5-Fluorouracil Induces an Acute Reduction in Neurogenesis and Persistent Neuroinflammation in a Mouse Model of the Neuropsychological Complications of Chemotherapy

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Abstract
The neuropsychological symptoms associated with chemotherapy treatment remain a major challenge with their prevention hampered by insufficient understanding of pathophysiology. While long-term neuroimmune changes have been identified as a hallmark feature shared by neurological symptoms, the exact timeline of mechanistic events preceding neuroinflammation, and the relationship between the glial cells driving this neuroinflammatory response, remain unclear. We therefore aimed to longitudinally characterize the neuroimmunological changes following systemic 5-fluorouracil (5-FU) treatment to gain insight into the timeline of events preceding the well-documented chronic neuroinflammation seen following chemotherapy. Eighteen female C57Bl/6 mice received a single intraperitoneal dose of 5-FU and groups were killed at days 1 and 2 (acute timepoint), days 4 and 8 (subacute timepoint), and days 16 and 32 (chronic timepoint). A further six mice were administered with vehicle control with tissues collected from three mice on day 1 and day 32 of the study. The expression of key genes of interest, BCL2, BDNF, TIMP1, MMP-9, MMP-2, TNFα, IL-1β, and IL-6R were assessed using real time polymerase chain reaction. Levels of neurogenesis were determined through immunofluorescent staining of doublecortin (DCX). The density of microglia and astrocytes were assessed using immunofluorescence staining of Iba1 and GFAP respectively. 5-FU treatment caused significant decreases to DCX staining at acute timepoints (p = 0.0030) which was positively correlated with BCL2 expression levels. An increase to microglial density was observed in the prefrontal cortex (p = 0.0256), CA3 region (p = 0.0283), and dentate gyrus (p = 0.0052) of the hippocampus at acute timepoints. 5-FU caused increases to astrocyte density, across multiple brains regions, at subacute and chronic timepoints which were positively correlated with TNFα, TIMP-1, MMP-2, and IL-6R expression. This study has identified acute objective neuroinflammatory changes suggesting that the role of early intervention should be explored to prevent the development of neuropsychological deficits in the longer-term following chemotherapy.

Keywords Chemistry · Neuropsychological deficits · Neuroinflammation · Neurogenesis · Supportive oncology

Introduction
Even in the era of novel therapies, chemotherapy remains the cornerstone of successful anti-cancer treatment for the majority of patients. Despite improvements in clinical efficacy, its use remains associated with severe side effects due to non-selective cytotoxicity that impacts nearly all body systems, including the central nervous system (CNS) [1]. The neuropsychological complications of chemotherapy encompass a constellation of neurological deficits that include typical cognitive impairments affecting memory, executive function, processing speeds and learning [2–4], in addition to psychological elements such as depression/anxiety, fear of...
recurrence and personality changes [5, 6]. These complications are particularly burdensome due to the chronicity of symptoms which persist long after treatment cessation, ultimately limiting employment prospects and severely impacting quality of life [7]. With cancer management increasingly being focussed on survivorship, there is now a heightened attention on minimising late treatment effects, with neuropsychological symptoms consistently identified as an unmet area of need by survivors of cancer [8].

Increasing evidence of neuropsychological symptom clusters has been observed in individuals treated with chemotherapy, suggestive of shared etiological mechanisms [9, 10]. Despite this, studies have failed to investigate the timeline of pathological events initiated by chemotherapy, which may be common to the global development of neuropsychological symptom clusters. Neuroimmune changes have been identified as a hallmark feature shared by these symptoms [11], suggesting that chemotherapy-induced neuroinflammation plays an important role in the pathophysiology of numerous neurological symptoms. Notably, chronic neuroinflammation has been strongly implicated in other clinical settings such as neurodegeneration and depression as well as fear and anxiety-based disorders [12–14]. While chronic neuroinflammation induced by chemotherapy treatment presents as a convincing mechanism, the exact timeline of potential mechanistic events occurring in the acute, subacute and/or chronic setting following initial exposure to chemotherapy agents, and their relationship with chronic neuroinflammation, remain unclear.

The neuroinflammation seen following chemotherapy has previously been linked to the action of microglia and astrocytes, key supportive cells within the CNS [15], with increases in their density and activation often described [16]. However, this role has largely been established through cross-sectional preclinical studies which often focus on one outcome measure, analyzed at one timepoint after chemotherapy. As such, the relationship between microglia and astrocytes following chemotherapy, including whether the chronic neuroinflammatory response is driven by one or both, remains to be defined. Furthermore, the cross-sectional design employed by preclinical studies has prevented investigation into the events preceding glial cell activation. This is particularly important to understand as gliosis most commonly occurs in response to a stress stimulus. Consequently, there is likely to be a triggering event responsible for glial cell migration and activation followed by subsequent neuroinflammation.

The migration and activation of glial cells can occur secondary to direct cytotoxic injury [6, 17], as seen in other organs systems and cell populations exposed to chemotherapy [18]. However, this mechanism remains controversial, as most chemotherapeutic drugs are not capable of crossing the highly restrictive blood brain barrier (BBB). Further, direct cytotoxic effects are difficult to rationalize given the propensity of chemotherapeutic drugs to induce cytotoxic injury to rapidly dividing cell populations, and the relatively low turnover rate of most CNS cells. While these strongly held beliefs have challenged our understanding of the etiology of neuropsychological symptoms, it is important to consider that (i) some chemotherapeutic drugs, particularly those with low molecular weights (e.g., 5-fluorouracil), are capable of crossing the BBB, (ii) BBB hyperpermeability has been reported after chemotherapy, increasing paracellular transport [19], and (iii) there are subpopulations of cells in the brain that are in fact highly proliferative.

Preclinical studies have shown certain chemotherapy drugs significantly reduce the number of actively proliferating neurons and immature neurons within the dentate gyrus of the hippocampus, a key site of adult neurogenesis [20–22], providing critical insight into the damaging effect of chemotherapy on proliferating neurons. Mechanistically, this direct cytotoxic injury may be sufficient to induce oxidative stress and resulting inflammation, which, with repeated doses of chemotherapy, would create sustained and chronic neuroinflammation. However, since analyses were only performed in the chronic setting following treatment, it is difficult to solely attribute causality to direct cytotoxicity when the prolonged stress and systemic inflammation commonly associated with chemotherapy may also contribute to impaired neurogenesis over such a sustained period of time [23–25]. We therefore aimed to longitudinally characterize the neuroimmunological changes of 5-fluorouracil (5-FU) treatment in order to gain insight into the timeline of events preceding the well-documented chronic neuroinflammation seen following chemotherapy. Specifically, we hypothesized that acute changes to neurogenesis would precede glial cell migration to brain regions of interest following administration of 5-FU.

**STAR Methods**

This study was reported using the STAR methods for structured, transparent and accessible reporting as well as the ARRIVE guidelines for the accurate and reproducible reporting of animal research.

**Experimental Model Details**

**Ethical Statement and Animal Husbandry**

The study was approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI, #SAM20-034) and complied with the National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014).
All experiments were conducted on female C57Bl/6 mice, obtained from the SAHMRI Bioresources Facility, aged 13 weeks at arrival and 14 weeks following 7 days of habituation prior to study commencement. Mice were group-housed (maximum of 5 animals/cage) in individually ventilated cages, under specific pathogen-free conditions at the SAHMRI Bioresources Facility (Adelaide, SA, Australia). They were housed under a 12-h light–dark cycle (lights on at 7:00 a.m.), at a constant temperature of 20–23 °C and humidity maintained between 45–75%, with ad libitum access to water and a standard chow diet (Teklad Global Diet #2918, Envigo). Sawdust and crinkle bedding, along with a toilet roll for enrichment, were provided in all cages.

To encourage normal feeding behavior following treatment, all mice were provided with soaked chow (standard chow softened with water to ease mastication) in a petri dish, on the cage floor as well as a heat mat to prevent hypothermia. All animals group-housed reached 10% weight loss (or above) often occurring 1 or 2 days following treatment. When all mice were provided with peanut butter (Bega Peanut Butter, Smooth) and DietGel 31 M (Clear H2O; 72–08-5022) in a petri dish, on the cage floor as well as a heat mat to prevent hypothermia.

**Study Design**

Mice were treated with a single 400 mg/kg dose of 5-FU (Sigma-Aldrich; F6627-10G), reconstituted at 10 mg/mL in sterile saline, via intraperitoneal (IP) injection (day 0). Following treatment, mice were randomly allocated into 6 experimental groups (N = 3/group), with tissues collected from each group at days 1, 2, 4, 8, 16, and 32 of the study. Data was pooled from animals with tissues collected on days 1 and 2, days 4 and 8, and days 16 and 32, to represent the acute (N = 6), subacute (N = 6), and chronic (N = 6) setting respectively following treatment. This was to ensure comprehensive coverage of the neuroimmune events occurring following 5-FU treatment within the experimental timeline. A further 6 mice, who received vehicle control administered via IP injection (day 0), were used to control for the effect of aging and stress due to repeated handling on outcome measures, with tissues being collected from N = 3 mice on day 1 and N = 3 mice on day 32 of the study (see supplementary Fig. 1).

**Tissue Collection**

All animals were anesthetized via inhalation isoflurane (2% in 1 L/min; Provet) and administered with 0.1 mL of sodium pentobarbital (325 g/mL, Lypard) intraperitoneally before transcardial perfusion with 20 mL of sterile saline (Vital Medical Supplies). The brains were dissected, with the left hemisphere snap frozen in liquid nitrogen and stored at −80 °C and the right hemisphere post-fixed in 10% formalin (Southern Cross Science) for 24 h prior to processing. The primary outcome measures for this study comprised of validated markers of neuroinflammation, neurogenesis and apoptosis. All analyses were performed blinded.

**Method Detail**

**RNA Extraction and Real-time Polymerase Chain Reaction**

RNA was extracted from the frontal cortex of the left hemisphere using the NuceloSpin RNA mini kit for RNA purification (Macherey-Nagel; 740,984.250) as per the manufacturer’s instructions. Briefly, frozen samples were homogenized, at room temperature, in 350μL of lysis buffer (Macherey-Nagel; 740,984.250) using the Qiagen TissueLyser LT (Qiagen) for 5 min at 50 Hz. Following a series of filtration, DNA digestion, and washing steps, highly purified RNA was eluted in RNase-free H2O and stored at −80 °C. Using a Nanodrop 100 spectrophotometer (Thermo Scientific), RNA yield and purity was determined. An amount of 1 μg of RNA from each sample was then reverse transcribed, as per the manufacturers’ instructions, using the iScript™ cDNA Synthesis Kit (BioRad; 1,706,891). RT-PCR was then performed using the Rotor-Gene 3000 (Corbett Research) to determine the level of expression of multiple genes of interest (Table 1). A total of 10 μL amplification mixes comprised of 1 μL of cDNA (prediluted to 100 ng/μL), 5 μL of QuantiTect SYBR green assay reagents (Qiagen; 204,143), 3 μL of RNase-free H2O, and 0.5 μL of both the forward and reverse primers for the gene of interest (prediluted to 50 pmol/μL). Primer sequences are detailed in Table 1. Thermal cycling conditions included an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 40 s. All samples were run in triplicate and cycle threshold (ct) values were calculated by the RotorGene 6 analysis software. The expression level for each gene of interest was calculated relative to the housekeeper gene β-actin using the Δct method, a variation of the Livak method [26].

**Immunofluorescence**

Formalin-fixed tissue was processed and embedded into paraffin wax. Using a rotary microtome (Leica; RM2235), 5-μM sagittal sections of the brain were cut and then mounted onto glass slides (Flex Plus Detection System, Dako; #K8020). Immunofluorescent analysis was performed for doublecortin (DCX), a protein expressed by neuronal precursor cells within the dentate gyrus of the hippocampus and commonly utilized as a marker of neurogenesis, as well as glial cell markers glial fibrillary acidic protein (GFAP) and ionized...
calcium-binding adaptor protein-1 (Iba1), which stain astrocytes and microglial respectively. Staining was performed using an automated system (AutostainerPlus, Dako; #AS480). Briefly, sections were dewaxed using xylene and rehydrated in graded ethanol (100%, 90%, and 70% v/v) before being subjected to heat-mediated antigen retrieval using the antigen retrieval buffers (Table 2) and the Dako PTLINK pre-treatment module (Dako; #PT101). Antigen retrieval buffers were preheated to 65 °C before immersion of slides. Buffer temperature was then brought to 97 °C for 20 min and cooled to 65 °C before removal of the slides. Following antigen retrieval, slides were placed in the Dako AutostainerPlus (Dako; #AS480) and stained using the manufacturer’s guidelines and the reagents (Table 2). All slides were counter-stained using the tertiary nuclei stain DAPI (4′, 6-diamidino-2-phenylindole, Sigma; D8417) at 1 μg/mL and cover slipped in aqueous mounting medium (Fluoroshield, Sigma; F6182). Negative controls were stained without a primary antibody. Slides were digitally scanned (Axio Scan.Z1, ZEISS) and assessed using the Zeiss Zen Blue 3.1 program. DCX staining was assessed in two regions of the dentate gyrus, the apex, and granule cells of the outer blade, with an average calculated to ensure an accurate representation of DCX expression within the entire dentate gyrus be obtained for each animal. Iba1 and GFAP staining was assessed in the hippocampus (CA1, CA3, and dentate gyrus), prefrontal cortex, midbrain, and hypothalamus. For staining within the

### Table 1 RT-PCR primer specifications

| Target | Sequence 5'-3' | Tm (°C) |
|--------|----------------|---------|
| BCL2   | F: TAC CGT CGT GAC TTC GCA GAG | 67.6; 70.2 |
|        | R: GCC AGG AGC AGG GTC TT     |         |
| BDNF   | F: TGC AGG GGC ATA GAC AAA AGG | 68.1; 68.3 |
|        | R: CTT ATG AAT CGC CAG CCA ATT CTC |        |
| TNFα   | F: CTG TAG CCC AGC TCG TAG C   | 56.4; 55.1 |
|        | R: TGG AGA TCC ATG CCG TGG    |         |
| IL-1β  | F: CAA CCA ACA AGT GAT ATT CTC CAT G | 60.7; 60.7 |
|        | R: GAT CCA CACT CTC CAG CTC CA |        |
| TIMP-1 | F: GGC ATC TGG CAT CCT CTG GT  | 66.5; 65.6 |
|        | R: CGC TGG TAT AAG GTG GTC TCG |         |
| IL-6R  | F: TGA ATG ATG ACC CCA GGC AC  | 57.5; 57.6 |
|        | R: ACA CCC ATC CGC TCT CTA CT  |         |
| MMP-2  | F: CTG ATA ACC TGG ATG CCG TCG | 68.7; 67.6 |
|        | R: CTG GTG TGC ATG GAA GAT     |         |
| MMP-9  | F: CGC TCA TGT ACC CGC TGT AT  | 64.9; 65.9 |
|        | R: CCG TGG GAG GTA TAG TGG GA  |         |
| B-actin| F: CTC TTC CAG CCT TTC TTC CT  | 56.4; 57.9 |
|        | R: AGC ACT GTG TGG CGT TAC AG  |         |

*Designed by PrimerBlast, analyzed using NetPrimer software, and synthesized by Sigma.

### Table 2 Reagents utilized for immunofluorescence and immunohistochemistry staining

| Marker | Antigen retrieval buffer | Blocking reagent | Primary antibody | Secondary antibody |
|--------|--------------------------|------------------|-----------------|--------------------|
| DCX    | Citrate buffer, pH 6.0 | Normal horse serum (10% v/v, Sigma; H0146) | Rabbit-anti-doublecortin (Abcam; ab18723) | Alexa fluor, donkey-anti-rabbit 568 (0.8 mg/mL, Life Technologies; A10042) |
|        | in 1× PBS               |                  | 1/250 in NHS (5% v/v, Sigma; H1270) | in BSA (1% v/v, Sigma; A604), in 1× PBS |
| Iba1   | EDTA buffer, pH 9.0     | Bovine serum albumin (BSA 5% v/v, Sigma; A604) in distilled H2O | Rabbit-anti-Iba1 (FUJIFILM Wako; 019–19741) at 1/200 in BSA (1% v/v, Sigma; H0146) | Alexa fluor, goat-anti-rabbit 488 (1/1000, Invitrogen; A11034) in BSA (1% v/v, Sigma; A604), in 1× PBS |
| GFAP   | Citrate buffer, pH 6.0 | Normal horse serum (10% v/v, Sigma; H0146) | Rabbit-anti-GFAP (Abcam; ab7260) | Alexa fluor, donkey-anti-rabbit 568 (0.8 mg/mL, Life Technologies; A10042) in BSA (1% v/v, Sigma; H0146) |
|        | in 1× PBS               |                  | 1/250 in NHS (5% v/v, Sigma; H1270) | in BSA (1% v/v, Sigma; H0146), in 1× PBS |
| Ki67   | EDTA buffer, pH 9.0     | Flex peroxidase-blocking reagent (Dako; K4011) | Rabbit-anti-Ki67 (Abcam; ab16667) | DAB-labelled substrate (Dako; K3468) |
|        | in 1× PBS               |                  | 1/250 in NHS (5% v/v, Sigma; H1270) | in 1× PBS |

DCX staining was assessed in two regions of the dentate gyrus, the apex, and granule cells of the outer blade, with an average calculated to ensure an accurate representation of DCX expression within the entire dentate gyrus be obtained for each animal. Iba1 and GFAP staining was assessed in the hippocampus (CA1, CA3, and dentate gyrus), prefrontal cortex, midbrain, and hypothalamus. For staining within the...
CA1 and CA3 region of the hippocampus, an average value was obtained for CA1a and CA1b as well as CA3a and CA3c respectively. All staining was quantified using Fiji software (ImageJ) to determine the percent area of positive staining.

**Immunohistochemistry**

Immunohistochemistry (IHC) was undertaken on 5-μm sagittal sections of the brain, cut on a rotary microtome (Leica; RM2235), and mounted on glass slides (Flex Plus Detection System, Dako; #K8020). IHC analysis was performed for Ki67 (Abcam; ab16667), a global marker of cellular proliferation, to supplement DCX analysis. Following tissue dewaxing in xylene, rehydration in graded ethanol (100%, 90%, and 70%) and antigen retrieval using an EDTA buffer (pH 9.0; 0.121% w/v Tris, 0.037% w/v EDTA, 0.03% v/v tween 20 in distilled H₂O) heated using the same protocol as described for immunofluorescence. Ki67 staining was performed using Dako reagents and an automated system (AutoStainerPlus, Dako; #AS480) following standard protocols supplied by the manufacturer. Negative controls were stained without a primary antibody. Slides were cover slipped using dibutylphthalate polystyrene xylene (DPX, Merck; 317,616) and scanned using a Nanozoomer (Hamamatsu Photonics). The dentate gyrus of each section was assessed, and cells positively stained for Ki67 counted by blinded investigators using the Nanozoomer Digital Pathology software view.2 program (Histalim).

**Quantification and Statistical Analysis**

All data were analyzed using GraphPad Prism version 9.0. Normality was assessed using a Shapiro–Wilks test. Following confirmation of normality, statistical significance between groups was identified with a one-way analysis of variance (ANOVA) and appropriate post hoc tests for multiple comparisons or a student’s T-test. A Pearson’s correlation coefficient was used to identify a statistical association between primary outcome measures which were graphed using a simple linear regression. A p value of <0.05 was considered statistically significant.

**Results**

**5-FU Significantly Increases TNFα Expression at Acute Timepoints, TIMP-1 Expression at Subacute Timepoints, and IL-6R Expression at Chronic Timepoints**

The expression of key neuroinflammatory genes of interest, including tumor necrosis factor α (TNFα; Fig. 1A), tissue inhibitor of metalloproteinase 1 (TIMP-1; Fig. 1B), matrix metalloproteinase 9 (MMP-9; Fig. 1C), MMP-2 (Fig. 1D), interleukin-6 receptor (IL-6R; Fig. 1E), interleukin-1β (IL-1β; Fig. 1F), B-cell lymphoma 2 (BCL2; Fig. 1G), and brain derived neurotrophic factor (BDNF; Fig. 1H), were assessed using RT-PCR. A significant increase in the expression of TNFα, a key hallmark of early neuroinflammatory events across varied neurodegenerative conditions [27], was seen in the acute setting following 5-FU treatment when compared to controls (Fig. 1A; p = 0.006). 5-FU treatment also caused a significant increase to TIMP-1 expression, which regulates the proteolytic activity of MMPs, at the subacute timepoint when compared to controls (Fig. 1B; p = 0.031). While there was no significant change to MMP-9 and MMP-2 expression following 5-FU (Fig. 1C, D respectively), TIMP-1 positively correlated with the combined expression of MMP-2 and MMP-9 (R² = 0.192, p = 0.037; data not shown). This suggests the increase in TIMP-1 expression may be a compensatory mechanism to account for the increased expression of combined MMPs and thus increased proteolytic activity within the CNS.

IL-6 is a pleiotropic cytokine which can have pro- or anti-inflammatory effects depending on the signalling pathway activated through binding to the membrane bound or soluble form of the IL-6R [28]. When bound to the soluble IL6R, IL-6 is capable of activating glycoprotein130 (gp130) to mediate its pro-inflammatory action [29]. Accordingly, the expression of IL-6R was quantified to investigate the impact of 5-FU on IL-6 signalling. 5-FU treatment caused a significant increase in IL-6R expression at chronic timepoints when compared to controls (Fig. 1E; p = 0.033), highlighting the potential for the involvement of IL-6 signalling in central neurotoxicity.

No significant differences were observed in IL-1β expression across timepoints when compared to controls (Fig. 1F) and although BCL2 and BDNF have established roles in protecting neurogenesis through anti-apoptotic and trophic actions respectively [30, 31], neither were found to be altered by 5-FU treatment (Fig. 1G, H).

**5-FU Markedly Reduces Neurogenesis at Acute Timepoints Following Treatment**

To investigate the possibility of direct neurotoxicity, the impact of 5-FU on neurogenesis was assessed using DCX, a specific marker of newly formed neurons within the dentate gyrus, a key site of hippocampal neurogenesis. 5-FU treatment significantly decreased positive DCX staining within the dentate gyrus at chronic timepoints compared to controls (Fig. 2A, B; p = 0.034). Clear clustering of day 1 and day 32 controls was observed, with the means of day 1 and day 32 controls being determined as significantly different by a student’s T-test (p = 0.008). As such, a sub-analysis was performed between day 1 controls (N = 3)
and the percent area of positive DCX stain seen at acute timepoints. This revealed that a significant decrease in DCX staining was present between time matched control and treated animals (Fig. 2C; \( p = 0.003 \)), highlighting that 5-FU decreases neurogenesis in the acute setting following 5-FU. All data is presented as mean ± SEM, black dot represents day 1 controls; white dot represents day 32 controls.

Given the role of BCL2 and BDNF in neurogenesis, the relationship between the expression of these two factors and DCX was investigated. A strong positive correlation was observed between BCL2 and DCX expression (\( R^2 = 0.422, p = 0.0006; \) data not shown). However, no correlation between DCX and BDNF expression was established.
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5-FU Increased Microglial Density at Acute Timepoints in Brain Regions Associated with Cognition

The density of microglia, which are known as first responders within the CNS and often initiate neuroinflammation when exposed to pathological stimuli [32], was assessed by determining the percent area of positive Iba1 stain (Fig. 3A) in the prefrontal cortex (Fig. 3B), hypothalamus (Fig. 3C), and midbrain (Fig. 3D), as well as the CA3 (Fig. 3E), CA1 (Fig. 3F), and dentate gyrus (Fig. 3G) regions of the hippocampus. Following 5-FU treatment, the expression of Iba1 significantly
Fig. 3 Representative images from the prefrontal cortex of control and treated animals at the acute timepoint show that 5-FU increased Iba1 expression (green; nuclei, DAPI blue, scale bar 50 μm) (A). Quantification of the percent area stain is presented for the prefrontal cortex (B), hypothalamus (C), and midbrain (D) as well as the CA3 (E), CA1 (F), and dentate gyrus (G) regions of the hippocampus. All data is presented as mean ± SEM, black dot represents day 1 controls; white dot represents day 32 controls.
increased at acute timepoints in the prefrontal cortex (Fig. 3B; \( p = 0.026 \)), as well as the CA3 region (Fig. 3E; \( p = 0.028 \)) and dentate gyrus (Fig. 3G; \( p = 0.005 \)) of the hippocampus when compared to controls, all of which are brain regions critical to cognitive function. Iba1 expression levels then decreased across subacute and chronic timepoints, with levels close to what was seen in controls observed in the chronic setting. This trend in microglial density was reflected in the hypothalamus (Fig. 3C), midbrain (Fig. 3D), and CA1 region of the hippocampus (Fig. 3F); however, results were not significantly different from those of controls.

**5-FU Increased Astrocyte Density Across Multiple Brain Regions, with the Highest Levels of GFAP Expression Seen at Chronic Timepoints**

The density of astrocytes, key supportive cells within the CNS that play a role in immune signalling, was assessed using GFAP (Fig. 4A) in the prefrontal cortex (Fig. 4B), hypothalamus (Fig. 4C), and midbrain (Fig. 4D) as well as the CA3 (Fig. 4E), CA1 (Fig. 4F), and dentate gyrus (Fig. 4G) regions of the hippocampus. Following 5-FU, the expression of GFAP significantly increased across multiple brain regions and at varying timepoints post-treatment when compared to controls. Specifically, in the prefrontal cortex, levels of GFAP expression were highest at the subacute timepoint (Fig. 4B; \( p < 0.0001 \)). This was reflected in the CA3 region of the hippocampus (Fig. 4E; \( p = 0.048 \)). All other regions displayed varying levels of GFAP expression within the acute and subacute setting, with highest levels of expression seen at chronic timepoints when compared to controls. Specifically, these regions included the hypothalamus (Fig. 4C; \( p = 0.0003 \)) and midbrain (Fig. 4D; \( p = 0.005 \)) as well as the CA1 region (Fig. 4F; \( p = 0.0003 \)) and dentate gyrus (Fig. 4G; \( p < 0.0001 \)) of the hippocampus.

**Astrocyte Density Shares a Negative Correlation with Neurogenesis Following 5-FU**

The relationship between the density of glial cells and neurogenesis, reflective of neuroinflammatory and neurodegenerative processes respectively, was investigated by assessing the correlation between the expression of Iba1 and GFAP with both DCX and BCL2 expression following 5-FU. A significant negative correlation was observed between the expression of GFAP and both DCX and BCL2 within the CA1 region (Fig. 6A; \( R^2 = 0.199, p = 0.029 & B; R^2 = 0.225, p = 0.019 \)) and the dentate gyrus (Fig. 6B; \( R^2 = 0.201, p = 0.028 & D; R^2 = 0.223, p = 0.020 \)) of the hippocampus, as well as the midbrain (Fig. 6C; \( R^2 = 0.166, p = 0.048 & F; R^2 = 0.221, p = 0.021 \)) and hippocampus (Fig. 6G; \( R^2 = 0.283, p = 0.008 & H; R^2 = 0.243, p = 0.014 \)). No significant correlation was established between the expression of GFAP and both DCX and BCL2 in the prefrontal cortex and CA3 region of the hippocampus (data not shown). Furthermore, no significant correlations were established between the expression of Iba1 and both DCX and BCL2 in any brain regions.

**Discussion**

The neuropsychological symptoms related to chemotherapy treatment remain a major, unresolved challenge with prevention hampered by insufficient understanding of their pathophysiology. Long-term neuroimmune changes have been identified as a hallmark feature shared by these symptoms [11], although the exact timeline of mechanistic events preceding neuroinflammation and the relationship between different glial cells driving this neuroinflammatory response remain unclear. Here, we provide a longitudinal study of region-specific, neuroimmune changes occurring in response to systemically administered 5-FU, and position impaired neurogenesis along with the action of microglia as key initiating events in the resulting neuroinflammatory sequelae, perpetuated by astrocytic function. We have shown for the first time in vivo that, after 5-FU, impaired neurogenesis may be related to reduced BCL2 expression and, following acute increases to microglial density, MMP activity, and TNFα and IL-6 signalling share a clear relationship with...
The presence of cytotoxic injury implies 5-FU has the capacity to access the brain to induce damage. Unlike many other chemotherapy drugs, 5-FU has a small molecular weight and therefore is capable of crossing the BBB [35]. However, despite 5-FU being routinely utilised for the treatment of CNS malignancies, its efficacy is somewhat limited by the agent’s high polarity [36]. As such, the concentration of 5-FU that truly accesses the brain and the capacity for this dose to induce neurotoxicity remains unclear. A further consideration in the ability of 5-FU to induce direct cytotoxicity is its short half-life of only 8 to 20 min in vivo, and its rapid catabolism in the liver [37]. As such, identifying the presence and distribution of 5-FU within the brain, using emerging techniques such as spatial metabolomics with mass spectrometry imaging, which provides greater sensitivity to low yield, small molecules compared to traditional techniques [38], is critical to understanding the role of direct cytotoxicity in the neuroimmune changes occurring post 5-FU treatment, as well as its distribution among critical brain regions such as the hippocampus.

While it is plausible that 5-FU enters the CNS to directly damage newly born neuronal progenitor cells and act on microglia, other triggers cannot be ignored, particularly given that we used only a single dose of 5-FU. Inflammatory processes are documented to induce neurodegeneration and cell death, with neuroinflammation being a hallmark feature shared by numerous neurodegenerative disorders [39]. Importantly, intense peripheral inflammation and the generation of associated danger signals (e.g., DAMPs, PAMPs and MAMPs) are commonly induced by a range of chemotherapy agents. These inflammatory responses are triggered by cytotoxic injury of chemotherapy drugs against tumor cells and the collateral damage they cause to healthy tissue, in particular the highly proliferative intestinal mucosa [18]. For example, gastrointestinal mucositis and breakdown of the mucosal barrier permits translocation of gut-derived inflammatory mediators and bacterial endotoxins such as lipopolysaccharide (LPS), into systemic circulation where they are able to impact BBB integrity [40]. Apoptosis within the intestinal mucosa and resulting peaks in peripheral endotoxin levels have been observed at 6 and 24 h following chemotherapy treatment respectively in mice. In that same study, damage to BBB integrity was identified at 24, 48, and 72 h post treatment, highlighting a clear time course of events facilitating peripheral to central communication [19]. With increases to BBB permeability and the movement of peripheral inflammatory mediators into the CNS, it is possible that these peripherally-derived mediators act to induce the acute damage to neuronal progenitor cells and migration of microglia seen following 5-FU treatment, or may work in conjunction with 5-FU to induce neuronal cell death [40–42].

After the transient increase to microglial density observed at acute timepoints following 5-FU, the downstream neuroinflammatory response characterized in our study involved increases to TIMP-1 and associated MMP expression as well as TNFα and IL-6R expression, with associated long-term increases to astrocyte density, suggestive of reactivity. A compensatory increase to TIMP-1 expression, reflective of increases to the overall levels of MMPs present within the CNS, is seen in astrocytes when exposed to conditioned...
media from activated microglial cell cultures [43]. We identified a positive correlation between TIMP-1 expression and astrocyte density within the prefrontal cortex and CA3 region of the hippocampus, with peaks in expression and density observed at subacute timepoints. Given that microglia are known to produce MMPs [44], it is likely that

Fig. 5 Pearson’s correlation established that the expression of GFAP, but not Iba1, positively correlates with TNFα expression in the hypothalamus (A) and dentate gyrus (B), IL-6R expression in the hypothalamus (C), MMP-2 expression in the prefrontal cortex (D), and TIMP-1 expression in both the CA3 region of the hippocampus (E) and prefrontal cortex (F)
the acute increases to microglial presence induced increased TIMP-1 expression, to compensate for increased MMP activity. In the chronic setting following 5-FU treatment, TIMP-1 expression returned to levels similar to those seen at baseline. However, TNFα and IL-6R expression peaked in the chronic setting and this was positively correlated with the level of astrocyte density observed in the hypothalamus and dentate gyrus. TNFα is a proinflammatory cytokine renowned for its role in initiating and perpetuating neuroinflammation, and notably can act on astrocytes to induce their switch to the reactive A1 phenotype [45]. In this study, we have established a positive correlation between the increased expression of TNFα and GFAP, suggesting that the astrocytes present at chronic timepoints are likely to be reactive. Trans signalling via the IL-6R is critical in mediating the pro-inflammatory action of IL-6 [29] and as such IL-6 signalling may also underlie the increase to astrocyte density and resulting neuroinflammation following 5-FU.

Fig. 6 Pearson’s correlation established that the expression of DCX and BCL2 negatively correlates with the expression of GFAP, but not Iba1, in the CA1 region (A, B) and dentate gyrus (C, D) of the hippocampus as well as the midbrain (E, F) and hypothalamus (G, H).

A

B

C

D

E

F

G

H

GFAP

Iba1
Therefore, our data imply that acute microglial and resulting MMP activities are initiating factors in downstream astrocyte activation and TIMP-1 expression within the subacute setting, with TNFα and IL-6 signalling as critical mediators perpetuating chronic neuroinflammation following 5-FU. Our evidence suggests that chronic neuropsychological symptoms have acute cytotoxic origins. This new knowledge challenges the current management guidelines which are fundamentally reactive. In addition to being variably implemented, neuropsychological symptoms are largely rehabilitated through group training, which entails repetitive completion of tasks posing a mental challenge, as well as psychological intervention and pharmacotherapy when symptoms develop [46]. If our data are indeed translatable to humans, we suggest that ongoing work should be focussed on protecting the brain in the acute phases of chemotherapy treatment by either reinforcing the BBB or mitigating peripheral signals that drive neurological changes.

Our study is the first to longitudinally characterize the neuroimmune changes occurring post 5-FU treatment; however, it is not without limitations. Notably, we did not undertake behavioral phenotyping in these animals as the main aim of this study was to investigate the timeline of molecular events preceding the chronic neuroinflammation seen following chemotherapy. While this neuroinflammatory response is proposed to underlie the neuropsychological side

**Fig. 7** Diagrammatic representation of the neuroimmune changes observed following 5-FU treatment. From baseline expression levels, DCX and associated BCL2 expression decreased at acute timepoints and maintained this level of expression over subacute and chronic timepoints. Microglial density and TNFα expression increased at acute timepoints but returned to what was seen at baseline in the chronic setting. TIMP-1 and associated MMP expression was highest at subacute timepoints. Astrocyte density and associated IL-6R expression, were highest in the chronic setting.

**Table: Neuroimmune Changes Following 5-FU Treatment**

| Timepoint   | DCX & BCL2 | Iba1 | TNFα | TIMP-1 & MMP | GFAP | IL-6R | Combined |
|-------------|------------|------|------|--------------|------|-------|----------|
| Baseline    | \(\downarrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) |
| Acute       | \(\downarrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) |
| Subacute    | \(\downarrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) |
| Chronic     | \(\downarrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) | \(\uparrow\) |
effects of chemotherapy treatment, the inclusion of behavioral analyses probing cognitive and psychological function would have introduced significant confounding factors without providing additional data relevant to our fundamental research question. As established by Mandillo et al., stress related to the repetitive handling required by a battery of behavioral assessments is the primary confounding variable affecting reliability and reproducibility of these tests [47, 48]. Given that stress can drive the development of neuronal damage, including both neuroinflammation and deficits in neurogenesis [23, 49], the inclusion of behavioral analyses and the resulting stress introduced by repeated handling may have masked the true effects of 5-FU on the outcome measures reported in this study. A further limitation is that all transcriptomic analyses were performed using the frontal cortex of the brain and as such no conclusions can be made regarding regional changes in the expression of the genes investigated. These analyses could, however, provide further clarification to the physiological changes occurring in critical brain regions, such as the hippocampus, following chemotherapy and thus is an avenue for future research to build on the time course of neurological events defined by the current study.

To our knowledge, this is the first study to provide in vivo evidence suggesting that microglia play a role in the acute, neuroimmune response to chemotherapy and astrocytes may drive the longitudinal changes seen in the chronic setting. However, it should be acknowledged that while this study provided evidence of significant increases to glial cell density in brain regions of interest, morphological analysis of both microglia and astrocytes in these same regions could provide additional insight into the activation state of glial cells. A further limitation is that the control group of this study included tissues collected from animals on day 1 and day 32 of the experimental timeline. Whilst time-matched controls would be preferable to minimize variability, the largest effect size in outcome measures would be observed at these timepoints, due to the stress of IP procedure and the effect of aging respectively. As such, controlling for these factors was prioritized within the experimental design and repetition with time-matched controls, and an increased sample size presents as an avenue for future research to build on the findings of this study. It should also be acknowledged that, given this study was performed in female mice, it is possible that the hormonal fluctuations of the menstrual cycle influenced neuroinflammatory events and repetition in males could allow additional insight. To provide clarity on the temporal relationship between the neuroimmune changes occurring from acute to subacute and chronic timepoints following chemotherapy treatment, a single bolus dose was utilized in this study design. The use of a multi-dose treatment schedule would have initiated injury at multiple timepoints, blurring the timeline of neuroimmune events occurring post-treatment. However, to improve translatability of findings, a multi-dose chemotherapy cycle would be more reflective of the patient experience in clinic.

Conclusions and Future Directions

The neuropsychological deficits associated with chemotherapy treatment remain a priority concern in the field of supportive oncology. This study has, for the first time, described the longitudinal timeline of neuroimmune events present following 5-FU treatment and identified impaired neurogenesis and acute increases to microglia density as probable initiating factors underlying downstream neuroinflammation, sustained by the action of astrocyte. These findings highlight the temporal dynamics of neurotoxicity and neuroinflammation and that significant and persistent neuroimmune changes can occur within the brain following a single dose of systemically administered chemotherapy. This strongly suggests that protecting the brain within the acute phases of treatment may be critical to preventing neuropsychological symptom development and warrants further research to identify modifiable triggers of acute neurotoxicity. We suggest that this preclinical model is well suited to enable continued investigations of this mechanism and test new neuroprotective interventions.

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Author Contribution Courtney Subramaniam: methodology, investigation, formal analysis, writing – original draft; Hannah Wardill: supervision, conceptualisation, formal analysis, writing – review & editing; Maya Davies: investigation; Vivien Heng: investigation; Marc Gladman: supervision, writing – review & editing; Joanne Bowen: supervision, conceptualisation, writing – review & editing.

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Data Availability The datasets generated within this study are not currently available to the public. However, can be made available upon request to the corresponding author.

Declarations

Ethics Approval The study was approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI, #SAM20-034) and complied with the National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014).
Consent to Participate N/A.

Consent to Publish N/A.

Competing Interests Joanne Bowen has received research funding from AstraZeneca, Helsinn Healthcare, Pfizer Pharmaceuticals, Pan-Theryx, and Puma Biotechnology Inc. Hannah Wardill has received research funding from Nutricia (Danone) Research. Remaining authors have no conflicts of interest to declare.

References
1. Nurgali K, Jagoe RT, Abalo R (2018) Editorial: adverse effects of cancer chemotherapy: anything new to improve tolerance and reduce sequelae? Front Pharmacol 9:245
2. Apple AC et al (2017) Subtle hippocampal deformities in breast cancer survivors with reduced episodic memory and self-reported cognitive concerns. Neuroimage Clin 14:685–691
3. Apple AC et al (2018) Hippocampal functional connectivity is related to self-reported cognitive concerns in breast cancer patients undergoing adjuvant therapy. Neuroimage Clin 20:110–118
4. Kesler SR, Kent JS, O’Hara R (2011) Prefrontal cortex and executive function impairments in primary breast cancer. Arch Neurol 68(11):1447–1453
5. Yang Y et al (2017) The relationship between cancer patient’s fear of recurrence and chemotherapy: a systematic review and meta-analysis. J Psychosom Res 98:55–63
6. Souza R et al (2020) Factors associated with sleep quality during chemotherapy: an integrative review. Nurs Open 7(5):1274–1284
7. Selamat MH et al (2014) Chemobrain experienced by breast cancer survivors: a meta-ethnography study investigating research and care implications. PLoS ONE 9(9):e108002
8. Ramsey I et al (2021) A core set of patient-reported outcomes for population-based cancer survivorship research: a consensus study. J Cancer Surviv 15(2):201–212
9. Huang J et al (2016) Symptom clusters in ovarian cancer patients with chemotherapy after surgery: a longitudinal survey. Cancer Nurs 39(2):106–116
10. Sullivan CW et al (2018) Stability of symptom clusters in patients with breast cancer receiving chemotherapy. J Pain Symptom Manage 55(1):39–55
11. Santos JC, Pyter LM (2018) Neuroimmunology of behavioral comorbidities associated with cancer and cancer treatments. Front Immunol 9:1195
12. Cherry JD, Olschowka JA, O’Banion MK (2014) Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation 11:98
13. Michopoulos V et al (2017) Inflammation in fear- and anxiety-based disorders: PTSD, GAD, and beyond. Neuropsychopharmacology 42(1):254–270
14. Troubat R et al (2021) Neuroinflammation and depression: a review. Eur J Neurosci 53(1):151–171
15. Stephenson J et al (2018) Inflammation in CNS neurodegenerative diseases. Immunology 154(2):204–219
16. George RP et al (2021) Neuroimmune reactivity marker expression in rodent models of chemotherapy-induced cognitive impairment: a systematic scoping review. Brain Behav Immun 94:392–409
17. Subramaniam CB et al (2020) The microbiota-gut-brain axis: an emerging therapeutic target in chemotherapy-induced cognitive impairment. Neurosci Biobehav Rev 116:470–479
18. Bowen J et al (2019) The pathogenesis of mucositis: updated perspectives and emerging targets. Support Care Cancer 27(10):4023–4033
19. Wardill HR et al (2016) Irinotecan-Induced Gastrointestinal Dysfunction and Pain Are Mediated by Common TLR4-Dependent Mechanisms. Mol Cancer Ther 15(6):1376–1386
20. Sritawan N et al (2020) Metformin alleviates memory and hippocampal neurogenesis decline induced by methotrexate chemotherapy in a rat model. Biomed Pharmacother 131:110651
21. Egeland M et al (2017) Depletion of adult neurogenesis using the chemotherapy drug temozolomide in mice induces behavioural and biological changes relevant to depression. Transl Psychiatry 7(4):e1101
22. Jiang ZG et al (2018) PAN-811 prevents chemotherapy-induced cognitive impairment and preserves neurogenesis in the hippocampus of adult rats. PLoS ONE 13(1):e0191866
23. Dioli C et al (2019) Chronic stress triggers divergent dendritic alterations in immature neurons of the adult hippocampus, depending on their ultimate terminal fields. Transl Psychiatry 9(1):143
24. Chesnokova V, Pechnick RN, Wawrowsky K (2016) Chronic peripheral inflammation, hippocampal neurogenesis, and behavior. Brain Behav Immun 58:1–8
25. Roxburgh CS, McMillan DC (2014) Cancer and systemic inflammation: treat the tumour and treat the host. Br J Cancer 110(6):1409–1412
26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402–408
27. Jung YJ et al (2019) Neuroinflammation as a factor of neurodegenerative disease: thalidomide analogs as treatments. Front Cell Dev Biol 7:313
28. Ertà M, Quintana A, Hidalgo J (2012) Interleukin-6, a major cytokine in the central nervous system. Int J Biol Sci 8(9):1254–1266
29. Rose-John S (2012) IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. Int J Biol Sci 8(9):1237–1247
30. Zhang R et al (2006) Bcl-2 enhances neurogenesis and inhibits apoptosis of newborn neurons in adult rat brain following a transient middle cerebral artery occlusion. Neurobiol Dis 24(2):345–356
31. Numakawa T, Odaka H, Adachi N (2017) Actions of brain-derived neurotrophic factor and glucocorticoid stress in neurogenesis. Int J Mol Sci 18(11):2312. https://doi.org/10.3390/ijms18112312
32. Cătălin B et al (2013) Microglia: first responders in the central nervous system. Rom J Morphol Embryol 54(3):467–472
33. Pereira Dias G et al (2014) Consequences of cancer treatments on adult hippocampal neurogenesis: implications for cognitive function and depressive symptoms. Neuro Oncol 16(4):476–492
34. Gibson EM et al (2019) Methotrexate Chemotherapy induces persistent tri-gliial dysregulation that underlies chemotherapy-related cognitive impairment. Cell 176(1–2):43-55.e13
35. Formica V et al (2006) 5-Fluorouracil can cross brain-blood barrier and cause encephalopathy: should we expect the same from capecitabine? A case report on capecitabine-induced central neurotoxicity progressing to coma. Cancer Chemother Pharmacol 58(2):276–278
36. Shinde G et al (2020) Enhanced brain targeting efficiency using 5-FU (fluorouracil) lipid-drug conjugated nanoparticles in brain cancer therapy. Prog Biomater 9(4):259–275
37. Diasio RB, Harris BE (1989) Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet 16(4):215–237
38. Alexandrov T (2020) Spatial Metabolomics and imaging mass spectrometry in the age of artificial intelligence. Annu Rev Biomed Data Sci 3:61–87
39. Guzman-Martínez L et al (2019) Neuroinflammation as a common feature of neurodegenerative disorders. Front Pharmacol 10:1008
40. Wardill HR et al (2016) Cytokine-mediated blood brain barrier disruption as a conduit for cancer/chemotherapy-associated neurotoxicity and cognitive dysfunction. Int J Cancer 139(12):2635–2645
41. Perez-Dominguez M et al (2019) The detrimental effects of lipopolysaccharide-induced neuroinflammation on adult hippocampal neurogenesis depend on the duration of the pro-inflammatory response. Neural Regen Res 14(5):817–825
42. Zhao J et al (2019) Neuroinflammation induced by lipopolysaccharide causes cognitive impairment in mice. Sci Rep 9(1):5790
43. Welser-Alves JV, Crocker SJ, Milner R (2011) A dual role for microglia in promoting tissue inhibitor of metalloproteinase (TIMP) expression in glial cells in response to neuroinflammatory stimuli. J Neuroinflammation 8:61
44. Könnecke H, Bechmann I (2013) The role of microglia and matrix metalloproteinases involvement in neuroinflammation and gliomas. Clin Dev Immunol 2013:914104
45. Liddelow SA et al (2017) Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541(7638):481–487
46. Runowicz CD et al (2016) American Cancer Society/American Society of Clinical Oncology Breast Cancer Survivorship Care Guideline. CA Cancer J Clin 66(1):43–73
47. Mandillo S et al (2008) Reliability, robustness, and reproducibility in mouse behavioral phenotyping: a cross-laboratory study. Physiol Genomics 34(3):243–255
48. Saré RM, Lemons A, Smith CB (2021) Behavior testing in rodents: highlighting potential confounds affecting variability and reproducibility. Brain Sci 11(4):522. https://doi.org/10.3390/brainsci11040522
49. Calcia MA et al (2016) Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness. Psychopharmacology 233(9):1637–1650

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