ANTICANCER RESEARCH *41*: 4053-4059 (2021)
doi:10.21873/anticanres.15206

Oral Administration of Lipopolysaccharide Prevents Cognitive Impairment in Streptozotocin-induced Diabetic Mice in a Blood Glucose-independent Manner

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Abstract. *Background/Aim: Diabetes is a risk factor for dementia. However, no radical preventive method for diabetes-associated dementia has yet been developed. Our previous study revealed that oral administration of lipopolysaccharide (LPS) prevents high-fat diet-induced cognitive impairment. Therefore, we investigated here whether oral administration of LPS (OAL) could also prevent diabetes-associated dementia. Materials and Methods: Diabetic mice were produced by intraperitoneal administration of streptozotocin (STZ), and then mice were orally administered LPS. Cognitive ability was evaluated using the Morris water maze, and gene expression was analyzed in isolated microglia. Results: OAL prevented STZ-induced diabetic cognitive impairment, but did not affect blood glucose levels. Moreover, OAL promoted the expression of neuroprotective genes in microglia, such as heat shock protein family 40 (HSP40) and chemokine CCL7. Conclusion: OAL prevents diabetes-associated dementia, potentially via promotion of HSP40 and CCL7 expression in microglia.*

Dementia is a prevalent disability in the elderly worldwide. According to a WHO report, 50 million people worldwide suffer from dementia, and 10 million new cases arise each year (1). As the number of patients with dementia continues to increase in our aging society, managing dementia is an urgent issue. However, there is still no standard of care for prevention and treatment of dementia.

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Key Words: Lipopolysaccharide (LPS), oral administration, cognitive impairment, dementia, diabetes, streptozotocin (STZ), microglia.

Recent studies have revealed that diabetes is an important risk factor for the development of dementia. It has been reported that the hazard ratio of developing Alzheimer's disease due to diabetes is 1.6, the hazard ratio of developing vascular dementia is 2.2, and the hazard ratio of developing all types of dementia, including these conditions, is 1.7 (2). A long-standing epidemiological study in Japan, known as Hisayama study, also demonstrated that diabetes significantly increases the risk of developing dementia (3). As a potential mechanism of diabetes-associated dementia, it has been reported that insulin resistance induces Alzheimer's disease pathology by promoting amyloid β accumulation and tau phosphorylation (4). In addition, diabetes also causes vascular lesions and metabolic dysregulation induced by glycotoxicity, oxidative stress, and advanced glycation end products. Thus, it is possible that the complex pathologies of circulatory disorders, metabolic disorders, and neurodegeneration can induce diabetes-associated dementia (5-7).

We have previously reported that oral administration of lipopolysaccharide (LPS) derived from *Pantoea agglomerans* prevents cognitive impairment in an Alzheimer's disease model using SAMP8 aging-accelerated mice administered high-fat diet (8). Therefore, we hypothesized that diabetes-associated dementia might also be prevented by oral administration of LPS (OAL). Here, we evaluated the preventive effect of OAL on diabetes-associated dementia using a general model of type I diabetes induced by intraperitoneal administration of streptozotocin (STZ) (9). Our results demonstrated that OAL prevented cognitive impairment in STZ-induced diabetic mice *via* a blood glucose-independent mechanism. Furthermore, our data suggest that the transformation of microglia, which are tissue-resident macrophages in the brain, is involved in this mechanism.

Materials and Methods

Animals. Male C57BL/6 mice were purchased from SLC, Inc., (Shizuoka, Japan) at 6 weeks of age (weighing 19-24 g) and acclimated for 1 week prior to use. All mice (5-6 mice per cage) were maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment under a 12-h light/dark cycle with unrestricted access to food and water. The animal experiments were reviewed and approved by the Animal Care and Use Committee of the Control of Innate Immunity TRA (Approval No. 18-04 and 18-12). Experiments were conducted according to the Law for the Humane Treatment and Management of Animals Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Ministry of the Environment, Japan), the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Japan), and the Guidelines for Proper Conduct of Animal Experiments (the Science Council of Japan). The health and well-being of all animals were assessed in accordance with the guidelines described above.

LPS treatment. LPS was orally administered to mice as described previously (8). Briefly, purified LPS derived from *Pantoea agglomerans* (Macrophi Inc., Kagawa, Japan) was dissolved in drinking water (sterile distilled water) at 1 mg/kg body weight (BW)/day. Drinking water was changed weekly, and the concentration of LPS was adjusted according to the average BW and water consumption. We have previously confirmed that LPS was not significantly degraded in drinking water after one week (10). LPS was orally administered to mice 1 week prior to STZ injection and until the end of the experiment.

Induction of type I diabetes by STZ. Mice were randomly divided into three groups (n=5-6). To induce type I diabetes, mice were intraperitoneally injected with STZ (Sigma-Aldrich, St Louis, MO, USA) at a dose of 200 mg/kg BW. After STZ injection, BW and blood glucose levels were measured regularly to confirm that mice developed diabetes. Non-fasting blood samples were collected from the tail vein and the blood glucose levels were monitored using an Accu-Chek Aviva blood glucose meter with Accu-Chek Aviva test strips (Roche Diagnostics K.K., Quebec, Canada).

Morris water maze (MWM) test. To assess spatial learning and memory, the MWM test was carried out 4 weeks after STZ injection as previously described (8), with minor modifications. Briefly, the area of the pool (100 cm in diameter and 40 cm in height) was conceptually divided into four equal quadrants, and cards with different shapes (circle, square, triangle or cross) were attached to the wall of each quadrant. A removable circular platform (10 cm in diameter) was placed 1 cm below the water surface, in a quadrant defined as the target quadrant. Each mouse consecutively received a pre-training session (1 day), training sessions (4 days), and a probe session (1 day). In the pre-training session, each mouse was put on the platform for 20 s, given a 30 s free swim, and then assisted in swimming back to the platform. In training sessions, mouse was released into the water at randomly assigned starting positions. Mice were given 60 s to find the platform, and were allowed to stay on it for 20 s. The spatial learning ability of each mouse was evaluated by the time elapsed between releasing and locating the platform, defined as escape latency. If the mouse failed to find the platform within 60 s, it was gently guided to the platform and kept there for 20 s, and the escape latency was recorded as 60 s. Each mouse underwent four trials per day, and the average value of the escape latency was calculated. Training sessions continued for four consecutive days. In the probe session, the probe test was performed to assess spatial reference memory. The platform was removed from the pool. Each mouse was placed in the pool at a starting position located opposite the target quadrant and allowed to swim freely for 60 s. The amount of time spent swimming in the target quadrant was recorded.

Isolation of microglia. Primary microglia cells were isolated from the brain by enzymatic digestion as described previously (11), with minor modifications. Briefly, brain tissue was chopped finely, and incubated in Dulbecco's Modified Eagle Medium containing 1.2 units/ml dispase II, 1 mg/ml papain (Sigma-Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), 20 units/ml RNase inhibitor (Promega, Madison, WI, USA), and 10 units/ml DNase I (Takara Bio, Shiga, Japan) for 30 min at 37˚C. Digestion was terminated by the addition of PBS containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), and the cell suspension was passed through a 70-μm cell strainer (Corning, Durham, NC, USA). After myelin was removed using the debris removal solution (Miltenyi Biotec, Bergisch Gladbach, Germany), the cells were incubated with anti-CD11b antibody-conjugated magnetic beads (20 μl/brain; Miltenyi Biotec) in PBS containing 0.5% BSA and 2 mM EDTA for 15 min at 4˚C. After washing, the CD11b+ microglia cells were separated using the autoMACS® proseparator (Miltenyi Biotec).

Quantitative RT-PCR. Quantitative RT-PCR was conducted as described previously (12). Briefly, RNA was extracted from isolated microglia using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was synthesized by reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturers' instructions. Real-time PCR assays were conducted using 2 μl of cDNA as the template and 10 μl of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a Stratagene Mx 3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The specific primers used in this study were as follows: 5'-TTACAAGGCGAGGAGAAGAC-3' and 5'-TTGACAA TCTGACCTGGATG-3' for *Dnaja1*, 5'-AAGATCCCCAAGAG GAATCTCA-3' and 5'-CAGACTTCCATGCCCTTCTTT-3' for *Ccl7*, and 5'-CGACTTCAACAGCAACTCCCACTCTTCC-3' and 5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3' for *Gapdh*. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to GADPH. The thermal cycling conditions for PCR were 95˚C for 10 min for polymerase activation, followed by 45 cycles at 95˚C for 15 s for denaturation and 60˚C for 1 min for extension.

Statistical analysis. Statistical analysis was conducted using the GraphPad Prism 6.0 software package (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as mean±standard error (SE). The differences between groups of mice were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test, or two-way ANOVA followed by Sidak multiple comparisons test. A student's *t*-test was used to compare the differences in the results between two independent groups. A p-value <0.05 was considered significantly different. Experiments were conducted independently in duplicate.

Results

OAL had no effect on weight loss or blood glucose levels in diabetic mice. The type I diabetes mouse model was induced by intraperitoneal injection of STZ. To confirm the development of diabetes, BW and non-fasting blood glucose were monitored. It was shown that STZ-injected mice lost BW, accompanied by polyuria. As shown in Figure 1A, the weight loss rate on day 31 after STZ injection was 20% (PBS-injected group, 28.2±1.3 g; STZ-injected group, 22.6±3.6 g). On the other hand, BW loss in LPS-administered group was comparable to that in STZ-injected group (LPS-administered group, 21.9±2.8 g). This result indicates that OAL does not affect STZ-induced BW loss.

Non-fasting blood glucose increased 1 week after STZ injection, and remained high until the end of the study (Figure 1B). The non-fasting blood glucose was 156±17 mg/dl in the control group, and increased to 412±138 mg/dl on the 31st day after STZ injection. In addition, non-fasting blood glucose in the LPS-administered group was not significantly different from that in STZ-injected group (459±172 mg/dl). This result indicates that OAL has also no effect on non-fasting blood glucose.

OAL prevented diabetes-related cognitive impairment. The cognitive ability of mice was evaluated by the MWM test. During the training period, escape latency was measured to evaluate spatial learning ability. As shown in Figure 2A, STZ injection prolonged escape latency (PBS-injected group, 8.2±3.4 s; STZ-injected group, 16.7±5.9 s), suggesting that spatial learning ability was impaired by STZ-induced diabetes. In contrast, escape latency of the LPS-administered group (5.0±1.2 s) was comparable to that of the control group. This result demonstrates that OAL prevents STZ-induced spatial learning ability impairment.

The time spent in the target quadrant was measured in the probe session to evaluate spatial reference memory. The result showed that STZ injection shortened the time spent in the target quadrant (PBS-injected group, 42.5±4.8 s; STZ-injected group, 20.1±10.7 s), which indicates the impairment of spatial reference memory (Figure 2B). On the other hand, STZ-induced shortening of the time spent in the target quadrant was rescued by OAL (32.3±5.1 s). The result revealed that OAL prevents STZ-induced spatial reference memory impairment. Therefore, we concluded that OAL prevents diabetes-induced cognitive impairment.

Prevention of diabetic cognitive impairment by OAL is independent of the blood glucose level. Based on the above results, the correlation between cognitive ability (time spent in the target quadrant in the probe session of the MWM) and non-fasting blood glucose level was evaluated. No correlation was found between cognitive ability and non-fasting blood glucose level (Figure 3). In the STZ-injected group, there was also no correlation between higher blood glucose level and lower cognitive ability. Therefore, STZ-induced diabetic cognitive impairment does not appear to occur in proportion to blood glucose levels.

Additionally, in the LPS-administered group, the prevention of cognitive impairment was not related to blood glucose levels, and even individuals with higher blood glucose levels showed high cognitive ability. Hence, OAL likely prevents diabetic cognitive impairment *via* a mechanism independent of blood glucose control.

OAL promotes neuroprotective gene expression in microglia. Our previous study suggested that microglia transformed by OAL contribute to the prevention of cognitive impairment (8). Therefore, in order to investigate the mechanism of prevention of diabetes-associated cognitive impairment by OAL, we analyzed gene expression in microglia isolated from naïve mice that were orally administered LPS for 6 days without STZ injection.

We observed that transcription of neuroprotective genes *Dnaja1* and *Ccl7* was promoted in microglia from LPS-administered mice (Figure 4). *Dnaja1* encodes heat shock protein family 40 member A1 (HSP40A1), and *Ccl7* encodes C-C Motif Chemokine Ligand 7 (CCL7). The promotion of these neuroprotective genes in microglia may be involved in the preventive mechanism of diabetes-associated cognitive impairment by OAL.

Discussion

In this study, we evaluated the effects of OAL on cognitive decline using a STZ-induced diabetes mouse model. STZ has selective toxicity toward pancreatic β-cells, and induces type I diabetes (9). We confirmed that STZ-injected mice showed typical diabetic symptoms, such as polyuria, weight loss, and increased blood glucose level (Figure 1A and B). However, these diabetic symptoms were not alleviated by OAL.

It is particularly interesting that OAL does not affect the increased blood glucose levels in type I diabetes, because our previous studies reported that high-fat diet-induced elevation of blood glucose in apolipoprotein E (ApoE)-deficient mice or SAMP8 mice was suppressed by OAL (8, 10). ApoE-deficient mice and SAMP8 mice, which exhibit hyperlipidemia-induced hyperglycemia, are considered to be models of type II diabetes (13, 14). It has been shown that hypoglycation as a result of OAL occurs in type II diabetes, in which pancreatic β cells survive, but not in type I diabetes, in which pancreatic β cells are depleted. Therefore, it is possible that pancreatic β-cells may be involved in the mechanism of the hypoglycemic effects of OAL.

Spatial cognitive ability in diabetic mice was evaluated by the MWM test. We observed cognitive impairment in STZ-

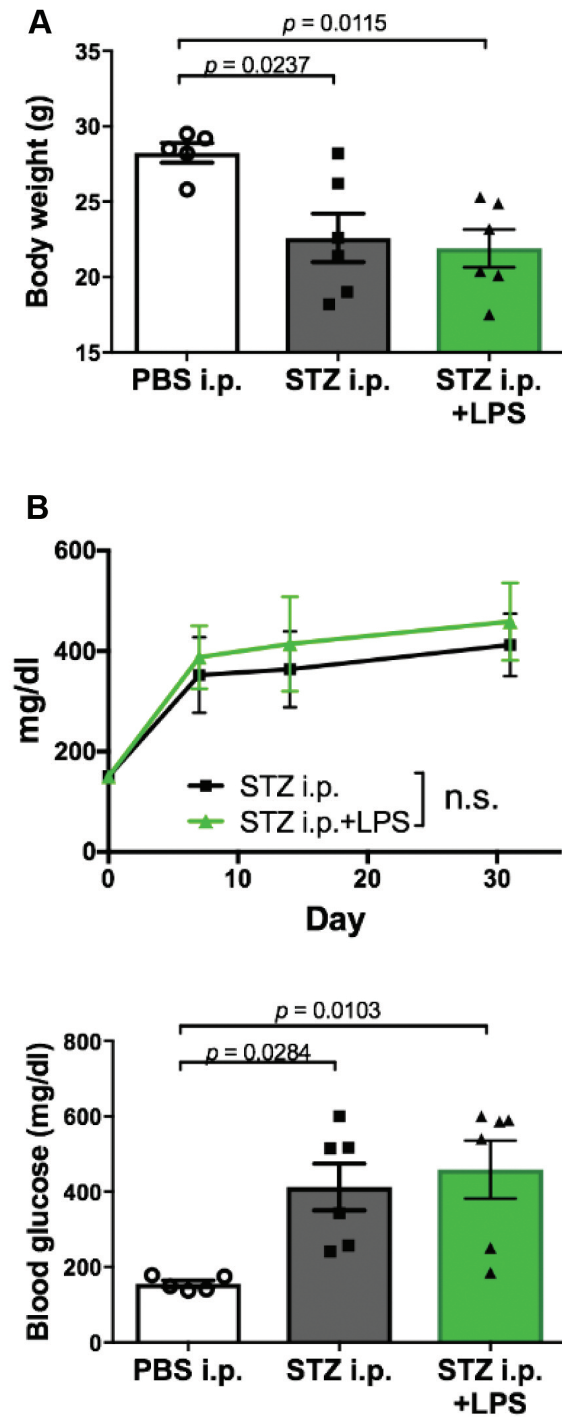

Figure 1. *Effect of oral administration of LPS on STZ-induced weight loss and elevation of blood glucose level. (A) Body weight 31 days after intraperitoneal injection of STZ. (B) Changes in non-fasting blood glucose after intraperitoneal injection of STZ (upper) and non-fasting blood glucose 31 days after intraperitoneal injection of STZ (lower). Data are presented as means±SE (n=5-6). *p<0.05 by one-way ANOVA with Tukey's multiple comparisons or two-way ANOVA with Sidak multiple comparisons. n.s.: Not significant; i.p.: intraperitoneal injection; LPS: lipopolysaccharide; STZ: streptozotocin.*

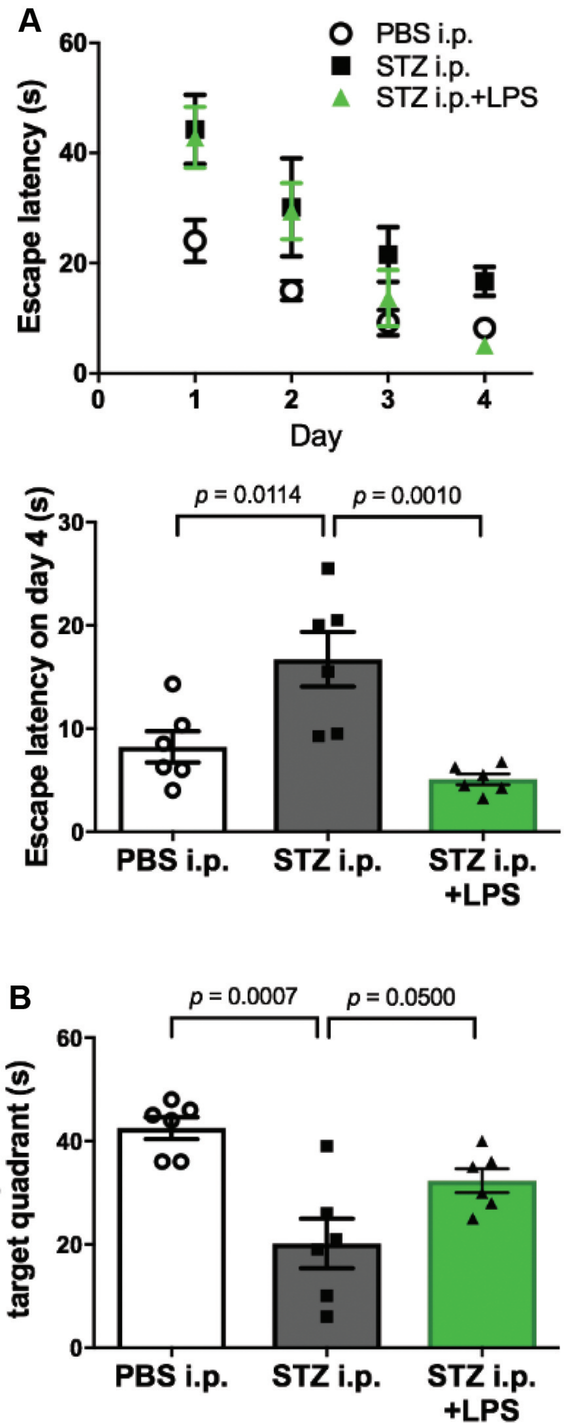

Figure 2. *Prevention of diabetic cognitive impairment by oral administration of LPS. Cognitive function was evaluated by the Morris water maze test 28 days after intraperitoneal injection of STZ. (A) Daily change in escape latency during training sessions (upper). Escape latency on day 4 (lower). (B)Time spent in the target quadrant in the probe test. Data are presented as Means±SE (n=5-6). *p<0.05 by one-way ANOVA with Tukey's multiple comparisons or two-way ANOVA with Sidak multiple comparisons. i.p.: Intraperitoneal injection; LPS: lipopolysaccharide; STZ: streptozotocin.*

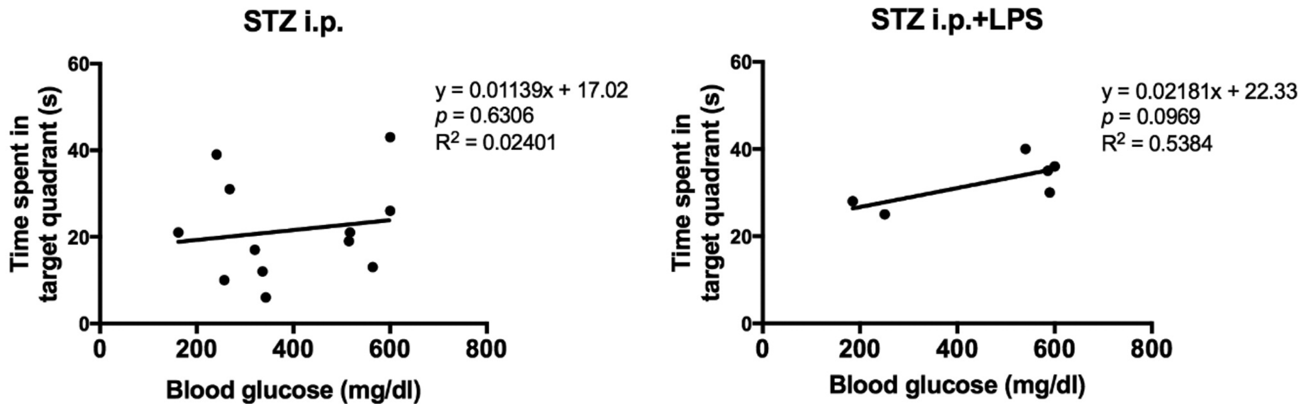

Figure 3. *Correlation between cognitive function and blood glucose. Scatter plot of time spent in the target quadrant of the Morris water maze and non-fasting blood glucose levels. Data are from the STZ-injected group (left) and oral administration of LPS group (right). Regression line equation, p-value, R^2 values are indicated. i.p.: Intraperitoneal injection: LPS: lipopolysaccharide; STZ: streptozotocin.*

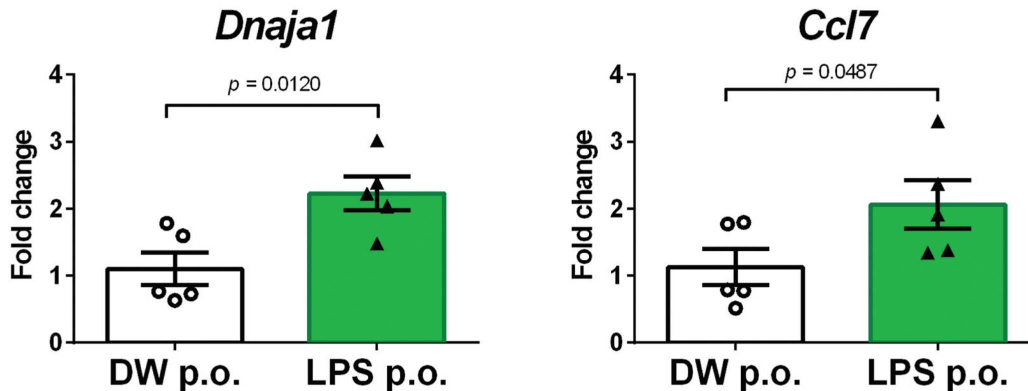

Figure 4. *Promotion of neuroprotective gene expression in microglia by oral administration of LPS. Naïve mice were orally administered LPS (1 mg/kg BW/day, for 6 days) without intraperitoneal STZ injection. Relative mRNA expression in isolated microglia was measured by real-time RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Data were normalized to Gapdh and expressed as relative fold change. Data are presented as means±SE (n=5). *p<0.05 using the Student's t-test. p.o.: Per os administration; LPS: lipopolysaccharide; STZ: streptozotocin.*

induced diabetic mice, similar to previous reports (15-17), however, OAL prevented the STZ-induced cognitive impairment (Figure 2A and B). This result was consistent with our previous study on the preventive effect of OAL on high-fat diet-induced cognitive impairment (8). Therefore, we conclude that OAL may represent a new preventive method for diabetes-associated dementia. Since we have already confirmed the safety of OAL based on Organization for Economic Co-operation and Development standards (18, 19), the clinical application of OAL is expected to be realistic.

Furthermore, in order to elucidate the mechanism of prevention of diabetes-associated dementia by OAL, the correlation between blood glucose level and cognitive ability was investigated. As shown in Figure 3, no correlation was found between cognitive ability (time spent in the target quadrant) and blood glucose level, indicating that STZ-induced diabetes-associated cognitive decline does not simply occur in proportion to blood glucose levels. In addition, LPS-administered mice showed high cognitive ability, even though their blood glucose levels were high, suggesting that OAL prevents diabetes-associated dementia by a mechanism other than blood glucose control.

Our previous studies suggested that OAL induces transformation of tissue-resident macrophages and contributes to disease prevention (8, 20). Microglia exist as tissue-resident macrophages in the brain, and maintain brain homeostasis by controlling innate immunity through transforming their characteristics (21-23). Therefore, we analyzed gene expression in microglia following OAL. We discovered that OAL promoted the expression of *Dnaja1* (HSP40A1) and *Ccl7* in

microglia (Figure 4). HSP40A1, known as a molecular chaperone, has been reported to promote microglial phagocytosis and neuroprotection when secreted (24). In addition, it has been reported that CCL7 promotes neural differentiation (25). Therefore, these neuroprotective molecules HSP40 and CCL7 promoted in microglia may be involved in the prevention of diabetes-associated dementia by OAL.

Our previous studies also suggest that promotion of microglia phagocytosis by OAL contributes to the prevention of high-fat diet-induced cognitive impairment (8). In agreement with the present study, it has been reported that microglia transformed by immune training with intraperitoneal administration of repetitive low-dose LPS prevent various neurological disorders, such as Alzheimer's disease, cerebral ischemia, brain damage, and epilepsy (26-29). Unlike so-called inflammatory microglia, which are induced by a single high-dose injection of LPS, repetitive low-dose LPS exposure induces the transformation to neuroprotective microglia (12, 30). Since the systemic injection of a single high dose of LPS induces potent inflammation, the stereotype that LPS only has pro-inflammatory functions remains pervasive. In contrast, our study shows that appropriate immune training with OAL may be an innovative treatment for various neurological disorders.

STZ is also used as an anticancer agent for pancreatic or gastrointestinal neuroendocrine tumors in clinical practice (31). The present study also indicates that the side effects of cognitive impairment due to the administration of the anticancer drug STZ can be prevented by OAL.

In conclusion, this study demonstrated that OAL prevents diabetes-associated dementia *via* a blood glucose-independent mechanism. Therefore, OAL might represent a novel preventive method for the prevention of diabetes-associated dementia. Furthermore, the expression of HSP40 and CCL7 promoted by transformed microglia may be involved in the mechanism of diabetes-associated dementia prevention by OAL. Further research will be required in the future to elucidate the full mechanism of prevention of diabetes-associated dementia by OAL.

Conflicts of Interest

HM, KY, MY, HI, and GS are employed by the Control of Innate Immunity, Collaborative Innovation Partnership. HI, CK, and GS are employed by Macrophi Inc. This does not alter the Authors' adherence to journal policies on sharing data and materials.

Authors' Contributions

HM, HI, and GS conceptualized the study and coordinated the experiments. HM, KY, and MY performed the experiments and HM performed data curation and formal analysis. HI and GS acquired the funding and administrated the project. HM wrote the manuscript supervised by HI, CK, and GS with contribution from all Authors.

Acknowledgements

This study was funded by Control of Innate Immunity Collaborative Innovation Partnership. This study was also supported by a grant of Cross-Ministerial Strategic Innovation Promotion Program (SIP-No. 14533073) from the Council for Science from Technology and Innovation (CSTI) in the Cabinet Office of Japanese Government and the National Agriculture and Food Research Organization (NARO). CSTI and NARO had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

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Received May 21, 2021
Revised June 3, 2021
Accepted June 4, 2021