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Lead exposure increases levels of β-amyloid in the brain and CSF and inhibits LRP1 expression in APP transgenic mice

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ABSTRACT

Lead (Pb) is an environmental factor suspected of contributing to neurodegenerative diseases such as Alzheimer's disease (AD). In AD, it has been postulated that increased production and/or decreased metabolism/clearance of β-amyloid (Aβ) may lead to amyloid plaque deposition as well as a cascade of other neuropathological changes. It has been suggested that Pb exposure may be associated with AD-like pathology and severe memory deficits in humans. Therefore, we investigated whether Pb exposure could induce Aβ accumulation in the brain. In this study, we demonstrated that acute Pb treatments lead to increased levels of Aβ in the cerebrospinal fluid (CSF) and brain tissues. Interestingly, Pb treatments did not affect Aβ production in brain neurons. Furthermore, Pb treatments significantly decreased LRP1 protein expression in the choroid plexus (CP). Our results suggest disrupted LRP1-mediated transport of Aβ in this region may be responsible for the Aβ accumulation in brain.

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. It is characterized by the progressive loss of memory and impairment of cognitive function. Accumulation of β-amyloid (Aβ) within extracellular spaces of the brain is believed to be an initial feature of AD pathogenesis [19]. Additionally, it has been reported that Aβ can stimulate hyperphosphorylation of tau that leads to formation of neurofibrillary tangles in the brain, another hallmark of Alzheimer disease (AD) [10].

Several processes may increase Aβ levels in the brains of AD patients including increased production of Aβ, decreased clearance within neurons, and disrupted influx or efflux transport of Aβ through brain barrier systems. The blood–brain barrier (BBB) and blood–cerebrospinal fluid (CSF) barrier (BCB) are two brain barriers that separate the systemic blood circulation from the brain. The choroid plexus (CP), located within brain ventricles, is the major BCB site. CSF is produced in the brain by the choroid plexus (CP) and absorbed by arachnoid granulations. Several studies had shown the presence of Aβ and its transport across the BCB [3,18,20–22]. Since the CP is in direct continuity with the cerebral interstitial fluid (ISF) and CSF, Aβ in the brain extracellular space can freely enter into the CSF [2]. The concentration ratio of $A\beta_{1-42}$ and $A\beta_{1-40}$ in CSF has been considered a potential biomarker for AD diagnosis [25]. Aβ has been detected in the CP of AD patients [13]. This tissue has been demonstrated to be immunoreactive to antibodies against Aβ and its precursor protein, APP [4,20], suggesting that the CP may be involved in Aβ brain clearance [1,3]. Thus, it is interesting to explore the role of the BCB in Aβ transport and metabolism.

The sporadic nature of most AD cases strongly suggests that environmental factors may play significant roles in AD pathogenesis. Lead (Pb) is an environmental neurotoxin known to produce detrimental effects in the nervous system [17]. Pb exposure is associated with both peripheral and central neurological effects including memory deficits and AD-like pathology [24,26]. Animal studies and human autopsy data have established a clear relationship between Pb exposure and the accumulation of Pb in the CP [16,30]. Accumulation of Pb in the CP reduces the tightness of BCB [23]. Recent evidence suggests that $A\beta_{1-40}$ is actively transported from the CSF to the blood via the CP [3] and another recent study showed an increased intracellular accumulation of Aβ in the CP of rats that had been pretreated with Pb for 24 h [1]. However, it remains unclear how brain Aβ levels are affected by Pb treatment.

Low-density lipoprotein receptor-related protein-1 (LRP1) is a member of the LDL receptor family that is involved in the clearance pathway of Aβ [11]. Evidence suggests that LRP-1 in the BBB may play a role in Aβ efflux from the brain to the blood [9]. Recently, it was reported that reduced LRP1 expression in the CP following Pb

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treatments may contribute to Pb-induced intracellular accumulation of Aβ, indicating this protein may be involved in Pb-inhibited Aβ efflux through the CP [1]. However, it remains to be determined whether and how the LRP1 expression in different types of brain cells is affected by Pb in transgenic AD mice.

The current study design uses a amyloid precursor protein (APP) transgenic mouse model overexpressing human APP with a mutation (V717F) that causes an autosomal dominant form of familial AD [6,8] in order to test whether acute Pb exposure increases brain levels of Aβ and disrupts its clearance by the CP. Studies were designed to investigate (1) the levels of Aβ in the CSF, blood and selected brain regions following acute exposure to Pb and (2) the expression of LRP1 in the CP and brain regions after Pb exposure. The outcomes of this study should help explore the mechanism whereby Pb exposure disturbs brain homeostasis of Aβ and its relationship to LRP1 in the BCB.

APP transgenic mice (APP V717F) and their wild type littermates (all on a C57BL/6 genetic background) were bred in the Animal Center at Indiana University School of Medicine. The mice were 2 months of age at the time of the experiment. The mice ($n = 9$ per group) were injected, i.p., with 50 mg Pb-acetate/kg (i.e., 27 mg Pb/kg) or with an equivalent molar concentration of Na-acetate, and were sacrificed 24 h later. At 24 h post injection, mice were euthanized. Whole blood, CSF and brain tissues were collected from each animal and the blood was centrifuged for 10 min ($2000 \times g$) to separate plasma and red blood cells (RBCs). Elemental profiling via inductively coupled plasma mass spectrometry (ICP-MS) was performed for Pb. Samples were transferred to a Teflon 96-well plate and digested with 0.15 mL of concentrated HNO_3 (Mallinckrodt, AR Select grade, Hazelwood, MO, USA) at 110˚ C for 4 h. Each sample was diluted to 1.45 mL with 18 MΩ water and analyzed on a PerkinElmer Elan DRCe ICP-MS. Indium (EM Science, Gibbstown, NJ, USA) was used as an internal standard [14]. Mice cortical and hippocampal neurons were generated from the forebrains of 1-day old pups of APP transgenic mice as previously described [7]. After three days in culture, neurons were treated with 1, 5, or 10 μM Pb for 24 h. Media were then collected to determine levels of $A\beta_{total}$. Levels of Aβ were assayed by sandwich ELISA as previously described [12]. Briefly, the tissue or cell samples were assayed using 96-well ELISA plates that were coated with antibodies, 2G3, 21F12, and 266.2 (generous gifts from Eli Lilly, Indianapolis, IN, USA) to determine $A\beta_{1-40}$, $A\beta_{1-42}$, and $A\beta_{total}$, respectively. Biotin-3D6 (another generous gift from Eli Lilly, Indianapolis, IN, USA) was used to detect Aβ. Total protein concentrations in the brain were determined using the Bradford protein assay, and the concentration of Aβ in the tissues was reported as ng/mg of total protein. Western blot analyses were performed using an antibody directly against LRP1 (1:250, Aviva Systems Biology, San Diego, CA, USA) on the mice CP, cortex, hippocampus and cerebellum. β-Actin was also assayed as a loading control. Band intensities were quantified and results were reported as the ratio of LRP1 to β-actin in CP [27]. Following Pb exposure, brains from APP transgenic mice were fixed and made into paraffin sections. The sections were stained with rabbit anti-mouse LRP1 (1:350, Aviva Systems Biology, San Diego, CA, USA), followed by a biotin goat-anti rabbit secondary antibody. After washing, the sections were incubated with fluorescein avidin DCS (1:500, Vector, Burlingame, CA, USA) for 30 min and visualized under the microscope [15]. The fluorescence intensity was quantified using Image J software and reported in arbitrary units (a.u.). The data were analyzed using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical analyses of the differences between groups were done using Mann–Whitney nonparametric tests. All data are expressed as mean ± SD. Quartiles were used to measure the spread of data distribution on APP mice brain Aβ levels. Differences between two means were considered significant when p was equal or less than 0.05. All statistical tests applied were 2-sided.

In order to examine whether Pb exposure affected brain levels of Aβ, we treated APP transgenic mice with a single injection of 50 mg/kg of Pb acetate (27 mg Pb/kg) or an equivalent molar concentration of Na-acetate, intraperitoneally (i.p.). This dose regimen has been shown to produce a significant accumulation of Pb in the CP during a short period of time [30]. After 24 h exposure, the levels of Pb in brain tissues and blood were measured by ICP-MS. The amount of Pb in control mice CP was 0.029 ± 0.018 μg/g tissue; cortex, 0.001 ± 0.000 μg/g; hippocampus, 0.001 ± 0.000 μg/g; cerebellum, 0.001 ± 0.000 μg/g and blood, 0.452 ± 0.291 μg/dL. In addition, The amount of Pb in Pb-treated mice was found to be about 10–200-fold greater than control mice, the CP was 0.2458 ± 0.1106 μg/g; cortex, 0.007 ± 0.002 μg/g; hippocampus, 0.014 ± 0.008 μg/g; cerebellum, 0.011 ± 0.004 μg/g and blood, 63.22 ± 15.70 μg/dL ($p < 0.05$, $n = 3$–6). Pb accumulated extensively in the CP after acute exposure. Levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in plasma, CSF and brain tissues were determined by ELISA. The CSF levels of $A\beta_{1-40}$ in Pb-treated mice and control mice were 0.590 ± 0.300 ng/ml (median, 0.571 [range, 0.280–0.919]) and 0.255 ± 0.120 ng/ml (median, 0.245 [range, 0.133–0.359]). The data showed a significant increase (131.4%, $p < 0.05$) in Pb-treated mice as compared to controls, whereas there was no significant difference in levels of $A\beta_{1-42}$ in the CSF (Fig. 1A). Additionally, hippocampal levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in Pb-treated mice were 0.013 ± 0.007 ng/mg (median, 0.013 [range, 0.008–0.015]) and 0.018 ± 0.007 (median, 0.019 [range, 0.014–0.021]) ng/mg; in control mice were 0.008 ± 0.004 (median, 0.013 [range, 0.008–0.015]) ng/mg and 0.013 ± 0.003 (median, 0.013 [range, 0.011–0.015]) ng/mg. Pb exposure significantly increased hippocampal levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ (62.5% in $A\beta_{1-40}$, and 38.5% in $A\beta_{1-42}$, $p < 0.05$) (Fig. 1B). Furthermore, cortical levels of $A\beta_{1-40}$ in Pb-treated mice were also higher than the control group, although the difference did not reach a statistical significance (Fig. 1B). There were no significant differences in the cortical level of $A\beta_{1-42}$ and cerebellar levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ between Pb-treated and control groups (data not shown). The plasma levels of Aβ in both treated and untreated mice were also not significantly different (Fig. 1A).

The increase of Aβ in the brain following Pb exposure may be the result of overproduction of Aβ in the brain or disrupted transport of Aβ out of brain by the brain barrier system. To test whether Pb exposure affected Aβ production in brain, we treated primary cultures of cortical and hippocampal neurons that were isolated from the cortices and hippocampi of 1-day old APP transgenic mice pups with Pb. Following incubation of cortical and hippocampal neurons with 1, 5, or 10 μM Pb for 24 h, the levels of $A\beta_{total}$ in culture media were not changed (Fig. 1C). The toxicity of Pb to neurons was also tested; the results indicated that Pb at these concentrations were not toxic to primary neurons.

Since it appeared that acute exposure of Pb did not affect Aβ production in brain cells, we hypothesized that the increases of brain Aβ levels following Pb exposure may result from disrupted Aβ transport at the BCB system. APP transgenic mice received acute Pb exposure (27 mg Pb/kg, i.p., 24 h) and the protein levels of LRP1 in the CP were determined by Western blot. Our results demonstrated that the CP levels of LRP1 were significantly lower in APP transgenic mice receiving acute Pb exposure than those without Pb treatment (27.1%, $p < 0.05$, Fig. 2A and B). To confirm this observation, the frozen brain sections of mice with or without Pb treatments were stained with immunofluorescence-labeled LRP1 antibody. Quantification of the fluorescence intensity using Image J software revealed a significant difference between control mice (36.9 ± 3.0) and Pb-treated mice (25.4 ± 2.7) ($p < 0.001$). Fluorescence intensity was expressed in arbitrary units (a.u.). The

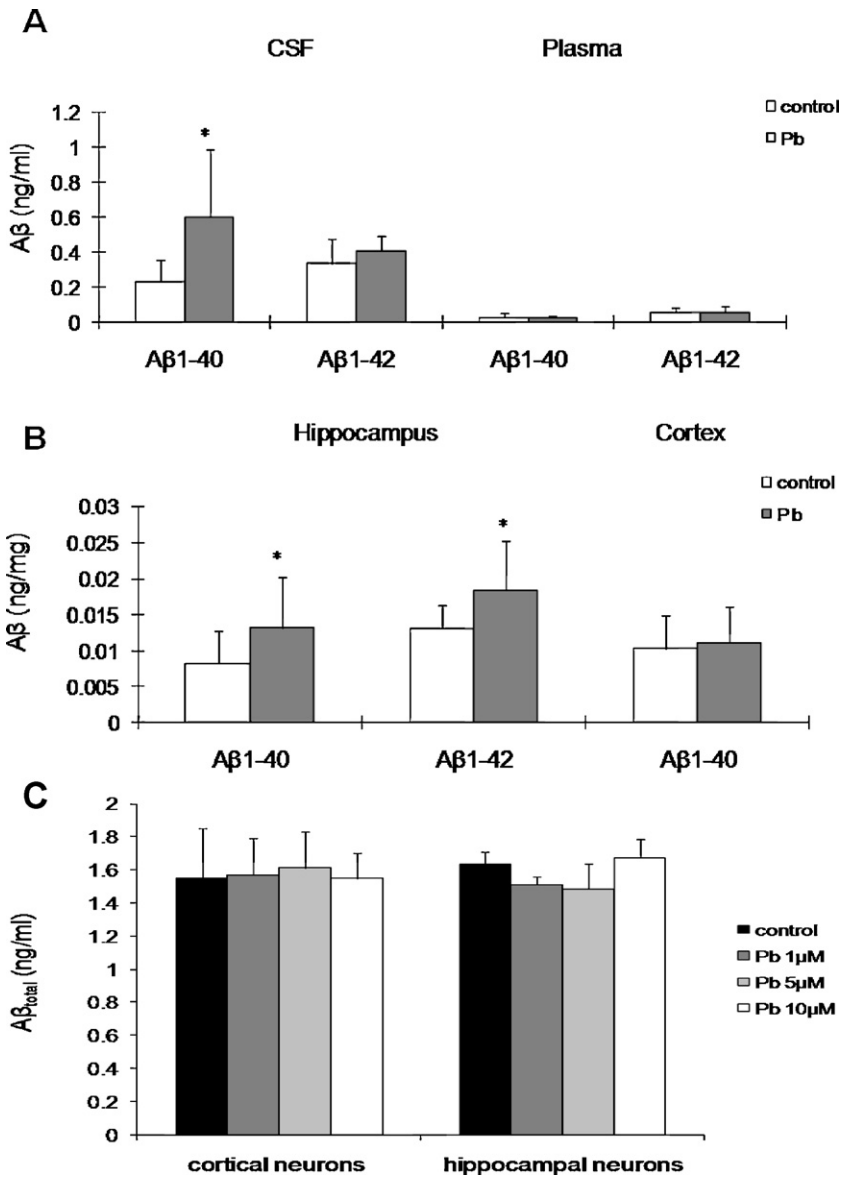

Fig. 1. (A and B) Alteration of Aβ levels in the CSF, plasma and brain tissues of APP transgenic mice following acute Pb exposure. APP transgenic mice received a single i.p. injection of 50 mg/kg of Pb acetate (27 mg Pb/kg) or with an equivalent molar concentration of Na-acetate as control. The levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ were determined by ELISA. (A) $A\beta_{1-40}$ and $A\beta_{1-42}$ in the CSF and plasma. (B) $A\beta_{1-40}$ and $A\beta_{1-42}$ in hippocampus and cortex. (C) Effect of Pb exposure on the production of $A\beta_{total}$ in primary culture of cortical and hippocampal neurons from APP transgenic mice. Cultured cells were treated with 1, 5, or 10 μM Pb for 24 h, followed by assessment of $A\beta_{total}$ level. Level of $A\beta_{total}$ in brain neuron conditional medium was measured by ELISA. The white bars represent controls and the grey bars represent Pb exposed mice. Data represent mean ± SD, $n = 8$ per group in (A), $n = 9$ per group in (B), $n = 6$ per group in (C); $^*p < 0.05$.

levels of LRP1 were markedly lower in Pb-treated mice than control mice (Fig. 2C). Protein levels of LRP1 in the brain regions were also determined by Western blot. There was no difference in LRP1 expression in the brains of control mice and Pb-treated mice, further suggesting LRP1 in CP may be involved in Pb-induced brain levels of Aβ.

Exposure to Pb has caused symptoms and features similar to AD including memory deficits and neurodegeneration [24,28]. However, it remains unclear whether Pb-associated memory deficits are due to Aβ accumulation in brain. The concentration of Pb in human blood at which results in Pb-induced neurotoxicity is 25 μg/dL or higher (>70 μg/dL) (also called an elevated blood lead level or EBLL). The CDC's National Surveillance Data (1997–2007) showed that some children have EBLL ≥ 70 μg/dL. In the present study, the blood Pb concentration in Pb-treated mice is about 60 μg/dL. Thus, compared to human exposure, the Pb dose used in this study is causes symptoms similar to those found in children. Animal studies and human autopsy data have shown that the CP is one of the brain targets of Pb accumulation [16,30]. Our data also demonstrate that after acute Pb exposure, Pb in the CP is increased compared to other brain regions. Our data clearly demonstrate that acute exposure to Pb increases levels of Aβ in the CSF and hippocampus in APP transgenic mice, which genetically over-express Aβ. This is the first study showing that Pb acute exposure increases brain Aβ levels in APP transgenic mice. In the present study, the CSF level of $A\beta_{1-40}$ showed a substantial elevation in Pb-treated mice as compared to controls. Additionally, both $A\beta_{1-40}$ and $A\beta_{1-42}$ were significantly increased in the hippocampus, a region with the most abundant Aβ software production. Interestingly, this phenomenon between Pb and Aβ was not found in primary cortical and hippocampal neuronal cultures. The results suggest that the increased brain levels of Aβ may be not a direct result of overproduction of Aβ in the brain. Similar to CSF, cortical levels of $A\beta_{1-40}$ in Pb-treated mice were also higher than the control group, albeit not significantly. Addi-

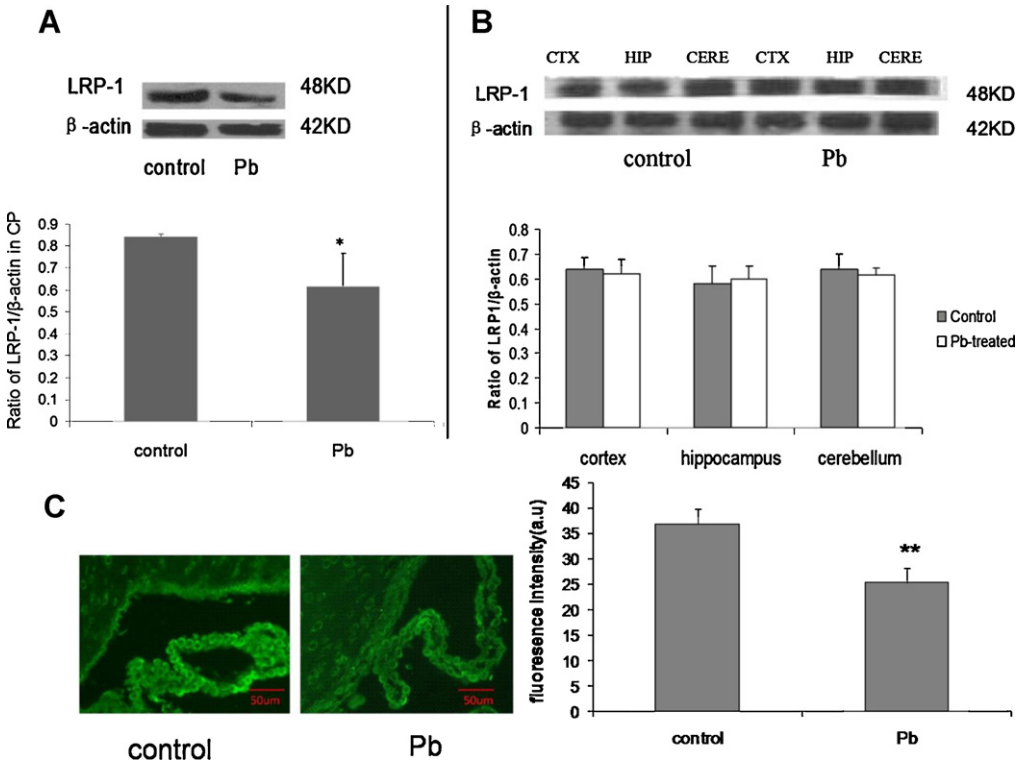

Fig. 2. Alteration of LRP1 protein expressions in the CP, cortex, hippocampus and cerebellum of APP transgenic mice following acute Pb exposure. LRP1 protein expression was determined by Western blot; β-actin was used as the protein loading control. Amounts of LRP1 were estimated from the corresponding band densities and normalized to those of β-actin. (A) LRP1 protein expression in the CP. (B) LRP1 protein expression in cortex, hippocampus and cerebellum. (C) Immunofluorescence staining of LRP1 in the CP of APP transgenic mice. Data represent mean ± SD, $n = 3$ per group; $^{*}p < 0.05$, $^{**}p < 0.001$.

tionally, there was no significant difference in the level of $A\beta_{1-42}$ in CSF and cortex. $A\beta_{1-40}$ is soluble and easily cleared by the brain barrier system, while $A\beta_{1-42}$ is more hydrophobic, rendering its transport through the brain barrier system to the plasma more difficult. Therefore, Pb exposure appears to inhibit primarily $A\beta_{1-40}$ not $A\beta_{1-42}$ transport in the brain barrier systems. In other words, this phenomenon suggests that Pb exposure may decrease or inhibit Aβ clearance systems. Noticeably, the plasma levels of Aβ in Pb exposed mice were not changed. Since the levels of Aβ in plasma were very low, it is possible that the subtle changes of eluted Aβ in the relatively large volume of blood following Pb treatments may be diluted below the detection limit in the current experiment. In order to further elucidate the relationship between Pb exposure and brain accumulation of Aβ, a chronic lead exposure experiment is currently underway.

LRP1 is considered to be an important protein that mediates the transport of Aβ through cellular membranes in the brain [11]. A previous study with rats showed that Pb pretreatment inhibits LRP-1 expression in the CP and this inhibition may cause an intra-cellular accumulation of infused synthetic Aβ [1]. However, it remains unclear whether acute exposure of Pb in transgenic APP animals with genetically over-expressed Aβ could also affect CP levels of LRP-1. Data presented in this study clearly indicate that exposure to Pb in APP transgenic mice results in a substantial decrease in the CP level of LRP1, which does not occur in other brain regions tested. This suggests that increased levels of Aβ after Pb treatment in transgenic APP mice is mediated, at least in part, by the inhibition of LRP1 in the CP, but not in the brain parenchyma. Conceivably, a decreased level of LRP1 may result in a functional deficit in CP transport of Aβ from the CSF to the blood stream as observed in our study with increased Aβ in mice CSF and brain tissue. Thus, it becomes logical that LRP1 plays an important role in transporting Aβ out of the CSF, and this process could be altered by Pb accumulation in the CP tissue. Interestingly, the promoter region of the LRP1 gene contains a Sp1-rich domain [5] and Pb has been shown to alter the binding of Sp1 to its targeted DNA sequences [29]. Thus, it is possible that Pb, by interfering with the Sp1 binding capacity, may interfere with gene expression of LRP1. Certainly, it remains to be determined why acute exposure of Pb only affects CP levels of LRP1. Furthermore, since CSF is reabsorbed at the granulationes arachnoideales, it is reasonable to investigate whether Aβ levels in that area are affected by Pb exposures in future.

In summary, acute exposure to Pb leads to increased CSF and hippocampal levels of Aβ in APP transgenic mice. This effect may be due to its inhibition of LRP1, a key Aβ transport protein in the CP. Our study suggests that Pb is a potential environmental factor in the dysregulation of Aβ homeostasis and may subsequently contribute to the pathogenesis of AD.

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