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ORIGINAL ARTICLE

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Characterization of the long-term effects of lethal total body irradiation followed by bone marrow transplantation on the brain of C57BL/6 mice

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ABSTRACT

Purpose: Total body irradiation (TBI) followed by bone marrow transplantation (BMT) is used in pre-clinical research to generate mouse chimeras that allow to study the function of a protein specifically on immune cells. Adverse consequences of irradiation on the juvenile body and brain are well described and include general fatigue, neuroinflammation, neurodegeneration and cognitive impairment. Yet, the long-term consequences of TBI/BMT performed on healthy adult mice have been poorly investigated.

Material and Methods: We developed a robust protocol to achieve near complete bone marrow replacement in mice using 2x550cGy TBI and evaluated the impact of the procedure on their general health, mood disturbances, memory, brain atrophy, neurogenesis, neuroinflammation and blood-brain barrier (BBB) permeability 2 and/or 16 months post-BMT.

Results: We found a persistent decrease in weight along with long-term impact on locomotion after TBI and BMT. Although the TBI/BMT procedure did not lead to anxiety- or depressive-like behavior 2- or 16-months post-BMT, long-term spatial memory of the irradiated mice was impaired. We also observed radiation-induced impaired neurogenesis and cortical microglia activation 2 months post-BMT. Moreover, higher levels of hippocampal IgG in aged BMT mice suggest an enhanced age-related increase in BBB permeability that could potentially contribute to the observed memory deficit.

Conclusions: Overall health of the mice did not seem to be majorly impacted by TBI followed by BMT during adulthood. Yet, TBI-induced alterations in the brain and behavior could lead to erroneous conclusions on the function of a protein on immune cells when comparing mouse chimeras with different genetic backgrounds that might display altered susceptibility to radiation-induced damage. Ultimately, the BMT model we here present could also be used to study the related long-term consequences of TBI and BMT seen in patients.

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Total body irradiation; bone marrow transplantation; mood disturbances; memory; neuroinflammation; neurodegeneration; neurogenesis

Introduction

Total body irradiation (TBI) preceding bone marrow transplantation (BMT) is used in the clinic as treatment for disseminated hematologic malignancies and can be combined with chemotherapy (Paix et al. 2018). Myeloablation is also used to ensure successful engraftment of donor bone marrow (BM) and to avoid the rejection of transplanted cells (Quast 2006; Paix et al. 2018). In preclinical research, however, TBI and BMT are widely used for the generation of mouse chimeras to study the role of immune cells in different pathologies but also the function of a protein specifically on immune cells (Reddy et al. 2008; Park et al. 2021).

Radiation has a serious impact on the organism (Zhao et al. 2019) and depending on the dose, dose fractionation and underlying comorbidities, the symptoms differ. Acute and late toxicities arise and have been linked to chronic oxidative stress and inflammation, both in mice and humans (Hayashi et al. 2012; Lumniczky et al. 2017; Paix et al. 2018; Sun et al. 2018, 2021). During TBI the brain is not protected, resulting in radiation-induced brain injury. Although inflammation is known to be involved, mechanisms underlying brain injury remain unclear (Lumniczky et al. 2017). A direct consequence of radiation is the destruction of neural progenitors and stem cells in the hippocampus, a notably radiosensitive region, that has been linked to progressive

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cognitive decline (Lumniczky et al. 2017). TBI also damages microvessels, thereby inducing a transient increase of the blood-brain barrier (BBB) permeability (Li et al. 2003; Lumniczky et al. 2017). Moreover, radiation-induced neuronal damage leads to the activation of glial cells (Lumniczky et al. 2017) that release proinflammatory cytokines, thereby further enhancing BBB permeability and attracting peripheral immune cells in the brain (Priller et al. 2001; Lumniczky et al. 2017). Infiltrating peripheral macrophages can ultimately engraft and differentiate into microglia (Priller et al. 2001; Morganti et al. 2014; Shemer et al. 2018).

Altogether, early and short-term consequences of TBI and BMT, mostly on the juvenile brain, are well described. However, to the best of our knowledge, the long-term effects of the BMT procedure applied to healthy adult mice have not been studied. Therefore, we first developed the optimal BMT procedure combining high and stable BM replacement with maximal survival. Next, we evaluated the general health of the BMT mice, the long-term engraftment of donor-derived BM cells in the brain and the permeability of the BBB, as well as the intermediate (2 months) and long-term (16 months) effects of TBI and BMT on anxiety- and depressive-like behavior, and memory function. Finally, the impact of TBI and BMT on brain atrophy, neurogenesis and neuroinflammation was evaluated at both timepoints.

Material and methods

Mice

Eight-weeks-old male Ly5.1 mice (B6.SJL-*PtprcaPepcb*/BoyCrl) were used as recipients and 14 to 16-weeks-old male C57BL/6J mice as BM donors. All mice were bred in the animal facility of the Vrije Universiteit Brussel, group-housed under standardized conditions (20–24 °C, 10/14h dark/light cycle, 45–65% humidity) with food and water ad libitum.

Optimization of the bone marrow transplantation procedure and replacement assessment

Ly5.1 mice received different irradiation regimens using a TrueBeam® system (Varian medical System), single or split dose, varying from 800 cGy to 12 Gy at a dose rate of 500 cGy/min, with or without exclusion of the head from the irradiation field (partial body irradiation, PBI, versus total body irradiation, TBI). BM cells of C57BL/6J mice were collected by flushing the tibiae and femur cavities with Hank's Balanced Salt Solution (HBSS, Lonza) supplemented with 2% fetal bovine serum (FBS, ThermoFisher Scientific). After red blood cell lysis on ice, BM cells were resuspended in HBSS + 2% FBS and 10×10^6 cells were injected in the tail vein in a maximal volume of 200 μ l.

Mice received 0.01% Enrofloxacin (Sigma-Aldrich) in their drinking water for one month following the procedure. For clarity in the text, the term 'BMT procedure' comprises TBI and BMT.

Ten weeks post-BMT, mice were sacrificed and chimerism was evaluated in the spleen and BM. Spleens were

homogenized through a 40 μ m nylon strainer (Corning Life Sciences) and erythrocytes discarded to obtain a single cell suspension. BM single cell suspensions were made as described above. Next, 5×10^5 cells were stained for 20 min at room temperature (RT) with eBioscience™ Fixable Viability Dye eFluor™ 780 (ThermoFisher Scientific), followed by 20 min incubation at 4 °C with the following antibodies (Table 1): PE anti-CD45.1 and BV421 anti-CD45.2. Samples were analyzed with a BD LSR Fortessa Cell Analyzer (BD Biosciences).

Experimental design

The optimal irradiation protocol consisted of TBI with a split dose of 2x550 cGy at 4h interval ($n = 14$ mice). Body weight (BW) of the mice was recorded 3x/week during the two first weeks following the BMT procedure and then weekly until the end of the experiment. Seven weeks post-BMT, submandibular blood was collected in heparin-coated capillaries (Hirschmann Laborgeräte) to evaluate the chimerism. Control mice ($n = 14$) were littermates from the irradiated recipients, transported to the TrueBeam® system, but not irradiated, and intravenously injected with saline (sham procedure).

As depicted in Figure 1, mice underwent all behavioral tests 2 months after BMT, whereas after 16 months only memory-related tests and the open field test were performed. After behavioral testing, all mice were sacrificed to collect plasma and brains. An additional cohort ($n = 6$ mice) underwent the BMT procedure to be sacrificed after 2 months.

Behavioral analysis

Behavioral tests were video-recorded and analyzed manually or with an automated video tracking system (Ethovision software, Noldus) by a researcher blinded to the treatment of the mice.

Spatial and working memory were evaluated using the Barnes maze and the spontaneous Y maze (Ugo Basile), as described before (De Bundel et al. 2011; Verbruggen et al. 2022). For the Barnes maze paradigm, the mice were habituated to the maze by placing them in the center of the platform and guiding them toward the escape box after 1 min. The acquisition phase consisted of five consecutive training days (3 min, 2x/day, with an inter-trial interval of 20 min) in which the mice were trained to seek the escape box underneath one of the 20 holes. Six days later, a test trial was performed to evaluate long-term memory, and the primary latency (duration before locating the target box for the first time) and primary distance (distance traveled to locate the target box for the first time) were measured as well as the search strategy the mice used to locate the target hole. The direct strategy was defined as the direct visit of the target or adjacent hole, with no more than three mistakes, the serial strategy as the consecutive visit of more than three holes in a serial way before finding the target hole, the random and the random/serial strategy as random crossings of the maze. A mouse that did not move (and thus did not locate the

Table 1. List of used antibodies.

Antibody	Reference	Company	Species	Dilution	Experiment
PE CD45.1	AB_313497, clone A20	Biolegend	Mouse	1/40	FC
BV421 CD45.2	AB_256551, clone 104	Biolegend	Mouse	1/80	FC
FITC CD11b	AB_312789, clone M1/70	Miltenyi Biotec	Human/mouse	1/50	FC
Doublecortin	Ab18723	Abcam	Rabbit	1/1000	IHC
Iba1	AB_839504	Wako Chemicals	Rabbit	1/1000	IHC
GFAP	AB_10013382	Dako Agilent	Rabbit	1/1000	IHC
NeuN	AB_2736206	ThermoFisher Scientific	Rabbit	1/1500	IF
IgG biotinylated (H + L)	BA-1000	Vector Laboratories	Goat anti-rabbit	1/200	IHC
Alexa Fluor 488 IgG	AB_2633280	ThermoFisher Scientific	Goat anti-rabbit	1/2000	IF
IgG HRP	P044	Dako Agilent	Goat anti-mouse	1/5000	WB

For IHC and IF, primary antibodies are diluted in blocking solution. For FC, antibodies are diluted in 1% bovine serum albumin and 0.02% sodium azide in phosphate buffered saline. IHC: immunohistochemistry; IF: immunofluorescence; FC: flow cytometry; WB: Western blotting.

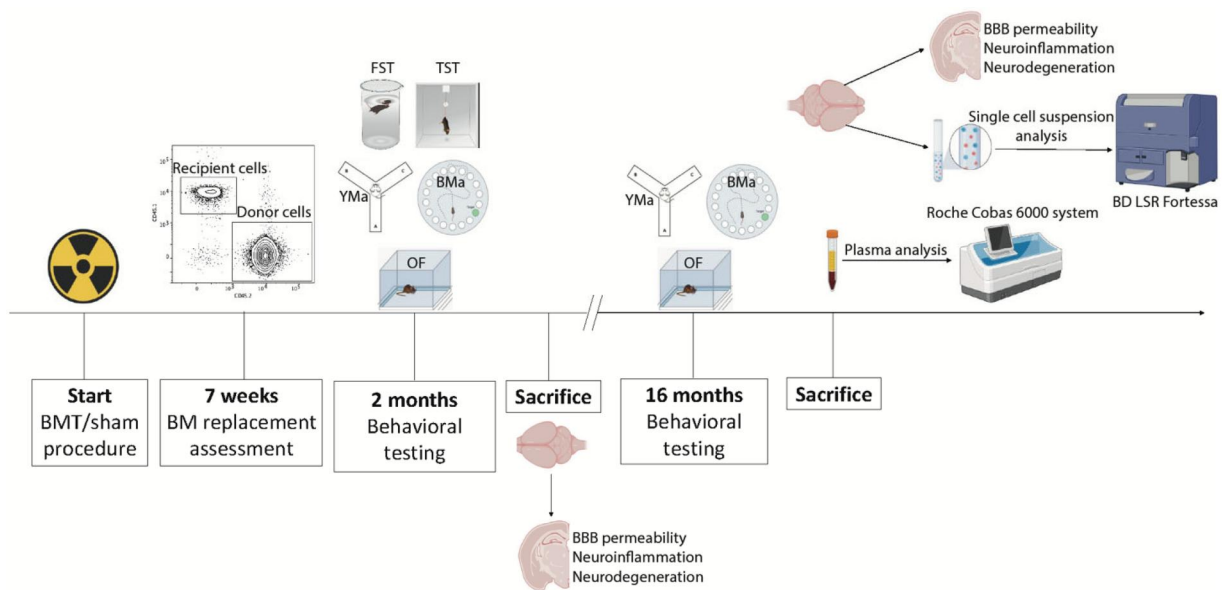


Figure 1. Experimental timeline. The experiment starts with 2x550cGy TBI followed by BMT or sham procedure. Seven weeks post-BMT, the percentage of chimerism is estimated and 2 months post-BMT, spontaneous locomotion and anxiety-like behavior are assessed using the open field test. Memory function is evaluated using the Y maze and Barnes maze task. Finally, the forced swim test and the tail suspension test are used to measure depressive-like behavior. Two months post-BMT, an additional group of mice is sacrificed for brain analysis. Sixteen months post-BMT, locomotor activity and anxiety-like behavior are assessed in the open field. Memory is again evaluated using the Y maze and the Barnes maze. Mice are sacrificed, and blood and brains collected for further analysis. BMT: bone marrow transplantation, TBI: total body irradiation, BM: bone marrow, OF: open field, Yma: Y maze, Bma: Barnes maze, FST: forced swim test, TST: tail suspension test, BBB: blood-brain barrier. Created with BioRender.com.

escape hole; primary latency = 90s), was excluded for the primary distance.

For the spontaneous Y maze task, the mouse was placed in the center of the maze and the sequence of arm entries was recorded for a duration of 8 min. One alternation is completed when the mouse visited the three arms in a consecutive manner. The percentage alternation was calculated as a measure for spatial working memory: (number of alternations/total possible alternations) x 100.

Spontaneous locomotion (distance traveled and velocity) and anxiety-like behavior (time spent in the 40cmx40cm center square) were assessed for 15 min in the open field, as described (Bentea et al. 2015).

Depressive-like behavior was studied using the tail suspension test and the forced swim test (Bentea et al. 2015; Albertini et al. 2018). In both tests, the mice were placed in an inescapable situation for 6 min and the time they spent

immobile during the last 5 min is a measure for depressive-like behavior.

Immunohistochemistry and immunofluorescence

Brains were post-fixed for three days in 4% paraformaldehyde (Sigma Aldrich) and sliced using a Leica VT 1000S vibratome (Leica Biosystems).

For each mouse, two 40 µm sections of the hippocampus were selected between -1.82 and -2.3 mm relative to bregma following the mouse brain atlas (Paxinos and Franklin 2019). Neurogenesis was studied using an antibody for doublecortin (DCX) and glial cell activation using antibodies for Iba1 (microglial marker) and GFAP (astrocytic marker), as previously described (Albertini et al. 2018; Verbruggen et al. 2022). After rinsing, sections were incubated in 3% H₂O₂ for 30 min, 25% pre-immune goat serum

in phosphate-buffered saline (PBS) for 30 min, followed by overnight incubation at 4°C with the primary antibodies (Table 1). The next day, the sections were stained using a Vectastain® ABC kit (Vector Laboratories) and visualized by the chromogen 3,30-diaminobenzidine intensified with nickel. Sections were scanned (40x magnification) using an Aperio GT450 (Leica Biosystems).

Neurogenesis was assessed in the subgranular zone of the hippocampal dentate gyrus (DG) by counting the number of DCX⁺ cells. Neural progenitor cells were identified as GFAP-expressing cells presenting a unipolar morphology (Garcia et al. 2004). To evaluate glial cell activation, the hilus of the DG as well as three adjacent regions in the cornu ammonis 1 (CA1) of the hippocampus and in the overlying cortex, respectively, were analyzed. The number of Iba1⁺ and GFAP⁺ cells was counted (averaged for the three regions in the CA1 and in the cortex) and the cell's area, perimeter and Feret's diameter (i.e., the longest distance between any two points along the selection boundary (Zanier et al. 2015)) were measured using ImageJ (National Institutes of Health).

To evaluate brain atrophy, sections were rinsed with PBS and blocked in 10% normal goat serum (Sigma-Aldrich) in 0.5% Triton-X-100 PBS for 1h before overnight incubation at RT with anti-NeuN antibody. Sections were next incubated with fluorescent secondary antibody for 1h at RT (Table 1), mounted with Fluoromount-G mounting medium (ThermoFisher Scientific) and scanned with the EVOS M7000 (ThermoFisher Scientific). Cortical thickness (three pooled measures/section), the surface area of the dorsal hippocampus, the pyramidal layer and the total width of the CA1 region (three pooled measures/section for each parameter) were quantified with ImageJ.

Quantification of recipient and donor-derived microglia using flow cytometry

Brains were quickly removed from the skull and the cerebellum was separated from the cerebrum. Next, tissue was dissociated using the 'Adult brain dissociation kit' to obtain a single cell suspension of brain tissue (Miltenyi Biotec). From this cell suspension, a small aliquot was used for flow cytometry and microglia were further isolated from the remaining single cell suspension using CD11b microbeads according to manufacturer's instructions (Miltenyi Biotec). Cell suspensions were stained with the fixable viability dye 780, PE anti-CD45.1 and BV421 anti-CD45.2 as described above, and with FITC anti-CD11b (Table 1). Samples were analyzed with a BD LSR Fortessa Cell Analyzer.

Western blotting

At dissection, blood vessels were flushed by transcardial perfusion with PBS. Snapfrozen hippocampal tissue was homogenized in 300 µl extraction buffer (2% SDS, 60 mM Tris base, 100 mM dithiothreitol, 1% phosphatase inhibitor cocktail 3, 1% protease inhibitor, pH 7.5) after which equal concentrations of protein were loaded onto a 4–12% Bis-Tris

gel (Bio-Rad Laboratories). Proteins were separated under reducing conditions and transferred to a polyvinylidene membrane. Nonspecific binding was blocked with a solution of 5% enhanced chemiluminescence (ECL) membrane blocking agent (GE Healthcare) before overnight incubation with an anti-mouse IgG coupled to horseradish peroxidase at 4°C (Table 1). Immunoreactive bands were visualized using ECL prime (GE Healthcare). A total protein stain (ServaPurple, Serva Electrophoresis GmbH) was performed to allow the normalization. The Western blot was repeated twice.

Plasma analysis

Cardiac blood was collected in a Li-heparin tube (BD Microtainer®) and centrifuged 3 min at 5500 rpm. Plasma was analyzed using the automated platform Roche Cobas 8000 system with the c702 chemistry module and the Cobas Pro system with the c503 chemistry module (Roche Diagnostic), as described before (Verbruggen et al. 2022).

Statistical analysis

Data are presented as mean ± SEM and analyzed with GraphPad Prism 9 software. An outlier test was performed, and significant outliers were removed (Grubb's test, α -value set at 0.05). Normality of the residuals was evaluated with the D'Agostino and Pearson omnibus normality test. Data were analyzed using an unpaired t-test (two groups with normally distributed data), a Mann-Whitney test (two groups with not normally distributed data) or Chi-square test (two groups; with Welch's correction for unequal variances), a three-way ANOVA (three variables), a two-way ANOVA or a mixed-effects model (two variables) followed by a Sidak's multiple comparisons test (MCT). In case of an interaction effect in the two-way ANOVA, only the most relevant post-hoc comparisons are shown in the graphs.

Results

Optimization of the irradiation protocol

To find the optimal irradiation protocol with the maximum replacement and minimum mortality, a pilot study testing different irradiation strategies (single dose 800 cGy and 950 cGy; split dose 2x450cGy, 2x550cGy and 2x600cGy) was conducted. Whereas we obtained only limited replacement in the spleen and BM using PBI, TBI at a dose of 2x550cGy resulted in 100% survival and a replacement of 73.8 ± 5.3% in the spleen and 87.8 ± 5.4% in the BM (Figure 2).

Next, two cohorts of mice underwent the BMT procedure: one large cohort kept until 16 months post-BMT (Figure 3(A), black dots) and a cohort of 6 mice sacrificed after 2 months (Figure 3(A), orange dots). Seven weeks after BMT, immune cell replacement in the blood reached 88.25 ± 6.3% and remained stable in the mice kept until 16 months post-BMT (85.38 ± 6.5% 16 months after BMT; Figure 3(A)). One out of 20 mice presented an initial poor

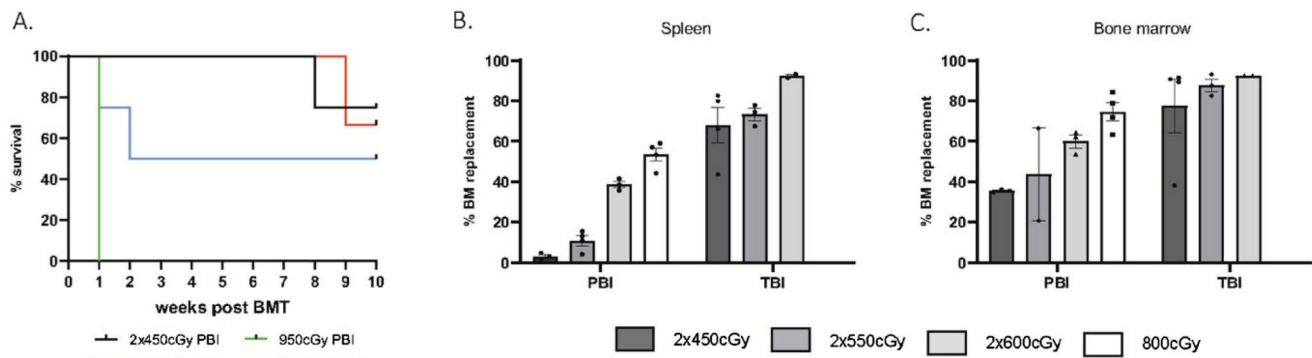


Figure 2. Optimization of the irradiation protocol. (A) Survival curves of the irradiation strategies leading to mortality. Ten weeks post-BMT, the percentage of chimerism in survivor mice was assessed (B) in the spleen and (C) in the BM. Data presented as mean $\pm$ SEM, $n = 3\text{--}4$ /condition. BMT: bone marrow transplantation, BM: bone marrow, PBI: partial body irradiation, TBI: total body irradiation.

replacement of 53% that further decreased to 7% 16 months post-BMT (excluded to calculate the average replacement).

General health status of the BMT mice

The BMT procedure did not increase the mortality rate of mice up to 16 months post-BMT. However, BW was impacted both by aging and BMT. The age effect was driven by the significant increase in the control group, while BW of BMT mice remained significantly lower than the weight of control mice at both timepoints (Figure 3(B)). Spontaneous locomotor activity was also affected both by age and BMT procedure. Overall, aged mice traveled a longer distance at higher speed (not shown), whereas the BMT procedure decreased the total distance traveled at both timepoints (Figure 3(C)). As locomotion is linked to skeletal muscle mass, we evaluated the relative weight of the gastrocnemius muscle. We found a significant age effect with an age-related reduction in the relative weight of the gastrocnemius, regardless of the irradiation status of the mice (Figure 3(D)). Moreover, we observed a trend toward a BMT effect (Figure 3(D)).

We also analyzed the plasma of the mice 16 months post-BMT to obtain insights in their general health status. Whereas no clinically relevant effects were seen on ion homeostasis and hepatic enzymes or biomarkers (Figure 3(E–G)), albumin and creatinine levels were significantly decreased (Figure 3(H)) and LDL cholesterol levels significantly increased in the plasma of aged BMT mice (Figure 3(I)).

Effect of BMT on BBB permeability and long-term macrophage engraftment in the CNS

Knowing the impact of irradiation on the BBB (Li et al. 2003; Lumniczky et al. 2017), we evaluated its permeability by quantifying the presence of IgG in the hippocampus of BMT versus non-irradiated mice 2 months and 16 months post-BMT. Whereas we could hardly detect immunoreactive signal in the homogenates 2 months post procedure (Figure 4(A)), IgG levels were increased in aged compared to adult mice, reflecting an age-related increase in BBB permeability which was significantly enhanced in irradiated mice (Figure 4(B)).

We also assessed the proportion of donor-derived engrafted microglia (originating from infiltrated donor-derived macrophages) relative to recipient microglia in the brain of BMT mice. As the cerebellum is more susceptible to peripheral immune cell infiltration following irradiation (Mildner et al. 2007), it was separated from the cerebrum. Donor-derived microglia were defined as live $\text{CD11b}^+\text{CD45.2}^{\text{int}}$ compared to $\text{CD11b}^+\text{CD45.1}^{\text{int}}$ recipient microglia (Figure 5(A)). In the cerebrum single cell suspension $9.9 \pm 2.4\%$ of microglia originated from the BM donor ($10.1 \pm 4.2\%$ in the purified microglia suspension) whereas in the total cerebellum, the proportion of donor-derived microglia cells was $17.3 \pm 1.9\%$ ($22.6 \pm 5.7\%$ in the purified microglia suspension) (Figure 5(B)).

BMT procedure impairs long-term spatial memory while anxiety- and depressive-like behavior are unaffected

The impact of the BMT procedure on long-term spatial memory was investigated using the Barnes maze test. Although all mice were able to learn the test, aging affected the learning curves of the mice with both an increased primary latency (Figure 6(A)) and primary distance (Figure 6(B)), at the end of the five-days training session (total of ten trials). Next, long-term spatial memory was evaluated six days after the last training session. BMT mice traveled a longer distance before locating the target hole, an effect that was mainly seen in aged mice (Figure 6(C)), but their primary latency was not affected by the procedure (Figure 6(D)). Moreover, 16 months post-BMT, a trend toward a significantly decreased use of the hippocampus-dependent direct search strategy was observed in BMT mice compared to controls (Figure 6(E)). Finally, short-term spatial working memory was evaluated in the spontaneous Y maze and was unaffected by age or BMT (Figure 6(F)).

At last, BMT mice were tested for the presence of mood disturbances. Anxiety-like behavior was evaluated by measuring the time the mice spent in the center of the open field, and neither aging nor BMT induced an anxiogenic effect (Figure 6(G)). Finally, no difference between control and BMT mice in the percentage of time spent immobile in the tail suspension or the forced swim test indicates the absence

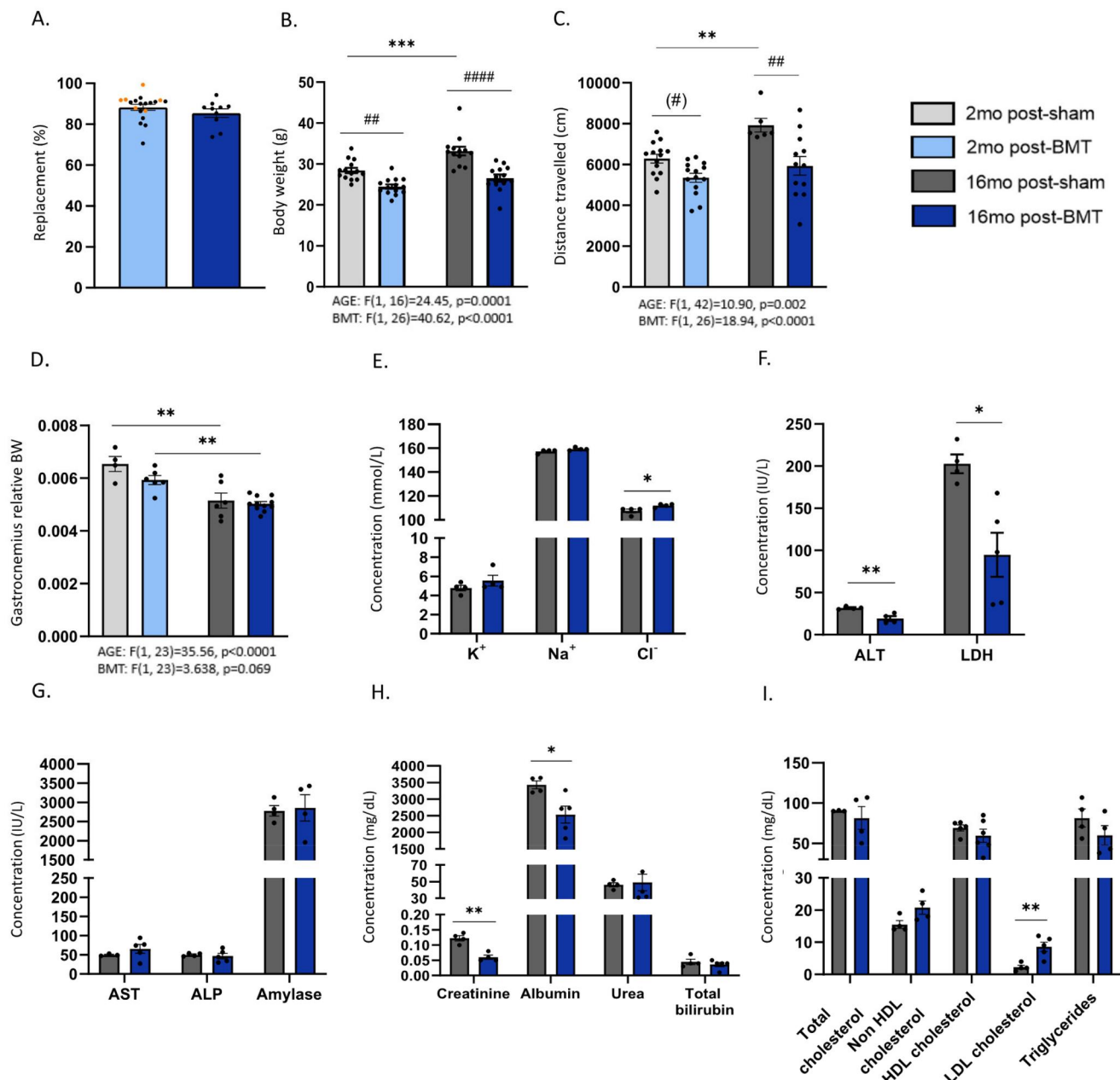


Figure 3. General health status of the BMT mice. (A) Seven weeks and 16 months post-BMT, BM replacement was evaluated by quantification of the percentage of CD45.2⁺ cells in the blood. The orange dots represent the mice sacrificed 2 months post-BMT and the black dots the mice kept until 16 months post-BMT. (B) Body weight was plotted 2 months and 16 months post-BMT. (C) At both timepoints, spontaneous locomotion was investigated by quantifying the total distance the mice traveled in the open field. (D) Postmortem evaluation of the relative mass of the gastrocnemius muscle 2 months and 16 months post procedure. Sixteen months post-BMT, plasma concentrations of (E) K⁺, Na⁺ and Cl⁻, (F) ALT and LDH, as well as (H) AST, ALP and amylase were assessed in addition to plasma levels of (H) creatinine, albumin, urea and total bilirubin, and (I) the different types of lipids. Data presented as mean $\pm$ SEM. Behavioral testing: 2 months post procedure $n = 14$ /group, 16 months post procedure $n = 6-11$ /group. Postmortem analysis: 2 months post procedure $n = 4-6$ /group, 16 months post procedure $n = 6-11$ /group. Statistical analysis was performed using a mixed effect models analysis followed by Sidak's MCT (B-C), a two-way ANOVA followed by Sidak's MCT (D), a Mann-Whitney test (E) and an unpaired t-test (F-I). Significant main effects of the two-way ANOVA are indicated on the figure. Sidak's MCT: * $p < .05$, ** $p < .01$, *** $p < .001$ (to compare adult versus aged mice), and (#) $p = 0.051$, ## $p < .01$, ### $p < .0001$ (to compare control versus BMT mice). BMT: bone marrow transplantation, OF: open field, BW: body weight, AST: aspartate aminotransferase, LDH: lactate dehydrogenase, ALT: alanine aminotransferase, ALP: alkaline phosphatase.

of increased depressive-like behavior in the BMT group (Figure 6(H,I)).

BMT procedure does not induce overt brain atrophy

Cortical thinning, typically observed with aging, can reflect neurodegeneration and underly cognitive impairment (Zarei

et al. 2013; Shaw et al. 2016). The overall age-related decrease in cortical thickness was more pronounced in the BMT groups (Figure 7(A)). Yet, no differences could be seen in the cortical thickness of BMT versus non-irradiated mice (Figure 7(A)). Next, we evaluated the impact of the procedure on the morphology of the hippocampus (Figure 7(B)), as it is a crucial brain region for spatial memory. No changes were seen

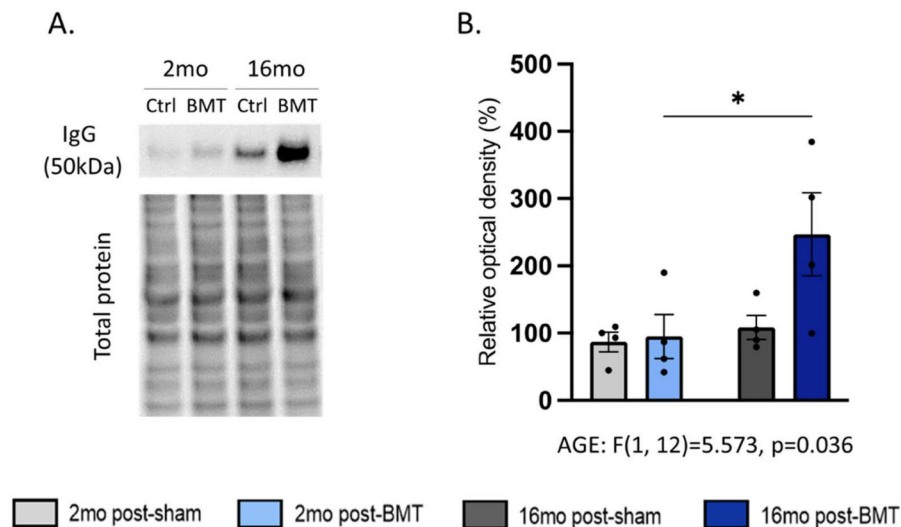


Figure 4. Enhanced age-related increase in BBB permeability of BMT mice. (A) Representative picture of a semi-quantitative Western blot and (B) its corresponding relative optical density to analyze the presence of IgG in hippocampal homogenates (as a measure for BBB permeability) 2 months and 16 months post-BMT. Data presented as mean $\pm$ SEM, $n=4-5$ /group. Statistical analysis was performed using a two-way ANOVA followed by Sidak's MCT, * $p < .05$. Significant main effects of the two-way ANOVA are indicated on the figure. BMT: bone marrow transplantation, BBB: blood-brain barrier.

in the dorsal hippocampus area (Figure 7(B,C)), the thickness of the CA1 pyramidal layer (Figure 7(B,D)) and the total width of the CA1 (Figure 7(B,E)).

BMT procedure impacts neurogenesis

Cognitive impairment is linked to reduced hippocampal neurogenesis (Greene-Schloesser et al. 2013). Therefore, we quantified the number of GFAP⁺ progenitor cells (Figure 7(F)) and DCX⁺ immature neurons (Figure 7(H)) in the subgranular zone of the hippocampus (Bond et al., 2015). The number of GFAP⁺ progenitor cells was comparable in all groups (Figure 7(G)). However, the BMT procedure induced a very pronounced loss of DCX⁺ cells after 2 months (Figure 7(I)). Sixteen months post-BMT, the number of immature neurons was significantly reduced in aged mice compared to the adult controls, independent of the BMT procedure (Figure 7(I)).

BMT procedure affects microglial cells in the cortex but not in the hippocampus

Finally, we quantified the number of microglia and astrocytes, and analyzed their morphology in the dorsal hippocampus and overlying cortex (Figure 8(A-C)). At the two timepoints studied, we did not find any evidence of microglia or astrocyte activation in the hippocampus of irradiated mice as no differences could be observed in the number of cells per mm² (Figure 8(D,E)) or in their morphology (Figure 8(H,I,L,M,P,Q)). On the contrary, the number of Iba1⁺ cells was significantly reduced in the cortex of BMT mice compared to control mice 2 months post procedure (Figure 8(F)), and these cells presented an activated morphology with a significantly increased area (Figure 8(J)), perimeter (Figure 8(N)) and Feret's diameter (Figure 8(R)). Sixteen months post procedure, Iba1⁺ cells presented an activated morphology -compared to the adult non-irradiated mice and regardless of the BMT treatment- as illustrated by

the significantly larger area (Figure 8(J)), perimeter (Figure 8(N)) and Feret's diameter (Figure 8(R)) of the cell body. Finally, we did not find any sign of astrocytic activation in the cortex of irradiated mice at the two timepoints studied (Figure 8(G,K,O,S)).

Discussion

Mouse chimeras are used to study the role of immune cells but also of specific proteins expressed on immune cells in physiological and pathological processes, including brain functioning. Hence, it is crucial to find the delicate balance between maximal BM replacement and minimal impact of irradiation on the brain, which would ideally be excluded from the irradiation field. However, by doing so, we obtained a poor BM replacement, confirming the important contribution of skull hematopoiesis (Mildner et al. 2011). Important irradiation doses are required to obtain near-complete myeloablation, thereby increasing the risk to die from radiation-induced toxicities (Down et al. 1991; Duran-Struuck and Dysko 2009). Fractioning high doses with an interval of a few hours or days decreases toxicities and mortality (Scanlon 1972; van Os et al. 1993; Tiku and Kale 2004), in line with our results where one dose of 950 cGy induced 100% mortality while all mice survived a split dose of 11 Gy.

BW was closely monitored, as gastrointestinal cells are very radiosensitive (Duran-Struuck and Dysko 2009). A transient reduction in BW was expected as irradiation happened during the fast-growing phase of the mice, inducing a delay in their growth (Yanai and Endo 2021). However, the difference between BMT and control mice persisted with aging. This long-term effect of BMT on BW has not been reported before and would require further in-depth analysis.

Reduced spontaneous locomotor activity together with decreased plasma creatinine, the waste product formed by muscles when creatine is broken down, might be linked to

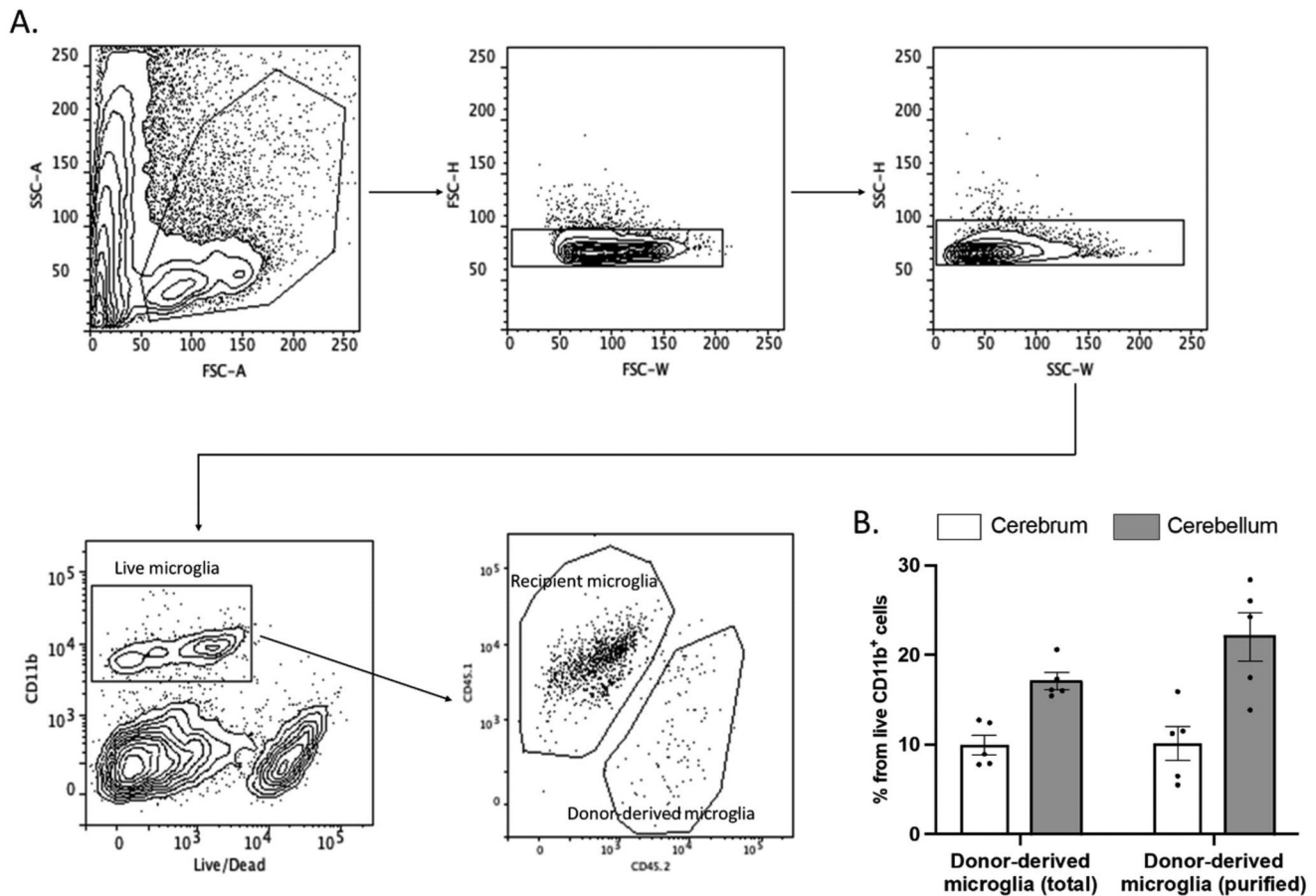


Figure 5. Evaluation of donor cell engraftment in the brain of aged BMT mice. (A) Cells were first gated from the whole population using the FSC and SSC. Next, singlets were isolated using the height and the width of the FSC and SSC. Finally, only live cells expressing CD11b were selected to further analyze the presence of the following markers: CD45.1 and CD45.2. Recipient and donor-derived microglia were identified by the common expression of CD11b and the respective expression of CD45.1^{int} and CD45.2^{int}. (B) Percentage of donor-derived relative to recipient microglia was estimated in the whole cell suspensions (total) as well as in purified microglia (purified) obtained from the cerebrum and cerebellum. FSC: forward scatter, SSC: side scatter.

the direct effect of irradiation on skeletal muscles, a phenomenon described before (Caiozzo et al. 2010; Cho-Lim et al. 2011; Hardee et al. 2014; Fu et al. 2015; Doreste et al. 2020). We do see a trend toward a reduced relative mass of the gastrocnemius muscle -that is crucial for walking- in the BMT mice. However, this is mainly the result of the difference observed 2 months post-BMT while the plasma analysis was conducted 16 months post procedure. This makes it unlikely that the changes in creatinine levels are related to muscle wasting. Yet, blood analysis of aged BMT mice also showed reduced albumin and increased LDL levels. While creatine and albumin are produced by the liver, and in mice circulating levels of LDL are controlled by fast hepatic clearance, our results could reflect radiation-induced liver dysfunction as seen in BMT patients and mice (e.g. BMT model for hepatic veno-occlusive disease) (Quimby and Luong 2007; Zeng et al. 2013; Kim and Jung 2017). Still, the absence of increased levels of the usual biomarkers for liver dysfunction (total bilirubin, ALT, AST and ASP) do not seem to support this hypothesis.

Conflicting findings have been published on the effects of irradiation on mood disturbances and memory function (Raber et al. 2004; Wong-Goodrich et al. 2010; Haridas et al. 2012; Constanzo et al. 2020; Dey et al. 2020; Parihar

et al. 2021). This is most probably related to differences in experimental set-up, including the irradiation strategy, the total dose received and the timepoints of behavioral testing. In our model, we could not observe BMT-related changes in anxiety- or depressive-like behavior. Yet, long-term spatial memory was impaired in BMT mice, similar to what was found 3 and 4 months after whole-brain radiation therapy or hippocampus-targeted radiation, using even lower doses than the one presented in this study (Raber et al. 2004; Wong-Goodrich et al. 2010). Long-term memory deficits have also been described in cancer patients who underwent whole-brain radiation therapy, whereas memory function is more preserved when patients are treated with hippocampal-avoidance whole-brain radiation therapy or stereotactic radiosurgery (Chang et al. 2009; Gondi et al. 2014; Farjam et al. 2015). Finally, it is noteworthy that, while we here present data in male mice only, it has been shown both in mice and humans that females are more prone to develop radiation-induced cognitive and memory decline (Raber 2010; Farjam et al. 2015).

Radiation-induced memory decline has been associated to structural brain damage and irremediable impairment of neurogenesis, which are known to be linked to neuroinflammation (Monje et al. 2003; Yuan et al. 2006; Lumniczky

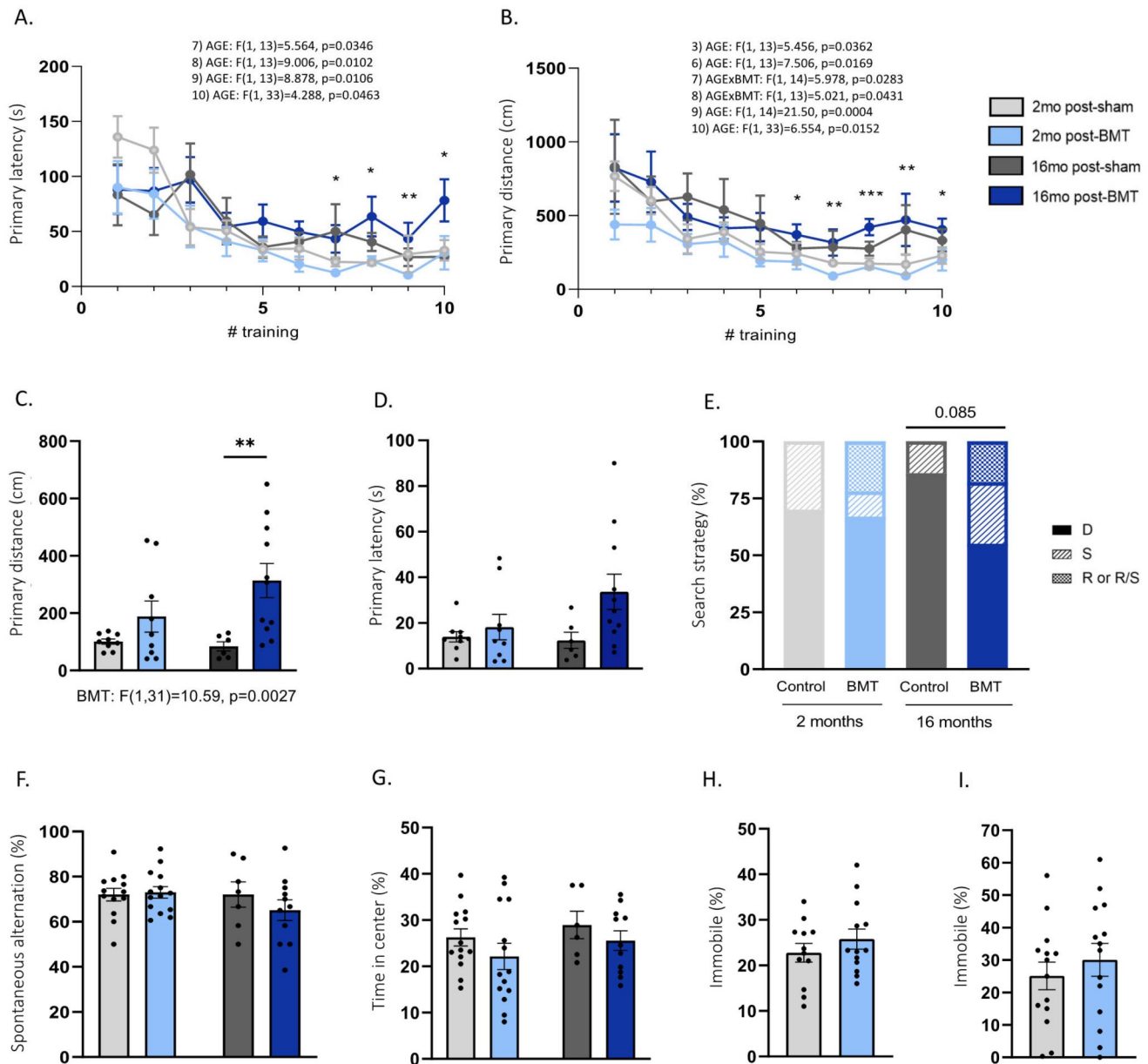


Figure 6. Impaired spatial memory in BMT mice with no change in anxiety- and depressive-like behavior. Two months and 16 months post procedure, long-term spatial memory was evaluated using the Barnes maze test. Learning curves depicting (A) the primary latency and (B) the primary distance are presented. Six days after the last training session, (C) the primary distance and (D) the primary latency were analyzed to evaluate long-term memory function. (E) Percentage of mice using the different search strategies during the long-term memory test, of which the direct search strategy is hippocampus-dependent. (F) Short-term spatial working memory was evaluated using the spontaneous Y maze. (G) Two months and 16 months post-BMT, anxiety-like behavior was evaluated in the open field through the percentage of time the mice spent in the center of the arena. (H-I) Two months post-BMT, depressive-like behavior was assessed by the percentage of time the mice spent immobile in (H) the tail suspension test and (I) the forced swim test. Data presented as mean $\pm$ SEM. The training of the mice was analyzed using a three-way ANOVA: trial effect $p < .0001$ (A,B) and a mixed-effects model analysis followed by Sidak's MCT was conducted at each individual timepoint and was only significant within the BMT groups. (C,D) The primary distance and latency and (F) the Y maze data were analyzed using a two-way ANOVA followed by Sidak's MCT. Significant main effects of the two-way ANOVA are indicated on the figure. * $p < .05$, ** $p < .01$, *** $p < .001$. (E) The search strategies were compared for each timepoint using a Chi-square test. For the mood disturbances, statistical analysis was performed using a mixed-effects model analysis (G) or an unpaired t-test (H-I). Two months post procedure $n = 14$ /group, 16 months post procedure $n = 7-11$ /group. BMT: bone marrow transplantation, D: direct, S: serial, R: random, R/S: random/serial.

et al. 2017). Using our BMT procedure, we did not observe any long-term gross structural changes in the cortex or hippocampus, but we did confirm the detrimental impact of irradiation on neurogenesis as seen by the strong reduction in the number of immature DCX⁺ neurons 2 months post-BMT (Conner et al. 2011; Park et al. 2012; Alaghband et al. 2020). Yet, the overall decreased neurogenesis with aging, as reported before (Walter et al. 2011; Wu et al. 2023), was not further impacted by the BMT procedure.

While the BMT procedure did not affect the number or morphology of glial cells in the hippocampus, it led to a substantial reduction in the number of cortical microglia together with an activated morphology 2 months post-BMT compared to control mice. After 16 months, cortical microglia morphology remained activated in BMT mice but to the same extent as the non-irradiated mice, confirming the known age-associated microglial activation (Edler et al. 2021). Most studies evaluating the impact of radiation on

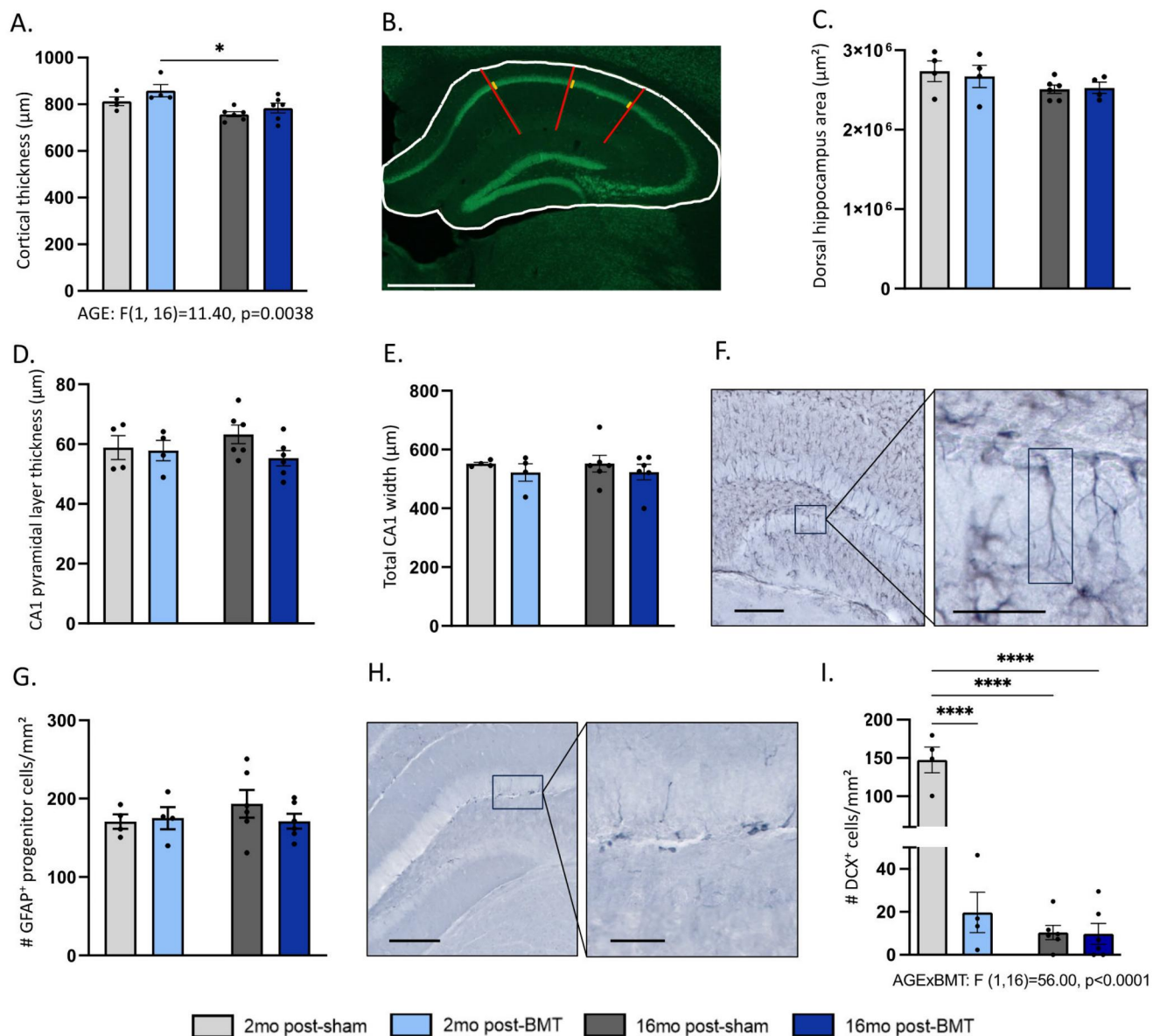


Figure 7. Short-term detrimental effect of BMT on neurogenesis but not on cortical thickness or hippocampal morphology. Evaluation of brain atrophy was performed 2 and 16 months post-BMT procedure by measuring (A) the cortical thickness, and (B-E) the morphology of the dorsal hippocampus. (B) Representative picture illustrating the parameters measured in the hippocampus: the white line represents (C) the area of the dorsal hippocampus, the three yellow lines were averaged to assess (D) the thickness of the CA1 pyramidal layer and the three red lines were averaged to calculate (E) the total thickness of the CA1. (F,H) Representative pictures of (F) GFAP⁺ progenitor cells and (H) doublecortin⁺ (DCX) cells in the subgranular zone of the DG of two adult control mice (2mo post-sham). (G,I) Number of (G) GFAP⁺ progenitor cells and (I) DCX⁺ cells per mm^2 counted in the subgranular zone of the DG. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using a two-way ANOVA followed by Sidak's MCT, * $p < .05$, **** $p < .0001$; $n=4-6/\text{group}$. Significant main effects are presented on the figure. BMT: bone marrow transplantation, CA1: cornu ammonis 1, DG: dentate gyrus, DCX: doublecortin. Scale bar: 650 μm (B), 200 μm (F,H left), 60 μm (F,H right).

the brain focus on the hippocampus, a region that is very sensitive to radiation-induced damage and involved in neurogenesis and cognition. In line with our results, Han et al. showed that microglia recruitment and activation in the hippocampus of adult mice are an early and transient process that peaks 6 hours after whole-brain radiation with a single dose of 8 Gy, while a long-lasting (up till 30 days have been studied) loss in hippocampal microglial numbers was seen (Han et al. 2016). Similarly, 30 days after a cranial single 10 Gy radiation dose, Hinkle et al. could not observe changes in microglial morphology. However, in their set-up, microglial activation markers were still upregulated in the hippocampus of male -but not female- mice (Hinkle et al. 2019). Similar studies performed on the rat brain also found

a persistent activation of microglia in the hilus of the DG, 2 and 3 months post radiation (Monje et al. 2002; Conner et al. 2011). These contradicting data can be explained by the different radiation doses applied and the techniques used to assess microglia activation, that were based on the one hand, on the morphological analysis of Iba1⁺ cells and quantification of CD68 marker and, on the other hand, on the quantification of ED-1⁺ cells. Whereas the literature regarding radiation's impact on hippocampal glia is abundant, it is much more limited for cortical glia, especially in a comparable set-up as ours. Loss of microglia following irradiation has indeed been reported in the mouse somatosensory cortex 16 days post whole-brain radiation therapy, the latest timepoint investigated, and was attributed to radiation-

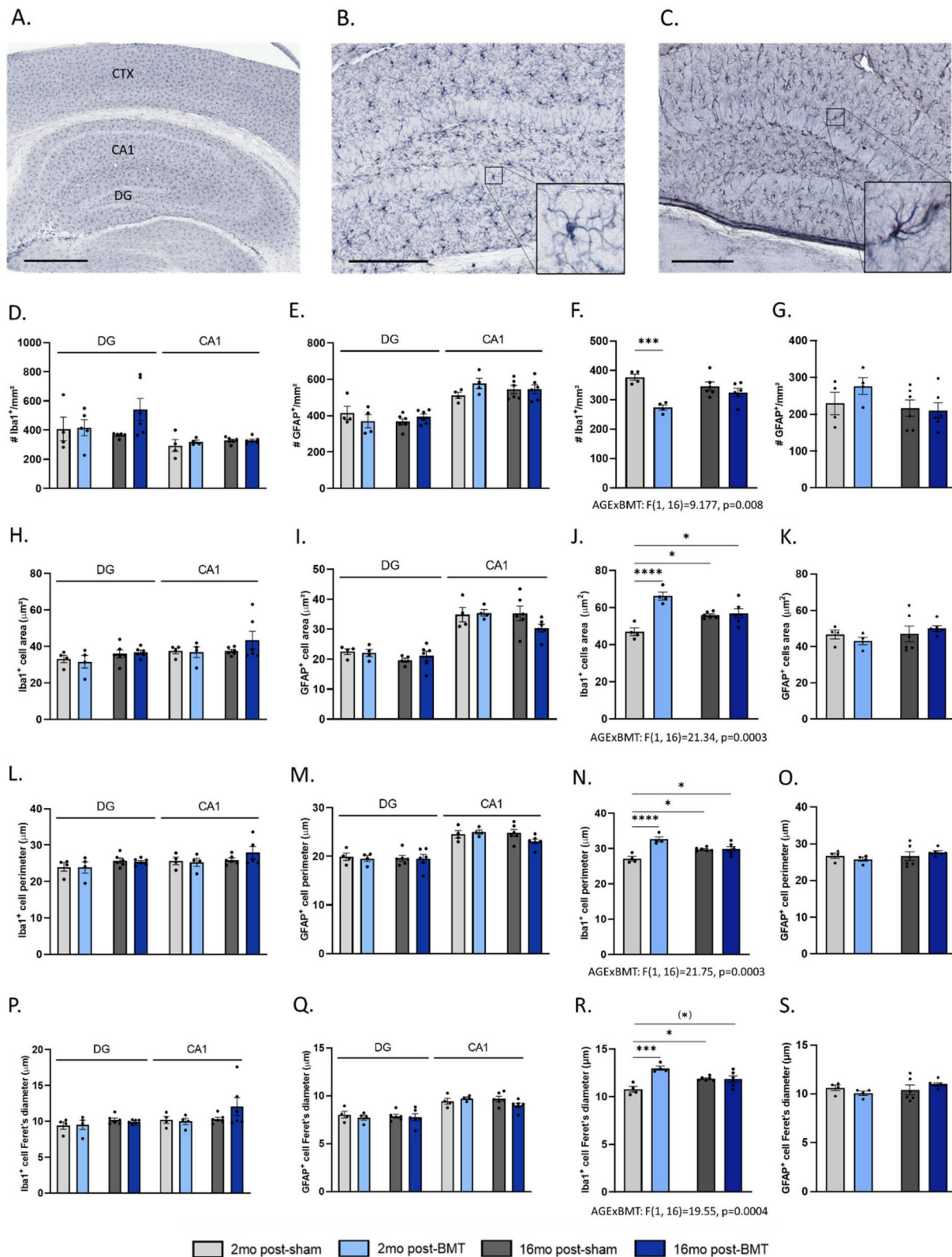


Figure 8. Early microglial activation in the cortex of BMT mice. (A-C) Illustration of (A) two regions -i.e. the dorsal hippocampus and its overlying cortex- that were analyzed to evaluate (B) microglial (Iba1 immunostaining) and (C) astrocytic (GFAP immunostaining) activation in an adult control mouse (2mo post-sham). (D-G) The number of (D,F) microglial cells (Iba1⁺) and (E,G) astrocytes (GFAP⁺) per mm² was calculated in (D,E) the hilus of the DG and the CA1 region of the hippocampus and in (F,G) the overlying cortex. (H-S) Glial cell activation in (H,I,L,M,P,Q) the hippocampus and (J,K,N,O,R,S) the overlying cortex was assessed through the analysis of (H,J) the area of Iba1⁺ and (I,K) GFAP⁺ cells, as well as their (L-O) perimeter and (P-S) Feret's diameter. Data presented as mean ± SEM. Statistical analysis was performed using a two-way ANOVA followed by Sidak's MCT, (*) $p < .05$, *** $p < .001$, **** $p < .0001$; $n = 4-6$ /group. Significant main effects are presented on the figure. BMT: bone marrow transplantation, CTX: cortex, CA1: cornu ammonis 1, DG: dentate gyrus, Iba1: ionized calcium-binding adapter molecule 1, GFAP: glial fibrillary acidic protein. Scale bar: 600μm (A), 200μm (B,C).

induced cell death (Whitelaw et al. 2021). Besides, an increased number of microglia with an activated morphology was observed in the medial prefrontal cortex 6 weeks post cranial irradiation (Acharya et al. 2016). To the best of our knowledge, we here report for the first time the consequences of TBI on cortical microglia 2 and 16 months post procedure.

The absence of hippocampal and cortical astrogliosis in our model is in line with a study evaluating the long-term effects of irradiation on the brain (up to 6,5 months after cranial irradiation) (Yuan et al. 2006). Overall, the adverse effects of the BMT procedure on neurogenesis and neuroinflammation might have paved the way to the impaired memory function in aged BMT mice.

Furthermore, the elevated presence of hippocampal IgG in aged BMT mice could reflect an enhanced age-related increase in BBB permeability after brain irradiation and additionally contribute to the observed memory deficit, as BBB breakdown has been linked to cognitive decline and neurodegenerative diseases (Farjam et al. 2015; Barisano et al. 2022).

To conclude, we here presented a broad characterization of the long-term consequences of TBI and BMT during adulthood and found a persistent delay in growth along with long-term impact on locomotion and memory. Because mouse chimeras are a tool used to investigate the interactions between the immune system and the CNS, and their potential consequences on behavior, unequal susceptibility of the recipient mouse strain to radiation-induced damage could bias the behavioral read-out of chimeras and should thus be considered while interpreting data. Ultimately, the BMT model we here present could also be used to investigate the long-term consequences of TBI and BMT seen in patients.

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Ethical approval

All experiments were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel and performed according to the national and European guidelines on animal experimentation.

Author contributions

PJ, LDP, IR, AM designed the experiments. MDR provided access to the TrueBeam® system and helped with the irradiation set-up. PJ and LDP generated the BMT mice, followed the welfare and health of the mice, assessed the chimerism in the blood at the timepoints mentioned in the manuscript. PJ and LDP conducted the behavioral experiments, and organized and coordinated the dissections. MM, OL, LM participated to the dissections and the immediate processing of the samples. BG was responsible for the blood chemistry analysis. PJ, LDP and JW performed and analyzed the immunostainings and Western blotting. PJ and GA optimized and performed the flow cytometry analysis of the brain samples. PJ, LDP and AM interpreted the data and wrote the paper. All authors discussed the results and gave feedback on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon request.

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