Simvastatin Therapy Attenuates Memory Deficits that Associate to Brain Monocyte Infiltration in Chronic Hypercholesterolemic ApoE$^{-/-}$ Mice

Short running title: Monocytes and memory deficits in hypercholesterolemia.

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Abstract

Aims: Metabolic and cardiovascular disease is the most prevalent disease burden in the developed world and risk factors for progressive cognitive decline. Effective treatments for cognitive deficits emanating from cardiovascular disease and its risk factors are missing due to limited understanding of underlying mechanisms. Several lines of evidence suggest association of cardiovascular risk factors to unfavorable immune system activation, neuro-inflammation and cognitive decline. Interestingly, cardiovascular therapeutics (e.g., statins and anti-hypertensive drugs) possess immune-modulatory functions in parallel to their principle cholesterol- or blood pressure-lowering properties. How their ability to modify immune responses affects cognitive function is unknown.

Methods and Results: By using multi-color flow cytometry, Elisa, qPCR, Western blotting and novel object recognition tasks, we examined the effect of chronic hypercholesterolemia on inflammation and memory function in Apolipoprotein E (ApoE) knockout mice and aged-matched wild-type (WT) controls. Chronic hypercholesterolemia associated to apparent immune system activation characterized by higher proportions of circulating pro-inflammatory Ly6Chi monocytes across different ages. This persistent low-grade immune activation facilitates the infiltration of pro-inflammatory Ly6Chi monocytes into the brain of aged ApoE$^{-/-}$ but not WT mice, potentially promoting the development of memory dysfunction associated to chronic hypercholesterolemia. Long-term therapeutic administration of an FDA-approved cholesterol- lowering drug (i.e. simvastatin) significantly reduced systemic and neuro-inflammation, and the occurrence of memory deficits in aged ApoE$^{-/-}$ mice.

Conclusion: Our study strongly suggests a causative link between chronic hypercholesterolemia, myeloid cell activation and neuro-inflammation with memory impairment. Cholesterol-lowering therapy provides

effectiveness to attenuate memory impairment and inflammatory events and hence, emerges as safe therapeutic strategy to combat hypercholesterolemia-associated memory decline. Moreover, our results support the notion that chronic rather than late-life exposure to cardiovascular risk factors contributes to the development of cognitive dysfunction in the elderly.

Translational Perspective: Our study links chronic hypercholesterolemia in mice to specific immune responses that may underlie the development of memory impairment. We describe favorable outcomes in respect to neuro-inflammation and memory function in chronically hypercholesterolemic mice after statin therapy. Thus, our study (**1**) could open the door for available CVD therapeutics with long-term safety profiles to managing cognitive dysfunction in patients suffering from CVD, and (**2**) stimulate targeted monitoring of the inflammatory signature in patients with high cardiovascular burden since it could be a surrogate biomarker of cognitive decline.

Keywords: chronic hypercholesterolemia, immune activation, pro-inflammatory monocytes, neuro-inflammation, memory impairment

Introduction:

Cognitive decline is an increasingly common problem that progresses with age. By gradually interfering with daily functioning and well-being, it poses an enormous burden on affected people and their environment.[1, 2] In recent years, the apparent role of cardiovascular risk factors as important modifiable elements in the development of cognitive decline has become subject of clinical as well as pre-clinical research efforts.[2-4] Over the past two decades, evidence accumulated suggesting a prominent link between mid-life chronic hypercholesterolemia and/or hypertension and the development of dementia later in life.[5, 6] As a consequence, the management of cardiovascular risk factors not only serves to preventing often detrimental acute consequences of for instance, hypertension or dyslipidemia but also to minimize potential adverse cognitive outcomes later on. A number of observational and randomized studies reported beneficial effects of antihypertensive therapies on cognitive outcome.[7-9] Nonetheless, existing distinctions regarding effectiveness between different classes of hypertension medication have been shown,[7] and longitudinal studies suggested risk-reducing effects of anti-hypertensive treatment rather than an efficacy to slowing or treating manifested hypertension-associated cognitive impairment.[10] Likewise, the use of statins in controlling risk factors for dementia prevention or treatment is controversially discussed. Their therapeutic efficacy that initially emerged from findings showing its positive effects on various factors related to memory function,[11, 12] such as brain derived neurotrophic growth factor (BDNF)[13, 14] was increasingly argued as case studies raised concerns regarding a contributory role of statins in development of cognitive problems.[15, 16] Due to the discrepancies observed between studies and the lack of knowledge regarding underlying mechanisms, strategies to combat cognitive impairment resulting from cardiovascular risk factors or cardiovascular disease (CVD) are yet to be established.

Immune system alterations and inflammation are not only hallmarks of many CVDs and their risk factors but have increasingly been recognized as contributors to impaired cognitive function in older people.[17-19] Pharmaceuticals directed against inflammation evidently improve cognitive alterations associated to normal aging, CVDs, and classical neurodegenerative diseases.[20, 21] Moreover, multiple drugs commonly used in the cardiovascular field possess immune-modulatory actions in parallel to their principle cholesterol- or blood pressure (BP)-lowering effects.[22, 23] How this ability of CVD therapeutics to modify immune responses affects cognitive function is unknown. One of the bottlenecks hampering the targeted use of CVD therapeutics as approach to minimize CVD-associated cognitive impairment is the lacking understanding of specific inflammatory signatures that relate to cognitive impairment in patients with increased cardiovascular burden.

The herein presented study investigated the effect of chronic exposure to cardiovascular risk factors on systemic and neuro-inflammation and memory function in ageing mice. Moreover, we tested the efficacy of CVD therapeutics as treatment for memory impairment associated to the increased cardiovascular burden in

aged Apolipoprotein E knockout (ApoE$^{-/-}$) mice. To this end, we therapeutically administered the antihypertensive agent hydralazine, known to reduce leukocyte migration in spontaneously hypertensive rats,[24] and/or the statin simvastatin that has been attributed a strong anti-inflammatory character by regulating proliferation and activation of macrophages.[25, 26] Our findings underscore a causative role of myeloid cell activation in the development of neuro-inflammation associated to chronic hypercholesterolemia as well as cell type-specific drug effects that may limit their efficacy.

Material and Methods:

An expanded Methods section is available in the online Data Supplement.

Materials: All chemical reagents and solutions were purchased from Fisher Scientific (Göteborg, Sweden), Saween & Werner (Limhamn, Sweden) or Sigma-Aldrich (Stockholm, Sweden) unlike otherwise stated. Commercially available primary antibodies against CD3 (Bio-techne, Abindon, UK), Ly6C, NeuN and BDNF (Abcam, Cambridge, UK), CD68 (Nordic Biosite, Täby, Sweden) were used for immunofluorescence. Secondary antibodies Alexa Fluor donkey anti-mouse, anti-rabbit (Life Technologies, Stockholm, Sweden) or Fluor 594 (Biolegend/Nordic Biosite, Täby, Sweden) were used for visualization. Primers for qPCR were purchased from Eurofins (Ebersberg, Germany).

Animals: This investigation conforms to the Guide for Care and Use of Laboratory Animals published by the European Union (**Directive 2010/63/EU**) and with the ARRIVE guidelines. All animal care and experimental protocols were approved by the institutional animal ethics committees at the University of Barcelona (CEEA) and Lund University (5.8.18-12657/2017) and conducted in accordance with European animal protection laws. Wild-type (WT) C57Bl/6J mice and Apolipoprotein E knockout (ApoE$^{-/-}$) mice (B6.129P2-Apoe (tm1Unc)/J) were obtained from Jackson Laboratories and bred in a conventional animal facility under standard conditions with a 12h:12h light-dark cycle, and access to food (standard rodent diet) and water *ad libitum*. Mice with a body weight BW $\geq$ 25g were housed in groups of 4-5 in conventional transparent polycarbonate cages with filter tops. At the age of 12 months, animals were randomly assigned to the following experimental groups using the computer software *Research Randomizer* (Research Randomizer (Version 3.0) retrieved from http://www.randomizer.org/): aged control, aged + hydralazine (25 mg/L), aged + simvastatin (2.5 mg/L), and aged + hydralazine/simvastatin combination. The calculated doses given to the mice were 5 mg/kg/d for hydralazine and 0.5 mg/kg/d for simvastatin, which corresponds to 10 mg/kg/d and 20 mg/kg/d in humans, respectively (allometric scaling was used to convert doses amongst species[27]). Treatment was administered via drinking water over a course of two months. ApoE$^{-/-}$ mice at the age of four months, and C57Bl/6J (WT) mice at the age of 4 and 14 months were used as controls. To ensure blinding behavioral assessment was performed after the animals had received codes that did not reveal the identity of the treatment. In order to obey the rules for animal welfare, we designed experimental groups in a way that minimizes stress for the animals and guarantees maximal information using the lowest group size possible when calculated with a type I error rate of $\alpha = 0.05$ (5%) and Power of $1-\beta > 0.8$ (80%) based on preliminary experiments.

Open Field testing: To test novel environment exploration, general locomotor activity, and screen for anxiety-related behavior mice were subjected to an open field exploration task using a video tracking system and the computer software EthoVision XT® (Noldus Information Technology, Wangeningen, Netherlands) as described before.[20] Mice were placed individually into an arena (56 × 56 cm), which was divided into a grid of equally sized areas. The software recorded each line crossing as one unit of exploratory activity. The following behavioral parameters were measured: activity, front to back count, active time, mobile time, slow activity, mobile counts, and slow mobile counts.

Novel object recognition (NOR): As previously described,[3, 20] a NOR task was employed to assess non-spatial memory components. Briefly, mice were habituated to the testing arena for 10 minutes over a period of 3 days. On test day, each mouse was exposed to two objects for 10 minutes. 5 min or 24 hrs later, mice were re-exposed to one object from the original test pair and to a novel object. The movements of the animal were

video tracked with the computer software EthoVision XT® (Noldus Information Technology, Wangeningen, Netherlands). A delay interval of 5 minutes was chosen to test short-term retention of object familiarity, and with a delay interval longer of 24 hrs, we tested long-term hippocampus-dependent memory function.[3, 28] A recognition index (RI) as the main index of retention was calculated by the time spent investigating the novel object relative to the total object investigation [RI = $T_{Novel}/(T_{Novel} + T_{Familiar})$].

Object placement task: To test spatial recognition memory, mice were placed individually into an arena (56 × 56 cm). Each mouse was exposed to two objects for 10 minutes. 5 min or 24 hrs later, mice were re-exposed to both objects, of which one remained in the original place and the second object was moved to a novel place. Exploration of the objects was assessed manually with a stopwatch when mice sniffed, whisked, or looked at the objects from no more than 1 cm away. The time spent exploring the objects in new (novel) and old (familiar) locations was recorded during 5 min. A location index was calculated RI = $T_{Novel}/(T_{Novel} + T_{Familiar})$, where T_{novel} is the time spent exploring the displaced object and $T_{familiar}$ is the time spent exploring the non-displaced object.

Fluorescence activated cell sorting: Before euthanasia through cervical dislocation, mice were sedated using inhalation anesthesia (isofluorane 2.5% at 1.5L/min in room air) for whole blood collection. Whole blood was collected in EDTA coated tubes and red blood cells were lysed before samples were incubated in F_c block solution; FACS buffer (PBS + 2 % FBS + 2 mM EDTA; pH 7.4) + anti-CD16/CD32, for 15 minutes followed by primary antibodies for 30 minutes at 4°C. After centrifugation, the supernatant was decanted, washed, and re-suspended in FACS buffer. Brain tissue was enzymatically digested and homogenized. After density separation using Percoll (GE Healthcare), pellets were reconstituted in F_c block and incubated for 15 minutes prior to staining with antibodies for 30 min (see data supplement for detailed list of antibodies). Data acquisition was carried out in a BD LSR Fortessa cytometer using FacsDiva software Vision 8.0 (BD Biosciences). Data analysis was performed with FlowJo software (version 10, TreeStar Inc., Ashland, OR, USA). Cells were plotted on forward versus side scatter and single cells were gated on FSC-A versus FSC-H linearity (see data supplement for gating strategy).

Cell culture: Bone-marrow-derived macrophages (BMDMs) were generated by culturing freshly isolated mouse bone marrow cells from C57BL/6J mice in IMDM medium (GIBCO, Life Technologies) supplemented with 10% FCS (GIBCO, Life Technologies), 100 U/ml penicillin (Sigma-Aldrich), and 100 U/ml streptomycin (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich) and 20% (v/v) L929 conditional medium for 7 days. Human monocytic THP-1 cells (ATCC #TIB-202) were cultured in RPMI-1640 containing 10% fetal bovine serum, 0.05 mM β-mercaptoethanol, and 1 % penicillin-streptomycin in large culture flasks. Cell cultures were maintained at 37°C with 5 % CO_2 and split 1:4 at a seeding density of 10^6 cells. For differentiation, THP-1 cells were seeded in 6-well plates at a density of $3x10^5$ cells and treated with 2.5 ng/ml phorbol-12-myristate-13-acetate (PMA) for 48 hrs. Prior to lipopolysaccharide (LPS) activation and treatment, cells were allowed to rest in culture media for 24 hrs. Human peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE healthcare) density gradient centrifugation from donated blood of healthy volunteers. After centrifugation, the PBMC layers were isolated and washed with PBS to remove erythrocytes and granulocytes. PBMCs purification was performed using anti-human CD14 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. Monocytes were maintained in RPMI 1640 (Gibco, Thermo Scientific) with 10 % FCS (Gibco, Thermo Scientific), 2 mM glutamine (Gibco, Thermo Scientific), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Scientific). For macrophage differentiation and maturation, human monocytes were plated onto tissue culture-treated 24-well plates at a density of $3×10^5$ cells/well. The medium was supplemented with 100 ng/mL recombinant human M-CSF using CellXVivo™ kit (R&D Systems) and differentiated for 5–7 days prior to use.

Cells were incubated with 1 µg/ml LPS (Invitrogen, Stockholm, Sweden) for 6 hrs prior to a 12 hrs treatment with 1 µM simvastatin (Bio-techne, Abindon, UK), 10 µM hydralazine (Sigma-Aldrich) and/or a combination of both. The concentrations were chosen based on the dosing regimen administered in the *in vivo* studies. After

incubation, cells were detached using cold PBS containing EDTA (5 mM) and either stained for flow cytometry or centrifuged and processed for RNA and protein isolation using the Trizol method.

Western Blotting, qPCR, ELISA and histological experiments: Standard biochemical procedures were utilized for experiments involving reverse transcription polymerase chain reaction, quantitative PCR, Western blotting and histological experiments. Methodological details are provided in the data supplement.

Statistical analysis: All data are expressed as mean ± SEM, where n is the number of animals. For comparison of multiple independent groups, the parametric one-way ANOVA test was used, followed by Tukey's post hoc test with exact P value computation as post hoc test. For comparison of two groups a two-tailed unpaired t-test was utilized. Differences were considered significant at error probabilities of $P \leq 0.05$.

Results

1. Chronic hypercholesterolemia contributes to the development of memory impairment: To investigate whether exposures to different cardiovascular risk factors affect important brain functions (i.e. cognition) during ageing, we tested memory function in WT and ApoE$^{-/-}$ mice at different ages (4 and 12 months; Fig.1A). In accordance with the increased cardiovascular burden in ApoE$^{-/-}$ compared to WT mice (i.e. significantly higher plasma cholesterol levels at 4 and 12 months of age, significantly increased BP at 12 months of age; **Table 1**), long-term (hippocampus-dependent) memory function deteriorated with age in ApoE$^{-/-}$ mice, resulting in significantly lower recognition indices (RI) compared to age-matched WT controls (Fig.1B). Although neither aged WT nor aged ApoE$^{-/-}$ mice presented with signs of short-term (rhino-cortical) memory impairment when compared to their young controls (Table S1), spatial short-term memory was compromised in aged ApoE$^{-/-}$ mice as evident by a lower RI obtained in an object placement test (Fig.1C). Taken together, these data imply negative effects of early chronic hypercholesterolemia on memory function later in life.

2. Pro-inflammatory monocytes infiltrate the brain of aged ApoE$^{-/-}$ but not WT mice: In chronically hypercholesterolemic ApoE$^{-/-}$ mice, ageing associated apparent immune system activation as evident by a higher number of circulating Ly6Chi monocytes (Fig.2A) and elevated plasma levels of interleukin (IL)12/23 (Fig.2B). The latter often secreted by activated monocytes/macrophages promote Th1 and Th17 priming of T-cells,[29] which secrete IL17 amongst other cytokines and are considered main contributors to tissue inflammation.[30] Elevated IL17 plasma levels in aged ApoE$^{-/-}$ mice (Fig.2C) led us to test immune cell infiltration into brain tissue. FACS analyses revealed an increase of Ly6Chi pro-inflammatory monocytes (Fig.2D) and an overall higher percentage of CD3+ cells in brain tissue of aged vs. young ApoE$^{-/-}$ mice (Fig.2E). In line with previous publications,[31] transcripts of key chemokines that regulate migration and infiltration of monocytes and macrophages, such as MCP-1 and CXCL2 were drastically elevated in brain tissue of the aged cohort compared to young control mice (**Table 2**). Moreover, an mRNA expression pattern typical of the pro-inflammatory M1 phenotype was predominant in brain tissue of aged ApoE$^{-/-}$ mice (**Table 2**: IL-6 (1.5-fold), Nos2 (3.1-fold), TNF-α (7.9-fold), and IL-12 (5.1-fold)) compared to young ApoE$^{-/-}$ controls.

Although WT mice presented with an age-related increase of circulating Ly6Chi pro-inflammatory monocytes (2.49± 0.31 vs. 1.37 ± 0.08, P<0.0001; Fig.S1), the percentage of Ly6Chi monocytes remained unaltered in the brain of aged WT mice (Fig.S2). Notably compared to WT mice, ApoE$^{-/-}$ mice already showed a significantly higher proportion of circulating Ly6Chi cells at young age (Fig.2F), suggesting a persistent pro-inflammatory state in chronically hypercholesterolemic mice, which may promote immune infiltration into the brain with implications for memory function. Correspondingly, the proportion of brain Ly6Chi monocytes was higher in aged ApoE$^{-/-}$ mice compared to age-matched WT mice (Fig.2G). Furthermore, brain tissue of aged ApoE$^{-/-}$ mice presented with significantly higher transcripts levels of chemokines MCP-1 and CXCL2 compared to aged-matched WT controls (**Table 3**). Taken together, these data suggest a key role for Ly6Chi monocyte activation and brain infiltration during chronic hypercholesterolemia. Interestingly, memory deficits were only observed in aged ApoE$^{-/-}$ mice.

3. Cholesterol- and/or BP-lowering therapy attenuates memory deficits in aged ApoE$^{-/-}$ mice. In order to assess the effect of cholesterol- and/or BP-lowering therapy on memory function, we subjected aged ApoE$^{-/-}$ mice to lipid-lowering and/or antihypertensive therapy for eight consecutive weeks, using the statin simvastatin (5 mg/kg/d), the muscle relaxant hydralazine (0.5 mg/kg/d), or a combination of the two. The doses administered correspond to therapeutic doses used in the clinic (10 mg/kg/d and 20 mg/kg/d, respectively). All treatment strategies proved similarly effective in lowering the age-related elevated systolic BP (Table S1). As expected, simvastatin treatment revealed significant cholesterol-lowering effects (Table S2). In line with other investigations,[32, 33] simvastatin treatment markedly reduced the age-related elevated heart-to-body weight ratio in our model without significantly affecting heart rate (HR) (Table S2). Interestingly, simvastatin's effects on heart hypertrophy and cholesterol were diminished in the presence of hydralazine (Table S2). Simvastatin revealed considerable anti-inflammatory capacity as evident by a reduction of circulating plasma IL12/23 levels (Fig.3A) and IL17 levels *in vivo* (Fig.3B). In the brain, the age-related increase of CD68 was significantly lower only in groups treated with simvastatin (Fig.3C). In accordance, brain tissue of simvastatin-treated mice revealed lower transcript levels of M1 markers, pro-inflammatory cytokines and chemokines, including IL6 and IL12, MCP-1 and CXCL2 (**Table 2**). Notably, hydralazine attenuated simvastatin's effects for some of the cytokines when administered in combination (**Table 2**). The age-related accumulation of CD3 was diminished in all treatment groups (Fig.3D).

In an open field test, simvastatin treatment significantly improved age-related impaired mobility in ApoE$^{-/-}$ mice (Fig.4A). Combination treatment with hydralazine revealed no apparent effects on mobility; yet, statistical analysis disclosed no difference to young control. Likewise, spatial short-term memory function (Fig.S3) and hippocampal-dependent memory function significantly improved in all treatment groups containing simvastatin (Fig.4B). The spatial component of long-term memory, however, was only improved in the group where simvastatin was given alone (Fig.4C). BDNF, an important regulator of white matter integrity, hippocampal long-term potentiation and consequently, learning and memory,[34] was negatively affected in aged ApoE$^{-/-}$ mice: chronic hypercholesterolemia associated to a lower brain BNDF mRNA expression compared to young controls (Fig.4D). Simvastatin but not hydralazine treatment increased BDNF expression (Fig.4D). Similarly, immune staining of hippocampal BDNF and the neuronal marker NeuN revealed evident differences between young and aged mice (Table S3, representative images shown in Fig.4E*i-v*). When normalized to the number of NeuN+ cells, BDNF expression was significantly lower in aged brains compared to young ApoE$^{-/-}$ mice (1.377 ± 0.054 vs. 0.781 ± 0.027; $P = 0.0286$), and only significantly higher after simvastatin treatment (Fig.4E).

3. Simvastatin and hydralazine exert cell-specific effects. The observed differences of simvastatin and hydralazine responses *in vivo*, led us to evaluate their effects *in vitro.* Utilizing human monocytic cells (THP-1) and freshly isolated human monocytes allowed us to assess drug effects not only in monocytes but also in monocyte-derived macrophages. In both cell systems, hydralazine significantly induced cell activation (i.e. increased CD69 expression) that affected monocytes with a higher magnitude than macrophages (2.0-fold vs. 1.3-fold and 1.8-fold vs. 1.2-fold in THP-1 cells and primary cells, respectively; Fig.5A*i-iv*). Simvastatin presented with no appreciable effects on CD69 expression (Fig.5B*i-ii*). Similar to CD69, hydralazine but not simvastatin increased monocyte and macrophage CD3 expression (Fig.S4), which has been shown to involve in the delivery of pro-inflammatory cytokines (i.e. tumor necrosis factor alpha, TNF-α) by macrophages.[35] Pro-inflammatory cytokine profiling in THP-1 cells revealed that LPS-induced augmentation of TNF-α protein expression only reversed with simvastatin but not hydralazine (Fig.5C). Interestingly, LPS-induced increases of intracellular IL6 protein abundance was not changed by simvastatin treatment but exacerbated in the presence of hydralazine (Fig.5D*i-v*). Hydralazine alone (without LPS pre-stimulation) disclosed similar pro-inflammatory effects (Fig.5E*i-iv*). Different from monocytic cells, LPS-induced IL6 expression was similarly reduced in the presence of simvastatin or hydralazine in PMA-differentiated THP-1 cells (Fig.S5), further indicating cell type-specific drug responses. In support of this, LPS-induced elevation of TNF-α and IL6 secretion in murine bone-marrow-derived macrophages (BMDMs) reduced with both simvastatin and hydralazine treatment (Fig.5F&G). Moreover, we detected lower surface expression of activation markers (i.e.,

CD86, CD80) in BMDMs of all treatment groups (Fig.S6). Additional flow cytometric analyses of monocytic or PMA-differentiated THP-1 cells revealed a strong pro-inflammatory effect of hydralazine in monocytic cells (i.e. augmentation of LPS-associated CD14+ CD16+ surface expression; 2.5-fold; Fig.S7) compared to PMA-differentiated THP-1 cells. Interestingly, none of the drugs showed effects on T-cell activation (Fig.S8). Together, these data suggest cell type-specific drug effects that may benefit simvastatin as it exerted anti-inflammatory effects in both monocytic cells and monocyte-derived macrophages. Hydralazine on the other hand, induced pro-inflammatory signatures especially in monocytic cells with potential implications for conditions characterized by elevated monocyte counts like chronic hypercholesterolemia, which may explain some of the effects we observe *in vivo*.

Discussion

In this present study, we provide compelling evidence that chronic hypercholesterolemia majorly contributes to the development of memory impairment, involving pro-inflammatory processes. Our data imply that mainly the modulation of monocyte activation and thus, the stimulation of related pro-inflammatory signaling mediators critically determine the effectiveness of CVD therapeutics for improving impaired memory function associated to chronic hypercholesterolemia and accompanying hypertension. Although our results indicate a link between hypercholesterolemia and hypertension, our data suggest that cholesterol-lowering rather than anti-hypertensive therapy effectively improves memory function in aged ApoE$^{-/-}$ mice.

The herein established link between chronically elevated plasma cholesterol and the development of memory impairment in aged mice is based on findings, showing that only early exposure to elevated plasma cholesterol, indicative of chronic hypercholesterolemia, results in reduced long-term and spatial memory function at an older age. This conclusion aligns with data obtained in epidemiological studies supporting a correlation between early- or mid-life cholesterol levels to dementia in later life.[36, 37] Similar to our findings obtained in aged WT mice, no association between cognitive decline and hypercholesterolemia was found in studies examining cholesterol levels in cohorts of older patients.[38, 39] Although hypercholesterolemia was generally accompanied by higher than normal BP in our mouse model, cholesterol-lowering rather than BP-lowering therapy significantly improved the impaired spatial memory of aged ApoE$^{-/-}$ mice. This further positions early exposure to cholesterol as major contributor to cognitive dysfunction in addition to potential hypertension-mediated structural and functional alterations in the brain.[3] It needs to be noted that unlike statins for dyslipidemia treatment, hydralazine is not the first line medication for treating hypertension. Nonetheless, hydralazine appears as first choice of BP-lowering therapy in numerous experimental studies where it has mostly been used preventatively to investigate the link between hypertension and inflammation or cognitive function,[3, 40, 41] and is often used to treat preeclampsia. Besides its vasorelaxant properties, its exact effects on the immune system remain to be determined. A few reports have assigned hydralazine with anti-inflammatory properties, resulting from reduced ICAM- and P-selectin- mediated T-cell transmigration[24] and induced T-cell apoptosis.[42] However, hydralazine-induced increases of cellular CD3-zeta chain content in T-cells are discussed to facilitate excessive drug-induced auto-immunity.[43] The pronounced enhancement of monocytic cell activation we observed in the presence of hydralazine might be resultant from reactive metabolites deriving from hydralazine oxidation[44] or from hydralazine-mediated inhibition of DNA methyltransferase,[45] which are postulated as major mechanisms in drug-induced auto-immunity,[44] and might limit its potency to resolve neuro-inflammatory processes and thus, improve memory deficits in our model. Moreover, it might limit therapeutic efficacy of other drugs when administered in combination. This effect calls for the need to test for potential interactions between BP lowering drugs of different nature and statins for potential consequences on immune regulation and cognitive impairment.

The activation of the immune system emerged as an important driver in the aged and vulnerable brain. Experimental studies verified the significance of inflammation in CVD progression[46, 47] and showed that CVD-associated inflammation negatively affects cognitive function.[20, 21, 48] However, precise pro-inflammatory mechanisms contributing to accelerated cognitive decline and dementia risk require further elucidation. An

experimental study by Dinh et. al convincingly associates high fat diet-induced hypercholesterolemia in aged ApoE$^{-/-}$ mice not only to elevated plasma levels of IL6, TNF- α, and INF-γ but also higher transcript levels of pro-inflammatory chemokine MCP-1 in brain tissue of these mice.[31] In our study, we observe a similar increase of such key chemokines responsible for regulating migration and infiltration of monocytes in brain tissue of standard chow-fed aged ApoE$^{-/-}$ but not WT mice. Consequently, the accumulation of pro-inflammatory Ly6Chi monocytes in the brain of chronically hypercholesterolemic aged ApoE$^{-/-}$ mice was absent in age-matched WT mice where plasma cholesterol levels only increase later in life. Thus, our findings position pro-inflammatory monocytes as important contributors to neuro-inflammation emanating from chronic hypercholesterolemia.

Our herein presented experimental strategy demonstrates that simvastatin therapy proves effective in reducing neuro-inflammation associated to chronic hypercholesterolemia in aged ApoE$^{-/-}$ mice. Simvastatin's anti-inflammatory character might be resultant from its potency to regulating proliferation and activation of monocytes and macrophages,[22, 25] and to modulating myeloid cell phenotype[49] and secretory function.[50, 51] Monocyte-derived immature dendritic cells and type 1 macrophages respond to simvastatin-induced down-regulation of MCP-1,[52] which may impact their chemotactic activity and thus, reduce their recruitment to sites of tissue injury. The herein presented *in vitro* findings describe simvastatin's direct therapeutic effect on monocyte and macrophage activation and extent previous reports that showed an effective reduction of cytokine secretion in PMA-differentiated THP-1 cells pre-treated with simvastatin.[50] In its role as modulator for IL secretion from monocytes and macrophages, simvastatin was shown to indirectly inhibit IL17 secretion from CD4+ T-cells.[53] These findings are in line with our herein presented data showing a reduction of IL17 plasma levels *in vivo* only after simvastatin treatment. Moreover, simvastatin has been shown to directly inhibit the expression of a transcription factor responsible for controlling IL17 production in CD4+ T-cells.[53] Because simvastatin has been associated to Th2 immune response and neuronal recovery[54, 55] we cannot exclude a potential therapeutic effect on T-cells in our model. Nonetheless, our results point to therapeutic effects through monocyte modulation, which aligns with studies conducted in patients at high risk for vascular events where a simvastatin-induced down-regulation of the angiotensin II type 1 receptor on monocytes but not T-cells significantly affected angiotensin II activity and thus, contributes to its anti-inflammatory profile and therapeutic effects in CVD.[56] Moreover, in patients with hypercholesterolemia, simvastatin was shown to reduce monocyte secretory function.[51, 57] Most interestingly in respect to neurodegeneration is a study reporting a lowering of the CD14+CD16+ "intermediate" monocyte subset in purified monocytes isolated from peripheral blood of HIV patients in response to an *ex vivo* treatment with simvastatin, as this particular subset of monocytes is closely linked to HIV-associated neurocognitive disorders.[49]

Despite the availability of case reports discussing adverse effects of statins on cognitive function,[15, 16] our study describes favorable outcomes for spatial memory function in aged ApoE$^{-/-}$ mice after statin therapy. Although lipophilic statins, such as simvastatin are thought to cross the blood–brain barrier, leading to a reduction of cholesterol availability and thereby, disturbing the integrity of the neuronal and glial cell membrane,[58] simvastatin treatment had no negative effects on hippocampal neurons, and significantly increased the BDNF expression in our model. However, it has been reported that hydrophilic statins exert negative effects on learning and recognition memory, which is reversed upon discontinuation.[59] In contrast to the published case reports showing statin-induced memory loss,[15, 16] two large randomized control trials on simvastatin[60] and pravastatin[61] did not identify a relationship between statin use and cognitive decline. Most beneficial effects of simvastatin in the brain are thought to root from the promotion of hippocampal neurogenesis, the inhibition of mesangial cell apoptosis,[62, 63] the enhancement of neurotrophic factors, and the restriction of inflammation.[64] Albeit not having increased the number of NeuN+ cells in hippocampus or cortex, simvastatin treatment markedly increased BDNF expression, which has been linked to improved functional recovery after stroke and the amelioration of depressive-like behavior.[13, 14]

Taken together, our study convincingly links chronic hypercholesterolemia to myeloid cell activation, neuro-

inflammation and memory impairment, supporting the notion that early rather than late-life exposure to cardiovascular risk factors promotes the development of cognitive dysfunction. Cholesterol-lowering therapy provides effectiveness to improving memory function potentially by reducing monocyte-driven inflammatory events and hence, emerges as safe therapeutic strategy to combat CVD-induced memory impairment.

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Conflict of Interest: None declared.

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Tables

Table 1: Hemodynamic data and plasma cholesterol levels of WT and ApoE$^{-/-}$ mice. Systolic BP (BP sys) and heart rate (HR) were measured in conscious mice using tail cuff plethysmography. Heart – body weight (BW) ratio calculated from animal weight (g) and respective heart weight (mg). Cholesterol levels were determined in plasma obtained from anaesthetized mice. *Values are expressed as mean +/- SEM. N=10 per group; * P ≤ 0.05 relative to respective young by one-way ANOVA followed by Tukey's post hoc test; + P ≤ 0.05 relative to aged group of respective other group by one-way ANOVA followed by Tukey's post hoc test.*

	ApoE$^{-/-}$ young	ApoE$^{-/-}$ aged	WT young	WT aged
BP_{sys} (mmHg)	104.8 ± 2.8	133.2 ± 4.4*	99.4 ± 3.4	114.6 ± 1.7*
HR (bpm)	489.1 ± 9.1	557.2 ± 11.0*	458.9 ± 17.7	556.9 ± 11.3*
Cholesterol (mg/L)	98.3 ± 2.4	181.7 ± 6.1*	74.2 ± 0.8$^{\&}$	99.8 ± 2.6*$^{+}$
Heart BW ratio	5.03 ± 0.19	5.91 ± 0.26*	5.34 ± 0.13	5.48 ± 0.27

Table 2: Brain mRNA expression of pro-inflammatory cytokines and chemokines in ApoE$^{-/-}$ mice. mRNA expression of prominent pro-inflammatory markers was determined in brain tissue isolated from the different experimental groups using quantitative real-time PCR. *Values are expressed as fold versus the mean value of the young group. Values show the mean +/- SEM. N=5 mice per group; * P ≤ 0.05 relative to young control by one-way ANOVA followed by Tukey's post hoc test; & P ≤ 0.05 relative to aged group by one-way ANOVA followed by Tukey's post hoc test.*

target	young	aged	aged + hydralazine	aged + simvastatin	aged + hydralazine/simvastatin
IL12	0.98 ± 0.18	16.84 ± 2.29 *	5.74 ± 2.04 *$^{\&}$	1.39 ± 0.39 $^{\&}$	6.15 ± 2.48*
MCP-1	0.93 ± 0.19	9.11 ± 2.64 *	1.02 ± 0.13 $^{\&}$	1.32 ± 0.27 $^{\&}$	2.75 ± 0.53 *$^{\&}$
TNF-a	0.94 ± 0.09	7.33 ± 1.57 *	2.16 ± 0.38 *$^{\&}$	1.05 ± 0.28 $^{\&}$	2.19 ± 0.14 *$^{\&}$
IL6	1.14 ± 0.13	2.95 ± 0.15 *	2.130± 0.37 *	1.62 ± 0.15 $^{\&}$	2.17 ± 0.23 *
CXCL2	0.68 ± 0.17	29.32 ± 7.97 *	11.60 ± 7.85 *$^{\&}$	3.09 ± 2.15 $^{\&}$	31.55 ± 6.70 *
iNOS	1.00 ± 0.17	3.06 ± 0.79 *	2.38± 0.58 *$^{\&}$	1.89 ± 0.48 $^{\&}$	2.59 ± 0.62 *

Table 3: Brain mRNA expression of inflammatory markers in aged ApoE$^{-/-}$ versus aged WT mice. Values are expressed as fold change compared to aged WT mice. *N=5 per group.*

gene	Fold change (ApoE$^{-/-}$ vs. WT)
MCP-1	5.673
CXCL2	4.924
GFAP	1.969
CD68	2.927
IL6	5.507

Figure 1: ***Chronic hypercholesterolemia impairs memory function.*** **(A)** Overview illustrating experimental timeline. BP, memory function and immune status in blood and brain were tested at 4, 12 and 14 months of age. **(B)** Schematic illustration of novel object recognition task with 24-h delay interval. Recognition index (RI) describing hippocampus-dependent long-term memory function (N=6-10 per group). **(C)** Schematic illustration of an object placement test with a 5 min delay interval. RI describing spatial short-term memory function (N=8 per group). In **B, C** * $P \leq 0.05$ relative to young control of same genotype by one-way ANOVA followed by Tukey's *post hoc* test; & $P \leq 0.05$ relative to WT young control by one-way ANOVA followed by Tukey's *post hoc* test. Schematics illustrated using BioRender.

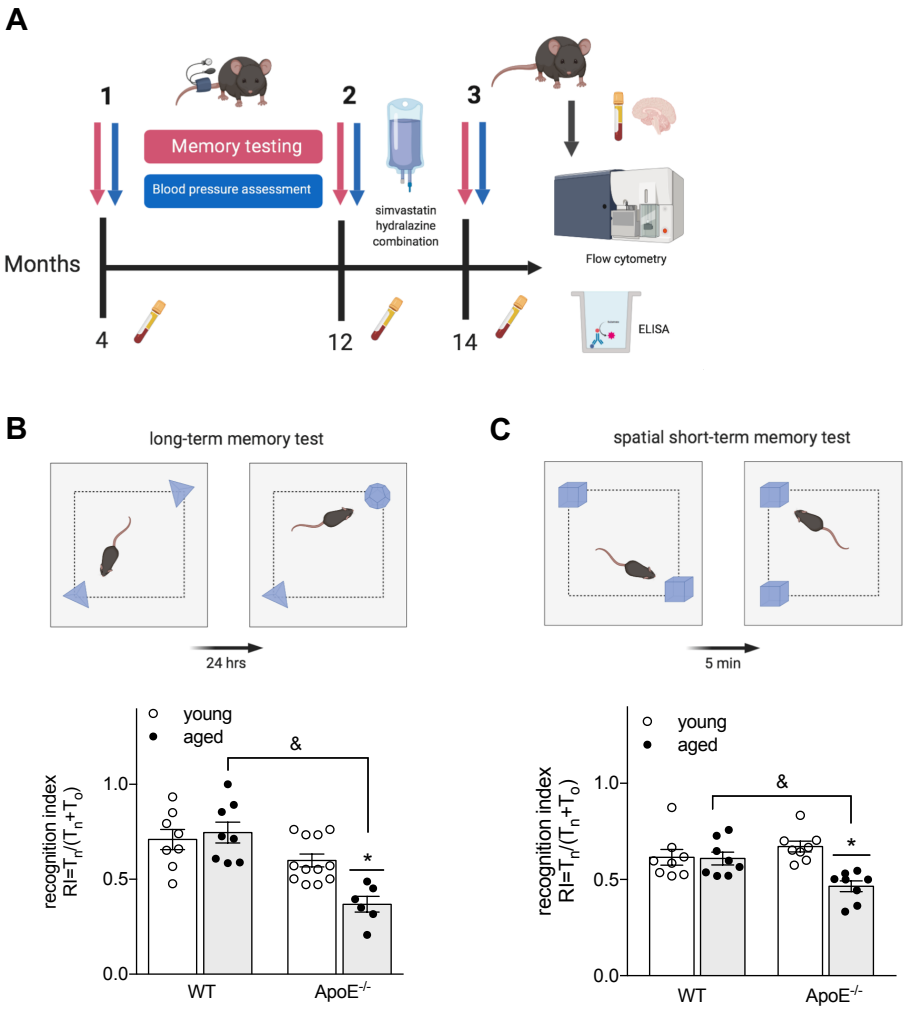

Figure 2: ***Immune system activation leads to leukocyte infiltration to the brain in aged ApoE$^{-/-}$ mice.*** **(A)** Percentage of circulating pro-inflammatory Ly6Chi monocytes determined by flow cytometry of young and aged ApoE$^{-/-}$ mice (N=10 per group) and representative images showing dot plots. **(B)** Plasma levels of pro-inflammatory IL-12/23 and **(C)** IL-17 of young and aged ApoE$^{-/-}$ mice (N=6-10 per group) determined by ELISA. **(D)** Percentage of Ly6Chi monocytes in brain tissue of young and aged ApoE$^{-/-}$ mice analyzed by flow cytometry (N=8-9 per group) and representative dot plots. **(E)** Percentage of CD3+ T-cells in brain tissue of young and aged ApoE$^{-/-}$ mice analyzed by flow cytometry (N=8-9 per group) and representative dot plots. **(F)** Percentage of circulating pro-inflammatory Ly6Chi monocytes in young WT and ApoE$^{-/-}$ mice (N=9-10 per group) determined by flow cytometry. **(G)** Percentage of Ly6Chi monocytes in brain tissue of aged WT and aged ApoE$^{-/-}$ mice (N=8 per group) analyzed by flow cytometry. In **A-G** * $P \leq 0.05$ by unpaired t-test.

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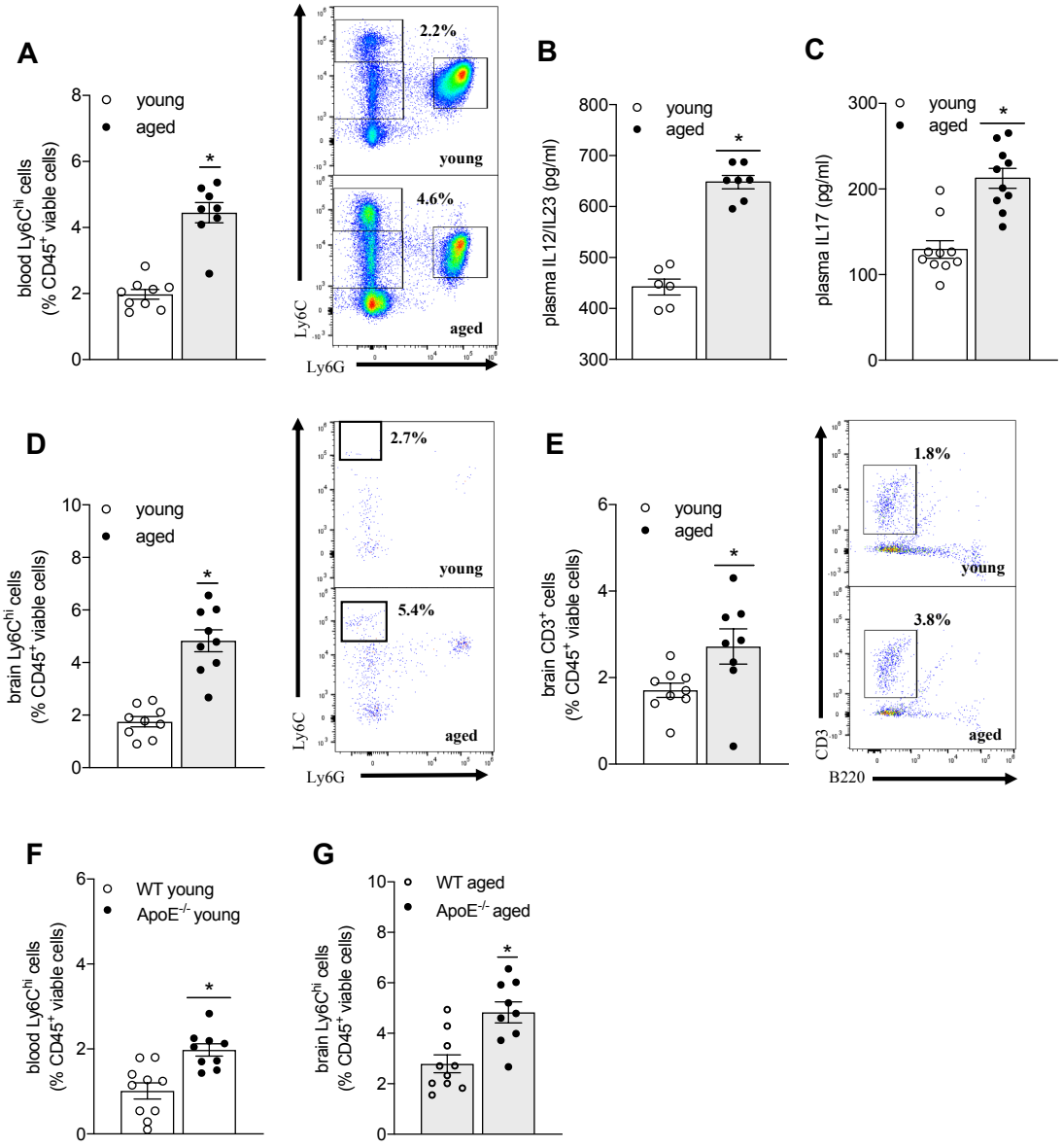

Figure 3: ***Monocytes infiltrate the brain of aged ApoE$^{-/-}$ mice but not aged WT mice.*** **(A)** Effect of cholesterol- and BP-lowering treatment on plasma levels of pro-inflammatory IL-12/23 (N=6-7 per group) and **(B)** IL-17 (N=4-10 per group) determined by ELISA. **(C)** Effect of cholesterol- and BP-lowering treatment on CD68 mRNA expression in whole brain lysates of aged ApoE$^{-/-}$ mice (N=8 per group). **(D)** Effect of cholesterol- and BP-lowering treatment on CD3 protein expression in whole brain lysates of aged ApoE$^{-/-}$ mice (N=4-5 per group). In **A, B, C** * $P \leq 0.05$ relative to young control by one-way ANOVA followed by Tukey's *post hoc* test; $^{\&}$ $P \leq 0.05$ relative to aged control by one-way ANOVA followed by Tukey's *post hoc* test; in **D** * $P \leq 0.05$ relative to control by one-way ANOVA followed by Tukey's *post hoc* test.

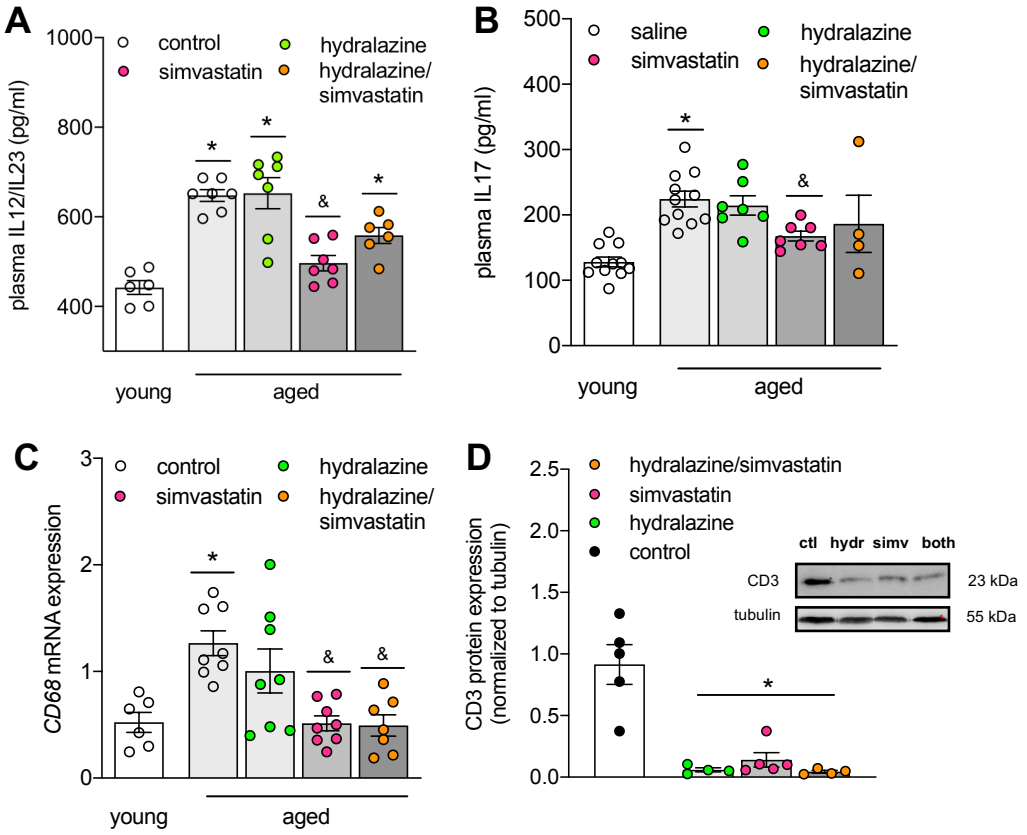

Figure 4: ***Simvastatin improves impaired memory function of aged ApoE$^{-/-}$ mice.*** **(A)** Quantification of open field test assessing mobility in aged ApoE$^{-/-}$ mice treated with hydralazine (green dots), simvastatin (pink dots) and a combination of both (orange dots) in comparison to young and aged control mice (black dots) (N=6-10 per group). Representative images showing (*i*) young control, (*ii*) aged control, (*iii*) aged + hydralazine, (*iv*) aged +simvastatin, and (*v*) aged + hydralazine/simvastatin. **(B)** Effect of lipid- and BP lowering treatment on long-term memory function (N=6-10 per group), and on **(C)** spatial long-term memory function (N=6 per group). **(D)** Treatment effects on BDNF mRNA expression in brain tissue (N=4-7 per group). Representative images of BDNF (green) and NeuN (red) immune staining showing the hippocampal CA2/3 region as illustrated in (*i*) HE-stained coronal brain section of an aged ApoE$^{-/-}$ mouse: (*ii*) aged control, (*iii*) aged + hydralazine, (*iv*) aged + simvastatin, and (*v*) aged + combined treatment. In **A-D** * $P \leq 0.05$ relative to young control by one-way ANOVA followed by Tukey's *post hoc* test and & $P \leq 0.05$ relative to aged control by one-way ANOVA followed by Tukey's *post hoc* test. Schematics illustrated using BioRender.

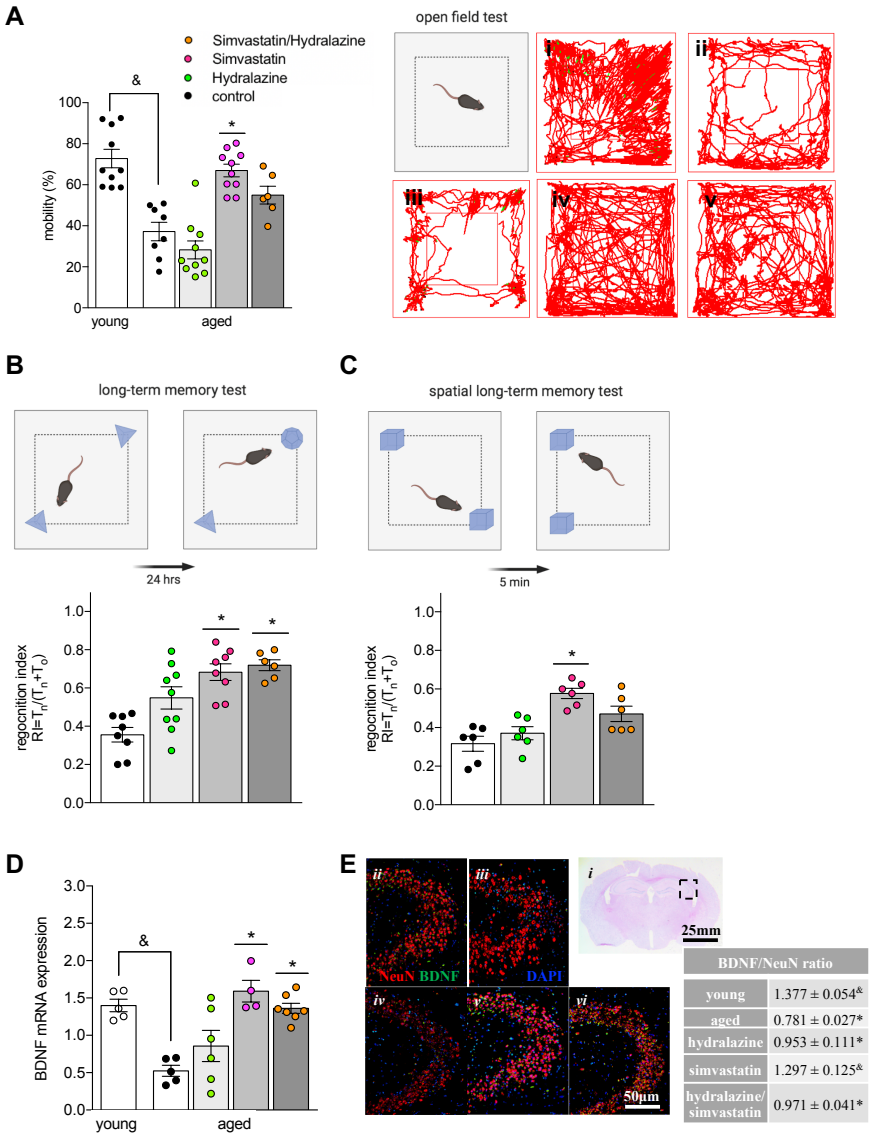

Figure 5: ***Hydralazine and simvastatin exert cell type-specific effects***. **(A)** Degree of myeloid cell activation in response to hydralazine determined by flow cytometric assessment of CD69 surface expression (expressed in fold change compared to control) in human monocytic THP-1 cells and PMA-treated monocyte derived macrophages compared to human primary monocytes and monocyte-derived macrophages (N=3 per group). Representative histograms showing changes in CD69 surface expression in (*i*) monocytic THP-1 cell, (*ii*) primary human monocytes, (*iii*) THP-1 macrophages and (*iv*) human monocyte-derived macrophages. **(B)** Degree of myeloid cell activation in response to simvastatin determined by flow cytometric assessment of CD69 surface expression (expressed in fold change compared to control) in human primary monocytes and monocyte-derived macrophages (N=3 per group). Representative histograms showing CD69 surface expression in (*i*) primary human monocytes and (*ii*) human monocyte-derived macrophages with and without simvastatin treatment. **(C)** Effect of hydralazine and simvastatin on LPS-induced TNF-a protein expression in human monocytic THP-1 cells (N=5 per group) determined by WB**. (D)** Effect of hydralazine and simvastatin on LPS-induced intracellular IL6 expression in human monocytic THP-1 cells (N=5 per group) determined by flow cytometry. Representative dot blots showing intracellular IL6 expression in (*i*) control cells and cells after (*ii*) LPS and subsequent (*iii*) hydralazine, (*iv*) simvastatin and (*v*) combination treatment. **(E)** Effect of hydralazine and simvastatin on intracellular IL6 expression in human monocytic THP-1 cells (N=5 per group) determined by flow cytometry. Representative dot blots showing intracellular IL6 expression in (*i*) control cells and cells after (*ii*) hydralazine, (*iii*) simvastatin and (*iv*) combination treatment. **(F)** Effect of hydralazine and simvastatin on LPS-mediated TNF-a release in murine bone marrow derived macrophages assessed by ELISA. **(G)** Effect of hydralazine and simvastatin on LPS-mediated IL6 release in murine bone marrow derived macrophages assessed by ELISA. In **A,** * $P \leq 0.05$ relative to respective control for unpaired t-test; in **C-G** * $P \leq 0.05$ relative to control by one-way ANOVA followed by Tukey's *post hoc* test; & $P \leq 0.05$ relative to LPS control by one-way ANOVA followed by Tukey's *post hoc* test.

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