Conditional Genetic Deletion of CSF1 Receptor in Microglia Ameliorates the Physiopathology of Alzheimer’s Disease

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Abstract

Background: Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the world. Microglia are the innate immune cells of CNS, their proliferation, activation and survival in pathologic and healthy brain have previously been shown to be highly dependent on CSF1R.

Methods: Here we investigate the impact of such receptor on AD etiology and microglia. We deleted CSF1R using Cre/Lox system, the knock-out (KO) is restricted to microglia in the APP/PS1 mouse model. We induced the knock-out at 3-month-old, before plaque formation and evaluated both 6 and 8-month-old groups of mice.

Results: Our findings demonstrated that CSF1R KO did not impair microglial survival and proliferation at 6 and 8 months of age in APP cKO compared to their littermate controls groups APP_{Swe/PS1}. We have also shown that cognitive decline is delayed in CSF1R-deleted mice. Ameliorations of AD etiology is associated with a decrease in plaque volume in cortex and hippocampus area. A compensating system seems to take place following the knock-out, since TREM2/β-Catenin and IL-34 expression are significantly increased. Such a compensatory mechanism may promote microglial survival and phagocytosis of Aβ in the brain.

Conclusions: Our results provide new insights on the role of CSF1R in microglia and how it interacts with the TREM2/β-Catenin and IL-34 system to clear Aβ and ameliorates the physiopathology of AD.

Background

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the world. The number of new cases is growing daily due to a lack of reliable biomarkers for diagnosis and efficient treatments. Beta amyloid (Aβ) is the hallmark of AD. Aβ aggregation leads to the formation of senile plaques. The cleavage of amyloid protein precursor (APP) by BACE1 and the enzymatic complex γ-secretase generates Aβ resulting in two main isoforms Aβ_{1–40} (Aβ_{40}) and Aβ_{1–42} (Aβ_{42}), the latter being the most toxic form. 4-6% of AD patients have genetic predisposition, early AD is associated with APP, PS1 and PS2 gene mutation, whereas late AD is related to a mutation of APOE 4.

Aβ deposits activate microglia, the innate immune cells of the central nervous system (CNS). Amyloid-exposed microglia synthetize tumor necrosis factor (TNF), which participates to the recruitment microglia and worsen inflammatory response. Microglia originate in the embryonic yolk sac and migrate to the brain between embryonic days 8.5 and 10 in mice. In adult CNS, microglia serve as brain macrophages, although they are distinct from other resident macrophages. Microglial cells dynamically survey the environment, they are responsible for the elimination of pathogens, cellular debris, dead cells, remodeling synapses, and the clearance of toxic proteins. They are partly dependent on colony-
stimulating factor-1 receptor (CSF1R) signaling for their maintenance, activation, proliferation and self-renewal (8,9).

CSF1R belongs to tyrosine kinase receptor family. It is broadly expressed in the organism by monocytes, macrophages, osteoclasts and microglia (10). CSF1R can bind two ligands namely macrophage-colony stimulating factor (mCSF) and Interleukine-34 (IL-34). They have different binding sites, but their roles are quite equivalent (11). In the brain, mCSF is primarily expressed by astrocytes, oligodendrocytes and microglia, whereas IL-34 is mainly expressed by neurons (Nandi et al. 2012). Previous studies have demonstrated that administration of mCSF can generate macrophages from bone-marrow precursor cells and a substantial increase in the number of blood monocytes (13–15). Blood levels of mCSF and other hematopoietic cytokines were found to decrease in AD patients, which was proposed as a good predictor for a rapid evolution from mild cognitive impairment pre-symptomatic to dementia (16). In 2009, Boissoneault and colleagues have performed a study using multiple systemic mCSF injections in APP_{swe/PS1} mice and they reported a powerful effect of such a treatment to prevent the cognitive decline even at a critical age of the disease. MCSF-treated mice presented a lower number of Aβ plaques, which were associated with an increased number of microglial cells in the brain. The cytokine stimulates the clearance of Aβ_{42} by microglia and infiltrating bone marrow-derived cells (17). In line with these findings, hippocampal injection of mCSF in AD mouse model induced the differentiation of bone marrow cells into bone marrow-derived microglia (BMDM), which resulted in improved cognitive decline (18).

IL-34, triggering receptor expressed on myeloid cells 2 (TREM2) and its adaptor DNAX-activating protein of 12 KDa (DAP12) have also an important role in the pathology. IL34 stimulates proliferation of monocytes and macrophages by binding CSF1R. Mizuno et al. have demonstrated that IL-34 injections ameliorated cognitive decline and reduced Aβ burden by up-regulating insulin-degrading enzyme (IDE) in a mouse model of AD (19). TREM2/DAP12 has a critical role in microglia survival and proliferation and their ability to phagocyte Aβ (20). A significant increase in TREM2 expression was found in plaque-associated microglia in AD patients (21–23). A mutation leading to a loss-of-function in TREM2 gene could decrease microglia survival and proliferation and a greater Aβ burden (24). Indeed, experiments have shown that TREM2 deficiency led to an impaired phagocytosis of Aβ by microglia in a mouse model of AD (25). β-Catenin has emerged as a crucial molecule for several pathologies (26–29).

CSF1R/TREM2/β-Catenin are linked by Src tyrosine kinase, the principal effector of CSF1R signaling that can phosphorylate DAP12. DAP12 downstream intermediate molecule Pyk2 promotes stabilization of β-Catenin (23,30). In AD, Wnt/β-Catenin is downregulated while Wnt antagonist Dkk1 is upregulated (26).

Some research groups have reported that restoring β-Catenin in adult hippocampus was able to reverse synaptic loss, whereas administration of Dkk1 provokes a decrease in β-Catenin, TCF, LEF, and PSD-95 protein levels (31,32).

Altogether these data suggest that CSF1R/TREM2/β-Catenin and IL-34 pathways are important to prevent cognitive impairment in AD. In this study, we investigated the role of CSF1R in a mouse model of AD using a conditional and specific microglial knock-out (KO). We have found that CSF1R gene deletion failed to affect microglia survival and activation probably due to several compensatory mechanisms. The
KO caused a significant increase in IL-34, TREM2 and β-Catenin gene expression together with improvement of cognitive decline and a decrease in Aβ burden. These molecules together with their signaling pathways may compensate for the suppression of CSF1R in microglia.

**Methods**

The aim of this study is to compare APP<sub>PS1/swe</sub> to APP CSF1R<sup>−/−</sup> to understand the role of CSF1R in Alzheimer’s disease.

**Animals:**

We used only male for this study.

Conditional CSF1R knock out mice CSF1R-lox/CX3CR1-Cre/ER (called, cKO) B6.Cg-CSF1R<sup>tm1jwp</sup>/J mice (JaxMice; stock number 02212) were crossed with the B6.129-Cx3cr1tm2.1 (CreER)Jung/Orl mice (EMMA mouse respiratory; EM:06350). The resulting mouse has a tamoxifen-inducible CRE activity specifically in microglial cells, leading to a non-functional CSF1R protein. Mice were injected with tamoxifen at 10-week-old.

Rosa<sup>red<sub>Tm</sub></sup>-CSF1R-lox/CX3CR1-Cre/ER mice: We crossed B6.Cg-Gt(ROSA)26Sor<sup>tm14(CAG-tdTomato)Hze</sup> (JaxMice cat# 007914) mice with CSF1R-lox/CX3CR1-Cre/ER to confirm the deletion of CSF1R. Mice were injected with tamoxifen at 10 weeks-old for 4 consecutive days.

APP<sub>Swe/PS1</sub> male transgenic mice bearing a chimeric human/mouse β-amyloid precursor protein (APP<sub>Swe</sub>) gene and the human presenelin 1 gene (A246E variant). These mice were purchased from The Jackson Laboratory [Strain: B6C3-Tg (APP695)3Dbo Tg(PSEN1)5Dbo/J] and maintained on a C57BL/6J background.

cKO mice and APP<sub>Swe/PS1</sub> mice were crossed to obtain an APP<sub>Swe/PS1-CSF1R-lox; CX3CR1-Cre/ER</sub> (called, APP cKO). The resulting mouse has a tamoxifen-inducible CRE activity specifically in microglial cells, leading to a non-functional CSF1R protein. Mice were injected with tamoxifen at 3-month-old.

All animals were acclimated to standard laboratory conditions (14 h light, 10 h dark cycle; lights on at 06:00 and off at 20:00 h) with free access to rodent chow and water. Protocols were conducted according to the Canadian Council on Animal Care guidelines, administered by the Laval University Animal Welfare Committee.

**Tamoxifen preparation and administration:**

Tamoxifen was dissolved in corn oil (Sigma-Aldrich cat#C8267) and Ethanol 100% for 1 h at 37 degrees Celsius, vortexed every 15 min. We used ~75 mg tamoxifen/kg body weight and 100 μl tamoxifen/corn oil solution was administered via intraperitoneal injection for 4 consecutive days.
Sacrifices:

All mice were deeply anesthetized with ketamine/xylazine (90:10) and sacrificed via intracardiac perfusion with 0.9% saline. Brains were retrieved and post-fixed 24 h in 4% PFA pH 7.4 and transferred in 4% PFA pH 7.4 + 20% sucrose for a minimum of 15 h. Brains were sliced in coronal sections of 20-μm thickness with a freezing microtome (Leica Microsystems), serially collected in anti-freeze solution and kept at −20 C until usage.

Western blot:

Brain protein lysate was extracted and quantified as previously described (63). Proteins were loaded in 8-16% agarose precast gels (BioRad) and electroblotted onto 0.45μm Immibilon PVDF membranes. Membranes were incubated with primary antibodies anti-b Catenin (Cell signaling rabbit 1:1000 cat#9562), TREM2 (R&D sheep 1:1000 cat#AF-1729), BDNF (Millipore rabbit 1:1000 cat#AB-1534), Syndecan-1 (Abcam mouse 1:1000 cat#AB-34164), IL-34 (R&D sheep 1:1000 cat#AF-5195), PSD95 (Millipore mouse 1:1000 cat#MAB-1596), BACE1 (Cerderlane rabbit 1:1000 AB-108394), APC (Novus biological rabbit 1:1000 cat#NBP-15422), ABCB1 (Novus biological rabbit 1:1000 cat#NB-100-80870), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and revealed by clarity (ECL) substrate (BioRad). Quantication was done by determining integrative density of bands using Thermo Scientific Pierce by Image Analysis software v2.0. Optical values were normalized over actin.

Immunofluorescent staining:

Brain sections were washed (4 x 5 minutes) in KPBS then blocked in KPBS containing 1% BSA and 1% Triton X-100 (Sigma-Aldrich cat#T8787). Tissues were incubated overnight 4 degrees Celsius with primary antibodies Iba1 (Wako rabbit 1:1000 cat#09-19741), 6E10 (Biolegend mouse 1:1000 cat#SIG-39320), CD31 (Ebioscience rat 1:1000 cat#11-0311-81). After washing sections in KPBS (4 x 5 minutes), tissues were incubated with the appropriate secondary antibodies for 2 hours at room temperature. Followed by washes in KPBS (4 x 5 minutes), tissues were incubated 10 minutes with DAPI (Molecular Probes 1:10000 cat#D3571). After washes in KPBS (4 x 5 minutes) sections were mounted onto MicroSlides Superforst® (Fisherbrand cat#22-037-246) and cover slipped (Globe scientific cat#1419-10) with Fluoromount-G (Electron microscopy sciences cat#17984-25).

Immunohistochemistry:

Brain sections were washed (4 x 5 minutes) in KPBS then blocked in KPBS containing 1% BSA and 1% Triton X-100 (Sigma-Aldrich cat#T8787). Tissues were incubated overnight 4 degrees Celsius with primary antibodies CSF1R (R&D sheep 1:750 cat#AF-3818). After washing sections in KPBS (4 x 5 minutes), tissues were incubated with the appropriate secondary antibodies for 2 hours at room temperature. Followed by washes in KPBS (4 x 5 minutes), tissues were incubated with ABC mix (Vector Laboratories Vectasin® Elite ABC-HPR Kit, Peroxidase (Standard) 2,25 μl/ml sol A and 2,5 μl/ml sol B
cat#PK-6100) for 1 hour at room temperature. After incubation sections were washed in KPBS (4 x 5 minutes) and incubated up to 15 minutes with a DAB solution (Sigma-Aldrich H₂O₂ 0.003% cat#H1009 and Sigma-Aldrich DAB 0.05% cat#D7304-1SET), followed by washed in KPBS (4 x 5 minutes). Sections were mounted onto MicroSlides Superforst® (Fisherbrand cat#22-037-246) and cover slipped (Globe scientific cat#1419-10) after dehydration with DPX (Electron microscopy sciences cat#13510).

Behavioral analyses:

Novel object recognition task protocol (34) consists of 3 phases: habituation, familiarization and test phase. The experimenter who observed and recorded the behavior was not aware of treatment and genotype of the tested animals (WT+vehicle, n=10, APP₅₆ₑ/PS1 + vehicle, n=10 and APP + tamoxifen, n = 10). Baseline data were obtained at 3 months of age, whereas the effects of the treatment were determined at 6 or 8 months. Tests were made at 15 lux.

Nesting protocol (64), mice were placed 1 per cage at 3 month-old. The experimenter who observed and recorded the behavior was not aware of treatment and genotype of the tested animals (WT+vehicle, n=10, APP₅₆ₑ/PS1 + vehicle, n=10 and APP + tamoxifen, n = 10). After each NOR task, cages were cleaned a new pad was placed inside. After 24h, the nests were analysed and scored. The scores vary from 1 (untouched pad) to 5 (perfect nest).

ELISA:

Total brain homogenates were used for all the ELISA. For the Aβ₁₋₄₀ (Millipore cat#EZHS40) we analyzed 50 μg per well following the protocol provided.

Unbiased Stereological count:

Brains were serially sectioned as previously described and were stained with Iba1, 6E10 and DAPI as previously described. We counted microglia and plaques at 20x magnification using an Axio Observer microscope equipped with Axiocam 503 monochrome camera and processed using with ZEN Imaging Software (Carl Zeiss Canada, Toronto, ON, Canada). Sampling characteristics for cortex sections: Sampling interval 12, total number of sections 7, section sampling interval 3 and starting selection 1. For hippocampal sections: Sampling interval 12, total number of sections 7, section sampling interval 2 and starting selection 1. After counting plaques using unbiased dissector, the mean plaque volume (MPV) was estimated using the rotator method. The estimated MPV was based on the length of line crossing each plaque using randomly oriented line. 4 locations for each hippocampus and 3 for each cortex were sampled in 7 sections Plaques and microglia were counted according to Gundersen unbiased counting rules, with optical fractionator method and sampling continued to a coefficient of error of 10% or less (65).

Image Acquisition:
Image acquisition of Fluorescent staining images was performed using a Zeiss LSM800 confocal microscope supported by the Zen software (2.3 system) using the 10×, 20×, and 40× lenses. Confocal images were then processed using Fiji (ImageJ Version 2.0.0-rc-43/1.51n). For analyses and bright field image acquisition of staining, Iba-1 and CSF1R, 8-bit grayscale TIFF images of the regions of interest were taken in a single sitting for whole protocols with a Qimaging camera (Qcapture program, version 2.9.10) attached to Nikon microscope (C-80) with the same gain/exposure settings for every image. To evaluate the level of Iba-1+ immune response in the regions of interest, the images were imported into ImageJ (1.37) and the percentage of area occupied by the staining was measured using the threshold parameter. Cell count was assed manually using ImageJ (1.37). Analysis was performed in double blinded to avoid bias of analysis. Fluorescent staining of images was performed using a Zeiss LSM800 confocal microscope supported by the Zen software (2.6 system). Confocal images were then processed using Fiji (ImageJ Version 2.0.0-rc-43/1.51n).

Statistical analyses and Figure preparation:

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were carried with the Prism software (version 8.0, GraphPad Software Inc.), comparing control groups vs. tested groups. Values were considered statistically significant if $p < 0.05$. All panels were assembled using Adobe Photoshop CC 2018 (version 19.1.0) and Adobe Illustrator CC 2018 (version 23.0.1).

Results

**CSF1R is solely deleted in microglia.**

CSF1R conditional knock-out (cKO) was made by a Cre/Lox system (Figure 1B). Cre recombinase is under the CX3CR1 promoter. Lox sites are on each side of the CSF1R Exon 5. When tamoxifen is injected, the Cre recombinase complex translocates to the nucleus to interact with lox sites thereby CSF1R gene is non-functional. Mice were injected with tamoxifen at 3-month-old (Figure 1 A). To determine whether the KO was efficient, we used Rosa<sup>red Tm</sup>-CSF1R-lox/CX3CR1-Cre/ER mice. Mice express robust red<sup>tm</sup> fluorescence following Cre-mediated recombination in CX3CR1 cells in the brain, meaning the knock-out is effective (Figure 1C, D). Quantification shows an endogenous activity of Cre-recombinase in mice without tamoxifen is around 20%, however, after induction the knock-out cells reach 89%. To confirm these results, we quantified by immunohistochemistry the percentage of CSF1R<sup>+</sup> cells in parenchyma of cKO mice. Results shown a robust decrease of CSF1R expression in cKO mice. These data indicate that our model is reliable, and strongly efficient to induce the knock-out selectively in microglia.

The deletion of CSF1R does not affect microglia survival and delay cognitive decline in APP cKO mice.

As previously described, CSF1R is largely depleted in microglia. Here we show using an unbiased stereological analysis of Iba1-positive cells, no significative difference in microglia number into hippocampus and cortex at 6 and 8-month-old in APP cKO compared to APP<sub>Swe/PS1</sub> (Figure 2A-C). These data are corroborating our previous findings, suggesting that KO has no impact on microglia survival in a
specific model of neuronal injury (33). To investigate the impact of the KO on cognition, the memory of APP cKO and their littermate control mice were tested at 3, 6 and 8-month-old using novel object recognition task (NOR). This test is a standard to evaluate the cognitive decline in mouse models of AD (34). The test measures the time spent on exploring the novel object compared to the common object. During the acquisition phase, every animal explored more than 10 seconds each object (Figure 2E). The test phase analysis shown no differences between groups at 3-month-old. However, at 6-month-old APP\textsubscript{Swe/PS1} mice present an expected sign of cognitive decline (***p=0.0002), which was also confirmed at 8-month-old (***p=0.0007). APP\textsubscript{Swe/PS1} mice spent equal time at exploring both objects rather than exploring the novel one. Interestingly, APP cKO mice did not have the same cognitive decline at 6 or 8-month-old, they spent an equivalent time to explore the novel object as the wild-type group did (Figure 2D). Our data suggest a protective effect of CSF1R gene deletion to prevent cognitive decline in APP cKO mice.

The nesting behavior was conducted to evaluate the effect of CSF1R knock-out on social withdrawal and apathy linked to AD. As depicted by the Figure 2F, the nest scores were equivalent between groups at 3 and 6-month-old. However, 8-month-old APP\textsubscript{Swe/PS1} mice had a lower score compared with both WT and APP cKO groups (Figure 2E, 2.5, APP\textsubscript{Swe/PS1} ***p=0.0009, 4.5, WT and 4.3 APP cKO). These data suggest that APP cKO do not exhibit a significant decrease in apathy and social withdrawal.

Altogether these results indicated that APP cKO mice do not present memory and social behavior impairment associated with AD. NOR and nesting tests did not show a significative difference between APP cKO mice and WT, unlike APP\textsubscript{Swe/PS1}, as expected arbor a robust cognitive decline. Moreover, the number of microglia was equal between APP cKO and their control APP\textsubscript{Swe/PS1}, suggesting CSF1R is not the only receptor involved in microglia proliferation and survival in this model.

Long-term knock-out reduces volume and plaque number, with the onset of cerebral amyloid angiopathy (CAA).

Cognitive impairment is correlated with the onset of A\textsubscript{β} plaque formation in the cortex and hippocampus in APP\textsubscript{Swe/PS1}. In this animal model, plaques are established and observable at 6-month-old. Since CSF1R-deleted mice performed as well as WT mice, they should have fewer amyloid deposits in hippocampus and cortex compared to APP mice. Sections were stained with Iba1 and anti-A\textsubscript{β} (6E10) at 6 and 8-month-old (Figure 3A). Here we observed a difference in plaque number and volume in the cortex area. Using unbiased stereology, we quantified the number of plaques per region and volume of A\textsubscript{β} deposit in APP cKO at 6 and 8-month-old and their littermate controls (Figure 3B-E). We observed a diminution by 2.1-fold of plaque volume in APP cKO group at 6-month-old (*p=0.0354 hippocampus, cortex *p=0.0479). We also wanted to see if the number of plaques in each structure was changed. For this matter we counted every plaque in both hippocampus and cortex and then normalized the data with the volume of these structures. Results are expressed in number of plaques per mm\textsuperscript{3}, the software gave an unbiased count on the whole brain. The relative number of plaques is significantly decreased by 1.8-fold in the cortex of the APP cKO 6-month-old group (*p=0.0270). At 8-month-old, the volume of plaques
is also reduced in hippocampus (*p=0.0320) and cortex (*p=0.0227) compared to their control littermates, respectively by 6.6-fold and 10-fold. Actually, the volume of plaque does not differ between 6 and 8 months in APP cKO. However, regarding the number of plaques in hippocampus or cortex, it remains similar in both groups at 8-month-old (Figure 3E).

These data provide clear evidence that deletion of CSF1R prevents the accumulation and/or induces a better clearance of Aβ in the brain.

The equilibrium between Aβ burden in the parenchymal and blood vessels is well described (35). AD patients present a diminution of the transporter ABCB1 that impairs the efflux of Aβ in blood vessels. According to previous data, we studied the vascular amyloid to see if this transport was maintained. We used Elisa kits to quantify Aβ40 levels in blood vessels. We did not observe any difference at 6 months in APP<sub>Swe/PS1</sub> or APP cKO groups. However, at 8-month-old, we detected and significant augmentation of Aβ40 in APP cKO group (**p=0.0098) (Figure 3F) associated with a stable expression of ABCB1 at 8 months for APP cKO (*p=0.023) (Figure 3G). These data indicate that CSF1R could play a role in CAA onset or at least it may contribute to down-regulate ABCB1. These results also show that peripheral immune system is not adequately activated to clear vascular amyloid.

Altogether these data indicate that the KO has a beneficial effect in this model on cerebral amyloid load by decreasing the volume of senile plaques (Figure 3B, E) and may accelerate vascular Aβ deposits and CAA (Figure 3F, G, H).

**TREM2/β-Catenin and IL-34 brain protein levels following CSF1R gene deletion.**

We have previously demonstrated that a genetic ablation of CSF1R in a non-inflammatory model did not affect microglia proliferation and activation. Moreover, CSF1R knockout microglia overexpress TREM2 following nerve section. However, in acute inflammatory model such as cuprizone, microglia are unable to proliferate and activate properly (33), indicating that the role of CSF1R is dependent on the microglial environment. Here, we detected the same amount of microglia in APP<sub>Swe/PS1</sub> and APP cKO and microgliosis around plaques was detected in both groups (Figure 2A, 3A). This suggests that the genetic deletion in this model did not impair microglial proliferation and survival that may depend on other factors or compensatory mechanisms due to the CSF1R gene deletion.

We then studied the expression of TREM2, β-Catenin and IL-34 in young WT and cKO 10-week-old mice, because we postulated that these molecules can play an important role in AD by compensating for the KO. TREM2 protein levels increased by 2-fold in cKO mice compared to littermate controls (*p=0.0384), which was associated with the stabilization of β-Catenin (*p=0.0360) and the diminution of adenomatous polyposis coli (APC), a member of β-Catenin destruction complex (**p=0.0023) (Figure 4A).

We then looked at our both groups of 6 and 8-month-old mice and found a robust expression of TREM2, especially at 6-month-old APP cKO mice compared to APP<sub>Swe/PS1</sub> age matched animals (Figure 4B) (*p=0.036). This was again associated with an augmentation of β-Catenin protein levels (*p=0.0027).
Such up-regulation of TREM2/β-Catenin may suggest that these pathways could compensate for the loss of CSF1R in order to keep microglia alive and functional. Interestingly, other very critical molecules increased in the 6 month-old cKO group, namely BDNF (**p=0.0071), Syndecan-1 (*p=0.0498), IL-34 (*p=0.0274) and PSD-95 (*p=0.0120) (Figure 4B). These increases suggest a beneficial effect of CSF1R deletion on neurons, synapses and microglia in the onset of the disease. Similar profile was found at 8-month-old (Figure 4C), at least for BDNF (*p=0.0494) and syndecan-1 (*p=0.0378), but not for PSD-95 and IL-34. It is noteworthy that BACE-1 significantly decreased (**p=0.001) in the brain of 8-month-old cKO mice (Figure 4C-D).

The number of microglia following knock-out induction remains unchanged.

Several studies have shown a robust microglia lethality following pharmacological inhibition of CSF1R (36,37). We have previously demonstrated that number of microglia in hippocampus and cortex between APP<sub>Swe/PS1</sub> and APP cKO is not statistically different (Figure 2A). We aimed to show whether microglia following the KO died and repopulated the brain or signalling pathways previously described are sufficient to keep microglia alive. We have injected tamoxifen in WT and cKO mice at 3-month-old every day for 4 days. We then have sacrificed mice every 2 days after the last injection, until 20<sup>th</sup> days post-injection. The count of microglia over the time indicates that microglia survived even depleted from CSF1R (Figure 5A). Interestingly, at D4 we can observe that almost all microglia are KO, unlike at D2 suggesting that tamoxifen induces KO within 4 days after the last injection (Figure 5B). These data strongly suggest that compensatory mechanisms must take place immediately following the conditional gene deletion to allow such microglial survival and activation in the brain of APP mice or CSF1R is far from being the only receptor involved in such process, at least in this mouse model of AD.

Discussion

In this article, we aimed to study the role of CSF1R in AD. For this purpose, we have bred APP<sub>Swe/PS1</sub> with CSF1R-lox/CX3CR1-Cre/ER mice. We induced the KO at 3-month-old by injecting tamoxifen, before plaque formation and we sacrificed animals at 6 or 8-month-old. AD is a multifactorial disease in which CSF1R could play a major role. MCSF/CSF1R axis promotes microglial proliferation, survival and activation (8). In 2003, Mitrasinovic and colleagues have stimulated microglia with mCSF and they found that the cytokine promoted phagocytosis of amyloid. In the same line, Boissonneault and colleagues found similar results by injecting mCSF in APP<sub>Swe/PS1</sub> mice. CSF1R stimulation has beneficial effects on Aβ clearance by microglia and bone marrow-derived microglia (BMDM) (17). However, growing evidence has shown recently that CSF1R inhibition could also prevent cognitive decline and amyloid deposition. Most of research groups used a molecule which inhibits CSF1R signalization, namely PLX5622. Interestingly, by impeding mCSF signal they observed a massive microglial depletion, suggesting a central role of mCSF/CSF1R axis in microglia survival (36,38–42). Lately, we have demonstrated the effect of CSF1R ablation on microglia is depending on the context. Actually, CSF1R-deleted microglia still survive and proliferate in a non-inflammatory environment, whereas they are unable to proliferate in a model of cuprizone-induced demyelination and inflammation (33).
In the present study, we used a Cre/Lox system to suppress CSF1R gene expression. The knock-out affects 89% of microglia in the brain (Figure 1D-F). Importantly, we observed a similar number of microglia following the KO (Figure 5A) indicating that CSF1R is not essential for microglia to survive and proliferate in APP/PS1 mice (Figure 2 A-C). These findings are contradictory with studies using PLX5622, raising the question about the specificity of CSF1R inhibitor molecules or that the proposed compensatory mechanisms may not take place following the chemical inhibition of the receptor. TREM2 and β-Catenin may have a critical role to allow the survival and proliferation of microglia in the CSF1R cKO APP mice. TREM2 is an immunoreceptor expressed in the brain by microglia (23) and activates survival pathway via β-Catenin (23,25,29,43). Moreover, TREM2/β-Catenin pathway is important for microglial survival and proliferation (43). Indeed, β-Catenin initiates the transcription of cyclin D1 and c-Myc. Interestingly, TREM2-deficient microglia have a reduced level of these controlling cell cycle molecules. In this model this signaling pathway may compensate CSF1R deletion. We observed that TREM2 increased by 2-fold in cKO mice compared to their littermate controls and this is accompanied with a stabilization of β-Catenin since there is a diminution by 2.2-fold of APC (Figure 4A). Adenomatous polyposis coli activation leads to β-Catenin degradation (44).

These data strongly suggest that the TREM2/β-Catenin signaling pathway compensates for the CSF1R KO in this mouse model of AD. Such augmentation of TREM2 and stabilization of β-Catenin at 6 and 8 months of age suggest a positive effect on microglia proliferation and survival, which has been found to be beneficial in a mouse model of AD (45). IL-34 and Syndecan-1 also increased in APP cKO, especially at 6 months of age and IL34 was recently proposed to be the main brain factor to stimulate proliferation of microglia in the ME7 model of prion disease (46). On interest, elevation of IL-34 and Syndecan-1 was also found in CSF1Rop/op mice. Indeed, osteoclasts compensate by overexpressing IL-34 indicating a crosstalk or a similar role of both cytokines (47). Furthermore, Mizuno and colleagues demonstrated that injections of IL-34 ameliorate cognitive decline and reduce Aβ burden in APP/PS1 mice (19). Importantly, CSF1R-deficient mice present different phenotypes whether CSF1R is constitutively deleted or not (48). Growing evidence show that IL-34 signaling could be more important in adulthood than mCSF (49). CSF1R is the main receptor of IL-34, but recently alternative receptors have been proposed. Syndecan-1 is one of them. In vitro syndecan-1 is positively expressed when IL-34 binds CSF1R in macrophages (50). In APP cKO model, IL-34/syndecan-1 could be an alternative pathway for survival and proliferation of microglia. Overexpression of both TREM2/β-Catenin and IL-34/syndecan-1 are in line with other findings, corroborated by NOR and nesting test (Figure 2 D, E). NOR shows a delayed cognitive decline in APP cKO 6 and 8-months-old associated with a decreased volume of plaques in hippocampus and cortex (Figure 3 B, C). Microglia may be more efficient to phagocyte amyloid due to the overexpression of TREM2. These data together with the fact that BDNF increased by 3.3-fold in APP cKO in 6-month-old and 1.8-fold in 8-month-old mice clearly underline the beneficial outcome of the compensatory mechanisms (Figure 4B, C). Of interest is the recent papers showing that TREM2 and β-Catenin up-regulation is linked to microglia expressing BDNF (51,52). Finally, the inverse correlation of BACE-1 and TREM2 in AD cKO mice is in line with the overall beneficial property of the conditional CSF1R gene deletion. In this regard is the ability of Aβ42 to stimulate NF-kB pathway and BACE-1 gene transcription together with TREM2 inhibition (53).
To summarize molecular expression changes, we proposed a representative diagram in Figure 5C.

Besides positive effects of CSF1R deletion on AD, we have observed CAA onset in APP cKO mice at 8-month-old. CAA is associated with AD in a large majority of cases (54). ABCB1 transports Aβ$_{40}$ from parenchyma to blood vessels and AD patients are known to present a lower expression level of ABCB1. This may allow the accumulation of Aβ in the brain (55–57). Our data indicate that APP CSF1R KO mice at 8 months of age tend to have a greater accumulation of Aβ$_{40}$ in blood vessels, due to a stable expression of ABCB1 compared to APP$_{Swe/PS1}$ at the same age (Figure 3F-H). β-catenin has a critical role in blood-brain barrier (BBB) regulation, its over-expression may limit BBB damages and prevent CNS immune cell infiltration (58). Moreover, β-catenin is downregulated in AD, but when this pathway is reactivated it restores ABCB1 (59,60). According to these data, the over-expression of β-catenin at 6 and 8-month-old in APP cKO groups can maintain ABCB1 expression and therefore relieve the brain from Aβ burden. Blood monocytes are more efficient to clear amyloid in blood vessels during AD course than microglia, however, phagocytic capacity of monocytes in AD patients is greatly reduced (61,62).

**Conclusions**

Our study aimed to understand the role of CSF1R in AD. With a conditional KO mouse model, we have induced CSF1R deletion at 3-month-old, before plaque formation. Here we demonstrated that strong compensatory pathways settled following the KO. Indeed, TREM2/β-Catenin and IL-34 expression increase leading to a reduction of plaque volume and a delayed cognitive decline. These ameliorations of mouse conditions are associated with the overexpression of molecules acting on maintenance and protection of neurons and synapses. We have also demonstrated that CSF1R deletion did not impair microglia proliferation and survival probably due to the compensating TREM2/β-Catenin and IL-34 pathways. These signaling pathways seem primordial for microglia and AD etiology when CSF1R gene is deleted in a conditional manner.

**List Of Abbreviations**

Aβ Amyloid-Beta

AD Alzheimer’s Disease

APC Adenomatous polyposis coli

APP Amyloid precursor protein

cKO Conditional knock-out

CSF1R Colony-stimulating factor-1 receptor

DAP12 DNAX-activating protein of 12 KDa
IL-34 Interleukin 34
KO Knock-out
mCSF Macrophage colony-stimulating factor-1 receptor
NOR Novel object recognition task
TNF Tumor necrosis factor
TREM2 Triggering receptor expressed on myeloid cells 2
WT Wild type

Declarations

Ethics approval and consent to participate:
The animal study was reviewed and approved by CPAUL3, Laval University

Consent for publication:
Not applicable

Availability of data and materials:
All data generated or analysed during this study are included in this published article

Competing interests:
The authors declare that they have no competing interest.

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Authors’ contributions:
VP participated in the design of experiments, analyzed and interpreted the data, wrote the manuscript, and assembled the Figures. PL and MMP participated in experiments. SR formulated the study concept, supervised the project, and revised the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

CSF1R deletion is solely deleted in microglia. A experimental design. B genetic construction of Cre/Lox system. C timeline starting at 3-month-old. D representative image of microglia (Iba1 green, Cre-recombination reporter gene Redtm) in WT and cKO. Scale bar = 200µm. E quantification of the average percentage of Redtm+/Iba1+ cells in RosaredTm-CSF1R-lox/CX3CR1-Cre/ER without tamoxifen (ctl cKOredtm) or with tamoxifen (cKOredtm) n=4 in each group. E representative image of CSF1R staining in
immunohistochemistry using 3-month-old WT and cKO mice. Scale bar = 200µm. F analysis of CSF1R staining at 3-month-old WT and cKO mice n=4 in each group. (****p<0.0001).

Figure 2

The deletion of CSF1R does not affect microglia survival and delay cognitive decline in APP cKO mice. A-B representative image of microglia (Iba1 green) in APPSwe/PS1 (6-8-month-old) and APP cKO (6-8-month-old). Scale bar = 500µm and 20µm. C unbiased stereological count of microglia in 6-8-month-old
APPswe/PS1 and APP cKO. APP 6m, n=6, APP 8m n=4, n=6 APP cKO 6m and n=4 APP cKO 8m. D NOR with 3-time points comparison of WT, APPswe/PS1 and APP cko from 3 to 8-month-old. (###, ## Discrimination differs from 0.5, APPswe/PS1 6-month-old ***p=0.0002, 8-month-old ***p=0.0007). n=10 for all groups. E average time of observation during NOR acquisition phase. F nesting with 3-time points comparison of WT, APPswe/PS1 and APP cKO from 3 to 8-month-old. Score range from 1 (untouched square) to 5 (perfect nest) (APPSwe/PS18-month-old ***p=0.0009). n=10 in all groups.

Figure 3
Long-term Knock-out reduces volume and plaque number, accompanied by cerebral amyloid angiopathy (CAA) onset. A hemisphere stitches microglia (Iba1 green) amyloid plaque (6E10 red) in 6 and 8-month-old APPSwe/PS1 and APP cKO Scale bar = 500µm. B-E Unbiased stereological analysis of plaques in hippocampus and cortex. Analysis has shown a reduction in plaque volume at 6 (hippocampus *p=0.0354, cortex *p=0.0479) and at 8-month-old (hippocampus *p=0.0320, cortex *p=0.0227) in APP cKO. A reduction in number of plaque/mm3 in cortex (*p=0.0270). APPSwe/PS1 6m, n=6, n=6 for APP cKO 6m. F Elisa Aβ40 APP cKO 6 and 8-month-old and their controls. APPSwe/PS1 (6-8m) n=6 and APP cKO (6-8m) n=7 (**p=0.0098). G Western blot analysis for ABCB1 between APP cKO (6-8m) and their control. APPSwe/PS1 (6-8m) n=6, n=7 APP cKO (6-8m) (*p=0.023). H Representative images of amyloid (6E10 red) on blood vessels (CD31 green) in APPSwe/PS1 and APP cKO at 6 and 8-month-old Scale bar = 50µm.
Figure 4

TREM2, β-Catenin and IL-34 can compensate CSF1R deletion. A western blot analysis of TREM2/β-Catenin pathway in WT and cKO 3-month-old. WT, n=6, n=7 for cKO. (TREM2, *p=0.0384, β-Catenin **p=0.0360, APC, **p=0.0023, Syndecan-1, *p=0.0498, IL-34, *p=0.0274). B western blot analysis on 6-month-old APP cKO and their littermate controls. APPSwe/PS1 and APP cKO 6-month-old, n=6 (TREM2, *p=0.0356, β-Catenin **p=0.0027, BDNF, **p=0.0071, Syndecan-1, *p=0.0498, IL-34, *p=0.0274, PSD-...
95, *p=0.0120) C western blot analysis on 8-month-old APP cKO and their littermate controls. APPSwe/PS1 and APP cKO 8-month-old, n=6. (TREM2, *p=0.0470, β-Catenin, *p=0.0352, BDNF, *p=0.0494, Syndecan-1, *p=0.0378, Bace-1, **p=0.0010) D representative image of BACE-1 (green) and 6E10 (red) in APPSwe/PS1 and APP cKO 8-month-old Scale bar = 50µm.

Figure 5
The knock-out has no impact on microglia survival over the time. A unbiased count of microglia in WT and cKO mice n=20 for each group. B representative image of microglia Iba+ (green) and knock-out cells (RedTm) for D2, D4, D10, D20 after tamoxifen injections. Scale bar = 100µm. n=4 per group for each time point. C fictive representation of molecular expression in APP cKO, APPSwe/PS1, cKO and WT over the time.