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The Therapeutic Effects of the Herbal Medicine, Juzen-taiho-to, on Amyloid-β Burden in a Mouse Model of Alzheimer's Disease

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Abstract. Innate immunity, especially that involving macrophage function, reportedly diminishes with advancing age and in patients with Alzheimer's disease (AD). In this study, we tried to elicit the non-specific activation of peripheral macrophages by oral administration of the herbal medicine Juzen-taiho-to (JTT), to assess its effect as a possible treatment for AD patients. Amyloid-β protein precursor transgenic mice were used as a model of AD to clarify the effect of JTT. Activated macrophages derived from bone marrow cross the blood-brain barrier, and then develop into microglia, which phagocytose aggregated amyloid-β (Aβ) in senile plaques. Here we show that orally administered JTT increased the number of CD11b-positive ramified microglia in the mouse brain. The immunohistochemical examination of brain sections stained with polyclonal anti-Aβ antibody showed reduced Aβ burden, and Aβ levels were also decreased in the insoluble fractions of brain homogenates, as determined by ELISA. Thus, the activation of peripheral macrophages by JTT might be a potential new therapeutic strategy for AD.

Keywords: Alzheimer's disease, herbal medicine, Juzen-taiho-to, macrophage, microglia

INTRODUCTION

Both adaptive and innate immunity are severely compromised with age. The remarkable age-related reduction in resistance to infection is mainly caused by a weakening of the acquired immune responses including immunological memory, which is maintained throughout life.

In patients with Alzheimer's disease (AD), innate immunity, especially macrophage functions, is also reported to decline. The etiology of AD remains unknown; however, based on the amyloid cascade theory [1], many reports have indicated the potential efficacy of immunotherapy for this disease [2,3]. The observations of these studies suggest that chronic exposure of the immune system to amyloid-β (Aβ) in humans might lead to poor humoral or cellular immune

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responses to Aβ itself, which might contribute to the development of AD. Simard and colleagues [4] reported that resident microglia in the central nervous system (CNS) could not clear Aβ; instead, bone marrow derived macrophages, which were chemoattracted into the brain by $A\beta_{40/42}$, disrupted Aβ fibrils and phagocytosed senile plaque amyloid. Moreover, Frenkel and collaborators [5] demonstrated that intranasal administration of copolymer 1 (glatiramer acetate) plus a proteosome-based adjuvant, IVX-908, activated microglia and reduced Aβ deposition.

The Japanese herbal medicine, Juzen-taiho-to (JTT), has long been used for patients with cancer and wasting caused by other chronic illnesses. JTT is known to have lipopolysaccharide (LPS)-like functions and exhibits anti-tumor effects by activating immune responses and enhancing phagocytosis [6,7]. In this study, we examined the effects of oral administration of JTT on the non-specific activation of macrophages and the clearance of Aβ depositions in the brains of amyloid-β protein precursor (AβPP) transgenic (Tg) mice (Tg2576), because these mice represent an animal model of AD in which Aβ is deposited at an abnormally high rate with aging.

MATERIALS AND METHODS

Animals and oral dosing of herbal medicine

The herbal medicine JTT (TJ-48) used in this study was obtained from Tsumura & Co., Ltd (Tokyo, Japan). Tg2576 mice were first described by Hsiao et al. [8] as expressing the Swedish mutation of AβPP (APPK670N, M671L) at high levels under control of the hamster prion protein (PrP) promoter. In the present study, they were obtained from the Mayo Foundation for Medical Education and Research (Taconic Corp, Hudson, NY). The 12-month-old Tg2576 mice were administered JTT in drinking water (0.1 mg/ml) for one month ($n = 4$ in each group). Control Tg2576 mice were given normal drinking water ($n = 4$ in each group).

Animals were kept in a specific-pathogen-free condition and fed *ad libitum*. After one month, they were sacrificed by deep anesthesia, the brain was cut in half, and each hemisphere was frozen.

Primary macrophage cultures

Mouse monocytes were isolated from the spleens of mice treated with JTT and controls by Ficoll density gradient centrifugation and suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The primary macrophage cultures were obtained by stimulation with M-CSF (20 ng/ml) and GM-CSF (2 ng/ml) for 4 days in 24-well plates. These macrophages were cultured with aggregated $A\beta_{1-42}$ (1 μM) for 3 h and were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and stained with anti-Aβ, -CD11b, and -LAMP-1 antibodies. Detection was performed with fluorescence-labeled (FITC, Cy-3) antibodies.

Antibodies

Aβ plaque-containing sections were stained with polyclonal rabbit anti-Aβ antibody (Senetek, Napa, CA). The following primary antibodies were used for immunohistochemistry or immunofluorescence: CD3, CD4, CD11b, CD19 (BD Biosciences Pharmingen, San Jose, CA; 1:50), Iba-1 (for microglia; kindly provided by Dr. U. Imai, National Institute of Neuroscience, NCNP, Tokyo, Japan), IL-1β (Rockland Immunochemicals, Inc, Gilbertsville, PA), IL-6 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), IL-12 (eBioscience, Inc, San Diego, CA), TNF-α, TGF-β (Santa Cruz Biotechnology Inc), Ki67 (abcam, Tokyo, Japan), and anti-glial fibrillary acidic protein (GFAP) (Sigma, Saint Louis, Missouri).

FITC-conjugated donkey anti-goat IgG and Cy3-conjyugated donkey anti-rabbit IgG were purchased from Millipore Corporation, Japan. FITC-conjugated goat anti-rat IgG was purchased from Santa Cruz Biotechnology Inc.

Immunohistochemistry

Brain cryosections of mice treated with JTT and controls were fixed for 15 min with 70% formic acid (for Aβ staining) or 4% paraformaldehyde in 0.1 M phosphate buffer and rinsed with PBS-Triton X-100 before incubation in 0.3% H_2O_2 in methanol for 30 min. Sections were incubated at room temperature for 2 h with antibody as indicated below. Sections were washed with PBS-Triton X-100 before incubation with secondary goat anti-mouse or rabbit antibodies for 2 h. After PBS-Triton X-100 washes, sections were stained by the avidin-biotin HRP/DAB method.

Quantitative analysis of Aβ burden was performed as described previously [9] in three different brain regions: the hippocampus, the frontal cortex, and the parietal association cortex of all examined mice. The Aβ burden was expressed as % of area of Aβ deposits in the whole brain tissue area examined.

Images were projected from an Olympus Vanox microscope onto a computer screen through an Olympus DP71 CCD Digital Camera. Images were captured and analyzed with an image analysis system (Win roof, Mitani Corporation, Fukui, Japan).

Immunofluorescence

Brain tissues were fixed in 10% formalin for 10 min, washed in PBS, and blocked for 1 h in PBS containing 10% goat serum. After incubation with primary antibodies for 1 h, the substrates were washed three times in PBS and incubated with anti-rat, anti-rabbit, or anti-goat IgG for 30 min, washed in PBS and mounted in VectorMount AQ mounting medium (Vector, Burlingame, CA). Images were captured using a Zeiss confocal microscope.

Thioflavin T fluorimetry

Aggregation of $A\beta_{1-40}$ peptide was measured by the thioflavin T (ThT) binding assay, in which the fluorescence intensity reflects the degree of Aβ fibrillar aggregation. Aqueous solutions of 0.12 mM $A\beta_{1-40}$ peptide (in 0.1 M Tris-HCl, pH7.1) were incubated in the presence of JTT (0∼1 mg/ml) for 48 h. The fluorescence was measured after addition of 1 ml of ThT (2 mM in 50 mM glycine, pH 9.0) by LSB-50 Perkin-Elmer spectrofluorimeter.

Aβ measurements of the brain tissue by ELISA

Using highly specific antibodies and a sensitive sandwich enzyme-linked immunosorbent assay (ELISA), we quantified insoluble $A\beta_{40}$ and $A\beta_{42}$ in brain homogenate fractions extracted with TBS (50 mM Tris-HCl, pH 7.6, 150 mM NaCl), or 2% SDS and 70% formic acid, respectively, as described previously [10]. A frozen hemisphere of brain tissue was homogenized with a homogenizer in 1 ml of TBS/complete protease inhibitor plus 20 μg/ml pepstatin A (Roche, Mannheim, Germany), then centrifuged at 100,000 g for 1 h at 4°C using an Optima TLX ultracentrifuge (Beckman Coulter Inc. CA). The TBS supernatants were stored at −80°C and the pellets were homogenized in 1 ml of 2% SDS/TBS with protease inhibitor (Roche), then centrifuged at 100,000 g for 1 h at 25 °C following a 15-min incubation at 37°C. The pellets (corresponding to the insoluble fraction) were washed once, then extracted further with 1 ml of 70% formic acid, and centrifuged at 100,000 g for 1 h. The supernatants of insoluble 70% formic acid extracts were neutralized with 1 M Tris-HCl, pH 8.0 at a dilution of 1:20. For quantification of the insoluble fractions, we used an Aβ ELISA Kit (Wako, Odawara, Japan). The supernatant was diluted with standard dilution buffer at 1:2000 ($A\beta_{40}$) or 1:400 ($A\beta_{42}$) and measured according to the manufacturer's instructions. The obtained values were corrected for the wet weight of each brain sample and expressed as pmol/g brain.

Statistical analysis

Results are expressed as the mean ± S.D. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Student's t test.

RESULTS

Activation of macrophages and enhanced phagocytosis of Aβ peptides by JTT in vitro

Primary bone marrow-derived macrophages isolated from the spleens of Tg2576 mice treated with JTT were cultured with aggregated $A\beta_{1-42}$ (1 μM) for 3 h and stained with anti-Aβ, -CD11b, and -LAMP-1 antibodies. Macrophages from JTT-treated mice showed higher expression of CD11b compared with those from control mice (Fig. 1A; a, b) and CD11b fluorescence intensity was notably increased (Fig. 1A; c). $CD11b^{high}$ macrophages effectively phagocytosed Aβ peptides (Fig. 1B; c, d, e), while macrophages from controls did not (data not shown). The phagocytosed Aβ in the cytoplasm was co-localized with the lysosomal marker LAMP-1 in macrophages from JTT-treated mice (Fig. 1B; f, g, h). Higher magnification images disclosed Aβ peptide in cytoplasmic lysosomes of macrophages (Fig. 1B; i, j). Quantitative image analysis of Aβ peptides co-localized in the lysosomes showed a significant increase ($p < 0.05$) of phagocytosed Aβ peptides by macrophages of JTT-treated mice *in vitro* (Fig. 1B; k).

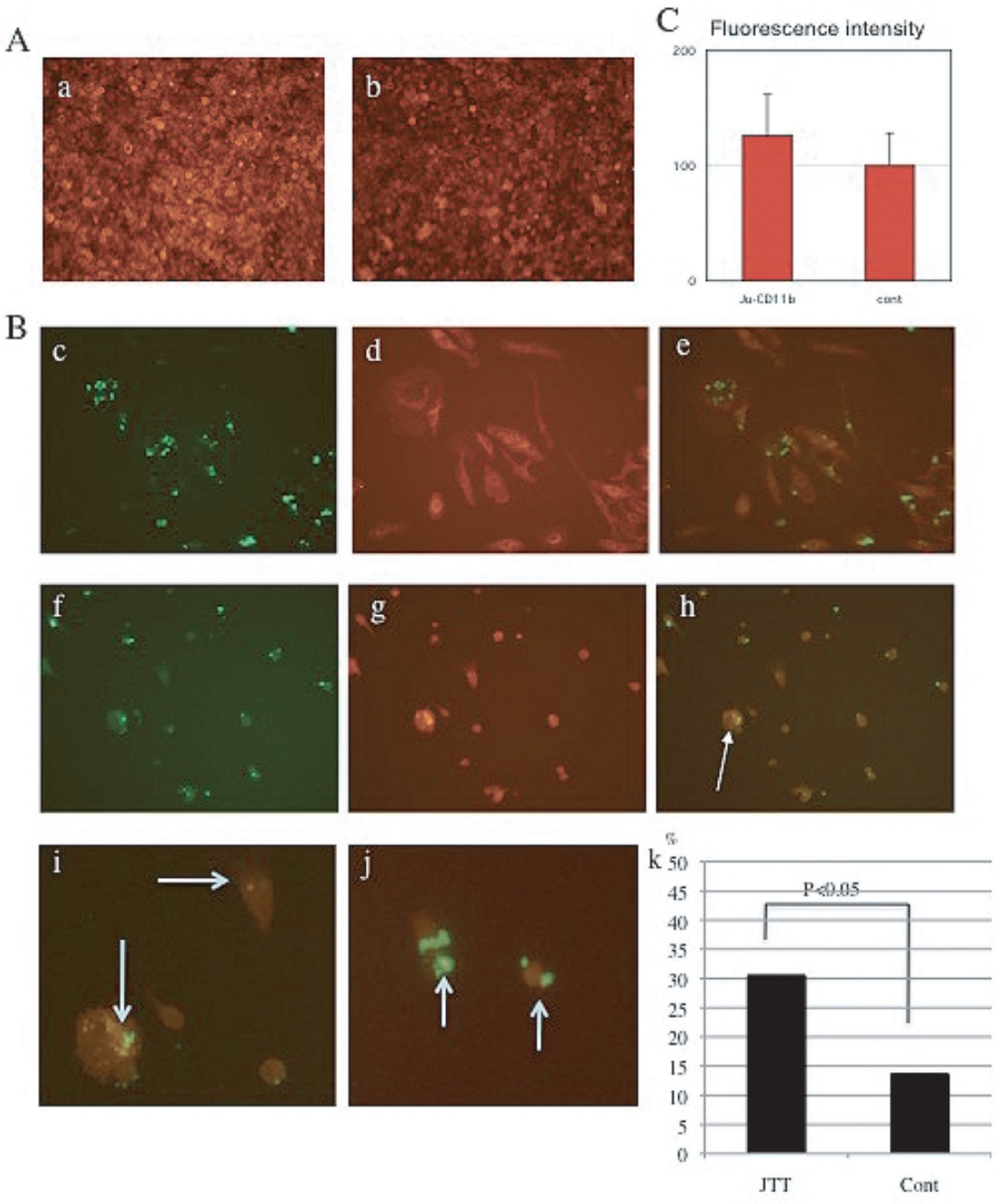

Fig. 1. A) Activation of bone marrow-derived macrophages and enhanced phagocytosis of Aβ peptides by JTT *in vitro*. Primary bone marrow derived macrophages isolated from spleens of control and JTT-treated Tg2576 mice were cultured with aggregated Aβ_{1-42} (1 μM) peptides for 3 h. Cell surface CD11b-Cy-3 staining of macrophages derived from (a) JTT-treated mice and (b) control mice. CD11b expression was notably increased in macrophages derived from JTT-treated mice compared with those from control mice (c). B) Macrophages derived from JTT-treated mice showed high expression of CD11b and uptake of Aβ peptides. (c) FITC-anti-Aβ staining, (d) CD11b-Cy-3, (e) Merge of Aβ and CD11b staining. Phagocytosed Aβ was co-localized with the lysosomal marker, LAMP-1, in the cytoplasm of macrophages derived from JTT-treated mice (f, g, h). (f) FITC-anti-Aβ staining, (g) Anti-LAMP-1-Cy-3, (h) merge of Aβ and LAMP-1 staining, (i, j) high magnification images of co-localization of Aβ in lysosomes (arrows). (k) Co-localization of Aβ peptides in the lysosomes was analyzed in 1×10^4 cells and showed a significant increase ($p < 0.05$) of Aβ peptides phagocytosed by macrophages derived from JTT-treated mice *in vitro*.

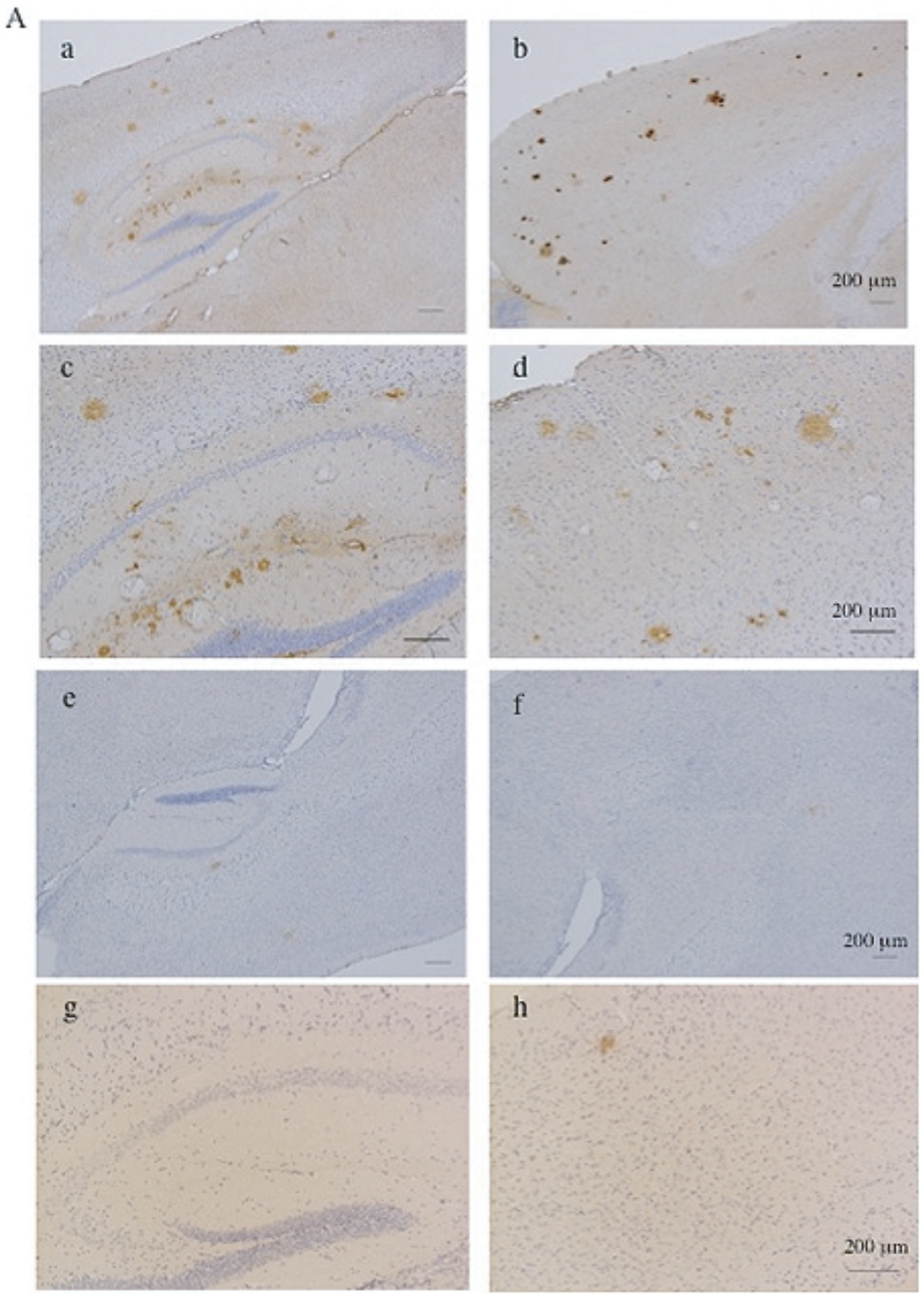

Fig. 2A. Reduction of Aβ burden in Tg2576 mice after oral administration of JTT. Sagittal brain sections of 13-month-old control AβPP Tg mice (a, b, c, d) and those treated with oral JTT (e, f, g, h) were stained for Aβ deposits with polyclonal anti-Aβ antibody in hippocampus (a, c, e, g) and frontal lobe (b, d, f, h) regions. Control Tg2576 mice showed diffuse and compact Aβ plaques throughout the hippocampus (a, c) and frontal lobes (b, d). On the other hand, JTT treated mice demonstrated almost complete clearance of Aβ plaques (e, f, g, h). Scale bar represents 200 μm.

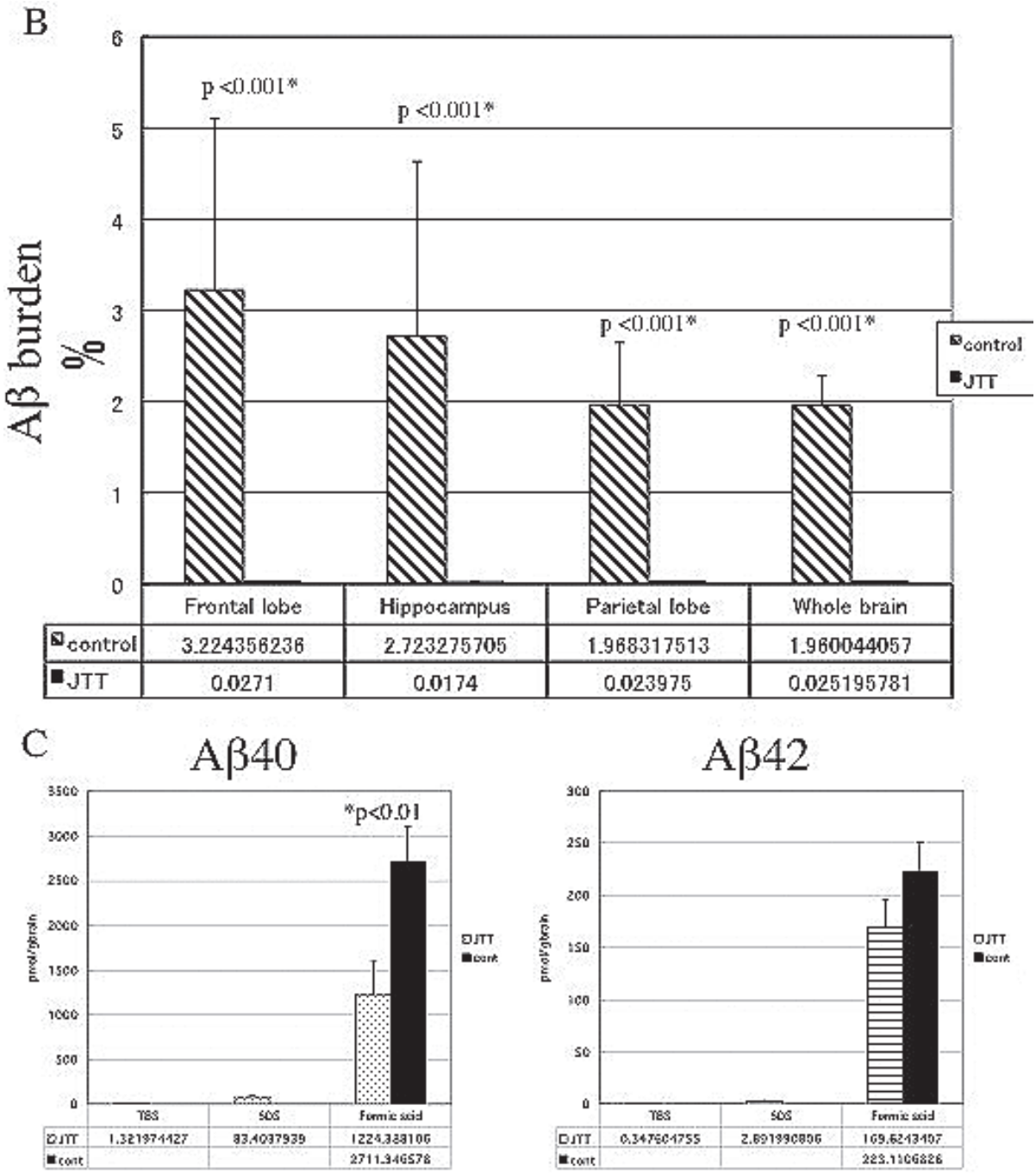

Fig. 2B-C. Quantitative image analysis of Aβ burden. The percentage of the area occupied by Aβ deposits was determined by quantitative image analysis in three brain different regions of all mice: the frontal cortex, parietal association cortex, and hippocampus. Aβ deposits were significantly reduced in the Tg2576 mice treated with oral JTT compared with non-treated control Tg2576 mice. Stereological quantification of Aβ burden throughout the frontal lobe, parietal lobe, and hippocampus also revealed significant Aβ clearance ($p < 0.001$) in the JTT-treated mice. All data are the mean $\pm$ SD from four different slices from each mouse. Aβ contents in the brain tissue. Using highly specific antibodies and a sensitive sandwich ELISA, we quantified soluble and insoluble Aβ_{40} and Aβ_{42} in brain hemisphere homogenate fractions extracted with TBS or 2% SDS and 70% formic acid, respectively. A significant reduction ($p < 0.01$) of Aβ_{40} concentration in the insoluble fraction (extracted with 70% formic acid) was observed in the brains of JTT-treated mice compared with those of control mice.

Effect of JTT on Aβ accumulation in Tg2576 mice

We investigated the anti-Aβ effect of JTT on the development of an AD-like neuropathology in 12-month-old Tg2576 mice. Oral dosing of JTT to Tg2576 mice markedly reduced the deposition of Aβ in the brain (Fig. 2A). Control Tg2576 mice showed diffuse and compact Aβ plaques throughout the hippocampus (Fig. 2A; a, c) and frontal lobes (Fig. 2A; b, d). On the other hand, almost complete clearance of Aβ plaques was seen in the JTT treated mice (Fig. 2A; e, f, g, h).

Quantitative image analyses were performed in the three different brain regions of control and JTT-treated 13-month-old Tg2576 mice (Fig. 2B). The JTT-treated group showed a marked decrease in Aβ burden compared with the control group. The mean Aβ burden in the JTT-treated group (frontal lobe: $0.027 \pm 0.0001\%$, parietal lobe: $0.024 \pm 0.0001\%$, hippocampus: $0.017 \pm 0.0001\%$) was significantly ($p < 0.001$) reduced compared with control mice (frontal lobe: $3.22 \pm 1.88\%$, parietal lobe: $1.97 \pm 0.677\%$, hippocampus: $2.72 \pm 1.91\%$). Stereological quantification of Aβ burden throughout the frontal lobe, parietal lobe, and hippocampus also revealed the significant Aβ clearance in the JTT-treated mice (Fig. 2B, table).

Soluble/insoluble $A\beta_{40}$ and $A\beta_{42}$ in brain homogenate fractions extracted with TBS or 2% SDS and 70% formic acid were quantified using the sandwich ELISA. Oral administration of JTT significantly reduced the concentrations of insoluble $A\beta_{40}$ and $A\beta_{42}$ in the insoluble fraction (Fig. 2C). In particular, $A\beta_{40}$ in the insoluble fraction was significantly ($p < 0.01$) decreased ($\sim$50%) in the JTT-treated group (1224.39 pmol/g brain) compared with the control group (2711.35 pmol/g brain).

To clarify why almost complete clearance of Aβ plaques was observed on immunohistochemistry despite the fact that ELISA did not show reduction of $A\beta_{42}$, we examined the direct effect of JTT on aggregation of Aβ peptides using the thioflavin T (ThT) reagent that binds specifically to the fibrillar amyloid structures. Aqueous solutions of 0.12 mM $A\beta_{1-40}$ peptide were incubated with various concentrations of JTT *in vitro* (Fig. 3, a). In the ThT fluorescence study, $>$ 0.125 mg/ml JTT directly inhibited Aβ aggregation. To exclude the possibility of binding ThT to JTT, we also measured the fluorescence intensity in the presence of ThT and JTT without Aβ peptides, and no increase of ThT binding was observed (Fig. 3, b).

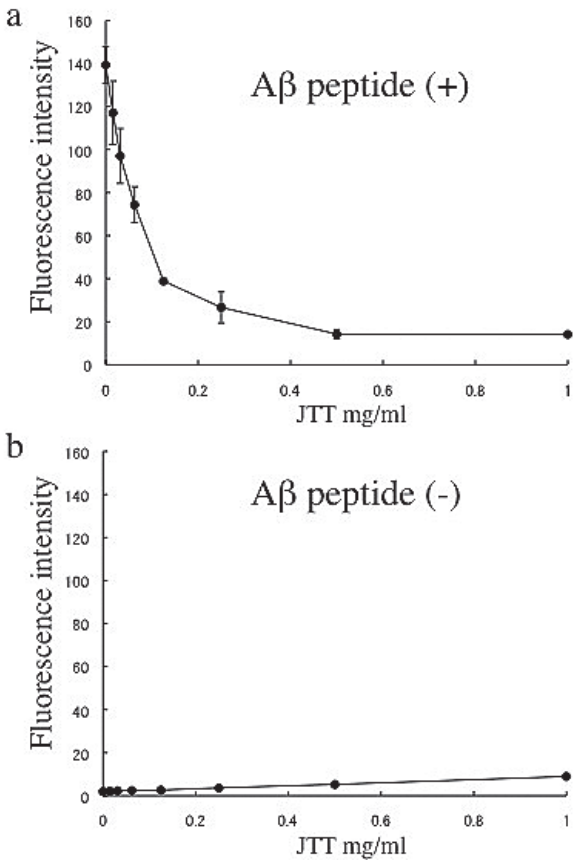

Fig. 3. The direct inhibitory effect of JTT on aggregation of Aβ peptides *in vitro*, using the thioflavin T (ThT) reagent that binds specifically to the fibrillar amyloid structures. Aqueous solutions of 0.12 mM $A\beta_{1-40}$ peptide were incubated in the presence of JTT (0$\sim$1 mg/ml) for 48 h. Fluorescence was measured after addition of 1 ml of ThT (2 mM in 50 mM glycine, pH 9.0). JTT at $>$ 0.125 mg/ml directly inhibited Aβ aggregation (a) and ThT did not bind to JTT (b).

Activation of bone marrow-derived macrophages after oral administration of JTT

We examined the effects of JTT on Aβ-induced macrophage attraction in 13-month-old Tg2576 mice. Control mice showed $CD11b^{low}$/Iba-1+ inactivated microglia around Aβ plaques in the frontal and parietal cortex (Fig. 4; a, c) and these nearly all of these microglia were negative for CD4 staining (Fig. 4; e). There were many cells positive for $CD11b^{high}$ (a marker of activated microglia) and Iba-1 in the brain parenchyma of JTT-treated mice (Fig. 4b, d). Iba-1 positive cells in JTT-treated mice contained CD4 positive cells (Fig. 4f). Immunofluorescence study showed the co-localization of CD11b and Iba-1 (Fig. 5; a, b, c). About 30% of Iba-1 positive microglia in the brain

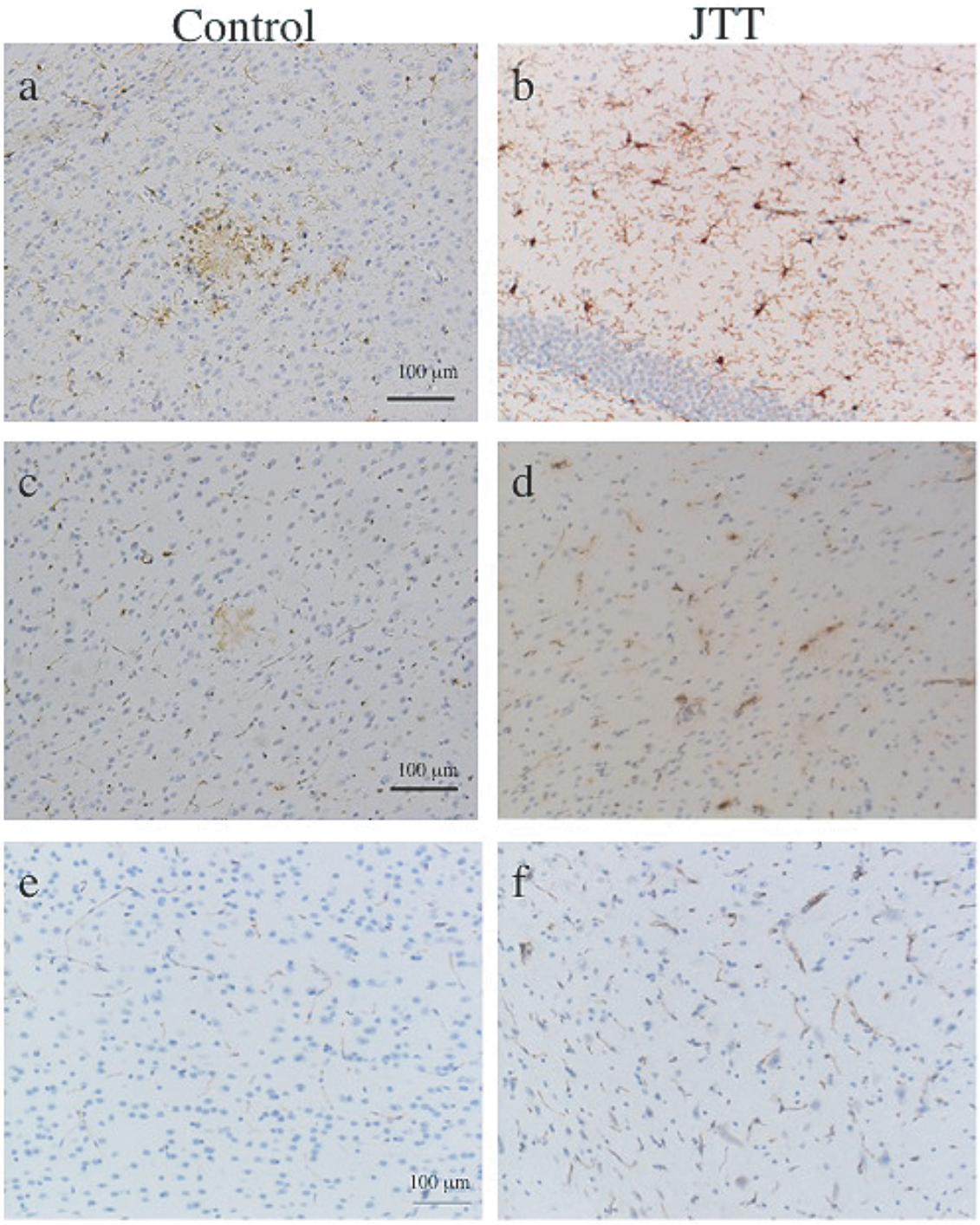

Fig. 4. Activated bone marrow derived macrophages in the brains of JTT-treated Tg2576 mice. Immunohistochemical examination of brain sections stained with (a, b) anti- Iba-1, (c, d) anti-CD11b and (e, f) anti-CD4 antibodies revealed activated Iba-1+/CD11b^{high}/CD4+ macrophages in JTT-treated mice (b, d, f). Because monocyte-derived microglia-like cells express higher CD4 *in vitro*, Iba-1+/CD4+ cells appeared to come from bone marrow cells. Iba-1+/CD11b^{low}/CD4- microglial cells surrounding Aβ plaques were observed in the control group (a, c, e). Scale bar represents 100 μm.

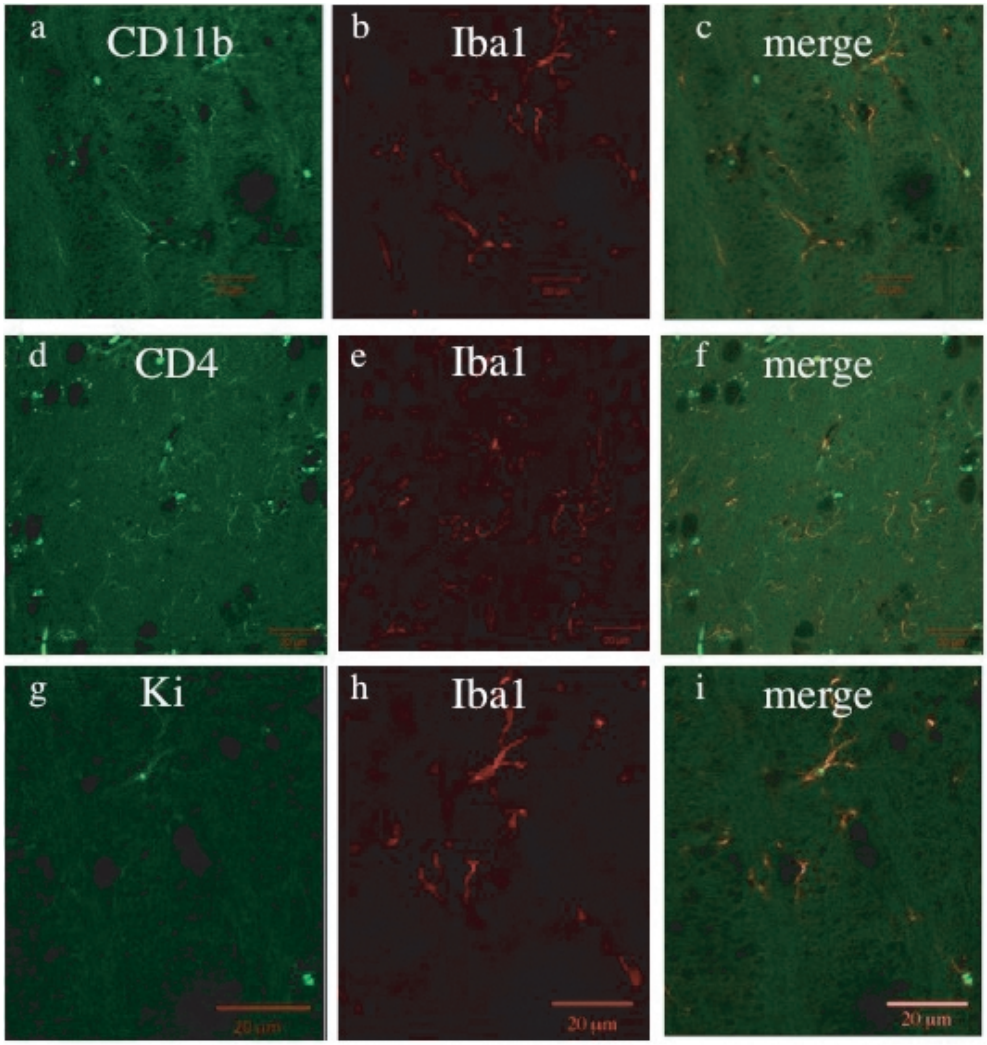

Fig. 5. Dual-immunofluorescence staining of brains from JTT-treated Tg2576 mice. Brain sections were stained with anti – CD11b (green), Iba-1 (red), CD4 (green), or Ki67 (green) antibodies together (merge; c, f, i). JTT-treated mice showed the co-localization of CD11b and Iba-1 (a, b, c). Iba-1 positive cells were also positive for CD4 (d, e, f), suggesting these cells were bone marrow-derived monocytes. There were Ki67/Iba-1 double positive cells (g, h, i) in JTT-treated mice, indicating the proliferation of bone marrow derived monocytes in the brain. Scale bar represents 20 μm.

of JTT treated mice expressed CD11b^{high}. Quantitative image analysis of CD11b/ Iba-1 double positive cells throughout the frontal lobe, parietal lobe, and hippocampus showed significant differences between control mice and JTT-treated mice (Fig. 6, a). These CD11b^{high}/Iba-1 positive cells were also positive for CD4 (Fig. 5;d, e, f), suggesting that they were derived from bone marrow monocytes, because monocyte-derived microglia-like cells express higher CD4 *in vitro* [11] and there were no infiltrations of lymphocytes such as CD3+ T cells or CD19+ B cells in the brain tissues (data not shown). Quantitative image analysis of CD4/ Iba-1 double positive cells throughout the frontal lobe, parietal lobe, and hippocampus indicated the increase of CD4/ Iba-1 cells in JTT-treated mice (Fig. 6, b). We also examined the expression of Ki67 antigen, the prototypic nuclear protein related to the cell cycle that is expressed by proliferating cells. JTT-treated mice showed the co-localization of Ki67 and Iba-1 in the brain (Fig. 5; g, h, i). However, control mice did not demonstrate Ki67/Iba-1 double positive cells (data not shown), indicating that Ki67/Iba-1 double positive cells found in JTT-treated mice were the result of proliferation of bone marrow derived macrophages rather than brain-endogenous microglia.

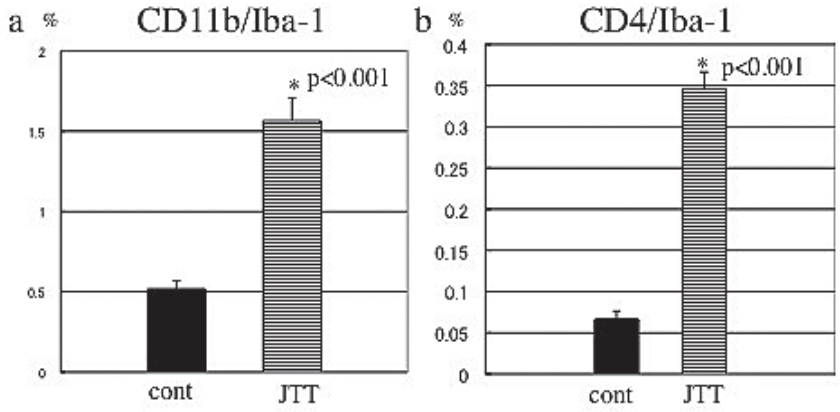

Fig. 6. Stereological quantitative image analysis of (a) CD11b/Iba-1 or (b) CD4/Iba-1 double positive cells throughout the frontal lobe, parietal lobe, and hippocampus revealed a significant increase of double positive cells in JTT-treated mice.

Cytokine expression in the brain after JTT treatment

To analyze the expression of cytokines in the brain after JTT treatment, we stained brain sections with anti-TNF-α, IL-12, IL-1β, TGF-β, and IL-6 antibodies. Control Tg2576 mice showed little expression of these cytokines (Fig. 7a). JTT-treated mice showed up-regulation of IL-6 in the brain (Fig. 7b), whereas inflammatory cytokines such as TNF-α, IL-12, and IL-1β were not detected (data not shown). To examine which cells express the increased amounts of IL-6, co-immunostaining with Iba-1 or GFAP were analyzed by immunofluorescence. About 90% of IL-6 producing cells were GFAP-positive astrocytes (Fig. 7; f, g, h). The remaining 10% of IL-6 producing cells were CD11b^{high}/Iba-1+ cells around the Aβ plaques (Fig. 7; c, d, e), indicating that the activated recruited microglia also produced IL-6 at the sites of Aβ burden. Quantitative image analysis of IL-6/Iba-1 double positive cells throughout the frontal lobe, parietal lobe, and hippocampus showed increased production of IL6 by microglia in JTT-treated mice (Fig. 7; i).

DISCUSSION

The Japanese herbal medicine JTT has LPS-like effects and stimulates immune functions by enhancing phagocytosis, cytokine production, and antibody production. JTT is often used to treat patients who are debilitated by chronic illness or cancer.

Simard and colleagues [4] reported that resident microglia in the brains of AβPP Tg mice could not clear Aβ; instead, bone marrow-derived macrophages, which were chemo-attracted into the brain by A$\beta_{40/42}$, disrupted Aβ fibrils and phagocytosed the senile plaque amyloid.

Recently, Liu et al. [12] reported that JTT activated microglia and induced microglial proliferation and activation without an increase in nitric oxide production. Furthermore, bone marrow derived macrophages from JTT-treated mice showed enhanced phagocytosis of fibrillar Aβ_{1-42}.

In the present study, we tried to elicit a non-specific activation of peripheral macrophages and to induce the clearance of Aβ depositions, without the use of antibody-mediated vaccination. After peroral administration of JTT to Tg2576 mice, a model of AD, we observed an increase in the number of CD11b-positive ramified microglia in the brain. These activated microglia also expressed CD4^{high} and seemed to be derived from bone marrow [11]. Immunohistochemical examination of brain sections stained with polyclonal anti-Aβ antibody revealed a reduction in extracellular Aβ deposition, and Aβ levels were also decreased in the insoluble fractions of AβPP Tg brain homogenates as measured by ELISA.

To clarify why almost complete clearance of Aβ plaques was observed on immunohistochemistry, we also examined the inhibitory effect of JTT on Aβ aggregation as analyzed by ThT binding assay and found that JTT directly inhibited Aβ aggregation *in vitro*.

Furthermore, there is a report of a comparison of a genomic-based (R1.40) and a cDNA-based (Tg2576) transgenic mouse model of AD in terms of Aβ production and deposition in the brain [13]. Significantly higher levels of Aβ_{40} were observed in the hippocampus and cortex in Tg2576 compared to R1.40 mice. Hence the large reduction of Aβ_{40} may be related to reduced formation of senile plaques in Tg2576 mice.

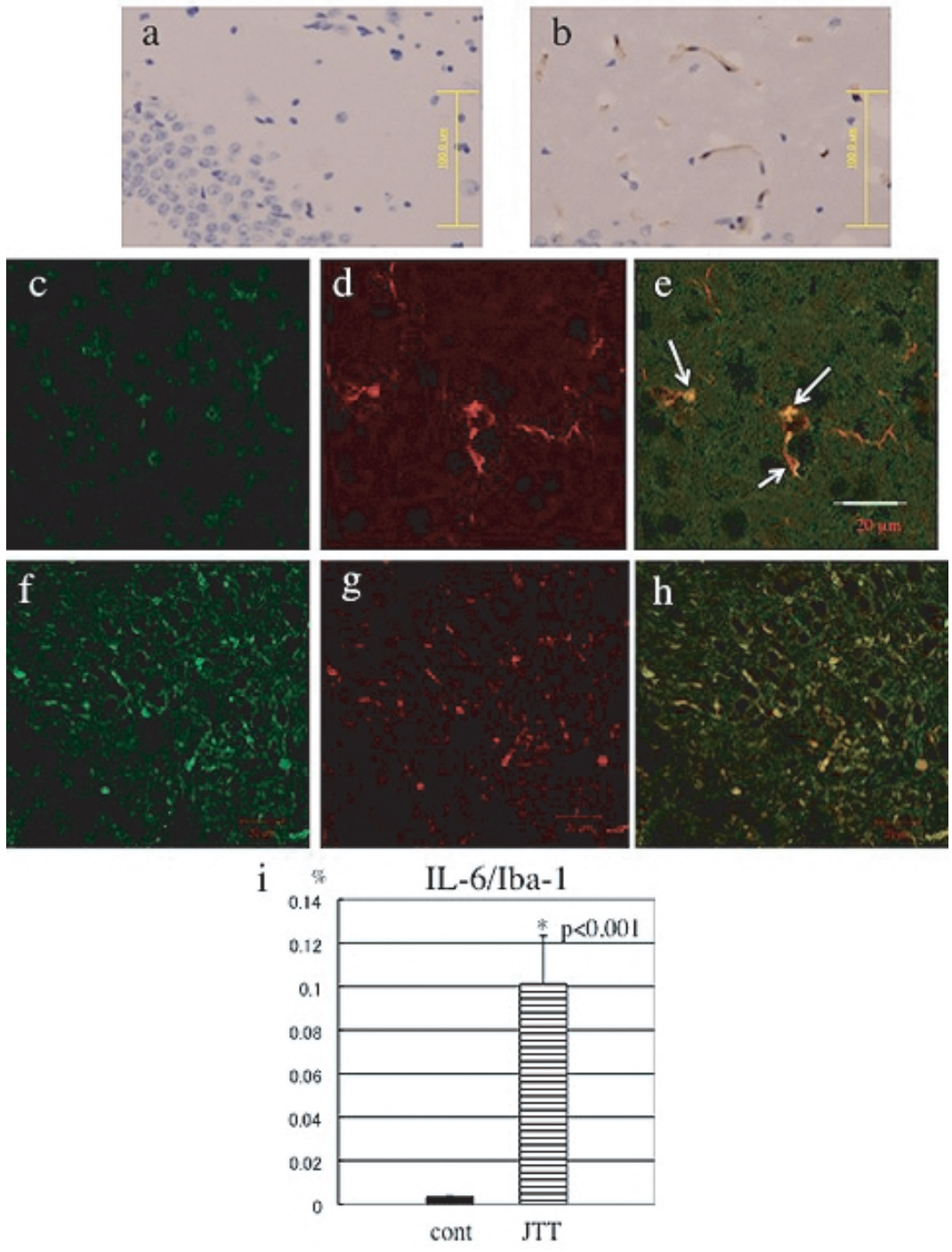

Fig. 7. Cytokine expression in the brains of Tg2576 mice after treatment with JTT. Brain sections of (a) control and (b) JTT-treated mice were stained with anti-IL-6 antibody. Up-regulation of IL-6 was observed in the brains of JTT-treated mice (b). Scale bar represents 100 $\mu\mu$m. Dual-immunofluorescence staining with IL-6 (green) and Iba-1 (red) revealed the production of IL-6 in some Iba-1+ microglia (c: IL-6, d: Iba-1, e:merge). Arrow indicates IL-6 producing microglia. GFAP positive (g: red) astrocytes mainly produced IL-6 (f; green) in the brain of JTT-treated mice (h; merge). Scale bar represents 20 μm. Quantitative image analysis of IL-6/Iba-1 double positive cells throughout the frontal lobe, parietal lobe, and hippocampus showed the increased production of IL6 by microglia in JTT-treated mice (i).

The induction of monocyte-derived microglia-like cells from the periphery and clearance of Aβ plaques, and the direct effect on Aβ aggregation by JTT might be the main reasons for the significant reductions of Aβ deposits in Tg2576 mice.

With regard to cytokines, upregulation of IL-6 was evident in microglia in the brains of JTT-treated mice. However, inflammatory cytokines such as TNF-α, IL-12, and IL-1β were not upregulated.

These findings strongly suggest that peripheral macrophages activated by JTT crossed the blood-brain barrier, and then developed into microglia that phagocytosed aggregated Aβ in the CNS.

The Phase II trial of the vaccine AN-1792, consisting of pre-aggregated Aβ peptides and QS21 adjuvant, was halted because of brain inflammation found in 18 of 300 (6%) AD patients [14]. QS21 is a strong T helper1 (Th1) polarizing adjuvant and Th1 responses have been implicated in many autoimmune diseases.

Frenkel and collaborators [5] reported that the activation of microglia could reduce brain Aβ levels in a mouse model of AD. They noticed that the meningoencephalitis that occurred in some patients involved in the clinical trial of AN-1792 resembled experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis. They then investigated whether AβPP Tg mice are vulnerable to EAE. All AβPP transgenic mice immunized with MOG peptides plus complete Freund's adjuvant (CFA) developed EAE. These Tg mice showed a reduction of Aβ burden in the CNS. Frenkel and coworkers then immunized B-cell deficient AβPP Tg mice with MOG and CFA, and found activation and proliferation of microglia plus reduction in amyloid load, compared with untreated mice. This indicated that the reduction of Aβ burden resulted from phagocytosis by microglia rather than activation of T cells or anti- Aβ antibodies. They showed that nasal vaccination with copolymer 1 (glatiramer acetate) plus the proteosome-based adjuvant, IVX-908, decreased Aβ plaques in AβPP Tg mice by activation and phagocytosis of microglia, without eliciting brain inflammation. However, moderate T cell infiltrations were noticed in these mice. The use of IVX-908 alone also reduced Aβ load, and this was considered to result from its LPS-like activity.

Akiyama et al. [15] also reported that incomplete ischemia elicited a local reduction in senile plaques in a neocortical region in an early AD case and suggested that their findings could be related to phagocytosis of amyloid by highly reactive microglia.

On the other hand, Nguyen and colleagues [16] addressed the effects of acute and chronic administration of LPS, a Gram-negative bacterial wall component, in a genetic model of neurodegeneration. Tg mice expressing a mutant form of the superoxide dismutase 1 (SOD1 G37R) linked to familial amyotrophic lateral sclerosis (ALS) were challenged intraperitoneally with single or repeated injections of LPS (1 mg/kg). At various ages, SOD1 G37R mice responded normally to acute endotoxemia. Remarkably, only a chronic challenge with LPS in pre-symptomatic 6-month-old SOD1 G37R mice hastened disease progression by 3 weeks and caused motor axon degeneration. This report clearly indicated the exacerbation of ALS symptoms by administration of LPS, suggesting that when using herbal medicine to activate immune function, we must select the target diseases carefully.

JTT is a traditional herbal medicine and has been shown to be safe and well-tolerated in patients with cancer, anorexia, and chronic illness. One study found that non-specific activation of bone marrow-derived macrophages is associated with the secretion of cytokines such as IL-6 and IL-12 [17]. The influence of macrophage-derived cytokines on neurons must be elucidated in further studies.

Since a limited number of animals per group were tested because of difficulty in procuring Tg2576 mice, the present research is a pilot study, and further investigation must be performed in larger groups.

In summary, JTT can activate and induce the proliferation of bone marrow-derived macrophages and clear Aβ depositions without eliciting antibody-mediated phagocytosis. In addition, JTT might have an inhibitory effect on Aβ aggregation. These findings suggest that JTT might reduce the severe side effects of meningoencephalitis observed in the AN-1792 trial [14] and could potentially be a new therapeutic approach for AD.

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All experiments were performed in accordance with the Guidelines for Animal Experiments of the NCGG/NILS animal experimentation committee. The procedures involving animals and the care of animals conformed to the international guidelines set out in the "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985).

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