Impact of Interleukin-34 on the Promotion of Bone Osteolysis and Neuroinflammation in Experimental Models of Alzheimer’s Disease

Anny Ho
Nova Southeastern University

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Thesis of
Anny Ho

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science
Biological Sciences

Nova Southeastern University
Halmos College of Arts and Sciences

April 2022

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This thesis is available at NSUWorks: https://nsuworks.nova.edu/hcas_etd_all/78
Impact of Interleukin-34 on the Promotion of Bone Osteolysis and Neuroinflammation in Experimental Models of Alzheimer’s Disease

By

Anny Ho

Submitted to the Faculty of
Halmos College of Arts and Sciences
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Biological Sciences

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May 2022
Abstract

Alzheimer’s disease (AD) is a growing health concern and is the most common type of dementia worldwide. Emerging evidence indicates that aggregated amyloid-beta (Aβ) peptides, one of the hallmark features of AD neuropathology, can increase RANKL-mediated osteoclast activity leading to the prevalence and severity of inflammatory osteolysis, e.g., osteoporosis and periodontal bone loss. Conversely, osteolytic lesions are associated with increased risk of dementia diagnosis indicating that there is a direct link between dementia and inflammatory osteolysis. It was demonstrated that the neuronal cells primarily produce interleukin-34 (IL-34) and microglia, macrophages, and osteoclast precursors express colony-stimulating factor 1 receptor (CSF-1R), a cognate receptor for IL-34. Similar to IL-34, macrophage colony stimulating factor (M-CSF) also promotes bone marrow (BM)-derived monocyte survival and differentiation into macrophages. While M-CSF has been extensively studied, the biology and functions of IL-34 are only now beginning to be uncovered. We recently reported the differences in the inflammatory signal transduction and polarization of IL-34 and M-CSF-macrophages in response to COVID-19 Spike protein. In addition, published observations demonstrated that IL-34 is relevant to innate immune responses in AD. Based on these lines of evidence, we hypothesized that IL-34 plays an essential role in the promotion of inflammatory bone osteolysis and neuroinflammation associated with AD. Using 3x-triple AD transgenic mice and their wild type, we found that the number of multinucleated osteoclasts were significantly increased from IL-34-polarized osteoclast precursors (OCPs) compared to M-CSF-OCPs in the presence of various Aβ peptides in vitro as well as in a mouse model of calvarial osteolysis. In addition, local calvaria injection of recombinant IL-34 protein increased immobility, fecal boli, and freezing behaviors in female 3x-Tg-AD mice in comparison to male. We also identified that IL-34 significantly elevated expression patterns of various markers associated with AD pathology, including Aβ, hyperphosphorylation of tau, Zonula occludin-1 (ZO-1), receptor-associated protein (RAP), receptor of advanced glycosylation end-products (RAGE), Occludin, and Neuronal nuclear protein (NeuN) in female 3x-Tg-AD mice. No or little effect of IL-34 was observed in male 3x-Tg-AD mice indicating that IL-34 promotes sex-dependent AD pathology and bone osteolysis. Furthermore, the amount of elevated production of senescence-associated secretory phenotype (SASP) markers of pro-inflammatory cytokines, e.g., TNF-α, IL-6 and IL-1β, IL-34-macrophages and -microglia cells were significantly elevated compared to those that were proliferated by M-CSF. Overall, our results highlight the challenges of targeting the IL-34 in the systemic and central compartments, important for framing any therapeutic effort to tackle bone loss, and neuroinflammation, and neurodegeneration during AD.

**Keywords:** Late-Onset Alzheimer’s Disease, Neuroinflammation, Amyloid-beta, Bone Osteolysis, Interleukin-34, Macrophage colony-stimulating factor, RANKL-mediated osteoclastogenesis, Osteoclast precursor, Cognitive behavior, Triple-transgenic mice
Acknowledgments

First and foremost, I would like to thank each and every person who has helped, guided, encouraged, and supported me through this project and my educational studies. This project would have not been possible if it were not for my support system both in the laboratory and at home. I would like to express my gratitude to my major advisor Dr. Alexandru Movila for opening the door to research for me. Thank you for all the advice, feedback knowledge and engagement throughout this project. Thank you for assisting me in all aspects of this project, from research planning to proposal and manuscript writing and everything in between. I would like to thank him for this incredible opportunity to join his lab. There are no words that can describe the value and dedication have been in mentoring me though the course of this program.

I would also like to thank my committee members, Dr. Lubov Nathanson for taking me under her wing, and provided me valuable feedback throughout the entire process of my project. I would like to thank my other committee members Dr. Andrew Ozga, and Dr. Veronica Fortino for their guidance, providing me with support and advice on my project. Thank you to my committee members for the additional perspectives and suggestion which were instrumental in furthering the quality of my thesis. In addition, I would like to thank Dr. William Kochen for helping and guiding me through the process of behavioral testing and providing feedback on my project. I appreciate the time it has taken for each and every one to examine my project.

I would like to thank my lab members Dr. Chiaki Yamada for his patience and kindness for teaching and helping me plan my experiments. Dr. Carolina Duarte for taking the time out of her schedule to help me with experiments and imaging, and Mr. Christopher Garcia and to our other current and past members in our lab. Thank you for your patience and guidance in helping me in the lab and data collection.

Lastly, thanks to my family, especially my parents Nhan Ho and Katty Nguyen, my sister for pushing and believing in me. My husband Christian Moffa for his unconditional support and encouragement throughout my journey. Thank you for celebrating with me every single little accomplishment, thank you for always believing in me and always encouraging me to challenge myself and strive for my dreams. Thank you to everyone for being a part of this journey and pushing me to work harder, to be a better person.

I would like to acknowledge that this research was supported by U.S. DHHS NIH- Impact of Aging RE-B (331635).
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<tr>
<td>3x-Tg-AD</td>
<td>Triple-Transgenic Mice</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced Glycation End-Products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APOE ε4</td>
<td>Apolipoprotein E4 Gene</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-beta peptide or β-amyloid peptide</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMDMs</td>
<td>Bone Marrow-Derived Macrophages</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF1</td>
<td>Colony Stimulating Factor 1</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Colony Stimulating Factor-1 Receptor</td>
</tr>
<tr>
<td>De-Stamp</td>
<td>Dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>E. Coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EZM</td>
<td>Elevated Zero Maze</td>
</tr>
<tr>
<td>fAD</td>
<td>Early-onset Familial Alzheimer's Disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-34</td>
<td>Interleukin-34</td>
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<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>LOAD</td>
<td>Late-Onset Alzheimer’s Disease</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRP1</td>
<td>Lipoprotein Receptor-Related Protein 1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal Nuclear Protein</td>
</tr>
<tr>
<td>O.C. T</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>OCPs</td>
<td>Osteoclast Precursor Cells</td>
</tr>
<tr>
<td>OFT</td>
<td>Open Field Test</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>p-Tau</td>
<td>Hyperphosphorylation of tau</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor of Advanced Glycosylation End-Products</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor Kappa-B Ligand</td>
</tr>
<tr>
<td>RAP</td>
<td>Receptor-Associated Protein</td>
</tr>
<tr>
<td>sAD</td>
<td>Sporadic Alzheimer’s Disease</td>
</tr>
<tr>
<td>SASPs</td>
<td>Senescence-Associated Secretory Phenotype</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SIM-A9</td>
<td>Spontaneously Immortalized Microglia-A9</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRAP+</td>
<td>Tartrate-resistant acid phosphatase positive cells</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludin-1</td>
</tr>
</tbody>
</table>
1. Introduction

1.1. Alzheimer’s disease

According to the Center for Disease Control and Prevention (CDC), Alzheimer’s disease (AD) is a multifactorial, highly heterogeneous, and complex age-dependent disorder that severely affects memory and cognitive functions of the patients, to the extent that they are completely dependent upon nursing care for the remainder of their life. AD is a complex neurodegenerative disorder that is characterized by progressive and severe dementia with neuropsychiatric symptoms. AD is the most common reason of progressive dementia in the elderly, accounting for approximately 70% of all dementia cases (Evans et al. 1989). It is now estimated that nearly 35.6 million patients are affected by AD worldwide and that about 4.6 million new cases are added each year, causing enormous societal and economic burden with an estimated cost to reach $1 trillion/year (Wimo, Winblad, and Jönsson 2010; Wimo et al. 2017).

The main risk factors for developing AD are not only just with age and but also gender. AD statistics reports that approximately 60–80% of individuals falls into the category of dementia cases, with an estimated prevalence of 4.4% among individuals that are around the age of 65 years or older (Niu et al. 2017). The prevalence of AD is increases with advancing age, statistics reports approximately 0.97% are around the ages of 65–74 years, it is approximately 7.7% for the ages around 75–84 years, and lastly, around 22.5% for greater than 85 years old (Collaborators 2019). In relation to age, while cognitive decline is one of the clinical hallmarks of AD, changes in physical health are also apparent during AD such as osteoporosis. AD and osteoporosis are both common degenerative diseases found in the elderly population. The prevalence of both of these diseases increases with age and poses a huge societal problem worldwide. Studies have shown how low amount of bone mineral density (BMD) is a related to the increased risk of the development of AD (Tan et al. 2005). To add, studies have demonstrated that AD patients tend to have elevated bone loss when compared to healthy individuals (Kumar et al. 2021; Kwon et al. 2021). From these data, bone loss has shown to occur in pre-clinical AD, and is frequently helping with early diagnosis, and consequently, may have prognostic significance in estimating AD risk.

As for gender, over 65% of people with Late-Onset Alzheimer’s Disease are women (Mielke, Vemuri, and Rocca 2014; Nebel et al. 2018; Bailly et al. 2019). There are recent findings that indicates that women account for nearly two-thirds of AD patients (Hebert et al. 2013).
Multiple factors have been proposed to explain the connection between bone loss and cognitive decline in women. Some of these factors that link the connection may include estrogen exposure, apolipoprotein E4 gene (APOE ε4), and lifestyle factors such as physical, diet, and environmental factors. In particularly, APOE ε4 allele gene is the consider one of the strongest heritable risk factors for Late-Onset Alzheimer’s Disease, or sporadic AD (Stocker et al. 2018). There have been animal studies that have shown that APOE ε4 allele gene deteriorates cognition behavior and amyloid-beta (Aβ) pathology more significantly in females than in males (Cacciottolo et al. 2016; Raber et al. 1998). There have been many epidemiologic studies demonstrating that neurodegeneration and clinical symptoms occur more rapidly for females once you are diagnosis (Laws, Irvine, and Gale 2016; F.-C. Lin et al. 2015; R. Li and Singh 2014).

Among those patients with AD, records show that only a very small percent of cases which are represented by 635 families worldwide suffer from early-onset familial AD (fAD); caused by missense mutations in the Presenilin 1 (PSEN1) gene (480 families), PSEN2 gene (34 families), and in the Amyloid Precursor Protein (APP) gene (121 families) (Cruts, Theuns, and Van Broeckhoven 2012). Thus, the vast majority of AD patients comprising nearly 99% of cases are designated as Late-Onset Alzheimer’s Disease (LOAD), or sporadic AD (sAD), not linked to any known genetic mutations. It was clearly demonstrated that both fAD and sAD (LOAD) patients share common pathological features, including, deposition of Aβ-protein containing amyloid plaques and neurofibrillary tangles, suggesting that alterations in APP protein metabolism are also crucial in the pathogenesis of LOAD. However, in addition to amyloidogenesis and hyperphosphorylation of tau (p-Tau), neuroinflammation is now considered as the third hallmark features of LOAD (Hollingworth et al. 2011; Krstic and Knuesel 2013; Lambert et al. 2009). The relationship between neuroinflammation and LOAD is further strengthened in a recent demonstration of positive correlation between cognitive decline in LOAD patients and both acute and chronic inflammation (Naj et al. 2011). Additionally, this evidence suggests that dysregulation of the immune system and inflammation contribute to the LOAD pathogenesis. Among the LOAD-associated risks factors, and possession of neurotoxic and neuroprotective properties of IL-34 have attracted much attention in recent years as key contributors in the pathology of AD because through proliferation of microglia and macrophages as they are able to affect both amyloid-beta protein generation and tau phosphorylation (Xie and Johnson 1997; Dunn et al. 2005; Mizuno et al. 2011) as well as inflammation and osteoclastogenesis (Kanzaki et al. 2017). Thus, there is an urgent need to identify
the exogenous risk factors that can affect neuroinflammation for the development of novel strategies to prevent and manage LOAD.

1.2. Hallmark factors of Alzheimer disease

AD is an irreversible, neurodegenerative disorder exhibiting a dangerous onset and a gradual progressive course. AD classical hallmarks include extracellular amyloid-beta (Aβ) deposits, intracellular aggregated phosphorylated tau, dystrophic neurites, synapses and neurons loss (Bedner et al. 2015). Neuropathologically, AD is depicted mostly by specific accumulation of both Aβ protein and hyperphosphorylation of tau protein (Rajmohan and Reddy 2017). They are considered as the key hallmarks of AD. The deposition of these proteins in the brain is considered one of the a significant pathological hallmark of AD (Murphy and Levine 2010). AD is characterized by the aggregation of Aβ-protein into soluble toxic oligomers (Mucke and Selkoe 2012) and the protein accumulation into senile plaques also known as extracellular deposits (Hardy and Selkoe 2002; Kowalska 2004; G. Chen et al. 2017). This also includes hyperphosphorylation of tau protein, which forms lesions and aggregates into neurofibrillary tangles and neuropil threads (Šimić et al. 2016; Iqbal et al. 2010). The hallmark physiopathological features of AD include Aβ-amyloid plaques, Aβ-protein, neurofibrillary tangles, and neuronal lesions that trigger a disturbance of the metabolic processes that can lead to a progressive cognition impairment (Association 2019).

APP is a membrane protein that expresses in many tissues and synapses of neurons. APP cleavage by β-secretase and then γ-secretase leads to pathological Aβ (Vallabhajosula 2011; Scheltens et al. 2016). The cleavage of these generated by Aβ results in two main isoforms known as Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42) peptides. Aβ40 is known to detected only in a subset of plaques (Iwatsubo et al. 1994; Mak et al. 1994; Miller et al. 1993) and Aβ42 is known to be one of the major component of amyloid plaques in AD brains (Miller et al. 1993; Iwatsubo et al. 1994). Aβ42 is known for being the most toxic form (A. Kimura, Hata, and Suzuki 2016; Jucker and Walker 2015). AD is caused from mutations in APP, PSEN1 or PSEN2 genes and these mutations are thought to cause AD at an early age due to the over production of Aβ, or an increase in Aβ42 to Aβ40 ratio (Bi, Bi, and Li 2019; Bekris et al. 2010; Kumar-Singh et al. 2006). Studies have demonstrated, that AD was associated with slowed clearance of both Aβ42 and Aβ40 (DeMattos et al. 2004), which implies that Aβ clearance mechanisms may be key in the development of AD.
There has also been developing evidence advocating that peripheral bone marrow-derived monocyte and macrophages play an important role in the biological clearance of cerebral Aβ (L. R. Zuroff et al. 2020a; 2020b) and the absence of Aβ in cerebral is associated in the pathogenesis of the common late-onset forms of AD (L. Zuroff et al. 2017).

1.3. Macrophages and Microglia influence on neuroinflammation

Cells of the mononuclear phagocytic lineage including monocytes, macrophages, Langerhans cells, osteoclasts and microglia (Gordon and Plüddemann 2017). All of these cells are known to be involved in tissue development, homeostasis and repair and serve as key regulators of immune function and are involved in numerous pathologies (Wynn, Chawla, and Pollard 2013). However, dysregulation of these cells contributes to a variety of diseases including inflammation, cancer, and bone disease. Macrophages play a key role in innate immune system and are present in every organ of the body (Hirayama, Iida, and Nakase 2018). Macrophages and macrophage-like resident central nervous system (CNS) immune cells, and microglia are key players for promotion and resolution of neuroinflammation (Sevenich 2018). Macrophages are an important factor and element in innate immunity and plays an essential role in inflammation and host defense (Gordon and Martinez 2010). As for microglia cells, they engage in neurotoxic and neuroprotective roles that play a critical factor in the development of AD (Mizuno et al. 2011). The activation of microglia has multiple effects on the progression of AD. These factors involve the activation of microglia that will lead to reducing Aβ accumulation by increasing its phagocytosis, clearance, and degradation of the brain. In this case, when it occurs, it will stop or inhibit the development of amyloid plaques in the brain (Perry, Nicoll, and Holmes 2010; Streit, Mrak, and Griffin 2004). Microglia cells are also involved in the neuroinflammation. When these cells are stimulated, they release or produce elevated levels of inflammatory mediators such as pro-inflammatory cytokines and chemokines (Meraz Rios et al. 2013; Morales et al. 2014).

Any changes in macrophage/microglia phenotypes could contribute to pathogenic forms of inflammation and neurodegenerative diseases. The production and secretion of pro-inflammatory mediators could promote neuroinflammation and neurodegeneration. There are ongoing developing studies that are happening to determine how the presence of stressed macrophages and microglia,
and how it will affect disease progression. Macrophages and microglia are very important in multiple neurodegenerative diseases and understanding how we can control its response will not only offer insights into the pathogenesis of CNS disorders, but it can help generate novel targets for drug development and the optimization of existing therapies for AD. To distinguish the different factors that are involved in the development and generation of macrophage and microglia, it is essential for determining their functions in physiological and pathological conditions relating to AD and neuroinflammation diseases.

1.4. Roles of recombinant protein M-CSF and IL-34

An important factor for the development, homeostasis, and function of mononuclear phagocytes is the colony stimulating factor-1 receptor (CSF-1R) (MacDonald et al. 2010). CSF-1R belongs to tyrosine kinase receptor family. It is generally expressed in the organism by monocytes, macrophages, osteoclasts, and microglia (Stanley and Chitu 2014). CSF-1R is involved in the development and existence of the microglia, these outcomes are linked with neuroinflammation (Janssen and Mach 2019). CSF-1R has two very known ligands which includes macrophage colony-stimulating factor (M-CSF), also known as colony stimulating factor 1 (CSF-1) and interleukin-34 (IL-34). These two cytokines play a significant role in the development and immunity by regulating tissue macrophages and osteoclasts, and of microglia of the brain (Yin et al. 2017).

M-CSF, also known as CSF-1 regulates the production, survival and function of macrophages through Fms, the receptor tyrosine kinase (X. Chen et al. 2008). M-CSF is the primary cytokine that helps promotes the survival, proliferation (found in mice), and differentiation for cells of the mononuclear phagocyte lineage such as monocytes, macrophages and osteoclasts (Hume and MacDonald 2012; Chitu and Stanley 2006; Hamilton 2008). It was also demonstrated that M-CSF helps regulate the development and function of mononuclear phagocytes, and plays vital roles in innate immunity, cancer and inflammation (Hamilton 2008; Hamilton and Achuthan 2013). This also includes number of studies that suggest the critical role for M-CSF in brain development and normal functioning and its relation to several disease processes which involves neuroinflammation.
IL-34 is a recently discovered cytokine that acts as a second ligand of CSF-1R comparable to M-CSF (H. Lin et al. 2008; Nakamichi, Udagawa, and Takahashi 2013; K. Kimura et al. 2015). IL-34 stimulates proliferation of monocytes and macrophages by binding to CSF-1R (W. Lin et al. 2019). IL-34 shares important functions of M-CSF such as regulating myeloid cell survival, differentiation, and proliferation (H. Lin et al. 2008). IL-34 was identified as an alternative in substitution of M-CSF in osteoclastogenesis (Baud’Huin et al. 2010). However, the current understanding of IL-34 in inflammatory bone-degenerative diseases is limited to a couple of reports concerning bone-osteolysis. IL-34 shares no sequence homology with M-CSF; however, it does resemble M-CSF. IL-34 also stimulates bone marrow-derived monocyte survival and differentiation into macrophages (Chihara et al. 2010; K. Kimura et al. 2015). IL-34 is also known to contribute to the upkeep of microglia in particular areas of the brain (Greter et al. 2012). There have been different studies that demonstrate the distinctive patterns of IL-34 and M-CSF expression are differentially controlled in the cortex of humans with AD and also found in transgenic mouse models (Olmos-Alonso et al. 2016; Walker, Tang, and Lue 2017).

Along with all the major roles these cytokines take part of, M-CSF and IL-34 is known for the ability to support cell growth or survival. Macrophage’s polarization and activation is also triggered by the ligation of M-CSF and IL-34, with the CSF-1R receptor. The macrophages are categorized as “classically activated” pro-inflammatory M1-macrophages or an “alternatively activated” anti-inflammatory M2-macrophages (Murray et al. 2014). These cytokines can differentiate into different activation states in relation to the cytokine balance in their microenvironment (Boulakirba et al. 2018). M1 phenotypic activation which aid in the development of inflammation by upregulating pro-inflammatory cytokines such as interferon gamma (IFNγ) and lipopolysaccharide (LPS) (Tanaka, Narazaki, and Kishimoto 2014). M2 phenotypic activation of anti-inflammation properties through enhancing the expression of anti-inflammation factors is stimulated by interleukin-4 (IL-4). The activated macrophages produce a distinct panel of pro-inflammatory cytokines, which includes tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), and collectively termed as senescence-associated secretory phenotype (SASP). However, the impact of IL-34 on the promotion of inflammation from derived M1 and M2 polarization of macrophages and microglia in the patients with AD remains elusive. There are very few studies on IL-34-polarized focused on being pro-inflammatory and anti-inflammatory macrophages and microglia of CNS (Boulakirba et al. 2018; Muñoz-Garcia et al. 2021). While M-
CSF has been extensively studied, the biology and functions of IL-34 are only now beginning to be uncovered. Thus, the aim of this study is to evaluate the role of IL-34 and how this cytokine associated and promotes with neuroinflammation, inflammation, and bone loss and how inflammation evaluates Alzheimer’s like symptoms compared to M-CSF.

1.5. **IL-34 relation with osteoclastogenesis and osteoporosis**

IL-34 has similar functions to M-CSF, which is a facilitator of inflammation, osteoclastogenesis, osteoporosis, and bone metastasis in cancer (Boström and Lundberg 2013; Tagoh et al. 2002). For a long period of time, it has been believed that the only cytokines required for osteoclast formation are M-CSF and RANKL. However, in newly discovered evidence proposed that the IL-34/ CSF-1R axis also promotes receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclastogenesis from mouse and human osteoclast precursor cells (OPCs) (Baghdadi et al. 2019; Baud’Huin et al. 2010). The identification of the RANKL is known as the key regulator for osteoclast differentiation for osteoclast differentiation. Osteoclasts are multinucleated giant cells that have the capability to resorb bone (Boström and Lundberg 2013; Z. Chen et al. 2011). RANKL/ RANK signaling regulates osteoclast formation, activation and survival in normal bone modeling and remodeling and osteoprotegerin (OPG) is known to protect bone from excessive resorption by binding to RANKL and preventing it from binding to RANK (Boyce and Xing 2008). Furthermore, the ligation of M-CSF with CSF-1R was significantly inhibited by IL-34 in macrophages (Chihara et al. 2010). Moreover, IL-34 has been identified in multiple myeloma, where it increases the rate of osteoclast differentiation and the degree of bone destruction by osteoclasts (Baghdadi et al. 2019), and it aggravates rheumatoid arthritis (RA)-associated bone loss by increasing the chemotactic migration of OCPs, as well as RANKL-induced osteoclastogenesis, in the absence of M-CSF (Hwang et al. 2012).

As mentioned above AD and osteoporosis are both common degenerative diseases found in the elderly population, and how Aβ is considered one of the hallmarks of AD. Studies have demonstrated that amyloid deposition in the brain and RANKL signaling are two seemingly independent pathways leading to AD and osteoporosis (Y.-H. Chen and Lo 2017; Kumar et al. 2021). It is known that M-CSF plays a key role in proliferation and survival of OCPs, as well as RANKL-mediated osteoclastogenesis, however the possible engagements of IL-34 in
osteoclastogenesis and recruitment of OCPs has remain to be elusive. As a ligand of CSF-1R, IL-34 may be relevant to innate immune responses in AD.

1.6. IL-34 pathology in Alzheimer disease

IL-34 is expressed by a variety of cells and tissues (Lelios et al. 2020), this expression is expected to be changed under different pathological conditions. Undeniably, there have been several studies have confirmed the enhancement of IL-34 expression at mRNA and protein levels in the context of various diseases including autoimmune disorders such as rheumatoid arthritis (Boström and Lundberg 2013; Bing Wang et al. 2017; 2018; F. Zhang et al. 2015), inflammation (Zwicker et al. 2015; Y. Wang et al. 2016; Franzè et al. 2015), neurological disorders (Mizuno et al. 2011; Jin et al. 2014; C. Zhu et al. 2016; Gómez-Nicola et al. 2013), and various types of cancer (Booker et al. 2015; Baud’Huin et al. 2010; Bo Wang et al. 2015; Franzè et al. 2017). At the cellular level, IL-34 is produced by a wide-ranging of cell types, these comprise of fibroblasts, immune cells, epithelial cells, neurons cells, adipocytes, Langerhans and cancer cells (Baud’Huin et al. 2010; Y. Wang et al. 2012; Franzè et al. 2015; 2016; 2020). In addition, studies have demonstrated a reduction in IL-34 expression that can be detected and observed in certain pathological conditions, such as in brain tissues in AD (Walker, Tang, and Lue 2017) and periodontal disease (Martinez et al. 2017). In IL-34 expression in cells and tissue, there have been a substantial amount of new evidence indicating the fundamental connection between changes in IL-34 expression with disease progression, pathogenesis, and severity (Baghdadi et al. 2017).

2. Statement of Purpose

M-CSF and IL-34 cytokines have remained a topic of research in the past years, however the understanding of IL-34 physiological remains elusive, including the potential role of IL-34 promotion of bone osteolysis and neuroinflammation and the difference between the connection of M-CSF and IL-34 pathways. As we know, IL-34 is a potential factor in the promotion of bone osteolysis, neuroinflammation, and neurodegeneration activity, indicating that IL-34-mediated inflammation can express hallmark features that can assist in the development of AD. Thus, we hypothesize that in the context of analyzing the impact of IL-34 on the promotion of bone osteolysis and neuroinflammation in experimental models of AD.
3. Material and Methods

3.1. Animals

Young (two-month-old) female and male triple-transgenic mouse model of AD (3x-Tg-AD) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The animals were kept on a 12-hour light-dark cycle at 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, and the experimental procedures were approved by the Institutional Animal Care and Use Committee at Nova Southeastern University.

3.2. Experimental Design in vivo and in vitro

For rigorous experimental design for robust and unbiased results in accordance with the NIH guidelines for preclinical research (Moher et al. 2015), all data will be analyzed by blinding procedures to avoid possible bias. We prepare appropriate controls for in vitro and in vivo experiments. All in vitro studies designed was repeated at least three times in an independent experiment, using five or more samples/condition. To reduce mouse-to-mouse variability in these parameters, all procedures and sampling for experiments using mice was performed at the same time of day to minimize circadian effects. The mice were handled every other day in the morning hours (between 8:00–17:00). The sample size of eight mice per group for in vivo experiments was determined based on 80% power at 0.05 significance using previous published data from a mouse model of ligature-induced periodontitis (Hirschfeld et al. 2017; Ishii et al. 2018; Movila et al. 2018; Wisitrasameewong et al. 2017; Yamaguchi et al. 2016).

Evaluation of the impact of mouse gender as a relevant biological variable on the IL-34-induced neuroinflammation.

For the evaluation of the impact of mouse gender as a relevant biological variable. The 3x-Tg-AD mouse line is widely used and accepted model of AD which shows the hallmark features of AD which includes the Aβ and phospho-tau. These factors have been fully characterized by several studies (Carroll et al. 2010; Billings et al. 2007). Since both LOAD AD patients (Bachman et al.
1992; Viña and Lloret 2010) and 3x-Tg-AD mice (Carroll et al. 2010; Billings et al. 2007) demonstrate gender-dependent pathological differences, in this case studies shows females displaying a significantly higher levels of Aβ, and phosphor-tau, we included both male and female mice, and the results will be analyzed separately. We believe that it will be of particular importance to test our central hypothesis that IL-34 contributes to promotion of bone osteolysis and neuroinflammation in experimental models of AD and serves as a risk factor for AD patients in association with gender.

3.3. Design for Behavioral Testing

To assess the effects of IL-34 on the learning and memory skills in mice, these tests were conducted one week prior to the first application of IL-34 injections and then every two weeks following the first application. Calvaria injections took place every other day for 7 weeks (52 days). We will evaluate the effects of IL-34 and the vehicle control on learning and memory skills, anxiety, exploratory behavior, and daily living activities in mice by I) Elevated Zero Maze, II) Open Field Test, and III) Y-Maze tests using a published protocol by Dr. William Kochen (Craven et al. 2018). The experimental timeline for behavioral testing is shown in Fig. 1. Behavioral testing will start at week 1, this will study the animals behavioral before calvaria injections, as mentioned before behavioral test will be every two-weeks, which occurred at week 2, 4 and 6. Animals will be habituated to the room for at least 15-30 minutes prior to behavioral testing. Before and after each trial, the surrounding surface area or arms of each test or maze were thoroughly cleaned with ethanol to remove any odors. After mice were habituated and acclimated, from a random generator male and female groups and mice were equally randomly selected. All behavioral testing will be video recorded and scored by blinded observers at a later date.

**Fig. 1** |Experimental timeline| for measuring anxiety-like behaviors before and after injections. EZM – Elevated Zero Maze; OFT – Open-Field Test and Y-Maze. IHC – immunohistochemistry.
3.4. Apparatus and Procedures for behavioral tests

Behavioral tests were performed in the following sequence: Elevated Zero Maze, Open Field Test, and Y-Maze. Mice were randomly assigned to be tested using a random generator, exclusively on either the EMZ, Open Field Test, or Y-Maze. Every two weeks, behavioral test treatment was taken place with total of 3 exposures to each of the apparatus. Experimental groups were represented in approximately equal numbers in respective cohorts. The numbers of male and female mice tested in each maze and at each testing interval are shown in Fig. 2.

<table>
<thead>
<tr>
<th>Testing Intervals</th>
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<tr>
<td>Daily</td>
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<tr>
<td>Male</td>
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<tr>
<td>Elevated Zero Maze</td>
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<tr>
<td>Open Field Exploratory Test</td>
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<tr>
<td>Y-Maze</td>
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<tr>
<td>Weekly</td>
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<tr>
<td>Male</td>
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<tr>
<td>Elevated Zero Maze</td>
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<tr>
<td>Open Field Exploratory Test</td>
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<td>Y-Maze</td>
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Fig. 2 | Testing Interval Diagram. The number of female and male mice tested in each apparatus and testing interval.

_Elevated Zero Maze_

Elevated Zero Maze (EZM) diagram shown in Fig. 3. EZM is a common test for anxiety and will be observed throughout the course of the test. For this test, mice are placed in the apparatus for 5 minutes after a 15-minute habituation period in the room. EZM testing took place in the mornings and in the same room. The apparatus was obtained from Stoeling (Wood Dale, IL, USA). EZM was elevated 50 cm above the floor. The EZM is an elevated circular platform (50 cm in diameter) divided into four equal quadrants. The two opposite quadrants were “open”, and the remaining two “closed” quadrants were surrounded by 15 cm high dark, opaque...
walls. Outer walls were constructed of gray plastic and the inner walls were black Plexiglas. Quadrant lanes were 5 cm in width.

**Open Field Test**

The Open-Field Test is a well-accepted assessment to evaluate anxiety, and locomotor activity and behavior in mice (Zhou et al. 2020). Following a 15-minute habituation period, animals were placed at the center of the square arena and consists of a blue plastic board (60 x 60 cm) surrounded by blue plastic walls (40 cm in height). The animals then were freely able to explore the maze for a period of 5 minutes and it was manually recorded. In Fig. 4 demonstrates an Open-Field diagram. Areas that the mice entered, number of center entries, number of fecal boli (the number of defecations or fecal boli deposits was manually counted by the observer), time spent in the center, amount of rearing (mice standing on hind limbs -unsupported by walls), and amount of time spent self-grooming will be assessed by later observers.

**Y-Maze**

The Y-maze (Fig. 5) (Stoelting, catalog number: 60180) has a grey, non-reflective base plate with the dimension’s lane width 5cm, arm length 35 cm and arm height 10 cm. The plastic fiber base plate makes it easy to disinfect between animals to remove any odors or fecal matter. The three arms were interconnected at an angle of 120°. Y-maze was initiated 32 days after IL-34 injections. An elevated

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**Fig. 4** | Open Field diagram, 3D models of Open Field Diagram. The animals were placed at the center of the square arena and consists of a plastic board (60 x 60 cm) surrounded by plastic walls (40 cm in height).

**Fig. 5** | Y-Maze diagram. Y-maze diagram depicting maze dimensions and zones used for monitoring the number of arm entries and exits.
Y-maze is used to evaluate spatial cognitive ability. It is commonly used to test for memory in mice. Animals were first placed at the center of the maze, where which the mice explored for a period of 6 minutes and was manually recorded. Spontaneous entry into and percentage of alternations between the three different arms were defined and recorded as continuous selection. The number of arm entries was also recorded.

3.5. Establish IL-34 simulated microglial activation using experimental models of LOAD-like neuroinflammation

A mouse model of LOAD-like neuroinflammation, altogether 32 young mice (2-month-old); 16 male and 16 female mice, an equivalent number of males and females will be employed in this study. The groups from male and female mice were separated into 2 groups, 8 mice per group, each which was injected with 1µg/ml, 100 µl per site. To induce LOAD-like neuroinflammation, a mixture of IL-34 with phosphate buffered saline (PBS) was injected over the calvaria using 3x-Tg-AD mice once every other day for 7 weeks (52 days). As for the control group, the animals will receive a mixture of PBS injections only.

3.6. Brain collection and histopathology

As for tissue harvesting once the cognitive tests and calvarial injections were completed at the end of week 7 (51 days). Brains from the non-treated and IL-34-treated mice were removed after exsanguination under anesthesia and fixed with 4% paraformaldehyde (PFA) in PBS and then transferred to 30% sucrose solution in PBS 4°C overnight, published protocol from Dr. Nacer and Dr. Movila (Nacer et al. 2012). Brains were prepped and put in a cryomold, then the brains were embedded in optimal cutting temperature (O.C.T) compound (Thermo Fisher Scientific) and sectioned (6 pm thickness) with a cryostat (Leica). The brain and chamber temperature were at -20°C respectively.

3.7. Immunohistochemistry of brain tissue isolated from mice

After the brains were collected and fixed, the brains were then slice into coronal sections and processed for staining. Slices were rinse with PBS 3x times every 10 minutes, and then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperate. Afterward, slices were
incubated with primary antibody overnight at 4°C. Antibodies used were as follows: Beta-Amyloid polyclonal antibody (CT695) (1:200, ThermoFisher), Phospho-Tau (Thr231) antibody (PA5-117230) (1:200, ThermoFisher), RAP recombinant rabbit monoclonal antibody (9) (1:200, ThermoFisher), RAGE polyclonal antibody (PA5-78736) (1:200, Invitrogen, ThermoFisher), NeuN antibody (14HCLC) ABfinity Rabbit Oligoclonal (1:200, ThermoFisher), ZO-1 antibody (PA5-85256) (1:200, ThermoFisher), Rabbit Anti-Tau oligomer antibody T22 (1:200, EMDMilipore) and Rabbit polyclonal Occludin antibody (NBP1-77037SS) (1:200, Novus Biologicals). Unspecific labeling was defined by omitting the primary antibody. After 24-hour, samples were rinse in 1% BSA in PBS 3x times for 10 minutes, and then secondary antibody Goat Anti-Rabbit IgG H&L (Alexa Fluo ® 594) (Abcam, UK, ab150080) was applied and incubated for 1-hour at room temperature. To label nuclei, the slices were incubated with Hoechst 33342 (1:2000, ImmunoChemistry Technologies) for 5 minutes. Slices were then rinse in 1% BSA in PBS 3x times for 10 minutes, and mounted in Aqua Poly/Mount (Polysciences, Inc.), and images were acquired with a EVOS Cell Imaging Systems and microscope (Thermofisher). Images were evaluated by Image J software. The scale bar was measured at 20x, 200 μm. The data was analyzed using PAST 2.1 statistical software. The statistical significance was evaluated using a one-way ANOVA with post hoc Tukey’s test. A p < 0.05 (*), 0.01 (**), and 0.001 (***)) was considered statistically significant.

3.8. Histology Tartrate-resistant acid phosphatase (TRAP) staining of Calvaria tissue isolated from mice

Tissue decalcification

Mice calvaria were dissected and fixed in 10% formaldehyde overnight at 4°C. The specimens were then washed in PBS and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 3 weeks or more at room temperature. Decalcified samples were dehydrated in graded alcohols and then embedded in paraffin. Frontal calvaria sections were slice at 6-μm in thickness centered on the sagittal suture and were obtained for histological analysis.


**TRAP staining**

For histological analysis, to stain for TRAP positive (TRAP+) osteoclast precursors cells, calvaria sections were first incubated in 0.2M acetate buffer containing 50 mM L-(-)-Tartaric acid (Sigma) at room temperature and then in TRAP staining solution (0.2 M acetate buffer, 50 mM L-(-)-Tartaric acid, 0.5 mg/ml Naphthol AS-MX phosphate, 1.1 mg/ml Fast Red AST salt; Sigma) at 37°C. Finally, the sections were counterstained with hematoxylin solution (Sigma) at room temperature. Immunofluorescence was observed with a Zeiss LSM780 Confocal Microscope or with EVOS Cell Imaging Systems and microscope (ThermoFisher). For bone histomorphometry, for each animal, had two slides, each containing four tissue sections with the largest number of bone marrow cells (twelve specimens total), were analyzed. TRAP positive (TRAP+) osteoclast precursors cells were counted manually, the statistical significance of the data was determined Student’s t-test. A $p < 0.05$ (*), 0.01 (**), and 0.001 (***) was considered statistically significant.

3.9. Cell cultures and reagents

**Establish the role of inflammation in IL-34-dependent macrophages/microglial activation in vitro**

Primary mouse bone marrow-derived macrophages (BMDMs) of 3x-Tg-AD mice and microglia cells will be proliferated using M-CSF and IL-34 mouse recombinant protein and then polarized into M1- using IFN-γ and LPS- *E. coli* and M2-subsets using IL-4, respectively (Yamaguchi et al. 2016). After, 24-hour stimulation, the culture supernatant, as well as cells total RNA and proteins will be collected, and evaluated for pro-inflammatory and anti-inflammatory cytokines using enzyme-linked immunosorbent assay (ELISA) and Real-Time PCR, respectively.

**Primary Bone Marrow Derived Macrophages**

To generate primary bone marrow-derived macrophages, BMDMs cells were isolated from the femurs and tibias of young (two-month-old) female and male 3x-Tg-AD mice and plated at a density of $5 \times 10^5$ cells per well, in alpha-MEM (Life Sciences) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1% Anti-Anti, 1% L-glutamine, 1% MEM-NEAA (Life Sciences).
**Mouse Microglia Cell Culture and Treatment**

Spontaneously Immortalized Microglia-A9 (SIM-A9) (ATCC® CRL-3265™) cell line was purchased 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® 30-2006™) supplemented with 10% FBS (Atlanta Biologicals), and 5% heat-inactivated horse serum (Gibco).

3.10. **Macrophage/Microglia polarization, differentiation, and activation**

To study inflammation markers on macrophage subtypes, macrophages are referred to as the cytokines used for their generation. M-CSF or IL-34 simulated macrophages were produced by exposing BMDMs. These cells were seeded at 5×10⁵ cells/ml in 24-well plate for 4 days in mouse recombinant protein of M-CSF or IL-34 (Biolegend) at 20 ng/ml, respectively. This will assist in monocytes differentiating into derived macrophages. On day 5, the derived-macrophages were polarized into M1 macrophages, M1 macrophages was stimulated on adherent culture media in the presence or absence of 10 ng/ml of IFN-γ and 10 ng/ml of LPS- E. coil (pro-inflammatory) and into M2 macrophages using 20 ng/ml IL-4 (anti-inflammatory), and a group were left untreated for the duration of the culture was considered M0 or as control. After 24h the samples were collected and measured in the collected supernatant using commercial sandwich ELISA kits (BioLegend and

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**Fig. 6 | Experimental Design for Macrophage Polarization in vitro.** (A) Extration of bone-marrow (BM) from 3x-Tg-AD mice. (B) Day 0: Isolated bone-marrow derived monocytes were seeded into 24-well plates and supplemented with either M-CSF or IL-34. (C) Day 3: BM-derived monocyte will successfully be differentiated into derived macrophages, fresh media was replaced with fresh M-CSF or IL-34 and stimulated with Pro-inflammatory (IFN-γ + LPS - E, coil) and anti-inflammatory (IL-4) cytokines and incubated for 24-hour, wells were split into one of the four treatment groups; the treatment condition was as follows: 1) Control or M-CSF or IL-34 only 2) M-CSF or IL-34 + M1, 3) M-CSF or IL-34 + M2. (D) Macrophages were successfully polarized into M1 Pro-inflammatory macrophages or M2 anti-inflammatory macrophages. Samples were collected and checked for pro-inflammatory and anti-inflammatory markers. (E) Legend of treatment groups.
R&D systems) according to the manufacturer’s instructions and Real-Time PCR, respectively. The experimental design for BMDMs is shown in Fig. 6.

As for mouse microglia SIM-A9 cells. These cells were seeded at 5x10^5 cells/ml in 24-well plate for 1 days in mouse recombinant protein of M-CSF or IL-34 (Biolegend) at 20 ng/ml, respectively. On day 2, the macrophages were polarized into M1 macrophages, M1 macrophages was stimulated on adherent culture media in the presence or absence using 10 ng/ml of IFN-γ and 10 ng/ml of LPS-E. coil (pro-inflammatory) and into M2 macrophages using 20 ng/ml of IL-4 (anti-inflammatory), and a group were left untreated for the duration of the culture was considered M0 or as control. After 24-hours the samples were collected and measured in the collected supernatant using commercial sandwich ELISA kits according to the manufacturer’s instructions and Real-Time PCR, respectively. The experimental design for SIM-A9 mouse microglia cells is shown in Fig. 7.

**Fig. 7 | Experimental Design for SIM-A9 Polarization in vitro.** (A) Day 0: SIM-A9 mouse microglia cells were seeded into 24-well plates and media was supplemented with either M-CSF or IL-34 for 24-hours. B) Day 2: Fresh media was replaced with fresh M-CSF and IL-34 and stimulated with Pro-inflammatory (IFN-γ + LPS-E, coil) and anti-inflammatory (IL-4) cytokines and incubated for 24-hour, wells were split into one of the four treatment groups; the treatment condition was as follows: 1) Control (cells only) 2) M-CSF or IL-34 only, 3) M-CSF or IL-34 + M1 and 4) M-CSF or IL-34 + M2. (C) Day 3: Microglia cells were successfully polarized into M1 Pro-inflammatory macrophages or M2 anti-inflammatory macrophages. Samples were collected and checked for pro-inflammatory and anti-inflammatory markers. (E) Legend of treatment groups.

3.11. **RANKL-Induced Osteoclastogenesis of Bone Marrow Derived Macrophages (BMDMs)**

BMDMs were isolated from the femurs and tibias of male 3x-Tg-AD mice (Jackson Laboratories) and plated at a density of 1x10^5 cells per well, in alpha- MEM (Corning) supplemented with 10% FBS (Atlanta Biologicals), 1% Anti-Anti, 1% L-glutamine, 1% MEM-
NEAA (all from Gibco), and 30 ng/ml mouse recombinant M-CSF or 30 ng/ml mouse recombinant IL-34 for 3 days (Duarte et al. 2021). To induce osteoclastogenesis, the proliferation media described above was supplemented with 10 ng/ml of recombinant mouse RANKL (Biolegend). In addition to RANKL, RANKL was also supplemented with 1 ng/ml or 10 ng/ml Aβ₄₀, and Aβ₄₂. The cells were cultured for seven days, and media was refreshed every two days. The samples were collected and measured in the collected supernatant using ELISA kits according to the manufacturer’s instructions. Wells were imaged with a 4x objective and followed by manual counting of osteogenic differentiation. Data were analyzed using PAST 2.1 statistical software. Statistical significance was evaluated using a one-way ANOVA with post hoc Tukey’s test. A p < 0.05 (*), 0.01 (**), and 0.001 (***) was considered statistically significant. Demonstrated in Fig. 8 is the experimental design.

Fig. 8 | Experimental Design for RANKL-induced Osteoclastogenesis in vitro. (A) Extraction of bone-marrow (BM) from 3x-Tg-AD mice. (B) Day 0: Isolated BM-derived monocytes were seeded into 24-well plates supplemented with either M-CSF or IL-34, cells were cultured for 7 days. (C) Day 3: BM-derived monocyte will successfully be differentiated into derived macrophages, fresh culture medium was supplemented with either M-CSF or IL-34 and stimulated with RANKL and various Aβ peptides. The treatment condition was as follows: 1) Control, 2) M-CSF or IL-34 RANKL alone, 3) M-CSF or IL-34 + RANKL + Aβ₄₀, 4) M-CSF or IL-34 + RANKL + Aβ₄₂. Media will be refreshed every 2 days. (D) Day 7: Cells will go through osteogenic differentiation and form osteoclast. Samples were collected and checked for osteogenic differentiation. (E) Legend of treatment groups.

3.12. Pit Formation Assay

3x-Tg-AD male BMDMs cells were preincubated with M-CSF or IL-34 in the presence or absence of RANKL alone, and two different concentrations of Aβ₄₀, and Aβ₄₂ in 96-well plates coated with calcium hydroxyapatite (Miyazaki et al. 2011). Ten days after RANKL, Aβ₄₀, and Aβ₄₂
addition, the plates were washed with sodium hypochlorite and air dried. Wells were imaged with a 10x objective using an EVOS cell imaging system. The total number of TRAP+ osteoclasts per 0.01 mm² was evaluated by Image J. Scale bar is measured at 50 μm. Data were analyzed using PAST 2.1 statistical software. Statistical significance was evaluated using a one-way ANOVA with post hoc Tukey’s test. A p < 0.05 (*), 0.01 (**), and 0.001 (*** ) was considered statistically significant.

3.13. Cytokine Analysis

Mouse TNF-α, IL-1β, and IL-6 levels in the culture supernatants were measured by ELISA using DuoSet kits from R&D Systems (Minneapolis, MN, USA) or BioLegend and following the manufacturer’s instructions. The absorbance (A) was detected at a single wavelength of 540 nm. The cytokine concentration was determined according to the standard curve.

3.14. Total RNA extraction and Real time PCR

Total RNA of BMDMs and microglia cells was extracted using the PureLinkTM RNA Mini Kit (Ambion, Life Technologies), according to the manufacturer’s instructions, and reverse transcription of 1 mg of total RNA was performed using the Verso cDNA Synthesis Kit (Thermo Scientific), following the manufacturer’s recommendations.

The gene expressions were measured using PowerUpTM SybrTM Green Master Mix (Applied Biosystems Diagnostics, Life Technologies) or TaqManTM Universal PCR Master Mix (Applied Biosystems, Life Technologies), in or StepOnePlus™ Real-Time PCR (Thermo Fisher) System or QuantStudio 3 Real-Time PCR (Thermo Fisher) System. The following primers were used: IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1) and TNF-α (Mm00443258_m1). Data were analyzed using the 2-ΔΔCt method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Mm 99999915_g1).

The gene expressions were measured using Dcstmap and Acp5/Trap were evaluated using the PowerUpTM SybrTM Green Master Mix (Applied Biosystems Diagnostics), in the AriaMx Real-time PCR System (Agilent, USA) and quantified using the AriaMX Software Version 1.3. The primer sequences used were Dcstamp (XM_006521519.3) (forward [F]: 5’-TCCTCCATGAACAAACAGTTCCA-3’, reverse [R]: 5’-AGACGTGGTTTAGGAATGCAGCT-3’), and Acp5/Trap (XM_006509945.3) (forward [F]: 5’
CCAGCGACAAGAGGTTCC-3’, reverse [R]: 5’-AGAGACGTTGCCAAGGTGAT-3’). The data was analyzed using the ΔΔCq method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH (NM_001289726.1) (forward [F]: 5’- AACTTTGCCATTGTGGAAGG-3’, reverse [R]: 5’-ATGCAGGGATGATGTTCTGG-3’).

3.15. Statistical analysis

Data are displayed as mean ± Standard Deviation (SD). Data were analyzed using PAST 2.1 statistical software. Statistical significance was evaluated using a one-way ANOVA with post hoc Tukey’s test. A p < 0.05 (*), 0.01 (**), and 0.001 (***)) was considered statistically significant. Analysis of the behavioral videos was completed by using ANY-maze Video Tracking System v7.07 (Stoelting). For data analysis, Statistics Package for the Social Sciences 27 (IBM) was used and for graphs we used the GraphPad v 9.2 software. EZM was analyzed by hand by blinded undergraduate students.

4. Results

4.1. Role of IL-34 and M-CSF in in RANKL-mediated osteoclastogenesis in vitro

The role of IL-34 and M-CSF in RANKL-mediated osteoclastogenesis, in the beginning of our study, in our preliminary research we initially assessed the effects of these cytokines on RANKL alone, RANKL simulated with Aβ40, and Aβ42 -mediated osteoclastogenesis in vitro using BMDMs isolated from male 3x-Tg-AD mice. To validate these measurements, we evaluated the expression pattern promoted from IL-34 with RANKL alone, RANKL with Aβ40, and Aβ42 in BMDMs, expression of mRNA for dendritic cell-specific transmembrane protein (Dc-stamp) and Acp5/Tartrate resistant acid phosphatase (TRAP) was analyzed using quantitative PCR.

The OCPs stimulated with IL-34 with RANKL alone, RANKL with Aβ40, and Aβ42 with 2 different concentrations showed significantly increased expression of some osteoclast-specific cell fusion and activity markers, including Dc-stamp and Acp5/TRAP, compared to those stimulated with a combination of M-CSF/RANKL alone and RANKL with Aβ40 (Fig. 9A, B), and Aβ42 (Fig. 9C, D). We then examined the ability of M-CSF and IL-34- proliferated OCPs to become multinucleated TRAP+ osteoclasts in the RANKL alone, RANKL with Aβ40, and Aβ42. IL-34/RANKL alone stimulation was significantly higher when compared to M-CSF/RANKL alone
IL-34/RANKL with Aβ40 1 ng/ml and 10 ng/ml stimulation was significantly higher when compared to M-CSF/RANKL with Aβ40 (Fig. 9F, 1 ng/ml \( p=0.0181 \), 10 ng/ml \( p=0.0033 \)). As for IL-34/RANKL with Aβ42 1 ng/ml and 10 ng/ml stimulation also demonstrated a significantly higher expression when compared to M-CSF/RANKL with Aβ42 (Fig. 9G, 1 ng/ml \( p=0.0016 \), 10 ng/ml \( p=0.0052 \)). Higher concentration of Aβ40 and Aβ42 with stimulated with IL-34 showed an increase of levels of IL-34. There was no TRAP+ multinucleated cells that were observed in the response of neither M-CSF nor IL-34 in the absence of RANKL alone, RANKL with Aβ40, and Aβ42.

Furthermore, the results for the number of pits formed, from mature osteoclast was accordingly increased in the presence of IL-34/RANKL alone (Fig. 9I, J, \( p=0.0197 \)), IL-34/RANKL with Aβ40 1 ng/ml (Fig. 9I, \( p=0.0001 \)), although IL-34 demonstrated more mature osteoclast, there was not a significant difference between M-CSF and IL-34 (Fig. 9I, \( p=0.1373 \)). As for Aβ42, mature osteoclast was accordingly increased in the presence of IL-34/RANKL alone ((Fig. 9I-L, \( p=0.0197 \)), IL-34/RANKL with Aβ40 1 ng/ml (Fig. 9J, \( p=0.0197 \)), although IL-34 demonstrated more mature osteoclast, there was not a significant difference between M-CSF and IL-34 (Fig. 9J, \( p=0.0938 \)).

As for the resorption area from mature osteoclast was accordingly increased in the presence of IL-34/RANKL alone (Fig. K, L, \( p=0.0388 \)). Although IL-34 demonstrated more mature osteoclast, there was not a significant difference between M-CSF and IL-34 RANKL with Aβ40 1 ng/ml (Fig. 9K, \( p=0.07414 \)). As for IL-34/RANKL + Aβ40 10 ng/ml there was an increase presence of mature osteoclast in (Fig.9K, \( p=0.00712 \)) in comparison to M-CSF/RANKL. As for Aβ42, there was significantly greater increase presence of mature osteoclast rather than the presence of M-CSF (Fig. 9L, 1ng/ml \( p=0.00712 \), 10 ng/ml \( p=0.00594 \)) in comparison to M-CSF/RANKL.
Fig. 9 | Effects of recombinant IL-34 and M-CSF proteins on RANKL-induced osteoclastogenesis in vitro. Expression profile of the osteoclast fusion and function genes, (A, B) Dc-stamp and (C, D) Acp5/Trap was quantified in IL-34- and M-CSF-proliferated BMDMs after 7 days culture in the presence or absence of recombinant RANKL. The difference in gene expression is shown as the fold change after normalization against GAPDH. Microscopic evaluation (E) of the TRAP staining and quantification of the number of TRAP+ multinucleated cells (F, G) in the M-CSF- and IL-34-stimulated OCPs exposed to RANKL, AβP 1–40, and AβP1–42 in vitro. Representative images of pit formation (H), number of pits formed (I, J) and pit area (K, L) quantified by Image J. n=3 samples/condition Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
4.2. Effect of IL-34 and M-CSF on BMDMs macrophages polarization into M1 or M2

Pro-inflammatory cytokines analysis: ELISA

The effect of IL-34 and M-CSF on BMDMs in M1 and M2 polarization of macrophages, the inflammatory responses of M1 and M2 polarization were assessed by measuring the levels of pro-inflammatory cytokines produced by BMDMs cells from 3x-Tg-AD. The supernatant collected from the BMDMs cells from 3x-Tg-AD mice was stimulated with M-CSF or IL-34 for 4 days, the cells that were cultured for M1 macrophage polarization was stimulated on adherent culture media in the presence or absence IFN-γ and LPS-E. coil and M2 macrophage polarization was supplemented with IL-4 for 24-hours. After 24-hours, the medium was collected and were subject to commercially available enzyme-linked immunosorbent assay (ELISA) for TNF-α, IL-6, and IL-1β.

Results from the measurements of supernatant from M1 from females and males 3x-Tg-AD BMDMs, TNF-α (Fig. 10G, S), IL-6 (Fig. 10H, T), and IL-1β (Fig. 10I, U) levels showed that IL-34/M1 was significantly elevated compared to all pro-inflammatory cytokine’s levels of M1/M-CSF. Comparing both genders, female showed IL-34/M1 and M-CSF/M1 demonstrated an upregulation of inflammation compared to males. Additionally, compared to M1 polarization (Fig 10G-I, S-T), female M2 polarization (Fig. 10J-L, V-X) demonstrated downregulation of inflammation. As for the production of TNF-α levels from M2 females (Fig. 10J) and males (Fig. 10V), M2/IL-34 showed prominently elevated prominently elevated TNF-α production. There was a significant difference effect from female TNF-α (Fig. 10J) between undifferentiated (M0) compared to M2/M-CSF and M2/ IL-34, and male (Fig. 10V) had a significant difference effect between undifferentiated (M0) compared to M2/M-CSF alone. This demonstrates that M1 simulated with these cytokines aid in inflammation, essential promoting proinflammatory responses and is expressing higher levels in females.

Analysis of MRNA expressions of Senescence-Associated Secretory (SASPs) Cytokines: qPCR

To validate these measurements, we evaluated the expression pattern of inflammation promoted from M1 and M2 in BMDMs, expression of mRNA for TNF-α, IL-6, and IL-1β was
analyzed using quantitative PCR. The relative ratio of the genes was compared to an internal control (GAPDH). Similar to ELISA data, the gene expressions from females and males 3x-Tg-AD BMDMs, TNF-α (Fig. 10A, M) IL-6 (Fig. 10B, N), and IL-1β (Fig. 10C, O), demonstrated that M1/IL-34 significantly upregulated these proinflammatory cytokines mRNA expression compared to M1/M-CSF.

Comparing both genders, female showed M1/IL-34 and M1/M-CSF demonstrated an increase of inflammation compared to males, except in IL-1β. Additionally, compared to M1 polarization (Fig. 10A-C, M-O), female M2 polarization (Fig. 10D-F, P-R) demonstrated decrease of inflammation. As for the production of TNF-α (Fig. 10A) and IL-6 (Fig. 10B), IL-1β (Fig. 10C) levels from M1 females M1/IL-34 showed prominently elevated production compared to the undifferentiated cells. There was a significant difference effect from female IL-1β (Fig. 10C) between undifferentiated (M0) compared to M1/M-CSF, M2/M-CSF and M2/ IL-34. Male TNF-α (Fig. 10M) and IL-1β (Fig. 10O) had a significant difference effect between undifferentiated (M0) compared to M1/M-CSF alone. Overall, results indicated that the additive effects between female M1/IL-34 and male M1/IL-34 in TNF-α, IL-6 and IL-1β elevated expression was mediated by SASPs receptors expressed of inflammation on macrophages in comparison to M-CSF.

Overall, the results exhibited that M1 proliferated macrophages induce IL-34 constantly expressed stronger production of pro-inflammatory cytokines like TNF-α and IL-6 at RNA and protein level compared to M-CSF. There was no significant effect on pro-inflammatory cytokine observed from M2 proliferated macrophages in M-CSF and IL-34 which was expected because M2 polarization are known as anti-inflammatory macrophages. The expression of TNF-α and IL-6 was significantly elevated in M1 and not M2 compared to control in both females and males. The expression of IL-1β was elevated by IL-34 only in males and not in females. These results demonstrated that M1-polarized macrophages stimulated with M-CSF or IL-34 cytokines help in inflammation promoting pro-inflammatory response compared to M2, and IL-34 demonstrated a stronger effect than M-CSF.
Fig. 10 | The effects of BMDMs polarization into M1 or M2 macrophages in vitro, on the expression Senescence-associated secretory phenotype (SASPs) markers in M-CSF- and IL-34- primed macrophages isolated from bone marrow of young female and male 3x-Tg-AD mice. The changes of mRNA gene expression of pro-inflammatory cytokines; and ELISA. Females (A-C, D-F) and Males (M-N, P-R) were assessed after 24-hour exposure M1 (IFN-γ 10 ng/ml / LPS-E. coli 10 ng/ml) and M2 (IL-4 20 ng/ml). n = 3 samples/condition; Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
4.3. Effects of IL-34 and M-CSF microglia polarization into M1 or M2 microglia in vitro

Pro-inflammatory cytokines analysis: ELISA

The inflammatory responses of M1 and M2 polarization were assessed by measuring the levels of pro-inflammatory cytokines produced by SIM-A9 mouse microglia cells. Supernatants collected from the SIM-A9 mouse microglia cells that were stimulated with M-CSF or IL-34 for 24-hours, after 24-hours the cells that were cultured for M1 macrophage polarization was stimulated on adherent culture media in the presence or absence IFN-γ and LPS E. coli and M2 macrophage polarization was supplemented with IL-4 for 24-hours. After 24-hours, the medium was collected and were subject to commercially available ELISA for TNF-α, IL-6, and IL-1β.

Results from measuring M1 from SIM-A9 microglia cells, TNF-α (Fig. 11G), IL-6 (Fig. 11H), and IL-1β (Fig. 11I) levels showed that M1/IL-34 was significantly elevated compared to all pro-inflammatory cytokine’s levels of M1/M-CSF. Additionally, compared to M1 polarization (Fig. 11G-I), female M2 polarization (Fig. 11J-L) demonstrated downregulation of inflammation. As for the production of TNF-α (Fig. 11G), IL-6 (Fig. 11H), and IL-1β (Fig. 11I) levels, M1/M-CSF and M1/IL-34 showed prominently elevated expressions compared to undifferentiated (M0) group. This demonstrates that M1 simulated with these cytokines aid in inflammation essential promoting proinflammatory responses and is expressing higher levels in M1/IL-34 than M1/M-CSF which can correlate to our data we got from expressions levels from BMDMs cells.

Analysis of mRNA expressions of Senescence-Associated Secretory (SASPs) Cytokines: qPCR

To validate these measurements, we evaluated the expression pattern of inflammation promoted from M1 and M2 in SIM-A9 mouse microglia cells, expression of mRNA for TNF-α, IL-6, and IL-1β was analyzed using quantitative PCR. The relative ratio of the genes was compared to an internal control (GAPDH). Similar to ELISA data from the expression’s levels from 3x-Tg-AD BMDMs and microglia data, TNF-α (Fig. 11A) IL-6 (Fig. 11B), and IL-1β (Fig. 11C) showed M1/IL-34 significantly upregulated these proinflammatory cytokines mRNA expression compared to M1/M-CSF.

SIM-A9 gene expression exhibited in M2/M-CSF and M2/IL-34 (Fig. 11D-F) demonstrated a downregulation of inflammation when compared to M1/IL-34 and M1/M-CSF (Fig. 11A-C).
Overall, results indicated that the additive effects compared to 3x-Tg-AD mice M1/IL-34 TNF-α, IL-6 and IL-1β expression in microglia cells was mediated by SASPs receptors expressed of inflammation on microglia.

Overall, SIM-A9 microglia cells are exhibiting similar results to primary cells M1 proliferated macrophages induced by IL-34. IL-34 constantly expressed stronger production of pro-inflammatory cytokine response than M-CSF. There was no significant effect on the pro-inflammatory response observed in M2 macrophages.

Fig. 11 | The effects of SIM-A9 mouse microglia cells polarization into M1 or M2 macrophages in vitro, on the expression Senescence-associated secretory phenotype (SASPs) markers in M-CSF- and IL-34- primed macrophages from SIM-A9. The changes of mRNA gene expression of pro-inflammatory cytokines (A-F); and ELISA (G-L) were assessed after 24-hours exposure M1 (IFN-γ 10 ng/ml / LPS-E. coli 10 ng/ml) and M2 (IL-4 20 ng/ml). n = 3 samples/condition; Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***).
4.4. The role of IL-34 in the development of peripheral bone lesions in vivo

To address whether IL-34 plays a role in the development of peripheral bone lesions in vivo, we first induced osteolysis lesion in the calvaria of 3x-Tg-AD mice via local injection of PBS (control) and IL-34 in PBS suspension. We examined the ability of proliferated OCPs to become TRAP+ osteoclasts in the presence of IL-34. The number of TRAP+ osteoclasts measured in a microscopic field (0.01 mm²) of TRAP-stained sections (Fig. 12A) from the OCPs in response to IL-34-treated mice was significantly higher when compared to non-treated mice (female p = 0.0004, male p= 0.0009). Additionally, we then examined the number of TRAP positive cells and inflammatory infiltrates at the lesion was found to be significantly elevated in the mice that received IL-34 compared to the groups that received control PBS injection alone (Fig. 12B). This indicates, there is a presence of bone loss after IL-34 injections and seen in female, they had a greater response to IL-34 that is significantly upregulated compared to male.

Fig. 12 | TRAP histochemical staining. Histological evaluation of TRAP+ osteoclasts in calvarial sections of control (PBS) and recombinant IL-34 protein in the calvaria section treated with PBS or IL-34 for female and male 3x-Tg-AD mice (A). Tartrate-resistant acid phosphatase (TRAP) purple staining indicates the deposits of osteoclast-like cell. The number of TRAP+ osteoclasts measured in a microscopic field (0.01 mm²) of TRAP-stained sections. (B). The total number of TRAP+ osteoclasts per 0.01 mm² was evaluated by Image J. Scale bar indicates 50 μm. n=3 samples/condition; Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
4.5. Behavioral Testing

**Elevated Zero Maze**

A two-way between subjects (sex × apparatus) ANOVA comparing activity levels of mice on EZM during the first exposure to the apparatus found that there was a significant interaction effect between gender and condition in this case male for number of entries into open arms \((p=0.014; \text{Fig. 13A})\). The total number of mice EZM entries in female mice indicates a decrease in IL-34 compared to its control. In males there was a significant different in the number of entries between the conditions, demonstrating control having an increase in entries. Comparing genders EZM entries, females had less entries than males, this can be indicative that females demonstrate an increased in stress and anxiety. As for EZM time, there was a significant interaction effect of gender on the amount of time spent in open arms \((p=0.022; \text{Fig. 13B})\). However, with there only being an effect of gender, it demonstrates that males spent more time in the open. This indicates there was more anxiety behavior overall expressed for males than females. For EZM head dips, there was a significant effect of the condition on the number of head dips into open arms \((p=0.022; \text{Fig. 13C})\), in both genders, there was a decrease from control compared to treated IL-34 and is an indication that animals had anxiety. Therefore, there was a significant interaction effect of conditions. There was a main effect of sex on mice movement in the maze during weekly testing, with female mice exploring the apparatus more than male mice.

![Fig. 13](image-url) | Behavior of female and male mice in the EZM during exposure. Maze comparison in untreated and treated 3x-Tg-AD mice female \((n=8)\) and male \((n=7)\) mice. The results were evaluated in parallel with control and IL-34. (A) The total amount of entries into the open and closed area of EZM during the experimental time. (B) EZM Time, the amount of exploration (total of time spent in open arms) (C) EZM Head Dips, the amount of time spent inside an enclosed area versus open regions in EZM during the entire experimental time. Student’s t-test and one-way ANOVA. \(p < 0.05 (*)\), 0.01 (**) and 0.001 (***).
Open Field Test

In the open-field test, overall, there was no significant effect in the number of fecal bolis between gender and condition (female: $p=0.4012$, male: $p=0.886$; Fig. 14A). Fecal bolis in female mice show an increase in IL-34-treated mice compared to non-treated. In males there was not a significant different in the number of fecal bolis between the conditions. Comparing genders, females had more fecal bolis than males, this can be indicative that females demonstrate an increased in anxiety. As for time immobile, there was not a significant effect between gender and condition (female: $p = 0.4084$, male: $p=0.8012$; Fig. 14B). As for the of the overall distance traveled, there was not a significant effect between gender and condition (female: $p = 0.8179$, male: $p=0.9469$; Fig. 14C). For the overall distance traveled, the females tended to be more active than males, displaying higher levels of movement activity, however these differences between gender and condition were not statistically significant. For episodes immobile, there was not a significant effect between gender and condition (female: $p = 0.458$, male: $p=0.6141$; Fig. 14D). For episodes immobile, the females showed a decline in IL-34-treated mice compared to non-treated mice. Males treated (IL-34) group showed to be more active. A decline in the female treated group compared to the males indicates a cognitive decline or expression of anxiety. However, these differences between gender and condition were not statistically significant, and overall, there was not any association from non-significant findings.

![Graphs showing the results of the open field test](image)

**Fig. 14** Behavior of 3x-Tg-AD mice in the Open Field Test during exposure. Maze comparison in untreated (control) and treated (IL-34) in 3x-Tg-AD mice. (A) Total number of fecal boli, (B) Time spent Immobile, (C) Total Distance Traveled, (D) Total number of Episodes spent Immobile. Female (n=8) and male (n=7) mice. Student’s t-test and one-way ANOVA. $p < 0.05 (*)$, 0.01 (**), and 0.001 (***).
**Y-Maze**

The Y-maze can be used to assess short term memory in mice (Kraeuter, Guest, and Sarnyai 2019). From what can be shown in **Fig. 15A, B**, it is a representative heatmaps of cumulative time exploration of start. Female IL-34-treated (**Fig. 15A**: right) 3x-Tg-AD mice visited significantly more arms compared to its control (**Fig. 15A**: left) and male 3x-Tg-AD mice (**Fig. 15B**). As for the percentage of alternations in the total number of entries was significantly increased from gender and condition (**Fig. 15C, p =0.042**). The test was taken place in a bright lit room. As for Female, IL-34 treated mice compared to male IL-34-treated mice display an increase in alterations percentage, this indicates that females could not inhibit their anxiety like behavior. Compared to male alone, IL-34-treated mice were more exploratory which represent the decrease in alteration percentage which indicates that there was a reeducation of anxiety and lack of excitement. For time spent immobile of non-treated mice and IL-34-treated mice in the Y-maze test there was a significant difference between non- treated and treated mice between gender and condition in the total time spend in maze (**Fig. 15D, p=0.015**). As for female alone, IL-34-treated mice exhibit a decrease compared to non-treated mice. This can indicate females is expressing stress or anxiety causing a decline in cognitive memory. When compared to males there is not a significant interaction effect been gender and condition.

![Fig. 15](image_url) | *Behavior of female and male mice in the Y-Maze during exposure.*  Y-maze comparison in untreated (control) and treated (IL-34). The Y-maze and the overall activities shown by heat maps (A) Female mouse, control mouse (left) and an IL-34 mouse (right), (B) Male mice, control mouse (left) and an IL-34 mouse (right), (C) Y-Maze Alterations, (D) Y-Maze Time Immobile. Female (n=8) and male (n=7) mice. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***).
4.6. *Immunofluorescence staining on IL-34 Injected 3x-Tg AD mice*

To demonstrate the development of inflammation in vivo, we injected IL-34 into young two-month-old female and male 3x-Tg-AD mice. Immunofluorescence staining of mouse antibodies in 3x-Tg-AD brains sections.

We used immunostaining to examine the expression of Aβ deposits area in the cortex and thalamus. IL-34-treated mice demonstrated positive Aβ deposits area in the cortex of 3x-Tg-AD mice by immunohistochemistry (Fig. 16A, B; bottom). Consistent with this observation, we did detect an elevated presence of Aβ deposits area of the IL-34-treated mice thalamus as well (Fig. 16D, E; bottom). As for the relative fluorescence intensity for cortex (Fig. 16C) demonstrated that IL-34-treated mice of both genders had an elevated amount of Aβ deposits levels compared to non-treated mice. Female IL-34 treated mice had a significant amount of Aβ deposits levels compared to non-treated mice ($p=0.0243$), there was not a significant amount of Aβ deposits levels within conditions for male ($p=0.8385$). As for the relative fluorescence intensity for thalamus (Fig. 16F), the expressions demonstrated in both genders that IL-34-treated mice had an elevated amount of Aβ deposits levels when compared to those of the non-treated mice. However, although female expressed an elevated amount of Aβ deposits levels between non-treated and IL-34-treated mice, there was not a significant difference ($p=0.3065$). As for male, IL-34-treated mice had a slight significant effect compared to non-treated mice ($p=0.0546$). Nevertheless, present in the cortex and thalamus, overall female IL-34 treated mice expressed a higher elevated presence of Aβ deposits than males.

We examined the immunohistochemistry for Phospho-Tau, and Phospho-Tau exhibited a similar elevated levels of immunopositivity in the cortex and thalamus for the IL-34-treated group. Phospho-Tau marker in the cortex, for both genders demonstrated that the treated IL-34-treated mice had expressed a higher expression compared to those in non-treated mice (Fig. 17A, B). The relative fluorescence intensity for cortex (Fig. 17C) demonstrated in both genders that IL-34-treated mice had a higher amount of Phospho-Tau marker compared to non-treated mice. Within gender conditions, for the cortex females and male IL-34-treated mice had a significant amount of Phospho-Tau marker compared to non-treated mice (female: $p=0.000018$, male: $p=0.0354$). As for the thalamus, females IL-34-treated mice had a significant amount of Phospho-Tau marker compared non-treated mice ($p=0.00035$, Fig. 17F), and for male, we found no significant difference.
in the expression of Phospho-tau in the brain tissue of non-treated mice and IL-34-treated mice ($p=0.47897$, Fig. 17F).

Next, we examined the immunohistochemistry for Anti-Tau, Anti-Tau also exhibited a similar high immunopositivity in the cortex and thalamus for the IL-34-treated mice, except in the cortex of male. In the cortex of male non-treated mice exhibited a higher expression level of Anti-Tau levels when compared to IL-34-treated mice. The Anti-Tau levels demonstrated in the cortex, female exhibited that the IL-34-treated mice had expressed a higher levels when compared to those non-treated mice (Fig. 18A, B). In the cortex of male mice, non-treated mice demonstrated a higher expression level of Anti-Tau compared to IL-34-treated mice. The relative fluorescence intensity for cortex, there was not find a difference in the expression of Anti-Tau levels in the brain tissue of both female and male non-treated mice and IL-34-treated mice (female: $p=0.10629$, male: $p=0.33744$, Fig. 18C). As for the Anti-Tau marker in the thalamus, for gender within conditions demonstrated that the treated IL-34-treated mice had expressed a higher expression level of Anti-Tau when compared to those in female and male non-treated mice (Fig. 18D, E). The relative fluorescence intensity for thalamus (Fig. 18F) demonstrated that IL-34-treated mice of female mice had a significant elevated amount of Anti-Tau levels when compared to non-treated mice ($p=0.00346$), and in males we did not find a difference in the expression of Anti-Tau ($p=0.30064$).

We then continue to proceed to examined other AD markers to confirm our findings. We examined the immunohistochemistry for Zonula occludin-1 (ZO-1) marker, ZO-1 also exhibited similar elevated levels of immunopositivity in the cortex and thalamus for the IL-34-treated mice when compared to non-treated mice. ZO-1 expressions in the cortex, IL-34-treated mice had demonstrated a higher expression compared to those in non-treated mice (Fig. 19A, B). The relative fluorescence intensity for cortex, for within gender conditions, female IL-34-treated mice was slightly significantly elevated compared to non-treated mice. As for male, IL-34-treated mice was significantly elevated compared to non-treated mice (female: $p=0.0424$, male: $p=0.0000036$ Fig. 19C). Overall, female expressed higher levels of ZO-1. As for ZO-1 expressions in the thalamus, for genders within conditions demonstrated that in female and male IL-34-treated mice had expressed a higher expression compared to those in non-treated mice (Fig. 19D, E). The relative fluorescence intensity for thalamus (Fig. 19F) showed that female IL-34-treated mice had a significantly elevated expressions of ZO-1 levels compared to non-treated mice ($p=0.0003$), and in males we did not find a difference in the expression of ZO-1 ($p=0.0958$).
In addition, we observed immunostaining for receptor-associated protein (RAP). RAP also exhibited a similar elevated immunopositivity in the cortex and thalamus of IL-34-treated mice. As for the RAP marker seen in the cortex (Fig. 20A, B) and thalamus (Fig. 20D, E), IL-34-treated mice had expressed a higher expression when compared to those in non-treated mice. The relative fluorescence intensity for cortex, for within gender conditions, female and male IL-34-treated mice was significantly elevated compared to non-treated mice (female: \( p= 0.0001 \), male: \( p= 0.0002 \), Fig. 20C). The relative fluorescence intensity for thalamus (Fig. 20F) likewise demonstrated that IL-34-treated mice of female and male mice had a significantly elevated amount of RAP expression levels when compared to non-treated mice (female: \( p= 0.00009 \), male: \( p= 0.00003 \), Fig. 20F). In general, we found that in the cortex and thalamus, IL-34-treated mice elevated the expression of RAP levels when compared to non-treated mice and female expressed higher levels of RAP in comparison to male.

We then observed the immunohistochemistry for receptor of advanced glycosylation end-products (RAGE). RAGE exhibited a similar elevated immunopositivity in the cortex and thalamus for the IL-34-treated mice in comparison to non-treated mice. RAGE expression exhibited an overexpression in IL-34-injected mice for the cortex (Fig. 21A, B) and thalamus (Fig. 21D, E) in contrast to non-treated mice for female and male. In particularly, the immunopositivity of the cortex was significantly evident in the region of the cortex of female IL-34-treated mice (Fig. 21A). This data was confirmed by the relative fluorescence intensity analysis for the cortex (Fig. 21C). The relative fluorescence intensity for cortex (Fig. 21C) demonstrated that females IL-34-treated mice had a significant amount of RAGE immunopositivity levels compared to male in the cortex. Between conditions, female IL-34-treated mice exhibited a significantly elevated expression when compared to non-treated mice (\( p= 0.00006 \)). Male IL-34-treated mice also exhibited a significant expression when compared to non-treated mice (\( p=0.0038 \)). As for the relative fluorescence intensity for thalamus (Fig. 21F), RAGE demonstrated in female and male that the IL-34- injected mice had a significant elevated expression of RAGE immunopositivity markers when compared to non-treated mice (female: \( p=0.0003 \), Fig. 21D, male: \( p= 0.0049 \) Fig. 21E), there was a non-significant difference seen between female and male mice.

Furthermore, from having similar characteristics to ZO-1, we also checked the expression of Occludin levels. Similar to other AD markers, Occludin also demonstrated that high immunopositivity in the cortex (Fig. 22A, B) and thalamus (Fig. 22D, E) for the IL-34-treated
mice. Occludin levels displayed in the cortex demonstrated in both genders of IL-34-treated mice expressed a higher expressed compared to those in non-treated mice. As for the relative fluorescence intensity for cortex (Fig. 22C), it demonstrated that IL-34-treated mice of both genders, had a significantly elevated amount of Occludin marker compared to non-treated mice (female: \( p = 0.00028 \), male: \( p = 0.0001 \), Fig. 22C). Occludin expression in the thalamus, for genders within conditions demonstrated that female and male IL-34-treated mice had demonstrated a higher expression of Occludin compared to those in non-treated mice (Fig. 22D, E). The relative fluorescence intensity for thalamus (Fig. 22F) demonstrated that IL-34-treated mice of both genders had a slightly significant elevated amount of Occludin expressions when compared to non-treated mice (female: \( p = 0.0124 \), male: \( p = 0.0474 \), Fig. 22F). Between the conditions, IL-34-treated mice expression of Occludin was significantly more elevated than those of the non-treated mice and for gender female expression of Occludin was significantly more elevated than males.

Lastly, we also examined the expression of the neuronal nuclear protein maker known as NeuN. NeuN levels in the cortex demonstrated immunopositivity of female IL-34-treated mice was significantly more elevated than those in in non-treated mice (\( p=0.00035 \), Fig. 23A). NeuN expression levels in the cortex male IL-34- treated mice demonstrated a higher expression when compared to those in non-treated mice, however there was not a significant difference between male conditions (\( p=0.16905 \), Fig. 23B). As for the expression of the NeuN seen in the thalamus, both genders, female and male IL-34-treated mice was significantly higher than those in in non-treated mice (female: \( p=0.00011 \), Fig. 23D, male: \( p= 0.0026 \) Fig. 23E). The relative fluorescence intensity for cortex can be seen in Fig. 23C and thalamus in Fig. 23F for both gender and conditions. From what can be understood from the immunohistochemistry of NeuN, the expression of NeuN levels was similar among other markers of AD that were examined. NeuN marker demonstrates an elevated expression of IL-34 when compared to non-treated mice and female expression of NeuN was significantly more elevated than males.
**Fig. 16 | Aβ immunofluorescence staining** A) Representative images of female non-treated (PBS) and IL-34-treated mice with amyloid β immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. Blue= Hoechst dye; nuclei staining, Red= Aβ deposits. (Scale bar, 1000 μm at 4x and 200 μm at 20x). (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image(J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
**Fig. 17** | *Phospho-Tau or Pho-Tau (Thr231) immunofluorescence staining.* A) Representative images of female non-treated (PBS) and IL-34-treated mice with Phospho-Tau immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining, Red= Pho-Tau deposits. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image(J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***).
Fig. 18 | *Anti-Tau oligomer antibody T22 immunofluorescence staining.* A) Representative images of female non-(PBS) and IL-34-treated mice with Anti-Tau immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining, Red= Anti-Tau deposits. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image (J) software. n = 3 sample/condition. Student’s t-test. *p<0.05, **p<0.01, ***p<0.001.
**Fig. 19 | ZO-1 immunofluorescence staining.** A) Representative images of female non-treated (PBS) and IL-34-treated mice with ZO-1 immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining. Red= ZO-1 deposits. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing ImageJ software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
**Fig. 20 | RAP immunofluorescence staining** A) Representative images of female non-treated (PBS) and IL-34-treated mice with RAP immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining, Red= RAP deposits. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image (J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***).
**Fig. 21 | RAGE immunofluorescence staining** A) Representative images of non-treated (PBS) and IL-34-treated mice with RAGE immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining. Red= RAGE immunopositivity. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image (J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
**Fig. 22 | Occludin immunofluorescence staining.** A) Representative images of female non-treated (PBS) and IL-34-treated mice with Occludin immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining, Red= Occludin immunopositivity. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image (J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
Fig. 23 | NeuN immunofluorescence staining. A) Representative images of female non-treated (PBS) and IL-34-treated mice with NeuN immunostaining in the cortex (B) male cortex, (D) female thalamus, (E) male thalamus of 2-month-old female and female 3x-Tg AD mice after PBS or IL-34 injection. (Scale bar, 1000 μm at 4x and 200 μm at 20x). Blue= Hoechst dye; nuclei staining. Red= NeuN deposits. (C, F) Percent area covered by amyloid β stain determined after threshold utilizing Image (J) software. n = 3 sample/condition. Student’s t-test and one-way ANOVA. p < 0.05 (*), 0.01 (**), and 0.001 (***)
5. Discussion

To the best of our knowledge, it is known that M-CSF and IL-34 share the same receptor, that is known as CSF-1R (H. Lin et al. 2008) and there is not an evident difference in the downstream signaling pathways triggered from both of these ligands in monocytes/macrophages have been reported so far (Chihara et al. 2010). However, there have been roughly a few genetic evidence that indicate that there could be differences in the effect of both cytokines in myeloid cells and in the brain that could lead to AD (Greter et al. 2012; Barve et al. 2013; Boulakirba et al. 2018).

In the present study, we concluded that IL-34-mediated conditions had a significant increase of inflammation compared to M-CSF-mediated conditions. There have been studies that established that both IL-34 and M-CSF are important in the differentiation of OCPs (Boström and Lundberg 2013), and that IL-34 can specifically bind to CSF-1 receptor similar to M-CSF (H. Lin et al. 2008). This led us to speculate on IL-34 potential overlapping effect with M-CSF. Previous studies we have examined demonstrated the role of M-CSF and IL-34 in RANKL-mediated osteoclastogenesis in vitro observations and it established that both M-CSF and IL-34 have the ability to promote osteoclastogenesis similar to the studies have shown (Duarte et al. 2021). In this study, it can confirm with the results that in fact in combination with RANKL, demonstrated that IL-34 induces elevated differentiation of osteoclasts in compared to M-CSF in 3x-Tg-AD BMDMs. In addition, as we mentioned before, it is known that the deposition of Aβ in the brain is a pathological hallmark of AD. There has been a link on the effect of Aβ40 and Aβ42 on the RANKL signaling network has been studied in vitro with Aβ40 and Aβ42 enhancing RANKL-induced osteoclastic bone resorption (S. Li et al. 2016). We examined the presence of various Aβ peptides protein known as Aβ40 and Aβ42 with the combination of M-CSF/RANKL and IL-34/RANKL. The results validates that there was a significant increase of osteoclastogenesis in IL-34-mediated condition in comparison to M-CSF. Furthermore, when compared to M-CSF, IL-34 elicited signals that promoted more active osteoclastogenesis in vitro, even when it was applied in equal concentration (Fig. 9.) Our data propose that IL-34 may also be a potential pharmaceutical target for the treatment of bone and inflammatory diseases, alongside with M-CSF. As we can speculate, IL-34 indeed does play an important role in regulating osteoclastogenesis and the promotion in comparison to M-CSF. Therefore, from our results IL-34 can be used in the substitution of M-CSF and link the role of IL-34 the directly to bone physiology and inflammation. This can open new possibilities to for new
potential clinical applications and the identification of such mechanisms and new avenues for therapeutic interventions to treat AD.

To further confirm this, we examined IL-34 role in vitro. Periodically 3x-Tg-AD were injected with IL-34 every other day into the subcutaneous tissues overlying mouse calvariae. As studies have demonstrated that 3x-Tg-AD is the one of the widely studied mouse model to exhibit both Aβ and tau pathology that is characteristic of the human form and is used to study AD (Elder, Gama Sosa, and De Gasperi 2010; Oddo et al. 2003), therefore our decision for choosing this animal strain. Histological characteristics of the in vivo model was based on 7 weeks (52 days) of consecutive injections of IL-34. The histological sections were prepared from the center of the calvaria lesion and was TRAP stain, and histomorphometry analysis was performed to quantify the osteoclast number and the area of bone resorption (Fig. 12). It demonstrated there was an elevated number of osteoclastogenesis produced in IL-34-treated samples compared to those of the non-treated samples. This can correlate to our previous results in vitro from Fig. 9 that demonstrated the presence of elevated osteoclast after IL-34 stimulation. This result can support that IL-34 significantly promotes inflammation in comparison to M-CSF. To make the connection of our results, IL-34-treated samples exhibited elevated osteoclast when compared to those of the non-treated samples, signifying the role IL-34 in increasing the numbers of osteoclast which can lead to bone loss. To correlate these result with recent studies, there has been accumulating number of results demonstrating Alzheimer’s patients have elevated bone loss comparing to healthy individuals (Loskutova et al. 2009; Başgöz et al. 2020; Kumar et al. 2021). Loskutova et al demonstrated the BMD of patients that was diagnose with AD was lower when compared to non-AD patients as the control. Our results expand this knowledge by demonstrating a significant advantage of IL-34 has over M-CSF in the production of osteoclastogenesis and that IL-34 is more pro-inflammatory when compared to M-CSF.

To connect these results, there have been several studies linking bone loss to neuroglial diseases such as AD (Kelly, Sidles, and LaRue 2020; Lv et al. 2018). The present study was established to test the impact of IL-34 activity will aid in mediating bone resorption/loss via calvarial injections and how potentially bone loss affects cognitive behavior in the 3x-Tg-AD and how it can help elevate Alzheimer’s like symptoms in vivo. 3x-Tg-AD mouse models are known for displaying deficits in spatial and contextual learning paradigms (Arsenault et al. 2011; Davis et al. 2013; Filali et al. 2012). To test this, non-treated mice with PBS injections was used for control,
and IL-34 protein injections was used treated group. Along with the injections, behavioral test was performed biweekly to monitor the cognitive performance of 3x-Tg-AD mice after IL-34 injections.

Subsequently, there a substantial studies on the influence of aging and sex on the behavioral parameters in murine experiments is well-established (Sutcliffe, Marshall, and Neill 2007; Frick et al. 1999) we investigated the impact of these factors in our experiments in order to get a gain a better understanding. In this study, IL-34-treated 3x-Tg-AD mice demonstrated a decline in cognitive performance and behavior. To test cognitive behavior, we conducted EZM, Open-Field, Y-Maze. EZM behavioral test are usually employed to assess anxiety-like behaviors in animals such as mice following experimental manipulations, or to test the effects of pharmacological agents (Tucker and McCabe 2017), in this case we used IL-34. The results showed that IL-34-treated mice had declined cognitive performance in EZM when compared to non-injected mice (Fig. 10) Through the analysis of gender and conditions, for the total number of entries that mice entered the EZM, in females there was significant declined, expressing an increased in stress and anxiety. Since there was a decline in behavior in EZM, this demonstrated that IL-34 was able to be suppressed the cognitive behavior and exhibiting signs of stress and anxiety.

The Open-Field test is a simple sensorimotor test used to determine general activity levels, gross locomotor activity, and exploration habits in mouse and rodent models of CNS disorders (Crusio 2001). The Open-Field behavioral inactivity observed in our mouse potentially reflected several co-existing psychological traits, including anxiety, depression, motivational deficits, and irritation (Lopatina et al. 2014; Seibenhener and Wooten 2015). After mice were removed from the test, the number of defecations or fecal boli deposits was manually counted by the observer. Usually, increased number of fecal bolis can be indication of increased anxiety and emotionality of the subject animal. From our results, IL-34-treated mice exhibited an increase in fecal boli when compared to non-mice (Fig.11). Overall, we did not find evidence of difference between the gender and conditions.

As for the Y-maze test are appropriate for determining spontaneous locomotor activity, exploratory behavior, and anxiety expression level (Borbély, Scheich, and Helyes 2013; Kraeuter, Guest, and Sarnyai 2019). In our case, we applied the Y-Maze to access short term memory in mice and factors such as spontaneous alternation or time immobile are some of the behaviors that can be observed. Our results demonstrate increased levels in the percentage of spontaneous alternation indicates animals stress and anxiety like behavior, a decline in the percentage of spontaneous
alternation indicates mice were more exploratory and presented lack of stress and anxiety. Female IL-34-treated mice exhibited higher levels compared to male. Our results for the time spent immobile of there was a significant difference, IL-34-treated mice expressed an increase of anxiety compared to non-treated mice in the total time spend in maze mostly in females (Fig. 13). This can indicate females is expressing stress or anxiety causing a decline in cognitive memory and when compared to males there is not a significant interaction effect between non-treated and treated mice.

To the best of our knowledge, this study that proves the association between bone health and AD. Interestingly, gathered from our previous results have demonstrated that IL-34 is linked to behavioral performance in 3x-Tg-AD mice after receiving IL-34 injections. This which can suggests a potentially important connection between the effects of bone loss and osteoclast production in Fig. 12, IL-34 demonstrated the ability to suppress and cause a decline in cognition memory and behavior in 3x-Tg-AD. The results of the behavioral test, correlate to these studies linking bone loss to decline in cognitive memory and behavior performance.

Furthermore, the results as shown by the behavioral studies demonstrated that the IL-34 significantly inhibited memory retention and increased anxiety and stress levels. Additionally, we decided to analyze the brains of the mice after injections. As one of the immunostainings, we examined Aβ, it is known that Aβ peptide deposition into insoluble plaques is a pathological hallmark of Alzheimer’s disease (AD), but soluble oligomeric Aβ is considered to be more potent and has been hypothesized to directly impair learning and memory. Moreover, evidence from some clinical studies indicated that Aβ oligomer formation is the major cause for early AD onset (Murphy and Levine 2010; Sharma et al. 2016). The immunofluorescence results demonstrate the presence of Aβ deposits were inside the brain after seven weeks of injection and confirmed by Aβ staining and the neurodegeneration induced by these deposits was confirmed by Aβ in thalamus and cortical regions. Aβ staining of IL-34 treated mice compared to non-treated mice demonstrated a significant expression of Aβ deposits (Fig. 16). Aβ deposition is in our results correlates with cognitive decline. Multiple evidence support that Aβ accumulation precedes and drives tau aggregation. However, Aβ seems to be responsible for synaptic failure and mitochondrial dysfunction that are observed in multiple brain regions in patients with AD (X. Zhang et al. 2018; d’Errico and Meyer-Luehmann 2020).

As previously stated Aβ drives tau aggregation, in relating to tau, in AD pathology, accumulation of the microtubule-associated protein tau takes place primarily in the neurons (Ren
and Sahara 2013; Lasagna-Reeves et al. 2012). There have been multiple transgenic mouse models that tau protein and demonstrated how tau pathology and neuronal progresses (Denk and Wade-Martins 2009). Phosphorylated Tau (Phospho-Tau) is found in neurofibrillary lesions in a range and other central nervous system disorders such as Pick's disease, frontotemporal dementia and cortico-basal degeneration (Alonso et al. 2018; Buée-Scherrer et al. 1996). In addition to that, there have studies that demonstrated phosphorylated tau protein as biological markers of neurodegeneration disease (Schraen-Maschke et al. 2008) linked to AD (Shoji et al. 1998; Arai et al. 1997; 1995). The immunofluorescence results demonstrate the presence of phospho-tau and anti-tau deposits was existent after IL-34 injections, this can be an indication of neurodegeneration induced by these deposits was confirmed by the staining in thalamus and cortical regions (Fig. 17). Phospho-tau and Anti-Tau staining of IL-34 treated mice compared to non-treated mice demonstrated a significant expression of tau deposits (Fig. 18). The development of multiple therapies that address the multiple physiological changes induced by tau could help to unravel the progression of neurodegeneration and reduce the number of patients affected, and IL-34 could be one of those factors.

Tight junctions (TJ) proteins contribute to upkeep of epithelial and endothelial barriers such as the intestinal barrier and the blood brain barrier (BBB) (Zenaro, Piacentino, and Constantin 2017; Vermette et al. 2018; Hernandez et al. 2022). BBB regulates transport of various molecules and maintains brain homeostasis and protecting the CNS (Kadry, Noorani, and Cucullo 2020). BBB damages have been associated with the pathogenesis of AD (Yamazaki and Kanekiyo 2017). ZO-1 is known as one of the essential components of BBB that form tight junctions and regulate BBB permeability (Lochhead et al. 2020). ZO-1 is one of the main TJ proteins, any changes in its levels are closely associated with BBB damage and it becomes a symbol of the BBB destruction. ZO-1 is associated with biomarkers of inflammation (Olsson et al. 2021; Ram, Pottakat, and Vairappan 2018; Kadry, Noorani, and Cucullo 2020). The expression of ZO-1 was strongly upregulated in the IL-34-treated mice (Fig. 19), suggesting the expression may decrease in permeability and begin the progression of AD-like symptoms.

Occludin is a transmembrane protein found in tight junctions, which binds to the cytoplasmic zona-occludens (ZO) proteins (Furuse et al. 1993; Schneeberger and Lynch 2004). Occludin is found in sections of the brain endothelial cells (Hirase et al. 1997). There have been studies of Occludin expression in primary and secondary cultures of neurons and astrocytes from
adult mouse (Bauer et al. 1999). To our knowledge, in this study we demonstration that Occludin-expressing neurons deposits was significant higher in IL-34 treated mice compared to non-treated mice (Fig. 22). Therefore, based on the results of this study, we can conclude that the presence of Occludin deposited found after IL-34 injections assisted in inflammation and can potentially be responsible for the increase expressions of Alzheimer like symptoms.

As we have mentioned before, increased aggregation of Aβ occurs in the brain of all patients with AD and is considered an important step in the disease pathogenesis (Hardy and Selkoe 2002; Kowalska 2004; G. Chen et al. 2017). There is significant amount of data that indicates certain components of the low-density lipoprotein (LDL) receptor family and their ligands are involved in Aβ aggregation and clearance and aid in CNS Function and neurodegeneration (Cam et al. 2005; Lane-Donovan, Philips, and Herz 2014; Kanekiyo and Bu 2014). An extended receptor from LDL called the low-density lipoprotein receptor-related protein 1 (LRP1) also plays an essential role in Aβ degeneration and transport across the blood–brain barrier (Liu et al. 2017; Ma et al. 2018). There have been recent studies that demonstrate that one of LRP1's major ligands known as the RAP is also capable of binding to Aβ and inhibiting its aggregation and toxicity (Kerr et al. 2010) and potentially main role in Aβ aggregation. One of the key reasons why we decided to examine the expressions of RAP in non-treated and IL-34-treated mice through immunohistochemistry. The result demonstrated that the presence of RAP deposits was significantly elevated in IL-34 treated mice compared to non-treated mice (Fig. 20).

Along with these confirmation, we decided to investigate the role of RAGE, there has been increasing evidence shows that RAGE levels are significantly elevated in patients with AD and AD models. (Cai et al. 2016; Paudel et al. 2020; C, Lukose, and Rani 2020). RAGE is an extension of advanced glycation end-products (AGEs). The formation of AGEs occurs in diverse settings such as diabetes, aging, renal failure, inflammation, and hypoxia (Ramasamy, Yan, and Schmidt 2012). RAGE and many other receptors activate several pathways involved in many human diseases characterized by a deregulation of collagen metabolism (Ramasamy, Yan, and Schmidt 2012; Yamagishi, Fukami, and Matsui 2015; Lohwasser et al. 2006) and effects the inflammatory signals (Senatus and Schmidt 2017). RAGE is expressed in multiple cell types at very low levels in the absence of disease with increased expression noted in a range of cell types and tissues in disease states, such as diabetes, neurodegenerative disorders, and autoimmune/inflammatory conditions (Lu et al. 2004). From the results we gathered, it is indicative to conclude the immunopositivity detected
after the staining RAGE in IL-34-treated mice was significant elevated compared to those in non-treated mice (Fig. 21).

Lastly, Neuronal nuclear protein also known as NeuN. NeuN is used to identify mature neurons in cell cultures and tissue sections through immunohistochemistry. NeuN can be used in combination with other cell type markers to study neural networks and neuronal differentiation and development (McKenzie et al. 2018; Y. Zhu et al. 2021). There have been studies that have suggested that quantitative changes in NeuN immunoreactivity can be a factor of neuronal loss in several pathologies including neurodegenerative diseases (Tippett et al. 2007). Using NeuN as an immunohistochemical method to identify neuronal cell bodies in histological, we found a significant difference in the number of neurons between the two genders, IL-34-treated mice was significant higher compared to those in non-treated mice (Fig. 23). These results can relate to neuronal loss that can correlate with development of AD-like symptoms.

Consequently, we can conclude that that IL-34 demonstrated in mouse models to have inflammatory properties, and through the analysis of several markers of inflammation exhibited an increased expression Aβ, Phospho-Tau, Anti-Tau, ZO-1, RAP, RAGE, Occludin, and NeuN in the cortex and thalamus of IL-34 treated 3x-Tg-AD mice as compared to non-treated mice. These results can link with the studies that have confirmed the decline of cognitive behavioral in AD compared to healthy patients (Murman 2015; Teri, Hughes, and Larson 1990). Therefore, confirming IL-34 role in neuroinflammation and inflammation that could be responses in the development of AD.

In response to different types of activation stimuli, macrophages can be polarized either to pro-inflammatory M1 or anti-inflammatory M2 with various biological functions. Importantly, only the M2 a subset plays an essential role in the inflammation resolution (W. Lin et al. 2019). It is true that the activated M1 produces a distinct panel of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, which are collectively termed SASPs. There have been studies indicating that M-CSF and IL-34-mediated intracellular signaling pathways, including auto-phosphorylation of the CSF-1R, caspase activities and autophagy are similar in monocytes induced to differentiate into macrophages, which plays essential roles in programmed cell death (Y. Zhang et al. 2012). This data suggest that the effects of IL-34 are mediated via the CSF-1R. We investigated the effects of M-CSF and IL-34 on macrophage polarization.
As our data show IL-34 aid in the promotion of osteoclastogenesis, therefore it is important to explore whether it can induce the differentiation of macrophages or monocytes in other tissues. Previous studies have suggested the critical role of RANKL in osteoclast differentiation. In addition, there have been several studies that reported IL-34 stimulates viability of monocytes and proliferation of macrophages from bone marrow cells (H. Lin et al. 2008; Duarte et al. 2021). To add on to our validation that IL-34 plays a significant role in inflammation compared to M-CSF, we investigated how IL-34 affects macrophage phenotype in response to structurally defined and stabilized biological components such as Aβ, in this study we will characterize bone marrow-derived macrophages cultured in media containing M-CSF or IL-34. We found that the immunological profile and activation phenotype of IL-34-stimulated BMDMs differed and was significantly elevated from those cultured with M-CSF alone. In this study, we first measured the level of mRNA expression and protein secretion of TNF-α, IL-1β, and IL-6 pro-inflammatory factors in monocyte-generated macrophages. M1-like macrophages of treated cells with IL-34 produced higher TNF-α levels compared to control macrophages for both genders. We did not find any remarkable differences in the gene expression and protein production of TNF-α, IL-1β, and IL-6 in M2-like macrophages between controls and IL-34. The gene expression of SASPs markers, TNF-α between genders were notably elevated in macrophages after their differentiation to M1 type in both controls and IL-34. In line with previous study, the level of gene expression and protein secretion of TNF-α and IL-6 was significantly upregulated in both gender subjects by polarization toward M1 type. However, we found that only M1 macrophages from treated cells and control macrophages upregulated the expression after polarization.

We also compared the level of inflammatory cytokines between controls and treated microglia. Although M1 microglia from IL-34 expressed and produced higher levels of TNF-α, IL-1β, and IL-6 compared to control M1 microglia, the increase did not reach statistical significance except for TNF-α expression. We also did find a significant difference in TNF-α production between control and treated microglia. Regarding our results, it seems that TNF-α expression and secretion are dysregulated in M1 microglia of treated cells. Polarization toward M2 type showed there was not a significant different between control and treated group. However, this concludes that M1 simulated with IL-34 in fact assist in the promotion of inflammation and essential promoting proinflammatory responses and expresses higher levels. Understanding the functions of
macrophages/microglia M1/M2 subtypes and their influence to health or disease contributes to the creation of new and novel strategies to delay or prevent disease advancement.

As studies have indicated that the main risk factors for developing AD is not only just age and but also gender. For the evaluation of the impact of mouse gender as a relevant biological variable on the in vivo studies, IL-34-induced neuroinflammation, when analyzing the data separately, female group, especially IL-34-treated mice demonstrated a significant higher levels of expression after immunohistochemistry staining in the cortex and thalamus. For the behavioral analysis, the age-dependent LOAD-like neuroinflammation was induced in the 3x-Tg-AD. Female 3x-Tg-AD mice exposed to IL-34 had a significant deficit in the learning and memory skills compared to young male mice. Since women are more affected by AD than men, it can be concluded from our results that that LOAD-like neuroinflammation in response to IL-34 significantly elevated in the female mice as compared to the male 3x-Tg-Ad mice. As for the in vitro, IL-34 significantly elevated M1 macrophages activation in females. This can confirm the previous studies validating gender effects on AD (R. Li and Singh 2014; Mielke, Vemuri, and Rocca 2014; Nebel et al. 2018). Overall, there has been diverse research designs as the steady accumulation of neurocognitive resources across the lifespan are being discovered. If we can determine the gender effects on AD, which can help determine the factors and preserve cognitive function by reducing the effects of age- and sex AD-related neuropathology.

6. Conclusion

To conclude, in accordance with the in vivo and in vitro results, all data analysis revealed after being treated with IL-34, IL-34 is relevant to innate immune responses in AD demonstrating bone osteolysis, neuroinflammation, inflammation. We have successfully evaluated the impact of IL-34 on the promotion of inflammatory osteolysis and neuroinflammation in experimental models of Alzheimer’s disease. Our study showed that IL-34 can replace M-CSF for osteoclast differentiation in mouse models. This provides experimental evidence supporting IL-34 as another ligand of CSF-1R. Collectively, therefore, the present studies detected a higher elevated production inflammation of IL-34-mediated conditions compared to M-CSF. Results also demonstrated that IL-34 significantly inhibits mice activity and productivity expressing fear, stress and anxiety in cognitive memory and behavior compared to those in the control. This can correlate with studies between healthy and AD patient’s cognitive behavioral test. In addition, besides IL-34 alone in
comparison to M-CSF, it also demonstrated that gender play a key role in inflammation. IL-34-treated female mice significantly had increase expressions in immunostaining, and M1/M2 polarization of macrophages compared to male. While more comprehensive assessments of the roles of M-CSF and IL-34-signaling in bone remodeling and cognitive behavior are needed for future studies. These results clearly indicate that IL-34-mediated inflammation in replacement of M-CSF can express hallmark features that can lead to AD and the immunological profile and activation phenotype of IL-34-stimulated BMDMs, and microglia differ significantly from those cultured with M-CSF alone. However, despite the results exhibiting the effect of IL-34 on M1/M2 macrophages/microglia proliferation, osteoclast differentiation, cognitive memory and behavioral activity, the function of this new cytokine is still largely unknown. Understanding the behavioral signs that occur in the models of AD may provide assistance in the early diagnosis and appropriate treatment of AD symptomology. Future investigations of IL-34 can lead to achieve a better understanding in in vitro and in vivo observations and shed new light on the pathogenesis of AD.
7. References


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Publications by the Author


