Decreased expression of the anti-inflammatory cytokine IL-4 in the hippocampus in response to tumours in a mouse model of breast cancer

Lisanne van de Bovekamp (5809088), Master Neuroscience and Cognition, Utrecht University
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First examiner: Dr Adam Walker, Laboratory of ImmunoPsychiatry – Neuroscience Research Australia
Second examiner: Dr Emma van Bodegraven, Department of Translational Neuroscience - Brain Division University Medical Center Utrecht

Abstract

Cancer patients with non-central nervous system (non-CNS) tumours often suffer from cognitive impairment. Previous studies have demonstrated cancer-related cognitive impairment in a 4T1.2 mouse model of breast cancer and identified inflammation as a causal mechanism. Here, we show that enhanced inflammation coincides with tumour growth. We investigated the relationship between inflammatory cytokine transcription in non-CNS tumours and the hippocampus and assessed its potential as a predictor of hippocampal inflammation. We observed increased transcription of pro-inflammatory cytokines in the microenvironment of 4T1.2 tumours. This transcription pattern was not translated to the hippocampi of 4T1.2 tumour-bearing mice as we found no difference in pro-inflammatory cytokines compared to tumour naïve controls. Interestingly, we did find decreased transcription of anti-inflammatory cytokine IL-4 in the hippocampus of tumour-bearing mice. Moreover, we showed that tumour growth and inflammation also coincide with decreased hippocampal IL-10 transcription, further implicating anti-inflammatory cytokines in cognitive impairment. Together, our findings suggest that systemic inflammation induced by tumours does not directly translate to inflammatory cytokine gene expression in the hippocampus. However, a dysregulated anti-inflammatory response in the hippocampus may contribute to cancer-related cognitive impairment.

Keywords: Cancer-related cognitive impairment, Cancer-induced cognitive impairment, cytokines, pro-inflammatory, anti-inflammatory, mammary tumour, tumour microenvironment, mice study, sickness response, IL-4 decrease, hippocampus, IL-10 decrease

1. Introduction

Cognitive impairment and depression are common in cancer patients with solid non-central nervous system (CNS) tumours (Yang *et al.,* 2013; Winocur *et al.,* 2018; Li *et al.,* 2019; Niedzwiedz *et al.,* 2019). Up to 70% of breast cancer survivors report deficits in concentration, multitasking, word-finding, learning and memory, often coupled with fatigue, depression, and anxiety (Somerset *et al.,* 2004; Krebber *et al.,* 2014; Janelsins *et al.,* 2014; Niedzwiedz *et al.,* 2019; Oppegaard *et al.,* 2021). These symptoms are partially attributed to the psychological impact of having a life-threatening illness (Yang & Hendrix, 2018). However, learning- and memory-related behavioural abnormalities have been reported in tumour-bearing rodents that do not experience the distress caused by a cancer diagnosis, suggesting that other factors besides psychological distress contribute to cancer-related cognitive impairment (CRCI) (Pyter *et al.,* 2010; Walker *et al.,* 2018). Moreover, cognitive impairment and depression in cancer patients are often associated with chemotherapy and were initially referred to as "chemo brain". However, clinical studies demonstrate cognitive impairment before any treatment, implicating cancer itself (Oppegaard *et al.,* 2021).

Neuroinflammation is one proposed mechanism for CRCI. Inflammation is a hallmark of cancer, and studies in non-cancer settings have demonstrated a correlation between inflammation and learning- and memory-related behavioural abnormalities (Hanahan & Weinberg, 2011; Singhal *et al.,* 2014). Several studies have reported an association between increased circulating inflammatory markers and cognitive symptoms in breast cancer patients (Patel *et al.,* 2015; Cheung *et al.,* 2015; Pomykala *et al.,* 2013; Lyon *et al.,* 2016). Several rodent models of breast cancer have demonstrated that neuroinflammation is associated with behavioural changes relevant to anxiety, stress-coping and cognitive deficits (Pyter *et al.,* 2009; Pyter *et al.,* 2010; Pyter *et al.,* 2017; Walker *et al.,* 2018). Using multiple mouse models of breast cancer, our laboratory has demonstrated increased pro-inflammatory cytokine protein secretion by tumour cells and an increase in hippocampal pro-inflammatory cytokines and microglial activation in response to mammary cancer (Walker *et al.,* 2018; McCaffrey *et al.,* 2022). In support of inflammation playing a causal role in cancer-related cognitive impairment, our laboratory has demonstrated that the anti-inflammatory drug aspirin improved memory function in tumour-bearing mice without affecting primary tumour growth or metastasis (Walker *et al.,* 2018). Likewise, retrospective epidemiological analyses in two Swedish cohort studies showed that prior aspirin use, especially long-term and low-dose use, was associated with a reduced rate of depression, anxiety, and stress-related disorders during the first year after cancer diagnosis (Hu *et al.,* 2020), as well as accidental deaths (e.g., falls and car accidents) which are associated with cognitive symptoms. Together, these findings implicate inflammation as a critical causal player in CRCI.

While our preclinical rodent models have shown that tumour-cell secreted factors, including pro-inflammatory cytokines interleukin 1 beta (IL-1β) or tumour necrosis factor-alpha (TNF-α), coincide with behavioural signs of sickness and worse memory (Walker *et al.,* 2018), other non-transformed cells capable of secreting inflammatory molecules are frequently seen in the tumour microenvironment. These include stromal cells like fibroblasts, mesenchymal stem cells, endothelial cells, pericytes, and immune cells like macrophages and lymphocytes (Whiteside, 2008). The interactions between tumours and non-transformed cells are crucial for determining how cancer develops. The cells can induce or inhibit tumour initiation, growth, migration, metastasis, and drug resistance and induce recruitment, activation, and differentiation of other cells in the tumour environment by secreting pro- and anti-inflammatory cytokines (Greten & Grivennikov, 2019). Cytokines secreted by the tumour

microenvironment can influence the brain resulting in behavioural signs of sickness, including fever, loss of interest in social and physical activities and fatigue (Dantzer *et al.,* 2008). After inflammation, these effects should subside. However, adaptive sickness behaviour in response to inflammation can switch to maladaptive anxiety and depression (Dantzer *et al.,* 2008). Similarly, pro-inflammatory cytokines like TNF-α and IL-1β can cause depressive-like behaviours when injected intracerebroventricularly (Palin *et al*., 2008; O'Connor *et al*., 2009a, b, c; Fu *et al*., 2010).

Once released from the tumour microenvironment, cytokines can circulate throughout the body and propagate to the brain via two known main routes, humoral and neural (Dantzer, 2008). With humoral signalling, cytokines can be transported into the brain by binding to cytokine transporters on endothelial cells at the blood-brain barrier that, in turn, release IL-1 and prostaglandins within the brain parenchyma (Dantzer, 2008). The neural route consists of signalling from vagal afferents that, when activated, express cytokine receptors, resulting in a neural signal to the brain. Another possibility is that cytokines or mRNA are transported to the brain from the tumour microenvironment through extracellular vesicles (Skog *et al.,* 2008; Meads *et al.,* 2009). These vesicles can directly cross the blood-brain barrier and release the cargo into the brain (Malhotra *et al.,* 2018). We sought to find if cytokines secreted by a solid, non-CNS tumour directly reflect the inflammatory response in the hippocampus in cancer-related cognitive impairment. To our knowledge, no study has compared the transcriptional profile in the brain with that of the tumour microenvironment to determine the extent to which tumour biopsies may serve as a biomarker of cancer-related brain inflammation.

To address this question, we will compare cytokine gene expression profiles found in the tumours and hippocampus of 4T1.2 mammary cancer-bearing mice. The hippocampus was selected because this region is essential in many of the symptoms observed in patients with cancer-related cognitive impairment, including function, concentration, attention, depression, anxiety, and earlier findings support that mammary tumours are sufficient to activate microglia, the primary immune cells in the CNS, in stress and anxiety-related neurocircuitry in this breast cancer model (McCaffery *et al.,* 2018). Pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α and anti-inflammatory cytokines IL-4, IL-5 and IL-10 were measured in the tumour microenvironment and the hippocampus because these specific cytokines were found the be involved in cognitive impairment, depression, and sickness behaviour (Dantzer*,* 2008; Li *et al.,* 2019; Renner *et al.,* 2022).

Aim: To determine if the magnitude of cytokines transcribed in the tumour micro-environment reflects the magnitude of cytokine transcription in the brain. Hypothesis: We expect that a bigger tumour correlates to more inflammation in the periphery resulting in a higher response in the hippocampus which would result in higher levels of inflammatory cytokine gene expression in this region, leading to cognitive impairments.

2. Methods

2.1 Animals and Ethics

Individually housed female BALB/c mice (n = 24, 6-8 weeks old at arrival) were kept in conventional shoebox cages in a controlled environment with a 12/12-h reversed light cycle (lights on at 19:00). Before the trial, mice were given a week to acclimate to the reversed light cycle and the NeuRA Animal Facility. Water and food were available *ad libitum* (standard chow, Gordons irradiated feed). Two equal groups of mice carrying tumours and mice without tumours were randomly assigned. The University of New South Wales Animal Ethics Committee and the National Health and Medical Research Council approved the protocols used for all mouse operations (ethics number: 4520210). The animals were monitored daily to ensure they were not in pain. The Morton (1997) Clinical Signs Severity Score checklist had humane objectives based on reduced body mass (>15%), tumour size (>1.3 cm^3), and a clinical severity score > 2 utilising body condition grading (Morton, 1997). At the end of the experiment, mice were euthanised using CO_2. There was no unexpected mortality.

2.2 Breast cancer model

2.2.1 Cell culture growth

4T1.2 mammary adenocarcinoma cells syngeneic to BALB/c mice were used in this experiment (Lelekakis *et al.*, 1999). Cell culture procedures were performed using aseptic techniques in a laminar flow hood (Thermo Fisher Hersasafe KS12). The tumour cells were retrieved from liquid nitrogen tanks and thawed at room temperature. To remove the storage solution (10% DMSO in FBS), cells were placed into a 15 mL falcon tube containing 9 mL of Dulbecco's Modified Eagle Medium (DMEM) with low glucose and free of serum, pyruvate, L-glutamine, HEPES and phenol red (Sigma-Aldrich) + 10% Fetal Bovine (FBS). The supernatant was collected after the cells were pelletised in a centrifuge (200 x g, 4 minutes, 22°C). The cells were seeded onto a T25 cell-culture flask, resuspended in 10 mL of 37°C DMEM with 10% FBS, and incubated for at least a week (37°C, 5% CO_2). To ensure I could culture cells without contamination and accurately assess cell division and expansion over time, a separate vial of 4T1.2 cells was thawed and lifted, counted using a haemocytometer and reseeded multiple times over a week before being discarded (Figure 1).

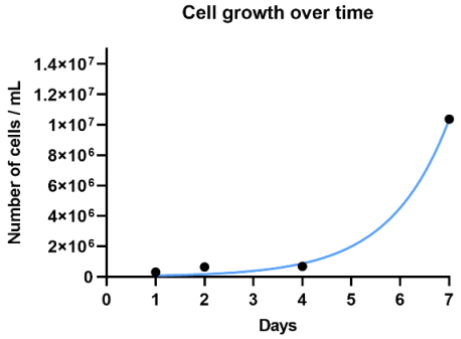

Figure 1: Cell growth over time of 4T1.2 cells

On the day of injection, the cells were washed with 5 mL of phosphate-buffered saline (PBS) and lifted using 5 mL PBS + 10% trypsin Ethylenediaminetetraacetic acid (T-EDTA) and incubated at 37°C for 5 minutes. After incubation, in a 1:1 ratio, fresh DMEM + 10% FBS was added to the T-EDTA to wash the flask. The cells were again pelletised in a centrifuge, the supernatant was removed, and the cells were resuspended in 1 mL fresh PBS. A haemocytometer was used to count the cells after diluting 10 µL of

resuspended cells 1:1 with Trypan Blue. After counting, the PBS volume was adjusted to 1 x 10^5cells per 20ul PBS.

2.2.2 Tumour induction

1 x 10^5 4T1.2 tumour cells cultured using aseptic methods were resuspended in 20 µl of sterile phosphate-buffered saline (PBS) (Invitrogen, USA) and injected into the fourth left mammary fat pad under 3% isoflurane anaesthesia (Walker *et al*., 2018). The control animals received injections of 20 µl sterile PBS under anaesthesia. Once the tumours were palpable, a digital calliper was used to assess primary tumour growth three times a week, and the tumour volume was determined using the formula (length x width^2)/2.

2.3 Tissue collection

Nineteen days after the tumour cell injection, mice were euthanised using a CO_2 chamber. The mice were perfused to drain the hippocampus from blood, so we are not measuring transcription levels from peripheral immune cells and circulating blood cells. Primary tumours and spleens were dissected, weighed, and frozen on dry ice. Of the control mice that received PBS, the fourth mammary fat pad was dissected, weighed and frozen as a control for the tumours. The hippocampi from tumour-bearing and non-tumour-bearing mice were dissected, weighed, and quickly frozen on dry ice. All tissues were stored at -80°C until processing.

2.4 Gene expression analysis

2.4.1 RNA extraction

600 µl of TRIzol reagent (Invitrogen, USA) was used to extract total RNA from the tumour and hippocampus according to the manufacturer's instructions (Life Technologies, USA). With axygen pestles, tissue samples were homogenised. Then 120 µl of chloroform (Sigma-Aldrich, Australia) was added to the homogenate to split into aqueous and phenol phases. The samples were centrifuged for 15 minutes at 12,000 g and 4°C. The aqueous phase was collected, placed into a new Eppendorf tube, and combined with 400 µl of 100% isopropanol to precipitate the RNA (Sigma-Aldirich, Australia). The supernatant from the tubes was discarded after a second centrifugation at 12,000 g for 10 minutes at 4°C. Then the pellet was rinsed in 800 µl of 70% ethanol (Ethanol absolute, Sigma-Aldirich, Australia). After centrifuging the tubes at 7,500 g, 4°C for five minutes, the ethanol supernatant was removed, and the tubes were air-dried. RNA pellets were dissolved in 30 µl of double-distilled water, and NanoDrop® ND100 was used to evaluate the purity and concentration of the RNA based on the 280/260 ratio, and a subset was confirmed to be of good quality. The extracted tumour-mRNA was additionally tested for RNA integrity numbers (RIN) (Agilent 2100 Bioanalyzer) but was not repeated for the fat pads or the hippocampi.

2.4.2 cDNA synthesis

Using the using SuperScript® IV (SSIV) First-Strand Synthesis Kit, cDNA was synthesised from 2 µg total RNA per sample following the manufacturer's protocol (Life Technologies, USA). A total of 11 mL of a mixture containing 2 g of total RNA diluted in water treated with diethyl procarbonate, 50 ng/mL random hexamers, and 10 nM deoxynucleotide triphosphate was pipetted to the bottom of a 96-well plate for primer annealing. The plate was heated to 65°C for 5 min using an Applied Biosystems GeneAmp PCR 9700 thermal cycler before being cooled at 4°C. To combine with the annealed mix, a reaction mix containing 4 µl of SSIV buffer, 1 µl of dithiothreitol, 1 µl of ribonuclease inhibitor, and 1 µl of SSIV reverse transcriptase was added to each well. The plate was then incubated for 10 minutes at

23°C, 55°C, and 80°C to deactivate it. No-reverse transcriptase and no-template controls were used on each plate to prevent contamination of genomic DNA and reagents.

2.4.3 RT-qPCR

RT-qPCR was used with 2 µg of total RNA to quantify changes in gene expression. RT-qPCR assays were conducted on seven pro- and anti-inflammatory genes (Table 1). Using pre-designed Taqman Gene Expression assays, the messenger RNA (mRNA) expression of genes was assessed in a GeneAmp PCR 9700 thermal cycler (Applied Biosystems, USA). 40 PCR amplification cycles of strand separation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 50 °C for 10 minutes were performed on the RNA and probe mixture.

The 2-ΔΔCT technique was used to normalise relative gene expression levels to the housekeeping gene actin beta (*Actb*). ΔΔCT scores are shown as a fold gene expression change relative to the control set to 1. In correlations, the ΔΔCT scores instead of fold change were plotted against the mass (Supplementary Figure 1).

Table 1: List of Taqman probes for gene expression assays

	Gene of interest	Taqman probe
Cytokines	Interleukin 1 alpha (*il1a*)	Mm00439620_m1
	Interleukin 1 beta (*il1b*)	Mm00434228_m1
	Interleukin 4 (*il4*)	Mm00445259_m1
	Interleukin 5 (*il5*)	Mm00439646_m1
	Interleukin 6 (*il6*)	Mm00446190_m1
	Interleukin 10 (*il10*)	Mm00439616_m1
	Tumour necrosis factor alpha (*TNF-α*)	Mm00443258_m1
Housekeeping gene	Actin beta (*Actb*)	Mm00607939_m1

2.5 Statistical analysis

IBM SPSS Statistics v.26 was used for the statistical analysis, and GraphPad Prism (9.2) was used to create the figures. Data on gene expression were analysed using a two-way ANOVA (ANOVA). Outliers were identified and eliminated based on whether they were more than two standard deviations from the mean. Using the Shapiro-Wilk test, the normality of the data was evaluated, and Levene's test for Equality of Error Variances was used to evaluate homogeneity. Nonparametric Mann-Whitney U tests were performed when the Log10 transformation did not provide a normal distribution. Post-hoc Fisher's least significant difference (LSD) tests with α adjusted to 0.05 were used to identify pairwise differences between groups or times when significant interaction effects were present. Significant values have been adjusted by the Hochberg correction for multiple ANOVA tests and the Bonferroni correction for multiple pairwise comparison tests. Pearson's correlation analyses were carried out between cycle threshold (Ct) values of the mRNA and tumour- and spleen mass in mg to ascertain if tumour or hippocampus gene expression changes were connected to tumour- or spleen mass changes.

3. Results

3.1 Mammary tumour-bearing mice show evidence of systemic inflammation

To explore the effect of peripheral tumours on hippocampal gene expression of inflammatory cytokines, BALB/c mice were injected with 4T1.2 tumour cells. Tumour volume was monitored using a digital calliper, and mice were euthanised 19 days after tumour cell inoculation. Tumours became palpable nine days after tumour cell injection and continued to grow until harvesting (Fig 1A). The mean tumour mass at day 19 reached 112.8 mg, SD = 61.8 mg. We evaluated if the mice showed spleen enlargement suggestive of myeloid cell number expansion indicative of systemic inflammation. We also assessed if the magnitude of spleen mass was associated with tumour size (Walker *et al.,* 2018). 4T1.2 tumours significantly increased spleen mass compared to cancer naïve control mice (Fig 1B, $t_{(21)}$=2.992, p=0.007). Furthermore, mammary tumour mass correlated with increased spleen mass, suggesting that the magnitude of cancer burden is associated with the extent of systemic inflammation (Fig 1C, $r_{(10)}$=0.78, p=0.005).

Cancer in its advanced stages frequently causes sickness behaviour and cachexia (Aoyagi *et al.,* 2015). Body weight measurements over the days after tumour cell injection revealed no change in body weight (Fig 1D, $F_{(1,23)}$=0.485, p=0.231) with extensive monitoring using the Animal Care and Ethics committee Cage / Phenotype Monitoring Sheets provided by the University of New South Wales. This indicates that the tumour-bearing mice displayed no sickness response in the context of weight loss as a result of reduced food intake.

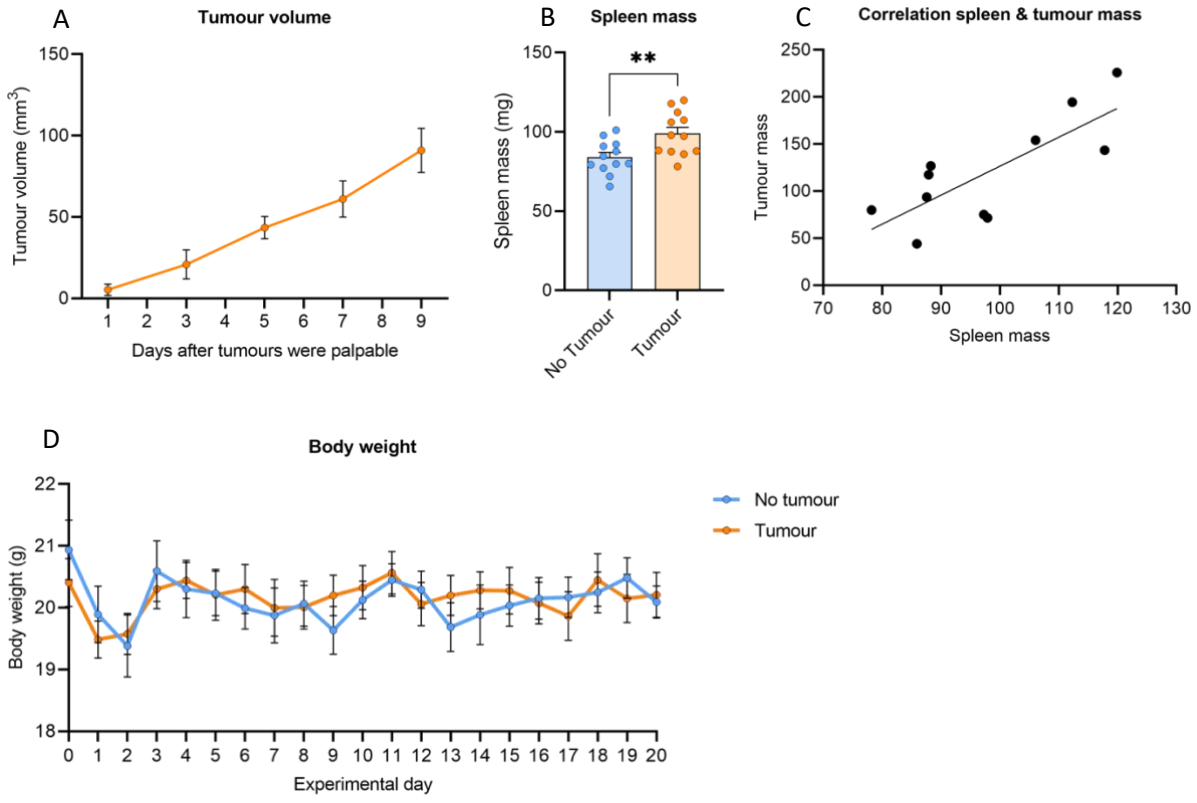

Figure 1: Mammary tumour growth-induced inflammation in mice. A: Tumour volume increased over time as measured by a digital calliper. **B:** Spleen mass was increased in tumour-bearing mice compared to control mice, $t_{(21)}$=2.992, p=0.007. **C:** Tumour and spleen mass were positively correlated, $r_{(10)}$=0.78, p=0.005. **D:** No difference in body weight between tumour-bearing mice and control mice, $F_{(1,23)}$=0.485, p=0.231.

3.2 Increased pro-inflammatory cytokine gene expression in the tumour microenvironment.

To explore the direct involvement of the tumour microenvironment in the systemic inflammatory response, we measured gene expression of pro-inflammatory cytokines (Figure 2A-2D) and anti-inflammatory cytokines (Figure 2E-2G) transcribed in the tumour microenvironment. The pro-inflammatory cytokines, also referred to as tumour-promoting cytokines, IL-1α (Figure 2A, $t_{(8)} = 5.836$, p=0.0004), IL-1β (Figure 2B, $t_{(10)} = 3.982$, p=0.01) and TNF-α (Figure 2D, $t_{(9)} = 6.912$, p=0.003) were all significantly upregulated in the tumour microenvironment compared to the mammary fat pad used as control tissue. Furthermore, a trend of upregulation of IL-6 was measured in the tumour microenvironment (Fig 2C, $t_{(9)} = 2.121$, p=0.06).

For anti-inflammatory cytokines, there was a trend of upregulation in IL-4 expression in the tumour microenvironment compared to the fat pad (Fig 2E, $t_{(8)} = 2.197$, p=0.06). However, there were no significant differences between gene expression in the tumour microenvironment and the control for IL-5 (Figure 2F, $t_{(7)} = 1.262$, p=0.09) and IL-10 (Figure 2G, $t_{(9)} = 1.498$, p=0.19).

Finally, we wanted to see if the size of the tumour relates to gene expression levels for inflammatory cytokines, as we hypothesised that the bigger tumour includes more malignant and immune cells in the tumour microenvironment, which could increase the gene expression of inflammatory cytokines. We did not find a correlation (Supplementary Figure 1).

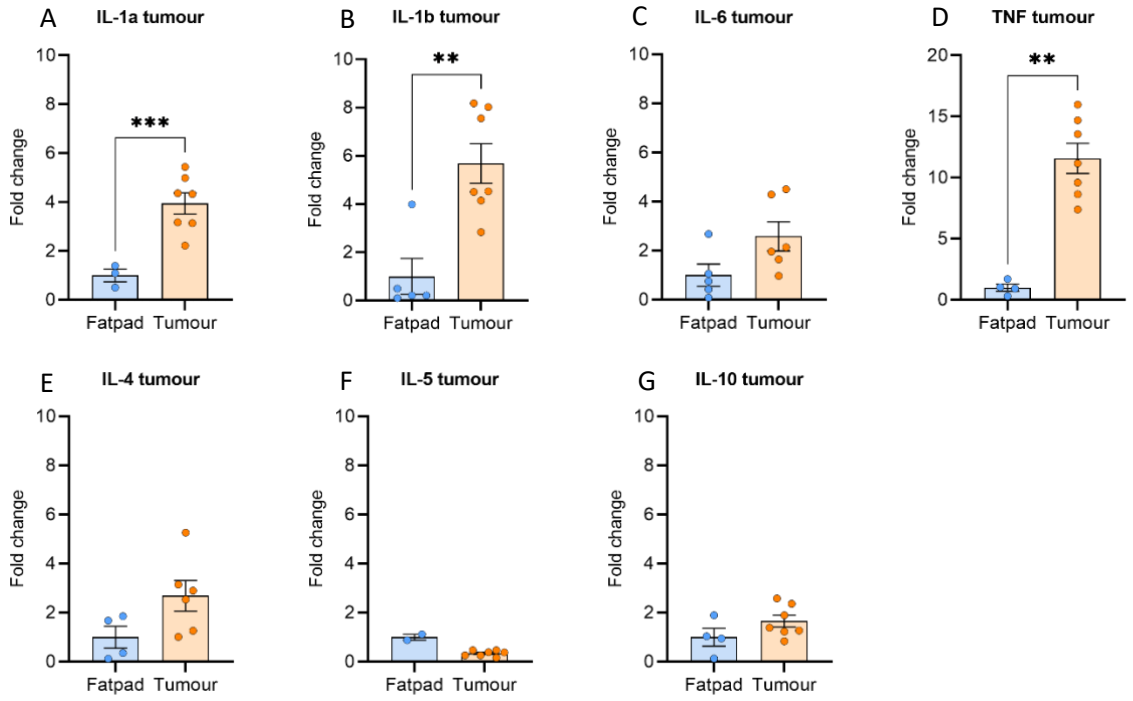

Figure 2: Upregulation of pro-inflammatory cytokines in tumour microenvironment. Data are shown in fold change with the fat pad data set to 1. **A:** Increased gene expression of the pro-inflammatory cytokine IL-1α in the tumour microenvironment compared to fat pad tissue, $t_{(8)} = 5.836$, p=0.0004. **B:** Upregulation of IL-1β in tumour microenvironment, $t_{(10)} = 3.982$, p=0.01. **C:** A trend in upregulation of IL-6 in the tumour microenvironment, $t_{(9)} = 2.121$, p=0.06. **D:** upregulation of TNF-α in the tumour microenvironment, $t_{(9)} = 6.912$, p=0.003. **E:** A trend in upregulation of the anti-inflammatory cytokine IL-4, $t_{(8)} = 2.197$, p=0.06. **F:** No significant difference in gene expression of IL-5, $t_{(7)} = 1.262$, p=0.09. **G:** No significant difference in gene expression of IL-10, $t_{(9)} = 1.498$, p=0.19.

3.3 Downregulation of anti-inflammatory cytokine IL-4 in the hippocampus of tumour-bearing mice

After we measured the increased gene expression of pro-inflammatory cytokines in the tumour microenvironment, we examined the gene expression of pro- and anti-inflammatory cytokines in the hippocampus. We are looking to see if the gene expression matches that measured in the tumour microenvironment. Inconsistent with our hypothesis, hippocampal mRNA levels for all pro-inflammatory cytokines measured did not significantly differ between 4T1.2 tumour-bearing mice and cancer naïve controls (IL-1α: Figure 3A, $t_{(20)} = 0.224$, p=0.825, IL-1β: Figure 3B, $t_{(19)} = 0.713$, p=0.485, IL-6: Figure 3C, $t_{(17)} = 0.669$, p=0.513, and TNF-α: Figure 3D, $t_{(16)} = 0.106$, p=0.917).

The anti-inflammatory cytokine IL-4 is significantly downregulated in the hippocampus of 4T1.2 tumour-bearing mice (Figure 3E, U=26, p=0.023). However, no effects were measured in the anti-inflammatory IL-5 ($t_{(22)} = 0.170$, p=0.86) and IL-10 ($t_{(15)}$=0.030, p=0.977) (Figure 3F, 3G).

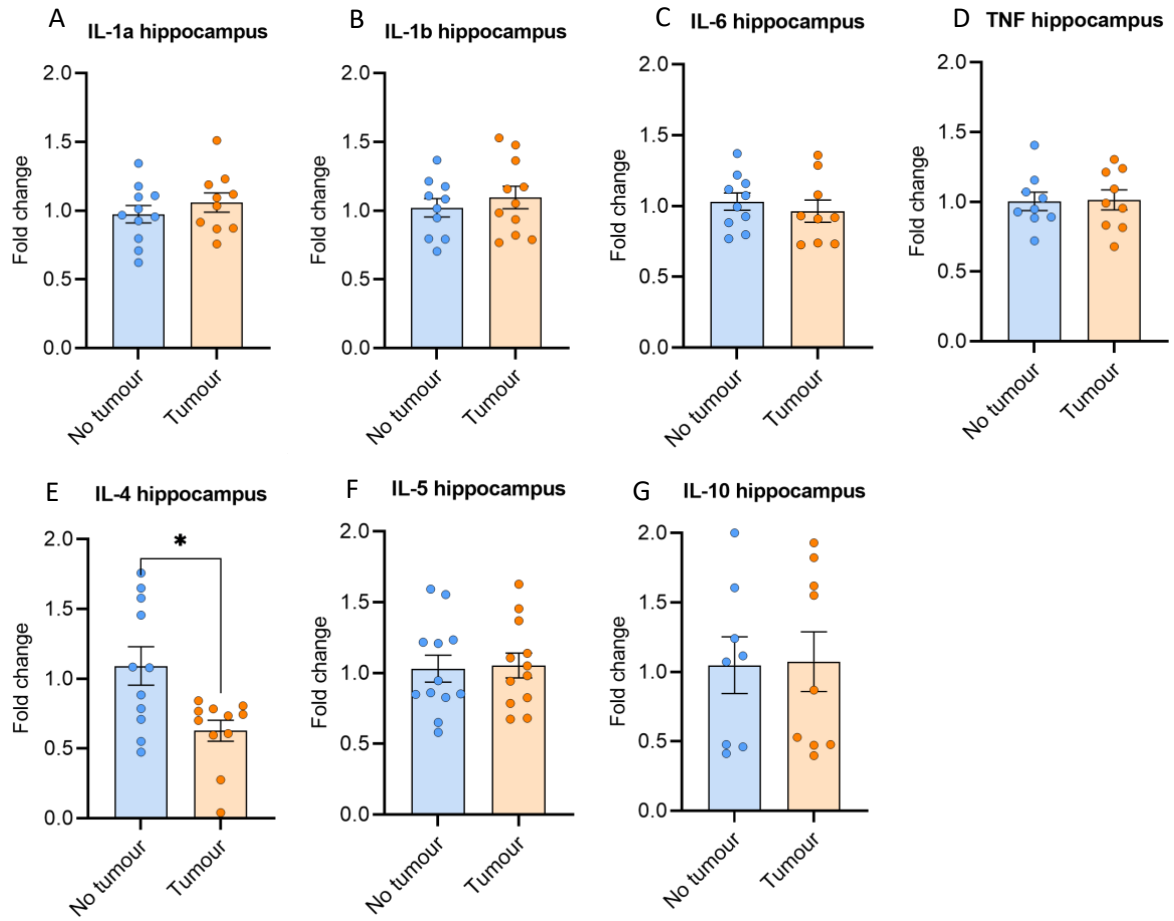

Figure 3: Cytokine gene expression in the hippocampus. Data are shown in fold change with the control group set to 1. **A:** No difference in hippocampal gene expression of IL-1α $t_{(20)}$=0.224, p=0.825. **B:** No difference in hippocampal gene expression of IL-1β, $t_{(19)}$=0.713, p=0.485. **C:** No difference in hippocampal gene expression of IL-6, $t_{(17)} = 0.669$, p=0.513. **D:** No difference in hippocampal gene expression of TNF-α, $t_{(16)} = 0.106$, p=0.917. **E:** Reduced hippocampal gene expression of IL-4 in 4T1.2 tumour-bearing mice compared to tumour naïve controls, U=26, p=0.023. **F:** No difference in hippocampal gene expression of IL-5, $t_{(22)} = 0.170$, p=0.86. **G:** No difference in hippocampal gene expression of IL-10, $t_{(15)}$=0.030, p=0.977.

3.4 Decreased IL-10 expression in the hippocampus with growing tumour size and inflammation

To determine if systemic inflammation can predict brain inflammation, we tested if gene expression of inflammatory cytokines in the hippocampus is associated with tumour- or spleen mass. We found no correlations for most inflammatory cytokines (Supplementary Figure 1). We did, however, find a correlation between higher CT scores indicating low expression of the anti-inflammatory IL-10 in the hippocampus and both spleen mass (Figure 4A, $r_{(12)}$=0.612, p=0.035) and tumour mass (Figure 4B, $r_{(10)}$=0.684, p=0.02). Besides, there was a trend of a negative relation between the CT scores of IL-10 in the tumour microenvironment and tumour mass, indicating higher IL-10 expression in the tumour microenvironment in bigger tumours (Figure 4C, $r_{(6)}$=-0.751, p=0.052) despite no significant effect on a group level for IL-10 expression (Figure 2G).

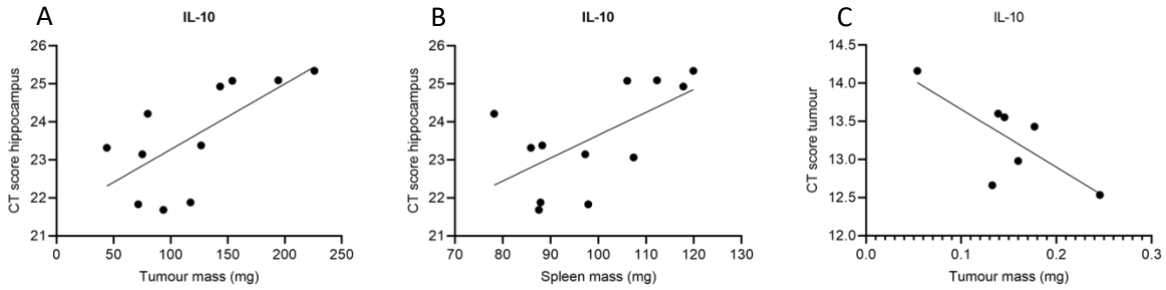

Figure 4: Correlations in hippocampal expression of IL-10 and increased systemic inflammation by increased tumour size. A: Tumour mass positively correlates with hippocampal CT score, $r_{(10)}$=0.684, p=0.02. **B:** Spleen mass positively correlates with hippocampal CT score, $r_{(12)}$=0.612, p=0.035. **C:** A correlative trend exists between tumour mass and CT score, $r_{(6)}$=-0.751, p=0.052.

4. Discussion

This study aimed to investigate the relationship between cytokine gene expression in the tumour microenvironment and the hippocampus in mammary tumour-bearing mice. We hypothesised that larger tumours would result in more inflammation, leading to higher levels of inflammatory cytokine gene expression in the hippocampus and subsequent cognitive impairments. We found that high levels of pro-inflammatory cytokines are expressed in the tumour microenvironment. However, this is not matched in the hippocampus as it showed a downregulation in the anti-inflammatory cytokine IL-4 but no difference in pro-inflammatory cytokine transcription. Furthermore, we found a relation between increased tumour and spleen mass, indicating systemic inflammation coinciding with cancer progression. Correlation analyses revealed that higher tumour and spleen mass were associated with decreased gene expression of IL-10 in the hippocampus. Thus far, studies measuring mRNA or protein levels in the brain of rodent models of cancer-related cognitive impairment only focussed on the involvement of pro-inflammatory cytokines but not enough on anti-inflammatory cytokines. With the findings in this study, we highlight the involvement of anti-inflammatory cytokines in the hippocampus of tumour-bearing mice. Furthermore, these findings suggest that tumour-induced systemic inflammation does not directly translate to inflammatory cytokine gene expression in the hippocampus.

Pro-inflammatory cytokines in the tumour microenvironment

Cancer-related cognitive impairment is suggested to be associated with the inflammatory response induced by a solid non-CNS tumour (Walker *et al.,* 2018). Tumours actively recruit immune cells from the surrounding tissues to create a favourable environment for their growth and survival using cytokines (Landskon *et al.,* 2014). Therefore, the environment surrounding the tumour comprises various immune cells, such as macrophages, neutrophils, and lymphocytes (Whiteside, 2008). Cancer cells can manipulate their microenvironment to promote inflammation. As a result, these immune cells often secrete pro-inflammatory cytokines as part of their response to the tumour. Cancer development is accelerated and promoted by cytokine production from the tumour and inflammation in the tumour microenvironment (Greten & Grivennikov, 2019). Our data found that the 4T1.2 tumours transcribe pro-inflammatory cytokines consistent with what is known about the role of pro-inflammatory cytokines in the tumour microenvironment.

Consistent with our findings, many studies report increased transcription of IL-1α, IL-1β, IL-6 and TNF-α in the tumour microenvironment (Dantzer *et al.,* 2008). Increased transcription of IL-1α in mammary tumours promotes the production of the cytokine thymic stromal lymphopoietin (TSLP) from tumour-infiltrating myeloid cells, which aids in cancer cell survival and metastasis (Kuan & Ziegler, 2018). IL-1β is suggested to induce breast tumour genesis by increasing IL-6 production via a transglutaminase 2/NF-B route or by employing the fibroblast growth factor receptor 1 (FGFR1)-induced mouse mammary carcinoma model, thereby implicating IL-1β-mediated expression of COX-2 (Oh *et al.,* 2016; Reed *et al.,* 2009). Upregulation of TNF-α in mammary tumours can activate various signalling pathways, including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), which promote tumour cell survival, proliferation, and angiogenesis (Landskron *et al.,* 2014). Furthermore, TNF-α can promote the recruitment of immune cells to the tumour site, including dendritic cells, T cells, and natural killer cells. However, it can also contribute to immune suppression by promoting the generation of regulatory T cells and myeloid-derived suppressor cells, which dampen anti-tumour immune responses (Landskron *et al.,* 2014). Upregulated IL-6 can contribute to tumour growth and survival by promoting cell proliferation, inhibiting apoptosis, and inducing angiogenesis (Tanaka *et al.,* 2014). Furthermore, IL-6

can enhance the recruitment and activation of immune cells, including macrophages and neutrophils, to the tumour microenvironment. IL-6 can also affect the differentiation and function of immune cells, such as T cells and regulatory T cells, potentially impacting anti-tumour immune responses (Tanaka *et al.,* 2014). In conclusion, the findings of this study provide further support for the increased transcription of pro-inflammatory cytokines in the tumour microenvironment, such as IL-1α, IL-1β, IL-6, and TNF-α, which play crucial roles in promoting tumour growth, survival, and immune modulation. These cytokines contribute to the complex interplay between cancer cells and the immune system, influencing tumour progression and the tumour microenvironment.

Anti-inflammatory cytokines in the tumour microenvironment

Anti-inflammatory cytokines can affect cell division and proliferation, initiate or alter specific cellular processes and inhibit tumour cell proliferation by promoting apoptosis or turning immune cells against the tumour cells (Kartikasari *et al.,* 2021). However, tumours have developed various mechanisms to evade immune surveillance and create an immunosuppressive microenvironment. By expressing anti-inflammatory cytokines, tumours can suppress immune responses and dampen the activity of immune cells (Landskron *et al.,* 2014). This immune suppression helps the tumour evade destruction by the immune system, effectively promoting its growth and survival. Our data demonstrated no significant expression of the anti-inflammatory cytokines IL-5 and IL-10. However, we found a trend in the upregulation of the anti-inflammatory IL-4 in the tumour microenvironment. Upregulation of IL-4 in the tumour microenvironment of mammary tumours may contribute to tumour growth and metastasis by enhancing cancer cell survival and invasive potential (Venmar *et al.,* 2014). Specifically, it can inhibit the activation and function of cytotoxic T cells and natural killer cells, essential for immune-mediated tumour control. Additionally, IL-4 can promote the generation and recruitment of immunosuppressive regulatory T cells and myeloid-derived suppressor cells, which dampen anti-tumour immune responses (Venmar *et al.,* 2014). Thus, we confirm a potential role for the immunosuppressive cytokine IL-4 in modulating the tumour microenvironment. A similar study demonstrated that levels of IL-1β, IL-6, TNF-α, and IL-4 were increased in the serum of mice with 4T1 tumours compared to controls (Walker *et al.,* 2017). However, unlike what many have reported before, the mammary tumours did not alter depressive-like behaviour or learned fear. In this current study, we have not performed any behavioural testing, but we did not observe a sickness response in the tumour-bearing mice as measured by body weight over time. Future studies should also aim to include behavioural responses to gauge the *in trans* effects of tumour cytokine expression accurately.

Pro-inflammatory cytokines in the hippocampus of mammary tumour-bearing mice

Our data show no differences in the transcription of any pro-inflammatory cytokines between the hippocampi in tumour-bearing and tumour naïve mice. Inconsistent with our mRNA data, in our unpublished protein data studying the same mouse model, we found that IL-1β protein levels were increased in the hippocampus of tumour-bearing mice. The IL-1β protein measured in the hippocampus may be circulating IL-1β that crosses the blood-brain barrier (Banks & Erickson, 2010; Wang *et al.,* 2015). However, in the same unpublished data, we found no difference in IL-1β protein levels in serum, suggesting that IL-1β might accumulate in the hippocampus. Additionally, we found that increased IL-6 protein levels in serum relate to increased IL-1β levels in the hippocampus. This might mean that circulating IL-6 could induce IL-1β through similar mechanisms in which IL-1β can induce IL-6 (Cahill & Rogers, 2008).

We should note, however, that protein measurement of cytokines is less sensitive than mRNA, can be timing-dependent and hence fluctuate dynamically. Furthermore, protein assessment of cytokines in the brain reflects locally synthesised cytokines and those that enter the brain from circulation. On the other hand, evaluating brain cytokine mRNA more accurately represents the transcriptional footprint of the brain parenchyma, with the caveat that resident immune cells in the brain may contribute to cytokine mRNA expression.

IL-4 mRNA was decreased in the hippocampus of mammary tumour-bearing mice

We found no effect on the transcription of the anti-inflammatory IL-5 and IL-10 in the hippocampus of tumour-bearing mice compared to tumour naïve mice. However, we did find decreased levels of IL-4 mRNA in the hippocampus of tumour-bearing mice. This is in line with findings from other rodent studies that show a reduction in IL-4 in the hippocampus of aged rodents and Alzheimer's disease mice models together with increased LTP deficits (Maher *et al.,* 2005; Boccardi *et al.,* 2019) and administration of IL-4 rescued the observed LTP deficits (Nolan *et al.,* 2005). Likewise, IL-4 protein levels were also reduced in anxious mice (Lee *et al.,* 2016) and an IL-4 injection attenuated anxiety-like behaviour (Han *et al.,* 2015). An IL-4 injection could be a valuable next step in researching the effect of homeostatic or increased hippocampal levels of IL-4 to assess the reduction in cancer-related cognitive impairment. Another study measuring the effects of mild stress in mice showed an increase in IL-4 expression in the hippocampus, suggesting that the effect of stress on the hippocampus is different to the other cognitively impaired models (You *et al.,* 2011). However, these findings are inconsistent with our earlier findings that show similar microglial activation in subcortical regions of tumour-bearing mice compared to mice subjected to chronic stress (McCaffrey *et al.,* 2022).

The downregulation of IL-4 in the hippocampus in mammary tumour-bearing mice may contribute to the chronic inflammation observed in cancer-related cognitive impairment via multiple mechanisms. Reduced IL-4 levels can disrupt the balance between pro-inflammatory and anti-inflammatory signals in the brain, potentially leading to neuronal dysfunction, impaired synaptic plasticity, and cognitive deficits (Maher *et al.,* 2005). However, the inflammation and immune response may be confined to specific brain regions without significant changes in systemic pro-inflammatory cytokine levels in the hippocampus (Silverman *et al.,* 2014). Therefore, while levels of pro-inflammatory cytokine expression may remain relatively stable, the hippocampus could still experience inflammation leading to a downregulation of IL-4. Additionally, cancer can alter the immune cell populations within the brain, including microglia and infiltrating immune cells, playing a critical role in regulating neuroinflammation and cytokine production (Landskon *et al.,* 2014; McCaffrey *et al.,* 2022). Dysregulation of these immune cells, even without significant changes in pro-inflammatory cytokine expression, could lead to altered cytokine profiles in the hippocampus, including reduced IL-4 expression. In addition, different cell types within the hippocampus may exhibit varied responses to cancer-related inflammation (Liu *et al.,* 2019). It is possible that specific cell populations within the hippocampus experience alterations in IL-4 expression while pro-inflammatory cytokines remain relatively unchanged. This cell-specific response could contribute to the downregulation of IL-4 in the hippocampus observed in this study. *In vitro* models, such as co-cultures of immune cells and neurons, can enable us to investigate the interactions between immune cells, cytokines, and neuronal function and which cell population specifically secretes which cytokines. By recreating the cell environment of the hippocampus and manipulating the levels of pro-inflammatory cytokines, we can assess the impact on IL-4 expression and its effects on neuronal function. These experiments could provide insights into the cell-specific effects and mechanisms underlying the downregulation of IL-4. Lastly, cytokine production and regulation can vary as the cancer

progresses (Eftekhari *et al.,* 2017). Pro-inflammatory cytokines may be elevated at specific time points or during acute phases of inflammation, while IL-4 downregulation occurs later or persists in a chronic state. Timing and duration of measurements may influence the apparent discrepancy between pro-inflammatory cytokines and IL-4 expression. Alternatively, single-cell RNA sequencing of resident brain immune cells may reveal changes in their transcriptional profiles in tumour-bearing mice *versus* naïve mice. In conclusion, the downregulation of IL-4 in the hippocampus of tumour-bearing mice may contribute to cancer-related cognitive impairment, potentially through disruption of the pro-inflammatory/anti-inflammatory balance, altered immune cell populations, cell-specific responses, and temporal dynamics of cytokine regulation. Further studies investigating the cell-specific effects, underlying mechanisms, and temporal changes in cytokine production are warranted better to understand the role of IL-4 in cancer-related cognitive impairment.

Inflammatory cytokine transcription in the hippocampus may depend on cancer progression.

For this project, tissue from two separate study replicates was assessed. mRNA levels in tumours and inflammatory cytokine protein levels in the hippocampus were measured in the same mice. mRNA levels in the hippocampus shown in this study were measured in other mice. The tumours from the second batch of mice were 1.7-fold smaller than those tested in this study. This suggests that the second mice were in an earlier stage of cancer progression than the first batch. This might affect the transcription in the tumour microenvironment (Eftekhari *et al.,* 2017). Additionally, the hippocampal response may vary depending on cancer progression. For future studies, I suggest dissecting the mice based on tumour volume instead of the number of days after tumour cell injection.

We cannot compare mRNA data from a tumour in an early stage of cancer development to protein levels in a later stage. Thus, mRNA levels should be measured in the tumours from the mice we measured the hippocampi from to dissect differences in tumour gene expression in different stages of cancer progression. Furthermore, because the tumours measured are not derived from the same mice in which the hippocampi were measured, we were not able to perform correlation analyses on the tumour transcription and the hippocampus transcription to assess if they match and thus answer the question if the tumour might be suited as a biomarker to predict cancer-related cognitive impairment. From the data gathered in this study, we can hypothesise that the IL-4 transcription is downregulated in the hippocampus early in tumour progression before the increase in IL-1β measured in other studies (Pyter *et al.,* 2009; Walker *et al.,* 2017). This downregulation may be because there is already a lot of IL-4 protein in the hippocampus as a protective response to the tumour-secreted inflammatory cytokines. This has to be confirmed by measuring protein levels in this stage of cancer progression. Then, later in cancer progression, as measured in our protein study, IL-1β levels are upregulated compared to the control. Previous studies found that IL-4 can inhibit the synthesis of IL-1β in circulating cells, but this observed downregulation in IL-4 gene expression in the hippocampus might lead to an increase in IL-1β expression (Maher *et al.,* 2005).

In conclusion, due to differences in cancer progression between the tumour and hippocampus measurements, we could not directly relate the gene expression in the tumour microenvironment to the hippocampus and determine if the tumour can serve as a biomarker for cancer-related cognitive impairment. However, our findings suggest a potential downregulation of IL-4 gene expression in the hippocampus early in tumour progression, which may contribute to the subsequent upregulation of IL-1β observed in later stages. Further investigations involving protein measurements and a more precise

alignment of tumour and hippocampus stages are necessary to validate these hypotheses and understand the dynamics of cytokine expression in cancer-related cognitive impairment.

Relationship between tumour size and inflammation

We found evidence that increased tumour size relates to increased spleen mass. 4T1.2 tumour cells are a validated model for breast cancer and are known to induce myeloid cell expansion, thereby increasing spleen size, indicative of systemic inflammation (Walker *et al.,* 2018). We found that the tumour and spleen mass correlate to IL-10 transcription in both the tumour microenvironment and the hippocampus. In the tumour microenvironment, increased tumour mass correlated to higher transcription of IL-10. The tumour and immune cells in the microenvironment can secrete IL-10 (Sabat *et al.,* 2010), where it can downregulate pro-inflammatory cytokine gene expression (Schottelius *et al.,* 1999). IL-10 may also reduce the presentation of antigens, cell maturation, and differentiation, enabling cancer cells to avoid immune defences (Hamidullah *et al.,* 2012). In the hippocampus, increased tumour and spleen mass correlated with decreased transcription of IL-10. Decreased IL-10 in the hippocampus may lead to reduced protection. It is possible that IL-10 from the tumour microenvironment reached the hippocampus, for example, through extracellular microvesicles, and the hippocampus responds by decreasing the transcription of IL-10. Reduced anti-inflammatory, including IL-4 and IL-10 levels in the brain, may make the brain lose its protective abilities, making it more vulnerable.

Alternative mediators of elevated inflammation in the brain in tumour-bearing mice

This study measured a subset of inflammatory cytokines in the hippocampus based on previous literature connecting these cytokines to cognitive impairment and depression (Dantzer, 2008; Li *et al.,* 2019; Renner *et al.,* 2022). We measured changes in all pro-inflammatory cytokines, but no anti-inflammatory cytokines in the tumour microenvironment but no effect in those same pro-inflammatory cytokines in the hippocampus. There might not exist a direct link between tumour-secreted cytokines and cytokines expressed in the hippocampus as a response. In our unpublished data measuring cytokine protein levels, we saw a correlation between serum IL-1α and hippocampal IL-6 and serum IL-6 and hippocampal TNF-α, demonstrating that there may not be a direct correlation between cytokine secretion in the tumour *versus* that in the hippocampus. Another way to measure if there is a direct response in the hippocampus to the tumour is to look at extracellular vesicles (EVs) secreted by the tumour microenvironment. EVs can be tagged and localised to see if they reach the hippocampus and can be compared to other tissues to see if transportation is brain-specific or 'normal' metastasis through EVs. Furthermore, other inflammatory candidates, including chemoattractants, chemokines and growth factors, might be key players in cancer-related cognitive impairment. Therefore, further studies should include more inflammatory markers in studying the inflammatory response in the hippocampus and other brain regions in a mouse model of breast cancer. Additionally, behavioural tests should be included to see if the measured molecular effects indeed result in cancer-related cognitive impairments similar to those seen in patients. In conclusion, our study suggests that there may not be a direct link between tumour-secreted cytokines and cytokines expressed in the hippocampus, highlighting the need for further investigation using alternative approaches, such as examining extracellular vesicles and exploring additional inflammatory markers to understand better the inflammatory response in the hippocampus and its implications for cancer-related cognitive impairments.

5. Conclusion

This study investigated the correlation between inflammatory cytokine gene expression in the tumour microenvironment and the hippocampus in 4T1.2 mammary tumour-bearing mice. The results revealed elevated expression of pro-inflammatory cytokines in the tumour microenvironment, consistent with their known role in promoting tumour growth. Surprisingly, there was no corresponding increase in pro-inflammatory cytokine gene expression in the hippocampus. Instead, the anti-inflammatory cytokine IL-4 showed downregulation in the hippocampus, suggesting an imbalance between pro-inflammatory and anti-inflammatory signals in the brain. We also found that tumour size correlated with systemic inflammation, as evidenced by increased spleen mass. Moreover, both tumour and spleen mass correlated with IL-10 gene expression in the tumour microenvironment and the hippocampus. These findings indicate that the tumour microenvironment can influence immune responses and cytokine production, potentially impacting cancer-related cognitive impairment. However, due to the utilisation of tissue from separate experiments with varying tumour sizes, it remains to be seen whether the magnitude of cytokine gene expression in the tumour microenvironment can predict the extent of cytokine transcription in the brain. Further investigations are necessary to fill in the gaps and determine whether tumours could serve as biomarkers for cancer-related cognitive impairment. It is important to note that the underlying mechanisms of cancer-related cognitive impairment and cytokine regulation in the brain are still actively researched. Multiple factors, including other cytokines, oxidative stress, neuroinflammation, and alterations in neurotransmitter systems, likely contribute to cognitive impairment in cancer patients. Ongoing studies aim to shed light on these mechanisms and identify potential therapeutic interventions for alleviating cognitive symptoms in cancer patients. Future research should focus on studying a wider range of inflammatory markers and incorporating behavioural tests to understand the inflammatory response and its impact on cognitive function in cancer-related cognitive impairment. Additionally, further investigations are needed to elucidate the specific mechanisms and cell types involved in the modulation of cytokine expression in the tumour microenvironment and the hippocampus.

6. Lay summary

Many cancer survivors, even years after treatment, struggle with cognitive impairment. These people have difficulties with short-term memory, organisational skills, problem-solving and learning and retaining information. When people have cancer, the body starts an inflammatory response which can reach the brain and trigger these symptoms. In this study, we wanted to see if we could predict if this person is at risk of developing cognitive impairment with a tumour biopsy. To test this, we looked at gene expression levels of inflammatory markers in the tumour and the hippocampus. The hippocampus is the brain region most likely to be affected in people with the symptoms of cognitive impairment. We injected mice with cancer cells in breast tissue, and after 19 days, we took the tumour and hippocampus to measure gene expression. We found that the tumour makes pro-inflammatory immune molecules that help them grow. However, we did not observe increased levels of pro-inflammatory cytokines when we examined the hippocampus. Interestingly, we found that these mice made less of the anti-inflammatory molecule IL-4 compared to mice without tumours. This may make the brain less capable of protecting itself against pro-inflammatory molecules. We also measured the size of the tumour and the spleen. The spleen can grow in size if the body is experiencing high levels of inflammation. We found that with a bigger tumour, the spleen is also bigger. This suggests that a bigger tumour creates a higher inflammatory response. We also found that with bigger tumours and bigger spleens, the gene expression of the anti-inflammatory IL-10 is lowered in the hippocampus but higher in the tumour. Thus, based on these findings, the anti-inflammatory molecules could be more informative in predicting or treating cancer-related cognitive impairment instead of the more commonly looked at pro-inflammatory molecules. This research provides insights into the complex relationship between cancer, inflammation, and cognitive impairment. While inflammation in the tumour microenvironment is present, the impact on brain inflammation and cognitive function is not straightforward. Further studies are needed to understand the underlying mechanisms and identify potential therapeutic targets for managing cognitive impairment in cancer patients.

7. References

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8. Supplementary data

Supplementary Table 1: Statistical output for repeated measures ANOVA on mice body weight and t-test on spleen mass

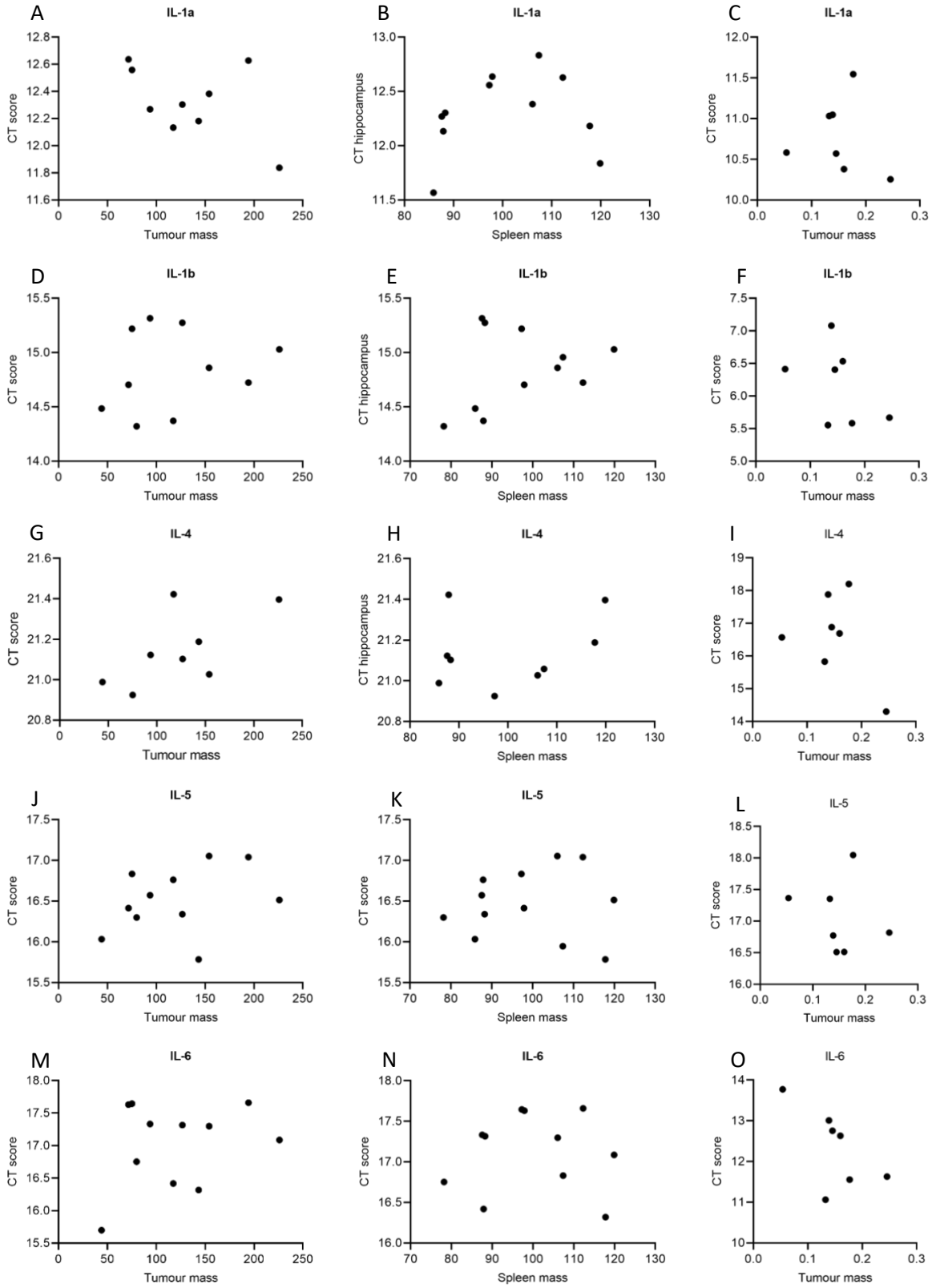
Main interaction/effect	Statistical output
Body weight tumour vs. no tumour with Epsilon Huynh-Feldt correction	$F_{(10.811)}$=1.615, p=0.097
Spleen mass tumour vs. no tumour	$t_{(22)}$=2.992, p=0.007

Supplementary Table 2: Statistical output for t-tests on gene expression

Gene	Main interaction/effect	Statistical output
IL1a	Tumour vs. fat pad	$t_{(8)}$=5.836, p=0.0004*
	Hippocampus tumour vs. no tumour	$t_{(20)}$=0.224, p=0.825
IL1b	Tumour vs. fat pad	$t_{(10)}$=3.982, p=0.013*
	Hippocampus tumour vs. no tumour	$t_{(19)}$=0.713, p=0.485
IL4	Tumour vs. fat pad	$t_{(8)}$=2.197, p=0.059
	Hippocampus tumour vs. no tumour	U=26, p=0.023*
IL5	Tumour vs. fat pad	$t_{(7)}$=4.981, p=0.088
	Hippocampus tumour vs. no tumour	$t_{(22)}$=0.170, p=0.866
IL6	Tumour vs. fat pad	$t_{(9)}$=8.768, p=0.064
	Hippocampus tumour vs. no tumour	$t_{(17)}$=0.669, p=0.513
IL10	Tumour vs. fat pad	$t_{(9)}$=5.752, p=0.187
	Hippocampus tumour vs. no tumour	$t_{(15)}$=0.030, p=0.977
TNF-α	Tumour vs. fat pad	$t_{(9)}$=6.912, p=0.003*
	Hippocampus tumour vs. no tumour	$t_{(16)}$=0.106, p=0.917

Supplementary Table 3: Statistical output for Pearson's correlation

Main interaction/effect	Statistical output
Correlation spleen mass and tumour mass	$r_{(10)}$=0.779, p=0.005*
Correlation IL-1α CT score tumour and tumour mass	$r_{(6)}$=-0.134, p=0.774
Correlation IL-1α CT score hippocampus and tumour mass	$r_{(8)}$=-0.497, p=0.173
Correlation IL-1α CT score hippocampus and spleen mass	$r_{(11)}$=0.180, p=0.596
Correlation IL-1β CT score tumour and tumour mass	$r_{(6)}$=-0.420, p=0.349
Correlation IL-1β CT score hippocampus and tumour mass	$r_{(10)}$=0.049, p=0.887
Correlation IL-1β CT score hippocampus and spleen mass	$r_{(11)}$=0.298, p=0.373
Correlation IL-4 CT score tumour and tumour mass	$r_{(6)}$=-0.392, p=0.385
Correlation IL-4 CT score hippocampus and tumour mass	$r_{(10)}$=0.373, p=0.258
Correlation IL-4 CT score hippocampus and spleen mass	$r_{(9)}$=0.248, p=0.520
Correlation IL-5 CT score tumour and tumour mass	$r_{(6)}$=-0.158, p=0.736
Correlation IL-5 CT score hippocampus and tumour mass	$r_{(10)}$=0.329, p=0.323
Correlation IL-5 CT score hippocampus and spleen mass	$r_{(12)}$=0.018, p=0.956
Correlation IL-6 CT score tumour and tumour mass	$r_{(6)}$=-0.641, p=0.121
Correlation IL-6 CT score hippocampus and tumour mass	$r_{(10)}$=-0.270, p=0.421
Correlation IL-6 CT score hippocampus and spleen mass	$r_{(11)}$=-0.004, p=0.990
Correlation IL-10 CT score tumour and tumour mass	$r_{(6)}$=-0.751, p=0.052
Correlation IL-10 CT score hippocampus and tumour mass	$r_{(10)}$=0.684, p=0.02*
Correlation IL-10 CT score hippocampus and spleen mass	$r_{(12)}$=0.612, p=0.035*
Correlation TNF-α CT score tumour and tumour mass	$r_{(6)}$=0.230, p=0.619
Correlation TNF-α CT score hippocampus and tumour mass	$r_{(10)}$=-0.011, p=0.975
Correlation TNF-α CT score hippocampus and spleen mass	$r_{(12)}$=-0.128, p=0.692


A
IL-1a
CT score
Tumour mass
B
IL-1a
CT hippocampus
Spleen mass
C
IL-1a
CT score
Tumour mass
D
IL-1b
CT score
Tumour mass
E
IL-1b
CT hippocampus
Spleen mass
F
IL-1b
CT score
Tumour mass
G
IL-4
CT score
Tumour mass
H
IL-4
CT hippocampus
Spleen mass
I
IL-4
CT score
Tumour mass
J
IL-5
CT score
Tumour mass
K
IL-5
CT score
Spleen mass
L
IL-5
CT score
Tumour mass
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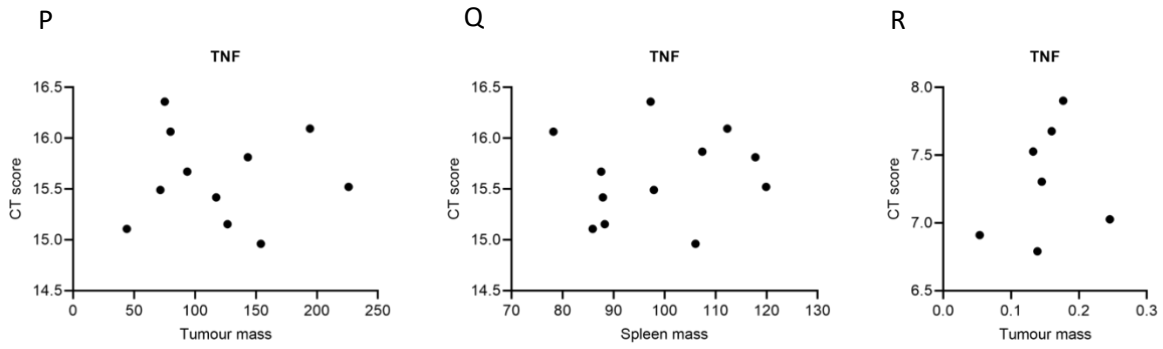
IL-6
CT score
Tumour mass
N
IL-6
CT score
Spleen mass
O
IL-6
CT score
Tumour mass



Supplementary Figure 1: Correlations of gene expression to tumour and spleen mass. A: IL-1α correlation tumour mass and hippocampal CT score, **B:** IL-1α correlation spleen mass and hippocampal CT score, **C:** IL-1α correlation tumour mass and tumour CT score, **D:** IL-1β correlation tumour mass and hippocampal CT score, **E:** IL-1β correlation spleen mass and hippocampal CT score, **F:** IL-1β correlation tumour mass and tumour CT score **G:** IL-4 correlation tumour mass and hippocampal CT score, **H:** IL-4 correlation spleen mass and hippocampal CT score, **I:** IL-4 correlation tumour mass and tumour CT score **J:** IL-5 correlation tumour mass and hippocampal CT score, **K:** IL-5 correlation spleen mass and hippocampal CT score, **L:** IL-5 correlation tumour mass and tumour CT score **M:** IL-6 correlation tumour mass and hippocampal CT score, **N:** IL-6 correlation spleen mass and hippocampal CT score, **O:** IL-6 correlation tumour mass and tumour CT score **P:** TNF-α correlation tumour mass and hippocampal CT score, **Q:** TNF-α correlation spleen mass and hippocampal CT score, **R:** TNF-α correlation tumour mass and tumour CT score.