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IL-4-Induced Selective Clearance of Oligomeric β-Amyloid Peptide_{1–42} by Rat Primary Type 2 Microglia

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A hallmark of immunopathology associated with Alzheimer’s disease is the presence of activated microglia (MG) surrounding senile plaque deposition of β-amyloid (Aβ) peptides. Aβ peptides are believed to be potent activators of MG, which leads to Alzheimer’s disease pathology, but the role of MG subtypes in Aβ clearance still remains unclear. In this study, we found that IL-4 treatment of rat primary-type 2 MG enhanced uptake and degradation of oligomeric Aβ_{1–42} (o-Aβ_{1–42}). IL-4 treatment induced significant expression of the scavenger receptor CD36 and the Aβ-degrading enzymes neprilysin (NEP) and insulin-degrading enzyme (IDE) but reduced expression of certain other scavenger receptors. Of cytokines and stimulants tested, the anti-inflammatory cytokines IL-4 and IL-13 effectively enhanced CD36, NEP, and IDE. We demonstrated the CD36 contribution to IL-4-induced Aβ clearance: Chinese hamster ovary cells overexpressing CD36 exhibited marked, dose-dependent degradation of o-Aβ_{1–42} compared with controls, the degradation being blocked by anti-CD36 Ab. Also, we found IL-4-induced clearance of o-Aβ_{1–42} in type 2 MG from CD36-expressing WKY/NCrj rats but not in cells from SHR/NCrj rats with dysfunctional CD36 expression. NEP and IDE also contributed to IL-4-induced degradation of Aβ_{1–42}, because their inhibitors, thiorphan and insulin, respectively, significantly suppressed this activity. IL-4-stimulated uptake and degradation of o-Aβ_{1–42} were selectively enhanced in type 2, but not type 1 MG that express CD40, which suggests that the two MG types may play different immunomodulating roles in the Aβ-overproducing brain. Thus, selective o-Aβ_{1–42} clearance, which is induced by IL-4, may provide an additional focus for developing strategies to prevent and treat Alzheimer’s disease. The Journal of Immunology, 2008, 181: 6503–6513.

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3 Abbreviations used in this paper: AD, Alzheimer’s disease; Aβ, β-amyloid; BBB, blood-brain barrier; CHO, Chinese hamster ovary; IDE, insulin-degrading enzyme; MG, microglia; NEP, neprilysin; o-Aβ_{1–42}, oligomeric Aβ_{1–42}; RAGE, receptor for advanced glycation end product; SRA, scavenger receptor class AI/All; SRA, scavenger receptor class B type I.

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MG-associated neuroprotection is suppression of the microglial inflammatory response with assistance of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (23).

IL-4 and other anti-inflammatory cytokines have been shown to promote alternative activation of MG (24–26). However, Landreth and colleague (27) reported that, in both BV-2 cells and mouse primary MG, IL-4 and other anti-inflammatory cytokines neither stimulated nor inhibited phagocytosis induced by phagocytic ligands including fibrillar Aβ. They suggested that these anti-inflammatory cytokines may oppose harmful effects of IL-1β, which stops phagocytic microspheres from forming inside cells and IFN-γ from preventing phagocytosis.

These studies, however, did not identify which type of MG possessed these neuroprotective functions. MG, as macrophage-like cells, are a heterogeneous population and several subsets were characterized as presenting different alloantigen molecules (e.g., CD40, MHC class II) during the establishment of cell lines (28). Alternative activation of MG to Th2-responsive cells is promoted by IL-4 and other anti-inflammatory cytokines (24–26). For greater understanding of the mechanisms important to amyloid vaccine approaches and alternatives to these approaches, it is necessary to ensure a Th2-type response as opposed to a Th1-type response. The prevailing working hypothesis in AD research, especially of groups studying amyloid vaccines, is that a shift to a Th2-type response may correct microglial dysfunction, reduce chronic inflammation, and enhance Aβ clearance and neuroprotective mechanisms.

We describe here a novel Aβ clearance mechanism involving type 2 MG, not type 1 MG, in primary culture after IL-4 treatment. We found clearance of o-Aβ1–42 with increased expression of scavenger receptor CD36 and the Aβ-degrading enzymes neprilysin (NEP) and insulin-degrading enzyme (IDE). We also discuss here the potential usefulness of these findings for developing anti-inflammatory therapeutic strategies against AD.

Materials and Methods

Chemicals and materials

The chloride form of Aβ1–42 was purchased from American Peptide. Thiorphan, fucoidan, and bovine insulin solution were purchased from Sigma-Aldrich. Proteinase K was obtained from Merck Biosciences. Na2HPO4 (3.7 Gb/ml) was from Amersham Pharmacia Biotech. Iodo-Gen was from Pierce Biotechnology. A mouse monoclonal anti-human Aβ1–16 was purchased from Chemicon International. Goat polyclonal anti-human CD36 Ab (1/1000 dilution), rabbit polyclonal anti-human NEP/CD10 (H-321), goat anti-rabbit, mouse CD40 Ab (T-20), and rabbit anti-mouse IL-4Rα Ab (S-20) were from Santa Cruz Biotechnology. A mouse monoclonal anti-IDE Ab (9B12) was from Covance. A rabbit polyclonal anti-mouse scavenger receptor class B type I (SR-BI) Ab (RED-1) was from Novus Biologicals. Rabbit polyclonal anti-mouse RAGE (5503) was from Transgenic. Mouse monoclonal anti-human SRA Ab (SRA-E5) described by Tomokiyo et al. (29) was a gift from Prof. M. Takeya (Kumamoto University, Kumamoto, Japan). Mouse monoclonal anti-human CD36 Ab (FA6-152) was from Immunotech. Goat anti-B7-2 (CD86) polyclonal Ab was purchased from Genzyme Technne. Mouse IgG (MOPC21) was obtained from Sigma-Aldrich. Chinese hamster ovary (CHO) cells overexpressing human CD36 (CD36-CHO) cells were provided by Prof. H. Arai (Tokyo University, Tokyo, Japan). Other chemicals were of the best grade available from commercial sources.

Preparation of oligomeric Aβ1–42

Oligomeric Aβ was prepared as previously reported (30), with a slight modification. The chloroform form of Aβ1–42 peptide was initially dissolved in hexafluoroisopropanol (Sigma-Aldrich) to achieve a concentration of 1 mM, and the solution was then separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was removed under vacuum in a SpeedVac concentrator and the peptide film was stored in desiccated form at −20°C. For the aggregation protocol on a preparative scale, the peptide was first resuspended in dry DMSO to a concentration of 5 mM. Ham’s F-12 (Invitrogen) was then added to bring the peptide to a final concentration of 500 μM, and the samples were rotated on a rotary shaker at 4°C for 24 h. o-Aβ1–42 was prepared as above (200 μg in PBS) was radiolabeled by the Iodo-Gen method with Na125I at 18.5 MBq. Excess 125I was equilibrated with PBS. The specific activity of the 125I-labeled Aβ1–42 was 856–1324 cpm/ng. Analytical scale aggregation protocols are described in Fig. 1 legend.

Cell culture and treatment

Rat primary MG were harvested from primary mixed glial cells prepared from neonatal Wistar rats, spontaneously hypertensive rats (SHR) (NCj), Wistar-Kyoto (WKY) (NCj) rats, or ddY mice as previously reported (31, 32). In brief, after meninges were carefully removed, the neonatal brain was dissociated by pipetting. The cell suspension was added to 75-cm² culture flasks at a density of five brains per 12 flasks (for rats) or one brain per flask (for mice) in 10 ml of Eagle’s MEM supplemented with 10% bovine serum, 5 μg/ml bovine insulin, and 0.2% glucose. Type 1 MG were isolated on days 14–16 by the “shaking off” method previously described (32). Type 2 MG were isolated on days 19–21 by harvesting with 5 mM EDTA in phosphate buffer solution via a modification of the mild trypsinization method (33). For some experiments, mixed MG that included both type 1 and type 2 cells were separately harvested on day 16 from a mixed glial cell culture with 5 mM EDTA in PBS via a modification of the mild trypsinization method (33). All MG preparations (mixed MG, type 1 MG, and type 2 MG) did not contain O2-A progenitors, oligodendrocytes, or astrocytes. CHO-K1 cells and human CD36-CHO cells were maintained as described previously (34). Rat primary MG were treated with Escherichia coli LPS (1 μg/ml, serotype 0127:B8; Sigma-Alldrich), rat IFN-γ (100 U/ml; PeproTech), mouse TNF-α (150 U/ml; Sigma-Alldrich), mouse M-CSF (50 U/ml; Sigma-Alldrich), rat IL-4 (5 ng/ml; PeproTech), rat IL-13 (5 ng/ml; PeproTech), or human TGF-β1 (2 ng/ml; PeproTech). Mouse primary MG were treated with mouse IL-4 (5 ng/ml; PeproTech).

Immunoblot analysis

Rat primary MG, mouse primary MG, and rat elicited macrophages (5 × 10⁶ cells/100-mm dish) were homogenized in 10 mM Tris-HCl (pH 7.6) containing 1% w/v SDS, 4 mM EDTA, 10% (v/v) glycerol, 1 mg/ml pepstatin A, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Aliquots of homogenates were saved for protein determination and, after addition of 25 mM DTT, samples were incubated at 60°C for 10 min. The samples (50 μg) were then subjected to SDS-PAGE and proteins were electrottransferred to polyvinylidene difluoride membranes. Membranes were incubated with TBS (pH 7.4) containing 2% dehydrated skimmed milk to block nonspecific protein binding. Membranes were then incubated with Abs to Aβ1–16 (6E10, 1/500 dilution), CD36 (L-17, 1/30 dilution), SRA (SRA-E5, 1/2000 dilution), SR-BI (RED-1, 1/1000 dilution), RAGE (5503, 1/560 dilution), NEP/CD10 (H-321, 1/100 dilution), IDE (9B12, 1/1000 dilution), CD40 (T-20, 1/100 dilution), CD86 (421340, 1/500 dilution), or β-actin (AC15, 1/2000 dilution), followed by secondary Abs: HRP-linked Abs against rabbit, goat, or mouse IgG (each diluted 1/1000). Bound HRP-labeled Abs were then detected via chemiluminescence (ECL kit).

Cell assay

For the Aβ degradation assay in medium, rat primary MG (see Fig. 2), rat primary type 2 MG (see Figs. 7 and 9), and mouse primary type 2 MG (see Fig. 10) were cultured in 24-well plates for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). Human CD36-CHO cells (34) and the control CHO-K1 cells (see Fig. 6) were grown in 24-well plates for 4–5 days before the start of the experiment. The cells were washed twice with labeling medium (DMEM containing 3% BSA) and then incubated with 125I-labeled o-Aβ1–42 for 6 h at 37°C in a 5% CO2-humidified air atmosphere. After the 6-h incubation, medium was removed from each well, and soluble radioactivity in TCA (degraded and extracellularly released peptide fragments) was determined as an index of Aβ degradation in medium as described previously (34). After cells were washed three times with 1 ml of labeling medium and then three more times with PBS, they were lysed with 1 ml of 0.1 M NaOH for 30 min at 37°C in deionized H2O and cell proteins were determined with the BCA Protein Assay Reagent (Pierce).

The Aβ degradation activity may thus include both receptor-mediated phagocytic activity and extracellular protease-mediated degradation. For inhibition assays, cells were incubated with or without Abs (10 μg/ml FA6-152 or control IgG for human CD36-CHO cells; see Fig. 6) or fucoidan (100 μg/ml), thiorphan (50 μM), or insulin (100 μg/ml) (see Figs. 7, 9, and 10). Radioactivity of these background dishes was subtracted from values for control experiments. Results are represented as the means ± SD (n = 3).
For the uptake assay, rat primary type 2 MG were cultured in 3.5-cm dishes (Western blotting; see Figs. 4 and 7) or 24-well plates (autogamma counter; see Fig. 9) for 96 h in MEM/10% FCS in the presence or absence of IL-4 (5 ng/ml). The cells were washed with labeling medium (DMEM containing 3% BSA) and then incubated with o-Aβ1–42 (see Figs. 4 and 7) or 125I-labeled o-Aβ1–42 (see Fig. 9) for 3 h at 37°C in the presence or absence of fucoidan (100 μg/ml). The cells were rinsed with ice-cold PBS, treated with proteinase K (100 μg/ml) or trypsin (2.5 mg/ml) at 4°C for 15 min, and then washed again with PBS, to remove all extracellular Aβ, as previously reported (35). For Figs. 4 and 7, proteins (10 μg) in the cell lysate were subjected to Western blot analysis using anti-Aβ1–17 Ab or anti-β-actin Ab. For Fig. 9, cells were lysed with 1 ml of 0.1 M NaOH for 30 min at 37°C, and cell-incorporated radioactivity and cell proteins were determined with an autogamma counter and the BCA Protein Assay Reagent (Pierce), respectively. Radioactivity of these background dishes was subtracted from values for control experiments.

Results

Preparation of o-Aβ1–42 and 125I-labeled Aβ1–42

To determine the microglial clearance of Aβ, o-Aβ1–42 was prepared by a method similar to that of Dahlgren et al. (30), but with a modification of incubation conditions. Fig. 1A shows that Aβ oligomers were formed as dimers to pentamers after 24 h of incubation at 4°C with the chloride form of Aβ1–42 at initial concentrations of 100–500 μM. After 48 h of incubation, the proportion of oligomers and monomers decreased, but fibrillar formation increased. Among the conditions tested, incubation of Aβ at 500 μM for 24 h gave the highest yield (30–40%) of Aβ oligomers. A portion of the oligomer preparation was further iodinated with Na125I via Iodo-Gen, and the resultant sample (namely, 125I-labeled o-Aβ1–42) had a similar electrophoretic pattern on SDS-PAGE (Fig. 1B). Starting with the trifluoroacetate form of Aβ1–42 under the same conditions (data not shown) resulted in a less efficient oligomer yield.

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IL-4 enhances uptake of o-Aβ₁₋₄₂ by MG

Fig. 2A shows endocytic degradation of ¹²⁵I-labeled o-Aβ₁₋₄₂ by primary mixed MG before and after treatment with IL-4. The degraded amount reached 120 ng/mg cell protein after treatment, which was 170% of the untreated control.

To determine which molecular species was incorporated into cells, cell lysate was subjected to Western blot analysis with anti-Aβ₁₋₁₇ Ab (Fig. 2B). Control MG incorporated mainly the Aβ monomer (4 kDa) and dimer (8 kDa) for 3–12 h. In contrast, IL-4-treated MG predominantly incorporated the dimer and tetramer (16 kDa) during the same period, with only slight incorporation of monomer.

The amount of uptake of each species was determined by densitometric scanning, normalized to the total amounts of the samples (control and IL-4 treated at 72 h) on the same blot sheet (Fig. 2B, right panel), and was expressed as a value relative to the total amounts incorporated at 6 h (Fig. 2C). Uptake of monomer reached a maximum around 50% of the total amounts in untreated MG during 3–12 h, which was 2.5 times greater than that of the IL-4-treated MG, and the uptake markedly decreased after 12 h in both samples. During the same period, uptake of dimer in both MG samples reached 40–50% of total incorporation and decreased considerably afterward. In contrast, uptake of tetramer was mainly observed in IL-4-treated MG and reached 30% of the total amount incorporated in 3 h. The uptake level by IL-4-treated MG, which was five times that of untreated MG, was maintained after 3 h. Overall, IL-4-treated MG exhibited preferential uptake of o-Aβ₁₋₄₂ tetramers and dimers to monomers for 3–12 h, compared with control MG that had a preference for monomers and dimers, and uptake by both MG samples decreased afterward.

Selective uptake of o-Aβ₁₋₄₂ by type 2 MG

Several subpopulations of MG are believed to exist in the CNS and may have different functions (36). We separated type 1 MG and type 2 MG from a mixed glial cell culture by using a previously described method (33) and compared uptake of o-Aβ₁₋₄₂ by both cell types. Successful separation of MG types was confirmed, because the type 2 MG obtained expressed essentially no CD40 and weakly expressed CD86 (Fig. 3A), as previously described (28). Using these MG preparations, we found greater uptake of the tetramer and monomer forms of Aβ₁₋₄₂ by IL-4-treated type 2 MG than type 1 MG (Fig. 3B, lane 7 vs lane 3). Fig. 3C gives quantified results after densitometric scanning. Uptake that increased 2.5-fold after IL-4 treatment was apparently suppressed by addition of fucoidan, a ligand for scavenger receptors, which suggests that this process involves a specific scavenger receptor.

Refinement of cellular uptake of oligomeric Aβ₁₋₄₂ by IL-4-treated type 2 MG

The apparent uptake shown in Fig. 2 may incorporate some nonspecific binding of Aβ₁₋₄₂ species to the cell surface, as mentioned by other investigators (35). To exclude nonspecific bound fractions, we performed additional experiments. Brief protease K treatment (4°C for 15 min) of type 2 MG, which had been preincubated with o-Aβ₁₋₄₂ (for 3 h at 37°C), markedly reduced the amounts of tetramer (85% reduction of control) and monomer (75% reduction of control) in IL-4-untreated control cells, as Fig. 4A (lane 3) shows. In IL-4-treated MG treated with protease K (Fig. 4, lane 4), however, the amounts of the Aβ components were almost unchanged (90% of control for the monomer) or weakly reduced (30% of control for the tetramer). These results indicate that the unfavorable nonspecific fractions can be removed by protease treatment and that the Aβ components observed after such treatment are believed to reflect cellular uptake. This result was confirmed by subsequent experiments (Fig. 4B). Treatments with proteinase K (Fig. 4, lanes 1–4) and trypsin (Fig. 4, lanes 5–8) gave similar patterns of o-Aβ₁₋₄₂ uptake: tetramer and monomer were the main uptake components, and IL-4 treatment enhanced uptake (Fig. 4, lane 3). Addition of fucoidan markedly suppressed uptake of both tetramer and monomer with either proteinase K or
Materials and Methods

Proteins (10 μg) in the cell lysate were subjected to Western blot analysis using anti-Aβ1-42 Ab or anti-β-actin Ab. Monomer and tetramer Aβ levels were measured by densitometric scanning and the results were plotted (right panel). The value for nonstimulated type 2 MG (lane 1) was set at 100%. B, Rat type 2 primary MG were cultured as described in A and were then incubated with o-Aβ1-42 (2 μg/ml) for 3 h at 37°C. The cells were treated with proteinase K (100 μg/ml) or trypsin (2.5 mg/ml) at 4°C for 15 min or were untreated, as described in Materials and Methods. Proteins (10 μg) in the cell lysate were subjected to Western blot analysis using anti-Aβ1-17 Ab or anti-β-actin Ab. Monomer and tetramer Aβ levels were measured by densitometric scanning and the results were plotted (right panel). The value for nonstimulated type 2 MG (lane 1) was set at 100%.

Change in expression of CD36 and SRA in type 1 and type 2 MG stimulated with various cytokines or stimulants

We investigated the effects of other cytokines and cell modulators on expression of the scavenger receptors CD36 and SRA and compared these effects in both types of MG. Among the molecules tested, CD36 was strongly induced by IL-4 in type 2 MG but moderately induced in type 1 MG (Fig. 5). Other modulators tested had no effect on the CD36 expression level. With regard to SRA expression, both MGs had similar responses to the effectors. IL-4 suppressed SRA expression to 82 and 83% of untreated controls in type 1 and type 2 MG, respectively. IFN-γ, M-CSF, and LPS caused little change in the SRA level. These results suggest that the two cell types regulate expression of CD36 and SRA differently.

Selective uptake as related to CD36 function

To demonstrate the contribution of CD36 to IL-4-induced clearance of o-Aβ1-42, we first evaluated CD36-CHO cells. The amount of 125I-labeled o-Aβ1-42 degraded by these cells was much higher than that of wild-type CHO cells (Fig. 6A). This dose-dependent increase to 600 ng/mg protein for 10 μg of 125I-labeled o-Aβ1-42 added was six times the level of the wild-type cells. Anti-CD36 Ab (FA6-152) inhibited this endocytic degradation by CD36-CHO cells by 55%, to a level similar to that of wild-type CHO cells (Fig. 6B). This dose-dependent inhibition was 55%, to a level similar to that of wild-type CHO cells (Fig. 6B). No inhibition was observed after addition of control IgG, which demonstrated that the endocytic degradation of 125I-labeled o-Aβ1-42 was mediated by CD36.

To confirm the contribution of CD36 to phagocytic clearance of o-Aβ1-42, type 2 MG from SHR rats with dysfunctional CD36 were compared with cells from wild-type WKY rats.
First, expression levels of CD36, SRA, SR-BI, and RAGE by both types of MG were examined via Western blotting (Fig. 7A). CD36 was clearly absent in SHR MG and was not induced even after IL-4 treatment, whereas it was significantly induced in WKY MG by IL-4. Among other scavenger receptors, SRA and RAGE were present in both MG types and were suppressed considerably after IL-4 treatment. SR-BI was absent in both MG types and was not induced by IL-4.

In a Western blot analysis of cells after proteinase K treatment (Fig. 7B), selective, marked uptake of o-Aβ_{1-42} (tetramer and monomer forms) was observed in IL-4-treated compared with untreated type 2 MG from WKY rats (Fig. 7B, lanes 7 vs 5) after 3 h of incubation with the o-Aβ_{1-42} preparation, but IL-4-treated and untreated MG from SHR rats did not differ in uptake (Fig. 7B, lanes 3 vs 1). IL-4-induced uptake of o-Aβ_{1-42} was markedly suppressed by addition of fucoidan (Fig. 7B, lane 8), which suggests again that a scavenger receptor, most likely CD36, is involved in cellular uptake. The degradation step was quantitatively evaluated by using ^125^I-labeled o-Aβ_{1-42} and type 2 MG from WKY and SHR rats were compared (Fig. 7C). After IL-4 treatment, MG from WKY rats increased degradation of o-Aβ_{1-42} 1.5-fold (Fig. 7C, rows 7 vs 5), whereas MG from CD36-dysfunctional SHR showed no change (Fig. 7C, rows 3 vs 1). Addition of fucoidan also significantly suppressed degraded amounts of o-Aβ_{1-42}, which suggests that a scavenger receptor (or receptors), including CD36, is also involved in the degradation step.

**Effects of cytokines on expression of CD36, NEP, and IDE in type 2 MG**

As a consequence of the finding that IL-4 induced expression of CD36 (Figs. 5B and 7A), and the concomitant increase in uptake of o-Aβ_{1-42} by type 2 MG, we examined whether IL-4 affected expression and activity of the Aβ-degrading enzymes NEP and IDE. We also examined whether the effect would be selectively induced in type 2 MG by particular cytokines. As shown in Fig. 8A, CD36 expression was similarly induced by IL-4 and IL-13, but less effectively by TNF-α. NEP expression was also enhanced by IL-4 and IL-13 but was suppressed by TNF-α and TGF-β1. IL-4 and TGF-β1 showed high potency for induction of IDE expression, followed by IL-13; TNF-α had the lowest potency. It is noteworthy that IL-4 and IL-13 enhanced CD36, NEP, and IDE, which were involved in phagocytosis and catabolism of o-Aβ_{1-42}.

The effect of IL-4 on expression of NEP and IDE was further investigated and compared with expression of the scavenger receptors CD36 and SRA. Expression of NEP and IDE increased considerably after addition of IL-4 and reached a maximum at 5 and 2 ng/ml, respectively (Fig. 8B). The increase in CD36 expression showed a similar dose dependence, whereas SRA expression was conversely decreased at 2–5 ng/ml IL-4 added. The effect of 5 ng/ml IL-4 on their expression changed over time (Fig. 8C): expression of both NEP and IDE uniformly increased in a time-dependent manner until day 10; CD36 expression increased more rapidly to reach a maximum at day 4, followed thereafter by a slight decrease; and SRA expression continued to decrease after addition of IL-4.

**Effects of various ligands on uptake and degradation of ^125^I-labeled o-Aβ_{1-42} in type 2 MG before and after IL-4 treatment**

To evaluate the contribution of NEP, IDE, and CD36 to o-Aβ_{1-42} uptake and degradation, we investigated the effects of their inhibitors on cellular uptake and degradation of ^125^I-labeled o-Aβ_{1-42} in type 2 MG. Effects before and after IL-4 treatment were analyzed and compared (Fig. 9).

Inhibitors of Aβ-degrading enzymes, thiopran as the NEP inhibitor and insulin as the IDE inhibitor, had no effects on the uptake step before and after IL-4 treatment (Fig. 9A). In the degradation step, however, both thiopran and insulin considerably inhibited degradation of o-Aβ_{1-42} at a level similar to that of fucoidan inhibition, in IL-4-treated cells (Fig. 9B, lanes 8 and 10). Without IL-4 treatment, thiopran and insulin also inhibited the degradation, which is likely due to inhibition of activities of NEP and IDE expressed at the original level.

Fucoidan, an inhibitor of various scavenger receptors, inhibited both cellular uptake and degradation of o-Aβ_{1-42}, but strong inhibition was observed for the uptake step (see also Fig. 7B) before and after IL-4 treatment. These data indicate that scavenger receptors function differently in both steps. However, details of this inhibition, such as which scavenger receptor is involved and the extent of its contribution, differ before and after IL-4 treatment. Before IL-4 treatment (control), fucoidan seemed to suppress SRA-mediated phagocytic uptake and degradation, because SRA was expressed at a high level but no CD36 was expressed (Figs. 5 and 8, B and C). In the IL-4-treated MG, however, cellular uptake and degradation increased 2.1 and 1.7 times, respectively (Fig. 9), and CD36-mediated uptake and degradation were suppressed by fucoidan, because expression of SRA was markedly reduced, whereas that of CD36 was enhanced (see also Fig. 8, B and C).
Addition of fucoidan resulted in a marked inhibitory effect on cellular uptake of α-β₄₁₋₄₂ and some inhibitory effect on its degradation in IL-4-treated type 2 MG, whereas thiorphan and insulin inhibited only degradation. These results indicated that both CD36-mediated uptake followed by intracellular degradation and α-β₄₁₋₄₂ catabolism by NEP and IDE are important for IL-4-induced clearance of α-β₄₁₋₄₂.

**Effect of IL-4 on mouse type 2 MG**

The effect of IL-4 on mouse MG was also assessed and compared with that on rat MG described above. IL-4 treatment markedly increased expression of CD36 but only slightly increased NEP expression (Fig. 10A). IL-4 also increased degradation of ¹²⁵I-labeled α-β₄₁₋₄₂ 1.8-fold. This degradation seen with IL-4 treatment was markedly inhibited by addition of fucoidan, whereas it was moderately inhibited by thiorphan (Fig. 10B). In contrast, addition of fucoidan or thiorphan resulted in partial or no inhibition of degradation, respectively, in control MG. These observations were very similar to data for type 2 MG from rat brains described above.

**Discussion**

Many observations have shown that activated MG may contribute significantly to the neuropathology of AD and other neurodegenerative diseases by induction of inducible NO synthase or production of TNF-α (for reviews, see Refs. 4–6). Recently, increasing
numbers of articles have claimed a neuroprotective role of MG in AD: for example, microglial activation by Aβ vaccination (14–16) or recruitment of bone marrow-derived MG (17, 18) contributed to Aβ clearance and concomitant neuroprotection. Among possible beneficial roles of MG in AD, we demonstrated here a novel Aβ clearance mechanism that was induced selectively in type 2 MG by IL-4 treatment.

Relevance of IL-4-induced clearance of o-Aβ₁₋₄₂ to expression of CD36, NEP, and IDE

The clearance selectivity for o-Aβ₁₋₄₂ is of great importance for prevention or treatment of AD, because this molecular species is believed to be more neurotoxic (1–3) than fibrillar Aβ₁₋₄₂. We demonstrated that CD36, NEP, and IDE were responsible, in their own ways, for clearance activity induced by IL-4. Two lines of evidence proved that CD36 played a major role: First, CD36-CHO cells, a CD36-overexpressing cell line, exhibited significant endocyotic degradative activity that was suppressed to the control level by addition of anti-CD36 Ab. Second, MG from SHR rats in which CD36 was dysfunctional did not show this clearance. We were unable to demonstrate directly that o-Aβ₁₋₄₂ was bound to CD36, because it was difficult to remove the considerable amount of nonspecific binding of [¹²⁵I]labeled o-Aβ₁₋₄₂ to CD36-CHO cells (data not shown). Instead, however, intracellular degradation was effectively suppressed by addition of anti-CD36 Ab (Fig. 6B), which suggests that the Ab inhibited o-Aβ₁₋₄₂ binding to CD36 and the subsequent uptake and degradation process. In addition, MG from CD36-dysfunctional SHR rats showed no apparent up-regulation or down-regulation of CD36 expression by MG in AD brains and could mediate production of reactive oxygen species in response to Aβ fibrils, but this role of CD36 apparently differs from our Aβ clearance function.

Several other scavenger receptors have been proposed to contribute to Aβ clearance by MG. SRA is one candidate (8), but the report that SRA knockout mice suppressed uptake of Aβ₁₋₄₀ by only 40% (10) suggested the participation of other molecules in Aβ clearance. Our finding of down-regulated SRA expression in IL-4-treated MG leads us to propose that SRA may not play a major role in IL-4-induced clearance of o-Aβ₁₋₄₂ as described here. This proposal is more likely in view of the effect of IL-4 treatment, which induced CD36 expression and led to higher clearance compared with no treatment. However, considerable SRA expression was retained, and we therefore do not exclude the possibility that CD36 may have an important function and cooperates...
with SRA during IL-4 stimulation. SR-BI is not a candidate, because IL-4 treatment did not induce its expression. RAGE has been reported to mediate Aβ transport across the BBB and accumulation of Aβ in the brain (38). RAGE may have functions different from Aβ-degrading enzymes NEP, even in mouse primary type 2 MG preparations: Landreth and colleague (27) previously showed that, in both BV-2 cells and mouse primary MG, IL-4, and other anti-inflammatory cytokines neither stimulated nor inhibited phagocytosis elicited by any phagocytic ligands tested including fibrillar Aβ (27). They suggested that these anti-inflammatory cytokines may oppose the harmful effects of IL-1β, which stops phagocytosis. However, our data clearly show that IL-4 treatment enhanced degradation of 125I-labeled Aβ1–42, by CD36-mediated intracellular phagocytosis and extracellular proteolysis involving Aβ-degrading enzymes such as NEP, even in mouse primary type 2 MG (see Fig. 10). This discrepancy may have two explanations. One is the difference in molecular species of Aβ used: Landreth and colleague (27) used primarily the fibrillar form, whereas we used mainly oligomers (monomer to pentamer), not the fibrillar form (see Fig. 1). The second is the difference between the MG preparations: Landreth and colleague (27) prepared samples by the simple shaking-off method, which is thought to provide mainly type 1 MG, whereas we prepared samples by using a modified mild trypsinization method, thereby producing predominantly type 2 MG (see Fig. 3A).

Selective Aβ clearance by type 2 MG

We demonstrated that two microglial preparations, type 1 and type 2, had different responses to IL-4 stimulation. Heterogeneous microglial populations are known. For example, Kanzawa et al. (28) reported no and lower expression of CD40 and CD86, respectively, in cell lines that were established as type 2 MG, which was in sharp contrast to type 1 MG. Although MG have been long believed to have functions in neuroinflammatory reactions as macrophage-like cells in the brain or in neuroimmunomodulation as APCs, neuroprotective roles of MG were claimed relatively recently (11, 12). However, it is still unclear whether all MG or only one or more subtypes (or subtypes) is responsible for such neuroprotective activity. A similar situation pertains for the microglial response to Aβ peptide: it was not clear whether one MG subtype had an inflammatory reaction to Aβ, whereas another subtype exhibited an anti-inflammatory response to Aβ or clearance of the peptide. In the present study, we demonstrated that type 2 MG induced CD36 more selectively than did type 1 MG after IL-4 stimulation and that type 2 MG showed clearance for Aβ1–42, by CD36-mediated intracellular phagocytosis (Fig. 9B). Insulin inhibited only the degradation process, suggesting cell surface receptors on which insulin may bind are not involved in the Aβ clearance. Insulin is considered to be an inhibitor for IDE but not specific for IDE, and other proteases that could be inhibited by insulin may not exclude the role for degrading Aβ. For human neprilysin, it is reported to degrade Aβ peptide not only in the monomeric form but also in the pathological oligomeric form (43). However, our data on degradation by IDE seem in conflict with data from Selkoe’s group (44) that showed that IDE can degrade monomer but not oligomer. Although our data on IL-4 treatment suggest it may lead to uptake of more oligomers, we assume that our Aβ preparation contains some monomer (Fig. 1). Together, these results suggest that IL-4 may be modulating extracellular degradation of monomer here, which was not exactly measured.

Landreth and colleague (27) previously showed that, in both BV-2 cells and mouse primary MG, IL-4, and other anti-inflammatory cytokines neither stimulated nor inhibited phagocytosis elicited by any phagocytic ligands tested including fibrillar Aβ (27). They suggested that these anti-inflammatory cytokines may oppose the harmful effects of IL-1β, which stops phagocytosis. However, our data clearly show that IL-4 treatment enhanced degradation of 125I-labeled Aβ1–42, by CD36-mediated intracellular phagocytosis and extracellular proteolysis involving Aβ-degrading enzymes such as NEP, even in mouse primary type 2 MG (see Fig. 10). This discrepancy may have two explanations. One is the difference in molecular species of Aβ used: Landreth and colleague (27) used primarily the fibrillar form, whereas we used mainly oligomers (monomer to pentamer), not the fibrillar form (see Fig. 1). The second is the difference between the MG preparations: Landreth and colleague (27) prepared samples by the simple shaking-off method, which is thought to provide mainly type 1 MG, whereas we prepared samples by using a modified mild trypsinization method, thereby producing predominantly type 2 MG (see Fig. 3A).
Pathophysiological significance of IL-4-induced Aβ clearance and source of IL-4 in the CNS

MG express a receptor for IL-4. IL-4 was shown in vitro to suppress microglial production of IFN-γ-induced MHC class II (46), TNF-α, NO (47), and IL-6 (23), but relatively little is known about its role in microglial function in the CNS, probably because generation of IL-4 in the CNS has not been proved. mRNA for IL-4 was reportedly expressed in human brain (23), and expression levels of mRNA and proteins were markedly decreased in Tg2576 mice, which served as an AD model (48). Decreased hippocampal IL-4 concentration and IL-4-stimulated signaling were also found in aged rats compared with young rats (49). Although the amount of IL-4 found (20–40 pg/mg homogenate) in the hippocampal tissue was smaller than the amount of IL-1β (400–800 pg/mg homogenate), it is noteworthy that the concentration was comparable to that (5 ng/ml) applied in our study.

Another feasible source of IL-4 in AD brains is Th2 cells that infiltrate the brain. Activated lymphocytes have been demonstrated to enter the CNS in the absence of overt inflammatory disease (50). Other reports further suggest that T cells are activated in AD patients and that these cells exist both in the periphery and as infiltrates in the brain (Refs. 51–53; for review, see Ref. 54) via a rather leaky BBB (55, 56). Their numbers, however, can be lower than those found in the neurodegenerative disorders multiple sclerosis and experimental autoimmune encephalitis, in which the CNS is severely injured and the BBB is disrupted.

With respect to cross-talk between MG and T cells, Monsonego et al. (57) showed in cultured cells that activated MG served as Aβ antigen-presenting cells and induced an adaptive immune response. They also demonstrated that Aβ-reactive Th1 cells underwent apoptosis after stimulation, accompanied by increased levels of IFN-γ, NO, and caspase 3. In contrast, MG-mediated proliferation of Aβ-reactive Th2 cells led to expression of the Th2 cytokines IL-4 and IL-10, which counteracted the toxic levels of NO induced by Aβ. As an aid to understanding the in vivo T cell response in the CNS of patients with AD, a typical example of the response is induction of meningeoclephalin after immunization of AD patients with Aβ1–42 (58).

Akiyama and McGeer (59) demonstrated involvement of MG in immunotherapeutic clearance of Aβ plaques. This report suggested that cross-talk between MG and T cells does exist and that therefore, in addition to the anti-inflammatory action of IL-4, IL-4-induced clearance by MG can be expected, if this IL-4 is derived from Th2 cells that infiltrate the AD brain. As a novel therapeutic method in AD, therefore, the use of agents to stimulate IL-4 production by either neuronal/glial cells or Th2 cells in the CNS, to augment Aβ-associated clearance activity, may be useful. However, IL-4 appears to contribute to pathogenesis of allergies and brain tumors, such as asthma (60) and glioma (61), respectively, and these effects should be kept in mind during development of IL-4 agonists for use as therapeutic agents in AD.

In conclusion, IL-4-stimulated microglial clearance of Aβ, which is mediated by induced CD36, NEP, and IDE, has potential utility for development of therapeutic strategies for AD, as evidenced by the following points: 1) induction of Aβ clearance by the anti-inflammatory cytokine IL-4, 2) simultaneous induction of three molecules (CD36, NEP, and IDE) that participate in clearance of Aβ1–42, and 3) preferential clearance of the highly neurotoxic α-Aβ1–42 which may prevent development and/or progression of AD. Among the different microglial phenotypes, type 2 MG was demonstrated to be sensitive to IL-4 and to have beneficial roles in Aβ clearance. In addition to the innovative method of mucosal Aβ vaccination (62), the technique of using agents to stimulate IL-4 production in the brain parenchyma or around the BBB, as based on the novel clearance mechanism of Aβ1–42 presented here, may spur development of new anti-inflammatory therapeutic strategies for AD.

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Disclosures

The authors have no financial conflict of interest.

References
