The spleen physiologically clears Aβ, and splenectomy aggravates Alzheimer-type pathogenesis

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Abstract

Background: A previous study demonstrated that nearly 40%-60% of brain Aβ flows out into the peripheral system for clearance. However, where and how circulating Aβ is cleared in the periphery remains unclear. The spleen acts as a blood filter and an immune organ. The aim of the present study was to investigate the role of the spleen in the clearance of Aβ in the periphery.

Methods: We investigated the physiological clearance of Aβ by the spleen and established a mouse model of AD and spleen excision by removing the spleens of APP/PS1 mice to investigate the effect of splenectomy on AD mice.

Results: We found that Aβ levels in the splenic artery were higher than those in the splenic vein, suggesting that circulating Aβ is cleared when blood flows through the spleen. Next, we found that splenic monocytes/macrophages could take up Aβ directly in vivo and in vitro. Splenectomy aggravated cognitive impairment, brain Aβ burden, and AD-related pathologies in AD mice.

Conclusion: Our study reveals for the first time that the spleen exerts a physiological function of clearing circulating Aβ in the periphery. Our study also suggests that splenectomy, which is a routine treatment for splenic rupture and hypersplenism, might accelerate the development of AD.

Introduction

Alzheimer's disease (AD) is the most common type of dementia and is characterized by progressive memory loss and cognitive deficits. Currently, there are no available disease-modifying treatments to halt or slow the progression of AD [1]. Extracellular senile plaques composed of amyloid-β (Aβ) are a main hallmark of AD. Aβ overproduction and impaired Aβ clearance have been suggested to play causative or pivotal roles in AD pathogenesis [2, 3]. However, only 1% of all AD cases are familial AD cases caused by overproduction of Aβ due to mutations in the amyloid precursor protein (APP) and PS1/2 genes. Dysfunction of Aβ clearance is believed to be the main cause of Aβ accumulation in sporadic AD, which accounts for 99% of all AD cases [3]. Thus, more attention should be paid to targeting Aβ clearance for the treatment of AD.

It is traditionally believed that Aβ is mainly cleared in the brain, such as through phagocytosis by microglia and subsequent autophagic elimination [4]. However, convincing evidence has indicated that the peripheral system plays a role in the clearance of Aβ [5–7]. Our and others' studies suggest that approximately 40–60% of brain-derived Aβ is transported to the peripheral system for clearance [7, 8]. However, the specific peripheral organs and tissues involved in Aβ clearance are currently unclear.

Similar in the structure to a large lymph node, the spleen acts primarily as a blood filter and exerts immunological functions. It removes old red blood cells and holds a reserve of blood. The spleen is composed of a variety of immune cells, with 7–8% of all cells being monocytes/macrophages. Monocytes/macrophages are an important part of the innate immune system and function as the first

line of defence in the host through various effector functions [9]. The capacity of blood monocytes/macrophages to clear Aβ has been confirmed [10]. Moreover, their ability to clear circulating Aβ is significantly decreased in patients with AD and during the course of ageing [11]. Whether the spleen has the physiological capacity to clear Aβ and its pathophysiological relevance to AD remain largely unknown.

In the present study, we investigated the physiological clearance of Aβ by the spleen and established a mouse model of AD and spleen excision by removing the spleens of APP/PS1 mice. We found that splenic monocytes/macrophages could take up Aβ directly in vivo and in vitro and that the removal of the spleen increased the brain Aβ burden in AD mice. In addition, we found that spleen excision decreased the number of monocytes/macrophages and resulted in higher levels of Aβ in the blood.

Methods And Materials

Animals

APP/PS1 transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J (wild-type, WT) mice were provided by the Animal Centre of Daping Hospital affiliated with the Third Military Medical University. All experimental procedures were approved by the Laboratory Animal Welfare and Ethics Committee of Daping Hospital. A total of 6 3-month-old female rabbits were used to investigate differences in Aβ levels between the splenic artery and splenic vein. The rabbits were anaesthetized with 5 ml/kg 25% urethane. Five millilitres of blood were collected from the splenic artery and from the splenic vein.

Splenectomy

Four-month-old AD mice underwent splenectomy. In brief, the mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg), and the skin was disinfected with iodophor three times. An approximately 8-cm-long incision was made in the centre of the abdomen. After exposing the spleen, the upper pole of the spleen and blood vessels were gently ligated with sutures, and the spleen was removed. If there was no bleeding at the vascular ligation site, the abdominal cavity was sutured layer by layer. After disinfecting the skin with iodophor, the mice were placed in a warm place for thermal resuscitation. In the sham operation, the abdominal cavity was opened but splenectomy was not performed, and the abdominal cavity was directly sutured.

Behavioural tests

Five months after spleen excision, all experimental mice were subjected to the Y-maze and open-field tests to investigate cognitive function according to our previous study [5, 12]. In brief, the open-field test was performed in a white opaque Plexiglas arena (45 cm × 45 cm × 40 cm). The experimental mice were placed in the empty arena and allowed to explore freely for 5 min. The path length and speed were recorded for analysis of spontaneous activity. The Y maze consisted of three identical arms connected in the centre at a 120 degrees angle. The Y maze test consisted of two parts. In the first part, spontaneous

alternations were analysed. Briefly, an experimental mouse was placed in a randomly selected arm and allowed to explore the apparatus freely. The sequence in which the mouse entered the three arms was recorded, and spontaneous alternations were analysed. In the other part, the novel arm exploration test, one arm (defined as the novel arm) was closed, and a mouse was placed in the maze for 5 min for the training trial. After training, the mice were returned to their home cages until the retrieval trial, during which the mice were allowed to explore all three arms freely for 5 min. The time spent in and percentage of entries into the novel arm were analysed. Performance was tracked with a computer tracking system (ANY-maze, Stoelting, Wood Dale, IL, USA).

Brain sampling

After the behavioural tests, all experimental mice were sacrificed, and brain and blood samples were collected according to our previous methods [13]. Briefly, blood was drawn from the eyes and centrifuged at 1800xg for 10 min. After intracardial perfusion with 0.1% $NaNO_2$ in normal saline, the right brain hemisphere was dissected and fixed with 4% paraformaldehyde, and coronal sections were cut with a frozen slicer at a thickness of 30 μm. The left brain hemisphere was snap-frozen in liquid nitrogen, ground into a powder, divided into three vials, weighed, and stored at -80°C for biochemical analysis.

Immunohistochemistry and Immunofluorescence

In vivo Aβ uptake by splenic monocytes/macrophages

100 μL FITC-Aβ (20 μg/ml) was injected into the tail vein of AD mice, and the spleen was sampled 1 hour after injecting. 10 μm spleen sections were stained with F4/80 antibody (1:200, Biolegend). Double immunofluorescence staining for FITC-Aβ and F4/80 was defined as the positive uptake.

Measurement of Aβ plaques and cerebral amyloid angiopathy (CAA)

Five sections spanning the entire brain were stained with a 6E10 antibody (1:1000, Biolegend) or Congo red (Sigma) to detect Aβ plaques in the brain (the 6E10 antibody was used to stain compact and diffuse Aβ; Congo red was used to stain for compact Aβ). CAA was manually assessed in Congo red-stained hippocampal sections under a microscope [14].

Investigation of Tau pathology, neuroinflammation and neurodegeneration

We conducted pT231 staining with an anti-pT231-tau antibody (1:400, Signalway antibody) to detect phosphorylated tau. Astrocytes were stained with an anti-glial fibrillary acidic protein (GFAP) antibody (1:500, Abcam). Ionized calcium binding adaptor molecule 1 (Iba1) staining and CD68 staining were used to detect total and activated microglia, respectively (Iba1: 1:1000, Wako; CD68: 1:200, Abcam). Neurodegeneration was indicated by neuronal loss, neurite degeneration and apoptosis of neurons. Neuronal loss and neurite degeneration in the CA1 region of the hippocampus were detected by double

immunofluorescence staining for NeuN and microtubule-associated protein (Map)-2 (NeuN: 1:500, Abcam; Map2: 1:2000, Abcam). Apoptosis of neurons in the CA3 region of the hippocampus was assessed by double immunofluorescence staining for NeuN and caspase-3 (NeuN: 1:500, Abcam; Caspase3: 1:500, Abcam).

All stained sections were photographed with a Zeiss microscope, and staining was quantified with ImageJ software. Histological staining was performed as described in our previous study [5, 13].

Western blotting

Brain samples were homogenized in ice-cold RIPA lysis buffer. Protein samples were loaded on a 4–20% SDS-polyacrylamide gel and then transferred onto nitrocellulose (NC) membranes. After blocking with 5% fat-free milk, the NC membranes were incubated with the following primary antibodies overnight at 4°C: anti-APP C-terminal (1:1000, Biolegend); anti-6E10 (1:400, Biolegend); anti-RAGE (1:1000, Millipore); anti-LRP-1 (1:1000, Abcam); anti-pS396-tau (1:1000, Abcam); anti-pT231-tau (1:1000, Signalway); anti-Tau5 (1:1000, Millipore); anti-Synapsin-1 (1:1000, Abcam); anti-PSD95 (1:1000, Millipore); and anti-β-actin (1:2000, Origene). The membranes were incubated with the corresponding IRDye 800 CW-conjugated secondary antibodies and scanned using an Odyssey fluorescent scanner. Relative band intensities were normalized to the band intensity of the internal reference protein for analysis.

ELISA

Proteins were extracted from brain tissues in Tris buffer solution (TBS), 2% sodium dodecyl sulfonate (SDS) and 70% formic acid (FA) to measure soluble and insoluble Aβ levels. The levels of human Aβ42 and Aβ40 in the TBS, SDS and FA fractions were measured using ELISA kits (Invitrogen, USA). The levels of inflammatory factors in brain homogenates, including TNF-α, IL-1β and IL-6, were measured with ELISA kits (Raybiotech, USA).

Flow cytometry

Blood was drawn 5 months after spleen excision and anticoagulated with heparin. Flow cytometry analysis of blood immune cells, including T cells, B cells, NK cells, DC cells and monocytes/macrophages, was performed as previously described [15]. In short, red blood cells were lysed (FACS lysing solution, Biolegend) for 9 min at room temperature, and other cells were stained with a BV786-CD3 antibody, a PE-CD19 antibody, a PE-CY7-NK1.1 antibody, a FITC-1A/1E antibody, an APC-CD11b antibody, a BV421-CD11c antibody, AF-700-GR-1 and 7-AAD. The cell suspensions were washed twice with PBS containing 1% foetal bovine serum (FBS). All cells were analysed by flow cytometry.

To test the uptake of Aβ, splenic cells were incubated with CD11b microbeads and passed through a magnetic activated cell sorting column. $CD11b^+$ cells were resuspended in RPMI medium with 10% FBS at a concentration of 10^6 cells/ml. FITC-labelled Aβ was added to the cell culture at a concentration of 0.2 μg/ml for 1 hour of incubation at 37°C in a 5% CO2 incubator. Adherent and suspended cells were collected and incubated with TruStainFcX™ PLUS (Biolegend) for Fc receptor blocking. Then, the cells

were stained with allophycocyanin (APC)-conjugated anti-mouse CD115. Following incubation, the cells were washed with PBS and subjected to flow cytometry (Beckman, USA) after appropriate compensation. The uptake of Aβ was determined by assessing the number of cells that were positive for both FITC and APC.

Statistical analysis

All data are presented as the means ± SEMs. All analyses were conducted with SPSS 22.0 software (SPSS, Chicago, IL, USA). Two-tailed independent t-test, paired t-test and the Mann-Whitney U test were used as appropriate. One-way ANOVA following the LSD test was used to compare multiple groups. $P < 0.05$ was considered statistically significant.

Results

Physiological clearance of circulating Aβ by the spleen

We found that splenic monocytes/macrophages phagocytosed FITC-conjugated Aβ in vitro and directly took up Aβ after FITC-conjugated Aβ was injected into the tail vein (Fig. 1A-C). We also compared the circulating Aβ levels in the rabbit splenic artery and vein; interestingly, the blood levels of Aβ42 and Aβ40 in the rabbit splenic vein were **11.7%** and **15.2%** lower, respectively, than those in the rabbit splenic artery **(**Fig. 1D). In addition, we investigated the blood Aβ level after spleen excision. We found that blood Aβ40 levels, but not blood Aβ42 levels were markedly higher in mice that underwent splenectomy than those in the control group (Fig. 1E). Taken together, our data suggested that monocytes/macrophages in the spleen physiologically clear circulating Aβ.

Spleen excision exacerbated cognitive deficits in AD mice

In the Y maze test, the percentage of entries into the novel arm and the ratio of time spent in the novel arm were not different between the sham group and mice that underwent splenectomy (Fig. 2A, B). Compared with sham mice, mice that underwent splenectomy seemed to show worse memory, as indicated by the percentage of spontaneous alterations, although the difference did not reach statistics significance (**$P = 0.08$**, Fig. 2C). In the open-field test, mice that underwent splenectomy travelled a longer distance than sham mice (**$P < 0.05$**, Fig. 2D).

Changes in the proportions of peripheral blood immune cells in AD mice after spleen excision

We investigated the proportions of peripheral blood immune cells in AD mice after splenectomy. Our data showed that the proportion of T cells in the AD sham control group was lower than that in WT mice (**$P < 0.05$**, Fig. 3), but there were no differences between the AD sham control and AD splenectomy groups (**$P > 0.05$**, Fig. 3). Additionally, the proportion of monocytes was higher in AD sham control mice than in WT mice and lower in AD splenectomy mice than WT mice (**$P < 0.05$**, Fig. 3). Compared with those in WT

mice, the proportions of other cells, including B cells, NK cells, DC cells and granulocytes, in the AD sham group and AD splenectomy group were not different (**P > 0.05**, Fig. 3).

Spleen excision aggravates the Aβ burden in the brains of AD mice

We performed Aβ 6E10 staining to detect total Aβ plaques and Congo red staining to detect compact Aβ plaques. Compared with sham control mice, mice that underwent splenectomy displayed a larger percentage area of both 6E10-positive and Congo red-positive plaques in the neocortex and hippocampus (**P < 0.01 for the neocortex; P < 0.05 for the hippocampus;** Fig. 4A, B). Mice that underwent splenectomy exhibited more Aβ deposition on vessel walls in the brain, which indicates the development of CAA, than AD sham control mice (**P < 0.05**, Fig. 4C).

Consistently, mice that underwent splenectomy had higher levels of total Aβ, Aβ42 and Aβ40 in the TBS, SDS and FA fractions of brain homogenates than AD sham controls (**P < 0.05**, Fig. 4D). To reveal the potential mechanism underlying the increased Aβ burden in the brain, we investigated Aβ production and the levels of Aβ transport-related proteins. There were no differences in the levels of APP itself or its metabolites, including CTFβ, CTFα, sAPPα, BACE1, ADAM10 and PS1, between mice that underwent splenectomy and sham control mice (**P > 0.05**, Fig. 4E), but the Aβ monomer level in mice that underwent splenectomy was higher than that in sham control mice (**P < 0.05**, Fig. 4E, F**)**. In addition, there were no differences in the levels of Aβ transport- and degradation-related proteins, including IDE, NEP, LRP1 and RAGE, between mice that underwent splenectomy and AD sham control mice (**P > 0.05**, Fig. 4G**)**. Taken together, these data suggest that the increased Aβ burden was not attributed to changes in Aβ production and clearance in the brain.

Spleen excision aggravates AD-type pathologies in AD mice

Compared with sham control mice, mice that underwent splenectomy exhibited more astrocytes in the neocortex, total microglia in both the neocortex and hippocampus, and activated microglia in the neocortex (**P < 0.05**, Fig. 5A, B**)**, and increased levels of proinflammatory factors, including TNF-α, IL-1β and IL-6 (**P < 0.05**, Fig. 5G).

There were more phospho-tau (pT231)-positive neurons in both the neocortex and hippocampus in the mice that underwent splenectomy than in sham mice (**P < 0.05**, Fig. 5C). The extent of tau phosphorylation at multiple epitopes, including Thr181 (pT181), Thr231 (pT231) and pS396, was increased in mice that underwent splenectomy compared with sham mice, whereas no differences in the levels of total tau (defined as tau5) were seen between the two groups (**P < 0.05**, Fig. 5D).

Compared with sham mice, mice that underwent splenectomy displayed less staining for markers of viable neurons (NeuN) and dendrites (Map-2) and more staining for an apoptosis-related protein (activated caspase-3) in the hippocampus (**P < 0.05**, Fig. 5E). The levels of synapse-related proteins, including PSD95, SYN and VAMP1, but not SNAP25, were reduced in the brains of mice that underwent splenectomy compared to those of sham mice (**P < 0.05**, Fig. 5F).

Taken together, our findings suggest that splenectomy worsens neuroinflammation, tau hyperphosphorylation and neurodegeneration in the brains of AD mice.

Discussion

In the present study, we found that the spleen physiologically clears circulating Aβ via phagocytosis of Aβ by local macrophages/monocytes in the spleen. Removal of the spleen aggravates cognitive deficits and AD-type pathologies in the brains of AD mice.

Previous studies have suggested that the peripheral system physiologically clears circulating Aβ derived from the brain. Where and how circulating Aβ is cleared in the periphery remains unclear [1, 6, 7]. In the present study, the Aβ level in the splenic artery was higher than that in the splenic vein, indicating that Aβ was cleared when it entered the spleen. The spleen is primarily considered a blood filter and an immune organ and is composed of a variety of immune cells, with monocytes/macrophages accounting for 7–8% of all cells. Monocytes and macrophages have been suggested to be able to clear circulating Aβ [10, 11]. In our study, macrophages/monocytes, but not other cells in the spleen, could take up Aβ in vitro and in vivo. These findings indicate that the spleen physiologically clears Aβ from the blood.

Whether elimination of splenic Aβ clearance can increase brain Aβ burden and exacerbate AD-related pathogenesis remains unclear. In our study, we removed the spleens of AD mice at 4 months of age, at which point no obvious Aβ plaques are present in the brain, and investigated brain Aβ burden 5 months after spleen excision. We found that Aβ levels in the blood of AD mice that underwent splenectomy were higher than those in the blood of control AD mice, indicating that peripheral clearance of Aβ decreased after spleen excision. Our and others' studies suggest that 40%-60% of Aβ in the brain is cleared via effusion into the peripheral blood [16–18]. In the present study, we found that spleen excision increased the brain Aβ burden by 13.1%, as measured by ELISA, implying that the spleen may clear approximately 13% of the Aβ produced in the brain. These findings suggest that the spleen exerts an important function in clearing brain-derived Aβ.

Splenectomy is a routine treatment for splenic rupture and hypersplenism. It has been reported that traumatic splenectomy increases the risk of various immune-related diseases, such as infection, tumours, type 2 diabetes, and liver abscess [19, 20] and induces a 1.9-3.4-fold increase in risk of hospitalization for pneumonia, meningitis, and septicemia [21]. Dysfunction of the immune system is involved in the development of AD [22]. In the present study, removal of the spleen increased Aβ levels in both the blood and brain and aggravated cognitive deficits in AD mice. It has been reported that the ability of monocytes to clear circulating Aβ is decreased during the course of ageing and reduced further in patients with AD [11], suggesting that deficits in the clearance of circulating Aβ may be involved in the pathogenesis of AD. To our knowledge, the impacts of spleen excision on cognition in humans have been rarely studied thus far. We speculate that excision of the spleen might impair Aβ clearance and increase the risk of the development of AD.

Conclusions

Our study revealed the physiological capacity of the spleen to clear circulating Aβ. The pathophysiological impacts of spleen disorders on brain health and whether the spleen is a potential therapeutic target for AD need to be investigated in the future.

Abbreviations

AD: Alzheimer's disease; Aβ: β-amyloid peptide;

APP: amyloid precursor protein; WT: wild-type;

GFAP: glial fibrillary acidic protein; Iba1: Ionized calcium binding adaptor molecule 1; Map: microtubule-associated protein; NC: nitrocellulose; TBS: Tris buffer solution SDS: sodium dodecyl sulfonate; FA: formic acid; APC: allophycocyanin; pT231: phosphorylation Thr231; pT181: phosphorylation Thr 181

Declarations

Ethics approval and consent to participate

All animal procedures were approved by Ethics Committee of Daping hospital affiliated to Third Military Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.J.W. and X.L.B. conceived and designed the project, Z.Y.Y., D.W.C., C.Y.H., G.H.Z., and C.R.T conducted animal and *in vitro* experiments, Z.Y.Y., D.W.C., and C.R.T analysed data, Z.Y.Y. and Y.J.W. wrote the

manuscript.

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Not applicable

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Figures

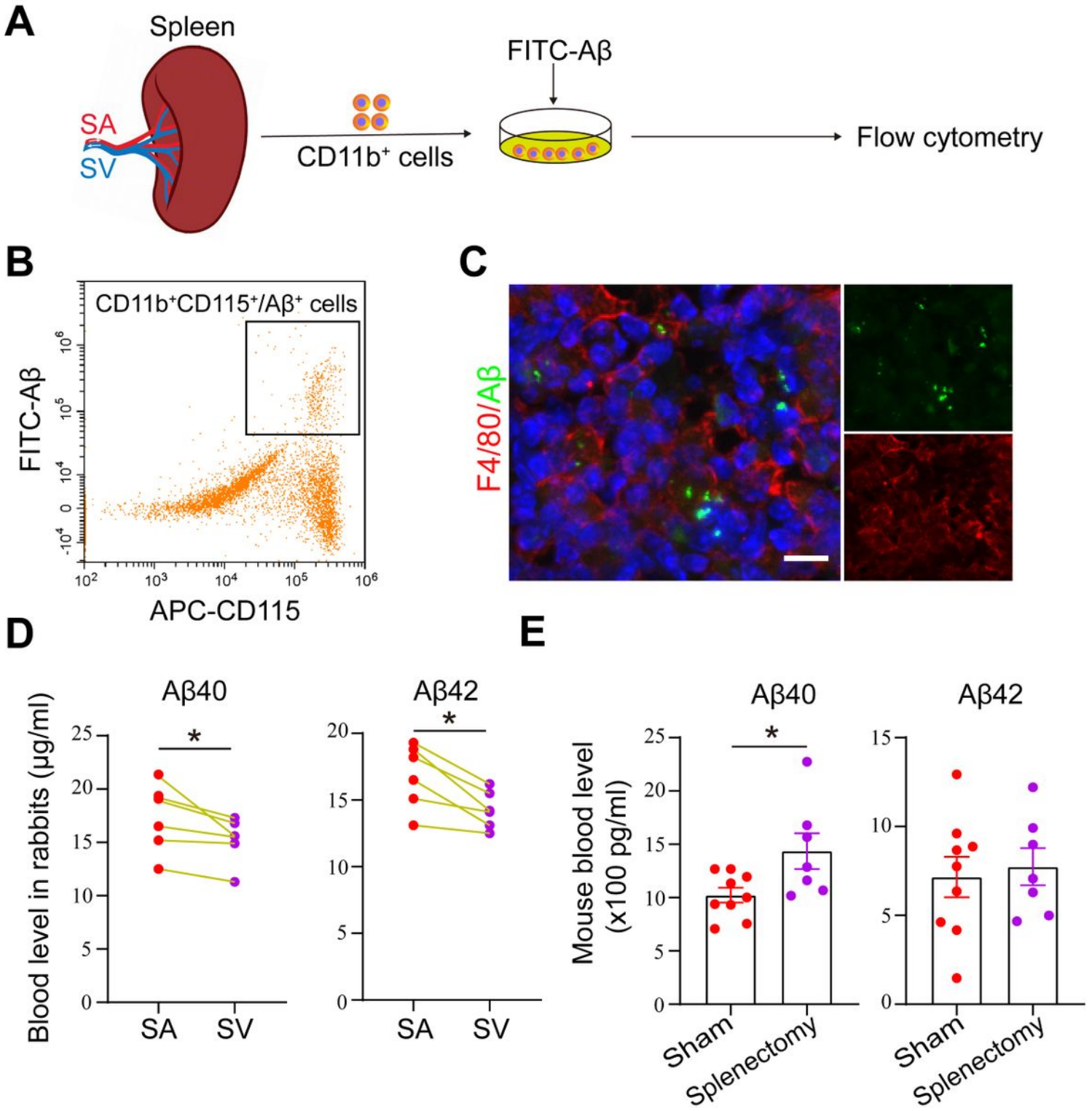

Figure 1

Physiological capacity for the spleen to clear Aβ. A and B. Schematic diagram and analysis of Aβ clearance by splenic monocytes/macrophages in vitro. C. Representative images of Aβ uptake by monocytes/macrophages in the mouse spleen after injection of FITC-Aβ into the tail vein. D. Comparison of Aβ levels in the splenic arteries (SAs) and splenic veins (SVs) of rabbits. E. Aβ levels in the blood of

mice that underwent splenectomy and sham control mice. n=6-9 per group; * indicates P < 0.05. Scale bar 100 μm. The error bars are the SEMs.

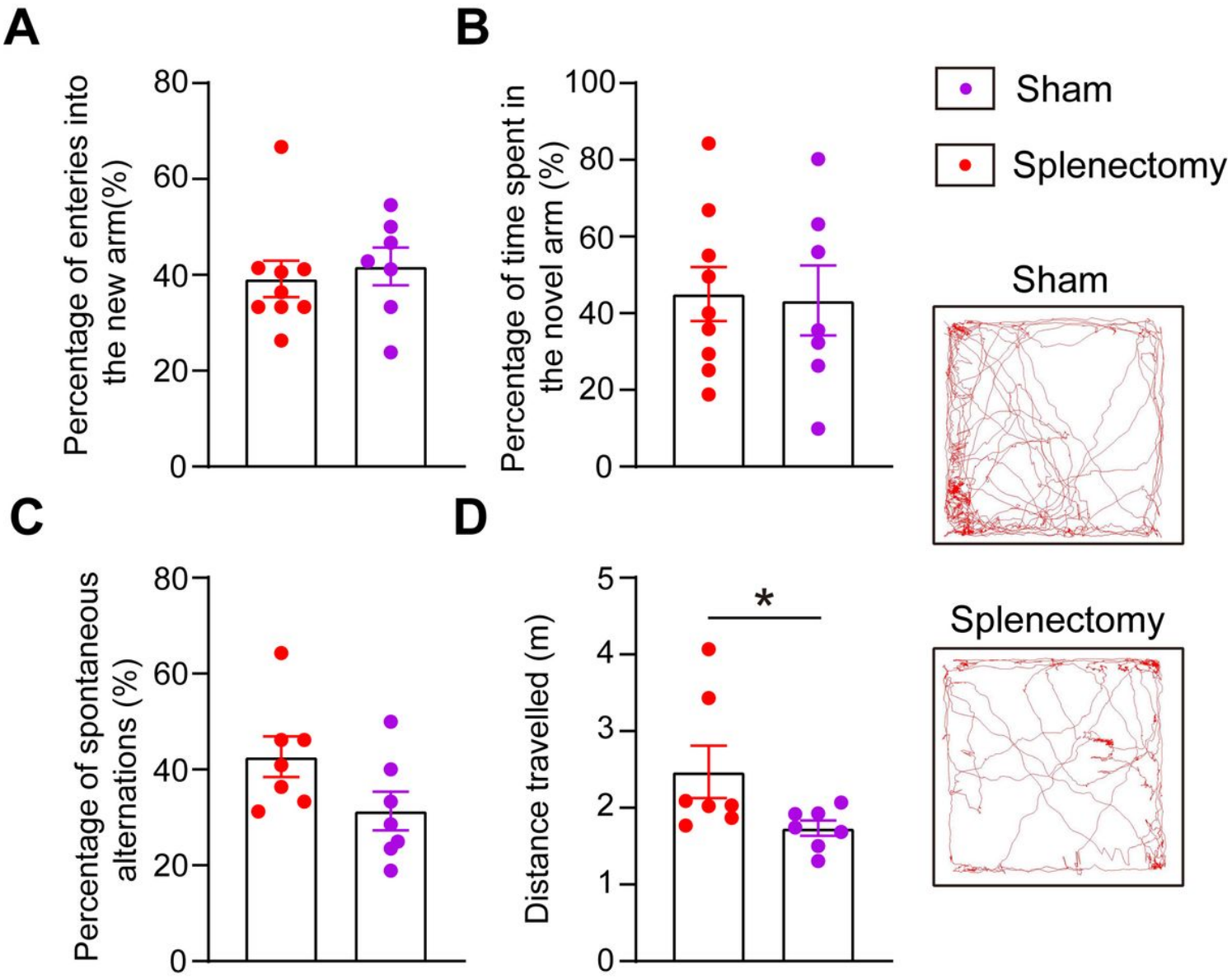

Figure 2

Effect of spleen excision on cognitive impairments in AD mice. A and B. Percentage of entries into the novel arm and percentage of time spent in the novel arm in the Y maze test. C. Spontaneous alterations in the Y maze test. D. Distance travelled in the open-field test. E. Representative track in the open-field test. n=7-9 per group; * indicates P < 0.05. The error bars are the SEMs.

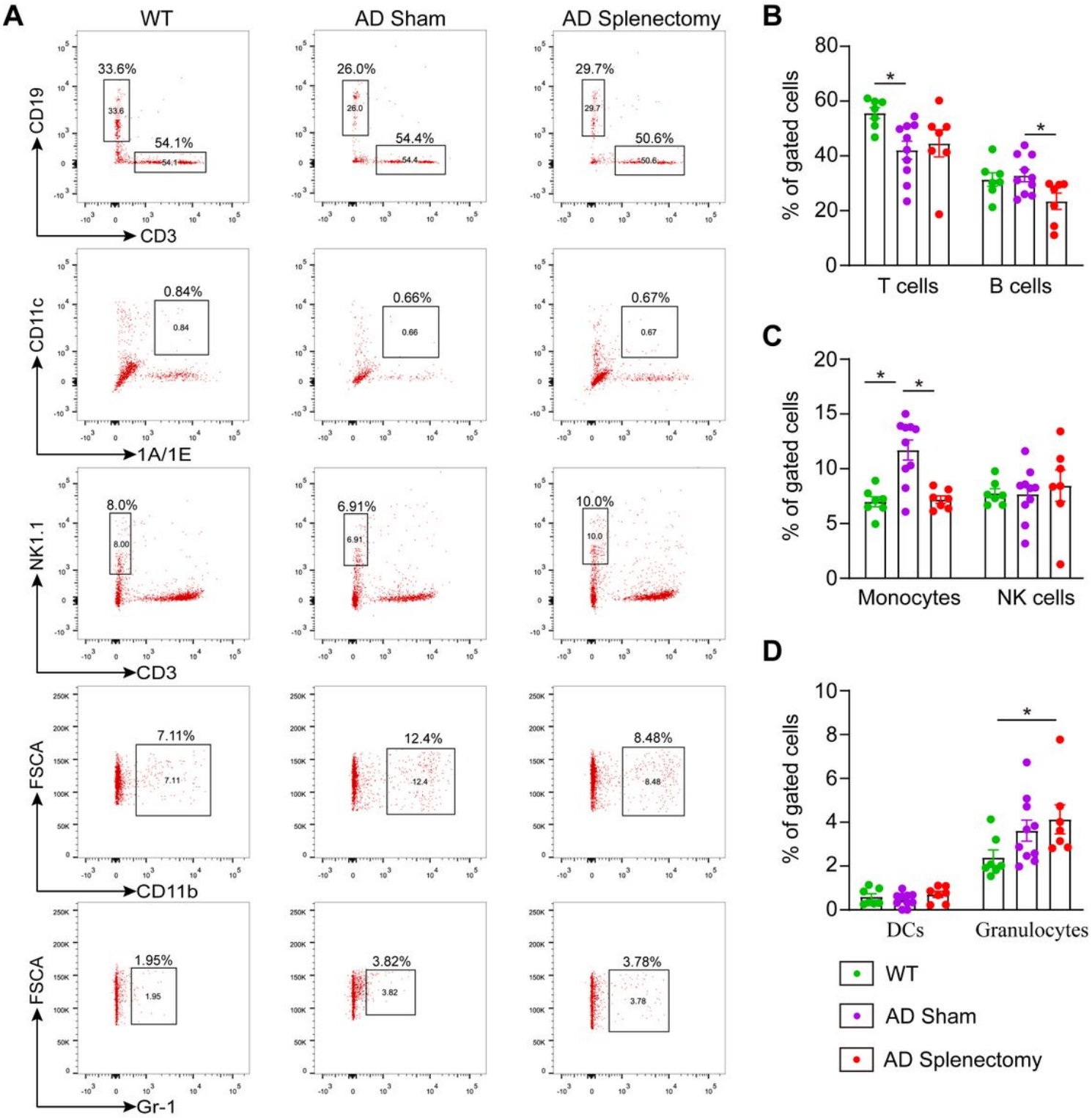

Figure 3

Effect of spleen excision on the proportions of peripheral blood immune cells in AD mice. A. Representative images of flow cytometry analysis of blood immune cells. B. Proportions of T cells and B cells in the peripheral blood of wild-type, AD sham mice and AD mice that underwent splenectomy. C. Proportions of monocytes and NK cells in peripheral blood of wild-type, AD sham mice and AD mice that underwent splenectomy. D. Proportions of DCs and granulocytes in peripheral blood of wild-type, AD

sham mice and AD mice that underwent splenectomy. DCs denotes dendrite cells, WT denotes wild-type. n=7-9 per group; * indicates P < 0.05. The error bars are the SEMs.

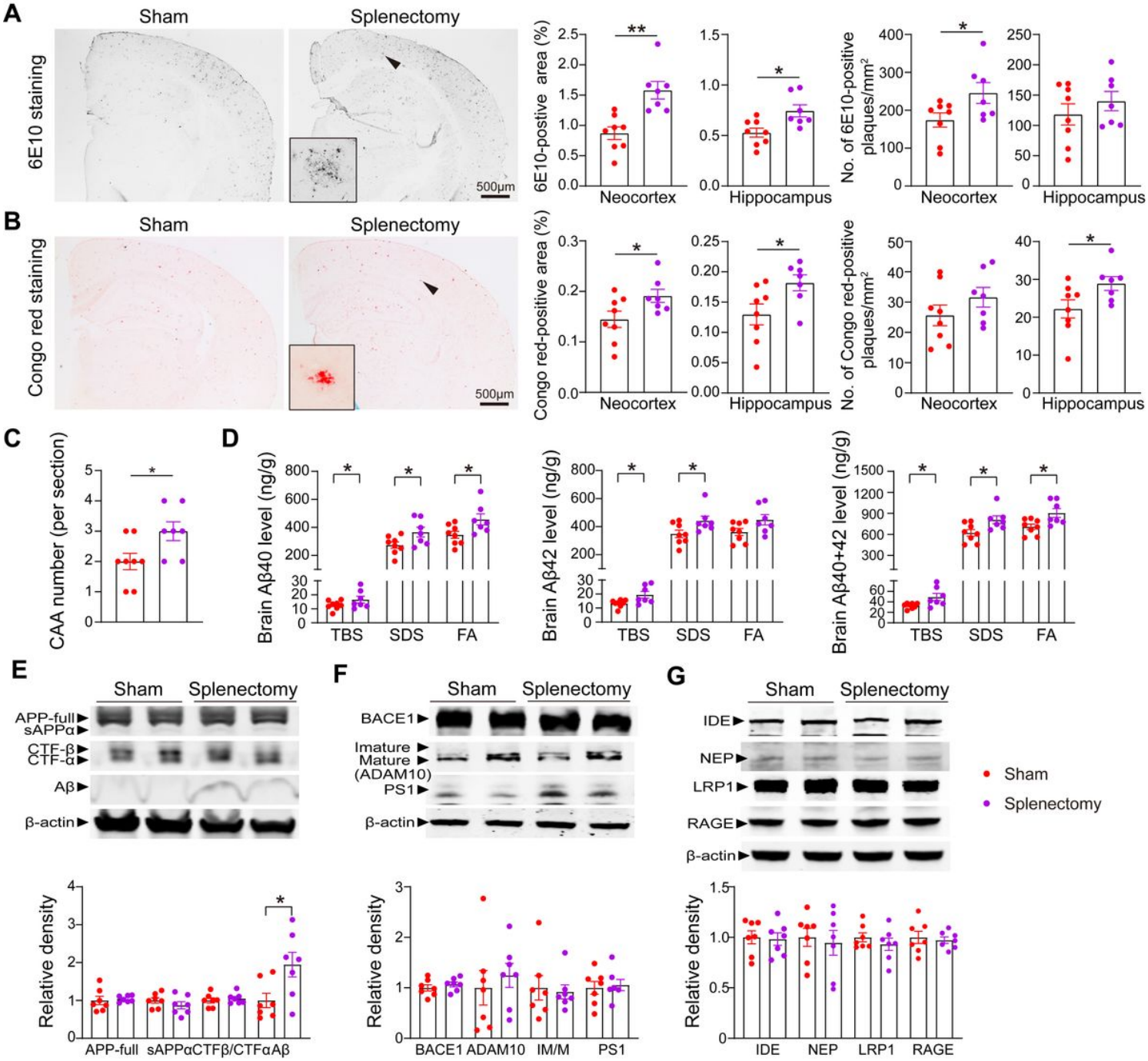

Figure 4

Spleen excision aggravates Aβ burden in AD mice. A and B. Immunostaining and quantification of Aβ plaques stained with 6E10 and Congo red in the neocortices and hippocampi of AD mice with or without splenectomy. C. Statistical analysis of cerebral amyloid angiopathy (CAA) number, as determined by Congo red staining. D. Comparison of Aβ40 and Aβ42 levels in the TBS, SDS and FA fractions of brain homogenates between the sham group and splenectomy group. E. Representative western blots and quantitative analysis of the levels of APP and its metabolites (sAPPα, CTFβ, CTFα, and Aβ) in brain

homogenates. F. Representative western blots and quantitative analysis of the levels of APP-metabolizing enzymes in brain homogenates. G. Representative western blot and quantitative analysis of the levels of Aβ-degrading enzymes and Aβ-transporting receptors in brain homogenates. n=6-9 per group; * indicates $P < 0.05$; ** indicates $P < 0.01$. The scale bars in A and B are 500 μm. The error bars are the SEMs.

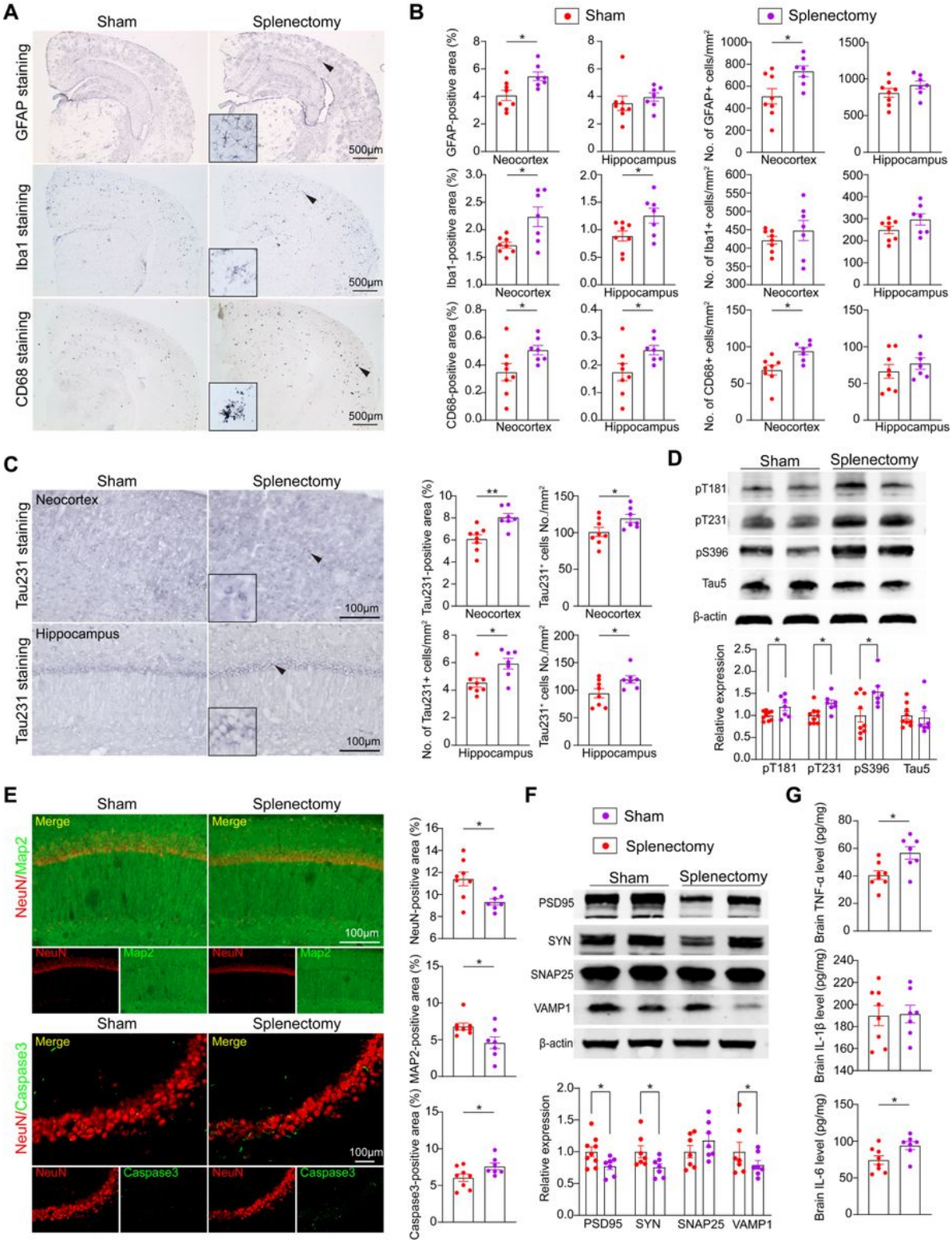

Figure 5

Spleen excision aggravates AD-related pathogenesis. A and B. Immunostaining of astrocytes and microglia with GFAP, Iba1 and CD68 in the neocortices and hippocampi of AD mice with or without splenectomy and quantification. C. Representative immunostaining images and quantification of pT231 expression. D. Representative western blots and quantitative analysis of phosphorylated Tau protein (pT181, pT231, PS396) and total Tau protein levels in brain homogenates. E. Representative images and quantification of neurons (NeuN, red) and dendrites (MAP2, green) in the CA1 region of the hippocampus and neural apoptosis (Caspase3, green) in the CA3 region of the hippocampus. F. Representative images and quantification of synaptic proteins (PSD95, Synapsin-1, SNAP25 and VAMP1). G. Statistical analysis of the levels of inflammatory factors, including TNF-α, IL-1β and IL-6. n=6-9 per group; * indicates $P < 0.05$; ** indicates $P < 0.01$. The scale bars in A and B are 500 μm, and the scale bar in E is 100 μm. The error bars are the SEMs.