Supplementary information

Naturally occurring antibodies against Bim are decreased in Alzheimer's disease and attenuate AD-type pathologies in a mouse model

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Supplementary 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. Subjects were excluded if they fulfil one of the following conditions: (1) Family history of dementia; (2) Concurrent neurological disease; (3) Severe heart, lung, liver, or kidney disease or any type of tumor; (4) Persistent mental illness (such as schizophrenia).

Dementia diagnosis

All patients received demographic information and medical history collection. The cognitive and functional status was assessed based on a neuropsychological battery. Patients with cognitive decline further received Pittsburgh compound B (PiB) positron emission tomography (PET) examination. The diagnosis of AD was made in accordance with the 2011 version of the NIA-AA standard (1) with PiB-PET positive. Patients with other types of dementia (such as frontotemporal dementia, Lewy body dementia, etc.) were confirmed by cognitive decline with PiB-PET negative. Fasting blood was collected between 07:00 and 09:00 to avoid the potential circadian rhythm influence. 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

Dilute the Bim protein (Sangon Biotech, China) powder to 200 μg/mL with PBS

and SDS loading buffer, 5 μL loaded into per well, and coagulate with sodium lauryl sulfate-polyacrylamide gel electrophoresis for separation. After transferring it 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. Mix random 3 healthy human serum samples 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.

Measurements of human plasma NAbs-Bim, Aβ, and tau levels

The relative levels of Naturally occurring Antibodies to Bim (NAbs-Bim) in human plasma were evaluated by enzyme-linked immunosorbent assay (ELISA). In short, the synthesized human Bim protein (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 at 4°C overnight. Blocked with 3% BSA at 37°C for 1 h, and then wash the wells with PBS containing 0.1% Tween 20 (PBST) 5 times. 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, add 100 μL of 3,3',5,5'-tetramethylbenzidine substrate (TMB Super Sensitive, Sigma, Germany)

to each well, and stop the enzymatic reaction 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. Plasma levels of Aβ42, Aβ40, 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 of 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 (2). In brief, Bim protein was dissolved in PBS to a final concentration of 0.5 mg/mL. After 50mg of Pierce NHS-Activated Agarose Dry Resin was placed to an empty spin column (Thermo Scientific Pierce Spin Columns), 750 µl of protein solutions were added. The coupling reaction was performed for 1 h at RT 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. Thereafter,10 mL IVIG was added to the column and incubated overnight with end-over-end mixing at 4℃. 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 M Tris;

pH 9) per 1 mL of 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 and stored at -80℃ for further use using a rotovap (Thermo Scientific).

Administrations of NAbs-Bim

8-month-old APPswe/PS1dE9 (APP/PS1) transgenic mice on C57BL/6 background and C57BL/6 wild-type (WT) mice were obtained from Jackson Laboratory and raised in the animal facility of Daping Hospital. Only female mice were used in this study to exclude the influence of gender on AD pathologies in the brain (3). Lateral ventricle stereotactic injection was performed in Tg mice. Tg mice 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 at the right cerebral hemisphere of the skull. Lateral ventricle injection was performed according to the following coordinates referred to the bregma point: anteroposterior, -2.46mm; lateral, -3.0mm; and ventral, 4.2mm. After the injection, the needle was left in place for 5 min before the withdrawal. All mouse husbandry procedures were approved by the Third Military Medical University Animal Welfare Committee.

Behavioral tests

Following the previously described procedure (4), Y maze and open field tests were performed on all mice. In the spontaneous alternate test, the mice were allowed to enter the three arms freely without repetition for 5 min. The percentage of rotation was

calculated as the total number of rotations×100%/ (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 entry 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. Recorded the distance traveled, the ratio of the time spent in the center, and the peripheral area, numbers of rearing, and grooming. Track performance through 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 injection, every mouse was killed humanly with an excess of 6% chloral hydrate (6 mL/kg). The blood collected from the eyes was centrifuged at 3000g at 4 °C for 10 min, and the aliquots were immediately frozen and stored at -80°C for subsequent analysis. All mice were perfused with 0.1% $NaNO_2$ saline, and the brains were sampled and weighed. The right hemisphere of each mouse was fixed in 4% paraformaldehyde for histological analysis, while the left hemisphere was frozen and ground into powder in liquid nitrogen and stored at -80°C. Homogenize the frozen brain in liquid nitrogen, and extract the protein with TBS and RIPA solution in sequence according to the method we described before for future analysis (5). This research and all experimental protocols were approved, and the methods were following the guidelines of the Animal Protection Committee Third Military Medical University.

ELISA assays

According to the instructions provided by the manufacturer, the levels of Aβ40 and Aβ42 in plasma and brain extracts were elevated by ELISA kits (Invitrogen, USA).

Histology and quantification

Histological staining and analysis were performed as previously described (6, 7). In short, the coronal section of the brain was cut into a thickness of 35 μm with a cryosectioning microtome. A series of five equidistant tissue sections (approximately 1.3 mm apart) spanning the entire brain were used for histological staining. Aβ plaques were observed 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 (1:500, ab104225, Abcam, UK) and caspase-3 (1:500, ab13847, Abcam, UK) double staining were used to detect neuronal cell apoptosis. NeuN and microtubule-associated protein (MAP)-2 (1:500, ab11267, Abcam, UK) double staining was used to detect the neuronal loss and synaptic degeneration. NeuN and Bim (1:500, ab32158, Abcam, UK) 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. Use Image-Pro Plus 6.0 (National Institutes of Health, USA) to quantify the positive staining.

Western blots of mice brain homogenates

The protein in mouse brain homogenate was extracted with RIPA lysate and then

subjected to SDS-PAGE gel electrophoresis and membrane transferation. The blot was probed with the following antibodies: anti-Aβ antibody 6E10 (803008, BioLegend, USA) that recognizes human Aβ and SAPPα, anti-Aβ antibody 171610 (751-770, Millipore, Germany) that recognizes human CTF-β, CTF-α, APP, Anti-Aβ antibody 22c11 (MAB348, Millipore, Germany) that recognizes human SAPPα/β, antibodies related to Aβ production, including anti-BACE-1 (B0681, Sigma, Germany), anti-ADAM10 (14194S, Cell Signaling Technology, USA), anti-PS1 (5643S, Cell Signaling Technology, USA), antibodies related to Aβ metabolism, including anti-IDE (ab32216, Abcam, UK), anti-NEP (AB5458, Millipore, Germany), antibodies related to Aβ transportation, 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. The band density was normalized to β-actin for analysis.

Cell line and cell culture

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 (8). SH/APPswe/PS1wtcells were cultured in Dulbecco's modified Eagle medium/F12 medium and supplemented with 10% fetal bovine serum, 1% fetal bovine serum geneticin (G418, 40 mg/mL, Invitrogen, USA), and 100 units /mL Penicillin/Streptomycin (Thermo Scientific, USA). Cells were plated in 6-well plates at a density of 1×10^5 cells per well. After overnight incubation, the cells were separately treated with Bim-antibody at dosages of 0.0563,0.563,5.63 μg/mL for 4 h, 50 mM SiRNA against Bim for 4 h, Bim protein at dosages of 0.0563,0.563,5.63 μg/mL for 48 h, and 5.63 μg/mL Bim protein plus with 5.63 μg/mL Bim-antibody for 48 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 CytoPainter Mitochondrial Staining Kit (ab112145, Abcam, UK). All the steps were performed according to the instructions provided by the manufacturer.

Cellular immunofluorescence

Cells were cultured in 6-well plates with carry sheet glasses at a density of 1×10^5 cells per well**.** After overnight incubation, the cells were given interventions as described above. Aspirate the medium, add 2 mL PBS, pH 7.4 to each well, and wash three times, after removal of last PBS wash, add 2 mL 2% paraformaldehyde to each well, fix the cells at room temperature for 10 min, then aspirate 2% paraformaldehyde and wash three times with PBS. Subsequently, add 2 mL 1% Triton to each well and let them react for 5 min at room temperature, remove 1% Triton and wash three times with

PBS, add 100 μL of 10% goat serum to each cell slide for blocking, after 30 min at 37℃, the blocking solution was recovered, then each cell slide was dripped with 100 μL of pre-prepared rabbit-derived anti-Bim antibody (1:200, ab32158, Abcam, UK). After 2 h incubation at room temperature, the primary antibody was recovered, each well was washed three times afterwards, then under dark conditions, each cell slide was dripped with rabbit-derived fluorescent secondary antibody Alex Flour 488 Rb (1:1000, A-11008, Invitrogen, USA), after incubating for 1 h at room temperature, wash each well three times. At the same conditions described before, add DAPI dye-containing aqueous mounting medium (sc-24941, Santa Cruz, USA) Onto the glass slides, invert the cell climbing slides in the 6-well plate on the glass slides. Quantitative analysis was performed by researchers who do not know the information of the sample groups. Quantify positive staining using Image-Pro Plus 6.0

Statistics

The results were presented as the mean ± SEM unless otherwise stated. Statistical comparisons between two groups were made using Student's t-test. One-way ANOVA or Kruskal-Wallis test were used to compare multiple groups, as applicable. Due to MMSE, SUVR, t-tau, and Average OD (NAbs-Bim) didn't conform to the normal distribution, Spearman correlation was used to evaluate the correlations between them. P values less than 0.05 (two-sided) were considered significant. All analyses were performed with SPSS version 25.0 (SPSS Inc., USA) and GraphPad Prism version 8.0 (GraphPad Software, USA).

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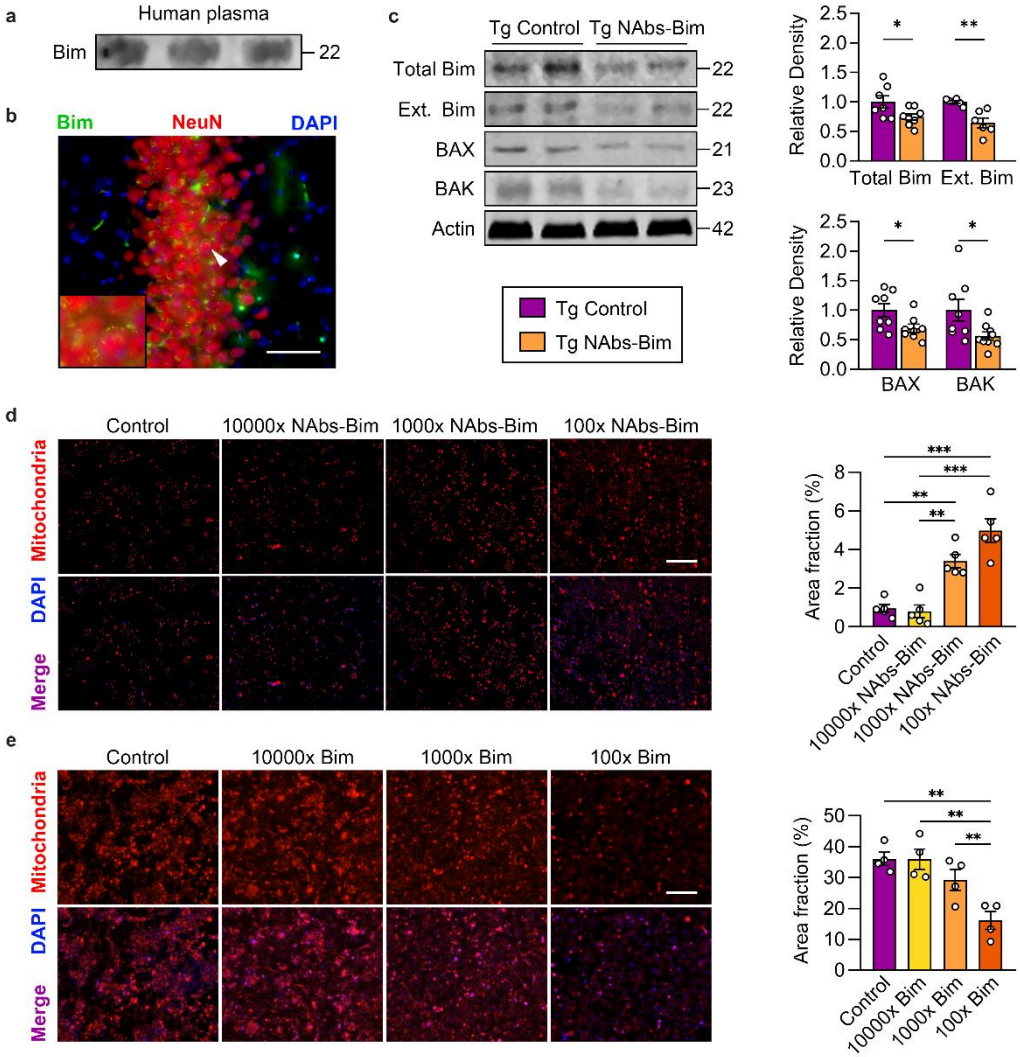
Supplementary Table 1. Characteristics of participants

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 ε4 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
Coexisting disorders, n (%)				
Diabetes	7(12.73)	3(10.71)	8(11.43)	0.7350
Hypertension	12(21.82)	7(25.00)	19(27.14)	0.9139
Dyslipidaemia	11(20.00) *	6(21.43) *	0(0) †	<0.0001
Coronary artery disease	12(21.82) *	4(14.29)	2(2.857) †	0.0005
Stroke history	4(7.273)	0(0)	1(1.429)	0.0560
Plasma NAbs-Bim OD, median (IQR)	0.2902(0.2396-0.3513) *	0.3631(0.2354-0.4308)	0.3822(0.2512-0.4978) †	0.0395
PiB-PET SUVR, median (IQR)	1.98(1.79-2.25)	1.0975(1.0545-1.2040) †	NA	<0.0001
Plasma Aβ42, mean±SEM, pg/mL	8.679±0.5557	11.55±1.230 †	NA	0.0165
Plasma Aβ40, mean±SEM, pg/mL	213.7±11.96	222.1±24.91	NA	0.7339
Plasma t-tau, median (IQR), pg/mL	4.733(3.424-6.084)	4.993(3.688-7.928)	NA	0.2400

Measurement data is represented by n (%); numeration data is 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 ε4: apolipoprotein E ε4 allele, MMSE: Mini-mental State

248 Examination, CDR: Clinical Dementia Rating, NAbs-Bim: Natural occurring Antibodies to Bim, OD: Optical density, SUVR: Standard Uptake

249 Value Ratio. * indicates P<0.05 in comparison with CN. † indicates P<0.05 in comparison with AD.



Supplementary Fig 1. NAbs-Bim exist in human blood and help control brain Bim burden. a Western blot for NAbs-Bim in human plasma. **b** Immunofluorescence co-staining of Bim, NeuN, and DAPI in the CA1 region of the hippocampus. Scale bars 10 μm. **c** Western blots and quantification for Total Bim, Ext. Bim, and its downstream protein in brain homogenates, including BAX, BAK. **d** Representative images and quantification of mitochondria and neurons stained with mitochondrial staining solution and DAPI immunofluorescence in control (PBS), 10000x Ab-Bim (0.0563 μg/mL), 1000x Ab-Bim (0.563 μg/mL), and 100x Ab-Bim (5.63 μg/mL) treated SH-SY5Y-APP695 cells. Scale bars 100 μm. **e** Representative images and quantification of

mitochondria and neurons stained with mitochondrial staining solution and DAPI immunofluorescence in control (PBS), 10000x Bim (0.0563 μg/mL), 1000x Bim (0.563 μg/mL), and 100x Bim (5.63 μg/mL) treated SH-SY5Y-APP695 cells. Scale bars 100 μm. Two-tailed t-test, one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. Ext. denotes extracellular. Bars express mean ± SEM.