Alzheimer's disease-associated protective variant Plcg2-P522R modulates peripheral macrophage function in a sex-dimorphic manner and confers functional advantage in female mice

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ABSTRACT

Genome-wide association studies have identified a protective mutation in the phospholipase C gamma 2 (*PLCG2*) gene which confers protection against Alzheimer's disease (AD)-associated cognitive decline. Therefore, PLCG2, which is primarily expressed in immune cells, has become a target of interest for potential therapeutic intervention. The protective allele, known as *P522R*, has been shown to be hyper-morphic in microglia, increasing phagocytosis of amyloid-beta (Aβ), and increasing the release of inflammatory cytokines. However, the effect of this protective mutation on peripheral tissue-resident macrophages, and the extent to which sex modifies this effect, has yet to be assessed. Herein, we show that peripheral macrophages from wild-type (WT) female and male mice harbor intrinsic differences, with macrophages from females exhibiting less phagocytosis and lysosomal protease activity, as well as a more pro-inflammatory phenotype upon stimulation compared to those from male mice. Furthermore, we demonstrate that the *P522R* mutation does indeed alter peripheral macrophage function in a sex-dependent manner, with more prominent effects seen in females, including pushing macrophages from females toward an M2 protective phenotype that more closely resembles macrophages from males. These results suggest that the *P522R* mutation may be partially conferring protection against protein aggregation via activation of peripheral immune cell function and underscore the importance of considering sex as a biological variable when assessing the viability of potential immune system-targeted therapies.

Keywords: Alzheimer's disease, macrophage, PLCG2, sex differences

42 **BACKGROUND**

43 Alzheimer's disease (AD) is a devastating neurodegenerative disorder that afflicts millions of
44 people worldwide over two-thirds of which are women (1). Classically, AD is histologically
45 characterized by the presence of amyloid-beta (Aβ) plaques extracellularly, and the formation of
46 tau tangles within neurons. However, over recent years, the immune system has been implicated
47 in the pathogenesis and progression of the disease. Of particular interest are cells of the innate
48 immune system, which includes both peripheral monocytes and macrophages, and the brain-
49 resident macrophages, microglia. These cells are considered to be the body's first line of defense,
50 and are main propagators of inflammation, which has been shown to exacerbate
51 neurodegeneration (2-4). Early in the course of AD, monocytes and macrophages have been
52 shown to be hyper-active, skewing towards a more pro-inflammatory phenotype, and performing
53 less phagocytosis (5). As the disease progresses, the cells of the immune system become
54 dysregulated, senescent, and associated with low-grade chronic inflammation (6). The lack of
55 disease-modifying interventions for AD until recently has fueled the search for identification of
56 dysregulated immune pathways as potential new targets to delay, arrest and perhaps prevent
57 disease development (7).

58 Genome-wide association studies identified a mutation within the phospholipase C
59 gamma 2 (*PLCG2*) gene, called *P522R*, that protects against AD-related cognitive decline (8, 9).
60 PLCG2 is highly expressed in immune cells including brain-resident microglia, peripheral
61 monocytes, and tissue-resident macrophages (10). Determining how this mutation confers its
62 protection in these cell populations will allow us to better understand how these cells play a role
63 in the progression of AD, and may open up new avenues for therapeutic development. PLCG2
64 has been shown to support numerous myeloid cell functions including phagocytosis, cytokine

65 secretion, survival, and lipid metabolism (11). To do so, it acts downstream of receptors such as
66 triggering receptor expressed on myeloid cells 2 (TREM2) and toll-like receptors (TLRs) to
67 cleave phosphatidylinositol (4,5) bisphosphate (PIP2) into diacylglycerol (DAG) and inositol
68 1,4,5 triphosphate (IP3). DAG and IP3 go on to both release intracellular calcium and activate
69 downstream pathways including nuclear factor light chain enhancer of activated B cells (NF-κB)
70 (11, 12). The *P522R* mutation has been shown to be hypermorphic, indicating an enhancement of
71 immune cell functions (13). As the primary brain cells that express PLCG2 are microglia, much
72 of the work aimed at investigating how its protective effect is conferred has focused on how it
73 alters microglial function. Studies have shown that the *P522R* mutation increases the release of
74 inflammatory cytokines by microglia, and has variable effects on phagocytosis depending on
75 cargo size (14). For example, studies showed that the *P522R* mutation reduced phagocytosis of
76 *Escherichia coli* but increased phagocytosis of Aβ (15, 16). Further *in vivo* work confirmed this
77 increased uptake of Aβ in a 5XFAD mouse model of amyloid-pathology (17).
78 However, the effect of this mutation in peripheral tissue-resident macrophages has yet to
79 be studied. As this protective pathway is of interest in the development of potential therapeutics,
80 it is important then to understand how those therapeutics may affect the immune system globally,
81 and not just within the brain. Furthermore, peripheral macrophages may not just be bystanders in
82 the progression of AD, as it has been shown that they are not only more efficient phagocytes of
83 Aβ than microglia, but they are also capable of traversing the blood-brain barrier during times of
84 injury and disease (18-22). Therefore, determining the effect of this protective mutation on
85 peripheral macrophage function is of vital importance. Additionally, given that women are at
86 higher risk for the development of AD, we posit that sex-dependent differences in the immune
87 system may be partially responsible for this increased risk. For instance, it has been well

88 established that females are at greater risk for autoimmune conditions due to an overactive and
89 inflammatory immune system (23). Given that activated microglia and infiltrating monocytes are
90 detectable in the early stages of AD (24), basal over-activation of innate immune cells in females
91 could be a potential link to the increased prevalence of AD in women. Further studies have also
92 shown that relative to healthy control females, females with AD possess a higher prevalence of
93 less phagocytic pro-inflammatory M1 macrophages and fewer M2-activated macrophages, which
94 are associated with phagocytosis, wound-healing, and anti-inflammatory properties (25). With
95 this in mind, we determined that sex as a biological variable should be investigated in the present
96 study, as sexually-dimorphic differences have been described for both immune function and risk
97 for AD.

98 The aims of our study were two-fold. First, we aimed to study the effect of the *P522R*
99 mutation on a peripheral macrophage population to determine how these cells may be
100 differentially affected by the mutation. To do so, we assessed changes in major macrophage
101 functions including phagocytosis and inflammatory cytokine secretion, as well as the
102 transcriptional changes that underlie these functions. We utilized peritoneal macrophages
103 (pMacs) which can be separated into two main populations, large peritoneal macrophages
104 (LPMs) and small peritoneal macrophages (SPMs). In broad terms, LPMs are known to be
105 resident to the peritoneal cavity, and SPMs are derived from blood-monocytes, which travel to
106 the peritoneal cavity upon infection or other inflammatory events (26). Recent studies have
107 shown that these recruited monocytes can, over time, differentiate into macrophages that closely
108 mimic the resident LPMs (27). Second, we aimed to address how sex may influence the effector
109 functions of this population of macrophages, both with and without the *P522R* mutation, as
110 knowing the baseline sex differences in wild-type (WT) mice would more easily allow us to

111 assess additional effects of the *P522R* mutation. We found that not only do pMacs exhibit
112 marked sex-differences, but that the *P522R* mutation has sexually dimorphic affects.
113 Mechanistically, we provide evidence that the *P522R* mutation may be selectively conferring its
114 protection in females by shifting macrophages from an inflammatory M1 phenotype to an
115 alternatively-activated M2 phenotype.
116

117 **METHODS**

118 ***Animals***

119 Congenic mice expressing the *P522R* mutation in the *Plcg2* gene were purchased from the
120 Jackson Laboratory (strain #029598) and housed at the University of Florida McKnight Brain
121 Institute vivarium. Mice heterozygous for the *P522R* mutation were produced by breeding
122 heterozygous mice and C57BL/6 control littermates. They were kept at 22°C in 60-70%
123 humidity and on a 12-hour light-dark cycle and fed *ad libitum*. All animal procedures were
124 approved by the University of Florida Institutional Animal Care and Use Committee and were in
125 accordance with the National Institute of Health Guide for the Care and Use of Laboratory
126 Animals (NIH Publications No. 80-23) revised 1996. Both male and female mice were bred and
127 used in these experiments and were 3-5 months old at the time of sacrifice. Mice were sacrificed
128 via cervical dislocation, and all experiments included 5-6 biological replicates.

129 ***pMac isolation and culture***

130 Three days prior to pMac extraction, mice were injected with 1mL of a 3% thioglycolate
131 (Brewer) solution. Thirty minutes prior to thioglycolate injection mice were injected with 50uL
132 of sustained-release Buprenorphine (ZooPharm) to alleviate pain. After three days, the mice were
133 sacrificed via cervical dislocation. The abdomen was sprayed with 70% ethanol and the skin was

134 split along the midline. Once the peritoneal cavity was exposed, a 10mL syringe filled with
135 10mL of cold RPMI (Gibco, 11875119) was injected into the peritoneal cavity using a 27G
136 needle, avoiding blood vessels. Once the 10mL of cold RPMI was injected, another empty 10mL
137 syringe with a 25-gauge needle was used to withdraw and place the peritoneal fluid into a 15mL
138 Falcon collection tube. Any remaining fluid was aspirated via transfer pipette and placed into the
139 collection tube. Once all mice were sacrificed and peritoneal fluid was collected into separate
140 collection tubes, the fluid was passed through a 70uM nylon filter pre-wet with 5mL of cold
141 HBSS-/- (Gibco, 14175103) into a 50mL conical tube. 5mL of cold HBSS-/- was then placed
142 into the collection tube to rinse any remaining cells and passed through the filter as well. The
143 filter was then washed with 5mL of HBSS-/- and the 50mL tube containing the fluid and HBSS-
144 /- were centrifuged at 400*g* for 5 minutes at 4°C to pellet the cells. If any blood cells were
145 present the pellet was resuspended in 1mL of ammonium-chloride-potassium lysis buffer for 1
146 minute and then centrifuged again at 400*g* for 5 minutes at 4°C. Once all cells were pelleted, the
147 supernatant was aspirated and each pellet was resuspended in 3mL of plating media (RPMI, 10%
148 fetal bovine serum, and 1% penicillin/streptomycin). Viable cells were identified and counted
149 using trypan-blue exclusion on an automated cell counter (Countess™). The volume of growth
150 media was adjusted so that cells were plated at a density appropriate for the intended assay
151 (detailed below). Cells were incubated at 37°C, 5% CO_2 for a minimum of 2-hours to allow
152 macrophages to adhere. Wells were washed with sterile DPBS (Gibco, 14190235) to remove any
153 non-adherent cells, and new, pre-warmed growth media was added.

154 ***Flow Cytometry***

155 For flow cytometry analysis, cells were plated at a density of $3x10^5$ cells/well in a 24-well plate
156 and treated with either vehicle (sterile phosphate buffered saline [PBS]) or 10ng/mL of LPS

157 (O111:B4 Sigma, L4391, Lot#0000185317) for 18 hours. 1 hour prior to collection, DQ-BSA
158 green (Invitrogen, D12050) was added to each well at a concentration of 10ug/mL to measure
159 protein degradation. After 1 hour, the cells were washed 3 times with sterile PBS (Gibco,
160 10010023) and then transferred to a 96-well v-bottom plate and centrifuged at 300*g* for 5 minutes
161 at 4°C. The cells were then resuspended in 50uL of PBS containing fluorophore-conjugated
162 antibodies for CD11b (1:100, Biolegend, 101216), MHCII (1:100, Biolegend, 107628), and
163 Live/Dead stain (1:2000, Fisher, 130113144). The cells were then incubated in the dark at 4°C for
164 20 minutes at which point they were centrifuged again at 300*g* for 5 minutes at 4°C. The cells were
165 then washed with sterile PBS, and this process was repeated twice. The cells were then fixed in
166 50uL per well of 1% paraformaldehyde in PBS for 30 minutes in the dark at 4°C. After 30 minutes
167 the cells were centrifuged at 300*g* for 5 minutes at 4°C, resuspended in 200uL of FACs buffer
168 (PBS, 0.5 mM EDTA, 0.1% sodium azide), and taken to the Macs quant analyzer (Miltenyi) for
169 flow cytometric analysis. A minimum of 100,000 events were captured per sample and data were
170 analyzed using FlowJo version 10.6.2 software (BD Biosciences). When validating flow cytometry
171 panels and antibodies, fluorescence minus one controls (FMOCs) were used to set gates and
172 isotype controls were used to ensure antibody-specific binding.

173 ***Phagocytosis Assay***

174 For phagocytosis studies, cells were plated at a density of $5x10^4$ cells/well in a 96-well black
175 wall plate (Cellvis, NC1648269) in triplicate. Cells were then treated with either vehicle or LPS
176 at a concentration of 10ng/mL for 6 hours at 37°C in a 5% CO_2 humidified cell culture incubator.
177 2mL of Live Cell imaging solution (Invitrogen, A59688DJ) was added to each vial of pHrodo
178 green *E. coli* bioparticles (Invitrogen, P35366), equating to a final concentration of 1mg/mL, and
179 these solutions were sonicated for 10 minutes. After sonication, the media was removed from the

180 cells in the plate and replaced with 100uL of *E. coli* bioparticle solution, at which point the plate
181 was placed into an onstage incubator connected to an EVOS M7000 microscope to maintain the
182 cells at 37°C and 5% CO_2 humidified atmosphere throughout the duration of the live-cell
183 imaging. GFP images were captured at 20X magnification with 5 images per well every 30
184 minutes for 15 hours within each well, and the EVOS Celleste (Version 5) software was used to
185 count cells and determine the integrated optical density of GFP fluorescence within each well
186 over time. Brightfield images were used for automated-focus throughout the time course.
187 Treatment conditions were performed in triplicate wells and averaged for analysis.

188 ***Multiplexed Immunoassays***

189 For inflammatory cytokine secretion, isolated pMacs were plated at a density of $1x10^6$ cells/well
190 in a 6-well plate and treated with either vehicle or LPS at a concentration of 10ng/mL. The cells
191 were then placed in a humidified cell culture incubator at 37°C and 5% CO2. Conditioned media
192 was collected at 2, 6, and 24 hours post stimulation for assessment of specific protein
193 concentrations of TNF, IL-6, IL-1B, KC/GRO, IL-4, IL-10, IFN-Y, IL-5, IL-2, and IL-12p70
194 (MSD, K15048D-2) using multiplexed immunoassays on the MesoScale Discovery (MSD)
195 platform. Samples were plated in duplicate at a 1:1 dilution with MSD diluent 41 and the rest of
196 the experimental procedure was performed according to the manufacturer's instructions. The
197 plate was then analyzed on the MSD Quickplex machine and MSD software (Discovery
198 Workbench Version 4.0). Cytokines with values below the lower limit of detection (LLOD) were
199 not quantified.

200 ***RNA Isolation***

201 For RNA extraction, cells were plated at $1x10^6$ cells/well in a 6-well plate and treated with either
202 vehicle or LPS at 10ng/mL. The cells were placed back in a humidified cell culture incubator at

203 37°C and 5% CO_2 for 6 hours. Buffer RLT from the RNeasy mini kit (Qiagen, 74104) was
204 combined with β-mercaptoethanol (Sigma, 63689) at a concentration of 20uL of BME per 1mL
205 of buffer RLT. Media was aspirated from the plate and then 350uL of RLT+BME solution was
206 then added to each well to lyse the cells. Each well was scraped with a mini cell scraper (Fisher,
207 NC0325221) and collected into a Qiashredder (Qiagen) column and centrifuged at 10,000*g* for 2
208 minutes. 350uL of 70% ethanol was added to the homogenized lysate and then transferred to a
209 RNeasy spin column. The columns were then centrifuged for 30 seconds at 10,000*g* and
210 afterwards the flow-through was discarded. 700uL of buffer RW1 was then added to each
211 column and the columns were centrifuged at 10,000*g* for 30 seconds and the flow-through was
212 discarded. 500ul of buffer RPE was added to each column and they were spun at 10,000*g* for 2
213 minutes and the flow-through was again discarded. This step was repeated twice, and the column
214 was then placed into a 1.5mL collection tube, 30uL of elution buffer was added to the column,
215 and then centrifuged for 1 minute at 10,000*g* to elute the RNA. Concentration of RNA was
216 analyzed on a spectrophotometer, and RNA quality was assessed using Bioanalyzer 2100
217 (Agilent, Denmark).

218 ***Gene expression analysis***

219 For quantification of mRNA transcripts in pMacs, the NanoString nCounter platform and Mouse
220 Immune Exhaustion Panel RNA (CAT#PSTD-M-EXHAUST-12) containing 785 genes was
221 used. 25ng/ul of RNA was loaded and hybridized to the provided capture and reporter probes
222 overnight at 65°C according to the manufacturer's instructions. The samples were then added
223 into the provided nCounter chip via the use of the NanoString MAX/FLEX
224 robot. Unhybridized capture and reporter probes were removed, and the hybridized mRNAs were
225 immobilized for transcript count quantification on the nCounter digital analyzer. The data was

226 then imported into the nSolver analysis software v4.0 for quality check and analysis according to
227 the manufacturer's instructions. Raw counts were normalized to the geometric mean of the
228 positive and negative controls and count expression was calculated via nSolver Advanced
229 Analysis v2.0.134. Gene expression was considered significant if $p < 0.05$ after adjustment using
230 the Benjamini-Hochberg method for false discovery rate.

231 ***Statistical Analysis***

232 Statistical analysis was performed using Prism Graphpad version 10.2.2. Differences between
233 groups was determined using 2-way ANOVA with multiple comparisons using Fisher's
234 uncorrected LSD. Results were considered statistically significant if $p<0.05$. Each graph depicts
235 individual data points with error bars representing the standard error of the mean (SEM).

236

237 **RESULTS**

238 ***The P522R mutation ablates sex-dependent population differences in pMacs***

239 Before addressing cellular functionality, it is beneficial to first understand and characterize the
240 macrophage populations present within our samples. As mentioned, pMacs are not a
241 homogenous population, with SPMs arising from blood monocytes and LPMs being resident to
242 the peritoneal cavity. To determine whether sex and/or the *P522R* mutation have any effect on
243 the frequencies of these cell populations we used flow cytometry to distinguish SPMs from
244 LPMs on the basis of CD11b expression, as has been previously described (Supp. Figure 1) (28).
245 We determined that sex significantly affects the frequencies of SPMs and LPMs present in the
246 peritoneal cavity. WT males exhibited a higher frequency of LPMs (Figure 1A) and WT females
247 exhibited a higher frequency of SPMs (Figure 1B). This was further confirmed via raw counts of
248 LPMs and SPMs (Supp. Figure 2A, B). Unexpectedly, these sex-dependent differences were not

observed in mice carrying the *P522R* mutation, as evident by the lack of significant differences in LPM and SPM frequencies between females and males (Figure 1A, B).

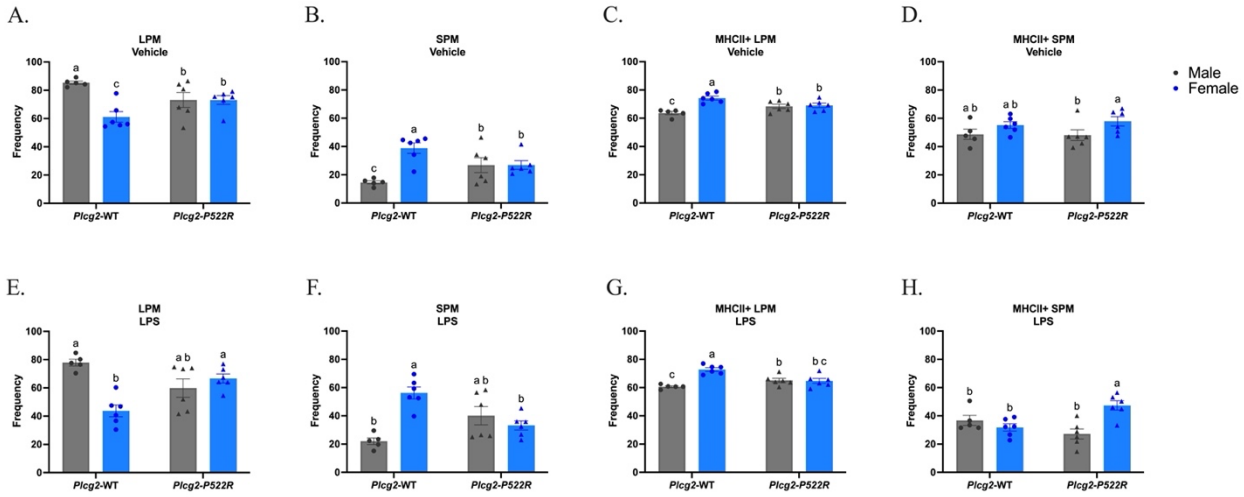


Figure 1: The P522R mutation ablates sex-dependent population differences in pMacs. pMacs from male and female, WT and *P522R* animals were isolated and populations within vehicle treated groups of (A) LPM (B) SPM (C) MHCII+ LPM and (D) MHCII+ SPM were quantified via flow cytometry. The same experiment was repeated but cells were treated with 10ng/mL of LPS and populations of (E) LPM (F) SPM (G) MHCII+ LPM and (H) MHCII+ SPM were quantified via flow cytometry. Data was quantified via two-way ANOVA with multiple comparisons using Fisher's uncorrected LSD. Groups that share a letter are considered non-significant ($p>0.05$) and groups that do not share a letter are considered significantly different ($p<0.05$).

After reviewing the raw counts, we determined that this effect was driven primarily by an increase in LPM count in pMacs from *P522R* females, while SPM counts did not change, leading to an increase in the total live cell counts of pMacs in *P522R* female mice (Supp. Figure 2). Next, we used major histocompatibility complex class II (MHCII) as a marker of antigen presentation. We found that LPMs from female WT mice displayed a higher frequency of MHCII+ cells, but that this difference did not persist in mice that carry the *P522R* mutation (Figure 1C). Finally, SPMs from female and male WT mice showed no differences in the frequency of MHCII+ cells, but when comparing mice carrying the *P522R* mutation, female mice showed a higher frequency of MHCII+ SPMs compared to males (Figure 1D). To evaluate the functional significance of these differences, cells were challenged with the inflammagen lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, to determine

271 whether these population differences still persisted after stimulation. We found that regardless of
272 LPS stimulation, all previously described macrophage population differences between male and
273 female WT mice were still evident, and the *P522R* mutation had the same effect of reducing
274 these population differences, aside from the higher frequency of MHCII+ SPMs which still
275 persisted as well after LPS stimulation (Figure 1E-H). These data indicate that female and male
276 WT mice harbor intrinsic differences in the makeup of their pMac populations, and the presence
277 of the *P522R* mutation ablates these sex-dependent differences.

278 ***pMacs from WT female mice exhibit increased expression of immune activation and***
279 ***inflammation-related genes***

280 Our previous data indicated that sex-dependent population differences were diminished in mice
281 that carry the *P522R* mutation. It could be assumed then that these shifts in population dynamics
282 would be followed by changes in macrophage function. To better assess how the *P522R*
283 mutation may alter macrophage function, we first aimed to understand how baseline sex
284 differences within these populations may be affecting function, as this will provide us a basis for
285 comparison. To do so, we performed a targeted transcriptomic analysis assessing genes
286 associated with immune cell function comparing baseline and LPS-stimulated gene expression in
287 pMacs from WT males and females. As transcription is the code for downstream functional
288 protein synthesis, obtaining data assessing gene expression can provide useful knowledge on
289 how these cells may be functioning. We found that compared to pMacs from WT male mice,
290 pMacs from WT females exhibit baseline up-regulation of numerous genes related to immune
291 activation and inflammatory processes, including but not limited to *Tnf, Tlr2, Ccl3, Ccl2, Cd14*,
292 and numerous genes related to *NF-kB* signaling, including *NF-kB2, NF-kBia, and NF-kBie*

(Figure 2A).

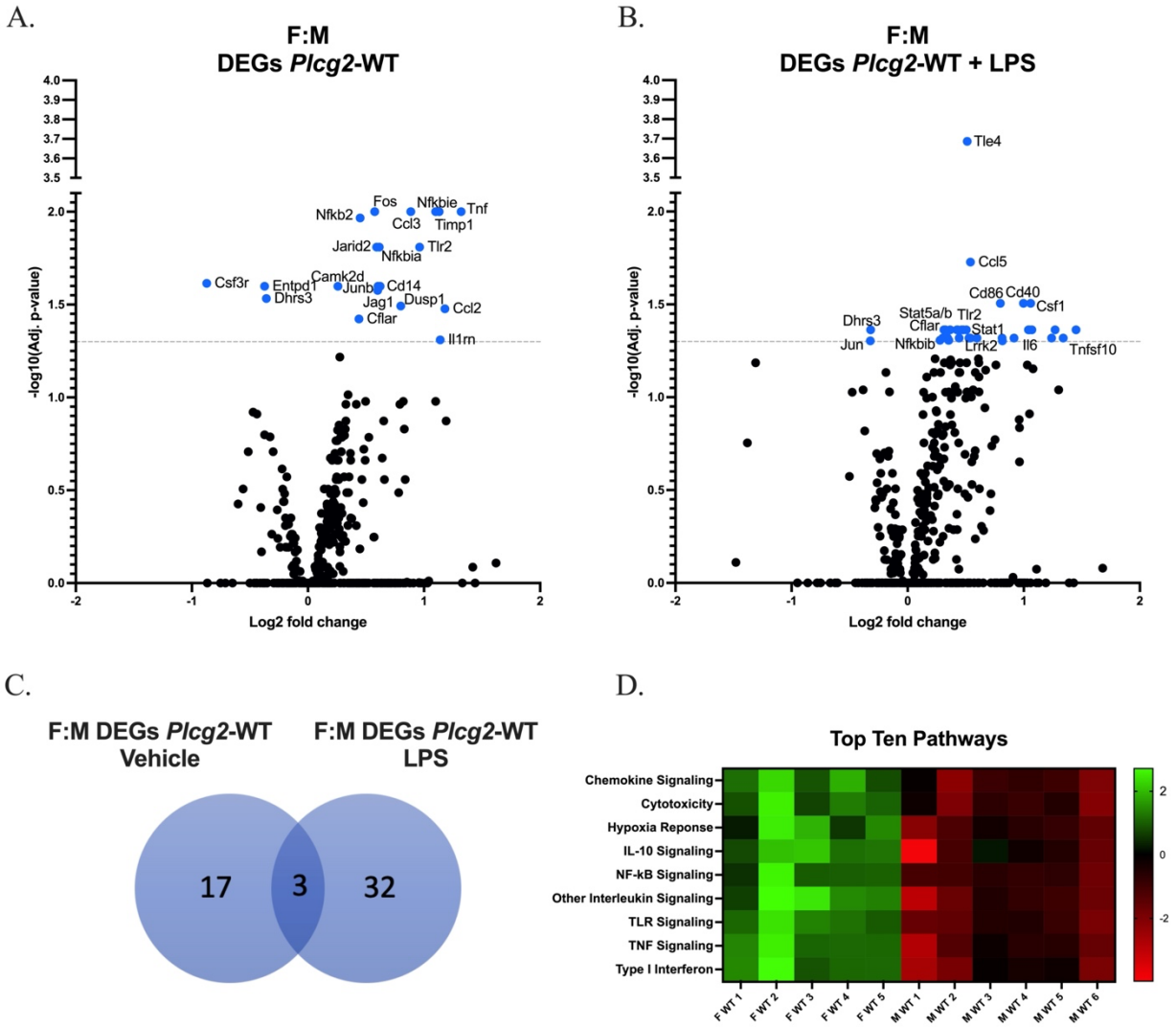


Figure 2: pMacs from WT female mice exhibit increased expression of immune activation and inflammation-related genes. pMacs from male and female WT mice were plated and treated with either vehicle or LPS and left to incubate for 6 hours. Cells were then collected and RNA was isolated for gene expression analysis. (A) Volcano plot comparing gene expression of pMacs from female WT mice to gene expression of pMacs from male WT mice, grey dashed line indicating adjusted $p<0.05$ (B) Volcano plot comparing gene expression of pMacs from female WT mice to gene expression of pMacs from male WT mice after LPS stimulation. (C) Venn diagram comparing number of differentially expressed genes between pMacs from WT females and pMacs from WT females after LPS stimulation. (D) Heatmap of the top ten pathways implicated in altered function between pMacs from male and female WT mice indicated by z-score.

When treated with LPS, the gene expression profile differs even more between the sexes, with WT females exhibiting increased expression of *Tlr2, Csf1, Cd86, Stat1, IL-6, Lrrk2,* and *NF-kBib* (Figure 2B). In total, pMacs from WT female mice exhibit 20 differentially expressed genes

compared to pMacs from WT males, and this difference widens after LPS treatment, with pMacs from WT females showing differential expression of 35 genes (Figure 2C). To further understand how these differentially expressed genes may be related to cellular functionality, we assessed the top ten pathways associated with these genes. We found that the up-regulated genes seen in pMacs from WT females are associated with pathways that implicate cytotoxicity, hypoxia response, and TLR, TNF, chemokine, and NF-kB signaling, all of which may be associated with the pro-inflammatory M1 macrophage phenotype (Figure 2D). These data reveal that pMacs in females may be primed toward a M1 inflammatory phenotype, and may respond more robustly to immune activators such as LPS.

The P522R mutation reduces differences in gene expression between the sexes

Based on the data showing that the *P522R* mutation is able to reduce pMac population differences between the sexes, we hypothesized that the gene expression profile would be more similar between pMacs from *P522R* females and males than it was between the WT groups. Indeed, we found that only 8 genes were differentially expressed in pMacs from *P522R* female mice compared to *P522R* males in the vehicle-treated condition, and only 1 gene was differentially expressed between these two groups in the LPS-treated condition (Figure 3A-C).

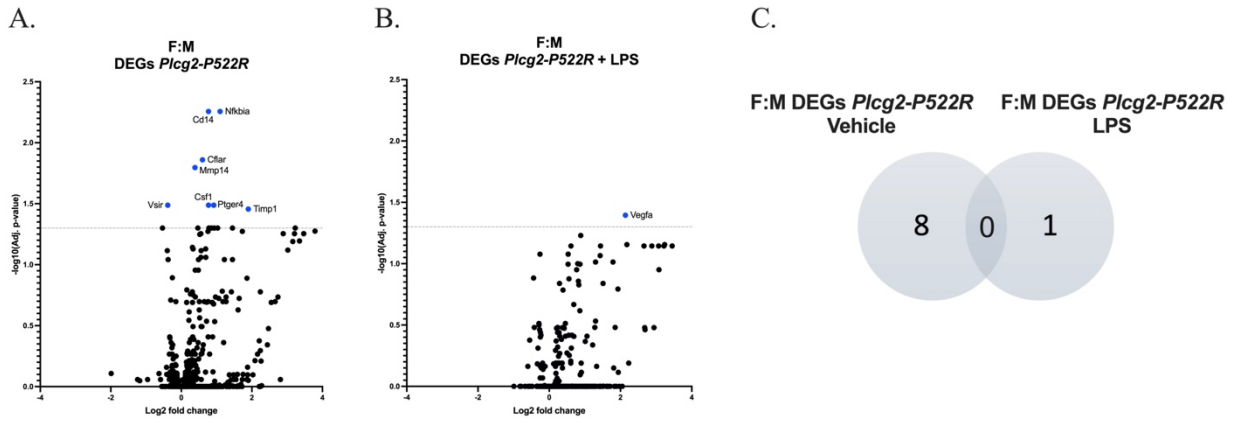


Figure 3: The P522R mutation reduces differences in gene expression between the sexes. pMacs from male and female *P522R* mice were plated and treated with either vehicle or LPS and left to incubate for 6 hours. Cells were

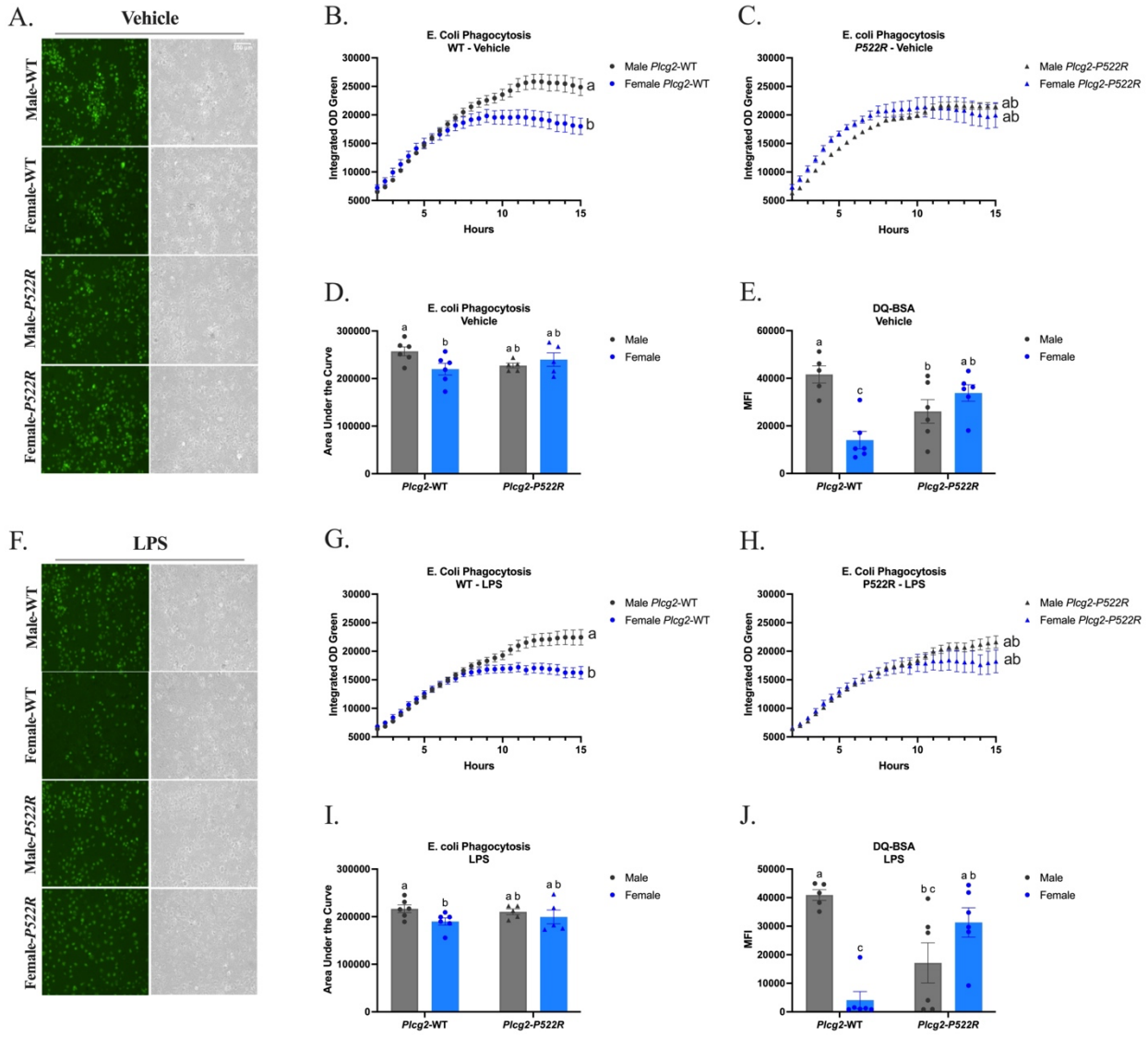
327 then collected and RNA was isolated for gene expression analysis. (A) Volcano plot comparing gene expression of
328 pMacs from female *P522R* mice to gene expression of pMacs from male *P522R* mice, grey dashed line indicating
329 adjusted $p<0.05$ (B) Volcano plot comparing gene expression of pMacs from female *P522R* mice to gene expression
330 of pMacs from male *P522R* mice after LPS stimulation. (C) Venn diagram comparing number of differentially
331 expressed genes between pMacs from *P522R* females and pMacs from *P522R* females after LPS stimulation.
332
333 Although the magnitude of differentially expressed genes is smaller, pMacs from *P522R* female
334 mice still exhibit more activation and inflammation-related genes than males, including *Cd14,*
335 *NF-kBia,* and *Csf1* (Figure 3A). After LPS treatment, the only gene that is upregulated in pMacs
336 from *P522R* females compared to males is vascular endothelial growth factor A (*Vegfa)*, which
337 has often been associated with the M2 macrophage phenotype (Figure 3B). Taken together, these
338 data indicate that the *P522R* protective mutation reduces some of the intrinsic sex differences
339 evident in peripheral macrophage populations at the transcriptional level and support that the
340 mutation shifts pMacs from female mice toward an M2 phenotype.

341 ***The P522R mutation alleviates sex-dependent differences in phagocytic and lysosomal***
342 ***function***

343 Given our findings that pMacs from WT females display a transcriptional profile that aligns
344 more closely with the canonical M1 activation phenotype, we hypothesized that this would lead
345 to differences in macrophage effector functions, such as phagocytosis, between pMacs from WT
346 males and females. As the M1 phenotype is characterized by lower levels of phagocytosis, we
347 hypothesized that pMacs from WT females may show deficiencies in phagocytic uptake
348 compared to pMacs from WT males. Furthermore, we hypothesized that the ability of the *P522R*
349 mutation to ablate population and transcriptional differences would also reduce functional
350 differences. To assess the phagocytic capabilities of these macrophages, we utilized fluorescently
351 labeled *E. coli* bioparticles which only fluoresce after being internalized and exposed to the low
352 pH associated with the lysosome, thereby reporting on the extent of bacterial uptake by the cells.
353 In accordance the lower abundance of M1 associated genes present in WT male mice, the pMacs

354 from WT male mice exhibited significantly higher levels of *E. coli* uptake compared to pMacs

355 from WT females (Figure 4A, B and D).



356

357 **Figure 4: The P522R mutation alleviates sex-dependent differences in phagocytic and lysosomal function**.
358 pMacs from WT males and females were plated, treated with vehicle, and incubated for 6 hours, at which point
359 200ug of *E. coli* was added to each experimental well. (A) GFP fluorescence was imaged at 20X magnification. (B)
360 Integrated optical density of GFP fluorescence was plotted over 15 hours. This experiment was repeated with *P522R*
361 carrier males and females and (C) Integrated optical density of GFP fluorescence was plotted over 15 hours of
362 pMacs from male and female *P522R* carriers. (D) Groups were compared via area under the curve. 1 hour prior to
363 cell collection DQ-BSA was added to the plate and then cells were collected for flow cytometry and (E) DQ-BSA
364 MFI is represented. pMacs from WT males and females were plated, treated with LPS, and incubated for 6 hours, at
365 which point 200ug of *E. coli* was added to each experimental well. (F) GFP fluorescence was imaged at 20X
366 magnification. (G) Integrated optical density of GFP fluorescence was plotted over 15 hours. This experiment was
367 repeated with *P522R* carrier males and females and (H) Integrated optical density of GFP fluorescence was plotted
368 over 15 hours of pMacs from male and female *P522R* carriers. (I) Groups were compared via area under the curve. 1
369 hour prior to cell collection DQ-BSA was added to the plate and then cells were collected for flow cytometry and (J)

370 DQ-BSA MFI is represented. Data were quantified via two-way ANOVA with multiple comparisons using Fisher's
371 uncorrected LSD. Groups that share a letter are considered non-significant ($p>0.05$) and groups that do not share a
372 letter are considered significantly different ($p<0.05$).
373
374 However, pMacs from *P522R* males and females did not show any significant difference in *E.*
375 *coli* uptake, consistent with our previous result showing that the *P522R* mutation was able to
376 decrease the differential expression of these M1 related genes in females (Figure 4A, C, and D).
377 Although the macrophages were able to take up the *E. coli* particles, we further assessed whether
378 pMacs from males and females displayed any differences in lysosomal function, or the ability to
379 successfully break down that which has been phagocytosed. To do so, we treated the cells with
380 DQ-BSA, a substrate for proteases that only fluoresces after it has been cleaved within the
381 lysosome, and quantified via flow cytometry. We found that similar to our previous result,
382 pMacs from WT male mice also displayed higher protease activity compared to pMacs from WT
383 females, but this difference was ablated by the presence of the *P522R* mutation (Figure 4E).
384 Next, we repeated the experiments with the addition of LPS stimulation and similar patterns
385 were detected with pMacs from WT male mice displaying higher *E. coli* uptake even after
386 stimulation compared to pMacs from WT female mice (Figure 4F, G and I), while pMacs from
387 *P522R* male and female mice showed no difference in *E. coli* uptake even after stimulation
388 (Figure 4F, H, and I). Additionally, even after LPS stimulation the *P522R* mutation was able to
389 rescue the lower lysosomal protease activity in females (Figure 4J). Specifically, these findings
390 revealed that pMacs from WT female mice had reduced phagocytic and lysosomal function
391 compared to pMacs from WT males, and that the *P522R* mutation alleviated this sex-dependent
392 difference.

393 ***The P522R mutation increases inflammatory cytokine release by pMacs from females but not***
394 ***from males***

Macrophages are known to be one of the major propagators of inflammation in response to immune activation, particularly via the M1 pro-inflammatory activation phenotype (29). As we saw that M1 associated genes were up-regulated in pMacs from WT female mice, we hypothesized that pMacs from WT females may be more pro-inflammatory in response to LPS stimulation. To test this, we treated pMacs from WT males and females with LPS and measured the protein concentration of cytokines within the media at 2, 6, and 24 hours post-LPS treatment. Concurrent with our hypothesis, we found that in general, pMacs from WT females displayed a stronger inflammatory response to LPS treatment compared to pMacs from WT males, with media from pMacs from WT females exhibiting higher concentrations of IL-1β and IL-12p70 by 6 hours post LPS, and higher concentrations of TNF, IL-6, IL-1β, IL-12p70, and IL-2 by 24 hours post LPS treatment (Figure 5A-E).

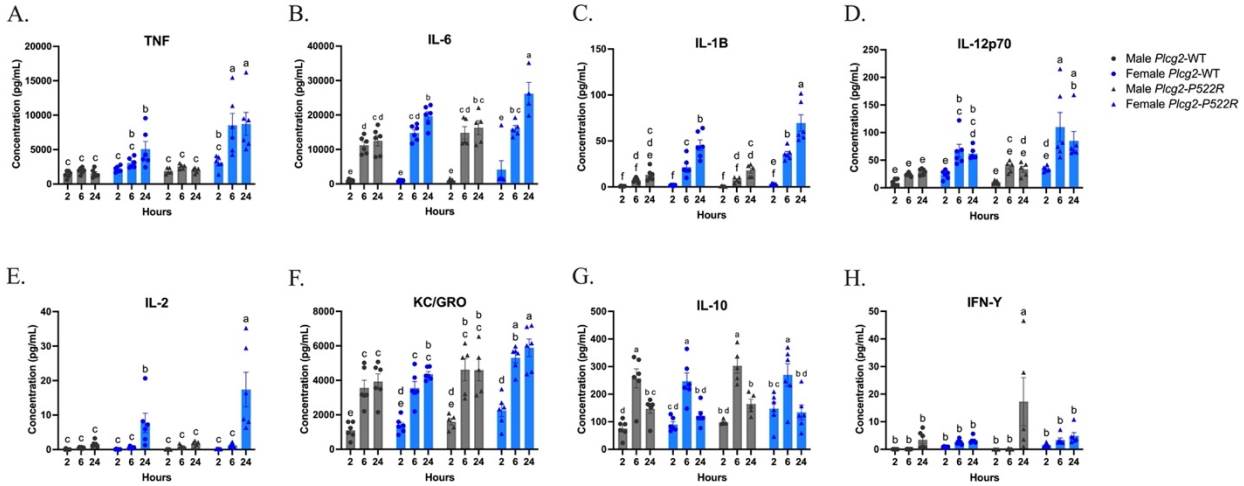


Figure 5: The P522R mutation increases inflammatory cytokine release by pMacs from females but not from males. pMacs from male and female WT and *P522R* mice were plated and treated with LPS, and their conditioned media was collected at 2, 6, and 24 hours post LPS stimulation. Concentrations of (A) TNF (B) IL-6 (C) IL-1B (D) IL-12p70 (E) IL-2 (F) KC/GRO (G) IL-10 and (H) IFN-Y were then assessed via MesoScale Discovery immunoassays. Data was quantified via two-way ANOVA with multiple comparisons using Fisher's uncorrected LSD. Groups that share a letter are considered non-significant ($p>0.05$) and groups that do not share a letter are considered significantly different ($p<0.05$).

Based on the finding that the *P522R* mutation reduced sex-related population, transcriptional, and functional differences, we originally hypothesized that this hyper-inflammatory phenotype

417 associated with pMacs from WT females would be reversed by the presence of the *P522R*
418 mutation. Surprisingly, we found that pMacs from *P522R* females exhibited an exacerbated
419 inflammatory phenotype relative to pMacs from WT females, with higher concentrations of
420 TNF, IL-1β, and IL-12p70 at 6 hours post LPS treatment, and higher concentrations of TNF, IL-
421 6, IL-1β, IL-12p70, KC/GRO, and IL-2 at 24 hours post LPS treatment (Figure 5A-F). Cytokine
422 release from WT male mice seemed to be largely unaffected by the *P522R* mutation, except for a
423 higher concentration of IFN-γ at 24 hours post LPS treatment (Figure 5H). Additionally, IL-10
424 secretion appeared to be similar, regardless of sex or genotype (Figure 5G). These results reveal
425 that pMacs from WT female mice are more pro-inflammatory in response to stimulation than
426 pMacs from WT males and this may be modified by genotype, raising the interesting possibility
427 that pMacs from male mice possess the ability to down-regulate cytokine production better than
428 pMacs from females.

429 ***Gene expression analysis reveals that pMacs from male mice regulate cytokine gene***
430 ***expression more effectively than those from female mice***

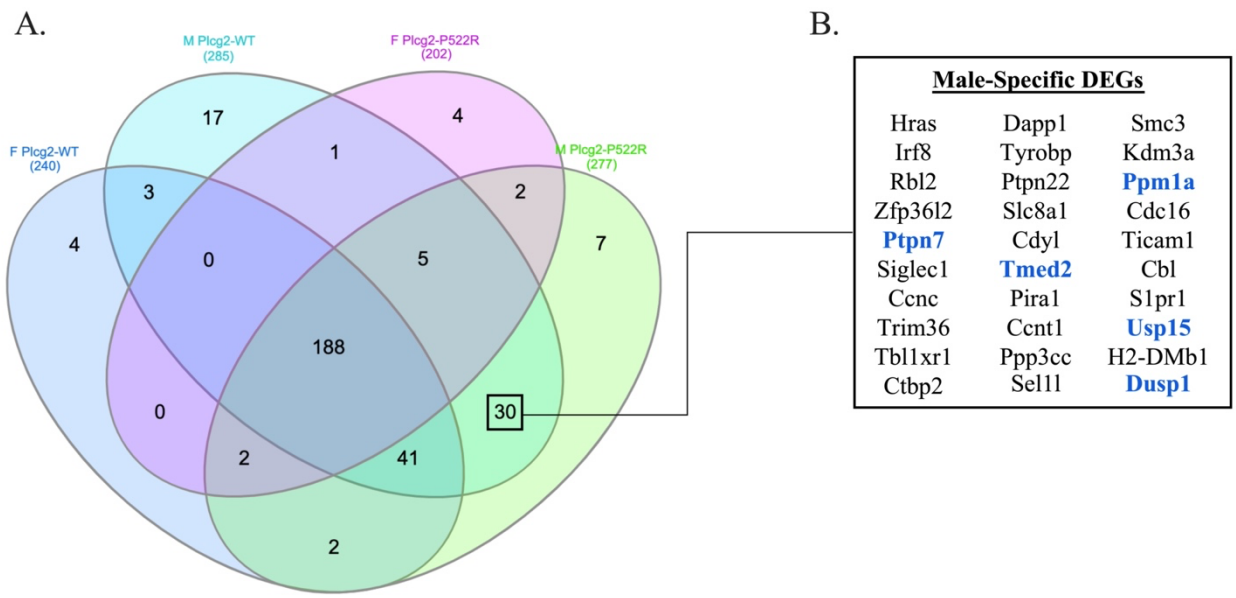
431 In every outcome measured, aside from cytokine secretion, the presence of the *P522R* mutation
432 was associated with reduced sex-dependent differences in macrophage function. As was
433 previously shown, pMacs from WT female mice are more pro-inflammatory in response to LPS
434 stimulation than pMacs from WT males (Figure 5). In contrast to most other outcome measures,
435 the presence of the *P522R* mutation did not ablate this difference, but rather exacerbated it, with
436 pMacs from *P522R* female mice producing more inflammatory cytokines than pMacs from either
437 WT females or *P522R* males (Figure 5). These observations raised the question as to whether
438 pMacs from male mice, regardless of genotype, possess a more effective method to regulate
439 cytokine expression in response to LPS stimulation compared to females that could explain these

440 findings. To determine this, we compared the overlap of all differentially expressed genes

441 (DEGs) from every group (male and female, WT and *P522R*) in response to LPS stimulation and

442 looked specifically at DEGs only shared between male groups. We found that 30 DEGs were

443 shared only by the pMacs in WT and *P522R* male groups (Figure 6A).



444

445 **Figure 6: Gene expression analysis reveals that pMacs from male mice regulate cytokine gene expression**
446 **more effectively.** pMacs from male and female WT and *P522R* mice were plated and treated with LPS and left to
447 incubate for 6 hours. Cells were then collected and RNA was isolated for gene expression analysis. (A) Venn
448 Diagram comparing overlap of differentially expressed genes after LPS treatment between all four groups. (B)
449 DEGs only shared by male groups.

450

451 Of these 30 genes, we discovered 5 up-regulated genes that have been associated with the down-

452 regulation of cytokine production in macrophages (Figure 6B). These data support a model in

453 which the *P522R* mutation exerts hyper-morphic effects, but peripheral macrophages in males

454 possess effective intrinsic mechanisms to effectively down-regulate the increased activity

455 conferred by the mutation, consistent with the differences observed in cytokine production

456 between males and females harboring the *P522R* mutation in this study.

457

458 **DISCUSSION**

459 Numerous studies have implicated the immune system as a player in the progression of
460 neurodegenerative disorders, both within the brain and in the periphery. This includes evidence
461 that peripheral myeloid cells, including monocytes and tissue-resident macrophages, are
462 dysregulated early in the course of AD, displaying lower phagocytosis and higher expression of
463 *NF-kB* (5). Over the course of disease progression immune cells become immunosenescent and
464 contribute to low-grade chronic inflammation (6). Therefore, it is of critical importance to
465 investigate the nature of these dysregulated immune mechanisms to identify potential new targets
466 for therapeutic intervention. With this objective in mind, many groups have studied the effects of
467 the *Plcg2-P522R* protective mutation in myeloid cells, primarily microglia, to try to determine
468 how this protection is being conferred since the effects manifest themselves in improved
469 cognitive function. However, the effect of the *P522R* mutation on peripheral tissue-resident
470 macrophages, which have become of increasing importance in the context of AD, had yet to be
471 determined. Additionally, we assessed how these protective effects may differ between sexes, as
472 it is well known that both AD risk and immune system function is highly affected by sex. Herein,
473 we show for the first time that the effects of the *P522R* mutation in the periphery seem to be
474 heavily influenced by sex-intrinsic differences in the regulation of immune-related genes and
475 functions and that these differences may underlie the protective mechanism exerted by the
476 *P522R* mutation against AD-related cognitive decline that is more prominent in women.

477 We found that pMac populations vary greatly depending on sex, with males exhibiting a
478 higher frequency of LPMs and females having a higher frequency of SPMs. Additionally,
479 although the frequency of LPMs was lower in females, more of them were MHCII+. This was in
480 accordance with recent studies showing that blood-derived monocytes more readily differentiate

481 into resident (LPM) macrophages in males (30). However, in mice that possess the *P522R*
482 mutation this negative female bias is ablated as males and females have similar frequencies of
483 LPMs and SPMs. As this effect was driven by an increase in LPMs in pMacs from *P522R* female
484 mice, we can deduce that the *P522R* mutation may enhance the ability of female pMacs to
485 differentiate into mature macrophages. These data reveal for the first time that the *P522R*
486 mutation has different effects depending on sex.

487 Based on this data, we sought to understand how these shifts in macrophage populations
488 might alter the functionality of these cells, and to do so we started with assessing and comparing
489 the transcriptional profiles of our samples. We found that pMacs from female mice display
490 increased expression of genes such as *Cd14, Tlr2, Ccl2, Ccl3, Csf1, Stat1,* and *NF-kB*-related
491 genes at baseline, and *Cd86* and *Lrrk2* after LPS stimulation than do pMacs from male mice.
492 Additionally, many of these genes up-regulated in pMacs from females correspond to the M1
493 macrophage phenotype, which is known to be pro-inflammatory and anti-microbial (31-35).
494 Particularly *NF-kB* genes, which are responsible for cytokine production (36, 37). Additionally,
495 our data showing the ability of the *P522R* mutation to significantly reduce M1 phenotype-related
496 gene expression down to just *Cd14* and *NF-kB* suggests that this mutation may be altering
497 macrophage function in females toward a more M2 alternately-activated phenotype. This is
498 supported also by the increased expression of *Vegfa* by pMacs from P522R females, which is
499 canonically associated with the tissue repair and regeneration phenotype M2 (38). Based on these
500 findings, we sought to determine whether these underlying changes in transcription would be
501 reflected in observable changes in function.

502 As pMacs from WT females exhibited increased expression of M1 activation-related
503 genes compared to males, we hypothesized that pMacs from WT females would display lower

504 levels of phagocytosis compared to males. In line with this, we observed that pMacs from male
505 mice exhibited both higher phagocytic capacity of *E. coli* and higher lysosomal protease activity
506 compared to pMacs from female mice. Additionally, in accordance with our data showing that
507 pMacs from *P522R* females no longer exhibited up-regulation of as many M1-associated genes
508 compared to pMacs from males, we observed that the sex-dependent deficits in phagocytic
509 capacity and lysosomal protease activity in females no longer persisted. The literature addressing
510 the effect of the *P522R* mutation on phagocytosis is inconsistent, showing both increased and
511 decreased phagocytosis (15-17). This discrepancy was hypothesized to be due to the size of the
512 cargo, with studies showing lower phagocytosis of larger particles such as zymosan and *E. coli*,
513 and more phagocytosis of smaller-sized dextrans (15, 16). We show here that the sex of the mice
514 from which the cells are taken can also influence the results. Additionally, we show that the
515 mutation may be more effective at improving phagocytosis and lysosomal function in females, as
516 pMacs from female mice seem to be more predisposed to deficiencies in these functions than
517 pMacs from males.

518 Previous studies have shown that post-pubescent females are more inflammatory in
519 response to injury and pathogens than males (5, 23, 39). Additionally, studies done in pMacs
520 specifically have shown that pMacs from female mice secrete more IL-6 and IL-1β in response
521 to stimulation than pMacs from male mice (40, 41). In this study, we were able to identify a
522 potential transcriptional pathway that may explain these higher incidences of inflammation in
523 females. This being the increased basal expression of the inflammation-associated M1 phenotype
524 related genes in macrophages from female mice. Additionally, we identified increased
525 expression of *Cd14*, a known co-receptor for LPS, suggesting another possible mechanism by
526 which pMacs in females might be more responsive to immune stimulation (42, 43). We

527 hypothesized based on these previous studies and our own transcriptional data that this increased
528 gene expression in pMacs from WT females would lead to a more pro-inflammatory response to
529 stimulation compared to pMacs from WT males. Our data addressing cytokine secretion after
530 LPS stimulation confirmed this, and showed that pMacs from female mice display a more pro-
531 inflammatory phenotype and increased release of not just IL-6 and IL-1β, but also TNF, IL-
532 12p70, and IL-2 across time than pMacs from male mice. Surprisingly, although our data
533 assessing population frequencies, transcription, phagocytosis and lysosomal activity showed that
534 the *P522R* mutation reduced differences between pMacs from male and female mice, this was
535 not the case for cytokine secretion. Our findings regarding cytokine secretion differences in spite
536 of the presence of the *P522R* mutation are particularly interesting and intriguing and as such
537 stood out from the rest of the data, as all other outcome measures we performed also suggested
538 the ability of the *P522R* mutation to reduce sex differences evident in pMacs. We discovered that
539 pMacs in female mice may be disproportionately pro-inflammatory compared to those in male
540 mice, regardless of genotype, due to the fact that male mice are able to upregulate genes
541 associated with cytokine regulation, thereby counteracting the hypermorphic effects of the
542 *P522R* mutation. These male-specific upregulated genes included *Dusp1* (also known as *MKP-*
543 *1*), *Usp15, Ptpn7, Ppm1a,* and *Tmed2*. All of these genes have been shown to regulate cytokine
544 production via inhibiting *NF-kB* or adjacent pathways (44-49). In line with previous studies
545 assessing the effect of the *P522R* mutation on cytokine release, pMacs from *P522R* mice
546 displayed a more pro-inflammatory phenotype than pMacs from WT mice, but our data showed
547 that this effect was restricted to females as males were largely unaffected by the mutation (14).
548 Taken together, these results highlight the importance of assessing sex as a biological
549 variable in research studies that look to identify potential therapeutic pathways, as sex will not

550 only affect the magnitude of an effect, but in some cases the directionality of an effect as well.
551 While it is well known that women are disproportionately affected by AD compared to males (1),
552 exactly how women are at higher risk for the development of AD is unknown; however, sex
553 differences in immune system function have been implicated in this difference. As has been
554 shown in our study and others, immune cells from females tend to mount a larger inflammatory
555 response to immunological stimuli (23, 39), several of which have been shown to enhance
556 chronic inflammation and increase risk for neurodegeneration (50-53). Additionally, studies have
557 shown that microglia from females display limited phagocytic capacity and a more pro-
558 inflammatory phenotype in most AD models, leading to increased accumulation of Aβ plaques
559 and chronic neuroinflammation (54, 55).

560 Based on our findings and the published literature on the effects of *P522R*, we propose
561 here that the protective effect of the *P522R* mutation may be partially mediated by pushing
562 macrophages in females away from the typical M1 phenotype and towards a more protective M2
563 phagocytic phenotype. Although cytokine secretion levels are higher in pMacs from *P522R*
564 female mice, the increased ability to phagocytose and degrade pathogens and physiologically
565 relevant proteins would likely mean that the inflammation would be acute, and therefore less
566 deleterious, and may even help remove amyloid plaques. Under normal physiological conditions,
567 macrophages typically only produce inflammatory cytokines in the presence of an immune
568 stimulus, and if the *P522R* mutation confers pMacs in females with the ability to more readily
569 resolve or process an immunological stimulus such as amyloid-beta oligomers or diffuse plaque
570 via phagocytosis and processing through endo-lysosomal pathways, their hyper-secretory
571 cytokine phenotype is likely to abate and/or aid in processing of toxic protein aggregates.
572 Importantly, it should be recognized that inflammation is an essential part of the body's

573 protective immune response in particular in the periphery, and therefore necessary and beneficial
574 as long as it does not become un-regulated and chronic. In summary, we provide herein novel
575 findings that the *P522R* mutation may confer differential advantages to peripheral macrophages,
576 and that this effect may be stronger in females. Further investigation is warranted to determine
577 whether the effects of the *P522R* mutation are also present in other peripheral myeloid cell
578 populations such as dendritic cells which have been implicated in the pathophysiology of AD
579 (56, 57).
580

581 **CONCLUSION**

582 Inflammation and the cells of the peripheral immune system have been implicated in the
583 progression of AD. Therefore, identifying targetable pathways that have the potential to
584 modulate these dysregulated immune cell functions has been of recent interest. Others have
585 demonstrated that the *P522R* mutation has the ability to enhance important immune functions in
586 microglia, such as phagocytosis and cytokine secretion. Herein we provide the first evidence that
587 the *P522R* mutation may be conferring its protective effect via modulating peripheral tissue-
588 resident macrophage functions, and that this effect is strongly seen in macrophages from
589 females. Taken together, the results suggest that peripheral immune cells may be able to become
590 a target for AD drug intervention and that sex as a biological variable should be an integral part
591 of any study looking to do so.
592

593 **LIST OF ABBREVIATIONS**

594 PLCG2, phospholipase C gamma 2
595 AD, Alzheimer's disease

596 Aβ, amyloid-beta

597 TREM2, triggering receptor expressed on myeloid cells 2

598 TLR, toll-like receptor

599 PIP2, phosphatidylinositol (4,5) bisphosphate

600 DAG, diacylglycerol

601 IP3, inositol 1,4,5 triphosphate

602 NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells

603 LPM, large peritoneal macrophage

604 SPM, small peritoneal macrophage

605 WT, wild-type

606 PBS, phosphate buffered saline

607 FMOC, fluorescence minus one control

608 MSD, MesoScale Discovery

609 LLOD, lower limit of detection

610 SEM, standard error of the mean

611 MHCII, major histocompatibility complex II

612 LPS, lipopolysaccharide

613 Vegfa, vascular endothelial growth factor A

614 DEG, differentially expressed gene

615

616 **DECLARATIONS**

617 **Ethics approval and consent to participate**

Mice were utilized in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Florida.

Consent for publication

Not applicable.

Availability of data and materials

All data are included in the present study or contained within the supplemental files. Raw data can be provided upon request.

Competing Interests

The authors declare no conflicts of interest.

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Authors Contributions

HAS and MGT conceived the study. HAS designed the experiments, performed them, and analyzed and interpreted the data. MLB, RLW, JJ, KBM, CC, NN, and AMT assisted with various experimental procedures. HAS designed all figures. HAS and MGT wrote and edited the manuscript. All authors approved the final version of the manuscript and figures.

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