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Development of a standardized monoclonal antibody to the inner lipoyl domain of PDC-E2 as a potential international AMA reference

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ABSTRACT

The detection of antimitochondrial antibodies (AMA) is the specific diagnostic marker for primary biliary cholangitis. Indeed, it is the most specific autoantibody in clinical autoimmunity, with a high titer directed response to the inner lipoyl domain of PDC-E2. The current international reference for AMA detection is based upon sera samples of PBC patients. In rheumatic diseases, i.e. rheumatoid arthritis, great efforts are placed at development of international standards. In this study, we report the development of a monoclonal chimeric IgG1 antibody as a reference for AMA testing. A monoclonal 4G6 antibody was constructed from a murine monoclonal antibody specific for the inner lipoyl domain (ILD) of PDC-E2, by combining the variable region with the constant region of human IgG1. The 4G6 antibody recognizes all AMA epitopes containing the ILD of PDC-E2, including the classical BPO recombinant antigen in all currently available diagnostic methods. The binding affinity of the 4G6 antibody to PDC-E2 and BPO antigen reaches K_D value of 7.22×10^{-11} M and 4.55×10^{-11} M, which is sufficient to use as a quantitative reference for all AMA tests. The unlimited availability of the 4G6 antibody makes it a promising candidate for use as an AMA reference or assay calibrator for the international community.

1. Introduction

The detection of antimitochondrial autoantibody (AMA) is the hallmark of primary biliary cholangitis (PBC) [1]. Since the initial identification of the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) as the target antigen for AMAs [2], three major antigens recognized by AMA have been defined as the E2 components of the 2-oxo-acid dehydrogenase family, which include PDC-E2, the E2 subunit of the branched-chain 2-oxo-acid dehydrogenase complex (BCOADC-E2), and the E2 subunit of the 2-oxo-glutarate dehydrogenase complex (OGDC-E2). AMAs from PBC patients primarily recognize the lipoyl domains of these three antigens, with the highest prevalence observed in PDC-E2 [3–6].

Initially the antibody was detected by indirect immunofluorescence

Abbreviations: AMA, antimitochondrial antibody; PBC, primary biliary cholangitis; ILD, inner lipoyl domain; CLIA, chemiluminescence immunoassay; LIA, line immunoassay; OLD, outer lipoyl domain; BLI, biolayer interferometry; IIF, indirect immunofluorescence; Mab, monoclonal antibody.

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(IIF) using as substrate thyroid, kidney and stomach unfixed tissue sections, which was reported in a landmark paper where the association of AMA with PBC was first demonstrated [7]. Later, rodent liver, kidney and stomach sections were commonly used as substrate for AMA detection. With the availability of purified recombinant antigens and the construction of antigens containing triple lipoyl domains of PDC-E2, BCOADC-E2, and OGDC-E2 (collectively known as BPO) [8,9], antigen-specific AMA analysis has been implemented in most clinical tests, including ELISA, chemiluminescence immunoassay (CLIA), and line immunoassay (LIA) [10–12].

In 2018, the Autoantibody Standardization Committee of the International Union of Immunological Societies (IUIS) developed an AMA reference serum for the international community toestablish internal reference standards for AMA testing. This reference serum was derived from a donor with high titers of AMAs against multiple components of the pyruvate dehydrogenase complex, including PDC-E2 [13]. Given the limited supply and eventual depletion of this individual serum, which restricts its use in routine clinical applications, we aimed to develop a chimeric monoclonal antibody that specifically recognizes the dominant AMA epitope as a potential future reference standard for AMA tests.

2. Materials and methods

2.1. Preparation of recombinant proteins

Sequences encoding the inner lipoyl domain (ILD) and outer lipoyl domain (OLD) of PDC-E2 (amino acids 91–294, A0A7P0TBE2), full-length PDC-E2, BCOADC-E2 (A0A7P0Z494), and OGDC-E2 (B7Z6J1) were amplified from cDNA prepared from HepG2 cells and cloned into pET28a plasmids with a C-terminal 6 × His tag. The coding sequence for the classical BPO antigen (containing the ILD of PDC-E2) [8] was synthesized by Genscript (Nanjing, China) and cloned into pET28a with a C-terminal 6 × His tag. All recombinant proteins were produced in *E. coli* BL21 strain using a standard protocol with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) induction at 37 °C for 4 h. The recombinant proteins were purified using Ni-TED Purose Fast Flow (A42302) from Qianchun Bio (Jiaxin, China).

2.2. Generation of mouse anti-PDC-E2 lipoic domain monoclonal antibody (Mab)

Pathogen-free female BALB/c mice (6 weeks of age) were housed in the animal facility at Southeast University, following approval from the ethical committee of Southeast University. A mixture of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) and purified ILD + OLD of PDC-E2 was used for the first two immunizations, administered at a 2-week interval. For the third immunization, Freund's incomplete adjuvant combined with ILD + OLD of PDC-E2 was used. Ten days later, a final boost with the antigen was given three days before the mice were sacrificed. The serum titer of the mice was determined by ELISA, using ILD + OLD of PDC-E2 as the antigen.

SP2/0 myeloma cells were fused with splenocytes from the immunized mice according to standard protocols. The hybridoma cells were cloned by limiting dilution and screened by ELISA. Positive clones were subjected to additional cloning through three rounds of limiting dilution. The supernatant was analyzed for IgG subtypes using a mouse Mab subtype characterization kit (Catalog No. PK20002, Proteintech, Wuhan, China).

2.3. Identification of the coding sequence of the variable regions of Mab

The cloned Mab cells were utilized for RNA preparation. RNA sequencing (RNA-seq) was conducted by Novogene using the Illumina sequencing platform. The heavy chain and light chain sequences were initially identified by searching against the constant region sequences of mouse heavy chain IgG1, IgG2a-b, kappa, and lambda chains. Sequence extensions were subsequently performed using the partially obtained sequences from the variable regions. All matching sequence fragments were aligned using Clustal Omega [14], and the complete sequence was assembled.

2.4. Expression and purification of the chimeric antibody

The coding sequence comprising the mouse heavy chain variable region and the human IgG1 heavy chain constant region, as well as the coding sequence for the mouse light chain variable region and the human kappa chain constant region, were synthesized by Genscript (Nanjing, China) and separately cloned into the pcDNA3.4/GS vector. Both heavy and light chain vectors were transfected into CHO-GS cells using electroporation (ECM830, BTX, USA). The transfected cells were cultured in serum-free Medium A CHO (PD Biosciences, Hangzhou, China) without the addition of glutamine, in a rotating cell culture incubator (IS-RDS6C5, Suzhou Jimei Electronic Co., China) at 37 °C and 125 rpm. After 12 days, the supernatant was harvested and subjected to antibody purification using BiogCap rProteinA beads (Biogenmicro, Suzhou, China).

2.5. Antibody affinity determination by biolayer interferometry (BLI)

Antibody binding affinities were assessed using BLI on the Octet® BLI system with Octet® Software (ForteBio, Inc., USA). The 6 × His-tagged BPO antigen was loaded onto an NTA biosensor (18–5019, Sartorius) at a concentration of 50 μg/mL in binding buffer (PBS containing 0.02 % Tween-20 and 0.05 % BSA). The sensor, immobilized with PDC-E2 or BPO, was dipped into the binding buffer for 60 s to establish a baseline, and then incubated in solutions of the 4G6 antibody at various concentrations (1:2 serial dilution from 1.5625 to 12.5 nM) to monitor the association phase. This was followed by observing the dissociation phase in the binding buffer. Data analysis was performed using the Octet System Data Acquisition Software (Release 7.1).

2.6. Indirect immunofluorescence (IIF)

HepG2 cells were cultured in a 12-well cell culture plate for 15–18 h and then fixed with 4 % paraformaldehyde for 20 min. After washing with PBS, the cells were permeabilized with 0.1 % Triton X-100 in PBS for 15 min and subsequently blocked with 10 % fetal calf serum in PBS for 2 h at room temperature. The cells were then incubated with human or mouse antibodies at various concentrations for 2 h. Following incubation, cells were washed with PBS and treated with TRITC-goat anti-human IgG (SA00007-10, Proteintech) or CoraLite594-conjugated goat anti-mouse IgG (SA00013-3, Proteintech) for 2 h. After the final wash, fluorescent images were acquired using a Zeiss LSM900 confocal microscope.

2.7. Western blotting and line-blotting analysis

Purified recombinant proteins or cell lysates from HuCC-T1, HepG2, and HEK293T cells were separated on a 12 % SDS-PAGE gel under denaturing conditions and then transferred onto a membrane. The chimeric 4G6 antibody targeting ILD was tested at concentrations ranging from 10 to 100 ng/mL. An AMA positive serum sample was diluted to 1:1000 for the assay, and a monoclonal anti-PDC-E2 antibody from Santa Cruz (sc-271534) was used as a control. Line-blotting was carried out using various concentrations of the 4G6 antibody on Liver-6s line blots from YHLO (Shenzhen, China), following the manufacturer's instructions.

2.8. ELISA assay

Purified BPO antigen was coated onto each well of MaxiSorp NUNC IMMUNO plates (Thermo Scientific, cat. 442404) at a concentration of

0.5 μg/mL overnight at 4 °C. The wells were then blocked with 1 % PBS at 37 °C for 2 h. The chimeric antibody was serially diluted with normal serum to obtain various concentrations. Following a 1:100 dilution with PBS-T buffer, the antibody was applied to each well and incubated for 1 h. After washing, peroxidase-labeled goat anti-human IgG (cat. A00166, Genscript, Nanjing, China) was added at a dilution of 1:50,000 and incubated for 1 h at room temperature. Following another wash, TMB substrate was added, and the reaction was terminated by the addition of 2 M H_2SO_4.

2.9. Chemiluminescent immunoassay (CLIA)

Purified 4G6 antibody was serially diluted with control serum to obtain various concentrations and tested using the YHLO AMA kit (cat. C89021G) with the iFlash 1200 Chemiluminescence Immunoassay Analyzer (YHLO, Shenzhen, China), following the manufacturer's instructions.

3. Results

3.1. Identification of mouse Mabs to ILD of PDC-E2

The purified lipoyl domains of the PDC-E2 recombinant protein was utilized for four rounds of immunization in BALB/c female mice. Initial screening of ten 96-well plates yielded 19 positive wells. The supernatants from these wells were used for IIF with HepG2 cells, revealing the strongest staining from clone 4G6. This clone underwent five rounds of limited dilution to isolate single clones. The purified 4G6 antibody was utilized for Western blotting analysis of cell lysates from three different cell lines. A commercial mouse monoclonal antibody to PDC-E2 served as a control, along with a previously identified AMA positive serum sample that was exclusively positive for PDC-E2. The 4G6 antibody displayed a staining pattern similar to that of both the commercial PDC-E2 monoclonal antibody and the PDC-E2 positive serum, indicating that 4G6, at a final concentration of 10 ng/mL, specifically recognizes PDC-E2 (Fig. 1). To further confirm the specificity of the 4G6 antibody for the ILD of PDC-E2, we evaluated its binding to full-length PDC-E2, BCOADC-E2, OGDC-E2, and BPO, which includes the lipoyl domains from BCOADC-E2 and OGDC-E2 along with the ILD from PDC-E2. The results demonstrated that the 4G6 antibody reacted exclusively with proteins containing the ILD of PDC-E2 (Fig. 2).

3.2. Characterization of affinity of chimeric 4G6 antibody to PDC-E2 and BPO antigens

According to the sequencing results, the 4G6 clone contains a mouse IgG1a heavy chain and kappa light chain. The variable regions of the heavy and light chains were synthesized in frame with the constant

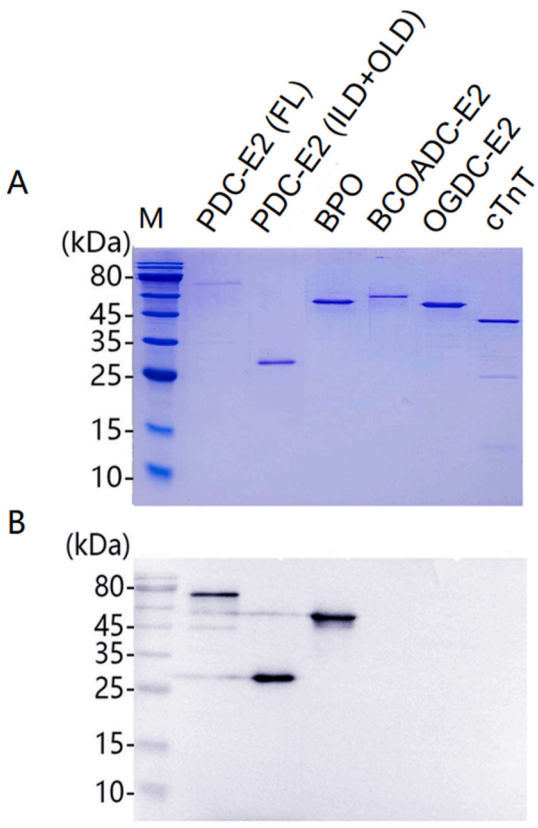


Fig. 2. Specificity of the 4G6 antibody against inner lipoyl domain of PDC-E2. Purified 7 pmole of different recombinant proteins were subjected to 12 % SDS-PAGE gel and stained with Coomassie Brillant Blue (A) or transferred to the PVDF membrane and immunoblotted against the 4G6 antibody (B). PDC-E2 (FL), full length PDC-E2; PDC-E2 (ILD + OLD), PDC-E2 with inner and outer lipoyl domains; BPO, recombinant AMA antigen containing only ILD of PDC-E2, and the lipoyl domains of both BCOADC-E2 and OGDC-E2; cTnT, cardiac troponin T. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regions of the human IgG1 heavy chain and kappa light chain, respectively. To produce the purified chimeric antibody, both vectors were transfected into CHO-GS cells for antibody production.

Given that PDC-E2 and BPO are the two most widely used antigens in the detection of AMA, we analyzed the binding affinity of the 4G6 antibody to both 6 × His-tagged PDC-E2 and BPO recombinant proteins. The binding tests were conducted using BLI. The 4G6 antibody exhibited "fast-on, slow-off" kinetics with respect to PDC-E2, showing an apparent

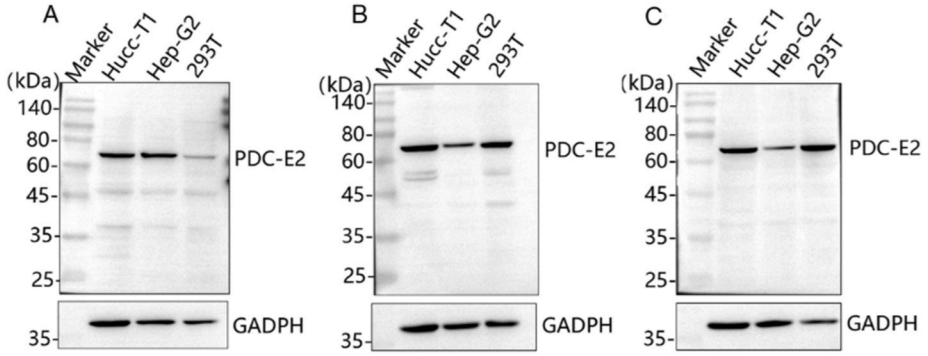


Fig. 1. Specificity of the 4G6 antibody against PDC-E2. Cell lysates (10 μg) from Hucc-T1, Hep-G2 and 293T cell line were subjected to 12 % SDS-PAGE gel, transferred to the PVDF membrane, and incubated with 100 ng/mL of the 4G6 antibody (A), Santa Cruz anti-PDC-E2 antibody sc-271534 (B), and a human serum sample (1:1000 dilution) specifically recognizing PDC-E2 (C).

association rate constant (k_{on}) of 1.50 × 10^6 (1/Ms) and a dissociation rate constant (k_{off}) of 1.08 × 10^{-4} (1/s). For BPO, the antibody demonstrated an apparent k_{on} value of 1.59 × 10^6 (1/Ms) and a k_{off} of 7.26 × 10^{-5} (1/s) (Fig. 3). The calculated binding affinities yielded dissociation constant (K_D) values of 7.22 × 10^{-11} M for PDC-E2 and 4.55 × 10^{-11} M for BPO, respectively. These results indicate that the 4G6 antibody possesses a strong affinity for both PDC-E2 and BPO.

3.3. Chimeric 4G6 antibody in IIF analysis

To verify whether the chimeric 4G6 antibody exhibits the typical cytoplasmic staining associated with AMA-positive serum, we conducted IIF staining using HepG2 cells. The 4G6 antibody demonstrated a cytoplasmic staining pattern identical to that observed with a commercial mouse monoclonal anti-PDC-E2 antibody from Santa Cruz. This staining pattern was also consistent with results from PDC-E2 antibody-positive serum reported by multiple research groups, as reviewed by Leung and colleagues [15–17]. Strong fluorescence signals were observed with both 8 μg/ml and 16 μg/ml final concentrations of the 4G6 antibody (Fig. 4a–d). No further dilutions of the antibody were tested in this analysis.

Additionally, we performed IIF analysis using commercially available monkey liver slides (Euroimmun, Hangzhou, China), where typical cytoplasmic staining was observed at a final concentration of 2.5 μg/ml. A strong staining signal was noted at a concentration of 10 μg/ml of the 4G6 antibody (Fig. 4e and f).

3.4. Chimeric 4G6 antibody in line-blotting assay

The chimeric 4G6 antibody was serially diluted to final concentrations of 5000, 500, 50, 5, 0.5, and 0.05 ng/ml using the reaction buffer provided by the manufacturer, and subsequently applied to the Liver-6s line blot analysis. A stepwise decrease in staining intensity was observed, with a lower detection limit established at a concentration of 5 ng/ml for the 4G6 antibody (Fig. 5). Importantly, no cross-reactivity with Sp100, gp210, LKM1, LC1, or SLA/LP was detected, even at a final concentration of 5 μg/ml in the reaction. These results indicate the high sensitivity and specificity of the 4G6 antibody in line-blotting analysis.

3.5. Chimeric 4G6 antibody in ELISA and CLIA

The classical BPO antigen was coated onto the ELISA plate at a concentration of 50 ng per well and subsequently tested with a series of dilutions of the 4G6 antibody. The 4G6 antibody was detectable at approximately 2 ng/ml, with saturation observed at concentrations between 40 and 60 ng/ml (Fig. 6a). In the CLIA, utilizing 100 ng of BPO antigen bound to magnetic beads, a broader quantitative detection range was achieved, with an upper limit of 1000 ng/ml for the 4G6 antibody in the final reaction (Fig. 6b).

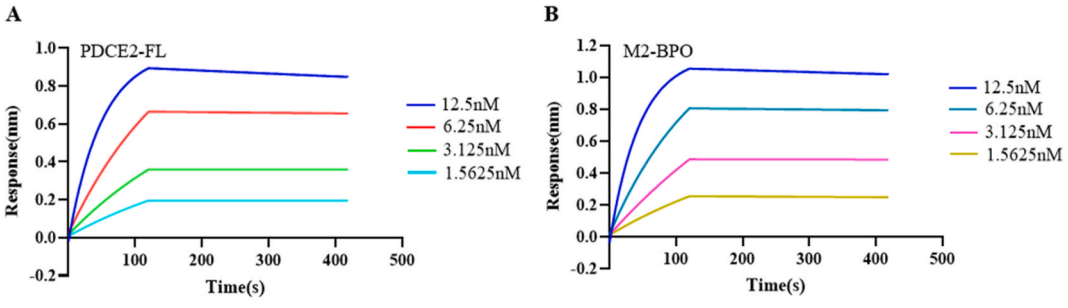
4. Discussion

The AMA is the most established serological marker for diagnosing PBC. The Autoantibody Standardization Committee of the International Union of Immunological Societies (IUIS) currently maintains a reference serum for AMA analysis [13]. This serum, derived from a patient with PBC, is capable of recognizing all three main antigens associated with the disease. However, a common issue with this reference serum is its inherently limited supply, which are often used only for calibrating secondary reference samples.

Due to the multi-antigenic nature of AMA, various manufacturers have developed assays with differing antigen compositions, resulting in significant variability in detection sensitivity. In clinical settings, AMAs are tested using different assays or methods. Consequently, the limited availability of the current IUIS AMA reference serum cannot adequately meet clinical demands. The antibody 4G6, reported in this study, aims to address this limitation.

Currently, AMA detection is performed using IIF, ELISA, and line blotting, which typically provide qualitative or semi-quantitative results. However, these methods are gradually being replaced by quantitative, automated assays employing magnetic or fluorescence beads. These newer approaches involve covalently or non-covalently attaching AMA antigens to beads, primarily through the -NH2 residues of lysine or the N-terminal amino acid. Given the high enrichment of lysines in the lipoic domains of PDC-E2, BCOADC-E2, and OGDC-E2, antigen coupling via lysines can result in steric hindrance, obstructing access to dominant AMA epitopes. Consequently, high-titer reference serum or AMA-positive serum, which also recognizes minor epitopes, may fail to detect significant variations with sufficient sensitivity. The 4G6 antibody, therefore, could serve as a calibration reagent to mitigate batch-to-batch variations during manufacturing.

In recent years, monoclonal antibodies have increasingly been used as quantitative references in autoantibody assays [18–20]. With advances in Chinese Hamster Ovary (CHO) cell-based antibody production, it is now possible to produce unlimited quantities of human monoclonal antibodies or chimeric antibodies that specifically recognize target autoantigens. This approach not only provides a universal



Antigen	K_{on}(1/Ms)	K_{off}(1/s)	K_D (M)	R^2
PDCE2-FL	$1.5\times10^{6}\pm1.18\times10^{4}$	$1.18\times10^{-4}\pm6.05\times10^{-6}$	$7.22\times10^{-11}\pm4.07\times10^{-12}$	0.9982
M2-BPO	$1.59\times10^{6}\pm9.82\times10^{3}$	$7.26\times10^{-5}\pm4.81\times10^{-6}$	$4.55\times10^{-11}\pm3.03\times10^{-12}$	0.9987

Fig. 3. Affinity analysis of chimeric 4G6 antibody with PDC-E2 and BPO. PDC-E2 and BPO proteins were captured to the NTA biosensor at the concentration of 50 μg/mL. The chimeric 4G6 antibody was applied from 1.5625 nM to 12.5 nM. k_{on} is the association rate constant, k_{off} is the dissociation rate constant. The binding affinity constant K_D was calculated as k_{off}/k_{on}.

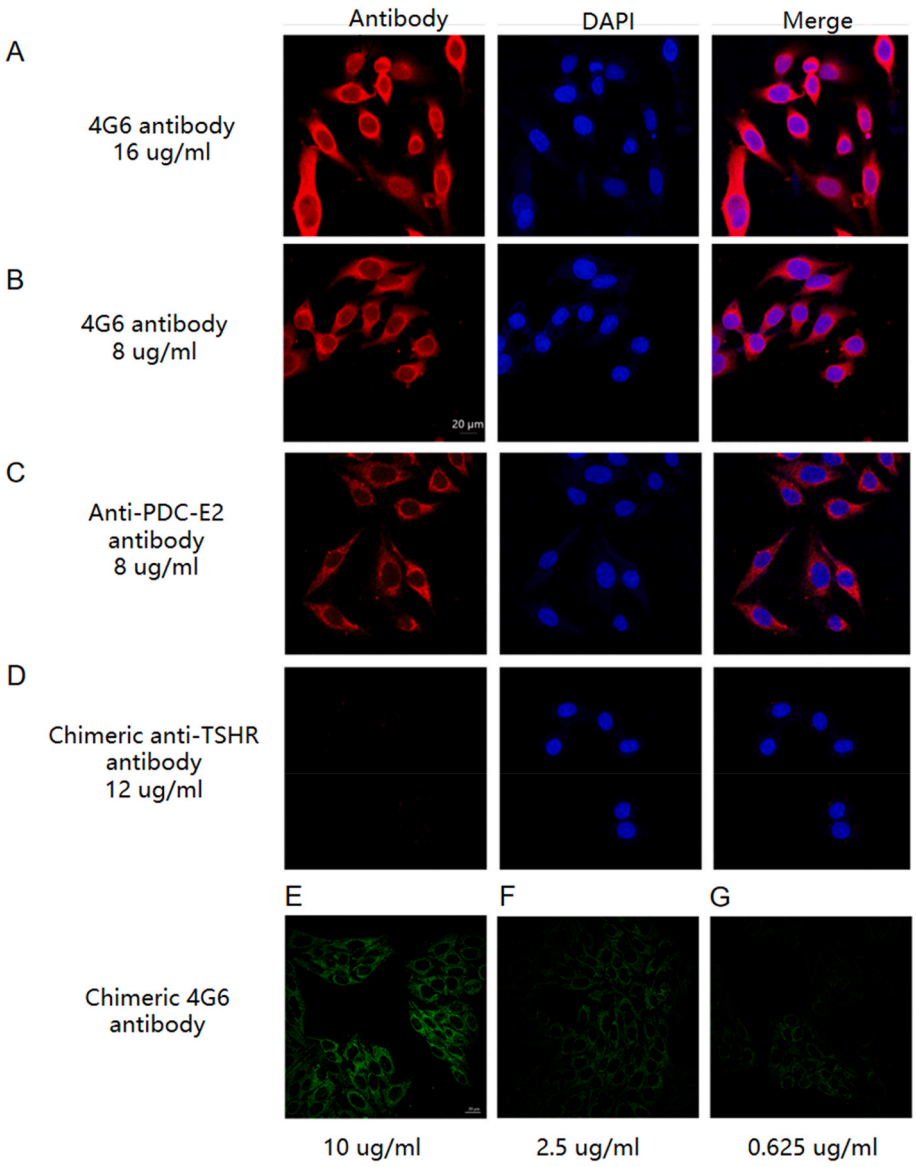


Fig. 4. Immunostaining pattern of the chimeric 4G6 antibody in HepG2 and primary liver cells. HepG2 cells were stained with chimeric 4G6 antibody at the concentration of 16 μg/mL (A) and 8 μg/mL, with Santa Cruz mouse anti-PDC-E2 antibody sc-271534 (C), and chimeric anti-TSHR antibody (12 μg/mL) (D). DAPI was used to stain the nucleus. The primary monkey liver cell slides from Euroimmune Inc., were incubated with 10, 2.5, and 0.625 μg/mL of chimeric 4G6 antibody (E–G).

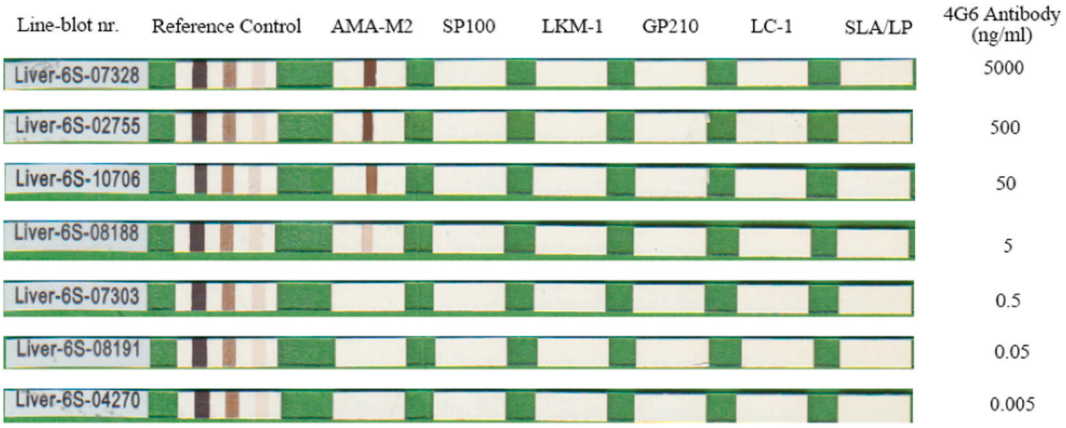


Fig. 5. Sensitivity and specificity of the chimeric 4G6 antibody in line-blotting assay. The Liver-6s line blots from YHLO were stained with 5000, 500, 50, 5, 0.5, 0.05 and 0.005 μg/mL of the chimeric 4G6 antibody.

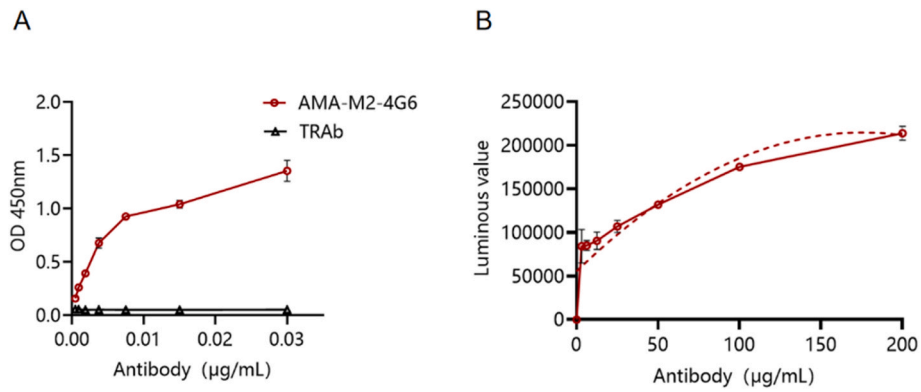


Fig. 6. Sensitivity and linear range of the chimeric 4G6 antibody in ELISA and CLIA analysis. A. BPO antigen was coated 50 ng per well in the 96-well plate and incubated with 30, 15, 7.5, 3.75, 1.875, 0.937, and 0.469 ng/mL of the 4G6 antibody and negative control anti-TRAb antibody. B. 100 ng of BPO antigen immobilized to magnetic beads was analyzed in each reaction with 200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL of the 4G6 antibody. The detection of luminous value was performed using YHLO AMA kit (cat. C89021G) with the iFlash 1200 Chemiluminescence Immunoassay Analyzer.

reference or standard for specific autoantibody detection but also facilitates the creation of universal calibrators for autoantibody detection products.

Key requirements for using monoclonal antibodies as references or standards include a high affinity for the target antigen, recognition of key or dominant epitopes, and applicability across multiple, significantly different assays. This has been a critical factor in the diverse range of autoantibody tests used in rheumatology. In this study, we generated a chimeric IgG1 subtype antibody, 4G6, which meets all these key requirements for AMA assays. This antibody has the potential to be used as a universal reference or standard in all current AMA analyses, including IIF, line blotting, ELISA, and CLIA, owing to its unlimited availability.

Despite its advantages, the polyclonal nature of AMA introduces potential limitations for 4G6 as a reference serum. Variability may arise from the antigens used in AMA detection. While all current AMA antigens include the ILD of PDC-E2, the exact ILD content of PDC-E2 may vary, particularly when comparing a mix of three antigens (PDC-E2, BCOADC-E2, and OGDC-E2) to the BPO antigen or a BPO + PDC-E2 combination. Another limitation of this study is the absence of detailed epitope mapping for the 4G6 antibody, which should be addressed in future research.

For future applications, large-scale production of the 4G6 antibody should be arranged for clinical testing across multiple laboratories, coordinated by an international reference group.

5. Conclusion

In summary, we characterized a high-affinity chimeric antibody targeting the dominant epitope of PDC-E2 for its potential application in AMA assays. Further evaluation is necessary to determine its suitability as a reference in clinical applications.

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CRediT authorship contribution statement

Zhuye Qin: Methodology, Investigation, Formal analysis, Data curation. **Fangming Cheng:** Methodology, Investigation, Data curation. **Mingming Zhang:** Methodology, Investigation, Formal analysis, Data curation. **Ruonan Qian:** Methodology, Investigation. **Hong Chen:** Methodology, Investigation, Data curation. **Yaqin Zhao:** Methodology, Investigation. **Youtao Zhang:** Methodology, Investigation, Data curation. **Yaping Dai:** Methodology, Investigation. **Chaochao Tang:** Methodology, Investigation. **Peng Jiang:** Methodology. **Xiaoli Hua:** Investigation. **Shen Li:** Investigation. **Bing Zheng:** Investigation. **Pin Yu:** Supervision, Methodology. **Xingjuan Shi:** Supervision. **Suraj Timilsina:** Writing – review & editing. **M. Eric Gershwin:** Writing – review & editing, Conceptualization. **Xiangdong Liu:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Chungen Qian:** Supervision, Conceptualization. **Fang Qiu:** Writing – original draft, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiangdong Liu reports financial support was provided by The National Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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