Lymphatic dysfunction sustains memory impairment despite Aβ reduction by reactive astrocytes in an Alzheimer’s disease model

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Research Article

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Abstract

The central nervous system is not an immune-privileged system and is expected to communicate with the peripheral immune system. To understand the effects of the immune system in Alzheimer's disease (AD), anti-CD4 antibody treatment and deep cervical lymph node ligation were performed in the 5XFAD mouse model. These mice were also treated with an anti-amyloid antibody and focused ultrasound with microbubbles. Based on the analysis of pathology and behavior tests, CD4 T cells modulated microglial reactivity and amyloid pathology in the AD model, and dCLN ligation induced changes in the microglial response to stimulation and memory impairment by reactive astrocytes. These results demonstrated that lymphocytes and the lymphatic system regulate glial cell status, amyloid pathology, and memory function in AD. Therefore, peripheral immune cells and lymphatic dysfunction can be used as targets for AD treatment, and these dysfunctions should be considered in the development of AD therapeutics.

Introduction

The discovery of meningeal lymphatic vessels, which are immune cells and fluid from CSF, ushered in a new era of the CNS immune system [1]. The meningeal lymphatic vessels are connected to the deep cervical lymph nodes (dCLN) to filter CNS antigens and communicate with immune cells[1]. B cells, neutrophils, and monocytes in CNS were supplied directly from the bone marrow of the skull, and B cells educated by CNS antigens help maintain the immune privilege of the brain[2, 3]. However, CD4 T cells in the cranial dura, spinal dura, skull bone marrow, femur BM, and vertebrae BM originate from blood [2]. In addition, infiltrated clonal B cells accumulate in the aged dura (20–25 months), and these blood-derived B cells in aged dura encounter antigens in the periphery [3]. The population of plasma cells also shift predominantly from IgA+ to IgG+ and IgM+ in aged dura, and these IgM-secreting plasma cells are expected to be terminally differentiated locally by CNS antigen from blood-derived B cells in aged dura [3].

The CNS immune system changes in the structure of lymphatic vessels as well as the composition of immune cells with aging. Dorsal meningeal lymphatic vessels regressed from zipper-like junctions between lymphatic endothelial cells and discontinuous vessel structures. Basal meningeal lymphatic vessels exhibited hyperplasia, aberrant lymphatic endothelial cell junctions and disrupted valves in aged mouse brains [4]. Meningeal lymphatic dysfunction in the Alzheimer's disease (AD) model led to amyloid accumulation in the meninges and parenchyma [5]. In addition, the effects of Aβ-targeted antibodies under ablation of meningeal lymphatic vessels in an AD model were impaired Aβ reduction, memory enhancement by microgliosis, and neurovascular dysfunction [6].

In neurodegenerative diseases, including AD, T cells increase around abnormal protein aggregates[7]. Increasing T cells are expected to contribute to disease pathogenesis and progression. In the tissues of Lewy body dementia, T cells are adjacent to Lewy bodies and dopaminergic neurons[8]. Communication with C-X-C motif chemokine receptor 4 (CXCR4) and C-X-C motif chemokine ligand 12 (CXCL12) is involved in the recruitment of pathological interleukin-17–producing T cells that cause neuroaxonal damage.[9] Increased T-cell infiltration in the brain upregulated MCH II expression in microglia. [8–10]
Changes in immune cell infiltration and lymphatic function communicate with glial cells. However, the relationship between brain cells and immune cell infiltration and lymphatic function in AD progression remains to be studied. In this study, to understand the effects of CD4 T cells on brain cells and lymphatic dysfunction in AD, blockage of the CD4 domain and dCLN ligation were performed. We also administered anti-Aβ immunotherapy and focused ultrasound with microbubbles. Blocking CD4 T cells reduced amyloid deposition and reactive microglia as well as memory impairment. In the dCLN-ligated AD model, memory impairment did not recover because reactive astrocytes persist despite a reduction in amyloid deposition and reactive microglia by CD4 blockade. LAMP1-positive dystrophic neurites around amyloid plaques correlated with astrocyte reactivity. Therefore, alterations in CNS immunity may contribute to the development or progression of AD by altering glial reactivity. It is necessary to consider various abnormalities in the CNS, including neuroimmunity, to understand AD and develop a treatment for AD.

Results

Blocking CD4 T cells ameliorated amyloid deposition

To mimic the AD environment, 5XFAD and dCLN-ligated 5XFAD (Supplementary Fig. 1a) were used as Alzheimer's disease models, and immunotherapy for Aβ (Aducanumab) and focused ultrasound with microbubbles (FUS) were used as therapeutic agents[11, 12] (Supplementary Fig. 1b). Aducanumab, a human IgG1 monoclonal antibody, was treated with an anti-CD4 antibody (αCD4) the day before administration to suppress the immune response (Supplementary Fig. 1c). After 4 weeks, both D54D2- and 6E10-positive Aβ areas decreased in the αCD4-treated groups. In particular, the D54D2-positive region that bound to Aβ, which encompasses a wide range of intracellular and soluble forms, was dramatically reduced compared to that of 6E10 (Fig. 1a-c). As a result, inactivating αCD4 reduced Aβ; thus, the effect of the AD therapeutics was not observed. The changed pattern of Aβ was also found in the entorhinal cortex (Supplementary Fig. 2). Therefore, CD4 T cells are expected to be involved in the production or clearance of soluble Aβ in AD. However, Aβ reduction was not observed in the dCLN-ligated AD model specifically treated with aducanumab.

As a result of confirming spatial working memory with the Y-maze, memory functions were improved in the αCD4-treated AD groups, except for the dCLN-ligated AD groups (Fig. 1d). Memory function was improved with Aβ reduction in the AD groups but was not restored despite the decrease in Aβ by αCD4 treatment after dCLN ligation (Fig. 1e). Therefore, the memory decline due to dCLN dysfunction was not related to amyloid deposition, and glial cells were observed to identify the cause.

Changes in the response of microglia to Aβ immunotherapy in the dCLN-ligated TG model

Microglia were changed to a homeostatic state-like morphology after αCD4 treatment, and the same change was observed in the dCLN-ligated AD model. However, in the aducanumab-treated groups after dCLN ligation, microglia showed reactive state-like morphology (Fig. 2a-b). The alteration of microglia was similarly observed in the entorhinal cortex (Supplementary Fig. 3). In particular, in the ipsilateral hemisphere given FUS, aducanumab was observed more often in the parenchyma than in the
contralateral hemisphere (Supplementary Fig. 4a). Comparing the ipsilateral and contralateral hemispheres, more reactive microglia and Aβ deposition were observed at the site of increased aducanumab in the dCLN-ligated AD model (Supplementary Fig. 4b). These results suggest that dCLN dysfunction altered the response of microglia to external stimuli and correlated with soluble amyloid deposition. Nevertheless, astrocytes were observed to affect the memory decline in the control and FUS-treated groups in dCLN-ligated AD, in which reactive microglia were reduced without external stimulation.

Dysfunction of lymphatic drainage affected the reactivity of astrocytes and neuronal processes

In the AD model, reactive astrocytes increased around Aβ, and the αCD4-treated groups showed a tendency to decrease reactivity. On the other hand, a decrease in reactive astrocytes was not observed in the dCLN-ligation group (Fig. 3a-b). The different patterns of reactive astrocytes exhibited by the AD and dCLN-ligated AD groups may contribute to different outcomes with regard to memory function.

In addition, LAMP1 staining was performed to identify abnormal neuronal processes[13] located around amyloid deposition. LAMP1-positive dystrophic neurites were dramatically reduced in the αCD4-treated groups. However, in the dCLN-ligated groups, there were no changes in dystrophic neurites despite treatment with αCD4 (Fig. 3c–d). Increased abnormal neurites correlate with memory impairment, suggesting that dCLN dysfunction is associated with reactive astrocytes and memory function in AD.

Correlation of cellular changes in treated groups

The correlation analysis of each value showed that TG (orange color) and αCD4-treated groups were different in every correlation graph, and dCLN-ligated groups were between TG and αCD4-treated groups (Fig. 4). Based on Fig. 3a-b analysis, dCLN ligation could not reduce astrocyte reactivity even though changes in microglial reactivity were evident (Fig. 4j). Figure 4d shows that GFAP reactivity and memory impairment were sustained after dCLN ligation with treated αCD4 and therapeutics compared with TG (Fig. 4d)

Discussion

Recent studies have shown that immune cells play a role in neurodegenerative diseases, including AD, Lewy body dementia, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis[14, 15]. Increased infiltration of CD4 T cells into the brain parenchyma was observed in aging and neurodegenerative diseases[16]. In this study, inactivating CD4 T cells modulated microglial status and reduced amyloid pathology in an AD model (Fig. 1, 2). Reduced amyloid deposition refers to changes in the production or removal of amyloid. Aβ removal may alter the clearance mechanism through degrading enzymes, phagocytosis by cells (microglia, astrocytes, and macrophages), efflux transporters in the BBB, and the perivascular drainage system[17]. The changes induced by CD4 T cells may be due to the binding to MHC2 observed in microglia surrounding CD4 T cells in the brains of those with neurodegenerative diseases[10]. In addition, changes caused by blocking CD4 T cells may be affected by various factors, such as changes in brain cells due to changes in cytokines/chemokines or changes in the balance of
other immune cells as well as MHC2 interactions[18]. After αCD4 treatment, the reactivity of microglia surrounding plaques decreased with the reduction in Aβ and maintained a homeostatic state. However, it remains to be confirmed whether this homeostatic state was restored after phagocytosis.

The decrease in Aβ may be due to decreased fragment formation by β- and γ-secretases[17]. LAMP-1-labeled organelles were observed at the swollen area in the axon of the neuron around the plaque, and LAMP-1-labeled organelles were confirmed to be protease-deficient vesicles with elevated BACE1 expression[13, 19]. A decrease in LAMP-1-labeled axonal dystrophy after αCD4 treatment indicates a decrease in BACE1 expression, and the possibility of reducing Aβ production can be considered. However, it is necessary to determine whether the LAMP1-labeled area decreased despite the Aβ reduction observed in the αCD4- and αCD4-FUS-treated groups among the dCLN-ligated groups (Fig. 3c-d). Therefore, it is necessary to investigate how various factors regulate amyloid pathology by CD4 T cells.

Lymphatic dysfunction leads to changes in microglial status by external stimuli. There were no particular changes in the absence of stimulation but were evident after the administration of an amyloid therapeutic antibody (Fig. 2, Supplementary Fig. 4). To generalize changes in microglia to external stimuli due to lymphatic system dysfunction, microglial responsiveness studies to various external stimuli should also be performed. Fundamentally, research on how changes in the lymphatic system that can occur due to aging or disease affect brain cells should be conducted. In addition, in the dCLN-ligation group, the failure to recover memory function despite the reduction of amyloid by αCD4 treatment seems to correlate with the tendency of astrocytes to not recover (Fig. 3a-b, 4d). Astrocytes play a role in memory function[20–22]. The role of astrocytes in AD has been reported; reactive astrocytes abnormally produce the inhibitory gliotransmitter GABA and produce APOE and APOJ lipoparticles with long-chain saturated lipids, and severely reactive astrocytes produce excessive hydrogen peroxide[23–25]. These functions lead to impairment of synaptic plasticity, learning and memory and eventual cell death[23–25]. Therefore, further studies are needed to understand how dysfunction of the lymphatic system affects astrocytes.

These results revealed that the CNS immune system modulates amyloid pathology by changing brain cells in AD, suggesting the need for research on the function of immune cells and the lymphatic system in AD. Through an understanding of the roles of various immune cells in AD, it is necessary to find appropriate immunomodulatory targets and therapies in AD. In addition, treatment with an αCD4 antibody and immunosuppressive agents in AD studies may change pathology; thus, caution is required in their use. There may be differences in the therapeutic effect depending on lymphatic function during the development of therapeutics for AD, and it is thus necessary to select patients by evaluating patients or to interpret the results by sorting the patient groups. In addition, research on various targets and therapeutics that can modulate the reactivity of astrocytes can be suggested as new therapeutic strategies, such as therapeutics or combination therapies. Taken together, these results suggest the need for research on changes in brain cells caused by immunity in AD and the direction of new treatment strategies based on the mechanism.

Methods
Animals and treatments

6.5-month-old male 5XFAD mice (Tg6799; on a C57/BL6-SJL background) containing five FAD mutations in human APP (the Swedish mutation, K670N/M671L; the Florida mutation, I716V; and the London mutation, V717I) and PS1 (M146L/L286V) were used, and age-matched littermates were used as wild-type mice. Male mice were used in all experiments. Mice of all strains were raised in a laboratory cage with controlled temperature and humidity and on a 12-h light-dark cycle with no restriction of standard feeding and water drinking. All experimental protocols and animal usage were approved by the Institutional Animal Care and Use Committee at Seoul National University.

dCLN ligation

The mice were anesthetized with isoflurane, and the incision site was shaved and sterilized. After a longitudinal incision of the neck from the mandible to the sternum, the muscles and fascia were separated from the carotid artery under a stereomicroscope. The dCLN, located around the carotid artery, was carefully dissected from surrounding tissues and ligated with a 9−0 black nylon suture (AILEE). After surgery, the animals were stabilized for 2 weeks. To confirm the ligation of lymphatics, 5 µl of 2% Evans blue was injected into the cisterna magna of mice, and the dCLN regions were compared before and after ligation (Supplementary Fig. 1a).

FUS-MB

For FUS, a focused transducer with a diameter of 10 cm and a focal length of 8 cm was used. At the center frequency of 715 kHz, the sonication was 60 s in duration and consisted of 20-ms bursts at a pulse repetition frequency of 1 Hz (2% duty cycle). The focal region at 3 dB was 1.8 mm in diameter and 10 mm in axial length. The sonication was delivered at an acoustic power of 0.15 W, which corresponded to the measured peak negative pressure amplitude in water of 0.42 MPa.

The mice were anesthetized with ketamine, and the fur was removed from the head. The tail vein catheter was inserted, and the mice were mounted on a stereotaxic frame to target the brain region for sonication. A total of 100 µl of MB (Bracco, SonoVue 8 µl/ml sulfur hexafluoride microbubbles) was continuously infused through the tail vein during sonication (60 s). To confirm the BBB opening effect of FUS-MB, 200 µl of 2% Evans blue was intravenously administered after sonication, and the targeted region was confirmed (Supplementary Fig. 1b).

Treatment with anti-APP therapeutic antibodies

Aducanumab (Creative Biolabs) is a recombinant human monoclonal antibody expressed in CHO that binds to human amyloid beta. This antibody is designed for the treatment of AD. Human aducanumab, as an anti-APP therapeutic antibody, was administered intravenously at a dose of 1 mg/kg after BBB opening with FUS-MB. Antibodies were injected every other week for a month, and the detailed injection schedule is described in the experimental scheme (Supplementary Fig. 1c). Antibodies entering the brain
were identified using fluorescently labeled secondary antibodies that capture human antibodies (Supplementary Fig. 4a).

Y-maze test

To evaluate short-term working memory, a Y-maze test was performed at 7.5 months after treatment for 1 month according to the experimental design (Supplementary Fig. 1c). The mice were habituated to the behavior room and Y-maze, including background white noise the day before behavior analysis. The maze consisted of three arms converged in the central area of an equilateral triangle. Each mouse was placed at the center of the Y-maze and allowed to move freely for 8 min. The number of arm entries was recorded to calculate the percentage of alternation.

Immunohistochemistry

Paraffin-embedded brain tissues were sectioned at a thickness of 4 µm. Deparaffinization was achieved with xylenes and decreasing concentrations of ethanol. Tissue sections were subjected to antigen retrieval using citrate buffer, pH 6.0, at boiling temperature for 10 min. Following rinsing with TBS, sections were incubated in blocking buffer containing TBS with 0.5% bovine serum albumin for 1 h at room temperature. Slides were then incubated with primary antibody in blocking buffer overnight at 4°C. The next day, the slides were washed with TBS and then stained with Alexa Fluor secondary antibodies (Invitrogen). The sections were rinsed again and stained with DAPI (1:100; Invitrogen) before being coverslipped with mounting medium (Vectashield). The primary antibodies used were rabbit-Iba1 (1:200; Abcam), rabbit-LAMP1 (1:50; Abcam), mouse-6E10 (1:100; BioLegend), rabbit-D54D2 (1:200; Cell Signaling) and mouse-GFAP (1:200; Cell Signaling). Confocal microscopy (Leica TCS SP8) was used to image the stained slides. To quantify the stained slice images, MetaMorph software (Molecular Devices) was used.

Abbreviations

AD: Alzheimer’s disease

CXCL12: C-X-C motif chemokine ligand 12

CXCR4: C-X-C motif chemokine receptor 4

dCLN: deep cervical lymph nodes

FUS: focused ultrasound with microbubbles

αCD4: anti-CD4 antibody

Declarations

Ethical Approval and Consent to participate
All animal experiments were approved by Institutional Animal Care and Use Committee at Seoul National University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

Y.C. designed the study. E.J.L. performed experiments and data analyses. E.J.P. contributed experiments for focused ultrasound. E.J.L. and Y.C. wrote the paper. D.S.L. performed data interpretation. All authors read and approved the final manuscript

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Not applicable

References


**Figures**

**Figure 1**

Inactivation of CD4 T cells contributes to the amelioration of AD pathology.

(a) Immunofluorescence images of the dentate gyrus from AD and dCLN-ligated AD models under different conditions are shown. Coronal brain sections were stained with D54D2 and 6E10 antibodies showing Aβ accumulation. (b, c) The total area of D54D2- and 6E10-positive signals in each stained image was quantified. The amount of Aβ was decreased with an anti-CD4 antibody but worsened when aducanumab was administered to dCLN-ligated models. (d) Memory function was evaluated using the Y-maze and expressed as alternating percentages. AD models showed improvement in memory function after drug administration, but the effect was not evident in dCLN-ligated AD models. (e) A graph of the correlation between the Y-maze and Aβ staining results is shown. The amounts of Aβ in the two groups were similar, but different behavioral characteristics were observed. Scale bar = 50 µm. (*p<0.05, **p<0.01 (compared to WT); #p<0.05, ##p<0.01 (compared to TG); *p<0.05, **p<0.01 (compared to dCLN-ligated TG); one-way ANOVA)
Figure 2

The response of microglia to Aβ immunotherapy was altered in the dCLN-ligated AD model.

(a) Immunofluorescence images of the dentate gyrus of coronal brain sections stained with Iba1 antibody showing activated microglia are shown. The changed morphology of microglia around Aβ was confirmed through the enlarged images. (b) The total area of Iba1-positive signals in each stained image was quantified. Activation levels were reduced under the influence of anti-CD4 antibody but were reactivated by aducanumab in dCLN-ligated AD models. Scale bar = 50 µm. (*p<0.05; #p<0.001 (compared to TG); +p<0.05 (compared to dCLN-ligated TG); one-way ANOVA)
Figure 3

Reactive astrocytes and dystrophic neurites decreased after anti-CD4 antibody treatment but not after dCLN ligation.

(a) Immunofluorescence images of the dentate gyrus were stained with GFAP antibody, indicating the reactivity of astrocytes. The changed morphology of astrocytes surrounding Aβ was confirmed through
magnified images. (b) The total area of GFAP-positive signals in each stained image was quantified. In the AD model, the reactivity was reduced in the drug-treated group, but there was no effect in the dCLN-ligated AD model. (c) Coronal brain sections were stained with LAMP1 antibody representing dystrophic neurites. LAMP1-positive signals were mainly found near Aβ and are shown through magnified images. (d) The total area of LAMP1-positive signals in each stained image was quantified. Alleviation of dystrophic neurites by drug effects was not reproduced in dCLN-ligated AD models. Scale bar = 50 µm. (*p<0.05, **p<0.01; one-way ANOVA)

Figure 4

The correlation of cellular changes with various treatments

The quantification values of immunofluorescence were plotted. Red spots indicate TG and dCLN-ligated TG. Open and closed dots indicate the presence or absence of dCLN ligation, respectively. The yellow circle represents the TG and dCLN-ligated TG groups. Green and blue circles represent various treatment groups in TG and dCLN-ligated TG, respectively. (d) dCLN dysfunction caused by ligation altered the therapeutic effect, and in particular, the reactive astrocytes changed dramatically.

Supplementary Files
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