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NEOD001 Binds a Wide Repertoire of Light Chain Sequences and Aggregation States Found in AL Amyloidosis

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INTRODUCTION

- Amyloid light chain (AL) amyloidosis is a rare, progressive, and typically fatal protein misfolding disease caused by extracellular deposition of aggregated, misfolded immunoglobulin light chains (LCs)[1-3]
  - An excess of LCs prone to misfolding are produced by clonal plasma cells
  - Soluble toxic aggregates and deposited fibrils (amyloid) lead to progressive failure of vital organs, including the heart, kidneys, and nervous system, causing significant morbidity and mortality[1-3]
  - Early intervention is critical for patients with AL amyloidosis, particularly those with cardiac involvement (as many as 74% of patients).[4,5] One study of 421 patients with AL amyloidosis followed up for a median of 4 years showed that patients with cardiac involvement have significantly shorter overall survival after treatment than patients without cardiac involvement (7.6 vs 3.4 years; $P < 0.0001$)[6]
- No therapies have been approved for AL amyloidosis, though patients may be treated with off-label therapies directed at the plasma cell dyscrasia[3,4,7]
  - ~75% of patients do not achieve cardiac organ response with plasma cell–directed therapies and have persistent organ dysfunction[8-12]
- There is a large unmet need for therapies that specifically target soluble toxic aggregates and deposited amyloid fibrils, thereby preserving and improving vital organ function
- NEOD001 is a first-in-class monoclonal antibody that specifically targets multiple forms of the disease-causing, misfolded LC aggregates in AL amyloidosis. Proposed mechanisms of action of NEOD001 include neutralizing soluble toxic aggregates and inducing the clearance of insoluble deposited fibrils (amyloid) through phagocytosis
- The specificity of NEOD001 depends on its binding to a cryptic epitope in kappa (κ) and lambda (λ) LC proteins that is uniquely exposed during misfolding and aggregation[13]

OBJECTIVE

- To further understand the characteristics of NEOD001 binding to a variety of LC aggregates and to provide insights into the exposure of the NEOD001 epitope during the processes of LC aggregation

METHODS

Patient Tissue Samples

- Tissue samples from patients with AL amyloidosis were obtained from Dr. Merrill Benson (Indiana University School of Medicine, Indianapolis, IN), Dr. Michaela Liedtke (Stanford University, Palo Alto, CA), Dr. Jon Wall (University of Tennessee, Knoxville, TN), and the National Disease Research Interchange (Philadelphia, PA). Fresh-frozen normal tissue was obtained from BioreclamationIVT (Baltimore, MD), and positive control tissue was purchased from American MasterTech (Lodi, CA)

Immunoassay of Peptide Sequences

- ELISA plates were coated with indicated peptides, blocked, and assayed with either NEOD001 or 2A4 (murine precursor of NEOD001), as indicated. After washing, appropriate horseradish peroxidase–conjugated secondary antibodies were applied. The plate was then washed and developed with *o*-phenylenediamine, and absorbance was read at 490 nm

Immunoassay of Patient Amyloid Samples

- Binding of 2A4 to amyloid extracts was measured by either time-resolved fluorescence immunoassay or by MSD electrochemiluminescence (ECL) assay, as previously described[23,24]
- When required, amino acid sequences of involved LCs were determined by laser capture microdissection–mass spectrometry (LCM-MS)
- Amino acid diversity of tissue samples with 2A4-positive binding was analyzed at the putative epitope at amino acid positions 81/82

Immunohistochemistry and Laser Capture Microdissection–Mass Spectrometry

- Fresh-frozen cryosections were assessed by immunohistochemistry; Congo red or ThT staining was used to detect amyloid
- LCM-MS isotyping was performed by Mayo Medical Laboratories. Samples were stained with Congo red to localize amyloid deposits, and deposits were excised by LCM. Isolated deposits were deparaffinized and solubilized before analysis and sequencing by laser capture microdissection–tandem mass spectrometry[14]

Recombinant LC Sequence Expression and Characterization

- For recombinant aggregation and binding experiments, an amyloidogenic LC sequence (containing both variable and constant regions) from a patient with AL amyloidosis and cardiac involvement[15] was recombinantly expressed in Chinese hamster ovary cells and purified by affinity chromatography. Mutations were introduced by site-directed mutagenesis and expressed and purified in an identical manner
- Aggregations were carried out for 120 hours at 57°C with orbital shaking (500 rpm). Unaggregated and aggregated LCs were characterized by SDS-PAGE, size-exclusion chromatography, ThT fluorescence, and electron microscopy

Bioinformatic Analysis of AL Amyloidosis–Associated LC Sequences

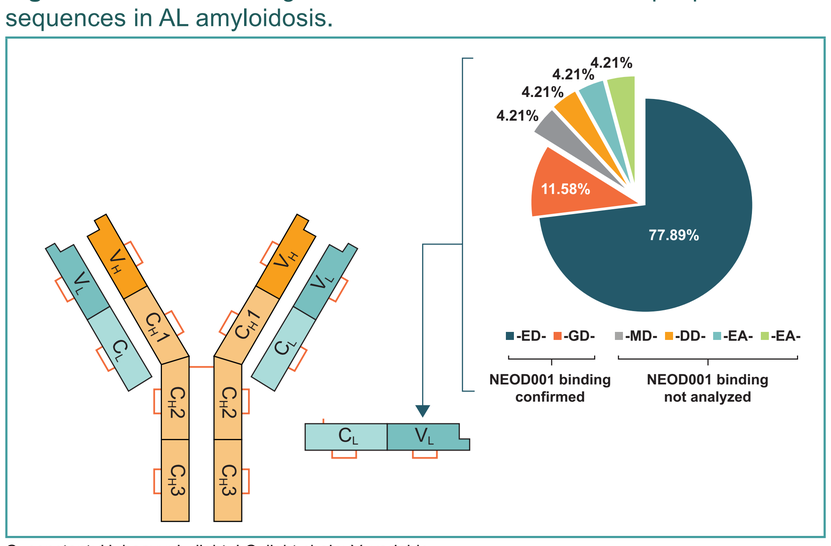
- The occurrence of different amino acids at positions 81 and 82 across the entire human V_H and V_L germline gene repertoire was determined by applying the Kabat numbering system to the LC sequences from the ImMunoGeneTics (IMGT) database and the amino acid sequence alignment using the MegAlign tool of LaserGene DNA software
- The frequency of gene subtypes and alleles that are prevalent in patients with AL amyloidosis was determined by bioinformatics analysis of the published LC sequences in ALBase (Boston University) and various publications.[16-22] The Kabat numbering system was applied, and the IMGT/DomainGapAlign software tool was used to identify gene subtypes and alleles

RESULTS

Putative Binding Site of NEOD001/2A4

- The putative binding site of NEOD001/2A4, determined by peptide analysis, was localized to the V_L region of an antibody light chain
- The predominant amino acid sequence of the putative NEOD001/2A4 binding site was -ED-/-GD- (**Figure 1**)

Figure 1. Putative binding site of NEOD001/2A4 and LC epitope sequences in AL amyloidosis.

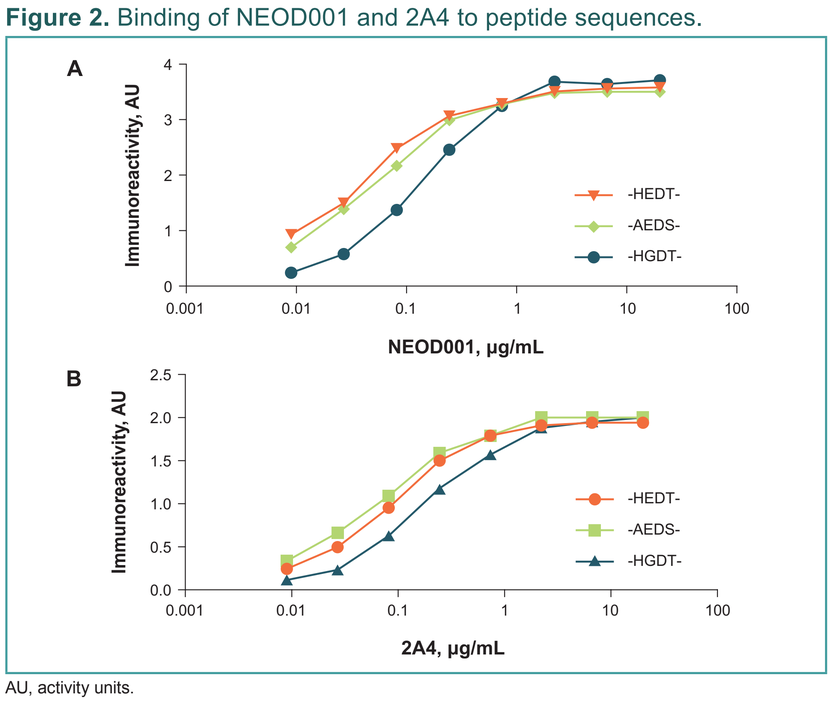


C, constant; H, heavy; L, light; LC, light chain; V, variable.

NEOD001 and 2A4 Bind the Peptide Sequences -XEDX- and -XGDX-, the Sequence Variants Found in LCs

- Binding of NEOD001 (**Figure 2A**) and 2A4 (**Figure 2B**) was comparable toward peptides containing residues -ED- and -GD- of the putative LC epitope and was measured by ELISA

Figure 2. Binding of NEOD001 and 2A4 to peptide sequences.

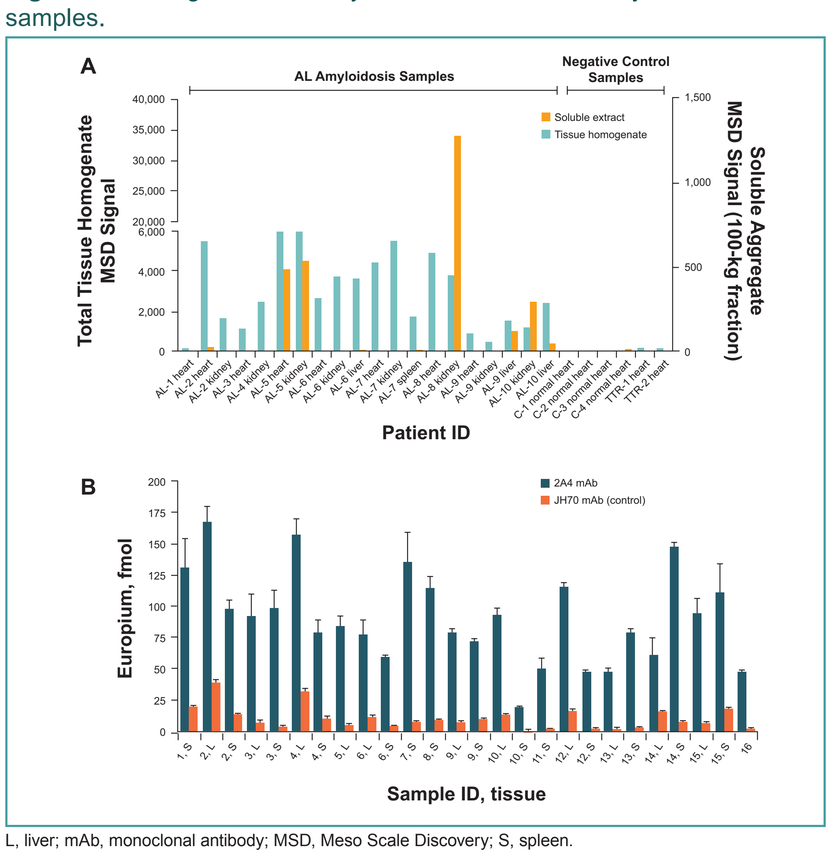


AU, activity units.

2A4 Binds a Wide Range of AL Amyloid Extracts of Varying AL Subtype

- 19 fresh-frozen samples from 10 patients with AL amyloidosis were processed to soluble and insoluble samples (representing both ALκ and ALλ), and 2A4 binding was assessed in the ECL immunoassay[23] (**Figure 3A**)
- Amyloid extracts from the spleens and livers of 15 patients with AL amyloidosis were assessed for 2A4 binding by time-resolved fluorescence immunoassay[24] (**Figure 3B**)

Figure 3. Binding of 2A4 to amyloid extracts from AL amyloidosis samples.



L, liver; mAb, monoclonal antibody; MSD, Meso Scale Discovery; S, spleen.

- LCM-MS and sequence analysis of tissue samples evaluated for 2A4 binding by immunoassay (**Figures 3A, 3B**) confirmed that a variety of LC subtypes were bound by 2A4 (**Table 1**)

Table 1. Summary of LCM-MS and Sequence Analysis of AL Amyloidosis Tissue Samples

AL Amyloidosis Patient Tissue					
Sample ID	LC Gene	Epitope Residues[a]	Sample ID	LC Gene	Epitope Residues[b]
AL-1	*ND*	ND	1	*LV2*	-ED-
AL-2	*LV1-44*	-ED-	2	*LV2*	-ED-
AL-3	*ND*	ND	3	*KV4*	-ED-
AL-4	*KV4-1*	-ED-	4	*LV2*	-ED-
AL-5	*LV6-57*	-ED-	5	*KV1*	ND[c]
AL-6	*LV6-57*	-ED-	6	*KV1*	ND[c]
AL-7	*LV2-18*	-ED-	7	*LV6*	-ED-
AL-8	*LV3-21*	-GD-	8	*lambda*	ND
AL-9	*ND*	ND	9	*LV3*	ND[d]
AL-10	*LV2-14*	-ED-	10	*LV2*	-ED-
—	—	—	11	*KV1*	ND[c]
—	—	—	12	*KV1*	ND[c]
—	—	—	14	*LV3*	ND[d]
—	—	—	15	*LV2*	-ED-
—	—	—	16	*LV6-57*	-ED-

LCM-MS, laser capture microdissection–mass spectrometry; ND, not determined.
[a]Epitope residues were determined by LCM-MS.
[b]Epitope residues were inferred from germline prevalence (only listed if present in all subtype genes).
[c]Epitope is -ED- in 91% of *KV1* sequences.
[d]Epitope is -ED- or -GD- in 94% of *LV3* sequences.

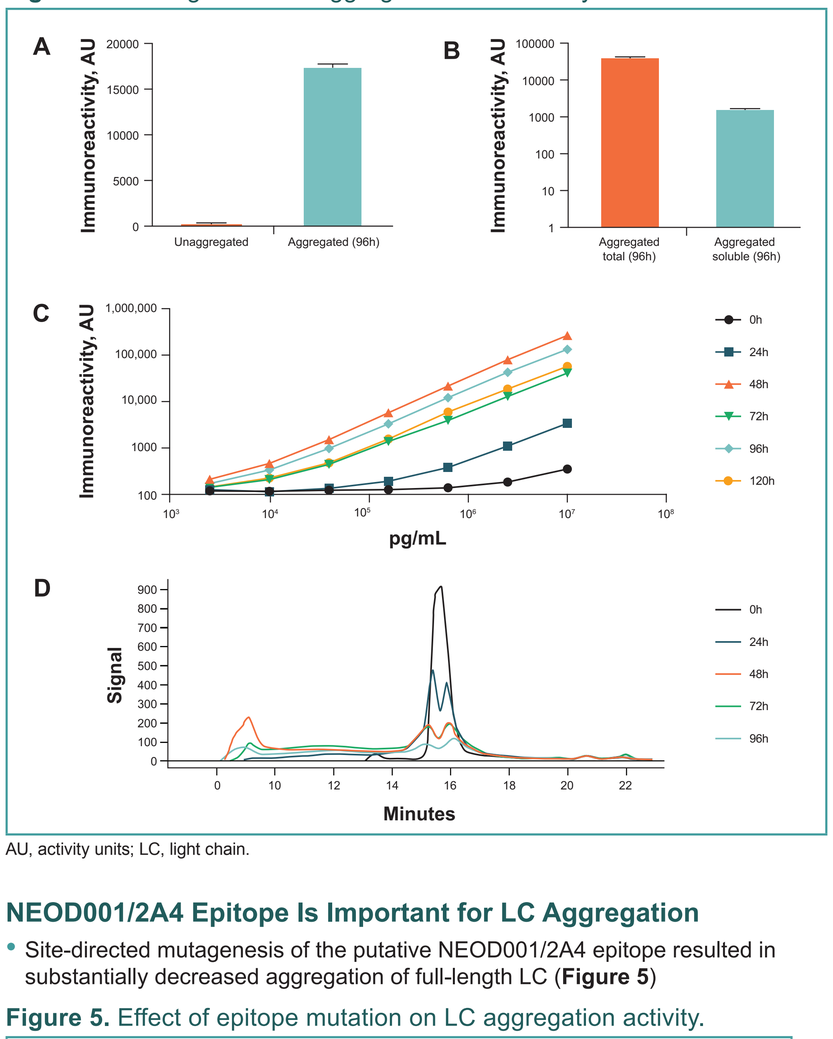
Analysis of Epitope Prevalence in AL-Associated LC Sequences Indicates That NEOD001/2A4 Has Ability to Bind ~90% of the LC Repertoire of AL Amyloidosis

- Amino acid occurrence at positions 81 and 82 in published patient-derived LC sequences (n = 95) was bioinformatically analyzed and graphed as a percentage of total sequences (**Figure 4**)

2A4 Binds Aggregated but Not Normally Folded LCs, and Binding Is Related to Aggregation Extent

- 2A4 bound both soluble and insoluble aggregated λ6-57 LCs but not normally folded LCs (**Figures 4A, 4B**)
- 2A4 bound increasingly aggregated λ6-57 LC, and binding was readily detectable at the first appearance of soluble aggregates detectable by size exclusion chromatography (**Figures 4C, 4D**)

Figure 4. Binding of 2A4 to aggregated and normally folded LCs.

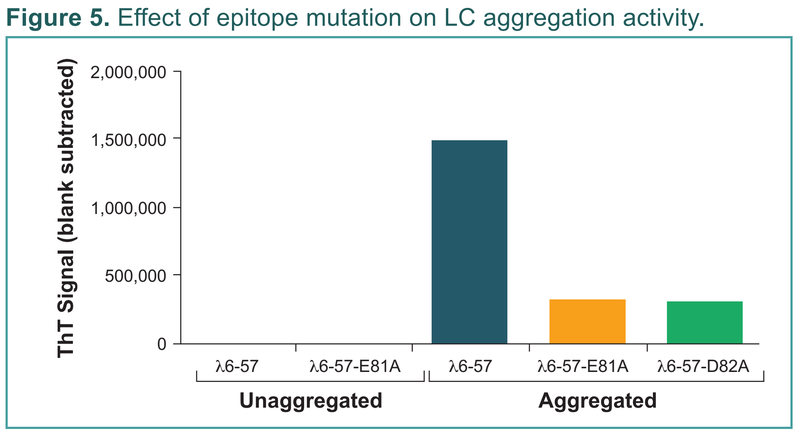


AU, activity units; LC, light chain.

NEOD001/2A4 Epitope Is Important for LC Aggregation

- Site-directed mutagenesis of the putative NEOD001/2A4 epitope resulted in substantially decreased aggregation of full-length LC (**Figure 5**)

Figure 5. Effect of epitope mutation on LC aggregation activity.



LC, light chain; ThT, thioflavin-T.

CONCLUSIONS

- NEOD001 bound to amino acid sequences that are highly conserved within the LCs involved in AL amyloidosis (both κ and λ)
- An epitope was exposed for NEOD001 binding during all stages of abnormal LC aggregation
- The NEOD001 epitope may be involved in the initiation of aggregation and the formation of amyloid

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ACKNOWLEDGMENTS

We thank Angela Dispenzieri and Surendra Dasari from the Mayo Clinic for bioinformatic analysis of LCM-MS samples, and John S. Wall of the University of Tennessee for his contribution of Figure 3B. Editorial assistance was provided by ApotheCom (San Francisco, CA) and was funded by Prothena Biosciences Inc.

Disclosures of Interest

All authors are employees of Prothena Biosciences Inc and own stock options in Prothena Corporation plc. This study was sponsored by Prothena Biosciences Inc, South San Francisco, CA, USA.

http://bit.ly/2p7TY2W

Presented at the XVIth International Symposium on Amyloidosis; March 26–29, 2018; Kumamoto, Japan