Protein denaturation conditions, such as high temperatures, high ionic strength, interactions with charged surfaces and even constant shaking can all contribute to FLC aggregation in vitro [7-10]. The mechanisms that promote aggregation in vivo are not well understood, although there is evidence to suggest that interactions with polyanions such as glycosaminoglycans and phospholipid membranes may contribute to this process [11-13].

There are two isotypes of LC, designated κ and λ, with approximately 61% of MM patients expressing the former [14]. Interestingly, κFLCs can be found on the surface of κLC expressing MM cells [15-17], and the anti-κFLC chimeric mAb, MDX-1097 [18], is currently being assessed in clinical trials as a possible therapy for MM (Australian New Zealand Clinical Trials Registry #12610000700033).

FLCs are secreted, so it was long presumed that membrane-associated κFLCs were bound to MM cells via a cell surface receptor [19]. However recent studies by our group have shown that κFLCs can bind directly to cell membranes, specifically, to saturated phosphocholine (PC) head group lipids such as sphingomyelin [15, 20]. Interestingly, membrane-associated κFLCs appeared to be aggregated, although only indirect methods were used to arrive at this conclusion. This led us to propose that κFLCs initially bind then aggregate to tightly associate with the cell membrane, possibly through a low affinity but high avidity interaction [15].

While it has been established that κFLCs are found on the surface of some MM cells [15, 16], it is not known if λFLCs behave in a similar fashion. In this study, we have shown for the first time that λFLCs are bound to the cell membrane of λLC isotype MM cells. Furthermore, membrane interaction studies reveal that just like their κ counterparts, λFLCs can bind saturated PC containing lipids. In addition, we tested a wide range of lipids and found high levels of binding to saturated phosphatidylethanolamine (PE), a lipid structurally related to PC head group lipids. Computational modelling suggests binding of PC by an acutely bent form of the λLC in a site formed by the variable and constant domains in a LC dimer. Finally, atomic force microscopy (AFM) was used to directly visualize FLCs bound to supported lipid bilayers. We observed large semi-elliptical aggregates of FLC on the surface of the bilayer. This finding supports the hypothesis that FLC aggregation is a requirement for stable association with MM cell membranes.
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MATERIALS AND METHODS

General reagents
RPMI8226 (ATCC No. CCL-155) cell line was from the American Type Culture Collection. JJN-3 (DSMZ No. ACC-541) and OPM-2 (DSMZ No. ACC-50) cell lines were from the German Resource Centre for Biological Material (DSMZ). Cell lines were maintained according to recommendations from the supplier. MM primary cells were from AllCells.

Anti-λFLC (clone: 4G7) was from Abcam. For immunofluorescence, the mAb was sulfhydryl labelled with allophycocyanin (APC) or AlexaFluor 488 (Life Technologies). FLCs were purified from urine of MM patients as previously described [21]. Phospholipids were from Avanti Polar and IgGλ was from Bethyl Laboratories.

Flow cytometry
MM cells were stained with anti-λFLC APC or anti-κFLC APC (clone: K-1-21) [18] as a negative isotype control for detection of membrane bound λFLC. For MM primary cells, samples were additionally stained with anti-CD38 PECy7 (clone: HIT2) and anti-CD45 PerCP (clone: TU116) mAbs (BD Biosciences). Stained samples were assessed on an LSR II flow cytometer (BD Biosciences).

3D-structured illumination microscopy
RPMI8226 MM cells were immobilized onto poly-L-lysine coated slides and stained with anti-λFLC AlexaFluor 488 for 30 min on ice. Cells were washed and fixed with 4% paraformaldehyde before nuclear staining with DAPI. Imaging was performed using a DeltaVision OMX 3D-structured illumination microscope (Applied Precision). Excitation was provided by solid-state lasers (405 and 488 nm) and images were captured using Photometrics Cascade (Photometrics) back-illuminated EMCCD cameras. All data was captured using a UPlanSApo X100 1.4NA oil objective (Olympus) with excitation and emission filter sets of (ex/em) 405/419-465 nm and 488/500-550 nm for DAPI and AlexaFluor 488 respectively. Raw 3-phase images were reconstructed as described previously [22, 23], and the reconstructed image was rendered in 3D using IMARIS software (version 7; Bitplane Scientific AG).

High resolution native PAGE (N-PAGE)
RPMI8226 cells were lysed by two rounds of liquid N2 freeze-thaw and two rounds of sonication. Cell membranes were purified by centrifugation at 20,000 g and washed twice with ddH2O to remove non-specifically bound material. Membrane proteins were solubilized in Native Sample Buffer (Life Technologies) with a final concentration of 0.5% digitonin. Debris was pelleted at 20,000 g for 10 min at 4°C and supernatant was loaded onto a native gel for 1D and 2D separation. N-PAGE running buffer (Life Technologies) was used for electrophoresis, however the cathode buffer also contained 0.5 mg/mL sodium deoxycholate. Samples were loaded and the gel was run for 1 h at 50 V, then the voltage increased to 100 V for 1 h and finally to 150 V until the dye front reached the bottom of the gel. For 1D, the gel was then transferred to PVDF for western blotting. For 2D N-PAGE/SDS-PAGE, immediately following the first dimension run, the gel was washed briefly with water and the lane to be run was excised. The entire lane was equilibrated in 10 mL sample equilibration buffer (6 M urea, 0.25 M Tris-HCl pH 6.8, Bromophenol Blue, 10% (w/v) SDS without the addition of reducing agents) for 20 min. The lane was loaded onto a single-well second dimension gel and separated by SDS-PAGE. Following electrophoresis, the gel was transferred to PVDF membrane, blocked then probed with anti-λLC alkaline phosphatase (Sigma) and developed with p-nitrophenyl phosphate (pNPP; Sigma).

Immunoprecipitation
RPMI8226 cells were washed three times in PBS followed by the addition of Sulfo-NHS-LC-Biotin (Thermo Scientific) to label cell surface proteins. Cells were incubated at 4°C for 1 h, washed three times with ice-cold PBS/0.1 M glycine and resuspended in PBS with protease inhibitors (Roche). Cells were lysed by sonication and centrifuged at 4,000 g for 10 min to remove nuclear material. The
supernatant was collected and centrifuged at 20,000 g for 1 h. The pellet containing purified cell membranes was washed twice with PBS with protease inhibitor and left to resolubilize in 0.5% digitonin (Sigma) in PBS with protease inhibitors overnight at 4°C. Sheep anti-mouse IgG immobilized to magnetic beads (Dynal Biotech) was used as a solid phase for immunoprecipitation. Briefly, ~1×10^8 beads were washed twice and resuspended in 500 µL of wash buffer (PBS/0.1% BSA). 5 µg of anti-human λFLC or IgG1 isotype control (clone: MOPC-21; Sigma) were incubated with the beads for 2 h at 4°C with constant rotation and washed three times with wash buffer. Beads were incubated with digitonin solubilized cell membranes for 1 h at room temperature, washed three times, eluted with 0.1 M glycine/HCl pH 2.5 and immediately neutralized with 1 M Tris pH 9. Eluates were separated by non-reducing SDS-PAGE and transferred to PVDF. Biotinylated proteins were probed with streptavidin peroxidase (GE Biosciences) and detected via chemiluminescence.

**Membrane extraction**

RPMI8226 cells were lysed by sonication and cell membranes purified by centrifugation at 20,000 g. The membrane pellet was washed three times in PBS, extracted with 0.1 M Na2CO3 pH 11 and centrifuged at 20,000 g. The supernatant was collected, which represented the peripheral membrane fraction, and the remaining pellet was washed in PBS before solubilising in 1% Triton X 100 to recover integral membrane proteins. For hydrophilic/hydrophobic phase separation, cell membranes were purified as before and resuspended in 4.5% Triton X 114/ PBS on ice for 10 min. The sample was heated to 30°C and centrifuged at 20,000 g for phase separation. Detergent and hydrophilic fractions were extracted. All samples were separated by SDS-PAGE, transferred to PVDF and probed with anti-λLC, anti-calnexin and anti-actin Abs (Sigma).

**Sucrose flotation assay**

Lipid stocks diluted in chloroform were mixed in a glass vial. Chloroform was rotary evaporated and the resulting lipid film was freeze-dried overnight to remove trace amounts of solvent. The film was re-hydrated with 100 mM Tris, 150 mM NaCl pH 7.5 (TBS), vigorously agitated and incubated for 1 h at 60°C. The lipid solution was extruded using a polycarbonate filter to create a homogenous solution of 100 µm large unilamellar vesicles (LUV). 5 mg/mL of LUVs were then incubated with 0.5 mg/mL of λFLC, BSA or mouse IgG1 K-1-21 Fab in TBS (400 µL volume) for 2 h at 37°C. The protein-LUV mixture was then overlaid with a discontinuous sucrose gradient and separated by centrifugation (100,000g for 2 h at 4°C). The top (LUV) fraction and the bottom fraction were collected and proteins were precipitated by trichloracetic acid. Proteins were then resolubilised in SDS-PAGE loading buffer, separated by SDS-PAGE and gels were developed by silverstain.

**Phospholipid ELISA**

Hydrophobic Poly-Sorp 96-well plates (Nunc) were coated in duplicate with 200 µL of 100 µg/mL phospholipids diluted in 95% ethanol. Phospholipids were dried under a stream of nitrogen gas overnight and then blocked in 1% BSA. Plates were washed and then incubated with serial dilutions of λFLC in 0.3% BSA-TBS. After 2 h, plates were washed and incubated with goat anti-λLC (Sigma) followed by a second incubation with rabbit anti-goat alkaline phosphatase (Sigma). Plates were developed with pNPP substrate (Sigma) and 405 nm absorbances were recorded on an Infinite 200 plate reader (Tecan).

**Molecular docking**

PC was extracted from the PDB 2MCP (Mc/Pc603 Fab-PC complex) and parameterized using the OPLS force field. Methyl-PC was drawn from the PC base structure using Maestro 9.2 [24]. The 2.0 Å resolution crystal structure of the Mcg λ LC dimer crystallized from ammonium sulfate (PDB 2MCG) [25] was obtained from the Protein Data Bank. The protein structure was prepared using the Protein Preparation Wizard in Maestro 9.2. Molecular docking was performed using the Standard Precision mode (SP) in Glide 5.7 [26, 27]. Scoring grids were set up within a cubic box (30 Å) centred at the centroid of two locations: 1) the ED motif, at positions 81 and 82, which were previously identified for PC binding to the κFLC dimer [15], and 2) the EAD motif at positions 83-85. Note that the Kabat numbering convention has been used throughout this manuscript [28].
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positions differ somewhat from the sequential numbering found in the Mcg crystal structure coordinate file 2MCG. Grids were generated only for the chain of the dimer that displayed an acutely bent conformation (chain ID 1; monomer 1) rather than the extended form (ID 2; monomer 2). With respect to the angles subtended by the V and C domains, monomer 1 has been compared to a heavy chain while monomer 2 is more similar in conformation to a typical LC of a conventional Fab molecule [29]. For analysis, the docked poses were clustered to an RMSD of 2.0 Å. All other settings in Glide were the defaults.

**Atomic force microscopy (AFM)**

For imaging of λFLCs, 30 µg/mL of λFLC was added to freshly cleaved mica for 30 min followed by washing in 100 mM Tris, 150 mM NaCl pH 7.5. For imaging on supported bilayers, LUVs were applied to freshly cleaved mica and incubated in a humidified box at 60°C for 30 min to allow LUVs to rupture and form uniform bilayers. Following washing of the mica surface, 250 µg/mL of λFLC or no protein control was added to the sample and incubated for 1-2 h. The sample was washed and imaged in a liquid cell using a Nanoscope IIIA MultiMode™ AFM equipped with an Extender™ electronics module (Veeco Instruments). Briefly, an E type scanner was used for imaging, having a maximum scan area of 12.5 μm squared and vertical height range of 3.4 μm. Cantilevers were the NP-S series narrow legged, V-shaped, 100 μm long oxide sharpened silicon nitride cantilevers, with integrated tips (Veeco Instruments) and a nominal spring constant, k, of 0.36 N/m. The AFM was driven in “Tapping mode™” at the typical cantilever resonance frequency (near 9 kHz) at ambient temperature. The piezo Z range was reduced to around 1 μm whilst scanning. The scan speeds ranged from 1 to 4 Hz. Data was analysed with Gwyddion Ver. 2.30 software. Full width at half height measurements were used to estimate the width and length of proteins. Individual λFLCs were found to adopt a rectangular conformation so the equation \( V = hw \) was used to calculate the protein volume. Membrane bound λFLCs were round but flattened on the membrane surface so the volume of a semi-ellipsoid, \( V = \frac{2}{3} \pi abh \), was used to approximate protein volumes.
RESULTS

λFLCs are found on the surface of λLC secreting MM cells

To establish if λFLCs were on the surface of the cells secreting them, we stained MM cell lines and primary cells with an anti-λFLC mAb and assessed expression by flow cytometry. Both the λLC isotype MM cell lines, RPMI8226 and OPM-2, as well as CD38+ CD45- MM primary cells were found to express cell surface associated λFLCs (Figure 1A, B & C). This was supported by super-resolution microscopy of anti-λFLC stained RPMI8226 cells which displayed a patchy and non-uniform distribution of cell membrane bound λFLCs (Figure 1D). In contrast, the κLC isotype MM cell line, JJN3, showed no evidence of λFLC expression while being positive for cell membrane-associated κFLC as defined by staining with an anti-κFLC mAb (Figure 1A). Together these findings highlight that λFLCs, like κFLCs [15, 16], can be found on the cell surface of MM cells.

Cell membrane-associated λFLCs reside in a high molecular weight complex

We next explored the molecular nature of cell membrane-associated λFLCs by high resolution N-PAGE and western blotting for λLC. In a membrane fraction from RPMI8226 cells, λLC were detected at ~700 kDa and 480 kDa, with the former showing the highest intensity. In contrast, soluble λFLC migrated at a size of ~50 kDa as expected for a FLC dimer (Figure 2A). To confirm that the 700 kDa and 480 kDa complexes consisted of λFLCs, these species were further separated by non-reducing SDS-PAGE and λLC-specific western blotting. λLCs migrated at 47 kDa with a weaker band also observed at 24 kDa, which corresponded to the 700 kDa and 480 kDa N-PAGE complexes respectively, thus confirming the presence of λFLC dimers and monomers within these high molecular weight complexes (Figure 2B). We then performed immunoprecipitation of biotinylated cell surface proteins with an anti-λFLC mAb to determine if co-precipitating proteins such as a λFLC receptor may be associated with the high molecular weight complexes found by N-PAGE. Four protein bands of Mr 60 kDa, 55 kDa, 47 kDa and 24 kDa were detected within the anti-λFLC precipitate. Three of these bands, namely 60 kDa, 55 kDa and 47 kDa were also found in the isotype control sample suggesting these are co-precipitating protein contaminants. Of note is that the observed band at 47 kDa was far darker in the anti-λFLC sample which corresponds to the size of a λFLC dimer. The 24 kDa band, which was only found in the anti-λFLC immunoprecipitate, is indicative of a λFLC monomer (Figure 2C). This data suggests that λFLCs are in direct association with the cell membrane rather than being bound by a protein receptor. Furthermore, the lack of co-precipitating proteins suggests that the 720 kDa complex identified by N-PAGE is a high molecular weight complex/aggregate of λFLCs, which is consistent with our previous findings for κFLCs [15].

To characterize the type of interaction that λFLCs have with cell membranes, a high ionic strength carbonate extraction protocol was used to strip peripherally associated proteins [30] from RPMI8226 cells and the aqueous and insoluble membrane fractions assessed by SDS-PAGE and western blotting. The majority of λFLC and calnexin, an integral membrane protein, were found in the insoluble fraction, indicating that these proteins are resistant to membrane extraction by the high ionic strength carbonate buffer. In contrast, actin was readily stripped from the membrane which is typical of its status as a peripheral membrane protein (Figure 2D).

To further understand the mode of membrane association by λFLCs, a Triton X 114 phase separation technique was employed to help distinguish between peripheral and integral membrane proteins [31] in the RPMI8226 cell membranes. As expected, actin was wholly recovered in the aqueous phase which is indicative of its status as a peripheral membrane protein. In contrast, calnexin, being an integral membrane protein, was mostly recovered in the Triton X 114 detergent phase although there was a small amount present in the aqueous phase. While the majority of λFLC was recovered in the aqueous fraction, a considerable amount partitioned into the detergent phase (Figure 2D). Together with the previous results, λFLCs appear to share properties of both integral and peripheral membrane proteins, the former based on resistance to carbonate extraction, and the latter based on its hydrophilic nature.
\( \lambda \)FLCs bind to saturated phospholipids

Our previous studies have shown that \( \kappa \)FLCs interact with saturated PC head group lipids such as sphingomyelin [15]. Until the present study, it remained unclear if \( \lambda \)FLCs can behave in a similar manner. To address this, we initially tested the binding of \( \lambda \)FLCs to LUVs composed of a mixture of lipids (3:3:2:2 molar ratio of DPPC, DPPE, POPS and cholesterol respectively) via a sucrose flotation assay. \( \lambda \)FLC was found within the top fraction of the density gradient indicating specific absorption to LUVs. In contrast, only a small amount of BSA was associated with the LUVs and there was no binding to LUVs by a murine Fab (Figure 3A). We then assessed the specificity of \( \lambda \)FLCs binding to common phospholipid species by ELISA. \( \lambda \)FLCs were found to interact with the saturated PC containing lipid, sphingomyelin, which is consistent with our previous results for \( \kappa \)FLC [15]. In contrast, little to no binding was observed for the unsaturated PC species, DOPC, the monosaturated phosphatidylserine (PS) lipid, POPS, or BSA. Surprisingly, \( \lambda \)FLCs also bound to the monosaturated PE lipid, POPE, which was not previously found for \( \kappa \)FLCs (Figure 3B).

To confirm that membrane-association is a property of FLCs rather than intact Ig, we tested the binding of IgG\( _\lambda \) to phospholipid monolayers at equivalent molar concentrations to \( \lambda \)FLCs. We detected minimal to no binding of IgG\( _\lambda \) to the various phospholipids with the exception of POPS where a low level of binding was detected (Figure 3C). The observed binding of IgG\( _\lambda \) to POPS is likely due to the presence of anti-PS Abs which are found in many individuals [32]. Based on the low to undetectable binding of IgG\( _\lambda \) to the various phospholipids, we conclude that membrane association is a property of \( \lambda \)FLCs rather than whole Ig.

Our ELISA results showed that \( \lambda \)FLCs associated with a saturated form of PC rather than the fully unsaturated PC lipid, DOPC, which suggests that the level of lipid saturation may be an important factor for membrane association by \( \lambda \)FLCs. ELISAs were performed to test the ability of \( \lambda \)FLC to interact with fully saturated, monosaturated and unsaturated species of PC and PE head group lipids. In regards to PC containing lipids, \( \lambda \)FLC bound to the fully saturated species, sphingomyelin and DPPC, but failed to bind monosaturated and unsaturated species, POPC and DOPC respectively (Figure 4A). Similarly, the saturated form of PE, DPPE, showed high levels of binding to \( \lambda \)FLC. The monosaturated PE lipid, POPE also bound to \( \lambda \)FLC, albeit at lower levels to DPPE (Figure 4B). These findings suggest that lipid saturation enhances binding of \( \lambda \)FLC to membranes. Of note is that the monosaturated PE containing lipid also showed some level of binding to \( \lambda \)FLC whereas there was minimal binding to the counterpart PC lipid. A possible explanation for this is that POPE displays a higher phase transition temperature than POPC (25°C and -2°C respectively). Consequently, POPE lipids are packed tighter and behave more as a fully saturated lipid than POPC at physiological conditions, which possibly facilitated the observed interaction with \( \lambda \)FLC.

Although acyl saturation is a factor that mediates \( \lambda \)FLC binding to membranes, it is less clear what the role of the lipid headgroup plays in this interaction. To gain a further understanding of the basis for the membrane interaction, we tested the ability of \( \lambda \)FLC to interact with a variety of different phospholipids. It was found that \( \lambda \)FLC showed the highest level of reactivity to the saturated phospholipids, DPPC and DPPE, and the least binding to the unsaturated PC containing lipid, DOPC, which confirms our previous results. Interestingly, \( \lambda \)FLC was also found to interact with fully saturated forms of phosphatidylserine (DPPS), phosphatidic acid (DPPA) and phosphatidylglycerol (DPPG), although the level of binding was not as high as DPPC or DPPE (Figure 4C). PC and PE containing lipids are zwitterionic and possess similar structured headgroups whereas PS, PA and PG lipid derivatives are all negatively charged. Our results therefore suggest that \( \lambda \)FLCs show a binding preference for saturated zwitterionic phospholipids over their negatively charged counterparts.

Molecular docking reveals a putative binding site for PC in \( \lambda \)FLC

Our phospholipid binding studies have demonstrated that \( \lambda \)FLCs show preferential association to saturated PC and PE containing lipids. These phospholipids are structurally similar and possess a positively charged head group, which is likely to interact with negatively charged residues on \( \lambda \)FLC.
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Interestingly, there is a negatively charged cavity within FLC variable domains that is structurally conserved between κ and λ FLCs, and in our previous study on κFLCs, we were able to successfully dock the trimethylammonium group of a PC ligand in this space [15]. Herein, we investigated this site as a possible region in λFLCs that could bind to zwitterionic phospholipids such as PC head group lipids. For λFLCs, this region comprises the residues EDEAD (residues 81-85). Molecular docking to the ED site (residues 81-82) in the Mcg λFLC dimer returned low scores, suggesting that PC binding is not likely to occur here (data not shown). Subsequently, our attention was focused on the EAD portion of this sequence (residues 83-85). The EAD sequence is inaccessible due to interactions with the constant domain in the extended, Fab-like state of the LC. In the acutely bent state, these interactions do not occur, and a cavity is revealed in the structure of the LC (Figure 5A & B). The proposed binding site features the side-chain of Glu83 pointing towards CL in the acutely bent LC (heavy chain analog) of the dimer. Thus, an extended cavity that is composed of residues from both the VL and CL domains is formed in the acutely bent λLC monomer.

The predicted binding mode of methyl-PC to the 2MCG structure features the PC portion making numerous contacts with LC residues (Figure 5C). The methyl groups of the trimethylammonium moiety interact with the carboxylate group of Glu83 and the main-chain carbonyl of Val806. At the other end of the binding pocket, the phosphate group interacts with a triad of constant domain residues: Tyr173, Lys866 and Tyr860. Despite the engagement of the phosphate group in these interactions, the predicted binding mode can still support the attachment of lipid tails, as demonstrated by the position of the capping methyl group in the predicted binding mode (Figure 5C & D).

Imaging membrane bound λFLC by atomic force microscopy

Electrophoretic (N-PAGE) analysis of purified membranes from RPMI8226 cells suggest that membrane-associated λFLCs are aggregated (Figure 2A & B). These results are consistent with our previous findings, where we proposed that membrane binding by κFLC was dependent on self-association into high molecular weight aggregates [15]. In both studies, indirect methods were used, and it was not directly confirmed that membrane bound FLCs are in an aggregated form. To gain further insight into the mode of membrane association by λFLCs, we used AFM to visualize λFLCs bound to supported membrane bilayers. Initially, λFLCs were imaged on freshly cleaved mica to define their native conformation by AFM. We found that the majority of λFLC were elongated suggesting they are lying on their side (Figure 6). The mean dimensions of λFLC were 3.0 nm, 9.3 nm and 16.1 nm for height, width and length respectively with an average protein volume of 458.7 nm³ (Table 1). The height dimension is slightly larger than what has previously been shown for IgG imaged by AFM which is approximately 2.4 nm [33]. In relation to the width and length values, to the best of our knowledge, this is the first study to approximate the sizes of individual FLCs by AFM. These values are almost twice the size of λFLC dimers in regards to X-ray crystal structures [34], however it must be noted that due to the pyramidal shape of the AFM tip, width and length measurements of proteins are overestimated as a result of broadening effects caused by the radius of the tip [35]. Nevertheless, the approximate dimensions of mica bound λFLCs suggest that these are individual proteins rather than oligomeric species.

Having established the approximate dimensions of native λFLC, we then set out to image λFLCs bound to the surface of a saturated DPPC/POPC lipid bilayers. Analysis of AFM images revealed a layer approximately 6 nm higher than the mica surface, which is the expected size of a membrane bilayer [36]. Furthermore, given that DPPC and POPC have different phases at room temperature, the bilayer was separated into a POPC region and DPPC region, the latter being approximately 0.5 nm taller as a result of having straight saturated acyl tails (Figure 7A). Following successful imaging of supported DPPC/POPC bilayers, λFLCs were added to the sample in order to characterize their association with membrane bilayers. λFLCs that had bound to the membrane surface appeared as large elliptical bumps found on the edges of the higher phase DPPC lipid. This confirms the phospholipid ELISA results where λFLCs bound to DPPC, but not POPC (Figure 3B). λFLCs were also found saturating the mica surface. The majority of these λFLCs were of small size and likely
single monomers or dimers, although there were occasional larger elliptical species which resembled those found on the supported bilayer (Figure 7B). Size distribution analysis revealed that membrane bound λFLC are large and varied in size with calculated means for width and height of 37.6 nm and 4.3 nm respectively. We also estimated the mean volume of each of these protein aggregates by assuming that these aggregates are semi-elliptical and lie flat on the membrane surface. Using the calculation for a semi-ellipsoid, the mean protein volume of these aggregates was 4153.3 nm³. Notably, the standard deviation for protein volume was very high, which is indicative of the large amount of variation in the size of these aggregates (Table 1). All of the lipid bound λFLC were substantially larger than individual λFLC bound to mica. Therefore, the AFM measurements support the conclusion that membrane bound λFLC are aggregated, which is consistent with our N-PAGE analysis.
DISCUSSION

The mAb, MDX-1097, is currently being assessed in a Phase II clinical trial as a potential therapy for MM. MDX-1097 targets κFLCs which are found on the surface of κ LC isotype MM cells [15, 16]. Despite MDX-1097’s clinical relevance, there have been limited studies on characterising the molecular nature of its antigen. Furthermore, it had not been previously determined if λFLCs behave in a similar fashion to κFLCs and whether these could be utilized as a potential target for the treatment of λ LC isotype MM. In the current study, we have described for the first time the presence of λFLCs on the surface of MM cell lines and primary cells. With similarities to κFLCs, λFLCs are bound to cell membranes in an aggregated form. In this case, analysis by N-PAGE revealed that the majority of membrane-associated λFLCs reside in large complexes at ~700 kDa, which differs from the previous study where κFLCs were found in complexes of ~480 kDa [15]. A likely explanation for these discrepancies is that the major building blocks for λFLC aggregates is a 47 kDa dimer, whereas κFLC aggregates contain LCs predominantly in a monomeric form. Thus, the two different isotypes of human FLCs may aggregate by slightly different molecular mechanisms. It is also worth noting that in these blots, the anti-λLC-specific region is a smear rather than a well-defined band, with λFLC species larger than 700 kDa also prominent. Thus, membrane-associated λFLC complexes appear to be aggregates of varied size consisting of numerous LCs rather than a defined oligomeric state. In support of this proposal, visualization of λFLC attached to artificial membrane bilayers by AFM revealed the presence of elliptical protein aggregates with a broad range of sizes.

FLCs belong to a family of proteins that readily form pathological aggregates. There are two types of FLC deposition disorders, namely LC amyloidosis (AL), where FLCs are deposited as ordered amyloid structures, or LC deposition disease (LCDD), which are characterized by the deposition of amorphous aggregates [4, 5]. Destabilising mutations within the FLC primary sequence are considered to be the major factor that promotes aggregation. However, it is also widely understood that changes in pH, ionic strength as well as a high local concentration of FLC can all contribute to the aggregation process [37, 38]. In fact, given the appropriate conditions, even non-pathogenic FLCs aggregate [8, 10]. A study by Sikkink and Ramirez-Alvarado compared the types of aggregates that are formed when FLCs are heated to their melting temperatures. They found that FLCs derived from AL and LCDD patients displayed their typical aggregation patterns of forming amyloids and amorphous deposits respectively. However, FLCs derived from MM patients without associated AL or LCDD (i.e. normal FLCs) formed elliptical aggregates of 100 to 150 nm in size [7]. Interestingly, in our AFM study, membrane-associated FLCs were also elliptical in nature, although these were of a smaller size ranging from 20 to 80 nm. The FLC used in our experiments was purified from a MM patient with no apparent deposition disorder. As such, future studies could explore whether pathological FLCs also bind membranes as elliptical-like complexes or form aggregates that are more reminiscent of their deposition disorder.

Testing of binding reactivity to a wide variety of membrane lipids revealed that λFLCs show a preference for saturated phospholipids bearing zwitterionic headgroups. This, in part, supports our findings on κFLCs where we also observed reactivity to saturated forms of PC containing lipids. However, a notable difference is that we found that λFLC associate with the monosaturated lipid, POPE, whereas in the previous study there was no binding to this lipid by κFLC [15]. A potential limitation of the κFLC study was that we labelled the protein with biotin in order to detect binding to membranes. Interestingly, if we take biotinylated λFLC, we see reduced binding to PE lipid derivatives, but retain binding to other lipids such as PC containing lipids. Furthermore, we have now tested non-biotinylated κFLCs derived from a number of MM patients and found that these also react with POPE, which suggests that PE recognition is a general property of FLCs (Supplemental Figure 1). It is not understood why biotinylation reduces binding to PE lipids, but it is thought that the biotin group introduces steric hindrance or alters the electrostatic properties of the FLC by capping positively charged amino groups (e.g. Lys side chains).
Although our results show that \( \lambda \)FLCs bind to saturated zwitterionic lipids, in a physiological setting, these phospholipids are likely to be surrounded by cholesterol in cell membranes. On that basis, we have tested the binding of \( \lambda \)FLCs to DPPC mixed with different concentrations of cholesterol. Interestingly, cholesterol appears to inhibit \( \lambda \)FLC binding to DPPC in a dose dependent manner, however \( \lambda \)FLCs retain the ability to bind DPPC at cholesterol levels equivalent to those found in B cell membranes (~20% of total lipids by molarity [39]; Supplemental Figure 2). In addition to this, \( \lambda \)FLCs were able to bind to LUVs composed of 20% cholesterol (Figure 3A), and we have also shown that both isotypes of FLC can interact with cell membranes from a variety of cell types [20]. Our studies therefore highlight that FLCs can interact with physiological compositions of cell membrane lipids.

Our molecular docking studies were focused on a conserved electronegative pocket found in FLC variable domains. While the major site identified for PC binding in this region is distinct to that identified in the \( \kappa \)FLC dimer Del [15], it has a few similarities. This site is not accessible in the LC of an intact immunoglobulin, but is only accessible in a LC present either as a free variable domain or as a dimer, where one of the chains can acutely bend to expose this site. However, the site identified in Del is accessible both in extended and acutely bent conformations of the LC, suggesting that LC bending is less likely to influence PC binding in Del compared to the \( \text{M} \)\( \text{c} \) \( \lambda \)FLC dimer. There are numerous positively charged residues near the negatively charged residues identified as the key PC-binding residues in Del and the local organization and interactions of these residues with one another may influence the accessibility of the site in Del. It is possible that these distinct mechanisms to expose the head group binding sites are generally utilized in \( \kappa \)FLCs and \( \lambda \)FLCs, although further structural studies are needed to establish this proposal.

It is not understood why FLC aggregates preferentially bind saturated phospholipids over their unsaturated counterparts. A possibility is that saturated lipids show high lateral packing, which reduces their surface hydrophobicity [40]. Consequently, electrostatic forces may predominate on the surface of a saturated bilayer, thus attracting hydrophilic proteins such as FLCs. An alternative explanation is that saturation decreases the fluidity of the membrane, resulting in a rigid electrostatic surface that may enhance the capacity for membrane interactions with FLCs. In addition, the finding that only aggregates are bound to membranes suggests that individual FLCs may only weakly interact with saturated zwitterionic lipids, perhaps through the pocket identified via molecular docking. However, if FLCs associate into a larger aggregate, the combined effects of these weak individual interactions with zwitterionic lipid head groups may contribute to a high avidity complex that is firmly associated with the membrane surface. In support of these arguments, the extraction studies suggest that \( \lambda \)FLCs associate with membranes through a combination of electrostatic and hydrophobic forces. Based on our hypothesis, the electrostatic properties could be explained through the interaction of the protein complex with zwitterionic head groups whereas the hydrophobic component may be as a result of FLC self-association into aggregates.

Although the anti-\( \kappa \)FLC specific mAb, MDX-1097, is currently being assessed in clinical trials for MM, there have been limited studies on defining the molecular nature of membrane associated FLCs. This is the first study to directly image FLCs bound to a supported bilayer which raises important insights into the possible mechanism of membrane association by this clinically important family of antigens. Furthermore, we describe for the first time the presence of membrane-associated \( \lambda \)FLCs on \( \lambda \)LC isotype secreting MM cells. Whether membrane-associated \( \lambda \)FLCs might make a potential therapeutic target for MM remains to be seen, as future studies will be required to determine the levels and frequency of \( \lambda \)FLC expression in patients with MM. However, both \( \kappa \) and \( \lambda \) FLCs warrant further investigation as they are highly atypical membrane proteins. For example, it is widely known that aggregated proteins can bind membranes yet, to the best of our knowledge, the only other example of an aggregate being found on the surface of a cell that also secretes the protein in its native form is \( \alpha \)-synuclein, the pathological aggregate in Parkinson’s disease [41]. As such, there may be many other proteins that interact with cell membranes via similar mechanisms, especially those that have a tendency to aggregate under physiological conditions.
FLC aggregates are found on the surface of myeloma cells

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AUTHOR CONTRIBUTION
ATH and PAR designed the study; ATH, AM, MBB, MA, JT, JLT and LT performed experiments; ATH, AM and PAR collected and analysed data; ATH and PAR wrote the manuscript; MBB, SPD, LT, CBW, ABE, DRJ and RLR gave technical support and conceptual advice.
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REFERENCES

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Table 1. Mean dimensions of $\lambda$FLC bound to mica or supported bilayer. Standard deviations in square brackets. $^\dagger$Volume of a rectangle $V=hl$. $^*$Volume of a semi-ellipsoid $V=\frac{2}{3}\pi abh$.
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**FIGURE LEGENDS**

**Figure 1** λFLCs are found on the surface of MM cells
(A) FACS analysis of MM cell lines stained with anti-λFLC APC (solid line) or anti-κFLC APC (isotype control; dashed line). Grey histogram denotes unstained cells. (B & C) FACS analysis of MM primary cells. MM cells are gated according to CD38+ CD45- phenotype. Solid line represents anti-λFLC stained cells, grey histogram represents isotype control stained cells. (D) 3D-structured illumination microscopy image of an RPMI8226 cell stained with DAPI (blue) and anti-λFLC AlexaFlour 488 (green).

**Figure 2** Membrane bound λFLC reside in a large molecular weight complex and are directly associated with the cell membrane
(A) RPMI8226 membrane proteins (lane 1) and soluble λFLC (lane 2) were separated by N-PAGE followed by western blotting to detect λLC. (B) RPMI8226 membrane proteins were separated by N-PAGE then subjected to non-reducing SDS-PAGE in the second dimension before western blotting for λLC. (C) Cell membranes were purified from cell surface biotinylated RPMI8226 cells and solubilized in digitonin. Membrane proteins were immunoprecipitated with anti-λFLC (lane 1) or isotype control (lane 2) and separated by non-reducing SDS-PAGE. Biotinylated proteins were detected by streptavidin peroxidase western blotting. (D) Left panel: Fractionation of RPMI8226 cell membranes in Na2CO3 pH 11 buffer. The sample was separated by centrifugation into an aqueous (Aq.) and insoluble (Ins.) fraction. The Right panel: Fractionation of RPMI8226 cell membranes in phase separated Triton X 114 (TX114) solution. Aq. fraction represents water soluble membrane proteins. The detergent (Det.) fraction represents TX114 soluble membrane proteins. All samples were separated by SDS-PAGE then probed for λLC, calnexin or actin by western blot.

**Figure 3** λFLCs bind to zwitterionic phospholipids
(A) Sucrose flotation assay testing λFLC, BSA and Fab binding to LUVs. The indicated proteins were incubated with (+) or without (-) LUVs for 2 h at 37°C then separated by centrifugation in a discontinuous sucrose gradient and assessed by SDS-PAGE and silverstain. ‘T’ and ‘B’ refers to top and bottom fractions respectively. Phospholipid ELISAs testing the ability of (B) λFLC and (C) IgGλ to interact with a variety of phospholipids. Note the concentration of λFLC used in this assay is equivalent to the concentration of IgGλ by molarity (IgGλ is approximately three times larger than λFLC in mass).

**Figure 4** Relative capacities of λFLC to interact with different saturated phospholipid
(A) Phospholipid ELISAs testing the reactivity of λFLC to saturated (sphingomyelin & DPPC), monosaturated (POPC) and unsaturated (DOPC) PC lipids. (B) Phospholipid ELISAs testing the reactivity of λFLC to saturated (DPPE), monosaturated (POPE) and unsaturated (DOPE) PE lipids. (C) Phospholipid ELISA testing the ability of λFLC to bind to saturated species of PC (DPPC), PE (DPPE), phosphatidylserine (DMPS), phosphatidic acid (DPPA) and phosphatidylglycerol (DPPG) as well as the unsaturated lipid, DOPC.

**Figure 5** Conformational properties of a λFLC dimer and molecular docking of PC into a putative phospholipid binding site
(A) Overlay of the extended LC of PDB 2MCG (grey) and the acutely bent light chain of PDB 2MCG (yellow). Only the VL portions of the structures were used for the overlay. The box represents the approximate location of panel B on the overlay. (B) Hydrogen bonding interactions (green dashes) taking place between the variable and constant domains in the extended LC of PDB 2MCG (grey). The acutely bent LC of PDB 2MCG (yellow) is shown for comparison. (C) Predicted binding mode of PC (carbon atoms in yellow) in the putative phospholipid binding site. Residues involved in interactions (carbon atoms in white) are shown, along with interaction distances. Hydrogen bonds are shown as green dashes; interactions between the choline group and electronegative atoms are shown as magenta dashes. (D) Ribbon representation of the Mcg λFLC dimer with PC bound to the putative phospholipid binding site of the acutely bent chain (yellow). PC is shown as space-filling spheres.
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Atom colorings for panels (B) and (C): grey/yellow/white – carbon, blue – nitrogen, red – oxygen, orange – phosphorus. Figures prepared in PyMOL.

**Figure 6 AFM images of individual λFLCs bound to mica**
λFLC absorbed to freshly cleaved mica were imaged by AFM in tapping mode. (A) 1 x 1 μm and (B) 0.25 x 0.25 μm sized images.

**Figure 7 AFM analysis of λFLCs bound to a supported lipid bilayer**
(A) 2D image of a mica supported membrane bilayer composed of equimolar amounts of DPPC and POPC lipids. Regions of DPPC, POPC and mica are indicated on the diagram for clarity. (B) 2D image of a mica supported bilayer after the addition of 250 μg/mL of λFLC for 2 h. (C) Size distribution profile of λFLCs bound to the mica-associated lipid bilayers. (D) 3D image of the sample shown in panel B.
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FIGURE 1

A

RPMI8226

B

CD38

22

17

12

7

2

OPM-2

C

CD45

CD38

10^0

10^1

10^2

10^3

10^4

10^5

50

40

30

20

10

5

0

CD45

CD38

10^0

10^1

10^2

10^3

10^4

10^5

60

50

40

30

20

10

5

0

JJN3

D

FLC

FLC

5μm
FLC aggregates are found on the surface of myeloma cells
FIGURE 3

FLC aggregates are found on the surface of myeloma cells
FIGURE 4

FLC aggregates are found on the surface of myeloma cells
FLC aggregates are found on the surface of myeloma cells
FIGURE 6

FLC aggregates are found on the surface of myeloma cells
FIGURE 7

FLC aggregates are found on the surface of myeloma cells.