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IL-34 exacerbates pathogenic features of Alzheimer's disease and calvaria osteolysis in triple transgenic (3x-Tg) female mice

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ABSTRACT

Hallmark features of Alzheimer's disease (AD) include elevated accumulation of aggregated Aβ40 and Aβ42 peptides, hyperphosphorylated Tau (p-Tau), and neuroinflammation. Emerging evidence indicated that interleukin-34 (IL-34) contributes to AD and inflammatory osteolysis *via* the colony-stimulating factor-1 receptor (CSF-1r). In addition, CSF-1r is also activated by macrophage colony-stimulating factor-1 (M-CSF). While the role of M-CSF in bone physiology and pathology is well addressed, it remains controversial whether IL-34-mediated signaling promotes osteolysis, neurodegeneration, and neuroinflammation in relation to AD. In this study, we injected 3x-Tg mice with mouse recombinant IL-34 protein over the calvaria bone every other day for 42 days. Then, behavioral changes, brain pathology, and calvaria osteolysis were evaluated using various behavioral maze and histological assays. We demonstrated that IL-34 administration dramatically elevated AD-like anxiety and memory loss, pathogenic amyloidogenesis, p-Tau, and RAGE expression in female 3x-Tg mice. Furthermore, IL-34 delivery promoted calvaria inflammatory osteolysis compared to the control group. In addition, we also compared the effects of IL-34 and M-CSF on macrophages, microglia, and RANKL-mediated osteoclastogenesis in relation to AD pathology *in vitro*. We observed that IL-34-exposed SIM-A9 microglia and 3x-Tg bone marrow-derived macrophages released significantly elevated amounts of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, compared to M-CSF treatment *in vitro*. Furthermore, IL-34, but not M-CSF, elevated RANKL-primed osteoclastogenesis in the presence of Aβ40 and Aβ42 peptides in bone marrow derived macrophages isolated from female 3x-Tg mice. Collectively, our data indicated that IL-34 elevates AD-like features, including behavioral changes and neuroinflammation, as well as osteoclastogenesis in female 3x-Tg mice.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly 65 + years old, characterized by progressive neurodegeneration and a gradual decline in memory and cognitive functions with elevated prevalence in females [1,2]. The hallmark features of AD

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neurodegeneration include elevated accumulation of aggregated amyloid beta (Aβ) peptides and hyperphosphorylated Tau protein, leading to the deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles, respectively, in the brain [3]. Furthermore, several studies increasingly recognized that neuroinflammation occurs earlier than neurodegeneration in patients with AD [4–6]. Neuroinflammation generally refers to elevated production of various pro-inflammatory cytokines, including tumor-necrosis factor α (TNF-α), Interleukin-1 beta (IL-1β), and Interleukin-6 (IL-6), within the central nervous system (CNS) [7]. In addition, compelling evidence suggests that the Receptor for Advanced Glycation End Products (RAGE) also serves as a cell surface receptor for Aβ promoting microglia and macrophage dysfunction in various experimental models of AD [8,9]. It is essential to mention that multiple systemic pathological insults, including infection, trauma, ischemia, and toxins, exacerbate neuroinflammation [10,11].

Published studies demonstrated that pro-inflammatory activation of macrophages and microglia represent key elements in neuroinflammation and the trajectory of AD. Signaling through the colony-stimulating factor-1 receptor (CSF-1r) is critical for maintaining the physiological and pathological signaling of bone marrow-derived macrophages and CNS microglia. The CSF-1r receptor is activated by two distinct cytokine ligands, namely macrophage colony-stimulating factor-1 (M-CSF) and interleukin-34 (IL-34) [12–15] that are essential for macrophages and microglia viability, development, and proliferation [16,17]. Ma et al. verified that IL-34-treated microglia show diminished neuroprotective effect compared to M-CSF-treated microglia [18]. Furthermore, it was demonstrated that ligation of M-CSF with CSF-1r was significantly inhibited by IL-34 in macrophages and osteoclast precursors cells [15], indicating a potential critical role of IL-34 in proliferation of microglia and macrophages. By contrast, the critical role of IL-34 in elevated RANKL-primed osteoclastogenesis was demonstrated [19]. Elevated bone loss in female patients with AD was also reported [20], indicating that our knowledge about the impact of IL-34 on inflammation, which is mediated in part by macrophages and CNS microglia, and inflammatory osteolysis in the context of sex-associated AD, remains limited.

In this study, we aimed to test the effects of local calvaria injection with mouse recombinant IL-34 protein on cognition and neuropathology as well as osteolysis associated with AD using male and female triple transgenic (3x-Tg) mouse model that is commonly used to assess potential therapies for the treatment of AD [21–23]. These mice feature three human mutations, including the Swedish amyloid precursor protein *(APP)* (KM670/671NL), the presenilin 1 *PSEN1* M146V, and the microtubule-associated protein tau *MAPT* P301L mutations [24], and demonstrate elevated RAGE-dependent neuroinflammation [25]. Using *in vitro* assays, we also compared the effects of IL-34 and M-CSF on pro-inflammatory (M1) and anti-inflammatory (M2)-proliferated microglia, bone marrow derived macrophages, and RANKL-primed osteoclastogenesis.

2. Material and methods

2.1. Animals

Female and male (two-month-old) 3x-Tg mice (B6;129-*Psen1tm1Mpm* Tg(APPSwe,tauP301L)1Lfa/Mmjax) were obtained from Mutant Mouse Resource and Research Centers supported by NIH. The animals were kept on a 12-hour light-dark cycle at a constant temperature, with free access to food and water. This study was conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Nova Southeastern University and Indiana University School of Medicine.

2.2. IL-34 treatment and behavioral assessment

To evaluate the effects of IL-34 on AD cognitive behavioral phenotype, 3x-Tg mice were randomly divided into two experimental groups (10 mice/sex/group). Starting at 3 months of age, group I was subcutaneously injected with 100 μl of mouse recombinant IL-34 protein (1 μg/ml) solution in PBS over the calvaria bone every other day for 42 days. Group II received PBS alone and served as control. Then, we conducted a battery of behavioral tests to evaluate the exploratory behavior, anxiety, and memory skills. Briefly, the Elevated Zero maze test was conducted first to assess the number of head dips on Day 43 as described [26,27]. The maze was constructed of black acrylic in a circular track 50 cm in diameter and elevated 50 cm from the floor (Stoelting). The maze was divided into four quadrants of equal length with two opposing open quadrants with 1 cm high clear acrylic curbs to prevent falls and two opposing closed quadrants with black acrylic walls. A 5 min trial under the same lighting conditions began with the animal placed in the center of a closed quadrant. Mouse behavior was recorded for 10 min, and the number of head dips was counted later.

At Day 44, we counted the number of fecal boli using the Open field maze [28,29]. The Open field test comprised a square arena (60 ×60 cm). Mice were placed at the center of the square arena consisting of a blue plastic board surrounded by blue plastic walls (40 cm in height). The test was initiated by placing a single mouse in the middle of the arena and letting it move freely for 5 min. Mouse behavior was video-recorded, and the number of fecal boli was later counted.

Finally, we evaluated working memory in 3x-Tg mice exposed to IL-34 and corresponding controls using spontaneous alternation behavior in a Y-maze on day 45. The test was performed in a symmetrical grey Plexiglas Y-Maze with three arms (20 cm long × 10 cm wide × 20 cm high) at 120° angles, designated A, B, and C. Mice were introduced to the center of the maze and then were allowed to explore the three arms for 6 min freely. The number of arm entries and the number of triads were recorded to calculate the percentage of alternation with the following formula:

$$\left(\frac{Alternations}{ArmEntry-2}\right) * 100$$

An entry occurred when all four limbs were within the arm.

Behavioral data obtained from the Elevated Zero maze were analyzed by blinded investigators. The Open field and Y maze spontaneous alterations data were evaluated using ANY-maze Video Tracking System v7.07 (Stoelting).

2.3. Brain collection and histopathology

After behavioral tests, brain samples were collected from Groups I and II as described [30]. Briefly, mice were anesthetized by intraperitoneal injection of a cocktail of 50 mg/kg ketamine and 10 mg/kg xylazine and exsanguinated. Then, brains were collected and fixed with 4% paraformaldehyde (PFA) in PBS overnight. Finally, samples were transferred to 30% sucrose solution in PBS, embedded in O.C.T (Thermo Fisher Scientific) and sectioned at 6 μm coronal thickness.

2.4. Immunofluorescence staining

Published clinical cohort studies were used to select AD markers for pre-clinical immunofluorescence staining of brain sections [31,32]. Briefly, brain sections were stained with the following diluted primary rabbit polyclonal antibodies against beta-amyloid precursor protein (CT695), phospho-Tau/Thr231 (PA5- 117230), and RAGE (PA5–78736) (dilution at 1:200, ThermoFisher). Then secondary antibody Goat anti-Rabbit IgG H&L (Alexa Fluo ® 594) (Abcam, UK, ab150080) was used. To label nuclei, the slices were incubated with Hoechst 33342 (1:2000, ImmunoChemistry Technologies) and then mounted in Aqua

Poly/Mount (Polysciences, Inc.). Images were acquired using EVOS Cell Imaging Systems microscope (Thermo Fisher Scientific) and evaluated by Image J.

2.5. Histological analysis of calvaria

Calvariae were dissected and fixed in 4% formaldehyde overnight and then decalcified in 10% EDTA (Thermo Fisher Scientific). The decalcified samples were dehydrated in graded alcohols and embedded in paraffin. Frontal calvarial Section 6 μm in thickness centered on the sagittal suture were obtained for histological analysis. Then, samples were stained for TRAP positive (TRAP+) osteoclasts and counted manually as described [33]. In addition, some sections were subjected to hematoxylin and eosin (H&E) staining.

2.6. Culture and treatment of microglia and bone marrow derived macrophages in vitro

Spontaneously Immortalized Microglia-A9 (SIM-A9) (ATCC® CRL-3265TM) cell line was obtained from American Type Culture Collection (ATCC). SIM-A9 cells were maintained using a complete medium growth consisting of Dulbecco's Modified Eagle Medium: F12 (DMEM: F-12 Medium) (ATCC® 30–2006TM) supplemented with 10% FBS (Atlanta Biologicals), 5% heat- inactivated horse serum (Gibco), and treated with 20 ng/ml recombinant mouse M-CSF or IL-34 proteins (BioLegend) for 5 days.

Bone marrow cells were isolated from the femur and tibia of female and male 3x-Tg mice using density gradient centrifugation in Histopaque 1083 (Sigma-Aldrich) as described elsewhere [33,34]. Then, cells were seeded at a density of 5×10^5 cells per well in alpha-MEM (Life Sciences) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1% antibiotic and antimycotic solution, 1% L-glutamine, 1% MEM-NEAA (Life Sciences), and treated with 20 ng/ml recombinant mouse M-CSF or IL-34 proteins (BioLegend) for 5 days.

Using M-CSF, our group and others reported that naïve (M0) macrophages and microglia could be polarized into two different phenotypes, *e.g.* proinflammatory (M1) or anti-inflammatory (M2) in response to a mixture of bacterial liposaccharides (LPS) and mouse recombinant IFN-γ protein or mouse recombinant IL-4 protein, respectively [34–37]. Therefore, to generate pro-inflammatory M1 and anti-inflammatory M2 populations, either M-CSF or IL-34 pretreated SIM-A9 microglia or bone marrow-derived macrophages (BMDM) were stimulated with mouse recombinant IFN-γ (BioLegend) (10 ng/ml) in the presence of lipopolysaccharide (LPS) (Sigma-Aldrich) (10 ng/ml LPS from *Escherichia coli* O26:B6) for M1 or with IL-4 alone (BioLegend) (20 ng/ml) for M2 for 24 h. As a control, non-polarized (M0) BMDM or SIM-9 were continuously stimulated with M-CSF or IL-34 alone. After 24 h, conditioned media was harvested from M0, M1, and M2 cells. Finally, concentrations of TNF-α, IL-1β, and IL-6 proteins were measured in the collected supernatant using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D) according to the manufacturer's instructions.

2.7. RANKL-mediated osteoclastogenesis in vitro

To generate osteoclasts, IL-34 or M-CSF proliferated BMDM isolated from 3x-Tg female mice were exposed to mouse recombinant RANKL protein (10 ng/ml) in the presence or absence of AD-associated Aβ40 and Aβ42 peptides (1 ng/ml or 10 ng/ml; Biolegend). Six days later, cells were stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma). TRAP-positive (TRAP+) cells with more than three nuclei were considered as mature osteoclasts. TRAP+ multinuclear cells were counted, and the results were expressed as numbers per well.

2.8. Real-Time PCR assay

Total RNA was isolated from IL-34- and M-CSF-treated M0, M1, and M2 macrophages and SIM-A9 cells using the PureLinkTM RNA Mini Kit (Ambion, Life Technologies), and reverse transcription of 1 mg of total RNA was performed using the Verso cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's recommendations. Using TaqManTM Universal PCR Master Mix (Applied Biosystems, Life Technologies) assay, we measured expression patterns of IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), and TNF-α (Mm00443258_m1).

Data were analyzed using the $2^{-\Delta\Delta Ct}$ method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm 99999915_g1). In addition, we measured relative expression of osteoclastogenic Acp5/Trap mRNA using Sybr™ Green Master Mix (Applied Biosystems Diagnostics) and results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer sequences were used: Acp5/Trap (XM_006509945.3) (F: 5'CCAGCGACAAGAGGTTCC-3', R: 5'-AGAGACGTTGCCAAGGTGAT-3'). GAPDH (F: 5'- AACTTTGGCATTGTGGAAGG-3', R: 5'-ATGCAGGGATGATGTTCTGG-3).

2.9. Statistical analysis

Data analyses were conducted using R (version 4.0.2, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism for MacOS Version 9.5.1 software (GraphPad; La Jolla, CA, USA). Mann-Whitney and parametric t-test with Welch's correction were used appropriately to compare treated groups, controls, and sex. The one-way ANOVA with post-hock Turkey's test was used to compare more than two experimental groups. Data are displayed as mean ± Standard Deviation (SD). Treatment differences with p-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Local calvaria injection with IL-34 recombinant protein promotes anxiety and pathological memory changes in 3x-Tg female mice

A number of studies have established that anxiety and behavioral alterations positively correlate with AD neuroinflammation [38,39]. In order to develop possible effects of IL-34 on AD pathology, we thought to evaluate whether local calvarial injection of recombinant IL-34 protein affects AD-like behavioral alterations and cognitive impairment in 3x-Tg mice [39]. Therefore, to test the long-term impact of subcutaneous injection of IL-34 recombinant protein over the calvarial bone on the emotion and levels of anxiety in female and male 3x-Tg mice, we first measured the number of head dips using an elevated zero test. As shown in Fig. 1A, the number of head dips decreased in female 3x-Tg mice exposed to IL-34 compared to the control group. In addition, a tendency to reduce the number of head dips in response to local calvaria injection with IL-34 was also observed in 3x-Tg male mice (Fig. 1A). Our data concur with previously published observations that chronic stress and inflammation reduced the number of head dips in AD-like APPS1 mouse mode [40]. Since head dipping is linked to exploratory behavior and anxiety in rodents [41], it is plausible that IL-34 reduces exploratory behavior in 3x-Tg mice. Typically, AD patients with concomitant apathy or depression interfering with their social interaction behavior display elevated serum and cerebrospinal fluid levels of inflammatory mediators [42,43]; however, no levels of IL-34 were evaluated in these published observations.

In addition to the reduced number of head dips, elevated defecation is another indicator of the anxiety behavior for mice exposed to a new and potentially dangerous environment [44–46]. Therefore, we next examined whether IL-34 promotes defecation in 3x-Tg mice using the open field test [28]. The number of defecation boli was significantly elevated in females exposed to IL-34 compared to their control groups

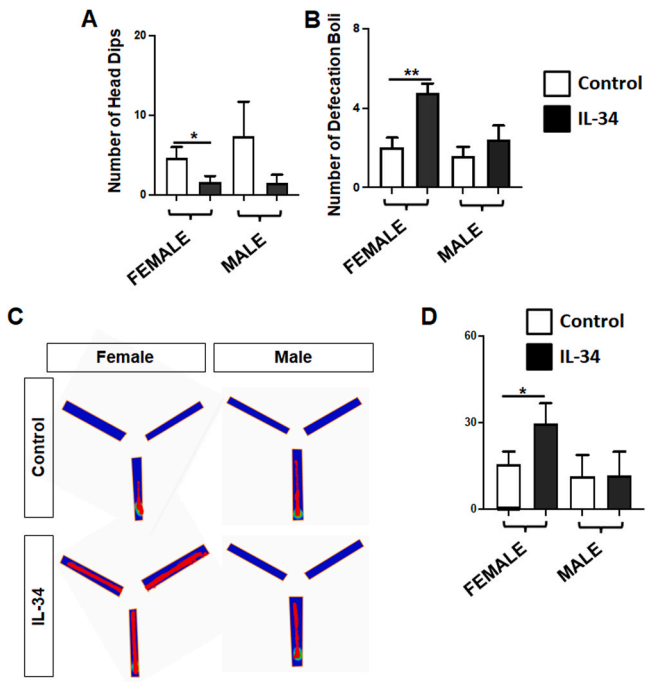

Fig. 1. Local subcutaneous calvaria injection with recombinant IL-34 protein elevates AD-like cognitive behavioral phenotype in 3x-Tg female mice. **A:** Number of head dips observed in female and males of 3x-Tg mice exposed to recombinant IL-34 protein using the Elevate Zero maze. **B:** Defecation levels observed in the Open Field maze; **C:** Y-maze spontaneous alterations heat maps of female and male 3x-Tg mice exposed to IL-34. **D:** The percentage of spontaneous alterations counted from the Y-maze heat maps. Data are expressed as the Mean ± SEM (n = 10/group). *p < 0.05, * *p < 0.01.

(Fig. 1B). In contrast, no significant changes in the number of defecation boli were detected between IL-34 exposed and a control group of male 3x-Tg mice, indicating that local calvarial injection with IL-34 recombinant protein elevates defecation and anxiety in 3x-Tg female mice. It is essential to mention that patients with dementia have an increased risk of fecal incontinence complications than healthy individuals [47]. Furthermore, positive correlation between elevated levels of inflammatory markers and a higher degree of anxiety was observed in AD patients [43]. Future studies might explore the potential implications of IL-34 in AD anxiety manifestations.

AD indeed correlates with pathophysilogical changes in brain regions involved in the learning and memory process [48–50]. Therefore, we also evaluated the spatial working memory in IL-34-exposed 3x-Tg mice using the Y-maze spontaneous alteration test [51]. It is expected that a mouse with intact working memory will remember the arms previously visited and demonstrate the tendency to enter a less recently visited arm [52]. However, we observed that the percentage of spontaneous alterations in IL-34-exposed female 3x-Tg mice was significantly elevated compared to males and corresponding control groups of mice (Fig. 1C&D). Since a study reported that wild-type mice have a reduced percentage of spontaneous alternation behaviors compared to 3x-Tg mice [53], our data indicated that IL-34 elevates cognitive deficits in female 3x-Tg mice. These results correlate with previous findings demonstrating elevated impairments in female 3x-Tg mice only [54]. On the other hand, various studies reported no sex differences in the 3x-Tg behavioral phenotype indicating that housing conditions play a critical role [53–55]. Specific housing conditions difference may critically contribute to the discrepancy between our results and previous Y maze spontaneous alterations test findings. Altogether, these behavioral results demonstrated that local calvaria injection with recombinant IL-34 protein promotes anxiety and pathological memory changes in female 3x-Tg mice.

3.2. IL-34 elevates accumulation of β-Amyloid precursor protein, p-Tau and RAGE in the brain cortex of 3x-Tg female mice

Published evidence demonstrated that amyloid beta (Aβ), hyperphosphorylation of tau (p-Tau), and RAGE are significant hallmarks of pathological features of neurodegeneration and neuroinflammation in patients with AD [3,8,56]. Since AD-related behavioral and cognitive changes often correlate with pathological changes in prefrontal cortex [57–59], we tested next the impact of IL-34 administration on the expression levels of β-Amyloid precursor protein, p-Tau/Thr231 in the brain cortex of female and male 3x-Tg mice using immunofluorescence assay. In this study we observed that the fluorescent intensity levels of β-Amyloid precursor protein and p-Tau/Thr231 were dramatically elevated in the brain cortex from the IL-34 exposed female group compared to males and corresponding controls of 4-month-old mice (Fig. 2A-D). Surprisingly, it was demonstrated that progressive amyloidogenesis appears between 6 and 12 months of age in relation to sex [24,60]. Sex-specific genetic studies point to a strong association between expression levels of AD genetic loci and Aβ42 and tau, as well as tangle density among female, but not male, AD patients [61]. In contrast, a recently published study demonstrated no sex differences in p-tau/Th231 levels in cerebrospinal fluid collected from patients with AD [31].

Our group and others previously demonstrated that elevated production of Aβ peptides from its precursor protein APP and tauopathy is associated with systemic inflammation [62–64]. Among multiple signaling pathways implicated in systemic inflammation, increasing evidence shows that RAGE signaling has been involved in AD and related dementia [8,65,66]. Fig. 2 (E&F) demonstrates that the immunohistochemical distribution of RAGE was significantly elevated in the brain cortex of 3x-Tg female mice, not males, exposed to IL-34 compared to their control at 4-month-old age. In other studies, it was reported that RAGE expression was increased in the cortex and hippocampus of the 22–24 months old 3x-Tg female mice [25]. Furthermore, a similar age-dependent increase in RAGE expression was reported in transgenic AD mice with overexpression of the Swedish mutant form of APP [25,67,68]. In postmortem brains of AD patients, hippocampal RAGE immunoreactivity shows an explicit disease stage dependency, with linearly increasing levels from early to advanced AD as a function of AD pathology severity [69]. Thus, our data indicate that IL-34 exacerbates the expression of RAGE in the brain cortex of 3x-Tg mice. Together, these observations suggested that locally injected recombinant IL-34 protein promotes neurodegeneration and neuroinflammation in female 3x-Tg mice *via* upregulation of amyloidogenesis, phosphorylation of tau, and RAGE.

3.3. Local injection of IL-34 elevates inflammatory osteolysis in 3x-Tg female calvaria

Since emerging evidence suggested that AD pathology correlates with elevated levels of osteolysis along with an increased number of inflammatory cell infiltrates and TRAP+ osteoclasts [70], we examined whether local administration of IL-34 promotes osteolysis in 3x-Tg mice. Histological images of calvarial tissues demonstrate elevated numbers of TRAP+ osteoclasts in IL-34-exposed female and male 3x-Tg mice compared to control groups, indicating that locally injected IL-34 recombinant protein accelerates osteolysis in 3x-Tg mice independently of sex (Fig. 3A&B). A study demonstrated that systemic administration of IL-34 to mice reduces trabecular bone mass in wild-type mice [19]. Our data agree with emerging evidence that chronic inflammatory osteolysis contributes to neuroinflammation and dementia, indicating that chronic peripheral inflammation mediated by IL-34 may be a novel therapeutic target for AD [71–73].

Our findings of osteolysis in 3x-Tg mice are also consistent with studies using different mouse models of AD. As an example, significant increases in osteoclastogenesis and bone resorption were observed in

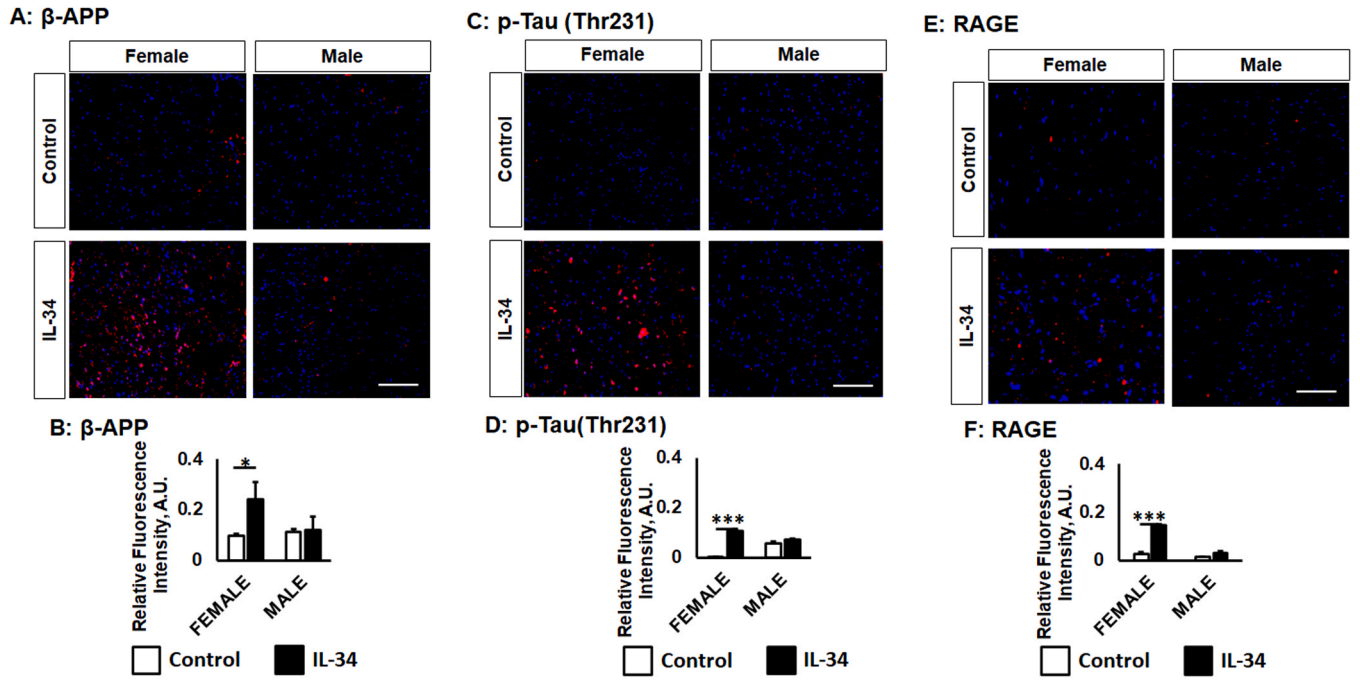

Fig. 2. Effect of local calvaria injection with recombinant IL-34 protein on the expression beta amyloid precursor protein (β-APP), phosphorylated Tau (Thr231), and RAGE (shown in Red) in the brain cortex of 3x-Tg mice. To label nuclei, the slices were incubated with Hoechst 33342 (Blue). T-test was used to evaluated statistical significance between sex-matched, IL-34 treated and control groups. Data are expressed as the Mean ± SD. * **p < 0.001.

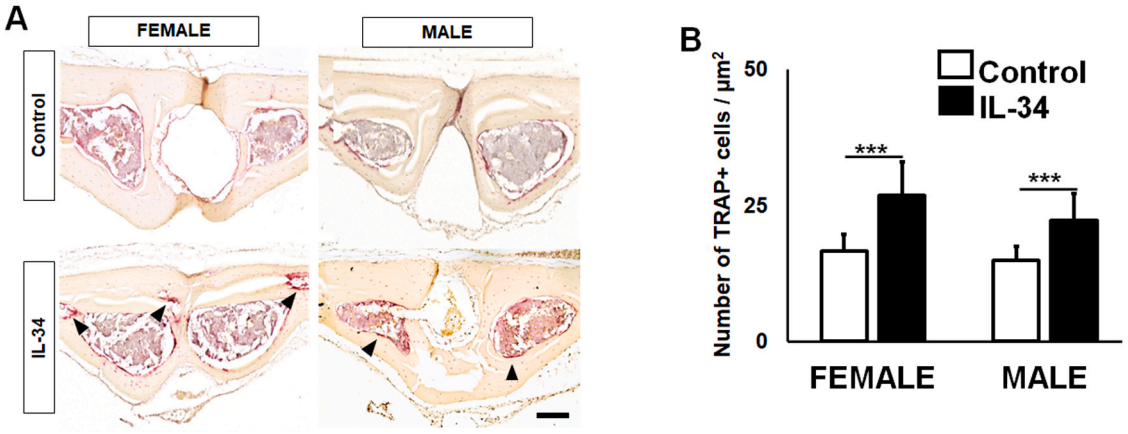

Fig. 3. Manifestations of osteolytic lesions found in calvaria locally exposed to recombinant IL-34 protein in female and male 3x-Tg mice. Histological evaluation of TRAP+ osteoclasts (A) counted in a microscopic field of TRAP-stained sections (B) in IL-34 exposed and control mice. Arrowheads demonstrate TRAP+ osteoclasts. Data are expressed as the Mean ± SD. T-test was used to evaluated statistical significance between sex-matched, IL-34 treated and control groups. Data are expressed as the Mean ± SD. * **p < 0.001. Scale bar = 100 µm.

Tg2576 mice in which the Swedish mutant APP is ubiquitously expressed under the control of a prion promoter [74]. In their studies they demonstrate that activation of the RAGE receptor in osteoclast lineage cells, leads to increased osteoclastogenesis, bone resorption, and an overall osteoporotic bone phenotype[74]. Additionally, the 5xFAD and hTau mouse models of AD have also been reported to exhibit osteoporotic phenotypes and numerous other genetically modified mice have been shown to have low bone mass and their genetic alterations are associated with AD as was recently reviewed [75–78]. Of note, the incidence of osteoporosis and AD increases during/following menopause, suggesting a role for sex-based hormones in these diseases. Indeed, hormones, including sex-based hormones such as follicle-stimulating hormone and estrogen have been strongly implicated in both AD and osteoporosis and treatments targeting this axis may improve bone health as well as AD. Important to these studies, estrogen and IL-34 levels appear to correlate in many inflammatory disorders, further suggesting that targeting IL-34 could be a novel method for improving both diseases.

3.4. IL-34 elevates the production of AD-associated pro-inflammatory cytokines from SIM-A9 microglia and macrophages in vitro

Our data indicated that local injection of IL-34 elevates brain inflammation in 3x-Tg mice (Fig. 2). Therefore, to extend our findings beyond the IL-34 barrier, we compared pro-inflammatory effects between IL-34 and M-CSF on non-treated M0, pro-inflammatory M1, and anti-inflammatory M2 SIM-9 microglia-like cells *in vitro*. Using Real-Time PCR, we evaluated the expression of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 mRNA, in the SIM-A9 microglia promoted toward M0, M1, and M2 phenotypes (Fig. 4A). Compared to

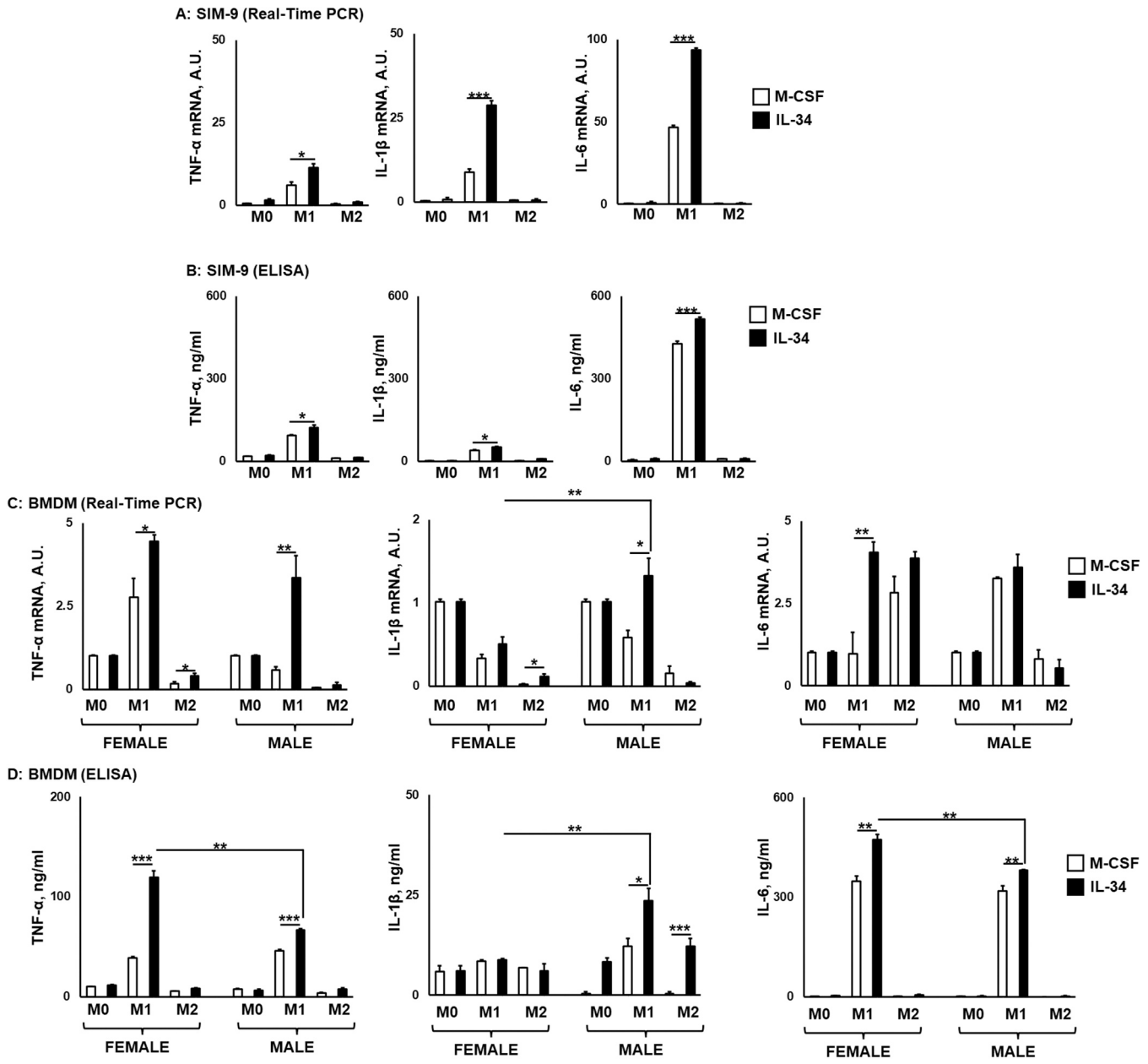

Fig. 4. Effects of IL-34 and M-CSF on the release of pro-inflammatory cytokines from SIM-9 microglia cell line and bone-marrow-derived macrophages isolated from 3x-Tg *in vitro*. mRNA expression and protein release of TNF-α, IL-1β, and IL-6 from SIM-A9 (A, B) and bone marrow derived macrophages, BMDM, (C, D) exposed either to IL-34 or M-CSF and then proliferated toward M0, M1, and M2 phenotypes. Specifically, we induced M1 proliferation using a mixture of LPS and IFN-γ (10 ng/ml each); M2 were proliferated using IL-4 (20 ng/ml); M0 are non-treated control cells. Comparisons among groups were performed with the one-way ANOVA followed by *post hoc* Tukey's test. Data are expressed as the Mean ± SD *p < 0.05, * *p < 0.01, * **p < 0.001.

M-CSF/M1, exposure to IL-34 significantly elevated TNF-α, IL-1β, and IL-6 mRNA expression patterns in M1 microglia. Using ELISA assay, we also observed that TNF-α, IL-1β, and IL-6 concentrations were significantly elevated in the culture supernatant compared to M-CSF/M1 microglia (Fig. 4B). However, no significant increase in those cytokine levels was detected in M0 or M2 SIM-9 cells exposed either to M-CSF or IL-34.

Next, BMDM isolated from female and male 3x-Tg mice were exposed either to mouse recombinant IL-34 or M-CSF and then induced toward M0, M1, and M2 macrophages *in vitro*. After 24 h, we observed that expression of TNF-α, IL-1β, and IL-6 mRNAs in IL-34/M1 macrophages was significantly elevated compared to M-CSF/M1 cells (Fig. 4C&D). It is important to note that we detected elevated release of TNF-α and IL-6 from female IL-34/M1 macrophages, while male IL-34/M1 demonstrated elevated production of IL1β. No or little effect on M2 macrophages was observed in response to M-CSF and IL-34.

It was also shown that recombinant IL-34 protein elevates systemic inflammation mediated by macrophages in wild-type mice compared to M-CSF-proliferated macrophages [79]. Furthermore, a recently published study demonstrated reduced clearance ability of Aβ pathological forms by IL-34 proliferated microglia compared to those proliferated by M-CSF in experimental models of AD [80]. A study demonstrated that expression patterns of CSF-1r and M-CSF are upregulated, while IL-34 was decreased in postmortem brains collected from patients with AD [81]. Furthermore, it was also reported that IL-34 induces differentiation of macrophages isolated from healthy human individuals and wild-type mice toward anti-inflammatory M2 phenotype [82]. In addition, IL-34 improves healing in wild-type rats [83], indicating that the

role of IL-34 in neuroinflammation and promotion of dementia pathologies remains controversial. Nonetheless, data from the current study indicate that the production of pro-inflammatory cytokines is elevated from IL-34/M1-proliferated SIM-9 microglia and bone marrow-derived macrophages isolated from 3x-Tg mice compared to those cells proliferated toward M-CSF/M1 *in vitro*.

3.5. *Aβ40 and Aβ42 exacerbates RANKL-primed osteoclastogenesis in the presence of IL-34 in vitro*

Because we observed that IL-34 elevates inflammatory osteolysis in 3x-Tg female mice, we compared the impact of IL-34 and M-CSF on RANKL-primed osteoclastogenesis in the presence of the AD-associated Aβ40 and Aβ42 peptides *in vitro* using BMDM isolated from 3x-Tg females. As illustrated in Fig. 5A-E, both Aβ40 and Aβ42 elevate expression patterns of pro-osteoclastogenic Acp5/TRAP mRNA and the number of TRAP+ osteoclasts in response to IL-34/RANKL axis compared to M-CSF/RANKL exposed BMDM. These data correlate well with an earlier report demonstrating that Aβ enhances RANKL-primed osteoclastogenesis [84]. In contrast, a study demonstrated that IL-34 regulates osteogenesis and enhances fracture healing in wild-type mice [83]. Surprisingly, it was also demonstrated that Aβ promotes bone formation in wild-type mice [85]. Therefore, further investigations are warranted to elucidate the impact of IL-34 and M-CSF in inflammatory osteolysis and bone remodeling in the context of AD and related dementia.

4. Conclusion

In this study, we demonstrated that local calvaria injection of

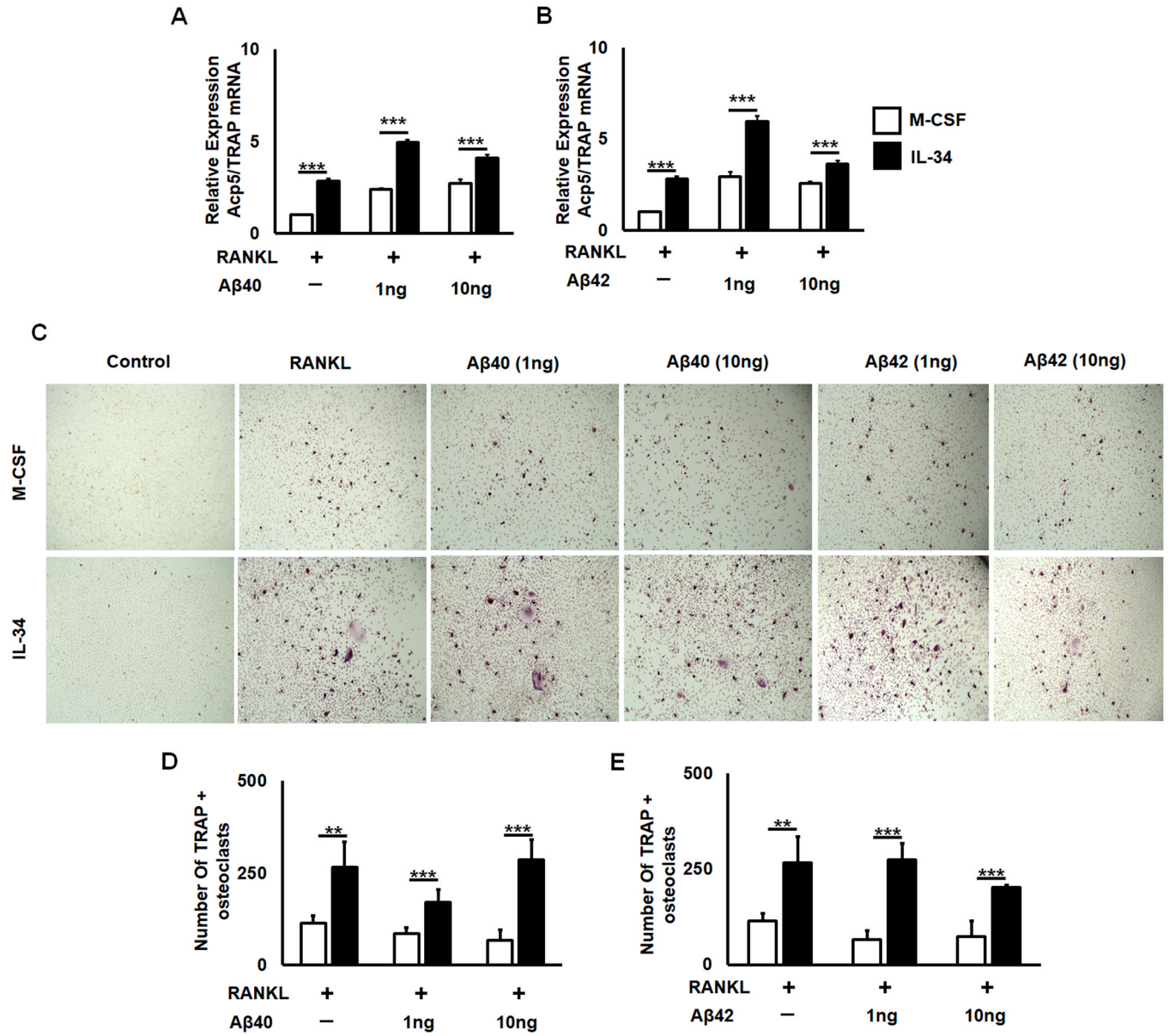

Fig. 5. Effects of IL-34 and M-CSF on RANKL-primed osteoclastogenesis exacerbated by AD-associated Aβ40 and Aβ42 peptides *in vitro*. Bone marrow cells were isolated from 3x-Tg female mice and then proliferated either with IL-34 or M-CSF for 3 days. Then, osteoclastogensis was mediated by mouse recombinant RANKL protein in the presence or absence of various concentrations of Aβ40 and Aβ42 peptides. Expression of osteoclastogenic ACP5/TRAP mRNA in RANKL-primed osteoclast precursors proliferated by IL-34 or M-CSF in the presence or absence of various concentration of Aβ40 (A) and Aβ42 (B) peptides. C: Microscopic evaluation of the TRAP staining and quantification of TRAP+ multinucleated cells in RANKL stimulated BMDM (D &E). Comparisons among groups were performed with T-test. Data are expressed as the Mean ± SD. * **p < 0.001.

recombinant IL-34 protein dramatically elevated AD-like behavior and neuroinflammation in 3x-Tg female mice. Furthermore, we also observed that IL-34 promoted calvaria inflammatory osteolysis compared to the sham control injected group of mice. Finally, we also demonstrated that IL-34-proliferated pro-inflammatory/M1 SIM-A9 microglia and BMDM isolated from 3x-Tg mice released significantly higher amounts of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, compared to M-CSF-proliferated/M1 cells *in vitro*. In addition, IL-34 elevates RANKL-primed osteoclastogenesis in the presence of Aβ40 and Aβ42 peptides *in vitro*. While a more comprehensive assessment of the IL-34 role in AD pathology is required in future studies, our data indicated that a novel therapeutic regimen targeting IL-34 could mitigate neuroinflammation, neurodegeneration, and elevated bone loss observed in patients with AD.

CRediT authorship contribution statement

Anny Ho, William Kochen, Stanislav Groppa, Melissa A. Kacena, Alexandru Movila: Designed the study and Methodology. Anny Ho, Bidii Ngala, Christopher Garcia, Carolina Duarte, Juliet Akkaoui, Dumitru Ciolac, Amilia Nusbaum, Chiaki Yamada, William Kochen, Lubov Nathanson, Alexandru Movila performed procedures with experimental animals, behavioral tests, data analysis, and sample collection. Anny Ho, Dumitru Ciolac, Lubov Nathanson, Stephanie Bissel, Adrian Oblak, Stanislav Groppa, Melissa A. Kacena, Alexandru Movila: Writing – original draft, Data interpretation, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Data Availability

Data will be made available on request.

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