Chapter 5

Neuroinflammatory response in aged mice after major surgery without sepsis – does age-related microglia priming matter?


Submitted
Abstract

Background: Ageing is associated with priming of microglia in the central nervous system of otherwise healthy individuals, which amplifies the neuroinflammatory response evoked by systemic inflammation and organ injury. Consequently, aged animals produce increased levels of cerebral pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, which can contribute to neurological dysfunction and disease progression rather than initiating a repair response. The aim of this study is to investigate whether a primed neuroinflammatory response in aged mice induces an excess neuroinflammatory response in a major surgery (MSu) model, i.e. a model without LPS administration or sepsis induction.

Methods: Aged male C57BL/6J mice of 14-16 months old and young adult male mice of 2-3 months old were subjected to a midline laparotomy and opening of the kidney capsule or sham procedure under ketamine anesthesia, or did not undergo any procedure (controls). Whole brain lysates were obtained at 4h and 24h post-surgery and levels of pro-inflammatory cytokines were analyzed by RT-qPCR, and proteolytic isoforms of Bdnf protein levels by Western blot. Microglia were sorted from brains of aged mice and the mRNA expression of inflammatory genes was evaluated by RT-qPCR.

Results: Plasma IL-6 levels significantly increased at 4h after MSu, both in aged and adult mice, indicating a systemic inflammatory response. MSu provoked an increase in brain Il-6 at 4h, which decreased again within 24h. Further, MSu induced a stronger increase in whole brain Il-6 levels in aged mice compared to their adult counterparts. Aged mice had significantly lower truncated Bdnf expression compared to adult mice at all time points, possibly indicating a decreased neuroregenerative stimulus, even without MSu. Also, MSu provoked an increase in microglial expression of Il-6 and Tnf-α mRNA, which decreased already at 24h, supporting the self-limiting nature of the inflammatory response.

Conclusion: Aged mice display higher overall amounts of pro-inflammatory cytokines in the brain at baseline and following MSu. At 4h MSu an increase in pro-inflammatory cytokine production was found in whole brain and in microglial cells, which decreased to below control levels at 24h MSu. Hence,
we conclude that major surgery without sepsis in aged mice does not provoke a long-lasting or grossly amplified neuroinflammatory response.

**Introduction**

Microglia are the resident innate immune cells of the central nervous system (CNS) and considered to be the most important cell type in the initiation of a neuroinflammatory response. Microglia may be activated in response to systemic inflammation (Hoogland et al., 2015), e.g. induced by a major surgical procedure. Once activated, microglia produce pro- and anti-inflammatory cytokines, such as interleukins and TNF-α, chemokines and proteases, and induce phagocytosis, in order to maintain neuronal plasticity and to restore tissue homeostasis. Under pathological conditions microglia may respond to underlying changes in the CNS by becoming primed (Perry et al., 2014), and respond with excessive neuroinflammation upon activation, possibly progressing neurological dysfunction. Ageing is suggested to cause priming of microglia in brains of otherwise healthy individuals (Dilger et al., 2008). Indeed, aged brains exhibit gene expression profiles indicative of increased microglial cell activation (Frank et al., 2006) and neuroinflammation (Godbout et al., 2005) in rodents. In addition, ageing decreases the neuroregenerative properties induced by neurotrophic factors, such as brain derived neurotrophic factor (BDNF)(Palomer et al., 2016; Tapia-Arancibia et al., 2008). Truncated Bdnf (tBdnf) is a proteolitic isoform of mature Bdnf protein with a molecular weight of 28kDa. It is suggested to have a specific role in age-related cognitive dysfunction through alternative splicing, as it has lower expression levels in patients with autism (Garcia et al., 2012) and in patients with higher cognitive impairment in schizophrenia (Carlino et al., 2011).

Elderly patients are at a higher risk to develop postoperative cognitive dysfunction (POCD) and postoperative delirium (Krenk & Rasmussen, 2011). POCD following major surgery is associated with glial cell activation in aged mice (Wan et al., 2010). However, experimental studies examining POCD and neuroinflammation in major surgery commonly use bacterial inflammatory co-
stimulation by administration of lipopolysaccharide (LPS) (Godbout et al., 2005), excess manipulation of the intestines (Rosczyk et al., 2008), clamping of mesenteric arteries (Hovens et al., 2013) or cecal ligation and puncture (Liu et al., 2014). Data regarding the neuroinflammatory response in major surgery without bacterial infestation or sepsis is scarce. A recent study in patients undergoing major knee surgery describes increased inflammatory markers in plasma and cerebrospinal fluid even after aseptic surgery (Hirsch et al., 2016). While the only patient in this study who developed postoperative delirium had a persistent increase in proinflammatory cytokines IL-6, IL-8, and MCP-1 in the cerebrospinal fluid, patients who developed POCD had decreased levels of several anti- and proinflammatory cytokines (Hirsch et al., 2016).

We hypothesize that priming of microglia increases the inflammatory response in aged mice after major surgery without sepsis induction. We therefore compared the (neuro)inflammatory response of aged (14-16 months) and young adult (2-3 months) male C57BL/6J mice at 4h and 24h post-surgery.
Material and methods

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Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen. Male aged C57BL/6J mice of 14-16 months old and adult mice of 2-3 months old were obtained from Envigo, The Netherlands. Aged mice had a body weight of 48.0 (42.0-54.5) g prior to surgery, whereas adult mice had a mean body weight of 26.5 (25.5-28.0) g (p<0.001). The duration of surgical or sham procedures was 66 (62-74) minutes on average. Animals were housed individually in a 12 hour light/dark cycle in a humidity controlled environment and were allowed free access to food and water throughout the entire experiment.

Anesthetic and surgical procedure

Mice were randomly assigned to undergoing major surgery (MSu) with follow-up of 4h or 24h, sham procedure with follow-up of 4h or to the untreated control group (n=4-5/group). Anaesthesia was induced with isoflurane 5%. Thereafter, the right jugular vein was cannulated to administer ketamine anaesthesia (70mg/ml, 0.1ml/hr), both in surgery and sham. In MSu, a midline laparotomy was performed as surgical intervention, including incision of the peritoneum, followed by cautious dislocation of the intestine outside the abdominal cavity in a wet compress (sterile 0.9% saline) and removal of the 1/3 caudal part of the capsule of the left kidney. The abdominal cavity was rinsed with sterile 0.9% saline at a temperature of 37°C, followed by closure of the peritoneum and the abdominal incision. Cardiorespiratory function was monitored throughout the procedure using the MouseOxPlus (Harvard apparatus, Germany). Rectal temperature was maintained at 36-37°C by using an electrical heating pad. The total time of the procedures was 65 min. Wounds were closed with 5-0 Vicryl sutures. Before postoperative housing at the animal care center, it was ensured that all animals were fully recovered from anesthesia and surgery. Animals were sacrificed 4h and 24h after wound closure by exsanguination through a cardiac puncture under isoflurane anesthesia. Organs
were flushed with saline via the abdominal aorta and then frozen in liquid nitrogen and stored at -80°C until further use.

Protein extraction and Western blot analysis

Brain samples were homogenized in RIPA-TBS buffer followed by centrifugation for 20 min at 14,000 rpm at 4°C. Protein concentration was determined in a Bradford protein assay (BioRad, The Netherlands) and equal amounts of total protein in SDS-PAGE sample buffer were separated on SDS-PAGE 4-20% PreciseTM Protein gels (Thermo Scientific, The Netherlands). After transfer to nitrocellulose membranes (BioRad, The Netherlands), proteins were blocked with 5% skimmed milk. Membranes were then incubated overnight at 4°C with primary antibodies, followed by incubation with secondary antibodies for 1h at room temperature. The following primary antibodies were used: rabbit polyclonal anti-Bdnf (ab72439, Abcam, UK), and mouse monoclonal β-actin (sc-47778 HRP, Santa Cruz, USA) as loading control. Secondary antibody used was horseradish peroxidase-conjugated anti-rabbit (Dako, Denmark). Signals were detected by Western Lightning Ultra (Perkin Elmer Inc., USA) and quantified by densitometry with software from Gene Gnome (Syngene, UK).

Isolation of microglia

Microglia were freshly isolated from half brains (sagittal incision) of aged mice. Brain tissue was homogenized and filtrated through a 70-µm cell strainer (BD FALCON) to obtain a single cell suspension. Subsequently, the cells were centrifuged at 220 rcf for 10 minutes at 4°C, and in order to remove myelin, the pellet was re-suspended in a solution of 22% Percoll (GE Healthcare), 40mM NaCl and 77% myelin gradient buffer containing 5.6 mM NaH₂PO₄•2H₂O, 140 mM NaCl, 5.4 mM KCl, 11 mM Glucose. PBS was added on top of the mixture and centrifuged at 950 rcf for 20 minutes at 4°C. Finally, the pellet was re-suspended in HBSS with 7.5 mM HEPES and 0.6% glucose (Sigma-Aldrich). For cell sorting and flow cytometry analysis, Fc receptors were blocked with anti-mouse CD16/CD32 (eBio-science) for 10 minutes on ice. Cells were thereafter incubated on ice for 45 minutes in the dark with CD11b PE (BioLegend), CD45 FITC (eBioscience), and Ly-6C APC (Biolegend). After washing with Medium A (HBSS containing 0.6% glucose and 15 mM HEPES
buffer) without phenol red, cells were resuspended in Medium A without phenol and passed through a 35-µm nylon mesh (BD Bioscience), and sorted (Beckman Coulter MoFloAstrios) immediately after addition of DAPI. Live microglia were identified as DAPI\(\text{neg}\) CD11b\(\text{high}\) CD45\(\text{int}\) Ly-6C\(\text{neg}\) cells. After collection of cells in RNAlater (Qiagen), tubes were centrifuges at 2000 rcf for 10 minutes at 4°C and the pellet lysed with 350 µl of RLT plus (Qiagen). The microglia lysates were stored at -80°C until further use.

**Inflammatory cytokines in brain and microglia**

The levels of pro-inflammatory cytokines Il-6, Il-1β, Tnf-α in brain tissue was determined by RT-qPCR. Half brains were disrupted on dry ice and mRNA was extracted from each sample using the Nucleospin tissue kit according to the manufacturer’s instructions (Macherey-Nagel, Germany). RNA from sorted microglia was extracted using the RNeasy micro plus kit according to the manufacturer’s instructions (Qiagen). Subsequently, mRNA (0.5 µg) was reverse transcribed in a reaction mixture (10 µl) containing 0.5 µl of random hexamers, 0.25 µl of RNase inhibitor, 0.1 µl of deoxynucleotide triphosphates, and 0.5 µl of reverse transcriptase (Thermo Scientific). Specific primers were used (Table 1). Housekeeping genes were Hmbs and Hprt.

Expression levels were subsequently quantified on the Bio-Rad CFX using GoTaq qPCR Master Mix (Promega, USA). The samples in the PCR 384-well plate were transferred to the thermal cycler and subjected to the following protocol: predenaturation at 95°C, 39 cycles of denaturation at 95°C (15 sec), annealing at 60°C, followed by a melting curve. All data were normalized to Hmbs expression.
Plasma IL-6 levels in mice were determined with an ELISA kit (BD Biosciences, Canada) according to the manufacturer’s instructions. In short, each sample of plasma was diluted 4 times with the Standard/Sample Diluent provided. All standards and samples were analyzed in duplo. Subsequently, 50 µl of ELISA Diluent and 50 µl of standard or sample was added to each well and incubated for 2 hours at room temperature. After 5 rounds of decanting and washing, 100 µl of Working Detector was added to each well and incubated for 1 hour at
room temperature. Seven rounds of decanting and washing followed, after which 100 µl of TMB One-Step Substrate was added to each well and the plate incubated for 30 minutes at room temperature in the dark. Finally, 50 µl of the Stop Solution was pipetted into each well. The absorbance was measured at 450 nm, with a wavelength correction of 570 nm using the Synergy H4 Hybrid Reader (Biotek, USA) and the All in One Microplate Reader Software Gen5, version 2.00.18.

Statistical analysis
Statistical analysis was performed using SPSS 22.0 for Windows. Sigmaplot 13.0 was used for generating the figures. Normality of distribution was tested by Kolmogorov-Smirnov tests. Differences in baseline characteristics were examined using ANOVA for continuous data, with Fisher’s LSD post-hoc testing. Data are presented as mean±SEM, or median (25th-75th percentile). Differences were considered significant when p<0.05.

Results
Systemic inflammatory response
To confirm induction of a systemic inflammatory response in the major surgery model, blood leukocyte numbers and plasma IL-6 levels were measured. Leucocyte count in adult mice did not change at all time points, compared to control. In aged mice, the variation in leukocyte count was much larger than in adult mice. Moreover, aged mice had a significantly higher leukocyte count than adult mice at 4h post-MSu (Figure 1A).

Adult and aged control mice animals showed similar basal levels of IL-6. In adult mice, MSu induced a significant increase in plasma Il-6 levels at 4h, which returned to baseline after 24h (Figure 1B). However, MSu in aged mice caused a prominent increase in plasma Il-6 levels at 4h, which persisted until 24h in 1 out of 5 mice. Il-6 levels between adult and aged mice did not differ at 4h or 24h. Collectively, these data demonstrate that MSu successfully induced a systemic inflammatory response, which seemed more prominent in aged compared to adult mice.
Figure 1. Leucocyte counts (panel A) and plasma IL-6 levels (panel B) and in adult and aged mice (n= 4-5/group). Significant differences are indicated with * for p<0.05, compared to adult control group. ## refers to a significant difference of p<0.01 in plasma levels between adult and aged mice.
**Pro-inflammatory response after major surgery in the brain**

MSu caused an increase in whole brain *Il-6* mRNA (Figure 2A), but not *Il-1β* and *Tnf-α* expression (Figures 2B,C). In adult mice, sham and MSu displayed an increase in brain *Il-6* levels at 4h, amounting respectively 1.7±0.2 fold (p=0.001) and 1.6±0.1 fold increases (p=0.002) compared to adult control animals (Figure 2A). Follow-up at 24h revealed a substantial decrease in the *Il-6* mRNA in MSu, amounting 0.7±0.1 fold of controls (p=0.034). Baseline levels of brain *Il-6* mRNA of control aged mice were 1.6 fold higher (p=0.047) compared to adult controls, and did not increase further in sham at 4h follow up. However, in aged mice subjected to MSu, the level of *Il-6* mRNA at 4h follow-up increased substantially by 2.2±0.2 fold (p=0.010) and subsequently decreased to 1.1±0.1 fold (p=0.045) compared to control aged mice. Thus, aged mice generally express *Il-6* at a higher level than adult mice, yet a significant decrease is seen in both aged and adult mice at 24h after MSu.

Levels of *Il-1β* remained unchanged in MSu and sham groups for adult mice. Relative mRNA expression of *Il-1β* in aged control mice was 1.4 fold increased, yet not significantly higher than adult control (p=0.334). At 4h MSu, we found relative levels of brain *Il-1β* for aged mice to be 2.1 (p=0.254), compared to 1.4 in adult mice (p=0.168). *Tnf-α* expression levels were also similar for MSu and sham groups in adult mice. However, there was a significantly induction of *Tnf-α* in aged mice at 24h post-MSu (p=0.031) compared to adult mice. Moreover, levels of pro-inflammatory cytokines in whole brain of aged mice are generally higher than in adult mice. After MSu we reported increased pro-inflammatory cytokines, but not exponentially or for a protracted time period when compared to adult counterparts (Figure 2).
Figure 2. RT-qPCR analysis for inflammatory markers in brain tissue from adult and aged mice. Significant differences are indicated with */** for p<0.05 and p<0.01 respectively, compared to control group. #/## refer to significant differences in relative expression between adult and aged mice. Relative expression is ΔCt, normalized to Hmbs.
Figure 3. Truncated Bdnf protein levels in whole brain lysates after major surgery in adult and aged mice. Significant differences are indicated with * for p<0.05, compared to adult control group. # refer to a significant differences in relative expression levels between adult and aged mice of p<0.05.

Cerebral tBdnf expression after major surgery

To specifically address neural supportive properties in response to major surgery in ageing, protein levels of tBdnf were determined in whole brain by western blotting (Figure 3). tBdnf expression in adult mice was similar to control in all treatment groups. In aged mice, cerebral tBdnf expression in control, 4h MSu and 24h MSu was significantly lower compared to the corresponding adult groups. However, sham groups of adult and aged mice did not differ and aged mice in the sham group had a significantly higher tBdnf expression than aged mice in the control group (p=0.033). tBdnf expression levels at 4h and 24h MSu were similar to control in aged and adult mice alike.

Taken together, we found increased levels of pro-inflammatory cytokines in the brain of aged mice combined with significantly lower levels of tBdnf expression. Microglia are the principal immune cells of the CNS and the first to respond to homeostatic disturbances. To determine if MSu-associated inflammation affected microglia, these cells were isolated from the CNS.
**Microglia specific analysis of inflammation and phagocytosis in aged mice**

Microglia were identified and isolated as CD11b\(^{\text{high}}\) CD45\(^{\text{int}}\) cells by FACS (Figure 4A). The number of viable microglial cells that could be isolated at 24h post-MSu was ~50% higher than the microglial cell number in the control group (Figure 4B).

![Figure 4](image)

**Figure 4.** Microglia sorted with fluorescence activated cell sorting. Microglial cells were identified by their level of CD11b and CD45 (A). The number of microglia in each group is quantified (B). Significant differences are indicated with ** for p<0.01, compared to control group.
Next, mRNA levels of inflammation related genes were measured in sorted microglia including *Il-6, Il-1β, Tnf-α, Axl, Mac2*, and *Bdnf* (Figure 5). Axl and Mac2 are proteins involved in phagocytosis and related to glial priming (Zagórska et al., 2014; Holtman et al., 2015). In line with the results of *Il-6* mRNA levels in whole brain, we detected a significant increase in microglial mRNA expression of *Il-6* at 4h MSu (p=0.045), followed by a strong decrease at 24h post-MSu (p=0.005; Figure 5A). Also similar to whole brain, both *Il-1β* and *Tnf-α* showed highest levels at 4h MSu, but differences did not reach statistical significance. *Tnf-α* expression sharply declined at 24h after MSu (p=0.027). Interestingly, sham procedure induced a substantial increase in *Axl* (p=0.023) at 4h compared to control, but was without effect on *Il-6, Il-1β* and *Tnf-α*. *Bdnf* expression in microglia was not significantly changed, but appeared increased in sham group.

**Discussion**

Our results demonstrate that major surgery invokes a modest systemic inflammatory response, which seems amplified and prolonged in aged male mice compared to young adult male mice. Further, aged mice display increased mRNA levels of brain pro-inflammatory cytokines at baseline compared to adults, which, however, did not result in an exacerbated increase by either sham or MSu. Aged mice display decreased levels of Bdnf protein levels at baseline, which persisted in mice subjected to sham and MSu, with a minor increase in sham operated mice compared to baseline. Finally, MSu in aged mice resulted in an increased number of microglia that could be isolated at 24h and the induction of mRNA expression of multiple inflammatory factors at 4h post-MSu. Taken together, although aged mice display activated microglia, MSu did not provoke an amplified or long-lasting neuroinflammatory response as in line with a primed microglia dependent immune response, despite a moderately increased and protracted systemic inflammatory response.
Figure 5. RT-qPCR analysis of isolated microglia from aged mice on pro-inflammatory cytokine mRNA of *Il-6*, *Il-1β*, and *Tnf-α* (Panel A-C), proteins involved in phagocytosis *Mac2* and *Axl* (Panel D, E), and neurotrophin *Bdnf* (Panel F). Relative expression is ∆Ct, normalized to *Hmbs*. Significant differences are indicated with */** for p<0.05 and p<0.01 respectively, compared to control group.
An innate immune response is not necessarily triggered by local pathogens (Rivest et al., 2009), but may also be induced by a systemic inflammatory response, as observed for the neuroinflammatory response (Hoogland et al., 2015; Ho et al., 2015). Apparently, the MSu procedure we applied -without stimulation with LPS or invoking sepsis - induced a modest neuroinflammatory response, which was detectable in the whole brain as a temporary and mild increase in Il-6 mRNA levels at 4h MSu. Notably, MSu did not induce a long-lasting neuroinflammatory response, as we found that at 24h post-MSu both the whole brain level of Il-6 in mice and microglial mRNA expression of Il-6 and Tnf-α had decreased significantly compared to control. This may indicate a negative feedback mechanism, which has been described prior in neuroinflammation-related cytokine production (Kiefer et al., 1993). Thus, the neuroinflammation triggered by sole MSu as performed in our study, is strikingly different from the one provoked by MSu involving LPS administration or sepsis, which not only induces a strong increase in pro-inflammatory cytokines in the acute postoperative phase at 3-7 hours (Griffin et al., 2013), but can last for up to 10 months in case of chronic neuroinflammation (Qin et al., 2007). Consequently, given its short timeframe, neuroinflammation resulting from MSu under sterile conditions would be expected to constitute a possible contributor to postoperative acute delirium rather than to postoperative cognitive dysfunction.

Microglial cells largely drive the neuroinflammatory response. The parallel of pro-inflammatory cytokines we found in whole brain and sorted microglia support this notion. Additionally, the amount of microglia sorted from the CNS increased significantly at 24h MSu (Figure 3B). Possibly, microglial cells proliferate during the 24h period post-MSu. Otherwise, as a consequence of morphological changes, activated glial cells might have a higher chance of passing through the flow cytometry procedure without apoptosis inducing cellular damage.

We found decreased tBdnf expression in aged mice compared to adult, suggesting that there may be changes in proteolytic processing of pro-Bdnf into truncated Bdnf in aged mice. Prior, a similar decrease of truncated proteolitic isoforms of Bdnf was observed in patients with autism (Garcia et al., 2012) and schizophrenia (Carlino et al., 2011), which might lead to changes in connectivity and synaptic plasticity and consequently in cognitive performance.
Increased levels of tBdnf, which is suggested to potentially enhance healthy aging and help to regenerate certain neuronal populations in some degenerative pathologies (Tapia-Arancibia et al., 2008), were found only in sham operated aged mice. Clearly, the age-related decrease in Bdnf production appears to originate mechanistically from decreased CaMKII activity in the NMDAR-CaMKII pathway (Palomer et al., 2016), but is not irreversible as it still can be enhanced in aged mice by ketamine anaesthesia.

Thus, aged mice display multiple potentially detrimental factors, being decreased tBdnf expression, and high pro-inflammatory cytokine levels at baseline. Hence, we investigated microglia of aged mice separately.

Although the production of cytokines may trigger apoptosis and secondary neuronal damage, microglial activation can induce the phenotypical shift towards phagocytosis in order to remove apoptotic cells and debris (Koizumi et al., 2007). Such a switch to a phagocytic phenotype following MSu seems however unlikely, as we did not find an increase of associated microglial markers, notably Mac2 or Axl. It also seems unlikely that the absence of increased Axl and Mac2 expression in MSu at 4h or 24h is caused by the timing of the collection of material, because we did find an increase in the sham group which was also collected at 4h after the procedure. Therefore, MSu might not have triggered an increase in apoptotic cells and debris to be cleared by phagocytosis, when compared to control. Diffuse traumatic brain injury is known to upregulate Mac2 after microglial activation (Venkatesan et al., 2010). In addition, microglial cells isolated from ERCC1Δ/Δ mice, a model for accelerated aging, express high levels of Mac2 (Raj et al., 2014) and maintain increased Mac2 expression and an activated phenotype even after being grafted into chimeric organotypic hippocampal slice cultures (Masuch et al., 2016). MSu might be too modest a trigger to induce Mac2, even when conducted in aged mice.

We observed significant differences in Il-6 expression in whole brain and in microglia. However, significant differences in the mRNA expression of Il-1β and Tnf-α at 4h MSu were not detected, possibly because of small sample sizes. We did not observe exponential increases in inflammatory processes on whole brain level, nor on the cellular level in microglia. It might be that this is already decreasing at the 4h MSu time point, and that the highest levels should be
expected earlier. However, studies using LPS show a moderate increase in neuroinflammation after 3h, reaching profound activation between 8h and 2 days after a single challenge (Hoogland et al., 2015).

Conclusion

Aged mice express higher amounts of pro-inflammatory cytokines in the entire brain, irrespective of a systemic inflammatory impulse. In addition, aged mice express truncated Bdnf at a lower level than adult mice, possibly indicating reduced neuroregenerative properties. After major surgery without sepsis, there is a short-lasting and modest increase in the pro-inflammatory cytokine Il-6. Apparently, primed microglia of aged mice are still capable of adequately responding to mild systemic inflammatory processes and Bdnf expression is still inducible by ketamine anaesthesia. Thus, our study suggests that as POCD occurs more often in elderly patients, it is unlikely that the neuroinflammatory response serves as a logical explanation for this.
Part B