Naturally-Occurring Antibodies Against Bim are Decreased in Alzheimer’s Disease and Attenuate AD-type Pathology in a Mouse Model

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Abstract Increased neuronal apoptosis is an important pathological feature of Alzheimer’s disease (AD). The Bcl-2-interacting mediator of cell death (Bim) mediates amyloid-beta (Aβ)-induced neuronal apoptosis. Naturally-occurring antibodies against Bim (NAbs-Bim) exist in human blood, with their levels and functions unknown in AD. In this study, we found that circulating NAbs-Bim were decreased in AD patients. Plasma levels of NAbs-Bim were negatively associated with brain amyloid burden and positively associated with cognitive functions. Furthermore, NAbs-Bim purified from intravenous immunoglobulin rescued the behavioral deficits and ameliorated Aβ deposition, tau hyperphosphorylation, microgliosis, and neuronal apoptosis in APP/PS1 mice. In vitro investigations demonstrated that NAbs-Bim were neuroprotective against AD through neutralizing Bim-directed neuronal apoptosis and the amyloidogenic processing of amyloid precursor protein. These findings indicate that the decrease of NAbs-Bim might contribute to the pathogenesis of AD and immunotherapies targeting Bim hold promise for the treatment of AD.

Keywords Alzheimer’s disease · Amyloid-beta · Bim · Naturally-occurring antibodies · Neuronal apoptosis

Introduction

Alzheimer’s disease (AD) is a devastating and incurable neurodegenerative disease that affects cognitive function in the older population [1]. Senile plaques comprising amyloid-beta (Aβ) peptide and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein are the major pathological hallmarks of AD. Most of the current therapeutic strategies attempt to target the two lesions, however, no clinical trial has been successful [2], partly because the dramatic neuronal loss is occurring when the interventions are given [3, 4]. These have led to increased attention to searching for alternative therapeutic targets of AD. Neuronal apoptosis is a terminal pathological process that contributes to cognitive impairment [5], thus rescuing neuronal apoptosis may be a potential therapeutic strategy for AD. Currently, several drugs in clinical trials are targeting this process [2].

Apoptosis is regulated by various proteins. Among those are primarily the B cell lymphoma-2 (Bcl-2) regulatory protein family [6]. The Bcl-2-interacting mediator of cell death (Bim) is a pro-apoptotic protein in the Bcl-2 family.
and is widely expressed in human tissues, including the central nervous system [7, 8]. Bim is an essential mediator of Aβ-induced neuronal apoptosis [9]. It has been suggested that the expression of Bim, but not other pro-apoptotic proteins of the Bcl-2 family, is selectively increased in the brain of an AD mouse model [10]. Postmortem studies have also found that Bim is elevated in the AD brain [11]. These findings indicate that Bim may play a pivotal role in the pathogenesis of AD.

A recent study screened out a panel of naturally-occurring antibodies that are altered in AD patients [12], suggesting that humoral autoimmunity may participate in the pathogenesis of AD. Besides, the permeability of the blood-brain barrier (BBB) increases with aging [13], thus facilitating the entry of autoantibodies into the brain to direct pathophysiological effects [14]. Naturally-occurring antibodies against Bim (NAbs-Bim) exist in human blood and may be involved in the pathogenesis of diseases such as malignant pleural effusion [15]. We found in this study that circulating NAbs-Bim were decreased in AD patients. We further investigated the clinical relevance of NAbs-Bim to AD and the functions of these antibodies in regulating AD-type pathologies.

Materials and Methods

Study Population

Patients with cognitive decline (including AD and other types of dementia) were recruited from the Department of Neurology, Daping Hospital, from December 2018 to May 2020. Age- and gender-matched controls with normal cognition were randomly recruited from the Health Examination Center, Daping Hospital. Participants were excluded if they fulfilled one of the following conditions: (1) a family history of dementia; (2) other concurrent neurological diseases that may affect cognition; (3) severe heart, lung, liver, or kidney disease, or any type of tumor; (4) persistent mental illness (such as schizophrenia). The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonisation Guidelines for Good Clinical Practice and was approved by the Institutional Review Board of Daping Hospital. The participants provided written informed consent to participate in this study.

Dementia Diagnosis

All patients provided demographic information and medical histories were collected. The cognitive and functional status was assessed based on a neuropsychological battery. Patients with cognitive decline further received a Pittsburgh compound B (PiB) positron emission tomography (PET) examination. The diagnosis of AD was made in accordance with the 2018 version of the NIA-AA standard [16, 17] with PiB-PET positive. Patients with other types of dementia (such as frontotemporal dementia or Lewy body dementia) were confirmed by cognitive decline with PiB-PET negative. Fasting blood was collected between 07:00 and 09:00 to avoid the potential influence of circadian rhythm. The blood samples were centrifuged at 2000g at 4 °C for 10 min, and the aliquots were then immediately frozen and stored at – 80 °C until use.

Western Blots of Human Plasma

The Bim protein (Sangon Biotech, China) powder was diluted to 200 μg/mL with PBS and SDS loading buffer, 5 μL loaded into each well, and coagulated with sodium lauryl sulfate-polyacrylamide gel electrophoresis for separation. After transfer to a nitrocellulose membrane (Bio-Rad Laboratory, USA), the membrane was blocked with 5% BSA blocking buffer at room temperature for 1 h and then incubated with a mixed human blood sample pool at 4 °C overnight. Three healthy human serum samples were randomly mixed into a sample pool (total capacity 8 mL). Then, the membrane was washed 3 times with PBS containing 0.1% Tween 20 (PBST) and incubated with IRDye 800 CW secondary antibodies (Li-Cor, USA). The membranes were scanned using the Odyssey fluorescent scanner (Li-Cor, USA).

Measurements of Human Plasma NAbs-Bim, Aβ, and Tau Levels

The relative levels of NAbs-Bim in human plasma were evaluated by enzyme-linked immunosorbent assay (ELISA). In short, the synthesized human Bim protein produced by E. coli (Sangon Biotech, China) was dissolved in the coating solution at a concentration of 50 μg/mL. The Bim protein diluent was in the form of a coating-unpacking pair, and 100 μL per well was loaded into a 96-well ELISA plate (Corning, Millipore, Germany) with high binding capacity and incubated at 4 °C overnight. After blocking with 3% BSA at 37 °C for 1 h, the wells were washed 5 times with PBST. Then, human plasma samples diluted at 1:200 in PBS were added to the coated wells (100 μL/well) and incubated overnight at 4 °C. After the same washing step, the wells were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-human IgG, 1:10000) at 37 °C for 30 min. Then, 100 μL of 3,3’,5,5’-tetramethylbenzidine substrate (TMB Super Sensitive,
Sigma, Germany) was added to each well, and the enzymatic reaction was stopped with 100 μL of ELISA stop solution (Sigma, Germany). The absorbance was measured at 450 nm and 620 nm with a SpectraMax luminometer (Molecular Devices, USA), and the relative level of NAbs-Bim was calculated as the difference between the optical density (OD) of the uncoated pair. The plasma levels of Aβ42, Aβ40, and total tau (t-Tau) were measured using the commercially available single-molecule array (SIMOA) Human Neurology 3-Plex A assay kit (Quanterix, UK) on-board the automated SIMOA HD-1 analyzer (Quanterix, UK).

**Purification of NAbs-Bim**

NAbs-Bim were purified from intravenous immunoglobulin (IVIg, Tonrol, China) according to previous protocols [18]. In brief, Bim protein was dissolved in PBS to a final concentration of 0.5 mg/mL. After 50 mg of Pierce NHS-Activated Agarose Dry Resin was placed in an empty spin column (Thermo Scientific Pierce Spin Columns), 750 μL of protein solution was added. The coupling reaction was allowed for 1 h at room temperature under gentle mixing, followed by 30 min reaction time without mixing. The column was packed into a collection tube and centrifuged at 1000×g for 1 min. The column was washed 3 times with 1 mL of coupling buffer. Nonspecific binding sites on the resin were blocked 2 times for 45 min with 1 M Tris, pH 7.4. After 3 washes, the resin (750 μL) was transferred into a 15 mL falcon vial using 5 mL PBS and mixed with 5 mL IVIg. After that, 10 mL IVIg was added to the column and incubated overnight with end-over-end mixing at 4 °C. After 3 washes, bound IgGs were eluted by applying 8 mL of Elution Buffer (Thermo Scientific, USA). The pH of eluted fractions was adjusted to neutral by adding 50 μL of Neutralization Buffer (1 mol/L Tris; pH 9) per 1 mL of the collected eluent. Antibody concentrations in the eluted fractions were determined by the Micro BCA Protein Assay Kit method (Pierce; Perbio, Bonn, Germany). Isolated antibodies were concentrated to a dry powder using a rotovap (Thermo Scientific, USA) and stored at −80 °C for further use.

**Specificity of NAbs-Bim**

Recombinant Bim protein (Sangon Biotech, China), irrelevant protein (Aβ42, GL Biochem, China), and a mixture of albumin (Solarbio, China) and Bim protein were diluted to a concentration of 500 μg/mL with PBS and SDS loading buffer. The diluted samples were loaded 10 μL per well, and coagulated with sodium lauryl sulfate-polyacrylamide gel electrophoresis for separation. After transfer to a nitrocellulose membrane (Bio-Rad Laboratory, USA), the membrane was blocked with 5% BSA blocking buffer at room temperature for 1 h and then incubated with monoclonal anti-Bim antibody (ab32158, Abcam, UK) or purified NAbs-Bim at 4 °C overnight. Then the membrane was washed 3 times with PBST and incubated with IRDye 800 CW secondary antibodies (Li-Cor, USA). The membranes were scanned using the Odyssey fluorescent scanner (Li-Cor, USA).

The protein in the mouse brain homogenates was subjected to SDS-PAGE gel electrophoresis and membrane transfer. Then the membrane was blocked with 5% BSA blocking buffer at room temperature for 1 h and incubated with monoclonal anti-Bim (ab32158, Abcam, UK), purified NAbs-Bim, or irrelevant IgG at 4 °C overnight. Then, the membrane was washed 3 times with PBST and incubated with IRDye 800 CW secondary antibodies (Li-Cor, USA). The membranes were scanned using the Odyssey fluorescent scanner (Li-Cor, USA).

**Administration of NAbs-Bim**

Eight-month-old APPswe/PS1dE9 (APP/PS1) transgenic (Tg) mice on the C57BL/6 background and C57BL/6 wild-type (WT) mice were obtained from the Jackson Laboratory and raised in the animal facility of Daping Hospital. Only female mice were used in this study to exclude the influence of sex on AD pathology in the brain [19]. Lateral ventricle stereotactic injection was performed in Tg mice, which were injected with 5 μL NAbs-Bim (0.563 μg/μL) as the Tg NAbs-Bim group (n = 9), or 5 μL PBS as the Tg control group (n = 10) once a week for 4 weeks. Briefly, the skin of the mouse head was cut along the sagittal line to expose the skull and a burr hole was drilled over the right cerebral hemisphere. Lateral ventricle injection was performed according to the following coordinates referred to bregma: anteroposterior, −2.46 mm; lateral, −3.0 mm; and ventral, 4.2 mm. After injection, the needle was left in place for 5 min before withdrawal. All mouse husbandry procedures were approved by the Third Military Medical University Animal Welfare Committee (Approval number: AMUWEC20191656).

**Behavioral Tests**

Following the previously described procedure [20], Y maze and open field tests were performed on all mice. In the spontaneous alternation test, the mice could enter the three arms freely without repetition for 5 min. The percentage of rotation was calculated as the total number of rotations.
x100%/(total number of arm entries–2). In the new arm exploration test, one arm (defined as the new arm) was blocked, and the mice were allowed to explore the other two arms (the family arm and the familiar arm) for 5 min. After 30 min, the mice were allowed to explore all three arms freely for 5 min. The novel arm entries and percentage were recorded and analyzed. In the open field test, mice were placed in the center of the field area and allowed to move freely for 5 min. We recorded the distance traveled, the ratio of the time spent in the center, the peripheral area, number of rearing and grooming. Performance was tracked using a computer tracking system (ANY-maze, Stoelting, USA). Age- and sex-matched WT mice were used as baseline controls.

Brain Sampling and Processing

Two weeks after the last injection, all mice were killed for biochemical and histological analysis. After anesthetization (0.7% pentobarbital sodium intraperitoneal injection), each mouse was intracardially perfused with 0.1% NaNO2 in 0.9% saline. The right hemisphere was dissected and fixed in 4% paraformaldehyde, while the left hemisphere was snap-frozen and ground into powder in liquid nitrogen and stored at −80°C. The frozen brain was homogenized in liquid nitrogen, and the extracellular protein was extracted with TBS solution and intracellular protein with RIPA solution in sequence according to the method we described previously [21]. This research and all experimental protocols were approved, and the methods followed the guidelines of the Animal Protection Committee of Third Military Medical University (Approval number: AMUWEC20191656).

Histology and Quantification

As previously described, histological staining and analysis were performed [22, 23]. In short, coronal sections were cut at 35 μm on a cryotome. A series of five equidistant sections (~1.3 mm apart) spanning the entire brain were used for histological staining. Aβ plaques were identified with 6E10 (mouse anti-human Aβ antibody, 1:200, 803008, BioLegend, USA). The dense Aβ plaques were stained with Congo red (048K0704V, Sigma-Aldrich, USA). Microglia were visualized with CD68 (1:200, ab213363, Abcam, UK), and astrocytes were visualized with GFAP (1:200, ab68428, Abcam, UK). Tau phosphorylation was detected with anti-pT231 (1:200, 11110, Signalway, USA). NeuN and caspase-3 (1:500, ab13847, Abcam, UK) double staining were used to detect neuronal apoptosis. NeuN and microtubule-associated protein (MAP)-2 (1:500, ab11267, Abcam, UK) double staining was used to detect neuronal loss and synaptic degeneration. NeuN and NAb-Bim double staining was used to detect the location of Bim protein in neurons. Quantitative analysis was performed by another researcher blinded to the group information. Image-Pro Plus 6.0 (National Institutes of Health, USA) was used to quantify the positive staining.

Western Blots of Mouse Brain Homogenates

The protein in mouse brain homogenates was extracted with RIPA lystate and then subjected to SDS-PAGE gel electrophoresis and membrane transfer. The blot was probed with the following antibodies: anti-Aβ 6E10 (803008, BioLegend, USA) that recognizes human Aβ and SAPPβ, anti-Aβ 171610 (751–770, Millipore, Germany) that recognizes human CTF-β, CTF-α, APP, anti-Aβ 22C11 (MAB348, Millipore, Germany) that recognizes human SAPPβ+β, antibodies associated with Aβ production, including anti- BACE-1 (B0681, Sigma, Germany), anti-ADAM10 (14194S, Cell Signaling Technology, USA), anti-PS1 (5643S, Cell Signaling Technology, USA), antibodies associated with Aβ metabolism, including anti-IDE (ab32216, Abcam, UK) and anti-NEP (AB5458, Millipore, Germany), antibodies associated with Aβ transport, including anti-LRP-1 (ab92544, Abcam, UK), anti-RAGE (ab3611, Abcam, UK), anti-phospho-tau antibodies, including anti-pS181 (11107, Signalway, USA), anti-pT231 (11110, Signalway, USA), anti-pS396 (11102, Signalway, USA), anti-total tau (Tau5, MAB361, Millipore, Germany), anti-synaptic related proteins, including anti-PSD-95 (MABN68, Millipore, Germany), anti-PSD-93 (ab151721, Abcam, UK), anti-SYN-1 (MABN894, Millipore, Germany), anti-Snap (MAB331-C, Millipore, Germany), anti-VAMP (SAB2107401, Sigma, Germany), anti-Bim and Bim downstream protein-related antibodies, including anti-Bim (ab32158, Abcam, UK), anti-BAX (ab32503, Abcam, UK), anti-BAK (ab104124, Abcam, UK), anti-FADD (ab124812, Abcam, UK), anti-FLIP (ab8421, Abcam, UK), and antibodies related to β-actin (A1978, Sigma-Aldrich, Germany). The membranes were incubated with IRDye 800 CW secondary antibodies (Li-Cor, USA) and scanned using the Odyssey fluorescent scanner (Li-COR, USA). The band density was normalized to β-actin for analysis.

Cell Lines and Cultures

Human neuroblastoma SH-SY5Y cells stably co-expressing human APP carrying the Swedish mutant gene (APPswe) and wild-type human PSEN1 (PS1wt), were
engineered as previously described [24]. SH/APPswe/PS1wt cells were cultured in Dulbecco’s modified Eagle medium/F12 and supplemented with 10% fetal bovine serum, 0.5% genetin (G418, 40 mg/mL, Invitrogen, USA), and 100 units/mL penicillin/streptomycin (Thermo Scientific, USA). Cells were plated in 6-well plates at 1 × 10^5 cells per well. After overnight incubation, the cells were separately treated with NAbs-Bim at 0.0563, 0.563, or 5.63 μg/mL for 4 h, Bim protein at 0.0563, 0.563, or 5.63 μg/mL for 48 h, 5.63 μg/mL Bim protein plus 5.63 μg/mL NAbs-Bim for 48 h, and 50 mM siRNA against Bim for 4 h. The cell lysates were subjected to Western blot for APP metabolism, Bim, and its downstream proteins.

Mitochondrial Staining

The mitochondria of cells were displayed using the CytoPainter Mitochondrial Staining Kit (ab112145, Abcam, UK). All the steps were performed according to the instructions provided by the manufacturer.

CCK8 Assay

Cell proliferation-toxicity was determined by Cell Counting Kit-8 assay (HY-K0301, MedChemExpress, USA). Cells were seeded and cultured at 3 × 10^4/well in 100 μL medium in 96-well microplates (ExCell Bio, China). After treatment, 10 μL of CCK-8 reagent was added to each well, and then cultured for 2 h. The absorbance was detected at 450 nm using a SpectraMax luminometer (Molecular Devices, USA), and wells without cells were used as blanks. Cell proliferation-toxicity was indicated by absorbance.

Cellular Immunofluorescence

Cells were cultured in 6-well plates on coverslips at 1 × 10^5 cells per well. After overnight incubation, the cells were treated as described above. The medium was aspirated, 2 mL PBS, pH 7.4 was added to each well, and washed three times. After the last PBS wash, 2 mL 2% paraformaldehyde was added to each well, the cells were fixed at room temperature for 10 min, then 2% paraformaldehyde was aspirated and washed three times with PBS. Subsequently, 2 mL 1% Triton was added to each well and allowed to react for 5 min at room temperature. The 1% Triton was removed, washed three times with PBS, 100 μL of 10% goat serum was added to each slide for blocking, and after 30 min at 37 °C, the blocking solution was recovered. Then 100 μL of pre-prepared rabbit-derived anti-Bim antibody (1:200, ab32158, Abcam, UK) was dripped onto each slide. After 2 h incubation at room temperature, the primary antibody was recovered and each well was washed three times. In darkness, rabbit-derived fluorescent secondary antibody Alex Flour 488 Rb (1:1000, A-11008, Invitrogen, USA) was dripped onto each slide and, after incubation for 1 h at room temperature, each well was washed three times. Under the same conditions, DAPI dye-containing aqueous mounting medium (sc-24941, Santa Cruz, USA) was added to the slides. Quantitative analysis was performed by researchers blinded to the sample groups. Positive staining was quantified using Image-Pro Plus 6.0 (National Institutes of Health, USA).

Statistics

The results are presented as the mean ± SEM unless otherwise stated. The normality of the data distribution was examined using the Shapiro-Wilk test and visual inspection of Q-Q plots. Age and plasma Aβ40 and Aβ42 levels were consistent with a normal distribution. Education, Mini-Mental State Examination (MMSE) scores, CDR scores, plasma t-Tau level, PIB-PET Standard Uptake Value Ratio (SUVR), and average OD of NAbs-Bim were not normally distributed. The χ² test was used to compare categorical variables. Statistical comparisons between two groups were made using Student’s t-test. One-way ANOVA or the Kruskal-Wallis test was used to compare the differences among multiple groups, as applicable. Spearman correlation was used to evaluate the correlations. Covariance analysis was used to compare NAbs-Bim levels between groups after adjusting for APOE ε4 carrier status and coexisting disorders. P values < 0.05 (two-sided) were considered significant. All analyses were performed with SPSS version 25.0 (SPSS Inc., USA).

Results

Plasma NAbs-Bim Levels are Reduced in AD Patients

To investigate whether plasma NAbs-Bim levels are altered in AD patients, 55 AD patients, 28 patients with non-AD dementia, and 70 cognitively normal (CN) participants were recruited. There was no significant difference in age, sex, education, and the frequencies of diabetes mellitus, hypertension, and stroke among the three groups (Table 1). The AD group had a higher proportion of APOE ε4 carriers and lower MMSE scores than the other two groups.

The presence of NAbs-Bim in human plasma was verified by Western Blot (Fig. S1A). Through ELISA analyses, we found that the plasma levels of NAbs-Bim in AD patients were significantly lower than in the CN group. AD patients also had slightly lower plasma levels of NAbs-
Bim than participants with non-AD dementia, but the difference was not significant (Fig. 1A). After adjusting for APOE e4 carrier status and co-existing disorders, the difference in plasma levels of NAbs-Bim between the AD and CN group was still significant.

We next investigated the associations of plasma NAbs-Bim levels with cognitive functions as determined by MMSE scores, brain amyloid burden as reflected by SUVR of PiB-PET, and plasma AD biomarkers. Plasma NAbs-Bim level showed a positive correlation with MMSE score (Fig. 1B), and a negative correlation with PiB-PET SUVR in AD patients and all participants with dementia (Fig. 1C). Besides, the plasma NAbs-Bim level was significantly correlated with the plasma Aβ42 level, but not with the Aβ40 or t-Tau level (Fig. 1D–F). The above findings indicated that NAbs-Bim are negatively associated with the severity of AD, suggesting a possible protective role of these antibodies in AD.

NAbs-Bim Rescue Behavioral Deficits in APP/PS1 Mice

NAbs-Bim interventions were applied to investigate the effects of NAbs-Bim on the behavioral performance of APP/PS1 mice. Five micrograms (at 1 μg/μL) of NAbs-Bim purified from IVIg or 5 μL PBS were injected into the right lateral ventricle of mice from 8 months of age. The mice were subjected to behavioral analyses at 9 months old when extensive Aβ pathology and significant behavioral deficits were evident. APP/PS1 mice treated with NAbs-Bim had more entries into the novel arm in the Y-maze test, reflecting a better spatial recognition memory. However, these mice did not show a significantly different performance in the spontaneous exploration test in the Y-maze (Fig. 2A). In open-field tests, NAbs-Bim-treated mice traveled a longer distance, had higher numbers of rearing and grooming, and had an increased ratio of time spent in the central zone to that in the peripheral zone than control APP/PS1 mice, indicating enhanced locomotor activity and reduced anxiety-like behavior (Fig. 2B, C). The above findings indicated that NAbs-Bim treatment protects against behavioral deficits in APP/PS1 mice.

NAbs-Bim Attenuate Aβ Pathology in APP/PS1 Mice

NAbs-Bim purified from IVIg bound to neuronal membranes, as reflected by the positive co-staining of NAbs-Bim, NeuN, and DAPI (Fig. S1B). Meanwhile, recombinant Bim was immunoblotted with NAbs-Bim, with

| Characteristics | AD (n = 55) | Non-AD Dementia (n = 28) | CN (n = 70) | P-value |
|-----------------|------------|--------------------------|------------|---------|
| Age, mean ± SEM, years | 66.50 ± 1.489 | 62.56 ± 2.093 | 62.77 ± 1.228 | 0.1133 |
| Female, n (%) | 29(52.73) | 20(71.43)* | 30(42.85) | 0.0381 |
| Education, median (IQR), years | 9(6–15) | 9(6.75–14.25) | 12(9–12) | 0.0755 |
| APOE e4 carriers, n (%) | 25(45.45)* | 4(14.28)† | 8(11.42)† | <0.0001 |
| MMSE scores, median (IQR) | 15(10–19)* | 22(18–28.5)*† | 27(25–29)† | <0.0001 |
| CDR scores, median (IQR) | 2(1–2)* | 1(0.5–1)* | 0† | <0.0001 |

Table 1 Characteristics of participants.

Measurement data are represented by n (%); numeration data are represented by mean ± SEM or median (IQR) according to the normality. P-value, one-way ANOVA, two-tailed t-test, or Kruskal Wallis test as appropriate. APOE e4: apolipoprotein E e4 allele, MMSE: Mini-mental State Examination, CDR: Clinical Dementia Rating, NAbs-Bim: naturally-occurring antibodies to Bim, OD: Optical density, SUVR: Standard Uptake Value Ratio. *P < 0.05 vs CN; †P < 0.05 vs AD.
monoclonal Bim antibody as the positive control. NAbs-Bim recognized recombinant Bim and a mixture of recombinant Bim and albumin, but did not recognize irrelevant protein (Aβ) (Fig. S1C). In addition, we tested the ability of NAbs-Bim to bind to endogenous Bim protein in brain homogenates, with monoclonal Bim antibody as a positive control.

Fig. 1 Plasma NAbs-Bim levels are reduced in AD patients and associated with cerebral amyloidosis. A Comparisons of the plasma NAbs-Bim levels among the AD, non-AD dementia, and CN groups (mean ± SEM, *P < 0.05, Kruskal-Wallis test). B Correlation of plasma NAbs-Bim levels with MMSE scores in the total cohort. C Correlation of plasma NAbs-Bim levels with PiB-PET SUVR in AD and non-AD dementia groups. D–F Correlation of plasma NAbs-Bim levels with plasma levels of Aβ42, Aβ40, and t-Tau in AD and non-AD dementia groups (n = 55 in AD group, n = 28 in non-AD dementia group, n = 70 in CN group; Spearman correlation analysis; shadows indicate 95% confidence interval). OD: optical density, CN: cognitively normal, MMSE: Mini-mental State Examination, SUVR: Standard Uptake Value Ratio.

Fig. 2 NAbs-Bim rescue behavioral deficits in APP/PS1 mice. A Percentage of novel arm entries and alternation in novel arm in Y-maze test. B Representative tracings in the open-field test. C Distance traveled, number of rearing and grooming, and ratio of time spent in central and peripheral areas in the open-field test. Mean ± SEM; n = 11 in Tg Control group, n = 9 for in NAbs-Bim group, n = 8 in WT group; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA. Tg: APP/PS1 transgenic mice, WT: wild-type mice.
positive control and irrelevant IgG as a negative control. We found that NAbs-Bim were also able to bind to endogenous Bim (Fig. S1D). These findings suggested that NAbs-Bim specifically bind to Bim protein.

To explore whether NAbs-Bim treatment could reduce Aβ deposition in the brain of APP/PS1 mice, we applied Aβ immunohistochemical (IHC) staining (6E10) for total Aβ plaques and Congo red staining for compact Aβ plaques. There was no significant difference in area fractions and the total plaque density of total Aβ plaques either in the neocortex or in the hippocampus between NAbs-Bim and the control group. Compared with APP/PS1

Fig. 3 NAbs-Bim attenuate Aβ pathologies in APP/PS1 mice. A, B Immunostaining and quantification of 6E10 and Congo red in the neocortex (Neoco.) and hippocampus (Hippo.) of 9-month controls and NAbs-Bim treated mice (scale bars, 500 μm; insets, representative morphology at a higher magnification). C Western blots and quantification for APP and its metabolites in brain homogenates. D Western blots and quantitative analysis for APP cleavage enzymes. E Western blots and quantitative analysis for Aβ-degrading enzymes and Aβ transporters. Mean ± SEM; n = 10 in Tg Control group, n = 9 in Tg NAbs-Bim group; *P <0.05, **P <0.01, two-tailed t-test. Neoco.: Neocortex, Hippo.: Hippocampus.
controls, mice treated with NAbs-Bim displayed a significant reduction in area fractions of compact plaque in the neocortex, but not in the hippocampus. No significant difference was found in the compact plaque density either in the neocortex or in the hippocampus (Fig. 3A, B). Taken together, our findings showed that NAbs-Bim attenuate compact Aβ plaques in the cortex of APP/PS1 mice.

We next measured amyloid precursor protein (APP) and its metabolites in brain homogenates from APP/PS1 mice. We found that Aβ, soluble APP (sAPPα+β), and C-terminal fragments (CTF)-β levels were significantly lower in the brain of mice treated with NAbs-Bim than in controls, while full-length APP (APPfl), sAPPα, and CTF-α did not differ (Fig. 3C). These results indicated that NAbs-Bim inhibits Aβ production via decreasing the amyloidogenic processing of APP.

We determined the levels of secretases responsible for APP processing. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is the major β-secretase that catalyzes β-cleavage and promotes the amyloidogenic processing of APP [25]. The NAbs-Bim treatment group had significantly lower levels of BACE1 in brain homogenates than APP/PS1 controls. However, no significant differences were found in the levels of Disintegrin and Metalloproteinase 10 or presenilin 1 (PS1, which is the catalytic subunit of γ secretase) (Fig. 3D). These results implied that the decrease in amyloidogenic processing of APP might be due to a reduction of BACE1. We also tested Aβ-degrading enzymes, including insulin-degrading...
enzyme (IDE) and neprilysin (NEP), and Aβ transporters, including low-density lipoprotein receptor-related protein-1 (LRP1) and receptor for advanced glycation end products (RAGE). RAGE was found to be lower in NAbs-Bim treated mice than in controls, while no differences were found in the IDE, NEP, and LRP1 levels (Fig. 3E), suggesting that NAbs-Bim reduces the receptor-mediated influx of Aβ through the BBB.

In addition, to confirm that NAbs-Bim inhibits the Bim-mediated apoptosis pathway in mice, we evaluated the abundance of Bim and its downstream protein in brain homogenates. The NAbs-Bim treated group had significantly lower levels of extracellular Bim, total Bim, BAX, and BAK levels than APP/PS1 controls (Fig. S2A).

**Bim Upregulates Aβ Production by Promoting BACE-1 Expression**

To explore the underlying mechanism by which NAbs-Bim attenuates Aβ pathology in the APP/PS1 mouse brain, we first investigated the effect of Bim on Aβ in vitro. Bim has been suggested to be a pro-apoptotic protein that acts mainly in intracellular compartments, which might not be accessible to NAbs-Bim. Furthermore, no evidence is now available regarding the role of Bim in APP metabolism. Therefore, to reveal the mechanisms of the protective effects of NAbs-Bim, we first investigated the pathological effects of endogenous Bim. To determine the concentration of NAbs-Bim and exogenous Bim protein with the best effect, we performed a preliminary experiment with gradient concentration treatment. As the dose increased, NAbs-Bim showed increased mitochondria-positive area fractions while the exogenous Bim showed decreased fractions (Fig. S2B, C). Therefore, we selected 100× (i.e., 5.63 μg/mL) of NAbs-Bim and exogenous Bim. SH-SY5Y-APP695 cells were treated with Bim, Bim plus NAbs-Bim, or PBS. In mitochondrial staining, the Bim-treated group displayed a significantly lower positive area fraction than the other two groups, which was consistent with the results of the CCK8 assay (Fig. 4A, B), indicating that extracellular Bim protein promotes the neuronal apoptosis pathway, which is antagonized by NAbs-Bim. Furthermore, the Bim-treated group showed higher levels of Aβ, CTF-β, sAPPα+β, and BACE-1 than the other groups, and this was attenuated by NAbs-Bim (Fig. 4C, D). These results indicated that extracellular Bim protein enhances Aβ generation by promoting the amyloidogenic processing of APP, and this is antagonized by NAbs-Bim.

**NAbs-Bim Antagonize Bim-induced Neuronal Apoptosis and Amyloidogenic Processing of APP in vitro**

To further investigate the effect of NAbs-Bim on endogenous extracellular Bim activity, we performed IF co-staining for Bim and DAPI. The group treated with siRNA-Bim, which inhibited Bim expression, was selected as a positive control. We found that the Bim-positive area fraction of the NAbs-Bim-treated group was significantly lower than the control group and higher than the siRNA-Bim-treated group (Fig. 5A). Furthermore, the NAbs-Bim group had a significantly higher mitochondria-positive area fraction than the control group but did not differ from the siRNA group (Fig. 5B). Similarly, the CCK8 assay showed that the cell activity in the NAbs-Bim-treated group was also significantly higher than that in the control group, but lower than that in the siRNA-Bim-treated group (Fig. 5C). These findings indicated that NAbs-Bim inhibits the activity of Bim and increases neuronal survival.

We next measured APP metabolites and APP cleavage enzymes in cell protein extracts. The NAbs-Bim group displayed lower levels of Aβ, sAPPα+β, CTF-β, and BACE-1 than controls. Compared with the siRNA group, the NAbs-Bim group had higher levels of sAPPα+β. No significant differences were found in other APP metabolites and APP cleavage enzymes among groups (Fig. 5D). The levels of Aβ-degrading enzymes (IDE and NEP) and Aβ transport receptors across the BBB (LRP-1 and RAGE) were also measured. The NAbs-Bim group and the siRNA group had significantly lower levels of RAGE than the control group, while there were no differences in other proteins among groups (Fig. 5E). In addition, we measured Bim and its downstream proteins, BAX and BAK, and found that the amount of Bim, BAX, and BAK was significantly lower in the NAbs-Bim- and siRNA-treated groups than in controls (Fig. 5F). This indicated that NAbs-Bim have siRNA-like effects, which inhibit the pro-
apoptotic pathway of Bim. These results were consistent with the in vivo experiments and supported the conclusion that NAbs-Bim promotes the survival of neurons by reducing Aβ production via decreasing the Bim-induced amyloidogenic processing of APP.

NAbs-Bim Promote Neuronal Survival and Increase Synaptic Density in APP/PS1 Mice

In vitro studies demonstrated that NAbs-Bim can attenuate neuronal apoptosis, and further, we investigated the effect of NAbs-Bim on neuronal apoptosis and synapses in the APP/PS1 mouse brain. The NAbs-Bim-treated mice displayed increased NeuN and Map-2 positive area fractions and a decreased activated caspase-3 positive area fraction in the hippocampus (Fig. 6A–D). The synapse-related proteins PSD93, PSD95, Snap, SYN1, and VAMP1 were also measured. There was a significant difference in PSD95 between NAbs-Bim-treated mice and controls (Fig. 6E). Taken together, these findings suggested that NAbs-Bim protects against neuronal apoptosis and synaptic degeneration in APP/PS1 mice.

NAbs-Bim Attenuate Neuroinflammation and Tau Hyperphosphorylation in APP/PS1 Mice

We then investigated whether NAbs-Bim affects other AD-type pathologies. Activated microglia (CD68+) was significantly decreased after NAbs-Bim treatment both in the neocortex and in the hippocampus (Fig. 7A). NAbs-Bim treatment mildly reduced the activation of astrocytes (GFAP+), but no significant differences were found either in the neocortex or in the hippocampus (Fig. 7B). NAbs-Bim significantly reduced the area fraction of pT231-positive neurons in the hippocampus of NAbs-Bim-treated mice compared to controls (Fig. 7C). Meanwhile, the levels
of total tau (Tau5) and phosphorylated tau at multiple epitopes, including pS396 and pT231, were reduced in the NAbs-Bim-treated group (Fig. 7D).

Collectively, the above findings suggested that extracellular Bim simultaneously promotes neuronal apoptosis and the amyloidogenic processing of APP. NAbs-Bim might act by antagonizing these pathological effects of extracellular Bim, thus exerting neuroprotective effects in AD.

**Discussion**

Over the past decades, the amyloid cascade hypothesis has gained traction as one of the major contributors to AD pathogenesis [26]. Cerebral Aβ accumulation is regarded as the initiator of AD and has been selected as one of the primary therapeutic targets in clinical trials, represented by anti-Aβ immunotherapies [27]. However, these clinical trials have not yet succeeded [28]. Therefore, therapeutic strategies targeting other pathological processes of AD are
emerging, including those targeting tau hyperphosphorylation, neuroinflammation, and oxidative stress [2]. Neuronal apoptosis is a key pathogenic pathway in AD [29], and it remains to be seen whether interventions targeting this pathway are disease-modifying. Bim plays a pivotal role in the transduction of pro-apoptotic signals upon the stimulation of pro-apoptotic factors such as Aβ [30]. Bim is upregulated in the AD brain [11], and inhibition of the Bim signaling pathway ameliorates AD-type pathology and rescues cognitive deficits [31], suggesting that Bim is an important pathogenic agent and a potential therapeutic target for AD.

Previous studies have identified a panel of autoantibodies associated with AD, such as autoantibodies against Aβ [32, 33]. Postmortem studies have found that brain-reactive autoantibodies are prevalent in human blood [34]. These findings suggest a potential role of humoral immunity in the pathogenesis of AD. NAbs-Bim have been suggested to exist in human blood, with their pathophysiological role unknown [11]. We found that the plasma levels of NAbs-Bim were decreased in AD patients and were negatively associated with the severity of AD, raising the possibility that NAbs-Bim exerts protective effects against AD. Therefore, we further investigated how NAbs-Bim might work in the AD brain. We believe that the most direct effect of NAbs-Bim is to antagonize the pathological effects of Bim via antibody-antigen interactions. Indeed, Bim levels were significantly reduced after treatment with NAbs-Bim both in vivo and in vitro. Bim directly binds and activates the pro-apoptotic effectors BAX and BAK, further activating the caspase cascade and inducing neuronal apoptosis [35]. NAbs-Bim significantly reduced the amounts of BAX and BAK, and finally rescued neurons from apoptosis. However, it has been suggested that Bim acts mainly in cellular compartments that might not be accessible to antibodies. We found in this study that Bim also exists in the extracellular space and extracellular Bim was neutralized by NAbs-Bim. Furthermore, extracellular Bim was also toxic to neurons, and its neurotoxicity was antagonized by NAbs-Bim. NAbs-Bim significantly reduced both total and extracellular Bim, thus ameliorating its pro-apoptotic effects.

We found here that extraneous Bim protein significantly promoted the expression of BACE1 and the production of Aβ in vitro. This finding is consistent with a previous study which found that BACE1 expression is increased during apoptosis [36]. Furthermore, a previous study has suggested that Bim interacts with Aβ and promotes the formation of Aβ protofibrils [37]. Therefore, Aβ overproduction and neuronal apoptosis may form a vicious cycle during the development of AD. We found that NAbs-Bim significantly reduced Bim-induced BACE1 upregulation and Aβ overproduction. These findings suggest another protective role of NAbs-Bim against AD through antagonizing the pro-amyloidogenic effects of Bim.

This study was conducted in APP/PS1 mice aged 8 months, at which stage amyloidosis has been initiated and a considerable amount of Aβ has been deposited. After 4 doses of intraventricular treatment, NAbs-Bim improved the behavioral deficits and reduced the amyloid burden of APP/PS1 mice. As described above, NAbs-Bim may help control brain amyloidosis by inhibiting the amyloidogenic process. Intraneuronal NFTs caused by tau hyperphosphorylation is another major pathological hallmark of AD [38, 39]. In this study, NAbs-Bim were found to reduce NFT formation and suppress tau hyperphosphorylation at several sites. Furthermore, other pathologies subsequent to Aβ or tau, including overactivation of glia cells, down-regulation of synaptic proteins, and dendritic damage were also improved after NAbs-Bim treatment. NAbs-Bim may act on Aβ and suppress its toxicity of triggering tau hyperphosphorylation and subsequent pathological changes. These findings imply that immunotherapies targeting the apoptotic process may hold promise for the treatment of AD.

**Limitations**

There are some limitations of this study. First, the clinical relevance of NAbs-Bim was investigated in a cross-sectional study, thus we could not address whether the decrease of NAbs-Bim would contribute to the disease progression from a longitudinal perspective. Second, we did not have a WT mouse set with treatment by NAbs-Bim to assess its effects on non-AD pathological states. Third, in the morphological analysis, Aβ pathology was ameliorated in the neocortex but not the hippocampus, but we did not perform biochemical analyses of different brain regions. Besides, results on tau phosphorylation may be confounded by anesthesia. Finally, we investigated the therapeutic effects of NAbs-Bim through ventricular administration at a much higher dose than its physical concentration. Further studies using peripheral administration of NAbs-Bim are needed to reach a more solid conclusion about the clinical potential of this antibody.

**Conclusions**

In this study, we reported that the plasma levels of NAbs-Bim were decreased in AD patients. These levels were negatively correlated with the severity of AD. NAbs-Bim rescued behavioral deficits and alleviated AD-type pathologies in an AD mouse model. Based on the neuroprotective effects of NAbs-Bim against AD, we supposed that the
decreased levels of NAbs-Bim in AD patients might contribute to the disease progression. Furthermore, this study reveals a potential therapeutic strategy by targeting neuronal apoptosis.

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Conflict of interest The authors declare no conflict of interest.

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