OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2025, **10(1),** bpaf001

https://doi.org/10.1093/biomethods/bpaf001
Advance Access Publication Date: 6 January 2025
Methods Article

Novel method for detection of Aβ and Iso-D7-Aβ N-terminus-specific B cells and Iso-D7-Aβ-specific antibodies

Elizaveta A. Kolobova[1,2], Irina Yu. Petrushanko[1,3], Vladimir A. Mitkevich[1], Alexander A. Makarov[1] and Irina L. Grigorova [1,2,*]

[1]Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991, Moscow, Russia
[2]Institute of Translational Medicine, Department of Molecular Technologies, Pirogov Russian National Research Medical University, 117513, Moscow, Russia
[3]Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991, Moscow, Russia

*Corresponding author. Institute of Translational Medicine, Department of Molecular Technologies, Pirogov Russian National Research Medical University, 117513, Moscow, Russia. Email: grig76@gmail.com

Abstract

Alzheimer's disease (AD) is a multifactorial systemic disease that is triggered, at least in part, by the accumulation of β-amyloid (Aβ) peptides in the brain, but it also depends on immune system-mediated regulation. Recent studies suggest that B cells may play a role in AD development and point to the accumulation of clonally expanded B cells in AD patients. However, the specificity of the clonally expanded B cells is unknown, and the contribution of Aβ-specific B cells to AD pathology development is unclear. In this study, we have developed a novel method to identify Aβ-specific B cells by flow cytometry using fluorescent tetramers. The suggested method also enables the identification of B-cell clones specific to a more pathology-provoking form of Aβ with an isomerized Asp7 residue (Iso-D7-Aβ) that accumulates in elderly people and in AD patients. The method has been verified using mice immunized with antigens containing the isomerized or non-isomerized Aβ N-terminus peptides. In addition, we describe a new method for the detection of Iso-D7-Aβ-specific antibodies, which was tested on mouse serum. These methods are of potential importance in research aimed at studying AD and may be also utilized for diagnostic and therapeutic purposes.

Keywords: Alzheimer's disease; β-amyloids; Iso-D7-Aβ; Aβ N-terminus-specific B cells; Iso-D7-Aβ-specific antibodies

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative pathology in the world, typically causing neuronal death and cerebral atrophy, accompanied by cerebral accumulation of senile plaques and neurofibrillary tangles, containing aggregated β-amyloids (Aβ) and hyperphosphorylated tau proteins correspondingly [1–7]. The initiation and development of the disease are associated with the accumulation and aggregation of Aβ peptides (most commonly $Aβ_{1–40}$ and $Aβ_{1–42}$) in the extracellular space of the brain [7]. Aggregating Aβ peptides promote neurotoxicity and induce local inflammation in the brain, thereby contributing to AD pathology development [8–10]. The current view in the field is that neurotoxicity is predominantly induced by soluble Aβ oligomers [11–13]. Importantly, some mutations and post-translational modifications in the N-terminus of Aβ can influence the rate of oligomerization and toxicity of Aβ. One of the most well-studied forms of posttranslationally modified Aβ is Iso-D7-Aβ, isomerized at the Asp7 residue, which accelerates oligomerization of Aβ and increases its cellular toxicity [14–18]. Iso-D7-Aβ, when administered to transgenic animals overexpressing human Aβ, sharply accelerates amyloidosis, potentially acting via amyloid seeding of endogenous Aβ [19, 20]. It has been theorized that as Iso-D7-Aβ accumulates in the brain with age, it is involved in triggering the progression of AD [21].

While the accumulation of Aβ in the brain triggers inflammation (innate immune response), recent studies in mouse models of AD suggest that B cells may also be important for the regulation of AD pathology [22, 23]. As human AD patients have increased titers of IgG autoantibodies to $Aβ_{1–12}$ (but not $Aβ_{19–30}$ or $Aβ_{25–36}$) that also correlate with increased brain amyloid burden and cognitive decline [24], it is plausible that this is accompanied by accumulation of B cells specific to the N-terminus of Aβ. While clonal B cells accumulate in the blood of human AD patients upon disease progression [25], it is unclear whether these B cells are specific to Aβ and whether they are involved in the regulation of the disease [26]. Experimental analysis of the Aβ-specific B cells has been limited due to the lack of approaches for their quick identification.

Herein, we describe a novel method that enables the identification of B cells specific to the N-terminus of Aβ in mice, by way of fluorescent tetramers that can bind in a specific way to B-cell receptors (BCRs) and flow cytometry approach. The method also enables the identification of B cells specific to Iso-D7-Aβ that are not cross-reactive to non-isomerized Aβ. In addition to the tetramer-specific analysis of Aβ N-terminus-specific B cells, in this study, we have developed an approach for the detection of serum antibodies to Iso-D7-Aβ that are not highly cross-reactive to non-isomerized Aβ.

Received: 25 October 2024. **Revised:** 19 December 2024. **Editorial decision:** 20 December 2024. **Accepted:** 4 January 2025

The methods described below may be useful for the analysis of Aβ and Iso-D7-Aβ-specific B cells and antibodies for AD research and diagnostic applications. Moreover, it should enable the sorting of Aβ-specific B cells for BCR variable region identification.

Materials and methods

Mouse immunization and tissue preparation

Materials

Antigens and adjuvant

- OVA-ovalbumin (Sigma-Aldrich, Cat# A5503).
- OVA-$A\beta_{1–16}$—ovalbumin conjugated with $A\beta_{1–16}$, labeling degree (1:17) (Peptide Specialty Laboratories GmbH, Heidelberg, Germany)
- OVA-Iso-D7-$A\beta_{1–16}$—ovalbumin conjugated with Iso-D7-$A\beta_{1–16}$, labeling degree (1:17) (Peptide Specialty Laboratories GmbH, Heidelberg, Germany).
- Ribi, sigma adjuvant system 1 mg/ml (Sigma-Aldrich, Cat# S6322).

Other materials

- Plastic:
  1.5 ml tubes (SSIbio, Cat# 1260-00),
  15 ml tubes (NEST, Cat# 601001), and
  cell strainer 70 μm (SPL, Cat# 93070).
- Liquids:
  DMEM medium without glutamine, 1 g/l glucose, 25 mM HEPES (Paneco, Cat# C450п) and
  erythrocyte lysis buffer (QIAGEN, Cat# 79217).
- Equipment:
  centrifuge Eppendorf 5804 (Eppendorf, RRID: SCR_019866),
  centrifuge Eppendorf 5424 (Eppendorf, RRID: SCR_019848), and
  TC20 automated cell-counter (BioRad, Cat# 1450102, RRID: SCR_025462)

Mice

Six- to 7-week-old female Balb/c mice were obtained from the Center for Collective Use of the Institute of Physiologically Active Compounds and housed under SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences (EIMB RAS). All animal procedures were performed in accordance with Russian regulations of animal protection and approved by the local Ethics Review Committee at EIMB RAS (Protocol No. 1 from 23/03/23).

Experimental procedures

Immunizations

Balb/c mice were immunized intraperitoneally with 50 μg of OVA, OVA-$A\beta_{1–16}$, or OVA-Iso-D7-$A\beta_{1–16}$ antigens mixed at 1:1 volume with Ribi adjuvant, 100 μl total. A booster immunization with the same antigens was performed 21 days later. Spleens and blood were collected from mice euthanized at 7 or 15 days after the booster immunization.

Serum preparation

A volume of 200 μl of blood was collected into 1.5 ml tubes without coagulant and left at room temperature. After 30–40 min, the tubes with blood were spun in a centrifuge at 1300*g* for 15 min. The serum was carefully collected, aliquoted, and frozen at −80C° for ELISA analysis.

Splenocyte preparation

Each isolated spleen was placed into 12 ml DMEM medium and ground through a 70 μm cell strainer. The resulting cell suspension was transferred into a 15 ml tube and spun in a centrifuge at 300*g* for 8 min at 4°C. The supernatant was drained, and precipitated cells were resuspended in 1 ml DMEM. Next, 11 ml of erythrocyte lysis buffer was added to the tube and mixed with the cells. Immediately after that, the cell suspension was centrifuged at 300*g* for 8 min at 4°C. Cell supernatant was drained, and the precipitated cells were resuspended in 12 ml DMEM and centrifuged at 300*g* for 8 min at 4°C. Cell supernatant was drained, and cells were resuspended in 1 ml DMEM and counted on the automated cell counter. Estimated splenocyte viability was above 80%.

B cells staining with Aβ-tetramers for flow cytometry analysis

Materials

Peptides and fluorophores for tetramers preparation

The following peptides were synthesized and biotinylated at the C-terminus by Peptide Specialty Laboratories GmbH, Heidelberg, Germany:

- $A\beta_{1–42}$-bio
- $A\beta_{1–16}$-bio
- Iso-D7-$A\beta_{1–16}$-bio

To generate tetramers, biotinylated peptides or biotin (Sigma-Aldrich, Cat# B4501) were conjugated to fluorescent streptavidins:

- SA-APC (BD Biosciences Cat# 554067, RRID: AB_10050396);
- SA-PE (BD Biosciences Cat# 554061, RRID: AB_10053328); and
- SA-FITC (BD Biosciences Cat# 554060, RRID: AB_10053373)

Antibodies

- Anti-mouse CD3 Alexa fluor 700 (clone: 17A2, BD Biosciences Cat# 561388, RRID: AB_10642588);
- Anti-mouse CD38 PerCP-Cy5.5 (clone: 90/CD38, BD Biosciences Cat# 562770, RRID: AB_2737782);
- Anti-mouse B220 V500 (clone: Ra3-6B2, BD Biosciences Cat# 561226, RRID: AB_10563910);
- Anti-mouse CD95 (FAS) PE-Cy7 (clone: Jo2, BD Biosciences Cat# 557653, RRID: AB_396768);
- Anti-mouse IgD APC-H7 (clone: 11-26c.2a, BD Biosciences Cat# 565348, RRID: AB_2739201); and
- Anti-mouse IgM BV421 (clone: R6-60.2, BD Biosciences Cat# 562595, RRID: AB_2737671).

Other materials

- Plastic:
  U-shape 96-well plate (Greiner, Cat# 650161),
  5 ml tubes for cytometry (SPL Lifesciences, Cat# 41005).
- Liquids:
  FACS buffer—DPBS without Ca^{2+} and Mg^{2+} (Paneco, Cat# P060), 0.5% FBS, 0.1% NaN_3
- Equipment:
  centrifuge Eppendorf 5804 (Eppendorf, RRID: SCR_019866),
  centrifuge Eppendorf 5424 (Eppendorf, RRID: SCR_019848), and
  BD FACSAria™ III Cell Sorter (BD Biosciences, RRID: SCR_016695).

Experimental procedures

Aβ and Iso-D7-Aβ-peptides preparation

Aβ peptides were dissolved in 25% NH_4OH. After a 15 min incubation period, solutions were aliquoted and lyophilized and stored at −20°C. Stock concentrations of peptides (0.15 mM) were prepared in phosphate-buffered saline (PBS, pH∼7.4).

Tetramers preparation

Tetramers were generated by conjugating each of the biotinylated peptides (or biotin only) with fluorescent streptavidin at a molar ratio of 4:1 or more. The following tetramers were prepared:

- biotin: SA-FITC,
- Iso-D7-$Aβ_{1–16}$-bio: SA-APC,
- $Aβ_{1–42}$-bio: SA-PE, or
- $Aβ_{1–16}$-bio: SA-PE.

The biotinylated peptides were mixed with SA-fluorophore and left to incubate on ice for 30 min. After that, the tetramers were mixed with each other and incorporated into the antibody mix. Cells should be stained with the antibody–tetramer mix immediately after tetramer addition.

Antibody–tetramer mix preparation

For B cell staining, 25 µl of antibody/tetramer mix in the FACS buffer was used per million of splenocytes. First, antibodies were mixed in the Fluorescent Activated Cell Sorting (FACS) buffer (for a final dilution of 1:100) and spun in a centrifuge at 15 000*g* for 10 min to pellet large particles. Supernatant with antibody mix was then collected. Mixed tetramers with SA-APC, SA-PE, and SA-FITC were added to the antibody mix in the supernatant for the final dilution of 1:100, 1:100, and 1:100 correspondingly.

Cells' staining

For analysis of Aβ-specific B cells, 2–10 million splenocytes were stained. Two million cells were stained in a U-shaped plate, while over 2 million cells were stained in 5 ml tubes. Cells were pelleted by centrifugation at 300*g* at 4°C (for 2 min in a plate and 4 min in a 5 ml tube) and resuspended in an appropriate amount of antibody–tetramer mix on ice for 30 min. The cells were washed two times with FACS buffer and resuspended in 300 µl (for 2 million cells) and 3 ml (for over 2 million of cells) correspondingly. The cell suspension was kept on ice until flow cytometry analysis on FACSAria™ III Cell Sorter via FACSDiva™ Software (BD, RRID: SCR_001456). Single-color cell stains for all fluorophores have been used for compensation. Flow cytometry analysis was carried out using FlowJo 10.8.1 (FlowJo, RRID: SCR_008520). For analysis of the flow cytometry data, see Fig. 1.

Iso-D7-Aβ-specific antibodies detection in serum via enzyme-linked immunosorbent assay (ELISA)

Materials

Antigens and antibodies

- BSA-$Aβ_{1–16}$—bovine serum albumin conjugated with $Aβ_{1–16}$, labeling degree (1:8) (Peptide Specialty Laboratories GmbH, Heidelberg, Germany).
- BSA-Iso-D7-$Aβ_{1–16}$—bovine serum albumin conjugated with Iso-D7-$Aβ_{1–16}$, labeling degree (1:8) (Peptide Specialty Laboratories GmbH, Heidelberg, Germany).
- Anti-mouse IgG-polyclonal (BioLegend Cat# 405303, RRID: AB_315006).
- SA-HRP (BD Biosciences Cat# 557630, RRID: AB_10065937).

Solutions

Bicarbonate buffer (pH 9.8):

- 1 × PBS—1000 ml (diaGene, Cat# 4436.0500).
- Sodium carbonate (Na_2CO_3, HiMedia Laboratories, Cat# MB253-1 kg)—2.33 g.
- Sodium hydrogen carbonate ($NaHCO_3$, CDH, Cat# PCT2535)—2.86 g.
- Magnesium chloride ($MgCl_2$, Amresco, Cat# Am-O288-0.1)—0.2 g.

Blocking buffer with gelatin:

- 1 × PBS—1000 ml (diaGene, Cat# 4436.0500).
- Fish gelatin powder—0.1% (Biotium, Cat# 22011).
- Sodium azide (NaN_3, dia-m, CAS 26628-22-8, Cat# 3598.0100)—0.1%.
- Tween-20 (Amresco, Cat# Am-O777-0.1)—0.05%.

PBST:

- 1 × PBS—1000 ml (diaGene, Cat# 4436.0500).
- Tween-20 (Amresco, Cat# Am-O777-0.1)—0.05%.

Stop solution:

- 0.16–0.20 mM H_2SO_4 (Sigmatek, Cat# TU 26 1212 0023 02).

TMB (AbiZyme, Cat# ZYM-011-3-100 ml).

Equipment

Horizontal shaker Biosan OS-20 (Biosan, Cat# BS-010108-AAG).
Orbital shaker Elmi RM-1L (Elmi, Cat# RM1L-30 × 14).

Experimental procedures

A new method was developed for the detection of serum antibodies specific to Iso-D7-$Aβ_{1–16}$ that are not highly cross-reactive to $Aβ_{1–16}$. The method is based on the pre-incubation of serum with soluble BSA-$Aβ_{1–16}$ to titrate away antibodies cross-reactive to $Aβ_{1–16}$ followed by indirect ELISA on the plates covered with BSA-$Aβ_{1–16}$ and BSA-Iso-D7-$Aβ_{1–16}$ antigens (Fig. 2).

Immobilization of BSA-$Aβ_{1–16}$ or BSA-Iso-D7-$Aβ_{1–16}$ antigens on the 96-well ELISA

1. 0.01 mg/ml solutions of BSA-$Aβ_{1–16}$ and BSA-Iso-D7-$Aβ_{1–16}$ antigens were prepared in bicarbonate buffer.
2. To cover 96-well ELISA plates with BSA-$Aβ_{1–16}$ or BSA-Iso-D7-$Aβ_{1–16}$ antigens, 50 µl of 0.01 mg/ml solutions (see step 1) were added to the wells of 96-well plates and incubated at room temperature for 1 h.
3. Antigen solutions were discarded with a sharp movement.
4. The wells were washed three times with 200 µl PBST solution by incubating them for 5 min (for each wash) on a horizontal shaker at 106–110 rpm. After each wash, the liquid was discarded as in step 3.
5. Three hundred microliter of the blocking buffer was added to the wells and incubated at room temperature for 1 h or overnight at +4°C.

Analysis of $Aβ_{1–16}$- and Iso-D7-$Aβ_{1–16}$-specific antibody titers in serum

6. Serial dilutions of serum were prepared in a blocking buffer. In our experiment, for the optimal data analysis, serial dilutions of serum from the OVA-$Aβ_{1–16}$ and OVA-

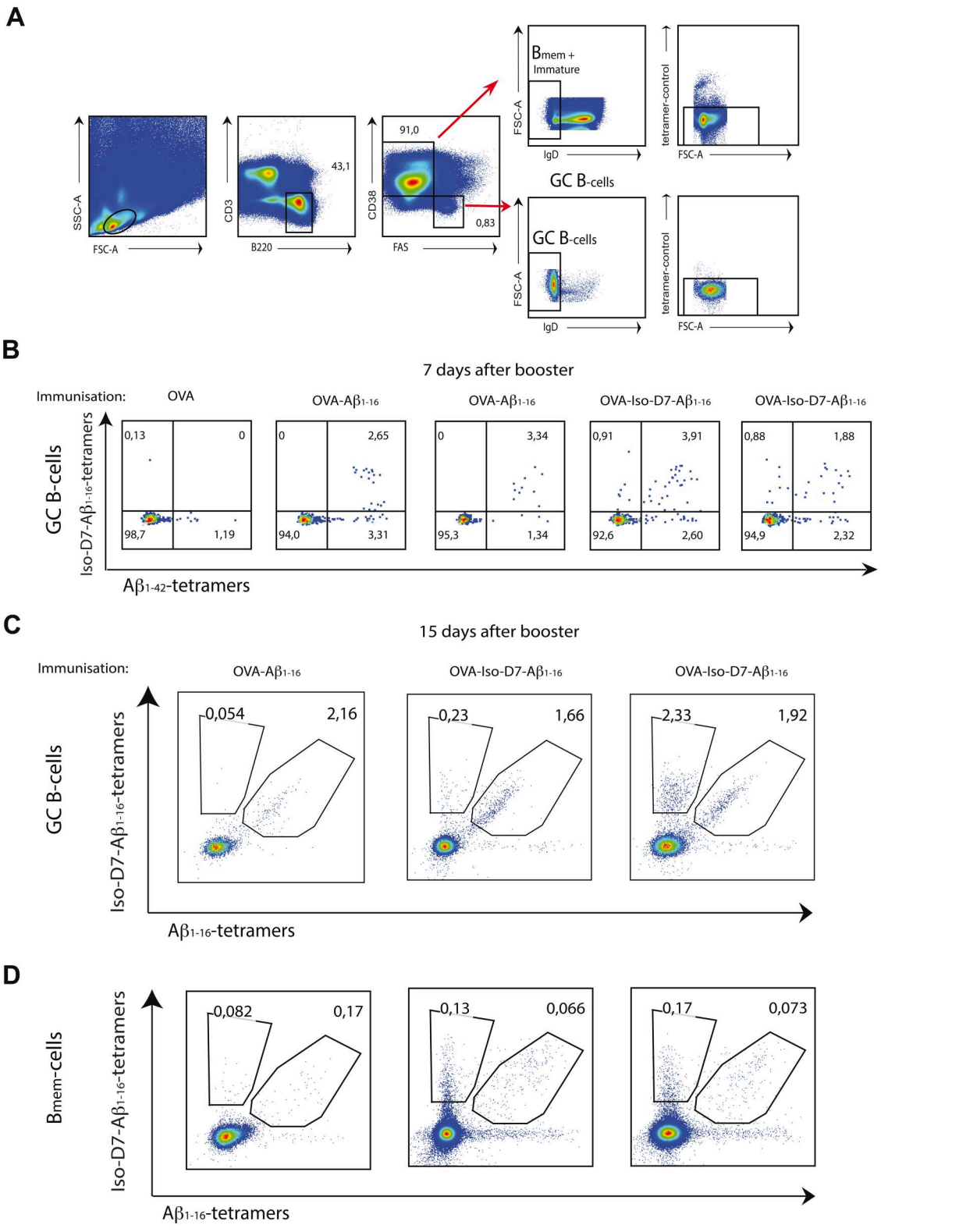

Figure 1 Identification of the Aβ and Iso-D7-Aβ N-terminus-specific B cells in mice. Flow cytometry analysis of the Aβ and Iso-D7-Aβ N-terminus-specific B cells was carried out on the splenocytes of mice immunized and boosted with OVA-$Aβ_{1–16}$, OVA-Iso-D7-$Aβ_{1–16}$ or OVA in Ribi adjuvant interperitoneally at 7 days (**A** and **B**) and 15 days (**C** and **D**) after the boost. (**A**) The gating strategy for GC B cells and class-switched memory + immature B cells. (**B–D**) Analysis of B cells staining with $Aβ_{1–42}$ and Iso-D7-$Aβ_{1–16}$ fluorescent tetramers at day 7 for GC B cells (for **B**), with $Aβ_{1–16}$ and Iso-D7-$Aβ_{1–16}$ fluorescent tetramers at day 15 for GC B cells (for **C**) and class-switched memory B cells (for **D**). B cells with BCRs cross-specific to Aβ and Iso-D7-$Aβ_{1–16}$ accumulate in the upper right corner/diagonal gate for both OVA-$Aβ_{1–16}$ and OVA-Iso-D7-$Aβ_{1–16}$ immunized mice. B cells with BCRs specific to Iso-D7-$Aβ_{1–16}$ and not highly cross-reactive to Aβ accumulate in the upper left corner for the OVA-Iso-D7-$Aβ_{1–16}$ immunized mice only. Data are representative of two independent experiments.

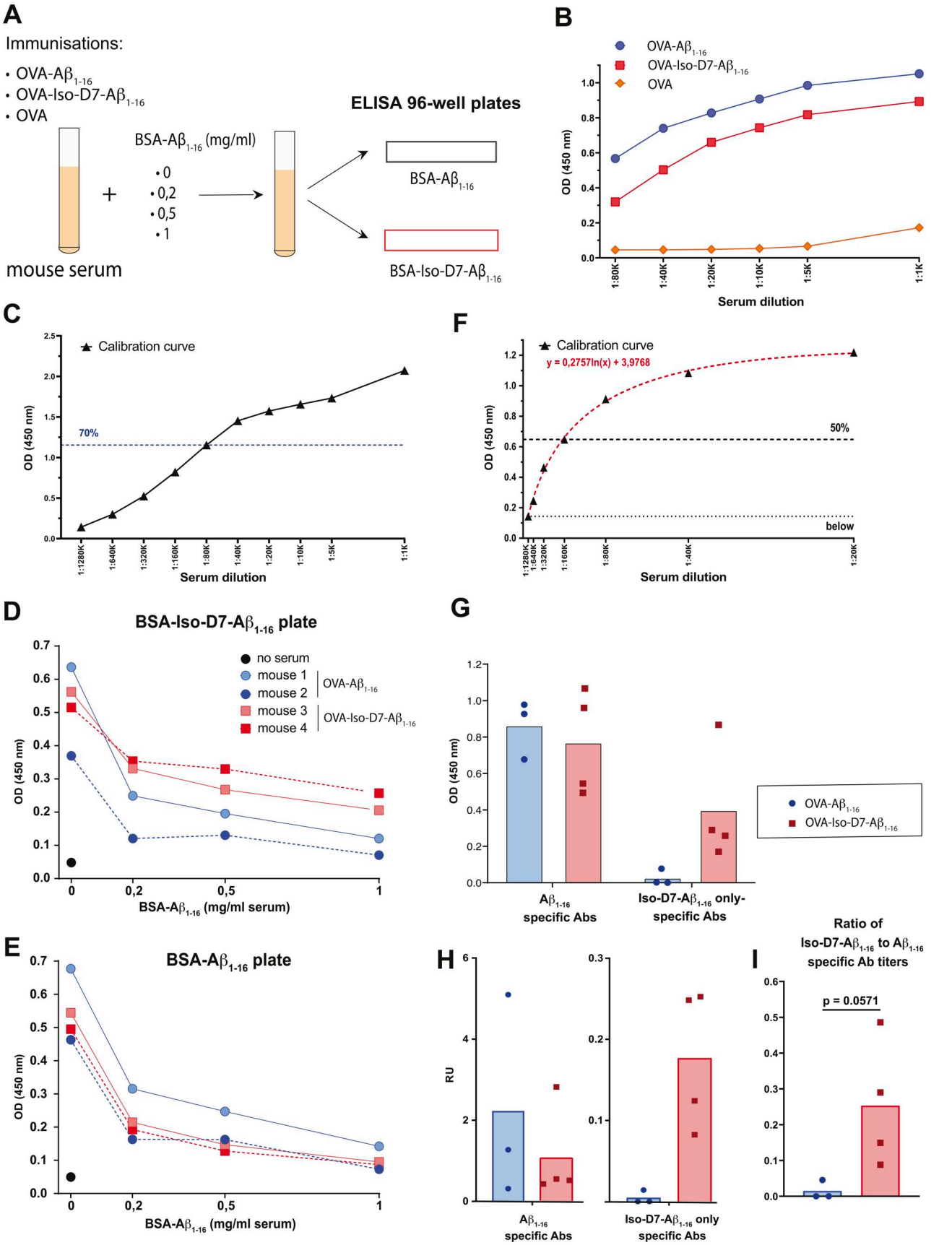

Figure 2 Measurement of the Iso-D7-Aβ N-terminus-specific antibody titers in mice. Competitive ELISA analysis of the Aβ and Iso-D7-Aβ N-terminus-specific antibodies in the serum of mice immunized and boosted with OVA-Aβ$_{1–16}$, OVA-Iso-D7-Aβ$_{1–16}$, or OVA in Ribi adjuvant interperitoneally. Blood was collected at 7 days after the boost. (**A**) The experimental scheme. (**B**) An example of serum dilution analysis of serum from the OVA-Aβ$_{1–16}$, OVA-Iso-D7-Aβ$_{1–16}$, and OVA-immunized mice on the BSA-Aβ$_{1–16}$-covered ELISA plates. (**C**) Serial dilutions of the serum selected as a standard for generating

Iso-D7-A$\beta_{1–16}$ immunized mice ranged from 1:5000 to 1:40 000 (Fig. 2B and C).

7. The blocking buffer was discarded from the plates with immobilized BSA-A$\beta_{1–16}$ or BSA-Iso-D7-A$\beta_{1–16}$ antigens, as described in step 3.
8. Fifty microliter of the serial serum dilutions were added in duplicates to the ELISA plates (both BSA-A$\beta_{1–16}$ covered plates and BSA-Iso-D7-A$\beta_{1–16}$ covered plates) and incubated for 1 h at room temperature or overnight at +4°C.
9. The liquid was drained, and the wells were washed as in steps 3 and 4.
10. Fifty microliters of secondary anti-mouse IgG-bio (1:1000 dilution in PBS) were added to each well and incubated at room temperature for 45 min.
11. The liquid was drained, and the wells were washed as in steps 3 and 4.
12. Fifty microliters of SA-HRP (1:1000 dilution in PBS) were added to each well and incubated at room temperature for 45 min.
13. The liquid was drained, and the wells were washed as in steps 3 and 4.
14. The wells were washed two more times with 200 µl of PBS per well (without 5-min incubation on the shaker).
15. One hundred microliters of TMB were added per well, and incubated until the blue staining of the wells became visible. In our experiment, for the optimal data analysis of serial dilutions, the incubation time was about 1.5 min.
16. One hundred microliters of stop solution were added to each well.
17. For the ELISA analysis, the optical density of the wells was measured at 450-nm wavelength using the automated enzyme immunoassay analyzer.
18. GraphPad Prism software was used for statistical analysis and data visualization.

Important notes for quantitative analysis

- To arrive at a correct range of serial dilutions for a selected group of serum samples and secondary antibody isotype, the initial analysis is usually performed for 1:100 (or 1:50) serum dilution with 5-fold dilution steps (1:100, 1:500, 1:2500, etc.). The desirable range of serial dilutions is then selected based on the range of OD_{450} signals, spanning from the threshold of signal detection through its linear range and to saturation. Two-fold serial dilutions of serum within that range could be selected for quantitative analysis.
- To compare between various samples/ELISA plates, serial dilutions from one selected serum with a prominent A$\beta_{1–16}$- and Iso-D7-A$\beta_{1–16}$-specific antibody response should be used to generate a standard curve for each ELISA plate/experiment (Fig. 2C).
- Each sample should be applied on the ELISA plate and tested in duplicate.
- For negative control, incubation with serum (step 8) should be skipped for two wells on the ELISA plate.
- For quantitative analysis, all wells on the plate should be incubated with TMB for the same duration of time before adding the stop solution.
- RUs of the N-terminus Aβ-specific antibody titers are defined as a standard curve dilution that corresponds to the sample serum OD_{450} signal (in the linear range) divided by the corresponding dilution of the sample serum (Fig. 2F).

Analysis of the Iso-D7-A$\beta_{1–16}$-specific antibodies in serum that are not highly cross-reactive to A$\beta_{1–16}$

To titrate away A$\beta_{1–16}$-specific antibodies, we have incubated mouse serum with various concentrations of soluble BSA-A$\beta_{1–16}$. Serum antibodies that are not efficiently titrated away with soluble BSA-A$\beta_{1–16}$ are defined here as not highly reactive or cross-reactive to A$\beta_{1–16}$. Following the incubation, serum and BSA-A$\beta_{1–16}$ mix was applied onto the ELISA plates covered with BSA-A$\beta_{1–16}$ and BSA-Iso-D7-A$\beta_{1–16}$. Below we describe an optimized semiquantitative approach for estimation of the Iso-D7-A$\beta_{1–16}$-specific antibodies titers when both Iso-D7-A$\beta_{1–16}$-specific and A$\beta_{1–16}$-specific antibodies are present in the serum.

19. First, for each serum sample, identify its serial dilution **D** that corresponds to about 70% of OD_{450}^{max} in the linear range of the BSA-A$\beta_{1–16}$-specific ELISA (Fig. 2C).
20. Serum should then be diluted with blocking buffer to a concentration 8× higher than the serial dilution **D** and incubated with soluble BSA-A$\beta_{1–16}$ at 0, 0.2, 0.5, and 1 mg BSA-A$\beta_{1–16}$ per 1 ml serum for 2 h at room temperature with constant rotation on the orbital shaker at 20 rpm.
21. Following the incubation with various concentrations of soluble BSA-A$\beta_{1–16}$, each sample should be diluted 8-fold with the blocking buffer and applied (50 µl) in duplicate on the BSA-A$\beta_{1–16}$- and BSA-Iso-D7-A$\beta_{1–16}$-covered ELISA plates prepared, as described in steps 1–5, and 7.
22. The standard curve samples should be added to the BSA-A$\beta_{1–16}$-covered ELISA plate at the same time.
23. The ELISA plates should be processed, as described in steps 9–17.
24. The data could be semiquantitatively analyzed as described below.

Semiquantitative analysis of Iso-D7-A$\beta_{1–16}$-specific antibody titers

Serum from the immunized mice may contain antibodies

i) specific to A$\beta_{1–16}$ peptides that do not recognize Iso-D7-A$\beta_{1–16}$.

Figure 2 Continued
calibration curve. For each serum sample, the initial serial dilution for the competition experiment is recommended at an OD_{450} around 70% of the calibration curve linear range. (**D** and **E**) Examples of the competition experiment results with serum from the OVA-A$\beta_{1–16}$ and OVA-Iso-D7-A$\beta_{1–16}$ immunized mice after serum preincubation (or not) with various doses of soluble BSA-A$\beta_{1–16}$ followed by analysis on the BSA-Iso-D7-A$\beta_{1–16}$- (in D) and BSA-A$\beta_{1–16}$- (in E) covered ELISA plates. (**F**) An example of fitting a standard calibration curve in the linear range (in Microsoft Excel). All serum sample values of OD_{450} below 0.14 are excluded from the semiquantitative analysis, as they fall below the linear range of the calibration curve. (**G–I**) Analysis of A$\beta_{1–16}$-specific and Iso-D7-A$\beta_{1–16}$-specific (not highly cross-reactive to A$\beta_{1–16}$) serum antibodies in the OVA-A$\beta_{1–16}$ and OVA-Iso-D7-A$\beta_{1–16}$ immunized mice. $N = 2$ independent experiments. Each point represents one mouse. In (**G**), OD_{450} corresponding to A$\beta_{1–16}$-specific antibody titers is equal to $OD^{A\beta_{1–16}}$ (serum) [see Equation (4)]; OD_{450} corresponding to Iso-D7-A$\beta_{1–16}$-specific antibody titers were calculated using Equations (8) and (9). In (**H**), the OD_{450} values obtained in G were converted into RU using the equations that fit the standard curve in the linear range for each experiment (see **F** for example) and serum dilution. In (**I**), each point represents the ratio of RU for Iso-D7-A$\beta_{1–16}$-specific to A$\beta_{1–16}$-specific antibody (shown in **H**) for each mouse.

ii) cross-specific to $A\beta_{1-16}$ and Iso-D7-$A\beta_{1-16}$ peptides.
iii) specific to Iso-D7-$A\beta_{1-16}$ that do not recognize $A\beta_{1-16}$ peptide.

$$A\beta \quad (1)$$
$$\text{cross} - \text{specific} \quad (2)$$
$$\text{Iso} - \text{D7} - A\beta \quad (3)$$

where Equations (1–3) are the ELISA OD_{450} absorbance signals for antibodies in the serum corresponding to (i), (ii), and (iii), respectively.

$$OD^{A\beta_{1-16}}(\text{serum}) = A\beta + \text{cross} - \text{specific} \quad (4)$$
$$OD^{A\beta_{1-16}}(\text{serum} + \text{sBSA} - A\beta_{1-16}) = (A\beta + \text{cross} - \text{specific}) \text{ x } (1-\gamma) \quad (5)$$
$$OD^{IsoA\beta_{1-16}}(\text{serum}) = \text{cross} - \text{specific} + \text{Iso} - \text{D7} - A\beta \quad (6)$$
$$OD^{IsoA\beta_{1-16}}(\text{serum} + \text{sBSA} - A\beta_{1-16}) = \text{cross} - \text{specific x } (1-\gamma) + \text{Iso} - \text{D7} - A\beta \quad (7)$$

Where $OD^{A\beta_{1-16}}$ * and $OD^{IsoA\beta_{1-16}}$ stand for the ELISA OD_{450} signals obtained with the plates covered with BSA-$A\beta_{1-16}$ and BSA-Iso-D7-$A\beta_{1-16}$ antigens correspondingly.

OD (serum+ sBSA-$A\beta_{1-16}$) or OD (serum) stands for the ELISA OD_{450} signal of the serum preincubated or not with soluble BSA-$A\beta_{1-16}$.

γ is the fraction of ELISA OD_{450} signal corresponding to the $A\beta_{1-16}$-specific and cross-specific antibodies titrated away by pre-incubation of serum with soluble BSA-$A\beta_{1-16}$. An assumption is that soluble BSA-$A\beta_{1-16}$ titrates away $A\beta_{1-16}$-specific and cross-specific antibodies with equivalent efficiency γ.

For semiquantitative analysis, select serum dilutions and soluble BSA-$A\beta_{1-16}$ co-incubations, after which the ELISA OD_{450} values: $OD^{A\beta_{1-16}}(\text{serum})$, $OD^{A\beta_{1-16}}(\text{serum} + \text{sBSA-}A\beta_{1-16})$, $OD^{IsoA\beta_{1-16}}(\text{serum})$, $OD^{IsoA\beta_{1-16}}(\text{serum} + \text{sBSA-}A\beta_{1-16})$ fall within the linear range of the standard curve (e.g. see Fig. 2C–F).

By solving the system of Equations (4–7), the following parameters can be derived:

$$\gamma = 1 - \frac{OD^{A\beta_{1-16}}(\text{serum} + \text{sBSA} - A\beta_{1-16})}{OD^{A\beta_{1-16}}(\text{serum})} \quad (8)$$

$$\text{Iso} - A\beta = \frac{OD^{IsoA\beta_{1-16}}(\textit{serum}) - OD^{IsoA\beta_{1-16}}(\text{serum}) - OD^{IsoA\beta_{1-16}}(\text{serum} + \text{sBSA} - A\beta_{1-16})}{\gamma} \quad (9)$$

The calculated Iso-Aβ values could be converted into the relative antibody titers using an equation that fits the standard curve in a linear range (see Fig. 2F–H). The calculated dilution of the standard sample corresponding to the Iso-Aβ OD_{450} value is then converted into the relative units (RUs) by dividing the calculated dilution by the actual dilution of the analyzed sample serum. $OD^{A\beta}_{1-16}$ values are converted in the RU using the same approach.

Results and discussion

Identification of $A\beta_{1-16}$- and iso-D7-$A\beta_{1-16}$-specific B cells

To induce B-cell immune response to the isomerized and non-isomerized N-terminus domain of Aβ, Balb/c mice were immunized with OVA-$A\beta_{1-16}$ or OVA-Iso-D7-$A\beta_{1-16}$ in Ribi adjuvant interperitoneally. For control, mice were immunized with OVA in Ribi. Booster immunizations were performed 21 days later. Germinal center (GC) B-cell response to foreign antigens usually peaks at 7–15 days after immunization in the antigen-draining secondary lymphoid organs (spleen for the intraperitoneal immunization), while memory B cells that differentiate from GC B cells accumulate over time. Therefore, we have focused on the analysis of splenic $A\beta_{1-16}$- and Iso-D7-$A\beta_{1-16}$-specific GC B cells at 7 and 15 days and memory B cells at 15 days after the booster immunization (Fig. 1).

For identification of the Iso-D7-Aβ- and Aβ-specific B cells, we generated fluorescent tetramers using streptavidin-phycoerythrin (SA-PE) conjugated to $A\beta_{1-42}$-bio or $A\beta_{1-16}$-bio and streptavidin-allophycocyanin (SA-APC) conjugated to Iso-D7-$A\beta_{1-16}$-bio. Splenocytes were labeled with a combination of $A\beta_{1-42}$ and Iso-D7-$A\beta_{1-16}$ (or $A\beta_{1-16}$ and iso-D7-$A\beta_{1-16}$) tetramers mixed with anti-mouse antibodies that enabled identification of the $B220^+CD3^-CD38^-FAS^{hi}IgD^-$ GC B cells and $B220^+CD3^-CD38^+FAS^-IgD^-$ B cells that include class-switched memory B cells and immature B cells. Incorporating the IgM-specific antibodies into the stain would help to further discriminate between class-switched memory B cells (IgM^-IgD^-) and immature B cells (IgM^+IgD^-) in the spleen. Biotin-streptavidin-FITC was included in the tetramers/antibody mix for exclusion of the streptavidin-specific B cells from the analysis (For gating strategy, see Fig. 1A). By use of the tetramers, we expected to identify two types of Aβ N-terminus-specific B cells actively participating in an immune response: (i) double-positive B cells for Aβ- and Iso-D7-$A\beta_{1-16}$-tetramers, which bind via their BCRs to $A\beta_{1-16}$ epitopes that are not perturbed by isomerization of D7 and (ii) only Iso-D7-$A\beta_{1-16}$-tetramer-positive B cells, which preferentially recognize the epitopes on Iso-D7-$A\beta_{1-16}$ that are not present in the non-isomerized $A\beta_{1-16}$.

Flow cytometry analysis of GC B cells from the splenocytes of immunized mice revealed the presence of the tetramer-binding B cells described above (Fig. 1B and C). OVA immunization was not expected to induce B-cell response to Aβ. Consistently, few, if any, Iso-D7-$A\beta_{1-16}$ tetramer binding or double-positive GC B cells were detected in the OVA-immunized mice (Fig. 1B). In Balb/c mice, GC B cells that are identified as tetramer positive may be considered background signal for the assay. In contrast, double-positive GC B cells were found in both OVA-$A\beta_{1-16}$ and OVA-Iso-D7-$A\beta_{1-16}$ immunized mice (Fig. 1B and C), while Iso-D7-$A\beta_{1-16}$ tetramer-only binding GC B cells were present in the OVA-Iso-D7-$A\beta_{1-16}$ immunized mice at both 7 and 15 days after the boost (Fig. 1B and C). Of note, tetramers with $A\beta_{1-16}$ and $A\beta_{1-42}$ peptides could be used interchangeably for the identification of cross-reactive to $A\beta_{1-16}$ B cells (Fig. 1B and C), indicating that N-terminus of full-length $A\beta_{1-42}$ peptides on the tetramers is accessible for binding to BCRs. Analysis of the Aβ tetramer-binding B cells also points to an accumulation of the Aβ N-terminus-specific class-switched memory cells at 15 days after the boost in both OVA-$A\beta_{1-16}$ and OVA-Iso-D7-$A\beta_{1-16}$ immunized mice, with increased frequency of the Iso-D7-$A\beta_{1-16}$ tetramer-binding B cells detected in the OVA-Iso-D7-$A\beta_{1-16}$ immunized mice (Fig. 1D).

Cumulatively, our data suggest that the selected combination of tetramers enables the detection of the $A\beta_{1-16}$-specific B cells. Moreover, it permits simultaneous identification of B cells with BCRs that recognize Iso-D7-$A\beta_{1-16}$ and are not significantly cross-reactive to $A\beta_{1-16}$.

Limitations of the approach

While our method enables the exclusion of streptavidin-specific B cells from the analysis, fluorescent tetramers may also label a few B cells with BCRs specific to fluorophores conjugated to the tetramers. When needed, to ensure BCR specificity to the selected Aβ peptide, two different kinds of tetramers with the same peptides, but different fluorophores, may be utilized for colabeling peptide-specific B cells.

Detection of Iso-D7-Aβ$_{1–16}$-specific antibodies in mouse serum

For the detection of Iso-D7-Aβ$_{1–16}$-specific antibodies that are not highly cross-reactive to Aβ$_{1–16}$, blood was collected from mice immunized with OVA-Iso-D7-Aβ$_{1–16}$, OVA-Aβ$_{1–16}$, and OVA at 7 days after the booster immunization, and mouse serum was prepared as described in "Materials and Methods" section. For analysis of Aβ$_{1–16}$ and Iso-D7-Aβ$_{1–16}$ serum antibodies titers, we performed indirect ELISA with BSA-Aβ$_{1–16}$ and BSA-Iso-D7-Aβ$_{1–16}$ antigens correspondingly as described in "Materials and Methods" section (Fig. 2A).

The initial ELISA analysis revealed the presence of Aβ$_{1–16}$ and Iso-D7-Aβ$_{1–16}$-specific antibodies in the serum of both OVA-Iso-D7-Aβ$_{1–16}$ and OVA-Aβ$_{1–16}$, but not OVA immunized mice (Fig. 2B). However, serum antibodies to Aβ$_{1–16}$ and Iso-D7-Aβ$_{1–16}$ may be cross-reactive if they bind to Aβ$_{1–16}$ epitopes unmodified by the isomerization of D7. To examine whether mouse serum also contained Iso-D7-Aβ$_{1–16}$-specific antibodies that are not highly cross-reactive to Aβ$_{1–16}$, we have utilized a competitive ELISA approach using soluble BSA-Aβ$_{1–16}$ to titrate away Aβ$_{1–16}$-specific antibodies as described in "Materials and Methods" section.

First of all, we titrated serum from all immunized mice, starting with a dilution of 1:5000 and followed by 2-fold dilutions and applied to a plate with BSA-Aβ$_{1–16}$ antigen (Fig. 2B). Based on the results obtained, one serum sample was selected and used as a calibration curve in subsequent experiments (Fig. 2C). Serum was incubated with increasing concentrations of soluble BSA-Aβ$_{1–16}$ as described in "Materials and Methods" section. Importantly, preincubation of serum from the OVA-Aβ$_{1–16}$ immunized mice with BSA-Aβ$_{1–16}$ led to comparable loss in serum antibody binding to both BSA-Aβ$_{1–16}$ and BSA-iso-D7-Aβ$_{1–16}$ covered plates (Fig. 2D and E). However, preincubation of serum from the OVA-Iso-D7-Aβ$_{1–16}$ immunized mice with soluble BSA-Aβ$_{1–16}$ resulted in less significant loss of serum antibody binding to BSA-Iso-D7-Aβ$_{1–16}$ than to BSA-Aβ$_{1–16}$ (Fig. 2D and E). These results suggest the availability of Iso-D7-Aβ$_{1–16}$-specific antibodies that are not highly cross-reactive to Aβ$_{1–16}$ in the OVA-Iso-D7-Aβ$_{1–16}$ but not OVA-Aβ$_{1–16}$ immunized mice.

For semiquantitative analysis of the Iso-D7-Aβ-specific antibodies in the serum, we have developed an approach (described in the "Materials and Methods" section) that enables estimation of the relative titers of Iso-D7-Aβ$_{1–16}$ specific antibodies that are not highly cross-reactive to Aβ$_{1–16}$. When applied to the ELISA analysis of serum from the OVA-Aβ$_{1–16}$ and OVA-Iso-D7-Aβ$_{1–16}$ immunized mice, the above semiquantitative approach suggests that non-highly cross-reactive Iso-D7-Aβ$_{1–16}$-specific antibodies comprise a significant fraction of the Aβ N-terminus-specific antibodies in mice immunized with OVA-Iso-D7-Aβ$_{1–16}$ (Fig. 2F–I).

To summarize, the ELISA method and analysis described above enable semiquantitative detection of the antibodies that are more specific to the unique epitopes on Iso-D7-Aβ$_{1–16}$ than to non-isomerized Aβ$_{1–16}$ even when both types of antibodies are present in serum.

Limitations of the approach

It should be noted that the method developed for the Iso-D7-Aβ-specific antibody detection could be utilized when such antibodies represent a significant fraction of antibodies specific to the N-terminus domain of Aβ in serum. Moreover, the suggested approach is not expected to work for the antibodies specific to the aggregated structures of Aβ- and Iso-D7-Aβ-peptide that hinder accessibility of the Aβ N-terminus.

Conclusions

The methods developed in this study may be useful for in-depth analysis of B cell and antibody responses to Aβ in various mouse models of AD. Moreover, these approaches could be further optimized for the examination of Aβ-specific N-terminus-specific B cells, Iso-D7-Aβ-specific B cells, and antibodies in humans for research, including the analysis of their correlates with the amelioration or aggravation of AD, as well as the development of new diagnostic applications for AD patients. Identification and sorting of the Aβ-specific B cells may also be instrumental for the analysis of their BCR variable regions and the generation of monoclonal Abs for both diagnostic and therapeutic purposes.

Acknowledgements

We thank Alexander Novoselov for technical assistance with the manuscript.

Author contributions

Elizaveta Kolobova (Conceptualization [supporting], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Writing—original draft [supporting]), Irina Petrushanko (Conceptualization [supporting], Data curation [supporting], Methodology [supporting], Writing—review & editing [equal]), Vladimir Mitkevich (Conceptualization [supporting], Funding acquisition [supporting], Project administration [supporting], Supervision [supporting], Writing—review & editing [equal]), Alexander A. Makarov (Conceptualization [supporting], Funding acquisition [lead], Project administration [lead], Resources [lead], Writing—review & editing [supporting]), and Irina L. Grigorova (Conceptualization [lead], Formal analysis [lead], Investigation[lead], Methodology [lead], Project administration [lead], Supervision [lead], Writing—original draft [lead])

Conflict of interest statement. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was funded by the Russian Science Foundation (Grant No. #19-74-30007) and the contract #075-15-2019-1660 from the Ministry of Science and Higher Education of the Russian Federation to the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS.

Data availability

Data are available at doi: 10.5061/dryad.44j0zpcqp.

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Biology Methods and Protocols, 2025, 10, 1–9
https://doi.org/10.1093/biomethods/bpaf001
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