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JLR RESEARCH ARTICLE

Generation and characterization of LPA-KIV9, a murine monoclonal antibody binding a single site on apolipoprotein (a)

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Abstract Lipoprotein (a) [Lp(a)] is a risk factor for CVD and a target of therapy, but Lp(a) measurements are not globally standardized. Commercially available assays generally use polyclonal antibodies that detect multiple sites within the kringle (K)IV$_2$ repeat region of Lp(a) and may lead to inaccurate assessments of plasma levels. With increasing awareness of Lp(a) as a cardiovascular risk factor and the active clinical development of new potential therapeutic approaches, the broad availability of reagents capable of providing isoform independence of Lp(a) measurements is paramount. To address this issue, we generated a murine monoclonal antibody that binds to only one site on apo(a). A BALB/C mouse was immunized with a truncated version of apo(a) that contained eight total KIV repeats, including only one copy of KIV$_2$. We generated hybridomas, screened them, and successfully produced a KIV$_2$-independent monoclonal antibody, named LPA-KIV9. Using a variety of truncated apo(a) constructs to map its binding site, we found that LPA-KIV9 binds to KIV$_9$ without binding to plasminogen. Fine peptide mapping revealed that LPA-KIV9 bound to the sequence ^{4076}LETPTVV^{4082} on KIV$_9$. In conclusion, the generation of monoclonal antibody LPA-KIV9 may be a useful reagent in basic research studies and in the clinical application of Lp(a) measurements.

Supplementary key words lipoprotein (a) • monoclonal antibody • isoform • kringle • cardiovascular disease • aortic stenosis • metabolism, therapy • lipoprotein (a)-kringle IV$_9$

Lipoprotein (a) [Lp(a)] is composed of apo(a) covalently bound to apoB-100 (1). Apo(a) consists of 10 unique kringle (K)IV repeats that are present in one copy, except for KIV$_2$, which is present in a variable number of identical copies at the protein level (1 to >40). It also contains one copy of KV and an inactive protease-like domain. The apo(a) protein displays broad size heterogeneity due to a variable number of KIV$_2$ repeats among individuals and populations. Plasma Lp(a) levels are genetically determined by the production and secretion rate of apo(a) by hepatocytes, with isoforms containing a small number of KIV$_2$ repeats being secreted more efficiently, leading to an inverse association of KIV$_2$ repeat number and plasma Lp(a) levels (2).

Elevated Lp(a) is highly prevalent, with an estimated 20% of the population having levels >50 mg/dl (>125 nmol/l), the threshold above which risk accrues in statin-treated patients (3, 4). Lp(a) is also a target of therapy, with recent studies showing that antisense oligonucleotides may lower Lp(a) by over 80% (5–7), and a phase 3 clinical trial that is now underway (https://clinicaltrials.gov/ct2/show/NCT04023552). Multiple national and international societies have suggested that Lp(a) be measured in individuals at moderate to high risk for CVD to enhance risk prediction or for considerations as a risk enhancer (8–14).

Many commercially available assays exist to measure plasma Lp(a), but assay reagents and methodologies vary among manufacturers and have not been globally standardized. It is anticipated that the testing of Lp(a) values will expand with recent European guidelines that have recommended that every adult person have Lp(a) measured at least once in their lifetime (14), as well as by the development of new therapies capable of substantially lowering Lp(a) (5). To date, only one isoform-independent antibody binding to KIV$_9$ on apo(a) has been described and well-characterized, but its peptide epitope has not been reported. This antibody has been used to develop an ELISA method, and the ELISA has been used to assign a target value to the WHO/IFCC reference material, and it is also used as a comparison method to validate commercial assays. However, while the method has been used in a variety of clinical studies, it is not at present commercially available (15). In this study, we describe the generation and characterization of a new murine monoclonal antibody that binds to a defined peptide sequence present only once

This article contains supplemental data.

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in KIV_9 that can be used in both basic and clinical aspects of Lp(a) research and Lp(a) measurements.

METHODS

Generation of immunogens and antigens

To generate immunogens, we used two approaches. First, we generated several truncated apo(a) proteins for use as immunogens. One construct spanned the entire KIV_{10}-KV sequence, and its generation and purification are described in the supplemental material. A second large apo(a) construct, designated 8K-IV, contained one copy of KIV_1, one copy of KIV_2, a fusion of KIV_3 and KIV_5, and individual kringles, KIV_6 to KIV_{10}, KV, and the protease-like domain, as previously described (16). Second, as a different strategy, we generated several unique short peptides derived from apo(a) but not present on plasminogen, which were conjugated to keyhole limpet hemocyanin for immunization. These included peptide GDGRSYRGISSTTVT present in one copy on KIV_9 and peptide MNPRKLFDYC present in one copy on KV.

Screening antigens included Lp(a) purified from a single donor as previously described (17), human plasminogen (R&D, Minneapolis, MN), and a variety of recombinant apo(a) constructs, including two KIV_2 constructs containing either three or five copies, various constructs spanning consecutively from KIV_6 to the protease domain ($KIV_{6\text{-}P}$, $KIV_{7\text{-}P}$, $KIV_{8\text{-}P}$, $KIV_{9\text{-}P}$, $KIV_{10\text{-}P}$, respectively), a 17 kringle human construct with eight KIV_2 repeats, a 17 kringle construct without the protease domain (17KΔP), and a 17 kringle construct without KV (17KΔV) (18, 19).

The disulfide bond between apoB-100 and apo(a) is composed of cysteine 4236 of apoB-100 and cysteine 4057 on apo(a) (20). Murine monoclonal antibody MB47, specific for human apoB-100 (21), whose epitope on apoB is between residues 3350 and 3506 (22), was used in an ELISA format to measure Lp(a). Similarly, murine monoclonal antibody LPA4 (23) that binds to the 14-amino acid epitope TRNYCRNPDAEIRP present on KIV_5, KIV_7, and KIV_8 of apo(a) was used in chemiluminescent ELISAs as previously described (24). The partial sequence of NYCRNPDA detected by LPA4 is also present on KIV_2 and is immunologically dominant as LPA4 also binds to KIV_2, as previously described (24).

Immunization of mice and generation and purification of monoclonal antibodies

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of California San Diego. BALB/C mice were immunized intraperitoneally with keyhole limpet hemocyanin-conjugated peptides GDGRSYRGISSTTVT and MNPRKLFDYC, as well as apo(a) construct KIV_{10}-KV, but these immunogens produced no specific antibodies or the resulting antibodies bound to KIV_2 and/or plasminogen, therefore no further work was performed with them and the results are not presented.

A BALB/C mouse was immunized with a similar protocol as previously described (25), with a primary boost of 125 μg of the 8K-IV apo(a) construct, followed at 2 week intervals with three intraperitoneal boosts with 75 μg. A final antigen boost of 25 μg of 8K-IV in PBS was administered intravenously 2 weeks after the third boost. Three days later, the mouse was euthanized, splenocytes were collected and fused with myeloma cells (p3X63Ag8.653) using a ClonaCell™-HY hybridoma kit (StemCell Technologies, Cambridge, MA), and the fused cells were resuspended in semi-solid HAT hybridoma selection medium. After 10–14 days, visible colonies were transferred to ClonaCell®-HY growth medium (DMEM, preselected serum, HT, gentamycin, and supplements).

To select colonies expressing antibodies specific to Lp(a) without binding to plasminogen or KIV_2, conditioned media from the hybridoma cells were screened by ELISA as described below. To ensure monoclonality, selected clones that bound Lp(a) but not KIV_2 or plasminogen were subcloned twice by limiting dilution in semi-solid gel, without HAT, and retested by ELISA. The resulting heavy and light chains of two colonies were sequenced (Genscript, Piscataway, NJ) and were found to be identical in sequence. The cells were expanded by BioXCell (West Lebanon, NH) in tissue culture in a stirred tank fermentation with Hybridoma-SFM medium supplemented with 1% Fetal Clone 3 (Life Technologies, Carlsbad, CA) and antibody purified with chromatography over Protein A/G resin. Determination of antibody isotype was carried out using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Vilvoorde, Belgium). This monoclonal antibody was designated as "LPA-KIV9".

ELISA with direct antigen plating to assess binding of hybridoma-generated antibodies

Titrations of antibodies present in immunized mouse plasma, screening of hybridoma-conditioned media, and testing the purified monoclonal antibody were performed by chemiluminescent ELISA as previously described (25). In brief, 96-well microtiter plates (Brand, Germany) were coated directly with Lp(a), apo(a) KIV_2 construct, or plasminogen at 5 μg/ml in PBS overnight at 4°C. Nonspecific sites were blocked with 1% BSA (Gemini Bioproducts, CA, USA) in TBS (1% BSA-TBS) and incubated for 30 min at room temperature. Subsequently, serial dilutions of mouse plasma (1:50 to 1:400) in 1% BSA-TBS or hybridoma-conditioned media (undiluted) were added and incubated for 60 min at room temperature. Bound IgG antibodies were detected using an anti-mouse IgG-alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO) and Lumi-Phos 530 substrate (Lumigen, Southfield, MI) on a luminescence plate reader (BioTek, Winooski, VT). Antibody binding was expressed as relative light units detected over 100 ms (RLU/100 ms).

Immunoblot analyses

An array of apo(a) constructs (18, 19, 26) was subjected to gel electrophoresis and immunoblot analysis with LPA-KIV9. Briefly, samples (250 ng/well) were loaded in reducing loading buffer and run on a 4–12% Bis-Tris gel (Life Technologies). Gels were transferred to a PVDF membrane (Life Technologies), and the membranes were blocked with 5% nonfat dry milk in TBS and Tween (TBST) for 1 h before incubation with primary antibodies (1 μg/ml in 1% BSA-TBST) overnight at 4°C, followed by incubation with secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature and by detection with KPL-TMB membrane peroxidase substrate (SeraCare, Milford, MA). A dot-blot was also performed with antigens in PBS (25 ng/dot) loaded directly on the membrane. Blocking and immunoblotting were done as described above and detected by a Super Signal West Dura substrate (Thermo Fisher Scientific, Waltham, MA) and an OptiChem HR imaging system (UVP, Upland, CA).

CDR sequences of LPA-KIV9

TRIzol® reagent (Ambion, #15596-026); PrimeScript™ 1st Strand cDNA synthesis kit (Takara, #6110A). Total RNA was isolated from the hybridoma cells following the technical manual of TRIzol® reagent. Total RNA was then reverse-transcribed into cDNA using either isotype-specific antisense primers or universal primers following the technical manual of PrimeScript™ 1st Strand cDNA synthesis kit. Antibody fragments of heavy chain and

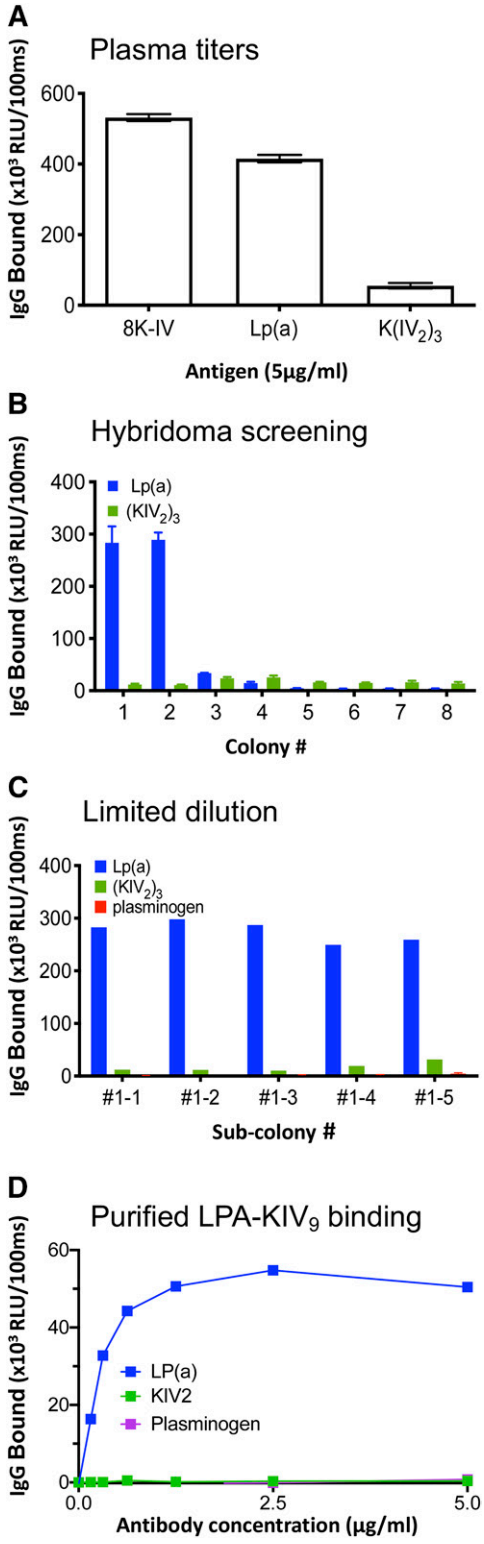


Fig. 1. Generation of monoclonal antibody LPA-KIV9 binding one site on apo(a). A: Mouse plasma titers of Lp(a)-specific antibodies. Following the first boost, target-specific antibody titers were determined by ELISA against immobilized antigen by screening serial dilutions of the serum. The graph represents the mean of technical triplicate measurements at 1:400 plasma dilution. B: Hybridoma screening: 576 hybridoma colonies were collected and expanded. Supernatants were screened by ELISA against the target antigen Lp(a) and counter-screened against antigen $K(IV_2)_3$. The graph shows a representative result of eight colonies and multiple screening rounds. C: Limiting dilution: To ensure monoclonality, 10 positive colonies were subjected to two cycles of limiting dilutions, using semi-solid gel without hypoxanthine-aminopterin-thymidine medium. Collected subclones were tested by ELISA. Colonies #1 and #2 were found to specifically bind to Lp(a), but not to plasminogen and KIV_2. These two colonies were found to have identical CDR3 sequences in their heavy and light chains and were thus derived from the same clone. Colony #1 was retested five times and designated LPA-KIV9. D: Purified LPA-KIV9 binding to Lp(a) (5 µg/ml) was tested by ELISA. The graph shows the mean of two to four technical replicates (mean ± SD).

light chain were amplified according to the standard operating procedure of rapid amplification of cDNA ends of GenScript. Amplified antibody fragments were cloned into a standard cloning vector separately. Colony PCR was performed to screen for clones with inserts of correct sizes. The consensus sequence was provided.

Peptide library array to determine peptide antigen of LPA-KIV9

A peptide library array spanning KIV_9, consisting of 100 overlapping 15-amino acid peptides differing by only one amino acid residue was synthesized on cellulose membranes (JPT, Germany). LPA-KIV9 binding was detected using anti-mouse IgG antibody conjugated with horseradish peroxidase and SuperSignal West Dura HRP chemiluminescent substrate (Thermo Scientific). Swiss-model structure online analysis software was used to simulate the epitope location on a three-dimensional structural model of KIV_8, KIV_9, KIV_{10} (27). The alignment template SMTL ID was 4dur.2.A, plasminogen, with 57.55% sequence identity. The alignment modeling sequence of apo(a) was from A3866 to V4190.

Statistics

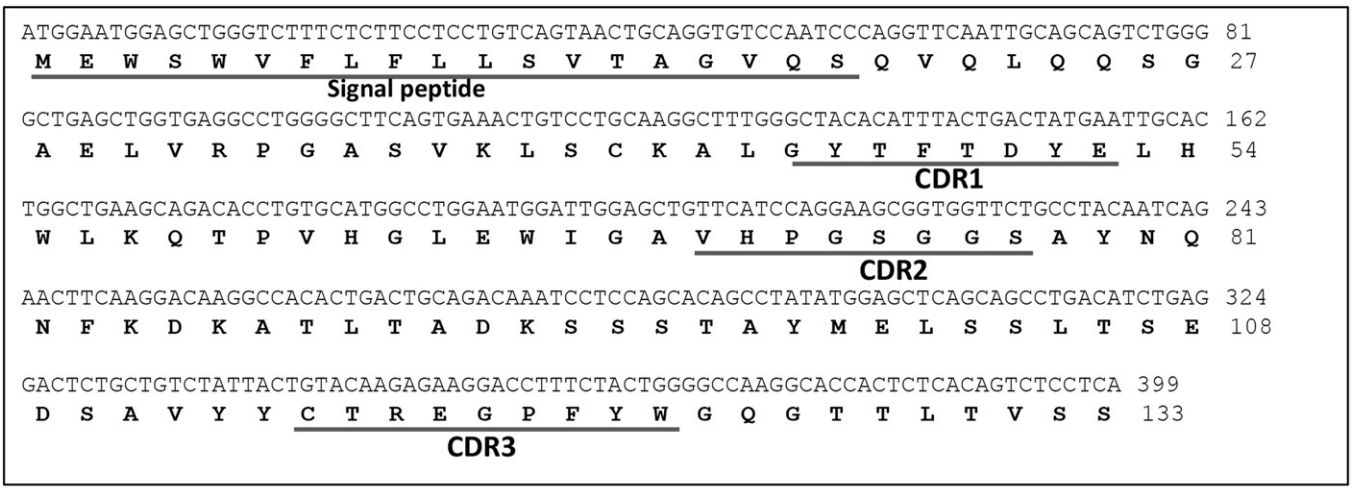
Descriptive statistical analysis and correlation analysis were performed with Spearman's rho test using SPSS version 26.

RESULTS

Generation and characterization of monoclonal antibodies

Following the immunization protocol using the 8K-IV apo(a) construct, mouse plasma was screened for the generation of IgG monoclonal antibodies binding Lp(a). Plasma contained strong binding to the 8K-IV apo(a) construct and Lp(a), with only background levels of binding to the $K(IV_2)_3$ construct (**Fig. 1A**). Hybridomas were then generated, and 576 colonies were collected, expanded, and screened by ELISA for production of IgG antibodies against Lp(a), resulting in 88 positive colonies that were expanded and retested. Two colonies were selected for their robust IgG binding to Lp(a) but not $K(IV_2)_3$ (Fig. 1B). Both colonies were subjected to two cycles of limiting dilution to ensure monoclonality. All daughter clones were positive for binding to Lp(a) but not to $K(IV_2)_3$ or plasminogen (Fig. 1C). On further analysis, the two clones were found to have identical CDR3 sequences in their heavy and light chains and thus were derived from the same clone. The CDR sequences are shown in **Fig. 2**. One colony was expanded in culture and the antibody LPA-KIV9 was purified and shown to be an IgG1 isotype. Testing by direct

LPA-KIV9 Heavy chain (IGHV1-15*01)



LPA-KIV9 Light chain (IGKV1-110*01)

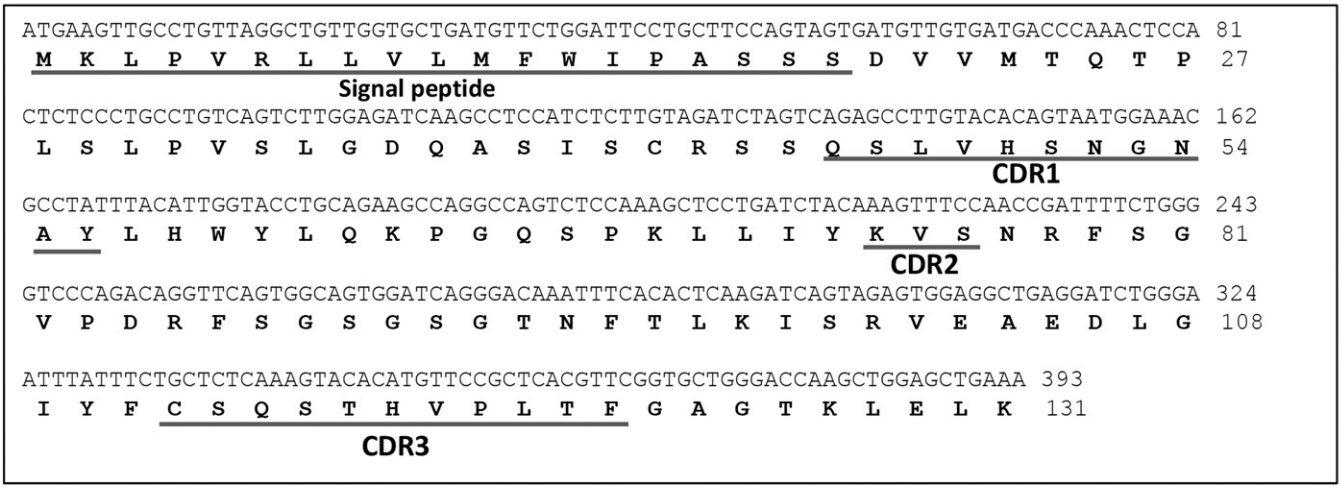


Fig. 2. CDR sequences of LPA-KIV9. LPA-KIV9 heavy chain (IGHV1-15*01) and light chain (IGKV1-110*01) and CDR1-3 sites are shown.

ELISA of the purified LPA-KIV9 showed a dose-dependent specific reactivity with Lp(a), with no cross-reactivity with $K(IV_2)_3$ or plasminogen (Fig. 1D).

Determination of the binding domain of LPA-KIV9 on apo(a)

To determine the site on apo(a) to which LPA-KIV9 bound, we tested its binding to an array of apo(a))-recombinant constructs by dot-blot (**Fig. 3A**) and Western blot (Fig. 3B). By dot-blot, LPA-KIV9 reacted with constructs containing various numbers of kringle segments, including $KIV_{6\text{-}P}$, $KIV_{7\text{-}P}$, $KIV_{8\text{-}P}$, and $KIV_{9\text{-}P}$ containing sequences from KIV_6, KIV_7, KIV_8, and KIV_9 to end of protease domain, respectively. It also recognized the 17 kringle full-length human construct, the 17KΔV construct missing KV, and the 17KΔP construct missing the protease-like domain (Fig. 3A). However, LPA-KIV9 did not react with $KIV_{10\text{-}P}$ and $K(IV_2)_5$, i.e., the only constructs not containing KIV_9, consistent with the epitope being present on KIV_9. This specific binding pattern was duplicated with Western blotting showing that the presence of KIV_9 was required for LPA-KIV9 immunoreactivity (Fig. 3B).

Determination of the specific peptide sequence on the epitope recognized by LPA-KIV9

A peptide library spanning KIV_9 consisting of 100 overlapping peptides, each 15 amino acids in length, was designed (**Fig. 4A**). Antibody LPA-KIV9 bound to nine overlapping peptide fragments on KIV_9 (Fig. 4B), which comprised the 7-amino acid epitope LETPTVV at positions 4076–4082 (Fig. 4C). This epitope is 19 amino acids downstream of cysteine 4057 on apo(a) (shown in orange in Fig. 4D) that creates the disulfide bond with cysteine 4236 of apoB-100 (20). The three-dimensional structure of the epitope on apo(a) was predicted by a Swiss-model server, showing that it is a loop surface epitope toward the C-terminal end of KIV_9 (shown in green in Fig. 4D) and three amino acids away from KIV_{10}.

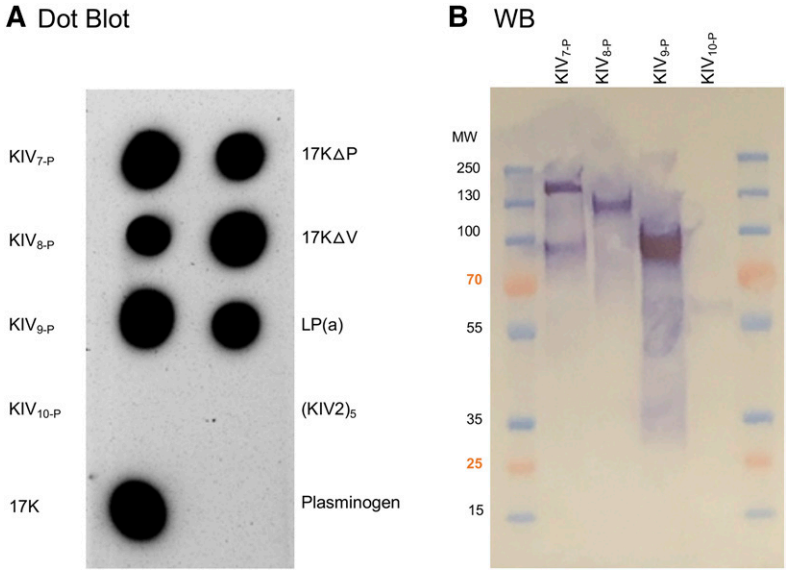


Fig. 3. Identification of the LPA-KIV9 epitope on apo(a). Lp(a) and array of recombinant apo(a) constructs were used to test LPA-KIV9 binding by dot-blot (25 ng protein/dot) (A) and Western blot (B) under reducing conditions (250 ng protein per well). LPA-KIV9 demonstrated a lack of reactivity with plasminogen or KIV_2, and high immunoreactivity with Lp(a) and all apo(a) constructs containing KIV_9.

DISCUSSION

This study describes the generation and characterization of a murine monoclonal antibody, LPA-KIV9, that detects a unique 7-amino acid epitope on KIV_9 of apo(a) present only in one copy. The generation of this new monoclonal antibody will hopefully allow for development of an isoform-independent assay for the measurement of plasma Lp(a) levels. By virtue of binding only one site on apo(a), it may address some of the limitations of clinical Lp(a) assays that utilize polyclonal antibodies binding multiple sites on apo(a) that can lead to either overestimation or underestimation of Lp(a) levels.

In 2018, an National Heart, Lung, and Blood Institute Working Group recommended the development of a globally standardized measurement of Lp(a) applicable to commercial laboratories (3) and that reporting total Lp(a) mass in milligrams per deciliter be discontinued and values reported in nanomoles per liter traceable to a common reference system. Furthermore, harmonization of results obtained by the different methods should be performed after an accuracy-based common calibration is performed.

A recent study evaluating Lp(a) levels by six commercially available methods concluded that current commercial immunological assays are differently calibrated and their biases differed significantly across the clinically relevant concentration range in a nonlinear manner not entirely dependent on apo(a) phenotypes (28). Most commercially available assays use polyclonal antibodies, which are a mixture of antibodies that may bind throughout the apo(a) protein, including the potential for binding multiple times within the KIV_2 region. Furthermore, assay calibrators are usually derived from pooled plasma of multiple donors but generally cannot represent all known apo(a) isoforms present in the samples being measured. Relative to the calibrators, polyclonal antibodies bind more frequently to larger apo(a) isoforms, which are found in subjects with low plasma Lp(a) and thus tend to overestimate these values. In contrast, such antibodies bind less frequently to small isoforms, which are found in Lp(a) in subjects with high plasma Lp(a) and thus tend to underestimate values (24, 29).

The difficulty in generating monoclonal antibodies unique to Lp(a) outside the KIV_2 region that also do not cross-react with plasminogen, which has substantial homology to apo(a) (30), suggests that only a limited number of antigenic sites are available to generate such antibodies. Consistent with this, in the current study, despite an intensive and wide-ranging approach to develop novel antibodies with multiple immunogens, only two clones were identified as being isoform independent and only binding one site, and both were identical in sequence. Three-dimensional mapping suggests that the epitope LETPTVV of LPA-KIV9 protrudes outward from the other kringles and may be immunologically more accessible.

For clinical applications, future efforts will be concentrated on developing high-throughput assay methods to measure Lp(a). Furthermore, validation of isoform independence in samples with known isoform size will be required once a clinically optimized assay method is developed.

In conclusion, the murine monoclonal antibody, LPA-KIV9, may be useful in developing optimized methods to measure Lp(a) in both research and clinical settings.

Data availability

Data that support the plots within this publication and other findings of this study are available from the corresponding author upon request.

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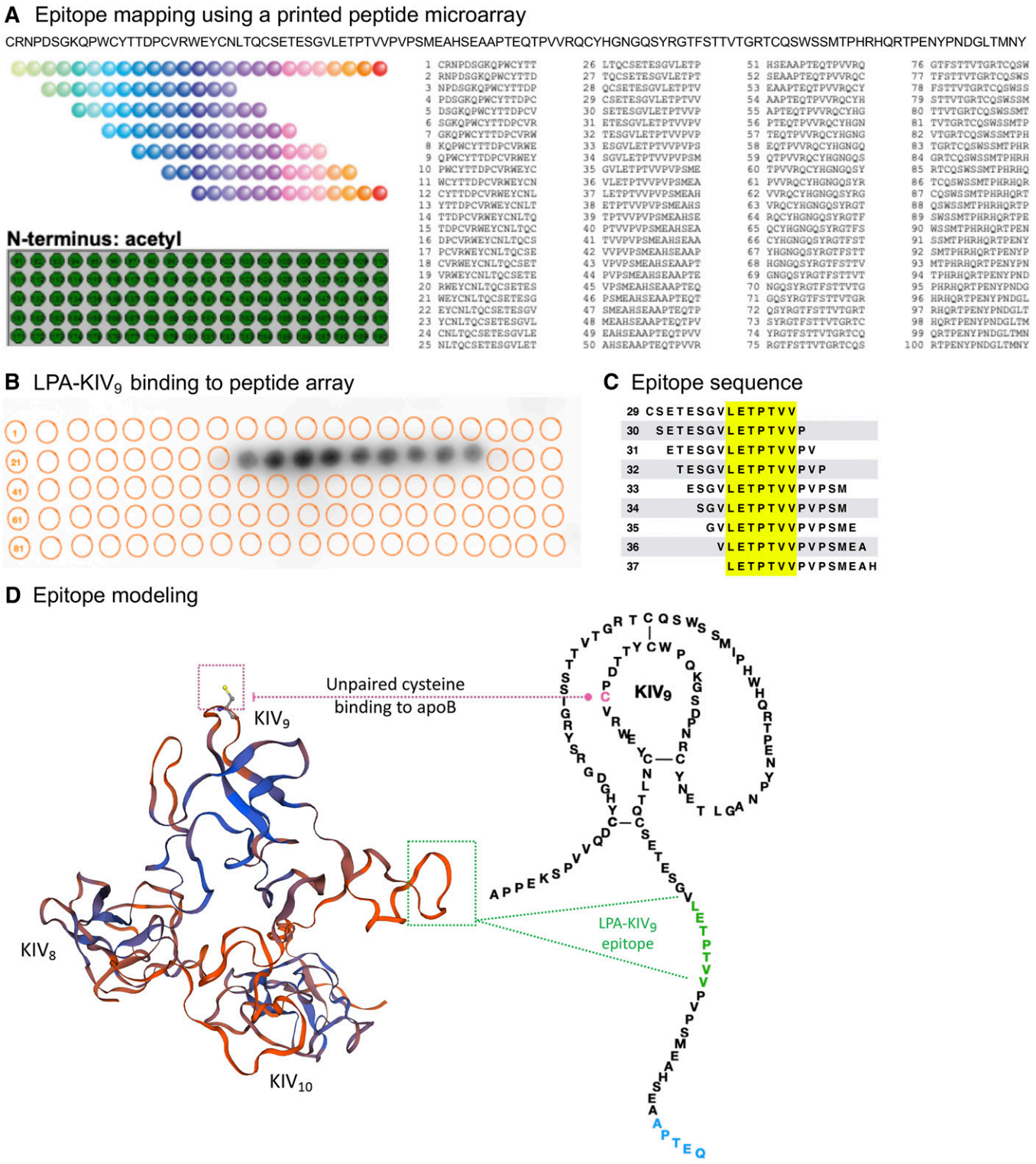

Fig. 4. LPA-KIV9 epitope mapping using a printed peptide microarray. A: One hundred overlapping 15-amino acid peptides differing by only one amino acid residue were synthesized on a membrane. B: LPA-KIV9 binding to peptides: LPA-KIV9 binding to peptide array was tested using biotin-anti-mouse-IgG antibody. C: LPA-KIV9 binds to peptides 29-37, which have sequence LETPTVV in common. D: Epitope modeling. The three-dimensional structure of the LPA-KIV9 epitope was predicted by the Swiss-model server. The alignment template SMTL ID was 4dur.2.A plasminogen, with 57.55% sequence identity. The alignment modeling sequence of apo(a) was from Q1386 to C1680. It is predicted that the whole sequence of epitope 4076LETPTVV4082 is a surface loop epitope.

Conflict of interest

A.G., S.T., and J.L.W. are co-inventors and receive royalties from patents owned by the University of California San Diego on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins. S.T. and J.L.W. are co-founders and have an equity interest in Oxitope, Inc. and its affiliates (Oxitope) as well as in Kleanthi Diagnostics, LLC (Kleanthi). Although these relationships have been identified for conflict of interest management based on the overall scope of the project and its potential benefit to Oxitope and Kleanthi, the research findings included in this particular publication may not necessarily relate to the interests of Oxitope and Kleanthi. The terms of this arrangement have been reviewed and approved by the University of California San Diego in accordance with its conflict of interest policies. S.T. has a dual appointment at University of California San Diego and Ionis Pharmaceuticals. J.L.W. is a consultant to Ionis Pharmaceuticals. M.L.K. is a paid consultant for Ayma Therapeutics and holds a research contract from Abcentra. M.B.B. is a paid consultant for Ayma Therapeutics. The other authors declare that they have no conflicts with the contents of this article.

Abbreviations

KIV, kringle IV; KV, kringle V; Lp(a), lipoprotein (a).

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