Familial Alzheimer's disease mutations impair neutrophil phagocytosis and release of immune modulators

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Illustration/figures: 4 main figures and 4 supplemental figures

Tables: 0

Word count: 3762

Keywords/phrases: age effect, AD neutrophils, phagocytosis, amyloid beta, cytokine production, Alzheimer's disease

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Abstract:

Recent reports show that neutrophil activity plays a role in the cognitive decline associated with Alzheimer's Disease (AD). Previous reports show that both amyloid precursor protein and Tau can be found in neutrophils. As such, the goal of this study was to determine if the presence of AD mutations can change neutrophil's function. Using a primary neuronal culture model, generated from 3xTg perinatal mice, we characterize the effect of AD mutation on neutrophils ability to remove pathogenic amyloid beta (Aβ) 42, phagocytose debris, and release immune modulators (cytokines, chemokines, growth factors, and neutrophil proteases). Our data show that primary neuronal cultures from 3xTg mice gradually increase the release of Aβ (40 and 42) as the culture ages. To address our experimental goals, Isolated neutrophils were added to the primary neuronal cultures. Culture media was collected and assessed for Aβ and immune modulator levels. Culture plates were fixed, stained, and assessed for neutrophil phagocytosis. AD neutrophils were isolated from 3xTg mice and the WT neutrophils from C57Bl6 mice. Neutrophils were isolated from young male/female mice (3-6 months of age) and aged male/female mice (16-18 months of age) of both genotypes. Our results show that the presence of AD mutation impairs the ability of neutrophils from young mice to remove Aβ. Their ability to phagocytose cellular debris is also impaired in a sex-dependent manner. Furthermore, the presence of AD mutations leads to an increased release of pro-inflammatory molecules by neutrophils from young mice. Neutrophils from aged WT mice increase the levels of Aβ in culture, while those from aged 3xTg mice lead to a decrease in Aβ levels. This was supported by the decrease in Aβ phagocytosis by neutrophils from aged WT mice. Neutrophils from aged 3xTg mice show a significant decrease in release of pro-inflammatory cytokines and proteases when compared to those of the aged WT mice. These data show that the presence of AD mutations impact neutrophil function and response in both young and old mice.

Introduction

With the increasing incidence of Alzheimer's Disease (AD), there is dire need to fully understand its etiology with the hope of finding effective treatments. It is understood that AD is a progressive disease with onset long before cognitive symptoms manifest. AD symptomology is characterized by abnormal cognitive and behavioral changes. These changes, including memory loss, diminishes a person's quality of life and contribution to society. AD is characterized, pathologically, by the accumulation of two key proteins, extracellular amyloid beta (Aβ) and intracellular hyperphosphorylated Tau (p-Tau) (1). These two proteins have been shown to be highly toxic (2). Recent clinical data showed that removal of these proteins late in disease has minimal effect on cognitive function.

The current theory in AD is that a trigger event is important to initiate the disease, especially in the case of sporadic AD. This is supported anecdotally by the fact that AD associated proteins are often characterized after disease symptoms have been observed. Indeed, in rodent models of AD, studies have focused primarily on characterization after pathogenesis is observed, be it the first presence of plaques or the presence of cognitive decline. The reliance on these timepoints disregards the inherent fact that all transgenic models of AD are generated through congenital mutations. Whether these AD mutations and/or protein directly affect immune function is yet unknown.

Recent studies have implicated inflammation as a key contributor to AD pathology. In the context of inflammation within the brain, there is evidence that early in the diseased brain, innate immune cells like microglia and astrocytes help remove Aβ proteins (3–5). Because the production of Aβ and p-Tau is continuous (6), the immune activity of astrocytes and microglia is sustained in the AD brain. This sustained inflammation contributes to disease pathology (7,8). There is mounting evidence of peripheral immune recruitment to the brain in AD (9). Neutrophils, a peripheral immune cell type, are found around Aβ deposits in the brain of AD patients (10). Several recent studies have demonstrated neutrophil recruitment to the brain vasculature in several rodent AD models (11–13). No studies have however determined whether the activity of neutrophils in these models is due to the presence of AD mutation in neutrophils. A recent study showed that the presence of the *APOE4* mutation, associated with increased AD risk (14), leads to changes in neutrophil function (15). Of note, a truncated amyloid precursor protein (cleavage leads to Aβ production) is present in neutrophil granules (16). These data suggest that the presence of amyloid mutations could have a direct effect on

neutrophil function. In the present project we hypothesize that the presence of AD mutation will increase the immune activity of neutrophils.

To test this hypothesis, we used the transgenic 3xTg mouse model of AD. This model contains 3 mutations associated with familial forms of AD. These mutations are the Swedish mutation in the amyloid precursor protein (K670N/M671L), a mutation in the presenilin protein (M146V), and a mutation in the Tau protein (P301L). Using cell culture, histology, cytokine assays, and amyloid ELISA, our data show that the presence of these mutations alters neutrophil phagocytosis and cytokine production in a sex and age dependent manner.

Material and methods

Animals:

Young (3-6 months) and aged (16-18 months), male and female 3xTg (B6;129-Tg(APPSwe,tauP301L)1Lfa *Psen1*^{tm1Mpm}/Mmjax) and C57Bl/6 wildtype mice were used for this project. Young male and female CVN (APPSwDI-Nos2$^{-/-}$) were also used in certain experiments. 3xTg and CVN breeder cages were kept to generate pups used for primary neuron cultures. Breeders are aged 3-9 months. Mice were kept on a 12 hour:12-hour light cycle at room temperature (22-25°C). Food and water were provided *ad libitum*. All experiments were done with the approval of West Virginia University Animal Care and Use Committee.

Primary Neuron cultures:

Brains of 3xTg and CVN P0 pups were collected to generate primary cortical neuronal cultures as previously described (17). Brains were removed from the pups. Brainstem and midbrain structures were discarded. Remaining brain sample was digested in a medium containing DMEM/F12, glucose, Glutamax, Na+/pyruvate, FBS and penicillin/streptomycin. Brain samples were centrifuged and resuspended in the same media. 12 well plates were pretreated with Poly-D-Lysine for one hour at 37 degrees Celsius. Neurons were seeded at a density of 2x10^5 cells/well in the pretreated 12-well plates. After a 2-hour incubation, media was removed and replaced with media containing Neurobasal, Glutamax, B27, and penicillin/streptomycin. The cultures were maintained for 4 weeks. One third of the culture media was replaced every 3-4 days. Starting at week 2, 500μL of media was collected from each well, weekly, and frozen for amyloid beta ELISA and Luminex assay.

Neutrophil isolation:

Neutrophil isolations were conducted using bone marrow from 3xTg, CVN, and wildtype mice at timepoints specified above. The animals were sacrificed using isoflurane and bone marrow was obtained from the tibia and femur. The neutrophils were isolated using the MojoSort Neutrophil Isolation Kit (BioLegend, 480058), according to the manufacturer's protocol. Isolated neutrophils were added to the primary neuron culture at the end of week 4. Neutrophils were seeded at a density of 2x10^5 cells/well. Co-cultures were incubated for 6 hours. Media was collected from each well after the incubation period, and plates were fixed with 4%PFA for IHC staining.

Elisa assays:

ELISA plates for Aβ40 (Abcam, ab193692) and Aβ42 (ThermoFisher, KHB3441) were run using supernatant samples from each pup collected from the cultures throughout the four weeks. The manufacturer protocols for the Aβ42 ELISA and Aβ40 ELISA plate kits were followed. Briefly, for both ELISA kit, Aβ42 or Aβ40 detection antibody solution was added to all wells (standards, controls, and samples) and incubated for 3 hours. The wells were washed, HRP-Streptavidin solution added, and incubated for 45 minutes. The plates were then washed and stabilized chromogen added. Plates were read using a plate reader (SpectraMax iD3) at 450nm absorbance.

Luminex:

15-plex Luminex assays (bio-techne, CUSTOM-LXSA-M15) were run using supernatants from the cultures, obtained at 4 weeks in vitro, to measure secretions of proteins. The proteins measured were pro-inflammatory cytokines (IL-1beta, IL-6, IL-17, TNFalpha, IL-alpha), anti-inflammatory cytokines (IL-4, IL-10), chemokines (CCL2, CXCL1, CXCL12), growth factors (G-CSF, IGF-1, beta-NGF), and proteases (S100A8, MMP-9). Luminex assays were run according to manufacturer's protocol by the Flow Cytometry and Single Cell core facility at West Virginia University Health Science Center. Assays were run on the Luminex MAG PiX instrument using the Xponent software version 4.3.

Immunohistochemistry Staining:

Paraformaldehyde fixed culture plates were stained using basic immunofluorescence staining protocols. The same staining protocol was applied to all plates. Primary and secondary antibody cocktails were procured to Aβ (Cell Signaling, 8243) and neutrophils (Ly6B; ThermoFisher, MA5-16541). All secondary antibodies were purchased from ThermoFisher. Plates were imaged

on the Echo microscope, converted to multi-channel images using ImageJ, and quantified using IMARIS.

Statistical Analysis:

One-way ANOVA was used for comparisons among the control and neutrophil groups for ELISA and Luminex data. Images were analyzed and quantified using IMARIS. A two-way ANOVA was used for comparisons among the control and neutrophil groups for internalized Aβ. Surface analysis on IMARIS was used to determine the number of neutrophils (Ly6B+) and the size distribution (fragment area, μm^2) of amyloid beta staining. Internalized and non-internalized Aβ fragments were determined by the distance between the neutrophil surface and the Aβ surface. Surfaces with distances more than 1μm apart were labeled outside neutrophils and amyloid beta surfaces below 0 μm (0 indicates the edge of the neutrophil surface) were labeled inside neutrophils. Student's T-test was performed for Luminex analyte concentrations that were normalized to control. One-way ANOVA was performed on raw analyte concentrations when the control value was 0, therefore unable to be normalized. Statistical analyses were performed using GraphPad Prism and significance ascribed at $p<0.05$.

Results

The presence of AD mutations directly alter female neutrophil's effect on Aβ levels in primary neuronal cultures.

To determine whether neutrophils interact with Amyloid beta in an in-vitro system, we generated a primary neuronal culture from 3xTg postnatal day 0 (P0) mice. Supernatant from cultures were collected at 2, 3, and 4 weeks in vitro. Our data shows that as early as 2 weeks in vitro, primary neuronal cultures release detectable levels of Aβ (Fig 1A). Characterization of two Aβ species, show a gradual increase of Aβ 42 (one-way ANOVA: $p=0.013$, $F(1.06, 17.01)=7.31$) and maintained levels of Aβ 40 (sup Fig 1A.). Further characterization shows Aβ42 levels to be significantly increased at both 3wks ($p=0.01$) and 4wks ($p=0.02$) from the 2wks time point in-vitro (Fig 1A).

To determine whether the presence of neutrophils would affect the levels of Aβ in the 3xTg primary neuronal cultures, at 4 wks in vitro cultures were exposed to neutrophils from WT and 3xTg mice. The addition of neutrophils to the cultures led to significant decrease in Aβ42 levels (One-way ANOVA: $p <0.0001$; $F(4,46)= 14.29$; Fig 1B) and no change to the Aβ40 levels (sup

Fig 1B). A closer look shows that neutrophils from young WT (p=0.010), young 3xTg (p=0.003), and aged 3xTg (p=0.00002) significantly decrease Aβ42 levels. On the other hand, neutrophils from WT aged mice did not alter Aβ42 levels in vitro (Fig 1B).

To determine if the sex of the neutrophils influenced Aβ levels in vitro, cultures were incubated with neutrophils isolated from male and female mice from WT/3xTg, young/aged mice. Our data shows that neutrophils from male (One-way ANOVA: p=0.000014; F(4,29)=11.10) and female (One-way ANOVA: p=0.00014; F(4,29)=7.082) mice both alter Aβ42 levels (Fig. 1C. and 1D.). For male derived neutrophils, cells from WT young (p= 0.039), 3xTg young (p= 0.005), and 3xTg aged (p=0.013) significantly decreased Aβ42 levels in culture. Interestingly, neutrophils from WT aged male mice increased the levels of Aβ42 levels in-vitro (p= 0.011). On the other hand, only neutrophils from 3xTg aged female mice significantly decreased Aβ42 levels (p=0.0001). Other than male neutrophils from 3xTg young mice, which significantly increased the levels of Aβ40 (one-way ANOVA: p=0.047; F(4, 29)=2.750; multiple comparison control vs 3xTg young p=0.012), neutrophils from male and female mice had no effect on the levels of Aβ40 in vitro (sup Fig 1C and 1D). These data suggest that the presence of AD associated mutations, as those present in the 3xTg mice, greatly affect the physiology of female neutrophils.

The presence of AD mutations alters neutrophil ability to phagocytose debris in young mice.

To fully understand the activity of neutrophils in the 3xTg primary neuronal cultures, we characterized neutrophil phagocytosis by determine the percentage of neutrophils with internalized Aβ fragments, the size distribution of internalized fragments, and the effect of neutrophils on the number of Aβ clusters found in our cultures. Our data show that on average approximately 60% of neutrophils derived from young mice contain Aβ fragments (Fig 2). About 75% of neutrophils from young 3xTg male mice contain fragments, which was significantly higher than neutrophils from male WT mice (Fig 2B; p=0.0287). Neutrophils from young 3xTg mice contained significantly more Aβ fragments of varying sizes (Fig 2C; p=0.0213) and significantly decrease the overall levels of Aβ clusters in culture (Fig 2C; compared to no neutrophil control p=0.0107). No changes were observed in the percentage of female neutrophils that contained fragments between genotypes (Fig 2D) or the number and distribution of Aβ clusters found in culture (Fig 2E). Interestingly, neutrophils from WT young female mice contained more fragments than those from controls (Fig 2E; p=0.0022).

To determine if these changes were specific to Aβ and the 3xTg mutations, we characterized the ability of neutrophils derived from the vascular AD model, CVN (18), on phagocytosing cellular debris. Of note, the CVN mouse contains 3 APP mutations. In addition to the Swedish mutation present in the 3xTg mouse, this mouse also has the Dutch (F693G) and the Iowa (D694N) mutations. Neutrophils isolated from the young male CVN mouse fail to clear cellular debris. Indeed, the WT derived cells remove a large number of debris in comparison (sup Fig2; $p<0.001$). Similarly, neutrophils from the WT female mice remove cellular debris efficiently, while those from the CVN mice fail to do so (sup Fig2; $p<0.0001$). These data show that there is both a genotype and sex difference in how neutrophils interact with Aβ fragments. Overall, our data show that presence of APP mutations directly affects neutrophil ability to phagocytose various biological debris.

Biological age further impairs neutrophil's ability to remove debris in a 3xTg primary neuronal culture.

On average about 60% of neutrophils from young mice show internalized Aβ fragments, while approximately 40-50% of those from aged mice show fragments (sup. Fig3; Two-way ANOVA: $p=0.0019$, $F(1,30)=11.63$)). There are significantly fewer neutrophils with internalized fragments from WT aged mice ($p=0.0150$) when compared to WT young. Though lowered, no significant differences were observed in the 3xTg mice. These data suggest that age alone significantly alters neutrophil's ability to phagocytose Aβ fragments.

Further characterization of the neutrophils from aged mice shows an abrogation of the changes observed in the cells derived from young mice (Fig2). Specifically, the genotype specific changes observed in the male mice were lost (Fig 3A & 3B). On the other hand, female mice, which showed no changes in the young mice, show a significant genotype difference in the percentage of neutrophils internalizing Aβ (Fig 3C; $p=0.0172$). This change is due to the neutrophils derived from aged 3xTg mice retaining the same percentage (60%) as the young, while the WT derived cells show a decrease. Interestingly, neutrophils from WT aged female mice leads to an increase in the number of Aβ clusters observed in cultures (Fig 3D.; compared to no neutrophil control $p=0.0010$). These data suggest that age directly affects neutrophil ability to regulate Aβ levels.

Both age and AD mutation significantly alter neutrophils release of immune modulators

Using a 15-plex immune array we characterized the production of pro-inflammatory, anti-inflammatory, growth factors, chemokines, and proteases (granule content) by neutrophils isolated from WT/3xTg, young/aged mice after exposure to 3xTg cultures containing both soluble and insoluble Aβ fragments. Of note, all data presented here was normalized to either the no neutrophil control protein levels or MFI values (as specified in the y-axis of each graph). If normalization was not possible (due to a 0 value for the control), raw MFI values are represented.

Neutrophils release both pro- and anti-inflammatory cytokines to modulate immune activity and response in tissue. Our data show that young WT neutrophils have very low production of pro-inflammatory cytokines (IL-1a, IL-17, IL-6) in 3xTg cultures, while the young 3xTg neutrophils have increased production of these cytokines (except for IL-6 in 3xTg young male neutrophils) (Fig 4A). Aged WT neutrophils also show a release of these cytokines. Though not significant, the data suggests a trend of a decrease of pro-inflammatory cytokines from aged 3xTg neutrophils from aged control neutrophils (Fig 4A). Interestingly, IL-1b and TNFa show the opposite effect, with young 3xTg neutrophils decreasing their production in culture (sup Fig4). These data suggest that the presence of AD mutations leads to altered production of inflammatory cytokines in neutrophils. These changes are abrogated by biological aging. Characterization of the anti-inflammatory cytokines only shows a significant increase in IL-10 production by young female 3xTg neutrophils (Fig 4B). No changes were observed in the aged neutrophils. This suggests that the production of anti-inflammatory cytokines may not be affected by the presence of AD mutations in neutrophils.

Neutrophils readily release chemokines to recruit other immune cells, including more neutrophils, to a site of inflammation. Our data show no change in the production of chemokines by young 3xTg neutrophils when compared to young WT neutrophils (Fig 4C). However, there is a significant loss in chemokine production by 3xTg neutrophils isolated from aged mice (Fig 4C). These data suggest that age 3xTg neutrophils show a defect in the ability to recruit other immune cells.

Recent studies show neutrophils can release various growth factors. In this study, we focused on the release of granulocyte-colony stimulating factor (G-CSF), a cytokine critical for the generation of neutrophils in the bone marrow (19), beta-nerve growth factor (beta-NGF), a growth factor critical for neuronal survival (20), and insulin-like growth factor-1 (IGF-1), a

cytokine showed to be released by neutrophils that can promote neuronal survival (21). Overall, the presence of AD mutation in neutrophils show very little effect on growth factor production by neutrophils derived from young mice (Fig 4D). On the other hand, aging significantly reduces the levels of beta-NGF from young male 3xTg neutrophils. Though not significant, there is a trend of decrease in production of both G-CSF, IGF1, and beta-NGF from aged neutrophils in all other conditions (Fig 4D; sup Fig4). These data suggest that both age and genotype hinder the ability of neutrophils to release growth factors in culture.

Neutrophil function is often linked to its secretion of various cytosolic or granule proteins. In this study, we focused on the release of S100A8, a calcium binding protein which release has been associated with the activation of NLRP3 inflammasomes (22), and MMP9 or gelatinase B, a secondary granule content that is associated with increased neutrophil infiltration and inflammation (23). Neutrophils from young WT mice show very low release of these proteins. However, the presence of 3xTg mutation leads to increase released of both S100A8 and MMP-9 from young neutrophils (Fig 4E). These data, in conjunction with those in Figure 4A, indicate a high inflammatory state in neutrophils from young 3xTg mice. Neutrophils from aged male WT mice show an elevated expression of both S100A8 and MMP-9 in culture, which is decreased in neutrophils from aged male 3xTg mice (Fig 4E). No changes were observed in the release of S100A8 from aged female neutrophils. However, a significant increase was observed in the release of MMP-9 from aged 3xTg female mice (Fig 4E). Similar to the cells from the young mice, these data, in conjunction to those in Figure 4A, suggest a decrease in inflammatory potential of 3xTg neutrophils with age. In all, our results show that immune modulation of neutrophils is greatly altered in the presence of AD mutations.

Discussion

The goals of the present study were to investigate the ability of neutrophils to clear neuron derived Aβ and determine how age and genotype influence basic neutrophil function. Especially, with the understanding that neutrophils express both amyloid precursor protein (16) and tau mRNA (15). Our data show a clear sex difference in young neutrophils ability to interact with the pathogenic form of Aβ. Neutrophils from young male mice, regardless of genotype, remove more Aβ than neutrophils from young female mice. This sex difference is lost with age. Indeed, in the aged-derived cells, only neutrophils from the 3xTg mice, male and female, significantly decrease Aβ levels. An impairment in neutrophil phagocytosis was evident by the

decrease clearance of cellular debris by CVN neutrophils. Our data also show that neutrophils from young 3xTg mice release more pro-inflammatory cytokines and proteases when compared to WT neutrophils. However, neutrophils from aged 3xTg mice show less release of pro-inflammatory cytokines, proteases, and chemokines compared to neutrophils from aged WT mice. To our knowledge, this study is the first to show that the presence of AD associated mutations alters neutrophil phenotype and function.

Recently, several reports have implicated significant contribution of neutrophils to AD pathology. These studies document neutrophils infiltration of the brain in various AD mouse models including APP/PS1, 3xTg, and 5xFAD (10,24,25). This infiltration has been linked to increased microglia activity via release of CAP37 in 3xTg mice at 12 months of age (10). Our data show that neutrophils from aged AD mice show increased protease activity, which can lead to increase microglia activity and inflammation (26). Neutrophil proteins have been shown to co-localize with amyloid beta plaques (24). It is yet unclear if this colocalization varies with sex. Neutrophils were shown to migrate towards amyloid beta plaques after infiltrating the AD brain (24). It was unclear what function they serve in plaques. Our study provides evidence that neutrophils not only interact but also phagocytose amyloid beta fragments. To our knowledge, we are the first to show, in vitro, that neutrophils can clear amyloid beta. Neutrophil phagocytosis has been investigated in the context of AD but in their ability to phagocytose *staphylococcus aureus*, not amyloid beta. In agreement with our data, this data show that neutrophils from AD patients show a disease and age alterations in neutrophil's ability to phagocytose (27). Of note, our study show that this defect is inherent in to all AD neutrophils, not only those from the aged animal. A previous study showed that MMP-9 can degrade Aβ peptides (28), suggesting that neutrophil release of MMP-9 could contribute to Aβ clearance. Our data show that aged 3xTg neutrophils decreased the concentration of Aβ without showing increased internalization. These aged neutrophils also show an increased release of MMP-9, which could account for the decreased observed in the cultures.

Recently, it was shown that neutrophils from aged AD patients (average age 67.4 years) and APP/PS1 mice (aged 12 months) show increased ROS production and NETosis (25) suggesting that neutrophils contributed to the inflammation observed in the AD brain. However, our data show that neutrophils from aged mice show a decreased expression of inflammatory markers. It is possible that were we to look at ROS production, we would have documented an increase in our model. Unfortunately, the previously mentioned studies do not address the inflammatory potential in neutrophils in these mice. Our data from young mice suggest a high inflammatory

potential of these cells. Finally, Although MPO has been observed in plaques in the AD brains, neutrophil extracellular traps have only been localized to brain vasculature (25). A previous report showed that extravasation can act as a mechanical inflammatory activation for neutrophils (29). The absence of endothelial cells in our cultures (experimental model) limits our ability to assess the effect extravasation has on neutrophils with AD mutations. Of note, many reports of neutrophils in various AD model show these cells to be mainly restricted to the aged brain microvasculature (9,25).

Conclusion

This study shows that there is a sex difference in how neutrophils interact with amyloid beta. This sex difference, present in neutrophils derived from young mice, is absent in cells from aged mice. Whether this sex difference plays a role in the sex vulnerability observed in AD (both mice and humans) remains to be determined. Our results also show that the presence of AD mutations, in our case Aβ and Tau mutations, greatly affect how neutrophils from both young and age mice interact with debris and release immune modulators. We propose that these data suggest a neutrophil dysfunction that is characteristic of AD. Whether these dysfunctions play a critical role in AD onset and disease progression remains to be determined.

Acknowledgements

The Luminex experiments listed here were conducted in the West Virginia University Health Sciences Center Flow Cytometry and Single Cell Core Facility (SCR_017738), which is funded by the TME CoBRE GM121322. Experiments that were relevant to the CVN mouse were funded by the NIH 1K22NS114363 awarded to Dr. Coulibaly.

Declaration of Interest

The authors declare no competing interests.

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Figure 1: **Both genotypes and sex affect Aβ-42 removal from primary neuronal cultures**. Primary neuronal cultures, generated from P0 3xTg mice, produce increasing levels of Aβ-42 over 4 weeks in vitro (A.). The addition of bone marrow neutrophils from young and aged mice significantly decreases Aβ42 levels in a 4weeks culture, except those derived from WT aged mice (B.). Further characterization shows that male neutrophils from WT/3xTg young and 3xTg aged neutrophils decrease the level of Aβ42 while WT aged neutrophils increases its levels (C.). For female mice, only neutrophils from aged 3xTg mice significantly decreased the levels of Aβ in cultures. *>0.05.

Figure 2: **Neutrophils derived from young mice are better at removing Aβ from culture**. A representative micrograph depicting the presence of Aβ plaques in 3xTg primary neuronal cultures after the addition of isolating neutrophils (A.). Significantly more neutrophils from young 3xTg male mice contained Aβ when compared to those from WT male mice (B.). Further analysis of the size of the Aβ fragments inside the cells show more fragments in the 3xTg derived neutrophils (C.). Overall, only young WT male neutrophils significantly decreased the number and Aβ fragment found in the cultures (C.). the addition of neutrophils from young WT and 3xTg female mice show no difference in their internalization of Aβ (D.). WT female neutrophils internalize more Aβ fragments than those from the 3xTg with no effect on the distribution of Aβ in the overall culture (E.). scale = 100 μm.

Figure 3: **Age significantly alters neutrophils ability to remove Aβ from culture**. A representation micrograph of the 3xTg primary neuronal culture used to assess the effect of age neutrophils on culture conditions (A.). similar percentage of neutrophils from WT aged or 3xTg aged mice contain Aβ fragments (B.). Genotype had no effect on the size of Aβ fragments inside aged neutrophils or their ability to alter overall Aβ deposits in cultures (C.). On the other hand, more neutrophils from aged 3xTg female mice contain Aβ fragments compared to those from the aged WT female (D.). Further analysis shows that more fragments were found inside the neutrophils from aged 3xTg female (E.). Interestingly, the presence of these cells led to an increase in the deposit of Aβ fragments in cultures (E.). Scale = 100 μm

Figure 4: **Age and AD mutations change the release of immune modulators from neutrophils**. Release of pro-inflammatory cytokines is increased in neutrophils isolated from young 3xTg mice (A.). This increase is abolished in the neutrophils from aged 3xtg mice (A.). The release of anti-inflammatory cytokine is unchanged in the neutrophils from young and aged mice, except for IL-10 which is increased in neutrophils from female 3xTg mice (B.). No changes were observed in the release of chemokines by neutrophils from young mice. However, neutrophils from aged 3xTg mice show a significant decrease in the release of chemokines (C.). no changes were observed in the release of growth factors by young and aged neutrophils between genotypes, except for a decrease in the expression of beta-NGF by neutrophils from aged male 3xTg mice (D.). Neutrophils from young 3xTg mice show an increase in the level of released proteases in cultures, while those from the from the 3xTg aged mice show a decrease (E.). except for MMP-9 which showed an increase release by neutrophils from aged 3xTg female mice (E.).

Supplemental figure 1: **Neutrophil genotype and sex have little effect on levels of Aβ40 in primary neuronal cultures**. 3xTg primary neuronal cultures show very little change in Aβ40 levels (A.). the addition of neutrophils to the cultures had little effect on Aβ40 levels at 4 weeks of incubation (B.). Only male neutrophils derived from young 3xTg mice increased the levels of Aβ40 (C.). female neutrophils had no effect on Aβ40 levels regardless of genotype (D.).

<u>Supplemental figure 2:</u> The presence of AD mutations alters neutrophil's ability to remove cellular debris. Neutrophils isolated from young CVN mice and incubated in culture plates with neuronal debris show that neutrophils derived from male and female CVN mice fail to remove cellular debris when compared to their WT counterparts.

<u>Supplemental figure 3:</u> The presence of AD mutation abolishes the effect of age on neutrophil phagocytosis. Aged WT neutrophils show a decrease in the percentage of neutrophils with internalized Aβ. However, neutrophils from 3xTg mice show similar internalization of Aβ as neutrophils from young mice.

<u>Supplemental figure 4:</u> AD mutation impairs the secretion of various immune modulators by young neutrophils. Yong neutrophils show a trend of decrease release of IL-1b, TNFa, and IGF-1 in 3xTg mice. no changes observed in the expression of these cytokines in aged mice.

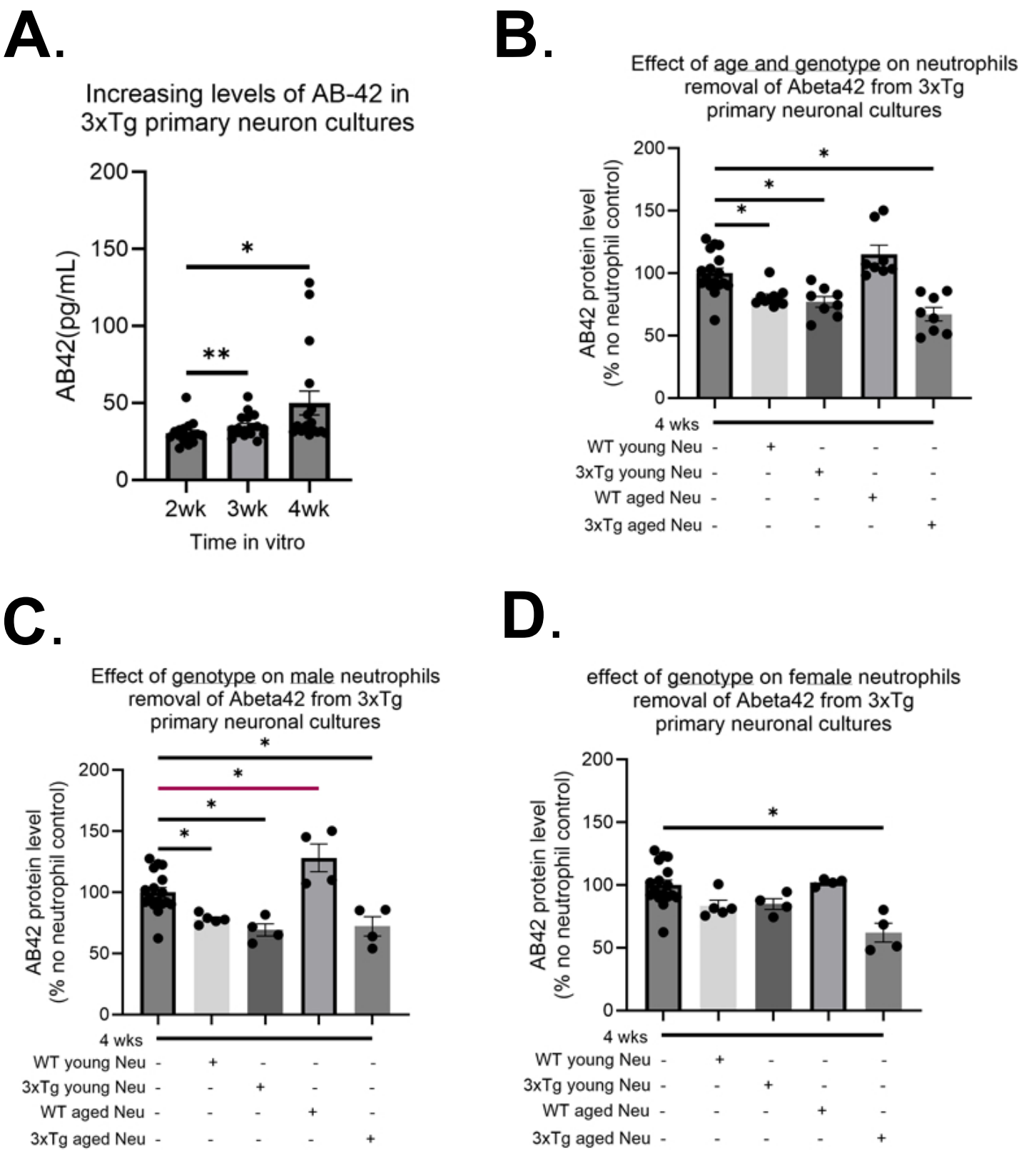

A.
Increasing levels of AB-42 in 3xTg primary neuron cultures
AB42(pg/mL)
200
150
100
50
0
*
**
2wk
3wk
4wk
Time in vitro
B.
Effect of age and genotype on neutrophils removal of Abeta42 from 3xTg primary neuronal cultures
AB42 protein level (% no neutrophil control)
200
150
100
50
0
*
*
*
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +
C.
Effect of genotype on male neutrophils removal of Abeta42 from 3xTg primary neuronal cultures
AB42 protein level (% no neutrophil control)
200
150
100
50
0
*
*
*
*
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +
D.
effect of genotype on female neutrophils removal of Abeta42 from 3xTg primary neuronal cultures
AB42 protein level (% no neutrophil control)
200
150
100
50
0
*
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +

Figure 1

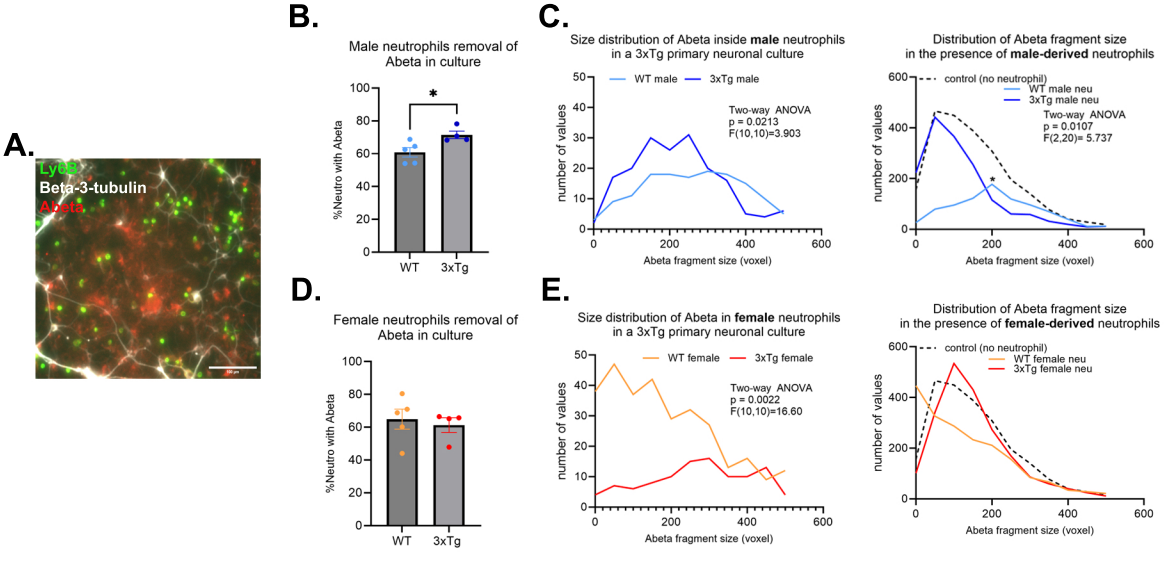

A.
Lyb9
Beta-3-tubulin
Abeta
B.
Male neutrophils removal of Abeta in culture
%Neutro with Abeta
WT
3xTg
C.
Size distribution of Abeta inside male neutrophils in a 3xTg primary neuronal culture
WT male
3xTg male
Two-way ANOVA
p = 0.0213
F(10,10)=3.903
number of values
Abeta fragment size (voxel)
Distribution of Abeta fragment size in the presence of male-derived neutrophils
control (no neutrophil)
WT male neu
3xTg male neu
Two-way ANOVA
p = 0.0107
F(2,20)= 5.737
D.
Female neutrophils removal of Abeta in culture
E.
Size distribution of Abeta in female neutrophils in a 3xTg primary neuronal culture
WT female
3xTg female
Two-way ANOVA
p = 0.0022
F(10,10)=16.60
Distribution of Abeta fragment size in the presence of female-derived neutrophils
WT female neu
3xTg female neu

Figure 2.

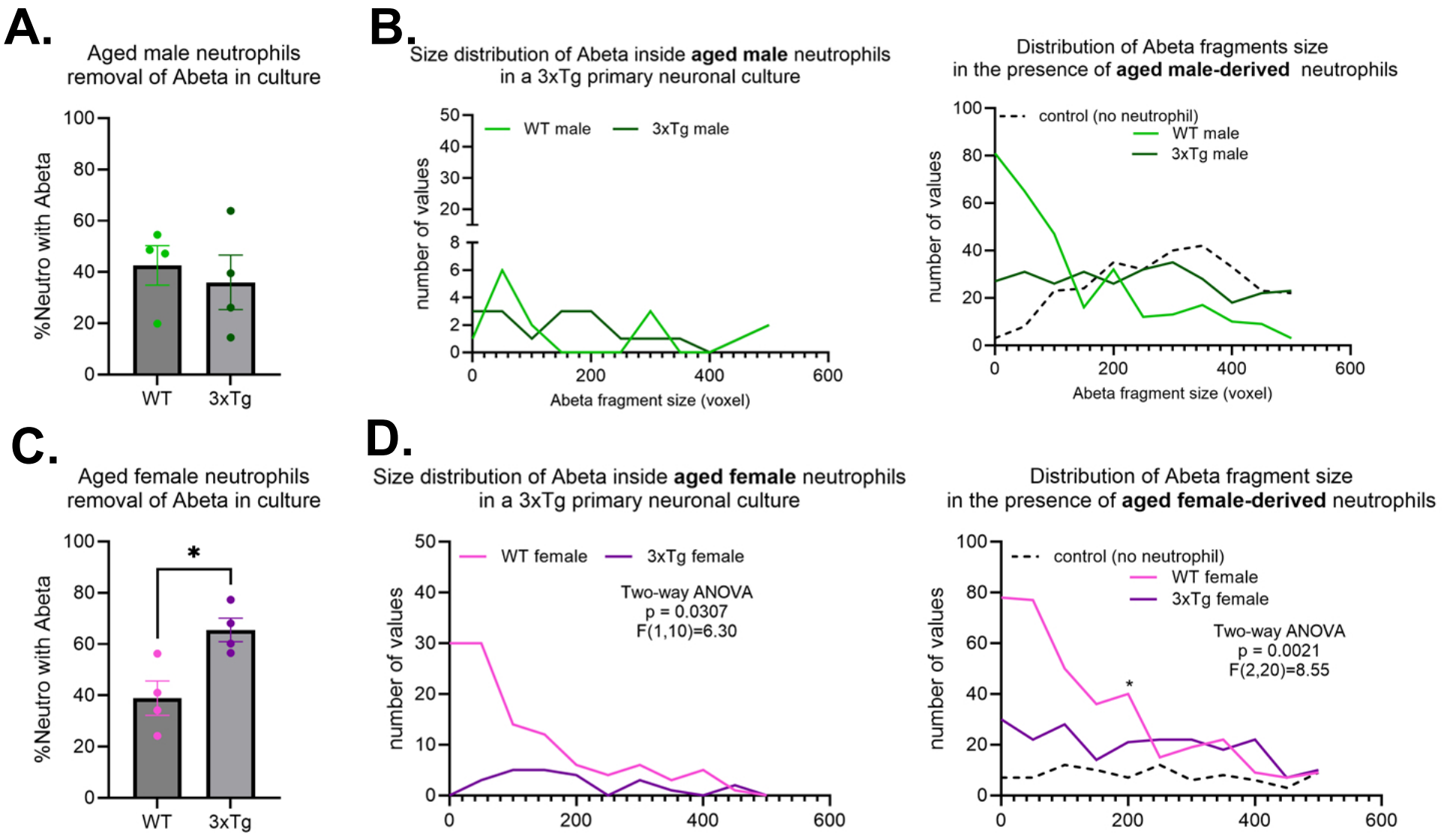

A.
Aged male neutrophils
removal of Abeta in culture
%Neutro with Abeta
100
80
60
40
20
0
WT
3xTg
B.
Size distribution of Abeta inside **aged male** neutrophils
in a 3xTg primary neuronal culture
WT male
3xTg male
number of values
50
40
30
20
8
6
4
2
0
0
200
400
600
Abeta fragment size (voxel)
Distribution of Abeta fragments size
in the presence of **aged male-derived** neutrophils
control (no neutrophil)
WT male
3xTg male
number of values
100
80
60
40
20
0
0
200
400
600
Abeta fragment size (voxel)
C.
Aged female neutrophils
removal of Abeta in culture
%Neutro with Abeta
100
80
60
40
20
0
*
WT
3xTg
D.
Size distribution of Abeta inside **aged female** neutrophils
in a 3xTg primary neuronal culture
WT female
3xTg female
Two-way ANOVA
p = 0.0307
F(1,10)=6.30
number of values
50
40
30
20
10
0
0
200
400
600
Distribution of Abeta fragment size
in the presence of **aged female-derived** neutrophils
control (no neutrophil)
WT female
3xTg female
Two-way ANOVA
p = 0.0021
F(2,20)=8.55
*
number of values
100
80
60
40
20
0
0
200
400
600

Figure 3.

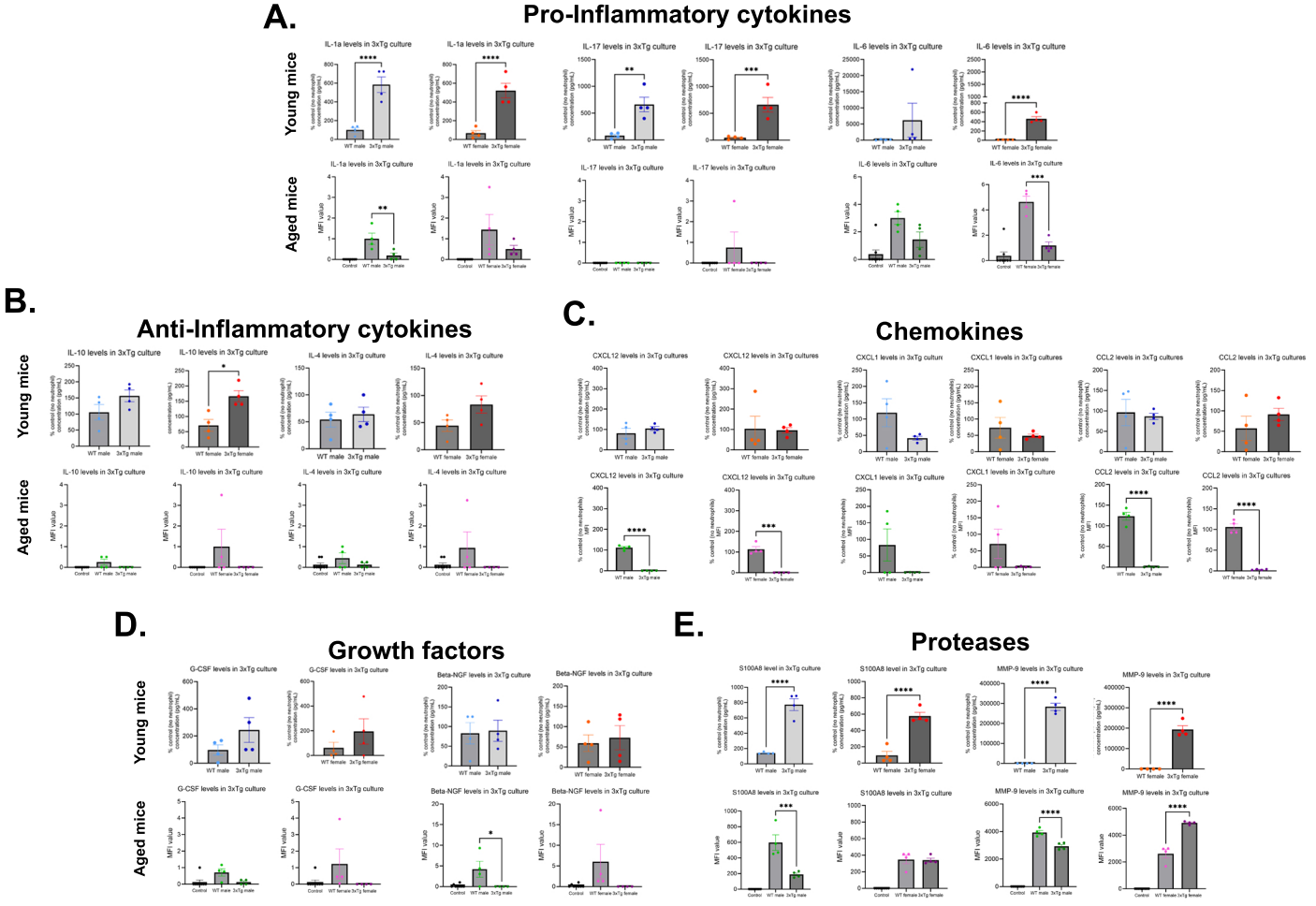

A.
Pro-Inflammatory cytokines
Young mice
Aged mice
IL-1a levels in 3xTg culture
IL-17 levels in 3xTg culture
IL-6 levels in 3xTg culture
B.
Anti-Inflammatory cytokines
IL-10 levels in 3xTg culture
IL-4 levels in 3xTg culture
C.
Chemokines
CXCL12 levels in 3xTg cultures
CXCL1 levels in 3xTg cultures
CCL2 levels in 3xTg cultures
D.
Growth factors
G-CSF levels in 3xTg culture
Beta-NGF levels in 3xTg culture
E.
Proteases
S100A8 level in 3xTg culture
MMP-9 levels in 3xTg culture

Figure 4.

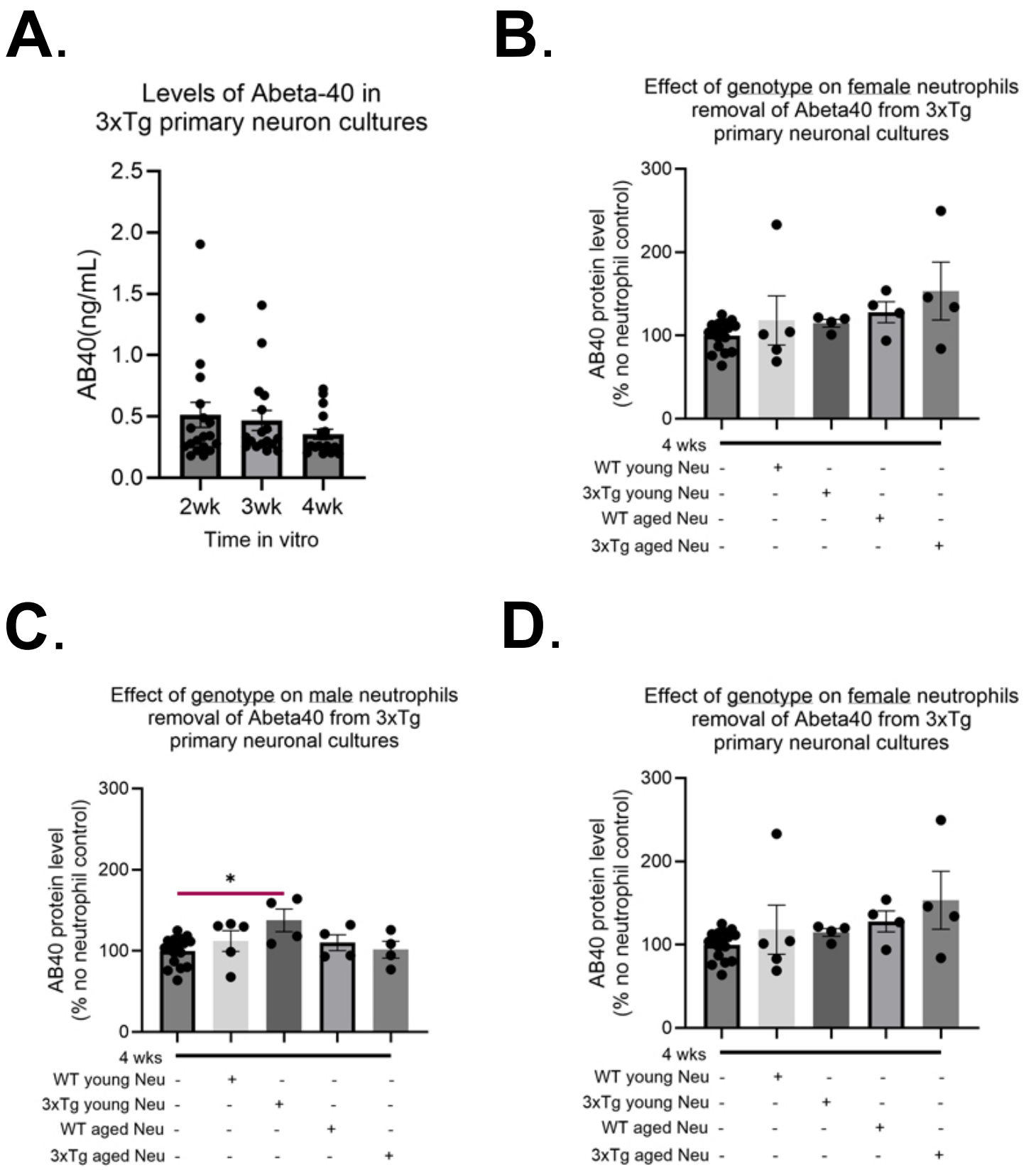
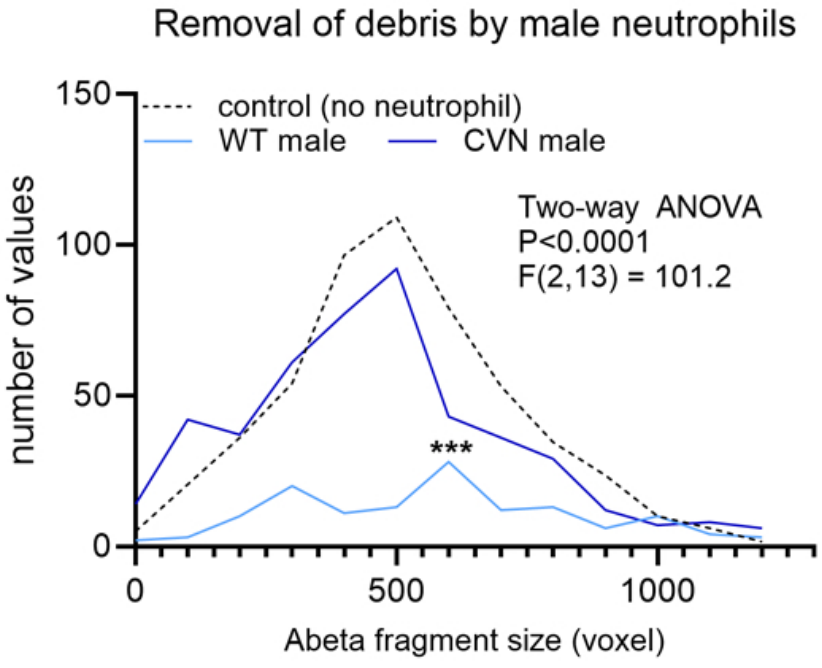
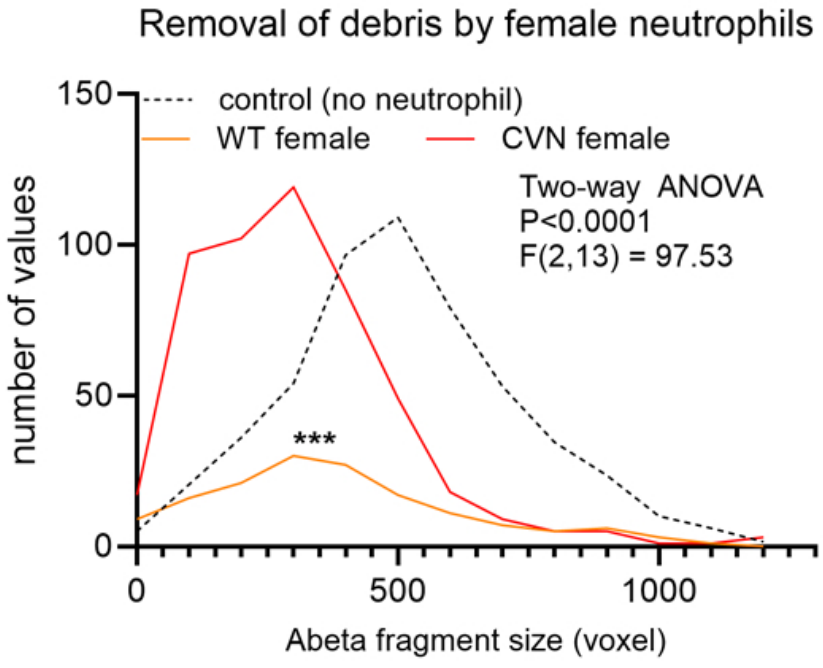
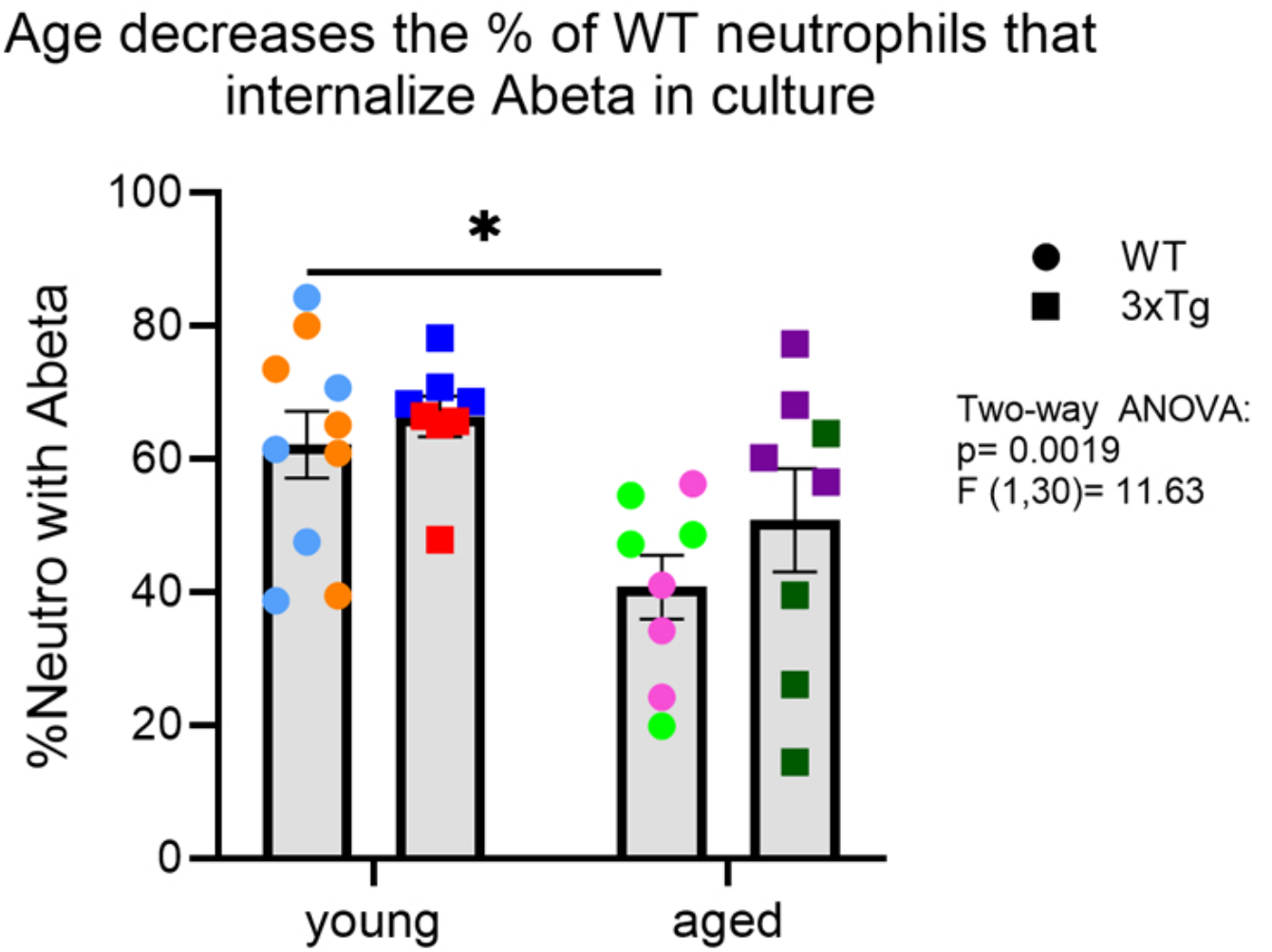

A.
Levels of Abeta-40 in
3xTg primary neuron cultures
AB40(ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
2wk
3wk
4wk
Time in vitro
B.
Effect of genotype on female neutrophils
removal of Abeta40 from 3xTg
primary neuronal cultures
AB40 protein level
(% no neutrophil control)
300
200
100
0
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +
C.
Effect of genotype on male neutrophils
removal of Abeta40 from 3xTg
primary neuronal cultures
AB40 protein level
(% no neutrophil control)
300
200
100
0
*
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +
D.
Effect of genotype on female neutrophils
removal of Abeta40 from 3xTg
primary neuronal cultures
AB40 protein level
(% no neutrophil control)
300
200
100
0
4 wks
WT young Neu - + - - -
3xTg young Neu - - + - -
WT aged Neu - - - + -
3xTg aged Neu - - - - +

Figure 1

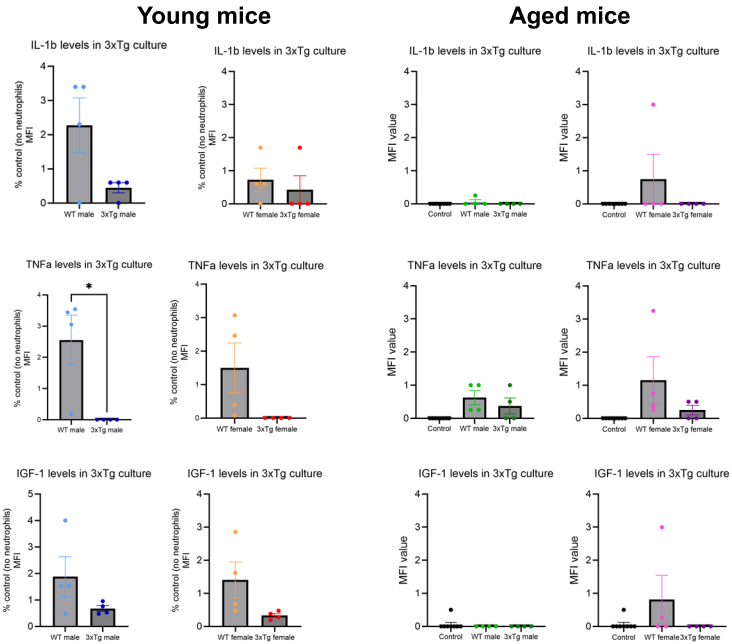




Supplemental figure 2



Supplemental Figure 3



Supplemental figure 4